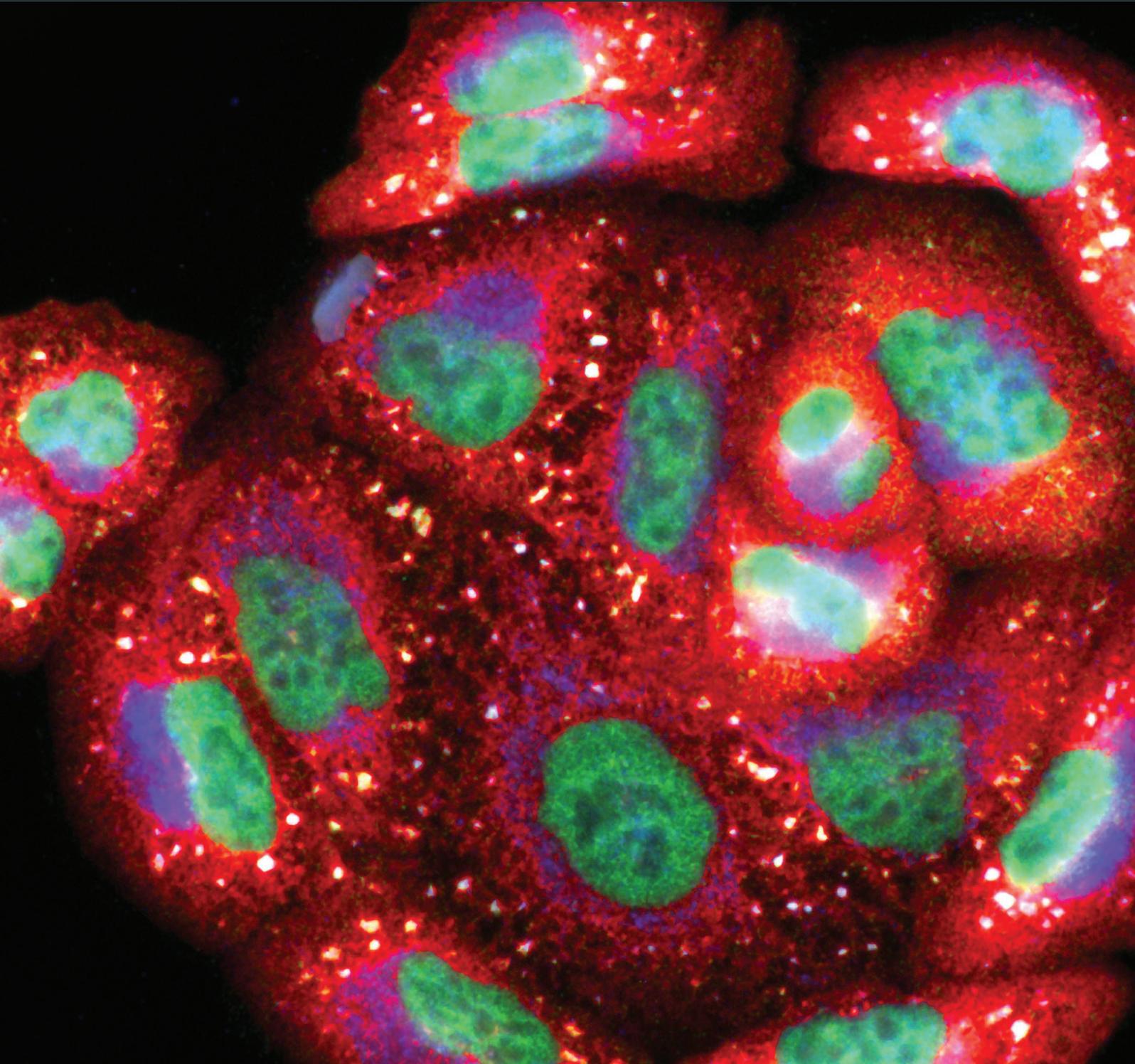


Harmful and Beneficial Role of ROS

Guest Editors: Sergio Di Meo, Tanea T. Reed, Paola Venditti, and Victor M. Victor





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Editorial

Harmful and Beneficial Role of ROS

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Reactive oxygen species (ROS) are an unavoidable byproduct of oxygen metabolism and their cellular concentrations are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes. For a long time ROS were thought to cause exclusively toxic effects which were associated with various pathologies, including carcinogenesis, neurodegeneration, atherosclerosis, diabetes, and aging. However, to date, it is known that while prolonged exposure to high ROS concentrations may lead to various disorders, low ROS concentrations exert beneficial effects regulating cell signaling cascades.

The papers reported in this issue focus attention on some aspects of ROS biology including the impact of ROS production on various body districts and the defense arising from endogenous and exogenous antioxidants, beneficial effects of ROS production, and ROS regulation of signaling pathways. However, the examination of the manuscripts clearly shows that the passage of time has partly changed the approach to various topics. For example, although in some works different substances, including antioxidants, have yet been used, in other works different procedures and substances, including products or extracts, have been successfully used in the treatment of oxidative stress and related disorders due to the complex bioactive compounds they contain.

Thus, A. V. Maksimenko studied the effects of intravenous injection of the superoxide dismutase-chondroitin sulfate-catalase (SOD-CHS-CAT) conjugate in a rat model of endotoxin shock. In this way he demonstrated the effectiveness of the conjugate in prevention and medication of oxidative stress damage, which is only partly due to prevention of NO conversion in peroxynitrite.

The review of V. D. Prokopieva et al. examined properties and biological effects of the antioxidant carnosine and presented data on successful use of carnosine in different pathologies. Such data show that carnosine is an effective antioxidant able to protect tissues against various adverse factors inducing development of oxidative stress.

S. Ponist et al. evaluated the therapeutic potential of carnosine in rat adjuvant arthritis. The results obtained on two animal models (model of local acute inflammatory reaction and subchronic model of rodent polyarthritis) showed that carnosine had systemic anti-inflammatory activity and protected rat brain and chondrocytes from oxidative stress.

A. Matuszyk et al. found that administration of exogenous obestatin accelerates the healing of acetic acid-induced colitis, an effect partly due to anti-inflammatory properties of obestatin that reduces IL-1 β concentration and myeloperoxidase activity in colonic mucosa.

C. Liu et al. used the aqueous extract of *Cordyceps militaris* fruit body in streptozotocin-induced diabetic rats and found that the extract displays antidiabetic and antinephrotic activity due to its ability to attenuate oxidative stress.

W. J. Bae et al. examined the effects of decursin extracted from *Angelica gigas* Nakai (AG) on antioxidant activity *in vitro* and in a cryptorchidism-induced infertility rat model. Their study suggests that decursin is able to reduce oxidative stress by Nrf2-mediated upregulation of heme oxygenase-1 (HO-1) in rat experimentally induced unilateral cryptorchidism and may improve cryptorchidism-induced infertility.

E. Kerasioti et al. studied the protective effect of sheep whey protein (SWP) against tert-butyl hydroperoxide-(tBHP-) induced oxidative stress in endothelial cells. Their

findings demonstrate that SWP protects endothelial cells from oxidative stress increasing GSH levels and decreasing GSSG, lipid peroxidation, protein oxidation, and ROS levels.

P. Boonruamkaew et al. studied the effect of an antioxidative nanoparticle (RNP^N) that they recently developed against APAP-induced hepatotoxicity in mice. Their findings lead to concluding that RNP^N possesses effective hepatoprotective properties and does not exhibit the notable adverse effects associated with NAC treatment.

M. J. Gomes et al. evaluated the influence of exercise on functional capacity, cardiac remodeling, and skeletal muscle oxidative stress in rats with aortic stenosis- (AS-) induced heart failure (HF). They found that exercise improves functional capacity in rats regardless of echocardiographic parameter changes. In soleus, exercise reduces oxidative stress, preserves antioxidant enzyme activity, and modulates mitogen-activated protein kinases (MAPK) expression.

S. Kremserova et al. evaluated the role of myeloperoxidase (MPO) in the regulation of acute lung inflammation and injury. They showed that MPO deficiency enhances neutrophilia during LPS-induced airway inflammation due to altered accumulation of proinflammatory cytokine RANTES (regulated on activation, normal T cell expressed and secreted) and reduces cell death of MPO deficient neutrophils. The role of MPO in the regulation of the course of pulmonary inflammation, independent of its putative microbicidal functions, can be potentially linked to its ability to modulate the life span of neutrophils and affect accumulation of chemotactic factors at the site of inflammation.

J. Petrović et al. investigated whether magnesium supplementation in sedentary and rugby players young men could protect peripheral blood lymphocytes (PBL) from hydrogen peroxide-induced DNA damage. They found that magnesium supplementation has marked effects in protecting the DNA from oxidative damage in both men with different lifestyles.

T. Kataoka et al. compared the mitigating effects on chronic constriction injury- (CCI-) induced neuropathic pain of radon inhalation and pregabalin administration and examined the combination effects of the treatments. They found that combined effect of radon and pregabalin is an additive effect because it has mitigative effect similar to the effects of remarkably higher dose of pregabalin. The possible mechanism is the activation of antioxidative functions induced by radon inhalation.

G. Espinha et al. found that the inhibition of RhoA GTPase, an enzyme overexpressed in highly aggressive metastatic tumors, increases sensitivity of melanoma cells to UV radiation effects, suggesting that this GTPase represents a potential inhibitory target for metastatic melanomas.

Some works have addressed the problem of the dual role played by ROS or ROS producing enzymes.

N. Kaludercic and V. Giorgio described mitochondria as a major site of production and as a target of ROS/RNS and discussed how the posttranslational modifications of ATP synthase due to ROS/RNS generation might play a dual role by promoting cell death or survival depending on their relative effects on mitochondrial ATP synthase catalysis and PTP.

H. Pei et al. summarized the present understanding of the role played by mitochondrial functional proteins such

as electron transport chain complexes, uncoupling proteins, mitochondrial dynamic proteins, translocases of outer membrane complex, and mitochondrial permeability transition pore in ROS production and in protection of mitochondrial integrity and function in ischemic heart diseases.

M. G. Battelli et al. reviewed the physiological and pathological roles of xanthine oxidoreductase- (XOR-) derived oxidant molecules showing that they may result in either harmful or beneficial outcomes. Indeed, XOR generates free radicals which are responsible for tissue damage in hypoxia/reoxygenation and ischemia/reperfusion, have proinflammatory activity, are involved in cancer pathogenesis, and favor the progression to malignancy by inducing angiogenesis and cell migration. On the other hand, XOR products may activate the expression of the proapoptotic protein p53 and transcription factors with antitumorigenic and antiproliferative activity.

H.-Y. Tan et al. reviewed the role of ROS in maintaining the homeostatic functions of macrophage and in particular macrophage polarization. They also reviewed the biology of macrophage polarization and the disturbance of the balance of the different functional phenotypes in human diseases.

J. A. Hernández et al. reviewed the role of lipids in the neuronal damage induced by ethanol-related oxidative stress and in the related compensatory or defense mechanisms. They showed that ethanol-induced neurodegeneration is at least partly the result of the equilibrium between the toxicity of signaling lipids and the protection that some lipids, such phosphatidylethanolamine and cholesterol, confer to the cell.

A. Schmidt et al. used a HaCaT keratinocyte cell culture model to investigate redox regulation and inflammation to periodic, low-dose oxidative challenge generated by recurrent incubation with cold physical plasma-treated cell culture medium. They investigated the HaCaT keratinocyte global transcriptomic profile over three months to identify genes responsible for adaptations to periodic oxidative stress as seen in redox-related diseases of the skin. Their results suggest that all keratinocytes may have adapted to redox stress over time, significantly altering their basal gene expression profile.

E. Ershova et al. studied the influence of a water-soluble fullerene derivative (F828) on serum-starving human embryo lung diploid fibroblasts HELFs. They found that F828 exerts a block on genotoxic effect of oxidative stress in serum-starving HELFs. The decrease in the number of double strand breaks and apoptosis was maximum at concentrations 0.2–0.25 μM , whereas, at concentrations higher than 0.5 μM , excessive ROS scavenging was accompanied by increased cell death rate.

The problem of the role of reactive species sources in health and disease was examined by S. Di Meo et al. They, after examining the cellular localization and supposed involvement of such sources in tissue dysfunction and protection, examined experimental evidence concerning their harmful and protective effects in a normal physiological activity, such as exercise, and in pathologic conditions, such as diabetes and neurodegenerative diseases.

Some works have faced different problems that are still united by the oxidative stress impact on various pathological conditions and the factors involved in the signaling pathways.

N. T. Costa et al. evaluated the involvement of TNF- α and insulin resistance (IR) in the inflammatory process, oxidative

stress, and disease activity in patients with rheumatoid arthritis (RA). They demonstrated that IR and TNF- α are important factors involved in redox imbalance in patients with RA which seems to be due to the maintenance of inflammatory state and disease activity.

S. Vranková et al. studied the effects of NF- κ B inhibition on ROS and NO generation and blood pressure (BP) regulation in hereditary hypertriglyceridemic rats. They found that NF- κ B inhibition leads to decreased ROS degradation by SOD followed by increased heart oxidative damage and BP elevation despite the increase in eNOS protein.

L. Minutoli et al. described the current knowledge on the role of NLRP3 inflammasome in some organs (brain, heart, kidney, and testis) after I/R injury, with particular regard to the role played by ROS in its activation. They conclude that a definite comprehension of the role of NLRP3 inflammasome in the host responses to different danger signals is still lacking.

S. Kazemi et al. investigated the effects of the xenoestrogenic chemical Bisphenol A (BPA) on hepatic oxidative stress-related gene expression in rats. Their finding demonstrated that BPA generates ROS and increases the expression of HO-1 and Gadd45b genes that cause hepatotoxicity.

A. N. Onyango reviewed the potential pathways of the endogenous formation of singlet oxygen and ozone, their relevance to human health, and how dietary factors affect generation and activity of such oxidants.

E. Gammella et al. described the multifaceted systems regulating cellular and body iron homeostasis and discussed how altered iron balance may lead to oxidative damage in some pathophysiological settings.

X. Wang et al. found that the transcription factor BTB and CNC homology 1 (Bach1) induces endothelial cell apoptosis and cell cycle arrest through ROS generation and, consequently, that functional downregulation of Bach1 may be a promising target for the treatment of vascular diseases.

M. Nita and A. Grzybowski, examining the current literature, showed that excessive production of reactive oxygen species and oxidative stress play important role in the pathogenesis of many age-related ocular diseases and other pathologies of the anterior and posterior eye segment in adults. ROS stimulate cells' death via apoptosis process, participate in the activation of proinflammatory and proangiogenic pathways, and are associated with the autophagy process.

T. R. S. Hamilton et al. evaluated lasting effects of heat stress-induced oxidative stress on ejaculated and epididymal sperm and found that it leads to rescuable alterations after one spermatogenic cycle in ejaculated sperm and also after 30 days in epididymal sperm.

M. L. Fanjul-Moles and G. O. López-Riquelme examined the relationships between oxidative stress, circadian rhythms, and retinal damage in humans, particularly those related to light and photodamage. Their review highlights the role of oxidative stress as one of the main causes of age-related macular degeneration (AMD) etiologies, a disease to which, in addition to genetic predispositions, at least four processes contribute: lipofuscinogenesis, drusenogenesis, local inflammation and neovascularization, and immunological mechanisms.

H. Nagahisa et al. studied structural and functional changes induced by SOD1 deficiency and their results suggested that muscle is damaged by ROS produced in the TypeIIx/b fibers of the gastrocnemius muscle, accelerating the proliferation and differentiation of satellite cells in SOD1 KO mice.

It is our opinion that the articles included in this special issue, despite dealing with so different topics, represent an important contribution to the knowledge of the harmful and beneficial effects of ROS in living organisms.

*Sergio Di Meo
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Research Article

Functionalized Fullerene Increases NF- κ B Activity and Blocks Genotoxic Effect of Oxidative Stress in Serum-Starving Human Embryo Lung Diploid Fibroblasts

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The influence of a water-soluble [60] fullerene derivative containing five residues of 3-phenylpropionic acid and a chlorine addend appended to the carbon cage (F-828) on serum-starving human embryo lung diploid fibroblasts (HELFs) was studied. Serum deprivation evokes oxidative stress in HELFs. Cultivation of serum-starving HELFs in the presence of 0.1–1 μ M F-828 significantly decreases the level of free radicals, inhibits autophagy, and represses expression of NOX4 and NRF2 proteins. The activity of NF- κ B substantially grows up in contrast to the suppressed NRF2 activity. In the presence of 0.2–0.25 μ M F-828, the DSB rate and apoptosis level dramatically decrease. The maximum increase of proliferative activity of the HELFs and maximum activity of NF- κ B are observed at these concentration values. *Conclusion.* Under the conditions of oxidative stress evoked by serum deprivation the water-soluble fullerene derivative F-828 used in concentrations of 0.1 to 1 μ M strongly stimulates the NF- κ B activity and represses the NRF2 activity in HELFs.

1. Introduction

Oxidative stress underlies the pathogenesis of some cardiovascular, oncologic, and neurologic diseases. Accumulation of reactive oxygen species (ROS) causes damage to the cellular DNA. In order to minimize the genotoxic effect, novel techniques for ROS reduction are under development.

Over the past decade, nanoparticles based on the molecular carbon form known as fullerenes are considered as promising and efficient ROS scavengers. Fullerenes represent very hydrophobic substances which are virtually not soluble in aqueous media. In order to improve their solubility, various substituents are attached to the carbon cage. Using various model systems, fullerene ability to penetrate through the cell

membrane and efficiently reduce the ROS level was unambiguously demonstrated [1–7]. It was revealed that different types of fullerene-based materials are able to intercept all of the major physiologically relevant ROS [8].

It is assumed that fullerenes reduce the ROS level not only due to direct chemical interaction with the reactive oxygen and nitrogen species, but also by influencing the signaling pathways, which regulate the ROS level and help the cells to survive in hostile environment. For example, experiments with polyhydroxylated fullerenes, also known as fullerlenols [$C_{60}(OH)_n$], have shown that $C_{60}(OH)_{24}$ can attenuate oxidative stress-induced apoptosis *via* augmentation of Nrf2-regulated antioxidant capacity of the cell [9]. Nuclear factor erythroid 2-related factor 2 (NRF2) is a basic leucine zipper

redox-sensitive transcriptional factor that plays a key role in ARE- (antioxidant response element-) mediated induction of phase II detoxification and activation of antioxidant enzymes. NRF2 mediates a set of adaptive responses to intrinsic and extrinsic cellular stresses [10]. Fullerene derivative $C_{60}(C(COOH)_2)_3$ (C_{60} -COOH) was investigated previously and it was demonstrated that C_{60} -COOH pretreatment attenuated the lipopolysaccharide-mediated activation of nuclear factor- (NF-) κ B and mitogen-activated protein kinase (MAPK) signaling, as well as the production of proinflammatory mediators [11]. Nuclear factor- κ B is an essential transcription factor, which controls the expression of genes involved in the immune and inflammatory responses. NF- κ B is known to regulate genes involved in apoptosis, cell proliferation, angiogenesis, and metastasis [12, 13]. The signaling pathways, where Nrf2 and NF- κ B factors are involved, interact in the cells [14, 15]. It is known that activation of Nrf2-antioxidant signaling attenuates the NF- κ B-inflammatory response and elicits apoptosis [14–16]. It suggests that water-soluble fullerenes can act as signaling pathway triggers in the cells, switching on the Nrf2-antioxidant signaling activity and blocking the NF- κ B activity. Such behavior of fullerene derivatives might allow their application as therapeutic agents for reducing the genotoxic effect accompanying oxidative stress of different origins and as anti-inflammatory agents.

It was previously shown that serum-free cultivation elicits chronic stress due to increased production of ROS as compared to the serum-supplemented cultures [17]. The increase in the production of free radicals is accompanied by an increase in the population of cells with numerous DSBs. We also observed an increase in the number of cells with condensed and fragmented chromatin due to cultivation in the absence of serum, which is an indicator of ongoing apoptosis. In the stressed, serum-starving fibroblasts, the level and activity of the transcriptional factor NF- κ B increase along with elevation of secreted concentrations of TNF α cytokine, while the NRF2 activity decreases [17]. Thus, serum-starving HELFs represent a good model to study water-soluble fullerene-mediated NRF2 induction and NF- κ B activity repression under the conditions of chronic oxidative stress, which provokes double-strand break formation in the cells. We studied the action of a water-soluble fullerene derivative F-828 carrying COOH groups [18, 19] upon serum-starving human embryo lung diploid fibroblasts (HELFs).

2. Methods

Chemical synthesis of a water-soluble fullerene derivative F-828 comprising five residues of 3-phenylpropionic acid and a chlorine atom arranged around one cyclopentadienyl unit on the fullerene cage carrying COOH groups was described by us in detail previously [18, 19].

2.1. Cell Culture. Human embryonic lung fibroblasts were obtained from the Research Centre for Medical Genetics RAMS collection. Ethical approval for the use of primary human cells was obtained from the Committee for Medical and Health Research Ethics of Research Centre for Medical Genetics, Russian Academy of Medical Sciences (approval

number 5). Before treatments, HELFs were subcultured with 10% serum at most four times. Cells were cultured in growth factor containing serum-free medium “Hybris” [20] that consists of the basal medium and a serum-free supplement containing purified human albumin and a growth factor cocktail (<http://www.paneco.ru/>). The cells were seeded at $5 \times 10^4/3$ mL of medium and then incubated for 2 h to ensure their attachment. Various F-828 concentrations were then introduced and the cells were incubated for 48 h.

2.2. Flow Cytometry (FCA). HELFs were washed in Versene solution and then treated with 0.25% trypsin, washed with culture media, and suspended in PBS. Staining of HELFs with various antibodies was also performed. To fix the cells, they were treated with paraformaldehyde (PFA, Sigma, 2%, 37°C, 10 min). The fixed cells were washed three times with 0.5% BSA-PBS and permeabilized with 0.1% Triton X-100 (PBS, 15 min, 20°C) or with 90% methanol (4°C). Afterwards, the cells were washed three times with 0.5% BSA-PBS and stained with 1 μ g/mL antibodies for 2 h (4°C) and then again washed thrice with 0.5% BSA-PBS. Then HELFs were incubated for 2 h (20°C) with the FITC goat anti-rabbit IgG. To quantify DNA, cells were treated with propidium iodide (PI) and RNase A.

The following primary antibodies were used: FITC- γ H2AX (pSer139) (Temecula, California); FITC-Ki-67, EEA1, PCNA, BECLIN, NRF2, NF- κ B (p65), NF- κ B (p65) (pSer529), NOX4, and FITC goat anti-rabbit IgG (Abcam). To quantify the background fluorescence, we stained a portion of the cells with secondary FITC-conjugated antibodies only.

The cells were analyzed using CyFlow Space (Partec, Germany).

2.3. ROS Assays. Cells were analyzed using total fluorescence assay in the 96-well plate format at $\lambda_{ex} = 488$ nm and $\lambda_{em} = 528$ nm (EnSpire Equipment, Finland). HELFs were treated with 5 μ M H_2 DCFH-DA (Molecular Probes/Invitrogen, CA, USA) for 10–60 min at 37°C. The constant of DCF generation rate was determined as a slope of the signal-time line.

2.4. Fluorescence Microscopy. The images of the cells were obtained using the AxioScope A1 microscope (Carl Zeiss). Immunocytochemistry: HELFs were fixed in 2% PFA (4°C, 20 min), washed with PBS, and then permeabilized with 0.1% Triton X-100 in PBS (15 min, 20°C), followed by blocking with 0.5% BSA in PBS (1 h, 4°C), and incubated overnight with rabbit polyclonal antibody against LC3 (Epitomics, Cambridge, MA), γ H2AX (pSer139), or NF- κ B (p65) (Abcam). After washing with 0.01% Triton X-100 in PBS HELFs were incubated for 2 h (20°C) with the FITC goat anti-rabbit IgG, washed with PBS, and then stained with DAPI or PI.

Nuclear fragmentation was examined by Hoechst 33342 (Sigma). Cells were washed and stained with Hoechst 33342 (10 μ g/mL) for 10 min at 37°C.

2.5. Quantification of mRNA Levels. Total mRNA was isolated using RNeasy Mini kit (Qiagen, Germany). After

the treatment with DNase I, RNA samples were reverse-transcribed by Reverse Transcriptase kit (Sileks, Russia). The expression profiles were obtained using qRT-PCR with SYBR Green PCR Master Mix (Applied Biosystems). The mRNA levels were analyzed using the StepOnePlus (Applied Biosystems); the technical error was approximately 2%. The following primers were used (Sintol, Russia):

BCL2 (F: GCCTTCTTTGAGTTTCGGTGG, R: ATC-TCCC GGTTGACGCTCT);

BCL2A1 (F: TACAGGCTGGCTCAGGACTAT R: CGCAACATTTTGTAGCACTCTG);

BCL2L1 (F: CGACGAGTTTGAAGTTCGGTA R: GGGATGTCAGGTCAGTGAATG);

BIRC2 (F: GAATCTGGTTTCAGCTAGTCTGG R: GGTGGGAGATAATGAATGTGCAA);

BIRC3 (F: AAGCTACCTCTCAGCCTACTTT R: CCACTGTTTTCTGTACCCGGA);

KEAP1 (F: GTGGTGTCCATTGAGGGTATCC, R: GCTCAGCGAAGTTGGCGAT);

NFKB1 (F: CAGATGGCCCATACCTTCAAAT, R: CGGAAACGAAATCCTCTCTGTT);

NFE2L2 (F: TCCAGTCAGAAACCAGTGGAT, R: GAATGTCTGCGCCAAAAGCTG);

NOX4 (F: TTGGGGCTAGGATTGTGTCTA; R: GAGTGTTCGGCACATGGGTA);

BAX (F: CCCGAGAGGTCTTTTCCGAG, R: CCA-GCCCATGATGGTTCTGAT);

TBP (reference gene) (F: GCCCGAAACGCCGAA-TAT, R: CCGTGGTTCGTGGCTCTCT). Standard curve method was used for the quantification of RNA levels.

2.6. Statistics. All the reported results were reproduced at least three times as independent biological replicates. In flow cytometry, the median of signal intensities was analyzed. The figures show the mean and standard deviation (SD) values. The significance of the observed differences was analyzed with nonparametric Mann-Whitney *U* tests. *p* values < 0.05 were considered statistically significant and marked in figures with (*). Data were analyzed with StatPlus 2007 professional software (<http://www.analystsoft.com/>).

3. Results

The effects of fullerene on human embryonic lung fibroblasts (HELFs) cultured in serum-free media were studied. We used specially formulated serum-free media “Hybris” containing a growth factor that allows cells to proliferate even in the absence of serum [20]. The molecular structure of the fullerene derivative F-828 comprising five residues of 3-phenylpropionic acid and a chlorine atom arranged around one cyclopentadienyl unit on the fullerene cage is shown in Figure 1. Compound F-828 is well soluble in water and culture medium in the presence as well as in the absence of serum. We used F-828 in concentrations ranging from 0.1 to 2.0 μM

for the analysis of its effects on proliferation of HELFs under the conditions of chronic oxidative stress induced by serum deprivation (the model elaborated and reported previously [17]). The fullerene derivative was added to the medium at the initial stage of HELF cultivation, which was as long as 48 hours.

We have revealed that aqueous solutions of this fullerene derivative demonstrate faint dark-red fluorescence when exposed to UV irradiation (300–400 nm). We used this property of F-828 for monitoring of its penetration through membranes and localization in serum-starving HELFs.

3.1. F-828 Penetrates through the Cell Membrane and Accumulates in HELFs. The fullerene localization inside the cell was visualized as dark-red fluorescent regions observed under the excitation at a wavelength of 350 nm (Figure 1, unfixed cells). It has been revealed that F-828 penetrates into the cells under our cultivation conditions and gets localized mainly in the area of cytoplasm adjacent to the cell nucleus. Some signals can be also detected in different areas of the cytoplasm. However, no F-828 fluorescence can be detected inside the nucleus. The dark-red fullerene fluorescence can be only observed in unfixed cells, which were analyzed immediately after a short wash-off of the slides with PBS. The signal intensity is considerably reduced when the cells are fixed with 3% paraformaldehyde. The signals completely disappeared after subsequent washing of the fixed cells with 0.1% solution of Triton X-100 (Figure 1, fixed cells). One can assume that the fullerene nanoparticles interact mainly with the membranes of cells and/or mitochondria. For instance, nanoparticles of soluble fullerene derivative $\text{C}_{60}(\text{OH})_{18-22}$ were earlier shown to interact with biomembranes [21]. Therefore, degradation of membranes as a result of the formaldehyde fixation and washing them away with a detergent solution also leads to the removal of fullerene nanoparticles from the cells. The revealed property of the F-828 nanoparticles to linger poorly in fixed cells after the cells are rinsed with Triton X-100 enables analyzing the cells with the use of antibodies labeled with fluorochrome, with no need to anticipate artefacts emerging due to fluorescence quenching by the nanoparticles.

3.2. F-828 Reduces Endocytosis in HELFs. Endocytosis is one of the common ways of delivery of exogenous compounds into the cell. The formation of novel endosomes is accompanied by an increase in expression of early endosome antigen 1 protein (EEA1), known as an early endosomal biomarker [22]. FITC-labeled antibodies to EEA1 (Figure 2) were used in the assay of early endosome quantity in HELFs using FCA technique. The amount of EEA1 protein increases in the cells under the conditions of serum deprivation. The induced stress leads to intensification of endocytosis. It is notable that approx. 60% of the cells demonstrate upregulated expression of EEA1 (gate R, Figure 2(a)). In the presence of 0.1–1 μM F-828 the percentage of the cells with high level of expression of EEA1 decreases. The mean FL1 signal intensity of the cell is reduced, respectively, by 10–40% (Figure 2(b)).

Thus, the fullerene derivative suppresses endocytosis proportionally to its concentration in the culture medium.

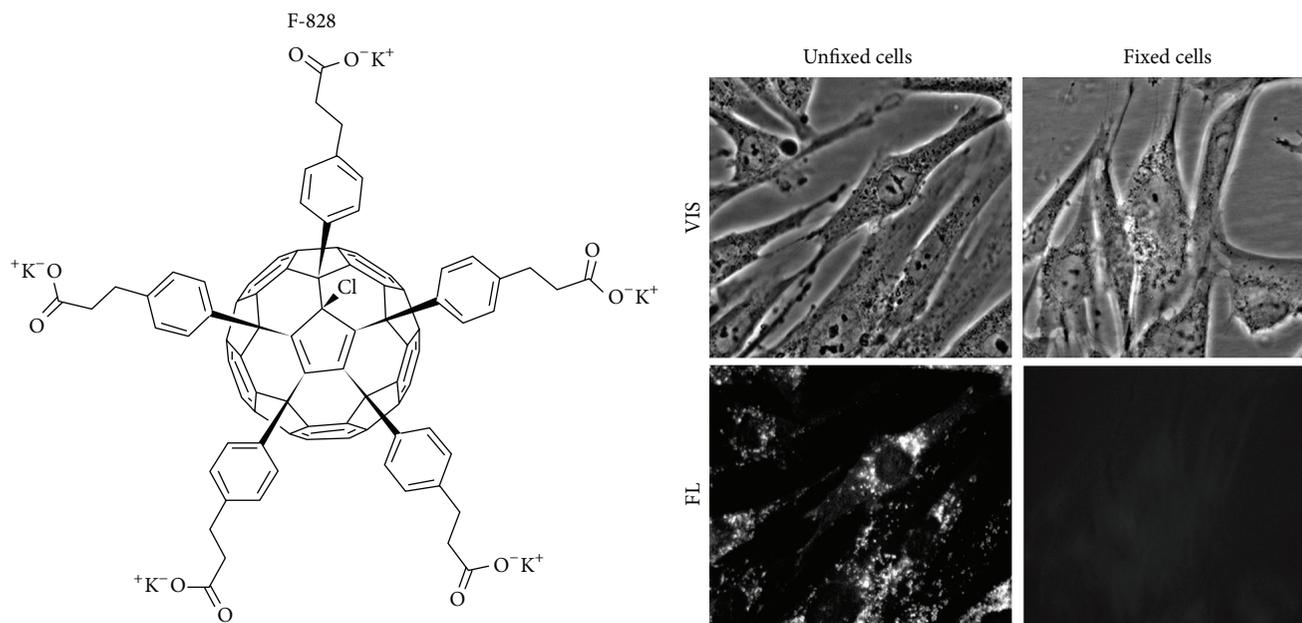


FIGURE 1: F-828 penetrates through the cell membrane and accumulates in HELFs. Optical microscopy image of HELFs treated with F-828 ($0.5 \mu\text{M}$). VIS: visible light; FL: fluorescence, $\lambda_{\text{ex}} = 350 \text{ nm}$. Magnification 40x.

Apparently, the fullerene itself does not penetrate the cells by means of endocytosis mechanism. Fullerenes are known to be able to interact efficiently with the cell membranes and to reach the inner membrane layer due to strongly pronounced amphiphilic properties of these compounds [21].

3.3. F-828 Has an Effect on HELF Cell Count. Figure 3(a) shows photographs of HELFs which were cultivated for 48 h in the presence of 2% FBS, in serum-free “Hybris” medium as well as in similar media containing also $0.5 \mu\text{M}$ and $2.0 \mu\text{M}$ F-828. Figure 3(b) presents the dependence of the cell count in the culture on F-828 concentration in the serum-free medium.

The cell count values increased approximately by a factor of 10.3 ± 0.8 in the HELF populations grown in the “Hybris” media supplemented with 2% FBS, while the growth in the serum-free media led to a modest increase by a factor of 3.2 ± 0.3 . It is rather remarkable that the number of cells was increased by 50% ($p < 0.01$) and became higher than that of the control experiment when similarly cultured serum-free cells had been exposed to F-828 ($0.2\text{--}0.5 \mu\text{M}$). However, no effects like that were observed in the presence of larger concentrations of F-828 (Figure 3(b)). A notable decrease in the cell counts was observed when using $1.0 \mu\text{M}$ of F-828. Moreover, the cells virtually did not divide in the presence of $2.0 \mu\text{M}$ F-828 (Figure 3(a)).

Cell count in culture depends on the rates of cell division and cell death. Therefore, we studied the influence of the fullerene derivative on the cell cycle and death of cells in order to explain the obtained results.

3.4. F-828 Affects Proliferative Activity of the HELFs. The cell cycle-related effects induced by F-828 were studied in HELFs

that were harvested 48 hours after addition of the fullerene derivative to the media (Figure 4). The cells were stained with antibodies specific to the proliferation markers Ki-67 and PCNA [23, 24] and enumerated by FCA. Additionally, the cell populations were also counted after DNA-specific propidium iodide (PI) treatment. Figure 4(b) shows the distribution of the cells with various Ki-67 contents. The serum starvation leads to a decrease in the proliferating cell fraction as compared to the FBS-supplemented control cells (Figure 4(b)). When serum-free HELFs were exposed to $0.1\text{--}0.25 \mu\text{M}$ F-828, the fractions of Ki-67-positive cells were increased as compared to the serum-free HELF controls. On the contrary, introduction of $0.5\text{--}1.0 \mu\text{M}$ of F-828 resulted in a decrease in the size of the Ki-67-expressing cell fractions in serum-free HELFs.

A similar analysis was performed for PCNA. When cultivating culture medium was augmented with 2% FBS, the proportion of PCNA-positive proliferating cells increased as compared to the cultures grown in serum-free media. When the cells were exposed to $0.1\text{--}0.75 \mu\text{M}$ of F-828, the proportions of PCNA-positive cells were increased as compared to the serum-free HELF controls (Figure 4(c)). An increase in the F-828 concentration resulted in less pronounced effects. Interestingly, the proportion of PCNA-positive cells increased when the cells were exposed to 0.5 and $0.75 \mu\text{M}$ of F-828, thus producing an overall picture, which is substantially different from that of Ki-67 staining (Figure 4(b)).

The increase in the HELF proliferative activity in the presence of small amounts of F-828 is confirmed by the data on the DNA content in the cells (Figure 4(a)). Fullerene derivative F-828 used in concentrations of $0.1\text{--}0.25 \mu\text{M}$ induced an increase in the number of cells in the S-phase

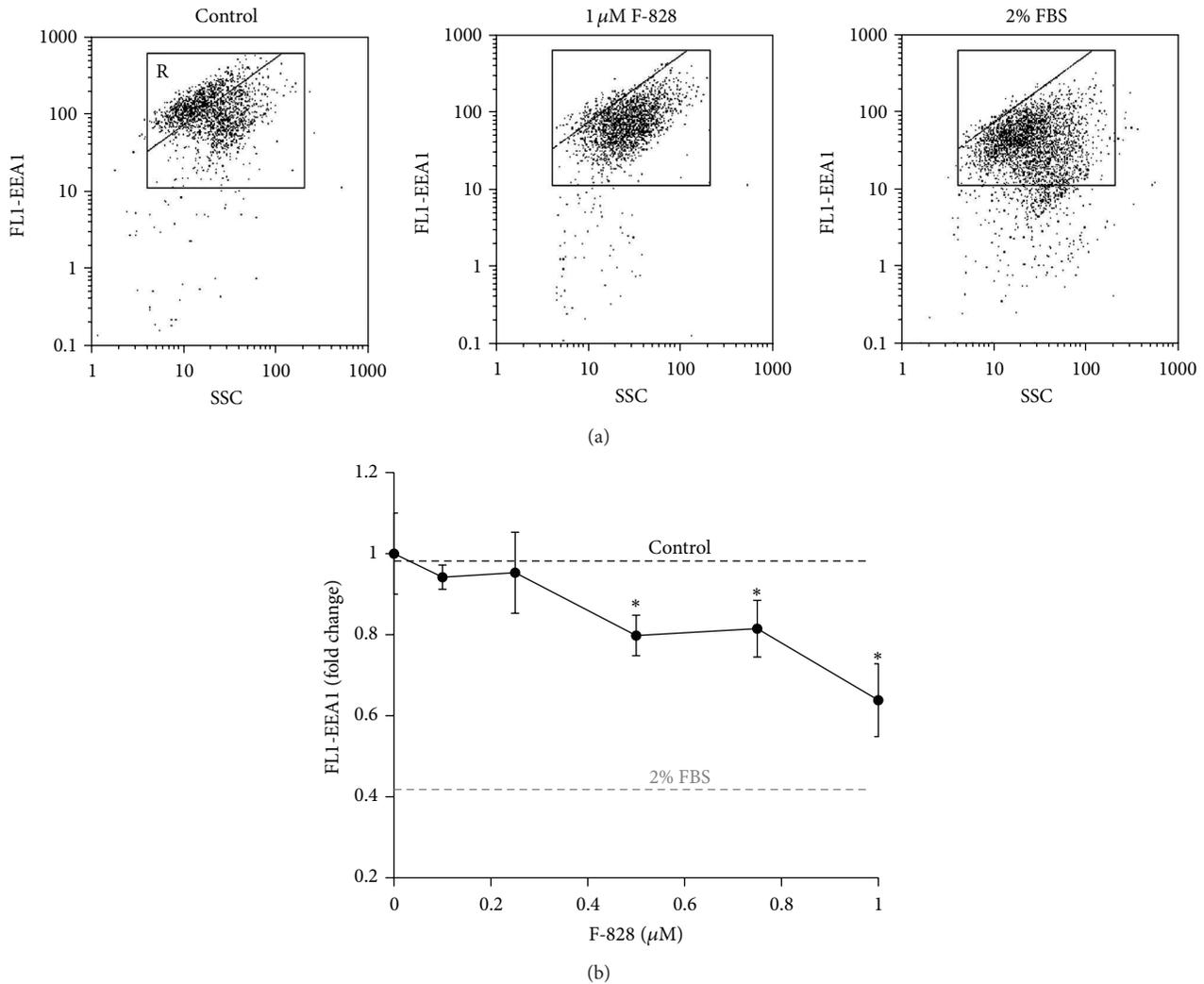


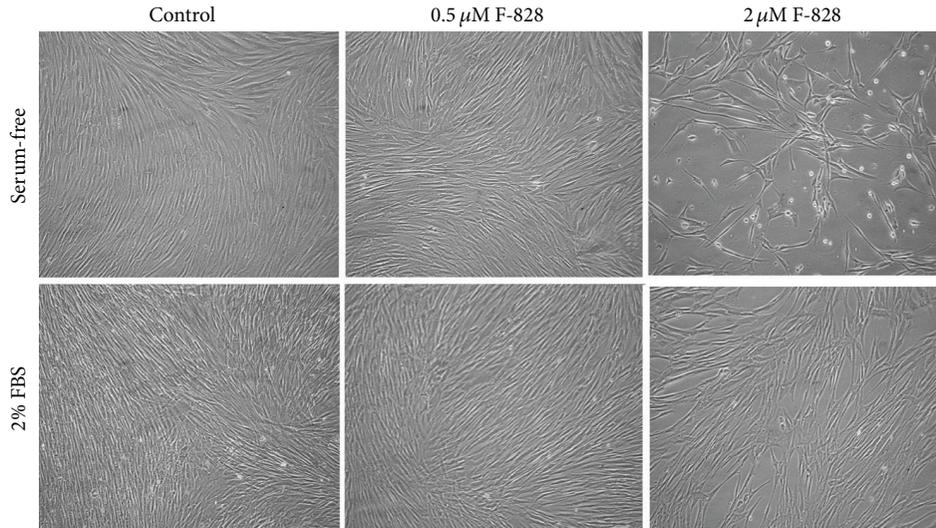
FIGURE 2: F-828 reduces endocytosis in HELFs. (a) (FCA): the FLI-EEA1 versus SSC plots. Gate R encircles the fraction of HELFs with elevated values of FLI-EEA1. (b) (FCA): dependence of the median values of FLI-EEA1 signals on the F-828 concentration. Concentrations of the fullerene derivative F-828 added to the medium are indicated in the graph.

($p < 0.01$). The ratio of the cells in the G0/G1 cycle phase decreases ($p < 0.05$).

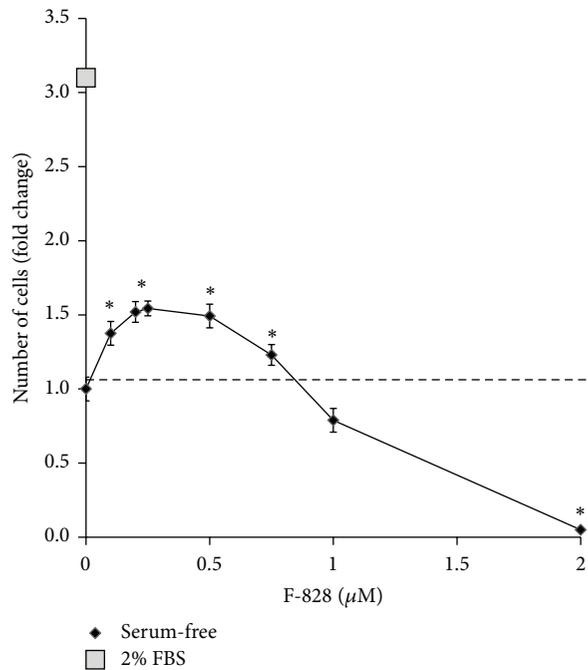
Propidium iodide staining for DNA content has revealed that HELF population grown in serum-free media shows an increased contribution from the G2/M cells (23% versus 7% for the medium with 2% FBS), Figure 4(a). An exposure of the cells to 0.1–0.25 μM F-828 leads to a decrease in the number of G2/M cells by ~50%. This fact suggests that low fullerene concentrations lead to cancelling the G2/M arrest of a considerable fraction of cells caused by cultivation under the specified stress conditions. The cells exposed to 0.5–1.0 μM F-828 show similar proportions of the G2/M cells to those of control serum-free cells.

3.5. F-828 Shifts the Levels of Autophagy and Death of the HELFs. Low cellular nutrient levels can activate autophagy, which acts to restore metabolic homeostasis through the

degradation of macromolecules to provide nutrients. Oxidative stress can boost the autophagy considerably. Autophagy, in turn, contributes to the reduction of oxidative damage by consuming the oxidized substances with their subsequent degradation [25]. Under the conditions of serum starvation stress HELFs demonstrate a significant activation of autophagy. A protein known as beclin is one of the autophagy markers. In order to perform an assay of this protein, antibodies to beclin and FAC technique were used. The quantity of beclin in the cells cultivated in the serum-starving medium is increased by a factor of 2 as compared to the cells cultivated in the presence of 2% FBS (Figure 5(a)). The introduction of the fullerene derivative to the culture medium in concentrations of 0.1–1.0 μM leads to a decrease in the amount of beclin in the cells by a factor of 1.1–2.0, respectively. We have also detected autophagosomes (puncta) in the cytoplasm after immunolabeling with the rabbit polyclonal antibody against LC3 [26]. The introduction of F-828 to the culture medium



(a)



(b)

FIGURE 3: F-828 has an effect on HELF cell count. (a) Microscopy image of HELFs treated with F-828 (magnification 20x). Top row stands for serum-free medium; bottom row shows 2% FBS added. Fullerene concentrations are indicated in the figure. Control means fullerene F-828 was not added. (b) Dependence of the cell count in the culture on the F-828 concentration.

at a concentration of $0.5 \mu\text{M}$ leads to a decrease in the amount of puncta in the cytoplasm of serum-starving HELFs (Figure 5(b)). Apparently, F-828 suppresses autophagy in the serum-starving HELFs.

To evaluate DNA damage degree in treated and control cells, we quantified the number of cells in the subG1 phase (Figure 4(a)(2)). Under the stress conditions the subG1 cell fraction was increased by a factor of 2 compared to the control cells cultivated in the presence of 2% of serum (8% versus 4%). The cells exposed to $0.1\text{--}0.25 \mu\text{M}$ F-828 showed

decreased contents of the subG1 fraction in comparison with the control serum-free cells. The cell cultures exposed to $1.0 \mu\text{M}$ F-828 did not demonstrate further decrease in the subG1 cell fraction.

We also investigated the appearance of cells exhibiting nuclear chromatin condensation using fluorescent microscopy in order to evaluate the damage degree in treated and control HELFs (Figure 5(d)). The Hoechst 33342 staining revealed that approx. 10% of the cells grown in the serum-free medium show typical morphological hallmarks of apoptosis,

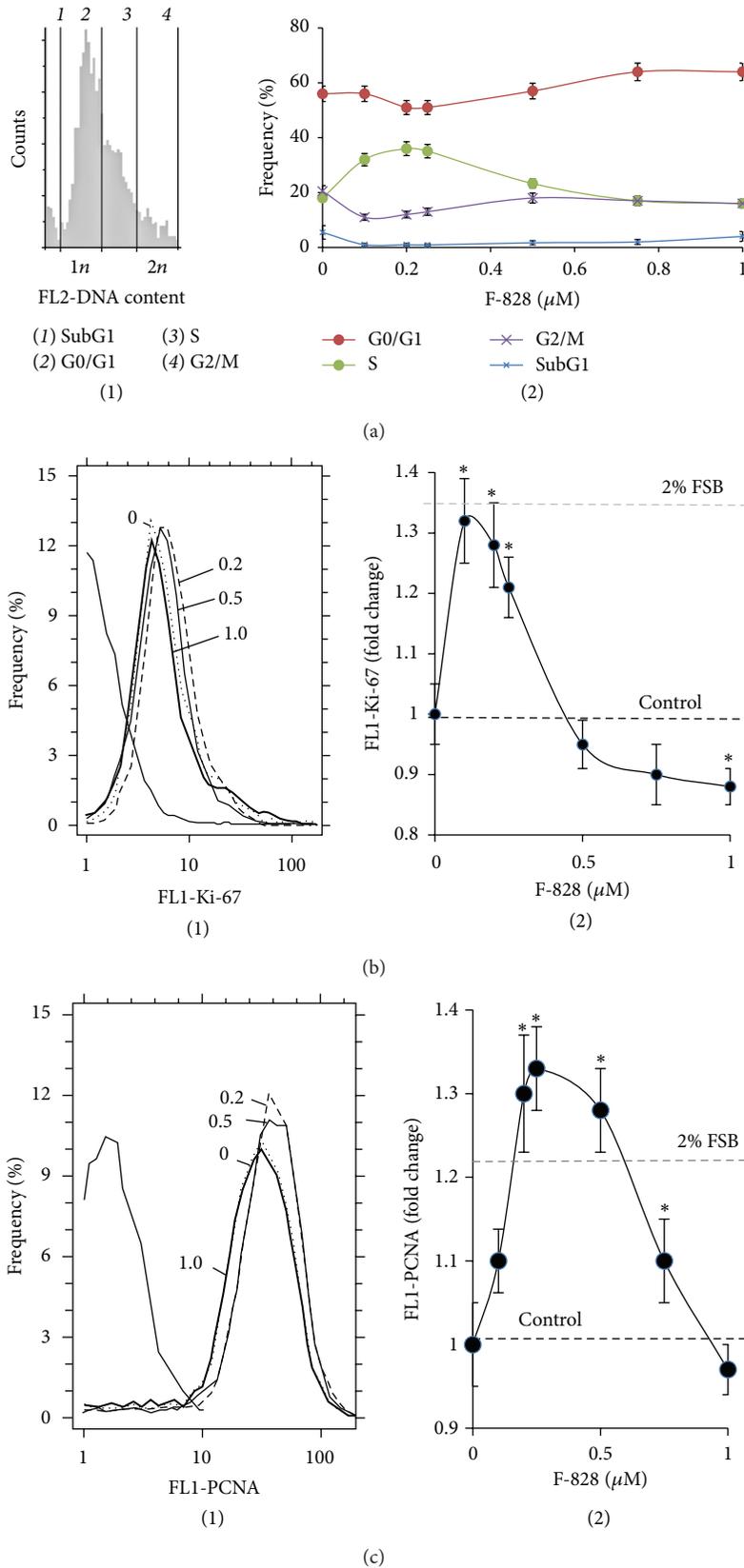


FIGURE 4: F-828 affects proliferative activity of HELFs. (a) (FCA): (1): distribution of fluorescence intensities of the cells stained with PI; (2): shifts of the proportions of cells with an amount of DNA corresponding to the G1-, S-, and G2/M-phases of cell cycle depending on the F-828 concentration in the culture medium. (b) (FCA): (1): distribution of the cells treated with F-828 according to the FL1-Ki-67 signal strength; (2): dependence of the median values of the FL1-Ki-67 signals on the fullerene concentration. (c) (FCA): (1): distribution of the cells treated with F-828 according to the FL1-PCNA signal strength; (2): dependence of the median values of FL1-PCNA signals on the F-828 concentration.

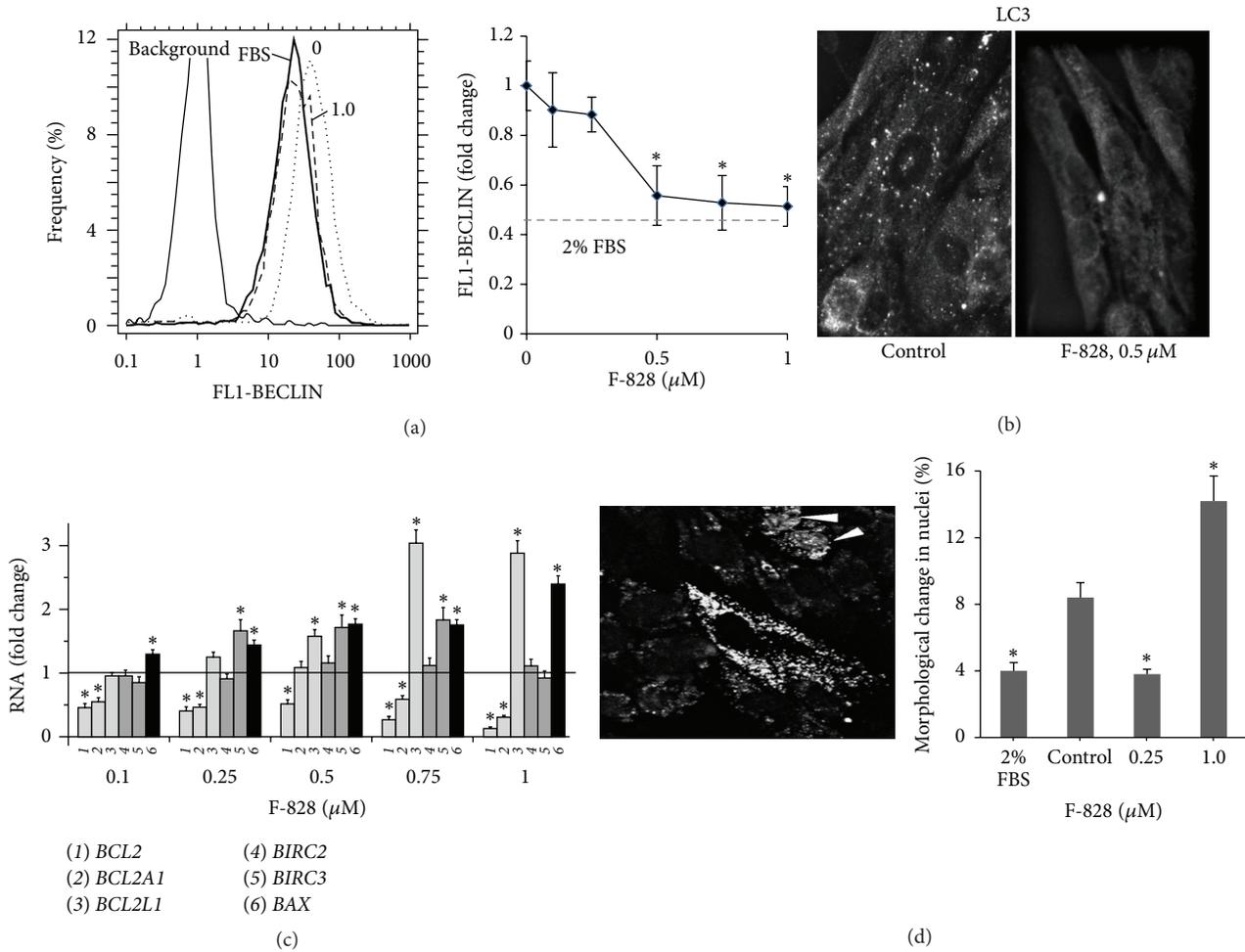


FIGURE 5: F-828 shifts the levels of autophagy and mortality of HELFs. (a) (FCA): distribution of the cells treated with F-828 according to the FL1-BECLIN signal strength; dependence of the median values of FL1-BECLIN signals on the F-828 concentration. (b) (Fluorescence microscopy): changes in the levels of LC3-positive puncta in the HELFs cells treated with $0.5 \mu\text{M}$ of F-828. (c) (qRT-PCR): changes in the levels of mRNAs encoding BCL2A1, BCL2, BCL2L1, BIRC2, BIRC3, and BAX in HELFs. (d) (Fluorescence microscopy): morphological changes in the HELFs nuclei. Cells were stained with Hoechst 33342. Photo: HELFs in serum-free media ($1 \mu\text{M}$ of F-828). Arrows point towards the cells, whose nuclei were entered by Hoechst 33342. The histogram presents the proportion of the cells with modified nuclei.

including nuclear chromatin condensation. The percentage of the damaged nuclei decreased when HELFs were exposed to $0.25 \mu\text{M}$ F-828. An exposure of the cells to $1.0 \mu\text{M}$ F-828 produced an opposite effect characterized by an elevated content of the damaged nuclei.

We analyzed changes in the expression patterns of five genes belonging to BIRC and BCL families, which participate in the antiapoptotic cell response (Figure 5(b)), and also BAX gene involved in the apoptosis induction. It was revealed that the level of the BAX mRNA increased in the presence of F-828. F-828 led to decreased expression of the antiapoptotic genes BCL2 and BCL2A1 in the cells. In parallel, the level of expression of the three other antiapoptotic genes, BCL2L1, BIRC2, and BIRC3, remains the same or becomes increased. The fact of different regulation of the expression of antiapoptotic genes in the presence of fullerene requires a special study.

3.6. F-828 Reduces the ROS Level in Serum-Starving HELFs.

Cultivation of HELFs in a serum-starving medium is known to result in a notable increase in the ROS level (Figure 6(a) and [17]). To study the possible influence of the fullerene on the intracellular ROS levels, the ROS were measured using dichlorodihydrofluorescein diacetate (H2DCFH-DA) dye. The experiment was carried out using a plate reader with a thermocontrolled platform. Figure 6(a) shows the results of the ROS assay performed on living cells. Figure 6(b) indicates relative values of the constants determined from the slopes of the straight lines that approximate dependence of DCF fluorescence intensity versus time (Figure 6(a)).

Fullerene derivative F-828 added at $0.2\text{--}2.0 \mu\text{M}$ suppresses the ROS formation in the cells. One can notice two discrete stair-like steps on the curve showing the dependence of the DCF formation rate on the fullerene concentration. When the fullerene concentration increases from 0.1 up to

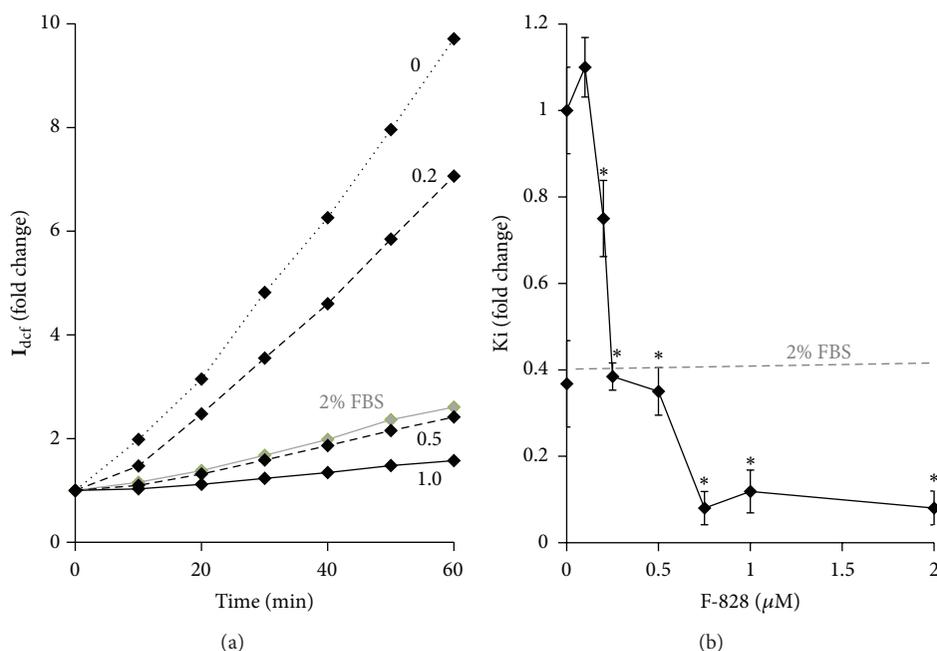


FIGURE 6: F-828 reduces the ROS level in serum-starving HELFs. (a) (Plate reader): the change of DCF fluorescence depending on the duration of the cell incubation in the presence of $10 \mu\text{M}$ of H₂DCFH-DA. (b) Dependence of the DCF synthesis rate constant in serum-starving HELFs on F-828 concentration. A square denotes DCF synthesis rate in the presence of 2% FBS.

$0.25 \mu\text{M}$, the reaction rate decreases by 60% and then stays almost constant in the range of concentrations between 0.25 and $0.5 \mu\text{M}$. An increase in the fullerene concentration up to $0.75 \mu\text{M}$ decreases further the DCF production rate by more than 30%.

There are several possible mechanisms, which can be responsible for the observed decrease in the ROS level in the presence of fullerene derivative. For instance, the fullerene derivative can harvest ROS directly *via* radical addition pathway. It is also possible that the fullerene derivative influences the enzymes and transcription factors responsible for ROS production and disposal in the cell.

3.7. F-828 Causes a Decrease in the Level of NOX4 Protein in Serum-Starving HELFs. It has been shown that production of cellular ROS is related to the action of NAD(P)H-oxidase type of enzymes, predominantly those encoded by NOX gene family [27]. NAD(P)H-oxidase 4 (NOX4) has been recognized recently as a major source of ROS in HELFs and it was shown to be implicated in the fibrogenic response to lung injury [28]. In living cells, NOX4 catalyzes the reaction responsible for the hydrogen peroxide formation.

The level of NOX4 protein was determined in HELFs using FCA and antibodies specific to NOX4 (Figure 7). The population of serum-starving HELFs comprises two cell fractions: one with elevated NOX4 (gate R on the plot of FL1-NOX4 versus SSC) representing about 60% of the total amount of the cells and the other with a lower NOX4 content. For comparison, HELFs cultivated in the presence of 2% FBS contain just 7% of cells with high level of NOX4 protein.

The mean level of NOX4 protein in HELFs cultivated under the serum starvation conditions is 3 times higher than that in the cells grown in the medium containing 2% of FBS (Figure 7(a)). Interestingly, the rate of DCF production in the serum-starved cells also appeared to be 3 times higher than in the control cells, which were cultivated in the presence of 2% of serum (Figure 6).

The addition of F-828 to the serum-free medium in concentrations of 0.1 – $1.0 \mu\text{M}$ induced a notable decrease in the content of NOX4 protein in the cells. The most prominent response was observed in the presence of $0.5 \mu\text{M}$ of F-828 when the average intensity of FL1-NOX4 decreased by 60% and the fraction of cells with high NOX4 level (gate R) decreased down to a remarkable value of 2%.

We also analyzed the evolution in the content of NOX4 mRNA in serum-starving HELFs (Figure 7(b)). It has been revealed that addition of the fullerene derivative to the medium led to an increase in the NOX4 mRNA content by a factor of 1.4 – 1.6 at any of the studied F-828 concentrations except for 0.1 and $1 \mu\text{M}$. This observation suggests that NOX4 gene expression is regulated presumably at the posttranscriptional level in the fullerene-treated cells.

Finally, we can emphasize that the revealed strong reduction of the content of NOX4 protein in the cells is one of the key factors responsible for the decrease of ROS in serum-starving HELFs in the presence of fullerene.

3.8. F-828 Reduces NRF2 in Serum-Starving HELFs. The NF-E2 related factor 2 (NRF2) regulates constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling [29, 30].

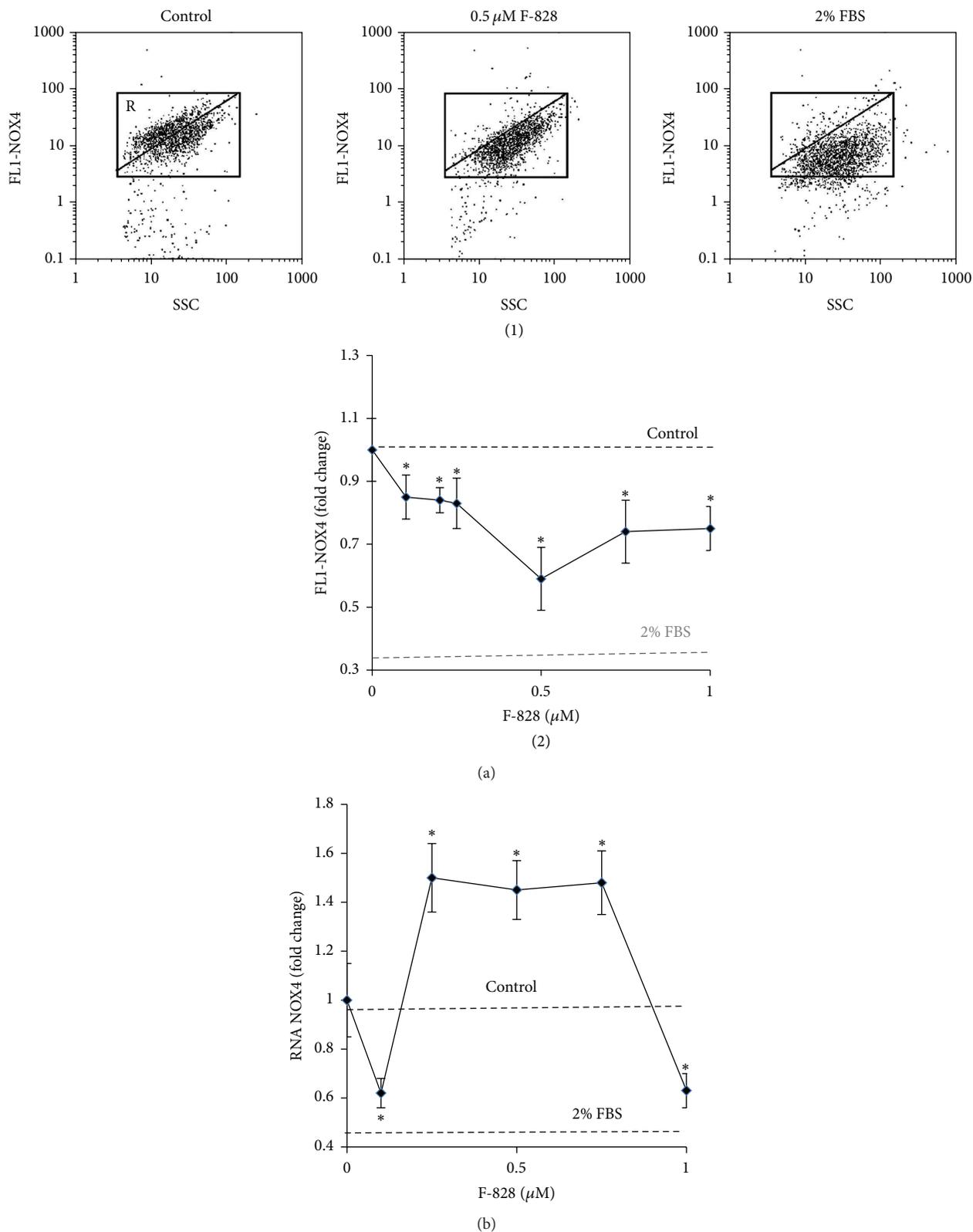


FIGURE 7: F-828 entails a decrease in the level of NOX4 protein in serum-starving HELFs. (a) (FCA): (1): the FL1-NOX4 versus SSC plots. Gate R encircles the fraction of HELFs with elevated values of FL1-NOX4; (2): dependence of the median values of the FL1-NOX4 signals on the fullerene concentration. (b) (qRT-PCR): changes in the levels of mRNAs encoding *NOX4* in HELFs.

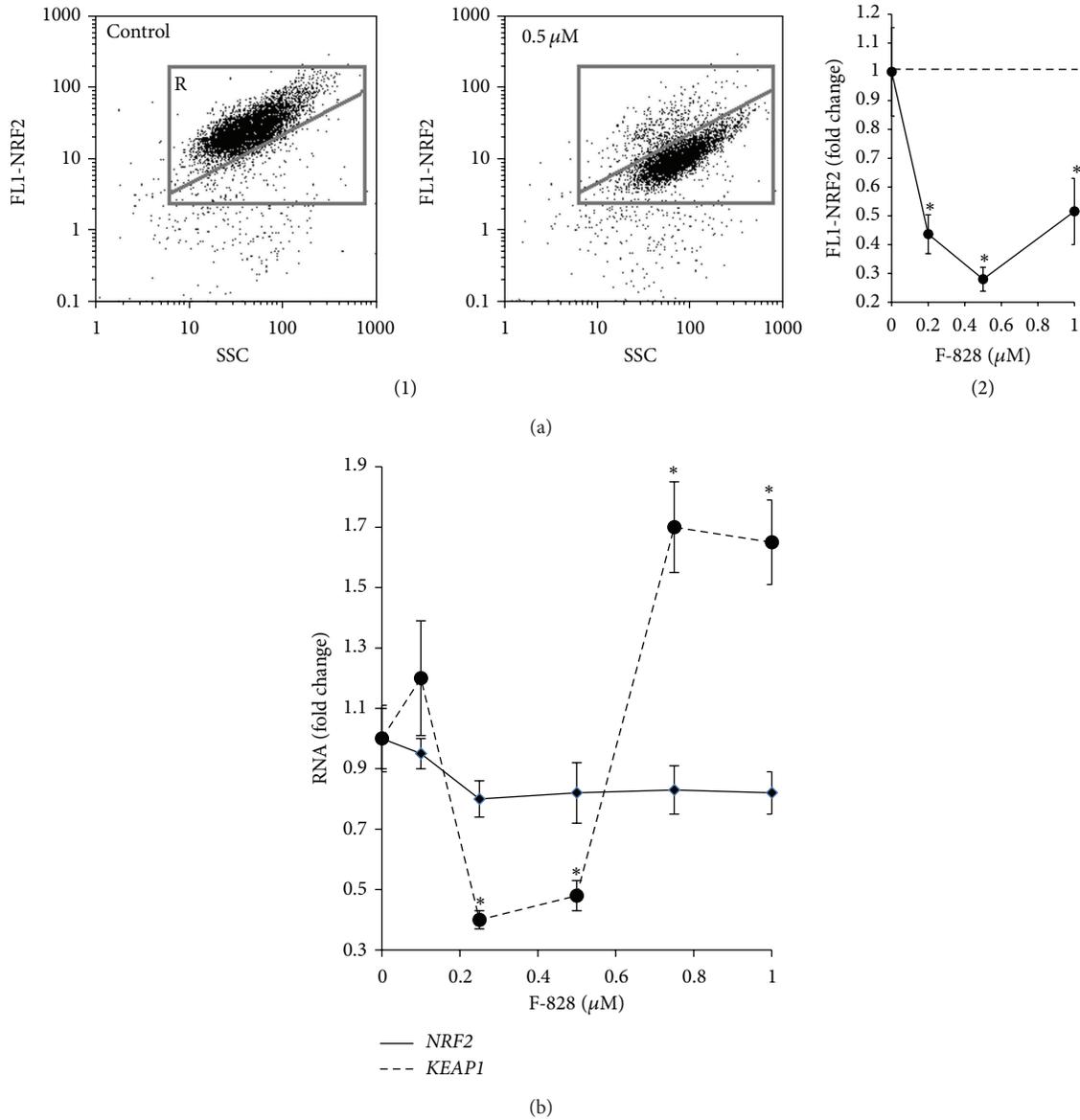


FIGURE 8: F-828 reduces NRF2 in serum-starving HELFs. (a) (FCA): (1): the FL1-NRF2 versus SSC plots. Gate R encircles the fraction of HELFs with elevated values of FL1-NRF2; (2): dependence of the median values of FL1-NRF2 signals on the fullerene concentrations. (b) (qRT-PCR): changes in the levels of mRNAs encoding *NRF2* and *KEAP1* in HELFs.

NRF2 controls ROS production involving mitochondria and NADPH oxidase [31]. NOX4-NRF2 imbalance is considered as an origin of pathological fibrosis [28]. NRF2 factor is expressed in serum-starving HELFs at the same level as in the cells growing in the presence of 2% of serum. However, this factor is not active and it is localized only in cytoplasm of the serum-starving HELFs [17]. In the cells cultivated in presence of serum NRF2 is located in the nucleus. This fact indicates an activation of NRF2 [17]. The content of NRF2 was measured using FCA and antibodies specific to NRF2 (Figure 8).

We observed that cultivating HELFs in the serum-starving medium in the presence of fullerene leads to a significant decline of NRF2 protein level in the cells (Figure 8(a)). Using fluorescence microscopy, it was shown that

NRF2 is still located solely in the cell cytoplasm; that is, it is inactive (data not shown). The observed lowering of the NRF2 protein content in the serum-starving HELFs in the presence of fullerene could be considered as a consequence of a considerable reduction in the level of ROS and NOX4 protein. It was previously shown that intracellular NOX4 reduction was accompanied by a decrease in the amount of NRF2 transcription factor [32, 33].

We also analyzed the quantity of mRNA encoding NRF2 protein in serum-starving HELFs (Figure 8(b)). The introduction of fullerene derivative to the medium resulted in a slight decrease in *NRF2* mRNA in the whole range of F-828 concentrations involved in our experiments. Presumably, the regulation of *NRF2* gene expression, as well as that of *NOX4*

gene, occurs at some posttranscriptional level in serum-starving cells treated with the fullerene derivative. NRF2 is known to be negatively regulated by cytoplasmic Kelch-like ECH-associated protein 1 (Keap1). We observed an increase in the concentration of mRNA for KEAP1 protein at 0.75 and 1 μM of F-828.

Thus, NRF2 transcription factor is not active in the serum-starving HELFs, which were cultivated in the presence of fullerene derivative. Therefore, NRF2 is neither a cause nor a factor of the observed significant decrease in ROS level in serum-starving HELFs cultivated in the presence of fullerene.

3.9. F-828 Affects the Content of Phosphorylated Form of H2AX Histone in Serum-Starving HELFs. One of the methods of revealing the DNA DSBs is based on the fact that a highly conservative histone protein (H2AX) is phosphorylated at the residue of serine 139 in the site where the development of DNA break starts with the participation of ATM, ATR, and DNA-PK kinases [34]. The reaction rapidly propagates engaging hundreds to thousands of molecules of H2AX, which can comprise up to several megabases of the chromatin DNA flanking the DSB site. Bounds with the labeled antibodies and phosphorylated histones called γH2AX foci are available for visualization in the cellular nucleus; their accumulation in great amounts indicates the beginning of the development of apoptosis in the cellular population.

The image of the fixed serum-starving HELFs stained with FITC-labeled antibodies specific to γH2AX is shown in Figure 9(a). The performed analysis revealed three types of cells: those without any label (3, Figure 9(a)), cells with small quantity of γH2AX (2), and those with a large number of overlapping spots due to the labeled γH2AX (1). FCA procedure (Figure 9(b)) also allowed us to find areas corresponding to these three cell fractions on the FL1 (γH2AX), SSC plot. R1 fraction (12% of the entire population) is represented by the cells with a high content of γH2AX , R2 fraction (39%) corresponds to the cells with a lower γH2AX content, and R3 fraction represents the cells, which have no γH2AX (51%). Thus, almost a half of serum-starving HELFs express detectable amounts of γH2AX .

The R1 fraction consists of the cells with a very high gamma-focus level. Apparently, they are cells undergoing apoptosis. In the presence of serum, the R1 fraction size reduces significantly from 12% down to 4%.

We registered elevation of the average number of γH2AX in the cells by a factor of 1.8–2.2 in the presence of 0.1, 0.5, 0.75, and 1.0 μM of the fullerene derivative (Figure 9(b)(2)). When the concentration of F-828 was equal to 0.2 or 0.25 μM , the average number of γH2AX in the cells was decreased by a factor of 1.5–2.0. One can notice in the histogram shown in Figure 9(b) that the cell counts corresponding to R1 and R2 fractions considerably decreased.

3.10. Exposure of the Cells to F-828 Stimulates an Increase in the Activity of NF- κB Factor. HELFs express relatively low amounts of NF- κB when they are grown in the presence of 2% serum [17]. The factor is inactive and located solely in cytoplasm. It can be assumed that serum contains some molecule(s) that strongly inhibit(s) NF- κB activation [35].

In serum-starving cells, the level of NF- κB (p65) increases to a substantial degree along with the translocation of NF- κB (p65) closer to the nucleus [17, 35]. Thus, the serum-starving stress induces the NF- κB activity in the cells. It is well known that NF- κB (p65) is activated by phosphorylation, which plays a key role in the regulation of its transcriptional activity and is associated with nuclear translocation of the factor [36]. Flow cytometry was applied for quantification of HELFs that contain phosphorylated Ser529 (p65) [17]. It has been shown that serum-starving entails a substantial increase in the proportion of the cells that contain phosphorylated Ser529 in p65, thus confirming that the transcriptional factor is active in these cells.

The introduction of the fullerene derivative F-828 in concentrations of 0.1–1.0 μM provokes a 2- to 3-fold increase of total p65 protein amount, while the content of its phosphorylated form Ser529 (p65) becomes 2.5 to 3.5 times higher. The maximum effect of F-828 was observed at a concentration of 0.25 μM (Figures 10(c) and 10(d)).

The elevation of p65 protein is also confirmed by fluorescence microscopy (Figure 10(b)). The fluorescence intensity of HELFs nuclei stained with antibodies specific to p65 gets considerably increased when the cells are cultivated in the presence of fullerene. It is notable that the protein in this case is located only in the cell nuclei.

4. Discussion

Here we studied the influence of water-soluble fullerene F-828 on human embryo lung fibroblasts cultivated under the conditions of chronic oxidative stress. The oxidative stress in HELFs was induced by serum deprivation. It is known that cell cultivation in a serum-starving medium drastically boosts the ROS production, particularly in mitochondria [37].

Previously, we described in detail the changes occurring in the HELFs cell culture under serum deprivation conditions [17]. Deficiency of nutrients in the environment slows down the cell proliferation and increases the proportions of SubG1/G0, G2/M, and apoptotic cells. Additionally, the number of double-strand DNA breaks grows up considerably in the cells. Intensification of endocytosis is observed in the cells as a response towards low-nutrient environment conditions. Elevated endocytosis is accompanied by a high level of EEAI protein, which is a marker of early endosomes (Figure 2).

The induction of oxidative stress is promoted by increased NOX4 gene expression leading to few times higher concentrations of the corresponding protein than those in the control cells (Figure 7). At the same time, the activity of transcription factor NRF2 is blocked. The level of this protein, which is the master switch of the cellular antioxidant response, is decreased, and moreover all the protein is located in cytoplasm. In the control cell culture cultivated in the presence of 2% serum, this factor is active and located in the cell nuclei [17]. An imbalance between the elevated NOX4 gene expression and blocked NRF2 activity is a specific feature of this model system.

Oxidative stress can also stimulate autophagy, which is a cellular process used to recycle the cytoplasmic components.

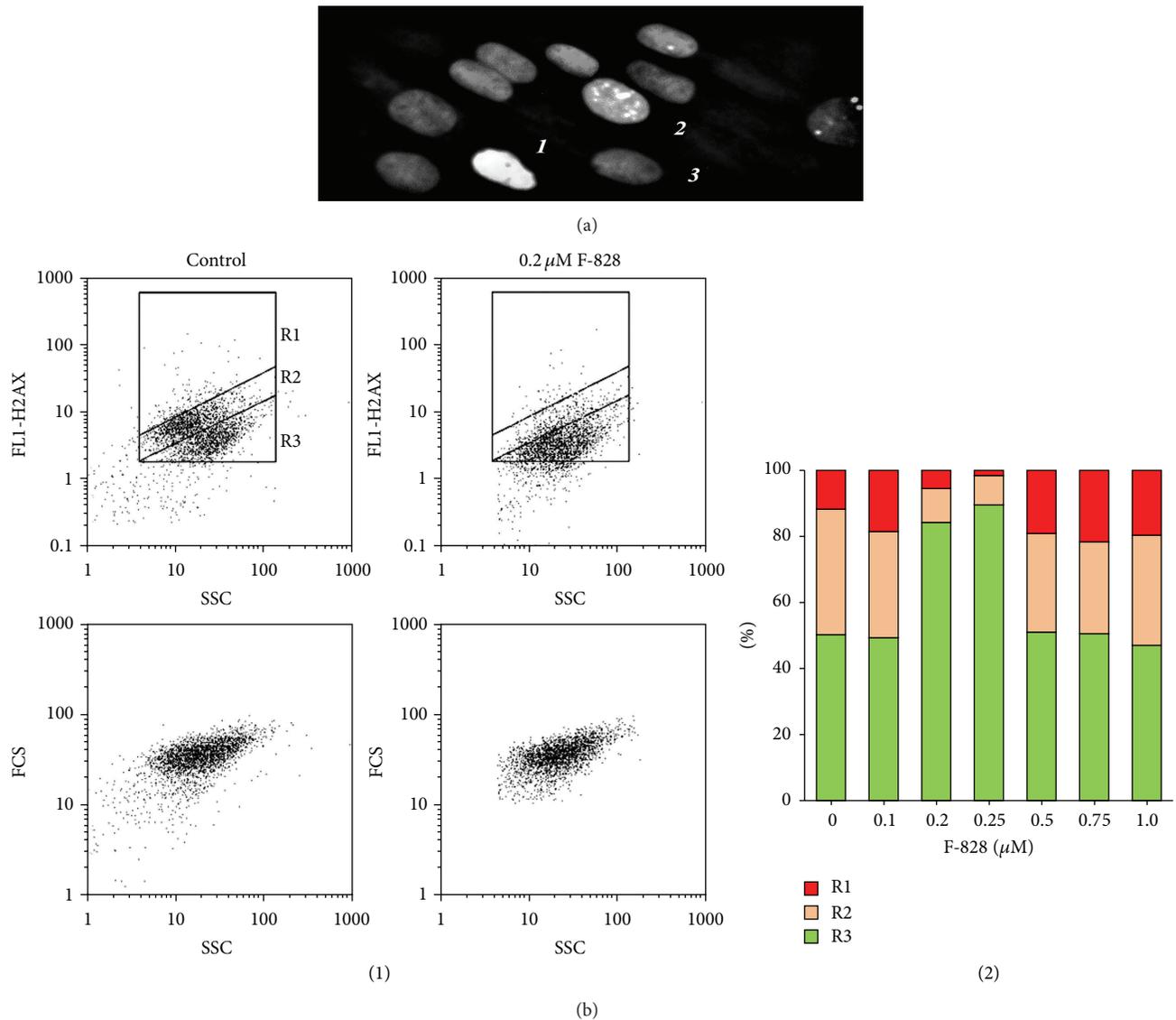


FIGURE 9: F-828 shifts the content of phosphorylated form of H2AX histone in serum-starving HELFs. (a) (Fluorescence microscopy, $\times 40$): cells were processed for immunofluorescence staining with anti- γ H2AX antibody and PI. Detected types of nuclei are denoted with digits: 1: nucleus with multiple dsDNA breaks; 2: nucleus with a few dsDNA breaks; 3: nucleus without gamma-foci. (b) (FCA): (1): the FL1- γ H2AX versus SSC plots. Gates R1, R2, and R3 encircle the fractions of HELFs with various content of γ H2AX. (2): changes of the proportions of three cell fractions in the HELF cell pool depending on the F-828 concentration.

Certain cellular stresses including nutrient depletion are known to induce autophagy [38]. Autophagy induced in the serum-starving HELFs is reflected in a twice higher concentration of autophagy marker Beclin 1 and higher number of LC3 puncta than in the control cells (Figures 5(a) and 5(b)). Recently, autophagy and Nrf2 were shown to be interconnected *via* a direct interaction between p62 (an autophagy adaptor protein) and Keap1 (the Nrf2 substrate adaptor for the Cul3 E3 ubiquitin ligase). Dysregulation of autophagy was shown to result in prolonged Nrf2 activation in a p62-dependent manner [39]. In the case of the investigated system (serum-starving HELFs), the elevated autophagy was followed by the expected decrease in the NRF2 activity.

Serum-starving stress is known to enhance the NF- κ B activity in the cells [17, 35]. Activation of NF- κ B can prevent cell death and promote cell growth. We also found that the serum-starving HELFs demonstrated elevated contents of total protein p65 and its active phosphorylated form compared with HELFs cultivated in the presence of 2% FBS. The observed localization of NF- κ B in cell nuclei confirms its activation. It was revealed previously that NF- κ B- and Nrf2-pathways can intersect [14–16]. Generally, an activation of either factor entails blocking the activity of the other one. Indeed, the factor NF- κ B is considerably activated, while the activity of NRF2 is blocked in serum-starving HELFs.

Considering the chemical structures of C₆₀ fullerene one can assume that this compound might demonstrate high

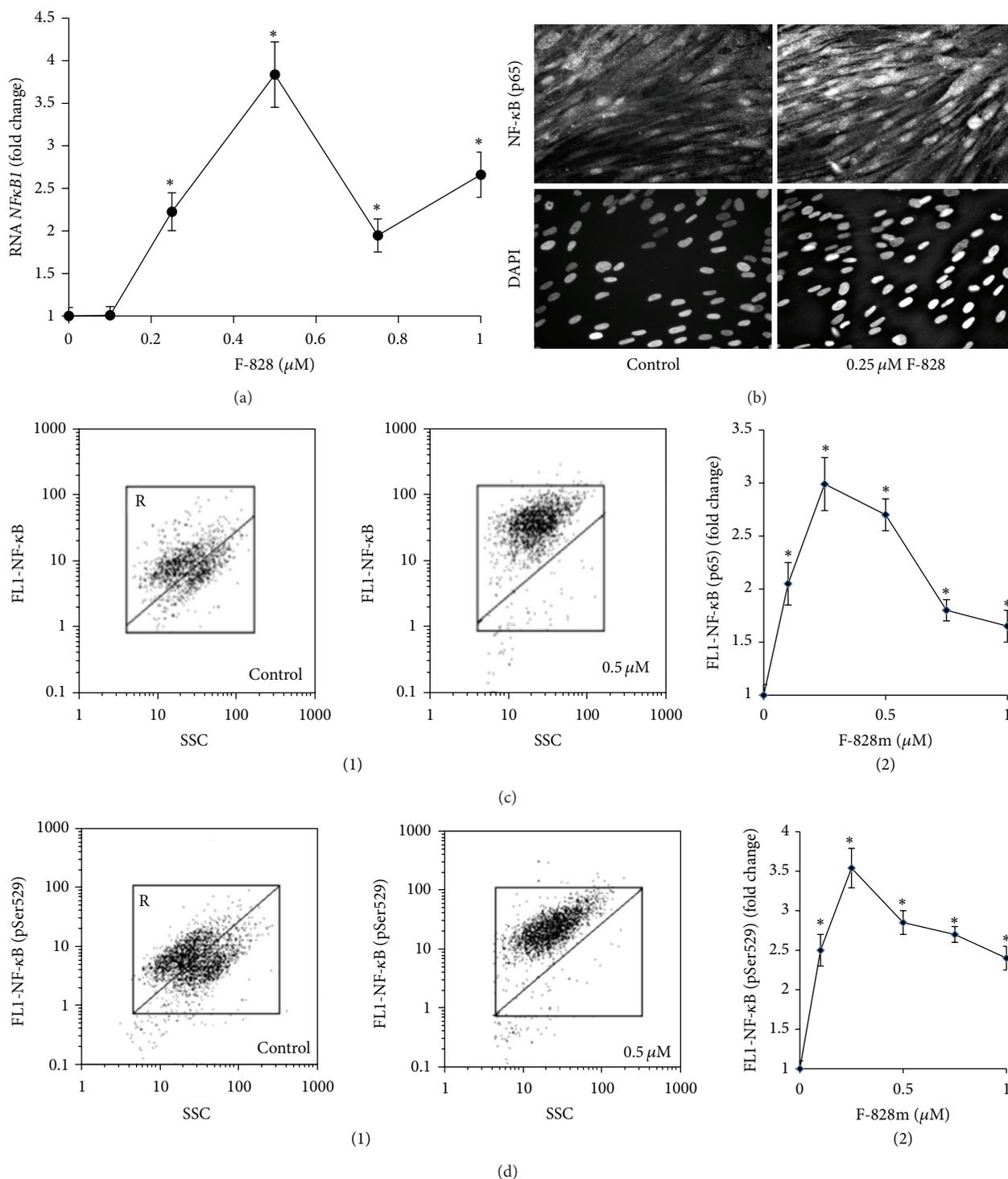


FIGURE 10: F-828 promotes an increase in the activity of NF-κB. (a) (qRT-PCR): changes in the levels of mRNAs encoding *NFκBI* in HELFs. (b) (Fluorescent microscopy): photo of the cells stained with anti-p65 (FITC) antibodies and DAPI, ×40. (c) (FCA): (1): the FL1-NF-κB (p65) versus SSC plots. Gate R encircles the fraction of HELFs with elevated values of FL1-NF-κB (p65). (2): dependence of the median values of FL1-NF-κB (p65) signals on the fullerene concentration. (d) (FCA): (1): the FL1-NF-κB (pSer529) versus SSC plots. Gate R encircles the fraction of HELFs with elevated values of FL1-NF-κB (pSer529). (2): median values of the FL1-NF-κB (pSer529) signals as a function of the fullerene concentration.

antioxidant capacity. The ability of the fullerene to bind and neutralize ROS directly can lead to the reduction of the ROS level in the serum-starving HELFs and improvement of cell culture's viability. Indeed, serum-starving HELFs appeared to be very sensitive to the low fullerene concentrations (0.2 to 1.0 μM), which indeed decreased the ROS level remarkably. The most pronounced effects were observed at a F-828 concentration above 0.5 μM (Figure 6). It is rather remarkable that the fullerene derivative not only binds ROS directly, but also affects signaling pathways involved in ROS production and scavenging. In particular, introduction of F-828 reduced the level of the NOX4 protein (Figure 7(a)).

The fullerene derivative reduced even to a greater extent the content of NRF2 factor, which is the master switch of antioxidant response (Figure 8(a)). These findings are very distinct from the previous report where a mixture of water-soluble fullerenols was shown to increase the activity of NRF2 factor [9]. It was assumed that $\text{C}_{60}(\text{OH})_{24}$ attenuates oxidative stress-induced apoptosis *via* augmentation of Nrf2-regulated cellular antioxidant capacity [9]. We have also shown that a decrease in the NRF2 activity is accompanied by reduced autophagy as concluded from the evolution of the Beclin 1 and LC3 puncta content in the cells. This is rather unexpected result, which warrants further investigations. It was shown previously that suppression of autophagy under the stress conditions is accompanied by an activation of NRF2-signaling pathway [39, 40].

The observed fullerene-induced decrease of ROS level in serum-starving HELFs considerably enhances the cell's viability. For instance, a 1.5-fold increase in the cell count was observed when F-828 was introduced in the culture of serum-starving HELFs in concentrations of 0.2–0.5 μM . This effect is caused by both enhancement of proliferative activity of the cells and lowering the level of the cell death. An exposure to F-828 at concentrations of 0.2 and 0.25 μM showed the minimum double-strand DNA break occurrence and the lowest cell mortality. It is notable that these positive effects were less pronounced at all the other concentrations of the fullerene derivative F-828 we used in the tests. Considerable improvement in the viability and survivability of the serum-starving HELFs in the presence of fullerene F-828 was accompanied by even more pronounced effects such as a strong increase in the activity.

It is important to note that elevated survival rate of serum-starving HELFs can be observed at F-828 concentrations from 0.2 up to 0.5 μM . At these fullerene concentrations, the ROS level found in serum-starving HELFs decreased down to the level registered in control cells (2% FBS). More effective ROS scavenging at a concentration of F-828 higher than 0.5 μM resulted in an increased cell death rate in comparison with the rate found in serum-starving HELFs without fullerene. A decrease in ROS level is accompanied by a decrease in the level of NF- κB factor and an increase in the number of DSBs and in the dead cell count. The findings suggest that even in oxidative stress conditions surplus ROS elimination may be associated with a decrease in the cell survival rate. The harmful effect could be due to a different, ROS-unrelated mechanism(s). This interesting fact warrants further investigation.

Additional Points

Highlights

- (i) The influence of a water-soluble [60] fullerene derivative (F-828) used in concentrations of 0.1 μM to 1 μM on serum-starving human embryo lung diploid fibroblasts (HELFs) was studied.
- (ii) Within a range of concentrations between 0.2 μM and 1 μM F-828 induced a decrease in the ROS level in HELFs.
- (iii) In concentrations of 0.2–0.25 μM F-828 enhanced proliferation of HELFs and decreased the DSB rate and apoptosis level.
- (iv) F-828 strongly stimulated NF- κB activity in serum-starving HELFs.
- (v) F-828 repressed NRF2 activity and NOX4 expression in serum-starving HELFs.
- (vi) In concentrations higher than 0.5 μM F-828 induced excessive ROS scavenging that resulted in an increased cell death rate as compared to the rate in serum-starving (stressed) HELFs without fullerene.

Disclosure

The authors approved the submission.

Competing Interests

The authors do not have any competing interests.

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References

- [1] R. Bakry, R. M. Vallant, M. Najam-ul-Haq et al., "Medicinal applications of fullerenes," *International Journal of Nanomedicine*, vol. 2, no. 4, pp. 639–649, 2007.
- [2] R. Qiao, A. P. Roberts, A. S. Mount, S. J. Klaine, and P. C. Ke, "Translocation of C_{60} and its derivatives across a lipid bilayer," *Nano Letters*, vol. 7, no. 3, pp. 614–619, 2007.
- [3] Z. Markovic and V. Trajkovic, "Biomedical potential of the reactive oxygen species generation and quenching by fullerenes (C_{60})," *Biomaterials*, vol. 29, no. 26, pp. 3561–3573, 2008.
- [4] F. Lao, L. Chen, W. Li et al., "Fullerene nanoparticles selectively enter oxidation-damaged cerebral microvessel endothelial cells inhibit JNK-related apoptosis," *ACS Nano*, vol. 3, no. 11, pp. 3358–3368, 2009.
- [5] S. S. Ali, J. I. Hardt, and L. L. Dugan, "SOD Activity of carboxy-fullerenes predicts their neuroprotective efficacy: a structure-activity study," *Nanomedicine*, vol. 4, no. 4, pp. 283–294, 2008.
- [6] F. Lao, W. Li, D. Han et al., "Fullerene derivatives protect endothelial cells against NO-induced damage," *Nanotechnology*, vol. 20, no. 22, Article ID 225103, 2009.

- [7] M. S. Misirkic, B. M. Todorovic-Markovic, L. M. Vucicevic et al., "The protection of cells from nitric oxide-mediated apoptotic death by mechanochemically synthesized fullerene (C₆₀) nanoparticles," *Biomaterials*, vol. 30, no. 12, pp. 2319–2328, 2009.
- [8] J.-J. Yin, F. Lao, P. P. Fu et al., "The scavenging of reactive oxygen species and the potential for cell protection by functionalized fullerene materials," *Biomaterials*, vol. 30, no. 4, pp. 611–621, 2009.
- [9] S. Ye, M. Chen, Y. Jiang et al., "Polyhydroxylated fullerene attenuates oxidative stress-induced apoptosis via a fortifying Nrf2-regulated cellular antioxidant defence system," *International Journal of Nanomedicine*, vol. 9, no. 1, pp. 2073–2087, 2014.
- [10] T. W. Kensler, N. Wakabayashi, and S. Biswal, "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway," *Annual Review of Pharmacology and Toxicology*, vol. 47, pp. 89–116, 2007.
- [11] S. Ye, T. Zhou, K. Cheng et al., "Carboxylic acid fullerene (C₆₀) derivatives attenuated neuroinflammatory responses by modulating mitochondrial dynamics," *Nanoscale Research Letters*, vol. 10, article 246, 2015.
- [12] G. Gloire, S. Legrand-Poels, and J. Piette, "NF- κ B activation by reactive oxygen species: fifteen years later," *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1493–1505, 2006.
- [13] M. S. Hayden and S. Ghosh, "NF- κ B, the first quarter-century: remarkable progress and outstanding questions," *Genes & Development*, vol. 26, no. 3, pp. 203–234, 2012.
- [14] L. M. Pedruzzi, M. B. Stockler-Pinto, M. Leite Jr., and D. Mafra, "Nrf2-keap1 system versus NF- κ B: the good and the evil in chronic kidney disease?" *Biochimie*, vol. 94, no. 12, pp. 2461–2466, 2012.
- [15] N. Wakabayashi, S. L. Slocum, J. J. Skoko, S. Shin, and T. W. Kensler, "When NRF2 talks, who's listening?" *Antioxidants & Redox Signaling*, vol. 13, no. 11, pp. 1649–1663, 2010.
- [16] W. Li, T. O. Khor, C. Xu et al., "Activation of Nrf2-antioxidant signaling attenuates NF κ B-inflammatory response and elicits apoptosis," *Biochemical Pharmacology*, vol. 76, no. 11, pp. 1485–1489, 2008.
- [17] S. V. Kostyuk, V. J. Tabakov, V. V. Chestkov et al., "Oxidized DNA induces an adaptive response in human fibroblasts," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 747–748, pp. 6–18, 2013.
- [18] A. B. Kornev, E. A. Khakina, S. I. Troyanov et al., "Facile preparation of amine and amino acid adducts of [60]fullerene using chlorofullerene C₆₀Cl₆ as a precursor," *Chemical Communications*, vol. 48, no. 44, pp. 5461–5463, 2012.
- [19] O. A. Troshina, P. A. Troshin, A. S. Peregudov, V. I. Kozlovskiy, J. Balzarini, and R. N. Lyubovskaya, "Chlorofullerene C₆₀Cl₆: a precursor for straightforward preparation of highly water-soluble polycarboxylic fullerene derivatives active against HIV," *Organic and Biomolecular Chemistry*, vol. 5, no. 17, pp. 2783–2791, 2007.
- [20] R. I. A. Podchernyaeva, O. V. Baklanova, L. A. Glushchenko et al., "Influenza virus reproduction in the MDCK cells adapted to growth in serum-free Hybris-2 medium," *Voprosy Virusologii*, vol. 55, no. 5, pp. 47–49, 2010.
- [21] S. M. Santos, A. M. Dinis, F. Peixoto, L. Ferreira, A. S. Jurado, and R. A. Videira, "Interaction of fullerene nanoparticles with biomembranes: from the partition in lipid membranes to effects on mitochondrial bioenergetics," *Toxicological Sciences*, vol. 138, no. 1, Article ID kft327, pp. 117–129, 2014.
- [22] J. J. Dumas, E. Merithew, E. Sudharshan et al., "Multivalent endosome targeting by homodimeric EEA1," *Molecular Cell*, vol. 8, no. 5, pp. 947–958, 2001.
- [23] P. Guillaud, S. du Manoir, and D. Seigneurin, "Quantification and topographical description of Ki-67 antibody labelling during the cell cycle of normal fibroblastic (MRC-5) and mammary tumour cell lines (MCF-7)," *Analytical Cellular Pathology*, vol. 1, no. 1, pp. 25–39, 1989.
- [24] S. N. Naryzhny, "Proliferating cell nuclear antigen: a proteomics view," *Cellular and Molecular Life Sciences*, vol. 65, no. 23, pp. 3789–3808, 2008.
- [25] L. Li, J. Tan, Y. Miao, P. Lei, and Q. Zhang, "ROS and autophagy: interactions and molecular regulatory mechanisms," *Cellular and Molecular Neurobiology*, vol. 35, no. 5, pp. 615–621, 2015.
- [26] D. J. Klionsky, K. Abdelmohsen, A. Abe et al., "Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition)," *Autophagy*, vol. 12, no. 1, pp. 1–222, 2016.
- [27] A. C. Cave, A. C. Brewer, A. Narayananicker et al., "NADPH oxidases in cardiovascular health and disease," *Antioxidants and Redox Signaling*, vol. 8, no. 5–6, pp. 691–728, 2006.
- [28] L. Hecker, N. J. Logsdon, D. Kurundkar et al., "Reversal of persistent fibrosis in aging by targeting Nox4-Nrf2 redox imbalance," *Science Translational Medicine*, vol. 6, no. 231, Article ID 231ra47, 2014.
- [29] T. Nguyen, P. J. Sherratt, P. Nioi, C. S. Yang, and C. B. Pickett, "Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1," *Journal of Biological Chemistry*, vol. 280, no. 37, pp. 32485–32492, 2005.
- [30] H. Dreger, K. Westphal, A. Weller et al., "Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for proteasome inhibitor-mediated cardioprotection," *Cardiovascular Research*, vol. 83, no. 2, pp. 354–361, 2009.
- [31] S. Kovac, P. R. Angelova, K. M. Holmström, Y. Zhang, A. T. Dinkova-Kostova, and A. Y. Abramov, "Nrf2 regulates ROS production by mitochondria and NADPH oxidase," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1850, no. 4, pp. 794–801, 2015.
- [32] S. Pendyala, J. Moitra, S. Kalari et al., "Nrf2 regulates hyperoxia-induced Nox4 expression in human lung endothelium: identification of functional antioxidant response elements on the Nox4 promoter," *Free Radical Biology and Medicine*, vol. 50, no. 12, pp. 1749–1759, 2011.
- [33] A. C. Brewer, T. V. A. Murray, M. Arno et al., "Nox4 regulates Nrf2 and glutathione redox in cardiomyocytes in vivo," *Free Radical Biology and Medicine*, vol. 51, no. 1, pp. 205–215, 2011.
- [34] M. Löbrich, A. Shibata, A. Beucher et al., "γH2AX foci analysis for monitoring DNA double-strand break repair: strengths, limitations and optimization," *Cell Cycle*, vol. 9, no. 4, pp. 662–669, 2010.
- [35] T. Kohno, Y. Kubo, K. Yasui et al., "Serum starvation activates NF- κ B through G protein β 2 subunit-mediated signal," *DNA and Cell Biology*, vol. 31, no. 11, pp. 1636–1644, 2012.
- [36] D. Wang and A. S. Baldwin Jr., "Activation of nuclear factor- κ B-dependent transcription by tumor necrosis factor- α is mediated through phosphorylation of RelA/p65 on serine 529," *Journal of Biological Chemistry*, vol. 273, no. 45, pp. 29411–29416, 1998.
- [37] A. V. Kuznetsov, I. Kehrer, A. V. Kozlov et al., "Mitochondrial ROS production under cellular stress: comparison of different detection methods," *Analytical and Bioanalytical Chemistry*, vol. 400, no. 8, pp. 2383–2390, 2011.

- [38] C. Liu, E. P. DeRoo, C. Stecyk, M. Wolsey, M. Szuchnicki, and E. G. Hagos, "Impaired autophagy in mouse embryonic fibroblasts null for Krüppel-like Factor 4 promotes DNA damage and increases apoptosis upon serum starvation," *Molecular Cancer*, vol. 14, no. 1, article 101, 2015.
- [39] T. M. Stepkowski and M. K. Kruszewski, "Molecular cross-talk between the NRF2/KEAP1 signaling pathway, autophagy, and apoptosis," *Free Radical Biology and Medicine*, vol. 50, no. 9, pp. 1186–1195, 2011.
- [40] N. Chen, L. Wu, H. Yuan, and J. Wang, "ROS/autophagy/Nrf2 pathway mediated low-dose radiation induced radio-resistance in human lung adenocarcinoma A549 cell," *International Journal of Biological Sciences*, vol. 11, no. 7, pp. 833–844, 2015.

Review Article

Role of ROS and RNS Sources in Physiological and Pathological Conditions

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There is significant evidence that, in living systems, free radicals and other reactive oxygen and nitrogen species play a double role, because they can cause oxidative damage and tissue dysfunction and serve as molecular signals activating stress responses that are beneficial to the organism. Mitochondria have been thought to both play a major role in tissue oxidative damage and dysfunction and provide protection against excessive tissue dysfunction through several mechanisms, including stimulation of opening of permeability transition pores. Until recently, the functional significance of ROS sources different from mitochondria has received lesser attention. However, the most recent data, besides confirming the mitochondrial role in tissue oxidative stress and protection, show interplay between mitochondria and other ROS cellular sources, so that activation of one can lead to activation of other sources. Thus, it is currently accepted that in various conditions all cellular sources of ROS provide significant contribution to processes that oxidatively damage tissues and assure their survival, through mechanisms such as autophagy and apoptosis.

1. Introduction

The existence of free radicals, known in chemistry since the beginning of the 20th century [1], was discovered in biological systems in 1954 [2]. In the same year, Gerschman et al. proposed that the noxious effects of hyperbaric oxygen and X irradiation had a common mechanism which involved radical and nonradical species, resulting from partial reduction of oxygen [3]. Soon after, Harman suggested that these species, at present referred to as reactive oxygen species (ROS), might play a role in the aging process [4].

Progress in the free radical knowledge occurred in 1969 when the enzyme superoxide dismutase (SOD) was isolated [5]. The SOD discovery inspired a large number of studies, which contributed to the knowledge of the ROS, even though for several decades they were thought to cause exclusively damaging effects. This view was mainly supported by the finding that ROS readily react with most biological macromolecules, causing their oxidative modification, ultimately

resulting in the loss of their function [6]. Actually, ROS include species, such as the hydroxyl radical ($\cdot\text{OH}$), whose reactivity is so high that it reacts very close to its site of formation [7], and other species, such as superoxide ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which are less reactive (Table 1). Thus, nitrogen containing species, which are now indicated as reactive nitrogen species (RNS), include nitric oxide (NO^{\cdot}), which is relatively unreactive, and its derivative the peroxynitrite (ONOO^-), a powerful oxidant, able to damage many biological molecules [8].

Nonetheless, ROS were considered as one of the key players in tissue injury, if occurring in organisms with a system of biochemical defenses to neutralize the oxidative effects of ROS [9], but the balance between ROS generation and antioxidant system activity is slightly tipped in favor of the ROS so that, in living systems, there is a continuous low level of oxidative damage. Moreover, when a greater imbalance occurs in favor of the ROS, oxidative stress ensues [10]. Oxidative stress is a phenomenon which was related to the

TABLE 1: Main reactive oxygen (ROS) and nitrogen (RNS) species.

Radicals	$O_2^{\bullet-}$	Superoxide anion
	$\bullet OH$	Hydroxyl
	$NO\bullet$	Nitric oxide
	$NO_2\bullet$	Nitrogen dioxide
Nonradicals	H_2O_2	Hydrogen peroxide
	$HOCl$	Hypochlorous acid
	$ONOO^-$	Peroxynitrite

development of many pathological conditions. Pathologies where ROS were identified as causal factors include cardiovascular disease, diabetes, rheumatoid arthritis, cancer, and neurodegenerative disorders [11] and the use of exogenous antioxidants was proposed for their treatment [12].

The finding that $\bullet OH$ radical stimulated formation of cyclic guanosine monophosphate (cGMP) [13] led to an opposing view about the ROS role in biological systems. Since then, it became clear that living systems not only adapted to a coexistence with free radicals but also developed methods to turn these toxicants to their own advantage utilizing them in critical physiological processes. This view has been supported by the observation that, at the cellular level, ROS regulate growth, apoptosis, and other signaling, while, at the system level, they contribute to complex functions, including blood pressure regulation, cognitive function, and immune function [14]. It has also been shown that whereas accumulation of oxidative damage results in organism death [15], several longevity-promoting interventions increase generation of ROS that activate stress responses that are beneficial to the organism and extend lifespan [16].

Like ROS, RNS play a dual role since they can be either harmful or beneficial to living systems. Nitric oxide, early identified as a signaling molecule in blood vessel modulation [17] and now known as a regulator of important physiological processes [18], can mediate cellular toxicity damaging metabolic enzymes and generating, by reaction with superoxide, peroxynitrite [19].

Although the role of ROS and RNS in cellular damage and signal transduction is well-established, several controversial questions remain open. At low concentrations, ROS and RNS play an important role as regulatory mediators in signaling processes, whereas, at moderate or high concentrations, they are harmful for living organisms inactivating important cellular molecules. This suggests that the concentrations of reactive species determine the shift from their advantageous to detrimental effects, but the concentrations to which this shift happens are not generally known. Moreover, given the wide range of possible targets and the general reactivity of cellular oxidants, it is unclear how any specificity in their opposite actions can be achieved. It has been suggested that contributing factors may include cell type, duration of oxidant production, reactive species produced, and the localization of their source and their targets [20], but our information about such a matter is still scarce and discordant. For example, it has often been assumed that mitochondria are the main cellular source of ROS in physiological and

pathological conditions [21], so that these organelles have been thought to play a crucial role in several human diseases and aging [22]. However, in the light of available data it seems that evidence that mitochondria are the main source of cellular ROS is lacking [23]. The issue is complicated by the existence of a strong interaction among the various sources of ROS generation in the cell [24, 25], which makes it difficult to establish what source of reactive species plays a main role in different physiological and pathological conditions.

To contribute to the understanding of the role of reactive species sources in health and disease, the present review, after examining the cellular localization and supposed involvement of such sources in tissue dysfunction and protection, examines experimental evidence concerning their harmful and protective effects on a normal physiological activity, such as exercise, and on pathologic conditions, such as diabetes and neurodegenerative diseases.

2. Cellular ROS and RNS Sources

In the living organisms, ROS are generated in several cellular systems localized on the plasma membrane, in the cytosol, in the peroxisomes, and on membranes of mitochondria and endoplasmic reticulum (Figure 1).

2.1. ROS Production in the Cytosol. Several soluble cell components, including thiols, hydroquinones, catecholamines, and flavins, can contribute to intracellular ROS production as they are able to undergo redox reactions [26]. Moreover, several cytosolic enzymes produce ROS during their catalytic activity. Probably the most studied ROS producing enzyme is xanthine oxidase (XO). In healthy tissues, the enzyme catalyzing oxidation of hypoxanthine to xanthine and xanthine to uric acid is xanthine dehydrogenase (XDH), which uses $NADP^+$ as an electron acceptor. Conversely, in damaged tissues, by either reversible oxidation of cysteine residues or irreversible Ca^{2+} -stimulated proteolysis, the enzyme is converted from the dehydrogenase form into the oxidase form, which transfers electrons to molecular oxygen producing the superoxide radical during xanthine or hypoxanthine oxidation [27].

2.2. ROS Production by Mitochondria. In aerobic cells, mitochondria are necessary for numerous fundamental functions, including respiration and oxidative energy production, regulation of the intracellular calcium concentration, and control of the fatty acid β -oxidation.

For a long time, mitochondria have only been considered for their role in energy production. They utilize about 95% of the oxygen assumed from aerobic animals to obtain energy by oxidizing substances contained in food by transfer of electrons to electron carriers such as NAD^+ , FMN, and FAD. The reduced forms of these molecules, in turn, transfer electrons to the components of the respiratory chain and finally to oxygen in a process which happens in a series of subsequent steps, so that the energy, resulting by the fall in the electron potential energy, is gradually released. In the process, several redox centers, in great part organized in four protein

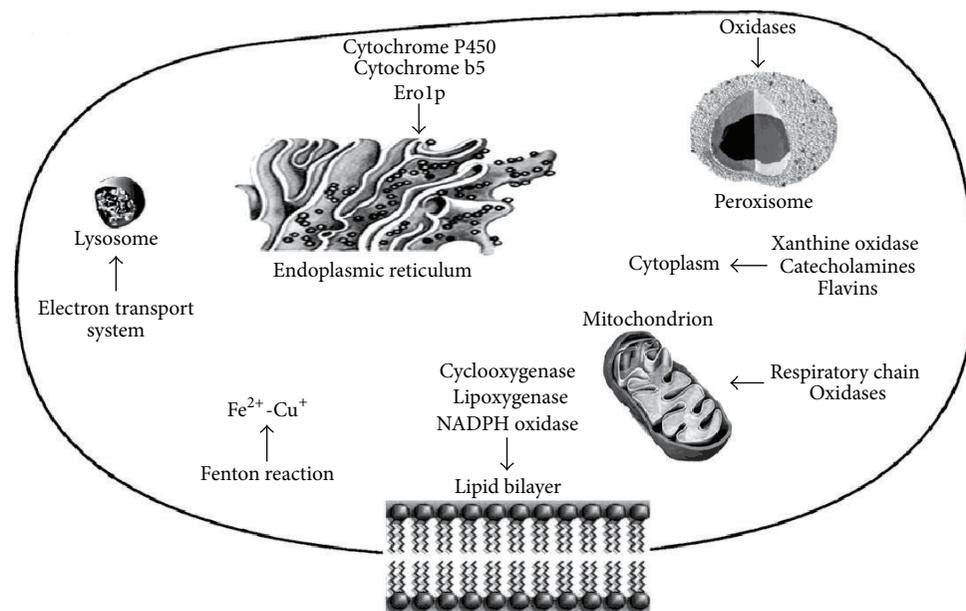


FIGURE 1: Cellular sources of ROS production. Subcellular organelles and structural and soluble cell components all contribute to production of a wide variety of reactive species (modified from Venditti et al. [116], with permission).

complexes inserted in the inner mitochondrial membrane, are involved [28]. Complexes I and II transfer electrons to the lipid-soluble carrier ubiquinone. From this, the electrons pass through Complex III, cytochrome c (another mobile carrier), and Complex IV, to the oxygen. The fall in electron potential energy is used to pump protons from the mitochondrial matrix to the intermembrane space, thus setting up a proton-motive force [29]. The process by which such a force drives protons back into the matrix through the mitochondrial ATP synthase leading to ATP synthesis is named oxidative phosphorylation [30].

2.2.1. Respiratory Chain. It has now been about 50 years since mitochondrial H₂O₂ production in the presence of respiratory substrates was first recorded [31], followed shortly after by the detection of mitochondrial generation of superoxide radical anion [32]. The discovery that electron-transfer along the inner mitochondrial membrane carriers is associated with formation of ROS suggested the mitochondrial involvement in degenerative processes linked to several diseases and aging.

The primary ROS generated within mitochondria by univalent autooxidation of electron carriers is O₂^{•-} [33], which is converted by mitochondrial SOD into H₂O₂, which can be turned into [•]OH radical via the Fenton reaction:



The main sites involved in mitochondrial ROS production are localized at Complexes I and III [34]. However, succinate dependent ROS production by Complex II from rat skeletal muscle [34] and glycerol 3-phosphate dependent production by Complex II from several rat tissues [35] have also been reported.

To date, the relative importance of each mitochondrial site to ROS production is still controversial, partly due to

utilization of different assays, substrates [36], and sources of mitochondria [37]. On the other hand, the localization of the generators is important for establishing ROS effects since it determines if O₂^{•-} is produced in the mitochondrial matrix or in the intermembrane space. Thus, both generators of Complex I and Complex III release O₂^{•-} into the matrix where it can damage mitochondrial DNA, whereas Complex III generator also releases O₂^{•-} into the intermembrane space, where it has easier access to the cytosol [36]. In their classic work, Boveris et al. [38] reported that H₂O₂ production by isolated liver mitochondria was about 1-2% of the total oxygen consumption during State 4 respiration and decreased during State 3 respiration. However, lower values, around 0.1-0.2% of the total oxygen consumption, have subsequently been found [39].

2.2.2. Other Mitochondrial Sites of ROS Production. Experiments with isolated enzymes or mitochondria have shown ROS production by several oxidoreductases located in mitochondrial membranes [40], whose contribution to mitochondrial ROS production is, however, unknown. They include monoamine oxidases, which produce H₂O₂ at rates that, in brain mitochondria, may be higher than those of other mitochondrial sources [41]; dihydroorotate dehydrogenase, which *in vitro*, in the absence of coenzyme Q, its natural electron acceptor, can produce H₂O₂ [42]; α -glycerophosphate dehydrogenase, which has been reported to produce H₂O₂ in mouse [37] and rat [34] mitochondria oxidizing glycerol-3-phosphate; succinate dehydrogenase, which produces ROS when isolated and incorporated in liposomes in the absence of coenzyme Q [43]; and α -ketoglutarate dehydrogenase complex, which has been found to generate both O₂^{•-} and H₂O₂ in isolated mouse brain mitochondria [44].

2.3. ROS Production by Peroxisomes. Although peroxisomes have long been known as organelles involved in cellular metabolism of H_2O_2 [45], it is now clear that they are involved in several metabolic pathways [46]. Important functions performed by peroxisomes include fatty acid β - and α -oxidation, amino acid and glyoxylate metabolism, and synthesis of lipidic compounds [47], and most enzymes catalyzing these processes produce ROS during their activity [48].

Early studies indicated that about 35% of the peroxide formed in rat liver is derived from peroxisomal oxidases [38]. Moreover, the observation that about 20–60% of the H_2O_2 generated inside peroxisomes diffused to the surrounding medium demonstrated that H_2O_2 can readily cross the peroxisomal membrane. The peroxide diffuses through the Pxmp2 channel permeable to small solutes [49], even though H_2O_2 generated by urate oxidase localized in the peroxisome core can be directly released in the cytosol through crystalloid core tubules [50]. In any case, it is apparent that, despite high content of catalase (CAT), peroxisomes are unable to prevent the H_2O_2 release.

Peroxisomes also contain xanthine oxidase [51] and the inducible form of nitric oxide synthase (see below) [52], which produce $O_2^{\bullet-}$ and NO^{\bullet} , respectively. Because such species react rapidly forming ONOO and H_2O_2 gives rise to $\bullet OH$ radicals via Fenton reaction, peroxisomes are a potential source of such highly reactive species. Because, if uncontrolled, ROS and RNS are very damaging, peroxisomes, in addition to catalase, contain other antioxidant enzymes [50]. However, in the light of their capacity to produce membrane permeant reactive species, such as H_2O_2 and NO^{\bullet} , it is highly likely that, under some physiological or pathological conditions, peroxisomes may act as a source of H_2O_2 and NO^{\bullet} in living cells [50].

2.4. ROS Production by Endoplasmic Reticulum. The endoplasmic reticulum (ER) is involved in multiple functions, such as synthesis, folding, and transport of Golgi, lysosomal, secretory, and cell-surface proteins [53], calcium storage [54], lipid metabolism, and, in some cell types, drug detoxification [55].

Smooth endoplasmic reticulum presents a chain of electron transport, constituted by two systems devoted to xenobiotic metabolism and introduction of double bonds in fatty acids, which are also able to produce ROS. Another microsomal system, which shares this ability, provides oxidative protein folding.

2.4.1. Xenobiotic Metabolism. The metabolism of xenobiotics generally occurs in two phases. Phase I reactions introduce a polar group in a lipophilic substrate (AH) using O_2 and a reducing agent (RH_2):



In the reaction, known as a monooxygenase reaction, it is involved in a system constituted by a flavoprotein (NADPH-cytochrome P450 reductase) and cytochromes known collectively as cytochromes P450 (CYPs).

The Phase II reactions are conjugation reactions in which an endogenous molecule is added to the Phase I reaction product or sometimes directly to the xenobiotic.

In whole, the process detoxifies xenobiotics converting them into species that are more water soluble and easier to excrete in urine or can be conjugated with substances which make their urinary or biliary excretion further easier.

The membranes of the endoplasmic reticulum system were recognized as a source of H_2O_2 in 1957 by Gillette et al. [56] that assumed that the NADPH-cytochrome c reductase might be the microsomal H_2O_2 generator. However, it was subsequently reported that $O_2^{\bullet-}$ [57] and H_2O_2 [58] could be formed by decay of two intermediates of the catalytic cycle.

The microsomal cytochrome P450-dependent monooxygenase system is one of the major producers of ROS in the liver cell [59]. Indeed, estimates performed using NADPH as substrate indicated that microsomes contribute to H_2O_2 production in rat liver by 45% [38].

2.4.2. Unsaturated Fatty Acid Production. In most organisms, unsaturated fatty acids are produced by desaturases, which convert a single bond between two carbon atoms in a fatty acyl chain into a double bond [60]. In the ER system, which allows for fatty acid desaturation, cytochrome b5 acts as an electron-transfer component, with two possible modes of action. First, desaturation can be carried out by a multienzyme system, composed of desaturase, NADH cytochrome b5 reductase, and cytochrome b5. In the reaction, cytochrome b5 transfers electrons by lateral diffusion, from NADH cytochrome b5 reductase to the desaturase which introduces carbon-carbon double bonds into fatty acids using one molecule of O_2 and forming two molecules of H_2O [61]. Secondly, many desaturases are modular proteins that are composed of desaturase and cytochrome b5 modules [62]. The fusion of the desaturase and cytochrome b5 domains makes the NADH cytochrome b5 reductase able to directly transfer electrons to the catalytic site of the cytochrome b5 fusion desaturases via the cytochrome b5-like domain without the requirement of an independent cytochrome b5 [63]. This can increase the rate of electron-transfer by presenting a correctly oriented heme group with respect to the dioxo-iron cluster, eliminating the need for diffusion and reorientation of the reduced cytochrome b5 [63].

The two electron transport systems do not act independently of each other and cytochrome b5 may play a role in the NADPH-dependent oxidation of xenobiotics. Indeed, cytochrome b5 exhibits a positive action on cytochrome P450 monooxygenase reaction, which is due to the transfer of the second of the two electrons necessary for molecular oxygen activation to cytochrome P450 [64]. This transfer makes the catalysis faster and reduces the time for formation of side products, such as H_2O_2 and $O_2^{\bullet-}$ [64]. Recently, it has also been demonstrated that NADH cytochrome b5 reductase can leak electrons to O_2 to make $O_2^{\bullet-}$ and this can be an additional source of $O_2^{\bullet-}$ *in vivo* [65].

2.4.3. Protein Folding. Most proteins synthesized in ER are stabilized by formation of intramolecular disulfide bonds, process that requires oxidation of free sulphhydryl groups.

The feasibility of protein oxidative folding in intraluminal ER milieu is ensured by an oxidized (GSSG) to reduced (GSH) glutathione ratio higher than that within the cytosol [66]. The formation of disulfide bonds in proteins is driven by protein disulfide isomerase (PDI), a member of the thioredoxin family, and endoplasmic reticulum-resident protein (Ero1p), which is tightly associated with FAD moiety [66]. Ero1p functions as an oxidase for PDI: oxidizing equivalents flow from Ero1p to substrate proteins via PDI, through direct dithiol-disulfide exchange between PDI and Ero1p [67].

The process of oxidative protein folding uses molecular oxygen as the source of oxidizing equivalents. Indeed, Ero1p transfers electrons from PDI to molecular oxygen by a FAD-dependent reaction, resulting in ER protein folding-induced oxidative stress [68]. By theoretical calculation, it was estimated that protein thiol oxidation via PDI and Ero1 could account for up to 25% of cellular ROS produced during protein synthesis [69].

2.5. ROS Production by Plasma Membrane. The plasma membrane is involved in several cellular processes such as cell adhesion, ion conductivity, and cell signaling. It is also a key site of free radical reactions because it is generally exposed to an oxidizing environment. ROS, which in tissues could be generated from dysfunctional cells [70], cause oxidative damage of membrane components unless efficient antioxidant systems are operative. The increase in membrane permeability, caused by oxidation of lipids or structurally important proteins, can result in a decrease in transmembrane ion gradients, loss of secretory functions, and inhibition of cellular metabolic processes.

Free radicals can be produced during the conversion of arachidonic acid into products, such as prostaglandins, thromboxanes, and leukotrienes, by membrane associated enzymes such as lipoxygenase and cyclooxygenase [71]. Such enzymes metabolize arachidonic acid released from membrane phospholipids via phospholipase A2 activity and generate ROS as by-products during arachidonic acid oxidation.

However, the main source of ROS is represented by $O_2^{\bullet-}$ production by the membrane-bound enzyme NADPH oxidases. $O_2^{\bullet-}$ production by the phagocyte enzyme is a well-known phenomenon, which helps to kill bacterial intruders [72].

The phagocyte NADPH oxidase is composed of two membrane proteins gp91phox (cytochrome b558 heavy chain, later designated as NOX2, which is the catalytic subunit of the enzyme) and p22phox, three cytosolic proteins p67phox, p47phox, and p40phox, and a small GTP binding protein Rac [73]. In resting cells, the enzyme is dormant, and its components are distributed between the cytosol and plasma membrane. Bacterial infection induces translocation of the cytosolic components to the phagosome membrane where they associate with cytochrome b558 and give rise to the catalytically active NADPH oxidase [73].

The presence of NOX2 homologs was firstly suggested by the observation that $O_2^{\bullet-}$ is produced in a NADPH-dependent manner in nonphagocytic cells, in which NOX2 is not expressed [74]. To date, five NOX isoforms (NOX1,

NOX2, NOX3, NOX4, and NOX5) and two related enzymes, DUOX1 and DUOX2, have been reported and most, if not all, isoforms were targeted to cellular membranes.

The NOX proteins constitute the only enzyme family with the sole function of producing ROS. These proteins have different regulation and specific subcellular localization and generate distinct ROS [75]. NOX1, present in smooth muscle cells and other vascular cells, NOX2, present in endothelial and phagocytic cells, and NOX3, found in the brain and inner ear, generate $O_2^{\bullet-}$. NOX4, constitutively expressed and active in vascular smooth muscle and endothelial cells, is responsible for the basal production of H_2O_2 . NOX5, identified in human immature lymphatic tissues and endothelial cells, produces H_2O_2 in a Ca^{2+} dependent fashion. The DUOX1 and DUOX2, originally isolated from the thyroid, produce H_2O_2 that oxidizes iodide during thyroid hormone synthesis.

The phagocyte NADPH oxidase, when activated, generates quantities of $O_2^{\bullet-}$ and H_2O_2 accounting for a significant fraction (10–90%) of total oxygen consumption of neutrophils, macrophages, and microglia, but the contribution of such and other NADPH oxidases to total cellular ROS production in resting or during activation is less clear [23].

It has also been reported that arachidonic acid [76] and its metabolites generated by lipoxygenase [77] and cyclooxygenase [78] stimulate the generation of ROS by NOXs, thus revealing the existence of an interconnecting signaling system between eicosanoids and NOXs.

2.6. ROS Production by Lysosomes. Although lysosomes are involved in several functions, until recently they were considered as pure sites for terminal degradation of macromolecules [79].

On rat liver membranes, flavins, ubiquinone, and a b-type cytochrome [80] form a functional electron transport system, starting with the donor NADH and ending to acceptor O_2 through the sequence FAD→cytochrome b→ubiquinone [81]. The role of this redox chain is to support proton accumulation within lysosomes [81] to maintain an optimal pH for the acidic hydrolases [79]. The electron transport chain appeared to give rise to $\bullet OH$ radical, which required the transfer of three electrons to molecular oxygen, whereas $O_2^{\bullet-}$ was not detected. This does not exclude the intermediate existence of $O_2^{\bullet-}$, because the acid pH-milieu inside lysosomes favors spontaneous dismutation of $O_2^{\bullet-}$ to H_2O_2 , which is cleaved by intralysosomal ferrous iron into $\bullet OH$ [82].

2.7. RNS Production. NO^{\bullet} is produced from the metabolism of the amino acid, L-arginine. The enzymes catalyzing this process, known as nitric oxide synthases (NOS), convert L-arginine into L-citrulline and NO^{\bullet} by a 5-electron oxidation of a guanidine nitrogen of L-arginine [83].

To date, three isoforms of nitric oxide synthase have been identified. Two isoforms, neuronal NOS (nNOS; type I NOS) and endothelial NOS (eNOS; type III NOS), are expressed constitutively and regulated by the interaction of Ca^{2+} with calmodulin [84]. The other isoform, inducible-NOS (iNOS; type II NOS), is induced in response to infection, inflammation, or trauma and is not regulated by Ca^{2+} because it forms a

complex with calmodulin at very low concentrations of Ca^{2+} [84].

NO^{\bullet} generated by the NOS isoforms located in different cell types plays different roles. NO^{\bullet} generated by nNOS in neurons serves in communication between nerve cells, whereas the free radical generated by iNOS in macrophages and smooth muscle cells contributes to their killing mechanism, and NO^{\bullet} generated by eNOS in endothelium, brain, and heart relaxes blood vessels and maintains normal blood pressure [84].

The subcellular distribution of NOS is dynamically regulated so that the enzymes are exposed to different concentrations of ROS depending on where in the cell they are localized. For example, eNOS is mainly found in plasma membranes of cardiac and endothelial cells and, in both cells, it is localized at the caveolae of the sarcolemma and T tubules, where it is associated with caveolin, the structural protein of caveolae [85]. However, as a result of different stimuli, eNOS shuttles between caveolae and distinct intracellular sites and it is likely that the selective movement of eNOS serves to determine specific responses to the agonists [86].

Interestingly, the study of the subcellular localization of iNOS showed that during sepsis a substantial amount of the enzyme of the rat hepatocytes localizes to peroxisomes [52], but subsequently it was found that only monomeric iNOS is associated with peroxisomes. Peroxisomal iNOS could be reactivated *in vitro*, but it had a lower specific activity than iNOS in the soluble pool [87]. Thus, uncoupled or deficient iNOS may be targeted to the peroxisome, even though it is possible that iNOS plays a role in the regulation of peroxisomal function. Furthermore, there is growing evidence supporting the existence of mitochondrial NOS (mtNOS) and its involvement in the regulation of mitochondrial as well as cellular functions in several tissues [88].

Some functions of NO^{\bullet} in signaling and regulation of cell function are performed through cGMP-independent pathways including those involving mitochondria [89]. At physiological concentrations, most mitochondrial effects of NO^{\bullet} are exerted on the respiratory chain. First, NO^{\bullet} competes with O_2 for the binding site at the binuclear center of cytochrome *c* oxidoreductase, leading to a reversible inhibition of cytochrome *c* oxidase activity [90]. Secondly, NO^{\bullet} , reacting with respiratory Complex III, inhibits electron-transfer and enhances $\text{O}_2^{\bullet-}$ production [91]. NO^{\bullet} also gives rise to protein nitrosation, reacting reversibly with the nucleophilic centers in protein thiol residues [92], and mitochondria, treated with NO^{\bullet} donors, exhibit S-nitrosation and inhibition of Complex I [93]. Moreover, the reaction of NO^{\bullet} with $\text{O}_2^{\bullet-}$, which is formed by the mitochondrial respiratory chain, leads to the switch from reversible inhibition of cellular respiration by NO^{\bullet} to the pathological inhibition of mitochondrial function by ONOO^- [94].

3. Role of ROS Sources in Oxidative Stress and Tissue Dysfunction

Tissue oxidative damage and consequent dysfunction shown in various pathological conditions depend on increased

cellular production of ROS and RNS or on impaired removal of such species. Extensive information is available on mitochondrial ROS production and its relationship with mitochondrial oxidative damage and dysfunction. Because mitochondria are required for oxidative energy production and multiple biosynthetic reaction pathways in aerobic cells, it is understandable that a disturbance of mitochondrial function can lead to impaired cell function and development of several pathologies [22].

However, the larger available information and relevance of mitochondrial function for cell viability do not make mitochondria the main source responsible for tissue dysfunction in conditions of oxidative stress. Indeed, even increases in reactive species production by other cellular sources can cause cellular alterations. On the other hand, available data about ROS and RNS production have mostly been obtained using isolated organelles or cellular cultures and, until recently, the direct measurement of ROS in living systems remained difficult due to the lack of adequate methodology. The recently introduced fluorescent protein-based probes for H_2O_2 and GSH/GSSG redox state [95] will likely facilitate reliable organelle-specific ROS measurements, but presently we are not able to provide a well-founded answer to the question concerning the role played by the various sources of reactive species in tissue dysfunction.

3.1. Mitochondria. Normally, the rate of mitochondrial ROS generation is rather low and results in minimal damage, because mitochondria have a highly efficient antioxidant defense system able to scavenge a large number of the ROS produced. However, in several circumstances, high rates of ROS production occur, so that a substantial part of oxidants may escape the scavenging systems and compromise important mitochondrial functions. Moreover, even though it is extremely unlikely that $\bullet\text{OH}$ radicals can be released by mitochondria, oxidative damage to components of cytoplasm and other cellular structures can result from mitochondrial leakage of other ROS, such as H_2O_2 that is able to readily cross mitochondrial membranes and reach such structures where, in the presence of Fe^{2+} ligands, it can generate $\bullet\text{OH}$ radical.

Information on the role of an increased ROS production in decline of mitochondrial function and cellular derangement is supplied by experimental work dealing with myocardial ischemia-reperfusion (IR) injury.

Myocardial ischemia occurs when myocardial oxygen demand exceeds oxygen supply. Unless reversed, this situation results in irreversible tissue injury and myocardial infarction. Although restoration of blood flow is necessary to salvage ischemic tissues, it may create another form of myocardial damage, termed “reperfusion injury” [96], which is partly due to the generation of toxic oxygen radicals [97].

Initially, $\text{O}_2^{\bullet-}$ was postulated to be the species responsible [98], but now it is clear that the several ROS and RNS are involved in reperfusion injury [99]. ROS are produced in reperfused myocytes from several sources, including xanthine oxidase, NADPH oxidase, and mitochondria. ROS may be produced by xanthine oxidase which is activated during hypoxia [98]. NADPH oxidases account for an important part of the ROS formed during ischemia-reperfusion [100].

However, there is now strong evidence that mitochondrial ROS generation plays a critical role in damaging cellular components and initiating cell death.

The proposal that the respiratory chain is a major source of ROS during reperfusion of ischemic myocardium [97] was supported by the observation that a generation of oxygen radicals was induced *in vitro* upon reoxygenation of mitochondria isolated from hearts that had been subjected to ischemia [101]. Further support was obtained demonstrating by electron paramagnetic resonance that resumption of mitochondrial oxidative phosphorylation upon postischemic reflow can be a source of oxygen radicals in intact rabbit hearts [102].

Because mitochondrial ROS generation depends on the degree of reduction of the autoxidizable electron carriers, the increased reduction of the respiratory chain associated with ischemia promotes ROS generation upon the respiration resumption. It was proposed that ROS generation is induced by interaction with ubiquinone, which accumulates in mitochondria during ischemia because of respiratory chain inhibition [103]. This ROS generation ends rapidly when the mitochondrial components of the respiratory chain are reoxidized. However, it is long-lasting mitochondria that have accumulated large amounts of reducing equivalents, so that the severity of reperfusion-induced oxidative damage and mitochondrial dysfunction should increase with ischemia duration. In fact, it was determined that mitochondrial function impairment was enhanced when coronary occlusion periods increased [104] and mitochondria lipid peroxidation increased gradually with ischemia duration [105]. These changes were well related to a gradual decline in mitochondrial respiration, which reflected damage to electron transport chain components. It was also shown that, after reperfusion of ischemic heart, functional recovery of the tissue was inversely correlated to mitochondrial derangement [105], supporting the idea that heart performance is strongly conditioned by mitochondrial functionality. Further support was provided by the observation that the antioxidant protection of mitochondrial function was associated with decreased impairment of cardiac function following ischemia-reperfusion [106]. Thus, mitochondria are a site of reperfusion-induced oxidative damage, whose severity increases with ischemia duration.

It is likely that mitochondrial oxidative damage and dysfunction is due to $\cdot\text{OH}$ radicals produced within mitochondria. These oxyradicals are highly reactive, short-lived species and are expected to cause damage at or near the site of formation. Therefore, they may inactivate components of the respiratory chain, enzymes of the Krebs cycle, and other mitochondrial proteins, leading to mitochondrial dysfunction.

NOS stimulation [107] and inhibition of mitochondrial function by both NO^{\bullet} [108] and ONOO^{-} [109] upon ischemia-reperfusion were reported, suggesting that the reduction in mitochondrial respiration induced by ischemia-reperfusion also depends on increased RNS production.

RNS involvement in reperfusion-linked alteration in mitochondrial and tissue function was demonstrated studying ischemia-reperfusion in the presence of the NOS inhibitor, N^{ω} -nitro-L-arginine (L-NNA) [110]. Indeed, L-NNA

improved heart functional recovery and mitochondrial respiration protecting mitochondria from the oxidative and nitrosative damage.

3.2. Strengthening of Mitochondrial Oxidative Damage. In light of the aforementioned results, the mechanism which, during reperfusion, causes tissue damage and dysfunction appears to be a positive feedback loop. Indeed, the concomitance of reflow-mediated perturbations, such as NOS activation and increased ROS production, strengthens mitochondrial damage and dysfunction thus leading to increased tissue derangement. Furthermore, there is evidence that other mechanisms, involving mitochondria, are able to alter the tissue susceptibility to stressful conditions, leading to pathological consequences.

A mechanism of ROS production strengthening is the process named ROS-induced ROS release (RIRR) [111]. RIRR is generated by circuits requiring mitochondrial membrane channels, including nonspecific mitochondrial channels called the mitochondrial permeability transition (MPT) pores [112] and the inner membrane anion channel (IMAC) [113].

A condition that leads to RIRR is the exposure to high oxidative stress resulting by an increase in ROS that reaches a threshold level that triggers the opening of MPT pore. Under oxidative stress, mitochondrial Ca^{2+} overload takes place, which depresses mitochondrial function [114] and triggers several processes, including MPT pore opening [111]. This, in turn, causes collapse of mitochondrial membrane potential and transient increase in ROS generation [111]. In addition to ROS effects in mitochondria where the RIRR is originated, the ROS release into cytosol, which seems to occur through IMAC [115], can lead to RIRR activation in the neighboring mitochondria. ROS trafficking between mitochondria could constitute a positive feedback mechanism for enhanced ROS production potentially leading to significant mitochondrial and cellular injury.

Although externally generated $\text{O}_2^{\bullet-}$ and H_2O_2 can readily cross mitochondrial membranes, it is likely that H_2O_2 is the messenger molecule leading to whole cell RIRR because of its longer lifetime in the cytosol and higher permeability in membrane lipids [116]. In addition, it is conceivable that a phenomenon similar to RIRR can also depend on NO^{\bullet} . Indeed, NO^{\bullet} diffuses from mitochondria to cytosol, as well as from cytosol to mitochondria, a process called mitochondria-cytosol NO^{\bullet} cross talk [117]. Within mitochondria, NO^{\bullet} is able to act as an inducer of permeability transition [118], through a direct effect on the MPT pore and an indirect effect secondary to oxidative phosphorylation inhibition [119].

Excess oxidants may also augment mitochondrial ROS by upregulating the expression of the lifespan regulator, the 66-kDa isoform of growth factor adaptor Shc (p66^{Shc}) protein, which has been implicated in the development of aging and aging-related diseases [120]. The protein resides mainly in the cytosol [121], with a small fraction localized in the mitochondrial intermembrane space [122]. The protein is kept by thioredoxin (TRX) 1 and glutathione in the inactive reduced state. However, stress factors, including ROS, can increase the

expression of the protein that is activated by thiol oxidation which causes a dimer-tetramer transition [123]. Activated p66^{Shc} translocates to the mitochondrial intermembrane space where it associates with cytochrome c producing H₂O₂, which can trigger MPT pore opening [121]. The importance of p66^{Shc} *in vivo* has been demonstrated by observation that knockout of p66^{Shc} increases lifespan, reduces H₂O₂ generation, and enhances survival to oxidant stress [120].

Mitochondrial ROS production can be increased by mitochondrial fission. As it is known, mitochondrial shape can be modified by fusion and fission, resulting in elongated, interconnected mitochondrial networks and fragmented, discontinuous mitochondria, respectively [124].

Specific changes in mitochondrial shape suggest that morphology and function of mitochondria are closely linked, so that loss of fusion or division activity results in dysfunctional mitochondria [125]. An explanation for the importance of mitochondrial fusion could be the need for exchange of intermembrane space and matrix contents between mitochondria, so that defects and transient stresses may be partially buffered. On the other hand, mitochondrial division should create organelles of the appropriate size for transport along actin or microtubule networks [125].

The fission process seems to have a remarkable impact on ROS metabolism. Indeed, it seems that oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins [126], leading to reduced respiratory capacity and enhanced ROS production [127]. It has also been suggested that changes in mitochondrial network structure provide an example of ROS-mediated ROS generation where ROS play a role in mitochondrial fission to augment ROS generation from restructured mitochondria [128].

NO[•] appears to play opposite roles in mitochondrial fission-fusion. Indeed, it may enhance mitochondrial fragmentation and cell death in neurodegenerative diseases by its effects on dynamin-related protein-1 (Drp1), which promotes mitochondrial fission [129]. In myogenesis, NO[•] has the opposite effect promoting the fusion of mitochondria into an elongated network by inhibiting Drp1-mediated fission [130].

3.3. Other Cellular ROS Sources. Until recently, the functional significance of ROS sources different from mitochondria has received lesser attention. However, in recent years, greater attention has been turned to the potential role of ROS produced by outer sources in cell signaling and dysfunction.

Moreover, there are reasons to think that ROS, released by mitochondria, interact not only with other mitochondria but also with other sources of ROS. It is now apparent that there is a substantial interplay between ROS sources, so that activation of one can lead to activation of the others, resulting in RIRR that further increases ROS production and oxidative stress (Figure 2).

3.3.1. Peroxisomes. Normally, peroxisomes display mechanisms to maintain the equilibrium between production and scavenging of ROS, but in some situations antioxidant system capacity is overwhelmed. One such situation is the increase in

peroxisome numbers stimulated by a heterogeneous class of chemicals, known as peroxisome proliferators, whose effects are mediated by peroxisome proliferator activated receptors (PPARs) which belong to the family of nuclear transcription factors [131]. Whereas the expression of the genes of the lipid β -oxidation, particularly of acyl-CoA oxidase, is induced 10–30-fold depending on compound and dosage, the catalase does not exceed 1–2-fold induction [132]. The disproportionate increase of H₂O₂-generating oxidases in comparison to H₂O₂-scavenging catalase was suggested to be responsible for oxidative stress leading to the development of hepatic tumors in rodents treated with peroxisome proliferating compounds [133].

The central event in the carcinogenesis seems to be the activation of PPAR α , because PPAR α -/- mice, fed a diet containing a potent nongenotoxic carcinogen, are refractory to both peroxisome proliferating effect and carcinogenesis [134]. This is consistent with resistance to the carcinogenic effect of peroxisome proliferators of primates and humans, which have low hepatic levels of PPAR α [135].

Peroxisomes rely heavily on cross talk with other subcellular organelles, notably mitochondria, to further metabolize the end products of their metabolism [136]. Peroxisomes and mitochondria also share an intricate redox sensitive relationship [137] and seem to cooperate in the maintenance of cellular ROS homeostasis. It has been suggested that when mitochondrial H₂O₂ generation increases and the system constituted by glutathione peroxidase (GPX) and glutathione reductase (GR), limited by GSH and NADPH levels, is unable to cope with the increased H₂O₂, the peroxide that escapes across the mitochondrial membrane may be degraded by catalase in the peroxisomes [138].

Actually, the very high content of catalase inside peroxisomes suggests that these organelles may serve as an intracellular sink for H₂O₂. This idea is supported by the finding that overexpression of catalase in pancreatic islets of transgenic mice produces a marked protection of islet insulin secretion against H₂O₂ [139]. However, other studies indicate that peroxisomes represent a potential source of oxidative stress, causing cell damage or modulating redox sensitive pathways [140], and disturbances in peroxisomal redox metabolism lead to mitochondrial oxidative stress [141]. For example, human fibroblasts treated with a catalase inhibitor not only exhibit high levels of cellular H₂O₂, protein carbonyls, and peroxisomal numbers but also increase mitochondrial ROS levels and decrease mitochondrial aconitase activity and inner membrane potential, demonstrating that peroxisome oxidative imbalance elicits oxidative damage throughout the cell and in particular to mitochondria [142]. Peroxisome dysfunction also has a profound impact on mitochondrial function, as demonstrated by the observation that Pex5p (peroxisomal cycling receptor) knockout mice possess increased levels of mitochondria, which show structural abnormalities and alterations in the expression and activity of respiratory chain complexes [143]. Mitochondrial oxidative phosphorylation is also impaired, with consequent increase in mitochondrial ROS generation, in X-linked adrenoleukodystrophy, the most common peroxisomal disorder [144].

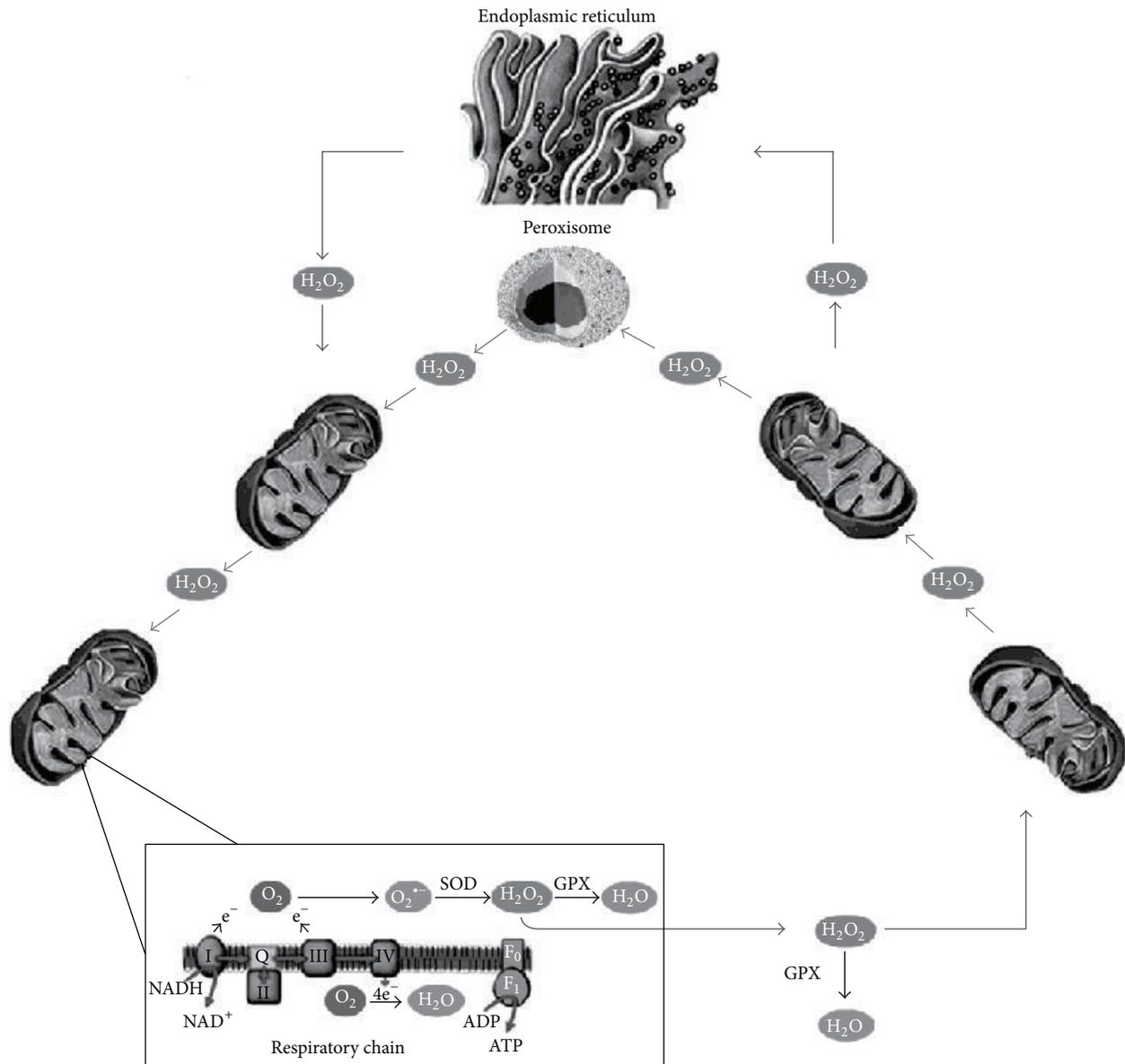


FIGURE 2: ROS propagation among mitochondria and other ROS sources. Mitochondrial ROS production can cause collapse of mitochondrial membrane potential and increase in ROS generation. ROS produced in only small number of mitochondria can influence neighboring mitochondria and other cellular organelles, eventually propagating the ROS surge to the whole cell (modified from Venditti et al. [116], with permission).

3.3.2. Endoplasmic Reticulum. ROS production in endoplasmic reticulum is normally neutralized by antioxidant system components, but in some conditions there is an excessive production of ROS leading to oxidative stress. An example is provided by the accumulation of unfolded and misfolded proteins in the ER lumen, a condition called ER stress, which has been associated with several diseases, such as neurodegenerative disorders, stroke, bipolar disorder, cardiac disease, cancer, diabetes, and muscle degeneration [145].

It is well documented that only properly folded proteins can be exported to the Golgi apparatus for further modification and translocated to their destined sites, while misfolded or incompletely folded proteins are retained in the ER [146].

ER stress can be due to extracellular stimuli and changes in intracellular homeostasis, including ER Ca²⁺, glycosylation, energy stores, redox state, and expression of proteins that are prone to misfolding. In response to ER stress, cells activate an adaptive response, the unfolded protein response (UPR), to resolve the protein folding defect. The UPR attenuates protein synthesis to reduce protein load, induces ER chaperone genes to accelerate protein folding, and degrades misfolded proteins by the ER-associated degradation (ERAD) machinery [24]. However, when ER stress is too severe or prolonged, processes need to be elicited to remove overstressed cells [147].

The ER protein folding homeostasis and ER redox state are closely linked since disulfide bond formation in the ER

lumen is highly sensitive to altered redox balance so that both reducing and oxidizing reagents disrupt protein folding and cause ER stress [148]. During oxidative protein folding, the thiol groups on cysteines of substrate peptides are oxidized and H_2O_2 is generated as a by-product. During ER stress, dysregulated disulfide bond formation and breakage may lead to oxidative stress by generating large amounts of H_2O_2 and depleting ER GSH [149].

Studies have indicated that ROS, produced in the ER during ER stress, subsequently caused mitochondrial dysfunction, impairing the respiratory chain, and increase mitochondrial ROS production particularly when ER stress is severe or sustained. Indeed, the increase in ROS levels causes a Ca^{2+} influx from the ER into the cytoplasm through the ER-localized channels and a large portion of the ion is taken up by the mitochondria resulting in ROS production [145]. Mitochondrial ROS can in turn increase further the ER stress response thereby amplifying ROS accumulation [150]. Thus, it seems that the ER is placed in a vicious cycle where ER stress can be caused by oxidative stress and increases the perturbed redox state. This process is likely favored by the existence of close ER-mitochondria contacts [151], which facilitate the ROS shift between organelle compartments.

ROS production can also be amplified when exposure to a variety of foreign compounds, such as phenobarbital (PB), increases the P450 levels. Induction by PB causes proliferation of the smooth endoplasmic reticulum with incorporation of the structural gene products (the P450s) into microsomal membrane [152]. In rat liver, increases in P450 levels, microsomal and mitochondrial protein content, and alteration of mitochondrial composition were reported [153]. PB-induced increase in P450 levels enhances H_2O_2 production by NADPH-supplemented liver microsomes [154] and gives rise to an increase in peroxidative reactions, involving the whole cell [153].

Although these results suggest a damaging role of P450 induction in hepatic tissue, a consequence of the high rates of production of ROS by P450 is its own labilization and subsequent rapid inactivation. Indeed, ROS, generated by some cytochrome P450 forms, are able to lead to oxidative inactivation of the P450 themselves modifying apoenzymes or oxidizing the heme groups. Attack on sulfur-containing amino acids results in the conversion of P450 into P420 [155], whereas attack on the heme moiety leads to its breakdown and loss of microsomal P450 [156]. Such a loss was observed during microsome catalytic turnover under the action of oxygen species generated at the hemoprotein active center [157]. The cytochrome P450 self-inactivation is important in situations in which increases in P450 levels occur due to uptake by the organism of enhanced amounts of xenobiotics.

Although P450 induction serves to help "detoxication" of xenobiotics, it can be considered an adaptive response that has survival value for the organism, as it can also increase "toxification" [158]. First, cytochrome P450 generates toxic chemically reactive intermediates from relatively unreactive compounds. Moreover, because cytochrome P450 inducibility is generally higher than the conjugating enzyme inducibility [159], there is a potential imbalance between the rate of generation of chemically reactive intermediates

and their rate of inactivation and removal. The reactive metabolites that are not conjugated and the ROS released by P450 may attack proteins, membrane components, or nucleic acids, leading to cytotoxicity, mutations, and cancer [160]. Therefore, P450 inactivation may be considered a mechanism to prevent cellular accumulation of high levels of the enzyme. Moreover, it appears to be part of a concerted adaptive response to oxidative stress, consisting of repression of ROS-generating systems and induction of antioxidant defenses, as observed in human hepatoma HepG2 cell lines, expressing CYP2E, the ethanol-inducible cytochrome P450 [161].

3.3.3. Lysosomes. Lysosomes contain the major pool of low mass redox-active intracellular iron, arising from the intralysosomal degradation of iron-containing proteins, such as ferritin, and iron-rich organelles, such as mitochondria [162]. Iron accumulation predisposes lysosomes to oxidant-induced damage and rupture with consequent cellular injury. Indeed, some H_2O_2 , formed outside the organelles and escaping the extralysosomal degradation, can diffuse into lysosomes and, together with that formed directly in the lysosomes, can generate $\cdot OH$ radicals by Fenton reaction. The ensuing oxidative damage on the lysosomal membranes leads to membrane permeabilization with release to cytosol of hydrolytic enzymes and low mass iron. This can relocate to other cellular sites, causing site-specific $\cdot OH$ production and oxidative damage in conditions of H_2O_2 production.

It seems that H_2O_2 formation and lysosomal destabilization are important for the radiation-induced cellular injury and death [163], which for a long time have been considered to depend on $\cdot OH$ formation due to radiolytic cleavage of water. Indeed, radiation, besides $\cdot OH$ radicals, produces significant amounts of H_2O_2 [164], which can enter the lysosomal compartment and cause membrane permeabilization. Employing a model of irradiated murine histiocytic lymphoma (J774) cells [163], it was found that the cells irradiated twice demonstrated progressive lysosomal damage from 2 h after the second irradiation, which in turn resulted in extensive cell death [163]. Irradiation-induced lysosomal disruption and cell death were significantly reduced by incubation with desferrioxamine conjugated with starch which forms a stable complex with iron and renders the metal inactive [163]. The protective effect of iron chelators against ionizing radiation damage was subsequently confirmed on several cell lines previously exposed to salicylaldehyde isonicotinoyl hydrazone, a lysosome targeted iron chelator [165].

3.3.4. NADH-Oxidase. Although multiple sources of ROS during ischemia-reperfusion have been identified, convincing evidence supports NADPH oxidases as important contributors to oxidant generation in several tissues, including cardiac tissue [166]. However, depending on the phase of IR injury, NADPH oxidases can be either detrimental or protective, shown to have a double-edged role. Low ROS levels are cardioprotective in pre- and postconditioning therapies, while high ROS levels are deleterious and lead to cardiomyocyte death [167]. In addition to the ROS level, the types of NOX expressed in cardiomyocytes and their localization

are also important in determining the cell fate. In cardiomyocytes, three NOXs are expressed, NOX1 and NOX2, predominantly localized to the plasma membrane, and NOX4, which is constitutively active and is localized on the intracellular membranes of organelles [168]. Although the role of NOX isoforms in ischemia-reperfusion has not yet clarified, a study showed that deleterious effects of NOX1 and NOX2 occurred during reperfusion phase in agreement with the idea that oxygen supply during reperfusion provides substrate for NOX-mediated ROS generation [169]. Moreover, indications were also obtained that NOX2 generated higher levels of ROS than NOX1, leading to direct myocardial damage [169].

Several examples of interplay between mitochondrial and NOX-derived ROS have been reported [170]. In the whole, they suggest the presence of a feedforward cycle in which NADPH oxidases increase mitochondrial ROS that further activate cytoplasmic NADPH oxidases and increase cellular ROS production.

The NADPH oxidase involvement in the ROS-induced ROS production is very well documented. For example, exposure of smooth muscle cells and fibroblasts to exogenous H_2O_2 activates these cells to produce $O_2^{\bullet -}$ via a NADPH oxidase [171]. This mechanism could help to explain why micromolar concentrations of H_2O_2 cause oxidant-mediated injury to many different types of cells during chronic oxidative stress. Moreover, H_2O_2 -induced NADPH oxidase activation in nonphagocytic cells could be an important mechanism by which the degree of oxidative stress, as well as the subsequent cellular damage, is amplified during inflammatory disorders.

It was also observed that endothelial levels of xanthine oxidase, a source of H_2O_2 and $O_2^{\bullet -}$, depend on NADPH oxidase [172]. The study of the mechanism showed that H_2O_2 was able to induce transformation of xanthine dehydrogenase into xanthine oxidase [173]. The observation that increase in $O_2^{\bullet -}$ production was a late effect of cell exposure to H_2O_2 suggested that the peroxide not only stimulated conversion of xanthine dehydrogenase into xanthine oxidase but also activated the NADPH oxidase, leading to prolonged H_2O_2 production and sustained xanthine dehydrogenase conversion [173].

4. ROS as Signaling Molecules

ROS are now appreciated as signaling molecules that regulate a wide variety of physiological functions. Indeed, they play crucial roles in gene activation [174], cellular growth [175], and modulation of chemical reactions in the cell [176]. They also participate in blood pressure control [177], are mediators in the biosynthesis of prostaglandins [178], function in embryonic development [179], and act as signaling molecules within the individual cell and among cells during their lifespan [180].

An important development in the field of ROS beneficial effects was the discovery that, in organisms from simple bacteria to complex mammals, ROS are able to induce redox sensitive signal cascades leading to increased expression of antioxidant enzymes. The increase in effectiveness of the antioxidant defense system provided by this genetic response

enables cells to survive an oxidant exposure that would normally be lethal.

In mammals, gene transcription determining cell survival can be activated by ROS in two ways: either via transcription factors, which can interact directly with specific DNA motifs on promoters of target genes, or via activation of mitogen-activated protein kinase cascades, which in turn activate transcription factors that trigger target gene transcription [181].

There are two ideas concerning the mechanism by which ROS initiate cellular signaling, namely, modification of target protein molecules and changes of intracellular redox state [182], even though the distinction between them is not easy.

While ROS, such as $\bullet OH$, may cause irreversible damage to macromolecules with low specificity, the main target of a mild oxidant, such as H_2O_2 , is thiol groups of protein cysteine residues. Oxidation of these residues forms reactive sulfenic acid (-SOH) that can form disulfide bonds with nearby cysteines (-S-S-) or undergo further oxidation to sulfinic (-SO₂H) or sulfonic (-SO₃H) acid. These modifications result in changes in structure and/or function of the protein. With the exception of sulfonic acid and to a lesser degree sulfinic acid the modifications are reversible by reducing systems such as thioredoxin and peroxiredoxins [183].

The cytosol redox state is normally achieved by the "redox-buffering" capacity of intracellular thiols, such as GSH and thioredoxin, which counteract cellular oxidative stress by reducing H_2O_2 . The high ratios of reduced to oxidized forms are maintained by the activity of GSH reductase and TRX reductase, respectively. Accumulating evidence suggests that GSH and TRX participate in cell signaling processes. GSH can regulate redox signaling by alterations both in the level of total GSH and in the ratio of its reduced to oxidized forms, while TRX can regulate the activity of some proteins by directly binding to them [182].

Whatever the mechanism by which ROS initiate cell signaling, there is increasing evidence that ROS cellular levels are strongly linked to the regulation of cellular antioxidant levels. A well-known example of this phenomenon is Nrf2. This transcription factor regulates the expression of several antioxidant and detoxifying genes by binding to promoter sequences containing a consensus antioxidant response element [184]. In turn, the subcellular localization and hence activity of Nrf2 are at least in part regulated by its interaction with specific reactive cysteine residues on an inhibitory protein called Keap1 [185].

Interestingly, ROS not only are involved in the regulation of the expression of antioxidant genes but also interact with critical signaling molecules such as MAP kinases, PI3 kinase, PTEN, and protein tyrosine phosphatases, to initiate signaling in several cellular processes, including proliferation and survival [186].

4.1. ROS Role in Protection against Tissue Excessive Dysfunction. An enhanced ROS production in a cellular site can involve further ROS production by other cellular sources giving rise to a self-destructive phenomenon as well as the propagation of a fire in a room containing inflammable material. If, as in the fire example, it occurs to confine the

more dangerous focuses and, above all, avoid that the fire reaches the other rooms and the entire house catches fire, the cell exposed to oxidative stress should be equipped with mechanisms able to avoid its death or that of surrounding cells, which should lead to excessive tissue dysfunction.

Several results indicate that ROS-linked mechanisms are operative to provide tissue protection against excessive dysfunction, in which mitochondrial systems seem to play a major role.

4.1.1. Mitoptosis. High mitochondrial ROS production triggers opening of the MPT pore leading to potentially significant mitochondrial and cellular injury. However, in many cases mitochondrial swelling due to megachannel opening is a signal for programmed mitochondrial destruction [187], a phenomenon called mitoptosis [188], which was proposed to represent a line of the antioxidant defense [70].

It is well-established that the mitochondrial population is heterogeneous with regard to its characteristics because it consists of fractions with different properties [189]. Studies on liver mitochondrial fractions, resolved by differential centrifugation, suggested that the light fractions, with low respiratory activity, contained transitional forms in the process of development into the heavy mitochondrial structures with high respiratory activity [190]. The heavy fraction also exhibited the lowest antioxidant level [190, 191] and the highest rates of H_2O_2 production and susceptibility to Ca^{2+} -induced swelling [190]. It is interesting that conditions leading to increased ROS production, such as exercise [192] and cold exposure [193, 194], decrease the amount of heavy mitochondria and increase that of light mitochondria in rat muscle and liver, respectively. This suggests that, in rat tissues, conditions of increased ROS production favor the substitution of the oldest ROS-overproducing mitochondria with neofomed mitochondria endowed with a smaller capacity to produce free radicals [190]. If so, the mechanism that, during oxidative stress, enhances the swelling of Ca^{2+} -loaded mitochondria constitutes a negative feedback loop. In fact, the perturbation itself, represented by an enhancement in ROS generation, should lead to accelerated mitoptosis, thus limiting tissue oxidative damage during oxidative stress.

4.1.2. Autophagy. Autophagy is a cytoprotective process by which organelles and bits of cytoplasm are sequestered in double-membrane vesicles, called autophagosomes, and subsequently delivered to lysosomes for hydrolytic digestion [195]. Autophagy serves as an adaptation strategy for stress conditions, such as amino acid starvation, unfolded protein response, or viral infection, and it is widely accepted that it regulates cell homeostasis by adjusting organelle number and clearing damaged structures. However, if autophagy prevents or promotes cell death and if it is a selective or nonelective process are still controversy questions. It is likely that autophagy can be involved in both survival and death. It assures cell survival when it removes damaged organelles that might activate programmed cell death as apoptosis (type I cell death) [196]. On the other hand, it promotes cell death when it is excessive and deregulated, since enzymes leaking

from lysosomes, such as cathepsins and other hydrolases, can initiate mitochondrial permeabilization and, eventually, apoptosis [197].

Clearly, autophagy leading to cell death is not selective, whereas the controversy persists about autophagy developing as a survival process. The observation that autophagosomes often contain various cytoplasmic elements, including cytosolic proteins and organelles [198], suggested that autophagy is a nonspecific form of lysosomal degradation. However, subsequent observations showed that the autophagy can be a selective process in which specific proteins or organelles are delivered to the autophagosome for degradation [197].

Such selective types of autophagy include selective degradation of mitochondria (mitophagy) [197], peroxisomes (pexophagy) [199], endoplasmic reticulum (reticulophagy) [200], or even nucleophagy during which parts of the nucleus are specifically degraded by an autophagic process [201].

Mitochondria subjected over time to multiple attacks become damaged and possibly dangerous to the cell, so that elimination of such mitochondria is essential to protect cells from the harm due to their disordered metabolism. The view that autophagic processes can remove damaged and dysfunctional mitochondria was directly confirmed by experiments in which selected mitochondria inside living hepatocytes were subjected to laser-induced photodamage [202]. Mitochondrial depolarization and inner membrane permeabilization seemed to be required for autophagy signaling [202] suggesting involvement of MPT pore opening and swelling in the mitophagy.

A growing body of evidence now suggests that processes of autophagy and/or apoptosis involving other cellular organelles are able to protect tissues in conditions leading to oxidative stress.

Peroxisomes were the first organelles for which selective organelle degradation by autophagy was described [203]. Studies on selective degradation of peroxisomes in methylotrophic yeasts showed that when grown on methanol as the carbon source, yeast species included several large peroxisomes containing the enzymes necessary to assimilate methanol. When methanol grown cells were shifted to a different carbon source, such as glucose or ethanol, whose metabolism did not involve these peroxisomal enzymes, pexophagy occurred [199]. Hence, this autophagic process occurred during a rapid intracellular remodeling process to remove organelles containing enzymes no longer needed for methanol utilization.

More recent studies indicate that autophagic degradation of peroxisomes in yeast also occurs as part of cellular house-keeping [204]. It was observed that, during chemostat cultivation of wild type *H. polymorpha* cells, entire peroxisomes were constitutively degraded by autophagy during normal vegetative growth, likely to enable the cells to rejuvenate the peroxisome population.

There is convincing evidence that ER stress and reticulophagy induction are strongly linked. For example, it was reported that, during unfolded protein response to ER stress, induced by DTT, portions of the ER were sequestered by double-membrane vesicles, similar to autophagosomes [200].

The presence of ribosomes on the outer membrane of these structures suggested a role of the ER as a membrane source for this type of autophagosomes, which subsequently fused with the vacuole releasing its content for degradation. This process was highly selective as cytosol and other organelles were not included into the vesicles [200]. Not much is known about ER degradation and its role in ER maintenance, but it is possible that this selective pathway serves to degrade damaged portions of the ER or resize it after the folding stress induced enlargement.

Although numerous factors and signaling pathways contribute to autophagy induction in different cellular contexts, ROS are indicated to be essential signals to activate autophagy stress by several stimulating conditions [205]. ROS appear to be implicated in the regulation of autophagy through distinct mechanisms, depending on cell types and stimulation. Autophagy, in turn, can reduce ROS production removing ROS-overproducing organelles.

It seems that ROS, generated from both mitochondria and NADPH oxidases, activate autophagy to protect cells from nutrient starvation, dysfunctional mitochondria, cell death, and invading pathogens [206]. The involvement of ROS as signaling molecules in starvation-induced autophagy was demonstrated for the first time showing that starvation triggered accumulation of ROS, most probably H_2O_2 , which was necessary for autophagosome formation and the resulting degradation pathway. The oxidative signal is partially dependent on phosphatidylinositol 3 kinase (PI3K), which plays a critical role in the early stages of autophagosome formation. Furthermore, a direct target for oxidation by H_2O_2 , the cysteine protease Atg4, has been identified [207]. Whereas mitochondria were found to be involved in starvation-induced autophagy [208], activation of antibacterial autophagy is due to NADPH oxidase that generates ROS necessary for targeting of the autophagic protein LC3 to the phagosome [205].

4.1.3. Autophagic and Apoptotic Cell Death. Autophagy usually promotes cell survival but when cellular damage is pronounced and survival mechanisms fail, death programs are activated in response to oxidative stress. The activation of the autophagic pathway beyond a certain threshold has recently been found to directly promote cell death by causing the collapse of cellular functions as a result of cellular atrophy (autophagic or type II cell death) [209]. This form of autophagic cell death, which is necessary under certain conditions, such as in apoptosis-defective cells [210], is activated in response to oxidative stress in nervous cells, as shown by oxidative stress and autophagic death induced in human neuroblastoma SH-SY5Y cells [211] and sympathetic neurons [212] by dopamine and growth factor deprivation, respectively.

Autophagy can also lead to the execution of apoptotic or necrotic (type III cell death) programs, likely via common regulators such as proteins from the Bcl-2 family [196]. Apoptotic death is an adaptive process, allowing for renewal of the organism constituents and life maintenance, which,

differently from necrosis, is a form of cell death that causes minimal damage to surrounding cells.

Apoptosis plays an important role in the elimination of unnecessary, damaged, or diseased cells during the whole lifespan and particularly during embryogenesis, when a majority of newly formed cells undergo programmed cell death. Although both types of cell death have been linked to autophagy, cell death does not necessarily result from a previous autophagic process. Depending on the cellular context and death trigger, apoptosis and necrosis either cooperate in a balanced interplay involving autophagy or are employed by cells in a complementary way to facilitate cellular destruction. A further complication results by the observation that apoptosis, necrosis, and autophagy are often regulated by similar pathways, engage the same subcellular sites and organelles, and share initiator and effector molecules.

An example is supplied by the cellular response to MPT pore opening. This process provides a common pathway leading to mitophagy, apoptosis, and necrosis [213]. With low intensity stress, limited MPT leads to mitoptosis, which is followed by the elimination of degraded mitochondria. Recent studies have shown that removal can occur through two different processes which rid cells of damaged mitochondria, the formation of mitoptotic bodies which are then extruded from the cell [214] or selective autophagy [215]. With increasing stress MPT involves an increasing proportion of mitochondria and cellular response progresses from mitoptosis to apoptosis driven by mitochondrial release of cytochrome c and other proapoptotic factors. This event occurs if permeabilization affects a great number of mitochondria, in the absence of excessive reduction in ATP levels, and can purify tissue from cells that produce large amounts of ROS [70]. Lastly, when extreme stress causes MPT pore opening in virtually all cellular mitochondria, ATP levels drop, and, because of bioenergetic failure, neither autophagy nor apoptosis can progress, and only necrotic cell death, which is not dependent on energy supply, ensues [197].

Another example involves cellular responses to ER stress, which include the activation of UPR, autophagy, and cell death [216]. These processes are not mutually exclusive, and there is significant cross talk between these cellular stress responses. Autophagy upregulation during ER stress is a pro-survival response directed to removal of unfolded proteins, protein aggregates, and damaged organelles, which is triggered in order to relieve the stress and restore ER homeostasis [217]. However, prolonged or unresolved ER stress results in apoptotic program activation [218]. Sometimes, the effect of autophagy on cell survival in ER stress also depends on the tissue type. In colon and prostate cancer cells, ER-induced autophagy protects against cell death, whereas in normal colon cells autophagy does not alleviate ER stress but rather contributes to ER-induced apoptosis [219].

Recent studies have shown several pathways that mediate the interplay between autophagy and apoptosis providing mechanistic insight into the network regulating both processes [220]. However, the mechanism by which ER stress induces apoptosis is not fully delineated, even though it is clear that mitochondria-dependent and independent cell

death pathways can mediate apoptosis in response to ER stress.

In ER-mitochondria-independent cell death pathway, caspases are required for apoptosis, and some members of this family of proteases are associated with the ER. In rodents, caspase-12 is ER-membrane proapoptotic protease specifically activated upon prolonged UPR. Several pathways involve caspase-12 activation in cell apoptosis after ER-induced stress. A pathway triggers apoptosis by ER stress-specific caspase cascade leading to the caspase-9 and caspase-3 activation in cytochrome c and apoptotic protease activating factor-1 (Apaf-1) independent manner [221].

ER stress can also trigger apoptosis by mitochondria-dependent pathways [222] which can share actors with mitochondria-independent pathways. The phosphorylation of inositol-requiring enzyme 1 (IRE1) leads to the phosphorylation of c-Jun N-terminal kinase (JNK) that, in turn, can activate p53 transcription factor. The p53-induced expression of a protein, the Bcl-2 interacting mediator of cell death (Bim) [223], leads to the formation of the BAX/BAK pore on the outer mitochondrial membrane and cytochrome c release resulting in activation of the canonical apoptosis pathway.

Whereas the relationship between mitochondrial oxidative stress and cell death is well-established [224], that of peroxisomes in cell death pathways is just beginning to emerge. In light of the strong impact of peroxisome dysfunction on mitochondria, it is possible that peroxisome-derived mediators of oxidative stress have an influence on mitochondria that would have profound implications for cell fate.

In fact, it has been reported that excess ROS generated inside peroxisomes quickly perturbs the mitochondrial redox balance and leads to remarkable mitochondrial fragmentation [225]. In a recent study using targeted variants of the photosensitizer Killer Red it has been showed that the phototoxic effects of peroxisomal Killer Red induce mitochondria-mediated apoptotic death and that this process is inhibited by targeted overexpression of antioxidant enzymes, including peroxisomal glutathione S-transferase kappa 1, superoxide dismutase 1, and mitochondrial catalase [226].

Over the last decades, the intricate molecular events underlying the process of apoptosis have been elucidated [227]. It is apparent that crucial steps involve mitochondrial release of proapoptotic factors, although the exact mechanisms involved in this release are less well understood. In this regard, it seems that, in some circumstances, the release into the cytosol of lysosomal constituents may be an initiating event in apoptosis and that mitochondrial release of proapoptotic factors might be a consequence of earlier lysosomal destabilization [228].

The destabilization of the lysosomal membrane is due to its vulnerability to oxidative stress, because agents, which induce oxidative stress, such as H_2O_2 [229] and radiation [230], also promote lysosomal membrane rupture. Different cell types but also cells of the same type and lysosomes of individual cells exhibit different lysosomal resistance to oxidative stress [231]. It has been suggested that such a resistance mainly depends on the capacity to degrade H_2O_2 before it reaches the acidic vacuolar compartment, resistance to ROS

of lysosomal membranes, and lysosomal content of redox-active iron [231]. Lysosomal membrane permeabilization per se triggers intracellular formation of ROS, a process which can be mediated by the action of lysosomal proteases, such as cathepsins B and D, which leak into the cytosol [208]. These enzymes affect mitochondria inducing further cytochrome c release and activation of caspase-mediated cell death [208, 228]. The involvement of lysosomes and their iron content in radiation-induced cell death is supported by the observation that cells are significantly protected from radiation damage if exposed to iron chelators [163, 165]. Conversely, the involvement of lysosomal proteases in cell death is supported by the observation that apoptosis inhibition by cyclosporin A-induced block of MPT pore opening favors the development of a necrotic form of cell death, which is attenuated by ROS scavengers and inhibition of cathepsin D activity [232].

5. Harmful and Beneficial Effects of ROS during Exercise

It is well documented that acute physical exercise can produce significant damage, including alterations in membranes of mitochondria and sarcoplasmic and endoplasmic reticulum [233–236] in skeletal muscles and other tissues. The contraction form most damaging to skeletal muscle is that in which the muscle is contracting while being lengthened (eccentric contraction). During such a contraction, disruption of cytoskeletal structures, loss of muscle force generation, and influx of phagocytic cells and neutrophils into the damaged fibers occur [237].

Different from acute exercise, aerobic physical activity regularly performed (training) induces adaptive responses in the whole organism and particularly in the cardiorespiratory and musculoskeletal systems [238], which lead to an increased ability to perform prolonged strenuous exercise [235]. Moreover, it has several healthy effects, including the maintenance of insulin sensitivity and cardiorespiratory fitness, so that it is able to prevent type 2 diabetes [239] and coronary heart diseases [240], and can also be used as adjunctive therapy in the treatment of patients with diabetes [241] and chronic heart failure [242].

To date, the idea that the opposite effects of acute exercise and training are in great part due to the ability of ROS to play a dual role in animal organisms is widely shared. In fact, it is well documented that, during a single session of prolonged aerobic exercise, ROS production increases and this can lead to cellular damage and dysfunction. On the other hand, some results suggest that the ROS produced during each session of a training program can act as factors inducing cellular adaptations to exercise.

During physical activity several ROS sources can be activated contributing to the oxidative damage and/or to the adaptive processes. It is reasonable to imagine that an initial source of ROS can activate the ROS release from other sources, inducing a positive feedback loop. In the next sections, we will point out our attention to what is known about production, sources, and double action of ROS during acute exercise and training.

6. Acute Exercise

6.1. ROS Production. ROS involvement in the tissue damage found after prolonged aerobic exercise was suggested in the late 1970s [243], but there is still no direct evidence that ROS production increases during exercise. Electron spin resonance (ESR) spectroscopy was able to furnish direct information of the presence of free radical species, but such a technique allows obtaining information on the ROS production after exercise. Davies et al. [244] first reported that signals of free radicals were enhanced in rat muscle and liver after a bout of exhaustive running. Subsequently, increased generation of free radical signals was observed in rat heart after an acute bout of exhaustive endurance exercise [245]. The idea that free radical activity might increase after extensive muscular activity was also confirmed on skeletal muscles subjected to electrical stimulation [246]. Using ESR spectroscopy in conjunction with the spin tapping technique, enhanced free radical concentration in human serum following exhaustive exercise was found [247, 248] which was prevented by ascorbic acid supplementation [249].

Indirect information on ROS production during acute exercise has been obtained by the changes in indexes of oxidative damage to lipids, proteins, and DNA and in the cellular redox state. A plethora of information exists concerning the increase in such indexes in various animal species, including human, but here we focus our attention on what is available in literature on rat.

Lipid oxidation can be evaluated measuring tissue levels of oxidized lipids, such as lipid hydroperoxides, or those coming from their degradation, such as malondialdehyde (MDA) and 4-hydroxyl-2-nonenal. Numerous studies showed increase in lipid oxidation markers in skeletal [244, 250–254] and cardiac muscle [251–253, 255], liver [244, 250–254, 256], brain [257], erythrocytes [258], and kidney [254] of untrained rats after acute exercise.

Exercise-induced increases in protein carbonyl content, a marker of protein oxidative damage, were reported in rat skeletal [253] and cardiac muscle [253, 255], liver [253, 256], and plasma [259]. Furthermore, increases in 8-hydroxy-2'-deoxyguanosine (8-OH-dG), a marker of oxidative damage to DNA, were also found in rat skeletal [260] and cardiac [255] muscle, liver, and lung [260] after acute exercise.

Some studies reported that the extent of oxidative damage depends on duration or intensity of exercise. One such study showed that exhaustive maximal exercise caused plasma MDA increase while short periods of submaximal exercise (less than 70% $\text{VO}_{2\text{max}}$) reduced lipid peroxidation [261]. Liver oxidative damage indexes did not change after 5 h of swimming and increased after 8 h of exercise [192]. Another study showed that an acute bout of a moderate as well as high intensity exercise led to an increase in malonaldehyde and lipid hydroperoxide levels in red vastus, white vastus, and soleus muscle [262]. The study also showed that when intensity of exercise was considerably decreased, lower MDA levels were found [262].

Reduced glutathione (GSH), a thiol-containing tripeptide playing a vital role in maintaining cells in the reduced state and in protecting tissues from oxidative stress [263],

is involved in reducing radicals arising from a variety of antioxidants, such as α -tocopherol and ascorbic acid, to the native structure [9]. GSH is oxidized to glutathione disulfide (GSSG) donating a pair of hydrogen atoms. The ratio of GSH to GSSG is used as an indicator of intracellular GSH redox status. A decrease in GSH/GSSG ratio suggests that the production of ROS goes over the reducing capacity of GSH and other antioxidants. Therefore, change of GSH redox status has been used as a footprint of oxidative stress during exercise. Several studies showed that prolonged exhaustive exercise promoted oxidation of GSH to form GSSG in the blood [264], liver [253, 264], heart [253, 265], and skeletal muscle [253, 264] of rats, even though it is possible that the GSH redox state in the muscle is not altered by short term exercise. Indeed, it was reported that physical exercise at submaximal level determined a progressive depletion of liver GSH to about 20% of the levels found in sedentary rats which persisted for several hours following the cessation of exercise [266]. However, skeletal muscle appeared to be spared by this severe depletion phenomenon, whereas the levels of plasma glutathione exhibited a transient increase at the beginning of the exercise bout followed by a linear decrease with increased running time [266]. In liver, the early decrease in GSH level during exercise did not depend on increased ROS production, even though it could be one of the factors inducing liver oxidative stress. Evidence that a decrease in liver GSH content precedes oxidative stress was supplied by the finding that prolonged exercise led to increased lipid peroxidation and decreased GSH content, while exercise of shorter duration was not able to induce oxidative stress in liver, although it reduced GSH content [192]. The liver GSH reduction can be due to several factors. It has been suggested that the exercise linked increase in plasma levels of glucagon, vasopressin, and epinephrine stimulates hepatic efflux of GSH which is delivered to tissues, such as skeletal muscle that necessitates a larger tripeptide supply and its uptake from the plasma [267, 268].

6.2. Sources of ROS during Physical Activity. Notwithstanding most work indicates that exercise increases ROS production in rat tissues, the debate about the cellular sources of such ROS is still open. Several intracellular sources of free radicals have been identified and it is possible that all contribute to the increased ROS production during exercise even though the extent of their contribution can depend on several factors including the type of tissue and exercise.

Mitochondria, NADPH oxidase, and xanthine oxidase are considered the main ROS sources during exercise but it is necessary to point out that reports concerning other cellular sources are scarce or lacking.

6.2.1. Mitochondria. Mitochondria were long considered the main source of ROS in the cell during physical activity. Initially, this belief stemmed from the widespread idea that in tissue free radical production was closely related to oxygen consumption [269]. Since over 90% of the oxygen consumed by a mammal is utilized in the mitochondria, which appear to generate free radicals in all tissues studied [270], it was speculated that the increase in muscular oxygen consumption

during exercise was associated with an increase in free radical production by the respiratory chain localized in the inner mitochondrial membrane [269].

During muscle contraction, ADP concentration increases and stimulates mitochondrial oxidative phosphorylation [271], shifting the mitochondrial respiration nearer to State 3 than to State 4 oxygen consumption. Therefore, the close link between ROS production and O_2 consumption should require that the percentage of total electron flow escaping from the respiratory chain to reduce O_2 to superoxide radical (the mitochondrial free radical leak) is not modified during the transition from State 4 to State 3 happening during muscle contraction. However, this idea is theoretically and experimentally inconsistent. The rate of the mitochondrial ROS generation is related to the degree of reduction of electron carriers able to donate electrons to oxygen, and such a degree of reduction decreases during transition from State 4 to State 3 [272]. In agreement with such prediction, measurements performed on isolated mitochondria show that ROS mitochondrial release is higher in State 4 and in all conditions when the rate of electron-transfer is lowered [272].

Nevertheless, results obtained measuring ROS release from mitochondria isolated from tissues of animals that exercised suggest that mitochondrial ROS release might increase during prolonged aerobic exercise not only in muscle but also in other tissues, such as liver and heart [272].

An increased release of ROS during basal and stimulated respiration, with respiratory substrates linked to Complex I (pyruvate plus malate) or Complex II (succinate), was reported for mitochondria isolated from the muscles of the hind limbs [273], gastrocnemius (red portion), heart, and liver [253] of rats subjected to prolonged swimming. Increased ROS release was also reported following prolonged treadmill running [274] in heart mitochondria. The increased ROS release was associated with an increase in the levels of oxidative stress markers in mitochondria isolated from cardiac and skeletal muscles and liver [253] of rats subjected to prolonged aerobic exercise. It was also accompanied by alterations in mitochondrial functionality. Indeed, exercise increased State 4 respiration in liver, muscle [253], and heart [253, 274] mitochondria and decreased State 3 respiration in liver and muscle mitochondria [253]. Such results suggest the possibility that, whatever the initial source of ROS during exercise is, such ROS can damage mitochondrial components inducing both functionality impairment and increasing ROS release. It was initially proposed that the ROS formation during exercise could involve the loss in the cytochrome oxidase activity and a consequent increase in the electron pressure within the respiratory chain [275]. The finding that in hind limb muscle mitochondria exercise-induced change in mitochondrial respiration is not associated with changes in the cytochrome oxidase activity [273] seems to exclude such a possibility suggesting that oxidative modifications of other components of mitochondrial membrane are involved. Oxidative modifications of lipids and proteins located in the inner mitochondrial membrane could be responsible for the increase in State 4 respiration. The increase in State 4 respiration rate represents a compensatory response to the increased leak of protons back in the mitochondrial matrix. It has

been proposed that adenine nucleotide translocase (ANT) and uncoupling proteins (UCPs) are involved in proton conductance of mitochondrial membrane [276]. However, the observation that in skeletal muscle ANT expression is not affected by acute exercise [277] excludes that ANT is responsible for the increase in State 4 respiration induced by exercise. Moreover, it is known that an uncoupling protein 1 (UCP1) catalyzes inducible proton conductance in brown adipose tissue (BAT) [278], and exercise upregulates UCP1 homologue expression in skeletal [279] and cardiac muscle [274]. However, whether in tissues differently from BAT UCP1 homologues are responsible for mitochondrial basal or inducible proton conductance is yet controversial [280].

More support is available for the idea that the observed enhancement in State 4 respiration is due to high production of ROS and RNS, which seem to be able to affect proton leak through an indirect mechanism. Both $O_2^{\bullet-}$ [281] and $ONOO^-$ [282] increase mitochondrial proton leak by enhancing the extent of peroxidative processes [282, 283]. Therefore, it is conceivable that the increase in State 4 respiration induced by exercise may be due to a lipid peroxidation-mediated increase in proton leak.

The decrease observed in State 3 respiration can be due to a direct action of ROS and/or RNS. Indeed, damage to respiratory chain components by ROS [284] and inhibition of mitochondrial function by $ONOO^-$ [109] have been reported. The decline of the respiration rate in mitochondria from rats that exercised is likely to involve $ONOO^-$, which during exercise could be formed in greater amount and cause irreversible inhibition of many mitochondrial components different from cytochrome aa3 [285].

Inhibition of mitochondrial function and increase in ROS release could also be due to the increase in mitochondrial Ca^{2+} content, which occurs in skeletal muscle after prolonged exercise [286] and leads to oxidative phosphorylation inhibition [287]. The Ca^{2+} effect on mitochondrial function results from induction of mitochondrial permeability transition (MPT) [114], which leads to degradation of the heaviest mitochondrial subpopulation characterized by high respiratory capacity and susceptibility to Ca^{2+} -induced swelling [190].

Following long-lasting exercise, a transfer of damaged mitochondria in the lightest fraction happened in rat liver, as demonstrated by the increase in protein percent content in such a fraction and its decrease in the heaviest one [192], whereas a similar transfer did not happen in skeletal muscle [273]. This agrees with the observation that acute exercise enhances mitochondrial resistance to Ca^{2+} overload in human skeletal muscle [277]. The Ca^{2+} tolerance of mitochondria after exercise could be due to the maintenance of relatively high levels of GSH, which should prevent mitochondrial membrane potential collapse [288] which precedes MPT pore opening. Although this can appear as a protective mechanism, it slows removal of ROS-overproducing mitochondria thus enhancing exercise-induced muscle oxidative damage and dysfunction.

6.2.2. NADPH Oxidases. In the cells of skeletal muscle, the isoform NOX2 is expressed [289] and protein subunits have

been identified in transverse tubules and triads obtained from rabbit skeletal muscle but not in sarcoplasmic reticulum vesicles [290]. Some authors suggested that this is one of the main sources of ROS during *in vitro* muscle contraction or electrical stimulation [291, 292]. Moreover, it was demonstrated that muscle contraction increased $O_2^{\bullet-}$ in cytosol and subsequently in mitochondria, suggesting that NADPH oxidase could be a potential primary source of ROS production during muscle contraction [293]. It is conceivable that during muscular activity the increased activity of NADPH oxidase can produce excessive ROS release which can contribute to damage cellular components. However, it is also possible that such a release can have beneficial roles. The increased level of ROS induces changes in intracellular calcium levels which are the result of oxidative modification of calcium channels or other proteins involved in calcium signaling [294] and it has been suggested that NOX2 ROS production may be necessary for the excitation-contraction coupling process [290].

The mechanism of activation of NADPH oxidase has not been completely defined but a recent paper suggests that this can involve the increase in ATP release. Electrical stimulation of adult muscular fibers isolated from the muscle flexor *digitorum brevis* activates a voltage gated L-type calcium channel (Cav1.1) with each depolarizing event. This activation induces ATP release via pannexin-1 (PnX1) channel which colocalizes in the transverse tubules with Cav1.1 [295]. These events, in turn, trigger a signaling cascade where, through ATP activation of a purinergic receptor (P2Y), phosphatidylinositol 3-kinase- γ and phospholipase C and consequently protein kinase C, which activates NOX2 oxidase and ROS release [296], are activated. It is conceivable that a similar activation can happen also in *in vivo* muscle because an increase in ATP concentration has been found in the interstitial muscular space after exercise and contraction [297, 298].

6.2.3. Xanthine Oxidases. Studies performed using XO inhibitors suggested a potential role of enzyme XO as a source of oxidative stress, during ischemia-reperfusion in various tissues, such as intestine, heart, lung, kidney, and liver [98, 299, 300]. In particular, XDH/XO activity in liver has been reported to be relatively higher with respect to other organs [301, 302]. On the other hand, it has been demonstrated that liver blood flow during severe exercise may be reduced to half of normal, indicating that exercise can induce ischemia or hypoxia in the hepatic tissue [303]. These findings would suggest that XO produces free radicals which may influence the function of hepatic cells during and/or after strenuous exercise. Furthermore, inosine and hypoxanthine produced by the skeletal muscle during severe exercise have been reported to be taken up by the liver via the blood stream and oxidized to uric acid mainly after exercise [304]. According to these observations, it was shown that the liver has a substantially higher risk of oxidative stress following a single bout of exhausting exercise, rather than during the exercise itself [305].

It has been reported that xanthine oxidase produces $O_2^{\bullet-}$ in the contracting rat [306] and human skeletal muscles [307]. However, there is controversy about the role xanthine

oxidase plays in $O_2^{\bullet-}$ production of human skeletal muscle during contraction, because such a muscle appears to possess low amounts of xanthine dehydrogenase or oxidase [308], even though these enzymes are present in associated endothelial cells. It has been speculated that postexercise oxidative stress in mouse skeletal muscles may be due to the conversion of XDH into XO in capillary endothelial cells [309] and enhanced adenosine 5'-triphosphate (ATP) degradation together [310].

More recently, it was found that the administration of allopurinol, XO inhibitor, did not prevent the increase in protein and lipid oxidative stress markers in rat plasma, erythrocytes, and gastrocnemius muscle due to swimming exhaustion [311]. Moreover, in rats treated with allopurinol alone, a similar increase in protein and lipid oxidative stress markers in erythrocytes and gastrocnemius muscle was found. The combination of allopurinol and exercise appeared to increase protein oxidative damage in plasma and protein and lipid oxidative damage in erythrocytes. Interestingly, allopurinol provoked a marked reduction in physical performance as demonstrated by the 35% decrease in the swimming time to exhaustion [311]. This result is in contrast with another work reporting a lack of effects of allopurinol administration on the time to exhaustion in rats performing treadmill running [305]. The discrepancy can be due to the different type of exercise and/or to the different doses of allopurinol. However, it is interesting that notwithstanding allopurinol seems to have intrinsic antioxidant properties being a potent hydroxyl radical scavenger [312, 313], it either did not change or decreased the time to exhaustion, differently from what was found administering antioxidants before an exhaustive exercise [314].

Another study [315] reported that, following acute exercise (60 min treadmill running 27 m/min, 5% incline), skeletal muscle oxidized glutathione (GSSG) significantly increased in allopurinol- and vehicle-treated rats despite XO activity and uric acid levels were unaltered. This suggests that XO was not the source of ROS during exercise. In the whole, the available data obtained by XO inhibition suggest that the enzyme can be a source of ROS during exercise but not the main.

7. Training

7.1. Effects of Training on Tissue Oxidative Damage. Contrary to acute exercise, aerobic exercise training induces adaptations which reduce liver [316] and skeletal [317] and cardiac muscle [318] oxidative damage of lipids and proteins. Such adaptations also render tissues less susceptible to the oxidative damage induced by conditions leading to oxidative damage. Indeed, training attenuates lipid and protein oxidative damage and glutathione depletion in rat heart subjected to ischemia-reperfusion [319]. Moreover, training prevents lipid peroxidation increase induced by moderate intensity exercise in rat liver and muscle [250]. However, other studies suggested that training does not affect the extent of lipid peroxidation due to exhaustive swimming but, delaying the rate of the peroxidative reactions, allows trained animals to

sustain the activity for a longer period before the fatigue becomes limiting [251, 252].

It is clear that training exerts protective effects reducing oxidative damage of tissues and increasing their resistance to oxidative challenges. Such effects seem to be associated with increased cellular antioxidant defenses. Several studies examined the effect of training on the activities of antioxidant enzymes. Much of these studies may not be directly compared to each other because of the differences in experimental design, animal model, and analytical procedures. However, in the whole they show that training results in an increase in skeletal [320] and cardiac [321] activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase, even though some studies failed to find enhanced antioxidant activity after training.

The training effect on liver antioxidant enzymes has been less studied and the results are rather contrasting. On the other hand, it was previously reported that liver total antioxidant capacity was increased by training as well as those of skeletal and cardiac muscle [251, 252].

Surprisingly, there are few studies concerning the effect of training on antioxidant enzyme expression. However, increases induced by training in CuZnSOD mRNA abundance in rat liver and heart [322] and Cu, ZnSOD, and MnSOD protein level only in some muscles but not in others [322, 323] were reported.

It is likely that training exerts its protective effects also decreasing H_2O_2 production, even though scarce information is available on training impact on cellular ROS sources. The rate of H_2O_2 release was decreased in liver [316], skeletal muscle [317, 324], and heart [318] mitochondria from rats trained to swim and in heart mitochondria [325] from rats trained to run. Conversely, no effect on H_2O_2 release was found in skeletal muscle mitochondria following voluntary wheel training [326].

Measurements of H_2O_2 release rate in the presence of respiratory inhibitors suggested that training reduces the concentration of the autoxidizable electron carriers located at Complexes I and III in the liver [316] and muscle [317] mitochondria and that of the autoxidizable electron carrier located at Complex III in the heart mitochondria [318]. However, other swim training-induced adaptations can contribute to the reduction of the H_2O_2 release rate found in the mitochondria isolated from liver heart and muscle. These could include the increased activity of the H_2O_2 metabolizing enzyme GPX, which is coupled with the increase in GR activity, in the mitochondria of the three tissues [316–318]. Conversely, it is unlikely that the decrease in H_2O_2 release is due to increased uncoupling of the inner mitochondrial membrane, since that training reduces mRNA expression of uncoupling protein 3 in skeletal muscle and uncoupling protein 2 in skeletal and cardiac muscles [327].

The effects of exercise training on NADPH oxidase activity are limited but some data suggest that exercise training is able to modulate NADPH oxidase activity. For example, it has been reported that exercise training mitigates age-related upregulation of NOX2 subunits gp91phox and p47phox in rat heart [328], reduces microvascular endothelial NOX content

in muscle biopsies from vastus lateralis of obese men [329] but not of lean men [330], and downregulates NADPH oxidase expression in obese rats [331]. Studies using XO inhibition by allopurinol show that such an inhibition produces complex effects that are not yet fully elucidated so that the relative data are not conclusive and do not allow us to point out the relevance of the ROS produced by XO for the training adaptations.

7.2. Mitochondrial Biogenesis. The improved cardiovascular function and aerobic capacity elicited by aerobic exercise training require an increased tissue metabolic activity. The first proof that the increase induced by the training in metabolic capacity was due to the increased tissue mitochondrial protein content was obtained in the skeletal muscle [332, 333]. Subsequent studies confirmed this finding [317] and showed that swim training increased mitochondrial protein content also in other tissues such as liver [316] and heart [318]. However, the changes in mitochondrial protein content seemed to differ in various tissues and were associated with different changes in the metabolic capacity of tissues and mitochondria. Indeed, in the skeletal muscle swim training increased tissue metabolic capacity by inducing a moderate increase in the tissue content of the mitochondrial population characterized by a lightly reduced aerobic capacity [317]. In liver, swim training did not induce increases in tissue metabolic capacity because the modest increase in mitochondrial population content was balanced by the reduction in mitochondrial metabolic capacity [316]. In heart, swim training increased tissue metabolic capacity by enhancing lightly both mitochondrial population content and mitochondrial respiratory capacity [318]. These different responses are consistent with the different functions of liver and muscles as energy supplier and consumers, respectively [334], during aerobic long-lasting exercise.

7.3. Mechanisms of Adaptive Response to Exercise. Mitochondrial biogenesis requires that expression of the mitochondrial genome and nuclear genes encoding mitochondrial proteins is finely organized. The process is controlled by the peroxisome proliferator-activated-receptor- γ coactivator 1 (PGC-1) which regulates the expression of transcription factors such as nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) [335]. NRF-1 and NRF-2 control the expression of many genes, among which are those implicated in mitochondrial biogenesis, adaptive thermogenesis, glucose and fatty acid metabolism, fiber type switching in skeletal muscle, and heart development [336].

PGC-1 expression increases quickly in muscle cells stimulated to contract [337], in rat skeletal muscle after a single bout of exercise [338] and in human skeletal muscle after endurance training [339]. A study using PGC-1 α knockout mice showed that in liver PGC-1 α plays a pivotal role in regulation of cytochrome c and cytochrome oxidase subunit I expression in response to a single bout of treadmill exercise and prolonged exercise training, which suggests that the exercise-induced changes in tissue oxidative capacity are regulated by PGC-1 α [340].

Increases in PGC-1 α expression were also found in heart after short term training to treadmill run [341] and in liver [316], skeletal muscle [317], and heart [318] after 10 weeks of training to swimming. In these tissues, the increases in PGC-1 α expression were associated with an increase in the expression of NRF-1 and NRF-2.

Interestingly, in addition to regulating mitochondrial biogenesis, PGC-1 is able to regulate endogenous antioxidant expression, such as Cu, ZnSOD, MnSOD, and GPX, in skeletal muscle [342, 343]. This coordination of the proliferation of ROS producing organelles with increase in antioxidant levels likely helps to maintain redox homeostasis. In addition, it has been shown that PGC-1 α promotes mSIRT3 gene expression, which is mediated by ER-binding element mapped to the SIRT3 promoter region [344]. In turn, SIRT3 binds to, deacetylates, and activates mitochondrial enzymes, including MnSOD, through a posttranslational mechanism [345].

PGC-1 α is also able to regulate the mRNA expression of uncoupling proteins 2 and 3 in cell culture [346], suggesting that PGC-1 α may also increase the uncoupling capacity and concomitantly reduce ROS production in the mitochondria [343].

Several initiating stimuli, activated during exercise, can contribute to eliciting the PGC-1 gene response. These include (i) increase in cytosolic calcium concentration, which activates various signaling pathways regulated by the calcineurin phosphatase and the calmodulin-modulated kinase, (ii) the decrease in levels of high-energy phosphates, leading to the activation of the AMP-sensitive kinase (AMPK), and (iii) stimulation of the adrenergic system, leading to cyclic AMP synthesis, and activation of protein kinase A and other kinases, such as mitogen-activated protein kinase (MAPK) [342]. However, it is necessary to point out that PGC-1 α regulation is not only due to variation in expression but also caused by covalent modifications among which are phosphorylation, acetylation methylation, and ubiquitination. Indeed, *in vitro* experiments showed that p38 MAPK and AMPK phosphorylate PGC-1 α producing a more active protein [342].

It seems that PGC-1 expression is also upregulated by ROS. Indeed, the observation that antioxidant incubation prevents the increase in PGC-1 α mRNA induced by electrical stimulation in rat skeletal muscle cell culture [347] indicates that increases in ROS may contribute to exercise-induced increases in skeletal muscle PGC-1 α mRNA content. Thus, the observation that the H₂O₂-induced increase in the mRNA content of SOD, catalase, and GPX in PGC-1 α KO fibroblasts is lower than that in wild type fibroblasts [348] indicates a role of PGC-1 α in the upregulation of ROS removing enzymes in response to increases in ROS.

Moreover, notwithstanding conflicting results exist in literature [349], it was reported that antioxidant supplementation attenuates the PGC-1 α expression increase due to training [316–318, 350–352]. It was also reported that vitamin E supplementation prevents the increase in activator and coactivator levels and mitochondrial population adaptation to physical training [316–318]. These results suggest that the ROS produced during each session of exercise training are able to regulate cellular functions acting as signals regulating

molecular events crucial for adaptive responses of liver, muscle, and heart.

The role of ROS as signaling molecules in the tissue adaptation induced by training seems to contrast with the oxidative damage and dysfunction elicited by acute exercise. However, this can be explained by differences in extent and temporal pattern of ROS generation. Thus, a moderate, intermittent ROS production during short time periods in a program of graduate aerobic training can activate signaling pathways leading to cellular adaptation and protection against future stresses. In contrast, moderate levels of ROS production over long time periods (e.g., hours) or high levels produced during brief exercise at high intensity may result in structural and functional tissue damage.

8. ROS Production and Type 2 Diabetes

Type 2 diabetes and other related diseases, such as metabolic syndrome and coronary heart disease, are a serious problem worldwide [353]. Specifically, diabetes is very closely related to microvascular and macrovascular complications that seriously affect the quality of life and life expectancy of patients. At present, there are around 350 million people worldwide with diabetes, a figure that will rise to 500–600 million over the following years. Diabetes, insulin resistance, and cardiometabolic diseases are associated and constitute an active field of research [354, 355].

Mitochondria are known to produce ATP after metabolism of nutrients and are capable of generating energy. In this sense, it has been demonstrated that mitochondrial dysfunction is characterized by decreased levels of ATP, inhibition of mitochondrial O₂ consumption, enhanced ROS production, a decrease in the antioxidant content, and alterations in mitochondrial membrane potential ($\Delta\Psi_m$). These effects are due mainly to an imbalance between energy intake and expenditure [356]. In fact, a decrease in the activity of the electron transport chain (ETC) complexes or increased uncoupling produced by the activity of uncoupling proteins or the ADP/ATP translocator (also called adenine nucleotide translocase, ANT) can induce changes in $\Delta\Psi_m$ that eventually lead to apoptosis [354, 355].

Different factors, both genetic and environmental (diet, exercise, and stress), have been shown to modulate mitochondrial function and alter insulin sensitivity [357, 358]. In this context, the presence of mitochondrial impairment has been demonstrated in different types of leukocytes [359] and tissues such as liver, lung, skeletal muscle, spleen, or heart in type 2 diabetes [360, 361], confirming the relationship between this condition and mitochondrial dysfunction.

Mitochondria are the main source of reactive oxygen species (ROS), as mentioned previously. ROS are key to the development of diabetic complications [362–364], and studies have demonstrated that the use of antioxidants such as lipoic acid (LA) can reduce insulin resistance and ROS production (by improving mitochondrial function) and prevent CVD in humans [365]. Recently, Faid et al. have demonstrated that resveratrol can alleviate diabetes-induced apoptosis by modulating caspase-3 activities, oxidative stress, and JNK signaling [366].

Electron transport chain (ETC) dysfunction is directly related to diabetes and its complications, including retinopathy, nephropathy, and neuropathy [355]. Furthermore, some studies have documented that deleterious genetic mutations related to a reduction in the activity of Complex I can lead to mitochondrial impairment and enhanced ROS production [367]. Therefore, mitochondria-targeted antioxidant therapy has been proposed as a beneficial tool in the treatment of mitochondria-related diseases [368].

Victor's group has performed several studies of type 2 diabetes patients which have shown that oxidative stress and mitochondrial dysfunction occur due to a decrease in O₂ consumption, Complex I activity, membrane potential, and glutathione levels and an increase in ROS production [359, 369–371], thus confirming mitochondria as a key target for diabetes treatment. In other related pathologies, such as polycystic ovary syndrome (PCOS), in which patients can develop insulin resistance, there is also an impairment of mitochondrial Complex I and an increase in leukocyte-endothelium interactions [372]. This mitochondrial dysfunction increases ROS production, reduces ATP and Ca²⁺, and alters membrane potential and mitochondrial morphology. However, in an animal model of diabetes (db/db mice), mitochondrial and renal function are improved in the presence of mitochondria-targeted antioxidants, such as CoQ10, highlighting the crucial role of mitochondria in the development and pathogenesis of diabetic nephropathy [373]. In addition, Victor's group has demonstrated that mitochondrial dysfunction and, especially, mitochondrial ROS production are related to the development of silent myocardial ischemia and endothelial dysfunction due to increased leukocyte/endothelium interactions [374].

Mitochondria-targeted antioxidants have been shown to have beneficial effects on conditions of oxidative stress. In this sense, MitoQ is an antioxidant which, due to a covalent attachment to the lipophilic triphenylphosphonium cation, is selectively taken up 1000-fold by mitochondria [363, 375]. Chacko et al. demonstrated an example of the beneficial effects of MitoQ on diabetes when they reported that MitoQ decreased urinary albumin levels to the same level as those of nondiabetic controls in a mouse model of diabetic nephropathy (Ins2(+/-)(Akita) mice) [376]. Furthermore, glomerular damage and interstitial fibrosis were significantly reduced in the treated animals, and there was a nuclear accumulation of the profibrotic transcription factors β -catenin and phospho-Smad2/3, which was prevented by MitoQ treatment. These results support the hypothesis that mitochondrially targeted therapies could be beneficial for the treatment of diabetic nephropathy.

8.1. Insulin Resistance and Mitochondrial Dysfunction. Glucose homeostasis is regulated by insulin. In addition, insulin has important cardiovascular, renal, and neural functions, which may explain why insulin resistance is a risk factor for microvascular complications such as retinopathy, nephropathy, hypertension, and CVD [25].

A series of conditions are related to the development of insulin resistance, such as obesity, changes in lipid and glucose metabolism, chronic inflammation, stress, or other

oxidative factors. In these conditions, the appearance of insulin resistance is frequently associated with a diminished capacity of tissues or cells to respond to levels of insulin [377]. This process is related to mitochondrial dysfunction, changes in mitochondrial dynamics, and enhanced ROS production.

In relation to this theory, it has been demonstrated that mitochondrial impairment, oxidative stress, excess energy intake, and lipodystrophy can enhance circulating free fatty acids (FFAs), which can lead to the accumulation of triglycerides, FFAs, and diacylglycerol (DG) in different tissular locations, including liver, skeletal muscle, heart, kidney, and β -cells. Furthermore, alterations in cholesterol subfractions, such as an increase in the atherogenic potential of small dense LDL, may be related to several metabolic properties of these particles, facilitating their transport into the subendothelial space [378], reducing LDL receptor affinity [379, 380], and increasing susceptibility to oxidative modifications [378, 381]. The cited studies feed into the idea that small dense LDL are related to arterial damage in patients with dyslipidemia associated with diabetes.

Insulin resistance, mitochondrial dysfunction, and enhanced production of ROS, which act as secondary messengers by activating serine kinases that phosphorylate IRS proteins, modulate the insulin response [377]. In addition, ROS can trigger the inflammatory process by activating IKK β , which phosphorylate IRS-1 [382]. Several studies have demonstrated that insulin sensitivity and mitochondrial function can be modulated by antioxidants, with a subsequent decrease in ROS production and an increase in the expression of UCP2/3 and a decrease in ROS levels [382]. However, the results of the said studies have generated some controversy [383].

Mitochondrial impairment and insulin resistance have also been shown to be related to diminishing levels of mitochondrial oxidative enzymes, which reduce mitochondrial complex activity, alter mitochondrial morphology, and limit mitochondrial number [384]. For example, mitochondrial oxidative capacity can correlate negatively with insulin sensitivity after the accumulation of intramyocellular lipids [385].

During obesity, there is an increase of triglycerides in adipose tissue, and consequently glucose metabolism is altered in other nonadipose tissues. In this sense, it has been speculated that lipodystrophy induces insulin resistance, mitochondrial dysfunction, and type 2 diabetes [386]. In diabetes and obesity, adipocytes can release high amounts of adipokines, such as resistin, leptin, adiponectin, and TNF- α , which can regulate metabolic pathways [387]. Furthermore, the number and morphology of mitochondria, as well as the expression of genes involved in mitochondrial biogenesis, are significantly decreased by the energetic alterations that appear as a result [388]. All of these studies support the idea that insulin resistance is present in lipodystrophy, obesity, and type 2 diabetes. This action leads to the accumulation of intracellular fatty acid metabolites (e.g., diacylglycerol, fatty acyl CoAs) in muscle and liver, which triggers the activation of a serine kinase cascade and finally induces defects in insulin signaling and insulin action in these tissues [389].

Oxidative stress, mitochondrial-endothelial dysfunction, and insulin resistance are very common in cardiovascular

diseases such as stroke, silent myocardial ischemia, coronary artery disease, or hypertension [390]. In this sense, type 2 diabetic patients exhibit high blood pressure, whose appearance is related to hyperglycaemia [391]. In relationship to this idea, Katz et al. have highlighted that diabetes is associated with a higher prevalence of calcified atherosclerotic plaque in the thoracic arteries [392]. Furthermore, endothelial impairment has been associated with intramyocardial lipid accumulation and glucose intolerance and, eventually, heart failure [393].

Not all organs are specifically protected against oxidative stress. For example, the heart, which has a high metabolic rate and high beta oxidation and ROS production, contains low levels of antioxidants, making it particularly susceptible to oxidative stress, mitochondrial dysfunction, and subsequent structural and functional abnormalities [394].

Mitochondrial oxidative stress damage and changes in the morphology/function of mitochondria have been reported in an animal model of obesity, namely, insulin-resistant obese Zucker rats [395]. Obesity and lipotoxicity can also enhance mitochondrial damage during the development of diabetic retinopathy [396]. Experiments involving transmission electron microscopic analysis of myocardial tissue have demonstrated an increase of abnormal mitochondria in an insulin-resistant rat model [397]. Another study showed an increase in the number of mitochondria in hypertrophied rat hearts under oxidative stress conditions [398]. In disagreement with these studies, others have failed to find changes in the number of mitochondria and their DNA content, while some have even reported reduced numbers in patients and animal models of pathological hypertrophy [399]. In conclusion, these results point out the importance of mitochondria in the heart and would suggest that they enhance CVD, including heart failure, stroke, cardiomyopathy, coronary heart disease, silent myocardial ischemia, and hypertension.

Insulin resistance is related to endothelial dysfunction [400], but the underlying mechanisms are yet to be confirmed. In the endothelium, mitochondria play an essential role by acting as sensors of local alterations in the concentration of O_2 and as regulators of intracellular Ca^{2+} concentrations [401]. Taking into account that mitochondrial dysfunction is related to endothelial dysfunction, different studies have demonstrated that blockade of ROS generation can improve endothelial function under hyperglycemia conditions [362, 402].

It is generally recognized that endothelial nitric oxide synthase (eNOS) plays a key role in the maintenance of vascular tone and insulin-stimulated NO^* production in the endothelium [403]. In fact, poor eNOS activity has been related to insulin resistance, hypertension, and dyslipidemia [404]. Therefore, there is an impairment in the NO^* production under insulin resistance conditions, which is related to the appearance of CVD such as coronary artery disease, heart failure, stroke, or silent myocardial ischemia.

Insulin-resistant patients can develop type 2 diabetes when there is impairment in β -cells, the result of which is an incapacity to sense glucose properly and release insulin and failed glucose homeostasis. In this sense, mitochondrial activity can modulate the potassium channels (K_{ATP}) modulating

ATP/ADP ratio. β -cell function and mitochondrial function are related through the ATP/ADP ratio [405]. Furthermore, mitochondrial Ca^{2+} levels are crucial to the maintenance of insulin secretion, as demonstrated by Han et al. who showed that taurine can enhance the glucose sensitivity of UCP2-overexpressing β -cells, probably by enhancing mitochondrial Ca^{2+} influx and subsequently increasing the ATP/ADP ratio and mitochondrial function [406]. Other studies have highlighted the importance of mitochondrial function in glucose homeostasis by using knockout Tfam (Transcription Factor A, Mitochondrial) mice, Tfam being a nuclear DNA-encoded mitochondrial protein, which results in a dramatic mtDNA depletion, a decreased of insulin secretion, reduced β -cell mass, and the development of diabetes [407]. In conclusion, all of the abovementioned studies highlight the fact that preserving mitochondrial function is essential for β -cell function under oxidative stress conditions and that mitochondrial impairment contributes to the pathogenesis of type 2 diabetes by interfering with insulin action and secretion. Furthermore, high levels of fatty acids can induce mitochondrial dysfunction and impair insulin signaling due to oxidative stress and enhanced ROS production. In summary, we consider that mitochondria should be considered a key target in therapy for insulin resistance in general and diabetes in particular.

Finally, we would like to mention that the possible beneficial effects of RNS and ROS can occur at low levels and can exert different physiological functions. Cells can produce H_2O_2 , $O_2^{\bullet-}$, or NO^* at physiological levels, but, in the case of diabetes, basal levels of ROS are elevated, and so these ROS and RNS are generally harmful. For example, in basal conditions, leukocytes kill pathogens by phagocytosis after which ROS are released. Basal levels of ROS can also trigger energy production by mitochondria, induce mitogenic responses, or activate the release of cytokines or nuclear transcription factors.

NO^* can also modulate vascular pressure, leukocyte adhesion, and angiogenesis. Furthermore, NO^* is an important neurotransmitter and is a key mediator of the immune response when generated by activated macrophages. Although different studies suggest that ROS act as secondary messengers, it is clear that they can be harmful when they accumulate and disrupt molecules and tissues [12, 408–410].

9. ROS and Neurodegeneration

The brain is composed of two main types of cells: glia and neurons. Glial cells encompass a wide variety of cells including astrocytes, microglia, and oligodendrocytes. Glia cells act as a neuronal support system and are the most abundant cells in the nervous system. Astrocytes support neurons in the brain and regulate the chemical and extracellular environment. They maintain low levels of ammonia and glutamate and produce neuroprotective enzymes. Upon activation, astrocytes repair cellular damage, mount an inflammatory response, and activate microglia. Microglia, the smallest glial cells, repair damage due to injury via phagocytosis. Oligodendrocytes are responsible for axon myelination,

a protective layer required for propagation of action potentials and maintenance of intracellular communication.

Neurons (nerve cells) are the basic structural elements of the nervous system. Their primary function is to transmit and receive information through nerve impulses, electrochemical signals that travel down the neuron. Neurons “sense” changes in environmental conditions and respond to such changes via neurotransmission. Neurons consist of three major components: the axon, dendrite, and perikaryon (or soma). The axon, typically ending at a specialized structure called the synapse, carries impulses to distant locations. The dendrite acts as the neuronal “receiver” but can also communicate via neurotransmitters to adjacent neurons. Dendrites become denser during neurogenesis. Small dendrites do not contain organelles, but large dendrites have neurofilaments (only found in neurons), microtubules, ribosomes, and endoplasmic reticulum. The perikaryon is the “metabolic hub” of the neuron. It houses mitochondria, ribosomes, Golgi apparatus, and endoplasmic reticulum, essential machinery in protein synthesis and energy production. Neurons are essential in contributing to emotions, perceptions, and memory and learning. Neuronal damage can alter these functions and ultimately lead to overall brain dysfunction and decline in the cognitive functions previously described. Neurodegeneration, an example of neuronal damage, is the loss of neuronal structures or function. During the aging process, myelin and neuronal loss occur, dendritic length and branching are decreased, and global brain volume is reduced. Consequences of these events include but are not limited to age associated cognitive decline, memory loss, epigenetic changes, reduced autophagy, and synaptic plasticity. Excessive neuronal death resulting in accelerated cognitive decline and memory loss has been observed in those suffering from neurodegenerative disorders. Recent findings from Villeda demonstrated a significant improvement in hippocampal learning exercises and contextual fear conditioning tasks when old mice were given plasma from young mice [411]. Other findings from this study showed an increase in dendritic spine number and synaptic plasticity in the old mice associated with reversal of cognitive decline.

Astrocytes are highly abundant throughout the central nervous system. Due to their extension-like end feet, they cover the free surfaces of neuronal dendrites and soma. Astrocytes also cover the inner surface of the one of the brain's most important meningeal membranes, the pia mater, and all blood vessels in the CNS. More importantly, these end feet surround the brain's capillary endothelial cells of the blood-brain barrier. They participate in neurotransmitter metabolism and play a pivotal role in glutamate uptake to prevent excitotoxicity. Glutamate is then converted into glutamine via glutamine synthetase, in which the basic amino acid is transported into the neuron. Furthermore, astrocytes maintain the pH of the extracellular space and ionic environment. In neurodegeneration, these cells can release cytokines which regulate the inflammatory response.

Microglia act as macrophages in the brain. Phenotypic characteristics include short spiny projections, which become enlarged under ROS conditions. Neurodegeneration is a

key promoter of microglial activation; therefore this phenomenon is observed in neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease [412]. Microglia also play a role in generating reactive nitrogen species as nitric oxide synthases, iNOS and NOX2, and NADPH oxidase are induced in these glial cells [413]. This NOX2 activation can lead to a respiratory burst of superoxide flooding the mitochondria further contributing to neurodegeneration. In addition to their inflammatory response, microglia also express multiple ion channels, namely, the sodium, proton, voltage gated Ca^{2+} and Cl^- , and potassium channels. Changes in ionic concentrations may play a role in depolarization and action potential initiation, which can trigger inflammation and neuronal activation.

Activation of these glial cells can have dramatic cellular effects including prompting an inflammatory response. This occurs in the normal aging process due to mitochondrial derived ROS production, which promotes inflammation and cytokine production [414]. This consequence can be observed as cognitive deficits as $\text{NF}\kappa\text{-B}$ is generated and neuroinflammation occurs. $\text{NF}\kappa\text{-B}$ serves as a proinflammatory agent and a prosurvival molecule by regulating the inflammatory response. This is an age-dependent process. If the $\text{NF}\kappa\text{-B}$ pathway is blocked in old mice, a reversal of gene expression occurs [415]. Interestingly, $\text{NF}\kappa\text{-B}$ is highly associated with RNS as this family of proteins induces nitric oxide synthase, further promoting nitrosative stress. Prostaglandin synthesis is also initiated through the $\text{NF}\kappa\text{-B}$ signaling cascade, propagating the inflammatory process. Neuroinflammation can be defined as the increased production of a multitude of proinflammatory molecules, mostly notably interleukin- 1β (IL- 1β), tumor necrosis factor α (TNF- α), and transforming growth factor β (TGF- β). IL- 1β is a proinflammatory agent that recruits neutrophils as part of the inflammatory response. This cytokine is frequently observed in several neurodegenerative disorders [416]. TNF- α is a key regulator of the immune system. It has been shown to be increased in microglia from aged mice in a lipopolysaccharide induced mouse model [417]. TNF- α is found predominantly in macrophages. It is released by activated microglia and astrocytes which perpetuates neurodegeneration and neuroinflammation by increasing levels of reactive oxygen species, specifically superoxide. The more the neuronal damage, the more frequent the neuroinflammation as microglia and astrocytes are constantly activated. In addition to the inflammatory properties of TNF- α , it directly activates NADPH oxidase which increases levels of superoxide in the cells similar to $\text{NF}\kappa\text{-B}$. NOX2 is highly expressed via TNF- α , which is linked to excess levels of RNS [418]. TGF- β regulates neuroinflammation and apoptosis by releasing inflammatory cytokines and reactive oxygen species [419]. TGF- β is mediated by the Smad3 pathway, which inhibits the production of free radicals which are normally promoted in the inflammatory pathway. This pathway is impaired during neurodegeneration and could contribute to the disease progression as neuronal loss and neuroinflammation are observed [420]. As these cytokines have been

tested as biomarkers of oxidative stress, a link between ROS, neurodegeneration, and neurodegenerative disorders can be further bolstered.

Oligodendrocytes. Oligodendrocytes are vulnerable to oxidative damage as they have a higher ATP requirement than other glial cells, contain low levels of glutathione, a potent antioxidant, and have a high intracellular iron level which can form prooxidants through Fenton chemistry [421]. The main function of oligodendrocytes is myelination. The myelin sheath is a necessary axonal component that increases saltatory conduction of action potentials, thereby stimulating neurotransmission. Demyelinating disorders such as multiple sclerosis exhibit motor function decline due to disruption of action potential propagation from the loss of myelin.

It has been well-established that, in neurodegenerative disease, neurodegeneration can occur as the result of oxidative stress, the imbalance of antioxidant and prooxidant levels [422]. Reactive oxygen species levels increase as a function of age and are even higher in age-related neurodegenerative disorders [423]; therefore oxidative stress can also occur if there is an excess of ROS/RNS production or an antioxidant deficiency [424]. This section of the review will focus on the interconnection between oxidative stress, reactive oxygen and nitrogen species, and neurodegeneration in the aforementioned cellular systems and research directed at neuroprotection, the delay or prevention of neurodegeneration.

9.1. Oxidative Stress. Mitochondria are the key source for free radicals [425, 426]. A minute amount of electrons leaks from the mitochondria and reacts with molecular oxygen to form superoxide. Other sources for free radicals can include environmental toxins [427], metal catalyzed reactions, certain enzymatic reactions (e.g., xanthine/xanthine oxidase), and cellular processes. During phagocytosis, oxidants are needed to ingest bacteria, viruses, and other pathogens [428].

9.1.1. Hypochlorous Acid. Hydrogen peroxide can react with a chloride anion to form hypochlorous acid (HOCl) via myeloperoxidase (MPO). Myeloperoxidase is largely present in neutrophils [429] but has also been located in neuronal cells under certain conditions [430]. Hypochlorous acid can further react with nitrogen dioxide (NO_2^*) to form nitryl chloride (NO_2Cl), a potent chlorinating and nitrating oxidant [431]. Phagocytes use HOCl as one of their agents [432]. Hydrogen sulfide (H_2S) is an inhibitor of HOCl from Cl^- . Myeloperoxidase in the presence of H_2O converts Cl^- into Cl^+ ; therefore hydrogen sulfide may have a protective effect. H_2S levels are lowered in Alzheimer's disease, a neurological disorder [433]:



9.1.2. Reactive Nitrogen Species. Similar to ROS, reactive nitrogen species are highly toxic [434]. Some examples of RNS, nitric oxide, and nitrogen dioxide will be discussed below. Reactive nitrogen species cause protein nitration by various methods, which can result in protein dysfunction and neuronal loss.

NO^* has been shown to play a role in neurodegenerative diseases by acting as a neurotoxin when excessively produced [435]. Hara has shown that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acts as NO^* sensor [436]. NO^* is involved in cell signaling pathways immune response and vasodilation. NO^* activates protein kinase C (ϵ isoform), which activates a specific family of tyrosine kinases that can stimulate apoptosis [437]. NO^* can also bind to glutamate channels and indirectly to calcium and potassium channels [438]. As glutamate is an excitatory neurotransmitter in the cell, depolarization of the membrane occurs. Glutamate binds to the NMDA receptor and an influx of Ca^{2+} enters the cell and causes a disruption in calcium homeostasis. This disruption can eventually lead to cell death.

NO_2^* acts as an outdoor and indoor air pollutant from car emissions, fossil fuels, cigarette smoke, heaters, and gas stoves, just to name a few [439]. NO_2^* is primarily found in the airways of the terminal bronchi; however, NO_2^* may be found in other areas of the respiratory tract. Minimal exposure to NO_2^* results in long morphological changes resulting in possible inflammation, pulmonary edema, and cellular injury [440]. Nitrogen dioxide exposure also leads to an increase in TBARS, a marker for lipid peroxidation in lung tissue, and vitamin E treatment showed a reduced pulmonary injury [441]. NO_2^* serves as an oxidant in inflammation mediated by the peroxidases, eosinophil peroxidase, and myeloperoxidase [442–446]. This gas can oxidize the antioxidant glutathione and increase activity of glutathione reductase and glutathione peroxidase [447, 448]. The depletion of glutathione shifts the balance to the side of nitrosative stress. NO_2^* can also be formed by the oxidation of ONOO^- , another potent reactive nitrogen species.

10. An Overview of Some Neurodegenerative Diseases

Neurodegenerative diseases are a classification of disorders in which neuronal loss and progressive cognitive decline are observed. These two consequences contribute to the memory loss exhibited by those with neurodegenerative disorders. This level of decline is accelerated in contrast to the normal aging process. Depending on the specific disease other characteristics and symptoms include dementia, a decline in motor function, and depression may be evident. The phenomenon of oxidative stress has been well-established in such neurodegenerative disorders as Huntington's disease [449, 450], Parkinson's disease [451], amyotrophic lateral sclerosis [452], and Alzheimer's disease [453–455]. This section will only highlight the correlation between autophagy, apoptosis, and oxidative stress in the following neurodegenerative diseases: Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

10.1. Alzheimer's Disease. Alzheimer's disease (AD), the most common form of dementia, is a neurodegenerative disease currently affecting millions of people worldwide. Age is one risk factor of AD, as the onset of disease typically occurs at about 65 years old. Although AD is an age-related

neurodegenerative disease, it can be inherited (familial AD) and sporadic. Familial AD (FAD) is very rare, as it affects less than 10% of those afflicted with the disease. There are four distinct types of FAD caused by the genetic factors described below [456–458]. Sporadic AD usually occurs in the late stage of AD and is associated with apolipoprotein E4 (APOE4) allele. Apolipoprotein E (APOE) helps transport cholesterol into the bloodstream. APOE4 is also involved in learning and memory as persons with the APOE4 allele showed a decline in processing new information [459]. APOE4 shows an increase of amyloid beta peptide and neurofibrillary tangles (NFT) [460, 461]. Increased oxidative stress is prevalent in AD and also linked to APOE4 [462, 463]. Ramassamy et al. demonstrated that tissue from patients with the APOE4 allele showed a decrease in activity of the antioxidant enzymes glutathione peroxidase and catalase [463]. Other risk factors include family history of disease, reduced brain volume, traumatic brain injury, and low education and mental ability early in life [464–468].

Extreme neurofibrillary tangles and senile plaques are the hallmarks of AD. These NFT are composed of paired helical filaments, which consist of hyperphosphorylated tau protein. The senile plaques are composed of amyloid beta ($A\beta$) peptide. Amyloid precursor protein (APP) is a transmembrane protein that plays a role in neuronal plasticity, long term potentiation, and memory loss [469]. APP is proteolytically cleaved by the enzymes β -secretase and γ -secretase to form a 40–42 amino acids' peptide, amyloid beta ($A\beta$) peptide. The two major forms of $A\beta$ found in human brain are $A\beta(1-40)$ and $A\beta(1-42)$. $A\beta(1-42)$ is the more toxic form of $A\beta$ and is the primary component of the senile plaque [470, 471]. $A\beta(1-42)$ induces oxidative stress *in vivo* [472, 473] and *in vitro* [474]. After this toxic peptide is formed, it can aggregate and these aggregates can accumulate outside or inside the cell contributing to the pathogenesis of AD.

There are three human causative genetic alterations in AD (APP; PS1; and PS2) and several genes associated with AD (APOE4, α -2 macroglobulin, chromosome 10, and chromosome 12). APP is a precursor to amyloid beta peptide. There are several APP mutations (i.e., Arctic, Flemish London, Dutch, Italian, Indiana, Iranian, etc.) that cause AD by many different mechanisms (i.e., protofibril formation, dense senile plaques, and increased $A\beta(1-42)$ production) [457, 475–478]. The highly characterized Swedish mutant Tg2576 transgenic mouse model for Alzheimer's disease contains human APP and has $A\beta$ plaques deposits as early as 9 months old [479, 480], leading to increased memory decline with age. Presenilin 1 (PS1) and presenilin 2 (PS2) are catalytic components of γ -secretase and are highly involved in APP processing. Mutations in PS1 or PS2 show an increase in $A\beta(1-42)$ production and are the cause of most FAD cases [456, 481–484]. Mutations in APP, PS1, and PS2 have been found in the first clinical stage of AD and MCI [485], early stage AD (EAD) [486], and late stage AD [487]. The APP/PS1 mutant mouse is a common model for AD as it exhibits early amyloid deposition and increased oxidative stress [488, 489]. A significant increase in levels of protein carbonyls, 4-hydroxynonenal, and 3-nitrotyrosine levels, markers of oxidative stress, was exhibited in APP/PS1 double mutant

neurons compared to wild type [489]. A triple transgenic (3XTg-AD) mouse model has been recently used to study the pathogenesis of Alzheimer disease. Similarly to the double APP/PS1 mouse, the 3XTg-AD mouse model exhibits oxidative imbalance as antioxidant levels are reduced and lipid peroxidation and overall brain oxidation are increased [490].

These mice have mutations in the PS1 gene and are homozygous for the Swedish APP mutation and tau P301L mutation, making them highly representative of this disorder and an excellent model to use as amyloid deposition is observed at 3 months, hippocampal hyperphosphorylated tau appears at 12–15 months, and synaptic transmission is impaired at an early age, making them ideal to study this neurodegenerative disorder.

Chromosome 10 codes several particular genes of interest as possible risk factors of AD [491]. Insulin degrading enzyme (IDE) is one such gene. IDE degrades and clears $A\beta$ in the brain [492, 493]. IDE levels are reduced in hippocampus [494] and this protein's catalytic activity is lowered in AD as well [495]. IDE, as the name infers, degrades insulin. Statin drugs have been shown to promote astrocytic IDE secretion in AD model to stimulate autophagy [496]. Abnormalities in insulin metabolism are associated with APOE status. AD patients with the APOE4 allele had higher insulin levels than patients without E4 allele [497]. Elevated plasma insulin levels correlate to increased $A\beta$ levels, which as previously mentioned has detrimental effects [498, 499]. Therefore, chromosome 10 mutations may be a possible risk factor in AD.

α -2-Macroglobulin (α_2M) is encoded by chromosome 12 and is thought to be a possible risk factor for AD as well [500]. α_2M protein binds to $A\beta$ and transports it from neurons into cells for degradation using the LRP receptor [501]. APOE uses this same receptor to enter into cells. Therefore, APOE4 or excess APOE may prevent the $A\beta/\alpha_2M$ complex from binding to the receptor and clearing for the neuron. α_2M has been shown to be neuroprotective against β amyloid fibrils *in vitro* [502] and *in vivo* [503] inhibiting toxicity. Mutations in this protein may result in $A\beta$ deposition and neuron death [504].

Microglial activation has been linked to $A\beta$ plaques in Alzheimer's disease. This activation results in a neuroinflammation and phagocytic impairment [505]. This inflammatory response leads to the release of TNF- α and interleukin-1 β [506]. Once the $A\beta$ peptide activates the microglia, nitric oxide is released [413], thereby promoting an oxidative stress via peroxynitrite elevation which can alter calcium homeostasis and promote cellular apoptosis [507].

10.2. Parkinson's Disease. Parkinson disease (PD), the second most prevalent age-dependent neurodegenerative disorder, is classified as a tauopathy, a neurological disorder that exhibits excessive levels of phosphorylated tau.

Tau is a microtubule-associated protein that is responsible for stabilizing microtubules. Microtubules are neuron-resident, cylindrically shaped, dynamic structures composed of alternating rows of α - and β -tubulin. Microtubules play a pivotal role in facilitating intracellular transport by assisting in motor protein-driven transport of vesicles, mitochondria, and other cargos in neurons. If tau is damaged, it can no longer stabilize these microtubules, thereby reducing

transport of critical factors to the neuron due to cytoskeletal disintegration. Ultimately, neurons become energy starved leading to apoptosis. Parkinson's disease is strongly correlated with neuroinflammation which is demonstrated by activated astrocytes and microglia in the central nervous system. The activation of these glial cells is detrimental to neurons and promotes neuronal loss.

Parkinson's disease presents as a decline in motor function in the form of resting tremors, muscle rigidity, and dyskinesia. This disease is attributed to protein aggregates of α -synuclein, a protein whose main function is mitochondrial functionality, vesicle trafficking, and synaptic vesicle formation [508, 509]. Alpha synuclein is predominantly located in the presynaptic terminals; thereby accumulation results in poor neurotransmission. These aggregates are the major component of Lewy bodies located primarily in the putamen and substantia nigra of the brain. These two regions are largely involved in motor movement and learning. Activated microglia are found largely in the substantia nigra and striatum of PD animals [510]. Oxidative stress in PD brain is evident by DNA damage [511] and increased levels of carbonylated proteins [512]. Neuronal dopamine, a key neurotransmitter involved in motor function, levels are significantly diminished in the substantia nigra causing substantial neuronal death. Aggregation also leads to Complex I impairment in dopaminergic neurons [513]. Protein aggregation in combination with dopamine loss causes a profound effect on the physical capabilities of persons suffering from Parkinson disease and late in the disease cognitive dysfunction is often observed. Posttranslational nitration of alpha synuclein [514] and Complex I [508] lead to altered energy metabolism which is evident in neurodegenerative disorders that are associated with oxidative stress.

The most commonly used models of PD include treatment with rotenone, 6-OHDA, or 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) induction. As muscle rigidity is a hallmark of PD, mitochondria are highly impacted by this neurodegenerative disease. Both the Krebs cycle and oxidative phosphorylation are the major energy generating processes of the mitochondria. When combined, both processes yield approximately 40 ATP molecules, providing the necessary energy for the body's daily function. Rotenone is an inhibitor to Complex I in the mitochondria, thereby reducing the proton gradient and energy production of the cell. Neurons treated with as little as 0.3 μ M of rotenone generated superoxide radicals, thereby promoting oxidative stress [515].

1-Methyl-4-phenyl-4-propionoxy-piperidine (MPPP) and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) have been shown to induce PD pathology [516]. Originally, they were discovered in heroin patients who developed Parkinsonian-like symptoms following use of a "synthetic heroin" distributed in California in the 1980s. This synthetic blend was a mixture of MPTP and MPPP. MPTP easily crosses the blood-brain barrier and is taken up by astrocytes and metabolized into 1-methyl-4-phenylpyridinium (MPP^+), which further breaks down into toxic compounds including 3,4-dihydroxyphenylacetaldehyde (DOPAL) [517], a compound that removes dopamine from synaptic vesicles. The oxidized product, MPP^+ , inhibits Complex I of the electron

transport chain working in a similar mechanism as rotenone. MPTP induces a slow progression of the nonmotor and motor symptoms of PD, allowing for longitudinal studies that demonstrate a realistic progression of PD. This is preferred by PD researchers over other toxins such as 6-hydroxydopamine (6-OHDA) [517].

Recent research has investigated the use of gliptins to prevent neuronal loss in animal models of Parkinson's disease. Gliptins are peptides that prevent neurodegeneration by preventing apoptosis and neuroinflammation by altering GLP-1, a glucagon-like peptide-1. Abdelsalam has shown that vildagliptin, a dipeptidyl peptidase, reduced iNOS, caspase-3, and myeloperoxidase levels. The antioxidant potential of this drug also blocked the RAGE/NFK- β cascade, thereby lessening neuroinflammation in the rodent rotenone Parkinsonian model [518]. Additionally, activation of aldehyde dehydrogenase, a mitochondrial enzyme responsible for detoxification of toxic aldehydes, has been shown to protect SH-SY5Y cells against rotenone-induced apoptotic cell death, thereby lowering oxidative stress levels [519]. Other studies involving the upregulation of antioxidants to promote cell survival in rotenone cell mediated toxicity and MPTP-induced Parkinson disease include ferulic acid (Ojha, DDT, 2015), catalase [520], a sirtuin, SIRT5 [521], pyrroloquinoline quinone [522], and the phenylpropanoid glycoside salidroside [523]. These results are promising in the development of a potential Parkinson's disease strategy.

The neurotoxin 6-hydroxydopamine (6-OHDA) is also capable of inducing Parkinson's-like symptoms, and its use in animal models of PD predates the discovery of MPTP's neurotoxic properties [524]. This destroys tyrosine hydroxylase (TH) containing neurons [517]. TH catalyzes the rate-limiting step for the production of dopamine, a pivotal neurotransmitter involved in motor functions. This neurotoxin plays a key role in modifying the nigrostriatal pathway in which dopaminergic neurons are produced.

Recently, Kostrzewa et al. have shown that early administration of 6-OHPA promotes a lifelong model for severe Parkinson's disease [525]. In this study, bilateral intracisternal or intracerebroventricular administration of 6-hydroxydopamine (6-OHDA) was given to perinatal rats. Elevated levels of hydroxyl radical and extreme loss of striatal dopaminergic neurons were observed. However, there were no differences in lifespan, feeding behavior, or motor function. This novel model promotes lifelong Parkinsonian symptoms without the characteristic motor deficits observed in PD, providing a truly unique system to study Parkinson's disease progression. Other studies aimed at reducing the oxidative stress promoted by 6-OHDA induction include the administration of antioxidants that also have anti-inflammatory properties such as echinacoside [526], PEG conjugated recombinant human FGF-2 [527], madopar (a combination of the dopamine precursor, levodopa, and benserazide, a decarboxylase inhibitor) [528], guanosine, an MPP^+ antagonist [529], and carnosine [530]. A safe, effective, combinatorial therapy of GAD and AAV2 vector which codes for the dopamine synthetic enzyme, aromatic-L-amino decarboxylase (AADC), has yielded encouraging results in

a small number of PD patients by improving motor performance [531, 532]. This drug is currently in Phase II clinical trial testing. These treatment strategies bolster the theory of oxidative stress as a contributor to neurodegeneration observed in neurodegenerative diseases.

10.3. Amyotrophic Lateral Sclerosis. Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, is a progressive neurodegenerative disorder in which all voluntary muscle movement is lost within 1–5 years after disease diagnosis. This disorder rapidly progresses to death within 2–5 years after the first symptoms are observed. ALS has two forms: sporadic or inherited (familial), with approximately 90% of all ALS cases classified as sporadic. Like PD, motor neurons are greatly affected in ALS resulting in muscle weakness, spasticity, and atrophy [533]. In the motor cortex, cerebellum, and parietal cortex, both forms of ALS display protein oxidation, DNA damage, and MDA modified proteins. Patients suffering from sporadic ALS showed a significant increase in these aforementioned oxidative insults compared to familial ALS subjects, thereby supporting the role of oxidative stress in amyotrophic lateral sclerosis [452]. Twenty percent of persons with inherited ALS have a mutation in the antioxidant enzyme superoxide dismutase, which leads to cellular toxicity [534]. This mutation lowers the ability to combat potentially harmful free radicals, thereby increasing levels of oxidative stress [535]. Mutant SOD1 can aggregate in the cytoplasm in motor neurons of inherited ALS patients and in various mouse models. These aggregates are capable of inducing apoptosis in cortical neurons of the G93A-SOD1 mouse, which has been widely studied as it has pathology similar to that of ALS. In the G93A-SOD1 mouse model, Ala for Gly substitution occurs at position 93. This transgenic mouse overexpresses mutant human SOD1 and exhibits the age-dependent motor neuronal characteristics associated with amyotrophic lateral sclerosis. In familial ALS patients with SOD1 mutations, a significant increase in oxidative stress, as indexed by protein carbonyls, was observed compared to control subjects [536].

It has been speculated that autophagy may play a direct role in neurodegeneration via this glutamate mechanism. This prevents the accumulation of proteins thereby reducing the risk of several neurodegenerative disorders including Alzheimer's disease (accumulation of A β), Parkinson's disease (accumulation of alpha synuclein), and Huntington's disease (accumulation of the huntingtin protein). Interestingly, Manchon has shown that the huntingtin protein is degraded by sphingosine kinase 1 in the sphingosine-1-phosphate pathway to promote cell survival via autophagy [537]. These results show a novel target for treatment of neurodegenerative disease. Defects in this process have been strongly associated with neurodegeneration [538, 539].

As ALS is a motor neuron disorder, the motor neurons of the central nervous system are greatly affected. Rojas et al. have shown that, in astrocytes of conditioned media that express human SOD1 in the G93A mouse model, c-Abl was activated [540]. This activation also caused opening of the mitochondrial permeability transition pore. C-Abl is a

tyrosine kinase that promotes apoptosis and ROS generation. Administration of the antioxidants trolox, esculetin, and tiron prevented c-Abl activation, thereby reducing oxidative stress and neuronal loss. In cell culture, it has been demonstrated that cells from the spinal cords of G93A mice overexpress transcription factor EB, which regulates autophagy. The expression of beclin-1 and LC3-II, both crucial autophagic markers, was observed as well in this study [541].

10.4. Mitochondrial Effect. Mitochondria are the powerhouse of ATP production in the cell. Mitochondria travel on microtubules with assistance of motor proteins (kinesin and dynein) to the presynaptic terminal and return to the soma. Mitochondrial dysfunction is a classic event observed in neurodegeneration as increased oxidative stress has deleterious effects on the mitochondria. Reduced energy metabolism has been observed in most neurodegenerative disorders. Dysregulation of mitochondria may also lead to alterations in the mitochondrial membrane potential which is an early characteristic of apoptosis. One leading theory to prevent neurodegeneration is reversing mitochondrial dysfunction. Several antioxidant therapies have been strategically targeted to the mitochondria to support this notion. Coenzyme Q₁₀ (CoQ₁₀) plays a key role in oxidative phosphorylation and has neuroprotective properties. It has been widely investigated in the treatment of neurodegenerative disorders [542, 543]. This quinone delays functional decline but does not increase dopamine levels. Although coenzyme Q₁₀ has shown promising results, its transport across the blood-brain barrier is poor. A new family of mitochondrial antioxidants, the Szeto-Schiller (SS) peptides, have shown an increase in motor function and cell survival [544]. These antioxidants have also protected dopaminergic neurons against MPTP neurotoxicity, thereby reducing effects of Parkinson's disease and stimulating the field of Parkinson's disease research. Another avenue of treatment includes stimulation of the high mobility group box 1 protein (HMGB-1). The high mobility group box 1 protein is a chromatin binding protein that recognizes DNA damage and promotes binding to p53 to stimulate an oxidative stress response, namely, autophagy or apoptosis. Studies investigating the correlation of HMGB-1 and mitochondrial dysfunction in 3-nitropropionic acid treated animals have been conducted to study the role HMGB-1 may have on striatal neurodegeneration *in vivo* and *in vitro* [545]. They demonstrated that HMGB-1 binds to beclin-1 to regulate autophagy, thereby establishing a new mechanism to study in striatal neurodegeneration via autophagy and apoptosis.

Oxidative stress is a well-established phenomenon that occurs in neurodegenerative disease. This coupled with an increase in apoptosis and autophagy contributes to the neurodegeneration and memory loss observed in Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Reactive oxygen and nitrogen species are highly abundant in these disorders as well. New antioxidant and mitochondrial based therapies show promise to reduce neuronal cell loss and promote neuroprotection, which will have a positive effect on patient outcomes.

11. Conclusions

In the whole, available data indicate that mitochondria are a significant source of ROS, but evidence for or against mitochondria being the main source of cellular ROS is lacking. However, there is convincing evidence that increases in mitochondrial ROS production can lead to mitochondrial and cellular oxidative stress and dysfunction, even though the same ROS can trigger mechanisms of tissue protection against excessive oxidative stress. Less information is available on role played by other cellular ROS sources in these processes. However, accumulating evidence favors the idea that, in many cells and conditions, such sources play a role in cellular oxidative damage as well as in survival mechanisms activated by oxidative stress, contributing to tissue rescue from excessive damage and dysfunction.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Sergio Di Meo, Tanea T. Reed, Paola Venditti, and Victor Manuel Victor contributed equally to this work.

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References

- [1] M. Gomberg, "An instance of trivalent carbon: triphenylmethyl," *Journal of the American Chemical Society*, vol. 22, no. 2, pp. 757–771, 1900.
- [2] B. Commoner, J. Townsend, and G. E. Pake, "Free radicals in biological materials," *Nature*, vol. 174, no. 4432, pp. 689–691, 1954.
- [3] R. Gerschman, D. L. Gilbert, S. W. Nye, P. Dwyer, and W. O. Fenn, "Oxygen poisoning and X-irradiation: a mechanism in common," *Science*, vol. 119, no. 3097, pp. 623–626, 1954.
- [4] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.
- [5] J. M. McCord and I. Fridovich, "Superoxide dismutase. An enzymic function for erythrocyte (hemocuprein)," *The Journal of Biological Chemistry*, vol. 244, no. 22, pp. 6049–6055, 1969.
- [6] G. Bartosz, "Reactive oxygen species: destroyers or messengers?" *Biochemical Pharmacology*, vol. 77, no. 8, pp. 1303–1315, 2009.
- [7] B. Halliwell, "Oxidants and human disease: some new concepts," *The FASEB Journal*, vol. 1, no. 5, pp. 358–364, 1987.
- [8] R. Radi, "Peroxynitrite, a stealthy biological oxidant," *The Journal of Biological Chemistry*, vol. 288, no. 37, pp. 26464–26472, 2013.
- [9] B. P. Yu, "Cellular defenses against damage from reactive oxygen species," *Physiological Reviews*, vol. 74, no. 1, pp. 139–162, 1994.
- [10] H. Sies, *Oxidative Stress, Oxidants and Antioxidants*, Academic Press, London, UK, 1991.
- [11] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry & Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.
- [12] H. Y. Aboul-Enein, P. Berczyński, and I. Kruk, "Phenolic compounds: the role of redox regulation in neurodegenerative disease and cancer," *Mini-Reviews in Medicinal Chemistry*, vol. 13, no. 3, pp. 385–398, 2013.
- [13] C. K. Mittal and F. Murad, "Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: a physiological regulator of guanosine 3',5'-monophosphate formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 74, no. 10, pp. 4360–4364, 1977.
- [14] K. Brieger, S. Schiavone, F. J. Miller Jr., and K.-H. Krause, "Reactive oxygen species: from health to disease," *Swiss Medical Weekly*, vol. 142, Article ID w13659, 14 pages, 2012.
- [15] D. Harman, "Origin and evolution of the free radical theory of aging: a brief personal history, 1954–2009," *Biogerontology*, vol. 10, no. 6, pp. 773–781, 2009.
- [16] M. Ristow and S. Schmeisser, "Extending life span by increasing oxidative stress," *Free Radical Biology and Medicine*, vol. 51, no. 2, pp. 327–336, 2011.
- [17] L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns, and G. Chaudhuri, "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 24, pp. 9265–9269, 1987.
- [18] C. Bogdan, "Nitric oxide and the regulation of gene expression," *Trends in Cell Biology*, vol. 11, no. 2, pp. 66–75, 2001.
- [19] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.
- [20] T. Finkel and N. J. Holbrook, "Oxidants, oxidative stress and the biology of ageing," *Nature*, vol. 408, no. 6809, pp. 239–247, 2000.
- [21] A. J. Lambert and M. D. Brand, "Reactive oxygen species production by mitochondria," *Methods in Molecular Biology*, vol. 554, pp. 165–181, 2009.
- [22] D.-F. Dai, Y. A. Chiao, D. J. Marcinek, H. H. Szeto, and P. S. Rabinovitch, "Mitochondrial oxidative stress in aging and healthspan," *Longevity & Healthspan*, vol. 3, no. 1, article 6, 22 pages, 2014.
- [23] G. C. Brown and V. Borutaite, "There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells," *Mitochondrion*, vol. 12, no. 1, pp. 1–4, 2012.
- [24] F. Camões, N. A. Bonekamp, H. K. Delle, and M. Schrader, "Organelle dynamics and dysfunction: a closer link between peroxisomes and mitochondria," *Journal of Inherited Metabolic Disease*, vol. 32, no. 2, pp. 163–180, 2009.
- [25] K. Vannuvel, P. Renard, M. Raes, and T. Arnould, "Functional and morphological impact of ER stress on mitochondria," *Journal of Cellular Physiology*, vol. 228, no. 9, pp. 1802–1818, 2013.
- [26] B. A. Freeman and J. D. Crapo, "Biology of disease: free radicals and tissue injury," *Laboratory Investigation*, vol. 47, no. 5, pp. 412–426, 1982.

- [27] J. M. McCord, R. S. Roy, and S. W. Schaffer, "Free radicals and myocardial ischemia. The role of xanthine oxidase," *Advances in Myocardiology*, vol. 5, pp. 183–189, 1985.
- [28] A. Navarro and A. Boveris, "The mitochondrial energy transduction system and the aging process," *American Journal of Physiology—Cell Physiology*, vol. 292, no. 2, pp. C670–C686, 2007.
- [29] P. Mitchell and J. Moyle, "Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria," *Nature*, vol. 208, no. 5006, pp. 147–151, 1965.
- [30] J. E. Walker, I. R. Collinson, M. J. Van Raaij, and M. J. Runswick, "Structural analysis of ATP synthase from bovine heart mitochondria," *Methods in Enzymology*, vol. 260, pp. 163–190, 1995.
- [31] P. K. Jensen, "Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles. I. pH dependency and hydrogen peroxide formation," *Biochimica et Biophysica Acta (BBA)—Enzymology and Biological Oxidation*, vol. 122, no. 2, pp. 157–166, 1966.
- [32] G. Loschen, A. Azzi, C. Richter, and L. Flohé, "Superoxide radicals as precursors of mitochondrial hydrogen peroxide," *FEBS Letters*, vol. 42, no. 1, pp. 68–72, 1974.
- [33] J. F. Turrens and A. Boveris, "Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria," *Biochemical Journal*, vol. 191, no. 2, pp. 421–427, 1980.
- [34] C. L. Quinlan, I. V. Perevoshchikova, M. Hey-Mogensen, A. L. Orr, and M. D. Brand, "Sites of reactive oxygen species generation by mitochondria oxidizing different substrates," *Redox Biology*, vol. 1, no. 1, pp. 304–312, 2013.
- [35] A. L. Orr, C. L. Quinlan, I. V. Perevoshchikova, and M. D. Brand, "A refined analysis of superoxide production by mitochondrial sn-glycerol 3-phosphate dehydrogenase," *The Journal of Biological Chemistry*, vol. 287, no. 51, pp. 42921–42935, 2012.
- [36] M. D. Brand, "The sites and topology of mitochondrial superoxide production," *Experimental Gerontology*, vol. 45, no. 7–8, pp. 466–472, 2010.
- [37] L. K. Kwong and R. S. Sohal, "Substrate and site specificity of hydrogen peroxide generation in mouse mitochondria," *Archives of Biochemistry and Biophysics*, vol. 350, no. 1, pp. 118–126, 1998.
- [38] A. Boveris, N. Oshino, and B. Chance, "The cellular production of hydrogen peroxide," *Biochemical Journal*, vol. 128, no. 3, pp. 617–630, 1972.
- [39] J. St-Pierre, J. A. Buckingham, S. J. Roeback, and M. D. Brand, "Topology of superoxide production from different sites in the mitochondrial electron transport chain," *Journal of Biological Chemistry*, vol. 277, no. 47, pp. 44784–44790, 2002.
- [40] A. Y. Andreyev, Y. E. Kushnareva, and A. A. Starkov, "Mitochondrial metabolism of reactive oxygen species," *Biochemistry*, vol. 70, no. 2, pp. 200–214, 2005.
- [41] N. Hauptmann, J. Grimsby, J. C. Shih, and E. Cadenas, "The metabolism of tyramine by monoamine oxidase A/B causes oxidative damage to mitochondrial DNA," *Archives of Biochemistry and Biophysics*, vol. 335, no. 2, pp. 295–304, 1996.
- [42] M. Löffler, C. Becker, E. Wegerle, and G. Schuster, "Catalytic enzyme histochemistry and biochemical analysis of dihydroorotate dehydrogenase/oxidase and succinate dehydrogenase in mammalian tissues, cells and mitochondria," *Histochemistry and Cell Biology*, vol. 105, no. 2, pp. 119–128, 1996.
- [43] L. Zhang, L. Yu, and C.-A. Yu, "Generation of superoxide anion by succinate-cytochrome c reductase from bovine heart mitochondria," *The Journal of Biological Chemistry*, vol. 273, no. 51, pp. 33972–33976, 1998.
- [44] A. A. Starkov, G. Fiskum, C. Chinopoulos et al., "Mitochondrial α -ketoglutarate dehydrogenase complex generates reactive oxygen species," *The Journal of Neuroscience*, vol. 24, no. 36, pp. 7779–7788, 2004.
- [45] C. De Duve and P. Baudhuin, "Peroxisomes (microbodies and related particles)," *Physiological Reviews*, vol. 46, no. 2, pp. 323–357, 1966.
- [46] M. Fransen, "Peroxisome dynamics: molecular players, mechanisms, and (Dys)functions," *ISRN Cell Biology*, vol. 2012, Article ID 714192, 24 pages, 2012.
- [47] I. Singh, "Biochemistry of peroxisomes in health and disease," *Molecular and Cellular Biochemistry*, vol. 167, no. 1–2, pp. 1–29, 1997.
- [48] V. D. Antonenkov, S. Grunau, S. Ohlmeier, and J. K. Hiltunen, "Peroxisomes are oxidative organelles," *Antioxidants and Redox Signaling*, vol. 13, no. 4, pp. 525–537, 2010.
- [49] A. Rokka, V. D. Antonenkov, R. Soininen et al., "Pxm2 is a channel-forming protein in mammalian peroxisomal membrane," *PLoS ONE*, vol. 4, no. 4, Article ID e5090, 2009.
- [50] R. Fritz, J. Bol, U. Hebling et al., "Compartment-dependent management of H₂O₂ by peroxisomes," *Free Radical Biology and Medicine*, vol. 42, no. 7, pp. 1119–1129, 2007.
- [51] S. Angermüller, G. Bruder, A. Völkl, H. Wesch, and H. D. Fahimi, "Localization of xanthine oxidase in crystalline cores of peroxisomes. A cytochemical and biochemical study," *European Journal of Cell Biology*, vol. 45, no. 1, pp. 137–144, 1987.
- [52] D. B. Stolz, R. Zamora, Y. Vodovotz et al., "Peroxisomal localization of inducible nitric oxide synthase in hepatocytes," *Hepatology*, vol. 36, no. 1, pp. 81–93, 2002.
- [53] J. Groenendyk and M. Michalak, "Endoplasmic reticulum quality control and apoptosis," *Acta Biochimica Polonica*, vol. 52, no. 2, pp. 381–395, 2005.
- [54] G. L. E. Koch, "The endoplasmic reticulum and calcium storage," *BioEssays*, vol. 12, no. 11, pp. 527–531, 1990.
- [55] A. E. Crib, M. Peyrou, S. Muruganandan, and L. Schneider, "The endoplasmic reticulum in xenobiotic toxicity," *Drug Metabolism Reviews*, vol. 37, no. 3, pp. 405–442, 2005.
- [56] J. R. Gillette, B. B. Brodie, and B. N. La Du, "The oxidation of drugs by liver microsomes: on the role of TPNH and oxygen," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 119, no. 4, pp. 532–540, 1957.
- [57] R. W. Estabrook, S. Kawano, J. Werringloer et al., "Oxycytochrome P-450: its breakdown to superoxide for the formation of hydrogen peroxide," *Acta Biologica et Medica Germanica*, vol. 38, no. 2–3, pp. 423–434, 1979.
- [58] H. Kuthan and V. Ullrich, "Oxidase and oxygenase function of the microsomal cytochrome P450 monooxygenase system," *European Journal of Biochemistry*, vol. 126, no. 3, pp. 583–588, 1982.
- [59] E. R. Stadtman, "Oxidation of proteins by mixed-function oxidation systems: implication in protein turnover, ageing and neutrophil function," *Trends in Biochemical Sciences*, vol. 11, no. 1, pp. 11–12, 1986.
- [60] P. S. Aguilar and D. de Mendoza, "Control of fatty acid desaturation: a mechanism conserved from bacteria to humans," *Molecular Microbiology*, vol. 62, no. 6, pp. 1507–1514, 2006.

- [61] M. A. Thiede, J. Ozols, and P. Strittmatter, "Construction and sequence of cDNA for rat liver stearyl coenzyme A desaturase," *The Journal of Biological Chemistry*, vol. 261, no. 28, pp. 13230–13235, 1986.
- [62] J. A. Napier, L. V. Michaelson, and O. Sayanova, "The role of cytochrome b5 fusion desaturases in the synthesis of polyunsaturated fatty acids," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 68, no. 2, pp. 135–143, 2003.
- [63] A. G. Mitchell and C. E. Martin, "A novel cytochrome b5-like domain is linked to the carboxyl terminus of the *Saccharomyces cerevisiae* Δ -9 fatty acid desaturase," *The Journal of Biological Chemistry*, vol. 270, no. 50, pp. 29766–29772, 1995.
- [64] J. B. Schenkman and I. Jansson, "The many roles of cytochrome b5," *Pharmacology & Therapeutics*, vol. 97, no. 2, pp. 139–152, 2003.
- [65] A. K. Samhan-Arias and C. Gutierrez-Merino, "Purified NADH-cytochrome b5 reductase is a novel superoxide anion source inhibited by apocynin: sensitivity to nitric oxide and peroxynitrite," *Free Radical Biology and Medicine*, vol. 73, pp. 174–189, 2014.
- [66] C. Hwang, A. J. Sinskey, and H. F. Lodish, "Oxidized redox state of glutathione in the endoplasmic reticulum," *Science*, vol. 257, no. 5076, pp. 1496–1502, 1992.
- [67] A. R. Frand and C. A. Kaiser, "Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum," *Molecular Cell*, vol. 4, no. 4, pp. 469–477, 1999.
- [68] B. P. Tu and J. S. Weissman, "The FAD- and O₂-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum," *Molecular Cell*, vol. 10, no. 5, pp. 983–994, 2002.
- [69] B. P. Tu and J. S. Weissman, "Oxidative protein folding in eukaryotes: mechanisms and consequences," *The Journal of Cell Biology*, vol. 164, no. 3, pp. 341–346, 2004.
- [70] V. P. Skulachev, "Why are mitochondria involved in apoptosis? Permeability transition pores and apoptosis as selective mechanisms to eliminate superoxide-producing mitochondria and cell," *FEBS Letters*, vol. 397, no. 1, pp. 7–10, 1996.
- [71] K.-J. Cho, J.-M. Seo, and J.-H. Kim, "Bioactive lipxygenase metabolites stimulation of NADPH oxidases and reactive oxygen species," *Molecules and Cells*, vol. 32, no. 1, pp. 1–5, 2011.
- [72] B. M. Babior, "NADPH oxidase: an update," *Blood*, vol. 93, no. 5, pp. 1464–1476, 1999.
- [73] S. J. Chanock, J. El Benna, R. M. Smith, and B. M. Babior, "The respiratory burst oxidase," *The Journal of Biological Chemistry*, vol. 269, no. 40, pp. 24519–24522, 1994.
- [74] Y.-A. Suh, R. S. Arnold, B. Lassegue et al., "Cell transformation by the superoxide-generating oxidase Mox1," *Nature*, vol. 401, no. 6748, pp. 79–82, 1999.
- [75] M. Katsuyama, "NOX/NADPH oxidase, the superoxide-generating enzyme: its transcriptional regulation and physiological roles," *Journal of Pharmacological Sciences*, vol. 114, no. 2, pp. 134–146, 2010.
- [76] C. Kim and M. C. Dinauer, "Impaired NADPH oxidase activity in Rac2-deficient murine neutrophils does not result from defective translocation of p47phox and p67 phox and can be rescued by exogenous arachidonic acid," *The Journal of Leukocyte Biology*, vol. 79, no. 1, pp. 223–234, 2006.
- [77] S. V. K. Mahipal, J. Subhashini, M. C. Reddy et al., "Effect of 15-lipoxygenase metabolites, 15-(S)-HPETE and 15-(S)-HETE on chronic myelogenous leukemia cell line K-562: reactive oxygen species (ROS) mediate caspase-dependent apoptosis," *Biochemical Pharmacology*, vol. 74, no. 2, pp. 202–214, 2007.
- [78] H.-Y. Hong, W.-K. Jeon, and B.-C. Kim, "Up-regulation of heme oxygenase-1 expression through the Rac1/NADPH oxidase/ROS/p38 signaling cascade mediates the anti-inflammatory effect of 15-deoxy- Δ 12,14-prostaglandin J2 in murine macrophages," *FEBS Letters*, vol. 582, no. 6, pp. 861–868, 2008.
- [79] G.-H. Sun-Wada, Y. Wada, and M. Futai, "Lysosome and lysosome-related organelles responsible for specialized functions in higher organisms, with special emphasis on vacuolar-type proton ATPase," *Cell Structure and Function*, vol. 28, no. 5, pp. 455–463, 2003.
- [80] K. Arai, T. Kanaseki, and S. Ohkuma, "Isolation of highly purified lysosomes from rat liver: identification of electron carrier components on lysosomal membranes," *Journal of Biochemistry*, vol. 110, no. 4, pp. 541–547, 1991.
- [81] L. Gille and H. Nohl, "The existence of a lysosomal redox chain and the role of ubiquinone," *Archives of Biochemistry and Biophysics*, vol. 375, no. 2, pp. 347–354, 2000.
- [82] H. Nohl and L. Gille, "The bifunctional activity of ubiquinone in lysosomal membranes," *Biogerontology*, vol. 3, no. 1-2, pp. 125–131, 2002.
- [83] B. Mayer and B. Hemmens, "Biosynthesis and action of nitric oxide in mammalian cells," *Trends in Biochemical Sciences*, vol. 22, no. 12, pp. 477–481, 1997.
- [84] C. Nathan and Q.-W. Xie, "Nitric oxide synthases: roles, tolls, and controls," *Cell*, vol. 78, no. 6, pp. 915–918, 1994.
- [85] O. Feron, L. Belhassen, L. Kobzik, T. W. Smith, R. A. Kelly, and T. Michel, "Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells," *The Journal of Biological Chemistry*, vol. 271, no. 37, pp. 22810–22814, 1996.
- [86] F. A. Sánchez, N. B. Savalia, R. G. Durán, B. K. Lal, M. P. Boric, and W. N. Durán, "Functional significance of differential eNOS translocation," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 291, no. 3, pp. H1058–H1064, 2006.
- [87] P. A. Loughran, D. B. Stolz, Y. Vodovotz, S. C. Watkins, R. L. Simmons, and T. R. Billiar, "Monomeric inducible nitric oxide synthase localizes to peroxisomes in hepatocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 39, pp. 13837–13842, 2005.
- [88] M. S. Parihar, R. R. Nazarewicz, E. Kincaid, U. Bringold, and P. Ghafourifar, "Association of mitochondrial nitric oxide synthase activity with respiratory chain complex I," *Biochemical and Biophysical Research Communications*, vol. 366, no. 1, pp. 23–28, 2008.
- [89] N. Paolocc, U. E. G. Ekelund, T. Isoda et al., "cGMP-independent inotropic effects of nitric oxide and peroxynitrite donors: potential role for nitrosylation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 279, no. 4, pp. H1982–H1988, 2000.
- [90] M. W. J. Cleeter, J. M. Cooper, V. M. Darley-Usmar, S. Moncada, and A. H. V. Schapira, "Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases," *FEBS Letters*, vol. 345, no. 1, pp. 50–54, 1994.
- [91] J. J. Poderoso, J. G. Peralta, C. L. Lisdero et al., "Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart," *American Journal of Physiology—Cell Physiology*, vol. 274, no. 1, pp. C112–C119, 1998.
- [92] D. I. Simon, M. E. Mullins, L. Jia, B. Gaston, D. J. Singel, and J. S. Stamler, "Polynitrosylated proteins: characterization,

- bioactivity, and functional consequences," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 10, pp. 4736–4741, 1996.
- [93] V. Borutaite, A. Budriunaite, and G. C. Brown, "Reversal of nitric oxide-, peroxynitrite- and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1459, no. 2-3, pp. 405–412, 2000.
- [94] R. Radi, A. Cassina, R. Hodara, C. Quijano, and L. Castro, "Peroxynitrite reactions and formation in mitochondria," *Free Radical Biology and Medicine*, vol. 33, no. 11, pp. 1451–1464, 2002.
- [95] K. A. Lukyanov and V. V. Belousov, "Genetically encoded fluorescent redox sensors," *Biochimica et Biophysica Acta—General Subjects*, vol. 1840, no. 2, pp. 745–756, 2014.
- [96] D. J. Hearse, "Reperfusion of the ischemic myocardium," *Journal of Molecular and Cellular Cardiology*, vol. 9, no. 8, pp. 605–616, 1977.
- [97] M. L. Hess and N. H. Manson, "Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 16, no. 11, pp. 969–985, 1984.
- [98] J. M. McCord, "Oxygen-derived free radicals in postischemic tissue injury," *The New England Journal of Medicine*, vol. 312, no. 3, pp. 159–163, 1985.
- [99] J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman, "Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 4, pp. 1620–1624, 1990.
- [100] V. Braunersreuther, F. Montecucco, M. Asrih et al., "Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 64, pp. 99–107, 2013.
- [101] H. Otani, H. Tanaka, T. Inoue et al., "In vitro study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury," *Circulation Research*, vol. 55, no. 2, pp. 168–175, 1984.
- [102] G. Ambrosio, J. L. Zweier, C. Duilio et al., "Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow," *The Journal of Biological Chemistry*, vol. 268, no. 25, pp. 18532–18541, 1993.
- [103] A. P. Halestrap, E. J. Griffiths, and C. P. Connern, "Mitochondrial calcium handling and oxidative stress," *Biochemical Society Transactions*, vol. 21, no. 2, pp. 353–358, 1993.
- [104] J. J. Kane, M. L. Murphy, J. K. Bisset, N. deSoyza, J. E. Doherty, and K. D. Straub, "Mitochondrial function, oxygen extraction, epicardial S-T segment changes and tritiated digoxin distribution after reperfusion of ischemic myocardium," *The American Journal of Cardiology*, vol. 36, no. 2, pp. 218–224, 1975.
- [105] P. Venditti, P. Masullo, and S. Di Meo, "Effects of myocardial ischemia and reperfusion on mitochondrial function and susceptibility to oxidative stress," *Cellular and Molecular Life Sciences*, vol. 58, no. 10, pp. 1528–1537, 2001.
- [106] P. S. Brookes, S. B. Digerness, D. A. Parks, and V. Darley-Usmar, "Mitochondrial function in response to cardiac ischemia-reperfusion after oral treatment with quercetin," *Free Radical Biology and Medicine*, vol. 32, no. 11, pp. 1220–1228, 2002.
- [107] P. Liu, C. E. Hock, R. Nagele, and P. Y.-K. Wong, "Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 272, no. 5, pp. H2327–H2336, 1997.
- [108] V. Borutaite and G. C. Brown, "Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide," *Biochemical Journal*, vol. 315, no. 1, pp. 295–299, 1996.
- [109] R. Radi, M. Rodriguez, L. Castro, and R. Telleri, "Inhibition of mitochondrial electron transport by peroxynitrite," *Archives of Biochemistry and Biophysics*, vol. 308, no. 1, pp. 89–95, 1994.
- [110] P. Venditti, R. De Rosa, L. Cigliano, C. Agnisola, and S. Di Meo, "Role of nitric oxide in the functional response to ischemia-reperfusion of heart mitochondria from hyperthyroid rats," *Cellular and Molecular Life Sciences*, vol. 61, no. 17, pp. 2244–2252, 2004.
- [111] D. B. Zorov, C. R. Filburn, L.-O. Klotz, J. L. Zweier, and S. J. Sollott, "Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes," *The Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1001–1014, 2000.
- [112] A. E. Vercesi, A. J. Kowaltowski, M. T. Grijalba, A. R. Meinicke, and R. F. Castilho, "The role of reactive oxygen species in mitochondrial permeability transition," *Bioscience Reports*, vol. 17, no. 1, pp. 43–52, 1997.
- [113] K. D. Garlid and A. D. Beavis, "Evidence for the existence of an inner membrane anion channel in mitochondria," *Biochimica et Biophysica Acta (BBA)—Reviews on Bioenergetics*, vol. 853, no. 3-4, pp. 187–204, 1986.
- [114] J. G. McCormack, A. P. Halestrap, and R. M. Denton, "Role of calcium ions in regulation of mammalian intramitochondrial metabolism," *Physiological Reviews*, vol. 70, no. 2, pp. 391–425, 1990.
- [115] M. A. Aon, S. Cortassa, E. Marbán, and B. O'Rourke, "Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes," *The Journal of Biological Chemistry*, vol. 278, no. 45, pp. 44735–44744, 2003.
- [116] P. Venditti, G. Napolitano, and S. Di Meo, "Role of mitochondria and other ROS sources in hyperthyroidism-linked oxidative stress," *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry*, vol. 15, no. 1, pp. 5–36, 2015.
- [117] S. Moncada and J. D. Erusalimsky, "Does nitric oxide modulate mitochondrial energy generation and apoptosis?" *Nature Reviews Molecular Cell Biology*, vol. 3, no. 3, pp. 214–220, 2002.
- [118] M. Yu. Balakirev, V. V. Khramtsov, and G. Zimmer, "Modulation of the mitochondrial permeability transition by nitric oxide," *European Journal of Biochemistry*, vol. 246, no. 3, pp. 710–718, 1997.
- [119] H. Vieira and G. Kroemer, "Mitochondria as targets of apoptosis regulation by nitric oxide," *IUBMB Life*, vol. 55, no. 10-11, pp. 613–616, 2003.
- [120] E. Migliaccio, M. Giogio, S. Mele et al., "The p66(shc) adaptor protein controls oxidative stress response and life span in mammals," *Nature*, vol. 402, no. 6759, pp. 309–313, 1999.
- [121] M. Giogio, E. Migliaccio, F. Orsini et al., "Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis," *Cell*, vol. 122, no. 2, pp. 221–233, 2005.

- [122] S. Nemoto, C. A. Combs, S. French et al., "The mammalian longevity-associated gene product p66shc regulates mitochondrial metabolism," *The Journal of Biological Chemistry*, vol. 281, no. 15, pp. 10555–10560, 2006.
- [123] M. Gertz, F. Fischer, D. Wolters, and C. Steegborn, "Activation of the lifespan regulator p66Shc through reversible disulfide bond formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 15, pp. 5705–5709, 2008.
- [124] A. E. Frazier, C. Kiu, D. Stojanovski, N. J. Hoogenraad, and M. T. Ryan, "Mitochondrial morphology and distribution in mammalian cells," *Biological Chemistry*, vol. 387, no. 12, pp. 1551–1558, 2006.
- [125] J. Nunnari and A. Suomalainen, "Mitochondria: in sickness and in health," *Cell*, vol. 148, no. 6, pp. 1145–1159, 2012.
- [126] S. Wu, F. Zhou, Z. Zhang, and D. Xing, "Mitochondrial oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins," *The FEBS Journal*, vol. 278, no. 6, pp. 941–954, 2011.
- [127] T. Yu, S.-S. Sheu, J. L. Robotham, and Y. Yoon, "Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species," *Cardiovascular Research*, vol. 79, no. 2, pp. 341–351, 2008.
- [128] D. E. Handy and J. Loscalzo, "Redox regulation of mitochondrial function," *Antioxidants and Redox Signaling*, vol. 16, no. 11, pp. 1323–1367, 2012.
- [129] D.-H. Cho, T. Nakamura, J. Fang et al., "S-Nitrosylation of Drp1 mediates β -amyloid-related mitochondrial fission and neuronal injury," *Science*, vol. 324, no. 5923, pp. 102–105, 2009.
- [130] C. De Palma, S. Falcone, S. Pisoni et al., "Nitric oxide inhibition of Drp1-mediated mitochondrial fission is critical for myogenic differentiation," *Cell Death & Differentiation*, vol. 17, no. 11, pp. 1684–1696, 2010.
- [131] I. Issemann and S. Green, "Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators," *Nature*, vol. 347, no. 6294, pp. 645–650, 1990.
- [132] S. M. Rao and J. K. Reddy, "Peroxisome proliferation and hepatocarcinogenesis," *Carcinogenesis*, vol. 8, no. 5, pp. 631–636, 1987.
- [133] J. K. Reddy, N. D. Lalvvai, and E. Farber, "Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans," *Critical Reviews in Toxicology*, vol. 12, no. 1, pp. 1–58, 1983.
- [134] F. J. Gonzalez, J. M. Peters, and R. C. Cattley, "Mechanism of action of the nongenotoxic peroxisome proliferators: role of the peroxisome proliferator-activated receptor α ," *Journal of the National Cancer Institute*, vol. 90, no. 22, pp. 1702–1709, 1998.
- [135] J. M. Peters, C. Cheung, and F. J. Gonzalez, "Peroxisome proliferator-activated receptor- α and liver cancer: where do we stand?" *Journal of Molecular Medicine*, vol. 83, no. 10, pp. 774–785, 2005.
- [136] R. J. A. Wanders, "Peroxisomes, lipid metabolism, and peroxisomal disorders," *Molecular Genetics and Metabolism*, vol. 83, no. 1–2, pp. 16–27, 2004.
- [137] M. Fransen, M. Nordgren, B. Wang, and O. Apanaset, "Role of peroxisomes in ROS/RNS-metabolism: Implications for human disease," *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1822, no. 9, pp. 1363–1373, 2012.
- [138] M. Schrader and Y. Yoon, "Mitochondria and peroxisomes: are the 'Big Brother' and the 'Little Sister' closer than assumed?" *BioEssays*, vol. 29, no. 11, pp. 1105–1114, 2007.
- [139] B. Xu, J. T. Moritz, and P. N. Epstein, "Overexpression of catalase provides partial protection to transgenic mouse beta cells," *Free Radical Biology and Medicine*, vol. 27, no. 7–8, pp. 830–837, 1999.
- [140] S. Mueller, A. Weber, R. Fritz et al., "Sensitive and real-time determination of H₂O₂ release from intact peroxisomes," *Biochemical Journal*, vol. 363, no. 3, pp. 483–491, 2002.
- [141] M. Nordgren and M. Fransen, "Peroxisomal metabolism and oxidative stress," *Biochimie*, vol. 98, no. 1, pp. 56–62, 2014.
- [142] P. A. Walton and M. Pizzitelli, "Effects of peroxisomal catalase inhibition on mitochondrial function," *Frontiers in Physiology*, vol. 3, article 108, 2012.
- [143] R. Dirx, I. Vanhorebeek, K. Martens et al., "Absence of peroxisomes in mouse hepatocytes causes mitochondrial and ER abnormalities," *Hepatology*, vol. 41, no. 4, pp. 868–878, 2005.
- [144] J. López-Erauskin, J. Galino, M. Ruiz et al., "Impaired mitochondrial oxidative phosphorylation in the peroxisomal disease X-linked adrenoleukodystrophy," *Human Molecular Genetics*, vol. 22, no. 16, pp. 3296–3305, 2013.
- [145] B. Bhandary, A. Marahatta, H.-R. Kim, and H.-J. Chae, "An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases," *International Journal of Molecular Sciences*, vol. 14, no. 1, pp. 434–456, 2013.
- [146] M. H. Smith, H. L. Plough, and J. S. Weissman, "Road to ruin: targeting proteins for degradation in the endoplasmic reticulum," *Science*, vol. 334, no. 6059, pp. 1086–1090, 2011.
- [147] I. Tabas and D. Ron, "Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress," *Nature Cell Biology*, vol. 13, no. 3, pp. 184–190, 2011.
- [148] J. D. Malhotra and R. J. Kaufman, "Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword?" *Antioxidants & Redox Signaling*, vol. 9, no. 12, pp. 2277–2293, 2007.
- [149] C. M. Haynes, E. A. Titus, and A. A. Cooper, "Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death," *Molecular Cell*, vol. 15, no. 5, pp. 767–776, 2004.
- [150] H. Yoon, D. Kim, G. Lee, K. Kim, H. Kim, and H. Chae, "Apoptosis induced by manganese on neuronal SK-N-MC cell line: Endoplasmic Reticulum (ER) stress and mitochondria dysfunction," *Environmental Health and Toxicology*, vol. 26, Article ID e2011017, 7 pages, 2011.
- [151] C. Franzini-Armstrong, "ER-mitochondria communication. How privileged?" *Physiology*, vol. 22, no. 4, pp. 261–268, 2007.
- [152] A. H. Conney, "Pharmacological implications of microsomal enzyme induction," *Pharmacological Reviews*, vol. 19, no. 3, pp. 317–366, 1967.
- [153] P. Venditti, C. M. Daniele, T. De Leo, and S. Di Meo, "Effect of phenobarbital treatment on characteristics determining susceptibility to oxidants of homogenates, mitochondria and microsomes from rat liver," *Cellular Physiology and Biochemistry*, vol. 8, no. 6, pp. 328–338, 1998.
- [154] A. G. Hildebrandt and I. Roots, "Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes," *Archives of Biochemistry and Biophysics*, vol. 171, no. 2, pp. 385–397, 1975.
- [155] C. A. Yu and I. C. Gunsalus, "Cytochrome P-450_{cam}. II. Interconversion with P-420," *The Journal of Biological Chemistry*, vol. 249, no. 1, pp. 102–106, 1974.
- [156] W. Levin, A. Y. H. Lu, M. Jacobson, R. Kuntzman, J. L. Poyer, and P. B. McCay, "Lipid peroxidation and the degradation of cytochrome P-450 heme," *Archives of Biochemistry and Biophysics*, vol. 158, no. 2, pp. 842–852, 1973.

- [157] I. I. Karuzina and A. I. Archakov, "The oxidative inactivation of cytochrome P450 in monooxygenase reactions," *Free Radical Biology and Medicine*, vol. 16, no. 1, pp. 73–97, 1994.
- [158] J. R. Mitchell, C. V. Smith, B. H. Lauterburg, H. Hughes, J. B. Corcoran, and E. C. Horning, "Reactive metabolites and the pathophysiology of acutelethal injury," in *Drug Metabolism and Drug Toxicity*, J. R. Mitchell and M. G. Horning, Eds., pp. 301–319, Raven Press, New York, NY, USA, 1984.
- [159] P. Talalay, H. J. Prochaska, and S. R. Spencer, "Regulation of enzymes that detoxify the electrophilic forms of chemical carcinogens," *Princess Takamatsu symposia*, vol. 21, pp. 177–187, 1990.
- [160] A. H. Conney, "Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons," *Cancer Research*, vol. 42, no. 12, pp. 4875–4917, 1982.
- [161] M. Mari and A. I. Cederbaum, "Induction of catalase, alpha, and microsomal glutathione S-transferase in CYP2E1 overexpressing HepG2 cells and protection against short-term oxidative stress," *Hepatology*, vol. 33, no. 3, pp. 652–661, 2001.
- [162] T. Kurz, A. Terman, B. Gustafsson, and U. T. Brunk, "Lysosomes in iron metabolism, ageing and apoptosis," *Histochemistry and Cell Biology*, vol. 129, no. 4, pp. 389–406, 2008.
- [163] H. L. Persson, T. Kurz, J. W. Eaton, and U. T. Brunk, "Radiation-induced cell death: importance of lysosomal destabilization," *Biochemical Journal*, vol. 389, no. 3, pp. 877–884, 2005.
- [164] E. Scherer, C. Streffer, and K. R. Trott, *Radiopathology of Organs and Tissues*, Springer, New York, NY, USA, 1991.
- [165] C. Berndt, T. Kurz, M. Selenius, A. P. Fernandes, M. R. Edgren, and U. T. Brunk, "Chelation of lysosomal iron protects against ionizing radiation," *Biochemical Journal*, vol. 432, no. 2, pp. 295–301, 2010.
- [166] T. Kahles and R. P. Brandes, "Which NADPH oxidase isoform is relevant for ischemic stroke? the case for Nox 2," *Antioxidants & Redox Signaling*, vol. 18, no. 12, pp. 1400–1417, 2013.
- [167] V. Braunersreuther and V. Jaquet, "Reactive oxygen species in myocardial reperfusion injury: from physiopathology to therapeutic approaches," *Current Pharmaceutical Biotechnology*, vol. 13, no. 1, pp. 97–114, 2012.
- [168] B. Lassègue, A. San Martín, and K. K. Griendling, "Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system," *Circulation Research*, vol. 110, no. 10, pp. 1364–1390, 2012.
- [169] V. Braunersreuther, F. Montecucco, M. Asrih et al., "Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/reperfusion injury," *Journal of Molecular and Cellular Cardiology*, vol. 64, pp. 99–107, 2013.
- [170] A. Daiber, "Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1797, no. 6–7, pp. 897–906, 2010.
- [171] W.-G. Li, F. J. Miller Jr., H. J. Zhang, D. R. Spitz, L. W. Oberley, and N. L. Weintraub, "H₂O₂-induced O₂—production by a non-phagocytic NAD(P)H Oxidase Causes Oxidant Injury," *The Journal of Biological Chemistry*, vol. 276, no. 31, pp. 29251–29256, 2001.
- [172] J. S. McNally, A. Saxena, H. Cai, S. Dikalov, and D. G. Harrison, "Regulation of xanthine oxidoreductase protein expression by hydrogen peroxide and calcium," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 8, pp. 1623–1628, 2005.
- [173] J. S. McNally, M. E. Davis, D. P. Giddens et al., "Role of xanthine oxidoreductase and NAD(P)H oxidase in endothelial superoxide production in response to oscillatory shear stress," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 285, no. 6, pp. H2290–H2297, 2003.
- [174] B. Kaltschmidt, T. Sparna, and C. Kaltschmidt, "Activation of NF- κ B by reactive oxygen intermediates in the nervous system," *Antioxidants & Redox Signaling*, vol. 1, no. 2, pp. 129–144, 1999.
- [175] R. E. Shackelford, W. K. Kaufmann, and R. S. Paules, "Oxidative stress and cell cycle checkpoint function," *Free Radical Biology and Medicine*, vol. 28, no. 9, pp. 1387–1404, 2000.
- [176] C. K. Sen, "Cellular thiols and redox-regulated signal transduction," *Current Topics in Cellular Regulation*, vol. 36, no. C, pp. 1–30, 2001.
- [177] M. S. Wolin, "Interactions of oxidants with vascular signaling systems," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 6, pp. 1430–1442, 2000.
- [178] F. A. Kuehl Jr. and R. W. Egan, "Prostaglandins, arachidonic acid, and inflammation," *Science*, vol. 210, no. 4473, pp. 978–984, 1980.
- [179] O. D. Saugstad, "Update on oxygen radical disease in neonatology," *Current Opinion in Obstetrics and Gynecology*, vol. 13, no. 2, pp. 147–153, 2001.
- [180] M. Benhar, D. Engelberg, and A. Levitzki, "ROS, stress-activated kinases and stress signaling in cancer," *EMBO Reports*, vol. 3, no. 5, pp. 420–425, 2002.
- [181] J. G. Scandalios, "Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses," *Brazilian Journal of Medical and Biological Research*, vol. 38, no. 7, pp. 995–1014, 2005.
- [182] V. J. Thannickal and B. L. Fanburg, "Reactive oxygen species in cell signaling," *American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 279, no. 6, pp. L1005–L1028, 2000.
- [183] G. Roos and J. Messens, "Protein sulfenic acid formation: from cellular damage to redox regulation," *Free Radical Biology and Medicine*, vol. 51, no. 2, pp. 314–326, 2011.
- [184] S. Singh, S. Vrishni, B. K. Singh, I. Rahman, and P. Kakkar, "Nrf2-ARE stress response mechanism: a control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases," *Free Radical Research*, vol. 44, no. 11, pp. 1267–1288, 2010.
- [185] D. D. Zhang and M. Hannink, "Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress," *Molecular and Cellular Biology*, vol. 23, no. 22, pp. 8137–8151, 2003.
- [186] P. D. Ray, B.-W. Huang, and Y. Tsuji, "Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling," *Cellular Signalling*, vol. 24, no. 5, pp. 981–990, 2012.
- [187] M. Zoratti and I. Szabò, "The mitochondrial permeability transition," *Biochimica et Biophysica Acta (BBA)—Reviews on Biomembranes*, vol. 1241, no. 2, pp. 139–176, 1995.
- [188] V. P. Skulachev, "Uncoupling: new approaches to an old problem of bioenergetics," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1363, no. 2, pp. 100–124, 1998.
- [189] E. L. Kuff and W. C. Schneider, "Intracellular distribution of enzymes. XII. Biochemical heterogeneity of mitochondria," *The Journal of Biological Chemistry*, vol. 206, no. 2, pp. 677–685, 1954.
- [190] P. Venditti, I. R. Costagliola, and S. Di Meo, "H₂O₂ production and response to stress conditions by mitochondrial fractions from rat liver," *Journal of Bioenergetics and Biomembranes*, vol. 34, no. 2, pp. 115–125, 2002.

