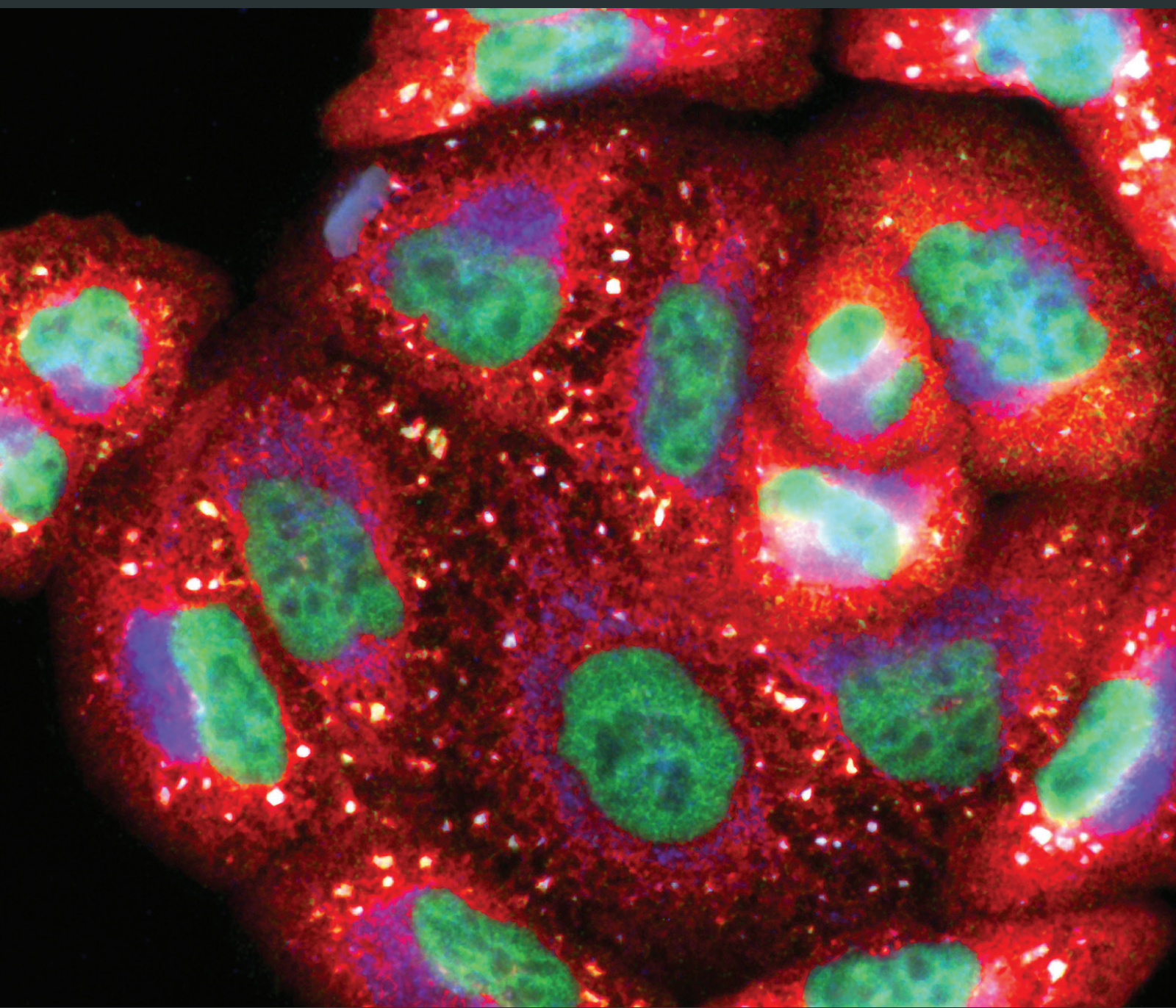
Oxidative Medicine and Cellular Longevity

Harmful and Beneficial Role of ROS 2017

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



Harmful and Beneficial Role of ROS 2017

Oxidative Medicine and Cellular Longevity

Harmful and Beneficial Role of ROS 2017

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

Editorial Board

Contents

Harmful and Beneficial Role of ROS 2017
Sergio Di Meo, Tanea T. Reed, Paola Venditti, and Victor M. Victor
Volume 2018, Article ID 5943635, 2 pages

The Good, the Bad, and the Ugly of ROS: New Insights on Aging and Aging-Related Diseases from Eukaryotic and Prokaryotic Model Organisms
Ana L. Santos, Sanchari Sinha, and Ariel B. Lindner
Volume 2018, Article ID 1941285, 23 pages

ROS-Induced DNA Damage Associates with Abundance of Mitochondrial DNA in White Blood Cells of the Untreated Schizophrenic Patients
I. V. Chestkov, E. M. Jestkova, E. S. Ershova, V. G. Golimbet, T. V. Lezheiko, N. Yu Kolesina, O. A. Dolgikh, V. L. Izhevskaya, G. P. Kostyuk, S. I. Kutsev, N. N. Veiko, and S. V. Kostyuk
Volume 2018, Article ID 8587475, 7 pages

Cadmium-Induced Oxidative Stress Impairs Glycemic Control in Adolescents
Gabriele Pizzino, Natasha Irrera, Alessandra Bitto, Giovanni Pallio, Federica Mannino, Vincenzo Arcoraci, Federica Aliquò, Letteria Minutoli, Chiara De Ponte, Paola D'andrea, Francesco Squadrito, and Domenica Altavilla
Volume 2017, Article ID 6341671, 6 pages

Effect of Bioactive Compound of *Aronia melanocarpa* on Cardiovascular System in Experimental Hypertension
Martina Cebova, Jana Klimentova, Pavol Janega, and Olga Pechanova
Volume 2017, Article ID 8156594, 8 pages

The Role of Oxidative Stress in Decreased Acetylcholinesterase Activity at the Neuromuscular Junction of the Diaphragm during Sepsis
Hua Liu, Jin Wu, Jun-yan Yao, Hong Wang, and Shi-tong Li
Volume 2017, Article ID 9718615, 6 pages

Molecular Mechanisms Responsible for Increased Vulnerability of the Ageing Oocyte to Oxidative Damage
Bettina P. Mihalas, Kate A. Redgrove, Eileen A. McLaughlin, and Brett Nixon
Volume 2017, Article ID 4015874, 22 pages

Oxidative Stress and Endometriosis: A Systematic Review of the Literature
Gennaro Scutiero, Piergiorgio Iannone, Giulia Bernardi, Gloria Bonaccorsi, Savino Spadaro, Carlo Alberto Volta, Pantaleo Greco, and Luigi Nappi
Volume 2017, Article ID 7265238, 7 pages

Benign Effect of Extremely Low-Frequency Electromagnetic Field on Brain Plasticity Assessed by Nitric Oxide Metabolism during Poststroke Rehabilitation
Natalia Cichoń, Piotr Czarny, Michał Bijak, Elżbieta Miller, Tomasz Śliwiński, Janusz Szemraj, and Joanna Saluk-Bijak
Volume 2017, Article ID 2181942, 9 pages

Cell Signaling with Extracellular Thioredoxin and Thioredoxin-Like Proteins: Insight into Their Mechanisms of Action
Thierry Léveillard and Najate Aït-Ali
Volume 2017, Article ID 8475125, 11 pages

Chronic Variable Stress Is Responsible for Lipid and DNA Oxidative Disorders and Activation of Oxidative Stress Response Genes in the Brain of Rats
Mariola Herbet, Agnieszka Korga, Monika Gawrońska-Grzywacz, Magdalena Izdebska, Iwona Piątkowska-Chmiel, Ewa Poleszak, Andrzej Wróbel, Włodzimierz Matysiak, Barbara Jodłowska-Jędrych, and Jarosław Dudka
Volume 2017, Article ID 7313090, 10 pages

The Contribution of Singlet Oxygen to Insulin Resistance
Arnold N. Onyango
Volume 2017, Article ID 8765972, 14 pages

Total Oxidant and Antioxidant Status in Prepubertal Children with Obesity
Grażyna Rowicka, Hanna Dyląg, Jadwiga Ambroszkiewicz, Agnieszka Riahi, Halina Weker, and Magdalena Chełchowska
Volume 2017, Article ID 5621989, 6 pages

Resveratrol-Enriched Rice Attenuates UVB-ROS-Induced Skin Aging via Downregulation of Inflammatory Cascades
Lalita Subedi, Taek Hwan Lee, Hussain Mustatab Wahedi, So-Hyeon Baek, and Sun Yeou Kim
Volume 2017, Article ID 8379539, 15 pages

Oxidative Stress: Harms and Benefits for Human Health
Gabriele Pizzino, Natasha Irrera, Mariapaola Cucinotta, Giovanni Pallio, Federica Mannino, Vincenzo Arcoraci, Francesco Squadrito, Domenica Altavilla, and Alessandra Bitto
Volume 2017, Article ID 8416763, 13 pages

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 5943635, 2 pages
https://doi.org/10.1155/2018/5943635

Editorial
Harmful and Beneficial Role of ROS 2017

Sergio Di Meo,[1] **Tanea T. Reed**,[2] **Paola Venditti**,[1] **and Victor M. Victor**[3,4]

[1]*Dipartimento di Biologia, Università di Napoli "Federico II", 80126 Napoli, Italy*
[2]*Department of Chemistry, Eastern Kentucky University, Richmond, KY 40475, USA*
[3]*Service of Endocrinology, Dr. Peset University Hospital, Foundation for the Promotion of Health and Biomedical Research in the Valencian Region (FISABIO), 46010 Valencia, Spain*
[4]*Department of Physiology, University of Valencia, Valencia, Spain*

Correspondence should be addressed to Sergio Di Meo; sergio.dimeo@unina.it

Received 31 January 2018; Accepted 1 February 2018; Published 2 April 2018

Over the years, the idea of a contrasting dual role played by free radicals in living organisms has become increasingly validated. On the one hand, a look at the recent literature confirms that free radicals are oxidizing agents implicated in tissue damage and that uncontrolled oxidative activity underlies various pathological conditions and can be countered by antioxidant treatment. On the other hand, research shows that oxidants exert beneficial effects by regulating cell signaling cascades. Further support for the idea of harmful and beneficial roles of free radicals is provided by the articles in the present special issue.

One paper examines extracellular thioredoxins, small oxidoreductase enzymes that control redox homeostasis in the cell via NADPH, which is generated by the pentose phosphate pathway as a cofactor of thioredoxin reductase. T. Léveillard and N. Aït-Ali chronologically reviewed key findings concerning extracellular thioredoxins. Starting with the discovery of human thioredoxin (TXN1), first identified as the adult T-cell leukemia-derived factor (ADF), a secreted protein, they go on to examine the extracellular truncated thioredoxin RdCVF encoded by the *NXNL1* gene, a unique example of a complete extracellular thioredoxin signaling system.

Several papers deal with the damaging effects of ROS and, in some cases, the protective effects of antioxidant intervention. The work by M. Cebova et al. shows that the polyphenol-rich extract of *Aronia melanocarpa* has a positive effect on blood pressure, NO-synthase activity, and proinflammatory processes in experimentally induced hypertension, a condition normally associated with endothelial dysfunction and oxidative stress.

I. V. Chestkov et al. show that the leukocytes of male paranoid schizophrenia patients contain more mitochondrial DNA (mtDNA) than those of both male patients treated with antipsychotic medication and healthy controls. Furthermore, the mtDNA content of unmedicated patients positively correlates with the level of 8-oxodG, a marker of DNA oxidation.

M. Herbet et al. studied biochemical and molecular changes associated with ROS generation in the brains of rats submitted to variable levels of chronic environmental stress. Their results show that this stress causes lipid and DNA oxidative damage and disruption of the antioxidant defense system, including decreased expression of gene encoding antioxidative transcriptional factors. Furthermore, they detected activation of oxidative stress-responsive genes such as 8-oxoguanine glycosylase1 and methionine sulfoxide reductase A, which play a role in triggering the oxidative DNA repair system.

L. Subedi et al. evaluated the efficacy of the antiaging compound resveratrol contained in genetically modified normal edible rice as a treatment for skin aging caused by chronic exposure to ultraviolet radiation. They found that this resveratrol-enriched rice overcomes the usual drawbacks of resveratrol and enhances its antiaging potential by controlling major pathways of skin aging.

H. Liu et al. investigated whether decreased AChE activity during sepsis is related to oxidative stress by observing AChE activity in different grades of sepsis induced by caecal ligation

and puncture. Their results show that AChE activity at the neuromuscular junction of the diaphragm decreases more significantly during severe sepsis. Furthermore, this activity is significantly and negatively correlated with the level of oxidative stress during sepsis.

The review by B. P. Mihalas et al. explores the reduced capacity of the aging oocyte to mitigate macromolecular damage arising from oxidative insults and highlights the dramatic consequences for oocyte quality and female fertility. It discusses how impaired ROS metabolism, decreased DNA repair, reduced sensitivity of the spindle assembly checkpoint, and decreased capacity for protein repair and degradation collectively render the aged oocyte acutely vulnerable to oxidative stress.

G. Pizzino et al. studied glycemic control and oxidative stress markers in male adolescents with increased urinary levels of cadmium. Their results indicate that cadmium burden alters glycemic control in adolescents and that oxidative stress plays a key role in cadmium-induced insulin resistance, thus increasing the risk of developing metabolic disorders.

G. Rowicka et al. evaluated the presence of oxidative stress in obese prepubertal children. They found a significant negative correlation between total antioxidant capacity and ox-LDL concentrations. Furthermore, obesity duration was positively correlated with total oxidative capacity level, which suggests that obesity-related oxidative stress already occurs in prepubescence.

The review by G. Scutiero et al. focuses on how a disruption of the balance between ROS production and antioxidant defenses and the oxidative stress, occuring as a consequence, affects the development and progression of endometriosis. The study of aspects such as iron metabolism, oxidative stress markers, genes involved in oxidative stress, endometriosis-associated infertility, and cancer development supports the role of oxidative stress in the development and progression of endometriosis.

A. N. Onyango reviews the literature which suggests that insulin-responsive cells such as endothelial cells, hepatocytes, adipocytes, and myocytes also produce singlet oxygen, thus contributing to insulin resistance, for example, by generating bioactive aldehydes, inducing endoplasmic reticulum (ER) stress, and modifying mitochondrial DNA.

One paper by N. Cichoń et al. examines the beneficial effect of nitrogen radicals such as nitric oxide ($NO^{\bullet}$). Indeed, it investigated the effect of an extremely low-frequency electromagnetic field (ELF-EMF) on the generation and metabolism of $NO^{\bullet}$ as a neurotransmitter on the rehabilitation of poststroke patients. They observed that the application of ELF-EMF significantly increased 3-nitrotyrosine and nitrate/nitrite levels, while *NOS2* expression was insignificantly decreased. Their results showed that ELF-EMF treatments also improve functional and mental status. The authors conclude that ELF-EMF therapy can promote recovery in poststroke patients.

Two other reviews deal with harmful and beneficial roles of ROS. A. L. Santos et al. summarize recent advances in research in the field and show that this dual role is found across species, from bacteria to humans, and in various aspects of cellular physiology. They also highlight the utility of bacterial models to elucidate the molecular pathways by which ROS mediate aging and aging-related diseases. G. Pizzino et al. describe recent findings regarding oxidative stress, highlighting both its positive and negative influence on human health. The authors show that oxidative stress and free radicals are generally detrimental to human health, contributing to the initiation and progression of several pathologies ranging from cardiovascular disease to cancer. On the other hand, they discuss how some prooxidant compounds or agents can benefit human health, particularly regarding cancer treatment.

Sergio Di Meo
Tanea T. Reed
Paola Venditti
Victor M. Victor

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 1941285, 23 pages
https://doi.org/10.1155/2018/1941285

Review Article

The Good, the Bad, and the Ugly of ROS: New Insights on Aging and Aging-Related Diseases from Eukaryotic and Prokaryotic Model Organisms

Ana L. Santos,[1] **Sanchari Sinha**,[2] **and Ariel B. Lindner**[1]

[1]*Institut National de la Santé et de la Recherche Médicale, U1001 & Université Paris Descartes, Sorbonne Paris Cité, Paris, France*
[2]*Defence Institute of Physiology and Allied Sciences, DRDO, New Delhi, India*

Correspondence should be addressed to Ana L. Santos; ana.santos@inserm.fr

Received 4 August 2017; Revised 18 December 2017; Accepted 2 January 2018; Published 18 March 2018

Academic Editor: Sergio Di Meo

Aging is associated with the accumulation of cellular damage over the course of a lifetime. This process is promoted in large part by reactive oxygen species (ROS) generated via cellular metabolic and respiratory pathways. Pharmacological, nonpharmacological, and genetic interventions have been used to target cellular and mitochondrial networks in an effort to decipher aging and age-related disorders. While ROS historically have been viewed as a detrimental byproduct of normal metabolism and associated with several pathologies, recent research has revealed a more complex and beneficial role of ROS in regulating metabolism, development, and lifespan. In this review, we summarize the recent advances in ROS research, focusing on both the beneficial and harmful roles of ROS, many of which are conserved across species from bacteria to humans, in various aspects of cellular physiology. These studies provide a new context for our understanding of the parts ROS play in health and disease. Moreover, we highlight the utility of bacterial models to elucidate the molecular pathways by which ROS mediate aging and aging-related diseases.

1. Introduction

Aging is characterized by a gradual loss of fitness over time. Aging is manifested as a series of dynamic changes at the molecular and macromolecular level over the course of a lifetime [1]. Faulty regulation of cellular processes can damage the cell's physiological integrity and subsequently lead to accumulation of damaged byproducts. Mankind has been fascinated with obtaining a better understanding of aging for many centuries, yet the exact mechanisms underlying the human aging process remain largely unclear. The aging process itself is complex due to several confounders, such as environmental factors, socioeconomic status, physical characteristics, and lifestyle [2].

Over the past few decades, life expectancy has increased linearly worldwide to an average of 60 years. The world's population over 60 is expected to increase from approximately 900 million people (12%) in 2015 to approximately 2 billion people (22%) in 2050 [3]. This increased life expectancy is associated with a reduced rate of child mortality, improved standards of living, and medical advancements, among others. Despite an increase in overall lifespan, aging and age-related diseases are major causes of mortality and morbidity worldwide [4]. Moreover, age-related disorders, such as Alzheimer's disease, dementia, cardiopulmonary disorders, diabetes, neurodegenerative and cognitive impairments, fragile physical condition, and psychosomatic disorders, are major causes of disability worldwide. These disorders account for over 20% of years lived with a disability [5]. Understanding the molecular mechanisms of aging is critical for developing therapeutic interventions that promote healthy aging.

Mitochondria often termed "the powerhouse of the cell," metabolize carbohydrates and fatty acids via oxidative phosphorylation. Through this process, the mitochondria can generate 32 to 34 adenosine triphosphate (ATP) molecules per molecule of glucose. The protein complexes in the inner mitochondrial membrane collectively form the

mitochondrial electron transport chain (ETC), which releases free radicals as byproducts of energy metabolism [6]. Harman originally proposed the free radical theory of aging in 1956 [7], according to which reactive oxygen species (ROS) are the primary mediators of the aging process. A brief overview of the sources of ROS and subsequent cellular responses is provided in Figure 1. The sources of ROS, antioxidant defenses, and subsequent biological effects have been reviewed elsewhere (e.g., [8]) and will not be covered in depth in this review. While extensive evidence indicates that enhanced ROS production and decreased ROS-scavenging ability shortens lifespan [9, 10], the free radical theory of aging has faced opposition, undermining the idea that ROS alone are responsible for the aging process. For instance, organisms can live a healthy lifespan in the absence of ROS scavengers [11–14]. Further, nutritional, pharmacological, and genetic interventions that increase production of ROS can promote longevity by activating mitochondrial oxidative phosphorylation and triggering downstream signaling pathways that promote an adaptive response [11, 15, 16], while pharmacological interventions that limit ROS production have been shown to shorten lifespan [11, 15].

While ROS and ROS-induced oxidative damage may not be the sole cause of the aging process, it is fairly consensual that ROS do play an important role in the molecular mechanisms that influence longevity. Thus, bridging the gap between the free radical theory and the current aging knowledge can help us to better understand how the interaction between ROS-induced oxidative damage and cellular metabolism affects aging and uncover genetic and pharmaceutical interventions that could modulate this interaction.

2. The Free Radical Theory of Aging and Beyond

Over the last few decades, the dominant aging model has been the free radical theory of aging. This theory states that organisms age because they accumulate oxidative damage produced by ROS. ROS are partially reduced metabolites of molecular oxygen generated by various metabolic reactions and cellular processes, such as respiration [11, 15]. Several studies support the free radical theory of aging. For instance, the garlic-derived thioallyl compounds S-allyl cysteine and S-allylmercaptocysteine have been shown to reduce ROS accumulation and increase *C. elegans* lifespan [17]. Similarly, treatment of *C. elegans* with four synthetic stilbene derivatives extended longevity by reducing ROS accumulation and oxidative stress [18].

However, recent research indicates that ROS play a more complex role in determining longevity than previously thought. For instance, *C. elegans* mutants lacking superoxide dismutase (SOD)—an enzyme that neutralizes the superoxide radical—while being more susceptible to multiple stressors, retain a normal lifespan [12]. In another study, deletion of the mitochondrial superoxide dismutase *sod-2* was actually found to extend the lifespan of *C. elegans* [19]. Furthermore, *C. elegans* lacking functional genes for subunits of the mitochondrial respiratory chain complexes I and III produce higher levels of superoxide, but they also have an extended lifespan. The extended lifespan of these knockouts can be completely abolished by treating them with the superoxide scavenger *N*-acetylcysteine [20]. Additionally, when wild-type *C. elegans* and the long-lived *clk-1* mitochondrial mutant were treated with paraquat, a superoxide generator, both the mean and maximum lifespan increased significantly [20].

2.1. Antioxidant Enzymes: Good or Bad? Antioxidant enzymes play a key role in the neutralization of various ROS. However, the relationship between antioxidant enzyme levels and lifespan is not straightforward.

Several studies investigating the role of the antioxidant defense system in regulating longevity have shown that increased resistance to oxidative stress can improve longevity in mice [21, 22]. For instance, Cu/Zn superoxide dismutase 1 knockout ($Sod1^{-/-}$) mice have significantly decreased lifespans. This reduced lifespan was associated with increased cellular senescence based on the increased expression of the senescence markers p16 and p21 [23]. Further, mitochondrial catalase overexpression has been connected to the increased median and maximum lifespan in transgenic mice overexpressing peroxisomal, nuclear, and mitochondrial catalases [24]. Mitochondrial catalase overexpression has also been shown to reduce various age-related pathological conditions, such as cardiac problems, inflammation-related disorders, and cancer [25].

However, other studies have found that increased antioxidant enzyme activity does not contribute to extended lifespan in rodents [26]. For instance, a study of transgenic mice overexpressing Cu/Zn SOD, Mn-SOD, and catalase, either alone or in combination, showed that overexpression of these enzymes did not significantly improve longevity compared with wild-type (WT) mice [27].

Glutathione peroxidase 1 (GPX1), the main isoform of the GPX protein family, is an important antioxidant enzyme that is ubiquitous in cells and plays an important role in the neutralization of hydrogen peroxides. While GPX1 expression has a protective effect against ROS-mediated cellular damage, *Gpx1*-knockout mice showed no evidence of increased oxidative damage to proteins and lipids, compared with their WT littermates [28]. By contrast, mice lacking both Mn-SOD and *Gpx1* had a higher level of oxidative DNA and protein damage, but their lifespan was not reduced compared with WT littermates [29]. Moreover, single-nucleotide polymorphisms of Mn-SOD and *Gpx1*genes have been shown to impact aging and longevity [30].

Another GPX family protein, GPX4, plays a major role in protecting the plasma membrane from peroxide-induced lipid damage. Null mutations of the *Gpx4* gene are lethal in mice. Ablation of GPX4 in a transgenic mice line (C57BL/6 background) resulted in increased oxidative damage in the brain as well as neuronal loss compared with WT mice [31]. Transgenic overexpression of GPX4 was shown to protect mice from the lethal null-mutation phenotype and prevented oxidative-stress-induced liver damage and cell death [32]. However, mice with reduced GPX4 expression and activity showed no significant differences in mean, median, and maximal lifespan compared with WT mice [33].

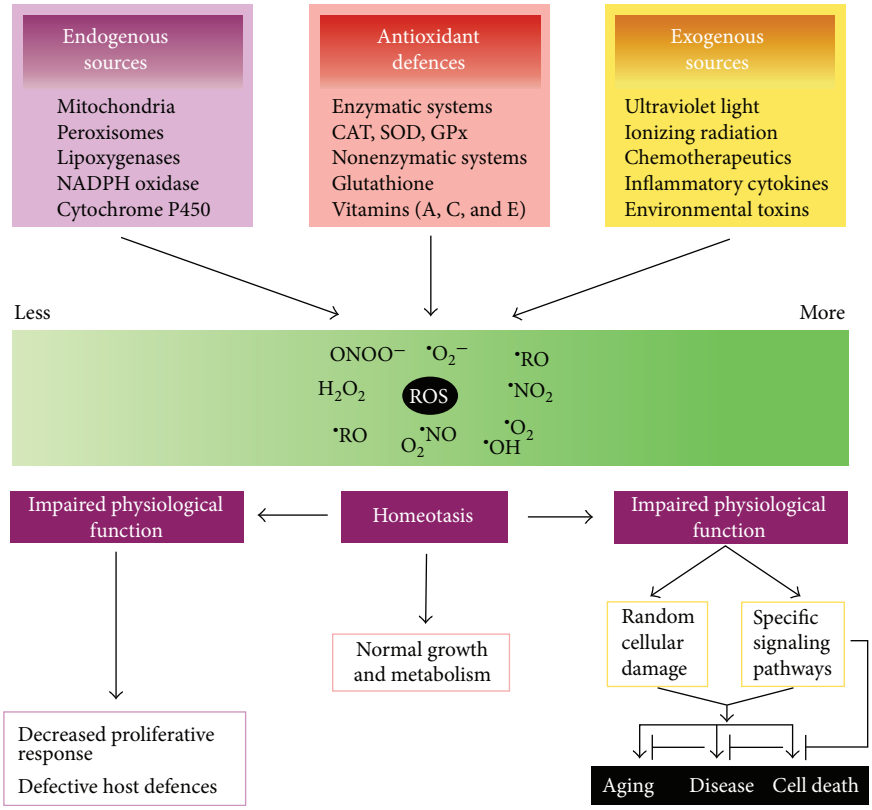


Figure 1: The sources and cellular responses to reactive oxygen species (ROS). Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS production. A sophisticated enzymatic and nonenzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defense. Similarly, increased ROS may also be detrimental and lead to cell death or to an acceleration in aging and age-related diseases. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids, and DNA. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redox-sensitive signaling pathways. Once activated, these diverse signaling pathways may have either damaging or potentially protective functions. Reproduced with permission from T. Finkel and N.J. Holbrook: Oxidants, oxidative stress and the biology of aging. *Nature*, vol. 408, no. 6809, pp.239-247, 2000.

Thioredoxin (Trx) is a redox protein that acts as a hydrogen donor in many reductive reactions in cells. It has two forms: cytoplasmic (Trx1) and mitochondrial (Trx2). Similar to *Gpx4*, *Trx2* null mutations are lethal in mice [34], and *Trx2* knockout impairs mitochondrial function by decreasing ATP production; increasing ROS production; inducing oxidative DNA, protein, and lipid damage in the liver; and increasing oxidative-stress-induced apoptosis of liver cells [35]. Trx1 overexpression (Tg(*TRX1*)$^{+/0}$) has been shown to protect against oxidative damage of cellular macromolecules and extend the earlier part of the lifespan in male mice; however, neither male nor female Tg(*TRX1*)$^{+/0}$ mice showed changes in maximum lifespan [36].

The cellular location of ROS production may determine whether ROS play a beneficial or detrimental role. For instance, deletion of mitochondrial *sod-2* in *C. elegans* has been shown to promote longevity, whereas deletion of cytoplasmic *sod-1* and *sod-5* limits lifespan [37]. ROS produced by mitochondrial respiratory complex I reverse electron transport have been shown to improve lifespan in *Drosophila* [38]. Moreover, respiration inhibition appears to activate the hypoxia-inducible factor-1 (HIF-1) by elevating ROS levels. This activation has been shown to increase longevity [17, 18]. Studies in genetically modified mice have shown that a moderately impaired mitochondrial function can result in healthier aging, whereas severely altered mitochondrial homeostasis can be detrimental [39, 40]. Based on these observations, it is clear that both the level and location of ROS production contribute to determining the role of ROS in regulating longevity [41].

3. Role of ROS in Nuclear and Mitochondrial DNA Damage

Nuclear and mitochondrial DNA damage caused by ROS contributes significantly to the aging process. Under normal physiological conditions, a myriad of DNA repair mechanisms work in harmony to keep damage contained. Base excision repair, mismatch repair, nucleotide excision repair, and double-strand-break repair all work rigorously to mend DNA damage induced by ROS, X-rays, UV and ionizing radiation, alkaline agents, replication errors, antitumor

agents, and various chemical agents [42]. Deficiencies in any of these repair mechanisms can accelerate the onset of aging [43].

The DNA theory of aging, first postulated by Szilard in 1959 [44], correlates the steady accumulation of DNA damage with imbalances in cellular function, ultimately leading to cell and organismal aging. Vilenchik and Knudson [45] calculated that the mammalian genome can sustain as many as 1000 lesions per hour per cell. These lesions include oxidative damage to bases, cross-linkages, and single-/double-strand breaks. Endogenous ROS usually cause the formation of abasic sites by breaking the glycosidic bonds between nucleotide bases and deoxyribose residues [46, 47]. Environmental agents like UV rays and chemical mutagens cause strand breaks through base modifications and intercalations [48, 49]. When unrepaired damage accumulates, it triggers the DNA damage response (DDR) [50, 51], which activates DNA repair systems [43]. Despite the number of lesions from which the genome suffers, the frequency of actual mutations is much lower, precisely because of these well-coordinated sensing and repair systems. However, when DNA repair mechanisms are overwhelmed or become dysfunctional, the DDR triggers senescence or apoptosis to suspend or eliminate the damaged cells, respectively. The accumulation of senescent cells in aging tissues [32] has been implicated as the driving force in the aging process, primarily through inflammatory pathways [33].

DNA repair can be divided into three types: base excision repair (BER), nucleotide excision repair (NER), and nonhomologous end joining (NHEJ). These processes have been reviewed exhaustively in the literature [52–54]. BER typically repairs oxidative DNA damage, most commonly the 8-oxoguanine lesion [55]. Briefly, DNA glycosylases excise the damaged base and a polymerase inserts the correct nucleotide in its place [56]. NER corrects more complex lesions not associated with oxidative damage, such as adduct formation between bases and UV-ray-induced cross-linkages [57]. While excision repairs primarily occur during replication, NHEJ can repair DNA double-strand breaks during the resting state as well [58]. NHEJ is a 3-step process that starts with the binding of the broken strand end to the Ku protein. The damaged and/or mismatched nucleotides are then removed, and the correct sequence is synthesized by DNA polymerase [59].

Unsurprisingly, studies have observed an age-related decline in DNA repair protein levels and activities [55]. Reduced BER activity has been reported in different tissues in older humans [60] and in mice lacking sirtuin 6, a histone deacetylase that is active during DNA repair [61]. Decreased levels of Ku protein and other NHEJ mediators are seen during normal human aging and in cases of Alzheimer's disease [62]. Similarly, NHEJ activity also decreases in aged rats that have accumulated DNA strand breaks in their neurons [63].

The strongest evidence for the DNA theory of aging comes from human progeroid (i.e., premature aging) syndromes, such as Werner syndrome (WS), Bloom's syndrome (BS), and xeroderma pigmentosum (XP). These syndromes are caused by genomic instability and an underlying defect in DNA repair. WS and BS are caused by loss-of-function mutations in the *WRN* and *BLM* genes, respectively [64, 65]. These genes encode RecQ helicases, which are involved in both DNA replication and repair and are known to interact with the Ku protein [66, 67]. Murine knockouts of WRN and BLM have significant genomic instability and impaired DNA repair mechanisms compared with WT mice [68, 69]. XP is characterized by a mutation in the excision repair cross-complementation group 1 xeroderma pigmentosum group F (ERCC1-XPF) nuclease, which plays an important role in both NER and NHEJ repairs [70]. Mice lacking *ERCC1* show accelerated skin aging and increased DNA damage and cellular senescence compared with WT mice [71]. Replicative telomere shortening has been implicated in aging based on studies in the telomerase-knockout mouse model. This mouse model exhibits progeria and accumulates extensive DNA damage (reviewed by [72]). Telomere shortening also accompanies human progeria syndromes, such as WS and BS. More recent studies have directly linked defective DNA repair—specifically BER and NER—to the sites of telomere-uncapping-induced DDR [73, 74]. Examples of specific DNA damage repair and response defects that lead to genetic disorders in humans are shown in Figure 2. Thus, there is substantial evidence linking impaired DNA repair with aging syndromes; however, further studies are needed to provide a direct mechanistic link.

Since mitochondria are the main sites of ROS production, mitochondrial DNA (mtDNA) contains higher levels of oxidative damage and its mutation rate is significantly greater than that of the nuclear DNA [75]. In addition to their proximity to the sites of ROS generation, it is likely that the mitochondrial genomes are more prone to oxidative damage because histones and other chromatin-associated proteins, present in nuclear genomes where they act as scavengers of oxygen radicals, are absent in the mitochondria. The existence of repair of oxidative damage to mtDNA, originally reported in the early '90s, is well established [76–78]. BER appears to be the only excision repair process active in the mitochondrial genomes. All mtDNA repair proteins are encoded by the nuclear genome and imported into the mitochondrial matrix. Most mtDNA repair proteins discovered so far are isoforms of the nuclear BER proteins arising from differential splicing or truncation of the terminal sequences [79, 80]. The mitochondrial DNA polymerase γ (Polγ) and mtDNA ligase (Lig IIIα), involved in mtDNA replication, appear to also be functional in mitochondrial BER [79, 80].

Accumulation of somatic mtDNA mutations has been found to accelerate normal aging [81–84], leading to oxidative damage, energy failure, increased production of ROS, and accumulation of amyloid-beta peptide (Abeta) [85, 86], a key molecule in Alzheimer disease (AD) [83]. A vicious cycle ensues which reinforces mtDNA damage, the impairment of the mitochondrial respiration, and oxidative stress.

4. Role of ROS in Protein Homeostasis

Similar to DNA damage, age-related protein damage and the accumulation of damaged protein products contribute to aging. Therefore, it is critical to understand how ROS

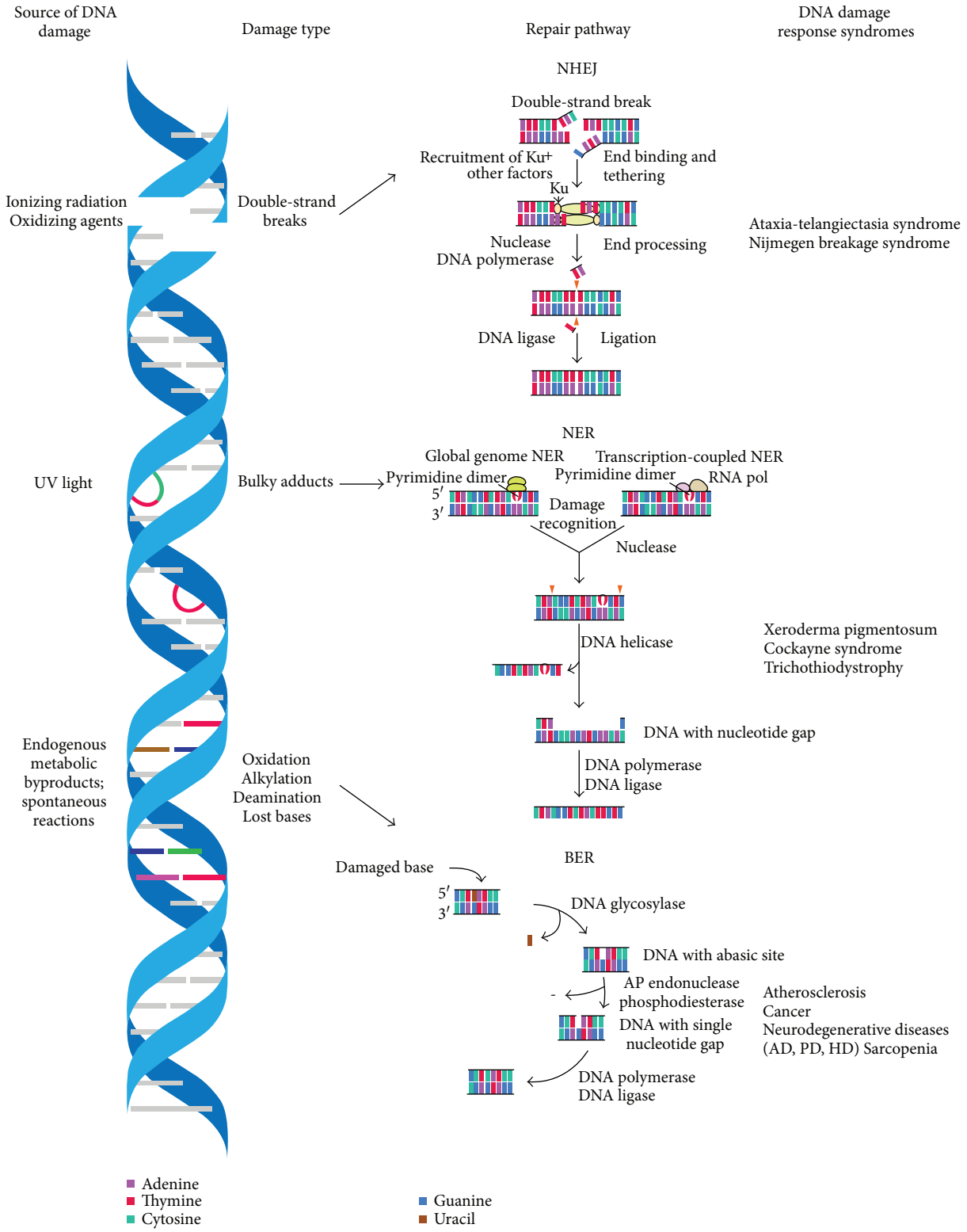


Figure 2: Examples of distinct DNA damage repair and response defects leading to genetic disorders in humans. Various damage types, including DNA double-strand breaks, bulky lesions, and base lesions, require nonhomologous end joining (NHEJ), nucleotide excision repair (NER), and base excision repair (BER), respectively. Defects in DNA-damage-response pathways lead to genome instability and, consequently, to complex syndromes characterized by tissue degeneration, cancer susceptibility, developmental defects, and premature aging. AD: Alzheimer's disease; PD: Parkinson's disease; HD: Huntington's disease.

contribute to an imbalance in cellular protein homeostasis and alter the aging process.

Free radicals can "attack" proteins, causing oxidative damage. Oxidative damage can alter protein function. Further, it can produce carbon-oxygen double bonds at arginine, lysine, proline, and threonine side chains, forming reactive ketones or aldehydes, known as protein carbonyls [87], normally considered to reflect the overall levels of cellular oxidative stress [88]. Protein carbonyls are associated with the production of aberrant protein isoforms [89, 90]. Unlike other oxidative modifications, such as disulfide bond formation, protein carbonylation is irreversible. Thus, the only means of limiting the damage caused by the affected proteins is their degradation. As more oxidative damage accumulates, proteins are more likely to misfold. Moderately oxidized proteins undergo degradation by the proteasome, the highly sophisticated protease complex designed to carry out selective, efficient, and processive degradation of short-lived, damaged, misfolded, or otherwise obsolete proteins [53]. However, heavily oxidized proteins can crosslink with other proteins, which prevents their degradation [54]. As a consequence, heavily damaged proteins accumulate within the cell, affecting its proper functioning. Accordingly, impaired proteostasis is a hallmark of many age-related diseases, including Alzheimer's and Parkinson's disease [91, 92].

Many studies have shown links between protein homeostasis, ROS, and oxidative stress. For instance, reducing insulin/IGF-1 signaling or inhibiting downstream mTOR signaling has been shown to improve the homeostasis of Alzheimer's disease-associated proteins, promoting longevity and protecting cognitive function in animal models [93]. Several studies in *C. elegans* have also shown that the heat shock factor 1 (HSF-1) works with the FOXO-like transcription factor, *daf-16*, to improve protein homeostasis and increase lifespan [94, 95]. Treating *C. elegans* with the amyloid-binding dye thioflavin T has been shown to reduce protein aggregation and extend lifespan via HSF-1- and SKN-1-/Nrf-mediated signaling [96]. Another study comparing the role of small heat shock proteins in *Drosophila* identified two proteins—CG14207 and HSP67BC—involved in proteostasis which mildly improved longevity when overexpressed in *Drosophila* [97].

Two important proteolytic pathways are the ubiquitin-proteasome pathway (UPP) and autophagy [98]. The UPP is a proteolytic system responsible for the majority of intracellular protein degradation. A key aspect of UPP-mediated proteolysis is the selective targeting of proteins for degradation via posttranslational modifications, particularly ubiquitination and sumoylation [77, 78]. Aging is associated with increased levels of ubiquitinated and sumoylated protein in various tissues [99–103], potentially as a result of age-dependent UPP malfunctioning [104, 105].

Ubiquitination pathways have been shown to play a significant role in regulating lifespan [106, 107]. In *Drosophila*, a loss-of-function mutation in the ubiquitin-activating enzyme Uba1 significantly reduced lifespan and weakened motor function [108]. In *C. elegans*, overexpression of the E3 ubiquitin ligase, WWP-1, increased lifespan via signaling mediated by the forkhead box A (FoxA) transcription factor [109].

Enhanced expression of the proteasome assembly protein Ump1 has also been associated with enhanced viability following exposure to various oxidative stress factors (e.g., menadione, hydrogen peroxide, and 4-hydroxynonenal) in *S. cerevisiae* [89]. This increased viability was associated with an enhanced preservation of proteasome-mediated protein degradation. Interestingly, cells expressing elevated levels of Ump1 also exhibited an enhanced preservation of proteasome-mediated protein degradation and enhanced viability during stationary-phase aging. Taken together, these data strongly support a key role of the proteasome during oxidative stress and aging [89].

Autophagy is also essential for maintaining protein homeostasis, as both cellular autophagy and mitophagy (autophagy of an entire mitochondrion) impact lifespan [90, 110]. Three autophagy proteins (LC3B, ATG5, and ATG12) play an important role in preserving mitochondrial integrity and lifespan [111]. In human umbilical vein endothelial cells, targeted mitochondrial damage was found to initiate a cascade of events involving a short-term increase in ROS production, followed by mitochondrial fragmentation and upregulation of LC3B, ATG5, and ATG12. This cascade significantly enhanced the replicative lifespan up to 150% and the number of population doublings up to 200% [111]. Additionally, in normal aging and during the progression of age-related pathologies, autophagy is responsible for the removal of proteins damaged by oxidation, for instance, from the brain to restore its proper function [112, 113].

During aging, mitochondria—the primary source of ROS—are often subjected to oxidative damage at a level that supersedes the protective capacity of the antioxidant response. In such cases, removal of damaged mitochondria through mitophagy is crucial to mitigate the detrimental effects on the organism [114]. Furthermore, in *C. elegans*, tight coupling between mitophagy and mitochondrial biogenesis is important for promoting longevity under stress conditions [115]. Also, in flies, overexpression of the mitophagy protein PARKIN has been shown to extend lifespan by enhancing mitochondrial turnover [116]. Therefore, mitophagy acts as a major marker of ROS-induced damage and plays a significant role in aging and various age-related disorders [117].

5. The Nucleus-Mitochondria Connection and the Importance of Mitochondrial Proteostasis

Nuclear DNA damage induces nuclear-to-mitochondrial signaling (NM signaling). This process plays a vital role in mitochondrial homeostasis and aging. Nuclear proteins (e.g., HIF-1α, proliferator-activated receptor gamma coactivator-1α (PGC-1α), forkhead box protein O (FOXO), and the sirtuin family) together with nuclear DNA damage repair proteins can affect mitochondrial integrity and contribute to age-related pathologies [118]. Recent studies have established an important connection between nicotinamide adenine

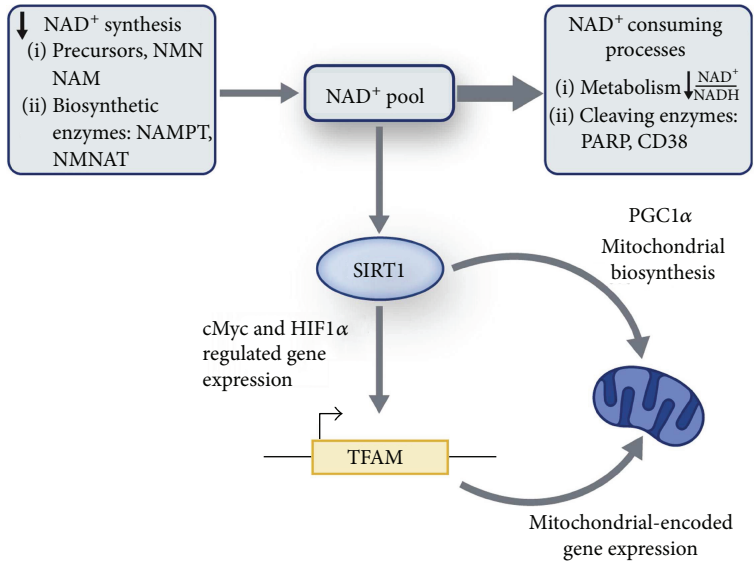


Figure 3: Age-dependent decline in NAD^+. Decreased NAD^+ synthesis and increased NAD^+ consumption with age may both contribute to a decrease in the NAD^+ pool. A reduction in NAD^+ levels leads to an age-related reduction of SIRT1 activity. Reduced SIRT1 activity impacts mitochondrial function through at least two mechanisms: (1) a reduction in biogenesis secondary due to a reduction in PGC1-α activity and (2) an impairment of mitochondrial function due to a reduction in mtDNA replication and transcription. Reproduced with permission from Prolla, T.A. and Denu, J.M., 2014. NAD+ deficiency in age-related mitochondrial dysfunction. Cell Metabolism, 19(2), pp.178-180.

dinucleotide (NAD^+) and DNA repair proteins in maintaining mitochondrial metabolism and increasing lifespan [119].

Sirtuins, NAD^+-dependent deacetylases, act as metabolic sensors that perceive imbalances in the NAD^+/NADH ratio. The inhibition of DNA repair proteins, specifically NAD^+-consuming poly (ADP-ribose) polymerase proteins (PARP-1 and PARP-2), increases cellular NAD^+ levels [119]. High NAD^+ levels subsequently activate sirtuins, which in turn promote higher mitochondrial content, increased energy expenditure, and protection against metabolic disease [119], ultimately extending longevity [120]. Furthermore, sirtuin activators, such as resveratrol, have been shown to promote longevity [121, 122] by inducing calorie restriction- (CR-) like effects in *C. elegans* [123].

However, both PARP and sirtuins must consume NAD^+ to be functional. Large amounts of PARP and sirtuins can deplete cellular NAD^+ levels. Depleted NAD^+ levels lead to sirtuin inactivation and excessive ROS production, which alters mitochondrial integrity [124]. Moreover, perturbations in the activity of sirtuins deactivate several enzymes including PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1α), forkhead box O (FOXO) transcription factors, hypoxia-inducible factor-1α (HIF-1α), and AMP-activated protein kinase (AMPK), which modulates the production of various antioxidative enzymes, affecting oxidative defense mechanisms [125].

DNA-damage-induced NM signaling through the PARP-NAD^+-sirtuin axis can accelerate the onset of aging by disrupting mitochondrial integrity. Thus, genetic or pharmacological interventions targeting proteins or metabolites involved in NM signaling can potentially promote longevity. For instance, in aging rats, treatment with the PARP inhibitor INO-1001 reduces cardiovascular disorders [126], and treatment with the PARP inhibitor PJ34 improves myocardial contractile function and restores endothelial function [127]. Furthermore, PARP-1 inhibition may protect against age-dependent endothelial dysfunction, potentially by regulating NO bioavailability via iNOS [128].

However, the beneficial role of PARP-1 inhibition in aging has been questioned [129]. For instance, PARP-1-null mice have a reduced lifespan, an earlier onset of aging, and an increased rate of spontaneous carcinogenesis compared with WT mice [130]. One explanation for discrepancies among studies is the dual role of PARP: while PARP contributes to maintain genomic stability and promote longevity, excessive PARP activity depletes cellular NAD^+ and triggers nuclear factor-κB- (NF-κB-) induced inflammation, leading to the rapid onset of aging and age-related disorders [131].

Aging is accompanied by decreased NAD^+ synthesis and increased NAD^+ consumption, resulting in a net decrease in the pool of available NAD^+ (Figure 3). Reduced NAD^+ levels lead to an age-related reduction of sirtuin 1 (SIRT1) activity. Reduced SIRT1 activity impacts mitochondrial function through at least two mechanisms: (1) reduced biogenesis secondary to a reduction in PGC1-α activity and (2) impaired mitochondrial function due to a reduction in mitochondrial DNA replication and transcription [132, 133]. Therefore, supplementation with NAD^+ or its precursors is hypothesized to promote healthy aging and longevity [134–136].

Experimental models have shown that NAD^+ supplementation is beneficial for maintaining carbohydrate metabolism, cardiovascular function, stem cell function, and longevity [137]. Moreover, nicotinamide prevents cellular senescence by reducing excessive ROS production [138, 139]. Several human clinical studies testing the efficacy of this compound are ongoing [140].

The NAD^+-mediated improvement in *C. elegans* lifespan was shown to involve a series of interconnected mechanisms

that include (1) activation of the worm sirtuin homolog Sir-2.1, (2) nuclear translocation and activation of the FOXO transcription factor *daf-16*, and (3) increased expression of antioxidative enzymes [141].

In a mouse model, treatment with the NAD^+ precursor nicotinamide riboside (NR) delayed muscle and neural stem cell senescence and increased longevity. This effect seemed to be mediated by the induction of the mitochondrial unfolded protein response (UPRmt) [142]. Involvement of the UPRmt in the lifespan-extending effect of NAD^+ has also been proposed in *C. elegans* [143].

The UPRmt is a form of retrograde signaling that contributes to ensuring the maintenance and functional integrity of the mitochondrial proteome [144]. Accumulation of misfolded proteins or unassembled complexes in the mitochondria beyond a certain threshold leads to altered proteostasis that can result in organelle/cell dysfunction [145]. Mitochondria relay this distress message to the cytosol and nucleus through various types of signals, and in response, the cell elicits a set of responses, including the production of mitochondrial localized molecular chaperones and proteases to promote the recovery of organellar protein homeostasis [91, 92, 146, 147].

An adaptive pathway triggered by a sirtuin-dependent UPRmt, which results in increased mitochondrial complex content and activity [143, 148], has been shown to lead to increased lifespan, at least in mice and flies [143, 142, 146]. Mitochondrial retrograde signaling to the nucleus via the mTOR pathway has also been found to extend normal human fibroblast lifespan, increase the mitochondrial membrane potential, reduce ROS level, and enhance autophagic flux [149]. ROS can exert an additional burden on the protein quality control system since protein chaperones themselves are susceptible to oxidative damage resulting in further damage accumulation and accelerated aging [4, 65].

Collectively, these studies establish a ROS-mediated connection between the mitochondria, the nucleus, and proteostasis.

6. Role of ROS in Nonpharmacological Strategies to Extend Lifespan

6.1. Calorie Restriction (CR). The term "caloric restriction" designates reduced energy intake without malnutrition, and it represents the most effective and reproducible dietary intervention known to promote healthy aging and slow down the manifestation of age-related disorders in various model organisms including yeast [150–153], nematodes [154, 155], fruit flies [156], mice [157–159], and primates [160]. CR regulates numerous physiological processes associated with aging, including metabolism [161–165], oxidative stress [166, 167], genomic stability [168], and growth signals [169–171].

Four major theories have been proposed to account for the beneficial effects of caloric restriction. According to the "oxidative damage attenuation" hypothesis, oxidative damage is decreased during caloric restriction (CR), through the decreased production of reactive oxygen species and the upregulation of protective enzymes, resulting in a decrease in DNA damage and increase in genomic stability [168, 172, 173]. The "glucose-insulin" hypothesis suggests that the decreased levels of circulating insulin and glucose that accompany CR lead to decreased cell growth and division, shifting the resources of the cell towards maintenance and repair [172, 173]. The related "insulin-like growth factor (IGF) 1" hypothesis suggests that decreased levels of growth hormone and IGF-1 in response to CR promote maintenance and repair activities [172, 173]. Finally, the "stress-adaptation" (or hormesis) hypothesis suggests that CR promotes a low level of stress which induces cross-adaptation to other stress factors by increasing the levels of antioxidant and DNA repair proteins [174].

Several molecular explanations for the lifespan-extending effects of CR have been proposed. However, much is still unknown about the precise contribution of each pathway to the lifespan-extension effect of CR. This understanding is further complicated by the extensive crosstalk between the different pathways and by the fact that some pathways are present in some model organisms but not in others. The complex network of pathways that are involved in the lifespan-extending effects of caloric restriction is depicted in Figure 4.

Two of the most studied pathways purportedly involved in the lifespan-mediated extension conferred by CR are those mediated by inhibition of insulin/IGF-1 signaling and inactivation of mTOR (mechanistic target of rapamycin). Both are considered nutrient-sensing pathways (insulin for glucose and mTOR for amino acids). Decreases in circulating levels of nutrients (amino acids, glucose, and even cholesterol)—all of which are also sensed by mTOR—contribute to decreased mTOR activity during CR [175]. mTOR inhibition leads to SKN-1-/Nrf- and *daf-16*-/FOXO-mediated activation of protective genes, resulting in an increase in stress resistance and longevity [176]. Additionally, inhibition of mTOR is known to induce autophagy, which has an important role in proteostasis during aging [177, 178]. The lifespan-extending effect of mTOR inhibition, either genetically or chemically, seems to be very conserved across different model organisms [159, 179–181]. The insulin pathway is mediated via several additional enzymes including PI3K/Akt/Ras and the forkhead O (FOXO) transcriptional factor [182–184].

The pathway mediated by adenosine monophosphate-activated protein kinase (AMPK) is a third possible CR-relevant pathway that can, in some organisms, crosstalk with the mTOR pathway. AMPK is a highly conserved sensor of increased levels of AMP and ADP originating from ATP depletion [185–187]. In general, activation of AMPK acts to maintain cellular energy stores, switching on catabolic pathways that produce ATP, mostly by enhancing oxidative metabolism and mitochondrial biogenesis, while switching off anabolic pathways that consume ATP. The importance of AMPK in determining lifespan is demonstrated by the fact that treatment with metformin, an AMPK activator, extends the lifespan of *C. elegans* and short-lived, cancer-prone mice strains [188–190].

One additional important pathway is that directed by sirtuins, the activity of which increases with CR. Association of sirtuins with decreased oxidative stress levels and increased

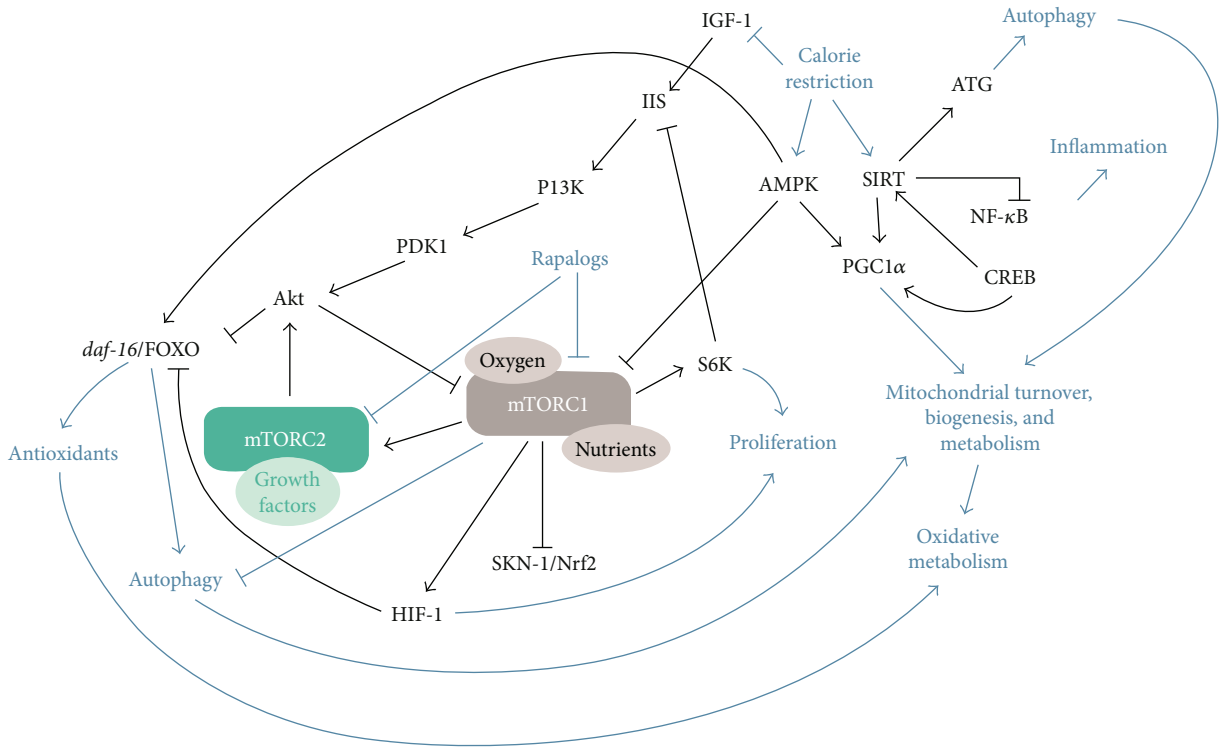


Figure 4: Crosstalk between mTOR and other longevity pathways. mTORC1 responds to a variety of environmental cues, including oxygen and nutrients, and communicates with several other known longevity factors in a complex network of interactions. Rapalogs inhibit mTORC and decrease its activity. Sensing of low oxygen levels stimulates mTORC1 to activate the hypoxic response by enhancing translation of HIF-1, which inhibits FOXO family members and increases longevity. mTORC1 inhibits the stress response transcription factor SKN-1/Nfr2, resulting in extended lifespan. Inhibition of the mTOR downstream effector ribosomal protein S6 kinase (S6K), involved in the regulation of protein translation, also results in extended lifespan. Caloric restriction can lower mTORC1 signaling partly through activation of AMPK, resulting in enhanced longevity, potentially via PGC1α-mediated increase in mitochondrial metabolism. Calorie restriction also inhibits IGF1-dependent signaling via PI3K/PDK1/Akt which inhibits FOXO, blocking the expression of antioxidants and autophagy. Calorie restriction leads to increased NAD^+/NADH ratio, which activates sirtuins, that in turn induce mechanisms to enhance cell protection, including enhanced antioxidant production and autophagy. Calorie restriction can also block inflammation via the effects of sirtuins on NF-κB. cAMP response element binding proteins (CREB) can also upregulate the transcription of sirtuins, slowing aging.

antioxidative defense has been proposed for several model organisms [191, 192], as well as humans, but the exact molecular mechanisms behind this association remain unclear. SIRT3 has been suggested as an essential player in enhancing the mitochondrial glutathione antioxidant defense system during caloric restriction [193]. SIRT3-dependent mitochondrial adaptation may also contribute to delaying aging in mammals [193].

Their role as mediators in the beneficial effects exerted by caloric restriction have made sirtuins promising pharmacological targets to delay aging and age-related diseases [194]. Resveratrol is a polyphenol antioxidant found in red wine and shown to activate sirtuins in several organisms, including humans [195, 196]. Resveratrol is also an AMPK activator, and this activity can also contribute to the beneficial effects of this polyphenol [197]. Purportedly, resveratrol upregulates antioxidant defense mechanisms and attenuates mitochondrial ROS production via sirtuin activation. Significant reduction of cellular hydrogen peroxide [198–200], upregulated MnSOD expression [195, 196], and increased cellular glutathione content [201] have been observed after resveratrol administration. The therapeutic potential of resveratrol has been the subject of intense research over the last decade (e.g., [195–198]).

CR has also been shown to reduce age-related accumulation of oxidative damage by decreasing mitochondrial respiration, membrane potential, and the rate of ROS production [166, 167], although CR seems to have only a minor effect on age-related changes in the mitochondrial proteome [202]. CR also increases mitochondrial biogenesis through the PGC-1α signaling pathway [203]. Moreover, other studies have also shown that CR protects from age-related vascular malfunctioning by increasing nitric oxide (NO) bioavailability, reducing ROS production, triggering anti-inflammatory responses, and preventing oxidative damage by activating the NRF-antioxidant response element (ARE) signaling pathway [204, 205].

Caloric restriction typically involves a 20–40% reduction of food consumption relative to normal intake. This is a rather severe intervention that can have detrimental effects [191]. Intermittent or periodic dietary restrictions without chronic caloric restriction have the potential to provide a significant health span increase while minimizing adverse effects. In fact, studies in rodents have shown that even a

10% decrease in food consumption can substantially affect lifespan [206]. *Sod*$^{-/-}$ mice show increased levels of oxidative stress, which in turn results in reduced lifespan. Dietary restriction (60% of ad libitum fed diet) was shown to increase the lifespan of *Sod*$^{-/-}$ mice by 30%, making it similar to that of wild-type, control mice fed ad libitum [207], by reducing lipid peroxidation in the liver and brain. The same dietary intervention was found to attenuate age-associated muscle atrophy by lowering oxidative stress in mice even in complete absence of the key antioxidant enzyme CuZnSOD [208].

6.2. Exercise. Exercise is another effective nonpharmacological means of delaying the negative effects of aging. Several studies reported elevated O_2 load in skeletal muscle fibers [209, 210] and increased ROS levels [209, 211] during exercise as a result of increased mitochondrial respiration required to generate ATP for muscle contractions. While mitochondrial oxidative phosphorylation is the primary source of exercise-induced ROS, xanthine oxidase and endothelial nitric oxide synthase (eNOS) also contribute to ROS generation during endurance training [212] and stretching exercises [40, 41]. Regular exercise has been associated with lowered mortality and incidence of age-related diseases [213–215]. Therefore, exercise interventions potentially could have benefits for older individuals through modulation of inflammatory and redox status, which can influence proteostasis, insulin sensitivity, body composition (e.g., adipose tissue), and hormone levels [216].

An aging-associated increase in ROS production in skeletal and cardiac muscle cells during rest and in a postexercise state has been reported [211, 217]. At the muscular level, age-related increases in ROS levels have been associated with various mechanisms, such as ETC dysregulation due to decreased activity of cytochrome c oxidase and other enzymes [218] and mitochondrial membrane disruption due to lipid peroxidation and unsaturation [44, 45].

However, conflicting results also have been reported. A study of the skeletal and cardiac muscle tissues of aged rats showed a significant increase in antioxidant enzymes, such as SOD, catalase, GPX [47–50], and glutathione (GSH) [51, 132]. Additionally, muscles that undergo chronic exercise show lower oxidative stress in terms of lipid, protein, and DNA damage in both humans and model organisms [38, 49, 133]. Accordingly, mitochondria isolated from trained muscle cells showed higher oxidative resistance *in vitro* [55, 219]. Studies also show an increase in SOD, GPX, and GSH levels following endurance training in both young and old individuals [56, 57]. These results suggest that regular physical exercise is accompanied by an adaptation of the cells to deal with oxidative stress, which in turn elicits beneficial effects, for example, in the immune system [220]. This idea is summarized by the concept of hormesis. Hormesis can be defined as the adaptive response seen in organisms continuously exposed to low to moderate levels of stress. Under these conditions, cells develop an adaptive response, including increased expression of antioxidant genes, which in turn makes them resistant to multiple stressors [221, 222].

The induction of hormesis is controlled by redox sensor pathways which, upon activation by oxidants, upregulate the antioxidant enzymatic system [223]. For example, intense physical exercise activates the mitogen-activated protein kinase (MAPK) and the NF-κB redox signaling pathways in both humans and rodents [60, 61]. The major targets of these pathways are antioxidant enzymes, including SOD, GPX, and GSH which contain NF-κB and activator protein-1 (AP-1) binding sites in their promoters [62–65] as well as responsive elements to various stimuli like proinflammatory cytokines, oxygen tension, and ROS [66–68]. In skeletal muscles, another crucial hormetic adaptation to oxidative stress is the increase in mitochondrial mass and protein content [69, 70], particularly the level of cytochrome c oxidase. Cytochrome c oxidase controls electron flow and the superoxide formation in the ETC [224]. These changes upregulate the expression of PGC-1, which drives mitochondrial biogenesis in skeletal muscles during exercise [225]. PGC-1 is also linked with reduced oxidative stress [226].

It has been hypothesized that this hormetic response to oxidative stress becomes impaired as skeletal muscles age [218, 227]. This hypothesis is supported by several studies reporting significantly lower NF-κB expression and activity in aged muscles [217, 228, 229]. By contrast, other studies have reported unchanged [230] or even higher [231] NF-κB levels at a resting state and decreased MAPK pathway activation postexercise in aged muscles of rodents and humans.

While exercise interventions have been proposed as effective, nonpharmacological means of delaying the negative effects of aging on functional and metabolic parameters [216], it is also well known that regular vigorous exercise can have detrimental effects, as evidenced by the enhanced susceptibility of elite athletes to infections [232]. This effect seems to be at least partly due to the detrimental effects of long-term exposure to the enhanced ROS production associated with intense exercise practice. For instance, chronic muscle injury, a common affliction of not only athletes but also older individuals, increases the production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which further contribute to oxidative stress that, in turn, exacerbates muscle inflammation, creating a vicious cycle of inflammation and oxidative damage [233].

Additional studies are needed to resolve the conflicting results regarding the effects of exercise and exercise-induced hormesis on the oxidative stress status skeletal muscles and its progression throughout the lifespan.

7. ROS versus Aging: May Bacteria Take the Stand

Until recently, dogma held that bacteria do not undergo any events that are equivalent to the aging process [234]. However, this viewpoint has changed over the last decade. Bacterial aging was first reported in the asymmetrically dividing *Caulobacter crescentus* [235, 236]. In this α-Proteobacteria, cell division is both morphologically and functionally asymmetric. This asymmetry produces a clear distinction between mother and daughter cells. Ackermann et al. [162] reported that, over multiple divisions, the time required for a mother cell to yield a new daughter cell doubled from 2.6 h to 5 h

per division cycle, a process similar to replicative aging in eukaryotes [235, 236]. Later, Stewart et al. [237] demonstrated that *Escherichia coli* also displayed features of replicative aging despite dividing by symmetrical, binary fission. Using automated time-lapse microscopy to image 8–10 reproduction cycles of individual cells, the authors observed that the old-pole (mother) cells showed a decreased growth fitness (e.g., growth rate) over successive generations, compared with their new-pole (sister) cells. The old-pole cells had reduced offspring formation and increased incidence of cell death. After approximately 100 divisions, the old-pole cells ceased to grow [237].

Subsequent research demonstrated that similar processes occur in *Bacillus subtilis* [238] and *Mycobacterium* spp. [239] (Figure 5). These observations confirm that aging in bacteria is a more general phenomenon than once thought, which affects not only microbes with distinct morphologies within the mother-to-daughter lineage but also those in which growth asymmetry is seen in the progeny at the functional/molecular level.

7.1. Aging and Conditional Senescence. During the stationary phase, as a result of nutrient limitation, *E. coli* cells enter a unique state known as conditional senescence [240]. Once rendered senescent, bacteria continuously lose their culturability and are unable to resume growth even when nutrients become available again. This feature makes conditional senescence very similar to human somatic cell senescence [100–103], and the replicative lifespan of yeast (*S. cerevisiae*), which is commonly used to model the aging process of mitotic tissues in higher organisms [241].

The observed functional asymmetry in bacterial division, initially reported by Stewart et al. [237], has been associated with asymmetric segregation of damaged cell components (e.g., protein aggregates) [242, 243], a process also present in eukaryotes [244–246]. Asymmetric protein damage aggregation seems to be an active process in yeast [87]. In bacteria, this process seems to be mainly passive and driven by molecular crowding [247].

Batch cultures of *E. coli* subjected to starvation-induced growth arrest exhibit markedly higher loads of damaged (carbonylated) proteins [248], a feature also present in aging eukaryotes [249, 250]. However, this load does not seem to be uniformly distributed in the population. Interestingly, low-carbonyl-load cells remained reproductively competent, whereas high-carbonyl-load cells were genetically dead (i.e., unable to be cultured). Whether this starvation-induced heterogeneity in carbonylation and fitness is programmed and whether it is the result of damage segregation during cytokinesis has not been elucidated. Bacterial cell senescence induced by other external stimuli, including UVA radiation, is also associated to the accumulation of protein carbonyls as a result of oxidative damage [251–253] (Figure 6).

Time-dependent accumulation of protein carbonyls has been observed during the stationary phase in *E. coli* [254]. The activities that contribute to protein oxidation during the stationary phase are shown in Figure 7. Given that one of the criteria for aging is an increase in mortality rate over time [255], this time-dependent accumulation of protein carbonyls provides a compelling argument that prokaryotes, such as *E. coli*, age. Some proteins, such as tricarboxylic acid (TCA) cycle enzymes, seem to be particularly susceptible to carbonylation [256]. Interestingly, cells lacking SOD-1 activity display higher amounts of protein carbonylation and lose viability more rapidly in the stationary phase [248]. Furthermore, stationary-phase populations incubated in the absence of oxygen have significantly extended lifespans compared to counterparts grown in the presence of oxygen [254]. These observations highlight the involvement of ROS and oxidative stress in stationary phase-associated senescence.

During the stationary phase and under stressful conditions, the oxidation of specific proteins in *E. coli* takes place. These proteins include DnaK (an Hsp70 chaperone), DNA-binding protein H-NS, universal stress protein A (UspA), the elongation factors EF-Tu and EF-G, glutamine synthetase, glutamate synthase, and aconitase [254, 256, 257]. Interestingly, some of these proteins are also carbonylated in yeast cells under oxidative stress [258], in aging flies [259, 260], and in the human brain of individuals with Alzheimer's disease [261]. These observations suggest that unchecked oxidative damage in the form of protein carbonylation could be the proximal cause of aging among stationary-phase *E. coli* populations [248]. However, there is no direct proof of this hypothesis.

Growth-arrested, stationary-phase *E. coli* develop resistance to heat and oxidative stress, a phenomenon known as stasis-induced cross-protection [262]. Cells starved of carbon or nitrogen are markedly more resistant to heat shock and oxidative stress than proliferating cells [262, 263]. An association between stress resistance and lifespan has been described in several eukaryotic model organisms [264–267]. These observations indicate that there might be an evolutionarily conserved mechanism channeling resources away from reproduction and toward maintenance and protective functions [268]. Similar to eukaryotes, the ability of cells to quench ROS may play a role in determining the bacterial lifespan [248].

However, as with eukaryotes, there are conflicting results regarding the contribution of ROS to bacterial senescence. For instance, reproductively arrested populations of *E. coli* have increased levels of oxidative defense proteins and increased population resistance to external oxidative stresses [138, 139]. However, these populations also display higher levels of damaged proteins [254, 256]. Additionally, no strict correlation has been observed between respiratory activity, protein oxidation, and the lifespan of growth-arrested *E. coli* [269]. Similar results have been observed in G0-growth-arrested yeast cells [270].

The first genes induced following growth arrest in bacteria play roles in countering stasis-induced senescence and death [262, 271]. Many of these genes encode proteins that protect the cell from external stresses, such as heat, oxidants, and osmotic challenge, which could account for stasis-induced cross-protection [262]. Cross-protection relies on the sigma factor Sigma-S [272]. Under not only starvation but also general stress conditions, the Sigma-S transcription factor accumulates, binds, and directs RNA polymerases toward more than 50 specific genes [272]. *E. coli* mutants

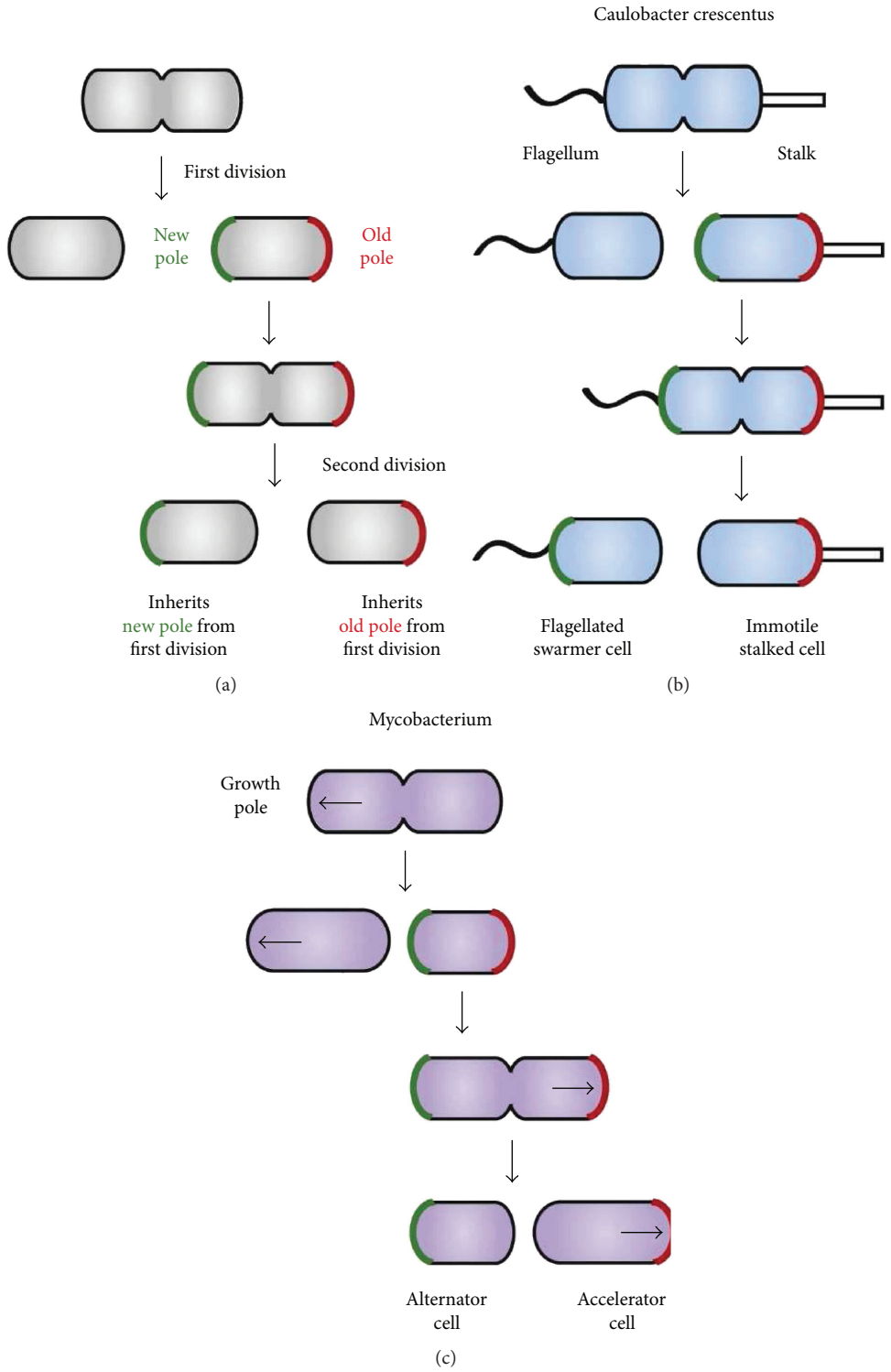


FIGURE 5: (a) All cell divisions in rod-shaped bacteria are asymmetric in that one daughter cell inherits the "new" pole (green) from a previous division and the other inherits the "old" pole (red). In some bacteria, this asymmetry is used to create functional specialization of daughter cells. (b) In *C. crescentus*, different polar appendages form at the new and old poles, leading to dimorphic daughter cells. (c) In *Mycobacterium*, cells preferentially grow at the old pole (marked with an arrow). Daughter cells that inherit the old pole, called accelerators, continue growing whereas those inheriting the new pole, called alternators, must form a new growth pole before elongating. Reproduced with permission from Aakre CD, Laub MT. Asymmetric cell division: a persistent issue? Developmental cell. 2012; 22 (2):235-236. doi:10.1016/j.devcel.2012.01.016.

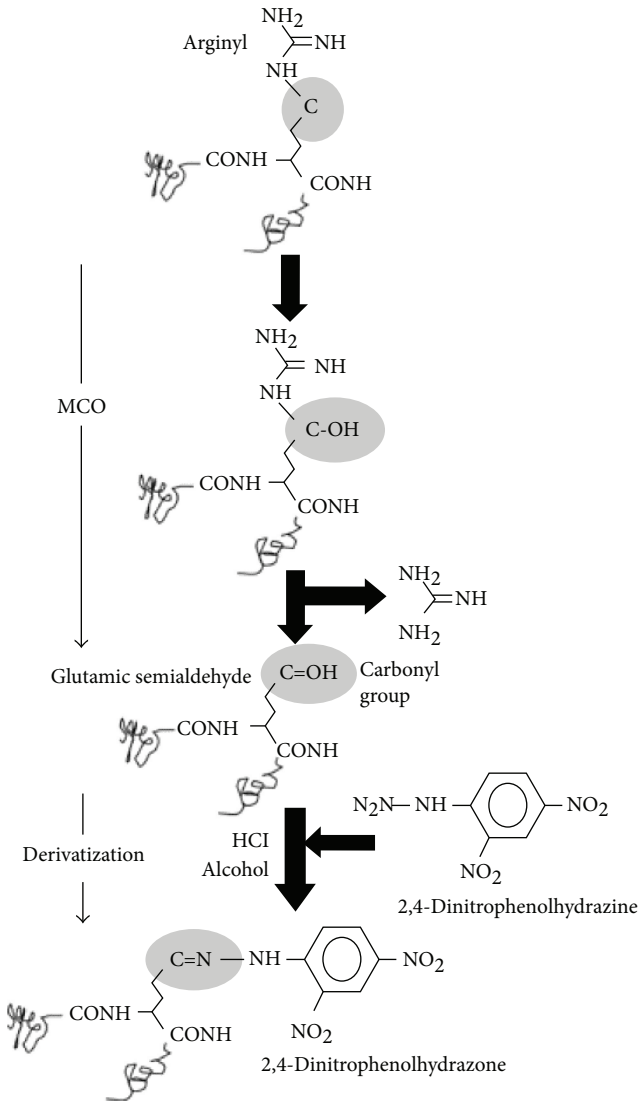


Figure 6: Carbonylation and derivatization of a protein amino acid side chain. A scheme for the formation of glutamic semialdehyde from an arginyl residue is depicted as a consequence of an MCO. For detection, the carbonyl group, in this case, glutamic semialdehyde, is subsequently derivatized by 2,4-dinitrophenolhydrazine. The resulting protein 2,4-dinitrophenolhydrazone can be detected by specific monoclonal or polyclonal antibodies [210]. Reproduced with permission from Nyström T. Role of oxidative carbonylation in protein quality control and senescence. The EMBO Journal. 2005; 24 (7):1311-1317. doi:10.1038/sj.emboj.7600599.

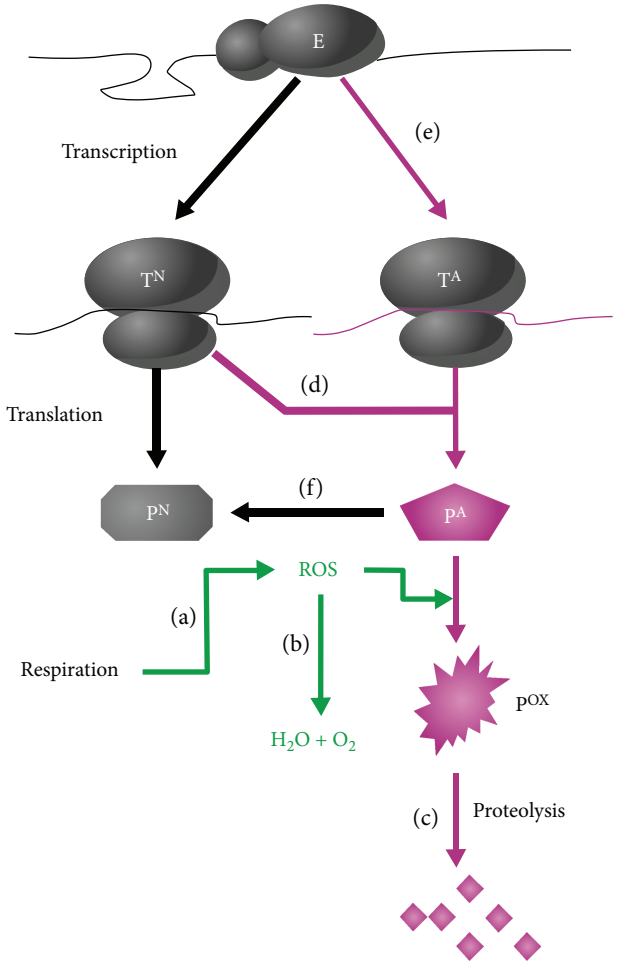


Figure 7: Activities of potential importance for stasis-induced oxidation of proteins. Traditionally, increased protein oxidation has been argued to be an effect of (a) increased production of reactive oxygen species (ROS), presumably derived from respiratory activity, (b) diminished activity or abundance of the antioxidant systems, or (c) reduced activity of the proteolysis or damage repair systems. Work on *E. coli* has highlighted the role of some alternative pathways in protein oxidation. These pathways relate to the production of aberrant proteins, which are highly susceptible to oxidative modification (carbonylation). Increased levels of such aberrant, malformed polypeptides can be the result of (d) reduced translational fidelity, (e) reduced transcriptional fidelity, or (f) diminished activity of the repair refolding apparatus. In the early stages of *E. coli* growth arrest, reduced translational fidelity appears to be the most important contributing factor to the elevated levels of oxidatively modified aberrant proteins. E, core RNA polymerase; P^A, aberrant protein; P^N, native protein; P^{ox}, oxidized protein; T^A, aberrant transcript; T^N, native transcript. Reproduced with permission from Nyström, Thomas. "Aging in bacteria." Current Opinion in Microbiology 5, no. 6 (2002): 596-601.

lacking Sigma-S have elevated levels of proteins with oxidative damage [254, 256] and accelerated senescence during growth arrest [272]. In *Salmonella* sp., both Sigma-S and Sigma-E are required for protection against oxidative damage in the stationary phase and mutants lacking Sigma-E have reduced survival and increased susceptibility to oxidative stress [273]. However, under anaerobic stationary-phase conditions, survival is completely preserved [273], indicating that oxidative injury is a major mechanism by which microbial viability is reduced during nutrient deprivation. Interestingly, members of the Sigma-S regulon include a diverse set of proteins with functions that overlap those of FOXO-/*daf-16*-regulated longevity genes in *C. elegans* [142, 147, 148]. Thus, functionally similar signaling pathways seem to regulate stress resistance, protein damage protection, and longevity in eukaryotes and prokaryotes. These pathways are pivotal for survival during periods of

starvation. They may have been evolutionarily conserved across different branches of the tree of life because they enhanced the maintenance capacity of the cell. Over time, they also may have become crucial for retarding aging in multicellular organisms [143, 149].

7.2. Genetic Determinants of Senescence and Aging in Bacteria. Literature investigating the genes that extend stationary-phase survival in bacteria is scarce. However, a few mutant strains that survive longer than WT have been reported. RssB, which regulates the stability of the sigma factor Sigma-S, has been found to play a key role in the survival of *E. coli*, potentially by increasing the cell's resistance to spontaneous, endogenous stresses [274].

More recently, a genome-wide screen for *E. coli* mutants with a prolonged stationary-phase survival phenotype identified three strains that lived longer than WT [275]. One of the strains, Δ*sdhA* (succinate dehydrogenase subunit A), displayed increased stress resistance and extended lifespan. Succinate dehydrogenase is a tetrameric protein complex that catalyzes the conversion of succinate to fumarate in the TCA cycle [276]. Subunit A, the enzymatically active part of the complex, is a well-established source of superoxide in the ETC of *E. coli* [277]. Purportedly, when this enzyme is absent, the rate of superoxide production is reduced, extending stationary-phase survival [275].

The two other mutants displaying extended stationary-phase survival were Δ*lipA* (lipoyl synthase) and Δ*lpdA* (dihydrolipoyl dehydrogenase) [275]. The authors attributed the enhanced lifespan of these two mutants to their reduced consumption of oxygen, compared to WT, which in turn increased the expression of the hypoxia transcription factor ArcA [278]. ArcA suppresses the expression of TCA cycle genes, such as citrate synthase (*gltA*), and activates the expression of genes required to generate energy under oxygen-limited conditions, extending stationary-phase survival [279]. These observations suggest that the extended lifespan observed in these mutants is associated with the induction of a physiological state typically associated with hypoxic conditions. These results are consistent with the lifespan-modulating role of HIF-1α in higher organisms [37, 162, 163]. In fact, ArcA could be considered a functional homolog of HIF-1α, although the two proteins do not share significant sequence similarity. This functional similarity points toward the adaptive response to oxygen-limited conditions as an evolutionarily conserved mechanism that can extend lifespan.

Given the conservation of key phenotypes associated with age-dependent macromolecular damage and the lifespan-extending role of genes that control the hypoxic response in both bacteria and higher eukaryotes, it is reasonable to hypothesize that the most fundamental mechanisms of aging might be conserved at all levels of life. Future studies will help to clarify what molecular processes underlying aging are similar between bacteria and eukaryotes. The results of these studies could open the possibility of using *E. coli* as a model organism of aging on which specific molecular mechanisms and evolutionary theories can be easily tested.

8. Conclusions and Future Perspectives

The progressive loss of mitochondrial function is a consistent and conserved hallmark of aging that impacts both cellular homeostasis and organismal health [134, 135]. While ROS contribute to aging, they also play a crucial role in cell signaling and development, thus serving a beneficial role. The mitochondrial theory of aging offers a conciliatory perspective of the dual role of ROS in the aging process by incorporating two important adaptive responses: (1) UPRmt-mediated retrograde signaling from the mitochondria to the nucleus to regulate aging and (2) ROS-mediated adaptive response to activate the antioxidant defense system of the cell. Interventions targeting either of these two adaptive pathways could be considered potential targets for antiaging and lifespan-promoting therapies.

Because the accumulation of oxidative damage throughout life is a major cause of aging, genetic or pharmacological interventions targeting oxidative damage repair or damage removal pathways themselves also have significant therapeutic potential. However, further research in humans and nonhuman primates is needed to gain insights into the clinical significance of potential genetic, pharmacological, and nonpharmacological interventions.

The observation that several of the processes that characterize eukaryotic aging can also be seen in bacteria highlights the potential of bacteria to serve as a simple model organism to study aging and age-related mechanisms. These tractable models might provide crucial assistance in the quest to uncover the genetic, molecular, and biochemical processes underlying aging and age-related diseases.

Disclosure

The funders had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript. Sanchari Sinha's current address is Independent Science Writer, Kolkata, India.

Conflicts of Interest

The authors declare no conflict of interest regarding the content of this paper.

Acknowledgments

Acknowledgments are due to the anonymous reviewer for a detailed analysis and helpful criticism of the previous version of this manuscript. The authors would also like to acknowledge Dr. Nathan Susnik (Hanover Medical School, Hanover, Germany) for his insightful comments and thoughtful advice. The authors gratefully acknowledge the AXA Research Fund (http://www.axa-research.org/) for their support.

References

[1] L. Partridge and D. Gems, "Mechanisms of ageing: public or private?," *Nature Reviews Genetic*, vol. 3, no. 3, pp. 165–175, 2002.

[2] P. Davalli, T. Mitic, A. Caporali, A. Lauriola, and D. D'Arca, "ROS, cell senescence, and novel molecular mechanisms in aging and age-related diseases," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 3565127, 18 pages, 2016.

[3] World Health Organization, "World report on aging and health," 2015.

[4] E. A. Kikis, T. Gidalevitz, and R. I. Morimoto, "Protein homeostasis in models of aging and age-related conformational disease," *Experimental Medicine and Biology*, vol. 694, pp. 138–159, 2010.

[5] K. Jin, J. W. Simpkins, X. Ji, M. Leis, and I. Stambler, "The critical need to promote research of aging and aging-related diseases to improve health and longevity of the elderly population," *Aging and Disease*, vol. 6, no. 1, pp. 1–5, 2015.

[6] R. J. Mailloux, "Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species," *Redox Biology*, vol. 4, pp. 381–398, 2015.

[7] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[8] K. Krumova and G. Cosa, "Chapter 1. Overview of reactive oxygen species," in *Singlet Oxygen: Applications in Biosciences and Nanosciences, Volume 1*, pp. 1–21, The Royal Society of Chemistry, 2016.

[9] V. K. Koltover, "Free radical timer of aging: from chemistry of free radicals to systems theory of reliability," *Current Aging Science*, vol. 10, no. 1, pp. 12–17, 2017.

[10] G. Barja, "Chapter one - The mitochondrial free radical theory of aging," in *The Mitochondrion in Aging and Disease*, pp. 1–27, Academic Press, 2014.

[11] 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.

[12] J. M. Van Raamsdonk and S. Hekimi, "Superoxide dismutase is dispensable for normal animal lifespan," *Proceedings of the National Academy of Sciences*, vol. 109, no. 15, pp. 5785–5790, 2012.

[13] J. Lapointe and S. Hekimi, "When a theory of aging ages badly," *Cellular and Molecular Life Sciences*, vol. 67, no. 1, pp. 1–8, 2010.

[14] D. Gems and R. Doonan, "Antioxidant defense and aging in *C. elegans*: is the oxidative damage theory of aging wrong?," *Cell Cycle*, vol. 8, no. 11, pp. 1681–1687, 2009.

[15] M. Ristow and K. Zarse, "How increased oxidative stress promotes longevity and metabolic health: the concept of mitochondrial hormesis (mitohormesis)," *Experimental Gerontology*, vol. 45, no. 6, pp. 410–418, 2010.

[16] 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.

[17] T. Ogawa, Y. Kodera, D. Hirata, T. K. Blackwell, and M. Mizunuma, "Natural thioallyl compounds increase oxidative stress resistance and lifespan in *Caenorhabditis elegans* by modulating SKN-1/Nrf," *Scientific Reports*, vol. 6, no. 1, pp. 1–13, 2016.

[18] N. Fischer, C. Büchter, K. Koch, S. Albert, R. Csuk, and W. Wätjen, "The resveratrol derivatives trans-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene and trans-2,4′,5-trihydroxystilbene decrease oxidative stress and prolong lifespan in *Caenorhabditis elegans*," *Journal of Pharmacy and Pharmacology*, vol. 69, no. 1, pp. 73–81, 2017.

[19] J. M. Van Raamsdonk and S. Hekimi, "Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in *Caenorhabditis elegans*," *PLoS Genetics*, vol. 5, no. 2, article e1000361, 2009.

[20] W. Yang and S. Hekimi, "A mitochondrial superoxide signal triggers increased longevity in *Caenorhabditis elegans*," *PLoS Biology*, vol. 8, no. 12, article e1000556, 2010.

[21] M. Holzenberger, J. Dupont, B. Ducos et al., "IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice," *Nature*, vol. 421, no. 6919, pp. 182–187, 2002.

[22] E. Migliaccio, M. Giorgio, S. Mele et al., "The p66shc adaptor protein controls oxidative stress response and life span in mammals," *Nature*, vol. 402, no. 6759, pp. 309–313, 1999.

[23] Y. Zhang, A. Unnikrishnan, S. S. Deepa et al., "A new role for oxidative stress in aging: the accelerated aging phenotype in *Sod1*$^{-/-}$ mice is correlated to increased cellular senescence," *Redox Biology*, vol. 11, pp. 30–37, 2017.

[24] S. E. Schriner, 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.

[25] P. M. Treuting, N. J. Linford, S. E. Knoblaugh et al., "Reduction of age-associated pathology in old mice by overexpression of catalase in mitochondria," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 63, no. 8, pp. 813–822, 2008.

[26] T. T. Huang, E. J. Carlson, A. M. Gillespie, Y. Shi, and C. J. Epstein, "Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 55, pp. B5–B9, 2000.

[27] V. I. Perez, H. Van Remmen, A. Bokov, C. J. Epstein, J. Vijg, and A. Richardson, "The overexpression of major antioxidant enzymes does not extend the lifespan of mice," *Aging Cell*, vol. 8, no. 1, pp. 73–75, 2009.

[28] Y. S. Ho, J. L. Magnenat, R. T. Bronson et al., "Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia," *Journal of Biological Chemistry*, vol. 272, no. 26, pp. 16644–16651, 1997.

[29] Y. Zhang, Y. Ikeno, W. Qi et al., "Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 64A, no. 12, pp. 1212–1220, 2009.

[30] M. Soerensen, K. Christensen, T. Stevnsner, and L. Christiansen, "The Mn-superoxide dismutase single nucleotide polymorphism rs4880 and the glutathione peroxidase 1 single nucleotide polymorphism rs1050450 are associated with aging and longevity in the oldest old," *Mechanisms of Ageing and Development*, vol. 130, no. 5, pp. 308–314, 2009.

[31] S.-E. Yoo, L. Chen, R. Na et al., "Gpx4 ablation in adult mice results in a lethal phenotype accompanied by neuronal loss in brain," *Free Radical Biology and Medicine*, vol. 52, no. 9, pp. 1820–1827, 2012.

[32] Q. Ran, H. Liang, M. Gu et al., "Transgenic mice overexpressing glutathione peroxidase 4 are protected against oxidative stress-induced apoptosis," *Journal of Biological Chemistry*, vol. 279, no. 53, pp. 55137–55146, 2004.

[33] V. I. Pérez, A. Bokov, H. Van Remmen et al., "Is the oxidative stress theory of aging dead?," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1790, no. 10, pp. 1005–1014, 2009.

[34] L. Nonn, R. R. Williams, R. P. Erickson, and G. Powis, "The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice," *Molecular and Cellular Biology*, vol. 23, no. 3, pp. 916–922, 2003.

[35] V. I. Pérez, C. M. Lew, L. A. Cortez et al., "Thioredoxin 2 haploinsufficiency in mice results in impaired mitochondrial function and increased oxidative stress," *Free Radical Biology and Medicine*, vol. 44, no. 5, pp. 882–892, 2008.

[36] V. I. Pérez, L. A. Cortez, C. M. Lew et al., "Thioredoxin 1 overexpression extends mainly the earlier part of life span in mice," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 66A, no. 12, pp. 1286–1299, 2011.

[37] C. E. Schaar, D. J. Dues, K. K. Spielbauer et al., "Mitochondrial and cytoplasmic ROS have opposing effects on lifespan," *PLOS Genetics*, vol. 11, no. 2, article e1004972, 2015.

[38] F. Scialò, A. Sriram, D. Fernández-Ayala et al., "Mitochondrial ROS produced via reverse electron transport extend animal lifespan," *Cell Metabolism*, vol. 23, no. 4, pp. 725–734, 2016.

[39] X. Liu, N. Jiang, B. Hughes, E. Bigras, E. Shoubridge, and S. Hekimi, "Evolutionary conservation of the clk-1-dependent mechanism of longevity: loss of mclk1 increases cellular fitness and lifespan in mice," *Genes & Development*, vol. 19, no. 20, pp. 2424–2434, 2005.

[40] C. Dell'Agnello, S. Leo, A. Agostino et al., "Increased longevity and refractoriness to Ca(2+)-dependent neurodegeneration in Surf1 knockout mice," *Human Molecular Genetics*, vol. 16, no. 4, pp. 431–444, 2007.

[41] J. M. Van Raamsdonk, Y. Meng, D. Camp et al., "Decreased energy metabolism extends life span in *Caenorhabditis elegans* without reducing oxidative damage," *Genetics*, vol. 185, no. 2, pp. 559–571, 2010.

[42] T. Lindahl and D. E. Barnes, "Repair of Endogenous DNA Damage," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 65, no. 0, pp. 127–134, 2000.

[43] H. L. Gensler and H. Bernstein, "DNA damage as the primary cause of aging," *The Quarterly Review of Biology*, vol. 56, no. 3, pp. 279–303, 1981.

[44] L. Szilard, "On the nature of the aging process," *Proceedings of the National Academy of Sciences*, vol. 45, no. 1, pp. 30–45, 1959.

[45] M. M. Vilenchik and A. G. Knudson, "Inverse radiation dose-rate effects on somatic and germ-line mutations and DNA damage rates," *Proceedings of the National Academy of Sciences*, vol. 97, no. 10, pp. 5381–5386, 2000.

[46] T. Lindahl and B. Nyberg, "Heat-induced deamination of cytosine residues in deoxyribonucleic acid," *Biochemistry*, vol. 13, no. 16, pp. 3405–3410, 1974.

[47] T. Lindahl and B. Nyberg, "Rate of depurination of native deoxyribonucleic acid," *Biochemistry*, vol. 11, no. 19, pp. 3610–3618, 1974.

[48] F. R. de Gruijl and H. Rebel, "Early events in UV carcinogenesis–DNA damage, target cells and mutant p53 foci," *Photochemistry and Photobiology*, vol. 84, no. 2, pp. 382–387, 2008.

[49] M. C. Poirier, R. M. Santella, and A. Weston, "Carcinogen macromolecular adducts and their measurement," *Carcinogenesis*, vol. 21, no. 3, pp. 353–359, 2000.

[50] K. A. Cimprich and D. Cortez, "ATR: an essential regulator of genome integrity," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 8, pp. 616–627, 2008.

[51] F. Lazzaro, M. Giannattasio, F. Puddu et al., "Checkpoint mechanisms at the intersection between DNA damage and repair," *DNA Repair*, vol. 8, no. 9, pp. 1055–1067, 2009.

[52] S. Dukan, A. Farewell, M. Ballesteros, F. Taddei, M. Radman, and T. Nyström, "Protein oxidation in response to increased transcriptional or translational errors," *Proceedings of the National Academy of Sciences*, vol. 97, no. 11, pp. 5746–5749, 2000.

[53] K. Tanaka, "The proteasome: overview of structure and functions," *Proceedings of the Japan Academy, Series B*, vol. 85, no. 1, pp. 12–36, 2009.

[54] T. Jung, N. Bader, and T. Grune, "Oxidized proteins: intracellular distribution and recognition by the proteasome," *Archives of Biochemistry and Biophysics*, vol. 462, no. 2, pp. 231–237, 2007.

[55] P. Fortini, B. Pascucci, E. Parlanti, M. D'Errico, V. Simonelli, and E. Dogliotti, "8-Oxoguanine DNA damage: at the crossroad of alternative repair pathways," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 531, no. 1-2, pp. 127–139, 2003.

[56] H. E. Krokan and M. Bjoras, "Base excision repair," *Cold Spring Harbor Perspectives in Biology*, vol. 5, no. 4, article a012583, 2013.

[57] O. D. Scharer, "Nucleotide excision repair in eukaryotes," *Cold Spring Harbor Perspectives in Biology*, vol. 5, no. 10, article a012609, 2013.

[58] M. R. Lieber, "The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway," *Annual Review of Biochemistry*, vol. 79, no. 1, pp. 181–211, 2010.

[59] J. K. Moore and J. E. Haber, "Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae," *Molecular and Cellular Biology*, vol. 16, no. 5, pp. 2164–2173, 1996.

[60] G. Xu, M. Herzig, V. Rotrekl, and C. A. Walter, "Base excision repair, aging and health span," *Mechanisms of Ageing and Development*, vol. 129, no. 7-8, pp. 366–382, 2008.

[61] R. Mostoslavsky, K. F. Chua, D. B. Lombard et al., "Genomic instability and aging-like phenotype in the absence of mammalian SIRT6," *Cell*, vol. 124, no. 2, pp. 315–329, 2006.

[62] J. Kanungo, "DNA-dependent protein kinase and DNA repair: relevance to Alzheimer's disease," *Alzheimer's Research & Therapy*, vol. 5, no. 2, p. 13, 2013.

[63] V. N. Vyjayanti and K. S. Rao, "DNA double strand break repair in brain: reduced NHEJ activity in aging rat neurons," *Neuroscience Letters*, vol. 393, no. 1, pp. 18–22, 2006.

[64] J. German, "Bloom syndrome: a mendelian prototype of somatic mutational disease," *Medicine*, vol. 72, no. 6, pp. 393–406, 1993.

[65] C. J. Epstein, G. M. Martin, A. L. Schultz, and A. G. Motulsky, "A review of its symptomatology, natural history, pathologic

features, genetics and relationship to the natural aging process," *Medicine*, vol. 45, no. 3, pp. 177–221, 1966.

[66] A. S. Balajee, A. Machwe, A. May et al., "The Werner syndrome protein is involved in RNA polymerase II transcription," *Molecular Biology of the Cell*, vol. 10, no. 8, pp. 2655–2668, 1999.

[67] B. Li and L. Comai, "Functional interaction between Ku and the werner syndrome protein in DNA end processing," *Journal of Biological Chemistry*, vol. 275, no. 37, pp. 28349–28352, 2000.

[68] P. Hasty, "The impact of DNA damage, genetic mutation and cellular responses on cancer prevention, longevity and aging: observations in humans and mice," *Mechanisms of Ageing and Development*, vol. 126, no. 1, pp. 71–77, 2005.

[69] J. H. J. Hoeijmakers, "DNA damage, aging, and cancer," *New England Journal of Medicine*, vol. 361, no. 15, pp. 1475–1485, 2009.

[70] K. Kashiyama, Y. Nakazawa, D. T. Pilz et al., "Malfunction of nuclease ERCC1-XPF results in diverse clinical manifestations and causes Cockayne syndrome, xeroderma pigmentosum, and Fanconi anemia," *The American Journal of Human Genetics*, vol. 92, no. 5, pp. 807–819, 2013.

[71] L. J. Niedernhofer, G. A. Garinis, A. Raams et al., "A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis," *Nature*, vol. 444, no. 7122, pp. 1038–1043, 2006.

[72] M. A. Blasco, "Telomere length, stem cells and aging," *Nature Chemical Biology*, vol. 3, no. 10, pp. 640–649, 2007.

[73] H. Vallabhaneni, F. Zhou, R. W. Maul et al., "Defective repair of uracil causes telomere defects in mouse hematopoietic cells," *Journal of Biological Chemistry*, vol. 290, no. 9, pp. 5502–5511, 2015.

[74] H. Vallabhaneni, N. O'Callaghan, J. Sidorova, and Y. Liu, "Defective repair of oxidative base lesions by the DNA glycosylase Nth1 associates with multiple telomere defects," *PLoS Genetics*, vol. 9, no. 7, article e1003639, 2013.

[75] C. Richter, J. W. Park, and B. N. Ames, "Normal oxidative damage to mitochondrial and nuclear DNA is extensive," *Proceedings of the National Academy of Sciences*, vol. 85, no. 17, pp. 6465–6467, 1988.

[76] S. P. Ledoux and G. L. Wilson, "Base excision repair of mitochondrial DNA damage in mammalian cells," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 68, pp. 273–284, 2001.

[77] W. J. Driggers, S. P. LeDoux, and G. L. Wilson, "Repair of oxidative damage within the mitochondrial DNA of RINr 38 cells," *Journal of Biological Chemistry*, vol. 268, no. 29, pp. 22042–22045, 1993.

[78] G. L. Dianov, N. Souza-Pinto, S. G. Nyaga, T. Thybo, T. Stevnsner, and V. A. Bohr, "Base excision repair in nuclear and mitochondrial DNA," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 68, pp. 285–297, 2001.

[79] J. A. Stuart, K. Hashiguchi, D. M. Wilson 3rd, W. C. Copeland, N. C. Souza-Pinto, and V. A. Bohr, "DNA base excision repair activities and pathway function in mitochondrial and cellular lysates from cells lacking mitochondrial DNA," *Nucleic Acids Research*, vol. 32, no. 7, pp. 2181–2192, 2004.

[80] R. Chattopadhyay, L. Wiederhold, B. Szczesny et al., "Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells," *Nucleic Acids Research*, vol. 34, no. 7, pp. 2067–2076, 2006.

[81] A. Bender, K. J. Krishnan, C. M. Morris et al., "High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease," *Nature Genetics*, vol. 38, no. 5, pp. 515–517, 2006.

[82] A. Trifunovic, A. Hansson, A. Wredenberg et al., "Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production," *Proceedings of the National Academy of Sciences*, vol. 102, no. 50, pp. 17993–17998, 2005.

[83] J. N. Octave, "Alzheimer disease: cellular and molecular aspects," *Bulletin et memoires de l'Academie royale de medecine de Belgique*, vol. 160, pp. 441–445, 2005.

[84] A. Chomyn and G. Attardi, "MtDNA mutations in aging and apoptosis," *Biochemical and Biophysical Research Communications*, vol. 304, no. 3, pp. 519–529, 2003.

[85] P. E. Coskun, M. F. Beal, and D. C. Wallace, "Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication," *Proceedings of the National Academy of Sciences*, vol. 101, no. 29, pp. 10726–10731, 2004.

[86] M. T. Lin, D. K. Simon, C. H. Ahn, L. M. Kim, and M. F. Beal, "High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain," *Human Molecular Genetics*, vol. 11, no. 2, pp. 133–145, 2002.

[87] T. Nystrom, "Role of oxidative carbonylation in protein quality control and senescence," *The EMBO Journal*, vol. 24, no. 7, pp. 1311–1317, 2005.

[88] Y. J. Suzuki, M. Carini, and D. A. Butterfield, "Protein carbonylation," *Antioxidants & Redox Signaling*, vol. 12, no. 3, pp. 323–325, 2010.

[89] Q. Chen, J. Thorpe, J. R. Dohmen, F. Li, and J. N. Keller, "Ump1 extends yeast lifespan and enhances viability during oxidative stress: central role for the proteasome?," *Free Radical Biology and Medicine*, vol. 40, no. 1, pp. 120–126, 2006.

[90] L. Knuppertz and H. D. Osiewacz, "Orchestrating the network of molecular pathways affecting aging: role of nonselective autophagy and mitophagy," *Mechanisms of Ageing and Development*, vol. 153, pp. 30–40, 2016.

[91] Q. Zhao, J. Wang, I. V. Levichkin, S. Stasinopoulos, M. T. Ryan, and N. J. Hoogenraad, "A mitochondrial specific stress response in mammalian cells," *The EMBO Journal*, vol. 21, no. 17, pp. 4411–4419, 2002.

[92] T. Yoneda, C. Benedetti, F. Urano, S. G. Clark, H. P. Harding, and D. Ron, "Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones," *Journal of Cell Science*, vol. 117, no. 18, pp. 4055–4066, 2004.

[93] C. O'Neill, A. P. Kiely, M. F. Coakley, S. Manning, and C. M. Long-Smith, "Insulin and IGF-1 signalling: longevity, protein homoeostasis and Alzheimer's disease," *Biochemical Society Transactions*, vol. 40, no. 4, pp. 721–727, 2012.

[94] J. F. Morley and R. I. Morimoto, "Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones," *Molecular Biology of the Cell*, vol. 15, no. 2, pp. 657–664, 2003.

[95] E. Cohen, J. Bieschke, R. M. Perciavalle, J. W. Kelly, and A. Dillin, "Opposing activities protect against age-onset proteotoxicity," *Science*, vol. 313, no. 5793, pp. 1604–1610, 2006.

[96] S. Alavez, M. C. Vantipalli, D. J. S. Zucker, I. M. Klang, and G. J. Lithgow, "Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan," *Nature*, vol. 472, no. 7342, pp. 226–229, 2011.

[97] M. J. Vos, S. Carra, B. Kanon et al., "Specific protein homeostatic functions of small heat-shock proteins increase lifespan," *Aging Cell*, vol. 15, no. 2, pp. 217–226, 2016.

[98] S. Kaushik and A. M. Cuervo, "Proteostasis and aging," *Nature Medicine*, vol. 21, no. 12, pp. 1406–1415, 2015.

[99] T. Grune, "Oxidative stress, aging and the proteasomal system," *Biogerontology*, vol. 1, no. 1, pp. 31–40, 2000.

[100] P. A. Szweda, M. Camouse, K. C. Lundberg, T. D. Oberley, and L. I. Szweda, "Aging, lipofuscin formation, and free radical-mediated inhibition of cellular proteolytic systems," *Ageing Research Reviews*, vol. 2, no. 4, pp. 383–405, 2003.

[101] Q. Ding, E. Dimayuga, W. R. Markesbery, and J. N. Keller, "Proteasome inhibition induces reversible impairments in protein synthesis," *The FASEB Journal*, vol. 20, no. 8, pp. 1055–1063, 2006.

[102] Q. Ding, E. Dimayuga, and J. N. Keller, "Proteasome regulation of oxidative stress in aging and age-related diseases of the CNS," *Antioxidants & Redox Signaling*, vol. 8, no. 1-2, pp. 163–172, 2006.

[103] A. L. Santos and A. B. Lindner, "Protein posttranslational modifications: roles in aging and age-related disease," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 5716409, 19 pages, 2017.

[104] J. N. Keller, F. F. Huang, and W. R. Markesbery, "Decreased levels of proteasome activity and proteasome expression in aging spinal cord," *Neuroscience*, vol. 98, no. 1, pp. 149–156, 2000.

[105] J. N. Keller, K. B. Hanni, and W. R. Markesbery, "Possible involvement of proteasome inhibition in aging: implications for oxidative stress," *Mechanisms of Ageing and Development*, vol. 113, no. 1, pp. 61–70, 2000.

[106] E. Kevei and T. Hoppe, "Ubiquitin sets the timer: impacts on aging and longevity," *Nature Structural & Molecular Biology*, vol. 21, no. 4, pp. 290–292, 2014.

[107] N. Hoe, C. M. Huang, G. Landis et al., "Ubiquitin overexpression phenotypes and ubiquitin gene molecular misreading during aging in Drosophila melanogaster," *Aging*, vol. 3, no. 3, pp. 237–261, 2011.

[108] H.-Y. Liu and C. M. Pfleger, "Mutation in E1, the ubiquitin activating enzyme, reduces *Drosophila* lifespan and results in motor impairment," *PLoS One*, vol. 8, no. 1, article e32835, 2013.

[109] A. C. Carrano, A. Dillin, and T. Hunter, "A Krüppel-like factor downstream of the E3 ligase WWP-1 mediates dietary-restriction-induced longevity in *Caenorhabditis elegans*," *Nature Communications*, vol. 5, 2014.

[110] R. A. Gottlieb and R. S. Carreira, "Autophagy in health and disease. 5. Mitophagy as a way of life," *American Journal of Physiology-Cell Physiology*, vol. 299, no. 2, pp. C203–C210, 2010.

[111] S. Mai, B. Muster, J. Bereiter-Hahn, and M. Jendrach, "Autophagy proteins LC3B, ATG5 and ATG12 participate in quality control after mitochondrial damage and influence lifespan," *Autophagy*, vol. 8, no. 1, pp. 47–62, 2014.

[112] J. N. Keller, E. Dimayuga, Q. Chen, J. Thorpe, J. Gee, and Q. Ding, "Autophagy, proteasomes, lipofuscin, and oxidative stress in the aging brain," *The International Journal of Biochemistry & Cell Biology*, vol. 36, no. 12, pp. 2376–2391, 2004.

[113] T. Hara, K. Nakamura, M. Matsui et al., "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice," *Nature*, vol. 441, no. 7095, pp. 885–889, 2006.

[114] M. Dodson, V. Darley-Usmar, and J. Zhang, "Cellular metabolic and autophagic pathways: traffic control by redox signaling," *Free Radical Biology and Medicine*, vol. 63, pp. 207–221, 2013.

[115] K. Palikaras, E. Lionaki, and N. Tavernarakis, "Coordination of mitophagy and mitochondrial biogenesis during ageing in *C. elegans*," *Nature*, vol. 521, no. 7553, pp. 525–528, 2015.

[116] A. Rana, M. Rera, and D. W. Walker, "Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan," *Proceedings of the National Academy of Sciences*, vol. 110, no. 21, pp. 8638–8643, 2013.

[117] 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.

[118] E. F. Fang, M. Scheibye-Knudsen, K. F. Chua, M. P. Mattson, D. L. Croteau, and V. A. Bohr, "Nuclear DNA damage signalling to mitochondria in ageing," *Nature Reviews Molecular Cell Biology*, vol. 17, no. 5, pp. 308–321, 2016.

[119] P. Bai, C. Cantó, H. Oudart et al., "PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation," *Cell Metabolism*, vol. 13, no. 4, pp. 461–468, 2011.

[120] S. Imai and L. Guarente, "NAD(+) and sirtuins in aging and disease," *Trends in Cell Biology*, vol. 24, no. 8, pp. 464–471, 2014.

[121] S. Park, R. Mori, and I. Shimokawa, "Do sirtuins promote mammalian longevity?: a critical review on its relevance to the longevity effect induced by calorie restriction," *Molecules and Cells*, vol. 35, no. 6, pp. 474–480, 2013.

[122] K. T. Howitz, K. J. Bitterman, H. Y. Cohen et al., "Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan," *Nature*, vol. 425, no. 6954, pp. 191–196, 2003.

[123] E. Morselli, M. C. Maiuri, M. Markaki et al., "Caloric restriction and resveratrol promote longevity through the sirtuin-1-dependent induction of autophagy," *Cell Death & Disease*, vol. 1, no. 1, article e10, 2010.

[124] E. Verdin, M. D. Hirschey, L. W. S. Finley, and M. C. Haigis, "Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling," *Trends in Biochemical Sciences*, vol. 35, no. 12, pp. 669–675, 2010.

[125] E. F. Fang, M. Scheibye-Knudsen, L. E. Brace et al., "Defective mitophagy in XPA via PARP1 hyperactivation and NAD+/SIRT1 reduction," *Cell*, vol. 157, no. 4, pp. 882–896, 2014.

[126] T. Radovits, L. Seres, D. Gerő et al., "Single dose treatment with PARP-inhibitor INO-1001 improves aging-associated cardiac and vascular dysfunction," *Experimental Gerontology*, vol. 42, no. 7, pp. 676–685, 2007.

[127] P. Pacher, J. G. Mabley, F. G. Soriano, L. Liaudet, K. Komjáti, and C. Szabó, "Endothelial dysfunction in aging animals: the role of poly(ADP-ribose) polymerase activation," *British Journal of Pharmacology*, vol. 135, no. 6, pp. 1347–1350, 2002.

[128] G. Zhang, M. Chao, L. Hui et al., "Poly(ADP-ribose)polymerase 1 inhibition protects against age-dependent endothelial dysfunction," *Clinical and Experimental Pharmacology and Physiology*, vol. 42, no. 12, pp. 1266–1274, 2015.

[129] S. Shall and G. de Murcia, "Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model?," *Mutation Research/DNA Repair*, vol. 460, no. 1, pp. 1–15, 2000.

[130] T. S. Piskunova, M. N. Yurova, A. I. Ovsyannikov et al., "Deficiency in poly(ADP-ribose) polymerase-1 (PARP-1) accelerates aging and spontaneous carcinogenesis in mice," *Current Gerontology and Geriatrics Research*, vol. 2008, Article ID 754190, 11 pages, 2008.

[131] A. Mangerich and A. Bürkle, "Pleiotropic cellular functions of PARP1 in longevity and aging: genome maintenance meets inflammation," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 321653, 19 pages, 2012.

[132] A. A. Freitas and J. P. de Magalhaes, "A review and appraisal of the DNA damage theory of ageing," *Mutation Research/Reviews in Mutation Research*, vol. 728, no. 1-2, pp. 12–22, 2011.

[133] N. J. Curtin, "DNA repair dysregulation from cancer driver to therapeutic target," *Nature Reviews Cancer*, vol. 12, no. 12, pp. 801–817, 2012.

[134] T. A. Prolla and J. M. Denu, "NAD^+ deficiency in age-related mitochondrial dysfunction," *Cell Metabolism*, vol. 19, no. 2, pp. 178–180, 2014.

[135] F. Li, Z. Z. Chong, and K. Maiese, "Cell life versus cell longevity: the mysteries surrounding the NAD(+) precursor nicotinamide," *Current Medicinal Chemistry*, vol. 13, no. 8, pp. 883–895, 2006.

[136] M. S. Bonkowski and D. A. Sinclair, "Slowing ageing by design: the rise of NAD+ and sirtuin-activating compounds," *Nature Reviews Molecular Cell Biology*, vol. 17, no. 11, pp. 679–690, 2016.

[137] S. Imai and L. Guarente, "It takes two to tango: NAD+ and sirtuins in aging/longevity control," *npj Aging and Mechanisms of Disease*, vol. 2, no. 1, article 16017, 2016.

[138] K. Maiese, Z. Z. Chong, J. Hou, and Y. C. Shang, "The vitamin nicotinamide: translating nutrition into clinical care," *Molecules*, vol. 14, no. 12, pp. 3446–3485, 2009.

[139] J. Y. Kwak, H. J. Ham, C. M. Kim, and E. S. Hwang, "Nicotinamide exerts antioxidative effects on senescent cells," *Molecules and Cells*, vol. 38, no. 3, pp. 229–235, 2015.

[140] K. Tsubota, "The first human clinical study for NMN has started in Japan," *npj Aging and Mechanisms of Disease*, vol. 2, no. 1, p. 16021, 2016.

[141] T. Hashimoto, M. Horikawa, T. Nomura, and K. Sakamoto, "Nicotinamide adenine dinucleotide extends the lifespan of *Caenorhabditis elegans* mediated by *sir-2.1* and *daf-16*," *Biogerontology*, vol. 11, no. 1, pp. 31–43, 2010.

[142] H. Zhang, D. Ryu, Y. Wu et al., "NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice," *Science*, vol. 352, no. 6292, pp. 1436–1443, 2016.

[143] L. Mouchiroud, R. H. Houtkooper, N. Moullan et al., "The NAD(+)/sirtuin pathway modulates longevity through activation of mitochondrial UPR and FOXO signaling," *Cell*, vol. 154, no. 2, pp. 430–441, 2013.

[144] M. B. Jensen and H. Jasper, "Mitochondrial proteostasis in the control of aging and longevity," *Cell Metabolism*, vol. 20, no. 2, pp. 214–225, 2014.

[145] T. Arnould, S. Michel, and P. Renard, "Mitochondria retrograde signaling and the UPR mt: where are we in mammals?," *International Journal of Molecular Sciences*, vol. 16, no. 8, pp. 18224–18251, 2015.

[146] M. Borch Jensen, Y. Qi, R. Riley, L. Rabkina, and H. Jasper, "PGAM5 promotes lasting FoxO activation after developmental mitochondrial stress and extends lifespan in Drosophila," *Elife*, vol. 6, 2017.