- [191] P. Venditti, M. C. Daniele, P. Masullo, and S. Di Meo, "Anti-oxidant-sensitive triiodothyronine effects on characteristics of rat liver mitochondrial population," *Cellular Physiology and Biochemistry*, vol. 9, no. 1, pp. 38–52, 1999.
- [192] P. Venditti, P. Masullo, and S. Di Meo, "Effect of exercise duration on characteristics of mitochondrial population from rat liver," *Archives of Biochemistry and Biophysics*, vol. 368, no. 1, pp. 112–120, 1999.
- [193] P. Venditti, R. De Rosa, G. Caldarone, and S. Di Meo, "Functional and biochemical characteristics of mitochondrial fractions from rat liver in cold-induced oxidative stress," *Cellular and Molecular Life Sciences*, vol. 61, no. 24, pp. 3104–3116, 2004.
- [194] P. Venditti, G. Napolitano, L. Di Stefano, and S. Di Meo, "Effect of vitamin E on characteristics of liver mitochondrial fractions from cold-exposed rats," *Journal of Bioenergetics and Biomembranes*, vol. 43, no. 4, pp. 387–397, 2011.
- [195] B. Levine and D. J. Klionsky, "Development by self-digestion: molecular mechanisms and biological functions of autophagy," *Developmental Cell*, vol. 6, no. 4, pp. 463–477, 2004.
- [196] J. Debnath, E. H. Baehrecke, and G. Kroemer, "Does autophagy contribute to cell death?" *Autophagy*, vol. 1, no. 2, pp. 66–74, 2005.
- [197] I. Kim, S. Rodriguez-Enriquez, and J. J. Lemasters, "Selective degradation of mitochondria by mitophagy," *Archives of Biochemistry and Biophysics*, vol. 462, no. 2, pp. 245–253, 2007.
- [198] J. Kopitz, G. Ø. Kisen, P. B. Gordon, P. Bohley, and P. O. Seglen, "Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes," *The Journal of Cell Biology*, vol. 111, no. 3, pp. 941–953, 1990.
- [199] W. A. Dunn Jr., J. M. Cregg, J. A. K. W. Kiel et al., "Pexophagy: the selective autophagy of peroxisomes," *Autophagy*, vol. 1, no. 2, pp. 75–83, 2005.
- [200] S. Bernales, S. Schuck, and P. Walter, "ER-phagy: selective autophagy of the endoplasmic reticulum," *Autophagy*, vol. 3, no. 3, pp. 285–287, 2007.
- [201] P. Roberts, S. Moshitch-Moshkovitz, E. Kvam, E. O'Toole, M. Winey, and D. S. Goldfarb, "Piecemeal microautophagy of nucleus in *Saccharomyces cerevisiae*," *Molecular Biology of the Cell*, vol. 14, no. 1, pp. 129–141, 2003.
- [202] I. Kim and J. J. Lemasters, "Mitophagy selectively degrades individual damaged mitochondria after photoirradiation," *Antioxidants & Redox Signaling*, vol. 14, no. 10, pp. 1919–1928, 2011.
- [203] C. Bormann and H. Sahm, "Degradation of microbodies in relation of activities of alcohol oxidase and catalase in *Candida boidinii*," *Archives of Microbiology*, vol. 117, no. 1, pp. 67–72, 1978.
- [204] E. B. Aksam, A. Koek, J. A. K. W. Kiel, S. Jourdan, M. Veenhuis, and I. J. Van Der Klei, "A peroxisomal Lon protease and peroxisome degradation by autophagy play key roles in vitality of *Hansenula polymorpha* cells," *Autophagy*, vol. 3, no. 2, pp. 96–105, 2007.
- [205] J. Huang, G. Y. Lam, and J. H. Brumell, "Autophagy signaling through reactive oxygen species," *Antioxidants & Redox Signaling*, vol. 14, no. 11, pp. 2215–2231, 2011.
- [206] R. Scherz-Shouval and Z. Elazar, "ROS, mitochondria and the regulation of autophagy," *Trends in Cell Biology*, vol. 17, no. 9, pp. 422–427, 2007.
- [207] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, and Z. Elazar, "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4," *The EMBO Journal*, vol. 26, no. 7, pp. 1749–1760, 2007.
- [208] A.-C. Johansson, H. Steen, K. Öllinger, and K. Roberg, "Cathepsin D mediates cytochrome c release and caspase activation in human fibroblast apoptosis induced by staurosporine," *Cell Death and Differentiation*, vol. 10, no. 11, pp. 1253–1259, 2003.
- [209] L. Galluzzi, J. M. Vicencio, O. Kepp, E. Tasdemir, M. C. Maiuri, and G. Kroemer, "To die or not to die: that is the autophagic question," *Current Molecular Medicine*, vol. 8, no. 2, pp. 78–91, 2008.
- [210] S. Shimizu, T. Kanaseki, N. Mizushima et al., "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes," *Nature Cell Biology*, vol. 6, no. 12, pp. 1221–1228, 2004.
- [211] C. Gómez-Santos, I. Ferrer, A. F. Santidrián, M. Barrachina, J. Gil, and S. Ambrosio, "Dopamine induces autophagic cell death and α -synuclein increase in human neuroblastoma SH-SY5Y cells," *Journal of Neuroscience Research*, vol. 73, no. 3, pp. 341–350, 2003.
- [212] R. A. Kirkland, R. M. Adibhatla, J. F. Hatcher, and J. L. Franklin, "Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a role for lipid peroxidation and autophagy," *Neuroscience*, vol. 115, no. 2, pp. 587–602, 2002.
- [213] J. J. Lemasters, A.-L. Nieminen, T. Qian et al., "The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1366, no. 1–2, pp. 177–196, 1998.
- [214] J. R. Jangamreddy and M. J. Los, "Mitoptosis, a novel mitochondrial death mechanism leading predominantly to activation of autophagy," *Hepatitis Monthly*, vol. 12, no. 8, Article ID e6159, 3 pages, 2012.
- [215] K. G. Lyamzaev, O. K. Nepryakhina, V. B. Saprunova et al., "Novel mechanism of elimination of malfunctioning mitochondria (mitoptosis): formation of mitoptotic bodies and extrusion of mitochondrial material from the cell," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1777, no. 7–8, pp. 817–825, 2008.
- [216] A. M. Gorman, S. J. M. Healy, R. Jäger, and A. Samali, "Stress management at the ER: regulators of ER stress-induced apoptosis," *Pharmacology and Therapeutics*, vol. 134, no. 3, pp. 306–316, 2012.
- [217] K. M. Doyle, D. Kennedy, A. M. Gorman, S. Gupta, S. J. M. Healy, and A. Samali, "Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 10, pp. 2025–2039, 2011.
- [218] E. Szegezdi, S. E. Logue, A. M. Gorman, and A. Samali, "Mediators of endoplasmic reticulum stress-induced apoptosis," *EMBO Reports*, vol. 7, no. 9, pp. 880–885, 2006.
- [219] W.-X. Ding, H.-M. Ni, W. Gao et al., "Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival," *The Journal of Biological Chemistry*, vol. 282, no. 7, pp. 4702–4710, 2007.
- [220] V. Nikolettou, M. Markaki, K. Palikaras, and N. Tavernarakis, "Crosstalk between apoptosis, necrosis and autophagy," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1833, no. 12, pp. 3448–3459, 2013.
- [221] N. Morishima, K. Nakanishi, H. Takenouchi, T. Shibata, and Y. Yasuhiko, "An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12," *The Journal of Biological Chemistry*, vol. 277, no. 37, pp. 34287–34294, 2002.

- [222] M. S. Ola, M. Nawaz, and H. Ahsan, "Role of Bcl-2 family proteins and caspases in the regulation of apoptosis," *Molecular and Cellular Biochemistry*, vol. 351, no. 1-2, pp. 41–58, 2011.
- [223] L. O'Connor, A. Strasser, L. A. O'Reilly et al., "Bim: a novel member of the Bcl-2 family that promotes apoptosis," *The EMBO Journal*, vol. 17, no. 2, pp. 384–395, 1998.
- [224] M. Ott, V. Gogvadze, S. Orrenius, and B. Zhivotovsky, "Mitochondria, oxidative stress and cell death," *Apoptosis*, vol. 12, no. 5, pp. 913–922, 2007.
- [225] O. Ivashchenko, P. P. Van Veldhoven, C. Brees, Y.-S. Ho, S. R. Terlecky, and M. Franssen, "Intraperoxisomal redox balance in mammalian cells: oxidative stress and interorganellar cross-talk," *Molecular Biology of the Cell*, vol. 22, no. 9, pp. 1440–1451, 2011.
- [226] B. Wang, P. P. Van Veldhoven, C. Brees et al., "Mitochondria are targets for peroxisome-derived oxidative stress in cultured mammalian cells," *Free Radical Biology and Medicine*, vol. 65, pp. 882–894, 2013.
- [227] S. H. Kaufmann and M. O. Hengartner, "Programmed cell death: alive and well in the new millennium," *Trends in Cell Biology*, vol. 11, no. 12, pp. 526–534, 2001.
- [228] M. Zhao, F. Antunes, J. W. Eaton, and U. T. Brunk, "Lysosomal enzymes promote mitochondrial oxidant production, cytochrome c release and apoptosis," *European Journal of Biochemistry*, vol. 270, no. 18, pp. 3778–3786, 2003.
- [229] F. Antunes, E. Cadenas, and U. T. Brunk, "Apoptosis induced by exposure to a low steady-state concentration of H₂O₂ is a consequence of lysosomal rupture," *Biochemical Journal*, vol. 356, no. 2, pp. 549–555, 2001.
- [230] Y. Ogawa, T. Kobayashi, A. Nishioka et al., "Reactive oxygen species-producing site in radiation-induced apoptosis of human peripheral T cells: involvement of lysosomal membrane destabilization," *International Journal of Molecular Medicine*, vol. 13, no. 1, pp. 69–73, 2004.
- [231] E. Nilsson, R. Ghassemifar, and U. T. Brunk, "Lysosomal heterogeneity between and within cells with respect to resistance against oxidative stress," *Histochemical Journal*, vol. 29, no. 11-12, pp. 857–865, 1997.
- [232] M.-A. Raymond, L. Mollica, N. Vigneault et al., "Blockade of the apoptotic machinery by cyclosporin A redirects cell death toward necrosis in arterial endothelial cells: regulation by reactive oxygen species and cathepsin D," *The FASEB Journal*, vol. 17, no. 3, pp. 515–517, 2003.
- [233] P. D. Gollnick and D. W. King, "Effect of exercise and training on mitochondria of rat skeletal muscle," *The American Journal of Physiology*, vol. 216, no. 6, pp. 1502–1509, 1969.
- [234] D. W. King and P. D. Gollnick, "Ultrastructure of rat heart and liver after exhaustive exercise," *The American Journal of Physiology*, vol. 218, no. 4, pp. 1150–1155, 1970.
- [235] C. B. Ebbeling and P. M. Clarkson, "Exercise-induced muscle damage and adaptation," *Sports Medicine*, vol. 7, no. 4, pp. 207–234, 1989.
- [236] L. J. McCutcheon, S. K. Byrd, and D. R. Hodgson, "Ultrastructural changes in skeletal muscle after fatiguing exercise," *Journal of Applied Physiology*, vol. 72, no. 3, pp. 1111–1117, 1992.
- [237] P. M. Clarkson, "Eccentric exercise and muscle damage," *International Journal of Sports Medicine, Supplement*, vol. 18, no. 4, pp. S314–S317, 1997.
- [238] D. E. R. Warburton, C. W. Nicol, and S. S. D. Bredin, "Health benefits of physical activity: the evidence," *Canadian Medical Association Journal*, vol. 174, no. 6, pp. 801–809, 2006.
- [239] S. R. Colberg, "Physical activity, insulin action, and diabetes prevention and control," *Current Diabetes Reviews*, vol. 3, no. 3, pp. 176–184, 2007.
- [240] S. G. Wannamethee, A. G. Shaper, and M. Walker, "Changes in physical activity, mortality, and incidence of coronary heart disease in older men," *The Lancet*, vol. 351, no. 9116, pp. 1603–1608, 1998.
- [241] D. J. O'Gorman and A. Krook, "Exercise and the treatment of diabetes and obesity," *Endocrinology and Metabolism Clinics of North America*, vol. 37, no. 4, pp. 887–903, 2008.
- [242] F. Edelmann, V. Grabs, and M. Halle, "Exercise training in heart failure," *Internist*, vol. 55, no. 6, pp. 669–675, 2014.
- [243] C. J. Dillard, R. E. Litov, W. M. Savin, E. E. Dumelin, and A. L. Tappel, "Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation," *Journal of Applied Physiology Respiratory Environmental and Exercise Physiology*, vol. 45, no. 6, pp. 927–932, 1978.
- [244] K. J. A. Davies, A. T. Quintanilha, G. A. Brooks, and L. Packer, "Free radicals and tissue damage produced by exercise," *Biochemical and Biophysical Research Communications*, vol. 107, no. 4, pp. 1198–1205, 1982.
- [245] C. T. Kumar, V. K. Reddy, M. Prasad, K. Thyagaraju, and P. Reddanna, "Dietary supplementation of vitamin E protects heart tissue from exercise-induced oxidant stress," *Molecular and Cellular Biochemistry*, vol. 111, no. 1-2, pp. 109–115, 1992.
- [246] M. J. Jackson, R. H. T. Edwards, and M. C. R. Symons, "Electron spin resonance studies of intact mammalian skeletal muscle," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 847, no. 2, pp. 185–190, 1985.
- [247] T. Ashton, C. C. Rowlands, E. Jones et al., "Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise," *European Journal of Applied Physiology and Occupational Physiology*, vol. 77, no. 6, pp. 498–502, 1998.
- [248] S. Mrakic-Spota, M. Gussoni, S. Porcelli et al., "Training effects on ROS production determined by electron paramagnetic resonance in master swimmers," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 804794, 8 pages, 2015.
- [249] T. Ashton, I. S. Young, J. R. Peters et al., "Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study," *Journal of Applied Physiology*, vol. 87, no. 6, pp. 2032–2036, 1999.
- [250] H. M. Alessio and A. H. Goldfarb, "Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training," *Journal of Applied Physiology*, vol. 64, no. 4, pp. 1333–1336, 1988.
- [251] P. Venditti and S. Di Meo, "Antioxidants, tissue damage, and endurance in trained and untrained young male rats," *Archives of Biochemistry and Biophysics*, vol. 331, no. 1, pp. 63–68, 1996.
- [252] P. Venditti and S. Di Meo, "Effect of training on antioxidant capacity, tissue damage, and endurance of adult male rats," *International Journal of Sports Medicine*, vol. 18, no. 7, pp. 497–502, 1997.
- [253] S. Carfagna, G. Napolitano, D. Barone, G. Pinto, A. Pollio, and P. Venditti, "Dietary supplementation with the microalga *Galdieria sulphuraria* (Rhodophyta) reduces prolonged exercise-induced oxidative stress in rat tissues," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 732090, 11 pages, 2015.
- [254] C.-C. Huang, T.-J. Lin, Y.-F. Lu, C.-C. Chen, C.-Y. Huang, and W.-T. Lin, "Protective effects of L-arginine supplementation against exhaustive exercise-induced oxidative stress in young rat tissues," *Chinese Journal of Physiology*, vol. 52, no. 5, pp. 306–315, 2009.

- [255] W. Ziolkowski, D. J. Flis, M. Halon et al., "Prolonged swimming promotes cellular oxidative stress and p66Shc phosphorylation, but does not induce oxidative stress in mitochondria in the rat heart," *Free Radical Research*, vol. 49, no. 1, pp. 7–16, 2015.
- [256] J. Liu, H. C. Yeo, E. Övervik-Douki et al., "Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants," *Journal of Applied Physiology*, vol. 89, no. 1, pp. 21–28, 2000.
- [257] M. Suzuki, S. Katamine, and S. Tatsumi, "Exercise-induced enhancement of lipid peroxide metabolism in tissues and their transference into the brain in rat," *Journal of Nutritional Science and Vitaminology*, vol. 29, no. 2, pp. 141–151, 1983.
- [258] Ü. K. Sentürk, F. Gündüz, O. Kuru et al., "Exercise-induced oxidative stress affects erythrocytes in sedentary rats but not exercise-trained rats," *Journal of Applied Physiology*, vol. 91, no. 5, pp. 1999–2004, 2001.
- [259] D. Ramos, E. G. Martins, D. Viana-Gomes, G. Casimiro-Lopes, and V. P. Salerno, "Biomarkers of oxidative stress and tissue damage released by muscle and liver after a single bout of swimming exercise," *Applied Physiology, Nutrition and Metabolism*, vol. 38, no. 5, pp. 507–511, 2013.
- [260] S. Asami, T. Hirano, R. Yamaguchi, Y. Tsurudome, H. Itoh, and H. Kasai, "Effects of forced and spontaneous exercise on 8-hydroxydeoxyguanosine levels in rat organs," *Biochemical and Biophysical Research Communications*, vol. 243, no. 3, pp. 678–682, 1998.
- [261] R. Lovlin, W. Cottle, I. Pyke, M. Kavanagh, and A. N. Belcastro, "Are indices of free radical damage related to exercise intensity," *European Journal of Applied Physiology and Occupational Physiology*, vol. 56, no. 3, pp. 313–316, 1987.
- [262] H. M. Alessio, A. H. Goldfarb, and R. G. Cutler, "MDA content increases in fast- and slow-twitch skeletal muscle with intensity of exercise in a rat," *The American Journal of Physiology*, vol. 255, no. 6, part 1, pp. C874–C877, 1988.
- [263] A. Meister and M. E. Anderson, "Glutathione," *Annual Review of Biochemistry*, vol. 52, pp. 711–760, 1983.
- [264] H. Lew, S. Pyke, and A. Quintanilha, "Changes in the glutathione status of plasma, liver and muscle following exhaustive exercise in rats," *FEBS Letters*, vol. 185, no. 2, pp. 262–266, 1985.
- [265] C. K. Sen, M. Atalay, and O. Hanninen, "Exercise-induced oxidative stress: glutathione supplementation and deficiency," *Journal of Applied Physiology*, vol. 77, no. 5, pp. 2177–2187, 1994.
- [266] S. Pyke, H. Lew, and A. Quintanilha, "Severe depletion in liver glutathione during physical exercise," *Biochemical and Biophysical Research Communications*, vol. 139, no. 3, pp. 926–931, 1986.
- [267] S. C. Lu, C. Garcia-Ruiz, J. Kuhlenkamp, M. Ookhtens, M. Salas-Prato, and N. Kaplowitz, "Hormonal regulation of glutathione efflux," *Journal of Biological Chemistry*, vol. 265, no. 27, pp. 16088–16095, 1990.
- [268] H. Galbo, *Hormonal Adaptations to Exercise*, Thieme, New York, NY, USA, 1983.
- [269] G. B. de Quiroga, "Brown fat thermogenesis and exercise: two examples of physiological oxidative stress?" *Free Radical Biology & Medicine*, vol. 13, no. 4, pp. 325–340, 1992.
- [270] B. Chance, H. Sies, and A. Boveris, "Hydroperoxide metabolism in mammalian organs," *Physiological Reviews*, vol. 59, no. 3, pp. 527–605, 1979.
- [271] H. J. Green, T. A. Duhamel, I. C. Smith et al., "Muscle metabolic, enzymatic and transporter responses to a session of prolonged cycling," *European Journal of Applied Physiology*, vol. 111, no. 5, pp. 827–837, 2011.
- [272] S. Di Meo and P. Venditti, "Mitochondria in exercise-induced oxidative stress," *Biological Signals and Receptors*, vol. 10, no. 1–2, pp. 125–140, 2001.
- [273] P. Venditti, A. Bari, L. Di Stefano, and S. Di Meo, "Role of mitochondria in exercise-induced oxidative stress in skeletal muscle from hyperthyroid rats," *Archives of Biochemistry and Biophysics*, vol. 463, no. 1, pp. 12–18, 2007.
- [274] H. Bo, N. Jiang, G. Ma et al., "Regulation of mitochondrial uncoupling respiration during exercise in rat heart: role of reactive oxygen species (ROS) and uncoupling protein 2," *Free Radical Biology and Medicine*, vol. 44, no. 7, pp. 1373–1381, 2008.
- [275] B. Sjödin, Y. Hellsten Westing, and F. S. Apple, "Biochemical mechanisms for oxygen free radical formation during exercise," *Sports Medicine*, vol. 10, no. 4, pp. 236–254, 1990.
- [276] M. D. Brand, "Uncoupling to survive? The role of mitochondrial inefficiency in ageing," *Experimental Gerontology*, vol. 35, no. 6–7, pp. 811–820, 2000.
- [277] M. Fernström, M. Tonkonogi, and K. Sahlin, "Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle," *The Journal of Physiology*, vol. 554, no. 3, pp. 755–763, 2004.
- [278] D. G. Nicholls and R. M. Locke, "Thermogenic mechanisms in brown fat," *Physiological Reviews*, vol. 64, no. 1, pp. 1–64, 1984.
- [279] M. Zhou, B.-Z. Lin, S. Coughlin, G. Vallega, and P. F. Pilch, "UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 279, no. 3, pp. E622–E629, 2000.
- [280] D. Ricquier and F. Bouillaud, "The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP," *Biochemical Journal*, vol. 345, no. 2, pp. 161–179, 2000.
- [281] D. A. Talbot, A. J. Lambert, and M. D. Brand, "Production of endogenous matrix superoxide from mitochondrial complex I leads to activation of uncoupling protein 3," *FEBS Letters*, vol. 556, no. 1–3, pp. 111–115, 2004.
- [282] P. S. Brookes, J. M. Land, J. B. Clark, and S. J. R. Heales, "Peroxynitrite and brain mitochondria: evidence for increased proton leak," *Journal of Neurochemistry*, vol. 70, no. 5, pp. 2195–2202, 1998.
- [283] K. S. Echtay, T. C. Esteves, J. L. Pakay et al., "A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling," *The EMBO Journal*, vol. 22, no. 16, pp. 4103–4110, 2003.
- [284] Y. Zhang, O. Marcillat, C. Giulivi, L. Ernster, and K. J. A. Davies, "The oxidative inactivation of mitochondrial electron transport chain components and ATPase," *The Journal of Biological Chemistry*, vol. 265, no. 27, pp. 16330–16336, 1990.
- [285] A. Cassina and R. Radi, "Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport," *Archives of Biochemistry and Biophysics*, vol. 328, no. 2, pp. 309–316, 1996.
- [286] K. Madsen, P. Ertbjerg, M. S. Djurhuus, and P. K. Pedersen, "Calcium content and respiratory control index of skeletal muscle mitochondria during exercise and recovery," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 271, no. 6, pp. E1044–E1050, 1996.
- [287] T. E. Gunter and D. R. Pfeiffer, "Mechanisms by which mitochondria transport calcium," *American Journal of Physiology—Cell Physiology*, vol. 258, no. 5, pp. C755–C786, 1990.
- [288] S. Vesce, M. B. Jekabsons, L. I. Johnson-Cadwell, and D. G. Nicholls, "Acute glutathione depletion restricts mitochondrial

- ATP export in cerebellar granule neurons," *The Journal of Biological Chemistry*, vol. 280, no. 46, pp. 38720–38728, 2005.
- [289] Q.-A. Sun, B. Wang, M. Miyagi, D. T. Hess, and J. S. Stampler, "Oxygen-coupled redox regulation of the skeletal muscle ryanodine receptor/ Ca^{2+} release channel (RyR1): sites and nature of oxidative modification," *The Journal of Biological Chemistry*, vol. 288, no. 32, pp. 22961–22971, 2013.
- [290] C. Hidalgo, G. Sánchez, G. Barrientos, and P. Aracena-Parks, "A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 S-glutathionylation," *The Journal of Biological Chemistry*, vol. 281, no. 36, pp. 26473–26482, 2006.
- [291] G. K. Sakellariou, A. Vasilaki, J. Palomero et al., "Studies of mitochondrial and nonmitochondrial sources implicate nicotinamide adenine dinucleotide phosphate oxidase(s) in the increased skeletal muscle superoxide generation that occurs during contractile activity," *Antioxidants & Redox Signaling*, vol. 18, no. 6, pp. 603–621, 2013.
- [292] A. Espinosa, A. Leiva, M. Peña et al., "Myotube depolarization generates reactive oxygen species through NAD(P)H oxidase; ROS-elicited Ca^{2+} stimulates ERK, CREB, early genes," *Journal of Cellular Physiology*, vol. 209, no. 2, pp. 379–388, 2006.
- [293] T. Pearson, T. Kabayo, R. Ng, J. Chamberlain, A. McArdle, and M. J. Jackson, "Skeletal muscle contractions induce acute changes in cytosolic superoxide, but slower responses in mitochondrial superoxide and cellular hydrogen peroxide," *PLoS ONE*, vol. 9, no. 5, Article ID e96378, 2014.
- [294] W. Dröge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, no. 1, pp. 47–95, 2002.
- [295] G. Jorquera, F. Altamirano, A. Contreras-Ferrat et al., "Cav1.1 controls frequency-dependent events regulating adult skeletal muscle plasticity," *Journal of Cell Science*, vol. 126, no. 5, pp. 1189–1198, 2013.
- [296] A. Díaz-Vegas, C. A. Campos, A. Contreras-Ferrat et al., "ROS production via P2Y_1 -PKC-NOX2 is triggered by extracellular ATP after electrical stimulation of skeletal muscle cells," *PLoS ONE*, vol. 10, no. 6, Article ID e0129882, 14 pages, 2015.
- [297] S. P. Mortensen, J. González-Alonso, J.-J. Nielsen, B. Saltin, and Y. Hellsten, "Muscle interstitial ATP and norepinephrine concentrations in the human leg during exercise and ATP infusion," *Journal of Applied Physiology*, vol. 107, no. 6, pp. 1757–1762, 2009.
- [298] Y. Hellsten, D. Maclean, G. Rådegran, B. Saltin, and J. Bangsbo, "Adenosine concentrations in the interstitium of resting and contracting human skeletal muscle," *Circulation*, vol. 98, no. 1, pp. 6–8, 1998.
- [299] T. G. McKelvey, M. E. Hollwarth, D. N. Granger, T. D. Engerson, U. Landler, and H. P. Jones, "Mechanisms of conversion of xanthine dehydrogenase to xanthine oxidase in ischemic rat liver and kidney," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 254, no. 5, part 1, pp. G753–G760, 1988.
- [300] D. A. Parks, T. K. Williams, and J. S. Beckman, "Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: a reevaluation," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 254, no. 5, pp. G767–G774, 1988.
- [301] E.-D. Jarasch, C. Grund, G. Bruder, H. W. Heid, T. W. Keenan, and W. W. Franke, "Localization of xanthine oxidase in mammary-gland epithelium and capillary endothelium," *Cell*, vol. 25, no. 1, pp. 67–82, 1981.
- [302] A. Kooij, M. Schijns, W. M. Frederiks, C. J. F. Van Noorden, and J. James, "Distribution of xanthine oxidoreductase activity in human tissues: a histochemical and biochemical study," *Virchows Archiv B: Cell Pathology Including Molecular Pathology*, vol. 63, no. 1, pp. 17–23, 1992.
- [303] P. Felig and J. Wahren, "Amino acid metabolism in exercising man," *The Journal of Clinical Investigation*, vol. 50, no. 12, pp. 2703–2714, 1971.
- [304] Y. Hellsten-Westing, L. Kaijser, B. Ekblom, and B. Sjödin, "Exchange of purines in human liver and skeletal muscle with short-term exhaustive exercise," *American Journal of Physiology*, vol. 266, no. 1, pp. R81–R86, 1994.
- [305] K. Koyama, M. Kaya, T. Ishigaki et al., "Role of xanthine oxidase in delayed lipid peroxidation in rat liver induced by acute exhausting exercise," *European Journal of Applied Physiology and Occupational Physiology*, vol. 80, no. 1, pp. 28–33, 1999.
- [306] M.-C. Gomez-Cabrera, C. Borrás, F. V. Pallardo, J. Sastre, L. L. Ji, and J. Viña, "Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats," *Journal of Physiology*, vol. 567, no. 1, pp. 113–120, 2005.
- [307] M.-C. Gómez-Cabrera, F. V. Pallardó, J. Sastre, J. Viña, and L. Garcia-Del-Moral, "Allopurinol and markers of muscle damage among participants in the tour de France," *Journal of the American Medical Association*, vol. 289, no. 19, pp. 2503–2504, 2003.
- [308] Y. Hellsten, F. S. Apple, and B. Sjödin, "Effect of sprint cycle training on activities of antioxidant enzymes in human skeletal muscle," *Journal of Applied Physiology*, vol. 81, no. 4, pp. 1484–1487, 1996.
- [309] J. A. R. Duarte, H.-J. Appell, F. Carvalho, M. L. Bastos, and J. M. C. Soares, "Endothelium-derived oxidative stress may contribute to exercise-induced muscle damage," *International Journal of Sports Medicine*, vol. 14, no. 8, pp. 440–443, 1993.
- [310] R. B. Armstrong, G. L. Warren, and J. A. Warren, "Mechanisms of exercise-induced muscle fibre injury," *Sports Medicine*, vol. 12, no. 3, pp. 184–207, 1991.
- [311] A. S. Veskokoukis, M. G. Nikolaidis, A. Kyparos et al., "Effects of xanthine oxidase inhibition on oxidative stress and swimming performance in rats," *Applied Physiology, Nutrition and Metabolism*, vol. 33, no. 6, pp. 1140–1154, 2008.
- [312] D. K. Das, R. M. Engelman, R. Clement, H. Otani, M. R. Prasad, and P. S. Rao, "Role of xanthine oxidase inhibitor as free radical scavenger: a novel mechanism of action of allopurinol and oxypurinol in myocardial salvage," *Biochemical and Biophysical Research Communications*, vol. 148, no. 1, pp. 314–319, 1987.
- [313] S. Lindsay, T.-H. Liu, J. Xu et al., "Role of xanthine dehydrogenase and oxidase in focal cerebral ischemic injury to rat," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 261, no. 6, pp. H2051–H2057, 1991.
- [314] G. P. Novelli, G. Bracciotti, and S. Falsini, "Spin-trappers and vitamin E prolong endurance to muscle fatigue in mice," *Free Radical Biology and Medicine*, vol. 8, no. 1, pp. 9–13, 1990.
- [315] G. D. Wadley, M. A. Nicolas, D. S. Hiam, and G. K. McConell, "Xanthine oxidase inhibition attenuates skeletal muscle signaling following acute exercise but does not impair mitochondrial adaptations to endurance training," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 304, no. 8, pp. E853–E862, 2013.
- [316] P. Venditti, G. Napolitano, D. Barone, and S. Di Meo, "Effect of training and vitamin e administration on rat liver oxidative metabolism," *Free Radical Research*, vol. 48, no. 3, pp. 322–332, 2014.
- [317] P. Venditti, G. Napolitano, D. Barone, and S. Di Meo, "Vitamin E supplementation modifies adaptive responses to training in

- rat skeletal muscle," *Free Radical Research*, vol. 48, no. 10, pp. 1179–1189, 2014.
- [318] P. Venditti, G. Napolitano, D. Barone, E. Pervito, and S. Di Meo, "Vitamin E-enriched diet reduces adaptive responses to training determining respiratory capacity and redox homeostasis in rat heart," *Free Radical Research*, vol. 50, no. 1, pp. 56–67, 2016.
- [319] P. Venditti, A. Bari, L. Di Stefano, C. Agnisola, and S. Di Meo, "Effect of T3 treatment on the response to ischemia-reperfusion of heart preparations from sedentary and trained rats," *Pflugers Archiv European Journal of Physiology*, vol. 455, no. 4, pp. 667–676, 2008.
- [320] S. K. Powers, L. L. Ji, and C. Leeuwenburgh, "Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review," *Medicine and Science in Sports and Exercise*, vol. 31, no. 7, pp. 987–997, 1999.
- [321] M. Atalay and C. K. Sen, "Physical exercise and antioxidant defenses in the heart," *Annals of the New York Academy of Sciences*, vol. 874, pp. 169–177, 1999.
- [322] M. Gore, R. Fiebig, J. Hollander, C. Leeuwenburgh, H. Ohno, and L. L. Ji, "Endurance training alters antioxidant enzyme gene expression in rat skeletal muscle," *Canadian Journal of Physiology and Pharmacology*, vol. 76, no. 12, pp. 1139–1145, 1998.
- [323] J. Hollander, R. Fiebig, M. Gore et al., "Superoxide dismutase gene expression in skeletal muscle: fiber-specific adaptation to endurance training," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 277, no. 3, pp. R856–R862, 1999.
- [324] P. Venditti, P. Masullo, and S. Di Meo, "Effect of training on H₂O₂ release by mitochondria from rat skeletal muscle," *Archives of Biochemistry and Biophysics*, vol. 372, no. 2, pp. 315–320, 1999.
- [325] J. W. Starnes, B. D. Barnes, and M. E. Olsen, "Exercise training decreases rat heart mitochondria free radical generation but does not prevent Ca²⁺-induced dysfunction," *Journal of Applied Physiology*, vol. 102, no. 5, pp. 1793–1798, 2007.
- [326] S. Servais, K. Couturier, H. Koubi et al., "Effect of voluntary exercise on H₂O₂ release by subsarcolemmal and intermyofibrillar mitochondria," *Free Radical Biology and Medicine*, vol. 35, no. 1, pp. 24–32, 2003.
- [327] O. Boss, S. Samec, D. Desplanches et al., "Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 in the rat," *The FASEB Journal*, vol. 12, no. 3, pp. 335–339, 1998.
- [328] Y. Lee, H.-B. Kwak, J. Hord, J.-H. Kim, and J. M. Lawler, "Exercise training attenuates age-dependent elevation of angiotensin II type 1 receptor and Nox2 signaling in the rat heart," *Experimental Gerontology*, vol. 70, pp. 163–173, 2015.
- [329] M. Cocks, C. S. Shaw, S. O. Shepherd et al., "Sprint interval and moderate-intensity continuous training have equal benefits on aerobic capacity, insulin sensitivity, muscle capillarisation and endothelial eNOS/NAD(P)H oxidase protein ratio in obese men," *Journal of Physiology*, vol. 594, no. 8, pp. 2307–2321, 2016.
- [330] M. Cocks, C. S. Shaw, S. O. Shepherd et al., "Sprint interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males," *Journal of Physiology*, vol. 591, no. 3, pp. 641–656, 2013.
- [331] S. Touati, A. C. Montezano, F. Meziri, C. Riva, R. M. Touyz, and P. Laurant, "Exercise training protects against atherosclerotic risk factors through vascular NADPH oxidase, extracellular signal-regulated kinase 1/2 and stress-activated protein kinase/c-Jun N-terminal kinase downregulation in obese rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 42, no. 2, pp. 179–185, 2015.
- [332] K. M. Baldwin, G. H. Klinkerfuss, R. L. Terjung, P. A. Molé, and J. O. Holloszy, "Respiratory capacity of white, red, and intermediate muscle: adaptive response to exercise," *The American Journal of Physiology*, vol. 222, no. 2, pp. 373–378, 1972.
- [333] K. J. A. Davies, L. Packer, and G. A. Brooks, "Biochemical adaptation of mitochondria, muscle, and whole-animal respiration to endurance training," *Archives of Biochemistry and Biophysics*, vol. 209, no. 2, pp. 539–554, 1981.
- [334] D. H. Wasserman and A. D. Cherrington, "Hepatic fuel metabolism during muscular work: role and regulation," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 260, no. 6, pp. E811–E824, 1991.
- [335] R. C. Scarpulla, "Nuclear activators and coactivators in mammalian mitochondrial biogenesis," *Biochimica et Biophysica Acta (BBA)—Gene Structure and Expression*, vol. 1576, no. 1–2, pp. 1–14, 2002.
- [336] H. Liang and W. F. Ward, "PGC-1 α : a key regulator of energy metabolism," *Advances in Physiology Education*, vol. 30, no. 4, pp. 145–151, 2006.
- [337] I. Irrcher, P. J. Adhichetty, T. Sheehan, A.-M. Joseph, and D. A. Hood, "PPAR γ coactivator-1 α expression during thyroid hormone- and contractile activity-induced mitochondrial adaptations," *American Journal of Physiology—Cell Physiology*, vol. 284, no. 6, pp. C1669–C1677, 2003.
- [338] K. Baar, A. R. Wende, T. E. Jones et al., "Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1," *The FASEB Journal*, vol. 16, no. 14, pp. 1879–1886, 2002.
- [339] L. Wang, H. Mascher, N. Psilander, E. Blomstrand, and K. Sahlin, "Resistance exercise enhances the molecular signaling of mitochondrial biogenesis induced by endurance exercise in human skeletal muscle," *Journal of Applied Physiology*, vol. 111, no. 5, pp. 1335–1344, 2011.
- [340] T. N. Haase, S. Ringholm, L. Leick et al., "Role of PGC-1 α in exercise and fasting-induced adaptations in mouse liver," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 301, no. 5, pp. R1501–R1509, 2011.
- [341] A. N. Kavazis, A. J. Smuder, and S. K. Powers, "Effects of short-term endurance exercise training on acute doxorubicin-induced FoxO transcription in cardiac and skeletal muscle," *Journal of Applied Physiology*, vol. 117, no. 3, pp. 223–230, 2014.
- [342] C. Kang and L. L. Ji, "Role of PGC-1 α signaling in skeletal muscle health and disease," *Annals of the New York Academy of Sciences*, vol. 1271, no. 1, pp. 110–117, 2012.
- [343] J. Olesen, K. Küllerich, and H. Pilegaard, "PGC-1 α -mediated adaptations in skeletal muscle," *Pflugers Archiv European Journal of Physiology*, vol. 460, no. 1, pp. 153–162, 2010.
- [344] X. Kong, R. Wang, Y. Xue et al., "Sirtuin 3, a new target of PGC-1 α , plays an important role in the suppression of ROS and mitochondrial biogenesis," *PLoS ONE*, vol. 5, no. 7, Article ID e11707, 2010.
- [345] T. Shi, F. Wang, E. Stieren, and Q. Tong, "SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes," *Journal of Biological Chemistry*, vol. 280, no. 14, pp. 13560–13567, 2005.
- [346] J. St-Pierre, J. Lin, S. Krauss et al., "Bioenergetic analysis of peroxisome proliferator-activated receptor γ coactivators 1 α and 1 β (PGC-1 α and PGC-1 β) in muscle cells," *The Journal of Biological Chemistry*, vol. 278, no. 29, pp. 26597–26603, 2003.
- [347] L. R. Silveira, H. Pilegaard, K. Kusuhara, R. Curi, and Y. Hellsten, "The effect of reactive oxygen species and antioxidants

- on basal and contraction-induced gene expression of PGC-1 α , UCP3 and HKII in primary rat skeletal muscle cells," *Biochemical and Biophysical Acta*, vol. 1763, pp. 969–976, 2006.
- [348] J. St-Pierre, S. Drori, M. Uldry et al., "Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators," *Cell*, vol. 127, no. 2, pp. 397–408, 2006.
- [349] K. Higashida, S. H. Kim, M. Higuchi, J. O. Holloszy, and D.-H. Han, "Normal adaptations to exercise despite protection against oxidative stress," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 301, no. 5, pp. E779–E784, 2011.
- [350] M.-C. Gomez-Cabrera, E. Domenech, M. Romagnoli et al., "Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance," *American Journal of Clinical Nutrition*, vol. 87, no. 1, pp. 142–149, 2008.
- [351] M. Ristow, K. Zarse, A. Oberbach et al., "Antioxidants prevent health-promoting effects of physical exercise in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 21, pp. 8665–8670, 2009.
- [352] G. Paulsen, K. T. Cumming, G. Holden et al., "Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind, randomised, controlled trial," *The Journal of Physiology*, vol. 592, no. 8, pp. 1887–1901, 2014.
- [353] B. M. Popkin, "Global nutrition dynamics: the world is shifting rapidly toward a diet linked with noncommunicable diseases," *The American Journal of Clinical Nutrition*, vol. 84, no. 2, pp. 289–298, 2006.
- [354] M. Rocha, N. Apostolova, J. R. Herance, S. Rovira-Llopis, A. Hernandez-Mijares, and V. M. Victor, "Perspectives and potential applications of mitochondria-targeted antioxidants in cardiometabolic diseases and type 2 diabetes," *Medicinal Research Reviews*, vol. 34, no. 1, pp. 160–189, 2014.
- [355] N. Apostolova and V. M. Victor, "Molecular strategies for targeting antioxidants to mitochondria: therapeutic implications," *Antioxidants & Redox Signaling*, vol. 22, no. 8, pp. 686–729, 2015.
- [356] A. Brehm, M. Krssak, A. I. Schmid, P. Nowotny, W. Waldhäusl, and M. Roden, "Increased lipid availability impairs insulin-stimulated ATP synthesis in human skeletal muscle," *Diabetes*, vol. 55, no. 1, pp. 136–140, 2006.
- [357] P. Ritz and G. Berrut, "Mitochondrial function, energy expenditure, aging and insulin resistance," *Diabetes and Metabolism*, vol. 31, no. 2, pp. S67–S73, 2005.
- [358] M. Frisard and E. Ravussin, "Energy metabolism and oxidative stress: impact on the metabolic syndrome and the aging process," *Endocrine*, vol. 29, no. 1, pp. 27–32, 2006.
- [359] A. Hernandez-Mijares, M. Rocha, N. Apostolova et al., "Mitochondrial complex I impairment in leukocytes from type 2 diabetic patients," *Free Radical Biology & Medicine*, vol. 50, no. 10, pp. 1215–1221, 2011.
- [360] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.
- [361] H. Ashrafian, M. P. Frenneaux, and L. H. Opie, "Metabolic mechanisms in heart failure," *Circulation*, vol. 116, no. 4, pp. 434–448, 2007.
- [362] T. Nishikawa, D. Edelstein, X. L. Du et al., "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage," *Nature*, vol. 404, no. 6779, pp. 787–790, 2000.
- [363] V. M. Victor, M. Rocha, R. Herance, and A. Hernandez-Mijares, "Oxidative stress and mitochondrial dysfunction in type 2 diabetes," *Current Pharmaceutical Design*, vol. 17, no. 36, pp. 3947–3958, 2011.
- [364] E. Tatsch, J. A. M. D. Carvalho, B. S. Hausen et al., "Oxidative DNA damage is associated with inflammatory response, insulin resistance and microvascular complications in type 2 diabetes," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 782, pp. 17–22, 2015.
- [365] I. Padmalayam, "Targeting mitochondrial oxidative stress through lipoic acid synthase: a novel strategy to manage diabetic cardiovascular disease," *Cardiovascular and Hematological Agents in Medicinal Chemistry*, vol. 10, no. 3, pp. 223–233, 2012.
- [366] I. Faid, H. Al-Hussaini, and N. Kilarkaje, "Resveratrol alleviates diabetes-induced testicular dysfunction by inhibiting oxidative stress and c-Jun N-terminal kinase signaling in rats," *Toxicology and Applied Pharmacology*, vol. 289, no. 3, pp. 482–494, 2015.
- [367] S. Verkaar, W. J. H. Koopman, J. Cheek et al., "Mitochondrial and cytosolic thiol redox state are not detectably altered in isolated human NADH:ubiquinone oxidoreductase deficiency," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1772, no. 9, pp. 1041–1051, 2007.
- [368] M. P. Murphy, "Antioxidants as therapies: can we improve on nature?" *Free Radical Biology and Medicine*, vol. 66, pp. 20–23, 2014.
- [369] S. Rovira-Llopis, M. Rocha, R. Falcon et al., "Is myeloperoxidase a key component in the ROS-induced vascular damage related to nephropathy in type 2 diabetes?" *Antioxidants & Redox Signaling*, vol. 19, no. 13, pp. 1452–1458, 2013.
- [370] S. Rovira-Llopis, N. Díaz-Morales, C. Bañuls et al., "Is autophagy altered in the leukocytes of type 2 diabetic patients?" *Antioxidants & Redox Signaling*, vol. 23, no. 13, pp. 1050–1056, 2015.
- [371] S. Rovira-Llopis, C. Bañuls, N. Apostolova et al., "Is glycemic control modulating endoplasmic reticulum stress in leukocytes of type 2 diabetic patients?" *Antioxidants and Redox Signaling*, vol. 21, no. 12, pp. 1759–1765, 2014.
- [372] V. M. Victor, M. Rocha, C. Bañuls et al., "Induction of oxidative stress and human leukocyte/endothelial cell interactions in polycystic ovary syndrome patients with insulin resistance," *Journal of Clinical Endocrinology and Metabolism*, vol. 96, no. 10, pp. 3115–3122, 2011.
- [373] M. F. Persson, S. Franzén, S.-B. Catrina et al., "Coenzyme Q10 prevents GDP-sensitive mitochondrial uncoupling, glomerular hyperfiltration and proteinuria in kidneys from db/db mice as a model of type 2 diabetes," *Diabetologia*, vol. 55, no. 5, pp. 1535–1543, 2012.
- [374] A. Hernandez-Mijares, M. Rocha, S. Rovira-Llopis et al., "Human leukocyte/endothelial cell interactions and mitochondrial dysfunction in type 2 diabetic patients and their association with silent myocardial ischemia," *Diabetes Care*, vol. 36, no. 6, pp. 1695–1702, 2013.
- [375] K. Green, M. D. Brand, and M. P. Murphy, "Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes," *Diabetes*, vol. 53, no. 1, pp. S110–S118, 2004.
- [376] B. K. Chacko, C. Reily, A. Srivastava et al., "Prevention of diabetic nephropathy in Ins2^{+/-Akita} mice by the mitochondria-targeted therapy MitoQ," *The Biochemical Journal*, vol. 432, no. 1, pp. 9–19, 2010.
- [377] K. Morino, K. F. Petersen, and G. I. Shulman, "Molecular mechanisms of insulin resistance in humans and their potential links

- with mitochondrial dysfunction,” *Diabetes*, vol. 55, supplement 2, pp. S9–S15, 2006.
- [378] T. Björnheden, A. Babyi, G. Bondjers, and O. Wiklund, “Accumulation of lipoprotein fractions and subfractions in the arterial wall, determined in an in vitro perfusion system,” *Atherosclerosis*, vol. 123, no. 1-2, pp. 43–56, 1996.
- [379] H. Campos, K. S. Arnold, M. E. Balestra, T. L. Innerarity, and R. M. Krauss, “Differences in receptor binding of LDL subfractions,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 16, no. 6, pp. 794–801, 1996.
- [380] N. F. Galeano, R. Milne, Y. L. Marcel et al., “Apoprotein B structure and receptor recognition of triglyceride-rich low density lipoprotein (LDL) is modified in small LDL but not in triglyceride-rich LDL of normal size,” *The Journal of Biological Chemistry*, vol. 269, no. 1, pp. 511–519, 1994.
- [381] D. L. Tribble, L. G. Holl, P. D. Wood, and R. M. Krauss, “Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size,” *Atherosclerosis*, vol. 93, no. 3, pp. 189–199, 1992.
- [382] T. Nishikawa and E. Araki, “Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications,” *Antioxidants & Redox Signaling*, vol. 9, no. 3, pp. 343–353, 2007.
- [383] M. Khodaeian, O. Tabatabaei-Malazy, M. Qorbani, F. Farzadfar, P. Amini, and B. Larijani, “Effect of vitamins C and E on insulin resistance in diabetes: A Meta-Analysis Study,” *European Journal of Clinical Investigation*, vol. 45, no. 11, pp. 1161–1174, 2015.
- [384] K. F. Petersen, S. Dufour, D. Befroy, R. Garcia, and G. I. Shulman, “Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes,” *The New England Journal of Medicine*, vol. 350, no. 7, pp. 664–671, 2004.
- [385] S. Oseid, H. Beck Nielsen, O. Pedersen, and O. Sovik, “Decreased binding of insulin to its receptor in patients with congenital generalized lipodystrophy,” *New England Journal of Medicine*, vol. 296, no. 5, pp. 245–248, 1977.
- [386] R. R. Banerjee, S. M. Rangwala, J. S. Shapiro et al., “Regulation of fasted blood glucose by resistin,” *Science*, vol. 303, no. 5661, pp. 1195–1198, 2004.
- [387] T. Yamauchi, J. Kamon, H. Waki et al., “The fat-derived hormone adiponectin reverses insulin resistance associated with both lipodystrophy and obesity,” *Nature Medicine*, vol. 7, no. 8, pp. 941–946, 2001.
- [388] I. Bogacka, H. Xie, G. A. Bray, and S. R. Smith, “Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo,” *Diabetes*, vol. 54, no. 5, pp. 1392–1399, 2005.
- [389] R. Parish and K. F. Petersen, “Mitochondrial dysfunction and type 2 diabetes,” *Current Diabetes Reports*, vol. 5, no. 3, pp. 177–183, 2005.
- [390] J. B. Kostis and M. Sanders, “The association of heart failure with insulin resistance and the development of type 2 diabetes,” *American Journal of Hypertension*, vol. 18, no. 5, pp. 731–737, 2005.
- [391] E. Ferrannini and W. C. Cushman, “Diabetes and hypertension: the bad companions,” *The Lancet*, vol. 380, no. 9841, pp. 601–610, 2012.
- [392] R. Katz, M. J. Budoff, K. D. O’Brien, N. D. Wong, and K. Nasir, “The metabolic syndrome and diabetes mellitus as predictors of thoracic aortic calcification as detected by non-contrast computed tomography in the Multi-Ethnic Study of Atherosclerosis,” *Diabetic Medicine*, vol. 33, no. 7, pp. 912–919, 2016.
- [393] J. M. McGavock, I. Lingvay, I. Zib et al., “Cardiac steatosis in diabetes mellitus: a 1H-magnetic resonance spectroscopy study,” *Circulation*, vol. 116, no. 10, pp. 1170–1175, 2007.
- [394] A. Whaley-Connell, G. Govindarajan, J. Habibi et al., “Angiotensin II-mediated oxidative stress promotes myocardial tissue remodeling in the transgenic (mRen2) 27 Ren2 rat,” *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 293, no. 1, pp. E355–E363, 2007.
- [395] P. V. G. Katakam, J. E. Jordan, J. A. Snipes, C. D. Tulbert, A. W. Miller, and D. W. Busija, “Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance,” *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 292, no. 2, pp. R920–R926, 2007.
- [396] B. Kumar, A. Kowluru, and R. A. Kowluru, “Lipotoxicity augments glucotoxicity-induced mitochondrial damage in the development of diabetic retinopathy,” *Investigative Ophthalmology & Visual Science*, vol. 56, no. 5, pp. 2985–2992, 2015.
- [397] Y. Nishio, A. Kanazawa, Y. Nagai, H. Inagaki, and A. Kashiwagi, “Regulation and role of the mitochondrial transcription factor in the diabetic rat heart,” *Annals of the New York Academy of Sciences*, vol. 1011, pp. 78–85, 2004.
- [398] N. Suematsu, H. Tsutsui, J. Wen et al., “Oxidative stress mediates tumor necrosis factor- α -induced mitochondrial DNA damage and dysfunction in cardiac myocytes,” *Circulation*, vol. 107, no. 10, pp. 1418–1423, 2003.
- [399] B. N. Finck and D. P. Kelly, “Peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease,” *Circulation*, vol. 115, no. 19, pp. 2540–2548, 2007.
- [400] J.-A. Kim, M. Montagnani, K. K. Kwang, and M. J. Quon, “Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms,” *Circulation*, vol. 113, no. 15, pp. 1888–1904, 2006.
- [401] S. M. Davidson and M. R. DuChen, “Endothelial mitochondria: contributing to vascular function and disease,” *Circulation Research*, vol. 100, no. 8, pp. 1128–1141, 2007.
- [402] Z. Zheng, H. Chen, G. Ke et al., “Protective effect of perindopril on diabetic retinopathy is associated with decreased vascular endothelial growth factor-to-pigment epithelium-derived factor ratio: involvement of a mitochondria-reactive oxygen species pathway,” *Diabetes*, vol. 58, no. 4, pp. 954–964, 2009.
- [403] M. Montagnani, H. Chen, V. A. Barr, and M. J. Quon, “Insulin-stimulated activation of eNOS is independent of Ca^{2+} but requires phosphorylation by Akt at Ser¹¹⁷⁹,” *The Journal of Biological Chemistry*, vol. 276, no. 32, pp. 30392–30398, 2001.
- [404] H. Duplain, R. Burcelin, C. Sartori et al., “Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase,” *Circulation*, vol. 104, no. 3, pp. 342–345, 2001.
- [405] P. Maechler and C. B. Wollheim, “Mitochondrial function in normal and diabetic β -cells,” *Nature*, vol. 414, no. 6865, pp. 807–812, 2001.
- [406] J. Han, J. H. Bae, S.-Y. Kim et al., “Taurine increases glucose sensitivity of UCP2-overexpressing β -cells by ameliorating mitochondrial metabolism,” *American Journal of Physiology—Endocrinology and Metabolism*, vol. 287, no. 5, pp. E1008–E1018, 2004.
- [407] A. Soejima, K. Inoue, D. Takai et al., “Mitochondrial DNA is required for regulation of glucose-stimulated insulin secretion in a mouse pancreatic beta cell line, MIN6,” *The Journal of Biological Chemistry*, vol. 271, no. 42, pp. 26194–26199, 1996.

- [408] T. P. A. Devasagayam, J. C. Tilak, K. K. Bloor, K. S. Sane, S. S. Ghaskadbi, and R. D. Lele, "Free radicals and antioxidants in human health: current status and future prospects," *The Journal of Association of Physicians of India*, vol. 52, pp. 794–804, 2004.
- [409] Y.-Z. Fang, S. Yang, and G. Wu, "Free radicals, antioxidants, and nutrition," *Nutrition*, vol. 18, no. 10, pp. 872–879, 2002.
- [410] H. M. Lander, "An essential role for free radicals and derived species in signal transduction," *FASEB Journal*, vol. 11, no. 2, pp. 118–124, 1997.
- [411] S. A. Villeda, K. E. Plambeck, J. Middeldorp et al., "Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice," *Nature Medicine*, vol. 20, no. 6, pp. 659–663, 2014.
- [412] D. K. Kaushik and A. Basu, "A friend in need may not be a friend indeed: role of microglia in neurodegenerative diseases," *CNS & Neurological Disorders—Drug Targets*, vol. 12, no. 6, pp. 726–740, 2013.
- [413] R. N. Saha and K. Pahan, "Regulation of inducible nitric oxide synthase gene in glial cells," *Antioxidants and Redox Signaling*, vol. 8, no. 5–6, pp. 929–947, 2006.
- [414] A. Aguzzi, B. A. Barres, and M. L. Bennett, "Microglia: scapegoat, saboteur, or something else?" *Science*, vol. 339, no. 6116, pp. 156–161, 2013.
- [415] A. S. Adler, T. L. A. Kawahara, E. Segal, and H. Y. Chang, "Reversal of aging by NF κ B blockade," *Cell Cycle*, vol. 7, no. 5, pp. 556–559, 2008.
- [416] A. K. Chauhan, N. Mittra, V. Kumar, D. K. Patel, and C. Singh, "Inflammation and B-cell lymphoma-2 associated X protein regulate zinc-induced apoptotic degeneration of rat nigrostriatal dopaminergic neurons," *Molecular Neurobiology*, 2015.
- [417] A. Sierra, A. C. Gottfried-Blackmore, B. S. Mcewen, and K. Bulloch, "Microglia derived from aging mice exhibit an altered inflammatory profile," *Glia*, vol. 55, no. 4, pp. 412–424, 2007.
- [418] R. P. Brandes, N. Weissmann, and K. Schröder, "Nox family NADPH oxidases: molecular mechanisms of activation," *Free Radical Biology and Medicine*, vol. 76, pp. 208–226, 2014.
- [419] K. Saud, R. Herrera-Molina, and R. Bernhardt, "Pro- and anti-inflammatory cytokines regulate the ERK pathway: implication of the timing for the activation of microglial cells," *Neurotoxicity Research*, vol. 8, no. 3–4, pp. 277–287, 2005.
- [420] I. Tesseur and T. Wyss-Coray, "A role for TGF- β signaling in neurodegeneration: evidence from genetically engineered models," *Current Alzheimer Research*, vol. 3, no. 5, pp. 505–513, 2006.
- [421] L. Fellner, K. A. Jellinger, G. K. Wenning, and N. Stefanova, "Glial dysfunction in the pathogenesis of α -synucleinopathies: emerging concepts," *Acta Neuropathologica*, vol. 121, no. 6, pp. 675–693, 2011.
- [422] B. Halliwell, "Oxidative stress and neurodegeneration: where are we now?" *Journal of Neurochemistry*, vol. 97, no. 6, pp. 1634–1658, 2006.
- [423] O. I. Aruoma, H. Kaur, and B. Halliwell, "Oxygen free radicals and human diseases," *Journal of the Royal Society of Health*, vol. 111, no. 5, pp. 172–177, 1991.
- [424] L. A. S. Brown, F. L. Harris, and D. P. Jones, "Ascorbate deficiency and oxidative stress in the alveolar type II cell," *American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 273, no. 4, pp. L782–L788, 1997.
- [425] J. Miquel, A. C. Economos, J. Fleming, and J. E. Johnson Jr., "Mitochondrial role in cell aging," *Experimental Gerontology*, vol. 15, no. 6, pp. 575–591, 1980.
- [426] J. Sastre, F. V. Pallardó, J. G. de la Asunción, and J. Viña, "Mitochondria, oxidative stress and aging," *Free Radical Research*, vol. 32, no. 3, pp. 189–198, 2000.
- [427] C. E. Thomas and S. D. Aust, "Free radicals and environmental toxins," *Annals of Emergency Medicine*, vol. 15, no. 9, pp. 1075–1083, 1986.
- [428] E. Niki, "Free radicals in the 1900's: from in vitro to in vivo," *Free Radical Research*, vol. 33, no. 6, pp. 693–704, 2000.
- [429] H. Schempp, E. Albrecht-Goepfert, and E. F. Elstner, "Detection of the production of reactive oxygen species by neutrophils in whole blood: modulation by adamantanes and triggering by Fe³⁺-ions," *Zeitschrift für Naturforschung*, vol. 54, no. 7–8, pp. 562–568, 1999.
- [430] B. S. van der, M. P. Veen, and P. Heeringa, "Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease," *Antioxidants & Redox Signaling*, vol. 11, pp. 2899–2937, 2009.
- [431] M. Whiteman, J. P. E. Spencer, A. Jenner, and B. Halliwell, "Hypochlorous acid-induced DNA base modification: potentiation by nitrite: biomarkers of DNA damage by reactive oxygen species," *Biochemical and Biophysical Research Communications*, vol. 257, no. 2, pp. 572–576, 1999.
- [432] M. Saran, I. Beck-Speier, B. Fellerhoff, and G. Bauer, "Phagocytic killing of microorganisms by radical processes: consequences of the reaction of hydroxyl radicals with chloride yielding chlorine atoms," *Free Radical Biology and Medicine*, vol. 26, no. 3–4, pp. 482–490, 1999.
- [433] K. Eto, T. Asada, K. Arima, T. Makifuchi, and H. Kimura, "Brain hydrogen sulfide is severely decreased in Alzheimer's disease," *Biochemical and Biophysical Research Communications*, vol. 293, no. 5, pp. 1485–1488, 2002.
- [434] L. Bergendi, L. Beneš, Z. Ďuracková, and M. Ferenčík, "Chemistry, physiology and pathology of free radicals," *Life Sciences*, vol. 65, no. 18–19, pp. 1865–1874, 1999.
- [435] J. B. Schulz, R. T. Matthews, and M. F. Beal, "Role of nitric oxide in neurodegenerative diseases," *Current Opinion in Neurology*, vol. 8, no. 6, pp. 480–486, 1995.
- [436] M. R. Hara, M. B. Cascio, and A. Sawa, "GAPDH as a sensor of NO stress," *Biochimica et Biophysica Acta (BBA)—Molecular Basis of Disease*, vol. 1762, no. 5, pp. 502–509, 2006.
- [437] A. Castrillo, D. J. Pennington, F. Otto, P. J. Parker, M. J. Owen, and L. Boscá, "Protein kinase C ϵ is required for macrophage activation and defense against bacterial infection," *The Journal of Experimental Medicine*, vol. 194, no. 9, pp. 1231–1242, 2001.
- [438] L. Fagni and J. Bockaert, "Effects of nitric oxide on glutamate-gated channels and other ionic channels," *Journal of Chemical Neuroanatomy*, vol. 10, no. 3–4, pp. 231–240, 1996.
- [439] R. L. Persinger, M. E. Poynter, K. Ckless, and Y. M. W. Janssen-Heininger, "Molecular mechanisms of nitrogen dioxide induced epithelial injury in the lung," *Molecular and Cellular Biochemistry*, vol. 234–235, pp. 71–80, 2002.
- [440] P. Moldéus, "Toxicity induced by nitrogen dioxide in experimental animals and isolated cell systems," *Scandinavian Journal of Work, Environment & Health*, vol. 19, supplement 2, pp. 28–36, 1993.
- [441] M. Sagai, T. Ichinose, and K. Kubota, "Studies on the biochemical effects of nitrogen dioxide. IV. Relation between the change of lipid peroxidation and the antioxidative protective system in rat lungs upon life span exposure to low levels of NO₂," *Toxicology and Applied Pharmacology*, vol. 73, no. 3, pp. 444–456, 1984.

- [442] J. Byun, D. M. Mueller, J. S. Fabjan, and J. W. Heinecke, "Nitrogen dioxide radical generated by the myeloperoxidase-hydrogen peroxide-nitrite system promotes lipid peroxidation of low density lipoprotein," *FEBS Letters*, vol. 455, no. 3, pp. 243–246, 1999.
- [443] E. A. Podrez, D. Schmitt, H. F. Hoff, and S. L. Hazen, "Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro," *The Journal of Clinical Investigation*, vol. 103, no. 11, pp. 1547–1560, 1999.
- [444] J. H. Wang, J. Duddle, J. L. Devalia, and R. J. Davies, "Nitrogen dioxide increases eosinophil activation in the early-phase response to nasal allergen provocation," *International Archives of Allergy and Immunology*, vol. 107, no. 1–3, pp. 103–105, 1995.
- [445] P. C. Dedon and S. R. Tannenbaum, "Reactive nitrogen species in the chemical biology of inflammation," *Archives of Biochemistry and Biophysics*, vol. 423, no. 1, pp. 12–22, 2004.
- [446] R. Zhang, M.-L. Brennan, Z. Shen et al., "Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation," *The Journal of Biological Chemistry*, vol. 277, no. 48, pp. 46116–46122, 2002.
- [447] C. K. Chow and A. L. Tappel, "Response of glutathione peroxidase to dietary selenium in rats," *The Journal of Nutrition*, vol. 104, no. 4, pp. 444–451, 1974.
- [448] P. J. Smith, A. L. Tappel, and C. K. Chow, "Glutathione peroxidase activity as a function of dietary selenomethionine," *Nature*, vol. 247, no. 5440, pp. 392–393, 1974.
- [449] M. F. Beal, "Aging, energy, and oxidative stress in neurodegenerative diseases," *Annals of Neurology*, vol. 38, no. 3, pp. 357–366, 1995.
- [450] A. H. Sharp and C. A. Ross, "Neurobiology of Huntington's disease," *Neurobiology of Disease*, vol. 3, no. 1, pp. 3–15, 1996.
- [451] A. C. Bowling and M. F. Beal, "Bioenergetic and oxidative stress in neurodegenerative diseases," *Life Sciences*, vol. 56, no. 14, pp. 1151–1171, 1995.
- [452] R. J. Ferrante, S. E. Browne, L. A. Shinobu et al., "Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis," *Journal of Neurochemistry*, vol. 69, no. 5, pp. 2064–2074, 1997.
- [453] D. A. Butterfield, J. Drake, C. Pocernich, and A. Castegna, "Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid β -peptide," *Trends in Molecular Medicine*, vol. 7, no. 12, pp. 548–554, 2001.
- [454] X. Zhu, H.-G. Lee, G. Casadesus et al., "Oxidative imbalance in Alzheimer's disease," *Molecular Neurobiology*, vol. 31, no. 1, pp. 205–217, 2005.
- [455] P. F. Good, P. Werner, A. Hsu, C. W. Olanow, and D. P. Perl, "Evidence of neuronal oxidative damage in Alzheimer's disease," *The American Journal of Pathology*, vol. 149, no. 1, pp. 21–28, 1996.
- [456] O. Nelson, H. Tu, T. Lei, M. Bentahir, B. De Strooper, and I. Bezprozvanny, "Familial Alzheimer disease-linked mutations specifically disrupt Ca^{2+} leak function of presenilin 1," *The Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1230–1239, 2007.
- [457] J. R. Murrell, A. M. Hake, K. A. Quaid, M. R. Farlow, and B. Ghetti, "Early-onset Alzheimer disease caused by a new mutation (V717L) in the amyloid precursor protein gene," *Archives of Neurology*, vol. 57, no. 6, pp. 885–887, 2000.
- [458] Y. Ji, Y. Gong, W. Gan, T. Beach, D. M. Holtzman, and T. Wisniewski, "Apolipoprotein E isoform-specific regulation of dendritic spine morphology in apolipoprotein E transgenic mice and Alzheimer's disease patients," *Neuroscience*, vol. 122, no. 2, pp. 305–315, 2003.
- [459] L. C. Baxter, R. J. Caselli, S. C. Johnson, E. Reiman, and D. Osborne, "Apolipoprotein E $\epsilon 4$ affects new learning in cognitively normal individuals at risk for Alzheimer's disease," *Neurobiology of Aging*, vol. 24, no. 7, pp. 947–952, 2003.
- [460] M. M. M. Wilhelmus, I. Otte-Höller, J. Davis, W. E. Van Nostrand, R. M. W. De Waal, and M. M. Verbeek, "Apolipoprotein E genotype regulates amyloid- β cytotoxicity," *The Journal of Neuroscience*, vol. 25, no. 14, pp. 3621–3627, 2005.
- [461] S. Ye, Y. Huang, K. Müllendorff et al., "Apolipoprotein (apo) E4 enhances amyloid beta peptide production in cultured neuronal cells: apoE structure as a potential therapeutic target," in *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, pp. 18700–18705, 2005.
- [462] C. M. Lauderback, J. Kanski, J. M. Hackett, N. Maeda, M. S. Kindy, and D. A. Butterfield, "Apolipoprotein E modulates Alzheimer's $\text{A}\beta(1-42)$ -induced oxidative damage to synaptosomes in an allele-specific manner," *Brain Research*, vol. 924, no. 1, pp. 90–97, 2002.
- [463] C. Ramassamy, D. Averill, U. Beffert et al., "Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain," *Neurobiology of Disease*, vol. 7, no. 1, pp. 23–37, 2000.
- [464] K. Blennow, M. J. de Leon, and H. Zetterberg, "Alzheimer's disease," *The Lancet*, vol. 368, no. 9533, pp. 387–403, 2006.
- [465] K. A. Jellinger, "Traumatic brain injury as a risk factor for Alzheimer's disease," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 75, no. 3, pp. 511–512, 2004.
- [466] K. A. Jellinger, W. Paulus, C. Wrocklage, and I. Litvan, "Traumatic brain injury as a risk factor for Alzheimer disease. Comparison of two retrospective autopsy cohorts with evaluation of ApoE genotype," *BMC Neurology*, vol. 1, no. 1, article 3, 2001.
- [467] J. A. Mortimer, A. R. Borenstein, K. M. Gosche, and D. A. Snowdon, "Very early detection of Alzheimer neuropathology and the role of brain reserve in modifying its clinical expression," *Journal of Geriatric Psychiatry and Neurology*, vol. 18, no. 4, pp. 218–223, 2005.
- [468] J. A. Mortimer, C. M. Van Duijn, V. Chandra et al., "Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies," *International Journal of Epidemiology*, vol. 20, no. 2, pp. S28–S35, 1991.
- [469] P. R. Turner, K. O'Connor, W. P. Tate, and W. C. Abraham, "Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory," *Progress in Neurobiology*, vol. 70, no. 1, pp. 1–32, 2003.
- [470] G. G. Glenner and C. W. Wong, "Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein," *Biochemical and Biophysical Research Communications*, vol. 122, no. 3, pp. 1131–1135, 1984.
- [471] G. G. Glenner, C. W. Wong, V. Quaranta, and E. D. Eanes, "The amyloid deposits in Alzheimer's disease: their nature and pathogenesis," *Applied Pathology*, vol. 2, no. 6, pp. 357–369, 1984.
- [472] D. Boyd-Kimball, A. Castegna, R. Sultana et al., "Proteomic identification of proteins oxidized by $\text{A}\beta(1-42)$ in synaptosomes: implications for Alzheimer's disease," *Brain Research*, vol. 1044, no. 2, pp. 206–215, 2005.
- [473] D. Boyd-Kimball, R. Sultana, H. Fai Poon et al., "Proteomic identification of proteins specifically oxidized by intracerebral injection of amyloid β -peptide (1–42) into rat brain: implications for Alzheimer's disease," *Neuroscience*, vol. 132, no. 2, pp. 313–324, 2005.

- [474] D. A. Butterfield and D. Boyd-Kimball, "Amyloid β -peptide(1-42) contributes to the oxidative stress and neurodegeneration found in Alzheimer disease brain," *Brain Pathology*, vol. 14, no. 4, pp. 426–432, 2004.
- [475] I. Dewachter, J. Van Dorpe, L. Smeijers et al., "Aging increased amyloid peptide and caused amyloid plaques in brain of old APP/V717I transgenic mice by a different mechanism than mutant presenilin1," *Journal of Neuroscience*, vol. 20, no. 17, pp. 6452–6458, 2000.
- [476] S. Kumar-Singh, P. Cras, R. Wang et al., "Dense-core senile plaques in the Flemish variant of Alzheimer's disease are vaso-centric," *The American Journal of Pathology*, vol. 161, no. 2, pp. 507–520, 2002.
- [477] P. Pasalar, H. Najmabadi, A. R. Noorian et al., "An Iranian family with Alzheimer's disease caused by a novel APP mutation (THr714Ala)," *Neurology*, vol. 58, no. 10, pp. 1574–1575, 2002.
- [478] S. Tsubuki, Y. Takaki, and T. C. Saido, "Dutch, Flemish, Italian, and Arctic mutations of APP and resistance of A β to physiologically relevant proteolytic degradation," *The Lancet*, vol. 361, no. 9373, pp. 1957–1958, 2003.
- [479] K. Hsiao, "Transgenic mice expressing Alzheimer amyloid precursor proteins," *Experimental Gerontology*, vol. 33, no. 7-8, pp. 883–889, 1998.
- [480] M. A. Westerman, D. Cooper-Blacketer, A. Mariash et al., "The relationship between A β and memory in the Tg2576 mouse model of Alzheimer's disease," *The Journal of Neuroscience*, vol. 22, no. 5, pp. 1858–1867, 2002.
- [481] J. L. Jankowsky, D. J. Fadale, J. Anderson et al., "Mutant presenilins specifically elevate the levels of the 42 residue β -amyloid peptide in vivo: evidence for augmentation of a 42-specific γ secretase," *Human Molecular Genetics*, vol. 13, no. 2, pp. 159–170, 2004.
- [482] W. D. Knight, J. Kennedy, S. Mead et al., "A novel presenilin 1 deletion (p.L166del) associated with early onset familial Alzheimer's disease," *European Journal of Neurology*, vol. 14, no. 7, pp. 829–831, 2007.
- [483] M. A. Leissring, I. Parker, and F. M. LaFerla, "Presenilin-2 mutations modulate amplitude and kinetics of inositol 1,4,5-trisphosphate-mediated calcium signals," *Journal of Biological Chemistry*, vol. 274, no. 46, pp. 32535–32538, 1999.
- [484] A. E. Shrimpton, R. L. Schelper, R. P. Linke et al., "A presenilin 1 mutation (L420R) in a family with early onset Alzheimer disease, seizures and cotton wool plaques, but not spastic paraparesis," *Neuropathology*, vol. 27, no. 3, pp. 228–232, 2007.
- [485] O. Almkvist, K. Axelman, H. Basun et al., "Clinical findings in nondemented mutation carriers predisposed to Alzheimer's disease: a model of mild cognitive impairment," *Acta Neurologica Scandinavica. Supplementum*, vol. 179, pp. 77–82, 2003.
- [486] C. Zekanowski, M. Styczyńska, B. Peplowska et al., "Mutations in presenilin 1, presenilin 2 and amyloid precursor protein genes in patients with early-onset Alzheimer's disease in Poland," *Experimental Neurology*, vol. 184, no. 2, pp. 991–996, 2003.
- [487] A. P. Grigorenko and E. I. Rogaev, "Molecular basics of Alzheimer's disease," *Molekuliarnaia Biologiia*, vol. 41, no. 2, pp. 331–345, 2007.
- [488] V. Calabrese, G. Scapagnini, C. Colombrita et al., "Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: a nutritional approach," *Amino Acids*, vol. 25, no. 3-4, pp. 437–444, 2003.
- [489] H. Mohmmad Abdul, R. Sultana, J. N. Keller, D. K. St Clair, W. R. Markesbery, and D. A. Butterfield, "Mutations in amyloid precursor protein and presenilin-1 genes increase the basal oxidative stress in murine neuronal cells and lead to increased sensitivity to oxidative stress mediated by amyloid β -peptide (1-42), H₂O₂ and kainic acid: implications for Alzheimer's disease," *Journal of Neurochemistry*, vol. 96, no. 5, pp. 1322–1335, 2006.
- [490] R. Resende, P. I. Moreira, T. Proença et al., "Brain oxidative stress in a triple-transgenic mouse model of Alzheimer disease," *Free Radical Biology & Medicine*, vol. 44, no. 12, pp. 2051–2057, 2008.
- [491] L. Bertram, D. Blacker, K. Mullin et al., "Evidence for genetic linkage of Alzheimer's disease to chromosome 10q," *Science*, vol. 290, no. 5500, pp. 2302–2303, 2000.
- [492] R. H. Fabian, J. R. Perez-Polo, and T. A. Kent, "Electrochemical monitoring of superoxide anion production and cerebral blood flow: effect of interleukin-1 β pretreatment in a model of focal ischemia and reperfusion," *Journal of Neuroscience Research*, vol. 60, no. 6, pp. 795–803, 2000.
- [493] W. Q. Qiu, D. M. Walsh, Z. Ye et al., "Insulin-degrading enzyme regulates extracellular levels of amyloid β -protein by degradation," *The Journal of Biological Chemistry*, vol. 273, no. 49, pp. 32730–32738, 1998.
- [494] D. G. Cook, J. B. Leverenz, P. J. McMillan et al., "Reduced hippocampal insulin-degrading enzyme in late-onset Alzheimer's disease is associated with the apolipoprotein E- ϵ 4 allele," *The American Journal of Pathology*, vol. 162, no. 1, pp. 313–319, 2003.
- [495] Y. O. Kim, H.-J. Kim, G. S. Kim et al., "Panax ginseng protects against global ischemia injury in rat hippocampus," *Journal of Medicinal Food*, vol. 12, no. 1, pp. 71–76, 2009.
- [496] S. M. Son, S. Kang, H. Choi, and I. Mook-Jung, "Statins induce insulin-degrading enzyme secretion from astrocytes via an autophagy-based unconventional secretory pathway," *Molecular Neurodegeneration*, vol. 10, article 56, 2015.
- [497] S. Craft, S. Asthana, G. Schellenberg et al., "Insulin metabolism in Alzheimer's disease differs according to apolipoprotein E genotype and gender," *Neuroendocrinology*, vol. 70, no. 2, pp. 146–152, 1999.
- [498] S. Craft, "Insulin resistance and Alzheimer's disease pathogenesis: potential mechanisms and implications for treatment," *Current Alzheimer Research*, vol. 4, no. 2, pp. 147–152, 2007.
- [499] N. Ertekin-Taner, N. Graff-Radford, L. H. Younkin et al., "Linkage of plasma A β 42 to a quantitative locus on chromosome 10 in late-onset Alzheimer's disease pedigrees," *Science*, vol. 290, no. 5500, pp. 2303–2304, 2000.
- [500] E. Van Uden, Y. Sagara, J. Van Uden et al., "A protective role of the low density lipoprotein receptor-related protein against amyloid β -protein toxicity," *Journal of Biological Chemistry*, vol. 275, no. 39, pp. 30525–30530, 2000.
- [501] R. D. Moir and R. E. Tanzi, "LRP-mediated clearance of A β is inhibited by KPI-containing isoforms of APP," *Current Alzheimer Research*, vol. 2, no. 2, pp. 269–273, 2005.
- [502] Y. Du, K. R. Bales, R. C. Dodel et al., " α 2-Macroglobulin attenuates β -amyloid peptide 1-40 fibril formation and associated neurotoxicity of cultured fetal rat cortical neurons," *Journal of Neurochemistry*, vol. 70, no. 3, pp. 1182–1188, 1998.
- [503] S. R. Hughes, O. Khorkova, S. Goyal et al., " α 2-macroglobulin associates with β -amyloid peptide and prevents fibril formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 6, pp. 3275–3280, 1998.
- [504] R. C. Dodel, Y. Du, K. R. Bales et al., " α 2 Macroglobulin and the risk of Alzheimer's disease," *Neurology*, vol. 54, no. 2, pp. 438–442, 2000.

- [505] G. J. Harry, C. Lefebvre d'Hellencourt, A. Bruccoleri, and D. Schmechel, "Age-dependent cytokine responses: trimethyltin hippocampal injury in wild-type, APOE knockout, and APOE4 mice," *Brain, Behavior, and Immunity*, vol. 14, no. 4, pp. 288–304, 2000.
- [506] S. Chakraborty, D. K. Kaushik, M. Gupta, and A. Basu, "Inflammation signaling at the heart of central nervous system pathology," *Journal of Neuroscience Research*, vol. 88, no. 8, pp. 1615–1631, 2010.
- [507] L. Qin, Y. Liu, C. Cooper, B. Liu, B. Wilson, and J.-S. Hong, "Microglia enhance β -amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species," *Journal of Neurochemistry*, vol. 83, no. 4, pp. 973–983, 2002.
- [508] S. J. Chinta, J. K. Mallajosyula, A. Rane, and J. K. Andersen, "Mitochondrial alpha-synuclein accumulation impairs complex I function in dopaminergic neurons and results in increased mitophagy in vivo," *Neuroscience Letters*, vol. 486, no. 3, pp. 235–239, 2010.
- [509] N. M. Bonini and B. I. Giasson, "Snaring the function of α -synuclein," *Cell*, vol. 123, no. 3, pp. 359–361, 2005.
- [510] A. Pisanu, D. Lecca, G. Mulas et al., "Dynamic changes in pro- and anti-inflammatory cytokines in microglia after PPAR- γ agonist neuroprotective treatment in the MPTP mouse model of progressive Parkinson's disease," *Neurobiology of Disease*, vol. 71, no. 1, pp. 280–291, 2014.
- [511] Z. I. Alam, A. Jenner, S. E. Daniel et al., "Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra," *Journal of Neurochemistry*, vol. 69, pp. 1196–1203, 1997.
- [512] Z. I. Alam, S. E. Daniel, A. J. Lees, D. C. Marsden, P. Jenner, and B. Halliwell, "A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease," *Journal of Neurochemistry*, vol. 69, no. 3, pp. 1326–1329, 1997.
- [513] D. S. Harischandra, H. Jin, V. Anantharam, A. Kanthasamy, and A. G. Kanthasamy, " α -Synuclein protects against manganese neurotoxic insult during the early stages of exposure in a dopaminergic cell model of Parkinson's disease," *Toxicological Sciences*, vol. 143, no. 2, pp. 454–468, 2015.
- [514] B. I. Giasson, J. E. Duda, I. V. J. Murray et al., "Oxidative damage linked to neurodegeneration by selective α -synuclein nitration in synucleinopathy lesions," *Science*, vol. 290, no. 5493, pp. 985–989, 2000.
- [515] M. Tiwari, M. Lopez-Cruzan, W. W. Morgan, and B. Herman, "Loss of caspase-2-dependent apoptosis induces autophagy after mitochondrial oxidative stress in primary cultures of young adult cortical neurons," *The Journal of Biological Chemistry*, vol. 286, no. 10, pp. 8493–8506, 2011.
- [516] J. W. Langston, P. Ballard, J. W. Tetrud, and I. Irwin, "Chronic parkinsonism in humans due to a product of meperidine-analog synthesis," *Science*, vol. 219, no. 4587, pp. 979–980, 1983.
- [517] V. Jackson-Lewis, J. Blesa, and S. Przedborski, "Animal models of Parkinson's disease," *Parkinsonism and Related Disorders*, vol. 18, supplement 1, pp. S183–S185, 2012.
- [518] R. M. Abdelsalam and M. M. Safar, "Neuroprotective effects of vildagliptin in rat rotenone Parkinson's disease model: role of RAGE-NF κ B and Nrf2-antioxidant signaling pathways," *Journal of Neurochemistry*, vol. 133, no. 5, pp. 700–707, 2015.
- [519] C.-C. Chiu, T.-H. Yeh, S.-C. Lai et al., "Neuroprotective effects of aldehyde dehydrogenase 2 activation in rotenone-induced cellular and animal models of parkinsonism," *Experimental Neurology*, vol. 263, pp. 244–253, 2015.
- [520] S. A. Eom, D. W. Kim, M. J. Shin et al., "Protective effects of PEP-1-Catalase on stress-induced cellular toxicity and MPTP-induced Parkinson's disease," *BMB Reports*, vol. 48, no. 7, pp. 395–400, 2015.
- [521] L. Liu, C. Peritore, J. Ginsberg, J. Shih, S. Arun, and G. Donmez, "Protective role of SIRT5 against motor deficit and dopaminergic degeneration in MPTP-induced mice model of Parkinson's disease," *Behavioural Brain Research*, vol. 281, pp. 215–221, 2015.
- [522] J. Qin, M. Wu, S. Yu et al., "Pyrroloquinoline quinone-conferred neuroprotection in rotenone models of Parkinson's disease," *Toxicology Letters*, vol. 238, no. 3, pp. 70–82, 2015.
- [523] S. Wang, H. He, L. Chen, W. Zhang, X. Zhang, and J. Chen, "Protective effects of salidroside in the MPTP/MPP⁺-induced model of Parkinson's disease through ROS-NO-related mitochondrion pathway," *Molecular Neurobiology*, vol. 51, no. 2, pp. 718–728, 2015.
- [524] J. Bové, D. Prou, C. Perier, and S. Przedborski, "Toxin-induced models of Parkinson's disease," *NeuroRx*, vol. 2, no. 3, pp. 484–494, 2005.
- [525] J. P. Kostrzewa, R. A. Kostrzewa, R. M. Kostrzewa, R. Brus, and P. Nowak, "Perinatal 6-hydroxydopamine to produce a lifelong model of severe Parkinson's disease," *Current Topics in Behavioral Neurosciences*, 2015.
- [526] Y.-H. Wang, Z.-H. Xuan, S. Tian, and G.-H. Du, "Echinacoside protects against 6-hydroxydopamine-induced mitochondrial dysfunction and inflammatory responses in PC12 cells via reducing ROS production," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 189239, 9 pages, 2015.
- [527] G. Zhu, G. Chen, L. Shi et al., "PEGylated rhFGF-2 conveys long-term neuroprotection and improves neuronal function in a rat model of Parkinson's disease," *Molecular Neurobiology*, vol. 51, no. 1, pp. 32–42, 2014.
- [528] X. Pan, C. Chen, J. Huang, H. Wei, and Q. Fan, "Neuroprotective effect of combined therapy with hyperbaric oxygen and madopar on 6-hydroxydopamine-induced Parkinson's disease in rats," *Neuroscience Letters*, vol. 600, pp. 220–225, 2015.
- [529] P. Giuliani, P. Ballerini, S. Buccella et al., "Guanosine protects glial cells against 6-hydroxydopamine toxicity," *Advances in Experimental Medicine and Biology*, vol. 837, pp. 23–33, 2015.
- [530] S. Afshin-Majd, M. Khalili, M. Roghani, N. Mehranmehr, and T. Baluchnejadmojarad, "Carnosine exerts neuroprotective effect against 6-hydroxydopamine toxicity in hemiparkinsonian rat," *Molecular Neurobiology*, vol. 51, no. 3, pp. 1064–1070, 2015.
- [531] M. G. Kaplitt, A. Feigin, C. Tang et al., "Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial," *The Lancet*, vol. 369, no. 9579, pp. 2097–2105, 2007.
- [532] P. A. LeWitt, A. R. Rezaei, M. A. Leehey et al., "AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial," *The Lancet Neurology*, vol. 10, no. 4, pp. 309–319, 2011.
- [533] D. W. Cleveland and J. D. Rothstein, "From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS," *Nature Reviews Neuroscience*, vol. 2, no. 11, pp. 806–819, 2001.
- [534] N. Nirmalanathan and L. Greensmith, "Amyotrophic lateral sclerosis: recent advances and future therapies," *Current Opinion in Neurology*, vol. 18, no. 6, pp. 712–719, 2005.
- [535] H. Mitsumoto, R. Santella, X. Liu et al., "Oxidative stress biomarkers in sporadic ALS," *Amyotrophic Lateral Sclerosis*, vol. 9, no. 3, pp. 177–183, 2008.

- [536] A. C. Bowling, J. B. Schulz, R. H. Brown Jr., and M. F. Beal, "Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis," *Journal of Neurochemistry*, vol. 61, no. 6, pp. 2322–2325, 1993.
- [537] J. F. Moruno Manchon, N.-E. Uzor, Y. Dabaghian, E. E. Furr-Stimming, S. Finkbeiner, and A. S. Tsvetkov, "Cytoplasmic sphingosine-1-phosphate pathway modulates neuronal autophagy," *Scientific Reports*, vol. 5, Article ID 15213, 2015.
- [538] D. C. Rubinsztein, "The roles of intracellular protein-degradation pathways in neurodegeneration," *Nature*, vol. 443, no. 7113, pp. 780–786, 2006.
- [539] E. Wong and A. M. Cuervo, "Autophagy gone awry in neurodegenerative diseases," *Nature Neuroscience*, vol. 13, no. 7, pp. 805–811, 2010.
- [540] F. Rojas, D. Gonzalez, N. Cortes et al., "Reactive oxygen species trigger motoneuron death in non-cell-autonomous models of ALS through activation of c-Abl signaling," *Frontiers in Cellular Neuroscience*, vol. 9, article A203, 2015.
- [541] Y. Chen, H. Liu, Y. Guan et al., "The altered autophagy mediated by TFEB in animal and cell models of amyotrophic lateral sclerosis," *American Journal of Translational Research*, vol. 7, no. 9, pp. 1574–1587, 2015.
- [542] J.-Y. Hwang, S.-W. Min, Y.-T. Jeon et al., "Effect of coenzyme Q₁₀ on spinal cord ischemia-reperfusion injury," *Journal of Neurosurgery: Spine*, vol. 22, no. 4, pp. 432–438, 2015.
- [543] L. Jing, M.-T. He, Y. Chang et al., "Coenzyme Q10 protects astrocytes from ROS-Induced damage through inhibition of Mitochondria-Mediated cell death pathway," *International Journal of Biological Sciences*, vol. 11, no. 1, pp. 59–66, 2015.
- [544] P. I. Moreira, X. Zhu, X. Wang et al., "Mitochondria: a therapeutic target in neurodegeneration," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1802, no. 1, pp. 212–220, 2010.
- [545] L. Qi, X. Sun, F.-E. Li et al., "Hmgbl promotes mitochondrial dysfunction-triggered striatal neurodegeneration via autophagy and apoptosis activation," *PLoS ONE*, vol. 10, no. 11, Article ID e0142901, 2015.

Clinical Study

Influence of Insulin Resistance and TNF- α on the Inflammatory Process, Oxidative Stress, and Disease Activity in Patients with Rheumatoid Arthritis

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The aim of this study was to evaluate the involvement of TNF- α and insulin resistance (IR) in the inflammatory process, oxidative stress, and disease activity in patients with rheumatoid arthritis (RA). This cross-sectional study included 270 subjects (control group, $n = 97$) and RA patients ($n = 173$). RA patients were divided into four groups: the first group without IR and not using antitumor necrosis factor- α (TNF- α) (G1, IR- TNF-); the second group without IR and using anti-TNF- α (G2, IR- TNF+); the third group with IR and not using anti-TNF- α (G3, IR+ TNF-); and the fourth group with IR and using anti-TNF- α (G4, IR+ TNF+). G3 and G4 had higher ($p < 0.05$) advanced oxidation protein products (AOPPs) and oxidative stress index (OSI) compared to G1. G4 group presented higher ($p < 0.05$) AOPPs and OSI than G2. TRAP was significantly lower in G3 compared to G1. Plasma TNF- α levels were significantly higher in G4 and G2 compared to G1 ($p < 0.0001$) and G3 ($p < 0.0001$ and $p < 0.01$, resp.). The presence of insulin resistance was robustly associated with both oxidative stress and TNF- α levels. More studies are warranted to verify if IR can be involved in therapeutic failure with TNF- α inhibitors. This trial is registered with Brazilian Clinical Trials Registry Register number RBR-2jvj92.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that leads to severe joint destruction. In addition, RA patients have higher risk of developing cardiovascular disease (CVD) and this is related to chronic inflammation [1] and corticosteroids treatment [2, 3]. Systemic chronic inflammation and proinflammatory cytokines have been proposed as major protagonists in the pathogenesis of insulin resistance (IR), an important factor for CVD [4, 5]. TNF- α plays a central

role in the pathogenesis of RA [6, 7] and has also been implicated in the development of IR [4, 8]. In addition, single infusion of the anti-TNF- α monoclonal antibody decreased insulin resistance in RA patients [9]. Abnormalities in glucose metabolism have been well documented in RA patients and may also correlate with Disease Activity Score evaluating 28 joints (DAS 28) [9].

Oxidative stress has a prominent role in the etiology and pathogenesis of joint tissue injury and chronic inflammation in patients with RA, which may lead to connective tissue

degradation and joint and periarticular deformities [10]. Reactive oxygen species (ROS) have been considered an enhancer factor for autoimmune disease risk [11]. ROS are important intracellular signaling molecules in the cells of the immune system that amplify the synovial inflammatory-proliferative response [12]. Previous studies showed that elevated levels of lipoperoxidation and decreased antioxidant system in RA are positively correlated with DAS 28 and high sensitivity C-reactive protein (hsCRP) [13, 14]. Tumor necrosis factor- α (TNF- α) can induce higher oxidative stress by initiators of the nuclear factor kappa B activation cascade and is under its transcriptional control, constituting a positive feedback loop [11]. Moreover, anti-TNF- α therapy can reduce oxidative stress in patients with RA [15, 16].

Our group has investigated the development of IR and the metabolic syndrome in chronic inflammatory diseases [17–20] and these reports have found an important role of oxidative stress in the development and maintenance of these conditions. Therefore, it seems that chronic inflammation and oxidative stress contribute to the pathogenesis of both RA and IR. Furthermore, previous studies have shown that IR [8, 21–23] and oxidative stress [15, 16, 24–26], independently, may impair disease activity in patients with RA.

Therefore, the aim of the present study was to verify the influence of insulin resistance and TNF- α on the inflammatory process, oxidative stress, and disease activity in patients with RA.

2. Patients and Methods

2.1. Subjects. This cross-sectional study included 270 subjects, healthy individuals (control group, $n = 97$) and RA patients ($n = 173$), aged between 18 and 70 years. The control group was selected from among blood donors of the University Hospital who did not present autoimmune disease, and RA patients were selected from among the Ambulatory of Rheumatology of the University Hospital of Londrina, Paraná, Brazil. RA patients were initially divided into two groups: the first group without IR (IR-, $n = 91$) and the second group with IR (IR+, $n = 82$). After that, to verify the influence of insulin resistance and also of anti-TNF- α therapy on anthropometric, biochemical, immunological, and oxidative stress parameters in patients with RA, they were divided into four groups: the first group (control group) without IR and not using anti-TNF- α therapy (G1, IR- TNF-, $n = 71$); the second group without IR and using anti-TNF- α therapy (G2, IR- TNF+, $n = 20$); the third group with IR and not using anti-TNF- α therapy (G3, IR+ TNF-, $n = 63$); and the fourth group with IR and using anti-TNF- α therapy (G4, IR+ TNF+, $n = 19$). RA patients (G2 and G4) were using anti-TNF- α therapy at least for six months. Sex, age, and ethnicity were controlled. RA was classified according to the 2010 rheumatoid arthritis classification criteria [27].

Disease activity status was determined using DAS 28 [9] and patients were classified into four different groups, namely, (1) remission group: $\text{DAS } 28 \leq 2.6$; (2) low disease activity group: $2.6 < \text{DAS } 28 \leq 3.2$; (3) moderate disease activity group:

$3.2 < \text{DAS } 28 \leq 5.1$; and (4) high disease activity group: $\text{DAS } 28 > 5.1$.

None of the subjects was receiving a specific diet. The individuals of both groups (control and RA) did not smoke and did not drink alcohol regularly. None of the participants in the study presented heart, thyroid, renal, hepatic, gastrointestinal, or oncological diseases, and none were receiving estrogen replacement therapy or drugs for hyperlipidemia or hyperglycemia or antioxidant supplements. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the Ethical Committee of the University of Londrina, Paraná, Brazil, approved all procedures involving human subjects and patients. Written informed consent was obtained from all subjects/patients.

2.2. Anthropometric Measurements. Body weight was measured in the morning to the nearest 0.1 kg by using an electronic scale with individuals wearing light clothing and without shoes; height was measured to the nearest 0.1 cm by using a stadiometer. Body mass index was calculated as weight (kg) divided by height (m) squared. Waist circumference (WC) was measured on standing subjects midway between the lowest rib and the iliac crest.

2.3. Biochemical, Immunological, and Hematological Biomarkers. After fasting for 12 hours, serum or plasma samples were obtained and the patients underwent the following laboratory blood analysis: glucose and uric acid (UA) were evaluated by a biochemical autoanalyzer (Dimension Dade AR, Dade Behring®, Deerfield, IL, USA), using Dade Behring kits; plasma insulin level and anticyclic citrullinated peptide (anti-CCP) antibody were determined by chemiluminescence microparticle immunoassay (Architect, Abbott Laboratory, Abbott Park, IL, USA). The homeostasis model assessment-IR (HOMA-IR) was used as a surrogate measurement of insulin resistance [28]. Consider the following: $\text{HOMA-IR} = \text{insulin fasting } (\mu\text{U/mL}) \times \text{glucose fasting (nmol/L)} / 22.5$. IR was considered when $\text{HOMA-IR} \geq 2.114$ [8]. Serum high sensitivity CRP (hsCRP) and rheumatoid factor (RF) were measured using a nephelometric assay (Behring Nephelometer II, Dade Behring, Marburg, Germany). TNF- α levels were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial immunoassay ELISA (Ready-SET-Go! Set, e-Bioscience, San Diego, California, USA). Erythrocyte sedimentation rate (ESR) was obtained by automated kinetic-photometric method (Ves-Matic CUBE 30, DIESSE, Siena, Italy).

2.4. Oxidative Stress Measurements. Samples for evaluating oxidative stress and total antioxidant capacity were performed with EDTA as anticoagulant and antioxidant. All samples were centrifuged at 3,000 rpm for 15 minutes and plasma aliquots stored at -70°C until assayed.

2.5. Tert-Butyl Hydroperoxide-Initiated Chemiluminescence (CL-LOOH). The CL-LOOH in plasma was evaluated as described previously by Gonzalez Flecha et al. [29]. For chemiluminescence (CL) measurement, reaction mixtures

TABLE 1: Clinical and laboratory data in patients with rheumatoid arthritis with (IR+) or without (IR-) insulin resistance.

	IR- (<i>n</i> = 91)	IR+ (<i>n</i> = 82)	<i>p</i>
Disease duration (years)	11.0 (5.0–18.3)	8.0 (4.0–20.3)	NS
RF (IU/mL)	48.3 (0.0–125.0)	26.9 (0.0–118.2)	NS
Anti-CCP (U/mL)	25.55 (0.13–120.10)	6.65 (0.50–131.40)	NS
DAS 28	3.51 (2.39–4.49)	3.76 (2.85–4.78)	0.043
DAS 28, <i>n</i> (%)			
Remission (<2.6)	27 (29.7%)	16 (19.5%)	
Low (2.6–3.2)	12 (13.2%)	11 (13.4%)	0.001
Moderate (3.2–5.1)	42 (46.1%)	39 (47.6%)	
High (>5.1)	10 (10.0%)	16 (19.5%)	
CPR (mg/L)	3.52 (1.31–12.38)	6.35 (2.51–11.08)	0.040
ESR (mm)	14.0 (6.0–22.0)	19.5 (9.3–35.5)	0.023
	<i>Therapy</i>		
Prednisone (Y/N)	64/27	54/28	NS
Antimalarials (Y/N)	38/53	32/50	NS
Anti-TNF- α (Y/N)	20/71	19/63	NS
Adalimumab	7	6	NS
Etanercept	13	13	
Methotrexate (Y/N)	57/34	62/20	NS
Leflunomide (Y/N)	40/51	35/47	NS

Chi-square test with Yates correction. Mann-Whitney test. Data are expressed as median (25–75%). Y, yes; N, no; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; DAS 28, Disease Activity Score evaluating 28 joints; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; and NS, not significant.

were placed in 20 mL scintillation vials (low-potassium glass) containing final concentrations of plasma (250 μ L), 30 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.4), and 120 mM KCl with 3 mM of tert-butyl hydroperoxide in a final volume of 2 mL. Tert-butyl hydroperoxide-initiated chemiluminescence was measured in Beckman LS 6000 Liquid Scintillation Counter set to the out-of-coincidence mode, with a response range from 300 to 620 nm. The vials were kept in the dark up to the moment of assay, and determination was carried out in a dark room at 30°C. The results are expressed in counts per minute (cpm).

2.6. Determination of Advanced Oxidation Protein Products (AOPPs). AOPPs were determined in the plasma using the semiautomated method described by Witko-Sarsat et al. [30]. AOPPs results of oxidation of amino acid residues such as tyrosine, leading to the formation of dityrosine-containing protein cross-linking products detected by spectrophotometry [17, 30]. AOPPs concentrations were expressed as micromoles per liter ($\mu\text{mol/L}$) of chloramines-T equivalents.

2.7. Total Radical-Trapping Antioxidant Parameter (TRAP). TRAP was determined as reported by Repetto et al. [31]. This method detects hydrosoluble and/or liposoluble plasma antioxidants by measuring the chemiluminescence inhibition time induced by 2,2-azobis(2-amidinopropane). The system was calibrated with vitamin E analog Trolox, and the values of TRAP are expressed in equivalent of μM Trolox/mg UA. TRAP analysis in conditions associated with hyperuricemia, as in patients with MetS, may be jeopardized because uric

acid concentration is responsible for 60% of plasma total antioxidant capacity. Thus, a correction of total antioxidant capacity based on uric acid concentration is needed [32, 33].

2.8. Oxidative Stress Index (OSI). Oxidative stress imbalance was verified when OSI was calculated as AOPPs ($\mu\text{mol/L}$) divided by TRAP (μM Trolox/mg UA), which indicates the oxidant-antioxidant ratio as a reflection of the cellular redox state.

2.9. Statistical Analysis. Distribution of sex, ethnicity, and therapy was analyzed by chi-square test with Yates correction. Comparisons between groups were performed using the Kruskal-Wallis test with Dunn's posttest and data were expressed as the median (25–75%). The results were considered significant when $p < 0.05$. To determine which factors were independently associated with IR in RA patients, the variables that presented $p < 0.10$ in univariate analyses were included in logistic regression model. Logistic regression analyses were performed with SPSS v20.0 (IBM, USA).

3. Results

Rheumatoid arthritis patients with or without IR were not statistically different in relation to disease duration and serum RF and anti-CCP levels and frequency in prednisone and antimalarials and methotrexate and leflunomide use and anti-TNF- α therapy (Table 1). However, IR+ group had an increased DAS 28 ($p = 0.043$) with enhanced frequency in patients with high disease activity. In addition, IR+ group

TABLE 2: Anthropometric, clinical, and laboratorial profile in healthy subjects (controls) and in patients with rheumatoid arthritis (RA) with or without insulin resistance (IR).

	Controls (<i>n</i> = 97)	RA+ IR– (<i>n</i> = 91)	RA+ IR+ (<i>n</i> = 82)	Control versus RA+ IR–	Control versus RA+ IR+	RA+ IR– versus RA+ IR+
Gender (F/M)	80/17	70/21	70/12	NS	NS	NS
Caucasian/not Caucasian	72/25	58/33	53/29	NS	NS	NS
Age (years)	51.0 (42.5–69.5)	56.0 (46.0–63.3)	57.5 (48.8–62.3)	NS	NS	NS
BMI (kg/m ²)	25.8 (23.8–28.0)	25.9 (22.8–29.3)	29.4 (25.3–33.4)	NS	<0.0001	<0.0001
WC (cm)	91.5 (87.0–97.3)	90.0 (82.0–97.3)	98.0 (91.0–107.3)	NS	<0.01	<0.0001
Glucose (mg/dL)	87.0 (82.8–95.0)	85.0 (80.0–90.0)	96.0 (88.9–113.0)	NS	<0.0001	<0.0001
Insulin (μU/mL)	6.35 (4.60–8.03)	6.70 (5.30–8.10)	13.95 (11.10–16.78)	NS	<0.0001	<0.0001
HOMA-IR	1.35 (1.01–1.69)	1.42 (1.07–1.75)	3.41 (2.71–4.46)	NS	<0.0001	<0.0001
CL-LOOH (cpm)	166.7 (141.9–179.0)	169.2 (150.0–198.9)	166.2 (152.6–201.5)	NS	NS	NS
AOPP (μmol/L of chloramines-T equivalents)	150.4 (118.4–209.6)	123.5 (100.4–171.3)	173.8 (123.9–238.7)	<0.05	NS	<0.0001
TRAP (μM Trolox/mg UA)	158.9 (122.2–200.9)	171.5 (146.1–207.9)	155.9 (121.0–177.3)	NS	NS	<0.05
OSI	0.228 (0.166–0.321)	0.762 (0.578–0.952)	1.183 (0.753–1.680)	<0.0001	<0.0001	<0.001

Kruskal-Wallis test with Dunn's posttest. Data are expressed as median (25–75%). BMI, body mass index; WC, waist circumference; HOMA-IR, homeostasis model assessment-insulin resistance; CL-LOOH, tert-butyl hydroperoxide-initiated chemiluminescence; AOPPs, advanced oxidation protein products; TRAP, total radical-trapping antioxidant parameter; and OSI, oxidative stress index. NS: not significant.

showed higher ESR ($p = 0.023$) and hsCRP ($p = 0.040$) compared to the IR– group (Table 1).

With regard to anthropometric and biochemical markers, IR+ group presented higher BMI ($p < 0.0001$, $p < 0.0001$), WC ($p < 0.01$; $p < 0.0001$), plasma glucose ($p < 0.0001$, $p < 0.0001$), and insulin ($p < 0.0001$, $p < 0.0001$) levels and HOMA-IR ($p < 0.0001$, $p < 0.0001$) compared to the control group and IR– group, respectively (Table 2).

In relation to oxidative stress markers, both IR– and RI+ groups had significantly higher OSI ($p < 0.0001$) compared to the control group, whereas IR– group showed lower AOPPs ($p < 0.05$) levels compared to the control group. Higher AOPPs ($p < 0.0001$) and OSI ($p < 0.001$) and lower TRAP ($p < 0.05$) were verified in the group composed of IR+ patients in relation to IR– group (Table 2). Plasma TNF- α levels were significantly higher both in IR– ($p < 0.01$) and in IR+ ($p < 0.0001$) groups compared to the control group (Figure 1). In addition, RI+ group had higher plasma TNF- α levels than IR– group ($p < 0.05$) (Figure 1).

Table 3 shows the differences when the groups were divided taking into account the presence or absence of IR and anti-TNF- α therapy. The groups composed of patients with IR, IR+ TNF– (G3) and IR+ TNF+ (G4), had higher ($p < 0.05$) AOPPs and OSI compared to G1 (control group). In addition, G4 group presented higher ($p < 0.05$) AOPPs and OSI than IR– TNF+ (G2) group. TRAP was significantly

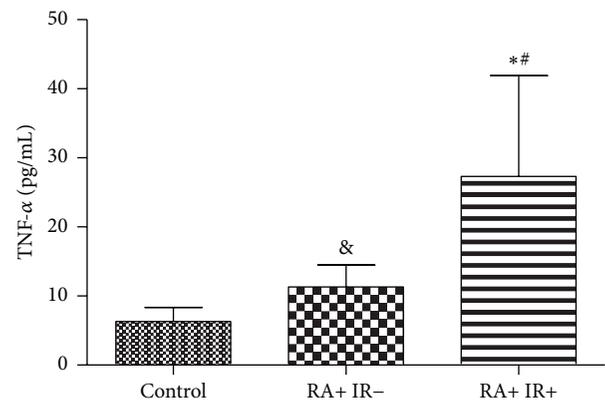


FIGURE 1: Plasma TNF- α levels in healthy subjects (controls) and in patients with rheumatoid arthritis with (IR+) or without (IR–) insulin resistance. Kruskal-Wallis test with Dunn's posttest. *IR+ versus control, $p < 0.0001$; &IR– versus control, $p < 0.01$; #IR+ versus IR–, $p < 0.05$.

lower in IR+ TNF– group (G3) in relation to G1. On the other hand, the groups without insulin resistance, G1 and G2, showed no differences in oxidative stress markers (Table 3). In relation to the inflammatory profile, ESR showed significantly higher ($p < 0.05$) levels in G3 and G4 than in G1, and G4

TABLE 3: Oxidative stress markers, disease activity, and inflammatory parameters in patients with rheumatoid arthritis with (IR+) or without (IR-) insulin resistance and using (TNF+) or not using (TNF-) anti-TNF- α .

	G1 (n = 71)	G2 (n = 20)	G3 (n = 63)	G4 (n = 19)
CL-LOOH (cpm)	170.7 (150.0–196.7)	167.4 (147.2–214.4)	165.7 (152.7–204.3)	166.2 (151.8–166.2)
AOPP (μ mol/L of chloramines-T equivalents)	124.5 (102.6–170.1)	123.2 (99.9–182.8)	173.3* (122.4–242.7)	173.8^{&} (124.4–222.4)
TRAP (μ M Trolox/mg UA)	175.4 (147.3–210.0)	164.7 (131.8–207.7)	150.8* (121.0–178.8)	159.2 (107.5–176.6)
OSI	0.73 (0.57–0.92)	0.85 (0.62–1.12)	1.21* (0.78–1.79)	1.18^{&} (0.69–1.53)
DAS 28	3.41 (2.23–4.57)	3.83 (3.08–4.89)	3.75 (2.87–4.80)	3.49 (2.78–4.30)
CRP (mg/dL)	4.74 (1.26–15.80)	2.75 (1.78–6.76)	6.63 (7.70–11.9)	4.66** (1.42–8.89)
ESR (mm)	14.0 (5.0–22.0)	14.5 (8.3–23.0)	19.0* (8.0–32.5)	26.0^{&#} (11.0–44.0)

Kruskal-Wallis test with Dunn's posttest. Data are expressed as median (25–75%). G1, IR- TNF-; G2, IR- TNF+; G3, IR+ TNF-; G4, IR+ TNF+; CL-LOOH, tert-butyl hydroperoxide-initiated chemiluminescence; AOPP, advanced oxidation protein product; TRAP, total radical-trapping antioxidant parameter; OSI, oxidative stress index; DAS 28, Disease Activity Score evaluating 28 joints; CRP, C-reactive protein; and ESR, erythrocyte sedimentation rate.

*G1 versus G3, $p < 0.05$; #G1 versus G4, $p < 0.05$; &G4 versus G2, $p < 0.05$; and **G4 versus G3.

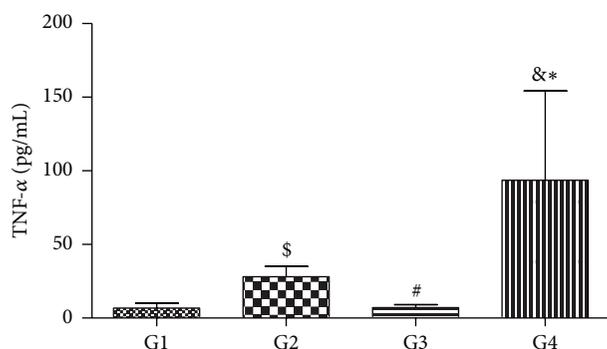


FIGURE 2: Plasma TNF- α levels in patients with rheumatoid arthritis with (IR+) or without (RI-) insulin resistance and using (TNF+) or not using (TNF-) anti-TNF- α . Kruskal-Wallis test with Dunn's posttest. G1: IR- TNF-; G2: IR- TNF+; G3: IR+ TNF-; and G4: IR+ TNF+. *G4 versus G1, $p < 0.0001$; &G4 versus G3, $p < 0.0001$; \$G2 versus G1, $p < 0.0001$; and #G2 versus G3, $p < 0.01$.

had also increased ESR ($p < 0.05$) levels compared to G2. There were significantly lower ($p < 0.05$) hsCRP levels in G4 compared to G3 (Table 3). Plasma TNF- α levels were significantly higher in patients who were using anti-TNF- α therapy, that is, G4 ($p < 0.0001$) and G2 ($p < 0.0001$), compared to G1 (Figure 2). Also, G4 and G2 had higher plasma TNF- α levels than G3 ($p < 0.0001$ and $p < 0.01$, resp.) (Figure 2).

Oxidative stress data according to anti-TNF- α therapy with etanercept or adalimumab are shown in Table 4. There was no significant difference in CL-LOOH, AOPPs, TRAP, or OSI values. However, AOPP levels showed an increase trend ($p = 0.071$) in patients using adalimumab and this trend was independent of BMI ($p = 0.047$, OR: 1.009, CI 95%: 1.000–1.018) (data not shown). In sum, presence of IR was related to increase in DAS 28 and ESR and hsCRP and TNF- α levels and AOPPs and OSI and decreased TRAP in patients with RA. On the other hand, IR did not have a role in changes related to RF and anti-CCP. In addition, TNF- α increase is related to IR development in patients with RA.

4. Discussion

Several reports have shown that IR is related to chronic inflammation [1, 32, 33] and corticosteroid treatment [2, 3]. Although previous articles have shown that corticosteroid may be involved in IR [21, 22, 34], this finding has not been verified in patients with RA, suggesting that corticosteroid beneficial anti-inflammatory effects would compensate the deleterious metabolic action [35, 36]. Penesová et al. [36] showed that low-dose glucocorticoid treatment with duration of 2–9 years is relatively safe and did not lead to glucose metabolism impairment. Independently of whether they had IR or not, in the present study the patients did not differ in the frequency they were using prednisone, showing that, in this cohort of RA patients, corticosteroid use does not seem to be a determinant factor for IR development. Moreover, patients used less than 7.5 mg/d corticosteroid (data not shown), which has been reported as safe [37].

Several reports have shown the association between chronic inflammatory disease states and IR [32, 33, 38]. Previous studies demonstrated that TNF- α may have an important role in the IR pathogenesis by multiple mechanisms, such as downregulation of genes that are required for normal insulin action, direct effects on insulin signaling, induction of elevated free fatty acids via stimulation of lipolysis, and negative regulation of peroxisome proliferator-activated receptor- γ (PPAR γ), an important insulin-sensitizing nuclear receptor [39]. In RA patients with severe and active disease even in the presence of anti-TNF- α therapy, high-grade inflammation was correlated negatively and independently with circulating adiponectin concentration [40], an important anti-inflammatory adipokine related to insulin resistance and metabolic syndrome [41]. *In vitro* studies have shown that TNF- α induced serine phosphorylation of insulin receptor substrate-1 (IRS-1) and inhibited insulin receptor tyrosine kinase, causing a change of the insulin signaling [40]. In the present study, patients using anti-TNF- α therapy, which is generally indicated to patients who have a severe disease not controlled by disease-modifying antirheumatic drugs (DMARDs), showed higher TNF- α levels. Even with anti-TNF- α therapy, TNF- α levels have not reached the values obtained by patients who control disease activity with

TABLE 4: Oxidative stress in patients with rheumatoid arthritis using adalimumab or etanercept.

Parameters	Etanercept <i>n</i> = 26	Adalimumab <i>n</i> = 13	<i>p</i>
CL-LOOH (cpm)	164.02 (145.51–187.05)	168.06 (162.71–197.90)	NS
AOPP ($\mu\text{mol/L}$ of chloramines-T equivalents)	127.44 (108.75–187.05)	167.80 (122.90–228.73)	0.071
TRAP (μM Trolox/mg UA)	157.27 (15.84–183.51)	159.70 (148.83–175.58)	NS
OSI	1.10 (0.77–1.31)	0.86 (0.74–1.37)	NS

Mann-Whitney test. Data are expressed as median (25–75%). CL-LOOH, tert-butyl hydroperoxide-initiated chemiluminescence; AOPP, advanced oxidation protein product; TRAP, total radical-trapping antioxidant parameter; OSI, oxidative stress index; and NS, not significant.

conventional therapy and DAS 28 maintained higher score (≥ 3.3) than the recommended one for patients using or not using biological agents [41]. Of note, the majority of our patients (66.7%), who were taking anti-TNF- α therapy, used etanercept, a soluble TNF- α receptor fusion protein. Etanercept prolongs the half-life of TNF- α with a subsequent rise in measured serum TNF- α levels; thus it renders TNF- α biologically inactive and unavailable to bind to its receptor [42–44]. In the current study, patients with IR had also higher ESR concomitantly to TNF- α increase, suggesting that chronic inflammatory process may be associated with IR development and maintenance in patients with RA. Regarding the present data, it is not possible to assure that etanercept changed TNF- α in a biologically inactive substance. However, it is conceivable to suggest that other proinflammatory cytokines, which were not evaluated in this study, may be involved in the inflammatory process verified in patients with IR.

The present study demonstrated that RA patients with IR have higher TNF- α levels and unfavorable oxidative status. Reactive oxygen species (ROS) damage directly cellular elements in cartilage and either directly or indirectly the components of the extracellular matrix by upregulating mediators of matrix degradation. ROS impair chondrocyte response to growth factors and migration to sites of cartilage injury. In addition, ROS inhibit the synthesis of matrix components including proteoglycans by chondrocytes [12]. In the present study, IR patients showed higher oxidative stress levels and DAS 28. The overproduction of TNF- α is thought to be the main contributor to increased ROS release in RA patients [24, 45, 46], leading to tissue damage and IR [47, 48]. Large amounts of ROS have been detected in the synovial fluid in RA [49], and this production can be induced by TNF- α stimulation [50]. TNF- α exerts its cytotoxic effects via generation of intracellular ROS that induce apoptosis [51, 52]. Moreover, TNF- α can induce ROS production from neutrophils through pathway activating phagocytic NADPH oxidases in mitochondria [53] and TNF- α combined with cytokines such as GM-CSF or G-CSF enhances O_2^- generation [54]. Of note, oxidative stress and IR are more closely associated and many evidences have shown that oxidative stress can lead to IR by promoting the expression of several proinflammatory cytokines, mainly TNF- α , interleukin 6 (IL-6), and interleukin 17 (IL-17), which can cause significant decline in insulin sensitivity [9]. On the other hand, ROS

may increase TNF- α levels because they function as a second messenger to stimulate nuclear factor kappa B dependent expression of proinflammatory cytokines [55]. Altogether, our data seem to suggest that higher TNF- α level can be involved in IR development and maintenance and have a direct influence on oxidative stress. It seems that a cyclic and complex relationship occurs between TNF- α , oxidative stress, and IR in patients with RA.

The administration of biological drugs seems to have a role in increasing the barrier which the body possesses against oxidative stress [56]. However, data about anti-TNF- α therapy remain a matter of controversy. Kageyama et al. [15] showed a decrease in oxidative stress markers after six months in 22 patients with RA using etanercept. In contrast, den Broeder et al. [46] did not find any significant changes in oxidative stress markers after two weeks in 21 patients with RA taking adalimumab, although marked reduction in neutrophil influx to synovial tissue with anti-TNF- α therapy was reported. Meanwhile, Binięcka et al. [45] evaluated oxidative stress, assessed by 4-hydroxy-2-nonenal (4-HNE) in the synovial tissue, after three months in 18 patients with RA using anti-TNF- α therapy. DAS 28 < 2.6 was found in seven patients who were considered as anti-TNF- α responders and DAS 28 ≥ 2.6 in 11 patients who were considered as anti-TNF- α nonresponders. There was a decrease in 4-HNE levels only in anti-TNF- α responders patients. The aforementioned study seems to suggest that anti-TNF- α therapy can decrease oxidative stress in RA patients by controlling the inflammatory process, and hence they do not act directly on the production of ROS. In the present study, most patients who used anti-TNF- α therapy were taking etanercept. Nevertheless, differently from Kageyama's et al. study [15], the patients did not show improvement in redox state. It is conceivable to suggest that this may have occurred because anti-TNF- α therapy maintained DAS 28 in similar values obtained by patients who were not using anti-TNF- α therapy. Meanwhile, inflammatory process shown by increased ESR and TNF- α levels, mainly in RA patients with IR, progressed in these patients being responsible for oxidative stress increase.

Upon looking at the results obtained in the present study, some limitations have to be considered. First, the cross-sectional design does not allow for inference causality. Second, although the minimum number of patients has been reached by the calculation of the sample size, a greater

number of patients would probably confer more strength to the statistical results.

This study corroborates with Binięckas et al.'s [45], which suggested that inflammatory state maintenance can be responsible for oxidative stress found in patients with RA. On the other hand, the data of the present study show that IR is involved in an unbalanced redox state, which possibly contributes to maintaining a vicious circle of high-grade inflammation.

5. Conclusions

This study demonstrates that IR and TNF- α are important factors involved in redox imbalance in patients with RA and it seems to be due to the maintenance of inflammatory state and disease activity. The data from the present study suggest a complex interaction of TNF- α , oxidative stress, and IR, but the presence of insulin resistance seems to be directly associated with both oxidative stress and TNF- α levels. The differences in oxidative stress markers in RA patients with or without IR could contribute to a better design for future drugs and/or nutritional interventional studies in this population. In addition, more studies are warranted to verify if IR can be involved in therapeutic failure with TNF- α inhibitors.

Abbreviations

Anti-CCP:	Anticyclic citrullinated peptide
AOPPs:	Advanced oxidation protein products
CL-LOOH:	Tert-butyl hydroperoxide-initiated chemiluminescence
hsCRP:	Highly sensitive C-reactive protein
DAS 28:	Disease Activity Score evaluating 28 joints
4-HNE:	4-Hydroxy-2-nonenal
CVD:	Cardiovascular disease
DMARDs:	Disease-modifying antirheumatic drugs
ESR:	Erythrocyte sedimentation rate
HOMA-IR:	Homeostasis model assessment-insulin resistance
IL-6:	Interleukin 6
IL-17:	Interleukin 17
IR:	Insulin resistance
OSI:	Oxidative stress index
PPAR γ :	Peroxisome proliferator-activated receptor- γ
RA:	Rheumatoid arthritis
RLU:	Relative luminescence units
RF:	Rheumatoid factor
ROS:	Reactive oxygen species
TRAP:	Total radical-trapping antioxidant parameter
TNF- α :	Tumor necrosis factor-alpha
UA:	Uric acid
WC:	Waist circumference.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Neide Tomimura Costa helped to analyze the data and to draft the paper. Andręa Name Colado Simão performed data analysis and together with Isaias Dichi helped to design the study and draft the paper and critically revised the paper for important intellectual content. Vinicius Daher Alvares Delfino helped to analyze the data and critically revised the paper. Tatiana Mayumi Veiga Iriyoda and Ricardo Braga Amin participated in clinical assessments. Francieli Delongui, Ana Paula Kallaur, Marcell Alysson Batisti Lozovoy, and Daniela Frizon Alfieri helped in laboratory analyses. All authors have given final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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References

- [1] I. D. del Rincón, K. Williams, M. P. Stern, G. L. Freeman, and A. Escalante, "High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors," *Arthritis and Rheumatism*, vol. 44, no. 12, pp. 2737–2745, 2001.
- [2] S. E. Gabriel, "Cardiovascular morbidity and mortality in rheumatoid arthritis," *The American Journal of Medicine*, vol. 121, no. 10, pp. S9–S14, 2008.
- [3] S. Haque, H. Mirjafari, and I. N. Bruce, "Atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus," *Current Opinion in Lipidology*, vol. 19, no. 4, pp. 338–343, 2008.
- [4] C. De Luca and J. M. Olefsky, "Inflammation and insulin resistance," *FEBS Letters*, vol. 582, no. 1, pp. 97–105, 2008.
- [5] A. M. El-Barbary, E. M. Kassem, M. A. S. El-Sergany, S. A. M. Essa, and M. A. Eltomey, "Association of anti-modified citrullinated vimentin with subclinical atherosclerosis in early rheumatoid arthritis compared with anti-cyclic citrullinated peptide," *The Journal of Rheumatology*, vol. 38, no. 5, pp. 828–834, 2011.
- [6] T. Saxne, M. A. Palladino Jr., D. Heinegard, N. Talal, and F. A. Wollheim, "Detection of tumor necrosis factor α but not tumor necrosis factor β in rheumatoid arthritis synovial fluid and serum," *Arthritis and Rheumatism*, vol. 31, no. 8, pp. 1041–1045, 1988.
- [7] I. B. McInnes and G. Schett, "The pathogenesis of rheumatoid arthritis," *The New England Journal of Medicine*, vol. 365, no. 23, pp. 2205–2219, 2011.
- [8] C. P. Chung, A. Oeser, J. F. Solus et al., "Inflammation-associated insulin resistance: differential effects in rheumatoid arthritis and systemic lupus erythematosus define potential mechanisms," *Arthritis and Rheumatism*, vol. 58, no. 7, pp. 2105–2112, 2008.
- [9] M. L. L. Prevoo, M. A. van't Hof, H. H. Kuper, M. A. van Leeuwen, L. B. A. van de Putte, and P. L. C. M. van Riel, "Modified disease activity scores that include twenty-eight-joint

- counts development and validation in a prospective longitudinal study of patients with rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 38, no. 1, pp. 44–48, 1995.
- [10] P. Vasanthi, G. Nalini, and G. Rajasekhar, "Status of oxidative stress in rheumatoid arthritis," *International Journal of Rheumatic Diseases*, vol. 12, no. 1, pp. 29–33, 2009.
- [11] L. I. Filippin, R. Vercelino, N. P. Marroni, and R. M. Xavier, "Redox signalling and the inflammatory response in rheumatoid arthritis," *Clinical and Experimental Immunology*, vol. 152, no. 3, pp. 415–422, 2008.
- [12] C. A. Hitchon and H. S. El-Gabalawy, "Oxidation in rheumatoid arthritis," *Arthritis Research and Therapy*, vol. 6, no. 6, pp. 265–278, 2004.
- [13] S. Taysi, F. Polat, M. Gul, R. Sari, and E. Bakan, "Lipid peroxidation, some extracellular antioxidants, and antioxidant enzymes in serum of patients with rheumatoid arthritis," *Rheumatology International*, vol. 21, no. 5, pp. 200–204, 2002.
- [14] S. Z. Hassan, T. A. Gheita, S. A. Kenawy, A. T. Fahim, I. M. El-Sorougy, and M. S. Abdou, "Oxidative stress in systemic lupus erythematosus and rheumatoid arthritis patients: relationship to disease manifestations and activity," *International Journal of Rheumatic Diseases*, vol. 14, no. 4, pp. 325–331, 2011.
- [15] Y. Kageyama, M. Takahashi, T. Nagafusa, E. Torikai, and A. Nagano, "Etanercept reduces the oxidative stress marker levels in patients with rheumatoid arthritis," *Rheumatology International*, vol. 28, no. 3, pp. 245–251, 2008.
- [16] S. Shahmohamadnejad, A. Vaisi-Raygani, Y. Shakiba et al., "Association between butyrylcholinesterase activity and phenotypes, paraoxonase192 rs662 gene polymorphism and their enzymatic activity with severity of rheumatoid arthritis: correlation with systemic inflammatory markers and oxidative stress, preliminary report," *Clinical Biochemistry*, vol. 48, no. 1-2, pp. 63–69, 2015.
- [17] M. A. B. Lozovoy, A. N. C. Simão, M. S. N. Hohmann et al., "Inflammatory biomarkers and oxidative stress measurements in patients with systemic lupus erythematosus with or without metabolic syndrome," *Lupus*, vol. 20, no. 13, pp. 1356–1364, 2011.
- [18] M. A. B. Lozovoy, A. N. C. Simão, S. R. Oliveira et al., "Relationship between iron metabolism, oxidative stress, and insulin resistance in patients with systemic lupus erythematosus," *Scandinavian Journal of Rheumatology*, vol. 42, no. 4, pp. 303–310, 2013.
- [19] S. R. Oliveira, A. N. Colado Simão, A. P. Kallaur et al., "Disability in patients with multiple sclerosis: influence of insulin resistance, adiposity, and oxidative stress," *Nutrition*, vol. 30, no. 3, pp. 268–273, 2014.
- [20] H. K. Morimoto, A. N. C. Simão, E. R. D. D. Almeida et al., "Role of metabolic syndrome and antiretroviral therapy in adiponectin levels and oxidative stress in HIV-1 infected patients," *Nutrition*, vol. 30, no. 11-12, pp. 1324–1330, 2014.
- [21] P. H. Desein and B. I. Joffe, "Insulin resistance and impaired beta cell function in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 9, pp. 2765–2775, 2006.
- [22] G. La Montagna, F. Cacciapuoti, R. Buono et al., "Insulin resistance is an independent risk factor for atherosclerosis in rheumatoid arthritis," *Diabetes and Vascular Disease Research*, vol. 4, no. 2, pp. 130–135, 2007.
- [23] G. Arcaro, "Insulin causes endothelial dysfunction in humans: sites and mechanisms," *Circulation*, vol. 105, no. 5, pp. 576–582, 2002.
- [24] Y. Kageyama, M. Takahashi, T. Ichikawa, E. Torikai, and A. Nagano, "Reduction of oxidative stress marker levels by anti-TNF- α antibody, infliximab, in patients with rheumatoid arthritis," *Clinical and Experimental Rheumatology*, vol. 26, no. 1, pp. 73–80, 2008.
- [25] O. Altindag, M. Karakoc, A. Kocyigit, H. Celik, and N. Soran, "Increased DNA damage and oxidative stress in patients with rheumatoid arthritis," *Clinical Biochemistry*, vol. 40, no. 3-4, pp. 167–171, 2007.
- [26] A. Nakajima, Y. Aoki, Y. Shibata et al., "Identification of clinical parameters associated with serum oxidative stress in patients with rheumatoid arthritis," *Modern Rheumatology*, vol. 24, no. 6, pp. 926–930, 2014.
- [27] D. Aletaha, T. Neogi, and A. J. Silman, "2010 rheumatoid arthritis classification criteria: An American College of Rheumatology/European League against rheumatism collaborative initiative," *Annals of the Rheumatic Diseases*, vol. 69, no. 10, pp. 1580–1588, 2010.
- [28] S. M. Haffner, H. Miettinen, and M. P. Stern, "The homeostasis model in the San Antonio Heart Study," *Diabetes Care*, vol. 20, no. 7, pp. 1087–1092, 1997.
- [29] B. Gonzalez Flecha, S. Llesuy, and A. Boveris, "Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle," *Free Radical Biology and Medicine*, vol. 10, no. 2, pp. 93–100, 1991.
- [30] V. Witko-Sarsat, M. Friedlander, T. N. Khoa et al., "Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure," *The Journal of Immunology*, vol. 161, no. 5, pp. 2524–2532, 1998.
- [31] M. Repetto, C. Reides, M. L. Gomez Carretero, M. Costa, G. Griemberg, and S. Llesuy, "Oxidative stress in blood of HIV infected patients," *Clinica Chimica Acta*, vol. 255, no. 2, pp. 107–117, 1996.
- [32] G. S. Hotamisligil, "Molecular mechanisms of insulin resistance and the role of the adipocyte," *International Journal of Obesity*, vol. 24, supplement 4, pp. S23–S27, 2000.
- [33] C. Popa, M. G. Netea, P. L. C. M. Van Riel, J. W. M. Van Der Meer, and A. F. H. Stalenhoef, "The role of TNF- α in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk," *Journal of Lipid Research*, vol. 48, no. 4, pp. 751–752, 2007.
- [34] P. H. Desein, B. I. Joffe, A. E. Stanwix, B. F. Christian, and M. Veller, "Glucocorticoids and insulin sensitivity in rheumatoid arthritis," *The Journal of Rheumatology*, vol. 31, no. 5, pp. 867–874, 2004.
- [35] K. L. G. Svenson, G. Lundqvist, L. Wide, and R. Hällgren, "Impaired glucose handling in active rheumatoid arthritis: effects of corticosteroids and antirheumatic treatment," *Metabolism*, vol. 36, no. 10, pp. 944–948, 1987.
- [36] A. Penesová, Z. Rádiková, M. Vlček et al., "Chronic inflammation and low-dose glucocorticoid effects on glucose metabolism in premenopausal females with rheumatoid arthritis free of conventional metabolic risk factors," *Physiological Research*, vol. 62, no. 1, pp. 75–83, 2013.
- [37] J. M. Sabio, J. A. Vargas-Hitos, N. Navarrete, C. Hidalgo-Tenorio, and J. Jiménez-Alonso, "Effects of low or medium-dose of prednisone on insulin resistance in patients with systemic lupus erythematosus," *Clinical and Experimental Rheumatology*, vol. 28, no. 4, pp. 483–489, 2010.
- [38] L.-S. Tam, B. Tomlinson, T. T. Chu, T. K. Li, and E. K. Li, "Impact of TNF inhibition on insulin resistance and lipids levels in patients with rheumatoid arthritis," *Clinical Rheumatology*, vol. 26, no. 9, pp. 1495–1498, 2007.

- [39] D. E. Moller, "Potential role of TNF- α in the pathogenesis of insulin resistance and type 2 diabetes," *Trends in Endocrinology and Metabolism*, vol. 11, no. 6, pp. 212–217, 2000.
- [40] T. Hayakawa, Y. Nagai, M. Taniguchi et al., "Tumor necrosis factor- β gene NcoI polymorphism decreases insulin resistance in Japanese men," *Metabolism: Clinical and Experimental*, vol. 49, no. 11, pp. 1506–1509, 2000.
- [41] J. A. Singh, D. E. Furst, A. Bharat et al., "2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis," *Arthritis Care & Research*, vol. 64, no. 5, pp. 625–639, 2012.
- [42] J. Lo, L. E. Bernstein, B. Canavan et al., "Effects of TNF- α neutralization on adipocytokines and skeletal muscle adiposity in the metabolic syndrome," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 293, no. 1, pp. E102–E109, 2007.
- [43] K. J. Grattendick, J. M. Nakashima, L. Feng, S. N. Giri, and S. B. Margolin, "Effects of three anti-TNF- α drugs: etanercept, infliximab and pirfenidone on release of TNF- α in medium and TNF- α associated with the cell *in vitro*," *International Immunopharmacology*, vol. 8, no. 5, pp. 679–687, 2008.
- [44] A. Bhatia and R. E. Kast, "Tumor Necrosis Factor (TNF) can paradoxically increase on etanercept treatment, occasionally contributing to TNF-mediated disease," *Journal of Rheumatology*, vol. 34, no. 2, pp. 447–449, 2007.
- [45] M. Biniecka, A. Kennedy, C. T. Ng et al., "Successful tumour necrosis factor (TNF) blocking therapy suppresses oxidative stress and hypoxia-induced mitochondrial mutagenesis in inflammatory arthritis," *Arthritis Research and Therapy*, vol. 13, no. 4, article R121, 2011.
- [46] A. A. den Broeder, G. J. A. Wanten, W. J. G. Oyen, T. Naber, P. L. C. M. Van Riel, and P. Barrera, "Neutrophil migration and production of reactive oxygen species during treatment with a fully human anti-tumor necrosis factor- α monoclonal antibody in patients with rheumatoid arthritis," *Journal of Rheumatology*, vol. 30, no. 2, pp. 232–237, 2003.
- [47] I. Stagakis, G. Bertias, S. Karvounaris et al., "Anti-tumor necrosis factor therapy improves insulin resistance, beta cell function and insulin signaling in active rheumatoid arthritis patients with high insulin resistance," *Arthritis Research and Therapy*, vol. 14, no. 3, article R141, 2012.
- [48] A. Stavropoulos-Kalinoglou, G. S. Metsios, V. F. Panoulas, P. Nightingale, Y. Koutedakis, and G. D. Kitas, "Anti-tumour necrosis factor alpha therapy improves insulin sensitivity in normal-weight but not in obese patients with rheumatoid arthritis," *Arthritis Research and Therapy*, vol. 14, no. 4, article R160, 2012.
- [49] P. Biemond, A. J. G. Swaak, and J. F. Koster, "Protective factors against oxygen free radicals and hydrogen peroxide in rheumatoid arthritis synovial fluid," *Arthritis and Rheumatism*, vol. 27, no. 7, pp. 760–765, 1984.
- [50] C.-H. Woo, T.-H. Kim, J.-A. Choi et al., "Inhibition of receptor internalization attenuates the TNF α -induced ROS generation in non-phagocytic cells," *Biochemical and Biophysical Research Communications*, vol. 351, no. 4, pp. 972–978, 2006.
- [51] K. Schulze-Osthoff, P. H. Kramer, and W. Dröge, "Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death," *The EMBO Journal*, vol. 13, no. 19, pp. 4587–4596, 1994.
- [52] K. Hirose, D. L. Longo, J. J. Oppenheim, and K. Matsushima, "Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation," *FASEB Journal*, vol. 7, no. 2, pp. 361–368, 1993.
- [53] R. Miesel, M. P. Murphy, and H. Kröger, "Enhanced mitochondrial radical production in patients with rheumatoid arthritis correlates with elevated levels of tumor necrosis factor alpha in plasma," *Free Radical Research*, vol. 25, no. 2, pp. 161–169, 1996.
- [54] E. Mur, A. Zabernigg, W. Hilbe, W. Eisterer, W. Halder, and J. Thaler, "Oxidative burst of neutrophils in patients with rheumatoid arthritis: influence of various cytokines and medication," *Clinical and Experimental Rheumatology*, vol. 15, no. 3, pp. 233–237, 1997.
- [55] G. Bonizzi, J. Piette, M.-P. Merville, and V. Bours, "Cell type-specific role for reactive oxygen species in nuclear factor-kappaB activation by interleukin-1," *Biochemical Pharmacology*, vol. 59, no. 1, pp. 7–11, 2000.
- [56] S. Coaccioli, A. Panaccione, R. Biondi et al., "Evaluation of oxidative stress in rheumatoid and psoriatic arthritis and psoriasis," *Clinica Terapeutica*, vol. 160, no. 6, pp. 467–472, 2009.

Research Article

Antidiabetic and Antinephritic Activities of Aqueous Extract of *Cordyceps militaris* Fruit Body in Diet-Streptozotocin-Induced Diabetic Sprague Dawley Rats

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Cordyceps militaris has long been used as a crude drug and folk tonic food in East Asia. The present study aims to evaluate the antidiabetic and antinephritic effects of the aqueous extract of the *Cordyceps militaris* fruit body (CM) in diet-streptozotocin- (STZ-) induced diabetic rats. During four weeks of continuous oral administration of CM at doses of 0.5, 1.0, and 2.0 g/kg and metformin at 100 mg/kg, the fasting blood glucose and bodyweight of each rat were monitored. Hypoglycemic effects of CM on diabetic rats were indicated by decreases in plasma glucose, food and water intake, and urine output. The hypolipidemic activity of CM was confirmed by the normalization of total cholesterol, triglycerides, and low- and high-density lipoprotein cholesterol in diabetic rats. Inhibitory effects on albuminuria, creatinine, urea nitrogen, and n-acetyl- β -d-glucosaminidase verified CM's renal protective activity in diabetic rats. Furthermore, CM exerted beneficial modulation of inflammatory factors and oxidative enzymes. Compared with untreated diabetic rats, CM decreased the expression of phosphor-AKT and phosphor-GSK-3 β in the kidneys. Altogether, via attenuating oxidative stress, CM displayed antidiabetic and antinephritic activities in diet-STZ-induced diabetic rats.

1. Introduction

The prevalence of diabetes and metabolic disease is increasing rapidly worldwide and has become a major health problem [1]. Currently, 387 million people are diagnosed with diabetes mellitus, 90% with type 2 diabetes mellitus (T2DM). A deficiency of insulin secretion leads to increased blood glucose levels and organ damage, which further disrupts the metabolism of the three major nutrients, namely, lipids, carbohydrates, and proteins [2, 3]. Various complications including nephropathy, neuropathy, retinopathy, and hyperlipemia are observed in most diabetic patients [4]. The longitudinal data predict that patients with T2DM will have a much more aggressive course of disease with greater risk of early hypertension and nephropathy compared with type 1 (T1DM) patients [5].

Diabetic nephropathy is a major cause of end-stage renal disease with high mortality and morbidity [6]. During the pathogenic process, microalbuminuria follows macroalbuminuria, leading to renal dysfunction. Multiple and complex mechanisms are involved in the pathogenesis of diabetic nephropathy, which is characterized by persistent albuminuria, elevated arterial blood pressure, and a decline in the glomerular filtration rate (GFR) [7]. In 2013, diabetic nephropathy accounted for over 25% of the incidence of end-stage renal disease (ESRD) in the UK, while over 40% of diabetic nephropathic patients in the United States receive dialysis [8].

Current therapy for diabetes focuses only on the recovery of pancreatic islet function and regulation of blood glucose, most of which fails to improve the symptoms of complications [9]. Poorly controlled blood pressure and cholesterol activate

inflammatory mediators, and genetic predisposition helps patients progress to an advanced stage of nephropathy. Insulin injection and commonly prescribed drugs such as metformin and pioglitazone produce adverse side effects, including insulin resistance, hypoglycemia, and gastrointestinal disturbances [10]. Due to the limited and unsatisfactory therapeutic effects of antidiabetic agents, alternative medications to treat diabetes and related nephropathy are highly desirable.

Herbs are a source of novel pharmaceuticals not only due to their potent efficacy with fewer side effects, but also due to the complex bioactive compounds they contain [11]. About 1,200 plants have been claimed to have antidiabetic properties, and over 400 plants and their bioactive compounds have been scientifically evaluated for T2DM treatment [12]. Due to its anti-inflammatory, antioxidant, and antitumor activities, *Cordyceps militaris* has been extensively used as a crude drug and folk tonic food in East Asia [13]. In our research group, *Cordyceps militaris* mycelium obtained via submerged fermentation has shown excellent antidiabetic and antinephropathic activities [14]. Aqueous extracts of *Cordyceps militaris* enhance insulin secretion and cholinergic activation in normal Wistar rats [15].

We therefore hypothesized that the polysaccharide-rich aqueous extract of the *Cordyceps militaris* fruit body (CM) may possess antidiabetic and antinephritic properties. A high-fat diet and streptozotocin- (STZ-) induced rat model was used to investigate the effects of CM on diabetes, renal injury, and other underlying mechanisms related to inflammatory factors and oxidative stress.

2. Materials and Methods

2.1. *Cordyceps militaris* Extract Preparation. *Cordyceps militaris* fruit body (purchased from Qianxiang Co., Ltd., Shenyang, China) was extracted with 10 volumes of double distilled (DD) water at 45°C for 3 h. After centrifugation, the residue was extracted at 80°C for another 3.5 h. After the two extracts were combined, the supernatant was sequentially concentrated in an evaporator under reduced pressure and then freeze-dried to produce a solid aqueous extract (CM). CM contained 29.1% polysaccharides, 20.5% total proteins, 6.1% cordycepic acid, 0.2% adenosine, and 0.4% cordycepin. The concentrations of adenosine and cordycepin were determined using HPLC methods and the results were shown in Figure 1S (in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/9685257>).

2.2. Animal Care. The experimental animal protocol used in the study was approved by the Institutional Animal Ethics Committee of Jilin University. Male Sprague Dawley rats weighing 180–220 g (SCXK(JI)-2014-0003) (purchased from the Norman Bethune College of Medicine, Jilin University, China) were maintained on a 12 h light/dark cycle (lights on 07:00–19:00) at 23 ± 1°C with water and food available *ad libitum*. All efforts were made to minimize animal suffering and reduce the number of animals used.

2.3. The Diet-Streptozotocin-Induced Diabetic Rat Model and Drug Administration Procedure. Rats were randomly divided into two groups and fed with either the standard control diet (normal control group, $n = 6$) or a high-fat diet (HFHSD, 12% protein, 5% fat, 67% carbohydrate, 5% cholesterol, and 5% other additives) ($n = 30$) for 8 weeks. HFHSD-treated rats were further intraperitoneally injected with 25 mg/kg STZ agent dissolved in a citrate buffer (0.1 mol/L sodium citrate and 0.1 mol/L citric acid, pH 4.5) for one week (once a day). Rats were defined as diabetic if their blood glucose levels 72 h after the last STZ injection were over 11.1 mmol/L.

Diabetic rats were randomly divided into five groups and orally treated with 2.0 mL/kg sterile saline (HFHSD+STZ diabetic model group, $n = 6$), 0.10 g/kg metformin hydrochloride (Met; from Beijing Jingfeng Zhiyao Co., Ltd, Beijing, China) (Met+HFHSD+STZ group, $n = 6$), and 0.5, 1.0, and 2.0 g/kg CM (CM+HFHSD+STZ group, $n = 6$). Normal rats, which received 2.0 mL/kg sterile saline, served as the normal control group. Over the four-week drug delivery period, bodyweight and blood glucose were recorded weekly. At the end of the experiment, the daily food intake, water intake, and 24 h urine output of each rat were recorded using the diuresis and metabolic cage method.

2.4. Oral Glucose Tolerance Test (OGTT) in Rats. After the last drug administration, the rats were fasted for 16 h, before undergoing a glucose tolerance test. Briefly, the rats were weighed and then orally given glucose (2.0 g/kg). Tail-vein blood samples were collected at intervals from 0 to 240 min and assayed via a fast blood glucose meter [16]. The area under the blood glucose curve (AUC) was calculated using the following [17]:

$$\begin{aligned} \text{AUC} = & (\text{basal glycaemia} + \text{glycaemia } 0.5 \text{ h}) \times 0.25 \\ & + (\text{glycaemia } 0.5 \text{ h} + \text{glycaemia } 1 \text{ h}) \times 0.25 \quad (1) \\ & + (\text{glycaemia } 1 \text{ h} + \text{glycaemia } 2 \text{ h}) \times 0.5. \end{aligned}$$

2.5. Sample Collection and Biochemical Analysis. Before sacrifice, blood was sampled from the heart of each rat under anesthesia. The blood samples were centrifuged at 3000 g for 10 min, and the serum was frozen at –80°C. After sacrifice, the kidneys were collected, and one part was homogenized in DD water (or RIPA buffer) with three washes in ice-cold physiological saline, while the other part was placed in 4% paraformaldehyde for histopathological examination.

The levels were then determined for serum pyruvate kinase (PK), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), creatinine (Scr), urea nitrogen (BUN), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and n-acetyl- β -d-glucosaminidase (NAG) and for albuminuria in urine, malondialdehyde (MDA) in serum and kidneys, and reactive oxygen species (ROS) in kidneys, using commercial kits (Nanjing Biotechnology Co., Ltd., Nanjing, China).

The serum levels of insulin (INS), interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and

TABLE 1: The effects of CM and Met on daily food intake, water intake, and urine output in each experimental rat.

		Food intake (g/100 g)	Water intake (g/100 g)	Urine output (mL/100 g)
CTRL	—	14.0 ± 2.0	14.5 ± 3.6	4.1 ± 1.2
Model	—	19.5 ± 3.2 [#]	63.8 ± 6.88 ^{##}	51.1 ± 5.8 ^{##}
	0.5	13.3 ± 1.9 [*]	42.3 ± 5.9 ^{###}	40.3 ± 6.7 ^{###}
CM (g/kg)	1.0	15.7 ± 1.8	32.6 ± 3.6 ^{###}	30.7 ± 7.1 ^{###}
	2.0	13.6 ± 0.7 [*]	37.1 ± 4.2 ^{###}	39.0 ± 3.6 ^{###}
Met (mg/kg)	100	16.6 ± 2.1	38.9 ± 6.4 ^{###}	41.7 ± 6.6 ^{###}

Daily food intake, water intake, and urine output were normalized to rat body weight, g/100 g or mL/100 g BW. Data are expressed as mean ± SEM ($n = 6$) and analyzed using one-way ANOVA. [#] $P < 0.05$ and ^{##} $P < 0.01$ versus normal controls. ^{*} $P < 0.05$ and ^{**} $P < 0.01$ versus model group.

6-keto-PGF were detected using enzyme-linked immunosorbent assay (ELISA) kits (Calbiotech, USA).

2.6. Histopathological Examination. The collected kidney tissue was immersed in 4% paraformaldehyde for 48 h and then dehydrated step by step using a gradient of ethanol (50%, 70%, 80%, 90%, 95%, and 100%). Samples were immersed in xylene for 30 min and incubated in paraffin at 65°C overnight. Once embedded in wax, the samples were cut serially into 5 μm thick sections using a microtome (Leica, Germany) and spread over microscopy slides. The sections were deparaffinized with fresh xylene for 10 min, rehydrated with a gradient of ethanol (100%, 90%, 80%, and 70%), and then washed three times with DD water. The sections were analyzed via hematoxylin and eosin (H&E) staining and examined with a light microscope digital camera (Nikon Instruments, Tokyo, Japan).

2.7. Western Blot. One part of the kidney tissue was homogenized in a radioimmunoprecipitation assay buffer (RIPA; Sigma-Aldrich, USA) containing 1% protease inhibitor cocktail and 2% phenylmethanesulfonyl fluoride (Sigma-Aldrich, USA). Protein concentrations were determined by the Bradford method, and 40 μg proteins were separated using 10% SDS-PAGE gel and transferred electrophoretically onto nitrocellulose membranes (0.45 μm; Bio Basic, Inc., USA). The transferred membranes were blotted with primary antibodies at 4°C overnight at a dilution of 1:1000: phospho-AKT (ab131443), total-AKT (ab200195), phospho-GSK-3β (ab75745), total-GSK-3β (#32391), and glyceraldehyde-3-phosphate dehydrogenase (#2118) (Abcam, Cambridge, UK) and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA). Chemiluminescence was detected using ECL detection kits (GE Healthcare, UK). The intensity of the bands was quantified by scanning densitometry using Image J software (National Institutes of Health, Bethesda, USA).

2.8. Statistical Analysis. All values were expressed as mean ± SEM. A one-way analysis of variance (ANOVA) was used to detect statistical significance followed by *post hoc* multiple comparisons (Dunn's test) using SPSS 16.0 software (IBM Corporation, Armonk, USA). A value of $P < 0.05$ was considered significant.

3. Results

3.1. Hypoglycemic Effects on Diabetic Rats. Compared with the normal control group, the diabetic rats clearly consumed more food and water and produced more urine ($P < 0.01$, Table 1). The four-week CM treatment at 1.0 g/kg strikingly decreased their urine output and water intake, and at 0.5 g/kg and 2.0 g/kg food intake was strongly reduced ($P < 0.01$, Table 1).

Reduced bodyweight and elevated blood glucose were observed after STZ treatment ($P < 0.01$, Table 2). Similar to Met, compared with the diabetic model rats, the maximum increase in bodyweight was nearly 31.3% in CM-treated diabetic rats ($P < 0.01$, Table 2). CM at doses of 0.5 and 1.0 g/kg reduced fasting blood glucose by 42.2% and 34.9%, respectively ($P < 0.05$, Table 2). However, only 0.5 g/kg CM clearly increased serum insulin compared with the diabetic model group ($P < 0.05$, Figure 1(a)). Both Met (100 mg/kg) and CM (1.0 g/kg) markedly increased PK activity in diabetic rats ($P < 0.05$, Figure 1(b)).

OGTT was applied to avoid false positive results from fasting blood glucose. Compared with the normal control rats, dramatically higher fasting blood glucose concentrations were noted in the diabetic rats from 0 to 240 min ($P < 0.01$, Figure 1(c)), with 1.0 g/kg CM significantly preventing blood glucose from shooting up at 30 to 240 min ($P < 0.05$, Figure 1(c)). The calculated AUC values for glucose response during the OGTT revealed a striking increment in the diabetic model group (43.3 ± 8.4 h·mmol/L) compared with the normal control group (10.9 ± 1.5 h·mmol/L). CM at 1.0 g/kg and Met at 100 mg/kg showed a significant reduction in AUC ($P < 0.05$, Figure 1(d)).

3.2. Hypolipidemic Effects in Diabetic Rats. Hyperlipidemia commonly accompanies diabetes mellitus [18]. Thus, a study was carried out to investigate whether CM beneficially affects the abnormal lipid profiles of diabetic rats. As with Met, CM at 1.0 and 2.0 g/kg significantly decreased TC and TG levels ($P < 0.05$, Figures 2(a) and 2(b)). Unlike Met, CM at 0.5 and 2.0 g/kg decreased LDL-C levels in diabetic rats ($P < 0.05$, Figure 2(c)). But only CM at 2.0 g/kg increased HDL-C levels in diabetic rats ($P < 0.05$, Figure 2(d)).

3.3. Renal Protection in Diabetic Rats. Albuminuria is traditionally considered a hallmark of diabetic nephropathy [19].

TABLE 2: The effects of CM and Met on bodyweight and fasting blood glucose levels in experimental rats.

		Initial	4-week drug treatment					
			8-week HFHSD feeding	7-day STZ injection	1	2	3	4
Body weight (g)	CTRL	140.2 ± 25.1	445.3 ± 33.9	468.3 ± 32.8	464.3 ± 29.8	470.3 ± 30.9	480.2 ± 28.4	485.2 ± 32.4
	Model	139.2 ± 26.8	450.1 ± 35.9	386.2 ± 35.1 [#]	326.0 ± 39.1 ^{##}	315.7 ± 25.9 ^{##}	303.9 ± 38.8 ^{##}	297.0 ± 32.4 [#]
	CM (g/kg)	145.1 ± 29.2	455.3 ± 33.8	381.7 ± 31.5 [#]	348.3 ± 32.7 ^{##}	339.2 ± 42.1 [#]	347.9 ± 59.2 [#]	361.7 ± 27.8 [#]
	Met (mg/kg)	143.2 ± 30.7	456.3 ± 30.0	388.6 ± 30.5 [#]	320.0 ± 42.6 ^{##}	335.7 ± 41.1 [#]	355.9 ± 31.9 [#]	373.7 ± 30.2 [#]
		140.1 ± 25.1	475.1 ± 35.9	385.1 ± 31.9 [#]	373.8 ± 23.5 [#]	355.2 ± 28.9 [#]	345.2 ± 30.7 [#]	390.0 ± 22.0 [#]
		139.6 ± 22.1	462.4 ± 32.3	391.9 ± 30.2 [#]	326.4 ± 37.9 ^{##}	345.1 ± 32.1 [#]	344.0 ± 38.2 [#]	349.0 ± 35.6 [#]
Fasting blood glucose (mmol/L)	CTRL	4.1 ± 0.7	4.3 ± 0.6	4.6 ± 0.8	4.6 ± 0.7	4.7 ± 0.6	4.7 ± 0.9	4.8 ± 0.7
	Model	4.2 ± 0.6	4.6 ± 0.5	18.1 ± 1.5 ^{##}	19.2 ± 1.4 ^{##}	19.1 ± 2.2 ^{##}	21.0 ± 1.9 ^{##}	18.9 ± 1.9 ^{##}
	CM (g/kg)	4.0 ± 0.5	4.5 ± 0.7	18.3 ± 1.6 ^{##}	15.2 ± 2.1 ^{##}	13.7 ± 1.7 ^{##}	12.3 ± 1.9 ^{##}	10.8 ± 1.8 [#]
	Met (mg/kg)	3.9 ± 0.7	4.2 ± 0.9	17.7 ± 1.6 ^{##}	16.1 ± 1.7 ^{##}	13.9 ± 1.4 ^{##}	11.6 ± 1.7 ^{##}	12.2 ± 1.9 [#]
		4.1 ± 0.7	4.5 ± 0.6	18.0 ± 2.1 ^{##}	14.8 ± 1.6 ^{##}	12.5 ± 1.9 ^{##}	11.9 ± 1.9 ^{##}	12.7 ± 1.7 ^{##}
		4.2 ± 0.613	4.4 ± 0.7	17.6 ± 2.1 ^{##}	17.1 ± 2.1 ^{##}	14.7 ± 1.6 ^{##}	13.8 ± 1.7 ^{##}	12.9 ± 1.8 [#]

Data are expressed as mean ± SEM ($n = 6$) and analyzed using one-way ANOVA. [#] $P < 0.05$ and ^{##} $P < 0.01$ versus normal controls. * $P < 0.05$ versus model group.

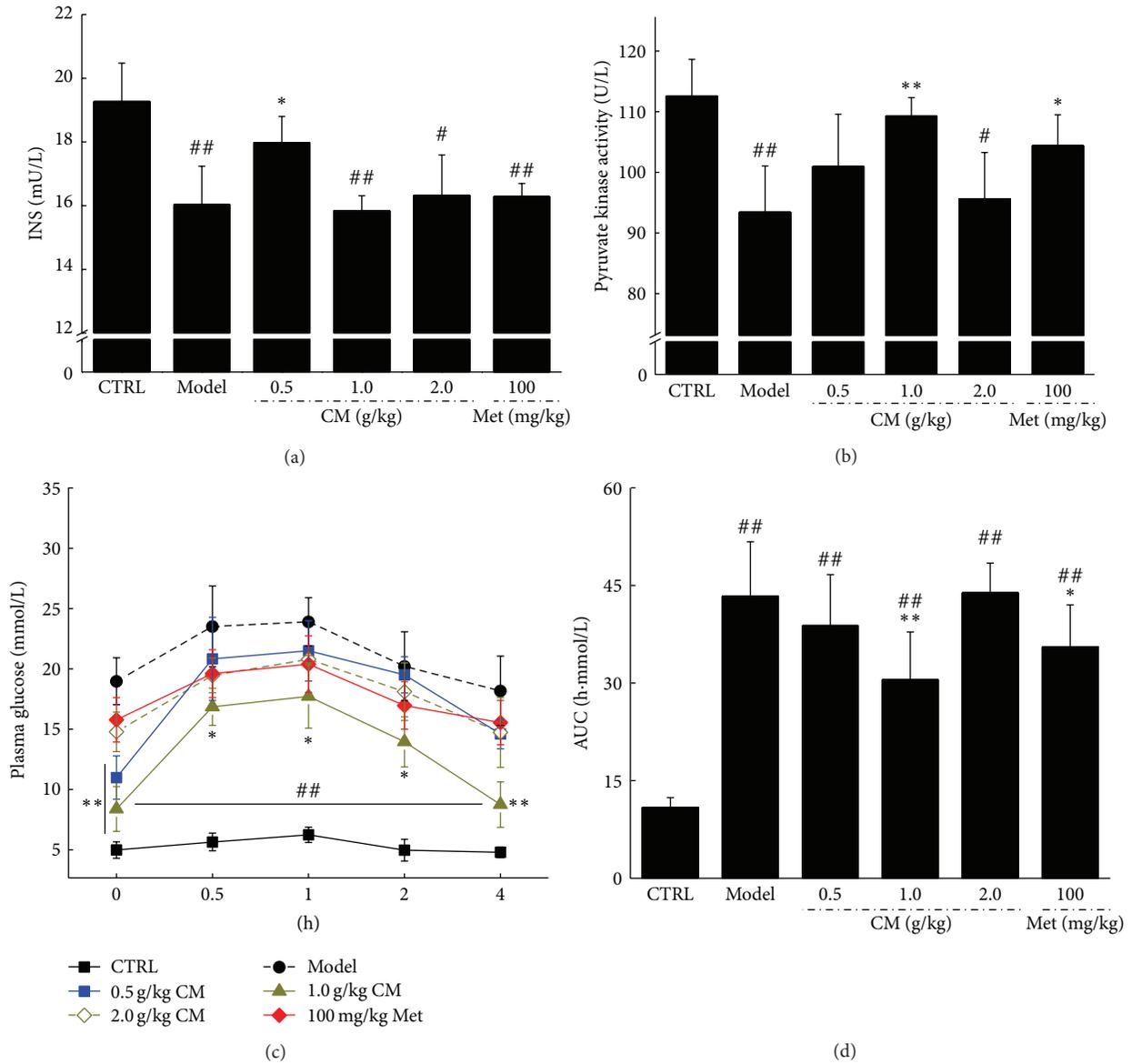


FIGURE 1: Diet-STZ-induced diabetic rats were treated with or without 100 mg/kg metformin (Met) and *Cordyceps militaris* water extract (CM) for four weeks. After the final drug treatment, the serum levels of insulin (a) and pyruvate kinase (b) were detected in all experimental rats. At the end of the experiment, after an oral treatment of 2 g/kg D-glucose in all experimental rats, the changes of plasma glucose (c) and area under the curve of glucose (d) were analyzed. Data are expressed as mean \pm SEM ($n = 6$) and analyzed using one-way ANOVA. * $P < 0.05$ and ** $P < 0.01$ versus normal controls. # $P < 0.05$ and ## $P < 0.01$ versus nontreated diabetic rats.

CM strongly suppressed the raised serum albuminuria levels of diabetic rats, especially at 1.0 g/kg ($P < 0.05$, Table 3). Abnormal BUN and Scr levels are recognized manifestations of renal dysfunction, and these were all reduced after four weeks of CM administration ($P < 0.05$, Table 3). However, Met and CM failed to influence serum NGA concentration in diabetic rats (Table 3).

Hyperglycemia and hyperlipidemia in T2DM always lead to toxicity in the kidneys, inducing renal damage associated with severe inflammation and characterized by the release of multiple inflammatory factors. Extremely high serum levels of IL-2, IL-6, TNF- α , and 6-keto-PGF were noted in the

diet-induced diabetic rats ($P < 0.05$, Figures 3(a)–3(d)). Met showed a suppressive effect on inflammatory cytokines ($P < 0.05$, Figures 3(a)–3(d)). Compared with the diabetic model group, CM at 1.0 g/kg reduced IL-2 and IL-6 levels by 35.1% and 27.1%, respectively ($P < 0.01$, Figures 3(a) and 3(b)). Additionally, serum TNF- α and 6-keto-PGF were reduced in CM-treated diabetic rats by up to 31.2% and 24.6%, respectively ($P < 0.01$, Figures 3(c) and 3(d)). CM treatment also significantly ameliorated the incidence of glomerular basement membrane thickening or mesangial proliferation and of inflammatory infiltrate injuries in the kidneys of diabetic rats (Figure 3(e)).

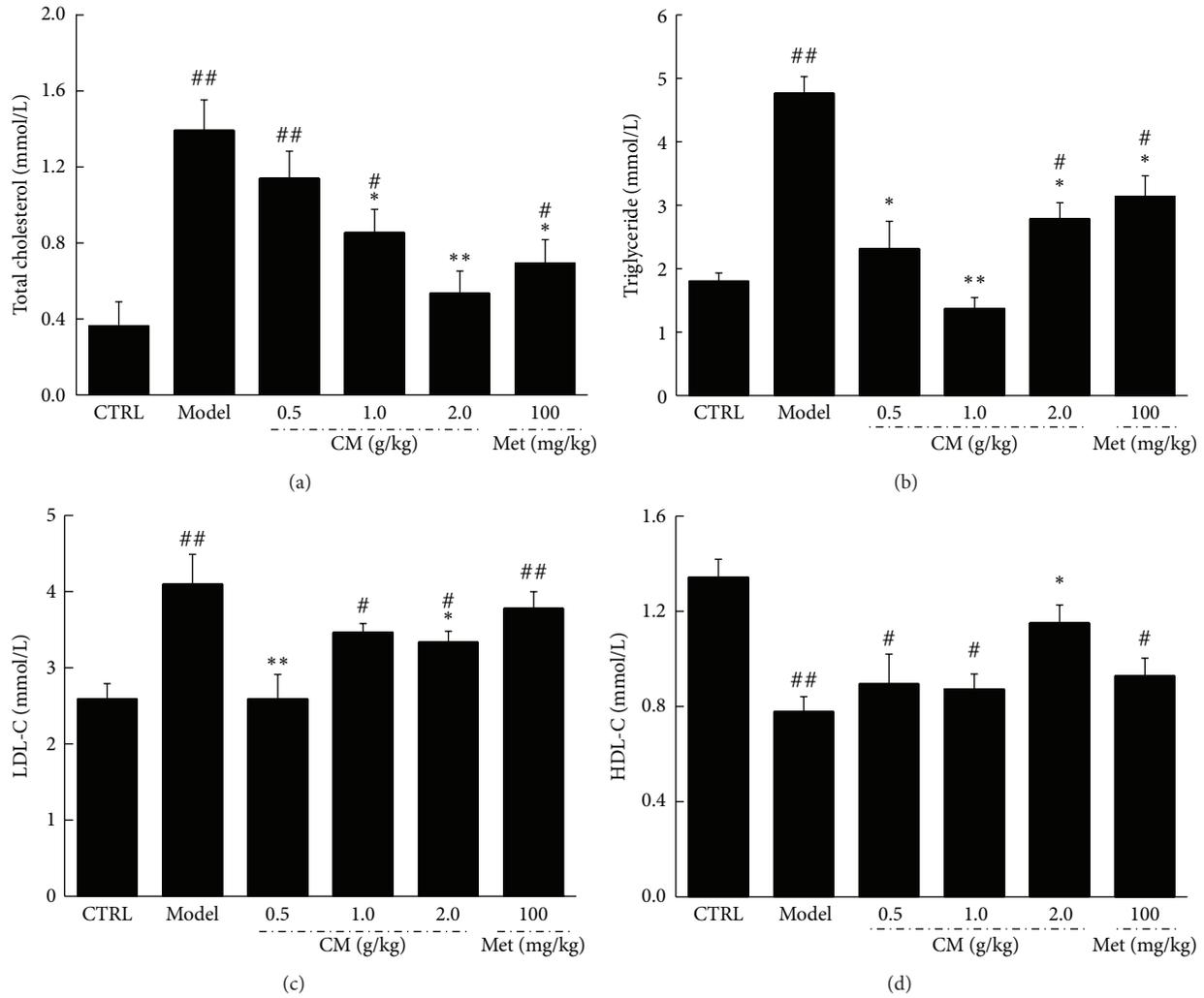


FIGURE 2: After four-week Met and CM treatment, the serum levels of total cholesterol (a), triglyceride (b), LDL-C (c), and HDL-C (d) in diet-STZ-induced diabetic rats were detected. Data are expressed as mean \pm SEM ($n = 6$) and analyzed using one-way ANOVA. # $P < 0.05$ and ## $P < 0.01$ versus control. * $P < 0.05$ and ** $P < 0.01$ versus model group.

TABLE 3: The effects of CM and Met on the levels of Scr, BUN, and albuminuria in serum and NAG in urine of diabetic rats.

		Scr ($\mu\text{mol/L}$)	BUN (mmol/L)	Albuminuria (mg/mL)	NAG (U/L)
CTRL	—	143.8 \pm 31.3	5.1 \pm 0.5	0.9 \pm 0.05	30.2 \pm 6.4
Model	—	338.8 \pm 32.3 ^{##}	10.2 \pm 0.7 ^{##}	2.6 \pm 0.3 ^{##}	75.7 \pm 7.7 ^{##}
CM (g/kg)	0.5	328.4 \pm 22.2 ^{##}	9.2 \pm 1.0 ^{##}	2.0 \pm 0.3 ^{##}	60.8 \pm 7.5 ^{##}
	1.0	228.5 \pm 54.6 ^{##*}	8.2 \pm 0.5 ^{##*}	1.8 \pm 0.2 ^{##*}	68.9 \pm 8.1 ^{##}
	2.0	226.3 \pm 32.1 ^{##*}	8.6 \pm 1.2 ^{##}	1.9 \pm 0.3 ^{##}	73.2 \pm 9.0 ^{##}
Met (mg/kg)	100	288.5 \pm 74.9 ^{##}	9.3 \pm 1.1 ^{##}	2.0 \pm 0.3 ^{##}	62.9 \pm 9.1 ^{##}

Data are expressed as mean \pm SEM ($n = 6$) and analyzed using one-way ANOVA. # $P < 0.05$ and ## $P < 0.01$ versus normal controls. * $P < 0.05$ and ** $P < 0.01$ versus model group.

3.4. Antioxidative Effects in Diabetic Rats. Oxidative stress underlies the development of T2DM and related complications [20]. Overproduction of intracellular ROS leads to oxidative stress and deleterious effects on tissues; however, antioxidant enzymes including GSH-Px and SOD prevent oxidative injury. The accumulation of ROS and MDA and low GSH-Px and SOD activity were noted in the serum and/or

kidneys of diabetic rats ($P < 0.05$, Table 4). CM enhanced GSH-Px and SOD activity and reduced the serum and kidney levels of ROS and MDA ($P < 0.05$, Table 4). Importantly, CM (2.0 g/kg) decreased ROS production in the kidneys by 12.4% ($P < 0.05$, Table 4). Met was also seen to modulate the oxidative factors in the serum and kidneys of diabetic rats ($P < 0.05$, Table 4).

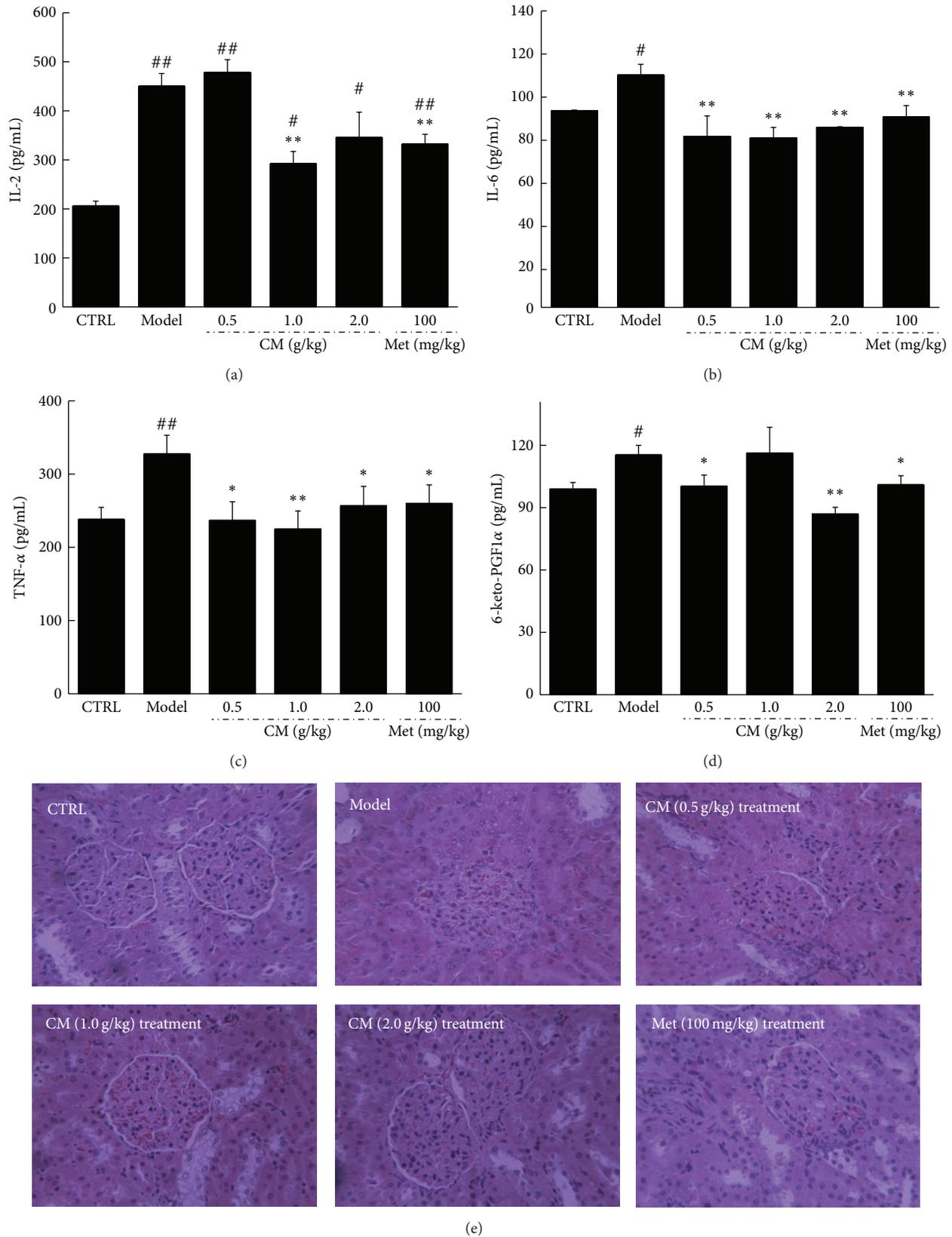


FIGURE 3: Diet-STZ-induced diabetic rats were orally treated with or without Met and CM at indicated doses for four weeks. The levels of IL-2 (a), IL-6 (b), TNF- α (c), and 6-keto-PGF (d) in serum were detected via ELISA method and histopathological changes in kidney collected from all experimental rats were observed through H&E staining ($n = 6, \times 400$) (e). Data are expressed as mean \pm SEM ($n = 6$) and analyzed using one-way ANOVA. # $P < 0.05$ and ## $P < 0.01$ versus control. * $P < 0.05$ and ** $P < 0.01$ versus model group.

TABLE 4: The regulatory effects of CM and Met on the oxidation related factors in serum and kidney of diabetic rats.

		CTRL	Model	CM (g/kg)			Met (mg/kg)
				0.5	1.0	2.0	100
Serum	SOD (U/mL)	245 ± 15	201 ± 12 [#]	223 ± 11	248 ± 16 ^{**}	237 ± 17 [*]	215 ± 17
	MDA (nmol/mL)	8.7 ± 0.8	28.0 ± 0.7 ^{##}	21.9 ± 2.7 ^{##}	12.4 ± 2.7 ^{***}	10.7 ± 1.8 ^{**}	15.0 ± 2.5 ^{***}
	GSH-Px (μmol/L)	1116 ± 41	944 ± 37 [#]	1000 ± 51	1053 ± 25 [*]	994 ± 33 [#]	994 ± 9 [#]
Kidney	SOD (U/mgprot)	134 ± 14	84 ± 11 ^{##}	110 ± 18 [#]	122 ± 13 ^{**}	105 ± 14 [#]	111 ± 11 ^{**}
	MDA (nmol/mgprot)	7.0 ± 1.4	11.3 ± 2.6 ^{##}	11.2 ± 2.7 ^{##}	8.2 ± 1.2 [*]	9.3 ± 2.0 [#]	8.5 ± 1.5 [*]
	GSH-Px (μmol/gprot)	6987 ± 318	4925 ± 402 ^{##}	5236 ± 210 [#]	6012 ± 462 [*]	5985 ± 433 [*]	5784 ± 223 ^{##}
	ROS (FI/gprot)	815 ± 32	988 ± 55 [#]	903 ± 31	895 ± 28 [*]	865 ± 48 [*]	875 ± 36 [*]

Data are expressed as mean ± SEM ($n = 6$) and analyzed using one-way ANOVA. [#] $P < 0.05$ and ^{##} $P < 0.01$ versus normal controls. ^{*} $P < 0.05$ and ^{**} $P < 0.01$ versus model group.

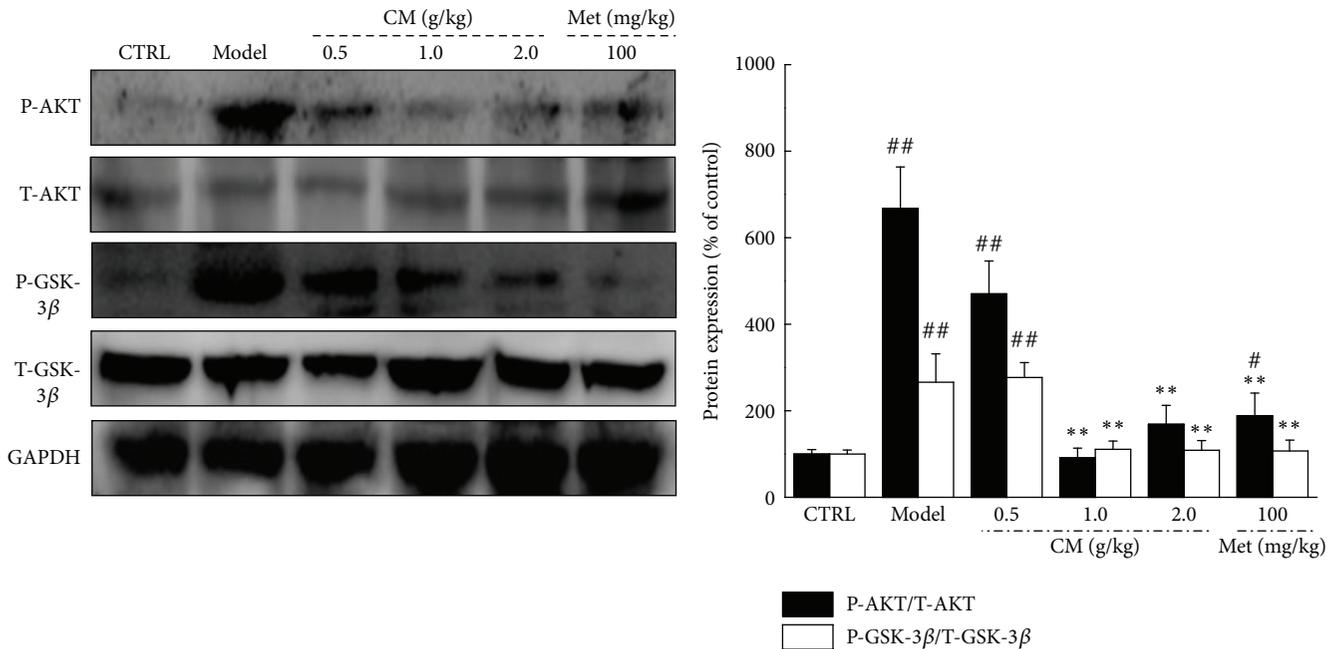


FIGURE 4: The expressions of T-AKT, P-AKT, T-GSK-3 β , and P-GSK-3 β in kidney were analyzed via western blot. Quantification data of the expression of P-AKT and P-GSK-3 β were normalized by corresponding T-AKT and T-GSK-3 β , respectively. Data are expressed as mean ± SEM ($n = 6$) and analyzed using one-way ANOVA. [#] $P < 0.05$ and ^{##} $P < 0.01$ versus control. ^{**} $P < 0.01$ versus model group.

3.5. Activation of AKT/GSK-3 β in Kidneys. The expression of P-AKT and P-GSK-3 β in the kidneys of diet-STZ-induced diabetic rats was significantly restored to normal levels after four weeks of CM and Met administration ($P < 0.01$, Figure 4).

4. Discussion

The HFHSD-STZ-induced diabetic rat model is closely analogous to the clinical situation of type 2 diabetes mellitus in humans [21]. Combined with the reduction in high fasting blood glucose levels, the modulation of OGTT, a more sensitive measure of early abnormality in glucose regulation [22], further verifies the hypoglycemic activity of CM. Abnormal changes in glucose metabolism are observed in diabetic patients, including decreased glycolysis, impeded glycogenesis, and increased gluconeogenesis [23]. Pyruvate

kinase is a key glycolytic enzyme for promoting glucose metabolism and energy production [24]. All of the data support the antidiabetic activity of CM in the diet-STZ-induced diabetic rat model.

Although the pathogenesis of T2DM-induced renal damage is multiple and complicated, dyslipidemia and subsequent lipotoxicity play important roles in this process and accelerate kidney injury. Dyslipidemia, defined as abnormal lipid profiles characterized by increased plasma and tissue levels of TG, TC, and LDL [21], is a major complication associated with high morbidity and mortality in diabetics [25]. Diabetes-related dyslipidemia is responsible for lipid accumulation in the kidney, which leads to insulin resistance, inflammation, and oxidative stress [26]. Gradually, insulin resistance results in the release of adipocytokines and relaxation of the afferent arteriole, finally causing glomerular hyperfiltration, angiogenesis, and mesangial cell proliferation [27, 28].

The antilipemic effect of CM plays an important role in renal protection in HFHSD-STZ-induced diabetic rats.

Oxidative stress has been singled out as a major cause of diabetic complications, especially nephropathy [29]. $O_2^{\cdot-}$ and nitric oxide (NO) levels are important in kidney and vascular function [30]. ROS, which is responsible for oxidative damage, degrades membrane polyunsaturated fatty acids through sequential peroxidation processes [31] and elevates MDA levels, which serve as biomarkers of tissue oxidative stress [32]. Excessive generation of ROS and MDA in the kidneys leads to tubular obstruction, back-leakage of renal tubules, and contraction of the mesangial cells, finally resulting in the abnormal expression of renal function markers such as Scr, BUN, albuminuria, and NAG [33]. However, cells defend themselves against oxidative stress via the activation of antioxidant enzymes. Antioxidant compounds are a common and effective way to prevent or inhibit pancreatic beta-cell destruction caused by alloxan [34]. SOD catalyzes the conversion of superoxides into hydrogen peroxide and oxygen, while GSH-Px scavenges the hydroxyl radicals [35]. The enhanced activity of SOD and GSH-Px in the serum and kidneys of CM-treated diabetic rats helps to maintain a balance of oxidants and antioxidants by causing the excretion of ROS. Therefore, CM improves renal function by scavenging free radicals, especially ROS and MDA. It is well known that inhibition of AKT phosphorylation downregulates GSK-3 β phosphorylation [36]. Gardenamide A is reported to attenuate ROS levels by promoting the phosphorylation of AKT, an effect that can be completely abrogated by the AKT inhibitor [37]. In CM-treated diet-STZ-reduced diabetic rats, decreased AKT and GSK-3 β phosphorylation is responsible for the transcriptional expression of multiple antioxidants to prevent diabetes-related oxidative damage.

Oxidative stress in T2DM favors the appearance of endothelial dysfunction, and oxidative production is an important step in inflammation [38]. Interleukins have important roles during inflammatory development, and the overexpression of IL-2 activates proinflammatory CD4+ T cells, exacerbating the glomerular damage by recruiting macrophages and neutrophils [39]. IL-6, secreted by the glomerular membrane system, is responsible for the proliferation of mesangial cells and the release of inflammatory mediators, including superoxide anions [40]. As reported, TNF- α upregulates IL-6 release by the podocytes in coculture with glomerular endothelial cells [41]. Previous studies have reported that oxidative stress is mediated in podocyte apoptosis in the process of diabetic nephropathy [42] and that the progression of renal interstitial fibrosis can be inhibited by suppressing oxidative stress [43]. Thus, CM exerts renal protection in diabetic rats via the regulation of inflammatory factors that are modulated by oxidative stress.

All of the data suggest that CM targets many molecules in the signaling of hyperglycemia, inflammation, and oxidative stress. This “systemic targeting” will completely eliminate the symptoms of diabetes and diabetic nephropathy in a much natural way, so that less adverse effect is expected. As a folk tonic food in China, CM has been emphasizing its safety with few adverse effects. Our subchronic toxic

test provides experimental basis for its safety indicating that CM showed no influences on bodyweights (Table 1S), organ indexes (Table 2S), and kidney structures in mice (Figure 2S). On the other hand, the crude drug nature of CM suggests multieffective components, which may show synergistic effect on the disease. It may explain that non-dose-dependent manner was the common way of action of some herbal medicines. Amount of natural productions is reported to show various pharmacological activities via non-dose-dependent manner [44, 45].

There is still a limitation in our present study. Although we confirmed the regulatory effects of Met and CM on inflammatory factors in serum, we failed to detect the related changes in kidney tissues. As reported, Met successfully regulates inflammatory cytokines associated with nephritis but shows no influences on kidney structure [46]. Our further study will focus on the effects of drugs on biochemical indices and pathological changes of kidney.

In summary, we successfully explored the antidiabetic and antinephritic effects of CM in diet-STZ-induced diabetic rats. During the experiment, CM exhibited the ability to reduce blood glucose, decrease blood lipids, reduce renal injury, and lower inflammatory factors through enhanced antioxidant expression and the attenuation of oxidative stress. *Cordyceps militaris* fruit body extract, a safe pharmaceutical agent, thus has great potential as a new treatment for diabetic patients, especially those with nephritis.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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References

- [1] S. M. Kabadi, B. K. Lee, and L. Liu, “Joint effects of obesity and vitamin D insufficiency on insulin resistance and type 2 diabetes: results from the NHANES 2001–2006,” *Diabetes Care*, vol. 35, no. 10, pp. 2048–2054, 2012.
- [2] Q. You, F. Chen, X. Wang, P. G. Luo, and Y. Jiang, “Inhibitory effects of muscadine anthocyanins on α -glucosidase and pancreatic lipase activities,” *Journal of Agricultural and Food Chemistry*, vol. 59, no. 17, pp. 9506–9511, 2011.
- [3] W. Kerner and J. Bruckel, “Definition, classification and diagnosis of diabetes mellitus,” *Experimental and Clinical Endocrinology & Diabetes*, vol. 122, no. 7, pp. 384–386, 2014.
- [4] G. Winkler, T. Hidvégi, G. Vándorfi, and G. Jermendy, “Risk-stratified screening for diabetes in adult subjects: results of the first investigation in Hungary,” *Orvosi Hetilap*, vol. 151, no. 17, pp. 691–696, 2010.

- [5] C. Solis-Herrera, C. L. Triplitt, and J. L. Lynch, "Nephropathy in youth and young adults with type 2 diabetes," *Current Diabetes Reports*, vol. 14, no. 2, p. 456, 2014.
- [6] K. Zhu, T. Takechi, M. Matsumoto et al., "NADPH oxidase NOX1 is involved in activation of protein kinase C and premature senescence in early stage diabetic kidney," *Free Radical Biology and Medicine*, vol. 83, pp. 21–30, 2015.
- [7] G. L. Bakris, "Recognition, pathogenesis, and treatment of different stages of nephropathy in patients with type 2 diabetes mellitus," *Mayo Clinic Proceedings*, vol. 86, no. 5, pp. 444–456, 2011.
- [8] J. Gilg, R. Pruthi, and D. Fogarty, "UK renal registry 17th annual report: chapter 1 UK renal replacement therapy incidence in 2013: national and centre-specific analyses," *Nephron*, vol. 129, supplement 1, pp. 1–29, 2015.
- [9] D. S. Kania, J. D. Gonzalvo, and Z. A. Weber, "Saxagliptin: a clinical review in the treatment of type 2 diabetes mellitus," *Clinical Therapeutics*, vol. 33, no. 8, pp. 1005–1022, 2011.
- [10] A. J. Scheen, "Antidiabetic agents in subjects with mild dysglycaemia: prevention or early treatment of type 2 diabetes?" *Diabetes and Metabolism*, vol. 33, no. 1, pp. 3–12, 2007.
- [11] T. Salihu Shinkafi, L. Bello, S. Wara Hassan, and S. Ali, "An ethnobotanical survey of antidiabetic plants used by Hausa-Fulani tribes in Sokoto, Northwest Nigeria," *Journal of Ethnopharmacology*, vol. 172, pp. 91–99, 2015.
- [12] J. Singh, E. Cumming, G. Manoharan, H. Kalasz, and E. Adeghate, "Medicinal chemistry of the anti-diabetic effects of *Momordica charantia*: active constituents and modes of actions," *Open Medicinal Chemistry Journal*, vol. 5, supplement 2, pp. 70–77, 2011.
- [13] S. K. Das, M. Masuda, A. Sakurai, and M. Sakakibara, "Medicinal uses of the mushroom *Cordyceps militaris*: current state and prospects," *Fitoterapia*, vol. 81, no. 8, pp. 961–968, 2010.
- [14] Y. Dong, T. Jing, Q. Meng et al., "Studies on the antidiabetic activities of *Cordyceps militaris* extract in diet-streptozotocin-induced diabetic Sprague-Dawley rats," *BioMed Research International*, vol. 2014, Article ID 160980, 11 pages, 2014.
- [15] Y.-W. Cheng, Y.-I. Chen, C.-Y. Tzeng et al., "Extracts of *Cordyceps militaris* lower blood glucose via the stimulation of cholinergic activation and insulin secretion in normal rats," *Phytotherapy Research*, vol. 26, no. 8, pp. 1173–1177, 2012.
- [16] C.-H. Ma, L.-L. Kang, H.-M. Ren, D.-M. Zhang, and L.-D. Kong, "Simiao pill ameliorates renal glomerular injury via increasing Sirt1 expression and suppressing NF- κ B/NLRP3 inflammasome activation in high fructose-fed rats," *Journal of Ethnopharmacology*, vol. 172, pp. 108–117, 2015.
- [17] Y. Dong, T. Jing, Q. Meng et al., "Studies on the antidiabetic activities of cordyceps militaris extract in diet-streptozotocin-induced diabetic sprague-dawley rats," *BioMed Research International*, vol. 2014, Article ID 160980, 11 pages, 2014.
- [18] S. O. Adewole, T. Adenowo, T. Naicker, and J. A. Ojewole, "Hypoglycaemic and hypotensive effects of *Ficus exasperata* vahl. (Moraceae) leaf aqueous extract in rats," *The African Journal of Traditional, Complementary and Alternative Medicines*, vol. 8, no. 3, pp. 275–283, 2011.
- [19] M. E. Williams, "Diabetic nephropathy: the proteinuria hypothesis," *American Journal of Nephrology*, vol. 25, no. 2, pp. 77–94, 2005.
- [20] S. Tangvarasittichai, "Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus," *World Journal of Diabetes*, vol. 6, no. 3, pp. 456–480, 2015.
- [21] M. Shao, X. Lu, W. Cong et al., "Multiple low-dose radiation prevents type 2 diabetes-induced renal damage through attenuation of dyslipidemia and insulin resistance and subsequent renal inflammation and oxidative stress," *PLoS ONE*, vol. 9, no. 3, Article ID e92574, 2014.
- [22] B. A. Sheikh, L. Pari, A. Rathinam, and R. Chandramohan, "Trans-anethole, a terpenoid ameliorates hyperglycemia by regulating key enzymes of carbohydrate metabolism in streptozotocin induced diabetic rats," *Biochimie*, vol. 112, pp. 57–65, 2015.
- [23] B. Huang, Z. Wang, J. H. Park et al., "Anti-diabetic effect of purple corn extract on C57BL/KsJ db/db mice," *Nutrition Research and Practice*, vol. 9, no. 1, pp. 22–29, 2015.
- [24] H. Wang, W. Chu, S. K. Das, Q. Ren, S. J. Hasstedt, and S. C. Elbein, "Liver pyruvate kinase polymorphisms are associated with type 2 diabetes in northern European Caucasians," *Diabetes*, vol. 51, no. 9, pp. 2861–2865, 2002.
- [25] N. K. Sambu, R. T. Kashinath, and J. G. Ambekar, "Effect of diallyl disulphide on diabetes induced dyslipidemia in male albino rats," *Journal of Clinical and Diagnostic Research*, vol. 9, no. 4, pp. BF01–BF03, 2015.
- [26] I. M. Wahba and R. H. Mak, "Obesity and obesity-initiated metabolic syndrome: mechanistic links to chronic kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 2, no. 3, pp. 550–562, 2007.
- [27] A. W. Miller, C. Dimitropoulou, G. Han, R. E. White, D. W. Busija, and G. O. Carrier, "Epoxyeicosatrienoic acid-induced relaxation is impaired in insulin resistance," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 281, no. 4, pp. H1524–H1531, 2001.
- [28] L. Piqueras, A. R. Reynolds, K. M. Hodivala-Dilke et al., "Activation of PPAR β/δ induces endothelial cell proliferation and angiogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 1, pp. 63–69, 2007.
- [29] A. P. Rolo and C. M. Palmeira, "Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress," *Toxicology and Applied Pharmacology*, vol. 212, no. 2, pp. 167–178, 2006.
- [30] A. Ragheb, A. Attia, W. S. Eldin, F. Elbarbry, S. Gazarin, and A. Shoker, "The protective effect of thymoquinone, an anti-oxidant and anti-inflammatory agent, against renal injury: a review," *Saudi Journal of Kidney Diseases and Transplantation*, vol. 20, no. 5, pp. 741–752, 2009.
- [31] O. R. Ayepola, M. E. Cerf, N. L. Brooks, and O. O. Oguntibeju, "Kolaviron, a biflavonoid complex of *Garcinia kola* seeds modulates apoptosis by suppressing oxidative stress and inflammation in diabetes-induced nephrotoxic rats," *Phytomedicine*, vol. 21, no. 14, pp. 1785–1793, 2014.
- [32] A. Goel, V. Dani, and D. K. Dhawan, "Protective effects of zinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture in chlorpyrifos-induced toxicity," *Chemico-Biological Interactions*, vol. 156, no. 2–3, pp. 131–140, 2005.
- [33] A. Y. Nasr and H. A. M. Saleh, "Aged garlic extract protects against oxidative stress and renal changes in cisplatin-treated adult male rats," *Cancer Cell International*, vol. 14, no. 1, article 92, 2014.
- [34] H. Sebai, S. Selmi, K. Rtibi, A. Souli, N. Gharbi, and M. Sakly, "Lavender (*Lavandula stoechas* L.) essential oils attenuate hyperglycemia and protect against oxidative stress in alloxan-induced diabetic rats," *Lipids in Health and Disease*, vol. 12, no. 1, article 189, 2013.
- [35] P. Borges, B. Oliveira, S. Casal, J. Dias, L. Conceição, and L. M. P. Valente, "Dietary lipid level affects growth performance

- and nutrient utilisation of Senegalese sole (*Solea senegalensis*) juveniles,” *British Journal of Nutrition*, vol. 102, no. 7, pp. 1007–1014, 2009.
- [36] C. Zhang, X. Lu, Y. Tan et al., “Diabetes-induced hepatic pathogenic damage, inflammation, oxidative stress, and insulin resistance was exacerbated in zinc deficient mouse model,” *PLoS ONE*, vol. 7, no. 12, Article ID e49257, 2012.
- [37] R. Wang, L. Peng, J. Zhao et al., “Gardenamide a protects RGC-5 cells from H₂O₂-induced oxidative stress insults by activating PI3K/Akt/eNOS signaling pathway,” *International Journal of Molecular Sciences*, vol. 16, no. 9, pp. 22350–22367, 2015.
- [38] A. Ceriello, A. Novials, E. Ortega et al., “Vitamin C further improves the protective effect of glucagon-like peptide-1 on acute hypoglycemia-induced oxidative stress, inflammation, and endothelial dysfunction in type 1 diabetes,” *Diabetes Care*, vol. 36, no. 12, pp. 4104–4108, 2013.
- [39] R. Bertelli, A. Di Donato, M. Cioni et al., “LPS nephropathy in mice is ameliorated by IL-2 independently of regulatory T cells activity,” *PLoS ONE*, vol. 9, no. 10, Article ID e111285, 2014.
- [40] L. Ma, Y. Gao, G. Chen et al., “Relationships of urinary VEGF/CR and IL-6/CR with glomerular pathological injury in asymptomatic hematuria patients,” *Medical Science Monitor*, vol. 21, pp. 356–362, 2015.
- [41] S. J. Kuravi, H. M. McGettrick, S. C. Satchell et al., “Podocytes regulate neutrophil recruitment by glomerular endothelial cells via IL-6-mediated crosstalk,” *The Journal of Immunology*, vol. 193, no. 1, pp. 234–243, 2014.
- [42] W. Hua, H.-Z. Huang, L.-T. Tan et al., “CD36 mediated fatty acid-induced podocyte apoptosis via oxidative stress,” *PLoS ONE*, vol. 10, no. 5, Article ID e0127507, 2015.
- [43] J. Qin, W.-J. Mei, Y.-Y. Xie et al., “Fluorofenidone attenuates oxidative stress and renal fibrosis in obstructive nephropathy via blocking NOX2 (gp91phox) expression and inhibiting erk/mapk signaling pathway,” *Kidney and Blood Pressure Research*, vol. 40, no. 1, pp. 89–99, 2014.
- [44] J. Wei, S. Wang, G. Liu et al., “Polysaccharides from *Enteromorpha prolifera* enhance the immunity of normal mice,” *International Journal of Biological Macromolecules*, vol. 64, pp. 1–5, 2014.
- [45] L. Ma, S. Zhang, and M. Du, “Cordycepin from *Cordyceps militaris* prevents hyperglycemia in alloxan-induced diabetic mice,” *Nutrition Research*, vol. 35, no. 5, pp. 431–439, 2015.
- [46] B. D. Sahu, M. Kuncha, U. K. Putcha, and R. Sistla, “Effect of metformin against cisplatin induced acute renal injury in rats: a biochemical and histoarchitectural evaluation,” *Experimental and Toxicologic Pathology*, vol. 65, no. 6, pp. 933–940, 2013.

Research Article

The Regulatory Role of Nuclear Factor Kappa B in the Heart of Hereditary Hypertriglyceridemic Rat

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Activation of nuclear factor- κ B (NF- κ B) by increased production of reactive oxygen species (ROS) might induce transcription and expression of different antioxidant enzymes and also of nitric oxide synthase (NOS) isoforms. Thus, we aimed at studying the effect of NF- κ B inhibition, caused by JSH-23 (4-methyl-*N*¹-(3-phenyl-propyl)-benzene-1,2-diamine) injection, on ROS and NO generation in hereditary hypertriglyceridemic (HTG) rats. 12-week-old, male Wistar and HTG rats were treated with JSH-23 (bolus, 10 μ mol, i.v.). After one week, blood pressure (BP), superoxide dismutase (SOD) activity, SOD1, endothelial NOS (eNOS), and NF- κ B (p65) protein expressions were higher in the heart of HTG rats compared to control rats. On the other hand, NOS activity was decreased. In HTG rats, JSH-23 treatment increased BP and heart conjugated dienes (CD) concentration (measured as the marker of tissue oxidative damage). Concomitantly, SOD activity together with SOD1 expression was decreased, while NOS activity and eNOS protein expression were increased significantly. In conclusion, NF- κ B inhibition in HTG rats led to decreased ROS degradation by SOD followed by increased oxidative damage in the heart and BP elevation. In these conditions, increased NO generation may represent rather a counterregulatory mechanism activated by ROS. Nevertheless, this mechanism was not sufficient enough to compensate BP increase in HTG rats.

1. Introduction

Increase in cell production of reactive oxygen species (ROS) leads to activation of intracellular signaling pathways, which in turn induce transcriptional changes that enable a cell to activate expression of a number of genes encoding antioxidant proteins, DNA repair proteins, stress-regulated chaperones, and antiapoptotic proteins. These genes are generally regulated by transcription factors whose structure, subcellular localization, or affinity for DNA is directly or indirectly regulated by the level of oxidative stress [1]. In such a way, ROS may serve as messenger molecules to activate adaptive responses, such as redox-sensitive nuclear factor kappa B (NF- κ B) signaling, which enhance gene expression of antioxidant enzymes in oxidatively stressed tissue [2, 3]. In

addition, NF- κ B may participate in regulation of nitric oxide synthase (NOS) isoforms expression including eNOS [4, 5].

The transcription factor NF- κ B has been shown to be cardioprotective after permanent coronary occlusion and late ischemic preconditioning. However, cell injurious effect of this factor after ischemia/reperfusion was shown in the heart as well. Tranter et al. identified 16 NF- κ B dependent cardioprotective genes that might contribute to understanding the mechanism of NF- κ B-induced myocardial salvage after permanent coronary occlusion [6].

NF- κ B belongs to the Rel family of transcriptional activator proteins and it exerts a variety of actions [7]. Sen et al. have found that NF- κ B responds directly to oxidative stress and its activation is controlled by the cell glutathione disulphide/glutathione (GSSG/GSH) ratio [8]. On the other

hand, *in vitro* inhibition of the transcriptional activity of NF- κ B may lead to accumulation of reactive oxygen species following oxidative damage [9]. The contradictory findings on the role of NF- κ B signaling reflect the diversity of cellular processes on molecular level and should be taken into account in different therapeutic settings.

It is evident that the model of nonobese hereditary hypertriglyceridemic (HTG) rats selected from the Wistar strain [10] represents a suitable model for the study of metabolic disturbances in relation to blood pressure as well as in the search for genetic determinants of these abnormalities [11]. Hereditary HTG rats exhibit insulin resistance, hyperinsulinemia, disturbances in glucose metabolism, hypertension, and different signs of oxidative stress, for example, increased lipoprotein oxidability and lipid peroxidation [12]. According to our knowledge, the role of NF- κ B signaling in response to increased oxidative damage in HTG rats was not studied as yet.

Shin et al. [13] showed that aromatic diamine, 4-methyl-N¹-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23), had an inhibitory effect on NF- κ B transcriptional activity in lipopolysaccharide- (LPS-) stimulated macrophages RAW 264.7. JSH-23 had inhibitory effects, in parallel, on LPS-induced DNA binding activity and nuclear translocation of NF- κ B p65. However, the compound JSH-23 did not influence LPS-induced inhibitory kappa B alpha protein (κ B α) degradation. These results indicate that the JSH-23 could inhibit nuclear translocation of NF- κ B p65 without affecting κ B α degradation, which is a very rare mode of action, lending JSH-23 a specific character of NF- κ B inhibition.

In this study we investigated the effect of NF- κ B inhibition (caused by JSH-23 injection) on heart reactive oxygen species level, superoxide dismutase and nitric oxide synthase activities, and blood pressure regulation in hereditary hypertriglyceridemic rats.

2. Material and Methods

2.1. Animals and Treatment. Male 12-week-old normotensive Wistar rats and Prague hereditary hypertriglyceridemic (HTG) rats (bred in the Institute of Physiology AS CR, Prague) were used in this study. All animals were kept under standard laboratory conditions (12 h light, 12 h darkness, 23 \pm 1°C, pelleted ST-1 diet, drinking *ad libitum*). All procedures and experimental protocols were approved by the Animal Care Ethical Committee of the Institute of Physiology AS CR in Prague and conformed to the European Convention on Animal Protection and Guidelines on Research Animal Use. Adult 12-week-old Wistar ($n = 18$) and HTG ($n = 18$) rats were included in the study. Nine Wistar rats and 9 HTG rats were taken as controls, whereas the remaining rats (9 Wistar and 9 HTG) were injected with JSH-23 (bolus, 10 μ mol, i.v.).

At the end of the experiment, one week after JSH-23 injection, blood pressure was measured by a direct puncture of the carotid artery under light ether anesthesia. Heart was dissected and left ventricle (LV) was taken for determination of biochemical parameters.

2.2. Biochemical Parameters. The concentration of conjugated dienes (CD) was measured in lipid extracts of heart homogenates [14]. After chloroform evaporation under inert atmosphere and addition of cyclohexane, conjugated diene concentrations were determined spectrophotometrically ($\lambda = 233$ nm, GBC 911A, Bio-Rad Laboratories).

Reduced glutathione (GSH) level was determined according to Ellman [15]. Samples of LV were homogenized in 1 mL of ice-cold 3% sulphosalicylic acid and, after centrifugation at 3,000 \times g for 5 min, GSH concentration was determined spectrophotometrically in the acid-soluble fractions ($\lambda = 412$ nm, GBC 911A, Bio-Rad Laboratories).

Total NO synthase (NOS) activity was determined in crude LV homogenates by measuring L-[³H]citrulline formation from L-[³H]arginine (Amersham, UK) as previously described by Bredt and Snyder [16] with minor modifications [17, 18].

Superoxide dismutase (SOD) activity was analyzed in LV homogenates spectrophotometrically using the SOD assay kit (Fluka, Switzerland). The absorbance was measured at 450 nm using a microplate reader (Thermo Scientific Multiscan FC, Finland). SOD activity was expressed in U/mg of protein in the tissues.

For Western blot analysis, samples of the LV were used and probed with polyclonal rabbit anti-eNOS (Santa Cruz Biotechnology, USA), anti-SOD1 (Santa Cruz Biotechnology, USA), anti-NF κ B (p65) (BioLegend, USA), and anti- β -actin (Santa Cruz Biotechnology, USA) antibodies.

2.3. Statistical Analysis. Results are expressed as means \pm SEM. One-way ANOVA and Bonferroni test were used for statistical analysis. $p < 0.05$ value was considered statistically significant.

3. Results

3.1. Biometric Parameters. At the end of experiment, mean blood pressure of control HTG rats was significantly increased (by 40%) in comparison with control Wistar rats. After JSH-23 treatment, blood pressure was increased significantly only in HTG rats compared with age-matched untreated rats (Table 1).

HTG rats had lower body weight and heart weight in comparison with normotensive Wistar rats. In HTG rats, HW/BW ratio was significantly higher than in Wistar rats. JSH-23 administration did not affect body weight, heart weight, and relative heart weight of Wistar as well as HTG rats (Table 1).

3.2. Biochemical Parameters

3.2.1. NF- κ B (p65) Expression and CD and GSH Concentration. The protein expression of NF- κ B (subunit p65) was significantly higher in HTG rats than in Wistar rats. JSH-23 treatment had no effect on NF- κ B protein expression in HTG as well as Wistar rats (Figure 1(a)).

The levels of conjugated dienes were increased significantly in HTG rats as compared to Wistar rats. JSH-23

TABLE 1: Body weight, heart weight, relative heart weight, and mean arterial pressure of Wistar rats. Wistar rats treated with JSH-23, hereditary hypertriglyceridemic (HTG) rats, and hereditary hypertriglyceridemic rats treated with JSH-23.

	BW (g)	HW (mg)	HW/BW mg/100 g	MAP (mmHg)
Wistar	435 ± 4	1074 ± 24	246 ± 4	95 ± 2
Wistar + JSH-23	426 ± 5	1039 ± 28	244 ± 4	98 ± 4
HTG	328 ± 6 ⁺	890 ± 32 ⁺	271 ± 7 ⁺	133 ± 4 ⁺
HTG + JSH-23	344 ± 10	965 ± 23	280 ± 3	144 ± 3 [*]

BW, body weight; HW, heart weight; HTG, hypertriglyceridemic; HW/BW, heart weight to body weight ratio; JSH-23, 4-methyl-*N*¹-(3-phenyl-propyl)-benzene-1,2-diamine; MAP, mean arterial pressure. Data are means ± SEM; significant differences ⁺*p* < 0.05 compared with Wistar rats; ^{*}*p* < 0.05 compared with control rats.

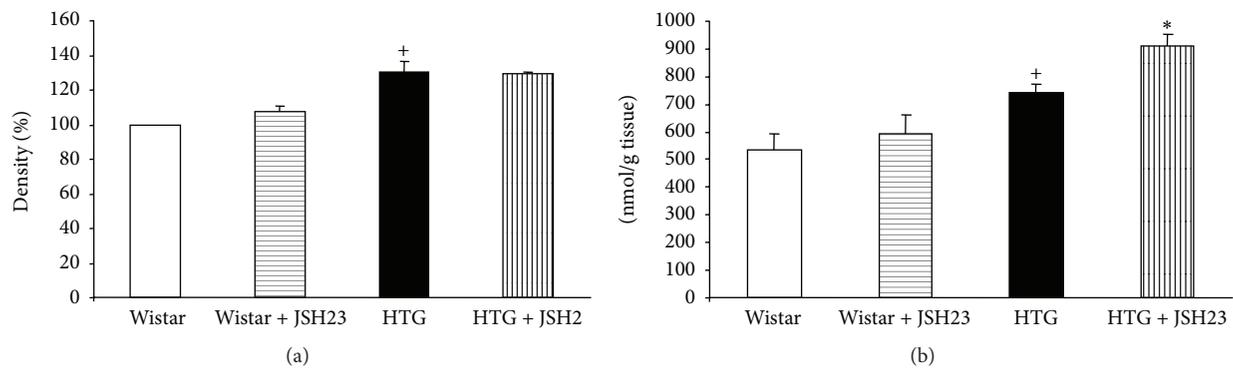


FIGURE 1: NF- κ B protein expression (a) and conjugated diene concentration (b) in the heart. HTG, hypertriglyceridemic; JSH-23, 4-methyl-*N*¹-(3-phenyl-propyl)-benzene-1,2-diamine. Data are means ± SEM (*n* = 9). ⁺*p* < 0.05 as compared to Wistar rats; ^{*}*p* < 0.05 as compared to controls.

increased concentration of conjugated dienes only in HTG rats (Figure 1(b)).

No significant changes in GSH levels were seen in HTG rats versus Wistar rats or in HTG rats and Wistar rats treated with JSH-23 (data not shown).

3.2.2. NOS Activity and eNOS Expression. Total NOS activity was significantly decreased in HTG rats in comparison with Wistar rats. JSH-23 treatment increased NOS activity in HTG rats and there were no significant changes in Wistar rats (Figure 2(a)).

Endothelial NO synthase (eNOS) protein expression was increased significantly in HTG rats. JSH-23 administration did not affect eNOS expression in control Wistar rats, but it was significantly increased in HTG rats (Figure 2(b)).

3.2.3. SOD Activity and SOD1 Expression. SOD activity was significantly increased in HTG rats in comparison with Wistar rats. However, SOD activity decreased significantly in HTG rats treated with JSH-23 as compared to untreated HTG rats. JSH-23 treatment had no significant effect on SOD activity in Wistar rats (Figure 3(a)).

Protein expression of SOD1 was increased in HTG rats (versus Wistar rats). JSH-23 attenuated the expression of SOD1 only in HTG rats (Figure 3(b)).

4. Discussion

In the present study, the aromatic diamine JSH-23 compound was used for the first time to inhibit NF- κ B transcriptional activity in the model of hereditary hypertriglyceridemic rats. In this model we detected increased BP and heart hypertrophy together with increased CD concentration, SOD activity, and SOD1, eNOS, and NF- κ B (p65) protein expressions. On the other hand, NOS activity was decreased significantly. NF- κ B inhibition led to additional increase in blood pressure and CD concentration, decrease in SOD1 expression and SOD activity, and, interestingly, increase in eNOS expression followed by elevated NOS activity. These results suggested rather regulatory than pathological role of NF- κ B in HTG rats.

Heart hypertrophy observed in HTG rats in our experiments is in accordance with previous studies, in which increase in blood pressure about 20–40 mmHg represents a hemodynamic overload that induced left ventricular hypertrophy [19]. The serious role of superoxides in blood pressure maintenance of moderately hypertensive HTG rats was demonstrated by Kuneš et al. using acute i.v. tempol administration in conscious animals [20]. Zicha et al. reported that the concentration of conjugated dienes, a marker of oxidative membrane damage, was significantly increased in the kidney

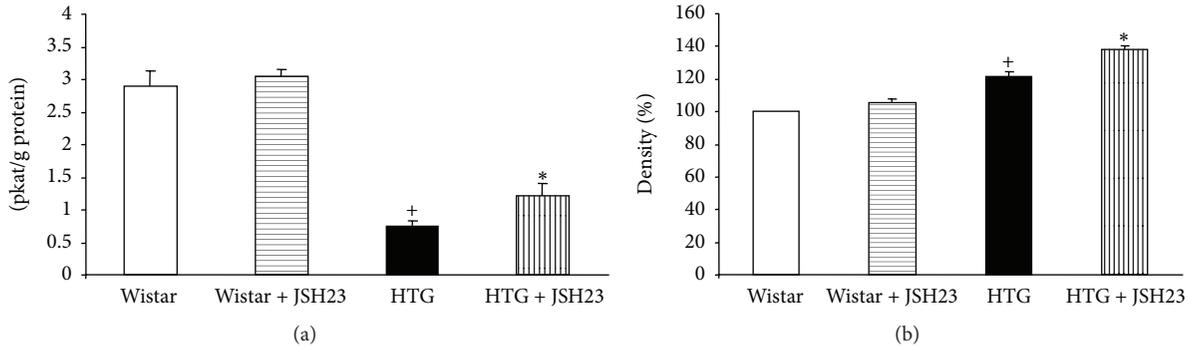


FIGURE 2: Total NOS activity (a) and endothelial NOS protein expression (b) in the heart. HTG, hypertriglyceridemic; JSH-23, 4-methyl- N^1 -(3-phenyl-propyl)-benzene-1,2-diamine. Data are means \pm SEM ($n = 9$). $^+p < 0.05$ as compared to Wistar rats; $^*p < 0.05$ as compared to controls.

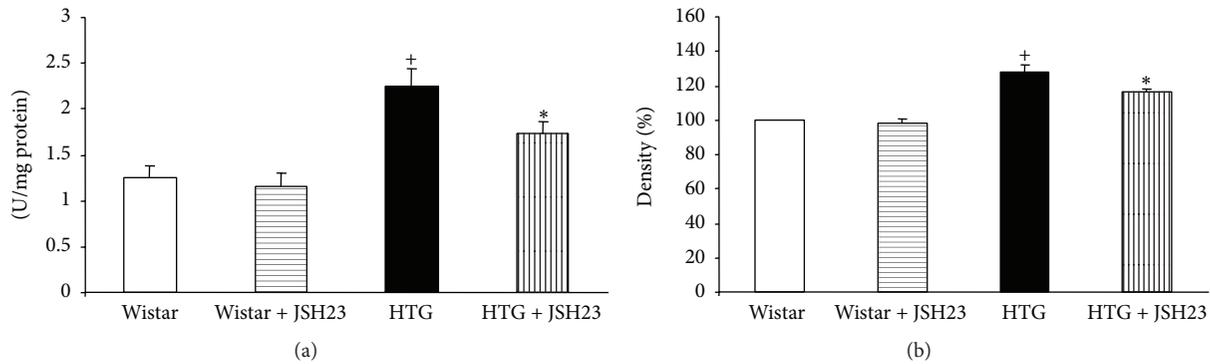


FIGURE 3: Superoxide dismutase activity (a) and SOD1 protein expression (b) in the heart. HTG, hypertriglyceridemic; JSH-23, 4-methyl- N^1 -(3-phenyl-propyl)-benzene-1,2-diamine. Data are means \pm SEM ($n = 9$). $^+p < 0.05$ as compared to Wistar rats; $^*p < 0.05$ as compared to controls.

of both 3- and 7-month-old HTG rats in comparison with Wistar rats. On the other hand, baseline GSH/GSSG ratio, as a marker of redox control, was significantly higher in 12-week-old HTG rats than in age-matched Wistar rats [11]. This increase was probably caused by activation of antioxidant mechanisms in hypertriglyceridemic animals, in which higher production of reactive oxygen species was documented [20, 21]. Oxidative stress manifestation was further enhanced by high-sucrose diet, as demonstrated by increased TBARS and conjugated diene concentration, decreased GSH levels, and decreased glutathione peroxidase activity in blood and liver of this respective animal model [22].

Increased level of reactive oxygen species may represent an initial step in the signal cascade of NF- κ B activation [2, 5]. Consequently, transcription factor NF- κ B enhances gene expression of antioxidant enzymes in oxidatively stressed tissue [2]. In our experiment, NF- κ B inhibition by JSH-23 showed decreased SOD1 expression and SOD activity together with increased CD concentration in the heart of HTG rats. On the other hand, GSH level was not affected by this treatment. Previously we have reported that chronic

NF- κ B inhibition with lactacystin also increased CD concentration in the heart of N^G -nitro-L-arginine methyl ester- (L-NAME-) treated rats [23]. Similarly, other authors showed that lactacystin treatment significantly increased oxidative protein damage (measured as the level of protein carbonyls), lipid peroxidation, and concentration of 3-nitrotyrosine in cell culture [24]. Higher levels of lactacystin increased the concentrations of 8-hydroxyguanine (a biomarker of oxidative DNA damage) and decreased GSH levels. Lactacystin treatment also decreased significantly activity of superoxide dismutase 1 and 2 [24]. Previously, Chen et al. documented accumulation of reactive oxygen species after inhibition of the NF- κ B transcriptional activity also [9].

It has been suggested that intracellular ROS overproduction may represent one of the causes leading to increased blood pressure in both experimental models and human hypertension [25–27]. Considering that the reactions between NO and superoxide anion are most likely a major cause of impaired endothelium dependent vasorelaxation in hypertension [28]. As mentioned above, a higher production of reactive oxygen species was also documented in HTG rats. In our study, NF- κ B inhibition by JSH-23 led to a slight but

significant blood pressure increase in HTG rats in comparison with untreated animals. We hypothesize that elevated production of ROS after NF- κ B inhibition might play an important role in blood pressure increase observed in this experimental model. Similarly, our recent results indicated that chronic NF- κ B inhibition with lactacystin increased blood pressure in L-NAME-treated rats [23]. Moreover, in this study, JSH-23 treatment increased total NOS activity in the heart of HTG rats. Similarly, lactacystin treatment led to increased production of nitric oxide, measured as levels of NO₂⁻ plus NO₃⁻ in cells [24]. Finally, we observed increased endothelial NO synthase expression in the heart of HTG rats treated with JSH-23. These changes could be explained as a compensatory mechanism activated due to the ROS and blood pressure increase.

While excessive amounts of ROS can be harmful within the cells [29], their increase to the regulatory level may trigger different signal transduction pathways [30, 31]. Dröge et al. [32] demonstrated that ROS elevated intracellular GSSG level and thereby acted indirectly on the signal cascade of NF- κ B activation, because NF- κ B activation requires an altered level of GSSG. Activated NF- κ B then enhances gene expression of antioxidant enzymes [2]. In our study, the inhibition of NF- κ B protein by JSH-23 caused inactivation of NF- κ B signaling, blocking presumably also the synthesis of antioxidants. Indeed, we detected decreased superoxide dismutase activity and SOD1 expression in the heart of HTG rats treated with JSH-23. Our present results confirm the role of NF- κ B signaling in gene expression of antioxidant enzymes in oxidatively stressed tissue. Cho et al. showed several possible mechanisms responsible for cellular ROS accumulation induced by NF- κ B inhibition. The most important ones involve impairment of the activities of antioxidant enzymes and glutathione depletion by NF- κ B inhibition [33]. Moreover, the production of antioxidant enzymes, such as ferritin heavy chain, manganese-dependent superoxide dismutase, and metallothionein, was found to exhibit NF- κ B-dependent manner [34]. A study by Cho et al. also reported that NF- κ B inhibition increased superoxide anion level and decreased GSH level in isolated human CD4⁺ T cells [33]. Reduced GSH is a major intracellular antioxidant and NF- κ B is the most important transcription factor, which induces the gene for glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis [35]. NF- κ B inhibitors, including pyrrolidine dithiocarbamate [36], parthenolide [37], gliotoxin [38], and proteasome inhibitor [24], were reported to cause GSH depletion. However, our present study did not confirm the decrease of GSH level after JSH-23 treatment. The unchanged GSH level along with increased eNOS protein expression after JSH-23 treatment in the heart may represent a compensatory mechanism activated due to ROS accumulation and blood pressure increase in HTG rats.

In conclusion, our data show that NF- κ B inhibition by JSH-23 leads to further increase of oxidative damage followed by increased blood pressure in the model of hereditary hyperglyceridemic rats. Under these conditions, increased NO production represents rather counterbalancing mechanism activated by blood pressure increase in this respective model.

Thus, NF- κ B inhibition under increased ROS level may not always have a beneficial effect in the heart.

Abbreviations

BP:	Blood pressure
CD:	Conjugated dienes
GSH:	Reduced glutathione
GSSG:	Glutathione disulphide
HTG:	Hypertriglyceridemic
JSH-23:	4-Methyl-N ¹ -(3-phenyl-propyl)-benzene-1,2-diamine
L-NAME:	N ^G -Nitro-L-arginine methyl ester
NF- κ B:	Nuclear factor κ B
NO:	Nitric oxide
NOS:	NO synthase
eNOS:	Endothelial NOS
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
TBARS:	Thiobarbituric Acid Reactive Substances.

Competing Interests

The authors declare that they have no competing interests.

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References

- [1] H. Liu, R. Colavitti, I. I. Rovira, and T. Finkel, "Redox-dependent transcriptional regulation," *Circulation Research*, vol. 97, no. 10, pp. 967–974, 2005.
- [2] M. Bar-Shai, E. Carmeli, P. Ljubuncic, and A. Z. Reznick, "Exercise and immobilization in aging animals: the involvement of oxidative stress and NF- κ B activation," *Free Radical Biology and Medicine*, vol. 44, no. 2, pp. 202–214, 2008.
- [3] J.-H. Kwak, J.-K. Jung, and H. Lee, "Nuclear factor-kappa B inhibitors; a patent review (2006–2010)," *Expert Opinion on Therapeutic Patents*, vol. 21, no. 12, pp. 1897–1910, 2011.
- [4] I. M. Grumbach, W. Chen, S. A. Mertens, and D. G. Harrison, "A negative feedback mechanism involving nitric oxide and nuclear factor kappa-B modulates endothelial nitric oxide synthase transcription," *Journal of Molecular and Cellular Cardiology*, vol. 39, no. 4, pp. 595–603, 2005.
- [5] M. J. Morgan and Z.-G. Liu, "Crosstalk of reactive oxygen species and NF- κ B signaling," *Cell Research*, vol. 21, no. 1, pp. 103–115, 2011.
- [6] M. Tranter, X. Ren, T. Forde et al., "NF- κ B driven cardioprotective gene programs; Hsp70.3 and cardioprotection after late ischemic preconditioning," *Journal of Molecular and Cellular Cardiology*, vol. 49, no. 4, pp. 664–672, 2010.
- [7] J. Napetschnig and H. Wu, "Molecular basis of NF- κ B signaling," *Annual Review of Biophysics*, vol. 42, no. 1, pp. 443–468, 2013.

- [8] C. K. Sen, S. Khanna, A. Z. Reznick, S. Roy, and L. Packer, "Glutathione regulation of tumor necrosis factor- α -induced NF- κ B activation in skeletal muscle-derived L6 cells," *Biochemical and Biophysical Research Communications*, vol. 237, no. 3, pp. 645–649, 1997.
- [9] F. Chen, V. Castranova, Z. Li, M. Karin, and X. Shi, "Inhibitor of nuclear factor kappa B kinase deficiency enhances oxidative stress and prolongs c-Jun NH2-terminal kinase activation induced by arsenic," *Cancer Research*, vol. 63, pp. 7689–7693, 2003.
- [10] A. Vrána and L. Kazdová, "The hereditary hypertriglyceridemic nonobese rat: an experimental model of human hypertriglyceridemia," *Transplantation Proceedings*, vol. 22, no. 6, p. 2579, 1990.
- [11] J. Zicha, O. Pecháňová, S. Čáčányiová et al., "Hereditary hypertriglyceridemic rat: a suitable model of cardiovascular disease and metabolic syndrome?" *Physiological Research*, vol. 55, supplement 1, pp. S49–S63, 2006.
- [12] L. Kazdová, A. Žák, and A. Vrána, "Increased lipoprotein oxidability and aortic lipid peroxidation in an experimental model of insulin resistance syndrome," *Annals of the New York Academy of Sciences*, vol. 827, pp. 521–525, 1997.
- [13] H.-M. Shin, M.-H. Kim, B. H. Kim et al., "Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF- κ B without affecting I κ B degradation," *FEBS Letters*, vol. 571, no. 1–3, pp. 50–54, 2004.
- [14] K. Kogure, B. D. Watson, R. Busto, and K. Abe, "Potentiation of lipid peroxides by ischemia in rat brain," *Neurochemical Research*, vol. 7, no. 4, pp. 437–454, 1982.
- [15] G. L. Ellman, "Tissue sulfhydryl groups," *Archives of Biochemistry and Biophysics*, vol. 82, no. 1, pp. 70–77, 1959.
- [16] D. S. Bredt and S. H. Snyder, "Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 2, pp. 682–685, 1990.
- [17] O. Pecháňová, I. Bernátová, V. Pelouch, and F. Šimko, "Protein remodelling of the heart in NO-deficient hypertension: the effect of captopril," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 12, pp. 3365–3374, 1997.
- [18] O. Pecháňová, J. Zicha, S. Kojšová, Z. Dobešová, L. Jendeková, and J. Kuneš, "Effect of chronic N-acetylcysteine treatment on the development of spontaneous hypertension," *Clinical Science*, vol. 110, no. 2, pp. 235–242, 2006.
- [19] F. Šimko, I. Luptak, J. Matuskova et al., "Heart remodeling in the hereditary hypertriglyceridemic rat: Effect of captopril and nitric oxide deficiency," *Annals of the New York Academy of Sciences*, vol. 967, pp. 454–462, 2002.
- [20] J. Kuneš, Z. Dobešová, and J. Zicha, "Altered balance of main vasopressor and vasodepressor systems in rats with genetic hypertension and hypertriglyceridaemia," *Clinical Science*, vol. 102, no. 3, pp. 269–277, 2002.
- [21] M. Žourek, P. Kyselová, J. Mudra et al., "The relationship between glycemia, insulin and oxidative stress in hereditary hypertriglyceridemic rat," *Physiological Research*, vol. 57, no. 4, pp. 531–538, 2008.
- [22] N. Škottová, L. Kazdová, O. Oliyarnyk, R. Večeřa, L. Sobolová, and J. Ulrichová, "Phenolics-rich extracts from *Silybum marianum* and *Prunella vulgaris* reduce a high-sucrose diet induced oxidative stress in hereditary hypertriglyceridemic rats," *Pharmacological Research*, vol. 50, no. 2, pp. 123–130, 2004.
- [23] S. Vranková, J. Parohová, A. Barta, P. Janega, F. Šimko, and O. Pecháňová, "Effect of nuclear factor kappa B inhibition on L-NAME-induced hypertension and cardiovascular remodelling," *Journal of Hypertension*, vol. 28, no. 1, pp. S45–S49, 2010.
- [24] M. H. Lee, D.-H. Hyun, P. Jenner, and B. Halliwell, "Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production," *Journal of Neurochemistry*, vol. 78, no. 1, pp. 32–41, 2001.
- [25] N. C. Ward and K. D. Croft, "Hypertension and oxidative stress," *Clinical and Experimental Pharmacology and Physiology*, vol. 33, no. 9, pp. 872–876, 2006.
- [26] S. Kojšová, L. Jendeková, J. Zicha, J. Kuneš, R. Andriantsitohaina, and O. Pecháňová, "The effect of different antioxidants on nitric oxide production in hypertensive rats," *Physiological Research*, vol. 55, supplement 1, pp. S3–S16, 2006.
- [27] C. S. Wilcox and A. Pearlman, "Chemistry and antihypertensive effects of tempol and other nitroxides," *Pharmacological Reviews*, vol. 60, no. 4, pp. 418–469, 2008.
- [28] B. Skibska and A. Goraca, "The protective effect of lipoic acid on selected cardiovascular diseases caused by age-related oxidative stress," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 313021, 11 pages, 2015.
- [29] P. X. Shaw, G. Werstuck, and Y. Chen, "Oxidative stress and aging diseases," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 569146, 2 pages, 2014.
- [30] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.
- [31] M. Woodward, K. D. Croft, T. A. Mori et al., "Association between both lipid and protein oxidation and the risk of fatal or non-fatal coronary heart disease in a human population," *Clinical Science*, vol. 116, no. 1, pp. 53–60, 2009.
- [32] W. Dröge, K. Schulze-Osthoff, S. Mihm et al., "Functions of glutathione and glutathione disulfide in immunology and immunopathology," *The FASEB Journal*, vol. 8, no. 14, pp. 1131–1138, 1994.
- [33] M.-L. Cho, Y.-M. Moon, Y.-J. Heo et al., "NF- κ B inhibition leads to increased synthesis and secretion of MIF in human CD4⁺ T cells," *Immunology Letters*, vol. 123, no. 1, pp. 21–30, 2009.
- [34] T. Sasazuki, T. Okazaki, K. Tada et al., "Genome wide analysis of TNF-inducible genes reveals that antioxidant enzymes are induced by TNF and responsible for elimination of ROS," *Molecular Immunology*, vol. 41, no. 5, pp. 547–551, 2004.
- [35] D. M. Townsend, K. D. Tew, and H. Tapiero, "The importance of glutathione in human disease," *Biomedicine and Pharmacotherapy*, vol. 57, no. 3, pp. 145–155, 2003.
- [36] M. C. Santos-Silva, M. S. D. Freitas, and J. Assreuy, "Involvement of NF- κ B and glutathione in cytotoxic effects of nitric oxide and taxol on human leukemia cells," *Leukemia Research*, vol. 30, no. 2, pp. 145–152, 2006.
- [37] S. Zhang, C.-N. Ong, and H.-M. Shen, "Critical roles of intracellular thiols and calcium in parthenolide-induced apoptosis in human colorectal cancer cells," *Cancer Letters*, vol. 208, no. 2, pp. 143–153, 2004.
- [38] J. G. Orr, V. Leel, G. A. Cameron et al., "Mechanism of action of the antifibrogenic compound gliotoxin in rat liver cells," *Hepatology*, vol. 40, no. 1, pp. 232–242, 2004.

Review Article

Widening and Elaboration of Consecutive Research into Therapeutic Antioxidant Enzyme Derivatives

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Undiminishing actuality of enzyme modification for therapeutic purposes has been confirmed by application of modified enzymes in clinical practice and numerous research data on them. Intravenous injection of the superoxide dismutase-chondroitin sulfate-catalase (SOD-CHS-CAT) conjugate in preventive and medicative regimes in rats with endotoxin shock induced with a lipopolysaccharide bolus has demonstrated that antioxidant agents not only effectively prevent damage caused by oxidative stress (as believed previously) but also can be used for antioxidative stress therapy. The results obtained emphasize the importance of investigation into the pathogenesis of vascular damage and the role of oxidative stress in it. The effects of intravenous medicative injection of SOD-CHS-CAT in a rat model of endotoxin shock have demonstrated a variety in the activity of this conjugate in addition to prevention of NO conversion in peroxynitrite upon interaction with $O_2^{\cdot -}$ superoxide radical. Together with the literature data, these findings offer a prospect for the study of NO-independent therapeutic effects of SOD-CHS-CAT, implying the importance of a better insight into the mechanisms of the conjugate activity in modeled cardiovascular damage involving vasoactive agents other than NO.

1. Introduction

A considerable role of enzymes in human body has prompted an extensive research of their derivatives for medical application. The intense initial period of the research promoted the development of “new biology” [1] and biopharmacology [2]. A decrease in the number of studies of proteins for therapeutic purposes was followed by great diversity of enzyme modification methods. Some enzyme derivatives have found successful clinical application. For instance, investigation and development of plasminogen activators provided a current set of protein preparations for clinical thrombolysis [3]. Their effectiveness urges further improvements, such as a decrease in molecular mass (compared with parent molecules) or an increase in it upon construction of new modified agents [4]. Fast development of glycobiology [5, 6] leads to new approaches and targets for the future protein therapy. Nanotechnologies allowed large biological conjugates for further investigation [3, 7–9], and “smart” biopolymers responding to temperature, pH, and effects of biomolecules [10] were

developed. New conceptions contributed to further diversification of protein agents, new protocols of their clinical trials, and appearance of novel aspects in the search for medical enzyme derivatives.

2. Improvement of Enzyme Agents by Modification

Modification of enzymes to overcome their disadvantages for medical use (low stability in the body, short retention period in the bloodstream, low resistance to inhibitors, etc.) and to add to them new properties (ability to concentrate in the damaged area, reduce toxicity, etc.) has been successfully used for a long time [3, 4]. Covalent binding of low-molecular-mass heparin to Cu, Zn-superoxide dismutase increased the enzyme stability in acid and alkaline media as well as its resistance to trypsinolysis and temperature [11]. Conjugation of bovine pancreatic ribonuclease with poly[N-(2-hydroxypropyl)methacrylamide] prolongs the enzyme retention in

the circulation and increases its proteolytic stability and resistance to inhibitors [12], thus enhancing antitumor activity of the ribonuclease. Antioxidant effect of ovalbumin and antimicrobial activity of lysozyme increase after glycosylation with galactomannan [13]. Covalent binding of peptides with albumin enhances their antiviral activity and *in vivo* half-life [14]. Polyethylene glycol (PEG) is often used to prolong the effects of enzyme derivatives in the organism. After PEGylation, recombinant human hyaluronidase markedly suppresses the growth of pancreatic tumor in mice [15]. PEGylated lysozyme displays activity in a wide pH range, stability at 50°C, and high resistance to proteolysis [16]. New methods for controlled PEGylation of peptides and proteins using transglutaminase have been suggested [17]. Modification with the amphiphilic polymer poly(N-acryloylmorpholine) was used as an alternative to PEGylation [18]. Recent evidence demonstrates the diversity and effectiveness of protein modifications for the development of new medical protein derivatives [3, 4, 19, 20] and evaluates the role of protein modifications in various physiological and pathological processes [21].

3. Diversity and Generalization of Interdisciplinary Studies of Protein Drugs

Optimization of technologies for production of protein derivatives and their clinical application have broadened the prospects of employing proteins as potential candidates for pharmacological agents. Protein-based products for biomedical purposes (compositions, combined drugs) [22, 23] were determined, new methods of their production and isolation were suggested [24], and the concordance between structural modifications of biopharmaceutics and their therapeutic effectiveness and safety was evaluated [25]. It should be noted that development of protein drugs has numerous stages, such as choice of modification method, optimization of modification, analysis of the active agent binding and release, cytotoxicity and toxicological parameters, statistically significant confirmation of therapeutic effects *in vivo*, study of biodistribution of derivatives, and evaluation of technological approaches [4, 10]. This has provoked a vast array of publications in biochemical, physiological, clinical, and biotechnological journals, which accelerated distribution of information but hampered a presentation of consecutive evidence concerning a given pharmaceutical agent. Therefore, papers selected due to semantic search technology (for a concrete test object) were provided by electron libraries [26–28]. An analysis and review of the results obtained in interdisciplinary study of protein derivatives for medical application seems relevant in this situation. The present review deals with consecutive studies of the superoxide dismutase-chondroitin sulfate-catalase (SOD-CHS-CAT) conjugate, a supramolecular modified enzyme derivative.

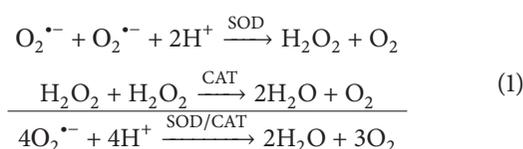
4. Combined Effect of Antioxidant Enzyme Conjugate in Oxidative Stress

In the norm the activity of oxidation-reduction (redox) system in the body is counterbalanced by that of antioxidant system

[29]. Shift in this balance towards overproduction of reactive oxygen species facilitates the development of oxidative stress with nonselective structural and functional damage to biological molecules and progression of pathology. Oxidative stress can be prevented and blocked by antioxidant therapeutic agents [30, 31], antioxidant enzymes among which being effective means to achieve this goal [32]. Modification of these enzymes improves their bioavailability, reduces effective dose, and increases the efficacy of therapy [4, 29, 30, 32–34].

The basis for successful antioxidant therapy is laid by the use of antioxidants at early stages of pathology, their localization and effective concentration in the damaged area, sufficient retention time in the body, safety, absence of toxic product accumulation, and beneficial interactions with body systems, for instance, immune system [29, 30, 32]. Neutralization of active oxygen species at the initial stages of their production is strongly associated with an effective blockade of damage induced by oxidative stress. This goal is achieved with help of superoxide radical scavengers at the early stages of chain transformations associated with oxidative stress. Superoxide dismutase (SOD), an enzyme that catalyzes the dismutation (or neutralization/partitioning) of the superoxide radical ($O_2^{\bullet -}$), can be employed as an interceptor. Hydrogen peroxide (H_2O_2) which in excessive amounts contributes to oxidative damage should be detoxified with catalase (CAT). This enzyme catalyzes H_2O_2 breakdown to harmless water and oxygen. Connected activities of SOD and CAT effectively block $O_2^{\bullet -}$ and H_2O_2 , when hydrogen peroxide, a product of SOD-catalyzed reaction, turns into substrate for CAT.

Schemes of SOD- and CAT-Catalyzed Reactions. Consider the following:



It is noteworthy that these conversions should be effectively realized in the damaged area which is often confined to the vascular wall. Antioxidant agents, including enzyme derivatives, have been used to prevent oxidative damage to blood vessels.

We have developed a covalent water-soluble bienzyme conjugate SOD-CHS-CAT [35, 36] which offers an effective defense of the vascular wall against oxidative stress damage. Some parameters of bienzyme SOD-CHS-CAT conjugate are given in Table 1. Coupling of SOD and CAT activities in the conjugate has provided higher efficiency in comparison with individual activities of SOD and CAT and their mixtures (Figure 1) [37]. This effect emphasizes the importance of simultaneous presence of these enzymes in the damaged area (focus of injury). Enhanced accumulation of chondroitin sulfate (CHS) in vascular zones of atherosclerotic lesions has determined the choice of this glycosaminoglycan of endothelial glycocalyx for preparation of the conjugate to potentiate its vasodilatory effect [38] and rooted antioxidant activity without accumulation of concomitant toxic products [37].

TABLE 1: Some characteristics of SOD-CHS-CAT derivative.

Parameters of bienzyme SOD-CHS-CAT conjugate	
Appearance of derivative	White-gray powder
Content of protein in preparation	5–7%
Specific SOD activity	43–47 U SOD/mg preparation
Specific CAT activity	70–75 U CAT/mg preparation
Effective dose for intravenous bolus administration <i>in vivo</i>	1–2 mg preparation
Recommended dose (according to enzyme activity) for acute injury <i>in vivo</i>	80–90 U SOD/kg
	145–185 U CAT/kg

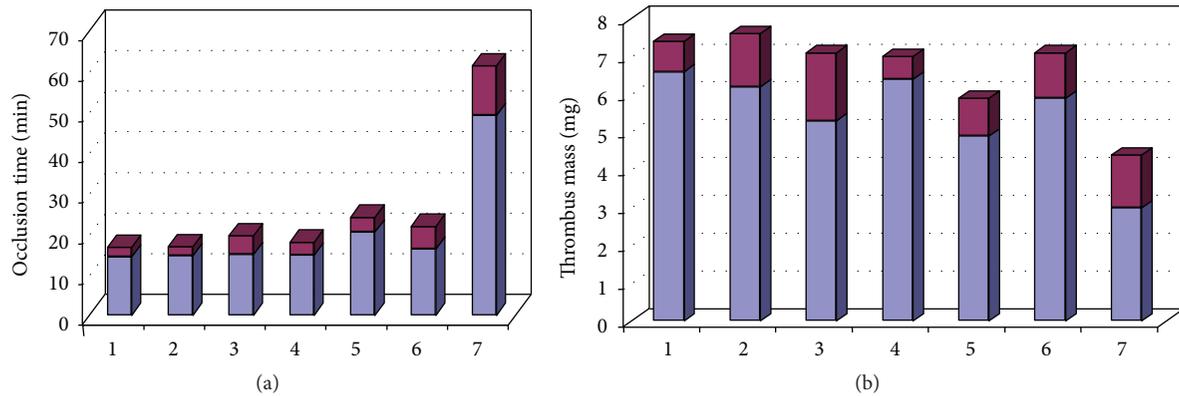


FIGURE 1: Dark areas at the top of bars demonstrate intervals of antithrombotic activities of various forms of SOD and CAT according to occlusion time (a) and thrombus mass (b). 1, control (normal saline); 2, mixture of native SOD and CAT; 3, mixture of native SOD and CAT with free CHS; 4, mixture of native SOD with CAT-CHS derivative; 5, mixture of SOD-CHS derivative with native CAT; 6, mixture of SOD-CHS and CAT-CHS derivatives; 7, SOD-CHS-CAT bienzyme derivative. Each combination of the derivatives was intravenously injected into rats with arterial thrombosis induced by ferric chloride (saturated solution). The dose was similar to that for SOD-CHS-CAT conjugate (37 ± 3 U SOD and 80 ± 3 U CAT activities).

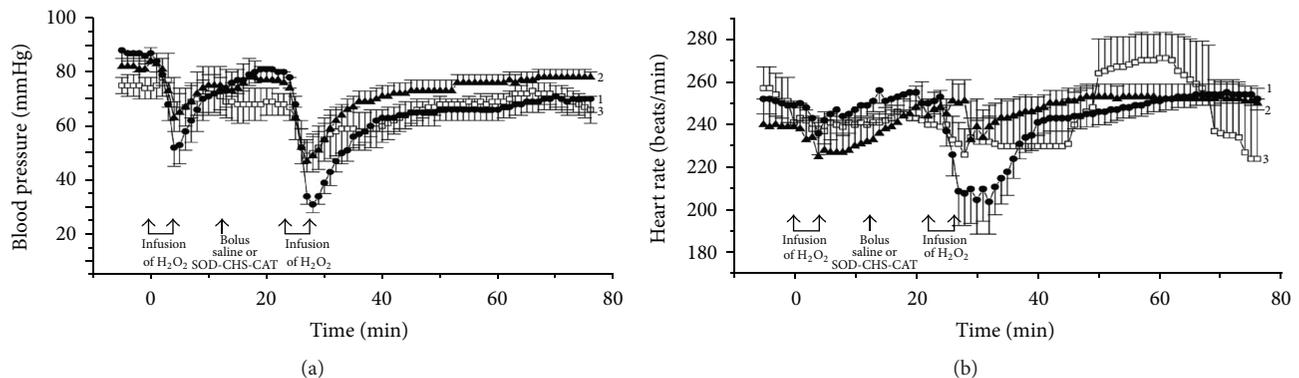


FIGURE 2: Arterial blood pressure (AP, (a)) and heart rate (HR, (b)) in anesthetized rabbits: control group (curve 1, bolus injection of normal saline), acute experiment group (curve 2, bolus injection of SOD-CHS-CAT conjugate), and prophylaxis experiment group (curve 3, injection of SOD-CHS-CAT 3 days before acute experiment with hydrogen peroxide). Arrows indicate initiation and termination of hydrogen peroxide infusion and bolus injection of normal saline or SOD-CHS-CAT conjugate (1.5 mg/kg).

Supramolecular structure of the conjugate has turned it into a nanoparticle that inhibits platelet aggregation induced by adenosine diphosphate (ADP), serotonin, or thrombin receptor agonist peptide (TRAP), which was never displayed by individual SOD and CAT [4, 39]. Preventive intravenous injection of SOD-CHS-CAT into rats 10 min before initialization of thrombosis in the carotid artery [37] or 10 min before

H₂O₂ infusion in a rabbit or rat model of oxidative stress [39] has demonstrated a statistically significant antithrombotic effect of the bienzyme conjugate and restoration of vital hemodynamic parameters: arterial blood pressure (AP) and heart rate (HR, Figure 2). Moreover, AP and HR decrease in response to the first injection of hydrogen peroxide was negligibly small in rabbits injected with SOD-CHS-CAT 3

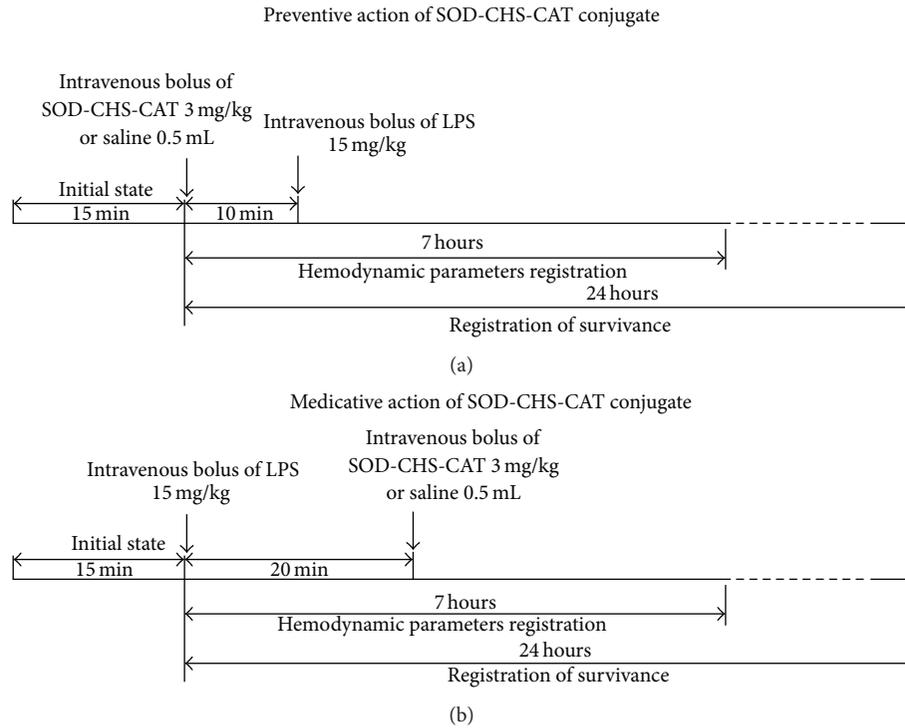


FIGURE 3: Experimental scheme with preventive (a) and medicative (b) regimens for intravenous injection of SOD-CHS-CAT bienzyme conjugate into rats.

days before the experiment. These findings point to the importance of preventive administration of the bienzyme conjugate and are consistent with the generally accepted concept of preventive (prior to the development of oxidative damage) application of antioxidants. Efficacy of small doses of SOD-CHS-CAT in a modeled oxidative stress [4, 37] urged for further biopharmaceutical studies of the conjugate.

5. Block of Oxidative Stress by Preventive Administration of SOD-CHS-CAT

Preventive effect of SOD-CHS-CAT bienzyme conjugate was examined in a rat model of endotoxic shock induced by intravenous bolus injection of 15 mg/kg lipopolysaccharide (LPS) isolated from *Salmonella enterica serotype Typhimurium* with subsequent monitoring of AP, HP, and mortality rate within a given time period. The experiments were performed in cooperation with Laboratory of Experimental Myocardial Pathology, Laboratory of Stem Cells, and Laboratory of Physico-Chemical Methods at the Institute of Experimental Cardiology.

Rapid production of reactive oxygen species (ROS) in oxidative stress causes nonselective damage to biological macromolecules, thus provoking progression of pathological disorders. Administration of antioxidants before the development of damage (Figure 3(a)) is aimed at its reduction [29, 32, 40]. Injection of SOD-CHS-CAT conjugate 10 min before LPS bolus increased 24 h survival rate to $69 \pm 8\%$ versus $43 \pm 8\%$ in the control ($p < 0.03$, Figure 4). The area under

the Kaplan-Meier curve in the control and experiment was 1.384 and 1.971, respectively, indicating statistically significant 1.4-fold increase in this parameter. This effect demonstrates that preventive administration of SOD-CHS-CAT increases rat viability in endotoxic shock.

6. Medicative Administration of SOD-CHS-CAT Is Effective against Oxidative Stress

It should be noted that endotoxin damage develops through two successive stages [40]. The first is referred to as neurological. It is associated with the nervous system reactions, develops within 20 min after LPS injection, and is blocked by injection of 2% lidocaine into the preoptic anterior hypothalamic area [41]. The second stage is referred to as cytokine-dependent, being associated with the release of the cytokines (bradykinin and $\text{TNF-}\alpha$, interleukins $\text{IL-1}\alpha$ and $\text{IL-1}\beta$, and chemokines IL-6 , IL-8 , and IL-18) as well as increasing blood concentration of nitric oxide. It develops 20–90 min after LPS bolus injection. The absence of differences between AP and HR in control and experimental rats during the neurological stage of oxidative damage development substantiates the actuality of studying medicative activity of SOD-CHS-CAT, implying an intravenous injection of the conjugate 20 min after, but not before, LPS (Figure 3(b)), thus bypassing the neurological stage.

After SOD-CHS-CAT injection according to the experiment scheme, survival rates of control and experimental

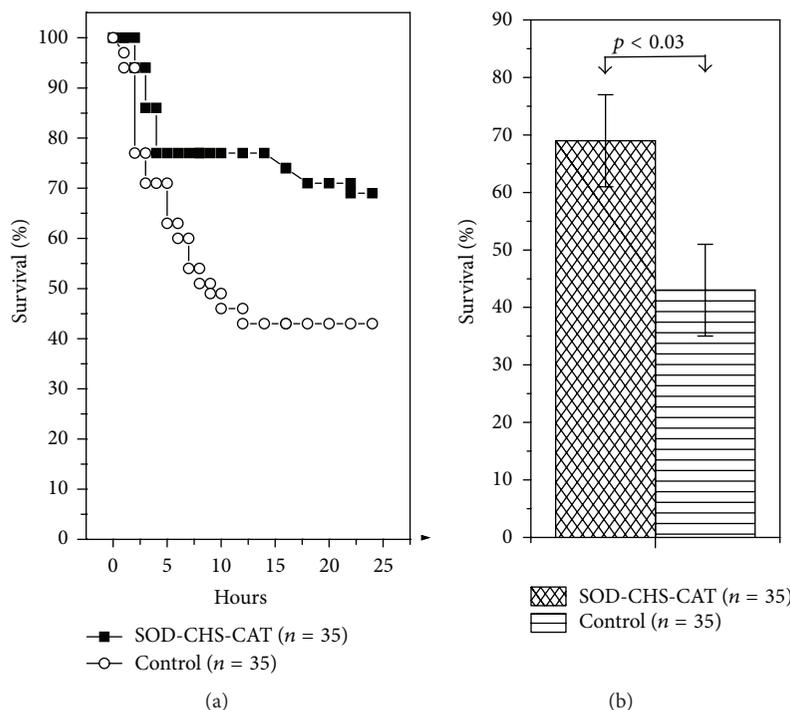


FIGURE 4: 24 h lethality of rats with endotoxic shock (left, (a) Kaplan-Meier curves; right, (b) diagram presentation of the areas under these curves) after preventive intravenous injection of SOD-CHS-CAT bienzyme conjugate. Here and in other figures n is the number of animals in groups.

groups were $35 \pm 9\%$ and $61 \pm 9\%$, $p < 0.04$, respectively (Figure 5). The area under the Kaplan-Meier curve was 1.129 in control and 1.643 in experimental rats, demonstrating a 1.46-fold increase in viability.

7. Effects of Preventive and Medicative SOD-CHS-CAT Injection in a Rat Model of Endotoxin Shock

The similarity between AP and HR changes after preventive and medicative injections of SOD-CHS-CAT with significantly enhanced survival of rats in experimental groups demonstrates that HR increase is a compensatory reaction to AP decrease in endotoxic shock. Restoration of AP was faster in experimental rats, while intergroup changes in HR were statistically insignificant [40]. The cytokine stage of oxidative damage development was chosen as a target for successful therapeutic correction with vascular antioxidants (SOD-CHS-CAT). Effectiveness of the conjugate in preventive and medicative regimens indicated its potential wide application. Direct antioxidant effect of SOD-CHS-CAT, particularly at the initial stages of oxidative damage [39], and remote/distant therapeutic effects [23, 33] actualized elucidation of mechanisms underlying these effects. This was facilitated by changing the administration route in a rat model of endotoxin shock.

It should be noted that oxidative stress has surely and soundly become a significant and attractive target for cardiovascular prevention and therapy [42]. A decrease in

oxidative stress demonstrated the efficacy of antioxidant therapy in STEMI (ST-segment-Elevation Myocardial Infarction) patients [34]. Systematic search for factors determining the success of biomedical data translation into clinical practice contributes to effective transition from the research to the clinic [43]. These trends stipulate efficacious formation of antioxidant therapy resources on the basis of biopharmacological investigations in animal [34, 42, 43].

8. Effect of Peroral Administration of SOD-CHS-CAT on the Development of Endotoxin Shock

Bearing in mind that peroral route of administration improves availability and spread of a drug, we designed an experimental protocol to test the effectiveness of the bienzyme conjugate administered perorally. Rats were given SOD-CHS-CAT with cottage cheese at a daily dose of 17.5 mg/kg body weight for 3 weeks (experimental group). Control rats did not receive the conjugate. The dynamics of weight gain was similar in both groups. AP, HR, and 24 h lethality were monitored after an intravenous LPS bolus injection (15 mg/kg).

There were no statistically significant intergroup differences in survival rate: $63 \pm 10\%$ (control) and $73 \pm 10\%$ (experiment) (Figure 6). It is noteworthy that lethal outcome was earlier in control rats, as evidenced by statistically significant differences 2, 4, and 5 h after LPS injection ($p < 0.05$). During a 5 h period the areas under the Kaplan-Meier curves

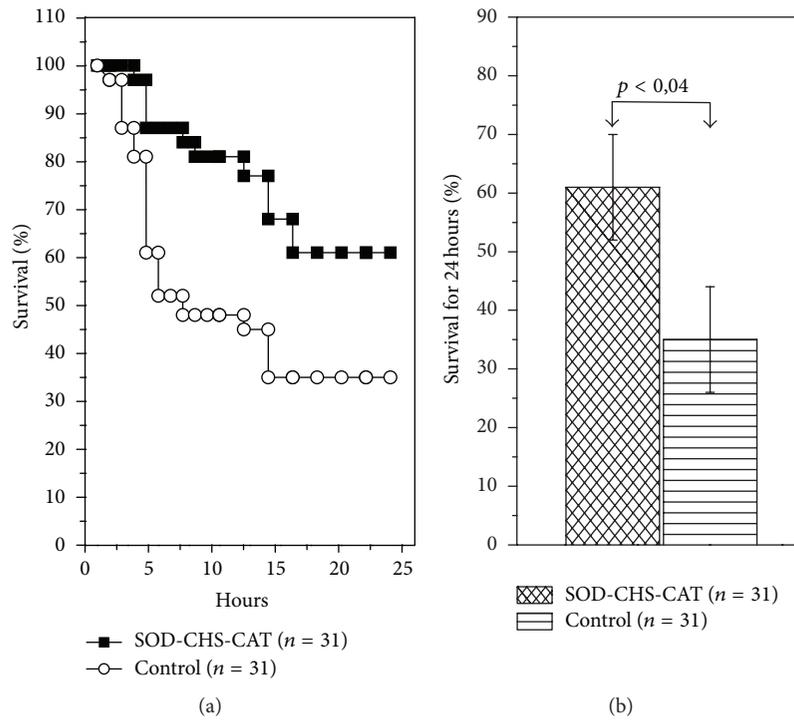


FIGURE 5: 24 h lethality of rats with endotoxic shock ((a) Kaplan-Meier curves and (b) diagram presentation of areas under these curves) after medicative intravenous injection of SOD-CHS-CAT bienzyme conjugate.

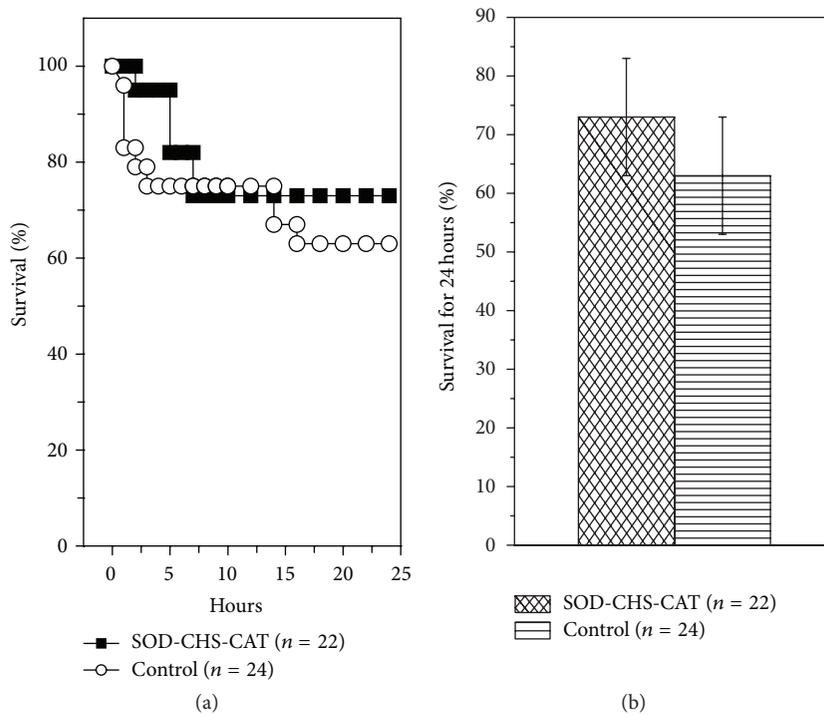


FIGURE 6: 24 h lethality of rats by Kaplan-Meier curves (a) and % surviving animals by the end of the observation period (b) after intravenous injection of LPS (15 mg/kg) into rats given SOD-CHS-CAT perorally (experimental group) and rats that did not receive the conjugate (control).

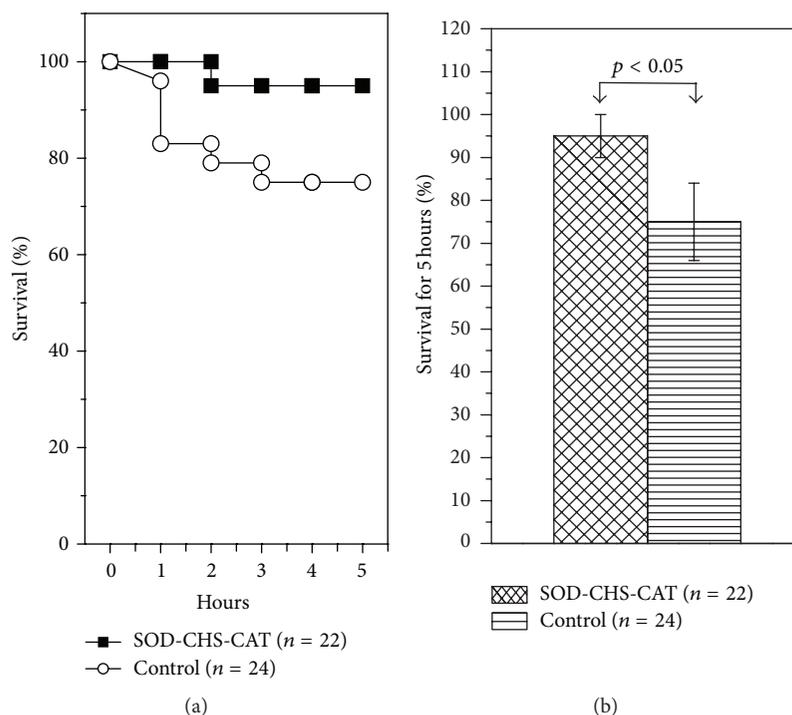


FIGURE 7: 5 h lethality of rats after intravenous injection of LPS (15 mg/kg) into rats given SOD-CHS-CAT perorally (experimental group) and rats that did not receive the conjugate (control). (a) Data according to Kaplan-Meier curves, (b) according to diagram presentation of areas under the curves.

were 0.671 ± 0.003 (control) and 0.800 ± 0.001 (experiment), that is, 1.192-fold greater ($p < 0.001$, Figure 7). These results indicate preservation of rat viability in the given period of endotoxic shock and point to protective fast-acting of SOD-CHS-CAT. A rapid therapeutic effect of the bienzyme conjugate suggests that its doses should be varied in an attempt to increase the effectiveness of peroral administration in a complex therapy.

The absence of statistically significant intergroup differences in 24 h lethality (Figure 6) can be associated with insufficient supply of SOD-CHS-CAT and its depletion/destruction in rat organism after peroral administration. A comparative *in vitro* study of trypsinolysis resistance of SOD-CHS-CAT and individual CAT and SOD has revealed a marked decrease in the enzyme activity of these compounds after 3 h incubation with trypsin. Destruction was confirmed by electrophoresis which demonstrated a wide band of small-molecular-weight fraction in comparison with initial samples. These findings indicate that SOD-CHS-CAT administered by peroral route is prone to proteolytic destruction.

9. Conclusion

The results obtained confirm statistically significant effect of experimental therapy with SOD-CHS-CAT in preventive and medicative regimens and substantiate the actuality of investigating the mechanisms responsible for this effect. Collected evidence points to a direct antioxidant effect of SOD-CHS-CAT [30, 39] and a possibility of increasing the level of

endogenous antioxidant biocatalysts after administration of therapeutic antioxidants [23, 33]. Moreover, it was reported that the size of myocardial infarction in rats can be reduced by NO-independent stimulation of guanylyl cyclase [44]. ROS generation was recorded at late stages of angiotensin II-induced hypertension [45] and mechanosensor regulation of angiotensin-converting enzyme was observed in response to endothelial shear stress [46, 47]. These data demonstrate NO-independent pathways for realization of vasodilatation/constriction. In addition to NO defense against conversion into peroxynitrite upon interaction with superoxide radical, the diversity of protective effects provided by antioxidants shows a prospect for a research in the mechanisms involving various vasoactive agents (not only NO) and determines the pathways for NO-dependent and NO-independent therapeutic effects of SOD-CHS-CAT bienzyme conjugate. The research into vascular damage pathogenesis and evaluation of the role of oxidative stress in it (time and place) are of paramount importance.

Production of novel protein drugs is strongly dependent on the development of biopharmaceutical industry. It has been generally accepted that producing companies should choose only two goals from the three major ones: high product quality, speedy development/production, and low cost of the product [48]. However, current situation urges the industry to achieve these three goals simultaneously. Undoubtedly, high quality, fast production, and reasonable price require advanced technologies and business strategies (the use of biomimetic metabolic pathways, breakthrough therapeutic

schemes, competition in the most promising areas, etc.). It has been suggested that this will be facilitated by smaller markets and small-scale products of biopharmaceutical industry for next ten years [48]. Such a development can accelerate production of drugs based on antioxidant enzymes.

Our findings demonstrate the effectiveness of SOD-CHS-CAT conjugate in prevention and medication of oxidative stress damage, attract attention to the elucidation of mechanism of its action (probably, especially via NO-independent pathway), and emphasize the increasing actuality of the research into pathogenesis of cardiovascular disorders and contribution of oxidative stress to them.

Abbreviations

ADP:	Adenosine diphosphate
AP:	Arterial pressure
CAT:	Catalase
CHS:	Chondroitin sulfate
HR:	Heart rate
LPS:	Lipopolysaccharide
PEG:	Polyethylene glycol
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
SOD-CHS-CAT:	Superoxide dismutase-chondroitin sulfate-catalase conjugate
TRAP:	Thrombin receptor agonist peptide.

Competing Interests

The author declares that there are no competing interests.

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References

- [1] B. E. Sobel, "Coronary thrombolysis and the new biology," *Journal of the American College of Cardiology*, vol. 14, no. 4, pp. 850–860, 1989.
- [2] A. V. Maksimenko and E. G. Tischenko, "New thrombolytic strategy: bolus administration of tPA and urokinase-fibrinogen conjugate," *Journal of Thrombosis and Thrombolysis*, vol. 7, no. 3, pp. 307–312, 1999.
- [3] A. V. Maksimenko, "Development and application of targeted therapeutic protein conjugates," *Russian Journal of General Chemistry*, vol. 84, no. 2, pp. 357–363, 2014.
- [4] A. V. Maksimenko, "Cardiological biopharmaceuticals in the conception of drug targeting delivery: practical results and research perspectives," *Acta Naturae*, vol. 4, no. 3, pp. 72–81, 2012.
- [5] Z. L. Wu, "Time of molecular glycobiology," *Glycobiology Journal*, vol. 1, no. 2, article e106, 2012.
- [6] C. M. West, "Latest advances in glycobiology highlighted and old challenges revisited at the 2013 annual conference of the society for glycobiology," *Glycobiology*, vol. 24, no. 3, pp. 218–219, 2014.
- [7] E. D. Hood, M. Chorny, C. F. Greineder, I. Alferiev, R. J. Levy, and V. R. Muzykantov, "Endothelial targeting of nanocarriers loaded with antioxidant enzymes for protection against vascular oxidative stress and inflammation," *Biomaterials*, vol. 35, no. 11, pp. 3708–3715, 2014.
- [8] P. U. Richard, J. T. Duskey, S. Stolarov, M. Spulber, and C. G. Paliyan, "New concepts to fight oxidative stress: nanosized three-dimensional supramolecular antioxidant assemblies," *Expert Opinion on Drug Delivery*, vol. 12, no. 9, pp. 1527–1545, 2015.
- [9] R. Sandhir, A. Yadav, A. Sunkaria, and N. Singhal, "Nano-antioxidants: an emerging strategy for intervention against neurodegenerative conditions," *Neurochemistry International*, vol. 89, pp. 209–226, 2015.
- [10] S. Kim, J.-H. Kim, O. Jeon, I. C. Kwon, and K. Park, "Engineered polymers for advanced drug delivery," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 71, no. 3, pp. 420–430, 2009.
- [11] H. W. Zhang, F. S. Wang, W. Shao et al., "Characterization and stability investigation of Cu,Zn-superoxide dismutase covalently modified by low molecular weight heparin," *Biochemistry (Moscow)*, vol. 71, supplement 1, pp. S96–S100, 2006.
- [12] J. Soucek, P. Poucková, J. Strohalm et al., "Poly[N-(2-hydroxypropyl)methacrylamide] conjugates of bovine pancreatic ribonuclease (RNase A) inhibit growth of human melanoma in nude mice," *Journal of Drug Targeting*, vol. 10, no. 3, pp. 175–183, 2002.
- [13] S. Nakamura and A. Kato, "Multi-functional biopolymer prepared by covalent attachment of galactomannan to egg-white proteins through naturally occurring Maillard reaction," *Nahrung*, vol. 44, no. 3, pp. 201–206, 2000.
- [14] D. Xie, C. Yao, L. Wang et al., "An albumin-conjugated peptide exhibits potent anti-HIV activity and long *in vivo* half-life," *Antimicrobial Agents and Chemotherapy*, vol. 54, no. 1, pp. 191–196, 2010.
- [15] A. Kultti, C. Zhao, N. C. Singha et al., "Accumulation of extracellular hyaluronan by hyaluronan synthase 3 promotes tumor growth and modulates the pancreatic cancer microenvironment," *BioMed Research International*, vol. 2014, Article ID 817613, 15 pages, 2014.
- [16] D. da Silva Freitas and J. Abrahão-Neto, "Biochemical and biophysical characterization of lysozyme modified by PEGylation," *International Journal of Pharmaceutics*, vol. 392, no. 1-2, pp. 111–117, 2010.
- [17] A. Mero, M. Schiavon, F. M. Veronese, and G. Pasut, "A new method to increase selectivity of transglutaminase mediated PEGylation of salmon calcitonin and human growth hormone," *Journal of Controlled Release*, vol. 154, no. 1, pp. 27–34, 2011.
- [18] O. Schiavon, P. Caliceti, P. Ferruti, and F. M. Veronese, "Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugated to linear or branched poly(ethylene glycol) and poly(N-acryloylmorpholine)," *II Farmaco*, vol. 55, no. 4, pp. 264–269, 2000.

- [19] M. Werle and A. Bernkop-Schnürch, "Strategies to improve plasma half life time of peptide and protein drugs," *Amino Acids*, vol. 30, no. 4, pp. 351–367, 2006.
- [20] M. Schlapschy, U. Binder, C. Börger et al., "PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins," *Protein Engineering, Design and Selection*, vol. 26, no. 8, pp. 489–501, 2013.
- [21] J. Wu, "New stories on protein modification," *Journal of Molecular Cell Biology*, vol. 8, no. 1, pp. 1–1, 2016.
- [22] A. Mire-Sluis, D. French, J. Mercer, G. Kleemann, and J. Dougherty, "Drug products for biological medicines: novel delivery devices, challenging formulations, and combination products, part 1," *BioProcess International*, vol. 11, no. 4, pp. 48–62, 2013.
- [23] Working Group on the Summit on Combination Therapy for Cardiovascular Diseases, "Combination pharmacotherapy to prevent cardiovascular disease: present status and challenges," *European Heart Journal*, vol. 35, no. 6, pp. 353–364, 2014.
- [24] T. Muller-Spath, N. Ulmar, L. Aumann et al., "Purifying common light-chain bispecific antibodies. A twin-column, counter-current chromatography platform process," *BioProcess International*, vol. 11, no. 5, pp. 36–44, 2013.
- [25] Z. Wei, E. Shacter, M. Schenerman, J. Dougherty, and L. D. McLeod, "The role of higher-order structure in defining biopharmaceutical quality," *BioProcess International*, vol. 9, no. 4, pp. 58–66, 2011.
- [26] I. F. Cooper and M. S. Siadaty, "Amino acids, peptides, or proteins associated with glycosaminoglycans binding," *BioMedLib Review*, vol. 705, pp. 864–909, 2014.
- [27] I. F. Cooper and M. S. Siadaty, "Therapeutic or preventive procedures associated with prourokinase," *BioMedLib Review*, vol. 705, pp. 305–490, 2014.
- [28] I. F. Cooper and M. S. Siadaty, "Enzymes associated with cell coat," *BioMedLib Review*, vol. 705, pp. 371–894, 2014.
- [29] A. V. Maksimenko, "Extracellular oxidative damage of vascular walls and their protection using antioxidant enzymes," *Pharmaceutical Chemistry Journal*, vol. 41, no. 5, pp. 235–243, 2007.
- [30] A. V. Maksimenko and A. V. Vavaev, "Antioxidant enzymes as potential targets in cardioprotection and treatment of cardiovascular diseases. Enzyme antioxidants: the next stage of pharmacological counterwork to the oxidative stress," *Heart International*, vol. 7, no. 1, pp. 14–19, 2012.
- [31] H. N. Siti, Y. Kamisah, and J. Kamsiah, "The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review)," *Vascular Pharmacology*, vol. 71, pp. 40–56, 2015.
- [32] A. V. Maksimenko, "Experimental antioxidant biotherapy for protection of the vascular wall by modified forms of superoxide dismutase and catalase," *Current Pharmaceutical Design*, vol. 11, no. 16, pp. 2007–2016, 2005.
- [33] J. Carillon, J.-M. Rouanet, J.-P. Cristol, and R. Brion, "Superoxide dismutase administration, a potential therapy against oxidative stress related diseases: several routes of supplementation and proposal of an original mechanism of action," *Pharmaceutical Research*, vol. 30, no. 11, pp. 2718–2728, 2013.
- [34] S. Ekelof, S. E. Jensen, J. Rosenberg, and I. Gogenur, "Reduced oxidative stress in STEMI patients treated by primary percutaneous coronary intervention and with antioxidant therapy: a systematic review," *Cardiovascular Drugs and Therapy*, vol. 28, no. 2, pp. 173–181, 2014.
- [35] A. V. Maksimenko and E. G. Tischenko, "Covalent modification of superoxide dismutase subunits by chondroitin sulfate," *Biochemistry (Moscow)*, vol. 62, no. 10, pp. 1163–1166, 1997.
- [36] A. V. Maksimenko and E. G. Tischenko, "Modification of catalase by chondroitin sulfate," *Biochemistry (Moscow)*, vol. 62, no. 10, pp. 1167–1170, 1997.
- [37] A. V. Maksimenko, V. L. Golubykh, and E. G. Tischenko, "The combination of modified antioxidant enzymes for anti-thrombotic protection of the vascular wall: the significance of covalent connection of superoxide dismutase and catalase activities," *Journal of Pharmacy and Pharmacology*, vol. 56, no. 11, pp. 1463–1468, 2004.
- [38] A. V. Vavaev, E. G. Tischenko, V. P. Mokh, and A. V. Maksimenko, "Effect of hydrogen peroxide on the tone of rat arterial fragment and its antioxidant protection with derivatives of catalase and superoxide dismutase," *Technol Living Systems*, vol. 6, no. 3, pp. 26–32, 2009 (Russian).
- [39] A. V. Maksimenko, A. V. Vavaev, L. I. Bouryachkovskaya et al., "Biopharmacology of enzyme conjugates: vasoprotective activity of supramolecular superoxide dismutase-chondroitin sulfate-catalase derivative," *Acta Naturae*, vol. 2, no. 4, pp. 82–94, 2010.
- [40] A. V. Maksimenko, A. V. Vavaeva, A. A. Abramov, A. V. Vavaev, and V. L. Lakomkin, "Medicative and preventive action of bienzyme superoxide dismutase-chondroitin sulfate-catalase conjugate at endotoxic shock," *Living Systems Technology*, vol. 11, no. 2, pp. 35–44, 2014 (Russian).
- [41] M. S. Yilmaz, W. R. Millington, and C. Feleder, "The preoptic anterior hypothalamic area mediates initiation of the hypotensive response induced by LPS in male rats," *Shock*, vol. 29, no. 2, pp. 232–237, 2008.
- [42] T. Münzel, T. Gori, R. M. Bruno, and S. Taddei, "Is oxidative stress a therapeutic target in cardiovascular disease?" *European Heart Journal*, vol. 31, no. 22, pp. 2741–2749, 2010.
- [43] G. P. van Hout, S. J. Jansen of Lorkeers, K. E. Wever et al., "Translational failure of anti-inflammatory compounds for myocardial infarction: a meta-analysis of large animal models," *Cardiovascular Research*, vol. 109, no. 2, pp. 240–248, 2016.
- [44] J. S. Bice, Y. Keim, J.-P. Stasch, and G. F. Baxter, "NO-independent stimulation or activation of soluble guanylyl cyclase during early reperfusion limits infarct size," *Cardiovascular Research*, vol. 101, no. 2, pp. 220–228, 2014.
- [45] S. Kimura, G.-X. Zhang, and Y. Abe, "Malfunction of vascular control in lifestyle-related diseases: oxidative stress of angiotensin II-induced hypertension: mitogen-activated protein kinases and blood pressure regulation," *Journal of Pharmacological Sciences*, vol. 96, no. 4, pp. 406–410, 2004.
- [46] V. G. Barauna, L. C. G. Campos, A. A. Miyakawa, and J. E. Krieger, "ACE as a mechanosensor to shear stress influences the control of its own regulation via phosphorylation of cytoplasmic ser¹²⁷⁰," *PLoS ONE*, vol. 6, no. 8, Article ID e22803, 2011.
- [47] I. Fleming, K. Kohlstedt, and R. Busse, "New fACEs to the renin-angiotensin system," *Physiology*, vol. 20, no. 2, pp. 91–95, 2005.
- [48] C. Scott, "Speeding development and lowering costs while enhancing quality: a BPI theater roundtable at the 2015 BIO convention," *BioProcess International*, vol. 13, supplement 4, pp. 23–25, 2015.

Research Article

Induction Effect of Bisphenol A on Gene Expression Involving Hepatic Oxidative Stress in Rat

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Background and Objective. Bisphenol A (BPA) is an abundantly used xenoestrogenic chemical which may cause various disorders in body. In the present study, we sought to investigate the effects of various doses of BPA on hepatic oxidative stress-related gene expression in rats. **Methods.** Male Wistar rats weighing 150–200 g were used in this study. Three doses of the BPA (5, 25, and 125 $\mu\text{g}/\text{kg}$) in corn oil were administered as gavage during 35 consecutive days. After the experiment, the rats were expired and the livers were removed and stored at -80°C freezer for RNA extraction. **Findings.** The Real Time PCR showed increased expression of HO-1 in the rats receiving BPA doses compared to the control group. This effect was dose-dependent and higher at doses of 25 and 125 $\mu\text{g}/\text{kg}$ than 5 $\mu\text{g}/\text{kg}$ of body weight ($p < 0.05$). It was also demonstrated that various doses BPA can increase GADD45B gene expression compared to control group. That expression was significantly dominant in the lowest dose (5 $\mu\text{g}/\text{kg}$) of the BPA ($p < 0.05$). The final body weights (168.0 ± 10.0 gr) in the treatment group [BPA (125 $\mu\text{g}/\text{kg}$)] showed a significant decrease compared to control group (191.60 ± 6.50 gr). **Conclusion.** These findings demonstrate that BPA generated ROS and increased the antioxidant gene expression that causes hepatotoxicity.

1. Introduction

Bisphenol A (BPA) is a synthetic xenoestrogenic compound as [1] which is widely used as a key monomer in production of various polycarbonate plastics and epoxy resins, such as food and drink containers, baby bottles, and dental sealants [2].

Although the BPA is not dangerous in polymeric form, it is unstable in acidic and basic solutions and exposure to UV light. Those conditions may convert the polymeric BPA to monomeric forms. In this condition BPA is released into food, beverages, or environment [3]. Due to long-term release of the BPA from food product containers, most individuals in general populations are widely exposed to this substance, according to the fact that every year hundreds of tons of the BPA are released into atmosphere [2]. It is water soluble as reported at high level in marine populations [4]. Many

investigations have been conducted to evaluate the toxic effects of the BPA on human health [5].

Several studies have revealed the toxic effect of BPA (even at low doses) on various organs by increasing oxidative stress [6]. The liver is an important organ that has been affected by the BPA through inducing oxidative stress [6–8]. Reactive oxygen species (ROS) such as superoxide, hydroxyl and proxy radicals, and hydrogen peroxide are cytotoxic agents that are able to stimulate oxidative stress by impairment of prooxidant/antioxidant balance [9, 10].

Up to date, so many investigations have demonstrated the impact of the BPA induced oxidative stress on various genes in liver [8, 11]. HO-1 and GADD45B are two genes which their expression can be affected on oxidative stress [9, 12–14]. HO-1 gene product is responsible for heme catabolism that may result in CO production which subsequently increase

TABLE 1: The primers and probes sequences for GAPDH, HO-1, and GADD45B genes.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	FAM Probe
GAPDH	CTACATGGCCTCCAAGGA-GTAAG	TGGAATTGTGAGGGAGAT-GCTC	ACCACCCAGCCCAGCAAGGATACT-TAMRA
HO-1	ACAGCATGTCCCAGGATT-TGTC	GGAGGCCATCACCAGCTT-AAAG	CCCTGGACACCTGACCCTTCTGAAAG-TAMRA
GADD45B	GAAGATGCAGGCGGTGAC-TG	CCTCCTCTTCTTCGTCTATGGC	CAGGCACAAGACCACGCTGTCCG-TAMRA

gene expression of interleukin-10 (IL-10) and interleukin-1 receptors (IL-1R). This process can reduce inflammation [15]. Another gene that its expression increases oxidative stress is GADD45B. This increased expression affects stopping cell cycle survival, apoptosis, and DNA repair. Its expression pattern change reveals rate of cell damage at gene level [14].

Based on the important role of two mentioned genes in oxidative stress condition, the present study was designed to investigate the impact of the BPA on expression of HO-1 and GADD45B and clarify whether they can be considered as appropriate biomarkers following the BPA hepatotoxicity.

2. Materials and Methods

2.1. Chemicals and Reagents. Bisphenol A with purity >99% (Daejung, Korea), tripure and DEPC (Roche, Sigma, Germany), and C-DNA kit (Amplisens Co., Russia) were obtained. The primers for RT-PCR analysis were synthesized by Bioneer, Korea. All other chemicals were purchased from local commercial sources.

2.2. Animals. Male Wistar rats (10 to 12 weeks old) were prepared from animal room of Babol University of Medical Science (Babol, Iran).

All rats were housed in plastic cages under a well-regulated light and dark schedule (12 h light:12 h dark) at $22 \pm 2^\circ\text{C}$, at humidity ($50 \pm 5\%$) environment, and with free access to chow and tap water *ad libitum*.

The University Ethics Committee approved the study and all the experiments were performed in accordance with the guidelines for Care and Use of Laboratory Animals.

2.3. Treatment. The rats were randomly divided into four groups ($n = 5$). The BPA was given in three doses (5, 25, and $125 \mu\text{g}/\text{kg}$ in corn oil as vehicle). It was orally administered every 24 hours for 35 days. The control group received olive oil alone.

2.4. Necropsy. All rats were fasted overnight after receiving the last dose of the BPA in corn oil. Then they were anesthetized using sodium thiopental and were finally sacrificed. Subsequently, the liver of each rat in specified group was removed and cleaned from adhering fat and connective tissues. The liver samples were quickly snap-frozen in liquid nitrogen and then were stored at -80°C for future experiments.

2.5. RNA Extraction. Total RNA was extracted from frozen rat liver tissues using tripure reagent kit and dissolved in diethylpyrocarbonate-treated deionized water; the quality and quantity of extracted RNA were assessed using Spectrophotometer (Thermo, USA) at 260 and 280 nm.

2.6. cDNA Synthesis and Real Time PCR Procedure. First-strand cDNA was synthesized by reverse transcription using Reverta-L RT Reagent kit, in accordance with the manufacturer's instruction. The thermocycler for cDNA synthesis was set up at 37°C for 30 min.

Quick PCR (qPCR) was performed using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Branchburg, NJ, USA) at three conditions, 95°C for 5 min, 45 cycles at 95°C for 30 s, and 60°C for 1 min. Expression levels of mRNA were normalized to GAPDH gene as the endogenous control. Then the relative differences between control and treatment groups were calculated and expressed as percentage of controls. Primers and probes for the qPCR were designed using Allele ID 6. All primers were listed in Table 1.

2.7. Statistical Analysis. All the analyses were performed using SPSS software version 19. Statistical analyses were performed using one-way ANOVA followed by *post hoc* Tukey test. The significance of differences between data was assumed at $p < 0.05$.

3. Result

3.1. Weight Changes. Body weight of the animals was recorded at the beginning of the experiment as baseline and before killing them. Based on the results, the weight was decreased in the BPA receiving groups compared to the control (Table 2 and Figure 1).

3.2. The Findings of Real Time PCR. The results of Real Time PCR showed that different concentrations of the BPA increased the expression of HO-1 compared to the control group ($p < 0.05$). This increased expression was dose-dependent and there was a significant difference ($p < 0.05$) between the effects of medium dose ($25 \mu\text{g}/\text{kg}$) and high dose ($125 \mu\text{g}/\text{kg}$). Despite increased expression of HO-1, no significant difference was observed after administering dose of $5 \mu\text{g}/\text{kg}$ BPA, compared to the control group (Figure 2).

TABLE 2: Mean \pm SD of the body weight of rat receiving the BPA and control.

Treatment groups	Body weight (g) at baseline	Body weight (g) after 35 days
Control	187 \pm 8.72	191.60 \pm 6.50
BPA (5 μ g/kg)	183 \pm 9.95	172.8 \pm 7.56*
BPA (25 μ g/kg)	175.80 \pm 9.01	160.4 \pm 5.03*
BPA (125 μ g/kg)	171.6 \pm 6.68	168.0 \pm 10.0*

* $p < 0.05$ significantly different from control group.

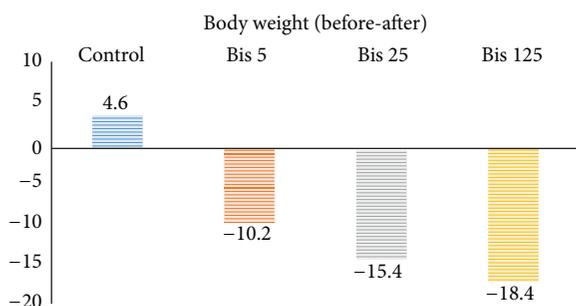


FIGURE 1: Mean of weight difference of baseline and end of treatment in control (olive oil) and bisphenol A receiving groups ($n = 5$). Bis 5: bisphenol A 5 μ g/kg, Bis 25: bisphenol 25 μ g/kg, and Bis 125: bisphenol 125 μ g/kg.

The results of Real Time PCR showed that administration of 5 and 25 μ g/kg doses of the BPA significantly increased GADD45B gene expression compared to the control ($p < 0.05$). This expression was significantly dominant in dose of 5 μ g/kg (Figure 3). Surprisingly enough, despite increase in expression of BPA concentration (25 and 125 μ g), the increased expression was not dependent on increased bisphenol A condensation; so gene expression was decreased at every level compared to previous level; and, contrary to GADD45B gene expression, the difference was not further significant at the concentration of 125 μ g of bisphenol A compared to the control group (Figure 3).

4. Discussion

The BPA is an estrogen-like chemical that can be released into the environment. The most studies have focused on its effects on reproductive system [16, 17]. The LD50 of the BPA (oral, rat) is 3.25 g/kg [18]. American environmental protection agency (EPA) has defined an acceptable daily dose of 50 μ g/kg of the BPA [19].

All three doses of the BPA induced a significant decrease in body weight in comparison to the control. Similar result was reported in a previous study on body weight loss [20].

It has been shown that the BPA by decreasing expression of the gene responsible for prevention of oxidative activity can induce production of ROS and subsequent hepatotoxicity [11].

HO-1 gene encodes an enzyme with the same name which can degrade heme molecule and help production of

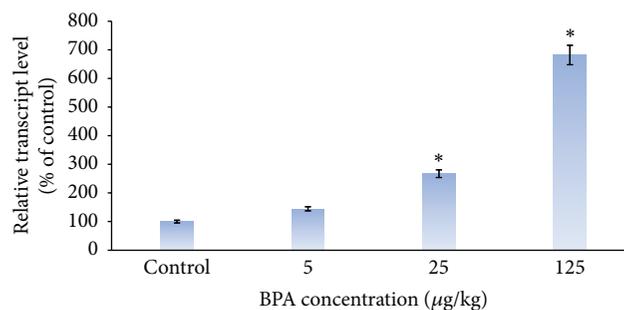


FIGURE 2: Dose-dependent increase in HO-1 gene expression in bisphenol A receiving groups compared to the control (olive oil). Higher effect is seen in dose of 125 μ g/kg bisphenol A receiving group. * $p < 0.05$ significantly different from control group.

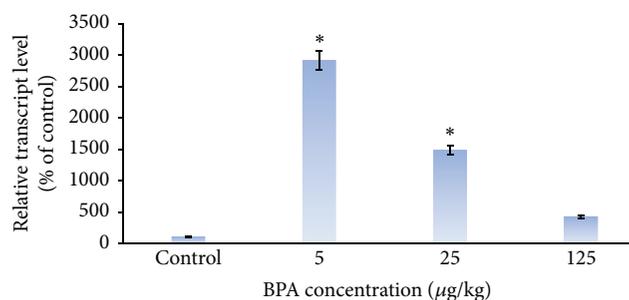


FIGURE 3: Dose-dependent increase in GADD45B gene expression in bisphenol A receiving groups compared to the control (olive oil). Higher effect is seen in dose of 5 μ g/kg bisphenol A receiving group. * $p < 0.05$ significantly different from control group.

compounds such as carbon monoxide and biliverdin. In addition to these activities, continuous expression of the gene in steady state may be associated with adverse effects on living cells [21].

Although there are limited data on the effect of different concentrations of the BPA on gene expression in the liver, the present study in order to assess its liver cell toxicity showed a dose-dependent profile of increased gene expression of HO-1 as compared to the control. This expression of HO-1 gene was the highest in the group receiving largest dose of the BPA (125 μ g/kg).

Several investigations have focused on the role of the BPA in expression of genes and proliferation of cancer cells by stimulating the MAPK signaling pathway [22]. It has been shown that the BPA can affect the expression of Fkbp5 gene and its methylation, indicating its effective role on stress responses in cells [23].

The impact of the BPA on antioxidant activity of genes in the liver was also examined through Real Time PCR method. The results showed that the BPA causes the production of ROS which can significantly reduce the expression of antioxidant genes, leading to liver toxicity [24]. A study conducted on rats, which were exposed to doses of 0.1, 3, and 10 mM of inorganic arsenic for 72 hours, showed an increment in HO-1 gene expression [12]. It was also shown

that HO-1 is a good biomarker to detect and define arsenic cell toxicity [25]. In another study on rats treated with various doses of the BPA (0.1, 1, 10, and 50 mg/kg), the compound led to changes in expression levels of antioxidant genes of glutathione, peroxidase (GSHPx), catalase (CAT), glutathione transferase (GST), and glutathione reductase (GR) in liver tissue. Expression of these antioxidant genes was decreased with increasing doses of the BPA. However, the impact of these doses of the BPA on biomarkers of oxidative stress showed a different result. With increasing doses of the BPA, the levels of TBARS and NO (x) were increased and GSH and SOD decreased [24].

Another result obtained in the present study showed that different doses of the BPA increased GADD45B gene expression compared to the control. This increased gene expression was significantly evident at the lowest doses (5 µg/kg), but the gene expression was decreased in larger doses of the BPA compared to the low doses. Although an increased expression of GADD45B gene was shown in comparison to the control group, this increment was not statistically significantly the highest doses of the BPA. One of the results of this study is the positive effect of expression of this gene on cell apoptosis. When the rats received higher doses of the BPA, Gadd45 gene expression is increased first. Increased expression of Gadd45 gene stimulates apoptosis and the percent of viable cells for expression of this gene is being decreased gradually. As a result, with increasing doses of the BPA, gene expression followed a decreased trend. In the case of HO-1 gene, no study has reported changes in GADD45B gene expression after administering BPA in rats.

5. Conclusion

According to the results, it is concluded that bisphenol A (BPA) increased expression of HO-1 gene more than Gadd45 gene at high doses. The BPA can induce dose-dependent liver damage. Increased reactive oxygen species (ROS) and oxidative reactions may be responsible for the toxicity.

Competing Interests

The authors declare that there are no competing interests.

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References

- [1] N. Ben-Jonathan and R. Steinmetz, "Xenoestrogens: the emerging story of bisphenol A," *Trends in Endocrinology & Metabolism*, vol. 9, no. 3, pp. 124–128, 1998.
- [2] C. Liu, W. Duan, R. Li et al., "Exposure to bisphenol A disrupts meiotic progression during spermatogenesis in adult rats through estrogen-like activity," *Cell Death and Disease*, vol. 4, article e676, 2013.
- [3] C. E. Talsness, A. J. M. Andrade, S. N. Kuriyama, J. A. Taylor, and F. S. V. Saal, "Components of plastic: experimental studies in animals and relevance for human health," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 364, no. 1526, pp. 2079–2096, 2009.
- [4] S. Mortazavi, A. Riyahi Bakhtiari, A. E. Sari, N. Bahramifar, and F. Rahbarizade, "Phenolic endocrine disrupting chemicals (EDCs) in Anzali Wetland, Iran: elevated concentrations of 4-nonylphenol, octylphenol and bisphenol A," *Marine Pollution Bulletin*, vol. 64, no. 5, pp. 1067–1073, 2012.
- [5] M. A. Kamrin, "Bisphenol A: a scientific evaluation," *MedGenMed*, vol. 6, no. 3, article 7, 2004.
- [6] M. K. Moon, M. J. Kim, I. K. Jung et al., "Bisphenol A impairs mitochondrial function in the liver at doses below the no observed adverse effect level," *Journal of Korean Medical Science*, vol. 27, no. 6, pp. 644–652, 2012.
- [7] A. Kourouma, C. Quan, P. Duan et al., "Bisphenol A induces apoptosis in liver cells through induction of ROS," *Advances in Toxicology*, vol. 2015, Article ID 901983, 10 pages, 2015.
- [8] V. Bindhumol, K. C. Chitra, and P. P. Mathur, "Bisphenol A induces reactive oxygen species generation in the liver of male rats," *Toxicology*, vol. 188, no. 2-3, pp. 117–124, 2003.
- [9] M. McMillian, A. Y. Nie, J. B. Parker et al., "A gene expression signature for oxidant stress/reactive metabolites in rat liver," *Biochemical Pharmacology*, vol. 68, no. 11, pp. 2249–2261, 2004.
- [10] L. A. Videla, "Oxidative stress signaling underlying liver disease and hepatoprotective mechanisms," *World Journal of Hepatology*, vol. 1, no. 1, pp. 72–78, 2009.
- [11] Z. K. Hassan, M. A. Eloheid, P. Virk et al., "Bisphenol A induces hepatotoxicity through oxidative stress in rat model," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 194829, 6 pages, 2012.
- [12] J. Liu, L. Yu, E. J. Tokar et al., "Arsenic-induced aberrant gene expression in fetal mouse primary liver-cell cultures," *Annals of the New York Academy of Sciences*, vol. 1140, no. 1, pp. 368–375, 2008.
- [13] M. Iida, C. H. Anna, W. M. Holliday et al., "Unique patterns of gene expression changes in liver after treatment of mice for 2 weeks with different known carcinogens and non-carcinogens," *Carcinogenesis*, vol. 26, no. 3, pp. 689–699, 2005.
- [14] J.-H. Kim, A. Qu, J. K. Reddy, B. Gao, and F. J. Gonzalez, "Hepatic oxidative stress activates the Gadd45b gene by way of degradation of the transcriptional repressor STAT3," *Hepatology*, vol. 59, no. 2, pp. 695–704, 2014.
- [15] C. A. Piantadosi, C. M. Withers, R. R. Bartz et al., "Heme oxygenase-1 couples activation of mitochondrial biogenesis to anti-inflammatory cytokine expression," *Journal of Biological Chemistry*, vol. 286, no. 18, pp. 16374–16385, 2011.
- [16] C. C. Willhite, G. L. Ball, and C. J. McLellan, "Derivation of a bisphenol A oral reference dose (RfD) and drinking-water equivalent concentration," *Journal of Toxicology and Environmental Health Part B: Critical Reviews*, vol. 11, no. 2, pp. 69–146, 2008.
- [17] T. E. Haavisto, N. A. Adamsson, S. A. Myllymäki, J. Toppari, and J. Paranko, "Effects of 4-tert-octylphenol, 4-tert-butylphenol, and diethylstilbestrol on prenatal testosterone surge in the rat," *Reproductive Toxicology*, vol. 17, no. 5, pp. 593–605, 2003.
- [18] R. E. Chapin, J. Adams, K. Boekelheide et al., "NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A," *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, vol. 83, no. 3, pp. 157–395, 2008.

- [19] B. S. Rubin, M. K. Murray, D. A. Damassa, J. C. King, and A. M. Soto, "Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels," *Environmental Health Perspectives*, vol. 109, no. 7, pp. 675–680, 2001.
- [20] K. Yamasaki, M. Sawaki, S. Noda, N. Imatanaka, and M. Takatsuki, "Subacute oral toxicity study of ethynylestradiol and bisphenol A, based on the draft protocol for the 'Enhanced OECD Test Guideline no. 407,'" *Archives of Toxicology*, vol. 76, no. 2, pp. 65–74, 2002.
- [21] Y. L. Tang, Y. Tang, Y. C. Zhang, K. Qian, L. Shen, and M. I. Phillips, "Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector," *Journal of the American College of Cardiology*, vol. 46, no. 7, pp. 1339–1350, 2005.
- [22] H.-C. Lan, I.-W. Lin, Z.-J. Yang, and J.-H. Lin, "Low-dose bisphenol A activates *Cyp11a1* gene expression and corticosterone secretion in adrenal gland via the JNK signaling pathway," *Toxicological Sciences*, vol. 148, no. 1, pp. 26–34, 2015.
- [23] E. Kitraki, I. Nalvarte, A. Alavian-Ghavanini, and J. Rüegg, "Developmental exposure to bisphenol A alters expression and DNA methylation of *Fkbp5*, an important regulator of the stress response," *Molecular and Cellular Endocrinology*, vol. 417, pp. 191–199, 2015.
- [24] Z. K. Hassan, M. A. Elobeid, P. Virk et al., "Bisphenol A induces hepatotoxicity through oxidative stress in rat model," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 194829, 6 pages, 2012.
- [25] J. Liu, M. B. Kadiiska, Y. Liu, T. Lu, W. Qu, and M. P. Waalkes, "Stress-related gene expression in mice treated with inorganic arsenicals," *Toxicological Sciences*, vol. 61, no. 2, pp. 314–320, 2001.

Review Article

The Reactive Oxygen Species in Macrophage Polarization: Reflecting Its Dual Role in Progression and Treatment of Human Diseases

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High heterogeneity of macrophage is associated with its functions in polarization to different functional phenotypes depending on environmental cues. Macrophages remain in balanced state in healthy subject and thus macrophage polarization may be crucial in determining the tissue fate. The two distinct populations, classically M1 and alternatively M2 activated, representing the opposing ends of the full activation spectrum, have been extensively studied for their associations with several disease progressions. Accumulating evidences have postulated that the redox signalling has implication in macrophage polarization and the key roles of M1 and M2 macrophages in tissue environment have provided the clue for the reasons of ROS abundance in certain phenotype. M1 macrophages majorly clearing the pathogens and ROS may be crucial for the regulation of M1 phenotype, whereas M2 macrophages resolve inflammation which favours oxidative metabolism. Therefore how ROS play its role in maintaining the homeostatic functions of macrophage and in particular macrophage polarization will be reviewed here. We also review the biology of macrophage polarization and the disturbance of M1/M2 balance in human diseases. The potential therapeutic opportunities targeting ROS will also be discussed, hoping to provide insights for development of target-specific delivery system or immunomodulatory antioxidant for the treatment of ROS-related diseases.

1. Introduction

Accumulating studies have implied the physiological role of reactive oxygen species (ROS) in various biological processes at distinct levels, for example, gene expression, protein translation, posttranslational modification, and protein interactions. Instead of merely being a harmful byproduct of metabolism, cell-derived ROS majorly derived from hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (OH^\cdot), is an independent or cooperative regulator for cellular signalling in response to environmental cues. For instance, H_2O_2 , due to its long-lived nature and ability to easily pass through cellular membrane, is an important secondary messenger in maintaining the cellular homeostasis under different conditions [1]. It may result in activation or shutdown of diverse cellular processes such as cell cycling,

chromatin remodeling, DNA repair, cell differentiation, and self-renewal [2]. The balance of intracellular ROS is therefore extremely important in maintaining normal physiology of human beings. While mitochondrial respiratory chain is the major component that cells used to produce intracellular ROS, cells develop a series of antioxidant enzymes to reduce redundant ROS. These enzymes, including catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR), can either grossly or specifically catalyze different types of ROS. The presence of other endogenous antioxidants such as peroxiredoxins and thioredoxins ensures critical monitoring of cellular ROS level and conserves the normal redox homeostasis. In some pathological conditions, the redox balance is disturbed when the intracellular redox system is shifted to oxidized state, which is defined as oxidative stress. During oxidative stress,

cells produce oxidized lipids, proteins, and damaged DNA which would consequently lead to cell death and tissue damage. In this regard, sustained oxidative stress has been linked with a series of human progressive diseases such as hepatitis, diabetes, and cancers.

Macrophages are long-lived innate immune cells that ubiquitously populate in almost all tissues. The general source of macrophages is bone marrow derived monocytes, which migrate to various tissues and adhere to become mature macrophages. However, studies also revealed that macrophages can be presented bypassing monocytic stages. In many human diseases, macrophages detect tissue injury or infections and process the damage or remodeling of wounded tissues [3]. Macrophages also sense the unfavourable conditions within human body including hypoxic and metabolic stresses where response of host defense and immune regulation are triggered [4, 5]. All these propose macrophages as an important regulator in multiple biological processes, including innate and adaptive immunity, angiogenesis, reproduction, and even malignancy [6]. The multiple function of macrophage is facilitated by its high plasticity in response to environmental- or self-derived stimulating signals [7]. This macrophage heterogeneity is reflected by functional polarization of differentiated macrophages and the dynamic switch between phenotypes. Under physiological condition, the M1/M2 population remains in mixture state while disoriented shift from M1 to M2 or vice versa results in disease progression. Multiple signalling pathways and several cellular stimuli, such as cAMP [8], phospholipid [9], and irons [10], have been found involved in the functional polarization of macrophages. To our knowledge, there are not many studies that highlighted the essential role of ROS in regulating the functional polarization of macrophages. Thus, in this review, we discussed the involvement of ROS in macrophage reprogramming. The biology of macrophage polarization, disturbance of M1/M2 balance in human diseases, and the role of ROS in macrophage homeostatic functions were addressed. How ROS drives macrophages polarization and the potential therapeutic opportunities targeting ROS were specifically reviewed and discussed.

2. Biology of Macrophage Polarization

One of the important hallmarks of macrophage is its high heterogeneity, allowing them to be activated to different functional statuses with particular properties upon exposure to endogenous and exogenous inducers in the microenvironment. Mirroring T_H1/T_H2 programming, macrophage could be reprogrammed to classically activated (M1) and alternatively activated (M2) subsets in response to the surrounding stimuli. Classically activated M1 macrophages are characterized by its high microbicidal function associated with the ability to secrete high amount of proinflammatory cytokines such as interleukin-1 β (IL-1 β), IL-12, tumor necrosis factor- α (TNF- α), and superoxide anions. As to alternatively activated M2 macrophages, their functionality is distinct from the classical one which produced high levels of anti-inflammatory mediators IL-10 and tumor growth factor- β (TGF- β) and express cell surface markers such as mannose

receptor (MR/CD206) and scavenger receptor (SR/CD163), which mainly participate in tissue remodeling, parasitic clearance, and resolution of inflammation. In healthy subject, macrophage remains in M1/M2 mixture state; therefore macrophage polarization is important to determine tissue fate. The switch of macrophages phenotypes towards extreme state of either M1 or M2 over time may result in several disease progressions such as obesity, cancer, and rheumatoid arthritis. Therefore, understanding the functionality of M1 and M2 macrophages and its role in disease progression may be crucial.

2.1. M1: The Classically Activated Macrophage. The major homeostatic function of naive macrophages is clearance of apoptotic debris, produced during the cellular process. In response to various endogenous danger signals, macrophage physiology is altered followed by production of proinflammatory mediators and modification of surface markers. Classical M1 macrophages could be activated by either interferon- γ (IFN γ) secreted by NK cells and adaptive T helper 1 (T_H1) cells or pathogen-associated molecular patterns (PAMPs) released by microorganisms through Toll-like receptor (TLRs) via MyD88 dependent manner [11]. Meanwhile, stimulation of TLR3 and TLR4 may also activate MyD88 independent pathway that induces IFN β secretion. This pathway is mediated by Toll/IL-1 receptor (TIR) domain-containing adaptor and knockdown of TIR led to blockade of TLR4 mediated inflammation [12].

The activation of classical M1 macrophages induces large amount of inflammatory genes and chemokines secretion which facilitates the antigen presentation and recruitment of T_H1 response for subsequent pathogen killing activity. Recent studies established the metabolic characteristics for M1 and M2 macrophages, suggesting that the macrophage metabolism under pathological condition also governs the functional phenotypic changes of macrophages [13]. Activation of M1 macrophages is associated with upregulation of iron storage protein H ferritin and reduction of ferroportin, leading to iron retention and inflammation [14]. The glycolytic flux is favourable in classical M1 macrophages phenotype that involves the expression changes of 6-phospho-2-kinase isoforms from liver-type to more active ubiquitous isoenzyme, thereby maintaining the level of fructose-2,6-bisphosphate [15]. Besides, M1 macrophage is associated with higher aerobic glycolysis and extracellular acidification rate [16]; increase of HIF-1 α further associates with IL1 β promoter and maintains IL1 β production in M1 macrophages. Through regulating glycolytic flux, blockade of carbohydrate kinase-like protein CARL triggers M1 polarization [17] while pyruvate dehydrogenase kinase 1 PDK1 promotes aerobic glycolysis in M1 macrophages [18]. Apart from glucose metabolism, activation of M1 macrophages is accompanied by increase of COX-2 and reduction of COX-1, thromboxane A synthase 1, arachidonate 5-lipoxygenase, and leukotriene A4 hydrolase [19].

2.2. M2: The Alternative Activated Macrophage. In contrast to classical activated macrophages, innate immune cells such

as basophils and mast cells and other adaptive cells produce IL-4 and IL-13 priming M2 alternative phenotype. IL-4 induced M2 macrophages expressed high concentration of IL-10, decoy receptor IL-1R, IL-1R antagonist, chemokines CCL22 and CCL17, and intracellular enzyme arginase-1. All of these ensue the recruitment and activation of T_H2 immune response and immune-suppressive function of M2 macrophages. In addition to T_H2 immune response, IL-4 induced macrophages stimulate arginase activity by converting arginine to polyamines and collagen precursors that are crucial for tissue modeling and wound healing. M2 macrophages also produce VEGFA, EGF, and IL-8 that are responsible for angiogenesis and lymphangiogenesis [7]. Apart from IL-4 induced phenotype, different schemes of M2 macrophages classification have been proposed due to the overlapping properties of alternative activated macrophages. The activation of M2 macrophages stimulated by addition of either IL-4 and IL-13, TGF β , immune complexes, glucocorticoids, or IL-10 may yield distinct activation profiles [20].

Cellular metabolism especially lipid metabolism also incurs important role in providing energy fuel for activation of alternative M2 macrophages. In iron metabolism, M2 macrophages increased ferroportin expressions, which further induced iron export [14]. As opposed to M1 classical activation, M2-regulated gene transcription occurs in condition favouring of mitochondrial metabolism and oxidative glucose metabolism, in which the M2 phenotype tends to be switched towards proinflammatory state under low oxygen condition [16]. In lipid metabolism, M2 macrophage activation is associated with fatty acid oxidation and its uptake. M2-secreted lysosomal acid lipase as well as its scavenger receptor CD36 facilitates uptake of triglycerols, LDL, and VLDL for β -oxidation and fatty acid generation [21]. Apart from LDL and VLDL, high density lipoproteins promote anti-inflammatory function through ATF3 dependent pathway [22]. Study by Prieur et al. [23] also postulated that short and saturated fatty acids favour M1 polarization, while longer and unsaturated fatty acids induce M2 anti-inflammatory phenotype. Therefore, M2 activation is accompanied by upregulation of arachidonic acid [19], omega-3 polyunsaturated fatty acids [24], sphingosine 1-phosphate [25], eicosapentaenoic acid, and docosahexaenoic acid [26]. All the correlation of alternative M2 activation with lipid metabolism also provides clue for the important role of M2 activation in atherosclerosis, obesity, and insulin sensitivity (as discussed later).

2.3. Disruption of M1/M2 Balance in Human Diseases. Classical M1 macrophages elicit major role in inducing inflammation and clearing of pathogens, whereas alternative M2 macrophages resolve inflammation and are crucial for the functions of tissue modeling and wound healing. They represent the opposing ends of the activation spectrum, and either accumulation of M1/M2 signals may lead to deterioration of the host. Extensive production of IL1, IL6, and IL23 by M1 macrophages incurs activation of T_H17 immune response and leads to autoimmune disorder progression [27]. Earlier studies may postulate that macrophage populations promote

disease progression but many recent studies have proposed that the proportions of different macrophage phenotypes contribute to disease progression. There is no absolute answer on which phenotype is “good” or “bad” as the switch of resident or recruited macrophages towards M2 phenotype may trigger tumor progression while accumulation of M1 macrophages leads to insulin resistance and atherosclerosis. Therefore, continuous activation of either states brings harm to the host and the understanding of how disruption of M1/M2 in regulating diseases may be essential. The involvement of M1/M2 in obesity, atherosclerosis, and cancer is briefly discussed below; the comprehensive reviews on human diseases have been published elsewhere [28–30].

2.3.1. Obesity and Insulin Resistance. Adipose tissue macrophages (ATM) are predominantly alternative M2 phenotype in lean subjects, and the ATM are reprogrammed to classically activated M1 phenotype with proinflammatory function upon exposure to free saturated fatty acids produced by adipose tissue, resulting in reduced insulin sensitivity in white adipose tissue. IL-10 secreted by M2 macrophages may be the responsible cytokine that protects adipocytes from obesity leading insulin resistance [31]. The free fatty acids will also trigger production of CCL2 by adipocytes and ATM in order to promote the recruitment of proinflammatory monocytes to the inflamed tissue [32]. Knockout of CCR2 in murine model effectively ameliorated the adipocyte inflammation, macrophage infiltration, and insulin resistance in obese mice [33].

2.3.2. Atherosclerosis. Atherosclerosis develops with the accumulation and trapping of low density lipoproteins (LDL) in the intima of the arteries, and the modified LDL-induced secretion of adhesion molecules by endothelial cells recruits monocytes with proinflammatory phenotype to atherosclerotic lesion, which eventually differentiate into lipid-laden foam cells [34]. Cholesterol crystal, IFN γ , LPS, and oxidized LDL are known to stimulate M1 activation and M1-activated cytokines further support the monocyte recruitment and macrophage retention at the plaque area [35]. Due to the involvement in plaque destabilization, the proatherogenic M1 populations are predominantly accumulated at the plaques where they are prone to rupture. As for M2 macrophages, they mostly reside at stable-cell enriched plaque and adventitia. M2 macrophages tend to prevent foam cell formation and protect against atherosclerosis.

2.3.3. Cancer. The growth and expansion of malignant cells is complex, which involves gene manipulation as well as establishment of microenvironment that favours tumor progression. Many recent studies have highlighted the role of tumor-associated macrophages in promoting cancer growth, angiogenesis, invasion, migration, and T cell suppression [36]. In most cancers, macrophages residing in tumor microenvironment exhibit M2 phenotype with immunosuppressive property. In contact with tumor cells, M2 macrophages tend to derive certain substances such as various growth factors,

chemokines, and proteases that maintain tumor growth and expansion [37]. Apart from mediating tumor growth and progression, M2 macrophages interact with other immune cells and suppress innate and adaptive antitumor immune response. Depending on the tumor environment, phenotype of tumor-associated macrophages would be reprogrammed to M1-like phenotype, which is characterized by the antigen presenting property and tumoricidal function that favours tumor regression [38]. M1 macrophages activation increases expressions of mediators that are responsible for antigen presentation and costimulation; this may further promote infiltration of neutrophils to the tumor area leading to neutrophil-targeted tumor regression [39].

3. Reactive Oxygen Species on Homeostasis Function of Macrophages

3.1. ROS in Regulating Phagocytosis of Macrophages. Numerous studies have unveiled the diverse regulations of ROS on the phagocytosis function of macrophage. The regulation may be controversial, but the discrete role of ROS on macrophages may be impacted by the different sources of ROS as well as plasticity of macrophage itself, which would be discussed in detail below. As macrophages are endogenous scavengers for dying cells in various pathological conditions, interaction between macrophages with compartments determines the phagocytic function of macrophages. Dying cells produce high levels of ROS, which are released into extracellular area when cellular membrane is degraded during cell death. Attachment of dying cells to macrophages requires intercellular communication in which ROS may play a role. On the other hand, extracellular and intracellular ROS may differentially control the phagocytosis process of macrophage by regulating the ability and capacity of macrophages in the uptake and degradation of dying compartments. In this regard, ROS plays a critical regulatory role in determining the initiation and outcome of cellular phagocytosis.

3.1.1. The Role of ROS from Dying Cells during Phagocytosis. Engulfment of cells undergoing apoptotic programmed cell death (PCD) by macrophage is initiated by the presentation of membrane signals to phagocytes that allows recognition of dying compartments. In macrophage-driven phagocytosis, these molecules include scavenger receptors, for example, CD36, immunoglobulin super-family molecules, CD31, complement receptors, such as C91, sugar and phospholipid-engaging molecules, for example, lectins and PSR, and some integrins such as $\alpha_v\beta_3$. The corresponding components on cellular membrane of apoptotic cells were less identified, but structures encompass lipid, carbohydrate, and protein which are exposed as molecules presenting find-me signals towards extracellular area during cell apoptosis [40]. These lipids and proteins presented on membranes of apoptotic cells are generally regarded as substrates of oxidation reaction containing dying cell-produced ROS. Indeed, experimental evidence has shown that oxidation of lipids and proteins by ROS confers recognition and attachment of macrophage towards

apoptotic cells. By using monoclonal antibody that blocks the epitopes of oxLDL of apoptotic cells, scientists observed failure of engulfment of dying cells by murine peritoneal macrophages [41]. It was further observed that surface presentation of oxidative modified phosphatidylserines (PS) on apoptotic cells is essential for macrophage engulfment [42]. The phagocytosis was blocked upon presence of lipoprotein-associated phospholipase A2 (Lp-PLA2), a secreted enzyme with high specificity towards PS metabolites. Oxidation of PS is often observed during cell apoptosis [43]. This gives rise to the mechanism underlying attenuated phagocytosis of etoposide-treated cells by macrophage as etoposide is able to suppress oxidation and externalization of PS of the apoptotic cells [44]. In addition, some phosphatidylcholine (PC) species, which are oxidized during early- to late-apoptotic process, present to the surface of apoptotic cells are recognized by C-reactive proteins, facilitating clearance of apoptotic cells by macrophage engulfment [41]. Study revealed that oxidation of PC (oxPC) was majorly processed by ROS, in virus-infected cells, and scavenging of ROS by NAC abrogated oxPC production [45]. Although macrophage receptors correspond to particular type of oxidized lipids and proteins have not been fully unveiled, all these studies have paved the importance of ROS-driven oxidation reaction in dying cells during initiation of phagocytosis.

3.1.2. The Role of Macrophage ROS in Regulating Phagocytosis. Besides dying cells, macrophage itself also produces intracellular ROS that is involved in the phagocytic process. It is a notion that ROS in macrophage is essential for uptake and clearance of apoptotic cells; however, maintaining high level of ROS may be harmful to macrophage as, in some studies, inducible ROS is sufficient to cause macrophage apoptosis [46]. Hypothesis of adaptive mechanism underlying survival of macrophage in high ROS condition was ever discussed, which included increase expressions of DNA repair proteins [47] and endogenous antioxidative enzymes [48] during monocyte-macrophage differentiation and classical activation of macrophage. Production of ROS by macrophage majorly relies on Nox2 gene, and study showed that activation of Nox2 gene in murine macrophage cell line RAW264.7 as well as primary peritoneal macrophages by carotenoid lutein induced ROS production that was responsible for the increased phagocytic activity [49]. Additionally, production of mitochondrial ROS (mROS) is able to increase phagocytosis of macrophages. In mROS-driven phagocytosis, intracellular fatty acids are utilized as fuel for oxidative phosphorylation by mitochondria-localizing enzyme encoded by Immuno-responsive gene 1 (IRG1). β -oxidation of fatty acids is associated with mROS production and augments the bactericidal activity of macrophages [50]. These observations give rise to the critical role of intracellular ROS in clearance of apoptotic cells by phagocyte. Besides, NO produced by phagocytosing macrophages is important for PS externalization of dying cells [51]. And this was further proved by the notion that phagocytosis entry requires class I PI3K product phosphatidylinositol 3,4,5-trisphosphate-induced ROS production in murine macrophages [52]. In fact, phagocytosis of macrophages requires Nox2-dependent

production of extracellular ROS [53], and clinical evidence of essential role of ROS was noted in chronic granulomatous disease patients, who lack Nox2, owning macrophages that failed to efficiently engulf apoptotic cells [54].

Additionally, oxidative burst is also required in the clearance of apoptotic cells by alternatively activated macrophages during wound healing process [53]. However, the increase of ROS in macrophage during early stage of apoptotic cell clearance is followed by attenuation of oxidative burst by PPAR γ activation. In PPAR γ -mutant macrophages, ROS level was restored due to constituting activation of PKC pathway during apoptotic cell clearance [55]. Further study revealed that resolvin D1 (RvD1), one of endogenous proresolving lipid mediators derived from docosahexaenoic acid, is able to inactivate Nox2 and inhibits production of ROS after engulfment of apoptotic cells by macrophages, which prevents macrophage from apoptotic cell death [56]. This indicates that ROS increase in macrophage is transient during phagocytic process of apoptotic cells. Indeed, intracellular level of ROS within macrophages can be triggered by cells undergoing apoptosis or necrosis. This oxidative burst system helps macrophages to recognize necrotic cells whose clearance requests an inflammatory reaction. In necrosis-related cell death, dying cells release high concentration of high-mobility group box1 (HMGB1), which triggers inflammatory response in macrophages [57]. Other molecules like calgranulins and adenosine triphosphate derived from necrotic cells trigger Nox2 activation in macrophage and produce more ROS [58, 59]. On the contrary, engulfment of apoptotic cells inhibits persistent ROS production thereby preventing activation of a secondary inflammation that is harmful to any bystander cells. This indicates a host response of engulfed compartment in the extent of oxidative burst. On the other hand, pathogens are able to develop mechanism in responding to macrophage-derived ROS, which was observed from the induced arginine-biosynthetic genes in *C. albicans* [60]. In summary, the phagocytosis process of macrophage can be regulated by ROS, which involves responses of host and engulfed cells towards oxidative burst.

3.2. ROS in the Control of Death of Macrophage. Increase of ROS during the differentiation and phagocytosis of macrophages may be harmful to the cells. Although increased levels of DNA repair proteins and ROS reductase in macrophage make it become highly ROS-resistant in fighting against overwhelmed intracellular ROS level [47, 48], it is still not completely prevented from ROS-associated death. Death of macrophage may not be favourable during diseases treatment, as dying macrophages may induce secondary response of necrotic cell death, which includes release of proinflammatory cytokines and proteolytic factors that further activate inflammation [61]. High level of circulating heme has been demonstrated to induce necrotic cell death of macrophage, which is associated with increased intracellular ROS level. And such macrophage death further affects the intracellular infection control of, for example, malaria and sepsis [62]. Study has revealed that a tissue damaging agent methemoglobin has toxicity towards murine macrophages

by increasing the ROS production. In this case, peripheral presentation of methemoglobin may lead to multiple tissues damage as well as immunosuppression [63]. These observations reveal that an overwhelmed ROS level in macrophage may lead to diseases-associated cell death. In fact, induction of cell death by ROS can be primarily due to Nox2 activity on phagosomes [64], as well as secondary responses towards several extracellular and intracellular factors. It was found that extracellular oxidatively modified high density lipoprotein induces ROS level in human-derived macrophage lineage cells, which is associated with macrophage death [65]. This was similarly observed in human-derived macrophages treated with oxidatively modified low density lipoproteins (oxLDL) [66], the mechanism of which may involve activation of Nox by lysophosphatidylcholine, a side product of LDL oxidation [67]. Interestingly, the cell death-inducing effect is not observed in either naive HDL or LDL. The phenomenon is commonly observed in atherogenesis, in which the persistent macrophage foam cell death and efferocytosis drive the formation of advanced lesions. Other factors that could result in ROS-associated cell death in macrophage include cytokines [68] and free cholesterol [69]. Additionally, some studies revealed that ROS-associated ER stress may lead to macrophage death upon being challenged by *Mycobacterium tuberculosis* and *Mycobacterium kansasii* [70, 71], which may be related to the presence of their heparin-binding haemagglutinin antigen [72], indicating that the ER stress may be the downstream event of elevated ROS in dying macrophage. Increased ER stress subsequently altered calcium homeostasis and activated Nox-mediated ROS formation, which eventually led to death of macrophages [61].

3.3. ROS on Monocyte Recruitment. The circulating monocytes, which derive from hematopoietic stem cells in the bone marrow and migrate to peripheral blood, have the capacity of differentiating into tissue macrophages. This is generally considered as the major population of macrophages involved in pathophysiological development of human diseases. However, tissue macrophages have distinct mechanisms of hematopoiesis, and embryonic macrophages even bypassed monocytic stages [73]. The diversity in strain of macrophages was reflected by their name in particular organs and tissues, for example, Kupffer cells in the liver and microglia in the brain. It is a notion that, during disease progression that involves inflammatory response, inflammatory monocytes are developed and exhibited migratory capacity towards primary sites of the diseases. Deficiency of CX₃CR₁ in mice suppressed the activation of monocyte-derived macrophages in periphery and reduced macrophage infiltration and microglia proliferation, which subsequently attenuated brain ROS level and neuron apoptosis [74]. This gives rise to the notion that oxidative stress may have correlation with monocyte recruitment in human diseases. Indeed, it was found that H₂O₂ may serve as chemoattractant to monocytes, as evidenced by the observation in zebrafish larvae whose wound produced H₂O₂ to facilitate rapid macrophage recruitment [75]. Overexpression of UCP2, which relieved oxidative stress and intracellular ROS

level of THP1 human monocytes, further reduced monocyte migration and adhesion through cellular monolayer [76]. Oxidative burst is therefore regarded as a favouring environment for monocyte activation. Previous study showed that ROS triggered CCL2-induced hyperalgesia in rats, which is attenuated in the presence of SOD confirming the role of ROS as facilitator in monocyte recruitment [77]. The authors also found that ROS level is not elicited in response to CCL2 in monocytes/macrophages with fewer expressions of CCL2 receptor, which indicated that intracellular ROS level may have an independent role in triggering recruitment of monocytes.

Moreover, metabolic stress may trigger expression of Nox4 in monocytes, increased intracellular H_2O_2 production, and thereby accelerated THP-1 monocyte migration [78]. The study also further explained why high fat diet-induced metabolic stress in mice had higher rate in monocyte chemotactic activity. The localization of Nox4 around focal adhesion and actin cytoskeleton of human-derived macrophages, together with the association with adhesion related proteins, supports the role of ROS in mediating macrophage motility [79]. The study also further postulated that ER stress stimulated THP-1 monocytes have augmented adhesion ability, and deletion of Nox4 blocked this activity. This further claimed that ER stress induced ROS production may be an endogenous factor in facilitating monocyte chemotaxis. Using Nox inhibitor apocynin or antioxidant catalase, recruitment of monocytes into atherosclerotic lesion was reduced, which suggested the central role of Nox-derived ROS in monocyte recruitment in atherosclerosis [80]. Very interestingly, ROS is associated with macrophage death (as discussed previously) driven by oxLDL and HDL in atherosclerosis; and also monocyte accumulation in diseased mice model proposes ROS as an important regulator for atherosclerosis progression.

Although the reported studies have highlighted ROS as a critical mediator of monocyte recruitment, the contribution of ROS as a chemoattractant of monocytes compared to others, as well as the factors that raise intracellular ROS in monocytes during pathophysiological process, remains unanswered. Previous study proposed that ROS-sensitized monocytes may have higher chemotactic response towards chemoattractants CCL5, CCL2, and PDGF- β [78], yet the study may not directly delineate the role of ROS in monocyte recruitment. There are few explanations for the difficulties of this investigation in deriving the cause-effect relationship: first, the reported models are insufficient to show the correlation of ROS and monocyte recruitment. Blocking ROS may drive the inhibition of other chemoattractants that further reduced monocyte motility; second, it is not clear whether increased monocyte recruitment is the consequences of enhanced monocyte proliferation and accumulation; thus the role of ROS in monocyte recruitment, accumulation, and proliferation needs to be established; third, monocyte is hypersensitive to ROS. Monocytes undergo extensive cell death in response to ROS in dose- and time-dependent manner [47]; proposed high levels of ROS are fatal to monocytes themselves. Therefore, the role of ROS in monocyte recruitment needs to be further deciphered.

3.4. The Role of ROS on Monocytes-to-Macrophages Differentiation. Studies have revealed that ROS drives monocytes-to-macrophages differentiation in *in vitro* culture of various types of monocytic cells, regardless of mouse or human origin. It was observed in human promyelocytic leukemia cell lines that production of ROS was induced in line with increased expression of macrophage marker CD14 during 1 α , 25-dihydroxyvitamin D (3) (VD3) induced differentiation. Regulation of monocyte differentiation is mediated through induction of ROS that further increases 5-lipoxygenase along with p38 MAPK activation [81]. In human promonocytic cell line U937, production of ROS was accelerated during macrophage differentiation induced by phorbol ester (PMA). This event was driven by NADPH oxidase, and it was observed that the persistent induction of Cox-2 along with monocyte differentiation was blocked by Nox inhibitors, suggesting the critical role of Nox in functional activation of proinflammatory gene Cox-2 [82]. Generation of ROS which serves as defense against invading microbes is regarded as the hallmark of monocyte/macrophage activation, though it is still not clear if ROS production directly results in macrophage maturation [47]. It was observed in the latter study that elimination of ROS by butylated hydroxyanisole (BHA) and other ROS inhibitors completely blocked monocyte/macrophage differentiation [83]. NADPH oxidase, in particular, is the molecule that primed to produce more ROS during macrophage differentiation. It is a typical ROS generator, and its expression as well as translocation to plasma membrane was induced by 1,25-dihydroxyvitamin D3 in human myeloid leukemia PLB-985 cell in line with increased surface markers CD11b and CD36 during macrophage differentiation [84]. The study also included *in vivo* intraperitoneal thioglycolate injection-induced mouse peritoneal macrophages model and findings showed that increased macrophage maturation is associated with enhanced cellular capacity in oxidizing LDL.

In lipopolysaccharide- (LPS-) induced macrophage differentiation model, it was observed that ROS production is essential in THP-1 differentiated macrophages for activation of HIF-1 α and acquired adaptive ability in hypoxic microenvironment of inflammatory site [85]. It is therefore reasonable that high ROS level is associated with diseased environment, mainly because ROS facilitates the differentiated macrophages survival under hypoxic condition. A similar observation is made on 12-O-tetradecanoylphorbol-13-acetate- (TPA-) induced THP-1 cells in which activation of NADPH oxidase-derived ROS is associated with monocytic differentiation [86]. Interestingly, TPA was found to decrease expression of endogenous antioxidant enzyme catalase during human U937 macrophage differentiation. By applying catalase in TPA-treated monocytes, the differentiation process as well as ROS production was blocked [87], further confirming the role of ROS in mediating macrophage differentiation. By silencing the important endogenous redox homeostasis regulator NF-E2-related factor 2 (NRF2) in PMA-induced U937 cells, the ROS level is maintained at high level during macrophage differentiation and this was followed by higher expressions of proinflammatory

cytokines [88]. Additionally, inactivation of oxidative stress-quenching molecule PPAR γ during saturated fatty acid-induced macrophage differentiation supports maintenance of high intracellular ROS level [89], and this process was found to involve an induced *de novo* synthesis of endogenous PPAR γ inhibitor ceramide [90]. Palmitate, the unsaturated fatty acid intervention, triggered ceramide production in macrophages and further derived the mitochondrial superoxide production that facilitates macrophage differentiation [91]. Interestingly, it was also reported that, in unprimed macrophages, differentiation towards alternative activated phenotype is ROS-dependent but not in classical activation of macrophages [83]. Consistent with the study, several studies also suggested that ROS-induced maturation of macrophages is associated with upregulation of proinflammatory gene expression [82, 88], the hallmark of classically activated macrophages. Though it is reasonable as macrophage is mainly present in inflammation site, this further renders the questions of correlation between ROS, inflammation, and macrophage maturation. Further discussion would be made in the latter section. Taken together, all these evidences have implied a reprogrammed redox homeostasis in differentiating macrophage from monocytes in terms of maintaining ROS level facilitating cell maturation.

4. Role of ROS in Macrophage Polarization

4.1. ROS Promotes M1. As mentioned above, M1 macrophages possess a high bactericidal function and defence against invading pathogens is the primary function of M1. To clear the site of injury, M1 macrophages tend to trigger the bactericidal response which involves the production of ROS and NO in contact with pathogen. The phagocytic function of M1 mainly depends on Nox2 gene, as discussed previously (Section 3.1.2). Production of ROS and NO by NADPH oxidase and nitric oxide synthase, in which both enzymes are generated from NADPH through pentose phosphate pathway [92], and Nox2 negatively regulates the phagosomal proteolysis [93]. It is also reported that M1 macrophages have reduced rate of acidification and proton-pumping activity compared to M2, which facilitates M1 macrophages to efficiently eliminate pathogens [94]. The effective microbicidal function of M1 macrophages requires continuous production of ROS followed by delayed maturation of phagosomes. *In vitro* stimulation of M1 macrophages with lipopolysaccharides (LPS) promotes recognition by TLRs, the primary LPS receptor, and, occasionally, LPS binds to phagocytic MAC1 receptor independent of TLRs [95]. The association of LPS with receptors drives the production of ROS and genes alterations. LPS-induced ROS generation is Nox-dependent and it further supports the ROS-induced TNF α production, which is evidenced from the reduced TNF α in PHOX $^{-/-}$ mice [96]. Although performed in human embryonic kidney cells HEK293T, previous study also proposed that LPS-induced ROS production is regulated by the direct association between cytoplasmic tail of TLR4 and COOH-terminal of Nox4. RNA interference against Nox4 on TLR4 expressing cells blocked LPS-induced ROS production [97]. Besides, ROS may serve as secondary messenger in

the LPS-induced signal transduction, facilitating the regulation of downstream pathways such as mitogen-activated protein kinase (MAPK) and NF- κ B [98]. Activation of these pathways by H₂O₂ promotes expression of proinflammatory genes.

Upon being challenged with TLRs ligands, activation of TLRs binding on macrophages triggers the translocation of TRAF6 from TLR signalling complex to evolutionarily conserved signalling intermediate in Toll pathways (ECSIT) on outer mitochondrial membrane that primes the generation of mitochondrial ROS and phagocytosis activity [99]. The stimulation of mitochondrial ROS production via electron transport chain in the inner mitochondrial membrane facilitates macrophage reprogramming towards M1 phenotype. It is evidenced by the murine model with overexpressing catalase in mitochondria, which showed increased bacterial loads after infection compared to wild type. Another study by Infantino et al. also links ROS production to homeostatic function of mitochondria in macrophages, in which it suggested that the mitochondrial citrate carrier that functions in transporting citrate into cytoplasm exerts important function in mediating ROS generation upon LPS induction. By either transient deactivation of citrate carrier or using citrate carrier inhibitor, BTA attenuated the production of nitric oxide, ROS, and prostaglandin. And the notion may be contributed by the acetyl-CoA and oxaloacetate, the cleavage product of citrate required for the production of ROS [100]. The production of mitochondrial ROS was also further explained to be mediated by immunoresponsive gene 1 (IRG1), which improved oxidative phosphorylation and thereby increased ROS production in phagosomes. Using zebrafish infection model, the study demonstrated that depletion of IRG-1 in macrophage lineage cells failed to employ fatty acid as their fuels and leads to impaired ROS production and bactericidal activity [50]. Using genetic deletion of p47^{PHOX} and gp91^{PHOX}, as well as apocynin, the NADPH inhibitor promoted the phenotypic changes of microglial towards M2-like phenotype and increased the M2-like genes response, which further evidenced the role of NADPH oxidase in maintaining the phenotype of M1 [101].

M1 macrophages activation is always correlated with upregulation of TNF α mediated inflammatory response. Activation of TNF α is deemed to depend on interaction of TNF with TNF receptors that triggers the downstream signalling, mitogen-activated protein kinases (MAPK) and I κ B-kinases (IKK), that activates NF- κ B signalling [98]. It is reported that H₂O₂ tends to accumulate in NF- κ B deficient cells when exposed to TNF; the H₂O₂ further oxidized the catalytic cysteine of MAPK phosphatases and triggered activation of MAPK cascades including JNK and p38 MAPK [102]. Excessive H₂O₂ also promotes I κ B-kinase activation and drives tyrosine phosphorylation of I κ B α , leading to stimulation of NF- κ B signalling [103]. Recent studies postulated that the macrophage reprogramming towards M1 phenotype along with proinflammatory gene expressions by small molecules is mediated by activation of MAPK and NF- κ B signalling cascades [104, 105], though involvement of

ROS was not mentioned. Given that ROS is closely related to the activation of MAPK and NF- κ B, ROS may partially regulate macrophage polarization towards M1. SIRT2, a NAD-dependent histone deacetylase, was also found to be involved in LPS-induced ROS generation in macrophages; deletion of SIRT2 inhibited NF- κ B p65 nuclear translocation and M1 related gene expressions [106]. Moreover, Nox-derived hydrogen peroxide H₂O₂ was believed to be the major ROS in response to microglial activation [107], as evidenced from the observation that catalase blocked the MAPK and NF- κ B mediated LPS-induced proinflammatory genes expressions, but not superoxide dismutase [108].

Rowlands et al. have postulated that TNF α in circulation increased mitochondrial Ca²⁺ and thereby triggers the endocytosis of TNF α receptor 1 mediated by TNF α converting enzyme TACE. The inflammatory response is further regulated through stimulation of mitochondrial complexes to generate ROS and binding of unattached TNF α R1 to soluble TNF α . It is a negative feedback loop in responding to TNF α induced inflammation, in which overexpression of catalase blocked mitochondrial H₂O₂ dependent TNF α R1 shedding and thus enhanced the inflammatory response [109]. This study is performed on lung endothelium, while another recent study also demonstrated the similar regulation on a mucin glycoprotein MUC1 expressed on alveolar macrophages. MUC1-expressing M1 macrophages activation increased MUC1 ectodomain shedding in TACE dependent manner; the upregulation of MUC1 is associated with blunted ROS production and phagocytic activity in M1 macrophages [110]. The findings also further revealed that MUC1-deficient M0 macrophage has augmented ROS and TNF α secretion, suggesting the tight regulation of ROS homeostasis in macrophages for maintaining the proper phagocytic activity and inflammatory response. The uncoupling protein 2 in inner mitochondrial membrane also interferes with ROS production; downregulation of UCP2 by LPS in murine bone marrow derived macrophages promotes proinflammatory cytokine secretions [111].

Besides, the activation of inflammasome followed by ROS production has been implicated in regulating proinflammatory cytokines, IL-1 β and IL18 production; it requires TLR ligands such as LPS for gene synthesis and second stimulus produced by DAMP for cleavage of caspase-1, which further stimulates the protein secretions [112]. The activation by second stimulus such as ATP will trigger ROS generation, followed by caspase-1 and inflammasome activation and cytokine production. Early study postulated that NADPH-derived ROS is responsible for the upstream of inflammasome activation. It is evidenced from the blockade of caspase-1 and cytokine secretion after addition of DPI, flavoprotein inhibitor of NADPH oxidase, which suggests that the interaction of ROS with inflammasome exerts important function for proinflammatory cytokines production [113]. However, there is contradicting study suggesting that superoxide dismutase 1, the antioxidant, also regulates caspase-1 activation [114]. Apart from that, mitochondrial derived ROS may also activate inflammasomes, in which the notion is further substantiated by addition of rotenone

and antimycin A; the respiratory chain inhibitors increased ROS production followed by IL-1 β secretion [115]. Deletion of dynamin-related protein 1 and the mitochondrial fission protein indirectly influences the localization of NLRP3 inflammasome, leading to caspase-1 activation and IL-1 β production [116]. Though ROS may be crucial for inflammasome activation and priming, there is also explanation proposing that redox signalling in macrophage may be derived from other cell types, and the inflammasome activation depends on the redox status in particular to cell types upon PAMP stimulation. In healthy macrophages, the antioxidant system is stimulated to counteract the high level of ROS and impaired antioxidant response leads to low inflammasome activation and IL-1 β production [117]. Nonetheless, redox signalling in inflammasome activation is far more complicated as postulated and studies reported on caspase-8-dependent inflammasome activation and ROS-induced NLRP3 inflammasome priming have been further proposed recently [118, 119]. Yet, there is no definite answer on how ROS impacts inflammasome activation or priming and more studies are needed to further justify the mechanisms involved. The mechanisms involved by M1 macrophages are illustrated in Figure 1.

4.2. ROS Promotes M2. Depending on the content of intracellular glutathione, the M1 and M2 macrophages are characterized as oxidative and reductive macrophages, suggesting the redox regulation in macrophages physiology [120]. In contrast to M1 macrophages, M2 activation stimulates increased arginase-1 activity and is accompanied by reduced ROS and NO generation. The functions of tissue remodeling and wound healing of M2 macrophages are explained to be attributed by the macrophages effect in expressing increased cathepsin S and cathepsin L and reduced NADPH oxidase (Nox2) activity, which all improved the phagosomal proteolytic activity of M2 (IL-4) macrophages. Reduced Nox2 also improved the wound healing functions of M2 in degrading disulphide protein [121]. Furthermore, the interaction between M2 macrophages with apoptotic bodies triggers instability of NADPH oxidase Nox2 mRNAs through binding blockade of RNA-binding protein SYNCRIP to Nox2 3'-UTR. And this further defects the ROS production and leads to M2 macrophage polarization [122]. In type I diabetes NOD murine model, the deficiency of NADPH oxidase-derived superoxide has rendered the skewing of islet resident macrophages towards M2 phenotype followed by downregulation of TNF α and IL1 β in surrounding environment and further protects β -cell from destruction [123]. Besides, the mutation of cytosolic protein of Nox2, p47^{phox}^{-/-}, also favours the macrophage reprogramming towards M2 phenotype together with upregulation of arginase-1, Ym1, and Fizz1 [124]. The notion is also further evidenced in diseased state of microglia, where deletion of p47^{phox} potentiates macrophages towards M2 upon LPS stimulation followed by increase of M2 markers IL-4R α , Ym1, Fizz1, Mrc1, CD163, and MARCO. Addition of apocynin, the inhibitor of NADPH oxidase, also gives similar trend of outcome and the effect is reversed upon intervention of IL-4 neutralising antibody [101]. All

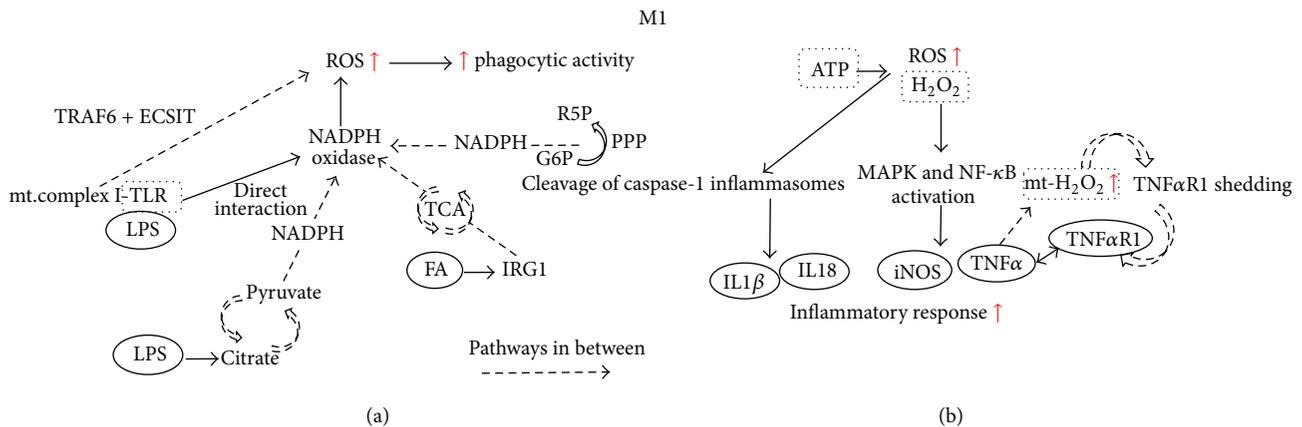


FIGURE 1: Involvement of ROS in regulating M1 responsible phagocytic activity and inflammatory response. (a) Multiple pathways are involved in generating NADPH, followed by ROS production by NADPH oxidase. The high ROS level mainly used to mediate the phagocytic activity of M1 macrophages. (b) ROS serves as second messenger mediating the inflammatory response of M1 macrophages, primarily through MAPK and NF- κ B as well as inflammasome activation. Mt, mitochondrial; FA, fatty acid; G6P, glucose-6-phosphate; R5P, ribulose-5-phosphate; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle.

these further propose depletion of Nox2 accompanied by ROS reduction which is important for reprogramming of M1 to M2 phenotype.

Apart from the suggested role of NADPH oxidase in regulating M2 macrophages, superoxide dismutase (SOD) enzyme, which catalyses superoxide dismutation and was associated with H₂O₂ production, also contributed to M2 macrophages activation. Study by He et al. postulated that Cu, Zn-SOD^{-/-} mice had abundance of alveolar macrophages in M1 phenotype, while Cu, Zn-SOD^{TG} mice predominantly expressed M2 macrophage markers. The function of Cu, Zn-SOD in modulating M2 alternative activation is regulated by redox-dependent STAT6 translocation [125]. Indeed, previous study revealed the absence of IL-4 related genes expressions in STAT6 deficient mice upon stimulation by IL-4. Silencing of STAT6 in T lymphocytes showed incapability to polarize to Th2 phenotype in the presence of IL-13 or IL-4. This also further postulated that STAT6 is implicated for M2 macrophage polarization, given that M2 macrophages have the same differentiation manner as Th2 lymphocytes [126]. Moreover, as opposed to M1 macrophages, extracellular ATP blocks IL-1 β in M2 macrophages. This was claimed to be mediated by two mechanisms, the direct blockade of ROS and inflammasome trapping through clustering of actin filaments, which are both associated with reduced ATP plasma membrane ion channel, P2X₇R [127]. Recent study also postulated the glucose metabolism related protein carbohydrate kinase-like protein (CARKL) promotes M2 activation. Reduced pentose phosphate pathway (PPP) flux in M2 macrophages is mediated by CARKL that catalyses the formation of sedoheptulose-7-phosphate, the intermediate of PPP. The reduced glucose metabolism activated M2 was evidenced by addition of metformin and rotenone which blocked (IL-4) M2 genes expressions. The study also further observed reduction in NAD by not NAD⁺ levels which suggested that CARKL may be important in regulating the redox balance in glucose metabolism [17].

Although impact of redox signalling or ROS production in M1 macrophages activation seems to interfere with M2 macrophage priming, study by Zhang et al. postulated that ROS production is also important for M2 macrophage differentiation. Intervention of antioxidant butylated hydroxyanisole BHA by inhibiting Nox-mediated O₂⁻ production before differentiation by M-CSF treatment blocked monocyte differentiation to M2, which suggests that ROS may implicate the early stage of M2 macrophage differentiation. Further intervention of BHA in urethane-induced murine lung cancer model also attenuated the occurrence of tumor-associated macrophages and thereby reduced tumor progression [83]. Also, study revealed that KLF4 triggers MCP-1-induced protein (MCP-1) to stimulate ROS production in IL-4-induced M2 macrophages and ROS attenuation blocked ER stress in M2 macrophages. Removal of the MCP-1 shifted the macrophage phenotype towards M1 with increasing phagocytic function [128]. These studies may render questions for activation and priming of alternative M2 macrophages, whether ROS may impact different stages of M2 macrophage manifestation, which need to be further investigated. The mechanisms involved by M2 macrophages are illustrated in Figure 2.

5. Discussion

5.1. ROS-Controlled Macrophage Polarization in Disease Progression: A Potential Drug Target? More evidences postulated that macrophage polarization played critical role in initiation and progression of multiple human diseases, as described above. In some cases, ROS plays a critical part in triggering disease-specific skewing of macrophages. This has been particularly observed in tumorigenesis and atherosclerosis in which the dysregulation caused by oxidative stress and inflammation have been extensively studied. However, the involvement of oxidative stress in diseases progression may be very broad and results in pathogenesis linking variety

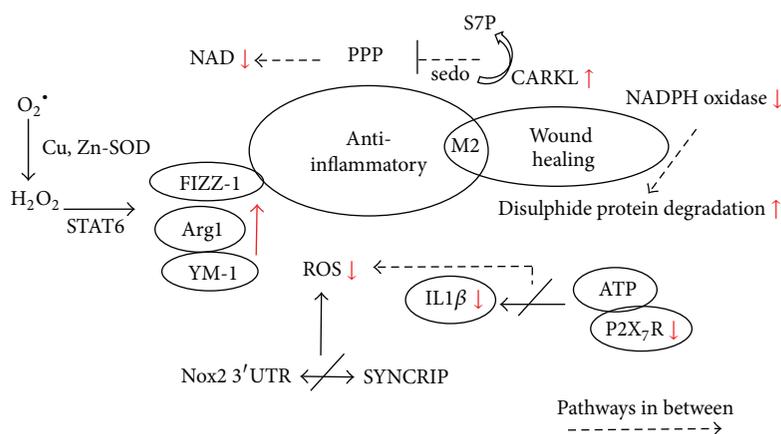


FIGURE 2: Involvement of ROS in regulating M2 responsible inflammation resolving and wound healing activities. Multiple pathways are involved in reducing NADPH and its oxidase followed by reduced ROS generation. The low ROS level was accompanied by reduced inflammatory mediators; increased M2-regulated genes responsible for inflammation resolution; and increased disulphide protein degradation which enhanced wound healing effect of M2. S7P, sedoheptulose-7-phosphate; sedo, sedoheptulose; PPP, pentose phosphate pathway; SYNCRIP, synaptotagmin binding, cytoplasmic RNA interacting protein; SOD, superoxide dismutase.

levels of biological processes; direct cues on ROS-directed macrophage polarization further dominate diseases progression which are still lacking. This is also not justified due to the nature of macrophages as effectors cells, whose function in part is antigen presenting. In this case, ROS-driven macrophage polarization is thus far difficult to become putative drug target owing to the lack of specificity. This shortage is reflected in multiple levels, and pharmaceutical companies have to develop systems that particularly target macrophage ROS as well as its polarization. This includes not only drug target study but also development of drug delivery system. Interestingly, recent studies have revealed that liposome specifically delivered molecules to macrophages [129], which allows molecules to be specifically targeted on macrophages in the body. And development of high-resolution transcriptome analysis allows differentiation of M1 macrophages from M2 phenotype by specific surface markers [130]. Development of particular antibodies also allows recognition of individual subtype of macrophages in human body. Potential molecules targeting ROS signalling and macrophage reprogramming could be further enriched in order to discover the target treatment. More experimental investigations as well as clinical trials shall be conducted to prove the hypothesis.

5.2. Antioxidative Herbal Supplements Regulating Macrophage Polarization: Any Clues for Diseases Treatment? There are a plenty of herbal supplements available for the indication of health improvement. Herbal supplements are commonly employed, by people not only in Asian countries with tradition of using herbal remedy for diseases treatment, but also in countries where use of herbal products is under strict control and closed monitoring, for example, Europe and US. There are an increasing number of populations who favour herbal products as dietary supplements due to the concrete health-improving effect of herbal supplements as demonstrated from the scientific evidences by both benchtop

and clinical studies. Most of these herbal supplements, such as *Ginkgo biloba*, lingzhi mushroom, baicalin, and some composite herbal formulae, exhibit excellent antioxidative effect in laboratory studies. Indeed, herbal supplements generally contain a series of flavone and phenol-like compounds that work as effective scavengers of ROS and that confer the health-improving effects. Interestingly, recent evidences demonstrated that some herbal supplements could regulate macrophage polarization in preclinical models of human diseases. Study by Lam et al. proved that PHY906, an herbal adjuvant derived from ancient Chinese Medicine formula Huangqin Decoction, has beneficial effect to cancer treatment. The tumor regression effect of PHY906 may be associated with its regulation on polarization of macrophage within tumor microenvironment. Tumor from mice receiving PHY906 showed more infiltrated M1 macrophages which facilitates tumor cells killing activity [131]. Our previous study on baicalin, which is the major compound in PHY906 and is used as calming and soothing supplement, could reskew M2 polarized macrophages towards M1 phenotype. This effect was reflected in tumor microenvironment, with more M1 but reduced M2 macrophages observed after baicalin treatment. Removal of macrophages attenuated tumor inhibition by baicalin [132]. Interestingly, baicalin is generally used to reduce inflammation. The property of baicalin and baicalin-containing herbal supplement in activating proinflammatory phenotype of tumor-associated macrophages may underscore a variety of mechanisms underlying effect of baicalin on macrophages. In fact, baicalin has been reported as a ROS generator as well as scavenger in macrophages [133, 134]. Given the complicated role of ROS as we discussed above, the dual role of baicalin in ROS homeostasis may be involved in baicalin-mediated proinflammatory macrophage skewing. More scientific evidences have to be acquired in order to prove if ROS involves baicalin-mediated macrophage functions, but this also further raises the consideration on the scientific scrutiny in consumption of herbal products as

daily dietary supplements, assuming that they may impact ROS-driven macrophages polarization. Similar cases are reported in other antioxidative herbal supplements, including bitter mushroom [135] and lingzhi mushroom [136], which facilitates the regulation of macrophage polarization towards particular phenotype. Nonetheless, the function of polarized macrophages with particular phenotype varies across different diseases, and there is no justifiable conclusion on the function of each individual phenotype of macrophages in all diseases. In this case, herbal supplements consumption without concerning their use in particular diseases may cause severe consequences, at least in the context of macrophage polarization. As we still could not deny the drug-like effect of many herbal supplements, their proper use shall be further supported by more scientific evidences.

6. Conclusion

In summary, macrophages are majorly diversified into two distinct phenotypes. A variety of peripheral and residence factors may determine the dominant phenotypes of macrophages within tissues. Although it is not yet concluded whether this polarization of macrophages is *in situ* reversible, it has been shown that the overall shift of macrophage phenotypes plays a critical role in the progression of various human diseases. Instead of merely being a harmful byproduct of metabolism, ROS has been shown to get involved in the functional and phenotypic regulation of macrophages. ROS is able to control the cell death, proliferation, motility, and phagocytic ability of macrophages. Intriguingly, it is recently observed that ROS may play a complicated role in regulating macrophage polarization, which implies the potent future therapeutic approaches for life-threatening diseases. Development of target-specific delivery system has been supportive for drug development, and natural antioxidants with immunomodulatory function shall be considered. More scientific evidences as well as clinical trials are imperative and emerging.

Competing Interests

The authors declare no competing interests.

Authors' Contributions

Hor-Yue Tan and Ning Wang drafted the paper; Sha Li, Ming Hong, and Xuanbin Wang revised the paper. Yibin Feng conceived, designed, revised, and finalized the paper. All authors discussed and confirmed the final paper.

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References

- [1] K. M. Holmström and T. Finkel, "Cellular mechanisms and physiological consequences of redox-dependent signalling," *Nature Reviews Molecular Cell Biology*, vol. 15, no. 6, pp. 411–421, 2014.
- [2] C. L. Bigarella, R. Liang, and S. Ghaffari, "Stem cells and the impact of ROS signaling," *Development*, vol. 141, no. 22, pp. 4206–4218, 2014.
- [3] D. Zhou, C. Huang, Z. Lin et al., "Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways," *Cellular Signalling*, vol. 26, no. 2, pp. 192–197, 2014.
- [4] D. Lecca, M. L. Trincavelli, P. Gelosa et al., "The recently identified P2Y-like receptor GPRI7 is a sensor of brain damage and a new target for brain repair," *PLoS ONE*, vol. 3, no. 10, Article ID e3579, 2008.
- [5] M. E. Rausch, S. Weisberg, P. Vardhana, and D. V. Tortoriello, "Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration," *International Journal of Obesity*, vol. 32, no. 3, pp. 451–463, 2008.
- [6] G. Dhabekar, R. Dandekar, and A. Kingaonkar, "Role of macrophages in malignancy," *Annals of Maxillofacial Surgery*, vol. 1, no. 2, pp. 150–154, 2011.
- [7] S. K. Biswas and A. Mantovani, "Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm," *Nature Immunology*, vol. 11, no. 10, pp. 889–896, 2010.
- [8] A. Sica and A. Mantovani, "Macrophage plasticity and polarization: in vivo veritas," *The Journal of Clinical Investigation*, vol. 122, no. 3, pp. 787–795, 2012.
- [9] M. J. Rauh, V. Ho, C. Pereira et al., "SHIP represses the generation of alternatively activated macrophages," *Immunity*, vol. 23, no. 4, pp. 361–374, 2005.
- [10] A. Kroner, A. D. Greenhalgh, J. G. Zarruk, R. PassosdosSantos, M. Gaestel, and S. David, "TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord," *Neuron*, vol. 83, no. 5, pp. 1098–1116, 2014.
- [11] K. Takeda, T. Kaisho, and S. Akira, "Toll-like receptors," *Annual Review of Immunology*, vol. 21, pp. 335–376, 2003.
- [12] M. Yamamoto, S. Sato, H. Hemmi et al., "Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway," *Science*, vol. 301, no. 5633, pp. 640–643, 2003.
- [13] S. K. Biswas and A. Mantovani, "Orchestration of metabolism by macrophages," *Cell Metabolism*, vol. 15, no. 4, pp. 432–437, 2012.
- [14] S. Recalcati, M. Locati, A. Marini et al., "Differential regulation of iron homeostasis during human macrophage polarized activation," *European Journal of Immunology*, vol. 40, no. 3, pp. 824–835, 2010.
- [15] J.-C. Rodríguez-Prados, P. G. Través, J. Cuenca et al., "Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation," *The Journal of Immunology*, vol. 185, no. 1, pp. 605–614, 2010.
- [16] E. Izquierdo, V. D. Cuevas, S. Fernández-Arroyo et al., "Reshaping of human macrophage polarization through modulation of

- glucose catabolic pathways,” *Journal of Immunology*, vol. 195, no. 5, pp. 2442–2451, 2015.
- [17] A. Haschemi, P. Kosma, L. Gille et al., “The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism,” *Cell Metabolism*, vol. 15, no. 6, pp. 813–826, 2012.
- [18] Z. Tan, N. Xie, H. Cui et al., “Pyruvate dehydrogenase kinase 1 participates in macrophage polarization via regulating glucose metabolism,” *The Journal of Immunology*, vol. 194, no. 12, pp. 6082–6089, 2015.
- [19] F. O. Martinez, S. Gordon, M. Locati, and A. Mantovani, “Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression,” *Journal of Immunology*, vol. 177, no. 10, pp. 7303–7311, 2006.
- [20] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati, “The chemokine system in diverse forms of macrophage activation and polarization,” *Trends in Immunology*, vol. 25, no. 12, pp. 677–686, 2004.
- [21] S. C.-C. Huang, B. Everts, Y. Ivanova et al., “Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages,” *Nature Immunology*, vol. 15, no. 9, pp. 846–855, 2014.
- [22] D. De Nardo, L. I. Labzin, H. Kono et al., “High-density lipoprotein mediates anti-inflammatory reprogramming of macrophages via the transcriptional regulator ATF3,” *Nature Immunology*, vol. 15, no. 2, pp. 152–160, 2014.
- [23] X. Prieur, C. Y. L. Mok, V. R. Velagapudi et al., “Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice,” *Diabetes*, vol. 60, no. 3, pp. 797–809, 2011.
- [24] D.-S. Im, “Functions of omega-3 fatty acids and FFA4 (GPR120) in macrophages,” *European Journal of Pharmacology*, 2015.
- [25] S.-J. Park, K.-P. Lee, S. Kang et al., “Sphingosine 1-phosphate induced anti-atherogenic and atheroprotective M2 macrophage polarization through IL-4,” *Cellular Signalling*, vol. 26, no. 10, pp. 2249–2258, 2014.
- [26] M. Masoodi, O. Kuda, M. Rossmeisl, P. Flachs, and J. Kopecky, “Lipid signaling in adipose tissue: connecting inflammation & metabolism,” *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, vol. 1851, no. 4, pp. 503–518, 2015.
- [27] A. Shiomi and T. Usui, “Pivotal roles of GM-CSF in autoimmunity and inflammation,” *Mediators of Inflammation*, vol. 2015, Article ID 568543, 13 pages, 2015.
- [28] M. J. Kraakman, A. J. Murphy, K. Jandeleit-Dahm, and H. L. Kammoun, “Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function?” *Frontiers in Immunology*, vol. 5, article 470, 2014.
- [29] G. Chinetti-Gbaguidi, S. Colin, and B. Staels, “Macrophage subsets in atherosclerosis,” *Nature Reviews Cardiology*, vol. 12, no. 1, pp. 10–17, 2015.
- [30] B. Ruffell, N. I. Affara, and L. M. Coussens, “Differential macrophage programming in the tumor microenvironment,” *Trends in Immunology*, vol. 33, no. 3, pp. 119–126, 2012.
- [31] C. N. Lumeng, J. L. Bodzin, and A. R. Saltiel, “Obesity induces a phenotypic switch in adipose tissue macrophage polarization,” *The Journal of Clinical Investigation*, vol. 117, no. 1, pp. 175–184, 2007.
- [32] A. Chawla, K. D. Nguyen, and Y. P. S. Goh, “Macrophage-mediated inflammation in metabolic disease,” *Nature Reviews Immunology*, vol. 11, no. 11, pp. 738–749, 2011.
- [33] S. P. Weisberg, D. Hunter, R. Huber et al., “CCR2 modulates inflammatory and metabolic effects of high-fat feeding,” *The Journal of Clinical Investigation*, vol. 116, no. 1, pp. 115–124, 2006.
- [34] S. Colin, G. Chinetti-Gbaguidi, and B. Staels, “Macrophage phenotypes in atherosclerosis,” *Immunological Reviews*, vol. 262, no. 1, pp. 153–166, 2014.
- [35] K. M. Botham and C. P. D. Wheeler-Jones, “Postprandial lipoproteins and the molecular regulation of vascular homeostasis,” *Progress in Lipid Research*, vol. 52, no. 4, pp. 446–464, 2013.
- [36] B.-Z. Qian and J. W. Pollard, “Macrophage diversity enhances tumor progression and metastasis,” *Cell*, vol. 141, no. 1, pp. 39–51, 2010.
- [37] H. Yang, C. Kim, M.-J. Kim et al., “Soluble vascular endothelial growth factor receptor-3 suppresses lymphangiogenesis and lymphatic metastasis in bladder cancer,” *Molecular Cancer*, vol. 10, article 36, 2011.
- [38] X. Tang, C. Mo, Y. Wang, D. Wei, and H. Xiao, “Anti-tumour strategies aiming to target tumour-associated macrophages,” *Immunology*, vol. 138, no. 2, pp. 93–104, 2013.
- [39] F. Eriksson, P. Tsagozis, K. Lundberg et al., “Tumor-specific bacteriophages induce tumor destruction through activation of tumor-associated macrophages,” *Journal of Immunology*, vol. 182, no. 5, pp. 3105–3111, 2009.
- [40] C. D. Gregory and A. Devitt, “The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically?” *Immunology*, vol. 113, no. 1, pp. 1–14, 2004.
- [41] M.-K. Chang, C. Bergmark, A. Laurila et al., “Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 11, pp. 6353–6358, 1999.
- [42] V. A. Tyurin, K. Balasubramanian, D. Winnica et al., “Oxidatively modified phosphatidylserines on the surface of apoptotic cells are essential phagocytic ‘eat-me’ signals: cleavage and inhibition of phagocytosis by Lp-PLA₂,” *Cell Death and Differentiation*, vol. 21, no. 5, pp. 825–835, 2014.
- [43] V. E. Kagan, G. G. Borisenko, B. F. Serinkan et al., “Appetizing rancidity of apoptotic cells for macrophages: oxidation, externalization, and recognition of phosphatidylserine,” *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 285, no. 1, pp. L1–L17, 2003.
- [44] Y. Y. Tyurina, F. B. Serinkan, V. A. Tyurin et al., “Lipid antioxidant, etoposide, inhibits phosphatidylserine externalization and macrophage clearance of apoptotic cells by preventing phosphatidylserine oxidation,” *The Journal of Biological Chemistry*, vol. 279, no. 7, pp. 6056–6064, 2004.
- [45] S. Hoshino, M. Konishi, M. Mori et al., “HIV-1 Vpr induces TLR4/MyD88-mediated IL-6 production and reactivates viral production from latency,” *Journal of Leukocyte Biology*, vol. 87, no. 6, pp. 1133–1143, 2010.
- [46] N. Dey, M. Sinha, S. Gupta et al., “Caspase-1/ASC inflammasome-mediated activation of IL-1 β -ROS-NF- κ B pathway for control of *Trypanosoma cruzi* replication and survival is dispensable in NLRP3^{-/-} macrophages,” *PLoS ONE*, vol. 9, no. 11, Article ID e111539, 2014.
- [47] M. Bauer, M. Goldstein, M. Christmann, H. Becker, D. Heylmann, and B. Kaina, “Human monocytes are severely impaired in base and DNA double-strand break repair that renders

- them vulnerable to oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 52, pp. 21105–21110, 2011.
- [48] S. Essler, N. Dehne, and B. Brüne, "Role of sestrin2 in peroxide signaling in macrophages," *FEBS Letters*, vol. 583, no. 21, pp. 3531–3535, 2009.
- [49] H.-M. Lo, C.-L. Chen, C.-M. Yang et al., "The carotenoid lutein enhances matrix metalloproteinase-9 production and phagocytosis through intracellular ROS generation and ERK1/2, p38 MAPK, and RAR β activation in murine macrophages," *Journal of Leukocyte Biology*, vol. 93, no. 5, pp. 723–735, 2013.
- [50] C. J. Hall, R. H. Boyle, J. W. Astin et al., "Immunoresponse gene 1 augments bactericidal activity of macrophage-lineage cells by regulating β -oxidation-dependent mitochondrial ROS production," *Cell Metabolism*, vol. 18, no. 2, pp. 265–278, 2013.
- [51] Y. Y. Tyurina, L. V. Basova, N. V. Konduru et al., "Nitrosative stress inhibits the aminophospholipid translocase resulting in phosphatidylserine externalization and macrophage engulfment: implications for the resolution of inflammation," *The Journal of Biological Chemistry*, vol. 282, no. 11, pp. 8498–8509, 2007.
- [52] T. Harada, T. Tanikawa, Y. Iwasaki, M. Yamada, Y. Imai, and M. Miyake, "Phagocytic entry of *Legionella pneumophila* into macrophages through phosphatidylinositol 3,4,5-trisphosphate-independent pathway," *Biological and Pharmaceutical Bulletin*, vol. 35, no. 9, pp. 1460–1468, 2012.
- [53] K. L. Brown, K. Christenson, A. Karlsson, C. Dahlgren, and J. Bylund, "Divergent effects on phagocytosis by macrophage-derived oxygen radicals," *Journal of Innate Immunity*, vol. 1, no. 6, pp. 592–598, 2009.
- [54] D. Sanmun, E. Witasp, S. Jitkaew et al., "Involvement of a functional NADPH oxidase in neutrophils and macrophages during programmed cell clearance: Implications for chronic granulomatous disease," *American Journal of Physiology—Cell Physiology*, vol. 297, no. 3, pp. C621–C631, 2009.
- [55] A. M. Johann, A. von Knethen, D. Lindemann, and B. Brüne, "Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-activated receptor- γ and attenuates the oxidative burst," *Cell Death and Differentiation*, vol. 13, no. 9, pp. 1533–1540, 2006.
- [56] H.-N. Lee and Y.-J. Surh, "Resolvin D1-mediated NOX2 inactivation rescues macrophages undertaking efferocytosis from oxidative stress-induced apoptosis," *Biochemical Pharmacology*, vol. 86, no. 6, pp. 759–769, 2013.
- [57] P. Scaffidi, T. Misteli, and M. E. Bianchi, "Release of chromatin protein HMGB1 by necrotic cells triggers inflammation," *Nature*, vol. 418, no. 6894, pp. 191–195, 2002.
- [58] S. Y. Lim, M. J. Raftery, J. Goyette, K. Hsu, and C. L. Geczy, "Oxidative modifications of S100 proteins: functional regulation by redox," *Journal of Leukocyte Biology*, vol. 86, no. 3, pp. 577–587, 2009.
- [59] S. F. Moore and A. B. MacKenzie, "NADPH oxidase NOX2 mediates rapid cellular oxidation following ATP stimulation of endotoxin-primed macrophages," *Journal of Immunology*, vol. 183, no. 5, pp. 3302–3308, 2009.
- [60] C. Jiménez-López, J. R. Collette, K. M. Brothers et al., "*Candida albicans* induces arginine biosynthetic genes in response to host-derived reactive oxygen species," *Eukaryotic Cell*, vol. 12, no. 1, pp. 91–100, 2013.
- [61] S. Tavakoli and R. Asmis, "Reactive oxygen species and thiol redox signaling in the macrophage biology of atherosclerosis," *Antioxidants and Redox Signaling*, vol. 17, no. 12, pp. 1785–1795, 2012.
- [62] G. B. Fortes, L. S. Alves, R. de Oliveira et al., "Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production," *Blood*, vol. 119, no. 10, pp. 2368–2375, 2012.
- [63] R. Deshmukh and V. Trivedi, "Methemoglobin exposure produces toxicological effects in macrophages due to multiple ROS spike induced apoptosis," *Toxicology in Vitro*, vol. 27, no. 1, pp. 16–23, 2013.
- [64] J. L. Miller, K. Velmurugan, M. J. Cowan, and V. Briken, "The type I NADH dehydrogenase of *Mycobacterium tuberculosis* counters phagosomal NOX2 activity to inhibit TNF- α -mediated host cell apoptosis," *PLoS Pathogens*, vol. 6, no. 4, Article ID e1000864, 2010.
- [65] V. S. Soumyarani and N. Jayakumari, "Oxidatively modified high density lipoprotein promotes inflammatory response in human monocytes-macrophages by enhanced production of ROS, TNF- α , MMP-9, and MMP-2," *Molecular and Cellular Biochemistry*, vol. 366, no. 1-2, pp. 277–285, 2012.
- [66] K. Clare, S. J. Hardwick, K. L. H. Carpenter, N. Weeratunge, and M. J. Mitchinson, "Toxicity of oxysterols to human monocyte-macrophages," *Atherosclerosis*, vol. 118, no. 1, pp. 67–75, 1995.
- [67] U. Rueckschloss, J. Galle, J. Holtz, H.-R. Zerkowski, and H. Morawietz, "Induction of NAD(P)H oxidase by oxidized low-density lipoprotein in human endothelial cells: antioxidative potential of hydroxymethylglutaryl coenzyme A reductase inhibitor therapy," *Circulation*, vol. 104, no. 15, pp. 1767–1772, 2001.
- [68] E. Lombardo, A. Alvarez-Barrientos, B. Maroto, L. Boscá, and U. G. Knaus, "TLR4-mediated survival of macrophages is MyD88 dependent and requires TNF- α autocrine signalling," *The Journal of Immunology*, vol. 178, no. 6, pp. 3731–3739, 2007.
- [69] S.-D. Ha, S. Park, C. Y. Han, M. L. Nguyen, and S. O. Kim, "Cellular adaptation to anthrax lethal toxin-induced mitochondrial cholesterol enrichment, hyperpolarization, and reactive oxygen species generation through downregulating MLN64 in macrophages," *Molecular and Cellular Biology*, vol. 32, no. 23, pp. 4846–4860, 2012.
- [70] Y.-J. Lim, J.-A. Choi, H.-H. Choi et al., "Endoplasmic reticulum stress pathway-mediated apoptosis in macrophages contributes to the survival of *Mycobacterium tuberculosis*," *PLoS ONE*, vol. 6, no. 12, Article ID e28531, 2011.
- [71] Y.-J. Lim, H.-H. Choi, J.-A. Choi et al., "*Mycobacterium kansasii*-induced death of murine macrophages involves endoplasmic reticulum stress responses mediated by reactive oxygen species generation or calpain activation," *Apoptosis*, vol. 18, no. 2, pp. 150–159, 2013.
- [72] J.-A. Choi, Y.-J. Lim, S.-N. Cho et al., "Mycobacterial HBHA induces endoplasmic reticulum stress-mediated apoptosis through the generation of reactive oxygen species and cytosolic Ca²⁺ in murine macrophage RAW 264.7 cells," *Cell Death and Disease*, vol. 4, article e957, 2013.
- [73] J. Y. Bertrand, A. Jalil, M. Klaine, S. Jung, A. Cumano, and I. Godin, "Three pathways to mature macrophages in the early mouse yolk sac," *Blood*, vol. 106, no. 9, pp. 3004–3011, 2005.
- [74] Z. Tang, Y. Gan, Q. Liu et al., "CX3CR1 deficiency suppresses activation and neurotoxicity of microglia/macrophage in experimental ischemic stroke," *Journal of Neuroinflammation*, vol. 11, article 26, 2014.
- [75] P. Niethammer, C. Grabher, A. T. Look, and T. J. Mitchison, "A tissue-scale gradient of hydrogen peroxide mediates rapid

- wound detection in zebrafish,” *Nature*, vol. 459, no. 7249, pp. 996–999, 2009.
- [76] J.-W. Ryu, K. H. Hong, J. H. Maeng et al., “Overexpression of uncoupling protein 2 in THP1 monocytes inhibits β 2 integrin-mediated firm adhesion and transendothelial migration,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 5, pp. 864–870, 2004.
- [77] D. Hackel, D. Pflücke, A. Neumann et al., “The connection of monocytes and reactive oxygen species in pain,” *PLoS ONE*, vol. 8, no. 5, Article ID e63564, 2013.
- [78] S. Ullevig, Q. Zhao, C. F. Lee, H. Seok Kim, D. Zamora, and R. Asmis, “NADPH oxidase 4 mediates monocyte priming and accelerated chemotaxis induced by metabolic stress,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 2, pp. 415–426, 2012.
- [79] C. F. Lee, S. Ullevig, H. S. Kim, and R. Asmis, “Regulation of monocyte adhesion and migration by Nox4,” *PLoS ONE*, vol. 8, no. 6, Article ID e66964, 2013.
- [80] D. Zhang, X. Jiang, P. Fang et al., “Hyperhomocysteinemia promotes inflammatory monocyte generation and accelerates atherosclerosis in transgenic cystathionine β -synthase-deficient mice,” *Circulation*, vol. 120, no. 19, pp. 1893–1902, 2009.
- [81] L. Stixová, J. Procházková, K. Souček, J. Hofmanová, and A. Kozubík, “5-Lipoxygenase inhibitors potentiate $1\alpha,25$ -dihydroxyvitamin D3-induced monocytic differentiation by activating p38 MAPK pathway,” *Molecular and Cellular Biochemistry*, vol. 330, no. 1-2, pp. 229–238, 2009.
- [82] S. S. Barbieri, S. Eligini, M. Brambilla, E. Tremoli, and S. Colli, “Reactive oxygen species mediate cyclooxygenase-2 induction during monocyte to macrophage differentiation: critical role of NADPH oxidase,” *Cardiovascular Research*, vol. 60, no. 1, pp. 187–197, 2003.
- [83] Y. Zhang, S. Choksi, K. Chen, Y. Pobezinskaya, I. Linnoila, and Z.-G. Liu, “ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages,” *Cell Research*, vol. 23, no. 7, pp. 898–914, 2013.
- [84] B. Fuhrman, M. Shiner, N. Volkova, and M. Aviram, “Cell-induced copper ion-mediated low density lipoprotein oxidation increases during in vivo monocyte-to-macrophage differentiation,” *Free Radical Biology and Medicine*, vol. 37, no. 2, pp. 259–271, 2004.
- [85] K. Nishi, T. Oda, S. Takabuchi et al., “LPS induces hypoxia-inducible factor 1 activation in macrophage-differentiated cells in a reactive oxygen species-dependent manner,” *Antioxidants and Redox Signaling*, vol. 10, no. 5, pp. 983–995, 2008.
- [86] J. Makino, T. Kamiya, H. Hara, and T. Adachi, “TPA induces the expression of EC-SOD in human monocytic THP-1 cells: involvement of PKC, MEK/ERK and NOX-derived ROS,” *Free Radical Research*, vol. 46, no. 5, pp. 637–644, 2012.
- [87] T. Yamamoto, N. Sakaguchi, M. Hachiya, F. Nakayama, M. Yamakawa, and M. Akashi, “Role of catalase in monocytic differentiation of U937 cells by TPA: hydrogen peroxide as a second messenger,” *Leukemia*, vol. 23, no. 4, pp. 761–769, 2009.
- [88] M.-G. Song, I.-G. Ryoo, H.-Y. Choi et al., “NRF2 signaling negatively regulates phorbol-12-myristate-13-acetate (PMA)-induced differentiation of human monocytic U937 cells into pro-inflammatory macrophages,” *PLoS ONE*, vol. 10, no. 7, Article ID e0134235, 2015.
- [89] C. Pararasa, J. Ikwuobe, S. Shigdar et al., “Age-associated changes in long-chain fatty acid profile during healthy aging promote pro-inflammatory monocyte polarization via PPAR γ ,” *Aging Cell*, vol. 15, no. 1, pp. 128–139, 2016.
- [90] C. Pararasa, C. Bailey, and H. Griffiths, “Macrophage polarization by fatty acids is PPAR γ -dependent,” *Free Radical Biology and Medicine*, vol. 75, supplement 1, pp. S31–S32, 2014.
- [91] J. D. Schilling, H. M. Machkovech, L. He et al., “Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages,” *Journal of Biological Chemistry*, vol. 288, no. 5, pp. 2923–2932, 2013.
- [92] B. Ghesquière, B. W. Wong, A. Kuchnio, and P. Carmeliet, “Metabolism of stromal and immune cells in health and disease,” *Nature*, vol. 511, no. 7508, pp. 167–176, 2014.
- [93] J. M. Rybicka, D. R. Balce, M. F. Khan, R. M. Krohn, and R. M. Yates, “NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the luminal redox environment of phagosomes,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 23, pp. 10496–10501, 2010.
- [94] J. Canton, R. Khezri, M. Glogauer, and S. Grinstein, “Contrasting phagosome pH regulation and maturation in human M1 and M2 macrophages,” *Molecular Biology of the Cell*, vol. 25, no. 21, pp. 3330–3341, 2014.
- [95] Z. Pei, H. Pang, L. Qian et al., “MAC1 mediates LPS-induced production of superoxide by microglia: the role of pattern recognition receptors in dopaminergic neurotoxicity,” *Glia*, vol. 55, no. 13, pp. 1362–1373, 2007.
- [96] L. Qin, Y. Liu, T. Wang et al., “NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia,” *Journal of Biological Chemistry*, vol. 279, no. 2, pp. 1415–1421, 2004.
- [97] H. S. Park, H. Y. Jung, E. Y. Park, J. Kim, W. J. Lee, and Y. S. Bae, “Cutting edge: Direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF- κ B,” *Journal of Immunology*, vol. 173, no. 6, pp. 3589–3593, 2004.
- [98] C. Kohchi, H. Inagawa, T. Nishizawa, and G.-I. Soma, “ROS and innate immunity,” *Anticancer Research*, vol. 29, no. 3, pp. 817–821, 2009.
- [99] A. P. West, I. E. Brodsky, C. Rahner et al., “TLR signalling augments macrophage bactericidal activity through mitochondrial ROS,” *Nature*, vol. 472, no. 7344, pp. 476–480, 2011.
- [100] V. Infantino, P. Convertini, L. Cucci et al., “The mitochondrial citrate carrier: a new player in inflammation,” *Biochemical Journal*, vol. 438, no. 3, pp. 433–436, 2011.
- [101] S.-H. Choi, S. Aid, H.-W. Kim, S. H. Jackson, and F. Bosetti, “Inhibition of NADPH oxidase promotes alternative and anti-inflammatory microglial activation during neuroinflammation,” *Journal of Neurochemistry*, vol. 120, no. 2, pp. 292–301, 2012.
- [102] H. Kamata, S.-I. Honda, S. Maeda, L. Chang, H. Hirata, and M. Karin, “Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases,” *Cell*, vol. 120, no. 5, pp. 649–661, 2005.
- [103] Y. Takada, A. Mukhopadhyay, G. C. Kundu, G. H. Mahabeleshwar, S. Singh, and B. B. Aggarwal, “Hydrogen peroxide activates NF- κ B through tyrosine phosphorylation of I κ B α and serine phosphorylation of p65: evidence for the involvement of I κ B α kinase and Syk protein-tyrosine kinase,” *The Journal of Biological Chemistry*, vol. 278, no. 26, pp. 24233–24241, 2003.
- [104] S. Hucke, M. Eschborn, M. Liebmann et al., “Sodium chloride promotes pro-inflammatory macrophage polarization thereby

- aggravating CNS autoimmunity,” *Journal of Autoimmunity*, vol. 67, pp. 90–101, 2016.
- [105] Y. Zhou, T. Zhang, X. Wang et al., “Curcumin modulates macrophage polarization through the inhibition of the toll-like receptor 4 expression and its signaling pathways,” *Cellular Physiology and Biochemistry*, vol. 36, no. 2, pp. 631–641, 2015.
- [106] A. S. Lee, Y. J. Jung, D. Kim et al., “SIRT2 ameliorates lipopolysaccharide-induced inflammation in macrophages,” *Biochemical and Biophysical Research Communications*, vol. 450, no. 4, pp. 1363–1369, 2014.
- [107] E. A. Bordt and B. M. Polster, “NADPH oxidase- and mitochondria-derived reactive oxygen species in proinflammatory microglial activation: a bipartisan affair?” *Free Radical Biology and Medicine*, vol. 76, no. 1, pp. 34–46, 2014.
- [108] S. Pawate, Q. Shen, F. Fan, and N. R. Bhat, “Redox regulation of glial inflammatory response to lipopolysaccharide and interferony,” *Journal of Neuroscience Research*, vol. 77, no. 4, pp. 540–551, 2004.
- [109] D. J. Rowlands, M. N. Islam, S. R. Das et al., “Activation of TNFR1 ectodomain shedding by mitochondrial Ca²⁺ determines the severity of inflammation in mouse lung microvessels,” *The Journal of Clinical Investigation*, vol. 121, no. 5, pp. 1986–1999, 2011.
- [110] K. Kato, R. Uchino, E. P. Lillehoj, K. Knox, Y. Lin, and K. C. Kim, “Membrane-tethered MUC1 mucin counter-regulates the phagocytic activity of macrophages,” *American Journal of Respiratory Cell and Molecular Biology*, vol. 54, no. 4, pp. 515–523, 2016.
- [111] Y. Emre, C. Hurtaud, T. Nübel, F. Criscuolo, D. Ricquier, and A.-M. Cassard-Doulcier, “Mitochondria contribute to LPS-induced MAPK activation via uncoupling protein UCP2 in macrophages,” *Biochemical Journal*, vol. 402, no. 2, pp. 271–278, 2007.
- [112] T. Strowig, J. Henao-Mejia, E. Elinav, and R. Flavell, “Inflammasomes in health and disease,” *Nature*, vol. 481, no. 7381, pp. 278–286, 2012.
- [113] C. M. Cruz, A. Rinna, H. J. Forman, A. L. M. Ventura, P. M. Persechini, and D. M. Ojcius, “ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages,” *The Journal of Biological Chemistry*, vol. 282, no. 5, pp. 2871–2879, 2007.
- [114] F. Meissner, K. Molawi, and A. Zychlinsky, “Superoxide dismutase 1 regulates caspase-1 and endotoxin shock,” *Nature Immunology*, vol. 9, no. 8, pp. 866–872, 2008.
- [115] R. Zhou, A. S. Yazdi, P. Menu, and J. Tschopp, “A role for mitochondria in NLRP3 inflammasome activation,” *Nature*, vol. 469, no. 7329, pp. 221–225, 2011.
- [116] S. Park, J.-H. Won, I. Hwang, S. Hong, H. K. Lee, and J.-W. Yu, “Defective mitochondrial fission augments NLRP3 inflammasome activation,” *Scientific Reports*, vol. 5, Article ID 15489, 2015.
- [117] A. Rubartelli, M. Gattorno, M. G. Netea, and C. A. Dinarello, “Interplay between redox status and inflammasome activation,” *Trends in Immunology*, vol. 32, no. 12, pp. 559–566, 2011.
- [118] F. Bauernfeind, E. Bartok, A. Rieger, L. Franchi, G. Núñez, and V. Hornung, “Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome,” *The Journal of Immunology*, vol. 187, no. 2, pp. 613–617, 2011.
- [119] R. Allam, K. E. Lawlor, E. C.-W. Yu et al., “Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming,” *EMBO Reports*, vol. 15, no. 9, pp. 982–990, 2014.
- [120] Y. Murata, T. Shimamura, and J. Hamuro, “The polarization of Th1/Th2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production,” *International Immunology*, vol. 14, no. 2, pp. 201–212, 2002.
- [121] D. R. Balce, B. Li, E. R. O. Allan, J. M. Rybicka, R. M. Krohn, and R. M. Yates, “Alternative activation of macrophages by IL-4 enhances the proteolytic capacity of their phagosomes through synergistic mechanisms,” *Blood*, vol. 118, no. 15, pp. 4199–4208, 2011.
- [122] L. Kuchler, A. K. Giegerich, L. K. Sha et al., “SYNCRIP-dependent Nox2 mRNA destabilization impairs ROS formation in M2-polarized macrophages,” *Antioxidants and Redox Signaling*, vol. 21, no. 18, pp. 2483–2497, 2014.
- [123] L. E. Padgett, A. R. Burg, W. Lei, and H. M. Tse, “Loss of NADPH oxidase-derived superoxide skews macrophage phenotypes to delay type 1 diabetes,” *Diabetes*, vol. 64, no. 3, pp. 937–946, 2015.
- [124] L. Yi, Q. Liu, M. S. Orandle et al., “P47(phox) directs murine macrophage cell fate decisions,” *American Journal of Pathology*, vol. 180, no. 3, pp. 1049–1058, 2012.
- [125] C. He, A. J. Ryan, S. Murthy, and A. B. Carter, “Accelerated development of pulmonary fibrosis via Cu,Zn-superoxide dismutase-induced alternative activation of macrophages,” *Journal of Biological Chemistry*, vol. 288, no. 28, pp. 20745–20757, 2013.
- [126] M. H. Kaplan, U. Schindler, S. T. Smiley, and M. J. Grusby, “Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells,” *Immunity*, vol. 4, no. 3, pp. 313–319, 1996.
- [127] P. Pelegrin and A. Surprenant, “Dynamics of macrophage polarization reveal new mechanism to inhibit IL-1 β release through pyrophosphates,” *The EMBO Journal*, vol. 28, no. 14, pp. 2114–2127, 2009.
- [128] N. Kapoor, J. Niu, Y. Saad et al., “Transcription factors STAT6 and KLF4 implement macrophage polarization via the dual catalytic powers of MCPiP,” *Journal of Immunology*, vol. 194, no. 12, pp. 6011–6023, 2015.
- [129] C. Kelly, C. Jefferies, and S. A. Cryan, “Targeted liposomal drug delivery to monocytes and macrophages,” *Journal of Drug Delivery*, vol. 2011, Article ID 727241, 11 pages, 2011.
- [130] M. Beyer, M. R. Mallmann, J. Xue et al., “High-resolution transcriptome of human macrophages,” *PLoS ONE*, vol. 7, no. 9, Article ID e45466, 2012.
- [131] W. Lam, Z. Jiang, F. Guan et al., “PHY906(KD018), an adjuvant based on a 1800-year-old Chinese medicine, enhanced the anti-tumor activity of Sorafenib by changing the tumor microenvironment,” *Scientific Reports*, vol. 5, article 9384, 2015.
- [132] H. Y. Tan, N. Wang, K. Man, S. W. Tsao, C. M. Che, and Y. Feng, “Autophagy-induced RelB/p52 activation mediates tumour-associated macrophage repolarisation and suppression of hepatocellular carcinoma by natural compound baicalin,” *Cell Death and Disease*, vol. 6, no. 10, Article ID e1942, 2015.
- [133] H.-Y. Lin, S.-C. Shen, C.-W. Lin, L.-Y. Yang, and Y.-C. Chen, “Baicalein inhibition of hydrogen peroxide-induced apoptosis via ROS-dependent heme oxygenase 1 gene expression,” *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1773, no. 7, pp. 1073–1086, 2007.
- [134] L.-L. Liu, L.-K. Gong, H. Wang et al., “Baicalin inhibits macrophage activation by lipopolysaccharide and protects mice

- from endotoxin shock," *Biochemical Pharmacology*, vol. 75, no. 4, pp. 914–922, 2008.
- [135] F. Sheu, P.-J. Chien, K.-Y. Hsieh et al., "Purification, cloning, and functional characterization of a novel immunomodulatory protein from *Antrodia camphorata* (Bitter Mushroom) that exhibits TLR2-dependent NF- κ B activation and M1 polarization within murine macrophages," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 10, pp. 4130–4141, 2009.
- [136] Y.-K. Zhang, J. Wang, L. Liu, R. C.-C. Chang, K.-F. So, and G. Ju, "The effect of *Lycium barbarum* on spinal cord injury, particularly its relationship with M1 and M2 macrophage in rats," *BMC Complementary and Alternative Medicine*, vol. 13, article 67, 2013.

Review Article

ROS-Mediated NLRP3 Inflammasome Activation in Brain, Heart, Kidney, and Testis Ischemia/Reperfusion Injury

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Ischemia and reperfusion (I/R) causes a reduction in arterial blood supply to tissues, followed by the restoration of perfusion and consequent reoxygenation. The reestablishment of blood flow triggers further damage to the ischemic tissue through reactive oxygen species (ROS) accumulation, interference with cellular ion homeostasis, and inflammatory responses to cell death. In normal conditions, ROS mediate important beneficial responses. When their production is prolonged or elevated, harmful events are observed with peculiar cellular changes. In particular, during I/R, ROS stimulate tissue inflammation and induce NLRP3 inflammasome activation. The mechanisms underlying the activation of NLRP3 are several and not completely elucidated. It was recently shown that NLRP3 might sense directly the presence of ROS produced by normal or malfunctioning mitochondria or indirectly by other activators of NLRP3. Aim of the present review is to describe the current knowledge on the role of NLRP3 in some organs (brain, heart, kidney, and testis) after I/R injury, with particular regard to the role played by ROS in its activation. Furthermore, as no specific therapy for the prevention or treatment of the high mortality and morbidity associated with I/R is available, the state of the art of the development of novel therapeutic approaches is illustrated.

1. Introduction

The term ischemia and reperfusion (I/R) indicates a reduction of arterial blood supply to tissues followed by the restoration of perfusion and consequent reoxygenation [1]. In humans, ischemia, with the consequent fall in blood supply, is generally induced by the presence of an arterial embolus, which induces a severe tissue hypoxia in a coexistent inflammatory environment secondary to different risk factors, such as diabetes, hyperlipidemia, and aging. Experimental studies examining the mechanisms and the consequences of I/R use surgical methods to block specific vessels in otherwise healthy animals [2], therefore, these models are indicated to better understand the mechanisms involved in the injury induced by I/R.

The restoration of blood flow causes further damage to the ischemic tissue through neutrophil infiltration, reactive oxygen species (ROS) accumulation, deregulation of cellular ion homeostasis, and cell death with consequent inflammatory responses. Furthermore, in addition to local damage, I/R can also induce deleterious remote effects, resulting in the development of systemic inflammatory responses and multiple organ dysfunction syndrome [3].

Reperfusion is characterized by an early and a late phase, during which, due to reduced adenylate cyclase activity and intracellular cAMP levels, free radicals, such as ROS, are generated [4], and the mechanisms of cell death are triggered.

ROS are free radicals containing the oxygen atom, among which there are hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and hydroxyl radical (OH^{\cdot}). They originate

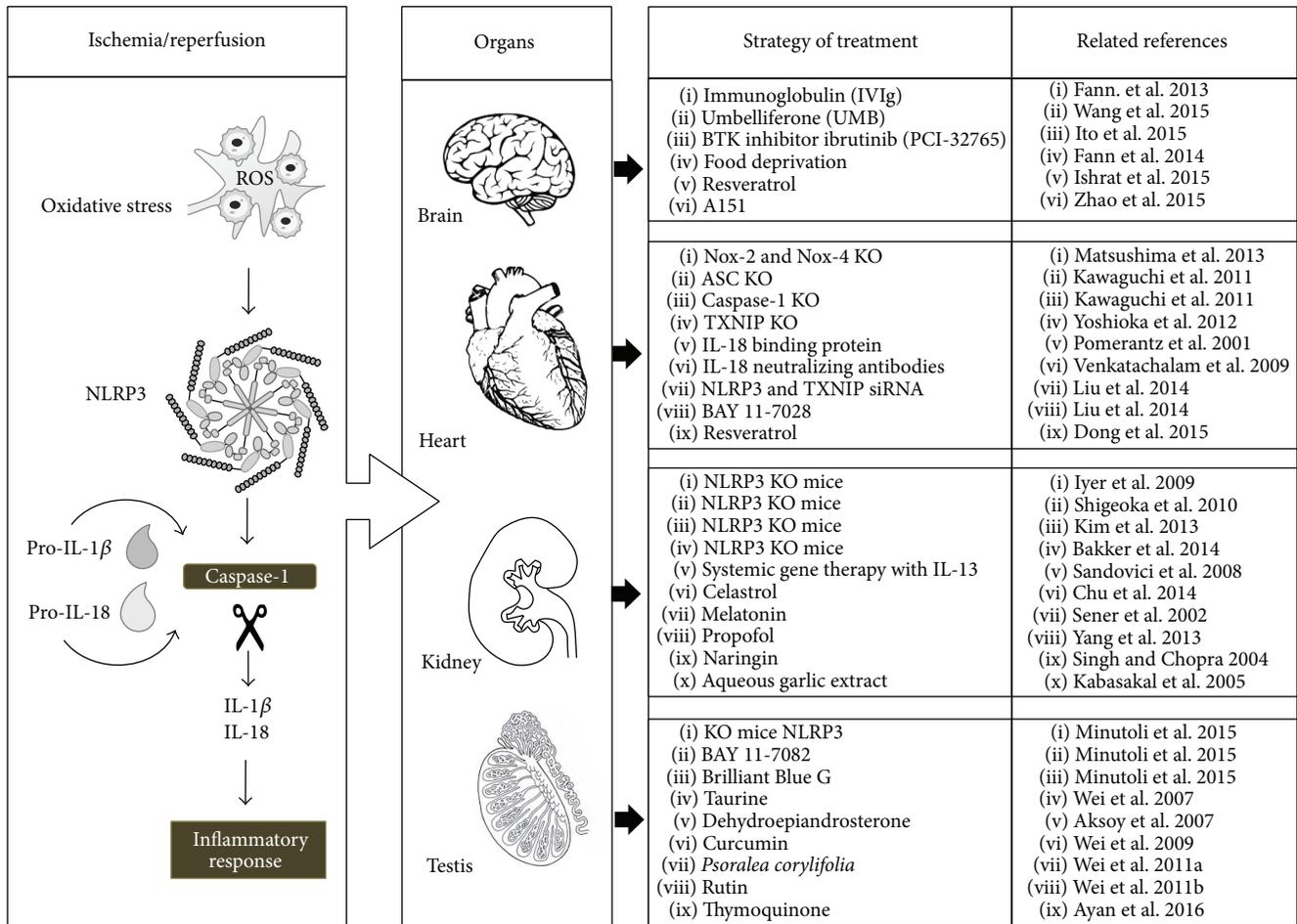


FIGURE 1: Schematic representation of ischemia/reperfusion injury and of potential therapeutic strategies to modulate oxidative stress and/or related NLRP3 activation.

mainly within the mitochondria, as a bioproduct of oxygen metabolism, but can also be generated by cellular enzymes, including lipoxygenase (LOX) and cyclooxygenase (COX) [5].

Under normal conditions, ROS have beneficial effects, as they regulate several important, physiological responses by redox-responsive signaling pathways. In fact, ROS control cellular growth, differentiation, and migration, regulate the vascular tone and cellular adhesion, contribute to the production of iNOS at transcriptional and posttranscriptional level by redox-dependent Nuclear Factor- κ B (NF- κ B) or mitogen activated protein kinases (MAPKs), and modulate immune response and control angiogenesis and apoptosis [5–7].

When ROS production is prolonged or elevated, detrimental events are observed with peculiar changes in cellular proteins, lipids, and ribonucleic acids, leading to cell dysfunction or death. Several enzymes with antioxidant activity are involved in neutralizing ROS: among them, superoxide dismutase (SOD), γ -glutamyltransferase (GGT), glutathione (GSH), glutathione reductase (GSSG-Rd), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), and catalase (CAT) are included [8]. All these antioxidant systems are differently expressed in various organs. In fact, the GSH

system shows moderate concentrations in kidney, heart, and brain, while GGT, GSSG-Rd, SOD, CAT, and GSH-Px are highest in the kidney, when compared to the brain [9], the heart [10], and the testis [10]. In addition, the antioxidant systems are differentially expressed in adult organs and in various embryonic stages [10].

The imbalance between ROS formation and the detoxifying action of these oxidizing radicals induces a cellular condition, called oxidative stress [11]. In particular, ROS, during I/R, promote tissue inflammation and activate immune response through NLRP3 inflammasome [11] (Figure 1).

The innate immune system is based on pattern-recognition receptors (PRRs) to sense pathogenic microbes and other endogenous or exogenous pathogens, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These immune activators initiate and regulate innate immune responses when identified by several classes of PRRs, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), and other DNA sensors [12, 13]. PRRs trigger the activation of specific signaling pathways, which induce the production of many

proinflammatory cytokines and chemokines and present antigens to the adaptive immune system for long-lasting protection.

NLRs form a large protein family of intracellular sensors, the members of which share a conserved central nucleotide-binding, oligomerization domain (NOD), a leucine-rich repeat (LRR) region, and a variable N-terminal effector domain [14, 15]. The family members of inflammasome are numerous [16], but NLRP3 inflammasome is the best characterized.

In a series of experimental models *in vitro* [17, 18], it was shown that two different steps, controlled by two different mechanisms, are required for the activation of NLRP3. The former is driven at transcriptional level by NF- κ B, whose increased production in I/R injury is activated via TLR signaling through Myd88-dependent pathways, with the subsequent stimulation of interleukin- (IL-) 1β and IL-18 gene expression [19, 20]. The latter is induced at posttranscriptional level and consists in the activation of NLRP3, driven by many activators, such as ROS in I/R injury [21].

When activated, the NLRP3 inflammasome is formed by the NLRP3 protein belonging to the family of NLRs, by the adapter protein apoptosis-associated speck-like protein (ASC), and by the procaspase-1 [15]. The assembly of NLRP3 moves procaspase-1 molecules near enough to transform them into active fragments [21], so that the conversion of the immature proinflammatory cytokines IL- 1β and IL-18 to their active forms is induced [22, 23]. These cytokines then initiate or amplify diverse downstream signaling pathways and drive proinflammatory processes [24], leading to cellular damage, such as autophagy and pyroptosis [22]. The former is a process of self-degradation of parts of the cells through sequestering of organelles or parts of the cytoplasm and subsequent fusion with lysosomes [25]. Pyroptosis is a programmed cell death with loss of the cellular membrane integrity, differently from apoptosis, associated with IL- 1β and IL-18 secretion; for this reason, it is considered an inflammatory form of cell death [26]. NLRP3 activation is able to promote also the initiation and the progression of different autoimmune and autoinflammatory diseases, such as metabolic disorders, inflammatory bowel syndrome [15], obesity, and cognitive diseases [27].

The mechanisms driving the activation of NLRP3, that is, NLRP3 oligomerization, ASC recruitment, and caspase-1 activation, are generally classified as noncanonical and canonical. The noncanonical pathway involves caspase-4 and caspase-5 in human cells and caspase-11 in mice and is activated by the identification of cell wall ligands, such as LPS, from phagocytized bacteria [28]. The canonical pathway is based on the recognition of general cellular stress, such as the oxidative stress induced by I/R, bacterial toxins, and particulate substances [28]. Recent studies showed that NLRP3 might sense the presence of ROS produced into the same cell by normal or malfunctioning mitochondria [29]. In particular, it was proposed that increased ROS are sensed by a complex of TRX and TRX-interacting protein (TXNIP) and induce the dissociation of the complex. In normal cells, TXNIP is constitutively connected to and kept in the reduced state by the ubiquitous TRX [30]. Following an increase

in cellular ROS concentration, this complex dissociates and TXNIP binds to the LRR region of NLRP3, leading to NLRP3 activation [31].

Given the growing evidence that NLRP3 inflammasome activation is involved in many systemic diseases [32, 33] and considering that conflicting data exist with respect to I/R injury, the present review was aimed to evaluate the current knowledge on the role of NLRP3 in some organs (brain, heart, kidney, and testis) after I/R injury, with particular regard to the role played by ROS in its activation. Furthermore, as no specific therapy exists for the prevention or treatment of the great mortality and morbidity associated with I/R, the state of the art of the development of therapeutic strategies was also evaluated.

2. ROS and NLRP3 in I/R Injury of the Brain

It is well known that reperfusion may exacerbate the brain injury initially caused by ischemia, producing an I/R injury [3]. Among the various underlying mechanisms of stroke, inflammation and oxidative stress are implicated in the pathogenesis of brain I/R, and an adequate regulation of inflammatory level may play a critical role in the prevention and treatment of stroke [34]. In the last years, the role of inflammasomes, particularly NLRP3, has been recognized in postischemic inflammation after stroke. Since the inflammasome initiates inflammation, modulation of NLRP3 inflammasome can regulate inflammatory response. However, from a molecular point of view, NLRP3 inflammasome pathway can be activated by a variety of molecular signaling systems in I/R brain and many mechanisms have not been fully defined yet.

IL- 1β is a crucial contributor to excitotoxic and ischemic brain injury and central inflammatory responses [35, 36]. Specifically, this cytokine is produced during a central nervous system disease or after a brain injury by macrophages or microglial cells [37] and molecular mediators as mitochondria-derived ROS and lysosomal protease cathepsin B are necessary for the microglial cell production of interleukin- 1β [38].

So far, NLRP3 inflammasome dependent responses *in vitro* are linked to an initial stimulus by a pathogen or damage-associated molecular pattern (DAMP) [18, 39, 40]. Recently, it has been shown that DAMPs could induce inflammatory responses through the production of IL-6 and chemokine (C-X-C motif) ligand 1 (CXCL1) and the release of cathepsin B, in the absence of any bacterial infection or products in cultured mouse mixed glia [41]. Savage and coworkers [41] also revealed that IL-1 production contributed to increase of IL-6 and CXCL1 levels following cerebral ischemia by middle cerebral artery occlusion in mice, confirming that DAMPs amplify brain inflammation by directly stimulating production of glial derived inflammatory mediators. In this context, acute phase protein serum amyloid A could act as a priming stimulus on glial cells [41]. Together, these data add new helpful information on molecular pathways of brain inflammation and/or ischemic brain damage that involve glial cells and NLRP3 inflammasome-activating DAMPs, in the absence of cell priming and in the presence of a relevant

endogenous priming stimulus, leading thus to new interesting perspectives from a therapeutic point of view.

So far, a number of recent studies focused on pathophysiological mechanism and/or potential therapeutic strategy to modulate inflammatory and oxidative stress pathways involving NLRP3 inflammasome in stroke brain damage (Figure 1).

Indeed, the expression pattern of the NLRP3 inflammasome in primary cortical neurons subjected to simulated ischemia, in a mouse model of focal ischemic stroke, and in brain tissue samples from stroke patients has been described [42]. Interestingly, these authors showed that intravenous immunoglobulin treatment protected brain cells in experimental stroke models by a mechanism involving suppression of NLRP3 activity [42].

Likewise, another interesting paper of Fann et al. [43] reported that 16 hours of food deprivation, daily, for 4 months, can attenuate the inflammatory response and tissue damage following focal ischemic stroke in mice through inhibition of NLRP3 inflammasome activity. Moreover, NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke [44].

Bruton's tyrosine kinase (BTK) is a tyrosine kinase involved in NLRP3 inflammasome activation leading, in turn, to caspase-1 activation and mature IL-1 β production in the ischemic brain [45]. Interestingly, Ito and coworkers [45] showed that ibrutinib (PCI-32765), a potent BTK inhibitor [46], suppresses NLRP3 inflammasome signal in a focal brain I/R model. Specifically, ibrutinib exerts neuroprotective effects through the suppression of IL-1 β maturation in infiltrating macrophages and neutrophils in the infarcted area.

As mentioned before, oxidative stress is a crucial hallmark in the pathophysiology of brain damage after stroke. To this purpose, it has been indicated that ROS are proximal signals for NLRP3 inflammasome activation in inflammatory diseases [47]. Experimental evidences demonstrate that an increase in ROS concentration following cellular stress leads to TRX oxidation, TXNIP recruitment of NLRP3, and consequent NLRP3 activation [48]. In fact, the action of curcumin in the hippocampus subjected to glutamate neurotoxicity was recently demonstrated [48]; *in vitro* and *in vivo* results showed that curcumin attenuated glutamate neurotoxicity by inhibiting endoplasmic reticulum stress-associated TXNIP/NLRP3 activation via the regulation of AMP-Activated Protein Kinase and thereby protected the hippocampus from ischemic insult [48].

Umbelliferone (UMB) is a natural compound belonging to the coumarin family with antioxidant properties. As a matter of fact, in an interesting paper of Wang et al. [49], pretreatment with UMB ameliorated the neurological outcomes, the infarct volume, and the brain edema in a rat model of focal cerebral ischemia induced by middle cerebral artery I/R. These results indicate that UMB exerts partly these neuroprotective effects through the inhibition of TXNIP/NLRP3 signal. Moreover, another study suggests that TXNIP plays a critical role in acute ischemic stroke because it is directly linked to redox imbalance and NLRP3 activation. The latter also suggests the importance of the antioxidant effect of resveratrol on the TRX/TXNIP system

in mice subjected to embolic middle cerebral artery occlusion [50].

Recently, it has been shown that A151, a synthetic oligodeoxynucleotide containing multiple telemeric TTAGGG motifs, reduces ischemic brain damage and NLRP3 mRNA levels in Stroke-Prone Spontaneously Hypertensive rats submitted to permanent middle cerebral artery occlusion [51].

Collectively, these data strongly confirmed that NLRP3 represents a potential therapeutic target in the management of ischemic stroke. Therefore, an appropriate treatment of brain I/R injury with compounds showing anti-inflammatory/antioxidant activity and targeting different and complex molecular pathways, also including NLRP3 activation, remains a big therapeutic challenge in translational medicine. In fact, it is nowadays difficult to establish the appropriate timing about their use and/or the duration of treatment to counteract the brain parenchymal damage in the setting of I/R injury.

3. ROS and NLRP3 in I/R Injury of the Heart

Myocardial I/R injury is a pathological process causing cardiac cells necrosis and apoptosis, in particular when the coronary perfusion is restored [52].

A large number of studies have demonstrated an increased ROS formation either during the ischemic phase or during the reperfusion period. Excessive ROS induce cell injury by disrupting cellular signaling transduction, activating inflammation factors, and inducing lipid peroxidation [53] and even cell death [54]. Additionally, ROS have been identified as an important NLRP3 inflammasome activator in cardiac diseases [55].

When activated, NLRP3 forms an inflammasome complex with the adaptor molecule ASC, thus controlling the activation of caspase-1; the latter cleaves pro-IL-1 β and pro-IL-18 into the biologically active forms, thus initiating the sterile inflammatory disease [56]. In a recent work [52], KO mice for NLRP3 showed larger infarct size than wild type, so a protective role of NLRP3 inflammasome was suggested. However, the results of the study were critically discussed and disproved by Toldo et al. [57].

IL-1 β and ASC are key players in I/R injury as they are important and early mediators of the inflammatory response in myocardial I/R injury (Figure 1). In fact, ASC deletion and IL-1 β inhibition protect the myocardium from I/R injury in mice [58, 59]. In myocardial I/R injury, an important role is played also by IL-18, whose expression is stimulated in cardiomyocytes by ROS [60]. Specifically, IL-18 may induce myocardial injury through the induction of inflammation, increased apoptosis, and changes in calcium overload [61]. The administration of human cardiomyocytes of IL-18 binding protein, a potent inhibitor of IL-18 activity, improved contractile function [58] and showed a protective role in the cardiac inflammatory response against I/R injury in mice [62]. Similarly, the treatment of mice with IL-18 neutralizing antibodies prior to I/R injury reduced the infarct size [60].

As to the mechanisms by which ROS induce inflammasome activation in the heart, many doubts are still present.

TXNIP is ubiquitously expressed in normal tissues and is an endogenous inhibitor of TRX as, when directly connected, it prevents TRX activity to scavenge ROS [63]. Therefore, TXNIP KO mice showed a protection from I/R injury in cardiomyocytes [64]. However, the mechanism by which TXNIP mediates cardiac injury is still not clear. Recently, in myocardial I/R injury a role of TXNIP in the activation of the NLRP3 inflammasome was proposed through a direct interaction in cardiac microvascular endothelial cells, after intramyocardial administration of NLRP3 and TXNIP siRNA and of BAY 11-7028, an inflammasome inhibitor [63] (Figure 1).

Many clinical studies examined the role of antioxidants in ROS-mediated I/R injury through the administration of antioxidant drugs after thrombolysis, but the results were not positive in reducing infarct size or enhancing heart function [65]. On the contrary, in mice KO for NADPH oxidases (Nox) 2 and 4, contributing to part of ROS production during I/R injury, a reduction in ROS production and a decrease of the infarct size were observed after I/R [66]. However, it was suggested that a total inhibition of Nox is not positive as it is involved in the physiological and beneficial production of ROS [66]. Among antioxidant drugs, resveratrol protects the heart during I/R injury by inhibition of NALP3 inflammasome and ROS production [67] (Figure 1).

4. ROS and NLRP3 in I/R Injury of the Kidney

I/R injury is one of the common causes of acute renal failure, thus playing a significant impact on patient morbidity and mortality [33]. Kidneys are particularly vulnerable to ischemia; therefore, I/R injury may cause early graft rejection in renal transplantation and induce structural damage after suprarenal aneurysm repair, renal artery reconstruction, contrast agent-induced nephropathy, cardiac arrest, and shock [68]. Even if ischemia initiates a complex, organized series of events, resulting in damage and death of renal cells due to the dramatic decrease in oxygen and nutrition, reperfusion, though essential for tissue survival, determines an exacerbation of tissue injury and a profound inflammatory response, leading to renal dysfunction [1]. Increasing ischemia time worsens the histological changes, which are particularly severe at 24–72 hours after reperfusion.

Epithelial cells, particularly those of the S3 segment of the proximal tubule in the outer renal medulla, are particularly exposed to both ischemia and reperfusion phases of I/R injury, which can lead to acute tubular necrosis [69, 70]; on the contrary, glomerular vessels degeneration was described only in the reperfusion phase [69]. Histopathological evaluation revealed the presence of extensive vascular dilatation, slight interstitial edema, tubular dilatation, tubular cell swelling, brush border, and nuclear loss [71]. Furthermore, I/R injury induces an early infiltration of inflammatory cells, mainly neutrophils [72], in addition to the rapid tubular necrosis determining an acute renal dysfunction [73]. However, it was observed that the less I/R-sensitive cells of the distal tubules might have a protective role, leading to reepithelialization of the injured tubules and preventing the progression to chronic kidney disease [74].

There is now substantial evidence that ROS and NLRP3 inflammasome have a key role, even if not coincident, in the primary mechanism through which I/R induces the above indicated kidney damage.

In fact, Iyer et al. [75] showed that nonlethal renal I/R injury resulted in a significant upregulation of NLRP3 gene expression, which was accompanied by pronounced acute tubular necrosis. It was suggested that specific forms of cellular injury result in the release of viable mitochondria into the extracellular space, triggering the activation of the NLRP3 inflammasome, in part through the release of ATP.

Furthermore, upregulation of NLRP3 activates caspase-1 and, subsequently, IL-1 β and IL-18; in addition, caspase-1 induces pyroptosis, a proinflammatory form of programmed cell death, characterized by pores in the plasma membrane at early time points [33].

Several pharmacological approaches were proposed to reduce the functional and morphological damage induced by I/R in kidney. In particular, the pathways of oxidative stress and NLRP3 inflammasome were examined by the administration of antioxidant drugs or by the study of NLRP3 knock-out (KO) animals (Figure 1).

As to the drugs acting on the ROS production and/or scavenging in the kidney, the pretreatment with the pineal hormone melatonin had a protective effect against oxidative damage caused by free radicals in a number of models both *in vivo* and *in vitro* of I/R injury: in particular, when administered prior to ischemia and immediately before the reperfusion, melatonin reduced the renal structural changes and limited the neutrophils infiltration [69].

Similar results were obtained from the pretreatment with naringin [76], aqueous garlic extract [77], rutin [78], propofol [79], celastrol [71], and allopurinol and apocynin, administered alone or together [80]; all these therapeutic strategies were based on the antioxidant properties of these substances, which are able to positively act on the exaggerated inflammatory responses and tissue damage dependent on the free radicals production and the inflammatory cells infiltration [53].

The pretreatment with modified adenovirus expressing IL-13, which is known to display antioxidant properties, diminished renal tubulointerstitial damage and inflammation induced by I/R [81].

Important data on the mechanisms involved in the I/R of kidney were obtained from the study of KO animals. In fact, in IL-18 KO mice, a reduced tubular damage and higher protection against I/R injury [82] were observed. In NLRP3 KO mice, an increased proliferation of tubular epithelial cells was observed, thus indicating that NLRP3 is detrimental to the repair response after the reperfusion phase of the I/R injury [83]. Similarly, in NLRP3 KO mice following I/R injury, an increased protection from lethal ischemic injury [56] and a reduced tubular necrosis and apoptosis, with consequent repopulation of the tubular epithelium, were observed after the reperfusion phase [84]. On the contrary, when ASC KO mice were examined, the protection from lethal ischemic injury was less pronounced [32, 75] and the tubular necrosis and apoptosis were higher and similar to those observed in WT mice [84]. It was evident that, at least

in the kidney, the NLRP3 protein, the essential component of the inflammasome, may play an independent role in injury signaling, different from that of the other components of the inflammasome (ASC, caspase-1).

5. ROS and NLRP3 in I/R Injury of the Testis

Testis torsion is a testicular lesion typical of the pediatric population and it is representative of the I/R injury observed in other organs [1, 85]. Under these circumstances, the testis produces several proinflammatory cytokines, whose increased levels can be considered an indirect evidence of tissue inflammation [86].

In normal conditions, enzymatic antioxidant defense systems, such as SOD, GPx, and CAT, protect testicular somatic and germinal cells from free radical damage. On the contrary, malondialdehyde (MDA) is an important indicator of lipid peroxidation induced by ROS [87].

I/R induces early tissue injuries, such as reactive oxygen species (ROS) generation [4], and a damaged barrier function of endothelial cells, resulting from reduced adenylate cyclase activity and intracellular cAMP levels. In particular, during I/R, nucleotides in the form of ATP stimulate tissue inflammation and trigger NLRP3 inflammasome [88]. This pathological cascade causes a decreased number of germ cells induced by an enhanced apoptosis, the vacuolization of the seminiferous epithelium, a reduced number of spermatozoa, and a recruitment of neutrophils [89]; at a later stage, testicular atrophy and impaired spermatogenesis are observed [90–92].

In order to prevent testicular I/R injury, the effects of different substances with antioxidant activities and the role of NLRP3 inflammasome have been investigated (Figure 1).

Among antioxidant substances, lipoic acid (LA) has ROS scavenging and metal chelating ability and regenerates endogenous antioxidants, such as glutathione and vitamins E and C [87]. The pretreatment with LA induced an increase of SOD and GPx activity, reduced MDA levels, and abated cellular damage.

Similar results have been obtained by the pre- or posttreatment with different antioxidant pharmacological approaches, including taurine [93], dehydroepiandrosterone [94], curcumin [95], *Psoralea corylifolia* [96], rutin [97], thymoquinone [98], and apocynin [99].

As to the role of NLRP3 inflammasome, specific inhibitors, such as BAY 11-7082 [100] and Brilliant Blue G (BBG) [101], are able to inhibit its effects in a testicular I/R model [102]. In fact, BAY 11-7082, an I- κ B kinase- β inhibitor, and BBG, blocking the membrane-bound purinergic P2X7 receptor, showed a significant reduction of IL-1 β and IL-18 mRNA expression, blunted caspase-1 and caspase-3 expression, minor histological damage, low TUNEL activity, and preserved spermatogenesis, indicating a selectively reduced NLRP3 inflammasome activity [102]. It was also observed that NLRP3 KO mice responded to I/R insult with a lower activation of the inflammatory and apoptosis cascade than WT animals.

Therefore, NLRP3 can be considered an interesting target for innovative drugs aimed at treating I/R injury after testicular torsion.

6. Conclusions

The restoration of blood flow as soon as possible is without any doubt the primary therapeutic approach to ischemia, even if reperfusion, although essential to restore oxygen and nutrients supply and to remove potentially harmful products of cellular metabolism, can induce further pathological processes in the same organ and tissue injuries in other organs.

The main points to be stressed as a conclusion of this review can be summarized as follows:

- (i) ROS have beneficial effects, as they regulate several important, physiological responses by redox-responsive signaling pathways, but, during I/R, they promote tissue inflammation and activate immune response through different pathways, including NLRP3 inflammasome.
- (ii) Not all organs demonstrate equal sensitivity to ischemia [2], the brain being the most sensitive to reductions in its blood supply; all organs demonstrate similar sensitivity to reperfusion injury, whose key events are inflammation and oxidative stress.
- (iii) The effects of ROS-mediated NLRP3 inflammasome activation in course of I/R injury in other experimental models are strongly suggested; in fact, by the examination of the existing literature, no data on limb, intestine, and ovary ischemia are present.
- (iv) In some of the already available experimental models, such as the heart, the testis, and the kidney, further studies using KO mice (NLRP3 or ASC) or antagonists of the NLRP3 cascade are needed to better understand the physiopathological events during I/R injury.

In conclusion, despite the increased literature of the past decade, a definite comprehension of the role of NLRP3 inflammasome in the host responses to different danger signals is still lacking. A detailed examination of the molecular mechanisms driving NLRP3 inflammasome transcription, assembly, and activation is needed to elucidate these processes in the different organs. This experimental approach, given the role of NLRP3 in several sterile inflammatory diseases, could be the basis for the design and elaboration of novel NLRP3 inflammasome inhibitors, thus avoiding the exclusive use of substances with antioxidant activity in patients with ischemic damage.

Abbreviations

- I/R: Ischemia and reperfusion
 ROS: Reactive oxygen species
 NLRP3: NOD-like receptor family pyrin domain containing 3

LOX:	Lipoxygenase
COX:	Cyclooxygenase
TRX:	Thioredoxin
CAT:	Catalase
PRRs:	Pattern-recognition receptors
PAMPs:	Pathogen-associated molecular patterns
DAMPs:	Damage-associated molecular patterns
TLRs:	Toll-like receptors
RLRs:	RIG-I-like receptors
NF- κ B:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLRs:	Nod-like receptors
ALRs:	AIM2-like receptors
CLRs:	C-type lectin receptors
IL-1 β :	Interleukin-1 β
IL-18:	Interleukin-18
TXNIP:	TRX-interacting protein
SOD:	Superoxide dismutase
GPx:	Glutathione peroxidase
MDA:	Malondialdehyde
KO:	Knock-out
BBG:	Brilliant Blue G.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

References

- [1] H. K. Eltzschig and T. Eckle, "Ischemia and reperfusion—from mechanism to translation," *Nature Medicine*, vol. 17, no. 11, pp. 1391–1401, 2011.
- [2] T. Kalogeris, C. P. Baines, M. Krenz, and R. J. Korthuis, "Cell biology of ischemia/reperfusion injury," *International Review of Cell and Molecular Biology*, vol. 298, pp. 229–317, 2012.
- [3] D. L. Carden and D. N. Granger, "Pathophysiology of ischaemia-reperfusion injury," *The Journal of Pathology*, vol. 190, no. 3, pp. 255–266, 2000.
- [4] X. Lei, H. Chao, Z. Zhang et al., "Neuroprotective effects of quercetin in a mouse model of brain ischemic/reperfusion injury via anti-apoptotic mechanisms based on the Akt pathway," *Molecular Medicine Reports*, vol. 12, no. 3, pp. 3688–3696, 2015.
- [5] F. Martinon, "Signaling by ROS drives inflammasome activation," *European Journal of Immunology*, vol. 40, no. 3, pp. 616–619, 2010.
- [6] P. D. Ray, B.-W. Huang, and Y. Tsuji, "Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling," *Cellular Signalling*, vol. 24, no. 5, pp. 981–990, 2012.
- [7] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.
- [8] J. Limón-Pacheco and M. E. Gonsebatt, "The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 674, no. 1–2, pp. 137–147, 2009.
- [9] R. Dringen, "Metabolism and functions of glutathione in brain," *Progress in Neurobiology*, vol. 62, no. 6, pp. 649–671, 2000.
- [10] J. Jurado, M.-J. Prieto-Álamo, J. Madrid-Risquez, and C. Pueyo, "Absolute gene expression patterns of thioredoxin and glutaredoxin redox systems in mouse," *The Journal of Biological Chemistry*, vol. 278, no. 46, pp. 45546–45554, 2003.
- [11] C. Bryant and K. A. Fitzgerald, "Molecular mechanisms involved in inflammasome activation," *Trends in Cell Biology*, vol. 19, no. 9, pp. 455–464, 2009.
- [12] C. Bourgeois and K. Kuchler, "Fungal pathogens—a sweet and sour treat for toll-like receptors," *Frontiers in Cellular and Infection Microbiology*, vol. 2, p. 142, 2012.
- [13] J. Cui, Y. Chen, H. Y. Wang, and R.-F. Wang, "Mechanisms and pathways of innate immune activation and regulation in health and cancer," *Human Vaccines & Immunotherapeutics*, vol. 10, no. 11, pp. 3270–3285, 2014.
- [14] F. Martinon, K. Burns, and J. Tschopp, "The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL- β ," *Molecular Cell*, vol. 10, no. 2, pp. 417–426, 2002.
- [15] B. Z. Shao, Z. Q. Xu, B. Z. Han, D. F. Su, and C. Liu, "NLRP3 inflammasome and its inhibitors: a review," *Frontiers in Pharmacology*, vol. 6, article 262, 2015.
- [16] E. Ozaki, M. Campbell, and S. L. Doyle, "Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives," *Journal of Inflammation Research*, vol. 8, pp. 15–27, 2015.
- [17] J. M. Kahlenberg, K. C. Lundberg, S. B. Kertesz, Y. Qu, and G. R. Dubyak, "Potentiation of caspase-1 activation by the P2X7 receptor is dependent on TLR signals and requires NF- κ B-driven protein synthesis," *Journal of Immunology*, vol. 175, no. 11, pp. 7611–7622, 2005.
- [18] F. G. Bauernfeind, G. Horvath, A. Stutz et al., "Cutting edge: NF- κ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression," *Journal of Immunology*, vol. 183, no. 2, pp. 787–791, 2009.
- [19] H. Zhao, J. S. Perez, K. Lu, A. J. T. George, and D. Ma, "Role of toll-like receptor-4 in renal graft ischemia-reperfusion injury," *American Journal of Physiology—Renal Physiology*, vol. 306, no. 8, pp. F801–F811, 2014.
- [20] G. Vilahur and L. Badimon, "Ischemia/reperfusion activates myocardial innate immune response: the key role of the toll-like receptor," *Frontiers in Physiology*, vol. 5, article 496, 2014.
- [21] A. Rubartelli, "Redox control of NLRP3 inflammasome activation in health and disease," *Journal of Leukocyte Biology*, vol. 92, no. 5, pp. 951–958, 2012.
- [22] K. Schroder and J. Tschopp, "The inflammasomes," *Cell*, vol. 140, no. 6, pp. 821–832, 2010.
- [23] Q. Zheng, Y. Ren, P. S. Reinach et al., "Reactive oxygen species activated NLRP3 inflammasomes prime environment-induced murine dry eye," *Experimental Eye Research*, vol. 125, pp. 1–8, 2014.
- [24] L. Niu, S. Zhang, J. Wu, L. Chen, and Y. Wang, "Upregulation of NLRP3 inflammasome in the tears and ocular surface of dry eye patients," *PLoS ONE*, vol. 10, no. 5, Article ID e0126277, 2015.
- [25] C. He and D. J. Klionsky, "Regulation mechanisms and signaling pathways of autophagy," *Annual Review of Genetics*, vol. 43, pp. 67–93, 2009.
- [26] H.-J. Anders and D. A. Muruve, "The inflammasomes in kidney disease," *Journal of the American Society of Nephrology*, vol. 22, no. 6, pp. 1007–1018, 2011.

- [27] A. J. S. Choi and S. W. Ryter, "Inflammasomes: molecular regulation and implications for metabolic and cognitive diseases," *Molecules and Cells*, vol. 37, no. 6, pp. 441–448, 2014.
- [28] J. Rivers-Auty and D. Brough, "Potassium efflux fires the canon: potassium efflux as a common trigger for canonical and non-canonical NLRP3 pathways," *European Journal of Immunology*, vol. 45, no. 10, pp. 2758–2761, 2015.
- [29] O. Gross, C. J. Thomas, G. Guarda, and J. Tschopp, "The inflammasome: an integrated view," *Immunological Reviews*, vol. 243, no. 1, pp. 136–151, 2011.
- [30] J. Tschopp and K. Schroder, "NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production?" *Nature Reviews Immunology*, vol. 10, no. 3, pp. 210–215, 2010.
- [31] J. Zhou and W.-J. Chng, "Roles of thioredoxin binding protein (TXNIP) in oxidative stress, apoptosis and cancer," *Mitochondrion*, vol. 13, no. 3, pp. 163–169, 2013.
- [32] H.-J. Kim, D. W. Lee, K. Ravichandran et al., "NLRP3 Inflammasome knockout mice are protected against ischemic but not cisplatin-induced acute kidney injury," *Journal of Pharmacology and Experimental Therapeutics*, vol. 346, no. 3, pp. 465–472, 2013.
- [33] C. M. Turner, N. Arulkumar, M. Singer, R. J. Unwin, and F. W. Tam, "Is the inflammasome a potential therapeutic target in renal disease?" *BMC Nephrology*, vol. 15, article 21, 2014.
- [34] M. Ahmad, N. J. Dar, Z. S. Bhat et al., "Inflammation in ischemic stroke: mechanisms, consequences and possible drug targets," *CNS and Neurological Disorders—Drug Targets*, vol. 13, no. 8, pp. 1378–1396, 2014.
- [35] H. Marini, D. Altavilla, M. Bellomo et al., "Modulation of IL-1 β gene expression by lipid peroxidation inhibition after kainic acid-induced rat brain injury," *Experimental Neurology*, vol. 188, no. 1, pp. 178–186, 2004.
- [36] B. Fogal and S. J. Hewett, "Interleukin-1 β : a bridge between inflammation and excitotoxicity?" *Journal of Neurochemistry*, vol. 106, no. 1, pp. 1–23, 2008.
- [37] A. Denes, P. Thornton, N. J. Rothwell, and S. M. Allan, "Inflammation and brain injury: acute cerebral ischaemia, peripheral, and central inflammation," *Brain, Behavior, and Immunity*, vol. 24, no. 5, pp. 708–723, 2010.
- [38] R. von Bernhardi, L. Eugenín-von Bernhardi, and J. Eugenín, "Microglial cell dysregulation in brain aging and neurodegeneration," *Frontiers in Aging Neuroscience*, vol. 7, article 124, 2015.
- [39] D. Brough, R. A. Le Feuvre, Y. Iwakura, and N. J. Rothwell, "Purinergic (P2X7) receptor activation of microglia induces cell death via an interleukin-1-independent mechanism," *Molecular and Cellular Neuroscience*, vol. 19, no. 2, pp. 272–280, 2002.
- [40] V. Hornung and E. Latz, "Critical functions of priming and lysosomal damage for NLRP3 activation," *European Journal of Immunology*, vol. 40, no. 3, pp. 620–623, 2010.
- [41] C. D. Savage, G. Lopez-Castejon, A. Denes, and D. Brough, "NLRP3-inflammasome activating DAMPs stimulate an inflammatory response in glia in the absence of priming which contributes to brain inflammation after injury," *Frontiers in Immunology*, vol. 3, article 288, 2012.
- [42] D. Yang-Wei Fann, S.-Y. Lee, S. Manzanero et al., "Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke," *Cell Death & Disease*, vol. 4, no. 9, article e790, 2013.
- [43] D. Y.-W. Fann, T. Santro, S. Manzanero et al., "Intermittent fasting attenuates inflammasome activity in ischemic stroke," *Experimental Neurology*, vol. 257, pp. 114–119, 2014.
- [44] F. Yang, Z. Wang, X. Wei et al., "NLRP3 deficiency ameliorates neurovascular damage in experimental ischemic stroke," *Journal of Cerebral Blood Flow & Metabolism*, vol. 34, no. 4, pp. 660–667, 2014.
- [45] M. Ito, T. Shichita, M. Okada et al., "Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury," *Nature Communications*, vol. 6, article 7360, 2015.
- [46] A. F. Herrera and E. D. Jacobsen, "Ibrutinib for the treatment of mantle cell lymphoma," *Clinical Cancer Research*, vol. 20, no. 21, pp. 5365–5371, 2014.
- [47] A. Abderrazak, T. Syrovets, D. Couchie et al., "NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases," *Redox Biology*, vol. 4, pp. 296–307, 2015.
- [48] Y. Li, J. Li, S. Li et al., "Curcumin attenuates glutamate neurotoxicity in the hippocampus by suppression of ER stress-associated TXNIP/NLRP3 inflammasome activation in a manner dependent on AMPK," *Toxicology and Applied Pharmacology*, vol. 286, no. 1, pp. 53–63, 2015.
- [49] X. Wang, R. Li, X. Wang, Q. Fu, and S. Ma, "Umbelliferone ameliorates cerebral ischemia-reperfusion injury via upregulating the PPAR gamma expression and suppressing TXNIP/NLRP3 inflammasome," *Neuroscience Letters*, vol. 600, pp. 182–187, 2015.
- [50] T. Ishrat, I. N. Mohamed, B. Pillai et al., "Thioredoxin-interacting protein: a novel target for neuroprotection in experimental thromboembolic stroke in mice," *Molecular Neurobiology*, vol. 51, no. 2, pp. 766–778, 2015.
- [51] J. Zhao, Y. Mou, J. D. Bernstock et al., "Synthetic oligodeoxynucleotides containing multiple telemeric TTAGGG motifs suppress inflammasome activity in macrophages subjected to oxygen and glucose deprivation and reduce ischemic brain injury in stroke-prone spontaneously hypertensive rats," *PLoS ONE*, vol. 10, no. 10, Article ID e0140772, 2015.
- [52] Ø. Sandanger, E. Gao, T. Ranheim et al., "NLRP3 inflammasome activation during myocardial ischemia reperfusion is cardioprotective," *Biochemical and Biophysical Research Communications*, vol. 469, no. 4, pp. 1012–1020, 2016.
- [53] S. M. Davidson, D. M. Yellon, M. P. Murphy, and M. R. Duchon, "Slow calcium waves and redox changes precede mitochondrial permeability transition pore opening in the intact heart during hypoxia and reoxygenation," *Cardiovascular Research*, vol. 93, no. 3, pp. 445–453, 2012.
- [54] K. Zhan, P. Yu, C. Liu, J. Luo, and W. Yang, "Detrimental or beneficial: the role of TRPM2 in ischemia/reperfusion injury," *Acta Pharmacologica Sinica*, vol. 37, no. 1, pp. 4–12, 2016.
- [55] Y. Wang, Y. Wu, J. Chen, S. Zhao, and H. Li, "Pirfenidone attenuates cardiac fibrosis in a mouse model of TAC-induced left ventricular remodeling by suppressing NLRP3 Inflammasome formation," *Cardiology*, vol. 126, no. 1, pp. 1–11, 2013.
- [56] M. Takahashi, "Role of the inflammasome in myocardial infarction," *Trends in Cardiovascular Medicine*, vol. 21, no. 2, pp. 37–41, 2011.
- [57] S. Toldo, C. Marchetti, and A. Abbate, "Re. 'NLRP3 inflammasome activation during myocardial ischemia reperfusion is cardioprotective,'" *Biochemical and Biophysical Research Communications*, vol. 470, no. 4, pp. 811–812, 2016.
- [58] B. J. Pomerantz, L. L. Reznikov, A. H. Harken, and C. A. Dinarello, "Inhibition of caspase 1 reduces human myocardial

- ischemic dysfunction via inhibition of IL-18 and IL-1 β ,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 5, pp. 2871–2876, 2001.
- [59] M. Kawaguchi, M. Takahashi, T. Hata et al., “Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury,” *Circulation*, vol. 123, no. 6, pp. 594–604, 2011.
- [60] K. Venkatachalam, S. D. Prabhu, V. S. Reddy, W. H. Boylston, A. J. Valente, and B. Chandrasekar, “Neutralization of interleukin-18 ameliorates ischemia/reperfusion-induced myocardial injury,” *The Journal of Biological Chemistry*, vol. 284, no. 12, pp. 7853–7865, 2009.
- [61] M. Wang, T. A. Markel, and D. R. Meldrum, “Interleukin 18 in the heart,” *Shock*, vol. 30, no. 1, pp. 3–10, 2008.
- [62] H. Gu, M. Xie, L. Xu, X. Zheng, Y. Yang, and X. Lv, “The protective role of interleukin-18 binding protein in a murine model of cardiac ischemia/reperfusion injury,” *Transplant International*, vol. 28, no. 12, pp. 1436–1444, 2015.
- [63] Y. Liu, K. Lian, L. Zhang et al., “TXNIP mediates NLRP3 inflammasome activation in cardiac microvascular endothelial cells as a novel mechanism in myocardial ischemia/reperfusion injury,” *Basic Research in Cardiology*, vol. 109, no. 5, p. 415, 2014.
- [64] J. Yoshioka, W. A. Chutkow, S. Lee et al., “Deletion of thioredoxin-interacting protein in mice impairs mitochondrial function but protects the myocardium from ischemia-reperfusion injury,” *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 267–279, 2012.
- [65] D. M. Yellon and D. J. Hausenloy, “Myocardial reperfusion injury,” *The New England Journal of Medicine*, vol. 357, no. 11, pp. 1074–1135, 2007.
- [66] S. Matsushima, J. Kuroda, T. Ago et al., “Broad suppression of NADPH oxidase activity exacerbates ischemia/reperfusion injury through inadvertent downregulation of hypoxia-inducible factor-1 α and upregulation of peroxisome proliferator-activated receptor- α ,” *Circulation Research*, vol. 112, no. 8, pp. 1135–1149, 2013.
- [67] W. Dong, R. Yang, J. Yang et al., “Resveratrol pretreatment protects rat hearts from ischemia/reperfusion injury partly via a NALP3 inflammasome pathway,” *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 8, pp. 8731–8741, 2015.
- [68] L. F. Tirapelli, D. F. Barione, B. F. M. Trazzi et al., “Comparison of two models for evaluation histopathology of experimental renal ischemia,” *Transplantation Proceedings*, vol. 41, no. 10, pp. 4083–4087, 2009.
- [69] G. Sener, A. Ö. Şehirli, M. Keyer-Uysal, S. Arbak, Y. Ersoy, and B. Ç. Yeğen, “The protective effect of melatonin on renal ischemia-reperfusion injury in the rat,” *Journal of Pineal Research*, vol. 32, no. 2, pp. 120–126, 2002.
- [70] R. W. Schrier, W. Wang, B. Poole, and A. Mitra, “Acute renal failure: definitions, diagnosis, pathogenesis, and therapy,” *The Journal of Clinical Investigation*, vol. 114, no. 1, pp. 5–14, 2004.
- [71] C. Chu, W. He, Y. Kuang, K. Ren, and X. Gou, “Celastrol protects kidney against ischemia-reperfusion-induced injury in rats,” *Journal of Surgical Research*, vol. 186, no. 1, pp. 398–407, 2014.
- [72] J. J. Friedewald and H. Rabb, “Inflammatory cells in ischemic acute renal failure,” *Kidney International*, vol. 66, no. 2, pp. 486–491, 2004.
- [73] J. V. Bonventre and A. Zuk, “Ischemic acute renal failure: an inflammatory disease?” *Kidney International*, vol. 66, no. 2, pp. 480–485, 2004.
- [74] G. C. Gobe and D. W. Johnson, “Distal tubular epithelial cells of the kidney: potential support for proximal tubular cell survival after renal injury,” *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 9, pp. 1551–1561, 2007.
- [75] S. S. Iyer, W. P. Pulsikens, J. J. Sadler et al., “Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 48, pp. 20388–20393, 2009.
- [76] D. Singh and K. Chopra, “The effect of naringin, a bioflavonoid on ischemia-reperfusion induced renal injury in rats,” *Pharmacological Research*, vol. 50, no. 2, pp. 187–193, 2004.
- [77] L. Kabasakal, Ö. Şehirli, Ş. Çetinel, E. Cikler, N. Gedik, and G. Şener, “Protective effect of aqueous garlic extract against renal ischemia/reperfusion injury in rats,” *Journal of Medicinal Food*, vol. 8, no. 3, pp. 319–326, 2005.
- [78] A. Korkmaz and D. Kolankaya, “Protective effect of rutin on the ischemia/reperfusion induced damage in rat kidney,” *Journal of Surgical Research*, vol. 164, no. 2, pp. 309–315, 2010.
- [79] S. Yang, W.-P. Chou, and L. Pei, “Effects of propofol on renal ischemia/reperfusion injury in rats,” *Experimental and Therapeutic Medicine*, vol. 6, no. 5, pp. 1177–1183, 2013.
- [80] E. K. Choi, H. Jung, K. H. Kwak et al., “Effects of allopurinol and apocynin on renal ischemia-reperfusion injury in rats,” *Transplantation Proceedings*, vol. 47, no. 6, pp. 1633–1638, 2015.
- [81] M. Sandovici, R. H. Henning, H. van Goor, W. Helfrich, D. de Zeeuw, and L. E. Deelman, “Systemic gene therapy with interleukin-13 attenuates renal ischemia-reperfusion injury,” *Kidney International*, vol. 73, no. 12, pp. 1364–1373, 2008.
- [82] H. Wu, M. L. Craft, P. Wang et al., “IL-18 contributes to renal damage after ischemia-reperfusion,” *Journal of the American Society of Nephrology*, vol. 19, no. 12, pp. 2331–2341, 2008.
- [83] P. J. Bakker, L. M. Butter, N. Claessen et al., “A tissue-specific role for Nlrp3 in tubular epithelial repair after renal ischemia/reperfusion,” *The American Journal of Pathology*, vol. 184, no. 7, pp. 2013–2022, 2014.
- [84] A. A. Shigeoka, J. L. Mueller, A. Kambo et al., “An inflammasome-independent role for epithelial-expressed Nlrp3 in renal ischemia-reperfusion injury,” *The Journal of Immunology*, vol. 185, no. 10, pp. 6277–6285, 2010.
- [85] L. Minutoli, P. Antonuccio, F. Polito et al., “Mitogen-activated protein kinase 3/mitogen-activated protein kinase 1 activates apoptosis during testicular ischemia-reperfusion injury in a nuclear factor- κ B-independent manner,” *European Journal of Pharmacology*, vol. 604, no. 1–3, pp. 27–35, 2009.
- [86] J. J. Lysiak, “The role of tumor necrosis factor-alpha and interleukin-1 in the mammalian testis and their involvement in testicular torsion and autoimmune orchitis,” *Reproductive Biology and Endocrinology*, vol. 2, article 9, 2004.
- [87] S. Ozbal, B. U. Ergur, G. Erbil, I. Tekmen, A. Bagryank, and Z. Cavdar, “The effects of α -lipoic acid against testicular ischemia-reperfusion injury in rats,” *The Scientific World Journal*, vol. 2012, Article ID 489248, 8 pages, 2012.
- [88] B. McDonald, K. Pittman, G. B. Menezes et al., “Intravascular danger signals guide neutrophils to sites of sterile inflammation,” *Science*, vol. 330, no. 6002, pp. 362–366, 2010.
- [89] J. G. Reyes, J. G. Farias, S. Henriquez-Olavarrieta et al., “The hypoxic testicle: physiology and pathophysiology,” *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 929285, 15 pages, 2012.

- [90] P. Antonuccio, L. Minutoli, C. Romeo et al., "Lipid peroxidation activates mitogen-activated protein kinases in testicular ischemia-reperfusion injury," *The Journal of Urology*, vol. 176, no. 4, part 1, pp. 1666–1672, 2006.
- [91] A. K. Srinivasan, J. Freyle, J. S. Gitlin, and L. S. Palmer, "Climatic conditions and the risk of testicular torsion in adolescent males," *The Journal of Urology*, vol. 178, no. 6, pp. 2585–2588, 2007.
- [92] L. Minutoli, P. Antonuccio, F. Squadrito et al., "Effects of polydeoxyribonucleotide on the histological damage and the altered spermatogenesis induced by testicular ischaemia and reperfusion in rats," *International Journal of Andrology*, vol. 35, no. 2, pp. 133–144, 2012.
- [93] S.-M. Wei, Z.-Z. Yan, and J. Zhou, "Beneficial effect of taurine on testicular ischemia-reperfusion injury in rats," *Urology*, vol. 70, no. 6, pp. 1237–1242, 2007.
- [94] H. Aksoy, T. Yapanoglu, Y. Aksoy, I. Özbey, H. Turhan, and N. Gursan, "Dehydroepiandrosterone treatment attenuates reperfusion injury after testicular torsion and detorsion in rats," *Journal of Pediatric Surgery*, vol. 42, no. 10, pp. 1740–1744, 2007.
- [95] S.-M. Wei, Z.-Z. Yan, and J. Zhou, "Curcumin attenuates ischemia-reperfusion injury in rat testis," *Fertility and Sterility*, vol. 91, no. 1, pp. 271–277, 2009.
- [96] S.-M. Wei, Z.-Z. Yan, and J. Zhou, "Psoralea corylifolia protects against testicular torsion/detorsion-induced ischemia/reperfusion injury," *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 568–574, 2011.
- [97] S.-M. Wei, Z.-Z. Yan, and J. Zhou, "Protective effect of rutin on testicular ischemia-reperfusion injury," *Journal of Pediatric Surgery*, vol. 46, no. 7, pp. 1419–1424, 2011.
- [98] M. Ayan, U. Tas, E. Sogut et al., "Protective effect of thymoquinone against testicular torsion induced oxidative injury," *Andrologia*, vol. 48, no. 2, pp. 143–151, 2016.
- [99] T. E. Şener, M. Yüksel, N. Özyılmaz-Yay et al., "Apocynin attenuates testicular ischemia-reperfusion injury in rats," *Journal of Pediatric Surgery*, vol. 50, no. 8, pp. 1382–1387, 2015.
- [100] C. Juliana, T. Fernandes-Alnemri, J. Wu et al., "Anti-inflammatory compounds parthenolide and bay 11-7082 are direct inhibitors of the inflammasome," *Journal of Biological Chemistry*, vol. 285, no. 13, pp. 9792–9802, 2010.
- [101] J. Zhao, H. Wang, C. Dai et al., "P2X7 blockade attenuates murine lupus nephritis by inhibiting activation of the NLRP3/ASC/caspase 1 pathway," *Arthritis & Rheumatism*, vol. 65, no. 12, pp. 3176–3185, 2013.
- [102] L. Minutoli, P. Antonuccio, N. Irrera et al., "NLRP3 Inflammasome involvement in the organ damage and impaired spermatogenesis induced by testicular ischemia and reperfusion in mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 355, no. 3, pp. 370–380, 2015.

Research Article

Antioxidant Effects of Sheep Whey Protein on Endothelial Cells

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Excessive production of reactive oxygen species (ROS) may cause endothelial dysfunction and consequently vascular disease. In the present study, the possible protective effects of sheep whey protein (SWP) from tert-butyl hydroperoxide- (tBHP-) induced oxidative stress in endothelial cells (EA.hy926) were assessed using oxidative stress biomarkers. These oxidative stress biomarkers were glutathione (GSH) and ROS levels determined by flow cytometry. Moreover, thiobarbituric acid-reactive substances (TBARS), protein carbonyls (CARB), and oxidized glutathione (GSSG) were determined spectrophotometrically. The results showed that SWP at 0.78, 1.56, 3.12, and 6.24 mg of protein mL⁻¹ increased GSH up to 141%, while it decreased GSSG to 46.7%, ROS to 58.5%, TBARS to 52.5%, and CARB to 49.0%. In conclusion, the present study demonstrated for the first time that SWP protected endothelial cells from oxidative stress. Thus, SWP may be used for developing food supplements or biofunctional foods to attenuate vascular disturbances associated with oxidative stress.

1. Introduction

Free radicals such as reactive oxygen species (ROS) can be generated in a wide variety of chemical and biological systems. ROS play an important role in body's immune response [1], redox regulation of gene transcription [2], and cell signaling [1]. On the other hand, the ensuing cascade of ROS can result in cellular damage including apoptosis, protein oxidation, DNA modification, and lipid peroxidation [3]. Under normal conditions ROS are controlled by antioxidant systems. When there is a disturbance between the prooxidant and antioxidant balance in favor of the former that leads to oxidative stress which can cause damage to all molecular targets [1], a range of antioxidants are active in the body including enzymatic and nonenzymatic antioxidants [4]. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) [4]. Non-enzymatic antioxidants include vitamin A, vitamin C, vitamin E, flavonoids, glutathione (GSH), uric acid, and bilirubin [5].

The endothelium lines the entire vascular system and is composed of a monolayer of endothelial cells. Endothelial cell structure and functional integrity are important in

the maintenance of the vessel wall and circulatory function. In addition to its role as a selective permeability barrier, endothelial cells are dynamic and are capable of conducting a variety of metabolic and synthetic functions and regulating homeostasis, immune, and inflammatory responses [6]. Endothelial cell injury or dysfunction is a hallmark of many pathologic conditions including atherosclerosis and thrombosis [6]. Excessive production of ROS may exceed the capacity of antioxidant mechanisms, thus contributing to vascular disease by induction of endothelial dysfunction through several pathways [6]. Endothelial dysfunction is considered largely as endothelial activation, which may eventually contribute to arterial disease [6]. Inflammatory cytokines, growth factors, and the interaction of the endothelium with leukocytes may induce ROS signaling in endothelial cells. Moreover, interaction between ROS and NO may cause a vicious circle leading to more endothelial activation and inflammation [6]. In addition, superoxide dismutase may use superoxide radical (O₂^{•-}) for producing hydrogen peroxide which can diffuse to the endothelial cells and damage proteins through reaction with cysteine groups [7]. Thus, continuous ROS signaling in endothelial cells can cause loss of integrity, progression to senescence, and detachment into the circulation [8].

Thus, there is a great interest for natural sources of antioxidants in order to enhance antioxidant mechanisms and protect the organism from the harmful effects of oxidative stress. For example, whey protein is a widely consumed supplement that is considered to increase the antioxidant defense [9, 10]. Whey protein is a by-product of cheese manufacturing, but it is used as a functional food with nutritional applications [11, 12]. The main components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropolymers, and lactose [13]. Some of these components act as antioxidants. For example, alpha-lactalbumin can chelate iron and thus result in the reduction of oxidative stress [14]. Moreover, whey protein has a high content in the sulphur-containing amino acids cysteine and methionine that enhance antioxidant mechanisms through intracellular conversion to glutathione [11].

In our previous studies, we have shown that a cake containing sheep whey protein (SWP) had antioxidant and anti-inflammatory activities in subjects submitted to intense exercise [9, 15]. We have also shown that SWP exerted antioxidant effects on C2C12 muscle cells [16]. The aim of the present study was to examine the possible protective effects of SWP against tert-butyl hydroperoxide- (tBHP-) induced oxidative stress in EA.hy926 endothelial cells.

2. Materials and Methods

2.1. Chemicals, Reagents, and Culture Medium. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), and L-glutamine and trypsin were purchased from Gibco (Grand Island, NY). Tert-butyl hydroperoxide (tBHP), 2,4-dinitrophenylhydrazine (DNPH), urea, oxidized glutathione (GSSG), nicotinamide adenine di-nucleotide phosphate (NADPH), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 2-vinyl pyridine, glutathione reductase, ethyl acetate, Bradford reagent, mercury orange, and 2,7-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), sodium hydroxide (NaOH), 2-thiobarbituric acid (TBA), and ethanol were purchased from Merck (Darmstadt, Germany). Cell proliferation kit II (XTT) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. Cell Culture. EA.hy926 endothelial cells were cultured as described previously in tissue culture flasks at 37°C in 5% CO₂ [17]. The medium used was DMEM, containing 10% (v/v) FBS, 2 mM L-glutamine, 100 units mL⁻¹ of penicillin, and 100 units mL⁻¹ of streptomycin (Gibco, UK).

2.3. Cell Viability Assay. Cell viability was assessed using the XTT assay kit (Roche, Germany) as described previously [17]. Briefly, EA.hy926 cells were subcultured into a 96-well plate with 1 × 10⁴ cells per well in DMEM medium. After 24 h of incubation, the cells were treated with increasing concentrations of SWP (0.78, 1.56, 3.12, and 6.24 mg of protein mL⁻¹) in serum-free DMEM medium for 24 h or tBHP (0.15, 0.3, 0.6, and 1.2 mM) for 1 h. Then, following manufacturer's instructions absorbance was measured at 450 nm and also

at 690 nm as a reference wavelength in a Bio-Tek ELx800 microplate reader (Winooski, VT, USA). Cell cultures in DMEM serum-free medium were used as a negative control. The absorbance of each SWP concentration alone in DMEM serum-free medium and XTT test solution was also tested at 450 nm. The absorbance values shown by the proteins alone were subtracted from those derived from EA.hy926 cell treated with proteins. Data were calculated as percentage of inhibition by the following formula:

$$\text{inhibition (\%)} = \left[\frac{(\text{O.D.}_{\text{control}} - \text{O.D.}_{\text{sample}})}{\text{O.D.}_{\text{control}}} \right] \times 100, \quad (1)$$

where O.D._{control} and O.D._{sample} indicated the optical density of the negative control and the tested compounds, respectively. All samples were measured in triplicate and at least in three independent experiments.

2.4. Determination of Conditions for the Treatment of EA.hy926 Cells with tBHP. In order to find out the appropriate conditions (i.e., dose, incubation time) at which tBHP-induced oxidative stress in EA.hy926 cells, the cells were seeded in 25 cm² culture flasks, and when cell confluency was 70–80% incubated with tBHP for 1/2 or 1 h at 0.15 and 0.3 mM. Then, oxidative stress markers (i.e., ROS and GSH levels) were evaluated using flow cytometry for assessing oxidative stress induction.

2.5. Treatment of EA.hy926 Cells with SWP. EA.hy926 cells were seeded in 25 cm² culture flasks for GSH and ROS determination and 75 cm² culture flasks for TBARS, protein carbonyls, and GSSG determination and were incubated for 24 h at 37°C in 5% CO₂. Then, at a cell confluency of 70–80%, the medium was removed and replaced with serum-free medium containing SWP at different concentrations (0–6.24 mg of protein mL⁻¹), followed by incubation for 24 h. The untreated cells were considered as controls. After incubation, SWP was removed and tBHP (0.3 mM) was added for 1 h. Then, the cells were trypsinized, collected, and centrifuged twice at 300 g for 10 minutes at 5°C. Each centrifugation was followed by supernatant dismissal and resuspension of cellular pellet in PBS. After the last centrifugation the cellular suspension was used for the measurement of oxidative stress markers, namely, GSH, ROS, TBARS, protein carbonyls, and GSSG.

2.6. Flow Cytometric Analysis of GSH and ROS Levels. The intracellular GSH and ROS levels were assessed by flow cytometry using mercury orange and DCF-DA, respectively, as described previously [17]. In particular, the fluorescent mercury orange binds directly to GSH, while DCF-DA within cells is deacetylated by esterases and further converted to fluorescent DCF by oxidative action of ROS. A 400 μM stock solution of mercury orange was made up in acetone and stored at 4°C, while a fresh 400 μM stock solution of DCF-DA was prepared in methanol. To assess the GSH and ROS levels, the cells were resuspended in PBS at 1 × 10⁶ cells per mL and incubated in the presence of mercury orange (40 μM) or DCF-DA (10 μM) in the dark at 37°C for 30 min. Then, the cells were washed, resuspended in PBS, and

submitted to flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, NJ, USA) with excitation and emission at 488 and 530 nm for ROS and at 488 and 580 nm for GSH. Also, forward angle and right angle light scattering showing the cells size and cell internal complexity, respectively, were measured. Cells were analyzed at a flow rate of 1000 events per second. Analyses were performed on 10000 cells per sample and fluorescence intensities were measured on a logarithmic scale of four decades of log of fluorescence. Data were analyzed by using BD Cell Quest software (Becton Dickinson). Each experiment was repeated at least three times.

2.7. Assessment of GSSG Levels. For the assessment of GSSG levels, cellular suspension was homogenized by sonication on ice. The resulting lysate was then centrifuged at 10,000 ×g for 10 min at 4°C. Afterwards, GSSG was measured in the supernatant according to the method of Reddy et al. [18]. Briefly, 50 μL of supernatant was neutralized to pH 7.0–7.5 with NaOH. Then, 5 μL of 2-vinyl pyridine (1:100 diluted) was added and the samples were incubated at room temperature for 2 h. Ten μL of the sample treated with 2-vinyl pyridine was mixed with 600 μL of 143 mM sodium phosphate (6.3 mM EDTA, pH 7.5), 100 μL of 3 mM NADPH, 100 μL of 10 mM DTNB, and 189 μL of H₂O. The samples were incubated for 10 min at room temperature. After the addition of 1 μL of glutathione reductase, the change in absorbance at 412 nm was read for 3 min. The assay requires more than 2–4 μg absolute amount of protein in the test sample. Total protein in cellular suspension was assayed using a Bradford reagent from Sigma-Aldrich. GSSG concentration was calculated using a standard sample containing 75 μL of 10 μmol L⁻¹ oxidized glutathione.

2.8. Assessment of TBARS Levels. For the assessment of TBARS levels, cellular suspension was homogenized by sonication on ice. Then, TBARS were measured in the resulting homogenate spectrophotometrically as previously described [16]. 400 μL of cellular suspension or 400 μL of PBS for blank was mixed with 500 μL of 35% TCA and 500 μL of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (200 mM, pH 7.4) and incubated for 10 min at room temperature. Afterwards, 1 mL of 2 M Na₂SO₄ and 55 mM TBA solution was added and the samples were incubated at 95°C for 45 min. The samples were cooled on ice for 5 min and were vortexed after adding 1 mL of 70% TCA. Then, the samples were centrifuged at 15,000 g for 3 min and the absorbance of the supernatant was read at 530 nm. The assay requires more than 30 μg absolute amount of protein in the test sample. Total protein in cellular suspension was assayed using a Bradford reagent from Sigma-Aldrich. Calculation of TBARS concentration was based on the molar extinction coefficient of malondialdehyde (MDA).

2.9. Assessment of Protein Carbonyl Levels. For the assessment of protein carbonyl levels, cellular suspension was homogenized by sonication on ice. Then, protein carbonyls were measured in the homogenate spectrophotometrically as previously described [17]. In this assay, 200 μL of 20% TCA

was added to 200 μL of cellular suspension and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded and 500 μL of DNPH [in 2.5 N hydrochloride (HCL)] for the sample or 500 μL 2.5 N HCL for the blank was added in the pellet. The samples were incubated in the dark for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed, and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded and 1 mL of ethanol-ethyl acetate (1:1 v/v) was added, vortexed, and centrifuged at 15,000 g for 5 min at 4°C. This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH 2.3) was added, vortexed, and incubated at 37°C for 15 min. The samples were centrifuged at 15,000 g for 3 min at 4°C and the absorbance was read at 375 nm. The assay requires more than 30 μg absolute amount of protein in the test sample. Total protein in cellular suspension was assayed using a Bradford reagent from Sigma-Aldrich. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH.

2.10. Statistical Analysis. Data were analyzed by one-way ANOVA followed by Tukey's test for multiple pairwise comparisons. The level of statistical significance was set at $P < 0.05$. For all statistical analyses SPSS, version 13.0 (SPSS Inc., Chicago, IL), was used. Data are presented as mean ± SEM.

3. Results

3.1. Determination of the Cytotoxic Activity of SWP and tBHP. In the present study, the SWP was examined at concentrations (0.78, 1.56, 3.12, and 6.24 mg protein/mL) that exhibited antioxidant activity *in vitro* [16]. The cytotoxic activity of SWP in EA.hy926 cells was examined using the XTT assay. The results showed that SWP had no cytotoxic effect at the examined concentrations (Figure 1(a)). Regarding tBHP, the results showed that there was no cytotoxicity at concentrations below 0.6 mM (Figure 1(b)).

3.2. Determination of the Conditions for the Treatment of EA.hy926 Cells with tBHP. In a previous study, we have found that tBHP-induced oxidative stress in mouse C2C12 myoblastoma cells at 0.3 mM after 1/2 h incubation time. Based on these results, for finding the appropriate conditions (i.e., incubation time and dose) for the treatment of EA.hy926 cells with tBHP, the following methodology was followed. At first, as mentioned above, tBHP's effects on viability of EA.hy926 cells were examined, so as the noncytotoxic concentrations to be used. The results showed that there was no cytotoxicity at concentrations below 0.6 mM of tBHP (Figure 1(b)). Then, noncytotoxic concentrations (i.e., 0.15 and 0.3 mM) of tBHP were used at two different incubation times, 1/2 and 1 h. At these incubation times, the effect of tBHP at different doses on GSH and ROS levels was assessed, so as to find out the appropriate concentration at which tBHP-induced oxidative stress. The results showed that there was a tBHP-induced decrease in GSH levels at 0.3 mM after 1 h of incubation (Figures 1(c), 1(d), 1(e), and 1(f)). For this

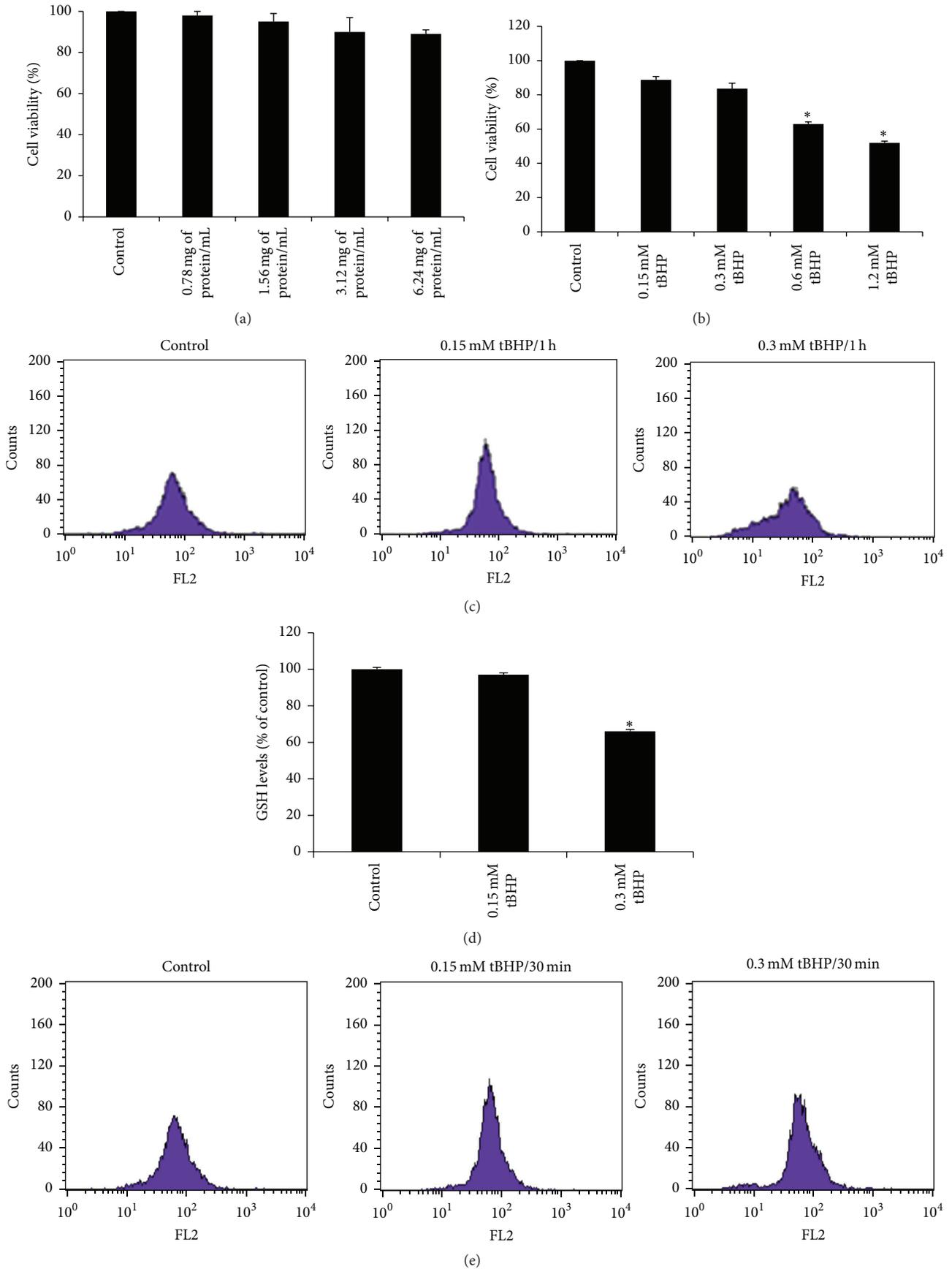


FIGURE 1: Continued.

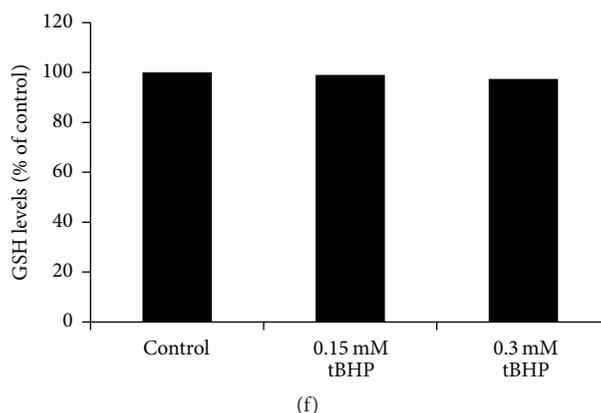


FIGURE 1: (a) Effects of whey protein on viability of EA.hy926 cells presented as % of control (untreated cells). (b) Effects of tBHP on viability of EA.hy926 cells presented as % of control (untreated cells). (c) The histogram of cell counts versus fluorescence of 10,000 cells analyzed by flow cytometer for the detection of GSH in EA.hy926 cells treated with tBHP at 0.15 and 0.3 mM for 1 h. FL2 represented the detection of fluorescence using 488 and 580 nm as the excitation and emission wavelength, respectively. (d) GSH levels in EA.hy926 cells treated with tBHP at 0.15 and 0.3 mM for 1 h, presented as % of control. (e) The histogram of cell counts versus fluorescence of 10,000 cells analyzed by flow cytometer for the detection of GSH in EA.hy926 cells treated with tBHP at 0.15 and 0.3 mM for 1/2 h. (f) GSH levels in EA.hy926 cells treated with tBHP at 0.15 and 0.3 mM for 1/2 h, presented as % of control. All values are presented as the mean \pm SEM of 3 experiments ($n = 9$ for cell viability assay; $n = 3$ for GSH assay). *Statistically significant compared to tBHP alone ($P < 0.05$).

reason, these conditions were selected for tBHP treatment. Moreover, although tBHP at 0.3 mM did not increase ROS levels (Figures 2(a) and 2(b)), it increased lipid peroxidation and protein oxidation (Figures 4(a) and 4(b), resp.).

3.3. Effects of Sheep Whey Protein on GSH and GSSG Levels in EA.hy926 Cells. For assessing the effects of SWP on GSH, ROS, and TBARS levels in EA.hy926 cells, noncytotoxic concentrations (0.78–6.24 mg of protein mL^{-1}) were used (Figure 1).

The GSH levels were evaluated by flow cytometry using mercury orange for staining. Histograms demonstrating the cell counts versus fluorescence of mercury orange are shown in Figure 2(a). The mean fluorescent intensity was evaluated using the BD Cell Quest software and the values are presented as percentage of the control (untreated cells) (Figure 2(b)). tBHP treatment decreased significantly GSH levels by 28.6% compared to controls (Figure 2(b)). However, treatment of EA.hy926 cells with SWP, at concentrations of 0.78, 1.56, 3.12, and 6.24 mg of protein mL^{-1} , before tBHP administration increased GSH levels by 56.2%, 82.6%, 141%, and 95.5%, respectively, compared to tBHP treatment alone (Figure 2(b)). Although there was an increase in GSH levels as SWP concentration increased, there were no statistically significant differences between GSH values at different SWP concentrations (Figure 2(b)).

The results showed that treatment of EA.hy924 cells with tBHP had no significant effect on GSSG levels compared to control. However, pretreatment with SWP at concentrations of 0.78, 1.56, 3.12, and 6.24 mg of protein mL^{-1} decreased GSSG levels by 40.5, 46.7, 28.1, and 32.5%, respectively, compared to tBHP treatment alone (Figure 2(c)). Similar to GSH assay, SWP-induced decrease in GSSG levels was not dose dependent (Figure 2(c)).

3.4. Effects of Sheep Whey Protein on ROS Levels in EA.hy926 Cells. ROS levels were evaluated by flow cytometry using DCF-DA for staining. Histograms demonstrating the cell counts versus fluorescence of DCF-DA are shown in Figure 3(a). The mean fluorescence intensity values were evaluated using the BD Cell Quest software and are expressed as percentage of the control (untreated cells) (Figure 3(b)). The administration of tBHP did not affect ROS levels compared to control. However, treatment of EA.hy926 cells with SWP at concentrations of 0.78, 1.56, 3.12, and 6.24 mg of protein mL^{-1} before tBHP administration decreased significantly ROS levels by 32.6, 57.8, 58.5, and 24.4%, respectively, compared to tBHP treatment alone (Figure 3(b)). In this assay, ROS values at 1.56 and 3.12 mg of protein mL^{-1} were significantly lower compared to 0.78 mg of protein mL^{-1} , indicating a dose dependent effect (Figure 3(b)).

3.5. Effects of Sheep Whey Protein on TBARS Levels in EA.hy926 Cells. The results showed that tBHP treatment increased significantly TBARS levels by 19.0% compared to control. Treatment of cells with SWP at 0.78–6.4 mg of protein mL^{-1} before tBHP administration led to a significant decrease in TBARS levels by 38.7, 39.4, 48.0, and 52.5%, respectively, compared to tBHP treatment alone (Figure 4(a)). Although there was a decrease in TBARS levels as SWP concentration increased, TBARS values were not significantly different among the different SWP concentrations (Figure 4(a)).

3.6. Effects of Sheep Whey Protein on Protein Carbonyl Levels in EA.hy926 Cells. Protein carbonyl levels were increased significantly by 60.0% after tBHP treatment compared to control. However, pretreatment of EA.hy926 cells with SWP at concentrations of 3.12 mg of protein mL^{-1} and 6.24 mg of

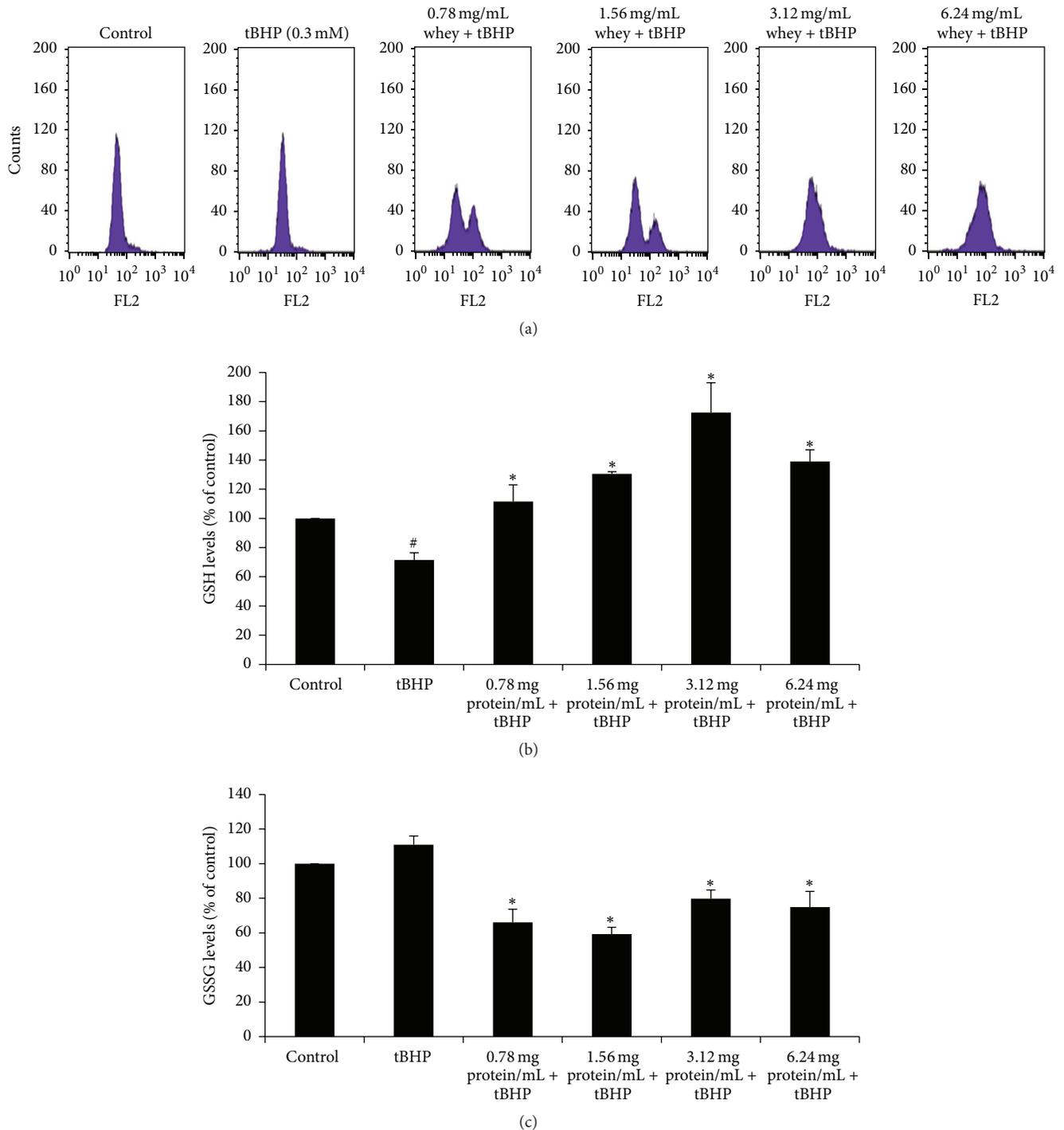


FIGURE 2: Effects of whey protein on GSH and GSSG levels in EA.hy926 cells. (a) The histogram of cell counts versus fluorescence of 10,000 cells analyzed by flow cytometer for the detection of GSH. FL2 represented the detection of fluorescence using 488 and 580 nm as the excitation and emission wavelength, respectively. (b) GSH levels in EA.hy926 cells presented as % of control. (c) GSSG levels as evaluated by spectrophotometer. Cells were studied under three conditions: under normal conditions (control), under treatment with tBHP (0.3 mM) for 1 h, and under the combination of whey protein (0.78–6.24 mg of protein mL^{-1}) for 24 h and tBHP (0.3 mM) for 1 h. All values are presented as the mean \pm SEM of 3 experiments ($n = 3$). * Statistically significant compared to tBHP alone ($P < 0.05$). # Statistically significant compared to control ($P < 0.05$).

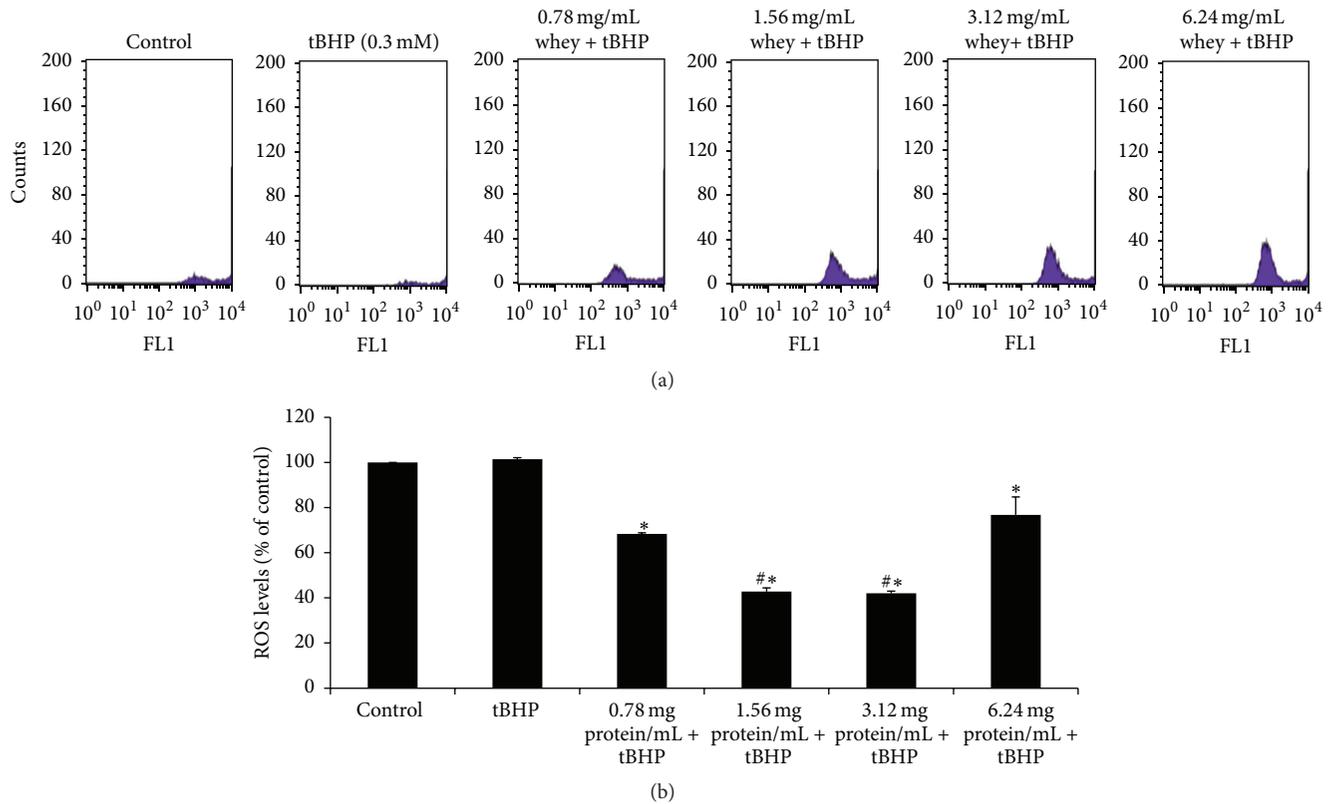


FIGURE 3: Effects of whey protein on ROS levels in EA.hy926 cells. (a) The histogram of cell counts versus fluorescence of 10,000 cells analyzed by flow cytometer for the detection of ROS. FL1 represented the detection of fluorescence using 488 and 530 nm as the excitation and emission wavelength, respectively. (b) ROS levels in EA.hy926 cells presented as % of control. Cells were studied under three conditions: under normal conditions (control), under treatment with tBHP (0.3 mM) for 1 h, and under the combination of whey protein (0.78–6.24 mg of protein mL^{-1}) for 24 h and tBHP (0.3 mM) for 1 h. All values are presented as the mean \pm SEM of 3 experiments ($n = 3$). *Statistically significant compared to tBHP alone ($P < 0.05$). #Statistically significant compared to 0.78 mg of protein mL^{-1} ($P < 0.05$).

protein mL^{-1} decreased significantly protein carbonyl levels by 22.0 and 49.0%, respectively, compared to tBHP treatment alone (Figure 4(b)). Moreover, there were significant differences in the protein carbonyl levels between 0.78 and 3.12 mg of protein mL^{-1} concentrations as well as between 1.56 and 6.24 mg of protein mL^{-1} concentrations suggesting a dose dependent effect of SWP (Figure 4(b)).

4. Discussion

An imbalance between ROS and antioxidants can lead to oxidative stress which causes lipid peroxidation, protein oxidation, and DNA damage, leading to several diseases [19]. All aerobic organisms including human have antioxidant mechanisms that protect against oxidative damage and repair damaged molecules. However, the natural antioxidant mechanisms may be insufficient and the supplementation with natural antioxidants through diet is of great interest. Such a natural product is whey protein, a by-product of cheese manufacturing, that is considered a functional food with a number of health benefits [11]. We have shown previously that SWP possesses antioxidant activity [9, 16]. In the present study, we investigated the protective effects of SWP against tBHP-induced oxidative stress in EA.hy926 endothelial cells.

For this purpose, the effects of SWP on GSH levels in EA.hy926 cells were examined. GSH is the most abundant antioxidant in aerobic cells, present in micromolar (μM) concentrations in bodily fluids and in millimolar (mM) concentrations in tissues [20]. Because of the cysteine residue, GSH is readily oxidized nonenzymatically to glutathione disulfide (GSSG) by electrophilic substances (e.g., ROS) resulting in their scavenging [5, 21]. The GSSG efflux from cells contributes to a net loss of intracellular GSH [5]. Thus, the decrease of GSH:GSSG ratio is considered an indicator of oxidative stress [22]. EA.hy926 cell treatment with tBHP decreased GSH levels and increased GSSG levels. It has been reported that tBHP oxidizes GSH through the activity of glutathione peroxidase (GP_x), thus leading to increased levels of GSSG [23]. However, pretreatment of cells with SWP before tBHP administration led to an increase in GSH levels and a decrease in GSSG levels compared to tBHP alone treatment. These results were consistent with those of other studies. For example, O’Keeffe and FitzGerald [24] have reported that incubation of human umbilical vein endothelial cells (HUVECs) with hydrolysate fractions of whey protein resulted in an increase in cellular glutathione by about 130%. In one of our previous studies, we have also shown that

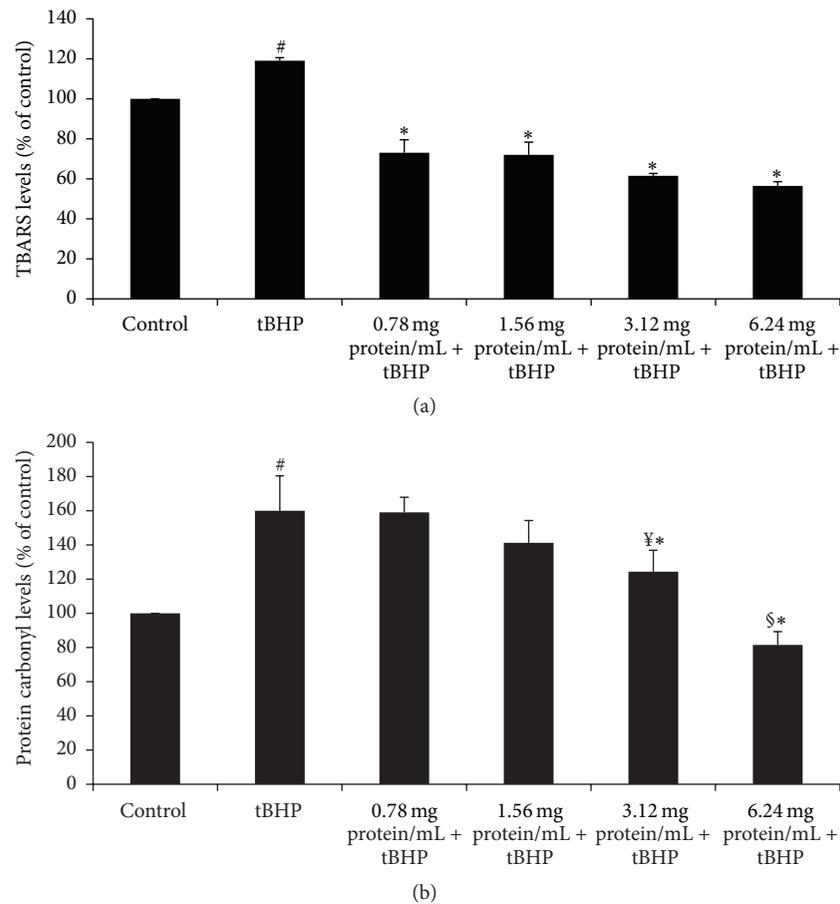


FIGURE 4: Effects of whey protein on (a) TBARS levels and (b) protein carbonyl levels as evaluated by spectrophotometer presented as % of control. Cells were studied under three conditions: under normal conditions (control), under treatment with tBHP (0.3 mM) for 1 h, and under the combination of whey protein (0.78–6.24 mg of protein mL⁻¹) for 24 h and tBHP (0.3 mM) for 1 h. All values are presented as the mean \pm SEM of 3 experiments ($n = 3$). *Statistically significant compared to tBHP alone ($P < 0.05$). #Statistically significant compared to control ($P < 0.05$). †Statistically significant compared to 0.78 mg of protein mL⁻¹ ($P < 0.05$). §Statistically significant compared to 1.56 mg of protein mL⁻¹ ($P < 0.05$).

treatment of C2C12 muscle cells with increasing concentrations of SWP (0.78–6.24 mg of protein mL⁻¹) before tBHP treatment increased GSH levels by 138% [16] and decreased GSSG levels by 31% (unpublished data) compared to tBHP treatment alone. Xu et al. [25] demonstrated that treatment of C2C12 cells with 0.5 mg mL⁻¹ whey protein, under the influence of hydrogen peroxide (H₂O₂), increased GSH levels by 341% compared to H₂O₂ alone treatment. In another study, administration of 0.1, 1, and 10 mg mL⁻¹ of whey protein, before ethanol exposure, increased GSH levels by 20.0%, 43.0%, and 98.0%, respectively, in the pheochromocytoma cell line PC12 [12]. The whey protein-induced increase in GSH levels is due probably to the contribution of cysteine residues that aid in the synthesis of GSH [13]. Furthermore, whey protein has been shown to induce the synthesis of GP_x eliminating hydroperoxides by oxidizing GSH to GSSG, which in turn is reduced to GSH by glutathione reductase (GR) [25].

Moreover, the effects of SWP on TBARS levels, a marker of lipid peroxidation, were examined. Treatment of EA.hy926

cells with tBHP increased significantly TBARS levels. It has been proposed that tBHP reacts with Fe²⁺ leading to the formation of tert-butyl hydroperoxide (tBO^{*}) radicals leading to lipid peroxidation [23, 26, 27]. The treatment of EA.hy926 cells with SWP decreased tBHP-induced increase of TBARS levels. Interestingly, in a previous study, we have found that pretreatment of C2C12 muscle cells with SWP (0.78–6.24 mg of protein mL⁻¹) decreased tBHP-induced increase of TBARS levels up to 25.5% [16]. Moreover, we have shown that a cake containing SWP decreased plasma TBARS levels in athletes after intense exercise [9]. In another study, diabetic rats supplemented with whey protein exhibited a significant decrease in the level of malondialdehyde (MDA) levels, a marker of lipid peroxidation [28]. Moreover, Xu et al. [25] showed that in C2C12 muscle cells whey protein at 0.5 mg mL⁻¹ is inhibited by 67% hydrogen peroxide-induced increase of MDA levels.

Furthermore, SWP treatment of EA.hy926 cells resulted in inhibition of tBHP-induced increase in protein oxidation, as shown by reduction in protein carbonyls. This effect is

important, since oxidative stress-induced carbonylation of proteins leads to the loss of their physiological function [29]. It is believed that there is an association between lipid and protein oxidation [30]. For example, tBHP has been suggested to lead to the formation of tBO[•] radicals that in turn lead to protein oxidation either directly by attacking the amino acyl side chains or indirectly by leading to lipid peroxidation [26]. Thus, the SWP-induced decrease in lipid peroxidation may also account, at least in part, for inhibiting tBHP-induced increase in protein oxidation. Moreover, Haraguchi et al. [31] have shown that whey protein precluded increases in muscle protein carbonyl content in exercised and sedentary animals.

Intriguingly, tBHP treatment did not affect ROS levels. As we have suggested previously, it seems that although tBHP produces free radicals, their “free form” cannot be observed [17]. This may be attributed to the reaction of free radicals with other molecules in the cells. For instance, the decrease in GSH and the increase in lipid peroxidation and protein oxidation after tBHP treatment suggested that free radicals may react with GSH, lipids, and proteins, respectively. However, treatment of EA.hy926 cells with SWP before tBHP administration led to a decrease in ROS levels up to 58.5% compared to tBHP alone treatment. In one of our previous studies, we have also found that pretreatment of C2C12 muscle cells with SWP (0.78–6.24 mg of protein mL⁻¹) decreased ROS levels to 41.3% [16]. Moreover, in another study, whey protein isolate (pWPI) and whey protein native hydrolysates (nWPI) at 2 mg mL⁻¹ inhibited H₂O₂-induced ROS formation by 76.0% and 32.5%, respectively, in human colonic adenocarcinoma (Caco-2) cell line [32]. Likewise, whey protein has been shown to decrease significantly ROS levels in diabetic rats [28]. The decrease in ROS levels may be explained by the SWP-induced increase in antioxidant defense mechanisms such as GSH. Interestingly, recent clinical studies have shown that a whey protein formulation reduced by almost twofold inflammatory oxidative damage (IOD) levels [33] and improved vascular functions [33, 34].

Although SWP treatment enhanced antioxidant capacity of EA.hy926 cells by either increasing antioxidant mechanisms or reducing ROS levels and oxidative stress-induced damage, these effects were not always dose dependent. Thus, SWP exhibited dose dependent decrease in ROS and CARB levels but SWP-induced increase in GSH or decrease in GSSG and TBARS levels was not dose dependent. This may be explained by the different mechanisms through which SWP affects each of the tested oxidative stress markers, and so its potency differs among the different assays. It seems that in GSH, GSSG, and TBARS assays, SWP's activity has already reached a plateau at the concentrations used and for this reason a dose dependent effect was not observed. Namely, if lower than the tested concentrations were used, it may also be a dose dependent effect in these assays.

In conclusion, the present study showed that SWP was effective to protect endothelial cells from oxidative stress-induced damage. SWP exerted its protective activity against oxidative stress, by increasing GSH levels and decreasing GSSG, lipid peroxidation, protein oxidation, and ROS levels in EA.hy926 cells. It should also be mentioned that SWP concentrations (i.e., from 0.78 to 6.24 mg/mL) used were not

cytotoxic. Moreover, in a previous study we have shown that at these concentrations SWP exhibited strong free radical scavenging activity and reducing power and enhanced the antioxidant capacity in mouse myoblastoma C2C12 cells [16]. Also, these concentrations are assimilated to the concentrations found in food. For example, the highest concentration of SWP was 6.24 mg/mL in the cell culture medium. Taken into account that the total plasma volume in human organism is about 3.5 L, then the concentration of 6.24 mg/mL of SWP would be achieved theoretically if about 20 g of SWP is consumed. This amount is within the range of the recommended intake doses of whey protein. Thus, since these whey protein concentrations can be found in blood, then the endothelial cells are possible to be exposed to them, since they are the main cells of the blood vessel walls. Thus, the findings of the present study suggest that SWP may be used as food supplement to attenuate vascular disturbances associated with oxidative stress.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

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References

- [1] B. Halliwell, “Free radicals and other reactive species in disease,” in *Encyclopedia of Life Sciences*, John Wiley & Sons, New York, NY, USA, 2001.
- [2] K. T. Turpaev, “Reactive oxygen species and regulation of gene expression,” *Biochemistry*, vol. 67, no. 3, pp. 281–292, 2002.
- [3] B. P. Chew and J. S. Park, “Carotenoid action on the immune response,” *Journal of Nutrition*, vol. 134, no. 1, pp. 257S–261S, 2004.
- [4] S. K. Powers and M. J. Jackson, “Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production,” *Physiological Reviews*, vol. 88, no. 4, pp. 1243–1276, 2008.
- [5] G. Wu, Y.-Z. Fang, S. Yang, J. R. Lupton, and N. D. Turner, “Glutathione metabolism and its implications for health,” *Journal of Nutrition*, vol. 134, no. 3, pp. 489–492, 2004.
- [6] J. E. Deanfield, J. P. Halcox, and T. J. Rabelink, “Endothelial function and dysfunction: testing and clinical relevance,” *Circulation*, vol. 115, no. 10, pp. 1285–1295, 2007.
- [7] S. G. Rhee, “H₂O₂, a necessary evil for cell signaling,” *Science*, vol. 312, no. 5782, pp. 1882–1883, 2006.
- [8] A. Woywodt, F. H. Bahlmann, K. De Groot, H. Haller, and M. Haubitz, “Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer,” *Nephrology Dialysis Transplantation*, vol. 17, no. 10, pp. 1728–1730, 2002.

- [9] E. Kerasiotti, A. Kiskini, A. Veskoukis et al., "Effect of a special carbohydrate-protein cake on oxidative stress markers after exhaustive cycling in humans," *Food and Chemical Toxicology*, vol. 50, no. 8, pp. 2805–2810, 2012.
- [10] W. J. Bartfay, M. T. Davis, J. M. Medves, and S. Lugowski, "Milk whey protein decreases oxygen free radical production in a murine model of chronic iron-overload cardiomyopathy," *Canadian Journal of Cardiology*, vol. 19, no. 10, pp. 1163–1168, 2003.
- [11] K. Marshall, "Therapeutic applications of whey protein," *Alternative Medicine Review*, vol. 9, no. 2, pp. 136–156, 2004.
- [12] Y.-M. Tseng, S.-K. Lin, J.-K. Hsiao et al., "Whey protein concentrate promotes the production of glutathione (GSH) by GSH reductase in the PC12 cell line after acute ethanol exposure," *Food and Chemical Toxicology*, vol. 44, no. 4, pp. 574–578, 2006.
- [13] R. L. Walzem, C. J. Dillard, and J. B. German, "Whey components: millennia of evolution create functionalities for mammalian nutrition: what we know and what we may be overlooking," *Critical Reviews in Food Science and Nutrition*, vol. 42, no. 4, pp. 353–375, 2002.
- [14] E. Ha and M. B. Zemel, "Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people," *Journal of Nutritional Biochemistry*, vol. 14, no. 5, pp. 251–258, 2003.
- [15] E. Kerasiotti, D. Stagos, A. Jamurtas et al., "Anti-inflammatory effects of a special carbohydrate-whey protein cake after exhaustive cycling in humans," *Food and Chemical Toxicology*, vol. 61, pp. 42–46, 2013.
- [16] E. Kerasiotti, D. Stagos, A. Priftis et al., "Antioxidant effects of whey protein on muscle C2C12 cells," *Food Chemistry*, vol. 155, pp. 271–278, 2014.
- [17] N. Goutzourelas, D. Stagos, N. Demertzis et al., "Effects of polyphenolic grape extract on the oxidative status of muscle and endothelial cells," *Human and Experimental Toxicology*, vol. 33, no. 11, pp. 1099–1112, 2014.
- [18] Y. N. Reddy, S. V. Murthy, D. R. Krishna, and M. C. Prabhakar, "Role of free radicals and antioxidants in tuberculosis patients," *Indian Journal of Tuberculosis*, vol. 51, pp. 213–218, 2004.
- [19] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.
- [20] J. B. Owen and D. A. Butterfield, "Measurement of oxidized/reduced glutathione ratio," *Methods in Molecular Biology*, vol. 648, pp. 269–277, 2010.
- [21] Y.-Z. Fang, S. Yang, and G. Wu, "Free radicals, antioxidants, and nutrition," *Nutrition*, vol. 18, no. 10, pp. 872–879, 2002.
- [22] K. Aquilano, S. Baldelli, and M. R. Ciriolo, "Glutathione: new roles in redox signalling for an old antioxidant," *Frontiers in Pharmacology*, vol. 5, p. 196, 2014.
- [23] C. F. Lima, M. Fernandes-Ferreira, and C. Pereira-Wilson, "Phenolic compounds protect HepG2 cells from oxidative damage: relevance of glutathione levels," *Life Sciences*, vol. 79, no. 21, pp. 2056–2068, 2006.
- [24] M. B. O'Keeffe and R. J. FitzGerald, "Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells," *International Dairy Journal*, vol. 36, no. 2, pp. 128–135, 2014.
- [25] R. Xu, N. Liu, X. Xu, and B. Kong, "Antioxidative effects of whey protein on peroxide-induced cytotoxicity," *Journal of Dairy Science*, vol. 94, no. 8, pp. 3739–3746, 2011.
- [26] S. Hix, M. B. Kadiiska, R. P. Mason, and O. Augusto, "In vivo metabolism of tert-butyl hydroperoxide to methyl radicals. EPR spin-trapping and DNA methylation studies," *Chemical Research in Toxicology*, vol. 13, no. 10, pp. 1056–1064, 2000.
- [27] M. Alía, S. Ramos, R. Mateos, L. Bravo, and L. Goya, "Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2)," *Journal of Biochemical and Molecular Toxicology*, vol. 19, no. 2, pp. 119–128, 2005.
- [28] H. Ebaid, A. Salem, A. Sayed, and A. Metwalli, "Whey protein enhances normal inflammatory responses during cutaneous wound healing in diabetic rats," *Lipids in Health and Disease*, vol. 10, article 235, 2011.
- [29] M. F. Beal, "Oxidatively modified proteins in aging and disease," *Free Radical Biology and Medicine*, vol. 32, no. 9, pp. 797–803, 2002.
- [30] Y. V. Vasil'ev, S.-C. Tzeng, L. Huang, and C. S. Maier, "Protein modifications by electrophilic lipoxidation products: adduct formation, chemical strategies and tandem mass spectrometry for their detection and identification," *Mass Spectrometry Reviews*, vol. 33, no. 3, pp. 157–182, 2014.
- [31] F. K. Haraguchi, M. E. Silva, L. X. Neves, R. C. Dos Santos, and M. L. Pedrosa, "Whey protein precludes lipid and protein oxidation and improves body weight gain in resistance-exercised rats," *European Journal of Nutrition*, vol. 50, no. 5, pp. 331–339, 2011.
- [32] A. F. Piccolomini, M. M. Iskandar, L. C. Lands, and S. Kubow, "High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells," *Food and Nutrition Research*, vol. 56, 2012.
- [33] I. M. Petyaev, P. Y. Dovgalevsky, V. A. Klochkov, N. E. Chalyk, and N. Kyle, "Whey protein lysosome formulation improves vascular functions and plasma lipids with reduction of markers of inflammation and oxidative stress in prehypertension," *The Scientific World Journal*, vol. 2012, Article ID 269476, 7 pages, 2012.
- [34] K. D. Ballard, B. R. Kupchak, B. M. Volk et al., "Acute effects of ingestion of a novel whey-derived extract on vascular endothelial function in overweight, middle-aged men and women," *British Journal of Nutrition*, vol. 109, no. 5, pp. 882–893, 2013.

Review Article

The Role of Mitochondrial Functional Proteins in ROS Production in Ischemic Heart Diseases

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Ischemic heart diseases (IHD) have become the leading cause of death around the world, killing more than 7 million people annually. In IHD, the blockage of coronary vessels will cause irreversible cell injury and even death. As the “powerhouse” and “apoptosis center” in cardiomyocytes, mitochondria play critical roles in IHD. Ischemia insult can reduce myocardial ATP content, resulting in energy stress and overproduction of reactive oxygen species (ROS). Thus, mitochondrial abnormality has been identified as a hallmark of multiple cardiovascular disorders. To date, many studies have suggested that these mitochondrial proteins, such as electron transport chain (ETC) complexes, uncoupling proteins (UCPs), mitochondrial dynamic proteins, translocases of outer membrane (Tom) complex, and mitochondrial permeability transition pore (MPTP), can directly or indirectly influence mitochondria-originated ROS production, consequently determining the degree of mitochondrial dysfunction and myocardial impairment. Here, the focus of this review is to summarize the present understanding of the relationship between some mitochondrial functional proteins and ROS production in IHD.

1. Introduction

As the leading cause of fatality worldwide, ischemic heart diseases (IHD) give rise to widespread loss of cardiomyocytes and subsequent adverse cardiac remodeling [1]. More than 17 million people had succumbed to IHD in 2008, and the number was estimated to be 23.6 million by 2030 [2]. Ischemia starves cardiomyocytes of vital oxygen, resulting in severe or irreversible injury to heart [3]. Abundant lines of evidence indicate that reactive oxygen species (ROS) overload that originates from mitochondria is closely linked with the pathogenesis of cardiovascular diseases, such as atherosclerosis, myocardial infarction, and heart failure [4]. In myocytes, mitochondria comprise more than 30% cell volume and generate about 90% of the ATP [5]. Accordingly, mitochondria are also the major source of ROS in the cardiovascular diseases [5, 6]. Numerous experimental and clinical studies have shown that ROS accumulation is significantly

exacerbated in the failing myocardium [7]. Chronic exposure of myocytes to ROS will lead to apoptosis, necrosis, and fibrosis, finally causing arrhythmias, impairment of excitation-contraction coupling, and cardiac remodeling [8–10]. Given that ROS affect virtually all aspects of cardiac myocyte physiology and that those lines of evidence support the notion that ROS are increased in IHD, the underlying mechanisms about ROS overproduction induced by mitochondrial abnormality should be further explored.

2. The Electron Transport Chain (ETC) Complexes and Uncoupling Proteins (UCPs)

Mitochondrial oxidative phosphorylation is the basic mechanism of ATP synthesis, in which the five enzyme complexes I–V in mitochondria are composed of multiple subunits. The respiratory chain, apart from energy production, plays a pivotal role in regulation of cardiac oxidative stress [11].

ETC damage will lead to a robust increase of ROS production in mitochondria [12]. In ischemic heart, oxygen delivery to myocardium is not sufficient to meet the demand of mitochondrial oxidation. Reintroduction of oxygen induced by reperfusion markedly enhances electron leakage from the ETC, resulting in overproduction of superoxide (O_2^-) and O_2^- -derived oxidants [12, 13]. Indeed, reduced activity of ETC subunits in heart failure patients has been confirmed independently of the etiology, notably of complex I [14], complex III [15], and complex IV [16]. Within the ETC, complexes I/III are regarded as the key contributors to ROS generation under stressful conditions [17, 18]. In the setting of myocardial ischemia/reperfusion (MI/R), oxidative injury of complex I can weaken complex interaction and enhance ROS production by complex III [19, 20]. Although the contribution of intact complex II seems to be negligible, some mutations in subunits of complex II have been linked to specific forms of cancer or neurodegeneration with increased ROS generation [21, 22]. Aldakkak et al. [23] revealed that pharmacologic blockade of electron transport attenuates ROS-induced cardiac injury. Consistently, nitrite can modulate mitochondrial resilience to reperfusion injury, perhaps via inhibiting complex I by posttranslational s-nitrosation to reduce ROS generation [24, 25]. Moreover, ROS scavenging ability may be impaired as an evident decrease of MnSOD activity in failing heart [26]. Coenzyme Q (CoQ) encompasses a collection of homologous molecules which consist of a benzoquinone ring linked to an isoprenoid side chain [27]. In the quinone form, CoQ serves as an electron carrier to transfer electrons in the mitochondrial ETC. In the quinol form (the reduced form of quinone), CoQ represents an efficient antioxidant in the body [28]. CoQ10 at high doses has also been proved to regulate gene transcription, including the genes important in lipid metabolism and the specific genes induced by ROS-sensitive intracellular pathways [29]. It is claimed that CoQ10 supplementation to obese rats attenuates age-related oxidative stress and preserves mitochondrial function in myocardium [30]. Moreover, CoQ10 deficiency is detrimental to the prognosis of chronic heart failure, and plasma concentration of CoQ10 may serve as an independent predictor of mortality [31]. Long-term supplementation of CoQ10 for patients with heart failure improves symptoms and reduces major adverse cardiovascular events [32].

In contrast, uncoupling proteins (UCPs) that are located in mitochondrial inner membrane exhibit opposite functions as complexes mentioned above. UCPs can reduce mitochondrial ROS production by dissipating the electrochemical gradient [33–36]. UCPs overexpression was found to salvage cardiomyocytes by preserving mitochondrial integrity [33, 36]. Specifically, mild to moderate mitochondrial uncoupling mediated by UCPs protects against MI/R injury by reducing ROS generation [37]. UCP1, characterized in brown adipocytes [38], has close relationship with endogenous CoQ to influence H^+ transport [39]. In the setting of hypoxia/reoxygenation, UCP1 overexpression confers cardioprotection [33]. The presence of UCP1 mitigates reperfusion-induced damage, probably by reducing mitochondrial hyperpolarization [40]. Moreover, UCP2 overexpression can exert cardioprotection perhaps by preventing

mitochondrial Ca^{2+} overload and attenuating ROS generation [41]. UCP3 has also been reported to play a critical role in cardioprotection during oxidative stress by suppressing detrimental ROS generation and maintaining myocardial high-energy phosphates [37]. In view of what was mentioned above, UCPs may be developed as protective targets for IHD patients.

3. Mitochondrial Dynamic Proteins

In order to adapt to stressful conditions, mitochondria dynamically undergo fission and fusion [42], which is basically regulated by some functional proteins [42, 43]. Mitofusin 1/2 (Mfn1/2) and optic atrophy 1 (Opal) are known to induce mitochondrial fusion [44, 45], while dynamin-related protein 1 (Drp1) is identified to interact with fission protein 1 (Fis1) to promote mitochondrial fission [46, 47]. In mammalian cells, mitochondrial function is largely governed by mitochondrial fission and fusion which is an important factor for the integrity, structure, and function of healthy mitochondria. Abnormal fission or fusion will impair mitochondrial function, for example, resulting in overload of mitochondrial Ca^{2+} , overproduction of free radicals, alteration of mitochondrial enzymatic activities, and reduction of ATP production [48].

Recent studies have highlighted the notion that defects in mitochondrial dynamics are relevant to various human disorders, including MI/R, heart failure, diabetes, and aging [49, 50]. Inhibition of mitochondrial fission has been considered to rescue myocardial infarction/heart failure [7]. The deletion of Mfn1/2 appears to render the heart less resistant to MPTP opening [51], whereas overexpression of Mfn1/2 prevents the opening of MPTP and reduces cell death following ischemia/reperfusion (I/R) [52, 53]. Opal mutation in myocardium results in mitochondrial dysfunction and increases ROS [54], while transfection of Opal in vivo protects mice from denervation-induced ischemic heart damage [55]. Moreover, inhibition of Drp1 significantly decreased I/R-induced mitochondrial fragmentation and cardiomyocytes apoptosis [52, 56]. Cumulative lines of evidence suggest that impaired mitochondrial dynamics are an early event in the progression of IHD that involve excessive mitochondrial fission and mitochondrial dysfunction, indicating that dynamics related proteins may be developed as new therapeutic targets against IHD.

4. The Translocases of Outer Membrane (Tom) Complex

The translocases of mitochondrial outer membrane (Tom) complex is a general entry gate for the importing of all mitochondrial preproteins. It comprises a central channel (formed by Tom40, Tom5, Tom6, and Tom7) and three receptors (Tom70, Tom20, and Tom22) [57]. Tom20 and Tom70 can recognize mitochondrial-targeting sequences on the precursor proteins and transfer them to Tom22 and the central channel [57, 58]. Tom20 and Tom22 mainly recognize the presequences of cleavable precursor proteins, while

Tom70 typically largely recognizes the internal targeting signals of hydrophobic precursor proteins, many of which are inner membrane metabolite carriers [58]. In addition, Tom70 can also function as a docking site for cytosolic chaperones, such as Hsp70 and Hsp90, in order to receive mitochondrial proteins [59].

It has been reported that mitochondrial Tom20 was reduced by ischemia, and the maintenance of Tom20 by preconditioning confers cardioprotection via improving mitochondrial structure and function [60]. Tom20 can also restore the translocation of Cx43 to the inner mitochondrial membrane to attenuate ischemic myocardial injury [60]. Conversely, proteasome inhibitors, such as MG115, proteasome inhibitor 1, and lactacystin, lead to higher levels of Tom20 and enhance the perinuclear accumulation of mitochondria, revealing the influence of cellular redox conditions on mitochondrial import [61]. Moreover, Tom70 is obviously suppressed in hypertrophic hearts, and genetic downregulation of Tom70 worsens pathological cardiomyocyte hypertrophy. The defective mitochondrial import of Tom70-targeted Opal triggered intracellular oxidative stress, which led to a pathological cellular response [62]. Adenosine administration in ischemic myocardium may exhibit protection by inducing the translocation of PKC ϵ to mitochondria in a Tom70/HSP90-dependent manner [63]. However, the detailed mechanisms about Tom complex in IHD remain obscured to date, so further studies are needed.

5. The Other Functional Proteins in Mitochondrion

5.1. Mitochondrial Permeability Transition Pore (MPTP). MPTP is a nonselective channel locating on the inner mitochondrial membrane. ROS overproduction and calcium overload can dissipate the proton electrochemical gradient and open the MPTP, leading to uncoupling of oxidative phosphorylation and further production of ROS [64, 65]. The opening of MPTP will release proapoptotic proteins to induce cell apoptosis or necrosis [13, 65]. Several studies have demonstrated that MPTP inhibition can mitigate cell loss in cardiac pathologies, including MI/R [66], heart failure [67], and diabetic cardiomyopathy [68]. Hausenloy and his colleagues revealed that inhibition of MPTP opening, during the first few minutes of reperfusion, protects myocytes from oxidative stress and finally limits infarct size [69]. However, Saotome et al. claimed that ROS-induced transient opening of MPTP protects from I/R-induced myocardium injury [70], revealing that the specific function of MPTP depends on the degree and the timing.

5.2. Mitochondrial Ca²⁺ Uniporter (MCU)/Mitochondrial Ca²⁺ Uptake 1 (MICU1)/MICU2. As a universal secondary messenger, Ca²⁺ plays a central role in a wide range of cellular processes, such as muscular contraction, synaptic transmission, cell migration, and cell proliferation [71]. In the heart, Ca²⁺ is central to cardiac excitation-contraction coupling and signaling networks in the regulation of pathological myocardial growth and remodeling [72]. Accumulating lines of

evidence indicate that Ca²⁺ overload is linked with mitochondrial dysfunction, contractile dysfunction, and cell death [23, 73–77]. Thus, maintenance of mitochondrial Ca²⁺ homeostasis is very important for the survival of cardiomyocytes under ischemic stress [78]. MCU is an inner mitochondrial membrane channel responsible for Ca²⁺ uptake into the matrix [79]. It plays a fundamental role in the control of aerobic metabolism as well as apoptosis [80]. MCU blockade may protect the heart from hypoxia/reoxygenation injury through suppressing mitochondria-originated production of ROS [81]. Recently, MICU1 and MICU2 are identified to function as the gatekeepers of MCU by the binding of Ca²⁺ to their EF hands [82]. And MICU2's activity requires the presence of MICU1 [83]. Under basal cytosolic [Ca²⁺] condition, Ca²⁺-free MICU1 and MICU2 can inhibit MCU function; at very high cytosolic [Ca²⁺] condition, all EF hands of MICU1 and MICU2 bind Ca²⁺ to activate MCU by dissociation of MICUs from MCU complex [82]. Consistently, Mallilankaraman et al. [84] prove that MICU1 is required to preserve mitochondrial [Ca²⁺], under basal condition. In the absence of MICU1, mitochondria suffers Ca²⁺ overload, leading to excessive ROS generation. In addition, Patron et al. [85] also reveal that MICU2 largely shuts down MCU activity at low cytosolic [Ca²⁺], whereas in response to high cytosolic [Ca²⁺], MICU1 appears to stimulate Ca²⁺ uptake by MCU. Very recently, Wang et al. claim that Mfn2 overexpression reduces the expression of MICU1 and MICU2 to trigger influx of Ca²⁺ into mitochondria [86], revealing the close relationship of mitochondrial dynamics with Ca²⁺ homeostasis. However, the full functions of MICU1 and MICU2 in cardiovascular diseases remain unclear yet, emphasizing an urgent need for deep research.

5.3. Connexin 43 (Cx43). Gap junction channels provide the basis for intercellular communication in cardiovascular systems, in order to keep metabolic interchange and maintain normal cardiac rhythm. In cardiomyocytes, Cx43 is the most abundant isoform. The alteration of Cx43 was observed in myocardium diseases, such as ischemia, hypertrophic cardiomyopathy, and heart failure [87]. The normal location of Cx43 at inner mitochondrial membrane is very important in the synchronization of contraction for cardiomyocytes [88, 89]. It was suggested that I/R injury is accompanied with the change of Cx43 expression [90]. Ischemic conditions can trigger Cx43 hemichannel opening, possibly mediated by the generation of ROS and nitrogen species [91]. The protection of preconditioning has been confirmed to depend on functional Cx43-formed channels [2]. Moreover, Cx43 deficiency will lead to ventricular arrhythmia which is the major cause of sudden death in heart failure. The proteasome inhibitor can be used to attenuate the degradation of Cx43 to prevent Cx43-mediated arrhythmia in heart failure [92]. Therefore, Cx43 may be developed as a potential therapeutic target for cardiovascular diseases [90].

5.4. Signal Transducer and Activator of Transcription 3 (STAT3). As a transcription factor, STAT3 has been implicated to protect hearts from acute ischemic injury under

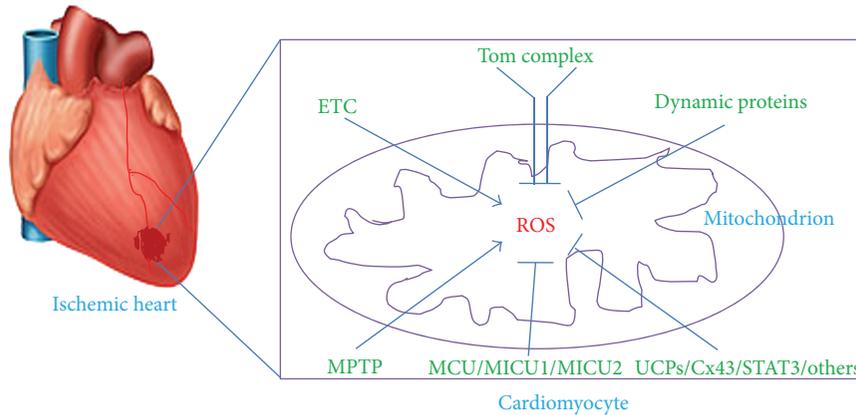


FIGURE 1: Schematic diagram indicating mitochondrial proteins' role in ROS generation in ischemic heart diseases. Under ischemic insult, the generation of ROS significantly increases in mitochondria of cardiomyocytes, which can be directly or indirectly influenced by mitochondrial functional proteins, including ETC complexes, UCPs, mitochondrial dynamic proteins, Tom complex, MPTP, MCU/MICU1/MICU2, Cx43, and STAT3. ROS: reactive oxygen species; ETC: electron transport chain; UCPs: uncoupling proteins; Tom: translocases of outer membrane; MPTP: mitochondrial permeability transition pore; MCU: mitochondrial Ca^{2+} uniporter; MICU1: mitochondrial Ca^{2+} uptake 1; MICU2: mitochondrial Ca^{2+} uptake 2; Cx43: Connexin 43; STAT3: signal transducer and activator of transcription 3.

stressful conditions [93], and loss of STAT3 will result in cardiomyopathy [94]. Accumulative lines of evidence also demonstrated that STAT3 serves as a protective molecule for the heart in hypertension and advanced age [95]. STAT3 is now known to be present in cardiac mitochondria and be able to regulate ROS generation and MPTP opening [96]. Wegrzyn and colleagues have proved that STAT3 deficiency can reduce the activities of complexes I and II [97]. Szczepanek et al. further revealed that mutation in the DNA binding domain of mitochondrial-targeted STAT3 can disrupt ETC activity in the heart [96]. Consistently, STAT3 transgene reduces the vulnerability of cardiac mitochondria to ischemia by restoring complex I activity and suppressing ROS generation [98]. Thus, in pathologic settings such as ischemia or early reperfusion, STAT3 may be an ideal therapeutic target to protect cardiac mitochondria.

6. Conclusion

In summary, mitochondrial functional proteins play critical roles in the production of ROS in IHD: (1) the defective ETC activity, notably of decreased activity of complex I, may form the pathological foundation for mitochondria-derived ROS overload; (2) the disruption of mitochondrial dynamics, especially depressed mitochondrial fusion, will aggravate mitochondrial ROS production; (3) Tom complex may possess important property in regulating oxidative stress, perhaps via influencing the translocation of mitochondrial proteins; (4) the other functional factors, such as MPTP, MCU/MICU1/MICU2, Cx43, and STAT3, play important roles in preserving mitochondrial integrity and function, directly or indirectly through inhibiting ROS overload (Figure 1).

In recent decades, there has been great progress in screening, identifying, and developing molecules as therapeutic targets to preserve mitochondrial integrity and prevent ROS

overload. As we understand more of the distinct abnormalities occurring in the mitochondria with IHD, the goal becomes to develop new methods to mitigate the mitochondrial abnormalities. Current medicines, such as beta blockers, statins, and nitrates, have improved the symptoms of IHD patients; however, these medicines' effects on mitochondrial impairment and mitochondrial functional proteins in IHD are far from clear yet. Undoubtedly, more work is needed to explore the fundamental roles of mitochondrial proteins in IHD, because they are attractive mechanistic targets for cardioprotection.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Haifeng Pei and Yi Yang contributed equally to this study.

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References

- [1] V. L. Roger, A. S. Go, D. M. Lloyd-Jones et al., "Executive summary: heart disease and stroke statistics—2012 update: a report from the American Heart Association," *Circulation*, vol. 125, no. 1, pp. 188–197, 2012.
- [2] H. He, N. Li, Z. Zhao, F. Han, X. Wang, and Y. Zeng, "Ischemic postconditioning improves the expression of cellular membrane connexin 43 and attenuates the reperfusion injury in rat acute

- myocardial infarction,” *Biomedical Reports*, vol. 3, pp. 668–674, 2015.
- [3] W. H. Townley-Tilson, X. Pi, and L. Xie, “The role of oxygen sensors, hydroxylases, and hif in cardiac function and disease,” *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 676893, 10 pages, 2015.
- [4] Z. W. Zhang, X. C. Xu, T. Liu, and S. Yuan, “Mitochondrion-permeable antioxidants to treat ROS-burst-mediated acute diseases,” *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 6859523, 10 pages, 2016.
- [5] E. Murphy and C. Steenbergen, “Preconditioning: the mitochondrial connection,” *Annual Review of Physiology*, vol. 69, pp. 51–67, 2007.
- [6] D. B. Zorov, C. R. Filburn, L.-O. Klotz, J. L. Zweier, and S. J. Sollott, “Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes,” *The Journal of Experimental Medicine*, vol. 192, no. 7, pp. 1001–1014, 2000.
- [7] A. A. Knowlton, L. Chen, and Z. A. Malik, “Heart failure and mitochondrial dysfunction: the role of mitochondrial fission/fusion abnormalities and new therapeutic strategies,” *Journal of Cardiovascular Pharmacology*, vol. 63, no. 3, pp. 196–206, 2014.
- [8] F. J. Giordano, “Oxygen, oxidative stress, hypoxia, and heart failure,” *The Journal of Clinical Investigation*, vol. 115, no. 3, pp. 500–508, 2005.
- [9] C. Maack, E. R. Dabew, M. Hohl, H.-J. Schäfers, and M. Böhm, “Endogenous activation of mitochondrial KATP channels protects human failing myocardium from hydroxyl radical-induced stunning,” *Circulation Research*, vol. 105, no. 8, pp. 811–817, 2009.
- [10] F. G. Akar, M. A. Aon, G. F. Tomaselli, and B. O’Rourke, “The mitochondrial origin of postischemic arrhythmias,” *The Journal of Clinical Investigation*, vol. 115, no. 12, pp. 3527–3535, 2005.
- [11] K. Schwarz, N. Siddiqi, S. Singh, C. J. Neil, D. K. Dawson, and M. P. Frenneaux, “The breathing heart—mitochondrial respiratory chain dysfunction in cardiac disease,” *International Journal of Cardiology*, vol. 171, no. 2, pp. 134–143, 2014.
- [12] T. Ide, H. Tsutsui, S. Kinugawa et al., “Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium,” *Circulation Research*, vol. 85, no. 4, pp. 357–363, 1999.
- [13] E. Murphy and C. Steenbergen, “Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury,” *Physiological Reviews*, vol. 88, no. 2, pp. 581–609, 2008.
- [14] R. J. Scheubel, M. Tostlebe, A. Simm et al., “Dysfunction of mitochondrial respiratory chain complex I in human failing myocardium is not due to disturbed mitochondrial gene expression,” *Journal of the American College of Cardiology*, vol. 40, no. 12, pp. 2174–2181, 2002.
- [15] D. Jarreta, J. Orús, A. Barrientos et al., “Mitochondrial function in heart muscle from patients with idiopathic dilated cardiomyopathy,” *Cardiovascular Research*, vol. 45, no. 4, pp. 860–865, 2000.
- [16] E. Arbustini, M. Diegoli, R. Fasani et al., “Mitochondrial DNA mutations and mitochondrial abnormalities in dilated cardiomyopathy,” *American Journal of Pathology*, vol. 153, no. 5, pp. 1501–1510, 1998.
- [17] M. P. Murphy, “How mitochondria produce reactive oxygen species,” *The Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.
- [18] M. D. Brand, “The sites and topology of mitochondrial superoxide production,” *Experimental Gerontology*, vol. 45, no. 7–8, pp. 466–472, 2010.
- [19] Q. Chen, A. K. S. Camara, D. F. Stowe, C. L. Hoppel, and E. J. Lesnefsky, “Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion,” *American Journal of Physiology—Cell Physiology*, vol. 292, no. 1, pp. C137–C147, 2007.
- [20] Q. Chen, C. L. Hoppel, and E. J. Lesnefsky, “Blockade of electron transport before cardiac ischemia with the reversible inhibitor amobarbital protects rat heart mitochondria,” *The Journal of Pharmacology and Experimental Therapeutics*, vol. 316, no. 1, pp. 200–207, 2006.
- [21] V. Yankovskaya, R. Horsefield, S. Törnroth et al., “Architecture of succinate dehydrogenase and reactive oxygen species generation,” *Science*, vol. 299, no. 5607, pp. 700–704, 2003.
- [22] T. M. Iverson, E. Maklashinas, and G. Cecchinis, “Structural basis for malfunction in complex II,” *The Journal of Biological Chemistry*, vol. 287, no. 42, pp. 35430–35438, 2012.
- [23] M. Aldakkak, D. F. Stowe, Q. Chen, E. J. Lesnefsky, and A. K. S. Camara, “Inhibited mitochondrial respiration by amobarbital during cardiac ischaemia improves redox state and reduces matrix Ca^{2+} overload and ROS release,” *Cardiovascular Research*, vol. 77, no. 2, pp. 406–415, 2008.
- [24] S. Shiva, M. N. Sack, J. J. Greer et al., “Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer,” *The Journal of Experimental Medicine*, vol. 204, no. 9, pp. 2089–2102, 2007.
- [25] S. M. Nadtochiy, L. S. Burwell, and P. S. Brookes, “Cardioprotection and mitochondrial S-nitrosation: effects of S-nitroso-2-mercaptopyrionyl glycine (SNO-MPG) in cardiac ischemia-reperfusion injury,” *Journal of Molecular and Cellular Cardiology*, vol. 42, no. 4, pp. 812–825, 2007.
- [26] F. Sam, D. L. Kerstetter, D. R. Pimental et al., “Increased reactive oxygen species production and functional alterations in antioxidant enzymes in human failing myocardium,” *Journal of Cardiac Failure*, vol. 11, no. 6, pp. 473–480, 2005.
- [27] J. L. Tarry-Adkins, H. L. Blackmore, M. S. Martin-Gronert et al., “Coenzyme Q10 prevents accelerated cardiac aging in a rat model of poor maternal nutrition and accelerated postnatal growth,” *Molecular Metabolism*, vol. 2, no. 4, pp. 480–490, 2013.
- [28] M. Bentinger, K. Brismar, and G. Dallner, “The antioxidant role of coenzyme Q,” *Mitochondrion*, vol. 7, supplement, pp. S41–S50, 2007.
- [29] C. Schmelzer, M. Kitano, K. Hosoe, and F. Döring, “Ubiquinol affects the expression of genes involved in PPAR α signalling and lipid metabolism without changes in methylation of CpG promoter islands in the liver of mice,” *Journal of Clinical Biochemistry and Nutrition*, vol. 50, no. 2, pp. 119–126, 2012.
- [30] J. J. Ochoa, J. L. Quiles, J. R. Huertas, and J. Mataix, “Coenzyme Q10 protects from aging-related oxidative stress and improves mitochondrial function in heart of rats fed a polyunsaturated fatty acid (PUFA)-rich diet,” *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 60, no. 8, pp. 970–975, 2005.
- [31] S. L. Molyneux, C. M. Florkowski, P. M. George et al., “Coenzyme Q10: an independent predictor of mortality in chronic heart failure,” *Journal of the American College of Cardiology*, vol. 52, no. 18, pp. 1435–1441, 2008.
- [32] S. A. Mortensen, F. Rosenfeldt, A. Kumar et al., “The effect of coenzyme Q₁₀ on morbidity and mortality in chronic heart

- failure: results from Q-SYMBIO: a randomized double-blind trial," *JACC: Heart Failure*, vol. 2, no. 6, pp. 641–649, 2014.
- [33] M. Bienengraeber, C. Ozcan, and A. Terzic, "Stable transfection of UCP1 confers resistance to hypoxia/reoxygenation in a heart-derived cell line," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 7, pp. 861–865, 2003.
- [34] C. J. McLeod, A. Aziz, R. F. Hoyt Jr., J. P. McCoy Jr., and M. N. Sack, "Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia," *The Journal of Biological Chemistry*, vol. 280, no. 39, pp. 33470–33476, 2005.
- [35] S. M. Nadtochiy, A. J. Tompkins, and P. S. Brookes, "Different mechanisms of mitochondrial proton leak in ischaemia/reperfusion injury and preconditioning: implications for pathology and cardioprotection," *The Biochemical Journal*, vol. 395, no. 3, pp. 611–618, 2006.
- [36] M. N. Sack, "Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance," *Cardiovascular Research*, vol. 72, no. 2, pp. 210–219, 2006.
- [37] C. Ozcan, M. Palmeri, T. L. Horvath, K. S. Russell, and R. R. Russell III, "Role of uncoupling protein 3 in ischemia-reperfusion injury, arrhythmias, and preconditioning," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 304, no. 9, pp. H1192–H1200, 2013.
- [38] S. Krauss, C.-Y. Zhang, and B. B. Lowell, "The mitochondrial uncoupling-protein homologues," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 3, pp. 248–261, 2005.
- [39] K. S. Echtay, E. Winkler, and M. Klingenberg, "Coenzyme Q is an obligatory cofactor for uncoupling protein function," *Nature*, vol. 408, no. 6812, pp. 609–613, 2000.
- [40] J. Hoerter, M.-D. Gonzalez-Barroso, E. Couplan et al., "Mitochondrial uncoupling protein 1 expressed in the heart of transgenic mice protects against ischemic-reperfusion damage," *Circulation*, vol. 110, no. 5, pp. 528–533, 2004.
- [41] Y. Teshima, M. Akao, S. P. Jones, and E. Marbán, "Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes," *Circulation Research*, vol. 93, no. 3, pp. 192–200, 2003.
- [42] R. J. Youle and A. M. van der Bliek, "Mitochondrial fission, fusion, and stress," *Science*, vol. 337, no. 6098, pp. 1062–1065, 2012.
- [43] M. Liesa, M. Palacín, and A. Zorzano, "Mitochondrial dynamics in mammalian health and disease," *Physiological Reviews*, vol. 89, no. 3, pp. 799–845, 2009.
- [44] T. Koshiba, S. A. Detmer, J. T. Kaiser, H. Chen, J. M. McCaffery, and D. C. Chan, "Structural basis of mitochondrial tethering by mitofusin complexes," *Science*, vol. 305, no. 5685, pp. 858–862, 2004.
- [45] S. Cipolat, O. Martins de Brito, B. Dal Zilio, and L. Scorrano, "OPA1 requires mitofusin 1 to promote mitochondrial fusion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 45, pp. 15927–15932, 2004.
- [46] E. Smirnova, L. Griparic, D.-L. Shurland, and A. M. van der Bliek, "Dynamamin-related protein Drp1 is required for mitochondrial division in mammalian cells," *Molecular Biology of the Cell*, vol. 12, no. 8, pp. 2245–2256, 2001.
- [47] Y. Yoon, E. W. Krueger, B. J. Oswald, and M. A. McNiven, "The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamamin-like protein DLP1," *Molecular and Cellular Biology*, vol. 23, no. 15, pp. 5409–5420, 2003.
- [48] P. H. Reddy, "Inhibitors of mitochondrial fission as a therapeutic strategy for diseases with oxidative stress and mitochondrial dysfunction," *Journal of Alzheimer's Disease*, vol. 40, no. 2, pp. 245–256, 2014.
- [49] W. W. Sharp, Y. H. Fang, M. Han et al., "Dynamamin-related protein 1 (Drp1)-mediated diastolic dysfunction in myocardial ischemia-reperfusion injury: therapeutic benefits of Drp1 inhibition to reduce mitochondrial fission," *The FASEB Journal*, vol. 28, no. 1, pp. 316–326, 2014.
- [50] J. Marín-García, A. T. Akhmedov, and G. W. Moe, "Mitochondria in heart failure: the emerging role of mitochondrial dynamics," *Heart Failure Reviews*, vol. 18, no. 4, pp. 439–456, 2013.
- [51] L. Chen, Q. Gong, J. P. Stice, and A. A. Knowlton, "Mitochondrial OPA1, apoptosis, and heart failure," *Cardiovascular Research*, vol. 84, no. 1, pp. 91–99, 2009.
- [52] S.-B. Ong, S. Subrayan, S. Y. Lim, D. M. Yellon, S. M. Davidson, and D. J. Hausenloy, "Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury," *Circulation*, vol. 121, no. 18, pp. 2012–2022, 2010.
- [53] K. N. Papanicolaou, R. J. Khairallah, G. A. Ngoh et al., "Mitofusin-2 maintains mitochondrial structure and contributes to stress-induced permeability transition in cardiac myocytes," *Molecular and Cellular Biology*, vol. 31, no. 6, pp. 1309–1328, 2011.
- [54] L. Chen, T. Liu, A. Tran et al., "OPA1 mutation and late-onset cardiomyopathy: mitochondrial dysfunction and mtDNA instability," *Journal of the American Heart Association*, vol. 1, Article ID e003012, 2012.
- [55] T. Varanita, M. E. Soriano, V. Romanello et al., "The OPA1-dependent mitochondrial cristae remodeling pathway controls atrophic, apoptotic, and ischemic tissue damage," *Cell Metabolism*, vol. 21, no. 6, pp. 834–844, 2015.
- [56] M. Sumida, K. Doi, E. Ogasawara et al., "Regulation of mitochondrial dynamics by dynamamin-related protein-1 in acute cardiorenal syndrome," *Journal of the American Society of Nephrology*, vol. 26, no. 10, pp. 2378–2387, 2015.
- [57] A. Chacinska, C. M. Koehler, D. Milenkovic, T. Lithgow, and N. Pfanner, "Importing mitochondrial proteins: machineries and mechanisms," *Cell*, vol. 138, no. 4, pp. 628–644, 2009.
- [58] O. Schmidt, N. Pfanner, and C. Meisinger, "Mitochondrial protein import: from proteomics to functional mechanisms," *Nature Reviews Molecular Cell Biology*, vol. 11, no. 9, pp. 655–667, 2010.
- [59] J. C. Young, N. J. Hoogenraad, and F. U. Hartl, "Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70," *Cell*, vol. 112, no. 1, pp. 41–50, 2003.
- [60] K. Boengler, P. Gres, A. Cabestrero et al., "Prevention of the ischemia-induced decrease in mitochondrial Tom20 content by ischemic preconditioning," *Journal of Molecular and Cellular Cardiology*, vol. 41, no. 3, pp. 426–430, 2006.
- [61] G. Wright, K. Terada, M. Yan, I. Sergeev, and M. Mori, "Oxidative stress inhibits the mitochondrial import of preproteins and leads to their degradation," *Experimental Cell Research*, vol. 263, no. 1, pp. 107–117, 2001.
- [62] J. Li, M. Qi, C. Li et al., "Tom70 serves as a molecular switch to determine pathological cardiac hypertrophy," *Cell Research*, vol. 24, no. 8, pp. 977–993, 2014.

- [63] Z. Yang, W. Sun, and K. Hu, "Molecular mechanism underlying adenosine receptor-mediated mitochondrial targeting of protein kinase C," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1823, no. 4, pp. 950–958, 2012.
- [64] D. J. Hausenloy and D. M. Yellon, "The mitochondrial permeability transition pore: Its fundamental role in mediating cell death during ischaemia and reperfusion," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 4, pp. 339–341, 2003.
- [65] C. P. Baines, "The molecular composition of the mitochondrial permeability transition pore," *Journal of Molecular and Cellular Cardiology*, vol. 46, no. 6, pp. 850–857, 2009.
- [66] J. Zhu, M. J. Rebecchi, P. S. A. Glass, P. R. Brink, and L. Liu, "Cardioprotection of the aged rat heart by GSK-3 β inhibitor is attenuated: age-related changes in mitochondrial permeability transition pore modulation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 3, pp. H922–H930, 2011.
- [67] E. N. Dedkova, L. K. Seidlmayer, and L. A. Blatter, "Mitochondria-mediated cardioprotection by trimetazidine in rabbit heart failure," *Journal of Molecular and Cellular Cardiology*, vol. 59, pp. 41–54, 2013.
- [68] P. J. Oliveira, R. Seiça, P. M. Coxito et al., "Enhanced permeability transition explains the reduced calcium uptake in cardiac mitochondria from streptozotocin-induced diabetic rats," *FEBS Letters*, vol. 554, no. 3, pp. 511–514, 2003.
- [69] D. J. Hausenloy, M. R. Duchon, and D. M. Yellon, "Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury," *Cardiovascular Research*, vol. 60, no. 3, pp. 617–625, 2003.
- [70] M. Saotome, H. Katoh, Y. Yaguchi et al., "Transient opening of mitochondrial permeability transition pore by reactive oxygen species protects myocardium from ischemia-reperfusion injury," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 296, no. 4, pp. H1125–H1132, 2009.
- [71] M. J. Berridge, P. Lipp, and M. D. Bootman, "The versatility and universality of calcium signalling," *Nature Reviews Molecular Cell Biology*, vol. 1, no. 1, pp. 11–21, 2000.
- [72] A. B. Aurora, A. I. Mahmoud, X. Luo et al., "MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca²⁺ overload and cell death," *The Journal of Clinical Investigation*, vol. 122, no. 4, pp. 1222–1232, 2012.
- [73] T. Liu, E. Takimoto, V. L. Dimaano et al., "Inhibiting mitochondrial Na⁺/Ca²⁺ exchange prevents sudden death in a Guinea pig model of heart failure," *Circulation Research*, vol. 115, no. 1, pp. 44–54, 2014.
- [74] P. S. Brookes, Y. Yoon, J. L. Robotham, M. W. Anders, and S.-S. Sheu, "Calcium, ATP, and ROS: a mitochondrial love-hate triangle," *American Journal of Physiology—Cell Physiology*, vol. 287, no. 4, pp. C817–C833, 2004.
- [75] M. Aldakkak, A. K. S. Camara, J. S. Heisner, M. Yang, and D. F. Stowe, "Ranolazine reduces Ca²⁺ overload and oxidative stress and improves mitochondrial integrity to protect against ischemia reperfusion injury in isolated hearts," *Pharmacological Research*, vol. 64, no. 4, pp. 381–392, 2011.
- [76] M. L. Riess, A. K. S. Camara, L. G. Kevin, J. An, and D. F. Stowe, "Reduced reactive O₂ species formation and preserved mitochondrial NADH and [Ca²⁺] levels during short-term 17 °C ischemia in intact hearts," *Cardiovascular Research*, vol. 61, no. 3, pp. 580–590, 2004.
- [77] M. L. Riess, A. K. S. Camara, E. Novalija, Q. Chen, S. S. Rhodes, and D. F. Stowe, "Anesthetic preconditioning attenuates mitochondrial Ca²⁺ overload during ischemia in Guinea pig intact hearts: reversal by 5-hydroxydecanoic acid," *Anesthesia and Analgesia*, vol. 95, no. 6, pp. 1540–1546, 2002.
- [78] R. Ferrari, P. Pedersini, M. Bongrazio et al., "Mitochondrial energy production and cation control in myocardial ischaemia and reperfusion," *Basic Research in Cardiology*, vol. 88, no. 5, pp. 495–512, 1993.
- [79] D. De Stefani, A. Raffaello, E. Teardo, I. Szabó, and R. Rizzuto, "A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter," *Nature*, vol. 476, no. 7360, pp. 336–340, 2011.
- [80] D. De Stefani, M. Patron, and R. Rizzuto, "Structure and function of the mitochondrial calcium uniporter complex," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1853, no. 9, pp. 2006–2011, 2015.
- [81] T. M. Ye, S. Z. Zhang, and Q. Xia, "Role of mitochondrial calcium uniporter in myocardial hypoxia/reoxygenation induced injury," *Chinese Journal of Applied Physiology*, vol. 22, pp. 136–140, 2006.
- [82] M. Ahuja and S. Muallem, "The gatekeepers of mitochondrial calcium influx: MICU1 and MICU2," *EMBO Reports*, vol. 15, no. 3, pp. 205–206, 2014.
- [83] K. J. Kamer and V. K. Mootha, "MICU1 and MICU2 play nonredundant roles in the regulation of the mitochondrial calcium uniporter," *EMBO Reports*, vol. 15, no. 3, pp. 299–307, 2014.
- [84] K. Mallilankaraman, P. Doonan, C. Cárdenas et al., "MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca²⁺ uptake that regulates cell survival," *Cell*, vol. 151, no. 3, pp. 630–644, 2012.
- [85] M. Patron, V. Checchetto, A. Raffaello et al., "MICU1 and MICU2 finely tune the mitochondrial Ca²⁺ uniporter by exerting opposite effects on MCU activity," *Molecular Cell*, vol. 53, no. 5, pp. 726–737, 2014.
- [86] W. Wang, Q. Xie, X. Zhou et al., "Mitofusin-2 triggers mitochondria Ca²⁺ influx from the endoplasmic reticulum to induce apoptosis in hepatocellular carcinoma cells," *Cancer Letters*, vol. 358, no. 1, pp. 47–58, 2015.
- [87] P. Michela, V. Velia, P. Aldo, and P. Ada, "Role of connexin 43 in cardiovascular diseases," *European Journal of Pharmacology*, vol. 768, pp. 71–76, 2015.
- [88] A. Rodriguez-Sinovas, K. Boengler, A. Cabestrero et al., "Translocation of connexin 43 to the inner mitochondrial membrane of cardiomyocytes through the heat shock protein 90-dependent TOM pathway and its importance for cardioprotection," *Circulation Research*, vol. 99, no. 1, pp. 93–101, 2006.
- [89] K.-G. Shyu, B.-W. Wang, W.-P. Cheng, and H.-M. Lo, "MicroRNA-208a increases myocardial endoglin expression and myocardial fibrosis in acute myocardial infarction," *Canadian Journal of Cardiology*, vol. 31, no. 5, pp. 679–690, 2015.
- [90] R. Schulz, P. M. Görges, A. Görbe, P. Ferdinandy, P. D. Lampe, and L. Leybaert, "Connexin 43 is an emerging therapeutic target in ischemia/reperfusion injury, cardioprotection and neuroprotection," *Pharmacology & Therapeutics*, vol. 153, pp. 90–106, 2015.
- [91] N. Wang, E. De Vuyst, R. Ponsaerts et al., "Selective inhibition of Cx43 hemichannels by Gap19 and its impact on myocardial ischemia/reperfusion injury," *Basic Research in Cardiology*, vol. 108, article 309, 2013.
- [92] G. Chen, J. Zhao, C. Liu, Y. Zhang, Y. Huo, and L. Zhou, "MG132 proteasome inhibitor upregulates the expression of connexin

- 43 in rats with adriamycin-induced heart failure," *Molecular Medicine Reports*, vol. 12, no. 5, pp. 7595–7602, 2015.
- [93] G. Heusch, J. Musiolik, N. Gedik, and A. Skyschally, "Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion," *Circulation Research*, vol. 109, no. 11, pp. 1302–1308, 2011.
- [94] K. Szczepanek, E. J. Lesnefsky, and A. C. Larner, "Multi-tasking: nuclear transcription factors with novel roles in the mitochondria," *Trends in Cell Biology*, vol. 22, no. 8, pp. 429–437, 2012.
- [95] F. A. Zouein, R. Altara, Q. Chen, E. J. Lesnefsky, M. Kurdi, and G. W. Booz, "Pivotal importance of STAT3 in protecting the heart from acute and chronic stress: new advancement and unresolved issues," *Frontiers in Cardiovascular Medicine*, vol. 2, article 36, 2015.
- [96] K. Szczepanek, Q. Chen, A. C. Larner, and E. J. Lesnefsky, "Cytoprotection by the modulation of mitochondrial electron transport chain: the emerging role of mitochondrial STAT3," *Mitochondrion*, vol. 12, no. 2, pp. 180–189, 2012.
- [97] J. Wegrzyn, R. Potla, Y.-J. Chwae et al., "Function of mitochondrial Stat3 in cellular respiration," *Science*, vol. 323, no. 5915, pp. 793–797, 2009.
- [98] K. Szczepanek, Q. Chen, M. Derecka et al., "Mitochondrial-targeted signal transducer and activator of transcription 3 (STAT3) protects against ischemia-induced changes in the electron transport chain and the generation of reactive oxygen species," *The Journal of Biological Chemistry*, vol. 286, no. 34, pp. 29610–29620, 2011.

Research Article

Redox Nanoparticle Therapeutics for Acetaminophen-Induced Hepatotoxicity in Mice

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The purpose of this study was to evaluate the hepatoprotective effect of an antioxidative nanoparticle (RNP^N) recently developed against APAP-induced hepatotoxicity in mice. The effects of oral administration of RNP^N to APAP-treated mice were assessed for various biochemical liver function parameters: alanine transaminase (ALT) activity, aspartate transaminase (AST) activity, alkaline phosphatase (ALP) activity, prothrombin time, and serum albumin (ALB) level. The treatment effects were assessed in terms of free radical parameters: malondialdehyde (MDA) accumulation, glutathione peroxidase (GPx) activity, % inhibition of superoxide anion (O₂^{•-}), and histopathological examination. The *N*-acetylcysteine (NAC)-treated group exhibited an enhanced prothrombin time relative to the control group, while RNP^N did not prolong prothrombin time. The RNP^N-treated animals exhibited lower levels of ALT, AST, and ALP, while increased ALB levels were measured in these animals compared to those in the other groups. The RNP^N-treated animals furthermore exhibited improved MDA levels, GPx activity, and % inhibition of O₂^{•-}, which relate to oxidative damage. Histological staining of liver tissues from RNP^N-treated animals did not reveal any microscopic changes relative to the other groups. The findings of this study suggest that RNP^N possesses effective hepatoprotective properties and does not exhibit the notable adverse effects associated with NAC treatment.

1. Introduction

Overdoses of acetaminophen (paracetamol, APAP, or *N*-acetyl-*p*-aminophenol) represent one of the most common pharmaceutical product poisonings in the world [1, 2]. Acetaminophen is widely used as an analgesic and antipyretic drug; however, it is also the leading major cause of acute liver failure (ALF) and acetaminophen overdose may lead to liver transplantation being required or even to death, since the early signs and symptoms of APAP-induced hepatotoxicity are not clear [3–8]. The main mechanism of acetaminophen-induced hepatotoxicity is an increase in oxidative stress and

subsequent saturation of the glucuronidation and sulfation pathways of hepatic elimination, leading to more APAP being metabolized to *N*-acetyl-*p*-benzoquinone imine (NAPQI). Finally, GSH depletion (~70–80%) occurs and NAPQI binds to liver cells, resulting in hepatotoxicity [9, 10].

N-Acetylcysteine (NAC), a precursor of glutathione (GSH), is the standard antidote administered to patients with APAP-induced hepatotoxicity [11, 12]; however, NAC has previously been shown to have adverse side effects including bruising, bleeding, nausea, vomiting, and diarrhea or constipation. Rarely, NAC also causes rashes, fever, headache, drowsiness, low blood pressure, and liver problems, while

NAC therapy was also shown to prolong prothrombin time and should thus be avoided in patients with coagulation disorders [13, 14]. Since NAC is a low molecular weight compound, it furthermore displays a low stability and nonspecific distribution *in vivo* physiological environments, resulting in a low therapeutic efficacy.

Recently, the development of nanotherapeutic strategies against APAP-induced liver injury has been the focus of several research studies [15–18]. We have developed a novel antioxidative nanoparticle, RNP^{N} , prepared by self-assembly of an amphiphilic block copolymer, methoxy-poly(ethylene glycol)-b-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl) aminomethylstyrene] (PEG-b-PMNT), which has nitroxide radicals on the side chain of the hydrophobic segment. Due to its amphiphilic nature, PEG-b-PMNT forms core-shell-type polymeric micelles with tens of nanometers in size in aqueous media [19]. We previously confirmed that the PEG-b-PMNT polymer internalized and circulated in the blood stream after oral administration of RNP^{N} and is subsequently disintegrated by the gastric acidity [20]. Since the nitroxide radicals in the PMNT segment catalytically eliminate reactive oxygen species (ROS), it functions as a strong antioxidant. However, due to the high molecular weight of the PEG-b-PMNT polymer, it is only minimally internalized in healthy cells, thereby avoiding marked disturbances to normal redox reactions such as those in the electron transport chain, which is adversely affected by conventional low molecular weight antioxidants [21]. On the basis of our previous investigations, therefore, the effects of RNP^{N} on APAP-induced liver injury must be investigated. We report here on both the therapeutic effect and the lack of adverse effects of orally administered RNP^{N} .

2. Materials and Methods

2.1. Materials. 1,1,3,3-Tetramethoxypropane (TMP), L-glutathione reduced, glutathione peroxidase (GPx), glutathione reductase (GR), xanthine, xanthine oxidase (XO), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium (β -NADPH) salt, APAP, and NAC were purchased from the Sigma Chemical Company, St. Louis, MO, USA. Other chemical reagents were analytical grade and milli-Q water was used all through the experiments.

2.2. Preparation and Characterization of RNP^{N} . PEG-b-PMNT was synthesized as previously described [19]. The molecular weight of the PEG segment and the degree of polymerization of PMNT were determined as 5,000 and 13, respectively. The RNP^{N} was prepared by self-assembly of the PEG-b-PMNT using the dialysis method (Figure 1) and the blank micelle (nRNP) was prepared in the same way using PEG-b-poly(chloromethylstyrene), a precursor of PEG-b-PMNT with the same molecular weight. The size and distribution of the resulting RNP^{N} and the nRNP in aqueous solution were determined by dynamic light scattering (DLS) measurements carried out in triplicate. Electron paramagnetic resonance (EPR) was used to quantify the amount of

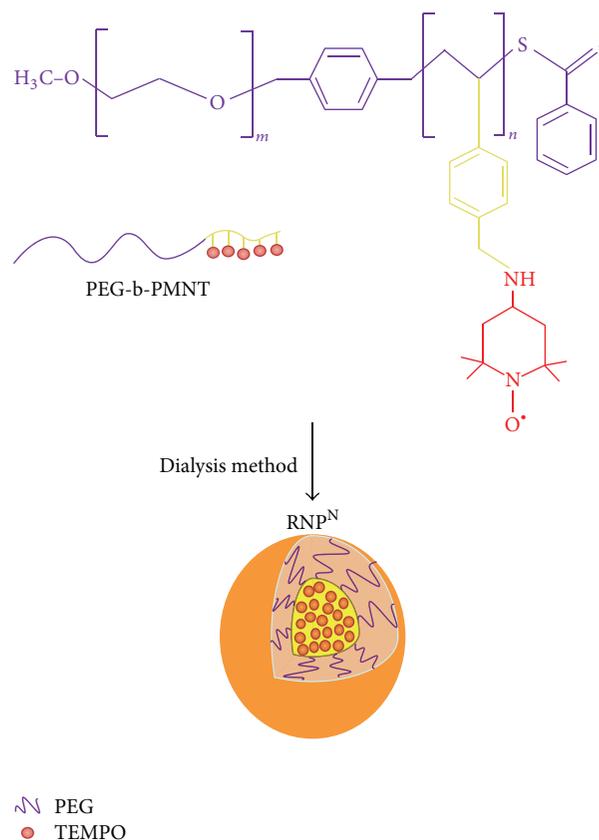


FIGURE 1: Schematic illustration of antioxidative RNP^{N} preparation.

2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) inside each RNP^{N} .

2.3. Animal Care Conditions. Animal experiments were carried out using male CD-1[®] IGS mice nomenclature: Crl:CD1(ICR) (10 weeks old, 40–50 g) purchased from Charles River Japan, Inc. (Yokohama, Japan). The mice ($n = 48$) were maintained in the Laboratory Animal Resource Center, University of Tsukuba (Japan), in six groups. The animals were housed in stainless steel cages and seven days prior to the experiment they were acclimated to the standard laboratory conditions of $23 \pm 1^\circ\text{C}$, ventilation with relative humidity of $50 \pm 5\%$, and a 12 h light/12 h dark cycle. The mice received food and filtered water *ad libitum* throughout the study. All animal experiments were undertaken in accordance with the criteria outlined in the license (numbers 15–434) approved by the Animal Ethics Committees of the University of Tsukuba.

2.4. APAP-Induced Hepatotoxicity Model. Mice were randomly divided into six groups of 8 animals each:

Group A: control, no treatment.

Group B: 0.9% normal saline (NSS) + APAP 2.5 g/kg body weight (BW)

(the APAP concentration has been used according to our pilot study).

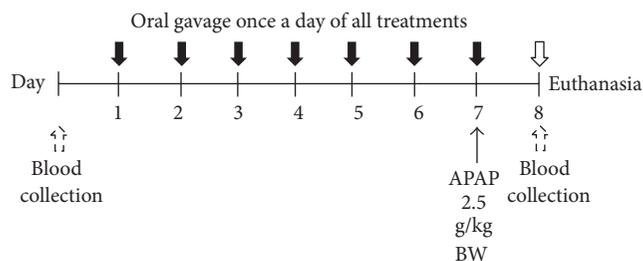


FIGURE 2: Experimental design of APAP-induced hepatotoxicity model.

Group C: N-acetylcysteine (NAC) 600 mg/kg BW + APAP 2.5 g/kg BW.

Group D: low molecular weight (LMW) TEMPO 15.4 mM + APAP 2.5 g/kg BW.

Group E: blank micelle (nRNP) 12.5 mg/mL + APAP 2.5 g/kg BW.

Group F: RNP^N 12.5 mg/mL (containing 4-amino TEMPO 15.4 mM) + APAP 2.5 g/kg BW.

Prior to the APAP-induced hepatotoxicity experiments, blood was collected from the tail veins of all mice to analyze prothrombin time. All mice in the treatment groups were orally administered their treatments once daily for 7 days after which hepatotoxicity was induced by oral gavage of 2.5 g/kg BW of APAP (Figure 2). After 24 h, the mice were rechecked of prothrombin time and anesthetized with intraperitoneal (i.p.) injection of pentobarbital 50 mg/kg BW. The blood was drawn and treated with anticoagulant to allow serum by centrifugation of the blood at 1600 g for 15 min at 4°C in order to determine the liver function. Animals were then sacrificed and their livers were removed. Half of each liver was fixed in 10% buffered formalin for histopathology analysis and the other half was homogenized (10% w/v) in ice cold phosphate buffer (50 mM, pH 7.4). The liver homogenates were centrifuged at 9,000 g for 15 min at 4°C and the resulting supernatants were frozen at -80°C until being subjected to oxidative stress analysis and lipid peroxidation assay.

2.5. Lipid Peroxidation (LPO) Assay. The levels of malondialdehyde (MDA), the product of lipid peroxidation, were assessed based on the method described by Ohkawa et al. [22]. Briefly, the samples were treated with thiobarbituric acid (TBA) to allow for thiobarbituric acid reactive substance (TBARS), a pink complex and indicator of lipid peroxidation, to be quantified. Absorbance levels at 532 nm were measured by spectrophotometer and the arbitrary values obtained were related to MDA concentration (nmol/g tissue) using a standard curve generated from the absorbance values obtained for standard solutions of 1,1,3,3-tetramethoxypropane (TMP).

2.6. Glutathione Peroxidase (GPx) Assay. GPx activity was determined according to the method described by Hussain et al. [23]. Glutathione peroxidase (GPx) is a radical-scavenging enzyme that catalyzes the reduction of hydrogen peroxide

(H₂O₂) and lipid peroxide (ROOH) using glutathione (GSH), resulting in the formation of oxidized glutathione (GSSH) and H₂O. Glutathione reductase (GR) subsequently catalyzes GSSH by reacting with nicotinamide adenine dinucleotide phosphate (NADPH) yielding GSH and NADP⁺. The generation of NADP⁺ was measured spectrophotometrically at 340 nm relative to a blank sample and a standard curve was generated from GPx solutions. GPx activity was expressed as units/mg protein, where protein concentration was measured using the method of Lowry et al. [24].

2.7. Superoxide (O₂^{•-}) Anion Assay. The O₂^{•-} levels in the samples were determined by spectrophotometric measurement based on the xanthine/xanthine oxidase (XO) system involving the conversion of yellow nitro blue tetrazolium (NBT) to blue formazan. Reactions contained EDTA, NBT, xanthine, XO, and samples were assessed at 560 nm relative to a standard curve generated using 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL). Data were expressed as % inhibition of O₂^{•-} [25].

2.8. Biochemical Assays in Plasma. Blood for biochemical analysis was collected of 100 μL from the animals prior to treatment via the lateral tail vein and 500 μL after APAP-induced hepatotoxicity induction through heart. The blood was centrifuged (1600 g, 15 min, 4°C) to isolate the serum, which was subsequently analyzed with a FUJI DRI-CHEM 7000V (Fujifilm, Japan) for biochemical liver activities of albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

2.9. Liver Histopathology. The liver tissues fixed in 10% neutral buffered formalin solution were cleaned in running water before being processed for further histopathological examination. Paraffin-embedded tissues were sectioned (5 μm thickness), stained with hematoxylin and eosin (H&E), assessed for histopathological changes by light microscopy, and graded blindly and independently using the modified method described by Wood et al. [26]. Grades (0–5) were as follows: 0, no lesions; 1, minimal lesions, only necrotic cells at the first cell level from the central vein; 2, mild lesions, necrotic cells extending two to three cell levels from the central vein; 3, moderate lesions, necrotic cells extending three to six cell levels within peripheral distribution; 4, marked lesions, the same as in 3 but with necrosis extruding to another central vein; and 5, lesions more severe than those in 4, with progressive centrilobular necrosis throughout the section.

2.10. Statistical Analysis. Data are expressed as mean ± SEM. Groups were compared by one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison test (Tukey's method) using SPSS version 16.0. Differences with $p < 0.05$ were considered statistically significant.

3. Results

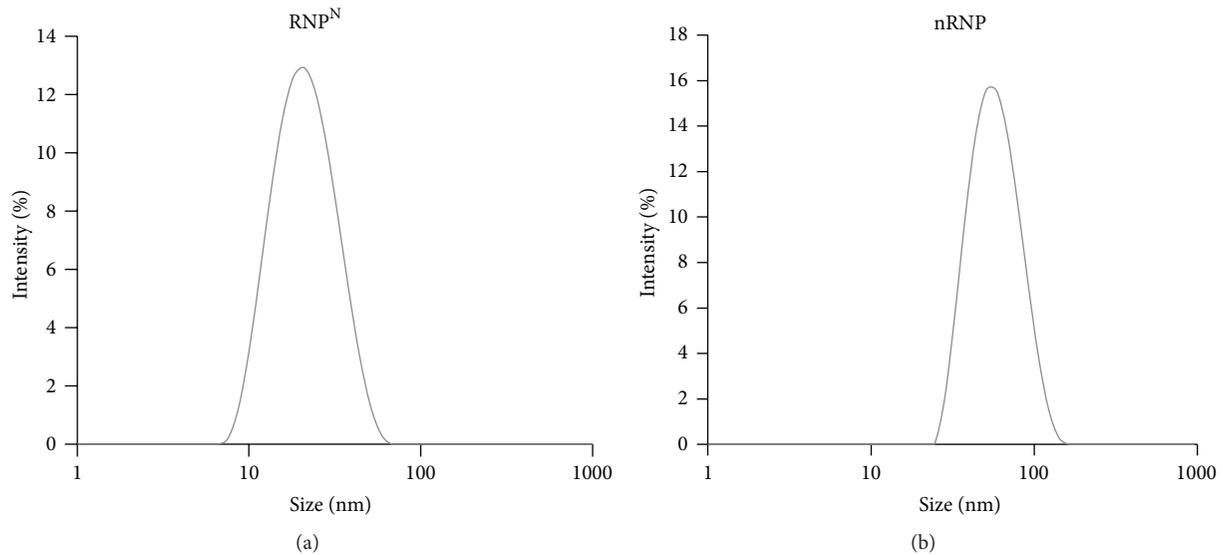


FIGURE 3: Size distribution of RNP^N (a) and nRNP (b) assessed by dynamic light scattering (DLS).

3.1. The Size and Distribution of RNP^N and nRNP. The DLS measurements confirmed the unimodal distribution of nanoparticles in the nanometer size range with no particle aggregation (Figure 3). The particle size and distribution were found to be 22.140 ± 0.307 nm and 0.139 ± 0.008 for RNP^N and 58.853 ± 0.696 nm and 0.205 ± 0.004 for nRNP, respectively. From the EPR spectrum of RNP^N, the amount of nitroxide radical in RNP^N (12.5 mg/mL) was 15.4 mM.

3.2. Effects of RNP^N on Serum ALB, ALP, ALT, and AST Levels. Hepatotoxicity was determined by quantitative analysis of ALB, ALT, AST, and ALP levels as shown in Figure 4. Serum ALB, ALT, AST, and ALP levels in control mice were 1.9875 ± 0.2997 g/dL, 23.75 ± 4.301 U/L, 91.25 ± 7.995 U/L, and 177.5 ± 10.915 U/L, respectively. The NSS + APAP-treated group exhibited decreased ALB levels, while the ALT, AST, and ALP levels in the serum of the animals in this group were significantly elevated compared to those measured for the control group ($p < 0.001$). Interestingly, the ALT, AST, and ALP levels in the RNP^N + APAP-treated animals did not differ from those in the NAC + APAP-treated group but differed significantly from those in the NSS + APAP-treated group ($p < 0.001$), indicating that RNP^N suppressed the adverse effects of APAP in mice. Despite the same dose of nitroxide radicals being administered in both groups, the protective effect of RNP^N was stronger than that of the low molecular weight (LMW) TEMPO, especially in terms of ALT, AST, and ALP levels.

3.3. Effects of Treatments on Prothrombin Time. All blood samples were tested for prothrombin time (Figure 5). The NAC + APAP-treated animals exhibited a significantly prolonged prothrombin time (24.778 ± 3.308 s) compared with the prothrombin times measured in the control ($13.444 \pm$

0.882 s) and NSS + APAP (13.375 ± 1.84 s) group animals ($p < 0.001$). Prothrombin time, one of the major side effects of NAC, results in hepatocellular necrosis or disseminated intravascular coagulation (DIC). Interestingly, RNP^N + APAP treatment did not prolong prothrombin time despite exhibiting strong therapeutic effects as stated above.

3.4. Effects on MDA Levels, GPx Activity, and % Inhibition of O₂^{•-}. The levels of MDA, the GPx activity, and the % inhibition of O₂^{•-} measured in the livers of all groups are presented in Figure 6. Liver MDA levels were found to be elevated in the NSS + APAP group compared with the control group, while GPx activity and % inhibition of O₂^{•-} were lower in the NSS + APAP group compared with the control group (13.251 ± 0.365 nmol/mg tissue versus 5.784 ± 1.015 nmol/mg tissue, 1.660 ± 0.207 units/mg protein versus 3.489 ± 0.168 units/mg protein, and $21.604 \pm 1.969\%$ versus $47.237 \pm 3.505\%$, resp.; $p < 0.001$). Remarkably, RNP^N + APAP induced a noticeable decrease in MDA levels relative to control levels, whereas GPx activity and % inhibition of O₂^{•-} were found to be elevated in the RNP^N + APAP group compared with the control group (7.558 ± 1.101 nmol/mg tissue versus 13.251 ± 0.365 nmol/mg tissue, 3.178 ± 0.201 units/mg protein versus 1.660 ± 0.207 units/mg protein, and $44.881 \pm 1.749\%$ versus $21.604 \pm 1.969\%$, resp.; $p < 0.01$). No significant differences in the MDA levels, the GPx activity, and the % inhibition of O₂^{•-} were observed between the RNP^N + APAP- and NAC + APAP-treated groups. These findings again demonstrate the protective effect of RNP^N to be significantly stronger than that of LMW TEMPO.

3.5. Histopathological Examinations of APAP-Induced Liver Toxicity. After seven days of treatment, liver tissues were taken from the animals of all groups and were subjected

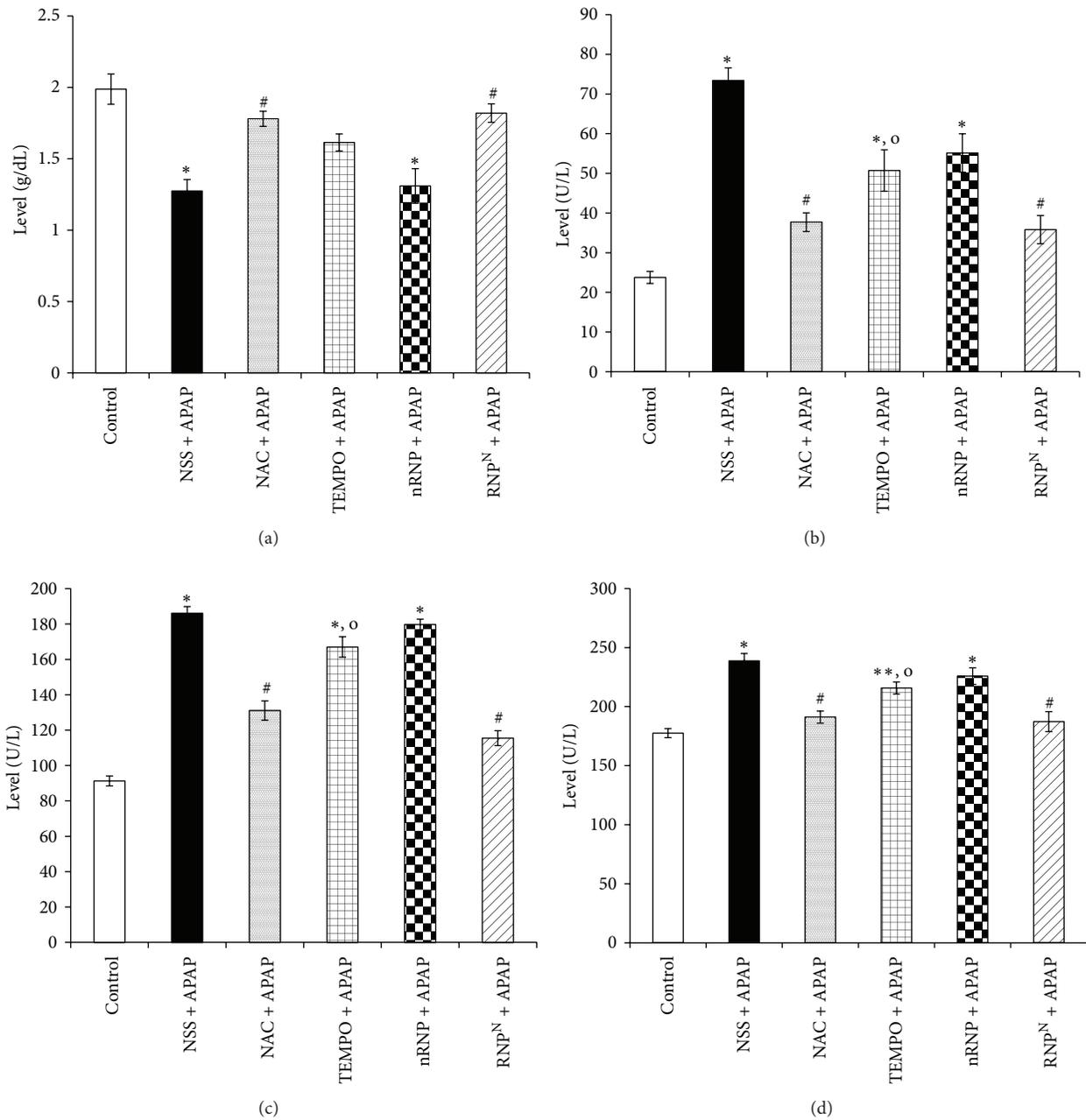


FIGURE 4: Biochemical liver function in terms of (a) ALB, (b) ALT, (c) AST, and (d) ALP activities after treatment for 7 days and APAP-induced hepatotoxicity. Data were expressed as mean \pm SEM, *versus control group, $p < 0.001$; **versus control group, $p < 0.01$; #versus NSS + APAP-treated group, $p < 0.001$; °versus RNP^N + APAP-treated group, $p < 0.05$; $n = 8$ mice/group.

to histological analysis (Figure 7). The livers from the animals in the control, NAC + APAP, and RNP^N + APAP groups had an overall smooth appearance and normal color. The control group livers were further found to have

normal lobular morphology and hepatocytes with well-defined sinusoids (Figure 7(a)). The hepatic injury in the mice treated with APAP (2.5 mg/kg BW dissolved in normal saline) manifested as inflammatory infiltration, swelling, hemorrhage, and necrosis involving mainly the centrilobular zone (Figure 7(b)). Mild congestion of sinusoidal spaces was observed in the centrilobular area of the livers of NAC + APAP-treated mice (Figure 7(c)). The livers of the mice

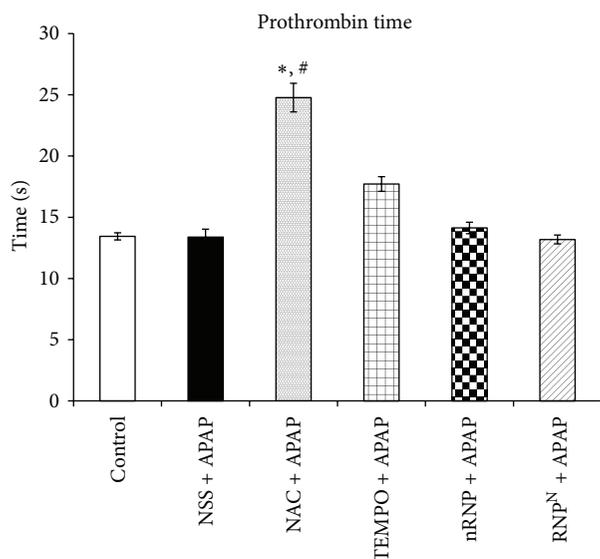


FIGURE 5: Effects of treatments on prothrombin time after treatment for 7 days. Data represent mean \pm SEM, *versus control group, $p < 0.001$; #versus NSS + APAP-treated group, $p < 0.001$; $n = 8$ mice/group.

treated with LMW TEMPO and nRNP appeared hyperemic, mottled and were fragile (Figures 7(d) and 7(e)). Remarkably, the livers of the mice treated with RNP^N did not reveal any significant microscopic changes relative to control tissue (Figure 7(f)). These findings are in agreement with the histopathological scale analysis of the liver sections (Figure 8): the RNP^N-treated group exhibited significantly less severe hepatic injury than the NSS, TEMPO, and blank micelle-treated groups ($p < 0.001$).