[147] C. J. Fiorese and C. M. Haynes, "Integrating the UPRmtinto the mitochondrial maintenance network," *Critical Reviews in Biochemistry and Molecular Biology*, vol. 52, no. 3, pp. 304–313, 2017.

[148] K. Gariani, K. J. Menzies, D. Ryu et al., "Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice," *Hepatology*, vol. 63, no. 4, pp. 1190–1204, 2016.

[149] C. Lerner, A. Bitto, D. Pulliam et al., "Reduced mammalian target of rapamycin activity facilitates mitochondrial retrograde signaling and increases life span in normal human fibroblasts," *Aging Cell*, vol. 12, no. 6, pp. 966–977, 2013.

[150] M. Kaeberlein, D. Hu, E. O. Kerr et al., "Increased life span due to calorie restriction in respiratory-deficient yeast," *PLoS Genetics*, vol. 1, no. 5, article e69, 2005.

[151] S.-J. Lin, E. Ford, M. Haigis, G. Liszt, and L. Guarente, "Calorie restriction extends yeast life span by lowering the level of NADH," *Genes & Development*, vol. 18, no. 1, pp. 12–16, 2004.

[152] P. Kyryakov, A. Beach, V. R. Richard et al., "Caloric restriction extends yeast chronological lifespan by altering a pattern of age-related changes in trehalose concentration," *Frontiers in Physiology*, vol. 3, 2012.

[153] A. Leonov, R. Feldman, A. Piano et al., "Caloric restriction extends yeast chronological lifespan via a mechanism linking cellular aging to cell cycle regulation, maintenance of a quiescent state, entry into a non-quiescent state and survival in the non-quiescent state," *Oncotarget*, vol. 8, no. 41, pp. 69328–69350, 2017.

[154] T. L. Kaeberlein, E. D. Smith, M. Tsuchiya et al., "Lifespan extension in *Caenorhabditis elegans* by complete removal of food," *Aging Cell*, vol. 5, no. 6, pp. 487–494, 2006.

[155] B. N. Heestand, Y. Shen, W. Liu et al., "Dietary restriction induced longevity is mediated by nuclear receptor NHR-62 in *Caenorhabditis elegans*," *PLoS Genetics*, vol. 9, no. 7, article e1003651, 2013.

[156] I. Bjedov, J. M. Toivonen, F. Kerr et al., "Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*," *Cell Metabolism*, vol. 11, no. 1, pp. 35–46, 2010.

[157] R. A. Miller, D. E. Harrison, C. M. Astle et al., "Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice," *The Journals of Gerontology: Series A*, vol. 66A, no. 2, pp. 191–201, 2011.

[158] V. N. Anisimov, M. A. Zabezhinski, I. G. Popovich et al., "Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice," *Cell Cycle*, vol. 10, no. 24, pp. 4230–4236, 2011.

[159] D. E. Harrison, R. Strong, Z. D. Sharp et al., "Rapamycin fed late in life extends lifespan in genetically heterogeneous mice," *Nature*, vol. 460, no. 7253, pp. 392–395, 2009.

[160] R. J. Colman, T. M. Beasley, J. W. Kemnitz, S. C. Johnson, R. Weindruch, and R. M. Anderson, "Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys," *Nature Communications*, vol. 5, 2014.

[161] C. K. Martin, L. K. Heilbronn, L. de Jonge et al., "Effect of calorie restriction on resting metabolic rate and

spontaneous physical activity," *Obesity*, vol. 15, no. 12, pp. 2964–2973, 2007.

[162] K. Hagopian, J. J. Ramsey, and R. Weindruch, "Enzymes of glycerol and glyceraldehyde metabolism in mouse liver: effects of caloric restriction and age on activities," *Bioscience Reports*, vol. 28, no. 2, pp. 107–115, 2008.

[163] S. Summermatter, D. Mainieri, A. P. Russell et al., "Thrifty metabolism that favors fat storage after caloric restriction: a role for skeletal muscle phosphatidylinositol-3-kinase activity and AMP-activated protein kinase," *The FASEB Journal*, vol. 22, no. 3, pp. 774–785, 2008.

[164] P. B. M. De Andrade, L. A. Neff, M. K. Strosova et al., "Caloric restriction induces energy-sparing alterations in skeletal muscle contraction, fiber composition and local thyroid hormone metabolism that persist during catch-up fat upon refeeding," *Frontiers in Physiology*, vol. 6, 2015.

[165] D. W. Lamming and R. M. Anderson, "Metabolic effects of caloric restriction," in *eLS. Chichester*, John Wiley & Sons Ltd, 2014.

[166] A. J. Donato, A. E. Walker, K. A. Magerko et al., "Life-long caloric restriction reduces oxidative stress and preserves nitric oxide bioavailability and function in arteries of old mice," *Aging Cell*, vol. 12, no. 5, pp. 772–783, 2013.

[167] X. Qiu, K. Brown, M. D. Hirschey, E. Verdin, and D. Chen, "Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation," *Cell Metabolism*, vol. 12, no. 6, pp. 662–667, 2010.

[168] A. R. Heydari, A. Unnikrishnan, L. V. Lucente, and A. Richardson, "Caloric restriction and genomic stability," *Nucleic Acids Research*, vol. 35, no. 22, pp. 7485–7496, 2007.

[169] W. E. Sonntag, C. D. Lynch, W. T. Cefalu et al., "Pleiotropic effects of growth hormone and insulin-like growth factor (IGF)-1 on biological aging: inferences from moderate caloric-restricted animals," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 54, no. 12, pp. B521–B538, 1999.

[170] I. Shimokawa, Y. Higami, T. Tsuchiya et al., "Life span extension by reduction of the growth hormone-insulin-like growth factor-1 axis: relation to caloric restriction," *The FASEB Journal*, vol. 17, no. 9, pp. 1108-1109, 2003.

[171] S. D. Hursting, S. M. Smith, L. M. Lashinger, A. E. Harvey, and S. N. Perkins, "Calories and carcinogenesis: lessons learned from 30 years of calorie restriction research," *Carcinogenesis*, vol. 31, no. 1, pp. 83–89, 2010.

[172] J. H. Um, S. J. Kim, D. W. Kim et al., "Tissue-specific changes of DNA repair protein Ku and mtHSP70 in aging rats and their retardation by caloric restriction," *Mechanisms of Ageing and Development*, vol. 124, no. 8-9, pp. 967–975, 2003.

[173] D. C. Cabelof, S. Yanamadala, J. J. Raffoul, Z. Guo, A. Soofi, and A. R. Heydari, "Caloric restriction promotes genomic stability by induction of base excision repair and reversal of its age-related decline," *DNA Repair*, vol. 2, no. 3, pp. 295–307, 2003.

[174] 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.

[175] M. V. Blagosklonny, "Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans)," *Cell Cycle*, vol. 9, no. 4, pp. 683–688, 2014.

[176] S. Robida-Stubbs, K. Glover-Cutter, D. W. Lamming et al., "TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO," *Cell Metabolism*, vol. 15, no. 5, pp. 713–724, 2012.

[177] S. L. Hands, C. G. Proud, and A. Wyttenbach, "mTOR's role in ageing: protein synthesis or autophagy?," *Aging*, vol. 1, no. 7, pp. 586–597, 2009.

[178] C. H. Jung, S.-H. Ro, J. Cao, N. M. Otto, and D.-H. Kim, "mTOR regulation of autophagy," *FEBS Letters*, vol. 584, no. 7, pp. 1287–1295, 2010.

[179] M. Kaeberlein, R. W. Powers 3rd, K. K. Steffen et al., "Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients," *Science*, vol. 310, no. 5751, pp. 1193–1196, 2005.

[180] P. Kapahi, B. M. Zid, T. Harper, D. Koslover, V. Sapin, and S. Benzer, "Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway," *Current Biology*, vol. 14, no. 10, pp. 885–890, 2004.

[181] T. Vellai, K. Takacs-Vellai, Y. Zhang, A. L. Kovacs, L. Orosz, and F. Muller, "Genetics: influence of TOR kinase on lifespan in *C. elegans*," *Nature.*, vol. 426, no. 6967, p. 620, 2003.

[182] L. Fontana, L. Partridge, and V. D. Longo, "Extending healthy life span—from yeast to humans," *Science*, vol. 328, no. 5976, pp. 321–326, 2010.

[183] C. Slack, M. E. Giannakou, A. Foley, M. Goss, and L. Partridge, "dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*," *Aging Cell*, vol. 10, no. 5, pp. 735–748, 2011.

[184] A. Ortega-Molina, A. Efeyan, E. Lopez-Guadamillas et al., "Pten positively regulates brown adipose function, energy expenditure, and longevity," *Cell Metabolism*, vol. 15, no. 3, pp. 382–394, 2012.

[185] M. M. Mihaylova and R. J. Shaw, "The AMPK signalling pathway coordinates cell growth, autophagy and metabolism," *Nature Cell Biology*, vol. 13, no. 9, pp. 1016–1023, 2011.

[186] D. G. Hardie, "AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function," *Genes & Development*, vol. 25, no. 18, pp. 1895–1908, 2011.

[187] G. R. Steinberg and B. E. Kemp, "AMPK in health and disease," *Physiological Reviews*, vol. 89, no. 3, pp. 1025–1078, 2009.

[188] B. Onken and M. Driscoll, "Metformin induces a dietary restriction–like state and the oxidative stress response to extend *C. elegans* healthspan via AMPK, LKB1, and SKN-1," *PLoS One*, vol. 5, no. 1, article e8758, 2010.

[189] J. Apfeld, G. O'Connor, T. McDonagh, P. S. DiStefano, and R. Curtis, "The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in *C. elegans*," *Genes & Development*, vol. 18, no. 24, pp. 3004–3009, 2004.

[190] V. N. Anisimov, "Metformin for cancer and aging prevention: is it a time to make the long story short?," *Oncotarget*, vol. 6, no. 37, pp. 39398–39407, 2015.

[191] M. Sorensen, A. Sanz, J. Gomez et al., "Effects of fasting on oxidative stress in rat liver mitochondria," *Free Radical Research*, vol. 40, no. 4, pp. 339–347, 2009.

[192] A. B. Crujeiras, D. Parra, E. Goyenechea, and J. A. Martinez, "Sirtuin gene expression in human mononuclear cells is modulated by caloric restriction," *Eur J Clin Invest.*, vol. 38, no. 9, pp. 672–678, 2008.

[193] S. Someya, W. Yu, W. C. Hallows et al., "Sirt3 mediates reduction of oxidative damage and prevention of age-related

hearing loss under caloric restriction," *Cell*, vol. 143, no. 5, pp. 802–812, 2010.

[194] S. Michan and D. Sinclair, "Sirtuins in mammals: insights into their biological function," *Biochemical Journal*, vol. 404, no. 1, pp. 1–13, 2007.

[195] M. T. Borra, B. C. Smith, and J. M. Denu, "Mechanism of human SIRT1 activation by resveratrol," *Journal of Biological Chemistry*, vol. 280, no. 17, pp. 17187–17195, 2005.

[196] M. Gertz, G. T. T. Nguyen, F. Fischer et al., "A molecular mechanism for direct sirtuin activation by resveratrol," *PLoS One*, vol. 7, no. 11, article e49761, 2012.

[197] B. Dasgupta and J. Milbrandt, "Resveratrol stimulates AMP kinase activity in neurons," *Proceedings of the National Academy of Sciences*, vol. 104, no. 17, pp. 7217–7222, 2007.

[198] L. M. V. de Almeida, M. C. Leite, A. P. Thomazi et al., "Resveratrol protects against oxidative injury induced by H2O2 in acute hippocampal slice preparations from Wistar rats," *Archives of Biochemistry and Biophysics*, vol. 480, no. 1, pp. 27–32, 2008.

[199] L. M. V. de Almeida, C. C. Piñeiro, M. C. Leite et al., "Protective effects of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures," *Neurochemical Research*, vol. 33, no. 1, pp. 8–15, 2008.

[200] L. Liu, L. Gu, Q. Ma, D. Zhu, and X. Huang, "Resveratrol attenuates hydrogen peroxide-induced apoptosis in human umbilical vein endothelial cells," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 1, pp. 88–94, 2013.

[201] L. M. V. de Almeida, C. C. Piñeiro, M. C. Leite et al., "Resveratrol increases glutamate uptake, glutathione content, and S100B secretion in cortical astrocyte cultures," *Cellular and Molecular Neurobiology*, vol. 27, no. 5, pp. 661–668, 2007.

[202] J. Chang, J. E. Cornell, H. Van Remmen, K. Hakala, W. F. Ward, and A. Richardson, "Effect of aging and caloric restriction on the mitochondrial proteome," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 62, no. 3, pp. 223–234, 2007.

[203] G. López-Lluch, N. Hunt, B. Jones et al., "Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency," *Proceedings of the National Academy of Sciences*, vol. 103, no. 6, pp. 1768–1773, 2006.

[204] Z. Ungvari, C. Parrado-Fernandez, A. Csiszar, and R. de Cabo, "Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging," *Circulation Research*, vol. 102, no. 5, pp. 519–528, 2008.

[205] L. Guarente, "NO link between calorie restriction and mitochondria," *Nature Chemical Biology*, vol. 1, no. 7, pp. 355-356, 2005.

[206] A. Richardson, S. N. Austad, Y. Ikeno, A. Unnikrishnan, and R. J. McCarter, "Significant life extension by ten percent dietary restriction," *Annals of the New York Academy of Sciences*, vol. 1363, no. 1, pp. 11–17, 2016.

[207] Y. Zhang, Y. Ikeno, A. Bokov et al., "Dietary restriction attenuates the accelerated aging phenotype of *Sod1*$^{-/-}$ mice," *Free Radical Biology and Medicine*, vol. 60, pp. 300–306, 2013.

[208] Y. C. Jang, Y. Liu, C. R. Hayworth et al., "Dietary restriction attenuates age-associated muscle atrophy by lowering oxidative stress in mice even in complete absence of CuZnSOD," *Aging Cell*, vol. 11, no. 5, pp. 770–782, 2012.

[209] M. Meydani and W. J. Evans, "Free radicals, exercise, and aging," in *Free Radicals in Aging*, B. P. Yu, Ed., pp. 183–204, CRC Press, Boca Raton, 1993.

[210] L. L. Ji, "Antioxidants and oxidative stress in exercise," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 222, no. 3, pp. 283–292, 1999.

[211] J. Bejma, P. R. Ramires, C. Donahue, and L. L. Ji, "Aging and acute exercise enhances free radical generation and oxidative damage in skeletal muscle," *Medicine & Science in Sports & Exercise*, vol. 30, Supplement, p. 322, 1998.

[212] M.-C. Gomez-Cabrera, C. Borrás, F. V. Pallardó, J. Sastre, L. L. Ji, and J. Viña, "Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats," *The Journal of Physiology*, vol. 567, no. 1, pp. 113–120, 2005.

[213] M. A. Naseeb and S. L. Volpe, "Protein and exercise in the prevention of sarcopenia and aging," *Nutrition Research*, vol. 40, pp. 1–20, 2017.

[214] A. T. Ludlow and S. M. Roth, "Physical activity and telomere biology: exploring the link with aging-related disease prevention," *Journal of Aging Research*, vol. 2011, Article ID 790378, 12 pages, 2011.

[215] B. Hurley and I. Reuter, "Aging, physical activity, and disease prevention," *Journal of Aging Research*, vol. 2011, Article ID 782546, 2 pages, 2011.

[216] N. Garatachea, H. Pareja-Galeano, F. Sanchis-Gomar et al., "Exercise attenuates the major hallmarks of aging," *Rejuvenation Research*, vol. 18, no. 1, pp. 57–89, 2015.

[217] J. Hollander, J. Bejma, T. Ookawara, H. Ohno, and L. L. Ji, "Superoxide dismutase gene expression in skeletal muscle: fiber-specific effect of age," *Mechanisms of Ageing and Development*, vol. 116, no. 1, pp. 33–45, 2000.

[218] A. McArdle and M. J. Jackson, "Exercise, oxidative stress and ageing," *Journal of Anatomy*, vol. 197, no. 4, pp. 539–541, 2000.

[219] Y.-J. Kim and D. M. Wilson III, "Overview of base excision repair biochemistry," *Current Molecular Pharmacology*, vol. 5, no. 1, pp. 3–13, 2012.

[220] M. Bar-Shai, E. Carmeli, and A. Z. Reznick, "The role of NF-κB in protein breakdown in immobilization, aging, and exercise: from basic processes to promotion of health," *Annals of the New York Academy of Sciences*, vol. 1057, no. 1, pp. 431–447, 2005.

[221] Z. Radak, H. Y. Chung, E. Koltai, A. W. Taylor, and S. Goto, "Exercise, oxidative stress and hormesis," *Ageing Research Reviews*, vol. 7, no. 1, pp. 34–42, 2008.

[222] 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.

[223] L. L. Ji, "Antioxidant signaling in skeletal muscle: a brief review," *Experimental Gerontology*, vol. 42, no. 7, pp. 582–593, 2007.

[224] B. Chance, H. Sies, and A. Boveris, "Hydroperoxide metabolism in mammalian organs," *Physiological Reviews*, vol. 59, no. 3, pp. 527–605, 1979.

[225] T. Akimoto, S. C. Pohnert, P. Li et al., "Exercise stimulates Pgc-1α transcription in skeletal muscle through activation of the p38 MAPK pathway," *Journal of Biological Chemistry*, vol. 280, no. 20, pp. 19587–19593, 2005.

[226] 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.

[227] L. L. Ji, "Exercise at old age: does it increase or alleviate oxidative stress?," *Annals of the New York Academy of Sciences*, vol. 928, pp. 236–247, 2001.

[228] Z. Radák, H. Y. Chung, H. Naito et al., "Age-associated increase in oxidative stress and nuclear factor κB activation are attenuated in rat liver by regular exercise," *The FASEB Journal*, vol. 18, no. 6, pp. 749-750, 2004.

[229] C. S. Broome, A. C. Kayani, J. Palomero et al., "Effect of lifelong overexpression of HSP70 in skeletal muscle on age-related oxidative stress and adaptation after nondamaging contractile activity," *The FASEB Journal*, vol. 20, no. 9, pp. 1549–1551, 2006.

[230] T. A. Hornberger, R. D. Mateja, E. R. Chin, J. L. Andrews, and K. A. Esser, "Aging does not alter the mechanosensitivity of the p38, p70S6k, and JNK2 signaling pathways in skeletal muscle," *Journal of Applied Physiology*, vol. 98, no. 4, pp. 1562–1566, 2005.

[231] D. Williamson, P. Gallagher, M. Harber, C. Hollon, and S. Trappe, "Mitogen-activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle," *The Journal of Physiology*, vol. 547, no. 3, pp. 977–987, 2003.

[232] M. Gleeson, N. C. Bishop, D. J. Stensel, M. R. Lindley, S. S. Mastana, and M. A. Nimmo, "The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease," *Nature Reviews Immunology*, vol. 11, no. 9, pp. 607–615, 2011.

[233] I. Gath, E. I. Closs, U. Gödtel-Armbrust et al., "Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: implications for contractile function," *The FASEB Journal*, vol. 10, no. 14, pp. 1614–1620, 1996.

[234] M. R. Rose, *Evolutionary biology of aging*, Oxford University Press, Oxford, UK, 1994.

[235] M. Ackermann, S. C. Stearns, and U. Jenal, "Senescence in a bacterium with asymmetric division," *Science*, vol. 300, no. 5627, p. 1920, 2003.

[236] M. Ackermann, L. Chao, C. T. Bergstrom, and M. Doebeli, "On the evolutionary origin of aging," *Aging Cell*, vol. 6, no. 2, pp. 235–244, 2007.

[237] E. J. Stewart, R. Madden, G. Paul, and F. Taddei, "Aging and death in an organism that reproduces by morphologically symmetric division," *PLoS Biology*, vol. 3, no. 2, article e45, 2005.

[238] J.-W. J. Veening, E. J. Stewart, T. W. Berngruber, F. Taddei, O. P. Kuipers, and L. W. Hamoen, "Bet-hedging and epigenetic inheritance in bacterial cell development," *Proceedings of the National Academy of Sciences*, vol. 105, no. 11, pp. 4393–4398, 2008.

[239] B. B. Aldridge, M. Fernandez-Suarez, D. Heller et al., "Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility," *Science*, vol. 335, no. 6064, pp. 100–104, 2012.

[240] T. Nyström, "Conditional senescence in bacteria: death of the immortals," *Molecular Microbiology*, vol. 48, no. 1, pp. 17–23, 2003.

[241] K. A. Steinkraus, M. Kaeberlein, and B. K. Kennedy, "Replicative aging in yeast: the means to the end," *Annual Review of Cell and Developmental Biology*, vol. 24, no. 1, pp. 29–54, 2008.

[242] J. Winkler, A. Seybert, L. König et al., "Quantitative and spatio-temporal features of protein aggregation in Escherichia coli and consequences on protein quality control and cellular ageing," *The EMBO Journal*, vol. 29, no. 5, pp. 910–923, 2010.

[243] A. B. Lindner, R. Madden, A. Demarez, E. J. Stewart, and F. Taddei, "Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation," *Proceedings of the National Academy of Sciences*, vol. 105, no. 8, pp. 3076–3081, 2008.

[244] M. Coelho and I. M. Tolic, "Asymmetric damage segregation at cell division via protein aggregate fusion and attachment to organelles," *Bioessays*, vol. 37, no. 7, pp. 740–747, 2015.

[245] R. Higuchi-Sanabria, W. M. A. Pernice, J. D. Vevea, D. M. Alessi Wolken, I. R. Boldogh, and L. A. Pon, "Role of asymmetric cell division in lifespan control in *Saccharomyces cerevisiae*," *FEMS Yeast Research*, vol. 14, no. 8, pp. 1133–1146, 2014.

[246] H. Aguilaniu, L. Gustafsson, M. Rigoulet, and T. Nyström, "Asymmetric inheritance of oxidatively damaged proteins during cytokinesis," *Science*, vol. 299, no. 5613, pp. 1751–1753, 2003.

[247] A.-S. Coquel, J.-P. Jacob, M. Primet et al., "Localization of protein aggregation in *Escherichia coli* is governed by diffusion and nucleoid macromolecular crowding effect," *PLoS Computational Biology*, vol. 9, no. 4, article e1003038, 2013.

[248] B. Desnues, C. Cuny, G. Grégori, S. Dukan, H. Aguilaniu, and T. Nyström, "Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable *Escherichia coli* cells," *EMBO Reports*, vol. 4, no. 4, pp. 400–404, 2003.

[249] I. Dalle-Donne, G. Aldini, M. Carini, R. Colombo, R. Rossi, and A. Milzani, "Protein carbonylation, cellular dysfunction, and disease progression," *Journal of Cellular and Molecular Medicine*, vol. 10, no. 2, pp. 389–406, 2006.

[250] I. Dalle-Donne, D. Giustarini, R. Colombo, R. Rossi, and A. Milzani, "Protein carbonylation in human diseases," *Trends in Molecular Medicine*, vol. 9, no. 4, pp. 169–176, 2003.

[251] T. Nyström, "The free-radical hypothesis of aging goes prokaryotic," *Cellular and Molecular Life Sciences (CMLS)*, vol. 60, no. 7, pp. 1333–1341, 2003.

[252] A. L. Santos, V. Oliveira, I. Baptista et al., "Wavelength dependence of biological damage induced by UV radiation on bacteria," *Archives of Microbiology*, vol. 195, no. 1, pp. 63–74, 2013.

[253] F. Bosshard, K. Riedel, T. Schneider, C. Geiser, M. Bucheli, and T. Egli, "Protein oxidation and aggregation in UVA-irradiated *Escherichia coli* cells as signs of accelerated cellular senescence," *Environmental Microbiology*, vol. 12, no. 11, pp. 2931–2945, 2010.

[254] S. Dukan, T. Nyström, and T. Nystro, "Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells," *Journal of Biological Chemistry*, vol. 274, no. 37, pp. 26027–26032, 1999.

[255] F. B. Johnson, D. A. Sinclair, and L. Guarente, "Molecular biology of aging," *Cell*, vol. 96, no. 2, pp. 291–302, 1999.

[256] S. Dukan and T. Nyström, "Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon," *Genes & Development*, vol. 12, no. 21, pp. 3431–3441, 1998.

[257] J. Tamarit, E. Cabiscol, 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.

[258] E. Cabiscol, E. Piulats, P. Echave, E. Herrero, and J. Ros, "Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*," *Journal of Biological Chemistry*, vol. 275, no. 35, pp. 27393–27398, 2000.

[259] R. S. Sohal, "Role of oxidative stress and protein oxidation in the aging process," *Free Radical Biology and Medicine*, vol. 33, no. 1, pp. 37–44, 2002.

[260] L.-J. Yan, R. L. Levine, and R. S. Sohal, "Oxidative damage during aging targets mitochondrial aconitase," *Proceedings of the National Academy of Sciences*, vol. 94, no. 21, pp. 11168–11172, 1997.

[261] A. Castegna, M. Aksenov, M. Aksenova et al., "Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1," *Free Radical Biology and Medicine*, vol. 33, no. 4, pp. 562–571, 2002.

[262] A. Matin, "The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*," *Molecular Microbiology*, vol. 5, no. 1, pp. 3–10, 1991.

[263] D. E. Jenkins, J. E. Schultz, and A. Matin, "Starvation-induced cross protection against heat or H2O2 challenge in Escherichia coli," *Journal of Bacteriology*, vol. 170, no. 9, pp. 3910–3914, 1988.

[264] R. A. Miller, "Cell stress and aging: new emphasis on multiplex resistance mechanisms," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 64A, no. 2, pp. 179–182, 2009.

[265] L. E. Enell, N. Kapan, J. A. E. Soderberg, L. Kahsai, and D. R. Nassel, "Insulin signaling, lifespan and stress resistance are modulated by metabotropic GABA receptors on insulin producing cells in the brain of *Drosophila*," *PLoS One*, vol. 5, no. 12, article e15780, 2010.

[266] S. Ayyadevara, M. R. Engle, S. P. Singh et al., "Lifespan and stress resistance of *Caenorhabditis elegans* are increased by expression of glutathione transferases capable of metabolizing the lipid peroxidation product 4-hydroxynonenal," *Aging Cell*, vol. 4, no. 5, pp. 257–271, 2005.

[267] M. Shaposhnikov, E. Proshkina, L. Shilova, A. Zhavoronkov, and A. Moskalev, "Lifespan and stress resistance in *Drosophila* with overexpressed DNA repair genes," *Scientific Reports*, vol. 5, no. 1, article 15299, 2015.

[268] V. D. Longo, J. Mitteldorf, and V. P. Skulachev, "Programmed and altruistic ageing," *Nature Reviews Genetics*, vol. 6, no. 11, pp. 866–872, 2005.

[269] M. Ballesteros, A. Fredriksson, J. Henriksson, and T. Nyström, "Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes," *The EMBO Journal*, vol. 20, no. 18, pp. 5280–5289, 2001.

[270] H. Aguilaniu, L. Gustafsson, M. Rigoulet, and T. Nyström, "Protein oxidation in G0 cells of *Saccharomyces cerevisiae* depends on the state rather than rate of respiration and is enhanced in *pos9* but not *yap1* mutants," *Journal of Biological Chemistry*, vol. 276, no. 38, pp. 35396–35404, 2001.

[271] T. Nyström, "Not quite dead enough: on bacterial life, culturability, senescence, and death," *Archives of Microbiology*, vol. 176, no. 3, pp. 159–164, 2001.

[272] A. Battesti, N. Majdalani, and S. Gottesman, "The RpoS-mediated general stress response in *Escherichia coli*," *Annual Review of Microbiology*, vol. 65, no. 1, pp. 189–213, 2011.

[273] T. L. Testerman, A. Vazquez-Torres, Y. Xu, J. Jones-Carson, S. J. Libby, and F. C. Fang, "The alternative sigma factor σ^E controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival," *Molecular Microbiology*, vol. 43, no. 3, pp. 771–782, 2002.

[274] F. Fontaine, E. J. Stewart, A. B. Lindner, and F. Taddei, "Mutations in two global regulators lower individual mortality in *Escherichia coli*," *Molecular Microbiology*, vol. 0, no. 0, pp. 2–14, 2007.

[275] S. Gonidakis, S. E. Finkel, and V. D. Longo, "Genome-wide screen identifies *Escherichia coli* TCA-cycle-related mutants with extended chronological lifespan dependent on acetate metabolism and the hypoxia-inducible transcription factor ArcA," *Aging Cell*, vol. 9, no. 5, pp. 868–881, 2010.

[276] L. Yu and C. Yu, "Interaction between succinate dehydrogenase and ubiquinone-binding protein from succinate-ubiquinone reductase," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 593, no. 1, pp. 24–38, 1980.

[277] K. R. Messner and J. A. Imlay, "Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase," *Journal of Biological Chemistry*, vol. 277, no. 45, pp. 42563–42571, 2002.

[278] S. Gonidakis, S. E. Finkel, and V. D. Longo, "*E. coli* hypoxia-inducible factor ArcA mediates lifespan extension in a lipoic acid synthase mutant by suppressing acetyl-CoA synthetase," *Biological Chemistry*, vol. 391, no. 10, pp. 1139–1147, 2010.

[279] A. S. Lynch and E. C. Lin, "Transcriptional control mediated by the ArcA two-component response regulator protein of Escherichia coli: characterization of DNA binding at target promoters," *Journal of Bacteriology*, vol. 178, no. 21, pp. 6238–6249, 1996.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2018, Article ID 8587475, 7 pages
https://doi.org/10.1155/2018/8587475

Research Article

ROS-Induced DNA Damage Associates with Abundance of Mitochondrial DNA in White Blood Cells of the Untreated Schizophrenic Patients

I. V. Chestkov,[1] E. M. Jestkova,[2] E. S. Ershova,[1,3] V. G. Golimbet,[4] T. V. Lezheiko,[4] N. Yu Kolesina,[4] O. A. Dolgikh,[1] V. L. Izhevskaya,[1] G. P. Kostyuk,[2] S. I. Kutsev,[1] N. N. Veiko,[1,3] and S. V. Kostyuk[4]

[1]*Research Centre for Medical Genetics (RCMG), Moscow 115478, Russia*
[2]*N. A. Alexeev Clinical Psychiatric Hospital №1 of Moscow Healthcare Department, Moscow 115447, Russia*
[3]*V. A. Negovsky Research Institute of General Reanimatology, Federal Clinical Research Center of Reanimatology and Rehabilitogy, Moscow 107031, Russia*
[4]*Mental Health Research Center, Moscow 115522, Russia*

Correspondence should be addressed to S. V. Kostyuk; svet.kostyuk@gmail.com

Received 31 July 2017; Revised 1 November 2017; Accepted 10 December 2017; Published 25 February 2018

Academic Editor: Tanea T. Reed

Objective. The aim of this study was (1) to examine the leukocyte mtDNA copy number (*CN*) in unmedicated (SZ (m−)) and medicated (SZ (m+)) male patients with paranoid schizophrenia (SZ) in comparison with the healthy male controls (HC) and (2) to compare the leukocyte mtDNA *CN* with the content of an oxidation marker 8-oxodG in lymphocytes of the SZ (m−) patients. *Methods*. We evaluated leukocyte mtDNA *CN* of 110 subjects with SZ in comparison with 60 male HC by the method qPCR (ratio mtDNA/nDNA (gene B2M) was detected). SZ patients were divided into two subgroups. The patients of the subgroups SZ (m+) ($N = 55$) were treated with standard antipsychotic medications in the hospital. The patients of the subgroup SZ (m−) ($N = 55$) were not treated before venous blood was sampled. To evaluate oxidative DNA damage, we quantified the levels of 8-oxodG in lymphocytes (flow cytometry) of SZ (m−) patients ($N = 55$) and HC ($N = 30$). *Results*. The leukocyte mtDNA *CN* showed no significant difference in SZ (m+) patients and HC. The mtDNA *CN* in the unmedicated subgroup SZ (m−) was significantly higher than that in the SZ (m+) subgroup or in HC group. The level of 8-oxodG in the subgroup SZ (m−) was significantly higher than that in HC group. *Conclusion*. The leukocytes of the unmedicated SZ male patients with acute psychosis contain more mtDNA than the leukocytes of the male SZ patients treated with antipsychotic medications or the healthy controls. MtDNA content positively correlates with the level of 8-oxodG in the unmedicated SZ patients.

1. Introduction

Approximately 1% of the world's population suffer from schizophrenia (SZ). Schizophrenia is a highly heritable neuropsychiatric disorder of complex genetic etiology. Mitochondrion (mt) is a cellular organelle involved in the regulation of a variety of complex cellular processes. Mitochondria, the cell energy source, have a crucial role in intracellular calcium homeostasis, producing ROS and activating the apoptotic pathway. MtDNA copy number (*CN*) variation has been suggested as a sensitive index of cellular oxidative stress, inflammation, and mitochondrial dysfunction [1, 2].

The accumulating morphological, genetic, and imaging data delineates mitochondrial multifaceted dysfunction as a pathological factor in schizophrenia [3]. Several studies have evaluated the influence of schizophrenia on mtDNA copy number. Results are controversial to each other. Some authors show lower mtDNA copy number in brain tissues and peripheral lymphocytes of schizophrenia patients compared with healthy controls (HC) [4–7]. Some studies

reported no anomalous mtDNA copy number in the tissues of schizophrenia patients [8, 9]. The reason for the observed contradictions may be the lack of sufficient data on the effect of antipsychotic therapy on mtDNA copy number. It is known that administration of antipsychotics involved mitochondrial functions, especially OXPHOS. The antipsychotics inhibit the mitochondrial respiratory chain [3]. However, there are few studies on the impact of antipsychotics on mtDNA content in schizophrenia patients. Li et al. recently had showed that antipsychotic risperidone causes a decrease in the number of mtDNA copies in leukocytes of patients with the first-episode antipsychotic-naïve schizophrenia [4]. Thus, the first objective of our work was to determine the effects of the standard antipsychotic therapy on the number of mtDNA copies in the cells of male patients with paranoid schizophrenia.

Systemic oxidative stress is associated with schizophrenia. Elevated reactive oxygen species (ROS) levels and declined antioxidant statuses have been reported in the brain and peripheral tissues of the patients with schizophrenia [10–12]. However, it is known that oxidative stress in the body is associated with the changes in mtDNA copy number. For example, oxidative stress caused by environmental exposure, for example, ionizing radiation, induces ROS-caused increase of the amount of mtDNA in the human and animal cells and organisms [13–15].

Thus, we can assume that a high level of ROS in the body of some schizophrenia patients should correlate with an increased mtDNA copy number. So, the second objective of our work was to test this hypothesis. We quantified mtDNA content and the levels of oxidative stress marker 8-oxodG in the white blood cells of male patients with untreated paranoid schizophrenia.

2. Methods

2.1. Subjects. This investigation was approved by the Regional Ethics Committee of RCMG. It was carried out in accordance with the latest version of the Declaration of Helsinki. One hundred ten male paranoid SZ patients with acute psychotic disorders were recruited from the general psychiatric units for treatment of acute forms of mental disorders (Psychiatric Hospital #14 of Moscow City Health Department and Mental Health Research Center, Moscow). All participants signed an informed written consent to participate in this investigation. Age of the patients: 38 ± 13 (19–62). Clinical symptoms have been measured using the positive and negative syndromes scale (PANSS) (Kay et al. [16]), a widespread instrument proven to be valid and suitable for evaluation of positive, negative, and general psychopathological items. It includes three subscales, totally 30 items: 7 for positive symptoms, 7 for negative, and 16 for general psychopathological ones. Each symptom has 7 ratings (1—absent, 2—questionable, 3—mild, 4—moderate, 5—severe, 6—markedly severe, and 7—extremely severe). The PANSS interviews, completed by a trained researcher, were conducted one week before the patient's discharge from the hospital. Patients were diagnosed with paranoid schizophrenia (F20.00 and F20.01 in the International Classification of Diseases 10th Revision (ICD-10)) using structured mini international neuropsychiatric interview (MINI). Diagnoses were also confirmed pursuant to DSM-IV criteria. The paranoid SZ duration was less than 3 years ($n = 24$, 22%), 3–10 years ($n = 20$, 18%), and more than 10 years ($n = 66$, 60%). No subjects suffered from any relevant disease.

SZ patients were divided into groups: SZ (m+) ($N = 55$) and SZ (m−) ($N = 55$). The patients in group SZ (m+) received standard antipsychotic therapy in the hospital for at least six weeks before the venous blood was sampled. They were clinically stable and their medications had not changed for at least one month. For the treatment of the acute disorders, standard antipsychotics were used: haloperidol, chlorpromazine, clozapine, risperidone, quetiapine, and olanzapine. Most of the patients of the group SZ (m−) refused to take antipsychotics in the home despite of the chronic course of the disease. These patients did not take antipsychotics for 3 to 8 months until a new hospitalization with acute psychosis. Some patients of the group SZ (m−) ($N = 12$, 21.8%) were diagnosed as the first-episode SZ and were never treated with antipsychotic medications.

From the SZ (m−) patients, the venous blood was collected on the day of hospitalization before starting any antipsychotic therapy. 20 mL of blood was collected from a peripheral vein of the subject using a syringe flushed with heparin under strict aseptic conditions. The control group consisted of 60 healthy males with no history of any psychiatric disorder, correlating with the patient group by age (37 ± 12 (17–73)) and by their smoking habits.

2.2. Isolating of DNA from the Blood. The leukocytes were isolated from 5 mL of blood by the method of Boyum [17]. 5 mL of the solution containing 2% sodium lauryl sarcosylate, 0.04 M EDTA, and 150 μg/mL RNAse A (Sigma, USA) was added to the fresh leucocytes for 45 min (37°C) and then was treated with proteinase K (200 μg/mL, Promega, USA) for 24 h at 37°C. The lysate samples were extracted with an equal volume of phenol, phenol/chloroform/isoamyl alcohol (25 : 24 : 1), and chloroform/isoamyl alcohol (24 : 1), respectively. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of ice-cold ethanol. For the extraction procedure, only freshly distilled solvents were used. Phenol was stabilized with 8-hydroxyquinoline. Finally, the DNA was collected by centrifugation at 10,000 g for 15 min at 4°C, washed with 70% ethanol (v/v), dried, and dissolved in water. The quantitation of the purified genomic DNA is performed using the PicoGreen dsDNA quantitation reagent from Molecular Probes (Invitrogen, CA, USA). The assay displays a linear correlation between dsDNA quantity and fluorescence over a wide range. The DNA concentration of the samples is calculated according to a DNA standard curve. We use EnSpire equipment (Finland) with the following parameters: excitation and emission wavelengths 488 and 528 nm, respectively.

2.3. Quantitative Real-Time PCR (qPCR). Serial qPCR assay was established using the StepOnePlus (Applied Biosystems). QPCR efficiencies for ten samples were determined by serially diluted genomic DNA. MtDNA copy number

determinations in the other samples were obtained using a calculated average efficiency (E + 1) of 2.0 ± 0.05 for gene B2M and 1.95 ± 0.03 for mtDNA. Each reaction contained 10 μL 2xSYBR Premix Ex Taq (PerfectRealTime™, Takara Bio), 2 μL primers (10 μM), and 8 μL of DNA (5 ng/μL) for a final volume of 20 μL. All reactions were performed in duplicates. PCR conditions were 6 min at 95°C initial denaturation, followed by 40 cycles of 30 s of denaturation at 95°C, 15 s of primer annealing at 60°C, and 10 s at 72°C of extension. The presence of unspecific amplicons was excluded by melting curve analysis and gel electrophoresis. The following primers (Malik et al. [18]) were used (Sintol, Russia):

Human mitochondrial genome NC_012920 (D-loop)
hmito (65) F CTTCTGGCCACAGCACTTAAAC; R GCTGGTGTTAGGGTTCTTTGTTTT
Human B2M (accession number M17987)
hB2M (95) F GCTGGGTAGCTCTAAACAATGTAT TCA; R CCATGTACTAACAAATGTCTAAAATGG

2.4. Flow Cytometry Analysis (FCA) of 8-oxodG. Method is described in detail earlier [19]. Briefly, lymphocytes were isolated from 15 mL of the fresh blood. To fix the cells, 2% paraformaldehyde (Sigma) was used (37°C, 10 min). Cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS (15 min, 25°C). Cells were stained with FITC-8-oxodG Abcam antibody (1 μg/mL, 4 h, 4°C). We stained a portion of the cells with secondary FITC-conjugated antibodies to determine the level of the background fluorescence. Cells were analyzed at CyFlow Space (Partec, Germany). Primary data are presented as median values of the signal FL1 (8-oxodG). Index 8-oxodG = (I−Ib)/Ib, where I and Ib are the experienced and the background signal.

2.5. Statistics. All reported results were reproduced at least two times as independent biological replicates.

The significance of the observed differences was analyzed using the nonparametric Mann–Whitney *U*-tests. All *p* values were two-sided and considered statistically significant at $\alpha < 0.05$.

For the statistical analysis was used Professional software StatPlus2007 (http://www.analystsoft.com/).

3. Results

3.1. Quantification of mtDNA in Leukocytes of HC and SZ Groups. The mtDNA *CN* in the leukocytes of SZ patients and HC detected by qPCR (mtDNA to nDNA (gene B2M) ratio was measured) is given in Figure 1. Comparison of the samples by the Mann–Whitney test is given in the table in Figure 1. In 12 patients of 55 (21.8%) from SZ (m−) group and only in 1 patients of 55 (1.7%) from SZ (m+) group, a considerable increase in mtDNA *CN* compared to the HC was found (Figure 1). Median mtDNA *CN* for the leukocytes of the unmedicated SZ (m−) patients was 2.8 or 2.2 times higher than the median mtDNA *CN* for the leukocytes of the medicated SZ (m+) or HC. The samples of HC and SZ (m+) did not differ among themselves in the content of mtDNA in leukocytes.

		Mt DNA CN			U-test
Group	N	Mean ± SD	Median	Range	HD-SZ
HD	60	153 ± 91	107	28 - 438	
SZ (m+)	55	141 ± 95	137	14 - 457	$p = 0.09$
SZ (m−)	55	311 ± 141	301	34 - 622	$p = 10^{-9}$

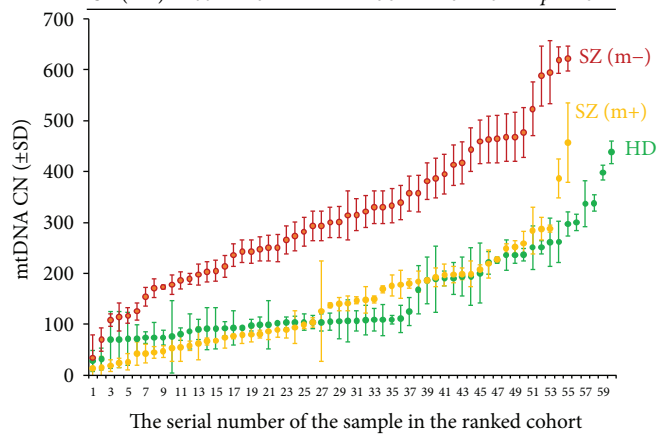


FIGURE 1: Leukocytes from unmedicated SZ patients with acute psychosis are enriched with mtDNA. Graph—Quantification of mtDNA in leukocytes of HC (green), SZ (m−) (red), and SZ (m+) (yellow) groups. The data in each group is ranked by the value of mtDNA *CN*. The mean and standard deviation for each DNA sample is given (the data of two independent experiments, three measurements in each experiment, $n = 6$). Table contains statistical data.

We did not find any significant correlation between the clinical symptoms (indexes of the scale PANSS) and the number of copies of mtDNA for the medicated SZ patients ($k < 0.2$; $p > 0.1$, linear regression method).

3.2. Quantification of 8-oxodG in Lymphocytes of HC and SZ (m−) Groups. FCA was employed in order to evaluate 8-oxodG content in the lymphocytes (Figure 2). Staining of the fixed cells with 8-oxodG antibodies was performed [19]. Figure 2 shows the mean values of the signal against the baseline values (8-oxodG index) for the groups HC and SZ (m−) (see table in Figure 2 for descriptive statistics). The 8-oxodG content in SZ (m−) lymphocytes was higher than in the control group ($p = 10^{-6}$). The patients from the SZ (m−) group were divided into subgroups: SZ-1(m−) ($N = 31$) with normal 8-oxodG content and SZ-2 (m−) ($N = 24$) with increased 8-oxodG content compared to control. These groups differ in the level of cellular DNA oxidation ($p = 10^{-10}$) (Figure 2).

3.3. The Dependence of mtDNA CN on 8-oxodG in HC and SZ (m−) Groups. Figure 3(a) presents dependence of mtDNA *CN* in the leukocytes on logarithm of the 8-oxodG content in the lymphocytes for control and SZ (m−) groups. In the control group ($N = 30$), correlation between the two parameters was found ($k = 0.47$; $p < 0.01$). In the total SZ (m−) group ($N = 55$), linear correlation between lg (8-oxodG) and mtDNA *CN* content was also found ($k = 0.44$; $p < 0.001$). The correlation between the analyzed parameters was detected for the total sample of healthy

Group	N	8-oxodG, arb.un. Mean ± SD	Median	Range	U-test HD-SZ
HD	30	0.6 ± 0.5	0.57	0.01 - 1.87	
SZ (m−)	55	3.2 ± 3.7	1.5	0.05 - 1.67	$p = 10^{-6}$
SZ-1 (m−)	31	0.9 ± 0.5	1.06	0.05 - 1.78	$p = 0.06$
SZ-2 (m−)	24	6.1 ± 3.9	4.6	1.99 - 16.7	$p = 10^{-10}$

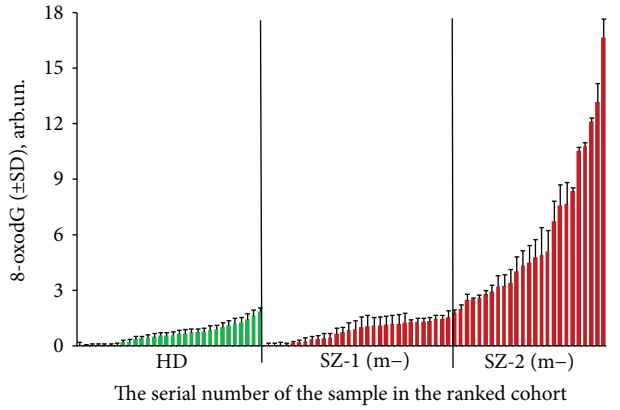


Figure 2: Lymphocytes from the unmedicated SZ patients with acute psychosis are enriched with 8-oxodG. Flow cytometry analysis of 8-oxodG. Index 8-oxodG (arb.un) is the ratio of the difference between the experienced and the background signal to the magnitude of the background signal. Graph—Quantification of 8-oxodG in lymphocytes of HC (green) and SZ (m−) (red) groups. The data in each group is ranked by the value of 8-oxodG. The mean and standard deviation for each cell sample is given (the data of three independent experiments, two measurements in each experiment, $n = 6$). Table contains statistical data.

and schizophrenic people ($N = 85$, $k = 0.53$, $p < 0.0001$). Thus, the greater the level of DNA oxidation in the human cells, the greater the content of mtDNA.

The ratio (8-oxodG/mtDNA *CN*) indicates the amount of 8-oxodG per one copy of mtDNA (Figure 3(b)). This ratio was significantly higher in the SZ-2 group than in the group SZ-1 or HC. For 11 patients of 55, this index reached very high values in comparison with the rest of the SZ (m−) sample. All these patients were sick with schizophrenia for more than 10 years and smoked more than 40 cigarettes a day. However, for the whole group SZ (m−), we did not find a pronounced dependence of the indices 8-oxodG, mtDNA *CN*, and (8-oxodG/mtDNA *CN*) on the duration of schizophrenia or on the intensity of smoking.

We also analyzed the indices 8-oxodG, mtDNA *CN*, and (8-oxodG/mtDNA *CN*) in connection with the 29 indices of standard biochemical analysis of venous blood. We did not find any significant correlations.

4. Discussion

SZ is increasingly considered as a systemic disorder, which is associated with biochemical disturbances not only in the CNS/brain cells [20]. In recent years, there has been an intensive search for blood-based biomarkers for SZ [21]. The study of white blood cells can give an understanding of how the whole body responds to disturbances that eventually lead to the disorders of a brain function.

4.1. Abundance of the mtDNA CN in Leucocytes of the Unmedicated SZ Patients. The first finding of this study is higher mtDNA *CN* in the unmedicated SZ patients than in the medicated patients and the healthy controls (Figure 1). We have shown that acute psychosis of the unmedicated SZ (m−) patients is accompanied by a significant increase in the mtDNA *CN* in leucocytes (Figure 1). The leukocytes of SZ (m+) patients with the antipsychotic medication contain the amount of mtDNA comparable to the control. There are few studies on the impact of antipsychotics on mtDNA content in SZ patients. Li et al. recently also demonstrated that antipsychotic risperidone causes a decrease in the mtDNA *CN* in leukocytes of patients with the first-episode antipsychotic-naïve schizophrenia [4].

The reason for reduction of mtDNA *CN* in the medicated SZ patients remains unknown. The patients in this study were treated with various typical and atypical antipsychotics. In a state of acute psychosis, patients usually were treated with typical antipsychotics. After improving the mental state of the patient, he was treated with atypical antipsychotics. This fact did not allow us to form large enough groups to analyze the effect of specific antipsychotics on mtDNA *CN*. Recent studies have suggested that some antipsychotic drugs have a useful therapeutic effect by reducing oxidative stress in schizophrenic patients [22, 23]. Perhaps these drugs cause a decrease in the mtDNA *CN* through the antioxidant effects. It is also possible that antipsychotics change mitophagy in the cells of SZ patients. Mitophagy is a highly specific quality control process which eliminates dysfunctional mitochondria and promotes mitochondrial turnover [24]. Further studies are needed to understand the reasons for reducing the number of copies of mtDNA in the treated patients.

4.2. Abundance of mtDNA in the SZ (m−) Patients Associates with Oxidative DNA Damage. The lymphocytes of some unmedicated SZ patients contain more 8-oxodG than the lymphocytes of healthy controls (Figure 2). 8-oxodG represents major oxidative DNA damage products. An elevated level of 8-oxodG in the blood and urine has been reported in SZ patients [25, 26]. Postmortem studies have revealed a higher level of 8-oxodG in the brains of SZ patients compared to the controls [27, 28]. So our findings are consistent with those of other author who showed a high level of ROS in the SZ patient's body.

An internal source of ROS is present in the body of a patient with SZ [10–12]. The main cause of oxidative stress in schizophrenia has not yet been found. Different mechanisms of oxidative stress in schizophrenia have been postulated: dopamine metabolism, NO metabolism, abnormalities in the mitochondrial electron transport chain patients with schizophrenia, mutations in the genes responsible for maintaining the required level of ROS in cells, and so on. However, regardless of the cause, the result is important: in the cells of some SZ patients, the level of ROS is significantly increased. We can compare this situation with the effect on the cells of an external source of ionizing radiation, which induces oxidative stress in human. This model is attractive because it is well studied [13–15, 29–32]. Moreover, some authors hypothesized that ionizing radiation

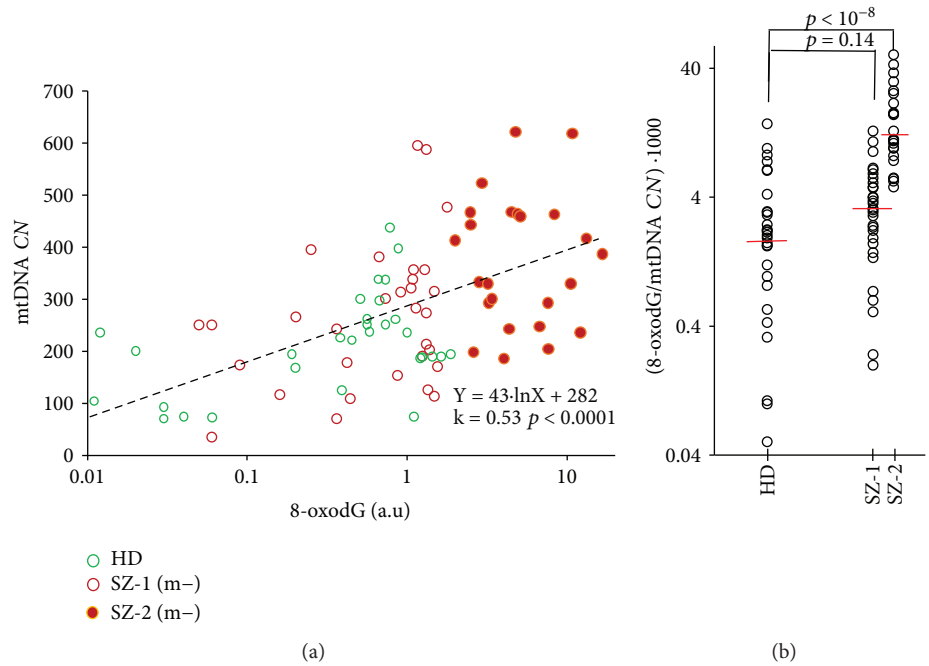


Figure 3: (a) The dependence of mtDNA *CN* on 8-oxodG (logarithmic scale) for unmedicated SZ (m−) and control groups. The SZ (m−) group was divided into two subgroups (SZ-1 and SZ-2). SZ-1 contains patients with normal content of 8-oxodG. Healthy donors belong to the same category. SZ-2 contains patients with high level of 8-oxodG. The graph shows the trend line and the linear regression equation. (b) Ratio 8-oxodG/mtDNA *CN* (logarithmic scale) is defined for three groups. Short lines denote medians. The comparison ratio 8-oxodG/mtDNA *CN* in the groups by the *U*-test is given.

may be an environmental trigger that can actualize a predisposition to human schizophrenia or indeed cause schizophrenia-like disorders [33, 34]. Technogenic catastrophes (Chernobyl and Fukushima), atomic explosions (Japan and the region of the Kazakhstan nuclear weapon testing area), and areas with high natural background radiation (in India) several times increase the risk of schizophrenia in the population. The recent SZ model study suggests that irradiation of rats in adulthood caused behavioral abnormalities relevant to schizophrenia [35].

In response to the oxidative stress, the cells of the unmedicated SZ patient (Figure 4) as well as cells of exposed to ionizing radiation human increase the amount of mtDNA. There is extensive literature on the increase in the number of mtDNA copies in response to ionizing radiation [13–15]. It is interesting to note that the effect of increasing mtDNA *CN* in untreated SZ-2 (m−) patients (average 2.8-fold compared with the control) is comparable to the effect of increasing mtDNA *CN* in leukemia patients undergoing total body irradiation therapy with a dose of 9 Gy (average 2.3-fold [36]). This may indicate that the levels of oxidative stress in some schizophrenia patient are comparable to the level of oxidative stress induced by sufficiently high doses of ionizing radiation.

It is currently unclear functional significance of an increased mitochondrial content induced by ROS. Some authors suggest that ROS could result in an adaptive response through enhanced production of mitochondria [15]. In conditions of oxidative stress, the transcriptional and replication machinery of mitochondrial biogenesis will be upregulated

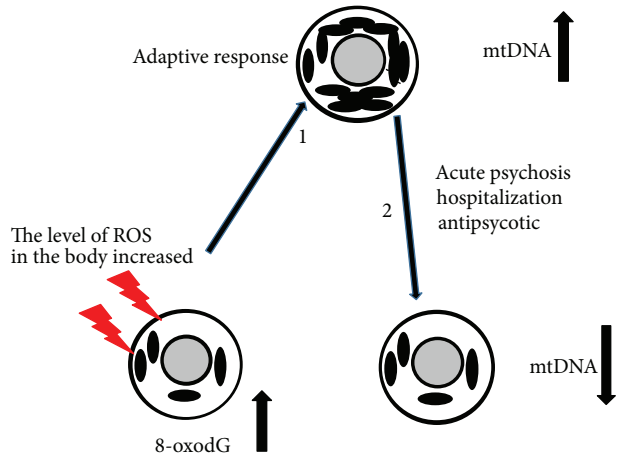


Figure 4: The scheme illustrates the choice of the parameters in the work. Schizophrenia is associated with elevated levels of ROS. Oxidative stress is known to cause the DNA damage. 8-oxodG is a marker of oxidative stress. (1) In response to increased levels of ROS and DNA damage, the cell increases the number of mitochondria. This response of the SZ patient's cells is similar to the response of healthy control's cells to oxidative stress induced by ionizing radiation [15]. (2) Hospitalization of the SZ patient and its treatment with antipsychotics leads to a decrease in the number of mtDNA in leukocytes.

resulting in increased mitochondrial biogenesis [15, 37, 38]. Some authors proposed that the stress response of cells in terms of mitochondrial copy numbers could be a key for the life or death of the cell under oxidative stress [4, 39–

41]. According to this hypothesis, an increase in mtDNA content may precede mitochondrial dysfunction as an adaptive response and could therefore be a predictive biomarker.

The above-mentioned hypothesis is confirmed by the data obtained in this study. Analyzing the oxidation marker 8-oxodG in SZ patients, we found that the unmedicated SZ patients can be distinctively divided into subgroups with different levels of 8-oxodG (Figure 2). MtDNA *CN* was much higher in the subgroup SZ-2 (m−) with a high level of 8-oxodG (Figure 3). On the one hand, the correlation between lg (8-oxodG) and mtDNA *CN* (Figure 3(a)) for HC and SZ(m−) can be explained by the preferential oxidation of mtDNA compared to nuclear DNA. MtDNA is highly susceptible to oxidative damage due to lack of protective histones and limited DNA repair capacity [42]. It can be assumed that the more copies of oxidized mtDNA in the cell, the higher the index 8-oxodG. However, very high values of the ratio 8-oxodG/mtDNA *CN* (Figure 3(b)) may indicate significant damage of the nuclear DNA. In the future, it is interesting to compare the ratio of 8-oxodG/mtDNA *CN* with the content of 8-oxodG in mtDNA and nuclear DNA using the PCR method.

It is still unclear whether peripheral findings reflect alterations in the brain of SZ patients at the time of an acute psychotic disorder. Nevertheless, we can suggest that enhanced copy number of mtDNA in the brain under the middle oxidative stress can contribute to abnormal behavioral symptoms in paranoid SZ. To answer the question about the role of an increased amount of mtDNA in patients with unmedicated acute psychosis, further studies are required.

Additional Points

Highlights. (i) Unmedicated SZ men contain more mtDNA in leukocytes than medicated ones and control. (ii) Unmedicated SZ men contain more 8-oxodG in lymphocytes than healthy control. (iii) MtDNA content positively correlates with the level of 8-oxodG in unmedicated SZ patients.

Disclosure

The small fragment of this work was presented at the International Symposium MAPEEG-2017 (Modern Achievements in Population, Evolutionary, and Ecological Genetics), Vladivostok, September 3–8, 2017; ISBN 978-5-8044-1648-6.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

T. V. Lezheiko, N. Yu Kolesina, I. V. Chestkov, E. M. Jestkova, and E. S. Ershova provided clinical samples, participated in designing, carried out most of the experimental procedures, and helped in the drafting of the manuscript. The software for "Imager 6" was written by R. Veiko. V. G. Golimbet, N. N. Veiko, and S. V. Kostyuk participated in performing the statistical analyses and the interpretation of results and writing the first draft of the manuscript. G. P. Kostyuk, V. L. Izhevskaya, and S. I. Kutsev participated in the coordination, in the conception, and in the design of the study and helped in the drafting of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the Russian Foundation for Basic Research (OFI Project Number 17-29-06017).