4. Discussion

The nanoparticle is one of the novel drug carriers for therapeutic and diagnostic objectives which has several potential effects in improving accumulation and bioavailability of drug in target side thereby suppressed immunogenicity or drug resistance and finally reducing adverse effects. Additionally, nanoparticles also promote drug solubility, controlled and sustained drug release, decreased drug elimination, and delivered more drugs combination treatment for synergistic effect [27–29].

Liver injury induced by APAP is one of the most causes of ALF worldwide and the mortality rate of ALF is ~20–40% [3, 4]. Characteristics of APAP-induced ALF include high levels of liver ALT, AST, and ALP enzymes after 24–72 h [30, 31]. Here, we show that NSS + APAP treatment raises the levels of ALT, AST, and ALP, while lowering the level of ALB, indicating successful preparation of the APAP-induced hepatotoxicity model. Treatment with NAC or RNP^N, on the other hand, was shown to suppress these APAP-induced increases. Treatment with LMW TEMPO and nRNP did not

have the same suppressive effect. In addition, our previous research finding found that RNP^N at dose of 300 mg/kg BW via oral administration for 1 month in mice did not show any toxicity in several organs including liver [20].

At therapeutic doses, about 90% of APAP is eliminated via sulfation or glucuronidation pathways [32, 33] and another 5% is metabolized by cytochrome P450 2E1 (CYP2E1) to NAPQI [34, 35]. The NAPQI subsequently binds to glutathione (GSH) to produce mercuric acid and cysteine conjugates before being eliminated from the body [11]. An overdose of APAP may result in the depletion of GSH and cause NAPQI-induced hepatic cell injury [11, 36–39]. Our GPx activity results showed that RNP^N treatment increased GPx activity compared to treatment with NSS, TEMPO, or nRNP, indicating the elevation of GSH level like NAC-treated group.

The production of ROS including hydrogen peroxide, hydroxyl radicals, and superoxide anions can be enhanced by NAPQI. Lipid peroxidation, DNA, and protein oxidation, as well as a decrease in radical-scavenging enzymes of GPx and superoxide dismutase (SOD) have also been reported in APAP-induced liver injury [40–42]. This mechanism has been proposed as a key player in the oxidative stress and hepatic injury in APAP-induced hepatotoxicity [39–43] and we therefore measured lipid peroxidation, and % inhibition of O₂^{••} in this study to confirm ROS production in our APAP-induced hepatotoxicity model. Interestingly, treatment with RNP^N was found to diminish lipid peroxidation compared to treatment with NSS, TEMPO, or nRNP, while % inhibition of O₂^{••} was also higher in the RNP^N-treated group compared with the NSS-, NAC-, TEMPO-, and nRNP-treated groups. Although LMW TEMPO, like RNP^N, possesses ROS scavenging activity, the scavenging efficiency of the LMW TEMPO is lower than that of the RNP^N, probably due to the rapid elimination, easy metabolism, and marked disturbance of normal redox reaction in normal cells. We have previously reported that only 5–7% of PEG-b-PMNT internalizes in blood stream by oral administration [20]. Prolonged circulation tendency of the internalized PEG-b-PMNT in blood stream might improve an access to liver tissue, which might improve therapeutic efficiency as compared with LMW TEMPO.

The standard treatment for APAP-induced liver toxicity is NAC, which acts as a GSH precursor to increase the GSH reservoir. Treatment with NAC, however, triggers an impaired coagulation cascade, which is the reason why prothrombin time was assessed in this study. Mice in the NAC + APAP-treated group were shown to have significantly prolonged prothrombin times compared to other groups ($p < 0.001$), while the mice in the RNP^N + APAP-treated group did not exhibit this side effect.

The findings from the hepatic function analyses in this study were confirmed by the histopathological changes observed by microscopic analysis. In the NSS + APAP,

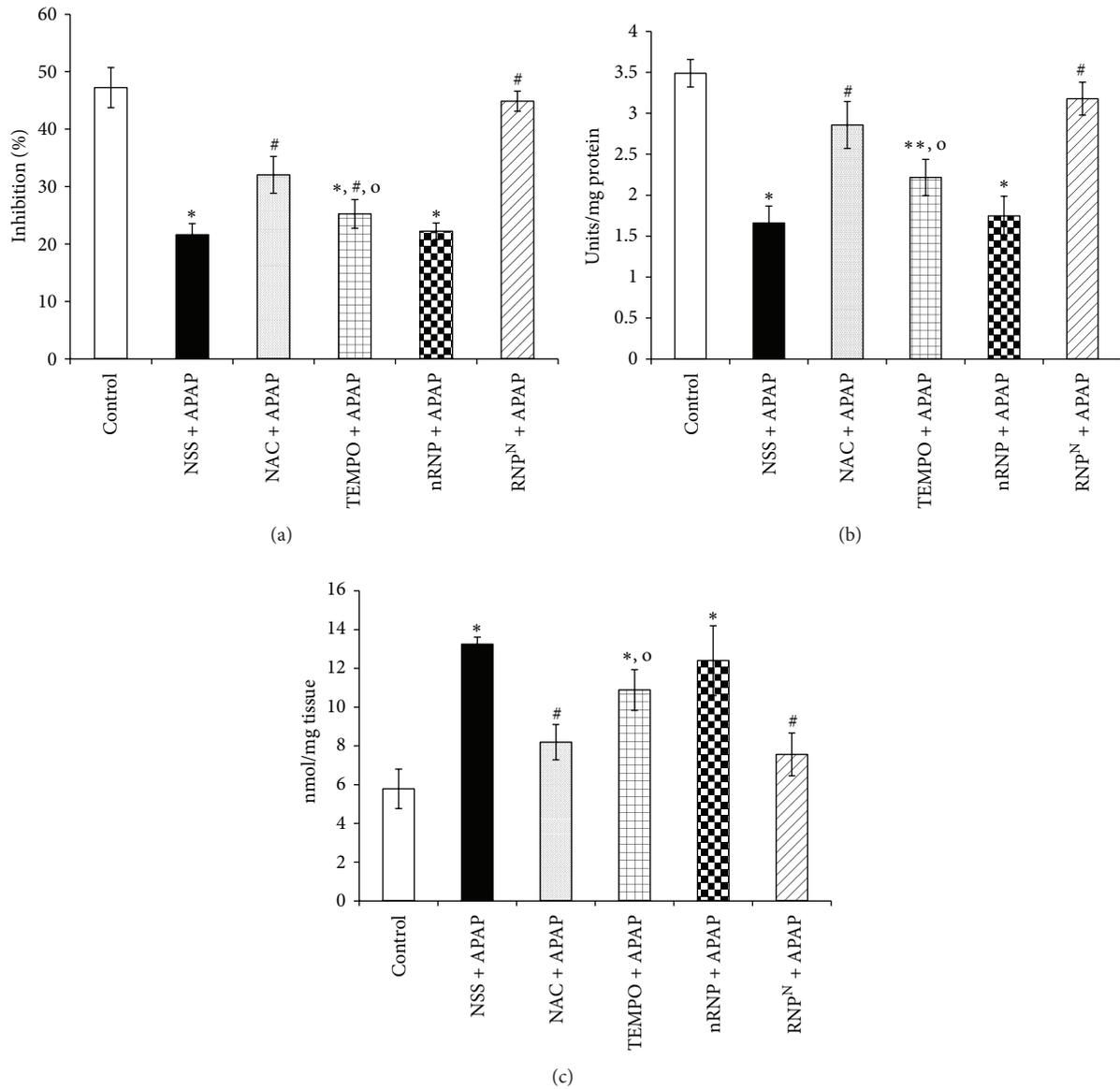


FIGURE 6: Effects of treatment for 7 days on (a) % inhibition of $O_2^{\bullet-}$, (b) GPx activity, and (c) MDA levels. Data represent mean \pm SEM, *versus control group, $p < 0.001$; **versus control group, $p < 0.01$; #versus NSS + APAP-treated group, $p < 0.01$; °versus RNP^N + APAP-treated group, $p < 0.05$; $n = 8$ mice/group.

TEMPO + APAP, and nRNP + APAP groups, inflammatory infiltration of lymphocytes, vacuolation, swelling, and centrilobular necrosis were observed, while pretreatment with RNP^N and NAC was found to prevent these histological changes. Mice in the NAC + APAP-treated group, however, exhibited mild changes in hepatocyte and sinusoid arrangement. Furthermore, the previous study showed that the RNP^N treatment ameliorates nonalcoholic steatohepatitis NASH fibrosis via the decrease of hepatic stellate cell activation marker of alpha-smooth muscle actin (α -SMA) [44].

We report here that RNP^N successfully ameliorated APAP-induced hepatotoxicity in mice as demonstrated by

decreased levels of the hepatic injury markers ALT, AST, and ALP and increased ALB levels. The observed protective effects of RNP^N may be due to antioxidant effects as shown by the reduced lipid peroxidation, increased GPx activity, and increased % inhibition of $O_2^{\bullet-}$. The histopathological analysis conducted in this study did not reveal any toxicity by RNP^N. Therefore, the patients who are long-term users of APAP need to suppress liver damage. So, combination of APAP with RNP^N is also very interesting strategy to suppress hepatotoxicity.

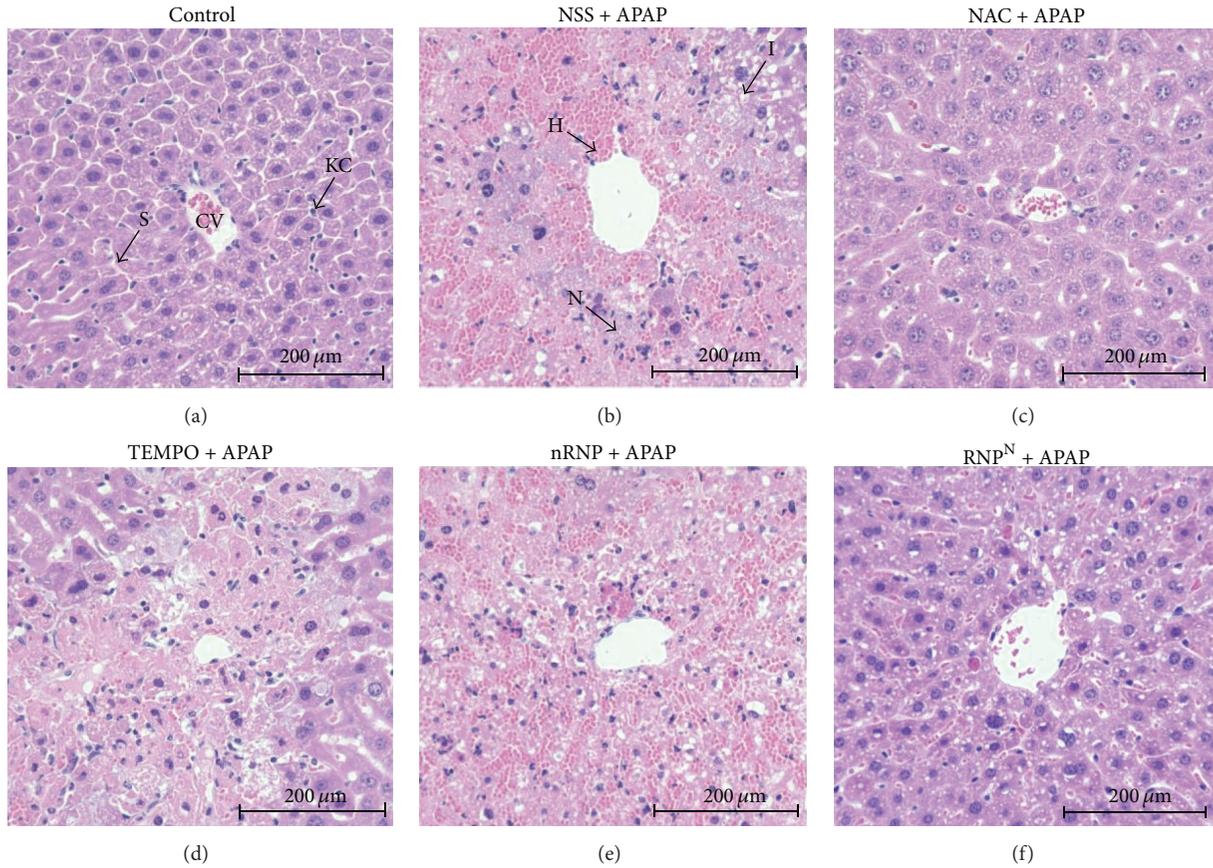


FIGURE 7: Hepatoprotective effect of treatments in mice with APAP-induced liver damage. (a) control group. (b) APAP-induced group. ((c)–(f)) APAP group treated with (c) 600 mg/kg BW of NAC, (d) 15.4 mM of LMW TEMPO, (e) 12.5 mg/mL of nRNP, (f) and 12.5 mg/mL of RNP^N. Liver sections were stained with hematoxylin and eosin (×400). CV: central vein; S: sinusoids; KC: Kupffer cell; N: necrosis; I: inflammation; H: hemorrhage.

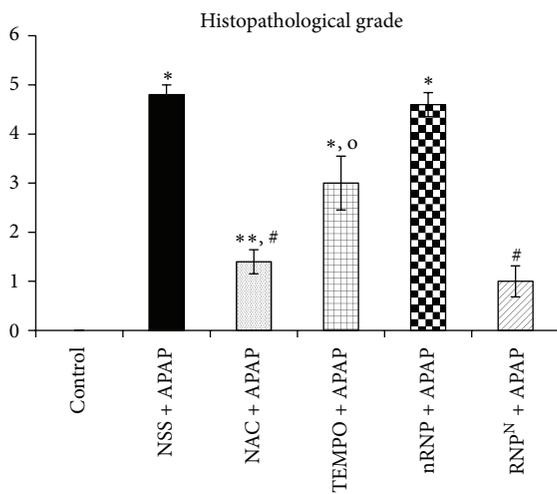


FIGURE 8: Histopathological changes in liver tissue due to APAP-induced hepatotoxicity. Data represent mean ± SEM, * versus control group, $p < 0.001$; ** versus control group, $p < 0.05$; # versus NSS + APAP-treated group, $p < 0.001$; ° versus RNP^N + APAP-treated group, $p < 0.001$; $n = 5$ samples/group.

5. Conclusion

The findings of this study indicate that RNP^N has a hepatoprotective effect against APAP-induced liver injury via antioxidant properties decreasing lipid peroxidation while increasing GPx activity and the % inhibition of O₂^{•-}. Treatment with RNP^N furthermore shows no side effect of coagulation cascade impairment, indicating that RNP^N may be a more effective treatment for APAP-induced hepatotoxicity than NAC. Our findings lead us to conclude that antioxidative nanomedicine is a promising strategy for improving therapeutic effects by suppressing disturbances of normal redox reactions in healthy cells.

Conflict of Interests

There is no conflict of interests to publish this paper.

Acknowledgments

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References

- [1] D. Gunnell, V. Murray, and K. Hawton, "Use of paracetamol (acetaminophen) for suicide and nonfatal poisoning: worldwide patterns of use and misuse," *Suicide and Life-Threatening Behavior*, vol. 30, no. 4, pp. 313–326, 2000.
- [2] T. L. Litovitz, W. Klein-Schwartz, G. C. Rodgers Jr. et al., "2001 Annual report of the American association of poison control centers toxic exposure surveillance system," *The American Journal of Emergency Medicine*, vol. 20, no. 5, pp. 391–452, 2002.
- [3] A. J. Makin, J. Wendon, and R. Williams, "A 7-year experience of severe acetaminophen-induced hepatotoxicity (1987–1993)," *Gastroenterology*, vol. 109, no. 6, pp. 1907–1916, 1995.
- [4] F. V. Schiødt, F. A. Rochling, D. L. Casey, and W. M. Lee, "Acetaminophen toxicity in an urban county hospital," *The New England Journal of Medicine*, vol. 337, no. 16, pp. 1112–1117, 1997.
- [5] G. Ostapowicz, R. J. Fontana, F. V. Schiødt et al., "Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States," *Annals of Internal Medicine*, vol. 137, no. 12, pp. 947–954, 2002.
- [6] W. M. Lee, "Acetaminophen and the U.S. acute liver failure study group: lowering the risks of hepatic failure," *Hepatology*, vol. 40, no. 1, pp. 6–9, 2004.
- [7] O. T. Ayonrinde, G. J. Phelps, J. C. Hurley, and O. A. Ayonrinde, "Paracetamol overdose and hepatotoxicity at a regional Australian hospital: a 4-year experience," *Internal Medicine Journal*, vol. 35, no. 11, pp. 655–660, 2005.
- [8] S. C. Cooper, R. C. Aldridge, T. Shah et al., "Outcomes of liver transplantation for paracetamol (acetaminophen)-induced hepatic failure," *Liver Transplantation*, vol. 15, no. 10, pp. 1351–1357, 2009.
- [9] A. M. Larson, "Acetaminophen hepatotoxicity," *Clinics in Liver Disease*, vol. 11, no. 3, pp. 525–548, 2007.
- [10] M. J. Hodgman and A. R. Garrard, "A review of acetaminophen poisoning," *Critical Care Clinics*, vol. 28, no. 4, pp. 499–516, 2012.
- [11] J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette, and B. B. Brodie, "Acetaminophen induced hepatic necrosis. IV. Protective role of glutathione," *Journal of Pharmacology and Experimental Therapeutics*, vol. 187, no. 1, pp. 211–217, 1973.
- [12] K. J. Heard, "Acetylcysteine for acetaminophen poisoning," *The New England Journal of Medicine*, vol. 359, no. 3, pp. 228–292, 2008.
- [13] T. T. Niemi, E. Munsterhjelm, R. Pöyhä, M. S. Hynninen, and M. T. Salmenperä, "The effect of N-acetylcysteine on blood coagulation and platelet function in patients undergoing open repair of abdominal aortic aneurysm," *Blood Coagulation & Fibrinolysis*, vol. 17, no. 1, pp. 29–34, 2006.
- [14] A. F. Pizon, D. H. Jang, and H. E. Wang, "The *in vitro* effect of N-acetylcysteine on prothrombin time in plasma samples from healthy subjects," *Academic Emergency Medicine*, vol. 18, no. 4, pp. 351–354, 2011.
- [15] F.-L. Yen, T.-H. Wu, L.-T. Lin, T.-M. Cham, and C.-C. Lin, "Nanoparticles formulation of *Cuscuta chinensis* prevents acetaminophen-induced hepatotoxicity in rats," *Food and Chemical Toxicology*, vol. 46, no. 5, pp. 1771–1777, 2008.
- [16] O. A. A. Ahmed, S. M. Badr-Eldin, M. K. Tawfik, T. A. Ahmed, K. M. El-Say, and J. M. Badr, "Design and optimization of self-nanoemulsifying delivery system to enhance quercetin hepatoprotective activity in paracetamol-induced hepatotoxicity," *Journal of Pharmaceutical Sciences*, vol. 103, no. 2, pp. 602–612, 2014.
- [17] K. Ganesh, D. Archana, and K. Preeti, "Galactosylated Albumin nanoparticles bearing Cimetidine for effective management of Acetaminophen induced hepatotoxicity," *International Journal of Nano Dimension*, vol. 5, no. 5, pp. 431–440, 2014.
- [18] Y. Umezaki, D. Iohara, M. Anraku et al., "Preparation of hydrophilic C₆₀(OH)₁₀/2-hydroxypropyl- β -cyclodextrin nanoparticles for the treatment of a liver injury induced by an overdose of acetaminophen," *Biomaterials*, vol. 45, pp. 115–123, 2015.
- [19] T. Yoshitomi, R. Suzuki, T. Mamiya, H. Matsui, A. Hirayama, and Y. Nagasaki, "pH-sensitive radical-containing-nanoparticle (RNP) for the L-band-EPR imaging of low pH circumstances," *Bioconjugate Chemistry*, vol. 20, no. 9, pp. 1792–1798, 2009.
- [20] P. Chonpathompikunlert, T. Yoshitomi, L. B. Vong et al., "Recovery of cognitive dysfunction via orally administered redox-polymer nanotherapeutics in SAMP8 mice," *PLOS ONE*, vol. 10, no. 5, Article ID e0126013, 2015.
- [21] T. Yoshitomi and Y. Nagasaki, "Reactive oxygen species-scavenging nanomedicines for the treatment of oxidative stress injuries," *Advanced Healthcare Materials*, vol. 3, no. 8, pp. 1149–1161, 2014.
- [22] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.
- [23] S. Hussain, W. Slikker Jr., and S. F. Ali, "Age-related changes in antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione in different regions of mouse brain," *International Journal of Developmental Neuroscience*, vol. 13, no. 8, pp. 811–817, 1995.
- [24] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of Biological Chemistry*, vol. 193, no. 1, pp. 265–275, 1951.
- [25] H. Ukeda, S. Maeda, T. Ishii, and M. Sawamura, "Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase," *Analytical Biochemistry*, vol. 251, no. 2, pp. 206–209, 1997.
- [26] M. Wood, M. L. Berman, R. D. Harbison, P. Hoyle, J. M. Phythyon, and A. J. Wood, "Halothane-induced hepatic necrosis in triiodothyronine-pretreated rats," *Anesthesiology*, vol. 52, no. 6, pp. 470–476, 1980.
- [27] E. Merisko-Liversidge, G. G. Liversidge, and E. R. Cooper, "Nanosizing: a formulation approach for poorly-water-soluble compounds," *European Journal of Pharmaceutical Sciences*, vol. 18, no. 2, pp. 113–120, 2003.

- [28] L. Zhang, F. X. Gu, J. M. Chan, A. Z. Wang, R. S. Langer, and O. C. Farokhzad, "Nanoparticles in medicine: therapeutic applications and developments," *Clinical Pharmacology & Therapeutics*, vol. 83, no. 5, pp. 761–769, 2008.
- [29] M. Ferrari, "Frontiers in cancer nanomedicine: directing mass transport through biological barriers," *Trends in Biotechnology*, vol. 28, no. 4, pp. 181–188, 2010.
- [30] D. G. Davidson and W. N. Eastham, "Acute liver necrosis following overdose of paracetamol," *The British Medical Journal*, vol. 5512, pp. 497–499, 1966.
- [31] D. C. Dahlin, G. T. Miwa, A. Y. Lu, and S. D. Nelson, "N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 5, pp. 1327–1331, 1984.
- [32] D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter, M. Hashimoto, and J. R. Mitchell, "Acetaminophen induced hepatic necrosis. VI. Metabolic disposition of toxic and nontoxic doses of acetaminophen," *Pharmacology*, vol. 12, no. 4-5, pp. 251–271, 1974.
- [33] J. T. Slattery and G. Levy, "Acetaminophen kinetics in acutely poisoned patients," *Clinical Pharmacology & Therapeutics*, vol. 25, no. 2, pp. 184–195, 1979.
- [34] S. D. Nelson, "Molecular mechanisms of the hepatotoxicity caused by acetaminophen," *Seminars in Liver Disease*, vol. 10, no. 4, pp. 267–278, 1990.
- [35] J. D. Gibson, N. R. Pumford, V. M. Samokyszyn, and J. A. Hinson, "Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress," *Chemical Research in Toxicology*, vol. 9, no. 3, pp. 580–585, 1996.
- [36] W. Z. Potter, S. S. Thorgeirsson, D. J. Jollow, and J. R. Mitchell, "Acetaminophen-induced hepatic necrosis. V. Correlation of hepatic necrosis, covalent binding and glutathione depletion in hamsters," *Pharmacology*, vol. 12, no. 3, pp. 129–143, 1974.
- [37] D. J. Miner and P. T. Kissinger, "Evidence for the involvement of N-acetyl-p-quinoneimine in acetaminophen metabolism," *Biochemical Pharmacology*, vol. 28, no. 22, pp. 3285–3290, 1979.
- [38] J. G. M. Bessems and N. P. E. Vermeulen, "Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches," *Critical Reviews in Toxicology*, vol. 31, no. 1, pp. 55–138, 2001.
- [39] L. P. James, P. R. Mayeux, and J. A. Hinson, "Acetaminophen-induced hepatotoxicity," *Drug Metabolism and Disposition*, vol. 31, no. 12, pp. 1499–1506, 2003.
- [40] S. L. Michael, N. R. Pumford, P. R. Mayeux, M. R. Niesman, and J. A. Hinson, "Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species," *Hepatology*, vol. 30, no. 1, pp. 186–195, 1999.
- [41] J. A. Hinson, T. J. Bucci, L. K. Irwin, S. L. Michael, and P. R. Mayeux, "Effect of inhibitors of nitric oxide synthase on acetaminophen-induced hepatotoxicity in mice," *Nitric Oxide*, vol. 6, no. 2, pp. 160–167, 2002.
- [42] J. A. Hinson, A. B. Reid, S. S. McCullough, and L. P. James, "Acetaminophen-induced hepatotoxicity: role of metabolic activation, reactive oxygen/nitrogen species, and mitochondrial permeability transition," *Drug Metabolism Reviews*, vol. 36, no. 3-4, pp. 805–822, 2004.
- [43] H. Jaeschke, T. R. Knight, and M. L. Bajt, "The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity," *Toxicology Letters*, vol. 144, no. 3, pp. 279–288, 2003.
- [44] A. Eguchi, T. Yoshitomi, M. Lazic et al., "Redox nanoparticles as a novel treatment approach for inflammation and fibrosis associated with nonalcoholic steatohepatitis," *Nanomedicine*, vol. 10, no. 17, pp. 2697–2708, 2015.

Review Article

The Dual Function of Reactive Oxygen/Nitrogen Species in Bioenergetics and Cell Death: The Role of ATP Synthase

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Reactive oxygen species (ROS) and reactive nitrogen species (RNS) targeting mitochondria are major causative factors in disease pathogenesis. The mitochondrial permeability transition pore (PTP) is a mega-channel modulated by calcium and ROS/RNS modifications and it has been described to play a crucial role in many pathophysiological events since prolonged channel opening causes cell death. The recent identification that dimers of ATP synthase form the PTP and the fact that posttranslational modifications caused by ROS/RNS also affect cellular bioenergetics through the modulation of ATP synthase catalysis reveal a dual function of these modifications in the cells. Here, we describe mitochondria as a major site of production and as a target of ROS/RNS and discuss the pathophysiological conditions in which oxidative and nitrosative modifications modulate the catalytic and pore-forming activities of ATP synthase.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important physiological functions but can also cause extensive cellular damage, in a balance that is determined by their relative rates of formation and removal. Usually, these species are removed rapidly before they cause cell dysfunction and death. Oxidative/nitrosative stress generated by an imbalance between formation of ROS/RNS and antioxidant defense capacity can affect major cellular components, including lipids, proteins, carbohydrates, and DNA. Mitochondria are recognized as a critical site in the cell for the formation of ROS/RNS and as their target.

Mitochondrial processes are highly compartmentalized because of the existence of two limiting membranes allowing the selective localization of proteins, nucleotides, and coenzymes in the intermembrane and matrix spaces. The outer mitochondrial membrane (OMM) is the interface between mitochondria and the cell components and its permeabilization is essential to allow the release of mitochondrial proteins involved in apoptosis such as cytochrome c [1]. The inner mitochondrial membrane (IMM), whose permeability to solutes is controlled by highly specific transporters and tightly regulated channels, is the site of coupling between

substrate oxidation and ATP synthesis in the process of oxidative phosphorylation. Mitochondria operate a sequence of energy conversion processes through which the exergonic flow of electrons along the respiratory complexes supports the endergonic pumping of protons from the matrix to the intermembrane space. The resulting proton motive force drives the rotation of the F_0 sector of ATP synthase leading to the synthesis of ATP in the F_1 sector, but the electron flow through the respiratory chain also generates ROS/RNS. In addition, the mitochondrial permeability transition pore (PTP), a large-conductance channel, is also located at the level of the IMM and prolonged opening of this channel leads to mitochondrial swelling, rupture of the OMM, and cell death [2]. PTP opening is dependent on the presence of matrix calcium, but the threshold calcium load which is required is modulated by inducers of the pore such as oxidants [3]. Although the existence of the PTP was established as early as the 1970s [4–6], its molecular nature has been the subject of controversy as many potential components were ruled out by the use of targeted gene deletion in mice [2]. The only candidate remaining is cyclophilin D (CyPD), which was found to act not as a structural component of the pore but as a modulator whose binding to the PTP decreases the threshold calcium concentration necessary to induce

permeability transition [7–10]. CyPD was shown to interact with the lateral stalk of the ATP synthase in mammals [11], a finding which was the basis for the characterization of the molecular structure of the PTP as formed by ATP synthase itself [12–16]. Genetic ablation of the *Ppif* gene (which encodes for CyPD) in the mouse or its displacement from the PTP by the treatment with cyclosporin A (CsA), a known inhibitor of the PTP, has been also used to demonstrate the important role of PTP in the pathophysiological mechanism of several diseases such as neurodegenerative diseases, muscular dystrophies, ischemia/reperfusion (I/R), and diabetes [2, 17, 18]. Besides the PTP, mitochondrial function and bioenergetics (including the modulation of the catalytic activity of ATP synthase) are also affected in most of these pathophysiological conditions and ROS/RNS are presumably involved as causative factors.

While numerous mechanisms of oxidant-induced injury have been identified, the impact of oxidants on the mitochondrial proteome has been investigated only recently. Oxidative or nitrosative stress may not only alter levels of mitochondrial proteins, but also induce posttranslational modifications of proteins. These modifications involve reversible changes at the level of cysteine, tyrosine, methionine, histidine, and tryptophan residues and irreversible protein carbonylation [19]. Thiol groups can be S-nitrosylated by nitric oxide (NO) or reversibly oxidized by ROS to form disulfide bonds or sulfenic acid; the latter can be further oxidized to sulfinic and sulfonic acids [20]. Sulfenic acid can also interact with glutathione to become glutathionylated. Tyrosine residues instead are target for peroxynitrite (ONOO⁻) which leads to irreversible formation of 3-nitrotyrosine. All these modifications lead to changes in protein structure and/or activity, thereby affecting their roles in cell function. In this review we discuss the role of mitochondria as a source and target of ROS/RNS during the switch between cell death and survival. We will focus on the effects of these reactive species on critical ATP synthase amino acid residues as part of the mechanism affecting ATP synthesis and/or permeability transition.

2. Mitochondria Are Sources of ROS/RNS

A number of sites responsible for ROS/RNS formation within the cell have been identified and shown to play a role in different pathologies. NADPH oxidases, xanthine oxidase, uncoupled nitric oxide synthase (NOS), and mitochondria are all relevant sources of ROS that in certain pathological conditions contribute to oxidative damage of cells and tissues. Indeed, these processes have been identified as disease-relevant enzymatic sources of ROS and their inhibition has been shown to afford protection in a number of diseases both in experimental models and in patients [21, 22].

2.1. Mitochondrial Sources of ROS. It is well accepted that mitochondria are a major source of ROS in the cell. These organelles contain several enzymes that catalyze ROS formation either as the obligatory product or as the result of an occasional, possibly undesired, reaction [23]. The best example of “accidental” ROS formation is represented by the mitochondrial respiratory chain. A small fraction of the

electrons (about 0.1%) flowing through the respiratory chain is diverted causing the partial reduction of O₂ into superoxide [24]. This process occurs at the level of the first three complexes where flavins or quinones are able to act as single electron donors. The electron detour at these sites is favored when flow is hampered downstream as a result of either protein alterations in respiratory complexes or their inhibition. Other mitochondrial enzymes, such as flavin containing glycerol-3-phosphate-, proline-, and dihydroorotate-dehydrogenase, and the electron transferring flavoprotein/ETF:Q oxidoreductase system of fatty acid β -oxidation have also been described as potential ROS producers [25]. Nevertheless, all these enzymes and respiratory complexes normally catalyze reactions required for energy metabolism, cell function, and viability maintenance, making it difficult to envision their inhibition as a potential therapeutic tool.

Mitochondria also contain other enzymes that may generate hydrogen peroxide (H₂O₂) as a direct and obligatory product. One such example is p66^{Shc}, cytosolic adaptor protein that upon stress translocates to mitochondria where it catalyzes electron transfer from cytochrome c to oxygen, a process that can result in the formation of ROS [37]. Genetic deletion of p66^{Shc} protects against I/R injury in mice [38, 39], obesity [40], and diabetic complications such as cardiomyopathy [41], nephropathy [42], delayed wound healing [43], pancreatic cell death [44], and endothelial dysfunction [45]. On the other hand, the observation that mice lacking p66^{Shc} actually live shorter when exposed to natural conditions (low temperatures and food competition) [46] may suggest that p66^{Shc} dependent ROS also serve a physiological role. NADPH oxidase 4 (Nox4), an enzyme belonging to the NADPH oxidase family, is another ROS generating enzyme that has been reported to localize in the plasma membrane and apparently also in the mitochondria, focal adhesions, nucleus, and endoplasmic reticulum [47]. This enzyme associates with p22^{phox} for its activation; it is constitutively active, and, unlike other members of the Nox family, generates H₂O₂ rather than superoxide [47, 48]. However, the relevance of Nox4 for mitochondrial ROS generation remains controversial. A recent study has shown that Nox4 can (in principle) interact with complex I subunits, but under physiological conditions Nox4 protein or ROS formation could not be detected in kidney or heart mitochondria [49]. Other studies have shown that cardiac-specific targeting of Nox4 can be both protective and harmful in different models of cardiac pressure overload [50, 51]. Moreover, while certain studies reported Nox4 to be deleterious, contributing to mitochondrial dysfunction and several pathologies such as ischemic stroke [52], diabetic cardiomyopathy [53], vascular inflammation, and remodeling [54], others concluded that Nox4 might be vascular-protective rather than vascular-damaging [55]. These apparently contradictory findings reflect the need for further investigation in order to address the pathophysiological role and regulation of Nox4. Another major source of H₂O₂ in the mitochondria is monoamine oxidase (MAO). Activation of this enzyme, localized at the level of the OMM, leads to H₂O₂ formation and has been shown to contribute to neuronal disorders, such as Parkinson's (PD) or Alzheimer's

disease (AD), most likely due to formation of ROS responsible for oxidative damage to neurons [56]. In recent years, the contribution of MAO to oxidative stress that ultimately leads to mitochondrial dysfunction and cell damage has been demonstrated also in the cardiovascular field where MAO inhibition has been shown to be protective in I/R injury, pressure overload, vascular damage, and diabetes [23, 57–62].

2.2. Mitochondrial Sources of RNS. In addition to ROS, cells are also capable of generating RNS. NOS exist in three isoforms (endothelial (eNOS), neuronal (nNOS), and inducible (iNOS)) which catalyze the conversion of L-arginine into citrulline and NO. This reaction also requires flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin (BH4), heme, and calmodulin. These cofactors are important (e.g., when BH4 levels are limited) because NOS may become uncoupled and generate superoxide instead of NO [63, 64]. Another possibility is that, in conditions of high oxidative stress, NO and superoxide interact to generate ONOO⁻, a very reactive species capable of nitrating tyrosine residues, thus amplifying oxidative damage.

It is generally accepted that NO has protective effects on mitochondria although it remains unclear whether mitochondria actually possess a mitochondrial NOS [65–69]. Nevertheless, in addition to being generated by NOS, NO can be formed from dietary inorganic nitrite (NO₂⁻) and nitrate (NO₃⁻) [70]. Several in vitro studies have demonstrated that mitochondria are able to metabolize NO₂⁻ into NO, since cytochrome c and respiratory chain complexes III and IV possess nitrite reductase activities that can be stimulated under hypoxic or acidic conditions [71–73]. Either way, the bioactivity of newly synthesized NO is rapidly terminated by its oxidation into NO₂⁻ and NO₃⁻, thus completing the NO cycle. The protective effects exerted by NO on mitochondria are due to S-nitrosylation of critical cysteine residues. One such example is represented by the mechanism through which MitoSNO, a mitochondria selective S-nitrosylating agent, affords protection in cardiac I/R injury in vivo [74]. Administration of MitoSNO within the first minutes of reperfusion temporarily inhibits complex I and thus blocks the reverse electron flow that leads to generation of ROS, oxidative damage, and tissue necrosis [74, 75]. The inhibition of complex I is due to S-nitrosylation of a critical cysteine residue (Cys39) within the ND3 subunit, underlining the importance of (i) the reversibility of cysteine modification and (ii) mitochondria as both source and target of ROS/RNS. This latter notion is further supported by the observations that mice lacking Mn superoxide dismutase (SOD), enzyme responsible for conversion of superoxide into H₂O₂, develop ROS toxicity and dilated cardiomyopathy [76] and that targeting catalase expression in mitochondria affords beneficial effects in a number of pathologies [77–80].

3. The Role of ATP Synthase in Mitochondrial ROS Formation

Recent evidence suggests that ATP synthase activity can modulate ROS formation in mitochondria. Although not a direct source of ROS, ATP synthase is able to regulate

energy metabolism and modulate pathways leading to ROS formation, cell death, and survival. A recent study by Ni et al. [81] very elegantly showed that α subunit is a target for calpain-1 in diabetic hearts, leading to its proteolytic degradation, reduction in ATP synthase activity, increase in mitochondrial superoxide formation, and diabetic cardiomyopathy in mice. Indeed, genetic inhibition of calpain-1 or upregulation of α subunit increased ATP synthase activity and reduced mitochondrial ROS generation and all the downstream changes occurring in diabetic hearts. It is likely that, besides insufficient ATP production that can directly contribute to myocardial dysfunction, disruption in ATP synthase activity leads to the accumulation of the electrons in the upstream complexes of the respiratory chain, promoting superoxide generation through complexes I and III [81].

Whereas inhibition of ATP synthase in normal cells leads to mitochondrial oxidative stress and cell death [81–83], most cancer cells are highly glycolytic and have adopted several molecular strategies to reduce oxidative phosphorylation including ATP synthase activity inhibition [84, 85], thus modulating the level of ROS in mitochondria. This does not only occur through downregulation of ATP synthase, but also through overexpression of its inhibitor protein (IF₁) [86]. IF₁ is the endogenous inhibitor of ATP synthase that reversibly binds to the enzyme. Its binding is promoted by low matrix pH and membrane potential, thereby limiting ATP hydrolysis and energy dissipation [87, 88]. In fact, its role has been extensively studied in myocardial ischemia and ischemic preconditioning (IPC), since IF₁ seems to mediate ATP synthase inhibition and thus sparing of ATP during myocardial preconditioning [89–92]. Moreover, IF₁ seems to regulate the oligomeric state of ATP synthase, increasing the density of cristae and formation of ATP synthase dimers [93–96]. Mitochondria from different types of carcinoma show a remarkable increase in IF₁ expression [86] that has been linked to the inhibition of ATP synthase, glycolytic switch in energy metabolism, and production of ROS [97–99]. A critical role of the phosphorylation status of IF₁ has been recently suggested in the modulation of ATP synthesis and respiration in tumors and heart [100]. ROS generated when ATP synthesis is inhibited are used as nuclear signals to initiate transcription of genes necessary to support tumor development [86, 97]. Indeed, IF₁ triggers ROS-induced activation of transcription factors (such as NF κ B and HIF1 α) in cancer cells causing enhanced proliferation, invasion, and survival [97]. These findings highlight how cancer cells, as opposed to normal cells, rely on a subtle mechanism of redox equilibrium: on the one hand, ROS enhance proliferation and favor genomic instability by damaging DNA, while on the other hand, excess ROS is harmful as it could promote PTP opening and cell death [101]. Moreover, it is tempting to speculate that PTP might be differently modulated by IF₁ and CyPD in cancer cells, as observed for CyPD in osteosarcoma and prostatic cell models [102]. This suggests that this different PTP modulation might render cancer cells more resistant to cell death. Nevertheless, the ROS signaling pathways triggered by IF₁ overexpression in many other cancer types still remain to be elucidated.

4. Mitochondrial ROS/RNS Induce Posttranslational Modification of ATP Synthase Residues

ROS/RNS induced posttranslational modifications are known to modulate the catalytic activity of ATP synthase in several pathophysiological conditions. Matrix calcium levels and oxidative/nitrosative stress play a crucial role also in the modulation of PTP. Recent finding that ATP synthase dimers form the PTP [12] makes the identification of the sites involved in the two different functions of ATP synthase more feasible (Figure 1). The most frequently modified sites of ATP synthase possibly involved in mitochondrial pathophysiology are listed in Table 1.

4.1. Matrix Residues. Critical thiols facing the matrix are involved in the modulation of ATP synthase catalysis. H_2O_2 inactivates F_1 -ATPase activity from bovine heart through formation of iron-protein adducts [103]. ROS- and RNS-mediated modifications responsible for ATPase inactivation are mostly localized at the level of cysteine residues in the α and γ subunits [104]. A special feature of higher plant CF_1 -ATPase is a regulatory domain in the γ subunit consisting of three methionines and a cysteine that were identified by mass spectrometry to be oxidized by ROS. Reduction of the disulfide bonds of γ subunit elevates ATP hydrolysis and synthesis [105].

Although in mammalian cells the chloroplast redox-sensitive γ subunit region is not present, mitochondrial ATP synthase F_1 domain might be a major site for oxidative/nitrosative posttranslational modifications. Garcia et al. [27] have reported that α subunit is S-glutathionylated under oxidative stress in rat brain or liver mitochondria leading to decreased ATPase activity. The same inhibitory effect due to α subunit S-glutathionylation was observed in transgenic mouse hearts overexpressing iNOS [28]. Sun et al. [29], on the other hand, reported inhibition of ATPase activity which is protective in preconditioned mouse hearts during myocardial ischemia due to α subunit S-nitrosylation and an additive cardioprotective effect of this posttranslational modification which causes desensitization of the PTP [30]. An age-associated decline of ATPase activity due to carbonylation of α subunit was also observed in mouse skeletal muscle [31]. An indirect effect of ROS on α subunit was also found following H_2O_2 treatment of mitochondria that caused tyrosine-phosphorylation of α (but not β) chain [32]. This is likely due to the inactivation of phosphatases mediated by H_2O_2 [106].

Two disulfide bonds were identified between cysteines of ATP synthase α and γ subunits and between those of α subunits in heart failure [26] and both S-glutathionylation and S-nitrosylation of α -cysteine 294 (corresponding to bovine Cys251, Figures 1 and 2) can prevent the formation of these disulfide bridges. Other selective targets of ROS in the matrix are tryptophan residues of d subunit of ATP synthase in mammals [36] or tryptophan 503 of α subunit in *Podospira anserina* [107]. In bovine heart mitochondria treated with $ONOO^-$, 3-nitrotyrosine modified residues were identified at the level of β and d subunits of ATP synthase [108, 109]. Although it is not yet known which of the tyrosine residues

present in this subunit were modified, one might speculate that Tyr345 and Tyr368 in the β subunit are involved, since they have been suggested to be a major target for nitrosative stress in rat liver under in vivo conditions [35] (Figure 3). Carbonylation at the level of β subunit was observed in *Escherichia coli* after treatment with H_2O_2 [110]. Whether sites targeted by ROS/RNS that modulate ATP synthase are also involved in PTP formation/modulation has not been clarified yet.

The first indication of the presence of ROS-sensitive modulatory thiols on the matrix-facing side of the PTP was revealed in isolated mitochondria through the use of chemical thiol-oxidants, such as diamide, arsenite anion (AsO), copper-*o*-phenanthroline, phenylarsine oxide (PhAsO), and β -hydroxybutyrate, or thiol-modifiers, such as *N*-ethylmaleimide (NEM) or monobromobimane (MBM) [111–114]. In these studies the authors proposed that the PTP can be modulated by oxidation-reduction effectors at two sites that can be distinguished experimentally. One site (which was called the “P site”) appears to be modulated through the oxidation-reduction state of pyridine nucleotides even when glutathione is fully reduced and can be blocked by NEM, but not by MBM. The other site (which was called the “S site”) coincides with the oxidation/reduction-sensitive dithiol(s) [111], and it can be activated by reaction with AsO or PhAsO. The S site can be blocked by both NEM and MBM. Irrespective of the precise mechanism by which glutathione and pyridine nucleotides affect the PTP, oxidative stress causes an increased probability of pore opening when the concentrations of reduced glutathione and pyridine nucleotides decrease.

Some of the above-mentioned oxidants and thiol-modifiers of the PTP sites are also known to be reagents effective on ATP synthase [115] and since the PTP is formed by ATP synthase dimers, an accurate analysis of the amino acid residues of ATP synthase might clarify the molecular localization for the S and P sensitive sites of the PTP. Moreover, the characterization of these residues could be useful to discriminate between the two effects of ROS/RNS on ATP synthase: the modulation of its catalytic activity and the induction of the PTP.

Upon treatment of beef heart mitochondria with radioactive NEM and dithiobis(nitrobenzoate), these radiolabeled compounds were incorporated into the oligomycin-sensitivity conferring protein (OSCP) subunit, which faces the matrix side, through its only cysteine (OSCP-Cys118 in Figure 1) without any detectable effect on the ATPase activity [34, 116]. Intriguingly, the OSCP subunit has been identified as the binding site for CyPD to ATP synthase [12], an interaction favored by phosphate [11]. In mouse liver mitochondria high phosphate (5 mM) protects PTP from the oxidative stress, something that is not observed in CyPD knockout (KO) mitochondria [8], suggesting that the binding of CyPD on OSCP, which is favored by phosphate, might affect PTP thiols availability. Taken together, these results suggest that the OSCP subunit in mammals might play a crucial modulatory role for PTP opening and ROS sensitivity. This hypothesis might explain the previous and never fully understood finding that overexpression of CyPD

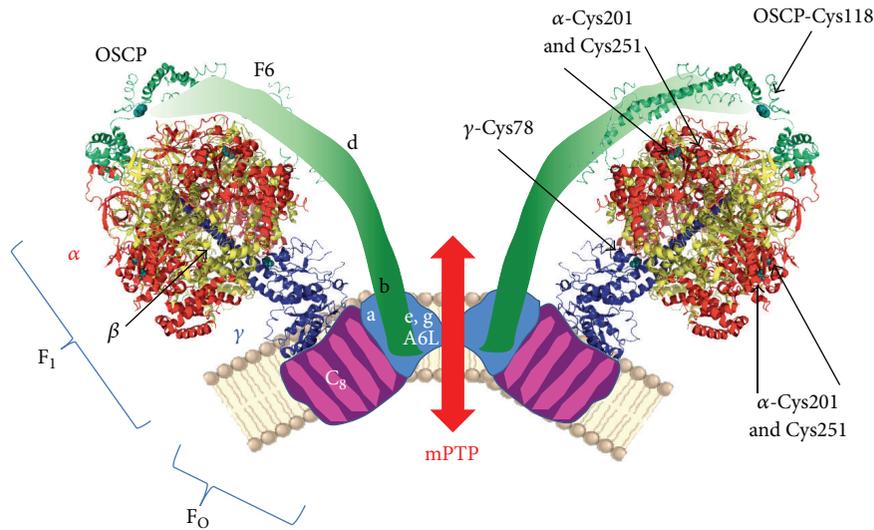


FIGURE 1: Schematic representation of ATP synthase and PTP. Dimers of ATP synthase that form the PTP are shown from a lateral view. F₁ catalytic part of ATP synthase is from bovine crystal structure (PDB 2WSS, modified by PyMOL 1.3 software) and is composed of α , β , and γ subunits in red, yellow, and blue, respectively, as indicated in the left part of the dimer. F₀ and lateral stalk subunits are also indicated in the left part in pink, light-blue, and green regions. On the right part of the dimer arrows indicate the critical cysteine residues modified by ROS/RNS in pathophysiological conditions. Cysteines are highlighted in cyan.

TABLE 1: Residues of ATP synthase modified by ROS/RNS in pathophysiology.

Residue	Condition	Modification	Reference(s)
α -Cys201	DHF	Oxidation, S-S bonds	[26]
	DHF	Oxidation, S-S bonds	[26]
	Rat brain and liver	S-glutathionylation	[27]
α -Cys251	Mouse heart overexpressing iNOS	S-glutathionylation	[28]
	Preconditioning and I/R	S-nitrosylation,	[29, 30]
	Aging-mouse skeletal muscles	Carbonylation,	[31]
	Rat brain mitochondria	Tyr-phosphorylation	[32]
α subunit	PD, <i>HtrA2</i> KO mouse	Truncation	[33]
γ -Cys78	DHF	Oxidation, S-S bonds	[26]
OSCP-Cys118	Bovine heart mitochondria	NEM incorporation	[34]
β -Tyr345	Rat liver	S-nitrosylation	[35]
β -Tyr368	Rat liver	S-nitrosylation	[35]
d-Trp	Human heart mitochondria	Oxidation	[36]

Residues refer to bovine ATP synthase subunits without mitochondrial targeting sequence.

DHF: dyssynchronous heart failure; iNOS: inducible nitric oxide synthase; I/R: ischemia/reperfusion; PD: Parkinson's disease; KO: knockout; OSCP: oligomycin-sensitivity conferring protein; and NEM: N-ethylmaleimide.

in HEK293 cells protects from oxidative stress and apoptosis [117].

4.2. Intermembrane Space/IMM Residues. Many possible sites of oxidation are present in the F₀ sector and in the noncatalytic subunits of ATP synthase in the IMM and facing the intermembrane space (Figure 1). These conserved cysteines are involved in dimer/oligomer formation.

Preferential interactions in yeast dimers occur through subunits e [118, 119], g [120], 6 [121], and 4 [122] and also through subunit h and the yeast-specific subunit i [123], most of which harbor cysteine residues. Among the mammalian corresponding subunits e, g, a, b, and F6 (Figure 1) only

b presents a conserved cysteine residue. However, in both yeast [121, 124] and mammals [95, 125, 126], the stabilizing contribution of the different subunits seems to be additive. In yeast mutants lacking e and/or g subunits, the PTP was desensitized to opening [14]. These data support the hypothesis that dimerization of ATP synthase is necessary for PTP formation. Moreover, the effect of ROS is conserved from human to yeast and *Drosophila* [127], since dimers extracted from blue native gels and tested for their channel activity generated currents in the presence of oxidants in all these species [12, 14, 15]. In yeast, both e and g subunits harbor a cysteine residue (eCys28 and gCys75) and form e/g interactions in the dimerization interface through GXXXG motifs [117].

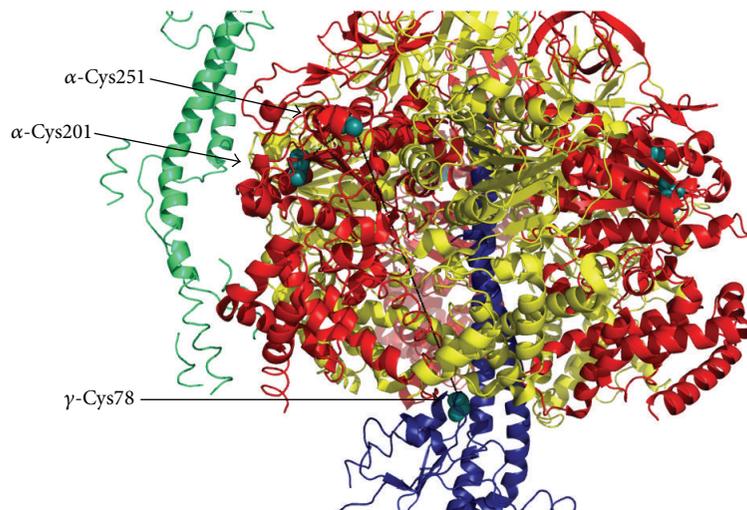


FIGURE 2: Lateral view of a section of the catalytic core of ATP synthase (PDB 2WSS, modified by PyMOL 1.3 software) composed of α , β , and γ subunits in red, yellow, and blue, respectively. Critical cysteine residues subjected to posttranslational modifications are highlighted in cyan. Distances between α -Cys251 and α -Cys201 or α -Cys251 and γ -Cys78 are indicated by black dashed lines and are 12 Å or 61.5 Å, respectively.

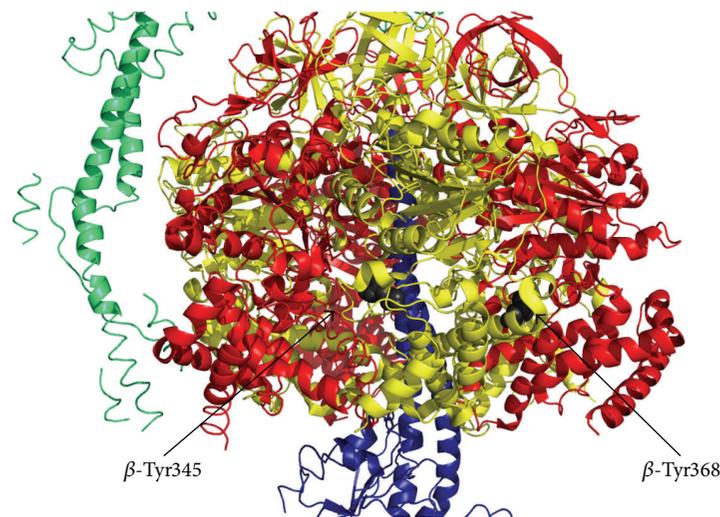


FIGURE 3: Lateral view of a section of the catalytic core of ATP synthase (PDB 2WSS, modified by PyMOL 1.3 software) composed of α , β , and γ subunits in red, yellow, and blue, respectively. Critical β -Tyr345 and β -Tyr368 residues that might be modified by RNS are highlighted in gray.

They also participate in the putative oligomerization interface through *e/e* and *g/g* interactions [124], as established in a yeast *gCys75Ser/Leu109Cys* mutant, which formed *eCys28-eCys28* and *gLeu109Cys-gLeu109Cys* cross-links [118, 120]. A similar arrangement of *e* and *g* subunits in the IMM of bovine heart has been hypothesized based on cross-linking experiments [128]. The treatment of yeast mitochondria lacking *e* and/or *g* subunits with copper(II) chloride (CuCl_2), which mimics the effect of oxidants, stabilized preexisting dimers by formation of disulfide bridges between adjacent monomers [14]. This suggests the involvement of species-specific cysteine residues located in subunits other than *e* and *g* that might stabilize dimers, as it was observed in subunit 6 in yeast [124] or factor B [129] and subunit *b* in bovine heart, whose modification also affects enzyme function [130–132].

Subunit *b* spans the membrane without contacting the c_8 -ring in the enzyme monomer, suggesting that two subunits *b* are in close proximity in the dimer [133].

It still remains to be defined whether these or other ATP synthase residues might coincide with cysteines of the PTP facing the intermembrane space showed to be sensitive to membrane-impermeant photooxidants in isolated mitochondria [134]. Moreover, their involvement in the modulation of the PTP in mitochondrial pathophysiology in humans remains to be addressed.

5. PTP Modulators as Targets for ROS/RNS

S-nitrosylation of CyPD cysteine 203 seems to be important for the modulation of the PTP during I/R injury [135–137],

since its modification inhibits PTP opening. Moreover, the substitution of this residue with a serine desensitizes PTP in MEF cells exposed to oxidative stress similarly to CyPD ablation [137]. Indeed, cysteine 203 has been identified as a major cysteine residue susceptible to oxidation in human cells, leading to conformational changes of CyPD and induction of PTP [138]. Another possible effect of ROS on the modulation of the PTP is the direct phosphorylation of CyPD by mitochondrial glycogen synthase kinase 3 (mGSK3) [102, 139]. This kinase has been involved in the regulation of PTP in pathological conditions, such as cardiac I/R injury or hypoxia [140–144], in tumor cells [102, 139], and in cells lacking mtDNA [145]. ROS-dependent activation of mGSK3 enhances cell death in neurons, probably following PTP induction [146, 147], and this suggests that mGSK3 coordinates diverse signaling pathways to connect PTP opening with stress and survival signals by modulating ROS and/or calcium threshold of PTP opening.

Another recently identified interactor of CyPD that might be involved in the modulatory effect of ROS on the PTP is the IMM protein spastic paraplegia 7 (SPG7), but the mechanisms of action through which it could sensitize the PTP to opening in the presence of oxidative stress need to be clarified [148].

6. The Dual Role of ATP Synthase in Cardiac Disease

The cardiac muscle heavily depends on mitochondrial bioenergetics and metabolism for its function. This becomes particularly evident during I/R, a scenario in which low levels of oxygen lead to the inhibition of electron flow through the respiratory chain and thus an impairment of energy conservation and oxidative metabolism. The resulting loss in proton motive force prevents ADP phosphorylation to ATP at the level of ATP synthase, which rather works in “reverse mode” coupling ATP hydrolysis to proton pumping. The net result is that mitochondria no longer produce ATP and become very powerful in hydrolyzing glycolytic ATP [149]. IMM permeabilization due to the opening of the PTP leads to depolarization and worsening of ATP depletion and precipitates cell death [150]. Indeed, the crucial role of the pore opening in mediating I/R induced damage was extensively corroborated by the protective effects of CsA [151] and lack of injury in CyPD KO mice [7, 9]. The opening of the PTP was thought for years to occur due to mitochondrial calcium overload, but this hypothesis was questioned recently by the observation that MCU KO mice are not protected from I/R injury [152]. Nevertheless, recent reports showed that cardiac-specific ablation of MCU affords protection from I/R injury [153, 154]. Considering that MCU KO MEFs are equally susceptible to oxidative damage as their wild type counterparts [152], it is tempting to speculate that PTP opening might be induced by oxidative stress independently of mitochondrial calcium overload, either by directly targeting critical cysteine residues of ATP synthase to induce conformational changes and dimer formation or through oxidative changes of CyPD. Other amino acid residues might also be involved in the oxidative modulation of the enzyme

activity and/or conformation, such as tyrosine nitration in the α subunit occurring in cardiac I/R injury [155] or tryptophan oxidation described at the level of subunits d and a [36]. The functional consequences resulting from these modifications are yet to be elucidated.

It is well accepted that low levels of ROS are key signals mediating physiological or cardioprotective responses, whereas high levels of ROS contribute to oxidative stress and cell or tissue damage. This dual effect has been named mitohormesis [156, 157]. One such example of beneficial effects of ROS is represented by IPC. IPC consists of brief periods of ischemia followed by brief periods of reperfusion, which prepare the heart and render cardiomyocytes more resistant to sustained ischemic insults. Although IPC involves complex mechanisms that are not completely understood, it has been established that ROS/RNS mediate signal transduction in the early phase of IPC through posttranslational modifications of redox-sensitive proteins [158]. Again, mitochondrial proteins come into play as both the source and the target of these species. More specifically, IPC resulted in the S-nitrosylation of subunit α [30]. S-nitrosylation has been suggested to be protective, shielding the cysteine residues from oxidation and thus protecting the proteins from irreversible oxidation occurring at the start of reperfusion. It has also been reported that S-nitrosoglutathione (GSNO) treatment, also protective against I/R injury, leads to S-nitrosylation of the α subunit and this results in decreased ATP hydrolytic activity [29]. Thus, in addition to shielding the cysteine residues from irreversible oxidative damage, S-nitrosylation might also afford protection by decreasing ATP synthase activity and therefore reduce the consumption of ATP following myocardial ischemia. S-nitrosylation of ATP synthase subunit α occurs in both IPC and postconditioning and is dependent on NOS activity [30, 159]. Moreover, cysteine 19 of the subunit ϵ was also found to be S-nitrosylated following postconditioning but the functional significance of this finding remains to be elucidated [159].

Oxidation and S-nitrosylation have also been shown to occur at the level of ATP synthase in a canine model of dyssynchronous heart failure (DHF) [26]. Indeed, DHF was correlated with disulfide bond formation in the ATP synthase complex occurring between either cysteine 294 of the neighboring α subunits or cysteine 294 of the α subunit and cysteine 103 of the γ subunit. This cross-linking was negatively correlated with ATP hydrolytic activity. Furthermore, cysteine 294 of the ATP synthase α subunit was also S-glutathionylated in DHF, another modification that correlated with a reduction in ATP synthase activity [104]. Interestingly, during chronic resynchronization therapy (CRT, the only clinically effective therapy for DHF) the disulfide bond at cysteine 294 was found to be reversed and partially replaced by S-nitrosylation, resulting in the recovery of ATPase activity. Indeed, the same group has shown that cysteine 294 is critical for ATP synthase function *in vitro* and that it may play a major role in redox regulation of ATP production acting as a redox-sensor [160]. Nevertheless, the S-nitrosylation of the α subunit and improvement in ATPase activity observed in this study are in contrast with the previously mentioned results from Sun et al. [29], showing that S-nitrosylation of ATP

synthase α subunit in IPC or after GSNO treatment leads to a reduction of its hydrolytic activity. One cannot exclude that different cysteine residues were modified in the two aforementioned studies, a fact that could explain this discrepancy. Another possible explanation is that cross-link reversal and recovery of ATPase activity observed in CRT occur at a higher level compared to the extent of S-nitrosylation, suggesting that the majority of cysteine residues are actually free in CRT hearts [26]. On the other hand, the distances between the two cysteines 294 of neighboring α subunits or between α cysteine 294 and γ cysteine 103 are more than 5 Å (corresponding to α -Cys251 and γ -Cys78, respectively, in Figures 1 and 2), making it unlikely that these disulfide bonds actually occur in the assembled complex. Rather, these bonds could form in the misfolded/aggregated enzyme, suggesting that the reversal of cysteine cross-links observed in CRT may promote the correct assembly of the complex, thus resulting in improved ATPase activity. Of note, CuCl_2 treatment of isolated ATP synthase complex from rat mitochondria in vitro showed that disulfide bonds can form also between α and OSCP subunits as well as between the two γ subunits and γ and OSCP [26]. The effect of these disulfide crossbridges on ATP synthase activity was not addressed, and whether these modifications could also occur in vivo still needs to be assessed. Further efforts should also be put in addressing the effect of these oxidative posttranslational modifications on PTP formation.

7. Neurodegenerative Diseases

Mitochondria have a central role in aging-related neurodegenerative diseases. Oxidative stress generated by mitochondria has been inversely correlated with longevity in model organisms [161, 162] and defects in mitochondrial bioenergetics have been implicated in a number of neurodegenerative diseases [163].

In vitro, ATP synthase is susceptible to ROS [103, 164, 165] and to oxidative/nitrosative stress associated with disorders of the central nervous system [166, 167] and aging [35, 168]. The fact that ROS/RNS modifications can alter the mitochondrial oxidative phosphorylation efficiency may explain the mitochondrial involvement in neurological diseases.

Moreover, the involvement of PTP in neurodegenerative diseases has been demonstrated by the use of CsA both in vitro and in vivo and the ablation in mouse models of its target, CyPD [17, 18]. CyPD plays an important dual function on the modulation of ATP synthase; on the one hand, it sensitizes PTP to matrix calcium, while, on the other hand, it inhibits both ATP hydrolysis and synthesis by 30% [11]. In cellular mechanisms of neurodegenerative diseases, when mitochondria are hydrolyzing ATP, CyPD binding and inhibition of ATP synthase can be an advantage avoiding ATP consumption and cell death, or a disadvantage in cases in which PTP is sensitized to calcium and the displacement of CyPD would promote cell survival. This is only one of the complex relationships between ATP synthase and PTP in neurodegeneration, and in this paragraph we attempt to summarize possible mechanisms linking ATP synthase/PTP and ROS/RNS modifications.

7.1. Amyotrophic Lateral Sclerosis. Amyotrophic lateral sclerosis (ALS) is a progressive degeneration of motor neurons. In familial ALS, mutations have been found in Cu,Zn-SOD that are suggested to increase generation of ROS [169, 170], RNS, and nitrosylation [171]. Furthermore, Beal et al. [172] detected increased levels of nitrotyrosine staining in motor neurons of both sporadic ALS and familial ALS, suggesting that ONOO⁻ mediated oxidative damage may play a role in both forms of the disease. Of note, as already mentioned, tyrosine modifications at the level of the β subunit of ATP synthase have been described to be modulatory of the catalytic ATPase activity in the presence of ROS/RNS [108, 109], even if their direct involvement in ALS remains to be addressed.

In light of the susceptibility of the mitochondrial respiratory chain to nitrosative stress, it is not surprising that mitochondrial function is impaired in ALS [173]. Spinal cord mitochondria in ALS mouse model display decreased calcium retention capacity long before the onset of motor weakness and neuronal death [174], and this was corrected by ablation of the *Ppif* gene which encodes CyPD [175]. In these mice, an improved mitochondrial ATP synthesis was matched by PTP inhibition and significant suppression of motor neuron death throughout disease, although survival was not improved confirming the role of ATP synthesis and permeability transition in ALS neuron cell death [176, 177]. Increased ATP synthesis in this mouse ALS model lacking CyPD could also be explained by our observation that ablation of CyPD increases the catalysis of ATP synthase [11].

7.2. Alzheimer's Disease. AD, the most common form of dementia in aged people, is characterized by deposition of amyloid plaques formed by the amyloid β peptide [178]. Amyloid β can be imported in mitochondria [179]. Several studies have observed activity changes in key mitochondrial enzymes in AD. While the exact mechanism for this loss of activity is unclear, evidence suggests that ROS/RNS production is increased in AD. Nitrotyrosine residues have been detected in postmortem Alzheimer's tissue but not in age-matched controls [180], indicating the presence of ONOO⁻. The induction of iNOS in cultured rat astrocytes causes NO-mediated neuronal death in a coculture system [181].

Amyloid β is also reported to stimulate glial NO production [182–184] and it has been shown to inhibit both purified complex IV [185] and complex IV in isolated brain mitochondria [186]. Additionally, in neuronal cultures amyloid β caused a loss of activity of all the mitochondrial complexes and a loss of mitochondrial integrity, due to PTP opening [187]. It has been shown that amyloid β oligomers alter calcium homeostasis [188].

Upon import in mitochondria, amyloid β interacts with CyPD and enhances PTP induction, since neurons from CyPD KO mice are protected from cell death induced by amyloid β dependent PTP opening [189]. Interestingly, a novel association with AD risk has been recently identified in the ATP synthase *ATP5H* locus, which encodes subunit d of the lateral stalk [190]. Intriguingly, in a proteomic analysis of human heart mitochondria in normal condition, a tryptophan residue at the level of d subunit was identified as a “hot spot” for oxidation [36], the latter being another

important pathophysiological factor in AD. Moreover, in the filamentous fungus *Podospora anserina*, a model with a clear defined mitochondrial etiology of aging, another tryptophan residue on the α subunit of ATP synthase (Trp503) has been described to be crucial for the selective targeting of oxidative damage [107]. Although this residue is not conserved in unicellular fungi and higher eukaryotes, the example of *P. anserina* is instructive because the authors proposed that oxidized cysteine and methionine can be efficiently reduced by repair mechanisms, whereas tryptophan oxidation products are irreversible and have the potential to form markers detected by the mitochondrial quality control system.

7.3. Multiple Sclerosis. In multiple sclerosis (MS), the myelin sheath of neurons in the central nervous system is destroyed leading to axonal degeneration [191]. This is associated with mitochondrial calcium overload and bioenergetic dysfunction [192]. CyPD KO mice with autoimmune encephalomyelitis display a marked protection from axonal degeneration and a milder clinical picture despite a normal inflammatory response, thus suggesting that PTP might be involved in disease pathogenesis [193], even if other effects on bioenergetics caused by CyPD ablation cannot be excluded. A further indication of the important role played by the PTP in MS and its activation by ROS is provided by the observation that axonal damage of mice undergoing experimental autoimmune encephalomyelitis is reduced by genetic ablation of p66^{Shc} [194]. Importantly, a proteomic study of experimental autoimmune encephalomyelitis identified the ATP synthase dimer-forming subunit e differentially expressed [195]. Moreover, mutations at the level of the genes encoding subunits a and A6L were observed in MS patients [196].

A large body of evidence exists implicating increased generation of RNS in MS. The observation that the concentration of NO₂⁻ plus NO₃⁻ in cerebrospinal fluid is elevated by 70% in MS patients supports this hypothesis [197]. Furthermore, increased iNOS activity and iNOS mRNA have been demonstrated in astrocytes associated with demyelinating lesions in postmortem MS brain [198] and in experimental models of demyelination [199, 200]. Nitrotyrosine residues indicating the presence of ONOO⁻ have also been detected in MS brain [201]. Whether NO-mediated mitochondrial damage is the cause of the disease remains to be established; however, the first direct evidence of altered mitochondrial function in MS came from a study by Lu et al. [202]. These authors described a decrease of respiratory complex I activity and a compensatory increase in complex IV in MS. Major targets for nitrosative stress in rat liver under in vivo conditions are tyrosine 345 and tyrosine 368 in the β subunit of the mitochondrial ATP synthase that is suggested as an early marker of nitrosative stress and aging [35]. A comparison between α and β subunits allowed the speculation that the latter is more accessible for RNS modification and that the catalytic conformation of this subunit also affects tyrosine residues exposure to nitration (higher exposure to RNS modifications in the absence of bound nucleotide). Moreover, Ding et al. [203] demonstrated in ND4 mice (an in vivo model for MS) an impaired transport of ATP5b mRNA (which generates subunit β) to mitochondria. This caused decreased ATP

synthesis in MS mice due to lower levels of this subunit and not to impaired ATP synthase assembly, as suggested by the authors. However, the possibility that tyrosine modifications in this subunit might affect enzyme activity cannot be excluded.

7.4. Parkinson's Disease. PD is caused by death of dopaminergic neurons in the *substantia nigra pars compacta*. A sensitization to PTP opening has been proposed as a major cause of neurodegeneration in several models of the disease characterized by altered homeostasis of intracellular calcium [204–206], including the forms caused by complex I inhibition [207, 208] and by inactivation of the Ser/Thr kinase PINK1 [209], in which changes in calcium storage capacity [210, 211], impairment of respiratory complex I [212], and altered mitophagy [213] are observed. Moreover, the PTP can be induced in dopaminergic neurons because of the inability to buffer increased intracellular ROS levels [214]. In a PD mouse model induced by parkin deficiency, state 3 and state 4 respiration rates were both affected indicating a more likely direct modulation of the respiratory chain compared to an effect on ATP synthase [215]. On the other hand, a mouse model lacking chaperone protein *Htra2* and showing a parkinsonian phenotype displays mitochondrial uncoupling at the level of ATP synthase and a truncated α subunit that might be involved in the neurodegeneration observed in these mice [33].

Postsynaptic density protein 95 (PSD-95) binds to neuronal nNOS and the neuroprotective effects of small-molecule inhibitors of this interaction were tested in an in vitro PD model. The observed protective effects were associated with suppressed mitochondrial dysfunction, as evidenced by decreased ROS generation, preserved ATP synthesis, and PTP inhibition [216]. Moreover, Darios et al. [217] demonstrated that in PC12 cells the overexpression of parkin protects from ceramide-induced swelling suggesting that parkin may act directly to prevent PTP mediated cell death, but the exact mechanism remains to be addressed.

8. Conclusions

Posttranslational modifications of ATP synthase due to ROS/RNS generation might play a dual role by promoting cell death or survival depending on their relative effects on mitochondrial ATP synthase catalysis and PTP. We have illustrated how ATP synthase is the target for oxidative/nitrosative modifications that affect its activity and might promote formation of the PTP. Identification of specific residues involved in the latter event is still lacking and will help to elucidate the mechanisms that mediate the role of ATP synthase in modulating cell survival or death. Finally, we discussed how changes in the ATP synthase activity regulate mitochondrial ROS formation and thus may represent an attractive strategy for the treatment of pathologies such as cancer.

Abbreviations

AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

AsO:	Arsenite anion
BH4:	Tetrahydrobiopterin
CRT:	Chronic resynchronization therapy
CsA:	Cyclosporin A
CuCl ₂ :	Copper(II) chloride
CyPD:	Cyclophilin D
DHF:	Dyssynchronous heart failure
eNOS:	Endothelial nitric oxide synthase
GSNO:	S-Nitrosoglutathione
H ₂ O ₂ :	Hydrogen peroxide
IF ₁ :	Inhibitor protein
IMM:	Inner mitochondrial membrane
iNOS:	Inducible nitric oxide synthase
IPC:	Ischemic preconditioning
I/R:	Ischemia/reperfusion
KO:	Knockout
MAO:	Monoamine oxidase
MBM:	Monobromobimane
mGSK3:	Mitochondrial glycogen synthase kinase 3
PTP:	Mitochondrial permeability transition pore
MS:	Multiple sclerosis
NEM:	N-Ethylmaleimide
nNOS:	Neuronal nitric oxide synthase
NO:	Nitric oxide
NO ₂ ⁻ :	Nitrite
NO ₃ ⁻ :	Nitrate
Nox4:	NADPH oxidase 4
OMM:	Outer mitochondrial membrane
ONOO ⁻ :	Peroxynitrite
OSCP:	Oligomycin-sensitivity conferring protein
PD:	Parkinson's disease
PhAsO:	Phenylarsine oxide
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
SPG7:	Spastic paraplegia 7

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] Å. B. Gustafsson and R. A. Gottlieb, "Bcl-2 family members and apoptosis, taken to heart," *The American Journal of Physiology—Cell Physiology*, vol. 292, no. 1, pp. C45–C51, 2007.
- [2] P. Bernardi, A. Rasola, M. Forte, and G. Lippe, "The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology," *Physiological Reviews*, vol. 95, no. 4, pp. 1111–1155, 2015.
- [3] K. Le-Quoc and D. Le-Quoc, "Crucial role of sulfhydryl groups in the mitochondrial inner membrane structure," *The Journal of Biological Chemistry*, vol. 260, no. 12, pp. 7422–7428, 1985.
- [4] R. A. Haworth and D. R. Hunter, "The Ca²⁺-induced membrane transition in mitochondria. II. Nature of the Ca²⁺ trigger site," *Archives of Biochemistry and Biophysics*, vol. 195, no. 2, pp. 460–467, 1979.
- [5] D. R. Hunter and R. A. Haworth, "The Ca²⁺-induced membrane transition in mitochondria. I. The protective mechanisms," *Archives of Biochemistry and Biophysics*, vol. 195, no. 2, pp. 453–459, 1979.
- [6] D. R. Hunter and R. A. Haworth, "The Ca²⁺-induced membrane transition in mitochondria. III. Transitional Ca²⁺ release," *Archives of Biochemistry and Biophysics*, vol. 195, no. 2, pp. 468–477, 1979.
- [7] C. P. Baines, R. A. Kaiser, N. H. Purcell et al., "Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death," *Nature*, vol. 434, no. 7033, pp. 658–662, 2005.
- [8] E. Basso, L. Fante, J. Fowlkes, V. Petronilli, M. A. Forte, and P. Bernardi, "Properties of the permeability transition pore in mitochondria devoid of cyclophilin D," *The Journal of Biological Chemistry*, vol. 280, no. 19, pp. 18558–18561, 2005.
- [9] T. Nakagawa, S. Shimizu, T. Watanabe et al., "Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death," *Nature*, vol. 434, no. 7033, pp. 652–658, 2005.
- [10] A. C. Schinzel, O. Takeuchi, Z. Huang et al., "Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 34, pp. 12005–12010, 2005.
- [11] V. Giorgio, E. Bisetto, M. E. Soriano et al., "Cyclophilin D modulates mitochondrial F₀F₁-ATP synthase by interacting with the lateral stalk of the complex," *The Journal of Biological Chemistry*, vol. 284, no. 49, pp. 33982–33988, 2009.
- [12] V. Giorgio, S. von Stockum, M. Antoniel et al., "Dimers of mitochondrial ATP synthase form the permeability transition pore," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 15, pp. 5887–5892, 2013.
- [13] M. Bonora, A. Bononi, E. De Marchi et al., "Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition," *Cell Cycle*, vol. 12, no. 4, pp. 674–683, 2013.
- [14] M. Carraro, V. Giorgio, J. Sileikyte et al., "Channel formation by yeast F-ATP synthase and the role of dimerization in the mitochondrial permeability transition," *The Journal of Biological Chemistry*, vol. 289, no. 23, pp. 15980–15985, 2014.
- [15] S. von Stockum, V. Giorgio, E. Trevisan et al., "F-ATPase of *Drosophila melanogaster* forms 53-picosiemens (53-pS) channels responsible for mitochondrial Ca²⁺-induced Ca²⁺ release," *The Journal of Biological Chemistry*, vol. 290, no. 8, pp. 4537–4544, 2015.
- [16] K. N. Alavian, S. I. Dworetzky, L. Bonanni et al., "The mitochondrial complex V—associated large-conductance inner membrane current is regulated by cyclosporine and dexramipexole," *Molecular Pharmacology*, vol. 87, no. 1, pp. 1–8, 2015.
- [17] V. Giorgio, M. E. Soriano, E. Basso et al., "Cyclophilin D in mitochondrial pathophysiology," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1797, no. 6–7, pp. 1113–1118, 2010.

- [18] J. W. Elrod and J. D. Molkentin, "Physiologic functions of cyclophilin D and the mitochondrial permeability transition pore," *Circulation Journal*, vol. 77, no. 5, pp. 1111–1122, 2013.
- [19] D. A. Butterfield and I. Dalle-Donne, "Redox proteomics: from protein modifications to cellular dysfunction and disease," *Mass Spectrometry Reviews*, vol. 33, no. 1, pp. 1–6, 2014.
- [20] S. F. Steinberg, "Oxidative stress and sarcomeric proteins," *Circulation Research*, vol. 112, no. 2, pp. 393–405, 2013.
- [21] A. I. Casas, V. T. Dao, A. Daiber et al., "Reactive oxygen-related diseases: therapeutic targets and emerging clinical indications," *Antioxidants & Redox Signaling*, vol. 23, no. 14, pp. 1171–1185, 2015.
- [22] V. T. Dao, A. I. Casas, G. J. Maghzal et al., "Pharmacology and clinical drug candidates in redox medicine," *Antioxidants & Redox Signaling*, vol. 23, no. 14, pp. 1113–1129, 2015.
- [23] N. Kaludercic, J. Mialet-Perez, N. Paolocci, A. Parini, and F. Di Lisa, "Monoamine oxidases as sources of oxidants in the heart," *Journal of Molecular and Cellular Cardiology*, vol. 73, pp. 34–42, 2014.
- [24] M. P. Murphy, "How mitochondria produce reactive oxygen species," *Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.
- [25] M. D. Brand, "The sites and topology of mitochondrial superoxide production," *Experimental Gerontology*, vol. 45, no. 7–8, pp. 466–472, 2010.
- [26] S.-B. Wang, D. B. Foster, J. Rucker, B. O'Rourke, D. A. Kass, and J. E. Van Eyk, "Redox regulation of mitochondrial ATP synthase: implications for cardiac resynchronization therapy," *Circulation Research*, vol. 109, no. 7, pp. 750–757, 2011.
- [27] J. Garcia, D. Han, H. Sancheti, L.-P. Yap, N. Kaplowitz, and E. Cadenas, "Regulation of mitochondrial glutathione redox status and protein glutathionylation by respiratory substrates," *Journal of Biological Chemistry*, vol. 285, no. 51, pp. 39646–39654, 2010.
- [28] M. B. West, B. G. Hill, Y.-T. Xuan, and A. Bhatnagar, "Protein glutathiolation by nitric oxide: an intracellular mechanism regulating redox protein modification," *The FASEB Journal*, vol. 20, no. 10, pp. 1715–1717, 2006.
- [29] J. Sun, M. Morgan, R.-F. Shen, C. Steenbergen, and E. Murphy, "Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport," *Circulation Research*, vol. 101, no. 11, pp. 1155–1163, 2007.
- [30] J. Sun, T. Nguyen, A. M. Aponte et al., "Ischaemic preconditioning preferentially increases protein S-nitrosylation in subsarcolemmal mitochondria," *Cardiovascular Research*, vol. 106, no. 2, pp. 227–236, 2015.
- [31] N. Das and C. K. Jana, "Age-associated oxidative modifications of mitochondrial α -subunit of F1 ATP synthase from mouse skeletal muscles," *Free Radical Research*, vol. 49, no. 8, pp. 954–961, 2015.
- [32] O. Augereau, S. Claverol, N. Boudes et al., "Identification of tyrosine-phosphorylated proteins of the mitochondrial oxidative phosphorylation machinery," *Cellular and Molecular Life Sciences*, vol. 62, no. 13, pp. 1478–1488, 2005.
- [33] H. Plun-Favreau, V. S. Burchell, K. M. Holmström et al., "HtrA2 deficiency causes mitochondrial uncoupling through the F(1)F(0)-ATP synthase and consequent ATP depletion," *Cell Death and Disease*, vol. 3, article e335, 2012.
- [34] A. Dupuis, J. Lunardi, J.-P. Issartel, and P. V. Vignais, "Interactions between the oligomycin sensitivity conferring protein (OSCP) and beef heart mitochondrial F1ATPase. 2. Identification of the interacting F1 subunits by cross-linking," *Biochemistry*, vol. 24, no. 3, pp. 734–739, 1985.
- [35] V. Haynes, N. J. Traaseth, S. Elfering, Y. Fujisawa, and C. Giulivi, "Nitration of specific tyrosines in FoF1 ATP synthase and activity loss in aging," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 298, no. 5, pp. E978–E987, 2010.
- [36] S. W. Taylor, E. Fahy, J. Murray, R. A. Capaldi, and S. S. Ghosh, "Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins," *Journal of Biological Chemistry*, vol. 278, no. 22, pp. 19587–19590, 2003.
- [37] M. Giorgio, E. Migliaccio, F. Orsini et al., "Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis," *Cell*, vol. 122, no. 2, pp. 221–233, 2005.
- [38] A. Carpi, R. Menabò, N. Kaludercic, P. Pelicci, F. Di Lisa, and M. Giorgio, "The cardioprotective effects elicited by p66Shc ablation demonstrate the crucial role of mitochondrial ROS formation in ischemia/reperfusion injury," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1787, no. 7, pp. 774–780, 2009.
- [39] R. D. Spescha, Y. Shi, S. Wegener et al., "Deletion of the ageing gene p66^{Shc} reduces early stroke size following ischaemia/reperfusion brain injury," *European Heart Journal*, vol. 34, no. 2, pp. 96–103, 2013.
- [40] S. Ciciliot, M. Albiero, L. Menegazzo et al., "p66Shc deletion or deficiency protects from obesity but not metabolic dysfunction in mice and humans," *Diabetologia*, vol. 58, no. 10, pp. 2352–2360, 2015.
- [41] M. Rota, N. LeCapitaine, T. Hosoda et al., "Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene," *Circulation Research*, vol. 99, no. 1, pp. 42–52, 2006.
- [42] S. Menini, L. Amadio, G. Oddi et al., "Deletion of p66Shc longevity gene protects against experimental diabetic glomerulopathy by preventing diabetes-induced oxidative stress," *Diabetes*, vol. 55, no. 6, pp. 1642–1650, 2006.
- [43] G. P. Fadini, M. Albiero, L. Menegazzo et al., "The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing," *Diabetes*, vol. 59, no. 9, pp. 2306–2314, 2010.
- [44] A. Natalicchio, F. Tortosa, R. Labarbuta et al., "The p66(Shc) redox adaptor protein is induced by saturated fatty acids and mediates lipotoxicity-induced apoptosis in pancreatic beta cells," *Diabetologia*, vol. 58, no. 6, pp. 1260–1271, 2015.
- [45] G. G. Camici, M. Schiavoni, P. Francia et al., "Genetic deletion of p66^{Shc} adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 12, pp. 5217–5222, 2007.
- [46] M. Giorgio, A. Berry, I. Berniakovich et al., "The p66^{Shc} knocked out mice are short lived under natural condition," *Aging Cell*, vol. 11, no. 1, pp. 162–168, 2012.
- [47] R. M. Touyz and A. C. Montezano, "Vascular Nox4: a multifarious NADPH oxidase," *Circulation Research*, vol. 110, no. 9, pp. 1159–1161, 2012.
- [48] K. D. Martyn, L. M. Frederick, K. von Loehneysen, M. C. Dinauer, and U. G. Knaus, "Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases," *Cellular Signalling*, vol. 18, no. 1, pp. 69–82, 2006.
- [49] C. Hirschihäuser, J. Bornbaum, A. Reis et al., "NOX4 in mitochondria: yeast two-hybrid-based interaction with complex I without relevance for basal reactive oxygen species?" *Antioxidants & Redox Signaling*, vol. 23, no. 14, pp. 1106–1112, 2015.
- [50] M. Zhang, A. C. Brewer, K. Schröder et al., "NADPH oxidase-4 mediates protection against chronic load-induced stress in

- mouse hearts by enhancing angiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 42, pp. 18121–18126, 2010.
- [51] J. Kuroda, T. Ago, S. Matsushima, P. Zhai, M. D. Schneider, and J. Sadoshima, "NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 35, pp. 15565–15570, 2010.
- [52] C. Kleinschnitz, H. Grund, K. Wingler et al., "Post-stroke inhibition of induced NADPH Oxidase type 4 prevents oxidative stress and neurodegeneration," *PLoS Biology*, vol. 8, no. 9, Article ID e1000479, 2010.
- [53] R. M. Maalouf, A. A. Eid, Y. C. Gorin et al., "Nox4-derived reactive oxygen species mediate cardiomyocyte injury in early type 1 diabetes," *The American Journal of Physiology—Cell Physiology*, vol. 302, no. 3, pp. C597–C604, 2012.
- [54] D. Sorescu, D. Weiss, B. Lassègue et al., "Superoxide production and expression of Nox family proteins in human atherosclerosis," *Circulation*, vol. 105, no. 12, pp. 1429–1435, 2002.
- [55] K. Schröder, M. Zhang, S. Benkhoff et al., "Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase," *Circulation Research*, vol. 110, no. 9, pp. 1217–1225, 2012.
- [56] M. B. H. Youdim, D. Edmondson, and K. F. Tipton, "The therapeutic potential of monoamine oxidase inhibitors," *Nature Reviews Neuroscience*, vol. 7, no. 4, pp. 295–309, 2006.
- [57] P. Bianchi, O. Kunduzova, E. Masini et al., "Oxidative stress by monoamine oxidase mediates receptor-independent cardiomyocyte apoptosis by serotonin and postschemic myocardial injury," *Circulation*, vol. 112, no. 21, pp. 3297–3305, 2005.
- [58] D. Pchejetski, O. Kunduzova, A. Dayon et al., "Oxidative stress-dependent sphingosine kinase-1 inhibition mediates monoamine oxidase A-associated cardiac cell apoptosis," *Circulation Research*, vol. 100, no. 1, pp. 41–49, 2007.
- [59] N. Kaludercic, A. Carpi, T. Nagayama et al., "Monoamine oxidase B prompts mitochondrial and cardiac dysfunction in pressure overloaded hearts," *Antioxidants and Redox Signaling*, vol. 20, no. 2, pp. 267–280, 2014.
- [60] N. Kaludercic, E. Takimoto, T. Nagayama et al., "Monoamine oxidase A-mediated enhanced catabolism of norepinephrine contributes to adverse remodeling and pump failure in hearts with pressure overload," *Circulation Research*, vol. 106, no. 1, pp. 193–202, 2010.
- [61] A. Sturza, M. S. Leisegang, A. Babelova et al., "Monoamine oxidases are mediators of endothelial dysfunction in the mouse aorta," *Hypertension*, vol. 62, no. 1, pp. 140–146, 2013.
- [62] P. Umbarkar, S. Singh, S. Arkat, S. L. Bodhankar, S. Lohidasan, and S. L. Sitasawad, "Monoamine oxidase-A is an important source of oxidative stress and promotes cardiac dysfunction, apoptosis, and fibrosis in diabetic cardiomyopathy," *Free Radical Biology and Medicine*, vol. 87, pp. 263–273, 2015.
- [63] E. Takimoto, H. C. Champion, M. Li et al., "Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load," *The Journal of Clinical Investigation*, vol. 115, no. 5, pp. 1221–1231, 2005.
- [64] E. Takimoto and D. A. Kass, "Role of oxidative stress in cardiac hypertrophy and remodeling," *Hypertension*, vol. 49, no. 2, pp. 241–248, 2007.
- [65] P. S. Brookes, "Mitochondrial nitric oxide synthase," *Mitochondrion*, vol. 3, no. 4, pp. 187–204, 2004.
- [66] P. Venkatakrisnan, E. S. Nakayasu, I. C. Almeida, and R. T. Miller, "Absence of nitric-oxide synthase in sequentially purified rat liver mitochondria," *The Journal of Biological Chemistry*, vol. 284, no. 30, pp. 19843–19855, 2009.
- [67] P. Venkatakrisnan, E. S. Nakayasu, I. C. Almeida, and R. T. Miller, "Arginase activity in mitochondria—an interfering factor in nitric oxide synthase activity assays," *Biochemical and Biophysical Research Communications*, vol. 394, no. 3, pp. 448–452, 2010.
- [68] S. L. Elfering, T. M. Sarkela, and C. Giulivi, "Biochemistry of mitochondrial nitric-oxide synthase," *The Journal of Biological Chemistry*, vol. 277, no. 41, pp. 38079–38086, 2002.
- [69] F. Forner, C. Kumar, C. A. Luber, T. Fromme, M. Klingenspor, and M. Mann, "Proteome differences between brown and white fat mitochondria reveal specialized metabolic functions," *Cell Metabolism*, vol. 10, no. 4, pp. 324–335, 2009.
- [70] T. R. Figueira, M. H. Barros, A. A. Camargo et al., "Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health," *Antioxidants and Redox Signaling*, vol. 18, no. 16, pp. 2029–2074, 2013.
- [71] A. V. Kozlov, K. Staniek, and H. Nohl, "Nitrite reductase activity is a novel function of mammalian mitochondria," *FEBS Letters*, vol. 454, no. 1-2, pp. 127–130, 1999.
- [72] H. Nohl, K. Staniek, and A. V. Kozlov, "The existence and significance of a mitochondrial nitrite reductase," *Redox Report*, vol. 10, no. 6, pp. 281–286, 2005.
- [73] S. Basu, N. A. Azarova, M. D. Font et al., "Nitrite reductase activity of cytochrome c," *The Journal of Biological Chemistry*, vol. 283, no. 47, pp. 32590–32597, 2008.
- [74] E. T. Chouchani, C. Methner, S. M. Nadtochiy et al., "Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I," *Nature Medicine*, vol. 19, no. 6, pp. 753–759, 2013.
- [75] E. T. Chouchani, V. R. Pell, E. Gaude et al., "Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS," *Nature*, vol. 515, no. 7527, pp. 431–435, 2014.
- [76] Y. Li, T.-T. Huang, E. J. Carlson et al., "Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase," *Nature Genetics*, vol. 11, no. 4, pp. 376–381, 1995.
- [77] D.-F. Dai, T. Chen, J. Wanagat et al., "Age-dependent cardiomyopathy in mitochondrial mutator mice is attenuated by overexpression of catalase targeted to mitochondria," *Aging Cell*, vol. 9, no. 4, pp. 536–544, 2010.
- [78] D.-F. Dai, L. F. Santana, M. Vermulst et al., "Overexpression of catalase targeted to mitochondria attenuates murine cardiac aging," *Circulation*, vol. 119, no. 21, pp. 2789–2797, 2009.
- [79] H.-Y. Lee, C. S. Choi, A. L. Birkenfeld et al., "Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance," *Cell Metabolism*, vol. 12, no. 6, pp. 668–674, 2010.
- [80] S. E. Schriener, N. J. Linford, G. M. Martin et al., "Extension of murine life span by overexpression of catalase targeted to mitochondria," *Science*, vol. 308, no. 5730, pp. 1909–1911, 2005.
- [81] R. Ni, D. Zheng, S. Xiong et al., "Mitochondrial calpain-1 disrupts ATP synthase and induces superoxide generation in type-1 diabetic hearts: a novel mechanism contributing to diabetic cardiomyopathy," *Diabetes*, vol. 65, no. 1, pp. 255–268, 2016.
- [82] K. M. Johnson, X. Chen, A. Boitano, L. Swenson, A. W. Opipari Jr., and G. D. Glick, "Identification and validation of the mitochondrial F1F0-ATPase as the molecular target of the

- immunomodulatory benzodiazepine Bz-423," *Chemistry and Biology*, vol. 12, no. 4, pp. 485–496, 2005.
- [83] G. Santamaría, M. Martínez-Diez, I. Fabregat, and J. M. Cuezva, "Efficient execution of cell death in non-glycolytic cells requires the generation of ROS controlled by the activity of mitochondrial H⁺-ATP synthase," *Carcinogenesis*, vol. 27, no. 5, pp. 925–935, 2006.
- [84] E. Gaude and C. Frezza, "Defects in mitochondrial metabolism and cancer," *Cancer & Metabolism*, vol. 2, article 10, 2014.
- [85] I. Martínez-Reyes and J. M. Cuezva, "The H(+)-ATP synthase: a gate to ROS-mediated cell death or cell survival," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1837, no. 7, pp. 1099–1112, 2014.
- [86] M. Sánchez-Aragó, L. Formentini, I. Martínez-Reyes et al., "Expression, regulation and clinical relevance of the ATPase inhibitory factor 1 in human cancers," *Oncogenesis*, vol. 2, article e46, 2013.
- [87] E. Cabezón, P. J. G. Butler, M. J. Runswick, and J. E. Walker, "Modulation of the oligomerization state of the bovine F₁-ATPase inhibitor protein, IF₁, by pH," *The Journal of Biological Chemistry*, vol. 275, no. 33, pp. 25460–25464, 2000.
- [88] J. R. Gledhill, M. G. Montgomery, A. G. W. Leslie, and J. E. Walker, "How the regulatory protein, IF₁, inhibits F₁-ATPase from bovine mitochondria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 40, pp. 15671–15676, 2007.
- [89] F. Di Pancrazio, I. Mavelli, M. Isola et al., "In vitro and in vivo studies of F₀F₁ ATP synthase regulation by inhibitor protein IF₁ in goat heart," *Biochimica et Biophysica Acta*, vol. 1659, no. 1, pp. 52–62, 2004.
- [90] K. Ylitalo, A. Ala-Rämi, K. Vuorinen et al., "Reversible ischemic inhibition of F₁F₀-ATPase in rat and human myocardium," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1504, no. 2-3, pp. 329–339, 2001.
- [91] D. W. Green and G. J. Grover, "The IF₁ inhibitor protein of the mitochondrial F₁F₀-ATPase," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1458, no. 2-3, pp. 343–355, 2000.
- [92] W. Rouslin and C. W. Broge, "Mechanisms of ATP conservation during ischemia in slow and fast heart rate hearts," *The American Journal of Physiology—Cell Physiology*, vol. 264, no. 1, pp. C209–C216, 1993.
- [93] M. Campanella, E. Casswell, S. Chong et al., "Regulation of mitochondrial structure and function by the F₁F₀-ATPase inhibitor protein, IF₁," *Cell Metabolism*, vol. 8, no. 1, pp. 13–25, 2008.
- [94] J. J. García, E. Morales-Ríos, P. Cortés-Hernández, and J. S. Rodríguez-Zavala, "The inhibitor protein (IF₁) promotes dimerization of the mitochondrial F₁F₀-ATP synthase," *Biochemistry*, vol. 45, no. 42, pp. 12695–12703, 2006.
- [95] E. Bisetto, M. Comelli, A. M. Salzano et al., "Proteomic analysis of F₁F₀-ATP synthase super-assembly in mitochondria of cardiomyoblasts undergoing differentiation to the cardiac lineage," *Biochimica et Biophysica Acta*, vol. 1827, no. 7, pp. 807–816, 2013.
- [96] E. Cabezón, I. Arechaga, P. Jonathan, G. Butler, and J. E. Walker, "Dimerization of bovine F₁-ATPase by binding the inhibitor protein, IF₁," *Journal of Biological Chemistry*, vol. 275, no. 37, pp. 28353–28355, 2000.
- [97] L. Formentini, M. Sánchez-Aragó, L. Sánchez-Cenizo, and J. M. C. Cuezva, "The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde pro-survival and proliferative response," *Molecular Cell*, vol. 45, no. 6, pp. 731–742, 2012.
- [98] M. Sánchez-Aragó, L. Formentini, and J. M. Cuezva, "Mitochondria-mediated energy adaptation in cancer: the H⁺-ATP synthase-gated switch of metabolism in human tumors," *Antioxidants & Redox Signaling*, vol. 19, no. 3, pp. 285–298, 2013.
- [99] L. Sánchez-Cenizo, L. Formentini, M. Aldea et al., "Up-regulation of the ATPase inhibitory factor 1 (IF₁) of the mitochondrial H⁺-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a Warburg phenotype," *Journal of Biological Chemistry*, vol. 285, no. 33, pp. 25308–25313, 2010.
- [100] J. Garcia-Bermudez, M. Sanchez-Arago, B. Soldevilla, A. Del Arco, C. Nuevo-Tapióles, and J. M. Cuezva, "PKA phosphorylates the ATPase inhibitory factor 1 and inactivates its capacity to bind and inhibit the mitochondrial H⁺-ATP synthase," *Cell Reports*, vol. 12, no. 12, pp. 2143–2155, 2015.
- [101] C. Gorrini, I. S. Harris, and T. W. Mak, "Modulation of oxidative stress as an anticancer strategy," *Nature Reviews Drug Discovery*, vol. 12, no. 12, pp. 931–947, 2013.
- [102] A. Rasola, M. Sciacovelli, F. Chiara, B. Pantic, W. S. Brusilow, and P. Bernardi, "Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 2, pp. 726–731, 2010.
- [103] G. Lippe, M. Comelli, D. Mazzilis, F. D. Sala, and I. Mavelli, "The inactivation of mitochondrial F₁ ATPase by H₂O₂ is mediated by iron ions not tightly bound in the protein," *Biochemical and Biophysical Research Communications*, vol. 181, no. 2, pp. 764–770, 1991.
- [104] S.-B. Wang, C. I. Murray, H. S. Chung, and J. E. Van Eyk, "Redox regulation of mitochondrial ATP synthase," *Trends in Cardiovascular Medicine*, vol. 23, no. 1, pp. 14–18, 2013.
- [105] F. Buchert, Y. Schober, A. Römpp, M. L. Richter, and C. Forreiter, "Reactive oxygen species affect ATP hydrolysis by targeting a highly conserved amino acid cluster in the thylakoid ATP synthase γ subunit," *Biochimica et Biophysica Acta*, vol. 1817, no. 11, pp. 2038–2048, 2012.
- [106] N. Kaludercic, S. Deshwal, and F. Di Lisa, "Reactive oxygen species and redox compartmentalization," *Frontiers in Physiology*, vol. 5, article 285, 2014.
- [107] S. Rexroth, A. Poetsch, M. Rögner et al., "Reactive oxygen species target specific tryptophan site in the mitochondrial ATP synthase," *Biochimica et Biophysica Acta*, vol. 1817, no. 2, pp. 381–387, 2012.
- [108] J. Murray, S. W. Taylor, B. Zhang, S. S. Ghosh, and R. A. Capaldi, "Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry," *Journal of Biological Chemistry*, vol. 278, no. 39, pp. 37223–37230, 2003.
- [109] S. Nesci, V. Ventrella, F. Trombetti, M. Pirini, and A. Pagliarini, "Preferential nitrite inhibition of the mitochondrial FF-ATPase activities when activated by Ca in replacement of the natural cofactor Mg," *Biochimica et Biophysica Acta*, vol. 1860, no. 2, pp. 345–353, 2015.
- [110] J. Tamarit, E. Cabisco, and J. Ros, "Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress," *Journal of Biological Chemistry*, vol. 273, no. 5, pp. 3027–3032, 1998.
- [111] V. Petronilli, P. Costantini, L. Scorrano, R. Colonna, S. Passamonti, and P. Bernardi, "The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols. Increase of the gating potential by oxidants and its reversal by reducing agents," *Journal of Biological Chemistry*, vol. 269, no. 24, pp. 16638–16642, 1994.

- [112] P. Costantini, B. V. Chernyak, V. Petronilli, and P. Bernardi, "Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites," *Journal of Biological Chemistry*, vol. 271, no. 12, pp. 6746–6751, 1996.
- [113] P. Costantini, B. V. Chernyak, V. Petronilli, and P. Bernardi, "Selective inhibition of the mitochondrial permeability transition pore at the oxidation-reduction sensitive dithiol by monobromobimane," *FEBS Letters*, vol. 362, no. 2, pp. 239–242, 1995.
- [114] P. Costantini, R. Colonna, and P. Bernardi, "Induction of the mitochondrial permeability transition by N-ethylmaleimide depends on secondary oxidation of critical thiol groups. Potentiation by copper-ortho-phenanthroline without dimerization of the adenine nucleotide translocase," *Biochimica et Biophysica Acta*, vol. 1365, no. 3, pp. 385–392, 1998.
- [115] S. Hong and P. L. Pedersen, "ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas," *Microbiology and Molecular Biology Reviews*, vol. 72, no. 4, pp. 590–641, 2008.
- [116] A. Dupuis, J.-P. Issartel, J. Lunardi, M. Satre, and P. V. Vignais, "Interactions between the oligomycin sensitivity conferring protein (OSCP) and beef heart mitochondrial F₁-ATPase. 1. Study of the binding parameters with a chemically radiolabeled OSCP," *Biochemistry*, vol. 24, no. 3, pp. 728–733, 1985.
- [117] D.-T. Lin and J. D. Lechleiter, "Mitochondrial targeted cyclophilin D protects cells from cell death by peptidyl prolyl isomerization," *The Journal of Biological Chemistry*, vol. 277, no. 34, pp. 31134–31141, 2002.
- [118] G. Arselin, M.-F. Giraud, A. Dautant et al., "The GxxxG motif of the transmembrane domain of subunit e is involved in the dimerization/oligomerization of the yeast ATP synthase complex in the mitochondrial membrane," *European Journal of Biochemistry*, vol. 270, no. 8, pp. 1875–1884, 2003.
- [119] V. Everard-Gigot, C. D. Dunn, B. M. Dolan, S. Brunner, R. E. Jensen, and R. A. Stuart, "Functional analysis of subunit e of the F₁F₀-ATP synthase of the yeast *Saccharomyces cerevisiae*: importance of the N-terminal membrane anchor region," *Eukaryotic Cell*, vol. 4, no. 2, pp. 346–355, 2005.
- [120] D. M. Bustos and J. Velours, "The modification of the conserved GXXXG motif of the membrane-spanning segment of subunit g destabilizes the supramolecular species of yeast ATP synthase," *The Journal of Biological Chemistry*, vol. 280, no. 32, pp. 29004–29010, 2005.
- [121] I. Wittig, J. Velours, R. Stuart, and H. Schagger, "Characterization of domain interfaces in monomeric and dimeric ATP synthase," *Molecular and Cellular Proteomics*, vol. 7, no. 5, pp. 995–1004, 2008.
- [122] C. Spannagel, J. Vaillier, G. Arselin, P.-V. Graves, X. Grandier-Vazeille, and J. Velours, "Evidence of a subunit 4 (subunit b) dimer in favor of the proximity of ATP synthase complexes in yeast inner mitochondrial membrane," *Biochimica et Biophysica Acta—Biomembranes*, vol. 1414, no. 1-2, pp. 260–264, 1998.
- [123] P. Paumard, G. Arselin, J. Vaillier et al., "Two ATP synthases can be linked through subunits i in the inner mitochondrial membrane of *Saccharomyces cerevisiae*," *Biochemistry*, vol. 41, no. 33, pp. 10390–10396, 2002.
- [124] J. Habersetzer, W. Ziani, I. Larrieu et al., "ATP synthase oligomerization: from the enzyme models to the mitochondrial morphology," *International Journal of Biochemistry and Cell Biology*, vol. 45, no. 1, pp. 99–105, 2013.
- [125] J. Habersetzer, I. Larrieu, M. Priault et al., "Human F₁F₀ ATP synthase, mitochondrial ultrastructure and OXPHOS impairment: a (super-)complex matter?" *PLoS ONE*, vol. 8, no. 10, Article ID e75429, 2013.
- [126] I. Wittig, B. Meyer, H. Heide et al., "Assembly and oligomerization of human ATP synthase lacking mitochondrial subunits a and A6L," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1797, no. 6-7, pp. 1004–1011, 2010.
- [127] L. Azzolin, S. von Stockum, E. Basso, V. Petronilli, M. A. Forte, and P. Bernardi, "The mitochondrial permeability transition from yeast to mammals," *FEBS Letters*, vol. 584, no. 12, pp. 2504–2509, 2010.
- [128] G. I. Belogradov, J. M. Tomich, and Y. Hatefi, "Membrane topography and near-neighbor relationships of the mitochondrial ATP synthase subunits e, f, and g," *The Journal of Biological Chemistry*, vol. 271, no. 34, pp. 20340–20345, 1996.
- [129] S. Joshi and J. B. Hughes, "Inhibition of coupling factor B activity by cadmium ion, arsenite-2,3-dimercaptopropanol, and phenylarsine oxide, and preferential reactivation by dithiols," *The Journal of Biological Chemistry*, vol. 256, no. 21, pp. 11112–11116, 1981.
- [130] F. Dabbeni-Sala, G. Lippe, and M. C. Sorgato, "Structural and functional modifications induced by diamide on the F₀ sector of the mammalian ATP synthase," *FEBS Letters*, vol. 281, no. 1-2, pp. 47–50, 1991.
- [131] G. Lippe, F. Dabbeni Sala, and M. C. Sorgato, "ATP synthase complex from beef heart mitochondria. Role of the thiol group of the 25-kDa subunit of F₀ in the coupling mechanism between F₀ and F₁," *The Journal of Biological Chemistry*, vol. 263, no. 35, pp. 18627–18634, 1988.
- [132] F. Zanotti, F. Guerrieri, G. Capozza, M. Fiermonte, J. Berden, and S. Papa, "Role of F₀ and F₁ subunits in the gating and coupling function of mitochondrial H⁺-ATP synthase. The effect of dithiol reagents," *European Journal of Biochemistry*, vol. 208, no. 1, pp. 9–16, 1992.
- [133] L. A. Baker, I. N. Watt, M. J. Runswick, J. E. Walker, and J. L. Rubinstein, "Arrangement of subunits in intact mammalian mitochondrial ATP synthase determined by cryo-EM," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 29, pp. 11675–11680, 2012.
- [134] V. Petronilli, J. Šileikyte, A. Zulian et al., "Switch from inhibition to activation of the mitochondrial permeability transition during hemoxylin-mediated photooxidative stress. Unmasking pore-regulating external thiols," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1787, no. 7, pp. 897–904, 2009.
- [135] M. J. Kohr, A. M. Aponte, J. Sun et al., "Characterization of potential S-nitrosylation sites in the myocardium," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 4, pp. H1327–H1335, 2011.
- [136] M. J. Kohr, J. Sun, A. Aponte et al., "Simultaneous measurement of protein oxidation and S-nitrosylation during preconditioning and ischemia/reperfusion injury with resin-assisted capture," *Circulation Research*, vol. 108, no. 4, pp. 418–426, 2011.
- [137] T. T. Nguyen, M. V. Stevens, M. Kohr, C. Steenbergen, M. N. Sack, and E. Murphy, "Cysteine 203 of cyclophilin D is critical for cyclophilin D activation of the mitochondrial permeability transition pore," *The Journal of Biological Chemistry*, vol. 286, no. 46, pp. 40184–40192, 2011.
- [138] D. Linard, A. Kandlbinder, H. Degand, P. Morsomme, K.-J. Dietz, and B. Knoops, "Redox characterization of human

- cyclophilin D: identification of a new mammalian mitochondrial redox sensor?" *Archives of Biochemistry and Biophysics*, vol. 491, no. 1-2, pp. 39–45, 2009.
- [139] J. Traba, A. Del Arco, M. R. Duchon, G. Szabadkai, and J. Satrústegui, "SCaMC-1 promotes cancer cell survival by desensitizing mitochondrial permeability transition via ATP/ADP-mediated matrix Ca^{2+} buffering," *Cell Death and Differentiation*, vol. 19, no. 4, pp. 650–660, 2012.
- [140] G. Chanoit, S. Lee, J. Xi et al., "Exogenous zinc protects cardiac cells from reperfusion injury by targeting mitochondrial permeability transition pore through inactivation of glycogen synthase kinase-3 β ," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 295, no. 3, pp. H1227–H1233, 2008.
- [141] M. Juhaszova, D. B. Zorov, Y. Yaniv, H. B. Nuss, S. Wang, and S. J. Sollott, "Role of glycogen synthase kinase-3 β in cardioprotection," *Circulation Research*, vol. 104, no. 11, pp. 1240–1252, 2009.
- [142] T. Miura and T. Miki, "GSK-3 β , a therapeutic target for cardiomyocyte protection," *Circulation Journal*, vol. 73, no. 7, pp. 1184–1192, 2009.
- [143] T. Miura and M. Tanno, "Mitochondria and GSK-3 β in cardioprotection against ischemia/reperfusion injury," *Cardiovascular Drugs and Therapy*, vol. 24, no. 3, pp. 255–263, 2010.
- [144] K. Otori, T. Miura, M. Tanno et al., "Ser9 phosphorylation of mitochondrial GSK-3 β is a primary mechanism of cardiomyocyte protection by erythropoietin against oxidant-induced apoptosis," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 295, no. 5, pp. H2079–H2086, 2008.
- [145] J. A. McCubrey, L. S. Steelman, W. H. Chappell et al., "Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1773, no. 8, pp. 1263–1284, 2007.
- [146] T. D. King, B. Clodfelder-Miller, K. A. Barksdale, and G. N. Bijur, "Unregulated mitochondrial GSK3 β activity results in NADH: ubiquinone oxidoreductase deficiency," *Neurotoxicity Research*, vol. 14, no. 4, pp. 367–382, 2008.
- [147] A. Petit-Paitel, F. Brau, J. Cazareth, and J. Chabry, "Involvement of cytosolic and mitochondrial GSK-3 β in mitochondrial dysfunction and neuronal cell death of MPTP/MPP+ treated neurons," *PLoS ONE*, vol. 4, no. 5, Article ID e5491, 2009.
- [148] S. Shanmughapriya, S. Rajan, N. Hoffman et al., "SPG7 is an essential and conserved component of the mitochondrial permeability transition pore," *Molecular Cell*, vol. 60, no. 1, pp. 47–62, 2015.
- [149] P. Bernardi, F. Di Lisa, F. Fogolari, and G. Lippe, "From ATP to PTP and back: a dual function for the mitochondrial ATP synthase," *Circulation Research*, vol. 116, no. 11, pp. 1850–1862, 2015.
- [150] F. Di Lisa and P. Bernardi, "Modulation of mitochondrial permeability transition in ischemia-reperfusion injury of the heart. Advantages and limitations," *Current Medicinal Chemistry*, vol. 22, no. 20, pp. 2480–2487, 2015.
- [151] E. J. Griffiths and A. P. Halestrap, "Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts," *Journal of Molecular and Cellular Cardiology*, vol. 25, no. 12, pp. 1461–1469, 1993.
- [152] X. Pan, J. Liu, T. Nguyen et al., "The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter," *Nature Cell Biology*, vol. 15, no. 12, pp. 1464–1472, 2013.
- [153] J. Q. Kwong, X. Lu, R. N. Correll et al., "The mitochondrial calcium uniporter selectively matches metabolic output to acute contractile stress in the heart," *Cell Reports*, vol. 12, no. 1, pp. 15–22, 2015.
- [154] T. S. Luongo, J. P. Lambert, A. Yuan et al., "The mitochondrial calcium uniporter matches energetic supply with cardiac workload during stress and modulates permeability transition," *Cell Reports*, vol. 12, no. 1, pp. 23–34, 2015.
- [155] B. Liu, A. K. Tewari, L. Zhang et al., "Proteomic analysis of protein tyrosine nitration after ischemia reperfusion injury: mitochondria as the major target," *Biochimica et Biophysica Acta*, vol. 1794, no. 3, pp. 476–485, 2009.
- [156] M. Ristow and K. Schmeisser, "Mitohormesis: promoting health and lifespan by increased levels of reactive oxygen species (ROS)," *Dose-Response*, vol. 12, no. 2, pp. 288–341, 2014.
- [157] J. Yun and T. Finkel, "Mitohormesis," *Cell Metabolism*, vol. 19, no. 5, pp. 757–766, 2014.
- [158] H. Otani, "Reactive oxygen species as mediators of signal transduction in ischemic preconditioning," *Antioxidants and Redox Signaling*, vol. 6, no. 2, pp. 449–469, 2004.
- [159] G. Tong, A. M. Aponte, M. J. Kohr, C. Steenbergen, E. Murphy, and J. Sun, "Postconditioning leads to an increase in protein S-nitrosylation," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 306, no. 6, pp. H825–H832, 2014.
- [160] H. S. Chung, S.-B. Wang, V. Venkatraman, C. I. Murray, and J. E. Van Eyk, "Cysteine oxidative posttranslational modifications: emerging regulation in the cardiovascular system," *Circulation Research*, vol. 112, no. 2, pp. 382–392, 2013.
- [161] R. S. Sohal and R. Weindruch, "Oxidative stress, caloric restriction, and aging," *Science*, vol. 273, no. 5271, pp. 59–63, 1996.
- [162] R. S. Balaban, S. Nemoto, and T. Finkel, "Mitochondria, oxidants, and aging," *Cell*, vol. 120, no. 4, pp. 483–495, 2005.
- [163] M. F. Beal, B. T. Hyman, and W. Koroshetz, "Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases?" *Trends in Neurosciences*, vol. 16, no. 4, pp. 125–131, 1993.
- [164] M. Comelli, G. Lippe, and I. Mavelli, "Differentiation potentiates oxidant injury to mitochondria by hydrogen peroxide in Friend's erythroleukemia cells," *FEBS Letters*, vol. 352, no. 1, pp. 71–75, 1994.
- [165] Y. Zhang, O. Marcillat, C. Giulivi, L. Ernster, and K. J. A. Davies, "The oxidative inactivation of mitochondrial electron transport chain components and ATPase," *Journal of Biological Chemistry*, vol. 265, no. 27, pp. 16330–16336, 1990.
- [166] H. F. Poon, V. Calabrese, M. Calvani, and D. A. Butterfield, "Proteomics analyses of specific protein oxidation and protein expression in aged rat brain and its modulation by l-acetylcarnitine: insights into the mechanisms of action of this proposed therapeutic agent for CNS disorders associated with oxidative stress," *Antioxidants and Redox Signaling*, vol. 8, no. 3-4, pp. 381–394, 2006.
- [167] R. Sultana, H. F. Poon, J. Cai et al., "Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach," *Neurobiology of Disease*, vol. 22, no. 1, pp. 76–87, 2006.
- [168] K. Groebe, F. Krause, B. Kunstmann et al., "Differential proteomic profiling of mitochondria from *Podospora anserina*, rat and human reveals distinct patterns of age-related oxidative changes," *Experimental Gerontology*, vol. 42, no. 9, pp. 887–898, 2007.

- [169] J. Grosskreutz, L. Van Den Bosch, and B. U. Keller, "Calcium dysregulation in amyotrophic lateral sclerosis," *Cell Calcium*, vol. 47, no. 2, pp. 165–174, 2010.
- [170] H. Kawamata and G. Manfredi, "Mitochondrial dysfunction and intracellular calcium dysregulation in ALS," *Mechanisms of Ageing and Development*, vol. 131, no. 7–8, pp. 517–526, 2010.
- [171] J. S. Beckman, M. Carson, C. D. Smith, and W. H. Koppenol, "ALS, SOD and peroxynitrite," *Nature*, vol. 364, no. 6438, p. 584, 1993.
- [172] M. F. Beal, R. J. Ferrante, S. E. Browne, R. T. Matthews, N. W. Kowall, and R. H. Brown Jr., "Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis," *Annals of Neurology*, vol. 42, no. 4, pp. 644–654, 1997.
- [173] J.-F. Collard, F. Cote, and J.-P. Julien, "Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis," *Nature*, vol. 375, no. 6526, pp. 61–64, 1995.
- [174] M. Damiano, A. A. Starkov, S. Petri et al., "Neural mitochondrial Ca^{2+} capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice," *Journal of Neurochemistry*, vol. 96, no. 5, pp. 1349–1361, 2006.
- [175] P. A. Parone, S. Da Cruz, J. S. Han et al., "Enhancing mitochondrial calcium buffering capacity reduces aggregation of misfolded SOD1 and motor neuron cell death without extending survival in mouse models of inherited amyotrophic lateral sclerosis," *Journal of Neuroscience*, vol. 33, no. 11, pp. 4657–4671, 2013.
- [176] P. Pasinelli and R. H. Brown, "Molecular biology of amyotrophic lateral sclerosis: insights from genetics," *Nature Reviews Neuroscience*, vol. 7, no. 9, pp. 710–723, 2006.
- [177] S. Boill e, C. Vande Velde, and D. W. Cleveland, "ALS: a disease of motor neurons and their nonneuronal neighbors," *Neuron*, vol. 52, no. 1, pp. 39–59, 2006.
- [178] C. Haass and D. J. Selkoe, "Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β -peptide," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 2, pp. 101–112, 2007.
- [179] C. A. Hansson Petersen, N. Alikhani, H. Behbahani et al., "The amyloid β -peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 35, pp. 13145–13150, 2008.
- [180] M. A. Smith, P. L. Richey Harris, L. M. Sayre, J. S. Beckman, and G. Perry, "Widespread peroxynitrite-mediated damage in Alzheimer's disease," *Journal of Neuroscience*, vol. 17, no. 8, pp. 2653–2657, 1997.
- [181] J. Hu, F. Castets, J. L. Guevara, and L. J. Van Eldiki, "S100 β stimulates inducible nitric oxide synthase activity and mRNA levels in rat cortical astrocytes," *The Journal of Biological Chemistry*, vol. 271, no. 5, pp. 2543–2547, 1996.
- [182] F. Rossi and E. Bianchini, "Synergistic induction of nitric oxide by β -amyloid and cytokines in astrocytes," *Biochemical and Biophysical Research Communications*, vol. 225, no. 2, pp. 474–478, 1996.
- [183] M. N. Wallace, J. G. Geddes, D. A. Farquhar, and M. R. Masson, "Nitric oxide synthase in reactive astrocytes adjacent to β -amyloid plaques," *Experimental Neurology*, vol. 144, no. 2, pp. 266–272, 1997.
- [184] K. T. Akama, C. Albanese, R. G. Pestell, and L. J. Van Eldik, "Amyloid β -peptide stimulates nitric oxide production in astrocytes through an NF κ B-dependent mechanism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 10, pp. 5795–5800, 1998.
- [185] C. S. Casley, L. Canevari, J. M. Land, J. B. Clark, and M. A. Sharpe, " β -Amyloid inhibits integrated mitochondrial respiration and key enzyme activities," *Journal of Neurochemistry*, vol. 80, no. 1, pp. 91–100, 2002.
- [186] L. Canevari, J. B. Clark, and T. E. Bates, " β -Amyloid fragment 25–35 selectively decreases complex IV activity in isolated mitochondria," *FEBS Letters*, vol. 457, no. 1, pp. 131–134, 1999.
- [187] C. S. Casley, J. M. Land, M. A. Sharpe, J. B. Clark, M. R. Duchon, and L. Canevari, " β -Amyloid fragment 25–35 causes mitochondrial dysfunction in primary cortical neurons," *Neurobiology of Disease*, vol. 10, no. 3, pp. 258–267, 2002.
- [188] A. Demuro, I. Parker, and G. E. Stutzmann, "Calcium signaling and amyloid toxicity in Alzheimer disease," *The Journal of Biological Chemistry*, vol. 285, no. 17, pp. 12463–12468, 2010.
- [189] H. Du, L. Guo, F. Fang et al., "Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease," *Nature Medicine*, vol. 14, no. 10, pp. 1097–1105, 2008.
- [190] M. Boada, C. Ant nez, R. Ram rez-Lorca et al., "ATP5H/KCTD2 locus is associated with Alzheimer's disease risk," *Molecular Psychiatry*, vol. 19, no. 6, pp. 682–687, 2014.
- [191] P. K. Stys, G. W. Zamponi, J. van Minnen, and J. J. G. Geurts, "Will the real multiple sclerosis please stand up?" *Nature Reviews Neuroscience*, vol. 13, no. 7, pp. 507–514, 2012.
- [192] E. M. Frohman, M. K. Racke, and C. S. Raine, "Multiple sclerosis—the plaque and its pathogenesis," *The New England Journal of Medicine*, vol. 354, no. 9, pp. 942–955, 2006.
- [193] M. Forte, B. G. Gold, G. Marracci et al., "Cyclophilin D inactivation protects axons in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 18, pp. 7558–7563, 2007.
- [194] C. Savino, P. Pelicci, and M. Giorgio, "The P66Shc/mitochondrial permeability transition pore pathway determines neurodegeneration," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 719407, 7 pages, 2013.
- [195] S. Azzam, L. Broadwater, S. Li, E. J. Freeman, J. McDonough, and R. B. Gregory, "A SELDI mass spectrometry study of experimental autoimmune encephalomyelitis: sample preparation, reproducibility, and differential protein expression patterns," *Proteome Science*, vol. 11, no. 1, article 19, 2013.
- [196] S. E. Ahari, M. Houshmand, M. S. Panahi, S. Kasraie, M. Moin, and M. A. Bahar, "Investigation on mitochondrial tRNA(Leu/Lys), NDI and ATPase 6/8 in Iranian multiple sclerosis patients," *Cellular and Molecular Neurobiology*, vol. 27, no. 6, pp. 695–700, 2007.
- [197] A. W. Johnson, J. M. Land, E. J. Thompson, J. P. Bolanos, J. E. Clark, and J. R. Heales, "Evidence for increased nitric oxide production in multiple sclerosis," *Journal of Neurology Neurosurgery and Psychiatry*, vol. 58, no. 1, p. 107, 1995.
- [198] L. B , T. M. Dawson, S. Wesselingh et al., "Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains," *Annals of Neurology*, vol. 36, no. 5, pp. 778–786, 1994.
- [199] Y. Okuda, Y. Nakatsuji, H. Fujimura et al., "Expression of the inducible isoform of nitric oxide synthase in the central nervous system of mice correlates with the severity of actively induced experimental allergic encephalomyelitis," *Journal of Neuroimmunology*, vol. 62, no. 1, pp. 103–112, 1995.
- [200] D. M. Grzybicki, K. Kwack, S. Perlman, and S. P. Murphy, "Nitric oxide synthase type II expression by different cell types in MHV-JHM encephalitis suggests distinct roles for nitric oxide

- in acute versus persistent virus infection," *Journal of Neuroimmunology*, vol. 73, no. 1-2, pp. 15–27, 1997.
- [201] O. Bagasra, F. H. Michaels, Y. M. Zheng et al., "Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 26, pp. 12041–12045, 1995.
- [202] F. Lu, M. Selak, J. O'Connor et al., "Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis," *Journal of the Neurological Sciences*, vol. 177, no. 2, pp. 95–103, 2000.
- [203] D. Ding, M. Enriquez-Algeciras, K. R. Dave, M. Perez-Pinzon, and S. K. Bhattacharya, "The role of deimination in ATP5b mRNA transport in a transgenic mouse model of multiple sclerosis," *EMBO Reports*, vol. 13, no. 3, pp. 230–236, 2012.
- [204] S. Büttner, L. Habernig, F. Broeskamp et al., "Endonuclease G mediates α -synuclein cytotoxicity during Parkinson's disease," *The EMBO Journal*, vol. 32, no. 23, pp. 3041–3054, 2013.
- [205] E. S. Luth, I. G. Stavrovskaya, T. Bartels, B. S. Kristal, and D. J. Selkoe, "Soluble, prefibrillar α -synuclein oligomers promote complex I-dependent, Ca^{2+} -induced mitochondrial dysfunction," *The Journal of Biological Chemistry*, vol. 289, no. 31, pp. 21490–21507, 2014.
- [206] L. J. Martin, S. Semenkov, A. Hanaford, and M. Wong, "The mitochondrial permeability transition pore regulates Parkinson's disease development in mutant α -synuclein transgenic mice," *Neurobiology of Aging*, vol. 35, no. 5, pp. 1132–1152, 2014.
- [207] J. T. Greenamyre, T. B. Sherer, R. Betarbet, and A. V. Panov, "Complex I and Parkinson's disease," *IUBMB Life*, vol. 52, no. 3–5, pp. 135–141, 2002.
- [208] T. A. Seaton, J. M. Cooper, and A. H. V. Schapira, "Cyclosporin inhibition of apoptosis induced by mitochondrial complex I toxins," *Brain Research*, vol. 809, no. 1, pp. 12–17, 1998.
- [209] E. M. Valente, P. M. Abou-Sleiman, V. Caputo et al., "Hereditary early-onset Parkinson's disease caused by mutations in PINK1," *Science*, vol. 304, no. 5674, pp. 1158–1160, 2004.
- [210] R. S. Akundi, Z. Huang, J. Eason et al., "Increased mitochondrial calcium sensitivity and abnormal expression of innate immunity genes precede dopaminergic defects in Pink1-deficient mice," *PLoS ONE*, vol. 6, no. 1, Article ID e16038, 2011.
- [211] S. Gandhi, A. Wood-Kaczmar, Z. Yao et al., "PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death," *Molecular Cell*, vol. 33, no. 5, pp. 627–638, 2009.
- [212] C. A. Gautier, E. Giaime, E. Caballero et al., "Regulation of mitochondrial permeability transition pore by PINK1," *Molecular Neurodegeneration*, vol. 7, article 22, 2012.
- [213] S. Kawajiri, S. Saiki, S. Sato, and N. Hattori, "Genetic mutations and functions of PINK1," *Trends in Pharmacological Sciences*, vol. 32, no. 10, pp. 573–580, 2011.
- [214] P. Brundin, J.-Y. Li, J. L. Holton, O. Lindvall, and T. Revesz, "Research in motion: the enigma of Parkinson's disease pathology spread," *Nature Reviews Neuroscience*, vol. 9, no. 10, pp. 741–745, 2008.
- [215] J. J. Palacino, D. Sagi, M. S. Goldberg et al., "Mitochondrial dysfunction and oxidative damage in parkin-deficient mice," *The Journal of Biological Chemistry*, vol. 279, no. 18, pp. 18614–18622, 2004.
- [216] W. Hu, L.-S. Guan, X.-B. Dang, P.-Y. Ren, and Y.-L. Zhang, "Small-molecule inhibitors at the PSD-95/nNOS interface attenuate MPP⁺-induced neuronal injury through Sirt3 mediated inhibition of mitochondrial dysfunction," *Neurochemistry International*, vol. 79, pp. 57–64, 2014.
- [217] F. Darios, O. Corti, C. B. Lücking et al., "Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death," *Human Molecular Genetics*, vol. 12, no. 5, pp. 517–526, 2003.

Clinical Study

Magnesium Supplementation Diminishes Peripheral Blood Lymphocyte DNA Oxidative Damage in Athletes and Sedentary Young Man

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Sedentary lifestyle is highly associated with increased risk of cardiovascular disease, obesity, and type 2 diabetes. It is known that regular physical activity has positive effects on health; however several studies have shown that acute and strenuous exercise can induce oxidative stress and lead to DNA damage. As magnesium is essential in maintaining DNA integrity, the aim of this study was to determine whether four-week-long magnesium supplementation in students with sedentary lifestyle and rugby players could prevent or diminish impairment of DNA. By using the comet assay, our study demonstrated that the number of peripheral blood lymphocytes (PBL) with basal endogenous DNA damage is significantly higher in rugby players compared to students with sedentary lifestyle. On the other hand, magnesium supplementation significantly decreased the number of cells with high DNA damage, in the presence of exogenous H₂O₂, in PBL from both students and rugby players, and markedly reduced the number of cells with medium DNA damage in rugby players compared to corresponding control nonsupplemented group. Accordingly, the results of our study suggest that four-week-long magnesium supplementation has marked effects in protecting the DNA from oxidative damage in both rugby players and in young men with sedentary lifestyle. Clinical trial is registered at ANZCTR Trial Id: ACTRN12615001237572.

1. Introduction

Modern age has brought upon a life style that is, in young people particularly, accompanied by lack of sleep, imbalanced diet including fast food consumption, excessive amount of stress, reduced physical activity, and alcohol abuse. All these factors contribute to excess inflammation and oxidative stress and exhibit detrimental influence on human genome [1–4]. Each day, the human genome suffers approximately one million lesions, including adducts, modifications, or fragmentation of the sugar phosphate backbone of DNA [5]. If left unrepaired, DNA damage can cause mutations such as base substitutions and chromosomal translocations that disrupt normal gene expression or create abnormal proteins that are detrimental to cellular function or viability [6].

Both endogenous and exogenous factors can increase production of reactive oxygen species (ROS) and induce DNA damage [7]. Endogenous factors include products of cellular metabolism, such as ROS created via mitochondrial oxidative respiration, or produced by lipid peroxidation, and during processes such as phagocytosis. Endogenous damage may also occur due to errors arising from normal cell replication [8, 9]. Exogenous factors, on the other hand, include improper diet, alcohol, cigarette smoking, and environmental toxins [8, 10]. Evidence indicates that oxidative stress induced DNA damage and impaired DNA repair mechanisms are involved in the pathogenesis of cancer, atherosclerosis, neurodegenerative disorders, and chronic lung diseases [11, 12].

Even though physical exercise is considered to have beneficial effects on health, many, but not all studies on this

topic, gave evidence that acute and strenuous exercise can induce oxidative stress [13]. Acute and strenuous exercise lead to oxidative stress by excessive production of ROS and reactive nitrogen species (RNS), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\cdot}), hypochlorous acid (HOCl), and nitric oxide (NO^{\cdot}) that exhibit detrimental effects on macromolecules such as lipids and proteins, but mainly the DNA [13, 14]. During exercise, oxygen consumption can increase up to 10- to 15-fold above resting levels, thus temporarily increasing the rate of mitochondrial free radicals production. Exercise may also induce inflammatory reactions similar to the acute phase response occurring in injury or infection [15].

Rugby matches are considered to be very demanding and cause both physical and psychological stress in participants [16, 17]. Various studies evaluated the changes in immunoendocrine and inflammatory markers, neutrophil function (production of ROS and phagocytic activity), and the recovery time-course of neuromuscular function, concentrations of testosterone and cortisol, mood impairments, and muscle damage after intense competitive physical performance, such as a rugby game [16, 18–21]. Nevertheless, there are few evidences concerning the correlation between rugby-induced stress and the level of oxidative damage [18, 21].

On the other hand, magnesium as an essential element is involved in regulation of cell cycle and apoptosis, stabilizes the structure of nucleic acids [22], protects DNA from alkylation, and has a role as a cofactor of the enzymes of nucleic acid metabolism: it is involved in DNA replication, DNA repair, and gene expression [23, 24].

Having all abovementioned in mind, the aim of this study was to investigate whether four-week-long magnesium supplementation in young men, with either sedentary life style such as students or athletes involved in strenuous exercise such as rugby players, could protect peripheral blood lymphocytes (PBL) from hydrogen peroxide-induced DNA damage evaluated by alkaline Comet test.

2. Materials and Methods

2.1. Subjects. Twenty-three healthy young male subjects volunteered for this study, thirteen were members of the same amateur rugby team, and the other ten were students of the University of Belgrade with sedentary life style. The participants were divided into four groups, as follows:

Group 1: students with a sedentary life style, without magnesium supplementation ($n = 5$).

Group 2: students with a sedentary life style, receiving 500 mg of magnesium per day divided in two doses separated by 12 h interval for 28 days ($n = 5$).

Group 3: rugby players, without magnesium supplementation ($n = 5$).

Group 4: rugby players receiving 500 mg of magnesium per day divided in two doses (one tablet of magnesium 250 mg[®]. Natural Wealth, NBTY Inc.) separated by 12 h interval for 28 days ($n = 8$).

Students had not been involved in any regular exercise for at least six months and they remained sedentary during the whole study. Rugby players trained three to four times a week for 2 hours and played a match on each Sunday in the early afternoon. Three months before and during the study, none of the participants was taking other vitamin or mineral supplements. All participants gave their written consent to participate after being fully informed of all experimental procedures. The investigation was carried out according to the guidelines and study protocol that has been approved by the Ethical Committee for Clinical trials of University of Belgrade, Faculty of Pharmacy, number 199/2.

2.2. Anthropometric Data. Rugby players involved in this study were 23.30 ± 0.93 years old and had average height 181.26 ± 1.30 cm, body mass 85.30 ± 2.31 kg, and body mass index (BMI) 25.91 ± 0.51 kg/m² (Mean \pm SD). Mean age of students was 22.6 ± 0.52 years, height was 186.9 ± 6.42 cm, body mass was 84.4 ± 6.60 kg, and BMI was 24.16 ± 1.41 kg/m².

2.3. Blood Collection. On the 29th day of experiment (first day after 28 days of supplementation) peripheral blood samples were collected from the participants of this study. In the morning from 09.00–10.00 a.m. (about 20 h after the rugby match for athletes), blood was drawn from an antecubital vein in sitting position into 2 mL EDTA Vacutainer tubes and comet assay was performed.

2.4. Comet Assay (Single Cell Gel Electrophoresis (SCGE)). The comet assay is highly reproducible, rapid, and sensitive method for measuring DNA damage [25, 26]. The comet test was conducted as described by Singh et al. [25]. Microscope slides were coated with a layer of 1% normal melting point agarose (Sigma-Aldrich, St. Louis, MO) and left to dry on room temperature. The 6 μ L of whole blood samples was suspended in 0.67% low melting point (LMP) agarose (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS, Torlak Institute of Immunology and Virology, Belgrade, Serbia), applied onto microscope slides (prepared on previously described way) and maintained on 4°C for 5 min, to solidify. Then a second layer of 0.5% LMP agarose was pipetted onto microscope slides. Every sample had a matching positive control that was treated with hydrogen peroxide (1.5 mM H_2O_2) and then after 5 min on 4°C with second layer of 0.5% LMP agarose. The following step included immersing all slides in lysing solution (2.5 M NaCl, 100 mM ethylene-diaminetetra-acetic acid, 10 mM Tris at pH 10, 1% Triton X-100 and 10% dimethylsulfoxide, pH 10 adjusted with NaOH) on 4°C and leaving them overnight. After that, DNA in lysed cells was allowed to unwind in alkali buffer (300 mM NaOH, 1 mM EDTA) for 30 min. Samples were then subjected to electrophoresis for another 30 min at 215 mA, 25 V, washed three times (for 5 min each slide) with a neutralizing buffer (0.4 M Tris, pH 7.5), and stained with ethidium bromide (20 μ g/mL). Cover slips were then placed on top of microscope slides and DNA damage was visually analyzed. Analyses were performed on Olympus BX 50 microscope (Olympus Optical Co., GmbH, Hamburg,

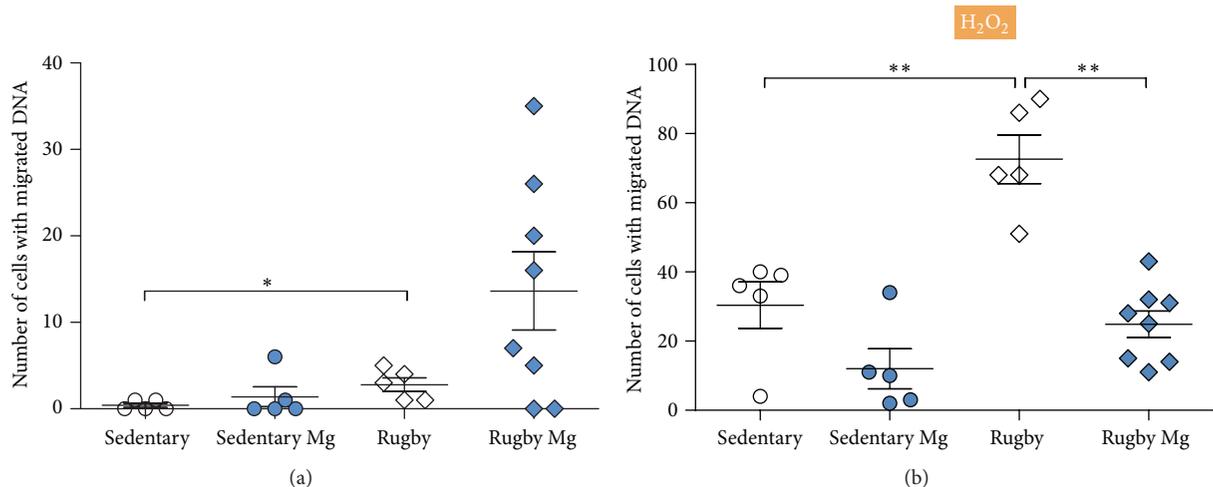


FIGURE 1: Effects of 4-week magnesium supplementation on number of peripheral blood lymphocytes (PBL) with DNA damage, scored by Comet assay. Participants were divided in four groups in total, due to Mg supplementation and level of physical activity: sedentary, sedentary with Mg supplementation (sedentary Mg), rugby players (rugby), and rugby players with Mg supplementation (rugby Mg). (a) Basal number of cells with migrated DNA. Number of participants per group: sedentary ($n = 5$), rugby ($n = 5$), sedentary Mg ($n = 5$), and rugby Mg ($n = 8$). (b) Number of cells with migrated DNA after exposure to 1.5 mM H_2O_2 . Number of participants per group: sedentary ($n = 5$), rugby ($n = 5$), sedentary Mg ($n = 5$), and rugby Mg ($n = 8$). Results are shown as means \pm SEM. The difference obtained was considered to be statistically significant when $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$).

Germany) equipped with a mercury lamp HBO (50 W, 516–560 nm, Zeiss) at 100x magnification.

Cells were graded by eye, as it was described by Anderson et al. [27], into 5 categories based on perceived comet tail length migration and relative proportion of DNA in the comet tail: no damage (<5%, class A); low level of damage (5–20%, class B); medium level of damage (20–40%, class C); high level of damage (40–95%, class D); and total damage (>95%, class E). For each blood sample comet assay was conducted on two microscope slides and 100 cells were analyzed (50 cells per slide).

2.5. Statistical Analysis. Statistical analysis was performed using SigmaPlot 11.0. Student's t -test and Mann-Whitney Rank Sum test were used in order to compare the difference between the groups and p values less than 0.05 were considered significant. The results were expressed as mean \pm SEM.

3. Results

Results from our study show that the level of basal endogenous DNA damage presented as number of cells with migrated DNA (Figure 1(a)) was higher in nonsupplemented rugby players compared to nonsupplemented students ($p = 0.042$). Likewise, in these groups, the levels of DNA damage in cells exposed to H_2O_2 were significantly higher in rugby players ($p = 0.002$) (Figure 1(b)).

After four-week-long magnesium supplementation there was no statistically significant difference between students with sedentary lifestyle and rugby players that received supplementation in the total number of cells with damaged nuclei, in the presence of H_2O_2 ($p = 0.080$) (Figure 1(b)). However, magnesium supplementation had

more pronounced effects in rugby players: athletes that received supplementation had significantly smaller total number of cells with DNA damage, in the presence of H_2O_2 , compared to rugby players from control group ($p = 0.002$) (Figure 1(b)).

Furthermore, the evaluation of degree of DNA damage was done, and scores were divided into low + medium damage and high + total damage and results are presented in Figure 2. There were no significant differences in the degree of DNA damage among groups in the absence of H_2O_2 . However, after four-week-long magnesium supplementation, the number of cells with low and medium damage (B + C), after exposure to H_2O_2 , was significantly smaller in rugby players compared to age-matched controls ($p = 0.002$) (Figure 2(a)). Interestingly, this effect was not observed in students compared to age-matched controls ($p = 0.099$) (Figure 2(a)). However, as presented in Figure 2(b), magnesium supplementation was effective in diminishing the number of cells with highly damaged DNA in both students and rugby players compared to respective controls. Results shown in this figure reveal that after H_2O_2 exposure the median number of cells with high and total damaged DNA (D + E category) is significantly lower in students and rugby players that received supplementation ($p = 0.025$ and $p = 0.002$, resp.). Also, the number of cells with highly and totally damaged nuclei is significantly higher in rugby players than students in groups that did not receive supplementation ($p < 0.0001$) (Figure 2(b)).

4. Discussion

In the present study DNA damage in PBL of rugby players and age-matched group of sedentary young man, university

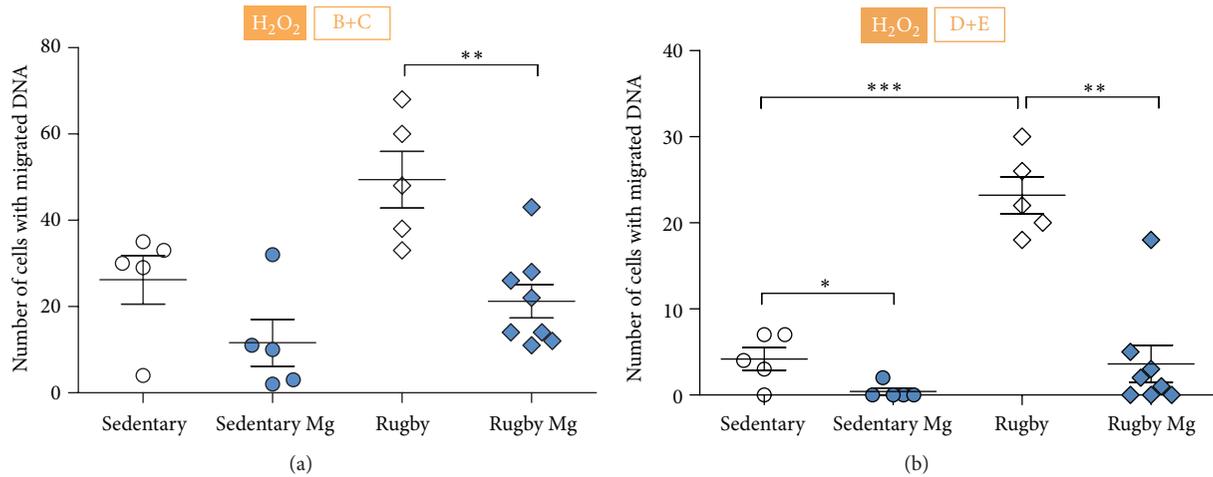


FIGURE 2: Effects of 4-week magnesium supplementation on the level of DNA damage induced by exposure to 1.5 mM H₂O₂ in peripheral blood lymphocytes (PBL), scored by Comet assay. Participants were divided into four groups in total, due to Mg supplementation and level of physical activity: sedentary, sedentary with Mg supplementation (sedentary Mg), rugby players (rugby), and rugby players with Mg supplementation (rugby Mg). (a) Number of cells with low and medium level of DNA damage (B and C). Number of participants per group: sedentary ($n = 5$), rugby ($n = 5$), sedentary Mg ($n = 5$), and rugby Mg ($n = 8$). (b) Number of cells with high and total level of DNA damage (D and E). Number of participants per group: sedentary ($n = 5$), rugby ($n = 5$), sedentary Mg ($n = 5$), and rugby Mg ($n = 8$). The difference obtained was considered to be statistically significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

students, was compared after four-week-long Mg supplementation. DNA damage was assessed by alkaline Comet test in the absence or presence of H₂O₂. Results of the study showed that PBL of rugby players were more susceptible to DNA damage compared to sedentary young man, both with and without H₂O₂ challenge. Furthermore, this study showed that even though Mg supplementation had no effect on DNA damage in unchallenged lymphocytes, it reduced level of high-damaged H₂O₂-treated cells in both groups of participants.

Comet assay is used in evaluating changes in DNA integrity such as strand breaks, alkali-labile sites, DNA cross-linking, and incomplete excision repair [28]. Different methods are used nowadays in assessing the level of DNA damage such as high performance liquid chromatography, 8-OH-deoxyguanosine assay, micronuclei test, chromosome aberration, and sister chromatid exchanges. Among them, comet test has a widespread use because of its sensitivity, simplicity, and reliability [29].

In our study, the level of DNA damage expressed as the number of cells with migrated DNA was significantly increased in rugby players on the day after the match compared to sedentary students, indicating that intense exercise exhibits negative effects on DNA integrity. Thus, this study is adding up to data that are proving that strenuous physical activity may lead to DNA instability, since it was demonstrated that acute and strenuous exercise lead to oxidative stress by excessive production of ROS and RNS [13, 14]. Nevertheless, effects of competitive exercise (trainings and matches) on DNA stability, based on the conducted studies, are still controversial. Niess et al. [30] reported an increase in DNA damage in 10 out of 12 participants in a half-marathon 24 h after the race. Hartmann et al. [31]

observed similar changes in DNA damage level. Using comet assay, they found elevated DNA migration in six athletes 24 h after a short-distance triathlon which is considered to be an endurance exercise. In both studies, blood was collected and DNA migration was compared 24 h before and after the race. Hartmann et al. [31] took blood samples seven times for the next 5 days and the level of DNA migration remained to be increased 5 days after race. To the best of our knowledge, the majority of studies have shown increased levels of DNA migration 24 h after competitive endurance exercise. However, Briviba et al. [32] found no change in the DNA strand breaks in ten participants, when conducting SCGE assays just after a half-marathon and a marathon race. Nevertheless, different protocols, small number of participants, and differences in the training status of the subjects involved in these studies represent limitations in interpreting the results [30–33].

We have demonstrated that athletes receiving magnesium supplementation for one month had significantly smaller number of PBLs with damaged DNA after H₂O₂ treatment, compared to rugby players devoid of supplementation. Magnesium supplementation apparently had protective effects on DNA against oxidative damage in rugby players, since both medium and high level of H₂O₂-induced damage were decreased compared to respective levels in nonsupplemented athletes. In addition, this effect was more pronounced in rugby players compared to students in which magnesium supplementation was accompanied by the decrease of DNA damage, but the effect was statistically significant only in decreasing the number of lymphocytes with highly damaged DNA. A possible explanation for the different effect of Mg on PBL DNA damage in rugby players and sedentary young men is the fact that intensive physical activity increases

magnesium requirement. Evidence indicates that both short-term high-intensity and long-term strenuous exercise cause significantly increased loss of magnesium through urine and sweat [34]. Also, during exercise, redistribution of magnesium to certain body compartments, with increased energy and ROS production, occurs [34–36]. Therefore, some authors propose that short- and long-term strenuous exercise should be accompanied with 10–20% higher magnesium intake compared to daily intake recommendations for the persons of the same age and sex with sedentary lifestyle [34].

Both animal and human studies indicated that magnesium deficiency has a negative impact on physical performance [34, 37]. However, that is not the only negative consequence of decreased level of magnesium in the body: studies on experimental animals demonstrated that magnesium deficiency leads to swollen mitochondria and disorganized sarcoplasmic reticulum in skeletal muscles, increases formation of lipid radicals and nitric oxide, and impairs endogenous protective mechanisms such as glutathione [38, 39]. It has also been shown that vigorous exercise induces inflammatory response because of tissue injury, consequently increasing the production of ROS, especially in activated phagocytes [15, 40–42]. Takahashi et al. [18] reported no significant change in ROS production in neutrophils right after a rugby game, but they also noticed that 4 h after the end of the game it had increased significantly. Nevertheless, some authors pointed out that chronic moderate exercise may induce adaptive responses in human organism by enhancing the expression of antioxidant enzymes such as Cu/Zn superoxide dismutase (SOD) and by reducing mitochondrial hydrogen peroxide [43, 44] in the muscle tissue. The effectiveness of the adaptive mechanisms in oxidative stress induced by regular physical activity also depends on the individual's lifestyle, nutrition, and expression of genes involved in DNA repair systems [29, 45]. Future studies investigating the effects of strenuous exercise on DNA stability should include longer observation periods and monitor the DNA integrity for at least five days after the match as some authors claim that for major alterations in DNA repair mechanisms take more than 24 hours [15, 33, 46, 47].

Furthermore, it might be taken into account that greater degree of magnesium deficiency which influences antioxidant activity in PBLs makes them more susceptible to oxidative stress caused by hydrogen peroxide, since previous data showed the harmful effect of magnesium deficiency on lipid peroxidation in the cardiovascular system [48], and that tissue homogenates from magnesium deficient animals were more susceptible to lipid peroxidation than animals fed diets adequate in magnesium [48]. Nevertheless, this assumption should be explored and possibly confirmed by estimating level of magnesium in PBLs of rugby players and sedentary students, in the future investigation on the larger population.

Results of this study point to the conclusion that strenuous exercise sensitizes PBLs to oxidative stress and that magnesium supplementation shows protective effects in reducing the level of H₂O₂-induced PBL DNA damage thus indicating the importance of adequate magnesium intake in both students with sedentary life style and physically active individuals.

Competing Interests

The authors stated that there is no conflict of interests whatsoever regarding the publication of this paper.

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References

- [1] M. E. Cogswell, P. Weisberg, and C. Spong, "Cigarette smoking, alcohol use and adverse pregnancy outcomes: implications for micronutrient supplementation," *The Journal of Nutrition*, vol. 133, no. 5, pp. 1722S–1731S, 2003.
- [2] B.-T. Ji, X.-O. Shu, M. S. Linet et al., "Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers," *Journal of the National Cancer Institute*, vol. 89, no. 3, pp. 238–244, 1997.
- [3] Y.-J. Kim, Y.-C. Hong, K.-H. Lee et al., "Oxidative stress in pregnant women and birth weight reduction," *Reproductive Toxicology*, vol. 19, no. 4, pp. 487–492, 2005.
- [4] S. Raimondi, S. Garte, R. J. Sram et al., "Effects of diet on biomarkers of exposure and effects, and on oxidative damage," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 620, no. 1-2, pp. 93–102, 2007.
- [5] J. M. Skoner, J. Sigmon, and L. L. Larcom, "Suppressed DNA repair capacity of peripheral lymphocytes in pregnant women," *Molecular and Cellular Endocrinology*, vol. 108, no. 1-2, pp. 179–183, 1995.
- [6] D. L. F. Furness, G. A. Dekker, and C. T. Roberts, "DNA damage and health in pregnancy," *Journal of Reproductive Immunology*, vol. 89, no. 2, pp. 153–162, 2011.
- [7] F. J. Jenkins, B. Van Houten, and D. H. Bovbjerg, "Effects on DNA damage and/or repair processes as biological mechanisms linking psychological stress to cancer risk," *Journal of Applied Biobehavioral Research*, vol. 19, no. 1, pp. 3–23, 2014.
- [8] Y. Gidron, K. Russ, H. Tissarchondou, and J. Warner, "The relation between psychological factors and DNA-damage: a critical review," *Biological Psychology*, vol. 72, no. 3, pp. 291–304, 2006.
- [9] J. H. J. Hoeijmakers, "Genome maintenance mechanisms for preventing cancer," *Nature*, vol. 411, no. 6835, pp. 366–374, 2001.
- [10] P. Møller, H. Wallin, and L. E. Knudsen, "Oxidative stress associated with exercise, psychological stress and life-style factors," *Chemico-Biological Interactions*, vol. 102, no. 1, pp. 17–36, 1996.
- [11] B. Halliwell and J. M. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, London, UK, 3rd edition, 1999.
- [12] L. L. Wu, C.-C. Chiou, P.-Y. Chang, and J. T. Wu, "Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics," *Clinica Chimica Acta*, vol. 339, no. 1-2, pp. 1–9, 2004.
- [13] K.-H. Wagner, S. Reichhold, C. Hölzl et al., "Well-trained, healthy triathletes experience no adverse health risks regarding oxidative stress and DNA damage by participating in an ultra-endurance event," *Toxicology*, vol. 278, no. 2, pp. 211–216, 2010.

- [14] L. Packer, E. Cadenas, and K. J. A. Davies, "Free radicals and exercise: an introduction," *Free Radical Biology & Medicine*, vol. 44, no. 2, pp. 123–125, 2008.
- [15] K. Tsai, T.-G. Hsu, K.-M. Hsu et al., "Oxidative DNA damage in human peripheral leukocytes induced by massive aerobic exercise," *Free Radical Biology & Medicine*, vol. 31, no. 11, pp. 1465–1472, 2001.
- [16] B. Cunniffe, A. J. Hore, D. M. Whitcombe, K. P. Jones, J. S. Baker, and B. Davies, "Time course of changes in immuneendocrine markers following an international rugby game," *European Journal of Applied Physiology*, vol. 108, no. 1, pp. 113–122, 2010.
- [17] T. Mashiko, T. Umeda, S. Nakaji, and K. Sugawara, "Position related analysis of the appearance of and relationship between post-match physical and mental fatigue in university rugby football players," *British Journal of Sports Medicine*, vol. 38, no. 5, pp. 617–621, 2004.
- [18] I. Takahashi, T. Umeda, T. Mashiko et al., "Effects of rugby sevens matches on human neutrophil-related non-specific immunity," *British Journal of Sports Medicine*, vol. 41, no. 1, pp. 13–18, 2007.
- [19] D. J. West, C. V. Finn, D. J. Cunningham et al., "Neuromuscular function, hormonal, and mood responses to a professional rugby union match," *Journal of Strength and Conditioning Research*, vol. 28, no. 1, pp. 194–200, 2014.
- [20] M. Elloumi, F. Maso, O. Michaux, A. Robert, and G. Lac, "Behaviour of saliva cortisol [C], testosterone [T] and the T/C ratio during a rugby match and during the post-competition recovery days," *European Journal of Applied Physiology*, vol. 90, no. 1-2, pp. 23–28, 2003.
- [21] A. Lindsay, J. Healy, W. Mills et al., "Impact-induced muscle damage and urinary pterins in professional rugby: 7,8-dihydropteridine oxidation by myoglobin," *Scandinavian Journal of Medicine & Science in Sports*, vol. 26, no. 3, pp. 329–337, 2016.
- [22] J. Anastassopoulou and T. Theophanides, "Magnesium-DNA interactions and the possible relation of magnesium to carcinogenesis. Irradiation and free radicals," *Critical Reviews in Oncology/Hematology*, vol. 42, no. 1, pp. 79–91, 2002.
- [23] A. Klungland, I. Rosewell, S. Hollenbach et al., "Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13300–13305, 1999.
- [24] P. Møller and H. Wallin, "Adduct formation, mutagenesis and nucleotide excision repair of DNA damage produced by reactive oxygen species and lipid peroxidation product," *Mutation Research/Reviews in Mutation Research*, vol. 410, no. 3, pp. 271–290, 1998.
- [25] N. P. Singh, M. T. McCoy, R. R. Tice, and E. L. Schneider, "A simple technique for quantitation of low levels of DNA damage in individual cells," *Experimental Cell Research*, vol. 175, no. 1, pp. 184–191, 1988.
- [26] M. Neri, D. Milazzo, D. Ugolini et al., "Worldwide interest in the comet assay: a bibliometric study," *Mutagenesis*, vol. 30, no. 1, pp. 155–163, 2015.
- [27] D. Anderson, T.-W. Yu, B. J. Phillips, and P. Schmezer, "The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay," *Mutation Research*, vol. 307, no. 1, pp. 261–271, 1994.
- [28] S.-Y. Park, E. Cho, E. Oh, and D. Sul, "Comet assay analysis of DNA damage in T- and B-lymphocytes separated by MACS for human biomonitoring studies," *Toxicology in Vitro*, vol. 26, no. 2, pp. 369–372, 2012.
- [29] B. Tomasello, S. Grasso, G. Malfa, S. Stella, M. Favetta, and M. Renis, "Double-face activity of resveratrol in voluntary runners: assessment of DNA damage by comet assay," *Journal of Medicinal Food*, vol. 15, no. 5, pp. 441–447, 2012.
- [30] A. M. Niess, M. Baumann, K. Roecker, T. Horstmann, F. Mayer, and H.-H. Dickhuth, "Effects of intensive endurance exercise on DNA damage in leucocytes," *The Journal of Sports Medicine and Physical Fitness*, vol. 38, no. 2, pp. 111–115, 1998.
- [31] A. Hartmann, S. Pfuhrer, C. Dennog, D. Germadnik, A. Pilger, and G. Speit, "Exercise-induced DNA effects in human leukocytes are not accompanied by increased formation of 8-hydroxy-2'-deoxyguanosine or induction of micronuclei," *Free Radical Biology and Medicine*, vol. 24, no. 2, pp. 245–251, 1998.
- [32] K. Briviba, B. Watzl, K. Nickel et al., "A half-marathon and a marathon run induce oxidative DNA damage, reduce antioxidant capacity to protect DNA against damage and modify immune function in hobby runners," *Redox Report*, vol. 10, no. 6, pp. 325–331, 2005.
- [33] S. Reichhold, O. Neubauer, C. Hoelzl et al., "DNA damage in response to an Ironman triathlon," *Free Radical Research*, vol. 43, no. 8, pp. 753–760, 2009.
- [34] F. H. Nielsen and H. C. Lukaski, "Update on the relationship between magnesium and exercise," *Magnesium Research*, vol. 19, no. 3, pp. 180–189, 2006.
- [35] K. Madsen, P. K. Pedersen, M. S. Djurhuus, and N. A. Klitgaard, "Effects of detraining on endurance capacity and metabolic changes during prolonged exhaustive exercise," *Journal of Applied Physiology*, vol. 75, no. 4, pp. 1444–1451, 1993.
- [36] A. Córdova, J. F. Escanero, and M. Gimenez, "Magnesium distribution in rats after maximal exercise in air and under hypoxic conditions," *Magnesium Research*, vol. 5, no. 1, pp. 23–27, 1992.
- [37] H. C. Lukaski and F. H. Nielsen, "Dietary magnesium depletion affects metabolic responses during submaximal exercise in postmenopausal women," *Journal of Nutrition*, vol. 132, no. 5, pp. 930–935, 2002.
- [38] E. Rock, C. Astier, C. Lab et al., "Dietary magnesium deficiency in rats enhances free radical production in skeletal muscle," *The Journal of Nutrition*, vol. 125, no. 5, pp. 1205–1210, 1995.
- [39] W. B. Weglicki, I. T. Mak, B. F. Dickens et al., "Neuropeptides, free radical stress and antioxidants in models of Mg-deficient cardiomyopathy," in *Magnesium: Current Status and New Developments*, T. Theophanides and J. Anastassopoulou, Eds., pp. 169–178, Kluwer Academic, Dordrecht, Netherlands, 1997.
- [40] B. Sjodin, Y. Hellsten Westing, and F. S. Apple, "Biochemical mechanisms for oxygen free radical formation during exercise," *Sports Medicine*, vol. 10, no. 4, pp. 236–254, 1990.
- [41] E. Zerba, T. E. Komorowski, and J. A. Faulkner, "Free radical injury to skeletal muscles of young, adult, and old mice," *The American Journal of Physiology—Cell Physiology*, vol. 258, no. 3, pp. C429–C435, 1990.
- [42] N. Zamzami, P. Marchetti, M. Castedo et al., "Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death," *Journal of Experimental Medicine*, vol. 182, no. 2, pp. 367–377, 1995.
- [43] C. A. Delaney, I. C. Green, J. E. Lowe et al., "Use of the comet assay to investigate possible interactions of nitric oxide and reactive oxygen species in the induction of DNA damage

- and inhibition of function in an insulin-secreting cell line," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 375, no. 2, pp. 137–146, 1997.
- [44] D. A. Wink and J. Laval, "The Fpg protein, a DNA repair enzyme, is inhibited by the biomediator nitric oxide in vitro and in vivo," *Carcinogenesis*, vol. 15, no. 10, pp. 2125–2129, 1994.
- [45] S. Goto, H. Naito, T. Kaneko, H. Chung, and Z. Radák, "Hormetic effects of regular exercise in aging: correlation with oxidative stress," *Applied Physiology, Nutrition and Metabolism*, vol. 32, no. 5, pp. 948–953, 2007.
- [46] S. Reichhold, O. Neubauer, V. Ehrlich, S. Knasmüller, and K.-H. Wagner, "No acute and persistent DNA damage after an ironman triathlon," *Cancer Epidemiology Biomarkers & Prevention*, vol. 17, no. 8, pp. 1913–1919, 2008.
- [47] A. Mastaloudis, T.-W. Yu, R. P. O'Donnell, B. Frei, R. H. Dashwood, and M. G. Traber, "Endurance exercise results in DNA damage as detected by the comet assay," *Free Radical Biology & Medicine*, vol. 36, no. 8, pp. 966–975, 2004.
- [48] J. Busserolles, E. Gueux, E. Rock, A. Mazur, and Y. Rayssiguier, "High fructose feeding of magnesium deficient rats is associated with increased plasma triglyceride concentration and increased oxidative stress," *Magnesium Research*, vol. 16, no. 1, pp. 7–12, 2003.

Review Article

Endogenous Generation of Singlet Oxygen and Ozone in Human and Animal Tissues: Mechanisms, Biological Significance, and Influence of Dietary Components

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Recent studies have shown that exposing antibodies or amino acids to singlet oxygen results in the formation of ozone (or an ozone-like oxidant) and hydrogen peroxide and that human neutrophils produce both singlet oxygen and ozone during bacterial killing. There is also mounting evidence that endogenous singlet oxygen production may be a common occurrence in cells through various mechanisms. Thus, the ozone-producing combination of singlet oxygen and amino acids might be a common cellular occurrence. This paper reviews the potential pathways of formation of singlet oxygen and ozone *in vivo* and also proposes some new pathways for singlet oxygen formation. Physiological consequences of the endogenous formation of these oxidants in human tissues are discussed, as well as examples of how dietary factors may promote or inhibit their generation and activity.

1. Introduction

Singlet oxygen ($^1\text{O}_2$) is an electronically excited form of oxygen which is well known to be formed when photosensitizers such as chlorophyll or the aromatic dye rose bengal absorb light energy and transfer some of that energy to molecular oxygen [1, 2]. Various nonphotosensitized mechanisms for its formation have also been reported and suggested to occur in biological systems, but the importance of such endogenous singlet oxygen formation has had a controversial history [1, 3]. Ozone (O_3) is best known as occurring in the stratosphere where it shields organisms on earth from ultraviolet C and much of ultraviolet B radiations, which are the most damaging UV components of solar radiations because they are readily absorbed by DNA [4, 5]. It is also known as a respiratory system-damaging pollutant in the troposphere and ironically as a therapeutic agent in alternative medicine [6]. More recently, it was shown that antibodies or amino acids catalyze the conversion of singlet oxygen ($^1\text{O}_2$) to ozone (O_3) and that this reaction occurs during the killing of bacteria by activated neutrophils [7, 8]. Since both singlet oxygen and ozone are highly reactive oxygen species, a full understanding of their

mechanisms of formation and action *in vivo* is necessary. Hence, this paper reviews the various reported mechanisms of the endogenous formation of these reactive oxygen species (ROS), the potential relevance of such pathways in human physiology, and how dietary factors affect the generation and activity of these oxidants.

2. Radiation-Induced Formation of Singlet Oxygen

Human beings are frequently exposed to natural and artificial radiation, and most of this interacts primarily with the skin. The spectrum of solar radiation at the earth's surface consists of ultraviolet (UV) radiation (UVB: 290–320 nm and UVA: 320–400 nm), visible radiation (VIS: 400–760 nm), and near infrared radiation (IRA: 760–1440 nm and IRB: 1440–3000 nm) [9]. UV, VIS, and IR contribute 7%, 39%, and 54% of the solar energy reaching the skin [10]. Direct absorption of UVB by cellular DNA leads to formation of cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone products, while UVA is not readily absorbed by DNA, and its direct damage to DNA is therefore not important [5].

Nevertheless, both UVA and UVB as well as visible light convert various photosensitizing compounds to excited states which transfer energy to triplet oxygen, thereby generating reactive oxygen species, particularly singlet oxygen.

UVA makes up 95% of the UV reaching the human skin, and up to 50% of it can penetrate to the dermis, unlike UVB that only penetrates the epidermis [11]. The human skin is rich in UVA and visible light (particularly the blue region) photosensitizers such as porphyrins, bilirubin, flavins, melanin and melanin precursors, pterins, B₆ vitamins, and vitamin K [12, 13]. The formation of singlet oxygen in the skin as a result of the interaction of UVA with these photosensitizers has been demonstrated directly by luminescence [14] and by detection of cholesterol-5-hydroperoxide which is preferentially generated by singlet oxygen but not by free radical mediated cholesterol oxidation [2]. The interaction between UVB and various vitamins and fatty acids also results in the generation of singlet oxygen, and some compounds including vitamin E that are ordinarily not UVA photosensitizers can be converted to UVA photosensitizers if they are preirradiated with UVB [11]. Photosensitized formation of singlet oxygen also occurs in the retina, which contains endogenous photosensitizers and is exposed to light [15]. One of the singlet oxygen-generating photosensitizers is lipofuscin, which forms in the retinal pigment epithelium with age or genetic disorders such as Stargardt's disease [15, 16]. Ground state oxygen can directly absorb visible light of 765 nm, even in mammalian cells, leading to formation of singlet oxygen without the involvement of a photosensitizer [17]. Similarly, IRB of 1268 nm can cause direct conversion of ground state oxygen to singlet oxygen [18].

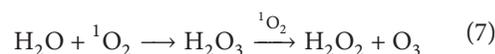
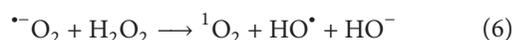
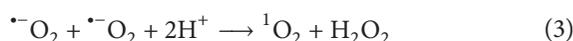
Both IRB and IRC penetrate the skin only shallowly, while IRA (which makes up 30% of the total IR radiation reaching the skin) penetrates deeply, with 65% of it reaching the dermis [19, 20]. Unlike UVA, IRA penetration of the skin does not cause photosensitized formation of singlet oxygen but initiates the formation of reactive oxygen species, mainly from the mitochondrial electron transport chain [9, 20, 21]. While singlet oxygen may be one of these ROS [9], its specific detection under such circumstances has not been studied. However, both UVA and IR induce upregulation of matrix metalloproteinases (MMPs) and thereby promote photoaging [9, 19]. The UV-induced MMP expression is dependent on cholesterol-5 hydroperoxide, a product of oxidation of cholesterol by singlet oxygen [2, 22]. Whether IR-induced metalloproteinase activation also depends to a great extent on singlet oxygen and cholesterol-5 hydroperoxide remains to be demonstrated. In this case, the role of IR in singlet oxygen formation may simply involve initiating the formation of superoxide anions, from which singlet oxygen would be generated by various types of radiation-independent reactions (vide infra). Singlet oxygen formation in organs other than the skin and eye mainly depends on such "dark" reactions.

Artificial sources of radiation may also contribute to endogenous singlet oxygen formation in humans. For example, during photodynamic therapy, a photosensitizer is inserted into cancerous tissue and irradiated with UV to produce singlet oxygen which serves the purpose of destroying cancer cells [23]. IR irradiation is commonly used

in medicine to warm muscle tissue [24] and might also contribute to singlet oxygen formation.

3. Leukocyte-Mediated Formation of Singlet Oxygen

Neutrophils, including human neutrophils, produce singlet oxygen [7, 35–37] and this has been suggested to be important for bacterial killing through the formation of ozone [7]. It is generally considered that production of singlet oxygen by neutrophils is dependent on myeloperoxidase (MPO) which catalyzes the formation of hypochlorous acid (HOCl) from hydrogen peroxide (H₂O₂) and chloride ion (see equation (1)), followed by reaction of HOCl with hydrogen peroxide anion (HO₂⁻) (see equation (2)) [7, 37]. However, the significance of the reaction between HO₂⁻ and HOCl under physiological environments such as the intraphagosomal milieu may be limited by the presence of other reactive partners for HOCl [36], and it was suggested that alternative pathways of singlet oxygen generation by neutrophils may exist, including the spontaneous dismutation of superoxide anions (see equation (3)) [36, 37]. However, the yield of singlet oxygen from the latter reaction was also found to be minor [38]. Peritoneal macrophages, which are MPO deficient, produce higher yield of singlet oxygen than neutrophils [37]. In the macrophage phagosome, the reaction between nitric oxide (NO[•]) and superoxide anion (⁻O₂) occurs at diffusion-controlled rates to form peroxyntirite (ONOO⁻) (see equation (4)) [39], which reacts with H₂O₂ to produce singlet oxygen (see equation (5)) [40]. The reaction of NO[•] with H₂O₂ was also found to generate singlet oxygen in a purely chemical system and in a superoxide generating system (see equation (6)) [41, 42]. The eosinophil peroxidase system generates singlet oxygen by a reaction between HOBr and HO₂⁻, analogously to (2) [30]:



4. Singlet Oxygen Formation by the Russel Mechanism

Russell [43] proposed the idea that two peroxy radicals can react to form an unstable tetroxide whose decomposition affords singlet oxygen, an alcohol, and a carbonyl compound, and this mechanism is now believed to contribute to singlet oxygen formation from various biomolecules including proteins, lipids, and nucleic acids [44]. The oxidation of DNA was

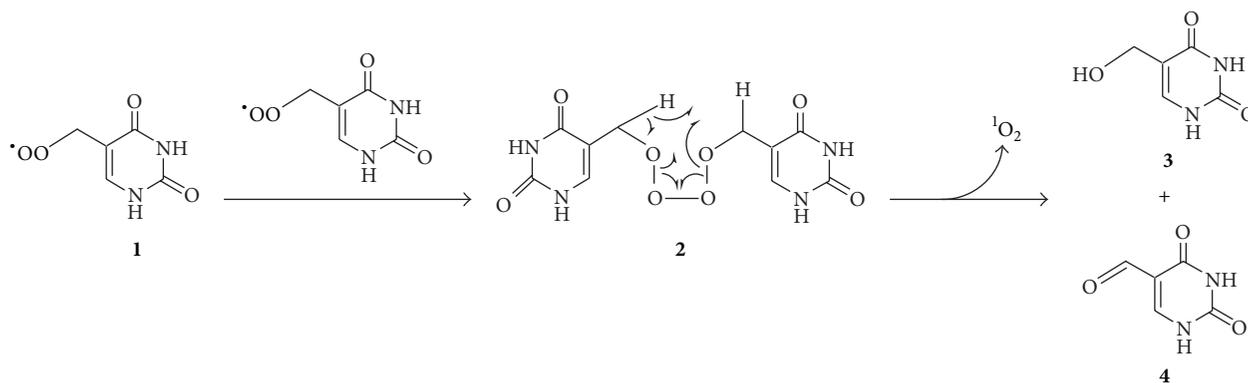


FIGURE 1: Formation of $^1\text{O}_2$ through reaction of thymine peroxy radicals (1) by the Russell mechanism [25].

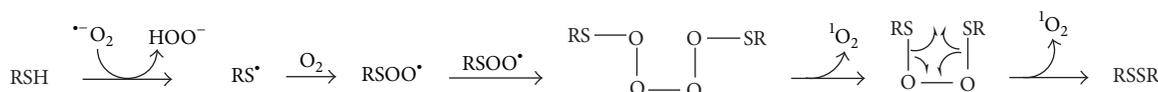


FIGURE 2: Russell-type mechanism for formation of $^1\text{O}_2$ from the reaction of glutathione (RSH) with superoxide anions ($\text{O}_2^{\cdot-}$) [26].

found to result in singlet oxygen by this mechanism as illustrated in Figure 1, whereby thymine peroxy radicals **1** react to generate tetroxide **2** whose decomposition produces alcohol **3**, carbonyl **4**, and $^1\text{O}_2$ [25].

Cysteine residues in glutathione (RSH) were found to be readily oxidized by superoxide anions to form singlet oxygen, glutathione disulfide (RSSR), and glutathione sulfonate (RSO_3^-) in a reaction that was suggested to involve the peroxy-sulphenyl radical (RSOO^\bullet) [26] and can be considered as a special type of Russell mechanism (Figure 2). This mechanism may also apply to cysteine residues in proteins. Hydroxyl radicals may also initiate the conversion of amino acids to peroxy radicals which then participate in the Russell mechanism [44].

As reviewed by Miyamoto et al. [45], various studies have demonstrated the formation of singlet oxygen by the Russell mechanism during the decomposition of lipid hydroperoxides (ROOH) in the presence of species such as Fe^{3+} , Cu^{2+} , peroxynitrite, HOCl, or cytochrome c, which oxidize the hydroperoxides to the corresponding peroxy radicals (ROO^\bullet). However, while singlet oxygen formation by the Russell mechanism in such purely chemical systems is established, its importance in tissues has been considered debatable because high concentrations of peroxy radicals are unlikely to develop under such systems [46, 47]. On the other hand, the fact that peroxy radicals derived from phospholipid and cholesterol hydroperoxides in liposomes produced singlet oxygen was considered as an indication that this phenomenon may occur in cellular membranes [45]. Moreover, cytochrome c was found to promote oxidation of polyunsaturated fatty acid-containing cardiolipin, with concomitant singlet oxygen formation, and this may be relevant in the mitochondria where both species exist [45].

Peroxynitrite (ONOO^-) reacts with glyoxal to produce singlet oxygen, and this was proposed to involve cleavage of glyoxal **6** to formic acid **7** and formyl radical **8**, with

subsequent conversion of the latter to peroxyformyl radical **9**, and reaction of two such peroxyacyl radicals by the Russell mechanism as shown in Figure 3 [27]. Even in the absence of peroxynitrite, aldehydes formed during lipid autoxidation are easily converted to the corresponding acids via acyl and peroxyacyl radicals [48], and these may similarly produce singlet oxygen, as illustrated for the conversion of lipid oxidation-derived formaldehyde **10** to formic acid and singlet oxygen (Figure 3).

5. Singlet Oxygen Formation via the Dismutation of Alkoxy Radicals

Two alkoxy radicals (RO^\bullet) can undergo dismutation to form a carbonyl and an alcohol (Figure 4), and some of the carbonyls are formed in the excited triplet state, with a yield of up to 8% [28]. The triplet carbonyls can transfer energy to triplet oxygen, thereby generating singlet oxygen [28]. Because alkoxy radicals are major intermediates during decomposition of biological hydroperoxides [28, 48], the potential contribution of this pathway to singlet oxygen formation cannot be ignored.

6. Singlet Oxygen Formation via the Oxidation of Phenolic Substances

Phenolic substances are important components of the human diet, and one of such compounds is the amino acid tyrosine. In many physiological situations, tyrosine **11** gets converted to the tyrosyl radical **12**, which in turn gets converted by superoxide anions to tyrosine hydroperoxide **13**, whose decomposition may produce singlet oxygen and regenerate tyrosine (Figure 5) [40]. However, tyrosine hydroperoxide **13** also gets converted to its bicyclic isomer **14**, whose decomposition does not produce singlet oxygen, and this greatly reduces the

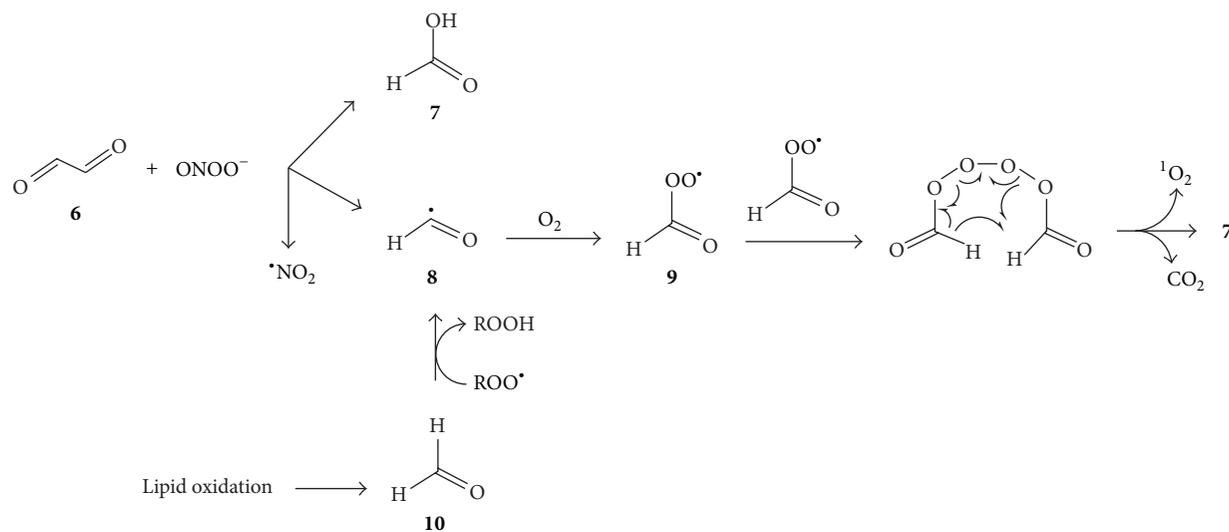


FIGURE 3: Russell-type mechanism for formation of $^1\text{O}_2$ upon reaction of glyoxal (6) with peroxynitrite (ONOO^-) via formyl radical (8) [27] and proposed occurrence of such a reaction via formyl radicals formed during lipid oxidation.

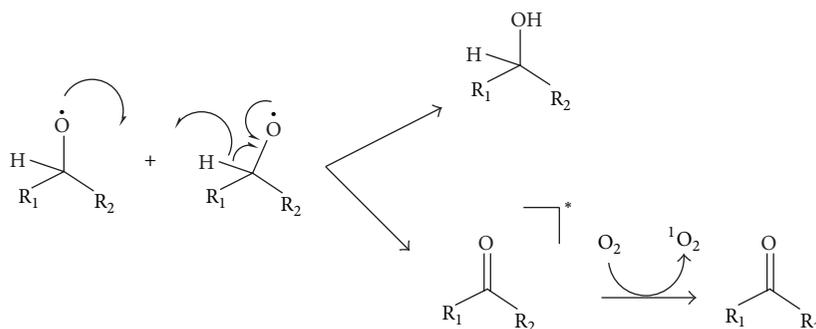


FIGURE 4: Formation of $^1\text{O}_2$ via dismutation of alkoxy radicals [28]. The asterisk indicates that the carbonyl is in excited state.

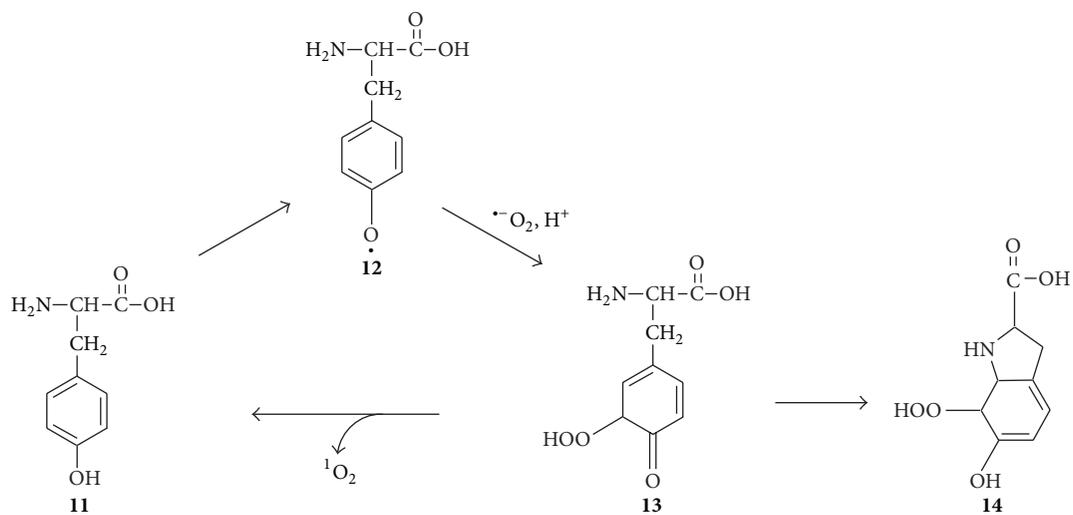


FIGURE 5: Formation of $^1\text{O}_2$ during the superoxide-dependent oxidation of tyrosine 11 [29].

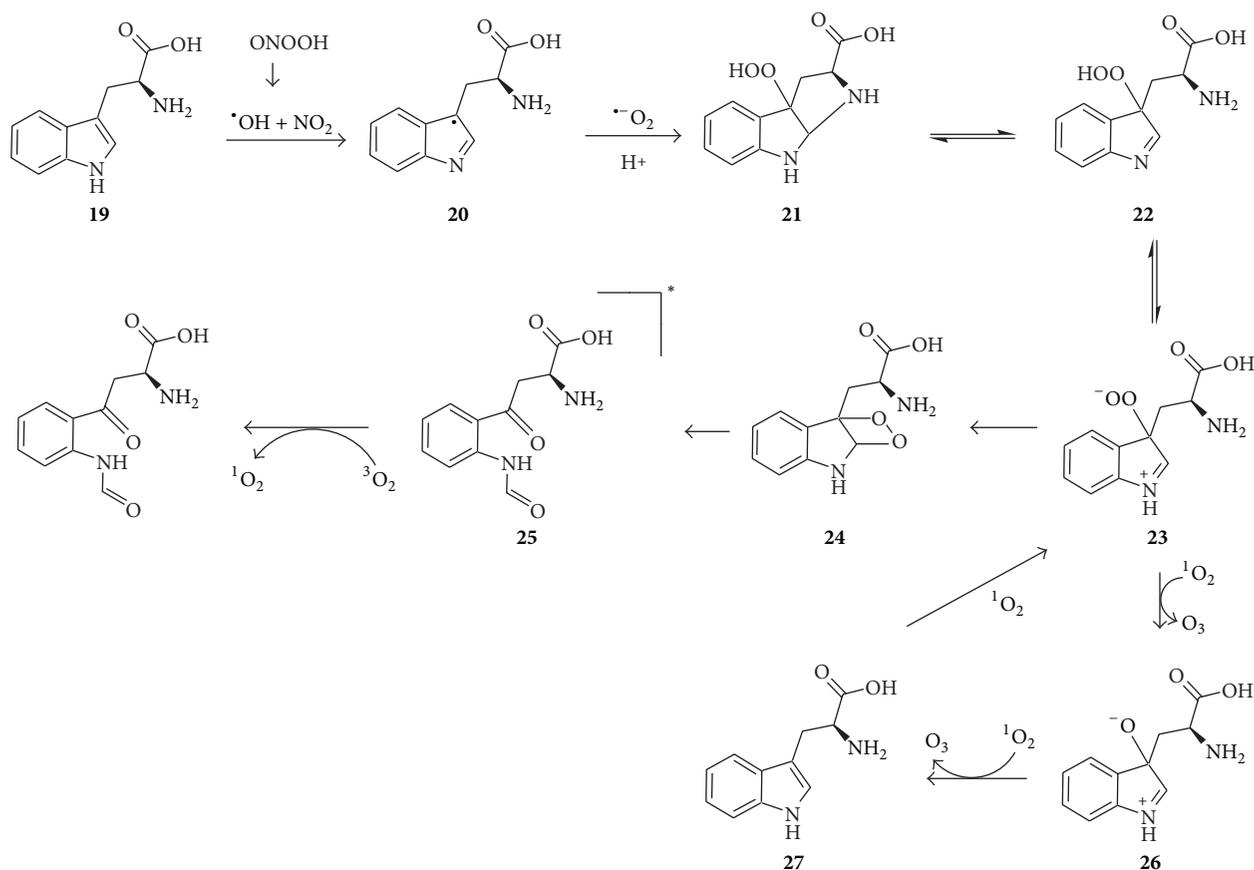


FIGURE 7: Formation of $^1\text{O}_2$ during the oxidation of tryptophan **19** via dioxetane **24** [32, 33] and formation of O_3 by $^1\text{O}_2$ -mediated deoxygenation of intermediates **23** and **26** [34]. The asterisk indicates that the carbonyl is in excited state.

The formation of some fatty acid oxidation products may be rationalized by pathways involving dioxetanyl derivatives formed by cyclization of peroxy radicals. The cyclization of a model peroxy radical derived from 3-hydroperoxy-2,3-dimethyl-1-butene (TMEOOH), and its subsequent conversion to excited carbonyls via a dioxetanyl intermediate was demonstrated by Timmins et al. [56]. Kaur et al. [49] postulated that such peroxy cyclization was an important pathway for the formation of major aldehydic products of linoleic acid oxidation, such as 9-oxononanoic acid and 4-hydroperoxy-2-nonenal. The latter is a precursor of the highly reactive and biologically active products, 4-hydroxy-2-nonenal and 4-oxo-2-nonenal, hence the great interest in its mechanism of formation [49, 50, 56–58]. It was proposed that linoleic acid **28** gets converted to peroxy radical **29**, whose cyclization affords dioxetanyl radical **30** as a precursor of hydroperoxy dioxetane **31**, whose decomposition affords 9-oxononanoic acid **32** and 4-hydroperoxy-2-nonenal **33** (Figure 8) [50]. One of the latter aldehydes may be in the triplet state and thus may be a source of energy for conversion of triplet oxygen to singlet oxygen. Lee et al. [57] found that the 13-hydroperoxide of linoleic acid (13-LA-OOH, **34**) was a major precursor of aldehydes **32** and **33**, with retention of the -OOH group of **34** in **33**. Schneider et al. [58] further found that, during conversion of 13-LA-OOH **34** to aldehydes **32** and **33**, there was facile conversion of hydroperoxide **34** via radicals **35** and **36**

to its 8,13-dihydroperoxy-derivative **37** (Figure 8) and 8-oxooctanoic acid was also formed. This is consistent with another postulated pathway for the formation of hydroperoxyaldehyde **33** involving cyclization of peroxy radical **36** to form dioxetanyl derivative **38**, whose decomposition affords 8-oxooctanoic acid **39** and radical **40**, a precursor **33** [50].

On the other hand, there is no evidence for the formation of dioxetanyl derivatives during cholesterol oxidation (Figure 9).

The autoxidation of cholesterol **41** proceeds via carbon-centered radical **42** and peroxy radical **43** to generate cholesterol-7-hydroperoxide **44** as a major product [59, 60]. While carbon-centered radical **42** might also be expected to isomerize and subsequently be converted via peroxy radical **45** to cholesterol 5-hydroperoxide **46**, formation of the latter during cholesterol autoxidation is negligible, even though **46** is the major product of cholesterol oxidation by singlet oxygen [59, 60]. Lack of formation of **46** during autoxidation has been attributed to a fast rate of dissociation of oxygen from peroxy radical **45** [60], indicating higher stability of radical **43** than **45**. Although peroxy radical **43** is easily converted to hydroperoxide **44**, there is no evidence that the former undergoes cyclization to form dioxetanyl derivatives **47** and **48**, because aldehydic products expected from the decomposition of the latter two have not been reported. This might likewise be due to a much higher stability of radical **43** than

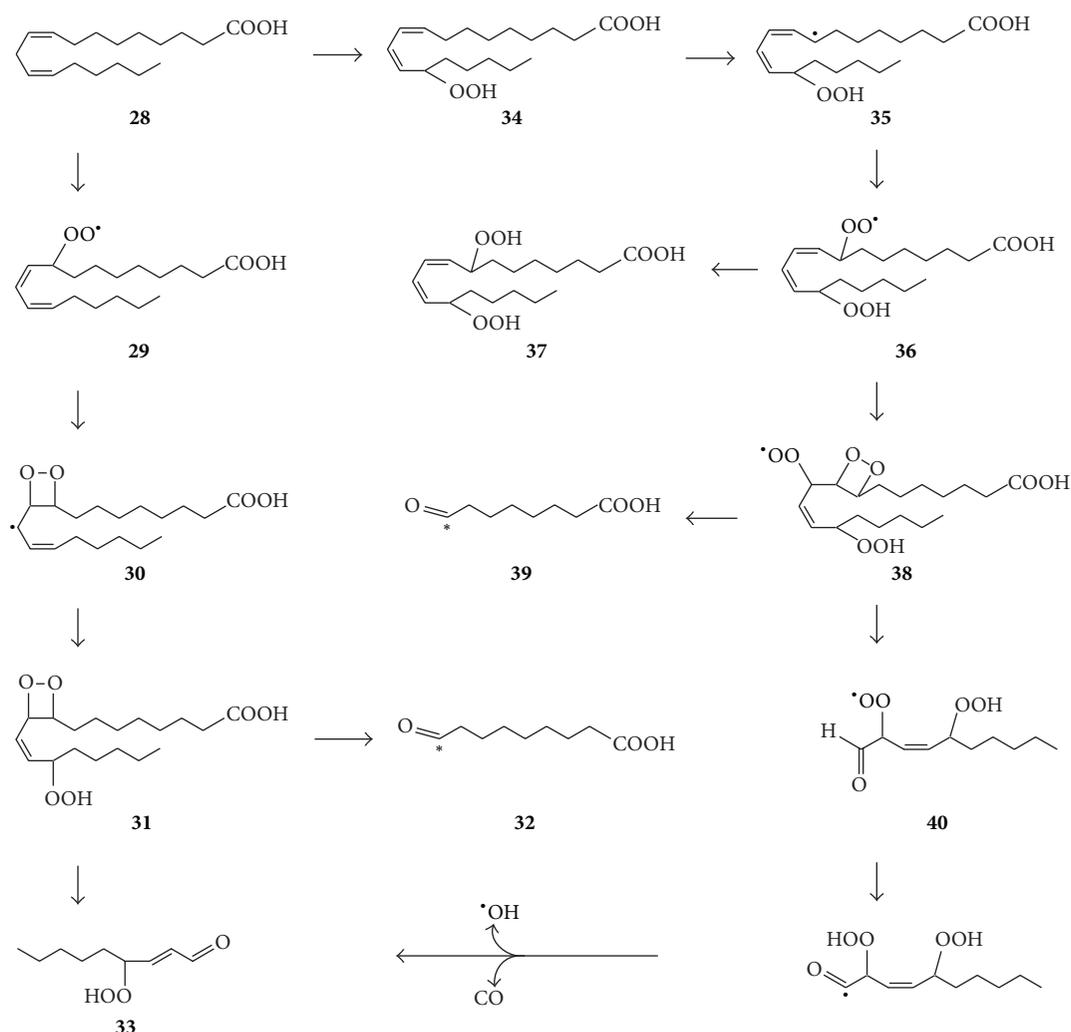


FIGURE 8: Postulated conversion of linoleic acid **28** via dioxetanyl derivative **31** to major products 9-oxononanoic acid **32** and 4-hydroperoxy-2-nonenal **33** [49] or via dioxetanyl radical **38** to hydroperoxyaldehyde **33** and 8-oxooctanoic acid **39** [50]. Decomposition of the dioxetanyl derivatives affords excited carbonyls that transfer energy to O_2 , thus forming 1O_2 . The asterisk indicates that the carbonyl is in excited state.

radicals **47** and **48**. Ozone directly converts cholesterol **41** to the secosterol aldehyde **49** (secosterol A), which undergoes some conversion to its aldolization product **50** (secosterol B) [61]. Hock cleavage of cholesterol 5-hydroperoxide **46** under acidic conditions affords mainly secosterol B **50** with minor amounts of secosterol A **49** [61]. However, no analogous C-C cleavage products attributable to decomposition of dioxetanyl derivatives arising from cholesterol peroxy radical cyclizations are known. Thus, decomposition of cholesterol hydroperoxides by the Russell mechanism [62] may be the only major pathway for singlet oxygen generation from cholesterol.

8. Singlet Oxygen Formation by the Reaction of Superoxide Anion with Hydrogen Peroxide

The reaction of superoxide anion with hydrogen peroxide to form singlet oxygen, hydroxyl radical, and hydroxide ion (see

equation (6)), a modified form of the Haber-Weiss reaction, was proposed by Kellogg and Fridovich [63] and demonstrated upon the reaction of potassium superoxide with hydrogen peroxide in a simple reaction system [64]. However, this reaction is controversial: Koppenol [65] registered strong disapproval for it, mainly based on the fact that various studies found that the rate constant for the Haber-Weiss reaction is in the order of $1 M^{-1} s^{-1}$ or less.

9. Singlet Oxygen Formation via Cytochrome c-Mediated Formation of Triplet Carbonyls

Cytochrome c converts carbonyls such as lipid-derived aldehydes to triplet carbonyls, which then transfer energy to oxygen, thus generating singlet oxygen [66]. In fact, singlet oxygen formation from a model membrane having polyunsaturated fatty acid-containing cardiolipin in association with cytochrome C was found to be more dependent on triplet

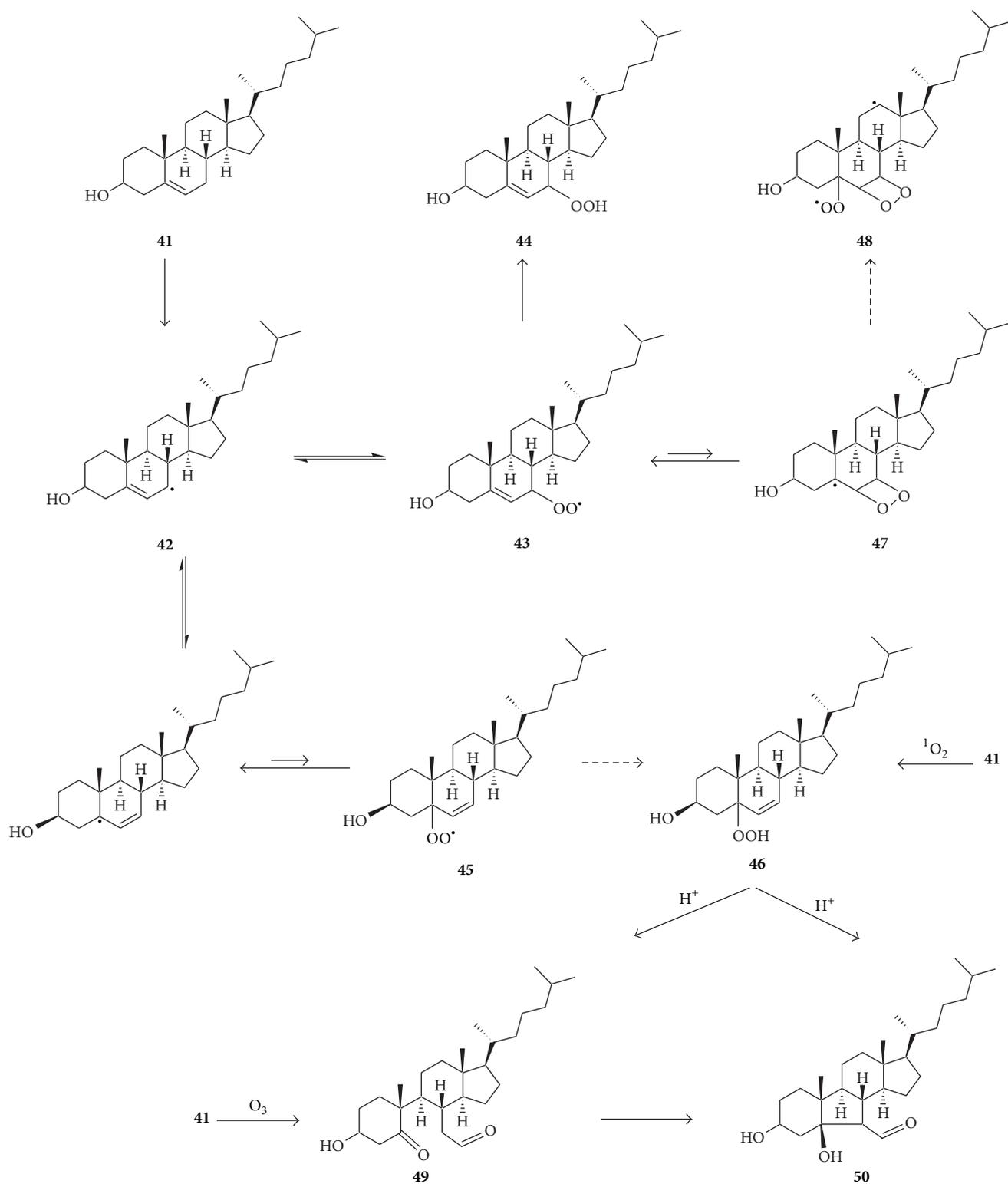


FIGURE 9: Oxidation pathways of cholesterol **41**, leading to the 7-hydroperoxide **44** during autoxidation, the 5-hydroperoxide **46** upon reaction with $^1\text{O}_2$, and secosterol A (**49**) upon reaction with O_3 . Aldolization of **49** produces secosterol B (**50**). Hock cleavage of **46** predominantly produces secosterol **50** and minor amounts of **49**. There is no evidence of formation of dioxetanyl derivatives.

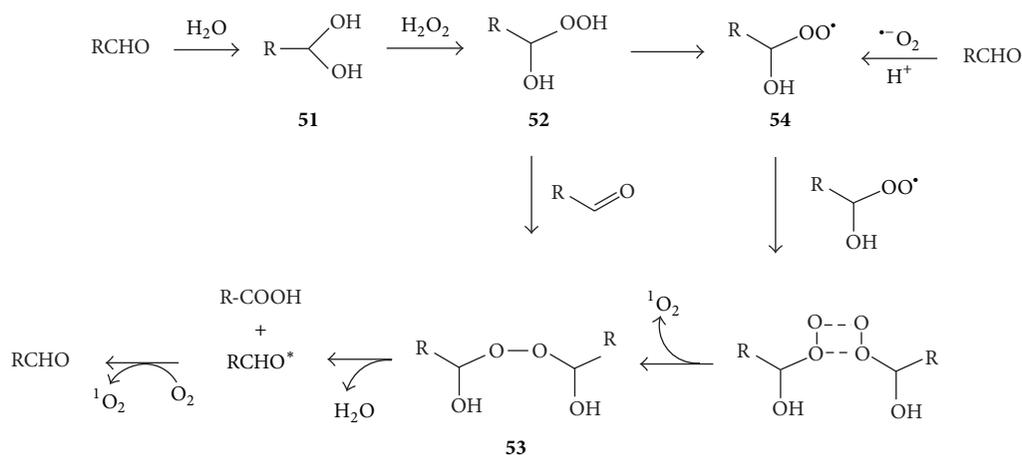


FIGURE 10: Mechanism of formation of ${}^1\text{O}_2$ and triplet carbonyls during reaction of H_2O_2 with carbonyls [51]. The asterisk indicates that the carbonyl is in excited state.

carbonyls than on the decomposition of hydroperoxides via the Russel mechanism [66].

10. Singlet Oxygen Formation by the Reaction of Hydroperoxides with Carbonyls

Under certain conditions such as in the presence of pyrogallol, lysine, tryptophan, or superoxide anions, the interaction of H_2O_2 with carbonyls such as formaldehyde, acetaldehyde, glyoxal, methyl-glyoxal, and even glucose was demonstrated to produce singlet oxygen and reactive aldehydes [51, 67–72], and such conditions should be common in vivo: considering that carbonyls are major lipid oxidation and glycoxidation products, all cells have formaldehyde generating pathways referred to as the formaldehydome [6, 69, 71], and hydrogen peroxide is also generated through many enzymatic and nonenzymatic reactions. The biological relevance of the reaction of H_2O_2 with carbonyls has been demonstrated in several studies. For example, brewed coffee and instant coffee give strong chemiluminescence due to singlet oxygen and reactive aldehydes [73], and the mutagenicity of coffee has been partly attributed to its content of both methylglyoxal and hydrogen peroxide [74]. The mutagenicity of glyoxal in *Salmonella* was found to be dependent on singlet oxygen generation and that catalase or scavengers of H_2O_2 reduced the mutagenic effect [75]. Kim et al. [76] found mixtures of glucose and lysine or arginine to be mutagenic and that such activity greatly depended on the formation of hydrogen peroxide and singlet oxygen. Maillard reaction products prepared by heating equimolar mixtures of glucose and amino acids, when incubated with DNA under physiological conditions, were reported to have DNA-strand breaking activity accompanied by singlet oxygen formation [77].

Hydroperoxides are very good nucleophiles because of the alpha effect, whereby interaction of lone electron pairs on two adjacent oxygen atoms increases nucleophilicity [78], and this explains the reactivity of hydrogen peroxide with aldehydes. Trézl and Pipek [51] proposed the pathways illustrated in Figure 10 for the generation of singlet oxygen during

such H_2O_2 -carbonyl reactions. First, the facile hydration of an aldehyde (RCHO) produces a gem diol **51** which reacts with H_2O_2 to form a 1-hydroxyalkylhydroperoxide **52**. The latter may react with another molecule of aldehyde to form bis-1-hydroxyalkylperoxide **53** whose decomposition affords an acid and an excited aldehyde which then participates in singlet oxygen formation. Alternatively the 1-hydroxyalkylhydroperoxide **52** is oxidized to form a 1-hydroxyalkylperoxy radical **54** that undergoes Russel-type decomposition. The oxidation of formaldehyde CH_2O by H_2O_2 in the presence of pyrogallol is called the Trautz-Schorigin reaction, a very efficient source of singlet oxygen whereby the semiquinone radical derived from pyrogallol is responsible for oxidizing the 1-hydroxyalkylhydroperoxide **52** to form **54** [79]. Superoxide anion also enhances formation of **54** [79].

The mechanism involved in the lysine or tryptophan-catalyzed formation of singlet oxygen from hydrogen peroxide and aldehyde has not been clearly defined. However, it is conceivable that this involves the pathways suggested in Figure 11, which is based on several known reactions.

First, the carbonyl (RCHO) reacts with the lysine (RNH_2) to form Schiff's base **55**, which then adds H_2O_2 to form hydroperoxide **56**. The latter reacts with another H_2O_2 molecule, resulting in regeneration of lysine and formation of a 1,1-dihydroperoxide **57**. Gem dihydroperoxide **57** may also be formed in an uncatalyzed reaction between H_2O_2 and 1-hydroxyhydroperoxide **52** [80, 81]. Hang et al. [82] reported that some monosubstituted 1,1-dihydroperoxides undergo decomposition to produce singlet oxygen in high yield. A reaction of **57** with a gem diol may generate such monosubstituted 1,1-dihydroperoxide **58** as a precursor of singlet oxygen. A reaction of **57** with Schiff's base may similarly lead to singlet oxygen formation via monosubstituted 1,1-dihydroperoxide **59**.

The reaction of H_2O_2 with formaldehyde or acrolein in the presence of lysine also leads to the formation of formyl lysine while acetaldehyde generates acetyl lysine [68]. Dehydration of hydroperoxide **56** may lead to such carbonylated

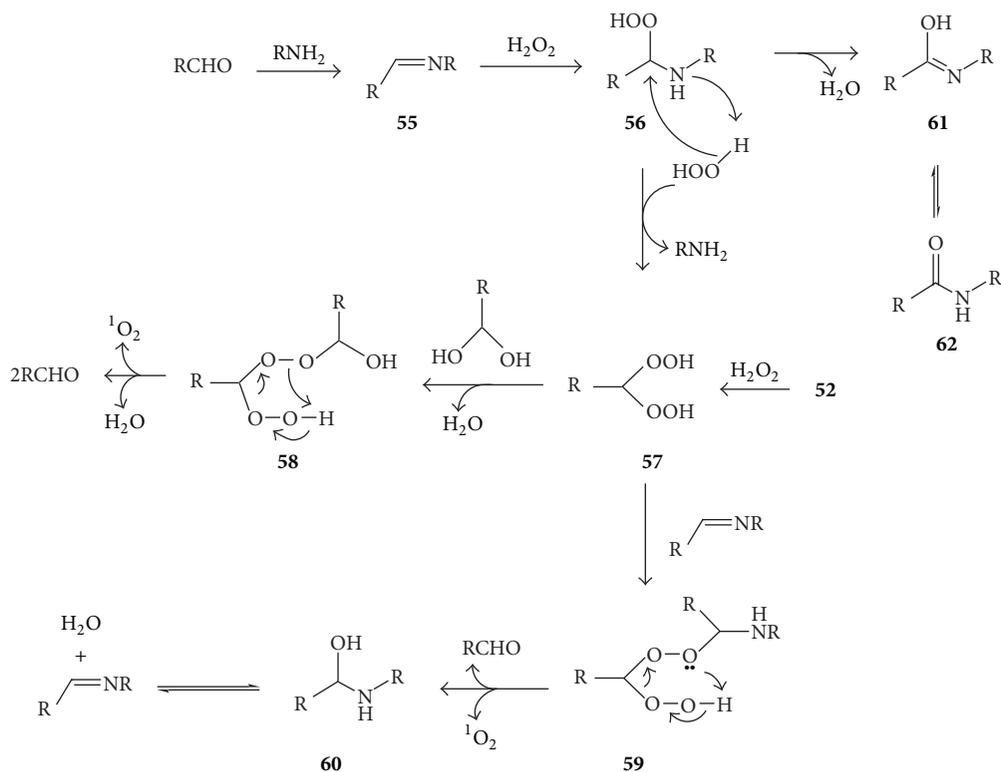


FIGURE 11: Proposed mechanisms of lysine- (RNH_2 -) promoted reaction of H_2O_2 with carbonyls to generate $^1\text{O}_2$ and the formation of carbonylated lysine products (**62**) under such conditions.

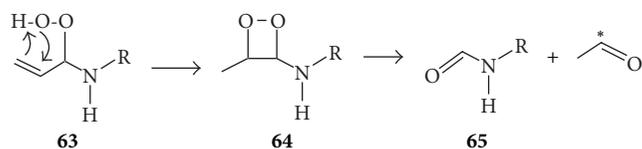


FIGURE 12: Proposed mechanism for conversion of acrolein-derived hydroperoxide **63** to formyl lysine **65** and acetaldehyde. The asterisk indicates that the carbonyl is in excited state.

lysine products represented by isomeric structures **61** and **62** (this involves a hydride transfer from carbon to oxygen in **56**). The fact that, like formaldehyde, acrolein (2-propenal) generates formyl lysine may be explained by the reaction illustrated in Figure 12, whereby the acrolein-derived hydroperoxide **63** rearranges to a dioxetane intermediate **64** whose decomposition affords formyl lysine **65** and a triplet acetaldehyde which may also be a source of energy for singlet oxygen production.

Eukaryotic cells synthesize polyamines such as spermine and spermidine, which are essential for normal cell growth and development [83, 84]. These compounds are catabolized by polyamine oxidases such as spermine oxidase (SMO) which catalyzes the conversion of spermine **66** to spermidine **67**, H_2O_2 , and 3-aminopropanal **68** (Figure 13) [83–85].

The coformation of these three products creates an ideal situation for singlet oxygen according to Figure 10 or Figure 11, since both aminopropanal and spermidine contain

the amino group, like lysine. This might be a key aspect in the mechanism of the known polyamine-dependent development of cancers such as gastric cancer [83, 84]. A recent study reported that Cu (II) polypyridyl complexes reduced the growth of breast cancer cells, and it was suggested that this was partly due to the production of singlet oxygen or a singlet oxygen-like compound that cleaved supercoiled DNA [86]. The expression of SMO in these cells also reduces their growth [87], which could likewise be due to singlet oxygen generation.

Neutrophils employ myeloperoxidase to oxidize nearly all amino acids found in plasma to aldehydes in high yield [88]. The reactions of these aldehydes with neutrophil-generated hydrogen peroxide may thus be another important mechanism for singlet oxygen production by neutrophils.

Organic hydroperoxides (ROOH) may participate in singlet oxygen generation through reactions related to Figures 11 and 12. For example, Kato et al. [89] reported that the 13-hydroperoxide of linoleic acid (13-hydroperoxy-9, 11-octadecadienoic acid, and HPODE) reacts with lysine to form N^ϵ -(hexanoyl) lysine and that this product is not formed by reaction of preformed aldehyde with lysine in the absence of the hydroperoxide. However, the exact mechanism of formation of this product, which is regarded to be proatherogenic and a marker of lipid hydroperoxide-derived modifications of biomolecules [89], has not been elucidated. As suggested in Figure 14, formation of this adduct may begin with lysine-catalyzed conversion of HPODE **34** to a dioxetane **69**, whose decomposition affords hexanal **70** and 12-oxo-9-dodecenoic

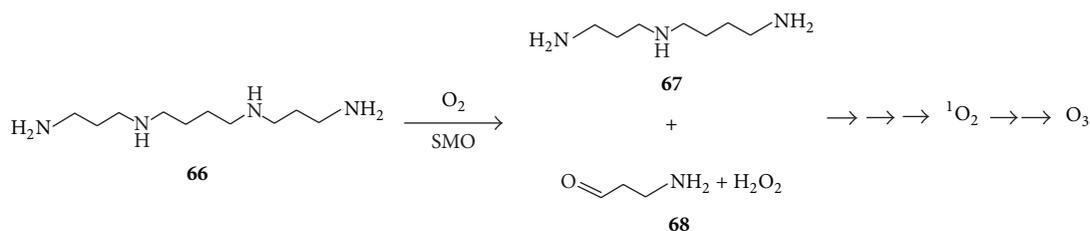


FIGURE 13: Spermine oxidase- (SMO-) catalyzed catabolism of spermine **66** to form H_2O_2 , spermidine **67**, and 3-aminopropanal **68**, which can participate in further reactions leading to $^1\text{O}_2$ and O_3 formation.

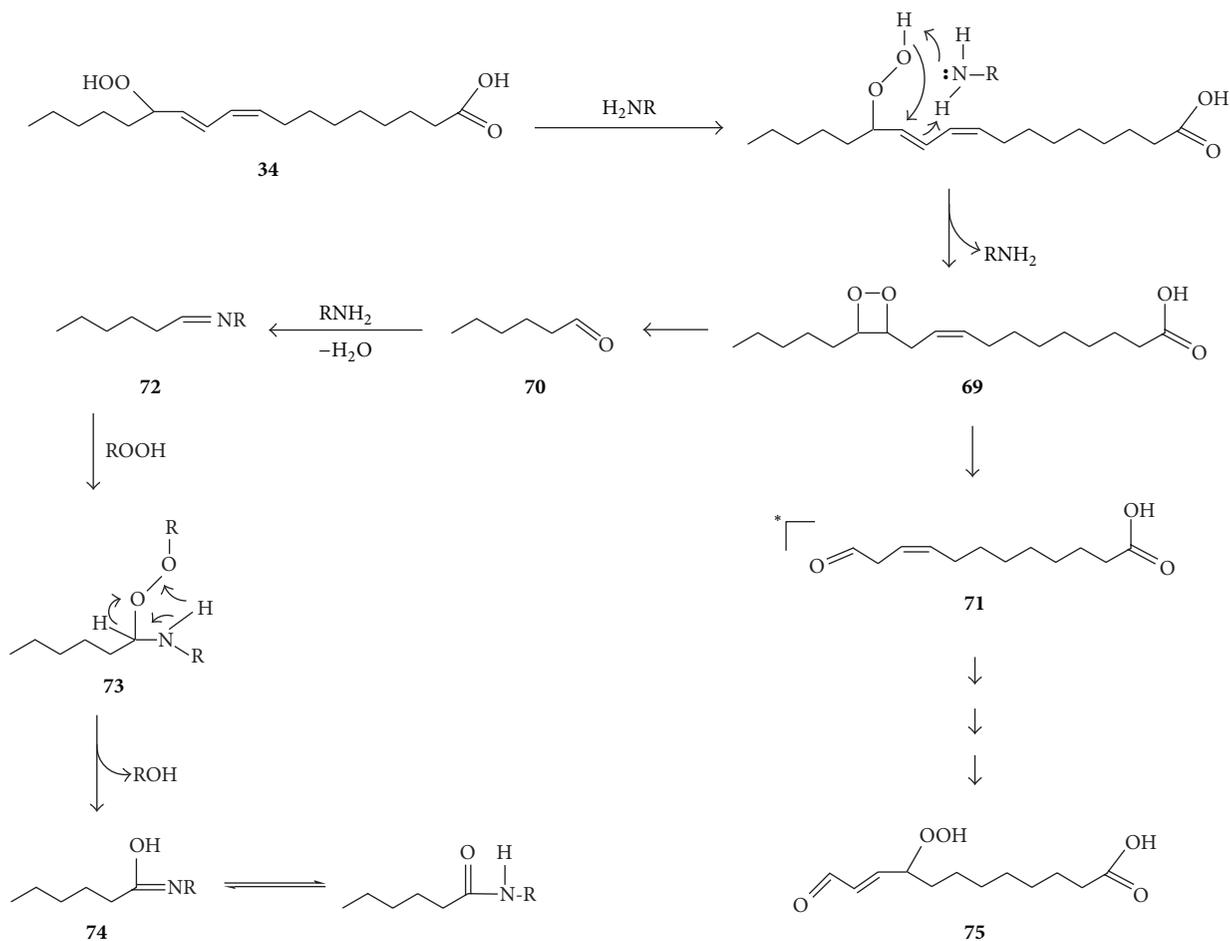


FIGURE 14: Proposed mechanism for the reaction of the 13-hydroperoxide of linoleic acid **34** with lysine (RNH_2) to form N^ϵ -(hexanoyl) lysine **74** via a dioxetane intermediate **69**. The asterisk indicates that the carbonyl is in excited state.

acid **71**, and one of these aldehydes may be in an excited state and thus contribute to singlet oxygen formation via energy transfer to triplet oxygen. Subsequent reaction of hexanal **70** with lysine (RNH_2) affords corresponding Schiff's base **72**, whose reaction with another HPODE molecule affords peroxide **73** which decomposes to form an alcohol and hexanoyl-lysine **74**. Formation of hexanoyl-lysine in this manner is analogous to the formation of formyl lysine in the reaction system consisting of formaldehyde, hydrogen peroxide, and lysine (vide supra, [68]). 12-oxo-9-dodecanoic acid **71** may subsequently undergo oxidation to form

12-oxo-9-hydroperoxy-dodecanoic acid **75** analogously to the known conversion of 3-nonenal to 4-hydroperoxy-2-nonenal [90]. The possibility that lysine catalyzes formation of dioxetane **69** suggests that proteins can promote formation of toxic aldehydic lipid oxidation products. It also indirectly supports the feasibility of cyclization of fatty acid peroxy radicals into dioxetanyl radicals as shown in Figure 8.

Wang et al. [91] recently found that thermal treatment of pure glucose or fructose solutions up to 70°C led to formation of both hydrogen peroxide and singlet oxygen. Using glucose as an example, the potential mechanism for singlet oxygen

$^1\text{O}_2$ and convert back to enediol **81**. Alternatively, **83** may release lysine and may be converted to dioxetane **84**, whose decomposition affords ketoacid **85** and formaldehyde, which may be in the excited state and thus contribute to singlet oxygen formation. Enediol **81** may also react with singlet oxygen to form dioxetane **86** whose decomposition produces erythronic acid **87** and carboxymethyllysine **88**. The latter is one of the most commonly formed advanced glycation end products (AGEs) that are known to contribute to various physiological disorders [94]. Dehydration of erythronic acid **87** may produce two regioisomeric deoxy-ketonic acids **89** and **90**. The latter may react with singlet oxygen to produce dioxetane **91** as a precursor of oxalic acid (HOOC-COOH), glycolaldehyde **92**, and glyoxal **93**, all of which are known products of glycooxidation [95]. Deoxy-ketonic acid **89** may, via its ketoform, decarboxylate to form monohydroxyacetone **94** (via the enol form of the latter), whose oxidation produces methylglyoxal **95**. Since the formation of the highly reactive glyoxal **93** and methylglyoxal **95** is accompanied by H_2O_2 formation, singlet oxygen formation according to Figures 10 and 11 will thus also occur during Maillard reaction.

Treatment of cultured U937 human leukemic cells or human multiple myeloma cells with H_2O_2 was found to cause singlet oxygen from both the cells and medium components, and this was not dependent on lipid oxidation [47, 96]. The mechanisms in Figures 10, 11, and 15 may be involved in such systems.

11. Evidence for Endogenous Ozone Formation and the Potential Mechanisms Involved

Wentworth et al. [7] were the first to suggest the possibility of the formation of ozone (O_3) in biological systems. One of their key pieces of evidence was that, in solutions of antibodies exposed to singlet oxygen, there was generation of a large amount of hydrogen peroxide, the occurrence of higher bactericidal activity than what could be attributed exclusively to H_2O_2 , as well as the oxidation of cholesterol to secosterol aldehyde A (**49** in Figure 9), a well-known product of the ozonolysis of cholesterol. They referred to the generation of H_2O_2 and ozone under such circumstances as the antibody-catalyzed water oxidation pathway and proposed the idea that this involves an initial reaction of water with $^1\text{O}_2$ to form dihydrogen trioxide (H_2O_3) and that decomposition of the latter affords H_2O_2 and O_3 (see equation (7)). This reaction was suggested to occur in a hydrophobic site in the antibody molecule, where the H_2O_3 would be shielded from hydrolysis and facilitated to undergo the conversion to H_2O_2 and O_3 [97]. Although antibodies produce much more H_2O_2 and O_3 than other proteins [7], Yamashita et al. [8] reported that antibody catalysis is not essential for this reaction, but rather the presence of one of four amino acids: histidine, tryptophan, cysteine, or methionine. On the other hand, various authors have expressed reservations concerning the generation of ozone under such systems, for example, based on the fact that the catalytic mechanisms for the antibody- or amino acid-catalyzed water oxidation remain ill-defined [1, 3, 35, 37]. Others have reported that cholesterol 5-hydroperoxide **46**, a product of the oxidation of cholesterol by singlet oxygen,

can also decompose to generate secosterol aldehydes [98, 99]. On the other hand, it has been shown that reaction of cholesterol with ozone predominantly generates secosterol A while decomposition of cholesterol-5 hydroperoxide predominantly generates secosterol B [61]. The fact that secosterol A is the predominant secosterol detected in human tissues and is formed by neutrophils in vitro thus supports the formation of endogenous ozone [61, 100]. There is also indirect evidence consistent with the formation of ozone in plant leaves or in the cyanobacterium *Synechocystis* PCC 6803 during light-induced damage to their PS II, because singlet oxygen and tryptophan or histidine residues, respectively, are involved [34]. Unlike other commonly generated ROS that only generate single strand breaks in DNA, ozone generates both single strand and double strand breaks [101, 102]. The addition of L-histidine to cultured mammalian cells exposed to H_2O_2 results in DNA double strand breaks [103–105], and this might be related to histidine-mediated ozone generation in the presence of singlet oxygen.

A BioArena system is an overpressured layer chromatography (OPLC) system which enables observation of biochemical interactions between microorganisms and biologically active compounds in an adsorbent layer covered with the microorganisms [106]. Using such a system, it was found that formaldehyde, singlet oxygen, and ozone are formed in the interaction between microorganisms and antibiotic substances such as resveratrol and cinnamic acid and that ozone-trapping compounds greatly reduce the antimicrobial effect [6, 69, 71, 72]. Based on results from the BioArena system, ozone is considered to be an indispensable endogenous molecule that can be detected and measured in practically all biological systems [6, 71, 72]. Moreover, in directly detecting ozone formation by plant leaves through GC-MS-SIM, Balla and Tyihák [4] have added direct proof for the formation of ozone in biological systems. Thus, the current evidence for endogenous ozone is enough to warrant further studies on the mechanisms of its formation and biological effects.

As already mentioned, a number of questions remain unanswered regarding the antibody-/amino acid-catalyzed water oxidation pathway for ozone generation. Moreover, decomposition of dihydrogen trioxide (H_2O_3), the proposed key precursor of ozone in both aqueous and organic solvents, has only been shown to produce singlet oxygen and water rather than hydrogen peroxide and ozone [107]. In response to these challenges, a new concept for ozone formation was recently suggested, involving the oxidation of organic substrates such as aldehydes or amino acids to form oxidized intermediates, and the subsequent singlet oxygen-mediated deoxidation of the oxidized intermediates to produce ozone, whose subsequent decomposition in water affords hydrogen peroxide [34]. For example, an aldehyde (RCHO) may be converted to a peroxyacid [RC(O)OOH] through a radical pathway [48] or by reacting with singlet oxygen [108], and the peroxyacid may undergo a Bayer-Villiger type reaction with singlet oxygen to produce an acid and O_3 (Figure 16) [34]. The significance of this mechanism is that it might generate ozone in many of the situations where singlet oxygen is formed in the presence of aldehydic compounds such as in Figures 10–15. Detailed potential mechanisms for histidine-,

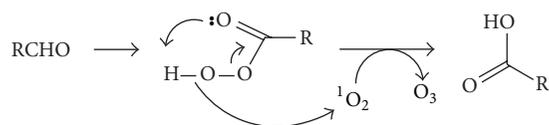


FIGURE 16: Mechanism of O_3 formation by 1O_2 -mediated deoxidation of a peroxyacid [34].

methionine-, and tryptophan-mediated ozone production have been proposed [34]. As an example, part of the reaction scheme for the tryptophan-dependent ozone generation is given in Figure 7. Thus, 1O_2 , acting as an electrophile, deoxidizes zwitterion **23** and alkoxide **26**, resulting in formation of two O_3 molecules and regeneration of tryptophan.

12. The Significance of Endogenous Singlet Oxygen and Ozone in Human Health

Harman [109] proposed the free radical theory of aging (FRTA) that considers free radical-induced damage to key biomolecules such as DNA and proteins as having a causative role in aging and reduced lifespan. A modified version of the FRTA is the mitochondrial free radical theory of aging (MFRTA), which considers the mitochondrion as the primary source and target of the damaging free radicals [110]. Formation of reactive oxygen species (ROS) from the electron transport chain (ECT) is the basis of the FRTA and MFRTA [111].

As high as 1–5% of consumed oxygen may be converted to superoxide anions, which are readily converted to H_2O_2 , and the latter is the principal mediator of cellular oxidative stress [111–113]. Reactive nitrogen species are also formed in the mitochondrion, since there is facile diffusion of nitric oxide (NO) to this organelle [114], and the presence of nitric oxide synthases has been demonstrated in mitochondria from various tissues in rats or mice [115, 116] as well as in cultured human cells [117]. In mitochondria, NO increases the formation of superoxide anions and H_2O_2 [118]. Thus, there exists suitable conditions in the mitochondria for the diffusion-controlled reaction of NO and superoxide anions to form peroxynitrite (see equation (4)) [114, 119] and subsequent reaction of the latter with H_2O_2 to generate singlet oxygen (see equation (5)) as one of the mitochondrial ROS. Peroxynitrite also initiates lipid oxidation [120] and may thus lead to further formation of singlet oxygen in the mitochondria by the Russel mechanism, or activation of lipid-derived carbonyls by cytochrome c oxidase, or the reaction of hydrogen peroxide with such carbonyls. In agreement with the operation of such mechanisms of singlet oxygen formation in the mitochondria, the superoxide anion-dependent formation of this ROS was demonstrated in mitochondria of rat liver and small intestine [121]. Additionally, Berneburg et al. [122] found that exposing normal human fibroblasts to sublethal doses of UVA led to singlet oxygen-dependent deletion of a 4,977-base pair in mitochondrial DNA, which is a common mutation associated with photoaging of human skin. The major product of mitochondrial DNA oxidation is

7,8-dihydro-8-oxoguanine (8-oxoG) [111, 123], and singlet oxygen contributes predominantly to the formation of this compound in DNA [124]. Although the reaction of hydroxyl radical with DNA also produces some 8-oxoG, this is a minor reaction [124, 125]. Thus, singlet oxygen should be an important contributor to aging according to the MFRTA. Besides, generation of singlet oxygen in the presence of carbonyls, amino acids, and proteins in the mitochondrion provides an environment for the generation of mitochondrial ozone, which might contribute to mitochondrial DNA double strand breaks.

The MFRTA has been used to explain the fact that dietary supplementation with antioxidants has not clearly shown antiaging effects, in that the dietary antioxidants may not effectively reach the mitochondria, the main sites of ROS generation, and age-related damage [110, 126]. In support of this, mice overexpressing mitochondria-targeted catalase were found to have improved lifespan, delayed cardiac pathology, and delayed cataract development [126, 127]. The beneficial effects of mitochondria-directed catalase might in part be due to reduced singlet oxygen and ozone, since H_2O_2 participates in a good number of the singlet oxygen-generating pathways (*vide supra*).

Another concept that is currently gaining ground is that of mitochondrial hormesis or mitohormesis, which suggests that low levels of reactive oxygen species such as superoxide anions are in fact part of normal physiology and are beneficial for longevity and metabolic health [126, 128–130]. The essence of this is that, at low levels, ROS act as signaling molecules that promote resistance to oxidative stress through increased endogenous antioxidant defense [126]. Hence, effects of ROS may be biphasic, whereby low levels are considered to be beneficial while high levels are detrimental [129]. Such an effect was demonstrated by treatment of elderly patients with a small amount of ozone by rectal sufflation, which resulted in improved antioxidant status and reduced biomarkers of lipid and protein oxidation [131]. A similar treatment was found to reduce the glycemic index and oxidative stress in diabetic patients [132]. Such beneficial effects of ozone therapy were suggested to be a result of the moderate oxidative stress under such conditions activating the nuclear factor-erythroid 2-related factor 2 (Nrf2), which then induces transcription of the antioxidant response elements (ARE), resulting in the production of numerous antioxidant enzymes including catalase as well as phase II detoxification enzymes and heat shock proteins [133]. However, more work is needed to determine the boundaries between the beneficial levels of ROS and the deleterious levels that promote physiological disorders.

As already mentioned, singlet oxygen generated photo-dynamically in the skin contributes to photoaging of the skin, and this at least partly occurs as follows: 1O_2 reacts with cholesterol to form cholesterol-5-hydroperoxide, which induces expression of matrix metalloproteinase-9 (MMP-9), which in turn degrades collagen and thereby induces the formation of wrinkles and sagging [2, 22]. Singlet oxygen also induces photoaging-associated mutations in mitochondrial DNA [122]. Porphyrins are rare diseases involving a disorder in heme synthesis and are manifested by accumulation of porphyrins in tissues [134]. During cutaneous porphyrias,

there is enhanced photosensitivity due to singlet oxygen formation [134].

Singlet oxygen-specific linoleic acid oxidation products, 10-hydroperoxy-8(E), 12(Z)-octadecadienoic acid (10-(E,Z)-HPODE), and 12-hydroperoxy-9(Z), 13(E)-octadecadienoic acid (12-(Z,E)-HPODE) have been found to be suitable biomarkers for the evaluation of the early stages of diabetes, underscoring the role of singlet oxygen in the pathogenesis of this disorder [135, 136]. Further, fasting levels of 10-(E,Z)-HPODE and 12-(Z,E)-HPODE, together with insulin and leptin/adiponectin, are excellent predictors of the risk for type II diabetes, glucose intolerance, and insulin resistance [137, 138]. Patients with diabetes are prone to other diseases and physiological disorders including atherosclerosis, cardiovascular diseases, chronic kidney disease, retinopathy, and skin disorders [139]. Therefore, by contributing to glucose intolerance, insulin resistance, and type II diabetes, singlet oxygen may be contributing to a much wider range of chronic diseases.

Recently, the catabolism of polyamines has been linked to the development of some cancers such as gastric cancer, prostate cancer, and colon cancer [84, 85, 140]. Human spermine oxidase isoforms were found to be localized in the nucleus where they generate reactive oxygen species close to DNA and nuclear proteins [85]. Spermine oxidase-catalyzed catabolism of spermine thus generates products that can react to produce singlet oxygen and ozone in the nucleus. Hence, singlet oxygen and ozone might play a key role in spermine oxidase-dependent cancers.

Since singlet oxygen is a precursor of endogenous ozone, some of the biological activities attributed to singlet oxygen might be directly mediated by ozone or ozonolysis products. High levels of cholesterol secosterols A and B have been detected in human atherosclerotic tissues and shown to be proatherogenic [141]. The fact that secosterol A is the predominant secosterol in human tissues [61, 62, 100] indicates a much greater role for ozone than singlet oxygen in the *in vivo* formation of the secosterols because even the secosterol B detected in such systems is at least partly formed by aldolization of secosterol A. A possible reason for the *in vivo* predominance of secosterol A is that, while cholesterol hydroperoxide-5, a direct precursor of secosterol B, may be easily formed in tissues, its Hock cleavage is favored by acidic conditions, which may not be common in most tissues. The fact that Tomono et al. [142] observed a time-dependent increase of the secosterols in plasma of mice after injection of lipopolysaccharide indicates that ozone formation is easily initiated during inflammation.

The secosterols A and B induce endothelial cell dysfunction by promoting apoptosis and inhibiting endothelial-dependent arterial relaxation [143]. These products are elevated in brain tissues of Alzheimer's disease patients, and it was demonstrated that they may trigger the disease by inducing protein misfolding [144]. Elevated levels of the secosterols are found in Lewy body dementia brain tissues, where they accelerate alpha-synuclein fibrillization [145]. The secosterols bind to the p53 protein and induce it to misfold and lose the ability to bind to a consensus DNA sequence, signifying that they may contribute to cancers where wild-type inactivation

of p53 occurs, such as breast cancer, colon cancer, colon adenoma, and neuroblastoma [146]. The secosterols and their acidic oxidation products are strongly cytotoxic against various human cell lines, and it was concluded that, during inflammation, cell death caused by them may contribute to further tissue damage and development of disease [147]. Formation of Schiff's bases between the secosterols and myelin basic protein (MBP) induces myelin instability and might contribute to the onset and progression of multiple sclerosis [148].

Despite the generally harmful effects of high levels of ROS, organisms employ high ROS levels in certain pathological situations in an attempt to restore normal physiological conditions. For example, singlet oxygen and ozone are produced by activated human neutrophils, where they are important for the destruction of bacteria during acute infections [7, 149]. The use of photodynamic therapy (PDT) in cancer treatment relies on the generation of singlet oxygen in the tissues to destroy cancer cells, and when PDT involves use of sensitizers conjugated to antibodies, ozone generation might be equally important [34].

13. The Influence of Dietary Factors on the Formation and Effects of Endogenous Singlet Oxygen and Ozone

Some dietary factors promote inflammation and oxidative stress, while others are anti-inflammatory and protective against oxidative stress. Generally, the proinflammatory factors may promote while the anti-inflammatory factors may inhibit the formation of singlet oxygen and ozone. More specifically, some of the dietary factors, such as carotenoids, act as quenchers of singlet oxygen. Such substances will prevent ozone formation and dysfunctions associated with singlet oxygen. Dietary carotenoids have been shown to easily accumulate in the skin, where they prevent the singlet oxygen-associated photoaging [2, 22, 150]. Just as singlet oxygen plays a role in the development of diabetes (*vide supra*), adequate dietary carotenoids have been reported to reduce the risk for type II diabetes and the metabolic syndrome [151–153]. Besides their singlet oxygen-quenching ability, carotenoids also have anti-inflammatory activity by interfering with the NF- κ B pathway [154]. Beta carotene was reported to prevent ozone-induced proinflammatory markers in murine skin [155].

On the other hand, alcohol consumption lowers the levels of carotenoids in the human skin and increases the risk for sunburn and potentially skin cancer [156]. Alcohol consumption and cigarette smoking also lower serum carotenoid levels [157]. By lowering carotenoids, alcohol consumption and cigarette smoking reduce singlet oxygen quenching and may promote ozone formation. Moreover, since ethanol is metabolized to acetaldehyde, there are increased blood levels of acetaldehyde after alcohol consumption [158] and this may promote production of ROS. Alcohol consumption is also associated with oroesophageal squamous cell carcinoma, gastric cancer, and colorectal cancer, which may be partly due to induction of ROS production by ethanol or its metabolite, acetaldehyde [159–161].

Helicobacter strains expressing the virulence factor cytotoxin-associated gene A (Cag-A) stimulate the expression of spermine oxidase in gastric epithelial cells and promote ROS generation and DNA damage in these cells, thereby increasing gastric cancer risk [83]. SMO expression also contributes to ulcerative colitis, an inflammatory condition that is associated with colon cancer [162]. The bacterium *Enterococcus faecalis* produces large amounts of superoxide anions and hydrogen peroxide in the colon and this causes DNA damage in luminal cells of the colon [163]. The large amounts of H₂O₂ may promote ¹O₂ and O₃ formation in that environment. In addition, some colon microorganisms convert bile components into metabolites that are carcinogenic, partly by inducing ROS generation [164]. Consumption of foods containing probiotic microorganisms may reduce oxidative stress in various ways including inhibiting the growth of oxidative stress-inducing microorganisms [164, 165].

High fat and high carbohydrate diets are associated with increased postprandial inflammation and oxidative stress [166]. Hence, such diets may promote the generation of singlet oxygen and ozone, besides other ROS. The consumption of foods rich in Maillard oxidation products may also contribute to endogenous singlet oxygen and ozone generation because dietary advanced glycation end products promote oxidative stress and inflammation through interaction with the receptor for advanced glycation products in a variety of cells, resulting in oxidative stress involving superoxide anion and H₂O₂ formation [167–169].

Various dietary phenolic substances are recognized as powerful antioxidants mainly by their scavenging of free radicals. On the other hand, these phenolic substances also have prooxidant activity in that they can react with oxygen to generate superoxide anions, hydrogen peroxide, and singlet oxygen [31, 52, 53]. Thus, consumption of coffee or tea, which is rich in these phytochemicals, also means consumption of substantial amounts of hydrogen peroxide and perhaps singlet oxygen [73, 74]. Nevertheless, the consumption of foods rich in such substances, such as fruits and vegetables, and even tea and coffee has mainly been associated with beneficial effects [170–172]. This is because, apart from direct antioxidant or prooxidant activity, these compounds also affect cellular physiology by signaling pathways. For example, they may inhibit redox-sensitive transcription factors and prooxidant enzymes such as xanthine oxidase or nitric oxide synthase [171] and/or induce expression of antioxidant enzymes and phase II detoxification enzymes [170–172]. Phytochemicals in virgin olive oil were found to lower postprandial inflammation by reducing postprandial plasma lipopolysaccharide levels [173].

14. Conclusion

Singlet oxygen may be commonly generated in tissues through a range of enzymatic and nonenzymatic reactions, and, at least based on the in vivo formation of cholesterol secosterol aldehydes, ozone formation also seems to be important. Endogenous overproduction of these two oxidants likely plays important roles in the pathogenesis of physiological disorders such as diabetes, cardiovascular diseases,

skin photoaging, and some cancers. Consumption of foods rich in singlet oxygen quenchers and components with anti-inflammatory activities, including probiotics, may help reduce the negative effects of high levels of these oxidants. Phytochemicals that generate low levels of these oxidants might also be useful for cellular adaptation to oxidative stress and prevention of physiological disorders.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

References

- [1] W. A. Pryor, K. N. Houk, C. S. Foote et al., “Free radical biology and medicine: it’s a gas, man!,” *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 291, no. 3, pp. R491–R511, 2006.
- [2] J. Terao, Y. Minami, and N. Bando, “Singlet molecular oxygen-quenching activity of carotenoids: relevance to protection of the skin from photoaging,” *Journal of Clinical Biochemistry and Nutrition*, vol. 48, no. 1, pp. 57–62, 2011.
- [3] L. L. Smith, “Oxygen, oxysterols, ouabain, and ozone: a cautionary tale,” *Free Radical Biology and Medicine*, vol. 37, no. 3, pp. 318–324, 2004.
- [4] J. Balla and E. Tyihák, “Direct measurement of emission of endogenous ozone from plants by GC-MS-SIM,” *Chromatographia*, vol. 71, supplement 1, pp. 87–91, 2010.
- [5] R. P. Rastogi, Richa, A. Kumar, M. B. Tyagi, and R. P. Sinha, “Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair,” *Journal of Nucleic Acids*, vol. 2010, Article ID 592980, 32 pages, 2010.
- [6] E. Tyihák, Á. M. Móricz, and P. G. Ott, “BioArena studies: unique function of endogenous formaldehyde and ozone in the antibiotic effect—a review,” *Medicinal Chemistry*, vol. 8, no. 1, pp. 75–84, 2012.
- [7] P. Wentworth Jr., J. E. McDunn, A. D. Wentworth et al., “Evidence for antibody-catalyzed ozone formation in bacterial killing and inflammation,” *Science*, vol. 298, no. 5601, pp. 2195–2199, 2002.
- [8] K. Yamashita, T. Miyoshi, T. Arai et al., “Ozone production by amino acids contributes to killing of bacteria,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 44, pp. 16912–16917, 2008.
- [9] M. Y. Akhalaya, G. V. Maksimov, A. B. Rubin, J. Lademann, and M. E. Darvin, “Molecular action mechanisms of solar infrared radiation and heat on human skin,” *Ageing Research Reviews*, vol. 16, no. 1, pp. 1–11, 2014.
- [10] I. E. Kochevar, M. A. Pathak, and J. A. Parrish, “Photophysics, photochemistry, and photobiology,” in *Fitzpatrick’s Dermatology in General Medicine*, I. M. Freedberg, A. Z. Eisen, K. Wolff et al., Eds., pp. 220–229, McGraw-Hill, New York, NY, USA, 1999.
- [11] A. Knak, J. Regensburger, T. Maisch, and W. Bäumler, “Exposure of vitamins to UVB and UVA radiation generates singlet oxygen,” *Photochemical and Photobiological Sciences*, vol. 13, no. 5, pp. 820–829, 2014.
- [12] G. T. Wondrak, M. K. Jacobson, and E. L. Jacobson, “Endogenous UVA-photosensitizers: mediators of skin photodamage and novel targets for skin photoprotection,” *Photochemical and Photobiological Sciences*, vol. 5, no. 2, pp. 215–237, 2006.

- [13] W. Bäumler, J. Regensburger, A. Knak, A. Felgenträger, and T. Maisch, "UVA and endogenous photosensitizers—the detection of singlet oxygen by its luminescence," *Photochemical & Photobiological Sciences*, vol. 11, no. 1, pp. 107–117, 2012.
- [14] J. Baier, T. Maisch, M. Maier, M. Landthaler, and W. Bäumler, "Direct detection of singlet oxygen generated by UVA irradiation in human cells and skin," *Journal of Investigative Dermatology*, vol. 127, no. 6, pp. 1498–1506, 2007.
- [15] M. Sun, S. C. Finnemann, M. Febbraio et al., "Light-induced oxidation of photoreceptor outer segment phospholipids generates ligands for CD36-mediated phagocytosis by retinal pigment epithelium," *Journal of Biological Chemistry*, vol. 281, no. 7, pp. 4222–4230, 2006.
- [16] M. J. Niedre, M. S. Patterson, A. Giles, and B. C. Wilson, "Imaging of photodynamically generated singlet oxygen luminescence in vivo," *Photochemistry and Photobiology*, vol. 81, no. 4, pp. 941–943, 2005.
- [17] M. Bregnhøj, A. Blázquez-Castro, M. Westberg, T. Breitenbach, and P. R. Ogilby, "Direct 765 nm optical excitation of molecular oxygen in solution and in single mammalian cells," *The Journal of Physical Chemistry B*, vol. 119, no. 17, pp. 5422–5429, 2015.
- [18] S. G. Sokolovski, S. A. Zolotovskaya, A. Goltsov, C. Pourreyaon, A. P. South, and E. U. Rafailov, "Infrared laser pulse triggers increased singlet oxygen production in tumour cells," *Scientific Reports*, vol. 3, article 3484, 2013.
- [19] A. M. Holzer, M. Athar, and C. A. Elmets, "The other end of the rainbow: infrared and skin," *Journal of Investigative Dermatology*, vol. 130, no. 6, pp. 1496–1499, 2010.
- [20] P. Schroeder, C. Calles, T. Benesova, F. MacAluso, and J. Krutmann, "Photoprotection beyond ultraviolet radiation—effective sun protection has to include protection against infrared a radiation-induced skin damage," *Skin Pharmacology and Physiology*, vol. 23, no. 1, pp. 15–17, 2010.
- [21] P. Schroeder, J. Lademann, M. E. Darvin et al., "Infrared radiation-induced matrix metalloproteinase in human skin: implications for protection," *Journal of Investigative Dermatology*, vol. 128, no. 10, pp. 2491–2497, 2008.
- [22] M. E. Darvin, W. Sterry, J. Lademann, and T. Vergou, "The role of carotenoids in human skin," *Molecules*, vol. 16, no. 12, pp. 10491–10506, 2011.
- [23] T. Rodrigues, L. P. de França, C. Kawai et al., "Protective role of mitochondrial unsaturated lipids on the preservation of the apoptotic ability of cytochrome c exposed to singlet oxygen," *The Journal of Biological Chemistry*, vol. 282, no. 35, pp. 25577–25587, 2007.
- [24] M. E. Darvin, I. Gersonde, H. Albrecht, L. Zastrow, W. Sterry, and J. Lademann, "In vivo Raman spectroscopic analysis of the influence of IR radiation on the carotenoid antioxidant substances beta-carotene and lycopene in the human skin. Formation of free radicals," *Laser Physics Letters*, vol. 4, no. 4, pp. 318–321, 2007.
- [25] F. M. Prado, M. C. B. Oliveira, S. Miyamoto et al., "Thymine hydroperoxide as a potential source of singlet molecular oxygen in DNA," *Free Radical Biology and Medicine*, vol. 47, no. 4, pp. 401–409, 2009.
- [26] H. Wefers and H. Sies, "Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen," *European Journal of Biochemistry*, vol. 137, no. 1–2, pp. 29–36, 1983.
- [27] J. Massari, R. Tokikawa, D. B. Medinas et al., "Generation of singlet oxygen by the glyoxal-peroxynitrite system," *Journal of the American Chemical Society*, vol. 133, no. 51, pp. 20761–20768, 2011.
- [28] G. D. Mendenhall, X. C. Sheng, and T. Wilson, "Yields of excited carbonyl species from alkoxyl and from alkylperoxyl radical dismutations," *Journal of the American Chemical Society*, vol. 113, no. 23, pp. 8976–8977, 1991.
- [29] P. Nagy, A. J. Kettle, and C. C. Winterbourn, "Superoxide-mediated formation of tyrosine hydroperoxides and methionine sulfoxide in peptides through radical addition and intramolecular oxygen transfer," *The Journal of Biological Chemistry*, vol. 284, no. 22, pp. 14723–14733, 2009.
- [30] J. R. Kanofsky, H. Hoogland, R. Wever, and S. J. Weiss, "Singlet oxygen production by human eosinophils," *The Journal of Biological Chemistry*, vol. 263, no. 20, pp. 9692–9696, 1988.
- [31] M. Akagawa, T. Shigemitsu, and K. Suyama, "Production of hydrogen peroxide by polyphenols and polyphenol rich beverages under quasi-physiological conditions," *Bioscience, Biotechnology and Biochemistry*, vol. 67, no. 12, pp. 2632–2640, 2003.
- [32] G. F. Fedorova, A. V. Trofimov, R. F. Vasilev, and T. L. Veprinstev, "Peroxy-radical-mediated chemiluminescence: mechanistic diversity and fundamentals for antioxidant assay," *ARKIVOC*, vol. Peroxy-radical-mediated chemiluminescence: mechanistic diversity and fundamentals for antioxidant assay, no. 8, pp. 163–215, 2007.
- [33] R. Michalski, J. Zielonka, E. Gapys, A. Marcinek, J. Joseph, and B. Kalyanaraman, "Real-time measurements of amino acid and protein hydroperoxides using coumarin boronic acid," *The Journal of Biological Chemistry*, vol. 289, no. 32, pp. 22536–22553, 2014.
- [34] A. N. Onyango, "Alternatives to the 'water oxidation pathway' of biological ozone formation," *Journal of Chemical Biology*, vol. 9, no. 1, pp. 1–8, 2016.
- [35] M. J. Steinbeck, A. U. Khan, and M. J. Karnovsky, "Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap," *The Journal of Biological Chemistry*, vol. 267, no. 19, pp. 13425–13433, 1992.
- [36] C. C. Winterbourn and A. J. Kettle, "Redox reactions and microbial killing in the neutrophil phagosome," *Antioxidants & Redox Signaling*, vol. 18, no. 6, pp. 642–660, 2013.
- [37] J. K. Hurst, "What really happens in the neutrophil phagosome?" *Free Radical Biology and Medicine*, vol. 53, no. 3, pp. 508–520, 2012.
- [38] C. S. Foote, F. C. Shook, and R. A. Abakerli, "Chemistry of superoxide ion. 4. Singlet oxygen is not a major product of dismutation," *Journal of the American Chemical Society*, vol. 102, no. 7, pp. 2503–2504, 1980.
- [39] M. J. Steinbeck, A. U. Khan, and M. J. Karnovsky, "Extracellular production of singlet oxygen by stimulated macrophages quantified using 9,10-diphenylanthracene and perylene in a polystyrene film," *The Journal of Biological Chemistry*, vol. 268, no. 21, pp. 15649–15654, 1993.
- [40] C. Prolo, M. N. Álvarez, and R. Radi, "Peroxynitrite, a potent macrophage-derived oxidizing cytotoxin to combat invading pathogens," *BioFactors*, vol. 40, no. 2, pp. 215–225, 2014.
- [41] P. Di Mascio, E. J. H. Bechara, M. H. G. Medeiros, K. Briviba, and H. Sies, "Singlet molecular oxygen production in the reaction of peroxynitrite with hydrogen peroxide," *FEBS Letters*, vol. 355, no. 3, pp. 287–289, 1994.

- [42] A. A. Noronha-Dutra, M. M. Epperlein, and N. Woolf, "Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing," *FEBS Letters*, vol. 321, no. 1, pp. 59–62, 1993.
- [43] G. A. Russell, "Deuterium-isotope effects in the autoxidation of aralkyl hydrocarbons—mechanism of the interaction of peroxy radicals," *Journal of the American Chemical Society*, vol. 79, no. 14, pp. 3871–3877, 1957.
- [44] S. Miyamoto, G. E. Ronsein, F. M. Prado et al., "Biological hydroperoxides and singlet molecular oxygen generation," *IUBMB Life*, vol. 59, no. 4–5, pp. 322–331, 2007.
- [45] S. Miyamoto, G. R. Martinez, M. H. G. Medeiros, and P. Di Mascio, "Singlet molecular oxygen generated by biological hydroperoxides," *Journal of Photochemistry and Photobiology B: Biology*, vol. 139, pp. 24–33, 2014.
- [46] K. M. Schaich, "Lipid oxidation: theoretical aspects," in *Baileys Industrial Oil and Fat Products*, F. Shahidi, Ed., vol. 1, pp. 269–355, Edible Oil and Fat Products: Chemistry, Properties and Health Effects, 6th edition, 2005.
- [47] M. Rác, M. Křupka, S. Binder et al., "Oxidative damage of U937 human leukemic cells caused by hydroxyl radical results in singlet oxygen formation," *PLOS ONE*, vol. 10, no. 3, Article ID e0116958, 2015.
- [48] A. N. Onyango and N. Baba, "New hypotheses on the pathways of formation of malondialdehyde and isofurans," *Free Radical Biology and Medicine*, vol. 49, no. 10, pp. 1594–1600, 2010.
- [49] K. Kaur, R. G. Salomon, J. O'Neil, and H. F. Hoff, "(Carboxyalkyl) pyrroles in human plasma and oxidized low-density lipoproteins," *Chemical Research in Toxicology*, vol. 10, no. 12, pp. 1387–1396, 1997.
- [50] A. N. Onyango and N. Baba, "A non-radical mechanism for the rearrangement of linoleic acid dihydroperoxides," *New Journal of Chemistry*, vol. 33, no. 8, pp. 1635–1636, 2009.
- [51] L. Trézl and J. Pipek, "Formation of excited formaldehyde in model reactions simulating real biological systems," *Journal of Molecular Structure: THEOCHEM*, vol. 170, pp. 213–223, 1988.
- [52] Y. Li, M. A. Trush, and J. D. Yager, "DNA damage caused by reactive oxygen species originating from a copper-dependent oxidation of the 2-hydroxy catechol of estradiol," *Carcinogenesis*, vol. 15, no. 7, pp. 1421–1427, 1994.
- [53] I. Kruk, K. Lichszteld, T. Michalska, J. Wrońska, and M. Bounias, "The formation of singlet oxygen during oxidation of catechol amines as detected by infrared chemiluminescence and spectrophotometric method," *Zeitschrift für Naturforschung C*, vol. 44, no. 11–12, pp. 895–900, 1989.
- [54] S. Ikeuci, Y. Miyamoto, T. Katoh, and K. Nishimura, "Action of ascorbic acid on a myosin molecule derived from carp," *Bio-science, Biotechnology and Biochemistry*, vol. 71, no. 8, pp. 2091–2094, 2007.
- [55] G. E. Ronsein, M. C. B. de Oliveira, M. H. G. de Medeiros, and P. di Mascio, "Mechanism of dioxindolylalanine formation by singlet molecular oxygen-mediated oxidation of tryptophan residues," *Photochemical and Photobiological Sciences*, vol. 10, no. 11, pp. 1727–1730, 2011.
- [56] G. S. Timmins, R. E. dos Santos, A. C. Whitwood et al., "Lipid peroxidation-dependent chemiluminescence from the cyclization of alkylperoxyl radicals to dioxetane radical intermediates," *Chemical Research in Toxicology*, vol. 10, no. 10, pp. 1090–1096, 1997.
- [57] S. H. Lee, T. Oe, and I. A. Blair, "Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins," *Science*, vol. 292, no. 5524, pp. 2083–2086, 2001.
- [58] C. Schneider, K. A. Tallman, N. A. Porter, and A. R. Brash, "Two distinct pathways of formation of 4-hydroxy-2-nonenal," *The Journal of Biological Chemistry*, vol. 276, no. 24, pp. 20831–20838, 2001.
- [59] R. C. Murphy and K. M. Johnson, "Cholesterol, reactive oxygen species, and the formation of biologically active mediators," *The Journal of Biological Chemistry*, vol. 283, no. 23, pp. 15521–15525, 2008.
- [60] H. Yin, L. Xu, and N. A. Porter, "Free radical lipid peroxidation: mechanisms and analysis," *Chemical Reviews*, vol. 111, no. 10, pp. 5944–5972, 2011.
- [61] A. D. Wentworth, B.-D. Song, J. Nieva, A. Shafton, S. Tripurenani, and P. Wentworth Jr., "The ratio of cholesterol 5,6-secosterols formed from ozone and singlet oxygen offers insight into the oxidation of cholesterol in vivo," *Chemical Communications*, no. 21, pp. 3098–3100, 2009.
- [62] M. Uemi, G. E. Ronsein, F. M. Prado et al., "Cholesterol hydroperoxides generate singlet molecular oxygen [O₂ (¹Δ_g)]: near-IR emission, ¹⁸O-labeled hydroperoxides, and mass spectrometry," *Chemical Research in Toxicology*, vol. 24, no. 6, pp. 887–895, 2011.
- [63] E. W. Kellogg and I. Fridovich, "Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system," *The Journal of Biological Chemistry*, vol. 250, no. 22, pp. 8812–8817, 1975.
- [64] A. U. Khan and M. Kasha, "Singlet molecular oxygen in the Haber-Weiss reaction," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 26, pp. 12365–12367, 1994.
- [65] W. H. Koppenol, "The Haber-Weiss cycle—70 years later," *Redox Report*, vol. 6, no. 4, pp. 229–234, 2001.
- [66] S. Miyamoto, I. L. Nantes, P. A. Faria et al., "Cytochrome c-promoted cardiolipin oxidation generates singlet molecular oxygen," *Photochemical and Photobiological Sciences*, vol. 11, no. 10, pp. 1536–1546, 2012.
- [67] E. E. Boh, W. H. Baricos, C. Bernofsky, and R. H. Steele, "Mitochondrial chemiluminescence elicited by acetaldehyde," *Journal of Bioenergetics and Biomembranes*, vol. 14, no. 2, pp. 115–133, 1982.
- [68] L. Trézl, G. Torok, G. Vasvari, J. Pipek, and L. Hullán, "Formation of burst of chemiluminescence, excited aldehydes, and singlet oxygen in model reactions and from carcinogenic compounds in rat liver S9 fractions," *Periodica Polytechnica-Chemical Engineering*, vol. 36, pp. 236–239, 1992.
- [69] E. Tyihák, S. Rozsnyay, É. Sárdi, G. Gullner, L. Trézl, and R. Gáborjányi, "Possibility of formation of excited formaldehyde and singlet oxygen in biotic and abiotic stress situations," *Acta Biologica Hungarica*, vol. 45, no. 1, pp. 3–10, 1994.
- [70] L. Trézl, L. Hullán, T. Szarvas, A. Csiba, and J. Pipek, "Analogies and differences in the excited reactions of formaldehyde and D-glucose," *Acta Biologica Hungarica*, vol. 49, no. 2–4, pp. 437–447, 1998.
- [71] E. Tyihák, A. M. Moricz, P. G. Ott, G. Katay, and E. Mincsovsics, "Biological characterization of ingredients in OPLC-BioArenagreenhouse-system: unique reactions of endogenous HCHO and O₃ in in vitro and in vivo conditions," *Chromatographia*, vol. 75, no. 17, pp. 983–990, 2012.
- [72] E. Tyihák, Á. M. Mócz, P. G. Ott, Z. Király-Véghely, G. Kátay, and E. Mincsovsics, "BioArena system for knowing and understanding the biological world: a review with new experimental results," *Journal of AOAC International*, vol. 96, no. 6, pp. 1189–1199, 2013.

- [73] T. Kato, K. Hiramoto, and K. Kikugawa, "Possible occurrence of new mutagens with the DNA breaking activity in coffee," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 306, no. 1, pp. 9–17, 1994.
- [74] Y. Fujita, K. Wakabayashi, M. Nagao, and T. Sugimura, "Implication of hydrogen peroxide in the mutagenicity of coffee," *Mutation Research Letters*, vol. 144, no. 4, pp. 227–230, 1985.
- [75] H. Ueno, K. Nakamuro, Y. Sayato, and S. Okada, "Characteristics of mutagenesis by glyoxal in *Salmonella typhimurium*: contribution of singlet oxygen," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 251, no. 1, pp. 99–107, 1991.
- [76] S.-B. Kim, I.-S. Kim, D.-M. Yeum, and Y.-H. Park, "Mutagenicity of Maillard reaction products from d-glucose-amino acid mixtures and possible roles of active oxygens in the mutagenicity," *Mutation Research/DNA Repair*, vol. 254, no. 1, pp. 65–69, 1991.
- [77] K. Hiramoto, T. Kato, and K. Kikugawa, "Generation of DNA-breaking activity in the Maillard reaction of glucose-amino acid mixtures in a solid system," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 285, no. 2, pp. 191–198, 1993.
- [78] J. Clayden, N. Greeves, and S. Warren, *Organic Chemistry*, Oxford University Press, Oxford, UK, 2nd edition, 2012.
- [79] I. Kruk, *Environmental Toxicology and Chemistry of Oxygen Species*, vol. 2, Springer, part 1, 1998.
- [80] N. Tada, L. Cui, H. Okubo, T. Miura, and A. Itoh, "A facile catalyst-free synthesis of gem-dihydroperoxides with aqueous hydrogen peroxide," *Chemical Communications*, vol. 46, no. 10, pp. 1772–1774, 2010.
- [81] K. S. Renar, S. Pečar, and J. Iskra, "Activation of aqueous hydrogen peroxide for non-catalyzed dihydroperoxidation of ketones by azeotropic removal of water," *Organic & Biomolecular Chemistry*, vol. 13, no. 36, pp. 9369–9372, 2015.
- [82] J. Hang, P. Ghorai, S. A. Finkenstaedt-Quinn et al., "Generation of singlet oxygen from fragmentation of monoactivated 1,1-dihydroperoxides," *Journal of Organic Chemistry*, vol. 77, no. 3, pp. 1233–1243, 2012.
- [83] R. Chaturvedi, T. de Sablet, R. Peek, and K. Wilson, "Spermine oxidase, a polyamine catabolic enzyme that links *Helicobacter pylori* CagA and gastric cancer risk," *Gut Microbes*, vol. 3, no. 1, pp. 48–56, 2012.
- [84] V. Battaglia, C. DeStefano Shields, T. Murray-Stewart, and R. A. Casero Jr., "Polyamine catabolism in carcinogenesis: potential targets for chemotherapy and chemoprevention," *Amino Acids*, vol. 46, no. 3, pp. 511–519, 2014.
- [85] T. Murray-Stewart, Y. Wang, A. Goodwin, A. Hacker, A. Meeker, and R. A. Casero Jr., "Nuclear localization of human spermine oxidase isoforms—possible implications in drug response and disease etiology," *FEBS Journal*, vol. 275, no. 11, pp. 2795–2806, 2008.
- [86] M. Salimi, K. Abdi, H. M. Kandelous et al., "Antiproliferative effects of copper(II)–polypyridyl complexes in breast cancer cells through inducing apoptosis," *BioMetals*, vol. 28, no. 2, pp. 267–278, 2015.
- [87] M. Cervelli, G. Bellavia, E. Fratini et al., "Spermine oxidase (SMO) activity in breast tumor tissues and biochemical analysis of the anticancer spermine analogues BEN_{Spm} and CPEN_{Spm}," *BMC Cancer*, vol. 10, article 555, 2010.
- [88] S. L. Hazen, A. d'Avignon, M. M. Anderson, F. F. Hsu, and J. W. Heinecke, "Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to oxidize α -amino acids to a family of reactive aldehydes. Mechanistic studies identifying labile intermediates along the reaction pathway," *Journal of Biological Chemistry*, vol. 273, no. 9, pp. 4997–5005, 1998.
- [89] Y. Kato, Y. Mori, Y. Makino et al., "Formation of N^ε-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification," *The Journal of Biological Chemistry*, vol. 274, no. 29, pp. 20406–20414, 1999.
- [90] W. Jian, S. H. Lee, J. S. Arora, M. V. S. Elipse, and I. A. Blair, "Unexpected formation of etheno-2'-deoxyguanosine adducts from 5(S)-hydroperoxyeicosatetraenoic acid: evidence for a bis-hydroperoxide intermediate," *Chemical Research in Toxicology*, vol. 18, no. 3, pp. 599–610, 2004.
- [91] Q. Wang, E. Durand, R. J. Elias, and R. V. Tikekar, "Generation of reactive oxidative species from thermal treatment of sugar solutions," *Food Chemistry*, vol. 196, pp. 301–308, 2016.
- [92] S. Kawakishi, Y. Okawa, and K. Uchida, "Oxidative damage of protein induced by the Amadori compound-copper ion system," *Journal of Agricultural and Food Chemistry*, vol. 38, no. 1, pp. 13–17, 1990.
- [93] A. Elgawish, M. Glomb, M. Friedlander, and V. M. Monnier, "Involvement of hydrogen peroxide in collagen cross-linking by high glucose in vitro and in vivo," *The Journal of Biological Chemistry*, vol. 271, no. 22, pp. 12964–12971, 1996.
- [94] S. Arena, A. M. Salzano, G. Renzone, C. D'Ambrosio, and A. Scaloni, "Non-enzymatic glycation and glycoxidation protein products in foods and diseases: an interconnected, complex scenario fully open to innovative proteomic studies," *Mass Spectrometry Reviews*, vol. 33, no. 1, pp. 49–77, 2014.
- [95] O. Novotný, K. Cejpek, and J. Velišek, "Formation of carboxylic acids during degradation of monosaccharides," *Czech Journal of Food Sciences*, vol. 26, no. 2, pp. 117–131, 2008.
- [96] M. Rác, M. Sedlářová, and P. Pospíšil, "The formation of electronically excited species in the human multiple myeloma cell suspension," *Scientific Reports*, vol. 5, article 8882, 2015.
- [97] X. Zhu, P. Wentworth Jr., A. D. Wentworth, A. Eschenmoser, R. A. Lerner, and I. A. Wilson, "Probing the antibody-catalyzed water-oxidation pathway at atomic resolution," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 8, pp. 2247–2252, 2004.
- [98] J. Brinkhorst, S. J. Nara, and D. A. Pratt, "Hock cleavage of cholesterol-5 α -hydroperoxide: an ozone-free pathway to the cholesterol ozonolysis products identified in arterial plaque and brain tissue," *Journal of the American Chemical Society*, vol. 130, no. 37, pp. 12224–12225, 2008.
- [99] M. Uemi, G. E. Ronsein, S. Miyamoto, M. H. G. Medeiros, and P. Di Mascio, "Generation of cholesterol carboxyaldehyde by the reaction of singlet molecular oxygen [O₂(¹Δg)] as well as ozone with cholesterol," *Chemical Research in Toxicology*, vol. 22, no. 5, pp. 875–884, 2009.
- [100] N. Miyoshi, L. Iuliano, S. Tomono, and H. Ohshima, "Implications of cholesterol autooxidation products in the pathogenesis of inflammatory diseases," *Biochemical and Biophysical Research Communications*, vol. 446, no. 3, pp. 702–708, 2014.
- [101] C. Hamelin, "Production of single- and double-strand breaks in plasmid dna by ozone," *International Journal of Radiation Oncology, Biology, Physics*, vol. 11, no. 2, pp. 253–257, 1985.
- [102] C. Hamelin and Y. S. Chung, "Repair of ozone-induced DNA lesions in *Escherichia coli* B cells," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 214, no. 2, pp. 253–255, 1989.

- [103] O. Cantoni, P. Sestili, and F. Cattabeni, "Induction of DNA double strand breaks in cultured mammalian cells exposed to hydrogen peroxide and histidine," *Cytotechnology*, vol. 5, no. 1, pp. 80–81, 1991.
- [104] O. Cantoni, P. Sestili, G. Brandi, and F. Cattabeni, "The L-histidine-mediated enhancement of hydrogen peroxide-induced cytotoxicity is a general response in cultured mammalian cell lines and is always associated with the formation of DNA double strand breaks," *FEBS Letters*, vol. 353, no. 1, pp. 75–78, 1994.
- [105] P. Sestili, O. Cantoni, F. Cattabeni, and D. Murray, "Evidence for separate mechanisms of cytotoxicity in mammalian cells treated with hydrogen peroxide in the absence or presence of L-histidine," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1268, no. 2, pp. 130–136, 1995.
- [106] I. Choma and W. Jesionek, "TLC-Direct bioautography as a high throughput method for detection of antimicrobials in plants," *Chromatography*, vol. 2, no. 2, pp. 225–238, 2015.
- [107] J. Cerkovnik and B. Plesničar, "Recent advances in the chemistry of hydrogen trioxide (HOOOH)," *Chemical Reviews*, vol. 113, no. 10, pp. 7930–7951, 2013.
- [108] M. Hajimohammadi, N. Safari, H. Mofakham, and A. Shaabani, "A new and efficient aerobic oxidation of aldehydes to carboxylic acids with singlet oxygen in the presence of porphyrin sensitizers and visible light," *Tetrahedron Letters*, vol. 51, no. 31, pp. 4061–4065, 2010.
- [109] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.
- [110] D. Harman, "The biologic clock: the mitochondria?" *Journal of the American Geriatrics Society*, vol. 20, no. 4, pp. 145–147, 1972.
- [111] I. Shokolenko, N. Venediktova, A. Bochkareva, G. I. Wilson, and M. F. Alexeyev, "Oxidative stress induces degradation of mitochondrial DNA," *Nucleic Acids Research*, vol. 37, no. 8, pp. 2539–2548, 2009.
- [112] A. Boveris, N. Oshino, and B. Chance, "The cellular production of hydrogen peroxide," *Biochemical Journal*, vol. 128, no. 3, pp. 617–630, 1972.
- [113] G. Loschen and A. Azzi, "On the formation of hydrogen peroxide and oxygen radicals in heart mitochondria," *Recent Advances in Studies on Cardiac Structure and Metabolism*, vol. 7, pp. 3–12, 1975.
- [114] V. Valez, A. Cassina, I. Batinic-Haberle, B. Kalyanaraman, G. Ferrer-Sueta, and R. Radi, "Peroxynitrite formation in nitric oxide-exposed submitochondrial particles: detection, oxidative damage and catalytic removal by Mn-porphyrins," *Archives of Biochemistry and Biophysics*, vol. 529, no. 1, pp. 45–54, 2013.
- [115] C. Giulivi, J. J. Poderoso, and A. Boveris, "Production of nitric oxide by mitochondria," *The Journal of Biological Chemistry*, vol. 273, no. 18, pp. 11038–11043, 1998.
- [116] M. Kirca, P. Kleinbongard, D. Soetkamp et al., "Interaction between Connexin 43 and nitric oxide synthase in mice heart mitochondria," *Journal of Cellular and Molecular Medicine*, vol. 19, no. 4, pp. 815–825, 2015.
- [117] G. B. Stefano, K. J. Mantione, L. Capellan et al., "Morphine stimulates nitric oxide release in human mitochondria," *Journal of Bioenergetics and Biomembranes*, vol. 47, no. 5, pp. 409–417, 2015.
- [118] T. Röszer, "Nitric oxide synthesis in the mitochondria of animal cells," in *The Biology of Subcellular Nitric Oxide*, pp. 169–178, Springer, Amsterdam, The Netherlands, 2012.
- [119] M. R. Ramis, S. Esteban, A. Miralles, D. Tan, and R. Reiter, "Protective effects of melatonin and mitochondria-targeted antioxidants against oxidative stress: a review," *Current Medicinal Chemistry*, vol. 22, no. 22, pp. 2690–2711, 2015.
- [120] R. Radi, J. S. Beckman, K. M. Bush, and B. A. Freeman, "Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide," *Archives of Biochemistry and Biophysics*, vol. 288, no. 2, pp. 481–487, 1991.
- [121] E. D. Kerver, I. M. C. Vogels, K. S. Bosch, H. Vreeling-Sindelárová, R. J. M. Van Den Munckhof, and W. M. Frederiks, "In situ detection of spontaneous superoxide anion and singlet oxygen production by mitochondria in rat liver and small intestine," *The Histochemical Journal*, vol. 29, no. 3, pp. 229–237, 1997.
- [122] M. Berneburg, S. Grether-Beck, V. Kürten et al., "Singlet oxygen mediates the UVA-induced generation of the photoaging-associated mitochondrial common deletion," *The Journal of Biological Chemistry*, vol. 274, no. 22, pp. 15345–15349, 1999.
- [123] R. De Bont and N. van Larebeke, "Endogenous DNA damage in humans: a review of quantitative data," *Mutagenesis*, vol. 19, no. 3, pp. 169–185, 2004.
- [124] J. Cadet, T. Douki, and J.-L. Ravanat, "Oxidatively generated damage to cellular DNA by UVB and UVA radiation," *Photochemistry and Photobiology*, vol. 91, no. 1, pp. 140–155, 2015.
- [125] A. Kumar, V. Pottiboyina, and M. D. Sevilla, "Hydroxyl radical (OH[•]) reaction with guanine in an aqueous environment: a DFT study," *The Journal of Physical Chemistry B*, vol. 115, no. 50, pp. 15129–15137, 2011.
- [126] D. Dai, Y. Chiao, D. J. Marcinek, H. H. Szeto, and P. S. Rabinovitch, "Mitochondrial oxidative stress in aging and healthspan," *Longevity & Healthspan*, vol. 3, article 6, 2014.
- [127] S. E. Schriener, N. J. Linford, G. M. Martin et al., "Medicine: extension of murine life span by overexpression of catalase targeted to mitochondria," *Science*, vol. 308, no. 5730, pp. 1909–1911, 2005.
- [128] P. C. Tapia, "Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: 'mitohormesis' for health and vitality," *Medical Hypotheses*, vol. 66, no. 4, pp. 832–843, 2006.
- [129] M. Ristow and K. Schmeisser, "Mitohormesis: promoting health and lifespan by increased levels of reactive oxygen species (ROS)," *Dose-Response*, vol. 12, no. 2, pp. 288–341, 2014.
- [130] K. Sharma, "Mitochondrial hormesis and diabetic complications," *Diabetes*, vol. 64, no. 3, pp. 663–672, 2015.
- [131] I. Wilkins-Peter, L. Delgado-Roche, J. M. Barrios, and G. B. Fabregas-Popoco, "Rectal insufflation of ozone attenuates chronic oxidative stress in elderly patients with cardiovascular diseases," *Oxidants and Antioxidants in Medical Science*, vol. 4, no. 1, pp. 23–27, 2015.
- [132] G. Martínez-Sánchez, S. M. Al-Dalain, S. Menéndez et al., "Therapeutic efficacy of ozone in patients with diabetic foot," *European Journal of Pharmacology*, vol. 523, no. 1–3, pp. 151–161, 2005.
- [133] M. Sagai and V. Bocci, "Mechanisms of action involved in ozone therapy: is healing induced via a mild oxidative stress?" *Medical Gas Research*, vol. 1, article 29, 2011.
- [134] R. P. E. Sarkany, "Making sense of the porphyrias," *Photodermatology Photoimmunology and Photomedicine*, vol. 24, no. 2, pp. 102–108, 2008.

- [135] A. Umeno, M. Shichiri, N. Ishida et al., "Singlet oxygen induced products of linoleates, 10- and 12-(Z,E)-hydroxyoctadecadienoic acids (HODE), can be potential biomarkers for early detection of type 2 Diabetes," *PLoS ONE*, vol. 8, no. 5, Article ID e63542, 2013.
- [136] K. Murotomi, A. Umeno, M. Yasunaga et al., "Switching from singlet-oxygen-mediated oxidation to free-radical-mediated oxidation in the pathogenesis of type 2 diabetes in model mouse," *Free Radical Research*, vol. 49, no. 2, pp. 133–138, 2015.
- [137] A. Umeno, K. Yoshino, Y. Hashimoto et al., "Multi-biomarkers for early detection of type 2 diabetes, including 10- and 12-(Z,E)-hydroxyoctadecadienoic acids, insulin, leptin, and adiponectin," *PLoS ONE*, vol. 10, no. 7, Article ID e0130971, 2015.
- [138] A. Umeno, H. Nagai, Y. Hagihara, and Y. Yoshida, "Demand for the early detection of diabetic risk at annual health examinations and a probable solution," *Journal of Diabetes and Metabolism*, vol. 6, article 589, 2015.
- [139] S. M. Grundy, "Pre-diabetes, metabolic syndrome, and cardiovascular risk," *Journal of the American College of Cardiology*, vol. 59, no. 7, pp. 635–643, 2012.
- [140] S. L. Nowotarski, P. M. Woster, and R. A. Casero Jr., "Polyamines and cancer: implications for chemotherapy and chemoprevention," *Expert Reviews in Molecular Medicine*, vol. 15, article e3, 2013.
- [141] P. Wentworth Jr., J. Nieva, C. Takeuchi et al., "Evidence for ozone formation in human atherosclerotic arteries," *Science*, vol. 302, no. 5647, pp. 1053–1056, 2003.
- [142] S. Tomono, N. Miyoshi, H. Shiokawa et al., "Formation of cholesterol ozonolysis products in vitro and in vivo through a myeloperoxidase-dependent pathway," *Journal of Lipid Research*, vol. 52, no. 1, pp. 87–97, 2011.
- [143] F. Luchetti, B. Canonico, E. Cesarini et al., "7-ketocholesterol and 5,6-secosterol induce human endothelial cell dysfunction by differential mechanisms," *Steroids*, vol. 99, pp. 204–211, 2015.
- [144] Q. Zhang, E. T. Powers, J. Nieva et al., "Metabolite-initiated protein misfolding may trigger Alzheimer's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 14, pp. 4752–4757, 2004.
- [145] D. A. Bosco, D. M. Fowler, Q. Zhang et al., "Elevated levels of oxidized cholesterol metabolites in Lewy body disease brains accelerate α -synuclein fibrilization," *Nature Chemical Biology*, vol. 2, no. 5, pp. 249–253, 2006.
- [146] J. Nieva, B.-D. Song, J. K. Rogel et al., "Cholesterol secosterol aldehydes induce amyloidogenesis and dysfunction of wild-type tumor protein p53," *Chemistry & Biology*, vol. 18, no. 7, pp. 920–927, 2011.
- [147] S. Tomono, Y. Yasue, N. Miyoshi, and H. Ohshima, "Cytotoxic effects of secosterols and their derivatives on several cultured cells," *Bioscience, Biotechnology and Biochemistry*, vol. 77, no. 3, pp. 651–653, 2013.
- [148] N. K. Cygan, J. C. Scheinost, T. D. Butters, and P. Wentworth, "Adduction of cholesterol 5,6-secosterol aldehyde to membrane-bound myelin basic protein exposes an immunodominant epitope," *Biochemistry*, vol. 50, no. 12, pp. 2092–2100, 2011.
- [149] Y. Nishinaka, T. Arai, S. Adachi, A. Takaori-Kondo, and K. Yamashita, "Singlet oxygen is essential for neutrophil extracellular trap formation," *Biochemical and Biophysical Research Communications*, vol. 413, no. 1, pp. 75–79, 2011.
- [150] J. Lademann, W. Köcher, R. Yu et al., "Cutaneous carotenoids: the mirror of lifestyle?" *Skin Pharmacology and Physiology*, vol. 27, no. 4, pp. 201–207, 2014.
- [151] I. Sluijjs, E. Cadier, J. W. J. Beulens, D. L. van der A, A. M. W. Spijkerman, and Y. T. van der Schouw, "Dietary intake of carotenoids and risk of type 2 diabetes," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 25, no. 4, pp. 376–381, 2015.
- [152] I. Sluijjs, J. W. J. Beulens, D. E. Grobbee, and Y. T. Van Der Schouw, "Dietary carotenoid intake is associated with lower prevalence of metabolic syndrome in middle-aged and elderly men," *Journal of Nutrition*, vol. 139, no. 5, pp. 987–992, 2009.
- [153] M. A. Beydoun, M. R. Shroff, X. Chen, H. A. Beydoun, Y. Wang, and A. B. Zonderman, "Serum antioxidant status is associated with metabolic syndrome among U.S. adults in recent national surveys," *The Journal of Nutrition*, vol. 141, no. 5, pp. 903–913, 2011.
- [154] A. Kaulmann and T. Bohn, "Carotenoids, inflammation, and oxidative stress-implications of cellular signaling pathways and relation to chronic disease prevention," *Nutrition Research*, vol. 34, no. 11, pp. 907–929, 2014.
- [155] G. Valacchi, A. Pecorelli, M. Mencarelli, E. Maioli, and P. A. Davis, "Beta-carotene prevents ozone-induced proinflammatory markers in murine skin," *Toxicology and Industrial Health*, vol. 25, no. 4-5, pp. 241–247, 2009.
- [156] M. E. Darvin, W. Sterry, J. Lademann, and A. Patzelt, "Alcohol consumption decreases the protection efficiency of the antioxidant network and increases the risk of sunburn in human skin," *Skin Pharmacology and Physiology*, vol. 26, no. 1, pp. 45–51, 2012.
- [157] K. Aoki, Y. Ito, R. Sasaki, M. Ohtani, N. Hamajima, and A. Asano, "Smoking, alcohol drinking and serum carotenoids levels," *Japanese Journal of Cancer Research*, vol. 78, no. 10, pp. 1049–1056, 1987.
- [158] S. Balbo, L. Meng, R. L. Bliss, J. A. Jensen, D. K. Hatsukami, and S. S. Hecht, "Time course of DNA adduct formation in peripheral blood granulocytes and lymphocytes after drinking alcohol," *Mutagenesis*, vol. 27, no. 4, pp. 485–490, 2012.
- [159] M. Tamura, H. Matsui, T. Kaneko, and I. Hyodo, "Alcohol is an oxidative stressor for gastric epithelial cells: detection of superoxide in living cells," *Journal of Clinical Biochemistry and Nutrition*, vol. 53, no. 2, pp. 75–80, 2013.
- [160] M. Tamura, H. Ito, H. Matsui, and I. Hyodo, "Acetaldehyde is an oxidative stressor for gastric epithelial cells," *Journal of Clinical Biochemistry and Nutrition*, vol. 55, no. 1, pp. 26–31, 2014.
- [161] Y. Liu, H. Chen, Z. Sun, and X. Chen, "Molecular mechanisms of ethanol-associated oro-esophageal squamous cell carcinoma," *Cancer Letters*, vol. 361, no. 2, pp. 164–173, 2015.
- [162] S.-K. S. Hong, R. Chaturvedi, M. B. Piazuelo et al., "Increased expression and cellular localization of spermine oxidase in ulcerative colitis and relationship to disease activity," *Inflammatory Bowel Diseases*, vol. 16, no. 9, pp. 1557–1566, 2010.
- [163] M. M. Huycke, V. Abrams, and D. R. Moore, "Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA," *Carcinogenesis*, vol. 23, no. 3, pp. 529–536, 2002.
- [164] M. A. Azcárate-Peril, M. Sikes, and J. M. Bruno-Bárcena, "The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer?" *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 301, no. 3, pp. G401–G424, 2011.
- [165] A. Mohammadi, S. Jazayeri, K. Khosravi-Darani et al., "Effects of probiotics on biomarkers of oxidative stress and inflammatory factors in petrochemical workers: a randomized, double-blind, placebo-controlled trial," *International Journal of Preventive Medicine*, vol. 6, no. 1, p. 82, 2015.

- [166] C. Patel, H. Ghanim, S. Ravishankar et al., "Prolonged reactive oxygen species generation and nuclear factor- κ B activation after a high-fat, high-carbohydrate meal in the obese," *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 11, pp. 4476–4479, 2007.
- [167] M. W. Poulsen, M. J. Bak, J. M. Andersen et al., "Effect of dietary advanced glycation end products on postprandial appetite, inflammation, and endothelial activation in healthy overweight individuals," *European Journal of Nutrition*, vol. 53, no. 2, pp. 661–672, 2014.
- [168] S. I. Yamagishi and T. Matsui, "Pathologic role of dietary advanced glycation end products in cardiometabolic disorders, and therapeutic intervention," *Nutrition*, vol. 32, no. 2, pp. 157–165, 2015.
- [169] G. Daffu, C. H. del Pozo, K. M. O'Shea, R. Ananthkrishnan, R. Ramasamy, and A. M. Schmidt, "Radical roles for RAGE in the pathogenesis of oxidative stress in cardiovascular diseases and beyond," *International Journal of Molecular Sciences*, vol. 14, no. 10, pp. 19891–19910, 2013.
- [170] K. B. Pandey and S. I. Rizvi, "Plant polyphenols as dietary antioxidants in human health and disease," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 5, pp. 270–278, 2009.
- [171] M. S. Butt, A. Imran, M. K. Sharif et al., "Black tea polyphenols: a mechanistic treatise," *Critical Reviews in Food Science and Nutrition*, vol. 54, no. 8, pp. 1002–1011, 2014.
- [172] V. Calabrese, C. Cornelius, A. Trovato-Salinaro et al., "The hormetic role of dietary antioxidants in free radical-related diseases," *Current Pharmaceutical Design*, vol. 16, no. 7, pp. 877–883, 2010.
- [173] A. Camargo, O. A. Rangel-Zuñiga, C. Haro et al., "Olive oil phenolic compounds decrease the postprandial inflammatory response by reducing postprandial plasma lipopolysaccharide levels," *Food Chemistry*, vol. 162, pp. 161–171, 2014.