References

[1] A. N. Malik and A. Czajka, "Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction?," *Mitochondrion*, vol. 13, no. 5, pp. 481–492, 2013.

[2] C. S. Liu, C. S. Tsai, C. L. Kuo et al., "Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes," *Free Radical Research*, vol. 37, no. 12, pp. 1307–1317, 2003.

[3] A. Rajasekaran, G. Venkatasubramanian, M. Berk, and M. Debnath, "Mitochondrial dysfunction in schizophrenia: pathways, mechanisms and implications," *Neuroscience & Biobehavioral Reviews*, vol. 48, pp. 10–21, 2015.

[4] Z. Li, M. Hu, X. Zong et al., "Association of telomere length and mitochondrial DNA copy number with risperidone treatment response in first-episode antipsychotic-naïve schizophrenia," *Scientific Reports*, vol. 5, no. 1, article 18553, 2015.

[5] S. M. Somerville, R. R. Conley, and R. C. Roberts, "Mitochondria in the striatum of subjects with schizophrenia," *The World Journal of Biological Psychiatry*, vol. 12, no. 1, pp. 48–56, 2011.

[6] R. C. Roberts, K. A. Barksdale, J. K. Roche, and A. C. Lahti, "Decreased synaptic and mitochondrial density in the postmortem anterior cingulate cortex in schizophrenia," *Schizophrenia Research*, vol. 168, no. 1-2, pp. 543–553, 2015.

[7] N. Uranova, P. Bonartsev, O. Brusov, M. Morozova, V. Rachmanova, and D. Orlovskaya, "The ultrastructure of lymphocytes in schizophrenia," *The World Journal of Biological Psychiatry*, vol. 8, no. 1, pp. 30–37, 2007.

[8] S. Sabunciyan, E. Kirches, G. Krause et al., "Quantification of total mitochondrial DNA and mitochondrial common deletion in the frontal cortex of patients with schizophrenia and bipolar disorder," *Journal of Neural Transmission*, vol. 114, no. 5, pp. 665–674, 2007.

[9] H. Torrell, E. Montaña, N. Abasolo et al., "Mitochondrial DNA (mtDNA) in brain samples from patients with major psychiatric disorders: gene expression profiles, mtDNA content and presence of the mtDNA common deletion," *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, vol. 162, no. 2, pp. 213–223, 2013.

[10] F. E. Emiliani, T. W. Sedlak, and A. Sawa, "Oxidative stress and schizophrenia: recent breakthroughs from an old story," *Current Opinion in Psychiatry*, vol. 27, no. 3, pp. 185–190, 2014.

[11] M. Boskovic, T. Vovk, B. Kores Plesnicar, and I. Grabnar, "Oxidative stress in schizophrenia," *Current Neuropharmacology*, vol. 9, no. 2, pp. 301–312, 2011.

[12] I. Smaga, E. Niedzielska, M. Gawlik et al., "Oxidative stress as an etiological factor and a potential treatment target of psychiatric disorders. Part 2. Depression, anxiety, schizophrenia and autism," *Pharmacological Reports*, vol. 67, no. 3, pp. 569–580, 2015.

[13] L. Malakhova, V. G. Bezlepkin, V. Antipova et al., "The increase in mitochondrial DNA copy number in the tissues of Γ-irradiated mice," *Cellular & Molecular Biology Letters*, vol. 10, no. 4, pp. 721–732, 2005.

[14] H. Zhang, D. Maguire, S. Swarts et al., "Replication of murine mitochondrial DNA following irradiation," *Advances in Experimental Medicine and Biology*, vol. 645, pp. 43–48, 2009.

[15] W. W.-Y. Kam and R. B. Banati, "Effects of ionizing radiation on mitochondria," *Free Radical Biology & Medicine*, vol. 65, pp. 607–619, 2013.

[16] S. R. Kay, A. Fiszbein, and L. A. Opler, "The positive and negative syndrome scale (PANSS) for schizophrenia," *Schizophrenia Bulletin*, vol. 13, no. 2, pp. 261–276, 1987.

[17] A. Boyum, "Separation of leukocytes from blood and bone marrow," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 21, pp. 90–109, 1968.

[18] A. N. Malik, R. Shahni, A. Rodriguez-de-Ledesma, A. Laftah, and P. Cunningham, "Mitochondrial DNA as a non-invasive biomarker: accurate quantification using real time quantitative PCR without co-amplification of pseudogenes and dilution bias," *Biochemical and Biophysical Research Communications*, vol. 412, no. 1, pp. 1–7, 2011.

[19] E. S. Ershova, E. M. Jestkova, I. V. Chestkov et al., "Quantification of cell-free DNA in blood plasma and DNA damage degree in lymphocytes to evaluate dysregulation of apoptosis in schizophrenia patients," *Journal of Psychiatric Research*, vol. 87, pp. 15–22, 2017.

[20] B. Kirkpatrick, "Schizophrenia as a systemic disease," *Schizophrenia Bulletin*, vol. 35, no. 2, pp. 381-382, 2009.

[21] C. Y. Lai, E. Scarr, M. Udawela, I. Everall, W. J. Chen, and B. Dean, "Biomarkers in schizophrenia: a focus on blood based diagnostics and theranostics," *World Journal of Psychiatry*, vol. 6, no. 1, pp. 102–117, 2016.

[22] S. W. Park, C. H. Lee, J. G. Lee et al., "Protective effects of atypical antipsychotic drugs against MPP^+-induced oxidative stress in PC12 cells," *Neuroscience Research*, vol. 69, no. 4, pp. 283–290, 2011.

[23] K. Kriisa, L. Haring, E. Vasar et al., "Antipsychotic treatment reduces indices of oxidative stress in first-episode psychosis patients," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 9616593, 7 pages, 2016.

[24] 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.

[25] U. Sertan Copoglu, O. Virit, M. Hanifi Kokacya et al., "Increased oxidative stress and oxidative DNA damage in non-remission schizophrenia patients," *Psychiatry Research*, vol. 229, no. 1-2, pp. 200–205, 2015.

[26] A. Jorgensen, K. Broedbaek, A. Fink-Jensen et al., "Increased systemic oxidatively generated DNA and RNA damage in schizophrenia," *Psychiatry Research*, vol. 209, no. 3, pp. 417–423, 2013.

[27] F. Cardozo-Pelaez, P. J. Brooks, T. Stedeford, S. Song, and J. Sanchez-Ramos, "DNA damage, repair, and antioxidant systems in brain regions: a correlative study," *Free Radical Biology & Medicine*, vol. 28, no. 5, pp. 779–785, 2000.

[28] N. Nishioka and S. E. Arnold, "Evidence for oxidative DNA damage in the hippocampus of elderly patients with chronic schizophrenia," *The American Journal of Geriatric Psychiatry*, vol. 12, no. 2, pp. 167–175, 2004.

[29] T. Yamamori, H. Yasui, M. Yamazumi et al., "Ionizing radiation induces mitochondrial reactive oxygen species production accompanied by upregulation of mitochondrial electron transport chain function and mitochondrial content under control of the cell cycle checkpoint," *Free Radical Biology & Medicine*, vol. 53, no. 2, pp. 260–270, 2012.

[30] T. Yamamori, T. Sasagawa, O. Ichii et al., "Analysis of the mechanism of radiation-induced upregulation of mitochondrial abundance in mouse fibroblasts," *Journal of Radiation Research*, vol. 58, no. 3, pp. 292–301, 2016.

[31] S. M. E. Nugent, C. E. Mothersill, C. Seymour, B. McClean, F. M. Lyng, and J. E. J. Murphy, "Increased mitochondrial mass in cells with functionally compromised mitochondria after exposure to both direct γ radiation and bystander factors," *Radiation Research*, vol. 168, no. 1, pp. 134–142, 2007.

[32] T. Yoshida, S. Goto, M. Kawakatsu, Y. Urata, and T.-s. Li, "Mitochondrial dysfunction, a probable cause of persistent oxidative stress after exposure to ionizing radiation," *Free Radical Research*, vol. 46, no. 2, pp. 147–153, 2012.

[33] K. N. Loganovsky and T. K. Loganovskaja, "Schizophrenia spectrum disorders in persons exposed to ionizing radiation as a result of the Chernobyl accident," *Schizophrenia Bulletin*, vol. 26, no. 4, pp. 751–773, 2000.

[34] K. N. Loganovsky, S. V. Volovik, K. G. Manton, D. A. Bazyka, and P. Flor-Henry, "Whether ionizing radiation is a risk factor for schizophrenia spectrum disorders?," *The World Journal of Biological Psychiatry*, vol. 6, no. 4, pp. 212–230, 2005.

[35] Y. Iwata, K. Suzuki, T. Wakuda et al., "Irradiation in adulthood as a new model of schizophrenia," *PLoS One*, vol. 3, no. 5, article e2283, 2008.

[36] Q. Wen, Y. Hu, F. Ji, and G. Qian, "Mitochondrial DNA alterations of peripheral lymphocytes in acute lymphoblastic leukemia patients undergoing total body irradiation therapy," *Radiation Oncology*, vol. 6, no. 1, p. 133, 2011.

[37] K. Palikaras and N. Tavernarakis, "Mitochondrial homeostasis: the interplay between mitophagy and mitochondrial biogenesis," *Experimental Gerontology*, vol. 56, pp. 182–188, 2014.

[38] S. Michel, A. Wanet, A. De Pauw, G. Rommelaere, T. Arnould, and P. Renard, "Crosstalk between mitochondrial (dys)function and mitochondrial abundance," *Journal of Cellular Physiology*, vol. 227, no. 6, pp. 2297–2310, 2012.

[39] H. C. Lee and Y. H. Wei, "Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress," *The International Journal of Biochemistry & Cell Biology*, vol. 37, no. 4, pp. 822–834, 2005.

[40] K. Singh, "Mitochondria damage checkpoint in apoptosis and genome stability," *FEMS Yeast Research*, vol. 5, no. 2, pp. 127–132, 2004.

[41] A. Rodell, L. J. Rasmussen, L. H. Bergersen, K. K. Singh, and A. Gjedde, "Natural selection of mitochondria during somatic lifetime promotes healthy aging," *Frontiers in Neuroenergetics*, vol. 5, p. 7, 2013.

[42] F. M. Yakes and B. Van Houten, "Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 2, pp. 514–519, 1997.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 6341671, 6 pages
https://doi.org/10.1155/2017/6341671

Research Article

Cadmium-Induced Oxidative Stress Impairs Glycemic Control in Adolescents

Gabriele Pizzino,[1] Natasha Irrera,[1] Alessandra Bitto,[1] Giovanni Pallio,[1] Federica Mannino,[1] Vincenzo Arcoraci,[1] Federica Aliquò,[1] Letteria Minutoli,[1] Chiara De Ponte,[1] Paola D'andrea,[2] Francesco Squadrito,[1] and Domenica Altavilla[3]

[1]*Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy*
[2]*Department of Pharmacy, University of Bari, Bari, Italy*
[3]*Department of Biomedical, Dental, Morphological and Functional Sciences, University of Messina, Messina, Italy*

Correspondence should be addressed to Francesco Squadrito; fsquadrito@unime.it

Received 26 May 2017; Revised 12 October 2017; Accepted 23 October 2017; Published 12 December 2017

Academic Editor: Victor M. Victor

Experimental evidence suggests that cadmium (Cd) boosts oxidative stress that may result in toxicity on the endocrine system also in humans. The aim of this study was to investigate the glycemic control and oxidative stress markers in male adolescents with increased urinary levels of cadmium. We investigated 111 males, aged 12–14 years, living in a polluted area of Sicily and a control age-matched population ($n = 60$) living 28–45 km far from the polluted site. Malondialdehyde (MDA), total antioxidant activity (TAC), metallothionein-1A (MT-1A) gene expression, insulin resistance by the homeostatic model assessment (HOMA-IR), and urinary cadmium were investigated. Cd levels were significantly higher in adolescents living in the polluted area than in control age-matched subjects. Adolescents with elevated Cd levels had a significant increase in MDA, MT-1A, and HOMA-IR and reduced TAC compared to the control group. A robust correlation was found between urinary cadmium and MT-1A, HOMA-IR, and MDA whereas an inverse correlation was identified between urinary cadmium and TAC. This study indicates that cadmium burden alters glycemic control in adolescents and suggests that oxidative stress plays a key role in cadmium-induced insulin resistance, increasing the risk of developing metabolic disorders.

1. Introduction

Air pollution represents an important risk factor for the development of diabetes and obesity. Air pollution, in fact, boosts a robust oxidative stress and a sustained activation of the inflammatory cascade that in turn causes lipogenesis, adipose tissue inflammation, and insulin resistance [1, 2]. Interestingly, ambient air pollution is also associated with predisposing conditions for diabetes and insulin resistance. Particulate matter (PM), a complex mixture of small particles of various sizes (ranging from 10 μm to 0.2 μm) formed by both numerous components (including nitrates, sulfates, organic chemicals, and heavy metals) and liquid droplets, is used currently to monitor outdoor air pollution. It has been shown that higher PM_{10} (particulate matter of 10 micrometers or less) levels were correlated with higher concentration of HbA_{1c} in Germans affected by type 2 diabetes (T2D), which may be considered as a read-out of the average glycemia during the previous 30–120 days [3]. Several pollutants were also reported to display negative effects on HbA_{1c} and fasting glucose levels in an elderly Taiwanese population [4]. A Korean study showed that subjects with a history of diabetes or with diabetes, exposed to nitric oxide (NO_2), had augmented homeostatic model assessment of insulin resistance (HOMA-IR) [5]. In agreement with these findings, it has been reported that healthy adults living in a nonpolluted area showed decreased insulin sensitivity after they were exposed to air pollution in a heavy-traffic urban area for 4-5 hours each day for five consecutive days [6]. Ambient air pollution also alters insulin

resistance in healthy children and adolescents: exposure to NO_2 and PM_{10} was, in fact, correlated with increased HOMA-IR in German adolescents [7]. Furthermore, HOMA-IR was augmented in 374 Iranian children aged 10–18 years following short-term coexposure to carbon monoxide (CO) and PM_{10} [8].

Generally speaking, there are poor data concerning children; this generates concerns. Indeed, children may absorb heavy metals, including Cd, more readily than adults and they are more susceptible to its toxicity because of biologic and developmental reasons. Cadmium (Cd) is an endocrine disruptor that interferes with metabolic homeostasis and normal development [9–11]. It is produced by the emission of the industrial plants, and it is taken up by the ecosystem components, entering the food chain. This explains why, besides the industrial workers, people living near polluted areas may be at enhanced risk of human exposure [12, 13].

The urban area of Milazzo-Valle del Mela (Sicily, Italy) has been indicated to be at high risk of environmental crisis by local government authorities because of the presence of several industrial plants nearby the residential area. We have previously shown that male adolescents living and residing in this area have enhanced urinary Cd levels and show delayed puberty and testis growth [14].

The aim of this study was to investigate insulin resistance by the means of HOMA-IR, a well-known predictor of T2D, and the possible correlation with oxidative stress markers in adolescents living in polluted areas.

2. Materials and Methods

2.1. Study Design and Population. This study was a part of a cross-sectional investigation aimed to evaluate the correlation between cadmium and pubertal development in adolescents. A population of 111 male children, aged 12–14 years of Caucasian origin, was recruited in the industrial area of Milazzo-Valle del Mela.

A control population ($n = 60$) race, sex, and age matched living 28–45 km far from the industrial site was also enrolled in a volunteer base. The study protocol was adherent with the principles of the Declaration of Helsinki, and all parents or legal tutor participants gave written informed consent. Only subjects of Sicilian origin, healthy and nonsmokers, living in the selected areas from at least 10 years were included in the study.

A medical visit was performed at enrollment, and all children were evaluated by specifically trained nurses and doctors that evaluated the height, weight, body mass index (BMI), and scored pubertal development, according to Tanner classification, as previously reported [14]. Testicular volume was assessed by ultrasound evaluation, and testosterone levels were determined as previously described [14]. A complete family history was obtained and routine evaluations were performed.

2.2. Cadmium Urine Levels. All children received urine collection containers for 24 h specimens, and their parents were tutored for apposite procedure and storage. Urines were collected 1 or 2 days before the medical visit and stored at 2–6°C to avoid contamination. Blinded technicians analyzed cadmium urine samples on coded samples using an atomic absorption spectrometer procedure, as previously reported [14].

2.3. Determination of Prooxidant Markers, Antioxidant Markers, and HOMA-IR. To assess oxidative stress, concentration of malondialdehyde (MDA), used as a marker of lipid peroxidation index, was measured in plasma using a colorimetric commercial kit (ab118970, Abcam plc, Cambridge, UK). MDA concentration was calculated from a standard curve and expressed as μmol/l.

The total antioxidant capacity (TAC) was determined by the ferric reducing ability of plasma (FRAP) method in which a colourless ferric tripyridyltriazine complex is reduced to a blue ferrous complex by the antioxidants in the serum [15]. Briefly, a mixed solution of 50 μl of serum and 50 μl of distilled water was added to 900 μl of FRAP reagent and incubated at 37°C for 25 min. The change in absorbance at 593 nm is directly related to the total reducing power of electron-donating antioxidants present in the serum. The results were expressed in μmol/dl.

Insulin resistance was assessed using the homeostasis model assessment for insulin resistance (HOMA-IR). HOMA-IR was calculated using the following formula: fasting glucose (mg/dl) × fasting insulin (μIU/ml)/22.5.

2.4. Metallothionein (MT-1A) Gene Expression. Total RNA was extracted from whole blood samples (250 μl) using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol, and was quantified with a spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific). Reverse transcription was carried out using 1 μg of RNA by using the SuperScript® VILO™ cDNA synthesis kit (Thermo Fisher Scientific) and random primers, following the manufacturer's protocol. 1 μl of total cDNA was used to quantify MT-1A (catalogue number: Hs00831826_s1), by real-time qPCR, using β-actin (catalogue number: 4310881E; Life Technologies) as reference gene. Reactions have been carried out in Singleplex in 96-well plates using the TaqMan Universal PCR master mix and premade hydrolysis probes (Thermo Fisher Scientific). PCR reaction was monitored by using the QuantStudio 6 Flex (Thermo Fisher Scientific), and results were quantified by the $2^{-\Delta\Delta Ct}$ method for both target and reference genes. As calibrator, a nonexposed volunteer was used.

2.5. Outcomes. Changes in HOMA-IR, plasma MDA, MT-1A gene expression, and TAC and their dependence to cadmium levels were evaluated.

2.6. Statistical Analysis. Standard descriptive statistical analyses were performed to evaluate basal demographic and clinical characteristics. All results were expressed as median with an interquartile range for continue variables, absolute and percentage frequencies for categorical variables.

The Kolmogorov-Smirnov test for normality was used to check data distribution. Because of some not-normal numerical variable, a nonparametric approach was used. The Mann–Whitney U test was applied to compare adolescents

Table 1: Characteristics of the study population.

	Exposed		Controls		
	Median	IQ range	Median	IQ range	p
Age (yrs)	13.1	12.7–13.8	13.0	12.5–13.6	0.326
Weight (kg)	51.0	44.0–63.0	52.0	48.6–56.5	0.623
Height (cm)	158.0	153.5–164.5	158.0	150.0–163.0	0.127
BMI	20.2	18.1–24.6	21.2	19.4–22.9	0.474
Tanner (stage)	3.0	2.0–3.0	3.0	3.0–4.0	<0.001
Testicular volume (ml)	6.2	3.9–8.7	11.3	9.4–15.2	<0.001
Testosterone (nmol/l)	1.0	0.2–1.7	9.5	6.7–15.9	<0.001
Cadmium (μg/L)	0.7	0.3–1.0	0.2	0.0–0.2	<0.001
HOMA-IR	2.3	1.0–4.6	0.9	0.4–1.2	<0.001
TAC μmol/dl	149.6	131.5–160.5	167.4	161.9–171.3	<0.001
MDA μmol/l	7.0	6.0–8.0	3.7	3.1–4.0	<0.001

living in the polluted area and controls with reference to quantitative characteristics.

The nonparametric two-tailed Spearman Rho test was estimated to assess possible associations between all covariates of interest.

Univariate linear regression models were used to assess the possible dependence of HOMA-IR, MDA, MT-1A, and TAC levels by cadmium exposition levels and by each covariate of interest. Moreover, predictors that emerged as significant using the univariate model were included in a multivariate linear regression model.

The nonparametric two-tailed Spearman Rho test was also used to estimate possible interdependence between all the predictors that emerged as significant using the univariate models. This has been done to avoid multicollinearity and, consequently, to better identify the key predictors to include in the multivariate model. Beta coefficient with 95% confidence interval (CI) was calculated for each covariate of interest.

Two-tailed p value was set at 0.05 to be considered statistically significant. Statistical analysis was performed by using Statistical Package for Social Science (SPSS Statistics 17.0, Chicago, IL) software.

3. Results

The characteristics of population included in the study are reported in Table 1. There were no statistical differences between adolescents living in the polluted area and controls regarding weight, height, and BMI, whereas tanner score, testicular volume, and testosterone were significantly lower in adolescents living in the polluted area. Cadmium urinary levels HOMA-IR and MDA resulted higher in exposed subjects, while TAC was significantly lower with respect to controls (Table 1).

Cadmium directly influenced HOMA-IR ($\beta = 2.48$ (95% CI 2.08–2.88); $p < 0.001$; Figure 1(a)) and MDA ($\beta = 2.37$ (95% CI 2.00–2.74); $p < 0.001$; Figure 1(b)), as assessed by the univariate linear regression models. On the contrary, TAC was inversely related to urinary cadmium levels ($\beta = -17.8$ (95% CI −23.85/−11.81); $p < 0.001$; Figure 1(c)).

Instead, HOMA-IR was inversely related to Tanner stage ($\beta = -0.90$ (95% CI −1.40/−0.40); $p = 0.001$), as well as testicular volume ($\beta = -0.19$ (95% CI −0.28/−0.09); $p < 0.001$), and testosterone levels ($\beta = -0.13$ (95% CI −0.20/−0.06); $p < 0.001$). On the contrary, TAC was directly influenced by testicular volume ($\beta = 1.22$ (95% CI 0.10–2.35); $p = 0.033$) and testosterone levels ($\beta = 1.18$ (95% CI 0.41–1.96); $p = 0.003$).

MDA was inversely related to Tanner stage ($\beta = -1.09$ (95% CI −1.47/−0.71); $p < 0.001$), testicular volume ($\beta = -0.25$ (95% CI −0.33/−0.17); $p < 0.001$), and testosterone levels ($\beta = -0.18$ (95% CI −0.23/−0.13); $p < 0.001$). On the contrary, age directly influenced MDA ($\beta = 0.99$ [95% CI 0.38–1.61]; $p = 0.002$).

The expression of the MT-1A gene coding for metallothionein (isoform 1A) was directly related to cadmium levels ($\beta = 1.12$ (95% CI 0.99/1.24); $p < 0.001$; Figure 1(d)) and inversely related to testis volume ($\beta = -0.04$ (95% CI −0.07/−0.01); $p = 0.005$), in the univariate model.

Because of interdependence between cadmium and Tanner stage (r_s −346; $p < 0.001$), testicular volume (r_s −456; $p < 0.001$), and testosterone levels (r_s −635; $p < 0.001$); and testicular volume and age (r_s 243; $p < 0.001$), Tanner stage (r_s 914; $p < 0.001$), and testosterone levels (r_s 755; $p < 0.001$), only cadmium and testicular volume were included in the multivariate linear regression model

Cadmium urinary levels resulted the only key predictors influencing HOMA-IR ($\beta = 2.41$ (95% CI 1.97–2.85); $p < 0.001$), TAC ($\beta = -17.81$ (95% CI −4.5/−11.12); $p < 0.001$), and MT-1A ($\beta = 1.16$ (95% CI 1.03/1.29); $p < 0.001$); whereas, cadmium urinary levels ($\beta = 2.03$ (95% CI 1.65–2.41); $p < 0.001$) and testicular volume ($\beta = -0.12$ (95% CI −0.17/−0.06); $p < 0.001$) are independent key predictors influencing MDA.

4. Discussion

The hyperglycemic potential of Cd has been demonstrated in both animal models and humans. Systemic cadmium exposure causes marked changes on several parameters of

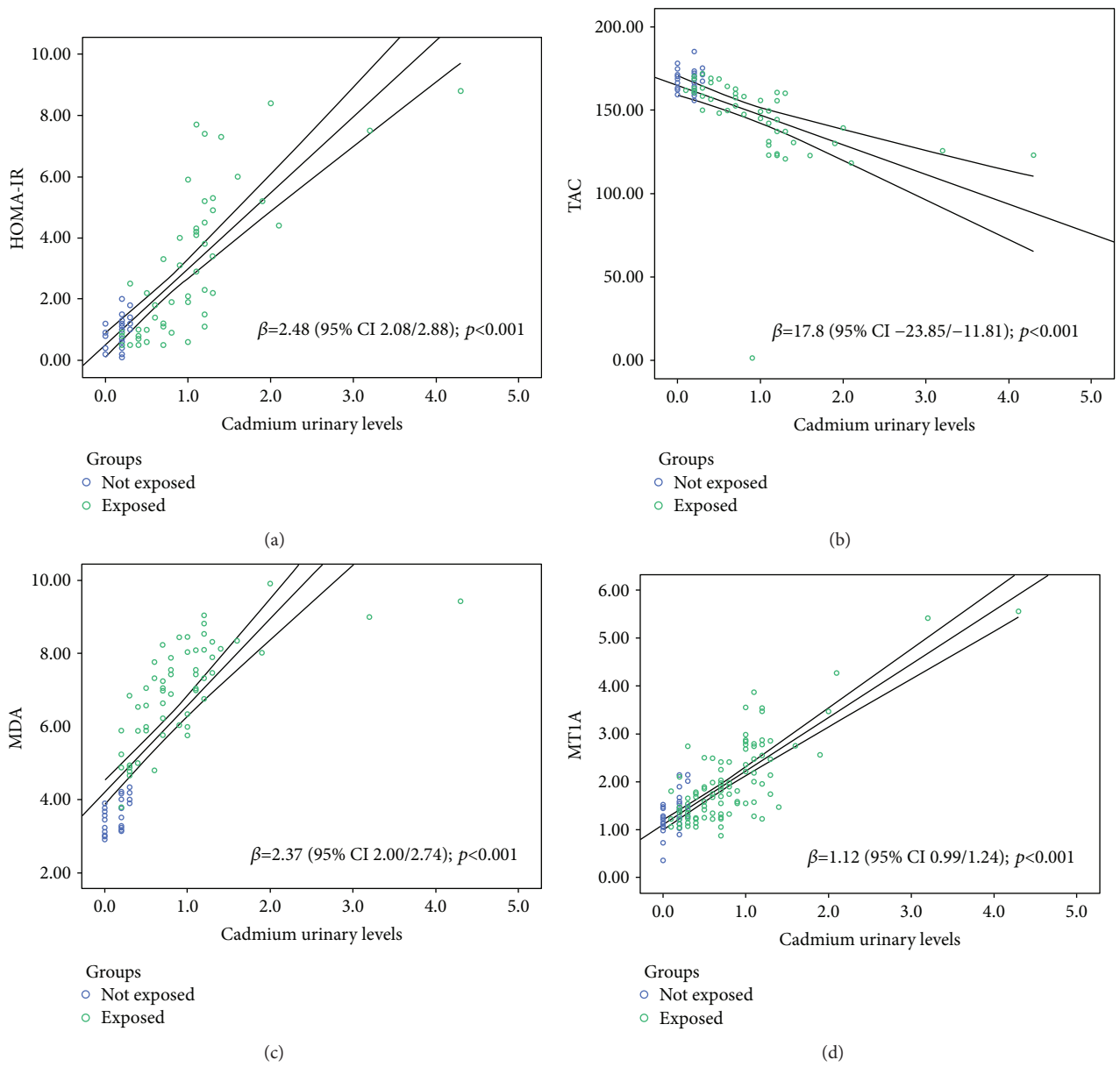


Figure 1: HOMA-IR (a), TAC (b), MDA (c), and MT-1A (d) dependence to cadmium urinary levels in subjects living or not in polluted area: univariate linear regression models.

glycemic metabolism that are abated by the administration of estradiol [16] and most interestingly by antioxidants [17].

In vitro, Cd also impairs insulin secretion by pancreatic beta cells and this is associated with an increase in metallothioneins, the most important cadmium-binding proteins [18]. As a matter of fact, the gene expression of metallothioneins increases following Cd exposure in adults and it has been related to renal toxicity [19]. The present data support these previous findings; in fact, the gene expression of MT-1A was significantly increased in Cd-exposed adolescents as compared to controls. In addition, this increase was related to a reduced testicular volume suggesting that cadmium not only reduces testis growth in adolescents but is also responsible for an increase in MT-1A as a compensatory detoxification mechanism.

An epidemiological study confirmed the Cd-disrupting effect on glycemic control: in fact, enhanced blood glucose level and reduced serum insulin levels were reported in smelter workers exposed to cadmium [20]. All these findings, taken together, strongly support the potential of Cd to cause insulin resistance, a predisposing condition for developing diabetes. Indeed, the adolescents exposed to cadmium revealed an altered insulin resistance that may actually predispose to diabetes. In addition, HOMA-IR also correlated with MDA and TAC variations that strongly indicates some level of Cd-induced systemic toxicity. However, as far as we know, no study has investigated the mechanisms underlying this Cd effect in humans and especially in adolescents.

Taking in consideration the results obtained so far, monitoring the internal exposure to chemicals in biological fluids

(human biomonitoring (HBM)) may be useful to study the possible effects of chronic low environmental exposure to pollutants in the general population of industrialized countries, especially those particularly susceptible such as adolescents. Using this methodology, we have shown that adolescents, living in the industrialized area of Milazzo-Valle del Mela in the north of Sicily, have increased urinary cadmium levels [21] that are robustly associated to a marked oxidative stress [22] and to a delayed puberty onset and testicular weight in males [14].

Considering the ability of cadmium to generate insulin resistance and reactive oxygen species, we investigated on a possible correlation between the heavy metal and these two variables. Our results suggest that adolescents with higher urinary levels of Cd had increased HOMA-IR and plasma malondialdehyde together with reduced total antioxidant activity. A robust correlation was also found between urinary cadmium and both HOMA-IR and MDA, whereas an inverse correlation was identified between urinary cadmium and TAC. As far as we know, this is the first study demonstrating a correlation between increased internal exposure of Cd and elevated insulin resistance. Furthermore, this study confirms the mechanistic role of oxidative stress in mediating the disrupting effect on glycemic control. More specifically, the results of the present data led us to speculate that cadmium causes, as suggested in experimental studies, insulin resistance by boosting the production of oxygen free radicals. However, our study has some limits such as this cross-sectional investigation has been carried out only in male adolescents and, at the present, we do not know whether the same conclusion can be extended to the female gender characterized by a different hormonal status. Furthermore, we lack information on the history of T2D in the family of enrolled adolescents. However, the presence of a control population that suffers from the same bias mitigates these methodological weaknesses.

5. Conclusion

This study, for the first time, indicates that cadmium burden alters glycemic control in adolescents and suggests that oxidative stress plays a key role in cadmium-induced insulin resistance that may augment at an older age, the risk of developing metabolic disorder.

Conflicts of Interest

The authors state no conflict of interest.

Authors' Contributions

Gabriele Pizzino and Natasha Irrera equally contributed to this paper.

Acknowledgments

The authors are thankful to all members of the Squadrito laboratory for technical support.

References

[1] T. Münzel, M. Sørensen, T. Gori et al., "Environmental stressors and cardio-metabolic disease: part I-epidemiologic evidence supporting a role for noise and air pollution and effects of mitigation strategies," *European Heart Journal*, vol. 38, no. 8, pp. 550–556, 2017.

[2] T. Münzel, M. Sørensen, T. Gori et al., "Environmental stressors and cardio-metabolic disease: part II-mechanistic insights," *European Heart Journal*, vol. 38, no. 8, pp. 557–564, 2017.

[3] T. Tamayo, W. Rathmann, U. Krämer, D. Sugiri, M. Grabert, and R. W. Holl, "Is particle pollution in outdoor air associated with metabolic control in type 2 diabetes?," *PLoS One*, vol. 9, no. 3, article e91639, 2014.

[4] K. J. Chuang, Y. H. Yan, S. Y. Chiu, and T. J. Cheng, "Long-term air pollution exposure and risk factors for cardiovascular diseases among the elderly in Taiwan," *Occupational & Environmental Medicine*, vol. 68, no. 1, pp. 64–68, 2011.

[5] J. H. Kim and Y. C. Hong, "*GSTM1*, *GSTT1*, and *GSTP1* polymorphisms and associations between air pollutants and markers of insulin resistance in elderly Koreans," *Environmental Health Perspectives*, vol. 120, no. 10, pp. 1378–1384, 2012.

[6] R. D. Brook, X. Xu, R. L. Bard et al., "Reduced metabolic insulin sensitivity following sub-acute exposures to low levels of ambient fine particulate matter air pollution," *Science of The Total Environment*, vol. 448, pp. 66–71, 2013.

[7] E. Thiering, J. Cyrys, J. Kratzsch et al., "Long-term exposure to traffic-related air pollution and insulin resistance in children: results from the GINIplus and LISAplus birth cohorts," *Diabetologia*, vol. 56, no. 8, pp. 1696–1704, 2013.

[8] R. Kelishadi, N. Mirghaffari, P. Poursafa, and S. S. Gidding, "Lifestyle and environmental factors associated with inflammation, oxidative stress and insulin resistance in children," *Atherosclerosis*, vol. 203, no. 1, pp. 311–319, 2009.

[9] O. Ademuyiwa, R. Agarwal, R. Chandra, and J. R. Behari, "Effects of sub-chronic low-level lead exposure on the homeostasis of copper and zinc in rat tissues," *Journal of Trace Elements in Medicine and Biology*, vol. 24, no. 3, pp. 207–211, 2010.

[10] P. Kakkar and F. N. Jaffery, "Biological markers for metal toxicity," *Environmental Toxicology and Pharmacology*, vol. 19, no. 2, pp. 335–349, 2005.

[11] E. Diamanti-Kandarakis, J. P. Bourguignon, L. C. Giudice et al., "Endocrine-disrupting chemicals: an Endocrine Society scientific statement," *Endocrine Review*, vol. 30, no. 4, pp. 293–342, 2009.

[12] Z. Mazej, S. Al Sayegh-Petkovsek, and B. Pokorny, "Heavy metal concentrations in food chain of lake Velenjsko jezero, Slovenia: an artificial lake from mining," *Archives of Environmental Contamination and Toxicology*, vol. 58, no. 4, pp. 998–1007, 2010.

[13] M. Qiao, C. Cai, Y. Huang, Y. Liu, A. Lin, and Y. Zheng, "Characterization of soil heavy metal contamination and potential health risk in metropolitan region of Northern China," *Environmental Monitoring and Assessment*, vol. 172, no. 1-4, pp. 353–365, 2011.

[14] M. Interdonato, G. Pizzino, A. Bitto et al., "Cadmium delays puberty onset and testis growth in adolescents," *Clinical Endocrinology*, vol. 83, no. 3, pp. 357–362, 2015.

[15] C. A. Pieme, J. A. Tatangmo, G. Simo et al., "Relationship between hyperglycemia, antioxidant capacity and some

enzymatic and non-enzymatic antioxidants in African patients with type 2 diabetes," *BMC Research Notes*, vol. 10, no. 1, p. 141, 2017.

[16] J. Mladenović, B. Ognjanović, N. Dorđević et al., "Protective effects of oestradiol against cadmium-induced changes in blood parameters and oxidative damage in rats," *Archives of Industrial Hygiene and Toxicology*, vol. 65, no. 1, pp. 37–46, 2014.

[17] I. Abdelaziz, M. I. Elhabiby, and A. A. Ashour, "Toxicity of cadmium and protective effect of bee honey, vitamins C and B complex," *Human & Experimental Toxicology*, vol. 32, no. 4, pp. 362–370, 2013.

[18] M. El Muayed, M. R. Raja, X. Zhang et al., "Accumulation of cadmium in insulin-producing β cells," *Islets*, vol. 4, no. 6, pp. 405–416, 2012.

[19] K. Boonprasert, R. Ruengweerayut, R. Aunpad, S. Satarug, and K. Na-Bangchang, "Expression of metallothionein isoforms in peripheral blood leukocytes from Thai population residing in cadmium-contaminated areas," *Environmental Toxicology and Pharmacology*, vol. 34, no. 3, pp. 935–940, 2012.

[20] Y. W. Chen, C. Y. Yang, C. F. Huang, D. Z. Hung, Y. M. Leung, and S. H. Liu, "Heavy metals, islet function and diabetes development," *Islets*, vol. 1, no. 3, pp. 169–176, 2009.

[21] M. Interdonato, A. Bitto, G. Pizzino et al., "Levels of heavy metals in adolescents living in the industrialised area of Milazzo-Valle del Mela (northern Sicily)," *Journal of Environmental and Public Health*, vol. 2014, Article ID 326845, 9 pages, 2014.

[22] G. Pizzino, A. Bitto, M. Interdonato et al., "Oxidative stress and DNA repair and detoxification gene expression in adolescents exposed to heavy metals living in the Milazzo-Valle del Mela area (Sicily, Italy)," *Redox Biology*, vol. 2, pp. 686–693, 2014.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8156594, 8 pages
https://doi.org/10.1155/2017/8156594

Research Article

Effect of Bioactive Compound of *Aronia melanocarpa* on Cardiovascular System in Experimental Hypertension

Martina Cebova,[1] Jana Klimentova,[1] Pavol Janega,[1,2] and Olga Pechanova[1]

[1]*Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovakia*
[2]*Department of Pathology, Faculty of Medicine, Comenius University, Bratislava, Slovakia*

Correspondence should be addressed to Martina Cebova; martina.cebova@savba.sk

Received 4 August 2017; Revised 10 October 2017; Accepted 18 October 2017; Published 30 November 2017

Academic Editor: Victor M. Victor

Aronia melanocarpa has attracted scientific interest due to its dense contents of different polyphenols. We aimed to analyse effects of *Aronia melanocarpa* (AME) extract on blood pressure (BP), lipid peroxidation, cytokine level, total NOS activity in the left ventricle (LV), and aorta of L-NAME-induced hypertensive rats. 12-week-old male WKY rats were assigned to the control group and groups treated with AME extract (57.90 mg/kg/day), L-NAME (40 mg/kg/day), or combination of L-NAME (40 mg/kg/day) and AME (57.90 mg/kg/day) in tap water for 3 weeks. NOS activity, eNOS protein expression, and conjugated diene (CD) concentration were determined in the LV and aorta. After 3 weeks of L-NAME treatment, BP was increased by 28% and concomitant treatment with AME reduced it by 21%. NOS activity of the LV and aorta in the L-NAME group was decreased by about 40%, while AME increased it almost on the control level. AME-induced eNOS upregulation may contribute to increase NOS activity. Moreover, AME decreased CD concentration in the LV and aorta and TNF-α and IL-6 production in the plasma were increased by L-NAME treatment. In conclusion, our results showed that active substances of *Aronia melanocarpa* may have a positive effect on blood pressure, NOS activity, and proinflammatory processes in L-NAME-induced hypertension.

1. Introduction

Hypertension is a cardiovascular risk factor associated with endothelial dysfunction and oxidative stress as well. This can lead to a reduction of NO availability and vasodilatation. Those processes participate in increasing systemic blood pressure and myocardial remodelling and in the development of cardiac hypertrophy [1, 2]. Inhibition of nitric oxide synthase by N^G-nitro-L-arginine methyl ester (L-NAME) is a well-established rat model of experimental hypertension with increased blood pressure and contractility in different parts of the vasculature, attenuated vascular relaxation, and decreased heart rate [3, 4]. The structural changes after long-term NO synthase inhibition in the cardiovascular system included left ventricle hypertrophy, remodelling of coronary arteries and aorta, as well as extensive areas of fibrosis and necrosis [4–6]. In hypertension, the increased production of oxygen-free radicals is a generally accepted fact. Usually, there is a balance between the antioxidants and the prooxidants *in vivo*, but several factors like stress, radiation, and nutrition may lead to the so-called oxidative stress, which imposes the necessity to contribute exogenous antioxidants with the diet. The injury caused by oxidative stress can affect all organ systems. Therefore, many studies are oriented on antioxidant treatment that may prevent the hypertension and associated organ alterations.

Increasing consumption of polyphenol-rich foods is a promising strategy to reduce cardiovascular risk. Increased flavonoid consumption correlated with positive effect on low-density lipoproteins, blood pressure, and flow-mediated dilation [7]. Polyphenols with numerous biological activities are used for their ability to act as an antioxidant either by scavenging reactive oxygen species (ROS) or inhibiting enzymes involved in the ROS production [1, 8, 9].

Aronia melanocarpa, also known as black chokeberry, belongs to the Rosaceae family, originally coming from

North America. Nowadays, it is commonly used also in Europe due to a high content of nutrients being beneficial for the health. The components of the aronia are dependent on many factors such as cultivar, fertilization, maturation of the berries, harvest date, or habitat/location [10, 11]. The most abundant components are anthocyanins and flavonoids [12]. Chokeberries with high resistance to frost and mechanized harvesting are widely used in processed and derived products including juices, wines, jellies, and tea [13, 14]. The protective role of the main components from aronia against cardiovascular diseases came up from clinical trials [15, 16] and animal studies [17–19]. Attention has been also focused on chokeberries due to their antioxidant properties related to the high polyphenolic content [20, 21].

The aim of our study was to determine the effect of nonalcohol *Aronia melanocarpa* extract on NO synthase activity, particularly NO synthase isoform protein expressions in the aorta and left ventricle, as well as their immunohistochemistry and on cytokine and conjugated diene levels on L-NAME-induced experimental model of arterial hypertension.

2. Materials and Methods

2.1. Chemicals. Most of the chemicals and reagents were obtained from Sigma-Aldrich; when not, the company is indicated.

2.2. Aronia melanocarpa Extract. Samples of *Aronia melanocarpa* wine (Winery Pereg Ltd.) were subjected to the process of dealcoholisation and concentration, producing an alcohol-free *Aronia melanocarpa* extract (AME) (previously described by Kondrashov [22]). The total phenolic content of AME was assessed according to the Folin-Ciocalteu method. Briefly, 1 mL of AME, 1 mL of Folin-Ciocalteu's reagent, and 5 mL of distilled water were mixed together. The solution was incubated for 5 min at a room temperature in the darkness. Then, 1 mL of 20% Na_2CO_3 was added. The solution was made up to 10 mL, mixed, and incubated for 1 h at a room temperature in the darkness. The absorbance of AME sample was measured at 765 nm against a blank (corresponding extraction mixture was used instead of algal extract) on UV/VIS spectrometer Lambda 25 (PerkinElmer, Waltham, MA, USA). Gallic acid was used as a standard to construct the calibration curve (20, 40, 60, 80, and 100 mg·L^{-1}). The total phenolic content of AME is expressed in mg·g^{-1} of gallic acid equivalent (GAE).

2.3. Animal Study

2.3.1. Animals and Treatment. All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology SAS and conform to the European Convention on Animal Protection and Guidelines on Research Animal Use.

12-week-old male Wistar Kyoto rats were divided into the control group, the group treated with AME in the dose 57.90 mg/kg/day, the group treated with N^G-nitro-L-arginine methyl ester (L-NAME) in the dose 40 mg/kg/day, and the group treated with L-NAME in the dose 40 mg/kg/day + AME 57.90 mg/kg/day. Each group obtained 6 animals. L-NAME and AME were administered *via* the drinking water from the 12th week of age for 3 weeks. Daily water consumption was estimated individually for every animal and adjusted, if necessary. All animals were housed at a temperature of 22–24°C and fed with a regular pellet diet *ad libitum*. Blood pressure (BP) was measured noninvasively, using tail-cuff plethysmography weekly. At the end of treatment, the animals were sacrificed; body weight (BW) and heart weight (HW) were determined. The HW/BW ratio was calculated. Samples of the left ventricle and aorta were used to determine NO synthase activity, eNOS and iNOS protein expressions (Western blot and immunohistochemistry), and conjugated diene level. Cytokine levels were measured in the plasma.

2.3.2. Total NOS Activity and Protein Expression. Total NO synthase activity was determined in crude homogenates of the left ventricle and aorta by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine (ARC, Montana, USA) as previously described and slightly modified by Pechanova [23]. [^{3}H]-L-citrulline was measured with the Quanta Smart TriCarb Liquid Scintillation Analyzer (Packard Instrument Company, Meriden, CT).

Protein expressions of eNOS and iNOS were determined in the aorta and left ventricle by Western blot analysis. The samples were probed with polyclonal rabbit, anti-eNOS, anti-iNOS, and anti-GAPDH antibodies (Abcam, Cambridge, UK). The intensity of bands was visualized using the enhanced chemiluminescence system (ECL, Amersham, UK), quantified by using ChemiDoc™ Touch Imagine System (Image Lab™ Touch software, Bio-Rad), and normalized to GAPDH bands.

2.3.3. Cytokine Level and CD Determination. Cytokine levels were determined by Bio-Plex Pro™ rat cytokine, chemokine, and growth factor assays in plasma.

The concentration of conjugated dienes (CD) was measured in lipid extracts of the left ventricle homogenates [24]. After chloroform evaporation under inert atmosphere and addition of cyclohexane, conjugated diene concentrations were determined spectrophotometrically ($\lambda = 233$ nm, GBC 911A, Bio-Rad Laboratories).

2.3.4. Immunohistochemical Analysis of eNOS and iNOS in LV. The tissue samples were processed in a standard manner, embedded in paraffin, and sectioned; 3 μm thick slices were deparaffinized and rehydrated in phosphate-buffered physiological saline solution (10 mM, pH 7.2). The tissue epitopes were damasked using the automated water bath heating process in Dako PT Link (Agilent, Santa Clara, California); the slides were incubated in citrate retrieval solution (10 mM citrate, pH 6.0) at 98°C for 20 minutes. The slides were subsequently incubated 2 hours at room temperature with the primary mouse monoclonal IgG2a antibody against eNOS or iNOS (Santa Cruz Biotechnology, Dallas, USA, sc-376751) diluted 1 : 100. The samples were immunostained using anti-mouse anti-rabbit immune-peroxidase polymer (Histofine, Nichirei Biosciences, Tokyo, Japan) for 30 minutes at a room temperature according to the

manufacturer's instructions. For visualization, the diaminobenzidine substrate-chromogen solution was used (DAB, Agilent, Santa Clara, California) for 5 minutes. The slides were counterstained with hematoxylin. The DAB positivity was evaluated by light microscopy and measured by histomorphometry using the Fiji morphometric software [25] based on ImageJ 1.51n platform [26]. The final results are expressed as proportional ratio comparing to the mean DAB positivity of evaluated marker in WKY controls (expressed as 1.00).

2.4. Statistics. The results are expressed as mean ± SEM. One-way analysis of variance and Duncan test were used for statistical analysis. Values were considered significant with a probability value $p < 0.05$.

3. Results

3.1. AME Content. The total content of nonalcohol extract of *Aronia melanocarpa* is shown in Table 1. The total phenolic content adjusted on gallic acid equivalent was 57,870 mg/L.

The most predominant mineral elements detected in AME were ferrum (Fe), zinc (Zn), and cuprum (Cu). The results are visualized in Table 2.

3.2. Animal Studies

3.2.1. Cardiovascular Parameters and Plasma Cytokine Level. After three weeks of L-NAME treatment, BP was increased by 28% in comparison to the control group. Concomitant treatment by AME reduced BP by 21% (Table 3). At the end of the experiment, HW/BW ratio (mg/g) was 2.72 ± 0.05 in the control group. This ratio was increased in L-NAME group (2.95 ± 0.10) versus control rats. AME was able to decrease this value on the control level (2.77 ± 0.02) (Table 3).

3.2.2. Cytokine and CD Concentration. AME was able to inhibit TNFα, and IL-6 production increased in the L-NAME group (Table 3). L-NAME administration increased the level of CD in both investigated tissues. Furthermore, AME was able to decrease the level of CD almost on the level of controls (Figures 1(a) and 1(b)).

3.2.3. Total NO Synthase Activity and Protein Expression. The total NO synthase activity was decreased after 3 weeks of L-NAME treatment in both the left ventricle and aorta. However, AME was able to increase NO synthase activity in the left ventricle on 90% of the control value (Figure 2(a)) and on control value in the aorta (Figure 2(b)).

Endothelial NOS protein expression was upregulated only in LV of AME group (Figure 3). The L-NAME administration alone and concomitant treatment with AME had the tendency to increase eNOS expression as well. We did not observe any changes of eNOS expression in the aorta.

Inducible NOS protein expression was not changed in any investigated tissue and group (data are not shown).

3.2.4. Immunohistochemical Analysis of eNOS and iNOS in LV. The eNOS showed diffuse and regular cytoplasmic positivity in cardiomyocytes of all experimental animals. Only the AME treatment without the L-NAME administration significantly increased the eNOS expression (Figures 4(a)–4(d)). Positivity of eNOS is well correlated with its protein expression analysed by Western blot. Similarly, as Western blot, immunohistochemistry did not show any changes in iNOS protein expression between the groups (data are not shown).

Table 1: Components of *Aronia melanocarpa* extract (in μg/g).

Component	($\mu g \cdot g^{-1}$)
Gallic acid	2.24 ± 0.01
Protocatechuic acid	340.86 ± 7.70
4-Hydroxybenzoic acid	21.44 ± 0.31
Epigallocatechin	354.81 ± 11.19
Catechin	149.68 ± 1.91
Chlorogenic acid	1948.60 ± 0.59
Vanillic acid	14.61 ± 0.11
Caffeic acid	4.78 ± 0.09
Syringic acid	4.32 ± 0.29
Epicatechin	29.24 ± 0.52
Trans-p-coum acid	19.37 ± 1.32
Ferulic acid	173.90 ± 1.94
Ellagic acid	42.78 ± 0.14
Rutin	17.44 ± 0.00
T-2-hydroxycinnamic acid	1.18 ± 0.12
Protocatechuic acid etylester	11.55 ± 0.39
Resveratrol	16.65 ± 0.32
Cinnamic acid	2.35 ± 0.00
Kaempferol	23.09 ± 0.79
Quercetin	0.00 ± 0.00

Data are expressed as mean values ($n = 3$) ±SD.

4. Discussion

Long-lasting inhibition of NO synthase induces hypertension, endothelial and contractile dysfunction, inflammation, and fibrosis enlargement [27]. Chronic inhibition of NO synthase by L-NAME causes increase of blood pressure associated with vascular structural changes [4, 28]. It has been also shown that L-NAME increased fibrosis and left ventricular hypertrophy [3, 29]. Thus, L-NAME-induced hypertension represents a useful tool for studying increase of blood pressure associated with inflammatory processes and fibrosis. Lowering blood pressure is one of the most important means to reduce cardiovascular morbidity and mortality. Our study showed that *Aronia melanocarpa* extract resulted in significant reduction of blood pressure in L-NAME-treated rats that may be due to high polyphenolic content. In our previous study, we have shown that polyphenols can act as antioxidants, due to their functional groups and aromatic structure [30]. Our finding is in agreement with Ciocoiu [31] in experimental hypertension in rats. They found reduction of systolic and diastolic pressures after treatment with *Aronia melanocarpa* extract. Other authors showed decreased blood

Table 2: Elemental profile of *Aronia melanocarpa* extract (in ng/mL).

Sample	K	Mg	P	Fe	Cu	Pb	Zn	Na
AME	12.12 ± 0.09	85.41 ± 0.82	14.47 ± 0.12	1960.71 ± 11.05	405.14 ± 2.87	28.62 ± 2.15	950.34 ± 4.35	12.19 ± 0.13

Data are expressed as mean values ($n = 3$) ± SD.

Table 3: Blood pressure (BP), relative heart weight (HW/BW), TNFα (pg/mL), and IL-6 level (pg/mL) of Wistar Kyoto rats treated with AME (57.90 mg/kg/day), with L-NAME (40 mg/kg/day), and with L-NAME (40 mg/kg/day) + AME (57.90 mg/kg/day) for 3 weeks and age-matched controls.

Group	BP (mmHg)	Hw/Bw (mg/g)	TNFα (pg/mL)	IL-6 (pg/mL)
WKY	118.39 ± 4.29	2.72 ± 0.05	12.11 ± 2.41	23.41 ± 3.78
WKY + AME	122.04 ± 1.81	2.55 ± 0.05	7.32 ± 2.12	22.14 ± 5.22
WKY + L-NAME	155.11 ± 3.71*	2.95 ± 0.10*	18.19 ± 1.38*	36.43 ± 3.08*
WKY + L-NAME + AME	129.22 ± 2.51	2.77 ± 0.02#	13.89 ± 1.29#	25.13 ± 2.11#

Data are means ± SEM, significant differences: *$P < 0.05$ compared to age-matched controls; #$P < 0.05$ compared to L-NAME group.

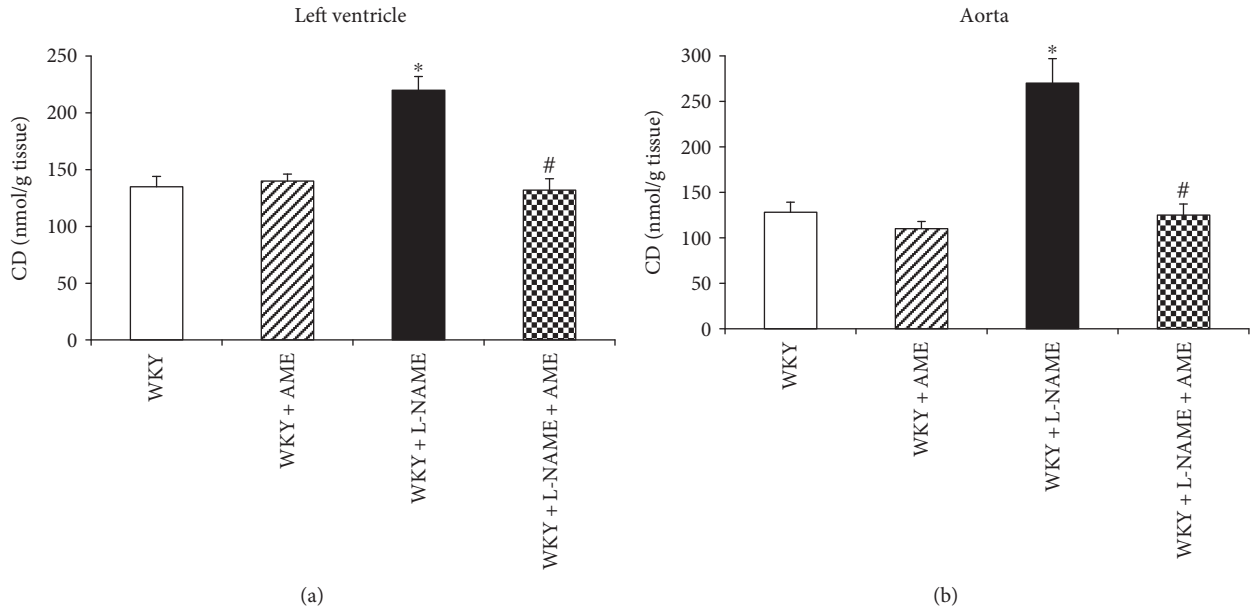


Figure 1: Conjugate diene (CD) level of the left ventricle (a) and aorta (b) of Wistar Kyoto rats treated with AME (57.90 mg/kg/day), with L-NAME (40 mg/kg/day), and with L-NAME (40 mg/kg/day) + AME (57.90 mg/kg/day) for 3 weeks and age-matched controls. Data are means ± SEM, significant differences: *$P < 0.05$ compared to age-matched controls; #$P < 0.05$ compared to L-NAME group.

pressure in SHR after the commercial *Aronia melanocarpa* extract *Aronox* treatment [32]. The hypotensive effect was revealed also by Naruszewicz [33] in patients after myocardial infarction with statin therapy and by Tjelle [34] in hypertensive volunteers.

Several studies showed that hypertension may lead to overproduction of free radicals, which could be the reason of biochemical, molecular, and behavioural changes [35, 36]. In this study, concentration of conjugated dienes (CD) was determined as a marker of oxidative damage and lipid peroxidation. Our previous results observed increased production of free radicals in the L-NAME model of hypertension [37]. We confirmed increased concentration of CD in the aorta and left ventricle after L-NAME treatment. AME extract was able to decrease the CD on the control level. Similarly, improved plasma and hepatic antioxidant function were found in apo $E^{-/-}$ mice after chokeberry extract treatment [38]. In the same pattern, IL-6 and TNF- α levels were decreased in the hypertensive group treated with AME. Han and Nicholson [39] showed significant drop of IL-6 as well as reduction in the level of oxidative stress in cardiac patients after chokeberry treatment. Nowadays, chokeberry consumption is in good relation with human immune system. Mechanisms of action are mediated by inhibition of cytokine IL-6, IL-8, and TNF-α in human monocytes as well as by activation of NFκB and prostaglandin E_2 [40].

The antioxidant activity of *Aronia melanocarpa* is mostly contributed by the phenolic compounds [40] and depends on

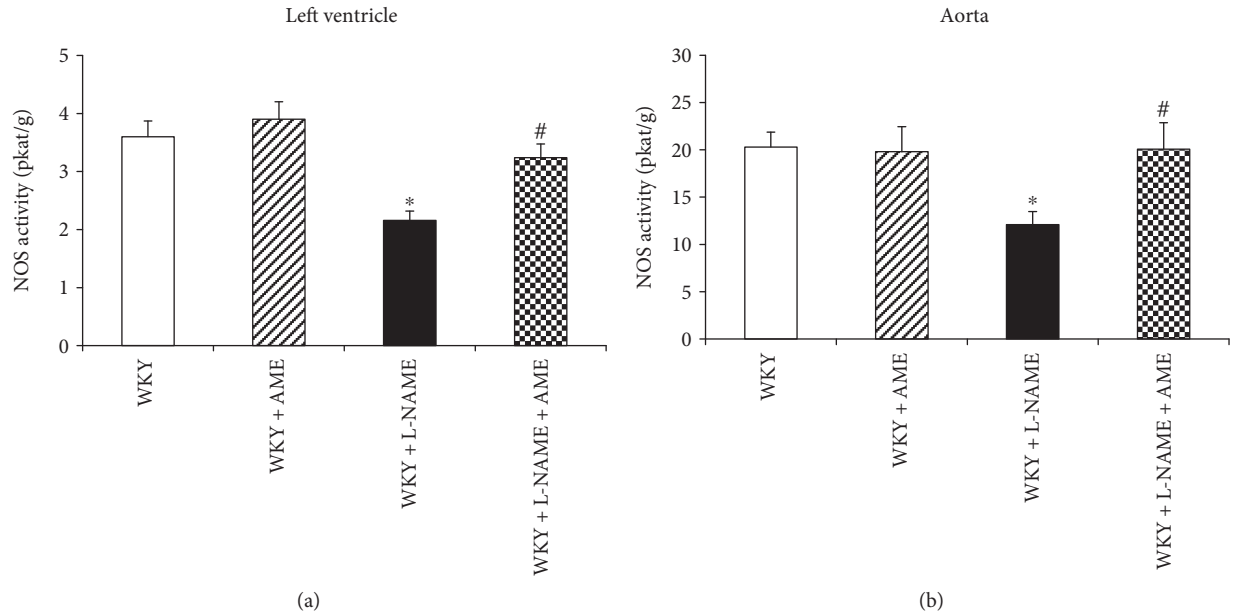


Figure 2: Nitric oxide synthase (NOS) activity of the left ventricle (a) and aorta (b) of Wistar Kyoto rats treated with AME (57.90 mg/kg/day), with L-NAME (40 mg/kg/day), and with L-NAME (40 mg/kg/day) + AME (57.90 mg/kg/day) for 3 weeks and age-matched controls. Data are means ± SEM, significant differences: $^{*}P < 0.05$ compared to age-matched controls; $^{\#}P < 0.05$ compared to L-NAME group.

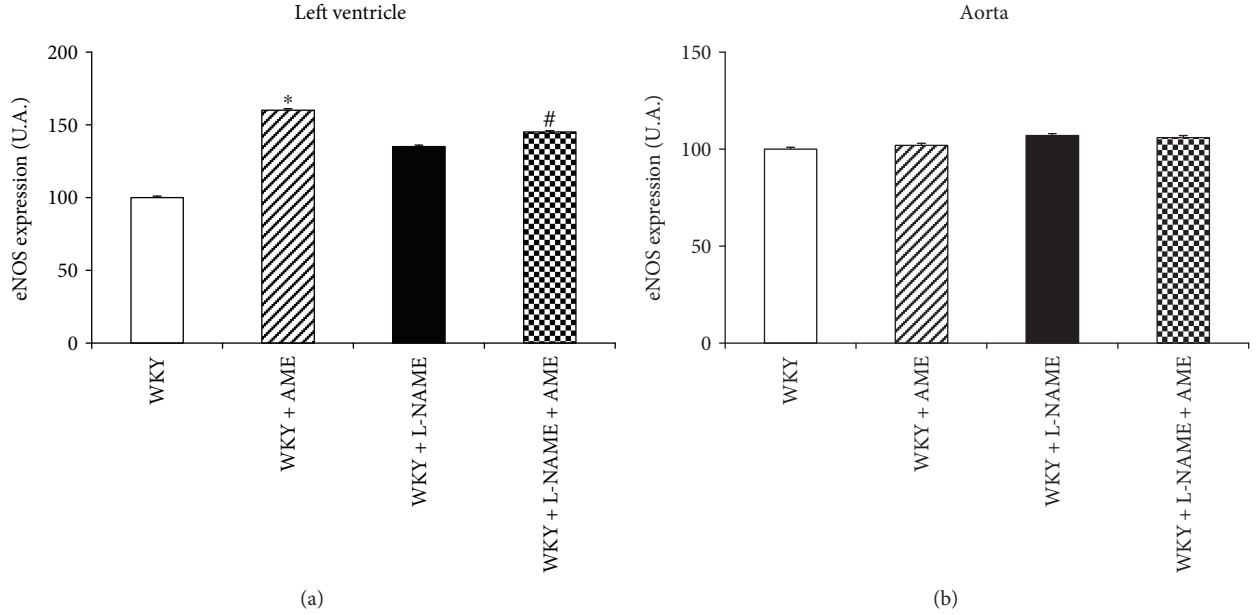


Figure 3: Endothelial NOS (eNOS) expression of the left ventricle (a) and aorta (b) of Wistar Kyoto rats treated with AME (57.90 mg/kg/day), with L-NAME (40 mg/kg/day), and with L-NAME (40 mg/kg/day) + AME (57.90 mg/kg/day) for 3 weeks and age-matched controls. Data are means ± SEM, significant differences: $^{*}P < 0.05$ compared to age-matched controls; $^{\#}P < 0.05$ compared to L-NAME group.

the structure of polyphenol functional groups [41]. It is also known from the literature that an increased level of free radicals leads to uncoupling of the NOS dimer to a monomer form, and an activity of this enzyme as well as production of NO is decreased. Moreover, under conditions of oxidative stress, NO synthase synthesizes rather superoxide radical than NO [37, 42]. This, besides direct L-NAME inhibition, may be one of the reasons of decreased NO synthase activity in our experiment. AME treatment in our experiment directly increased NO synthase activity probably by both decreasing oxidative stress by polyphenolic group function and stabilizing NO synthase dimer by addition of Zn.

This study provides also the evidence that active compounds of AME are likely to stimulate the expression of eNOS in myocardium; however, in combination with L-NAME, this stimulation is not significant. Those results

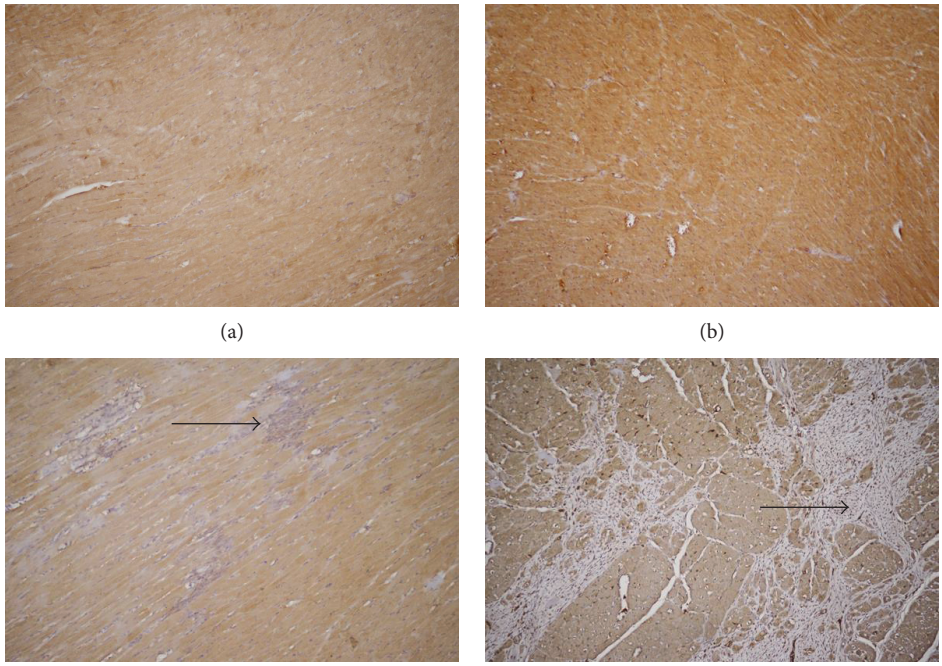


Figure 4: eNOS expression in heart tissue. Diffuse cytoplasmic positivity of eNOS was found in all samples. Only the AME treatment without the L-NAME administration significantly increased the eNOS expression. (a) WKY, (b) AME, (c) L-NAME, (d) L-NAME + AME, eNOS, Ab-Poly, DAB, 200x.

are in good agreement with Wallerath [43] who found increased eNOS expression in humans after polyphenol treatment. The positive effect on endothelial formation of NO in coronary arteries was observed also by Kim [38]. Authors showed that phosphorylation of eNOS via redox-sensitive activation of Src/PI3/Akt pathway is mostly by conjugated cyanidins and chlorogenic acids, which are also the most, represented content of our extract.

5. Conclusions

In conclusion, AME treatment was able to reduce blood pressure in L-NAME-induced hypertension by decreasing oxidative stress and increasing NO production. Both direct contribution of polyphenolic groups and Zn addition may be responsible for NO synthase upregulation in LV. Moreover, in the same tissue, AME alone was able to increase eNOS protein expression and had a tendency to increase it during the concomitant treatment with L-NAME which may further contribute to NO synthase activity increase.

Disclosure

An earlier version of this work was presented before at the "25th Anniversary of the FEPS, 168th Anniversary of French Physiological Society, 2016," and published as an abstract in the "Acta Phyiologica" Official Journal of the Federation of European Physiological Societies in 2016.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The study was supported by Scientific Grant Agency VEGA (nos. 2/0170/17 and 2/0165/15) and Slovak Research and Development Agency APVV (no. APVV-14-0932).

References

[1] O. Pechanova, Z. V. Varga, M. Cebova, Z. Giricz, P. Pacher, and P. Ferdinandy, "Cardiac NO signalling in the metabolic syndrome," *British Journal of Pharmacology*, vol. 172, no. 6, pp. 1415–1433, 2015.

[2] M. Rodriguez-Porcel, X. Y. Zhu, A. R. Chade et al., "Functional and structural remodeling of the myocardial microvasculature in early experimental hypertension," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 290, no. 3, pp. H978–H984, 2006.

[3] P. Babal, O. Pechanova, and I. Bernatova, "Long-term administration of D-NAME induces hemodynamic and structural changes in the cardiovascular system," *Physiological Research*, vol. 49, no. 1, pp. 47–54, 2000.

[4] F. Kristek and M. Cebova, "Long-term effect of exogenous NO donors on geometry of conduit arteries in NO-deficient and spontaneously hypertensive rats," in *Experimental Hypertension and Ischemic Heart Diseases*, pp. 175–191, Publishing House of the Slovak Academy of Sciences, Bratislava, 2005.

[5] F. Kristek and M. Gerova, "Long-term NO synthase inhibition affects heart weight and geometry of coronary and

carotid arteries," *Physiological Research*, vol. 45, no. 5, pp. 361–367, 1996.

[6] P. Babal, O. Pechanova, I. Bernatova, and S. Stvrtina, "Chronic inhibition of NO synthesis produces myocardial fibrosis and arterial media hyperplasia," *Histology and Histopathology*, vol. 12, no. 3, pp. 623–629, 1997.

[7] L. Hooper, P. A. Kroon, E. B. Rimm et al., "Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials," *The American Journal of Clinical Nutrition*, vol. 88, no. 1, pp. 38–50, 2008.

[8] A. Agouni, H. A. Mostefai, A. H. Lagrue et al., "Paradoxical effect of nonalcoholic red wine polyphenol extract, Provinols™, in the regulation of cyclooxygenases in vessels from Zucker fatty rats (*fa*/*fa*)," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 8536910, 12 pages, 2017.

[9] J. A. Ross and C. M. Kasum, "Dietary flavonoids: bioavailability, metabolic effects, and safety," *Annual Review of Nutrition*, vol. 22, no. 1, pp. 19–34, 2002.

[10] N. Jeppsson and R. Johansson, "Changes in fruit quality in black chokeberry (*Aronia melanocarpa*) during maturation," *The Journal of Horticultural Science and Biotechnology*, vol. 75, no. 3, pp. 340–345, 2000.

[11] K. Skupien and J. Oszmianski, "The effect of mineral fertilization on nutritive value and biological activity of chokeberry fruit," *Agricultural and Food Science*, vol. 16, pp. 46–55, 2007.

[12] S. E. Kulling and H. M. Rawel, "Chokeberry *(Aronia melanocarpa)* – a review on the characteristic components and potential health effects," *Planta Medica*, vol. 74, no. 13, pp. 1625–1634, 2008.

[13] I. Ochmian, J. Grajkowski, and M. Smolik, "Comparison of some morphological features, quality and chemical content of four cultivars of chokeberry fruits (*Aronia melanocarpa*)," *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, vol. 40, pp. 253–260, 2012.

[14] K. Wilkes, L. R. Howard, C. Brownmiller, and R. L. Prior, "Changes in chokeberry (*Aronia melanocarpa* L.) polyphenols during juice processing and storage," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 18, pp. 4018–4025, 2014.

[15] M. Broncel, M. Kozirog, P. Duchnowicz, M. Koter-Michalak, J. Sikora, and J. Chojnowska-Jezierska, "*Aronia melanocarpa* extract reduces blood pressure, serum endothelin, lipid, and oxidative stress marker levels in patients with metabolic syndrome," *Medical Science Monitor*, vol. 16, no. 1, pp. CR28–CR34, 2010.

[16] S. Pascual-Teresa and M. T. Sanchez-Ballesta, "Anthocyanins: from plant to health," *Phytochemistry Reviews*, vol. 7, no. 2, pp. 281–299, 2008.

[17] S. Valcheva-Kuzmanova, K. Kuzmanov, S. Tancheva, and A. Belcheva, "Hypoglycemic effects of *Aronia melanocarpa* fruit juice in streptozotocin-induced diabetic rats," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 29, no. 2, pp. 101–105, 2007.

[18] L. I. Mennen, D. Sapinho, A. de Bree et al., "Consumption of foods rich in flavonoids is related to a decreased cardiovascular risk in apparently healthy French women," *The Journal of Nutrition*, vol. 134, no. 4, pp. 923–926, 2004.

[19] M. Cebova, A. Barta, Z. Matuskova, R. Rehakova, M. Kosutova, and O. Pechanova, "The effect of bioactive compound of *Aronia melanocarpa* on cardiovascular system in experimental hypertension," *Acta Physiologica*, vol. 217, Supplement 708, p. 49, 2016.

[20] B. W. Bolling, R. Taheri, R. Pei et al., "Harvest date affects aronia juice polyphenols, sugars, and antioxidant activity, but not anthocyanin stability," *Food Chemistry*, vol. 187, pp. 189–196, 2015.

[21] T. Jurikova, J. Mlcek, S. Skrovankova et al., "Fruits of black chokeberry *Aronia melanocarpa* in the prevention of chronic diseases," *Molecules*, vol. 22, no. 6, p. 944, 2017.

[22] A. Kondrashov, S. Vrankova, I. Dovinova et al., "The effects of new Alibernet red wine extract on nitric oxide and reactive oxygen species production in spontaneously hypertensive rats," *Oxidative Medicine and Cellular Longevity*, vol. 2012, Article ID 806285, 8 pages, 2012.

[23] O. Pechanova, I. Bernatova, V. Pelouch, and F. Simko, "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.

[24] 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.

[25] J. Schindelin, I. Arganda-Carreras, E. Frise et al., "Fiji: an open-source platform for biological-image analysis," *Nature Methods*, vol. 9, no. 7, pp. 676–682, 2012.

[26] M. D. Abramoff, P. J. Magelhaes, and S. J. Ram, "Image processing with ImageJ," *Biophotonics International*, vol. 11, no. 7, pp. 36–42, 2004.

[27] S. Moncada, R. M. Palmer, and E. A. Higgs, "Nitric oxide: physiology, pathophysiology, and pharmacology," *Pharmacological Reviews*, vol. 43, no. 2, pp. 109–142, 1991.

[28] O. Pechánová and I. Bernátová, "Effect of long-term NO synthase inhibition on cyclic nucleotide content in rat tissues," *Physiological Research*, vol. 45, no. 4, pp. 305–309, 1996.

[29] A. Michalska and A. N. Schechter, "Dietary nitrite and nitrate: a review of potential mechanisms of cardiovascular benefits," *European Journal of Nutrition*, vol. 50, pp. 293–303, 2011.

[30] O. Pechánová, R. Rezzani, P. Babál, I. Bernátová, and R. Andriantsitohaina, "Beneficial effects of Provinols™: cardiovascular system and kidney," *Physiological Research*, vol. 55, Supplement 1, pp. S17–S30, 2006.

[31] M. Ciocoiu, M. Badescu, O. Badulescu, D. Tutunaru, and L. Badescu, "Polyphenolic extract association with renin inhibitors in experimental arterial hypertension," *Journal of Biomedical Science and Engineering*, vol. 6, no. 4, Article ID 30525, 5 pages, 2013.

[32] J. K. Hellstrom, A. N. Shikov, M. N. Makarova et al., "Blood pressure-lowering properties of chokeberry (*Aronia mitchurinii*, var. Viking)," *Journal of Functional Food*, vol. 2, no. 2, pp. 163–169, 2010.

[33] M. Naruszewicz, I. Laniewska, B. Millo, and M. Dłużniewski, "Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (MI)," *Atherosclerosis*, vol. 194, no. 2, pp. e179–e184, 2007.

[34] T. E. Tjelle, L. Holtung, S. K. Bøhn et al., "Polyphenol-rich juices reduce blood pressure measures in a randomised controlled trial in high normal and hypertensive volunteers," *The British Journal of Nutrition*, vol. 114, no. 07, pp. 1054–1063, 2015.

[35] D. Berg, M. B. Youdim, and P. Riederer, "Redox imbalance," *Cell and Tissue Research*, vol. 318, no. 1, pp. 201–213, 2004.

[36] 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.

[37] O. Pechanova, J. Matuskova, D. Capikova, L. Jendekova, L. Paulis, and F. Simko, "Effect of spironolactone and captopril on nitric oxide and S-nitrosothiol formation in kidney of L-NAME-treated rats," *Kidney International*, vol. 70, no. 1, pp. 170–176, 2006.

[38] J. H. Kim, C. Auger, I. Kurita et al., "*Aronia melanocarpa* juice, a rich source of polyphenols, induces endothelium-dependent relaxations in porcine coronary arteries via the redox-sensitive activation of endothelial nitric oxide synthase," *Nitric Oxide*, vol. 35, pp. 54–64, 2013.

[39] J. Han and A. C. Nicholson, "Lipoproteins modulate expression of the macrophage scavenger receptor," *The American Journal of Pathology*, vol. 152, no. 6, pp. 1647–1654, 1998.

[40] K. Appel, P. Meiser, E. Millán et al., "Chokeberry (*Aronia melanocarpa* (Michx.) Elliot) concentrate inhibits NF-κB and synergizes with selenium to inhibit the release of pro-inflammatory mediators in macrophages," *Fitoterapia*, vol. 105, pp. 73–82, 2015.

[41] T. Hussain, B. Tan, Y. Yin, F. Blachier, M. C. Tossou, and N. Rahu, "Oxidative stress and inflammation: what polyphenols can do for us?," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 7432797, 9 pages, 2016.

[42] U. Landmesser, S. Dikalov, S. R. Price et al., "Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension," *The Journal of Clinical Investigation*, vol. 111, no. 8, pp. 1201–1209, 2003.

[43] T. Wallerath, D. Poleo, H. Li, and U. Förstermann, "Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects," *Journal of the American College of Cardiology*, vol. 41, no. 3, pp. 471–478, 2003.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 9718615, 6 pages
https://doi.org/10.1155/2017/9718615

Research Article

The Role of Oxidative Stress in Decreased Acetylcholinesterase Activity at the Neuromuscular Junction of the Diaphragm during Sepsis

Hua Liu,[1,2] Jin Wu,[1] Jun-yan Yao,[1] Hong Wang,[1] and Shi-tong Li[1]

[1]*Department of Anesthesiology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China*
[2]*Department of Anesthesiology, The Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China*

Correspondence should be addressed to Shi-tong Li; lishitongs@yahoo.com

Received 13 July 2017; Accepted 17 October 2017; Published 5 November 2017

Academic Editor: Paola Venditti

Our recent study demonstrated that acetylcholinesterase (AChE) activity at the neuromuscular junction (NMJ) of the diaphragm decreased during sepsis. However, the mechanisms were not clearly identified. In this study, we aimed to investigate whether the decreased AChE activity was related to oxidative stress by observing AChE activity in different grades of sepsis induced by caecal ligation and puncture (CLP). At 24 h after surgery, an assay of thiobarbituric acid reactive species (TBARS) and protein carbonyls, as well as the myeloperoxidase (MPO), superoxide dismutase (SOD), and catalase (CAT) activity, was conducted. AChE activity was measured by biochemical and histological detection. AChE and CAT activity in the diaphragm decreased, while the contents of TBARS and protein carbonyls, the activity of MPO and SOD, and the SOD/CAT ratios increased. The above changes were much more significant in the mid-grade septic group than in the low-grade septic group. The colour of the AChE activity staining at the NMJ gradually lightened from the sham surgery group to the mid-grade septic group. AChE activity was significantly negatively correlated with the levels of TBARS and protein carbonyls. We consider that oxidative stress might be responsible for decreased AChE activity in the diaphragms of rats induced with sepsis.

1. Introduction

Sepsis is a clinical syndrome caused by severe infection, leading to multiple organ dysfunction [1, 2]. Our recent study has reported that acetylcholinesterase (AChE) activity at the neuromuscular junction (NMJ) of the diaphragm was inhibited during sepsis [3]. However, the effects of different grades of sepsis on the activity of AChE were not illustrated in that study. Moreover, the mechanisms through which AChE activity was inhibited during sepsis were not clearly identified.

Previous studies have demonstrated that several factors, including inflammatory, immune, hormonal, metabolic, and bioenergetic responses, were involved in the pathogenesis of sepsis [4–6]. One of the crucial factors in these processes is the loss of balance between the reactive species (ROS) and antioxidant systems, leading to a state of irreversible oxidative stress [7]. ROS contain a series of active oxygen free radical or molecule, including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and hypochlorous acid (HOCl) [8, 9], which can attack proteins, carbohydrates, nucleic acids, and unsaturated lipids, subsequently causing tissue and organism injury [10]. Several previous studies have found that ROS can inhibit AChE in various tissues [11–17].

These findings raise the possibility that oxidative stress might be a vital contributor to the inhibition of AChE activity during sepsis. In the current study, we detected AChE activity

in two different grades of sepsis to more intensively determine the effects of sepsis on AChE activity. Meanwhile, we measured the indicators of oxidative injury (thiobarbituric acid reactive species (TBARS) and protein carbonyl) to observe the correlation between AChE activity and oxidative stress during sepsis. We aimed to clarify the role of oxidative stress in the decreased AChE activity at the NMJ of diaphragm during sepsis.

2. Materials and Methods

2.1. Animals and Experimental Protocol. Our study was approved by the Animal Care and Use Committee of Shanghai General Hospital Affiliated to Shanghai Jiao Tong University (Grant number 2017DW001). Experiments were performed using a total of 28 adult male Sprague-Dawley rats weighing between 220 and 260 g. The animals were allowed unrestrained access to food and water and housed at an ambient temperature of 23–25°C with 12 h light-dark cycles throughout the study. The rats were randomly divided into three groups: (1) the sham group (S group, $n = 8$), (2) the low-grade sepsis group (L group, $n = 8$), and (3) the midgrade sepsis group (M group, $n = 12$ due to an expected mortality rate of approximately 25% within the first 24 h in our previous study). The rats were anaesthetized with intraperitoneal injection of pentobarbital (50 mg/kg). In the L and M groups, sepsis was surgically induced using the CLP method, as it is widely used and known to closely mimic the pathophysiology of septic patients [18]. Under aseptic conditions, a 3 cm midline abdominal incision was performed to expose the caecum, with adjoining intestine. The caecum in the L group was ligated near the distal pole comprising 10% of the caecum, whereas the caecum in the M group was ligated in the middle portion [19]. Then, the caecum was perforated once with an 18-gauge needle. Faecal droplets were squeezed out of the caecum through penetration holes. The caecum was then relocated into the abdominal cavity, and the laparotomy was closed with 3-0 silk sutures. In the S group, a midline abdominal incision was made, and the caecum was exposed but not ligated or perforated. All rats were immediately resuscitated with prewarmed (37°C) normal saline (50 ml/kg subcutaneous). All animals were allowed to return to their cages, with free access to food and water after surgery.

At 24 h after surgery, the surviving rats were euthanized through intraperitoneal injection of pentobarbital (100 mg/kg), and their diaphragms were harvested. The right hemidiaphragm was removed and immediately stored at −80°C until being assayed for AChE activity, as well as the contents of TBARS formation and protein carbonyls, and MPO, SOD, and CAT activities. The ventral costal region of the left hemidiaphragm was immediately removed for histological detection of AChE activity at the NMJ.

2.2. Histological Detection of AChE Activity at the NMJ. The AChE activity at the NMJ of the diaphragm was detected through a modified Karnovsky and Roots method by referencing our previous study [3]. The brown insoluble products on the muscle fibres indicated AChE activity [20].

2.3. Biochemical Measurement of AChE Activity. The method is based on the fact that AChE can hydrolyse acetylcholine to form acetic acid and choline. The latter can react with a mercapto colour reagent to form a yellow symmetrical trinitrobenzene compound. Measuring the colour depth of the compound can reflect the activity of AChE. The AChE activity assay test was carried out using an AChE assay kit from the Nanjing Jiancheng Bioengineering Institute (A024, Nanjing, China) according to the manufacturer's instructions [21]. The results were determined by measuring the absorbance at 412 nm and expressed as units of AChE activity per milligram of protein.

2.4. TBARS Measurement. As an indicator of lipid peroxidation in oxidative stress, we determined the formation of TBARS during an acid-heating reaction, as previously described [22]. Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% purchased from Sigma Chemical (St. Louis, MO, USA) and 1 ml thiobarbituric acid 0.67%, and then, the mixture was heated in a boiling water bath for 15 mins. TBARS was determined by measuring the absorbance at 535 nm, and the results are expressed as malondialdehyde (MDA) equivalents per milligram of protein.

2.5. Measurement of Protein Carbonyls. As a marker of the oxidative damage to proteins, protein carbonyl groups were measured based on the reaction with dinitrophenyl hydrazine, as previously described [23]. Briefly, the proteins were precipitated by adding 20% trichloroacetic acid and redissolved in dinitrophenyl hydrazine. Eventually, the protein carbonyls were determined by reading the absorbance at 375 nm, and the results were expressed as nmols of protein carbonyls per milligram of protein.

2.6. MPO Activity Assay. MPO activity in homogenates of diaphragm tissue was determined using a test kit from Nanjing Jiancheng Bioengineering Institute (A044, Nanjing, China). Activity was measured spectrophotometrically as the change in absorbance at 412 nm, using a Spectramax microplate reader. The results are expressed as units of MPO activity per gram of tissue wet weight.

2.7. Measurement of CAT and SOD Activities. The above-prepared supernatant was used for CAT and SOD activity assay according to the instructions of their corresponding test kit purchased from Nanjing Jiancheng Bioengineering Institute (CAT: A007-1; SOD: A001-1, Nanjing, China). Both results were expressed as units of enzyme activity per gram of tissue wet weight. Finally, the ratio of SOD/CAT was calculated and recorded.

2.8. Statistical Analyses. SPSS (version 19.0; SPSS Inc., Chicago, Illinois, USA) was used for the statistical analysis. Values are expressed as the mean ± standard deviation (SD). The data were tested for normality and equality of variance. Between-group comparisons for each dependent variable were assessed using analysis of variance (ANOVA) with the least significant difference (LSD) test. Pearson's correlation analysis was used to determine the association

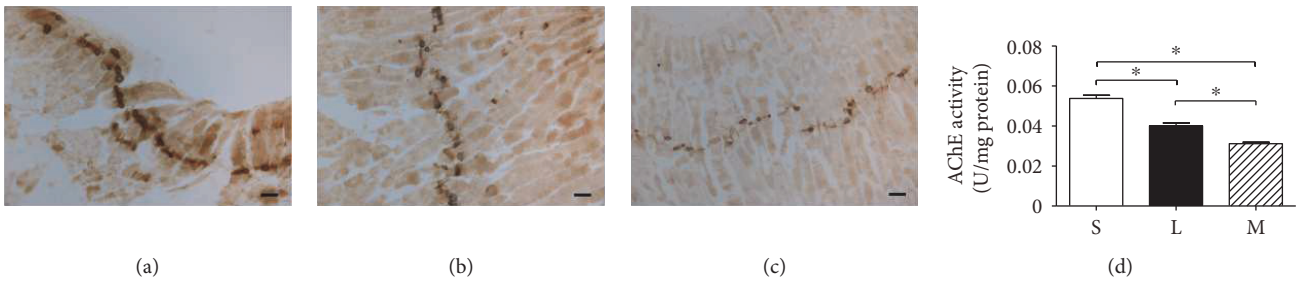


Figure 1: AChE staining at neuromuscular junction in sham group (a), low-grade septic group (b), and midgrade septic group (c). Bars = 50 μm. Comparison of AChE activity through biochemical measurement in three groups of rat diaphragm (d). The values are expressed as means ± SD. $n = 8$, $^*P < 0.01$. S: sham group; L: low-grade septic group; M: midgrade septic group; SD: standard deviation.

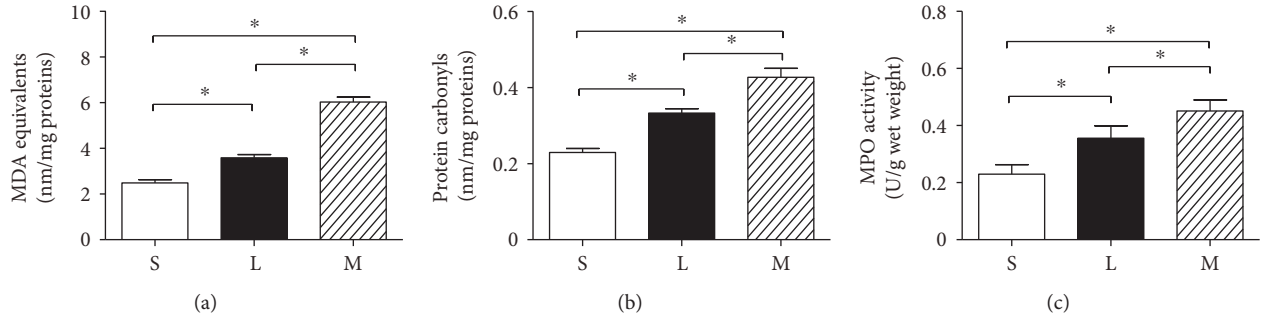


Figure 2: Comparison of thiobarbituric acid reactive species (a), protein carbonyls (b), and MPO activity (c) in three groups of rat diaphragm. The values are expressed as means ± SD. $n = 8$, $^*P < 0.01$. S: sham group; L: low-grade septic group; M: midgrade septic group; SD: standard deviation.

between AChE activity and TBARS or protein carbonyls, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Mortality within 24 h. Four of the 12 rats in the M group died within 24 h, and all other rats survived during the first 24 h. In addition, the rats in midgrade septic group developed more severe septic symptoms, such as shortness of breath, hair erection, subconjunctival haemorrhage, diarrhoea, and loss of movement, than those in low-grade septic group.

3.2. AChE Activity Staining at the NMJ. As shown in Figure 1, AChE staining was detected at motor endplate regions in rat diaphragms from all three groups. Observed from the slices, the brown insoluble products of AChE staining in the S group were deepest, while the brown in M group was lightest (Figures 1(a), 1(b), and 1(c)).

3.3. AChE Activity in the Diaphragm. Compared with the S group, AChE activity decreased significantly in both the L and M groups ($P < 0.01$). Furthermore, the decline of AChE activity in the M group was much more significant than that in the L group ($P < 0.01$) (Figure 1(d)).

3.4. Oxidative Parameters at the Diaphragm

3.4.1. TBARS and Protein Carbonyls in the Diaphragm. Twenty-four hours after CLP (in both the L and M groups), TBARS and protein carbonyls increased significantly in the diaphragm ($P < 0.01$). Additionally, the levels of TBARS and protein carbonyls were higher in the M group than those in the L group ($P < 0.01$) (Figures 2(a) and 2(b)).

3.4.2. MPO Activity in the Diaphragm. As illustrated in Figure 2(c), MPO activity was significantly elevated in rats in both the L and M groups compared to rats in the S group ($P < 0.01$). Moreover, MPO activity was higher in the M group than that in the L group ($P < 0.01$) (Figure 2(c)).

3.5. Antioxidant Enzyme Activities

3.5.1. SOD and CAT Activities and SOD/CAT Ratios. SOD activity increased in both the L and M groups ($P < 0.01$), and the increase of SOD activity was higher in the M group than that in the L group (Figure 3(a)). In contrast, a significant decrease was observed in CAT activity in the L ($P < 0.05$) and M groups ($P < 0.01$). CAT activity decreased more in the M group than in the L group (Figure 3(b)). The ratio of SOD and CAT increased significantly in the L group ($P < 0.05$) and M group ($P < 0.01$). Meanwhile, this ratio was higher in the M group than that in the L group ($P < 0.01$) (Figure 3(c)).

3.6. Correlation between AChE Activity and TBARS or Protein Carbonyls. AChE activity was significantly and negatively correlated with the levels of TBARS ($r = -0.839$, $P < 0.01$) (Figure 4(a)) and protein carbonyls ($r = -0.857$, $P < 0.01$) (Figure 4(b)).

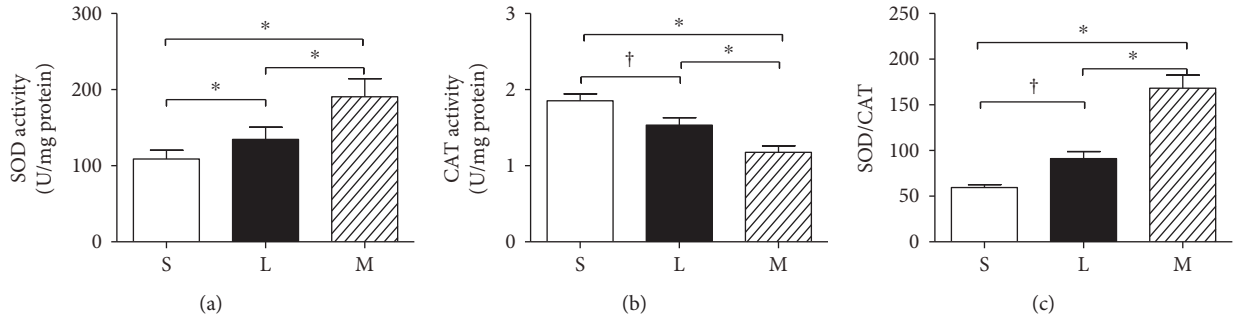


Figure 3: Comparison of SOD activity (a), CAT activity (b), and SOD/CAT (c) in three groups of rat diaphragms. The values are expressed as the means ± SD. $n = 8$, $^{*}P < 0.01$, $^{\dagger}P < 0.05$. S: sham group; L: low-grade septic group; M: midgrade septic group; SD: standard deviation.

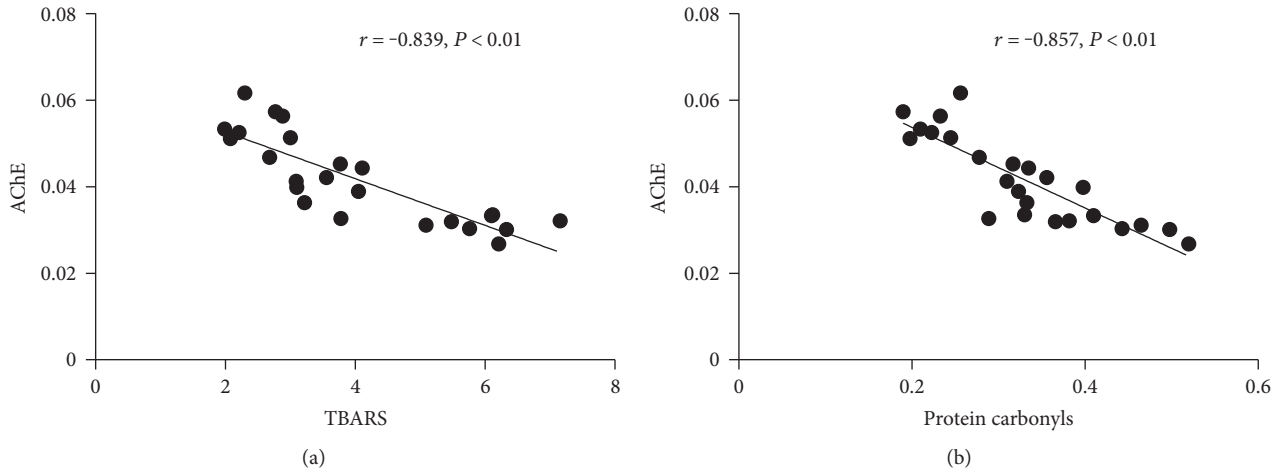


Figure 4: Correlation between AChE activity and TBARS (a) or protein carbonyls (b).

4. Discussion

Using low-grade and midgrade models of sepsis in rats, this study found that the AChE and CAT activities in the diaphragm decreased, while the contents of TBARS and protein carbonyls, the activity of MPO and SOD, and the SOD/CAT ratios increased at 24 h after CLP surgery. Furthermore, the above changes were much more significant in the midgrade septic group than those in the low-grade septic group. Strikingly, we found that AChE activity was significantly and negatively correlated with the levels of TBARS and protein carbonyls in rats.

In this study, low-grade and midgrade sepsis was established by ligating different caecum lengths, as described in previous studies [19]. The rats in the midgrade septic group had a higher mortality rate and developed more severe septic symptoms than those in the low-grade septic group. Moreover, TBARS and protein carbonyls, which represent the lipid and protein injury, respectively, in oxidative stress increased more in the midgrade septic group than in the low-grade septic group. These results illustrated that our sepsis models of different severity grades were successfully created.

AChE activity was found to be significantly and negatively correlated with the levels of TBARS and protein carbonyls, which indicated that oxidative stress may contribute to the decreased AChE activity during sepsis. It is believed that ROS plays an important role in the oxidative stress to cause cellular injury during sepsis. ROS in the diaphragm may primarily be derived from mitochondrial respiration chain impairment and infiltrating inflammatory cells [24, 25]. $O_2^{\bullet-}$ produced through the above two manners can be converted to H_2O_2 by SOD. H_2O_2 will be converted to harmless H_2O and O_2 through the action of CAT. Otherwise, it will allow neutrophils to oxidize chloride ions into HOCl through MPO [26]. SOD and CAT are two oxidative enzymes that play vital roles in eliminating ROS. Some research has revealed that SOD activity increased without a proportional increase in CAT activity during sepsis [27, 28]. In our study, the imbalance also existed, and it was much more serious in midgrade sepsis than in low-grade sepsis. The different modulation of SOD and CAT during sepsis may rise from some reasons below. SOD activity increased in the CLP surgery probably as a response to oxidative stress induced by sepsis. SOD activation during sepsis could be a compensatory response to the overproduction of mitochondrial $O_2^{\bullet-}$ so as to eliminate excessive $O_2^{\bullet-}$[28]. Additionally, the increase of interleukin-1 (IL-1), tumor necrosis factor (TNF), and lipopolysaccharide (LPS) during sepsis could also activate SOD [29, 30]. Unlike SOD, the previous study demonstrated that excessive $O_2^{\bullet-}$ or

H_2O_2 could oxidize CAT active site leading to enzymatic inactivation [31]. The imbalance between SOD and CAT may result in the accumulation of excessive H_2O_2 in the diaphragm [27, 32]. Additionally, our results indicated that MPO activity increased in both septic groups, and the activity increased higher in midgrade sepsis than in low-grade sepsis. MPO, synthesized and secreted by neutrophils, is a marker of inflammation and neutrophil infiltration in tissues [33]. As described above, increased MPO can further convert excessive H_2O_2 into HOCl. Moreover, excessive H_2O_2 will react with iron to generate $^{\bullet}OH$ through Fenton chemistry [28].

Early in 1966, O'Malley et al. found that both H_2O_2 and peroxides could inhibit erythrocyte AChE [11]. A study from Danylovych reported that AChE of myometrium sarcolemma could be inhibited by H_2O_2 [12]. Accumulation of H_2O_2 in the mM range can decrease epidermal AChE expression and inhibit human recombinant AChE activity [13, 14]. Additionally, it has been verified that $^{\bullet}OH$ inhibits rat brain AChE and human recombinant AChE activity [15, 16]. In addition, HOCl has been found to be a strong inhibitor of AChE [17]. A kinetic analysis using pure recombinant human AChE and a molecular modelling based on the established 3D structure of human AChE supported that ROS-mediated oxidation of Trp432, Trp435, and Met436 moves and disorients the active site His440 of AChE, leading to deactivation of the protein [13, 14]. Moreover, TBARS can decrease membrane fluidity and decrease AChE activity through lipid-protein interactions [15]. These results provided a molecular basis through which oxidative stress inhibits AChE activity during sepsis.

However, our study has two limitations. First, we failed to observe the conformation change of AChE induced by ROS directly because of the limitations of the experimental methods. Second, we did not detect the quantity of AChE expression simultaneously, although recent data from another study in our laboratory revealed that such data may be another factor affecting the decrease of AChE activity (unpublished data).

5. Conclusions

We believe that there were at least two highlights in the present study. First, we found that AChE activity at the NMJ of diaphragm decreased more significantly during severe sepsis for the first time. Second, AChE activity at the NMJ of diaphragm is found to be significantly and negatively correlated with level of oxidative stress during sepsis.

In conclusion, our study verified that oxidative stress might be responsible for the decreased activity of the AChE at the NMJ in the diaphragm. Furthermore, the effects of antioxidant measurements on AChE activity during sepsis should be investigated in the future.

Conflicts of Interest

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

Authors' Contributions

Hua Liu and Jin Wu contributed equally.

Acknowledgments

This study was supported by a grant from the National Natural Science Foundation of China (Grant no. 81171845).

References

[1] M. Singer, C. S. Deutschman, and C. W. Seymour, "The third international consensus definitions for sepsis and septic shock (sepsis-3)," *JAMA*, vol. 315, no. 8, pp. 801–810, 2016.

[2] A. Demoule, B. Jung, H. Prodanovic et al., "Diaphragm dysfunction on admission to ICU: prevalence, risk factors and prognostic impact - a prospective study," *American Journal of Respiratory and Critical Care Medicine*, vol. 88, no. 2, pp. 213–219, 2013.

[3] J. Wu, T. Jin, H. Wang, and S.-T. Li, "Sepsis strengthens antagonistic actions of neostigmine on rocuronium in a rat model of cecal ligation and puncture," *Chinese Medical Journal*, vol. 129, no. 12, pp. 1477–1482, 2016.

[4] R. C. Bone, C. J. Grodzin, and R. A. Balk, "Sepsis: a new hypothesis for pathogenesis of the disease process," *Chest*, vol. 112, no. 1, pp. 235–243, 1997.

[5] R. C. Bone, R. A. Balk, F. B. Cerra, R. P. Dellinger, A. M. Fein, and W. A. Knaus, "Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis," *Chest*, vol. 101, no. 6, pp. 1644–1655, 1992.

[6] D. Rittirsch, M. A. Flierl, and P. A. Ward, "Harmful molecular mechanisms in sepsis," *Nature Reviews Immunology*, vol. 8, no. 10, pp. 776–787, 2008.

[7] J. Duran-Bedolla, M. A. Montes de Oca-Sandoval, and V. Saldaña-Navor, "Sepsis, mitochondrial failure and multiple organ dysfunction," *Clinical and Investigative Medicine*, vol. 37, no. 2, pp. E58–E69, 2014.

[8] M. P. Fink, "Role of reactive oxygen and nitrogen species in acute respiratory distress syndrome," *Current Opinion in Critical Care*, vol. 8, no. 1, pp. 6–11, 2002.

[9] H. Bayir, "Reactive oxygen species," *Critical Care Medicine*, vol. 33, no. 12, pp. S498–S501, 2005.

[10] C. A. Prauchner, "Oxidative stress in sepsis: pathophysiological implications justifying antioxidant co-therapy," *Burns*, vol. 43, no. 3, pp. 471–485, 2017.

[11] B. W. O'Malley, C. E. Mengel, and W. D. Meriwether, "Inhibition of erythrocyte acetylcholinesterase by peroxides," *Biochemistry*, vol. 5, no. 1, pp. 40–44, 1966.

[12] D. IuV, "Hydrogen peroxide inhibits acetylcholinesterase of myometrium sarcolemma," *UkrBiokhim Zh*, vol. 81, no. 4, pp. 32–38, 2009.

[13] K. U. Schallreuter, S. M. Elwary, and N. C. Gibbons, "Activation/deactivation of acetylcholinesterase by H2O2: more evidence for oxidative stress in vitiligo," *Biochemical and Biophysical Research*, vol. 315, no. 2, pp. 502–508, 2004.

[14] K. U. Schallreuter and S. Elwary, "Hydrogen peroxide regulates the cholinergic signal in a concentration dependent manner," *Life Sciences*, vol. 80, no. 24-25, pp. 2221–2226, 2007.

[15] S. Tsakiris, P. Angelogianni, K. H. Schulpis, and J. C. Stavridis, "Protective effect of L phenylalanine on rat brain

acetylcholinesterase inhibition induced by free radicals," *Clinical Biochemistry*, vol. 33, no. 2, pp. 103–106, 2000.

[16] A. Méndez-Garrido, M. Hernández-Rodríguez, R. Zamorano-Ulloa et al., "In vitro effect of H2O2, some transition metals and hydroxyl radical produced via Fenton and Fenton-like reactions, on the catalytic activity of AChE and the hydrolysis of ACh," *Neurochemical Research*, vol. 39, no. 11, pp. 2093–2104, 2014.

[17] G. J. den Hartog, E. Vegt, and W. J. van der Vijgh, "Hypochlorous acid is a potent inhibitor of acetylcholinesterase," *Toxicology and Applied Pharmacology*, vol. 181, no. 3, pp. 228–232, 2002.

[18] D. Rittirsch, M. S. Huber-Lang, M. A. Flierl, and P. AWard, "Immunodesign of experimental sepsis by cecal ligation and puncture," *Nature Protocols*, vol. 4, no. 1, pp. 31–36, 2009.

[19] S. Ruiz, F. Vardon-Bounes, V. Merlet-Dupuy, J.-M. Conil, M. Buléon, and O. Fourcade, "Sepsis modeling in mice: ligation length is a major severity factor in cecal ligation and puncture," *Intensive Care Medicine Experimental*, vol. 4, no. 1, p. 22, 2016.

[20] M. J. Karnovsky and L. Roots, "A "direct-coloring" thiocholine method for cholinesterases," *The Journal of Histochemistry and Cytochemistry*, vol. 12, no. 3, pp. 219–221, 1964.

[21] S. Ma, S. Xu, B. Liu et al., "Long-term treatment of l-3-n-butylphthalide attenuated neurodegenerative changes in aged rats," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 379, no. 6, pp. 565–574, 2009.

[22] H. H. Draper and M. Hadley, "Malondialdehyde determination as index of lipid peroxidation," *Methods in Enzymology*, vol. 186, pp. 421–431, 1990.

[23] R. L. Levine, D. Garland, and C. N. Oliver, "Determination of carbonyl content in oxidatively modified proteins," *Methods in Enzymology*, vol. 186, pp. 464–478, 1990.

[24] D. E. Taylor, A. J. Ghio, and C. A. Piantadosi, "Reactive oxygen species produced by liver mitochondria of rats in sepsis," *Archives of Biochemistry and Biophysics*, vol. 316, no. 1, pp. 70–76, 1995.

[25] Y. V. Lerman and M. Kim, "Neutrophil migration under normal and sepsis conditions," *Cardiovascular & Hematological Disorders Drug Targets*, vol. 15, no. 1, pp. 19–28, 2015.

[26] J. P. Gaut, G. C. Yeh, and H. D. Tran, "Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 21, pp. 11961–11966, 2001.

[27] C. Ritter, M. E. Andrades, A. Reinke, S. Menna-Barreto, J. C. Moreira, and F. Dal-Pizzol, "Treatment with N-acetylcysteine plus deferoxamine protects rats against oxidative stress and improves survival in sepsis," *Critical Care Medicine*, vol. 32, no. 2, pp. 342–349, 2004.

[28] C. Ritter, M. Andrades, and M. L. Frota Júnior, "Oxidative parameters and mortality in sepsis induced by cecal ligation and perforation," *Intensive Care Medicine*, vol. 29, pp. 1782–1789, 2003.

[29] A. Masuda, D. L. Longo, and Y. Kobayashi, "Induction of mitochondrial manganese superoxide dismutase by interleukin 1," *The FASEB Journal*, vol. 2, pp. 3087–3091, 1988.

[30] G. A. Visner, W. C. Douglas, J. M. Wilson, I. A. Burr, and H. S. Nick, "Regulation of manganese superoxide dismutase by lipopolysaccaride, interleukin-1, and tumor necrosis factor," *The Journal of Biological Chemistry*, vol. 265, pp. 2856–2286, 1990.

[31] T. G. Gantchev and J. E. van Lier, "Catalase inactivation following photosensitization with tetrasulfonated metallophthalocyanines," *Photochemistry and Photobiology*, vol. 62, pp. 123–134, 1995.

[32] C. A. Prauchner, S. Prestes Ade, and J. B. da Rocha, "Effects of diphenyl diselenide on oxidative stress induced by sepsis in rats," *Pathology, Research and Practice*, vol. 207, pp. 554–558, 2011.

[33] M. Faith, A. Sukumaran, A. B. Pulimood, and M. Jacob, "How reliable an indicator of inflammation is myeloperoxidase activity?," *Clinica Chimica Acta*, vol. 396, pp. 23–25, 2008.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 4015874, 22 pages
https://doi.org/10.1155/2017/4015874

Review Article

Molecular Mechanisms Responsible for Increased Vulnerability of the Ageing Oocyte to Oxidative Damage

Bettina P. Mihalas,[1] Kate A. Redgrove,[1] Eileen A. McLaughlin,[1,2] and Brett Nixon[1]

[1]*Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia*
[2]*School of Biological Sciences, University of Auckland, Auckland, New Zealand*

Correspondence should be addressed to Bettina P. Mihalas; bettina.mihalas@uon.edu.au and Brett Nixon; brett.nixon@newcastle.edu.au

Received 24 May 2017; Accepted 3 August 2017; Published 18 October 2017

Academic Editor: Tanea T. Reed

In their midthirties, women experience a decline in fertility, coupled to a pronounced increase in the risk of aneuploidy, miscarriage, and birth defects. Although the aetiology of such pathologies are complex, a causative relationship between the age-related decline in oocyte quality and oxidative stress (OS) is now well established. What remains less certain are the molecular mechanisms governing the increased vulnerability of the aged oocyte to oxidative damage. In this review, we explore the reduced capacity of the ageing oocyte to mitigate macromolecular damage arising from oxidative insults and highlight the dramatic consequences for oocyte quality and female fertility. Indeed, while oocytes are typically endowed with a comprehensive suite of molecular mechanisms to moderate oxidative damage and thus ensure the fidelity of the germline, there is increasing recognition that the efficacy of such protective mechanisms undergoes an age-related decline. For instance, impaired reactive oxygen species metabolism, decreased DNA repair, reduced sensitivity of the spindle assembly checkpoint, and decreased capacity for protein repair and degradation collectively render the aged oocyte acutely vulnerable to OS and limits their capacity to recover from exposure to such insults. We also highlight the inadequacies of our current armoury of assisted reproductive technologies to combat age-related female infertility, emphasising the need for further research into mechanisms underpinning the functional deterioration of the ageing oocyte.

1. Introduction

The developmental potential of the mammalian oocyte markedly decreases with increasing maternal age, culminating in elevated rates of miscarriage, birth defects, and ultimately reduced fertility [1–4]. This loss of fecundity becomes evident when a woman reaches her midthirties. In particular, the incidence of chromosome abnormalities increases from approximately 2% for women in their twenties to 35% and 50% in their forties and fifties, respectively [3, 4]. Despite public misconceptions, current IVF technologies are unable to recover the fertility of older women with the live birthrate per oocyte steadily decreasing from 26% in younger women (<35) to just 1% for women at 42 [5]. The need to elucidate the mechanisms by which advanced maternal age negatively affects oocyte quality has become particularly pressing owing to the recent trend for women in developed countries to delay child bearing several years beyond that of their peak reproductive capacity. For example, in Australia, the average childbearing age increased from 27.7 years in 1987 to 30.7 years in 2008 [6]. In addition, the percentage of women having children later in life has also risen, with 8.5% of mothers being ≥35 in 1987 increasing to 24.4% in 2008 in Australia. Similar trends have also been observed in other developed countries including the UK, US, and Japan [6, 7].

More than two decades after being first proposed, the free radical theory of ageing remains a leading hypothesis to explain the deterioration of the ageing oocyte [8–11]. Indeed,

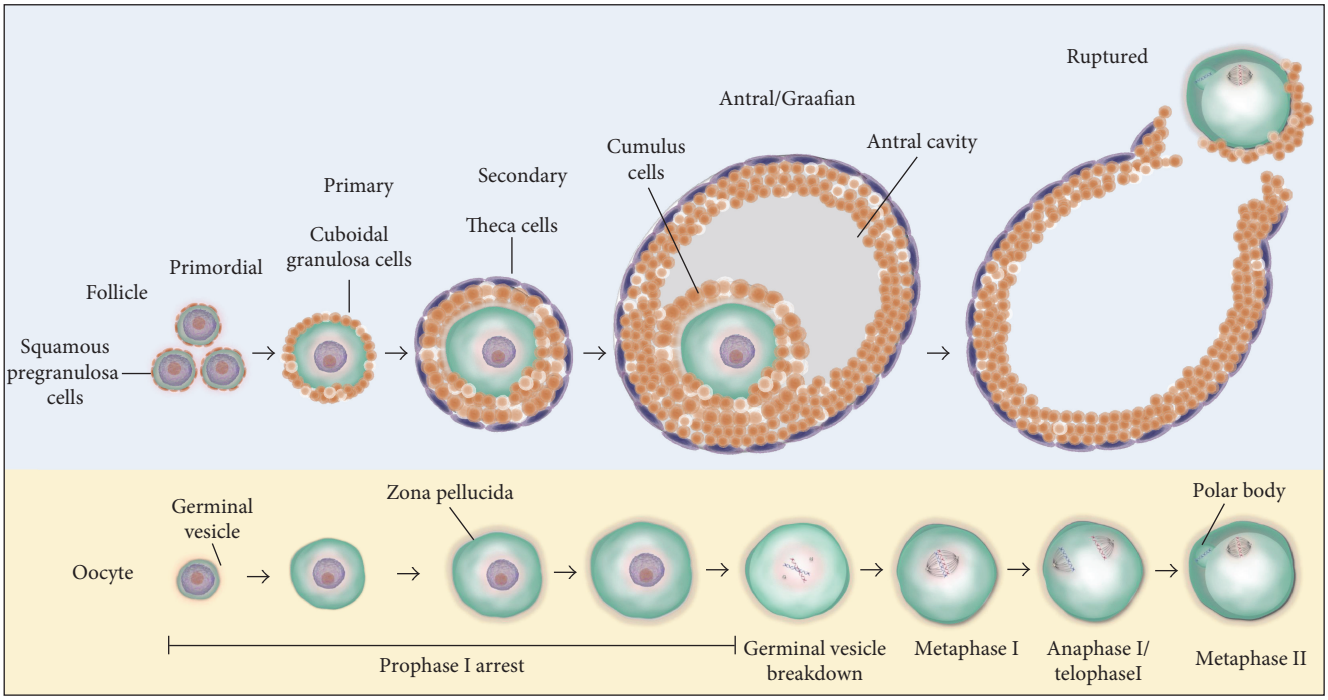


Figure 1: Stages of folliculogenesis and oocyte maturation. Primordial follicles consist of an immature GV oocyte arrested at prophase I, which is encapsulated by pregranulosa cells. Activation of primordial follicles to primary follicles is marked by a morphological change of pregranulosa cells from squamous to cuboidal. The development of the secondary follicle is marked by the acquisition of two or more layers of granulosa cells and the presence of a theca layer and contains an oocyte with a completely formed zona pellucida. The antral or Graafian follicle is the last stage of follicular development. This stage is marked by the presence of a follicular fluid-filled antral cavity adjacent to the oocyte. In the final stage of folliculogenesis, the oocyte achieves meiotic resumption, undergoing germinal vesical breakdown, and progresses through anaphase I and telophase I to complete meiosis I. At the completion of the first meiotic division, the first polar body is extruded and the ovulated oocyte becomes arrested once more at metaphase II until after fertilisation. Once the follicle is ruptured to release the mature oocyte, the remaining granulosa and theca cells differentiate into the corpus luteum.

an increase in intraovarian reactive oxygen species (ROS) has been convincingly correlated with increasing maternal age [12–15]. Moreover, several studies have drawn a compelling link between oxidative stress (OS) and the decline in oocyte quality [16–21] as well as *in vitro* fertilisation (IVF) and pregnancy success rates [15, 21–25]. The devastating consequences of OS on oocyte quality and female fertility have been comprehensively reviewed [9, 26–29]. Despite this, the molecular mechanisms that underpin the increased vulnerability of the aged oocyte to oxidative insults are still being elucidated. In this review, we provide a new perspective on reproductive ageing by exploring the underlying mechanisms behind the increased vulnerability of the ageing mammalian oocyte to OS. We consider the origin of elevated ROS in ageing oocytes but focus on the simultaneous decrease in the capacity of the oocyte to mitigate the detrimental impact of such oxidative insults. We also discuss the current means by which OS can be prevented or delayed in older mothers.

2. Overview of Oocyte and Follicular Development

The synergetic processes of folliculogenesis and oocyte maturation are required to produce oocytes capable of fertilisation. Primordial germ cells (PGCs) undergo mitotic proliferation to form a finite number of oogonia during prenatal life. In humans, folliculogenesis commences in utero in the second trimester with the recruitment of pregranulosa cells to the germ cells forming the primary functional unit of the ovary, the ovarian follicle [30] (Figure 1). These primordial follicles remain meiotically arrested in an extended prophase I, also known as germinal vesicle (GV) arrest, until they are recruited into the growing follicle pool for maturation and subsequent ovulation [31]. Upon activation, the primordial follicle experiences a period of follicle stimulating hormone- (FSH-) mediated follicular growth through primary, secondary, and antral follicle stages. This growth is accompanied by an accumulation of granulosa cell layers surrounding the oocyte, formation of the zona pellucida (ZP), and the differentiation of steroid-secreting theca cells at the basement membrane [30, 31]. Continual follicular growth sees the formation of the preovulatory follicle with the presence of an antral cavity containing plasma fluid and steroid hormones that are excreted by the granulosa cells, adjacent to the oocyte [32]. The oocytes populating preovulatory follicles remain in prophase I arrest yet have experienced significant growth, completed nuclear and cytoplasmic maturation, and are now surrounded by cumulus cells—a granulosa

cell subtype [31]. A subsequent surge of luteinising hormone (LH) is responsible for initiating meiotic resumption and ovulation. During ovulation, the basement membrane of the follicle ruptures releasing a mature oocyte equipped for fertilisation [32]. The remaining granulosa and theca cells differentiate into the corpus luteum, which produces progesterone for pregnancy maintenance, or alternatively undergoes luteolysis [33]. Meanwhile, in the preovulatory follicle, the oocyte undergoes germinal vesicle breakdown (GVBD) and entry into the first meiotic division (MI) [34], during which chromosomes attach to the meiotic spindle and line up on the metaphase plate. Chromosomes then separate, with one pair of sister chromatids retained within the oocyte and the other extruded in the first polar body [35]. Following the first meiotic division, the oocyte becomes arrested once more, at metaphase II of meiosis (MII), whereupon transcription is temporarily repressed until after fertilisation at the 2-cell stage in mice or 4-cell stage in humans [33].

3. Aetiology of Elevated Cytosolic ROS in Ageing Oocytes

Traditional paradigms hold that the primary mechanism behind the age-related decline in oocyte quality is the accumulation of spontaneous damage to the mitochondria arising from ROS produced by the mitochondria themselves during daily biological metabolism [36]. Furthermore, there are several additional sources of ROS, attributed to either exogenous (i.e., lifestyle, stress conditions, and environmental factors) [37, 38] and/or endogenous factors (i.e., inflammation, cell proliferation, and apoptosis) [39], that can impact the ovarian environment and contribute to cellular ageing. While each of these factors have featured in elegant reviews [28, 40, 41], it has also recently been shown that the ovarian specific surges in ROS that accompany ovulation and luteolysis can act as a potent source of intraovarian OS with the potential to contribute to age-related decline in oocyte quality [20, 42]. Moreover, additional age-associated sources of ovarian ROS have been suggested to arise from the accelerated production of advanced glycation end-products (AGEs) and an accompanying decrease in the efficiency of perifollicular vascularisation [43, 44].