Research Article

Bach1 Induces Endothelial Cell Apoptosis and Cell-Cycle Arrest through ROS Generation

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The transcription factor BTB and CNC homology 1 (Bach1) regulates genes involved in the oxidative stress response and cell-cycle progression. We have recently shown that Bach1 impairs cell proliferation and promotes apoptosis in cultured endothelial cells (ECs), but the underlying mechanisms are largely uncharacterized. Here we demonstrate that Bach1 upregulation impaired the blood flow recovery from hindlimb ischemia and this effect was accompanied both by increases in reactive oxygen species (ROS) and cleaved caspase 3 levels and by declines in the expression of cyclin D1 in the injured tissues. We found that Bach1 overexpression induced mitochondrial ROS production and caspase 3-dependent apoptosis and its depletion attenuated H₂O₂-induced apoptosis in cultured human microvascular endothelial cells (HMVECs). Bach1-induced apoptosis was largely abolished when the cells were cultured with N-acetyl-L-cysteine (NAC), a ROS scavenger. Exogenous expression of Bach1 inhibited the cell proliferation and the expression of cyclin D1, induced an S-phase arrest, and increased the expression of cyclin E2, which were partially blocked by NAC. Taken together, our results suggest that Bach1 suppresses cell proliferation and induces cell-cycle arrest and apoptosis by increasing mitochondrial ROS production, suggesting that Bach1 may be a promising treatment target for the treatment of vascular diseases.

1. Introduction

The transcription factor BTB and CNC homology 1 (Bach1) regulates genes involved in apoptosis, the oxidative stress response, mitotic chromatin dynamics, and the cell-cycle [1–5]. Previous studies suggest that Bach1 deficiency may protect against oxidative tissue damage in murine models of lung, liver, intestine, pancreas, and cardiovascular disease [6–11], and we have shown that Bach1 suppresses angiogenesis in mice with surgically induced hindlimb ischemia (HLI) [2]. Our results also indicate that Bach1 disrupts Wnt/ β -catenin signaling and that this disruption reduces the proliferation, migration, and tube formation activity of endothelial cells (ECs) [12]. Bach1 upregulation also increased apoptosis in cultured ECs, but the mechanisms involved in Bach1-induced EC apoptosis are largely uncharacterized.

Physiological levels of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radical, are required for normal endothelial activity [13]; for example, we have shown that inhibition of NADPH oxidase subunit 4, which is among the most prominent sources of ROS production in the endothelium [14], impairs the migration, proliferation, and tube formation of human microvascular endothelial cells (HMVECs) [15]. However, the high ROS levels caused by inflammation and the response to ischemic injury appeared to impair neovascularization in the ischemic limbs of mice [16, 17] by inducing endothelial dysfunction and apoptosis [17]. The role of ROS in Bach1-induced apoptosis has not been investigated. Thus, in the present study, we conducted a series of analyses in a murine HLI model and in cultured HMVECs to determine whether the proapoptotic activity of Bach1 in ECs is mediated by ROS production.

2. Materials and Methods

2.1. Reagents. N-Acetyl-L-cysteine (A7250), H₂O₂ (88597), and the β -actin antibody (A5316) were purchased from Sigma-Aldrich (St. Louis, MO). Dihydroethidium (DHE, D1168) and MitoSOX Red (M36008) were obtained from Invitrogen (Carlsbad, CA). Antibodies against Bach1 (sc-14700), Bcl2 (sc-7382), Bcl-xL (sc-8392), HO-1 (sc-1796), cyclin D1 (sc-20044), cyclin E2 (sc-28351), cyclin B1 (sc-595), CDK2 (sc-163), CDK4 (sc-601), CDK6 (sc-177), p21 (sc-397), p53 (sc-126), the human Bach1 siRNA (sc-37064), and the control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cleaved caspase 3 (9664) and cyclin A2 (4656) were obtained from Cell Signaling (Beverly, MA). The recombinant adenoviruses coding for human Bach1 (Ad-GFP-Bach1) or GFP (Ad-GFP) were purchased from GenePharma (Shanghai, China).

2.2. Cells. Human microvascular endothelial cells (HMVECs) were immortalized with the human telomerase catalytic protein (hTERT) [18] and obtained as a kind gift from Dr. Rong Shao (University of Massachusetts, Springfield, MA). The cells were cultured as described previously.

2.3. Mice. Eight-week-old male C57BL/6J mice were obtained from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and housed in a standard, pathogen-free facility. All experimental procedures were approved by the Ethics Committee of Experimental Research at Fudan University Shanghai Medical College and were consistent with US National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

2.4. Transfection and Viral Transduction of ECs. HMVECs were transfected with the Bach1 siRNA (100 nmol/L) or the nontargeting negative control siRNA (100 nmol/L) using the Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The transfection medium was replaced after six hours by EBM-2 medium and the cells were incubated for another 72 hours. Transfection efficiency was verified using reverse transcription polymerase chain reaction and Western blotting. For the viral transduction, cells were infected with the adenoviruses (Ad-Bach1 or Ad-GFP) at an MOI of 25. At 72 h after infection, cell viability was assessed. No detectable cellular toxicity was observed. Transduction was verified via GFP expression.

2.5. Cell Proliferation. EC proliferation was determined by bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA. HMVECs were seeded in a 96-well plate in culture medium; then BrdU was added to each well and incubated with the cells for six hours. BrdU incorporation was evaluated according to the manufacturer's instructions (Calbiochem, San Diego, CA). The absorbance was measured at 450 nm using a spectrophotometer microplate reader. Each experiment was performed in triplicate, and each assay was performed three independent times.

2.6. Cell-Cycle Profile. HMVECs were harvested and fixed in 70% ethanol for 1 h at 4°C and then stained with a solution containing 0.05 mg/mL propidium iodide, 1 mg/mL RNase A, and 0.3% Triton X-100 in the dark for 1 h. The percentage of cells in different phases of the cell-cycle was examined by measuring the DNA content (propidium iodide intensity) with a flow cytometer (Beckman Coulter, Brea, CA), and populations of G1, S, and G2/M phase cells were determined with the ModFIT software. Each experiment was repeated three independent times.

2.7. Apoptosis Analysis. Cells were incubated with propidium iodide and annexin V-PE for 15 min at 37°C; then, the samples were analyzed with a flow cytometer (Beckman Coulter), and the percentage of cells that were positive for annexin V was calculated. Each experiment was performed in triplicate, and each assay was performed three times. To evaluate apoptosis in ischemic muscles, cleaved caspase 3 levels in skeletal muscle tissue homogenates were measured using Western blotting.

2.8. Measurement of Intracellular ROS Levels. ROS production was monitored via the dihydroethidium (DHE) assay as described previously [19]. Briefly, the cells were incubated with DHE (5 μ M) for 20 min in a light-protected humidified chamber at 37°C and then harvested and resuspended in PBS; then, DHE fluorescence was excited at 535 nm and monitored at 610 nm with a flow cytometer (Beckman Coulter). The ROS levels were expressed as the fold change relative to the control. MitoSOX Red was used to measure mitochondrial ROS production [20]. HMVECs were loaded with MitoSOX Red (5 μ M) for 15 min in a light-protected humidified chamber at 37°C, followed by washout. Cells were then incubated with Hoechst 33258 before image acquisition. Confocal images were obtained by excitation at 510 nm and measuring the emitted light at 580 nm with identical parameters for all samples. MitoSOX Red fluorescence was measured in five different areas in each sample. Immunofluorescence intensity of MitoSOX was analyzed by ImageJ software (NIH). Fluorescent levels were expressed as percent increase over the control. Each experiment was performed three times in three replicate wells.

2.9. Western Blot Analysis. Western blot analysis was performed as described previously [21]. Briefly, cells or tissue homogenates were lysed with RIPA buffer; then, the proteins were separated via SDS-PAGE, labeled with the corresponding antibodies and detected via chemiluminescence with a Tanon-5500 Imaging System (Tanon Science & Technology Ltd., Shanghai, China). Band intensities were quantified with the ImageJ software and normalized to the control. Each experiment was performed three times.

2.10. Murine Ischemic Hindlimb Model. Unilateral hindlimb ischemia was induced via femoral artery ligation as described previously [12], and then the animals were randomly assigned to treatment with intramuscular injections of Ad-Bach1 or Ad-GFP (2×10^8 plaque-forming units in 40 μ L per mouse;

$n = 12$ per experimental group). Injections were administered in the gastrocnemius muscle and the adductor muscle immediately after HLI induction.

Blood flow measurements were performed at the indicated time points with a MoorLDI2-2 laser Doppler imaging system (Moor Instruments, Devon, UK); the mice were euthanized with sodium pentobarbital (50 mg/kg i.p.) and maintained at 37°C on a heating plate to minimize temperature variation. Measurements in the ischemic limb were normalized to measurements in the nonischemic, contralateral limb.

Mice were sacrificed on day seven or 14 postsurgery and adductor muscles were harvested and snap frozen in OCT compound for cryosectioning. ROS levels were determined via DHE fluorescence. Briefly, the unfixed tissues were cut into 10 μM thick sections and incubated with DHE (2 μM) at 37°C for 30 min in a light-protected humidified chamber. Pictures from five random fields of each section and four sections per mouse were taken using a fluorescence microscope. Positive staining was quantified by measuring the percentage of positive staining/ mm^2 using the ImageJ software.

2.11. Statistical Analysis. Data are presented as the mean \pm SEM. Comparisons between 2 groups were evaluated for significance with the *t*-test. Differences among groups were determined with one-way analysis of variance followed by Bonferroni post hoc test. Differences between groups were considered significant when $P < 0.05$. Analyses were performed using GRAPHPAD Prism Version 5.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Bach1 Overexpression Promotes ROS Production and Apoptosis in the Ischemic Limbs of Mice. Recently, we have shown that Bach1 disrupts Wnt/ β -catenin signaling and impedes angiogenesis in a murine model of hindlimb ischemia (HLI) [12]; however, Bach1 is also known to participate in the oxidative stress response [6], cell-cycle regulation [3, 5], and apoptosis [8]. Thus, we investigated whether mechanisms involved in ROS production, cell-cycle progression, and apoptosis can be altered by upregulating Bach1 in the ischemic limbs of mice. Unilateral HLI was surgically induced by ligating the femoral artery in one hind limb, and then the injured limb was treated with intramuscular injections of adenoviruses coding for Bach1 (Ad-Bach1) or GFP (Ad-GFP) or an equal volume of normal saline (NS); two weeks later, perfusion measurements confirmed that blood flow was significantly lower in the injured limbs of Ad-Bach1 mice than in the injured limbs of mice treated with Ad-GFP or NS (Figure 1(a)). Cells with elevated ROS levels were significantly more common after treatment with Ad-Bach1 than after Ad-GFP or NS injection (Figures 1(b) and 1(c)), and Ad-Bach1 treatment was associated with significantly greater levels of cleaved caspase 3 (Figure 1(d)), which promotes apoptosis, and with lower levels of cyclin D1 (Figure 1(d)), which is required for progression through the G1 phase of the cell cycle. Thus, Bach1 overexpression appears to impede the angiogenic response to ischemic injury by increasing cellular

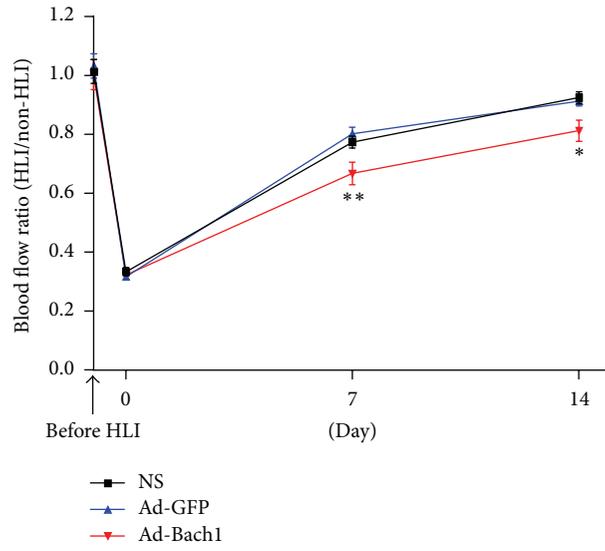
ROS production, promoting apoptosis, and disrupting cell-cycle progression.

3.2. Bach1 Promotes Mitochondrial ROS Production and Apoptosis in Cultured ECs. The results from our previous investigation suggested that when Ad-Bach1 was delivered to the ischemic limbs of mice, the vectors tended to be expressed by ECs [12]. Thus, we performed a series of in vitro experiments to determine whether ROS levels, apoptosis, and cell-cycle progression can be altered in ECs by manipulating Bach1 expression. The effect of Bach1 upregulation was evaluated by comparing assessments in Ad-Bach1 infected and Ad-GFP infected HMVECs, while Bach1 downregulation was evaluated by performing experiments in HMVECs that had been transfected with Bach1 siRNA (Bach1siRNA) or a control siRNA (Con siRNA).

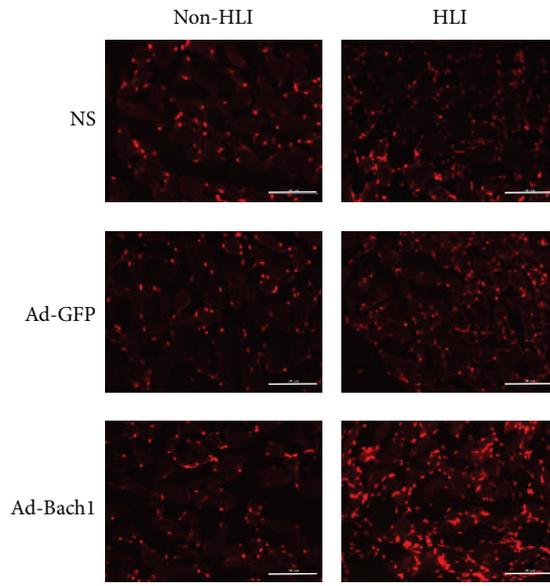
Bach 1 overexpression appeared to promote apoptotic nuclear condensation (Figure 2(a)) and cell apoptosis (Figure 2(b)), and cleaved caspase 3 levels (Figure 2(c)) and intracellular ROS levels (Figure 3(a)) were significantly higher in populations of Ad-Bach1 HMVECs than in Ad-GFP HMVECs. MitoSOX Red, a redox fluorophore detecting selectively mitochondrial superoxide, was used to evaluate mitochondrial ROS generation in HMVECs. The fluorescence intensity of MitoSOX Red was significantly higher in Ad-Bach1 HMVECs than that in Ad-GFP HMVECs (Figure 3(b)), indicating that Bach1 increases mitochondrial ROS levels. The higher levels of Bach1 expression was also associated with declines in expression of the apoptosis inhibitors Bcl2, Bcl-xL, and heme oxygenase 1 (HO-1) (Figure 2(c)). However, when Ad-Bach1 HMVECs were cultured with the ROS scavenger N-acetyl-L-cysteine (NAC; 10 mM), mitochondrial ROS levels (Figure 3(b)), cell apoptosis (Figure 3(c)), and cleaved caspase 3 levels (Figure 3(d)) declined significantly.

Measurements in Bach1siRNA- and Con siRNA-transfected cells were generally similar under normal culture conditions, but when oxidative stress was induced by culturing the cells with 500 μM hydrogen peroxide, Bach1 silencing was associated with significant declines in ROS levels (Figure 4(a)), cell apoptosis (Figure 4(b)), and cleaved caspase 3 levels (Figure 4(c)). Collectively, these observations suggest that Bach1 activates ROS-dependent apoptotic signaling pathways in ECs.

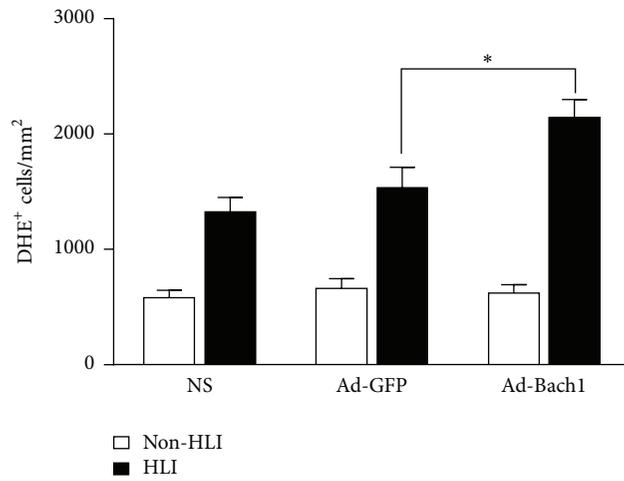
3.3. Bach1 Disrupts Cell-Cycle Progression and Limits Proliferation in Cultured ECs. Bach1 overexpression was associated with a significant decline in cell proliferation (Figure 5(a)), and this decline was partially abolished by culturing the cells with NAC (Figure 5(b)). However, cell proliferation was significantly greater in Bach1siRNA than in Con siRNA HMVECs (Figure 5(c)), even though apoptosis rates for the two cell populations were similar (Figure 4(b)). Bach1 overexpression also caused a decrease of cells in the G0/G1 phase and an increase of cells in the S phase, and this S-phase cell-cycle arrest was partially blocked by concurrent treatment with NAC (Figures 5(d) and 5(e)). Furthermore, cyclin D1 levels were significantly reduced in Ad-Bach1 HMVECs but



(a)



(b)



(c)

FIGURE 1: Continued.

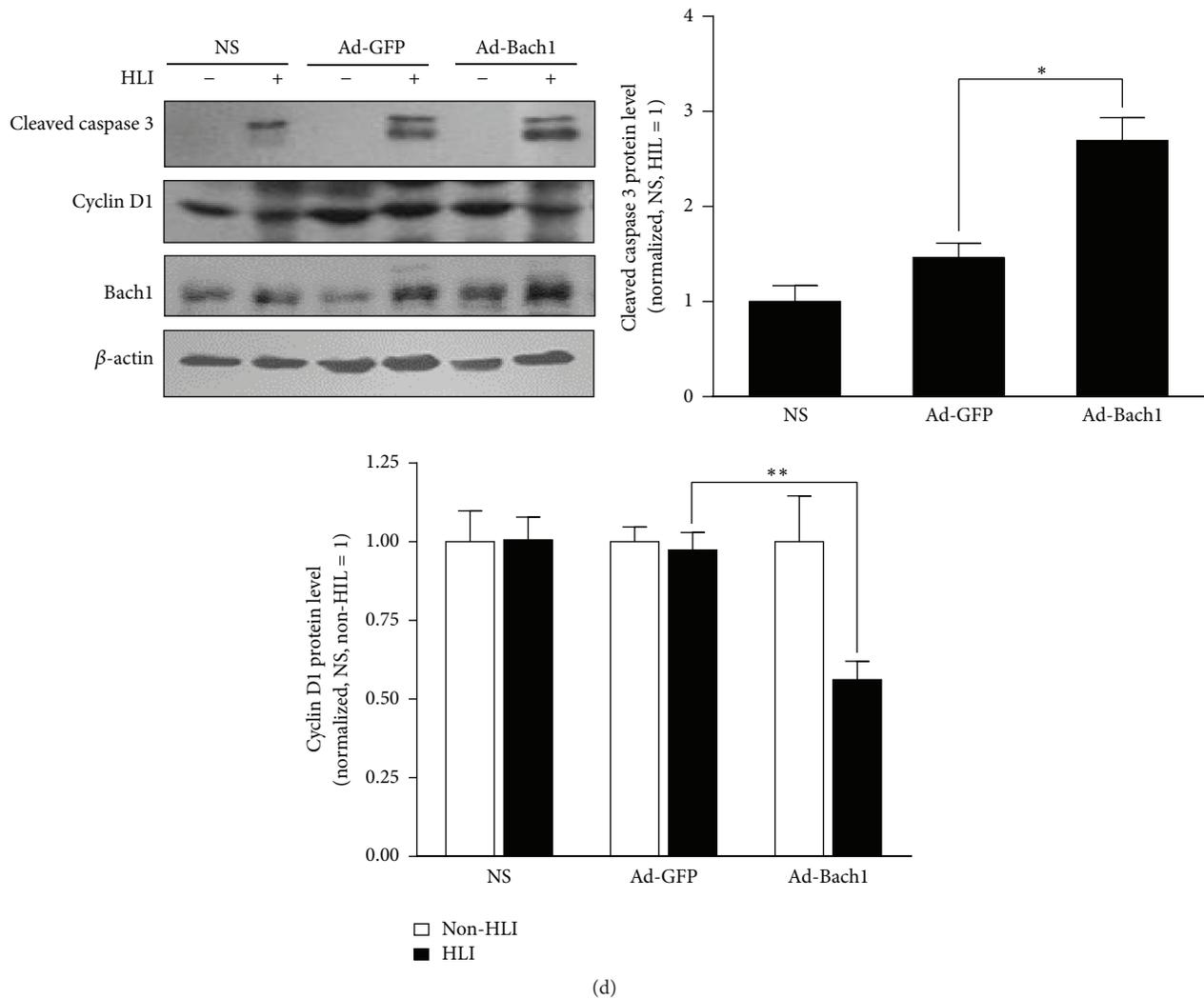


FIGURE 1: Bach1 overexpression promotes ROS production and apoptosis in the ischemic limbs of mice. Eight-week-old male C57BL/6 mice were subjected to hindlimb ischemia (HLI). GFP, Bach1 adenovirus, or saline (NS) was injected into the adductor and gastrocnemius muscles immediately after surgery. (a) Bach1 inhibited blood flow restoration after HLI. Blood flows of ischemic and nonischemic limb were measured with a laser Doppler imaging system at 0, 7, and 14 days after surgery. Ratios of blood flow from ischemic hindlimb to nonischemic hindlimb are shown ($n = 12$; * $P < 0.05$, ** $P < 0.01$ versus Ad-GFP). ((b) and (c)) ROS level in nonischemic and ischemic muscle tissue on day 7 was determined by an in situ detection of superoxide with dihydroethidium (DHE) fluorescence. Bars, 100 μm (b). DHE-positive cells were quantified as DHE-positive cells/ mm^2 . Data from four sections of each mouse muscle tissue are shown in graphics and $n = 6$ for each group ((c), * $P < 0.05$ versus Ad-GFP). (d) Seven days after HLI, cleaved caspase 3 and cyclin D1 protein levels were evaluated in HLI and non-HLI limbs via Western blot ($n = 6$; * $P < 0.05$, ** $P < 0.01$ versus Ad-GFP).

increased significantly when the cells were cultured with NAC (Figures 6(a) and 6(b)). Cyclin E2 levels were significantly greater in Ad-Bach1 HMVECs but decreased markedly when the cells were treated with NAC (Figures 6(a) and 6(b)). Thus, the decline in cell proliferation associated with Bach1 overexpression likely evolved, at least in part, from ROS-induced changes in cell-cycle progression.

4. Discussion

Bach1 is known to regulate the expression of genes involved in the oxidative stress response and cell-cycle progression [1–5], and we have recently shown that Bach1 impairs proliferation

and promotes apoptosis in cultured ECs [12]. Thus, we manipulated the level of Bach1 expression in the limbs of mice with HLI and in cultured HMVECs to determine whether the proapoptotic activity of Bach1 is mediated by ROS production. Our results indicated that Bach1 upregulation impaired the blood flow recovery from hindlimb ischemia and this effect was accompanied both by increases in ROS and cleaved caspase 3 levels and by declines in the expression of cyclin D1 in the injured tissues. Bach1 also promoted mitochondrial ROS production and the levels of cleaved caspase 3 in cultured HMVECs and disrupted cell-cycle progression, and these effects were largely abolished when the cells were cultured with the ROS scavenger NAC. Thus,

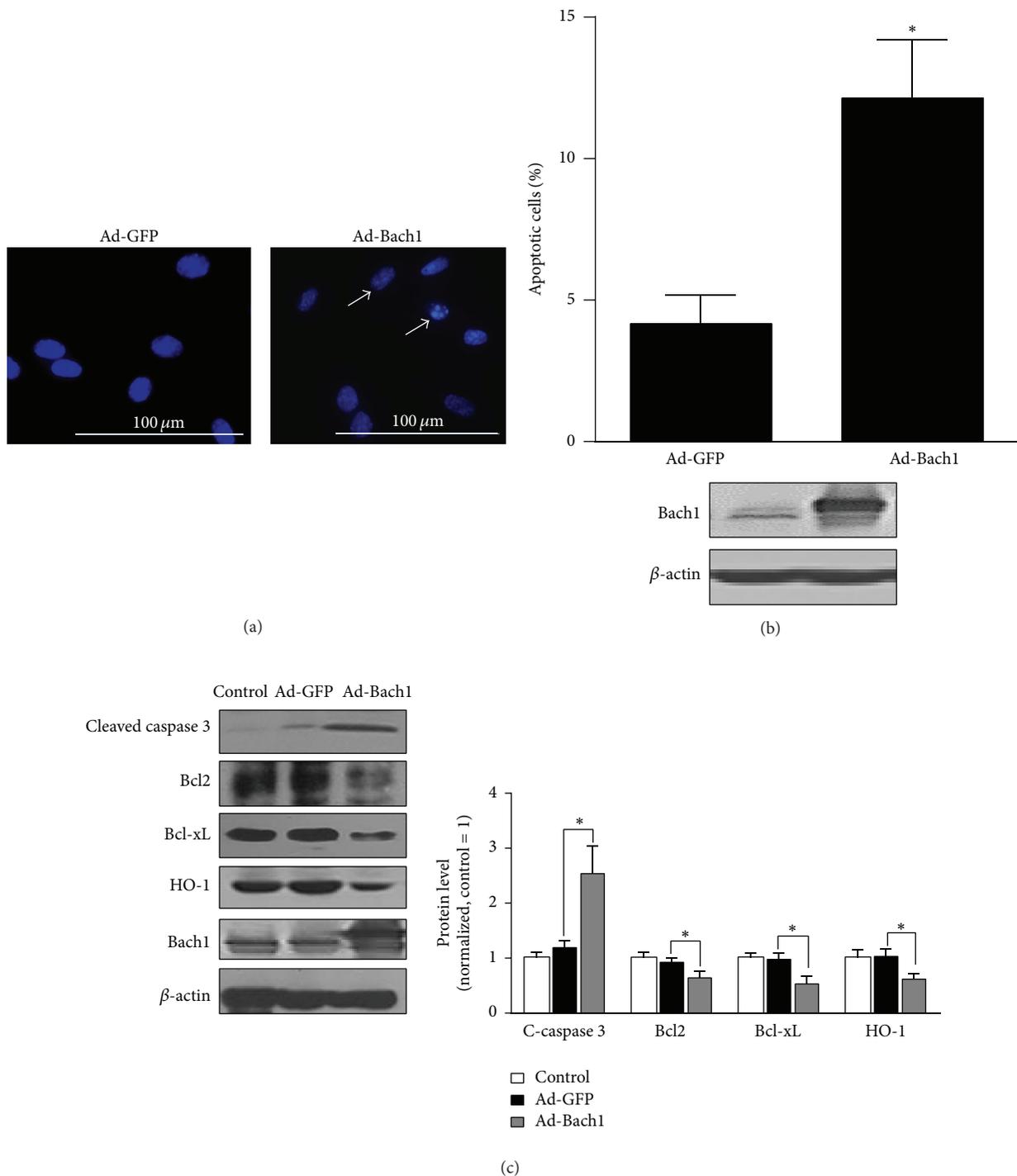


FIGURE 2: Bach1 promotes apoptosis in cultured HMVECs. (a) HMVECs were infected with the adenoviruses (Ad-GFP or Ad-Bach1); then cells were fixed at 72 hours after infection, and nuclei were visualized by Hoechst 33342. Cells with nuclear condensation are indicated by white arrows. Bars, 100 μm . (b) Ad-GFP- and Ad-Bach1-infected HMVECs were seeded in 60 mm dishes, cultured for 72 hours, and then harvested and labeled with annexin V and PI. Cell apoptosis was quantified by identifying positively annexin V labeled cells via flow cytometry ($n = 3$; * $P < 0.05$ versus Ad-GFP, upper panel). Bach1 protein levels were evaluated via Western blot (lower panel). (c) Cleaved caspase 3, Bcl2, Bcl-xL, HO-1, and Bach1 protein levels were determined via Western blot in HMVECs that had been infected with Ad-GFP or Ad-Bach1 ($n = 3$; * $P < 0.05$ versus Ad-GFP).

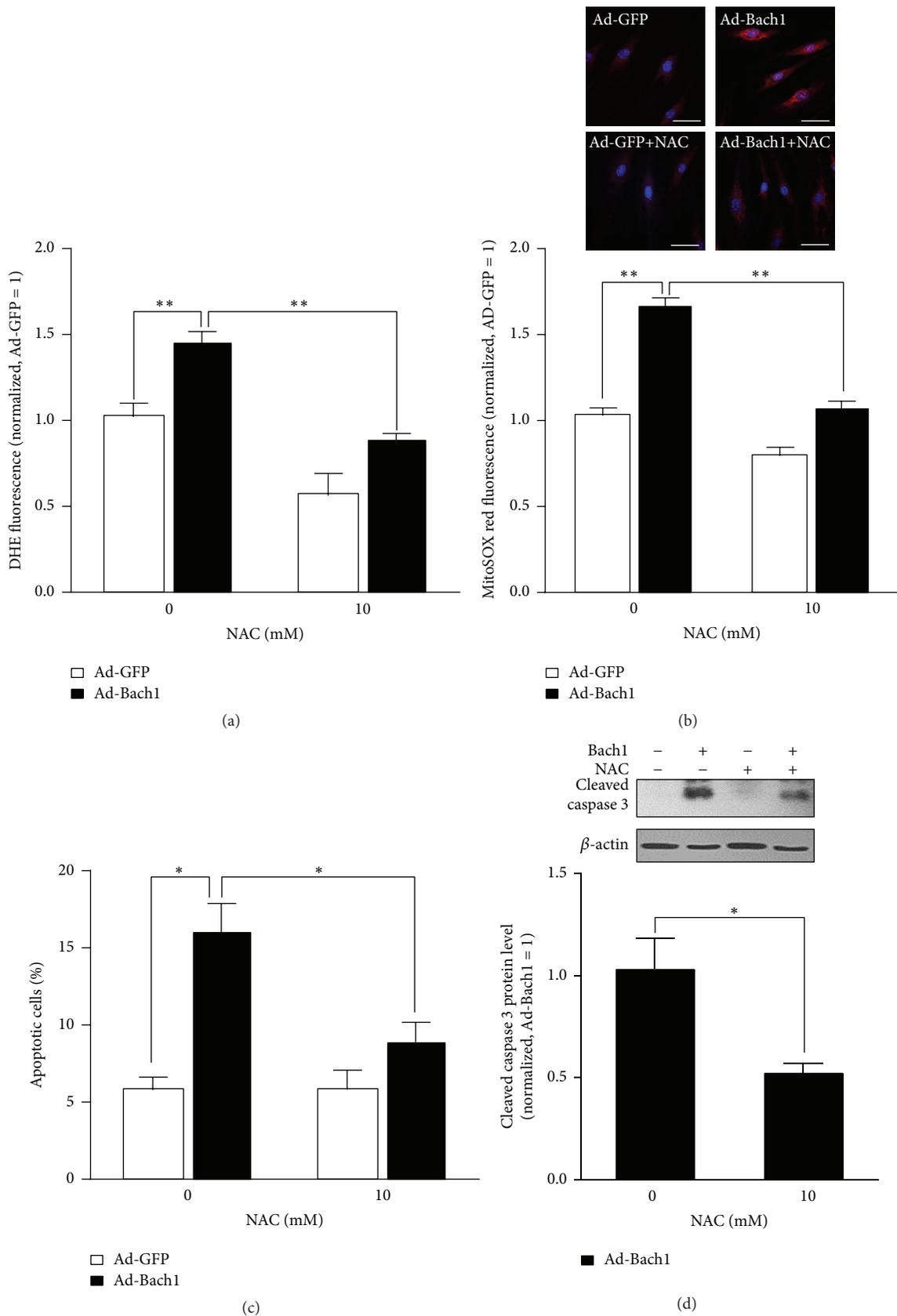


FIGURE 3: Bach1 induces apoptosis through mitochondrial ROS production. Ad-GFP- and Ad-Bach1-infected HMVECs were incubated with or without NAC (10 mM) for 48 hours, ROS production was then determined by the detection of dihydroethidium (DHE) fluorescence (a), or mitochondrial ROS production was determined by MitoSOX red fluorescence (b). Bars, 50 μ m. ($n = 3$; $**P < 0.01$). Cell apoptosis (c) and cleaved caspase 3 protein levels (d) were evaluated ($n = 3$; $*P < 0.05$).

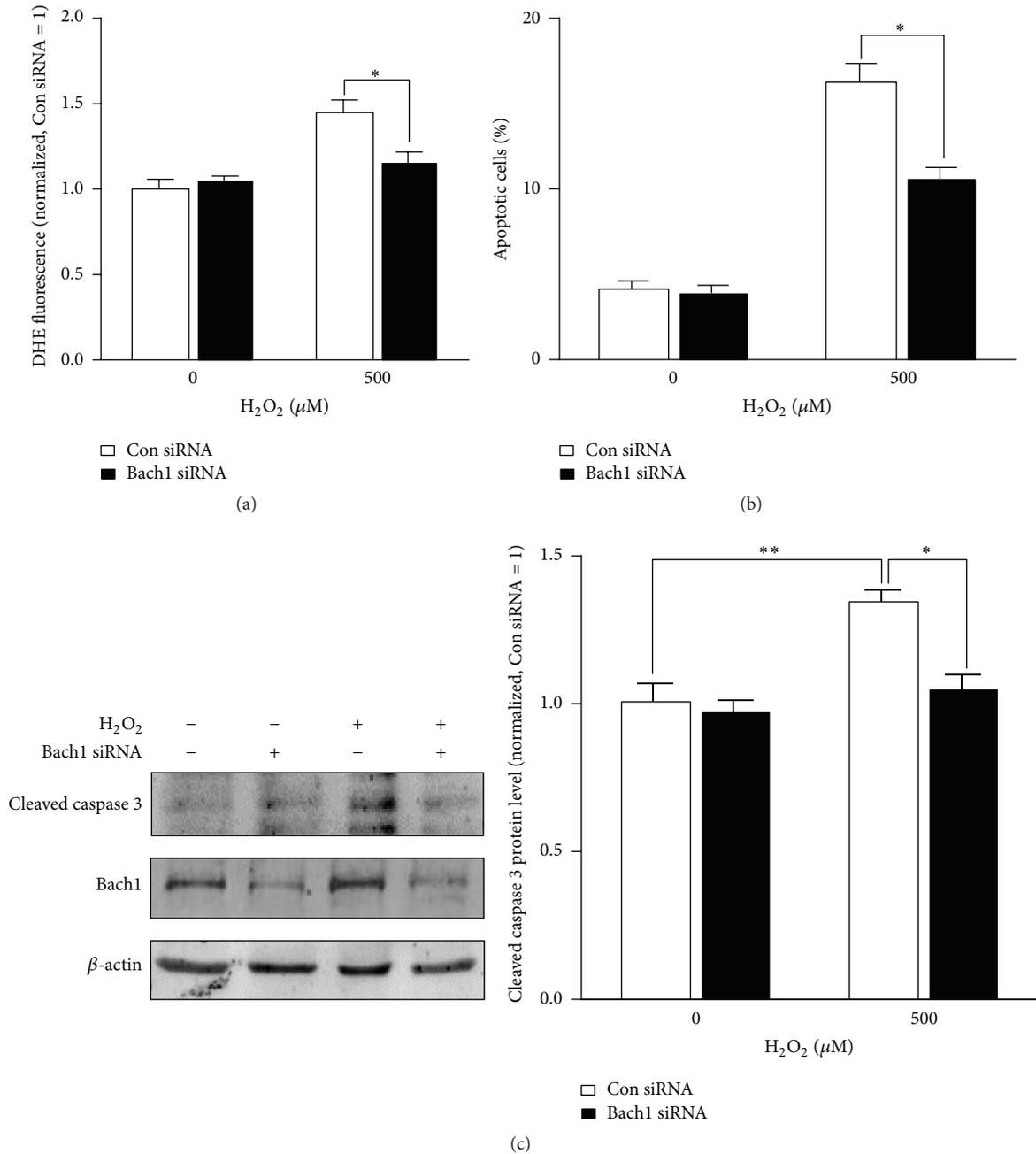


FIGURE 4: Bach1 downregulation attenuates H₂O₂-induced cell apoptosis. HMVECs were transfected with Con siRNA or Bach1 siRNA for 72 hours and then treated with or without H₂O₂ (500 μM) for 12 hours; the levels of ROS (a) cell apoptosis (b) and the protein levels of cleaved caspase 3 and Bach1 (c) were evaluated ($n = 3$; * $P < 0.05$, ** $P < 0.01$).

Bach1 appears to impede cell-cycle progression and induce apoptosis in ECs through increases in mitochondria ROS production (Figure 7).

Bach1 target involved in the oxidative stress response includes heme oxygenase-1 (HO-1). We found that HO-1 was downregulated in Bach1-overexpressing cells, which plays a crucial role in protection from oxidative stress and apoptosis [22, 23], and HO-1 catabolizes heme into ferrous iron, carbon monoxide, and biliverdin, which have antioxidant and antiapoptotic properties in vivo [24]. Another

Bach1 target involved in the oxidative stress response is the glutamate-cysteine ligase modifier subunit (GCLM) [25], which increases synthesis of the antioxidant glutathione and was upregulated after Bach1 knockdown [3]. Thus, Bach1 likely increases ROS generation by inhibiting antioxidants, at least, including HO-1. Most ROS are generated in cells by the mitochondrial respiratory chain. Mitochondrial ROS production is modulated largely by the rate of electron flow through respiratory chain complexes [26]. We showed that mitochondria were the major source of Bach1-induced ROS

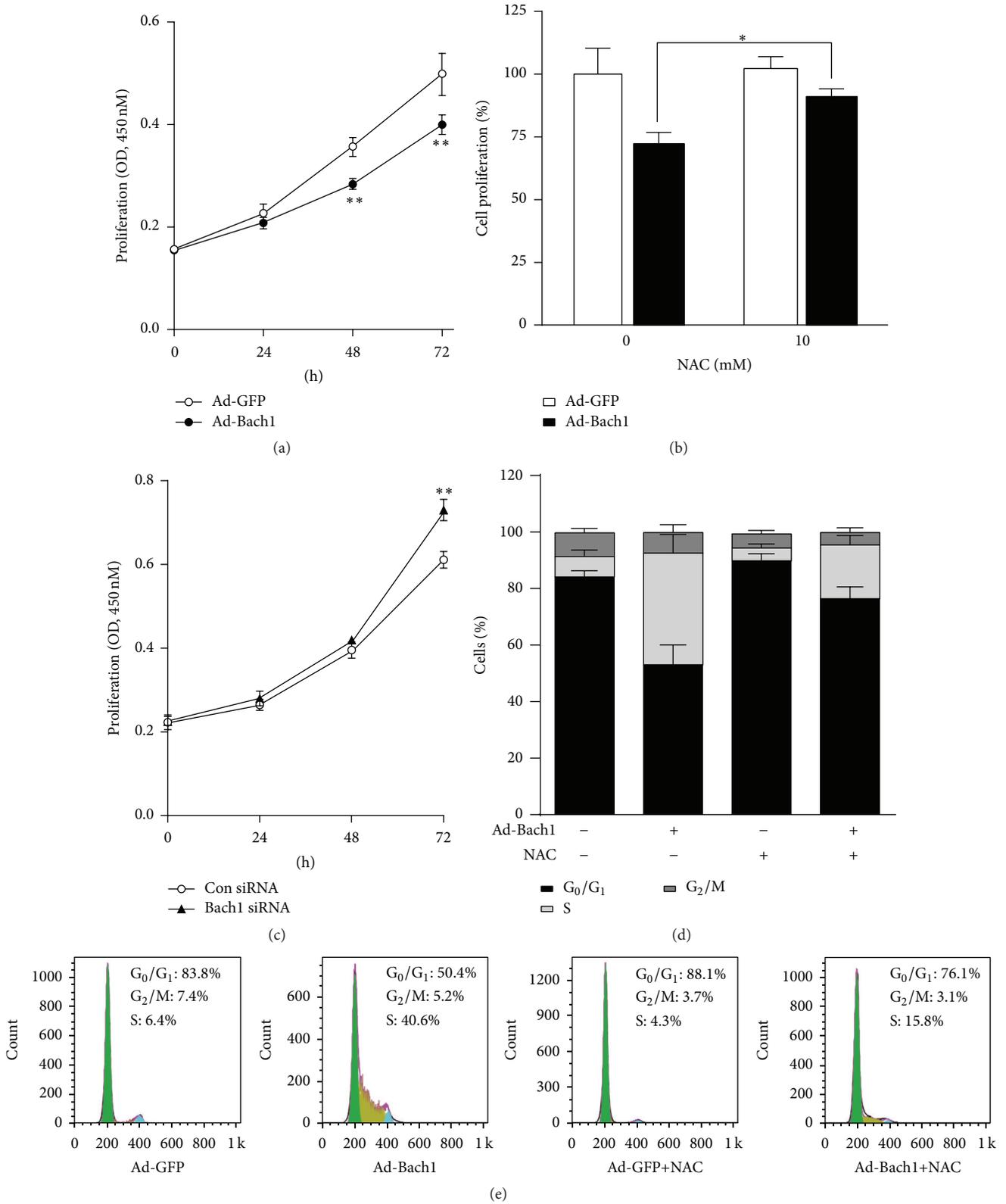


FIGURE 5: Bach1 disrupts cell-cycle progression and inhibits cell proliferation through ROS production. Cell proliferation was evaluated in (a) HMVECs that had been infected with Ad-GFP or Ad-Bach1, in (b) Ad-GFP- and Ad-Bach1-infected HMVECs treated with or without NAC (10 mM) for 48 hours, and in (c) HMVECs that had been transfected with Bach1siRNA or Con siRNA. Cells were cultured in 96-well plates, and BrdU incorporation assessments were performed via optical density measurements (450 nm wavelength) at the indicated time points ($n = 3$; * $P < 0.05$, ** $P < 0.01$). ((d) and (e)) HMVECs were infected with Ad-GFP or Ad-Bach1 and then incubated with or without NAC (10 mM) for 48 hours; then cells were harvested. The cell-cycle analysis was conducted using flow cytometry and quantified ($n = 3$).

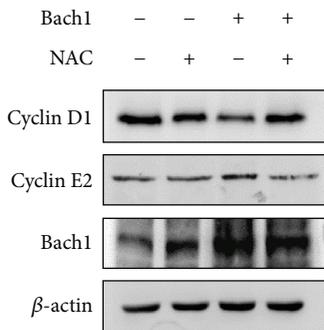
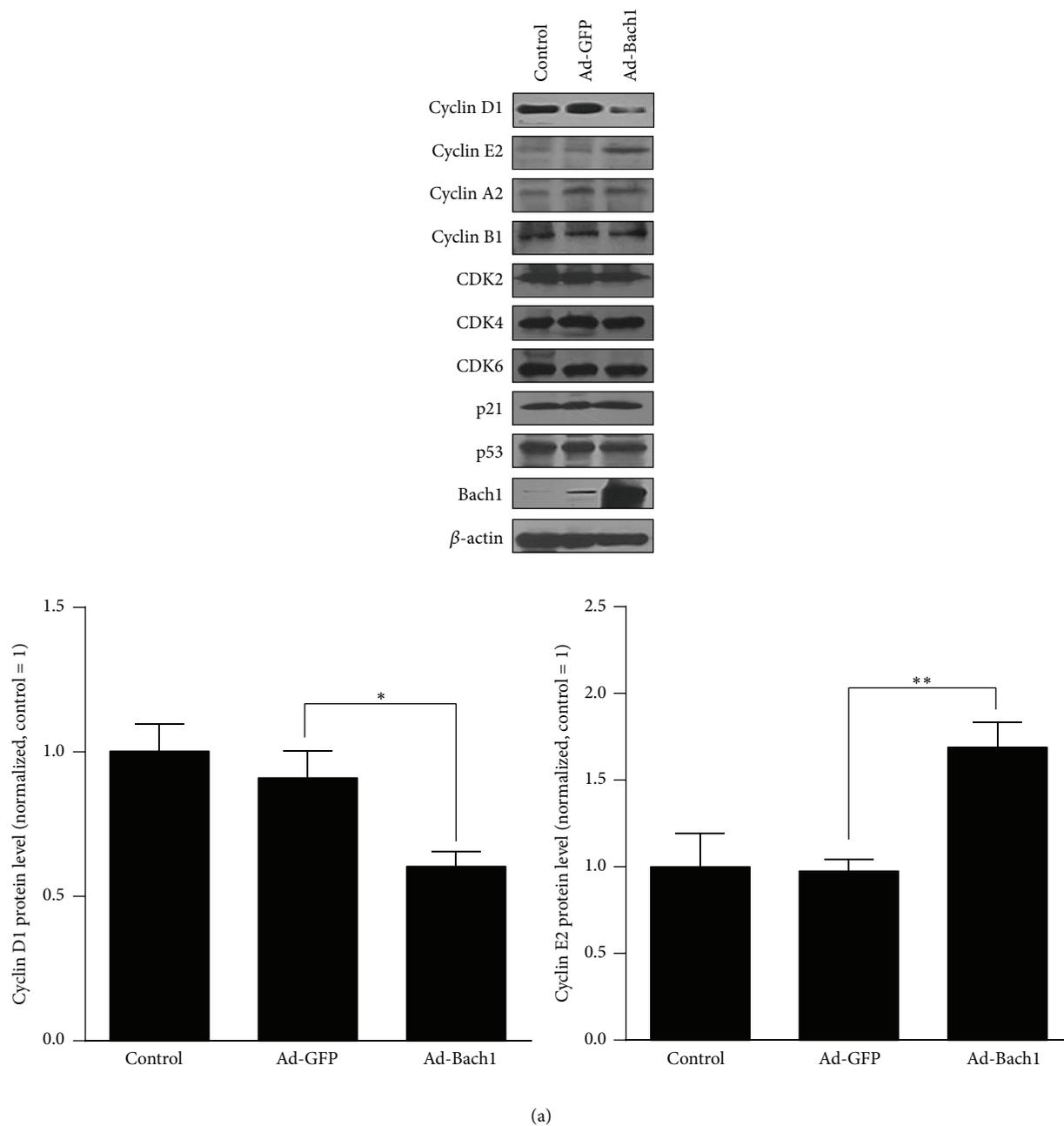


FIGURE 6: Continued.

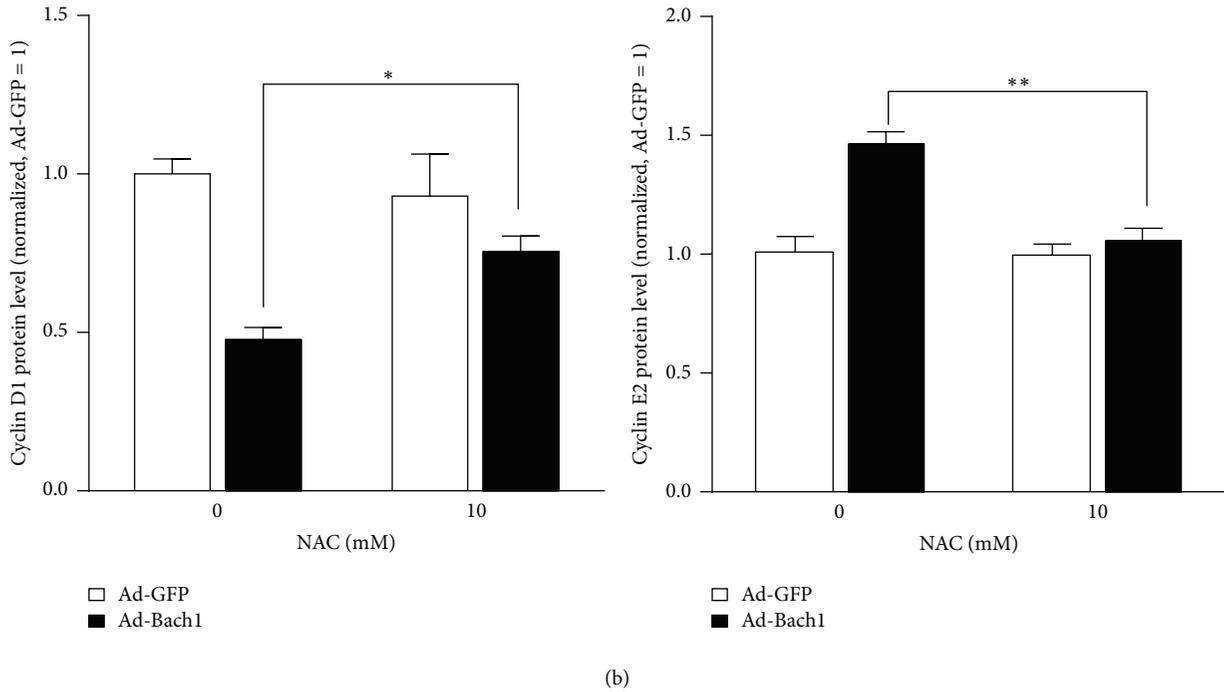


FIGURE 6: Bach1 regulates the expression of cell-cycle regulatory proteins through ROS production. (a) Western blot analysis of cell-cycle regulatory proteins (cyclin D1, cyclin E2, cyclin A2, cyclin B1, CDK2, CDK4, CDK6, p21, and p53) in Ad-GFP- and Ad-Bach1-infected HMVECs ($n = 3$; $*P < 0.05$, $**P < 0.01$). (b) HMVECs were infected with Ad-GFP or Ad-Bach1 and then incubated with or without NAC (10 mM) for 48 hours; then cells were harvested. Cyclin D1, cyclin E2, and Bach1 protein levels were evaluated ($n = 3$; $*P < 0.05$, $**P < 0.01$).

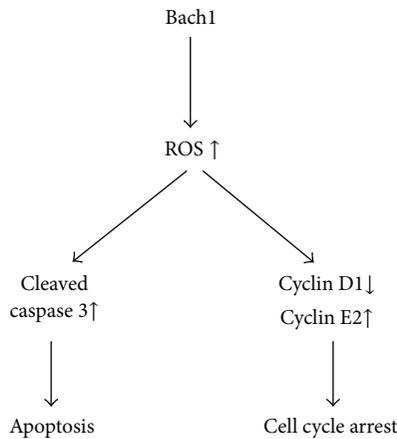


FIGURE 7: Mechanisms for the Bach1-induced cell-cycle arrest and apoptosis in ECs. Bach1 induces caspase 3-dependent apoptosis by ROS production in ECs; Bach1 also regulates the expression of cell-cycle regulatory proteins (cyclin D1 and cyclin E2) and promotes cell-cycle arrest through ROS generation.

generation in HMVECs. ROS can also be generated by the activation of various enzymes, including mitochondrial oxidases, the NADPH oxidases, NO synthases, and xanthine oxidase [27]. The specific mechanisms linking Bach1 and ROS production have yet to be identified in ECs.

We have recently demonstrated that Bach1 upregulation can impair angiogenesis in murine models of periph-

eral ischemic injury [12]. In the present study, we found that Bach1 overexpression enhanced ROS production and induced apoptosis in ischemic mouse hindlimbs. High levels of ROS have been shown to increase apoptosis and impair neovascularization in the ischemic limbs of mice [16, 17]. Thus, Bach1 likely inhibits angiogenesis at least partially by increasing ROS generation and apoptosis.

Apoptosis can be induced either through an extrinsic pathway, which is triggered by the binding of apoptosis-inducing ligands to cell surface receptors, or through an intrinsic pathway, which is regulated by the balance between proapoptotic and antiapoptotic Bcl2 proteins at the mitochondria [28–30]. In the present study, both Bcl2 and Bcl-xL levels declined in response to Bach1 upregulation in HMVECs. ROS have been shown to induce mitochondrial injury by reducing mitochondrial membrane potential and decreasing the expression ratio of Bcl2/Bax [31, 32]. Thus, the suppression of Bcl2 and Bcl-xL by Bach1 overexpression may be due to the increase of ROS production in cells. In addition, we also found that Bach1 triggered caspase 3 activation, and caspase 3 can be cleaved and activated by components of both pathways [33, 34]. Thus, the relationship between Bach1 levels and ROS production could contribute to both extrinsic and intrinsic apoptotic signaling.

Cyclin D1 and E2 were not among the direct Bach1-targeted cell-cycle-related proteins identified in HEK293 cells [3]. However, we have previously shown that Bach1 suppresses the Wnt/ β -catenin pathway [12], which is known to regulate cyclin D1, and previous studies have linked increases

in cyclin E expression with S-phase arrest [35]. Thus, Bach1 appears to inhibit cell-cycle progression and proliferation in ECs through a network of at least three overlapping mechanisms: (1) declines in Wnt-mediated cyclin D1 expression, (2) increases in ROS-mediated cyclin D1 repression, and (3) upregulation of cyclin-E2-induced S-phase arrest.

In conclusion, our results indicate that Bach1 inhibits cell proliferation and induces cell-cycle arrest and apoptosis by increasing mitochondria derived ROS production and, consequently, that functional downregulation of Bach1 may be a promising treatment target for the treatment of vascular diseases.

Disclosure

Xinhong Wang and Junxu Liu share first authorship.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Dan Meng and Kang Yao designed this study. Xinhong Wang, Junxu Liu, and Li Jiang performed the experiments. Xiangxiang Wei, Cong Niu, Rui Wang, and Jianyi Zhang analyzed the data. Dan Meng wrote the paper. Xinhong Wang and Junxu Liu contributed equally to this paper.

Acknowledgments

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References

- [1] T. Oyake, K. Itoh, H. Motohashi et al., "Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site," *Molecular and Cellular Biology*, vol. 16, no. 11, pp. 6083–6095, 1996.
- [2] K. Igarashi and J. Sun, "The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation," *Antioxidants & Redox Signaling*, vol. 8, no. 1-2, pp. 107–118, 2006.
- [3] H.-J. Warnatz, D. Schmidt, T. Manke et al., "The BTB and CNC homology 1 (BACH1) target genes are involved in the oxidative stress response and in control of the cell cycle," *The Journal of Biological Chemistry*, vol. 286, no. 26, pp. 23521–23532, 2011.
- [4] K. Ogawa, J. Sun, S. Taketani et al., "Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1," *The EMBO Journal*, vol. 20, no. 11, pp. 2835–2843, 2001.
- [5] J. Li, T. Shiraki, and K. Igarashi, "Transcription-independent role of Bach1 in mitosis through a nuclear exporter Crml-dependent mechanism," *FEBS Letters*, vol. 586, no. 4, pp. 448–454, 2012.
- [6] K. Kondo, Y. Ishigaki, J. Gao et al., "Bach1 deficiency protects pancreatic β -cells from oxidative stress injury," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 305, no. 5, pp. E641–E648, 2013.
- [7] M. Inoue, S. Tazuma, K. Kanno, H. Hyogo, K. Igarashi, and K. Chayama, "Bach1 gene ablation reduces steatohepatitis in mouse MCD diet model," *Journal of Clinical Biochemistry and Nutrition*, vol. 48, no. 2, pp. 161–166, 2011.
- [8] A. Harusato, Y. Naito, T. Takagi et al., "Suppression of indomethacin-induced apoptosis in the small intestine due to Bach1 deficiency," *Free Radical Research*, vol. 45, no. 6, pp. 717–727, 2011.
- [9] T. Tanimoto, N. Hattori, T. Senoo et al., "Genetic ablation of the Bach1 gene reduces hyperoxic lung injury in mice: role of IL-6," *Free Radical Biology and Medicine*, vol. 46, no. 8, pp. 1119–1126, 2009.
- [10] Y. Watari, Y. Yamamoto, A. Brydun et al., "Ablation of the Bach1 gene leads to the suppression of atherosclerosis in Bach1 and apolipoprotein E double knockout mice," *Hypertension Research*, vol. 31, no. 4, pp. 783–792, 2008.
- [11] Y. Yano, R. Ozono, Y. Oishi et al., "Genetic ablation of the transcription repressor Bach1 leads to myocardial protection against ischemia/reperfusion in mice," *Genes to Cells*, vol. 11, no. 7, pp. 791–803, 2006.
- [12] L. Jiang, M. Yin, X. Wei et al., "Bach1 represses Wnt/beta-catenin signaling and angiogenesis," *Circulation Research*, vol. 117, no. 4, pp. 364–375, 2015.
- [13] R. S. Frey, M. Ushio-Fukai, and A. B. Malik, "NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology," *Antioxidants and Redox Signaling*, vol. 11, no. 4, pp. 791–810, 2009.
- [14] J. Streeter, W. Thiel, K. Brieger, and F. J. Miller Jr., "Opportunity nox: the future of NADPH oxidases as therapeutic targets in cardiovascular disease," *Cardiovascular Therapeutics*, vol. 31, no. 3, pp. 125–137, 2013.
- [15] D. Meng, A. Mei, J. Liu et al., "NADPH oxidase 4 mediates insulin-stimulated HIF-1 α and VEGF expression, and angiogenesis in vitro," *PLoS ONE*, vol. 7, no. 10, Article ID e48393, 2012.
- [16] T. G. Ebrahimian, C. Heymes, D. You et al., "NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes," *American Journal of Pathology*, vol. 169, no. 2, pp. 719–728, 2006.
- [17] H. W. Kim, A. Lin, R. E. Guldborg, M. Ushio-Fukai, and T. Fukai, "Essential role of extracellular SOD in reparative neovascularization induced by hindlimb ischemia," *Circulation Research*, vol. 101, no. 4, pp. 409–419, 2007.
- [18] R. Shao and X. Guo, "Human microvascular endothelial cells immortalized with human telomerase catalytic protein: a model for the study of in vitro angiogenesis," *Biochemical and Biophysical Research Communications*, vol. 321, no. 4, pp. 788–794, 2004.
- [19] D. Meng, D.-D. Lv, and J. Fang, "Insulin-like growth factor-I induces reactive oxygen species production and cell migration through Nox4 and Rac1 in vascular smooth muscle cells," *Cardiovascular Research*, vol. 80, no. 2, pp. 299–308, 2008.

- [20] J. Fauconnier, D. C. Andersson, S.-J. Zhang et al., "Effects of palmitate on Ca^{2+} handling in adult control and *ob/ob* cardiomyocytes: impact of mitochondrial reactive oxygen species," *Diabetes*, vol. 56, no. 4, pp. 1136–1142, 2007.
- [21] D. Meng, X. Shi, B.-H. Jiang, and J. Fang, "Insulin-like growth factor-I (IGF-I) induces epidermal growth factor receptor transactivation and cell proliferation through reactive oxygen species," *Free Radical Biology and Medicine*, vol. 42, no. 11, pp. 1651–1660, 2007.
- [22] C. D. Ferris, S. R. Jaffrey, A. Sawa et al., "Haem oxygenase-1 prevents cell death by regulating cellular iron," *Nature Cell Biology*, vol. 1, no. 3, pp. 152–157, 1999.
- [23] N. Yun, H.-A. Eum, and S.-M. Lee, "Protective role of heme oxygenase-1 against liver damage caused by hepatic ischemia and reperfusion in rats," *Antioxidants and Redox Signaling*, vol. 13, no. 10, pp. 1503–1512, 2010.
- [24] L. E. Otterbein, M. P. Soares, K. Yamashita, and F. H. Bach, "Heme oxygenase-1: unleashing the protective properties of heme," *Trends in Immunology*, vol. 24, no. 8, pp. 449–455, 2003.
- [25] A. K. MacLeod, M. McMahon, S. M. Plummer et al., "Characterization of the cancer chemopreventive NRF2-dependent gene battery in human keratinocytes: demonstration that the KEAP1-NRF2 pathway, and not the BACH1-NRF2 pathway, controls cytoprotection against electrophiles as well as redox-cycling compounds," *Carcinogenesis*, vol. 30, no. 9, pp. 1571–1580, 2009.
- [26] R. O. Poyton, K. A. Ball, and P. R. Castello, "Mitochondrial generation of free radicals and hypoxic signaling," *Trends in Endocrinology & Metabolism*, vol. 20, no. 7, pp. 332–340, 2009.
- [27] P. L. Hordijk, "Regulation of NADPH oxidases: the role of Rac proteins," *Circulation Research*, vol. 98, no. 4, pp. 453–462, 2006.
- [28] L. Coultas and A. Strasser, "The role of the Bcl-2 protein family in cancer," *Seminars in Cancer Biology*, vol. 13, no. 2, pp. 115–123, 2003.
- [29] N. N. Danial and S. J. Korsmeyer, "Cell death: critical control points," *Cell*, vol. 116, no. 2, pp. 205–219, 2004.
- [30] D. R. Green and G. Kroemer, "The pathophysiology of mitochondrial cell death," *Science*, vol. 305, no. 5684, pp. 626–629, 2004.
- [31] T. Adachi, H. Tanaka, S. Nonomura, H. Hara, S.-I. Kondo, and M. Hori, "Plasma-activated medium induces A549 cell injury via a spiral apoptotic cascade involving the mitochondrial-nuclear network," *Free Radical Biology and Medicine*, vol. 79, pp. 28–44, 2015.
- [32] P. Wyrach, C. Blenn, J. Bader, and F. R. Althaus, "Cell death and autophagy under oxidative stress: roles of poly(ADP-Ribose) polymerases and Ca^{2+} ," *Molecular and Cellular Biology*, vol. 32, no. 17, pp. 3541–3553, 2012.
- [33] E. Frejlich, J. Rudno-Rudzińska, K. Janiszewski et al., "Caspases and their role in gastric cancer," *Advances in Clinical and Experimental Medicine*, vol. 22, no. 4, pp. 593–602, 2013.
- [34] J.-H. Zhang, Y. Zhang, and B. Herman, "Caspases, apoptosis and aging," *Ageing Research Reviews*, vol. 2, no. 4, pp. 357–366, 2003.
- [35] J.-W. Park, Y.-J. Choi, M.-A. Jang et al., "Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells," *Cancer Letters*, vol. 163, no. 1, pp. 43–49, 2001.

Review Article

Dual Role of ROS as Signal and Stress Agents: Iron Tips the Balance in favor of Toxic Effects

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Iron is essential for life, while also being potentially harmful. Therefore, its level is strictly monitored and complex pathways have evolved to keep iron safely bound to transport or storage proteins, thereby maintaining homeostasis at the cellular and systemic levels. These sequestration mechanisms ensure that mildly reactive oxygen species like anion superoxide and hydrogen peroxide, which are continuously generated in cells living under aerobic conditions, keep their physiologic role in cell signaling while escaping iron-catalyzed transformation in the highly toxic hydroxyl radical. In this review, we describe the multifaceted systems regulating cellular and body iron homeostasis and discuss how altered iron balance may lead to oxidative damage in some pathophysiological settings.

1. Introduction

In eukaryotes, oxygen utilization by the mitochondria, NADPH oxidase enzymes, cytochromes, and so forth leads to the generation of reactive oxygen species (ROS), unstable and reactive molecules formed by one-electron transfers from a redox donor to molecular oxygen. The first of such products is anion superoxide (O_2^-), which can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase enzymes. Metal-catalyzed oxidation of H_2O_2 can then originate hydroxyl radicals (HO^\bullet), the most reactive ROS. Redox balance, that is, regulated production of ROS, is essential for normal cellular physiology, as deregulation in the production of oxidative species, that is, oxidative stress, causes DNA damage, lipid peroxidation, and aberrant posttranslational modification of proteins, thus leading to injury, cell death, and disease [1]. Conversely, accumulating evidence indicates that physiological concentrations of ROS are necessary to support redox signaling events that are involved in important physiological functions and adaptive cell responses, such as chemotaxis, hormone synthesis, immune response, cytoskeletal remodeling, and calcium homeostasis [2]. To be considered as signaling biological messengers, ROS should meet precise spatial and regulatory criteria; that is, they should be

produced enzymatically and their levels should be regulated by intracellular molecular mechanisms [3, 4]. Therefore, O_2^- and H_2O_2 , particularly H_2O_2 that is more stable than O_2^- and can cross membranes, are considered signaling molecules because their levels under normal conditions remain under a physiological threshold and their synthesis is enzymatically regulated, whereas HO^\bullet is deemed to be a highly toxic reactant leading to permanent modifications of target molecules that can impact cellular function and life. In the cell, rapid and efficient conversion of H_2O_2 to HO^\bullet through the Fenton reaction requires the presence of transition metals, that is, copper and iron. While copper is a more efficient catalyst, iron is much more abundant and thus it is the actual key player in the so-called metal-catalyzed oxidative reactions [5].

While metal-dependent ROS production has historically been associated with necrosis, a form of passive cell death characterized by dissolution of cellular structures, new evidence suggests a role in regulated necrosis. In fact, a form of regulated cell death, termed ferroptosis because it requires iron, has been recently identified [6]. Interestingly, ferroptosis is induced following inhibition of cystine import and downstream glutathione synthesis, leading to the accumulation of intracellular ROS and lipid peroxidation [7]. Further evidence for the role of iron-mediated disruption

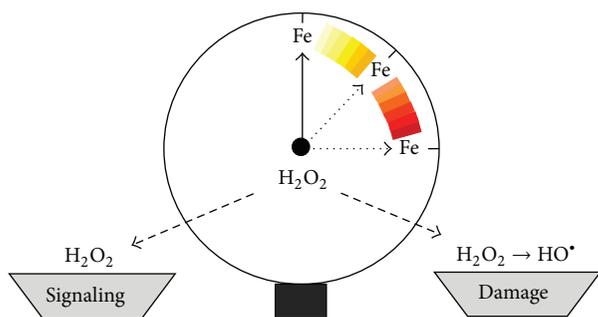


FIGURE 1: Dual role of ROS as signaling and toxic molecules, iron tips the scale for damage. By triggering Fenton chemistry, increased iron (Fe) availability may change the role of H₂O₂ from a relatively safe compound involved in cell signaling to a source of the highly toxic HO^{*}.

of cellular redox homeostasis in ferroptosis was provided by the essential regulatory function in ferroptotic cell death of glutathione peroxidase [8], which is the only known enzyme able to reduce phospholipid hydroperoxides and thus one of the most important antioxidant enzymes, and by the demonstration that transferrin-mediated iron import is essential for ferroptosis [9]. Moreover, in hepatocellular carcinoma cells, knockdown of H ferritin, one of the two subunits that compose the iron storage protein ferritin, promoted ferroptosis in response to classical inducers [10].

During the slow and long process of oxygen accumulation that led to the present 20.9% atmospheric oxygen content initiated about 1.5 billion years ago by photosynthesizing cyanobacteria, all life forms evolved mechanisms to exploit the chemical reactivity of iron for efficient aerobic reactions. Indeed, the number of iron-containing enzymes is striking and includes proteins of vital physiological significance involved in functions such as oxygen transport, cell respiration, and DNA synthesis [11, 12]. Living organisms were also forced to concurrently evolve protection systems against highly damaging HO^{*} radicals produced by the iron-catalyzed conversion of superoxide and H₂O₂. To this aim, a complex regulatory pathway formed by a variety of proteins that bind, transport, and store iron has been developed, in order to maintain an appropriate iron balance in both the individual cells and the whole body [13, 14]. Over the last years, the mechanisms by which iron homeostasis at the cellular and organismal levels is regulated have been elucidated. In this review, we have summarized recent advances in the control of iron homeostasis and how changes in availability of poorly liganded ferrous iron are related to ROS production. Moreover, we propose that whenever the efficiency of the network controlling iron balance is compromised, the role of ROS switches from signaling to damage (Figure 1).

2. The IRE/IRP Regulatory Pathway Controls Cellular Iron Homeostasis

The challenging task of maintaining intracellular iron levels sufficient for essential cellular functions, including ROS-dependent cell signaling, but as low as possible to avoid

ROS-mediated injury, is controlled at multiple steps but primarily accomplished by iron regulatory proteins (IRP1 and IRP2), which strictly control intracellular iron metabolism by posttranscriptionally regulating the coordinated expression of proteins involved in iron utilization (e.g., erythroid 5 aminolevulinic acid synthase, mitochondrial aconitase, and *Drosophila* succinate dehydrogenase), uptake (transferrin receptor (TfR1) and divalent metal transporter (DMT1)), storage (H and L ferritin subunits), and export (ferroportin) [15, 16] (Figure 2). IRP1 and IRP2 recognize and bind conserved 25–30 nucleotides-long RNA stem-loop structures named iron responsive elements (IREs) in the untranslated regions of the mRNAs coding for these proteins. It has also been shown that IRPs can bind the mRNAs for other proteins not directly related to iron homeostasis [15, 16].

The activity of IRP1 and IRP2 is dictated by the size of the cellular labile iron pool (LIP), a pool of iron in the low μ M range bound to low molecular weight compounds like citrate or glutathione [17], which is in continuous equilibrium with the sites of iron utilization or storage. When cells are iron deficient and thus the size of the LIP is shrunk, IRP1 and IRP2 bind to IREs located in the 3' region of transcripts and stabilize the mRNA for TfR1 and DMT1, thus increasing the uptake of both transferrin-bound and unbound iron. At the same time, binding to 5' IRE impairs translation of mRNAs for ferroportin, the only iron exporter, and ferritin, which sequesters iron in a catalytically inactive form. This coordinate regulation eventually expands the cellular LIP. Conversely, when the LIP is large, the IRE-binding activity of both IRPs is decreased, resulting in efficient translation of ferritin and ferroportin mRNAs and lower stability of TfR1 and DMT1 mRNAs, ultimately enhancing iron storage and release over uptake [15, 16] (Figure 2).

Both IRPs are homologous to the mitochondrial TCA cycle enzyme aconitase that converts citrate to isocitrate using an iron sulfur cluster (4Fe–4S) as a cofactor, but only IRP1 can assemble a cluster when sufficient iron is available, thus functioning as cytosolic aconitase, which is the prevailing form in most cells. Conversely, in conditions of iron deficiency, the cluster is disassembled and the IRP1 apoform binds IRE [15, 16]. IRP2 accumulates in iron-deficient cells where it binds IRE motifs with affinity and specificity similar to that of IRP1, whereas in iron-replete cells it is rapidly targeted for proteasomal degradation by an E3 ubiquitin ligase complex that comprises FBXL5, a recently identified protein containing an hemerythrin-like domain that is involved in its regulation according to iron (and oxygen) availability [18]. Under conditions of iron scarcity, the assembly of the di-iron center in the hemerythrin-like domain is impaired and FBXL5 is polyubiquitinated and degraded by the proteasome, thereby leading to IRP2 stabilization. Conversely, when iron is abundant, FBXL5 levels in the cell increase promoting IRP2 (and apo-IRP1) proteasomal degradation [19, 20].

The respective role and importance of IRPs have been revealed by studies involving gene deletion [21, 22]. Ablation of both IRP1 and IRP2, which are ubiquitously expressed, is early embryonic lethal whereas single knockout mice are viable, indicating that the two IRPs can compensate for each

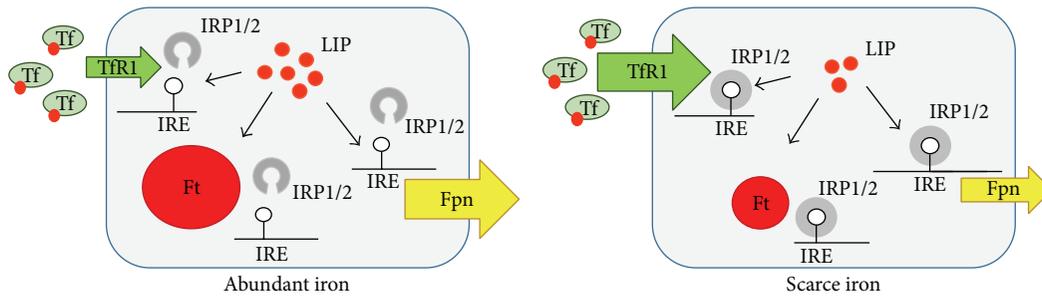


FIGURE 2: Simplified model of IRP-dependent regulation of intracellular iron homeostasis. The IRE/IRP machinery posttranscriptionally controls the expression of the major proteins of intracellular iron import (transferrin receptor, TfR1), export (ferroportin, Fpn), and storage (ferritin, Ft). Under conditions of iron excess in the labile iron pool (LIP), IRPs lose their RNA-binding capacity, and hence TfR1 mRNA is degraded (small arrow) while Fpn and Ft mRNAs are actively translated (big arrow and circle, resp.). The opposite occurs under conditions of iron deficiency: IRP1 and IRP2 binding to iron responsive elements (IREs) stabilize TfR1 mRNA (big arrow) and prevent the translation of Fpn and Ft mRNAs (small arrow and circle, resp.); this response increases iron availability.

other and confirming the *in vitro* data which suggested essential but largely overlapping functions. However, early studies did not show an apparent phenotype in IRP1 single knock-out mice, whereas hematopoietic defects and late onset neurodegeneration were described in mice lacking IRP2. These findings showing a dominant role for IRP2 in the regulation of iron homeostasis in mice were in line with a study indicating that IRP2 is the major regulator of intracellular iron metabolism in humans [23]. However, recent results showed that IRP1 predominantly binds specific IRE-containing mRNAs, such as those coding for erythroid aminolevulinic synthase and hypoxia inducible factor 2 α (HIF2 α) [24].

Although iron is the major regulator of the IRE/IRP network, other signals and conditions can modulate the activity of both IRPs, including oxygen tension and nitric oxide (reviewed in [16]). Not surprisingly given the relationship between ROS and iron, IRPs are both targets and modulators of free radical reactions and IRP activity is altered under conditions of oxidative stress. Despite reports showing IRP1 activation in cells exposed to H₂O₂, which may be a phenomenon more correlated to the signaling role of H₂O₂ rather than to oxidative stress, it appears that IRP1 inactivation occurs in response to ROS both *in vitro* and *in vivo* [15, 16]. Overall, since IRP2 is highly susceptible to ROS-mediated downregulation, it is possible to conclude that inhibition of IRP1 and IRP2 binding activity, with ensuing ferritin induction and reduction of LIP, may represent a protective strategy to prevent amplification of oxidative injury (discussed in [25]). The role of ferritin as an antioxidant protein is underscored by the multiplicity of mechanisms leading to its upregulation in response to oxidative challenge; in fact, it has been demonstrated that stressful conditions transcriptionally activate ferritin expression in various cell types, and ferritin overexpression protects from oxidative stress [26].

Readers can refer to recent excellent and comprehensive reviews for specific aspects of the IRE/IRP regulatory network [24, 27–29].

3. Conditions in Which Disruption of Cellular Iron Homeostasis Leads to Oxidative Damage

3.1. Neurodegenerative Disorders. Abundant evidence shows that a number of neurodegenerative disorders are characterized by regional iron accumulation in particular areas of the central and/or peripheral nervous systems [30, 31]. This is often caused by cellular iron redistribution and may result in iron-catalyzed Fenton chemistry. For instance, increased iron levels in specific regions of the brain are a hallmark of Parkinson disease [32].

Friedrich's ataxia (FRDA) is a paradigmatic example because the disruption of iron homeostasis in this disease has been well defined [33]. FRDA, which is the most prevalent form of hereditary ataxia in Caucasians, is characterized by progressive degeneration of large sensory neurons in the central and peripheral nervous systems leading to neurological impairment. In addition to neurological symptoms like spinocerebellar and sensory ataxia, FRDA patients also suffer from important nonneurological manifestations, in particular hypertrophic cardiomyopathy. The disease results from loss of function mutations (most often triplet expansion) in the FXN gene that lead to decreased expression of frataxin, a mitochondrial iron-binding protein that interacts with proteins involved in the mitochondrial Fe-S cluster biogenesis [34]. In patients, frataxin deficiency results in disruption of Fe-S cluster biosynthesis, severe mitochondrial iron overload, a hallmark of Fe-S defects, and increased sensitivity to oxidative stress [35].

Although several studies provided evidence that ROS generated through Fenton reaction play a role in FRDA, the primary involvement and the importance of ROS in the pathophysiology of FRDA are still debated [33]. However, the protection afforded by mitochondrial ferritin, which has a strong antioxidant role [36], in yeast, mammalian cells, and fibroblasts from FRDA patients was accompanied by reduced ROS level, thus strongly indicating the involvement of toxic free radicals [37]. Since mitochondrial ferritin plays

a protective role in several pathological conditions by sequestering iron [36], these data suggest that iron chelators are of particular interest as therapeutic approaches for FRDA. However, FRDA and other regional sideroses (i.e., iron accumulation in particular tissues or cell compartments) will require novel chelation modalities [38].

3.2. Role of Iron and ROS in Anthracycline Cardiotoxicity.

The role of iron in anthracycline cardiotoxicity is another illuminating example of the complex interplay and synergism between iron and free radicals as causative factors of apoptosis or other forms of cell damage. Doxorubicin (DOX) is an antineoplastic drug of the anthracyclines family, which plays a recognized key role in the chemotherapy for several types of cancer. However, anthracyclines have established risks of cardiotoxicity, as their chronic administration induces cardiomyopathy and congestive heart failure [39]. This dose-dependent side effect limits the clinical use of DOX in cancer patients. Multiple mechanisms of cardiotoxicity induced by DOX have been described, but activation of the mitochondrial intrinsic pathway of apoptosis seems to represent a major response to anthracycline treatment. The development of anthracycline-induced cardiomyopathy has been found to depend on drug metabolism [40]; in fact, in addition to the role of reductive activation of the quinone moiety of DOX discussed below, a correlation exists between toxicity and myocardial accumulation of anthracycline secondary alcohol metabolites [39]. Conversely, anthracycline oxidative degradation may serve as a salvage pathway for diminishing the levels and toxicity of DOX in cardiomyocytes (reviewed in [41]).

The involvement of iron in DOX-induced cardiac damage is well established, and cardiotoxicity induced by DOX may occur at lower cumulative doses under conditions of iron overload [42]. The adverse role of iron has been suggested by several lines of evidence: in particular, a number of studies showed the protecting efficacy of iron chelators both in patients and in animal models, while others demonstrated that primary and secondary iron overload exacerbated the cardiotoxic effects of the drug, but the underlying molecular mechanisms remain to be fully understood (see [39, 41, 42] for review). Iron has been proposed to act as a catalyst of ROS formation in reactions primed by DOX. In fact, DOX is a redox compound, as NAD(P)H reductases catalyze one-electron reduction of the quinone moiety of the tetracycline ring to the semiquinone free radical, which can regenerate the parent quinone reacting with molecular oxygen. The latter reaction generates O_2^- and its dismutation product H_2O_2 , which can then be transformed into the more potent HO^\bullet by reactions catalyzed by iron. In turn, HO^\bullet can damage DNA and proteins and initiate membrane lipid peroxidation, thus leading to cardiomyocyte death [42, 43].

However, since antioxidants did not offer protection in clinical settings, the apparently obvious explanation for the aggravating role of iron in DOX cardiotoxicity based on increased iron-catalyzed ROS formation has been called into question [39, 41]. In line with this view, we showed that DOX doses in the range of the plasma levels found in patients undergoing chemotherapy were able to cause apoptotic death of cardiac-derived H9c2 myocytes in the

absence of ROS production [44]. Moreover, we provided evidence that activation of the HIF pathway contributes to the cardioprotective effect of the iron chelator dexrazoxane, thus suggesting that the protective capacity of iron chelators against DOX toxicity may be mediated by mechanisms not related to the prevention of ROS formation [45].

We also showed that anthracycline cardiotoxicity is related to ROS-dependent and ROS-independent disruption of cardiac iron homeostasis due to targeted interaction of DOX with IRP1, which leads to a “null” IRP1 devoid of both its functions and hence it is unable to sense iron levels and to regulate iron homeostasis. Moreover, DOX triggers IRP2 degradation, which may serve as a protective role by favoring iron sequestration in newly formed ferritin [25, 46]. Indeed, it has been shown that ferritin is induced in H9c2 cardiomyocytes [47] and mouse hearts [48] exposed to DOX and protects cardiac cells against iron toxicity. Moreover, ferritin H chain plays an important role in the preventive effect of metformin against DOX cardiotoxicity in isolated cardiomyocytes [49]. DOX treatment also results in iron storage by inducing mechanisms leading to higher accumulation of iron into ferritin [50]. Overall, these results suggest that the role of iron in anthracycline-dependent cardiotoxicity may extend beyond the formation of ROS.

On the other hand, recent results regarding mitochondrial ferritin (FtMt), a ferritin type particularly expressed in mitochondria-rich tissues, including the heart, where it prevents iron-mediated oxidative damage [51], reinforce the idea that ROS are involved in the mechanisms linking iron and anthracycline cardiotoxicity. FtMt expression was induced in the heart of mice exposed to DOX [52], and FtMt-deficient mice exposed to DOX are more sensitive to ROS-mediated heart damage and death [53]. In addition, mice with heart-specific deletion or overexpression of ABCB8, which exports iron out of the mitochondria, were more sensitive or resistant, respectively, to DOX cardiotoxicity [52].

The importance of methodological aspects introduces some cautionary issues that should be taken into account when considering the discrepancies reported above regarding the pathophysiological relevance of iron-mediated ROS production in DOX toxicity. In fact, one should keep in mind that the model systems in which the mechanisms of DOX cardiotoxicity have been characterized have inherent limitations in representing the human chronic cardiomyopathy. Moreover, the dual role of ROS in signaling events and cell damage should be considered when evaluating if iron contributes to chronic cardiomyopathy by mechanisms not related to its ability to generate HO^\bullet .

4. Systemic Iron Metabolism

Given the dual role of iron, elegant control mechanisms have evolved to maintain appropriate body iron levels by means of a complex network of transporters, storage molecules, and regulators. Intestinal iron absorption and iron recycling in reticuloendothelial cells are coordinately orchestrated in order to maintain iron levels in the circulation adequate for the needs of the various tissues and organs but insufficient to activate dangerous ROS production [13, 14].

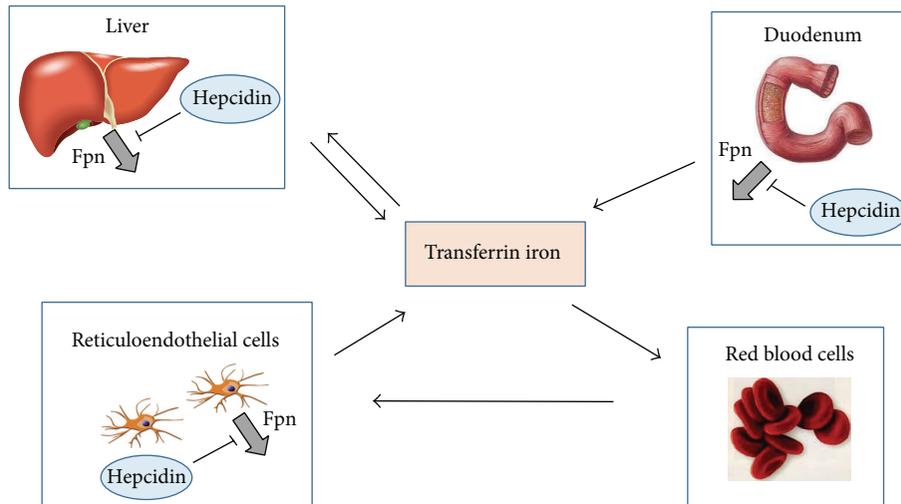


FIGURE 3: Hepcidin regulates transferrin-mediated body iron traffic. The interaction of hepcidin with ferroportin inhibits the flow of iron into plasma and thereby regulates the transferrin-mediated distribution of iron in the body from sites of iron absorption (duodenum) and recycling (reticuloendothelial cells in spleen and liver) to tissues where it is used (e.g., for the synthesis of hemoglobin in red blood cells) or stored (e.g., liver).

The task of keeping circulating iron in a safe but readily available form is performed by transferrin (Tf), a protein synthesized and secreted by the liver that binds up to two ferric iron atoms with high affinity and is the major iron transport protein. Under physiological conditions, Tf-bound iron is the main source of iron for the majority of tissues, primarily for bone marrow erythroid precursors, which consume iron for hemoglobin biosynthesis (Figure 3).

Tf incorporates iron coming from two major sources: dietary iron (both inorganic and heme iron) absorbed in the duodenum to compensate for daily iron loss and iron derived from destruction of old and effete erythrocytes by reticuloendothelial cells in the spleen and liver (Figure 3). After reduction by the reductase DcytB, dietary iron is transported across the apical membrane of absorptive epithelial cells by DMT1 [54]. In intestinal enterocytes, most iron is then exported to the blood at the basolateral surface by ferroportin, assisted by the function of oxidases (circulating ceruloplasmin and membrane-bound hephaestin) that convert ferrous iron to ferric iron and thus permit the incorporation of iron into Tf. Although various transporters have been identified [54], the mechanisms of intestinal uptake and release of heme iron are less clearly understood, but the majority of heme is degraded in the enterocytes and iron is released by ferroportin, as ferroportin-deficient mice are not viable [55].

Spleen and liver macrophages specialized in recycling iron obtained from the phagocytosis and destruction of senescent erythrocytes are the main iron supplier for hemoglobin synthesis. The major pathway of heme iron recycling involves hemoglobin degradation by cytosolic heme oxygenase-1 and export of heme-derived iron into the circulation by ferroportin [56].

Recent studies in mice with disrupted IRP1 and/or IRP2 in the entire organism or specific tissues have shown that IRPs are important regulators also of systemic homeostasis [22], but hepcidin, a peptide hormone produced and secreted by

the liver, can be considered the key regulator of body iron balance [57, 58]. That hepcidin is most important regulator of body iron homeostasis which is also indicated by a number of studies showing that disruption of hepcidin regulation is involved in a variety of disorders associated with iron deficiency (e.g., anemia) or overload (e.g., siderosis). In particular, inadequate hepcidin levels in relation to body iron stores characterize most hereditary iron overload diseases [59]. Hepcidin controls plasma iron concentration and body iron balance by regulating the expression of ferroportin, the only known cellular iron exporter (Figure 3). The binding of hepcidin to ferroportin on the plasma membrane induces its internalization and degradation, thereby blocking iron release [60]. Hepcidin expression is regulated at multiple levels: the expression of hepcidin is induced by iron overload, inflammatory stimuli, or endoplasmic reticulum stress [57, 58, 61]; this mechanism stops the efflux of unwanted iron in the circulation by negatively modulating iron absorption by enterocytes, iron recycling by reticuloendothelial cells, and iron mobilization from hepatic stores. Conversely, increased erythropoietic activity under conditions of iron deficiency, anemia, and hypoxia represses hepcidin, thereby leading to higher iron availability for new erythrocytes synthesis [20, 62]. Among the positive regulators of hepcidin, iron exerts its effect through the BMPs/SMAD dependent pathway, while inflammatory cytokines, especially IL-6, activate the JAK2-STAT3 signaling cascade. Erythroferrone, produced by erythroid precursors in the marrow and the spleen in response to erythropoietin, seems to be the major inhibitor of hepcidin production when erythropoiesis is stimulated [63].

4.1. Iron Overload Conditions. Two mechanisms protect the cells from the damaging effects of excess body iron: the partial saturation of Tf at the systemic level and the IRP-dependent regulated expression of TfR1 at the cellular level. Under normal conditions, Tf saturation is around 30% and

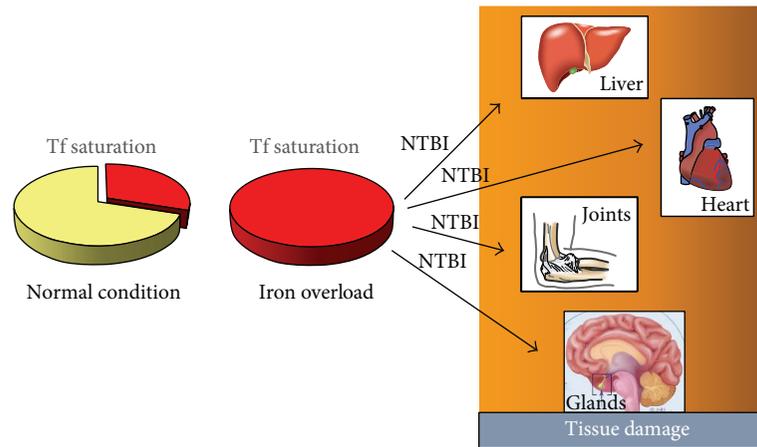


FIGURE 4: Mechanisms of iron overload mediated tissue damage. Whenever the iron-binding capacity of transferrin (Tf) is exceeded, non-transferrin-bound iron (NTBI) forms, penetrates into the cells, and undergoes redox cycling, ultimately leading to cell injury and organ damage.

the protein can therefore bind excess iron entering the circulation, thus functioning as a protective shield against HO^\bullet production. However, under conditions of heavy systemic iron overload (primarily due to insufficient hepcidin production), the iron-binding capacity of Tf is exceeded, and non-transferrin-bound iron (NTBI) is formed (Figure 4). Despite the downregulation of TfR1, cells become iron-loaded because NTBI, that is, iron associated with various low molecular weight plasma components, like citrate, phosphates, and proteins, lacks a regulated uptake system and hence is able to penetrate into the cells [38]. The consequent decrease in IRP binding activity leads to efficient ferritin mRNA translation to promote iron storage, but ultimately the high capacity of ferritin is exceeded as this unabated flux of NTBI continues. NTBI is clearly involved in tissue siderosis, but the incomplete characterization of the iron species involved prevents a clear understanding of how it enters the cell under different pathological conditions. NTBI enters into cells mainly through divalent cation transporters such as DMT1 and ZIP14, which require previous reduction to ferrous iron [64, 65] or Ca^{2+} channels, either L-type or T-type, particularly in cardiomyocytes [66]. NTBI toxicity within cells derives from the fact that iron in the expanded LIP is no longer protected from redox cycling; hence, it accelerates the catalysis of reactions that produce HO^\bullet , which then cause lipid peroxidation and organelle damage and ultimately cell death (Figure 4). In particular, mitochondria appear to be primarily affected by iron-mediated HO^\bullet production. In fact, it has been demonstrated that mitochondria rapidly acquire NTBI, and this, along with their high generation of ROS [67], props up oxidative damage. Apparently, the variety of enzymatic and nonenzymatic antioxidant defense systems is inadequate to prevent metal-catalyzed oxidative injury once iron in the LIP is increased [66]. Although production of hydroxyl radical and lipid peroxidation are important in the initiation of iron overload pathology, additional mechanisms involving apoptosis and fibrosis can account for its complex pathophysiology that leads to organ failure [58].

The most important clinical conditions involving primary and secondary iron overload leading to iron-mediated tissue damage are genetic hemochromatosis and transfusional siderosis, respectively. Hereditary hemochromatosis linked to mutations in HFE, a MHC class I-like protein that is a necessary component of the iron-sensing machinery controlling hepcidin expression, is the most common genetic disease in Caucasians and presents a multisystem involvement: although iron overload first affects the liver, in hemochromatosis patients endocrine abnormalities, cardiac problems, and arthropathy are also common [68]. Loss of HFE function leads to inappropriately low hepcidin production and unneeded iron release in the bloodstream from the duodenum and reticuloendothelial system. Consequent NTBI formation and iron deposition in parenchymal cells lead to oxidative damage and determine the clinical features of hemochromatosis [69].

Secondary iron overload is mainly observed in association with transfusion-dependent diseases [70]. Since our body lacks any regulated mechanism to effectively excrete excess iron, long-term blood transfusion inescapably results in iron overload in patients. Transfusional iron overload affects particularly patients with inherited hemoglobinopathies, such as β thalassemia, which is the secondary iron overload condition more closely linked to tissue iron overload [71, 72]. However, the adverse effects of iron overload are also found in patients with a variety of conditions (e.g., Blackfan-Diamond anemia, aplastic anemia, sideroblastic anemia, myelodysplasia, etc.).

With continued transfusion, reticuloendothelial cells can no longer safely store all the excess iron, which thus enters the circulation in amounts that exceed the binding capacity of Tf, and NTBI develops [69]. It should be noted that in conditions such as β thalassemia hepcidin levels are paradoxically low because erythropoiesis-dependent downregulation prevails over the upregulation associated with body iron levels; therefore, high intestinal iron absorption contributes to iron overload [20, 62]. While in hemochromatosis patients

excess iron is removed by phlebotomy, the treatment of transfusional iron overload is mainly based on iron chelation therapy [61]. Iron chelators can be considered as antioxidants, but not all the possible molecules able to bind iron can be regarded as safe antioxidants. Since all the six iron atoms have to be bound to the chelator to form a stable complex, incomplete iron-chelate complexes (e.g., iron-EDTA) can undergo redox cycling and generate harmful free radicals. Therefore, for efficient and safe scavenging of excess iron, chelating molecules and/or dosages should be carefully evaluated to raise the thermodynamic stability of the iron-ligand complex.

5. Conclusions

Presently, it is not completely clear as to why in some conditions ROS are associated with cell damage and scavenging high ROS levels improves metabolic homeostasis, whereas in other settings ROS exert signaling functions important for essential cell activities. Possible explanations for these distinct biological specificities of ROS action include differences in the amount, sources, duration, and localization of ROS production; in addition, certainly, the higher ROS reactivity is, the greater toxicity is, while signaling capacity is diminished. Therefore, H_2O_2 , which is relatively stable and diffusible, is indeed a mild oxidant but is suitable for signaling. However, in the presence of ferrous iron, H_2O_2 can generate the highly reactive and toxic HO^\bullet . Therefore, we propose that increased availability of iron not bound to proteins specifically evolved to transport or store this essential metal can make the critical difference between the two opposite functions of ROS. The view that iron plays a main role in the scenario leading ROS to become signal or stress agents is supported by the sophisticated systems regulating intracellular and systemic iron homeostasis that we have summarized in this review. Additional evidence indicating the contribution of iron emerges from the number of studies showing the occurrence of ROS-mediated tissue damage whenever the control of iron metabolism is disrupted, conditions of which we selectively highlighted a few illustrative examples. Improved understanding of the complex interplay between iron metabolism and redox homeostasis will clarify these pathways and their relevance in pathophysiology. In consideration of the disappointing results of antioxidant therapy for a variety of diseases [73], targeting iron will possibly represent a more advanced therapeutic approach aimed at preventing the harmful effects of ROS while permitting their physiological role in cell signaling.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] B. Halliwell and J. M. C. Gutteridge, "Oxygen toxicity, oxygen radicals, transition metals and disease," *Biochemical Journal*, vol. 219, no. 1, pp. 1–14, 1984.
- [2] D. Trachootham, W. Lu, M. A. Ogasawara, N. R.-D. Valle, and P. Huang, "Redox regulation of cell survival," *Antioxidants and Redox Signaling*, vol. 10, no. 8, pp. 1343–1374, 2008.
- [3] T. Finkel, "Signal transduction by mitochondrial oxidants," *The Journal of Biological Chemistry*, vol. 287, no. 7, pp. 4434–4440, 2012.
- [4] G. Shadel and T. Horvath, "Mitochondrial ROS signaling in organismal homeostasis," *Cell*, vol. 163, no. 3, pp. 560–569, 2015.
- [5] B. Harwell, "Biochemistry of oxidative stress," *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1147–1150, 2007.
- [6] S. J. Dixon, K. M. Lemberg, M. R. Lamprecht et al., "Ferroptosis: an iron-dependent form of nonapoptotic cell death," *Cell*, vol. 149, no. 5, pp. 1060–1072, 2012.
- [7] S. J. Dixon, D. Patel, M. Welsch et al., "Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis," *eLife*, vol. 2014, no. 3, 2014.
- [8] J. P. Friedmann Angeli, M. Schneider, B. Proneth et al., "Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice," *Nature Cell Biology*, vol. 16, no. 12, pp. 1180–1191, 2014.
- [9] M. Gao, P. Monian, N. Quadri, R. Ramasamy, and X. Jiang, "Glutaminolysis and transferrin regulate ferroptosis," *Molecular Cell*, vol. 59, no. 2, pp. 298–308, 2015.
- [10] X. Sun, Z. Ou, R. Chen et al., "Activation of the p62-Keap1-NRF2 pathway protects against ferroptosis in hepatocellular carcinoma cells," *Hepatology*, vol. 63, no. 1, pp. 173–184, 2016.
- [11] G. Cairo, F. Bernuzzi, and S. Recalcati, "A precious metal: iron, an essential nutrient for all cells," *Genes & Nutrition*, vol. 1, no. 1, pp. 25–39, 2006.
- [12] A. D. Sheftel, A. B. Mason, and P. Ponka, "The long history of iron in the Universe and in health and disease," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1820, no. 3, pp. 161–187, 2012.
- [13] N. C. Andrews, "Forging a field: the golden age of iron biology," *Blood*, vol. 112, no. 2, pp. 219–230, 2008.
- [14] M. W. Hentze, M. U. Muckenthaler, B. Galy, and C. Camaschella, "Two to tango: regulation of Mammalian iron metabolism," *Cell*, vol. 142, no. 1, pp. 24–38, 2010.
- [15] G. Cairo and S. Recalcati, "Iron-regulatory proteins: molecular biology and pathophysiological implications," *Expert Reviews in Molecular Medicine*, vol. 9, no. 33, pp. 1–13, 2007.
- [16] S. Recalcati, G. Minotti, and G. Cairo, "Iron regulatory proteins: from molecular mechanisms to drug development," *Antioxidants and Redox Signaling*, vol. 13, no. 10, pp. 1593–1616, 2010.
- [17] R. C. Hider and X. Kong, "Iron speciation in the cytosol: an overview," *Dalton Transactions*, vol. 42, no. 9, pp. 3220–3229, 2013.
- [18] T. Moroishi, M. Nishiyama, Y. Takeda, K. Iwai, and K. I. Nakayama, "The FBXL5-IRP2 axis is integral to control of iron metabolism in vivo," *Cell Metabolism*, vol. 14, no. 3, pp. 339–351, 2011.
- [19] J. C. Ruiz and R. K. Bruick, "F-box and leucine-rich repeat protein 5 (FBXL5): sensing intracellular iron and oxygen," *Journal of Inorganic Biochemistry*, vol. 133, pp. 73–77, 2014.
- [20] S. Recalcati, E. Gammella, and G. Cairo, "New perspectives on the molecular basis of the interaction between oxygen

- homeostasis and iron metabolism,” *Hypoxia*, vol. 3, pp. 93–103, 2015.
- [21] M. U. Muckenthaler, B. Galy, and M. W. Hentze, “Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network,” *Annual Review of Nutrition*, vol. 28, pp. 197–213, 2008.
- [22] N. Wilkinson and K. Pantopoulos, “The IRP/IRE system in vivo: insights from mouse models,” *Frontiers in Pharmacology*, vol. 5, article 176, 2014.
- [23] S. Recalcati, A. Alberghini, A. Campanella et al., “Iron regulatory proteins 1 and 2 in human monocytes, macrophages and duodenum: expression and regulation in hereditary hemochromatosis and iron deficiency,” *Haematologica*, vol. 91, no. 3, pp. 303–310, 2006.
- [24] D.-L. Zhang, M. C. Ghosh, and T. A. Rouault, “The physiological functions of iron regulatory proteins in iron homeostasis—an update,” *Frontiers in Pharmacology*, vol. 5, article 124, 2014.
- [25] G. Cairo, S. Recalcati, A. Pietrangelo, and G. Minotti, “The iron regulatory proteins: targets and modulators of free radical reactions and oxidative damage,” *Free Radical Biology and Medicine*, vol. 32, no. 12, pp. 1237–1243, 2002.
- [26] P. Arosio, F. Carmona, R. Gozzelino, F. Maccarinelli, and M. Poli, “The importance of eukaryotic ferritins in iron handling and cytoprotection,” *Biochemical Journal*, vol. 472, no. 1, pp. 1–15, 2015.
- [27] C. P. Anderson, M. Shen, R. S. Eisenstein, and E. A. Leibold, “Mammalian iron metabolism and its control by iron regulatory proteins,” *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research*, vol. 1823, no. 9, pp. 1468–1483, 2012.
- [28] K. Pantopoulos, S. K. Porwal, A. Tartakoff, and L. Devireddy, “Mechanisms of mammalian iron homeostasis,” *Biochemistry*, vol. 51, no. 29, pp. 5705–5724, 2012.
- [29] L. C. Kühn, “Iron regulatory proteins and their role in controlling iron metabolism,” *Metallomics*, vol. 7, no. 2, pp. 232–243, 2015.
- [30] T. A. Rouault, “Iron metabolism in the CNS: implications for neurodegenerative diseases,” *Nature Reviews Neuroscience*, vol. 14, no. 8, pp. 551–564, 2013.
- [31] G. Isaya, “Mitochondrial iron-sulfur cluster dysfunction in neurodegenerative disease,” *Frontiers in Pharmacology*, vol. 5, article 29, 2014.
- [32] L. Zecca, M. B. H. Youdim, P. Riederer, J. R. Connor, and R. R. Crichton, “Iron, brain ageing and neurodegenerative disorders,” *Nature Reviews Neuroscience*, vol. 5, no. 11, pp. 863–873, 2004.
- [33] A. Martelli and H. Puccio, “Dysregulation of cellular iron metabolism in Friedreich ataxia: from primary iron-sulfur cluster deficit to mitochondrial iron accumulation,” *Frontiers in Pharmacology*, vol. 5, article 130, 2014.
- [34] V. Campuzano, L. Montermini, M. D. Moltò et al., “Friedreich’s ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion,” *Science*, vol. 271, no. 5254, pp. 1423–1427, 1996.
- [35] R. A. Vaubel and G. Isaya, “Iron-sulfur cluster synthesis, iron homeostasis and oxidative stress in Friedreich ataxia,” *Molecular and Cellular Neuroscience*, vol. 55, pp. 50–61, 2013.
- [36] P. Arosio and S. Levi, “Cytosolic and mitochondrial ferritins in the regulation of cellular iron homeostasis and oxidative damage,” *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1800, no. 8, pp. 783–792, 2010.
- [37] A. Campanella, G. Isaya, H. A. O’Neill et al., “The expression of human mitochondrial ferritin rescues respiratory function in frataxin-deficient yeast,” *Human Molecular Genetics*, vol. 13, no. 19, pp. 2279–2288, 2004.
- [38] Z. I. Cabantchik, “Labile iron in cells and body fluids: physiology, pathology, and pharmacology,” *Frontiers in Pharmacology*, vol. 5, article 45, 2014.
- [39] G. Minotti, P. Menna, E. Salvatorelli, G. Cairo, and L. Gianni, “Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity,” *Pharmacological Reviews*, vol. 56, no. 2, pp. 185–229, 2004.
- [40] P. Menna, S. Recalcati, G. Cairo, and G. Minotti, “An introduction to the metabolic determinants of anthracycline cardiotoxicity,” *Cardiovascular Toxicology*, vol. 7, no. 2, pp. 80–85, 2007.
- [41] E. Gammella, S. Recalcati, I. Rybinska, P. Buratti, and G. Cairo, “Iron-induced damage in cardiomyopathy: oxidative-dependent and independent mechanisms,” *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 230182, 10 pages, 2015.
- [42] G. Minotti, G. Cairo, and E. Monti, “Role of iron in anthracycline cardiotoxicity: new tunes for an old song?” *The FASEB Journal*, vol. 13, no. 2, pp. 199–212, 1999.
- [43] M. Štěrba, O. Popelová, A. Vávrová et al., “Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection,” *Antioxidants and Redox Signaling*, vol. 18, no. 8, pp. 899–929, 2013.
- [44] F. Bernuzzi, S. Recalcati, A. Alberghini, and G. Cairo, “Reactive oxygen species-independent apoptosis in doxorubicin-treated H9c2 cardiomyocytes: role for heme oxygenase-1 downmodulation,” *Chemico-Biological Interactions*, vol. 177, no. 1, pp. 12–20, 2009.
- [45] R. D. Spagnuolo, S. Recalcati, L. Tacchini, and G. Cairo, “Role of hypoxia-inducible factors in the dexrazoxane-mediated protection of cardiomyocytes from doxorubicin-induced toxicity,” *British Journal of Pharmacology*, vol. 163, no. 2, pp. 299–312, 2011.
- [46] G. Minotti, R. Ronchi, E. Salvatorelli, P. Menna, and G. Cairo, “Doxorubicin irreversibly inactivates iron regulatory proteins 1 and 2 in cardiomyocytes: evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy,” *Cancer Research*, vol. 61, no. 23, pp. 8422–8428, 2001.
- [47] G. Corna, P. Santambrogio, G. Minotti, and G. Cairo, “Doxorubicin paradoxically protects cardiomyocytes against iron-mediated toxicity: role of reactive oxygen species and ferritin,” *The Journal of Biological Chemistry*, vol. 279, no. 14, pp. 13738–13745, 2004.
- [48] G. Corna, B. Galy, M. W. Hentze, and G. Cairo, “IRP1-independent alterations of cardiac iron metabolism in doxorubicin-treated mice,” *Journal of Molecular Medicine*, vol. 84, no. 7, pp. 551–560, 2006.
- [49] M. C. Asensio-López, J. Sánchez-Más, D. A. Pascual-Figal et al., “Involvement of ferritin heavy chain in the preventive effect of metformin against doxorubicin-induced cardiotoxicity,” *Free Radical Biology and Medicine*, vol. 57, pp. 188–200, 2013.
- [50] J. C. Kwok and D. R. Richardson, “Examination of the mechanism(s) involved in doxorubicin-mediated iron accumulation in ferritin: studies using metabolic inhibitors, protein synthesis inhibitors, and lysosomotropic agents,” *Molecular Pharmacology*, vol. 65, no. 1, pp. 181–195, 2004.
- [51] J. Drysdale, P. Arosio, R. Invernizzi et al., “Mitochondrial ferritin: a new player in iron metabolism,” *Blood Cells, Molecules & Diseases*, vol. 29, no. 3, pp. 376–383, 2002.

- [52] Y. Ichikawa, M. Ghanefar, M. Bayeva et al., "Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation," *The Journal of Clinical Investigation*, vol. 124, no. 2, pp. 617–630, 2014.
- [53] F. Maccarinelli, E. Gammella, M. Asperti et al., "Mice lacking mitochondrial ferritin are more sensitive to doxorubicin-mediated cardiotoxicity," *Journal of Molecular Medicine*, vol. 92, no. 8, pp. 859–869, 2014.
- [54] S. Gulec, G. J. Anderson, and J. F. Collins, "Mechanistic and regulatory aspects of intestinal iron absorption," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 307, no. 4, pp. G397–G409, 2014.
- [55] J. E. Levy, O. Jin, Y. Fujiwara, F. Kuo, and N. C. Andrews, "Transferrin receptor is necessary for development of erythrocytes and the nervous system," *Nature Genetics*, vol. 21, no. 4, pp. 396–399, 1999.
- [56] E. Gammella, F. Maccarinelli, P. Buratti, S. Recalcati, and G. Cairo, "The role of iron in anthracycline cardiotoxicity," *Frontiers in Pharmacology*, vol. 5, article 25, 2014.
- [57] T. Ganz, "Systemic iron homeostasis," *Physiological Reviews*, vol. 93, no. 4, pp. 1721–1741, 2013.
- [58] A. Pietrangelo, "Pathogens, metabolic adaptation, and human diseases—an iron-thrifty genetic model," *Gastroenterology*, vol. 149, no. 4, pp. 834–838, 2015.
- [59] A. Pietrangelo, "Hepcidin in human iron disorders: therapeutic implications," *Journal of Hepatology*, vol. 54, no. 1, pp. 173–181, 2011.
- [60] E. Nemeth, M. S. Tuttle, J. Powelson et al., "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization," *Science*, vol. 306, no. 5704, pp. 2090–2093, 2004.
- [61] C. Camaschella, "Iron-deficiency anemia," *The New England Journal of Medicine*, vol. 373, no. 5, pp. 485–486, 2015.
- [62] A. Kim and E. Nemeth, "New insights into iron regulation and erythropoiesis," *Current Opinion in Hematology*, vol. 22, no. 3, pp. 199–205, 2015.
- [63] L. Kautz, G. Jung, E. V. Valore, S. Rivella, E. Nemeth, and T. Ganz, "Identification of erythroferrone as an erythroid regulator of iron metabolism," *Nature Genetics*, vol. 46, no. 7, pp. 678–684, 2014.
- [64] J. P. Liuzzi, F. Aydemir, H. Nam, M. D. Knutson, and R. J. Cousins, "Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 37, pp. 13612–13617, 2006.
- [65] S. Ludwiczek, I. Theurl, M. U. Muckenthaler et al., "Ca²⁺ channel blockers reverse iron overload by a new mechanism via divalent metal transporter-1," *Nature Medicine*, vol. 13, no. 4, pp. 448–454, 2007.
- [66] G. Y. Oudit, H. Sun, M. G. Trivieri et al., "L-type Ca²⁺ channels provide a major pathway for iron entry into cardiomyocytes in iron-overload cardiomyopathy," *Nature Medicine*, vol. 9, no. 9, pp. 1187–1194, 2003.
- [67] P. Venditti, L. Di Stefano, and S. Di Meo, "Mitochondrial metabolism of reactive oxygen species," *Mitochondrion*, vol. 13, no. 2, pp. 71–82, 2013.
- [68] A. Pietrangelo, "Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment," *Gastroenterology*, vol. 139, no. 2, pp. 393.e1–408.e2, 2010.
- [69] G. Sebastiani and K. Pantopoulos, "Disorders associated with systemic or local iron overload: from pathophysiology to clinical practice," *Metallomics*, vol. 3, no. 10, pp. 971–986, 2011.
- [70] R. E. Fleming and P. Ponka, "Iron overload in human disease," *The New England Journal of Medicine*, vol. 366, no. 4, pp. 348–359, 2012.
- [71] J. B. Porter and M. Garbowski, "The pathophysiology of transfusional iron overload," *Hematology/Oncology Clinics of North America*, vol. 28, no. 4, pp. 683–701, 2014.
- [72] V. Berdoukas, T. D. Coates, and Z. I. Cabantchik, "Iron and oxidative stress in cardiomyopathy in thalassemia," *Free Radical Biology and Medicine*, vol. 88, pp. 3–9, 2015.
- [73] J. M. C. Gutteridge and B. Halliwell, "Antioxidants: molecules, medicines, and myths," *Biochemical and Biophysical Research Communications*, vol. 393, no. 4, pp. 561–564, 2010.

Research Article

Lung Neutrophilia in Myeloperoxidase Deficient Mice during the Course of Acute Pulmonary Inflammation

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Systemic inflammation accompanying diseases such as sepsis affects primarily lungs and induces their failure. This remains the most common cause of sepsis induced mortality. While neutrophils play a key role in pulmonary failure, the mechanisms remain incompletely characterized. We report that myeloperoxidase (MPO), abundant enzyme in neutrophil granules, modulates the course of acute pulmonary inflammatory responses induced by intranasal application of lipopolysaccharide. MPO deficient mice had significantly increased numbers of airway infiltrated neutrophils compared to wild-type mice during the whole course of lung inflammation. This was accompanied by higher levels of RANTES in bronchoalveolar lavage fluid from the MPO deficient mice. Other markers of lung injury and inflammation, which contribute to recruitment of neutrophils into the inflamed lungs, including total protein and other selected proinflammatory cytokines did not significantly differ in bronchoalveolar lavage fluid from the wild-type and the MPO deficient mice. Interestingly, MPO deficient neutrophils revealed a decreased rate of cell death characterized by phosphatidylserine surface expression. Collectively, the importance of MPO in regulation of pulmonary inflammation, independent of its putative microbicidal functions, can be potentially linked to MPO ability to modulate the life span of neutrophils and to affect accumulation of chemotactic factors at the inflammatory site.

1. Introduction

Inflammation is protective and vital to health, but when acute inflammation is unrestrained in amplitude or duration, it can lead to disease [1]. In most instances, the molecular and cellular events during acute inflammation are successful in limiting the inciting injury or infection and restoring tissue homeostasis. However, overwhelming inflammation can lead to fatal consequences. The lungs are generally the first to undergo failure, and this remains the most common cause of acute life-threatening pathologies such as septic shock and/or

multiple organ failure induced mortality [2, 3]. Although acute inflammation is generally self-limited, alternate fates include abscess formation, fibrosis, or conversion to chronic inflammation [1].

While it has become clear that neutrophil granulocytes, the most abundant subpopulation of polymorphonuclear granulocytes, play a key role in pulmonary failure during sepsis and development of chronic inflammation, the mechanisms remain incompletely characterized [2, 4]. The progression and control of inflammation are significantly affected by mediators produced by leukocytes accumulated at the site of

inflammation [1]. One of them is myeloperoxidase (MPO), an abundant hemoprotein of neutrophils, which is typically perceived to primarily mediate host defense reactions [5–7]. However, there is increasing evidence showing a novel regulatory role of MPO not directly related to host defense. MPO accumulated in the lungs could significantly modulate redox-sensitive cellular signaling pathways controlling inflammatory processes among others through the catabolism of nitric oxide (NO), induction of wide range of posttranslational modification of proteins, and modulation of metabolism of arachidonic and linoleic acid derived mediators [5–10].

However, the importance of MPO in various inflammatory conditions is questioned and is highly dependent on the type of the inflammatory model. Consistent with microbicidal function of MPO, the MPO deficient mice were more likely than the wild-type mice to be infected or die from infection employing various models, suggesting that the MPO-dependent oxidative system is important for host defense against fungi and bacteria [5, 6, 9]. However, the MPO importance varies by pathogen species [11]. In contrast, in the cases of inflammatory response to noninfectious stimuli or chronic inflammation in the absence of live pathogens, MPO can damage host tissue through the generation of oxidants. This has been observed in many chronic inflammatory diseases, including cardiovascular diseases, rheumatoid arthritis, and kidney diseases (see reviews [5–7, 9]). Thus, based on the assumption of detrimental effect of MPO during chronic inflammation, it can be expected that the inflammation should actually be reduced in MPO deficient mice. On the contrary, experimental data from various studies employing MPO deficient mice suggest that the MPO deficiency is connected with dysregulated inflammatory response. These studies show significant adverse effects of MPO deficiency in various inflammatory models induced by noninfectious stimuli including nonviable *Candida albicans* [12], experimental autoimmune encephalomyelitis [13], ischemic brain injury [14], lung dysfunction after allogeneic bone marrow transplantation [15], ultraviolet- (UV-) exposed skin inflammation [16], atherosclerotic lesions development [17], skin delayed-type hypersensitivity and antigen-induced arthritis [18], and different types of autoimmune renal diseases [19, 20]. These data support the suggestion of a protective, anti-inflammatory role of MPO in pathologies characterized by complex inflammatory response in the absence of infectious agent.

Here, a role of MPO in the regulation of acute lung inflammation and injury was evaluated. Model of acute airway inflammation induced by intranasal administration of lipopolysaccharide (LPS) was employed in both the wild-type and the MPO deficient mice. Various markers of inflammation and injury in lung tissues or lung lavage fluids were determined. Such LPS instillation is known to cause an acute inflammatory response with transient extravasation of primarily neutrophils in the airways [21–23]. The temporal course of inflammation was evaluated at different time points (8 h, 24 h, 48 h, and 72 h) following application of LPS. Our results indicate that MPO deficiency enhances neutrophilia during LPS-induced airway inflammation due to altered

accumulation of proinflammatory cytokine RANTES and reduced cell death of MPO deficient neutrophils.

2. Materials and Methods

2.1. Animal Exposure to LPS. Male C57BL/6J wild-type and MPO deficient mice MPO^{tmilus} (MPO deficient) (The Jackson Laboratory, USA) [8] both of age 12–16 weeks and of weight 25–30 g were subjected to brief anesthesia with ketamine-xylazine, and 50 μ L of LPS (from *Escherichia coli* serotype 055:B5, Sigma-Aldrich, USA) solution in phosphate-buffered saline (PBS) was instilled directly into their nostrils to reach a dose of 0.3 mg/kg LPS as described previously [23]. Previous studies demonstrated that a significant fraction of intranasally administered LPS will reach the lungs and that such instillation evokes an acute transient inflammatory response [21–23]. Mice in the control group received a similar volume of sterile PBS. The experiments were approved by the Animal Care Committee and were in accordance with the EU and NIH Guide for Care and Use of Laboratory Animals.

2.2. Collection of Bronchoalveolar Lavage Fluid (BALF). At various time points after LPS instillation, mice were deeply anesthetized by intraperitoneal administration of ketamine-xylazine and blood was collected from the heart into heparinized syringes. The tracheae were cannulated, and the lungs were lavaged with 2 consecutive washes with 1 mL of PBS, which were pooled to a total recovered BALF of 1.6–1.8 mL. The BALF was used for the cell analysis after pelleting (5 min, 250 g, 4°C) or the cell-free BALF was obtained after centrifugation (4 min, 2 000 g, 4°C) and stored at –80°C until further analysis.

2.3. Determination of Inflammatory Cells in BALF and Their Differentiation Count. Total cell count in the collected lavage samples and total leukocyte count in blood were determined with a Coulter Counter Z1 after lysis of RBCs by Zap-Oglobin II lytic reagent (Coulter, USA). Cell differential counts were determined on blood smears or on slides after cytopspin centrifugation stained with Giemsa stain (Diff-Quick, ThermoFisher Scientific, USA).

2.4. Inflammatory Cytokines and Total Proteins in BALF. To quantitate proinflammatory cytokine production in the lung tumor necrosis factor- (TNF-) α , monocyte chemoattractant protein- (MCP-) 1, Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), interleukin- (IL-) 6, IL-10, and IL-12 were determined in BALF by sandwich enzyme-linked immunosorbent assay (ELISA) using murine-specific commercial kits (Quantikine Mouse cytokine assay kit, R&D Systems, USA).

As a marker of epithelial injury and lung permeability, total protein concentration within BALF was measured with a Protein Assay Kit (Bio-Rad, USA) based on the method of Bradford, with bovine serum albumin as a standard.

2.5. Analysis of NO Production in Lungs. To quantitate the production of NO in the lungs, its metabolites nitrates/nitrites were measured in BALF by chemical reduction of

nitrites and nitrites to NO in 120 mM vanadium (III) in 2 M HCl at 95°C and analysis by ozone-enhanced chemiluminescence (ANTEK Instruments Inc., Model 7020, USA).

2.6. Expression of CD11b on Neutrophil Surface. Cells were washed with PBS (5 min, 250 g, 4°C) and incubated with fluorescein-conjugated anti-mouse CD11b (Invitrogen, USA) antibodies at 4°C in the dark for 30 min, consequently washed with PBS (5 min, 250 g, 4°C), and analyzed using a BD FACSVerse™ flow cytometer (BD Biosciences, USA) [24]. Cells incubated with appropriate isotype control were used to determine the nonspecific background signal. The data analysis was performed using Flowing Software (<http://www.flowingsoftware.com>).

2.7. Histological Analysis of Lungs. Following euthanasia, the lungs were instilled with OCT Compound (Sakura, The Netherlands). The lungs were subsequently removed and placed into OCT Compound and immediately frozen in liquid nitrogen. 7 μm sections were stained with H&E stain and histological analysis was performed by light microscopy (10x objective). Series of pictures were obtained from the different types of samples that were evaluated and typical examples are represented.

2.8. Analysis of MPO Enzymatic Activity. Isolated cells were washed with PBS (5 min, 250 g, 4°C) and the remaining erythrocytes were lysed by hypotonic lysis using distilled water for 5 seconds. Cells were washed with PBS (5 min, 250 g, 4°C) and subsequently lysed in PBS with 0.1% Triton X-100 (Sigma-Aldrich, USA). Peroxidase activity was measured as the oxidation of tetramethylbenzidine (TMB, 2 mM, Sigma-Aldrich, USA) in 300 mM sodium acetate buffer (pH 5.4) in the presence of 0.075 mM hydrogen peroxide (H₂O₂) (Sigma-Aldrich, USA) within 20 min as described previously [24]. The formation of the reaction product was determined spectrophotometrically as the increase in absorption at 350 nm using an Infinite M200 microplate spectrofluorometer (Tecan, Switzerland).

2.9. Caspase 3 Activity Assay. Caspase 3 activity was analyzed as described previously [25]. Isolated cells were washed twice with PBS (5 min, 250 g, 4°C) and lysed (50 mM HEPES; 5 mM CHAPS; 5 mM DTT) on ice for 20 min and centrifuged (15 min, 15 000 g, 4°C). Lavage fluid was diluted 1:1 with 2x concentrated lysing buffer. The proteins present in supernatants were quantified using Coomassie® Protein Assay (Bio-Rad, USA) and diluted to an equal concentration. 5 μg of protein samples was incubated in an assay buffer in parallels (20 mM HEPES; 2.5 mM CHAPS, 5 mM DTT, 2 mM EDTA) containing 50 μM of caspase 3 (Ac-DEVD-AMC) substrate (Sigma-Aldrich, USA) at 37°C for 4 h. The level of fluorescence was determined using microplate reader (Infinite 200, Tecan, Switzerland; 360 nm excitation, 460 nm emission).

2.10. Phosphatidylserine (PS) Externalization (Annexin V/Propidium Iodide Assay). The presence of cells with

permeable membrane (dead cells) and cells with surface expression of PS was evaluated by flow cytometry using propidium iodide (PI) and Annexin V staining, respectively [26]. Cells were washed with PBS (5 min, 250 g, room temperature), resuspended in 100 μL Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), and incubated for 15 min with 1 μL Annexin V-FITC (Apronex Ltd., Czech Republic). PI (1 μg/mL) was added 1 min before analysis. Flow cytometric analysis of the stained cells was performed using a BD FACSVerse flow cytometer (BD Biosciences, USA). The cell population for the analysis was gated using forward- versus side-scatter parameters to exclude any debris. Per sample, 10 000 cells were collected. Three different populations can be identified using this assay: intact viable cells are negative for both PI and Annexin V-FITC, apoptotic cells are positive for Annexin V-FITC but negative for PI, and dead cells positive for both Annexin V-FITC and PI. The data analysis was performed using Flowing Software (<http://www.flowingsoftware.com>).

2.11. DNA Fragmentation. 3 * 10⁶ cells isolated from BALF were washed with PBS (5 min, 250 g, 4°C) and DNA was isolated using the Invisorb Apoptosis Detection Kit II (Invitex; Invitex GmbH, Germany). Gel electrophoresis was performed in 1.5% agarose (Sigma-Aldrich, USA), using 1 kbp DNA ladder as a marker (ThermoFisher Scientific, USA). DNA was stained with ethidium bromide (Sigma-Aldrich, USA) and scanned on UV-transilluminator using the Scion Image software (Scion Corporation, USA).

2.12. Statistical Analysis. Statistical comparisons were analyzed with the Student *t*-test for pairwise-dependent samples. A *p* value equal to or lower than 0.05 was considered statistically significant. Data are presented as the means ± standard error of the mean (SEM). All statistical analyses were carried out with Statistica 10 (StatSoft, USA).

3. Results

3.1. The LPS-Induced Airway Neutrophilia. Intranasal instillation of LPS (0.3 mg/kg) in the wild-type and the MPO deficient mice induced acute airway inflammation, characterized by an increase of the lung lavage cell numbers. This accumulation of cells in lungs was maximal 48 h after LPS instillation and declined thereafter (Figure 1(a)). As expected, while nucleated cells in BALF of untreated mice almost exclusively represented alveolar macrophages (Figure 1(b)), increased total numbers of nucleated cells in lung lavage of both wild-type and MPO deficient mice were almost exclusively due to neutrophils (Figure 1(c)) as assessed by Wright-Giemsa stain of cytospin samples. The number of lymphocytes, basophils, eosinophils, or other undetermined nucleated cells in BALF did not exceed 4% at any time point (data not shown). Interestingly, in contrast to expectations, the total numbers of nucleated cells in lavage were significantly lower in the wild-type mice compared to the MPO deficient mice at last two evaluated time points 48 h and 72 h after LPS instillation (Figure 1(a)). This was solely due to

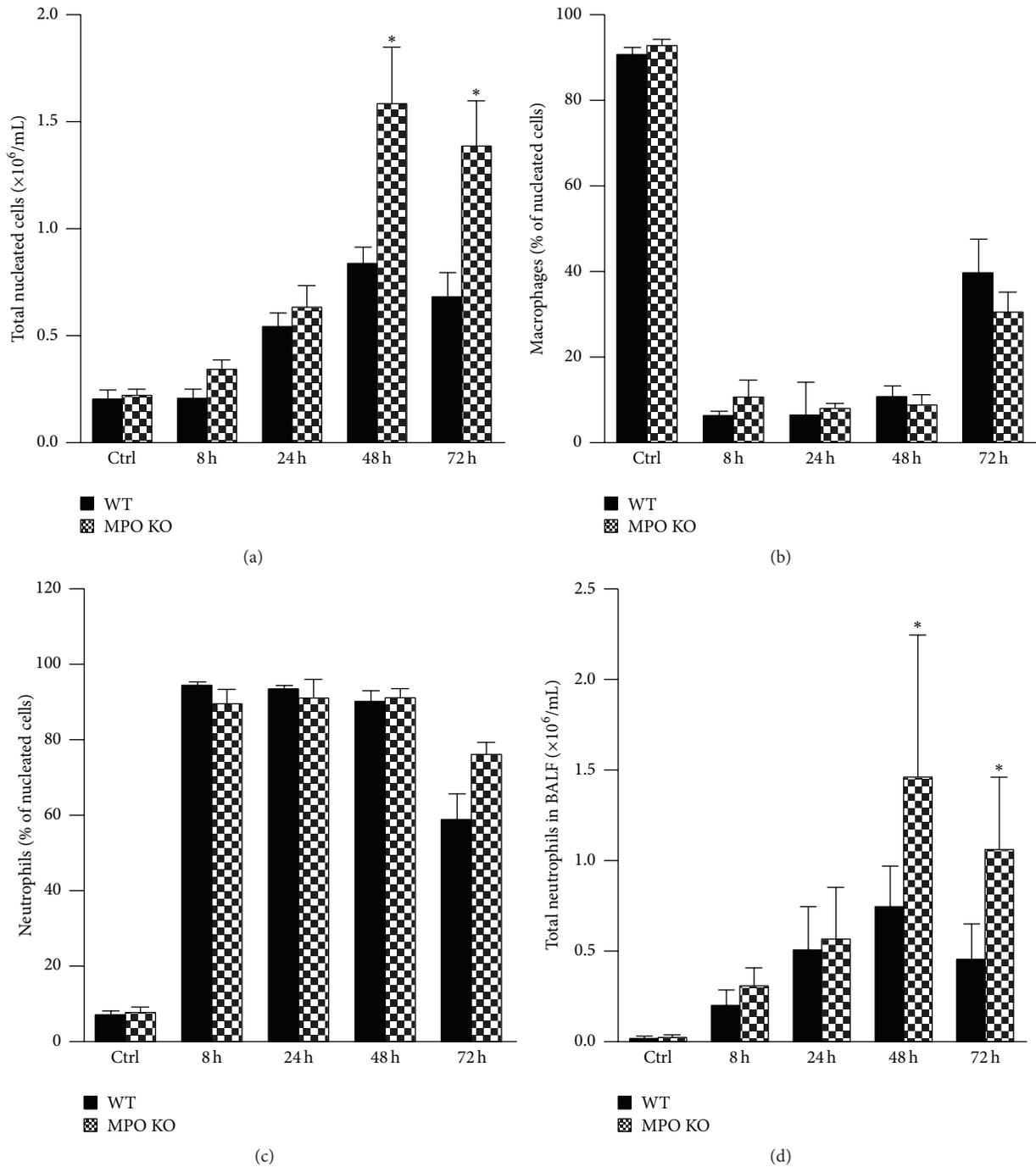


FIGURE 1: Acute lung inflammation induces accumulation of neutrophils in lungs. (a) Total nucleated cell counts, (b) relative count of airway macrophages, (c) relative count of neutrophils, and (d) total neutrophils in BALF collected at various times (8 h, 24 h, 48 h, and 72 h) after instillation of LPS (0.3 mg/kg) or PBS (control at time 0 h) in wild-type (WT; black bars) and MPO deficient (MPO KO; grey bars) mice. Values represent mean \pm SEM from 8–10 mice with significant difference between WT and MPO KO mice (* $p < 0.05$).

accumulation of neutrophils in lungs of the MPO deficient mice (Figure 1(d)), since as mentioned above, the differential count of cells in BALF did not differ significantly between the wild-type and the MPO deficient mice (Figures 1(b) and 1(c)). The determination of peroxidase activity in nucleated cells isolated from BALF of wild-type and MPO deficient mice

clearly confirmed the absence of peroxidase activity in cells isolated from BALF of MPO deficient mice (Figure 2).

3.2. Abundance and Activation Status of Neutrophils in Peripheral Blood. Extravasation of neutrophils from peripheral circulation into the lungs is dependent on the number of

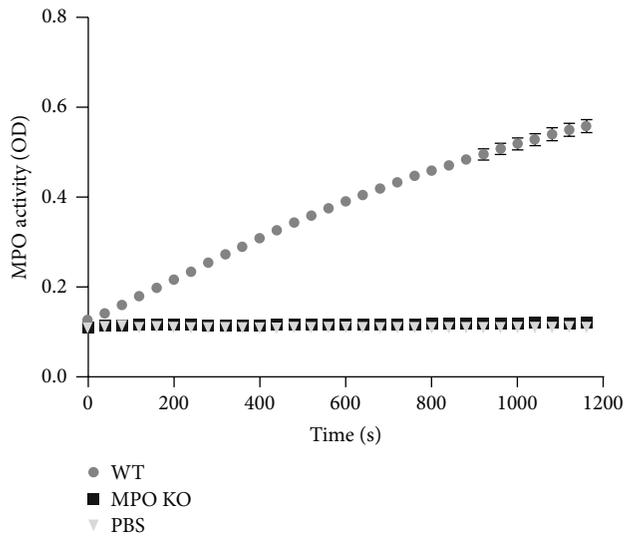


FIGURE 2: Peroxidase enzymatic activity of MPO in nucleated cells isolated from inflamed lungs of wild-type and MPO deficient mice. It was detected by spectrophotometry employing TMB. Data are presented as the increase in absorption at 350 nm and are expressed as mean \pm SEM ($n = 5$).

neutrophils in the blood and activation status corresponding with a level of the surface expression of molecules (such as CD11b) involved in adhesion and migration of neutrophils through a vessel wall to extravascular space. Interestingly, in contrast to lung neutrophilia, the total neutrophil count in blood of wild-type and MPO deficient mice before and at different time points of LPS-induced lung inflammation did not significantly differ (Figure 3(a)). Similarly, the surface expression of CD11b did not differ significantly between blood neutrophils of the wild-type and the MPO deficient mice at any time point (Figure 3(b)). Further, the CD11b surface expression was also analyzed on cells isolated from BALF. Corresponding to peripheral circulation, the CD11b surface expression was the same in the wild-type and the MPO deficient animals (data not shown).

3.3. Alternations in the Lung Epithelial Cell Barrier Permeability and Increased Accumulation of NO Metabolites in BALF. Accumulation of neutrophils in lungs during the course of acute inflammation is also modulated by permeability of lung epithelium and capillaries of peripheral circulation. In our model, the increase in lung permeability, which is a marker of injury of lung epithelial cell barrier, was determined by measuring the total protein in BALF. The total protein concentration in BALF was significantly increased during acute lung inflammation with maximal levels at 48 h after LPS application (Figure 4). However, the total protein in BALF did not significantly differ between the MPO deficient and the wild-type mice at any time point. These findings suggest that significantly higher accumulation of neutrophils in the MPO deficient mice could not be explained by different permeability of lungs. The intranasal administration of LPS induced production of NO (determined as stable products

nitrites and nitrates in BALF). The analysis revealed increased levels of NO production in both the MPO deficient and the wild-type mice, however, without significant differences between these groups (Figure 5). Finally, histological staining of lung sections showed no significant differences among lungs of wild-type and MPO deficient mice either in control groups or after 48 h of LPS instillation (Figure 6).

3.4. LPS-Induced Accumulation of Proinflammatory Cytokines in BALF. Corresponding with the course of inflammatory process, the proinflammatory cytokines were significantly increased in BALF after LPS instillation. TNF- α and IL-6 reached the maximal levels at the earliest time point after LPS instillation and decreased over the following days without significant differences between the MPO deficient and the wild-type mice (Figures 7(a) and 7(b)). IL-12 and MCP-5 reached maximal levels later, 48 h after the LPS instillation, and remained increased also at 72 h after LPS instillation but did not differ between MPO deficient and wild-type mice (Figures 7(c) and 7(d)). Levels of other potent chemoattractant RANTES increased from the first time point and in contrast to other evaluated cytokines were significantly higher in BALF of the MPO deficient mice compared to the wild-type mice (Figure 7(e)).

3.5. Increased Viability in BALF Cells from MPO Deficient Mice. The accumulation of inflammatory cells can be mediated by alternated apoptosis of extravasated inflammatory cells. Thus, to further evaluate the observed phenomenon of higher accumulation of neutrophils in lungs of the MPO deficient mice after LPS instillation, the cells in BALF were examined for programmed cell death markers and for their ability to die after incubation *in vitro*. Interestingly, the cells isolated from BALF of the MPO deficient mice revealed a decrease of number of cells positive for Annexin V staining and cells positive for Annexin V/PI staining with permeable membrane (dead cells) (Figures 8(a) and 8(b)). After prolonged 5 h incubation of cells *ex vivo*, the number of Annexin V positive cells and dead cells increased in BALF cells from the wild-type mice whereas in the case of cells from the MPO deficient mice the number of these types of cells remained low (Figures 8(a) and 8(b)). Interestingly, there was only background caspase 3 activity either directly in BALF (data not shown) or in BALF cell lysate, either from the MPO deficient or from the wild-type mice (Figure 9(a)). Similarly, we did not observe any fragmentation of DNA either in freshly isolated cells or after prolonged 5 h incubation of cells *in vitro* (Figure 9(b)).

4. Discussion

In this study, the importance of MPO in the course of acute lung inflammation was evaluated. In contrast to our previous study showing MPO mediated potentiation of neutrophils extravasation into the site of inflammation in various other tissues, such as livers [27], herein the data show that the MPO deficient mice had significantly increased numbers of airway infiltrated neutrophils compared to the wild-type

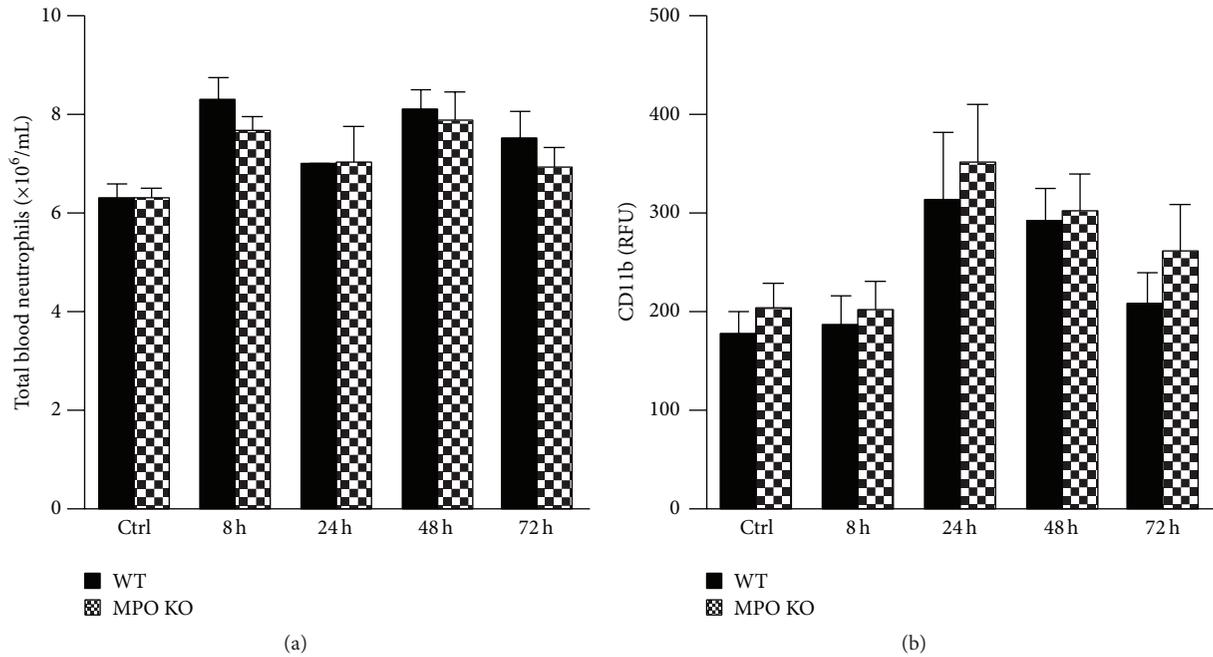


FIGURE 3: Total blood neutrophil count and surface expression of CD11b by neutrophils during the course of acute lung inflammation. (a) Total blood count of neutrophils was determined from total blood leukocytes count and their relative differentiation count in blood samples taken at various times (8 h, 24 h, 48 h, and 72 h) after instillation of LPS (0.3 mg/kg) or PBS (control at time 0 h) in both WT (black bars) and MPO KO (grey bars) mice. (b) Expression of surface CD11b receptor on blood neutrophils was determined by flow cytometer with a use of fluorescent labeled anti-CD11b monoclonal antibodies in blood samples described above. Results are expressed as relative fluorescence units (RFU). Values represent mean \pm SEM from 8–10 mice.

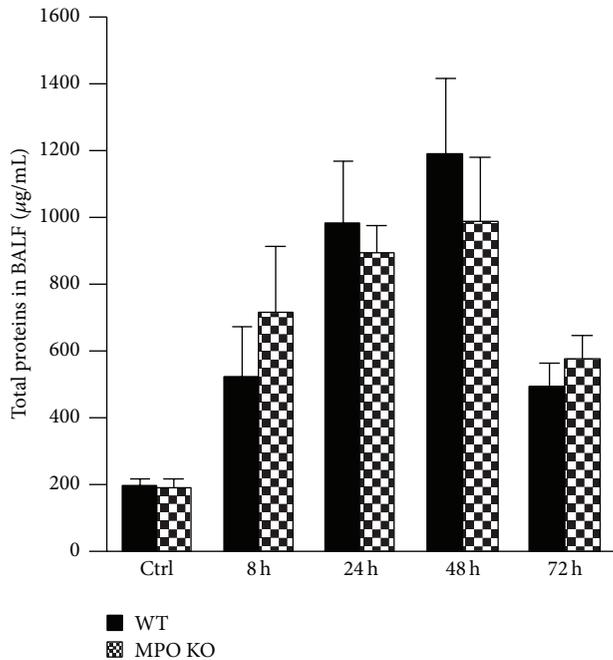


FIGURE 4: Total protein concentration during the course of acute lung inflammation. Total protein concentration in BALF at various times (8 h, 24 h, 48 h, and 72 h) after instillation of LPS (0.3 mg/kg) or PBS (control at time of 0 h) in both WT (black bars) and MPO KO (grey bars) mice. Values represent mean \pm SEM from 8–10 mice.

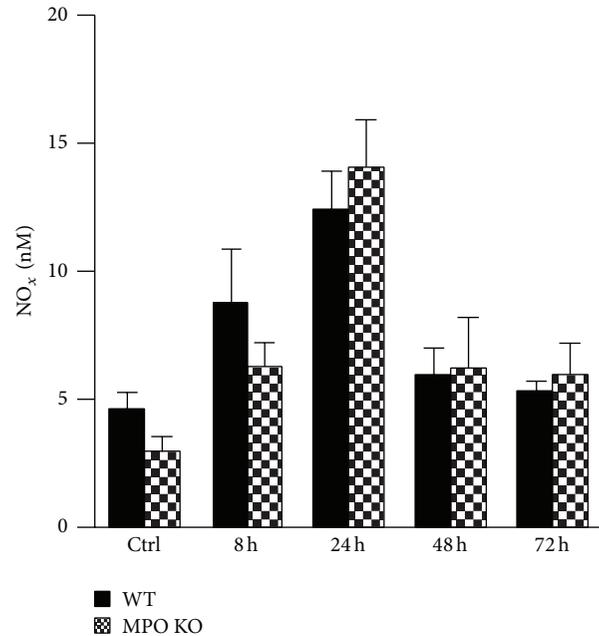


FIGURE 5: Accumulation of NO metabolites in BALF. Nitrates/nitrites in BALF at various time points (8 h, 24 h, 48 h, and 72 h) after instillation of LPS (0.3 mg/kg) or PBS (control at time 0 h) in both WT (black bars) and MPO KO (grey bars) mice. Values represent mean \pm SEM from 8–10 mice.

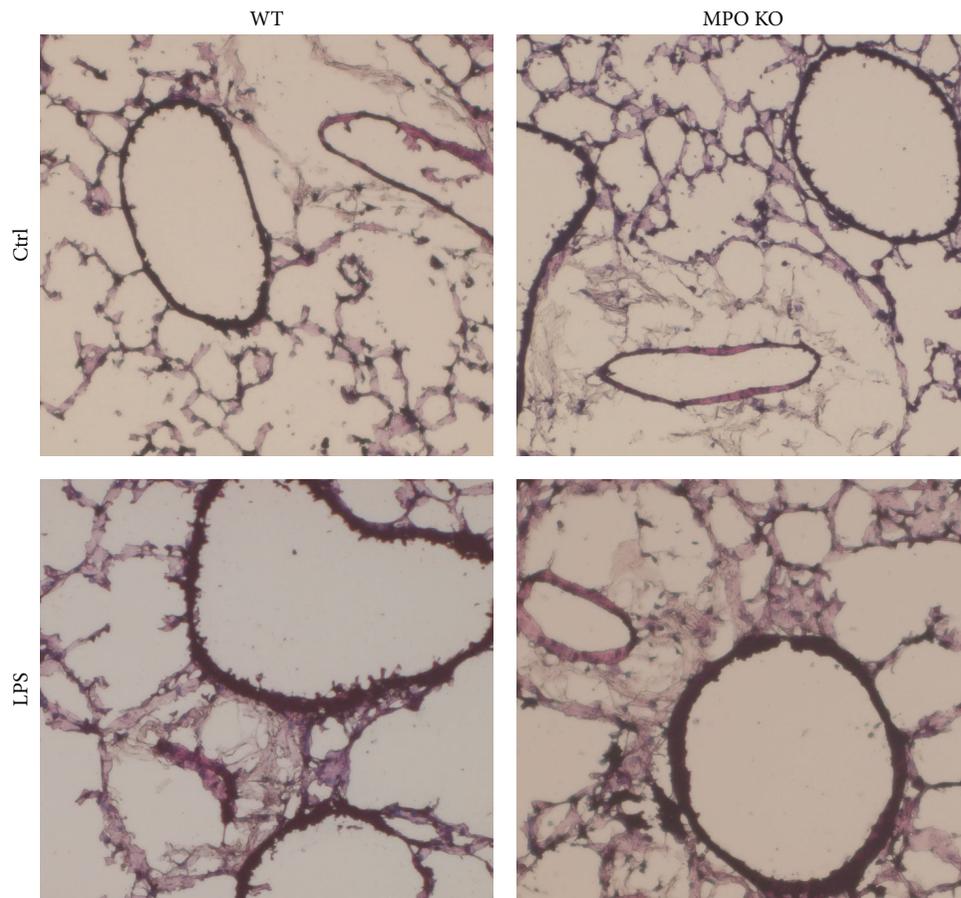


FIGURE 6: Lung histopathology. H&E staining of lung sections of WT and MPO KO mice 48 h after instillation of LPS (0.3 mg/kg) or PBS (ctrl).

mice during the later course of lung inflammation induced by intranasal application of LPS. Interestingly, most markers characterizing status of lung injury and inflammation including total protein and selected proinflammatory cytokines in BALF did not significantly differ between the wild-type and the MPO deficient mice. The only exception was the BALF levels of RANTES that were increased in the MPO deficient mice. Interestingly, analysis of the cell death characterized by robust cell surface expression of PS revealed the significant delay of this process in BALF cells from the MPO deficient mice.

We did not observe any significant differences in the percentage of inflammatory cells in BALF between MPO deficient and wild-type mice. In agreement with our findings, Milla et al. did not observe any dissimilarities between wild-type and MPO deficient mice in BALF cell differentiation count in a model of transplantation induced lung inflammation [15]. The large numbers of neutrophils accumulated in the airspaces pose a major challenge for the host in resolving inflammation. Interestingly, the significantly more profound accumulation of leukocytes at the site of inflammation in MPO deficient mice was observed also by other authors using different types of murine inflammatory models. Takeuchi et al. reported that zymosan mediated inflammation led

to greater neutrophil infiltration into the lungs of MPO deficient mice [28]. Further, this group showed that the MPO deficient mice that received nonviable *Candida albicans* showed more severe pneumonia with significantly higher numbers of alveolar neutrophils than wild-type mice [12]. Other studies revealed that MPO deficient mice exhibited accelerated lung dysfunction connected with increased number of inflammatory cells compared to wild-type mice after allogeneic bone marrow transplantation [15]. Also, recruitment of leukocyte into the peritoneum and glomerular accumulation of leukocytes including neutrophils were increased in pristine induced inflammation in MPO deficient mice [20]. On the other hand, Haegens and coauthors showed reduced inflammation in lungs of MPO deficient mice compared with wild-type mice despite employing a similar model of acute lung inflammation induced by intranasal LPS instillation [29]. However, these authors used different techniques for the estimation of lung infiltration and used higher dose of LPS compared to our study, which could result in a significantly higher level of inflammatory processes with a higher level of inflammation based injury. Under these conditions, other mechanisms responsible for the accumulation of neutrophils in lungs could predominate relative to the mechanisms suggested in our model. In general, the variability in results

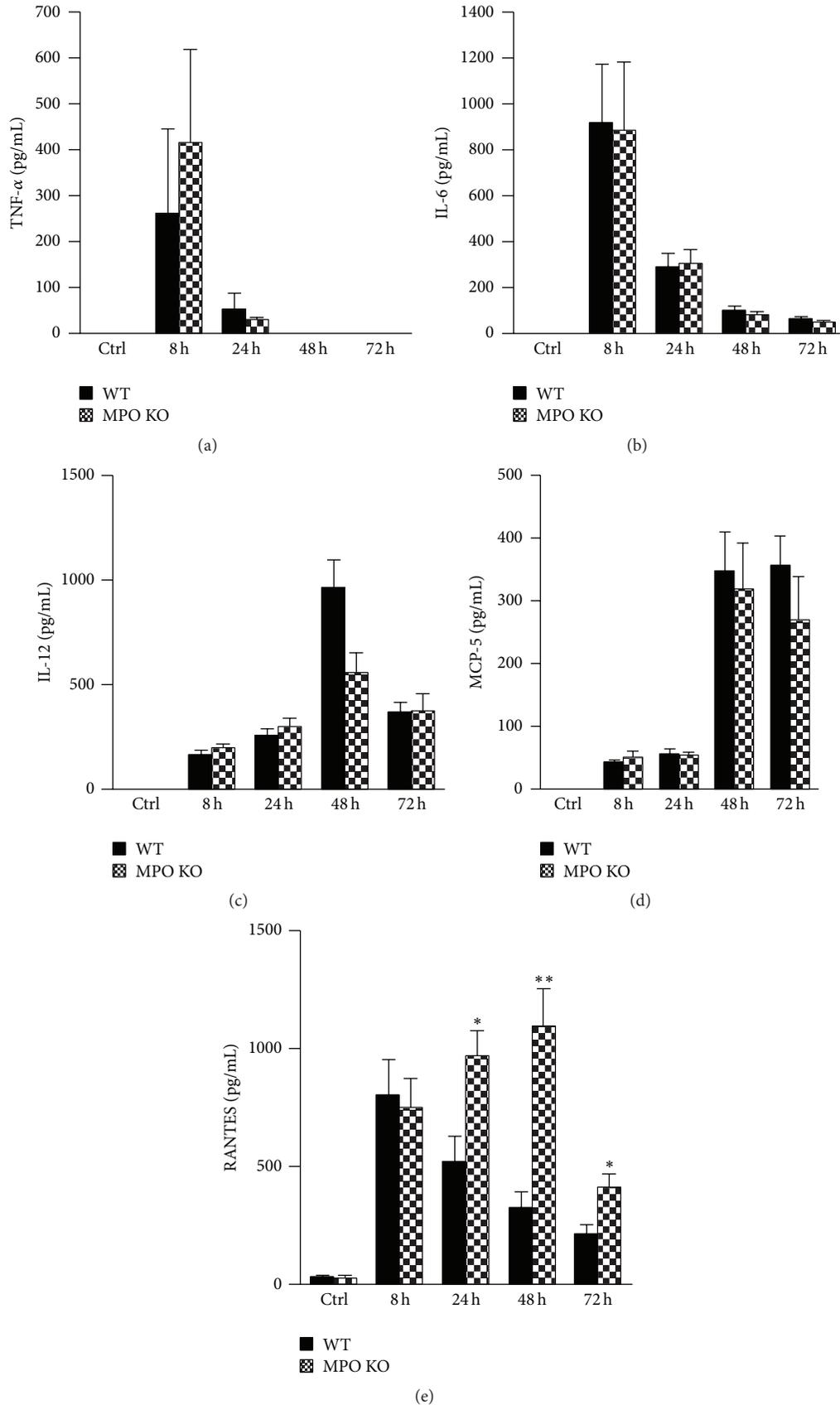


FIGURE 7: Levels of proinflammatory cytokines during the course of acute lung inflammation. (a) TNF- α , (b) IL-6, (c) IL-12, (d) MCP-5, and (e) RANTES concentrations in BALF were determined by commercial ELISA kits in samples described above. Values represent mean \pm SEM from 8–10 mice with significant difference between WT and MPO KO mice (* $p < 0.05$; ** $p < 0.01$).

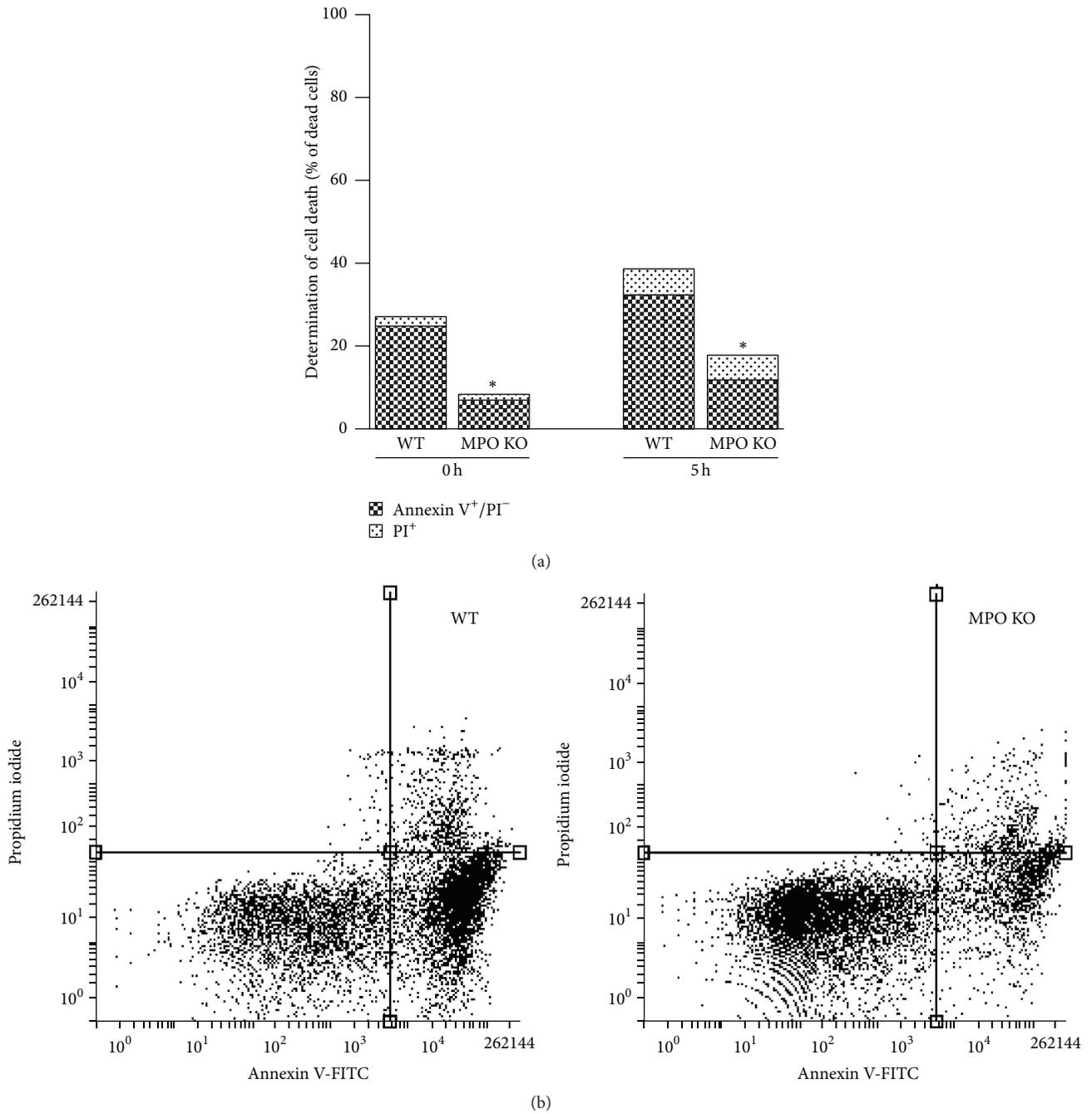


FIGURE 8: Determination of cell death in BALF cells. (a) Freshly isolated nucleated cells (1×10^6 cells/mL) from BALF of WT and MPO KO mice treated for 36 h by intranasal application of LPS (0.3 mg/kg) or cells 5 h after isolation were evaluated by flow cytometry using Annexin V/PI staining. Values represent mean from 4 mice with significant difference between Annexin V⁺ cells in BALF from WT and MPO KO mice (* $p < 0.05$). (b) Representative scatterplots from flow cytometric analysis. Cells were classified as either viable (lower left quartile, Annexin V⁻/PI⁻) cells with detectable expression of PS (lower right quartile, Annexin V⁺/PI⁻) or dead cells with permeable membrane (upper left and right quartile, PI⁺).

has to be examined in the context of the complexity of the lung inflammatory process when the infiltration and the accumulation of inflammatory cells in lungs are orchestrated by numerous factors [1, 3]. Thus, herein, a selection of these factors was determined to systematically evaluate this process to uncover factors that are affected by MPO and are

connected or responsible for the observed alternations in lung inflammatory response in MPO deficient mice.

The extravasation of neutrophils into the site of injury is significantly affected by numbers of neutrophils in the peripheral circulation and their activation status. The employed MPO deficient mice were reported to have total

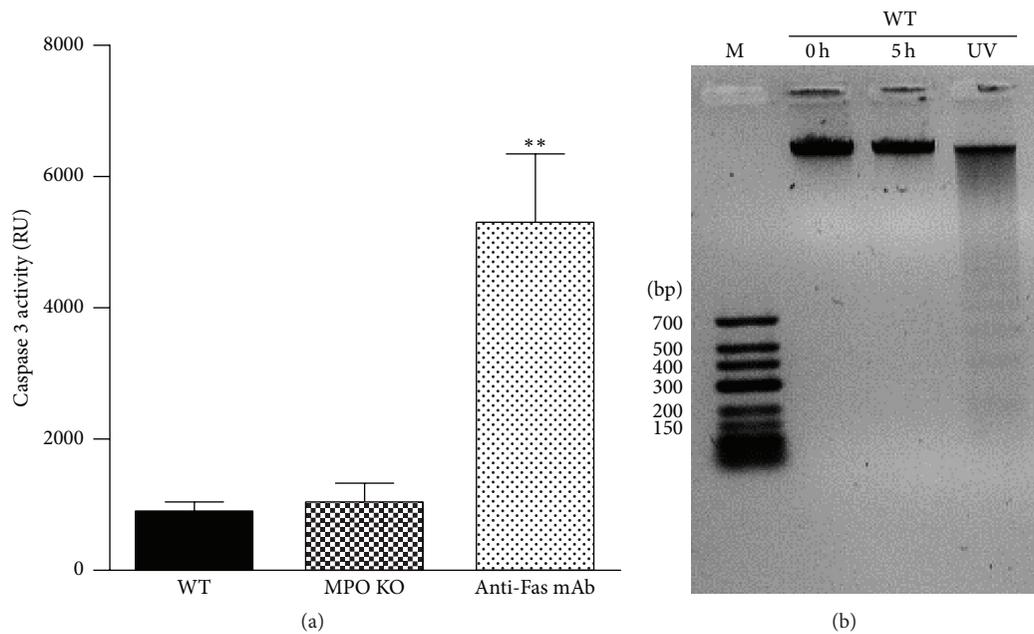


FIGURE 9: Determination of caspase 3 activity and DNA fragmentation. (a) Freshly isolated nucleated cells or cells 5 h after isolation (5×10^6 cells/mL) from BALF of WT and MPO KO mice treated for 36 h by intranasal application of LPS (0.3 mg/kg) were lysed and caspase 3 activity was determined by enzymatic assay. Cells treated with anti-Fas antibody were used as a positive control. Values represent mean \pm SEM from 8–10 mice with significant difference between WT or MPO KO mice and positive control (** $p < 0.01$). (b) DNA extracted from freshly isolated cells (0 h) or cells incubated *in vitro* for 5 h (5 h). Cells were isolated from BALF of WT mice treated for 36 h by intranasal application of LPS (0.3 mg/kg). UV-irradiated BALF cells were used as a positive control.

white blood cell counts and differentials similar to the wild-type animals [17], which was confirmed in our study in both controls and during the course of acute lung inflammation. Similarly, other authors have not found any significant differences in the numbers of leukocytes and neutrophils between the wild-type and the MPO deficient mice in various inflammatory models [8, 15, 30]. Further, we did not observe any differences in the activation status of neutrophils determined based on the surface expression of the CD11b among the wild-type and the MPO deficient mice of either neutrophils in peripheral circulation or neutrophils obtained from lungs. The expression of CD11b was selected as a sensitive marker of neutrophil activation and also an important player of the neutrophil interaction with endothelium responsible for the neutrophil extravasation into the site of inflammation [31]. Further, the neutrophil influx into the lungs is affected by the permeability of the lung epithelium, which was determined by total protein concentration in BALF, primarily reflecting the leakage of albumin from lung capillaries of peripheral circulation into the alveolar space. However, this parameter did not differ significantly between the wild-type and the MPO deficient mice. In contrast, BALF protein levels in the model of transplantation induced lung inflammation were significantly higher in the MPO deficient compared with the wild-type mice [15].

The key role in the induction of neutrophil extravasation into the site of injury is played by proinflammatory cytokines, particularly with chemotactic potential. Corresponding to the course of inflammatory process, BALF levels of the proinflammatory cytokines were significantly increased after LPS

instillation including two potent chemoattractants. MCP-5 increased later after LPS instillation but did not differ between the MPO deficient and the wild-type mice. In contrast, levels of RANTES were higher in BALF of the MPO deficient mice. RANTES is not a primary chemokine responsible for the extravasation of neutrophils at the site of inflammation; nevertheless, the correlation between levels of RANTES in lung lavage fluid and lung neutrophilia was presented also by the authors Lee et al. [32]. Thus, differences in levels of chemotactic RANTES could potentially contribute to an increased extravasation of neutrophils from blood periphery to the lungs and contribute to observed higher neutrophilia in the MPO deficient mice. Interestingly, authors from laboratory of Dr. Aratani reported that in model of zymosan induced lung inflammation the lavage from the MPO deficient mice contained significantly higher levels of macrophage inflammatory protein-2 (MIP-2) [28]. In their next study employing model of lung inflammation induced by nonviable *Candida albicans*, these authors showed that the MPO deficient mice had significantly increased production of MIP-2 and keratinocytes derived chemokine relative to the wild-type mice [12]. Furthermore, the MPO deficient mice had even significantly higher BALF concentrations of TNF- α and IL-1 β than the wild-type mice [12]. Exploring the source of MIP-2, these authors showed that the MPO deficient neutrophils produce greater amount of MIP-2 *in vitro* than do the wild-type neutrophils when stimulated with zymosan or *Candida albicans* [12, 28, 33]. Interestingly, the MIP-2 production was reduced when MPO was added to the MPO deficient neutrophils exogenously. These authors

speculate that both the lack of hypochlorous acid and the accumulation of H_2O_2 due to MPO deficiency contribute to the upregulation of MIP-2 production in the mouse MPO deficient neutrophils. Similarly, in the model of transplantation induced lung inflammation the MPO deficient mice exhibited higher levels of $TNF-\alpha$ and the chemoattractant MCP-1 compared to the wild-type mice [15]. The importance of this phenomenon of higher levels of chemotactic factors in lungs of the MPO deficient mice for the observed lung neutrophilia was shown by Homme et al. when the neutralization of MIP-2 *in vivo* significantly reduced neutrophil infiltration [12]. In context of our study, the presence of higher levels of RANTES could increase extravasation of neutrophils into the lungs and increased survival of neutrophils in lungs.

Another key factor affecting the accumulation of inflammatory cells in the inflamed lungs is the clearance of these cells from alveolar space. Thus, induction of regulated neutrophil cell death, apoptosis, is a critical event in the downregulation and resolution of inflammation [34, 35]. Therefore, the presence of dying cells in BALF from the MPO deficient and the wild-type mice was assessed by various methods including Annexin V staining, caspase 3 activity determination, and the DNA fragmentation. During apoptosis, PS residues on the inner leaflet of the cell membrane are externalized, providing a marker that can be detected by binding of Annexin V. Interestingly, significantly fewer cells positive for Annexin V and a delay in onset of PS externalization and cell permeability after *ex vivo* incubation were detected in BALF cells isolated from the MPO deficient mice. Substantial delay of the induction of cell death in the MPO deficient neutrophils strongly suggests possible mechanism for increased neutrophilia in the MPO deficient mice based on the accumulation of the MPO deficient neutrophils in the inflamed lungs. Interestingly, the defect of apoptotic induction in neutrophils from the MPO deficient mice was already suggested by other authors as well. Tsurubuchi et al. showed that the MPO deficient neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA) or H_2O_2 underwent apoptosis significantly slower compared to normal neutrophils during interval up to 3 h of incubation [36]. However, in their other study employing zymosan induced lung inflammation they did not observe this phenomenon since they did not find an obvious difference in cell death between the wild-type and the MPO deficient neutrophils cultured for 6 h in the presence of zymosan [28]. Another model of the transplantation induced lung injury resulted in the increased number of inflammatory cells in BALF from the MPO deficient mice that was associated with suppressed apoptosis of BALF inflammatory cells [15].

Interestingly, in our study the observed neutrophil cell death is challenging to classify as conventional apoptosis since the dying neutrophils showed only one type of apoptotic marker, the expression of PS. Other markers of apoptosis, the caspase 3 activity, and DNA fragmentation were not present. In general, it can be suggested that the type of neutrophil cell death depends on the way of neutrophil activation. Interestingly, similar to our results Aratani and colleagues observed delayed PS externalization in PMA activated neutrophils from the MPO deficient mice that was not associated with

caspase 3 activation [36, 37]. Moreover, Fadeel et al. showed the caspases activations in neutrophils treated by Fas ligand; however, it was absent in neutrophils stimulated with PMA [38]. In agreement with our results, DNA fragmentation was not observed in neutrophils incubated for 6 h alone or stimulated with PMA [39]. The PS surface exposure is known as one of the earliest markers of apoptosis and it precedes the morphologic appearances of apoptosis and changes in membrane permeability and the characteristic DNA fragmentation. Importantly, the PS exposure is a key mechanism by which apoptotic cells are recognized by macrophages, targeted for ingestion, and clearance from lungs [34, 35]. These results indicate that MPO probably participates in process of regulated cell death and contributes to clearance of neutrophils from lungs and reduction of airway neutrophilia. Taking into account our previous observations suggesting that MPO potentiate neutrophils to extravasate into site of inflammation in various other tissues, such as livers [27], we assume that in this case the higher number of neutrophils in the MPO deficient mice is not associated with the higher extent of LPS-induced neutrophil influx into the lungs. In contrast, we suggest that MPO deficiency is responsible for the suppressed cell death of lung infiltrating neutrophils.

Interestingly, the MPO effect on neutrophil presence in the lungs can be both dependent on and independent of MPO enzymatic activity. MPO enzymatic activity would be crucial also in the case of direct contribution of MPO as a cytotoxic agent that contributes to the induction of the death of cells accumulated in lungs. We can speculate that MPO enzymatic activity can decrease the formation of chemotactic mediators, as suggested by Tateno et al. [33]. Further, cytotoxic potential of MPO derived intermediates is also suggested to be important for the observed different sensitivity of MPO deficient neutrophils to apoptosis compared to the neutrophils of wild-type animals. MPO deficiency is suggested to be connected with suppressed regulated cell death in neutrophils by Milla et al. [15]. Furthermore, delayed PS externalization in PMA stimulated leukocytes from MPO deficient mice compared to leukocytes from wild-type mice was observed by Tsurubuchi et al. [36]. Likewise, pretreatment of neutrophils with the MPO inhibitor 4-ABAH blocked apoptosis induced by coactivation of neutrophils by $TNF-\alpha$ and H_2O_2 [40]. On the other hand, current data presented by Metzler et al. also suggest the importance of MPO independent of enzymatic activity in the specific regulated cell death NETosis [41]. Based on their observations, MPO is required independently of enzymatic activity for the release of proteases across intact membranes, which is key for the activation of specific proteases during NETosis. However, the importance of this newly suggested function of MPO independent of enzymatic activity has to be proven also in other types of regulated cell death that are relevant for the clearance of neutrophils from inflamed lungs.

In conclusion, these results suggest that neutrophil-derived MPO may play an important role in regulating the course of pulmonary inflammation, independent of its putative microbicidal functions. Because the MPO deficient neutrophils undergo delayed apoptosis *in vitro*, it is possible

that these neutrophils remain alive longer at sites of inflammation. As a result, they would continue to release various ROS, inflammatory cytokines, and cytotoxic enzymes for a longer time, eventually resulting in tissue damage. However, further work evaluating the time course of appearance of apoptotic neutrophils is required to confirm the role of MPO in regulated cell death and to determine whether such defective functions of neutrophils are involved in the pathology of various inflammatory conditions. Knowledge gained from this research will help to determine more extensively the biological functions of MPO in inflammatory human lung disease and will aid in the development of potential pharmacological treatments for both acute and chronic lung injury.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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References

- [1] B. D. Levy and C. N. Serhan, "Resolution of acute inflammation in the lung," *Annual Review of Physiology*, vol. 76, pp. 467–492, 2014.
- [2] R. G. Khadaroo and J. C. Marshall, "ARDS and the multiple organ dysfunction syndrome: common mechanisms of a common systemic process," *Critical Care Clinics*, vol. 18, no. 1, pp. 127–141, 2002.
- [3] J. Cohen, "The immunopathogenesis of sepsis," *Nature*, vol. 420, no. 6917, pp. 885–891, 2002.
- [4] J. P. Burns, "Septic shock in the pediatric patient: pathogenesis and novel treatments," *Pediatric Emergency Care*, vol. 19, no. 2, pp. 112–115, 2003.
- [5] S. J. Klebanoff, A. J. Kettle, H. Rosen, C. C. Winterbourn, and W. M. Nauseef, "Myeloperoxidase: a front-line defender against phagocytosed microorganisms," *Journal of Leukocyte Biology*, vol. 93, no. 2, pp. 185–198, 2013.
- [6] W. M. Nauseef, "Myeloperoxidase in human neutrophil host defence," *Cellular Microbiology*, vol. 16, no. 8, pp. 1146–1155, 2014.
- [7] C. Nussbaum, A. Klinke, M. Adam, S. Baldus, and M. Sperandio, "Myeloperoxidase: a leukocyte-derived protagonist of inflammation and cardiovascular disease," *Antioxidants and Redox Signaling*, vol. 18, no. 6, pp. 692–713, 2013.
- [8] L. Kubala, K. R. Schmelzer, A. Klinke et al., "Modulation of arachidonic and linoleic acid metabolites in myeloperoxidase-deficient mice during acute inflammation," *Free Radical Biology and Medicine*, vol. 48, no. 10, pp. 1311–1320, 2010.
- [9] J. Arnhold and J. Flemmig, "Human myeloperoxidase in innate and acquired immunity," *Archives of Biochemistry and Biophysics*, vol. 500, no. 1, pp. 92–106, 2010.
- [10] L. Kubala, H. Kolářová, J. Vítěček et al., "The potentiation of myeloperoxidase activity by the glycosaminoglycan-dependent binding of myeloperoxidase to proteins of the extracellular matrix," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1830, no. 10, pp. 4524–4536, 2013.
- [11] Y. Aratani, F. Kura, H. Watanabe et al., "Differential host susceptibility to pulmonary infections with bacteria and fungi in mice deficient in myeloperoxidase," *Journal of Infectious Diseases*, vol. 182, no. 4, pp. 1276–1279, 2000.
- [12] M. Homme, N. Tateno, N. Miura, N. Ohno, and Y. Aratani, "Myeloperoxidase deficiency in mice exacerbates lung inflammation induced by nonviable *Candida albicans*," *Inflammation Research*, vol. 62, no. 11, pp. 981–990, 2013.
- [13] M.-L. Brennan, A. Gaur, A. Pahuja, A. J. Lusis, and W. F. Reynolds, "Mice lacking myeloperoxidase are more susceptible to experimental autoimmune encephalomyelitis," *Journal of Neuroimmunology*, vol. 112, no. 1-2, pp. 97–105, 2001.
- [14] S. Takizawa, Y. Aratani, N. Fukuyama et al., "Deficiency of myeloperoxidase increases infarct volume and nitrotyrosine formation in mouse brain," *Journal of Cerebral Blood Flow and Metabolism*, vol. 22, no. 1, pp. 50–54, 2002.
- [15] C. Milla, S. Yang, D. N. Cornfield et al., "Myeloperoxidase deficiency enhances inflammation after allogeneic marrow transplantation," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 287, no. 4, pp. L706–L714, 2004.
- [16] J. Komatsu, H. Koyama, N. Maeda, and Y. Aratani, "Earlier onset of neutrophil-mediated inflammation in the ultraviolet-exposed skin of mice deficient in myeloperoxidase and NADPH oxidase," *Inflammation Research*, vol. 55, no. 5, pp. 200–206, 2006.
- [17] M.-L. Brennan, M. M. Anderson, D. M. Shih et al., "Increased atherosclerosis in myeloperoxidase-deficient mice," *The Journal of Clinical Investigation*, vol. 107, no. 4, pp. 419–430, 2001.
- [18] D. Odobasic, A. R. Kitching, Y. Yang et al., "Neutrophil myeloperoxidase regulates T-cell-driven tissue inflammation in mice by inhibiting dendritic cell function," *Blood*, vol. 121, no. 20, pp. 4195–4204, 2013.
- [19] D. Odobasic, A. R. Kitching, T. J. Semple, and S. R. Holdsworth, "Endogenous myeloperoxidase promotes neutrophil-mediated renal injury, but attenuates T cell immunity inducing crescentic glomerulonephritis," *Journal of the American Society of Nephrology*, vol. 18, no. 3, pp. 760–770, 2007.
- [20] D. Odobasic, R. C. Muljadi, K. M. O'Sullivan et al., "Suppression of autoimmunity and renal disease in pristane-induced lupus by myeloperoxidase," *Arthritis & Rheumatology*, vol. 67, no. 7, pp. 1868–1880, 2015.
- [21] T. Okamoto, K. Gohil, E. I. Finkelstein, P. Bove, T. Akaike, and A. Van Der Vliet, "Multiple contributing roles for NOS₂ in LPS-induced acute airway inflammation in mice," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 286, no. 1, pp. L198–L209, 2004.
- [22] M. E. Poynter, C. G. Irvin, and Y. M. W. Janssen-Heininger, "A prominent role for airway epithelial NF- κ B activation in

- lipopolysaccharide-induced airway inflammation,” *The Journal of Immunology*, vol. 170, no. 12, pp. 6257–6265, 2003.
- [23] D. Viackova, M. Pekarova, T. Crhak et al., “Redox-sensitive regulation of macrophage-inducible nitric oxide synthase expression in vitro does not correlate with the failure of apocynin to prevent lung inflammation induced by endotoxin,” *Immunobiology*, vol. 216, no. 4, pp. 457–465, 2011.
- [24] H. Kolarova, A. Klinke, S. Kremserova et al., “Myeloperoxidase induces the priming of platelets,” *Free Radical Biology and Medicine*, vol. 61, pp. 357–369, 2013.
- [25] A. Klinke, A. Möller, M. Pekarova et al., “Protective effects of 10-nitro-oleic acid in a hypoxia-induced murine model of pulmonary hypertension,” *American Journal of Respiratory Cell and Molecular Biology*, vol. 51, no. 1, pp. 155–162, 2014.
- [26] L. Sviháková-Sindlerová, V. Foltinová, A. Vaculová et al., “LA-12 overcomes confluence-dependent resistance of HT-29 colon cancer cells to Pt (II) compounds,” *Anticancer Research*, vol. 30, no. 4, pp. 1183–1188, 2010.
- [27] A. Klinke, C. Nussbaum, L. Kubala et al., “Myeloperoxidase attracts neutrophils by physical forces,” *Blood*, vol. 117, no. 4, pp. 1350–1358, 2011.
- [28] K. Takeuchi, Y. Umeki, N. Matsumoto et al., “Severe neutrophil-mediated lung inflammation in myeloperoxidase-deficient mice exposed to zymosan,” *Inflammation Research*, vol. 61, no. 3, pp. 197–205, 2012.
- [29] A. Haegens, P. Heeringa, R. J. van Suylen et al., “Myeloperoxidase deficiency attenuates lipopolysaccharide-induced acute lung inflammation and subsequent cytokine and chemokine production,” *The Journal of Immunology*, vol. 182, no. 12, pp. 7990–7996, 2009.
- [30] M.-L. Brennan, W. Wu, X. Fu et al., “A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species,” *Journal of Biological Chemistry*, vol. 277, no. 20, pp. 17415–17427, 2002.
- [31] E. Kolaczowska and P. Kubes, “Neutrophil recruitment and function in health and inflammation,” *Nature Reviews Immunology*, vol. 13, no. 3, pp. 159–175, 2013.
- [32] C. S. Lee, E. H. Yi, J.-K. Lee et al., “Simvastatin suppresses RANTES-mediated neutrophilia in polyinosinic-polycytidylic acid-induced pneumonia,” *European Respiratory Journal*, vol. 41, no. 5, pp. 1147–1156, 2013.
- [33] N. Tateno, N. Matsumoto, T. Motowaki, K. Suzuki, and Y. Aratani, “Myeloperoxidase deficiency induces MIP-2 production via ERK activation in zymosan-stimulated mouse neutrophils,” *Free Radical Research*, vol. 47, no. 5, pp. 376–385, 2013.
- [34] C. Haslett, “Granulocyte apoptosis and its role in the resolution and control of lung inflammation,” *American Journal of Respiratory and Critical Care Medicine*, vol. 160, no. 5, part 2, pp. S5–S11, 1999.
- [35] T. R. Martin, M. Nakamura, and G. Matute-Bello, “The role of apoptosis in acute lung injury,” *Critical Care Medicine*, vol. 31, no. 4, pp. S184–S188, 2003.
- [36] T. Tsurubuchi, Y. Aratani, N. Maeda, and H. Koyama, “Retardation of early-onset PMA-induced apoptosis in mouse neutrophils deficient in myeloperoxidase,” *Journal of Leukocyte Biology*, vol. 70, no. 1, pp. 52–58, 2001.
- [37] T. Saito, H. Takahashi, H. Doken, H. Koyama, and Y. Aratani, “Phorbol myristate acetate induces neutrophil death through activation of p38 mitogen-activated protein kinase that requires endogenous reactive oxygen species other than HOCl,” *Bio-science, Biotechnology and Biochemistry*, vol. 69, no. 11, pp. 2207–2212, 2005.
- [38] B. Fadeel, A. Åhlin, J.-I. Henter, S. Orrenius, and M. B. Hampton, “Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species,” *Blood*, vol. 92, no. 12, pp. 4808–4818, 1998.
- [39] H. Takei, A. Araki, H. Watanabe, A. Ichinose, and F. Sendo, “Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis,” *Journal of Leukocyte Biology*, vol. 59, no. 2, pp. 229–240, 1996.
- [40] A. Kanayama and Y. Miyamoto, “Apoptosis triggered by phagocytosis-related oxidative stress through FLIPS down-regulation and JNK activation,” *Journal of Leukocyte Biology*, vol. 82, no. 5, pp. 1344–1352, 2007.
- [41] K. D. Metzler, C. Goosmann, A. Lubojemska, A. Zychlinsky, and V. Papayannopoulos, “A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis,” *Cell Reports*, vol. 8, no. 3, pp. 883–896, 2014.

Research Article

Protective Effect of Decursin Extracted from *Angelica gigas* in Male Infertility via Nrf2/HO-1 Signaling Pathway

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Higher testicular temperature results in altered spermatogenesis due to heat-related oxidative stress. We examined the effects of decursin extracted from *Angelica gigas* Nakai on antioxidant activity *in vitro* and in a cryptorchidism-induced infertility rat model. TM3 Leydig cell viability was measured based on oxidative stress according to treatment. Either distilled water or AG 400 mg/kg of *A. gigas* extract was administered orally for 4 weeks after unilateral cryptorchidism was induced. After 1, 2, and 4 weeks, six rats from the control group and six rats from treatment group were sacrificed. Testicular weight, semen quality, antioxidant activities, nuclear factor erythroid 2-related factor 2 (Nrf2) protein, and mRNA expression of Nrf2-regulated genes were analyzed. Treatment with *A. gigas* extract (1) protected TM3 cells against oxidative stress in a dose-dependent manner, (2) improved the mean weight of the cryptorchid testis, (3) maintained sperm counts, motility, and spermatogenic cell density, (4) decreased levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and increased levels of superoxide dismutase (SOD), (5) significantly increased Nrf2 and heme oxygenase-1 (HO-1), and (6) significantly decreased apoptosis. This study suggests that decursin extracted from *A. gigas* is a supplemental agent that can reduce oxidative stress by Nrf2-mediated upregulation of HO-1 in rat experimentally induced unilateral cryptorchidism and may improve cryptorchidism-induced infertility.

1. Introduction

Infertility, which can be explained as the failure of a couple in trying to conceive after one year of frequent, unprotected sexual intercourse, is a serious clinical issue that affects 13–15% of couples worldwide [1]. Male infertility is responsible for 60% of cases involving conceptive couples with pregnancy-related problems [2]. Sperm is produced by the highly complicated process of spermatogenesis, and partial or complete interruption of spermatogenesis ultimately leads to oligospermia or azoospermia.

Observation study on male infertility with oligozoospermia or azoospermia, in particular, suggests that some patients

may have testicular heat exposure due to an intrinsic defect in scrotal thermoregulation, varicocele, or work hazard [3]. Several studies report that testicular hyperthermia above normal ranges causes impaired spermatogenesis due to heat-related oxidative stress on the seminiferous tubules [4, 5]. Moreover, nuclear factor erythroid 2-related factor 2 (Nrf2) plays a significant role in preventing the development of oxidative stress in spermatogenesis [6]. Yu et al. [7] also demonstrated a strong correlation between functional discrepancy in Nrf2 promoter gene and abnormal spermatogenesis in humans.

Decursin, a major active ingredient from *Angelica gigas* Nakai (Apiaceae), has been reported to inhibit the growth of various cancer cells through cell cycle arrest and apoptosis

[8, 9]. In addition, a protective effect of decursin has been suggested against the neurotoxicity in animal cortical cells [10]. Even further, decursin plays a major role as free radical scavenger and activated the upregulation of heme oxygenase-1 (HO-1) expression through stimulation of Nrf2, conferring protection against oxidative damage in rat pheochromocytoma (PC12) cells [11].

We examined the effects of decursin extracted from *Angelica gigas* on antioxidant activity *in vitro* and in a cryptorchidism-induced infertility rat model. We hypothesized that decursin-induced HO-1 expression would protect against heat stress-induced degeneration of testicular germ cells and apoptosis.

2. Methods

2.1. Preparation of *Angelica gigas* Extract and Characterization of Decursin. The extract of *Angelica gigas* used in our study was produced using the following method: commercial *Angelica gigas* roots were extracted with 12,000 mL of 30% ethanol for 3 hours at 90–100°C. The extracts were filtered twice through a 50 μm and a 1 μm filter and concentrated in vacuo and then lyophilized. Decursin in the *Angelica gigas* extract was analyzed and quantified by high performance liquid chromatography (HPLC) using Waters 2695 Preparation Module HPLC system (Waters Corporation, MA, USA). Several peaks were obtained in the HPLC chromatogram by diode array detection (DAD) at 230 nm. The major peak was identified as decursin by comparison with the standard compound (Figure 1). As a result of this assay, decursin content was quantified as 37.6 ± 2.2 mg/g.

2.2. Cell Viability Test In Vitro. TM3 cells, an immature mouse Leydig cell line (Korean Cell Line Bank, Seoul, Korea), were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (GIBCO, Life Technologies Co., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) at 37°C. Cells (75,000 cells/mL medium/well) were plated in 24-well cluster dishes, unless otherwise specified. They were seeded on 96-well plates in 10% FBS/DMEM/F-12 and bred for 24 hours. They were pretreated with the *Angelica gigas* extract for two hours and treated with 40 μM hydrogen peroxide (H_2O_2) for two hours to create oxidative cellular stress. Afterwards, alamarBlue (Invitrogen, USA) was added to the cells, and the intensity of the presented color was measured at 570 nm using ELISA Reader (Molecular Devices, USA) after incubating for 3 hours. Cell viability was calculated as previously described [12].

2.3. Animal Groups and Treatment Protocol. This study was investigated in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee in School of Medicine, The Catholic University of Korea (CUMC-2012-0168-01). Thirty-six 8-week-old male Sprague-Dawley rats were treated under an approved

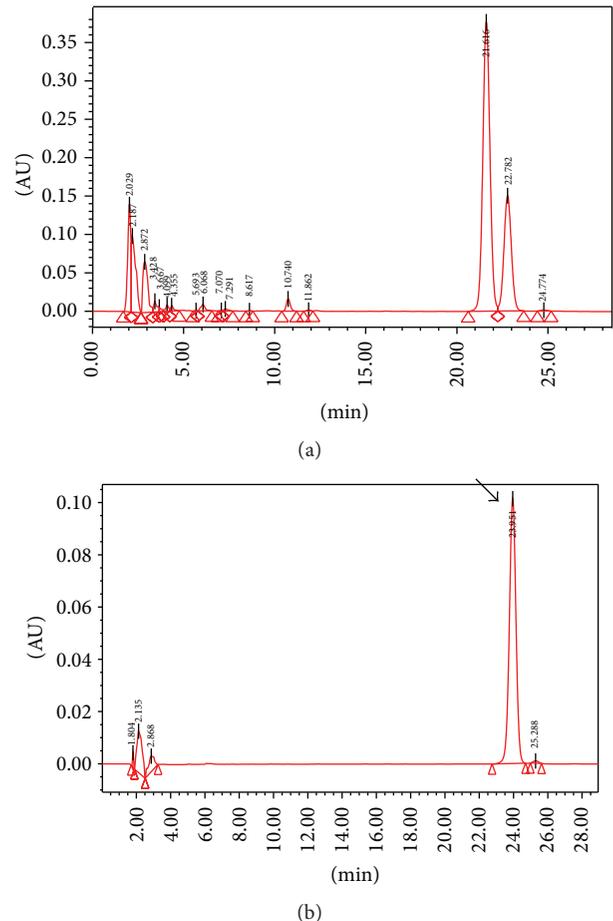


FIGURE 1: HPLC chromatogram of the extract of *Angelica gigas* (a) and the standard solution (b). The peak with the arrow indicates decursin in standard compounds. A corresponding peak was seen in the extract HPLC chromatogram.

protocol. Unilateral cryptorchidism in rats was surgically induced as previously described [13]. Animals were anesthetized, and a midline abdominal incision was performed. The gubernaculum on the left side was cut to displace the testis into the abdomen, and the inguinal canal was closed. The contralateral testis was sham-operated to act as the paired control; the gubernaculum was cut and the inguinal canal reconstructed. Either distilled water ($n = 18$, control groups) or 400 mg/kg of *Angelica gigas* extract ($n = 18$, treatment groups) was administered orally for 4 weeks after unilateral cryptorchidism was induced. *Angelica gigas* extract was dissolved in distilled water and administered orally once a day. After 1, 2, and 4 weeks, six rats from the control group and six rats from treatment group were sacrificed and blood samples were collected. The testes and epididymides were resected after anesthesia and measured.

2.4. Evaluation of Cauda Epididymal Sperm Count and Motility. Samples of spermatozoa were collected from the caudal region of epididymis by mincing it finely in normal saline containing 0.5% bovine serum albumin at 37°C and then

TABLE 1: Primers used for real-time PCR.

Gene	GenBank accession number	Sequence (5' → 3')	Length of DNA product (bp)
Beta-actin (ACTB)	NM_007393	F: CTGTCCCTGTATGCCTCTG R: ATGTCACGCACGATTTC	218
Nuclear factor erythroid 2-related factor 2	NM_010902	F: CAGTGCTCCTATGCGTGAA R: GCGGCTTGAATGTTTGTC	109
Heme oxygenase-1	NM_010442	F: ACAGATGGCGTCACTTCG R: TGAGGACCCACTGGAGGA	128

were filtered. Sperm suspensions were analyzed as previously described [14]. The sperm count represents the number of sperms in 1 mL of the medium. Sperm motility is expressed as the percentage of sperm that showed any movement.

2.5. Measurement of Spermatogenic Cell Density. Testicular tissues procured were fixed, embedded in paraffin, stained with haematoxylin-eosin, and inspected under a light microscope at $\times 400$ magnification. Ten characteristic sites in seminiferous tubules were selected randomly and spermatogenic cell density was measured. It was calculated as the diameter of germinal cell layer divided by the width of the seminiferous tubule [15].

2.6. Measurement of Oxidative Stress. Oxidative stress was assessed by measuring the 8-hydroxy-2-deoxyguanosine (8-OHdG) content and superoxide dismutase (SOD) activity quantitatively. Total DNA was extracted from the testis using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). The level of 8-OHdG was measured with a DNA oxidation kit (Highly Sensitive 8-OHdG Check ELISA; Japan Institute for the Control of Aging, Fukuroi, Japan). After the final color was developed with the addition of 3,3',5,5'-tetramethylbenzidine, absorbance was measured at 450 nm. Tissue sample concentration was measured from a standard curve and corrected for DNA concentration. SOD activity (CuZnSOD and Mn SOD) in tissue was determined using SOD Assay Kit-WST (Dojindo) and the decrease in the rate of superoxide-mediated reduction of nitroblue tetrazolium monitored at 450 nm using a spectrophotometer.

2.7. Western Blot Analysis. Western blot was performed by the standard method. Equal amounts of proteins were fractionated by SDS-PAGE gel electrophoresis and electrotransferred to Immun-Blot PVDF membrane (0.2 μ M pore size, Bio-Rad). Membranes were blocked overnight at 4°C in Tris-buffered saline (TBS), 0.05% (v/v) Tween-20, 150 mM NaCl, and 5% (w/v) bovine serum albumin (BSA, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by 2 hours of incubation with primary antibody diluted in the same buffer. Immunoblot analysis was carried out using anti-Nrf2 (1/250), anti-HO-1 (1/1000), anti-Bax (1/1000), and anti-Bcl-2 (1:1000) polyclonal antibody (Abcam Co., UK). After washing with TBS-T (TBS, 0.1% Tween 20), the membrane was incubated with anti-rabbit IgG AP-linked secondary antibody and then washed with the same

buffer. The immunoblotted membrane was developed with BCIP/NBT color-developing solution. The blots in the samples were quantified by densitometry analysis using PDQuest software (Version 7.0, Bio-Rad, Hercules, CA, USA).

2.8. Quantitative Real-Time PCR. The frozen testes were homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA, US) to extract mRNA and cDNA synthesis was performed using the SuperScript 3 First-Strand kit (Invitrogen, Carlsbad, CA, US) according to the manufacturer's information. Gene-specific primers were determined based on the corresponding mRNA sequences with Primer Version 5.0 (Table 1). PCR amplification of cDNA was performed in a real-time PCR machine step on plus (Applied Biosystems) with SYBR Green PCR Master Mix (Invitrogen) as indicated: 2 minutes at 50°C for dUTP activation and 10 minutes at 95°C for initial denaturation of cDNA followed by 40 cycles, each consisting of 15 s of denaturation at 95°C and 60 s at 60°C for primer annealing and chain extension.

2.9. Statistical Analysis. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, USA). The data was expressed as mean \pm standard deviation. Statistical significance was analyzed by ANOVA test and $p < 0.05$ was considered to be significant.

3. Results

3.1. Protective Effect against Oxidative Stress in TM3 Cells. To evaluate whether *Angelica gigas* extract could protect TM3 cells against H₂O₂-induced damage, alamarBlue assay was performed. Incubation with H₂O₂ significantly decreased cell viability by 40% compared to untreated cells. Increased cell viability was found in a dose-dependent manner (Figure 2). The viabilities were increased to 140% and 165% by treatment with *Angelica gigas* extract concentrations of 10 μ g/mL and 50 μ g/mL, respectively.

3.2. Body and Testes Weights. There was no significant difference in contralateral testicular weights for four weeks. Upon cryptorchidism induction in the first week, there were also no significant differences in left testicular weights between the control group and the treatment group. However, the mean weight of the left testes from the treatment group was significantly larger compared with the control group on

TABLE 2: Comparisons of parameters of the cryptorchid testicular health.

		Left testicular weight (g)	Sperm count ($\times 10^6$ /g cauda)	% of motile spermatozoa	Spermatogenic cell density
1st week	Control	1.526 \pm 0.225	350.2 \pm 10.9	20.7 \pm 3.3	0.343 \pm 0.016
	AG	1.612 \pm 0.033	360.6 \pm 14.0	22.5 \pm 5.2	0.305 \pm 0.031
2nd week	Control	1.232 \pm 0.165	240.2 \pm 13.6	7.6 \pm 2.3	0.275 \pm 0.043
	AG	1.515 \pm 0.367*	300.8 \pm 12.5*	14.5 \pm 2.7*	0.314 \pm 0.074*
4th week	Control	0.858 \pm 0.285	75.2 \pm 18.2	5.7 \pm 3.1	0.154 \pm 0.028
	AG	1.433 \pm 0.634*	310.5 \pm 14.7*	13.2 \pm 8.2*	0.269 \pm 0.052*

Data show the mean \pm SD AG, the extract of *Angelica gigas* treatment.

* $p < 0.05$ as compared with the control group at the same period of time.

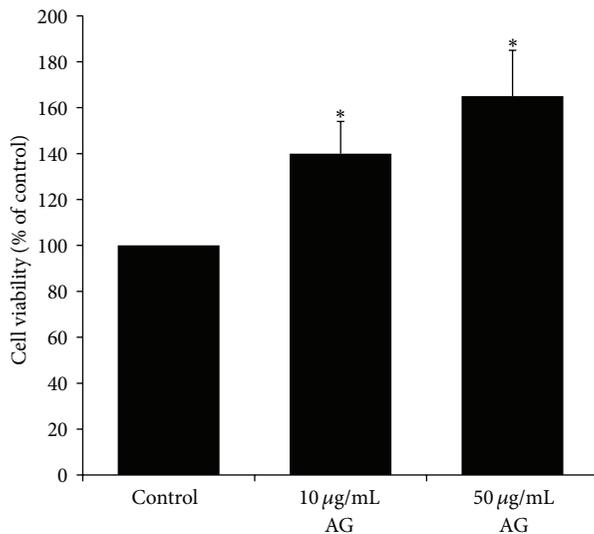


FIGURE 2: Protective effects of *Angelica gigas* extract against H_2O_2 . The cells were pretreated with the extract of *Angelica gigas* 2 hours before H_2O_2 treatment. * $p < 0.05$ as compared with the control group.

the second and fourth weeks ($p < 0.05$). The mean weights of the left testes are listed in Table 2.

3.3. Sperm Counts and Motility. Mean sperm counts and the percentage of motile spermatozoa in the left epididymis are shown in Table 2. There were no significant differences between the control and treatment groups in the first week, but mean sperm counts and the percentage of motile spermatozoa from the control group were significantly lower than those in the treatment group by the second and fourth weeks ($p < 0.05$).

3.4. Spermatogenic Cell Density. Upon cryptorchidism induction in the first week, considerable spermatocytes lined up the germinal cell layer in the control and the treatment groups (Figure 3). On the other hand, the seminiferous tubule is shrunk and the germinal cell layers are decreased in the control group after 2 weeks. The germinal cell layer in the treatment group was thicker compared with that of the control group by the second and fourth weeks (Table 2,

$p < 0.05$). There was no significant difference in contralateral testicular spermatogenic cell density.

3.5. Measurement of Oxidative Stress. Mean expression of oxidative stress markers in the left testes is shown in Figure 4. There were no significant differences in 8-OHdG and SOD expression between the two groups in the first week. A time-dependent increase in 8-OHdG and a decrease in SOD were examined in the control group, but oxidative stress was observed to be significantly lower in the treatment group at the same period of time ($p < 0.05$).

3.6. Expression of Nrf2 and HO-1. We found that significant increases of Nrf2 and HO-1 proteins were exhibited in the treatment group compared with the control group by the second and fourth weeks (Figure 5(a)). There were no significant differences in Nrf2 and HO-1 mRNA in the control group across time points, but mRNA transcript levels were significantly higher in the treatment group compared with the control group by the second and fourth weeks (Figure 5(b)). Moreover, the progressive increase in Nrf2 levels was firmly associated with an increase in HO-1 expression.

3.7. Apoptosis-Related Protein Expression. The expression of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins was investigated. Treatment with *Angelica gigas* extract significantly declined the level of Bax protein with a collateral increase in Bcl-2 protein compared with the control group over the whole period (Figure 6(a)). These changes decreased the Bax/Bcl-2 ratio, which is considered to be an index to evaluate apoptosis (Figure 6(b)).

4. Discussion

The main findings of our study were as follows. The treatment with *Angelica gigas* extract (1) protected TM3 cells against H_2O_2 -induced oxidative stress in a dose-dependent manner, (2) improved the mean weight of the cryptorchid testis, (3) maintained sperm counts, motility, and spermatogenic cell density, (4) decreased levels of 8-OHdG and increased levels of SOD, demonstrating its antioxidant effect, (5) significantly increased Nrf2 and HO-1, and (6) significantly decreased apoptosis.

We evaluated the protective effect of *Angelica gigas* extract against H_2O_2 -induced oxidative stress in TM3 cells.

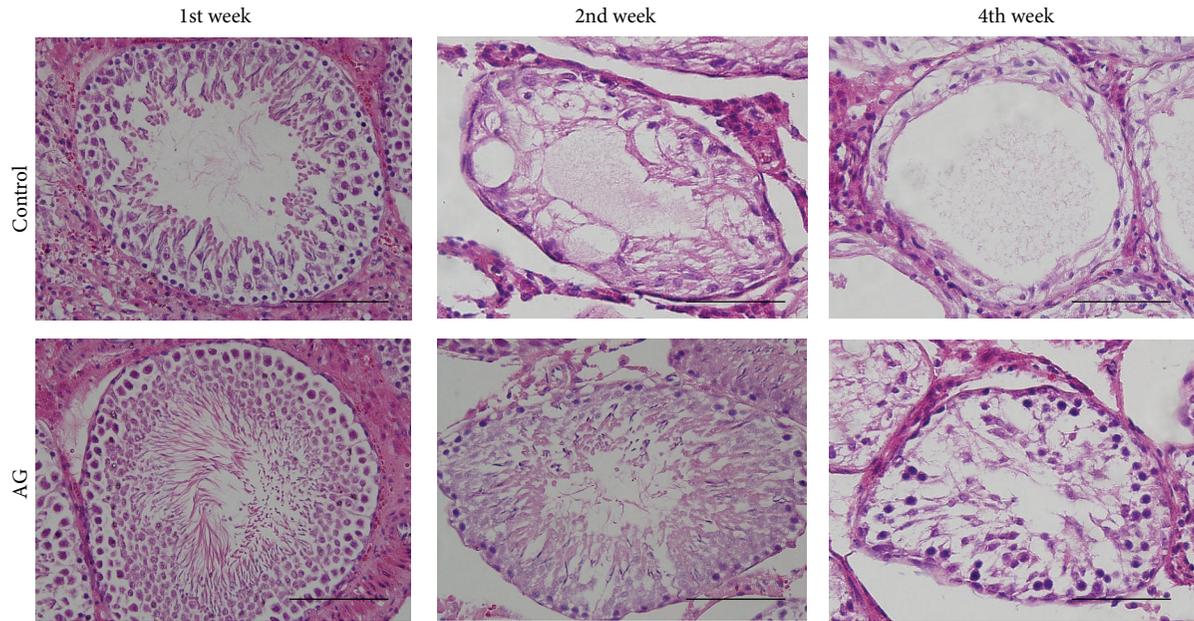


FIGURE 3: Time-dependent histopathological findings of left testis (haematoxylin and eosin stain). Scale bars shown in each figure represent 100 μm . AG, the extract of *Angelica gigas* treatment.

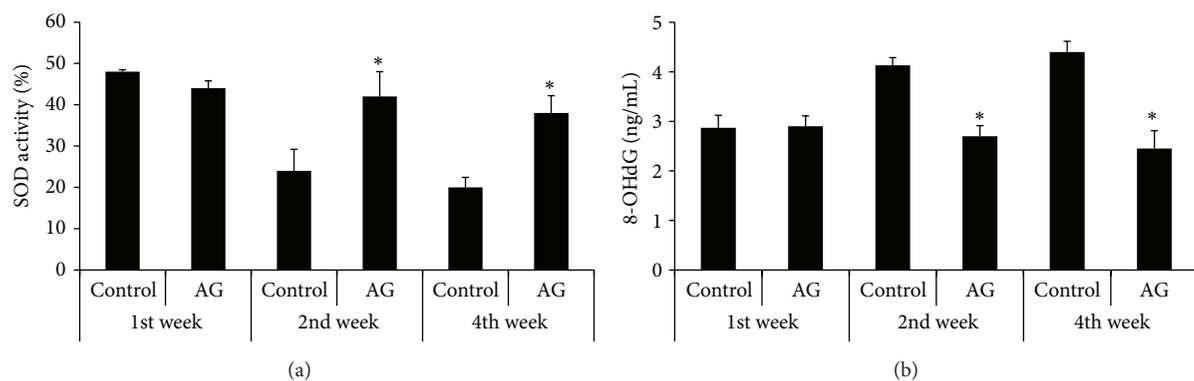


FIGURE 4: Comparison of the expression levels of SOD (a) and 8-OHdG (b). AG, the extract of *Angelica gigas* treatment. * $p < 0.05$ as compared with the control group at the same period of time.

In addition, we demonstrated that the mean weight of the cryptorchid testis in the control group was significantly decreased compared with that of the treatment group by the second and fourth weeks. Most mammal testes are more sensitive to heat than other organs, and scrotal temperature is lower than core body temperature [16, 17]. Elevated intratesticular temperature induces oxidative stress, resulting in apoptosis and impairment of spermatogenesis [18, 19]. It is correlated with a decrease in cellular antioxidant defenses or an increase in the production of reactive oxygen species (ROS) [20]. Several studies demonstrated that increased ROS impaired the physiological processes such as capacitation, hyperactivation, acrosome reactions, and signaling processes to provide appropriate fertilization [21]. Thus, antioxidant supplement may help improve the imbalance of an excessive ROS and restore sperm parameters.

Oral supplements and herbal medicines have been proposed to recover male factor infertility [22]. These supplements were reported to improve sperm quality, and their antioxidants are thought to decrease ROS in oligoasthenospermia patients [23–26]. We found that treatment with *Angelica gigas* extract decreases oxidative stress in cryptorchidism-induced rats and posit that the curative effects may contribute to suppression of ROS production. In addition, we examined the detailed mechanism of the antioxidant activity in *Angelica gigas* extract.

HO-1 is a stress-responsive protein shortly activated by variable noxious stimuli as well as oxidative stress. HO-1 has been known as the cytoprotective effect resistant to oxidative stress [27]. The Nrf2 antioxidant system also has been recognized as an important therapeutic target against oxidative stress, producing expression of cytoprotective enzymes and

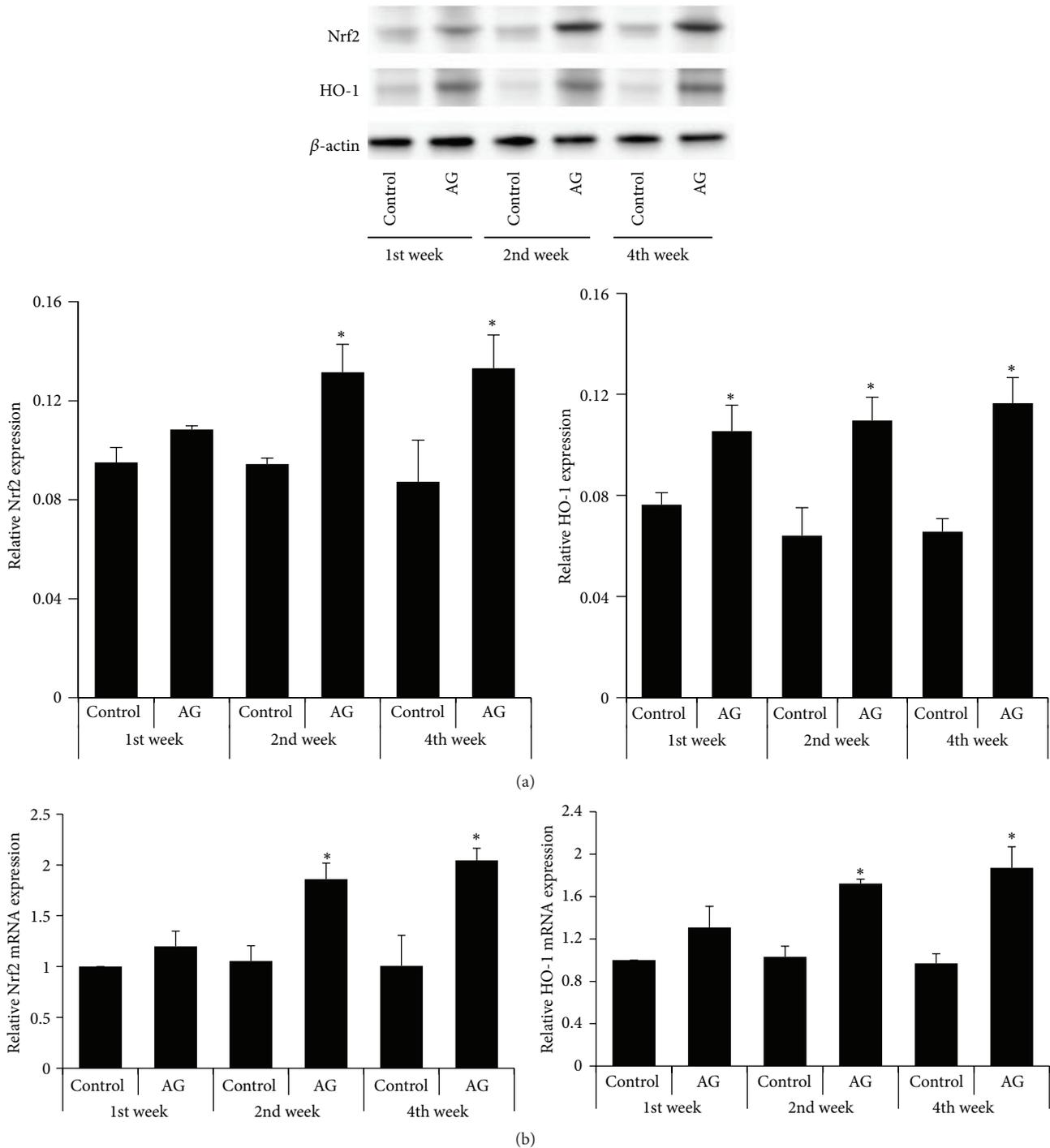


FIGURE 5: Effect of *Angelica gigas* extract on protein expression (a) and mRNA expression (b) of Nrf2-regulated genes. AG, the extract of *Angelica gigas* treatment; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2. * $p < 0.05$ as compared with the control group at the same period of time.

related proteins [28]. Previous studies have reported that oxidative stress resulted in lower epididymal sperm motility in Nrf2 knockout mice [6]. Nrf2 is released and transmits the stress signal to the nucleus for activation of a specific set of genes encoding phase II antioxidant enzymes as well as stress responsive proteins such as HO-1 [29].

Li et al. [30] studied about the oxidative damage on male reproductive organ. The whole body heat stress upregulated Nrf2 expression for about a week, but the significant increased oxidative stress was identified thereafter. Several supplements (e.g., sulforaphane, curcumin, and caffeic acid phenethyl ester) activate the Nrf2 antioxidant response element (ARE)

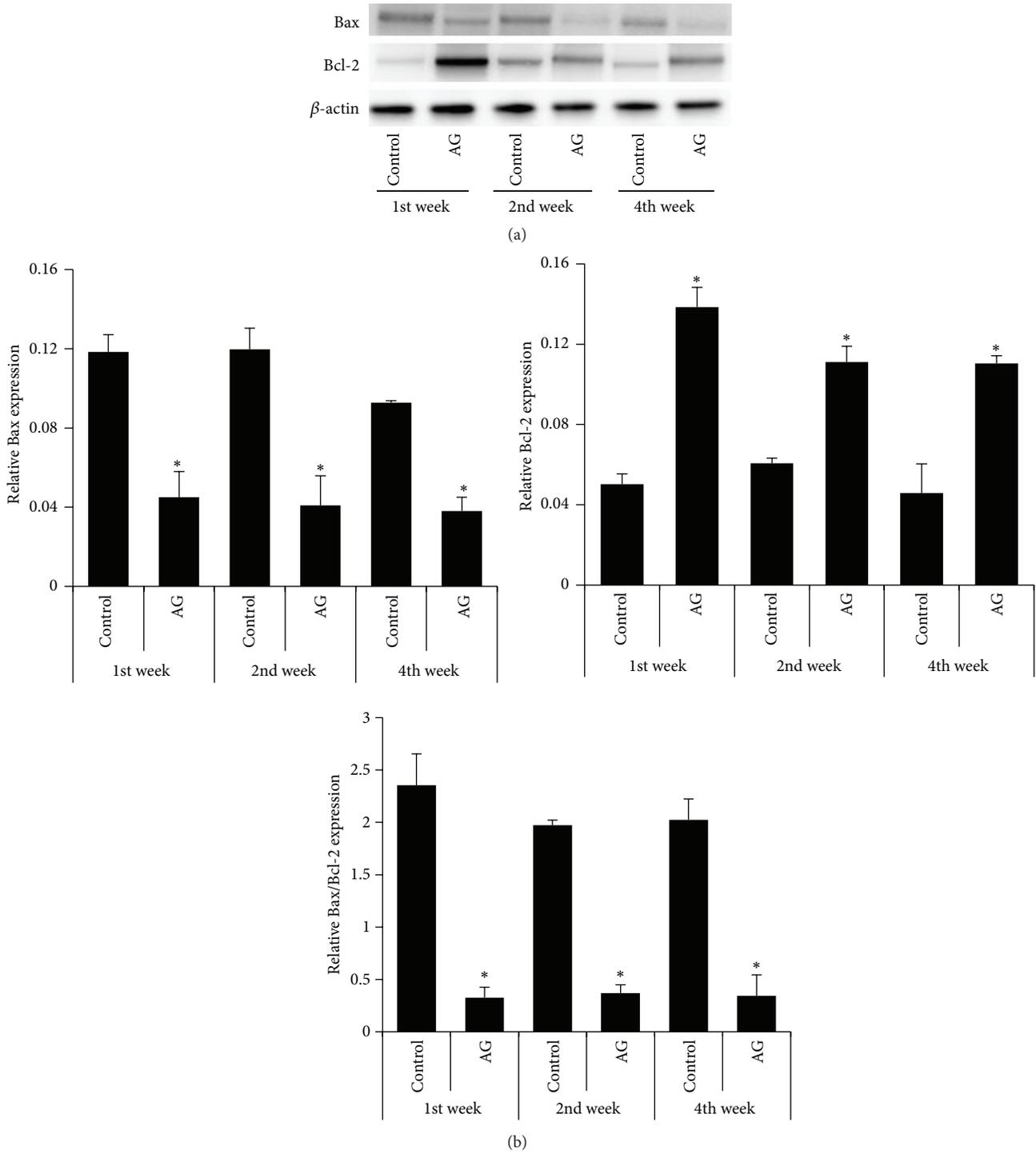


FIGURE 6: Comparison of the expression levels of Bax and Bcl-2 expression in the testicular tissue. (a) Western blot analysis of Bax and Bcl-2. (b) Densitometric analysis of Bax, Bcl-2, and Bax/Bcl-2 ratio relative to beta-actin. AG, the extract of *Angelica gigas* treatment. * $P < 0.05$ as compared with the control group at the same period of time.

system [31, 32]. Li et al. [11] reported that decursin treatment leads to the upregulation of Nrf2/HO-1 expression in PC12 cells. Decursin may have the possibilities to prevent chemotherapy-induced cytotoxicity via the activation

of antioxidant enzyme. We found that decursin extracted from *Angelica gigas* increased Nrf2 and HO-1 in the cryptorchid testis. Moreover, a prevention of decrease in SOD was observed in the treatment group. Therefore, our results

suggest that antioxidant defense is reinforced by decursin via activation of Nrf2 and upregulation of antioxidant enzyme activity.

The Bcl-2 family and related proteins are key regulators of apoptosis [33]. They can be classified into two groups: Bcl-2, an antiapoptotic protein, and Bax, a proapoptotic protein [34]. Knudson et al. [35] demonstrated that the Bcl-2 family mainly plays a role during spermatogenesis in Bax-deficient mice. Several studies have reported the importance of the Bcl-2 family in heat-induced oxidative stress on male reproductive function [36, 37]. They suggested the possible involvement of Bcl-2 and Bax in the germ cell apoptosis. We also investigated the germ cell apoptosis induced by cryptorchidism and the antiapoptotic effect of decursin extracted from *Angelica gigas*.

We examined the role of decursin extracted from *Angelica gigas* as a supplemental agent to prevent heat-induced oxidative stress in cryptorchidism. Our limitation is that the more accurate infertility model due to oxidative stress is needed. More defense mechanisms such as Nrf2 and related antioxidative enzyme are required if oxidative stress causes impaired semen parameters, DNA damage, and apoptosis. Antioxidant supplementation as well as lifestyle change like no smoking or balanced diet can be helpful for the natural balance between ROS and antioxidant. Therefore our study may support appropriate evidence for the use of a new complementary and alternative medicine for treating male infertility. In addition, future work should investigate a detailed mechanism of decursin with or without Nrf2 related enzyme activity.

The present study suggests that decursin extracted from *Angelica gigas* is a supplemental agent that can reduce oxidative stress by Nrf2-mediated upregulation of HO-1 and may improve cryptorchidism-induced infertility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

- [1] J. P. Jarow, I. D. Sharlip, A. M. Belker et al., "Best practice policies for male infertility," *The Journal of Urology*, vol. 167, no. 5, pp. 2138–2144, 2002.
- [2] P. Thonneau, S. Marchand, A. Tallec et al., "Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988-1989)," *Human Reproduction*, vol. 6, no. 6, pp. 811–816, 1991.
- [3] R. Dada, N. P. Gupta, and K. Kucheria, "Spermatogenic arrest in men with testicular hyperthermia," *Teratog Carcinog Mutagen*, supplement 1, pp. 235–243, 2003.
- [4] A. Kumagai, H. Kodama, J. Kumagai et al., "Xanthine oxidase inhibitors suppress testicular germ cell apoptosis induced by experimental cryptorchidism," *Molecular Human Reproduction*, vol. 8, no. 2, pp. 118–123, 2002.
- [5] R. M. Viguera-Villaseñor, I. Ojeda, O. Gutierrez-Pérez et al., "Protective effect of α -tocopherol on damage to rat testes by experimental cryptorchidism," *International Journal of Experimental Pathology*, vol. 92, no. 2, pp. 131–139, 2011.
- [6] B. N. Nakamura, G. Lawson, J. Y. Chan et al., "Knockout of the transcription factor NRF2 disrupts spermatogenesis in an age-dependent manner," *Free Radical Biology and Medicine*, vol. 49, no. 9, pp. 1368–1379, 2010.
- [7] B. Yu, H. Lin, L. Yang et al., "Genetic variation in the Nrf2 promoter associates with defective spermatogenesis in humans," *Journal of Molecular Medicine*, vol. 90, no. 11, pp. 1333–1342, 2012.
- [8] J. Guo, C. Jiang, Z. Wang et al., "A novel class of pyranocoumarin anti-androgen receptor signaling compounds," *Molecular Cancer Therapeutics*, vol. 6, no. 3, pp. 907–917, 2007.
- [9] H. J. Lee, H. J. Lee, E. O. Lee et al., "In vivo anti-cancer activity of Korean *Angelica gigas* and its major pyranocoumarin decursin," *The American Journal of Chinese Medicine*, vol. 37, no. 1, pp. 127–142, 2009.
- [10] S. Y. Kang and Y. C. Kim, "Decursinol and decursin protect primary cultured rat cortical cells from glutamate-induced neurotoxicity," *Journal of Pharmacy and Pharmacology*, vol. 59, no. 6, pp. 863–870, 2007.
- [11] L. Li, J.-K. Du, L.-Y. Zou, T. Wu, Y.-W. Lee, and Y.-H. Kim, "Decursin isolated from *angelica gigas* Nakai rescues PC12 cells from amyloid β -protein-induced neurotoxicity through Nrf2-mediated upregulation of heme oxygenase-1: potential roles of MAPK," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 467245, 14 pages, 2013.
- [12] H. J. Lee, J.-H. Bach, H.-S. Chae et al., "Mitogen-activated protein kinase/extracellular signal-regulated kinase attenuates 3-hydroxykynurenine-induced neuronal cell death," *Journal of Neurochemistry*, vol. 88, no. 3, pp. 647–656, 2004.
- [13] W. J. Bae, U. S. Ha, K. S. Kim et al., "Effects of KH-204 on the expression of heat shock protein 70 and germ cell apoptosis in infertility rat models," *BMC Complementary and Alternative Medicine*, vol. 14, article 367, 2014.
- [14] K. W. Ko, J. Y. Chae, S. W. Kim et al., "The effect of the partial obstruction site of the renal vein on testis and kidney in rats: is the traditional animal model suitable for varicocele research?" *Korean Journal of Urology*, vol. 51, no. 8, pp. 565–571, 2010.
- [15] G. Türk, M. Sönmez, M. Aydin et al., "Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats," *Clinical Nutrition*, vol. 27, no. 2, pp. 289–296, 2008.
- [16] B. P. Setchell, "The Parkes lecture. Heat and the testis," *Journal of Reproduction and Fertility*, vol. 114, no. 2, pp. 179–194, 1998.
- [17] P. J. Hansen, "Effects of heat stress on mammalian reproduction," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 364, no. 1534, pp. 3341–3350, 2009.
- [18] C. Paul, S. Teng, and P. T. K. Saunders, "A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death," *Biology of Reproduction*, vol. 80, no. 5, pp. 913–919, 2009.

- [19] K. Shiraiishi, H. Takihara, and H. Matsuyama, "Elevated scrotal temperature, but not varicocele grade, reflects testicular oxidative stress-mediated apoptosis," *World Journal of Urology*, vol. 28, no. 3, pp. 359–364, 2010.
- [20] R. J. Aitken and S. D. Roman, "Antioxidant systems and oxidative stress in the testes," *Oxidative Medicine and Cellular Longevity*, vol. 1, no. 1, pp. 15–24, 2008.
- [21] A. Agarwal, G. Virk, C. Ong, and S. S. du Plessis, "Effect of oxidative stress on male reproduction," *The World Journal of Men's Health*, vol. 32, no. 1, pp. 1–17, 2014.
- [22] N. A. Clark, M. Will, M. B. Moravek, and S. Fisseha, "A systematic review of the evidence for complementary and alternative medicine in infertility," *International Journal of Gynecology and Obstetrics*, vol. 122, no. 3, pp. 202–206, 2013.
- [23] A. Agarwal and L. H. Sekhon, "The role of antioxidant therapy in the treatment of male infertility," *Human Fertility*, vol. 13, no. 4, pp. 217–225, 2010.
- [24] E. Greco, M. Iacobelli, L. Rienzi, F. Ubaldi, S. Ferrero, and J. Tesarik, "Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment," *Journal of Andrology*, vol. 26, no. 3, pp. 349–353, 2005.
- [25] A. E. Omu, M. K. Al-Azemi, E. O. Kehinde, J. T. Anim, M. A. Oriowo, and T. C. Mathew, "Indications of the mechanisms involved in improved sperm parameters by zinc therapy," *Medical Principles and Practice*, vol. 17, no. 2, pp. 108–116, 2008.
- [26] M. R. Safarinejad, "Efficacy of coenzyme Q10 on semen parameters, sperm function and reproductive hormones in infertile men," *Journal of Urology*, vol. 182, no. 1, pp. 237–248, 2009.
- [27] L. E. Otterbein and A. M. K. Choi, "Heme oxygenase: colors of defense against cellular stress," *American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 279, no. 6, pp. L1029–L1037, 2000.
- [28] T. Ohta, K. Iijima, M. Miyamoto et al., "Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth," *Cancer Research*, vol. 68, no. 5, pp. 1303–1309, 2008.
- [29] K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, T. O'Connor, and M. Yamamoto, "Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles," *Genes to Cells*, vol. 8, no. 4, pp. 379–391, 2003.
- [30] Y. Li, Y. Huang, Y. Piao et al., "Protective effects of nuclear factor erythroid 2-related factor 2 on whole body heat stress-induced oxidative damage in the mouse testis," *Reproductive Biology and Endocrinology*, vol. 11, article 23, 2013.
- [31] C. Chen and A.-N. T. Kong, "Dietary chemopreventive compounds and ARE/EpRE signaling," *Free Radical Biology and Medicine*, vol. 36, no. 12, pp. 1505–1516, 2004.
- [32] Y. Morimitsu, Y. Nakagawa, K. Hayashi et al., "A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway," *The Journal of Biological Chemistry*, vol. 277, no. 5, pp. 3456–3463, 2002.
- [33] J. M. Adams and S. Cory, "The Bcl-2 protein family: arbiters of cell survival," *Science*, vol. 281, no. 5381, pp. 1322–1326, 1998.
- [34] Y. Akao, Y. Otsuki, S. Kataoka, Y. Ito, and Y. Tsujimoto, "Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes," *Cancer Research*, vol. 54, no. 9, pp. 2468–2471, 1994.
- [35] C. M. Knudson, K. S. K. Tung, W. G. Tourtellotte, G. A. J. Brown, and S. J. Korsmeyer, "Bax-deficient mice with lymphoid hyperplasia and male germ cell death," *Science*, vol. 270, no. 5233, pp. 96–99, 1995.
- [36] C. M. Yamamoto, A. P. Sinha Hikim, P. N. Huynh et al., "Redistribution of Bax is an early step in an apoptotic pathway leading to germ cell death in rats, triggered by mild testicular hyperthermia," *Biology of Reproduction*, vol. 63, no. 6, pp. 1683–1690, 2000.
- [37] Z.-H. Zhang, X. Jin, X.-S. Zhang et al., "Bcl-2 and Bax are involved in experimental cryptorchidism-induced testicular germ cell apoptosis in rhesus monkey," *Contraception*, vol. 68, no. 4, pp. 297–301, 2003.

Research Article

Periodic Exposure of Keratinocytes to Cold Physical Plasma: An *In Vitro* Model for Redox-Related Diseases of the Skin

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Oxidative stress illustrates an imbalance between radical formation and removal. Frequent redox stress is critically involved in many human pathologies including cancer, psoriasis, and chronic wounds. However, reactive species pursue a dual role being involved in signaling on the one hand and oxidative damage on the other. Using a HaCaT keratinocyte cell culture model, we investigated redox regulation and inflammation to periodic, low-dose oxidative stress after two, six, eight, ten, and twelve weeks. Chronic redox stress was generated by recurrent incubation with cold physical plasma-treated cell culture medium. Using transcriptome microarray technology, we identified both acute ROS-stress responses as well as numerous adaptations after several weeks of redox challenge. We determined a differential expression (2-fold, $FDR < 0.01$, $p < 0.05$) of 260 genes that function in inflammation and redox homeostasis, such as cytokines (e.g., IL-6, IL-8, and IL-10), growth factors (e.g., CSF2, FGF, and IGF-2), and antioxidant enzymes (e.g., HMOX, NQO1, GPX, and PRDX). Apoptotic signaling was affected rather modestly, especially in p53 downstream targets (e.g., BCL2, BBC3, and GADD45). Strikingly, the cell-protective heat shock protein HSP27 was strongly upregulated ($p < 0.001$). These results suggested cellular adaptations to frequent redox stress and may help to better understand the inflammatory responses in redox-related diseases.

1. Introduction

The skin protects against environmental assaults and is crucial for maintaining the redox homeostasis [1]. The redox state is determined by the balance between pro- and antioxidative processes involving reactive oxygen and nitrogen species (ROS/RNS) [2]. Excessive presence of ROS/RNS is associated with a number of pathologies of the skin. This includes cutaneous malignancies [3], chronic wounds [4], and psoriasis [5]. There were an estimated 3.45 million new cases of cancer and 1.75 million deaths from cancer in Europe in 2012 [6]. The incidence of chronic wounds varies [7] but is expected to affect about 6.5 million patients in the United States [8]. The prevalence of psoriasis ranges from 0% to 8.5% according to age and geographic region [9]. Yet, it is important to recognize the dual role of reactive species, also in the human skin [10]. At low concentrations, they serve as signaling molecules [11] while at higher concentrations they become increasingly cytotoxic [12].

Generally, oxidants can be of exogenous or endogenous origin. The former derive from, for example, ozone, ionizing and nonionizing radiation, cigarette smoke, or invasion of pathogens and their associated infections [13]. The latter spring from oxygen-metabolizing enzymatic reactions (oxidases) and the leakage of superoxide from mitochondria [14]. Upon stimulation, the inducible nitric oxide (NO) synthase contributes to RNS production by generating large amounts of NO for defense or signaling purposes [15], also in keratinocytes [16]. Disturbance of the physiological balance between formation and removal of ROS/RNS (oxidative stress) disrupts the accurate interplay between the affiliated cells [17]. However, most toxic effects are counterbalanced by the complex and finely tuned antioxidative defense system [18].

We here attempted to investigate the redox response of HaCaT keratinocytes over three months. Frequent exposure to reactive species was generated using cold physical plasma. Cold plasma has been shown to have therapeutic benefit in

diseases of the skin, such as chronic wounds and psoriasis [19–21]. The advantage of cold physical plasma over single oxidative agents is that it provides a more complex mixture of reactive molecules. Plasma was used to mimic the frequent redox stress apparent in redox-related diseases on the one hand and to investigate the effect of its long-term use on the other. We hypothesized that the cellular responses would substantially differ in cells exposed to rather acute (up to two weeks) or chronic (up to twelve weeks) oxidative challenge. Understanding these differences may help to better understand the skin tissue response in pathologies that involve chronic redox stress. Several studies have investigated acute oxidative stress conditions in keratinocyte skin cell culture models [22–24]. In contrast to primary skin tissue, *in vitro* cultures obviously allow the investigation of cells with the same genetic background over long investigation times and under highly controlled conditions.

Following repeated redox challenge, we used transcriptome microarray technology to study the molecular mechanisms and to identify markers of cellular redox modulation in keratinocytes. Periodic redox stress was applied over 3 months and coincided with an altered gene expression linked to cell metabolism, inflammation, and general stress response. Our model system was designed to emphasize mild redox conditions rather than applying cytotoxic dosages. We were able to show distinct transcript profiles of cytokines, keratins, and growth factors as well as an imperative involvement of junctional proteins in redox adaptations. Collectively, the presented system-wide modifications suggest that the simultaneous alteration of multiple pathways provides an important in-depth transcriptome overview linking permanent plasma-evoked oxidative stress effects with cell responses and adaptation mechanisms, conceivably having implications in redox-based diseases of the skin.

2. Material and Methods

2.1. Cell Culture and Cold Physical Plasma. Three million epithelial keratinocyte HaCaT cells [25] were cultured in 75 cm² dishes using RPMI1640 cell culture medium supplemented with 8% bovine calf serum (Sigma, USA), 0.1 mg/mL penicillin/streptomycin, and 2 mM L-glutamine (Lonza, Switzerland). Cold physical plasma-treated cell culture medium was generated by exposure of 5 mL of fully supplemented medium to an atmospheric pressure argon plasma jet (60 s; *kINPen 09*; neoplas tools, Germany) [26]. Three standard liters per minute of argon (99.99%; Air Liquide, France) were used as feed gas. The plasma was generated by applying a voltage of 2–6 kV_{pp} with a frequency of 1.0–1.1 MHz to the central electrode. Three million HaCaT keratinocytes were exposed to 15 mL of plasma-treated medium twice a week and passaged once a week (Figure 2(a)). After two, six, eight, ten, and twelve weeks, cells were either subjected to microscopic experiments or RNA or protein isolation.

2.2. Flow Cytometry. Keratinocytes were loaded with 5 μM of CM-H₂DCF-DA or mitotracker deep red (MTDR; both

life technologies, USA) according to the manufacturer's instructions. After exposure to plasma-treated medium, cells were trypsinized and green fluorescence was quantified using a *Gallios* flow cytometer (Beckman-Coulter, USA). For assessment of apoptosis, keratinocytes were plasma-treated and incubated for 18 h at 37°C. Etoposide (50 μM) was used as positive control (BioVision, USA). Subsequently, cells were loaded with active caspase 3 detection reagent (Enzo, USA) according to the vendor's protocol. Keratinocytes were then trypsinized and subjected to flow cytometric evaluation. Data analysis was conducted using *Kaluza* software (Beckman-Coulter).

2.3. Transcriptomic Analysis. RNA was purified according to the manufacturer's instructions (Bio&Sell, Germany) and RNA quality was maximal (scores of 10 out of 10; data not shown) as evaluated using low-volume gel electrophoresis (Bioanalyzer 2100; Agilent, USA). For cRNA generation, RNA of two independent experiments was pooled for each sample. cRNA was conjugated to Cy3, and transcriptional analysis was carried out using microarray-based exon analysis (Agilent) as described before [27]. Briefly, samples were hybridized onto SurePrint G3 custom GE 8 × 60 K chips (OakLabs, Germany) for 17 h at 65°C in a hybridization oven (Agilent). After washing, fluorescence intensities were recorded using a gene chip scanner (Agilent). Background-corrected signal intensities were determined and processed using feature extraction software (Agilent). Normalization, statistical tests, clustering, and filtering methods were conducted using gene expression analysis software (Partek, USA). Microarray data were deposited into gene expression omnibus database (GSE4876). Data were grouped according to their respective treatment regime (2, 6, 8, 10, and 12 weeks) and analyzed statistically using multiple testing corrections to identify differentially expressed genes (at least ±2-fold with $p < 0.05$). Also expression changes of less than ±2-fold are shown if target expression at one or more time points was greater than ±2-fold. Additionally, sets of coregulated genes were functionally clustered, and their biological relevance was analyzed using PANTHER software.

2.4. Protein Analysis. Instead of validating a large number of microarray results, we rather chose protein targets based on their significance within main cellular responses. These included oxidative defense (PRDX2 was among the 333 always differentially expressed genes (Figure 2(c)); HSP27 and its phosphorylated form were previously found to be central, studying the redox response in THP-1 monocytes [28]; NQO1 was also found to be redox-controlled using cold plasma [27]), cellular damage and apoptosis (histone H2AX and its phosphorylation are prominent markers of genomic DNA damage; Puma [29] and p21 [30] are involved in redox-related apoptosis induction), and cellular structure (keratin 1 showed the strongest regulation among all keratins (Table 2); β-actin served as housekeeping protein). For protein isolation, cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (cComplete Mini, phosSTOP; Roche, Germany) and 2 mM phenylmethanesulfonyl fluoride (Roth,

Germany). For western blotting, proteins of cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on precast 10% gels (AbCam, UK) and transferred to PVDF membranes (Roth). Immunoblots of whole protein extracts (20 μ g) were performed with antibodies against Puma and E-cadherin (all Cell Signaling, USA); occludin and ZO-1 (both life technologies, USA); and CTNNB1, β -actin, Hsp27, phospho-Hsp27, PRDX2, H2AX, and phospho-H2AX (all Santa Cruz, USA). Antibody binding was followed by three washing steps and incubation with horseradish peroxidase-coupled secondary antibodies before chemiluminescence (Light Polarix; Serva, Germany) detection (ImageQuant; GE Healthcare, USA). Band intensities were quantified using ImageQuantTL Software (GE Healthcare), normalized to total protein level, and expressed as fold change compared to the corresponding control.

2.5. Cell Migration and Immunofluorescence Microscopy. One million HaCaT keratinocytes were seeded onto 60 mm plastic culture dishes and incubated overnight to permit cell adhesion. Scratches were performed using a 10 μ L pipette tip. Gap closure was followed by time lapse microscopy (Zeiss, Germany) and three gap distances per samples were evaluated at different time points using *Axio Vision* software (Zeiss). For immunofluorescence, HaCaT keratinocytes were grown on glass coverslips for 24 h prior to fixation (4% paraformaldehyde; Sigma) and permeabilization (PBS with 0.1% Triton X-100; Sigma). Samples were subsequently incubated with primary antibodies (1:500; cell signaling technologies, USA) targeted at zona occludens protein 1 (ZO-1) overnight at 4°C. Cells were washed twice and incubated with appropriate Alexa Fluor 488-conjugated secondary antibodies (1:700; life technologies) for 1 h. Coverslips were washed and mounted onto glass microscope slides using mounting medium containing DAPI (VectaShield; Biozol, Germany) prior to analysis using an *Axio Observer Z.1* (Zeiss).

3. Results

3.1. Cold Plasma Induced Redox Changes but Only Modestly Impacted Cell Viability. Plasma generates reactive species of various kinds and we found a fluorescence increase of intracellular DCF indicative of redox stress (Figures 1(a) and 1(b)). Then, we investigated cell viability after single application of plasma-treated medium. In contrast to positive control (etoposide), we identified an only subtle, nonsignificant increase in apoptotic cells receiving plasma-treated medium (Figures 1(c) and 1(d)). Thus, the plasma treatment time (60 s) was mediating a rather nontoxic oxidative challenge to keratinocyte cells.

3.2. HaCaT Keratinocyte Gene Expression Was Affected by Periodic Redox Challenge. Next, we repeatedly exposed HaCaT keratinocytes to cold physical plasma-treated (60 s) cell culture medium over three months to assess its impact on global gene expression (Figure 2(a)). Hierarchical clustering

illustrated the statistically relevant changes in gene expression between oxidatively challenged compared to untreated cells (Figure 2(b)). We detected a total of approximately 3,000 genes with at least ≥ 2 -fold increase or decrease of expression. Venn diagrams were constructed to identify the number of exclusively up- or downregulated genes corresponding to the different sampling time points. Overlapping differences shared among more than one sample group comparison are represented in the areas of intersection between two or more circles. The Venn diagram showed the overlapping genes among the 2,068, 2634, 1602, 1319, and 1052 transcripts differentially expressed between the w2 and w12 groups (Figure 2(c)). The 333 transcripts in the center of the Venn diagram represent genes that are differently expressed among all treatment groups (in contrast to control). A total of 123 genes with ID classification (from these 333 transcripts) were identified using PANTHER software (Supplemental Table 1A) (see Supplementary Material available online at <http://dx.doi.org/10.1155/2016/9816072>) and were further subdivided into several functional and protein classes (Supplemental Table 1B). In total, 411, 1079, 709, 453, and 321 transcripts were upregulated, and 496, 1241, 850, 527, and 391 transcripts were downregulated in our different groups from w2 to w12 and in comparison to untreated keratinocytes, respectively. In comparison to w8–12, the number of differentially expressed genes was higher at the relatively earlier time points (w2 and w6) (Figure 2(d)). The differentially expressed genes with a known function were assigned to gene ontology (GO) and protein classes (Figure 3). Significant GO terms ($p < 0.05$; PANTHER database) were “immune system” (324 genes) and “response to stimulus” (457 genes) (Figures 3(a) and 3(b)). Within the latter, a large number of genes were involved in “stress responses” (134 genes) and “immunological processes” (128 genes). Further, there were a number of genes related to signaling (Figure 3(c)), such as protein kinases, phosphatases, growth factors, cytokines, and chemokines. We also identified a large set of genes belonging to oxidoreductases (Figure 3(d)). Taken together, the transcriptomic investigation revealed a number of modulations following cyclic oxidative stress.

3.3. The Antioxidative Defense System Was Activated by Frequent Redox Stress. We extended our transcriptomic view to enzymes involved in resolving redox stress (Figure 4). Stress signaling on downstream factors, such as heme oxygenases (HMOX) and NADPH quinone oxidoreductase 1 (NQO1), was at least in part significantly increased (Figure 4(a)). Initial (week 2) superoxide dismutases 1 and 3 (SOD1/3) expression was unaffected whereas their expression reached a maximum at week 6 of periodic oxidative challenge (Figure 4(b)). Expression of catalase was moderately but significantly increased after ten weeks of frequent plasma exposure. Glutathione peroxidases (GPX1, GPX5, and GPX8) showed an overall modest upregulation while GPX3 was found to be constantly downregulated (Figure 4(c)). Peroxiredoxin (PRDX) 1, 2, 4, and 6 expressions were rather

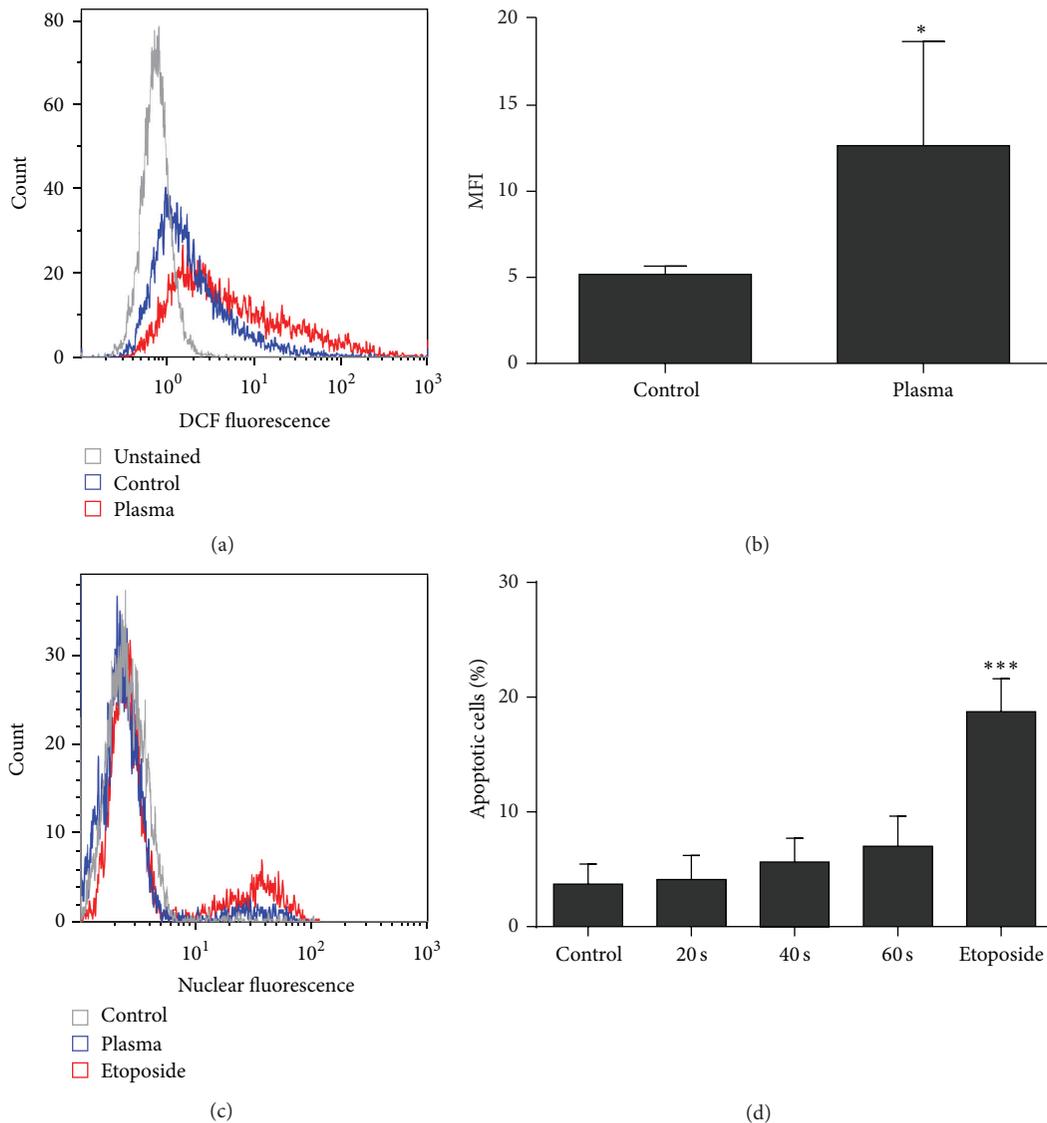


FIGURE 1: Exposure to plasma-treated medium evoked acute oxidative stress but was not cytotoxic. HaCaT keratinocytes were loaded with CM-H₂DCF-DA and exposed to plasma-treated medium (60 s). Cells were subsequently trypsinized and fluorescence was measured using flow cytometry (a). Compared to control cells, intracellular mean fluorescence intensity (MFI) of DCF was significantly increased in treated keratinocytes (b). To assess viability, keratinocytes were exposed to plasma-treated medium and incubated for 18 h. Cells were stained for active caspase 3 and analyzed by flow cytometry (c). The number of apoptotic cells was significantly increased in etoposide control but not in cells exposed to plasma-treated medium (d). Data are presented as one representative (a, c) or mean + SD (b, d) of two independent experiments. Statistical comparison was done using *Student's t*-test (b) or one-way ANOVA with *Dunnett* corrections for multiple comparison to untreated control (d) (* $p < 0.05$, *** $p < 0.001$).

enhanced, especially after week 6. A significant increase of expression of members of the antioxidative defense system was most prominent after 6 weeks of frequent exposure to plasma-treated medium (Figure 4(d)), indicating strong adaptation responses of keratinocytes.

3.4. Apoptosis Pathways. Only a small number of genes involved in apoptotic signaling were found in our transcriptomic studies with 31 genes being positive and 17 genes being negative regulators of apoptosis (Figure 5(a)). BCL2

and BBC3 (both p53-upregulated inducers of apoptosis) transcript levels were only nonsignificantly increased throughout the three months of treatments. Similarly, downregulation of the cell cycle gate-keeper CDKN1A (p21) was steady but modest (Figure 5(b)) while upregulation of the DNA repair enzyme GADD45 was significant at w2 and w6. Heat shock proteins protect cells from excessive protein stress and subsequently from apoptosis, and we found several candidates (HSP90A, HSP90AB, and HSP90B) to be significantly upregulated at w2 and/or w6 (Figure 5(c)). In contrast, actin mRNA

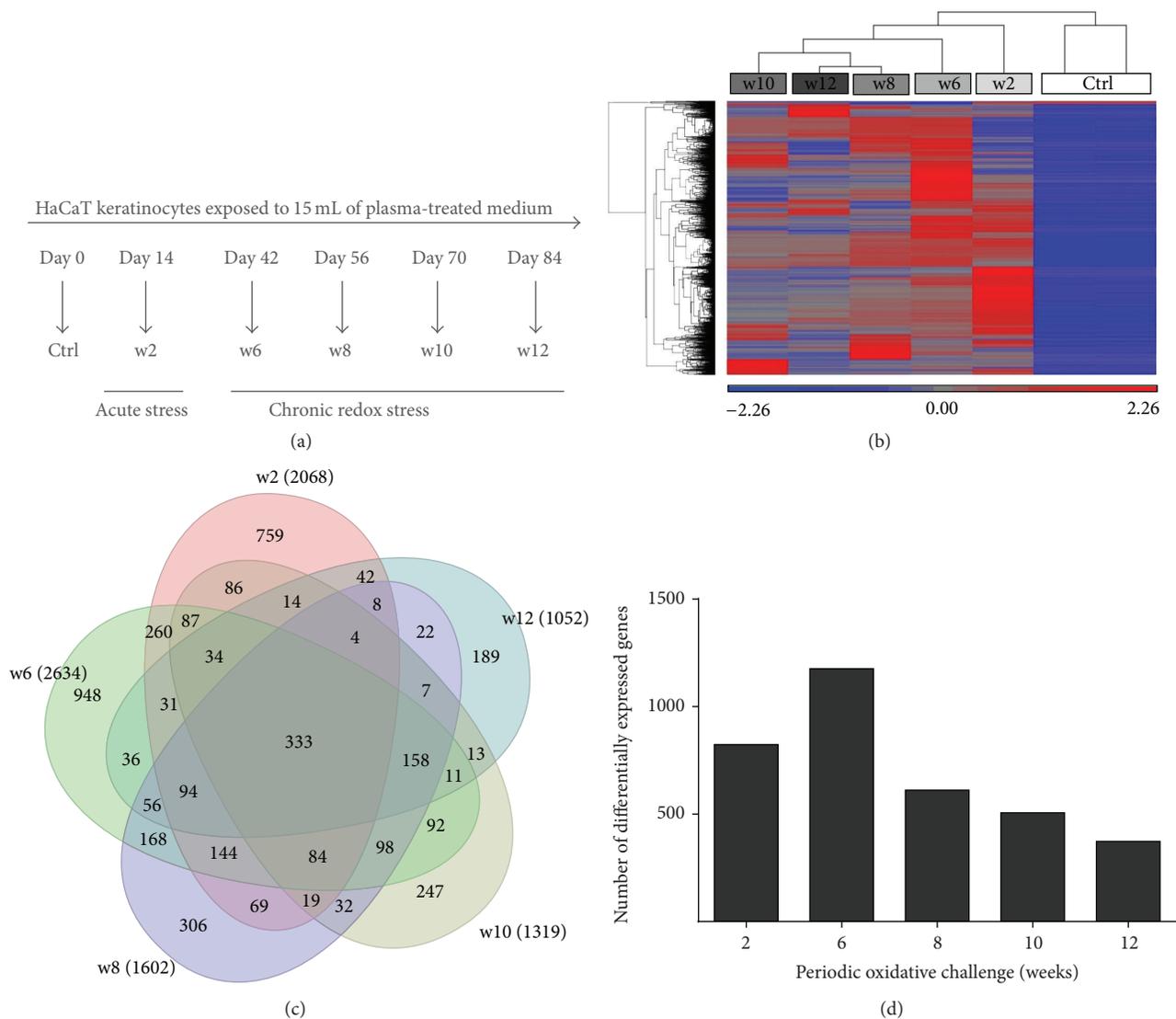


FIGURE 2: Repeated redox challenge modulated HaCaT keratinocyte gene expression profile. HaCaT keratinocytes were exposed to plasma-treated (60 s) medium twice per week, and gene expression was determined after acute (w2) and chronic (w6, w8, w10, and w12) exposure to redox stress (a). Hierarchical clustering of differentially expressed genes (upregulated: red; downregulated: blue) after several weeks of plasma-mediated redox challenge (w2 to w12) is shown (b). The Venn diagram visualizes the overlap of the differentially expressed genes (number in parentheses) between different groups (in color) compared to untreated HaCaT keratinocytes (c). Numbers of differentially expressed genes with unambiguous identification are given for each treatment regimen and according to PANTHER classification (d).

and protein expression remained relatively unchanged (data not shown). Therefore, β -actin was used as loading control in western blot analysis (Figure 6(a)(I)). Correlating to transcriptomics, western blot analysis of the antioxidative enzymes NQO1 and PRDX2 confirmed a slight or significant upregulation, respectively (Figures 6(a)(II) and 6(a)(III)). Contrasting data on mRNA expression, a strong upregulation was seen for Puma (BBC3) and p21 (CDKN1A). Their protein level remained significantly elevated at weeks 8 to 10 of cyclic oxidative challenges with p21 but not Puma decreasing after that (Figures 6(b)(I) and 6(b)(II)). For H2AX, we found a twofold repression of its total protein (Figure 6(c)(I)) amount whereas its phosphorylated form (γ -H2AX) and the ratio

between phosphorylated and total protein were increased (Figures 6(c)(II) and 6(c)(III)), indicating cellular perception of redox stress. Moreover, we detected a significant increase of the cytoprotective HSP27 total protein as well as its phosphorylated form throughout this study from w2 to w12 (Figures 6(d)(I) and 6(d)(II)). The p-HSP27/HSP27 ratios negatively correlated to number of passages periodically receiving oxidative challenge (Figure 6(d)(III)).

3.5. Gene Expression of Structural Proteins Was Strongly Affected with Frequent Redox Stress. Structural proteins serve as important barriers and mediate cell-cell contact. We were able to identify a differential expression of occludin (OCLN),

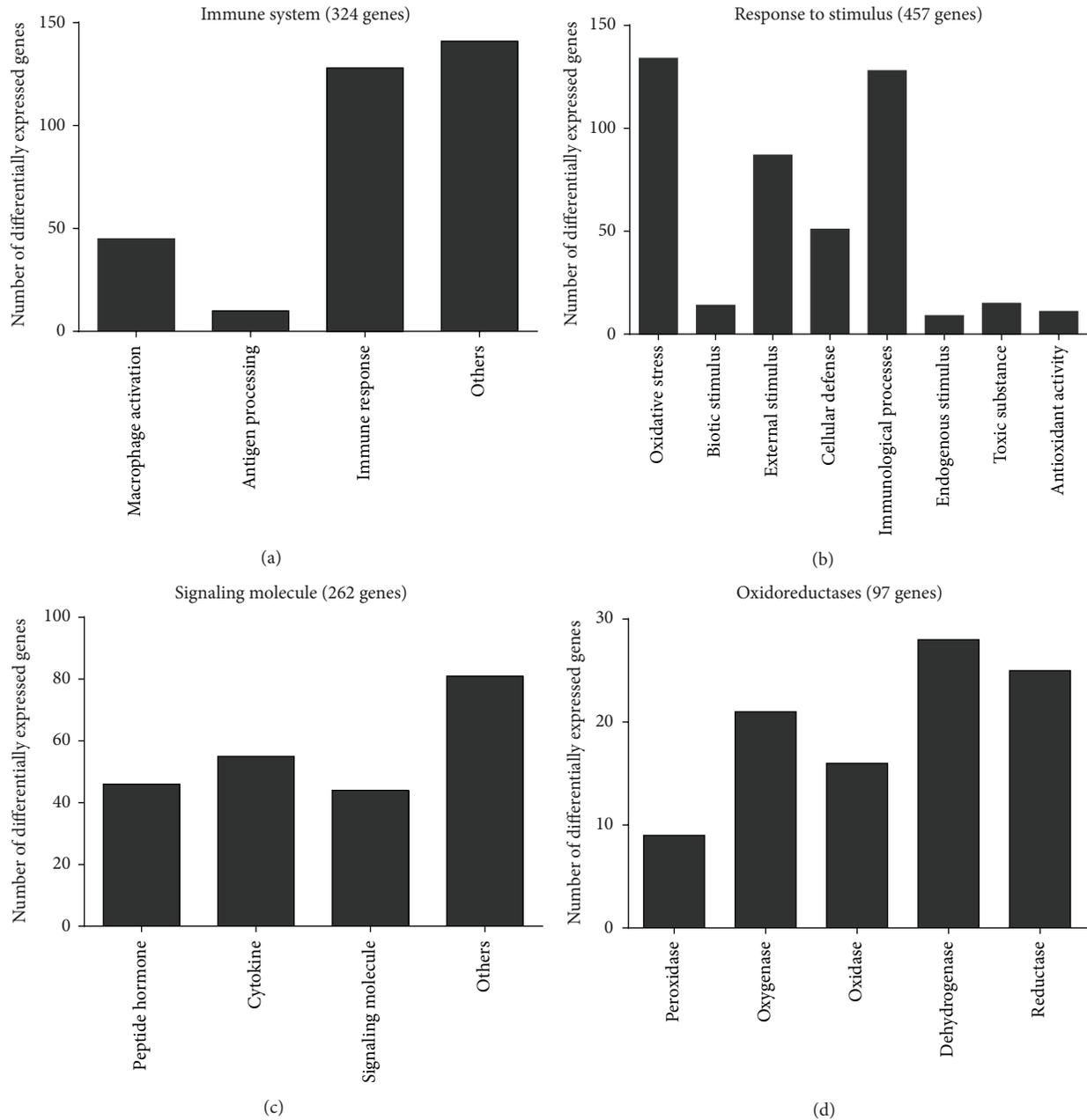


FIGURE 3: Gene ontology (GO) classification of the identified genes differentially expressed. PANTHER classification system was used to analyze the gene lists for each experimental group (w2–w12 versus untreated control) to list the categories with the “biological process” functions domain of GO ($p < 0.05$). Differentially expressed genes in the categories “immune system” (a) and “response to stimulus” (b) are shown. Top hits of the protein classes associated with the repeated redox challenge include signaling molecules (c) and oxidoreductases (d). The total number of modulated genes is given in parentheses.

several claudins (CLDN 1–4, 7, 8, 12, 17–19, 22, and 23), and zonula occludens (ZO) tight junction proteins (ZO-1, ZO-2, and ZO-3) (Table 1). Interestingly, ZO-1 protein was only upregulated after initial redox challenges up to 6 weeks. CDHs are linked to actin via β -catenin (CTNNB; cadherin-associated protein), and mRNA expression of the latter was also enhanced following repeated exposure to redox stress. We also discovered transcriptional changes

in a striking number of keratins (KRT) and their associated proteins (KRTAP) as well as matrix-metalloproteinases (MMPs) and other structural proteins (Table 2). A significant decline was seen with KRT1, KRT4, KRT13, and KRT77 while others were strongly upregulated (e.g., KRT35, KRT38, KRT72, KRT82, and KRT83) after redox stress. KRT1 is a major epithelial keratin and western blotting confirmed its significantly lower expression after periodic oxidative

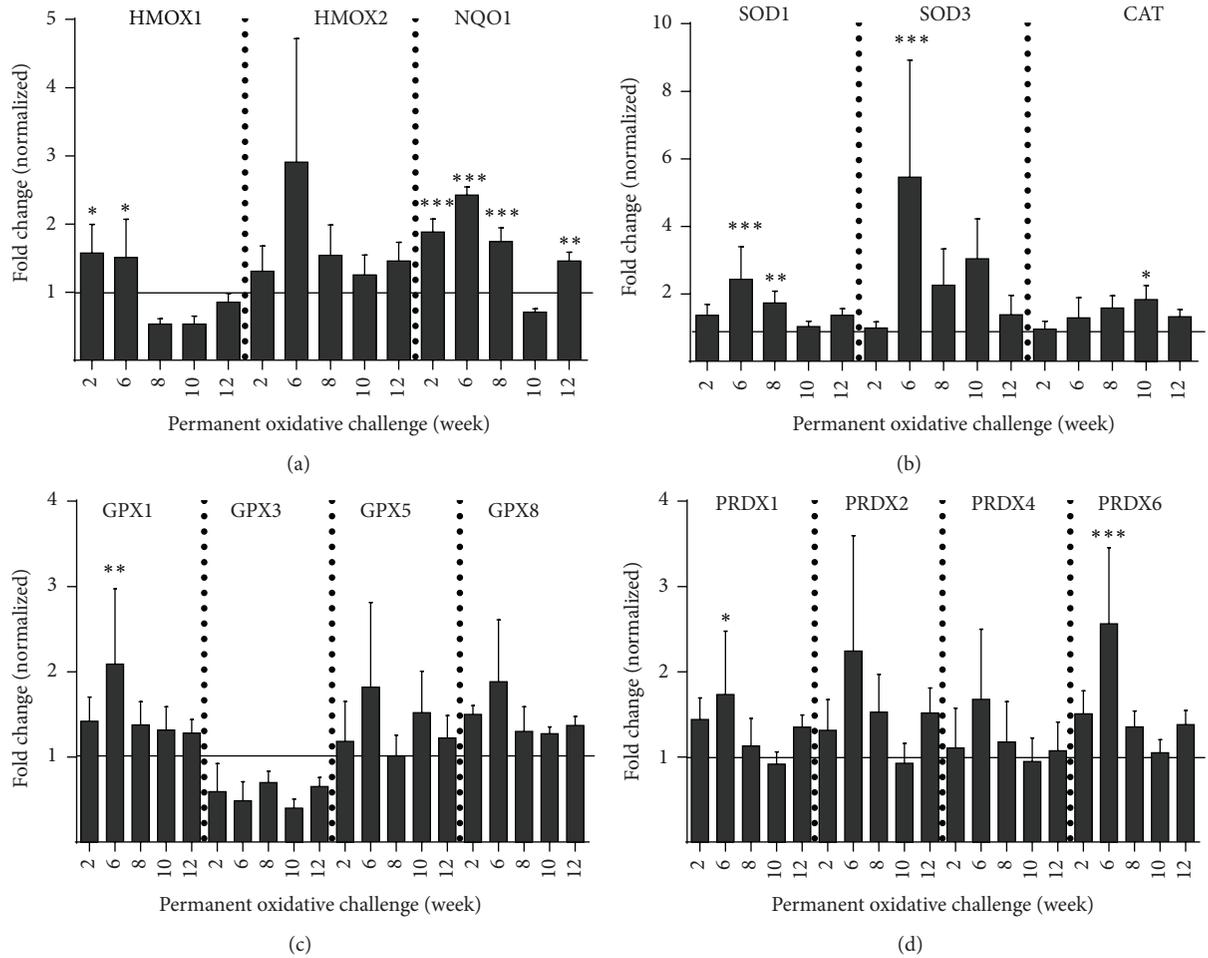


FIGURE 4: Transcriptional response of the antioxidant defense system to acute and chronic redox challenge. Transcription of several antioxidant enzymes in HaCaT keratinocytes was measured using microarray technology. HMOX2 and NQO1 were mainly upregulated over three months in contrast to a transient upregulation of HMOX1 during acute stress phases followed by downregulation after chronic stress exposure (a). SOD1, SOD3, and catalase were mainly upregulated (b). GPX1, GPX5, and GPX8 mRNA copy numbers were enhanced whereas those of GPX3 were found to be reduced (c). Expression of several peroxiredoxins was increased after periodic oxidative stress (d). Data are presented as mean + SD of two analyses. Statistical analysis was done using one-way ANOVA with *Dunnett* corrections for multiple comparisons to untreated, normalized control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

challenge (Figure 6(b)(III)). The MMPs investigated seemed to be always upregulated in oxidative stress conditions. While acute stress only modulated few targets investigated (20/66 in w2), chronic stress affected a larger number of transcripts (47/66 in w6) with declining numbers after that (32/66 in w8; 31/66 in w10; 21/66 in w12).

3.6. Redox Stress Reduced HaCaT Keratinocyte Motility and Induced Morphological Changes. Scratch assay was performed to determine keratinocyte motility using time lapse video microscopy (Figure 7(a)). Repeated plasma treatment significantly repressed keratinocyte motility following oxidative stress (Figure 7(b)). Interestingly, repression peaked at w2 but was partially reversed after further redox challenges (w6 and w8). Visually, periodic exposure to oxidative stress

apparently enlarged cell size as shown using immunofluorescence staining with ZO-1 (Figure 7(c)). Cell size enhancement may be linked to a higher number of mitochondria. We therefore assessed the total mitochondrial content. Mito-tracker fluorescence was measured in periodically plasma-treated cells and compared with untreated keratinocytes. Using flow cytometry, a moderate but significant increase in mitochondrial content in stressed cells supported the notion of enlarged cell bodies after oxidative stress challenge (Figure 7(d)).

3.7. Plasma-Treated Medium Elicited a Distinct Secretory and Inflammatory Profile. Secretory products, such as cytokines, growth factors, and other inflammatory mediators, serve to mediate autocrine and paracrine communication between

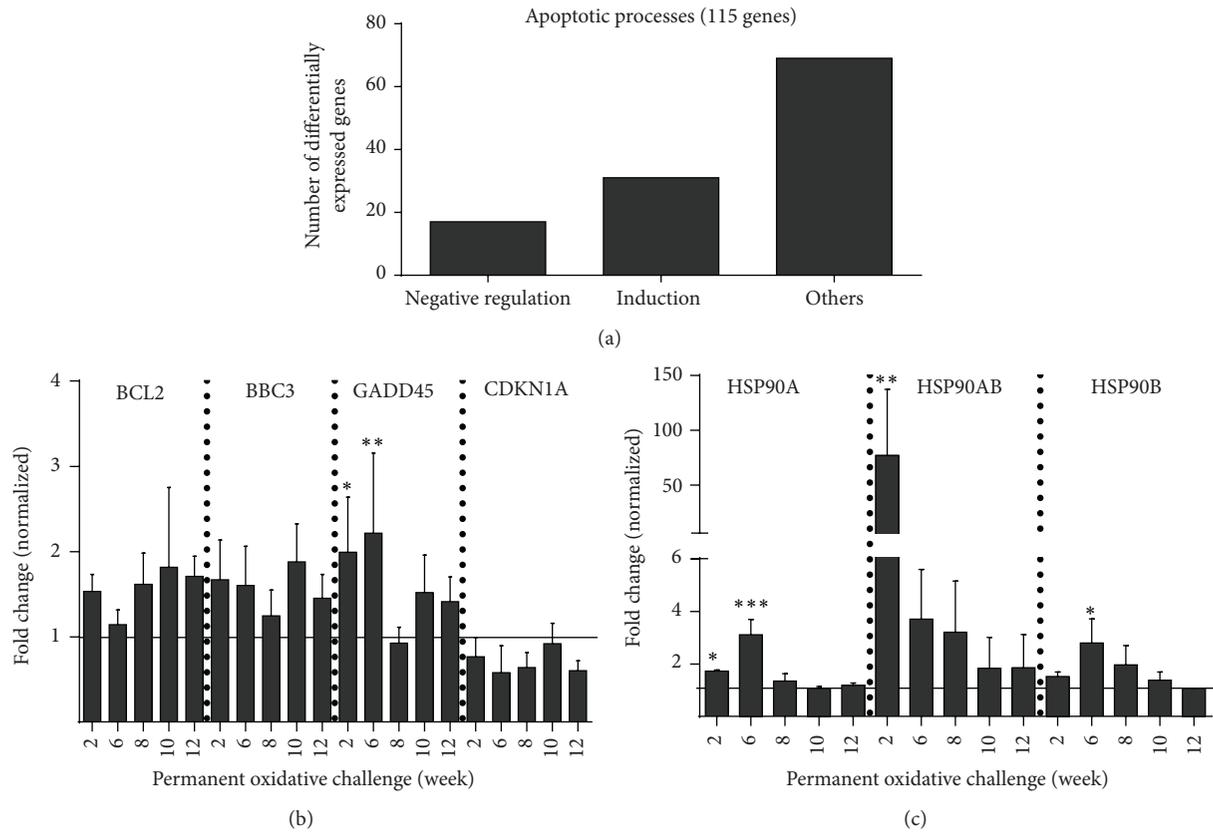


FIGURE 5: Apoptosis-related gene expression in response to periodic redox challenge. One hundred and fifteen apoptosis-related genes were associated with frequent redox challenges whereas 17 and 31 genes belonged to negative or positive regulation apoptosis, respectively (a). Downstream targets of p53 signaling pathway included BCL2 and BBC3 which represent the proapoptotic p53 pathway and GADD45 that is involved in DNA repair. All of them were mainly upregulated. CDKN1A plays a role in cell cycle control and senescence and was slightly downregulated (b). Expression of stress-related heat shock proteins was partially enhanced as well (c). Data are presented as mean + SD of two analyses (b, c). Statistical analysis was done using one-way ANOVA with *Dunnett* corrections for multiple comparisons to untreated, normalized control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

cells. An overview of the altered cytokine expression is given (Table 3). Cold plasma induced not only proinflammatory (e.g., IL-4, IL-10, and TGF β) but also anti-inflammatory (e.g., IL-6, IL-8, and TNF α) cytokines. Moreover and in response to plasma-mediated oxidative stress, HaCaT keratinocytes contained significantly increased levels of several growth factor transcripts (e.g., CSF2, GAS1, FGF6, and IGFs).

4. Discussion

We investigated the HaCaT keratinocyte global transcriptomic profile over three months to identify genes reflecting adaptations to periodic oxidative stress as seen in redox-related diseases of the skin. The frequent redox challenge was applied using cold physical plasma-treated medium as in previous proteomics studies [31]. Its use is advantageous over addition of single oxidants as it provides a complex mixture of reactive components that either originate directly from the plasma gas phase or are formed during secondary reactions with ambient air or water molecules [32]. This includes the deposition of biologically active reactive molecules, such as superoxide, hydroxyl radicals, nitrite, nitrate, peroxynitrite, and hydrogen

peroxide [33–35]. Accordingly, and in line with previous results using other cell types [36–38], plasma-treated medium led to intracellular oxidation (Figure 1). It is important to consider the dual role exhibited by ROS and RNS [39]. While cytotoxic effects occur at high doses [40], low-dose redox stimulation is imperative in cell physiology [41]. Activation of kinases and inhibition of phosphatases are critically regulated by reactive species [42]. Reactive species also play a crucial role in wound healing [43]. In particular hydrogen peroxide is an important second messenger for HaCaT keratinocyte cell growth [44]. Cellular RNS are mainly produced secondary to signaling events initiated upon ROS [45]. We here present a group of interesting targets in response to repetitive redox stress.

Insufficient removal of reactive intermediates leads to intracellular accumulation of ROS and results in oxidative stress [46–48]. This may result in induction of apoptosis and p53 activation through phosphorylation of MAP kinases and activation of Bcl-2 family proteins [49–51]. Within the transcriptome, our continuous plasma treatment targeted apoptotic processes in HaCaT cells only to a minor extent. By contrast, Puma and p21 protein were upregulated from

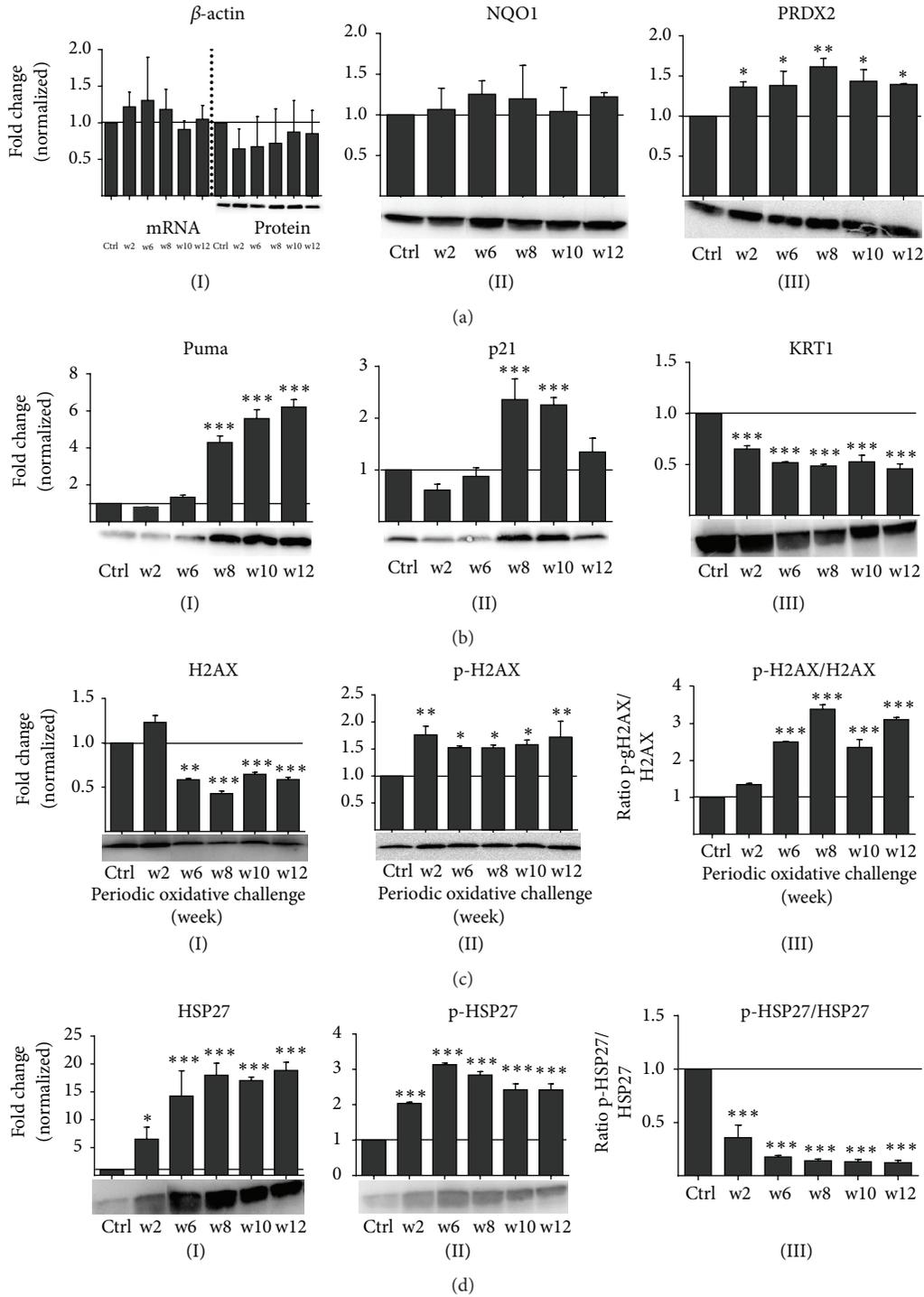


FIGURE 6: Western blot analysis showed a differential expression of several redox-related proteins. Western blot analysis confirmed the RNA expression profiles of β -actin ((a)(I), no change) and of the antioxidative enzymes NQO1 ((a)(II)) and PRDX2 ((a)(III)). Puma and p21 ((b)(I), (b)(II)) were upregulated after chronic but not acute oxidative challenge whereas keratin 1 was downregulated ((b)(III)). The total amount of H2AX protein was twofold downregulated after periodic oxidative challenge in contrast to acute stress ((c)(I)) while its phosphorylated form γ -H2AX was slightly upregulated in response to oxidative stress ((c)(II)). The ratio of p-H2AX to the total protein amount of H2AX was significantly enhanced after chronic oxidative challenge ((c)(III)). The total amount ((d)(I)) and the phosphorylated form ((d)(II)) of the stress-related protein HSP27 was significantly enhanced after repeated plasma treatment. The ratio of p-HSP27 to total protein amount of HSP27 was significantly decreased ((d)(III)). Representative western blot images are shown. Data in diagrams are presented as mean + SD of two independent experiments. Statistical analysis was performed using one-way ANOVA with *Dunnett* corrections for multiple testing to untreated, normalized control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

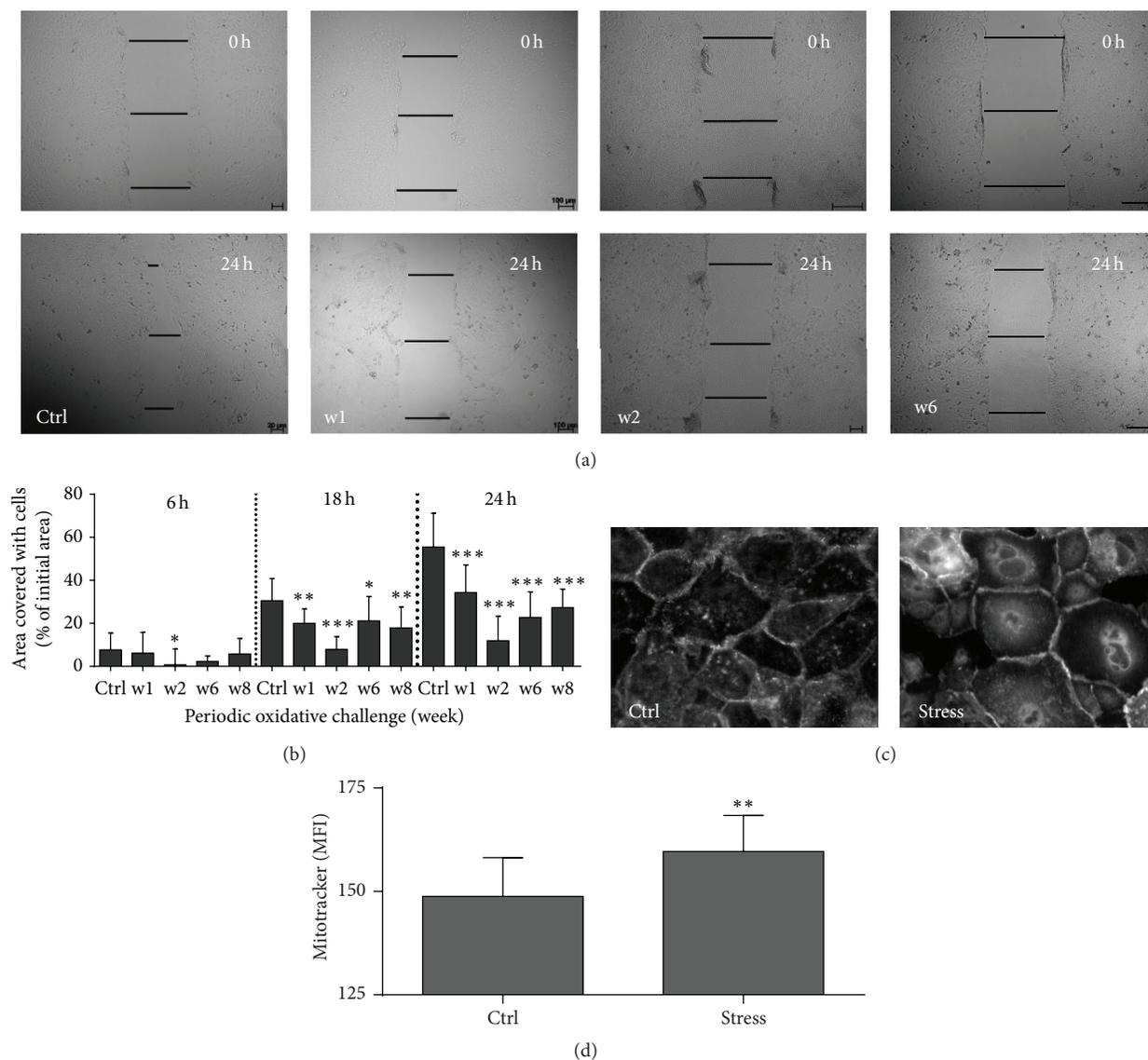


FIGURE 7: Periodic oxidative redox challenges decelerated keratinocytes motility and migration. Scratch assays were performed to monitor artificial wound healing over 24 h. Representative images are shown for controls and repeatedly (1–8 weeks) plasma-treated cells (a). There was a significant decrease in cell motility following plasma induced redox stress (b). Compared to control cells (ctrl) plasma-treated cells (w2) displayed a notable enlargement in size as seen after staining cell margins for ZO-1 (c). Mitotracker red fluorescence (MFI), which is a marker for total mitochondrial content, increased after chronic stress and was measured using flow cytometry (d). Data are presented as mean + SD of two to four experiments and statistical comparison was done using ANOVA with *Dunnnett* correction for multiple comparison to untreated, normalized control (b), or *Student's t*-test (d) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

w6 to w12, indicating a differential apoptotic programming with long-term redox challenges [52, 53]. GADD45 modifies DNA-accessibility if damage is anticipated and its upregulation was only transient (w2 to w6) suggesting that adaptive mechanisms take place with frequent redox challenges. The ROS/RNS-associated, antiapoptotic heat shock proteins are mediators between p53 and mitogen-activated protein (MAP) kinase signaling [54]. The cell stress chaperone HSP90, which is essential for protein folding and transports within the cell [55], and both total HSP27 protein and its phosphorylated form, which function in signal transduction

and inhibition of apoptosis [56], were significantly upregulated during frequent oxidative challenge. Both molecules are promising clinical targets to reduce oxidative stress [57, 58]. H2AX facilitates recruitment of DNA repair proteins [59] and becomes phosphorylated (γ -H2AX) as a downstream effect of the apoptosis cascade and in response to DNA replication stress in a ATR-dependent manner [60]. The increased presence of γ -H2AX suggests that genomic stress was perceived throughout the 3 months of treatment. We could not find a significant modulation of ATR expression (data not shown) which corroborates the results of a previous

TABLE 1: Expression (fold change compared to untreated control) of tight and adherent junction proteins with at least one significant modulation throughout the time course of plasma treatment. Actin expression remained relatively unchanged.

Gene name	Gene ID	w2	w6	w8	w10	w12
Occludin	OCLN	1.32	-1.15	-2.11	-2.02	-1.86
Claudin 1	CLDN1	-1.11	-2.32	-1.38	-1.01	1.04
Claudin 2	CLDN2	2.17	2.11	1.40	1.48	-1.26
Claudin 3	CLDN3	1.97	1.68	-4.12	-2.41	-1.27
Claudin 4	CLDN4	1.66	3.19	-1.02	1.00	1.08
Claudin 6	CLDN6	1.59	2.52	-1.16	1.37	-1.99
Claudin 7	CLDN7	1.07	-1.38	-2.03	-1.56	-1.08
Claudin 8	CLDN8	1.05	-10.46	-1.86	-1.31	1.06
Claudin 12	CLDN12	1.42	2.21	1.67	1.78	1.28
Claudin 17	CLDN17	1.86	-1.70	-1.93	-2.66	-2.61
Claudin 18	CLDN18	4.86	-1.11	-1.27	-1.74	-1.81
Claudin 19	CLDN19	1.86	-2.46	-2.41	-1.04	1.38
Claudin 22	CLDN22	17.47	2.93	-1.66	1.26	-1.52
Claudin 23	CLDN23	-1.24	-1.68	-3.49	-2.51	-1.69
Zonula occludens protein 1	TJP1 (ZO-1)	1.68	3.06	1.14	-1.09	1.10
Zonula occludens protein 2	TJP2 (ZO-2)	-1.05	-2.27	-2.26	-1.53	-1.07
Zonula occludens protein 3	TJP3 (ZO-3)	-1.62	-2.60	-1.45	-1.08	-1.39
Cadherin 2	CDH2	4.78	46.49	11.74	1.56	6.26
Cadherin 3	CDH3	1.37	2.10	1.70	1.26	1.25
Cadherin 4	CDH4	1.66	3.19	-1.02	1.00	1.08
Cadherin 5	CDH5	1.36	2.06	-1.06	1.78	1.15
Cadherin 7	CDH7	1.85	1.97	1.32	2.29	2.27
Cadherin 9	CDH9	2.53	-3.54	-3.35	-2.47	-2.51
Cadherin 10	CDH10	2.14	1.32	1.31	1.63	1.20
Cadherin 13	CDH13	-1.19	2.95	3.40	3.34	-1.09
Cadherin 16	CDH16	3.55	-1.47	-1.42	-1.82	1.11
Cadherin 18	CDH18	2.04	-1.14	2.48	1.18	-1.02
Cadherin 19	CDH19	2.32	-1.35	-1.34	-1.35	-1.31
Cadherin 20	CDH20	5.80	1.76	2.35	1.67	-1.14
Cadherin 23	CDH23	3.07	1.02	2.48	1.47	1.90
Cadherin 24	CDH24	1.53	2.83	1.61	1.25	1.06
α -actin 2	ACTA2	1.10	-1.21	2.70	1.41	1.06
β -actin	ACTB	1.34	1.35	1.23	1.04	1.02
β -catenin 1	CTNNB1	1.62	2.05	1.27	1.03	1.21

study investigating the transcriptome in cold plasma-treated corneas [61].

Oxidative or nitrosative stress is usually counteracted by enzymatic scavengers while silencing of antioxidative defense enzymes enhances cellular damage and cancer formation [62]. We found an upregulation of several peroxiredoxins and glutathione peroxidases except for GPX3 (Figure 4). GPX1, GPX5, and GPX8 prevent peroxide-induced oxidative damage, lipid peroxidation, and protein degradation [63–65]. They were upregulated rather at the beginning of chronic oxidative challenge (w6) suggesting an adaption of keratinocytes within the antioxidative defense system during the later course of culture. The upregulation of PRDX2, another important antioxidative enzyme, has also been found in psoriasis [66]. The downmodulation of GPX3, the only extracellular member of the glutathione peroxide

family [67], may have been compensated by the concomitant upregulation of the other antioxidative enzymes. The overall upregulation of the antioxidative system corroborates our recent results involving a redox-based activation of the Nrf2 pathway controlling HMOX1 expression [27]. Our results also correlate well with previous studies that identified a subtle decrease of HMOX1 but not HMOX2 [68] following redox stress.

Keratinocytes appear to play an important role in differentiation, migration, and reepithelialization in the final stage of wound closure [69]. For acute redox stress, plasma-treated medium notably repressed cell migration which is in agreement with our previous results using a carcinoma cell line [70]. Interestingly, cell migration activity partly recovered afterwards, pointing to an adaption of keratinocytes to periodic redox challenges. Proliferation is tightly linked to

TABLE 2: Expression (fold change compared to untreated control) of matrix-metalloproteinases and fibrous structural proteins (keratins) with at least one significant modulation throughout the time course of plasma treatment.

Gene name	Gene ID	w2	w6	w8	w10	w12
Matrilysin	MMP7	53.36	3.49	3.41	2.65	2.34
Matrix metalloproteinase 27	MMP27	3.00	4.16	3.81	2.74	3.16
Stromelysin 2	MMP10	2.08	124.31	2.81	2.10	2.27
Keratin I cytoskeletal 13	KRT13	-1.22	-5.77	-1.46	-1.35	-1.03
Keratin I cuticular Ha5	KRT35	1.43	12.00	1.71	1.61	1.20
Keratin I cuticular Ha8	KRT38	12.15	3.24	11.82	2.67	1.25
Keratin II cytoskeletal 1	KRT1	-1.21	-87.56	-2.72	-1.57	1.13
Keratin II cytoskeletal 1b	KRT77	-1.78	-41.78	-4.12	-2.51	-1.65
Keratin II cytoskeletal 4	KRT4	1.20	-3.27	-1.20	-1.15	-1.03
Keratin II cytoskeletal 8	KRT8	1.46	3.42	1.68	1.26	1.28
Keratin II cytoskeletal 72	KRT72	5.70	3.19	3.04	2.23	3.78
Keratin II cytoskeletal 79	KRT79	-1.08	-1.87	-1.63	6.24	-1.56
Keratin II cuticular Hb2	KRT82	1.35	2.86	2.74	1.94	3.03
Keratin II cuticular Hb3	KRT83	1.79	2.77	1.18	-1.24	1.28
Keratin-associated 3-3	KRTAP3-3	5.60	1.42	2.04	1.48	-1.01
Keratin-associated 4-1	KRTAP4-1	-1.18	-1.12	5.29	-1.11	1.21
Keratin-associated 4-2	KRTAP4-2	-1.10	-2.06	1.76	11.54	-2.28
Keratin-associated 10-7	KRTAP10-7	-1.76	3.76	2.81	3.08	3.84
Keratin-associated 10-10	KRTAP10-10	-1.11	-2.03	3.13	-1.62	-1.13
Keratin-associated 10-11	KRTAP10-11	5.66	3.17	-1.40	-1.42	1.72
Keratin-associated 10-12	KRTAP10-12	-1.27	8.01	4.78	2.68	1.25
Keratin-associated 13-3	KRTAP13-3	1.19	35.43	-1.23	-1.26	1.54
Keratin-associated 16-1	KRTAP16-1	-1.22	-1.30	-1.20	25.27	1.49
Keratin-associated 22-2	KRTAP22-2	-1.29	5.20	-1.54	-1.54	1.89
Filaggrin	FLG	-14.0	1.05	-1.23	-3.29	-1.41
Laminin subunit alpha-1	LAMA1	2.05	2.09	1.79	1.31	1.19
Late CE protein 1A	LCE1A	1.71	7.89	3.03	2.05	1.65
Loricrin	LOR	-1.68	-6.88	-2.35	-3.90	-1.18
Protein S100-A4	S100A4	-1.44	-4.27	-2.75	-1.14	-1.17
Protein S100-A6	S100A6	-1.06	-3.53	-1.65	-1.53	-1.25
Protein S100-A7	S100A7	1.23	-4.63	-4.54	1.28	1.51
Protein S100-A8	S100A8	1.02	-8.31	-3.31	1.21	1.17
Protein S100-12	S100A12	-1.39	-4.62	-2.42	1.28	1.28

cell-cell contacts and adhesion molecules, such as tight junctions [71–73]. Downregulation of junctional proteins (e.g., occludins and claudins) is often associated with impairment of barrier function and disease [74–76]. Several of such transcripts were modulated by repetitive redox challenges, possibly indicating a reorganization of the junctional network in response to ROS as also suggested by others [77–79]. Keratins also contribute to the barrier properties [80] and are associated with regulatory functions [81], forming a signaling network with kinases [82]. A large number of keratin transcripts were differentially expressed following repeated exposure to oxidative mediators, possibly being responsible for the observed differences in cellular migration. Moreover, they may be linked to the observed increase in cell size. This corroborates our previous findings in plasma-treated THP-1 cells [83] and links to ROS-mediated increase in keratinocyte cell size and differentiation [84]. In support

of this notion, LCE1A, which is expressed in late stages of keratinocyte maturation [85], was significantly upregulated. Filaggrin (FLG), another important component of protective skin layers of the epidermis was downregulated. A loss of FLG function is associated with several skin diseases such as *ichthyosis vulgaris* [86] and atopic dermatitis [87]. Both LCE1A and FLG belong to the cornified envelope which was recently shown to be highly involved in redox regulation via ROS quenching [88].

We identified a number of secretory factor transcripts being regulated via frequent redox stress. The extracellular matrix is important for keratinocyte migration in wound healing but also prone to MMP digestion [89]. As such, MMPs participate in physiological (e.g., angiogenesis and wounds healing) and pathological (e.g., cancer and non-healing wounds) processes [90–92]. MMP2 (collagenase IV) and MMP16 (activates MMP2) expression was significantly

TABLE 3: Expression (fold change compared to untreated control) of cytokines and growth factors with at least one significant modulation throughout the time course of plasma treatment.

Gene name	Gene ID	w2	w6	w8	w10	w12
Interleukin-1 α	IL1A	11.85	17.09	4.01	1.36	3.18
Interleukin-1 β	IL1B	4.30	5.65	2.30	-1.01	2.14
Interleukin-4	IL4	5.72	-1.34	1.05	-1.33	-1.77
Interleukin-5	IL5	5.51	-1.06	-2.05	-2.34	-1.92
Interleukin-6	IL6	3.38	3.95	1.28	1.82	3.48
Interleukin-8	IL8	2.25	2.83	1.28	-1.84	-1.25
Interleukin-9	IL-9	-1.39	-1.12	2.85	-1.06	-1.26
Interleukin-10	IL10	3.81	1.68	2.08	1.36	1.15
Interleukin-11	IL-11	1.02	-2.34	-1.50	-1.39	-1.80
Pro-interleukin-16	IL16	3.02	-1.45	-1.42	1.98	1.79
Interleukin-17A	IL-17A	1.74	2.74	1.35	2.56	1.39
Interleukin-17B	IL17B	13.90	3.20	2.94	2.07	7.54
Interleukin-17D	IL17D	2.91	11.47	3.16	-1.41	1.55
Interleukin-18BP	IL-18BP	-2.24	1.12	1.41	1.46	-1.74
Interleukin-17F	IL17F	-1.46	11.22	-1.55	-1.09	-1.13
Interleukin-19	IL19	2.09	2.51	1.03	2.05	-1.32
Interleukin-24	IL24	1.16	3.53	3.13	2.21	2.27
Interleukin-31	IL31	1.85	16.15	13.18	13.64	1.49
Interleukin-32	IL32	2.25	1.82	-1.07	1.59	1.29
Interleukin-33	IL33	2.87	2.23	1.19	1.30	-1.70
Interleukin-34	IL34	2.01	2.46	-1.07	2.39	-1.20
Interleukin-36 α	IL36A	4.38	1.70	-1.23	1.13	-1.01
Interleukin-36 β	IL36B	6.59	2.31	2.33	1.25	9.74
Interleukin-37	IL37	1.17	3.11	1.36	-1.06	1.13
Colony stimulating factor 2	CSF2	1.69	6.91	2.54	1.71	1.67
Fibroblast growth factor 2	FGF2	-3.02	2.57	-1.24	1.80	1.36
Fibroblast growth factor 5	FGF5	8.95	-1.22	-1.16	1.03	9.55
Fibroblast growth factor 6	FGF6	1.58	1.20	7.19	1.45	3.01
Fibroblast growth factor 9	FGF9	-1.34	3.59	-1.51	1.77	2.68
Fibroblast growth factor 14	FGF14	1.13	-1.08	1.08	-1.06	3.10
Fibroblast growth factor 18	FGF18	-1.24	13.76	1.68	-1.09	-1.86
Fibroblast growth factor 21	FGF21	1.74	-1.40	-1.30	6.29	1.47
Fibroblast growth factor 22	FGF22	3.02	3.03	-1.33	-1.37	1.47
Growth arrest-specific protein 1	GAS1	3.56	2.33	1.97	1.86	2.36
Growth arrest-specific protein 2	GAS2	-1.22	-1.14	-1.03	8.87	1.32
Growth/differentiation factor 11	GDF11	-1.04	3.04	1.68	1.21	1.04
Insulin-like growth factor I	IGF1	7.10	2.70	2.35	1.88	1.14
Insulin-like growth factor II	IGF2	2.45	7.87	-3.18	-3.43	-1.21
TGF β	TGFB1	1.61	2.80	1.71	-1.35	-1.06
TNF α	TNFA	1.97	7.57	1.67	1.57	2.10

upregulated at all time points, suggesting a detrimental role in wound healing processes [93]. Moreover, we found a differential regulation of transcripts associated with inflammatory processes in the skin (e.g., S100A) [94], which are associated with an altered cellular phenotype, and enhanced expression of inflammatory mediators, for example, cytokines [95]. Such immune mediators are often dysregulated in chronic wounds [96]. IL-19 transcript numbers were increased in response to

periodic redox stress and were previously reported to upregulate the expression of proinflammatory IL-6 and TNF α which together with IL-1 and IL-8 were also found to be upregulated in our study [97]. These mediators were shown to be elevated in nonhealing wounds [98–101]. Also highly proinflammatory neutrophils are chemotactic for CSF2 [102], and in line with previous studies [103] CSF2 was found to be constantly elevated in response to redox stress. However,

periodic redox stress also increased the transcription of anti-inflammatory mediators. IL-37 is a fundamental inhibitor of innate immunity [104] and keratinocyte-derived IL-4 and IL-10 mediate immune suppression [105]. TGF β has been used in clinical trials combating deficient wound healing [106] and was partially increased following plasma as well. Frequent redox challenges thus generated not only a pro- but also anti-inflammatory cytokine signature and thus may have a complex impact on the quality of inflammation in redox-related diseases.

We can only speculate why there was no consistent up- and downregulation patterns over time in the targets investigated in this study. First, gene expression may be heterogeneous within cultured cells. Frequent cold plasma treatment may have promoted growth of cells that are better equipped against oxidative stress, consequently overgrowing the cells that are not. For example, single cold plasma treatment of keratinocytes led to G2/M-phase cell cycle arrest but this was dependent on the total treatment time and was not present in all cells [107]. Alternatively, all keratinocyte cells may have adapted to redox stress over time, significantly altering their basal gene expression profile.

5. Conclusion

Using cold physical plasma-derived reactive species, our keratinocyte-based *in vitro* model aimed to mimic the characteristics of chronic oxidative stress in redox-related disorders of the skin to pinpoint biomarkers for molecular therapies. Redox processes are important decision makers in skin disease and tumorigenesis and here affected cell-cell communication, cellular proliferation, and inflammatory processes. The identification of genes and proteins whose expression is altered following permanent oxidative challenge is an important step to better understand redox regulations in clinical settings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] D. R. Bickers and M. Athar, "Oxidative stress in the pathogenesis of skin disease," *Journal of Investigative Dermatology*, vol. 126, no. 12, pp. 2565–2575, 2006.
- [2] F. Q. Schafer and G. R. Buettner, "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple," *Free Radical Biology and Medicine*, vol. 30, no. 11, pp. 1191–1212, 2001.
- [3] C. S. Sander, F. Hamm, P. Elsner, and J. J. Thiele, "Oxidative stress in malignant melanoma and non-melanoma skin cancer," *British Journal of Dermatology*, vol. 148, no. 5, pp. 913–922, 2003.
- [4] T. J. James, M. A. Hughes, G. W. Cherry, and R. P. Taylor, "Evidence of oxidative stress in chronic venous ulcers," *Wound Repair and Regeneration*, vol. 11, no. 3, pp. 172–176, 2003.
- [5] Q. Zhou, U. Mrowietz, and M. Rostami-Yazdi, "Oxidative stress in the pathogenesis of psoriasis," *Free Radical Biology and Medicine*, vol. 47, no. 7, pp. 891–905, 2009.
- [6] J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent et al., "Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012," *European Journal of Cancer*, vol. 49, no. 6, pp. 1374–1403, 2013.
- [7] N. Graves and H. Zheng, "The prevalence and incidence of chronic wounds: a literature review," *Wound Practice and Research*, vol. 22, no. 1, pp. 14–19, 2014.
- [8] C. K. Sen, "Wound healing essentials: let there be oxygen," *Wound Repair and Regeneration*, vol. 17, no. 1, pp. 1–18, 2009.
- [9] R. Parisi, D. P. M. Symmons, C. E. M. Griffiths, and D. M. Ashcroft, "Global epidemiology of psoriasis: a systematic review of incidence and prevalence," *Journal of Investigative Dermatology*, vol. 133, no. 2, pp. 377–385, 2013.
- [10] D. Darr and I. Fridovich, "Free radicals in cutaneous biology," *Journal of Investigative Dermatology*, vol. 102, no. 5, pp. 671–675, 1994.
- [11] C. Lehner, R. Gehwolf, H. Tempfer et al., "Oxidative stress and blood-brain barrier dysfunction under particular consideration of matrix metalloproteinases," *Antioxidants and Redox Signaling*, vol. 15, no. 5, pp. 1305–1323, 2011.
- [12] K. R. Martin and J. C. Barrett, "Reactive oxygen species as double-edged swords in cellular processes: low-dose cell signaling versus high-dose toxicity," *Human and Experimental Toxicology*, vol. 21, no. 2, pp. 71–75, 2002.
- [13] R. Kohen and A. Nyska, "Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification," *Toxicologic Pathology*, vol. 30, no. 6, pp. 620–650, 2002.
- [14] E. H. Sarsour, M. G. Kumar, L. Chaudhuri, A. L. Kalen, and P. C. Goswami, "Redox control of the cell cycle in health and disease," *Antioxidants and Redox Signaling*, vol. 11, no. 12, pp. 2985–3011, 2009.
- [15] S. J. Green, L. F. Scheller, M. A. Marletta et al., "Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens," *Immunology Letters*, vol. 43, no. 1-2, pp. 87–94, 1994.
- [16] M.-M. Cals-Grierson and A. D. Ormerod, "Nitric oxide function in the skin," *Nitric Oxide*, vol. 10, no. 4, pp. 179–193, 2004.
- [17] J. J. P. Gille, P. Pasman, C. G. M. van Berkel, and H. Joenje, "Effect of antioxidants on hyperoxia-induced chromosomal breakage in Chinese hamster ovary cells: protection by carnosine," *Mutagenesis*, vol. 6, no. 4, pp. 313–318, 1991.
- [18] A. T. Black, J. P. Gray, M. P. Shakarjian, D. L. Laskin, D. E. Heck, and J. D. Laskin, "Increased oxidative stress and antioxidant expression in mouse keratinocytes following exposure to paraquat," *Toxicology and Applied Pharmacology*, vol. 231, no. 3, pp. 384–392, 2008.
- [19] F. Brehmer, H. A. Haenssle, G. Daeschlein et al., "Alleviation of chronic venous leg ulcers with a hand-held dielectric barrier discharge plasma generator (PlasmaDerm[®] VU-2010): results of a monocentric, two-armed, open, prospective, randomized and controlled trial (NCT01415622)," *Journal of the European*

- Academy of Dermatology and Venereology*, vol. 29, no. 1, pp. 148–155, 2015.
- [20] M. Klebes, J. Lademann, S. Philipp et al., “Effects of tissue-tolerable plasma on psoriasis vulgaris treatment compared to conventional local treatment: a pilot study,” *Clinical Plasma Medicine*, vol. 2, no. 1, pp. 22–27, 2014.
- [21] H. R. Metelmann, T. von Woedtke, R. Bussiahn et al., “Experimental recovery of CO₂-laser skin lesions by plasma stimulation,” *American Journal of Cosmetic Surgery*, vol. 29, pp. 52–56, 2012.
- [22] S. K. Mantena and S. K. Katiyar, “Grape seed proanthocyanidins inhibit UV-radiation-induced oxidative stress and activation of MAPK and NF- κ B signaling in human epidermal keratinocytes,” *Free Radical Biology and Medicine*, vol. 40, no. 9, pp. 1603–1614, 2006.
- [23] N. Kanda and S. Watanabe, “17 β -estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bcl-2 expression,” *Journal of Investigative Dermatology*, vol. 121, no. 6, pp. 1500–1509, 2003.
- [24] A. A. Shvedova, C. Kommineni, B. A. Jeffries et al., “Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes,” *Journal of Investigative Dermatology*, vol. 114, no. 2, pp. 354–364, 2000.
- [25] P. Boukamp, R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig, “Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line,” *The Journal of Cell Biology*, vol. 106, no. 3, pp. 761–771, 1988.
- [26] K.-D. Weltmann, E. Kindel, R. Brandenburg et al., “Atmospheric pressure plasma jet for medical therapy: plasma parameters and risk estimation,” *Contributions to Plasma Physics*, vol. 49, no. 9, pp. 631–640, 2009.
- [27] A. Schmidt, S. Dietrich, A. Steuer et al., “Non-thermal plasma activates human keratinocytes by stimulation of antioxidant and phase II pathways,” *The Journal of Biological Chemistry*, vol. 290, no. 11, pp. 6731–6750, 2015.
- [28] L. Bundscherer, K. Wende, K. Ottmüller et al., “Impact of non-thermal plasma treatment on MAPK signaling pathways of human immune cell lines,” *Immunobiology*, vol. 218, no. 10, pp. 1248–1255, 2013.
- [29] A. Maillat and S. Pervaiz, “Redox regulation of p53, redox effectors regulated by p53: a subtle balance,” *Antioxidants & Redox Signaling*, vol. 16, no. 11, pp. 1285–1294, 2012.
- [30] A. Matsuzawa and H. Ichijo, “Stress-responsive protein kinases in redox-regulated apoptosis signaling,” *Antioxidants and Redox Signaling*, vol. 7, no. 3–4, pp. 472–481, 2005.
- [31] K. Wende, A. Barton, S. Bekeschus et al., “Proteomic tools to characterize non-thermal plasma effects in eukaryotic cells,” *Plasma Medicine*, vol. 3, no. 1–2, pp. 81–95, 2013.
- [32] A. Schmidt-Bleker, J. Winter, S. Iseni, M. Dünbnier, K.-D. Weltmann, and S. Reuter, “Reactive species output of a plasma jet with a shielding gas device—combination of FTIR absorption spectroscopy and gas phase modelling,” *Journal of Physics D: Applied Physics*, vol. 47, no. 14, Article ID 145201, 2014.
- [33] H. Tresp, M. U. Hammer, K.-D. Weltmann, and S. Reuter, “Effects of atmosphere composition and liquid type on plasma-generated reactive species in biologically relevant solutions,” *Plasma Medicine*, vol. 3, no. 1–2, pp. 45–55, 2013.
- [34] H. Tresp, M. U. Hammer, J. Winter, K.-D. Weltmann, and S. Reuter, “Quantitative detection of plasma-generated radicals in liquids by electron paramagnetic resonance spectroscopy,” *Journal of Physics D: Applied Physics*, vol. 46, no. 43, Article ID 435401, 2013.
- [35] S. Bekeschus, S. Iseni, S. Reuter, K. Masur, and K.-D. Weltmann, “Nitrogen shielding of an argon plasma jet and its effects on human immune cells,” *IEEE Transactions on Plasma Science*, vol. 43, pp. 776–781, 2015.
- [36] S. Bekeschus, J. Kolata, A. Müller et al., “Differential viability of eight human blood mononuclear cell subpopulations after plasma treatment,” *Plasma Medicine*, vol. 3, no. 1–2, pp. 1–13, 2013.
- [37] X. Yan, Z. Xiong, F. Zou et al., “Plasma-induced death of HepG2 cancer cells: intracellular effects of reactive species,” *Plasma Processes and Polymers*, vol. 9, no. 1, pp. 59–66, 2012.
- [38] M. Yokoyama, K. Johkura, and T. Sato, “Gene expression responses of HeLa cells to chemical species generated by an atmospheric plasma flow,” *Biochemical and Biophysical Research Communications*, vol. 450, no. 4, pp. 1266–1271, 2014.
- [39] L. A. Pham-Huy, H. He, and C. Pham-Huy, “Free radicals, antioxidants in disease and health,” *International Journal of Biomedical Science*, vol. 4, no. 2, pp. 89–96, 2008.
- [40] L. Bundscherer, S. Bekeschus, H. Tresp et al., “Viability of human blood leukocytes compared with their respective cell lines after plasma treatment,” *Plasma Medicine*, vol. 3, no. 1–2, pp. 71–80, 2013.
- [41] B. D’Auréaux and M. B. Toledano, “ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis,” *Nature Reviews Molecular Cell Biology*, vol. 8, no. 10, pp. 813–824, 2007.
- [42] T.-C. Meng, T. Fukada, and N. K. Tonks, “Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo,” *Molecular Cell*, vol. 9, no. 2, pp. 387–399, 2002.
- [43] C. K. Sen and S. Roy, “Redox signals in wound healing,” *Biochimica et Biophysica Acta—General Subjects*, vol. 1780, no. 11, pp. 1348–1361, 2008.
- [44] A. E. K. Loo and B. Halliwell, “Effects of hydrogen peroxide in a keratinocyte-fibroblast co-culture model of wound healing,” *Biochemical and Biophysical Research Communications*, vol. 423, no. 2, pp. 253–258, 2012.
- [45] R. B. Mikkelsen and P. Wardman, “Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms,” *Oncogene*, vol. 22, no. 37, pp. 5734–5754, 2003.
- [46] H. J. Ahn, K. I. Kim, G. Kim, E. Moon, S. S. Yang, and J.-S. Lee, “Atmospheric-pressure plasma jet induces apoptosis involving mitochondria via generation of free radicals,” *PLoS ONE*, vol. 6, no. 11, Article ID e28154, 2011.
- [47] S. Bekeschus, T. von Woedtke, A. Kramer, K.-D. Weltmann, and K. Masur, “Cold physical plasma treatment alters redox balance in human immune cells,” *Plasma Medicine*, vol. 3, no. 4, pp. 267–278, 2013.
- [48] K. Priya Arjunan and A. Morss Clyne, “Hydroxyl radical and hydrogen peroxide are primarily responsible for dielectric barrier discharge plasma-induced angiogenesis,” *Plasma Processes and Polymers*, vol. 8, no. 12, pp. 1154–1164, 2011.
- [49] J. A. Daniel, M. Pellegrini, B.-S. Lee et al., “Loss of ATM kinase activity leads to embryonic lethality in mice,” *Journal of Cell Biology*, vol. 198, no. 3, pp. 295–304, 2012.
- [50] S. Ditch and T. T. Paull, “The ATM protein kinase and cellular redox signaling: beyond the DNA damage response,” *Trends in Biochemical Sciences*, vol. 37, no. 1, pp. 15–22, 2012.

- [51] J. R. Jeffers, E. Parganas, Y. Lee et al., "Puma is an essential mediator of p53-dependent and -independent apoptotic pathways," *Cancer Cell*, vol. 4, no. 4, pp. 321–328, 2003.
- [52] M. Ott, V. Gogvadze, S. Orrenius, and B. Zhivotovsky, "Mitochondria, oxidative stress and cell death," *Apoptosis*, vol. 12, no. 5, pp. 913–922, 2007.
- [53] T. Finkel, "Oxidant signals and oxidative stress," *Current Opinion in Cell Biology*, vol. 15, no. 2, pp. 247–254, 2003.
- [54] L. Xu, S. Chen, and R. C. Bergan, "MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer," *Oncogene*, vol. 25, no. 21, pp. 2987–2998, 2006.
- [55] L. Whitesell and S. L. Lindquist, "HSP90 and the chaperoning of cancer," *Nature Reviews Cancer*, vol. 5, no. 10, pp. 761–772, 2005.
- [56] J. Huot, F. Houle, D. R. Spitz, and J. Landry, "HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress," *Cancer Research*, vol. 56, no. 2, pp. 273–279, 1996.
- [57] S. Tukaj, D. Grüner, D. Zillikens, and M. Kasperkiewicz, "Hsp90 blockade modulates bullous pemphigoid IgG-induced IL-8 production by keratinocytes," *Cell Stress and Chaperones*, vol. 19, no. 6, pp. 887–894, 2014.
- [58] E. A. Voll, I. M. Ogden, J. M. Pavese et al., "Heat shock protein 27 regulates human prostate cancer cell motility and metastatic progression," *Oncotarget*, vol. 5, no. 9, pp. 2648–2663, 2014.
- [59] E. P. Rogakou, D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner, "DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139," *The Journal of Biological Chemistry*, vol. 273, no. 10, pp. 5858–5868, 1998.
- [60] I. M. Ward and J. J. Chen, "Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress," *The Journal of Biological Chemistry*, vol. 276, no. 51, pp. 47759–47762, 2001.
- [61] U. Rosani, E. Tarricone, P. Venier et al., "Atmospheric-pressure cold plasma induces transcriptional changes in ex vivo human corneas," *PLoS ONE*, vol. 10, no. 7, Article ID e0133173, 2015.
- [62] B. Halliwell, "Oxidative stress and cancer: have we moved forward?" *Biochemical Journal*, vol. 401, no. 1, pp. 1–11, 2007.
- [63] J. B. De Haan, P. J. Crack, N. Flentjar, R. C. Iannello, P. J. Hertzog, and I. Kola, "An imbalance in antioxidant defense affects cellular function: the pathophysiological consequences of a reduction in antioxidant defense in the glutathione peroxidase-1 (Gpx1) knockout mouse," *Redox Report*, vol. 8, no. 2, pp. 69–79, 2003.
- [64] R. J. Aitken, "Gpx5 protects the family jewels," *Journal of Clinical Investigation*, vol. 119, no. 7, pp. 1849–1851, 2009.
- [65] T. Rammung, H. G. Hansen, K. Nagata, L. Ellgaard, and C. Appenzeller-Herzog, "GPx8 peroxidase prevents leakage of H₂O₂ from the endoplasmic reticulum," *Free Radical Biology and Medicine*, vol. 70, pp. 106–116, 2014.
- [66] J. Ryu, S. G. Park, B. C. Park, M. Choe, K.-S. Lee, and J.-W. Cho, "Proteomic analysis of psoriatic skin tissue for identification of differentially expressed proteins: up-regulation of GSTP1, SFN and PRDX2 in psoriatic skin," *International Journal of Molecular Medicine*, vol. 28, no. 5, pp. 785–792, 2011.
- [67] Y. Dincer, Y. Erzin, S. Himmetoğlu, K. N. Gunes, K. Bal, and T. Akçay, "Oxidative DNA damage and antioxidant activity in patients with inflammatory bowel disease," *Digestive Diseases and Sciences*, vol. 52, no. 7, pp. 1636–1641, 2007.
- [68] A. Pellacani, P. Wiesel, A. Sharma et al., "Induction of heme oxygenase-1 during endotoxemia is downregulated by transforming growth factor- β 1," *Circulation Research*, vol. 83, no. 4, pp. 396–403, 1998.
- [69] T. Jacks and R. A. Weinberg, "Cell-cycle control and its watchman," *Nature*, vol. 381, no. 6584, pp. 643–644, 1996.
- [70] A. Schmidt, S. Bekeschus, T. von Woedtke, and S. Hasse, "Cell migration and adhesion of a human melanoma cell line is decreased by cold plasma treatment," *Clinical Plasma Medicine*, vol. 3, no. 1, pp. 24–31, 2015.
- [71] J. M. Brandner, S. Kief, C. Grund et al., "Organization and formation of the tight junction system in human epidermis and cultured keratinocytes," *European Journal of Cell Biology*, vol. 81, no. 5, pp. 253–263, 2002.
- [72] L. Langbein, C. Grund, C. Kuhn et al., "Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom," *European Journal of Cell Biology*, vol. 81, no. 8, pp. 419–435, 2002.
- [73] C. M. Nelson and C. S. Chen, "Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal," *FEBS Letters*, vol. 514, no. 2-3, pp. 238–242, 2002.
- [74] N. S. Harhaj and D. A. Antonetti, "Regulation of tight junctions and loss of barrier function in pathophysiology," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 7, pp. 1206–1237, 2004.
- [75] S. Zeissig, N. Bürgel, D. Günzel et al., "Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease," *Gut*, vol. 56, no. 1, pp. 61–72, 2007.
- [76] M. P. Fink and R. L. Delude, "Epithelial barrier dysfunction: a unifying theme to explain the pathogenesis of multiple organ dysfunction at the cellular level," *Critical Care Clinics*, vol. 21, no. 2, pp. 177–196, 2005.
- [77] C. Coisne and B. Engelhardt, "Tight junctions in brain barriers during central nervous system inflammation," *Antioxidants and Redox Signaling*, vol. 15, no. 5, pp. 1285–1303, 2011.
- [78] K. D. Rochfort, L. E. Collins, R. P. Murphy, and P. M. Cummins, "Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions," *PLoS ONE*, vol. 9, no. 7, Article ID e101815, 2014.
- [79] R. Rao, "Oxidative stress-induced disruption of epithelial and endothelial tight junctions," *Frontiers in Bioscience*, vol. 13, no. 18, pp. 7210–7226, 2008.
- [80] L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal, "Global cancer statistics, 2012," *CA: A Cancer Journal for Clinicians*, vol. 65, no. 2, pp. 87–108, 2015.
- [81] P. A. Coulombe and C.-H. Lee, "Defining keratin protein function in skin epithelia: epidermolysis bullosa simplex and its aftermath," *Journal of Investigative Dermatology*, vol. 132, no. 3, pp. 763–775, 2012.
- [82] M. Moravcová, A. Libra, J. Dvořáková et al., "Modulation of keratin 1, 10 and involucrin expression as part of the complex response of the human keratinocyte cell line HaCaT to ultraviolet radiation," *Interdisciplinary Toxicology*, vol. 6, no. 4, pp. 203–208, 2013.
- [83] S. Bekeschus, A. Schmidt, L. Bethge et al., "Redox stimulation of human THP-1 monocytes in response to cold physical plasma," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 5910695, 11 pages, 2016.

- [84] S. Tamiji, J.-C. Beauvillain, L. Mortier et al., "Induction of apoptosis-like mitochondrial impairment triggers antioxidant and Bcl-2-dependent keratinocyte differentiation," *Journal of Investigative Dermatology*, vol. 125, no. 4, pp. 647–658, 2005.
- [85] J. S. Breitenbach, M. Rinnerthaler, A. Trost et al., "Transcriptome and ultrastructural changes in dystrophic epidermolysis bullosa resemble skin aging," *Aging*, vol. 7, no. 6, pp. 389–411, 2015.
- [86] F. J. D. Smith, A. D. Irvine, A. Terron-Kwiatkowski et al., "Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris," *Nature Genetics*, vol. 38, no. 3, pp. 337–342, 2006.
- [87] J. P. Thyssen and S. Kezic, "Causes of epidermal filaggrin reduction and their role in the pathogenesis of atopic dermatitis," *Journal of Allergy and Clinical Immunology*, vol. 134, no. 4, pp. 792–799, 2014.
- [88] W. P. Vermeij, A. Alia, and C. Backendorf, "ROS quenching potential of the epidermal cornified cell envelope," *Journal of Investigative Dermatology*, vol. 131, no. 7, pp. 1435–1441, 2011.
- [89] E. A. O'Toole, "Extracellular matrix and keratinocyte migration," *Clinical and Experimental Dermatology*, vol. 26, no. 6, pp. 525–530, 2001.
- [90] W. C. Parks, "Matrix metalloproteinases in repair," *Wound Repair and Regeneration*, vol. 7, no. 6, pp. 423–432, 1999.
- [91] W. C. Parks, Y. S. López-Boado, and C. L. Wilson, "Matri lysin in epithelial repair and defense," *Chest*, vol. 120, no. 1, supplement, pp. 36S–41S, 2001.
- [92] A. B. Wysocki, L. Staiano-Coico, and F. Grinnell, "Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9," *Journal of Investigative Dermatology*, vol. 101, no. 1, pp. 64–68, 1993.
- [93] J. Rohl and R. Murray, "Matrix metalloproteinases during wound healing—a double edged sword," *Wound Practice and Research*, vol. 21, no. 4, pp. 174–182, 2013.
- [94] C. Gebhardt, J. Németh, P. Angel, and J. Hess, "S100A8 and S100A9 in inflammation and cancer," *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1622–1631, 2006.
- [95] D. N. Granger, T. Vowinkel, and T. Petnehazy, "Modulation of the inflammatory response in cardiovascular disease," *Hypertension*, vol. 43, no. 5, pp. 924–931, 2004.
- [96] B. Behm, P. Babilas, M. Landthaler, and S. Schreml, "Cytokines, chemokines and growth factors in wound healing," *Journal of the European Academy of Dermatology and Venereology*, vol. 26, no. 7, pp. 812–820, 2012.
- [97] Y.-C. Liao, W.-G. Liang, F.-W. Chen, J.-H. Hsu, J.-J. Yang, and M.-S. Chang, "IL-19 induces production of IL-6 and TNF- α and results in cell apoptosis through TNF- α ," *Journal of Immunology*, vol. 169, no. 8, pp. 4288–4297, 2002.
- [98] R. W. Tarnuzzer and G. S. Schultz, "Biochemical analysis of acute and chronic wound environments," *Wound Repair and Regeneration*, vol. 4, no. 3, pp. 321–325, 1996.
- [99] C. C. Finnerty, D. N. Herndon, R. Przkora et al., "Cytokine expression profile over time in severely burned pediatric patients," *Shock*, vol. 26, no. 1, pp. 13–19, 2006.
- [100] B. A. Mast and G. S. Schultz, "Interactions of cytokines, growth factors, and proteases in acute and chronic wounds," *Wound Repair and Regeneration*, vol. 4, no. 4, pp. 411–420, 1996.
- [101] M. C. Peral, M. M. Rachid, N. M. Gobbato, M. A. Huaman Martinez, and J. C. Valdez, "Interleukin-8 production by polymorphonuclear leukocytes from patients with chronic infected leg ulcers treated with *Lactobacillus plantarum*," *Clinical Microbiology and Infection*, vol. 16, no. 3, pp. 281–286, 2010.
- [102] R. H. Weisbart, L. Kwan, D. W. Golde, and J. C. Gasson, "Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants," *Blood*, vol. 69, no. 1, pp. 18–21, 1987.
- [103] A. Barton, K. Wende, L. Bundscherer et al., "Non-thermal plasma increases expression of wound healing related genes in a keratinocyte cell line," *Plasma Medicine*, vol. 3, no. 1-2, pp. 125–136, 2013.
- [104] M. F. Nold, C. A. Nold-Petry, J. A. Zepp, B. E. Palmer, P. Bufler, and C. A. Dinarello, "IL-37 is a fundamental inhibitor of innate immunity," *Nature Immunology*, vol. 11, no. 11, pp. 1014–1022, 2010.
- [105] V. Shreedhar, T. Giese, V. W. Sung, and S. E. Ullrich, "A cytokine cascade including prostaglandin E2, IL-4, and IL-10 is responsible for UV-induced systemic immune suppression," *The Journal of Immunology*, vol. 160, no. 8, pp. 3783–3789, 1998.
- [106] S. Barrientos, O. Stojadinovic, M. S. Golinko, H. Brem, and M. Tomic-Canic, "Growth factors and cytokines in wound healing," *Wound Repair and Regeneration*, vol. 16, no. 5, pp. 585–601, 2008.
- [107] K. Wende, S. Straßenburg, B. Haertel et al., "Atmospheric pressure plasma jet treatment evokes transient oxidative stress in HaCaT keratinocytes and influences cell physiology," *Cell Biology International*, vol. 38, no. 4, pp. 412–425, 2014.

Review Article

Use of Carnosine for Oxidative Stress Reduction in Different Pathologies

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The main properties and biological effects of the antioxidant carnosine, the natural dipeptide β -alanyl-L-histidine, are considered. Data on the effective use of carnosine in different pathologies are presented. Special attention is paid to issues of use of carnosine in neurologic and mental diseases, in alcoholism as well as in physiological states accompanied by activation of free-radical processes and formation of oxidative stress.

1. Oxidative Stress and Its Correction by Antioxidants

The pathogenesis of most diseases involves excess activation of free-radical processes and disturbance of functioning of the organism's antioxidant protection systems. This leads to increase in the level of reactive oxygen species (ROS) and forming of oxidative stress (OS). Mechanisms of OS formation in different pathologies are quite universal and are especially linked with disturbance of homeostasis and redox processes. The characterization of ROS, their types, main sources of formation in the organism, properties, and transformations are well described in various publications [1–3]. The main targets of damage under conditions of OS are proteins, lipids, carbohydrates, and nucleic acids.

It is well known that under normal physiological conditions ROS carry out important regulatory functions in the organism [1, 4]. However, under uncontrolled increase in ROS they interact with biomolecules, leading to their oxidative modifications. Products of such modification usually lose ability to carry out their functions. These products serve as “markers of oxidative stress,” and they include carbonylated, nitrosylated, and glycated proteins; aggregates due to crosslinking of protein molecules; products of lipid peroxidation (malondialdehyde, diene conjugates, hydroxynonenal, etc.); various types of hybrid adducts; advanced glycation end

products (AGE products); dihydroguanosine, homocysteine, and so forth [1, 5]. All these products of oxidative damage of biomolecules are resistant to destructions and accumulate in cells, complicating their vital functions. Their neutralization can play an important role in correction of oxidative stress.

The search for and development of ways to correct oxidative stress is a relevant problem of modern medicine. One way that can be effective under clinical conditions involves the use of the so-called antioxidants, substances that neutralize ROS, reducing their reactivity in the organism. Despite the very large number of known antioxidants, to choose one for effective use in a specific clinical situation is quite difficult. This is caused by the abundance of factors of modification of macromolecules under OS. In addition, the mechanism of action of an antioxidant changes depending on its chemical structure, bioavailability, and damage rate of redox processes and severity of oxidative stress in the organism.

It was found that under conditions of oxidative stress, the endogenous systems of antioxidant response of the organism are activated through transcription factor Nrf2 [6]. Consequently, expression of endogenous antioxidant enzyme genes increased, increasing cellular defenses against detrimental redox modulations [7, 8].

The human endogenous antioxidant response system can regulate the amount of reactive species tightly and

minimize related cellular damage. But the role of exogenous antioxidants is also important. It was found that exogenous antioxidants have a priming effect on the antioxidant response system [9]. Working together with the endogenous antioxidant response system, exogenous antioxidants allow for a more enhanced and efficient defense against detrimental redox modulations.

Extensive experimental and clinical material on the use of antioxidants has accumulated. In medicine, they are mainly used as additional agents to basic therapy. Many medications, in addition to their main therapeutic effect, also manifest antioxidant properties. However, depending on conditions and concentration, antioxidants can also show the opposite to antioxidant effect, that is, prooxidant action. Carotenes are polyunsaturated compounds; therefore, they can be oxidized via a radical mechanism and act as prooxidants [10]. Under certain conditions, for example, in the presence of mixed-valence metal ions, prooxidative effect is shown by ascorbate. Vitamin E as an antioxidant is most effective in a complex with other fat- and water-soluble reductants (ascorbic acid, ubiquinol, and flavonoids) in whose absence it is quickly inactivated or transforms into tocopheryl radical capable of initiating new chains of oxidation of unsaturated lipids; that is, it becomes a prooxidant as well [5, 11].

Adhering to the correct dosage of an antioxidant, as with every pharmacologically active compound, is very important. There are examples of ineffective use of antioxidants in the treatment of some pathologies accompanied by decrease in the level of antioxidants in blood plasma. So, clinical trials of the treatment of Alzheimer's disease with the addition of the well-known antioxidants lycopene and vitamins A, C, and E did not show positive results and even showed progressive decrease in cognitive function in the study participants in some cases [12]. Though these results do not favor antioxidant therapy, it might be due to prooxidative effects of these antioxidants under these conditions as well as terms and the scheme of their administration.

The choice of a specific antioxidant and exact indications and contraindications are still insufficiently developed for every specific disease. There is no information on the interaction of pharmaceuticals of natural origin with synthetic medications. In addition, antioxidants can cause allergic reactions, be toxic, and show low efficiency, and standardization is not always possible; the possibility of overdose also remains. Therefore, the search for substances with maximum antioxidant action and minimum side effects under conditions of OS continues and remains an important problem. Ideally, the antioxidant should show considerable antioxidant action within a broad range of concentration, be natural and hydrophilic, have good bioavailability, be nontoxic, not form toxic products during interaction with reactive oxygen species, not have negative effects in case of overdose, and have good compatibility with other medications.

In spite of the fact that the use of antioxidants in clinical practice does not always show positive results, the concept of use of antioxidant therapy is still relevant and has the potential for effective treatment of a number of disorders accounting for pathophysiological mechanisms of their forming and development.

2. Main Properties and Biological Effects of Carnosine

Numerous references as well as our own work experience indicate that the antioxidant carnosine, the natural dipeptide β -alanyl-L-histidine, meets almost all requirements for an ideal antioxidant. It is synthesized and contained in human muscle and nervous tissues, is easily absorbed in the digestive tract, penetrates through blood-brain barrier, and has high bioavailability and membrane-stabilizing action. Carnosine is a low molecular weight hydrophilic antioxidant of direct action, though it can also have an impact on the antiradical protection system of the organism [13]. Results of experiments on rats showed that carnosine accelerates the metabolizing of cortisol and noradrenaline released into blood of animals under stress, showing the mediation effect of carnosine [14]. Decrease in level of stress hormones in blood leads to a decrease in the severity of OS. In addition, carnosine is not addictive; there is no danger of overdose, and it does not accumulate in the organism during long-term administration because its surplus is cleaved by the enzyme carnosinase into amino acids that are easily eliminated from the organism [13]. However, it is noteworthy that there are cases of development of carnosinemia, a rare autosomal recessive [15] metabolic disorder [16] caused by a deficiency of carnosinase. This disorder results in an excess of carnosine in the urine, blood, and nervous tissue [17], and variety of neurological symptoms have been associated with carnosinemia [16, 18]; that is, under certain conditions, carnosine can exert negative effects.

There are publications in which positive biological effects of carnosine are explained by its pH-buffering properties [19]. However, carnosine is a buffer not only for protons, but also a buffer for mixed-valence metal ions and reactive oxygen species [20]. The ability of carnosine to form complexes with bivalent metals is known: with ions of copper, cobalt, manganese, and cadmium [21]. In another work, it was shown that carnosine binds iron ions [22]. Because ions of metals take an active part in many metabolic processes and can activate free-radical processes, the ability of carnosine to regulate the level of mixed-valence metal ions in the organism is one more important property of carnosine that confirms its antioxidant status.

Further, the antiglycating [23, 24] and the anticrosslinking [25] properties of carnosine have been shown, which are, in essence, reflections of its antioxidant effects, the ability to block oxidation of biomolecules.

Great contributions to the study of molecular mechanisms of protection of biomolecules by carnosine were made by Aldini et al. [26, 27]. Using liquid chromatography/electrospray ionization tandem mass spectrometry, they showed that carnosine and related peptides act as quenchers of reactive and cytotoxic carbonyl species through its ability to form adducts with them. This suggested that carnosine is a protector of biomolecules from oxidative/carbonyl stress. The ability of carnosine to react with carbonyls of proteins (termed "carnosinylation" of proteins) was reported by other authors [28], who considered this property of carnosine important for inactivation/removal of damaged proteins.

In culture of human cells, it has been shown that addition of carnosine into the medium at concentrations close to physiological (20–50 mM) increases longevity of the cells [29]. This was attributed to either reduction of the length of telomere fragments of chromosomes, lost by the cell during every doubling, or decrease in methylation of DNA. It could not be ruled out that carnosine decreases the accumulation of some other changes in DNA, whose accumulation above a critical point leads to termination of divisions.

It has also been reported that carnosine prevents toxic effects of hyperhomocysteinemia in rats [30]. It is known that homocysteine is a potent initiator of oxidative stress in many tissues. However, the molecular mechanism of such protection is not clear. Perhaps carnosine modulates affinity of glutamate receptors to homocysteine, prevents accumulation of ROS, or has other protective mechanisms. But it has been shown that these effects of carnosine are not connected with the improvement of homocysteine metabolism or the decrease in its concentration.

Data on the study of biological effects of carnosine shows that molecular mechanisms of its effects cannot always be explained only by antioxidant action. The exact molecular mechanisms of some effects of carnosine observed in experiment should be found. At the same time, obvious positive effect of this dipeptide allows using carnosine widely already in routine clinical practice.

Prospects for the use of carnosine in the treatment of some pathologies are reported in a report by Quinn et al. [31]. Data on the possible physiological role of carnosine based on its biochemical properties and study of the therapeutic potential of carnosine in a number of pathologies accompanied by oxidative or carbonyl stress are presented in a review by Boldyrev et al. [32].

3. Clinical Use of Carnosine

Researchers of the Kharkov Physiotherapeutic Institute were the creators of the first injection dosage form of carnosine. During subcutaneous injection of 0.5–1.0 mg, high therapeutic effectiveness in treatment of infectious and rheumatic polyarthritides and ulcer of the gastrointestinal tract was obtained [33]. Later, positive effect of carnosine in healing wounds of lung tissue was shown [34]. Japanese researchers played a great role in the study of a wound healing effect of carnosine. They created the agent Z-103 based on a complex of carnosine and zinc ions (L-carnosine- Zn^{2+}) that has considerable antiulcer effect and reduces damage to the stomach lining induced by different forms of stress and chemical agents [35]. Japanese scientists also have priority for the use of carnosine in cancer diseases [36]. Carnosine combined with radiotherapy in treatment of patients with breast cancer considerably reduced side effects of radiation, injury of skin and intoxication of the organism, and increases immunity and increases likelihood of healing of treatment severalfold. Carnosine was effective also for the prevention of the cachexia caused by chemotherapy in cancer therapy [36]. In experimental studies on cultures of tumor cells, it has been shown that carnosine suppresses proliferation

of human glioblastoma completely, and it decreases the level of reactive oxygen species and increases the activity of mitochondrial superoxide dismutase in tumor cells [37]. Possible mechanisms of inhibition of tumor cells growth by carnosine were considered recently [38].

The ability of carnosine to prevent age-related phacocytosis of the eye has been shown. Free-radical reactions leading to oxidative modification of lipids and proteins of crystallins of tissues of the eye are a basic reason for phacocytosis in senile cataract. In the development of cataract in the crystalline lens, a considerable decrease in the endogenous antioxidants glutathione and carnosine occurs. In clinical trials, efficiency of the agent in the form of eye drops for treatment of cataract containing 5% solution of carnosine has been shown. Later, when developing eye drops, a natural dipeptide, the relative of carnosine N-acetylcarnosine [13, 39], was successfully applied. Also, Chinese authors report the ability of carnosine to prevent development of cataract [40].

Carnosine in the form of 5% solution was also successfully used for the treatment of seasonal allergic rhinoconjunctivitis; thus, the need for additional administration of antihistaminic medications disappeared [13]. Carnosine found application also for the treatment of inflammatory diseases of parodontium for patients with fixed orthodontic designs: 5% solution of the dipeptide had a substantial immunocorrecting effect and increased activity of enzymes of antioxidant protection in saliva [41].

Carnosine was effective in treatment of diabetic complications in experimental studies on rats with streptozotocin-induced diabetes. It was found that treatment with carnosine (1 g/kg body weight per day) restored carnosine kidney levels, prevented podocyte loss, restrained glomerular apoptosis, and reduced expression of Bax and cytochrome C [42]. In rats with experimental diabetic retinopathy, carnosine exerted considerable protective effect on cells of capillaries of the retina [43]. Administration of carnosine (100 mg/kg injected daily) to mice with type 2 diabetes, to which experiment wounds were made (6 millimeters), enhanced significantly healing of wounds, which was accompanied by increased expression of growth factors and cytokine genes involved in wound healing [44].

Carnosine is applied successfully in cardiological practice. Addition of L-carnosine in cardioplegic solution during stopped heart operations allows increasing the operation duration severalfold without signs of necrotic damage of tissues of the heart in the operative field [45].

In experiments on rats with isoproterenol-induced myocardial infarction, it was shown that preliminary administration of carnosine (250 mg/kg/day i.p.) reduces cardiac toxicity of isoproterenol due to reduction of oxidative stress [46]. Use of carnosine in metabolic syndrome is promising, a state accompanied by oxidative stress and inflammation leading to development of diabetes and cardiovascular diseases [47]. There are also data indicating that carnosine has nephroprotective properties [48]. That report provides results of studies concerning the role of carnosine in kidney diseases, particularly in ischemia/reperfusion induced acute renal

failure, diabetic nephropathy, gentamicin-induced nephrotoxicity, and blood pressure regulation.

Currently in Russia, a tableted dietary supplement under the name Sevitin is applied as a source of carnosine. It has been shown that this agent promotes recovery of cerebral circulation in chronic discirculatory encephalopathy and has a regulating effect on the activity of the immune system [49]. Studies are carried out focusing on obtaining new carnosine-containing agents for use under clinical conditions. There is a report on the creation and testing of nanocomplexes containing carnosine included in the structure of phospholipid nanostructures [50]. Use of such nanocomplexes provides resistance of carnosine to the action of carnosinase during its supply to the destination, which can significantly increase the influence of the dipeptide.

Recently, the question of reaching the effective concentrations of carnosine in tissues during its injection into the organism was specially studied on mice of C57 Black/6 line. It was shown that, after intraperitoneal administration of the agent at dose 1g/kg, its maximum concentration in blood plasma is reached in 15 minutes. It was found that administration of exogenous carnosine could considerably increase its concentration in the brain: the maximum concentration of carnosine in the brain is reached 6 hours after injection, when the concentration of the agent in blood is the minimum [51].

4. Use of Carnosine in Neurologic and Mental Disorders

It is known that OS develops in Parkinson's and Alzheimer's diseases [52], acute ischemic stroke [53], schizophrenia [54], depression [55], addictive disorders, alcoholism [56–58], and so forth. Cells of the nervous system are very sensitive to free-radical oxidation due to many factors: high intensity of metabolic processes and high level of oxygen consumption; large amounts of lipids with polyunsaturated fatty acids; increased content of bound iron ions (oxidation inducers); low content of its transporters; formation of ROS during cellular metabolism of secondary messengers in neuronal cells; participation of free radicals in neuroregulation; low level of antioxidant protection in comparison with cells of other organs. This initiates an excitotoxic "chain reaction" in which neurons continually experience excessive extracellular glutamate levels and so forth [3, 5, 59].

This determines the special need for the protection of cells of nervous tissue against free-radical oxidation by natural antioxidants able to penetrate through the blood-brain barrier, such as carnosine.

Positive results were obtained during carnosine addition (2.0 g/day) to basic therapy of patients with chronic discirculatory encephalopathy. Such treatment led to increase in resistance of lipoproteins of blood plasma against Fe^{2+} -induced oxidation, stabilization of erythrocytes against acid-induced hemolysis, intensification of respiratory burst of leukocytes, strengthening of endogenous antioxidant protection of the organism, and improvement of cognitive functions of the brain of patients [49], that is, carnosine exerted antioxidant,

membrane-stabilizing, and immunomodulatory effects in this pathology.

Considerable improvement of clinical state of patients was observed during administration of carnosine at dose of 1.5 g/days for 30 days in addition to traditional therapy in treatment of Parkinson's disease [60]. Use of carnosine reduced toxic effects of basic therapy (side effects of antiparkinsonian agents). In patients, a statistically significant reduction of neurologic symptoms (improvement of coordination of movements) was observed. Positive correlation between activation of antioxidant enzyme of superoxide dismutase in erythrocytes and decrease in neurologic symptoms was revealed. Addition of carnosine in the scheme of treatment led to reliable decrease in hydroperoxides in lipoproteins of blood plasma and considerably increased the resistance low-density and very-low-density lipoproteins against Fe^{2+} -induced oxidation and also reduction in amount of oxidized proteins in blood plasma. Thus, addition of carnosine to basic therapy not only improved considerably clinical indices, but also elevated the antioxidant status of the organism in patients with Parkinson's disease.

Carnosine was reported to have application also in schizophrenia. A randomized double-blind placebo-controlled study revealed that carnosine inclusion (2.0 g/days) as an addition to basic therapy in treatment of patients with schizophrenia improved their cognitive functions [61].

The protective activity of carnosine against zinc-induced neurotoxicity and its molecular mechanisms such as cellular Zn influx and Zn-induced gene expression were investigated using hypothalamic neurons (GT1-7 cells) [62]. The findings showed that carnosine could be effective in the treatment of vascular dementia, as Zn-induced neurotoxicity plays a crucial role in the pathogenesis of this disorder, and carnosine inhibits Zn-induced neuronal death.

Dietary supplementation with carnosine has been shown to suppress stress in animals and improve behaviour, cognition, and well-being in human subjects [63]. These results allow with great confidence assuming efficiency of treatment using carnosine for stress-related and depressive disorders.

5. Correction of Oxidative Stress with Carnosine in Alcoholic Patients

It has been reported that in alcoholic patients oxidative stress contributes strongly to forming somatic complications [64], disturbance of immune status [65], and induction of apoptosis [66]. In alcoholism, formation of OS can be increased by ethanol, the concentration of which significantly exceeds the norm in patients, as well as the toxic metabolite of ethanol-acetaldehyde, whose level also increases in the organism during alcoholic intoxication. Acetaldehyde can bind with many biological molecules (proteins of plasma, hemoglobin, factors of coagulant system of blood, lipids, etc.), forming with them aldehydic adducts that are deposited and accumulated in different tissues (liver, brain, heart, muscles, and intestines) [67, 68].

High indices of oxidative modification of biomolecules of membranes of erythrocytes and blood serum were found in alcoholic patients who were in the state of abstinence [69]. In other works, elevated content of carbonylated proteins and activity of aminotransferases of blood serum were revealed in patients with alcoholic delirium who were infected with hepatitis C or HIV virus [70]. The relationship between level of oxidation (carbonylation) of proteins of blood plasma and severity of manifestations of abstinence syndrome in patients was reported [71]. There is an opinion that a metabolic basis of developing alcoholic psychosis is the accumulation of acetaldehyde which, interacting with serotonin, forms toxic products having hallucinogenic properties [72]. It is known that, in patients with alcohol addiction, hyperhomocysteinemia is observed [73, 74]. The elevated concentrations of homocysteine stimulate entrance of Ca^{2+} and increase in ROS in the cytoplasm of neurons, which aggravates the state of OS. It has been reported that, in homocysteinemia, the functional activity of both nervous and immune systems of the organism decreases [30].

Thus, the activation of free-radical processes leading to accumulation of products of oxidative modification of biomolecules contributes considerably to the clinical course of alcoholism and can determine its features, which makes the study of effects of antioxidants in this pathology extremely important.

We have carried out several investigations on the effects of carnosine in alcoholism. In experiments *in vitro*, it is shown that addition of carnosine in tests with blood of alcoholics leads to increase in resistance of erythrocytes to acid hemolysis, promoting preservation of normal morphology of these cells [75].

A placebo-controlled study of the efficiency of carnosine in correction of OS in patients with alcohol addiction at the stage of forming of remission has been published [76]. Patients after basic treatment received carnosine at a dose of 1.2 g/day for one month before being released from the hospital. It was found that, after treatment in hospital, OS remained at a high level in patients. One month afterwards, during investigation in comparison group (patients who did not receive any agents at the stage of remission formation), severity of OS remained at the same level, as at baseline. In the patient group who received carnosine, reliable decrease in carbonylated proteins and products of lipid peroxidation (LP) in blood plasma to values corresponding to healthy persons was found. Intake of carnosine by patients for one month also led to an increase in the activity of superoxide dismutase of plasma and decrease in the activity of aminotransferases of blood serum. These results show that intake of carnosine effectively reduces severity of OS in the organism of alcoholic patients. Undesirable side effects were not observed. The mechanism of positive effect of carnosine on severity of OS in alcoholic patients remains unclear. However, our data on the ability of carnosine to prevent oxidative damage of proteins and lipids of blood induced by ethanol or acetaldehyde *in vitro* [77] show the ability of this dipeptide to protect biomolecules against direct toxic effects of ethanol and its metabolites.

6. Use of Carnosine in Physiological States Accompanied by Activation of Free-Radical Processes

Oxidative stress can develop not only in pathological processes, but also during considerable physical loads and during physiological aging of the organism. Therefore, carnosine now finds broad application as a general health-improving agent for healthy people under conditions of physical and psychological stress, during the impact of various adverse factors, and under extreme conditions. Carnosine is applied for the acceleration of recovery of tired muscles and increase in their working capacity in athletes [78] and in healthy elderly persons with active lifestyle [79]. Under experimental conditions, the geroprotective effect of carnosine has been shown. In experiments with the use of a specially bred line of rapidly aging mice, it was found that inclusion of carnosine in their diet leads to delay of aging of the animals due to increase in their antioxidant status [80].

The geroprotective effect of carnosine is mentioned in many publications where antioxidant, antiglycating, and anti-crosslinking properties of carnosine are considered, because it was proven in the course of aging of the organism products of carbonylation, glycation, and cross-linking accumulate, which are well neutralized by carnosine.

Developments on the use of carnosine in the cosmetic industry are promising, which is confirmed by the available data on the ability of carnosine to prevent structural changes of collagen in skin and to prevent loss of its elasticity [81].

The cited data on successful use of carnosine in various pathologies and in physiological states accompanied by activation of free-radical oxidation shows prospects for its use as an effective antioxidant, a protector of tissues against various adverse factors inducing development of oxidative stress. Carnosine reduces action of factors whose excess in a cell has toxic effects.

Abbreviations

ROS:	Reactive oxygen species
OS:	Oxidative stress
AGE products:	Advanced glycation end products
Nrf2:	Nuclear factor (erythroid-derived 2) like 2.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, UK, 4th edition, 2007.
- [2] M. T. Lin and M. F. Beal, "Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases," *Nature*, vol. 443, no. 7113, pp. 787–795, 2006.
- [3] A. A. Boldyrev, "Oxidative stress and brain," *Sorosovskij Obrazovatel'nyj Zhurnal*, vol. 7, no. 4, pp. 21–28, 2001 (Russian).
- [4] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.
- [5] E. B. Menshchikova, V. Z. Lankin, and N. K. Zenkov, *Oxidative Stress. Pro-Oxidants and Antioxidants*, Slovo, Moscow, Russia, 2006 (Russian).
- [6] T. W. Kensler, N. Wakabayashi, and S. Biswal, "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway," *Annual Review of Pharmacology and Toxicology*, vol. 47, pp. 89–116, 2007.
- [7] J. W. Kaspar, S. K. Niture, and A. K. Jaiswal, "Nrf2:Keap1 signaling in oxidative stress," *Free Radical Biology and Medicine*, vol. 47, no. 9, pp. 1304–1309, 2009.
- [8] C. Gorrini, I. S. Harris, and T. W. Mak, "Modulation of oxidative stress as an anticancer strategy," *Nature Reviews Drug Discovery*, vol. 12, no. 12, pp. 931–947, 2013.
- [9] S. K. Niture, R. Khatri, and A. K. Jaiswal, "Regulation of Nrf2—an update," *Free Radical Biology and Medicine*, vol. 66, pp. 36–44, 2014.
- [10] G. M. Lowe, K. Vlismas, and A. J. Young, "Carotenoids as prooxidants?" *Molecular Aspects of Medicine*, vol. 24, no. 6, pp. 363–369, 2003.
- [11] V. W. Bowry, K. U. Ingold, and R. Stocker, "Vitamin E in human low-density lipoprotein. When and how this antioxidant becomes a pro-oxidant," *Biochemical Journal*, vol. 288, no. 2, pp. 341–344, 1992.
- [12] T. Persson, B. O. Popescu, and A. Cedazo-Minguez, "Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail?" *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 427318, 11 pages, 2014.
- [13] A. A. Boldyrev, *Carnosine—Unraveled Mystery of Nature*, IKAR, Moscow, Russia, 2009 (Russian).
- [14] K. Nagai, T. Suda, K. Kawasaki, and Y. Yamaguchi, "Acceleration of metabolism of stress-related substances by L-carnosine," *Journal of the Physiological Society of Japan*, vol. 52, no. 7, pp. 221–228, 1990.
- [15] S. M. Willi, Y. Zhang, J. B. Hill, M. C. Phelan, R. C. Michaelis, and K. R. Holden, "A deletion in the long arm of chromosome 18 in a child with serum carnosinase deficiency," *Pediatric Research*, vol. 41, no. 2, pp. 210–213, 1997.
- [16] T. L. Perry, S. Hansen, B. Tischler, R. Bunting, and K. Berry, "Carnosinemia—a new metabolic disorder associated with neurologic disease and mental defect," *The New England Journal of Medicine*, vol. 277, no. 23, pp. 1219–1227, 1967.
- [17] L. R. Gjessing, H. A. Lunde, L. Mørkrid, J. F. Lenney, and O. Sjaastad, "Inborn errors of carnosine and homocarnosine metabolism," *Journal of Neural Transmission*, vol. 29, pp. 91–106, 1990.
- [18] K. Wisniewski, L. Fleisher, D. Rassin, and H. Lassmann, "Neurological disease in a child with carnosinase deficiency," *Neuropediatrics*, vol. 12, no. 2, pp. 143–151, 1981.
- [19] V. P. Skulachev, "Carnosine and anserine as specialised pH-buffers—hydrogen ions transporters," *Biohimija*, vol. 57, pp. 1311–1316, 1992 (Russian).
- [20] S. E. Severin, A. A. Boldyrev, and A. M. Dupin, "Biological role of histidine dipeptides in excitable tissues," *Voprosy Medicinskoj Chimii*, vol. 3, no. 3, pp. 32–36, 1984 (Russian).
- [21] C. E. Brown and W. E. Antholine, "Chelation chemistry of carnosine. Evidence that mixed complexes may occur in vivo," *Journal of Physical Chemistry*, vol. 83, no. 26, pp. 3314–3319, 1979.
- [22] Y. A. Vladimirov, "Studies of the antioxidant activity by measuring chemiluminescence kinetics," in *Proceedings of the International Symposium on Natural Antioxidants: Molecular Mechanisms and Health Effects*, L. Parcker, M. G. Traber, and W. Xin, Eds., pp. 125–144, CRC Press, Champaign, Ill, USA, 1996.
- [23] A. R. Hipkiss, J. Michaelis, and P. Syrris, "Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent," *FEBS Letters*, vol. 371, no. 1, pp. 81–85, 1995.
- [24] V. P. Reddy, M. R. Garrett, G. Perry, and M. A. Smith, "Carnosine: a versatile antioxidant and antiglycating agent," *Science of Aging Knowledge Environment*, vol. 2005, no. 18, article 12, 2005.
- [25] L. J. Hobart, I. Seibel, G. S. Yeorgans, and N. W. Seidler, "Anticrosslinking properties of carnosine: significance of histidine," *Life Sciences*, vol. 75, no. 11, pp. 1379–1389, 2004.
- [26] G. Aldini, R. M. Facino, G. Beretta, and M. Carini, "Carnosine and related dipeptides as quenchers of reactive carbonyl species: from structural studies to therapeutic perspectives," *BioFactors*, vol. 24, no. 1–4, pp. 77–87, 2005.
- [27] G. Vistoli, M. Orioli, A. Pedretti et al., "Design, synthesis, and evaluation of carnosine derivatives as selective and efficient sequestering agents of cytotoxic reactive carbonyl species," *ChemMedChem*, vol. 4, no. 6, pp. 967–975, 2009.
- [28] C. Brownson and A. R. Hipkiss, "Carnosine reacts with a glycated protein," *Free Radical Biology and Medicine*, vol. 28, no. 10, pp. 1564–1570, 2000.
- [29] R. Holliday and G. A. McFarland, "A role for carnosine in cellular maintenance," *Biochemistry*, vol. 65, no. 7, pp. 843–848, 2000.
- [30] A. A. Boldyrev, "Why is homocysteine toxic?" *Priroda*, vol. 10, pp. 18–23, 2009 (Russian).
- [31] P. J. Quinn, A. A. Boldyrev, and V. E. Formazuyk, "Carnosine: its properties, functions and potential therapeutic applications," *Molecular Aspects of Medicine*, vol. 13, no. 5, pp. 379–444, 1992.
- [32] A. A. Boldyrev, G. Aldini, and W. Derave, "Physiology and pathophysiology of carnosine," *Physiological Reviews*, vol. 93, no. 4, pp. 1803–1845, 2013.
- [33] P. R. Normark, Z. S. Edel, and I. S. Leonov, "Experience of use of carnosine per se and in combination with diathermia in infectious and rheumatoid polyarthritis," *Vrachebnoedelo*, vol. 11–12, pp. 741–744, 1940 (Russian).
- [34] M. I. Perelman, Z. Kh. Kornilova, V. S. Paukov, A. K. Boikov, and A. A. Priimak, "Influence of carnosine on healing of wound of lung," *Bulleten' Jeksperimental'noj Biologii i Mediciny*, vol. 108, no. 9, pp. 352–355, 1989 (Russian).
- [35] M. Seiki, S. Ueki, Y. Tanaka et al., "Studies on anti-ulcer effects of a new compound, zinc L-carnosine (Z-103)," *Folia Pharmacologica Japonica*, vol. 95, no. 5, pp. 257–269, 1990.
- [36] S. Shimanaka, "Treatment of cancer with carnosine," in *Proceedings of the 31st International Congress of Physiological Sciences*, p. 3306, Helsinki, Finland, 1989.

- [37] Y. S. Rybakova and A. A. Boldyrev, "Effect of carnosine and related compounds on proliferation of cultured rat pheochromocytoma PC-12 cells," *Bulletin of Experimental Biology and Medicine*, vol. 154, no. 1, pp. 136–140, 2012 (Russian).
- [38] A. R. Hipkiss and F. Gaunitz, "Inhibition of tumour cell growth by carnosine: some possible mechanisms," *Amino Acids*, vol. 46, no. 2, pp. 327–337, 2014.
- [39] M. A. Babizhayev, "Biomarkers and special features of oxidative stress in the anterior segment of the eye linked to lens cataract and the trabecular meshwork injury in primary open-angle glaucoma: challenges of dual combination therapy with *N*-acetylcarnosine lubricant eye drops and oral formulation of nonhydrolyzed carnosine," *Fundamental and Clinical Pharmacology*, vol. 26, no. 1, pp. 86–117, 2012.
- [40] Y. Guo and H. Yan, "Preventive effect of carnosine on cataract development," *Yan Ke Xue Bao*, vol. 22, no. 2, pp. 85–88, 2006 (Chinese).
- [41] Yu. A. Gioeva, E. A. Bazikyan, and D. A. Seleznev, "Use of carnosine for treatment of gingivitis in patients under orthodontic therapy," *Ortodontija*, vol. 3, p. 60, 2005 (Russian).
- [42] E. Riedl, F. Pfister, M. Braunagel et al., "Carnosine prevents apoptosis of glomerular cells and podocyte loss in stz diabetic rats," *Cellular Physiology and Biochemistry*, vol. 28, no. 2, pp. 279–288, 2011.
- [43] F. Pfister, E. Riedl, Q. Wang et al., "Oral carnosine supplementation prevents vascular damage in experimental diabetic retinopathy," *Cellular Physiology and Biochemistry*, vol. 28, no. 1, pp. 125–136, 2011.
- [44] I. Ansurudeen, V. G. Sunkari, J. Grünler et al., "Carnosine enhances diabetic wound healing in the db/db mouse model of type 2 diabetes," *Amino Acids*, vol. 43, no. 1, pp. 127–134, 2012.
- [45] L. A. Bokeriya, A. A. Boldyrev, P. P. Movsesyan et al., "Cardioprotective effect of histidine-containing dipeptides in pharmacological cold cardioplegia," *Bulletin of Experimental Biology and Medicine*, vol. 145, no. 3, pp. 291–295, 2008.
- [46] B. Evran, H. Karpuzoğlu, S. Develi et al., "Effects of carnosine on prooxidant-antioxidant status in heart tissue, plasma and erythrocytes of rats with isoproterenol-induced myocardial infarction," *Pharmacological Reports*, vol. 66, no. 1, pp. 81–86, 2014.
- [47] B. C. Song, N.-S. Joo, G. Aldini, and K.-J. Yeum, "Biological functions of histidine-dipeptides and metabolic syndrome," *Nutrition Research and Practice*, vol. 8, no. 1, pp. 3–10, 2014.
- [48] K. Kiliś-Pstrusińska, "Carnosine, carnosinase and kidney diseases," *Postępy Higieny i Medycyny Doswiadczalnej*, vol. 66, pp. 215–221, 2012 (Polish).
- [49] T. N. Fedorova, M. S. Belyaev, O. A. Trunova, V. V. Gnezditsky, M. Y. Maximova, and A. A. Boldyrev, "Neuropeptide carnosine increases stability of lipoproteins and red blood cells as well as efficiency of immune competent system in patients with chronic discirculatory encephalopathy," *Biochemistry (Moscow) Supplement Series A*, vol. 3, no. 1, pp. 62–65, 2009.
- [50] E. V. Konovalova, L. V. Karpova, S. L. Stvolinsky, and A. A. Boldyrev, "Carnosine containing nanoliposomes protect PC-12 cells and neurons from oxidative stress in vitro," in *Proceedings of the International Congress on Carnosine in Exercise and Disease*, Abstract Book, p. 47, Ghent, Belgium, July 2011.
- [51] A. K. Sariiev, D. A. Abaimov, M. V. Tankevich et al., "Experimental study of the basic pharmacokinetic characteristics of dipeptide carnosine and its efficiency of penetration into brain tissues," *Experimental and Clinical Pharmacology*, vol. 78, no. 3, pp. 30–35, 2015.
- [52] S. Cahill-Smith and J.-M. Li, "Oxidative stress, redox signalling and endothelial dysfunction in ageing-related neurodegenerative diseases: a role of NADPH oxidase 2," *British Journal of Clinical Pharmacology*, vol. 78, no. 3, pp. 441–453, 2014.
- [53] I. M. Cojocaru, M. Cojocaru, V. Sapira, and A. Ionescu, "Evaluation of oxidative stress in patients with acute ischemic stroke," *Romanian Journal of Internal Medicine*, vol. 51, no. 2, pp. 97–106, 2013.
- [54] C. Fendri, A. Mechri, G. Khiari, A. Othman, A. Kerkeni, and L. Gaha, "Oxidative stress involvement in schizophrenia pathophysiology: a review," *Encephale*, vol. 32, no. 2, part 1, pp. 244–252, 2006.
- [55] T. M. Michel, D. Pülschen, and J. Thome, "The role of oxidative stress in depressive disorders," *Current Pharmaceutical Design*, vol. 18, no. 32, pp. 5890–5899, 2012.
- [56] V. D. Prokopieva, N. A. Bokhan, E. V. Patysheva et al., "Assessment of severity of oxidative stress in alcoholic patients and its correction with Sevitin," *Sibirskij Vestnik Psikiatrii Narkologii*, vol. 2, pp. 37–40, 2007 (Russian).
- [57] M.-C. Huang, C.-H. Chen, F.-C. Peng, S.-H. Tang, and C.-C. Chen, "Alterations in oxidative stress status during early alcohol withdrawal in alcoholic patients," *Journal of the Formosan Medical Association*, vol. 108, no. 7, pp. 560–569, 2009.
- [58] T. Zima, L. Fialová, O. Mestek et al., "Oxidative stress, metabolism of ethanol and alcohol-related diseases," *Journal of Biomedical Science*, vol. 8, no. 1, pp. 59–70, 2001.
- [59] B. Halliwell, "Oxidative stress and neurodegeneration: where are we now?" *Journal of Neurochemistry*, vol. 97, no. 6, pp. 1634–1658, 2006.
- [60] A. Boldyrev, T. Fedorova, M. Stepanova et al., "Carnosine increases efficiency of DOPA therapy of Parkinson's disease: a pilot study," *Rejuvenation Research*, vol. 11, no. 4, pp. 821–827, 2008.
- [61] K. N. R. Chengappa, S. R. Turkin, S. Desanti et al., "A preliminary, randomized, double-blind, placebo-controlled trial of L-carnosine to improve cognition in schizophrenia," *Schizophrenia Research*, vol. 142, no. 1–3, pp. 145–152, 2012.
- [62] D. Mizuno, K. Konoha-Mizuno, M. Mori et al., "Protective activity of carnosine and anserine against zinc-induced neurotoxicity: a possible treatment for vascular dementia," *Metalomics*, vol. 7, no. 8, pp. 1233–1239, 2015.
- [63] A. R. Hipkiss, "Possible benefit of dietary carnosine towards depressive disorders," *Aging and Disease*, vol. 6, no. 5, pp. 300–303, 2015.
- [64] L. F. Panchenko, B. V. Davydov, N. N. Terebilina, V. Yu. Baronets, and T. A. Naumova, "Oxidative stress in the pathogenesis of alcoholic liver disease," *Voprosy Narkologii*, vol. 2, pp. 82–91, 2013 (Russian).
- [65] S. A. Ivanova, T. P. Vetlugina, N. A. Bokhan, and O. I. Epstein, "Immunobiology of addictive disorders: mechanisms of psychoneuroimmunomodulation," *Sibirskij Vestnik Psikiatrii Narkologii*, vol. 1, pp. 50–57, 2002 (Russian).
- [66] S. A. Ivanova, N. M. Vyalova, E. V. Zhernova, and N. A. Bokhan, "Spontaneous and in vitro induced apoptosis of lymphocytes and neutrophils in patients with alcohol dependence," *Bulletin of Experimental Biology and Medicine*, vol. 149, no. 2, pp. 246–249, 2010.
- [67] O. Niemelä, "Acetaldehyde adducts in circulation," *Novartis Foundation Symposium*, vol. 285, pp. 183–192, 2007.
- [68] M. Setshedi, J. R. Wands, and S. M. de la Monte, "Acetaldehyde adducts in alcoholic liver disease," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 3, pp. 178–185, 2010.

- [69] N. A. Bokhan and V. D. Prokopieva, *Molecular Mechanisms of Influence of Ethanol and its Metabolites on Erythrocytes in Vitro and in Vivo*, House of Tomsk University, Tomsk, Russia, 2004.
- [70] V. E. Tseilikman, K. A. Babin, D. B. Vinogradov et al., "Features of oxidative stress in patients with delirium tremens infected with hepatitis C and human immunodeficiency virus," *Kazanskij Medicinskij Zhurnal*, vol. 94, no. 5, pp. 778–781, 2013 (Russian).
- [71] A. Kh. Mingazov, E. N. Krivulin, K. A. Babin, Yu. M. Shatrova, and D. B. Vinogradov, "Gender characteristics of blood plasma oxidative protein modification among older drinkers," *Sibirskij Vestnik Psikiatrii Narkologii*, vol. 3, pp. 9–13, 2013 (Russian).
- [72] I. E. Kovalev, N. E. Kovalev, and N. G. Seleznev, "Reaction of acetylation in chronic alcoholism," *Zhurnal Nevropatologii i Psikiatrii Imeni S.S. Korsakova*, vol. 84, no. 2, pp. 232–234, 1984 (Russian).
- [73] S. Bleich, D. Degner, K. Javaheripour, C. Kurth, and J. Kornhuber, "Homocysteine and alcoholism," *Journal of Neural Transmission, Supplement*, vol. 60, pp. 187–196, 2000.
- [74] S. Bleich, D. Degner, W. Sperling, D. Bönsch, N. Thürauf, and J. Kornhuber, "Homocysteine as a neurotoxin in chronic alcoholism," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 28, no. 3, pp. 453–464, 2004.
- [75] V. D. Prokopieva, N. A. Bohan, P. Johnson, H. Abe, and A. A. Boldyrev, "Effects of carnosine and related compounds on the stability and morphology of erythrocytes from alcoholics," *Alcohol and Alcoholism*, vol. 35, no. 1, pp. 44–48, 2000.
- [76] E. G. Yarygina, V. D. Prokopieva, M. B. Arzhanik, L. G. Molkina, and N. A. Bokhan, "Randomized placebo-controlled investigation of efficacy of application of Sevitin in alcoholic patients at the stage of formation of remission," *Sibirski Jmedicinskij Zhurnal*, vol. 25, no. 1–4, pp. 84–88, 2010 (Russian).
- [77] E. G. Yarygina and V. D. Prokopieva, "Protection of blood plasma proteins and lipids against damage induced by ethanol and acetaldehyde," *Sibirskij Vestnik Psikiatrii Narkologii*, vol. 3, pp. 5–8, 2015 (Russian).
- [78] A. Baguet, J. Bourgois, L. Vanhee, E. Achten, and W. Derave, "Important role of muscle carnosine in rowing performance," *Journal of Applied Physiology*, vol. 109, no. 4, pp. 1096–1101, 2010.
- [79] S. Del Favero, H. Roschel, M. Y. Solis et al., "Beta-alanine (Carnosyn[®]) supplementation in elderly subjects (60–80 years): effects on muscle carnosine content and physical capacity," *Amino Acids*, vol. 43, no. 1, pp. 49–56, 2012.
- [80] A. A. Boldyrev, S. L. Stvolinsky, T. N. Fedorova, and Z. A. Suslina, "Carnosine as a natural antioxidant and geroprotector: from molecular mechanisms to clinical trials," *Rejuvenation Research*, vol. 13, no. 2–3, pp. 156–158, 2010.
- [81] C. Rona, F. Vailati, and E. Berardesca, "The cosmetic treatment of wrinkles," *Journal of Cosmetic Dermatology*, vol. 3, no. 1, pp. 26–34, 2004.

Research Article

Beneficial Effects of Physical Exercise on Functional Capacity and Skeletal Muscle Oxidative Stress in Rats with Aortic Stenosis-Induced Heart Failure

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Objective. We evaluated the influence of exercise on functional capacity, cardiac remodeling, and skeletal muscle oxidative stress, MAPK, and NF- κ B pathway in rats with aortic stenosis- (AS-) induced heart failure (HF). **Methods and Results.** Eighteen weeks after AS induction, rats were assigned into sedentary control (C-Sed), exercised control (C-Ex), sedentary AS (AS-Sed), and exercised AS (AS-Ex) groups. Exercise was performed on treadmill for eight weeks. Statistical analyses were performed with Goodman and ANOVA or Mann-Whitney. HF features frequency and mortality did not differ between AS groups. Exercise improved functional capacity, assessed by maximal exercise test on treadmill, without changing echocardiographic parameters. Soleus cross-sectional areas did not differ between groups. Lipid hydroperoxide concentration was higher in AS-Sed than C-Sed and AS-Ex. Activity of antioxidant enzymes superoxide dismutase and glutathione peroxidase was changed in AS-Sed and restored in AS-Ex. NADPH oxidase activity and gene expression of its subunits did not differ between AS groups. Total ROS generation was lower in AS-Ex than C-Ex. Exercise modulated MAPK in AS-Ex and did not change NF- κ B pathway proteins. **Conclusion.** Exercise improves functional capacity in rats with AS-induced HF regardless of echocardiographic parameter changes. In soleus, exercise reduces oxidative stress, preserves antioxidant enzyme activity, and modulates MAPK expression.

1. Introduction

Heart failure is an important public health issue due to its high prevalence and poor prognosis [1]. Heart failure is clinically characterized by a reduced exercise capacity with the early occurrence of fatigue and dyspnea [2, 3]. Skeletal muscle abnormalities can contribute to the impaired ability to perform physical activities. Muscle changes include

wasting and fibrosis, changes in fiber type and myosin heavy chain composition, decrease in oxidative capacity, increase in oxidative stress, and contractile dysfunction [4–8].

Current guidelines strongly recommend regular physical exercise for patients with stable heart failure to prevent and/or attenuate cardiac remodeling and skeletal muscle alterations [9, 10]. Clinical and experimental studies have shown that physical exercise attenuates abnormal cardiac remodeling,

reduces muscle wasting and local inflammation, improves muscle capillarization, blood flow, and oxygen utilization, and increases functional capacity, exercise duration, and quality of life [11–16].

However, most studies on the effects of exercise have evaluated postmyocardial infarction-induced heart failure. The influence of exercise during conditions characterized by persistent left ventricular pressure-overload such as aortic stenosis remains unsettled. As life expectancy is increasing and aortic stenosis prevalence augments with age, the number of patients with pressure-overload-induced heart failure will grow [17]. Experimental studies on the effects of exercise on cardiac and skeletal muscle during aortic stenosis have shown controversial results. Mice with mild or severe aortic stenosis subjected to short-term voluntary rotating wheel exercise presented unchanged left ventricular function with a trend towards aggravated ventricular dysfunction in severe cases [18]. On the other hand, we observed that aerobic treadmill training attenuated systolic dysfunction during transition from compensated cardiac dysfunction to heart failure in aortic stenosis rats [19]. Furthermore, exercise prevented skeletal muscle atrophy through anticatabolic effects [19].

Ascending aortic stenosis in rats has been used to induce persistent and chronic pressure overload. In this model, 3–4 week-old rats are subjected to a clip placement around the ascending aorta. After clip placement, aorta diameter is preserved; as rats grow, stenosis progressively develops. The model has the advantage that, despite rapid left ventricular hypertrophy onset, ventricular dysfunction and heart failure occur slowly [19], similar to what is observed in human chronic pressure overload. In this study we evaluated the influence of physical exercise on functional capacity, cardiac remodeling, and skeletal muscle phenotype and oxidative stress in rats with aortic stenosis-induced heart failure. Since the NADPH oxidase (NOX) family is an important source of reactive oxygen species in various tissues [20] and skeletal muscle expresses two NADPH oxidase isoforms, we analyzed NADPH oxidase activity and gene expression of its NOX2 and NOX4 subunits. As mitogen-activated protein kinases (MAPK) and the nuclear factor-kappa B (NF- κ B) pathway may be involved in skeletal muscle response to oxidative stress [21], we also evaluated protein expression of these pathways.

2. Materials and Methods

2.1. Experimental Animals and Study Protocol. Male Wistar rats weighing 90–100 g were purchased from the Central Animal House, Botucatu Medical School, UNESP. All experiments and procedures were approved by the Animal Experimentation Ethics Committee of Botucatu Medical School, UNESP, SP, Brazil, which follows the guidelines established by the Brazilian College for Animal Experimentation (protocol number 999/2013).

Rats were anaesthetized with a mixture of ketamine hydrochloride (50 mg/kg, i.m.) and xylazine hydrochloride (10 mg/kg, i.m.) and aortic stenosis (AS) was induced by placing a 0.6 mm stainless-steel clip on the ascending aorta via a thoracic incision according to a previously described

method [19]. Sham operated rats were used as controls. All animals were housed in a temperature controlled room at 23°C and kept on a 12-hour light/dark cycle. Food and water were supplied *ad libitum*. Eighteen weeks after surgery, rats were subjected to transthoracic echocardiogram to evaluate degree of cardiac injury and assigned to four groups: sedentary control (C-Sed, $n = 22$), exercised control (C-Ex, $n = 23$), sedentary aortic stenosis (AS-Sed, $n = 25$), and exercised aortic stenosis (AS-Ex, $n = 27$) for eight weeks. At the end of the experimental period, rats were subjected to transthoracic echocardiogram and euthanized the next day. During euthanasia, we determined the presence or absence of clinical and pathologic heart failure features. The clinical finding suggestive of heart failure was tachypnea/labored respiration. Pathologic assessment of heart failure included pleuropericardial effusion, left atrial thrombi, ascites, hepatic congestion, pulmonary congestion (lung weight/body weight ratio higher than 2 standard deviations above the C-Sed group mean), and right ventricular hypertrophy (right ventricle weight/body weight ratio higher than 0.8 mg/g) [22, 23].

2.2. Exercise Testing. Functional capacity was assessed before and after the exercise period. Rats underwent 10 min/day testing environment adaption for one week before evaluations. Each animal was tested individually. The test consisted of an initial 5 min warm-up at 5 m/min on treadmill. The rats were then subjected to interval exercise at a speed of 8 m/min followed by 3 m/min increases in speed every 3 min until exhaustion. Exhaustion was determined when the animal refused to run even after electric stimulation or was unable to coordinate steps [24]. Maximum running speed was recorded and total distance calculated.

2.3. Exercise Training Protocol. Exercise was performed on a treadmill five times a week for eight weeks [14, 25]. There was an initial adaptation period, with a gradual increase in speed and exercise time. Speed from the 1st to the 3rd week was 5, 7.5, and 10 m/min and then remained constant until the end of the protocol. Exercise duration from the 1st to the 6th week was 10, 14, 18, 22, 26, and 30 min and then remained constant until the end of the experiment. In the first two weeks of training, animals were subjected to low-voltage electrical stimulation to start exercise. No animals were lost during exercise training.

2.4. Echocardiography. Cardiac structures and left ventricular function were evaluated by transthoracic echocardiogram and tissue Doppler imaging using a commercially available echocardiograph (General Electric Medical Systems, Vivid S6 model, Tirat Carmel, Israel) equipped with a 5–11.5 MHz multifrequency transducer as previously described [26–29]. After anesthesia with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (1 mg/kg) intramuscularly, the rats were placed in the left lateral decubitus. A two-dimensional parasternal short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles. M-mode tracings were obtained from short-axis views of the LV at or just below the tip of the mitral-valve leaflets and

at the level of the aortic valve and left atrium. M-mode images of the LV were printed on a black-and-white thermal printer (Sony UP-890MD) at a sweep speed of 100 mm/s. All LV structures were manually measured by the same observer (KO). Values obtained were the mean of at least five cardiac cycles on M-mode tracings. The following structural variables were measured: left atrium diameter (LA), LV diastolic and systolic diameters (LVDD and LVSD, resp.), LV diastolic (D) and systolic (S) posterior wall thickness (PWT) and septal wall thickness (SWT), and aortic diameter. Left ventricular mass (LVM) was calculated using the formula $[(LVDD + DPWT + DSWT)^3 - LVDD^3] \times 1.04$. LV relative wall thickness (RWT) was calculated by the formula $2 \times DPWT/LVDD$. Left ventricular function was assessed by the following parameters: endocardial fractional shortening (EFS), midwall fractional shortening (MFS), ejection fraction (EF), posterior wall shortening velocity (PWSV), early and late diastolic mitral inflow velocities (E and A waves), E/A ratio, E-wave deceleration time (EDT), and isovolumetric relaxation time (IVRT). A joint assessment of diastolic and systolic LV function was performed using the myocardial performance index (Tei index). The study was complemented with evaluation by tissue Doppler imaging (TDI) of systolic (S'), early diastolic (E'), and late diastolic (A') velocity of the mitral annulus (arithmetic average travel speeds of the lateral and septal walls) and E/E' ratio.

2.5. Collection of Skeletal Muscle and Other Tissues for Analysis. Biological tissue collection was performed in the Experimental Research Laboratory (UNIPEX), Botucatu Medical School, UNESP. One day after final echocardiogram, the rats were weighed and anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) and euthanized. After blood collecting, hearts were removed by thoracotomy. Atria and ventricles were dissected and weighed separately. Soleus muscles of the right and left hind limbs were dissected, immediately weighed and frozen in liquid nitrogen, and stored at -80°C . Lung weight was used to assess the degree of pulmonary congestion. Fragments of lung and liver were weighed before and after drying sessions (65°C for 72 h) to evaluate wet/dry weight ratio.

2.6. Morphologic Study. Serial transverse $10\ \mu\text{m}$ thick sections of soleus muscles were cut in a cryostat cooled to -20°C and stained with hematoxylin and eosin. Measurements were performed using a microscope (Leica DML5; Nussloch, Germany) attached to a computerized imaging analysis system (Media Cybernetics, Silver Spring, MD, USA). At least 150 cross-sectional fiber areas were measured from each soleus muscle.

2.7. Oxidative Stress Evaluation

2.7.1. Antioxidant Enzymes Activity and Lipid Hydroperoxide Concentration. Soleus muscle samples ($\sim 200\ \text{mg}$) were homogenized in 5 mL of cold 0.1 M phosphate buffer, pH 7.0. Tissue homogenates were prepared in a motor-driven Teflon-glass Potter-Elvehjem, tissue homogenizer. The homogenate

was centrifuged at 10,000 g, for 15 min at 4°C , and the supernatant was assayed for total protein, lipid hydroperoxide (LOOH), and glutathione peroxidase (GSH-Px, E.C.1.11.1.9), catalase (E.C.1.11.1.6.), and superoxide dismutase (SOD, E.C.1.15.1.1.) activities by spectrophotometry [7]. Enzyme activities were analyzed at 25°C using a microplate reader ($\mu\text{Quant-MQX 200}$) with Kcjunior software for computer system control (Bio-Tec Instruments, Winooski, Vermont, USA). Spectrophotometric determinations were performed in a Pharmacia Biotech spectrophotometer with temperature-controlled cuvette chamber (UV/visible Ultrospec 5000 with Swift II applications software for computer system control, Cambridge, UK). All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.7.2. Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Gene expression of NADPH oxidase subunits (NOX2, NOX4, $p22^{\text{phox}}$, and $p47^{\text{phox}}$) and reference genes cyclophilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by RT-PCR according to a previously described method [30].

Total RNA was extracted from soleus muscles with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and treated with DNase I (Invitrogen Life Technologies). One microgram of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit, according to standard methods (Applied Biosystems, Foster City, CA, USA). Aliquots of cDNA were then submitted to real-time PCR reaction using customized assay containing sense and antisense primers and Taqman (Applied Biosystems, Foster City, CA, USA) probes specific to each gene: NOX2 (Rn00576710_m1), NOX4 (Rn00585380_m1), $p22^{\text{phox}}$ (Rn00577357_m1), and $p47^{\text{phox}}$ (Rn00586945_m1). The amplification and analysis were performed using Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data expression was normalized to reference gene expressions: cyclophilin (Rn00690933_m1) and GAPDH (Rn01775763_g1). Reactions were performed in triplicate and expression levels calculated using the CT comparative method ($2^{-\Delta\Delta\text{CT}}$).

2.7.3. NADPH Oxidase Activity. NADPH oxidase activity was evaluated in membrane-enriched cellular fraction by reduction of lucigenin detected by luminometer. Soleus muscle was carefully washed in PBS to remove blood. Muscle fragments ($\sim 200\ \text{mg}$) were homogenized in 1 mL of ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 100 mM DTPA, 0.1% β -mercaptoethanol, and protease inhibitors. Samples were then sonicated (3 cycles of 10 s at 8 W) and centrifuged at 1,000 g for 3 min, at 4°C . Supernatant was transferred to another microtube and centrifuged at 18,000 g for 10 min, at 4°C , and transferred again to ultracentrifuge tubes and centrifuged at 100,000 g for 45 min, at 4°C . The supernatant was then discarded and the precipitate resuspended in 100 μL of lysis buffer [31]. Total protein content was quantified by the Bradford method. Subsequently, 30 μg of membrane-enriched cellular fraction was incubated in PBS (pH 7.4, containing EDTA 10 μM) and 150 μL of NADPH 2 mM in plastic

tubes in a Berthold Sirius Luminometer, which performs automatic 20 μL injections of 0.25 mM lucigenin into each tube. Luminescence data were collected every 2 s for 5 min using the FB12/Sirius program. NADPH oxidase activity was shown as the area under the curve of luminescence data obtained during the reaction.

2.7.4. ROS Generation. Muscle fragment (~100 mg) was washed in PBS and incubated in a solution containing PBS/DTPA and 150 μM dihydroethidium (DHE) for 25 min at 37°C in a dark room. The muscle fragment was then washed in PBS, transferred to liquid nitrogen, and homogenized with mortar and pestle. The homogenate was resuspended in acetonitrile (0.5 mL), sonicated (3 cycles at 8 W for 10 s), and centrifuged (12,000 g for 10 min at 4°C). Supernatant was dried under vacuum (Speed Vac Plus model SC-110A, Thermo Savant) and pellets were maintained at 20°C in the dark until analysis. Samples were resuspended in 80 μL deionized water and injected into an HPLC system. Total ROS generation was evaluated by quantification of two DHE oxidation-derived fluorescent compounds, 2-hydroxyethidium (EOH) and ethidium, using the HPLC according to a previously described method [31, 32]. EOH is generated when DHE is oxidized by anion superoxide, while ethidium production is associated to heme proteins levels and peroxidase activity. DHE-derived products were expressed as ratios of generated EOH and ethidium over consumed DHE (initial DHE concentration minus remaining DHE).

2.8. Western Blotting. Soleus muscle protein levels were analyzed by Western blotting according to a previously described method [33] using specific anti-JNK (JNK 1/2 D-9 sc-137019), p-JNK (p-JNK G-7 sc-6254), p38 (p38 α/β A-12 sc-7972), p-p38 (p-p38 Thr 180/Tyr 182-R sc-17852-R), ERK (ERK 1 C-16 sc-93), p-ERK (p-ERK 1/2 Thr 202/Tyr 204 sc-16982), NF- κB (p65 NF- κB sc-7151), p-NF- κB (Ser 536 p-p65 NF- κB sc-33020), I κB (I κB - α sc-1643), and p-I κB (p-I κB - α sc-101713) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein levels were normalized to GAPDH (6C5 sc-32233, Santa Cruz Biotechnology).

Muscle protein was extracted using RIPA buffer and supernatant protein content was quantified by Bradford assay. Samples were separated on a polyacrylamide gel and then transferred to a nitrocellulose membrane. After blockade, membrane was incubated with the primary antibodies. Membrane was then washed with TBS and Tween 20 and incubated with secondary peroxidase-conjugated antibodies. Super Signal[®] West Pico Chemiluminescent Substrate (Pierce Protein Research Products, Rockford, USA) was used to detect bound antibodies.

2.9. Statistical Analysis. Data are expressed as the mean \pm standard deviation or median and percentiles. Comparisons between groups were performed by analysis of variance (ANOVA) for a 2 \times 2 factorial design followed by the Tukey test or Mann-Whitney test ($p < 2$ value). Comparisons of interest are as follows: C-Ex versus C-Sed, AS-Sed versus

TABLE 1: Frequency of heart failure features in the aortic stenosis rats.

	Frequency (%)	
	AS-Sed ($n = 18$)	AS-Ex ($n = 18$)
Tachypnea/labored respiration	22.2	16.7
Left atrial thrombi	33.3	38.9
Pleuropericardial effusion	66.7	55.6
Hepatic congestion	16.7	11.1
Pulmonary congestion	66.7	66.7
Right ventricular hypertrophy	72.2	61.1
Ascites	22.2	5.60

AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; n : number of animals. Goodman test; $p > 0.05$.

C-Sed, AS-Ex versus AS-Sed, and AS-Ex versus C-Ex. Mortality and frequency of heart failure features were compared between AS-Ex and AS-Sed groups using the Goodman test. The level of significance was set at 5%.

3. Results

3.1. Experimental Groups and Anatomic Parameters. At the end of the experimental period, C-Sed group contained 22 and C-Ex 19 rats. One rat from C-Sed had ascites and one from C-Ex presented pleural effusion. AS-Sed and AS-Ex groups had 18 rats each at the end of the experiment. Mortality did not statistically differ between groups. The frequency of heart failure features did not differ between AS-Ex and AS-Sed groups (Table 1).

Anatomical variables are presented in Table 2. Final body weight did not differ between groups. Left ventricle, right ventricle, atria, and lungs weights, absolute or normalized to body weight values, were higher in AS-Sed and AS-Ex than their respective controls. Soleus muscle weight was lower in the AS-Sed than C-Sed and gastrocnemius weight was lower in AS-Sed and AS-Ex than controls.

3.2. Exercise Testing. At initial exercise test, AS rats presented worse functional capacity than control groups, characterized by less run distance and time spent on the treadmill. At the end of the experiment, functional capacity was better in exercised than sedentary rats and worse in AS than control rats (Table 3).

3.3. Echocardiographic Evaluation. Before the exercise protocol, groups with aortic stenosis presented concentric left ventricular hypertrophy with mild systolic dysfunction and diastolic dysfunction. All parameters were similar in AS-Ex and AS-Sed groups (data not shown).

At the end of the experiment, structural variables did not differ between C-Ex and C-Sed group, except for reduced LV systolic posterior wall thickness in C-Ex. Both AS-Ex and AS-Sed presented higher LV systolic and diastolic diameters, LV wall thickness, relative wall thickness, aorta and left atrium diameters, and LV mass than their respective controls (Table 4). Functionally, there were no differences between C-Ex and C-Sed groups. AS-Ex and AS-C groups had decreased

TABLE 2: Anatomical data.

	C-Sed (<i>n</i> = 22)	C-Ex (<i>n</i> = 19)	AS-Sed (<i>n</i> = 18)	AS-Ex (<i>n</i> = 18)
BW (g)	454 ± 57.4	466 ± 64.6	435 ± 35.8	439 ± 47.8
LVW (mg)	0.92 (0.86–0.99)	0.96 (0.84–1.04)	1.40 (1.28–1.73)*	1.36 (1.23–1.50) [†]
LVW/BW (mg/g)	1.95 (1.78–1.99)	1.88 (1.67–2.11)	3.17 (2.67–3.86)*	3.35 (2.74–3.84) [†]
RVW (mg)	0.24 (0.21–0.25)	0.23 (0.21–0.28)	0.45 (0.31–0.55)*	0.43 (0.26–0.54) [†]
RVW/BW (mg/g)	0.48 (0.42–0.51)	0.50 (0.41–0.56)	0.99 (0.76–1.22)*	0.98 (0.62–1.28) [†]
Atria (mg)	0.09 (0.07–0.10)	0.09 (0.08–0.11)	0.31 (0.27–0.38)*	0.27 (0.15–0.35) [†]
Atria/BW (mg/g)	0.19 (0.14–0.21)	0.19 (0.16–0.23)	0.68 (0.59–0.87)*	0.68 (0.59–0.87) [†]
Lung (mg)	1.84 (1.65–2.02)	1.78 (1.48–2.03)	3.35 (2.31–4.08)*	3.15 (2.11–3.50) [†]
Lung/BW (mg/g)	3.84 (3.54–4.25)	3.60 (2.98–4.12)	6.58 (4.40–8.10)*	7.36 (4.83–10.25) [†]
Soleus (g)	0.25 ± 0.05	0.22 ± 0.05	0.22 ± 0.04*	0.21 ± 0.03
Soleus/BW (mg/g)	0.50 ± 0.10	0.45 ± 0.11	0.48 ± 0.07	0.49 ± 0.09
Gastro (g)	2.67 ± 0.33	2.70 ± 0.29	2.44 ± 0.33*	2.42 ± 0.27 [†]
Gastro/BW (mg/g)	5.39 ± 0.41	5.51 ± 0.44	5.33 ± 0.54	5.54 ± 0.41

C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; BW: body weight; LVW: left ventricle weight; RVW: right ventricle weight; Gastro: gastrocnemius muscle weight. Data are mean ± SD or median and percentiles; ANOVA and Tukey or Mann-Whitney; * $p < 0.05$ versus C-Sed; [†] $p < 0.05$ versus C-Ex.

TABLE 3: Maximal exercise test before and after physical training.

	C-Sed (<i>n</i> = 22)	C-Ex (<i>n</i> = 19)	AS-Sed (<i>n</i> = 18)	AS-Ex (<i>n</i> = 18)
Distance				
Before	257 ± 86.5	247 ± 55.8	171 ± 59.7*	163 ± 35.5 [†]
After	225 ± 64.8	304 ± 47.5*	154 ± 48.9*	254 ± 47.2 ^{†‡}
Time				
Before	22.8 ± 4.10	22.7 ± 2.53	18.5 ± 3.16*	18.1 ± 2.16 [†]
After	21.7 ± 3.51	25.1 ± 1.94*	18.0 ± 3.05*	22.7 ± 2.61 ^{†‡}

C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group. Data are mean ± SD; ANOVA and Tukey; * $p < 0.05$ versus C-Sed; [†] $p < 0.05$ versus C-Ex; [‡] $p < 0.05$ versus AS-Sed.

TABLE 4: Echocardiographic structural data.

	C-Sed (<i>n</i> = 22)	C-Ex (<i>n</i> = 19)	AS-Sed (<i>n</i> = 18)	AS-Ex (<i>n</i> = 18)
LVDD (mm)	8.30 ± 0.55	8.50 ± 0.55	9.02 ± 0.85*	9.17 ± 0.74 [†]
LVDD/BW (mm/kg)	16.6 ± 2.05	16.8 ± 1.91	18.9 ± 2.69*	20.4 ± 3.09 [†]
LVSD (mm)	4.05 ± 0.55	4.30 ± 0.60	4.81 ± 1.17*	5.27 ± 0.98 [†]
DPWT (mm)	1.42 (1.38–1.46)	1.38 (1.31–1.44)	2.06 (1.84–2.12)*	1.99 (1.84–2.17) [†]
SPWT (mm)	3.01 (2.76–3.14)	2.69 (2.65–3.00)*	3.60 (2.86–3.79)*	3.49 (2.91–3.72) [†]
DSWT (mm)	1.43 (1.40–1.46)	1.38 (1.34–1.46)	2.11 (1.84–2.16)*	2.01 (1.84–2.17) [†]
SSWT (mm)	2.52 (2.42–2.65)	2.42 (2.33–2.59)	2.79 (2.59–3.16)*	2.93 (2.68–3.25) [†]
RWT	0.34 (0.33–0.36)	0.33 (0.31–0.34)	0.44 (0.38–0.48)*	0.42 (0.40–0.50) [†]
AO (mm)	4.02 (3.83–4.16)	4.02 (3.83–4.16)	3.94 (3.80–4.16)	3.94 (3.80–4.09)
LA (mm)	5.29 (4.93–5.69)	5.66 (5.47–5.99)	8.54 (7.88–8.69)*	8.14 (6.79–8.54) [†]
LA/AO	1.32 (1.26–1.38)	1.43 (1.30–1.52)	2.17 (1.83–2.29)*	2.09 (1.67–2.24) [†]
LA/BW (mm/kg)	10.3 (9.5–12.1)	10.9 (10.0–12.3)	17.3 (14.8–19.5)*	17.3 (15.7–18.9) [†]
LVM (g)	0.82 (0.77–0.89)	0.83 (0.74–0.93)	1.62 (1.18–1.87)*	1.38 (1.32–1.93) [†]
LVMI (g/kg)	1.69 (1.44–1.88)	1.64 (1.47–1.85)	2.81 (2.69–3.67)*	3.40 (2.66–4.16) [†]

C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; LVDD and LVSD: left ventricular (LV) diastolic and systolic diameter, respectively; BW: body weight; DPWT and SPWT: LV diastolic and systolic posterior wall thickness, respectively; DSWT and SSWT: LV diastolic and systolic septal wall thickness, respectively; RWT: relative wall thickness; AO: aorta diameter; LA: left atrial diameter; LVM: LV mass; LVMI: LVM index. Data are mean ± SD or median and percentiles; ANOVA and Tukey or Mann-Whitney; * $p < 0.05$ versus C-Sed; [†] $p < 0.05$ versus C-Ex.

TABLE 5: Echocardiographic evaluation of left ventricle systolic function.

	C-Sed (<i>n</i> = 22)	C-Ex (<i>n</i> = 19)	AS-Sed (<i>n</i> = 18)	AS-Ex (<i>n</i> = 18)
HR (bpm)	282 (262–318)	312 (255–341)	294 (280–325)	312 (290–318)
EFS %	51.6 (46.6–55.8)	50.0 (46.2–51.5)	46.6 (38.9–54.8)	43.6 (38.3–47.4) [†]
MFS %	29.5 (27.6–32.4)	29.7 (28.4–31.7)	28.6 (21.1–32.7)	24.5 (21.6–26.6) [†]
EF	0.89 (0.85–0.91)	0.87 (0.84–0.89)	0.85 (0.77–0.91)	0.82 (0.76–0.85) [†]
PWSV (mm/s)	41.1 ± 5.27	41.4 ± 7.10	31.0 ± 7.14*	29.1 ± 6.66 [†]
Tei index	0.44 ± 0.08	0.43 ± 0.07	0.41 ± 0.08	0.42 ± 0.11
TDI S' (average, mm/s)	3.76 ± 0.72	3.58 ± 0.35	2.96 ± 0.69*	2.94 ± 0.49 [†]

C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; HR: heart rate; EFS: endocardial fractional shortening; MFS: midwall fractional shortening; EF: ejection fraction; PWSV: posterior wall shortening velocity; Tei index: myocardial performance index; TDI S': tissue Doppler imaging of systolic velocity of the mitral annulus. ANOVA and Tukey or Mann-Whitney; * *p* < 0.05 versus C-Sed; [†] *p* < 0.05 versus C-Ex.

TABLE 6: Echocardiographic evaluation of left ventricle diastolic function.

	C-Sed (<i>n</i> = 22)	C-Ex (<i>n</i> = 19)	AS-Sed (<i>n</i> = 18)	AS-Ex (<i>n</i> = 18)
Mitral E (cm/s)	79.5 (73.0–84.0)	81.5 (76.0–89.0)	142 (89.0–165)*	145 (85.0–159) [†]
Mitral A (cm/s)	59.5 (51.0–67.0)	60.0 (51.0–76.0)	24.0 (22.0–47.5)*	22.5 (16.0–59.0) [†]
E/A	1.40 (1.22–1.53)	1.34 (1.17–1.60)	5.85 (1.73–7.15)*	7.50 (1.47–9.16) [†]
IVRT (ms)	24.0 (22.0–26.0)	22.0 (21.2–26.0)	18.0 (15.0–22.0)*	16.0 (15.0–22.0) [†]
IVRTn	53.0 ± 7.25	51.2 ± 8.37	42.6 ± 11.4*	39.0 ± 10.2 [†]
EDT (ms)	45.0 (41.0–52.0)	47.0 (41.0–53.0)	28.0 (23.0–35.0)*	29.0 (24.5–33.0) [†]
TDI E' (average mm/s)	4.37 (3.95–4.95)	4.50 (4.35–4.92)	4.25 (3.04–4.90)	4.17 (3.50–5.25)
TDI A' (average mm/s)	4.50 ± 1.37	4.37 ± 1.21	3.79 ± 1.47	4.12 ± 1.07
E/TDI E' (average)	17.9 (15.8–20.0)	18.4 (15.7–20.2)	33.0 (25.7–41.3)*	32.1 (24.8–40.6) [†]

C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group. E/A: ratio between early- (E-) to-late (A) diastolic mitral inflow; IVRT: isovolumetric relaxation time; IVRTn: IVRT normalized to heart rate; EDT: E wave deceleration time; TDI E' and A': tissue Doppler imaging (TDI) of early (E') and late (A') diastolic velocity of mitral annulus. ANOVA and Tukey or Mann-Whitney; * *p* < 0.05 versus C-Sed; [†] *p* < 0.05 versus C-Ex.

posterior wall shortening velocity, TDI systolic velocity of the mitral annulus, mitral A wave, isovolumetric relaxation time, and E wave deceleration time and increased mitral E wave, E/A ratio, and E wave/TDI early average mitral annulus diastolic velocity compared to respective controls. AS-Ex presented reduced endocardial and mesocardial fractional shortening and ejection fraction compared to C-Ex. We observed no differences between AS-Ex and AS-C groups (Tables 5 and 6).

3.4. Morphometric Analysis. Soleus muscle trophicity was evaluated on hematoxylin and eosin stained sections. Fiber cross-sectional area did not statistically differ between groups (C-Sed 3980 ± 231; C-Ex 4113 ± 253; AS-Sed 3541 ± 200, AS-Ex 3941 ± 253 μm²; *p* > 0.05).

3.5. Oxidative Stress Evaluation. We used lipid hydroperoxide concentration as an oxidative stress biomarker in soleus muscle. The concentration was higher in AS-Sed than C-Sed and AS-Ex (C-Sed 136 ± 36.0; C-Ex 141 ± 31.2; AS-Sed 197 ± 37.8; AS-Ex 143 ± 35.0 nmol/g tissue; Figure 1(a)).

Glutathione peroxidase activity was lower in C-Ex and AS-Sed than C-Sed and higher in AS-Ex than AS-Sed (C-Sed 85.6 ± 12.3; C-Ex 70.2 ± 14.8; AS-Sed 45.2 ± 9.73; AS-Ex 74.7 ± 10.6 nmol/mg protein, *p* < 0.001; Figure 1(b)). Superoxide

dismutase activity was higher in AS-Sed than C-Sed and AS-Ex (C-Sed 8.52 ± 1.95; C-Ex 8.09 ± 0.94; AS-Sed 12.7 ± 1.55, AS-Ex 8.60 ± 0.93 nmol/mg protein, *p* < 0.001; Figure 1(c)). Catalase activity did not differ between groups (C-Sed 34.9 ± 9.20; C-Ex 41.8 ± 10.2; AS-Sed 52.9 ± 10.9; AS-Ex 43.6 ± 15.2 μmol/g tissue; Figure 1(d)).

NADPH oxidase complex subunit p22^{phox} gene expression was higher in AS-Ex than C-Ex. Subunits p47^{phox}, NOX2, and NOX4 gene expression did not differ between groups (Table 7). NADPH oxidase activity did not differ between groups (Figure 2(a)).

Total reactive oxygen species generation was evaluated in soleus muscle by quantification EOH and ethidium, two fluorescent compounds derived from DHE oxidation. The EOH/DHE ratio was lower in AS-Ex than C-Ex and did not differ between AS-Ex and AS-Sed (Figure 2(b)). The ethidium/DHE ratio did not differ between groups (Figure 2(c)).

3.6. Western Blotting. MAPK protein expression is shown in Figure 3. Phosphorylated ERK was higher in AS-Ex than AS-Sed. Total JNK was lower in AS-Ex than C-Ex and AS-Sed. Protein expression of p38 did not differ between groups. Expression of total (p65 NF-κB: C-Sed 1.00 ± 0.42, C-Ex 1.05 ± 0.34, AS-Sed 1.00 ± 0.65, and AS-Ex 0.83 ± 0.40) and phosphorylated [p-p65 NF-κB: C-Sed 0.96 (0.87–1.06),

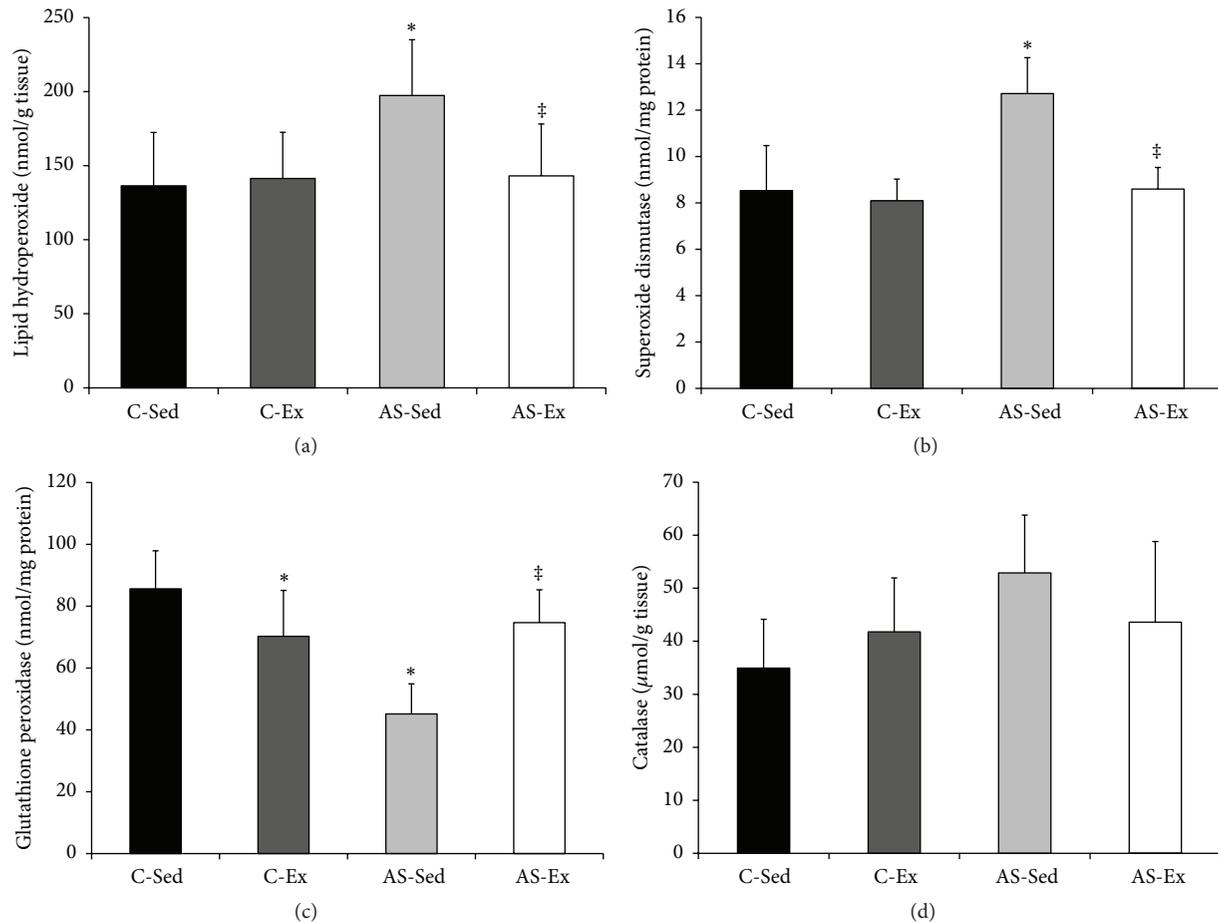


FIGURE 1: Lipid hydroperoxide concentration (a) and antioxidant enzymes activity in soleus muscle ((b) to (d)). C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; n : number of animals. Data are mean \pm SD ($n = 8$); ANOVA and Tukey; * $p < 0.05$ versus C-Sed; $^{\ddagger}p < 0.05$ versus AS-Sed.

TABLE 7: Gene expression of NADPH oxidase subunits.

	C-Sed ($n = 8$)	C-Ex ($n = 8$)	AS-Sed ($n = 8$)	AS-Ex ($n = 8$)
p22 ^{phox}	1.00 \pm 0.40	0.44 \pm 0.15	1.10 \pm 0.66	1.17 \pm 0.70 [†]
p47 ^{phox}	1.00 \pm 0.53	1.52 \pm 0.49	0.84 \pm 0.48	1.21 \pm 0.65
NOX2	1.00 (0.37–1.39)	0.33 (0.26–0.75)	0.82 (0.53–2.16)	0.63 (0.42–1.07)
NOX4	1.00 \pm 0.52	0.70 \pm 0.46	0.79 \pm 0.22	0.98 \pm 0.32

C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group. ANOVA and Tukey or Mann-Whitney; [†] $p < 0.05$ versus C-Ex.

C-Ex 0.99 (0.67–1.16), AS-Sed 0.71 (0.49–1.44), and AS-Ex 1.08 (0.76–1.17)] NF- κ B subunit, and total I κ B [C-Sed 0.97 (0.83–1.14), C-Ex 0.79 (0.68–0.87), AS-Sed 1.32 (1.13–1.84), and AS-Ex 1.53 (0.90–2.08)] did not differ between groups. Phosphorylated I κ B was lower in C-Ex and AS-Sed than in C-Sed (C-Sed 1.00 \pm 0.21, C-Ex 0.71 \pm 0.17, AS-Sed 0.54 \pm 0.24, and AS-Ex 0.67 \pm 0.39).

4. Discussion

In this study, we showed that physical exercise improves functional capacity in rats with aortic stenosis-induced heart

failure regardless of changes in cardiac structures or left ventricular function. Additionally, we performed the first assessment of the influence of physical exercise on oxidative stress and MAPK and NF- κ B pathways in soleus muscle from aortic stenosis rats.

Aortic stenosis in young rats leads to early left ventricular hypertrophy, gradual ventricular dysfunction, and heart failure, similarly to human chronic pressure overload. After developing hypertrophy, rats remain compensated for approximately 20 to 28 weeks [19]. They then begin to present clinical and pathological heart failure features such as tachypnea, ascites, pleural effusion, atrial thrombus, lung

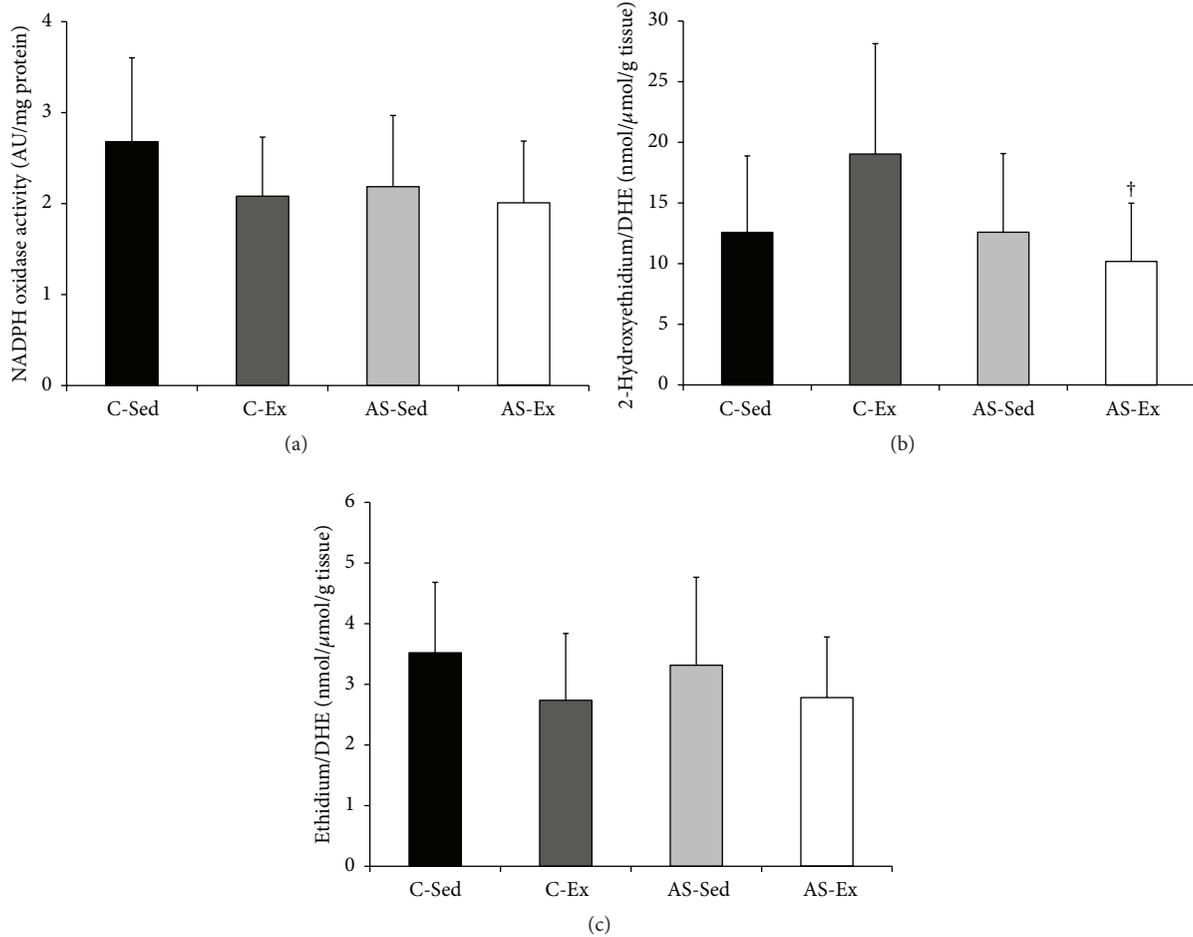


FIGURE 2: Soleus muscle NADPH oxidase activity analyzed by reduction of lucigenin (a), total reactive species generation by quantifying two dihydroethidium (DHE) oxidation-derived fluorescent compounds, 2-hydroxyethidium and ethidium, using HPLC ((b) and (c)). C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; n : number of animals. Data are mean \pm SD ($n = 8$); ANOVA and Tukey; [†] $p < 0.05$ versus C-Ex.

congestion, and right ventricular hypertrophy. Left untreated, rats evolve to death within two to four weeks [34]. We therefore initiated the exercise protocol 18 weeks after aortic stenosis induction when no rat had tachypnea.

We used an exercise protocol adapted from studies with rats subjected to different models of cardiac injury such as myocardial infarction [35], arterial hypertension [14, 36], diabetes mellitus [25], and aortic stenosis [19]. All these conditions are characterized by a moderate-to-severe reduction in physical exercise tolerance. In spontaneously hypertensive rats [37, 38], voluntary wheel running, which is characterized by short periods of high intensity activity, has been associated with impaired cardiac remodeling [38]. Furthermore, untreated spontaneously hypertensive rats experienced sudden death at a running speed of 17.5 m/min [39]. We therefore used a low intensity aerobic exercise protocol, subjecting our rats to physical exercise at a maximum speed 10 m/min, a tolerable intensity for all rats.

Despite the low intensity of the aerobic exercise, it was effective in improving functional capacity of both control and aortic stenosis groups. At the end of the experiment, time on

the treadmill and distance run were significantly higher in exercised than sedentary rats.

Body weight did not differ between groups. Decrease in body weight, known as cardiac cachexia, is often observed in advanced stages of heart failure in humans and is associated with poor prognosis, independently of important variables such as age, ejection fraction, exercise capacity, or functional class [40]. In this study, body weight preservation suggests that rats surviving to the end of the study were not in severe heart failure.

We performed transthoracic echocardiogram and tissue Doppler imaging before the exercise protocol to evaluate the degree of cardiac injury induced by aortic stenosis and assure homogeneity between groups. Both aortic stenosis groups presented concentric ventricular hypertrophy with mild systolic dysfunction and diastolic dysfunction. Cardiac remodeling with concentric hypertrophy and mild left ventricular dysfunction is usually observed in compensated chronic pressure overload [41].

After exercise, the aortic stenosis groups maintained the pattern of concentric left ventricular hypertrophy with slight

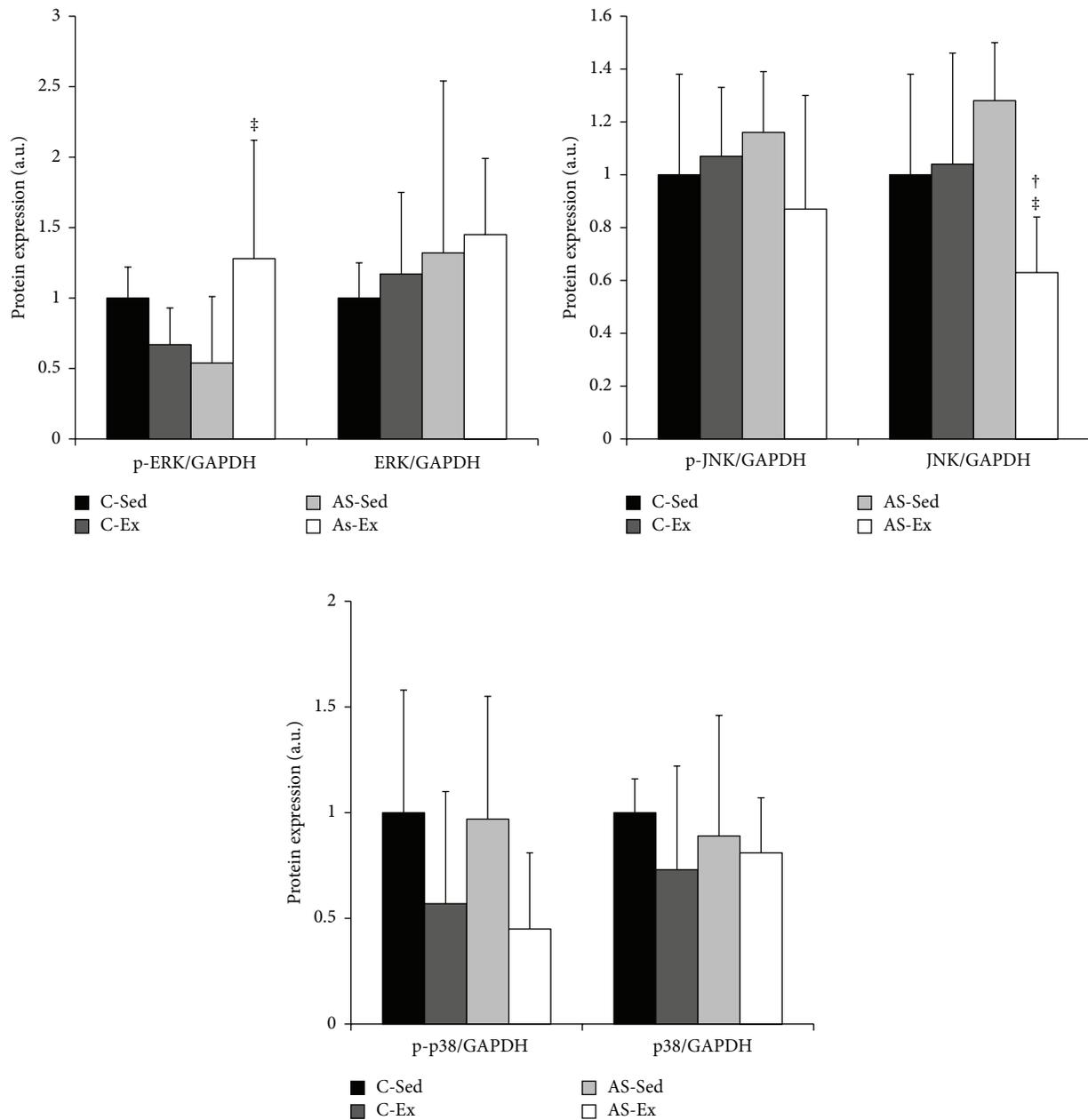


FIGURE 3: Protein levels of mitogen-activated protein kinases analyzed by Western blotting. Protein levels were normalized to GAPDH levels. C-Sed: sedentary control group; C-Ex: exercised control group; AS-Sed: sedentary aortic stenosis group; AS-Ex: exercised aortic stenosis group; n : number of animals. Data are mean \pm SD ($n = 7$); ANOVA and Tukey; [†] $p < 0.05$ versus C-Ex; [‡] $p < 0.05$ versus AS-Sed.

ventricular dilatation and mild systolic dysfunction. Diastolic dysfunction was characterized by increased E/A ratio, which establishes the restrictive advanced pattern of diastolic dysfunction. We did not detect any significant differences between EA-Ex and AS-Sed groups. We therefore conclude that a light exercise protocol improves functional capacity without changing structural or functional heart parameters in aortic stenosis rats. The effects of exercise on the heart with aortic stenosis are not completely understood. Mice with mild or severe aortic stenosis subjected to voluntary rotating wheel exercise for eight weeks presented unchanged ventricular

function with a trend towards aggravated LV dysfunction in severe aortic stenosis [18]. On the other hand, continuous treadmill aerobic exercise attenuated systolic dysfunction in aortic stenosis rats [19].

As cardiac function did not differ between AS-Ex and AS-Sed, the better physical capacity of exercised rats was probably related to functional improvement in skeletal muscles properties.

Skeletal muscles are composed of tissue which can change their metabolic, morphological, and functional characteristics after training or injury [42]. We evaluated the soleus,

a muscle with predominantly slow twitch fibers and oxidative metabolism. Previous studies have shown that this muscle is sensitive to alterations induced by acute or chronic heart failure [6–8]. Furthermore, skeletal muscles preferentially composed of oxidative fibers are more sensitive to the effects of aerobic exercise than muscles preferably composed of glycolytic fibers [43].

We first evaluated redox status. Although the mechanisms involved in muscle alterations are not completely understood, it is well established that increased oxidative stress plays a role in muscle abnormalities [7, 44].

Lipid hydroperoxide concentration was greater in AS-Sed than C-Sed and AS-Ex, showing that muscle oxidative stress is increased during heart failure and normalized by exercise. Cellular response to oxidative stress depends on the intensity of the reactive oxygen species generation. At low concentrations, reactive oxygen species stimulates antioxidant enzymes and receptors; at high concentrations, however, they inhibit enzymatic activity leading to cellular damage [45]. In the AS-Sed group, increased oxidative stress was combined with increased superoxide dismutase activity and reduced glutathione peroxidase activity. Exercise prevented changes in antioxidant enzymes in the AS-Ex group. Reduction of antioxidant enzyme activity has been described in skeletal muscle during heart failure [7, 35, 44]. In addition, clinical and experimental studies have shown that exercise induces antioxidant properties in skeletal muscle during heart failure [35, 46]. Our results therefore show that aerobic exercise prevented increases in oxidative stress and changes in antioxidant enzymes.

The NADPH oxidase complex is an important source of reactive oxygen species. To determine whether this complex is involved in increased oxidative stress and its modulation by exercise, we evaluated NADPH oxidase activity and gene subunit expression. We analyzed gene expression of transmembrane subunits NOX2, NOX4, and p22^{phox} and the cytosolic subunit p47^{phox}. NADPH oxidase activity and its subunits expression did not differ between groups, except for higher p22^{phox} subunit expression in AS-Ex than C-Ex. Despite this increased p22^{phox} gene expression, AS-Ex group NADPH oxidase activity was unchanged. We evaluated NADPH oxidase activity by measuring the light emitted by a reaction between reduced lucigenin and superoxide anion, which is a product of NADPH oxidase [31, 32]. The role of NADPH oxidase in muscle oxidative stress during heart failure is still unclear. In infarcted rats [21] and mice [47], increased NADPH oxidase activity was observed in the plantaris and hindlimb muscles. On the other hand, by evaluating NADPH oxidase activity using dihydroethidium, a molecule oxidized by superoxide anion, we found unchanged activity in soleus from rats with myocardial infarction-induced heart failure [7]. Different animal species, experimental model, or muscle evaluated may be responsible for divergent results.

Total reactive oxygen species generation was assessed by quantifying two fluorescent compounds derived from dihydroethidium oxidation: 2-hydroxyethidium and ethidium. Hydroxyethidium detection by HPLC fluorescence was proposed to more precisely measure superoxide production

in blood vessels and was later adapted for use in striated muscle [31, 32]. Total reactive oxygen species generation did not change between groups, except for a lower hydroxyethidium/dihydroethidium ratio in AS-Ex than C-Ex. This result is in accordance with the lower lipid hydroperoxide concentration in the AS-Ex group.

Intracellular reactive oxygen species signaling pathways are not completely understood. Studies suggest that MAPK and the NF- κ B pathway are involved in muscle response to oxidative stress [21, 48]. MAPKs consist of four members: extracellular signal-regulated kinase (ERK) 1/2, p38, c-Jun NH2-terminal kinase (JNK), and ERK 5 [49]. Expression of the MAPK proteins did not differ between AS-Sed and C-Sed. Despite ERK expression being approximately 46% lower in AS-Sed than C-Sed, this difference did not reach statistical significance. Exercise reduced total JNK expression in AS-Ex compared to C-Ex and AS-Sed and increased phosphorylated ERK in AS-Ex compared to AS-Sed. While activation of JNK and p38 is associated with cell response to stress and muscle loss, activation of ERK is related to anabolic processes such as cell division, growth, and differentiation [49]. Therefore, in this study exercise modulated total JNK and phosphorylated ERK towards preserving protein synthesis and muscle mass. We have previously observed MAPK changes in diaphragm and soleus muscle in infarcted rats with heart failure [7, 50]. Except for reduced I κ B expression in C-Ex and AS-Sed compared to C-Sed, NF- κ B pathway protein expression remained unchanged in this experimental model.

5. Conclusion

Exercise improves the functional capacity of rats with aortic stenosis-induced heart failure regardless of changes in cardiac structures or left ventricular function. In soleus muscle, exercise reduces oxidative stress, preserves antioxidant enzyme activity, and modulates JNK and p-ERK expression with no changes in NADPH oxidase activity, NADPH oxidase subunits gene expression, or NF- κ B pathway protein expression.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgments

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References

- [1] D. Mozaffarian, E. J. Benjamin, A. S. Go et al., "Heart disease and stroke statistics—2015 update: a report from the American Heart Association," *Circulation*, vol. 131, no. 4, pp. e29–e322, 2015.

- [2] S. Mandic, J. Myers, S. E. Selig, and I. Levinger, "Resistance versus aerobic exercise training in chronic heart failure," *Current Heart Failure Reports*, vol. 9, no. 1, pp. 57–64, 2012.
- [3] S. A. Phillips, K. Vuckovic, L. P. Cahalin, and T. Baynard, "Defining the system: contributors to exercise limitations in heart failure," *Heart Failure Clinics*, vol. 11, no. 1, pp. 1–16, 2015.
- [4] R. L. Damatto, P. F. Martinez, A. R. R. Lima et al., "Heart failure-induced skeletal myopathy in spontaneously hypertensive rats," *International Journal of Cardiology*, vol. 167, no. 3, pp. 698–703, 2013.
- [5] J. Downing and G. J. Balady, "The role of exercise training in heart failure," *Journal of the American College of Cardiology*, vol. 58, no. 6, pp. 561–569, 2011.
- [6] A. R. R. Lima, P. F. Martinez, K. Okoshi et al., "Myostatin and follistatin expression in skeletal muscles of rats with chronic heart failure," *International Journal of Experimental Pathology*, vol. 91, no. 1, pp. 54–62, 2010.
- [7] P. F. Martinez, C. Bonomo, D. M. Guizoni et al., "Influence of N-acetylcysteine on oxidative stress in slow-twitch soleus muscle of heart failure rats," *Cellular Physiology and Biochemistry*, vol. 35, no. 1, pp. 148–159, 2015.
- [8] P. F. Martinez, K. Okoshi, L. A. Zornoff et al., "Chronic heart failure-induced skeletal muscle atrophy, necrosis, and changes in myogenic regulatory factors," *Medical Science Monitor*, vol. 16, no. 12, pp. BR374–BR383, 2010.
- [9] J. J. V. McMurray, S. Adamopoulos, S. D. Anker et al., "ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC," *European Heart Journal*, vol. 33, no. 14, pp. 1787–1847, 2012.
- [10] C. W. Yancy, M. Jessup, B. Bozkurt et al., "2013 ACCF/AHA guideline for the management of heart failure: executive summary: a report of the American College of Cardiology Foundation/American Heart Association task force on practice guidelines," *Circulation*, vol. 128, no. 16, pp. 1810–1852, 2013.
- [11] K. E. Flynn, I. L. Piña, D. J. Whellan et al., "Effects of exercise training on health status in patients with chronic heart failure," *The Journal of the American Medical Association*, vol. 301, no. 14, pp. 1451–1459, 2009.
- [12] P. Giannuzzi, P. L. Temporelli, U. Corrà, and L. Tavazzi, "Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the exercise in left ventricular dysfunction and chronic heart failure (ELVD-CHF) trial," *Circulation*, vol. 108, no. 5, pp. 554–559, 2003.
- [13] C. E. Negrao, H. R. Middlekauff, I. L. Gomes-Santos, and L. M. Antunes-Correa, "Effects of exercise training on neurovascular control and skeletal myopathy in systolic heart failure," *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 308, no. 8, pp. H792–H802, 2015.
- [14] L. U. Pagan, R. L. Damatto, M. D. Cezar et al., "Long-term low intensity physical exercise attenuates heart failure development in aging spontaneously hypertensive rats," *Cellular Physiology and Biochemistry*, vol. 36, no. 1, pp. 61–74, 2015.
- [15] U. Wisløff, A. Støylen, J. P. Loennechen et al., "Superior cardiovascular effect of aerobic interval training versus moderate continuous training in heart failure patients. A randomized study," *Circulation*, vol. 115, no. 24, pp. 3086–3094, 2007.
- [16] J. C. Campos, T. Fernandes, L. R. Bechara et al., "Increased clearance of reactive aldehydes and damaged proteins in hypertension-induced compensated cardiac hypertrophy: impact of exercise training," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 464195, 11 pages, 2015.
- [17] D. J. Duncker, E. D. van Deel, M. C. de Waard, M. de Boer, D. Merkus, and J. van der Velden, "Exercise training in adverse cardiac remodeling," *Pflügers Archiv*, vol. 466, no. 6, pp. 1079–1091, 2014.
- [18] E. D. van Deel, M. de Boer, D. W. Kuster et al., "Exercise training does not improve cardiac function in compensated or decompensated left ventricular hypertrophy induced by aortic stenosis," *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 6, pp. 1017–1025, 2011.
- [19] R. W. A. Souza, W. P. Piedade, L. C. Soares et al., "Aerobic exercise training prevents heart failure-induced skeletal muscle atrophy by anti-catabolic, but not anabolic actions," *PLoS ONE*, vol. 9, no. 10, Article ID e110020, 2014.
- [20] K. Bedard and K.-H. Krause, "The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology," *Physiological Reviews*, vol. 87, no. 1, pp. 245–313, 2007.
- [21] L. R. G. Bechara, J. B. N. Moreira, P. R. Jannig et al., "NADPH oxidase hyperactivity induces plantaris atrophy in heart failure rats," *International Journal of Cardiology*, vol. 175, no. 3, pp. 499–507, 2014.
- [22] M. D. M. Cezar, R. L. Damatto, P. F. Martinez et al., "Aldosterone blockade reduces mortality without changing cardiac remodeling in spontaneously hypertensive rats," *Cellular Physiology and Biochemistry*, vol. 32, no. 5, pp. 1275–1287, 2013.
- [23] P. F. Martinez, K. Okoshi, L. A. M. Zornoff et al., "Echocardiographic detection of congestive heart failure in postinfarction rats," *Journal of Applied Physiology*, vol. 111, no. 2, pp. 543–551, 2011.
- [24] O. C. Mendes, M. M. Sugizaki, D. S. Campos et al., "Exercise tolerance in rats with aortic stenosis and ventricular diastolic and/or systolic dysfunction," *Arquivos Brasileiros de Cardiologia*, vol. 100, no. 1, pp. 44–51, 2013.
- [25] C. Gimenes, R. Gimenes, C. M. Rosa et al., "Low intensity physical exercise attenuates cardiac remodeling and myocardial oxidative stress and dysfunction in diabetic rats," *Journal of Diabetes Research*, vol. 2015, Article ID 457848, 10 pages, 2015.
- [26] K. Okoshi, J. R. Fioretto, M. P. Okoshi et al., "Food restriction induces in vivo ventricular dysfunction in spontaneously hypertensive rats without impairment of in vitro myocardial contractility," *Brazilian Journal of Medical and Biological Research*, vol. 37, no. 4, pp. 607–613, 2004.
- [27] K. Okoshi, L. S. Matsubara, M. P. Okoshi et al., "Food restriction-induced myocardial dysfunction demonstrated by the combination of in vivo and in vitro studies," *Nutrition Research*, vol. 22, no. 11, pp. 1353–1364, 2002.
- [28] K. Okoshi, H. B. Ribeiro, M. P. Okoshi et al., "Improved systolic ventricular function with normal myocardial mechanics in compensated cardiac hypertrophy," *Japanese Heart Journal*, vol. 45, no. 4, pp. 647–656, 2004.
- [29] C. Di Filippo, M. C. Trotta, R. Maisto et al., "Daily oxygen/O₃ treatment reduces muscular fatigue and improves cardiac performance in rats subjected to prolonged high intensity physical exercise," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 190640, 11 pages, 2015.
- [30] C. M. Rosa, N. P. Xavier, D. H. Campos et al., "Diabetes mellitus activates fetal gene program and intensifies cardiac remodeling and oxidative stress in aged spontaneously hypertensive rats," *Cardiovascular Diabetology*, vol. 12, article 152, 2013.
- [31] F. R. M. Laurindo, D. C. Fernandes, and C. X. C. Santos, "Assessment of superoxide production and NADPH oxidase

- activity by HPLC analysis of dihydroethidium oxidation products,” *Methods in Enzymology*, vol. 441, pp. 237–260, 2008.
- [32] D. C. Fernandes, J. Wosniak Jr., L. A. Pescatore et al., “Analysis of DHE-derived oxidation products by HPLC in the assessment of superoxide production and NADPH oxidase activity in vascular systems,” *The American Journal of Physiology—Cell Physiology*, vol. 292, no. 1, pp. C413–C422, 2007.
- [33] X. Yan, A. J. T. Schuldt, R. L. Price et al., “Pressure overload-induced hypertrophy in transgenic mice selectively overexpressing AT₂ receptors in ventricular myocytes,” *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 294, no. 3, pp. H1274–H1281, 2008.
- [34] M. O. Boluyt, K. G. Robinson, A. L. Meredith et al., “Heart failure after long-term supravalvular aortic constriction in rats,” *The American Journal of Hypertension*, vol. 18, no. 2, pp. 202–212, 2005.
- [35] J. B. N. Moreira, L. R. G. Bechara, L. H. M. Bozi et al., “High-versus moderate-intensity aerobic exercise training effects on skeletal muscle of infarcted rats,” *Journal of Applied Physiology*, vol. 114, no. 8, pp. 1029–1041, 2013.
- [36] A. J. Chicco, S. A. McCune, C. A. Emter et al., “Low-intensity exercise training delays heart failure and improves survival in female hypertensive heart failure rats,” *Hypertension*, vol. 51, no. 4, pp. 1096–1102, 2008.
- [37] R. L. Schultz, E. L. Kullman, R. P. Waters et al., “Metabolic adaptations of skeletal muscle to voluntary wheel running exercise in hypertensive heart failure rats,” *Physiological Research*, vol. 62, no. 4, pp. 361–369, 2013.
- [38] R. L. Schultz, J. G. Swallow, R. P. Waters et al., “Effects of excessive long-term exercise on cardiac function and myocyte remodeling in hypertensive heart failure rats,” *Hypertension*, vol. 50, no. 2, pp. 410–416, 2007.
- [39] C. A. Emter, S. A. McCune, G. C. Sparagna, M. J. Radin, and R. L. Moore, “Low-intensity exercise training delays onset of decompensated heart failure in spontaneously hypertensive heart failure rats,” *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 289, no. 5, pp. H2030–H2038, 2005.
- [40] S. D. Anker, A. Negassa, A. J. S. Coats et al., “Prognostic importance of weight loss in chronic heart failure and the effect of treatment with angiotensin-converting-enzyme inhibitors: an observational study,” *The Lancet*, vol. 361, no. 9363, pp. 1077–1083, 2003.
- [41] M. D. Cezar, R. L. Damatto, L. U. Pagan et al., “Early spironolactone treatment attenuates heart failure development by improving myocardial function and reducing fibrosis in spontaneously hypertensive rats,” *Cellular Physiology and Biochemistry*, vol. 36, no. 4, pp. 1453–1466, 2015.
- [42] P. Bonaldo and M. Sandri, “Cellular and molecular mechanisms of muscle atrophy,” *Disease Models and Mechanisms*, vol. 6, no. 1, pp. 25–39, 2013.
- [43] A. P. Russell, V. C. Foletta, R. J. Snow, and G. D. Wadley, “Skeletal muscle mitochondria: a major player in exercise, health and disease,” *Biochimica et Biophysica Acta—General Subjects*, vol. 1840, no. 4, pp. 1276–1284, 2014.
- [44] A. Linke, V. Adams, P. C. Schulze et al., “Antioxidative effects of exercise training in patients with chronic heart failure: increase in radical scavenger enzyme activity in skeletal muscle,” *Circulation*, vol. 111, no. 14, pp. 1763–1770, 2005.
- [45] Z. Radak, H. Y. Chung, and S. Goto, “Exercise and hormesis: oxidative stress-related adaptation for successful aging,” *Biogerontology*, vol. 6, no. 1, pp. 71–75, 2005.
- [46] A. V. N. Bacurau, M. A. Jardim, J. C. B. Ferreira et al., “Sympathetic hyperactivity differentially affects skeletal muscle mass in developing heart failure: role of exercise training,” *Journal of Applied Physiology*, vol. 106, no. 5, pp. 1631–1640, 2009.
- [47] Y. Ohta, S. Kinugawa, S. Matsushima et al., “Oxidative stress impairs insulin signal in skeletal muscle and causes insulin resistance in postinfarct heart failure,” *The American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 5, pp. H1637–H1644, 2011.
- [48] M.-S. Byun, K.-I. Jeon, J.-W. Choi, J.-Y. Shim, and D.-M. Jue, “Dual effect of oxidative stress on NF- κ B activation in HeLa cells,” *Experimental and Molecular Medicine*, vol. 34, no. 5, pp. 332–339, 2002.
- [49] H. Shi, J. M. Scheffler, C. Zeng et al., “Mitogen-activated protein kinase signaling is necessary for the maintenance of skeletal muscle mass,” *American Journal of Physiology—Cell Physiology*, vol. 296, no. 5, pp. C1040–C1048, 2009.
- [50] A. R. R. Lima, P. F. Martinez, R. L. Damatto et al., “Heart failure-induced diaphragm myopathy,” *Cellular Physiology and Biochemistry*, vol. 34, no. 2, pp. 333–345, 2014.

Research Article

Effect of Carnosine in Experimental Arthritis and on Primary Culture Chondrocytes

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Carnosine's (CARN) anti-inflammatory potential in autoimmune diseases has been but scarcely investigated as yet. The aim of this study was to evaluate the therapeutic potential of CARN in rat adjuvant arthritis, in the model of carrageenan induced hind paw edema (CARA), and also in primary culture of chondrocytes under H₂O₂ injury. The experiments were done on healthy animals, arthritic animals, and arthritic animals with oral administration of CARN in a daily dose of 150 mg/kg b.w. during 28 days as well as animals with CARA treated by a single administration of CARN in the same dose. CARN beneficially affected hind paw volume and changes in body weight on day 14 and reduced hind paw swelling in CARA. Markers of oxidative stress in plasma and brain (malondialdehyde, 4-hydroxynonenal, protein carbonyls, and lag time of lipid peroxidation) and also activity of gamma-glutamyltransferase were significantly corrected by CARN. CARN also reduced IL-1alpha in plasma. Suppression of intracellular oxidant levels was also observed in chondrocytes pretreated with CARN. Our results obtained on two animal models showed that CARN has systemic anti-inflammatory activity and protected rat brain and chondrocytes from oxidative stress. This finding suggests that CARN might be beneficial for treatment of arthritic diseases.

We dedicate this paper to the memory of Professor Alexander A. Boldyrev with respect and gratitude.

1. Introduction

Carnosine (CARN) is dipeptide consisting of β -alanine and L-histidine. It was shown to be a specific constituent of excitable tissues of all vertebrates accumulating in amounts exceeding that of ATP [1]. The antioxidant capacity of this compound is well documented, as well as its pH buffering, osmoregulating, and metal-chelating abilities [2]. However, a more recent study showed unexpectedly lower binding constant values of carnosine and therefore a relatively minor role of the transition metal ion chelation in its antioxidant abilities [3]. We previously showed inhibitory properties of CARN against degradation of hyaluronan solutions at

experimental conditions *in vitro*. In the reaction system with the prevalence of hydroxyl and/or peroxy-type radicals, CARN in 200 μ mol/L concentration tested exerted a protective action on hyaluronan degradation [4]. CARN, when compared with ascorbate, was more effective in inhibition of lipid peroxidation in meat [5]. At physiological concentrations, CARN was found to directly react with superoxide anion comparably to superoxide dismutase and the constant for interaction of carnosine with O₂^{•-} was calculated to be 10⁵ M⁻¹·s⁻¹ and not significantly different in respect to that of ascorbic acid and α -tocopherol [6]. Also, a sparing or regenerating effect of carnosine towards endogenous antioxidants was demonstrated in the liver of rats treated with

carnosine or L-histidine. Both compounds increased the liver content of glutathione and α -tocopherol [7]. CARN seems to be similarly effective endogenous antioxidant as ascorbate and α -tocopherol. A potentially useful characteristic of CARN is its ability to act as an antiglycating agent [8–10], to quench superoxide anion and hydroxide radical [11–13], and to neutralize 4-hydroxy-nonenal (HNE) and other toxic aldehydes [14–16]. CARN may be pluripotent with respect to its ability to suppress proteotoxic stress and aging-associated phenomena [17]. CARN may suppress glycolysis, similar to the effects of rapamycin, by inhibiting TOR activity which reduces glycolytic flux, thereby decreasing the potential for methylglyoxal generation [18]. It is possible that carnosine can promote catabolism of altered proteins. Preliminary evidence was obtained suggesting that carnosine stimulates catabolism of slowly turning over proteins in aged human fibroblasts following growth for many generations with the dipeptide [19]. Oxygen metabolism has an important role in the pathogenesis of rheumatoid arthritis (RA). Reactive oxygen species (ROS) produced in the excessive amounts under some pathological states exceed the physiological ROS buffering capacity and result in oxidative stress (OS). Excessive production of ROS can damage proteins, lipids, nucleic acids, and matrix components [20, 21]. OS and decreased antioxidant status are present in patients with RA, as observed in recent years [22].

The aim of this study was to evaluate whether administration of CARN in carrageenan induced hind paw edema (model of local acute inflammatory reaction) and in adjuvant arthritis (subchronic model of rodent polyarthritis) would ameliorate inflammation, OS, and disease progression. Furthermore, in order to elucidate a potential contribution of antioxidant mechanism to these effects at cellular level, *in vitro* evaluation of the efficacy of CARN to reduce OS markers of rat primary chondrocytes exposed to hydrogen peroxide was performed, along with the assessment of their viability protection.

2. Methods

2.1. Animals, Experimental Design, and Treatments. Male Lewis rats, weighing 160–180 g, were obtained from the Breeding Farm Dobra Voda (Slovakia). The rats had free access to standard pellet diet and tap water. The animal facilities comply with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes. The experimental protocol was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology and by the Slovak State Veterinary Committee of Animal Experimentation. AA was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* (MB) in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). The injection was performed near the tail base. The experiments included healthy animals (CO), arthritic animals (AA) not treated, and arthritic animals given 150 mg/kg b.w. of CARN daily for 28 days using gastric gavages (AA-CARN). In the acute model of

inflammation, hind paw edema was induced by intraplantar injection of 0.1 mL of 1% water solution of carrageenan type IV (CARA) into the right hind paw of Lewis rats. The experiment included animals with carrageenan induced hind paw edema without any drug administration (CARA) and animals with carrageenan induced hind paw edema given carnosine (CARA-CARN). CARN was given one hour before induction of CARA in a single oral dose of 150 mg/kg b.w.

2.2. Blood and Tissue Collection. After the animals had been sacrificed under deep ketamine/xylazine anesthesia, blood for plasma preparation and tissues for brain, spleen, and hind paw joint homogenate preparation were taken on day 28. Heparinized plasma and tissue were stored at -70°C until biochemical and immunological analysis.

2.3. Clinical Parameters: Hind Paw Volume and Body Mass. Hind paw volume (HPV) increase was calculated as the percentage increase in HPV on a given experimental day relative to the HPV at the beginning of the experiment. HPV was recorded on days 1, 14, and 28 with the use of an electronic water plethysmometer (UGO BASILE). Change of body mass—CBM (g)—was measured on days 1, 14, and 28. We monitored the HPV in the CARA model at minutes 0, 30, 60, 90, 120, and 240, using the same water plethysmometer.

2.4. Tissue Activity of Cellular γ -Glutamyltransferase in Joint and Spleen Tissue. The activity of cellular γ -glutamyltransferase (GGT) in hind paw joint and spleen tissue homogenates was measured by the method of Orłowski and Meister [23] as modified by Ondrejickova et al. [24]. Samples were homogenized in a buffer (2.6 mM NaH_2PO_4 , 50 mM Na_2HPO_4 , 15 mM EDTA, and 68 mM NaCl; pH 8.1) at 1:9 (w/v) by UltraTurax TP 18/10 (Janke & Kunkel, Germany) for 1 min at 0°C . Substrates (8.7 mM γ -glutamyl-p-nitroanilide, 44 mM methionine) were added to 65% isopropylalcohol to final concentrations of 2.5 mM and 12.6 mM, respectively. After incubation for 60 min at 37°C , the reaction was stopped with 2.3 mL cold methanol and the tubes were centrifuged for 20 min at 5000 rpm. Absorbance of supernatant was measured in a Specord 40 spectrophotometer in a 0.5 cm cuvette at 406 nm. Reaction mixtures in the absence of either substrate or acceptor were used as reference samples.

2.5. Malondialdehyde and 4-Hydroxynonenal in Plasma and Brain Homogenates

(1) *Malondialdehyde.* ELISA (Enzyme Linked Immunosorbent Assay) Kit (Cusabio catalogue number CSB-E08557h) for the quantitative determination of endogenous MDA concentrations in plasma and tissue homogenates was used. Procedure was done according to manufacturer instructions.

(2) *4-Hydroxynonenal.* ELISA Kit (Cusabio catalogue number CSB-E16214h) for the quantitative determination of endogenous 4-hydroxynonenal (HNE) concentrations in plasma and tissue homogenates was used. Procedure was done according to manufacturer instructions.

2.6. Lag Time of Fe^{2+} -Induced Chemiluminescence of Plasma and Brain Homogenate. Lag time of Fe^{2+} -induced chemiluminescence (LTC) of plasma was analyzed using the signal derived from addition of ferrous ions to plasma. After addition of 100 μ L of 25 mM $FeSO_4$ to the plasma sample, the lag period between initial fast flash and the following slow rising chemiluminescence signal reflecting the rate of lipid oxidation was measured. The lag time is referring to the stability of the sample to the Fe^{2+} -induced oxidation (the longer the lag period, the more stable the resistance of the biological material to oxidation) being dependent on intrinsic antioxidant capacity of plasma and brain homogenate. Chemiluminescence signal was monitored using LKB 1251 Chemiluminometer [25].

2.7. Protein Carbonyls in Plasma and Brain Homogenate

2.7.1. Blood Plasma. ELISA was used for quantitative determination of protein carbonyls in plasma [26]. Protein samples were derivatized with dinitrophenylhydrazine (DNPH) and adsorbed in multiwell plates (NuncImmunosorp plates, Roskilde, Denmark). A biotin-conjugated anti-dinitrophenyl rabbit IgG (Sigma, USA) was used as the primary antibody and a peroxidase conjugated monoclonal anti-rabbit-IgG antibody (Sigma, USA) as the secondary antibody. The development was performed with orthophenylenediamine.

2.7.2. Brain Tissue Homogenates. The amount of 250 μ L of 10% homogenate was added into three 2 mL test tubes for tissue blank and two parallel measurements of the sample. A 20% solution of trichloroacetic acid was added to each tube. The solutions were centrifuged for 10 min at 14 500 rpm. 200 μ L of 0.2 M NaOH was added into each tube. After complete dissolution of pellets, a volume of 200 μ L of 2 M HCl was added into each tube. 200 μ L of 10 mM dinitrophenylhydrazine solution was added only to sample tubes and incubated for 30 min. The samples were centrifuged for 10 min at 14 500 rpm. After centrifugation, the samples were washed four times with 700 μ L of a cooled solution of 98% ethanol:ethyl acetate (1:1). To each tube, 250 μ L of 6 M guanidine solution was added. Pellets were left to dissolve in guanidine overnight at 4°C. The concentration of proteins in each tube was measured at 280 nm and protein carbonyls were determined at 369 nm.

2.8. Glutathione Reductase in Brain Homogenate. The hemisphere of brain was homogenized (5% w/v) in 0.1 M sodium phosphate buffer (pH 7.0) and following centrifugation (14 000 rpm for 30 min) the supernatant was used for glutathione reductase (GR) analysis. GR activity was determined using a modification of the method described by Barker et al. [27]. The reaction mixture contained 200 μ L 0.1 M sodium phosphate buffer (pH 7.0), 30 μ L 0.1 mM NADPH, and 60 μ L of 5% homogenate. The reaction was initiated by the addition of 10 μ L GSSG (110 mg/1 mL). The oxidation of NADPH was followed at 340 nm. The molar extinction coefficient of 6.27×10^3 M/cm was used to determine GR activity, and one unit of

activity was defined as the number of μ M of NADPH oxidized $\text{min}^{-1} \cdot \text{mg}^{-1}$ proteins.

2.9. Proinflammatory Cytokine IL-1 α in Plasma. For determination of IL-1 α concentration in plasma, ELISA Kit from R&D Systems Quantikine for IL-1 was used. Assay procedure was performed as described in the product manual. The results were calculated from standard calibration curve on internal standards.

2.10. Primary Chondrocytes. Primary chondrocytes were isolated from the normal articular cartilage of Wistar rats by collagenase II (0.1%) and trypsin (0.25%) digest and cultured in DMEM/Ham's F-12 supplemented with 1 mmol/L glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum in CO_2 incubator at 37°C in a humidified atmosphere containing 5% CO_2 . The culture medium was refreshed every 48 h. When cells reached the confluence, they were detached by 0.05% trypsin and subcultured up to the third passage. Chondrocytes from the 2nd and the 3rd passages were used for the experiments.

2.11. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) Assay. Rat primary chondrocytes were grown on 96-well microplates until confluency in 200 μ L DMEM/Ham's F-12. The MTT colorimetric assay was performed as previously described [28]. Briefly, the cells were preincubated for 24 hours with or without different concentrations of CARN followed by the incubation with H_2O_2 in DMEM/Ham's F-12 for 60 min at 37°C. MTT was added to the final concentration of 0.5 mg/mL. For the compound cytotoxicity assays, MTT was added to the wells directly following the 24-hour incubation with the substance tested. After 4 hrs, 100 μ L of 10% sodium dodecyl sulfate in HCl (0.01 mol/L) was added and the cells were thoroughly resuspended. The absorbance was spectrophotometrically recorded at 570 nm on Tecan Infinite 200 instrument.

2.12. Cell Viability Evaluation. Rat primary chondrocytes were grown on 96-well microplates until confluency in 200 μ L DMEM/Ham's F-12. The MTT colorimetric assay was performed as described above. The assessment of viability of primary chondrocytes was completed by fluorescence microscopy technique. The cells were grown in 96-well plates. Following incubation with H_2O_2 , a medium was replaced by fresh medium for 5 hrs. Ethidium bromide (EB) and acridine orange dye mix (3 μ L) was added to each well and cells were viewed under the XDS-2 inverted fluorescence microscope [29]. Each image was collected with excitation at 488 nm. Calculations were done minimum in quadruplicate, counting a minimum of 100 total cells each.

2.13. Production of Intracellular Oxidants. Cellular oxidant production was determined by using dichlorodihydrofluorescein-diacetate (DCFH-DA) with a modified method as described by Giardina and Sait Inan [30]. The cells were grown in 96-well plates until confluency and then preincubated for 24 hours with CARN. Chondrocytes were then

preincubated with solution of DCFH-DA (15 $\mu\text{mol/L}$) for 30 min in Krebs-Ringer buffer (KRB; 10 mmol/L HEPES, 2 g/L BSA (bovine serum albumin), and pH 7.4). After the probe loading, the cells were washed with KRB and incubated with H_2O_2 . The fluorescence of the generated dichlorofluorescein was measured on Tecan Infinite 200 instrument at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

2.14. Statistical Analysis

2.14.1. Cell Cultures and Relevant Assays. Each experiment was performed at least three times. Results are expressed as median. For cell culture experiments, Levene's test of equality of variances between compared groups was applied. Since the groups showed equal variances, the means were compared using unpaired Student's *t*-test. The untreated cells were compared with H_2O_2 exposed cells without addition of CARN (*), CARN treated cells were compared with H_2O_2 exposed cells (+), and statistical significance was expressed as extremely significant ($p < 0.001$), highly significant ($p < 0.01$), significant ($p < 0.05$), and not significant ($p > 0.05$).

2.14.2. Measurements from In Vivo Experiments. Mean and S.E.M. values were calculated for each parameter in each group (8–10 animals in each experimental group). Data in figures are expressed as mean \pm S.E.M. The untreated arthritis group was compared with healthy control animals (*); treated arthritis groups were compared with untreated arthritic animals (+). Statistically significant differences among treated group, untreated group, and control groups were tested using parametric Analysis of Variance (ANOVA). Alternatively, nonparametric Kruskal-Wallis test (K-W) in case of non-normal distributed data was used. Post hoc tests (Tukey-Kramer (ANOVA), Dwass-Steel-Critchlow-Fligner (K-W)) were applied in situation where differences among groups were significant at level of significance $\alpha = 0.05$. After *post hoc* testing, the following significance designations were specified as follows: extremely significant ($p < 0.001$), highly significant ($p < 0.01$), significant ($p < 0.05$), and not significant ($p > 0.05$).

3. Results

3.1. Adjuvant Arthritis and Carrageenan Induced Hind Paw Edema. CARN beneficially affected clinical parameters (change of body mass and hind paw volume) in the model of AA measured on days 14 and 28 (Figures 1 and 2) and hind paw volume significantly at day 14 (Figure 2). CARN also reduced hind paw volume in the model of CARRA during the whole experiment: 30 min–240 min (Figure 3), most effectively at 240 min. Reduction of hind paw volume by CARN treatment in AA on day 14 was 42% and in CARRA model 34% at 240 min, when compared to untreated arthritic animals. Activity of GGT in joint and spleen homogenates from arthritic animals was reduced by CARN administration (Figures 4(a) and 4(b)) significantly in spleen tissue—35% (Figure 4(b)). CARN decreased lipid peroxidation in plasma assessed as MDA and HNE (Figures 5(a) and 5(b)). Moreover,

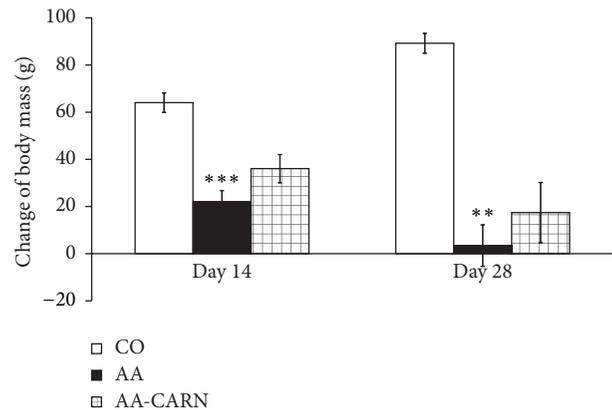


FIGURE 1: Changes in body mass of animals in adjuvant arthritis. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. *** $p < 0.001$ and ** $p < 0.01$ with respect to control healthy animals. The experiment included healthy intact animals as reference controls (CO), arthritic animals without any drug administration (AA), and arthritic animals with the administration of carnosine (AA-CARN).

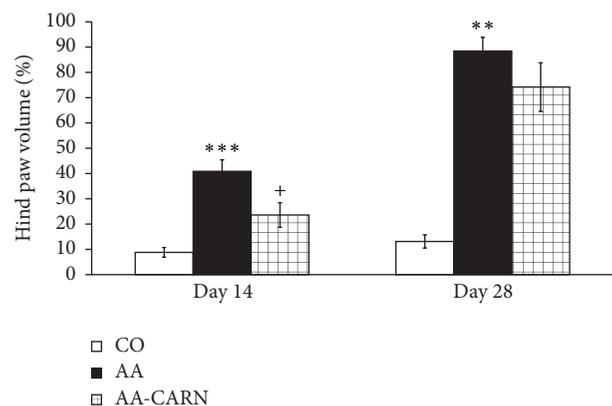


FIGURE 2: Changes in hind paw volume of animals in adjuvant arthritis. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. *** $p < 0.001$ and ** $p < 0.01$ with respect to control healthy animals; + $p < 0.05$ with respect to untreated arthritic animals. Groups of animals are the same as in Figure 1.

plasmatic proteins were protected against oxidation occurring in AA development very effectively by CARN administration (Figure 5(d)). Also total resistance of plasma against Fe^{2+} -induced oxidation was significantly increased by CARN measured as lag time of chemiluminescence (Figure 5(c)). AA increased the levels of inflammation marker IL-1 α in plasma (Figure 6). Our results point out to connection between OS and immune response in AA, because the decrease of OS markers (MDA, HNE, LTC, and protein carbonyls) was simultaneously accompanied by reduction of immunological marker IL-1 α in plasma by CARN. This result is showing that CARN can have also anti-inflammatory activity (Figure 6). AA caused increased oxidative stress in brain tissue measured by MDA, HNE, and protein carbonyls. CARN completely prevented the damage done by oxidative

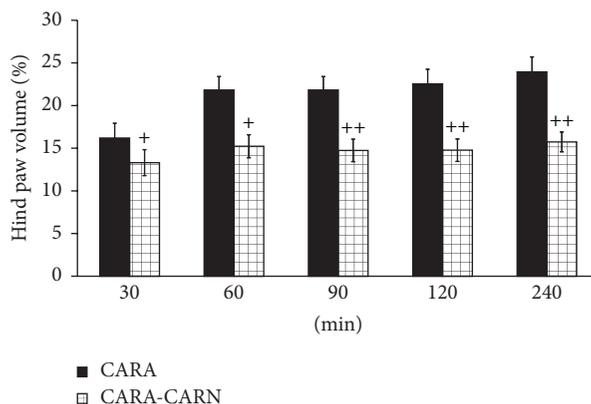


FIGURE 3: Changes in carrageenan induced edema of hind paws of Lewis rats. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. $^+p < 0.05$ and $^{++}p < 0.01$ with respect to untreated animals with carrageenan induced hind paw edema. The experiment included animals with carrageenan induced hind paw edema without any drug administration (CARA), and animals with carrageenan induced hind paw edema with the administration of carnosine (CARA-CARN).

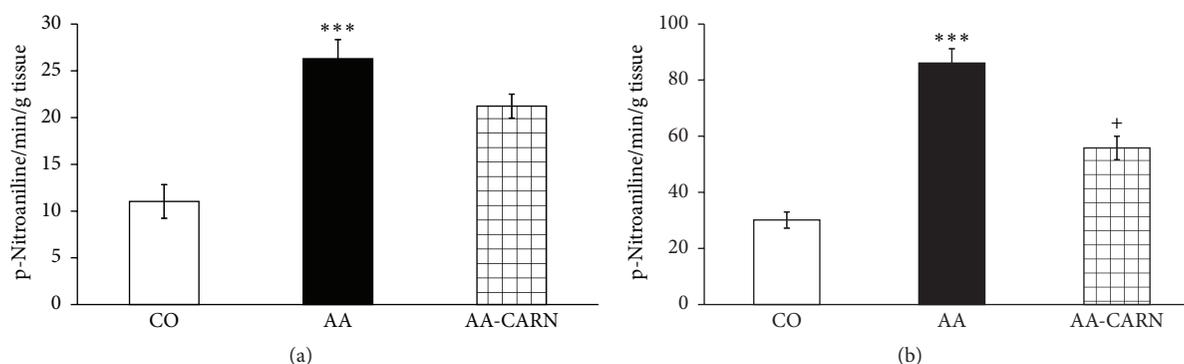


FIGURE 4: Activity of GGT in joint (a) and spleen (b) tissue homogenates in the adjuvant arthritis. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. $^{***}p < 0.001$ with respect to control healthy animals; $^+p < 0.05$ with respect to untreated arthritic animals. Groups of animals are the same as in Figure 1.

stress to lipids and proteins (Figures 7(a), 7(b), and 7(c)). Lowered antioxidant capacity of brain tissue was measured as decreased lag time of chemiluminescence and was corrected by CARN administration (Figure 8(a)). Activity of GR in brain tissue homogenates of arthritic animals was significantly elevated and was decreased by CARN to control values (Figure 8(b)).

3.2. Primary Cell Culture of Chondrocytes. Cytotoxicity of hydrogen peroxide to primary chondrocytes showed a clear concentration-dependent profile (Figure 9(a)). The MTT viability was reduced to $43.6 \pm 0.6\%$ of control formazan production upon treatment with maximum 5 mM H_2O_2 (Figure 9(a)). In addition, representative image (Figure 9(c)) shows the concomitant increase of EB-positive dead cells (by $48.4 \pm 4.6\%$) in primary chondrocytes exposed to H_2O_2 (5 mM) in comparison to untreated cells (0%, Figure 8(b)). An apparent lack of mitotic cells was also observed in chondrocytes exposed to H_2O_2 (yielding $0.9 \pm 0.5\%$, Figure 9(c)) in contrast to a noticeable number of dividing cells in untreated control ($11.2 \pm 1.8\%$, calculated as mother cells and daughter pair cells with bright condensed nuclear

chromatin, Figure 8(b)). In spite of a remarkable decrease of MTT reduction by chondrocytes treated with 1 mM H_2O_2 ($55.7 \pm 0.0\%$), only a negligible increase of EB-positive cells was observed at this concentration (data not shown). Pretreatment with CARN reduced considerably the intracellular levels of oxidants in 5 mM H_2O_2 -stressed primary chondrocytes (Figure 10(a)), which was accompanied with a moderate but significant prevention of viability injury of the cells (Figure 10(b)). For the concentration range tested, no dose dependency was found.

4. Discussion

The reactive oxygen and nitrogen species can react with lipids, proteins, and nucleic acids and are thought to be of importance for the etiology of chronic inflammatory rheumatic diseases [20]. One approach to counteract this OS situation is the use of antioxidants as therapeutic agents. CARN was found to have neuroprotective, hepatoprotective, and antiaging abilities [31] as well as antiradical activity [12, 13]. Nevertheless, its anti-inflammatory potential in

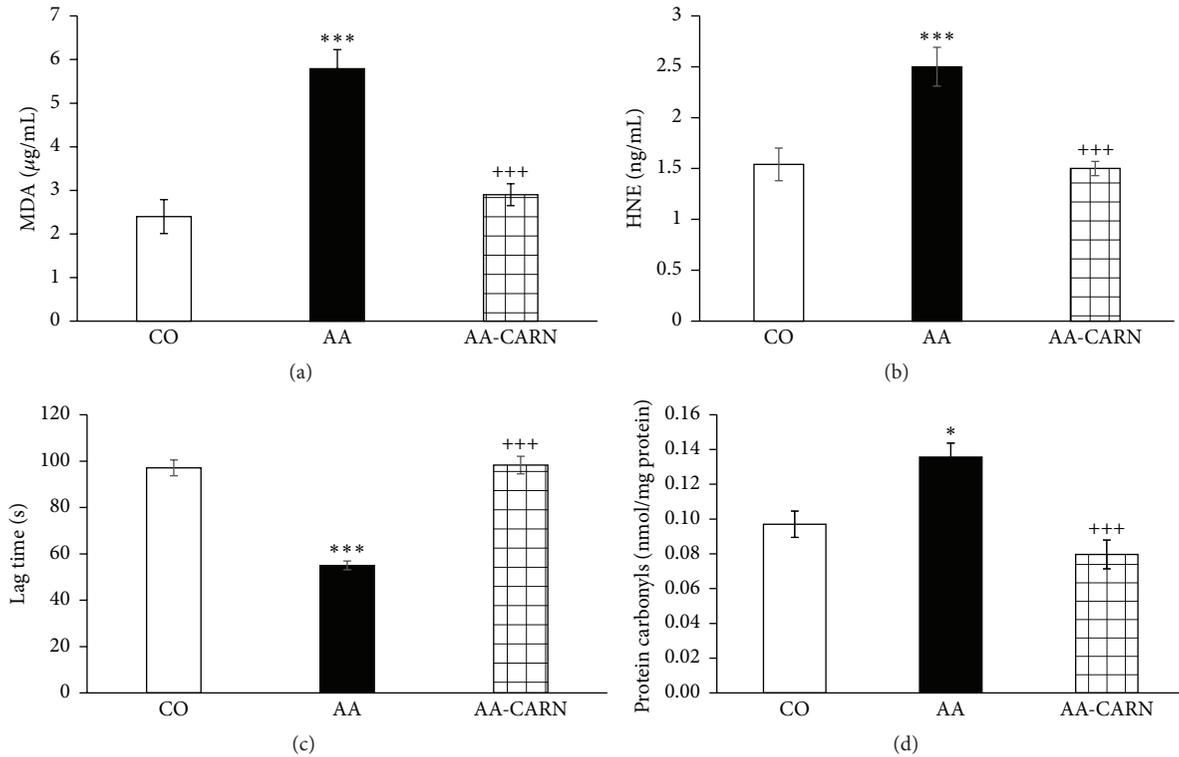


FIGURE 5: Markers of oxidative stress measured in the plasma of rats with adjuvant arthritis. Level of MDA (a) and HNE (b), lag time of Fe^{2+} -induced chemiluminescence (c), and levels of protein carbonyls (d). The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. *** $p < 0.001$ and * $p < 0.05$ with respect to control healthy animals; and +++ $p < 0.001$ with respect to untreated arthritic animals. Groups of animals are the same as in Figure 1.

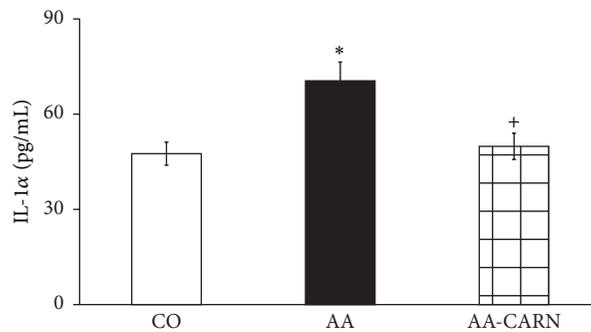


FIGURE 6: Level of IL-1 α in plasma in the adjuvant arthritis. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. * $p < 0.05$ with respect to control healthy animals; + $p < 0.05$ with respect to untreated arthritic animals. Groups of animals are the same as in Figure 1.

autoimmune systemic inflammatory diseases, as RA, has been scarcely investigated as yet.

The GGT activity was elevated in peripheral joint and spleen tissue. CARN effectively reduced the activity of GGT in spleen homogenates and slightly in joint. In our previous studies with coenzyme Q_{10} [32] and glucomannan [33], the reduction of activity of GGT in spleen was accompanied also by beneficial improvement of clinical markers of AA disease. Basaran-Küçükgergin et al. [34] showed the ability of CARN to decrease the GGT activity in serum of diethylnitrosamine-induced OS and tissue injury in liver of

rats. RA was associated with significant depletion (ca. 50%) in glutathione levels compared with normal control subjects. Serum levels of the detoxifying enzymes GR and glutathione peroxidase decreased by ca. 50% and 45%, respectively. These results support a hypothesis that defense mechanisms against reactive oxygen species are impaired in RA [35–37]. In our experiment, we found increased activity of GR in brain tissue of arthritic animals, which was decreased to basal values after administration of CARN. This result is suggesting a higher turnover of glutathione in brain during AA, probably due to increased OS also in this tissue (see below the increased

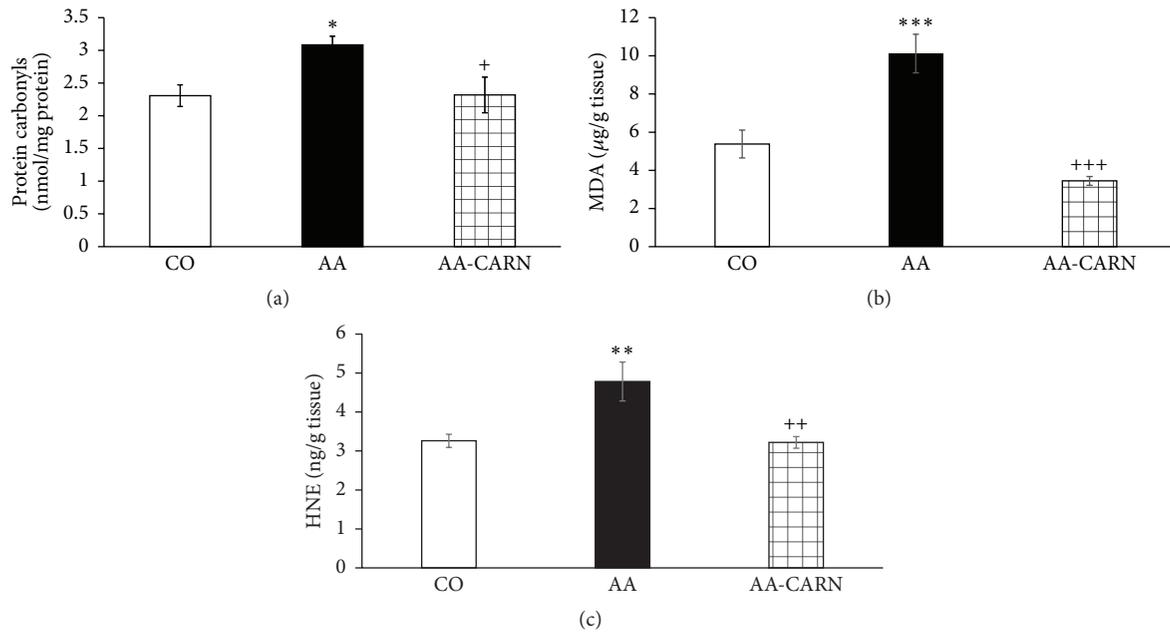


FIGURE 7: Level of protein carbonyls (a), MDA (b), and HNE (c) in brain tissue of rats with adjuvant arthritis. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ with respect to control healthy animals; + $p < 0.05$, ++ $p < 0.01$, and +++ $p < 0.001$ with respect to untreated arthritic animals. Groups of animals are the same as in Figure 1.

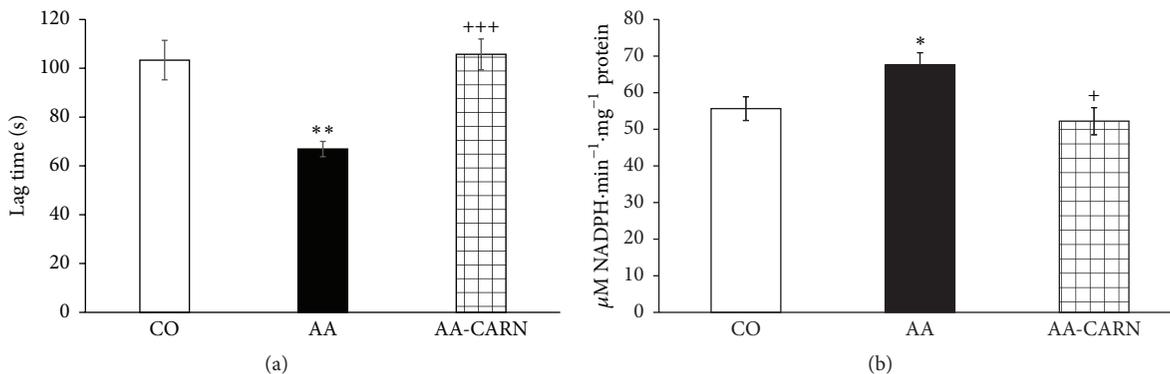


FIGURE 8: Lag time of chemiluminescence (a) and activity of glutathione reductase (b) in brain tissue of rats with adjuvant arthritis. The data are expressed as arithmetic mean with S.E.M. Each group contained 8–10 animals. * $p < 0.05$, ** $p < 0.01$ with respect to control healthy animals; + $p < 0.05$, +++ $p < 0.001$ with respect to untreated arthritic animals. Groups of animals are the same as in Figure 1.

protein carbonyl level, MDA, HNE, and LTC in brain tissue). Unfortunately, there is a lack of studies about CARN affecting GR; thus the mechanism how CARN decreases GR activity remains unclear. CARN increased the LTC in plasma samples, which refers to its ability to restore the systemic antioxidant capacity of plasma. CARN has shown a good protective activity against LTC as human plasma lipoproteins *in vitro* and brain of experimental animals [38]. In the present experiment, we report for the first time on the protective effect of CARN on plasmatic LTC and on GR activity in brain of rats with AA. In animal models of AA, the level of MDA was elevated in the plasma [39, 40]. Administration of CARN lowered the level of secondary products of lipid peroxidation in plasma measured as MDA and HNE. There is only little information about OS and brain damage in the

literature [41, 42]. For the first time, we evidenced increased HNE and reduced lag time of chemiluminescence in rat brain during AA. Although RA is not a typical CNS involvement disease, brain dysfunctions occur in 20 to 30% of rheumatic patients [43]. In the hippocampus of AA animals, upregulation of mRNA for IL-1 β , IL-6, and markers of oxidative stress-inducible NO synthase and NADPH oxidase-1 were observed within four days. The changes correlated with anxiety-like behavior [42]. Elevated levels of protein carbonyls were found in experimental animals [44]. In this paper, we report for the first time a significant elevation of protein carbonyls in brain of rats with AA. This novel finding emphasizes the systemic role of OS in chronic inflammatory diseases such as AA with oxidatively modified proteins not in directly affected tissues only (cartilage, bone, and skeletal muscle).

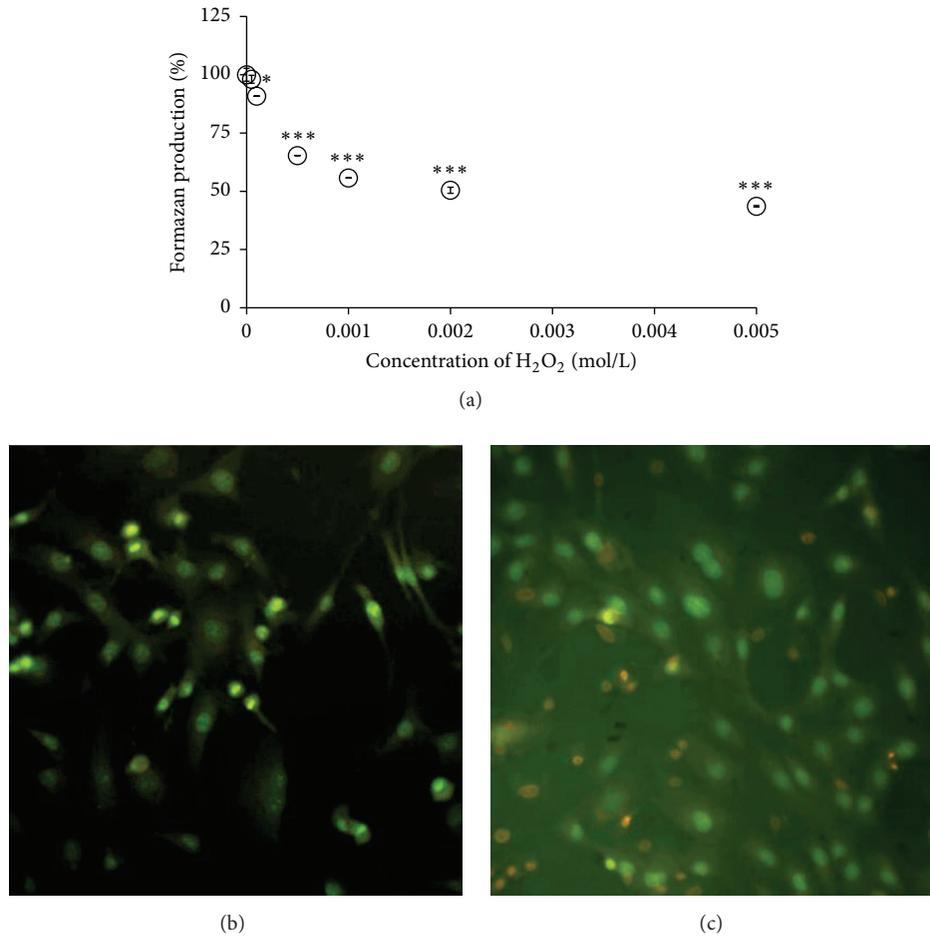


FIGURE 9: Vitality changes of primary rat chondrocytes incubated with hydrogen peroxide. Concentration-dependent effect of H₂O₂ on MTT viability (a) and combined fluorescence labeling of the cells with ethidium bromide and acridine orange (b, c). Images were captured 5 hours following incubation without (b) or with H₂O₂ (5 mmol/L, 400x) (c). Results are expressed as arithmetic mean with S.E.M. * $p < 0.05$ and *** $p < 0.001$ with respect to control.

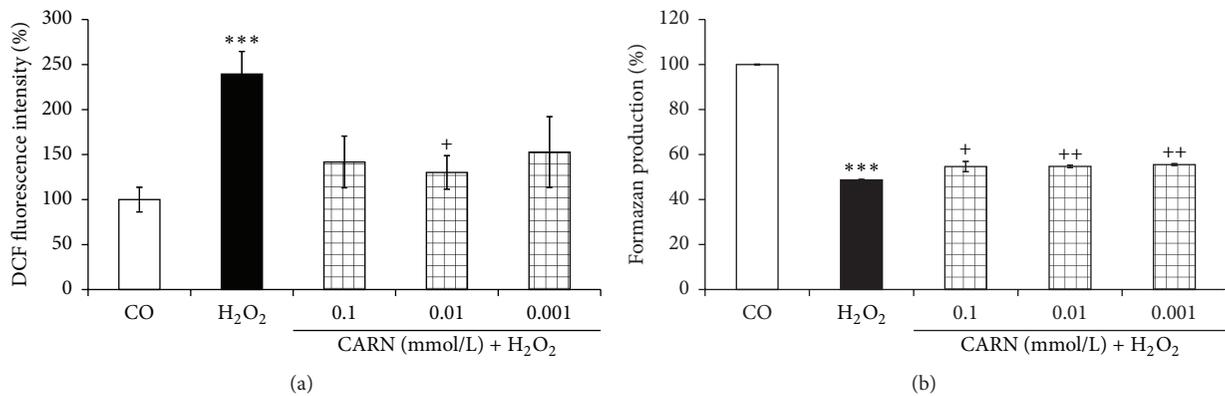


FIGURE 10: Effect of CARN on intracellular oxidant levels (a) and on viability injury (b) in rat primary chondrocytes exposed to H₂O₂. The cells were preincubated for 20 hours with the compound tested. Results are expressed as median. *** $p < 0.001$ with respect to control and ++ $p < 0.01$ and + $p < 0.05$ with respect to H₂O₂ control.

Other authors also found CARN to reduce protein carbonyls. CARN decreased protein carbonyl levels in both liver and brain tissues in several tissues of rats exposed to chronic cold plus immobilization stress [45]. In our experiment, CARN showed complex protective effects against OS induced depletion of plasma antioxidants assessed by LTC, lipid peroxidation measured as MDA and HNE, and protein carbonylation.

The outcome of our *in vitro* experiments shed light partially also on an alternative mechanism of antioxidant action of CARN. Hydrogen peroxide, a highly reactive substance involved in the pathogenesis of RA, caused a concentration-dependent viability decrease of primary chondrocytes (principal components of articular cartilage). Thus CARN may provide also prevention of oxidative damage and tissue injury in RA development. We did not find typical dose-dependent effect for the concentration range tested, suggesting indirect mechanism of protection by CARN against free radical damage derived from H₂O₂ likely linked to its referred hormetic properties [46]. In agreement with works reporting on a biphasic dose-response curve for the substances inducing hormesis [47], we found the maximum suppression of ROS levels in the cells pretreated with the middle concentration tested (10 μM) of CARN.

The action of CARN resulted in decreased systemic inflammation in AA, monitored by plasmatic level of proinflammatory cytokine IL-1α. Recent research has shown that in the processes of RA IL-1 is one of the pivotal cytokines in initiating the disease. In patients with RA and related spondyloarthropathies, IL-1 and TNFα are key contributors [48, 49]. In AA, CARN significantly reduced the level of IL-1α in plasma, but this effect resulted only in mild reduction of HPV on day 28. However, while inhibition of IL-1, TNFα, or both yields a significant anti-inflammatory effect in rats with AA, residual disease persists [32]. Beneficial effects of CARN manifested in reduction of systemic OS and reduced level of IL-1α in plasma were accompanied also by reduction of HPV and CBM. CARN beneficially affected HPV and CBM measured on day 14 and on day 28, significantly on day 14 when the clinical manifestation of the disease started. CARN was able to delay the disease onset. CARN also reduced HPV in the model of CARA during the whole experiment, 30 min–240 min, and was more effective in this animal model of acute inflammation. One of the possible explanations of HPV reduction is that restoration of redox balance in AA could have inhibitory effects on some immune cells and cytokine signaling involved in disease.

5. Conclusion

We showed a protective effect of CARN against oxidative damage on chondrocytes, which may be helpful in preventing cartilage degradation in “arthritic” joint. CARN also reduced the level of protein carbonyls and the activity of glutathione reductase in the brain of animals with AA, which is a unique finding for this animal model of arthritis. Our results from two animal models indicate that CARN may have also systemic anti-inflammatory effects. However, it still remains unclear if the ability of CARN to restore redox balance is

the only mechanism responsible for its anti-inflammatory effects in AA and CARA. Nevertheless, CARN administered together with standard antirheumatic therapy could enhance its effectivity and it might become a potential candidate to enrich the repertoire of anti-inflammatory drugs in the future.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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References

- [1] A. A. Boldyrev and S. E. Severin, “The histidine-containing dipeptides, carnosine and anserine: distribution, properties and biological significance,” *Advances in Enzyme Regulation*, vol. 30, pp. 175–193, 1990.
- [2] A. A. Boldyrev, “Retrospectives and perspectives on the biological activity of histidine-containing dipeptides,” *International Journal of Biochemistry*, vol. 22, no. 2, pp. 129–132, 1990.
- [3] S. Velez, N. G. Nair, and V. P. Reddy, “Transition metal ion binding studies of carnosine and histidine: biologically relevant antioxidants,” *Colloids and Surfaces B: Biointerfaces*, vol. 66, no. 2, pp. 291–294, 2008.
- [4] F. Drafi, K. Bauerova, K. Valachova et al., “Carnosine inhibits degradation of hyaluronan induced by free radical processes in vitro and improves the redox imbalance in adjuvant arthritis in vivo,” *Neuro Endocrinology Letters*, vol. 31, supplement 2, pp. 96–100, 2010.
- [5] B. J. Lee, G. D. Hendricks, and P. D. Cornforth, “A comparison of carnosine and ascorbic acid on color and lipid stability in a ground beef patty model system,” *Meat Science*, vol. 51, no. 3, pp. 245–253, 1999.
- [6] G. I. Klebanov, Y. O. Teselkin, I. V. Babenkova et al., “Evidence for a direct interaction of superoxide anion radical with carnosine,” *Biochemistry and Molecular Biology International*, vol. 43, no. 1, pp. 99–106, 1997.
- [7] S.-L. Yan, S.-T. Wu, M.-C. Yin, H.-T. Chen, and H.-C. Chen, “Protective effects from carnosine and histidine on acetaminophen-induced liver injury,” *Journal of Food Science*, vol. 74, no. 8, pp. H259–H265, 2009.
- [8] A. Boldyrev, “Carnosine as natural antioxidant and neuroprotector: biological functions and possible clinical use,” in *Free Radicals, Nitric Oxide, and Inflammation: Molecular, Biochemical, and Clinical Aspects*, A. Tomasi, T. Ozben, and V. Skulachev,

- Eds., pp. 202–217, IOS Press, Amsterdam, The Netherlands, 2002.
- [9] A. R. Hipkiss and C. Brownson, “Carnosine reacts with protein carbonyl groups: another possible role for the anti-ageing peptide?” *Biogerontology*, vol. 1, no. 3, pp. 217–223, 2000.
 - [10] A. R. Hipkiss, V. C. Worthington, D. T. J. Himsworth, and W. Herwig, “Protective effects of carnosine against protein modification mediated by malondialdehyde and hypochlorite,” *Biochimica et Biophysica Acta*, vol. 1380, no. 1, pp. 46–54, 1998.
 - [11] A. A. Boldyrev, *Carnosine and Oxidative Stress in Cells and Tissues*, Nova Science Publishers, New York, NY, USA, 2007.
 - [12] A. R. Pavlov, A. A. Revina, A. M. Dupin, A. A. Boldyrev, and A. I. Yaropolov, “The mechanism of interaction of carnosine with superoxide radicals in water solutions,” *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1157, no. 2, pp. 304–312, 1993.
 - [13] A. M. Rubtsov, M. Schara, M. Sentjurc, and A. A. Boldyrev, “Hydroxyl radical-scavenging activity of carnosine: a spin trapping study,” *Acta Pharmaceutica Jugoslavica*, vol. 41, no. 4, pp. 401–407, 1991.
 - [14] G. Aldini, M. Carini, G. Beretta, S. Bradamante, and R. M. Facino, “Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction?” *Biochemical and Biophysical Research Communications*, vol. 298, no. 5, pp. 699–706, 2002.
 - [15] Y. Liu, G. Xu, and L. M. Sayre, “Carnosine inhibits (E)-4-hydroxy-2-nonenal induced protein cross-linking: structural characterization of carnosine-HNE adducts,” *Chemical Research in Toxicology*, vol. 16, no. 12, pp. 1589–1597, 2003.
 - [16] M. Štrosová, I. Tomašková, S. Poništ et al., “Oxidative impairment of plasma and skeletal muscle sarcoplasmic reticulum in rats with adjuvant arthritis—effects of pyridoindole antioxidants,” *Neuroendocrinology Letters*, vol. 29, no. 5, pp. 706–711, 2008.
 - [17] A. R. Hipkiss, “Energy metabolism, proteotoxic stress and age-related dysfunction—protection by carnosine,” *Molecular Aspects of Medicine*, vol. 32, no. 4–6, pp. 267–278, 2011.
 - [18] K. M. Desai, T. Chang, H. Wang et al., “Oxidative stress and aging: is methylglyoxal the hidden enemy?” *Canadian Journal of Physiology and Pharmacology*, vol. 88, no. 3, pp. 273–284, 2010.
 - [19] A. R. Hipkiss, “On the enigma of carnosine’s anti-ageing actions,” *Experimental Gerontology*, vol. 44, no. 4, pp. 237–242, 2009.
 - [20] K. Bauerová and Š. Bezek, “Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis,” *General Physiology and Biophysics*, vol. 18, pp. 15–20, 1999.
 - [21] C. Y. Chuang, G. Degendorfer, and M. J. Davies, “Oxidation and modification of extracellular matrix and its role in disease,” *Free Radical Research*, vol. 48, no. 9, pp. 970–989, 2014.
 - [22] S. Kundu, P. Ghosh, S. Datta, A. Ghosh, S. Chattopadhyay, and M. Chatterjee, “Oxidative stress as a potential biomarker for determining disease activity in patients with Rheumatoid Arthritis,” *Free Radical Research*, vol. 46, no. 12, pp. 1482–1489, 2012.
 - [23] M. Orlowski and A. Meister, “The gamma-glutamyl cycle: a possible transport system for amino acids,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 67, no. 3, pp. 1248–1255, 1970.
 - [24] O. Ondrejickova, A. Ziegelhoeffer, I. Gabauer et al., “Evaluation of ischemia-reperfusion injury by malondialdehyde, glutathione and gamma-glutamyl transpeptidase: lack of specific local effects in diverse parts of the dog heart following acute coronary occlusion,” *Cardioscience*, vol. 4, no. 4, pp. 225–230, 1993.
 - [25] T. N. Fedorova, A. A. Boldyrev, and I. V. Gannushkina, “Lipid peroxidation in experimental ischemia of the brain,” *Biochemistry*, vol. 64, no. 1, pp. 75–79, 1999.
 - [26] H. Buss, T. P. Chan, K. B. Sluis, N. M. Domigan, and C. C. Winterbourn, “Protein carbonyl measurement by a sensitive ELISA method,” *Free Radical Biology and Medicine*, vol. 23, no. 3, pp. 361–366, 1997.
 - [27] J. E. Barker, S. J. R. Heales, A. Cassidy, J. P. Bolaños, J. M. Land, and J. B. Clark, “Depletion of brain glutathione results in a decrease of glutathione reductase activity; an enzyme susceptible to oxidative damage,” *Brain Research*, vol. 716, no. 1–2, pp. 118–122, 1996.
 - [28] A. Cumaoglu, L. Rackova, M. Stefek, M. Kartal, P. Maechler, and Ç. Karasu, “Effects of olive leaf polyphenols against H₂O₂ toxicity in insulin secreting β -cells,” *Acta Biochimica Polonica*, vol. 58, no. 1, pp. 45–50, 2011.
 - [29] L. Račková, V. Šnirc, T. Jung, M. Štefek, C. Karasu, and T. Grune, “Metabolism-induced oxidative stress is a mediator of glucose toxicity in HT22 neuronal cells,” *Free Radical Research*, vol. 43, no. 9, pp. 876–886, 2009.
 - [30] C. Giardina and M. Sait Inan, “Nonsteroidal anti-inflammatory drugs, short-chain fatty acids, and reactive oxygen metabolism in human colorectal cancer cells,” *Biochimica et Biophysica Acta*, vol. 1401, no. 3, pp. 277–288, 1998.
 - [31] A. A. Boldyrev, “Protection of proteins from oxidative stress: a new illusion or a novel strategy?” *Annals of the New York Academy of Sciences*, vol. 1057, pp. 193–205, 2005.
 - [32] K. Bauerova, E. Paulovicova, D. Mihalova et al., “Combined methotrexate and coenzyme Q10 therapy in adjuvant-induced arthritis evaluated using parameters of inflammation and oxidative stress,” *Acta Biochimica Polonica*, vol. 57, no. 3, pp. 347–354, 2010.
 - [33] K. Bauerova, S. Ponist, J. Navarova et al., “Glucomannan in prevention of oxidative stress and inflammation occurring in adjuvant arthritis,” *Neuroendocrinology Letters*, vol. 29, no. 5, pp. 691–696, 2008.
 - [34] C. Basaran-Küçükgergin, I. Bingül, M. S. Tekkesin, V. Olgaç, S. Dogru-Abbasoglu, and M. Uysal, “Effects of carnosine, taurine, and betaine pretreatments on diethylnitrosamine-induced oxidative stress and tissue injury in rat liver,” *Toxicology and Industrial Health*, 2014.
 - [35] P. B. Desai, S. Manjunath, K. Sumangala, K. Chetana, and J. Vanishree, “Oxidative stress and enzymatic antioxidant status in rheumatoid arthritis: a case control study,” *European Review for Medical and Pharmacological Sciences*, vol. 14, no. 11, pp. 959–967, 2010.
 - [36] M. Q. Hassan, R. A. Hadi, Z. S. Al-Rawi, V. A. Padron, and S. J. Stohs, “The glutathione defense system in the pathogenesis of rheumatoid arthritis,” *Journal of Applied Toxicology*, vol. 21, no. 1, pp. 69–73, 2001.
 - [37] U. Tarp, K. Stengaard-Pedersen, J. C. Hansen, and E. B. Thorling, “Glutathione redox cycle enzymes and selenium in severe rheumatoid arthritis: lack of antioxidative response to selenium supplementation in polymorphonuclear leucocytes,” *Annals of the Rheumatic Diseases*, vol. 51, no. 9, pp. 1044–1049, 1992.
 - [38] T. N. Fedorova, “Application of chemiluminescent analysis for comparative assessment of antioxidant activity of some pharmacological compounds,” *Eksperimental’naia i Klinicheskaia Farmakologija*, vol. 66, no. 5, pp. 56–58, 2003.

- [39] K. Bauerova, J. Kucharska, D. Mihalova, J. Navarova, A. Gvozdjakova, and Z. Sumbalova, "Effect of coenzyme Q₁₀ supplementation in the rat model of adjuvant arthritis," *Biomedical Papers*, vol. 149, no. 2, pp. 501–503, 2005.
- [40] Y.-H. He, J. Zhou, Y.-S. Wang et al., "Anti-inflammatory and anti-oxidative effects of cherries on Freund's adjuvant-induced arthritis in rats," *Scandinavian Journal of Rheumatology*, vol. 35, no. 5, pp. 356–358, 2006.
- [41] M. Skurlova, A. Stofkova, A. Kiss et al., "Transient anorexia, hyper-nociception and cognitive impairment in early adjuvant arthritis in rats," *Endocrine Regulations*, vol. 44, no. 4, pp. 165–173, 2010.
- [42] M. Skurlova, A. Stofkova, and J. Jurcovicova, "Anxiety-like behavior in the elevated-plus maze tests and enhanced IL-1 β , IL-6, NADPH oxidase-1, and iNOS mRNAs in the hippocampus during early stage of adjuvant arthritis in rats," *Neuroscience Letters*, vol. 487, no. 2, pp. 250–254, 2011.
- [43] S. Appenzeller, M. B. Bértolo, and L. T. L. Costallat, "Cognitive impairment in rheumatoid arthritis," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 26, no. 5, pp. 339–343, 2004.
- [44] G. Kogan, A. Stasko, K. Bauerova, M. Polovka, L. Soltes, and V. Brezova, "Antioxidant properties of yeast (1 \rightarrow 3)- β -D-glucan studied by electron paramagnetic resonance spectroscopy and its activity in the adjuvant arthritis," *Carbohydrate Polymers*, vol. 61, pp. 18–28, 2005.
- [45] E. B. Kalaz, B. Evran, S. Develi-İş, P. Vural, S. Dogru-Abbasoglu, and M. Uysal, "Effect of carnosine on prooxidant-antioxidant balance in several tissues of rats exposed to chronic cold plus immobilization stress," *Journal of Pharmacological Sciences*, vol. 120, no. 2, pp. 98–104, 2012.
- [46] V. Calabrese, C. Cornelius, S. Cuzzocrea, I. Iavicoli, E. Rizzarelli, and E. J. Calabrese, "Hormesis, cellular stress response and vitagenes as critical determinants in aging and longevity," *Molecular Aspects of Medicine*, vol. 32, no. 4–6, pp. 279–304, 2011.
- [47] E. J. Calabrese, "Hormesis: once marginalized, evidence now supports hormesis as the most fundamental dose response," in *Hormesis: A Revolution in Biology, Toxicology and Medicine*, M. P. Mattson and E. J. Calabrese, Eds., pp. 15–56, Humana Press, 2010.
- [48] F. M. Brennan, M. Field, C. Q. Chu, M. Feldmann, and R. N. Maini, "Cytokine expression in rheumatoid arthritis," *British Journal of Rheumatology*, vol. 30, pp. 76–80, 1991.
- [49] C. Keller, A. Webb, and J. Davis, "Cytokines in the seronegative spondyloarthropathies and their modification by TNF blockade: a brief report and literature review," *Annals of the Rheumatic Diseases*, vol. 62, no. 12, pp. 1128–1132, 2003.

Review Article

The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults

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The reactive oxygen species (ROS) form under normal physiological conditions and may have both beneficial and harmful role. We search the literature and current knowledge in the aspect of ROS participation in the pathogenesis of anterior and posterior eye segment diseases in adults. ROS take part in the pathogenesis of keratoconus, Fuchs endothelial corneal dystrophy, and granular corneal dystrophy type 2, stimulating apoptosis of corneal cells. ROS play a role in the pathogenesis of glaucoma stimulating apoptotic and inflammatory pathways on the level of the trabecular meshwork and promoting retinal ganglion cells apoptosis and glial dysfunction in the posterior eye segment. ROS play a role in the pathogenesis of Leber's hereditary optic neuropathy and traumatic optic neuropathy. ROS induce apoptosis of human lens epithelial cells. ROS promote apoptosis of vascular and neuronal cells and stimulate inflammation and pathological angiogenesis in the course of diabetic retinopathy. ROS are associated with the pathophysiological parainflammation and autophagy process in the course of the age-related macular degeneration.

1. Introduction

The reactive oxygen species (ROS) form as products under normal physiological conditions due to the partial reduction of molecular oxygen. ROS, that is, superoxide anion (O_2^-), hydroxyl radical (OH^\bullet), hydrogen peroxide (H_2O_2), and singlet oxygen (1O_2), arise in many ways, as a product of the respiratory chain in mitochondria, in photochemical and enzymatic reactions, as a result of the exposure to UV light, ionizing radiation, or heavy metal ions. Superoxide is generated directly from the reduction of oxygen and then dismutated to hydrogen peroxide. Hydrogen peroxide is a molecule with low reactivity, but it can readily penetrate cell's membranes and generate the most reactive form of oxygen, the hydroxyl radical, via Fenton's reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^\bullet$).

Low levels of ROS production are required to maintain physiological functions, including proliferation, host defense, signal transduction, and gene expression [1]. ROS are produced mainly by mitochondrion. The mitochondrial electron transport chain generates superoxide radicals through the single-electron leak at respiratory complexes I and III of the oxidative phosphorylation (OXPHOS) pathway [1, 2]; however, flavin-dependent enzymes in the mitochondrial matrix may produce the reactive oxygen species at much higher rates than complex I [3]. Under physiological conditions there is a cellular balance between ROS generation and clearance, since eukaryotic cells have several antioxidative defense mechanisms, including enzymes and antioxidants. There are five major types of primary intracellular antioxidant enzymes, that is, Cu/Zn-superoxide dismutase (Cu/Zn-SOD, SOD1) in the cytosol, manganese superoxide dismutase (Mn-SOD,

SOD2) in the mitochondrial matrix, catalase, glutathione peroxidase (GPx), and glutathione reductase (GR). The SODs dismutate superoxide to oxygen and hydrogen peroxide, while catalase and GPx convert hydrogen peroxide into H₂O and O₂. Apart from the antioxidant enzymes, small molecular weight and nonenzymatic antioxidants are also involved in the protection of the intracellular components against the reactive oxygen species. However, when ROS cellular overproduction overwhelms intrinsic antioxidant capacity, then the oxidative stress occurs and next the damage to the biomolecules of normal cells and tissues may occur [1].

The oxidative stress usually results from either excessive ROS production, mitochondrial dysfunction, impaired antioxidant system, or a combination of these factors. The prooxidative/antioxidative cellular imbalance between the ROS production and ability of the biological systems' defense mechanisms to eliminate the cellular stress disturbances leads to the vicious circle, since the oxidative stress reciprocally aggravates ROS production. ROS can be generated at elevated rates under normal aging, as well as in acute or chronic pathophysiological conditions [4–6]. The excessiveness of ROS causes oxidative damage to the deoxyribonucleic acid (DNA), proteins, and lipids. ROS can react with the nucleic acids attacking the nitrogenous bases and the sugar phosphate backbone and can evoke single- and double-stranded DNA breaks. Human mitochondrial DNA (mtDNA) is a covalently closed, double-stranded molecule, encoding 13 proteins of the oxidative phosphorylation chain, 22 tRNAs, and 2 rRNAs. mtDNA is more susceptible to the oxidative damage than its nuclear counterpart, since it is located in close vicinity to the inner mitochondrial membrane; a major site of ROS production is not protected by histones or other associated proteins, has intronless regions and a high transcription rate, and has a higher susceptibility to the oxidative modifications in its coding region. DNA damage induced by the oxidative stress may affect the protein-coding region of mtDNA and influence oxidative phosphorylation. mtDNA mutations can cause disturbances in the respiratory chain as well as the loss of control of ROS production. The much less effective repair system for mtDNA damage may be the cause for accumulating the oxidative stress together with its consequences. ROS also attack structural and enzymatic proteins by the oxidation of residual amino acids, prosthetic groups, formation of cross links, protein aggregates, and proteolysis. The inactivation of the key proteins can lead to the serious consequences in the vital metabolic pathways. Lipid peroxidation (autooxidation) is a process of oxidation of polyunsaturated fatty acids due to the presence of several double bonds in their structure and it involves production of peroxides (chemical compounds in which two oxygen atoms are linked together by a single covalent bond), ROS, and other reactive organic free radicals. There are several markers of oxidative damage, including the following: 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative damage to DNA; protein carbonyl groups, a marker of protein oxidation; malondialdehyde (MDA), a marker of lipid peroxidation; and 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation [4–6].

The cell's inability to repair the incurred damage may cause genetically programmed cell's death (apoptosis) or mutations in the DNA, which leads to carcinogenesis or development of many neurodegenerative diseases. Increased ROS levels and oxidative damage of the cellular components play an important role in the senescence process. The amount of accumulated damage increases with the age due to impairs in the DNA repair system and the intensified oxidative stress as well as with the decreased antioxidant defense. Mutations in the key DNA repair genes result in an impaired recognition system and an inefficient repair of DNA damage, which accelerates the aging of the organism, leading to the age-related disruptions in cellular and tissue functions. The aging of the organism is an inevitable process since the formation of ROS is a result of normal daily cellular metabolism. Therefore, cells have complex defense mechanisms to combat both the formation of ROS and the impacts of their actions. The oxidative stress leads to apoptosis when antioxidant capacity is insufficient. The oxidative stress can induce apoptosis by damaging mtDNA, inhibiting the mitochondrial respiratory chain transition, and increasing mitochondrial membrane permeability [4, 7]. Cell's death induced by the excessive ROS production and the oxidative stress is involved in pathomechanism of many general neurodegenerative pathologies such as Alzheimer's disease [8], Parkinson's disease [9], prion disease [10], protein misfolding diseases [11], and ophthalmological diseases [12].

2. The Role of ROS and the Oxidative Stress in the Cornea Diseases

The cornea, an avascular tissue which maintains transparency at the frontal surface of the eye, contains three major layers, that is, the outer epithelium, a thick stroma with corneal fibroblasts, and the inner endothelium.

In the cornea, the source of oxidative stimuli is solar ultra violet (UV) radiation [13]; the human cornea absorbs 92% of UV-B, that is, 280–315 nm sunlight radiation reaching the eye [14] and atmospheric oxygen, mainly dioxygen [13]. Due to its localization and function, the cornea is chronically exposed to ROS accumulation as well as to the oxidative stress [13]; however, normal corneas have well-developed antioxidant defense systems which contain direct free radical scavengers, including nonenzymatic, low molecular weight antioxidants covering vitamin C, vitamin E, β-carotene [15, 16], reduced glutathione (GSH) [17], ferritin [18], α-tocopherol [19], and several indirect, enzymatic high molecular weight antioxidants, that is, catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase [20]. With the age, the malfunction of the corneal antioxidant defense mechanisms leads to ROS accumulation and the oxidative stress. The prooxidant/antioxidant imbalance is due to the inactivation of the antioxidant enzymes, mainly catalase, glutathione peroxidase, and lactate dehydrogenase, and leads to the functional and structural changes in the corneal tissue [21]. In response to the oxidative stress and with the age, through the apoptotic process the number of corneal fibroblasts and corneal endothelial cells declines in normal

human corneas [22]. However, in specific conditions the increased ROS production and accumulation, the oxidative stress, and the prooxidant/antioxidant imbalance lead to the corneal pathologies.

The increased ROS levels and the oxidative stress both play a crucial role in the development of Fuchs endothelial corneal dystrophy, keratoconus, and granular corneal dystrophy type 2.

2.1. Fuchs Endothelial Corneal Dystrophy (FECD). FECD is an oxidative stress disorder which leads to the age-related gradual loss of corneal endothelial cells (CECs), resulting in corneal edema and loss of vision. FECD affects approximately 4% of the population in the fourth or fifth decade of life and is characterized by an accelerated decrease of postmitotic endothelial cells density caused by apoptosis, as well as formation of posterior excrescences of Descemet's membrane, termed guttae, which arise as abnormal accumulation of subendothelial deposition of profibrotic extracellular matrix [23, 24]. Nondividing nature, positioned within the light path and high metabolic activity, predisposes CECs, mainly from the corneal center to the oxidative stress. Oxidative stress is the major contributor to the slow developing of CECs degeneration, loss of their hexagonal shape, and density in FECD patients [25]. FECD corneas exhibit increased accumulation of ROS (and RNS) in comparison with the normal tissues [21]. Central areas of FECD corneal buttons showed increased level of 8-hydroxy-2'-deoxyguanosine (8-OHdG), decreased numbers of mitochondria, and reduced activity of cytochrome oxidase (the major respiratory chain enzyme) in Jurkunas et al.'s study. According to the authors, increased level of oxidative mtDNA damage exhibited next to corneal guttae confirms association between macromolecular damage triggered by the oxidative stress and the endothelial cells apoptosis. Endothelial oxidative DNA damage is caused by imbalance of oxidant/antioxidant factors in CECs, based on an abnormal response of the transcription factor Nrf2 and its antioxidant targets, including superoxide dismutases [26]. Nrf2, nuclear factor-erythroid 2-related factor-2, is a key nuclear transcription factor coordinating upregulation of antioxidant defense in response to cellular stress. Nrf2 shows a high affinity to the antioxidant response element (ARE), which is involved in transcriptional activation of genes encoding proteins important for the protection against the oxidative stress, including peroxiredoxin, glutathione S-transferases, heme oxygenase-1 (HO-1), thioredoxin reductase 1 (TXNRD1), and ferritin [27]. Aberrant Nrf2 response influences the expression of multiple antioxidants in FECD corneal endothelium, causing accumulation of free radicals and other reactive species [23, 28]. Peroxiredoxins, which are involved in the removal of hydrogen peroxide from the cells and in the inhibition of ROS-induced apoptosis, show decreased expression in corneal endothelial cells and Descemet's membrane of FECD corneas [28]. Matthaai et al. showed decreased transcriptional levels of superoxide dismutase 1 and superoxide dismutase 2 in FECD endothelial samples and overexpression of NOX4 enzyme (NADPH oxidase 4) [29]. NADPH oxidase, (NOX) family, is a reduced form of nicotinamide adenine dinucleotide

phosphate and one of the most important sources for ROS generation [30]. Decreased levels of SOD1 and SOD2, decreased Nrf2-expression, and augmented NOX4 activity significantly exacerbate antioxidant/oxidant imbalance and contribute to consecutive induction senescence of CECs in FECD patients [29]. Lack of Nrf2 leads to perturbation of tissue homeostasis and activates p53-dependent apoptotic pathway in FECD endothelial cells [31]. On the other hand, experimental applying of sulfolane, which is Nrf2 agonist, caused cytoprotective effect by significant upregulation of major ARE-dependent antioxidants, decreased intracellularly ROS production, and ameliorated oxidative stress-induced CECs apoptosis in FECD corneas [32].

2.2. Keratoconus (KC). KC is a degenerative disorder characterized by the corneal ectasia (stromal thinning leads to cone-shaped protrusion, often in the inferotemporal quadrant), associated with breaks in Bowman's layer and Fleischer's ring-iron deposits in the basal layer of the epithelium. Irregular astigmatism, myopia, and cornea scarring reduce the visual acuity. KC is mostly bilateral, with the onset usually in puberty and arresting around the fourth decade of life, affecting both genders and all ethnicities [33]. Visual impairment may be alleviated by spectacles or specialized contact lenses in most of the patients and in a part of them may be employed riboflavin-ultraviolet-A-induced collagen cross-linking therapy, which causes covalent bonding between the collagen fibrils and biomechanically strengthens the diseased cornea [34]; however, 10–20% of affected patients may necessitate the corneal transplantation [35]. Oxidizing UV radiation and blue light, genetic predispositions, and the environmental mechanical influences such as contact lens wear, atopy, and eye rubbing play a role in KC pathogenesis [36–38].

In comparison with the normal cornea samples, human KC corneas exhibit an increased stress-induced ROS generation, including superoxide [39], as well as the accumulation of nitrotyrosine, a marker for the formation of peroxynitrite, and increased production of nitric oxide (NO) radicals; elevated amounts of endothelial nitric oxide synthesis (eNOS) were detected at the site of Bowman's layer breaks [21]. Increased ROS and RNS formation lead to oxidative stress [37] and cause mtDNA impairment in keratoconus corneas.

Apparent and important role of mtDNA damage in the development of KC pathogenesis confirm many studies. In comparison with the age-matched normal corneas, keratoconus corneas present an increased level of mtDNA damage, which affects the protein-coding mtDNA region and disturbs the mitochondrial process of oxidative phosphorylation. Aberrations in the expression of oxidative phosphorylation proteins lead to improper ATP synthesis, increased ROS/RNS formation, and then in turn the further oxidative damage and increased ROS/RNS formation. KC corneas exhibit decreased activity of complex IV subunit 1 (CO I) in areas of corneal thinning [40]. KC stromal fibroblasts also show in vitro increased mitochondrial cytochrome oxidase subunit 2 (CO II) RNA levels in comparison with the normal cultures [41]. Han Chinese population exhibited a significant ($p = 0.0002$) decrease of leukocyte mtDNA copy number in KC patients compared to control subjects, which remained even

when age, gender, and mtDNA genetic background were considered. Leukocytes are normal tissues in keratoconic corneas and leukocyte mtDNA copy number represents the general mtDNA copy numbers of the individual. There was no correlation between mtDNA haplogroup and the risk of keratoconus in this group [42], contrary to Saudi Arabian population, in which increased risk to develop KC in individuals correlated with the mitochondrial haplogroups H and R [43]. According to the authors, decreased leukocyte mtDNA copy number in keratoconus patients represents a genetic susceptibility to KC. Moreover, it is a predisposing factor for disease development by influencing oxidative stress, since decreased mtDNA copy number sustains imbalance between damaged and normal mtDNA, which favours further increasing ROS formation and additional oxidative stress in KC cornea [42].

KC corneas exhibit disturbance in the level of transcripts and/or activities of different antioxidant enzymes [44]. The activity of extracellular superoxide dismutase [45] and content of glutathione [37] are decreased in KC corneas in comparison to the normal samples.

Increased ROS formation, oxidative stress, and mtDNA damage and decreased antioxidant defenses in KC corneas cause keratocyte apoptosis and unfavourable changes in extracellular matrix (ECM), which finally lead to thinning and deformation of keratoconus corneas. Oxidative stress accelerates keratocyte apoptosis [41]. Integrity of mtDNA plays an important role in viability of cells and the mitochondrial dysfunction contributes to KC deformation [40].

Oxidative stress changes expression of two structural components of ECM, that is, collagen type XVIII/endostatin and collagen type XV. Remodeling of the extracellular matrix makes stroma more susceptible to degradation and results in its thinning [46]. Degradation of collagen components of the extracellular matrix in keratoconus corneas favours changes in the expression of ECM enzymatic regulators, that is, gelatinase A and matrix metalloproteinase 2 (MMP-2), which digest the main structural elements of the ECM, namely, collagen IV, collagen V, fibronectin, and laminin [47, 48], and MMP-2 inhibitor, that is, tissue inhibitor metalloproteinase 1 (TIMP-1). KC corneas exhibit increased activity of gelatinase A and decreased mRNA expression and protein levels of TIMP-1 [49]. ROS/nitric oxide pathway degrade TIMP-1 and increase MMP-2 activity [50]. TIMP-1 plays a role in the inhibition of apoptosis in a variety of cell types; therefore lower amount of this protein is associated with fragmentation of the epithelium and stroma thinning of KC corneas [51].

In some patients, their genetic predisposition to keratoconus, that is, polymorphisms in COL4A3 and COL4A4 genes, encoding components of type IV collagen, a major corneal structural protein [52], and/or mutation in the superoxide dismutase 1 gene [53] may accelerate the corneal changes. However, Stabic-Silih et al. did not confirm correlations between mutation of SOD1 gene and keratoconus [54].

2.3. Granular Corneal Dystrophy Type 2 (GCD2). GCD2 is an autosomal dominant disorder caused by point mutations (R124H) in transforming growth factor- β -induced gene-h3 (BIGH3) and is characterized by age-dependent progressive

accumulation of transforming growth factor- β -induced protein (TGFBIp) deposits in the corneal epithelia and stroma, which interferes with corneal transparency [55].

Choi et al. confirmed that the oxidative stress is also involved in the pathogenesis of GCD2, since GCD2 primary cultured corneal fibroblasts demonstrate increased intracellular ROS and H₂O₂ generation and they are highly susceptible to the oxidative stress-induced cell's death in comparison with the normal primary cultured corneal fibroblasts [56]. Choi et al. showed in the other study that melatonin, which is involved in the control of various physiological functions and also has antioxidant and antiapoptotic properties, protected GCD2 corneal fibroblasts against the paraquat (PQ-) induced oxidative stress, since it reduced intracellular levels of H₂O₂ and increased expression of Cu/Zn-superoxide dismutase and glutathione reductase in fibroblasts GCD2 corneas [57].

Increased ROS production and increased level of the oxidative stress play role in the etiology of the superficial punctate keratopathy [58] and impair the corneal wound healing [59, 60].

3. The Role of ROS and the Oxidative Stress in Glaucoma Pathogenesis

Glaucoma, an age-dependent disease being more common in the elderly population, is one of the leading causes of irreversible blindness [61]. Glaucoma is an optic neuropathy characterized by the progressive degeneration of retinal ganglion cells (RGCs), which die through an apoptotic process [62]. Increased intraocular pressure (IOP) is a consequence of abnormal high resistance to aqueous humor drainage via the trabecular meshwork, causing anterograde/retrograde axoplasmic flow impairment (the mechanical theory of glaucoma), and it is the leading risk factor for RGCs apoptosis in glaucoma [63]. However, several concomitant factors such as increased ROS production and oxidative retina damage and imbalance between prooxidative and antioxidant capacity have been postulated as the crucial factors in early retinal injury [64], together with the reduced perfusion pressure in the blood vessels (the vascular theory of glaucoma), which also significantly contribute to the glaucomatous neurodegeneration [65]. In the glaucoma pathogenesis are involved the trabecular meshwork in the anterior chamber of the eye; RGCs and their axons in the posterior eye segment; and the lateral geniculate nuclei and the visual cortex in the central nervous system [66–68].

3.1. The Influence of ROS and the Oxidative Stress on the Human Trabecular Meshwork. The human trabecular meshwork (TM) is the most sensitive tissue of the anterior chamber to the oxidative damage, since it is hidden in the sclerocorneal angle and not directly exposed to light and in consequence has fewer, than cornea and iris, antioxidant defence [69]. ROS induced by light change the oxidant/antioxidant balance in the aqueous humor. The oxidative stress stimulates enzymatic antioxidant defence systems and decreases the total antioxidant potential in aqueous humor; therefore, the level and activity of protecting superoxide dismutase and glutathione

peroxidase decrease and such oxidant/antioxidant imbalance causes TM cells impairment [70]. Human TM cells are in contact with the relatively high concentrations of hydrogen peroxide and such exposure to H_2O_2 has no effect on outflow in normal eyes; however, it causes a 33% decrease in outflow in reduced glutathione-depleted eyes [71]. Patients with primary open-angle glaucoma (POAG) expose higher susceptibility to oxidative damage, since their total reactive antioxidant potential is reduced by 60–70%, although the activity of antioxidative enzymes is increased by the same amount [70], and they show the reduced levels of glutathione in plasma [72]. POAG patients display a genetic background rendering them susceptible to ROS-induced damage, since there is a more frequent deletion of the gene encoding for GSH S-transferase compared with the control individuals [66].

Increased hydrogen peroxide levels and the oxidative stress damage mainly structural and functional components of mtDNA in TM endothelial cells; however, damage of proteins and membrane lipids also occurs [73].

Increased level of 8-OH-dG derived from guanosine oxidation is an established biomarker of oxidative DNA damage. In Sorkhabi et al.'s study, both aqueous and serum 8-OH-dG levels were significantly higher in glaucoma patients than in the control group (4.61 ± 2.97 ng/mL versus 1.98 ± 0.70 ng/mL, $p = 0.002$, and 17.80 ± 8.06 ng/mL versus 13.63 ± 3.54 ng/mL, $p = 0.046$, resp.), and total antioxidant status determined in serum and in aqueous humor was significantly lower in glaucoma patients than in control group (0.55 ± 0.13 mmol/lit. versus 0.70 ± 0.14 , $p = 0.001$, and 0.23 ± 0.13 mmol/lit. versus 0.34 ± 0.15 , $p = 0.001$, resp.) [74]. Trabecular meshwork samples of patients with POAG, obtained during the filtration surgery, also exhibited increased level of 8-OH-dG [75]. More recently, the altered stability of mRNAs in human TM cells exposed to oxidative stress has been reported as well [76]. Mitochondrial dysfunction and oxidative mtDNA impairment of the human TM endothelial cells occur in POAG and pseudoexfoliative glaucoma, however not in other types of glaucoma [77], and are proportional to the clinical symptoms of the POAG, that is, intraocular pressure elevation and visual fields damage [75], and contribute to POAG progression [73].

Elevated ROS concentration influences TM activating nuclear factor- κ B (NF- κ B) pathway and causes oxidative/peroxynitrite stress [78].

NF- κ B is a family of transcription factors which play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival. Increased ROS generation results in sustained NF- κ B activation, which in turn induces the expression of proinflammatory markers, including endothelial leukocyte adhesion molecule-1 (ELAM-1), interleukin-(IL-) 1α , IL-6, and IL-8 [79]. Short-term ROS/NF- κ B pathway activation contributes to the decrease of IOP [80]; however, its chronic stimulation exerts pathological effects on the TM and leads to glaucoma progression [81]. Enhanced level of superoxide anions induces overproduction of nitric oxide (NO), which in turn reacts with H_2O_2 and produces toxic metabolites, reactive peroxynitrite (ONOO⁻). Physiological levels of NO play an important role in controlling ocular vascular tone and the blood flow. NO is synthesized from

l-arginine by a family of nitric oxide synthase (NOS) isozymes which includes neuronal (n)NOS, endothelial (e)NOS, and inducible (i)NOS. nNOS and eNOS are constitutive Ca^{2+} (calcium)/calmodulin-dependent enzymes and are tightly controlled by mechanisms regulating physiological intracellular Ca^{2+} levels, whereas iNOS is Ca^{2+} -independent [82]. The human TM endothelium cells, involved in modulating the permeability of the endothelial barrier, express mainly eNOS isoform (and significantly lower amount of nNOS), which physiologically regulates aqueous outflow by maintaining endothelial cell function. The oxidative/peroxynitrite stress leads to eNOS deficit, which alters TM mobility and causes its contractile dysfunction [83]. Moreover, ROS/ONOO⁻ stress induces and sustains inflammation and proliferation of TM endothelial cells [64], as well as breakage of mtDNA and impairment of mitochondrial respiration, and the energy failure leads finally to the endothelial cells damage [64, 84].

Increased hydrogen peroxide levels and the oxidative stress cause remodeling of TM cytoarchitecture and this leads to TM enlargement or collapse. The oxidative stress stimulates mobility (migration) of human TM cells in vitro, which causes trabecular thickening and fusion and contributes to trabecular enlargement [85]. Human TM cellularity declines linearly in relation to age; however, glaucomatous subjects have lower TM cellularity than nonglaucomatous subjects at the same age [86]. Human TM cells exposed to 1 mmol of H_2O_2 stress show reduced adhesiveness to the extracellular matrix structural components (to collagen types I and IV, laminin, and fibronectin) and such rearrangement of cytoskeletal structures may also lead to endothelial cells loss and cause TM disruption and collapse [87]. The exact mechanism of TM cells loss and the environmental factors contributing to it are not known yet; however, exogenous influence of higher H_2O_2 levels, combined with the insufficient glutathione level, may induce collagen matrix remodeling and cause trabecular cell apoptosis, independently of mitochondria [84]. TM plays a major role in the regulation of aqueous outflow [88]; therefore, its oxidative-related enlargement or collapse leads to the increased outflow resistance and elevation of IOP (Figure 1) [84].

3.2. The Influence of ROS and the Oxidative Stress on the Development of Glaucomatous Neurodegeneration. Increased IOP and/or hypoxia stimulate ROS production in glaucoma patients. Amplified ROS generation causes chronic stress state of the retina and of the optic nerve head tissue. ROS and the oxidative stress constitute an important noxious stimulus, which leads to progressive retinal ganglion cells (RGCs) loss in apoptotic and autophagic process, causes retinal and optic nerve glial dysfunction, and dysregulates ocular hemodynamics. In patients with glaucomatous neurodegeneration, progressive loss of RGCs and optic nerve axons leads finally to characteristic optic nerve atrophy and visual field defects.

3.2.1. The Role of ROS and the Oxidative Stress in the Pathomechanism of RGCs Death. Oxidative mitochondrial dysfunction caused by amplified ROS generation (as well as nitric oxide-induced damage) is a part of the pathway for

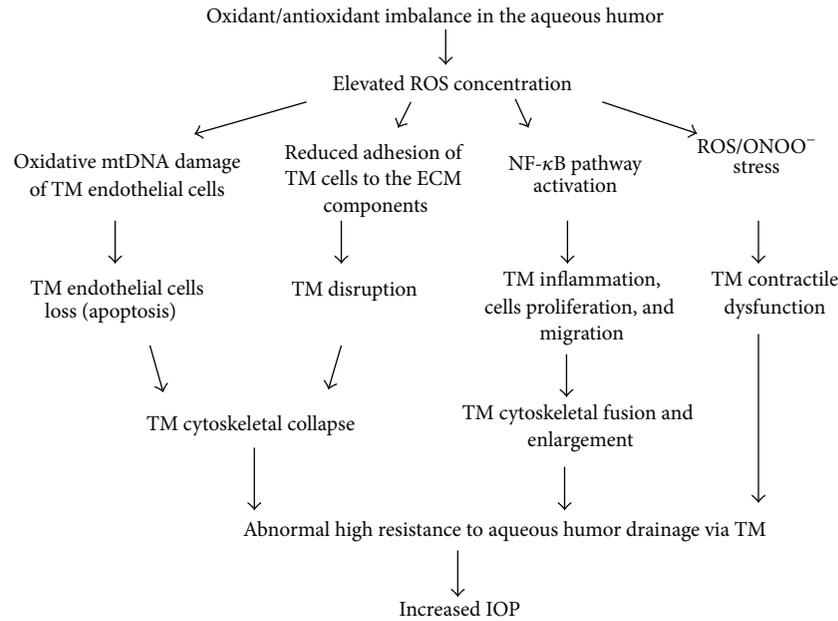


FIGURE 1: Schematic overview of the harmful influence of ROS and the oxidative stress on the trabecular meshwork structure and its function in glaucoma. ROS, reactive oxygen species; mtDNA, mitochondrial deoxyribonucleic acid; TM, trabecular meshwork; ECM, extracellular matrix; NF- κ B, nuclear factor- κ B; ROS/ONOO⁻, reactive oxygen species/peroxynitrite; IOP, intraocular pressure.

RGCs death and plays role in the development of glaucomatous neurodegeneration. Glaucomatous neurodegeneration means the impairment of RGCs (soma), as well as dendrites in the retina, axons in the optic nerve, and synapses in the brain. ROS may act as the direct cytotoxic stimulus causing RGC loss through apoptosis and as intracellular signaling molecules (second messenger parallel to neurotrophin deprivation) for RGCs death after axonal injury, responsible for transforming the information from the damaged axon into RGCs soma. ROS may be directly cytotoxic to RGCs in a caspase-dependent manner or may function in caspase-independent pathway. Caspase-independent apoptotic pathways can be activated by apoptosis-inducing factor. Extracellular ROS released from stressed cells into the extracellular milieu may facilitate RGCs degeneration additionally to the neurodegenerative injury induced by the intracellular ROS attack. Moreover, ROS released by the neighboring cells may also be cytotoxic to the primarily undamaged RGCs [12, 89].

In glaucoma patients, RGCs may also die in the course of dysregulated basal autophagic process [90] and ROS and the oxidative stress induce autophagy [91]. Autophagy refers to lysosomal degradation of cell's own constituents. This is highly conserved adaptive metabolic process, which permits the degradation and recycling of cellular constituents (including long-lived proteins and organelles), and is crucial for maintaining cellular homeostasis and cell survival under stressful conditions. Autophagy is triggered in response to various stressful conditions such as oxidative stress, hypoxia, ischemia/reperfusion injury, growth factor withdrawal, and nutrient deficiency. The autophagosome, a double membrane structure containing engulfed cytoplasm and its organelle content, fuses with lysosome(s) to create an autophagolysosome within which

the endocytosed contents can be degraded by lysosomal enzymes. There are predominantly 3 autophagic pathways, that is, macroautophagy (autophagy), microautophagy, and chaperone-mediated autophagy (chaperones are a functionally related group of proteins assisting protein folding in the cell under physiological and stress conditions). Autophagy is primarily a nonselective degradation pathway but also different kinds of selective autophagy exist, that is, mitophagy, reticulophagy, pexophagy, xenophagy, and nucleophagy, which, respectively, refer to the selective removal of mitochondria, endoplasmic reticulum, peroxisomes, intruding microorganisms, and nuclei. Intralysosomal waste material accumulated with age and damaged cellular components, which are no longer functional, are also degraded by this self-eating process. If the autophagic degradative pathway is faulty, an accumulation of damaged proteins as aggregated deposits takes place which may cause the anatomical obstacles to physiological processes. Diminished autophagic activity plays a major role in aging and age-related diseases [92]. Mizushima subclassified autophagy into "baseline" and "induced." Neuronal autophagy is protective at basal levels and prevents the aggregation of damaged organelles and the accumulation of proteins in neurons, thus promoting axonal homeostasis and efficient clearance of cellular soma. However, with the progression of autophagy, neurons undergo stimulation, which deregulates the basal autophagic mechanism and converts it to induced autophagy [93].

Superoxide and hydrogen peroxide play a dual role signaling autophagy process. Through influence on BECN1 (Beclin 1, autophagy-related) ROS activate formation of class III phosphatidylinositol 3-kinase (PtdIns3K) complexes and positively regulate autophagy; PtdIns3K is an intracellular energy sensor, which specifically responds to the energy

depletion [94]. BECN1 contributes to the early formation of autophagic vesicles [95] and is predominantly located in RGCs layer of the retina [96]. However, ROS also can activate BCL2 (B-cell CLL/lymphoma 2) family proteins, which after binding to BECN1 disorganize the formation of PtdIns3K and disrupt the induction of autophagy [94].

Both chronic hypertensive glaucoma and/or conditions of retinal transient ischemia, induced by acutely increasing IOP, stimulate ROS production and dysregulate basal autophagy. Autophagy is originally activated in the dendrites of RGCs to promote cellular protection and maintain intracellular homeostasis. Axons contain a large number of mitochondria, which make them more sensitive to chronic ischemia. Neuroprotective effect of autophagy in axons lessens after 4 weeks of progressive increase of IOP. Thereafter, autophagy is predominantly activated in the neuronal soma, which disrupts homeostasis, decreases cell viability, and triggers autophagic RGC death [90, 96]. If the IOP increases acutely, autophagy is enhanced immediately and occurs mostly in cell bodies, which induces neuronal cell death in a relatively short time [90].

3.2.2. The Influence of ROS and the Oxidative Stress on Glial Cells of the Retina and Optic Nerve Head. Under oxidative conditions glial cell lessens its neuroprotective ability and may even become neurodestructive. ROS and the oxidative stress cause supportive glial dysfunction, which lead to the secondary RGCs apoptosis. ROS stimulate pathological glial activity (increase secretion of TNF- α and nitric oxide), cause oxidation of its protein (glutamine synthetase), and damage glial cells through activation of AGE/RAGE signaling pathway. ROS induce glial immune system as well, which additionally facilitate the progression of glaucomatous neurodegeneration [12].

ROS and aging promote accumulation of advanced glycation end products (AGEs), a hallmark of many chronic neurodegenerative diseases. AGEs stimulate ROS production and assist development of glaucomatous neurodegeneration [12]. AGEs are proteins, lipids, or nucleic acids formed by nonenzymatic glycation or glycooxidated, after the exposure to the aldose sugars. The oxidative stress increases with age and, furthermore, the ability to respond to the oxidative stress declines with age, mostly due to the imbalance between increasing oxidant production and decreasing antioxidant capacity and AGEs accumulate in various tissues in the course of physiological aging. Due to their synergism with the oxidative stress, AGEs production is promoted by the oxidative stress; while AGEs lead to ROS generation, AGEs are commonly implicated as factor which exacerbates progression of many neurodegenerative diseases. These detergent insoluble and protease-resistant, nondegradable AGEs aggregates may impair normal cellular/tissue functions directly, or indirectly, by AGE/RAGE pathway after binding to the specific receptors for advanced glycation end products (RAGEs). AGE/RAGE pathway results in the cell activation, proliferation, apoptotic cell death, chemotaxis, angiogenesis, and ROS generation. As a consequence of AGEs accumulation, many proteins lose their function, including proteins involved in the regulation of gene transcription [97].

In glaucoma, AGEs are accumulated predominantly extracellularly in lamellar cribriform plates and blood vessels of the optic nerve head, mainly on many long-lived macromolecules like collagen [98]. Extracellular aggregates increase tissue rigidity and mechanical strength and increase impairment of microcirculation, which additionally facilitate injury of axons damaged by increased IOP [12]. AGEs are also accumulated intracellularly in RGCs, glial cells, and axons as well [98], which cause intracellular protein traffic and impair axonal transport [12]. Receptors for AGEs are upregulated predominantly on Müller cells of retinal glial cells and on RGCs [98]. It makes glial cells and RGCs particularly susceptible to AGE/RAGE signaling pathway and such receptor-mediated signaling may amplify direct cytotoxic effect caused by extracellular/intracellular AGEs accumulation [12].

The retina and optic nerve glial cells vigorously respond to ROS stimulation. Activated autoimmune response may facilitate primary and/or secondary RGCs degeneration through stimulation of an aberrant immune response [12]. In Tezel et al.'s study, glial cells exposed to ROS upregulated major histocompatibility complex (MHC) class II molecules, important in autoimmune response. T cells recognize antigens in the form of small peptides tightly bound to MHC class II molecules displayed on the surfaces of antigen-presenting cells and MHC complex interacts with antigen-specific receptors on T cells to induce an antigen-specific reaction. Compared with the control, glial cells in ROS-generating systems were more potent inducers of T cell activation in a cell density- and time-dependent manner, assessed by increased T cell proliferation (approximately threefold) and TNF- α secretion (approximately sixfold), $p < 0.01$ [99].

McElnea et al. confirmed the influence of ROS overproduction, oxidative stress, and mitochondrial dysfunction (as well as impaired calcium extrusions) on glial cells of the optic nerve head in glaucomatous patients. The compare levels of the oxidative stress, mitochondrial function (as well as calcium homeostasis) in glial fibrillary acid-negative protein lamina cribrosa cells obtained from the optic nerve head region of glaucomatous lamina cribrosa (GLC), and normal lamina cribrosa (NLC) human donor eyes showed that intracellular ROS production was increased in GLC compared to NLC ($27.19 \pm 7.05 \mu\text{M}$ MDA versus $14.59 \pm 0.82 \mu\text{M}$ MDA, $p < 0.05$); malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation used as an indicator of the oxidative stress. Moreover, mitochondrial membrane potential was lower in GLC ($57.5 \pm 6.8\%$) compared to NLC ($41.8 \pm 5.3\%$), expression of the antioxidants (aldo-keto reductase family 1 member C1 and glutamate cysteine ligase catalytic subunit) was significantly ($p = 0.02$) lower in GLC compared to NLC control, and intracellular calcium (Ca^{2+}) levels were significantly higher ($p < 0.001$) in GLC cells compared to NLC [100].

ROS and the oxidative stress have influence on the optic nerve tissue remodeling, since they trigger neuronal loss, disorganize lamellar cribriform plates secondary to extracellular AGEs accumulation, and stimulate matrix metalloproteinases (MMPs) for digestion of extracellular matrix (ECM) in glaucomatous eyes. Degradation of the optic nerve neurons

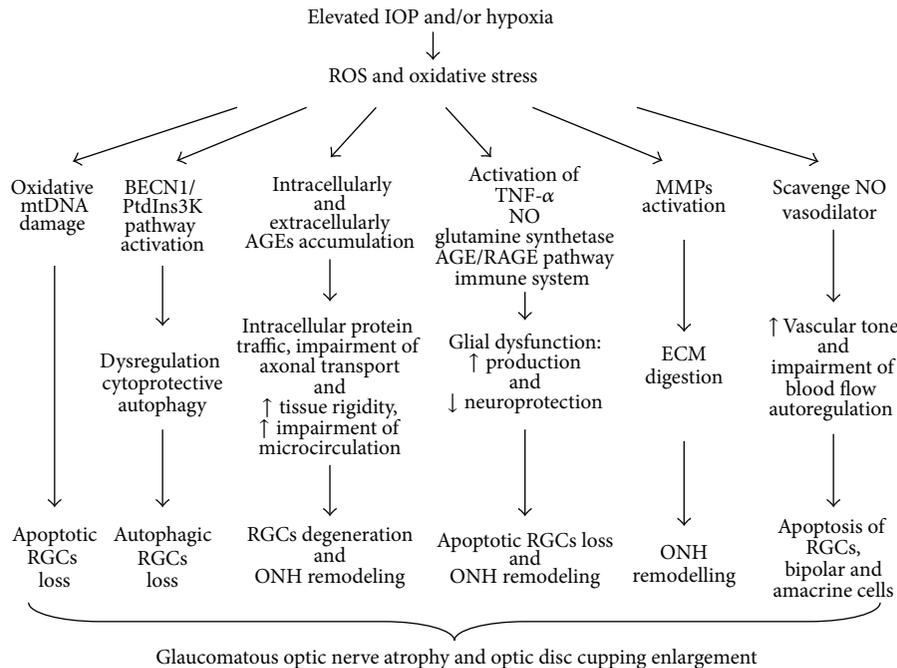


FIGURE 2: Schematic overview of the influence of ROS and the oxidative stress on the retina and the optic nerve head changes in the course of glaucomatous neurodegeneration. IOP, intraocular pressure; ROS, reactive oxygen species; mtDNA, mitochondrial deoxyribonucleic acid; RGCs, retinal ganglion cells; BECN1/PtdIns3K, Beclin 1/phosphatidylinositol 3-kinase; AGEs, advanced glycation end products; ONH, optic nerve head; TNF- α , tumor necrosis factor alpha; NO, nitric oxide; AGE/RAGE, advanced glycation end product/receptor for advanced glycation end product; MMPs, matrix metalloproteinases; ECM, extracellular matrix.

progresses and optic disc cupping enlarges despite glial activation and increased glial production of the extracellular matrix molecules [12].

3.2.3. The Influence of ROS and the Oxidative Stress on Ocular Hemodynamics. ROS and the oxidative stress lead to dysregulation of ocular hemodynamics, which also contribute to RGCs apoptosis. In the eye, the nature of disturbed hemodynamics is characterized as an altered regulation of perfusion in the terminal or preterminal arterial vasculature, however not as an obstructive vascular disease like arteriosclerosis [101]. ROS scavenge NO radicals and thus remove an important vasodilator resulting in vasoconstriction. NO is a potent signaling molecule in the blood vessels, where its continuous formation from endothelial cells acts on the underlying smooth muscle to maintain vasodilatation and blood flow and plays an important role in controlling ocular vascular tone and blood flow in the human eye [102]. ROS increase vascular tone and impair autoregulation of blood flow. In Zeitz et al.'s study, ROS induced in vitro a transient increase of vascular tone (transient contractions) of isolated rings of porcine posterior ciliary arteries. The shape of these contractions had parallels with vasospasms. Short-time exposure alters vascular tone which was totally reversible and the maximal force generation potential was unchanged; however, the arterial ring preparation lost its excitability after the prolonged ROS exposure [103]. Glaucoma progression is associated with the decreased blood flow velocities in the short posterior ciliary artery [104]. Excessive elevation of the intraocular pressure leads to retinal ischemia-reperfusion

(I/R) insult. This strong prooxidant condition stimulates ROS production and predisposes retina to the oxidative damage. In animal models, transient acute I/R injury and impair blood flow dynamics resulted in necrosis and apoptosis of cells in both the ganglion cell layer and inner nuclear layer [105]. The schematic overview of the role of ROS in the development of glaucomatous neurodegeneration is presented in Figure 2.

4. The Role of ROS and Oxidative Stress in Leber's Hereditary Optic Neuropathy and in the Traumatic Optic Neuropathy

4.1. Leber's Hereditary Optic Neuropathy (LHON). LHON is an acute or subacute bilateral central vision loss, due to optic nerve degeneration, and occurring predominantly in young males. LHON is the most frequent mitochondrial disease, due to mtDNA point mutations (positions 11778, 3460, and 14484) coding for proteins in mitochondrial electron transport chain complexes I and III. mtDNA mutations lead to loss of axons and their RGCs soma in apoptosis process [106, 107]. The primary cause of the disease is clearly known; however, the mechanism of relatively selective loss of the smaller RGCs and their axons remains enigmatic, and enhanced H_2O_2 production by the mutant mitochondrial complexes has been hypothesized as etiological factor [107], since (i) mitochondrial complexes I and III are the main sources of basal superoxide production and aberrant production of H_2O_2 from mutated METC components may cause RGCs death [106], (ii) RGCs use superoxide as an intracellular signal for initiating the apoptosis [108], probably by oxidizing critical

sulfhydryls in signaling macromolecules [109], and (iii) RGCs death is triggered when the mitochondrial superoxide levels are increased by knocking down mitochondrial superoxide dismutase 2 [110].

The effect of LHON mutations has been studied by producing transmitochondrial cybrids in human cell lines, which demonstrate upregulation of some mtDNA transcripts and exhibit increased superoxide production compared to wild-type cells. In Giordano et al.'s study, LHON cybrids presented overproduction of ROS, as well as decrease in mitochondrial membrane potential, increased apoptotic rate, loss of cell viability, and hyperfragmented mitochondrial morphology compared with control cybrids [111]. In another study, LHON cybrids carrying the np11778 mutation became selectively more H₂O₂ sensitive compared with the parental cell line. They contained a decreased cellular glutathione pool (49%, $p \leq 0.05$), despite 1.5-fold enhanced expression of the regulatory subunit of γ -glutamylcysteine synthetase ($p \leq 0.05$). The capacity to detoxify H₂O₂ was reduced although the activity of superoxide dismutase, glutathione peroxidase, and glutathione reductase was unchanged [107].

4.2. ROS Overproduction Contributes to the Traumatic Optic Neuropathy (TON) Pathogenesis. TON means partial or complete loss of optic nerve function due to the direct or indirect optic nerve injury, after head trauma sequelae such as edema, hemorrhage, and concussion [112]. RGCs apoptosis after optic nerve injury is caused by lack of neurotrophin support, increased extracellular glutamate levels, disruption of cellular homeostasis, and damage from free radicals. Apoptotic processes are also activated by microglial cells, which release inflammatory mediators (cytokines, prostaglandins, and complement molecules) and reactive oxygen species [113]. Ahmad et al. confirmed the increased oxidative stress in mice retina with TON [114]; however, treatment with ABT702 (pharmacological adenosine kinase inhibitor) attenuated neurotoxicity and significantly decreased levels of the oxidative stress markers, that is, superoxide anion, iNOS/nNOS, and nitrotyrosine, and attenuated inflammation (decreased expression of many inflammatory molecules mediated by adenosine) in retinal sections of mouse with TON [115]. ROS are also overproduced during secondary degeneration following neurotrauma, which means that the precise impairment of only dorsal axons of optic nerve causes secondary degeneration of intact ventral axons [116]. In experimental study, the level of ROS (and nitrogen species) increased at 1, 3, and 7 days in ventral optic nerve after dorsal injury. Immunoreactivity for glutathione peroxidase and heme oxygenase-1 increased in ventral optic nerve at 3 and 7 days after injury, respectively. Despite the increased antioxidant immunoreactivity, DNA oxidation was evident just since the 1st day, lipid oxidation after 3 days, and protein nitration after 7 days since the injury. Oxidative (and nitrosative) damage was particularly evident in CCl₄-positive oligodendrocytes [117].

5. The Role of ROS and the Oxidative Stress in Cataractogenesis

Cataract, the opacification of the crystalline lens, is one of the leading causes of blindness in the world and aging is

the greatest risk factor for noncongenital cataract formation. However, it is a multifactorial optic disorder and other factors like exposure to sunlight UV radiation, smoking, diabetes, malnutrition, myopia, and drug (steroid) use also contribute significantly to cataractogenesis [118].

The progressive loss of lens transparency associated with the increasing age is a cumulative physiological response to toxic environmental factors leading to an excessive generation of ROS in the lens epithelium cells (LECs) and in the superficial lens fiber cells, as well as in the aqueous humor [119]. UV-induced oxidative damage is a significant contributory factor to cataractogenesis. The eyes are continuously exposed to solar radiation, which can be divided into five regions in increasing order of wavelengths, that is, ultraviolet UVC, UVB, UVA, visible range, and infrared range. Among these radiations, UVC and UVB are responsible for photochemical reactions. UVA, visible range, and infrared range radiation are traditionally thought to be less damaging [120]. The major effect of UV radiation is through photochemical generation of ROS, including superoxide and its derivatization to other potent entities such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen in the lens and in aqueous humor, which lead to oxidative damage of the lens tissue [120, 121]. The incidence of cataract is higher in the population which is more exposed to the sunlight [122].

Under normal conditions the lens is well equipped and uses multiple physiologic defense strategies to scavenge ROS and to maintain them at low levels to protect the lens from the toxic effects of oxidative damage. The lens defense system constitutes enzymatic antioxidants, that is, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) utilizing H₂O₂, chemical antioxidants, that is, α -tocopherol, beta-carotene, ascorbate, and GSH, structural antioxidants, that is, cholesterol and membrane protein, and transition metal-sequestering protein including aqueous and plasma ceruloplasmin [123, 124]. Enzymatic defense system also protects the lens from lipid-derived hydrogen peroxide [123]. However, protective systems decrease with the age and long-term exposure to oxidative stress predispose lens cells at risk for cumulative oxidative damage and cataract formation [118]. The effectiveness of SOD and other antioxidant enzymes is limited to several reasons such as the following: (i) their deactivation with aging and their selective distribution and availability in various cellular compartments, (ii) being macromolecules, where they cannot penetrate the certain sensitive sites of oxidation in nucleic acids and in proteins, (iii) the fact that the lens is surrounded and bathed by aqueous and the vitreous humors, fluids which lack the enzymatic defenses. Therefore the lens cell membranes, which are continuously exposed to a photochemical oxidative environment due to the continued light penetration during the long periods of photopic vision, remain susceptible to photo damage [121]. Human mature cataractous lenses show decreased activity of SOD, glutathione peroxidase (reducing organic hydrogen peroxide, including hydrogen peroxide of lipids) and glutathione reductase, and however no signs of deficiency in activities of catalase [123]. Genetic variations in the antioxidant genes coding for the SOD, CAT, and GPX enzymes may also lead to decreased or impaired regulation

of their enzymatic activity and alter ROS detoxification. Zhang et al.'s study showed that the G/G genotype of the SOD1-251A/G polymorphism may be associated with an increased risk of cataract. However, in CAT-21A/T and GPX1-198C/T polymorphisms, there were no significant differences in the variant homozygous frequencies in age-related cataract patients in comparison to controls [125].

5.1. The Role of ROS in Oxidative Stress-Induced Mitochondria-Dependent Apoptosis in Human Lens Epithelial Cells. Oxidative stress of the lens is not only the result of an imbalance between lens oxidants and antioxidants but also the consequence of cellular redox status imbalance in the lens [123]. The short range packing of the crystallins, which make up over 90% of the soluble lens, must exist in a homogenous state, that is, in the redox balance, to maintain lens transparency, which means the necessity to maintain continuously the thiol (sulfhydryl) groups of proteins of the lens center in a nearly 100% reduced state to prevent formation of high molecular weight protein aggregates, which contributes to the cataract formation [118]. LECs are the center of lens metabolic activity and their oxidative damage plays a significant role in cataractogenesis [126]. Mitochondria are abundant in the lens but only within the epithelium and differentiating fibers; mature fibers in the core of the lens lack mitochondria [127]. Mitochondria of LECs and superficial fiber cells consume 90% of the oxygen entering the lens and are major endogenous sources of ROS [118, 128]. It is estimated that up to 1% to 5% of oxygen consumed by the lens mitochondria is converted to ROS [129].

Age-related mitochondrial dysfunction and ROS imbalance induce oxidative damage of cellular components and play crucial role in the pathogenesis of senile cataract development [118]. ROS are formed in LECs mitochondria as a byproduct of normal metabolism and as a consequence of exposure to environmental compounds and if not eliminated cause oxidative damage of DNA, proteins, and lipids [130].

Nucleic acids are prone to oxidative damage by ROS. Continuous attack of ROS leads to DNA oxidation. OH[•] modify guanine of DNA and form 8-hydroxyguanine. 8-OHdG is highly mutagenic, causes GC to TA transversions, and has been commonly quantified as a steady-state estimate of oxidative stress in tissues [131]. The extent of lens DNA damage caused by direct ROS attack can be assessed by 8-hydroxyguanine assay and comet assay. Cultured normal human LECs show increase of 8-hydroxyguanine marker in response to the oxidative stress [132]. Quantitative assessment of DNA damage in LECs achieved from senile cataract patients and performed in comet assay showed smearing of DNA fragments instead of bands in the tail, which indicate random (nonenzymatic) damage with ROS, which act by chemical reaction [133]. Oxidative mtDNA damage is a causative factor in aging and a wide variety of degenerative diseases. mtDNA damage is more extensive and persists longer than nDNA damage because of its close proximity to ROS generation through the respiratory chain and its paucity of protective histones. A vicious cycle of mtDNA damage and ROS production established within cells leads to loss of the mitochondrial membrane potential and release of

cytochrome c, resulting in the cell apoptosis [130]. Oxidative stress can disrupt the balance between ROS production and the radical scavenging effect and lead to apoptotic cell death through the mitochondrial apoptosis pathway. Numerous studies of human cataractous lenses confirmed extensive oxidative damage of mtDNA and membrane pumps of lens cells, as well as increased unscheduled expression of genes stimulated by excessive ROS production. mtDNA damage and pathological gene expression are both responsible for loss of LECs viability and their death by apoptotic and necrotic mechanisms. LECs death by apoptosis plays key role in the pathogenesis of noncongenital cataract development in human [118, 134–136].

Increased accumulation of oxidized proteins, mainly methionines and cysteine residues, also is linked to cataractogenesis and confirms age-related increase in rates of ROS generation, decrease in antioxidant activity, and loss in the capacity to degrade oxidized proteins [137]. Moreover, excessive ROS production and oxidative stress lead to formation of lipid peroxides, which contribute to pathological processes of aging and play role in systemic (diabetes, atherosclerosis, chronic renal failure, and inflammation) and retinal degenerative diseases, and they are statistically significant risk factors for cataract development [123]. Lipid peroxides impair both cell membrane and cytosol regions [138], damage DNA [139], induce a drop in total glutathione and dramatic change in the redox ratio of glutathione, and lead to the appearance of new fluorophores and large protein aggregates with low solubility (clouding matrix) in the lens matter [140].

6. The Role of ROS and the Oxidative Stress in Diabetic Retinopathy

Diabetes mellitus (DM) is a chronic and progressive neurodegenerative disease, characterized by chronic hyperglycemia and altered cellular homeostasis, which lead to the diffuse microvascular and macrovascular damage, numerous complications, and multiorgan dysfunction. During the course of DM, every cell is exposed to the abnormally high glucose concentrations; however, high glucose-related damage only targets specific tissues, that is, retina, nerve tissues, and kidney, since these tissues are deficient in the ability to change glucose transport rates when faced with the elevated extracellular glucose concentrations [141].

Diabetic retinopathy (DR) is a chronic and progressive complication in the course of diabetes mellitus type 1 or type 2 and the major cause of blindness in people of working age. It develops over approximately 10 to 25 years, and during the first two decades of the disease, nearly all individuals with type 1 and approximately 60% of individuals with type 2 diabetes will have some degree of retinopathy [142]. Diabetic retinopathy is one of the microvascular diabetes complications, characterized by gradual and progressive alterations in the retinal microvasculature with accompanying damage of glia and neurons [143]. DR results from capillaries damage. Capillaries are lined with endothelial cells, surrounded by smooth muscle cells and sealed by pericytes, which provide tone to the vessels and create a blood barrier for closed capillaries in the retina and in the choroid. In early DR

stage, pericyte and endothelial cells undergo accelerated death by apoptosis, which leads to the reduction of pericyte numbers manifested by their degeneration (ghost cells) or loss, followed by the increased numbers of acellular-occluded capillaries, microaneurysms, and capillary basement membrane thickening. Noncapillary cells (Müller cells and other glial cells) are also lost selectively via apoptosis. Unsealed capillaries begin to leak plasma and erythrocytes into the surrounding retinal tissue, resulting in edema and intraretinal hemorrhages. Endothelial cells try to repair the damage by multiplying on the inner membrane; however, it leads to the capillaries occlusion and ischemic retina releases the growth factors leading to the pathological angiogenesis [144]. Based on the extent of microvascular damage, DR is classified into either nonproliferative (mild, moderate, or preproliferative, characterized by cotton wool spots, venous beading and loops, blood vessel closure, tissue ischaemia, and the formation of intraretinal microvascular abnormalities) or proliferative. In proliferative DR, pathological angiogenesis is driven by the vascular endothelial growth factor (VEGF) sourced from retinal vascular pericytes, retinal ganglion cells, and glia. Vision loss occurs from breakdown of the blood-retinal barrier, resulting in macular edema, inner retinal and vitreous hemorrhages, and tractional retinal detachment [144].

6.1. The Interaction between Hyperglycemia, ROS Stress, and Hyperglycemia-Induced Metabolite Pathways. Hyperglycemia stimulates overproduction of mitochondrial ROS and generates the oxidative stress. Increased mitochondrial ROS levels activate the poly-ADP-ribose polymerase (PARP) pathway, which reduces glyceraldehydes 3-phosphate dehydrogenase (GAPDH) activity. Decreased GAPDH level in turn contributes to overactivation of four classic hyperglycemia-induced metabolite mechanisms, that is, the polyol pathway, the protein kinase C (PKC) pathway, AGEs pathway, and the hexosamine pathway. The reduction of GAPDH activity can be prevented by MnSOD [145–147].

According to Brownlee, all four classic hyperglycemia-induced pathways are activated by a single upstream event; that is, the mitochondrial overproduction of ROS and all four pathways become the source of increased ROS production and stimulation of the oxidative stress [145, 146]. All classic hyperglycemia-induced metabolite mechanisms result in decreased NADPH levels and increased NADPH oxidase (Nox) levels (see below). NADPH regenerate glutathione, an important scavenger of ROS. Therefore, decreased levels of NADPH are responsible for the increased ROS accumulation and the oxidative stress damage. Moreover, decreased NADPH levels lead to the inhibition of GAPDH, which in turn activate four hyperglycemia-induced pathways [145, 146]. The unifying mechanism proposed by Brownlee interconnects increased ROS production and the oxidative stress with four main hyperglycemia-induced processes and explains the hyperglycemia-induced endothelial cells damage (apoptosis) and the progression of diabetic retinopathy [145].

ROS and the oxidative stress contribute to “metabolic memory” or “legacy effect.” It means that the diabetic retinopathy progress, even after glycemia, has been normalized and well controlled. Mitochondrial abnormalities are

irreversible, even after hyperglycemia stress is terminated and these impaired mitochondria are source of permanent ROS overproduction [145]. In laboratory conditions, human ARPE-19 retinal cells [148] and retinal pericytes [145] continue ROS overproduction even after the glucose normalization. Hyperglycemia, increased ROS production, the oxidative stress, and excessive AGE formation are causally associated with and show linear relationship in the early years of diabetes. ROS, which act at the mitochondrial level, are associated with “micro” metabolic memory and form the lowest denominator of diabetic complications. However, persistent mtDNA damage and respiratory chain protein glycation generate AGEs, which stimulate ROS production, and more ROS amplified AGEs formation and such vicious cycle acts independently of hyperglycemia level in advanced diabetes stage. AGEs, which are result of chronic interaction with the oxidative stress at tissue/vessel level, are associated with “macro” metabolic memory and form the bridge between micro- and macrovascular diabetic damage [149].

Intracellular AGEs formed via nonenzymatic glycation and glycooxidation processes [146] on short half-life proteins lead to endothelial cells dysfunction, loss of pericytes, and neuronal cells damage. They also reduce platelet survival, increase platelet aggregation, promote a procoagulant state, lead to ischemia, and induce growth factors, which stimulate pathological angiogenesis [150]. AGEs formed on long half-life proteins like collagen modify extracellular matrix and cause loss of charge and structural distortions associated with a lower integrin binding affinity, which leads to cell detachment, basement membrane thickening, and their resistance to proteolytic digestion [151]. AGEs/RAGE pathway increases cytosolic ROS level, activates NF- κ B pathway, and increases expression of cytokine and adhesion molecule. RAGEs activate indirectly the toll-like receptor 4 (TLR-4), which can trigger interaction with an innate immune system as well in type 2 diabetic patients [152].

6.2. The Influence of ROS and the Oxidative Stress on Endothelial Cells Apoptosis in Diabetic Retinopathy. Sustained hyperglycemia and increased chronic local oxidative stress disrupt retinal metabolism and accelerate premature endothelial cells apoptosis via mitochondrial dysfunction in both type 1 and type 2 diabetes retinopathy. In the early stages of diabetes, increased mtDNA biogenesis and repair compensate the ROS-induced damage. However, while it sustained insulting, this mechanism is overwhelmed and both the function and structure of mtDNA are damaged (mitochondrial electron transport chain is highly sensitive to the oxidative stress) [153–155]. The compromised electron transport chain propagates a vicious cycle of ROS and the dysfunctional mitochondria fuel loss of capillary endothelial cells by initiating their apoptosis [153–155]. Poor glycemia control and chronically increased intracellular glucose flux decrease retinal mtDNA copy number [156]. In diabetic retinopathy, the mtDNA damage at the regulatory region (the displacement loop) is considerably higher in comparison to other mtDNA portions [156]. The enzymes important for mtDNA repair, that is, 8-oxoguanine DNA glycosylase (OGG1), MutY homolog, and thymine DNA glycosylase,

become subnormal, and the transcription and replication mechanisms including mitochondrial transcription factor A (TFAM) and polymerase gamma (POLG) are also compromised [156, 157].

In diabetic retinopathy, increased ROS expression and the oxidative stress may induce endothelial cells senescence via downregulation of Sirt6. Sirt6 is a nuclear chromatin-bound protein, which regulates glucose homeostasis [158], has antiaging and anti-inflammatory properties, and is involved in the oxidative stress-induced endothelial cells senescence pathomechanism [159, 160]. In Liu et al.'s study, Sirt6 protein was markedly reduced in endothelial cells activated by H₂O₂, and overexpression of Sirt6 partially reversed H₂O₂-induced endothelial cells dysfunction and senescence symptoms like decrease in endothelial cells growth, proliferation and angiogenic ability, loss of eNOS protein, and increase in senescence markers. According to the authors, induced by the oxidative stress, downregulation of Sirt6 may be involved in the pathogenesis of diabetic retinopathy [161].

6.3. The Influence of ROS on Retinal Neuronal Cells Apoptosis in Diabetic Retinopathy. ROS not only influence retinal vasculature but also exert neurodegenerative impact on diabetic retina [162, 163], which confirm results of the experimental studies.

In the streptozotocin- (STZ-) induced type 1 diabetes model mouse, cross-talk between ROS and renin-angiotensin system led to the reduction level of synaptophysin (synaptic vesicle protein for neurotransmitter release), most likely through excessive protein degradation by the ubiquitin-proteasome system. Moreover, ROS also decreased brain-derived neurotrophic factor (BDNF), which regulates axonal growth, synaptic activity, and neuronal survival. The damage of synaptic transmitter and degradation of neurotrophic factor, stimulated by the excessive ROS level, caused neuronal cells apoptosis and visual impairment [162]. However, constant lutein (antioxidant) treatment of the STZ-induced diabetes model mice (which presented synaptophysin and BDNF reduction caused by H₂O₂ stimulation) suppressed decreasing of synaptophysin protein and electroretinography impairment and preserved neuronal cells survival [163]. Lutein is a yellow pigment, which filters the high-energy blue light being toxic to the retina. The preventive effect of lutein observed in the mice diabetes model occurred by lutein antioxidative influence and the ROS reduction and not because of the filtering light of high energy [164].

Experimental studies show that AGEs also affect adversely the whole diabetic neurosensory retina. Intracellular AGEs accelerate directly neuronal cells apoptosis and extracellular AGEs (which alter metabolism of neuroretinal supporting cells) accelerate indirectly neuronal apoptosis [165]. Müller cells play fundamental role in retinal physiology. However, macroglia stimulated by hyperglycemia increases ROS production and amplifies AGEs formation and becomes dysfunctional due to increase glial fibrillary acidic protein (GFAP) expression, NO production, and glutamate synthesis (as a function of glutamate transporter disruption), and in consequence Müller cells contribute indirectly with retinal neurons excitotoxicity to the diabetic retina [166]. In

connection with above Chilelli et al. suggest that diabetic retinopathy can be a sensory neuropathy, like peripheral diabetic neuropathy [149].

6.4. The Role of ROS in Stimulating Local Inflammation and Pathological Angiogenesis in Diabetic Retinopathy. The oxidative stress and inflammatory processes play the important roles in the development of microvascular lesions characteristic for diabetic retinopathy. The oxidative stress regulates expression of proinflammatory proteins [167, 168]. The oxidative stress and inflammation promote endothelial cells senescence [169] and pathological angiogenesis characteristic for proliferative diabetic retinopathy [170].

Reactive oxygen species in the retina may stimulate retinal angiogenesis by many molecular pathomechanisms. ROS participate in the activation of proinflammatory NF- κ B pathway, which in turn leads to the production of tumor necrosis factor alpha (TNF- α) and subsequent generation of inflammatory and angiogenic mediators such as interleukin 6 (IL-6), interleukin 8 (IL-8), cyclooxygenase 2 (COX-2), intercellular adhesion molecule 1 (ICAM-1), monocyte chemoattractant protein 1 (MCP-1), and VEGF [170].

Mitochondrial derived ROS trigger pathological angiogenesis by stabilization HIF-1 α factor. Hypoxia-inducible factor-1 (HIF-1), the main regulator of oxygen homeostasis, consists of HIF-1 α and HIF-1 β subunits. Under hypoxic conditions, HIF-1 activates the transcription of a broad variety of genes, including those encoding erythropoietin, glucose transporters, glycolytic enzymes, inducible nitric oxide synthase, heme oxygenase-1, VEGF, and others, to ensure cell survival under conditions of hypoxic stress and to restore O₂ homeostasis [171, 172]. Under normoxic conditions, HIF-1 α is conserved by HIF prolyl hydroxylases (PHDs), which allows them to be rapidly degraded. However, under hypoxic conditions PHD is inhibited, since it requires oxygen for functioning and this results in the stabilization of HIF-1 α . The stabilization of HIF-1 α leads to the upregulation of many hypoxic-sensitive genes such as angiopoietin, erythropoietin, VEGF, and stromal cell derived factor-1 (SCDF-1). All of them exhibit angiogenic properties in the retina, resulting in pathological angiogenesis and vascular leakage [170, 171]. ROS can directly be ligated to the active ferrous iron center of PHDs and promote phosphorylation-dependent stabilization of HIF-1 α [170, 172], which trigger pathological angiogenesis. Moreover, the relationships between the NF- κ B and HIF-1 pathways result in the amplification of signals of both pathways [170, 173].

ROS derived from the family of NADPH oxidase (Nox) enzymes may also activate NF- κ B and HIF-1 pathways and participate in the development of proliferative diabetic retinopathy. The Nox family, important source of ROS production, consists of seven isoforms named Nox1–5, Duox (dual oxidase) 1, and Duox2 and contributes to vascular injury. Nox1, Nox2, and Nox4 participate in pathological angiogenesis. The RAAS (renin-angiotensin-aldosterone system), and particularly AngII (angiotensin II), is a key stimulator of Nox. RAAS exists in the retina and it is a blockade of AngII and aldosterone attenuates pathological angiogenesis of the retina. However, it is not fully recognized

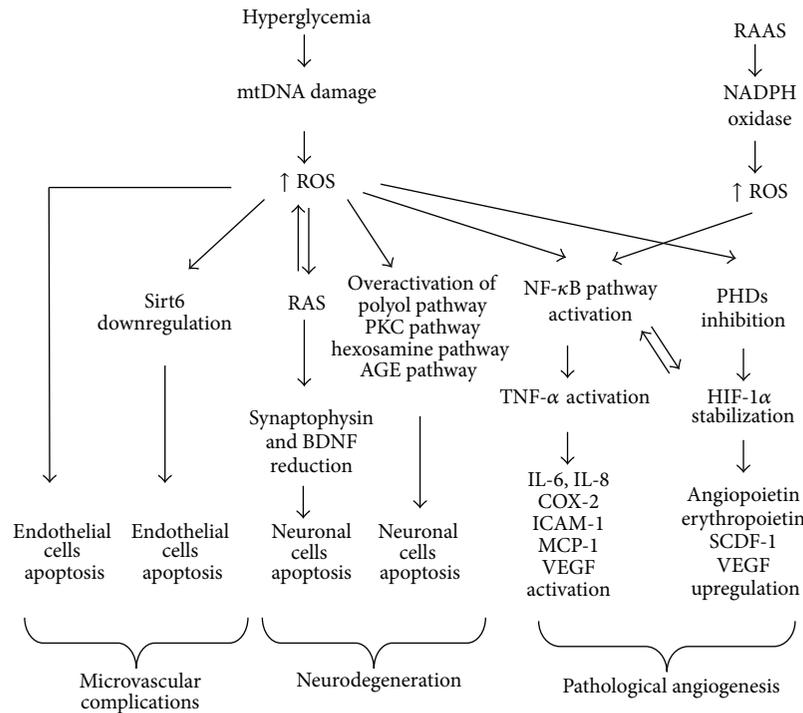


FIGURE 3: Schematic overview of the ROS influence on the development of microvascular complications, neurodegeneration, and pathological angiogenesis in the course of diabetic retinopathy. mtDNA, mitochondrial deoxyribonucleic acid; ROS, reactive oxygen species; Sirt6, the name of a nuclear chromatin-bound protein; RAS, renin-angiotensin system; BDNF, brain-derived neurotrophic factor; PKC, the protein kinase C, AGEs, advanced glycation end products; NF- κ B, nuclear factor- κ B; TNF- α , tumor necrosis factor alpha; IL-6, IL-8, interleukins 6 and 8; COX-2, cyclooxygenase 2; ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; VEGF, vascular endothelial growth factor; PHDs, prolyl hydroxylases; HIF-1, hypoxia-inducible factor-1; SCDF-1, stromal cell derived factor-1; RAAS, rennin-angiotensin-aldosterone system; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase.

if RAAS has influence on the production of ROS derived from Nox in diabetic retinopathy [170]. The schematic overview of the role of ROS in the development of diabetic retinopathy is presented in Figure 3.

ROS take part in the pathogenesis of cystoid macular edema (CME). CME is caused by inflammatory breakdown of blood-retinal barrier, which results in the accumulation of fluid and protein. Edema and thickening of the macula lead to decrease of vision acuity [174]. In Samanta et al.'s study, both diabetic and normal patients with cystoid macular edema after uncomplicated standardized phacoemulsification surgery exhibited significantly increased activity of ROS determined in the serum samples, in comparison with diabetic and normal patients without CMO after uncomplicated cataract surgery [175].

7. The Role of ROS in the Pathomechanism of the Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of permanent, irreversible, central blindness (scotoma in the central visual field makes impossible the following: reading and writing, stereoscopic vision, recognition of colours and details) in patients over the age of 50 in developed countries. It is estimated that approximately 50 million

old people suffer from AMD worldwide [176]. The major pathological changes associated with AMD are observed in the functionally and anatomically related tissues including photoreceptors, retinal pigment epithelium (RPE), Bruch's membrane, and choriocapillaris. Soft drusen and/or pigmentary abnormalities are clinically visible symptoms of early AMD. Late (or advanced) AMD occurs in two distinct forms. Visual loss is caused by the "geographic atrophic" death of photoreceptors and retinal pigment epithelium (RPE) cells, so-called "dry-AMD," GA/AMD, or by formation of the choroidal neovascular membrane (CNV), as a result of pathological angiogenesis, so-called "exudative- or wet-AMD," CNV/AMD [177, 178].

AMD is a complex chronic neurodegenerative and progressive disease of multifactorial etiology [179]. Advanced age and its related physiological cell apoptosis and tissue involution, together with genetic predisposition and epigenetic modifications, are the strongest risk factors (epigenetics refers to heritable changes in gene expression that do not involve changes to the underlying DNA sequence, a change in phenotype without a change in genotype, and are regular and natural occurrence but can also be influenced by age, the environment/lifestyle, and disease state). However, other factors such as sex and environmental influences such as smoking cigarettes, heart and vascular disorders, hypertension, dyslipidemia/hypercholesterolemia, diabetes, obesity,

improper diet, sedentary lifestyle, and phototoxic exposure are also important [179–181].

Excessive ROS production and accumulation together with the oxidative stress seem to play a pivotal role in AMD pathogenesis and RPE cells are critical site of injury in AMD [182]. ROS levels increase in the aging retina, although the retina and RPE cells are rich in both enzymatic and nonenzymic antioxidants. Augmented level of ROS and attenuated antioxidant cell defense systems lead to the oxidative stress and result in damage of photoreceptors, RPE cells, and choriocapillaris in apoptosis process [183, 184].

7.1. The Reasons of ROS Accumulation in Outer Part of the Retina in the Course of AMD. The retinal tissue is abundant in ROS, since (i) in the retina is the highest oxygen consumption among all human tissues, (ii) RPE and photoreceptors of the macula are exposed to high-energy light, (iii) the cell membranes of photoreceptors are rich in polyunsaturated fatty acids (PUFA), which are readily oxidized, (iv) there are many photosensitizers in photoreceptors and RPE, and (v) phagocytosis of photoreceptor outer segments (POS) conducted by RPE cells is accompanied by a respiratory burst and rapid eruption of ROS [185].

Photoreceptors are cells of high metabolic activity and high demand for oxygen and nutrients delivered from the blood vessels. Due to the high consumption of oxygen, their supply in the retina is higher than in other tissues [186]. The retina oxygen tension is 70 mmHg [187]. The high partial pressure of oxygen promotes generation of ROS in the retina [185].

Radiation reaching the eye is partly absorbed by the cornea and lens, whereas the rest of it (400–760 nm) penetrates the eye reaching the retina. At the retina level, exposure to visible light stimulates RPE cells to phagocytosis (ingestion). The digestion of photoreceptors' outer segments induces formation of superoxide anion in the RPE cells. Epidemiological evidences suggest a direct relationship between phototoxicity (cumulative light exposure) and the development of AMD and susceptibility to the blue light-mediated damage represents one of the aspects of AMD pathogenesis [188]. The blue portion of the visible spectrum of light (441 nm) is dangerous for RPE cells, since it is the most energetic radiation reaching the macula and because it promotes photooxidation of lipofuscin generating the reactive photoproducts including N-retinylidene-N-retinylethanolamine (A2E), DNA oxidation, and cells apoptosis [189, 190]. Blue light leads to disturbances of the outer blood-retinal barrier and damage of POS and alterations in the RPE and choroidal cells are similar to atrophic changes in GA/AMD [191]. Tissues with a high tissue oxygen concentration and a high proportion of membrane lipids are most sensitive to the damage by increased level of ROS and the oxidative stress [192]. Photoreceptors rich in PUFAs are particularly vulnerable to the lipid peroxidation, since the susceptibility of unsaturated fatty acids to oxidation increases with the number of double bonds. The oxidation of PUFAs leads to the development of peroxides and organic radicals [193], as well as other products such as carboxyethylpyrrole (CEP) and 4-hydroxy-2-nonenal (4-HNE), which form adducts with

proteins and are accumulated in the outer retina and in drusen [194]. The age-dependent susceptibility of the macula to the lipid peroxidation and its products is connected with the attenuation of antioxidant defense systems with aging. Oxidation of PUFAs lasts many years and leads to the functional and structural impairment of cells membranes and finally to degeneration of photoreceptors [195]. Along with the growth of age, oxidated PUFAs are not efficiently digested in the lysosomes of aged RPE cells and become deposited in the form of lipofuscin. Lipofuscin is a chromophore, serving as the main RPE photosensitizer, which after absorbing a high-energy photon, especially that of blue light, undergoes a variety of photochemical reactions involving ROS formation, which in turn evoke photochemical damage in the retina and RPE cells [196]. A2E is a major hydrophobic fluorophore of RPE lipofuscin, which forms through a multistep biosynthetic pathway, starting with reactions between phosphatidylethanolamine and all-trans-retinal. Upon blue light excitation, A2E acts as a photosensitive generator of singlet oxygen and superoxide, which connect at the carbon-carbon double bonds to form harmful epoxides [197, 198]. A2E-epoxides also accelerate ROS generation and initiate RPE cells damage [199].

The retina is particularly susceptible to aging [1] and vulnerable to the oxidative stress [4], since its two vital components are highly metabolically active and composed of postmitotic cells. Nondividing photoreceptors and RPE cells are particularly prone to the accumulate mtDNA damage due to their inability to reduce defective mitochondria during mitosis. Mitochondria impairment correlates with increased sensitivity of aging RPE cells to the oxidative stress. Changes in mitochondrial number, size, shape, matrix density, cristae architecture, and membrane integrity were more distinct in RPE cells obtained from donors aged 60 and more in comparison with younger individuals. In older donors mitochondria were more elongated, however less numerous [200]. With age, mitochondrial dysfunctions are associated with low ATP level, attenuated mitochondrial membrane potential, reduced cytoplasmic Ca^{2+} , and augmented mitochondrial Ca^{2+} sequestration. The decrease level of mitochondrial superoxide dismutase, stimulated long time by the mitochondrial oxidative stress, leads to the increase in superoxide anion, shortening and disorganization of the photoreceptors' outer and inner segments, degeneration of RPE cells, thickening of Bruch's membrane, and finally apoptotic cells death in AMD process [201]. The analysis of the mitochondrial proteomics of RPE cells in advanced stages of AMD showed that the distribution of mitochondrial mutations is qualitatively different in AMD compared to that in normal aging [202, 203].

Chronic low-grade inflammation [204] and hypoxia [205] presented in the aging retina also are the source of ROS production and accumulation.