3.1. Ovulation-Induced ROS. Following FSH-mediated follicular maturation, an LH surge stimulates LH receptors on granulosa and cumulus cells, resulting in the generation of ROS and the concomitant depletion of antioxidant defences. This response is essential for promotion of granulosa cell apoptosis and breakdown of the follicular wall to permit oocyte release during ovulation [45–48]. The ROS generated have also been linked to integral roles in meiotic resumption via activation of the maturation-promoting factor (MPF) [48, 49]. Additionally, progesterone-induced OS is required for the induction of luteal cell apoptosis; a prerequisite for luteolysis with *in vitro* exposure of corpus luteum to ROS scavengers and antioxidants leading to a potent inhibition of this process [45, 50]. The potential damage arising from chronic exposure of oocytes to OS generated during recurrent cycles of stimulated ovulation has been highlighted in mouse models. Indeed, the induction of as few as three to six concurrent stimulated ovulatory cycles has been shown to elicit increased mitochondrial aggregation and mitochondrial DNA (mtDNA) mutations in oocytes, as well as oxidative damage to nuclear DNA, lipids, and proteins—lesions that collectively result in degenerative embryos and failure to reach blastulation [42]. Comparable phenotypes have also been documented after the induction of five sequential stimulated ovulation cycles in mice. In this study, it was determined that oocytes were of poorer functional quality and possessed significantly more mitochondrial defects, resulting in an accentuation of the level of intracellular OS [20]. Notably, these defects were ameliorated upon administration of the antioxidant L-carnitine throughout the repetitive ovulation cycles, indicating that repeated exposure to elevated ROS generated via folliculogenesis can certainly stimulate OS and precipitate a decline in oocyte quality [20]. In addition, repeated ovarian stimulation has been linked to progressive increases in spindle abnormalities, detached chromosomes, and cytoplasmic asters. Interestingly, these data were only observed after *in vivo* maturation (IVO), indicative of a compromised intrafollicular milieu [51]. In contrast, after four weeks of stimulation, an alternative mouse study reported a decline in meiotic competence during *in vitro* maturation (IVM), but not IVO. The latter was however associated with decreased ATP content in GV and IVO MII oocytes. Despite this, the authors failed to record any observable impact on implantation or reabsorption rates upon mating [52].

The injurious effects of repeated ovarian stimulation have also been demonstrated in several human studies of ovarian hyperstimulation, with consequential decreases in the frequency of implantation and pregnancy rates having been reported [53–55]. However, this evidence must be considered against that of other studies, which have failed to document any significant decline in ovarian response to repeated stimulation, including reports of no difference in the number of oocytes retrieved, embryo morphology, fertilisation and implantation, or pregnancy rates [56–59]. Despite the conflicting evidence emerging from human studies, it remains possible that oocytes from women of advanced maternal age, who will likely have experienced monthly ovulatory cycles for anywhere between three to four decades as well as a concomitant decrease in antioxidant defences (detailed below), are placed at heightened vulnerability upon exposure to additional source(s) of ROS.

3.2. Advanced Glycation End Products and Altered Vascularisation. An age-related increase in the levels of advanced glycation end products (AGEs) in the ovarian microenvironment has been newly postulated to contribute to ovarian ageing through the induction of elevated ROS [43, 44]. AGEs act to induce the generation of intracellular ROS by binding and activating ligand transmembrane receptors, known as RAGE (receptor for advanced glycation end products). Indeed, upon binding, RAGE triggers the downstream activation of NAD(P)H oxidase, mitogen-activated protein kinases (MAPKs), and the transcription factor

nuclear factor kappa B (NF-κB) [60–62]. This leads to an increase in intracellular ROS as well as upregulated expression of growth factors, cell-adhesion molecules, and proinflammatory cytokines and RAGE [63–67]. This positive feedback cycle ultimately culminates in a proinflammatory response and an increase in OS [64, 65, 68].

In support of the contribution of AGEs in ovarian ageing, Takeo et al. have recently reported significantly higher levels of AGEs in follicular fluid derived from aged cows when compared to their younger counterparts [14]. Furthermore, elevated levels of AGEs were associated with increased ROS as well as additional age-associated events including accelerated nuclear maturation, abnormal fertilisation, and decreased blastulation rates [14]. A corroborative study also reported an age-associated accumulation of AGEs and RAGE in human ovarian granulosa-lutein and monocytes [69]. Moreover, Tatone et al. documented reduced expression and activity of detoxifying methylglyoxal (an AGE precursor) in the ovaries of aged mice compared to those of young mice [70]. Taken together, these studies implicate AGEs as a contributor to the accelerating ROS generation observed in the ageing ovary.

Notably, AGEs have also been implicated in propagating age-related cellular damage in a more direct manner, independent of ROS induction, by inducing protein crosslinking [71]. Proteins with long half-lives such as collagen are the most vulnerable to this type of modification, leading to collateral damage in the form of altered vascular structure and function [72, 73]. Accordingly, it been suggested that the weakened efficiency of perifollicular vascularisation can be attributed to collagen damage induced by AGEs [43, 44]. Normal perifollicular vascularisation is essential to meet the demand for oxygen supply to the oocyte. This is particularly the case in advanced phases of follicular development where the oocyte relies on an ingrowth of capillaries into the surrounding theca cells [74]. It follows that impaired vascularisation resulting from inadequate capillary ingrowth can lead to a state of hypoxia, which in itself can trigger the generation of ROS and contribute to the pathology of age-related ovarian dysfunction [75]. The implications of inefficient vascularisation have been clinically demonstrated as oocytes derived from such follicles have reduced oxygen content (<3%) and lower fertilisation and developmental potential [76]. Accordingly, a negative correlation has been established between a woman's age and the degree of vascularisation observed in her late-stage ovarian follicles [77]. The converse is also true, whereby a positive correlation exists between highly vascularised oocytes and live birth rates resulting from IVF procedures [78, 79]. Further highlighting the potential significance of insufficient vascularisation in the aged ovary is the demonstration that oocytes retrieved from Graafian follicles with reduced vascularisation commonly present with spindle and chromosomal alterations similar to those witnessed in aged MII oocytes [80, 81].

3.3. Mitochondrial Defects. Several studies have converged on the notion that dysfunctional mitochondria represent the main source of elevated ROS within the ageing oocyte [36, 82]. Mitochondria hold central roles in calcium homeostasis, initiation of apoptosis, and cellular energetic metabolism within oocytes [83]. A key element of this metabolic strategy is the electron transport chain (ETC) that resides in the mitochondria and is responsible for the bulk of ATP generation within the oocyte; energy that is essential for the successful completion of meiosis [84]. Indeed, during spindle assembly in the MI and MII phases of oocyte maturation, mitochondria relocalise in dense clusters around the spindles in order to meet the enhanced demand for ATP [85]. This redistribution of mitochondria results in a burst of ATP production during oocyte maturation [86], the importance of which is highlighted by the fact that mitochondrial damage potently comprises GVBD, formation of the meiotic spindles, chromosome segregation, and polar body extrusion [87].

Mitochondria possess their own maternally transmitted multicopy genome (mtDNA) that acts independently of nuclear DNA. Oxidative damage to mtDNA is of particular importance as, unlike genomic DNA, these organelles lack protective histones and encompass limited mtDNA repair enzymes [88]. This underpins the hypothesis for why mtDNA have a 10- to 25-fold increased mutation and deletion rate relative to their nuclear DNA counterparts [89–91]. Accordingly, unfertilised oocytes retrieved from older women present with a higher incidence of chromosomal deletions and mtDNA point mutations [92, 93]. Such an increase in mtDNA mutations poses a significant threat to the health of the oocyte as it could lead to impairment of several mitochondrial encoded components of the ETC.

Maternal ageing has also been linked to mitochondrial dysfunction resulting in decreased oxidative phosphorylation and ATP generation [94, 95]. Indeed, global transcript analyses of human and mouse oocytes have revealed that a large proportion of age-related changes in transcript expression are associated with mitochondrial activity, including the downregulation of succinate dehydrogenase complex flavoprotein subunit A (*Sdha*) and genes coding for proteins associated with ATP production [3, 96, 97].

Additionally, Ben-Meir et al. demonstrated that generation of the coenzyme Q10 (CoQ_{10}), which fulfils an essential role in the transport of electrons within the ETC, is compromised in the oocytes of ageing humans and mice. Furthermore, disruption of CoQ_{10} production elicits phenotypic changes that mimic the ageing effect in ovaries, with reduced ovarian reserve, decreased ATP production, and increased spindle abnormities resulting in infertility. Interestingly, oral administration of CoQ_{10} can reportedly reduce the level of the age-related decline in oocyte quality and quantity [98].

Similarly, Wilding et al. also reported that altered mitochondrial morphology and lower electron potential at the inner mitochondrial membrane in aged human oocytes was negatively correlated with the rate of embryo development [99]. Moreover, a decrease in mtDNA copy number has been recorded in bovine, mouse, hamster, and human oocytes with increasing maternal age and has been associated with a concomitant decrease in ATP production

Table 1: Alterations in gene expression of pathways involved in mitigating oxidative damage in the aged oocyte.

Category	Genes	Cellular compartment	References
Antioxidants	↓*Sod1*, ↓*Sod2*, and ↓*Cat*	Human granulosa cells	[112]
	↑*Gpx1*, ↓*Gstm2*, ↓*Prdx3*, and ↓*Txn2*	Mouse ovaries	[13]
	↓*Sod1*, ↓*Txn1*, ↓Txndc9, and ↓*Apacd*	Mouse MII oocytes	[97]
	↓*Apacd*, ↓*Glrx*, ↓*N33*, ↓*Pdcl*, ↓*Grp58*, and ↓*Pdia6*	Human MII oocytes	[96]
	↓*Txnrd1 and* ↓ *Sod1*	Mouse GV oocytes	[3]
	↓*Txnrd1*, ↓*Txnrd3*, and ↑*Sod2*	Mouse MII oocytes	[3]
Sirtuin proteins	↑*Sirt1*	Mouse GV oocyte	[127]
	↓*Sirt3*	Human granulosa and cumulus cells	[133]
	↓*Sirt2* and ↓*Sirt6*	Mouse cumulus cells	[136]
DNA repair/checkpoint	↓*Brca1*, ↓*Mre11*, ↓*ATM*, and ↓*Rad51*	Mice and human primordial follicles and GV oocytes	[142]
	↓*Brca1*	Mouse MII oocytes	[3]
	↓*Atr*, ↓ *Chek1*, ↓*Nbs1*, and ↓*Rad17*	Human MII oocytes	[96]
	↓*Tert*	Mouse MII oocytes	[148]
Ubiquitin proteasome system	↓*Psmb2*, ↓*Psmb5*, ↓*Psmc2*, ↓*Psmd4*, ↓*Psmd8*, ↓*Ubap1*, ↑*Ube2h*, ↑*Usp15*, ↓*Usp2*, ↓*Usp31*, ↑*Usp7*, and ↑*Usp8*	Mouse GV oocytes	[3]
	↑*Psma4*, ↑*Psmb4*, ↑*Psmc2*, ↓*Psmc5*, ↓*Psmd9*, ↑*Psmd11*, ↓*Psme3*, ↓*Psmf1*, ↑*Ubap1*, ↓*Ube1c*, ↑*Ubc*, ↑*Ube2a*, ↓*Ube2d1*, ↓*Ube2d2*, ↓*Ube2h*, ↓*Ube3a*, ↑*Usp15*, and ↑*Usp8*	Mouse MII oocytes	[3]
	↓*Anapc4*, ↓*Hip2*, ↓*Ubc*, ↓*Ube1c*, ↓*Ube2a*, ↓*Ube2e3*, ↓*Ube2g1*, ↓*Usp1*, ↓*Usp30*, ↓*Psma6*, ↓*Psmb1*, ↓*Psmb4*, ↓*Psmc2*, ↓*Psmc3*, ↓*Psmd12*, and ↓*Siah2*	Mouse MII oocytes	[97]
	↓*Hip2*, ↓*Psmc2*, ↓*Psmc6*, ↑*Psmd2*, ↑*Psmd5*, ↑*Psmd1*, ↑*Psmd9*, ↑*Ubch9*, ↓*Ube2n*, ↓*Ube2e*, ↓*Ube2g1*, ↑*Ube2h*, ↑*Ube2v*, ↑*Ube3a*, ↓*Usp1*, ↓*Usp8*, and ↓*Usp9x*	Human MII oocytes	[96]
Chaperones	↓*Cct2*, ↓*Cct3*, ↓*Cct5*, ↓*Tra1*, ↓*Dnaj1*, ↓*Hsp86-1*, ↓*Hspa4*, ↓*Hsp70-4*, ↓*Hspa8*, ↓*Vbp1*, ↓*Mmp2*, ↓*Skt25*, ↓*Map4k5*, and ↓*Ndr4*	Mouse MII oocytes	[97]
	↓*Cct2*, ↓*Dnajb1*, ↓*Dnajb6*, ↓*Dnajc5*, ↓*Hspa14*, ↓*Hspa1b*, ↓*Hspa8*, *and* ↓*Hspcb*	Mouse GV oocytes	[3]
	↑*Cct2*, ↓*Cct7*, ↑*Dnaja1*, ↓*Dnaja2*, ↓*Dnaja4*, ↓*Dnajb10*, ↓*Dnajb11*, ↓*Dnajc3*, ↓*Dnajc8*, ↓*Hspa1b*, ↓*Hspa1b*, ↓*Hspa9a*, ↑*Hspb1*, ↓*Hspcb*, *and* ↓*Mmp2*	Mouse MII oocytes	[3]

[93, 94, 100, 101]. In addition, oocyte mitochondria in aged mice and hamsters also exhibit altered morphology with vacuolisation, cristae alterations, and changes in cytoplasmic lamellae [94].

Among the most insidious consequences of mitochondrial dysfunction is an increase in ROS leakage from the ETC. Such elevated concentrations of ROS have the potential to set in train a cascade of events that culminate in a state of auto-oxidation whereby the mitochondria are unable to regulate the events of the ETC, thus exacerbating damage to mtDNA and proteins. During mitochondrial ATP production, ROS are released locally as a by-product, becoming a major source of intracellular ROS. Under normal physiological conditions, the mitochondria only reduce 0.1% of all oxygen entering the ETC into the prooxidant $O_2^{\cdot-}$, which serves as a precursor to the majority of biological ROS [18, 102]. However, damage to the ETC has the potential to elevate $O_2^{\cdot-}$ generation, leading to increased cellular oxidation. Studies from our research group have also established that in MII mouse oocytes, OS-catalyzed lipid peroxidation is capable of initiating cyclic ROS propagation via direct damage to mitochondrial components [103]. Specifically, we have shown that covalent modification of SDHA by the lipid aldehyde 4-hydroxynonenal (4-HNE) results in the auto-oxidation and subsequent transference of electrons to oxygen rather than reduction via CoQ_{10}. This, in turn, leads to a decrease in mitochondrial membrane potential, an increase in OS, and the propagation of the lipid peroxidation cycle. As a consequence, oocytes experience DNA fragmentation and increased apoptosis [103]. Independent research has also established that exposure of mouse oocytes to exogenous H_2O_2 leads to the dissipation of mitochondrial membrane potential and a concomitant decrease in cytoplasmic ATP levels and the disassembly of MII spindles [104]. However, supplementation of the antioxidant *N*-acetylcysteine is able to ameliorate such damage [104].

Table 2: Alterations in protein expression and activity of pathways involved in mitigating oxidative damage in the aged oocyte.

Category	Proteins/hormones	Cellular compartment	References
Antioxidants	↓SOD1, ↓SOD2, and ↓CAT	Human granulosa cells	[112]
	↓SOD1 and ↓SOD (activity)	Human cumulus cells	[113]
	↓GST (activity and expression), ↓CAT (activity), and ↑SOD (activity)	Human follicular fluid	[114]
	↓GST (activity), ↓GR (activity), and ↓GPX (activity)	Human follicular fluid	[24]
	↓SOD and ↓GPX	Human ovaries	[115]
	↓TXN1 and ↓GSTM5	Mouse MII oocytes	[116]
	↓GST (activity)	Mouse MII oocytes	[117]
	↓Melatonin	Human follicular fluid	[121]
Sirtuin proteins	↓SIRT1	Mouse GV oocyte	[127]
	↓SIRT2	Mouse MII oocyte	[137]
	↓SIRT3	Human granulosa and cumulus	[133]
DNA repair/ checkpoint	↓Telomerase (activity)	Human ovary	[147]
	↓Telomerase (activity) and ↓TERT	Mouse MII oocytes	[148]
UPS	↓PSMD12 and ↓USP15	Mouse MII oocytes	[116]
Chaperones	↑Dnajc19 and ↑Dnajc11	Mouse MII oocytes	[116]

4. Increased Vulnerability of the Ageing Oocyte to Oxidative Damage

4.1. Reduced Antioxidant Capacity and ROS Metabolism. Mammalian cells are endowed with a wide array of antioxidants with the ability to scavenge ROS, including nonenzymatic antioxidants such as vitamins A, C, and E and glutathione (GSH) as well as several enzymatic antioxidants including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST), peroxiredoxin (PRDX), and thioredoxin (TXN) [105–110]. Enhanced levels of cellular OS with ageing have been, at least partially, attributed to the weakening of the antioxidant enzymatic defences present within the cell [111]. In combination with age-associated increases in prooxidants, it is likely that this scenario may enable free radicals to evade cellular defences and subsequently cause damage to a suite of macromolecules that are required to maintain oocyte viability. While reports of the impact of ageing on the expression and activity of various antioxidants are plagued by considerable variability, there is a general consensus amongst these studies that dysregulation of ROS metabolism is a feature of the ageing ovary and oocyte (Tables 1 and 2).

Antioxidant enzymes within granulosa cells, cumulus cells, and follicular fluid each play a critical role in the protection of the oocyte, owing to their ability to facilitate the scavenging of ROS, particularly during steroid hormone synthesis. Of concern, however, is that age-dependent dysregulation of antioxidant enzyme activity and/or expression has been reported in every one of these cellular compartments. For instance, a study of patients undergoing IVF reported lower gene and protein expression of SOD1, SOD2, and CAT in cultured granulosa cells from 38- to 42-year-old IVF patients compared to equivalent cells obtained from a younger patient cohort aged between 27 and 32 years [112] (Tables 1 and 2). Total SOD activity, as well as SOD1 protein expression, has also been shown to decrease within the cumulus cells surrounding ovulated oocytes from IVF patients of advanced maternal age. Moreover, such changes have been correlated with unsuccessful IVF outcomes [113]. In human follicular fluid, Carbone et al. observed a decrease in the levels of GST and CAT activities, higher SOD activity, and a decrease in GST protein expression in IVF patients aged between 39 and 45 years compared to younger patients aged between 27 and 32 years [114]. Decreased activity of GST, glutathione reductase (GR), and GPX is also characteristic of the follicular fluid recovered from poor IVF responders and has, in turn, been positivity correlated with elevated nitric oxide and the lipid peroxidation products of malondialdehyde (MDA) and 4-HNE [24].

Differing ovarian gene expression levels of critical antioxidant enzymes have also been documented in aged versus young mice and attributed to notable increases in lipid, protein, and DNA oxidation. This ageing phenomenon is true for both ovarian interstitial cells and the follicles themselves [13]. For instance, the expression of the cytosolic antioxidant *Gpx1* has been documented to increase in aged mouse ovaries, while the expression of glutathione *S*-transferase mu 2 (*Gstm2*) apparently decreases in this tissue. Additionally, the mitochondrial antioxidants *Prdx3* and *Txn2* also experience decreased expression in such models [13]. Similarly, significant attenuation of the activity of both SOD and GPX has been recorded in ovarian homogenates from premenopausal to menopausal women [115].

Global gene expression analysis of aged oocytes also revealed reduced expression of *Sod1* and *Txn* family members in ovulated mouse and human oocytes [3, 96, 97]. At the protein level, only modest decreases have been detected in the expression of GSTM5 and TXN1 in MII oocytes recovered from mature versus aged mice [116]. Additionally, the activities of GST and thiols have been reported to decrease

in aged MII mouse oocytes [117]. In alternative model species such as the pig, chemical inhibition of SOD activity in oocytes has been shown to elicit a reduction in meiotic progression, decreased GSH levels, and diminished rates of cleavage and blastocyst formation [118]. Such defects have also been documented in the hamster and bovine, where the depletion of GSH has been associated with altered spindle morphology, disturbed microtubule function, and chromosome clumping in MII oocytes [119, 120].

More recently, the hormone melatonin, which has strong antioxidant capacity, has also been shown to be downregulated upon ageing. Indeed, reduced levels of melatonin have been recorded in the follicular fluid of women of advanced maternal age [121]. The authors of these studies also reported a positive correlation between melatonin levels and IVF outcomes, with higher ovarian reserves, numbers of collected oocytes, fertilised oocytes, cleaved zygotes, high-quality embryos, blastocysts, and embryos suitable for transplantation all being documented in parallel with elevated melatonin. Accordingly, an inverse correlation has been established between lower levels of melatonin and higher levels of the lipid peroxidation product MDA in the serum obtained from infertile female patients [122].

4.2. Downregulation of Sirtuin Proteins. The sirtuin (SIRT) family of proteins have been newly established as having strong antioxidant capacity, an important biochemical property in the context of the protection they afford to oocytes from oxidative insults [123]. SIRT proteins possess either NAD^+-dependent deacetylases or mono-ADP-ribosyltransferase activity and have been shown to modulate ageing and cell metabolism, primarily by guarding cells against the damaging impact of oxidative insults [123–125]. SIRT proteins have proven to be essential in oocytes, with inhibition of total SIRT activity resulting in the disruption of meiotic maturation, the formation of the actin cap and the cortical granule-free domain, and induced spindle defects and chromosome misalignments [126].

A decrease in SIRT1 protein expression and an increase in *Sirt1* gene expression have been observed in GV oocytes of aged mice [127]. Di Emidio et al. also detected a relocalisation of SIRT1 and the upregulation of *Sirt1* expression in response to the induction of OS in mouse GV oocytes. These changes occurred commensurate with a decrease in *miR-132*, a microRNA implicated in the posttranscriptional regulation of the *Sirt1* transcript. Interestingly, aged oocytes were found to be characterised by a lower basal expression of *miR-132* and, upon exposure to oxidative insults, did not experience a further reduction in *miR-132* or concomitant increase in *Sirt1*, equivalent to the response recorded in the oocytes of younger females. Together, these data suggest that this stress response mechanism is compromised during maternal ageing. The authors of this study also reported a dose-dependent increase in intracellular ROS and a decrease in the number of oocytes reaching the MII phase of development, upon inhibition of SIRT1 activity [127]. SIRT1 inhibition also prevented the upregulation of the antioxidant *Sod2*, in response to OS, indicating that the protein likely acts upstream to mediate *Sod2* gene expression. In additional studies, an age-dependent decrease in SIRT1 protein expression has been linked to chromatin disorganisation in the GV oocyte, a defect possibly arising from an inability to modulate the activity of histone methyl-transferase and subsequent trimethylation of histones [128, 129].

Remarkably, transgenic mice engineered to overexpress SIRT1 presented with a pronounced delay in reproductive ageing accompanied by a decreased time to conception compared to that of wild-type control mice [130]. Similarly, resveratrol-mediated upregulation of SIRT1 in IVM bovine oocytes has resulted in an improved fertilisation rate and blastocyst numbers and increased mtDNA copy number, membrane potential, and ATP content in the mature oocyte [131]. Furthermore, a rapamycin-mediated elevation of SIRT1 and SIRT6 expression in rat ovaries has also been associated with preservation of the primordial follicle pool [132]. Overall, these studies suggest that a SIRT1-mediated decrease in ROS may contribute to the preservation of fertility under conditions of ageing and oxidative stress.

In addition to SIRT1, the SIRT3 isoform also has distinct roles in the deacetylation and activation of diverse mitochondrial enzymes involved in antioxidant protection (e.g., glutamate dehydrogenase 1), the metabolism of amino acids and fatty acids, and in the electron transport chain [133, 134]. Indeed, an age-dependent decrease in SIRT3 gene and protein expression in human granulosa and cumulus cells of IVF patients has been associated with attenuation of GSH deacetylation, mitochondrial activation, and altered ROS metabolism, thus contributing to a depleted ovarian reserve [133]. Moreover, pan SIRT inhibition, siRNA-induced knockdown of *Sirt3* in fertilised eggs, and the targeted ablation of *Sirt3* in global knockout mouse models have each been shown to increase mitochondrial ROS production and repress blastulation and postimplantation development in mouse embryos generated via IVF [135]. Interestingly, the same study also reported an upregulation of SIRT3 protein and *Sirt3* mRNA expression in response to OS. Moreover, under conditions of low oxygen, 2-cell embryo and blastocyst formation were unaffected by siRNA knock down, with ROS levels also remaining low [135]. These findings highlight the importance of SIRT3 activity under conditions that lead to an induction of OS.

In terms of alternative isoforms of the sirtuin family, the expression of both *Sirt2* and *Sirt6* has also been shown to decrease in the cumulus cells of aged mice, suggesting they too may contribute to the hierarchy of mechanisms that protect the quality of oocytes in young animals [136]. Accordingly, SIRT2 depletion in mouse oocytes has been linked to spindle defects, chromosome disorganisation, and impaired microtubule-kinetochore interactions [137]. A similar spectrum of lesions (i.e., spindle defects, chromosome misalignment, impaired kinetochore-microtubule interactions, and aneuploidy during meiosis) has also been reported in the oocytes of mice targeted for *Sirt6* knockdown [138]. Conversely, a range of age-related oocyte pathologies can be mitigated via the overexpression of SIRT2 in these cells [137]. Taken together, these data highlight the important role that several members of the SIRT family of proteins hold in protecting the oocyte from oxidative stress and regulating

oocyte meiotic events, and the detrimental pathologies that develop if these proteins are downregulated during the ageing process.

4.3. Compromised Oxidative DNA Damage Repair Pathways. An additional characteristic of the aged oocyte that renders them particularly vulnerable to oxidative attack is the downregulation of genes involved in DNA damage repair. DNA damage can result in alterations in gene expression mediated through epigenetic modifications and mutagenesis [139–141]. Oocytes are acutely susceptible to accumulating DNA damage due to their extended prophase arrest. Indeed, an increase in DNA double stand breaks (DSBs) has been detected in aged mouse and human primordial follicles as well as in GV oocytes [142–144]. Alternatively, the interstitial tissue of ageing mouse ovaries has also been shown to accumulate a higher proportion of oxidative DNA damage, detected via measurement of the DNA oxidation marker 8-hydroxy-2′-deoxyguanosine (8-OHdG), than that of an equivalent tissue in young animals [13]. This is particularly concerning as elevated intrafollicular levels of 8-OHdG lesions positively correlate with high rates of degenerative oocytes in women [21]. The causative nature of these phenotypes is suggested by evidence that antioxidant treatments aimed at ameliorating 8-OHdG concentrations can enhance a woman's chances of conceiving and maintaining pregnancy, as has been documented amongst a cohort of women that had previously experienced failed pregnancies arising from IVF embryo transfers [21]. In rat models, it has been shown that GV oocytes subjected to exogenous H_2O_2 experience an increase in DNA fragmentation and eventually succumb to apoptosis [17]. Studies of this nature serve to illustrate the pervasive nature OS and the capacity of this insult to elicit DNA damage and compromise the fidelity of the ageing oocyte.

Such findings take on added significance in view of the fact that the efficacy of DNA DSB repair mechanisms become attenuated in aged oocytes. Indeed, an increase in the expression of the DNA DSB damage maker γH2AX in the primordial follicles and GV oocytes from aged mice and humans correlates with a decline in the expression of several DNA DSB repair genes including, *Brca1*, *Mre11*, *ATM*, and *Rad51* [142]. The decrease in *Brca1* expression in the MII oocytes of aged mice was also observed by Pan et al. with the RNAi-mediated reduction of *Brca1* resulting in abnormal spindle formation, chromosome misalignment, and a significant increase in hyperploid oocytes [3]. Interestingly, microarray analysis of human MII oocytes recovered from aged versus young donors has also revealed that the former are characterised by an apparent decrease in several genes associated with the DNA damage checkpoint, including *Atr*, *Chek1*, *Nbs1*, and *Rad17* [96]. The importance of oxidative DNA damage repair during ageing has been further highlighted in senescence-accelerated mice (SAM) models. Using this mouse strain, it has been demonstrated that increased oxidative damage brought about by mutations in mtDNA and the oxidative DNA repair enzyme OGG1 leads to accelerated ageing phenotypes including spindle and chromosomal abnormalities [145, 146]. While the consequential reduction in DNA repair capacity argues that oxidative insults could elicit a higher level of DNA damage in an aged oocyte, this has yet to be experimentally confirmed.

Aside from DNA damage, telomere shortening has also been documented as a consequence of both ageing and OS in the ovarian environment [147, 148]. Telomeres are comprised of repetitive DNA nucleotide sequences and associated proteins localised to the end of eukaryotic chromosomes. These entities serve a predominantly protective function to maintain chromosomal integrity and prevent end-to-end chromosome binding [149]. During ageing, telomere length gradually shortens due to repeated cycles of DNA replication and the adverse effects of a variety of genotoxic agents including OS [150]. Although oocytes and their surrounding granulosa cells are among a limited number of normal adult cell populations endowed with telomerase enzymes to counteract telomere shortening and ensure genetic stability, the telomerase activity of these ovarian cells is reduced in women of advanced maternal age [147, 151]. Similarly, in aged mouse models, oocytes experience a reduction in gene and protein expression of telomerase reverse transcriptase (TERT), a catalytic subunit of telomerase, as well as a consequential attenuation of telomerase activity [148]. Telomeres are a primary target of DNA damage in ageing human somatic cells, and severely shortened or uncapped telomeres result in genetic instability and cellular senescence [152]. By analogy, telomere shortening in oocytes during reproductive ageing may also predict developmental competence.

4.4. Reduced Fidelity of the Spindle Assembly Checkpoint. Stringent molecular mechanisms exist to prevent oocytes with significant DNA damage from progressing through meiosis and/or embryo development. Damage to genomic content of primordial and primary follicles ultimately leads to the induction of an apoptotic cascade via activation of the transcription factor transformation-related protein 63 (TAP63). However, oocytes that have proceeded to secondary and more advanced stages of development fail to express TAP63 and are therefore reliant on the meiotic spindle assembly checkpoint (SAC) to halt the development of any cells compromised by DNA damage and/or chromosomal abnormalities [153, 154]. In oocytes harbouring DNA damage induced by chemical agents and UV radiation, SAC activity increases resulting in MI arrest [155–157].

Despite this checkpoint, in ageing mothers, an increase in DNA damage and abnormal MII oocytes and embryos is more likely to occur than in their younger counterparts. This is consistent with evidence that the fidelity of SAC is compromised in aged oocytes, leading to a situation in which aged oocytes harbouring DNA damage are able to more readily evade MI arrest [3, 155]. This suggests that SAC failure is a likely contributor to the increased incidence of chromosome abnormalities documented in oocytes and embryos from older women. Concomitantly, there is mounting evidence from somatic cells that even modest concentrations of H_2O_2 can compromise the stringency of the SAC [158]. By analogy, it is tempting to speculate that oxidative induced DNA damage, which escapes the SAC in aged mothers, could increase the incidence of chromosomal defects in oocytes and embryos.

4.5. Downregulation of Reversible Protein Oxidation Repair. Oocytes are also equipped with stringent repair and proteolytic pathways to mitigate the impact of oxidatively damaged proteins. Upon oxidation, targeted proteins are subject to repair or, in the event that the damage is too great to mount complete repair, they are selectively degraded [159, 160]. Unfortunately, only limited oxidative-based modifications of the sulfur-containing amino acids, cysteine, and methionine possess the capacity for reversibility via reduction back to their native form [161, 162]. For instance, oxidation of the sulfhydryl groups in cysteine can be reduced by the thioredoxin-thioredoxin reductase system and glutaredoxin-glutathione-glutathione reductase system. Glutaredoxin and thioredoxin reduce disulfide bonds of cysteine by acting as electron doners and are subsequently reduced by glutathione-glutathione reductase and thioredoxin reductase, respectively, in an NADPH-dependent manner. An alternative class of enzymes, the methionine sulfoxide reductases (MSRS), are responsible for the conversion of methionine sulfoxides back to methionine, with the resulting sulfenic acid intermediate being reduced by the thioredoxin-thioredoxin reductase system [162, 163].

The importance of a fully functioning thioredoxin-thioredoxin reductase system is further emphasised by the fact that homozygous KO of either *Txn1* or *Txn2* is embryonically lethal, with cells derived from the inner cell mass of *Txn1* KO animals unable to proliferate [164, 165]. It is therefore of concern that the gene expression of the thioredoxin family has been shown to decease in the ovaries and oocytes of aged mice. Indeed, members of the thioredoxin family such as *Txn1* and thioredoxin domain-containing protein 9 (*Txndc9)* are each characterised by decreased expression in the MII oocytes of 42- to 45-week-old mice compared to that of younger, 5- to 6-week-old animals [97]. Similar reduced expression profiles have also been reported for thioredoxin reductase 1 (*Txnrd1*) and *Txnrd3* in the GV and MII oocytes of 66-week-old mice versus that of 6-week-old animals [3]. This trend also applies to *Txn2*, which experiences a notable decrease in ovarian expression between 2- and 12-month-old mice [13]. Consequently, a marked decrease in reduced sulfhydryl groups, indicative of an elevated level of protein oxidation, has been observed in follicular fluid recovered from aged women [43]. Taken together, these data indicate that the capacity to repair reversibly oxidised proteins may be compromised in the aged oocyte owing to the downregulation of the reductase systems tasked with this role, which could manifest in catastrophic consequences for the oocyte.

4.6. Compromised Degradation of Irreversible Protein Oxidation. In contrast to the situation described in the previous section, the majority of nonenzymatic oxidative protein modifications are actually irreversible, a situation that prevents their repair and instead targets the damaged proteins for proteolysis [166, 167]. In terms of the nature of these modifying agents, it is well-established that they include a variety of highly reactive aldehyde species that are formed as secondary products of the lipid peroxidation cycle, including 4-HNE (Figure 2) [168–172]. Indeed, an elevation in the levels of 4-HNE has been demonstrated in whole ovary and the interstitial tissue of aged mice ovaries [13, 173]. Similarly, in recent work conducted in our laboratory, this oxidative lesion also appeared to be concentrated in the vicinity of the oocyte [174]. Accordingly, we identified a significant increase in the level of 4-HNE-modified proteins in GV and MII stage mouse oocytes between 4–6 weeks and 14 months of age [174]. Moreover, direct exposure of oocytes from these young mice to exogenous 4-HNE resulted in a range of defects that mirrored those witnessed in aged oocytes. Such changes included decreased meiotic completion, increased spindle abnormities, chromosome misalignments, and elevated aneuploidy rates. Our study also identified α-, β-, and γ-tubulin as major targets for 4-HNE modifications in both aged oocytes as well as young oocytes exposed to exogenous oxidative insult in the form of either H_2O_2 or 4-HNE. On the basis of these data, we infer that the susceptibility of key meiotic proteins, such as tubulins, to 4-HNE modification contributes to the increased aneuploidy rates recorded in aged oocytes.

In addition to tubulin, several other functionally important proteins have also been shown to be vulnerable to oxidative adduction by reactive aldehyde species. In the case of mouse oocytes, previous work from our laboratory has identified SDHA, a component of complex II of the mitochondrial electron transport chain, as a proven target for adduction by 4-HNE, and possibly MDA and acrolein, thus further implicating these reactive aldehydes in the deteriorating quality of the postovulatory aged eggs [103] (Figure 2). Interestingly, both alpha-tubulin and SDHA appear to be conserved targets for 4-HNE adduction in alternative cell types such as the male gamete [175, 176]. However, additional targets in these cells are known to include the molecular chaperone, HSPA2, which facilitates ZP binding, as well as dynein heavy chain, cAMP-dependent protein kinase, alpha-catalytic subunit (PRKACA), and tubulin polymerisation-promoting protein family member 2 (TPPP2), each of which are each important for sperm functionally [175–177]. The detrimental impact of each of these phenomena on cellular proteostasis highlights the impetus for rapid clearance of the recipients of oxidative damage.

The degradation of irreversibly damaged protein is most often mediated by the ubiquitin-proteasome system (UPS) [178–180]. In the UPS, proteins are covalently modified with multiubiquitin chains for targeted degradation by the 26S proteasome holoenzyme. This enzyme comprises a 19S regulatory subunit and a 20S catalytic core, each of which are composed of multiple subunits. In its regulatory capacity, the 19S subunit is responsible for controlling the entrance of ubiquitin-tagged proteins into the 20S core as well as the removal of the ubiquitinated chains thereby permitting target protein degradation by the 20S core. Despite this gate-keeper role, there is accumulating evidence to suggest that the predominant pathway utilised for the removal of oxidatively damaged proteins is via the 20S proteasome alone, independent of the 19S subunit, ATP, and/or ubiquitin [179, 180] (Figure 3).

In cells functioning under normal physiological parameters, the activity of the proteasome is upregulated in response to OS in order to resolve concomitant increases in dysfunctional and/or misfolded proteins [181–184].

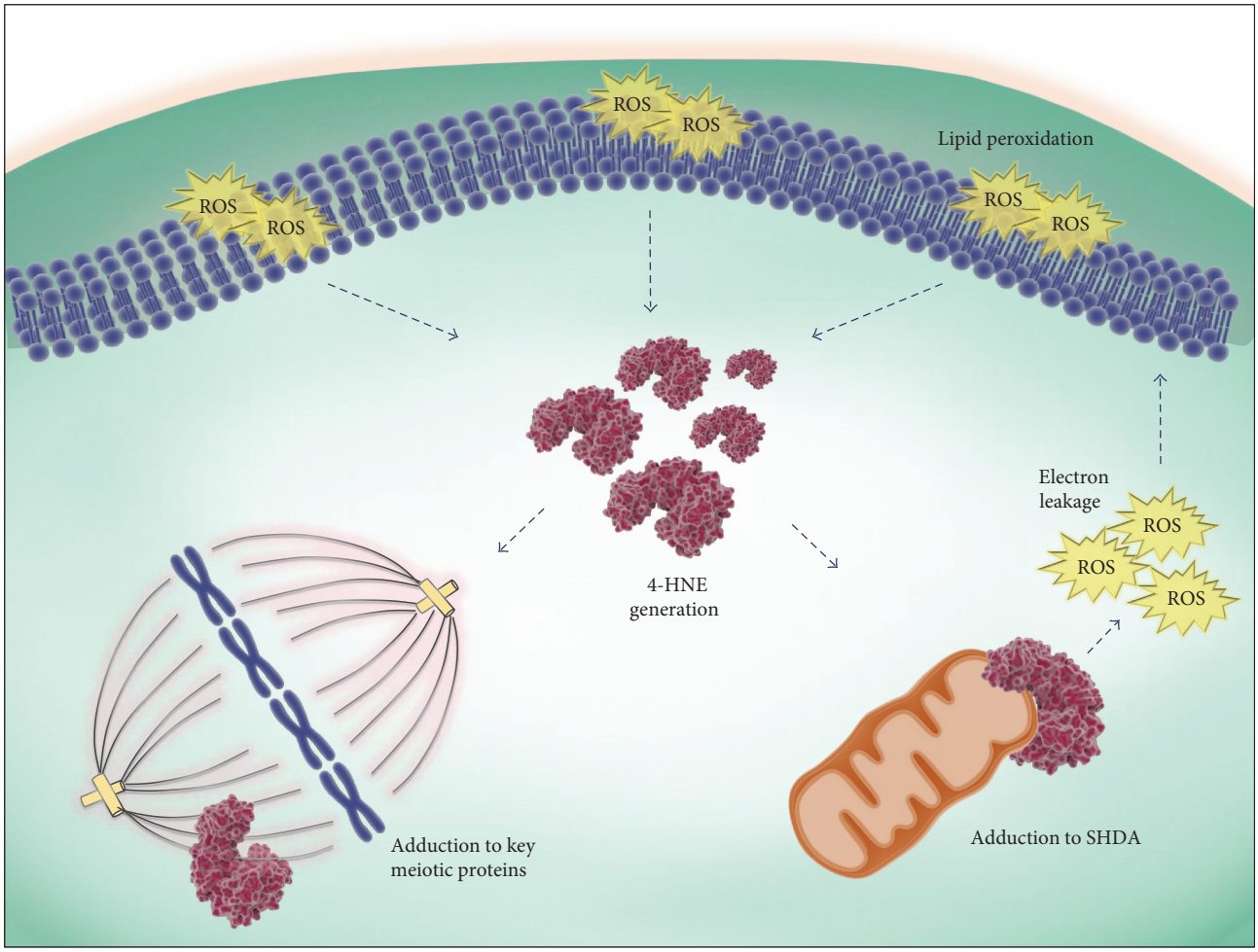


Figure 2: Cyclic propagation OS via lipid peroxidation impacts oocyte quality. Upon the induction of OS, ROS can instigate the peroxidation of lipids and the subsequent generation of highly electrophilic lipid aldehyde by-products such as 4-HNE. 4-HNE has the capacity to covalently modify and damage a wide array of proteins, including those essential for meiosis [174]. Additionally, adduction of mitochondrial SDHA impairs the ETC chain leading to electron leakage and the initiation of a positive feedback loop resulting in the generation of more ROS and lipid aldehydes [103].

Nevertheless, it has been convincingly demonstrated that the proteasomal system is generally less active in ageing somatic cells, thus decreasing the rate of oxidised protein resolution and creating an imbalance between ROS production and ROS clearance, factors that ultimately contribute to increased aggregate formation and accentuation of cellular damage [185–187]. Furthermore, this imbalance leads to a decrease in the ability of the ageing cell to prevail through oxidative attack.

Though it has yet to be proven directly, the activity of the proteasome is also likely to be downregulated in ageing oocytes owing to a decrease in transcript expression of several constituents of the UPS as detected via global comparative transcript analyses conducted on young versus aged mouse oocytes (GV and MII). Candidate genes identified in these studies included those encoding ubiquitin-activating enzymes (*Ube1c*), ubiquitin-conjugating enzymes (*Hip2*, *Ube2a*, *Ube2e3*, and *Ube2g1*), ubiquitin-ligases (*Siah2*), and ubiquitin itself (*Ubc*), as well other ubiquitination promoting enzymes (*Anapc4*). Proteasomal components appear to be equally susceptible to age-dependent decline, with documentation of a downregulation of transcripts encoding for 20S proteasome subunits (*Psma6*, *Psmb1*, *Psmb2*, *Psmb4*, and *Psmb5*), ATPase (*Psmc2*, *Psmc3*, and *Psmc5*), as well as the non-ATPase subunits of the 19S regulator (*Psmd4*, *Psmd8*, *Psmd9*, and *Psmd12*) [3, 97] (Table 1). Moreover, several of these transcripts appear to be similarly dysregulated in the MII oocytes of aged human donors [96] (Table 1). Although not as comprehensive, an equivalent decrease in the levels of proteasomal proteins, such as PMSD12, has also been detected in the MII oocytes of aged mice [116].

In contrast to this hypothesis, Tsakiri et al. demonstrated that the gonads and maturing oocytes of naturally aged *D. melanogaster* retain relatively high activity of the 26S proteasome and consequently accumulate less oxidatively damaged proteins than those of equivalently aged somatic tissues [187]. In addition, a study in *C. elegans* revealed that carbonylated proteins are abruptly eliminated by the proteasome

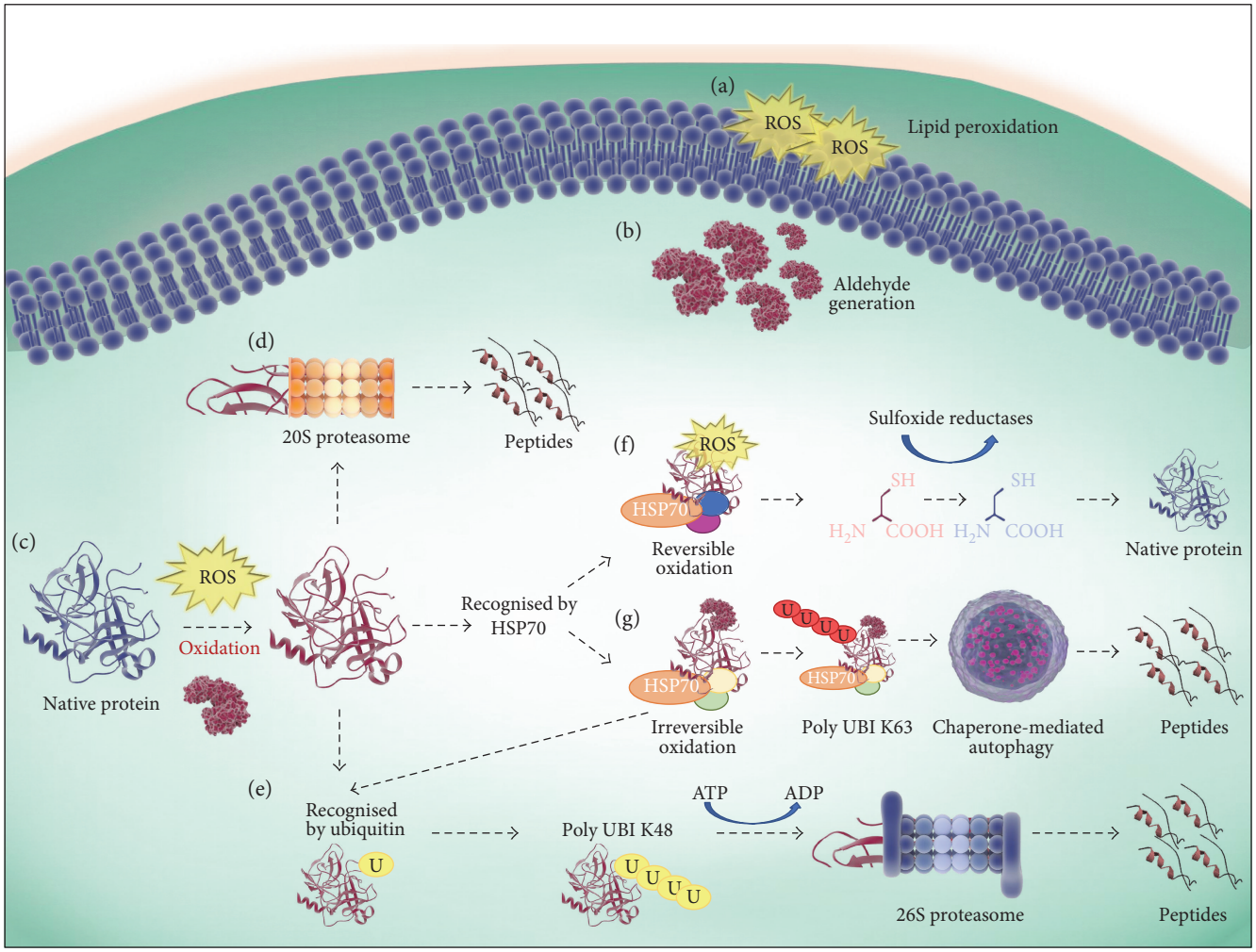


Figure 3: Repair and degradation mechanisms of oxidised proteins. (a) Elevated ROS induces a state of OS resulting in the peroxidation of lipids and (b) the generation of lipid aldehyde by-products such as 4-HNE. (c) During OS, native proteins can be oxidised directly by ROS or by secondary by-products of oxidation, such as 4-HNE. There are several pathways for the resolution of oxidised proteins; (d) oxidised protein can be degraded into peptides directly by the proteasomes 20S catalytic core, or (e) can be modified by ubiquitin and polyubiquitinated via K48 to be recognised and degraded by the 26S proteasome in an ATP-dependent manner. Alternatively, the oxidised protein can also be recognised by HSP70s. (f) In the case of revisable oxidation, HSP70s, in combination with cochaperones, mediate protein reduction and refolding back to their native form. (g) Where HSP70 recognition occurs and the oxidative modification is irreversible, such as 4-HNE adduction, the HSP70 and an alternate subset of cochaperones act to facilitate protein degradation via mediating polyubiquitination via K63 (recognised by the autophagy machinery) or K48 (recognised by the 26 proteasome).

in maturing oocytes, irrespective of the age of the mother [188]. While it is difficult to refute the possibility that such conflicting data may simply reflect species-specific responses, it must also be considered that the influence of ageing on proteasome activity does vary between distinct cell types. Indeed, the maintenance of proteasome activity within the oocyte may very well act as a compensatory mechanism to repair and protect against damage to the germline. Considering this, investigation into the existence of such potential compensatory mechanisms in mammalian ovarian tissue is clearly required.

Notwithstanding the obvious importance of the UPS, it has been suggested that the proteasome is only capable of degrading mildly oxidised proteins stemming from the fact that proteins must have the capacity to be unfolded prior to entering the relatively narrow 20S catalytic core [189]. Thus, the degradation of moderately to severely oxidised proteins that are unable to be sufficiently linearized rests with alternative lysosomal-dependent pathways [189]. Indeed, through the use of inhibitors capable of selectively, and independently, targeting the proteasomal or lysosomal pathways, it has been demonstrated in somatic systems that proteins bearing more extensive modifications are preferentially directed away from the proteasome and toward lysosomal degradation pathways whereupon they are recycled via the action of acidic lysosomal hydrolases [190]. This pathway has been demonstrated in *Xenopus* oocytes wherein lysosomal activity was stimulated upon intracellular accumulation of damaged proteins [191].

The lysosomal pathways can, in turn, act via direct engulfment of the substrate by the lysosomal membrane, or alternatively, substrates can be delivered to the lysosome by either molecular chaperone proteins or by a double membraned autophagosome that forms around cytoplasmic material [192, 193]. The latter catabolic mechanism, also known as autophagy, serves to compensate for the impairment of UPS activity under high oxidative burden [194, 195]. Interestingly, autophagy was shown to be upregulated in oocytes obtained from cows aged between 25 and 167 months, potentially suggesting that autophagic compensation may indeed be occurring in the context of the ageing oocyte. Curiously, chemical stimulation of autophagy in aged oocytes has been associated with an increase in oocyte quality [196].

Ubiquitination has also been implicated in coordinating the catabolism of proteins via selective autophagy, thus acting as a unifying factor linking the UPS, autophagy, and lysosomal pathways of degradation [197]. Accordingly, linker molecules exist with the capacity for interaction between both ubiquitin and components of the autophagy machinery. Indeed, under situations in which a cell's proteasomal capacity is overwhelmed by an accumulation of misfolded proteins, polyubiquitinated proteins tend to aggregate into large-scale aggresomes, which are subsequently degraded by autophagy. The formation of these aggresomes is critical for cell survival owing to their ability to mitigate the cytotoxic effects of free-floating misfolded proteins [198]. Illustrative of this process, the adaptor molecules HDAC6 and p62 have the capacity to bind mono and polyubiquitinated proteins and subsequently translocate them to aggresomes and to autophagic machinery, respectively [199–202]. Taken together, it is tempting to speculate that the dysregulation of ubiquitin and ubiquitin-related enzymes in the ageing oocyte might also compromise the efficiency of aggresome formation and the selective autophagy of oxidised proteins in oocytes.

4.7. The Role of Molecular Chaperones. Molecular chaperones are defined by their ability to confer resistance to environmental stressors and as such are often referred to the cell stress response or heat shock proteins (HSPs) [203, 204]. HSPs act by transiently associating with client proteins to mediate conformational stabilisation and aggregate prevention, relocalisation, or degradation [205]. The decision as to whether a protein will undergo protein repair and refolding or proteolysis is regulated in part by HSPs and their cochaperones. Indeed, it has been suggested that the HSP70 and HSP90 chaperone families and ubiquitin compete for the binding of a substrate, with those interactions favouring substrate-ubiquitin adhesion resulting in proteolysis [206]. In contrast, substrate-chaperone interactions can direct proteins towards either refolding and repair or proteolysis, with the protein's fate being largely arbitrated by cochaperones. Interactions with cochaperones that promote client binding and/or ATPase activity often facilitate protein repair and refolding [206, 207]. However, in situations where the substrate is unable to resume its folded conformation, chaperones can maintain the folded intermediate in a soluble form for recognition and catabolism by proteolytic machinery [208]. Conversely, interaction between the HSP70-HSP90 complex and the cochaperones BAG1 and CHIP, which can also act as an E3 ubiquitin ligase to catalyse the transfer of ubiquitin to the protein substrate, results in the inactivation of the HSPs ATPase folding activity, leading to client proteins being ubiquitinated and directed towards proteasomal degradation [206, 209].

The importance of HSPs in maintaining proteostasis within oocytes takes on added significance in light of evidence that oocytes obtained from aged mice exhibit a marked decrease in the gene expression of Hsp40s (*Dnaja2*, *Dnaja4*, *Dnajb1*, *Dnajb6*, *Dnajb10*, *Dnajb11*, *Dnajc3*, *Dnajc5*, *Dnajc8*, and *Dnajc21*), Hsp70s (*Hspa1b*, *Hspa4*, *Hspa8*, *Hspa9a*, *Hspa14*, and *Hsp70–3*), Hsp90s (*Hspcb*), and components of the chaperonin containing TCP1 complex (*Cct1–3* and *Cct5*) (Table 1) [3, 97]. Such reductions are particularly concerning given the vital role that HSPs play in survival and recovery from oxidative stress. Accordingly, several studies have reported an increase in gene and protein expression of HSPs in response to oxidative stress [207, 210, 211]. Conversely, it has also been shown that oxidative stress can impair the heat shock response, thus delaying resolution of unfolded proteins [212]. HSP70-mediated autophagy has also been demonstrated to be induced during oxidative stress and shown to accelerate the degradation of specific oxidized proteins [213, 214]. Moreover, HSP70 proteins have been shown to mediate dissociation and reassociation of the 26S proteasome during adaptation to oxidative stress [215]. Furthermore, HSP70 and HSP90 are known to interact with misfolded proteins to avert aggregation and initiate substrate refolding in an ATP- and cochaperone-dependent manner [216] with an elevation of HSP70 resulting in reduced aggregate formation via proteasome stimulation [217]. Ultimately, an age-related deterioration in the capacity of the oocyte to produce active heat shock proteins could contribute to an accumulation of oxidatively damaged proteins and their aggregation in ageing oocytes.

5. Therapeutic Interventions to Combat Age-Associated Decline in Oocyte Quality

5.1. Antioxidant Treatments. In recognition of the fundamental role that OS holds in the aetiology of ageing, in the following studies, the administration of antioxidant therapies has proven successful in improving both the quality and quantity of oocytes recovered from aged mice. In this context, potent antioxidants such as resveratrol have been successful in counteracting the ageing phenotype in mouse ovaries. Indeed, 12 months of resveratrol treatment, initiated at the time of weaning, resulted in an elevated follicle pool, decreased spindle aberrations, and decreased chromosome misalignments, which together culminated in increased litter sizes when compared to nontreated females of the same age [19]. Notably, telomerase activity was elevated and telomere length was also preserved in resveratrol-treated mice [19]. Liu et al. were also able to delay ovarian ageing with short-term treatment (2 months) of the antioxidant N-acetyl-L-cysteine (NAC), reporting an increase in zygote quality and early embryo development as well as increased telomerase

activity and longer telomere length. Furthermore, long-term (12 month) administration of low doses of NAC also resulted in increased litter size [218]. Complementing these promising data, mice fed a diet supplemented with antioxidants, vitamins C and E, from weaning onwards also experienced a significant decrease in age-related aneuploidy in their oocytes [219]. Two additional studies from this group also served to demonstrate that either early-onset (from weaning) or late-onset (from 22 to 33 weeks) oral administration of a combinatorial treatment of pharmacological doses of vitamins C and E was successful in ameliorating age-associated decrease in oocyte quantity and quality. Indeed, both timing regimens resulted in increase in ovulated eggs and normal chromosomal alignment at MII [220, 221]. Conversely, experimental mouse models of accelerated ageing (e.g., achieved through the administration of D-galactose) have also shown improvement upon treatment with radical-scavenging agents such as the biliprotein C-phycocyanin [222]. In this context, phycocyanin treatment led to decreased oocyte fragmentation and aneuploidy relative to that of females receiving D-galactose only. The phycocyanin-treated animals also responded with elevating levels of SOD in oocytes such that they were indistinguishable from those levels found untreated control females [222].

In a clinical context, supplementation of the antioxidant melatonin during ovarian stimulation in women undergoing IVF resulted in efficient accumulation into follicular fluid and improved oocyte and embryo quality in aged women [223]. In mice, the long-term dietary supplementation of melatonin, between 10 and 43 weeks of age, was also able to mitigate the phenotype of the ageing oocyte with the restoration of oocyte numbers, fertilisation rate, and blastulation rate. At a molecular level, melatonin largely preserved the mRNA reserve of aged oocytes and stimulated SIRT expression, antioxidant capacity, and ribosome function and maintained telomere length [224]. Similar beneficial effects have also been demonstrated in a bovine model, with the addition of melatonin during bovine IVF and embryo culture promoting blastocyst quality and yield [225].

Notwithstanding these positive outcomes, it is important to note that high doses of antioxidant therapy can have negative effects on female fertility. For instance, Tarin et al. revealed the risks associated with the pharmacological doses of antioxidant required to ameliorate the effects of ageing on oocyte quality. In this study, administration of vitamins C and E had a negative impact on uterine function and resulted in decreased litter sizes, reduced frequency of litters, and poor survival of male pups. The authors attributed these adverse outcomes to an inability of the corpus luteum to support pregnancy, thus leading to an increase in foetal resorption [226]. Additionally, high concentrations of eicosatetraynoic and eicosatriynoic acids, as well as lipoxygenase inhibitors, can reversibly block GVBD in mouse oocytes through their antioxidant action [227, 228]. Furthermore, administration of broad-spectrum ROS scavengers into the ovarian bursa have been shown to significantly decrease ovulation rates in hormonally stimulated mice. This response is apparently mediated by inhibition of LH driven upregulation of ovulation related genes [46]. This collective evidence showcases the delicate balance of pro- and antioxidants required for normal ovarian function and highlights the need for careful screening of safe and efficient antioxidant treatments before such a strategy can be reasonably transferred into a clinical setting.

5.2. Mitochondrial Replacement Therapy. In recent years, several groups have advocated for age-related infertility to be treated with mitochondrial replacement therapy (MRT). MRT has, as recently as December 2016, been legally approved in the UK for the treatment of mitochondrial disease [229]. The two most well-studied techniques include maternal spindle transfer (MST) and pronuclear transfer (PNT) [229, 230]. Briefly, MST involves the removal of the spindle-chromosome complex of a donor MII stage oocyte and its replacement with the spindle contents of the intended mother prior to fertilisation. In contrast, PNT involves the removal of the female and male pronuclei from the donor oocyte following fertilisation and before pronuclei fusion has occurred. The pronuclei are then replaced with those of the intended parents.