The products of the oxidative stress trigger chronic low-grade inflammation (pathophysiological parainflammation) process in AMD patients. Pathophysiological parainflammation process mediated by many factors and stimulated by complement system, especially its alternative pathway, and carried out in Bruch's membrane leads to early and

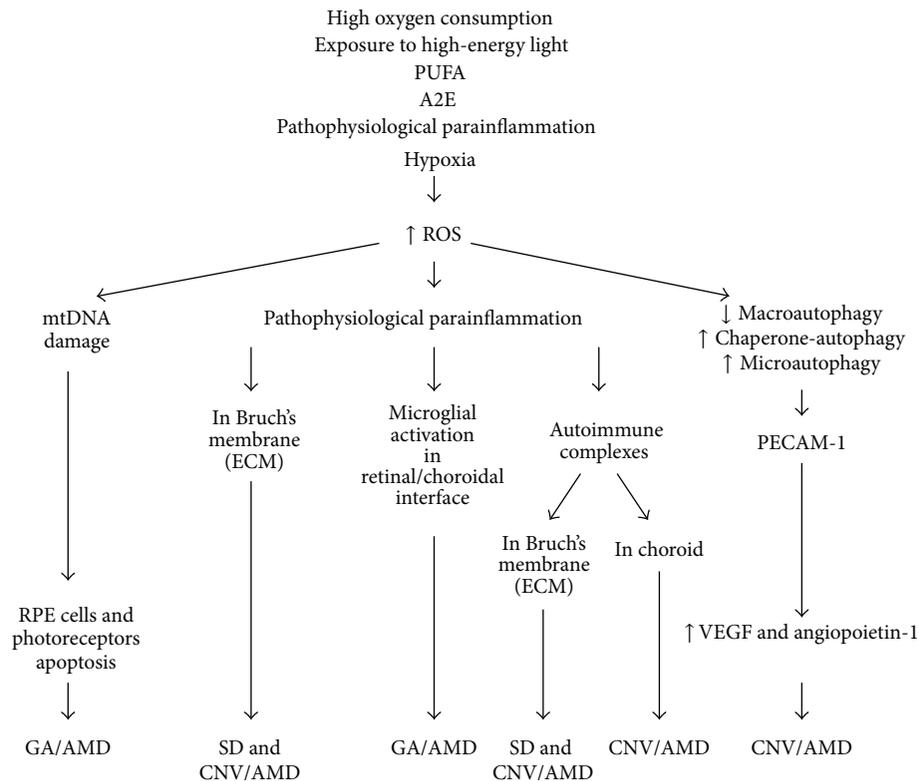


FIGURE 4: Schematic overview of the ROS influence on the development of early and advanced forms of age-related macular degeneration. PUFA, polyunsaturated fatty acid; A2E, a component of retinal pigmented epithelial cell (RPE) lipofuscin; ROS, reactive oxygen species; mtDNA, mitochondrial deoxyribonucleic acid; ECM, extracellular matrix; PECAM-1, platelet endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor; SD, soft drusen; GA/AMD, geographic atrophy/age-related macular degeneration; CNV/AMD, choroidal neovascularization/age-related macular degeneration.

advanced CNV/AMD forms. Pathophysiological parainflammation process is connected with the microglial activation carried out in retinal/choroidal interface and leads to the advanced atrophic AMD form (GA/AMD). Moreover, it is connected with autoantibodies and formation of immune complexes carried out in Bruch's membrane, which leads to the early and advanced AMD, as well as with choroidal macrophages infiltration, which leads to CNV/AMD [204]. ROS impair cells function not only by reacting with nucleic acids, proteins, and lipids but also by inducing production of proinflammatory cytokine [206] and angiogenic signals [207].

In AMD eyes hypoxia is the result of diminished choroidal blood circulation, which confirms measurements of oxygen tension, perfusion pressure, and blood flow rate [208, 209]. The inner part of the retina is better protected from ischemic stress than the outer retina layers, which means that photoreceptors and RPE cells are capable of recovering after an acute hypoxic insult, however not after chronic retinal ischemia and hypoxia, which can lead to cell death and irreversible visual impairment [209, 210]. The retinal blood flow is disturbed in both dry and CNV/AMD type [208]; the reduction in choroidal perfusion has been positively correlated with the disease progression [205].

During inflammation, hypoxia in the retinal cells may result from increased consumption of oxygen due to the increased metabolic activity of the inflamed retina [205].

Superoxide anions are involved in the regulation of cells adaptation to hypoxia via HIF-1 α factor [211] and are involved in the regulation of mitochondrial autophagy process [212, 213].

Chronic elevated ROS levels and the oxidative stress, pathophysiological parainflammation, and long stay hypoxia decrease the ability of RPE cells to remove damaged or nonfunctional proteins via the lysosomal clearance system, including macroautophagy [214]. In aged RPE cells the substrate for autophagy is degraded by lysosomal acid hydrolases, including cathepsins D, B, and L, after autophagolysosome, and Rab7, LAMP-2A, and SNAREs proteins are critical for the fusion of lysosome and autophagosome. Ubiquitin (Ub), LC3II, and p62 complexed to the substrate connect autophagy with the proteasomal clearance system [214].

A marked reduction of macroautophagic activity with aging has been associated with an increase in chaperone-mediated autophagy [215]. Experimental studies confirm that ROS also take part in microautophagy and disturb endothelial reticulum (ER) in AMD process. Human RPE cells exhibited in vitro ROS accumulation and subsequent

elevation of GRP78 and CHOP expression (indicators of ER stress) after A2E and blue light-induced damage. Moreover, N-acetylcysteine (NAC), ROS scavenger, diminished expression protein of ER stress [216]. In another study, t-butylhydroperoxide induced the oxidative stress which led also to the accumulation of ROS in the internal space (lumen) of the endoplasmic reticulum and disturbed ER homeostasis in RPE cells [217].

According to Blasiak et al., triplet consists of the oxidative stress, hypoxia, and autophagy which play an important role in CNV/AMD pathogenesis [214]. Pathological angiogenesis in AMD is connected with the activity of VEGF and angiopoietin-1 (and its receptor) [218]. Two proteins, that is, platelet endothelial cell adhesion molecule (PECAM-1) and thrombospondin-1 (TSP-1), act as linkage molecules, which have a reciprocal relationship with autophagy and angiogenesis mediated by angiopoietin 1 [214]. TSP-1, a common denominator between autophagy, angiogenesis, and AMD, acts as antiangiogenic molecule (and a target for antineovascular therapy). Impaired expression of thrombospondin-1 in Bruch's membrane and choroidal vessels was shown in the rodent eyes with age-related macular degeneration [219]. The schematic overview of the role of ROS in the development of early and advanced AMD is presented in Figure 4.

8. Conclusions

Excessive production of the reactive oxygen species and the oxidative stress play important role in the pathogenesis of many age-related ocular diseases and other pathologies of the anterior and posterior eye segment in adults.

ROS stimulate cells' death via apoptosis process, participate in the activation of proinflammatory and proangiogenic pathways, and are associated with the autophagy process.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] W. Dröge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, no. 1, pp. 47–95, 2002.
- [2] M. D. Brand, "The sites and topology of mitochondrial superoxide production," *Experimental Gerontology*, vol. 45, no. 7-8, pp. 466–472, 2010.
- [3] C. L. Quinlan, R. L. S. Goncalves, M. Hey-Mogensen, N. Yadava, V. I. Bunik, and M. D. Brand, "The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I," *The Journal of Biological Chemistry*, vol. 289, no. 12, pp. 8312–8325, 2014.
- [4] H. Cui, Y. Kong, and H. Zhang, "Oxidative stress, mitochondrial dysfunction, and aging," *Journal of Signal Transduction*, vol. 2012, Article ID 646354, 13 pages, 2012.
- [5] T. Finkel, "Signal transduction by reactive oxygen species," *The Journal of Cell Biology*, vol. 194, no. 1, pp. 7–15, 2011.
- [6] M. D. Evans, M. Dizdaroglu, and M. S. Cooke, "Oxidative DNA damage and disease: Induction, repair and significance," *Mutation Research*, vol. 567, no. 1, pp. 1–61, 2004.
- [7] R. Gredilla, "DNA damage and base excision repair in mitochondria and their role in aging," *Journal of Aging Research*, vol. 2011, Article ID 257093, 9 pages, 2011.
- [8] D. A. Butterfield, J. Drake, C. Pocernich, and A. Castegna, "Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid β -peptide," *Trends in Molecular Medicine*, vol. 7, no. 12, pp. 548–554, 2001.
- [9] I. G. Onyango, "Mitochondrial dysfunction and oxidative stress in Parkinson's disease," *Neurochemical Research*, vol. 33, no. 3, pp. 589–597, 2008.
- [10] J. I. Kim, S. I. Cho, N. H. Kim et al., "Oxidative stress and neurodegeneration in prion diseases," *Annals of the New York Academy of Sciences*, vol. 928, pp. 182–186, 2001.
- [11] B. J. Tabner, O. M. A. El-Agnaf, M. J. German, N. J. Fullwood, and D. Allsop, "Protein aggregation, metals and oxidative stress in neurodegenerative diseases," *Biochemical Society Transactions*, vol. 33, no. 5, pp. 1082–1086, 2005.
- [12] G. Tezel, "Oxidative stress in glaucomatous neurodegeneration: mechanisms and consequences," *Progress in Retinal and Eye Research*, vol. 25, no. 5, pp. 490–513, 2006.
- [13] A. Shoham, M. Hadziahmetovic, J. L. Dunaief, M. B. Mydlarski, and H. M. Schipper, "Oxidative stress in diseases of the human cornea," *Free Radical Biology and Medicine*, vol. 45, no. 8, pp. 1047–1055, 2008.
- [14] H. L. Chandler, K. S. Reuter, L. T. Sinnott, and J. J. Nichols, "Prevention of UV-induced damage to the anterior segment using class I UV-absorbing hydrogel contact lenses," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 1, pp. 172–178, 2010.
- [15] M. H. Suh, J.-W. Kwon, W. R. Wee, Y. K. Han, J. H. Kim, and J. H. Lee, "Protective effect of ascorbic acid against corneal damage by ultraviolet B irradiation: a pilot study," *Cornea*, vol. 27, no. 8, pp. 916–922, 2008.
- [16] H. Sies and W. Stahl, "Vitamins E and C, beta-carotene, and other carotenoids as antioxidants," *The American Journal of Clinical Nutrition*, vol. 62, pp. 1315S–1321S, 1995.
- [17] M. Araie, E. Shirasawa, and M. Hikita, "Effect of oxidized glutathione on the barrier function of the corneal endothelium," *Investigative Ophthalmology and Visual Science*, vol. 29, no. 12, pp. 1884–1887, 1988.
- [18] T. F. Linsenmayer, C. X. Cai, J. M. Millholland, K. E. Beazley, and J. M. Fitch, "Nuclear ferritin in corneal epithelial cells: tissue specific nuclear transport and protection from UV-damage," *Progress in Retinal and Eye Research*, vol. 24, no. 2, pp. 139–159, 2005.
- [19] R. Brancato, T. Fiore, L. Papucci et al., "Concomitant effect of topical ubiquinone Q10 and vitamin E to prevent keratocyte apoptosis after excimer laser photocoagulation in rabbits," *Journal of Refractive Surgery*, vol. 18, no. 2, pp. 135–139, 2002.
- [20] S. A. Marchitti, Y. Chen, D. C. Thompson, and V. Vasiliou, "Ultraviolet radiation: cellular antioxidant response and the role of ocular aldehyde dehydrogenase enzymes," *Eye and Contact Lens*, vol. 37, no. 4, pp. 206–213, 2011.
- [21] R. Buddi, B. Lin, S. R. Atilano, N. C. Zorapapel, M. C. Kenney, and D. J. Brown, "Evidence of oxidative stress in human corneal diseases," *Journal of Histochemistry and Cytochemistry*, vol. 50, no. 3, pp. 341–351, 2002.

- [22] J. Berlau, H.-H. Becker, J. Stave, C. Oriwol, and R. F. Guthoff, "Depth and age-dependent distribution of keratocytes in healthy human corneas: a study using scanning-slit confocal microscopy in vivo," *Journal of Cataract and Refractive Surgery*, vol. 28, no. 4, pp. 611–616, 2002.
- [23] T. Schmedt, M. M. Silva, A. Ziaei, and U. Jurkunas, "Molecular bases of corneal endothelial dystrophies," *Experimental Eye Research*, vol. 95, no. 1, pp. 24–34, 2012.
- [24] H. Elhalis, B. Azizi, and U. V. Jurkunas, "Fuchs endothelial corneal dystrophy," *Ocular Surface*, vol. 8, no. 4, pp. 173–184, 2010.
- [25] N. C. Joyce, "Proliferative capacity of corneal endothelial cells," *Experimental Eye Research*, vol. 95, no. 1, pp. 16–23, 2012.
- [26] U. V. Jurkunas, M. S. Bitar, T. Funaki, and B. Azizi, "Evidence of oxidative stress in the pathogenesis of Fuchs endothelial corneal dystrophy," *American Journal of Pathology*, vol. 177, no. 5, pp. 2278–2289, 2010.
- [27] J. M. Lee, M. J. Calkins, K. Chan, Y. W. Kan, and J. A. Johnson, "Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis," *The Journal of Biological Chemistry*, vol. 278, no. 14, pp. 12029–12038, 2003.
- [28] U. V. Jurkunas, I. Rawe, M. S. Bitar et al., "Decreased expression of peroxiredoxins in Fuchs' endothelial dystrophy," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 7, pp. 2956–2963, 2008.
- [29] M. Matthaehi, A. Y. Zhu, L. Kallay, C. G. Eberhart, C. Cursiefen, and A. S. Jun, "Transcript profile of cellular senescence-related genes in Fuchs endothelial corneal dystrophy," *Experimental Eye Research*, vol. 129, pp. 13–17, 2014.
- [30] Y. Gorin and K. Block, "Nox4 and diabetic nephropathy: with a friend like this, who needs enemies?" *Free Radical Biology and Medicine*, vol. 61, pp. 130–142, 2013.
- [31] B. Azizi, A. Ziaei, T. Fuchsluger, T. Schmedt, Y. Chen, and U. V. Jurkunas, "p53-regulated increase in oxidative-stress-induced apoptosis in Fuchs endothelial corneal dystrophy: a native tissue model," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 13, pp. 9291–9297, 2011.
- [32] A. Ziaei, T. Schmedt, Y. Chen, and U. V. Jurkunas, "Sulforaphane decreases endothelial cell apoptosis in fuchs endothelial corneal dystrophy: a novel treatment," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 10, pp. 6724–6734, 2013.
- [33] T. Sherwin and N. H. Brookes, "Morphological changes in keratoconus: pathology or pathogenesis," *Clinical and Experimental Ophthalmology*, vol. 32, no. 2, pp. 211–217, 2004.
- [34] B. J. Dahl, E. Spotts, and J. Q. Truong, "Corneal collagen cross-linking: an introduction and literature review," *Optometry*, vol. 83, no. 1, pp. 33–42, 2012.
- [35] J. Colin and S. Velou, "Current surgical options for keratoconus," *Journal of Cataract and Refractive Surgery*, vol. 29, no. 2, pp. 379–386, 2003.
- [36] K. P. Burdon and A. L. Vincent, "Insights into keratoconus from a genetic perspective," *Clinical and Experimental Optometry*, vol. 96, no. 2, pp. 146–154, 2013.
- [37] E. Arnal, C. Peris-Martínez, J. L. Menezo, S. Johnsen-Soriano, and F. J. Romero, "Oxidative stress in keratoconus?" *Investigative Ophthalmology and Visual Science*, vol. 52, no. 12, pp. 8592–8597, 2011.
- [38] M. Edwards, C. N. J. McGhee, and S. Dean, "The genetics of keratoconus," *Clinical and Experimental Ophthalmology*, vol. 29, no. 6, pp. 345–351, 2001.
- [39] M. Chwa, S. R. Atilano, V. Reddy, N. Jordan, D. W. Kim, and M. C. Kenney, "Increased stress-induced generation of reactive oxygen species and apoptosis in human keratoconus fibroblasts," *Investigative Ophthalmology & Visual Science*, vol. 47, no. 5, pp. 1902–1910, 2006.
- [40] S. R. Atilano, P. Coskun, M. Chwa et al., "Accumulation of mitochondrial DNA damage in keratoconus corneas," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 4, pp. 1256–1263, 2005.
- [41] M. Chwa, S. R. Atilano, D. Hertzog et al., "Hypersensitive response to oxidative stress in keratoconus corneal fibroblasts," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 10, pp. 4361–4369, 2008.
- [42] X. D. Hao, A. P. Chen, Y. Wang, S.-X. Li, and L.-X. Xie, "Mitochondrial DNA copy number, but not haplogroup is associated with keratoconus in Han Chinese population," *Experimental Eye Research*, vol. 132, pp. 59–63, 2015.
- [43] K. K. Abu-Amero, T. A. Azad, T. Sultan, H. Kalantan, A. A. Kondkar, and A. M. Al-Muammar, "Association of mitochondrial haplogroups H and R with keratoconus in Saudi Arabian patients," *Investigative Ophthalmology and Visual Science*, vol. 55, no. 5, pp. 2827–2831, 2014.
- [44] M. C. Kenney and D. J. Brown, "The cascade hypothesis of keratoconus," *Contact Lens and Anterior Eye*, vol. 26, no. 3, pp. 139–146, 2003.
- [45] E. M. Olofsson, S. L. Marklund, F. Pedrosa-Domellöf, and A. Behndig, "Interleukin-1 α downregulates extracellular-superoxide dismutase in human corneal keratoconus stromal cells," *Molecular Vision*, vol. 13, pp. 1285–1290, 2007.
- [46] M. Määttä, R. Heljasvaara, R. Sormunen, T. Pihlajaniemi, H. Autio-Harmanen, and T. Tervo, "Differential expression of collagen Types XVIII/endostatin and XV in normal, keratoconus, and scarred human corneas," *Cornea*, vol. 25, no. 3, pp. 341–349, 2006.
- [47] M. Nita, B. Strzałka-Mrozik, A. Grzybowski, U. Mazurek, and W. Romaniuk, "Age-related macular degeneration and changes in the extracellular matrix," *Medical Science Monitor*, vol. 20, pp. 1003–1016, 2014.
- [48] H. Nagase, R. Visse, and G. Murphy, "Structure and function of matrix metalloproteinases and TIMPs," *Cardiovascular Research*, vol. 69, no. 3, pp. 562–573, 2006.
- [49] M. C. Kenney, M. Chwa, S. R. Atilano et al., "Increased levels of catalase and cathepsin V/12 but decreased TIMP-1 in keratoconus corneas: evidence that oxidative stress plays a role in this disorder," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 3, pp. 823–832, 2005.
- [50] D. J. Brown, B. Lin, M. Chwa, S. R. Atilano, D. W. Kim, and M. C. Kenney, "Elements of the nitric oxide pathway can degrade TIMP-1 and increase gelatinase activity," *Molecular Vision*, vol. 10, pp. 281–288, 2004.
- [51] V. A. Smith, H. B. Hoh, M. Littleton, and D. L. Easty, "Overexpression of a gelatinase A activity in keratoconus," *Eye*, vol. 9, no. 4, pp. 429–433, 1995.
- [52] M. Stabuc-Silih, M. Ravnik-Glavac, D. Glavac, M. Hawlina, and M. Strazisar, "Polymorphisms in COL4A3 and COL4A4 genes associated with keratoconus," *Molecular Vision*, vol. 15, pp. 2848–2860, 2009.
- [53] S. Saeed-Rad, H. Hashemi, M. Miraftab et al., "Mutation analysis of VSX1 and SOD1 in Iranian patients with keratoconus," *Molecular Vision*, vol. 17, pp. 3128–3136, 2011.

- [54] M. Stabuc-Silih, M. Strazisar, M. Ravnik-Glavac, M. Hawlina, and D. Glavac, "Genetics and clinical characteristics of keratoconus," *Acta Dermatovenerologica Alpina, Panonica, et Adriatica*, vol. 19, pp. 3–10, 2010.
- [55] E. Korvatska, H. Henry, Y. Mashima et al., "Amyloid and non-amyloid forms of 5q31-linked corneal dystrophy resulting from kerato-epithelin mutations at Arg-124 are associated with abnormal turnover of the protein," *Journal of Biological Chemistry*, vol. 275, no. 15, pp. 11465–11469, 2000.
- [56] S. I. Choi, T. I. Kim, K. S. Kim et al., "Decreased catalase expression and increased susceptibility to oxidative stress in primary cultured corneal fibroblasts from patients with granular corneal dystrophy type II," *The American Journal of Pathology*, vol. 175, no. 1, pp. 248–261, 2009.
- [57] S. I. Choi, S. Dadakhujaev, H. Ryu, T. I. Kim, and E. K. Kim, "Melatonin protects against oxidative stress in granular corneal dystrophy type 2 corneal fibroblasts by mechanisms that involve membrane melatonin receptors," *Journal of Pineal Research*, vol. 51, no. 1, pp. 94–103, 2011.
- [58] S. Nakamura, M. Shibuya, H. Nakashima et al., "Involvement of oxidative stress on corneal epithelial alterations in a blink-suppressed dry eye," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 4, pp. 1552–1558, 2007.
- [59] N. Bryan, H. Ahswain, N. Smart, Y. Bayon, S. Wohlert, and J. A. Hunt, "Reactive oxygen species (ROS)—a family of fate deciding molecules pivotal in constructive inflammation and wound healing," *European Cells and Materials*, vol. 24, pp. 249–265, 2012.
- [60] M. Schäfer and S. Werner, "Oxidative stress in normal and impaired wound repair," *Pharmacological Research*, vol. 58, no. 2, pp. 165–171, 2008.
- [61] H. A. Quigley and A. T. Broman, "The number of people with glaucoma worldwide in 2010 and 2020," *British Journal of Ophthalmology*, vol. 90, no. 3, pp. 262–267, 2006.
- [62] R. N. Weinreb and P. T. Khaw, "Primary open-angle glaucoma," *The Lancet*, vol. 363, no. 9422, pp. 1711–1720, 2004.
- [63] M. C. Moreno, J. Campanelli, P. Sande, D. A. Sáenz, M. I. Keller Sarmiento, and R. E. Rosenstein, "Retinal oxidative stress induced by high intraocular pressure," *Free Radical Biology and Medicine*, vol. 37, no. 6, pp. 803–812, 2004.
- [64] M. Aslan, A. Cort, and I. Yucel, "Oxidative and nitrative stress markers in glaucoma," *Free Radical Biology and Medicine*, vol. 45, no. 4, pp. 367–376, 2008.
- [65] M. Mozaffarieh, M. C. Grieshaber, and J. Flammer, "Oxygen and blood flow: players in the pathogenesis of glaucoma," *Molecular Vision*, vol. 14, pp. 224–233, 2008.
- [66] S. C. Saccà and A. Izzotti, "Oxidative stress and glaucoma: injury in the anterior segment of the eye," *Progress in Brain Research*, vol. 173, pp. 385–407, 2008.
- [67] G. R. Howell, R. T. Libby, T. C. Jakobs et al., "Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma," *Journal of Cell Biology*, vol. 179, no. 7, pp. 1523–1537, 2007.
- [68] N. Gupta, L. C. Ang, L. Noël de Tilly, L. Bidaisee, and Y. H. Yücel, "Human glaucoma and neural degeneration in intracranial optic nerve, lateral geniculate nucleus, and visual cortex," *British Journal of Ophthalmology*, vol. 90, no. 6, pp. 674–678, 2006.
- [69] A. Izzotti, S. C. Saccà, M. Longobardi, and C. Cartigl, "Sensitivity of ocular anterior chamber tissues to oxidative damage and its relevance to the pathogenesis of glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 50, no. 11, pp. 5251–5258, 2009.
- [70] S. M. Ferreira, S. F. Lerner, R. Brunzini, P. A. Evelson, and S. F. Llesuy, "Oxidative stress markers in aqueous humor of glaucoma patients," *American Journal of Ophthalmology*, vol. 137, no. 1, pp. 62–69, 2004.
- [71] M. G. Kahn, F. J. Giblin, and D. L. Epstein, "Glutathione in calf trabecular meshwork and its relation to aqueous humor outflow facility," *Investigative Ophthalmology and Visual Science*, vol. 24, no. 9, pp. 1283–1287, 1983.
- [72] D. Gherghel, H. R. Griffiths, E. J. Hilton, I. A. Cunliffe, and S. L. Hosking, "Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 3, pp. 877–883, 2005.
- [73] A. Izzotti, S. C. Saccà, M. Longobardi, and C. Cartiglia, "Mitochondrial damage in the trabecular meshwork of patients with glaucoma," *Archives of Ophthalmology*, vol. 128, no. 6, pp. 724–730, 2010.
- [74] R. Sorkhabi, A. Ghorbanihaghjo, A. Javadzadeh, N. Rashtchizadeh, and M. Moharrery, "Oxidative DNA damage and total antioxidant status in glaucoma patients," *Molecular Vision*, vol. 17, pp. 41–46, 2011.
- [75] S. C. Saccà, A. Pascotto, P. Camicione, P. Capris, and A. Izzotti, "Oxidative DNA damage in the human trabecular meshwork: clinical correlation in patients with primary open-angle glaucoma," *Archives of Ophthalmology*, vol. 123, no. 4, pp. 458–463, 2005.
- [76] H. Mochizuki, C. J. Murphy, J. D. Brandt, Y. Kiuchi, and P. Russell, "Altered stability of mRNAs associated with glaucoma progression in human trabecular meshwork cells following oxidative stress," *Investigative Ophthalmology and Visual Science*, vol. 53, no. 4, pp. 1734–1741, 2012.
- [77] A. Izzotti, M. Longobardi, C. Cartiglia, and S. C. Saccà, "Mitochondrial damage in the trabecular meshwork occurs only in primary open-angle glaucoma and in pseudoexfoliative glaucoma," *PLoS ONE*, vol. 6, no. 1, Article ID e14567, 2011.
- [78] S. C. Saccà and A. Izzotti, "Focus on molecular events in the anterior chamber leading to glaucoma," *Cellular and Molecular Life Sciences*, vol. 71, no. 12, pp. 2197–2218, 2014.
- [79] G. Li, C. Luna, P. B. Liton, I. Navarro, D. L. Epstein, and P. Gonzalez, "Sustained stress response after oxidative stress in trabecular meshwork cells," *Molecular Vision*, vol. 13, pp. 2282–2288, 2007.
- [80] P. B. Liton, C. Luna, M. Bodman, A. Hong, D. L. Epstein, and P. Gonzalez, "Induction of IL-6 expression by mechanical stress in the trabecular meshwork," *Biochemical and Biophysical Research Communications*, vol. 337, no. 4, pp. 1229–1236, 2005.
- [81] P. B. Liton, C. Luna, P. Challa, D. L. Epstein, and P. Gonzalez, "Genome-wide expression profile of human trabecular meshwork cultured cells, nonglaucomatous and primary open angle glaucoma tissue," *Molecular Vision*, vol. 12, pp. 774–790, 2006.
- [82] D. S. Brecht and S. H. Snyder, "Nitric oxide: a physiologic messenger molecule," *Annual Review of Biochemistry*, vol. 63, pp. 175–195, 1994.
- [83] J. A. Nathanson and M. McKee, "Alterations of ocular nitric oxide synthase in human glaucoma," *Investigative Ophthalmology and Visual Science*, vol. 36, no. 9, pp. 1774–1784, 1995.
- [84] S. C. Saccà, A. Izzotti, P. Rossi, and C. Traverso, "Glaucomatous outflow pathway and oxidative stress," *Experimental Eye Research*, vol. 84, no. 3, pp. 389–399, 2007.

- [85] P. Hogg, M. Calthorpe, M. Batterbury, and I. Grierson, "Aqueous humor stimulates the migration of human trabecular meshwork cells in vitro," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 5, pp. 1091–1098, 2000.
- [86] J. Alvarado, C. Murphy, and R. Juster, "Trabecular meshwork cellularity in primary open-angle glaucoma and nonglaucomatous normals," *Ophthalmology*, vol. 91, no. 6, pp. 564–579, 1984.
- [87] L. Zhou, Y. Li, and B. Y. J. T. Yue, "Oxidative stress affects cytoskeletal structure and cell-matrix interactions in cells from an ocular tissue: the trabecular meshwork," *Journal of Cellular Physiology*, vol. 180, no. 2, pp. 182–189, 1999.
- [88] J. A. Alvarado, R. G. Alvarado, R. F. Yeh, L. Franse-Carman, G. R. Marcellino, and M. J. Brownstein, "A new insight into the cellular regulation of aqueous outflow: how trabecular meshwork endothelial cells drive a mechanism that regulates the permeability of Schlemm's canal endothelial cells," *British Journal of Ophthalmology*, vol. 89, no. 11, pp. 1500–1505, 2005.
- [89] G. Tezel and X. Yang, "Caspase-independent component of retinal ganglion cell death, in vitro," *Investigative Ophthalmology & Visual Science*, vol. 45, no. 11, pp. 4049–4059, 2004.
- [90] W. J. Lin and H. Y. Kuang, "Oxidative stress induces autophagy in response to multiple noxious stimuli in retinal ganglion cells," *Autophagy*, vol. 10, no. 10, pp. 1692–1701, 2014.
- [91] J. Lee, S. Giordano, and J. Zhang, "Autophagy, mitochondria and oxidative stress: cross-talk and redox signaling," *Biochemical Journal*, vol. 441, no. 2, pp. 523–540, 2012.
- [92] P. Jiang and N. Mizushima, "Autophagy and human diseases," *Cell Research*, vol. 24, no. 1, pp. 69–79, 2014.
- [93] N. Mizushima, "The pleiotropic role of autophagy: from protein metabolism to bactericide," *Cell Death and Differentiation*, vol. 12, no. 2, pp. 1535–1541, 2005.
- [94] M. Høyer-Hansen, L. Bastholm, P. Szyniarowski et al., "Control of macroautophagy by calcium, calmodulin-dependent kinase-beta, and Bcl-2," *Molecular Cell*, vol. 25, no. 2, pp. 193–205, 2007.
- [95] M. V. Guillot-Sestier, C. Sunyach, C. Druon, S. Scarzello, and F. Checler, "The α -secretase-derived N-terminal product of cellular prion, N1, displays neuroprotective function in vitro and in vivo," *The Journal of Biological Chemistry*, vol. 284, no. 51, pp. 35973–35986, 2009.
- [96] R. Russo, L. Berliocchi, A. Adornetto et al., "Calpain-mediated cleavage of Beclin-1 and autophagy deregulation following retinal ischemic injury in vivo," *Cell Death and Disease*, vol. 2, article e144, 2011.
- [97] A. M. Schmidt, S. D. Yan, S. F. Yan, and D. M. Stern, "The biology of the receptor for advanced glycation end products and its ligands," *Biochimica et Biophysica Acta*, vol. 1498, no. 2-3, pp. 99–111, 2000.
- [98] C. Luo, X. Yang, and G. Tezel, "Accelerated aging in glaucoma: immunohistochemical assessment of advanced glycation end products in the human retina and optic nerve head," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 3, pp. 1201–1211, 2007.
- [99] G. Tezel, X. Yang, C. Luo, Y. Peng, S. L. Sun, and D. Sun, "Mechanisms of immune system activation in glaucoma: oxidative stress-stimulated antigen presentation by the retina and optic nerve head glia," *Investigative Ophthalmology & Visual Science*, vol. 48, no. 2, pp. 705–714, 2007.
- [100] E. M. McElnea, B. Quill, N. G. Docherty et al., "Oxidative stress, mitochondrial dysfunction and calcium overload in human lamina cribrosa cells from glaucoma donors," *Molecular Vision*, vol. 17, pp. 1182–1191, 2011.
- [101] J. Flammer, M. Pache, and T. Resink, "Vasospasm, its role in the pathogenesis of diseases with particular reference to the eye," *Progress in Retinal and Eye Research*, vol. 20, no. 3, pp. 319–349, 2001.
- [102] R. M. Mann, C. E. Riva, R. A. Stone, G. E. Barnes, and S. D. Cranston, "Nitric oxide and choroidal blood flow regulation," *Investigative Ophthalmology and Visual Science*, vol. 36, no. 5, pp. 925–930, 1995.
- [103] O. Zeitz, L. Wagenfeld, N. Wirtz et al., "Influence of oxygen free radicals on the tone of ciliary arteries: a model of vasospasms of ocular vasculature," *Graefes Archive for Clinical and Experimental Ophthalmology*, vol. 245, no. 9, pp. 1327–1333, 2007.
- [104] O. Zeitz, P. Galambos, L. Wagenfeld et al., "Glaucoma progression is associated with decreased blood flow velocities in the short posterior ciliary artery," *British Journal of Ophthalmology*, vol. 90, no. 10, pp. 1245–1248, 2006.
- [105] S. Y. Li, Z. J. Fu, H. Ma et al., "Effect of lutein on retinal neurons and oxidative stress in a model of acute retinal ischemia/reperfusion," *Investigative Ophthalmology and Visual Science*, vol. 50, no. 2, pp. 836–843, 2009.
- [106] M. J. Hoegger, C. J. Lieven, and L. A. Levin, "Differential production of superoxide by neuronal mitochondria," *BMC Neuroscience*, vol. 9, article 4, 14 pages, 2008.
- [107] S. Schoeler, K. Winkler-Stuck, R. Szibor et al., "Glutathione depletion in antioxidant defense of differentiated NT2-LHON cybrids," *Neurobiology of Disease*, vol. 25, no. 3, pp. 536–544, 2007.
- [108] C. J. Lieven, M. J. Hoegger, C. R. Schlieve, and L. A. Levin, "Retinal ganglion cell axotomy induces an increase in intracellular superoxide anion," *Investigative Ophthalmology & Visual Science*, vol. 47, no. 4, pp. 1477–1485, 2006.
- [109] K. I. Swanson, C. R. Schlieve, C. J. Lieven, and L. A. Levin, "Neuroprotective effect of sulfhydryl reduction in a rat optic nerve crush model," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 10, pp. 3737–3741, 2005.
- [110] X. Qi, A. S. Lewin, W. W. Hauswirth, and J. Guy, "Optic neuropathy induced by reductions in mitochondrial superoxide dismutase," *Investigative Ophthalmology and Visual Science*, vol. 44, no. 3, pp. 1088–1096, 2003.
- [111] C. Giordano, M. Montopoli, E. Perli et al., "Oestrogens ameliorate mitochondrial dysfunction in Leber's hereditary optic neuropathy," *Brain*, vol. 134, no. 1, pp. 220–234, 2011.
- [112] K. D. Steinsapir and R. A. Goldberg, "Traumatic optic neuropathy: an evolving understanding," *American Journal of Ophthalmology*, vol. 151, no. 6, pp. 928–933, 2011.
- [113] S. M. Lucas, N. J. Rothwell, and R. M. Gibson, "The role of inflammation in CNS injury and disease," *British Journal of Pharmacology*, vol. 147, supplement 1, pp. S232–S240, 2006.
- [114] S. Ahmad, N. Fatteh, N. M. El-Sherbiny et al., "Potential role of A_{2A} adenosine receptor in traumatic optic neuropathy," *Journal of Neuroimmunology*, vol. 264, no. 1-2, pp. 54–64, 2013.
- [115] S. Ahmad, N. M. Elsherbiny, K. Bhatia, A. M. Elsherbini, S. Fulzele, and G. I. Liou, "Inhibition of adenosine kinase attenuates inflammation and neurotoxicity in traumatic optic neuropathy," *Journal of Neuroimmunology*, vol. 277, no. 1-2, pp. 96–104, 2014.
- [116] M. Fitzgerald, C. A. Bartlett, L. Evill, J. Rodger, A. R. Harvey, and S. A. Dunlop, "Secondary degeneration of the optic nerve following partial transection: the benefits of lomerizine," *Experimental Neurology*, vol. 216, no. 1, pp. 219–230, 2009.

- [117] R. L. O'Hare Doig, C. A. Bartlett, G. J. Maghzal et al., "Reactive species and oxidative stress in optic nerve vulnerable to secondary degeneration," *Experimental Neurology*, vol. 261, pp. 136–146, 2014.
- [118] R. J. W. Truscott, "Age-related nuclear cataract—oxidation is the key," *Experimental Eye Research*, vol. 80, no. 5, pp. 709–725, 2005.
- [119] J. Pande, E. B. Hanlon, and A. Pande, "A comparison of the environment of thiol groups in bovine and human γ crystallins using Raman spectroscopy," *Experimental Eye Research*, vol. 75, no. 3, pp. 359–363, 2002.
- [120] M. J. Davies and R. J. W. Truscott, "Photo-oxidation of proteins and its role in cataractogenesis," *Journal of Photochemistry and Photobiology B: Biology*, vol. 63, no. 1–3, pp. 114–125, 2001.
- [121] S. D. Varma, S. Kovtun, and K. R. Hegde, "Role of ultraviolet irradiation and oxidative stress in cataract formation—medical prevention by nutritional antioxidants and metabolic agonists," *Eye and Contact Lens*, vol. 37, no. 4, pp. 233–245, 2011.
- [122] C. Delcourt, I. Carrière, A. Ponton-Sanchez, A. Lacroux, M.-J. Covacho, and L. Papoz, "Light exposure and the risk of cortical, nuclear, and posterior subcapsular cataracts: the Pathologies Oculaires Liees à l'âge (POLA) Study," *Archives of Ophthalmology*, vol. 118, no. 3, pp. 385–392, 2000.
- [123] M. A. Babizhayev, "Mitochondria induce oxidative stress, generation of reactive oxygen species and redox state unbalance of the eye lens leading to human cataract formation: disruption of redox lens organization by phospholipid hydroperoxides as a common basis for cataract disease," *Cell Biochemistry and Function*, vol. 29, no. 3, pp. 183–206, 2011.
- [124] Z. Yildirim, F. Yildirim, N. I. Ucgun, and N. Kilic, "The evaluation of the oxidative stress parameters in nondiabetic and diabetic senile cataract patients," *Biological Trace Element Research*, vol. 128, no. 2, pp. 135–143, 2009.
- [125] Y. Zhang, L. Zhang, D. L. Sun, Z. S. Li, L. Wang, and P. Liu, "Genetic polymorphisms of superoxide dismutases, catalase, and glutathione peroxidase in age-related cataract," *Molecular Vision*, vol. 17, pp. 2325–2332, 2011.
- [126] M. Kernt, C. Hirneiss, A. S. Neubauer, M. W. Ulbig, and A. Kampik, "Coenzyme Q10 prevents human lens epithelial cells from light-induced apoptotic cell death by reducing oxidative stress and stabilizing BAX/Bcl-2 ratio," *Acta Ophthalmologica*, vol. 88, no. 3, pp. e78–e86, 2010.
- [127] Z. Huang, J. Jiang, V. A. Tyurin et al., "Cardiolipin deficiency leads to decreased cardiolipin peroxidation and increased resistance of cells to apoptosis," *Free Radical Biology and Medicine*, vol. 44, no. 11, pp. 1935–1944, 2008.
- [128] L. Huang, M. C. Yappert, M. M. Jumblatt, and D. Borchman, "Hyperoxia and thyroxine treatment and the relationships between reactive oxygen species generation, mitochondrial membrane potential, and cardiolipin in human lens epithelial cell cultures," *Current Eye Research*, vol. 33, no. 7, pp. 575–586, 2008.
- [129] V. Bantsev, D. McCanna, A. Banh et al., "Mechanisms of ocular toxicity using the in vitro bovine lens and sodium dodecyl sulfate as a chemical model," *Toxicological Sciences*, vol. 73, no. 1, pp. 98–107, 2003.
- [130] J. H. Santos, L. Hunakova, Y. Chen, C. Bortner, and B. Van Houten, "Cell sorting experiments link persistent mitochondrial DNA damage with loss of mitochondrial membrane potential and apoptotic cell death," *The Journal of Biological Chemistry*, vol. 278, no. 3, pp. 1728–1734, 2003.
- [131] B. S. Mandavilli, J. H. Santos, and B. Van Houten, "Mitochondrial DNA repair and aging," *Mutation Research*, vol. 509, no. 1–2, pp. 127–151, 2002.
- [132] N. C. Taarnhoj, A. C. Shenl, D. F. Gebhard, B. D. Brees, and C. J. Soms, "Investigating markers of DNA oxidation, lipid peroxidation and stress response in human lens epithelial cell lines," *Investigative Ophthalmology & Visual Science*, vol. 46, pp. 3850–3854, 2005.
- [133] K. Sorte, P. Sune, A. Bhake, V. B. Shivkumar, N. Gangane, and A. Basak, "Quantitative assessment of DNA damage directly in lens epithelial cells from senile cataract patients," *Molecular Vision*, vol. 17, pp. 1–6, 2011.
- [134] K. Yao, P. P. Ye, L. Zhang, J. Tan, X. J. Tang, and Y. D. T. Zhang, "Epigallocatechin gallate protects against oxidative stress-induced mitochondria-dependent apoptosis in human lens epithelial cells," *Molecular Vision*, vol. 14, pp. 217–223, 2008.
- [135] W. C. Li, J. R. Kuszak, K. Dunn et al., "Lens epithelial cell apoptosis appears to be a common cellular basis for non-congenital cataract development in humans and animals," *The Journal of Cell Biology*, vol. 130, no. 1, pp. 169–181, 1995.
- [136] J. G. Jose and K. L. Yielding, "'Unscheduled' DNA synthesis in lens epithelium following ultraviolet irradiation," *Experimental Eye Research*, vol. 24, no. 2, pp. 113–119, 1977.
- [137] E. R. Stadtman, "Protein oxidation and aging," *Free Radical Research*, vol. 40, no. 12, pp. 1250–1258, 2006.
- [138] M. A. Babizhayev and A. I. Deyew, "Lens opacity induced by lipid peroxidation products as a model of cataract associated with retinal disease," *Biochimica et Biophysica Acta*, vol. 1004, no. 1, pp. 124–133, 1989.
- [139] J. W. Park and R. A. Floyd, "Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA," *Free Radical Biology and Medicine*, vol. 12, no. 4, pp. 245–250, 1992.
- [140] A. H. Shin, C. J. Oh, and J.-W. Park, "Glycation-induced inactivation of antioxidant enzymes and modulation of cellular redox status in lens cells," *Archives of Pharmacal Research*, vol. 29, no. 7, pp. 577–581, 2006.
- [141] C. J. Nolan, P. Damm, and M. Prentki, "Type 2 diabetes across generations: from pathophysiology to prevention and management," *The Lancet*, vol. 378, no. 9786, pp. 169–181, 2011.
- [142] C. P. Wilkinson, F. L. Ferris III, R. E. Klein et al., "Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales," *Ophthalmology*, vol. 110, no. 9, pp. 1677–1682, 2003.
- [143] E. L. Fletcher, J. A. Phipps, M. M. Ward, T. Puthussery, and J. L. Wilkinson-Berka, "Neuronal and glial cell abnormality as predictors of progression of diabetic retinopathy," *Current Pharmaceutical Design*, vol. 13, no. 26, pp. 2699–2712, 2007.
- [144] R. N. Frank, "Diabetic retinopathy," *The New England Journal of Medicine*, vol. 350, no. 1, pp. 48–58, 2004.
- [145] Y. Wu, L. Tang, and B. Chen, "Oxidative stress: implications for the development of diabetic retinopathy and antioxidant therapeutic perspectives," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 752387, 12 pages, 2014.
- [146] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.
- [147] X. Du, T. Matsumura, D. Edelstein et al., "Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells," *The Journal of Clinical Investigation*, vol. 112, no. 7, pp. 1049–1057, 2003.

- [148] M. A. Ihnat, J. E. Thorpe, C. D. Kamat et al., "Reactive oxygen species mediate a cellular "memory" of high glucose stress signalling," *Diabetologia*, vol. 50, no. 7, pp. 1523–1531, 2007.
- [149] N. C. Chillelli, S. Burlina, and A. Lapolla, "AGEs, rather than hyperglycemia, are responsible for microvascular complications in diabetes: a 'glycooxidation-centric' point of view," *Nutrition, Metabolism & Cardiovascular Diseases*, vol. 23, no. 10, pp. 913–919, 2013.
- [150] D. A. Antonetti, A. J. Barber, S. K. Bronson et al., "Diabetic retinopathy: seeing beyond glucose-induced microvascular disease," *Diabetes*, vol. 55, no. 9, pp. 2401–2411, 2006.
- [151] B. Duran-Jimenez, D. Dobler, S. Moffatt et al., "Advanced glycation end products in extracellular matrix proteins contribute to the failure of sensory nerve regeneration in diabetes," *Diabetes*, vol. 58, no. 12, pp. 2893–2903, 2009.
- [152] F. Piarulli, G. Sartore, A. Ceriello et al., "Relationship between glyco-oxidation, antioxidant status and microalbuminuria in type 2 diabetic patients," *Diabetologia*, vol. 52, no. 7, pp. 1419–1425, 2009.
- [153] J. M. Santos, S. Tewari, and R. A. Kowluru, "A compensatory mechanism protects retinal mitochondria from initial insult in diabetic retinopathy," *Free Radical Biology and Medicine*, vol. 53, no. 9, pp. 1729–1737, 2012.
- [154] Q. Zhong and R. A. Kowluru, "Diabetic retinopathy and damage to mitochondrial structure and transport machinery," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 12, pp. 8739–8746, 2011.
- [155] S. G. Jarrett, H. Lin, B. F. Godley, and M. E. Boulton, "Mitochondrial DNA damage and its potential role in retinal degeneration," *Progress in Retinal and Eye Research*, vol. 27, no. 6, pp. 596–607, 2008.
- [156] S. Tewari, J. M. Santos, and R. A. Kowluru, "Damaged mitochondrial DNA replication system and the development of diabetic retinopathy," *Antioxidants and Redox Signaling*, vol. 17, no. 3, pp. 492–504, 2012.
- [157] J. M. Santos, S. Tewari, A. F. X. Goldberg, and R. A. Kowluru, "Mitochondrial biogenesis and the development of diabetic retinopathy," *Free Radical Biology and Medicine*, vol. 51, no. 10, pp. 1849–1860, 2011.
- [158] L. Zhong, A. D'Urso, D. Toiber et al., "The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1 α ," *Cell*, vol. 140, no. 2, pp. 280–293, 2010.
- [159] M. Lappas, "Anti-inflammatory properties of sirtuin 6 in human umbilical vein endothelial cells," *Mediators of Inflammation*, vol. 2012, Article ID 597514, 11 pages, 2012.
- [160] D. B. Lombard, B. Schwer, F. W. Alt, and R. Mostoslavsky, "SIRT6 in DNA repair, metabolism and ageing," *Journal of Internal Medicine*, vol. 263, no. 2, pp. 128–141, 2008.
- [161] R. Liu, H. Liu, Y. Ha, R. G. Tilton, and W. Zhang, "Oxidative stress induces endothelial cell senescence via downregulation of Sirt6," *BioMed Research International*, vol. 2014, Article ID 902842, 13 pages, 2014.
- [162] Y. Ozawa, T. Kurihara, M. Sasaki et al., "Neural degeneration in the retina of the streptozotocin-induced type 1 diabetes model," *Experimental Diabetes Research*, vol. 2011, Article ID 108328, 7 pages, 2011.
- [163] M. Y. Sasaki, Y. Ozawa, T. Kurihara et al., "Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes," *Diabetologia*, vol. 53, no. 5, pp. 971–979, 2010.
- [164] P. Bhosale, B. Li, M. Sharifzadeh et al., "Purification and partial characterization of a lutein-binding protein from human retina," *Biochemistry*, vol. 48, no. 22, pp. 4798–4807, 2009.
- [165] A. W. Stitt, "AGEs and diabetic retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 10, pp. 4867–4874, 2010.
- [166] S. Genuth, W. Sun, P. Cleary et al., "Glycation and carboxymethyllysine levels in skin collagen predict the risk of future 10-year progression of diabetic retinopathy and nephropathy in the Diabetes Control and Complications Trial and Epidemiology of Diabetes Interventions and Complications participants with type 1 diabetes," *Diabetes*, vol. 54, no. 11, pp. 3103–3111, 2005.
- [167] J. Tang and T. S. Kern, "Inflammation in diabetic retinopathy," *Progress in Retinal and Eye Research*, vol. 30, no. 5, pp. 343–358, 2011.
- [168] L. Zheng and T. Kern, "Role of nitric oxide, superoxide, peroxynitrite and poly (ADPribose) polymerase in diabetic retinopathy," *Frontiers in Bioscience*, vol. 14, pp. 3974–3987, 2006.
- [169] R. A. Kowluru and P.-S. Chan, "Oxidative stress and diabetic retinopathy," *Experimental Diabetes Research*, vol. 2007, Article ID 43603, 12 pages, 2007.
- [170] J. L. Wilkinson-Berka, I. Rana, R. Armani, and A. Agrotis, "Reactive oxygen species, Nox and angiotensin II in angiogenesis: implications for retinopathy," *Clinical Science*, vol. 124, no. 10, pp. 597–615, 2013.
- [171] O. Arjamaa and M. Nikinmaa, "Oxygen-dependent diseases in the retina: role of hypoxia-inducible factors," *Experimental Eye Research*, vol. 83, no. 3, pp. 473–483, 2006.
- [172] T. Kietzmann and A. Görlach, "Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression," *Seminars in Cell and Developmental Biology*, vol. 16, no. 4-5, pp. 474–486, 2005.
- [173] A. Görlach and S. Bonello, "The cross-talk between NF- κ B and HIF-1: further evidence for a significant liaison," *The Biochemical Journal*, vol. 412, no. 3, pp. e17–e19, 2008.
- [174] A. Augustin, A. Loewenstein, and B. D. Kuppermann, "General pathophysiology," *Developments in Ophthalmology*, vol. 47, pp. 10–26, 2010.
- [175] A. Samanta, P. Kumar, S. Machhua, G. N. Rao, and A. Pal, "Incidence of cystoid macular oedema in diabetic patients after phacoemulsification and free radical link to its pathogenesis," *British Journal of Ophthalmology*, vol. 98, no. 9, pp. 1266–1272, 2014.
- [176] A. Gordois, H. Cutler, L. Pezzullo et al., "An estimation of the worldwide economic and health burden of visual impairment," *Global Public Health*, vol. 7, no. 5, pp. 465–481, 2012.
- [177] I. Bhutto and G. Luttj, "Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex," *Molecular Aspects of Medicine*, vol. 33, no. 4, pp. 295–317, 2012.
- [178] P. T. V. M. De Jong, "Age-related macular degeneration," *The New England Journal of Medicine*, vol. 355, no. 14, pp. 1474–1485, 2006.
- [179] K. A. Kaarniranta, A. Salminen, A. Haapasalo, H. Soininen, and M. Hiltunen, "Age-related macular degeneration (AMD): Alzheimer's disease in the eye?" *Journal of Alzheimer's Disease*, vol. 24, no. 4, pp. 615–631, 2011.
- [180] J. Blasiak, A. Salminen, and K. Kaarniranta, "Potential of epigenetic mechanisms in AMD pathology," *Frontiers in Bioscience*, vol. 5, no. 2, pp. 412–425, 2013.

- [181] R. D. Jager, W. F. Mieler, and J. W. Miller, "Age-related macular degeneration," *The New England Journal of Medicine*, vol. 358, no. 24, pp. 2606–2617, 2008.
- [182] K. Kaarniranta, A. Salminen, E.-L. Eskelinen, and J. Kopitz, "Heat shock proteins as gatekeepers of proteolytic pathways—implications for age-related macular degeneration (AMD)," *Ageing Research Reviews*, vol. 8, no. 2, pp. 128–139, 2009.
- [183] L. Lu, S. F. Hackett, A. Mincey, H. Lai, and P. A. Campochiaro, "Effects of different types of oxidative stress in RPE cells," *Journal of Cellular Physiology*, vol. 206, no. 1, pp. 119–125, 2006.
- [184] M. A. Zarbin, "Current concepts in the pathogenesis of age-related macular degeneration," *Archives of Ophthalmology*, vol. 122, no. 4, pp. 598–614, 2004.
- [185] P. Tokarz, K. Kaarniranta, and J. Blasiak, "Role of antioxidant enzymes and small molecular weight antioxidants in the pathogenesis of age-related macular degeneration (AMD)," *Biogerontology*, vol. 14, no. 5, pp. 461–482, 2013.
- [186] D. Y. Yu and S. J. Cringle, "Oxygen distribution and consumption within the retina in vascularised and avascular retinas and in animal models of retinal disease," *Progress in Retinal and Eye Research*, vol. 20, no. 2, pp. 175–208, 2001.
- [187] A. J. Whitehead, J. A. Mares, and R. P. Danis, "Macular pigment: a review of current knowledge," *Archives of Ophthalmology*, vol. 124, no. 7, pp. 1038–1045, 2006.
- [188] J. J. Hunter, J. I. W. Morgan, W. H. Merigan, D. H. Sliney, J. R. Sparrow, and D. R. Williams, "The susceptibility of the retina to photochemical damage from visible light," *Progress in Retinal and Eye Research*, vol. 31, no. 1, pp. 28–42, 2012.
- [189] J. R. Sparrow, J. Zhou, S. Ben-Shabat, H. Vollmer, Y. Itagaki, and K. Nakanishi, "Involvement of oxidative mechanisms in blue-light-induced damage to A2E-laden RPE," *Investigative Ophthalmology & Visual Science*, vol. 43, no. 4, pp. 1222–1227, 2002.
- [190] J. R. Sparrow, J. Zhou, and B. Cai, "DNA is a target of the photodynamic effects elicited in A2E-laden RPE by blue-light illumination," *Investigative Ophthalmology and Visual Science*, vol. 44, no. 5, pp. 2245–2251, 2003.
- [191] W. T. Ham Jr., J. J. Ruffolo Jr., H. A. Mueller, A. M. Clarke, and M. E. Moon, "Histologic analysis of photochemical lesions produced in rhesus retina by short-wave-length light," *Investigative Ophthalmology and Visual Science*, vol. 17, no. 10, pp. 1029–1035, 1978.
- [192] M. A. De la Paz and R. E. Anderson, "Lipid peroxidation in rod outer segments: role of hydroxyl radical and lipid hydroperoxides," *Investigative Ophthalmology and Visual Science*, vol. 33, no. 7, pp. 2091–2096, 1992.
- [193] A. U. Arstila, M. A. Smith, and B. F. Trump, "Microsomal lipid peroxidation: morphological characterization," *Science*, vol. 175, no. 4021, pp. 530–533, 1972.
- [194] J. G. Hollyfield, V. L. Bonilha, M. E. Rayborn et al., "Oxidative damage-induced inflammation initiates age-related macular degeneration," *Nature Medicine*, vol. 14, no. 2, pp. 194–198, 2008.
- [195] M. A. De la Paz and R. E. Anderson, "Region and age-dependent variation in susceptibility of the human retina to lipid peroxidation," *Investigative Ophthalmology and Visual Science*, vol. 33, no. 13, pp. 3497–3499, 1992.
- [196] J. R. Sparrow and M. Boulton, "RPE lipofuscin and its role in retinal pathobiology," *Experimental Eye Research*, vol. 80, no. 5, pp. 595–606, 2005.
- [197] Y. Wu, E. Yanase, X. Feng, M. M. Siegel, and J. R. Sparrow, "Structural characterization of bisretinoid A2E photocleavage products and implications for age-related macular degeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 16, pp. 7275–7280, 2010.
- [198] J. R. Sparrow, K. Nakanishi, and C. A. Parish, "The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 7, pp. 1981–1989, 2000.
- [199] J. R. Sparrow, H. R. Vollmer-Snarr, J. Zhou et al., "A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation," *The Journal of Biological Chemistry*, vol. 278, no. 20, pp. 18207–18213, 2003.
- [200] Y. He, J. Ge, J. M. Burke, R. L. Myers, Z. Z. Dong, and J. Tombran-Tink, "Mitochondria impairment correlates with increased sensitivity of aging RPE cells to oxidative stress," *Journal of Ocular Biology, Diseases, and Informatics*, vol. 3, no. 3, pp. 92–108, 2011.
- [201] V. Justilien, J.-J. Pang, K. Renganathan et al., "SOD2 knockdown mouse model of early AMD," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 10, pp. 4407–4420, 2007.
- [202] P. P. Karunadharm, C. L. Nordgaard, T. W. Olsen, and D. A. Ferrington, "Mitochondrial DNA damage as a potential mechanism for age-related macular degeneration," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 11, pp. 5470–5479, 2010.
- [203] C. L. Nordgaard, P. P. Karunadharm, X. Feng, T. W. Olsen, and D. A. Ferrington, "Mitochondrial proteomics of the retinal pigment epithelium at progressive stages of age-related macular degeneration," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 7, pp. 2848–2855, 2008.
- [204] M. Nita, A. Grzybowski, F. J. Ascaso, and V. Huerva, "Age-related macular degeneration in the aspect of chronic low-grade inflammation (pathophysiological parainflammation)," *Mediators of Inflammation*, vol. 2014, Article ID 930671, 10 pages, 2014.
- [205] O. Arjamaa, M. Nikinmaa, A. Salminen, and K. Kaarniranta, "Regulatory role of HIF-1 α in the pathogenesis of age-related macular degeneration (AMD)," *Ageing Research Reviews*, vol. 8, no. 4, pp. 349–358, 2009.
- [206] E. Naik and V. M. Dixit, "Mitochondrial reactive oxygen species drive proinflammatory cytokine production," *Journal of Experimental Medicine*, vol. 208, no. 3, pp. 417–420, 2011.
- [207] H. Wang, E. S. Wittchen, and M. E. Hartnett, "Breaking barriers: insight into the pathogenesis of neovascular age-related macular degeneration," *Eye and Brain*, vol. 2011, no. 3, pp. 19–28, 2011.
- [208] T. I. Metelitsina, J. E. Grunwald, J. C. DuPont, G.-S. Ying, A. J. Brucker, and J. L. Dunaief, "Foveolar choroidal circulation and choroidal neovascularization in age-related macular degeneration," *Investigative Ophthalmology and Visual Science*, vol. 49, no. 1, pp. 358–363, 2008.
- [209] J. E. Grunwald, T. I. Metelitsina, J. C. DuPont, G.-S. Ying, and M. G. Maguire, "Reduced foveolar choroidal blood flow in eyes with increasing AMD severity," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 3, pp. 1033–1038, 2005.
- [210] T. Bek, "Inner retinal ischaemia: current understanding and needs for further investigations," *Acta Ophthalmologica*, vol. 87, no. 4, pp. 362–367, 2009.
- [211] J. W. Kim, I. Tchernyshyov, G. L. Semenza, and C. V. Dang, "HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia," *Cell Metabolism*, vol. 3, no. 3, pp. 177–185, 2006.
- [212] G. Bellot, R. Garcia-Medina, P. Gounon et al., "Hypoxia-induced autophagy is mediated through hypoxia-inducible

- factor induction of BNIP3 and BNIP3L via their BH3 domains,” *Molecular and Cellular Biology*, vol. 29, no. 10, pp. 2570–2581, 2009.
- [213] H. Zhang, M. Bosch-Marce, L. A. Shimoda et al., “Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia,” *Journal of Biological Chemistry*, vol. 283, no. 16, pp. 10892–10903, 2008.
- [214] J. Blasiak, G. Petrovski, Z. Veréb, A. Facskó, and K. Kaarniranta, “Oxidative stress, hypoxia, and autophagy in the neovascular processes of age-related macular degeneration,” *BioMed Research International*, vol. 2014, Article ID 768026, 7 pages, 2014.
- [215] N. Rodríguez-Muela, H. Koga, L. García-Ledo et al., “Balance between autophagic pathways preserves retinal homeostasis,” *Aging Cell*, vol. 12, no. 3, pp. 478–488, 2013.
- [216] J. Feng, X. Chen, X. Sun, F. Wang, and X. Sun, “Expression of endoplasmic reticulum stress markers GRP78 and CHOP induced by oxidative stress in blue light-mediated damage of A2E-containing retinal pigment epithelium cells,” *Ophthalmic Research*, vol. 52, pp. 224–233, 2014.
- [217] S. He, J. Yaung, Y. H. Kim, E. Barron, S. J. Ryan, and D. R. Hinton, “Endoplasmic reticulum stress induced by oxidative stress in retinal pigment epithelial cells,” *Graefe’s Archive for Clinical and Experimental Ophthalmology*, vol. 246, no. 5, pp. 677–683, 2008.
- [218] A. Otani, H. Takagi, H. Oh, S. Koyama, M. Matsumura, and Y. Honda, “Expressions of angiopoietins and Tie2 in human choroidal neovascular membranes,” *Investigative Ophthalmology and Visual Science*, vol. 40, no. 9, pp. 1912–1920, 1999.
- [219] K. Uno, I. A. Bhutto, D. S. McLeod, C. Merges, and G. A. Luty, “Impaired expression of thrombospondin-1 in eyes with age related macular degeneration,” *British Journal of Ophthalmology*, vol. 90, no. 1, pp. 48–54, 2006.

Research Article

Evaluation of Lasting Effects of Heat Stress on Sperm Profile and Oxidative Status of Ram Semen and Epididymal Sperm

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Higher temperatures lead to an increase of testicular metabolism that results in spermatid damage. Oxidative stress is the main factor responsible for testicular damage caused by heat stress. The aim of this study was to evaluate lasting effects of heat stress on ejaculated sperm and immediate or long-term effects of heat stress on epididymal sperm. We observed decrease in motility and mass motility of ejaculated sperm, as well as an increase in the percentages of sperm showing major and minor defects, damaged plasma and acrosome membranes, and a decrease in the percentage of sperm with high mitochondrial membrane potential in the treated group until one spermatid cycle. An increased enzymatic activity of glutathione peroxidase and an increase of stressed cells were observed in ejaculated sperm of the treated group. A decrease in the percentage of epididymal sperm with high mitochondrial membrane potential was observed in the treated group. However, when comparing immediate and long-term effects, we observed an increase in the percentage of sperm with low mitochondrial membrane potential. In conclusion, testicular heat stress induced oxidative stress that led to rescuable alterations after one spermatid cycle in ejaculated sperm and also after 30 days in epididymal sperm.

1. Introduction

The testicle is the organ of the male reproductive tract responsible for spermatogenesis. In mammals, the testis temperature must range from 2 to 8°C below body temperature to ensure successful spermatogenesis [1]. The lower temperature is maintained by a cooling system comprising the scrotum, pampiniform plexus, and muscles [1]. Higher temperatures would lead to an increase of testicular metabolism without a corresponding increase in blood supply, resulting in local hypoxia and deleterious effects for the tissue [2, 3]. Moreover, similar to organ transplantation procedures, a phenomenon known as hypoxia-reperfusion injury may occur [3, 4]. In this condition, the oxidative imbalance may occur after the reestablishment of the normal temperature and tissue reperfusion. This situation has been described in studies

where suppression of testicular function under heat stress led to a decrease in fertility in ruminants [5, 6], murine [7], and human affected by varicocele [8]. These studies concluded that oxidative stress is the main factor responsible for damage caused by heat stress.

Oxidative stress is defined as the damage caused to biomolecules by the imbalance between prooxidative molecules overlapping antioxidative molecules [9]. The increase in reactive oxygen species (ROS) or decrease in antioxidant levels could happen after heat stress; however, the exact mechanism is still unknown. The use of experimental models is important due to obvious impossibility of human experimentation. In this context, the ram would be an interesting model based on the ease of maintenance and genetic proximity with human [10].

Spermatozoa are very sensitive to oxidative damage due to the high levels of polyunsaturated fatty acids (PUFAs) in the plasma membrane [11]. In addition, the reduced cytoplasm limits the intracellular antioxidant levels [11]. Structure and function of the sperm membrane are affected by oxidative stress and this compromises fertilization [12]. For instance, loss of membrane fluidity causes a decrease in sperm motility and impairs sperm-oocyte fusion [13, 14]. ROS not only affect the sperm membrane, as high levels in seminal plasma were negatively correlated with sperm motility and positively correlated with the incidence of sperm DNA fragmentation in infertile men [15]. Ram spermatozoa produce high levels of hydrogen peroxide, especially due to high amounts of polyunsaturated/saturated fatty acids and low proportions of cholesterol/phospholipids in the plasma membrane when compared with other species [16]. These ratios are responsible for an increased susceptibility to oxidative damage in the presence of ROS, and subsequent loss of membrane and acrosome integrity [16].

A variety of enzymatic and nonenzymatic antioxidants present in the plasma allows neutralization of ROS. Indeed, studies on lipid peroxidation and antioxidant enzymes in fertile and infertile men have shown an increase in superoxide dismutase (SOD) activity [17]. The epididymis is an important source for the antioxidant content in the seminal plasma, protecting sperm cell from oxidative damage during storage [18]. In this context, the study of the antioxidant activity in the epididymal environment could bring information about protection mechanisms after oxidative stress induced by heat stress. The relationship between spermatoc attributes and the antioxidant activity present in the seminal plasma or in the sperm cell under oxidative stress conditions is poorly described in ovine.

The antioxidant response to a stressful event may involve an immediate response in cases of acute situations, accomplished mainly by protein activation. On the other hand, a long-term response is also important, which would require gene activation and translation of new proteins [19, 20]. Little is known about the contribution of the epididymis to the maintenance of oxidative balance in immediate or long-term response to stressful conditions. The dynamics of antioxidant responses are critical during spermatogenesis once it is a cyclical and continuous event. Therefore, when considering the testicular and epididymal environments, the longitudinal effect of this oxidative balance must be performed considering the sperm cycle.

In this context, the aim of this study was to evaluate how heat stress, induced by ram testicular insulation, affects sperm profile and the enzymatic antioxidant activity in ejaculated sperm during consecutive weeks or in epididymal sperm immediately after insulation or in a long-term response.

2. Material and Methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemicals (St. Louis, MO). All experiments were performed using fresh ram semen collected using an artificial vagina. Semen collections were performed weekly, during nine weeks, from twelve mature (8 months old) Santa

Ines rams. Animals belonged to the Department of Animal Reproduction of the School of Veterinary Medicine and Animal Science from the University of Sao Paulo. The animals were submitted to uniform nutritional conditions, and the experiments were approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Sciences, University of Sao Paulo (protocol number 2445-2011).

2.1. Reagents and Solutions. All chemical reagents and solutions used in this study were purchased from Sigma-Aldrich (St. Louis, MO, EUA) unless otherwise stated.

2.2. Experiment 1: Lasting Effects of Heat Stress on Sperm Profile and Oxidative Status on Ejaculated Sperm. The animals were randomly divided into two groups: animals undergoing testicular insulation (treated, $n = 6$) and control group ($n = 6$). An insulating bag was placed in the testicles of animals from the treated group to induce heat stress effects on spermatogenesis. The bags were kept for 288 consecutive hours, and during this period the internal temperature of each bag and environmental temperature were monitored using a digital thermometer. After the removal of the bags, semen was collected weekly, during 9 weeks.

2.2.1. Immediate and Morphological Sperm Evaluations. The following evaluations were immediately performed: seminal volume (mL), motility (%), and mass motility (0–5). Sperm concentration count was performed using a hemocytometer. Sperm morphological abnormalities were assessed in a phase contrast microscope at 1000x magnification under oil using 10 μ L of fresh semen fixed in 1 mL of buffered formalin (Phosphate-Buffered Saline, PBS, Gibco, Life Technologies, Carlsbad, USA, with 2% of formalin 37%). Sperm abnormalities were quantified and classified into major and minor defects, and the sum of defects was considered as total defects [21].

A total of 200 cells per sample were evaluated.

2.2.2. Flow Cytometry (Plasma and Acrosome Membranes Integrity, Mitochondrial Membrane Potential, and Oxidative Status). Plasma membrane and acrosome integrities were evaluated by propidium iodide (PI) and fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA), respectively. The association of these fluorescent probes divides sperm populations into four groups: intact membrane and intact acrosome (IMIA), intact membrane and damaged acrosome (IMDA), damaged membrane and intact acrosome (DMIA), and damaged membrane and damaged acrosome (DMDA). The procedure was performed with 200,000 cells diluted in SP-Talp [Modified Tyrode's Albumin Lactate Pyruvate (NaCl 0.1 M, KCl 0.003 M, MgCl₂ 0.0004 M, NaH₂PO₄ 0.0003 M, NaHCO₃ 0.025 M, CaCl₂·H₂O 0.003 M, Ácido Láctico Syr 0.3% v/v, Hepes 0.01 M, pH 7.4, and Osm 295–300)] and stained for 5 minutes with 0.5 mg/mL PI and 100 μ g/mL FITC-PSA. Samples were analyzed by flow cytometry, using a 488 nm excitation laser and emission was detected at 630–650 nm (PI) and 515–530 nm (FITC). Mitochondrial membrane potential was evaluated

using JC-1 probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride, Invitrogen, Eugene, OR, USA). This probe emits green fluorescent light from cells with low (LMM) and medium (MMM) mitochondrial potential or red-orange fluorescent from cells with high mitochondrial potential (HMP). The procedure was performed with 200,000 cells diluted in SP-Talp and stained with 76.5 μM JC-1 for 5 minutes. Samples were analyzed by flow cytometry, excited at 488 nm, and detected at 590 nm. Sperm oxidative stress was assessed using fluorescent probe dichlorofluorescein (2',7'-dichlorofluorescein diacetate, DCF). DCF emits fluorescence when in contact with free radicals [14, 22, 23]. Evaluations were performed by flow cytometry. In brief, approximately 4,000 spermatozoa were resuspended in 1 μL of TALP medium and incubated with 3.5 μL of 1 mM fluorescent probe DCF for 5 minutes. Propidium iodide (PI; 0.5 mg/mL, 0.5 μL) was added simultaneously to identify and exclude cells with damaged membrane, as this condition does not allow proper function of DCF stain. Flow cytometer analysis was performed as described above using the 525 nm detector (green fluorescence). The same protocol was performed in Experiment 2.

Flow cytometry analysis of sperm samples was performed using Guava EasyCyte Mini System (Guava Technologies, Hayward, CA, USA). A total of 10,000 events per sample were analyzed and data corresponding to yellow (PM1 photodetector—583 nm), red (PM2 photodetector—680 nm), and green (PM3 photodetector—525 nm) fluorescent signals were recorded after a logarithmic amplification. For analysis, cell doublets and debris were excluded using PM3/FSC (forward scatter). All data was analyzed by FlowJo version 8.7 software.

2.2.3. Seminal Plasma Enzymatic Activity. Seminal plasma was obtained by centrifuging 500 μL of fresh semen at 5°C for 10 minutes at 660 g. The supernatant was recovered and stored at -20°C for further analysis. Measurements were performed based on the rate of substrate consumption in reactions catalyzed by each antioxidant enzyme in a given time interval using a spectrophotometer (Evolution 300 UV-Vis, Thermo Scientific, Waltham, MA, USA). Activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GRD) were determined as described previously [24]. In addition, catalase activity was determined by evaluating the consumption of hydrogen peroxide for 3 minutes at 242 nm, and the $18.6 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ molar extinction coefficient was used.

2.2.4. Quantification of Antioxidant Enzymes. We used SDS-polyacrylamide gel electrophoresis and western blotting to quantify the levels of antioxidant enzymes in the seminal plasma. Total protein concentration (Protein Assay, Bio-Rad, Hercules, CA, USA) in seminal plasma was determined by the Bradford method [25]. Then, 20 mg of protein was mixed with 5 μL of loading buffer (0.045 M Tris/HCl, 0.8 mM EDTA, 3% SDS 10% glycerol, 5% β -mercaptoethanol, and 0.004% bromophenol blue) and loaded into wells. Proteins were separated by dimension on 12% polyacrylamide gel (v/v) by standard SDS-PAGE using a Mini Protean III System

(Bio-Rad, Hercules, CA, USA). A mixture of prestained protein standards was used as marker, with molecular weights ranging from 10 to 250 kDa (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed for 90 minutes at 130 V at 4°C. Subsequently, proteins were blotted onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo for 10 minutes at 2.5 A, 25 V (Bio-Rad, Hercules, CA, USA). After air-drying the membrane, blocking of nonspecific sites was performed with 5% BSA in PBS for 2 hours. Membranes were incubated overnight at 4°C with primary antibodies; anticatalase (SC 50508, H-300, Santa Cruz Biotechnology, Dallas, Texas, USA), anti-SOD (SOD-3, SC 67088, H-90, Santa Cruz Biotechnology), anti-GPx (GPx-5, SC 50498, H-45, Santa Cruz Biotechnology), and anti-GRD (antigluthathione reductase antibody ab84963, Abcam, Cambridge, FL, UK) diluted in 1/1000 PBS-Tween with 1% BSA. After 3 washes every 5 minutes, membranes were incubated with FITC conjugated secondary donkey anti-rabbit antibody (Li-COR Biotechnology, Bad Homburg, Germany, 1/15000 in PBS-Tween with 1% BSA) for 75 minutes at room temperature and protected from light. Quantifications of signal intensities and areas of bands were performed by scanning the membranes using Odyssey CLX (Li-Cor Biotechnology, Bad Homburg, Germany). Results were expressed considering the relationship between signal (pixel) and band area.

2.2.5. Measurement of Seminal Plasma (Spontaneous) and Sperm (Induced) Thiobarbituric Acid Reactive Substances (TBARS). This technique is based on methodology previously described by Ohkawa et al. [26], in which two thiobarbituric acid molecules reacted with one molecule of malondialdehyde, producing a pink color complex, which was measured spectrophotometrically at 532 nm. This reaction occurs between 90 and 100°C at an acidic pH. For the determination of spontaneous TBARS in seminal plasma, aliquots of 300 μL of fresh semen associated with 600 μL of 10% trichloroacetic acid solution were centrifuged at 5°C, 16,000 g, for 10 minutes to precipitate the proteins. After precipitation of proteins, approximately 700 μL of the supernatant was frozen at -20°C for further analysis. For quantification of induced TBARS, about 1 million sperm diluted in 200 μL of PBS were incubated with 4 mM of ferrous sulfate (50 μL) and 20 mM ascorbate (50 μL) at 37°C for 1.5 hours, as described by Simões et al. [27]. Immediately after ROS induction, 600 μL of 10% (v/v) trichloroacetic was added to the mixture (2:1) in order to precipitate proteins and cellular debris. Samples were centrifuged (16,000 g, for 10 minutes) and the supernatant was recovered (500 μL) and stored (-20°C). TBARS seminal and sperm samples were thawed and incubated with 500 μL of a 1% thiobarbituric acid solution (in NaOH 0.05 M) for 10 minutes at 90–100°C. Reaction was stopped by placing samples on ice. Levels of TBARS were assessed using a spectrophotometer at 532 nm. Results were compared to a standard curve previously prepared with malondialdehyde. Malondialdehyde is the major substance that reacts with thiobarbituric acid, and the TBARS concentration was determined using the value of $1.56 \times 10^5 \text{ M}^{-1} \times \text{mL}^{-1}$ as the malondialdehyde molar extinction

coefficient. Lipid peroxidation in semen is expressed in TBARS/mL nanograms of seminal plasma (spontaneous) or for each 10^6 sperm (induced). The same protocol for induced TBARS was performed in Experiment 2.

2.3. Experiment 2: Study of Immediate and Long-Term Effects of Heat Stress on Epididymal Sperm. The same animals distributed in the same experimental groups were subjected to a second period of testicular insulation, which lasted 10 days (240 hours). This was performed 60 days after the end of the first experiment when the sperm profile returned to results similar to those observed in the beginning of the first experiment. The early and the late effect of heat stress on epididymal sperm were evaluated in this study. Thus, each animal was subjected to two unilateral orchiectomies: the first one 24 hours after the removal of insulation bag (D0) and the second one in 30 days after the first orchiectomy (D30).

2.3.1. Epididymis Sperm Collection. After surgery, epididymides were immediately taken to the laboratory and washed in saline solution at 37°C . To collect epididymis semen samples, small incisions were performed with a scalpel blade in the epididymis tail, and pressure was applied on its base using hemostats and sperm collected with the aid of automatic pipette [28]. Sperm were resuspended in PBS for sperm concentration assessment.

2.3.2. Computer Assisted Sperm Analysis (CASA) System. Motility parameters of epididymis semen were performed by Computer Assisted Sperm Analysis System (CASA, Hamilton Thorne IVOS). The chambers (Standard Count 4-chamber slide, 20 microns, Leja), heated at 37°C , were filled with $6\ \mu\text{L}$ of sample (approximately 5×10^7 cells/mL), and 5 fields were selected for the analysis, in which approximately 1×10^6 sperm cells were analyzed. The setup used was as follows: image capture: frames per sec = 60 Hz, and number of frames = 45; cell detection: minimum contrast = 70, minimum cell size = 5 pix; defaults: cell size = 10 pix, cell intensity = 80; progressive cells: path velocity (VAP) = $50\ \mu\text{s}$, straightness (STR) = 80%; slow cells: VAP cutoff = $20\ \mu\text{s}$, VSL cutoff = $5\ \mu\text{s}$; static intensity gates = minimum 0.20 and maximum 1.92; static intensity gates = minimum 0.60 and maximum 4.32; and static elongation gates = minimum 7 and maximum 91. The following parameters were evaluated: mean average velocity (VAP, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), linearity coefficient (LIN, %), straightness coefficient (STR, %), amplitude of lateral head displacement (ALH, μm), beating cross frequency (BCF, Hz), total (%) and progressive (%) motility, and percentage of cells with fast, medium, slow, and static movement.

2.3.3. Epididymal Sperm Enzymatic Activity. After extraction, epididymal sperm were diluted in BotuBov (Botupharma Animal Biotechnology, Botucatu-SP, Brazil) and frozen at -196°C . Standard freezing curve was performed (37°C to 5°C in 2 hours with a decrease of -0.25°C/min ; 3 hours and 45 minutes in balance time, -20°C with a decrease of

-5°C/min , -196°C with a decrease of 125°C/min). Straws were thawed at 37°C (90 seconds) and removal of the diluent was performed by addition of 1 mL of semen diluted in SP-Talp ($750\ \mu\text{L}$ of semen in 3 mL of SP-Talp) carefully on 7.5 mL of sucrose solution (0.9% NaCl, 7.5% sucrose, and 0.18% glucose). After two centrifugations (200 g/5 minutes and 900 g/10 min), the supernatant was discarded and 1 mL of the sediment was incubated with $200\ \mu\text{L}$ 4% Triton during 30 minutes in water bath with agitation. Samples were centrifuged (600 g/8 minutes), and the supernatant was removed and stored at -20°C for further analysis. Quantification of intracellular antioxidant activity of SOD and GPx enzymes was performed according to Nichi et al. [5]. SOD activity was measured indirectly by reduction of cytochrome c by superoxide (O_2^-) generated by xanthine oxidase/xanthine system. The SOD present in the sample competes with cytochrome c by converting superoxide into hydrogen peroxide. We observed absorbance for 5 minutes in spectrophotometer at 470 nm at 25°C . Enzymatic activity of GPx was based on the consumption of NADPH by GSSH conversion into GSH. A reaction was induced between hydrogen peroxide and reduced glutathione (GSH), catalyzed by GPx and the enzyme glutathione reductase (GSR). NADPH consumption was detected at a wavelength of 340 nm, for 10 minutes at 37°C (measurements at every 5 seconds). SOD and GPx activity results were expressed in IU/ 10^6 sperm. Molar attenuation coefficient of NADPH ($6.22\ \text{mM}^{-1}\ \text{cm}^{-1}$) was used for determination of values.

3. Statistical Analysis

The dependent variables were analyzed by Statistical Analysis System 9.3 (SAS Institute, Cary, NC). All data were tested for normality of residues and homogeneity of variance. Variables that did not comply with these statistical premises were subjected to transformations. In Experiment 1, the MIXED procedure was used for analysis of variance with repeated measures over time. Comparisons of means were performed using *least square means* (LS means) for different dependent variables and for each condition of the statistical model (treatment, week, and treatment \times week). For nonparametric data, we used nonparametric analysis of variance (Kruskal Wallis, through NPARIWAY procedure), and for comparison of means we used comparison between two groups at a time (Wilcoxon). The parametric results are presented as mean \pm standard error. The nonparametric results are presented as median (low quartile, high quartile). In Experiment 2, the analysis of variance was carried using GLM procedure considering the 2×2 factorial. Factors considered treatment effect (treated group versus control group), and immediate and long-term effect of the damage induced by heat stress (first and second unilateral orchiectomies), with subsequent comparison of means by the LSD method. A 5% significance level was used to reject the hypothesis of nullity.

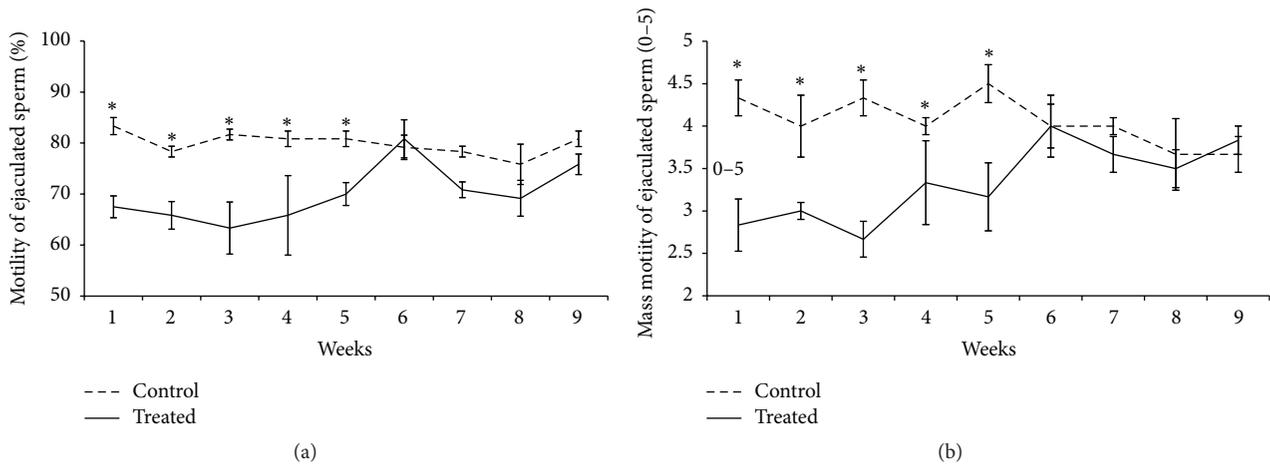


FIGURE 1: Comparison of motility (a) and mass motility (b) of ejaculated sperm considering the interaction effect between treatment and week in rams submitted or not to heat stress. The results are presented as means \pm SEM. Asterisk represents significant differences ($p < 0.05$).

4. Results

4.1. Experiment 1

4.1.1. Testicular and Environment Temperature. The mean internal temperature was $33.29^{\circ}\text{C} \pm 0.34$ for treated and $28.05^{\circ}\text{C} \pm 1.30$ for control group. The mean environment temperature and relative humidity in this period were $17.01^{\circ}\text{C} \pm 0.52$ and $78.08\% \pm 0.73$, respectively.

4.1.2. Immediate Evaluation and Sperm Morphology. An interaction between treatment and week of sample collection was observed for the variables motility ($p = 0.007$, Figure 1(a)), mass motility ($p = 0.0001$, Figure 1(b)), and percentage of sperm with minor defects ($p < 0.05$). Considering motility and mass motility, a decrease in the percentage of mobile cells was verified in the treated group until the fifth experimental week (Figures 1(a) and 1(b)), one spermatid cycle. From the sixth week onwards, differences between groups were no longer observed, which may indicate a recovery response to heat stress. In regard to minor defects, the treated group presented higher percentages of defects when compared to the control group in the fourth experimental week (Figure 2). In regard to sperm major defects, a treatment effect was observed, as a significant increase in the number of defects was detected in the treated group [2.5 (1.5; 4.5)] when compared to the control [2 (1.5; 3)]. Media values, standard errors, and p values of all variable measured to immediate evaluation and sperm morphology for treatment, week, and interaction effect are present in Supplementary Material (see Tables S1, S2, and S3 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/1687657>).

4.1.3. Evaluation of Plasma and Acrosome Membranes Integrity, Mitochondrial Membrane Potential, and Intracellular Marking of Free Radicals in Sperm. An interaction between treatment and week of sample collection was observed in the percentage of IMIA ($p = 0.028$, Figure 3(a)) and DMDA ($p < 0.05$, Figure 3(b)). The treated group presented an

increase in the percentage of DMDA sperm compared with control group during the first sixth experimental weeks. We also verified a reduction in the percentage of IMIA sperm in the treated group too in the same experimental weeks. Considering the percentage of sperm only with acrosome damage (IMDA), treatment effects and interactions were not observed, while effect of time was present (weeks, Table S2). It is noteworthy that this IMDA percentage was higher in the seventh week when compared to the other weeks. Concerning the percentage of sperm only with membrane damage (DMIA, Table S2), interaction and treatment effects were not observed, while effect of time was present when the treated group presented higher percentages at the sixth week when compared to others. In regard to mitochondrial membrane potential, an interaction was observed. Treated group showed lower percentages of cells with high mitochondrial membrane potential at weeks 3 and 6 when compared to control (Figures 4(a) and 4(b)). An increase in the percentage of cells displaying oxidative stress (stained by dichlorofluorescein) was observed in the treated group when compared to the control group (5.60 ± 2.09 versus 3.35 ± 0.6 , resp., Figure 5). Media values, standard errors, and p values of all variables measured to plasma and acrosome membranes integrity, mitochondrial membrane potential, and intracellular marking of free radicals in semen sperm for treatment, week, and interaction effect are present in Supplementary Material (Tables S1, S2, and S3).

4.1.4. Enzymatic Activity and Western-Blot in Seminal Plasma. We observed one protein band of 23 kDa and one between 50 and 75 kDa (Figure 6(a)) corresponding to GPxBI and GPxBS, respectively [29, 30]. GDR exhibited one strong protein band of 56 kDa (Figure 6(b)), catalase showed one protein band of 64 kDa (Figure 6(c)), and SOD presented one protein band of 70 kDa (Figure 6(d)). An increase in the enzymatic activity of GPx and GRD was detected in the treated group when compared to the control (GPx: 0.00120 ± 0.000069 UI/mL versus 0.00096 ± 0.000070 UI/mL, Figure 7(a); GRD: 0.000081 ± 0.0000048 UI/mL versus

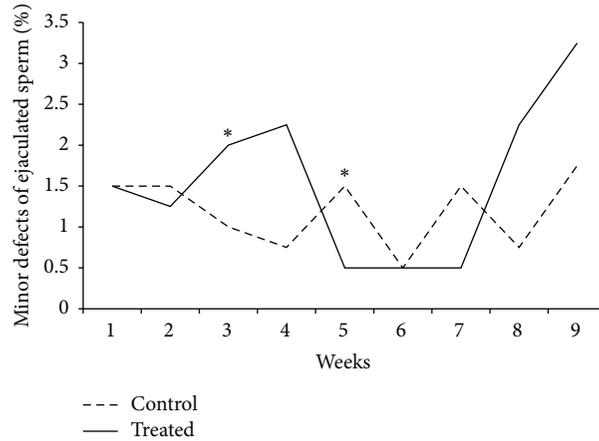
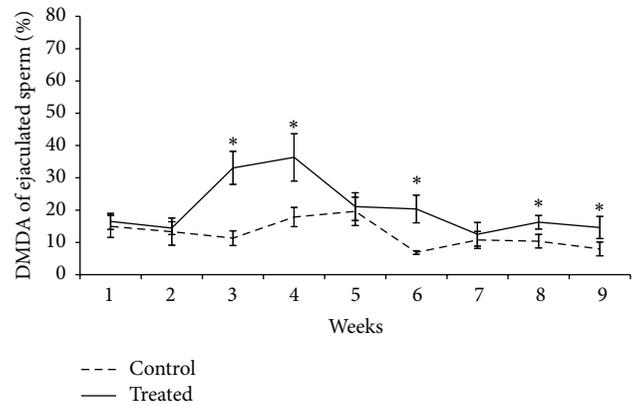
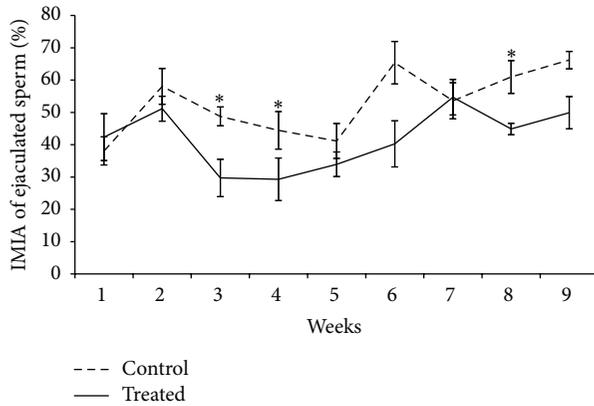


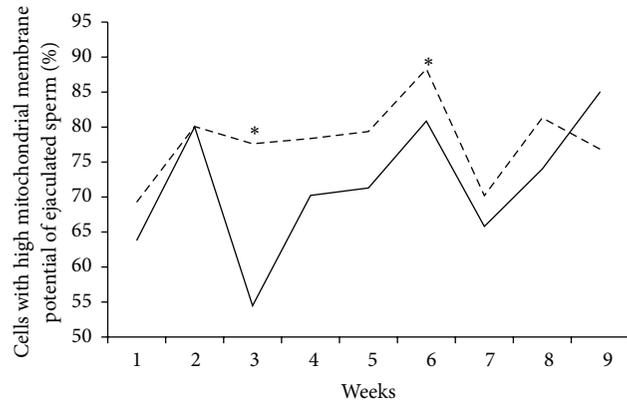
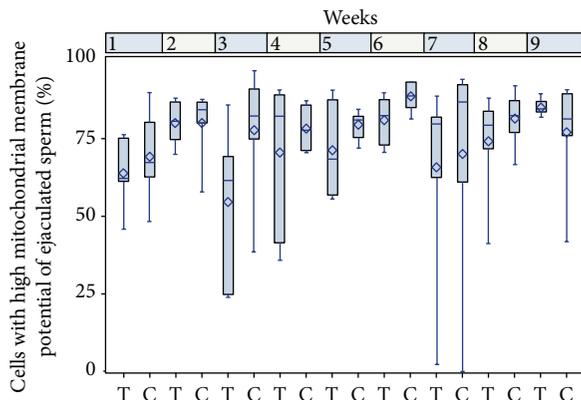
FIGURE 2: Percentages of minor defects observed in ejaculated sperm considering the interaction effect between treatment and week in rams submitted or not to heat stress. Graphic presented as mean. Asterisk represents significant differences ($p < 0.05$). T: treated, C: control.



(a)

(b)

FIGURE 3: Percentages of IMIA (a) and DMDA (b) of ejaculated sperm considering the interaction effect between treatment and week in rams submitted or not to heat stress. Graphic presented as means \pm SEM. Asterisk represents significant differences ($p < 0.05$). IMIA: intact membrane and intact acrosome sperm, DMDA: damaged membrane and damaged acrosome sperm.



(a)

(b)

FIGURE 4: Percentages of cells with high mitochondrial membrane potential of ejaculated sperm considering the interaction effect between treatment and week in rams submitted or not to heat stress. (a) Boxplot presented as median (superior quartile, inferior quartile). (b) Data presented as mean. Asterisk represents significant differences ($p < 0.05$). T: treated, C: control.

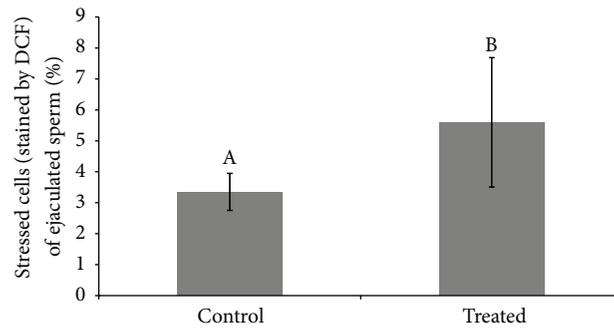


FIGURE 5: Percentage of stressed cells (stained by dichlorofluorescein, DCF) of ejaculated sperm considering the treatment effect between treated and control group in rams submitted or not to heat stress. Data presented as means \pm SEM. Different superscript letters in each bar represent significant differences ($p < 0.05$).

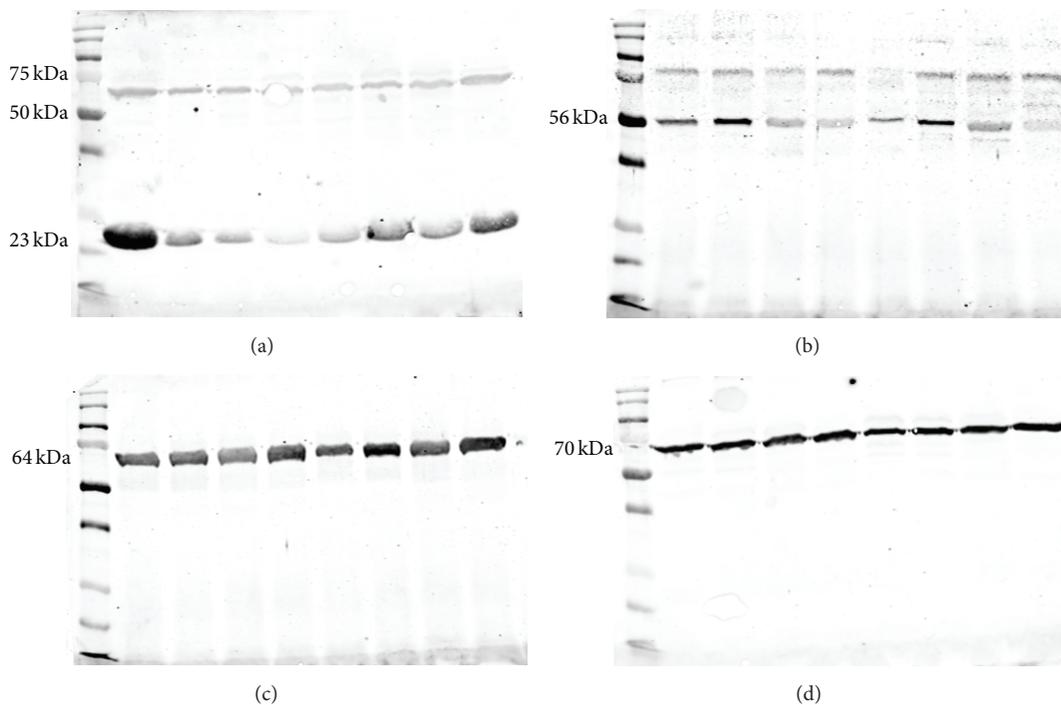


FIGURE 6: Immunoblotting detection of glutathione peroxidase, GPx (a), glutathione reductase, GDR (b), catalase (c), and superoxide dismutase, SOD (d), in seminal plasma of ejaculated sperm in rams submitted or not to heat stress.

0.000043 ± 0.00000221 UI/mL, Figure 7(b)). No differences were observed between SOD and catalase enzymatic activities. Quantification of enzymes through western blotting did not allow observation of interaction or treatment effects for GPx, GRD, SOD, and catalase. We observed an effect of time when quantifying GPx (GPxBI $p = 0.003$, GPxBS $p = 0.002$) and catalase ($p < 0.001$) (Table S2). Media values, standard errors, and p values of all variables measured to enzymatic activity and western-blot in seminal plasma for treatment, week, and interaction effect are present in Supplementary Material (Tables S1, S2, and S3).

4.1.5. Lipid Peroxidation in Seminal Plasma and Sperm. No differences on TBARS levels in seminal plasma were observed between treated and control groups. However, an effect of

week for quantification of TBARS in sperm cell ($p < 0.0001$) and seminal plasma ($p < 0.0001$) was observed (Table S2). Media values, standard errors, and p values of all variables measured to enzymatic activity and western-blot in seminal plasma for treatment, week, and interaction effect are present in Supplementary Material (Tables S1, S2, and S3).

4.2. Experiment 2

4.2.1. Computerized Motility Analysis. No differences in any of the CASA parameters measured were observed between treatments or in immediate and long-term effects of heat stress in the testicle (treatment, week, and interaction effect are present in Supplementary Material, Tables S4, S5, and S6).

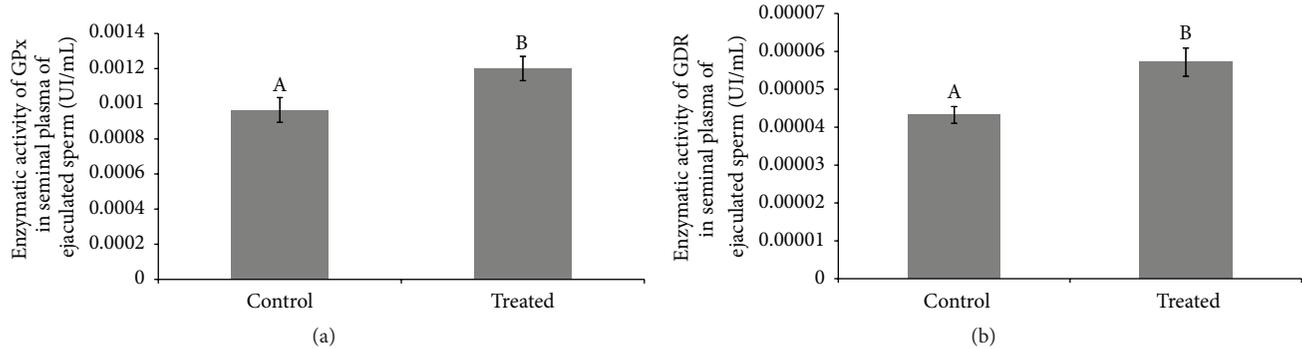


FIGURE 7: Comparison of enzymatic activity of glutathione peroxidase, GPx (a), and glutathione reductase, GDR (b), in seminal plasma of ejaculated sperm considering the treatment effect between treated and control group in rams submitted or not to heat stress. Graphic presented as means \pm SEM. Different superscript letters in each bar represent significant differences ($p < 0.05$).

4.2.2. Evaluation of Plasma and Acrosomal Membranes Integrity, Mitochondrial Membrane Potential, and Intracellular Marking of Free Radicals in the Epididymal Sperm. No differences were observed between groups and between immediate and long-term effects of heat stress on epididymal sperm in any categories of plasma and acrosome membranes integrity (treatment, week, and interaction effect are present in Supplementary Material, Tables S4, S5, and S6). Nonetheless, the treated group displayed a decrease in the percentage of cells with high mitochondrial membrane potential ($61 \pm 5.62\%$ versus $78.39 \pm 2.29\%$, Figure 8(a)). Furthermore, the treated group presented an increase in the percentage of cells with intermediary ($29.96 \pm 4.71\%$ versus $18.41 \pm 1.45\%$, Figure 8(b)) and low ($9.04 \pm 2.06\%$ versus $4.15 \pm 1.35\%$, Figure 8(c)) mitochondrial membrane potential, when compared to the control. No differences were observed between immediate and long-term effects of heat stress in high and intermediate mitochondrial membrane potential categories. We observed a decrease in low mitochondrial membrane potential in epididymal sperm considering the long-term response compared to immediate response to heat stress ($2.75 \pm 1.16\%$ versus $10.44 \pm 176\%$). Considering oxidative stress, there was an increase in the percentage of cells positive for DCF staining in the treated group when compared to the control ($2.27 \pm 0.56\%$ versus $1.10 \pm 0.33\%$, Figure 8(d)). No differences were observed between immediate and long-term effects of heat stress in stressed cells stained by DCF.

4.2.3. Intracellular Enzymatic Activity and Lipid Peroxidation in Epididymal Sperm. No differences were observed between groups and between immediate and long-term effects of heat stress considering GPx and SOD activity in epididymal sperm (treatment, week, and interaction effect are present in Supplementary Material, Tables S4, S5, and S6). Regarding lipid peroxidation, no differences were observed between groups (control versus treated), and between immediate and long-term effects of heat stress (treatment, week, and interaction effect are present in Supplementary Material, Tables S4, S5, and S6) when considering TBARS.

5. Discussion

Heat stress has been related to decrease in sperm motility, concentration, and viability in mice [31], bull [5, 32–34], men [35], and ram [36, 37]. In our study, we observed a decrease in motility, vigor, and mass motility and an increase in the percentage of cells with major and minor defects up to the fifth experimental week. Similar results were observed when rams were submitted to heat stress for 14 or 28 days [38, 39]. Also, in the present study, acrosome and plasma membrane damage were observed in the sperm from the ejaculate after induced heat stress. In this case, the heat stress impaired sperm quality for approximately 1 sperm cycle (47 days). From the sixth experimental week onwards, no more differences were observed between groups for these variables, indicating a recovery of damage possibly caused by heat stress.

Lipids such as PUFAs are the most susceptible molecules to peroxidation in the sperm plasma membrane [40]. Peroxidation of PUFAs has been associated as the main cause of decrease in sperm motility due to the increase of ROS concentrations [41–44]. The increase of ROS has also been associated with sperm morphological alterations and teratospermia [44]. The extent of the damage depends on the nature and quantity of ROS involved, duration of exposure, and extracellular factors such as temperature and oxygen tension [40]. In our study, despite differences observed in sperm from the ejaculate, no differences were observed in epididymal sperm after the second testicular insulation. It is possible that cells susceptible to ROS damage were eliminated after the stress from the first insulation (Experiment 1), while cells more resistant to heat stress remained in the second induction (Experiment 2). These cells seem to have passed undamaged, with no alterations in motility and plasma and acrosome membrane integrity. Similar results were found in studies of renal patients, where the highest degree of apoptosis correlated with improved renal function six months after kidney transplant [45]. Apoptosis could be a mechanism involved in the elimination of susceptible cells [45].

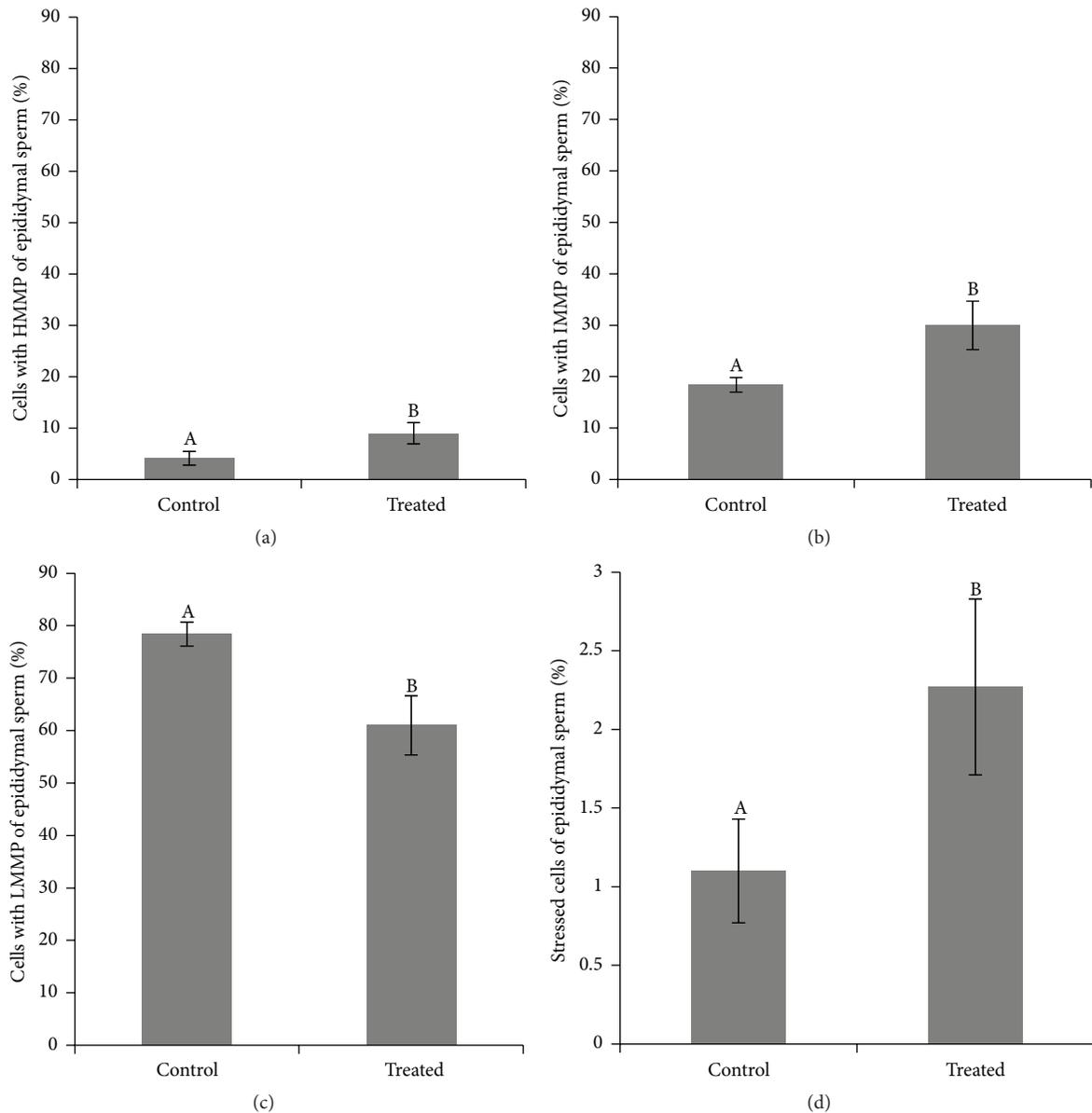


FIGURE 8: Comparison of percentage of high, HMMP (a), intermediary, IMMP (b), low, LMMP (c), mitochondrial membrane potential and percentage of stressed cells (stained by dichlorofluorescein (d)) of epididymal sperm considering the treatment effect between treated and control groups in rams submitted or not to heat stress. Graphic presented as means \pm SEM. Different superscript letters in each bar represent significant differences ($p < 0.05$).

In our study, heat stress treatment effectively increased ROS levels in both experiments as observed by DCF staining [23, 46, 47]. Furthermore, although a significant difference cannot be verified, there was a fourfold increase ($8.89 \pm 0.42\%$ in the treated group versus $2.05 \pm 3.42\%$ in the control group, $p = 0.24$) in cell susceptibility to oxidative stress, quantified by induced TBARS. This evidence corroborates the idea that heat stress damage to the spermatid cell is mediated by oxidative stress. Regarding oxidative status evaluations, no difference was observed in TBARS quantification in seminal plasma; however, an increase of enzymatic activity of GPx and GRD was observed in the treated group when compared

to the control. Some studies have correlated increases in GPx activity to situations in which oxidative stress is the reason of the pathological processes, such as in women with preeclampsia [48], patients with Down Syndrome [49], postexercise stress [50, 51], and even infertility [52, 53]. A study found a tenfold increase in GPx activity in infertile men compared to fertile ones [53], indicating that this enzyme may serve as a marker of oxidative imbalance. The increase of GPx activity would be a response to the increase of ROS, in particular, hydrogen peroxide [54].

Despite the increase in the enzymatic activity of GPx in seminal plasma, no differences were observed in the

immunodetection. One hypothesis could be that GPx is present in the epididymis in an inactive state, becoming active when necessary [9]. In epididymal environment, this activation mechanism would be essential for sperm protection, since activation of already translated antioxidant enzymes would be faster and more effective than the synthesis of new ones, preventing oxidation reactions faster. Supporting this hypothesis, GPx activity was correlated with seminal characteristics in infertile men but it was not correlated to mRNA levels of GPx [54]. According to these authors, there is a posttranscriptional control of GPx activity, but the mechanisms are still unknown. Further studies are necessary to clarify how the activation of these antioxidant enzymes occurs in the seminal plasma. In epididymal environment, there were no differences between groups and between immediate and long-term responses to antioxidant enzyme activity quantified in sperm cells.

In this work, in both sperm from the ejaculate and epididymal sperm, there was a decrease in the percentage of cells with high potential of mitochondrial membrane in the treated group, when compared to the control, suggesting that mitochondrial damage can be the source of oxidative damage. Mitochondrial metabolism is possibly correlated to pathophysiology of oxidative homeostasis imbalance, caused by heat stress. Several studies have observed a clear relationship between sperm oxidative stress and mitochondrial activity [8, 43, 55]. Mitochondria present in sperm are the main factor responsible for ATP production by oxidative phosphorylation and thus are responsible for the production of ROS. Physiologically, about 1% of the oxygen formed is converted to superoxide anion, originating the building ROS chain [56, 57], which is fundamental to several physiological functions [58]. In the case of mitochondrial damage, ROS production may be exacerbated by the release of more prooxidative factors [55–59]. More studies contemplating mitochondrial fraction [60] and possible signaling pathways involved in mitochondrial function must be conducted to clarify how heat stress alters oxidative balance, such as cytochrome c levels by western blotting [61], disturbances in mitochondrial enzyme complexes (I–IV), and decrements in tricarboxylic acid cycle enzymes [62].

One may conclude that heat stress causes alterations in sperm during a spermatogenic cycle and disruption of oxidative homeostasis, due to oxidative stress, observed by increase in DCF staining and GPx enzymatic activity. This stress may possibly be caused by a mitochondrial alteration, once sperm from the ejaculate and epididymis presented a decrease in the high potential of mitochondrial membrane when the rams were submitted to heat stress. In addition, the epididymal immediate and long-term response to the heat stress do seem to be similar.

Conflict of Interests

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Authors' Contribution

Dr. Thais Rose dos Santos Hamilton, Mayra Elena Ortiz D'Ávila Assumpção, and Marcílio Nichi designed the experimental study; Dr. Thais Rose dos Santos Hamilton, Leticia Signori de Castro, Patrícia Monken de Assis, Juliana de Carvalho Delgado, Adriano Felipe Perez Siqueira, and Camilla Mota Mendes performed animal management and analyzed sperm. Dr. Thais Rose dos Santos Hamilton performed enzymatic activities, western-blot, and statistical analyses. Dr. Teresa Muiño-Blanco and José Álvaro Cebrián-Pérez performed and assisted in enzymatic activities and western-blot analyses. Dr. Thais Rose dos Santos Hamilton, Marcílio Nichi, Marcelo Demarchi Goissis, and José Antonio Visintin drafted the paper, whereas Dr. Mayra Elena Ortiz D'Ávila Assumpção provided financial support and completed critical revision and approval of the paper.

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References

- [1] P. L. Senger, "The organization and function of the male reproductive system," in *Pathways to Pregnancy and Parturition*, Pullman: Current Conceptions, pp. 44–79, 4th edition, 2003.
- [2] R. J. Aitken and S. D. Roman, "Antioxidant systems and oxidative stress in the testes," *Oxidative Medicine and Cellular Longevity*, vol. 1, no. 1, pp. 15–24, 2008.
- [3] J. G. Reyes, J. G. Farias, S. Henríquez-Olavarrieta et al., "The hypoxic testicle: physiology and pathophysiology," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 929285, 15 pages, 2012.
- [4] W. Hou, Y. Dong, J. Zhang et al., "Hypoxia-induced deacetylation is required for tetraploid differentiation in response to testicular ischemia-reperfusion (IR) injury," *Journal of Andrology*, vol. 33, no. 6, pp. 1379–1386, 2012.
- [5] M. Nichi, P. E. J. Bols, R. M. Züge et al., "Seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions," *Theriogenology*, vol. 66, no. 4, pp. 822–828, 2006.
- [6] J. S. Fleming, F. Yu, R. M. McDonald et al., "Effects of scrotal heating on sperm surface protein PH-20 expression in sheep," *Molecular Reproduction and Development*, vol. 68, no. 1, pp. 103–114, 2004.
- [7] C. Paul, S. Teng, and P. T. K. Saunders, "A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death," *Biology of Reproduction*, vol. 80, no. 5, pp. 913–919, 2009.

- [8] C. G. Blumer, R. M. Fariello, A. E. Restelli, D. M. Spaine, R. P. Bertolla, and A. P. Cedenho, "Sperm nuclear DNA fragmentation and mitochondrial activity in men with varicocele," *Fertility and Sterility*, vol. 90, no. 5, pp. 1716–1722, 2008.
- [9] B. Halliwell and J. M. C. Gutteridge, "Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death," in *Free Radicals in Biology and Medicine*, pp. 187–268, Oxford University Press, Oxford, UK, 4th edition, 2007.
- [10] L. Schibler, D. Vaiman, A. Oustry, C. Giraud-Delville, and E. P. Cribsu, "Comparative gene mapping: a fine-scale survey of chromosome rearrangements between ruminants and humans," *Genome Research*, vol. 8, no. 9, pp. 901–915, 1998.
- [11] P. Vernet, R. J. Aitken, and J. R. Drevet, "Antioxidant strategies in the epididymis," *Molecular and Cellular Endocrinology*, vol. 216, no. 1, pp. 31–39, 2004.
- [12] F. M. Flesch and B. M. Gadella, "Dynamics of the mammalian sperm plasma membrane in the process of fertilization," *Biochimica et Biophysica Acta—Reviews on Biomembranes*, vol. 1469, no. 3, pp. 197–235, 2000.
- [13] R. Jones and T. Mann, "Damage to ram spermatozoa by peroxidation of endogenous phospholipids," *Journal of Reproduction and Fertility*, vol. 50, no. 2, pp. 261–268, 1977.
- [14] A. J. Koppers, G. N. De Iuliis, J. M. Finnie, E. A. McLaughlin, and R. J. Aitken, "Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa," *The Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 8, pp. 3199–3207, 2008.
- [15] R. Mahfouz, R. Sharma, A. Thiyagarajan et al., "Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species," *Fertility and Sterility*, vol. 94, no. 6, pp. 2141–2146, 2010.
- [16] J. G. Alvarez and B. T. Storey, "Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation," *Journal of Andrology*, vol. 13, no. 3, pp. 232–241, 1992.
- [17] S. P. Dandekar, G. D. Nadkarni, V. S. Kulkarni, and S. Punekar, "Lipid peroxidation and antioxidant enzymes in male infertility," *Journal of Postgraduate Medicine*, vol. 48, no. 3, pp. 186–189, 2002.
- [18] R. J. Potts, T. Mjefferies, and L. J. Notarianni, "Antioxidant capacity of the epididymis," *Human Reproduction*, vol. 14, no. 10, pp. 2513–2516, 1999.
- [19] J. Djordjevic, A. Djordjevic, M. Adzic, A. Niciforovic, and M. B. Radojic, "Chronic stress differentially affects antioxidant enzymes and modifies the acute stress response in liver of wistar rats," *Physiological Research*, vol. 59, no. 5, pp. 729–736, 2010.
- [20] A. Rahal, A. Kumar, V. Singh et al., "Oxidative stress, prooxidants, and antioxidants: the interplay," *BioMed Research International*, vol. 2014, Article ID 761264, 19 pages, 2014.
- [21] E. Blom, "The ultrastructure of some characteristic sperm defects and a proposal for a new classification of the bull spermogram," *Nordisk Veterinaermedicin*, vol. 25, no. 7-8, pp. 383–391, 1973.
- [22] A. Gomes, E. Fernandes, and J. L. F. C. Lima, "Fluorescence probes used for detection of reactive oxygen species," *Journal of Biochemical and Biophysical Methods*, vol. 65, no. 2-3, pp. 45–80, 2005.
- [23] S.-H. Kim, D.-H. Yu, and Y.-J. Kim, "Apoptosis-like change, ROS, and DNA status in cryopreserved canine sperm recovered by glass wool filtration and Percoll gradient centrifugation techniques," *Animal Reproduction Science*, vol. 119, no. 1-2, pp. 106–114, 2010.
- [24] J. I. Martí, E. Martí, J. A. Cebrián-Pérez, and T. Muiño-Blanco, "Survival rate and antioxidant enzyme activity of ram spermatozoa after dilution with different extenders or selection by a dextran swim-up procedure," *Theriogenology*, vol. 60, no. 6, pp. 1025–1037, 2003.
- [25] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.
- [26] H. Ohkawa, N. Ohishi, and K. Yagi, "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction," *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.
- [27] R. Simões, W. B. Feitosa, A. F. P. Siqueira et al., "Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome," *Reproduction*, vol. 146, no. 5, pp. 433–441, 2013.
- [28] M. Nichi, I. G. F. Goovaerts, C. N. M. Cortada, V. H. Barnabe, J. B. P. De Clercq, and P. E. J. Bols, "Roles of lipid peroxidation and cytoplasmic droplets on in vitro fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34°C," *Theriogenology*, vol. 67, no. 2, pp. 334–340, 2007.
- [29] C. N. Vaisberg, L. V. Jelezarsky, B. Dishlianova, and T. A. Chaushev, "Activity, substrate detection and immunolocalization of glutathione peroxidase (GPx) in bovine reproductive organs and semen," *Theriogenology*, vol. 64, no. 2, pp. 416–428, 2005.
- [30] J. R. Drevet, "The antioxidant glutathione peroxidase family and spermatozoa: a complex story," *Molecular and Cellular Endocrinology*, vol. 250, no. 1-2, pp. 70–79, 2006.
- [31] M. Pérez-Crespo, B. Pintado, and A. Gutiérrez-Adán, "Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ration in mice," *Molecular Reproduction and Development*, vol. 75, no. 1, pp. 40–47, 2008.
- [32] M. J. Fields, W. C. Burns, and A. C. Warnick, "Age, season and breed effects on testicular volume and semen traits in young beef bulls," *Journal of Animal Science*, vol. 48, no. 6, pp. 1299–1304, 1979.
- [33] C. M. Barros, M. F. Pegorer, J. L. M. Vasconcelos, B. G. Eberhardt, and F. M. Monteiro, "Importance of sperm genotype (*indicus* versus *taurus*) for fertility and embryonic development at elevated temperatures," *Theriogenology*, vol. 65, no. 1, pp. 210–218, 2006.
- [34] M. B. Rahman, L. Vandaele, T. Rijsselaere et al., "Scrotal insulation and its relationship to abnormal morphology, chromatin protamination and nuclear shape of spermatozoa in Holstein-Friesian and Belgian Blue bulls," *Theriogenology*, vol. 76, no. 7, pp. 1246–1257, 2011.
- [35] P. Thonneau, L. Bujan, L. Multigner, and R. Miesusset, "Occupational heat exposure and male fertility: a review," *Human Reproduction*, vol. 13, no. 8, pp. 2122–2125, 1998.
- [36] J. K. Voglmayr, B. P. Setchell, and I. G. White, "The effects of heat on the metabolism and ultrastructure of ram testicular spermatozoa," *Journal of Reproduction and Fertility*, vol. 24, no. 1, pp. 71–80, 1971.
- [37] P. Williamson, "The fine structure of ejaculated ram spermatozoa following scrotal heating," *Journal of Reproduction and Fertility*, vol. 40, no. 1, pp. 191–195, 1974.
- [38] S. W. Byers, "Effect of scrotal insulation on the ability of ram testes to produce testosterone in vitro," *Journal of Reproduction and Fertility*, vol. 71, no. 1, pp. 17–21, 1984.

- [39] S. W. Byers and T. D. Glover, "Effect of scrotal insulation on the pituitary-testicular axis of the rat," *Journal of Reproduction and Fertility*, vol. 71, no. 1, pp. 23–31, 1984.
- [40] R. Walczak-Jedrzejowska, J. K. Wolski, and J. Slowikowska-Hilczner, "The role of oxidative stress and antioxidants in male fertility," *Central European Journal of Urology*, vol. 66, no. 1, pp. 60–67, 2013.
- [41] S. A. Suleiman, M. Elamin Ali, Z. M. S. Zaki, E. M. A. El-Malik, and M. A. Nasr, "Lipid peroxidation and human sperm motility: protective role of vitamin E," *Journal of Andrology*, vol. 17, no. 5, pp. 530–537, 1996.
- [42] S. I. Peris, J.-F. Bilodeau, M. Dufour, and J. L. Bailey, "Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm," *Molecular Reproduction and Development*, vol. 74, no. 7, pp. 878–892, 2007.
- [43] A. Ferramosca, S. P. Provenzano, D. D. Montagna, L. Coppola, and V. Zara, "Oxidative stress negatively affects human sperm mitochondrial respiration," *Urology*, vol. 82, no. 1, pp. 78–83, 2013.
- [44] A. Agarwal, E. Tvrda, and R. Sharma, "Relationship amongst teratozoospermia, seminal oxidative stress and male infertility," *Reproductive Biology and Endocrinology*, vol. 12, article 45, 2014.
- [45] U. Ott, A. Aschoff, R. Fünfstück, G. Jirikowski, and G. Wolf, "DNA fragmentation in acute and chronic rejection after renal transplantation," *Transplantation Proceedings*, vol. 39, no. 1, pp. 73–77, 2007.
- [46] R. Mahfouz, R. Sharma, J. Lackner, N. Aziz, and A. Agarwal, "Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa," *Fertility and Sterility*, vol. 92, no. 2, pp. 819–827, 2009.
- [47] N. Aziz, J. Novotny, I. Oborna, H. Fingerova, J. Brezinova, and M. Svobodova, "Comparison of chemiluminescence and flow cytometry in the estimation of reactive oxygen and nitrogen species in human semen," *Fertility and Sterility*, vol. 94, no. 7, pp. 2604–2608, 2010.
- [48] J. B. Sharma, A. Sharma, A. Bahadur, N. Vimala, A. Satyam, and S. Mittal, "Oxidative stress markers and antioxidant levels in normal pregnancy and pre-eclampsia," *International Journal of Gynecology and Obstetrics*, vol. 94, no. 1, pp. 23–27, 2006.
- [49] I. Garaiová, J. Muchová, M. Šustrová et al., "The relationship between antioxidant systems and some markers of oxidative stress in persons with Down syndrome," *Biologia*, vol. 59, no. 6, pp. 787–794, 2004.
- [50] R. P. Barcelos, M. A. Souza, G. P. Amaral et al., "Caffeine supplementation modulates oxidative stress markers in the liver of trained rats," *Life Sciences*, vol. 96, no. 1-2, pp. 40–45, 2014.
- [51] M. A. Bouzid, O. Hammouda, R. Matran, S. Robin, and C. Fabre, "Changes in oxidative stress markers and biological markers of muscle injury with aging at rest and in response to an exhaustive exercise," *PLoS ONE*, vol. 9, no. 3, Article ID e90420, 2014.
- [52] A. Giannattasio, M. De Rosa, R. Smeraglia et al., "Glutathione Peroxidase (GPX) activity in seminal plasma of healthy and infertile males," *Journal of Endocrinological Investigation*, vol. 25, no. 11, pp. 983–986, 2002.
- [53] M. Maiorino, V. Bosello, F. Ursini et al., "Genetic variations of gpx-4 and male infertility in humans," *Biology of Reproduction*, vol. 68, no. 4, pp. 1134–1141, 2003.
- [54] N. Garrido, M. Meseguer, C. Simon, A. Pellicer, and J. Remohi, "Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility," *Asian Journal of Andrology*, vol. 6, no. 1, pp. 59–65, 2004.
- [55] H. D. Guthrie, G. R. Welch, and J. A. Long, "Mitochondrial function and reactive oxygen species action in relation to boar motility," *Theriogenology*, vol. 70, no. 8, pp. 1209–1215, 2008.
- [56] A. L. Agarwal, K. Makker, and R. Sharma, "Clinical relevance of oxidative stress in male factor infertility: an update," *American Journal of Reproductive Immunology*, vol. 59, no. 1, pp. 2–11, 2008.
- [57] R. J. Aitken, J. S. Clarkson, and S. Fishel, "Generation of reactive oxygen species, lipid peroxidation, and human sperm function," *Biology of Reproduction*, vol. 41, no. 1, pp. 183–197, 1989.
- [58] R. J. Aitken and M. A. Baker, "Reactive oxygen species generation by human spermatozoa: a continuing enigma," *International Journal of Andrology*, vol. 25, no. 4, pp. 191–194, 2002.
- [59] R. J. Aitken and J. S. Clarkson, "Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa," *Journal of Reproduction and Fertility*, vol. 81, no. 2, pp. 459–469, 1987.
- [60] K. Stephan, M. Chang, E. P. Brass, and C. L. Hoppel, "Decreased activities of ubiquinol: ferricytochrome c oxidoreductase (complex III) and ferrocycytochrome c: oxygen oxidoreductase (complex IV) in liver mitochondria from rats with hydroxycobalamin[c-lactam]-induced methylmalonic aciduria," *The Journal of Biological Chemistry*, vol. 266, no. 31, pp. 20998–21003, 1991.
- [61] N. J. Waterhouse, J. C. Goldstein, O. Von Ahsen, M. Schuler, D. D. Newmeyer, and D. R. Green, "Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process," *The Journal of Cell Biology*, vol. 153, no. 2, pp. 319–328, 2001.
- [62] P. Mahaboob Basha and S. M. Saumya, "Suppression of mitochondrial oxidative phosphorylation and TCA enzymes in discrete brain regions of mice exposed to high fluoride: amelioration by *Panax ginseng* (Ginseng) and *Lagerstroemia speciosa* (Banaba) extracts," *Cellular and Molecular Neurobiology*, vol. 33, no. 3, pp. 453–464, 2013.

Review Article

Lipids and Oxidative Stress Associated with Ethanol-Induced Neurological Damage

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The excessive intake of alcohol is a serious public health problem, especially given the severe damage provoked by chronic or prenatal exposure to alcohol that affects many physiological processes, such as memory, motor function, and cognitive abilities. This damage is related to the ethanol oxidation in the brain. The metabolism of ethanol to acetaldehyde and then to acetate is associated with the production of reactive oxygen species that accentuate the oxidative state of cells. This metabolism of ethanol can induce the oxidation of the fatty acids in phospholipids, and the bioactive aldehydes produced are known to be associated with neurotoxicity and neurodegeneration. As such, here we will review the role of lipids in the neuronal damage induced by ethanol-related oxidative stress and the role that lipids play in the related compensatory or defense mechanisms.

1. Introduction

A clear relationship has been established between ethanol intake, addiction and dependency [1–3], and several risk factors for chronic disease and injury [4]. Indeed, the public health problem associated with increased alcohol consumption and alcoholism [5, 6] is becoming ever more severe due to the increased economic burden of the complications on the health national systems and the cost of the relevant treatments [4, 7–10]. Alcoholism provokes high rates of mortality and it increases in risk of several disabling disorders [4, 11]. Such damage can be classified in function of the organs involved (liver, kidney, heart, brain, etc.), the type of intake (acute or chronic), or the subject's age at the time of exposure to ethanol (prenatal, neonatal, or adult). In summary, ethanol has several negative health effects, especially if we consider prenatal exposure where the brain is a major target for the damage provoked.

2. Effects of Ethanol in the Brain

Ethanol has many effects in the brain depending on the age of exposure (prenatal, postnatal, or adult). For example,

aggressive behavior and depression are observed after acute postnatal exposure to ethanol, possibly due to a decrease in circulating tryptophan, followed by the depletion of serotonin in the brain [12, 13]. Another effect of acute postnatal alcohol exposure is related to impaired impulsive and control behavior [14, 15], although few *in vivo* studies have focused on this issue. Cognitive performance has been associated with specific prefrontal cortical regions in Rhesus Macaque monkeys [11] and GABA receptors in this structure have been implicated in the effects of acute postnatal ethanol exposure [16–18]. Indeed, GABA was found to be a mediator in ethanol-induced ataxia [18, 19].

The most severe alcohol-related damage is found following acute prenatal or chronic pre- and postnatal ethanol exposure, effects that have been associated with a loss of neurons (Table 1). In terms of prenatal exposure, the babies born to women that drink alcohol excessively during pregnancy may suffer from fetal alcohol syndrome [20], a condition characterized by specific craniofacial abnormalities, pre- and postnatal growth deficiencies, and nervous system dysfunction that is manifested as persistent intellectual, behavioral, and neurological defects [5, 21]. These latter symptoms have

TABLE 1: Summary of the neurological effects induced by *in vivo* ethanol administration at different ages.

Age of exposure	Species (model)	Frequency of administration	Structural CNS changes	Behavioral, intellectual, or other effects	References
Prenatal	Rat	Chronic	Reduced number of neurons and dendritic spines in the hippocampus and pyramidal tracts	Memory, spatial learning	[22]
			The cerebellum is most sensitive to alcohol-induced Purkinje cell loss	Cerebellar disorders (ataxia, cognitive, behavioral, and affective disturbances)	[23, 24]
			Cholinergic neurons loss	Spontaneous alternation, spatial working memory	[25, 26]
			Reduction in gray and white matter in the hippocampus, amygdala, thalamus, caudate, putamen, and globus pallidus	Cognitive, behavioral, and neurological impairments	[27, 28]
Young	Human	Chronic	Reduced white matter, corpus callosum, and hippocampal volumes	Impairment in neurocognitive tests, including those measuring memory, attention, visuospatial skills, and executive function	[29, 30]
			Reduced oxygen consumption in the subcallosal, anterior cingulate, left prefrontal, and bilateral insular regions	Dysfunction during spatial working memory and simple motor tasks	[31, 32]
Adult	Human	Chronic	Reduced volume in the diencephalon, cerebral cortex, hippocampus, and white matter	Progressive cognitive dysfunction and loss of neural plasticity due to reduced GABAergic inhibition and increased glutamatergic excitation	[33]
			Corpus callosum ultrastructure	Cognitive and motor function	[34]
			Frontal and temporal lobes	Attention, impulsivity, verbal memory, and impaired cognition	[35, 36]
			Hypothalamus D3 and 5HT _{1A} neuronal receptors	Alcohol dependency	[37, 38]
	Human or monkey	Chronic	Nucleus basalis Meynert	Loss cognitive disorders and dementia	[39–42]
			Cerebellar atrophy, Purkinje cell loss	Wernicke's encephalopathy, cognitive and emotional dysfunction	[43, 44]
			Peripheral nerves stimulation	Withdrawal-induced hyperalgesia	[45]
			Hemorrhage in the ventral diencephalon, mesencephalon, and Basal ganglia, and severe white matter edema in the cerebral hemispheres and pontine nuclei and medullary tegmental	Cognitive impairment, necrosis, and death	[46, 47]

been related to neurodegeneration in experimental animal models (see Table 1 for a summary of some of the available literature).

Chronic alcohol exposure has been associated with permanent neuronal loss in brain regions like the hippocampus and cerebellum. Moreover, *in vivo* studies have demonstrated neurological effects following chronic ethanol exposure in young and adult populations, with deterioration in memory, motor function, cognition, and so forth. All these effects could be due to neurotoxicity or neurodegeneration, and there is evidence that oxidative stress associated with ethanol metabolism is involved.

3. The Pharmacokinetics of Ethanol

The ethanol concentration that can be found in blood following its ingestion depends on its pharmacokinetics (PK). PK determines not only the time-course and persistence of ethanol in blood but also the amount of alcohol and its metabolic products that accumulate in different tissues, and hence their pharmacological and toxicological responses [48].

3.1. Absorption. In adults, the ethanol ingested is almost completely and instantly absorbed by passive diffusion, reaching a peak concentration in humans between 30 and 90 min. Absorption is more efficient in the small intestine than in stomach [49], a difference in absorption that is due to two factors. First, the thickness of mucus that protects the stomach appears to have a resistance ~16 times greater than that which protects the small intestine [49, 50], which also has a greater intestinal absorption surface due to the presence villi and microvilli [51]. The second difference reflects the speed of stirring caused by peristalsis, which is more important in the small intestine than in the stomach, playing a role in gastric emptying and in the intestinal transit time [49].

In addition, the presence of food is another factor that modifies the absorption rate [52], mainly as food reduces gastric emptying and ethanol is absorbed more slowly [53]. Solid food intake can reduce the ethanol absorption rate by 30% and it has been suggested that this effect is due to the need for food digestion prior to absorption process. As such, if food is taken in as a liquid then it would not produce this effect [49, 54]. Moreover, a small amount of ethanol can be oxidized to acetaldehyde by alcohol dehydrogenase (ADH) classes I and IV [52, 55] in the stomach and intestine. This acetaldehyde can be absorbed along with ethanol and metabolized by the liver or other tissues.

3.2. First Pass Metabolism and the Distribution of Ethanol. The amount of alcohol in any given tissue depends on its relative concentration in the blood, which is a function of first pass metabolism [49], that is, the oxidation of ethanol in the stomach, intestine, and liver.

Most first pass metabolism occurs in the liver [49, 55] and the rate-limiting step is the oxidation of ethanol to acetaldehyde. This reaction is catalyzed by proteins of the ADH family [56], of which class I (ADH1) and III (ADH3) enzymes metabolize ethanol in the liver [57, 58]. These two

types of enzymes differ in their K_m , with ADH1 having a low K_m while ADH3 has a high K_m value [57, 59]. Consequently ADH3 plays a more important role in the metabolism of alcohol at high concentrations. In addition, microsomal ethanol oxidizing system (MEOS) and catalase contribute to the metabolism of alcohol in specific circumstances, such as high ethanol concentrations [48, 60].

The acetaldehyde produced by the oxidation of ethanol is thereafter transformed to acetate by aldehyde dehydrogenase (ALDH) [61], which can be further metabolized through the tricarboxylic acid cycle to generate energy, or these metabolites can be deposited in the plasma [62, 63]. Indeed, increases in acetate but not acetaldehyde can be detected in human plasma after ethanol intake [64, 65] (Figure 1).

The efficiency of ethanol metabolism is dependent on the enzymatic activity and pathways involved. It has been reported that ADH, cytochrome P450 (CYP), and ALDH show genetic variations (ADH1B, ALDH2, CYP2E1*6, and CYP2E1*7B besides others) that affect enzymatic activity in the liver and alcohol metabolism [66–68]. As a result, ethanol's pharmacokinetic and pharmacodynamic properties are affected by this genetic variation, as reflected in interracial and ethnic pharmacological differences [56, 66–71]. Consequently the risk of developing diseases may increase in certain populations, including that of hypertension [70], alcohol dependence, and several types of alcohol-related cancer [60, 72–75].

After first pass metabolism, the remaining ethanol and its metabolites are distributed in different tissue, and the excess alcohol is excreted in the breath, urine, and sweat [56]. The distribution of ethanol throughout the body is driven in direct proportion to water content of each tissue, especially at the ethanol steady-state. Since ethanol is a small, polar molecule, the distribution volume of ethanol is dependent on the total body water of an individual (50 to 60% lean body weight) [76–78]. The variation in the distribution volume of ethanol has been evaluated for women and men, and in both sexes, the distribution volume decreases as the body mass index increases [79].

Alcohol-driven physiological changes, such as vascular effects (vasodilation) or changes in cardiac output, can also modify tissue blood flow and ethanol distribution [78]. Since the blood flow to the brain remains relatively constant, changes in the blood concentration of ethanol are the most relevant factor influencing the amount of ethanol delivered to the brain and therefore for the different levels of brain intoxication [78–80].

The distribution of ethanol is also particularly relevant during pregnancy, as 1–2 hours after maternal alcohol ingestion the fetal alcohol concentrations reach levels that are nearly equivalent to the maternal levels [81]. The elimination of ethanol by the fetus is impaired due to its reduced metabolic capacity. Thus, fetal exposure is prolonged through the reuptake of amniotic-fluid containing ethanol [81]. Ultimately, the elimination of alcohol from the fetus relies on the mother's metabolic capacity, which inevitably is a process that occurs late, meaning that the fetus is exposed to the toxicological effects of alcohol [82]. Therefore, many of the physical effects of ethanol on brain structure not only

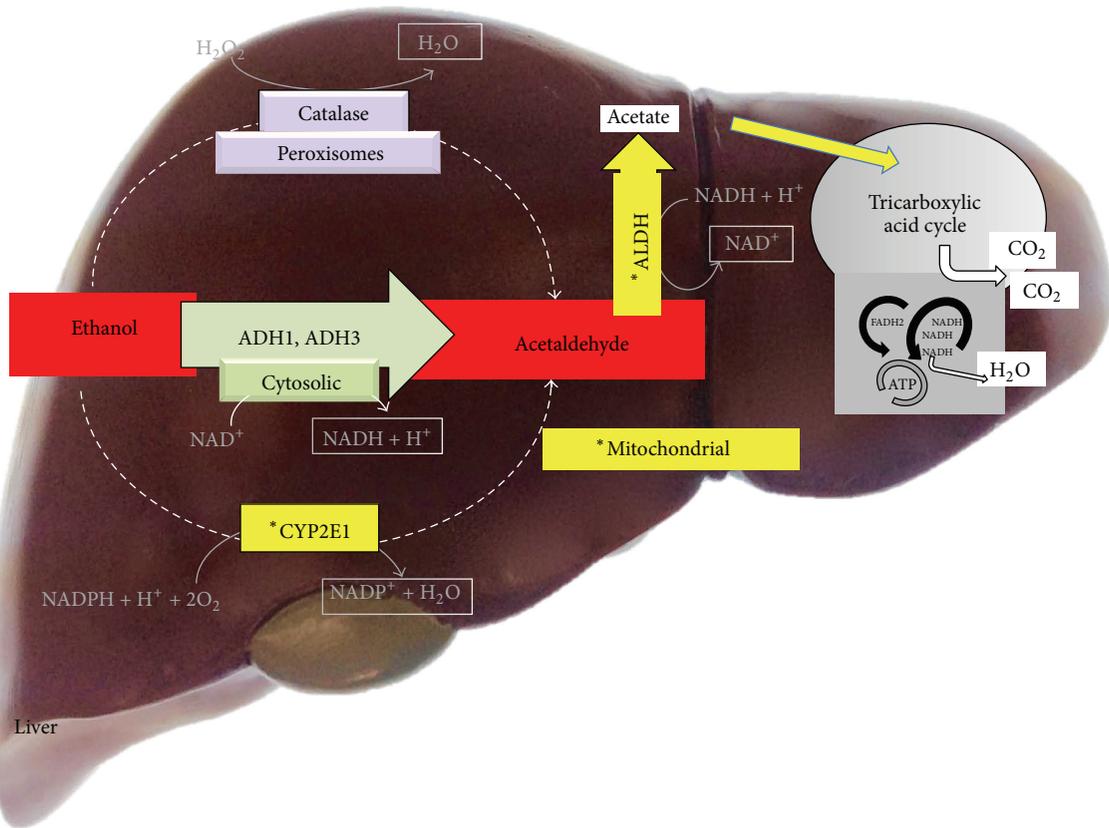


FIGURE 1: Mechanisms of ethanol metabolism in the liver. Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are the main enzymes that convert ethanol to acetate in the liver.

affect neurobehavioral features during fetal development but may also persist into childhood, potentially enduring until adulthood [82, 83].

3.3. Ethanol and Acetate Can Reach the Brain. Ethanol can cross the blood-brain barrier and it can be metabolized in the brain. Indeed, ethanol has been found in the human brain after alcohol intake [84], although metabolites of ethanol, like acetate, can also reach the brain as products of first pass metabolism [85]. Recently, the metabolism of [2-(13)C]-ethanol was evaluated in the brains of rats, and products such as labeled acetate, glutamate, glutamine, and GABA were detected found [86].

4. Metabolism of Ethanol and Acetate in the Brain

The oxidation of ethanol to acetaldehyde can occur in the brain through pathways that involve catalase, cytochrome CYP2E1, and ADH. The main pathway to metabolize ethanol in the liver is that involving ADH, although it has not been definitively shown to play a role in ethanol metabolism in the brain. In certain regions of the adult rat, mouse, and human brain it has been possible to identify ADH mRNA transcripts, with ADH1 and ADH4 expressed at distinct sites [87, 88], yet with no detectable activity after exposure to ethanol. Nonetheless, ADH4 inhibition avoids the synaptic dysfunction associated with severe alcohol intoxication in the

hippocampus [89]. Moreover ADH activity (ADH1, ADH3, and ADH4) was found in the human brain but under pathological process like brain cancer [73] and Alzheimer's disease [90], and not induced by alcohol intake. In addition, and despite fulfilling a less prominent role in ethanol metabolism [85, 91], ADHs have been related to enhanced voluntary alcohol intake in rats [92].

Other pathways metabolize ethanol in the brain. Catalase and CYP2E1 are the main pathways; there is evidence that they do indeed play an important role in ethanol oxidation to acetaldehyde in the brain [91]. Indeed, acetaldehyde production in the brain *in vivo* depends on catalase activity [85, 93] and catalase appears to be expressed in all neural cells. Peroxisomal catalase is a tetrameric, heme-containing enzyme that, in addition to converting hydrogen peroxide (H₂O₂) to water and oxygen, can also oxidize ethanol to acetaldehyde. The discovery of the catalase pathway for acetaldehyde formation in the brain represented an important first step in our understanding of the role of acetaldehyde in the effects of ethanol in the brain [94]. Studies using inhibitors of catalase and acatalasemic mice revealed that catalase is responsible for approximately half of the ethanol metabolism occurring in the CNS [91]. Indeed, inhibitors of catalase are also effective in inhibiting the production of acetaldehyde.

The cytochrome P450 enzymes (CYP2E1) that are involved in ethanol metabolism in the liver have also been implicated in its metabolism in the brain. CYP2E1 reduces

molecular oxygen to water and thus ethanol is oxidized to acetaldehyde. This enzyme is induced in response to chronic drinking and it may contribute to the increased rates of ethanol elimination in heavy drinkers. Some endogenous substrates for CYP2E1 include acetone and fatty acids, both of which are abundant in the brain [95]. The CYP2E1 system fulfills an important role in the generation of reactive oxygen species (ROS) and exposure to ethanol is related to the accumulation of ROS, which in rat brain homogenates may be attributed to the induction of CYP2E1 [96]. Not only ethanol but many other substrates are also metabolized by CYP2E1, including neurotoxins or procarcinogens, producing reactive intermediates [97, 98]. Moreover, in human neurons CYP2E1 is known to generate ROS and nitric oxide through the induction of NADPH/xanthine oxidase and nitric oxide synthase [99].

Therefore, CYP2E1 and catalase are the main pathways in the brain that metabolize ethanol to acetaldehyde, while ADH appears to play a minor role. Acetaldehyde is a biologically active compound and it has been implicated in alcohol addiction [100, 101], as well as inducing euphoria at low concentrations [102]. The effects of ethanol are modulated by acetaldehyde [100, 103], which in turn may react with endogenous substances to form other biologically active compounds. Acetaldehydes along with other proteins (adducts) were found in mice brain after alcohol consumption and in alcoholic human brains, suggesting they are involved in neural damage [104, 105]. Moreover adducts like salsolinol (formed when acetaldehyde binds to dopamine) were also seen to be involved in neurotoxicity [106] and in reinforcing addictive ethanol conduct [107]. Salsolinol has been identified in the brain and cerebrospinal fluid of patients with Parkinson disease, and it has been proposed to increase ROS production along with a reduction of glutathione [108], as well as reducing intracellular ATP and thereby acting as an inhibitor of mitochondrial energy supply. Thus, acetaldehyde reinforces its own effects or enhances the addictive action of ethanol [109, 110].

As a result, acetaldehyde oxidation is required for detoxification and it can be metabolized to acetate by ALDH [111]. ALDH is critically important and the risk of alcohol-induced toxicity in individuals with mutant ALDH2 increases remarkably [112], while ALDH2 overexpression diminishes alcohol-related ROS production [113]. However, the accumulation of NADH increases in association with ALDH activity [114] and if the NAD^+/NADH ratio decreases, the amounts of superoxide radicals increase [115, 116]. Moreover, although ALDH activity has beneficial effects, such as in the reduction of acetaldehyde, it also produces free radicals. Finally, the acetate produced by ALDH is metabolized in the Krebs cycle to produce energy or provide intermediaries for other molecules. Recent research showed that oxidation of $[^{13}\text{C}]$ -acetate generates specific neurotransmitters, as $[^{13}\text{C}]$ -glutamine, glutamate, and GABA levels were higher in chronic ethanol-exposed rats than in controls [86]. The production of these molecules may be related to the known effects of GABA receptors [16, 17, 19, 117], although other receptors are also involved in the effects of ethanol, such

as dopamine, acetylcholine, and NMDA receptors [118–120] (Figure 2).

5. Oxidative Stress Produced by Ethanol

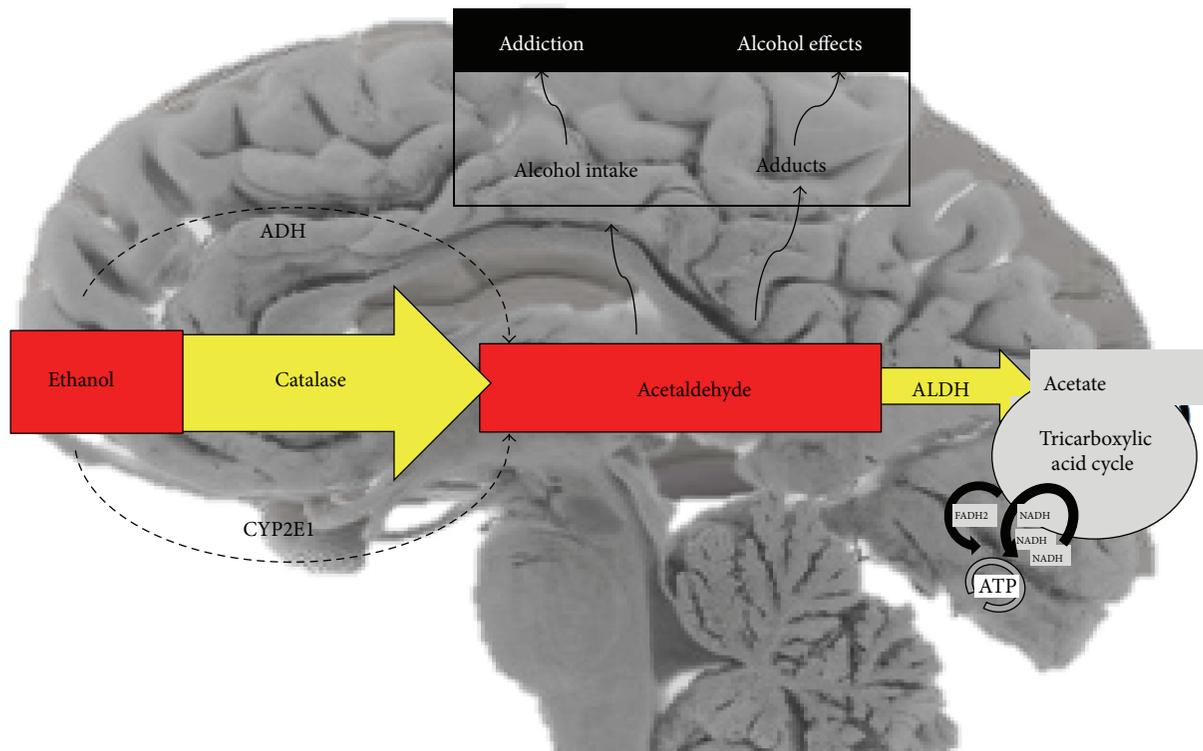
ROS are produced by exposure to ethanol [85] and they are associated with the effects of ethanol in the brain [92, 99, 101, 121–125], where ROS-related damage is due to oxidative stress [99, 124, 126–128]. The oxidative balance is a result of the amount of ROS that accumulates and the activity of antioxidant enzymes. In the brain, antioxidant enzymes are present in the cortex, cerebellum, hypothalamus, striatum, and spinal cord, and they include glutathione peroxidase, superoxide dismutase, glutathione reductase, and peroxiredoxin [129]. When the oxidative balance is disturbed, oxidative stress develops that affects the cell as a whole, as well as proteins, lipids, and DNA individually, provoking neurotoxicity or neurodegeneration.

6. The Antioxidant System and the Effects of Ethanol

The formation of ROS accompanies many physiological processes, such that the body has developed a system of antioxidant protection against their harmful effects. In the brain, where the generation of free radicals is particularly severe, it is essential that the antioxidant system functions correctly [130]. Antioxidant activity is considered as enzymatic or nonenzymatic based on the mechanism of action involved.

6.1. Superoxide Dismutase (SOD). It is an enzyme that catalyzes the dismutation of the superoxide anion to hydrogen peroxide, which is then decomposed by catalases primarily located in the peroxisomes. There are two main SOD isoenzymes found in the CNS of mammals: Mn-SOD (dependent on mitochondrial manganese ions) and Cu, Zn-SOD (SOD-1) present in the cytoplasm, microsomes, and synaptosomes [131]. Increased SOD activity is considered to be an adaptive response to oxidative stress, such as that induced by acute ethanol toxicity in the cerebral cortex [132]. However, acute ethanol intoxication reduces the activity of Cu, Zn-SOD in the cytosolic and microsomal fraction of the rat brain, and Mn-SOD activity in the mitochondria [131]. SOD interacts closely with catalase, which catalyzes the deprotonation of peroxide hydrogen and the oxidation of substances like methanol, ethanol, formate, nitrite, and quinones.

6.2. Catalase. In mammals, catalase is primarily located in the liver, erythrocytes, kidneys, and CNS. In the CNS, it can be found in microsomes [133] and it has been shown that, in acute ethanol poisoning, there is an increase of catalase activity in the cytosol, microsomes, and synaptosomes, as well as a reduction in the mitochondria of the rat CNS [131]. The increase in catalase activity following ethanol intake and its effects in the CNS are associated with weak ADH activity. This increase in catalase activity in the CNS may be adaptive processes induced by the increase in the hydrogen peroxide



Enzyme	ADH	Catalase	CYP2E1	ALDH
Principal role	Alcohol intake	Alcohol metabolism	Alcohol metabolism-ROS	Acetaldehyde metabolism

FIGURE 2: Enzymes related to ethanol metabolism in the brain and their principal role. Note the importance of acetaldehyde in ethanol metabolism.

generated, as what occurs in the CNS of animals exposed to high concentrations of ethanol [134].

6.3. *Glutathione Peroxidase (GSH-Px)*. It is present in many tissues, as well as in the neurons and glia of the CNS [135, 136]. The role of GSH-Px is limited to the reduction of peroxides in which glutathione participates, which is accompanied by the formation of glutathione disulfide. In the rat and human CNS, the greatest glutathione peroxidase activity is observed in the gray and white matter of the cerebral cortex [137, 138].

6.4. *Glutathione Reductase (GRed)*. It is an enzyme present in the cytosol and in the mitochondria of most cells, catalyzing the regeneration of reduced glutathione oxidation at the expense of NADPH. Most GRed activity is found in neurons and glial cells [139], and acute ethanol poisoning significantly dampens GRed activity in the cerebral cortex [140].

The activity of antioxidant enzymes is significantly altered in the CNS of animals chronically intoxicated with ethanol. The antioxidative capacity of the CNS also depends on exogenous antioxidants obtained by the organism through its dietary intake. The most important exogenous antioxidant

in the CNS is vitamin E, and both vitamin E and vitamin C content in the CNS falls after ethanol consumption, whereas vitamin A content increases [131].

7. Oxidized Fatty Acids as a Consequence of Oxidative Stress

Lipid peroxidation affects polyunsaturated fatty acids in membrane phospholipids as oxidative stress increases, producing bioactive aldehydes like 4-hydroxyalkenals and malondialdehyde [141]. Oxidative stress and the products of lipid peroxidation, 4-hydroxynonenal (HNE) [99, 142–145] or malondialdehyde [141, 146, 147], have been related to decreased neuronal viability in some studies. Ethanol-induced lipoperoxidation by oxidative stress [142] and its products decrease the intracellular reduced glutathione and increase its oxidized form [148]. HNE has also been associated with increases in mitochondrial permeability and cytochrome c release [143, 149, 150], the latter triggering apoptotic cell death by activating caspases [145, 150]. Interestingly, the toxicity mediated by the product of lipoperoxidation was weaker when glutathione transferase A4-4 activity

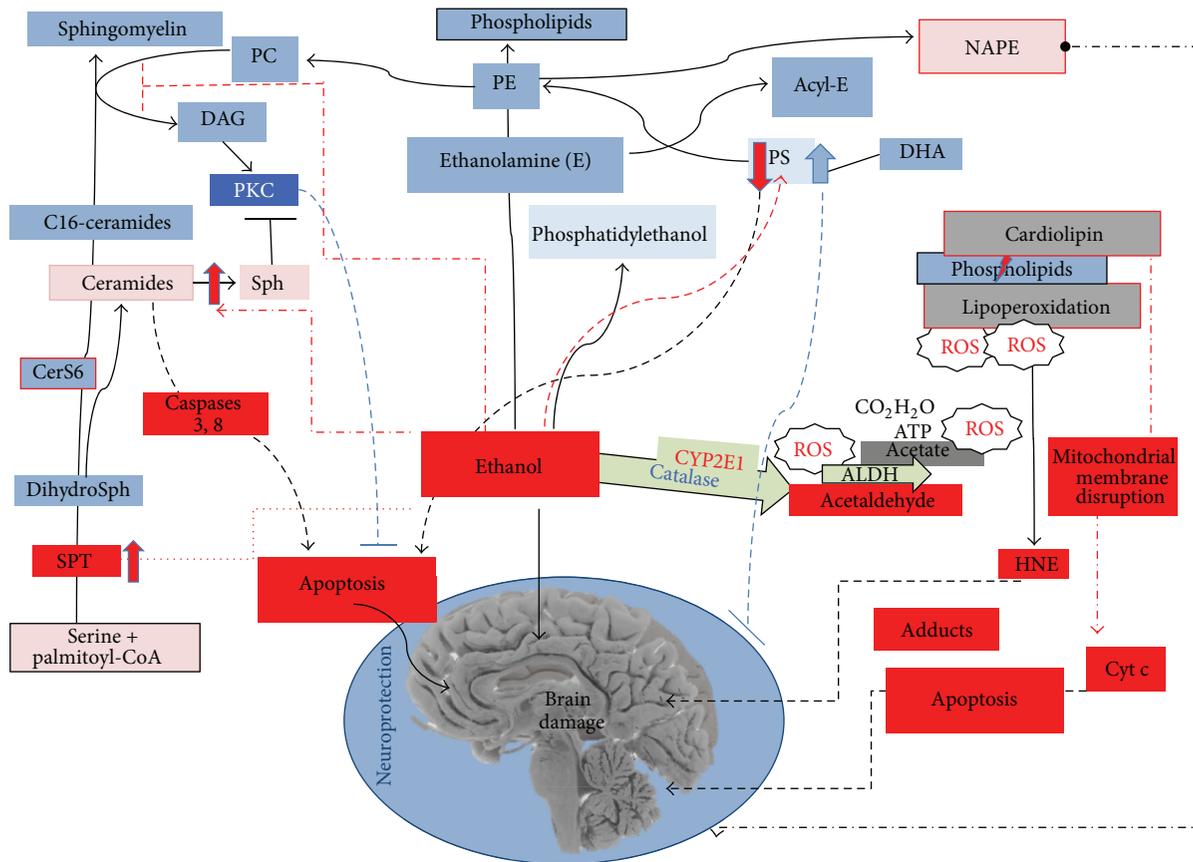


FIGURE 3: The role of lipids in ethanol-induced damage. Lipid metabolic pathways may be involved in neurodegeneration, such as lipoperoxidation, reduced phosphatidylserine (PS), N-acyl-PE (NAPE), and ceramide/Sph (sphingosine). Some lipids are produced as a compensatory mechanism and they fulfill a protective role, such as c16-ceramide, PS, sphingomyelin (SM), phosphatidyl ethanolamine (PE), and phosphatidylethanol.

was enhanced and glutathionyl-HNE was produced, avoiding the accumulation of HNE [150, 151] and possibly serving as a mechanism of tolerance. However, the activation of glutathione transferase A4-4 was suppressed in the presence of anionic phospholipids like cardiolipin [152]. Furthermore, the ability of HNE to produce glutathionyl-HNE was prevented by a PLA2 inhibitor [153], suggesting a role of PLA2 in the production of HNE.

8. The Role of Phospholipids in Stress Damage

Cardiolipin is a phospholipid and it is the major component of mitochondrial membranes, although ethanol-induced oxidative stress provokes a loss of this lipid [152, 154–157] in conjunction with the appearance of HNE [157, 158]. Therefore, cardiolipin oxidation occurs following ethanol ingestion and consequently its fatty acids are released from phospholipids by PLA2. When cardiolipin is affected by ethanol, mitochondrial function is impaired and the outer mitochondrial membrane may disintegrate [157, 159], which could induce the release of cytochrome c from the mitochondria and trigger an apoptotic cascade mediated by caspases [158, 160]. Interestingly, the neurodegeneration induced by ethanol can be prevented by an inhibitor of PLA2 *in vitro* [153, 161].

Phosphatidylserine (PS) has also been shown to play a role in apoptotic signaling, and both the reduction in PS and the enhanced neuronal cell death that ensues during the developmental period may contribute to the brain defects often observed in fetal alcohol syndrome [162]. Meanwhile, docosahexaenoic acid (DHA: 22:6n-3) prevents neuronal apoptosis by promoting PS accumulation [162], while conversely, PLA2 activity and oxidation-mediated HNE production may diminish the levels of PS.

9. Ceramide Related to Neurodegeneration

Ceramides are produced in the central nervous system by *de novo* synthesis or sphingomyelin hydrolysis [163]. Ceramide has been shown to accumulate in mitochondria upon the induction of apoptotic processes related to neurodegeneration [164–175]. The expression of serine palmitoyltransferase was localized in neurons and it was enhanced in caspase 3-positive neurons induced by ethanol [172], indicating that *de novo* ceramide synthesis participates in ethanol-induced apoptotic neurodegeneration in the brain. Although ceramide synthase 6 (CerS6) fulfills a protective role, this enzyme produces C16-ceramides and they are the precursors of other sphingolipids, such as sphingomyelin and glucosylceramide. Interestingly, CerS6 is enhanced within hours

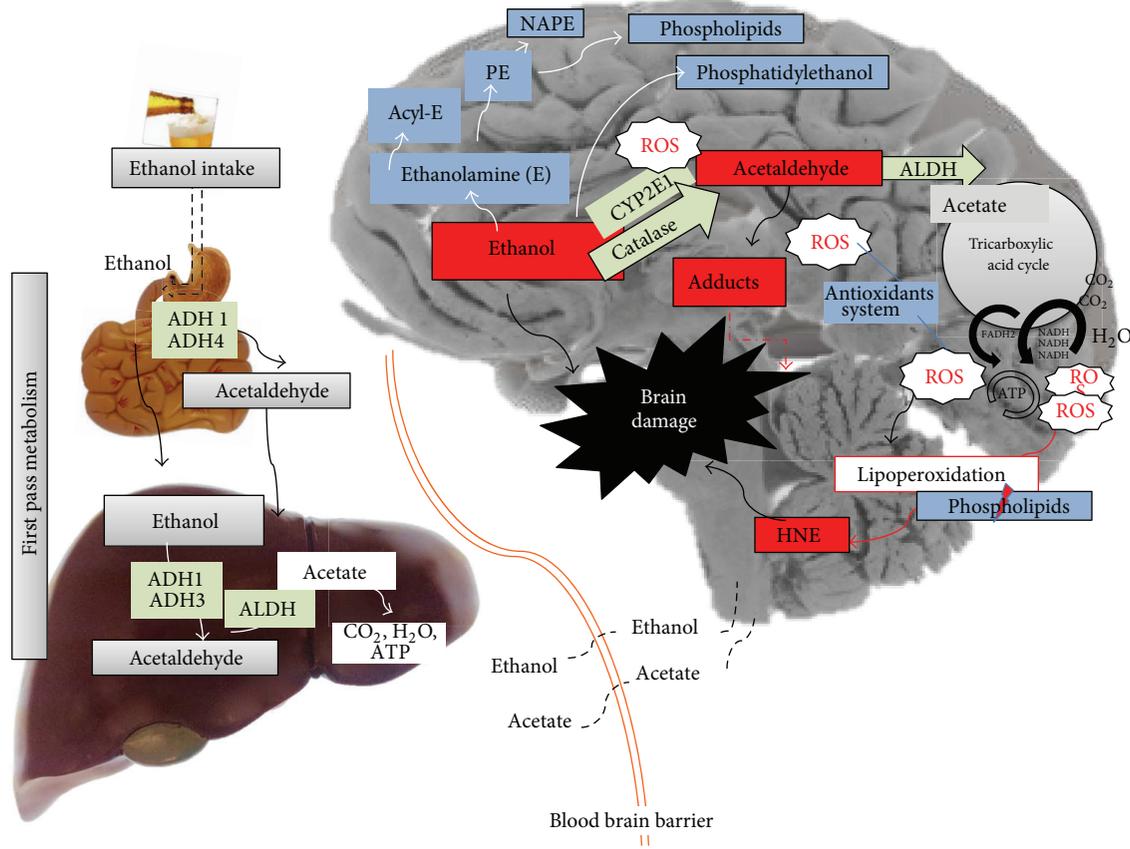


FIGURE 4: Oxidative stress and the role of lipids related to ethanol metabolism in the brain. Ethanol intake undergoes first pass metabolism in the stomach, intestine, and liver, although excess ethanol reaches the brain. Ethanol metabolism increases oxidative stress and lipid oxidation occurs, affecting mitochondrial membrane phospholipids and provoking cell death, thereby provoking damage in the brain. However ethanol-induced damage can be avoided by the activation of compensatory mechanisms involving lipids: E (ethanolamine), PE (phosphatidylethanolamine), acyl-E (acyl-ethanolamine), and NAPE (N-acyl-phosphatidylethanolamine).

of ethanol withdrawal as a compensatory effect [176]. In summary, ceramide is an apoptotic signal [173] but it is also necessary for the sphingomyelin synthesis required to produce diacylglycerol (DAG), which in turn activates PKC [177], thereby avoiding apoptosis [178].

10. Lipids Potentially Involved in the Compensatory Mechanisms Protecting against Ethanol-Induced Damage

While some lipids are altered to signal cells for destruction, others seem to offset some of the effects that occur due to oxidation. For example, there is more cholesterol in neuron membranes exposed to ethanol [155]. Cholesterol is known to provide rigidity to membranes and ethanol is effective in disrupting unstable lipid membranes. Hence, an increase in the cholesterol present in membranes may represent a compensatory mechanism to combat ethanol damage. Indeed, when mitochondrial cardiolipin is oxidized and its fatty acid released, membranes become unstable due to a loss of rigidity.

Other lipids can also reduce the availability or the effects of metabolites of ethanol, such as phosphatidylethanolamine, phosphatidylethanol, and acylethanolamine. Ethanol exposure augments the amount of phosphatidylethanolamine due

to the attachment of aminated ethanol to citidylphosphate [152, 179], resulting in the production of phosphatidylethanolamine through the Kennedy pathway [180]. Moreover, phosphatidylethanolamine can serve as a substrate for acyltransferases and indeed N-acylphosphoethanolamine (NAPE) is produced following ethanol exposure [168]. The amount of NAPE in membranes augments under cellular stress and as a result of tissue damage [181–184], and NAPE represents a precursor of the N-acylethanolamines [185] involved in learning and memory [186], neuroinflammation [187], oxidative stress, neuroprotection, and neurogenesis. Palmitoylethanolamine treatment of cultured cells produces neuroprotection against oxidative stress, impeding apoptosis [187–189] and protection in mice with chronic constriction injury [190]. Moreover, the endocannabinoid anandamide is also involved in neurodegeneration and thus acylethanolamines, and especially palmitoylethanolamine, appear to play an important role as neuroprotectors. Acylethanolamines can be found in the mitochondria *in vitro* [191] and palmitoyl requires carnitine to enter mitochondria. When cells or animals receive carnitine it acts as a neuroprotective agent, preventing ethanol-induced damage [147]. Furthermore, ω type-3 unsaturated fatty acids and DHA provide neuroprotection in conjunction with an increase in

the formation of acylethanolamine [161, 162], suggesting that the formation of the latter prevents the damage caused by the oxidative metabolism of ethanol. Finally, ethanol can also be metabolized as phosphatidylethanol, a molecule found in the brain of rats [192] that is possibly formed to avoid ethanol oxidation.

11. Conclusions

Lipid metabolism is clearly affected by exposure to ethanol (Figure 3), and the alterations to lipid components like cardiolipin and some phospholipids in response to ethanol provide evidence of cell damage. The formation of oxidized species, abnormal lipids, and dysfunctional membranes due to ethanol uptake also provokes cell degeneration. However, compensatory mechanisms exist to dampen the effects of these metabolic events and to minimize cell damage, as reflected by the neuroprotective activities of natural lipids like DHA, esters, vitamin E, and so forth. Thus, ethanol-induced neurodegeneration is at least partly the result of the equilibrium maintained between the toxicity of signaling lipids and the protection they confer on the cell (Figure 4).

Conflict of Interests

The authors have no conflict of interests to declare.

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References

- [1] K. G. Chartier, M. N. Hesselbrock, and V. M. Hesselbrock, "Ethnicity and gender comparisons of health consequences in adults with alcohol dependence," *Substance Use and Misuse*, vol. 48, no. 3, pp. 200–210, 2013.
- [2] K. J. Kiecolt, S. H. Aggen, and K. S. Kendler, "Genetic and environmental influences on the relationship between mastery and alcohol dependence," *Alcoholism: Clinical and Experimental Research*, vol. 37, no. 6, pp. 905–913, 2013.
- [3] S. Alaux-Cantin, V. Warnault, R. Legastelois et al., "Alcohol intoxications during adolescence increase motivation for alcohol in adult rats and induce neuroadaptations in the nucleus accumbens," *Neuropharmacology*, vol. 67, pp. 521–531, 2013.
- [4] M. Roerecke and J. Rehm, "Cause-specific mortality risk in alcohol use disorder treatment patients: a systematic review and meta-analysis," *International Journal of Epidemiology*, vol. 43, no. 3, Article ID dyu018, pp. 906–919, 2014.
- [5] F. Calhoun, M. L. Attilia, P. A. Spagnolo, C. Rotondo, R. Mancinelli, and M. Ceccanti, "National Institute on Alcohol Abuse and Alcoholism and the study of fetal alcohol spectrum disorders. The International Consortium," *Annali dell'Istituto Superiore di Sanita*, vol. 42, no. 1, pp. 4–7, 2006.
- [6] D. Dias, M. C. Mendonça, F. C. Real, D. N. Vieira, and H. M. Teixeira, "Suicides in the Centre of Portugal: seven years analysis," *Forensic Science International*, vol. 234, no. 1, pp. 22–28, 2014.
- [7] T. M. Wickizer, "State-level estimates of the economic costs of alcohol and drug abuse," *Journal of Health Care Finance*, vol. 39, no. 3, pp. 71–84, 2013.
- [8] G. Scally, "Crunch time for the government on alcohol pricing in England: backtracking on the minimum unit price pledge would be a public health disaster," *British Medical Journal*, vol. 346, no. 7900, Article ID f1784, 2013.
- [9] M. P. Mundt, S. Parthasarathy, F. W. Chi, S. Sterling, and C. I. Campbell, "12-Step participation reduces medical use costs among adolescents with a history of alcohol and other drug treatment," *Drug and Alcohol Dependence*, vol. 126, no. 1–2, pp. 124–130, 2012.
- [10] J. Rehm, C. Mathers, S. Popova, M. Thavorncharoensap, Y. Teerawattananon, and J. Patra, "Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders," *The Lancet*, vol. 373, no. 9682, pp. 2223–2233, 2009.
- [11] H. P. Jedema, M. D. Carter, B. P. Dugan, K. Gurnsey, A. S. Olsen, and C. W. Bradberry, "The acute impact of ethanol on cognitive performance in rhesus macaques," *Cerebral Cortex*, vol. 21, no. 8, pp. 1783–1791, 2011.
- [12] A. A.-B. Badawy, C. J. Morgan, J. W. T. Lowett, D. M. Bradley, and R. Thomas, "Decrease in circulating tryptophan availability to the brain after acute ethanol consumption by normal volunteers: implications for alcohol-induced aggressive behaviour and depression," *Pharmacopsychiatry*, vol. 28, supplement 2, pp. 93–97, 1995.
- [13] A. A.-B. Badawy, D. M. Dougherty, D. M. Marsh-Richard, and A. Steptoe, "Activation of liver tryptophan pyrrolase mediates the decrease in tryptophan availability to the brain after acute alcohol consumption by normal subjects," *Alcohol and Alcoholism*, vol. 44, no. 3, pp. 267–271, 2009.
- [14] S. Loeber and T. Duka, "Extinction learning of stimulus reward contingencies: the acute effects of alcohol," *Drug and Alcohol Dependence*, vol. 102, no. 1–3, pp. 56–62, 2009.
- [15] D. M. Dougherty, D. M. Marsh-Richard, E. S. Hatzis, S. O. Nouvion, and C. W. Mathias, "A test of alcohol dose effects on multiple behavioral measures of impulsivity," *Drug and Alcohol Dependence*, vol. 96, no. 1–2, pp. 111–120, 2008.
- [16] H. M. Haughey, L. A. Ray, P. Finan, R. Villanueva, M. Niculescu, and K. E. Hutchison, "Human γ -aminobutyric acid A receptor $\alpha 2$ gene moderates the acute effects of alcohol and brain mRNA expression," *Genes, Brain and Behavior*, vol. 7, no. 4, pp. 447–454, 2008.
- [17] A. Wadleigh and C. F. Valenzuela, "Ethanol increases GABAergic transmission and excitability in cerebellar molecular layer interneurons from GAD67-GFP knock-in mice," *Alcohol and Alcoholism*, vol. 47, no. 1, pp. 1–8, 2012.
- [18] V. Tiwari, P. Veeraiah, V. Subramaniam, and A. B. Patel, "Differential effects of ethanol on regional glutamatergic and GABAergic neurotransmitter pathways in mouse brain," *Journal of Neurochemistry*, vol. 128, no. 5, pp. 628–640, 2014.
- [19] G. Wu, H. Liu, J. Jin et al., "Ethanol attenuates sensory stimulus-evoked responses in cerebellar granule cells via activation of GABA_A receptors in vivo in mice," *Neuroscience Letters*, vol. 561, pp. 107–111, 2014.
- [20] H. L. Halliday, M. M. Reid, and G. McClure, "Results of heavy drinking in pregnancy," *British Journal of Obstetrics and Gynaecology*, vol. 89, no. 11, pp. 892–895, 1982.
- [21] J. R. West and C. A. Blake, "Fetal alcohol syndrome: an assessment of the field," *Experimental Biology and Medicine*, vol. 230, no. 6, pp. 354–356, 2005.

- [22] R. F. Berman and J. H. Hannigan, "Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy," *Hippocampus*, vol. 10, no. 1, pp. 94–110, 2000.
- [23] D. H. Lindquist, G. Sokoloff, E. Milner, and J. E. Steinmetz, "Neonatal ethanol exposure results in dose-dependent impairments in the acquisition and timing of the conditioned eyeblink response and altered cerebellar interpositus nucleus and hippocampal CA1 unit activity in adult rats," *Alcohol*, vol. 47, no. 6, pp. 447–457, 2013.
- [24] A. Ramezani, I. Goudarzi, T. Lashkarboluki, M. T. Ghorbanian, K. Abrari, and M. E. Salmani, "Role of oxidative stress in ethanol-induced neurotoxicity in the developing cerebellum," *Iranian Journal of Basic Medical Sciences*, vol. 15, no. 4, pp. 965–974, 2012.
- [25] J. D. Thomas, E. J. Abou, and H. D. Dominguez, "Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats," *Neurotoxicology and Teratology*, vol. 31, no. 5, pp. 303–311, 2009.
- [26] M. Krishnamoorthy, B. A. Gerwe, C. D. Scharer et al., "Low ethanol concentration alters CHRNA5 RNA levels during early human development," *Reproductive Toxicology*, vol. 30, no. 3, pp. 489–492, 2010.
- [27] A. Nardelli, C. Lebel, C. Rasmussen, G. Andrew, and C. Beaulieu, "Extensive deep gray matter volume reductions in children and adolescents with fetal alcohol spectrum disorders," *Alcoholism: Clinical and Experimental Research*, vol. 35, no. 8, pp. 1404–1417, 2011.
- [28] D. Kuehn, S. Aros, F. Cassorla et al., "A prospective cohort study of the prevalence of growth, facial, and central nervous system abnormalities in children with heavy prenatal alcohol exposure," *Alcoholism: Clinical and Experimental Research*, vol. 36, no. 10, pp. 1811–1819, 2012.
- [29] S. F. Tapert, C. Pulido, M. P. Paulus, M. A. Schuckit, and C. Burke, "Level of response to alcohol and brain response during visual working memory," *Journal of Studies on Alcohol*, vol. 65, no. 6, pp. 692–700, 2004.
- [30] D. B. Clark, D. L. Thatcher, and S. F. Tapert, "Alcohol, psychological dysregulation, and adolescent brain development," *Alcoholism: Clinical and Experimental Research*, vol. 32, no. 3, pp. 375–385, 2008.
- [31] S. F. Tapert, G. G. Brown, M. V. Baratta, and S. A. Brown, "fMRI BOLD response to alcohol stimuli in alcohol dependent young women," *Addictive Behaviors*, vol. 29, no. 1, pp. 33–50, 2004.
- [32] S. F. Tapert, A. D. Schweinsburg, V. C. Barlett et al., "Blood oxygen level dependent response and spatial working memory in adolescents with alcohol use disorders," *Alcoholism: Clinical and Experimental Research*, vol. 28, no. 10, pp. 1577–1586, 2004.
- [33] A. M. White, "What happened? Alcohol, memory blackouts, and the brain," *Alcohol Research and Health*, vol. 27, no. 2, pp. 186–196, 2003.
- [34] X. He, E. V. Sullivan, R. K. Stankovic, C. G. Harper, and A. Pfefferbaum, "Interaction of thiamine deficiency and voluntary alcohol consumption disrupts rat corpus callosum ultrastructure," *Neuropsychopharmacology*, vol. 32, no. 10, pp. 2207–2216, 2007.
- [35] S. A. Magrys and M. C. Olmstead, "Alcohol intoxication alters cognitive skills mediated by frontal and temporal brain regions," *Brain and Cognition*, vol. 85, pp. 271–276, 2014.
- [36] C. F. Zorumski, S. Mennerick, and Y. Izumi, "Acute and chronic effects of ethanol on learning-related synaptic plasticity," *Alcohol*, vol. 48, no. 1, pp. 1–17, 2014.
- [37] D. Erritzoe, A. Tziortzi, D. Bargiela et al., "In vivo imaging of cerebral dopamine D3 receptors in alcoholism," *Neuropsychopharmacology*, vol. 39, no. 7, pp. 1703–1712, 2014.
- [38] E. J. Burnett, K. A. Grant, A. T. Davenport, S. E. Hemby, and D. P. Friedman, "The effects of chronic ethanol self-administration on hippocampal 5-HT_{1A} receptors in monkeys," *Drug and Alcohol Dependence*, vol. 136, pp. 135–142, 2014.
- [39] R. P. Vetreno, J. M. Hall, and L. M. Savage, "Alcohol-related amnesia and dementia: animal models have revealed the contributions of different etiological factors on neuropathology, neurochemical dysfunction and cognitive impairment," *Neurobiology of Learning and Memory*, vol. 96, no. 4, pp. 596–608, 2011.
- [40] R. P. Vetreno, R. L. Ramos, S. Anzalone, and L. M. Savage, "Brain and behavioral pathology in an animal model of Wernicke's encephalopathy and Wernicke-Korsakoff syndrome," *Brain Research*, vol. 1436, pp. 178–192, 2012.
- [41] J. Akai and K. Akai, "Neuropathological study of the nucleus basalis of meynert in alcoholic dementia," *Arukuru Kenkyuto Yakubutsu Ison*, vol. 24, no. 2, pp. 80–88, 1989.
- [42] Z. Zhang, Y. Liu, B. Zhou et al., "Altered functional connectivity of the marginal division in Alzheimer's disease," *Current Alzheimer Research*, vol. 11, no. 2, pp. 145–155, 2014.
- [43] B. B. Andersen, "Reduction of Purkinje cell volume in cerebellum of alcoholics," *Brain Research*, vol. 1007, no. 1-2, pp. 10–18, 2004.
- [44] G. Halliday, K. Cullen, and A. Harding, "Neuropathological correlates of memory dysfunction in the Wernicke-Korsakoff syndrome," *Alcohol and Alcoholism Supplement*, vol. 2, pp. 245–251, 1994.
- [45] T. Jochum, M. K. Boettger, C. Burkhardt, G. Juckel, and K.-J. Bär, "Increased pain sensitivity in alcohol withdrawal syndrome," *European Journal of Pain*, vol. 14, no. 7, pp. 713–718, 2010.
- [46] M. Mizuguchi, M. Tomonaga, T. Fukusato, and M. Asano, "Acute necrotizing encephalopathy with widespread edematous lesions of symmetrical distribution," *Acta Neuropathologica*, vol. 78, no. 1, pp. 108–111, 1989.
- [47] L. Riethdorf, R. Warzok, and G. Schwesinger, "Alcoholic encephalopathies in autopsy material," *Zentralblatt für Pathologie*, vol. 137, no. 1, pp. 48–56, 1991.
- [48] V. A. Ramchandani, W. F. Bosron, and T. K. Li, "Research advances in ethanol metabolism," *Pathologie Biologie*, vol. 49, no. 9, pp. 676–682, 2001.
- [49] M. D. Levitt, R. Li, E. G. Demaster, M. Elson, J. Furne, and D. G. Levitt, "Use of measurements of ethanol absorption from stomach and intestine to assess human ethanol metabolism," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 273, no. 4, pp. G951–G957, 1997.
- [50] M. D. Levitt, C. Fine, J. K. Furne, and D. G. Levitt, "Use of maltose hydrolysis measurements to characterize the interaction between the aqueous diffusion barrier and the epithelium in the rat jejunum," *The Journal of Clinical Investigation*, vol. 97, no. 10, pp. 2308–2315, 1996.
- [51] K. S. Pang, "Modeling of intestinal drug absorption: roles of transporters and metabolic enzymes (for the Gillette review series)," *Drug Metabolism and Disposition*, vol. 31, no. 12, pp. 1507–1519, 2003.
- [52] C. M. Oneta, U. A. Simanowski, M. Martinez et al., "First pass metabolism of ethanol is strikingly influenced by the speed of gastric emptying," *Gut*, vol. 43, no. 5, pp. 612–619, 1998.

- [53] J. Shultz, H. Weiner, and J. Westcott, "Retardation of ethanol absorption by food in the stomach," *Journal of Studies on Alcohol*, vol. 41, no. 9, pp. 861–870, 1980.
- [54] A. J. Sedman, P. K. Wilkinson, E. Sakmar, D. J. Weidler, and J. G. Wagner, "Food effects on absorption and metabolism of alcohol," *Journal of Studies on Alcohol*, vol. 37, no. 9, pp. 1197–1214, 1976.
- [55] S.-L. Lee, G.-Y. Chau, C.-T. Yao, C.-W. Wu, and S.-J. Yin, "Functional assessment of human alcohol dehydrogenase family in ethanol metabolism: significance of first-pass metabolism," *Alcoholism: Clinical and Experimental Research*, vol. 30, no. 7, pp. 1132–1142, 2006.
- [56] Å. Norberg, W. A. Jones, R. G. Hahn, and J. L. Gabrielsson, "Role of variability in explaining ethanol pharmacokinetics: research and forensic applications," *Clinical Pharmacokinetics*, vol. 42, no. 1, pp. 1–31, 2003.
- [57] J. S. Deetz, C. A. Luehr, and B. L. Vallee, "Human liver alcohol dehydrogenase isozymes: reduction of aldehydes and ketones," *Biochemistry*, vol. 23, no. 26, pp. 6822–6828, 1984.
- [58] T. Haseba, Y. Tomita, M. Kurosu, and Y. Ohno, "Dose and time changes in liver alcohol dehydrogenase (ADH) activity during acute alcohol intoxication involve not only class I but also class III ADH and govern elimination rate of blood ethanol," *Legal Medicine*, vol. 5, no. 4, pp. 202–211, 2003.
- [59] T. Haseba, K. Kameyama, K. Mashimo, and Y. Ohno, "Dose-dependent change in elimination kinetics of ethanol due to shift of dominant metabolizing enzyme from ADH 1 (Class I) to ADH 3 (Class III) in mouse," *International Journal of Hepatology*, vol. 2012, Article ID 408190, 8 pages, 2012.
- [60] T.-K. Li, S.-J. Yin, D. W. Crabb, S. O'Connor, and V. A. Ramchandani, "Genetic and environmental influences on alcohol metabolism in humans," *Alcoholism: Clinical and Experimental Research*, vol. 25, no. 1, pp. 136–144, 2001.
- [61] T. N. Smolen, A. Smolen, and J. L. van de Kamp, "Developmental profile of hepatic alcohol and aldehyde dehydrogenase activities in long-sleep and short-sleep mice," *Alcohol*, vol. 7, no. 1, pp. 69–74, 1990.
- [62] F. Lundquist, N. Tygstrup, K. Winkler, K. Mellemgaard, and S. Munck-Petersen, "Ethanol metabolism and production of free acetate in the human liver," *The Journal of Clinical Investigation*, vol. 41, pp. 955–961, 1962.
- [63] K. O. Lindros, A. Stowell, P. Pikkarainen, and M. Salaspuro, "Elevated blood acetaldehyde in alcoholics with accelerated ethanol elimination," *Pharmacology Biochemistry and Behavior*, vol. 13, supplement 1, pp. 119–124, 1980.
- [64] J. G. Puig and I. H. Fox, "Ethanol-induced activation of adenine nucleotide turnover. Evidence for a role of acetate," *The Journal of Clinical Investigation*, vol. 74, no. 3, pp. 936–941, 1984.
- [65] T. Sarkola, M. R. Iles, K. Kohlenberg-Mueller, and C. J. P. Eriksson, "Ethanol, acetaldehyde, acetate, and lactate levels after alcohol intake in white men and women: effect of 4-methylpyrazole," *Alcoholism: Clinical and Experimental Research*, vol. 26, no. 2, pp. 239–245, 2002.
- [66] Z. Kayaalti and T. Soylemezoglu, "Distribution of *ADH1B*, *ALDH2*, *CYP2E1* *6, and *CYP2E1* *7B genotypes in Turkish population," *Alcohol*, vol. 44, no. 5, pp. 415–423, 2010.
- [67] A. J. Montano Loza, M. T. Ramirez Iglesias, I. Perez Diaz et al., "Association of alcohol-metabolizing genes with alcoholism in a Mexican Indian (Otomi) population," *Alcohol*, vol. 39, no. 2, pp. 73–79, 2006.
- [68] J. García-Bañuelos, A. Panduro, D. Gordillo-Bastidas et al., "Genetic polymorphisms of genes coding to alcohol-metabolizing enzymes in Western Mexicans: association of *CYP2E1* *c2/*CYP2E1* *5B allele with cirrhosis and liver function," *Alcoholism: Clinical and Experimental Research*, vol. 36, no. 3, pp. 425–431, 2012.
- [69] K. Y. Seng, L. M. G. Limenta, D. Heng, and E. J. D. Lee, "Population pharmacokinetics and pharmacogenetics of alcohol in Chinese and Indians in Singapore," *Journal of Clinical Pharmacy and Therapeutics*, vol. 38, no. 2, pp. 141–149, 2013.
- [70] Y. Yamada, F. Sun, I. Tsuritani, and R. Honda, "Genetic differences in ethanol metabolizing enzymes and blood pressure in Japanese alcohol consumers," *Journal of Human Hypertension*, vol. 16, no. 7, pp. 479–486, 2002.
- [71] A. Lorenzo, T. Auguet, F. Vidal et al., "Polymorphisms of alcohol-metabolizing enzymes and the risk for alcoholism and alcoholic liver disease in Caucasian Spanish women," *Drug and Alcohol Dependence*, vol. 84, no. 2, pp. 195–200, 2006.
- [72] Y.-C. Chen, G.-S. Peng, M.-F. Wang, T.-P. Tsao, and S.-J. Yin, "Polymorphism of ethanol-metabolism genes and alcoholism: correlation of allelic variations with the pharmacokinetic and pharmacodynamic consequences," *Chemico-Biological Interactions*, vol. 178, no. 1–3, pp. 2–7, 2009.
- [73] M. Laniewska-Dunaj, W. Jelski, K. Orywal, J. Kochanowicz, R. Rutkowski, and M. Szmitkowski, "The activity of class I, II, III and IV of alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) in brain cancer," *Neurochemical Research*, vol. 38, no. 7, pp. 1517–1521, 2013.
- [74] R. Sanchez-Alvarez, U. E. Martinez-Outschoorn, Z. Lin et al., "Ethanol exposure induces the cancer-associated fibroblast phenotype and lethal tumor metabolism: implications for breast cancer prevention," *Cell Cycle*, vol. 12, no. 2, pp. 289–301, 2013.
- [75] I. S. Chan, C. D. Guy, M. V. Machado et al., "Alcohol activates the hedgehog pathway and induces related procarcinogenic processes in the alcohol-preferring rat model of hepatocarcinogenesis," *Alcoholism: Clinical and Experimental Research*, vol. 38, no. 3, pp. 787–800, 2014.
- [76] J. M. Cowan Jr., A. Weathermon, J. R. McCutcheon, and R. D. Oliver, "Determination of volume of distribution for ethanol in male and female subjects," *Journal of Analytical Toxicology*, vol. 20, no. 5, pp. 287–290, 1996.
- [77] H. G. E. Endres and O. Grüner, "Comparison of D₂O and ethanol dilutions in total body water measurements in humans," *The Clinical Investigator*, vol. 72, no. 11, pp. 830–837, 1994.
- [78] A. F. Pizon, C. E. Becker, and D. Bikin, "The clinical significance of variations in ethanol toxicokinetics," *Journal of Medical Toxicology*, vol. 3, no. 2, pp. 63–72, 2007.
- [79] K. E. Maudens, L. Patteet, A. L. N. van Nuijs, C. Van Broekhoven, A. Covaci, and H. Neels, "The influence of the Body Mass Index (BMI) on the volume of distribution of ethanol," *Forensic Science International*, vol. 243, pp. 74–78, 2014.
- [80] M. Marxen, G. Gan, D. Schwarz et al., "Acute effects of alcohol on brain perfusion monitored with arterial spin labeling magnetic resonance imaging in young adults," *Journal of Cerebral Blood Flow and Metabolism*, vol. 34, no. 3, pp. 472–479, 2014.
- [81] L. Burd, J. Blair, and K. Dropps, "Prenatal alcohol exposure, blood alcohol concentrations and alcohol elimination rates for the mother, fetus and newborn," *Journal of Perinatology*, vol. 32, no. 9, pp. 652–659, 2012.
- [82] E. M. Moore and E. P. Riley, "What happens when children with fetal alcohol spectrum disorders become adults?" *Current Developmental Disorders Reports*, vol. 2, no. 3, pp. 219–227, 2015.

- [83] A. G. Skorput, V. P. Gupta, P. W. Yeh, and H. H. Yeh, "Persistent interneuronopathy in the prefrontal cortex of young adult offspring exposed to ethanol *in utero*," *The Journal of Neuroscience*, vol. 35, no. 31, pp. 10977–10988, 2015.
- [84] G. Fein and D. J. Meyerhoff, "Ethanol in human brain by magnetic resonance spectroscopy: correlation with blood and breath levels, relaxation, and magnetization transfer," *Alcoholism: Clinical and Experimental Research*, vol. 24, no. 8, pp. 1227–1235, 2000.
- [85] S. M. Zimatkin and A. I. Buben, "Ethanol oxidation in the living brain," *Alcohol and Alcoholism*, vol. 42, no. 6, pp. 529–532, 2007.
- [86] J. Wang, H. Du, X. Ma et al., "Metabolic products of [2–13C]ethanol in the rat brain after chronic ethanol exposure," *Journal of Neurochemistry*, vol. 127, no. 3, pp. 353–364, 2013.
- [87] S. E. Martínez, J. Vaglenova, J. Sabrià, M. Carmen Martínez, J. Farrés, and X. Parés, "Distribution of alcohol dehydrogenase mRNA in the rat central nervous system. Consequences for brain ethanol and retinoid metabolism," *European Journal of Biochemistry*, vol. 268, no. 19, pp. 5045–5056, 2001.
- [88] D. Galter, A. Carmine, S. Buervenich, G. Duester, and L. Olson, "Distribution of class I, III and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain," *European Journal of Biochemistry*, vol. 270, no. 6, pp. 1316–1326, 2003.
- [89] K. Tokuda, Y. Izumi, and C. F. Zorumski, "Locally-generated acetaldehyde contributes to the effects of ethanol on neurosteroids and LTP in the hippocampus," *Neurology and Clinical Neuroscience*, vol. 1, no. 4, pp. 138–147, 2013.
- [90] E. Borger, L. Aitken, H. Du, W. Zhang, F. J. Gunn-Moore, and S. S. Du Yan, "Is amyloid binding alcohol dehydrogenase a drug target for treating Alzheimer's disease?" *Current Alzheimer Research*, vol. 10, no. 1, pp. 21–29, 2013.
- [91] S. M. Zimatkin, S. P. Pronko, V. Vasiliou, F. J. Gonzalez, and R. A. Deitrich, "Enzymatic mechanisms of ethanol oxidation in the brain," *Alcoholism: Clinical and Experimental Research*, vol. 30, no. 9, pp. 1500–1505, 2006.
- [92] E. Karahanian, M. E. Quintanilla, L. Tampier et al., "Ethanol as a prodrug: brain metabolism of ethanol mediates its reinforcing effects," *Alcoholism: Clinical and Experimental Research*, vol. 35, no. 4, pp. 606–612, 2011.
- [93] D. E. Rhoads, C. Contreras, and S. Fathalla, "Brain levels of catalase remain constant through strain, developmental, and chronic alcohol challenges," *Enzyme Research*, vol. 2012, Article ID 572939, 6 pages, 2012.
- [94] C. M. G. Aragon, F. Rogan, and Z. Amit, "Ethanol metabolism in rat brain homogenates by a catalase-H₂O₂ system," *Biochemical Pharmacology*, vol. 44, no. 1, pp. 93–98, 1992.
- [95] C. S. Lieber, "Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968–1998)—a review," *Alcoholism: Clinical and Experimental Research*, vol. 23, no. 6, pp. 991–1007, 1999.
- [96] Y. Zhong, G. Dong, H. Luo et al., "Induction of brain CYP2E1 by chronic ethanol treatment and related oxidative stress in hippocampus, cerebellum, and brainstem," *Toxicology*, vol. 302, no. 2–3, pp. 275–284, 2012.
- [97] D. R. Koop, "Oxidative and reductive metabolism by cytochrome P450 2E1," *The FASEB Journal*, vol. 6, no. 2, pp. 724–730, 1992.
- [98] T. Leung, R. Rajendran, S. Singh, R. Garva, M. Krstic-Demonacos, and C. Demonacos, "Cytochrome P450 2E1 (CYP2E1) regulates the response to oxidative stress and migration of breast cancer cells," *Breast Cancer Research*, vol. 15, no. 6, article R107, 2013.
- [99] J. Haorah, S. H. Ramirez, N. Floreani, S. Gorantla, B. Morsey, and Y. Persidsky, "Mechanism of alcohol-induced oxidative stress and neuronal injury," *Free Radical Biology and Medicine*, vol. 45, no. 11, pp. 1542–1550, 2008.
- [100] G. A. Deehan Jr., S. R. Hauser, J. A. Wilden, W. A. Truitt, and Z. A. Rodd, "Elucidating the biological basis for the reinforcing actions of alcohol in the mesolimbic dopamine system: the role of active metabolites of alcohol," *Frontiers in Behavioral Neuroscience*, vol. 7, article 104, 2013.
- [101] X.-S. Deng and R. A. Deitrich, "Putative role of brain acetaldehyde in ethanol addiction," *Current Drug Abuse Reviews*, vol. 1, no. 1, pp. 3–8, 2008.
- [102] C. J. Eriksson, "The role of acetaldehyde in the actions of alcohol (update 2000)," *Alcoholism Clinical and Experimental Research*, vol. 25, no. 5, supplement, pp. 15s–32s, 2001.
- [103] A. I. Cederbaum, "Alcohol metabolism," *Clinics in Liver Disease*, vol. 16, no. 4, pp. 667–685, 2012.
- [104] K. Nakamura, K. Iwahashi, A. Furukawa et al., "Acetaldehyde adducts in the brain of alcoholics," *Archives of Toxicology*, vol. 77, no. 10, pp. 591–593, 2003.
- [105] K. Nakamura, K. Iwahashi, M. Itoh et al., "Immunohistochemical study on acetaldehyde adducts in alcohol-fed mice," *Alcoholism, Clinical and Experimental Research*, vol. 24, no. 4, supplement, pp. 93S–96S, 2000.
- [106] G. Xie, K. Krnjević, and J.-H. Ye, "Salsolinol modulation of dopamine neurons," *Frontiers in Behavioral Neuroscience*, vol. 7, article 52, 2013.
- [107] M. E. Quintanilla, M. Rivera-Meza, P. A. Berríos-Cárcamo et al., "Salsolinol, free of isosalsolinol, exerts ethanol-like motivational/sensitization effects leading to increases in ethanol intake," *Alcohol*, vol. 48, no. 6, pp. 551–559, 2014.
- [108] S. Wanpen, P. Govitrapong, S. Shavali, P. Sangchot, and M. Ebadi, "Salsolinol, a dopamine-derived tetrahydroisoquinoline, induces cell death by causing oxidative stress in dopaminergic SH-SY5Y cells, and the said effect is attenuated by metallothionein," *Brain Research*, vol. 1005, no. 1–2, pp. 67–76, 2004.
- [109] A. T. Peana and E. Acquasb, "Behavioral and biochemical evidence of the role of acetaldehyde in the motivational effects of ethanol," *Frontiers in Behavioral Neuroscience*, vol. 7, article 86, 2013.
- [110] M. E. Quintanilla, M. Rivera-Meza, P. Berríos-Cárcamo, B. K. Cassels, M. Herrera-Marschitz, and Y. Israel, "(R)-Salsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake," *Addiction Biology*, 2015.
- [111] A. Kikonyogo and R. Pietruszko, "Aldehyde dehydrogenase from adult human brain that dehydrogenates γ -aminobutyraldehyde: purification, characterization, cloning and distribution," *Biochemical Journal*, vol. 316, part 1, pp. 317–324, 1996.
- [112] Y. Zhang and J. Ren, "ALDH2 in alcoholic heart diseases: molecular mechanism and clinical implications," *Pharmacology and Therapeutics*, vol. 132, no. 1, pp. 86–95, 2011.
- [113] J. Bai and Y. Mei, "Overexpression of aldehyde dehydrogenase-2 attenuates neurotoxicity induced by 4-hydroxynonenal in cultured primary hippocampal neurons," *Neurotoxicity Research*, vol. 19, no. 3, pp. 412–422, 2011.
- [114] S. Manzo-Avalos and A. Saavedra-Molina, "Cellular and mitochondrial effects of alcohol consumption," *International Journal of Environmental Research and Public Health*, vol. 7, no. 12, pp. 4281–4304, 2010.

- [115] J. Antosiewicz, J. H. Spodnik, M. Teranishi et al., "NADH-generating substrates reduce peroxyl radical toxicity in RL-34 cells," *Folia Morphologica*, vol. 68, no. 4, pp. 247–255, 2009.
- [116] M. Velayutham, C. Hemann, and J. L. Zweier, "Removal of H₂O₂ and generation of superoxide radical: role of cytochrome c and NADH," *Free Radical Biology and Medicine*, vol. 51, no. 1, pp. 160–170, 2011.
- [117] B. M. Walker and G. F. Koob, "The γ -aminobutyric acid-B receptor agonist baclofen attenuates responding for ethanol in ethanol-dependent rats," *Alcoholism: Clinical and Experimental Research*, vol. 31, no. 1, pp. 11–18, 2007.
- [118] E. B. Rex, M. L. Rankin, M. A. Ariano, and D. R. Sibley, "Ethanol regulation of D₁ dopamine receptor signaling is mediated by protein kinase C in an isozyme-specific manner," *Neuropsychopharmacology*, vol. 33, no. 12, pp. 2900–2911, 2008.
- [119] L. M. Hendrickson, P. Gardner, and A. R. Tapper, "Nicotinic acetylcholine receptors containing the α 4 subunit are critical for the nicotine-induced reduction of acute voluntary ethanol consumption," *Channels*, vol. 5, no. 2, pp. 124–127, 2011.
- [120] E. Villegas, R. Estruch, G. Mengod, and R. Cortés, "NMDA receptors in frontal cortex and hippocampus of alcohol consumers," *Addiction Biology*, vol. 16, no. 1, pp. 163–165, 2011.
- [121] M. Correa, M. N. Arizzi, A. Betz, S. Mingote, and J. D. Salamone, "Open field locomotor effects in rats after intraventricular injections of ethanol and the ethanol metabolites acetaldehyde and acetate," *Brain Research Bulletin*, vol. 62, no. 3, pp. 197–202, 2003.
- [122] N. D. Volkow, S. W. Kim, G.-J. Wang et al., "Acute alcohol intoxication decreases glucose metabolism but increases acetate uptake in the human brain," *NeuroImage*, vol. 64, no. 1, pp. 277–283, 2013.
- [123] G. U. Corsini, A. Zuddas, U. Bonuccelli, S. Schinelli, and I. J. Kopin, "1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity in mice is enhanced by ethanol or acetaldehyde," *Life Sciences*, vol. 40, no. 9, pp. 827–832, 1987.
- [124] L. Heap, R. J. Ward, C. Abiaka et al., "The influence of brain acetaldehyde on oxidative status, dopamine metabolism and visual discrimination task," *Biochemical Pharmacology*, vol. 50, no. 2, pp. 263–270, 1995.
- [125] J. Y. Wan, J. Y. Wang, Y. Wang, and J. Y. Wang, "A comparison between acute exposures to ethanol and acetaldehyde on neurotoxicity, nitric oxide production and NMDA-induced excitotoxicity in primary cultures of cortical neurons," *The Chinese Journal of Physiology*, vol. 43, no. 3, pp. 131–138, 2000.
- [126] F. T. Crews and K. Nixon, "Mechanisms of neurodegeneration and regeneration in alcoholism," *Alcohol and Alcoholism*, vol. 44, no. 2, pp. 115–127, 2009.
- [127] C. Ikonomidou and A. M. Kaindl, "Neuronal death and oxidative stress in the developing brain," *Antioxidants and Redox Signaling*, vol. 14, no. 8, pp. 1535–1550, 2011.
- [128] J. Luo, "Mechanisms of ethanol-induced death of cerebellar granule cells," *Cerebellum*, vol. 11, no. 1, pp. 145–154, 2012.
- [129] S.-Y. Shim and H.-S. Kim, "Oxidative stress and the antioxidant enzyme system in the developing brain," *Korean Journal of Pediatrics*, vol. 56, no. 3, pp. 107–111, 2013.
- [130] A. Augustyniak, K. Michalak, and E. Skrzydlewska, "The action of oxidative stress induced by ethanol on the central nervous system (CNS)," *Postępy Higieny i Medycyny Doświadczalnej*, vol. 59, pp. 464–471, 2005.
- [131] S. K. Reddy, K. Husain, E. C. Schlorff, R. B. Scott, and S. M. Somani, "Dose response of ethanol ingestion on antioxidant defense system in rat brain subcellular fractions," *NeuroToxicology*, vol. 20, no. 6, pp. 977–987, 1999.
- [132] M. Enache, V. Van Waes, E. Vinner, M. Lhermitte, S. Maccari, and M. Darnaudéry, "Impact of an acute exposure to ethanol on the oxidative stress status in the hippocampus of prenatal restraint stress adolescent male rats," *Brain Research*, vol. 1191, pp. 55–62, 2008.
- [133] L. Hipolito, M. J. Sanchez, A. Polache, and L. Granero, "Brain metabolism of ethanol and alcoholism: an update," *Current Drug Metabolism*, vol. 8, no. 7, pp. 716–727, 2007.
- [134] Y. Fukui and H. Sakata-Haga, "Intrauterine environment-genome interaction and children's development (1): ethanol: a teratogen in developing brain," *Journal of Toxicological Sciences*, vol. 34, supplement 2, pp. SP273–SP278, 2009.
- [135] F. Bosch-Morell, F. Martínez-Soriano, A. Colell, J. C. Fernández-Checa, and F. J. Romero, "Chronic ethanol feeding induces cellular antioxidants decrease and oxidative stress in rat peripheral nerves. Effect of S-adenosyl-L-methionine and N-acetyl-L-cysteine," *Free Radical Biology and Medicine*, vol. 25, no. 3, pp. 365–368, 1998.
- [136] M. Chalimoniuk, S. Jagsz, E. Sadowska-Krepa, S. J. Chrapusta, B. Klapinska, and J. Langfort, "Diversity of endurance training effects on antioxidant defenses and oxidative damage in different brain regions of adolescent male rats," *Journal of Physiology and Pharmacology*, vol. 66, no. 4, pp. 539–547, 2015.
- [137] K. A. Ansari, D. Bigelow, and E. Kaplan, "Glutathione peroxidase activity in surgical and autopsied human brains," *Neurochemical Research*, vol. 10, no. 5, pp. 703–711, 1985.
- [138] J. Huang and M. A. Philbert, "Distribution of glutathione and glutathione-related enzyme systems in mitochondria and cytosol of cultured cerebellar astrocytes and granule cells," *Brain Research*, vol. 680, no. 1-2, pp. 16–22, 1995.
- [139] R. Dringen and J. Hirrlinger, "Glutathione pathways in the brain," *Biological Chemistry*, vol. 384, no. 4, pp. 505–516, 2003.
- [140] S. M. Somani and K. Husain, "Interaction of exercise training and chronic ethanol ingestion on antioxidant system of rat brain regions," *Journal of Applied Toxicology*, vol. 17, no. 5, pp. 329–336, 1997.
- [141] S. Pizzimenti, E. Ciamporcerro, M. Daga et al., "Interaction of aldehydes derived from lipid peroxidation and membrane proteins," *Frontiers in Physiology*, vol. 4, article 242, 2013.
- [142] J. J. Chen, S. Schenker, and G. I. Henderson, "4-Hydroxynonenal levels are enhanced in fetal liver mitochondria by in utero ethanol exposure," *Hepatology*, vol. 25, no. 1, pp. 142–147, 1997.
- [143] V. Ramachandran, A. Perez, J. Chen, D. Senthil, S. Schenker, and G. I. Henderson, "In utero ethanol exposure causes mitochondrial dysfunction, which can result in apoptotic cell death in fetal brain: a potential role for 4-hydroxynonenal," *Alcoholism: Clinical and Experimental Research*, vol. 25, no. 6, pp. 862–871, 2001.
- [144] V. Ramachandran, L. T. Watts, S. K. Maffi, J. Chen, S. Schenker, and G. I. Henderson, "Ethanol-induced oxidative stress precedes mitochondrially mediated apoptotic death of cultured fetal cortical neurons," *Journal of Neuroscience Research*, vol. 74, no. 4, pp. 577–588, 2003.
- [145] S. K. Maffi, M. L. Rathinam, P. P. Cherian et al., "Glutathione content as a potential mediator of the vulnerability of cultured fetal cortical neurons to ethanol-induced apoptosis," *Journal of Neuroscience Research*, vol. 86, no. 5, pp. 1064–1076, 2008.
- [146] A. Rendón-Ramírez, M. Cortés-Couto, A. B. Martínez-Rizo, S. Muñoz-Hernández, and J. B. Velázquez-Fernández, "Oxidative

- damage in young alcohol drinkers: a preliminary study," *Alcohol*, vol. 47, no. 7, pp. 501–504, 2013.
- [147] A. Augustyniak and E. Skrzydlewska, "The influence of L-carnitine supplementation on the antioxidative abilities of serum and the central nervous system of ethanol-induced rats," *Metabolic Brain Disease*, vol. 25, no. 4, pp. 381–389, 2010.
- [148] W. Liu, M. Kato, A. A. Akhand et al., "4-hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death," *Journal of Cell Science*, vol. 113, no. part 4, pp. 635–641, 2000.
- [149] A. Mahr, F. Batteux, S. Tubiana et al., "Brief report: prevalence of antineutrophil cytoplasmic antibodies in infective endocarditis," *Arthritis & Rheumatology*, vol. 66, no. 6, pp. 1672–1677, 2014.
- [150] H. Raza and A. John, "4-Hydroxynonenal induces mitochondrial oxidative stress, apoptosis and expression of glutathione S-transferase A4-4 and cytochrome P450 2E1 in PC12 cells," *Toxicology and Applied Pharmacology*, vol. 216, no. 2, pp. 309–318, 2006.
- [151] K. E. McElhanon, C. Bose, R. Sharma, L. Wu, Y. C. Awasthi, and S. P. Singh, "4 null mouse embryonic fibroblasts exhibit enhanced sensitivity to oxidants: role of 4-hydroxynonenal in oxidant toxicity," *Open Journal of Apoptosis*, vol. 2, no. 1, pp. 1–11, 2013.
- [152] M. Shimoji, N. Imaizumi, and Y. Aniya, "Modulation of membrane-bound glutathione transferase activity by phospholipids including cardiolipin," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 2, pp. 209–213, 2011.
- [153] K.-H. Moon, N. Tajuddin, J. Brown, E. J. Neafsey, H.-Y. Kim, and M. A. Collins, "Phospholipase A2, oxidative stress, and neurodegeneration in binge ethanol-treated organotypic slice cultures of developing rat brain," *Alcoholism: Clinical and Experimental Research*, vol. 38, no. 1, pp. 161–169, 2014.
- [154] M. A. C. Rodrigues, J. L. Rodrigues, N. M. Martins et al., "Carvedilol protects against cisplatin-induced oxidative stress, redox state unbalance and apoptosis in rat kidney mitochondria," *Chemico-Biological Interactions*, vol. 189, no. 1-2, pp. 45–51, 2011.
- [155] G. Barceló-Coblijn, L. E. Wold, J. Ren, and E. J. Murphy, "Prenatal ethanol exposure increases brain cholesterol content in adult rats," *Lipids*, vol. 48, no. 11, pp. 1059–1068, 2013.
- [156] V. D. Reddy, P. Padmavathi, G. Kavitha, B. Saradamma, and N. Varadacharyulu, "Alcohol-induced oxidative/nitrosative stress alters brain mitochondrial membrane properties," *Molecular and Cellular Biochemistry*, vol. 375, no. 1-2, pp. 39–47, 2013.
- [157] G. Petrosillo, P. Portincasa, I. Grattagliano et al., "Mitochondrial dysfunction in rat with nonalcoholic fatty liver. Involvement of complex I, reactive oxygen species and cardiolipin," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1767, no. 10, pp. 1260–1267, 2007.
- [158] W. Liu, N. A. Porter, C. Schneider, A. R. Brash, and H. Yin, "Formation of 4-hydroxynonenal from cardiolipin oxidation: intramolecular peroxy radical addition and decomposition," *Free Radical Biology and Medicine*, vol. 50, no. 1, pp. 166–178, 2011.
- [159] E. Rubin and H. Rottenberg, "Ethanol-induced injury and adaptation in biological membranes," *Federation Proceedings*, vol. 41, no. 8, pp. 2465–2471, 1982.
- [160] Y. A. Vladimirov, E. V. Proskurnina, and A. V. Alekseev, "Molecular mechanisms of apoptosis. Structure of cytochrome c-cardiolipin complex," *Biochemistry*, vol. 78, no. 10, pp. 1086–1097, 2013.
- [161] J. Brown III, N. Achille, E. J. Neafsey, and M. A. Collins, "Binge ethanol-induced neurodegeneration in rat organotypic brain slice cultures: effects of PLA2 inhibitor mepacrine and Docosahexaenoic Acid (DHA)," *Neurochemical Research*, vol. 34, no. 2, pp. 260–267, 2009.
- [162] M. Akbar, J. Baick, F. Calderon, Z. Wen, and H.-Y. Kim, "Ethanol promotes neuronal apoptosis by inhibiting phosphatidylserine accumulation," *Journal of Neuroscience Research*, vol. 83, no. 3, pp. 432–440, 2006.
- [163] C. R. Horres and Y. A. Hannun, "The roles of neutral sphingomyelinases in neurological pathologies," *Neurochemical Research*, vol. 37, no. 6, pp. 1137–1149, 2012.
- [164] M. Pascual, S. L. Valles, J. Renau-Piqueras, and C. Guerri, "Ceramide pathways modulate ethanol-induced cell death in astrocytes," *Journal of Neurochemistry*, vol. 87, no. 6, pp. 1535–1545, 2003.
- [165] M. Saito, M. Saito, T. B. Cooper, and C. Vadasz, "Ethanol-induced changes in the content of triglycerides, ceramides, and glucosylceramides in cultured neurons," *Alcoholism: Clinical and Experimental Research*, vol. 29, no. 8, pp. 1374–1383, 2005.
- [166] B. Schatter, S. Jin, K. Löffelholz, and J. Klein, "Cross-talk between phosphatidic acid and ceramide during ethanol-induced apoptosis in astrocytes," *BMC Pharmacology*, vol. 5, article 3, 2005.
- [167] S. Dasgupta, J. A. Adams, and E. L. Hogan, "Maternal alcohol consumption increases sphingosine levels in the brains of progeny mice," *Neurochemical Research*, vol. 32, no. 12, pp. 2217–2224, 2007.
- [168] M. Saito, G. Chakraborty, R.-F. Mao et al., "Ethanol alters lipid profiles and phosphorylation status of AMP-activated protein kinase in the neonatal mouse brain," *Journal of Neurochemistry*, vol. 103, no. 3, pp. 1208–1218, 2007.
- [169] N. A. Babenko and E. G. Shakhova, "Effects of flavonoids on sphingolipid turnover in the toxin-damaged liver and liver cells," *Lipids in Health and Disease*, vol. 7, article 1, 2008.
- [170] S. A. Novgorodov, T. I. Guduz, and L. M. Obeid, "Long-chain ceramide is a potent inhibitor of the mitochondrial permeability transition pore," *The Journal of Biological Chemistry*, vol. 283, no. 36, pp. 24707–24717, 2008.
- [171] S. M. de La Monte, L. Longato, M. Tong, S. Denucci, and J. R. Wands, "The liver-brain axis of alcohol-mediated neurodegeneration: role of toxic lipids," *International Journal of Environmental Research and Public Health*, vol. 6, no. 7, pp. 2055–2075, 2009.
- [172] M. Saito, G. Chakraborty, M. Hegde et al., "Involvement of ceramide in ethanol-induced apoptotic neurodegeneration in the neonatal mouse brain," *Journal of Neurochemistry*, vol. 115, no. 1, pp. 168–177, 2010.
- [173] G. Wang and E. Bieberich, "Prenatal alcohol exposure triggers ceramide-induced apoptosis in neural crest-derived tissues concurrent with defective cranial development," *Cell Death and Disease*, vol. 1, no. 5, article e46, 2010.
- [174] T.-X. Deng, Z.-X. Wang, X.-Q. Gao et al., "Alcohol-induced proliferation of neurons in mouse hippocampal dentate gyrus: a possible role of ceramide," *Sheng Li Xue Bao*, vol. 63, no. 6, pp. 479–490, 2011.
- [175] T. Ramirez, L. Longato, M. Tong, J. R. Wands, S. M. de la Monte, and M. Dostalek, "Insulin resistance, ceramide accumulation and endoplasmic reticulum stress in experimental chronic alcohol-induced steatohepatitis," *Alcohol and Alcoholism*, vol. 48, no. 1, pp. 39–52, 2013.

- [176] M. Bae, V. V. R. Bandaru, N. Patel, and N. J. Haughey, "Ceramide metabolism analysis in a model of binge drinking reveals both neuroprotective and toxic effects of ethanol," *Journal of Neurochemistry*, vol. 131, no. 5, pp. 645–654, 2014.
- [177] J. Cerbón and R. D. C. López-Sánchez, "Diacylglycerol generated during sphingomyelin synthesis is involved in protein kinase C activation and cell proliferation in Madin-Darby canine kidney cells," *Biochemical Journal*, vol. 373, no. 3, pp. 917–924, 2003.
- [178] S. A. Novgorodov, D. A. Chudakova, B. W. Wheeler et al., "Developmentally regulated ceramide synthase 6 increases mitochondrial Ca²⁺ loading capacity and promotes apoptosis," *The Journal of Biological Chemistry*, vol. 286, no. 6, pp. 4644–4658, 2011.
- [179] Z. Wen and H.-Y. Kim, "Alterations in hippocampal phospholipid profile by prenatal exposure to ethanol," *Journal of Neurochemistry*, vol. 89, no. 6, pp. 1368–1377, 2004.
- [180] F. Gibellini and T. K. Smith, "The Kennedy pathway—de novo synthesis of phosphatidylethanolamine and phosphatidylcholine," *IUBMB Life*, vol. 62, no. 6, pp. 414–428, 2010.
- [181] S. Subbanna, M. Shivakumar, D. Psychoyos, S. Xie, and B. S. Basavarajappa, "Anandamide-CB1 receptor signaling contributes to postnatal ethanol-induced neonatal neurodegeneration, adult synaptic, and memory deficits," *The Journal of Neuroscience*, vol. 33, no. 15, pp. 6350–6366, 2013.
- [182] R. Taléns-Visconti, I. Sanchez-Vera, J. Kostic et al., "Neural differentiation from human embryonic stem cells as a tool to study early brain development and the neuroteratogenic effects of ethanol," *Stem Cells and Development*, vol. 20, no. 2, pp. 327–339, 2011.
- [183] J. Suárez, Y. Romero-Zerbo, L. Márquez et al., "Ulcerative colitis impairs the acylethanolamide-based anti-inflammatory system reversal by 5-aminosalicylic acid and glucocorticoids," *PLoS ONE*, vol. 7, no. 5, Article ID e37729, 2012.
- [184] A. K. Gebeh, J. M. Willets, M. Bari et al., "Elevated anandamide and related *N*-acylethanolamine levels occur in the peripheral blood of women with ectopic pregnancy and are mirrored by changes in peripheral fatty acid amide hydrolase activity," *Journal of Clinical Endocrinology and Metabolism*, vol. 98, no. 3, pp. 1226–1234, 2013.
- [185] K. Tsuboi, Y. Okamoto, N. Ikematsu et al., "Enzymatic formation of *N*-acylethanolamines from *N*-acylethanolamine plasmalogen through *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D-dependent and -independent pathways," *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids*, vol. 1811, no. 10, pp. 565–577, 2011.
- [186] N. Maroof, M. C. Pardon, and D. A. Kendall, "Endocannabinoid signalling in Alzheimer's disease," *Biochemical Society Transactions*, vol. 41, no. 6, pp. 1583–1587, 2013.
- [187] P. Garg, R. S. Duncan, S. Kaja, and P. Koulen, "Intracellular mechanisms of *N*-acylethanolamine-mediated neuroprotection in a rat model of stroke," *Neuroscience*, vol. 166, no. 1, pp. 252–262, 2010.
- [188] G. Lombardi, G. Miglio, F. Varsaldi, A. Minassi, and G. Appendino, "Oxyhomologation of the amide bond potentiates neuroprotective effects of the endolipid *N*-palmitoylethanolamine," *Journal of Pharmacology and Experimental Therapeutics*, vol. 320, no. 2, pp. 599–606, 2007.
- [189] R. S. Duncan, K. D. Chapman, and P. Koulen, "The neuroprotective properties of palmitoylethanolamine against oxidative stress in a neuronal cell line," *Molecular Neurodegeneration*, vol. 4, no. 1, article 50, 2009.
- [190] L. Di Cesare Mannelli, G. D'Agostino, A. Pacini et al., "Palmitoylethanolamide is a disease-modifying agent in peripheral neuropathy: pain relief and neuroprotection share a PPAR- α -mediated mechanism," *Mediators of Inflammation*, vol. 2013, Article ID 328797, 12 pages, 2013.
- [191] R. S. Duncan, H. Xin, D. L. Goad, K. D. Chapman, and P. Koulen, "Protection of neurons in the retinal ganglion cell layer against excitotoxicity by the *N*-acylethanolamine, *N*-linoleoylethanolamine," *Clinical Ophthalmology*, vol. 5, no. 1, pp. 543–548, 2011.
- [192] C. Alling, L. Gustavsson, J.-E. Månsson, G. Benthin, and E. Änggård, "Phosphatidylethanol formation in rat organs after ethanol treatment," *Biochimica et Biophysica Acta (BBA)—Lipids and Lipid Metabolism*, vol. 793, no. 1, pp. 119–122, 1984.

Review Article

Xanthine Oxidoreductase-Derived Reactive Species: Physiological and Pathological Effects

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Xanthine oxidoreductase (XOR) is the enzyme that catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid and is widely distributed among species. In addition to this housekeeping function, mammalian XOR is a physiological source of superoxide ion, hydrogen peroxide, and nitric oxide, which can function as second messengers in the activation of various pathways. This review intends to address the physiological and pathological roles of XOR-derived oxidant molecules. The cytotoxic action of XOR products has been claimed in relation to tissue damage, in particular damage induced by hypoxia and ischemia. Attempts to exploit this activity to eliminate unwanted cells via the construction of conjugates have also been reported. Moreover, different aspects of XOR activity related to phlogosis, endothelial activation, leukocyte activation, and vascular tone regulation, have been taken into consideration. Finally, the positive and negative outcomes concerning cancer pathology have been analyzed because XOR products may induce mutagenesis, cell proliferation, and tumor progression, but they are also associated with apoptosis and cell differentiation. In conclusion, XOR activity generates free radicals and other oxidant reactive species that may result in either harmful or beneficial outcomes.

1. Introduction

The enzyme xanthine oxidoreductase (XOR) has a wide distribution throughout living organisms and is highly conserved in prokaryotic, plant, and animal species (reviewed in [1]). XOR is a dimeric metalloflavoprotein comprising two identical subunits of approximately 145 kDa each, including one molybdenum-containing molybdopterin cofactor (Mo-co) and one flavin adenine dinucleotide (FAD) cofactor, as well as two nonidentical iron-sulfur redox centers. The purine oxidation occurs at the Mo-co site, while the FAD site is the oxidized nicotinamide adenine dinucleotide (NAD⁺) and O₂ reduction sites. The electron flux moves between the Mo-co and FAD cofactors through the two iron-sulfur clusters (reviewed in [2]).

XOR catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid, which are the last two steps of purine catabolism in the highest primates. XOR has the rate-limiting function of generating irreversible products, thus precluding the salvage pathway of purine nucleotides.

Additionally, different endogenous metabolites and various xenobiotics can be oxidized by XOR. Uric acid and its oxidized derivatives may exert prooxidant activity, mainly within the cell; however, it has *in vivo* antioxidant activity, mainly in body fluids. This scavenger action is supposed to provide an evolutionary advantage to primates that lost their uricase activity via mutation and acquired a crucial defense against oncogenesis by free radicals [3].

XOR is highly regulated at both the transcriptional and posttranslational levels. XOR activity is present in all mammalian tissue and fluids, although, in most of them, it is expressed at very low levels because the human XOR gene is usually subjected to a repressing regulation at the transcriptional level [4]. The highest XOR levels are expressed in liver, intestine, kidney, and lactating mammary gland epithelial cells and in vascular endothelial cells (reviewed in [5]). XOR expression may be increased by various stimuli, such as hormones, growth factors, inflammatory cytokines, and low oxygen tension. At the posttranslational level, XOR

is modulated with both quantitative and qualitative changes in its activity. XOR protein may be produced in demolybdo- and/or desulfo-forms, which are inactive in xanthine catalysis at the Mo-co site, although they can oxidize the reduced nicotinamide adenine dinucleotide (NADH) at FAD site. These defective XOR forms are present in varying percentages in milk and could be reactivated with the reinsertion of the lacking atoms at the active site. XOR activity was observed to increase in response to hypoxia without changes in the levels of mRNA or enzyme protein, indicating a posttranslational regulation of XOR (reviewed in [6]). However, the most peculiar modulation of XOR activity in mammals consists of the conversion from the dehydrogenase to the oxidase form. This transition occurs in various pathological conditions (reviewed in [7]).

In all organisms, XOR is present in its constitutively active dehydrogenase form, whereas, only in mammals, the NAD^+ -dependent xanthine dehydrogenase (XDH, EC 1.1.1.204) can be converted to the oxidase form (XO, EC 1.1.3.22) through sulfhydryl group oxidation or limited proteolysis [8]. XO delivers electrons directly to molecular oxygen (O_2), thus generating the reactive oxygen species (ROS), superoxide anion ($\text{O}_2^{\bullet-}$), and hydrogen peroxide (H_2O_2), via a one-electron and a two-electron reduction, respectively. This gives rise to the hydroxyl radical (HO^\bullet) in the presence of iron via the Haber-Weiss and Fenton reactions. The percentage of divalent versus univalent electron transfer to O_2 and the relative quantities of $\text{O}_2^{\bullet-}$ and H_2O_2 generated by XO are dependent upon O_2 tension, pH, and purine concentration. Thus, under normal physiological conditions, H_2O_2 is the major reactive product derived from the XO-catalyzed O_2 reduction. H_2O_2 formation is further favored when both the O_2 levels and pH are reduced, such as under ischemic and/or hypoxic conditions (reviewed in [9]). Under hypoxic conditions, these ROS can also be produced by XDH, which, at the FAD site, can oxidize NADH. Hypoxia-mediated acidic pH and low O_2 tension lessen the nitric oxide (NO) formation by NO synthase and increase its potential to uncouple and produce $\text{O}_2^{\bullet-}$. These conditions reduce XOR affinity for xanthine while increasing affinity for nitrites, which compete with xanthine at the Mo-co site and can be reduced to NO. Under the same conditions the amount of $\text{O}_2^{\bullet-}$ formation by XOR is sufficient to react with NO and generate reactive nitrogen species (RNS), particularly peroxynitrite (ONOO^-). Both free radicals, such as $\text{O}_2^{\bullet-}$, HO^\bullet , and NO, and nonradical forms, such as H_2O_2 and ONOO^- , have an oxidizing effect, thereby contributing to oxidative stress (reviewed in [10]).

The generation of these oxidants may be only partially blocked by allopurinol, which inhibits the Mo-co site in a competitive manner but does not inhibit the catalytic activity at the FAD site. All together, these products are responsible for XOR cytotoxic and proinflammatory activities and for pro- and antitumorigenic effects, in both physiological and pathological conditions. The various XOR functions are dependent on (i) the level of ROS production, as in the case of cytotoxic effects; (ii) the type of the prevalent product, for instance, NO in the presence of high nitrate level; (iii) the specificity of different cell types, such as phagocytes

in inflammation; (iv) the level of XOR gene expression, in particular in cancer.

2. Cytotoxicity of Xanthine Oxidoreductase Products

XOR cytotoxicity received much attention during the second half of last century, together with the circumstances of the conversion from XDH to XO. An elevated XO/XDH activity ratio has been reported in different pathological conditions, which were characterized by tissue damage and cell necrosis. In particular, the XDH to XO shift was observed in a variety of hypoxic/ischemic conditions (reviewed in [6]), including organ transplantation (reviewed in [11]). In such circumstances, any reoxygenation/reperfusion could increase the supply of oxygen for the formation of oxidants, but it was not strictly required. Additionally, the conversion from XDH to XO was not necessary for ROS generation, as discussed above, especially in the presence of low oxygen tension that favors the NADH oxidase activity of XOR. However, the formation of XOR-derived ROS was indicated as the causal agent of the injury or, at least, of the damage amplification, although more than one source of ROS could be implicated (reviewed in [12]).

The mechanism of ROS cytotoxicity is attributed to peroxidation of membrane lipids, DNA damage, and protein oxidation, which impair mitochondrial function and lead to apoptosis (reviewed in [13]) (Figure 1(a)). Indeed, DNA damage and the consequent loss of cloning efficiency occurred in a Burkitt lymphoma-derived cell line via XOR activity through the production of ROS [14]. Apoptosis and necrosis were induced to proliferating human lymphocytes by XOR-derived oxidative stress, which was prevented by catalase [15]. Additionally, oxidative DNA damage, consequent to the ROS generated by XOR activity, provoked cell death in a nasopharyngeal carcinoma cell line [16]. Accordingly, XOR-derived ROS caused DNA double-strand breaks that were associated with p53 function/expression and caspase-dependent apoptosis in primary human lung microvascular endothelial cells that were exposed to cigarette smoke extract [17].

The oxidative stress could be utilized to eliminate unwanted cells, particularly cancer cells. An attempt to take advantage of the cytotoxicity of XOR products was performed by conjugating the XOR protein to monoclonal antibodies, with the intent of delivering XOR activity to the antigen-bearing cell. XOR-containing conjugates recognizing B lymphocyte antigens were prepared with the purpose of autologous bone marrow grafting. These conjugates selectively killed B lymphoma cell lines [18] without reducing normal myeloid clonogenic efficiency [19] and were effective in bone marrow purging from malignant B lymphocytes [20]. XOR immunotargeting was also studied in an experimental model to eliminate T lymphocytes from bone marrow for heterologous transplantation [21]. The cytotoxicity and selectivity of conjugated XOR were enhanced by the addition of chelated iron that potentiates the free radical formation (reviewed in [22]). The efficacy of XOR activity was proven in conditions that were very similar to the *ex vivo* treatment for bone marrow purging from multiple myeloma cells, with

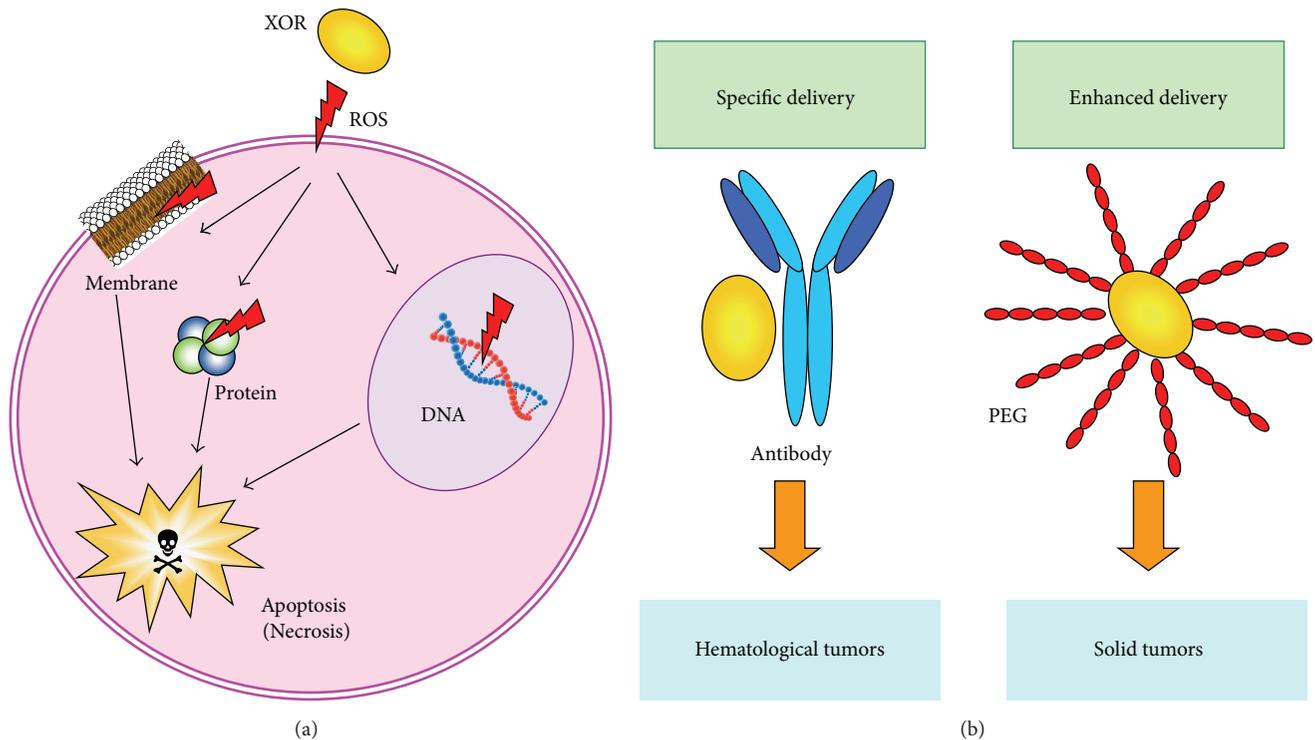


FIGURE 1: Pharmaceutical applications of xanthine oxidoreductase (XOR) cytotoxicity. (a) Mechanisms of ROS cytotoxicity: ROS induce peroxidation of membrane lipids, DNA damage, and protein oxidation and lead to cell death, mainly via apoptosis through impaired mitochondrial function (reviewed in [13]). (b) XOR was conjugated to carriers for the experimental elimination of specific target cells. Selective cell killing was obtained by conjugating XOR to an antibody that was able to specifically deliver reactive oxygen species (ROS) to target cells [23]. Enhanced ROS delivery to solid tumors was achieved by XOR conjugation to polyethylene glycol (PEG) [24].

a XOR/antibody conjugate or with a free monoclonal antibody followed by a XOR/anti-antibody conjugate. Both direct and indirect methods induced a prevalence of apoptotic death over necrosis in malignant B lymphocytes [23] (Figure 1(b)).

To improve the ROS delivery efficiency to solid tumors, XOR was conjugated to polyethylene glycol [24], which (i) confers superior *in vivo* pharmacokinetic characteristics by increasing the blood half-life of the enzyme; (ii) counteracts the aspecific adhesiveness of XOR to the vascular inner surface; and (iii) concentrates XOR in cancer tissues by exploiting the enhanced permeability and retention effect of macromolecules and lipids in solid tumors (reviewed in [25]) (Figure 1(b)).

3. Proinflammatory Activity of Xanthine Oxidoreductase Products

The evolution of XOR from the highly conserved dehydrogenase to the interconvertible mammalian oxidase form confers to its enzyme activity a new role of producing physiologic signal transduction that is mediated by ROS as secondary messengers (reviewed in [26, 27]).

XOR activity is known to be upregulated in response to inflammatory cytokines [28], which induce the XDH to XO transition and also increase the XOR level in plasma [11], supporting the hypothesis that XOR is a component of the innate immune system (reviewed in [29]). Indeed, XOR

has been implicated in the defense against infectious diseases because of its capability of activating the cellular phlogistic response at various levels (reviewed in [30]). XOR-derived ROS promote leukocyte-endothelial cell interactions by increasing the adhesion of phagocytes [31]. They also induce the production of cytokines [32], thus amplifying the inflammatory response, and chemotactic factors [33], which cause the accumulation of polymorphonuclear granulocytes in the microvasculature [34]. The bactericidal activity of XOR may contribute to the oxygen-dependent cell killing during leukocyte phagocytosis through ROS and ONOO⁻ production [35]. The antibacterial properties of XOR suggest that its abundance in milk could have the role of a natural antibiotic, representing one of the reasons to encourage breastfeeding by mothers [36] (Figure 2(a)).

The usually very low XOR serum level in humans may become more elevated in pathological circumstances that cause tissue damage and the release of XOR from cells into the bloodstream. Circulating XOR is converted to the oxidase form and binds to endothelial cells, even at distant sites, inducing proinflammatory signaling or even remote organ injury (reviewed in [11]). The proinflammatory activity exerted by the XOR-derived ROS may affect the microvascular lining by inducing endothelium permeabilization, which begins both the physiological cascade of immune response and the pathological events that induce atheromatous plaque formation (reviewed in [37]) (Figure 2(b)). The XOR

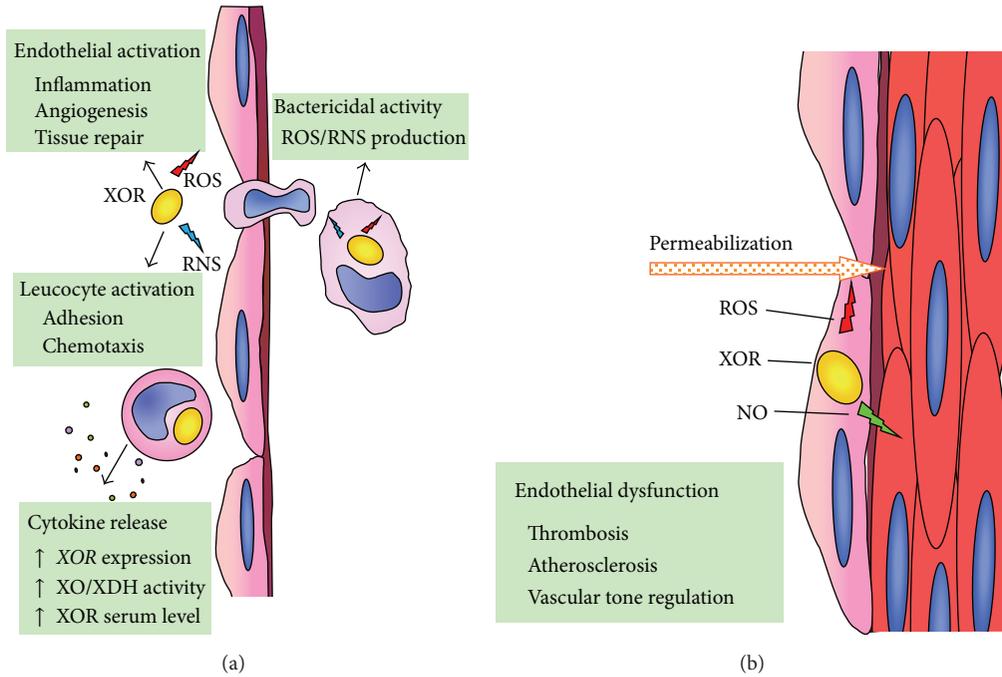


FIGURE 2: Prophlogistic action of reactive oxygen (ROS) and nitrogen (RNS) species. (a) Interferon and other cytokines increase xanthine oxidoreductase gene (XOR) expression as well as the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) and XOR serum level (reviewed in [11]). XOR-derived ROS and RNS mediate the endothelial and phagocytic cell activation that is functional in antibacterial defense (reviewed in [30]). (b) XOR products induce endothelial permeabilization and dysregulation of vascular tone, which may lead to thrombosis and atherosclerosis (reviewed in [37]).

products together with the oxidants generated by NAD(P)H oxidase and NO synthase may also modulate another endothelial cell function, the regulation of arteriolar tone via NO production, which has local and systemic vasodilating activity and causes XOR inhibition (Figure 2(b)). NO is produced by endothelial NO synthase that is inhibited either by ROS or under hypoxic conditions. In these circumstances, NO generation is assured by the nitrite reductase activity of both XOR and NAD(P)H oxidase, which undergo reciprocal activation by generating $O_2^{\bullet-}$ (reviewed in [38]). As the activities of these enzymes are interdependent in the endothelium, the final outcome is the result of a physiopathological balance amongst their activities. Thus, it is not surprising that both XOR activity and its inhibition by allopurinol may induce endothelial dysfunction and promote platelet aggregation, as well as aggravating hypertension and cardiovascular diseases [39].

In patients with coronary disease, the treatment with the angiotensin receptor blocker losartan reduced the endothelium-bound XOR activity and XOR inhibition with oxypurinol improved endothelium-dependent vasodilation, suggesting that endothelial dysfunction in coronary disease is at least in part dependent on angiotensin II-dependent endothelial XOR activation [40]. In patients with metabolic syndrome, XOR inhibition by allopurinol reduces myeloperoxidase and malondialdehyde blood levels, while increasing the flow-mediated dilation, suggesting that XOR-induced oxidative stress contributes to endothelial vasomotor dysfunction [41]. The underlying mechanism is supposed to be

the reduced bioavailability of NO due to the reaction of NO with $O_2^{\bullet-}$ (reviewed in [39]). However, in grade 1 drug-naïve hypertensive subjects a dietary nitrate load reduces systolic and diastolic blood pressure. This effect is related to an increased NO generation, which is significantly attenuated by allopurinol and is associated with higher levels of erythrocytic XOR expression and nitrite reductase activity in hypertensive patients in comparison to normotensives volunteers [42]. The effects of ROS generated by human XOR on cardiovascular disease have been detailed in two recent publications (reviewed in [11, 37]).

XOR may produce ROS and NO, which are both required for the formation of normal granulation tissue and wound healing. In vitro keratinocytes and endothelial cell proliferation and migration were increased by H_2O_2 and nitrite. XOR expression was upregulated shortly after wounding at the wound edge. Locally applied allopurinol, as well as a tungsten-enriched diet that drastically lowered XOR activity, significantly delayed wound healing in mice. The effect was reversed and angiogenesis improved with the topical H_2O_2 administration, strongly suggesting that XOR contributes to wound repair [43].

4. Pro- and Antitumorigenic Activity of Xanthine Oxidoreductase Products

In both experimental and clinical pathology, the level of XOR expression was often found to be higher or lower in cancer tissues compared with the corresponding normal tissue or to

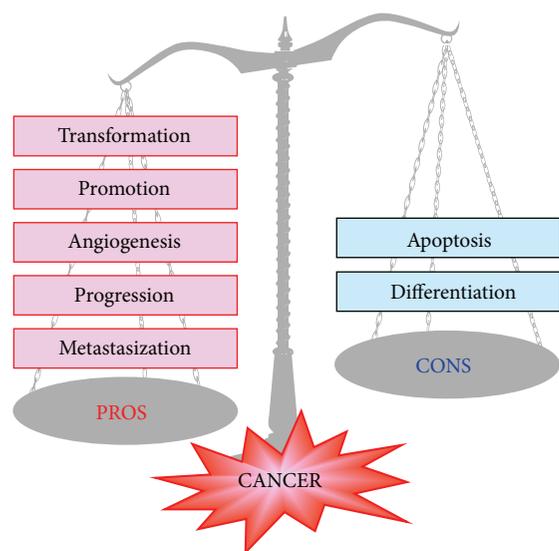


FIGURE 3: Cancer pathogenesis: ambiguous role of xanthine oxidoreductase (XOR). XOR-derived ROS may activate genes responsible for each phase of cancer development (reviewed in [47]) as well as genes that promote antioncogenic activities (reviewed in [45]).

the normal tissues bordering cancerous tissues (reviewed in [44]). In particular, XOR expression and activity in neoplastic human tissues have been recently addressed and discussed together with the XOR role in differentiation and oncogenesis (reviewed in [45]). Moreover, XOR products have been associated with both the process of oncogenesis ([46], reviewed in [47]) and its prevention ([48], reviewed in [49]) (Figure 3).

The level of XOR activity was higher than normal and that of paraoxonase I, a free radical scavenger enzyme, was lower in the serum of patients with various cancer illnesses [50]. A low activity of various oxidative enzymes, in particular XOR, has been reported to correlate with cell proliferation in different settings, including cancer, and a hypothesis has been formulated that a low level of free radicals may stimulate cancer cell growth [51]. XOR can also confer a cancer-promoting action through the above-discussed proinflammatory activities of ROS and RNS.

The analysis of a vast cohort of women followed for 11 years showed a dose-dependent risk of breast cancer by alcohol consumption [52] and a mechanism involving XOR-derived ROS has been proposed for the pathogenesis of this cancer. XDH is expressed at high levels by mammary epithelium, particularly in relation to lactation, and can produce ROS by oxidizing ethanol [53] as well as acetaldehyde and NADH, which are generated by alcohol catabolism. ROS can be responsible for DNA damage, mutagenesis, and neoplastic transformation, especially in aged breast tissue with high iron levels and low antioxidant levels (reviewed in [54, 55]).

XOR is an upstream regulator of various molecules with transduction signal functions in different pathways, which may result in either pro- or antitumorogenic signaling.

In human lung microvascular endothelial cells, XOR was shown to increase the expression of the tumor suppressor protein p53, which is very often mutated and deactivated in

human cancer. XOR induced oxidative stress, DNA damage, and the ROS-dependent upregulation of p53 protein, with the consequent activation of the caspase enzymatic cascade and apoptosis [17].

In 3T3-L1 murine cells, the ROS produced by the NADH-oxidizing activity of XOR were able to stimulate the activation of peroxisome proliferator-activated receptor-gamma (PPAR- γ), which belongs to the nuclear hormone receptor superfamily. This ligand-activated intracellular transcription factor has antiproliferative and antioncogenic activities because it can favor cell differentiation and inhibit angiogenesis [56].

XOR-derived ROS can modulate the expression of the inflammation mediator, cyclooxygenase-2 (COX-2), by either increasing or decreasing its expression. The XOR-dependent COX-2 expression in newborn mice was essential for regular kidney development, and the lack of XOR was associated with renal hypoplasia and dysplasia [57]. Additionally, XOR depletion in primary renal epithelial cells induced positive immunostaining for mesenchymal cell type markers and the lack of reactivity to E-cadherin associated with cell morphology changes from a cuboidal to myofibroblastic shape, which indicated epithelial to mesenchymal transition [58]. However, a high XOR level in human mammary epithelium lowered the COX-2 and matrix metalloprotease expression levels, which are crucial for cell migratory activity and thus for tumor progression and ability of metastasis formation [48].

XOR-generated oxidants can turn on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) in rat liver both during ischemia [59] and in type 1 diabetes [60]. NF- κ B is a transcription factor that is usually activated during chronic inflammation and in cancer, where it promotes the production of immunological cytokines and the expression of a set of antiapoptotic genes.

In U251-MG cells, derived from human brain, chemically induced hypoxia increased XOR activity and the level of XOR-derived ROS, which upregulated hypoxia-inducible factor-1 alpha (HIF-1 α) [61]. This transcription factor is overexpressed in hypoxia and induces angiogenesis, as well as cancer invasion, thus contributing to both tumor development and progression.

5. Conclusions

Mammal XOR is the end product of a complicated evolutionary process leading to a hyperregulated enzyme with low specificity and highly versatile activity. In mammals, XOR has acquired many functions through the production of ROS, NO, and RNS, whereby it is involved in the triggering of key biological cell pathways and in the regulation of several physiological and pathological conditions. For these reasons, XOR represents the two faces of free radicals, which can have either negative or positive effects. XOR-derived RNS and ROS may have a cytotoxic effect. This activity may be responsible for tissue damage in hypoxia/reoxygenation and ischemia/reperfusion injury. However, this cytotoxic effect can be pharmacologically exploited to obtain selective cancerous cell killing by conjugating XOR to a specific antibody.

XOR activity increases during infectious diseases and its cytotoxic action is useful for the defenses against bacteria. Additionally, XOR-derived NO and ROS have proinflammatory activity because they regulate endothelial functions, by both increasing the permeability of vascular lining and modulating the arteriolar tone. For this reason, XOR has been implicated in hypertension, cardiovascular diseases, and atherosclerosis. XOR-derived ROS are also involved in cancer pathogenesis because they may promote neoplastic transformation by activating target genes with proinflammatory, antiapoptotic, and proliferative actions. Moreover, they favor the progression to malignancy by inducing angiogenesis and cell migration. On the other hand, XOR products may activate the expression of the proapoptotic protein p53 and of transcription factors belonging to the nuclear hormone receptor superfamily with antitumorogenic and antiproliferative activity, promoting cell differentiation and inhibiting angiogenesis.

Highlights

- (i) XOR-derived ROS, NO, and RNS have proinflammatory and bactericidal activities.
- (ii) XOR products may be cytotoxic in many circumstances.
- (iii) XOR products modulate endothelial function and arteriolar tone.
- (iv) XOR products may induce mutagenesis, cell proliferation, and tumor progression.
- (v) XOR products are associated with apoptosis and cell differentiation.

Abbreviations

COX-2:	Cyclooxygenase-2
FAD:	Flavin adenine dinucleotide
HO [•] :	Hydroxyl radical
H ₂ O ₂ :	Hydrogen peroxide
Mo-co:	Molybdenum-containing molybdopterin cofactor
NAD ⁺ :	Oxidized nicotinamide adenine dinucleotide
NADH:	Reduced nicotinamide adenine dinucleotide
NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO:	Nitric oxide
ONOO ⁻ :	Peroxynitrite
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
O ₂ :	Molecular oxygen
O ₂ ^{•-} :	Superoxide anion
XDH:	Xanthine dehydrogenase
XO:	Xanthine oxidase
XOR:	Xanthine oxidoreductase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

- [1] D. A. Parks and D. N. Granger, "Xanthine oxidase: biochemistry, distribution and physiology," *Acta Physiologica Scandinavica*, vol. 126, no. 548, pp. 87–99, 1986.
- [2] R. Hille and T. Nishino, "Xanthine oxidase and xanthine dehydrogenase," *The FASEB Journal*, vol. 9, no. 11, pp. 995–1003, 1995.
- [3] B. N. Ames, R. Cathcart, E. Schwiers, and P. Hochstein, "Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 11, pp. 6858–6862, 1981.
- [4] P. Xu, P. LaVallee, and J. R. Hoidal, "Repressed expression of the human xanthine oxidoreductase gene. E-box and TATA-like elements restrict ground state transcriptional activity," *The Journal of Biological Chemistry*, vol. 275, no. 8, pp. 5918–5926, 2000.
- [5] A. Kooij, "A re-evaluation of the tissue distribution and physiology of xanthine oxidoreductase," *Histochemical Journal*, vol. 26, no. 12, pp. 889–915, 1994.
- [6] C. E. Berry and J. M. Hare, "Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications," *The Journal of Physiology*, vol. 555, no. 3, pp. 589–606, 2004.
- [7] A. Boueiz, M. Damarla, and P. M. Hassoun, "Xanthine oxidoreductase in respiratory and cardiovascular disorders," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 294, no. 5, pp. L830–L840, 2008.
- [8] E. Della Corte and F. Stirpe, "The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme," *Biochemical Journal*, vol. 126, no. 3, pp. 739–745, 1972.
- [9] N. Cantu-Medellin and E. E. Kelley, "Xanthine oxidoreductase-catalyzed reactive species generation: a process in critical need of reevaluation," *Redox Biology*, vol. 1, no. 1, pp. 353–358, 2013.
- [10] R. Harrison, "Structure and function of xanthine oxidoreductase: where are we now?" *Free Radical Biology and Medicine*, vol. 33, no. 6, pp. 774–797, 2002.
- [11] M. G. Battelli, A. Bolognesi, and L. Polito, "Pathophysiology of circulating xanthine oxidoreductase: new emerging roles for a multi-tasking enzyme," *Biochimica et Biophysica Acta*, vol. 1842, no. 9, pp. 1502–1517, 2014.
- [12] C. Li and R. M. Jackson, "Reactive species mechanisms of cellular hypoxia-reoxygenation injury," *American Journal of Physiology: Cell Physiology*, vol. 282, no. 2, pp. C227–C241, 2002.
- [13] A. H. Bhat, K. B. Dar, S. Anees et al., "Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight," *Biomedicine & Pharmacotherapy*, vol. 74, pp. 101–110, 2015.
- [14] M. Chiricolo, P. L. Tazzari, A. Abbondanza, A. Dinota, and M. G. Battelli, "Cytotoxicity of, and DNA damage by, active oxygen species produced by xanthine oxidase," *FEBS Letters*, vol. 291, no. 2, pp. 173–176, 1991.
- [15] M. G. Battelli, S. Musiani, P. L. Tazzari, and F. Stirpe, "Oxidative stress to human lymphocytes by xanthine oxidoreductase activity," *Free Radical Research*, vol. 35, no. 6, pp. 665–679, 2001.

- [16] C.-C. Huang, K.-L. Chen, C. H. A. Cheung, and J.-Y. Chang, "Autophagy induced by cathepsin S inhibition induces early ROS production, oxidative DNA damage, and cell death via xanthine oxidase," *Free Radical Biology and Medicine*, vol. 65, pp. 1473–1486, 2013.
- [17] B. S. Kim, L. Serebreni, O. Hamdan et al., "Xanthine oxidoreductase is a critical mediator of cigarette smoke-induced endothelial cell DNA damage and apoptosis," *Free Radical Biology and Medicine*, vol. 60, pp. 336–343, 2013.
- [18] M. G. Battelli, A. Abbondanza, P. L. Tazzari et al., "Selective cytotoxicity of an oxygen-radical-generating enzyme conjugated to a monoclonal antibody," *Clinical and Experimental Immunology*, vol. 73, no. 1, pp. 128–133, 1988.
- [19] P. L. Tazzari, M. G. Battelli, A. Abbondanza et al., "Targeting of a plasma cell line with a conjugate containing xanthine oxidase and the monoclonal antibody 62B1," *Transplantation*, vol. 48, no. 1, pp. 119–122, 1989.
- [20] A. Dinota, P. L. Tazzari, A. Abbondanza, M. G. Battelli, M. Gobbi, and F. Stirpe, "Bone marrow purging by a xanthine oxidase-antibody conjugate," *Bone Marrow Transplantation*, vol. 6, no. 1, pp. 31–36, 1990.
- [21] M. G. Battelli, A. Abbondanza, P. L. Tazzari, A. Bolognesi, R. M. Lemoli, and F. Stirpe, "T lymphocyte killing by a xanthine-oxidase-containing immunotoxin," *Cancer Immunology, Immunotherapy*, vol. 35, no. 6, pp. 421–425, 1992.
- [22] S. J. Dixon and B. R. Stockwell, "The role of iron and reactive oxygen species in cell death," *Nature Chemical Biology*, vol. 10, no. 1, pp. 9–17, 2014.
- [23] M. G. Battelli, L. Polito, F. Falà et al., "Toxicity of xanthine oxidoreductase to malignant B lymphocytes," *Journal of Biological Regulators & Homeostatic Agents*, vol. 19, no. 3–4, pp. 120–129, 2005.
- [24] T. Sawa, J. Wu, T. Akaike, and H. Maeda, "Tumor-targeting chemotherapy by a xanthine oxidase-polymer conjugate that generates oxygen-free radicals in tumor tissue," *Cancer Research*, vol. 60, no. 3, pp. 666–671, 2000.
- [25] J. Fang, T. Seki, and H. Maeda, "Therapeutic strategies by modulating oxygen stress in cancer and inflammation," *Advanced Drug Delivery Reviews*, vol. 61, no. 4, pp. 290–302, 2009.
- [26] H. Sauer, M. Wartenberg, and J. Hescheler, "Reactive oxygen species as intracellular messengers during cell growth and differentiation," *Cellular Physiology and Biochemistry*, vol. 11, no. 4, pp. 173–186, 2001.
- [27] A. Meneshian and G. B. Bulkley, "The physiology of endothelial xanthine oxidase: from urate catabolism to reperfusion injury to inflammatory signal transduction," *Microcirculation*, vol. 9, no. 3, pp. 161–175, 2002.
- [28] S. Page, D. Powell, M. Benboubetra et al., "Xanthine oxidoreductase in human mammary epithelial cells: activation in response to inflammatory cytokines," *Biochimica et Biophysica Acta—General Subjects*, vol. 1381, no. 2, pp. 191–202, 1998.
- [29] A. Agarwal, A. Banerjee, and U. C. Banerjee, "Xanthine oxidoreductase: a journey from purine metabolism to cardiovascular excitation-contraction coupling," *Critical Reviews in Biotechnology*, vol. 31, no. 3, pp. 264–280, 2011.
- [30] H. M. Martin, J. T. Hancock, V. Salisbury, and R. Harrison, "Role of xanthine oxidoreductase as an antimicrobial agent," *Infection and Immunity*, vol. 72, no. 9, pp. 4933–4939, 2004.
- [31] M. Suzuki, M. B. Grisham, and D. N. Granger, "Leukocyte-endothelial cell adhesive interactions: role of xanthine oxidase-derived oxidants," *Journal of Leukocyte Biology*, vol. 50, no. 5, pp. 488–494, 1991.
- [32] R. Shenkar and E. Abraham, "Plasma from hemorrhaged mice activates CREB and increases cytokine expression in lung mononuclear cells through a xanthine oxidase-dependent mechanism," *American Journal of Respiratory Cell and Molecular Biology*, vol. 14, no. 2, pp. 198–206, 1996.
- [33] W. F. Petrone, D. K. English, K. Wong, and J. M. McCord, "Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 2, pp. 1159–1163, 1980.
- [34] M. J. Müller, B. Vollmar, H.-P. Friedl, and M. D. Menger, "Xanthine oxidase and superoxide radicals in portal triad cross-clamping-induced microvascular reperfusion injury of the liver," *Free Radical Biology and Medicine*, vol. 21, no. 2, pp. 189–197, 1996.
- [35] E. Tubaro, B. Lotti, C. Santiangelli, and G. Cavallo, "Xanthine oxidase: an enzyme playing a role in the killing mechanism of polymorphonuclear leucocytes," *Biochemical Pharmacology*, vol. 29, no. 21, pp. 3018–3020, 1980.
- [36] C. R. Stevens, T. M. Millar, J. G. Clinch, J. M. Kanczler, T. Bodamyali, and D. R. Blake, "Antibacterial properties of xanthine oxidase in human milk," *The Lancet*, vol. 356, no. 9232, pp. 829–830, 2000.
- [37] M. G. Battelli, L. Polito, and A. Bolognesi, "Xanthine oxidoreductase in atherosclerosis pathogenesis: not only oxidative stress," *Atherosclerosis*, vol. 237, no. 2, pp. 562–567, 2014.
- [38] H. Cai and D. G. Harrison, "Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress," *Circulation Research*, vol. 87, no. 10, pp. 840–844, 2000.
- [39] N. Cantu-Medellin and E. E. Kelley, "Xanthine oxidoreductase-catalyzed reduction of nitrite to nitric oxide: insights regarding where, when and how," *Nitric Oxide*, vol. 34, pp. 19–26, 2013.
- [40] U. Landmesser, S. Spiekermann, C. Preuss et al., "Angiotensin II induces endothelial xanthine oxidase activation: role for endothelial dysfunction in patients with coronary disease," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 4, pp. 943–948, 2007.
- [41] O. Yiginer, F. Ozelik, T. Inanc et al., "Allopurinol improves endothelial function and reduces oxidant-inflammatory enzyme of myeloperoxidase in metabolic syndrome," *Clinical Research in Cardiology*, vol. 97, no. 5, pp. 334–340, 2008.
- [42] S. M. Ghosh, V. Kapil, I. Fuentes-Calvo et al., "Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential," *Hypertension*, vol. 61, no. 5, pp. 1091–1102, 2013.
- [43] M. C. Madigan, R. M. McEnaney, A. J. Shukla et al., "Xanthine oxidoreductase function contributes to normal wound healing," *Molecular Medicine*, vol. 21, no. 1, pp. 313–322, 2015.
- [44] G. Weber, "Biochemical strategy of cancer cells and the design of chemotherapy: G.H.A. Clowes memorial lecture," *Cancer Research*, vol. 43, no. 8, pp. 3466–3493, 1983.
- [45] M. G. Battelli, L. Polito, M. Bortolotti, and A. Bolognesi, "Xanthine oxidoreductase in cancer: more than a differentiation marker," *Cancer Medicine*, In press.
- [46] I. A. Shmarakov and M. M. Marchenko, "Xanthine oxidase activity in transplantable Guerin's carcinoma in rats," *Voprosy Onkologii*, vol. 55, no. 3, pp. 345–350, 2009.
- [47] K. Balamurugan, "HIF-1 at the crossroads of hypoxia, inflammation, and cancer," *International Journal of Cancer*, 2015.
- [48] M. A. Fini, D. Orchard-Webb, B. Kosmider et al., "Migratory activity of human breast cancer cells is modulated by differential

- expression of xanthine oxidoreductase," *Journal of Cellular Biochemistry*, vol. 105, no. 4, pp. 1008–1026, 2008.
- [49] M. Valko, C. J. Rhodes, J. Moncol, M. Izakovic, and M. Mazur, "Free radicals, metals and antioxidants in oxidative stress-induced cancer," *Chemico-Biological Interactions*, vol. 160, no. 1, pp. 1–40, 2006.
- [50] Z. Q. Samra, S. Pervaiz, S. Shaheen, N. Dar, and M. A. Athar, "Determination of oxygen derived free radicals producer (xanthine oxidase) and scavenger (paraoxonase) enzymes and lipid parameters in different cancer patients," *Clinical Laboratory*, vol. 57, no. 9-10, pp. 741–747, 2011.
- [51] A. S. Sun and A. I. Cederbaum, "Oxidoreductase activities in normal rat liver, tumor-bearing rat liver, and hepatoma HC-252," *Cancer Research*, vol. 40, no. 12, pp. 4677–4681, 1980.
- [52] S. A. Smith-Warner, D. Spiegelman, S.-S. Yaun et al., "Alcohol and breast cancer in women: a pooled analysis of cohort studies," *The Journal of the American Medical Association*, vol. 279, no. 7, pp. 535–540, 1998.
- [53] G. D. Castro, A. M. A. Delgado de Layo, M. H. Costantini, and J. A. Castro, "Cytosolic xanthine oxidoreductase mediated bioactivation of ethanol to acetaldehyde and free radicals in rat breast tissue. Its potential role in alcohol-promoted mammary cancer," *Toxicology*, vol. 160, no. 1–3, pp. 11–18, 2001.
- [54] R. M. Wright, J. L. McManaman, and J. E. Repine, "Alcohol-induced breast cancer: a proposed mechanism," *Free Radical Biology and Medicine*, vol. 26, no. 3-4, pp. 348–354, 1999.
- [55] R. G. Dumitrescu and P. G. Shields, "The etiology of alcohol-induced breast cancer," *Alcohol*, vol. 35, no. 3, pp. 213–225, 2005.
- [56] K. J. Cheung, I. Tzamelis, P. Pissios et al., "Xanthine oxidoreductase is a regulator of adipogenesis and PPAR γ activity," *Cell Metabolism*, vol. 5, no. 2, pp. 115–128, 2007.
- [57] T. Ohtsubo, I. I. Rovira, M. F. Starost, C. Liu, and T. Finkel, "Xanthine oxidoreductase is an endogenous regulator of cyclooxygenase-2," *Circulation Research*, vol. 95, no. 11, pp. 1118–1124, 2004.
- [58] T. Ohtsubo, K. Matsumura, K. Sakagami et al., "Xanthine oxidoreductase depletion induces renal interstitial fibrosis through aberrant lipid and purine accumulation in renal tubules," *Hypertension*, vol. 54, no. 4, pp. 868–876, 2009.
- [59] N. Matsui, I. Satsuki, Y. Morita et al., "Xanthine oxidase-derived reactive oxygen species activate nuclear factor kappa B during hepatic ischemia in rats," *The Japanese Journal of Pharmacology*, vol. 84, no. 3, pp. 363–366, 2000.
- [60] M. Romagnoli, M.-C. Gomez-Cabrera, M.-G. Perrelli et al., "Xanthine oxidase-induced oxidative stress causes activation of NF- κ B and inflammation in the liver of type I diabetic rats," *Free Radical Biology and Medicine*, vol. 49, no. 2, pp. 171–177, 2010.
- [61] C. E. Griguer, C. R. Oliva, E. E. Kelley, G. I. Giles, J. R. Lancaster Jr., and G. Y. Gillespie, "Xanthine oxidase-dependent regulation of hypoxia-inducible factor in cancer cells," *Cancer Research*, vol. 66, no. 4, pp. 2257–2263, 2006.

Review Article

Relationship between Oxidative Stress, Circadian Rhythms, and AMD

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This work reviews concepts regarding oxidative stress and the mechanisms by which endogenous and exogenous factors produce reactive oxygen species (ROS). It also surveys the relationships between oxidative stress, circadian rhythms, and retinal damage in humans, particularly those related to light and photodamage. In the first section, the production of ROS by different cell organelles and biomolecules and the antioxidant mechanisms that antagonize this damage are reviewed. The second section includes a brief review of circadian clocks and their relationship with the cellular redox state. In the third part of this work, the relationship between retinal damage and ROS is described. The last part of this work focuses on retinal degenerative pathology, age-related macular degeneration, and the relationships between this pathology, ROS, and light. Finally, the possible interactions between the retinal pigment epithelium (RPE), circadian rhythms, and this pathology are discussed.

1. Introduction

Over millions of years of evolution, organisms have developed diverse protective systems to control excess reactive oxygen species (ROS), which produce oxidative stress (OS). This term refers to elevated intracellular levels of ROS that cause damage to lipids, proteins, and DNA, a process that has been considered to be linked to a myriad of pathologies in humans.

The mechanisms of ROS production (e.g., via aerobic respiration or flavin-containing oxidases) and its rapid removal (e.g., via catalase) are present in almost all of the cell types found in organisms. OS effects depend on the intensity of damage induced by the ROS in the cell and the cellular response to this damage; if the cell is unable to overcome the damage and recover its function or if exogenous and endogenous antioxidant defenses (AOXs) cannot counter it, the cell can die. However, ROS have also been shown to function as second messengers by transducing extracellular signals to generate specific cellular responses. Proteins and other molecules that participate in signaling pathways can

be modified by redox changes [1]. Numerous studies have substantially contributed to the development of the concepts of OS and the mechanisms involved in the production and regulation of ROS as well as their participation in cellular signaling processes (for a review, see [2]). The present work briefly reviews some of the mechanisms of ROS production and scavenging in addition to the participation of ROS in complex processes such as aging and biological rhythms. Due to the growing interest in the involvement of ROS in degenerative pathologies, the last part of this review is focused on the effects of OS on retinal degenerative processes, particularly on age-related macular degeneration (AMD). Due to the importance of circadian rhythms in the development of degenerative pathologies, a short survey of the possible relationships between circadian rhythms and AMD is included in the last part of this work.

2. Reactive Oxygen Species

ROS are formed either during metabolic processes that are linked to life-sustaining, enzyme-catalyzed reactions, such as

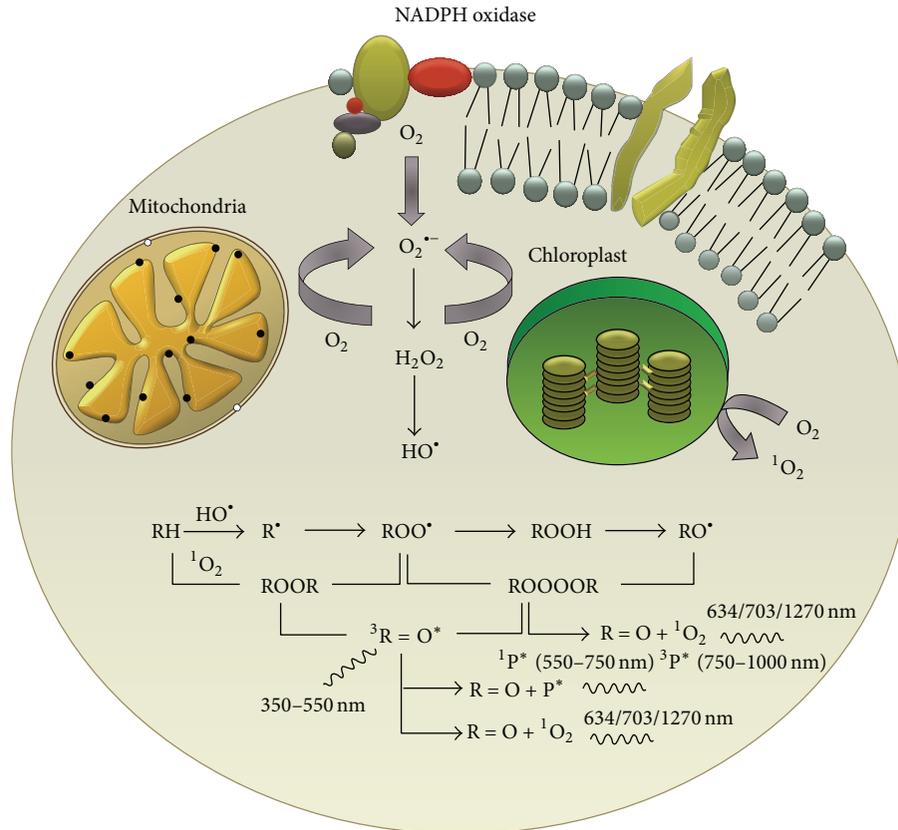


FIGURE 1: A model showing the formation of reactive oxygen species (ROS) in different organelles of the cell. Superoxide anion radical ($O_2^{\cdot-}$) is produced via the membrane-bound enzyme complex NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) which is found embedded within the plasma membranes and membranes of various organelles such as mitochondria, chloroplasts, and phagosomes. The dismutation of $O_2^{\cdot-}$ is accompanied by the formation of hydrogen peroxide (H_2O_2) and then the hydroxyl radical (HO^{\cdot}) via the Fenton reaction. The highly reactive HO^{\cdot} has the capability to oxidize all types of biomolecules such as lipids, proteins, and nucleic acids. The oxidation of biomolecules is accompanied by the formation of high-energy intermediates such as dioxetane (ROOR) and tetroxide (ROOOOR), which upon further decomposition, generate electronically excited species such as triplet excited carbonyl, singlet and triplet excited pigments, and singlet oxygen (1O_2). From Pospišil et al. [7] with the permission of the authors and Elsevier.

aerobic respiration, or during responses to stress reactions when organisms, including humans, are exposed to biotic and abiotic stress factors, such as situations like hypoxia or anoxia. ROS encompass a variety of diverse chemical species including singlet oxygen, superoxide anion radical, hydroxyl radical (OH), hydrogen peroxide (H_2O_2), hydroxylperoxyl radical, alkoxy radicals, and peroxy radicals. Superoxide anion ($O_2^{\cdot-}$) is converted to H_2O_2 by the enzyme superoxide dismutase (SOD), and the hydroxyl radical (OH) is a byproduct of the Fenton reaction. Nitric oxide (NO) and singlet oxygen are examples of reactive species. Some of these species, such as superoxide or hydroxyl radicals, are extremely unstable, whereas others, such as H_2O_2 , are freely diffusible and relatively long-lived. These various radical species can be generated either exogenously by physical or chemical factors that induce stress reactions or through cell-dependent mechanisms via several different mechanisms, such as cytosolic enzyme systems or mitochondrial mechanisms. The cytosolic systems include, among others, the family of NADPH oxidases (NOX) [3], whereas the production of mitochondrial superoxide radicals occurs primarily

at two discrete points in the electron transport chain (ETC), namely, complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase) [4]. Mitochondrial production of ROS will be discussed in the next section. Some of the exogenous chemical sources of ROS include the xanthine/xanthine oxidase system, which produces $O_2^{\cdot-}$; the Fenton reagent, which generates HO^{\cdot} ; and photosensitizers such as rose bengal and benzoporphyrin derivatives, which produce 1O_2 upon photosensitization [5, 6]. Physical abiotic factors such as UV radiation and visible light result in the formation of radical ($O^{\cdot-}$ and H^{\cdot}) and nonradical (1O_2) ROS by Type I and Type II reactions, respectively (for a review, see [7]) (Figure 1). Various endogenous pigments in organisms, such as porphyrins, bilirubins, melanins, and pterins, are known to act as photosensitizers by absorbing radiation or visible light. This leads to the formation of the singlet photosensitizer state, which forms the triplet excited state. The excited photosensitizer undergoes either electron transport, forming $O_2^{\cdot-}$, H_2O_2 , and HO^{\cdot} or energy transfer, forming 1O_2 (Type II reaction) [8, 9]. In Type I reaction,

electron transport leads to the production of O_2^- via the formation of photosensitizer anion radicals and substrate cation radicals or *vice versa* [10]. Spontaneous or enzymatically driven dismutation of O_2^- leads to the formation of H_2O_2 , which subsequently forms OH^\bullet via the Fenton reaction or other metal-catalyzed reactions. In a Type II reaction, triplet-singlet energy transfer from the excited photosensitizer to molecular oxygen forms 1O_2 [11].

Among all of the biomolecules that can be attacked by ROS, lipids are likely the most susceptible to oxidation. All cell membranes are rich in polyunsaturated fatty acids (PUFAs), which are easily injured by oxidizing agents. Peroxidation of cellular lipids modifies membrane properties and produces cytotoxic compounds, although some peroxidation products also play useful roles. Lipid peroxidation (autoxidation), a process that leads to the oxidation of PUFAs due to the presence of several double bonds in their structure, involves the production of peroxides and reactive organic free radicals (FRs). The latter can then react with other fatty acids, initiating a FR reaction cascade.

ROS can also react with nucleic acids by attacking nitrogenous bases and the sugar phosphate backbone. Furthermore, some of their primary effects include basic DNA damage and single- and double-strand DNA breaks. To a large extent, the oxidative radical damage that occurs in nucleic acids results from the reaction of DNA with radicals such as H^\bullet and $^\bullet OH$. Hydroxyl radicals are known to attack DNA, causing single- and/or double-strand breaks as well as pyrimidine and purine lesions, both of which can affect the integrity of the genome [12]. The inability of cells to repair the damage incurred in this process may lead to their death; alternatively, mutations may occur in the DNA, leading to carcinogenesis or the development of neurodegenerative diseases [13].

Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage than its nuclear counterpart. Mutations in mtDNA can cause disturbances in the respiratory chain and loss of the control of ROS production. mtDNA is not protected by histones or other associated proteins, and because it has intronless regions and a high transcription rate, mtDNA is more susceptible to oxidative modifications in its coding regions. The reduced effectiveness of the repair system for mtDNA damage may be the cause of the accumulation of OS and its consequences. The formation of carbonyl derivatives is the most widely studied OS-induced protein modification [14]. Carbonyl formation can occur through a variety of mechanisms, including the direct oxidation of certain amino acid side chains and oxidation-induced peptide cleavage. Although all organs and proteins can potentially be modified by OS, certain tissues and protein targets may be especially susceptible [3]. Other protein modifications are NO-dependent. For example, NO reacts with $O_2^{\bullet-}$ to generate $ONOO^-$, which is capable of initiating further protein oxidation and nitration [15, 16].

The nitrogen dioxide radical, which is biologically formed from the reaction of N_2 with oxygen or by the decomposition of $ONOO^-$, reacts with tyrosine residues, resulting in the formation of 3-nitro-tyrosine. The addition of NO to the thiol

groups of proteins via S-nitrosation (also referred to as S-nitrosylation) has also been reported to be associated with neurodegenerative diseases [16].

2.1. Mitochondria and ROS. Mitochondria are a major intracellular generator of ROS (mitoROS) [17]. Multiple producers of O^- have been reported in mitochondria, including flavins in complexes I and II; ubiquinone binding sites in complexes I, II, and III; flavoprotein-Q oxidoreductase-mediated fatty acid beta-oxidation; and glycerol-3-phosphate, 2-oxoglutarate, and pyruvate dehydrogenases [18–21]. The major players in mitoROS production are complexes I and III of the mitochondrial ETC [18], although other ROS-producing mitochondrial sites include mitochondrial glycerol-3-phosphate dehydrogenase, the electron-transferring flavoprotein/ETF: ubiquinone oxidoreductase system of fatty acid oxidation, dihydroorotate dehydrogenase, the dihydrolipoamide dehydrogenase, and 2-oxoacid dehydrogenase complexes. In addition, the 2-oxoglutarate dehydrogenase complex, the branched-chain 2-oxoacid dehydrogenase complex, the pyruvate dehydrogenase complex, and proline dehydrogenase, among others, have been implicated in ROS production (for a review, see [22]). The mitochondrial ETC consists of four mitochondrial redox carriers that are known as complexes I, II, III, and IV. Electrons that are donated by NADH and $FADH_2$ toward complexes I and II, respectively, are transferred to complex III and then eventually to complex IV to reduce oxygen molecules to water [23]. In complex I, O^- production is driven by the presence of NADH, which donates electrons to a series of redox enzymes within the complex itself; this series includes flavin mononucleotide, $Fe-S$ clusters, and coenzyme Q10 (ubiquinone) [24]. O^- production has been linked to two sites within complex I: reduced flavin mononucleotides and quinone binding sites [24]. In isolated mitochondria, complex I can reduce O_2 to O^- under two different conditions: (1) when ATP is in low demand and the proton motive force (PMF) is maximal and (2) when the NADH/NAD⁺ ratio is high and ATP is continuously synthesized, resulting in a low PMF [25]. The first mechanism, which is also known as reverse electron transfer (RET), occurs when the PMF is maximal or when electrons donated from succinates in complex II are reverse transferred to complex I. This action causes electron binding to quinone binding sites, which allows electrons to leak out and reduce oxygen molecules [25]. The second mechanism, which is known as forward electron transfer, requires the donation of electrons from reduced form to oxygen molecules, producing O^- . This process depends on a high NADH/NAD⁺ ratio or inhibition of the respiratory chain through mitochondrial damage, ischemia, the loss of cytochrome c, or mutations. It also causes the loading of electrons onto flavin and the subsequent leakage of electrons that reduce oxygen molecules.

In addition to the NADH-driven ROS production from complex I and, subsequently, complex III, complex II has also been demonstrated to be a source of ROS production [26, 27]. Complex II, or succinate dehydrogenase, is composed of four subunits: flavoprotein, the $Fe-S$ cluster, and two transmembrane cytochrome b heme subunits [21, 28]. In

complex II, O^- production is driven by the presence of succinate, which sequentially donates two electrons to flavin, the Fe-S cluster and ubiquinone [27, 29, 30]. Complex IV, in contrast, has yet to be shown to directly produce ROS, although it affects the course of O^- production by complexes I, II, and III and seems to be the terminal and rate-limiting complex that influences the flow of electrons across the ETC.

2.2. Autophagy and ROS. Autophagy is a lysosome-mediated degradation process for nonessential or damaged cellular components [31]. Physiologically, autophagy preserves the balance between organelle biogenesis and both the synthesis and clearance of proteins. This process is emerging as an important mediator of pathological responses because it involves cross talk between ROS and RNS (reactive nitrogen species) during both cell signaling and protein damage. Dysregulated redox signaling or mitochondrial dysfunction can also influence autophagic activities. OS is inseparably linked to mitochondrial dysfunction because mitochondria are both generators and targets of reactive species. Mitochondrial turnover depends on autophagy, which declines with age and is frequently dysfunctional in neurodegenerative diseases [32]. OS can also lead to nonspecific posttranslational modification of proteins, and it contributes to protein aggregation (for a review, see [33]).

2.3. Antioxidants. Organisms have natural AOXs that diminish the harmful effects of continuous ROS production. There are both endogenous and exogenous defense mechanisms against oxidative attack. OS and its biomarkers are the biochemical products of an imbalance between ROS production and the ability of biological AOXs to counteract the effects of ROS metabolites [34]. These defenses are located in the cytoplasm, the cellular membrane, and the extracellular space, and they include enzymatic defense systems, FR scavengers, and chelating agents for transitional metals. The enzymatic systems consist of intracellular molecules, such as SODs, catalase, glutathione peroxidase (GPx), and reductase [35]. FR scavengers slow down oxidation reactions, trapping FRs and transforming them into less aggressive compounds. They can be hydrosoluble and cytosolic (e.g., GSH and vitamin C) or liposoluble and membrane-bound (e.g., vitamin E and carotenoids). Vitamin C is considered to be the main hydrosoluble AOX system, and it may also regenerate tocopherol (vitamin E), which is one of the main liposoluble AOXs in cellular membranes, along with carotenoids (β -carotene, lutein, zeaxanthin, and lycopenes). GSH is considered the most abundant AOX system in the cytosol, nucleus, and mitochondria. The relevant chelating agents include molecules that bind to iron and copper as well as flavonoids. In this way, these chelating agents prevent these metals from participating in Fenton and Haber-Weiss reactions. Depending on their source, AOX agents can be either endogenous, as in GSH, SOD, and CAT, or exogenous (only obtained from nutrients), as in vitamins C and E, carotenoids, flavonoids, and oligo elements [36].

2.3.1. Melatonin as an Antioxidant. Melatonin (*N*-acetyl-5-methoxytryptamine) is an important AOX molecule. It

is a ubiquitous substance that is secreted by the pineal gland of all mammals, including humans. In addition, its presence has been confirmed in many animals, plants, and unicellular organisms [37–39]. Melatonin participates in diverse functions in the body, including sleep, circadian rhythm regulation, and immunoregulation, and it may have anticarcinogenic effects [39]. As a chelating agent, melatonin is a potent FR scavenger and a regulator of redox-active enzymes [40]. This hormone is secreted during darkness and plays a key role in various physiological responses, including the regulation of circadian rhythms, sleep, homeostasis, retinal neuromodulation, and vasomotor responses. It scavenges hydroxyl, carbonate, and various organic radicals and a number of RNS. Melatonin also enhances the AOX potential of cells by stimulating the synthesis of AOX enzymes, including SOD, GPx, and glutathione reductase, and by augmenting glutathione levels [41]. This hormone preserves mitochondrial homeostasis, reduces FR generation, and protects mitochondrial ATP synthesis by stimulating complexes I and IV, thereby counteracting oxidative mtDNA damage and restoring the mitochondrial respiratory control system [41]. Because reduced complex I activity and is a sign of enhanced electron leakage, the resulting increase in OS is sufficient to induce apoptosis. The ability of melatonin to return complex I activity to normal levels suggests its significance in overall health via the prevention of age-associated degenerative changes [42].

2.4. ROS and Aging. Aging has been considered to involve the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death that accompanies advancing age. In 1956, Harman [43] proposed that endogenously generated oxygen FRs induce the macromolecular oxidative damage that is responsible for senescence (for a review, see [44]) and is associated with a decline in physiological fitness during aging. This “free radical hypothesis” was modified and merged with the “oxidative stress hypothesis,” resulting in the term “oxidative stress” [45], which was defined as a disturbance in the pro-oxidant-antioxidant balance that results in a cellular state in which the AOXs are insufficient for complete eradication of various ROS [44]. Both propositions postulate that the progression of age-related deleterious alterations is a function of the imbalance between ROS fluxes and AOXs, and both predict that the narrowing of this gap should reduce the amount of structural damage and thereby prolong the life span. “Therefore, from this historical perspective, the postulated mechanism by which ROS are implicated in the aging process can be aptly characterized as the structural damage-based oxidative stress hypothesis” [44]. Over the past several decades, however, there has been a shift in ideology concerning the role of ROS in cell physiology. Some oxidants, particularly H_2O_2 , have now been recognized as essential for cell survival due to their regulatory roles in a wide range of functions, including gene regulation, cell signaling, protein activation/deactivation, cellular differentiation, and apoptosis (reviewed in [46, 47]). Now, ROS have been recognized to act as signals [48, 49]. Although some of these signaling pathways promote cell death [50], others, such as

oxygen-sensing and hypoxic responses [51] or the induction of autophagy [52], promote cell survival. Therefore, in theory, low (nontoxic) levels of ROS can promote lifespan extension by activating pathways that promote cellular resistance to various stresses.

Although there is little doubt that high levels of ROS are detrimental, mounting evidence indicates that mild to moderate ROS elevation extends lifespan. Dietary caloric restriction, inhibition of insulin-like growth factor-I signaling, and inhibition of the nutrient-sensing mechanistic target of rapamycin are robust longevity-promoting interventions [53] that all appear to elicit retrograde mitochondrial signaling processes that may even spread to other cells.

Other factors, such as biological rhythms, also seem to contribute to aging [54]. Age-associated changes in the day/night rhythm of melatonin production have been identified, with phase advances encountered more frequently in the elderly compared with young women [55]. Suprachiasmatic nucleus (SCN) function has also been shown to decline with age, particularly in patients with aging-associated neurodegenerative disorders, which are a major cause of dementia, and other poor health conditions that are common in elderly populations [56, 57]. A decline in melatonin production and altered melatonin rhythms can be major contributing factors to increased levels of OS and the associated degenerative changes that are observed in the elderly. Nevertheless, individuals of the same chronological age can exhibit dissimilar degrees of senescence-associated functional impairment, differences that may be attributable to well-documented interindividual variations in melatonin levels [58–61]. Variations in the degenerative changes that occur in cells and tissues have been attributed to variations in melatonin production; these changes are more often determined by an individual's physiological age rather than his chronological age [62]. Recently, genetic variation in the enzyme ASMT (HIOMT), which performs a metabolic step that determines the amount of melatonin produced [63], has been demonstrated.