Considering that aneuploidy at the MII phase of oocyte development is a primary source of age-related infertility, it is apparent that MRT using already segregated nuclear material from oocytes at MII or beyond would not be sufficient in overcoming this obstacle. As such, the benefit of similar techniques for the treatment of age-related infertility remains an active area of research. Indeed, germinal vesicle transfer (GVT) is one promising development whereby the cytoplasmic contents of the oocyte are replaced before the onset of chromosome segregation. GVT involves the removal and replacement of the entire nuclear content including diploid chromosome sets as well as the surrounding nuclear material from GV stage oocytes [231]. A recent study by Nakagawa and FitzHarris was able to demonstrate the value of this technique with the restoration of spindle dynamics in the aged oocyte following reciprocal transfer of nuclei between young and aged GV mouse oocytes [232]. Encouragingly, the efficacy of this technique appears to be conserved in human oocytes as demonstrated in a preclinical study where reconstructed oocytes successfully overcome age associated aneuploidy with a euploidy rate of 80% [233]. However, the authors of this study did offer the cautionary note that the results were based on small oocyte numbers and thus argued for more research in this field. In an alternative study, the GV from young and aged human oocytes were reciprocally transplanted into ooplasts from the opposing age group [234]. Remarkably, 71% of aged GV to young ooplast transfers displayed a normal karyotype compared to only 25% of young GV to aged ooplast transfers [234]. Such findings indicate that the cytoplasmic content of the young oocyte contains the factors necessary to reduce damage in aged oocytes. Furthermore, developmental and fertilisation potential can be restored when the GV of mitochondrially damaged oocytes are transferred into donor ooplasts [87]. Given the electron leakage associated with mitochondrial damage, it could be assumed that the oxidative burden is reduced upon GVT of aged oocytes into young ooplasts and leads us to once again highlight the contribution of OS to the

age-related deterioration of the oocyte. Despite these encouraging results, considerable research is still required before MRT is likely to receive widespread adoption as a therapeutic intervention of choice to treat age-related female infertility.

5.3. Calorie Restriction. Dietary caloric restriction (CR) in adult life could be an alternate mechanism to reduce OS within the oocytes of older women. Calorie restriction has been linked to a decrease in free radical production by the mitochondria [1, 235–237]. After as little as 6 weeks of 40% CR in rats, ROS production was reduced by 45% and mtDNA damage by 30% in cardiac cells [238]. While translation into nonhuman primates has been controversial, there is evidence to suggest that CR also delays the onset of age-related disorders in rhesus monkeys [239]. Moreover, the application of the same CR regimen for an extended period of 7.5 months was shown to maintain oocyte quality in 12-month-old mice. Indeed, oocytes from the 12-month-old CR mice presented with significantly decreased aneuploidy, meiotic spindle abnormalities, mitochondrial dysfunction, and chromosomal misalignment compared to noncalorie-restricted age-matched control animals [1]. In seeking to account for this positive effect, the authors posited that it was likely attributed to a decrease in the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a transcriptional regulator of several genes associated with mitochondrial respiration. It is therefore possible that reduction in mitochondrial-derived ROS mediated by CR in adult life could be an effective means of improving the fertility of women of advancing maternal age. However, this study was performed for over 40% of the animal's lifetime and therefore a more practical model would need to be developed for human use.

6. Concluding Remarks

The causative relationship between the age-related decline in oocyte quality and increases in oxidative stress (OS) is well accepted. A concomitant decline in the fidelity of the protective mechanisms that oocytes are capable of mounting to mitigate oxidative insults only acts to perpetuate the intensity of the oxidative damage. Indeed, an attenuation of the efficacy of ROS metabolism, DNA repair, spindle assembly checkpoint, capacity for protein repair, and/or degradation collectively render the aged oocyte acutely vulnerable to oxidative insult. While antioxidant treatments, mitochondrial replacement therapy, and calorie restriction offer the potential for therapeutic intervention for the treatment of age-related infertility in women of advanced maternal age, it is clear that each of these strategies would benefit from further refinement before their full potential can be realised. In this review, we conclude that the accumulation of oxidative damage in the maternally aged oocyte likely results from the convergence of an increase in ROS production as well as a decreased capacity of the oocyte to mitigate oxidative damage. We also urge further basic research into therapeutic interventions to enhance the activity of oxidative repair pathways in the oocyte or otherwise ameliorate the damaging impact of OS lesions in these cells.

Conflicts of Interest

The authors have no conflicts to declare.

Acknowledgments

This work was supported by the University of Newcastle's Priority Research Centre for Reproductive Science and the Hunter Medical Research Institute's Pregnancy and Reproduction Program. Bettina P. Mihalas is a recipient of a Research Training Program Scholarship and an Emlyn and Jennie Thomas Postgraduate Medical Research Scholarship.

References

[1] K. Selesniemi, H.-J. Lee, A. Muhlhauser, and J. L. Tilly, "Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies," *Proceedings of the National Academy of Sciences*, vol. 108, no. 30, pp. 12319–12324, 2011.

[2] Y. L. Miao, K. Kikuchi, Q. Y. Sun, and H. Schatten, "Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility," *Human Reprodution Update*, vol. 15, no. 5, pp. 573–585, 2009.

[3] H. Pan, P. Ma, W. Zhu, and R. M. Schultz, "Age-associated increase in aneuploidy and changes in gene expression in mouse eggs," *Developmental Biology*, vol. 316, no. 2, pp. 397–407, 2008.

[4] T. Chiang, R. M. Schultz, and M. A. Lampson, "Meiotic origins of maternal age-related aneuploidy," *Biology of Reproduction*, vol. 86, no. 1, pp. 1–7, 2012.

[5] S. J. Silber, K. Kato, N. Aoyama et al., "Intrinsic fertility of human oocytes," *Fertility and Sterility*, vol. 107, no. 5, pp. 1232–1237, 2017.

[6] M. Carolan and D. Frankowska, "Advanced maternal age and adverse perinatal outcome: a review of the evidence," *Midwifery*, vol. 27, no. 6, pp. 793–801, 2011.

[7] K. M. Benzies, "Advanced maternal age: are decisions about the timing of child-bearing a failure to understand the risks?," *Canadian Medical Association Journal*, vol. 178, no. 2, pp. 183-184, 2008.

[8] J. J. Tarin, "Aetiology of age-associated aneuploidy: a mechanism based on the 'free radical theory of ageing'," *Human Reproduction*, vol. 10, no. 6, pp. 1563–1565, 1995.

[9] J. J. Tarin, "Potential effects of age-associated oxidative stress on mammalian oocytes/embryos," *Molecular Human Reproduction*, vol. 2, no. 10, pp. 717–724, 1996.

[10] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[11] D. Harman, "The biologic clock: the mitochondria?," *Journal of the American Geriatrics Society*, vol. 20, no. 4, pp. 145–147, 1972.

[12] S. E. Elizur, O. Lebovitz, R. Orvieto, J. Dor, and T. Zan-Bar, "Reactive oxygen species in follicular fluid may serve as biochemical markers to determine ovarian aging and follicular metabolic age," *Gynecological Endocrinology*, vol. 30, no. 10, pp. 705–707, 2014.

[13] J. Lim and U. Luderer, "Oxidative damage increases and antioxidant gene expression decreases with aging in the mouse ovary," *Biology of Reproduction*, vol. 84, no. 4, pp. 775–782, 2011.

[14] S. Takeo, K. Kimura, K. Shirasuna, T. Kuwayama, and H. Iwata, "Age-associated deterioration in follicular fluid induces a decline in bovine oocyte quality," *Reproduction Fertility and Development*, vol. 29, no. 4, pp. 759–767, 2017.

[15] Z. Wiener-Megnazi, L. Vardi, A. Lissak et al., "Oxidative stress indices in follicular fluid as measured by the thermochemiluminescence assay correlate with outcome parameters in in vitro fertilization," *Fertility and Sterility*, vol. 82, Supplement 3, pp. 1171–1176, 2004.

[16] W. J. Choi, J. Banerjee, T. Falcone, J. Bena, A. Agarwal, and R. K. Sharma, "Oxidative stress and tumor necrosis factor-α-induced alterations in metaphase II mouse oocyte spindle structure," *Fertility and Sterility*, vol. 88, 4 Supplement, pp. 1220–1231, 2007.

[17] S. K. Chaube, P. V. Prasad, S. C. Thakur, and T. G. Shrivastav, "Hydrogen peroxide modulates meiotic cell cycle and induces morphological features characteristic of apoptosis in rat oocytes cultured in vitro," *Apoptosis*, vol. 10, no. 4, pp. 863–874, 2005.

[18] A. P. Goud, P. T. Goud, M. P. Diamond, B. Gonik, and H. M. Abu-Soud, "Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid," *Free Radical Biology & Medicine*, vol. 44, no. 7, pp. 1295–1304, 2008.

[19] M. Liu, Y. Yin, X. Ye et al., "Resveratrol protects against age-associated infertility in mice," *Human Reproduction*, vol. 28, no. 3, pp. 707–717, 2013.

[20] K. Miyamoto, E. F. Sato, E. Kasahara et al., "Effect of oxidative stress during repeated ovulation on the structure and functions of the ovary, oocytes, and their mitochondria," *Free Radical Biology & Medicine*, vol. 49, no. 4, pp. 674–681, 2010.

[21] H. Tamura, A. Takasaki, I. Miwa et al., "Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate," *Journal of Pineal Research*, vol. 44, no. 3, pp. 280–287, 2008.

[22] V. Babuska, M. Cedikova, D. Rajdl et al., "Comparison of selective oxidative stress parameters in the follicular fluid of infertile women and healthy fertile oocyte donors," *Ceská Gynekologie*, vol. 77, no. 6, pp. 543–548, 2012.

[23] S. Das, R. Chattopadhyay, S. Ghosh et al., "Reactive oxygen species level in follicular fluid—embryo quality marker in IVF?," *Human Reproduction*, vol. 21, no. 9, pp. 2403–2407, 2006.

[24] R. Nuñez-Calonge, S. Cortés, L. M. Gutierrez Gonzalez et al., "Oxidative stress in follicular fluid of young women with low response compared with fertile oocyte donors," *Reproductive Biomedicine Online*, vol. 32, no. 4, pp. 446–456, 2016.

[25] A. Agarwal, A. Aponte-Mellado, B. J. Premkumar, A. Shaman, and S. Gupta, "The effects of oxidative stress on female reproduction: a review," *Reproductive Biology and Endocrinology*, vol. 10, pp. 49–49, 2012.

[26] C. M. H. Combelles, S. Gupta, and A. Agarwal, "Could oxidative stress influence the in-vitro maturation of oocytes?," *Reproductive Biomedicine Online*, vol. 18, no. 6, pp. 864–880, 2009.

[27] S. Prasad, M. Tiwari, A. N. Pandey, T. G. Shrivastav, and S. K. Chaube, "Impact of stress on oocyte quality and reproductive outcome," *Journal of Biomedical Science*, vol. 23, p. 36, 2016.

[28] E. H. Ruder, T. J. Hartman, J. Blumberg, and M. B. Goldman, "Oxidative stress and antioxidants: exposure and impact on female fertility," *Human Reproduction Update*, vol. 14, no. 4, pp. 345–357, 2008.

[29] A. Agarwal, S. Gupta, and R. K. Sharma, "Role of oxidative stress in female reproduction," *Reproductive Biology and Endocrinology*, vol. 3, pp. 28–28, 2005.

[30] M. K. Skinner, "Regulation of primordial follicle assembly and development," *Human Reproduction Update*, vol. 11, no. 5, pp. 461–471, 2005.

[31] E. A. McLaughlin and S. C. McIver, "Awakening the oocyte: controlling primordial follicle development," *Reproduction*, vol. 137, no. 1, pp. 1–11, 2009.

[32] A. N. Hirshfield, "Development of follicles in the mammalian ovary," *International Review of Cytology*, vol. 124, pp. 43–101, 1991.

[33] K. J. Hutt and D. F. Albertini, "An oocentric view of folliculogenesis and embryogenesis," *Reproductive Biomedicine Online*, vol. 14, no. 6, pp. 758–764, 2007.

[34] J. E. Holt, S. I. Lane, and K. T. Jones, "The control of meiotic maturation in mammalian oocytes," *Current Topics in Developmental Biology*, vol. 102, pp. 207–226, 2013.

[35] A. P. Sobinoff, J. M. Sutherland, and E. A. McLaughlin, "Intracellular signalling during female gametogenesis," *Molecular Human Reproduction*, vol. 19, no. 5, pp. 265–278, 2013.

[36] Y. Bentov, T. Yavorska, N. Esfandiari, A. Jurisicova, and R. F. Casper, "The contribution of mitochondrial function to reproductive aging," *Journal of Assisted Reproduction and Genetics*, vol. 28, no. 9, pp. 773–783, 2011.

[37] N. J. Camlin, A. P. Sobinoff, J. M. Sutherland et al., "Maternal smoke exposure impairs the long-term fertility of female offspring in a murine model," *Biology of Reproduction*, vol. 94, no. 2, p. 39, 2016.

[38] A. P. Sobinoff, V. Pye, B. Nixon, S. D. Roman, and E. A. McLaughlin, "Adding insult to injury: effects of xenobiotic-induced preantral ovotoxicity on ovarian development and oocyte fusibility," *Toxicological Sciences*, vol. 118, no. 2, pp. 653–666, 2010.

[39] L. Sominsky, A. P. Sobinoff, M. S. Jobling, V. Pye, E. A. McLaughlin, and D. M. Hodgson, "Immune regulation of ovarian development: programming by neonatal immune challenge," *Frontiers in Neuroscience*, vol. 7, p. 100, 2013.

[40] E. H. Ruder, T. J. Hartman, and M. B. Goldman, "Impact of oxidative stress on female fertility," *Current Opinion in Obstetrics and Gynecology*, vol. 21, no. 3, pp. 219–222, 2009.

[41] K. C. Kregel and H. J. Zhang, "An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations," *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*, vol. 292, no. 1, pp. R18–R36, 2007.

[42] H. T. Chao, S. Y. Lee, H. M. Lee, T. L. Liao, Y. H. Wei, and S. H. Kao, "Repeated ovarian stimulations induce oxidative damage and mitochondrial DNA mutations in mouse ovaries," *Annals of the New York Academy of Sciences*, vol. 1042, pp. 148–156, 2005.

[43] C. Tatone, F. Amicarelli, M. C. Carbone et al., "Cellular and molecular aspects of ovarian follicle ageing," *Human Reproduction Update*, vol. 14, no. 2, pp. 131–142, 2008.

[44] Q. Li, X. Geng, W. Zheng, J. Tang, B. Xu, and Q. Shi, "Current understanding of ovarian aging," *Science China Life Sciences*, vol. 55, no. 8, pp. 659–669, 2012.

[45] A. Rizzo, M. T. Roscino, F. Binetti, and R. L. Sciorsci, "Roles of reactive oxygen species in female reproduction," *Reproduction in Domestic Animals*, vol. 47, no. 2, pp. 344–352, 2012.

[46] K. Shkolnik, A. Tadmor, S. Ben-Dor, N. Nevo, D. Galiani, and N. Dekel, "Reactive oxygen species are indispensable in ovulation," *Proceedings of the National Academy of Sciences*, vol. 108, no. 4, pp. 1462–1467, 2011.

[47] A. N. Pandey, A. Tripathi, K. V. PremKumar, T. G. Shrivastav, and S. K. Chaube, "Reactive oxygen and nitrogen species during meiotic resumption from diplotene arrest in mammalian oocytes," *Journal of Cellular Biochemistry*, vol. 111, no. 3, pp. 521–528, 2010.

[48] A. Tripathi, S. Khatun, A. Pandey et al., "Intracellular levels of hydrogen peroxide and nitric oxide in oocytes at various stages of meiotic cell cycle and apoptosis," *Free Radical Research*, vol. 43, no. 3, pp. 287–294, 2009.

[49] A. N. Pandey and S. K. Chaube, "A moderate increase of hydrogen peroxide level is beneficial for spontaneous resumption of meiosis from diplotene arrest in rat oocytes cultured in vitro," *BioResearch Open Access*, vol. 3, no. 4, pp. 183–191, 2014.

[50] M. Tanaka, T. Miyazaki, S. Tanigaki et al., "Participation of reactive oxygen species in PGF2alpha-induced apoptosis in rat luteal cells," *Journal of Reproduction and Fertility*, vol. 120, no. 2, pp. 239–245, 2000.

[51] J. V. Blerkom and P. Davis, "Differential effects of repeated ovarian stimulation on cytoplasmic and spindle organization in metaphase II mouse oocytes matured in vivo and in vitro," *Human Reproduction*, vol. 16, no. 4, pp. 757–764, 2001.

[52] C. M. Combelles and D. F. Albertini, "Assessment of oocyte quality following repeated gonadotropin stimulation in the mouse," *Biology of Reproduction*, vol. 68, no. 3, pp. 812–821, 2003.

[53] T. Silberstein, J. R. Trimarchi, L. Gonzalez, D. L. Keefe, and A. S. Blazar, "Pregnancy outcome in in vitro fertilization decreases to a plateau with repeated cycles," *Fertility and Sterility*, vol. 84, no. 4, pp. 1043–1045, 2005.

[54] J. G. Wang, N. C. Douglas, C. Dicken, G. S. Nakhuda, M. M. Guarnaccia, and M. V. Sauer, "Cryopreservation of supernumerary high quality embryos predicts favorable outcomes for patients undergoing repeated cycles of in vitro fertilization," *Fertility and Sterility*, vol. 89, no. 2, pp. 368–374, 2008.

[55] B. S. Shapiro, K. S. Richter, D. C. Harris, and S. T. Daneshmand, "Dramatic declines in implantation and pregnancy rates in patients who undergo repeated cycles of in vitro fertilization with blastocyst transfer after one or more failed attempts," *Fertility and Sterility*, vol. 76, no. 3, pp. 538–542, 2001.

[56] C. Caligara, J. Navarro, G. Vargas, C. Simón, A. Pellicer, and J. Remohí, "The effect of repeated controlled ovarian stimulation in donors," *Human Reproduction*, vol. 16, no. 11, pp. 2320–2323, 2001.

[57] E. M. Kolibianakis and P. Devroey, "No decrease occurs in the number of COCs retrieved with repeated IVF cycles," *Human Reproduction*, vol. 19, no. 8, pp. 1927-1928, 2004.

[58] N. Doldi, P. Persico, L. D. Santis, E. Rabellotti, E. Papaleo, and A. Ferrari, "Consecutive cycles in in vitro fertilization–embryo transfer," *Gynecological Endocrinology*, vol. 20, no. 3, pp. 132–136, 2005.

[59] F. Hoveyda, L. Engmann, J. Steele, A. L. Bernal, and D. H. Barlow, "Ovarian response in three consecutive in vitro fertilization cycles," *Fertility and Sterility*, vol. 77, no. 4, pp. 706–710, 2002.

[60] H. M. Lander, J. M. Tauras, J. S. Ogiste, O. Hori, R. A. Moss, and A. M. Schmidt, "Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress," *The Journal of Biological Chemistry*, vol. 272, no. 28, pp. 17810–17814, 1997.

[61] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[62] D. Xu and J. M. Kyriakis, "Phosphatidylinositol 3'-kinase-dependent activation of renal mesangial cell Ki-Ras and ERK by advanced glycation end products," *The Journal of Biological Chemistry*, vol. 278, no. 41, pp. 39349–39355, 2003.

[63] J. Li and A. M. Schmidt, "Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products," *The Journal of Biological Chemistry*, vol. 272, no. 26, pp. 16498–16506, 1997.

[64] A. K. Mohamed, A. Bierhaus, S. Schiekofer, H. Tritschler, R. Ziegler, and P. P. Nawroth, "The role of oxidative stress and NF-κB activation in late diabetic complications," *BioFactors*, vol. 10, no. 2-3, pp. 157–167, 1999.

[65] A. Neumann, R. Schinzel, D. Palm, P. Riederer, and G. Munch, "High molecular weight hyaluronic acid inhibits advanced glycation endproduct-induced NF-κB activation and cytokine expression," *FEBS Letters*, vol. 453, no. 3, pp. 283–287, 1999.

[66] G. Basta, A. M. Schmidt, and R. D. Caterina, "Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes," *Cardiovascular Research*, vol. 63, no. 4, pp. 582–592, 2004.

[67] A. Goldin, J. A. Beckman, A. M. Schmidt, and M. A. Creager, "Advanced glycation end products: sparking the development of diabetic vascular injury," *Circulation*, vol. 114, no. 6, pp. 597–605, 2006.

[68] A. M. Schmidt, S. D. Yan, S. F. Yan, and D. M. Stern, "The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses," *Journal of Clinical Investigation*, vol. 108, no. 7, pp. 949–955, 2001.

[69] M. H. Stensen, T. Tanbo, R. Storeng, and P. Fedorcsak, "Advanced glycation end products and their receptor contribute to ovarian ageing," *Human Reproduction*, vol. 29, no. 1, pp. 125–134, 2014.

[70] C. Tatone, M. C. Carbone, G. Campanella et al., "Female reproductive dysfunction during ageing: role of methylglyoxal in the formation of advanced glycation endproducts in ovaries of reproductively-aged mice," *Journal of Biological Regulators and Homeostatic Agents*, vol. 24, no. 1, pp. 63–72, 2010.

[71] Z. Merhi, "Advanced glycation end products and their relevance in female reproduction," *Human Reproduction*, vol. 29, no. 1, pp. 135–145, 2014.

[72] M. B. Yim, H. S. Yim, C. Lee, S. O. Kang, and P. B. Chock, "Protein glycation: creation of catalytic sites for free radical generation," *Annals of the New York Academy of Sciences*, vol. 928, pp. 48–53, 2001.

[73] G. Soldatos and M. E. Cooper, "Advanced glycation end products and vascular structure and function," *Current Hypertension Reports*, vol. 8, no. 6, pp. 472–478, 2006.

[74] O. Oktem and B. Urman, "Understanding follicle growth in vivo," *Human Reproduction*, vol. 25, no. 12, pp. 2944–2954, 2010.

[75] M. Tafani, L. Sansone, F. Limana et al., "The interplay of reactive oxygen species, hypoxia, inflammation, and sirtuins in cancer initiation and progression," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 3907147, 18 pages, 2016.

[76] S. Huey, A. Abuhamad, G. Barroso et al., "Perifollicular blood flow Doppler indices, but not follicular pO_2, pCO_2, or pH, predict oocyte developmental competence in in vitro fertilization," *Fertility and Sterility*, vol. 72, no. 4, pp. 707–712, 1999.

[77] M. F. Costello, S. M. Shrestha, P. Sjoblom et al., "Power doppler ultrasound assessment of the relationship between age and ovarian perifollicular blood flow in women undergoing *in vitro* fertilization treatment," *Journal of Assisted Reproduction and Genetics*, vol. 23, no. 9-10, pp. 359–365, 2006.

[78] P. S. Bhal, N. D. Pugh, L. Gregory, S. O'Brien, and R. W. Shaw, "Perifollicular vascularity as a potential variable affecting outcome in stimulated intrauterine insemination treatment cycles: a study using transvaginal power Doppler," *Human Reproduction*, vol. 16, no. 8, pp. 1682–1689, 2001.

[79] A. Borini, A. Maccolini, A. Tallarini, M. A. Bonu, R. Sciajno, and C. Flamigni, "Perifollicular vascularity and its relationship with oocyte maturity and IVF outcome," *Annals of the New York Academy of Sciences*, vol. 943, pp. 64–67, 2001.

[80] J. V. Blerkom, M. Antczak, and R. Schrader, "The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics," *Human Reproduction*, vol. 12, no. 5, pp. 1047–1055, 1997.

[81] J. V. Blerkom, "The influence of intrinsic and extrinsic factors on the developmental potential and chromosomal normality of the human oocyte," *Journal of the Society for Gynecologic Investigation*, vol. 3, no. 1, pp. 3–11, 1996.

[82] P. May-Panloup, L. Boucret, J.-M. Chao de la Barca et al., "Ovarian ageing: the role of mitochondria in oocytes and follicles," *Human Reproduction Update*, vol. 22, no. 6, pp. 725–743, 2016.

[83] R. Dumollard, M. Duchen, and J. Carroll, "The role of mitochondrial function in the oocyte and embryo," *Current Topics in Developmental Biology*, vol. 77, pp. 21–49, 2007.

[84] S. Chappel, "The role of mitochondria from mature oocyte to viable blastocyst," *Obstetrics and Gynecology International*, vol. 2013, Article ID 183024, 10 pages, 2013.

[85] S. Nagai, T. Mabuchi, S. Hirata et al., "Oocyte mitochondria: strategies to improve embryogenesis," *Human Cell*, vol. 17, no. 4, pp. 195–202, 2004.

[86] Y. Yu, R. Dumollard, A. Rossbach, F. A. Lai, and K. Swann, "Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation," *Journal of Cellular Physiology*, vol. 224, no. 3, pp. 672–680, 2010.

[87] T. Takeuchi, Q. V. Neri, Y. Katagiri, Z. Rosenwaks, and G. D. Palermo, "Effect of treating induced mitochondrial damage on embryonic development and epigenesis," *Biology of Reproduction*, vol. 72, no. 3, pp. 584–592, 2005.

[88] M. Alexeyev, I. Shokolenko, G. Wilson, and S. LeDoux, "The maintenance of mitochondrial DNA integrity—critical analysis and update," *Cold Spring Harbor Perspectives in Biology*, vol. 5, no. 5, article a012641, 2013.

[89] R. W. Taylor, M. J. Barron, G. M. Borthwick et al., "Mitochondrial DNA mutations in human colonic crypt stem cells," *The Journal of Clinical Investigation*, vol. 112, no. 9, pp. 1351–1360, 2003.

[90] H.-C. Lee and Y.-H. Wei, "Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging," *Experimental Biology and Medicine*, vol. 232, no. 5, pp. 592–606, 2007.

[91] M. Lynch, B. Koskella, and S. Schaack, "Mutation pressure and the evolution of organelle genomic architecture," *Science*, vol. 311, no. 5768, pp. 1727–1730, 2006.

[92] D. L. Keefe, T. Niven-Fairchild, S. Powell, and S. Buradagunta, "Mitochondrial deoxyribonucleic acid deletions in oocytes and reproductive aging in women," *Fertility and Sterility*, vol. 64, no. 3, pp. 577–583, 1995.

[93] C. C. Chan, V. W. Liu, E. Y. Lau, W. S. Yeung, E. H. Ng, and P. C. Ho, "Mitochondrial DNA content and 4977 bp deletion in unfertilized oocytes," *Molecular Human Reproduction*, vol. 11, no. 12, pp. 843–846, 2005.

[94] F. Simsek-Duran, F. Li, W. Ford, R. J. Swanson, H. W. Jones Jr., and F. J. Castora, "Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes," *PLoS One*, vol. 8, no. 5, article e64955, 2013.

[95] J. V. Blerkom, J. Sinclair, and P. Davis, "Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy," *Human Reproduction*, vol. 13, no. 1o, pp. 2857–2868, 1998.

[96] N. M. Steuerwald, M. G. Bermudez, D. Wells, S. Munne, and J. Cohen, "Maternal age-related differential global expression profiles observed in human oocytes," *Reproductive Biomedicine Online*, vol. 14, no. 6, pp. 700–708, 2007.

[97] T. Hamatani, G. Falco, M. G. Carter et al., "Age-associated alteration of gene expression patterns in mouse oocytes," *Human Molecular Genetics*, vol. 13, no. 19, pp. 2263–2278, 2004.

[98] A. Ben-Meir, E. Burstein, A. Borrego-Alvarez et al., "Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging," *Aging Cell*, vol. 14, no. 5, pp. 887–895, 2015.

[99] M. Wilding, B. Dale, M. Marino et al., "Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos," *Human Reproduction*, vol. 16, no. 5, pp. 909–917, 2001.

[100] H. Iwata, H. Goto, H. Tanaka et al., "Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes," *Reproduction, Fertility and Development*, vol. 23, no. 3, pp. 424–432, 2011.

[101] E. Babayev, T. Wang, K. Szigeti-Buck et al., "Reproductive aging is associated with changes in oocyte mitochondrial dynamics, function, and mtDNA quantity," *Maturitas*, vol. 93, pp. 121–130, 2016.

[102] I. Martin and M. S. Grotewiel, "Oxidative damage and age-related functional declines," *Mechanisms of Ageing and Development*, vol. 127, no. 5, pp. 411–423, 2006.

[103] T. Lord, J. H. Martin, and R. J. Aitken, "Accumulation of electrophilic aldehydes during postovulatory aging of mouse oocytes causes reduced fertility, oxidative stress, and apoptosis," *Biology of Reproduction*, vol. 92, no. 2, p. 33, 2015.

[104] X. Zhang, X. Q. Wu, S. Lu, Y. L. Guo, and X. Ma, "Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles," *Cell Research*, vol. 16, no. 10, pp. 841–850, 2006.

[105] E. F. Sato, H. Kobuchi, K. Edashige et al., "Dynamic aspects of ovarian superoxide dismutase isozymes during the ovulatory process in the rat," *FEBS Letters*, vol. 303, no. 2-3, pp. 121–125, 1992.

[106] C. H. Hsieh, S. P. Tsai, H. I. Yeh, T. C. Sheu, and M. F. Tam, "Mass spectrometric analysis of rat ovary and testis cytosolic glutathione S-transferases (GSTs): identification of a novel class-Alpha GST, rGSTA6*, in rat testis," *Biochemical Journal*, vol. 323, Part 2, pp. 503–510, 1997.

[107] R. F. Aten, K. M. Duarte, and H. R. Behrman, "Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing hormone and prostaglandin F2 α," *Biology of Reproduction*, vol. 46, no. 3, pp. 401–407, 1992.

[108] G. Leyens, B. Verhaeghe, M. Landtmeters, J. Marchandise, B. Knoops, and I. Donnay, "Peroxiredoxin 6 is upregulated in bovine oocytes and cumulus cells during in vitro maturation: role of intercellular communication," *Biology of Reproduction*, vol. 71, no. 5, pp. 1646–1651, 2004.

[109] L. J. Osborne, K. F. Tonissen, V. H. Tang, and F. M. Clarke, "Expression and localisation of thioredoxin in mouse reproductive tissues during the oestrous cycle," *Molecular Reproduction and Development*, vol. 58, no. 4, pp. 359–367, 2001.

[110] U. Luderer, T. J. Kavanagh, C. C. White, and E. M. Faustman, "Gonadotropin regulation of glutathione synthesis in the rat ovary," *Reproductive Toxicology*, vol. 15, no. 5, pp. 495–504, 2001.

[111] T. C. Brink, L. Demetrius, H. Lehrach, and J. Adjaye, "Age-related transcriptional changes in gene expression in different organs of mice support the metabolic stability theory of aging," *Biogerontology*, vol. 10, no. 5, pp. 549–564, 2009.

[112] C. Tatone, M. C. Carbone, S. Falone et al., "Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells," *Molecular Human Reproduction*, vol. 12, no. 11, pp. 655–660, 2006.

[113] L. Matos, D. Stevenson, F. Gomes, J. L. Silva-Carvalho, and H. Almeida, "Superoxide dismutase expression in human cumulus oophorus cells," *Molecular Human Reproduction*, vol. 15, no. 7, pp. 411–419, 2009.

[114] M. C. Carbone, C. Tatone, S. Delle Monache et al., "Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes," *Molecular Human Reproduction*, vol. 9, no. 11, pp. 639–643, 2003.

[115] Y. Okatani, N. Morioka, A. Wakatsuki, Y. Nakano, and Y. Sagara, "Role of the free radical-scavenger system in aromatase activity of the human ovary," *Hormone Research*, vol. 39, Supplement 1, pp. 22–27, 1993.

[116] C. Schwarzer, M. Siatkowski, M. J. Pfeiffer et al., "Maternal age effect on mouse oocytes: new biological insight from proteomic analysis," *Reproduction*, vol. 148, no. 1, pp. 55–72, 2014.

[117] J. J. Tarin, V. Gomez-Piquer, J. F. Pertusa, C. Hermenegildo, and A. Cano, "Association of female aging with decreased parthenogenetic activation, raised MPF, and MAPKs activities and reduced levels of glutathione S-transferases activity and thiols in mouse oocytes," *Molecular Reproduction and Development*, vol. 69, no. 4, pp. 402–410, 2004.

[118] H. Tatemoto, N. Muto, I. Sunagawa, A. Shinjo, and T. Nakada, "Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid," *Biology of Reproduction*, vol. 71, no. 4, pp. 1150–1157, 2004.

[119] E. C. Curnow, J. Ryan, D. Saunders, and E. S. Hayes, "Bovine in vitro oocyte maturation as a model for manipulation of the γ-glutamyl cycle and intraoocyte glutathione," *Reproduction, Fertility and Development*, vol. 20, no. 5, pp. 579–588, 2008.

[120] K. A. Zuelke, D. P. Jones, and S. D. Perreault, "Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes," *Biology of Reproduction*, vol. 57, no. 6, pp. 1413–1419, 1997.

[121] J. Tong, S. L. Sheng, Y. Sun et al., "Melatonin levels in follicular fluid as markers for IVF outcomes and predicting ovarian reserve," *Reproduction*, vol. 153, no. 4, pp. 443–451, 2017.

[122] S. Soleimani Rad, S. Abbasalizadeh, A. Ghorbani Haghjo, M. Sadagheyani, A. Montaseri, and J. Soleimani Rad, "Evaluation of the melatonin and oxidative stress markers level in serum of fertile and infertile women," *Iranian Journal of Reproductive Medicine*, vol. 13, no. 7, pp. 439–444, 2015.

[123] C. Tatone, G. D. Emidio, M. Vitti et al., "Sirtuin functions in female fertility: possible role in oxidative stress and aging," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 659687, 11 pages, 2015.

[124] X. Guo, M. Kesimer, G. Tolun et al., "The NAD^+-dependent protein deacetylase activity of SIRT1 is regulated by its oligomeric status," *Scientific Reports*, vol. 2, p. 640, 2012.

[125] P. O. Hassa, S. S. Haenni, M. Elser, and M. O. Hottiger, "Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going?," *Microbiology and Molecular Biology Reviews*, vol. 70, no. 3, pp. 789–829, 2006.

[126] L. Zhang, R. Ma, J. Hu, X. Ding, and Y. Xu, "Sirtuin inhibition adversely affects porcine oocyte meiosis," *PLoS One*, vol. 10, no. 7, article e0132941, 2015.

[127] G. D. Emidio, S. Falone, M. Vitti et al., "SIRT1 signalling protects mouse oocytes against oxidative stress and is deregulated during aging," *Human Reproduction*, vol. 29, no. 9, pp. 2006–2017, 2014.

[128] I. Manosalva and A. González, "Aging changes the chromatin configuration and histone methylation of mouse oocytes at germinal vesicle stage," *Theriogenology*, vol. 74, no. 9, pp. 1539–1547, 2010.

[129] A. Vaquero, M. Scher, H. Erdjument-Bromage, P. Tempst, L. Serrano, and D. Reinberg, "SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation," *Nature*, vol. 450, no. 7168, pp. 440–444, 2007.

[130] L. Bordone, D. Cohen, A. Robinson et al., "SIRT1 transgenic mice show phenotypes resembling calorie restriction," *Aging Cell*, vol. 6, no. 6, pp. 759–767, 2007.

[131] S. Takeo, K. Kimura, Y. Monji, T. Kuwayama, R. Kawahara-Miki, and H. Iwata, "Resveratrol improves the mitochondrial function and fertilization outcome of bovine oocytes," *Journal of Reproduction and Development*, vol. 60, no. 2, pp. 92–99, 2014.

[132] X.-m. Zhang, L. Li, J.-j. Xu et al., "Rapamycin preserves the follicle pool reserve and prolongs the ovarian lifespan of female rats via modulating mTOR activation and sirtuin expression," *Gene*, vol. 523, no. 1, pp. 82–87, 2013.

[133] L. Pacella-Ince, D. Zander-Fox, and M. Lane, "Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age," *Human Reproduction*, vol. 29, no. 7, pp. 1490–1499, 2014.

[134] B. Kincaid and E. Bossy-Wetzel, "Forever young: SIRT3 a shield against mitochondrial meltdown, aging, and neurodegeneration," *Frontiers in Aging Neuroscience*, vol. 5, p. 48, 2013.

[135] Y. Kawamura, Y. Uchijima, N. Horike et al., "Sirt3 protects in vitro-fertilized mouse preimplantation embryos against oxidative stress-induced p53-mediated developmental arrest," *The Journal of Clinical Investigation*, vol. 120, no. 8, pp. 2817–2828, 2010.

[136] N. Okamoto, K. Kawamura, N. Kawamura et al., "Effects of maternal aging on expression of sirtuin genes in ovulated oocyte and cumulus cells," *Journal of Mammalian Ova Research*, vol. 30, no. 1, pp. 24–29, 2013.

[137] L. Zhang, X. Hou, R. Ma, K. Moley, T. Schedl, and Q. Wang, "Sirt2 functions in spindle organization and chromosome alignment in mouse oocyte meiosis," *The FASEB Journal*, vol. 28, no. 3, pp. 1435–1445, 2014.

[138] L. Han, J. Ge, L. Zhang et al., "Sirt6 depletion causes spindle defects and chromosome misalignment during meiosis of mouse oocyte," *Scientific Reports*, vol. 5, article 15366, 2015.

[139] M. Yasui, Y. Kanemaru, N. Kamoshita, T. Suzuki, T. Arakawa, and M. Honma, "Tracing the fates of site-specifically introduced DNA adducts in the human genome," *DNA Repair*, vol. 15, pp. 11–20, 2014.

[140] J. Li, A. Braganza, and R. W. Sobol, "Base excision repair facilitates a functional relationship between guanine oxidation and histone demethylation," *Antioxidants & Redox Signaling*, vol. 18, no. 18, pp. 2429–2443, 2013.

[141] N. Nishida, T. Arizumi, M. Takita et al., "Reactive oxygen species induce epigenetic instability through the formation of 8-hydroxydeoxyguanosine in human hepatocarcinogenesis," *Digestive Diseases*, vol. 31, no. 5-6, pp. 459–466, 2013.

[142] S. Titus, F. Li, R. Stobezki et al., "Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans," *Science Translational Medicine*, vol. 5, no. 172, article 172ra121, 2013.

[143] H. Li, J. R. Mitchell, and P. Hasty, "DNA double-strand breaks: a potential causative factor for mammalian aging?," *Mechanisms of Ageing and Development*, vol. 129, no. 7-8, pp. 416–424, 2008.

[144] G. A. Garinis, G. T. v. d. Horst, J. Vijg, and J. H. Hoeijmakers, "DNA damage and ageing: new-age ideas for an age-old problem," *Nature Cell Biology*, vol. 10, no. 11, pp. 1241–1247, 2008.

[145] L. Liu and D. L. Keefe, "Ageing-associated aberration in meiosis of oocytes from senescence-accelerated mice," *Human Reproduction*, vol. 17, no. 10, pp. 2678–2685, 2002.

[146] L. Liu and D. L. Keefe, "Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes," *Reproductive Biomedicine Online*, vol. 16, no. 1, pp. 103–112, 2008.

[147] C. Kinugawa, T. Murakami, K. Okamura, and A. Yajima, "Telomerase activity in normal ovaries and premature ovarian failure," *The Tohoku Journal of Experimental Medicine*, vol. 190, no. 3, pp. 231–238, 2000.

[148] T. Yamada-Fukunaga, M. Yamada, T. Hamatani et al., "Age-associated telomere shortening in mouse oocytes," *Reproductive Biology and Endocrinology*, vol. 11, p. 108, 2013.

[149] M. S. Rhyu, "Telomeres, telomerase, and immortality," *Journal of the National Cancer Institute*, vol. 87, no. 12, pp. 884–894, 1995.

[150] D. Keefe, L. Liu, and K. Marquard, "Telomeres and aging-related meiotic dysfunction in women," *Cellular and Molecular Life Sciences*, vol. 64, no. 2, pp. 139–143, 2007.

[151] S. Ozturk, B. Sozen, and N. Demir, "Telomere length and telomerase activity during oocyte maturation and early embryo development in mammalian species," *Molecular Human Reproduction*, vol. 20, no. 1, pp. 15–30, 2014.

[152] G. Hewitt, D. Jurk, F. D. Marques et al., "Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence," *Nature Communications*, vol. 3, p. 708, 2012.

[153] E. K. Suh, A. Yang, A. Kettenbach et al., "p63 protects the female germ line during meiotic arrest," *Nature*, vol. 444, no. 7119, pp. 624–628, 2006.

[154] J. B. Kerr, K. J. Hutt, E. M. Michalak et al., "DNA damage-induced primordial follicle oocyte apoptosis and loss of fertility require TAp63-mediated induction of Puma and Noxa," *Molecular Cell*, vol. 48, no. 3, pp. 343–352, 2012.

[155] P. Marangos, M. Stevense, K. Niaka et al., "DNA damage-induced metaphase I arrest is mediated by the spindle assembly checkpoint and maternal age," *Nature Communications*, vol. 6, article 8706, 2015.

[156] J. K. Collins, S. I. Lane, J. A. Merriman, and K. T. Jones, "DNA damage induces a meiotic arrest in mouse oocytes mediated by the spindle assembly checkpoint," *Nature Communications*, vol. 6, article 8553, 2015.

[157] J. Y. Ma, Y. C. O. Yang, Z. W. Wang et al., "The effects of DNA double-strand breaks on mouse oocyte meiotic maturation," *Cell Cycle*, vol. 12, no. 8, pp. 1233–1241, 2013.

[158] V. D'Angiolella, C. Santarpia, and D. Grieco, "Oxidative stress overrides the spindle checkpoint," *Cell Cycle*, vol. 6, no. 5, pp. 576–579, 2007.

[159] A. Hohn, J. Konig, and T. Grune, "Protein oxidation in aging and the removal of oxidized proteins," *Journal of Proteomics*, vol. 92, pp. 132–159, 2013.

[160] R. S. Sohal, "Role of oxidative stress and protein oxidation in the aging process," *Free Radical Biology & Medicine*, vol. 33, no. 1, pp. 37–44, 2002.

[161] A. Holmgren, C. Johansson, C. Berndt, M. E. Lönn, C. Hudemann, and C. H. Lillig, "Thiol redox control via thioredoxin and glutaredoxin systems," *Biochemical Society Transactions*, vol. 33, no. 6, pp. 1375–1377, 2005.

[162] N. Brot and H. Weissbach, "Peptide methionine sulfoxide reductase: biochemistry and physiological role," *Peptide Science*, vol. 55, no. 4, pp. 288–296, 2000.

[163] S. Boschi-Muller, A. Olry, M. Antoine, and G. Branlant, "The enzymology and biochemistry of methionine sulfoxide reductases," *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, vol. 1703, no. 2, pp. 231–238, 2005.

[164] M. Matsui, M. Oshima, H. Oshima et al., "Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene," *Developmental Biology*, vol. 178, no. 1, pp. 179–185, 1996.

[165] L. Nonn, R. R. Williams, R. P. Erickson, and G. Powis, "The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice," *Molecular and Cellular Biology*, vol. 23, no. 3, pp. 916–922, 2003.

[166] A. Höhn, J. König, and T. Grune, "Protein oxidation in aging and the removal of oxidized proteins," *Journal of Proteomics*, vol. 92, pp. 132–159, 2013.

[167] B. Friguet, "Oxidized protein degradation and repair in ageing and oxidative stress," *FEBS Letters*, vol. 580, no. 12, pp. 2910–2916, 2006.

[168] S. Pizzimenti, E. S. Ciamporcero, M. Daga et al., "Interaction of aldehydes derived from lipid peroxidation and membrane proteins," *Frontiers in Physiology*, vol. 4, p. 242, 2013.

[169] S. Dalleau, M. Baradat, F. Guéraud, and L. Huc, "Cell death and diseases related to oxidative stress: 4-hydroxynonenal (HNE) in the balance," *Cell Death and Differentiation*, vol. 20, no. 12, pp. 1615–1630, 2013.

[170] J. A. Doorn and D. R. Petersen, "Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4-hydroxy-2-nonenal and 4-oxo-2-nonenal," *Chemical Research in Toxicology*, vol. 15, no. 11, pp. 1445–1450, 2002.

[171] L. M. Sayre, D. Lin, Q. Yuan, X. Zhu, and X. Tang, "Protein adducts generated from products of lipid oxidation: focus on HNE and one," *Drug Metabolism Reviews*, vol. 38, no. 4, pp. 651–675, 2006.

[172] H. Esterbauer, R. Schaur Jr., and H. Zollner, "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes," *Free Radical Biology & Medicine*, vol. 11, no. 1, pp. 81–128, 1990.

[173] C. E. Aiken, J. L. Tarry-Adkins, and S. E. Ozanne, "Transgenerational developmental programming of ovarian reserve," *Scientific Reports*, vol. 5, article 16175, 2015.

[174] B. P. Mihalas, G. N. D. Luliis, K. A. Redgrove, E. A. McLaughlin, and B. Nixon, "The lipid peroxidation product 4-hydroxynonenal contributes to oxidative stress-mediated deterioration of the ageing oocyte," *Scientific Reports*, vol. 7, no. 1, article 6247, 2017.

[175] M. A. Baker, A. Weinberg, L. Hetherington et al., "Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function," *Bioliogy of Reproduction*, vol. 92, no. 4, p. 108, 2015.

[176] R. J. Aitken, S. Whiting, G. N. D. Iuliis, S. McClymont, L. A. Mitchell, and M. A. Baker, "Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase," *The Journal of Biological Chemistry*, vol. 287, no. 39, pp. 33048–33060, 2012.

[177] E. G. Bromfield, R. J. Aitken, A. L. Anderson, E. A. McLaughlin, and B. Nixon, "The impact of oxidative stress on chaperone-mediated human sperm-egg interaction," *Human Reproduction*, vol. 30, no. 11, pp. 2597–2613, 2015.

[178] F. Shang, T. R. Nowell Jr, and A. Taylor, "Removal of oxidatively damaged proteins from lens cells by the ubiquitin-proteasome pathway," *Experimental Eye Research*, vol. 73, no. 2, pp. 229–238, 2001.

[179] K. J. A. Davies, "Degradation of oxidized proteins by the 20S proteasome," *Biochimie*, vol. 83, no. 3-4, pp. 301–310, 2001.

[180] R. Shringarpure, T. Grune, J. Mehlhase, and K. J. Davies, "Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome," *The Journal of Biological Chemistry*, vol. 278, no. 1, pp. 311–318, 2003.

[181] A. M. Pickering, A. L. Koop, C. Y. Teoh, G. Ermak, T. Grune, and K. J. Davies, "The immunoproteasome, the 20S proteasome and the PA28$\alpha\beta$ proteasome regulator are oxidative-stress-adaptive proteolytic complexes," *Biochemical Journal*, vol. 432, no. 3, pp. 585–594, 2010.

[182] Q. Ding, K. Reinacker, E. Dimayuga et al., "Role of the proteasome in protein oxidation and neural viability following low-level oxidative stress," *FEBS Letters*, vol. 546, no. 2-3, pp. 228–232, 2003.

[183] S. A. Hussong, R. J. Kapphahn, S. L. Phillips, M. Maldonado, and D. A. Ferrington, "Immunoproteasome deficiency alters retinal proteasome's response to stress," *Journal of Neurochemistry*, vol. 113, no. 6, pp. 1481–1490, 2010.

[184] Q. Ding, S. Martin, E. Dimayuga, A. J. Bruce-Keller, and J. N. Keller, "LMP2 knock-out mice have reduced proteasome activities and increased levels of oxidatively damaged proteins," *Antioxidants & Redox Signaling*, vol. 8, no. 1-2, pp. 130–135, 2006.

[185] F. Shang, X. Gong, H. J. Palmer, T. R. Nowell Jr., and A. Taylor, "Age-related decline in ubiquitin conjugation in response to oxidative stress in the lens," *Experimental Eye Research*, vol. 64, no. 1, pp. 21–30, 1997.

[186] F. Shang and A. Taylor, "Oxidative stress and recovery from oxidative stress are associated with altered ubiquitin conjugating and proteolytic activities in bovine lens epithelial cells," *Biochemical Journal*, vol. 307, Part 1, pp. 297–303, 1995.

[187] E. N. Tsakiri, G. P. Sykiotis, I. S. Papassideri, V. G. Gorgoulis, D. Bohmann, and I. P. Trougakos, "Differential regulation of proteasome functionality in reproductive vs. somatic tissues of Drosophila during aging or oxidative stress," *The FASEB Journal*, vol. 27, no. 6, pp. 2407–2420, 2013.

[188] J. Goudeau and H. Aguilaniu, "Carbonylated proteins are eliminated during reproduction in *C. elegans*," *Aging Cell*, vol. 9, no. 6, pp. 991–1003, 2010.

[189] R. A. Dunlop, U. T. Brunk, and K. J. Rodgers, "Oxidized proteins: mechanisms of removal and consequences of accumulation," *IUBMB Life*, vol. 61, no. 5, pp. 522–527, 2009.

[190] K. J. Rodgers, H. Wang, S. Fu, and R. T. Dean, "Biosynthetic incorporation of oxidized amino acids into proteins and their cellular proteolysis," *Free Radical Biology & Medicine*, vol. 32, no. 8, pp. 766–775, 2002.

[191] I. C. Bathurst, D. M. Errington, R. C. Foreman, J. D. Judah, and R. W. Carrell, "Human Z α1-antitrypsin accumulates intracellularly and stimulates lysosomal activity when synthesised in the Xenopus oocyte," *FEBS Letters*, vol. 183, no. 2, pp. 304–308, 1985.

[192] S. J. Orenstein and A. M. Cuervo, "Chaperone-mediated autophagy: molecular mechanisms and physiological relevance," *Seminars in Cell & Developmental Biology*, vol. 21, no. 7, pp. 719–726, 2010.

[193] D. Glick, S. Barth, and K. F. Macleod, "Autophagy: cellular and molecular mechanisms," *The Journal of Pathology*, vol. 221, no. 1, pp. 3–12, 2010.

[194] W.-X. Ding, H.-M. Ni, W. Gao et al., "Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability," *The American Journal of Pathology*, vol. 171, no. 2, pp. 513–524, 2007.

[195] U. B. Pandey, Z. Nie, Y. Batlevi et al., "HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS," *Nature*, vol. 447, no. 7146, pp. 859–863, 2007.

[196] M. Sugiyama, R. Kawahara-Miki, H. Kawana, K. Shirasuna, T. Kuwayama, and H. Iwata, "Resveratrol-induced mitochondrial synthesis and autophagy in oocytes derived from early antral follicles of aged cows," *Journal of Reproduction and Development*, vol. 61, no. 4, pp. 251–259, 2015.

[197] V. Kirkin, D. G. McEwan, I. Novak, and I. Dikic, "A role for ubiquitin in selective autophagy," *Molecular Cell*, vol. 34, no. 3, pp. 259–269, 2009.

[198] J. A. Johnston, C. L. Ward, and R. R. Kopito, "Aggresomes: a cellular response to misfolded proteins," *The Journal of Cell Biology*, vol. 143, no. 7, pp. 1883–1898, 1998.

[199] Y. Kawaguchi, J. J. Kovacs, A. McLaurin, J. M. Vance, A. Ito, and T. P. Yao, "The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress," *Cell*, vol. 115, no. 6, pp. 727–738, 2003.

[200] A. Iwata, B. E. Riley, J. A. Johnston, and R. R. Kopito, "HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin," *The Journal of Biological Chemistry*, vol. 280, no. 48, pp. 40282–40292, 2005.

[201] V. I. Korolchuk, F. M. Menzies, and D. C. Rubinsztein, "Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems," *FEBS Letters*, vol. 584, no. 7, pp. 1393–1398, 2010.

[202] J. Long, T. R. A. Gallagher, J. R. Cavey et al., "Ubiquitin recognition by the ubiquitin-associated domain of p62 involves a novel conformational switch," *Journal of Biological Chemistry*, vol. 283, no. 9, pp. 5427–5440, 2008.

[203] B. Nixon, E. G. Bromfield, M. D. Dun, K. A. Redgrove, E. A. McLaughlin, and R. J. Aitken, "The role of the molecular chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition," *Asian Journal of Andrology*, vol. 17, no. 4, pp. 568–573, 2015.

[204] F. Ritossa, "Discovery of the heat shock response," *Cell Stress & Chaperones*, vol. 1, no. 2, pp. 97-98, 1996.

[205] H. R. Saibil, "Chaperone machines in action," *Current Opinion in Structural Biology*, vol. 18, no. 1, pp. 35–42, 2008.

[206] C. Marques, W. Guo, P. Pereira et al., "The triage of damaged proteins: degradation by the ubiquitin-proteasome pathway or repair by molecular chaperones," *FASEB Journal*, vol. 20, no. 6, pp. 741–743, 2006.

[207] B. Kalmar and L. Greensmith, "Induction of heat shock proteins for protection against oxidative stress," *Advanced Drug Delivery Reviews*, vol. 61, no. 4, pp. 310–318, 2009.

[208] B. Bercovich, I. Stancovski, A. Mayer et al., "Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70," *Journal of Biological Chemistry*, vol. 272, no. 14, pp. 9002–9010, 1997.

[209] P. Connell, C. A. Ballinger, J. Jiang et al., "The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins," *Nature Cell Biology*, vol. 3, no. 1, pp. 93–96, 2001.

[210] C. P. Liu, J. Fu, F. P. Xu, X. S. Wang, and S. Li, "The role of heat shock proteins in oxidative stress damage induced by Se deficiency in chicken livers," *Biometals*, vol. 28, no. 1, pp. 163–173, 2015.

[211] X. H. Gu, Y. Hao, and X. L. Wang, "Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress1," *Poultry Science*, vol. 91, no. 4, pp. 790–799, 2012.

[212] M. Adachi, Y. Liu, K. Fujii et al., "Oxidative stress impairs the heat stress response and delays unfolded protein recovery," *PLoS One*, vol. 4, no. 11, article e7719, 2009.

[213] R. Kiffin, C. Christian, E. Knecht, and A. M. Cuervo, "Activation of chaperone-mediated autophagy during oxidative stress," *Molecular Biology of the Cell*, vol. 15, no. 11, pp. 4829–4840, 2004.

[214] A. M. Cuervo and J. F. Dice, "Lysosomes, a meeting point of proteins, chaperones, and proteases," *Journal of Molecular Medicine*, vol. 76, no. 1, pp. 6–12, 1998.

[215] T. Grune, B. Catalgol, A. Licht et al., "HSP70 mediates dissociation and reassociation of the 26S proteasome during adaptation to oxidative stress," *Free Radical Biology & Medicine*, vol. 51, no. 7, pp. 1355–1364, 2011.

[216] K. Liberek, A. Lewandowska, and S. Ziętkiewicz, "Chaperones in control of protein disaggregation," *The EMBO Journal*, vol. 27, no. 2, pp. 328–335, 2008.

[217] J. L. Dul, D. P. Davis, E. K. Williamson, F. J. Stevens, and Y. Argon, "Hsp70 and antifibrillogenic peptides promote degradation and inhibit intracellular aggregation of amyloidogenic light chains," *The Journal of Cell Biology*, vol. 152, no. 4, pp. 705–716, 2001.

[218] J. Liu, M. Liu, X. Ye et al., "Delay in oocyte aging in mice by the antioxidant N-acetyl-L-cysteine (NAC)," *Human Reproduction*, vol. 27, no. 5, pp. 1411–1420, 2012.

[219] J. J. Tarin, F. J. Vendrell, J. Ten, and A. Cano, "Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes," *Molecular Human Reproduction*, vol. 4, no. 3, pp. 281–288, 1998.

[220] J. J. Tarin, S. Perez-Albala, and A. Cano, "Oral antioxidants counteract the negative effects of female aging on oocyte quantity and quality in the mouse," *Molecular Reproduction and Development*, vol. 61, no. 3, pp. 385–397, 2002.

[221] J. Tarin, J. Ten, F. J. Vendrell, M. N. de Oliveira, and A. Cano, "Effects of maternal ageing and dietary antioxidant supplementation on ovulation, fertilisation and embryo development in vitro in the mouse," *Reproduction Nutrition Development*, vol. 38, no. 5, pp. 499–508, 1998.

[222] Y. J. Li, Z. Han, L. Ge et al., "C-phycocyanin protects against low fertility by inhibiting reactive oxygen species in aging mice," *Oncotarget*, vol. 7, no. 14, pp. 17393–17409, 2016.

[223] A. Pacchiarotti, "Antioxidative capacity of melatonin in follicular fluid of aged IVF patients: beneficial effects on oocytes and embryo," *Journal of Gynecology & Neonatal Biology*, vol. 1, no. 2, pp. 1–5, 2015.

[224] H. Tamura, M. Kawamoto, S. Sato et al., "Long term melatonin treatment delays ovarian aging," *Journal of Pineal Research*, vol. 62, no. 2, 2017.

[225] F. Wang, X. Tian, Y. Zhou et al., "Melatonin improves the quality of *in vitro* produced (IVP) bovine embryos: implications for blastocyst development, cryotolerance, and modifications of relevant gene expression," *PLoS One*, vol. 9, no. 4, article e93641, 2014.

[226] J. J. Tarin, S. Perez-Albala, J. F. Pertusa, and A. Cano, "Oral administration of pharmacological doses of vitamins C and E reduces reproductive fitness and impairs the ovarian and

uterine functions of female mice," *Theriogenology*, vol. 57, no. 5, pp. 1539–1550, 2002.

[227] M. Takami, S. L. Preston, V. A. Toyloy, and H. R. Behrman, "Antioxidants reversibly inhibit the spontaneous resumption of meiosis," *American Journal of Physiology*, vol. 276, no. 4, Part 1, pp. E684–E688, 1999.

[228] M. Takami, S. L. Preston, and H. R. Behrman, "Eicosatetraynoic and eicosatriynoic acids, lipoxygenase inhibitors, block meiosis via antioxidant action," *American Journal of Physiology Cell Physiology*, vol. 278, no. 4, pp. C646–C650, 2000.

[229] A. S. Reznichenko, C. Huyser, and M. S. Pepper, "Mitochondrial transfer: implications for assisted reproductive technologies," *Applied & Translational Genomics*, vol. 11, pp. 40–47, 2016.

[230] S. Mitalipov and D. P. Wolf, "Clinical and ethical implications of mitochondrial gene transfer," *Trends in Endocrinology and Metabolism*, vol. 25, no. 1, pp. 5–7, 2014.

[231] J. Zhang, "Revisiting germinal vesicle transfer as a treatment for aneuploidy in infertile women with diminished ovarian reserve," *Journal of Assisted Reproduction and Genetics*, vol. 32, no. 2, pp. 313–317, 2015.

[232] S. Nakagawa and G. FitzHarris, "Intrinsically defective microtubule dynamics contribute to age-related chromosome segregation errors in mouse oocyte meiosis-I," *Current Biology*, vol. 27, no. 7, pp. 1040–1047, 2017.

[233] J. Zhang, C. W. Wang, L. Krey et al., "In vitro maturation of human preovulatory oocytes reconstructed by germinal vesicle transfer," *Fertility and Sterility*, vol. 71, no. 4, pp. 726–731, 1999.

[234] G. D. Palermo, T. Takeuchi, and Z. Rosenwaks, "Technical approaches to correction of oocyte aneuploidy," *Human Reproduction*, vol. 17, no. 8, pp. 2165–2173, 2002.

[235] G. Barja, "Aging in vertebrates, and the effect of caloric restriction: a mitochondrial free radical production-DNA damage mechanism?," *Biological Reviews*, vol. 79, no. 2, pp. 235–251, 2004.

[236] R. S. Sohal, H. H. Ku, S. Agarwal, M. J. Forster, and H. Lal, "Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse," *Mechanisms of Ageing and Development*, vol. 74, no. 1-2, pp. 121–133, 1994.

[237] G. Barja, "Endogenous oxidative stress: relationship to aging, longevity and caloric restriction," *Ageing Research Reviews*, vol. 1, no. 3, pp. 397–411, 2002.

[238] R. Gredilla, A. Sanz, M. Lopez-Torres, and G. Barja, "Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart," *FASEB Journal*, vol. 15, no. 9, pp. 1589–1591, 2001.