3. Circadian Clocks and ROS

Circadian rhythmicity is a fundamental biological phenomenon that is universally important. This endogenous, innate oscillation with a period of approximately one day has been identified in all of the organisms that have been studied to date, from bacteria to eukaryotes. Temporal variations that are driven by a circadian oscillator are evident in many cellular functions including gene expression, metabolic flux rates, concentrations of signaling molecule, and cellular substructures. In multicellular organisms, circadian rhythms can be studied at different integration levels, from cell-to-cell interactions to organ physiology and from endocrine and neural communications to behavior. Although the control and coordination of circadian rhythms in metazoans are typically organized by specialized pacemaker structures, primary oscillations are generated at the cellular level. These rhythms are widely accepted to be genetically determined, and several genetic clocks have been identified in different taxa, including unicellular organisms [64].

The core of the circadian clock is based on an intracellular time-tracking system that enables organisms to anticipate environmental changes and thereby adapt their behavior and physiology to the appropriate time of day [65]. It is well known that in some animals, such as insects and mammals, a specific set of transcription factors constitutes the molecular architecture of the circadian clock. These factors are organized into positive and negative regulatory feedback loops that function in a cell-autonomous manner and that are rhythmically controlled by a master oscillatory system, which coordinates tissue-specific rhythms according to the input it receives from the rhythms of the outside world [66].

The one or more endogenous oscillators that function to generate a free-running period that is close to 24 h when the organism is maintained under constant environmental conditions are a central component of the circadian system. At the molecular level, these oscillators are based both on the products of “clock genes,” which are organized into transcriptional-translational feedback loops (TTLs), and on oscillations in posttranslational modifications of proteins, which contribute significantly to circadian oscillations [67]. Some of the clock genes encode transcriptional activators, whereas others encode negative feedback elements that inhibit their own expression by disrupting the activity of their activators. Meanwhile, kinases and phosphatases regulate the speed and precision of the clock [68]. Components of the oscillators receive environmental information through input pathways, allowing the oscillators to remain synchronized with the 24 h solar day. This time-of-day information from the oscillator(s) is then relayed through output pathways to regulate the expression of circadian clock-controlled genes and overt rhythmicity. One mechanism by which the output pathways are predicted to be rhythmically controlled is through transcription factors or signaling molecules that are themselves components of the oscillator. These factors, which are activated by the circadian clock, may in turn regulate the downstream clock control genes in a time-of-day-specific manner [69].

The internal clock would be useless if it was not able to synchronize with environmental time or if the cells within a tissue were not synchronized to each other. Therefore, input pathways to the circadian oscillator are vital to maintain the proper timing of the oscillator with respect to the environment. In a process called entrainment, input pathways reset the oscillator so that the period of the oscillator conforms to the 24 h period of the environment [69]. Input pathways detect environmental cues and utilize various mechanisms to increase or decrease the levels or activity of components of the molecular oscillator to set the clock to the correct time of day. One of the most ubiquitous time-giving cues is light, but nonphotic environmental cues, including nutrition, temperature, and social interactions, can also entrain the circadian clock [70–73]. In addition, the clock utilizes a strategy called gating to restrict responses to environmental cues at certain times of day. For example, diurnal mammals are typically insensitive to a light pulse during the day, but, during the night, a light pulse can advance or delay the clock to synchronize it with the environment [74], just as we would adjust our watches to match the local time. In

organisms of various complexities, cells vary in their ability to support a molecular oscillator that can be entrained by environmental signals. In unicellular organisms, each cell has a fully entrainable oscillator that primarily responds to light [75]. However, in complex multicellular organisms, not all cell types have the necessary sensory capabilities, such as photoreception, to entrain the circadian oscillator. The cellular oscillators and overall rhythmicity of the organism are broken down into components that include a master pacemaker and peripheral oscillators [76]. To integrate multiple sensory inputs, organisms that possess a nervous system typically delegate the ability to sense environmental cues to a central oscillator or pacemaker rather than to individual cells. In mammals, sensory inputs to the clock are integrated in the brain, where signals from the master pacemaker entrain the oscillators in other tissues throughout the organism. Light is perceived by nonvisual retinal ganglion cells that transmit information via neural connections to the master pacemaker, which is located in a region of the hypothalamus, the SCN. The SCN pacemaker synchronizes oscillators in other tissues by a mechanism that utilizes circadian input pathways from the SCN to individual cells in the periphery. In addition to maintaining the entrainment of peripheral oscillators by the environment, this system ensures that cellular oscillations within tissues are properly in phase to provide resonance between individual cellular rhythms [77].

Melatonin acts as an important synchronizer in mammals and provides temporal feedback to oscillators within the SCN, which regulates the circadian phase and maintains rhythmic stability [78]. Various studies in birds and mammals, both *in vitro* and *in vivo*, have demonstrated that melatonin adjusts the circadian “clock” by acting directly on the molecular timing system [78, 79]. In humans, as in other mammals, melatonin is presumed to influence circadian rhythms by acting directly on receptors in the SCN [80].

For at least three decades, the cellular redox state in plants and animals has been known to change over circadian time [81], although many chronobiologists have long assumed that metabolic rhythms are a functional readout of the circadian clock and that redox oscillators simply provide feedback to the central TTL pacemaker [82].

Recent discoveries have, however, uncovered redox-based circadian oscillators that are conserved across both eukaryotic and prokaryotic species. Circadian rhythms in ROS generation and scavenging have been observed in a variety of species, including fungi [83], plants [84], and animals [85]. Further evidence of the involvement of the circadian clockwork in the regulation of redox systems is supported by studies of mutants, where the deletion or disruption of clock genes also results in the perturbation of redox systems. In mammals, 24 h oscillations in concentrations of oxidized NADPH and the reduced form of FAD have been observed in organotypic slices of the rodent SCN [86]. The rhythms of these coenzymes are believed to be dependent on the molecular clockwork because *Bmal1*^{-/-} mice exhibit stochastic, but not circadian, FAD, and NADPH rhythms. These studies also revealed links between the redox state and the membrane excitability of SCN neurons, given that

oxidizing and reducing agents can produce hyperpolarization and depolarization, respectively. Redox-dependent modulation of K⁺ channel conductance is believed to underlie these oscillations [86]. All of these findings support complex interdependence among the redox state, cellular energetics, and circadian clockwork in mammals.

The links between circadian physiology, prooxidizing changes in the redox state, as reflected by a decline in redox potential, and the process of aging seem to be coherent and well established [87]. Hence, in the present work, the OS damage generated in the human eye and its relationship with AMD is briefly discussed. Here, the impact of OS from the accumulation of ROS, which is probably due to circadian alterations, and its relevance to chronic pathologies, is briefly reviewed, with a particular emphasis on retinal neurodegenerative diseases such as AMD.

4. The Retina and Oxidative Stress

In humans, the retina is very prone to the generation of ROS compared with other tissues. This structure is a photosensitive tissue with high oxygen levels in the choroid and a high metabolic rate that is also exposed to light. Furthermore, the retina contains a higher concentration of PUFAs than other body tissues [88]. Lipids in the outer segment membranes of photoreceptors can be oxidized by radicals produced during photonic activation, and the endogenous oxygen species that are generated in the eyes through this process can induce ROS-related acute or chronic retinal damage. All of these factors, combined with the very high oxygen levels in the choroid, the high metabolic rate, and the exposure to light, make our retinas vulnerable to the effects of light, especially to light of shorter wavelengths [89]. “Each day, the retina of the average human absorbs approximately 10¹² to 10¹⁵ photons, and this amount can be greatly increased by the workplace, sunlight exposure, or medical imaging of the retina during an eye examination. Such high levels of exposure to visible light can cause irreparable damage to the retina” [90]. Recently, Roehlecke et al. [91] demonstrated that ROS are generated and OS occurs directly in the outer segments of photoreceptors after blue light irradiation.

Among all of the retinal cell organelles, the mitochondria are particularly sensitive to OS due to their handling of electrons in the respiratory chain. In addition, after blue light exposure, more electrons deviate from the respiratory chain in the mitochondria, resulting in further damage. In fact, inhibiting the mitochondrial transport chain in retinal pigment epithelium (RPE) cells or adding mitochondria-specific AOXs blocks ROS formation and cell death [89]. Furthermore, chromophores in general and cytochromes in particular can be sources of ROS [36]. OS-induced inflammation initiates a functional decline in tear production, and dry eye is the first symptom of this type of damage to the eye [92].

In the human eye, OS in the RPE is widely accepted as a contributing factor for retinal disorders. The RPE, a monostratified cell layer, constitutes the outer blood-retinal barrier (BRB), controls fluid and metabolic exchange between the retina and the choriocapillaris, and participates in several

functions linked to photoreceptor physiology, such as the phagocytosis of shed photoreceptor outer segments and transportation of molecules to and from the retina [93].

In addition, RPE cells release factors that control neuronal survival and angiogenesis (e.g., pigment epithelium-derived factor, PEDF) and that promote photoreceptor survival and have an antiangiogenic effect on the choriocapillaris [94, 95]. The RPE secretes PEDF on the apical side; in contrast, the angiogenic factor vascular endothelial growth factor (VEGF) is secreted on the basolateral side to maintain fenestration of the choriocapillaris. An imbalance in the expression of PEDF and VEGF appears to be involved in ocular pathologies. Because PEDF has antioxidative and antiangiogenic properties, this factor may protect against vision-threatening angiogenic mechanisms. In contrast, VEGF is generally accepted as the most potent inducer of endothelial activation and angiogenesis, a process in which new vessels develop from the preexisting vasculature (for a review, see [96]). Under ischemic or hypoxic conditions, which occur in many neovascular diseases, retinal expression and production of VEGF are dramatically increased [97, 98].

The oxidation of polyunsaturated fats in the retina leads to lipid peroxidation products such as carboxyethylpyrrole and 4-hydroxy-2-nonenal, which can form adducts with proteins and accumulate in the outer retina and in drusen [99]. The levels of AOX proteins such as catalase and SOD are increased in RPE homogenates derived from the eyes of late-stage AMD patients, indicating a response to increased OS in the eyes of these patients [100].

As indicated in previous sections, the NOX [101] family of enzymes has recently been recognized as a generator of ROS in rod or cone photoreceptors after they are damaged due to serum deprivation in a model of retinitis pigmentosa [102]. Roehlecke et al. [103] further demonstrated that blue light irradiation results in increased superoxide anion production.

Lipofuscin is the generic name given to a heterogeneous group of complex and autofluorescent bisretinoids, lipid peroxides, and proteins and to various fluorescent compounds that are formed from modified lipids or that are derived from vitamin A. The major substrate for lipofuscin in the RPE is a nondegradable end product that results from phagocytosis of the photoreceptor outer segments, which are rich in PUFAs and vitamin A. This substrate is located within the RPE, where it accumulates in lysosomes with age. Lipofuscin is a byproduct of the visual cycle [104, 105] that is produced when phagocytosed material is not entirely degraded within the RPE lysosomes, resulting in the accumulation of this complex over time. Lipofuscin is continually exposed to high oxygen tensions (70 mmHg) and to visible light (400–700 nm) during daylight hours, creating a prime environment for the generation of ROS that have the potential to damage cellular proteins and lipid membranes. Thus, lipofuscin is a photoinducible generator of ROS that increases the risk of oxidative injury [106]. A lipofuscin fluorophore, A2E, is now known to mediate the blue light-induced apoptosis of RPE cells, which inhibits the degradative capacity of lysosomes and disrupts membrane integrity [107, 108]. This complex phototoxic effect is wavelength-dependent; more superoxide anions are generated in granules exposed to blue

light (400–520 nm) than in granules exposed to red light (660–730 nm) or full white light [109]. Lipofuscin induces the apoptosis of cultured RPE cells, leading to a decline in mitochondrial activity that is associated with the translocation of cytochrome c, an apoptosis-inducing factor, and two apoptosis-inducing proteins into the cytoplasm and nucleus [110].

However, whether A2E or lipofuscin may impair lysosomal function is still controversial. Recently, Saadat et al. [111] demonstrated that A2E and impaired autophagy mediate RPE cell damage. These authors proposed that decreased lysosomal capacity might also result in decreased autophagy in RPE cells, which would not affect autophagosome biogenesis or fusion with lysosomes but would instead impair the terminal stage of the degradation process. Thus, the accumulation of A2E in aged RPE cells, in which autophagy is already impaired, may result in RPE cell damage. The authors also considered the possibility that A2E inhibited lysosomal function, which then resulted in the accumulation of autophagosomes rather than an increase in autophagic flux through this pathway.

Some authors have maintained [112] that the RPE secretes apolipoprotein B particles into Bruch's membrane and that these particles accumulate with age and may in turn form a lipid wall, a precursor of the basal linear deposit. Then, certain constituents of the described aggregates may interact with ROS, resulting in proinflammatory, peroxidized lipids and leading to the upregulation of cytokines/chemokines, which then promotes neovascularization. However, recent publications indicate that, in the RPE, OS is also capable of inducing protective pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor erythroid-2-related factor 2 pathways. In addition, VEGF and neuroprotectin D1 signaling act to protect the retina [113, 114].

The phagocytosis of oxidized fatty acids from photoreceptor outer segments contributes to metabolic failure in the RPE [115]. Importantly, the FR byproducts of mitochondrial energy metabolism damage the RPE [116]. In addition, the mitochondrial ETC generates superoxide radicals through single-electron leaks at respiratory complexes I and III [18].

Flavin-dependent enzymes in the mitochondrial matrix may also be large contributors of ROS [22]. Superoxide can directly damage mitochondrial DNA, proteins, and lipids, and it can also be converted to H₂O₂ by manganese SOD in the mitochondria [22]. The potential for RPE light damage is influenced by a number of factors, including age, diurnal fluctuations, and pathology [117].

Otherwise, circadian photoreception decreases with age due to disruptions to circadian rhythms, age-related pupillary miosis and reduced crystalline lens transmission, particularly of blue light. Circadian studies have revealed loss of the control of pupil size and the crystalline lens in aging subjects [118].

5. ROS and Age-Related Macular Degeneration (AMD)

AMD, a major cause of visual impairment in the elderly, is linked to pathological changes involving the RPE. AMD

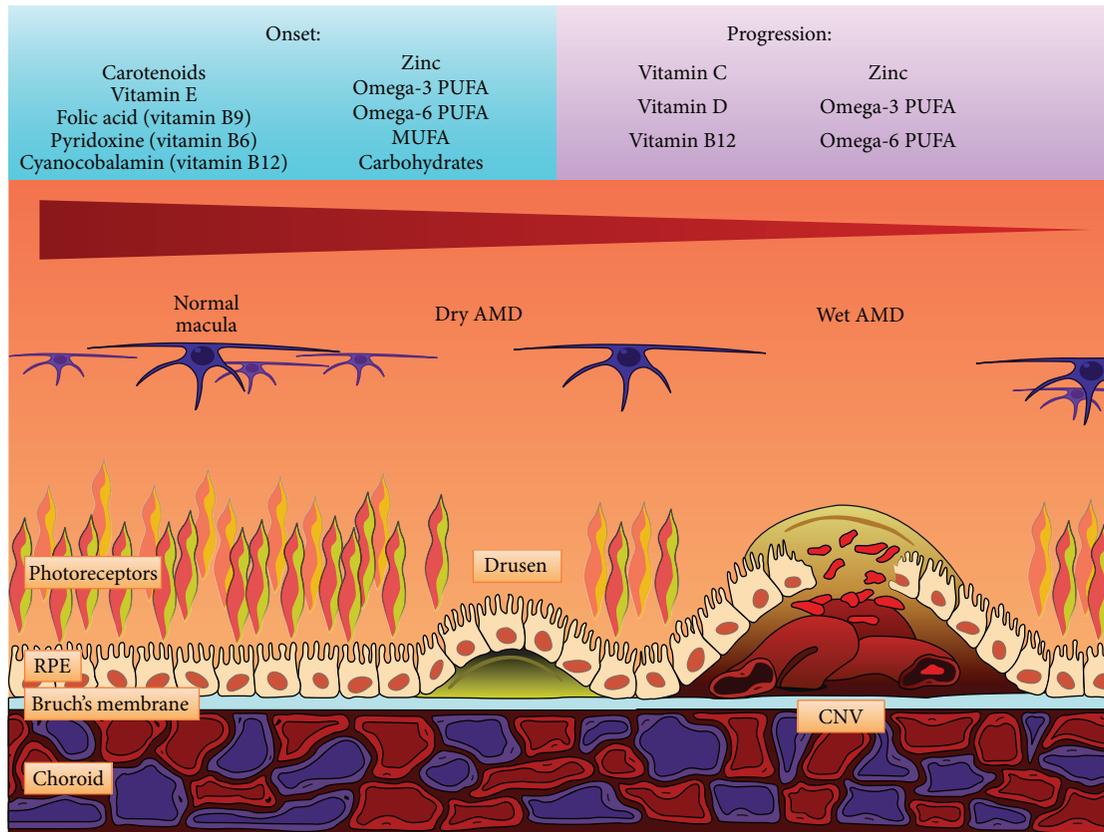


FIGURE 2: Development of AMD. AMD is mainly due to photochemical damage and oxidative stress. In the figure, the progression of AMD is represented by the cellular structure of the normal macula (left side), the dry AMD (center), and the wet AMD (right side). In the left side of the image, the cells in the macula are normal. In the dry form of AMD (center), drusen impair the metabolic connection between the choroid and the upper layers of macula, leading to the degeneration of the RPE and photoreceptors. In the wet form of AMD (right side), the production of neovascular factors results in the formation of choroidal neovascularization (CNV) with subsequent fluid leakage and major degeneration of the RPE and photoreceptors. In the normal ageing process, lipids accumulate in Bruch's membrane, causing the membrane to thicken and improving the oxidative distress. Moreover, Bruch's membrane lacks an adequate intrinsic antioxidant system. Furthermore, the lipids can bind the macrophages, inducing the secretion of vascular endothelial growth factor (VEGF). The production of inflammatory factors improves the damage to the RPE and photoreceptors and induces the formation of functional microvascular networks (choroidal neovascularization). Antioxidant factors might prevent the disease and delay its progression. In the upper portion of the image, the pool of nutrients involved in the onset (left side) and progression (right side) of AMD are listed. From Zampatti et al. [131], with the permission of the authors and Elsevier. Rightlinks License number is 3679500345328.

is a progressive neurodegenerative disease of the central retinal area (macula lutea) and represents the most common cause of legal blindness in industrialized countries [119]. Epidemiologic studies from several countries have shown a dramatic increase in the prevalence and severity of AMD with age. Despite intensive basic and clinical research, its pathogenesis remains unclear, likely due to its multifactorial nature [120]. In addition to the strong age dependence of the disease, complex interactions between metabolic, functional, genetic, and environmental factors create a platform for the development of chronic changes in the ocular structures of the macular region (e.g., choriocapillaris, Bruch's membrane, RPE, and photoreceptors), and changes in each of these structures may contribute to varying degrees to the onset of AMD (Figure 2). Photoreceptors renew their light-sensitive outer segments continuously and shed their distal tips daily. The adjacent RPE efficiently consumes shed photoreceptor outer segment fragments (POSSs) and recycles or digests their

components via phagocytosis [121]. Outer segment renewal is crucial for the photoreceptor function and survival. Experimental studies have demonstrated that a lack of efficient POS phagocytosis by RPE cells in some rat strains causes rapid photoreceptor degeneration [122]. A delay in POS digestion can directly cause the accumulation of undigested POS materials, such as lipofuscin, in the RPE, which is detrimental to the RPE and the retina and may contribute to the development or progression of AMD [123, 124].

Based on its clinical presentation, AMD is categorized into early, intermediate, and late stages [125]. Early and intermediate AMD are characterized by soft, yellowish deposits that vary in size from small to large (drusen) and by pigmentation changes in the macula, with little or no visual loss. In late AMD, visual loss appears in two forms, that is, neovascular AMD (also called "wet" or "exudative" AMD), in addition to drusen and atrophy. This form of AMD is characterized by the presence of edema and hemorrhage

within or below the retina or RPE and geographic atrophy (also called “dry” AMD). Currently, there are no treatments for geographic atrophy, but neovascular AMD is treated with VEGF inhibitors, which, although not curative, are often effective in preventing severe visual loss [126]. The dry form, also known as age-related maculopathy, is characterized by the presence of drusen under the RPE that is accompanied by either the loss or focal accumulation of melanin pigment. This form of AMD is typically characterized by a progressive course that leads to degeneration of the RPE and photoreceptors. The exudative form is linked to choroidal neovascularization that is directed toward the subretinal macular region, with subsequent bleeding and/or fluid leakage that can result in a sudden loss of central vision. It is the most rapidly progressing form of AMD. Both the atrophic and exudative forms are associated with severe visual impairment [119]. The pathophysiology of AMD is complex, and, in addition to genetic predispositions, at least 4 processes contribute to the disease: lipofuscinogenesis, drusogenesis, local inflammation and neovascularization (in the case of the wet form), and immunological mechanisms [127].

The current pathophysiological conception of AMD assigns a primary role to age-related, cumulative oxidative damage to the RPE due to an imbalance between the generation and elimination of ROS [128]. In particular, lipofuscin has been hypothesized to be the primary source of the ROS responsible for both the cellular and extracellular matrix alterations found in AMD [129, 130].

The accumulation of lipofuscin and other lipid peroxides and potentially toxic substances may dramatically influence RPE physiology, as described above. This accumulation greatly reduces the phagocytic capacity, lysosomal enzyme activities, and AOX potential of the human RPE in vitro [133, 134]. The first clinical sign of early AMD is drusen, as mentioned above. Drusen are lipid-rich, sub-RPE deposits that contain a variety of proteins, including vitronectin, components of the terminal complement cascade, and β -amyloid [135, 136]. Microscopic analysis of eyes donated by patients with AMD revealed lipid deposits within Bruch's membrane and apoptosis of RPE cells as features of the disease that are distinct from normal aging. The advanced form of dry AMD, which leads to geographic atrophy, was characterized by breakdown of the RPE, the choriocapillaris, and photoreceptors in regions of the retina, often where large drusen were present [137]. Dysregulation of the phagocytosis of oxidized fatty acids from photoreceptor outer segments is a contributing factor to the metabolic failure of RPE cells [115, 123]. In addition to all of these factors, the damage caused by the FR byproducts of mitochondrial energy metabolism has been implicated in age-related damage to the RPE [116].

As reviewed above, the mitochondrial ETC generates superoxide radicals through single-electron leaks at respiratory complexes I and III [18], and flavin-dependent enzymes in the mitochondrial matrix may be large contributors of ROS [29].

Otherwise, starvation and hypoxia, which can result from poor perfusion, are generally associated with increased amounts of ROS, which promote autophagy via several complex signaling mechanisms [138] (Figure 3). In response

to OS, autophagy is significantly increased in an attempt to remove oxidatively damaged organelles such as mitochondria. At this time, accumulating evidence linking the impairment of autophagy with a range of age-related neurodegenerative diseases, including AMD, has suggested that autophagy occurs in the RPE to maintain homeostasis because these cells are exposed to sustained OS. However, insufficient digestion due to impaired autophagy or lysosomal degradation in the RPE can lead to an accumulation of damaged organelles, toxic proteins (including lipofuscin), and extracellular drusen deposits, all of which can contribute to RPE dysfunction or RPE cell death, which have been associated with the pathogenesis of AMD [139]. In addition, the AOXs of the retina (e.g., via macular molecules such as lutein and zeaxanthin) are reduced in AMD [140].

5.1. VEGF and AMD. VEGF is the most potent inducer of endothelial activation and angiogenesis. It is mainly expressed in retinal neurons and glial cells and is present in only scant amounts in blood vessels [141]. Under ischemic conditions, retinal expression and production of VEGF is increased [142]. This factor has also been implicated in the development of retinal neovascularization in ischemic retinopathies such as AMD [98]. Through a paracrine mechanism, VEGF binds to its cell-surface receptors, including VEGFR1/Flt-1, VEGFR2/Flk-1/KDR, and VEGFR3, and promotes endothelial cell survival, proliferation, migration, and tubular structure formation [142]. Among these receptors, VEGFR2 is the crucial receptor that mediates angiogenic and vascular permeability, whereas VEGFR3 mainly mediates lymphangiogenic functions. The activation of VEGFR1 plays a dual role and can either stimulate or inhibit angiogenesis, whereas the activation of VEGFR2 seems to only stimulate angiogenesis [143, 144].

Upon binding VEGF, VEGFR2 undergoes dimerization and autophosphorylation, resulting in the activation of its downstream kinases, including mitogen-activated protein kinase (MAPK), ERK1/2, p38, JNK, and PI3K/Akt, and of endothelial NO synthase (e-NOS), which may lead to further alterations in endothelial cell survival, proliferation, and migration. Recent reviews provide further information on this important factor [145, 146].

A constant oxygen supply is clearly essential for proper homeostasis and normal functioning in all retinal tissues. Cellular responses to reduced oxygen levels are mediated by the transcriptional regulator hypoxia-inducible factor-1 (HIF-1), a heterodimeric protein complex that consists of an oxygen-dependent subunit (HIF-1 α) and a constitutively expressed nuclear subunit (HIF-1 β). Under normoxic conditions, de novo synthesized cytoplasmic HIF-1 α is degraded by the 26S proteasome. Under hypoxic conditions, HIF-1 α is stabilized, binds to HIF-1 β , and activates the transcription of various target genes. These genes play a key role in the regulation of angiogenesis in various visual pathologies, such as AMD [147].

5.2. AMD and the Complement Pathway. Growing evidence indicates that AMD is downstream of a chronic inflammatory condition in which activation of the immune system plays

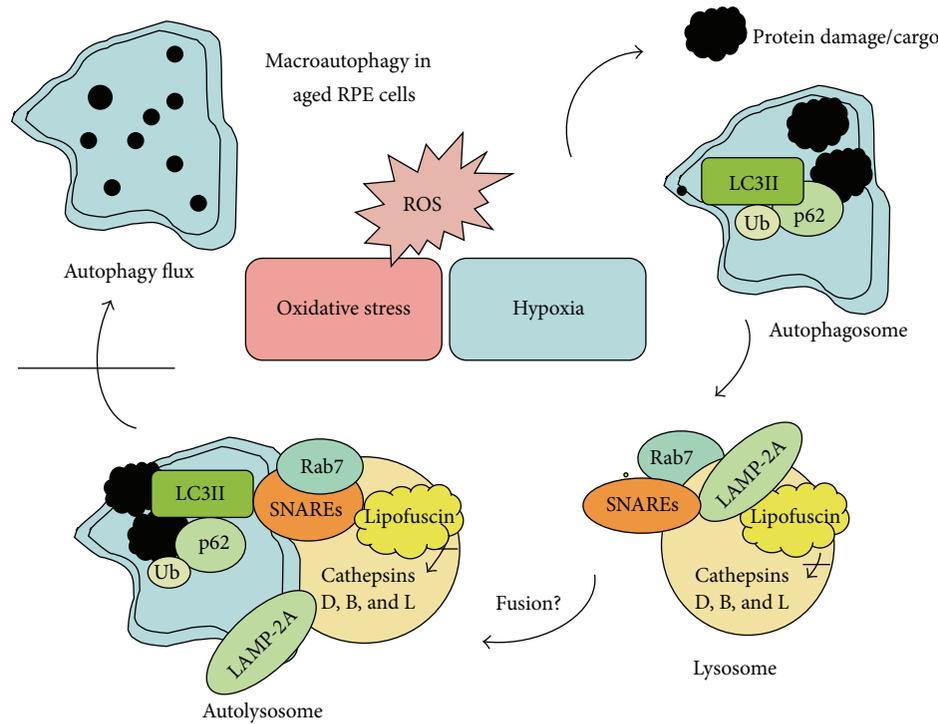


FIGURE 3: Schematic presentation of the macroautophagy process in aged retinal pigment epithelial (RPE) cells. Oxidative stress, (ROS), and hypoxia lead to protein damage and aggregation, which induces autophagy. The substrate (cargo) for autophagy is degraded by lysosomal acid hydrolases, including cathepsins D, B, and L, after the fusion of lysosomes and autophagosomes to form autolysosomes. Rab7, LAMP-2A, and SNARE proteins are critical for the lysosome and autophagosome fusion process. Ubiquitin (Ub), LC3II, and p62 are complexed to the cargo and connect autophagy to the proteasomal clearance system. Macroautophagy is prevented in AMD because lysosomal lipofuscin disturbs cathepsin activity and autophagy flux. Fusion mechanisms in the RPE cells are under investigation. From Blasiak et al. [132], with the permission of the authors.

an important role. Metabolic products accumulate in the extracellular space between Bruch's membrane and the RPE and activate the complement system through a significant increase in OS, similar to the processes that occur in atherosclerosis or Alzheimer's disease [148]. These findings as well as those of many studies over the past decade have changed the understanding of the molecular mechanisms underlying AMD and led scientists to explore the targeting of specific molecular components of the complement pathway [149–153]. The complement system is a major component of innate immunity that plays a role in defense against invading microorganisms, the clearance of apoptotic cells, and the modulation of immune responses [154]. The complement cascade's four activation pathways converge upon a common terminal pathway that culminates in the formation of the cytolytic membrane attack complex (MAC). Binding of circulating C1q to antigen antibody complexes activates the *classical* pathway. The *lectin* pathway is activated by mannan-binding lectin following its recognition of, and binding to, molecular patterns on pathogen surfaces. The recently characterized *intrinsic* pathway is activated by proteases that cleave C3 and C5 directly. In contrast to the other three pathways, the *alternative* pathway is continuously active at a low level and is characterized by the spontaneous hydrolysis of C3 into the C3a and C3b fragments. C3b binds complement factor B (CFB), and once bound, CFB is cleaved by complement

factor D (CFD) into Ba and Bb, thereby forming the active C3 convertase (C3bBb). C3bBb cleaves additional C3 molecules, which generates more C3a and C3b and thereby promotes further amplification of the cascade. In addition to the 30 or more complement components and fragments, numerous soluble and membrane-bound regulatory proteins modulate the complement system [153]. Complement factor H (CFH) is an important regulatory complement protein and is a major inhibitor of the alternative complement cascade that prevents excessive activation of the complement components. CFH regulates complement activity by inhibiting the activation of C3 to C3a and C3b and by inactivating existing C3b [154].

The discovery that drusen contain alternative complement pathway proteins led to the hypothesis that drusen could be involved in local complement-mediated inflammation [153]. The reports of an association between AMD and genetic variants in the *cfh* gene, a major inhibitor of the alternative pathway, support the inflammation model [154]. Other AMD risk variants have been found in genes underlying the alternative pathway, principally the formation of unstable C3 convertase, C3bBb, which cleaves C3 to generate the active segment C3b. Deposition of C3b on the target surface triggers the effector molecules C3a and C5a and the MAC, resulting in inflammation and cell lysis. In addition to CFH, several other AMD risk variants have been identified in genes underlying the alternative pathway. Variations in the

factor B (BF) and complement component 2 (C2) genes are also associated with AMD [155].

A third line of evidence in support of complement involvement in AMD was provided by studies that showed that AMD patients have higher levels of complement activation products in their blood [156].

In 2001, data collected from the Age-Related Eye Disease Study (AREDS) revealed that patients who were treated with zinc, either alone or in combination with vitamins, displayed reduced progression to advanced AMD. AREDS results led to the recommendation that persons who are older than 55 years of age and who are at risk of developing advanced AMD should consider taking vitamin supplements plus zinc. A previous report published by the Blue Mountains Eye Study, a population-based study, confirmed the beneficial effect of zinc in AMD patients [157]. A recent study also provided evidence that daily administration of 50 mg of zinc sulfate can inhibit complement involvement in AMD patients who have increased complement activation [158].

5.3. AMD, RPE, and Daily and Circadian Rhythms. The retinal circadian system involves a unique structure. It contains a complete circadian system with multiple generation sites of numerous circadian rhythms, each of which deserves its own review. However, in the vertebrate retina, the intimate reciprocal relationship that exists between the neural retina and the underlying RPE is crucial for vision, while the diurnal and circadian rhythmicity of the RPE is critical for photoreceptor support and retinal function. Thus, in this section, we briefly review some of the rhythmic functions of the RPE that contribute to normal and pathological vision.

The association between AMD and biological rhythms has been poorly studied; however, there is a strong link between ocular physiology and circadian rhythms in both humans and animals. The renewal and elimination of aged photoreceptor outer segment tips by cells from the RPE is a daily rhythmic process that is crucial for long-term vision. Photoreceptors indefinitely renew their light-sensitive outer segments by disk shedding and the subsequent formation of new disks from the cilium of the inner segment. In higher vertebrates, outer segment renewal is synchronized by circadian rhythms [121, 159].

This shedding occurs once per day. In mice and rats, rod shedding is synchronized with light onset [160, 161]. To maintain the constant length of photoreceptors, the outer segment needs to be shed, and the formation of new outer segments must be coordinated.

The task of the adjacent RPE is to absorb shed POSs by phagocytosis and to recycle or digest their components. Outer segment renewal is crucial for photoreceptor function and survival, and a lack of efficient phagocytosis is sufficient to cause rapid photoreceptor degeneration by disk shedding [121, 162]. Photoreceptor disk shedding and subsequent phagocytosis by the RPE must be precisely regulated [163]. Alterations, such as delayed termination of shedding or defective digestion in the RPE, can cause the accumulation of lipofuscin [123]. Outer segment renewal and RPE phagocytosis are synchronized under circadian control and are triggered by the dark/light periods of the daily rhythm [163, 164].

Studies in higher vertebrates have revealed differences between cone- and rod-dominant species. Rod shedding mainly occurs in the morning, resulting in complementary RPE phagocytic activity by an increased number of phagosomes within the first 2 h after light onset, whereas cone shedding is more variable and mainly occurs either during the night or during the first 2 h after light onset [163, 164]. Any disruption in this process causes photoreceptor dysfunction and blindness in animal models and retinal disease in humans [93]. The synchronization of shedding with light seems to be crucial to photoreceptor physiology and survival because the accumulation of undigested material is detrimental to the RPE and retina and may contribute to the development or progression of AMD [123, 124]. These rhythms that synchronize with light are perceived by photosensitive retinal ganglion cells that contain the pigment melanopsin. Daily information is transmitted to the master circadian oscillator located in the SCN via the retinohypothalamic tract [66]. The action spectrum of light information for the circadian biological rhythm shows a peak at a shorter wavelength (464 nm) than that for visual information (approximately 555 nm).

Pivotal studies [161–164] have conclusively demonstrated that, in the rat retina, the diurnal rhythm of rod POS shedding and RPE phagocytosis is under circadian regulation. The mammalian retina displays persistent rhythmic activity even when it is isolated from the brain. In rats with transected nerves, POS shedding and RPE phagocytosis continue diurnally despite the loss of synaptic connections between the eye and the brain, suggesting that this rhythm is generated and controlled locally in the eye [165]. However, this rhythm cannot be reset by light unless the optic nerve remains intact [166]. Thus, the circadian renewal of POS involves both local control within the retina and central regulation by the brain.

The cellular and molecular mechanisms that underlie circadian regulation of shedding/phagocytosis are complex and are not the subject of this review. Numerous genes, proteins and signaling pathways play important roles in the engulfment of POS and the subsequent lysosomal degradation of spent photoreceptor disks within the RPE.

The lack of POS phagocytosis or digestion leads to photoreceptor dystrophy and blindness [122] due to the absence of the engulfment activity of the RPE. In rats that are deficient in MerTK, this absence causes dramatic and early onset retinal degeneration [122, 167]. Because the ingestion rate of POS by RPE cells exhibits a pronounced circadian rhythm that peaks around subjective dawn in both rat and mouse strains [159, 168], Prasad and colleagues [169] suggested that a feature of the TAM receptor system, such as ligand and/or receptor expression levels, might be regulated as a function of position in the circadian cycle.

Despite our vast knowledge of circadian biology and angiogenesis, the role of the circadian clock in the regulation of angiogenesis and vascular patterning remains poorly understood. Experimental animal models may help define the relationships between circadian rhythms and some retinal pathologies, such as AMD. Jensen et al. [170] showed that disruption of the circadian clock by both constant exposure to light and genetic manipulation of key genes in zebrafish

led to impaired developmental angiogenesis. The disruption of crucial circadian regulatory genes, including *Bmall* and *Period2*, resulted in either marked impairment or enhancement of vascular development. At the molecular level, these authors showed that the circadian regulator *Bmall* directly targets the promoter region of the *vegf* gene in zebrafish, leading to elevated VEGF expression. Interestingly, deletion of these E-boxes in the promoter region of the zebrafish *vegf* gene resulted in inactivation of the promoter. These findings can be reasonably extended to developmental angiogenesis in mammals and even to pathological angiogenesis in humans [171]. Disruption of the circadian clock system not only affects the physiological activity of an organism but also often leads to the onset, development and progression of various diseases [172].

An important circadian hormone that is involved in vertebrate retinal circadian rhythms is melatonin, which is synthesized and produced by photoreceptors and shows a clear daily rhythm, with an acrophase at night [173]. This indolamine seems to have protective effects on other retinal cell types, including RPE cells and photoreceptors. Melatonin protects cultured RPE cells from OS and ischemia-induced cell death [174, 175]. Several studies have reported that melatonin is involved in the pathogenesis of AMD. In 2005, Yi et al. [176] reported that daily administration of melatonin (3 mg) may protect the retina and delay the progression of AMD. Rosen et al. [177] reported that the production of melatonin is decreased in AMD patients compared with age-matched controls, suggesting that a deficiency in melatonin may play a role in the occurrence of AMD. A further indication of the possible role of melatonin in age-related pathologies is the observation that retinal melatonin synthesis decreases during aging. In 2012, Tosini et al. [178] proposed that melatonin could affect the circadian clocks in photoreceptors and RPE cells and could thereby affect metabolism in these cells.

Melatonin and dopamine, two regulatory signals that play important roles in retinal physiology, have been proposed to be involved in the control of circadian POS shedding and RPE phagocytosis. In the retina, the production and release of melatonin and dopamine are under circadian control [179, 180]. Although there is insufficient clinical and experimental evidence to demonstrate a direct relationship between melatonin, circadian rhythms, and AMD, some reports have suggested that the melatonin rhythm is reversed in AMD patients [181].

6. Conclusion

This review highlights the role of OS as one of the main causes of AMD etiologies. In this retinal disease, as in other metabolic and degenerative pathologies, complex interactions among metabolic, functional, genetic, and environmental factors create a platform for the development of chronic changes in the ocular structures of the macular region in addition to a strong age dependence. In addition to genetic predispositions, at least four processes contribute to the disease: lipofuscinogenesis, drusenogenesis, local inflammation and neovascularization (in the case of the wet form), and immunological mechanisms.

The current pathophysiological conception of AMD assigns a primary role to the age-related, cumulative oxidative damage to the RPE that occurs due to an imbalance between the generation and elimination of ROS. In particular, lipofuscin has been hypothesized to be the primary source of ROS and to be responsible for both the cellular and extracellular matrix alterations in AMD. However, there may also be an association between the increasing levels of environmental sun radiation, especially short wavelengths in the violet and blue spectrum, due to both the ozone hole and climate change over the last decades, particularly given that AMD remains the leading cause of irreversible vision loss among the elderly in developed nations. In 2004, the overall global prevalence of AMD was approximately 8.7 percent, and the number of AMD patients was projected to rise to 196 million people worldwide by 2020 and to 288 million by 2040 [182]. Interestingly, the association between AMD and circadian rhythms, particularly different RPE rhythms, suggests a role for the circadian clock in AMD-related circadian abnormalities, which have generally been considered to be a consequence of neurodegeneration. However, recent evidence suggests that circadian disruption might actually contribute to the degenerative process and thus might be a modifiable cause of cell or neural injury.

Circadian clock genes have been shown to regulate VEGF signaling in tumorigenesis [183], and dopamine has been shown to modulate the effects of VEGF receptor activation on vascular endothelial cells [184, 185]. Recent results also indicate that *Period* genes may play a similar role in regulating vascularization signals in the retina in retinopathy disease models [186]. Regardless, retinal clock gene expression is disrupted in proliferative neovascularizing diseases [187].

Although circadian disturbances due to aging and neurodegenerative diseases have been duly noted, a key question is whether these disturbances influence the pathology of AMD. This question deserves further investigation.

List of Abbreviations

AMD:	Age-related macular degeneration
AOX:	Antioxidant defense
BRB:	Blood-retinal barrier
CFH:	Complement factor H
ETC:	Electron transport chain
FR:	Free radical
GPx:	Glutathione peroxidase
HIF-1:	Hypoxia-inducible factor-1
Melatonin:	<i>N</i> -acetyl-5-methoxytryptamine
MAC:	Membrane attack complex
mitoROS:	Mitochondrially generated ROS
mtDNA:	Mitochondrial DNA
NOX:	NADPH oxidases
OS:	Oxidative stress
PEDF:	Pigment epithelium-derived factor
PMF:	Proton motive force
POSs:	Photoreceptor outer segment fragments
PUFAs:	Polyunsaturated fatty acids
RET:	Reverse electron transfer
RNS:	Reactive nitrogen species

ROS: Reactive oxygen species
 RPE: Retinal pigment epithelium
 SCN: Suprachiasmatic nucleus
 SODs: Superoxide dismutases
 TTL: Transcriptional-translational feedback loop
 VEGF: Vascular endothelial growth factor.

Disclosure

María Luisa Fanjul-Moles is a retired Professor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] B. Halliwell, "The antioxidant paradox: less paradoxical now?" *British Journal of Clinical Pharmacology*, vol. 75, no. 3, pp. 637–644, 2013.
- [2] M. Schieber and N. S. Chandel, "ROS function in redox signaling and oxidative stress," *Current Biology*, vol. 24, no. 10, pp. R453–R462, 2014.
- [3] T. Finkel and N. J. Holbrook, "Oxidants, oxidative stress and the biology of ageing," *Nature*, vol. 408, no. 6809, pp. 239–247, 2000.
- [4] J. F. Turrens, "Superoxide production by the mitochondrial respiratory chain," *Bioscience Reports*, vol. 17, no. 1, pp. 3–8, 1997.
- [5] P. Kuppusamy and J. L. Zweier, "Characterization of free radical generation by xanthine oxidase. Evidence for hydroxyl radical generation," *Journal of Biological Chemistry*, vol. 264, no. 17, pp. 9880–9884, 1989.
- [6] M. C. DeRosa and R. J. Crutchley, "Photosensitized singlet oxygen and its applications," *Coordination Chemistry Reviews*, vol. 233–234, pp. 351–371, 2002.
- [7] P. Pospíšil, A. Prasad, and M. Rác, "Role of reactive oxygen species in ultra-weak photon emission in biological systems," *Journal of Photochemistry and Photobiology B: Biology*, vol. 139, pp. 11–23, 2014.
- [8] B. I. Kruff and A. Greer, "Photosensitization reactions in vitro and in vivo," *Photochemistry and Photobiology*, vol. 87, no. 6, pp. 1204–1213, 2011.
- [9] F. Liebel, S. Kaur, E. Ruvolo, N. Kollias, and M. D. Southall, "Irradiation of skin with visible light induces reactive oxygen species and matrix-degrading enzymes," *Journal of Investigative Dermatology*, vol. 132, no. 7, pp. 1901–1907, 2012.
- [10] G. R. Buettner, "Molecular targets of photosensitization—some biological chemistry of singlet oxygen," in *Photobiology Sciences on Line*, K. C. Smith, Ed., American Society for Photobiology, 2011, <http://www.photobiology.info/Buettner.html>.
- [11] J. R. Kanofsky, "Measurement of singlet-oxygen in vivo: progress and pitfalls," *Photochemistry and Photobiology*, vol. 87, no. 1, pp. 14–17, 2011.
- [12] D. Ziech, R. Franco, A. Pappa, and M. I. Panayiotidis, "Reactive oxygen species (ROS)—induced genetic and epigenetic alterations in human carcinogenesis," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 711, no. 1–2, pp. 167–173, 2011.
- [13] M. D. Evans, M. Dizdaroglu, and M. S. Cooke, "Oxidative DNA damage and disease: induction, repair and significance," *Mutation Research/Reviews in Mutation Research*, vol. 567, no. 1, pp. 1–61, 2004.
- [14] I. Dalle-Donne, R. Rossi, D. Giustarini, A. Milzani, and R. Colombo, "Protein carbonyl groups as biomarkers of oxidative stress," *Clinica Chimica Acta*, vol. 329, no. 1–2, pp. 23–38, 2003.
- [15] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, no. 1, pp. 315–424, 2007.
- [16] B. G. Hill, B. P. Dranka, S. M. Bailey, J. R. Lancaster Jr., and V. M. Darley-Usmar, "What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology," *The Journal of Biological Chemistry*, vol. 285, no. 26, pp. 19699–19704, 2010.
- [17] J. F. Turrens, "Mitochondrial formation of reactive oxygen species," *The Journal of Physiology*, vol. 552, no. 2, pp. 335–344, 2003.
- [18] M. D. Brand, "The sites and topology of mitochondrial superoxide production," *Experimental Gerontology*, vol. 45, no. 7–8, pp. 466–472, 2010.
- [19] J. Guo and B. D. Lemire, "The ubiquinone-binding site of the *Saccharomyces cerevisiae* succinate-ubiquinone oxidoreductase is a source of superoxide²," *Journal of Biological Chemistry*, vol. 278, no. 48, pp. 47629–47635, 2003.
- [20] M. P. Paranagama, K. Sakamoto, H. Amino, M. Awano, H. Miyoshi, and K. Kita, "Contribution of the FAD and quinone binding sites to the production of reactive oxygen species from *Ascaris suum* mitochondrial complex II," *Mitochondrion*, vol. 10, no. 2, pp. 158–165, 2010.
- [21] V. Yankovskaya, R. Horsefield, S. Törnroth et al., "Architecture of succinate dehydrogenase and reactive oxygen species generation," *Science*, vol. 299, no. 5607, pp. 700–704, 2003.
- [22] C. L. Quinlan, R. L. S. Goncalves, M. Hey-Mogensen, N. Yadava, V. I. Bunik, and M. D. Brand, "The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I," *Journal of Biological Chemistry*, vol. 289, no. 12, pp. 8312–8325, 2014.
- [23] Y. Liu, G. Fiskum, and D. Schubert, "Generation of reactive oxygen species by the mitochondrial electron transport chain," *Journal of Neurochemistry*, vol. 80, no. 5, pp. 780–787, 2002.
- [24] J. Hirst, J. Carroll, I. M. Fearnley, R. J. Shannon, and J. E. Walker, "The nuclear encoded subunits of complex I from bovine heart mitochondria," *Biochimica et Biophysica Acta*, vol. 1604, no. 3, pp. 135–150, 2003.
- [25] M. P. Murphy, "How mitochondria produce reactive oxygen species," *Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.
- [26] C. Gleason, S. Huang, L. F. Thatcher et al., "Mitochondrial complex II has a key role in mitochondrial-derived reactive oxygen species influence on plant stress gene regulation and defense," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 26, pp. 10768–10773, 2011.
- [27] C. L. Quinlan, A. L. Orr, I. V. Perevoshchikova, J. R. Treberg, B. A. Ackrell, and M. D. Brand, "Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions," *Journal of Biological Chemistry*, vol. 287, no. 32, pp. 27255–27264, 2012.
- [28] C. Hägerhäll, "Succinate: quinone oxidoreductases: variations on a conserved theme," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1320, no. 2, pp. 107–141, 1997.
- [29] R. F. Anderson, R. Hille, S. S. Shinde, and G. Cecchini, "Electron transfer within complex II. Succinate:ubiquinone oxidoreductase of *Escherichia coli*," *Journal of Biological Chemistry*, vol. 280, no. 39, pp. 33331–33337, 2005.

- [30] J. Zhang, F. E. Frerman, and J.-J. P. Kim, "Structure of electron transfer flavoprotein-ubiquinone oxidoreductase and electron transfer to the mitochondrial ubiquinone pool," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 44, pp. 16212–16217, 2006.
- [31] J. Lee, S. Giordano, and J. Zhang, "Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling," *Biochemical Journal*, vol. 441, no. 2, pp. 523–540, 2012.
- [32] K. A. Malkus, E. Tsika, and H. Ischiropoulos, "Oxidative modifications, mitochondrial dysfunction, and impaired protein degradation in Parkinson's disease: how neurons are lost in the Bermuda triangle," *Molecular Neurodegeneration*, vol. 4, article 24, 24 pages, 2009.
- [33] S. J. Chong, I. C. C. Low, and S. Pervaiz, "Mitochondrial ROS and involvement of Bcl-2 as a mitochondrial ROS regulator," *Mitochondrion*, vol. 19, pp. 39–48, 2014.
- [34] W. Dröge, "Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants," *Experimental Gerontology*, vol. 37, no. 12, pp. 1333–1345, 2002.
- [35] E. Birben, U. M. Sahiner, C. Sackesen, S. Erzurum, and O. Kalayci, "Oxidative stress and antioxidant defense," *World Allergy Organization Journal*, vol. 5, no. 1, pp. 9–19, 2012.
- [36] K. Rahman, "Studies on free radicals, antioxidants and co-factors," *Clinical Interventions in Aging*, vol. 2, no. 2, pp. 219–236, 2007.
- [37] I. Balzer and R. Hardeland, "Photoperiodism and effects of indoleamines in a unicellular alga, *Gonyaulax polyedra*," *Science*, vol. 253, no. 5021, pp. 795–797, 1991.
- [38] R. Hardeland, "Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance," *Endocrine*, vol. 27, no. 2, pp. 119–130, 2005.
- [39] S. R. Pandi-Perumal, V. Srinivasan, G. J. M. Maestroni, D. P. Cardinali, B. Poeggeler, and R. Hardeland, "Melatonin: nature's most versatile biological signal?" *FEBS Journal*, vol. 273, no. 13, pp. 2813–2838, 2006.
- [40] A. Galano, D. X. Tan, and R. J. Reiter, "Melatonin as a natural ally against oxidative stress: a physicochemical examination," *Journal of Pineal Research*, vol. 51, no. 1, pp. 1–16, 2011.
- [41] H.-M. Zhang and Y. Zhang, "Melatonin: a well-documented antioxidant with conditional pro-oxidant actions," *Journal of Pineal Research*, vol. 57, no. 2, pp. 131–146, 2014.
- [42] D. Acuña-Castroviejo, A. Coto-Montes, M. G. Monti, G. G. Ortiz, and R. J. Reiter, "Melatonin is protective against MPTP-induced striatal and hippocampal lesions," *Life Sciences*, vol. 60, no. 2, pp. PL23–PL29, 1996.
- [43] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.
- [44] R. S. Sohal and W. C. Orr, "The redox stress hypothesis of aging," *Free Radical Biology & Medicine*, vol. 52, no. 3, pp. 539–555, 2012.
- [45] H. Sies, "Biochemistry of oxidative stress," *Angewandte Chemie International Edition in English*, vol. 25, no. 12, pp. 1058–1071, 1986.
- [46] N. Brandes, S. Schmitt, and U. Jakob, "Thiol-based redox switches in eukaryotic proteins," *Antioxidants & Redox Signaling*, vol. 11, no. 5, pp. 997–1014, 2009.
- [47] C. Klomsiri, P. A. Karplus, and L. B. Poole, "Cysteine-based redox switches in enzymes," *Antioxidants & Redox Signaling*, vol. 14, no. 6, pp. 1065–1077, 2011.
- [48] Y. S. Bae, H. Oh, S. G. Rhee, and Y. D. Yoo, "Regulation of reactive oxygen species generation in cell signaling," *Molecules and Cells*, vol. 32, no. 6, pp. 491–509, 2011.
- [49] T. Finkel, "Signal transduction by mitochondrial oxidants," *Journal of Biological Chemistry*, vol. 287, no. 7, pp. 4434–4440, 2012.
- [50] H. Kamata, S.-I. Honda, S. Maeda, L. Chang, H. Hirata, and M. Karin, "Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases," *Cell*, vol. 120, no. 5, pp. 649–661, 2005.
- [51] N. S. Chandel, E. Maltepe, E. Goldwasser, C. E. Mathieu, M. C. Simon, and P. T. Schumacker, "Mitochondrial reactive oxygen species trigger hypoxia-induced transcription," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11715–11720, 1998.
- [52] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, and Z. Elazar, "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4," *The EMBO Journal*, vol. 26, no. 7, pp. 1749–1760, 2007.
- [53] Y. C. Long, T. M. C. Tan, I. Takao, and B. L. Tang, "The biochemistry and cell biology of aging: metabolic regulation through mitochondrial signaling," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 306, no. 6, pp. E581–E591, 2014.
- [54] G. Banks, I. Heise, B. Starbuck et al., "Genetic background influences age-related decline in visual and nonvisual retinal responses, circadian rhythms, and sleep," *Neurobiology of Aging*, vol. 36, no. 1, pp. 380–393, 2015.
- [55] D. J. Skene and D. F. Swaab, "Melatonin rhythmicity; effect of age and Alzheimer's disease," *Experimental Gerontology*, vol. 38, no. 1-2, pp. 199–206, 2003.
- [56] S. R. Pandi-Perumal, L. K. Seils, L. Kayumov et al., "Senescence, sleep, and circadian rhythms," *Ageing Research Reviews*, vol. 1, no. 3, pp. 559–604, 2002.
- [57] Y.-H. Wu and D. F. Swaab, "Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease," *Sleep Medicine*, vol. 8, no. 6, pp. 623–636, 2007.
- [58] E. Ferrari, L. Cravello, F. Falvo et al., "Neuroendocrine features in extreme longevity," *Experimental Gerontology*, vol. 43, no. 2, pp. 88–94, 2008.
- [59] R. C. Travis, N. E. Allen, P. H. M. Peeters, P. A. H. Van Noord, and T. J. Key, "Reproducibility over 5 years of measurements of 6-sulphatoxymelatonin in urine samples from postmenopausal women," *Cancer Epidemiology Biomarkers & Prevention*, vol. 12, no. 8, pp. 806–808, 2003.
- [60] E. Grof, P. Grof, G. M. Brown, M. Arato, and J. Lane, "Investigations of melatonin secretion in man," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 9, no. 5-6, pp. 609–612, 1985.
- [61] J. D. Bergiannaki, C. R. Soldatos, T. J. Paparrigopoulos, M. Syrengelas, and C. N. Stefanis, "Low and high melatonin excretors among healthy individuals," *Journal of Pineal Research*, vol. 18, no. 3, pp. 159–164, 1995.
- [62] N. Barzilay and I. Gabriely, "Genetic studies reveal the role of the endocrine and metabolic systems in aging," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 10, pp. 4493–4500, 2010.
- [63] C. Pagan, H. Goubran-Botros, K. Poirier et al., "Mutation screening of ASMT, the last enzyme of the melatonin pathway, in a large sample of patients with intellectual disability," *BMC Medical Genetics*, vol. 12, no. 1, article 17, 2011.
- [64] S. R. Mackey, S. S. Golden, and J. L. Ditty, "The itty-bitty time machine: genetics of the cyanobacterial circadian clock," *Advances in Genetics*, vol. 74, pp. 13–53, 2011.

- [65] J. C. Dunlap, "Molecular bases for circadian clocks," *Cell*, vol. 96, no. 2, pp. 271–290, 1999.
- [66] S. M. Reppert and D. R. Weaver, "Coordination of circadian timing in mammals," *Nature*, vol. 418, no. 6901, pp. 935–941, 2002.
- [67] P. E. Hardin, "The circadian timekeeping system of *Drosophila*," *Current Biology*, vol. 15, no. 17, pp. R714–R722, 2005.
- [68] J. T. Vanselow and A. Kramer, "Posttranslational regulation of circadian clocks," *Protein Reviews*, vol. 12, pp. 79–104, 2010.
- [69] C. H. Johnson, J. A. Elliott, and R. Foster, "Entrainment of circadian programs," *Chronobiology International*, vol. 20, no. 5, pp. 741–774, 2003.
- [70] B. T. S. Carneiro and J. F. Araujo, "Food entrainment: major and recent findings," *Frontiers in Behavioral Neuroscience*, vol. 6, article 83, 2012.
- [71] I. Palma-Anzures, J. Prieto-Sagredo, and M. L. Fanjul-Moles, "Temperature pulses synchronise the crayfish locomotor activity rhythm," *Biological Rhythm Research*, vol. 43, no. 1, pp. 15–24, 2012.
- [72] G. Bloch, E. D. Herzog, J. D. Levine, and W. J. Schwartz, "Socially synchronized circadian oscillators," *Proceedings of the Royal Society of London B: Biological Sciences*, vol. 280, no. 1765, Article ID 20130035, 2013.
- [73] N. Mrosovsky, "Locomotor activity and non-photoc influences on circadian clocks," *Biological Reviews*, vol. 71, no. 3, pp. 343–372, 1996.
- [74] C. Pittendrigh, "Circadian systems: entrainment," in *Handbook of Behavioral Neurobiology, Vol 4 Biological Rhythms*, J. Aschoff, Ed., pp. 95–124, Plenum Press, New York, NY, USA, 1981.
- [75] D. Bell-Pedersen, V. M. Cassone, D. J. Earnest et al., "Circadian rhythms from multiple oscillators: lessons from diverse organisms," *Nature Reviews Genetics*, vol. 6, no. 7, pp. 544–556, 2005.
- [76] J. A. Mohawk, C. B. Green, and J. S. Takahashi, "Central and peripheral circadian clocks in mammals," *Annual Review of Neuroscience*, vol. 35, pp. 445–462, 2012.
- [77] C. Dibner, U. Schibler, and U. Albrecht, "The mammalian circadian timing system: organization and coordination of central and peripheral clocks," *Annual Review of Physiology*, vol. 72, pp. 517–549, 2009.
- [78] V. M. Cassone, "Effects of melatonin on vertebrate circadian systems," *Trends in Neurosciences*, vol. 13, no. 11, pp. 457–464, 1990.
- [79] G. C. Wagner, J. D. Johnston, B. B. Tournier, F. J. P. Ebling, and D. G. Hazlerigg, "Melatonin induces gene-specific effects on rhythmic mRNA expression in the pars tuberalis of the Siberian hamster (*Phodopus sungorus*)," *European Journal of Neuroscience*, vol. 25, no. 2, pp. 485–490, 2007.
- [80] D. R. Weaver, J. H. Stehle, E. G. Stopa, and S. M. Reppert, "Melatonin receptors in human hypothalamus and pituitary: implications for circadian and reproductive responses to melatonin," *Journal of Clinical Endocrinology & Metabolism*, vol. 76, no. 2, pp. 295–301, 1993.
- [81] R. Hardeland, A. Coto-Montes, and B. Poeggeler, "Circadian rhythms, oxidative stress, and antioxidative defense mechanisms," *Chronobiology International*, vol. 20, no. 6, pp. 921–962, 2003.
- [82] M. Meroow and T. Roenneberg, "Circadian clocks: running on redox," *Cell*, vol. 106, no. 2, pp. 141–143, 2001.
- [83] Y. Yoshida, H. Iigusa, N. Wang, and K. Hasunuma, "Cross-talk between the cellular redox state and the circadian system in *Neurospora*," *PLoS ONE*, vol. 6, no. 12, Article ID e28227, 2011.
- [84] A. G. Lai, C. J. Doherty, B. Mueller-Roeber, S. A. Kay, J. H. M. Schippers, and P. P. Dijkwel, "Circadian clock-associated 1 regulates ROS homeostasis and oxidative stress responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 42, pp. 17129–17134, 2012.
- [85] M. L. Fanjul-Moles, "ROS signaling pathways and biological rhythms: perspectives in crustaceans," *Frontiers in Bioscience*, vol. 18, no. 2, pp. 665–675, 2013.
- [86] T. A. Wang, Y. V. Yu, G. Govindaiah et al., "Circadian rhythm of redox state regulates excitability in suprachiasmatic nucleus neurons," *Science*, vol. 337, no. 6096, pp. 839–842, 2012.
- [87] A. Bednářová, D. Kodrlik, and N. Krishnan, "Nature's timepiece-molecular coordination of metabolism and its impact on aging," *International Journal of Molecular Sciences*, vol. 14, no. 2, pp. 3026–3049, 2013.
- [88] N. G. Bazan, "Survival signaling in retinal pigment epithelial cells in response to oxidative stress: significance in retinal degenerations," *Advances in Experimental Medicine and Biology*, vol. 572, pp. 531–540, 2005.
- [89] A. King, E. Gottlieb, D. G. Brooks, M. P. Murphy, and J. L. Dunaief, "Mitochondria-derived reactive oxygen species mediate blue light-induced death of retinal pigment epithelial cells," *Photochemistry and Photobiology*, vol. 79, no. 5, pp. 470–475, 2004.
- [90] J. J. Hunter, J. I. W. Morgan, W. H. Merigan, D. H. Sliney, J. R. Sparrow, and D. R. Williams, "The susceptibility of the retina to photochemical damage from visible light," *Progress in Retinal and Eye Research*, vol. 31, no. 1, pp. 28–42, 2012.
- [91] C. Roehlecke, U. Schumann, M. Ader et al., "Stress reaction in outer segments of photoreceptors after blue light irradiation," *PLoS ONE*, vol. 8, no. 9, Article ID e71570, 2013.
- [92] Y. Uchino, T. Kawakita, M. Miyazawa et al., "Oxidative stress induced inflammation initiates functional decline of tear production," *PLoS ONE*, vol. 7, no. 10, Article ID e45805, 2012.
- [93] O. Strauss, "The retinal pigment epithelium in visual function," *Physiological Reviews*, vol. 85, no. 3, pp. 845–881, 2005.
- [94] D. W. Dawson, O. V. Volpert, P. Gillis et al., "Pigment epithelium-derived factor: a potent inhibitor of angiogenesis," *Science*, vol. 285, no. 5425, pp. 245–248, 1999.
- [95] G. L. King and K. Suzuma, "Pigment-epithelium-derived factor—a key coordinator of retinal neuronal and vascular functions," *The New England Journal of Medicine*, vol. 342, no. 5, pp. 349–351, 2000.
- [96] A. N. Witmer, G. F. J. M. Vrensen, C. J. F. Van Noorden, and R. O. Schlingemann, "Vascular endothelial growth factors and angiogenesis in eye disease," *Progress in Retinal and Eye Research*, vol. 22, no. 1, pp. 1–29, 2003.
- [97] Y. Yoshida, S.-I. Yamagishi, T. Matsui et al., "Protective role of pigment epithelium-derived factor (PEDF) in early phase of experimental diabetic retinopathy," *Diabetes/Metabolism Research and Reviews*, vol. 25, no. 7, pp. 678–686, 2009.
- [98] J. Y. Do, Y. K. Choi, H. Kook, K. Suk, I. K. Lee, and D. H. Park, "Retinal hypoxia induces vascular endothelial growth factor through induction of estrogen-related receptor γ ," *Biochemical and Biophysical Research Communications*, vol. 460, no. 2, pp. 457–463, 2015.
- [99] J. G. Hollyfield, V. L. Bonilha, M. E. Rayborn et al., "Oxidative damage-induced inflammation initiates age-related macular degeneration," *Nature Medicine*, vol. 14, no. 2, pp. 194–198, 2008.
- [100] A. Decanini, C. L. Nordgaard, X. Feng, D. A. Ferrington, and T. W. Olsen, "Changes in select redox proteins of the retinal pigment epithelium in age-related macular degeneration,"

- American Journal of Ophthalmology*, vol. 143, no. 4, pp. 607–615, 2007.
- [101] K. Bedard and K.-H. Krause, “The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology,” *Physiological Reviews*, vol. 87, no. 1, pp. 245–313, 2007.
- [102] L. Bhatt, G. Groeger, K. McDermott, and T. G. Cotter, “Rod and cone photoreceptor cells produce ROS in response to stress in a live retinal explant system,” *Molecular Vision*, vol. 16, pp. 283–293, 2010.
- [103] C. Roehlecke, U. Schumann, M. Ader, L. Knels, and R. H. W. Funk, “Influence of blue light on photoreceptors in a live retinal explant system,” *Molecular Vision*, vol. 17, pp. 876–884, 2011.
- [104] H. E. P. Bazan, N. G. Bazan, L. Feeney-Burns, and E. R. Berman, “Lipids in human lipofuscin-enriched subcellular fractions of two age populations. Comparison with rod outer segments and neural retina,” *Investigative Ophthalmology & Visual Science*, vol. 31, no. 8, pp. 1433–1443, 1990.
- [105] K.-P. Ng, B. Gugiu, K. Renganathan et al., “Retinal pigment epithelium lipofuscin proteomics,” *Molecular & Cellular Proteomics*, vol. 7, no. 7, pp. 1397–1405, 2008.
- [106] J. Wassell, S. Davies, W. Bardsley, and M. Boulton, “The photoreactivity of the retinal age pigment lipofuscin,” *The Journal of Biological Chemistry*, vol. 274, no. 34, pp. 23828–23832, 1999.
- [107] F. Schütt, S. Davies, J. Kopitz, M. Boulton, and F. G. Holz, “A retinoid constituent of lipofuscin, A2-E, is a photosensitizer in human retinal pigment epithelial cells,” *Der Ophthalmologe*, vol. 97, no. 10, pp. 682–687, 2000.
- [108] J. R. Sparrow, K. Nakanishi, and C. A. Parish, “The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells,” *Investigative Ophthalmology & Visual Science*, vol. 41, no. 7, pp. 1981–1989, 2000.
- [109] M. Boulton, A. Dontsov, J. Jarvis-Evans, M. Ostrovsky, and D. Svistunenko, “Lipofuscin is a photoinducible free radical generator,” *Journal of Photochemistry and Photobiology B: Biology*, vol. 19, no. 3, pp. 201–204, 1993.
- [110] M. Suter, C. Remé, C. Grimm, M. Suter et al., “Age-related macular degeneration. The lipofuscin component *N*-retinyl-*N*-retinylidene ethanamine detaches proapoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cell,” *The Journal of Biological Chemistry*, vol. 275, no. 50, pp. 39625–39630, 2000.
- [111] K. A. S. M. Saadat, Y. Murakami, X. Tan et al., “Inhibition of autophagy induces retinal pigment epithelial cell damage by the lipofuscin fluorophore A2E,” *FEBS Open Bio*, vol. 4, pp. 1007–1014, 2014.
- [112] C. A. Curcio, M. Johnson, M. Rudolf, and J.-D. Huang, “The oil spill in ageing Bruch membrane,” *British Journal of Ophthalmology*, vol. 95, no. 12, pp. 1638–1645, 2011.
- [113] Z. Faghiri and N. G. Bazan, “PI3K/Akt and mTOR/p70S6K pathways mediate neuroprotectin D1-induced retinal pigment epithelial cell survival during oxidative stress-induced apoptosis,” *Experimental Eye Research*, vol. 90, no. 6, pp. 718–725, 2010.
- [114] A. Klettner, “Oxidative stress induced cellular signaling in RPE cells,” *Frontiers in Bioscience*, vol. 4, no. 2, pp. 392–411, 2012.
- [115] K. Kaarniranta, D. Sinha, J. Blasiak et al., “Autophagy and heterophagy dysregulation leads to retinal pigment epithelium dysfunction and development of age-related macular degeneration,” *Autophagy*, vol. 9, no. 7, pp. 973–984, 2013.
- [116] S. G. Jarrett and M. E. Boulton, “Consequences of oxidative stress in age-related macular degeneration,” *Molecular Aspects of Medicine*, vol. 33, no. 4, pp. 399–417, 2012.
- [117] V. McConnell and G. Silvestri, “Age-related macular degeneration,” *Ulster Medical Journal*, vol. 74, no. 2, pp. 82–92, 2005.
- [118] P. L. Turner and M. A. Mainster, “Circadian photoreception: ageing and the eye’s important role in systemic health,” *British Journal of Ophthalmology*, vol. 92, no. 11, pp. 1439–1444, 2008.
- [119] M. van Lookeren Campagne, J. LeCouter, B. L. Yaspan, and W. Ye, “Mechanisms of age-related macular degeneration and therapeutic opportunities,” *Journal of Pathology*, vol. 232, no. 2, pp. 151–164, 2014.
- [120] J. Z. Nowak, “Age-related macular degeneration (AMD): pathogenesis and therapy,” *Pharmacological Reports*, vol. 58, no. 3, pp. 353–363, 2006.
- [121] R. W. Young and D. Bok, “Participation of the retinal pigment epithelium in the rod outer segment renewal process,” *The Journal of Cell Biology*, vol. 42, no. 2, pp. 392–403, 1969.
- [122] R. J. Mullen and M. M. LaVail, “Inherited retinal dystrophy: primary defect in pigment epithelium determined with experimental rat chimeras,” *Science*, vol. 192, no. 4241, pp. 799–801, 1976.
- [123] S. C. Finnemann, L. W. Leung, and E. Rodriguez-Boulan, “The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 6, pp. 3842–3847, 2002.
- [124] J. R. Sparrow and M. Boulton, “RPE lipofuscin and its role in retinal pathobiology,” *Experimental Eye Research*, vol. 80, no. 5, pp. 595–606, 2005.
- [125] F. L. Ferris III, C. P. Wilkinson, A. Bird et al., “Clinical classification of age-related macular degeneration,” *Ophthalmology*, vol. 120, no. 4, pp. 844–851, 2013.
- [126] A. W. Scott and S. B. Bressler, “Long-term follow-up of vascular endothelial growth factor inhibitor therapy for neovascular age-related macular degeneration,” *Current Opinion in Ophthalmology*, vol. 24, no. 3, pp. 190–196, 2013.
- [127] J. Zhou, Y. P. Jang, S. R. Kim, and J. R. Sparrow, “Complement activation by photooxidation products of A2E, a lipofuscin constituent of the retinal pigment epithelium,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 44, pp. 16182–16187, 2006.
- [128] A. Wiktorowska-Owczarek and J. Z. Nowak, “Oxidative damage in age-related macular degeneration (AMD) and antioxidant protection as a therapeutic strategy,” *Polish Journal of Environmental Studies*, vol. 15, pp. 69–72, 2006.
- [129] J. G. Hollyfield, R. G. Salomon, and J. W. Crabb, “Proteomic approaches to understanding age-related macular degeneration,” *Advances in Experimental Medicine and Biology*, vol. 533, pp. 83–89, 2003.
- [130] G. Wolf, “Lipofuscin and macular degeneration,” *Nutrition Reviews*, vol. 61, no. 10, pp. 342–346, 2003.
- [131] S. Zampatti, F. Ricci, A. Cusumano, L. T. Marsella, G. Novelli, and E. Giardina, “Review of nutrient actions on age-related macular degeneration,” *Nutrition Research*, vol. 34, no. 2, pp. 95–105, 2014.
- [132] J. Blasiak, G. Petrovski, Z. Veréb, A. Facskó, and K. Kaarniranta, “Oxidative stress, hypoxia, and autophagy in the neovascular processes of age-related macular degeneration,” *BioMed Research International*, vol. 2014, Article ID 768026, 7 pages, 2014.
- [133] F. A. Shamsi and M. Boulton, “Inhibition of RPE lysosomal and antioxidant activity by the age pigment lipofuscin,” *Investigative*

- Ophthalmology and Visual Science*, vol. 42, no. 12, pp. 3041–3046, 2001.
- [134] S. Sundelin, U. Wihlmark, S. E. G. Nilsson, and U. T. Brunk, “Lipofuscin accumulation in cultured retinal pigment epithelial cells reduces their phagocytic capacity,” *Current Eye Research*, vol. 17, no. 8, pp. 851–857, 1998.
- [135] J. W. Crabb, M. Miyagi, X. Gu et al., “Drusen proteome analysis: an approach to the etiology of age-related macular degeneration,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 23, pp. 14682–14687, 2002.
- [136] L. Wang, M. E. Clark, D. K. Crossman et al., “Abundant lipid and protein components of drusen,” *PLoS ONE*, vol. 5, no. 4, Article ID e10329, 2010.
- [137] F. G. Holz, E. C. Strauss, S. Schmitz-Valckenberg, and M. Van Lookeren Campagne, “Geographic atrophy: clinical features and potential therapeutic approaches,” *Ophthalmology*, vol. 121, no. 5, pp. 1079–1091, 2014.
- [138] L. Galluzzi, F. Pietrocola, B. Levine, and G. Kroemer, “Metabolic control of autophagy,” *Cell*, vol. 159, no. 6, pp. 1263–1276, 2014.
- [139] S. K. Mitter, H. V. Rao, X. Qi et al., “Autophagy in the retina: a potential role in age-related macular degeneration,” in *Retinal Degenerative Diseases*, vol. 723 of *Advances in Experimental Medicine and Biology*, pp. 83–90, Springer, Berlin, Germany, 2012.
- [140] J. Wu, S. Seregard, and P. V. Alverge, “Photochemical damage of the retina,” *Survey of Ophthalmology*, vol. 51, no. 5, pp. 461–481, 2006.
- [141] E. Famiglietti, E. G. Stopa, E. D. McGookin, P. Song, V. LeBlanc, and B. W. Streeten, “Immunocytochemical localization of vascular endothelial growth factor in neurons and glial cells of human retina,” *Brain Research*, vol. 969, no. 1-2, pp. 195–204, 2003.
- [142] S. A. Vinore, A. I. Youssri, J. D. Luna et al., “Upregulation of vascular endothelial growth factor in ischemic and non-ischemic human and experimental retinal disease,” *Histology and Histopathology*, vol. 12, no. 1, pp. 99–109, 1997.
- [143] Y. Cao, “Positive and negative modulation of angiogenesis by VEGFR1 ligands,” *Science Signaling*, vol. 2, no. 59, article rel, 2009.
- [144] P. R. Somanath, N. L. Malinin, and T. V. Byzova, “Cooperation between integrin alphavbeta3 and VEGFR2 in angiogenesis,” *Angiogenesis*, vol. 12, no. 2, pp. 177–185, 2009.
- [145] J. Li, J. J. Wang, and S. X. Zhang, “NADPH oxidase 4-derived H₂O₂ promotes aberrant retinal neovascularization via activation of VEGF receptor 2 pathway in oxygen-induced retinopathy,” *Journal of Diabetes Research*, vol. 2015, Article ID 963289, 13 pages, 2015.
- [146] C. S. Metzger, D. Koutsimpelas, and J. Brieger, “Transcriptional regulation of the VEGF gene in dependence of individual genomic variations,” *Cytokine*, vol. 76, no. 2, pp. 519–526, 2015.
- [147] R. K. Vadlapatla, A. D. Vadlapudi, and A. K. Mitra, “Hypoxia-inducible factor-1 (HIF-1): a potential target for intervention in ocular neovascular diseases,” *Current Drug Targets*, vol. 14, no. 8, pp. 919–935, 2013.
- [148] A. W. A. Weinberger, C. Eddahabi, D. Carstesen, P. F. Zipfel, P. Walter, and C. Skerka, “Human complement factor H and factor H-like protein 1 are expressed in human retinal pigment epithelial cells,” *Ophthalmic Research*, vol. 51, no. 2, pp. 59–66, 2014.
- [149] M. Ansari, P. M. Mckeigue, C. Skerka et al., “Genetic influences on plasma CFH and CFHR1 concentrations and their role in susceptibility to age-related macular degeneration,” *Human Molecular Genetics*, vol. 22, no. 23, pp. 4857–4869, 2013.
- [150] P. F. Zipfel and C. Skerka, “Complement regulators and inhibitory proteins,” *Nature Reviews Immunology*, vol. 9, no. 10, pp. 729–740, 2009.
- [151] P. F. Zipfel and N. Lauer, “Defective complement action and control defines disease pathology for retinal and renal disorders and provides a basis for new therapeutic approaches,” *Advances in Experimental Medicine and Biology*, vol. 734, pp. 173–187, 2013.
- [152] M. A. Khan, A. M. Assiri, and D. C. Broering, “Complement and macrophage crosstalk during process of angiogenesis in tumor progression,” *Journal of Biomedical Science*, vol. 22, no. 1, p. 58, 2015.
- [153] D. H. Anderson, M. J. Radeke, N. B. Gallo et al., “The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited,” *Progress in Retinal and Eye Research*, vol. 29, no. 2, pp. 95–112, 2010.
- [154] R. J. Klein, C. Zeiss, E. Y. Chew et al., “Complement factor H polymorphism in age-related macular degeneration,” *Science*, vol. 308, no. 5720, pp. 385–389, 2005.
- [155] B. Gold, J. E. Merriam, J. Zernant et al., “Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration,” *Nature Genetics*, vol. 38, no. 4, pp. 458–462, 2006.
- [156] R. Reynolds, M. E. Hartnett, J. P. Atkinson, P. C. Giclas, B. Rosner, and J. M. Seddon, “Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes,” *Investigative Ophthalmology & Visual Science*, vol. 50, no. 12, pp. 5818–5827, 2009.
- [157] W. Smith, P. Mitchell, K. Webb, and S. R. Leeder, “Dietary antioxidants and age-related maculopathy: the Blue Mountains Eye Study,” *Ophthalmology*, vol. 106, no. 4, pp. 761–767, 1999.
- [158] D. Smailhodzic, F. Van Asten, A. M. Blom et al., “Zinc supplementation inhibits complement activation in age-related macular degeneration,” *PLoS ONE*, vol. 9, no. 11, Article ID e112682, 2014.
- [159] M. M. LaVail, “Rod outer segment disk shedding in rat retina: relationship to cyclic lighting,” *Science*, vol. 194, no. 4269, pp. 1071–1074, 1976.
- [160] R. W. Young, “The renewal of photoreceptor cell outer segments,” *Journal of Cell Biology*, vol. 33, no. 1, pp. 61–72, 1967.
- [161] A. I. Goldman, P. S. Teirstein, and P. J. O’Brien, “The role of ambient lighting in circadian disc shedding in the rod outer segment of the rat retina,” *Investigative Ophthalmology & Visual Science*, vol. 19, no. 11, pp. 1257–1267, 1980.
- [162] R. W. Young, “The daily rhythm of shedding and degradation of rod and cone outer segment membranes in the chick retina,” *Investigative Ophthalmology & Visual Science*, vol. 17, no. 2, pp. 105–116, 1978.
- [163] M. M. LaVail, “Circadian nature of rod outer segment disc shedding in the rat,” *Investigative Ophthalmology & Visual Science*, vol. 19, no. 4, pp. 407–411, 1980.
- [164] C. Bobu, C. M. Craft, M. Masson-Pevet, and D. Hicks, “Photoreceptor organization and rhythmic phagocytosis in the Nile rat *Arvicanthis ansorgei*: a novel diurnal rodent model for the study of cone pathophysiology,” *Investigative Ophthalmology & Visual Science*, vol. 47, no. 7, pp. 3109–3118, 2006.

- [165] J. S. Terman, C. E. Remé, and M. Terman, "Rod outer segment disk shedding in rats with lesions of the suprachiasmatic nucleus," *Brain Research*, vol. 605, no. 2, pp. 256–264, 1993.
- [166] P. S. Teirstein, A. I. Goldman, and P. J. O'Brien, "Evidence for both local and central regulation of rat rod outer segment disc shedding," *Investigative Ophthalmology and Visual Science*, vol. 19, no. 11, pp. 1268–1273, 1980.
- [167] P. M. D'Cruz, D. Yasumura, J. Weir et al., "Mutation of the receptor tyrosine kinase gene *Mertk* in the retinal dystrophic RCS rat," *Human Molecular Genetics*, vol. 9, no. 4, pp. 645–651, 2000.
- [168] D. Gibbs, J. Kitamoto, and D. S. Williams, "Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the Usher syndrome 1B protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 11, pp. 6481–6486, 2003.
- [169] D. Prasad, C. V. Rothlin, P. Burrola et al., "TAM receptor function in the retinal pigment epithelium," *Molecular and Cellular Neuroscience*, vol. 33, no. 1, pp. 96–108, 2006.
- [170] L. D. Jensen, Z. Cao, M. Nakamura et al., "Opposing effects of circadian clock genes *bmal1* and *period2* in regulation of VEGF-dependent angiogenesis in developing zebrafish," *Cell Reports*, vol. 2, no. 2, pp. 231–241, 2012.
- [171] L. D. Jensen and Y. Cao, "Clock controls angiogenesis," *Cell Cycle*, vol. 12, no. 3, pp. 405–408, 2013.
- [172] S. Sahar and P. Sassone-Corsi, "Circadian clock and breast cancer: a molecular link," *Cell Cycle*, vol. 6, no. 11, pp. 1329–1331, 2007.
- [173] G. Tosini and M. Menaker, "Circadian rhythms in cultured mammalian retina," *Science*, vol. 272, no. 5260, pp. 419–421, 1996.
- [174] F.-Q. Liang, L. Green, C. Wang, R. Alssadi, and B. F. Godley, "Melatonin protects human retinal pigment epithelial (RPE) cells against oxidative stress," *Experimental Eye Research*, vol. 78, no. 6, pp. 1069–1075, 2004.
- [175] Y. Fu, M. Tang, Y. Fan, H. Zou, X. Sun, and X. Xu, "Anti-apoptotic effects of melatonin in retinal pigment epithelial cells," *Frontiers in Bioscience*, vol. 17, no. 4, pp. 1461–1468, 2012.
- [176] C. Yi, X. Pan, H. Yan, M. Guo, and W. Pierpaoli, "Effects of melatonin in age-related macular degeneration," *Annals of the New York Academy of Sciences*, vol. 1057, no. 1, pp. 384–392, 2005.
- [177] R. Rosen, D.-N. Hu, V. Perez et al., "Urinary 6-sulfatoxymelatonin level in age-related macular degeneration patients," *Molecular Vision*, vol. 15, pp. 1673–1679, 2009.
- [178] G. Tosini, K. Baba, C. K. Hwang, and P. M. Iuvone, "Melatonin: an underappreciated player in retinal physiology and pathophysiology," *Experimental Eye Research*, vol. 103, pp. 82–89, 2012.
- [179] G. Tosini and J. C. Dirden, "Dopamine inhibits melatonin release in the mammalian retina: in vitro evidence," *Neuroscience Letters*, vol. 286, no. 2, pp. 119–122, 2000.
- [180] S. E. Doyle, M. S. Grace, W. McIvor, and M. Menaker, "Circadian rhythms of dopamine in mouse retina: the role of melatonin," *Visual Neuroscience*, vol. 19, no. 5, pp. 593–601, 2002.
- [181] K. E. Schmid-Kubista, C. G. Glittenberg, M. Cezanne, K. Holzmann, B. Neumaier-Ammerer, and S. Binder, "Daytime levels of melatonin in patients with age-related macular degeneration," *Acta Ophthalmologica*, vol. 87, no. 1, pp. 89–93, 2009.
- [182] W. L. Wong, X. Su, X. Li et al., "Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis," *The Lancet Global Health*, vol. 2, no. 2, pp. e106–e116, 2014.
- [183] S. Koyanagi, Y. Kuramoto, H. Nakagawa et al., "A molecular mechanism regulating circadian expression of vascular endothelial growth factor in tumor cells," *Cancer Research*, vol. 63, no. 21, pp. 7277–7283, 2003.
- [184] C. Sarkar, D. Chakroborty, R. B. Mitra, S. Banerjee, P. S. Dasgupta, and S. Basu, "Dopamine in vivo inhibits VEGF-induced phosphorylation of VEGFR-2, MAPK, and focal adhesion kinase in endothelial cells," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 287, no. 4, pp. H1554–H1560, 2004.
- [185] S. Sinha, P. K. Vohra, R. Bhattacharya, S. Dutta, S. Sinha, and D. Mukhopadhyay, "Dopamine regulates phosphorylation of VEGF receptor 2 by engaging Src-homology-2-domain-containing protein tyrosine phosphatase 2," *Journal of Cell Science*, vol. 122, no. 18, pp. 3385–3392, 2009.
- [186] A. D. Bhatwadekar, Y. Yan, X. Qi et al., "Per2 mutation recapitulates the vascular phenotype of diabetes in the retina and bone marrow," *Diabetes*, vol. 62, no. 1, pp. 273–282, 2013.
- [187] J. V. Busik, M. Tikhonenko, A. Bhatwadekar et al., "Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock," *The Journal of Experimental Medicine*, vol. 206, no. 13, pp. 2897–2906, 2009.

Research Article

Inhibition of the RhoA GTPase Activity Increases Sensitivity of Melanoma Cells to UV Radiation Effects

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Ultraviolet radiation is the main cause of DNA damage to melanocytes and development of melanoma, one of the most lethal human cancers, which leads to metastasis due to uncontrolled cell proliferation and migration. These phenotypes are mediated by RhoA, a GTPase overexpressed or overactivated in highly aggressive metastatic tumors that plays regulatory roles in cell cycle progression and cytoskeleton remodeling. This work explores whether the effects of UV on DNA damage, motility, proliferation, and survival of human metastatic melanoma cells are mediated by the RhoA pathway. Mutant cells expressing dominant-negative (MeWo-RhoA-N19) or constitutively active RhoA (MeWo-RhoA-V14) were generated and subjected to UV radiation. A slight reduction in migration and invasion was observed in MeWo and MeWo-RhoA-V14 cells but not in MeWo-RhoA-N19 cells, which presented inefficient motility and invasiveness associated with stress fibers fragmentation. Proliferation and survival of RhoA-deficient cells were drastically reduced by UV compared to cells displaying normal or high RhoA activity, suggesting increased sensitivity to UV. Loss of RhoA activity also caused less efficient DNA repair, with elevated levels of DNA lesions such as strand breaks and cyclobutane pyrimidine dimers (CPDs). Thus, RhoA mediates genomic stability and represents a potential target for sensitizing metastatic tumors to genotoxic agents.

1. Introduction

Among the broad range of skin cancers, melanoma accounts for less than 2% of skin cancer cases. However, melanoma is the cause of the vast majority of skin cancer-related deaths. According to the American Cancer Society, approximately 76,100 new melanoma cases were diagnosed and approximately 9,710 people were expected to die of this type of skin cancer in the United States in 2014 (<http://www.cancer.org/cancer/skincancer-melanoma/detailedguide/melanoma-skin-cancer-key-statistics>). The rate of melanoma has been dramatically increasing over the last thirty years, and even more alarmingly the incidence of melanoma is growing in children [1, 2].

Exposure to solar radiation is a major cause of skin cancers [3]. Within the spectrum of electromagnetic radiation comprising the solar spectrum, the ultraviolet (UV) region is

considered to be highly genotoxic [4]. UV radiation exposure causes damage to many different biomolecules, but DNA is by far the most affected molecule. The promotion of DNA damage by nonionizing radiation, such as UV light, primarily induces lesions via the direct absorption of photons by DNA bases. The ultraviolet radiation spectrum is divided into UVA radiation (315–400 nm), UVB radiation (270–315 nm), and UVC radiation (100–280 nm). UVB and UVC light induce the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (6-4 PPs), whereas UVA light primarily causes oxidative DNA damage via the formation of 8-oxo-7,8-dihydroguanine (8-oxoG) and cyclobutane thymidine dimers [5, 6], potentially leading to single-strand breaks and other interstrand cross-links (ICLs) in DNA [7].

UVB radiation, which has been associated with the induction of nonmelanoma skin cancer, is considered to be

more carcinogenic than UVA radiation. UVA radiation is more abundant in sunlight and can penetrate deeper into the skin compared to UVB radiation. However, UVA radiation is not significantly absorbed by native DNA and is less efficient in inducing direct DNA damage. UVA radiation might indirectly damage DNA via its absorption by non-DNA endogenous sensitizers and via the formation of reactive oxygen species [8, 9]. UVC radiation, which is generally absorbed by oxygen and ozone in the atmosphere, does not reach the surface of the earth and is less harmful to human's skin. Although UVC radiation does not generate reactive oxygen species, this type of radiation has been found to be highly energetic and has become a useful tool for the destruction of many microorganisms, as it is technically simple to generate high doses of UVC radiation at a wavelength (254 nm) approximating the absorption maximum of DNA [10].

The development of metastatic melanoma from normal melanocytes, which typically adhere to the basal membrane of normal skin, is initiated by the selection of a common acquired benign nevus that exhibits aberrant proliferation and that overcomes cellular senescence, resulting in dysplasia. Subsequently, these cells progress to a superficial spreading stage (radial growth phase, RGP) that is confined to the epidermis, and these cells show low invasive potential. However, RGP cells acquire the ability to invade the dermis (vertical growth phase, VGP) and to metastasize [11, 12]. It has long been suggested that motility is necessary and obligatory for tumor cell metastasis [13]. After passing through the basal lamina, tumor cells migrate through the extracellular matrix over long distances for efficient dissemination via blood and lymphatic vessels. Based on the formation of F-actin-rich protrusions that enable forward extension to adhere to their surroundings followed by contraction of their trailing end, tumor cells use both collective motility and single-cell motility based on *in vivo* experiments. The formation of membrane protrusions requires actin polymerization, and in invasive tumor cells this signaling pathway is altered to increase motility [14, 15].

Rho-family GTPases have been directly associated with motility and protrusion formation via the activation of signaling targets that direct upstream actin cytoskeleton-modifying proteins. Among the 20 members of this GTPase family, RhoA has been shown to play key roles in cytoskeletal dynamics, such as the regulation of cell adhesion and migration [16]. However, RhoA exerts pleotropic effects on cellular metabolism via the regulation of gene transcription, cell differentiation and proliferation, and the cell cycle, and these effects are particularly obvious during the establishment and development of human and mouse tumors [17].

However, the involvement of RhoA in melanoma cell metastasis following exposure to UV light deserves further exploration and understanding. The first report associating Rho GTPase activity with UV radiation-induced DNA damage in human cells and DNA repair signaling pathways showed that RhoB is an early-response gene induced by DNA damage agents which participates in the initial signaling events in response to genotoxic stress promoted by UVB radiation [18]. Studies have also shown that, in keratinocytes,

RhoE acts as a protective factor against UVB radiation-induced damage [19], and it was only recently shown that miR-340 regulates UVB light-induced dendrite formation via the downregulation of RhoA protein and mRNA expression in melanocytes [20]. Moreover, cross talk between DNA damage and cytoskeletal dynamics directly involving RhoA and the regulation of cell proliferation and survival were shown in two reports using bacterial cytolethal distending toxins (CDTs) as cytotoxic agents for the promotion of DNA double-strand breaks, which, in turn, led to ATM- and FEN1-dependent RhoA activation under conditions of carcinogenesis triggered by chronic bacterial infection [21, 22].

Based on this strong experimental evidence, the aims of the present study were to examine the correlations between RhoA activity and DNA damage and repair under genotoxic stress promoted by radiation consisting of each one of the three UV wavelengths (A, B, or C) and to determine whether the modulation of RhoA impacts on the motility, invasiveness, and proliferation of human melanoma cell lines. Thus, the cross talk between RhoA activity and genomic stability may suggest this GTPase as a potential target for the sensitization of melanomas to radio-chemotherapies for cancer treatments [23, 24].

2. Materials and Methods

2.1. Cell Culture. The human melanoma cell line, which was derived from a metastatic site on a lymph node (MeWo lineage, HTB-65), was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) at 37°C in 5% CO₂ in a Sanyo model MCO-19AIC (UV) incubator (Sanyo, Osaka, Japan). MeWo cell clones were generated via transfection (using Lipofectamine) with the packaging cell line Φ NX-Ampho (Phoenix) and a plasmid containing RhoA cDNA (mutated at the V14 position (active RhoA) or at the N19 position (dominant-negative RhoA)) cloned into the retroviral vector pCM (pCLNCX backbone). The plasmids were packaged into retroviral particles contained in the viral vector supernatant ($>10^6$ c.f.u.), and these retroviral particles were used to transduce or infect MeWo cells in the presence of 4 μ g/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Infected cells (clones) were selected in culture medium containing the antibiotic G418 (400 μ g/mL) because the pCM vector carries a neomycin resistance gene [25]. Mutant clonal cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 100 μ g/mL G418.

2.2. UV Radiation Treatments. MeWo cells and mutant cells expressing dominant-negative RhoA (MeWo-RhoA-N19) or constitutively active RhoA (MeWo-RhoA-V14) were subjected to UV irradiation using the following procedure: the cell culture medium was replaced with PBS, and the cells were exposed to UV radiation lamps at specific wavelengths corresponding to UVA (365 nm), UVB (302 nm), or UVC (260 nm) radiation for the necessary exposure duration to

reach an intensity of 50 kJ/m² UVA, 80 J/m² UVB, or 4 J/m² UVC, respectively. These conditions were determined and monitored using a model VLX-3W dosimeter (Vilber Lourmat, Eberhardzell, Baden-Württemberg, Germany) coupled to specific probes for each wavelength; this instrument displayed an accuracy of +/-5%. Following each treatment, the culture medium was replaced, and the cells were incubated for the indicated periods prior to further analyses.

2.3. Generation of Rhotekin-Binding Domain-Glutathione S-Transferase (RBD-GST) Fusion Proteins. *E. coli* (BL21) bacteria were transformed with the plasmid carrying RBD-GST (a kind donation from Gary M. Bokoch of the Scripps Research Institute, La Jolla, CA, USA) via thermal shock. Subsequently, the transformed bacteria were plated on LA medium containing 100 µg/mL ampicillin and incubated at 37°C. A colony of transformed *E. coli* (BL21) bacteria was inoculated into 200 mL of LB medium. The inoculum was incubated for 18 h at 37°C under constant agitation (200 rpm). Subsequently, this culture was inoculated into 2 L of LB medium, and the cell culture was maintained at 37°C under constant agitation until reaching an optical density of approximately 0.6. RBD-GST expression was induced by adding isopropyl β-D thio-galactopyranoside (IPTG, 0.5 mM), followed by incubation at 37°C for 2 h, and the cells were recovered via centrifugation (8,000 rpm for 10 min at 4°C). The pellet was resuspended in 20 mL of lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 5 mM MgCl₂; 1% Triton X-100; 1 mM DTT; 10 µg/mL aprotinin; 10 µg/mL leupeptin; and 1 mM PMSF) and sonicated on ice by applying 8 cycles of 2 min at 50% amplitude and a pulse protocol of 15 sec on and 30 sec off. Following lysis, the suspension was centrifuged at 14,000 rpm for 30 min at 4°C, and the soluble fraction containing the RBD-GST fusion protein was collected. Approximately 12 mL of this soluble fraction was incubated in 500 µL of glutathione-Sepharose 4B resin (GE Healthcare, Pittsburgh, PA, USA) for 90 min at 4°C under constant agitation. Subsequently, the resin containing the bound fusion protein was washed (3,000 rpm for 3 min) 6 times with wash buffer (50 mM Tris, pH 7.5; 0.5% Triton X-100; 150 mM NaCl; 5 mM MgCl₂; 1 mM DTT; 1 µg/mL aprotinin; 1 µg/mL leupeptin; and 0.1 mM PMSF), and the beads were resuspended in 5 mL of wash buffer containing 10% glycerol, followed by aliquoting and storage at -80°C [26].

2.4. RhoA GTPase Activity Assay. To obtain protein lysates, the cells were plated on 10 mm dishes at approximately 60% confluence. Following radiation treatment for the specified durations and at the specified doses, the cells were washed twice with ice-cold PBS and disrupted with RIPA lysis buffer (50 mM Tris, pH 7.2; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 500 mM NaCl; 10 mM MgCl₂; 1 mM Na₃VO₄; 1 mM NaF; 1 mM PMSF; and 10 µg/mL each of aprotinin and leupeptin) and stored at -20°C. The protein concentration was quantified using the Bradford colorimetric method (Bio-Rad). A 500 µg sample of the total lysate was subsequently incubated in 25 µg of RBD-GST at 4°C for 90 min. Then, the beads were centrifuged at 4°C for 3 min, washed three times with buffer B (Tris buffer containing 1%

Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 1 mM Na₃VO₄, 1 mM NaF, 1 mM PMSF, and 10 µg/mL each of aprotinin and leupeptin), and intercalated via centrifugation at 3,000 rpm for 3 min in a cold room. The active RhoA protein (RhoA-GTP) bound to the glutathione-Sepharose beads was detected via Western blotting [26].

2.5. Western Blotting for RhoA. To analyze the obtained proteins, electrophoresis was performed under denaturing conditions using polyacrylamide gels consisting of 5% acrylamide in the stacking gel and 13% acrylamide in the separating gel. The proteins were separated via SDS-PAGE at a constant voltage (120 V) and were then transferred to a nitrocellulose membrane (Merck-Millipore, Billerica, MA, USA) using a dry system (Bio-Rad, Hercules, CA, USA) at 300 mA for 90 min. The membrane was blocked with 5% milk in TBS-T (20 mM Tris, pH 7.6; 137 mM NaCl; and 0.1% Tween) for 1 h with stirring at room temperature, followed by three washes with TBS-T. Subsequently, the membrane was incubated for 3 h at room temperature in a monoclonal primary antibody against RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBS-T. The membrane was incubated in the fluorescent secondary antibody IRDye 680CW for 1 h, and the bands were visualized using an Odyssey Infrared Imaging System (Li-Cor, Bad Homburg, Germany). The obtained bands were quantified using Odyssey V3.0 software (Li-Cor, Bad Homburg, Germany).

2.6. Stress Fiber, RhoA, and CPD Staining for Immunofluorescence. On the day before the experiment, the cells were plated on glass coverslips at ~25% confluence, maintained under culture conditions described above and subjected to UV irradiation. The cells were subsequently washed twice with PBS and fixed at room temperature with 3% formaldehyde/2% sucrose/PBS (Phalloidin) or 10% TCA/PBS (RhoA) for 10 min, followed by two additional washes with PBS and permeabilization with PBS containing 0.5% Triton X-100, 6.84% sucrose, and 3 mM MgCl for 5 min on ice. Subsequently, the cells were treated with blocking buffer (PBS containing 3% BSA and 10% FBS) for 30 min at room temperature, followed by incubation for 2 h in an anti-Phalloidin antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) diluted 1:500 in blocking buffer (stress fibers) or in a mouse anti-RhoA antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation in an Alexa Fluor 680 anti-mouse antibody (1:15,000; Invitrogen, Carlsbad, CA, USA) for 1 h in a dark chamber at room temperature in a humidified atmosphere (RhoA). To stain for CPD, coverslips containing an approximately 80% confluent cell monolayer were UV-irradiated and subsequently collected at 0, 6, 24, or 48 h, followed by fixation with 4% paraformaldehyde, permeabilization with 0.5% Triton X-100, and genomic DNA denaturation in the presence of 2 M HCl. The coverslips were incubated for 2 h in a rabbit anti-CPD primary antibody (Cosmo Bio Co., Ltd., Japan) diluted 1:200 in blocking buffer and then for 1 h at room temperature in an Alexa Fluor 568 anti-rabbit secondary antibody. The cells were subsequently mounted on glass slides using VECTASHIELD

containing 4',6-diamidino-2-phenylindole (DAPI; 1 $\mu\text{g}/\text{mL}$). The images were visualized and captured using a Zeiss LSM-510 microscope. Quantitation of the fluorescence per cell was performed using ZEN software (Zeiss, Oberkochen, Germany), and at least 50 cells per condition were individually quantified.

2.7. Cell Migration Assay. Approximately 1.5×10^6 cells were plated on 35 mm plates and incubated until reaching 100% confluence. After various radiation exposure treatments, the plates were diametrically scratched using a sterile pipette tip. Cell migration was assessed by comparing the cell invasion area of the scratch at the initial time (0 h) with the cell invasion area of the scratch at the ending time (24 h). Several micrographs were obtained along the scratch at 200x magnification using an inverted Olympus microscope, and the cell migration or invasion measurements were conducted using appropriate tools provided in cell-F software (Olympus, Shinjuku, Tokyo, Japan) [27].

2.8. Matrigel Invasion Assay. MeWo cells suspended in serum-free medium were plated in the upper chamber of a BD BioCoat Matrigel Invasion Chamber (BD Biosciences, San Jose, CA, USA) (10^5 cells in 100 μL) and were allowed to invade for 24 h at 37°C in 5% CO_2 in the presence or absence of the matrix metalloproteinases (MMP) inhibitor GM6001 (Calbiochem, Billerica, MA, USA) at a concentration of 25 μM . The lower chamber was filled with complete medium as a chemoattractant for cellular invasion. At the end of the experiment, the upper sides of the inserts were scraped with cotton swabs, and the cells on the bottom side of the membrane were fixed in 3.7% formaldehyde, subjected to nuclear staining with DAPI (Sigma), and photographed. Cells were counted at 20x magnification in 10 different optical fields per insert.

2.9. Growth Curves. The role of RhoA protein in cell proliferation following UV irradiation was observed using growth curves. MeWo cells and RhoA mutant clones (3.5×10^4) were plated on 35 mm plates at 24 h before treatment. Subsequently, the cells were trypsinized, fixed in a formaldehyde/PBS solution, and counted in a Fuchs-Rosenthal chamber every 24 h for five consecutive days.

2.10. Clonogenic Assay. Each cell line used in this study was plated at a low density (2×10^3 cells/plate) on 60 mm plates at 24 h before the radiation treatments. Subsequently, the cells were irradiated as previously described and provided with fresh medium, which was replaced every three days until the cell colonies were visible (approximately 10–12 days). The colonies were fixed with 10% formaldehyde/PBS and stained with a 0.5% crystal violet solution for visualization, followed by manual counting and plate scanning.

2.11. Single-Cell Gel Electrophoresis or Comet Assay. Parental MeWo cells and MeWo-RhoA-N19 and MeWo-RhoA-V14 mutant cells were plated at a density of 2×10^5 cells/plate on 35 mm plates 24 h before UVA, UVB, or UVC irradiation.

Following each specific treatment, the cells were collected via trypsinization and mixed with 0.5% low-melt agarose at 37°C. This mixture was applied to glass slides covered with a thin layer of 1.5% agarose and incubated at 4°C for 15 min for jellification. The cells were subsequently lysed in lysis solution (10 mM Tris, pH 10; 2.5 M NaCl; 100 mM EDTA; 1% Triton X-100; and 10% DMSO) for 24 h at 4°C. Following lysis, the slides were placed in a horizontal electrophoresis tank, immersed in electrophoresis buffer (300 mM NaOH and 1 mM EDTA), and incubated for 30 min to denature the DNA. The slides were subjected to electrophoresis at 1 V/cm and 300 mA for 30 min. Subsequently, the slides were incubated in neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min and fixed in absolute ethanol for 5 min, followed by DNA staining with 2 $\mu\text{g}/\text{mL}$ ethidium bromide and visualization under a fluorescence microscope (Olympus BX51). The results of the DNA damage analysis assay were expressed as the olive tail moment, which was obtained using Komet 6.0 software (Andor Technology, Oxford, UK), and 100 cells per sample were analyzed (50 cells per slide) [28].

2.12. Statistical Analysis. The treatments were compared to determine significant differences using Student's *t*-test for paired data, and statistical significance was assumed at $P < 0.05$. ANOVA was used for comparing the means of two or more groups.

3. Results

3.1. Generation and Characterization of MeWo-RhoA Mutant Clones and Investigation of the Effects of UV Irradiation on Cell Migration and Invasion. In the present study, we used the MeWo cell line, an adherent cell line with fibroblastic morphology derived from the lymph nodes of patients with malignant melanoma [29–32]. This cell line was subjected to retroviral transduction with a construct containing the RhoA-N19 (dominant-negative) or the RhoA-V14 (constitutively active) mutant [33] to obtain clonal lines ectopically expressing each RhoA GTPase variant to interfere with the endogenous activity of RhoA in MeWo cells. Nine MeWo-RhoA-N19 clones and six MeWo-RhoA-V14 clones were isolated, and the migration ability of these cells was tested using scratch wound healing assays in the presence or absence of serum (results not shown). We selected the two most representative clones from each mutation and measured the basal levels of RhoA and RhoA-GTP to examine functionality. We demonstrated that the MeWo-RhoA-N19 mutant cells displayed a reduced basal level of RhoA-GTP compared with the MeWo cells and the MeWo-RhoA-V14 cells, which displayed the highest levels of RhoA activity, as expected (Supplementary Figure S1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/2696952>).

These MeWo-RhoA clones were exposed to different doses (not shown) of UV (UVA, UVB, or UVC) radiation and examined for stress fiber formation to assess RhoA functionality (Figure 1(a)). The results showed that the RhoA-deficient MeWo-RhoA-N19 clones contained less filamentous actin (F-actin), which was stained with high affinity using Phalloidin, and exhibited a more fragmented morphology

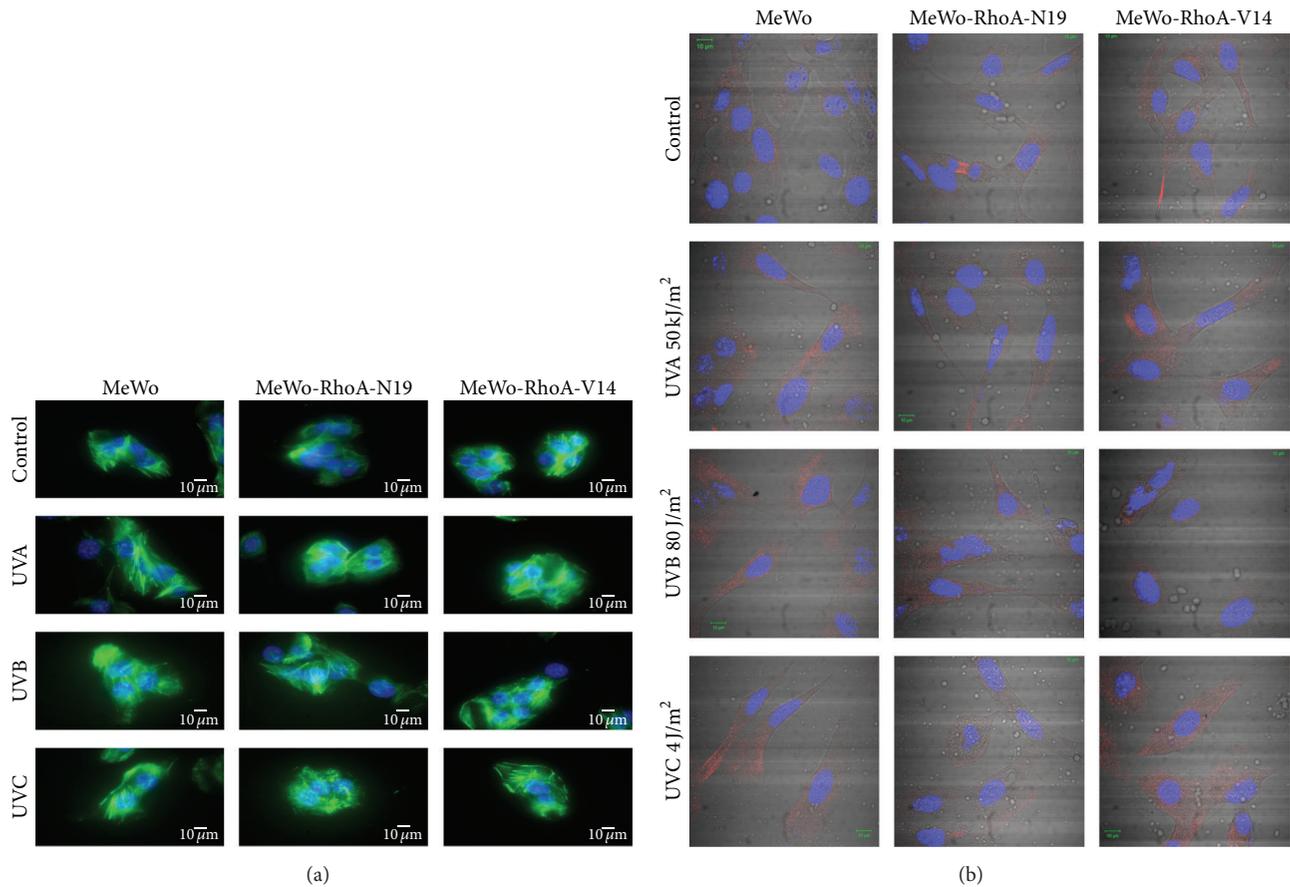


FIGURE 1: Immunofluorescence analysis was performed on the parental MeWo clone and the MeWo-RhoA-N19 and MeWo-RhoA-V14 mutant clones to evaluate the profile of stress fiber formation and RhoA distribution after damage induced by 50 kJ/m² UVA, 80 J/m² UVB, or 4 J/m² UVC irradiation. A total of 200,000 cells were seeded on 35 mm culture dishes at 24 h before treatment. At 1 h after the given radiation treatment, the cells were fixed in 3% paraformaldehyde/2% sucrose/PBS buffer (a) or 10% TCA/PBS (b) and permeabilized with 0.5% Triton X-100/6.84% sucrose/3 mM MgCl₂/PBS buffer. Subsequently, the cells were blocked in 3% BSA/PBS for 30 min and incubated in 1:500 Alexa Fluor 488 Phalloidin (Invitrogen, Carlsbad, CA, USA) for 1 h at 4°C (a) or in 1:250 mouse anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h followed by 1:15000 Alexa Fluor 680 secondary antibody (Invitrogen, Carlsbad, CA, USA) (b). After washing with PBS, the cells were mounted on coverslips in VECTASHIELD medium containing DAPI, and images were acquired using a Zeiss LSM 510 laser confocal microscope. The photomicrographs are representative of three different fields in two independent experiments.

than the parental and MeWo-RhoA-V14 clones, particularly after UVC irradiation. The MeWo cell line and the constitutively active RhoA-expressing MeWo-RhoA-V14 clones displayed cytoskeletal features characteristic of physiologic actin function and exhibited normal RhoA levels and stress fiber integrity regardless of the UV treatment applied. Another fundamental morphological characteristic of the MeWo-RhoA mutant cells was the fact that the MeWo-RhoA-N19 cells were thinner and more elongated but the MeWo-RhoA-V14 cells were more spread out and flattened than the parental MeWo cells (Figure 1(b), bright field micrographs). Importantly, the classical and expected cytoplasmic distribution of RhoA was not affected in any of the three cell lines investigated and was not altered by the three different UV treatments applied, as shown in the immunofluorescence microscopy experiments (Figure 1(b), RhoA stained in red).

Considering the previously described behavior of stress fiber assembly, the effect of UV on the motility of these cells

was evaluated using scratch wound healing assays in the presence of 10% serum. The results for the three cell lines highlighted the inhibition (28% maximum) of cell migration following exposure to each of the three UV wavelengths compared with no treatment, and the most pronounced effect was consistently triggered by UVC irradiation (Figures 2(a) and 2(b)). In addition to their expected reduced migration, the RhoA-deficient MeWo-RhoA-N19 cells were less sensitive to the effects of UV irradiation on motility than the cells displaying high levels of RhoA activity. To avoid inaccurate conclusions regarding whether the dominant-negative RhoA-expressing clones were migrating or proliferating within 24 h in the wound healing assays, new migration assays were performed in the presence of two different doses of mitomycin C, and the cells were evaluated after 16 and 24 h. The results showed no differences in cell migration in the presence (Supplementary Figure S2) or absence of mitomycin C (Figure 2) based on a comparison of the three cell lines.

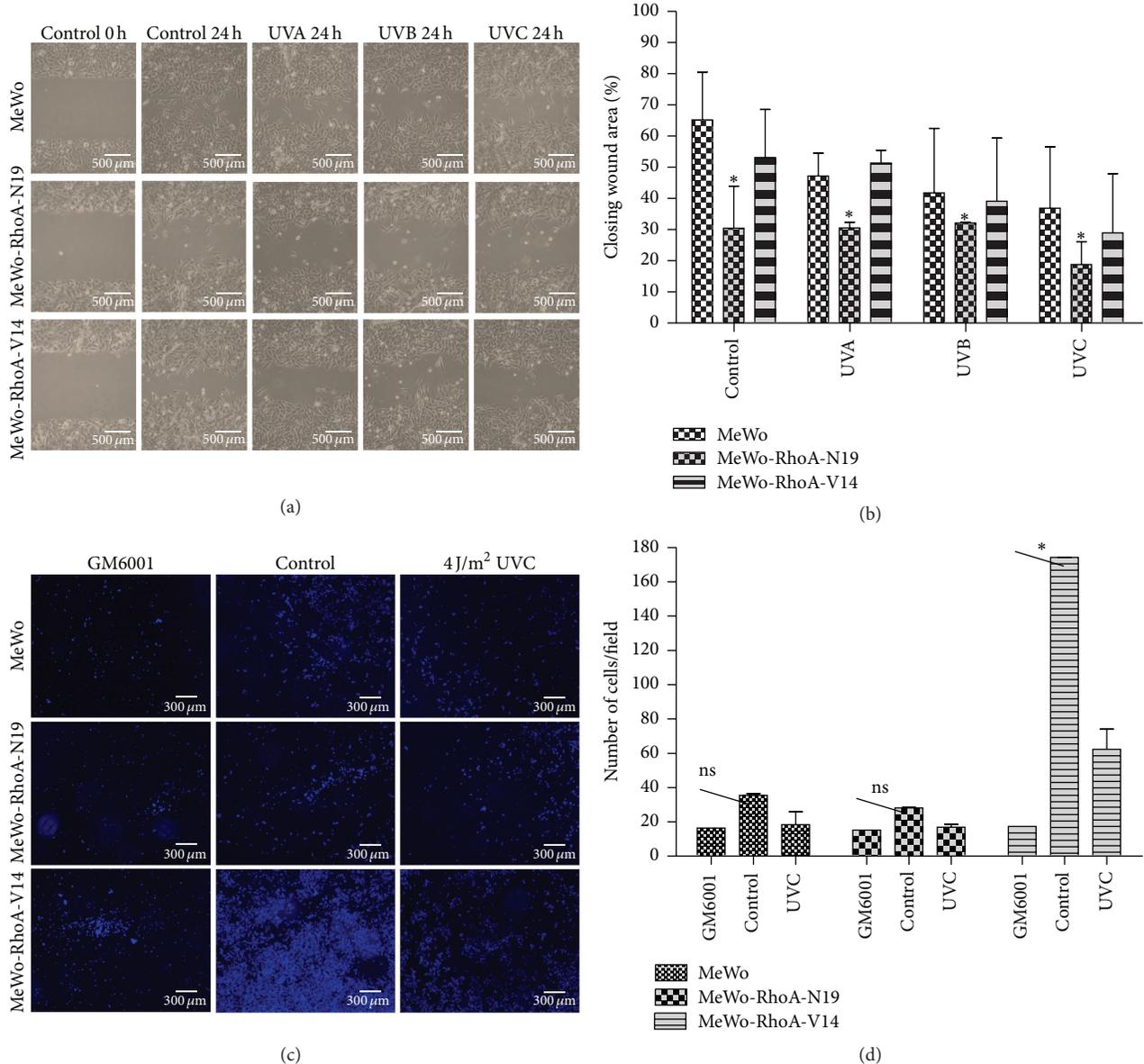


FIGURE 2: Scratch wound healing and Matrigel invasion assays for MeWo, MeWo-RhoA-N19, and MeWo-RhoA-V14 cells treated with UV radiation. (a) A scratch-like wound was made in a monolayer of cells on 100% confluent plates using a micropipette tip (time zero) prior to irradiation treatment (50 kJ/m^2 UVA, 80 J/m^2 UVB, or 4 J/m^2 UVC). The cells were photographed at 0 and 24 h after treatment at 20x magnification using an inverted microscope (Olympus, Tokyo, Japan), and representative micrographs are shown. (b) Measurements of the initial and final open areas were calculated using cell-F software (Olympus, Tokyo, Japan) and were plotted in bar graphs as the percentage of the closed area. The results are presented as the mean and standard deviation from at least three independent images captured at 24 h after treatment. Two-way ANOVA was performed to compare the RhoA-N19 clone with the two other clones treated according to the same specified conditions. * $P = 0.005$. (c) Representative micrographs of the Matrigel invasion assay for MeWo, MeWo-RhoA-N19, and MeWo-RhoA-V14 cells untreated (control) or pretreated with 4 J/m^2 UVC or with $25 \mu\text{M}$ of a broad-spectrum MMP inhibitor (GM6001). Nuclei of the invasive cells were visualized using DAPI (4x magnification). (d) Quantification of invasive cells shown in 2C. A t -test was performed to compare the control cells with the UVC radiation-treated cells from two independent experiments. * $P = 0.004$; ns, nonsignificant.

This finding confirmed that the MeWo-RhoA-N19 clone was clearly less motile than the other two clones, independent of the presence of the antiproliferative agent mitomycin C.

To determine whether RhoA-dependent sensitivity to UVC radiation treatment also influences the invasiveness of MeWo cells in vitro, the capacity of the MeWo clones to invade through Matrigel was evaluated using Transwell

invasion assays (Figures 2(c) and 2(d)). In the absence of UV irradiation, the invasive capacity of the MeWo cells directly correlated with their RhoA activity levels (MeWo-RhoA-V14 > MeWo > MeWo-RhoA-N19). These data were supported by preliminary spheroid invasion assays (multicellular tumor spheroids (MTS) formed via the spontaneous aggregation of 10,000 cells/well and embedded in 3D rat-tail

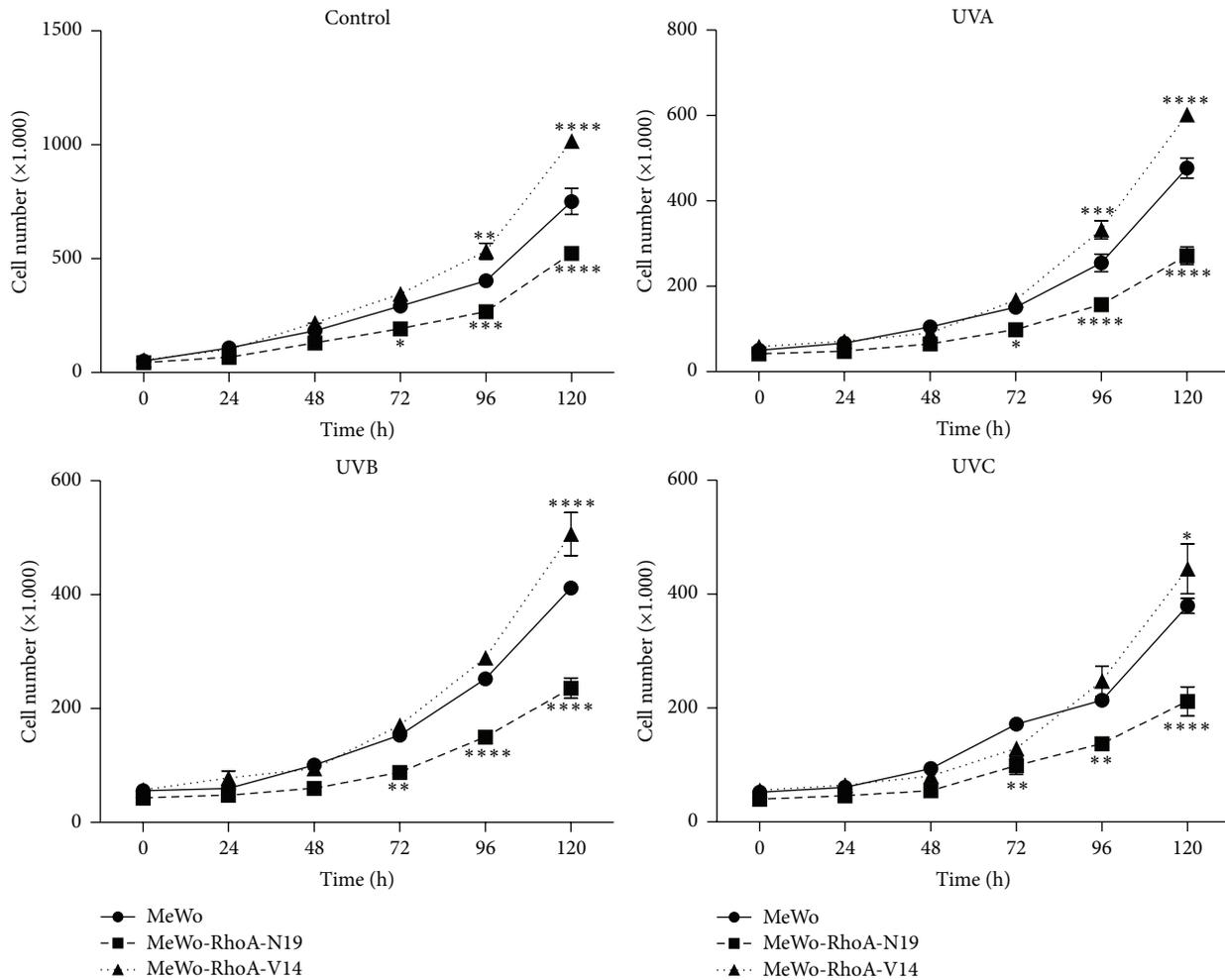


FIGURE 3: Proliferation curves for the MeWo, MeWo-RhoA-N19, and MeWo-RhoA-V14 clones exposed to genotoxic-equivalent doses of UV radiation. The cells were seeded at a density of 35×10^3 cells per 35 mm culture dish at 24 h before treatment and were exposed to 50 kJ/m^2 UVA, 80 J/m^2 UVB, or 4 J/m^2 UVC radiation. The cells were collected every 24 h for five consecutive days and were counted daily in a Fuchs-Rosenthal chamber. The graphs are representative of three independent experiments, and the standard deviation and statistical significance are shown for only the fifth day. Two-way ANOVA was performed to compare the mutant clones with the MeWo clone treated according to the same specified conditions. * $P = 0.01$; ** $P = 0.005$; *** $P = 0.0001$; **** $P < 0.0001$.

type-1 collagen matrices), showing that the constitutively active RhoA-expressing MeWo clones exhibited an invasive phenotype, in contrast to the RhoA-deficient MeWo clones (Supplementary Figure S3). Notably, pretreatment with a broad-spectrum MMP inhibitor (GM6001) robustly suppressed the invasiveness of the three cell lines, including the highly invasive MeWo-RhoA-V14 cells, indicating that MeWo invasion through Matrigel is MMP-dependent. In agreement with the results of the migration assays (Figure 2(b)), the inhibitory effects of UVC irradiation on cell invasion were clearly the least pronounced in the cells displaying the lowest RhoA activity levels (Figures 2(c) and 2(d)).

3.2. The Proliferation and Survival of RhoA-Deficient MeWo-RhoA-N19 Cells Are More Strongly Affected by UV Radiation Than Cells Displaying Normal RhoA Activity. The measurements of cellular proliferative capacity after any type of genotoxic stress, such as UV radiation of any of the three

wavelengths applied, revealed how the cells recovered in response to damage to DNA or other biomolecular structures to escape death or to enter cell cycle arrest. Thus, proliferation curves were generated for the MeWo clone and the two RhoA mutant MeWo clones for five consecutive days following exposure to UVA, UVB, or UVC radiation or no treatment (Figure 3). The initial results showed that the three cell lines responded more effectively as the energy of the applied UV radiation decreased; that is, $\text{UVA} < \text{UVB} < \text{UVC}$. The MeWo-RhoA-V14 clone exhibited higher proliferative capacity, independent of the treatment, closely followed by the parental MeWo clone; however, these two cell lines, which displayed high levels of RhoA activity, were much more resistant to the deleterious effects of UV irradiation on cell proliferation than the RhoA-deficient clone. These results were confirmed by the cell cycle distribution of the cell population, as analyzed by flow cytometry, which showed a discrete and expected perturbation of the cell

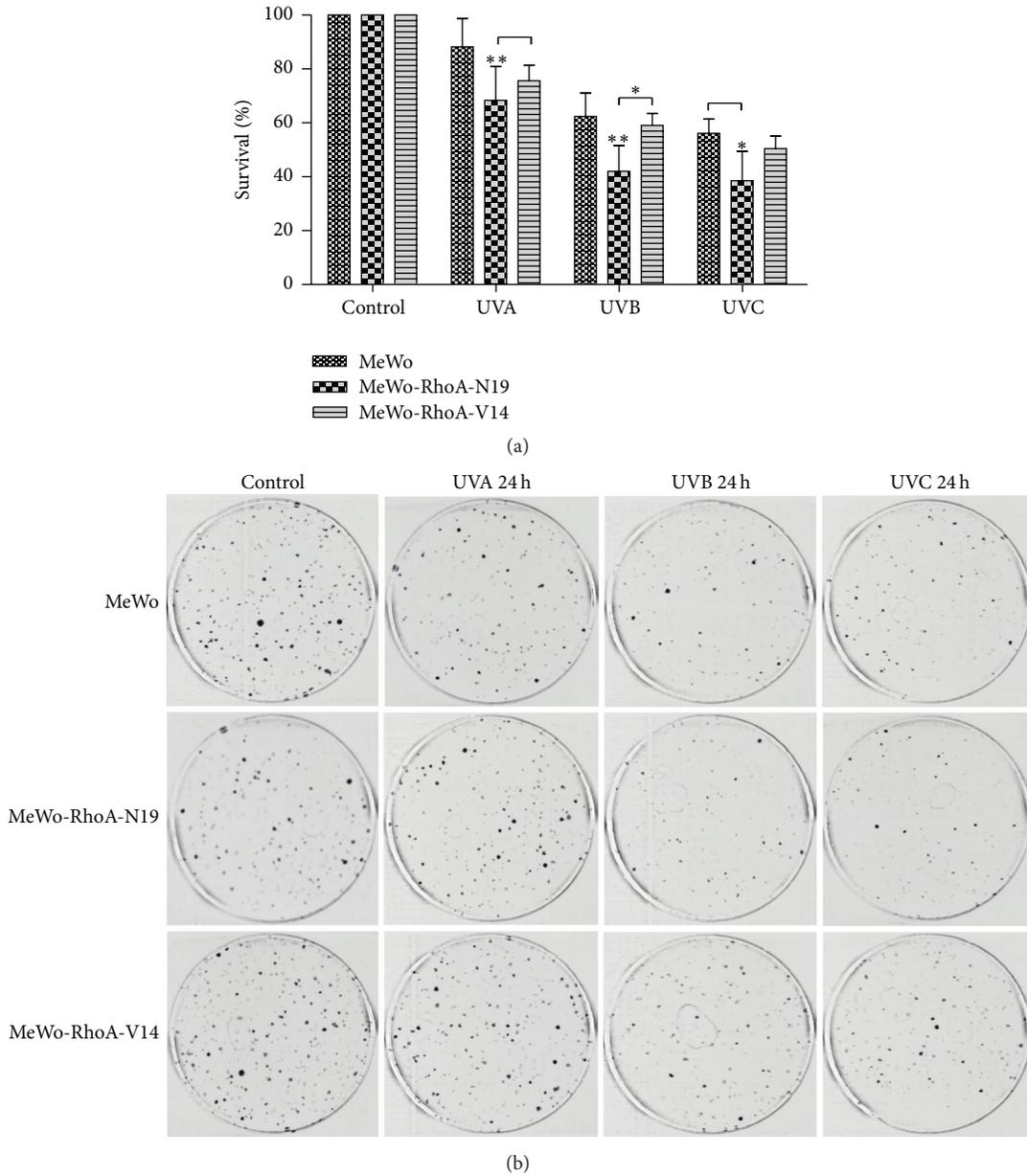


FIGURE 4: Clonogenic assays showing the highly superior survival of MeWo and MeWo-RhoA-V14 cells, which displayed high levels of RhoA activity, compared with RhoA-deficient MeWo-RhoA-N19 cells after exposure to different types of UV radiation. The bars represent the average and standard deviation of at least three independent experiments. Two-way ANOVA was performed to compare the mutant cells with the MeWo cells treated according to the same specified conditions. * $P < 0.01$; ** $P < 0.005$.

cycle distribution in the cells displaying high RhoA activity (Supplementary Figure 4). However, the MeWo-RhoA-N19 cells were the most sensitized after treatment with all three UV wavelengths, and these cells exhibited a reduction in proliferation of approximately 50% compared with untreated cells (Figure 3). This behavior was fully complemented by the flow cytometry results, which showed an accumulation of cells in G1 phase and a concomitant reduction of cells in G2-M phase at only 24 h after UV treatment in the RhoA-deficient clones (Supplementary Figure 4). This delay in

the cell cycle suggests that more of these cells are arrested at the G1 checkpoint, likely reflecting inefficient DNA repair.

When examined for a longer period (15 days) and when seeded at a much lower density (2,000 compared to 50,000 cells) in colony formation assays, the proliferation and survival capacities showed similar results (Figure 4). Thus, cells exhibiting high levels of RhoA activity (MeWo and MeWo-RhoA-V14) are more resistant to UVA, UVB, or UVC irradiation, resulting in enhanced survival, whereas RhoA-deficient MeWo cells (MeWo-RhoA-N19) are more sensitive to UV

irradiation, resulting in reduced survival. The different chemical and physical effects of the three UV wavelengths applied apparently equivalently affected the three cell lines, as each respective cell line responded similarly to all UV radiation treatments.

Consistent with cell proliferation, acute cell death, particularly apoptosis, was evaluated and Annexin V labeling experiments were performed on the same cell lines under the same treatment conditions [34]. As a positive control, H_2O_2 was used to produce approximately 50% apoptotic cells. UVA, UVB, or UVC radiation treatment resulted in higher apoptosis in MeWo-RhoA-N19 cells than in MeWo and MeWo-RhoA-V14 cells, which exhibited even lower apoptosis than the parental MeWo cells (Supplementary Figure 5). As the loss of plasma membrane asymmetry is an early reversible event in apoptosis that results in the exposure of phosphatidylserine (PS) residues on the outer plasma membrane [35], these preliminary results show that RhoA deficiency increases the sensitivity of MeWo cells to UV irradiation and renders these cells more susceptible to apoptotic cell death.

3.3. MeWo Cells Displaying High RhoA Activity Are Much Less Affected by UV Radiation-Induced Damage, Such DNA Strand Breaks and CPDs, and Exhibit More Efficient DNA Repair Than RhoA-Deficient MeWo Cells. Previous results showed an evident association between reduced proliferative ability and cellular recovery from UV radiation-induced damage, as reflected by the reduced levels of RhoA activity in the MeWo-RhoA-N19 mutant clones. The multiple DNA lesions promoted by UV radiation are well known; therefore, we next explored another potential correlation between DNA damage and RhoA activity in MeWo melanoma cells. For this investigation, we used the alkaline comet assay, which is a general assay for DNA damage, to detect both single and double DNA strand breaks after exposure to the three UV wavelengths (Figure 5). Moreover, using kinetics experiments to detect damage over time, it is possible to infer repair ability over a period of up to 6 h after the irradiation of the cells. For example, the improved ability of MeWo-RhoA-V14 clones to repair UV radiation-promoted DNA damage has been observed, as these cells completely recover to the initial conditions by 6 h after treatment. Additionally, in MeWo cells displaying high levels of RhoA activity, this recovery capacity is highly similar. However, the RhoA-deficient MeWo-RhoA-N19 clone exhibited higher basal damage under the control conditions (minimum of 30% more damage), peaking at 30 min after UV radiation and increasing to approximately 40–50% of that in the other two clones. Intriguingly, the RhoA deficiency of these cells likely reflects their inability to recover from DNA damage up to 6 h after treatment or even to prevent the accumulation of these lesions over time (Figure 5).

The DNA lesions promoted by different UV wavelengths and detected using alkaline comet assays indicate a strong correlation between RhoA activity and DNA damage and repair. Thus, we further measured the formation of CPDs, which are specific, highly toxic, and mutagenic DNA lesions promoted by UV radiation. Using a specific antibody to detect CPDs in the nucleus of the three cell lines, which was

delimited based on nuclear staining using DAPI via confocal microscopy, we quantified the DNA lesions after UV irradiation for different periods (Figure 6(a)). Consistent with the results of the comet assay, we observed that the RhoA-deficient MeWo-RhoA-N19 cells exhibited higher levels of CPDs, particularly after UVB or UVC irradiation, and the most striking results were the accumulation of CPDs up to 48 h after either UVA, UVB, or UVC irradiation. Alternatively, the MeWo and MeWo-RhoA-V14 cells exhibited lower CPD staining and greater recovery at 24 h after all three treatments. All of these results were clearly observed directly on the micrographs shown in Figure 6(b), showing a direct correlation between RhoA activity and the function of the nucleotide excision repair (NER) pathway, which is the main pathway responsible for the recovery from CPD lesions.

4. Discussion

Approximately ten years ago, the biochemical functions of RhoA (and the typical GTPases) were associated with the regulation of the actin cytoskeleton, the microtubule cytoskeleton, gene expression, and certain uncommon enzymatic activities (involving lipid metabolism and ROS generation). These GTPases were responsible for biological functions such as cell cycle control (G1 progression, mitosis, and cytokinesis), cell morphogenesis (cell-cell interactions and cell polarity), and cell migration (movement and directional sensing) [36]. However, recently, novel RhoA functions similar to those of the Ras homolog were found to be regulated by reactive oxygen species [37, 38], and this regulation may be particularly relevant to some pathological conditions, such as genotoxic stress-induced DNA damage [39]. Thus, RhoA and certain subfamily members were reported to mediate genomic stability or integrity via their indirect involvement in DNA repair mechanisms [18, 19, 21, 22]. Additionally, it was recently shown that RhoA activation is mediated by its physical interaction with the OGG1 protein, a key enzyme in the DNA repair of 8-oxoG modifications [40].

Thus, taking advantage of the well-known roles of RhoA (and other GTPases) in the regulation of actin polymerization and in the metastasis of many types of aggressive tumors [13, 15, 16], including melanomas [11], and considering that the mutagenic effects of UV radiation on melanocytes and keratinocytes trigger metastasis [12, 41], we explored the potential cross talk between the small GTPase RhoA, UV damage and melanoma cell migration, invasion, proliferation, and DNA repair.

These studies were performed in the human metastatic melanoma cell line MeWo upon exposure to three different wavelengths of UV light, as the oxidative stress generated via UVA and UVB radiation and the high energy of UVC radiation induce direct electron transfer/rearrangements in DNA, resulting in serious consequences for the cell cycle [42, 43]. First, we generated cellular models of MeWo cells expressing either constitutively active RhoA (MeWo-RhoA-V14) or deficient RhoA (MeWo-RhoA-N19) for comparisons with parental MeWo cells. These clones were characterized using three different methodologies: (i) pull-down assays, which showed higher levels of RhoA-GTP and stronger

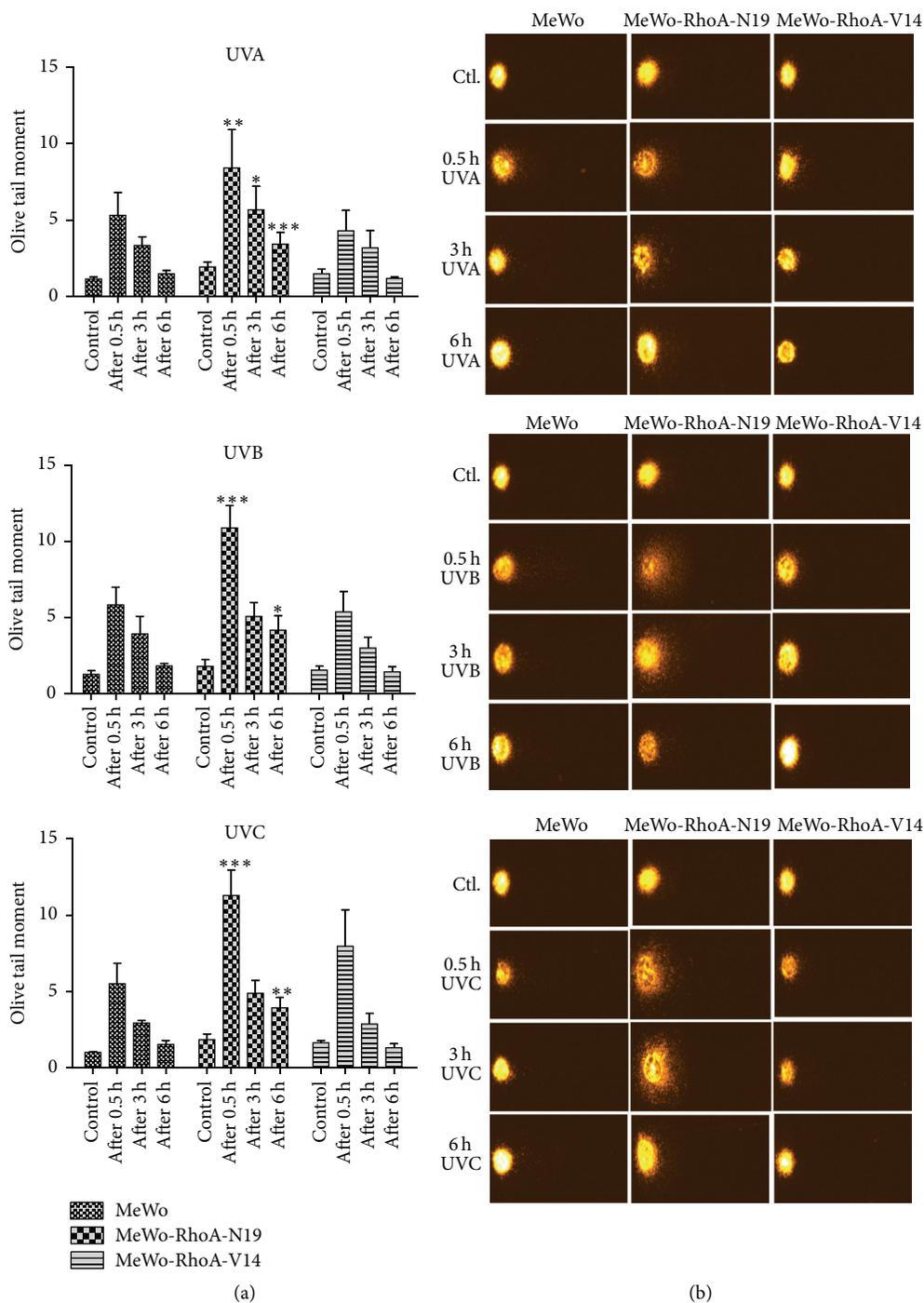


FIGURE 5: Alkaline comet assays showing DNA single- and double-strand break formation and DNA repair kinetics up to 6 h after exposing MeWo, MeWo-RhoA-V14, and MeWo-RhoA-N19 clonal cells to UVA, UVB, or UVC radiation. In these experiments, control (Ctl.) refers to 0 h or non-UV radiation treatment. A total of 2×10^4 cells were plated on 35 mm culture dishes 24 h before the treatment, followed by trypsinization, suspension in low-melting point agarose, and spreading onto glass slides. After 2 h of electrophoresis in an appropriate buffer, the cell nuclei were stained with ethidium bromide (see details in Materials and Methods), as shown in (b), and many different parameters were acquired using a Nikon microscope controlled by Komet 6.0 software (Andor Technology, Oxford, UK). The most relevant parameter, the olive tail moment, was used to quantify DNA damage and repair in the cells under these conditions (bar graphs in (a)). The graphs represent the average and standard deviation of at least three independent experiments, and the statistical significance of the results was obtained by comparing the effects of different treatments between the MeWo cells and the mutant cells using two-way ANOVA. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

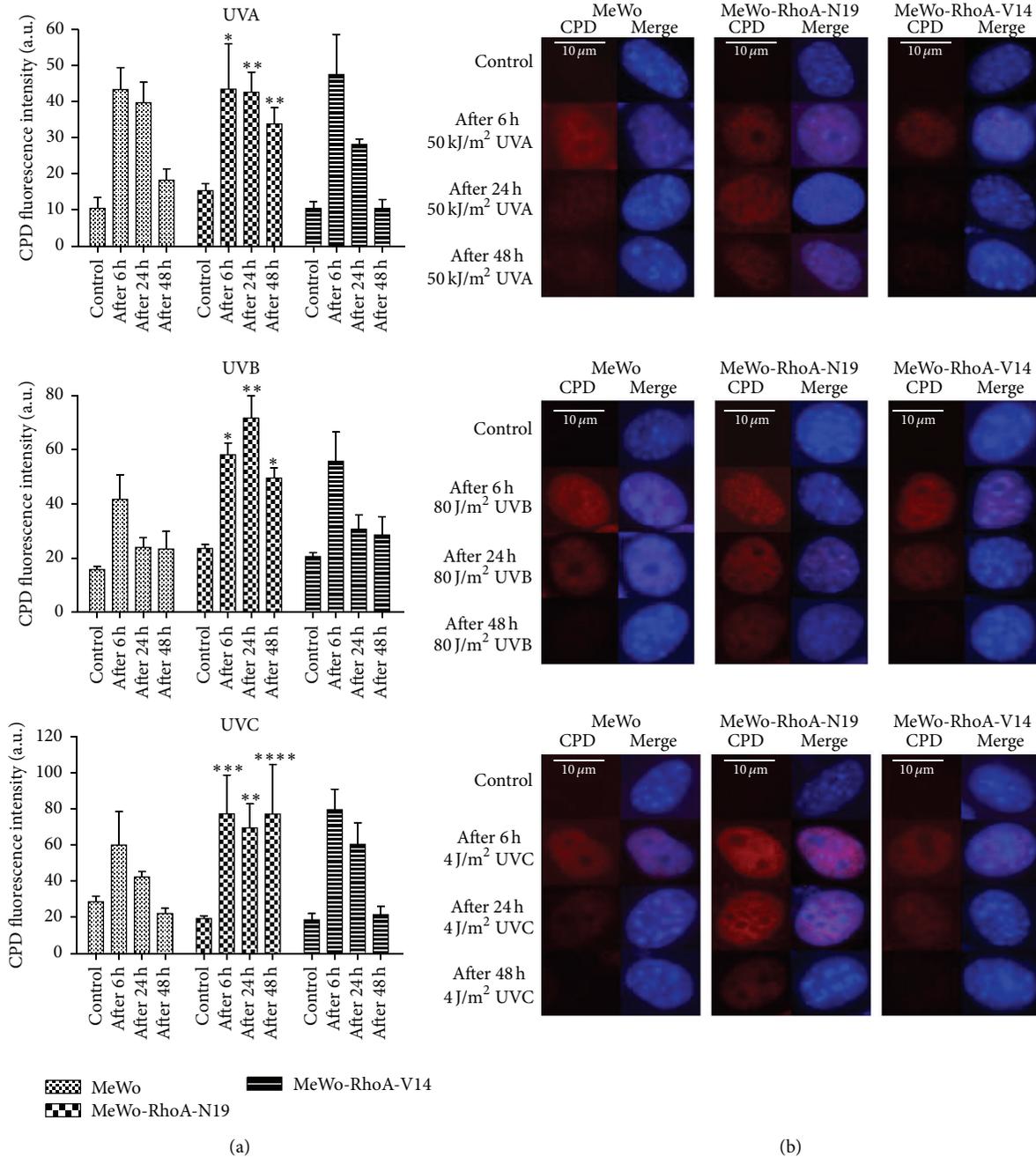


FIGURE 6: Immunofluorescence analysis for the detection of cyclobutane pyrimidine dimers (CPDs) generated in the MeWo tumor cell line and in the MeWo-RhoA-N19 and MeWo-RhoA-V14 clones after treatment with UV radiation. Coverslips containing an approximately 80% confluent cell monolayer were exposed to UV radiation and collected after 0 (control or non-UV radiation treatment), 6, 24, or 48 h, followed by fixation with 4% paraformaldehyde, permeabilization with 0.5% Triton X-100, and genomic DNA denaturation in the presence of 2 M HCl. The coverslips were incubated for 2 h in an anti-CPD primary antibody (diluted 1 : 200) and then for 1 h at room temperature in an Alexa Fluor 568 secondary antibody (a). Approximately 50 single cells were photographed at 100x magnification and were quantified in sequence using Zen software (Zeiss). The bars represent the average and standard deviation from three independent experiments (b). Two-way ANOVA was performed to compare the mutant clones with the MeWo clone treated according to the same specified conditions. * $P = 0.01$; ** $P = 0.005$; *** $P = 0.0001$; **** $P < 0.0001$.

responses to serum deprivation for the MeWo and MeWo-RhoA-V14 clones than for the RhoA-deficient MeWo clones (Supplementary Figure 1); (ii) Phalloidin labeling of F-actin fibers, which showed reduced levels of stress fibers and

shortened and fragmented cell morphology in the MeWo-RhoA-V14 clones compared with the other cell lines (Figure 1(a)), despite normal subcellular RhoA distribution (Figure 1(b)); and (iii) monolayer migration and 3D matrix

penetration assays, which showed reduced motility and invasion capacity of MeWo-RhoA-N19 clones compared with the parental and MeWo-RhoA-V14 clones under control conditions in the presence of serum (Figure 2 and Supplementary Figure S3).

Second, the effects of UV radiation treatment on the three cell lines with respect to stress fiber formation, cell shape, RhoA distribution, migration, and invasion were examined to confirm the deleterious effects of these genotoxic stressors. We observed that UVA, UVB, or even UVC irradiation did not lead to any detectable change in the cell edge shape or the stress fiber morphology in the MeWo or MeWo-RhoA-V14 cells (Figure 1). The migration of these cells was slightly reduced after 24 h, particularly under UVB or UVC radiation treatment (Figure 2(a)), and their invasion capacity was slightly reduced by UVC radiation treatment (Figure 2(b)); these effects were not strictly dependent on the actin-myosin cytoskeleton and were potentially caused by many other factors [44]. Conversely, negative effects on stress fiber morphology and content were observed in RhoA-deficient MeWo-RhoA-N19 clones (Figure 1(a)), very likely reflecting their reduced motility (Figures 2(a) and 2(b)) and invasion ability (Figures 2(c) and 2(d)). The three evaluated cytoskeletal features (Phalloidin staining of actin-myosin fibers, cell edge shape, and RhoA subcellular distribution) are in agreement with each other and with the biological responses of motility and invasiveness characteristic of the aggressive phenotype of melanoma cells and, moreover, with the modulation of RhoA activity. Corroborating the effects of UV radiation treatment on cell migration, a significant reduction in the MMP-dependent invasive capacity of the MeWo cells was observed in all experimental groups following UVC radiation treatment. However, this suppressive effect appeared to be less pronounced in the RhoA-deficient MeWo-RhoA-N19 clones, and this result supports the hypothesis that the inhibitory effects of UV radiation on melanoma cell invasion are partially dependent on RhoA activity.

We next confirmed the well-known antiproliferative effects of UV radiation on melanomas and the potential role of RhoA modulation in this process [17, 24]. Growth curves and colony formation assays confirmed the higher resistance of MeWo-RhoA-V14 and MeWo cells (to a lesser extent) to UV radiation treatment compared with RhoA-deficient cells (Figures 3 and 4), as the MeWo-RhoA-V14 and MeWo cells recovered even after UVB and UVC radiation-induced damage. The opposite effects were observed for the RhoA-deficient clones; that is, these cells exhibited approximately 50% higher sensitivity to UV radiation-induced damage.

As previously reported, these results likely reflect that RhoA affects the efficiency of DNA repair mechanisms [18, 40]. Thus, to assess DNA integrity and specific UV radiation-promoted damage, we performed alkaline comet assays and CPD formation experiments. We showed that the inability to remove damage over time clearly reflects cell proliferation via the modulation of RhoA activity-proficient and activity-deficient MeWo clones. Measurements of single- and double-strand breaks showed that cells displaying high RhoA activity exhibit less damage at 0.5 h (peak) after treatment with the three types of UV radiation and exhibit more efficient repair,

completely recovering to the basal levels after 6 h. In contrast, dominant-negative RhoA-expressing cells showed an accumulation of damage from 0.5 to 6 h after injury induced by UVA, UVB, or UVC radiation (Figure 5). Intriguingly, the levels of CPDs peaked at approximately 6 h after UV radiation in all three cell lines, irrespective of RhoA activity, but these lesions were almost completely removed after 48 h in cells exhibiting high levels of RhoA activity. In addition, in the RhoA-deficient MeWo cells (MeWo-RhoA-N19 clones), the accumulation of CPD lesions remained high for up to 48 h, independently of treatment with UVA, UVB, or UVC radiation. Thus, this specific UV radiation-induced damage accumulates in the cellular background of low RhoA activity (Figure 6).

As predicted from the results of other previous studies and confirmed in the present study, the higher the RhoA activity the more efficient the DNA repair; this phenomenon is common to many human tumor cells [45]. UV radiation induces DNA damage, such as single-strand breaks, pyrimidine dimers, and 6-4 PPs, which induce mutations that are characteristic of the promotion, establishment, and development of tumors [46]. These forms of damage are typically repaired by the NER cascade [47], and failures in this repair machinery result in many diseases, such as xeroderma pigmentosum [3]. Thus, in the present study, we established a strong correlation between RhoA activity and the efficiency of the repair of UV radiation-induced damage to melanoma cells, suggesting that NER pathway function might be affected by RhoA-transduced signals that activate cellular responses, such as gene transcription, cell proliferation, and cell death [17]. Although additional molecular studies are needed, RhoA represents a potential target for UV radiation-induced carcinogenesis in skin [48], as well as for gamma radiation-induced damage in cervix carcinomas, where RhoA was also shown to mediate double-strand breaks repair [49]. Similar results, in the same cellular models, were also found for the Rac1 GTPase [50] again suggesting actin cytoskeleton remodeling signals towards the nuclear machineries in charge of the genomic stability.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Conception, design, and supervision were done by Fabio Luis Forti. Development of methodology was done by Gisele Espinha, Juliana Harumi Osaki, and Erico Tosoni Costa. Acquisition of data was done by Gisele Espinha and Juliana Harumi Osaki. Analysis and interpretation of data were performed by Gisele Espinha, Erico Tosoni Costa, and Fabio Luis Forti. Writing and revision of the paper were made by Gisele Espinha and Fabio Luis Forti.

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References

- [1] K. Ferguson, "Melanoma," *The Journal of Continuing Education in Nursing*, vol. 36, no. 6, pp. 242–243, 2005.
- [2] J. J. Strouse, T. R. Fears, M. A. Tucker, and A. S. Wayne, "Pediatric melanoma: risk factor and survival analysis of the surveillance, epidemiology and end results database," *Journal of Clinical Oncology*, vol. 23, no. 21, pp. 4735–4741, 2005.
- [3] S. Hu, F. Ma, F. Collado-Mesa, and R. S. Kirsner, "UV radiation, latitude, and melanoma in US Hispanics and blacks," *Archives of Dermatology*, vol. 140, no. 7, pp. 819–824, 2004.
- [4] R. G. Zepp, D. J. Erickson III, N. D. Paul, and B. Sulzberger, "Interactive effects of solar UV radiation and climate change on biogeochemical cycling," *Photochemical and Photobiological Sciences*, vol. 6, no. 3, pp. 286–300, 2007.
- [5] T. Douki, A. Reynaud-Angelin, J. Cadet, and E. Sage, "Bipyrimidine photoproducts rather than oxidative lesions are the main type of DNA damage involved in the genotoxic effect of solar UVA radiation," *Biochemistry*, vol. 42, no. 30, pp. 9221–9226, 2003.
- [6] S. Mouret, C. Baudouin, M. Charveron, A. Favier, J. Cadet, and T. Douki, "Cyclobutane pyrimidine dimers are predominant DNA lesions in whole human skin exposed to UVA radiation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 37, pp. 13765–13770, 2006.
- [7] J. Cadet, T. Douki, J.-L. Ravanat, and P. Di Mascio, "Sensitized formation of oxidatively generated damage to cellular DNA by UVA radiation," *Photochemical and Photobiological Sciences*, vol. 8, no. 7, pp. 903–911, 2009.
- [8] A. Besaratinia, S. E. Bates, T. W. Synold, and G. P. Pfeifer, "Similar mutagenicity of photoactivated porphyrins and ultraviolet A radiation in mouse embryonic fibroblasts: involvement of oxidative DNA lesions in mutagenesis," *Biochemistry*, vol. 43, no. 49, pp. 15557–15566, 2004.
- [9] H.-C. DeFedericis, H. B. Patrzyc, M. J. Rajecki et al., "Singlet oxygen-induced DNA damage," *Radiation Research*, vol. 165, no. 4, pp. 445–451, 2006.
- [10] G. P. Pfeifer, Y.-H. You, and A. Besaratinia, "Mutations induced by ultraviolet light," *Mutation Research*, vol. 571, no. 1–2, pp. 19–31, 2005.
- [11] A. J. Miller and M. C. Mihm Jr., "Melanoma," *The New England Journal of Medicine*, vol. 355, no. 1, pp. 51–65, 2006.
- [12] M. R. Zaidi, C.-P. Day, and G. Merlino, "From UVs to metastases: modeling melanoma initiation and progression in the mouse," *The Journal of Investigative Dermatology*, vol. 128, no. 10, pp. 2381–2391, 2008.
- [13] L. A. Liotta, "Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award lecture," *Cancer Research*, vol. 46, no. 1, pp. 1–7, 1986.
- [14] W. Wang, S. Goswami, K. Lapidus et al., "Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors," *Cancer Research*, vol. 64, no. 23, pp. 8585–8594, 2004.
- [15] W. Wang, J. B. Wyckoff, S. Goswami et al., "Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors," *Cancer Research*, vol. 67, no. 8, pp. 3505–3511, 2007.
- [16] J. J. Bravo-Cordero, L. Hodgson, and J. Condeelis, "Directed cell invasion and migration during metastasis," *Current Opinion in Cell Biology*, vol. 24, no. 2, pp. 277–283, 2012.
- [17] R. Karlsson, E. D. Pedersen, Z. Wang, and C. Brakebusch, "Rho GTPase function in tumorigenesis," *Biochimica et Biophysica Acta*, vol. 1796, no. 2, pp. 91–98, 2009.
- [18] G. Fritz, B. Kaina, and K. Aktories, "The Ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments," *The Journal of Biological Chemistry*, vol. 270, no. 42, pp. 25172–25177, 1995.
- [19] S. A. Boswell, P. P. Ongusaha, P. Nghiem, and S. W. Lee, "The protective role of a small GTPase RhoE against UVB-induced DNA damage in keratinocytes," *The Journal of Biological Chemistry*, vol. 282, no. 7, pp. 4850–4858, 2007.
- [20] Q. Jian, Q. An, D. Zhu et al., "MicroRNA 340 is involved in UVB-induced dendrite formation through the regulation of RhoA expression in melanocytes," *Molecular and Cellular Biology*, vol. 34, no. 18, pp. 3407–3420, 2014.
- [21] T. Frisan, X. Cortes-Bratti, E. Chaves-Olarte, B. Stenerlöw, and M. Thelestam, "The *Haemophilus ducreyi* cytolethal distending toxin induces DNA double-strand breaks and promotes ATM-dependent activation of RhoA," *Cellular Microbiology*, vol. 5, no. 10, pp. 695–707, 2003.
- [22] L. Guerra, R. Guidi, I. Slot et al., "Bacterial genotoxin triggers FEN1-dependent RhoA activation, cytoskeleton remodeling and cell survival," *Journal of Cell Science*, vol. 124, no. 16, pp. 2735–2742, 2011.
- [23] S. Pavey, L. Spoerri, N. K. Haass, and B. Gabrielli, "DNA repair and cell cycle checkpoint defects as drivers and therapeutic targets in melanoma," *Pigment Cell and Melanoma Research*, vol. 26, no. 6, pp. 805–816, 2013.
- [24] V. Sanz-Moreno and C. J. Marshall, "Rho-GTPase signaling drives melanoma cell plasticity," *Cell Cycle*, vol. 8, no. 10, pp. 1484–1487, 2009.
- [25] F. L. Forti and H. A. Armelin, "Vasopressin triggers senescence in K-ras transformed cells via RhoA-dependent downregulation of cyclin D1," *Endocrine-Related Cancer*, vol. 14, no. 4, pp. 1117–1125, 2007.
- [26] X.-D. Ren, W. B. Kiosses, and M. A. Schwartz, "Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton," *The EMBO Journal*, vol. 18, no. 3, pp. 578–585, 1999.

- [27] C.-C. Liang, A. Y. Park, and J.-L. Guan, "In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro," *Nature Protocols*, vol. 2, no. 2, pp. 329–333, 2007.
- [28] N. P. Singh, M. T. McCoy, R. R. Tice, and E. L. Schneider, "A simple technique for quantitation of low levels of DNA damage in individual cells," *Experimental Cell Research*, vol. 175, no. 1, pp. 184–191, 1988.
- [29] T. E. Carey, T. Takahashi, L. A. Resnick, H. F. Oettgen, and L. J. Old, "Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 73, no. 9, pp. 3278–3282, 1976.
- [30] J. Fogh, J. M. Fogh, and T. Orfeo, "One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice," *Journal of the National Cancer Institute*, vol. 59, no. 1, pp. 221–226, 1977.
- [31] J. Fogh, W. C. Wright, and J. D. Loveless, "Absence of HeLa cell contamination in 169 cell lines derived from human tumors," *Journal of the National Cancer Institute*, vol. 58, no. 2, pp. 209–214, 1977.
- [32] W. C. Wright, W. P. Daniels, and J. Fogh, "Distinction of seventy-one cultured human tumor cell lines by polymorphic enzyme analysis," *Journal of the National Cancer Institute*, vol. 66, no. 2, pp. 239–247, 1981.
- [33] É. T. Costa, F. L. Forti, T. G. F. Matos et al., "Fibroblast growth factor 2 restrains Ras-driven proliferation of malignant cells by triggering RhoA-mediated senescence," *Cancer Research*, vol. 68, no. 15, pp. 6215–6223, 2008.
- [34] M. van Engeland, L. J. W. Nieland, F. C. S. Ramaekers, B. Schutte, and C. P. M. Reutelingsperger, "Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure," *Cytometry*, vol. 31, no. 1, pp. 1–9, 1998.
- [35] G. Kroemer, L. Galluzzi, P. Vandenabeele et al., "Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009," *Cell Death and Differentiation*, vol. 16, no. 1, pp. 3–11, 2009.
- [36] A. B. Jaffe and A. Hall, "Rho GTPases: biochemistry and biology," *Annual Review of Cell and Developmental Biology*, vol. 21, pp. 247–269, 2005.
- [37] J. Heo, K. W. Raines, V. Mocanu, and S. L. Campbell, "Redox regulation of RhoA," *Biochemistry*, vol. 45, no. 48, pp. 14481–14489, 2006.
- [38] K. W. Raines, M. G. Bonini, and S. L. Campbell, "Nitric oxide cell signaling: S-nitrosation of Ras superfamily GTPases," *Cardiovascular Research*, vol. 75, no. 2, pp. 229–239, 2007.
- [39] A. Aghajanian, E. S. Wittchen, S. L. Campbell, and K. BurrIDGE, "Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif," *PLoS ONE*, vol. 4, no. 11, Article ID e8045, 2009.
- [40] J. Luo, K. Hosoki, A. Bacsı et al., "8-Oxoguanine DNA glycosylase-1-mediated DNA repair is associated with Rho GTPase activation and alpha-smooth muscle actin polymerization," *Free Radical Biology and Medicine*, vol. 73, pp. 430–438, 2014.
- [41] C. Jhappan, F. P. Noonan, and G. Merlino, "Ultraviolet radiation and cutaneous malignant melanoma," *Oncogene*, vol. 22, no. 20, pp. 3099–3112, 2003.
- [42] W. K. Kaufmann, "The human intra-S checkpoint response to UVC-induced DNA damage," *Carcinogenesis*, vol. 31, no. 5, pp. 751–765, 2010.
- [43] S. Miwa, S. Yano, Y. Hiroshima et al., "Imaging UVC-induced DNA damage response in models of minimal cancer," *Journal of Cellular Biochemistry*, vol. 114, no. 11, pp. 2493–2499, 2013.
- [44] K. Kaibuchi, S. Kuroda, and M. Amano, "Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells," *Annual Review of Biochemistry*, vol. 68, pp. 459–486, 1999.
- [45] E. Sahai, M. F. Olson, and C. J. Marshall, "Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility," *The EMBO Journal*, vol. 20, no. 4, pp. 755–766, 2001.
- [46] A. Besaratinia, J.-I. Yoon, C. Schroeder, S. E. Bradforth, M. Cockburn, and G. P. Pfeifer, "Wavelength dependence of ultraviolet radiation-induced DNA damage as determined by laser irradiation suggests that cyclobutane pyrimidine dimers are the principal DNA lesions produced by terrestrial sunlight," *The FASEB Journal*, vol. 25, no. 9, pp. 3079–3091, 2011.
- [47] R. P. Rastogi, Richa, A. Kumar, M. B. Tyagi, and R. P. Sinha, "Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair," *Journal of Nucleic Acids*, vol. 2010, Article ID 592980, 32 pages, 2010.
- [48] A. Brozyna, B. Zbytek, J. Granese, J. A. Carlson, J. Ross, and A. Slominski, "Mechanism of UV-related carcinogenesis and its contribution to nevi/melanoma," *Expert Review of Dermatology*, vol. 2, no. 4, pp. 451–469, 2007.
- [49] J. H. Osaki, G. Espinha, Y. T. Magalhaes, and F. L. Forti, "Modulation of RhoA GTPase activity sensitizes human cervix carcinoma cells to γ -radiation by attenuating DNA repair pathways," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 6012642, 11 pages, 2016.
- [50] G. Espinha, J. H. Osaki, Y. T. Magalhaes, and F. L. Forti, "Rac1 GTPase-deficient HeLa cells present reduced DNA repair, proliferation, and survival under UV or gamma irradiation," *Molecular and Cellular Biochemistry*, vol. 404, no. 1, pp. 281–297, 2015.

Research Article

Activation of Antioxidative Functions by Radon Inhalation Enhances the Mitigation Effects of Pregabalin on Chronic Constriction Injury-Induced Neuropathic Pain in Mice

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Radon inhalation brings pain relief for chronic constriction injury- (CCI-) induced neuropathic pain in mice due to the activation of antioxidative functions, which is different from the mechanism of the pregabalin effect. In this study, we assessed whether a combination of radon inhalation and pregabalin administration is more effective against neuropathic pain than radon or pregabalin only. Mice were treated with inhaled radon at a concentration of 1,000 Bq/m³ for 24 hours and pregabalin administration after CCI surgery. In mice treated with pregabalin at a dose of 3 mg/kg weight, the 50% paw withdrawal threshold of mice treated with pregabalin or radon and pregabalin was significantly increased, suggesting pain relief. The therapeutic effects of radon inhalation or the combined effects of radon and pregabalin (3 mg/kg weight) were almost equivalent to treatment with pregabalin at a dose of 1.4 mg/kg weight or 4.1 mg/kg weight, respectively. Radon inhalation and the combination of radon and pregabalin increased antioxidant associated substances in the paw. The antioxidant substances increased much more in radon inhalation than in pregabalin administration. These findings suggested that the activation of antioxidative functions by radon inhalation enhances the pain relief of pregabalin and that this combined effect is probably an additive effect.

1. Introduction

Radon therapy is performed for mainly pain-related diseases using radon hot springs in Japan [1] and Europe [2] and using mines in Europe [2]. In both cases, patients inhale radon through their nose because radon is a radioactive gas. The conditions of radon therapy in the Misasa Medical Center in Japan and the hospitals in Europe are slightly different [3]. The radon concentration in the treatment room of the Misasa Medical Center is approximately 2,000 Bq/m³ [1], while the concentration in Europe is twenty-five times higher [2]. However, the beneficial effects of radon therapy show that there is not much difference between Misasa and Europe in terms of alleviation of pain-related diseases. A report suggested that radon and thermal therapy using hot springs alleviated osteoarthritis [1]. One of the possible reasons for the effects is the prevention of peroxidation reactions and

immune depression. Another possible reason is an increase in tissue perfusion because vasoactive-associated substance decreased and pain-associated substance increased by radon therapy. Another report suggested that not only radon and thermal therapy, but also thermal therapy alleviated ankylosing spondylitis [4]. However, radon and thermal therapy continue the alleviation effect longer than thermal therapy. Radon therapy is also effective against rheumatic diseases [5]. In addition, radon therapy reduced the dosage of medicine [6]. These findings indicated that radon therapy has a positive effect on pain-related diseases such as osteoarthritis, ankylosing spondylitis, and rheumatic diseases. Although it is likely that radon therapy has a positive effect against pain-related diseases, it is insufficient to understand the mechanisms of radon therapy. To investigate the mechanisms, we previously reported that radon inhalation has preventive and curative effects against chronic constriction injury (CCI) in mice

due to the reduction of reactive oxygen species (ROS) by the activation of antioxidative functions following radon inhalation [7].

Pregabalin is a medicine that relieves pain. Toth reviewed the clinical implications for the management of neuropathic pain [8]. Clinical studies suggested that administration of pregabalin is effective against diabetic peripheral neuropathy. But the doses of up to 150 mg/day are inefficacious [9]. Several adverse central nervous system effects, such as dizziness and somnolence, and adverse systemic effects, such as peripheral edema, were observed [8]. The incidence of these adverse effects increases with larger pregabalin doses [8, 10].

It is well known that radon inhalation increases lung cancer risk [11]. Therefore, a combination of pregabalin and radon treatment has beneficial effects to reduce the adverse effects of pregabalin and the lung cancer risk caused by radon inhalation. The purpose of this study was to compare the mitigating effects on CCI of radon inhalation and pregabalin administration and to examine the combination effects of radon and pregabalin on CCI-induced neuropathic pain. We assayed the behavioural response to evaluate the pain and the following biochemical parameters to assess the effects of radon and pregabalin treatment: SOD activity, catalase activity, total glutathione content (t-GSH), and lipid peroxide level in paw.

2. Materials and Methods

2.1. Animals. Male ICR mice (age, 8 weeks; body weight, approximately 33–40 g) were obtained from Charles River (Yokohama, Japan). Ethical approval for all protocols and experiments was obtained from the Animal Experimentation Committee of Okayama University. The mice were housed under a 12:12 h artificial light cycle (8:00 a.m. to 8:00 p.m.) at a temperature of $22 \pm 2^\circ\text{C}$.

2.2. Behavioral Testing: von Frey Test. The behavioral response of mice to mechanical stimuli was assessed using the von Frey test [12]. von Frey tests were conducted once a day before (2 or 3 days) and after (2 or 3 days) CCI surgery and at 30, 60, 90, and 120 min after pregabalin administration, 24-hour radon inhalation, or a combination of radon inhalation and pregabalin administration. In the group treated with radon and pregabalin, mice were administered pregabalin immediately after radon inhalation. Mice were individually placed in plastic cages with a wire-mesh floor (1 mm diameter wire placed 5 mm apart). The paw was touched with 1 of a series of 9 von Frey hairs (0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, and 4 g) (North Coast Medical Inc., CA, USA). A positive response was noted when the paw was sharply withdrawn. The 50% withdrawal threshold was determined using the up-down method [12]. Briefly, the behavioral test was initiated with the 0.6 g von Frey hair, representing the middle of the series. A stronger stimulus was chosen if the paw showed a negative response. A weaker stimulus was chosen if the paw showed a withdrawal response. The critical 6 data points were noted after the response threshold was first crossed.

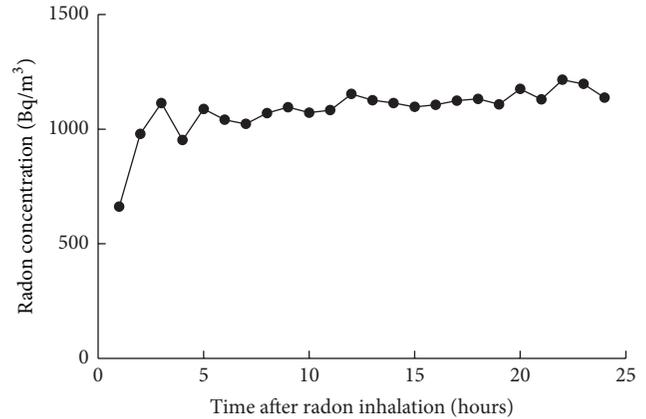


FIGURE 1: Changes in radon concentration in the mouse cage.

50% g threshold = $10^{X_f + k\delta}$, where X_f is log units value of the final von Frey hair used; k is tabular value for the pattern of positive or negative responses [13]; and δ is mean difference (in log units) between stimuli.

2.3. CCI Surgery. Mice received a unilateral CCI after a pretest for mechanical sensitivity using a von Frey test. Briefly, the right sciatic nerve was exposed at the midhigh level and was then constricted loosely with three ligations using 4-0 chromic gut, each spaced about 1 mm apart under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The same surgical operation was performed, without CCI, on a group of animals that served as sham-operated controls.

2.4. Radon Inhalation. To generate conditions for inhalation of specified radon concentrations, our radon exposure original system was used as described in a previous report [14]. The radon concentration in the mouse cage was measured using a radon monitor (CMR-510, femto-TECH Inc., Ohio, USA). The mean concentration of radon was approximately $1,000 \text{ Bq/m}^3$ (Figure 1). The mice inhaled radon at a concentration of $1,000 \text{ Bq/m}^3$ for 24 hours after CCI while having free access to food and water during radon inhalation.

2.5. Pregabalin Treatment. Pregabalin (1, 3, or 10 mg/kg body weight; Sigma-Aldrich Japan Co. LLC., Tokyo, Japan) was injected into the peritoneum of the mice after the CCI operation. To examine the combination effects of radon and pregabalin, mice were administered pregabalin at a dose of 3 mg/kg of body weight immediately after radon inhalation.

2.6. Biochemical Assays. Paws were homogenized on ice in 10 mM phosphate buffer (PBS; pH 7.4). The homogenates were used for the assays of SOD and catalase.

SOD activity was assayed by the nitroblue tetrazolium (NBT) reduction method using the Wako-SOD test (Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan) [15]. Briefly, the homogenates were centrifuged at $12,000 \times g$ for 45 min at 4°C and the supernatants were used to assay SOD activity. SOD activity in the paw was measured by the extent of

inhibition of the reduction in NBT measured at 560 nm using a spectrophotometer. One unit of enzyme activity was defined as 50% inhibition of NBT reduction.

Catalase activity was measured as the hydrogen peroxide (H_2O_2) reduction rate at 37°C and was assayed at 240 nm using a spectrophotometer [16]. The assay mixture consisted of 50 μL of 1 M Tris-HCl buffer containing 5 mM ethylenediaminetetraacetic acid (pH 7.4), 900 μL of 10 mM H_2O_2 , 30 μL deionized water, and 20 μL paw supernatant. Activity was calculated using a molar extinction coefficient of $7.1 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$. The changes of absorbance were observed for a minute.

t-GSH content was measured using the Bioxytech GSH-420 assay kit (OXIS Health Products, Inc., Portland, OR, USA). Briefly, tissue samples from the paw were homogenized in 10 mM PBS (pH 7.4) and then mixed with ice-cold 7.5% trichloroacetic acid solution. The homogenates were centrifuged at $3,000 \times g$ for 10 min. Assays were performed on tissue supernatants. This assay is based on the formation of a chromophoric thione, the absorbance of which can be measured at 420 nm and is directly proportional to the t-GSH concentration.

Lipid peroxide levels were assayed using the Bioxytech LPO-586 assay kit (OXIS Health Products, Inc.). Briefly, the paw samples were homogenized in 10 mM phosphate buffer (PBS; pH 7.4) on ice. Prior to homogenization, 10 μL of 0.5 M butylated hydroxytoluene in acetonitrile was added per 1 mL of the buffer-tissue mixture. After homogenization, the homogenate was centrifuged at $15,000 \times g$, for 10 min at 4°C, and the supernatant was used for the assay. The lipid peroxide level assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with malondialdehyde and 4-hydroxyalkenals at 45°C. The optical density of the colored products was read at 586 nm in a spectrophotometer.

The protein content in each sample was measured by the Bradford method, using the Protein Quantification Kit-Rapid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) [17].

2.7. Statistical Analyses. The data are presented as the mean \pm standard error of the mean (SEM). Each experimental group consisted of samples from 5-6 animals. Statistically significant differences were determined using an unpaired *t*-test for comparisons between two groups and Tukey's tests for multiple comparisons where appropriate. *P* values were considered significant at $P < 0.05$.

3. Results

3.1. Effect of Sham-Operation, Radon Inhalation, and Pregabalin Administration on 50% Paw Withdrawal Threshold following CCI Surgery. We first confirmed whether the sham-operation decreased the 50% paw withdrawal threshold or not. Results showed that the sham-operation did not decrease the 50% paw withdrawal threshold whereas CCI surgery significantly decreased it (Figure 2(a)).

Next, we examined dose- and time-dependent changes in the 50% paw withdrawal threshold following pregabalin

administration. Results showed that the peak of the mitigative effects was at around one hour after pregabalin administration. In addition, mechanical allodynia was mitigated in a dose-dependent manner at 60 minutes after pregabalin administration (Figure 2(b)).

Then, we compared the mitigative effects of radon inhalation and pregabalin administration at one hour after treatment of radon or pregabalin because the peak of mitigative effects is at around one hour after pregabalin administration. As a result, radon inhalation has a mitigating effect against mechanical allodynia similar to the effects of approximately 1.4 mg/kg of body weight of pregabalin (Figure 2(c)).

3.2. Effect of Pregabalin Administration on Antioxidative Functions in Paw. To clarify the changes in the antioxidant associated substances after pregabalin administration, SOD, catalase, t-GSH, and lipid peroxide level were assayed.

Although no significant changes were observed in the activities of SOD and catalase, pregabalin administration increased t-GSH content in paw. The lipid peroxide level in the paw of mice, which were administered pregabalin at a dose of 10 mg/kg of body weight, significantly decreased (Figure 3).

3.3. Combined Effects of Radon and Pregabalin on CCI-Induced Neuropathic Pain. To clarify the combined effects of radon and pregabalin on neuropathic pain, von Frey tests were conducted.

The fifty percent paw withdrawal threshold was significantly decreased by CCI surgery. The 50% paw withdrawal threshold of mice treated with pregabalin (60 min) or radon and pregabalin (30 min, 60 min) was significantly increased. Radon inhalation also increased the 50% paw withdrawal threshold, but this difference was not significant (Figure 4).

From the formula in Figure 2(c), we estimated the combined effects of radon and pregabalin (3 mg/kg of body weight) on CCI-induced neuropathic pain. As a result, the combination of radon and pregabalin has a mitigative effect against mechanical allodynia similar to the effects of approximately 4.1 mg/kg of body weight of pregabalin. Therefore, this combined effect is probably an additive effect because of the mitigating effect of radon similar to the effects of approximately 1.4 mg/kg of body weight of pregabalin.

3.4. Effect of Sham-Operation, Radon Inhalation, and Pregabalin Treatment on Antioxidative Functions in Paw. To clarify the involvement of the antioxidant effects, antioxidant associated substances, such as SOD, catalase, t-GSH, and lipid peroxide level were assayed.

SOD activity in paw of mice that received CCI surgery significantly decreased. The SOD activity of mice administered pregabalin following CCI surgery was at the same level as that of mice that had received CCI surgery. However, the SOD activities of radon or radon and pregabalin treated mice were at the same level as that of sham-operated mice (Figure 5).

Catalase activity in the paw of mice that received CCI surgery decreased, but this difference was not significant. The catalase activity of mice administered pregabalin following

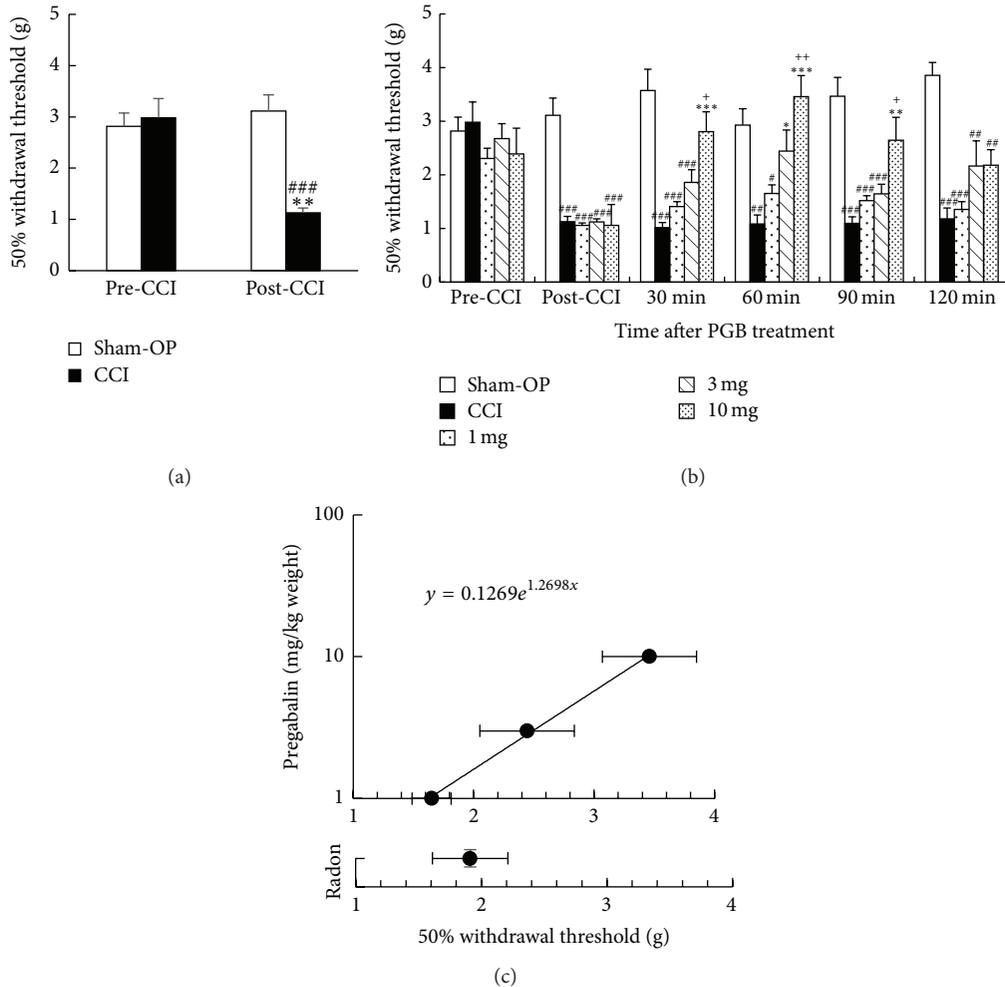


FIGURE 2: Changes in 50% paw withdrawal threshold following pregabalin (PGB) administration. (a) Changes in sham-operation (Sham-OP) or CCI surgery on 50% paw withdrawal threshold. ** $P < 0.01$ versus pre-CCI (CCI), *** $P < 0.001$ versus post-CCI (Sham-OP). (b) Time- and dose-dependent changes of 50% paw withdrawal threshold. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus each CCI, # $P < 0.01$, ## $P < 0.01$, and ### $P < 0.01$ versus each sham-OP, and + $P < 0.05$, ++ $P < 0.01$ versus each 1 mg. (c) Effectiveness comparison between radon and pregabalin. Values are presented as the mean \pm SEM of data from 5-6 animals.

CCI surgery was at the same level as that of mice that had received CCI surgery. However, the catalase activities of radon inhaled mice were significantly increased compared with CCI surgery received mice. In addition, the catalase activity of radon and pregabalin treated mice was at the same level as that of radon treated mice (Figure 5).

The t-GSH content in the paw of mice that had received CCI surgery significantly decreased. The t-GSH contents of mice treated with pregabalin, radon, or radon and pregabalin following CCI surgery were significantly increased compared with that of CCI surgery received mice (Figure 5).

The lipid peroxide level in the paw of mice that had received CCI surgery increased, but this difference was not significant. The lipid peroxide levels of mice treated with pregabalin, radon, or radon and pregabalin following CCI surgery were lower than that of CCI surgery received mice (Figure 5).

4. Discussion

The possible mechanisms of action for pregabalin are not completely understood [8]. It has been reported that pregabalin binds with high affinity to the calcium channel $\alpha_2\text{-}\delta$ site [8]. Reducing the stimulated synaptic influx of calcium reduces the stimulated release of transmitters such as glutamate, noradrenaline, GABA, and acetylcholine at the neuromuscular junction and spinal inhibitory glycine [18]. To our knowledge, there are no reports that low-dose irradiation including radon inhalation has effects similar to pregabalin as described above or that pregabalin has antioxidative effects. Therefore, we assumed that the combination of radon inhalation and pregabalin administration has additive or synergetic effects on CCI-induced neuropathic pain. In this study, we focused on the antioxidative functions.

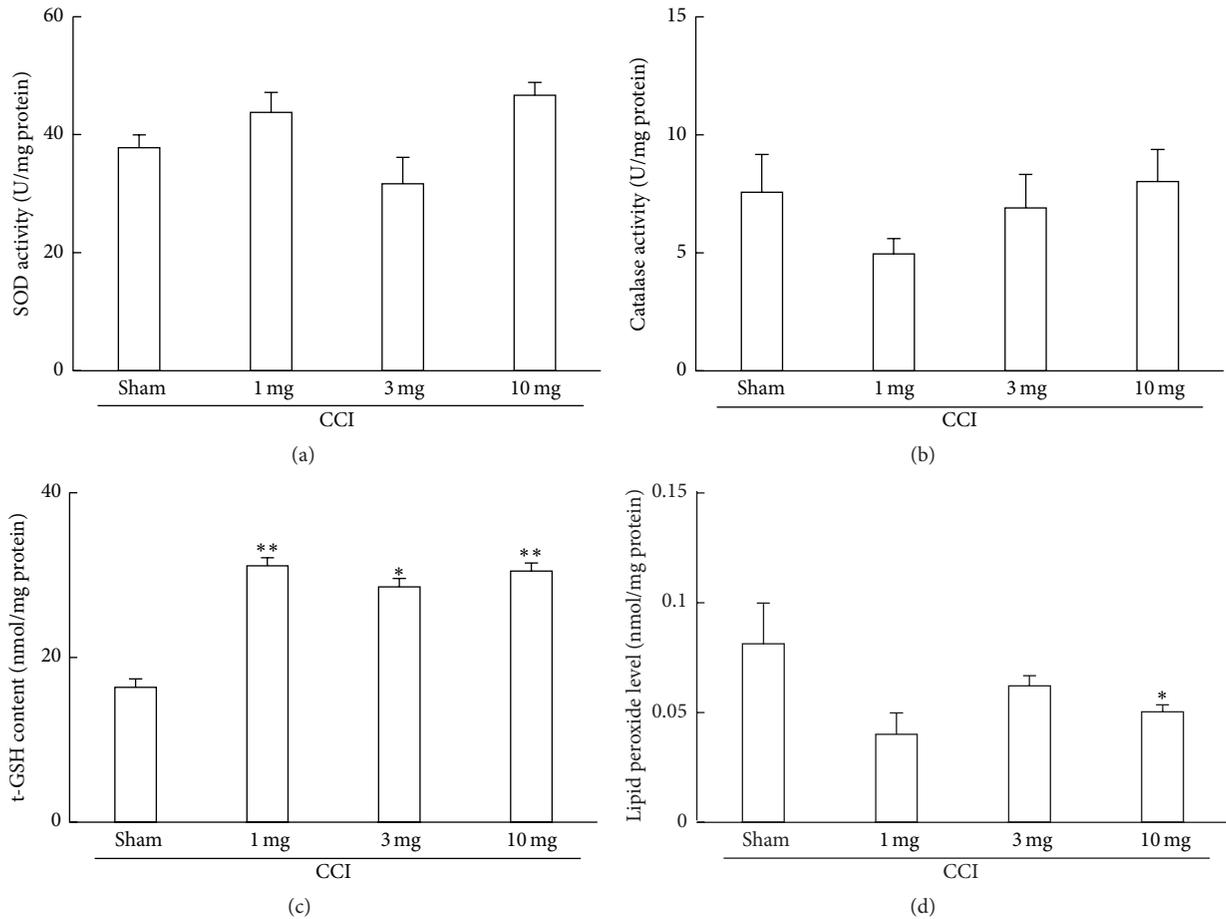


FIGURE 3: Changes in antioxidant-associated parameters in the paw following CCI surgery and pregabalin administration. Sham; no pregabalin administration. Values are presented as the mean \pm SEM of data from 5-6 animals. * $P < 0.05$, ** $P < 0.01$ versus sham (CCI only; no administration of pregabalin).

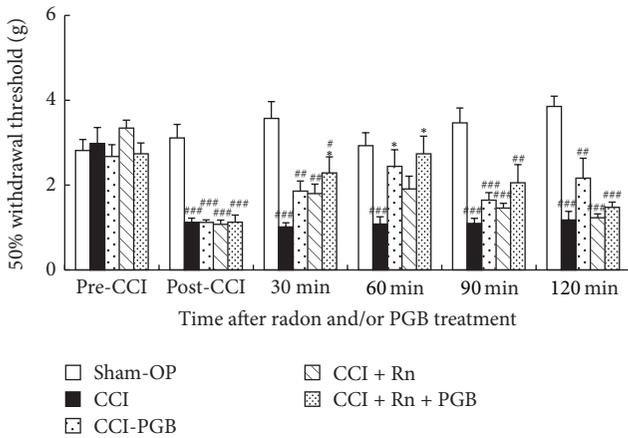


FIGURE 4: Effectiveness comparison among radon (Rn), pregabalin, and combination of radon and pregabalin. Sham-OP: sham-operation, PGB—3 mg/kg weight, Rn—1,000 Bq/m³, and Rn + PGB—1,000 Bq/m³ + 3 mg/kg weight. Values are presented as the mean \pm SEM of data from 6 animals. * $P < 0.05$ versus each CCI, ## $P < 0.01$, and ### $P < 0.01$ versus each sham-OP.

Low-dose X- or γ -irradiation has a stimulatory effect on animals, and the stimulation induced the activation of antioxidative functions [19, 20] and immune functions [21, 22]. These activations contribute to the inhibition of oxidative stress induced damages. For example, continuous low-dose-rate γ -irradiation ameliorates diabetic nephropathy in mice through the activation of antioxidative functions in the kidney [23]. Another report suggested that low-dose γ -ray irradiation attenuates collagen-induced arthritis by suppressing proinflammatory cytokines and autoantibody production and by inducing regulatory T cells [24]. Although radon is a gas and emits α -ray, similar effects were observed. Radon inhalation increases SOD activity in many organs of mice [25] and inhibits some kinds of oxidative damage [3]. However, the estimated absorbed doses of these organs by radon inhalation are much smaller than those by X- or γ -irradiation [26]. To clarify the radon effects, future research is required because there are no data to explain why radon inhalation increases antioxidative functions.

Oxidative stress is involved in the neuropathic pain conditions. For example, N-acetyl-L-cysteine (NAC), which

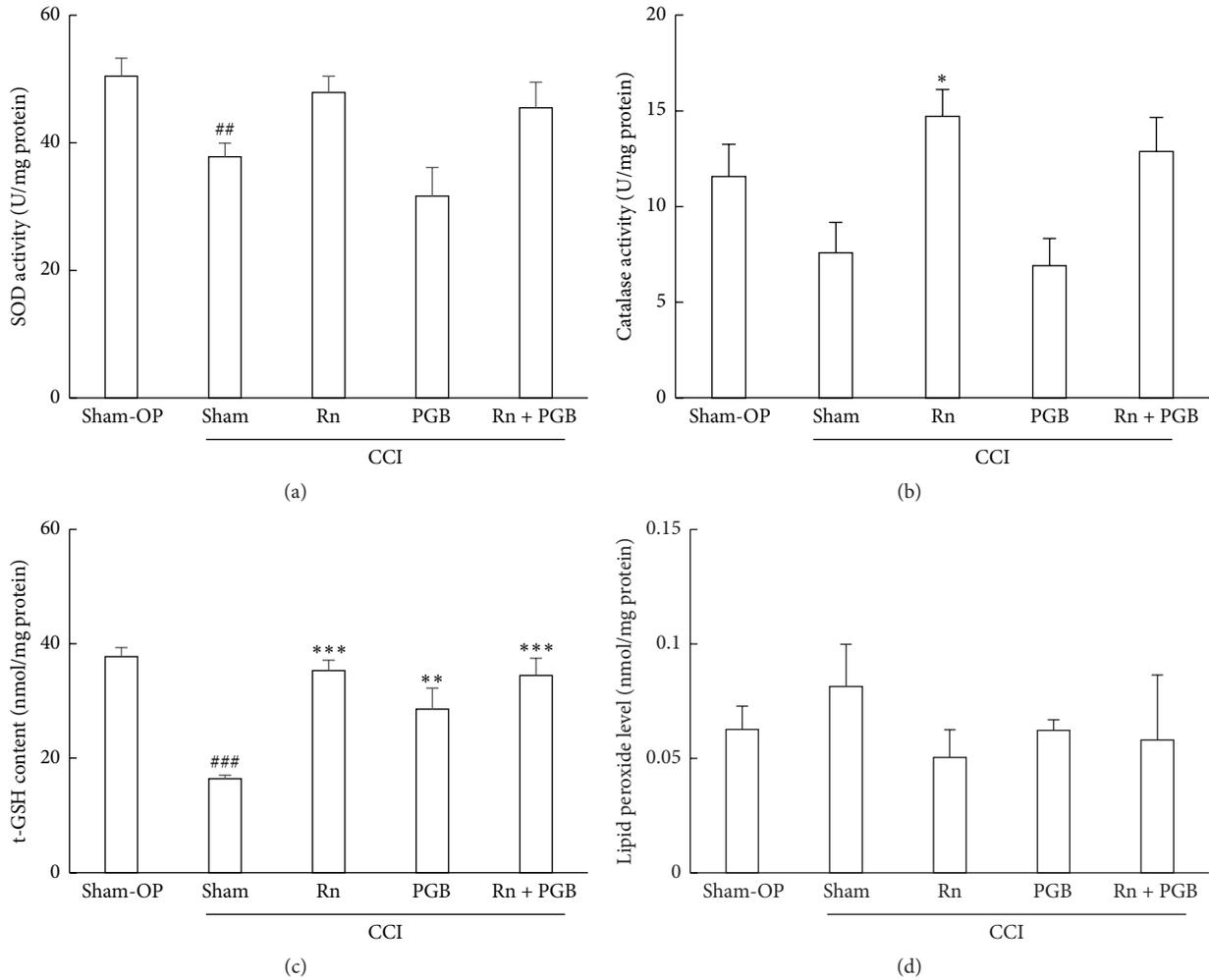


FIGURE 5: Changes in antioxidant-associated parameters in the paw. Values are presented as the mean \pm SEM of data from 6 animals. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus sham (CCI only; no treatment with either radon or pregabalin, pregabalin; 3 mg/kg weight); # $P < 0.01$, ## $P < 0.01$, ### $P < 0.001$ versus sham-OP.

acts as a cysteine donor, resulted in significant reduction of hyperalgesia in CCI-induced neuropathic pain in rats. This report also suggested that glutathione plays an important role in the inhibition of neuropathic pain because of its capacity to donate cysteine amino acid, a component of glutathione [27]. We previously reported that radon inhalation brings pain relief for CCI-induced neuropathic pain in mice due to the activation of antioxidative functions [7]. This activation is involved in the decrease in the inflammatory leukocytes migration in the paw, indicating the anti-inflammatory effects of radon inhalation. These findings suggest that inhibition of overproduction of ROS plays an important role in the mitigation of CCI-induced neuropathic pain. In this study, antioxidant associated substances were increased by radon inhalation and 50% paw withdrawal thresholds were increased. Interestingly, antioxidant associated substances of mice treated with a combination of radon and pregabalin were increased. Although the t-GSH of mice treated with pregabalin increased, the activities of SOD and catalase did not increase. These findings suggested that antioxidative

functions activate much more in radon inhalation than pregabalin administration and that the mitigation mechanisms of radon and pregabalin are different.

Our previous report suggested that radon inhalation at a concentration of $2,000 \text{ Bq/m}^3$ is more effective in CCI-induced neuropathic pain in mice than that of $1,000 \text{ Bq/m}^3$ [7]. In this study, we made a choice of a lower radon concentration because lower radon concentration can reduce the absorbed dose. The absorbed dose from radon inhalation should be reduced to reduce lung cancer risk. In this study, the combination of radon and pregabalin enhanced the mitigative effect against mechanical allodynia. These findings indicate the usefulness of the combination of radon and pregabalin.

In conclusion, radon inhalation at a concentration of $1,000 \text{ Bq/m}^3$ for 24 hours has a mitigative effect against mechanical allodynia similar to the effects of approximately 1.4 mg/kg weight of pregabalin. The combined effect of radon and pregabalin is an additive effect because the combination of radon and pregabalin has a mitigative effect against

mechanical allodynia similar to the effects of approximately 4.1 mg/kg weight of pregabalin. The possible mechanism of the additive effect is the activation of antioxidative functions induced by radon inhalation. However, the effects of radon inhalation on the nerve system have yet to be confirmed. They provide a substantial basis for future studies aimed at assessing the detailed mechanisms of the additive effects of neuropathic pain.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgments

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References

- [1] K. Yamaoka, F. Mitsunobu, K. Hanamoto, S. Mori, Y. Tanizaki, and K. Sugita, "Study on biologic effects of radon and thermal therapy on osteoarthritis," *The Journal of Pain*, vol. 5, no. 1, pp. 20–25, 2004.
- [2] A. Falkenbach, J. Kovacs, A. Franke, K. Jörgens, and K. Ammer, "Radon therapy for the treatment of rheumatic diseases—review and meta-analysis of controlled clinical trials," *Rheumatology International*, vol. 25, no. 3, pp. 205–210, 2005.
- [3] T. Kataoka, "Study of antioxidative effects and anti-inflammatory effects in mice due to low-dose X-irradiation or radon inhalation," *Journal of Radiation Research*, vol. 54, no. 4, pp. 587–596, 2013.
- [4] A. van Tubergen, R. Landewé, D. van der Heijde et al., "Combined spa-exercise therapy is effective in patients with ankylosing spondylitis: a randomized controlled trial," *Arthritis & Rheumatology*, vol. 45, no. 5, pp. 430–438, 2001.
- [5] A. Franke, L. Reiner, H. G. Pratzel, T. Franke, and K. L. Resch, "Long-term efficacy of radon spa therapy in rheumatoid arthritis—a randomised, sham-controlled study and follow-up," *Rheumatology*, vol. 39, no. 8, pp. 894–902, 2000.
- [6] A. Franke, L. Reiner, and K.-L. Resch, "Long-term benefit of radon spa therapy in the rehabilitation of rheumatoid arthritis: a randomised, double-blinded trial," *Rheumatology International*, vol. 27, no. 8, pp. 703–713, 2007.
- [7] K. Yamato, T. Kataoka, Y. Nishiyama, T. Taguchi, and K. Yamaoka, "Preventive and curative effects of radon inhalation on chronic constriction injury-induced neuropathic pain in mice," *European Journal of Pain*, vol. 17, no. 4, pp. 480–492, 2013.
- [8] C. Toth, "Pregabalin: latest safety evidence and clinical implications for the management of neuropathic pain," *Therapeutic Advances in Drug Safety*, vol. 5, no. 1, pp. 38–56, 2014.
- [9] J. Satoh, S. Yagihashi, M. Baba et al., "Efficacy and safety of pregabalin for treating neuropathic pain associated with diabetic peripheral neuropathy: a 14 week, randomized, double-blind, placebo-controlled trial," *Diabetic Medicine*, vol. 28, no. 1, pp. 109–116, 2011.
- [10] D. Semel, T. K. Murphy, G. Zlateva, R. Cheung, and B. Emir, "Evaluation of the safety and efficacy of pregabalin in older patients with neuropathic pain: results from a pooled analysis of 11 clinical studies," *BMC Family Practice*, vol. 11, article 85, 2010.
- [11] World Health Organization (WHO), *WHO Handbook on Indoor Radon*, World Health Organization, Geneva, Switzerland, 2009.
- [12] W. J. Dixon, "Efficient analysis of experimental observations," *Annual Review of Pharmacology and Toxicology*, vol. 20, pp. 441–462, 1980.
- [13] S. R. Chaplan, F. W. Bach, J. W. Pogrel, J. M. Chung, and T. L. Yaksh, "Quantitative assessment of tactile allodynia in the rat paw," *Journal of Neuroscience Methods*, vol. 53, no. 1, pp. 55–63, 1994.
- [14] T. Kataoka, J. Teraoka, A. Sakoda et al., "Protective effects of radon inhalation on carrageenan-induced inflammatory paw edema in mice," *Inflammation*, vol. 35, no. 2, pp. 713–722, 2012.
- [15] R. L. Baehner, S. K. Murrmann, J. Davis, and R. B. Johnston Jr., "The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions," *The Journal of Clinical Investigation*, vol. 56, no. 3, pp. 571–576, 1975.
- [16] H. Aebi, S. R. Wyss, B. Scherz, and J. Gross, "Properties of erythrocyte catalase from homozygotes and heterozygotes for Swiss-type acatalasemia," *Biochemical Genetics*, vol. 14, no. 9–10, pp. 791–807, 1976.
- [17] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1–2, pp. 248–254, 1976.
- [18] C. P. Taylor, "Mechanisms of analgesia by gabapentin and pregabalin—calcium channel $\alpha_2\text{-}\delta$ [$\text{Ca}_v\alpha_2\text{-}\delta$] ligands," *Pain*, vol. 142, no. 1–2, pp. 13–16, 2009.
- [19] S. Kojima, O. Matsuki, T. Nomura et al., "Induction of mRNAs for glutathione synthesis-related proteins in mouse liver by low doses of γ -rays," *Biochimica et Biophysica Acta—General Subjects*, vol. 1381, no. 3, pp. 312–318, 1998.
- [20] S. Kojima, O. Matsuki, T. Nomura et al., "Localization of glutathione and induction of glutathione synthesis-related proteins in mouse brain by low doses of γ -rays," *Brain Research*, vol. 808, no. 2, pp. 262–269, 1998.
- [21] S. Kojima, K. Nakayama, and H. Ishida, "Low dose gamma-rays activate immune functions via induction of glutathione and delay tumor growth," *Journal of Radiation Research*, vol. 45, no. 1, pp. 33–39, 2004.
- [22] Y. Kawakita, M. Ikekita, R. Kurozumi, and S. Kojima, "Increase of intracellular glutathione by low-dose gamma-ray irradiation is mediated by transcription factor AP-1 in RAW 264.7 cells," *Biological and Pharmaceutical Bulletin*, vol. 26, no. 1, pp. 19–23, 2003.
- [23] T. Nomura, X.-H. Li, H. Ogata et al., "Suppressive effects of continuous low-dose-rate γ irradiation on diabetic nephropathy in type II diabetes mellitus model mice," *Radiation Research*, vol. 176, no. 3, pp. 356–365, 2011.
- [24] H. Nakatsukasa, M. Tsukimoto, Y. Ohshima, F. Tago, A. Masada, and S. Kojima, "Suppressing effect of low-dose gamma-ray irradiation on collagen-induced arthritis," *Journal of Radiation Research*, vol. 49, no. 4, pp. 381–389, 2008.

- [25] T. Kataoka, A. Sakoda, Y. Ishimori et al., "Study of the response of superoxide dismutase in mouse organs to radon using a new large-scale facility for exposing small animals to radon," *Journal of Radiation Research*, vol. 52, no. 6, pp. 775–781, 2011.
- [26] A. Sakoda, Y. Ishimori, A. Kawabe, T. Kataoka, K. Hanamoto, and K. Yamaoka, "Physiologically based pharmacokinetic modeling of inhaled radon to calculate absorbed doses in mice, rats, and humans," *Journal of Nuclear Science and Technology*, vol. 47, no. 8, pp. 731–738, 2010.
- [27] A. K. Naik, S. K. Tandan, S. P. Dudhgaonkar et al., "Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by *N*-acetyl-L-cysteine in rats," *European Journal of Pain*, vol. 10, no. 7, pp. 573–579, 2006.

Research Article

Characteristics of Skeletal Muscle Fibers of SOD1 Knockout Mice

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Cu/Zn superoxide dismutase (SOD1) knockout (KO) mice are known as an aging model in some aspects, but the damage and regeneration process of each fiber type have not been sufficiently studied. In this study, we investigated the damage and satellite cell state of the gastrocnemius muscle in SOD1 KO mice (6 months old) using immunohistochemical staining and real-time RT-PCR. The proportion of central nuclei-containing Type IIX/b fibers in the deep and superficial portions of the gastrocnemius muscle was significantly higher in SOD1 KO than control mice. The number of satellite cells per muscle fiber decreased in all muscle fiber types in the deep portion of the gastrocnemius muscle in SOD1 KO mice. In addition, the mRNA expression levels of Pax7 and myogenin, which are expressed in satellite cells in the activation, proliferation, and differentiation states, significantly increased in the gastrocnemius muscle of SOD1 KO mice. Furthermore, mRNA of myosin heavy chain-embryonic, which is expressed in the early phase of muscle regeneration, significantly increased in SOD1 KO mice. It was suggested that muscle is damaged by reactive oxygen species produced in the mitochondrial intermembrane space in Type IIX/b fibers, accelerating the proliferation and differentiation of satellite cells through growth factors in SOD1 KO mice.

1. Introduction

Reactive oxygen species (ROS) are associated with cardiovascular and neurodegenerative diseases developing with aging [1]. ROS also inhibit the normal skeletal muscle regeneration process, being involved in age-related skeletal muscle weakness, that is, sarcopenia [2]. Generally, when skeletal muscle is damaged by stimulation, such as an overload, satellite cells, which are dormant myogenic stem cells, are activated. Activated satellite cells supply myonuclei to damaged muscle fibers through proliferation and differentiation, contributing to skeletal muscle repair. Some proliferated satellite cells do not differentiate into myonuclei, and they return to the dormant state and maintain a satellite cell pool for regeneration as a self-renewal system. Since ROS production markedly increases with aging [3], interference with the regeneration process described above by ROS may be a cause of skeletal muscle weakness.

Many tissues including skeletal muscle possess a superior system to control ROS, and superoxide dismutase (SOD) is one of the enzymes playing a control role. SOD mediates the disproportional reaction of converting oxygen, a species of ROS, to hydrogen peroxide in the body. There are isoforms of SOD: Cu-Zn SOD (SOD1) localized in the cytoplasm and mitochondrial intermembrane space and Mn SOD (SOD2) localized in mitochondria. Previous studies reported that SOD1 deficiency accelerated aging-related muscle weight reduction and the accumulation of oxidative damage in mice [4], suggesting that these were caused by superoxide increased by SOD1 deficiency and its secondary product. Thus, SOD1 KO mice are a useful model to investigate the influence of ROS and aging on the regeneration process of skeletal muscle. However, the muscle fiber regeneration process with satellite cell dynamics in SOD1 KO mice has not been sufficiently studied. In this study, SOD1 deficiency-induced structural and functional changes in muscle cells

were investigated using immunohistochemical techniques and real-time RT-PCR.

2. Materials and Methods

All procedures were approved by the Animal Welfare and Ethics Committee of the Yamaguchi University and followed the American Physiological Society's Animal Care Guidelines.

2.1. Animals and Muscle Sampling. Seven *SOD1*^{-/-} (*SOD1* KO) mice [5] and seven control (CTL; C57BL/6 strain) mice were used in this study (males, 6.2 ± 0.2 months old). *SOD1*^{+/-} b129Sv mice purchased through Jackson Laboratories (Bar Harbor, ME, USA) were backcrossed more than 8 times with C57BL/6 males and bred at our institute. All mice were maintained in a room controlled temperature and 12 h:12 h light/dark cycle and unrestricted access to food and water. All animals were anaesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally), and then left and right gastrocnemius muscles were removed. All muscle samples were frozen by liquid nitrogen and stored at -80°C until analyzed.

2.2. Immunohistochemical Analysis. Serial 10 μm cross sections of the right muscle were obtained on a cryostat (CM510; Leica, Wetzlar, Germany) at -20°C . The sections were warmed to room temperature (RT) and then preincubated in 1% normal goat serum (EMD Millipore, Billerica, MA) in 0.1M phosphate buffered saline (PBS; pH 7.6) at RT for 10 min. The primary monoclonal antibody was then applied: either (1) fast myosin (1:2000; Sigma, St. Louis, MO), which specifically reacts with the myosin heavy chain- (MHC-) IIa and IIx, or (2) SC-71 (1:1000; Developmental Studies Hybridoma Bank, Iowa City, IA), which specifically reacts with MHC-IIa. The sections were incubated in these primary antibodies overnight at RT and incubated with a secondary antibody (goat anti-mouse IgG) conjugated with horseradish peroxidase (HRP, Bio-Rad, Hercules, CA, 1:1,000) at RT for 3 hours. Diaminobenzidine tetrahydrochloride was used as a chromogen to localize HRP. Images of the stained muscle fibers were recorded with a photomicroscopic (E600; Nikon, Tokyo, Japan) image processing system (DS-U1; Nikon). The fibers were classified as Type I, IIa, or IIx/b fibers based on their immunohistochemical staining properties, and population and cross-sectional areas (CSAs) of each muscle fiber type were calculated in deep and superficial portions (Figure 1(a)).

2.3. Muscle Nuclei and Satellite Cell Identification. In another serial section, hematoxylin and eosin (HE) staining was conducted based on standard procedures. Number of nuclei and percentage of fibers with central nuclei were calculated for the 3 fiber types separately (Figures 1(b) and 1(c)).

Another serial section was fixed in 4% paraformaldehyde in 0.1M PBS at RT for 10 min. These sections were preincubated in blocking solution containing 10% normal goat serum (EMD Millipore) and 2% bovine serum albumin (BSA;

Sigma) in PBS at RT for 30 min. Each section was incubated for 1 hour at RT in the primary antibodies, a mouse anti-Pax7 (1:1000; Developmental Studies Hybridoma Bank) and a rabbit anti-laminin (1:1000; Sigma) diluted in 2% bovine serum albumin/PBS. The sections were incubated in appropriate secondary antibodies: Cy3-conjugated AffiniPure goat anti-mouse IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) for Pax7 and AlexaFluor488 goat anti-rabbit IgG (1:1000; Molecular Probes, Eugene, OR) for laminin, respectively. After incubation, the sections were stained with 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) diluted in PBS at RT for 5 min. Images for anti-Pax7, antilaminin, and DAPI were merged with an image processing software (Adobe Photoshop software CS2) and used for quantification of satellite cells. Satellite cells were identified as stained positive for both DAPI and Pax7 at the periphery of each fiber beneath the basal lamina. The numbers of satellite cells/fiber were calculated for the 3 fiber types separately (Figure 1(d)).

2.4. RNA Isolation and Real-Time RT-PCR. Total RNA was extracted from the left muscles with TRIZOL reagent (Invitrogen, Carlsbad, CA). The purity and quantity of total RNA were determined by measuring the absorbance of aliquots at 260 and 280 nm. Total RNA was then treated for 30 min at 37°C with TURBO DNase (Ambion-Life Technologies, Austin, TX) to remove genomic DNA from samples. DNase-treated RNA (0.5 μg) was used to synthesize first-strand cDNA with an Exscript RT reagent Kit (TaKaRa Bio, Otsu, Japan). Thereafter, the cDNA products were analyzed by real-time PCR using the SYBR Green PCR Master Mix protocol in a StepOne Real-Time PCR System (Applied Biosystems Japan, Japan).

The amplification program included an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 sec, and annealing/extension at 58°C for 1 min. The amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was estimated as an internal control. Each mRNA was normalized to GAPDH by subtracting the cycle threshold (Ct) value of GAPDH from the Ct value of the gene target [ΔCt (target)]. The relative expression of the target gene was calculated as the relative quantification (RQ) value for CTL value. Following the relative expression, dissociation-curve analysis detected no nonspecific amplification in cDNA samples.

The sequences of the specific primers used in this study were presented in Table 1. Each PCR primer was designed by Primer Express software (v3.0; Applied Biosystems), and the oligonucleotides were purchased from FASMAC (Kanagawa, Japan).

2.5. Statistics. All data are presented as the mean \pm SE. Data obtained from the histochemical analysis were analyzed with one-way ANOVA followed by *t*-test with Bonferroni adjustment. A Wilcoxon's signed-rank test was used to compare differences in mRNA expressions between *SOD1* KO and CTL mouse. Statistical significance was set at $P < 0.05$.

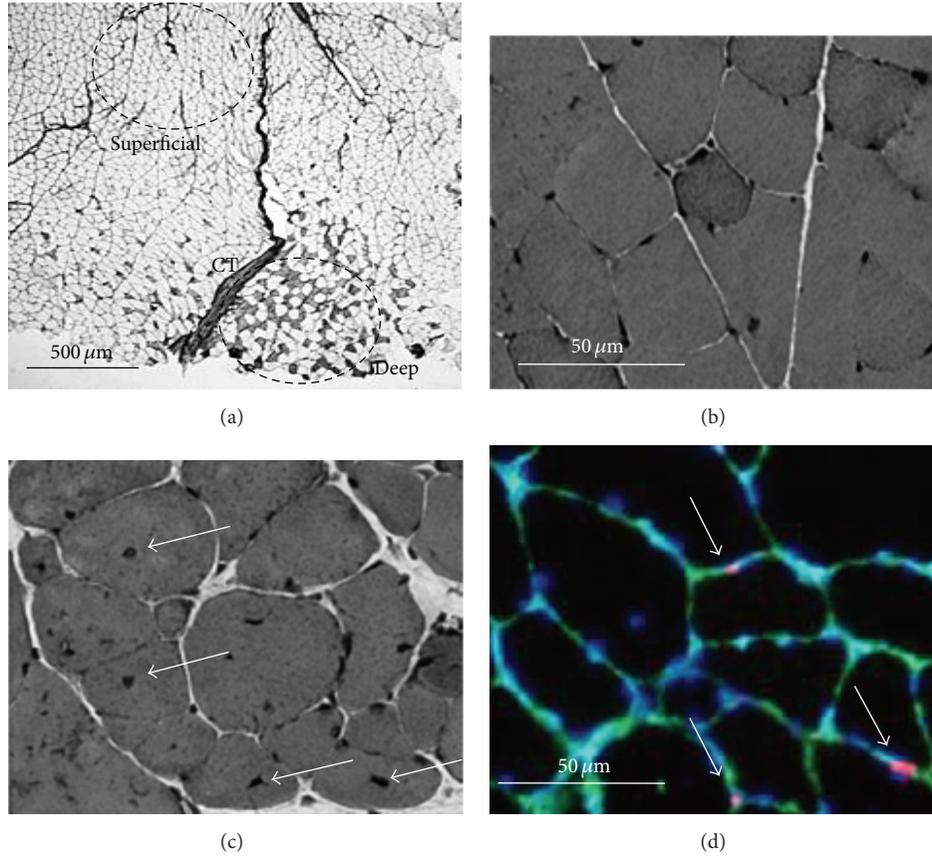


FIGURE 1: Histochemical staining sections of gastrocnemius muscle in mice. (a) Antimysin heavy chain IIa; (b) and (c) hematoxylin and eosin staining; (d) identification of satellite cells by immunohistochemical triple staining. Only Type IIx/b fibers were present in the muscle (most superficial portion in (a)), while muscle fibers in the deep portion consisted of Types I, IIa, and IIx/b (small portion deeper than connective tissue (CT)). Many fibers with central nuclei (white arrows) were found in Cu/Zn superoxide dismutase (SOD1) knockout (KO) mice (c) but not in control (CTL) mice (b). Satellite cells (white arrows) were identified as stained positive for both DAPI (blue) and Pax7 (red) at the periphery of each fiber beneath the basal lamina (green) in (d).

TABLE 1: Real-time RT-PCR primer sequences.

GAPDH	F	CATGGCCTTCCGTGTTCCCTA
	R	GCGGCACGTCAGATCCA
SOD1	F	GCCCGGCGGATGAAG
	R	CCTTTCAGCAGTCACATTGC
Myogenin	F	AGCATCACGGTGGAGGATATG
	R	CAGTTGGGCATGGTTTCGT
IL-6	F	CCACGGCCTTCCCTACTTC
	R	TTGGGAGTGGTATCCTCTGTGA
Pax7	F	AAAAAACCCCTTCCCTTCTACA
	R	AGCATGGGTAGATGGCACACT
MyoD	F	GCCGGTGTGCATTCCA
	R	CACTCCGGAACCCCAACAG
MHC-e	F	GAGCAGCTGGCGCTGAA
	R	TCTGATCCGTGCTCCAGTTTCT

SOD1: superoxide dismutase 1; IL-6: interleukin-6; Pax7: paired box 7 protein; MyoD: myogenic determination; MHC-e: myosin heavy chain-embryonic.

3. Results

3.1. Body and Muscle Weights. Although the difference was not significant, the body weight was 17% lower in SOD1 KO than CTL mice (CTL: 33.2 ± 2.4 g, SOD: 27.4 ± 2.0 %). The muscle weight also tended to decrease (CTL: 62.8 ± 4.9 mg, SOD: 55.6 ± 3.3 mg), and the relative muscle weight for the body weight was about 0.2% in both groups.

3.2. Muscle Fiber Type Population and Area. Based on the results of immunohistochemical staining, the gastrocnemius muscle was divided into the deepest and superficial portions, and 300 fibers were analyzed in each portion, setting the baseline to the parameters in connective tissue in the deep portion.

Regarding the muscle fiber type population, Type IIa fibers increased and Type IIx/b fibers decreased in the deep portion of the gastrocnemius muscle in SOD1 KO mice compared to those in CTL mice. No change was noted in Type I fiber composition. Only Type IIx/b fibers were present in

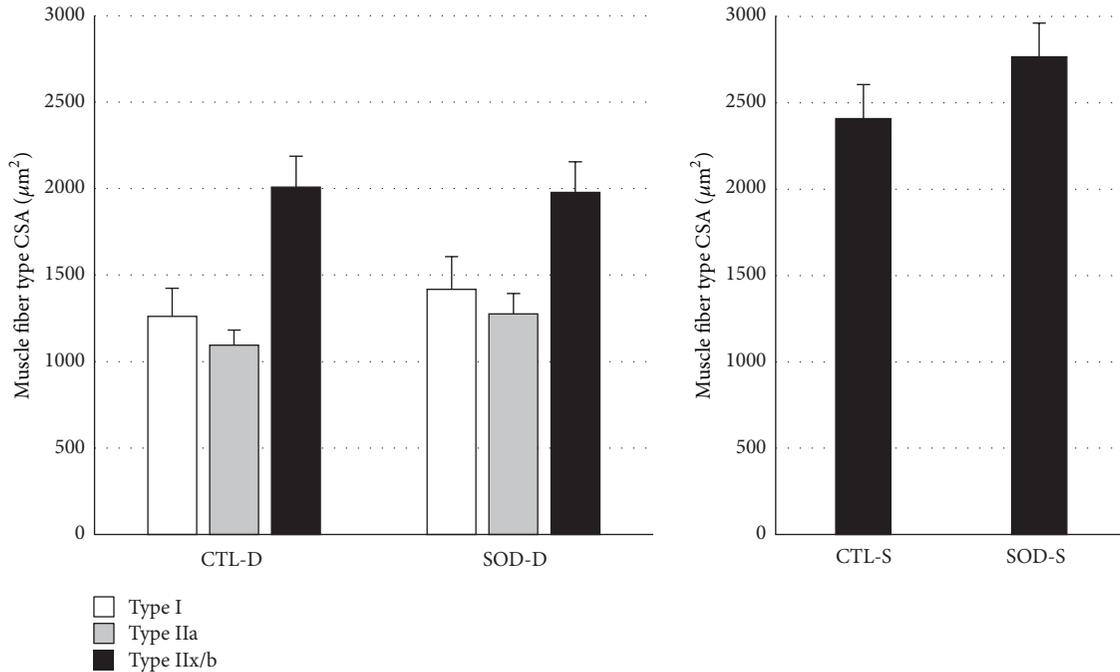


FIGURE 2: Comparison of cross-sectional area (CSA) in each fiber type between CTL and SOD1 KO mice. No significant differences were noted in the CSA of all fiber types in both portions. D: deep portion; S: superficial portion. Values are mean \pm SE.

the superficial portion of the gastrocnemius muscle in both mouse groups.

As shown in Figure 2, the CSA in the deep portion of the gastrocnemius muscle tended to increase in SOD1 KO mice compared to those in CTL mice, but no significant difference was noted in any muscle fiber type. Similarly, no significant change was noted in Type IIx/b CSA in the superficial portion.

3.3. Myonuclei and Satellite Cells. As shown in Figure 3, the tendency of the myonuclear number was similar to that of the CSA: the myonuclear number increased in all fiber types in the deep portion in SOD1 KO mice compared to those in CTL mice, but the increases were not significant. A similar tendency was noted in Type IIx/b fibers occupying the superficial portion.

As shown in Figure 4, the proportion of Type IIx/b fibers containing a central nuclei (%) in the deep portion was significantly higher in SOD1 KO than CTL mice. No central nuclei was present in Type I fibers in the deep portion in either group, but it tended to increase in Type IIa fibers in SOD1 KO mice compared to that in CTL mice. In addition, the proportion of central nuclei-containing Type IIx/b fibers in the superficial portion was significantly higher in SOD1 KO than CTL mice.

As shown in Figure 5, the number of satellite cells per specified number of muscle fibers tended to decrease in SOD1 KO mice compared to that in CTL mice in all muscle fiber types. A similar tendency was noted in Type IIx/b fibers in the superficial portion.

3.4. Satellite Cell-Related mRNA Expression. Since it is difficult to accurately divide the deep and superficial portions for real-time RT-PCR, the 2 regions were combined in this analysis, and the expression level was presented as a value relative to that in CTL mice (Figure 6). SOD1 mRNA SOD1 expression was not detected in SOD1 KO mice. Expressions of Pax7, which is expressed in the activation and proliferation states of satellite cells, and myogenin, which is expressed in the differentiation state, were significantly enhanced in SOD1 KO mice compared to those in CTL mice. Similarly, the expression level of MyoD, which is expressed in the proliferation and differentiation states, tended to increase. The expression level of an inflammatory cytokine, IL-6, which is a satellite cell activator, tended to increase in SOD1 KO mice. The expression level of MHC-embryonic (MHC-e), which is considered to be expressed in the early phase of muscle regeneration, significantly increased in SOD1 KO mice.

4. Discussion

4.1. SOD1 Deficiency-Associated Muscle Fiber Damage. The rate of central nuclei-containing Type II fibers in the superficial and deep portion of the gastrocnemius muscle was higher in SOD1 KO than CTL mice, and the rate of central nuclei-containing Type IIx/b fibers was significantly higher, being consistent with the results of previous studies in which central nucleus increased in the flexor digitorum brevis muscle in SOD1 KO mice [6]. Generally, central nuclei are observed in muscle fibers in the regeneration process. Therefore, the high proportion of central nuclei-containing fibers in SOD1 KO

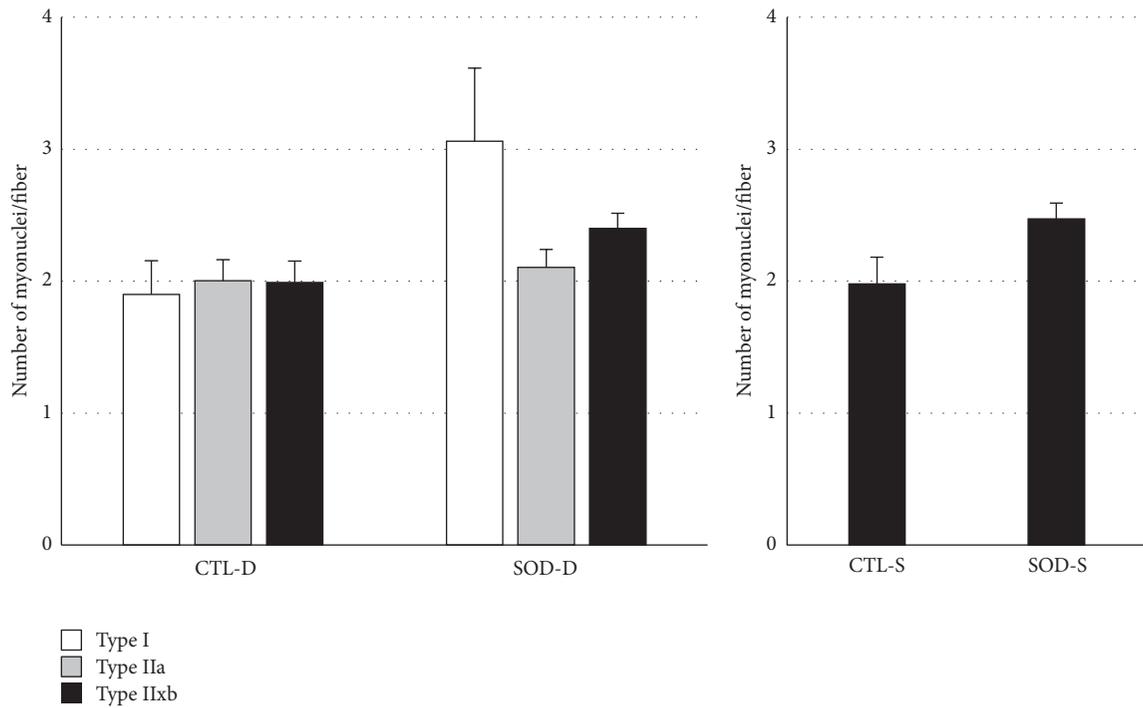


FIGURE 3: Comparison of number of myonuclei in each fiber type between CTL and SOD1 KO mice. Myonuclear number of all fiber types tended to increase in both portions in SOD1 KO mice compared to those in CTL mice. D: deep portion; S: superficial portion. Values are mean \pm SE.

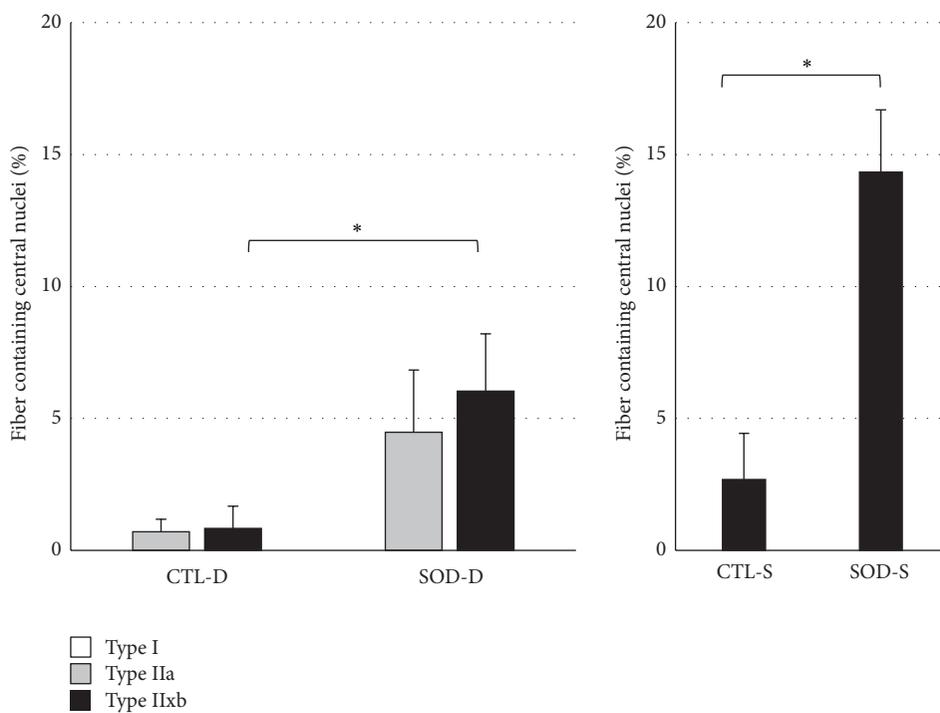


FIGURE 4: Comparison of proportion of central nuclei-containing muscle fibers in both portions between CTL and SOD1 KO mice. The proportions in Type IIx/b fibers were significantly higher in SOD1 KO than CTL mice. D: deep portion; S: superficial portion. Values are mean \pm SE, * $P < 0.05$.

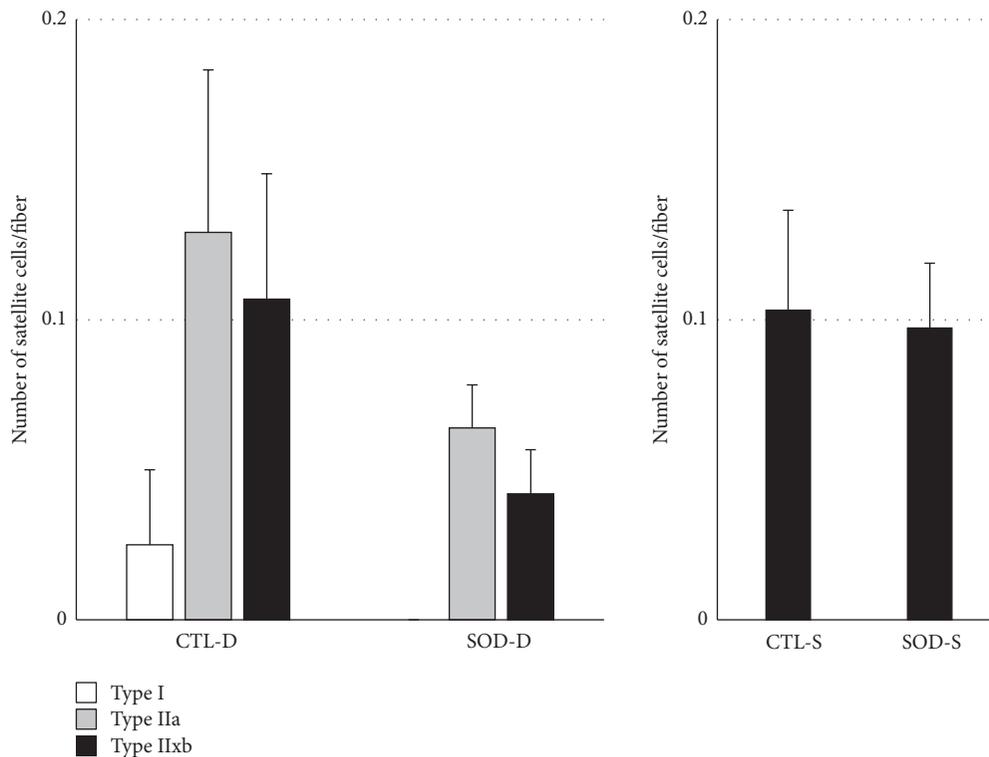


FIGURE 5: Comparison of number of satellite cells in both portions between CTL and SOD1 KO mice. Number of satellite cells per specified number of muscle fibers tended to decrease in SOD1 KO mice compared to that in CTL mice in all muscle fiber types. D: deep portion; S: superficial portion. Values are mean \pm SE.

mice may have been due to marked oxidative damage by ROS compared to that in CTL mice. High H_2O_2 production in Type II fibers, particularly Type IIb fibers, was demonstrated in a preceding study [7], in which it was also suggested that superoxide production occurs in the intermembrane space in Type IIb fibers, not in the matrix, being different from that in other fiber types. These findings suggest that the necessity of SOD1 present in the mitochondrial intermembrane space is higher for Type IIb than other fiber types. However, high ROS production in Type IIb fibers contradicts the fact that Type I fibers contain more mitochondria, the main source of ROS production. This contradiction may be related to differences in the partial pressure of oxygen among muscle fiber types and ROS production under a hypoxic condition. The partial pressure of oxygen in the microvasculature is low in the gastrocnemius muscle, rich in Type II fibers, compared to the soleus muscle and diaphragm, rich in Type I fibers, and a lower partial pressure of oxygen in a region containing many fast muscle fibers than that in a region containing less fast muscle fibers within the gastrocnemius muscle has been reported [8]. It is also known that ROS production is inhibited by lowering the electron current passing through the electron transport chain to adapt to a hypoxic condition of the tissue. Under a hypoxic condition, the mitochondrial electron transport efficiency decreases, and ROS are produced through passing electrons from complexes I and III to molecules different from normal receiver

molecules [9]. In addition, an increase in ROS by a complex III inhibitor, antimycin, and the prevention of oxidative stress-associated ischemia-reperfusion injury by decreasing metabolic dependence on the electron transport chain have been clarified [10, 11]. Furthermore, it has been shown that the ability of oxidative fibers to upregulate NO-induced antioxidative enzymes is higher than that of glycolytic fibers [12]. Considering these comprehensively, the differences in the number of central nucleus among the fiber types in SOD1 KO mice may have been due to hypoxia-induced or low antioxidative enzyme level-associated ROS production in the mitochondrial intermembrane space in Type II fibers, particularly Type IIx/b fibers.

4.2. Influence of SOD1 Deficiency on Satellite Cells. On analysis of mRNA expression using real-time RT-PCR, significant increases in the Pax7, myogenin, and MHC-e mRNA levels and a slight increase in the MyoD mRNA level were observed in the gastrocnemius muscle of SOD1 KO mice. Pax7, MyoD, and myogenin are markers detected in the resting/proliferation, proliferation/differentiation, and differentiation states of satellite cells, respectively. MHC-e is expressed in the fetal period and muscle regeneration before differentiation into adult fiber types. Since these factors were upregulated, satellite cells may have been activated in SOD1 KO mice compared to those in CTL mice, and the muscle regeneration process may have been enhanced.

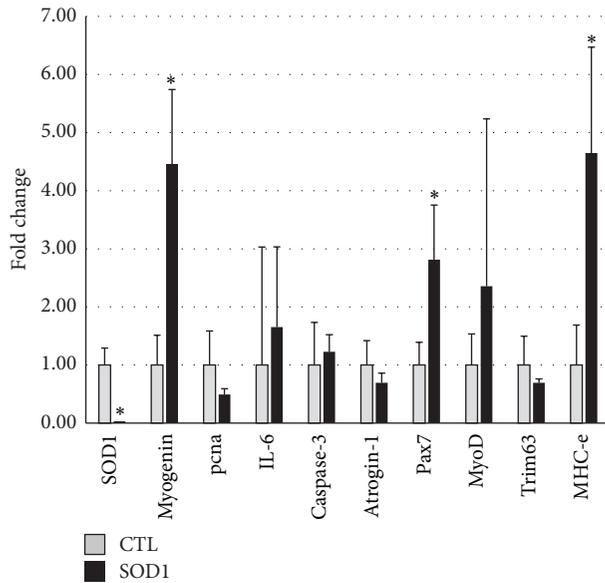


FIGURE 6: Comparison of mRNA expressions between CTL and SOD1 KO mice. Expressions of the target gene in SOD1 KO were presented as the relative values (fold change) for CTL mouse. Expressions of myogenin, Pax7, and MHC-e were significantly enhanced in SOD1 KO mice compared to those in CTL mice. Values are mean \pm SE. * $P < 0.05$, versus CTL.

Actually, the myonuclear number per muscle fiber tended to increase in SOD1 KO mice, which may have resulted from the differentiation of satellite cells into myonuclei.

There are many common points between SOD1 deficiency and aging-related changes, such as increases in ROS production and inflammatory cytokines and reduction of the muscle function [2]. A study on satellite cell activation and the stem cell niche clarified that MyoD and myogenin mRNA expressions in satellite cells are more markedly enhanced in senescence compared to those in early life, showing that the activation and differentiation of satellite cells are promoted in senescence, whereas the Pax7-positive rate of satellite cells decreased in senescence, suggesting that the replication competence of satellite cells decreases with aging, reducing the number of satellite cells [13]. In addition, stem cell niche-derived fibroblast growth factor-2 (FGF2) was identified as the main cause of these aging-related changes in the study. In our study, the number of satellite cells per muscle fiber decreased in SOD1 KO mice compared to that in the CTL mice. It has also been suggested that an increase in ROS production leads to an increase in FGF2 production in sheep pulmonary arterial smooth muscle cells [14]. An increase in the IL-6 mRNA expression level was observed in our study. IL-6 is a satellite cell activator through the JAK/STAT3 signaling pathway (Janus kinase/signal transducer and activators of transcription 3) [15, 16], and it has been demonstrated to be a regulatory factor essential for skeletal muscle hypertrophy in a compensatory overload experiment using IL-6 KO mice [17].

Therefore, enhanced MyoD, myogenin, and MHC-e mRNA expressions in SOD1 KO mice observed in our study

may have been due to the activation of satellite cells by constantly increased FGF2 and IL-6 induced by constantly increased ROS.

4.3. Specificity of SOD KO Mouse Model. SOD1 KO mice are considered a useful model to investigate the association between an increase in ROS production and muscular atrophy, but responses specific to this model are also observed. For example, increases in the PGC1 α mRNA expression level and mitochondria in SOD1 KO mice have been reported [18], whereas reduction of the PGC1 α expression level and mitochondrial function by increased ROS production in response to aging-related changes, disuse, and hypoxic stimulation has been observed [18–23]. Moreover, slowed muscle contraction-inducing adaptive responses [24] and the accumulation of mitochondria below the muscle cell membrane have been observed in SOD1 KO mice [18, 25]. It has been suggested that constant ROS production interferes with ROS production during muscle contraction, slowing muscle contraction-inducing adaptive responses [24]. Actually, another study [6] reported the absence of an increase in ROS after contraction in SOD1 KO mice. It has also been clarified that the muscle contraction-inducing ROS production in CTL mice is higher than that in resting SOD1 KO mice [6], and expression of proteins with antioxidative actions, such as SOD2 and heat shock proteins, is enhanced in SOD1 KO mice compared to those in control mice [24]. These phenomena indicate that constant ROS production markedly changes the antioxidative defense system, to which attention should be paid when SOD1 KO mice are considered as an aging model.

5. Conclusion

The proportion of central nuclei-containing Type IIx/b fibers in the deep and superficial portions of the gastrocnemius muscle was significantly higher in SOD1 KO than CTL mice. The mRNA expression levels of Pax7, myogenin, and MHC-embryonic significantly increased in SOD1 KO mice. It was suggested that muscle is damaged by ROS produced in Type IIx/b fibers, accelerating the proliferation and differentiation of satellite cells in SOD1 KO mice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

- [1] D. H. Kang and S. W. Kang, "Targeting cellular antioxidant enzymes for treating atherosclerotic vascular disease," *Biomolecules and Therapeutics*, vol. 21, no. 2, pp. 89–96, 2013.
- [2] A. Vasilaki and M. J. Jackson, "Role of reactive oxygen species in the defective regeneration seen in aging muscle," *Free Radical Biology and Medicine*, vol. 65, pp. 317–323, 2013.
- [3] S. Fulle, F. Protasi, G. Di Tano et al., "The contribution of reactive oxygen species to sarcopenia and muscle ageing," *Experimental Gerontology*, vol. 39, no. 1, pp. 17–24, 2004.
- [4] F. L. Muller, W. Song, Y. Liu et al., "Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy," *Free Radical Biology and Medicine*, vol. 40, no. 11, pp. 1993–2004, 2006.
- [5] M. M. Matzuk, L. Dionne, Q. Guo, T. R. Kumar, and R. M. Lebovitz, "Ovarian function in superoxide dismutase 1 and 2 knockout mice," *Endocrinology*, vol. 139, no. 9, pp. 4008–4011, 1998.
- [6] G. K. Sakellariou, D. Pye, A. Vasilaki et al., "Role of superoxide-nitric oxide interactions in the accelerated age-related loss of muscle mass in mice lacking Cu,Zn superoxide dismutase," *Aging Cell*, vol. 10, no. 5, pp. 749–760, 2011.
- [7] E. J. Anderson and P. D. Neuffer, "Type II skeletal myofibers possess unique properties that potentiate mitochondrial H₂O₂ generation," *The American Journal of Physiology—Cell Physiology*, vol. 290, no. 3, pp. C844–C851, 2006.
- [8] L. F. Ferreira, P. McDonough, B. J. Behnke, T. I. Musch, and D. C. Poole, "Blood flow and O₂ extraction as a function of O₂ uptake in muscles composed of different fiber types," *Respiratory Physiology and Neurobiology*, vol. 153, no. 3, pp. 237–249, 2006.
- [9] G. L. Semenza, "Hypoxia-inducible factors in physiology and medicine," *Cell*, vol. 148, no. 3, pp. 399–408, 2012.
- [10] W. W. Wheaton and N. S. Chandel, "Hypoxia. 2. Hypoxia regulates cellular metabolism," *American Journal of Physiology—Cell Physiology*, vol. 300, no. 3, pp. C385–C393, 2011.
- [11] N. S. Chandel, E. Maltepe, E. Goldwasser, C. E. Mathieu, M. C. Simon, and P. T. Schumacker, "Mitochondrial reactive oxygen species trigger hypoxia-induced transcription," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11715–11720, 1998.
- [12] Z. Yu, P. Li, M. Zhang, M. Hannink, J. S. Stamler, and Z. Yan, "Fiber type-specific nitric oxide protects oxidative myofibers against cachectic stimuli," *PLoS ONE*, vol. 3, no. 5, Article ID e2086, 2008.
- [13] J. V. Chakkalakal, K. M. Jones, M. A. Basson, and A. S. Brack, "The aged niche disrupts muscle stem cell quiescence," *Nature*, vol. 490, no. 7420, pp. 355–360, 2012.
- [14] S. M. Black, J. M. DeVol, and S. Wedgwood, "Regulation of fibroblast growth factor-2 expression in pulmonary arterial smooth muscle cells involves increased reactive oxygen species generation," *American Journal of Physiology—Cell Physiology*, vol. 294, no. 1, pp. C345–C354, 2008.
- [15] M. Masuda, M. Suzui, R. Yasumatu et al., "Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma," *Cancer Research*, vol. 62, no. 12, pp. 3351–3355, 2002.
- [16] M. Ernst and B. J. Jenkins, "Acquiring signalling specificity from the cytokine receptor gp130," *Trends in Genetics*, vol. 20, no. 1, pp. 23–32, 2004.
- [17] A. L. Serrano, B. Baeza-Raja, E. Perdiguero, M. Jardí, and P. Muñoz-Cánoves, "Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy," *Cell Metabolism*, vol. 7, no. 1, pp. 33–44, 2008.
- [18] Y. C. Jang, M. S. Lustgarten, Y. Liu et al., "Increased superoxide in vivo accelerates age-associated muscle atrophy through mitochondrial dysfunction and neuromuscular junction degeneration," *The FASEB Journal*, vol. 24, no. 5, pp. 1376–1390, 2010.
- [19] T. Wenz, "Regulation of mitochondrial biogenesis and PGC-1 α under cellular stress," *Mitochondrion*, vol. 13, no. 2, pp. 134–142, 2013.
- [20] M. Picard, D. Ritchie, M. M. Thomas, K. J. Wright, and R. T. Hepple, "Alterations in intrinsic mitochondrial function with aging are fiber type-specific and do not explain differential atrophy between muscles," *Aging Cell*, vol. 10, no. 6, pp. 1047–1055, 2011.
- [21] E. E. Talbert, A. J. Smuder, K. Min, O. S. Kwon, H. H. Szeto, and S. K. Powers, "Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant," *Journal of Applied Physiology*, vol. 115, no. 4, pp. 529–538, 2013.
- [22] J. L. Gamboa and F. H. Andrade, "Mitochondrial content and distribution changes specific to mouse diaphragm after chronic normobaric hypoxia," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 298, no. 3, pp. R575–R583, 2010.
- [23] J. Magalhães, A. Ascensão, J. M. C. Soares et al., "Acute and severe hypobaric hypoxia increases oxidative stress and impairs mitochondrial function in mouse skeletal muscle," *Journal of Applied Physiology*, vol. 99, no. 4, pp. 1247–1253, 2005.
- [24] A. Vasilaki, J. H. van der Meulen, L. Larkin, and et al, "The age-related failure of adaptive responses to contractile activity in skeletal muscle is mimicked in young mice by deletion of Cu,Zn superoxide dismutase," *Aging Cell*, vol. 9, no. 6, pp. 979–990, 2010.
- [25] T. Y. Kostrominova, K. A. Pasyk, H. Van Remmen, A. G. Richardson, and J. A. Faulkner, "Adaptive changes in structure of skeletal muscles from adult Sod1 homozygous knockout mice," *Cell and Tissue Research*, vol. 327, no. 3, pp. 595–605, 2007.

Research Article

Obestatin Accelerates the Healing of Acetic Acid-Induced Colitis in Rats

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Obestatin, a 23-amino acid peptide derived from the proghrelin, has been shown to exhibit some protective and therapeutic effects in the gut. The aim of present study was to determine the effect of obestatin administration on the course of acetic acid-induced colitis in rats. *Materials and Methods.* Studies have been performed on male Wistar rats. Colitis was induced by a rectal enema with 3.5% acetic acid solution. Obestatin was administered intraperitoneally twice a day at a dose of 8 nmol/kg, starting 24 h after the induction of colitis. Seven or 14 days after the induction of colitis, the healing rate of the colon was evaluated. *Results.* Treatment with obestatin after induction of colitis accelerated the healing of colonic wall damage and this effect was associated with a decrease in the colitis-evoked increase in mucosal activity of myeloperoxidase and content of interleukin-1 β . Moreover, obestatin administration significantly reversed the colitis-evoked decrease in mucosal blood flow and DNA synthesis. *Conclusion.* Administration of exogenous obestatin exhibits therapeutic effects in the course of acetic acid-induced colitis and this effect is related, at least in part, to the obestatin-evoked anti-inflammatory effect, an improvement of local blood flow, and an increase in cell proliferation in colonic mucosa.

1. Introduction

Obestatin is a 23-amino acid peptide derived from preproghrelin, a common prohormone for ghrelin and obestatin [1–3]. Obestatin was originally extracted from the rat stomach and the stomach seems to be a major source of circulating obestatin [1, 2, 4]. Secretion of obestatin is pulsative and displays an ultradian rhythmicity similar to ghrelin and growth hormone [5]. In contrast to ghrelin, obestatin has been reported to be an anorexic hormone, reducing food

intake, gastric emptying time, jejunal motility, and body weight gain [2, 3].

Previous studies have shown that pretreatment with ghrelin, an alternative product of posttranslational processing of preproghrelin, protects gastric mucosa against damage evoked by different noxious factors [6–8] and inhibits the development of experimental acute pancreatitis [9, 10]. Moreover, apart from its protective effect, ghrelin exhibits therapeutic effect in the gut. It accelerates the healing of gastric [11], duodenal [11, 12] and oral ulcers [13], and colonic

inflammation [14, 15]. Therapeutic effect of ghrelin has been also shown in experimental models of acute pancreatitis [16–19].

In the case of obestatin, there are studies which have shown that also this peptide exhibits some protective and therapeutic effects in the gut [3]. It has been demonstrated so far that preventive administration of obestatin inhibits the development of cerulein- and ischemia/reperfusion-induced acute pancreatitis [20, 21]. Moreover, Granata et al. have reported that obestatin promotes survival of pancreatic islets, especially β -cell [22]. In the stomach, it has been shown that treatment with obestatin accelerates the healing of acetic acid-induced gastric ulcers [23]. Moreover, previous studies have suggested that endogenous and exogenous obestatin may affect or be related to the development of colitis. Alexandridis et al. have found that the ratio of serum level of obestatin to ghrelin in patients with active inflammatory bowel disease (IBD) is significantly lower than in patients with remission [24]. This observation was confirmed by Jung et al. and they have suggested that the obestatin/ghrelin ratio may be useful in monitoring of remission in the course of IBD [25]. Furthermore, one experimental study has shown protective effect of obestatin in the colon. Pamukcu et al. have reported that administration of obestatin before and during the development of dextran sodium sulfate-induced colitis reduces the severity of this inflammation [15].

The objective of the present research was to determine whether administration of obestatin after the development of colitis exhibits therapeutic effect in this disease.

2. Material and Methods

2.1. Animals and Treatment. Studies were performed on 80 male Wistar rats weighing 270–320 g and were conducted following the experimental protocol approved by the 1st Local Committee of Ethics for the Care and Use of Laboratory Animals in Cracow (Permit number 2/2013 released on January 16, 2013). During the experiments animals were kept in cages placed in room temperature with a 12 h light-darkness cycle. Animals were fasted, with free access to water, for 18 h prior to induction of colitis. Later, water and food were available *ad libitum*.

The animals were randomly divided into four groups: (1) control rats without colitis induction treated intraperitoneally (i.p.) with saline; (2) rats without colitis induction treated i.p. with obestatin; (3) rats with colitis treated i.p. with saline; (4) rats with colitis treated i.p. with obestatin.

In the rats anesthetized with pentobarbital (30 mg/kg i.p., Vetbutal, Biowet, Puławy Poland), colitis was induced by a rectal enema with 1 mL of 3.5% (v/v) acetic acid diluted in saline. Acetic acid solution was administered through a polyethylene catheter inserted into the rectum. There are different models of acetic acid-induced colitis and the tip of catheter can be positioned from 1.2 [26] to 8 cm [27] proximal to the anus verge. For this reason we have chosen an intermediate depth of catheter insertion, 4.5 cm from the anus. Rats without induction of colitis obtained rectal enema with an aqueous saline solution administered at the same

manner as a solution of acetic acid in animals with induction of colitis.

Starting 24 hours after a rectal enema with saline or acetic acid, the rats were treated with saline (groups 1 and 3) or obestatin (groups 2 and 4) administered i.p. twice a day. Rat obestatin (Yanaihara Institute, Shizuoka, Japan) was given at a dose of 8 nmol/kg. This dose was chosen because previous studies have shown that obestatin given at a dose 8 nmol/kg exhibits strong and repeatable therapeutic effect in the healing of chronic gastric ulcers [23]. Rats from each experimental group were randomly divided into two subgroups. In the first subgroup, the healing rate of the colon was evaluated 7 days after the acetic acid enema. In the second subgroup evaluation was performed 7 days later. Each subgroup consisted of 10 animals.

2.2. Measurement of Colonic Blood Flow and Colonic Damage. At the end of the experiments, animals were anesthetized again with pentobarbital. After opening the abdominal cavity and exposure of the colon, the rate of colonic blood flow was measured using laser Doppler flowmeter (PeriFlux 4001 Master monitor, Perimed AB, Järfälla, Sweden), in accordance with the methodology described before [28]. The measurement of mucosal blood flow was performed every time in five parts of the descending and sigmoid colon and the main value of five records was expressed as the percentage of the value obtained in the animals from the control group. After the measurement of colonic blood flow, anesthetized animals were euthanized by exsanguination from the abdominal aorta. Then, the area of mucosal damage was measured, using a computerized planimeter (Morphomat, Carl Zeiss, Berlin, Germany), in accordance with the method described earlier [12]. Briefly, the large bowel was removed from the body, opened along of its long axis on the side opposite to the mesentery, and washed with ice-cold saline. The colon samples were flattened and carefully sandwiched between two layers of transparent plastic folder. The area of damage was measured by tracing its perimeter.

2.3. Biochemical Analysis. The dynamics of DNA replication in the colon mucous membrane was marked with radioisotope method through measurement of tritium-labeled thymidine incorporation into DNA, according to the methodology described before [29]. A mucous membrane sample was fragmented with scissors, subsequently, the tissue was incubated in 37°C for 45 minutes in 2 mL of nutritive solution containing tritium-marked thymidine ([6-³H] thymidine, 20–30 Ci/mmol; Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic), with activity of 8 μ Ci/mL. Incorporation of [³H]-thymidine into DNA was determined by counting 0.5 mL DNA-containing supernatant in liquid scintillation system. The rate of DNA synthesis was expressed as disintegration per minute [³H]-thymidine per microgram DNA (dpm/ μ g DNA).

Samples of the colonic mucosa, in which the concentration of interleukin-1 β was measured, were homogenized in phosphate buffer at 4°C. Then the homogenate was centrifuged and the concentration of interleukin-1 β in the

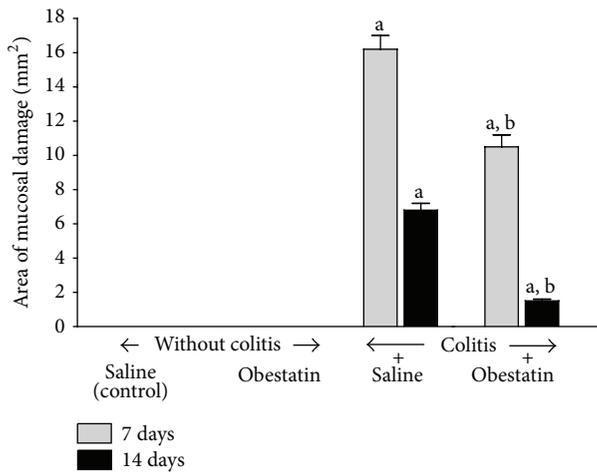


FIGURE 1: Effect of saline or obestatin given intraperitoneally for 7 or 14 days on the area of colonic lesions in rats without or with acetic acid-induced colitis. Mean value \pm SEM. $N = 10$ animals in each experimental group and each time of observation. ^a $P < 0.05$ compared to control at the same time of observation; ^b $P < 0.05$ compared to colitis plus saline at the same time of observation.

supernatant was determined using the Rat IL-1 β Platinum Elisa (Bender MedSystem GmbH, Vienna, Austria). The concentration of interleukin-1 β of the colonic mucosa was expressed in nanograms per 1 gram of tissue.

Biopsy samples for measurement of mucosal myeloperoxidase activity were homogenized in ice-cold potassium phosphate and, until the marking was done, stored at the temperature of -60°C . Marking myeloperoxidase activity was performed with the use of a modification of the method described by Bradley et al. [30]. Results obtained in units per gram of tissue were finally expressed as a percentage of the value observed in the control group.

2.4. Statistical Analysis. Statistical analysis of the data was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad-Prism (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when P was less than 0.05.

3. Results

Figure 1 shows the influence of obestatin administration on the healing of acetic acid-induced mucosal damage in the colon. In saline- and obestatin-treated rats without induction of colitis no mucosal damage was observed. In saline-treated rats, 7 days after the induction of colitis, the mucosal damage area was $16.2 \pm 0.8 \text{ mm}^2$, whereas 7 days later it was reduced to $6.8 \pm 0.4 \text{ mm}^2$ as a result of spontaneous regeneration. Treatment with obestatin for 7 days after administration of acetic acid accelerated a reduction in the mucosal damage area by 35.2%. After the next 7 days of treatment with obestatin, the area of colonic damage was reduced by around 78% when compared to lesions observed in the animals

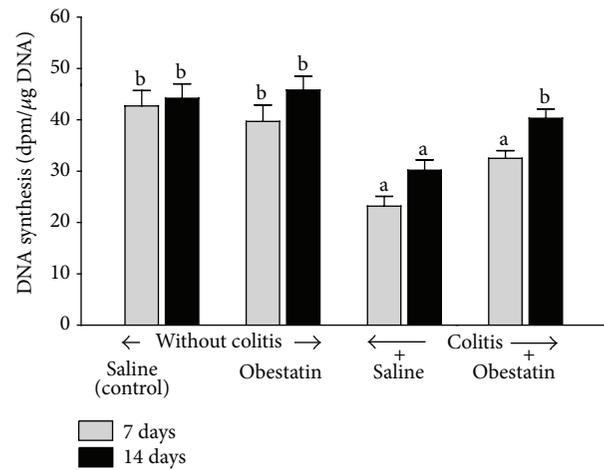


FIGURE 2: Effect of saline or obestatin given intraperitoneally for 7 or 14 days on the rate of DNA synthesis in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value \pm SEM. $N = 10$ animals in each experimental group and each time of observation. ^a $P < 0.05$ compared to control at the same time of observation; ^b $P < 0.05$ compared to colitis plus saline at the same time of observation.

treated with saline. The healing promoting effect of obestatin was statistically significant at both periods of observation, after 7 and 14 days of obestatin administration (Figure 1).

In the rats without induction of colitis, administration of obestatin at a dose used failed to affect significantly DNA synthesis in colonic mucosa (Figure 2). Induction of colitis by an enema with acetic acid led to reduction in mucosal DNA synthesis in the colon. DNA synthesis in the colonic mucosa was significantly reduced by around 45 and 32% at the 7th and 14th day after induction of colitis, respectively. Treatment with obestatin partly reversed the colitis-evoked reduction in DNA synthesis in the colonic mucosa and this effect was statistically significant after 14 days of obestatin administration (Figure 2).

In the groups of animals without induction of colitis, intraperitoneal administration of obestatin for 7 or 14 days failed to affect mucosal blood flow in the colon (Figure 3). In the rats with colitis, 7 days after an enema with acetic acid, blood flow through the colonic mucosa was significantly reduced by around 50%, when compared to the value observed in the control animals without colitis. After the next seven days, mucosal blood flow in the colon of the animals with colitis was almost fully restored and no significant difference was observed in comparison to a value in control group of animals. In the rats with colitis, administration of obestatin caused an improvement of mucosal blood flow in the colon and this effect was statistically significant at the 7th day after the induction of colitis (Figure 3).

In the rats without colitis, administration of obestatin for 7 or 14 days at the dose used was without any effect on mucosal concentration of interleukin-1 β (IL-1 β) in the colon (Figure 4). Induction of colitis significantly increased mucosal concentration of IL-1 β in the colon. As shown in Figure 4, rats with colitis demonstrated more than 10-fold and

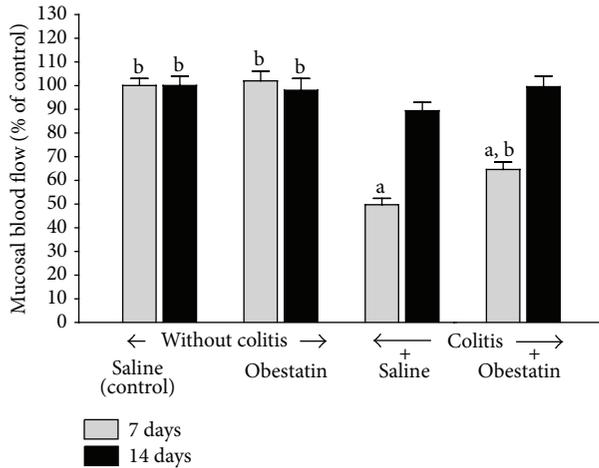


FIGURE 3: Effect of saline or obestatin given intraperitoneally for 7 or 14 days on mucosal blood flow in the colon rats without or with acetic acid-induced colitis. Mean value \pm SEM. $N = 10$ animals in each experimental group and each time of observation. ^a $P < 0.05$ compared to control at the same time of observation; ^b $P < 0.05$ compared to colitis plus saline at the same time of observation.

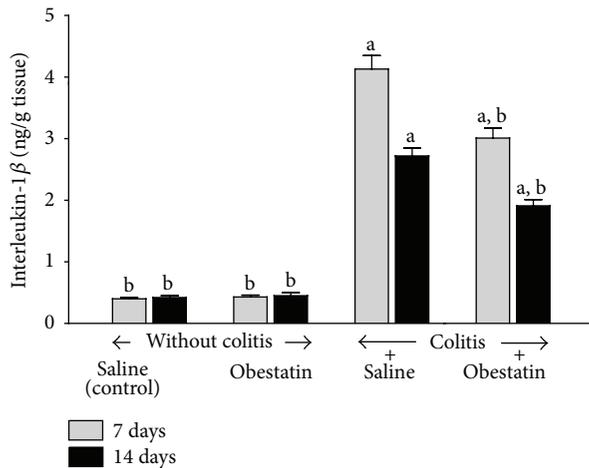


FIGURE 4: Effect of saline or obestatin given intraperitoneally for 7 or 14 days on interleukin-1 β concentration in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value \pm SEM. $N = 10$ animals in each experimental group and each time of observation. ^a $P < 0.05$ compared to control at the same time of observation; ^b $P < 0.05$ compared to colitis plus saline at the same time of observation.

6-fold increase in this parameter at the 7th day and 14th after induction of colitis, respectively. Administration of obestatin at the dose used partly reversed the colitis-evoked increase in mucosal concentration of IL-1 β and this effect was statistically significant in both periods of observation (Figure 4).

Administration of obestatin for 7 or 14 days was without effect on mucosal myeloperoxidase activity in the colon in the rats without colitis induction (Figure 5). In rats treated with saline after induction of colitis, 7 days after induction of this inflammation, myeloperoxidase activity in colonic mucosa

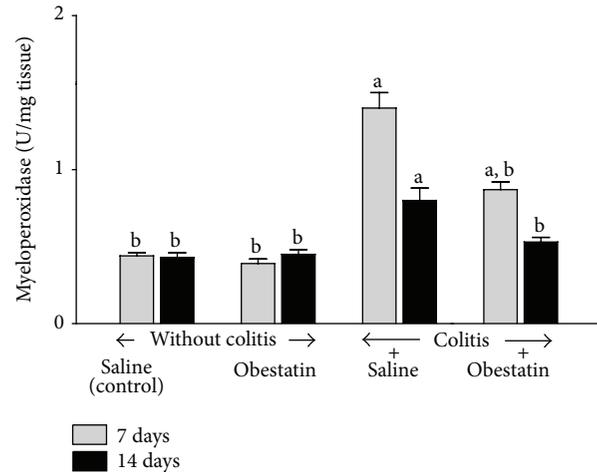


FIGURE 5: Effect of saline or obestatin given intraperitoneally for 7 or 14 days on myeloperoxidase activity in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value \pm SEM. $N = 10$ animals in each experimental group and each time of observation. ^a $P < 0.05$ compared to control at the same time of observation; ^b $P < 0.05$ compared to colitis plus saline at the same time of observation.

was increased by 318%. Also, after the next 7 days, almost 2-fold increase in myeloperoxidase activity was still observed in colonic mucosa in rats with colitis treated with saline. Treatment with obestatin reduced the colitis-evoked increase in myeloperoxidase activity in the colonic mucosa. This effect was statistically significant in both periods of observation, 7 and 14 days after induction of (Figure 5).

4. Discussion

In this study, we have investigated the influence of treatment with obestatin on the course of acetic acid-induced colitis. We have found that intraperitoneal administration of obestatin given at the dose 8 nmol/kg significantly accelerates the recovery of colonic wall integrity in rats with this model of experimental colitis. Maintaining the integrity of the digestive tract mucous membrane depends on preservation of balance between proliferation and loss of epithelial cells. A disruption of that balance, as a result of a decrease in cell proliferation, apoptosis, and/or increased cell loss, leads to mucosal atrophy and/or ulceration [31, 32], whereas an increase in cell division may lead to mucosal hypertrophy. On the other hand, stimulation of cell proliferation typically leads to increase in the protection of the digestive tract mucous membrane against damaging factors and accelerates the healing of mucosal damage [33–35]. Synthesis of DNA is an indispensable process for mitosis to take place [36]. Therefore, assessment of DNA synthesis dynamics through measurement of incorporation of tritium-marked thymidine into cellular DNA reflects vitality of cells and cellular division dynamics.

In our present study, we have found that administration of obestatin in animals without induction of colitis failed

to significantly affect mucosal DNA synthesis in the colon. This leads to the conclusion that, in animals with normal colonic mucous membrane, treatment with obestatin given at the dose of 8 nmol/kg does not stimulate DNA synthesis in colonic mucosa and therefore this peptide seems to be safe and does not bring a risk of hyperplasia and hypertrophy of colonic mucosa. On the other hand, administration of obestatin in rats with colitis led to a considerable improvement of mucosal DNA synthesis in the colon. This observation indicates that therapeutic effect of obestatin in the course of acetic acid-induced colitis is, at least in part, a result of increase in cell vitality in colonic mucosa.

Another factor that plays a role in maintaining mucosal integrity is suitable, adequate for the demand, organ blood flow. The important role of vascular mechanisms in protection and healing of gastrointestinal mucosa has been previously described [37–39]. Experimental studies have demonstrated that exposure of the stomach to damaging factors leads to small mucosal damage, as long as a sufficient blood flow is maintained. A similar significance of sufficient blood flow in maintaining mucosal integrity was also demonstrated in other parts of the digestive tract, including the oral cavity [13], esophagus [38], duodenum [11, 12] and colon [39]. In our present study, we have observed that intrarectal administration of acetic acid solution decreases mucosal blood flow in the colon. Seven days after induction of colitis mucosal blood flow was decreased by around 50% in comparison with a value observed in control group without colitis. After the next 7 days, blood flow in colonic mucosa in rats with colitis was still lower than in control group, but this difference was statistically insignificant.

Administration of obestatin in the dose used did not exhibit any influence on blood flow in the colonic mucous membrane in animals without colitis. In contrast to that, administration of obestatin in animals with colitis led to a significant improvement in blood flow in the colonic mucous membrane, and this effect was statistically significant at the 7th day after induction of colitis. After the next 7 days a value of blood flow in rats with colitis treated with obestatin was similar to that recorded in control animals without colitis. Moreover, this obestatin-evoked improvement of mucosal blood flow was connected with a decrease in the area and severity of colonic mucosal damage. This observation indicates that therapeutic effect of obestatin in acetic acid-induced colitis involves an improvement of mucosal blood flow in the large bowel.

The next interesting finding originated from our present study is the influence of intraperitoneal administration of obestatin on the concentration of interleukin-1 β (IL-1 β) and myeloperoxidase activity in colonic mucosa of animals with colitis. Activation of inflammatory cells with subsequent release of proinflammatory cytokines is responsible for intensity of local and systemic inflammatory response [40]. Between proinflammatory cytokines, IL-1 β plays a fundamental role in the initiation of biochemical cascade of inflammation [40–45]. Results obtained in our present study have demonstrated that acetic acid-induced colitis led to a tenfold and sixfold increase in IL-1 β concentration in colonic mucosa at the 7th and 14th day after induction of this

inflammation, respectively. Intraperitoneal administration of obestatin partially but significantly reduced that increase. On the other hand, administration of obestatin failed to affect IL-1 β concentration in the mucous membrane in rats without colitis.

Myeloperoxidase (MPO) is an enzyme which is present in azurophilic granules of neutrophil granulocytes. Antimicrobial function of neutrophils is related, among others, to their possibility to generate reactive oxygen species (ROS) during the respiratory burst [46]. On the other hand, excess of ROS leads to oxidative damage of own tissues on cellular and subcellular level resulting in destruction of proteins, nucleic acids, and lipids [46–49]. MPO is released by activated neutrophils and reflects the degree of tissue infiltration by those granulocytes [46, 50]. Those data are in agreement with our present observation. Seven days after rectal enema with acetic acid, we have observed a threefold increase in MPO activity in the colonic mucous membrane in rats treated with saline. After the next 7 days mucosal activity of MPO in colon of rats with colitis was still significantly increased above a level observed in control rats without induction of colitis. Intraperitoneal administration of obestatin in animals with acetic acid-induced colitis caused a considerable decrease in colonic MPO activity. In contrast to that, mucosal activity of MPO in the colon of rats without colitis was low and administration of obestatin was without effect on colonic MPO activity in those rats.

Our findings mentioned above concerning the inhibitory effect of treatment with obestatin on IL-1 β concentration and MPO activity in colonic mucosa in rats with acetic acid-induced colitis indicate that the healing promoting effect in this kind of colitis is, at least in part, related to anti-inflammatory properties of obestatin. On the other hand, obestatin failed to affect mucosal IL-1 β concentration and MPO activity in the colon of rats without induction of colitis. This observation suggests that obestatin does not disturb the immune system in normal circumstances, without inflammation.

As stated in the introduction, ghrelin and obestatin are peptides encoded by the same gene and derived from a common prohormone [1–3]. Ghrelin exhibits protective and therapeutic effect in various organs and experimental models of organ damage [6–19]. Data on the effects of obestatin are less numerous. Previous studies have demonstrated that this peptide demonstrates protective effect in the large bowel and its earlier application reduces damage in colitis induced by dextran sodium sulfate (DSS) [15]. Our present studies have shown that obestatin also exhibits a therapeutic effect in the course of colitis and administration of this peptide after the development of acetic acid-induced colitis accelerates the healing of inflammation. Differences between ghrelin and obestatin are based on their action mechanisms. Ghrelin acts directly via activation of GHS-R receptor and indirectly via a release of growth hormone and IGF-1 [2, 3, 51]. In the case of obestatin, its receptor is still unknown. Initially it was thought that obestatin acts by binding to the G protein-coupled receptor 39 (GRP39) [1], but later studies do not seem to confirm this hypothesis [52]. Also there is another effect of these peptides on food intake. Ghrelin stimulates food

intake, whereas, in the case of obestatin, its inhibitory effect on appetite has been postulated [1–3].

There are numerous experimental models of inflammatory bowel disease, because each of these models is not perfect and does not fully meet the clinical condition. DSS-induced colitis is reproducible and one of widely used animal models of IBD. DSS mainly affects the large bowel, but some studies have reported that DSS also affects the distal parts of small intestine [27]. In mice or rats, administration of DSS causes hematochezia, body weight loss, shortening of intestine, mucosal ulcers, and infiltration of neutrophils [53]. DSS causes erosion with complete loss of surface epithelium because of its deleterious effect on epithelial cells [27]. Acetic acid-induced colitis is an animal model of colitis that shows close resemblance to human IBD in terms of pathogenesis, morphological features, and inflammatory factors involved in this kind of inflammation [27]. Intrarectal enema with acetic acid solution causes inflammation characterized by neutral infiltration into intestinal tissue, massive necrosis of mucosal and submucosal tissues, edema, vascular dilatation, and submucosal ulceration. Those findings are similar to that observed in human colitis [27].

In conclusion, we can say that treatment with obestatin accelerates the healing of acetic acid-induced colitis and this effect seems to be related to the obestatin-evoked anti-inflammatory effect, an improvement of local mucosal blood flow, and an increase in cell proliferation in colonic mucosa.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] J. V. Zhang, P.-G. Ren, O. Avsian-Kretchmer et al., “Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin’s effects on food intake,” *Science*, vol. 310, no. 5750, pp. 996–999, 2005.
- [2] C.-Y. Chen, A. Asakawa, M. Fujimiya, S.-D. Lee, and A. Inui, “Ghrelin gene products and the regulation of food intake and gut motility,” *Pharmacological Reviews*, vol. 61, no. 4, pp. 430–481, 2009.
- [3] P. Ceranowicz, Z. Warzecha, and A. Dembinski, “Peptidyl hormones of endocrine cells origin in the gut—their discovery and physiological relevance,” *Journal of Physiology and Pharmacology*, vol. 66, no. 1, pp. 11–27, 2015.
- [4] M. W. Furnes, B. Stenström, K. Tømmerås et al., “Feeding behavior in rats subjected to gastrectomy or gastric bypass surgery,” *European Surgical Research*, vol. 40, no. 3, pp. 279–288, 2008.
- [5] P. Zizzari, R. Longchamps, J. Epelbaum, and M. T. Bluet-Pajot, “Obestatin partially affects ghrelin stimulation of food intake and growth hormone secretion in rodents,” *Endocrinology*, vol. 148, no. 4, pp. 1648–1653, 2007.
- [6] V. Sibilgia, G. Rindi, F. Pagani et al., “Ghrelin protects against ethanol-induced gastric ulcers in rats: studies on the mechanisms of action,” *Endocrinology*, vol. 144, no. 1, pp. 353–359, 2003.
- [7] T. Brzozowski, P. C. Konturek, S. J. Konturek et al., “Exogenous and endogenous ghrelin in gastroprotection against stress-induced gastric damage,” *Regulatory Peptides*, vol. 120, no. 1–3, pp. 39–51, 2004.
- [8] S. Ö. İşeri, G. Şener, M. Yüksel et al., “Ghrelin against alendronate-induced gastric damage in rats,” *Journal of Endocrinology*, vol. 187, no. 3, pp. 399–406, 2005.
- [9] A. Dembinski, Z. Warzecha, P. Ceranowicz et al., “Ghrelin attenuates the development of acute pancreatitis in rats,” *Journal of Physiology and Pharmacology*, vol. 54, no. 4, pp. 561–573, 2003.
- [10] A. Dembiński, Z. Warzecha, P. Ceranowicz et al., “Role of growth hormone and insulin-like growth factor-1 in the protective effect of ghrelin in ischemia/reperfusion-induced acute pancreatitis,” *Growth Hormone and IGF Research*, vol. 16, no. 5–6, pp. 348–356, 2006.
- [11] P. Ceranowicz, Z. Warzecha, A. Dembinski et al., “Treatment with ghrelin accelerates the healing of acetic acid-induced gastric and duodenal ulcers in rats,” *Journal of Physiology and Pharmacology*, vol. 60, no. 1, pp. 87–98, 2009.
- [12] Z. Warzecha, D. Ceranowicz, A. Dembiński et al., “Ghrelin accelerates the healing of cysteamine-induced duodenal ulcers in rats,” *Medical Science Monitor*, vol. 18, no. 5, pp. BR181–BR187, 2012.
- [13] Z. Warzecha, P. Kownacki, P. Ceranowicz, M. Dembinski, J. Cieszkowski, and A. Dembinski, “Ghrelin accelerates the healing of oral ulcers in non-sialoadenectomized and sialoadenectomized rats,” *Journal of Physiology and Pharmacology*, vol. 64, no. 5, pp. 657–668, 2013.
- [14] P. C. Konturek, T. Brzozowski, M. Engel et al., “Ghrelin ameliorates colonic inflammation. Role of nitric oxide and sensory nerves,” *Journal of Physiology and Pharmacology*, vol. 60, no. 2, pp. 41–47, 2009.
- [15] O. Pamukcu, Z. N. O. Kumral, F. Ercan, B. Ç. Yegen, and D. Ertem, “Anti-inflammatory effect of obestatin and ghrelin in dextran sulfate sodium-induced colitis in rats,” *Journal of Pediatric Gastroenterology and Nutrition*, vol. 57, no. 2, pp. 211–218, 2013.
- [16] Z. Warzecha, P. Ceranowicz, A. Dembinski et al., “Therapeutic effect of ghrelin in the course of cerulein-induced acute pancreatitis in rats,” *Journal of Physiology and Pharmacology*, vol. 61, no. 4, pp. 419–427, 2010.
- [17] D. Ceranowicz, Z. Warzecha, A. Dembinski et al., “Role of hormonal axis, growth hormone—IGF-1, in the therapeutic effect of ghrelin in the course of cerulein-induced acute pancreatitis,” *Journal of Physiology and Pharmacology*, vol. 61, no. 5, pp. 599–606, 2010.
- [18] J. Bukowczan, Z. Warzecha, P. Ceranowicz, B. Kusnierz-Cabala, R. Tomaszewska, and A. Dembinski, “Therapeutic effect of ghrelin in the course of ischemia/reperfusion-induced acute pancreatitis,” *Current Pharmaceutical Design*, vol. 21, no. 17, pp. 2284–2290, 2015.
- [19] P. Ceranowicz, J. Cieszkowski, Z. Warzecha, B. Kuśnierz-Cabala, and A. Dembiński, “The beginnings of pancreatology as a field of experimental and clinical medicine,” *BioMed Research International*, vol. 2015, Article ID 128095, 5 pages, 2015.
- [20] P. Ceranowicz, Z. Warzecha, A. Dembinski et al., “Pretreatment with obestatin inhibits the development of cerulein-induced pancreatitis,” *Journal of Physiology and Pharmacology*, vol. 60, no. 3, pp. 95–101, 2009.
- [21] J. Bukowczan, Z. Warzecha, P. Ceranowicz, B. Kuśnierz-Cabala, R. Tomaszewska, and A. Dembinski, “Pretreatment

- with obestatin reduces the severity of ischemia/reperfusion-induced acute pancreatitis in rats," *European Journal of Pharmacology*, vol. 760, pp. 113–121, 2015.
- [22] R. Granata, F. Settanni, D. Gallo et al., "Obestatin promotes survival of pancreatic β -cells and human islets and induces expression of genes involved in the regulation of β -cell mass and function," *Diabetes*, vol. 57, no. 4, pp. 967–979, 2008.
- [23] A. Dembiński, Z. Warzecha, P. Ceranowicz et al., "Administration of obestatin accelerates the healing of chronic gastric ulcers in rats," *Medical Science Monitor*, vol. 17, no. 8, pp. BR196–BR200, 2011.
- [24] E. Alexandridis, A. Zisimopoulos, N. Liratzopoulos, I. Katsos, K. Manolas, and G. Kouklakis, "Obestatin/ghrelin ratio: a new activity index in inflammatory bowel diseases," *Inflammatory Bowel Diseases*, vol. 15, no. 10, pp. 1557–1561, 2009.
- [25] J. Y. Jung, J. B. Jeong, J. W. Kim et al., "Circulating ghrelin levels and obestatin/ghrelin ratio as a marker of activity in ulcerative colitis," *Intestinal Research*, vol. 13, no. 1, pp. 68–73, 2015.
- [26] B. R. MacPherson and C. J. Pfeiffer, "Experimental production of diffuse colitis in rats," *Digestion*, vol. 17, no. 2, pp. 135–150, 1978.
- [27] P. K. Randhawa, K. Singh, N. Singh, and A. S. Jaggi, "A review on chemical-induced inflammatory bowel disease models in rodents," *Korean Journal of Physiology and Pharmacology*, vol. 18, no. 4, pp. 279–288, 2014.
- [28] K. Johansson, H. Ahn, J. Lindhagen, and O. Lundgren, "Tissue penetration and measuring depth of laser doppler flowmetry in the gastrointestinal application," *Scandinavian Journal of Gastroenterology*, vol. 22, no. 9, pp. 1081–1088, 1987.
- [29] A. Dembiński, Z. Warzecha, S. J. Konturek, R. Z. Cai, and A. V. Schally, "The effects of antagonists of receptors for gastrin, cholecystokinin and bombesin on growth of gastroduodenal mucosa and pancreas," *Journal of Physiology and Pharmacology*, vol. 42, no. 2, pp. 195–209, 1991.
- [30] P. P. Bradley, D. A. Priebe, R. D. Christensen, and G. Rothstein, "Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker," *Journal of Investigative Dermatology*, vol. 78, no. 3, pp. 206–209, 1982.
- [31] P. Greant, G. Delvaux, and G. Willems, "Influence of stress on epithelial cell proliferation in the gut mucosa of rats," *Digestion*, vol. 40, no. 4, pp. 212–218, 1988.
- [32] P. A. Hall, P. J. Coates, B. Ansari, and D. Hopwood, "Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis," *Journal of Cell Science*, vol. 107, no. 12, pp. 3569–3577, 1994.
- [33] S. J. Konturek, A. Dembinski, Z. Warzecha, T. Brzozowski, and H. Gregory, "Role of epidermal growth factor in healing of chronic gastroduodenal ulcers in rats," *Gastroenterology*, vol. 94, no. 6, pp. 1300–1307, 1988.
- [34] T. Brzozowski, P. C. Konturek, S. J. Konturek et al., "Effect of local application of growth factors on gastric ulcer healing and mucosal expression of cyclooxygenase-1 and -2," *Digestion*, vol. 64, no. 1, pp. 15–29, 2001.
- [35] S. Beckert, N. Class, F. Farrahi, and S. Coerper, "Growth hormone enhances gastric ulcer healing in rats," *Medical Science Monitor*, vol. 10, no. 8, pp. BR255–BR258, 2004.
- [36] D. K. Podolsky, "Regulation of intestinal epithelial proliferation: a few answers, many questions," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 264, no. 2, pp. G179–G186, 1993.
- [37] H. Sørbye and K. Svanes, "The role of blood flow in gastric mucosal defence, damage and healing," *Digestive Diseases*, vol. 12, no. 5, pp. 305–317, 1994.
- [38] R. C. Orlando, "The integrity of the esophageal mucosa. Balance between offensive and defensive mechanisms," *Best Practice and Research: Clinical Gastroenterology*, vol. 24, no. 6, pp. 873–882, 2010.
- [39] F. W. Leung, K. C. Su, J. M. Pique, G. Thieflin, E. Passaro Jr., and P. H. Guth, "Superior mesenteric artery is more important than inferior mesenteric artery in maintaining colonic mucosal perfusion and integrity in rats," *Digestive Diseases and Sciences*, vol. 37, no. 9, pp. 1329–1335, 1992.
- [40] C. A. Dinarello, "Immunological and inflammatory functions of the interleukin-1 family," *Annual Review of Immunology*, vol. 27, pp. 519–550, 2009.
- [41] C. A. Dinarello, A. Simon, and J. W. M. van der Meer, "Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases," *Nature Reviews Drug Discovery*, vol. 11, no. 8, pp. 633–652, 2012.
- [42] J. Norman, M. Franz, J. Messina et al., "Interleukin-1 receptor antagonist decreases severity of experimental acute pancreatitis," *Surgery*, vol. 117, no. 6, pp. 648–655, 1995.
- [43] C. A. Dinarello, "A clinical perspective of IL-1 β as the gatekeeper of inflammation," *European Journal of Immunology*, vol. 41, no. 5, pp. 1203–1217, 2011.
- [44] H. D. De Koning, J. Schalkwijk, J. van der Ven-Jongekrijg, M. Stoffels, J. W. M. van der Meer, and A. Simon, "Sustained efficacy of the monoclonal anti-interleukin-1 beta antibody canakinumab in a 9-month trial in Schnitzler's syndrome," *Annals of the Rheumatic Diseases*, vol. 72, no. 10, pp. 1634–1638, 2013.
- [45] T. Herlin, B. Fiirgaard, M. Bjerre et al., "Efficacy of anti-IL-1 treatment in Majeed syndrome," *Annals of the Rheumatic Diseases*, vol. 72, no. 3, pp. 410–413, 2013.
- [46] S. J. Klebanoff, "Myeloperoxidase: friend and foe," *Journal of Leukocyte Biology*, vol. 77, no. 5, pp. 598–625, 2005.
- [47] F. J. Romero, F. Bosch-Morell, M. J. Romero et al., "Lipid peroxidation products and antioxidants in human disease," *Environmental Health Perspectives*, vol. 106, no. 5, pp. 1229–1234, 1998.
- [48] M. D. Evans and M. S. Cooke, "Factors contributing to the outcome of oxidative damage to nucleic acids," *BioEssays*, vol. 26, no. 5, pp. 533–542, 2004.
- [49] S. Kwiecién, K. Jasnos, M. Magierowski et al., "Lipid peroxidation, reactive oxygen species and antioxidative factors in the pathogenesis of gastric mucosal lesions and mechanism of protection against oxidative stress—induced gastric injury," *Journal of Physiology and Pharmacology*, vol. 65, no. 5, pp. 613–622, 2014.
- [50] K. M. Mullane, R. Kraemer, and B. Smith, "Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium," *Journal of Pharmacological Methods*, vol. 14, no. 3, pp. 157–167, 1985.
- [51] Z. Warzecha and A. Dembiński, "Protective and therapeutic effects of ghrelin in the gut," *Current Medicinal Chemistry*, vol. 19, no. 1, pp. 118–125, 2012.
- [52] B. Holst, K. L. Egerod, E. Schild et al., "GPR39 signaling is stimulated by zinc ions but not by obestatin," *Endocrinology*, vol. 148, no. 1, pp. 13–20, 2007.
- [53] A. R. Jurjus, N. N. Houry, and J.-M. Reimund, "Animal models of inflammatory bowel disease," *Journal of Pharmacological and Toxicological Methods*, vol. 50, no. 2, pp. 81–92, 2004.