[239] J. A. Mattison, R. J. Colman, T. M. Beasley et al., "Caloric restriction improves health and survival of rhesus monkeys," *Nature Communications*, vol. 8, article 14063, 2017.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7265238, 7 pages
https://doi.org/10.1155/2017/7265238

Review Article

Oxidative Stress and Endometriosis: A Systematic Review of the Literature

Gennaro Scutiero,[1] Piergiorgio Iannone,[1] Giulia Bernardi,[1] Gloria Bonaccorsi,[1] Savino Spadaro,[2] Carlo Alberto Volta,[2] Pantaleo Greco,[1] and Luigi Nappi[3]

[1]*Department of Morphology, Surgery and Experimental Medicine, Section of Obstetrics and Gynecology, Azienda Ospedaliero-Universitaria S. Anna, University of Ferrara, Via Aldo Moro, 8, 44121 Cona, Ferrara, Italy*
[2]*Department of Morphology, Surgery and Experimental Medicine, Section of Anaesthesia and Intensive Care University of Ferrara, Azienda Ospedaliero-Universitaria S. Anna, Via Aldo Moro, 8, 44121 Cona, Ferrara, Italy*
[3]*Department of Medical and Surgical Sciences, Institute of Obstetrics and Gynecology, University of Foggia, Viale L. Pinto, 71100 Foggia, Italy*

Correspondence should be addressed to Piergiorgio Iannone; pg.iannone88@gmail.com

Received 23 May 2017; Revised 22 June 2017; Accepted 24 August 2017; Published 19 September 2017

Academic Editor: Victor M. Victor

Endometriosis is one of the most common gynaecologic diseases in women of reproductive age. It is characterized by the presence of endometrial tissue outside the uterine cavity. The women affected suffer from pelvic pain and infertility. The complex etiology is still unclear and it is based on three main theories: retrograde menstruation, coelomic metaplasia, and induction theory. Genetics and epigenetics also play a role in the development of endometriosis. Recent studies have put the attention on the role of oxidative stress, defined as an imbalance between reactive oxygen species (ROS) and antioxidants, which may be implicated in the pathophysiology of endometriosis causing a general inflammatory response in the peritoneal cavity. Reactive oxygen species are intermediaries produced by normal oxygen metabolism and are inflammatory mediators known to modulate cell proliferation and to have deleterious effects. A systematic review was performed in order to clarify the different roles of oxidative stress and its role in the development of endometriosis. Several issues have been investigated: iron metabolism, oxidative stress markers (in the serum, peritoneal fluid, follicular fluid, peritoneal environment, ovarian cortex, and eutopic and ectopic endometrial tissue), genes involved in oxidative stress, endometriosis-associated infertility, and cancer development.

1. Introduction

Endometriosis is an estrogen-dependent pelvic inflammatory disease characterized by implantation and growth of endometrial tissue (glands and stroma) outside the uterine cavity [1]. It affects about 10–15% of women of reproductive age [2, 3]. The most common symptoms of the disease are pelvic pain and infertility [1]. In fact, the prevalence of endometriosis in women with pelvic pain ranges from 30 to 45% of infertile population [4]. However, endometriosis can be also asymptomatic or accompanied by symptoms such as dysmenorrhea and dyspareunia [3, 5, 6]. The etiology of endometriosis is still unclear: the implantation theory of Sampson, the coelomic metaplasia theory of Mayer, and the theory of induction are the three classic theories that have tried to designate the definitive pathogenetic mechanism of endometriosis but they have failed to establish it [7, 8]. Recent studies have addressed the role of other factors in the development of endometriotic lesions such as familiar tendency and genetic predisposition [9]. It is now widely accepted that oxidative stress, defined as an imbalance between reactive oxygen species (ROS) and antioxidants, may be implicated in the pathophysiology of endometriosis causing a general inflammatory response in the peritoneal cavity [10–12]. Reactive oxygen species are intermediaries produced by normal oxygen metabolism and are inflammatory mediators known to modulate cell proliferation and to have deleterious effects [13]. Indeed, cells have developed a

wide range of antioxidant system, such as superoxide dismutase, catalase and glutathione peroxidase, and vitamin E and vitamin C, to limit ROS production, inactivate them, and repair cell damage; however, oxidative stress may occur when the balance between ROS production and antioxidant defence is disrupted [14]. Macrophages, erythrocytes, and apoptotic endometrial tissue that transplant into the peritoneal cavity through retrograde menstruation are well known inducers of oxidative stress; therefore, peritoneal production of ROS may be involved in endometriosis. Indeed, activated macrophages play an important role in the degradation of erythrocytes that release prooxidant and proinflammatory factors such as heme and iron, implicated in the formation of deleterious ROS [15].

2. Materials and Methods

A review of the literature was conducted in order to identify the most relevant studies reported in the English language. We searched PubMed MEDLINE electronic database (https://www.ncbi.nlm.nih.gov/pubmed) published until March 2017. The keywords used were as follows: "endometriosis," "oxidative stress," "oxidative stress markers," "reactive oxygen species," "inflammation," and "iron." Different combinations of the terms were used. Moreover, references in each article were searched to identify potentially missed studies.

3. Results and Discussion

3.1. Role of Iron. Recent findings have put the attention on the role of altered iron metabolism in the endometriosis development [16]. The presence of iron overload in the different components of the peritoneal cavity of endometriosis patients has been widely studied; however, it remains strongly localized in the pelvic cavity and does not affect body iron content [16].

Higher levels of iron, ferritin, and haemoglobin have been found in the peritoneal fluid of affected women than controls [17]. The stroma of endometriotic lesions and peritoneum also revealed the presence of iron conglomerates. Peritoneal iron overload may be a consequence of increased influx caused by erythrocyte degradation, resulting either from more abundant menstrual reflux or bleeding lesions or from a deficiency in the peritoneal iron metabolism system [14].

Iron metabolism by macrophages appears to be enhanced in endometriosis. In fact, siderophages, macrophages storing iron, are heavily laden with hemosiderin inside the pelvic cavity [17]. Moreover, macrophages express more transferrin receptors and are more haptoglobin-saturated [14].

Overwhelming iron can act as a catalyst in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$) to potentiate oxygen and nitrogen toxicity by the generation of a wide range of ROS, inducing oxidative injury to cells [17].

Oxidative stress is responsible for local destruction of the peritoneal mesothelium, producing adhesions for ectopic endometrial cells. Iron-binding protein haemoglobin has been identified as one of the menstrual effluent factors potentially harmful to mesothelium, leading to adhesion formation [14].

Defrere et al. showed that epithelial cells in endometriotic lesions increase the proliferative activity after erythrocyte injection in murine model, whereas desferrioxamine administration, an iron chelator, inhibits this process, suggesting that iron may contribute to endometriotic lesion growth [18].

Nuclear factor-kappa B (NF-kappa B) is a transcriptional factor that plays a role in the immune and inflammatory response. In vivo and in vitro studies have demonstrated its inflammatory activation in endometriotic cells. ROS production by iron overload induces an increase of NF-kappa B in peritoneal macrophages, leading to proinflammatory, growth, and angiogenic factors in endometriosis women than healthy controls [14].

3.2. Oxidative Stress Markers. The progression of endometriosis is clearly related to oxidative stress. The connection between endometriosis and the ROS production is widely accepted and deeply studied [19].

In endometriotic cells as in tumor cells, the increased production of ROS is associated with an increase in the proliferation rate [20]. For twenty years, researchers have paid attention to the oxidative stress markers in endometriotic disease. These markers studied have been collected from different samples, which can be divided in 5 main groups:

(i) Serum

(ii) Peritoneal fluid

(iii) Follicular fluid

(iv) Ovarian cortex and endometrial tissue (ectopic and eutopic)

3.2.1. Serum. Women affected by endometriosis show a higher level of oxidative stress markers than women who are not affected.

Heat shock proteins (HSPs) are intracellular proteins induced to protect cells from various insults during stress status caused by infection or inflammation. HSP70 is a stress-inducible member of HSP family. HSP70 is a chaperone protein which prevents abnormal interactions during protein synthesis. Lambrinoudaki et al. showed that women with endometriosis have a higher serum level of HSP70 [21]. The expression increase is also present in the eutopic endometrium of women affected [22].

Lipid metabolism and its connections with inflammation factors might play a role in the genesis of oxidative stress. Lipid levels have been evaluated in women with endometriosis resulting in a higher level of triglyceride, total cholesterol, and low-density lipoprotein (LDL) in serum of affected women. On the other side, lower levels of high-density lipoprotein (HDL) have been observed [23].

The increase of lipid peroxides can be considered as an oxidative stress marker. Malondialdehyde (MDA) has been evaluated as an index of lipid peroxides. Nasiri et al. observed a higher level of MDA in the serum of women with endometriosis than healthy controls [24].

Lipid peroxidation leads also to the generation of lipid hydroperoxides (LOOHs). These compounds derive from unsaturated phospholipids, glycolipids, and cholesterol. Women with endometriosis show a higher level of LOOHs than controls [23].

The concentration of vitamin E, which is a natural antioxidant, is higher in endometriosis women serum, but this finding has not been clearly explained [25, 26].

Catalase is described as an intracellular antioxidant enzyme in hepatic pathogenesis; its concentration is found to be higher in endometriosis women than healthy controls [27].

Paraoxonase-1 (PON-1) is a HDL-associated antioxidant enzyme, which is considered as a strong predictor of coronary artery disease (CAD). PON-1 shows a significant decreased activity in the serum of women with endometriosis [23, 25]. Though its activity is decreased, there is no correlation between PON-1 and the stage of the disease [28].

Another enzyme involved in oxidative stress is superoxide dismutase (SOD). SOD is an important antioxidant system. It catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen. SDO shows a decreased activity in the plasma of women of endometriosis, suggesting a decreased antioxidant capacity in these women [26].

Another oxidative stress marker which appears to be diminished in the serum of women with endometriosis is 8-F2-isoprostane [25]. Thiols are molecules, which can react with oxidizing agents and mediate the formation of reversible disulphide bonds. Turgut et al. studied the antioxidant system in women with endometriosis and showed that the total antioxidant system (TAS) and the native thiol levels in the serum of women with endometriosis are significantly lower than those of controls [27, 29]. They also described higher levels of copper and ceruloplasmin in the serum of women with endometriosis, although Turkyilmaz found lower levels in contrast to the previous finding [27]. These findings support the hypothesis that low antioxidant levels are linked to the pathogenesis of endometriosis.

Andrisani et al. examined the possible involvement of carbonic anhydrase activation in response to oxidative stress in red blood cells of women with endometriosis. They found an increased enzyme activity together with a membrane increase of glutathionylated protein and a cytosolic decrease of glutathione content than control serum. The oxidation-induced activation of carbonic anhydrase is also positively correlated to glutathione content in red blood cells of women with endometriosis [30]. Most of these studies are either observational or case-control studies; moreover, measurement of biomarkers is subject to interlaboratory variations and interobserver differences. A uniform method should be used so that the results can be compared across studies.

3.2.2. Peritoneal Fluid. Peritoneal oxidative stress is currently thought to be a major constituent of the endometriosis-associated inflammation. The development of peritoneal endometriotic lesions involve multiple factors based on immunological and inflammatory etiology. Peritoneal oxidative stress regulates expression of numerous genes encoding immunoregulators, cytokines, and cell adhesion molecules. Peritoneal concentration of macrophages appears to be higher in women with endometriosis and they may release prostaglandins, cytokines, growth factors, and other enzymes. It has been theorized that macrophages play an important role in the initiation, maintenance, and progression of endometriotic disease [14, 31].

Santulli et al. explored the peritoneal fluid protein oxidative status in women with endometriosis. They found higher levels of advanced oxidation protein products (AOPP) than controls. In the same way, concentrations of nitrates and nitrites are higher in affected patients than controls. Moreover, AOPP and nitrates/nitrites are higher in patients with deep infiltrating endometriosis, especially those with intestinal involvement [32]. The higher concentration of nitrates probably derives from the augmented nitric oxide (NO) activity of the peritoneal macrophages, as already described by Osborn, who observed also a higher activity of nitric oxide synthase 2 (NOS 2) [33]. Interestingly, they also observed a significant correlation between pelvic pain symptom scores and peritoneal protein oxidative stress markers in women with endometriosis [32]. Oxidative mechanisms involving LDL are higher in women with endometriosis. It consists in the oxidation of polyunsaturated fatty acid containing lipids of the lipoprotein. Murphy et al. showed higher levels of oxidized LDL (ox-LDL) than controls in peritoneal fluid [34]. Polak et al. noted that ox-LDL concentrations are higher in women with severe endometriosis [35].

MDA and LOOHs peritoneal levels are higher in women with endometriosis [36]. As a confirmation of this hypothesis, Mier-Cabrera et al. observed a decrease in the concentrations of MDA and LOOHs in women with endometriosis both in serum and peritoneal fluid after the supplementation of vitamins C and E, natural antioxidants, whose levels are low in affected women. These findings support the hypothesis of decreased antioxidant activity in peritoneal fluid of women with endometriosis [36–38]. Other oxidative stress markers found higher in peritoneal fluid of women with endometriosis are 8-hydroxy-2-deoxyguanosine, 8-isoprostane, 8-iso prostaglandin F2α, and 25-hydroxycholesterol [35, 39].

3.2.3. Follicular Fluid. Follicular fluid (FF) plays a crucial role in the reproductive performance of oocyte. An imbalance between ROS and antioxidant systems in the FF could be responsible for abnormal oocyte development, causing DNA, cytoskeleton, and cell membrane damage, which would result in lower egg quality and endometriosis-associated infertility [24, 26].

Women with endometriosis show higher levels of lipid peroxide (LPO) and lower levels of total antioxidant capacity (TAC) than controls [24].

Singh et al. widely evaluated the FF oxidative stress of women with endometriosis. They found higher levels of ROS, MDA, and NO in the affected women FF. High concentrations of ROS and NO were found to be corresponding to immature oocytes and poor-quality embryos. The antioxidant system is less active in women with endometriosis. Antioxidant enzymatic activity, such as SOD, catalase, glutathione peroxidase, and glutathione reductase are found to be

lower in studied FF. Also, nonenzymatic activity is less expressed. Vitamins A, C, and E concentration in FF of endometriosis women are significantly decreased than controls [26, 40]. Interestingly, however, the oxidative stress and antioxidant system in FF in patients with unilateral endometrioma is similar to those who do not have endometrioma [41].

3.2.4. Ovarian Cortex and Endometrial Tissue. Oxidative stress induces ovarian damage. In fact, granulosa cells in patients with endometriosis show more signs of oxidative DNA damage than controls. Granulosa cells of women with endometriosis exhibit higher incidence of apoptotic bodies and nytrotyrosine than controls [42]. Ovarian cortex of women affected is also damaged by oxidative stress. Matsuzaki et al. demonstrated that ovarian cortex of women with endometriosis express higher levels of 8-hydroxy-2-deoxyguanosine than those of women with dermoid and serous cysts [43].

Oxidative stress activity and ROS levels are high in endometriosis, and their main effects on cells are translated into damage and proliferation [44]. Ngô et al. have evaluated oxidative stress level from biopsies of eutopic endometrium and endometriotic lesions. They observed a higher concentration of superoxide anions in both samples, whereas hydrogen peroxide is higher in endometriotic cells than in controls and endometrial cells. The detoxification of hydrogen peroxide is achieved through two different enzymatic systems: glutathione peroxidase and catalase. Glutathione peroxidase expression is higher in endometriotic cells than in controls, whereas catalase concentration is lower in endometriotic cells than in controls. Also SDO activity appears augmented in endometriotic lesions than healthy controls [45]. These findings show the role of oxidative stress in the control of endometriotic cell proliferation [20].

Oxidative stress plays its role through mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) pathway for survival and proliferation of endometriotic lesions through expression and action of c-Fos and c-Jun. The ERK signalling pathway is involved in the proliferative response induced by endogenous ROS [44, 46]. Activation of ERK pathway and its connection to deep infiltrating endometriosis (DIE) is established through a specific inhibitor of phosphorylation of the protein tyrosine kinase ERK [47]. The endogenous activation of the mammalian target of rapamycin (mTOR)/AKT pathways is also involved in the development of DIE [44]. Among oxidative stress markers, 8-hydroxy-2-deoxyguanosine and MDA are higher in endometriotic lesions than healthy controls [48]. MDA levels are also positively correlated with plasma 17β-estradiol (E2) concentrations in the ectopic endometrioma [45]. The endometrial cells of endometriosis women subjected to both E2 and hydrogen peroxide show increased ERK phosphorylation. These results show the connection between E2 and apoptosis resistance and endometriotic lesion progression [46].

Toll-like receptors (TLR) are endogenous ligands of endometrium. TLR3 and TLR 4 are predominantly expressed in healthy endometrium and in endometriotic tissue; TLR 4 seems to promote cell growth in endometriosis [49].

3.3. Oxidative Stress and Endometriosis-Associated Infertility. The association between endometriosis and infertility is well established in literature. The monthly fecundity rate in infertile women with endometriosis is from 2 to 10%, whereas in healthy women, the rate is between 15 and 20% [50]. In the literature, it is well explained how ROS might affect a variety of physiologic functions such as oocyte maturation, ovarian steroidogenesis, ovulation, implantation, formation of blastocyst, luteolysis, and luteal maintenance in pregnancy. Oxidative stress affects fertility in women with endometriosis in either natural or assisted conception [11]. The imbalance between ROS and antioxidant mechanisms leads to oxidative stress status in peritoneal environment, follicular fluid, and ovary surrounding, which can partly explain the infertility status associated to endometriosis.

3.4. Oxidative Stress and Genes. The roles of molecular alteration such as genomic instability and cell survival are debated aspects of the pathogenesis of endometriosis. Recent genetic studies have put the attention on several elements which can be related to oxidative stress, such as cell cycle checkpoint sensors, hepatocyte nuclear factor (HNF), forkhead transcription factor (FOX), and microRNAs [51].

The role of ROS, iron, and superoxide might be an epigenetic modulation. Superoxide plays an important role in epigenetic process under physiologic and pathologic conditions and regulates main epigenetic processes of DNA methylation, histone methylation, and histone acetylation [52].

Recent studies have observed aberrant histone modifications in the promoter regions of the cell cycle checkpoint kinase genes. Thus, oxidative stress stimulates cell cycle progression and enhances cellular transformation [51].

Ito et al. suggested that iron, heme, and hemoglobin accumulation leads to oxidative stress causing DNA hypermethylation and histone modifications. DNA hypermethylation is linked to defective endometrium development in endometriosis patient [52].

HFN overexpression is linked to endometriotic cell survival probably through detoxification and antiapoptotic activated pathways [53].

FOX activity is controlled by ROS-induced posttranslational modifications. Loss of FOX disables the ability of cells to arrest at checkpoint, thereby facilitating lesion development. FOX levels are lower in endometriosis women than in controls [51].

MicroRNAs are a class of noncoding small RNAs that regulates hundreds of gene expression via both posttranslational inhibition and mRNA degradation. MicroRNAs control development, differentiation, apoptosis, proliferation, and cell survival. MicroRNAs have been studied in endometriosis with up- and downregulated levels in women affected. MicroRNA dysfunction results in immune alterations and inflammatory cytokine production [51].

Moreover, microRNAs responsible for targeting nociceptive and inflammatory molecules are downregulated in women with endometriosis, thus playing a role in the etiology of endometriotic pain [54].

Hevir et al. evaluated several genes expression involved in oxidative metabolism of estrogens. Increased expression of

CYP1A1, CYP3A7, and COMT was observed in endometriosis. Expression of SULT1E1, SULT2B1, UGT2B7, NQO1, and GSTP1 was decreased. These findings exhibit a disturbed balance between phase I and II metabolizing enzymes in endometriosis, leading to excessive hydroxy-estrogen and altered ROS formation, and stimulation of ectopic endometrium proliferation [55].

3.5. Oxidative Stress and Endometriosis-Associated Cancer Development. Oxidative stress within endometriosis is likely to contribute to the malignant transformation process. Data from literature show at least a two-step explanation which might lead to cancer. The first step is as follows: the generation of oxidative stress-induced DNA damage evokes enhanced cell apoptosis and survival in endometriotic cells. The second step is as follows: cancer progression may be associated with persistent antioxidant production favouring a protumoral microenvironment [56]. Oxidant/antioxidant balance function is a double-edged sword, promoting cell death or carcinogenesis. Upregulation of antioxidant functions in endometriosis may result in restoration of cell survival and subsequent malignant transformation [57].

4. Conclusions

Reactive oxygen species have an important role in modulating many physiological functions in reproduction as well as in conditions such as endometriosis and infertility; a delicate balance exists between ROS and antioxidants in the female reproductive process that maintains redox homeostasis [58]. Oxidative stress occurs when this balance between ROS production and antioxidant defence is disrupted, and it may be due to either inadequate antioxidant protection or excess production of ROS.

Various lines of evidence support the role of oxidative stress in the development and progression of endometriosis [12, 14, 15, 59, 60]. This observation may open the way to evaluation of therapeutic approaches targeting oxidative imbalance: the oxidative stress status may represent the key to treat and, eventually, to prevent endometriosis. In particular, in the future, clinical trials will help to better clarify the efficacy of antioxidants as potential therapies of endometriosis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

The project was designed and executed by all authors. The paper was revised and approved by all authors.

Acknowledgments

The present study was supported by the Department of Morphology, Surgery and Experimental Medicine, Azienda Ospedaliero-Universitaria S. Anna, University of Ferrara, Via Aldo Moro, 8, 44121 Cona, Ferrara.

References

[1] S. Kennedy, A. Bergqvist, C. Chapron et al., "ESHRE guideline for the diagnosis and treatment of endometriosis," *Human Reproduction*, vol. 20, no. 10, pp. 2698–2704, 2005.

[2] A. Augoulea, A. Alexandrou, M. Creatsa, N. Vrachnis, and I. Lambrinoudaki, "Pathogenesis of endometriosis: the role of genetics, inflammation and oxidative stress," *Archives of Gynecology and Obstetrics*, vol. 286, no. 1, pp. 99–103, 2012.

[3] N. Singh, K. Lata, M. Naha, N. Malhotra, A. Tiwari, and P. Vanamail, "Effect of endometriosis on implantation rates when compared to tubal factor in fresh non donor in vitro fertilization cycles," *Journal of Human Reproductive Sciences*, vol. 7, no. 2, pp. 143–147, 2014.

[4] D. E. Houston, "Evidence for the risk of pelvic endometriosis by age, race and socioeconomic status," *Epidemiologic Reviews*, vol. 6, pp. 167–191, 1984.

[5] P. Bellelis, J. A. Dias Jr., S. Podgaec, M. Gonzales, E. C. Baracat, and M. S. Abrao, "Epidemiological and clinical aspects of pelvic endometriosis-a case series," *Revista da Associacao Medica Brasileira (1992)*, vol. 56, no. 4, pp. 467–471, 2010.

[6] P. G. Signorile and A. Baldi, "Endometriosis: new concepts in the pathogenesis," *The International Journal of Biochemistry & Cell Biology*, vol. 42, no. 6, pp. 778–780, 2010.

[7] J. A. Sampson, "Metastatic or embolic endometriosis, due to the menstrual dissemination of endometrial tissue into the venous circulation," *The American Journal of Pathology*, vol. 3, no. 2, pp. 93–110.43, 1927.

[8] D. Vinatier, G. Orazi, M. Cosson, and P. Dufour, "Theories of endometriosis," *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, vol. 96, no. 1, pp. 21–34, 2001.

[9] S. Kennedy, H. Mardon, and D. Barlow, "Familial endometriosis," *Journal of Assisted Reproduction and Genetics*, vol. 12, no. 1, pp. 32–34, 1995.

[10] G. Christodoulakos, A. Augoulea, I. Lambrinoudaki, V. Sioulas, and G. Creatsas, "Pathogenesis of endometriosis: the role of defective 'immunosurveillance'," *The European Journal of Contraception & Reproductive Health Care*, vol. 12, no. 3, pp. 194–202, 2007.

[11] A. Augoulea, G. Mastorakos, I. Lambrinoudaki, G. Christodoulakos, and G. Creatsas, "The role of the oxidative-stress in the endometriosis-related infertility," *Gynecological Endocrinology*, vol. 25, no. 2, pp. 75–81, 2009.

[12] A. Agarwal, S. Gupta, and R. K. Sharma, "Role of oxidative stress in female reproduction," *Reproductive Biology and Endocrinology*, vol. 3, p. 28, 2005.

[13] S. Gupta, J. M. Goldberg, N. Aziz, E. Goldberg, N. Krajcir, and A. Agarwal, "Pathogenic mechanisms in endometriosis-associated infertility," *Fertility and Sterility*, vol. 90, no. 2, pp. 247–257, 2008.

[14] J. C. Lousse, A. Van Langendonckt, S. Defrere, R. G. Ramos, S. Colette, and J. Donnez, "Peritoneal endometriosis is an inflammatory disease," *Frontiers in Bioscience (Elite Edition)*, vol. 4, pp. 23–40, 2012.

[15] A. Van Langendonckt, F. Casanas-Roux, and J. Donnez, "Oxidative stress and peritoneal endometriosis," *Fertility and Sterility*, vol. 77, no. 5, pp. 861–870, 2002.

[16] M. M. Wolfler, I. M. Meinhold-Heerlein, C. Henkel et al., "Reduced hemopexin levels in peritoneal fluid of patients with endometriosis," *Fertility and Sterility*, vol. 100, no. 3, pp. 777–781, 2013.

[17] S. Defrere, J. C. Lousse, R. Gonzalez-Ramos, S. Colette, J. Donnez, and A. Van Langendonckt, "Potential involvement of iron in the pathogenesis of peritoneal endometriosis," *Molecular Human Reproduction*, vol. 14, no. 7, pp. 377–385, 2008.

[18] S. Defrere, A. Van Langendonckt, S. Vaesen et al., "Iron overload enhances epithelial cell proliferation in endometriotic lesions induced in a murine model," *Human Reproduction*, vol. 21, no. 11, pp. 2810–2816, 2006.

[19] L. F. Carvalho, A. N. Samadder, A. Agarwal, L. F. Fernandes, and M. S. Abrao, "Oxidative stress biomarkers in patients with endometriosis: systematic review," *Archives of Gynecology and Obstetrics*, vol. 286, no. 4, pp. 1033–1040, 2012.

[20] C. Ngo, C. Chereau, C. Nicco, B. Weill, C. Chapron, and F. Batteux, "Reactive oxygen species controls endometriosis progression," *The American Journal of Pathology*, vol. 175, no. 1, pp. 225–234, 2009.

[21] I. V. Lambrinoudaki, A. Augoulea, G. E. Christodoulakos et al., "Measurable serum markers of oxidative stress response in women with endometriosis," *Fertility and Sterility*, vol. 91, no. 1, pp. 46–50, 2009.

[22] H. Ota, S. Igarashi, J. Hatazawa, and T. Tanaka, "Distribution of heat shock proteins in eutopic and ectopic endometrium in endometriosis and adenomyosis," *Fertility and Sterility*, vol. 68, no. 1, pp. 23–28, 1997.

[23] F. F. Verit, O. Erel, and N. Celik, "Serum paraoxonase-1 activity in women with endometriosis and its relationship with the stage of the disease," *Human Reproduction*, vol. 23, no. 1, pp. 100–104, 2008.

[24] N. Nasiri, A. Moini, P. Eftekhari-Yazdi, L. Karimian, R. Salman-Yazdi, and A. Arabipoor, "Oxidative stress statues in serum and follicular fluid of women with endometriosis," *Cell Journal*, vol. 18, no. 4, pp. 582–587, 2017.

[25] L. W. Jackson, E. F. Schisterman, R. Dey-Rao, R. Browne, and D. Armstrong, "Oxidative stress and endometriosis," *Human Reproduction*, vol. 20, no. 7, pp. 2014–2020, 2005.

[26] L. Prieto, J. F. Quesada, O. Cambero et al., "Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis," *Fertility and Sterility*, vol. 98, no. 1, pp. 126–130, 2012.

[27] E. Turkyilmaz, M. Yildirim, B. D. Cendek et al., "Evaluation of oxidative stress markers and intra-extracellular antioxidant activities in patients with endometriosis," *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, vol. 199, pp. 164–168, 2016.

[28] F. B. Bragatto, C. P. Barbosa, D. M. Christofolini et al., "There is no relationship between paraoxonase serum level activity in women with endometriosis and the stage of the disease: an observational study," *Reproductive Health*, vol. 10, p. 32, 2013.

[29] A. Turgut, A. Ozler, N. Y. Goruk, S. Y. Tunc, O. Evliyaoglu, and T. Gul, "Copper, ceruloplasmin and oxidative stress in patients with advanced-stage endometriosis," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 11, pp. 1472–1478, 2013.

[30] A. Andrisani, G. Dona, A. M. Brunati et al., "Increased oxidation-related glutathionylation and carbonic anhydrase activity in endometriosis," *Reproductive Biomedicine Online*, vol. 28, no. 6, pp. 773–779, 2014.

[31] J. Donnez, M. M. Binda, O. Donnez, and M. M. Dolmans, "Oxidative stress in the pelvic cavity and its role in the pathogenesis of endometriosis," *Fertility and Sterility*, vol. 106, no. 5, pp. 1011–1017, 2016.

[32] P. Santulli, S. Chouzenoux, M. Fiorese et al., "Protein oxidative stress markers in peritoneal fluids of women with deep infiltrating endometriosis are increased," *Human Reproduction*, vol. 30, no. 1, pp. 49–60, 2015.

[33] B. H. Osborn, A. F. Haney, M. A. Misukonis, and J. B. Weinberg, "Inducible nitric oxide synthase expression by peritoneal macrophages in endometriosis-associated infertility," *Fertility and Sterility*, vol. 77, no. 1, pp. 46–51, 2002.

[34] A. A. Murphy, N. Santanam, and S. Parthasarathy, "Endometriosis: a disease of oxidative stress?," *Seminars in Reproductive Endocrinology*, vol. 16, no. 4, pp. 263–273, 1998.

[35] G. Polak, B. Barczynski, W. Kwasniewski et al., "Low-density lipoproteins oxidation and endometriosis," *Mediators of Inflammation*, vol. 2013, Article ID 624540, 4 pages, 2013.

[36] J. Mier-Cabrera, L. Jimenez-Zamudio, E. Garcia-Latorre, O. Cruz-Orozco, and C. Hernandez-Guerrero, "Quantitative and qualitative peritoneal immune profiles, T-cell apoptosis and oxidative stress-associated characteristics in women with minimal and mild endometriosis," *BJOG: An International Journal of Obstetrics and Gynaecology*, vol. 118, no. 1, pp. 6–16, 2011.

[37] J. Mier-Cabrera, M. Genera-Garcia, J. De la Jara-Diaz, O. Perichart-Perera, F. Vadillo-Ortega, and C. Hernandez-Guerrero, "Effect of vitamins C and E supplementation on peripheral oxidative stress markers and pregnancy rate in women with endometriosis," *International Journal of Gynaecology and Obstetrics*, vol. 100, no. 3, pp. 252–256, 2008.

[38] D. Vinatier, M. Cosson, and P. Dufour, "Is endometriosis an endometrial disease?," *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, vol. 91, pp. 113–125, 2000, Ireland.

[39] I. Sharma, L. K. Dhaliwal, S. C. Saha, S. Sangwan, and V. Dhawan, "Role of 8-iso-prostaglandin F2alpha and 25-hydroxycholesterol in the pathophysiology of endometriosis," *Fertility and Sterility*, vol. 94, no. 1, pp. 63–70, 2010.

[40] A. K. Singh, R. Chattopadhyay, B. Chakravarty, and K. Chaudhury, "Markers of oxidative stress in follicular fluid of women with endometriosis and tubal infertility undergoing IVF," *Reproductive Toxicology*, vol. 42, pp. 116–124, 2013.

[41] K. Nakagawa, M. Hisano, R. Sugiyama, and K. Yamaguchi, "Measurement of oxidative stress in the follicular fluid of infertility patients with an endometrioma," *Archives of Gynecology and Obstetrics*, vol. 293, no. 1, pp. 197–202, 2016.

[42] P. T. Goud, A. P. Goud, N. Joshi, E. Puscheck, M. P. Diamond, and H. M. Abu-Soud, "Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis," *Fertility and Sterility*, vol. 102, no. 1, pp. 151–159.e5, 2014.

[43] S. Matsuzaki and B. Schubert, "Oxidative stress status in normal ovarian cortex surrounding ovarian endometriosis," *Fertility and Sterility*, vol. 93, no. 7, pp. 2431-2432, 2010.

[44] C. Tosti, S. Pinzauti, P. Santulli, C. Chapron, and F. Petraglia, "Pathogenetic mechanisms of deep infiltrating endometriosis," *Reproductive Sciences*, vol. 22, no. 9, pp. 1053–1059, 2015.

[45] Y. Oner-Iyidogan, H. Kocak, F. Gurdol, D. Korkmaz, and F. Buyru, "Indices of oxidative stress in eutopic and ectopic endometria of women with endometriosis," *Gynecologic and Obstetric Investigation*, vol. 57, no. 4, pp. 214–217, 2004.

[46] S. S. Andrade, C. Azevedo Ade, I. C. Monasterio et al., "17β-estradiol and steady-state concentrations of H2O2: antiapoptotic effect in endometrial cells from patients with

endometriosis," *Free Radical Biology & Medicine*, vol. 60, pp. 63–72, 2013.

[47] C. Ngo, C. Nicco, M. Leconte et al., "Protein kinase inhibitors can control the progression of endometriosis in vitro and in vivo," *The Journal of Pathology*, vol. 222, no. 2, pp. 148–157, 2010.

[48] S. H. Kao, H. C. Huang, R. H. Hsieh, S. C. Chen, M. C. Tsai, and C. R. Tzeng, "Oxidative damage and mitochondrial DNA mutations with endometriosis," *Annals of the New York Academy of Sciences*, vol. 1042, pp. 186–194, 2005.

[49] H. Kajihara, Y. Yamada, S. Kanayama et al., "New insights into the pathophysiology of endometriosis: from chronic inflammation to danger signal," *Gynecological Endocrinology*, vol. 27, no. 2, pp. 73–79, 2011.

[50] M. G. Da Broi and P. A. Navarro, "Oxidative stress and oocyte quality: ethiopathogenic mechanisms of minimal/mild endometriosis-related infertility," *Cell and Tissue Research*, vol. 364, no. 1, pp. 1–7, 2016.

[51] H. Shigetomi, Y. Higashiura, H. Kajihara, and H. Kobayashi, "A potential link of oxidative stress and cell cycle regulation for development of endometriosis," *Gynecological Endocrinology*, vol. 28, no. 11, pp. 897–902, 2012.

[52] F. Ito, Y. Yamada, A. Shigemitsu et al., "Role of oxidative stress in epigenetic modification in endometriosis," *Reproductive Sciences*, vol. 60, 2017.

[53] Y. Yamada, H. Shigetomi, A. Onogi et al., "Redox-active iron-induced oxidative stress in the pathogenesis of clear cell carcinoma of the ovary," *International Journal of Gynecological Cancer*, vol. 21, no. 7, pp. 1200–1207, 2011.

[54] K. R. Wright, B. Mitchell, and N. Santanam, "Redox regulation of microRNAs in endometriosis-associated pain," *Redox Biology*, vol. 12, pp. 956–966, 2017.

[55] N. Hevir, M. Ribic-Pucelj, and R. T. Lanisnik, "Disturbed balance between phase I and II metabolizing enzymes in ovarian endometriosis: a source of excessive hydroxy-estrogens and ROS?," *Molecular and Cellular Endocrinology*, vol. 367, no. 1-2, pp. 74–84, 2013.

[56] H. Kobayashi, "Potential scenarios leading to ovarian cancer arising from endometriosis," *Redox Report*, vol. 21, no. 3, pp. 119–126, 2016.

[57] T. Iwabuchi, C. Yoshimoto, H. Shigetomi, and H. Kobayashi, "Oxidative stress and antioxidant defense in endometriosis and its malignant transformation," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 848595, 7 pages, 2015.

[58] A. Agarwal, S. Gupta, L. Sekhon, and R. Shah, "Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications," *Antioxidants & Redox Signaling*, vol. 10, no. 8, pp. 1375–1404, 2008.

[59] A. Agarwal, A. Aponte-Mellado, B. J. Premkumar, A. Shaman, and S. Gupta, "The effects of oxidative stress on female reproduction: a review," *Reproductive Biology and Endocrinology*, vol. 10, p. 49, 2012.

[60] A. Harlev, S. Gupta, and A. Agarwal, "Targeting oxidative stress to treat endometriosis," *Expert Opinion on Therapeutic Targets*, vol. 19, no. 11, pp. 1447–1464, 2015.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 2181942, 9 pages
https://doi.org/10.1155/2017/2181942

Research Article

Benign Effect of Extremely Low-Frequency Electromagnetic Field on Brain Plasticity Assessed by Nitric Oxide Metabolism during Poststroke Rehabilitation

Natalia Cichoń,[1] Piotr Czarny,[2] Michał Bijak,[1] Elżbieta Miller,[3,4] Tomasz Śliwiński,[5] Janusz Szemraj,[2] and Joanna Saluk-Bijak[1]

[1]*Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, Lodz, Poland*
[2]*Department of Medical Biochemistry, Medical University of Lodz, Mazowiecka 6/8, Lodz, Poland*
[3]*Department of Physical Medicine, Medical University of Lodz, Pl. Hallera 1, Lodz, Poland*
[4]*Neurorehabilitation Ward, III General Hospital in Lodz, Milionowa 14, Lodz, Poland*
[5]*Department of Molecular Genetics, Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Pomorska 141/143, Lodz, Poland*

Correspondence should be addressed to Natalia Cichoń; natalia.cichon@biol.uni.lodz.pl

Received 12 May 2017; Revised 2 July 2017; Accepted 14 August 2017; Published 12 September 2017

Academic Editor: Tanea T. Reed

Nitric oxide (NO) is one of the most important signal molecules, involved in both physiological and pathological processes. As a neurotransmitter in the central nervous system, NO regulates cerebral blood flow, neurogenesis, and synaptic plasticity. The aim of our study was to investigate the effect of the extremely low-frequency electromagnetic field (ELF-EMF) on generation and metabolism of NO, as a neurotransmitter, in the rehabilitation of poststroke patients. Forty-eight patients were divided into two groups: ELF-EMF and non-ELF-EMF. Both groups underwent the same 4-week rehabilitation program. Additionally, the ELF-EMF group was exposed to an extremely low-frequency electromagnetic field of 40 Hz, 7 mT, for 15 min/day. Levels of 3-nitrotyrosine, nitrate/nitrite, and TNFα in plasma samples were measured, and NOS2 expression was determined in whole blood samples. Functional status was evaluated before and after a series of treatments, using the Activity Daily Living, Geriatric Depression Scale, and Mini-Mental State Examination. We observed that application of ELF-EMF significantly increased 3-nitrotyrosine and nitrate/nitrite levels, while expression of NOS2 was insignificantly decreased in both groups. The results also show that ELF-EMF treatments improved functional and mental status. We conclude that ELF-EMF therapy is capable of promoting recovery in poststroke patients.

1. Introduction

Cardiovascular diseases, including ischemic stroke (IS), are a serious problem of the modern age, killing 4 million people each year in Europe [1]. Stroke is caused by ischemia of brain tissue. Brain structure damage occurring during ischemia/reperfusion is due to the generation of significant amounts of reactive oxygen species and inflammatory mediators [2]. Damage to brain tissue as a result of a stroke cannot be undone. However, the most important part of poststroke therapy is immediate and long-term rehabilitation, considering the enormous plasticity of the brain [3]. Although extremely low-frequency electromagnetic field (ELF-EMF) therapy is not a standard treatment in the poststroke rehabilitation, some authors suggest its increased positive effect on patients [4]. ELF-EMF treatment is based on regeneration, osteogenesis, analgesics, and anti-inflammatory action. Its biological effect is related to processes of ion transport, cell

proliferation, apoptosis, protein synthesis, and changes in the transmission of cellular signals [5]. The regenerative and cytoprotective effect of ELF-EMF is based on mechanism associated with nitric oxide induction, collateral blood flow, opioids, and heat shock proteins [6].

Nitric oxide (NO) is an unstable, colourless, water-soluble gas with a short half-life (3–6 sec). The compound has one unpaired electron, which makes it a highly reactive free radical. It is characterized by the multiplicity of action in the body, in both physiological and pathological conditions [7]. Synthesis of NO in the organism is catalysed by nitric oxide synthase (NOS), occurring in three isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), encoded by different genes whose expression is subject to varying regulation. The constituent isoforms of NOS are eNOS and nNOS, whose activity is associated with concentration of calcium ions and the level of calmodulin in a cell, as well as with hypoxia, physical activity, and the level of certain hormones, that is, oestrogens [8]. In contrast, because it is closely related with the calmodulin, iNOS does not require a high concentration of calcium ions but is regulated by various endogenous and exogenous proinflammatory factors [9].

The two-stage synthesis of NO consists of the oxidation of L-arginine to Nω-hydroxy-L-arginine and, under the influence of NOS and oxygen, formation of L-citrulline and release of NO. All isoforms of NOS require the same cofactors: nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH4), iron protoporphyrin IX (heme), and O_2 [7].

Nitric oxide is one of the most important signal molecules, involved in both physiological and pathological processes. One of the major functions of NO is as a potent vasodilation, increasing the blood flow and regulation of blood pressure, which has been used in clinical practice for many years. Deficiency of this compound is observed in various disorders of many systems: cardiovascular, gastrointestinal, respiratory, and genitourinary [10]. The beneficial effects of NO lie in its platelet inhibition, macrophage cytotoxicity (antibacterial, antiviral, and antiparasitic), and protection of the mucosal lining of the digestive system. On the other hand, excessive expression of iNOS can be disadvantageous, for example, during sepsis. The adverse action of NO is associated with the production of superoxide anions and subsequent generation of peroxynitrite and hydroxyl radicals, which are highly toxic [11].

In the central nervous system, NO as a neurotransmitter regulates cerebral blood flow, as well as neurogenesis and synaptic plasticity. Furthermore, neuronal death is caused by high concentrations of NO by caspase-dependent apoptosis process and promotion of inflammation. Elevated levels of nitric oxide promote necrosis by energy depletion. On the basis of these mechanisms, NO is involved in the etiology of many neurological diseases, such as major depression, schizophrenia, epilepsy, anxiety, and drug addiction [12].

Our study was designed to investigate the effect of ELF-EMF on the metabolism of NO, as a signal molecule in the central nervous system, in the rehabilitation of acute poststroke patients.

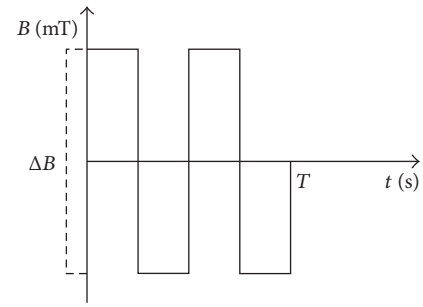


Figure 1: ELF-EMF description. $\Delta B = 5$ mT; $T = 1.3$ sec.

2. Materials and Methods

2.1. Blood Sample Collection. Blood samples were collected into $CPDA_1$-containing tubes (Sarstedt, Nümbrecht, Germany). Immediately upon doing so, a portion of the sample was frozen at −80°C and the rest of the samples centrifuged to isolate the plasma (15 min, 1500g) at 25°C. Blood samples were collected twice, at an interval of 14 days before and after a standard 10 sessions of therapy. For additional analysis of 3-nitrotyrosine levels, the blood samples were collected three times, at an interval of 28 days: before treatment, after 10 treatments, and after 20 treatments. All blood samples were taken in the morning (between 7 am and 9 am) under patient fasting condition and stored using the same protocol.

2.2. Subject Presentation. Forty-eight poststroke patients were enrolled in the study. Participants were randomly divided into two groups: ELF-EMF ($n = 25$) and non-ELF-EMF ($n = 23$). Patients with metal and/or electronic implants (pacemakers, etc.) were excluded from the ELF-EMF group, for safety reasons. The ELF-EMF group had already undergone ELF-EMF therapy with specific parameters (40 Hz frequency, magnetic induction of 5 mT (ΔB), rectangular and bipolar waveforms) (Figure 1), which was conducted using a Magnetronic MF10 generator (EiE Elektronika i Elektromedycyna, Otwock, Poland). The parameters were selected on the basis of the fact that low-intensity stimuli improve the vital functions of the body. In addition, rectangular pulses are more intense than sinusoidal and trapezoid, while bipolar pulses show more range of changes than unipolar pulses [13]. The ELF-EMF and non-ELF-EMF groups were treated for the same amount of time (15 minutes). The non-ELF-EMF subjects were given only sham exposure. The pelvic girdle of the patients was exposed to the electromagnetic field, because exposure of the head to ELF-EMF can affect the activation of the epilepsy focus in the brain. The same therapeutic program was used for both subject groups. This consisted of aerobic exercise (30 min), neurophysiological routines (60 min), and psychological therapy (15 min). Poststroke patients with moderate stroke severity according to NIHSS scores of 4.9 ± 3.1 in the ELF-EMF group (aged 48.8 ± 7.7) and 5.4 ± 2.9 (aged 44.8 ± 8.0) in the non-ELF-EMF group were enrolled in the study. Table 1 shows the clinical and demographic characteristics. Participants with haemorrhagic stroke, dementia, chronic or significant acute inflammatory factors, decreased consciousness, and/

Table 1: Clinical demographic characteristics.

		Control $n = 23$	Study group $n = 25$	p
Demographics	Age (mean ± SD)	44.8 ± 7.7	48.0 ± 8.0	0.84
	Sex: man versus female (%)	48 versus 52	60 versus 40	0.27
	Living alone (%)	32.1	34.2	0.59
Vascular risk	Hypertension (%)	97.3	98.5	0.07
	Diabetes (%)	31.4	39.2	0.21
	Dyslipidemia (%)	78.8	72.2	0.7
	BMI ≥ 30 (%)	21	34	0.78
Concomitant medications	Antidepressants (%)	29	34	0.5
	ASA (%)	70	65	0.42
	NSAID (%)	25	27	0.8
Stroke characteristics	Weeks since stroke (mean ± SD)	3.9 ± 0.6	3.2 ± 0.4	
	NIHSS scores (mean ± SD)	5.4 ± 2.9	4.9 ± 3.1	
	ADL (mean ± SD)	8.89 ± 2.87	9.95 ± 2.35	0.22
Lesion location	Anterior (n)	3	5	
	Posterior (n)	7	6	
	Intermediate (n)	13	14	
Lesion side	Left (n)	15	13	
	Right (n)	8	12	

or neurological illness other than stroke in their medical prestroke history were excluded. The subjects had undergone neurorehabilitation for 4 weeks in Neurorehabilitation Ward III of the General Hospital in Lodz, Poland, as well as internal and neurological examinations. The Bioethics Committee of the Faculty of Biology and Environmental Protection of The University of Lodz, Poland, approved the protocol with resolution numbers 28/KBBN-UŁ/II/2015 and 13/KBBN-UŁ/II/2016. All participants provided written informed consent prior to participation. Depression was screened in both groups using the Geriatric Depression Scale (GDS). Cognitive status was estimated in a Mini-Mental State Examination (MMSE), and functional status using the Barthel Index of Activities of Daily Living (ADL). The GDS, ADL, and MMSE were administered either on the same day as the blood sampling or on the afternoon before.

2.3. Magnetronic MF10 Devices. ELF-EMF therapy was performed by a Magnetronic MF10 generator as per accepted guidelines. This device is able to produce pulses in rectangular, trapezoid, and sinusoidal shapes. The pulses were applied using an AS-550 applicator (EiE, Otwock, Poland), which has the following properties: 550 mm in diameter, 270 mm in length, and 5 layers of 187 turns of 1.45 mm twin-parallel wires. Magnetic induction was set at 5 mT. The electromagnetic field intensity was not uniformed; its distribution is vertical, while the induction coils are set horizontally. Induction of the electromagnetic field of 5 mT is present at the geometric center of the applicator, and the value increases in the proximity to the surface about 7 mT. Other factors that could affect EMF were eliminated (electronic measuring instruments occurring in rehabilitation room and other electronic equipment).

2.4. Immunodetection of 3-Nitrotyrosine by c-ELISA. Levels of 3-NT-containing proteins in plasma were determined using a modified c-ELISA method, as described by Khan et al. [14]. 96-well microtiter plates were coated with nitro-fibrinogen (nitro-Fg) (1 mg/mL) and kept overnight at 4°C. Concentrations of nitrated proteins inhibiting the binding of anti-nitrotyrosine antibodies were assessed from the standard curve (10–100 nM nitro-Fg equivalents) and expressed as nitro-Fg equivalents [15].

2.5. Nitrate/Nitrite Estimation. Plasma samples were diluted twice before the measurement of nitrate/nitrite concentration using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, USA), based on the two-step Griess method. In the first step, the nitrate is converted to nitrite with nitrate reductase, while in the second step, after addition of the Griess reagent, the nitrite is converted to a deep purple azo compound. The absorbance measurement was performed at 540 nm in a 96-well microplate reader (SPECTROstarNano, BMG Labtech, Ortenberg, Germany) [16].

2.6. Determination of NOS2 Expression in Whole Blood Samples. RNA was isolated from the frozen whole blood samples (−80°C), in accordance with the manufacturer's protocol using TRI Reagent® (Sigma-Aldrich, USA). The aqueous phase was purified in accordance with the manufacturer's protocol using an InviTrap Spin Universal RNA Mini Kit (Stratec Biomedical Systems, Germany). The purity and quantity of isolated RNA were assessed using a Synergy HTX Multi-Mode Microplate Reader equipped with a Take3 Micro-Volume Plate and connected to a PC running Gen5 Software (BioTek Instruments Inc., Winooski, VT, USA). Isolated RNA (20 ng/μL) was transcribed onto cDNA with

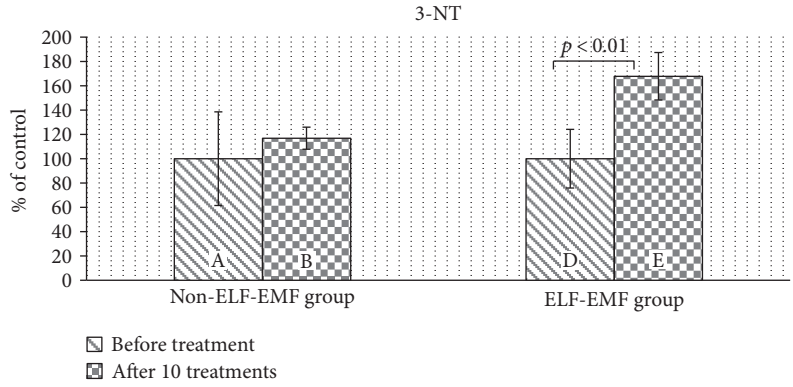


Figure 2: The comparison of 3-NT levels in plasma proteins obtained from the ELF-EMF group versus those from the non-ELF-EMF group. Statistical significance between the ELF-EMF and non-ELF-EMF groups: B versus D ($p < 0.05$).

a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA). Quantitative assays were executed using a TaqMan Hs01075529_m1 probe for human *NOS2* genes and an Hs02786624_g1 for endogenous control, which was *GAPDH* (Life Technologies). Reactions were carried out using a TaqMan Universal Master Mix II, without UNG (Life Technologies) in a BioRad CFX96 real-time PCR system (BioRad Laboratories, Hercules, CA, USA), all in accordance with the manufacturers' protocols. Relative expression of *NOS2* was obtained using the equation $2^{-\Delta Ct}$, where ΔCt is the threshold cycle (Ct) value for the target gene minus Ct values obtained for the housekeeping gene *GAPDH* [17].

2.7. Determination of TNFα. Measurements of human tumour necrosis factor alpha (TNFα) in plasma samples were made with a Human TNFα ELISA development kit (MABTECH, Cincinnati, OH, USA), in accordance with the manufacturer's protocol. The combination of two coating antibodies (TNF3 and TNF4) were used for the analysis. The absorbance was measured at 450 nm, and TNFα concentration was expressed as pg/mL [18].

2.8. Data Analysis. Biochemical and clinical data were expressed as mean ± SD. All measurements were executed in duplicate. Output value (100%) was determined for each measured parameter of each patient before treatment. Data from tests performed on the same study subjects after therapy constituted a percentage of the output value. Percentage values were presented as mean ± SD. Statistical analyses were performed using the Statistica 12 statistical software (StaftSoft Inc.). A Shapiro-Wilk test was used to analyse for normality. A paired Student *t*-test was used to the calculate differences between the values obtained for subjects before and after therapy, whereas unpaired Student *t*-test or Mann–Whitney *U* tests were used to determine differences between the ELF-EMF and non-ELF-EMF groups. *p* values of 0.05 were accepted as statistically significant for all analyses.

3. Results

Our comparative analysis demonstrated an increased level of 3-nitrotyrosine (3-NT) ($p < 0.05$) (Figure 2) and an elevated nitrate/nitrite concentration ($p < 0.01$) (Figure 3) in the plasma of patients from the ELF-EMF group. The gain in the 3-NT level was significantly higher with an increased amount of sessions (Figure 2). In the non-ELF-EMF group, we saw that the effect of rehabilitation on nitrative stress was largely weaker and not statistically significant ($p > 0.05$) (Figures 2 and 3). The 3-NT level increased more in the ELF-EMF group than in the non-ELF-EMF after 10 treatments (68% versus 17%, $p < 0.05$) (Figure 2). The level of nitrate/nitrite in the non-ELF-EMF group even decreased after 10 treatments (although not statistically significantly) (Figure 3).

In the next set of experiments, we determined the effect of magnetotherapy on gene expression in the whole blood samples of *NOS2* mRNA. Its expression was unmeasurable in 35% of subjects from both the ELF-EMF and non-ELF-EMF groups. We observed a statistically insignificant decrease in the level of *NOS2* mRNA expression after treatment in both the ELF-EMF and non-ELF-EMF groups (Figure 4).

Subsequently, we determined the concentration of proinflammatory cytokine TNFα. We found that the concentration of TNFα was comparable before treatment in both the ELF-EMF and non-ELF-EMF-groups. The cytokine level did not change in either groups after rehabilitation (Figure 5).

The ADL, MMSE, and GDS were used to evaluate the functional and mental status of poststroke patients undergoing rehabilitation. We demonstrated that treatment using ELF-EMF improves their clinical parameters, particularly in cognitive and psychosomatic functions.

Motor abilities estimated by ADL score changed at similar levels in both groups, with the observed improvement being statistically significant in all rehabilitated patients ($p < 0.001$) (Table 2).

The baseline MMSE values before treatment in both groups were comparable, but statistically different ($p < 0.05$) after rehabilitation. After 2 weeks of rehabilitation, MMSE parameters improved markedly in the ELF-EMF group ($p = 0.002$), while a small increase in the non-ELF-EMF group was not statistically significant ($p = 0.2$) (Table 2).

Depression syndrome expressed by GDS improved significantly in both groups after rehabilitation. However, the ΔGDS value reached about a 60% lower result in the ELF-EMF group than in the non-ELF-EMF group

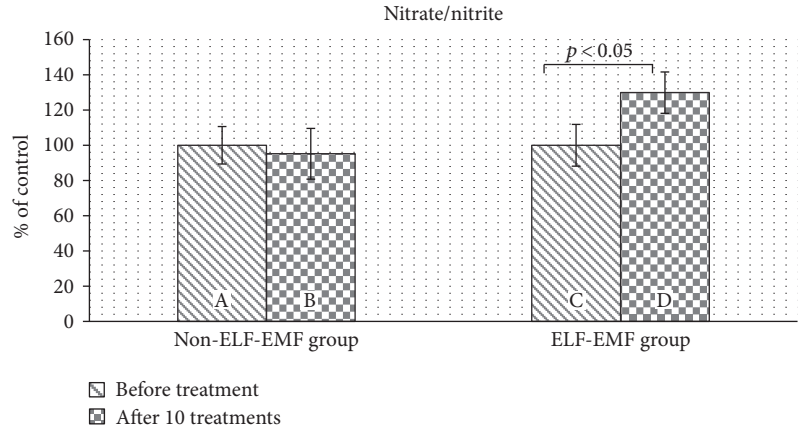


Figure 3: The comparison of nitrate/nitrite levels in plasma proteins obtained from the ELF-EMF group versus those from the non-ELF-EMF group. Statistical significance between ELF-EMF and non-ELF-EMF groups: B versus D ($p < 0.05$).

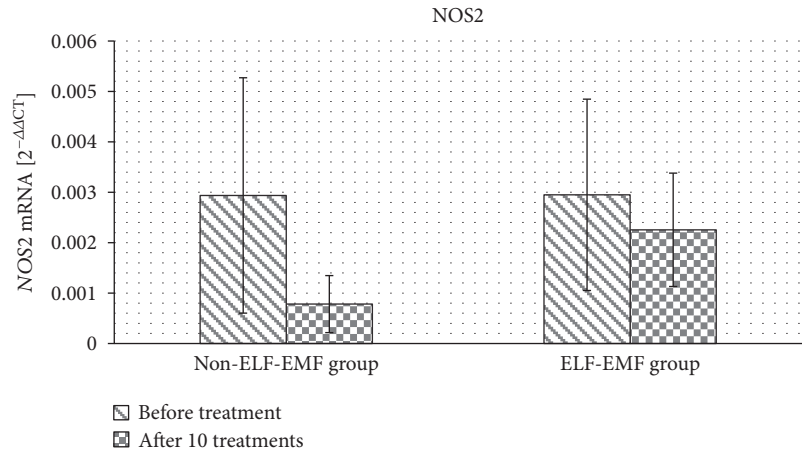


Figure 4: The comparison of *NOS2* mRNA expression obtained from the ELF-EMF group versus that from the non-ELF-EMF group.

($p = 0.018$), starting from a similar base level in both groups ($p > 0.05$) (Table 2).

4. Discussion

In this study, we provide the evidence that application of extremely low-frequency electromagnetic field increases nitric oxide generation and its metabolism, as well as improving the effectiveness of poststroke ischemic patients' treatments.

Ischemic stroke is one of the major causes of morbidity and mortality in the world's population and is one of the main causes of long-term disability. The mechanisms of neurological function recovery after brain injury associated with neuroplasticity (cortical reorganization) are still insufficiently understood. Poststroke neurorehabilitation is designed to provide external stimuli, improving the effectiveness of compensatory plasticity [19].

In the central nervous system, NO is both a pre- and postsynaptic signal molecule. The activity of NO is associated with a cGMP-mediated signalling cascade. The presynaptic excitatory action of NO is related to the phosphorylation of synaptophysin by the cGMP-dependent protein kinase G (PKG) pathway and the subsequent potentates of glutamatergic neurotransmission [20]. On the other hand, NO causes a neurotransmission inhibition through gamma-aminobutyric acid- (GABA-) ergic synaptic communication. It is associated with ion exchange and regulation of membrane excitation [21, 22]. Moreover, NO as an important vasodilation factor mediates neurovascular coupling. The enlargement of vessel diameter is caused by increasing metabolic consumption as a result of neuronal activity. Neurovascular coupling maintains functional and structural brain integrity [23].

This study was designed to investigate the impact of ELF-EMF on the metabolism of nitric oxide in the rehabilitation of acute poststroke patients.

In our study, we demonstrate that poststroke rehabilitation increases the level of 3-NT and nitrate/nitrite concentrations. Due to its vasodilating and proangiogenic effects, NO serves as a protective function during cerebral ischemia. Su et al. investigated the role of simvastatin-regulated TRPV1 receptors (transient receptor potential vanilloid type 1) in NO bioavailability, activation of eNOS, and angiogenesis in

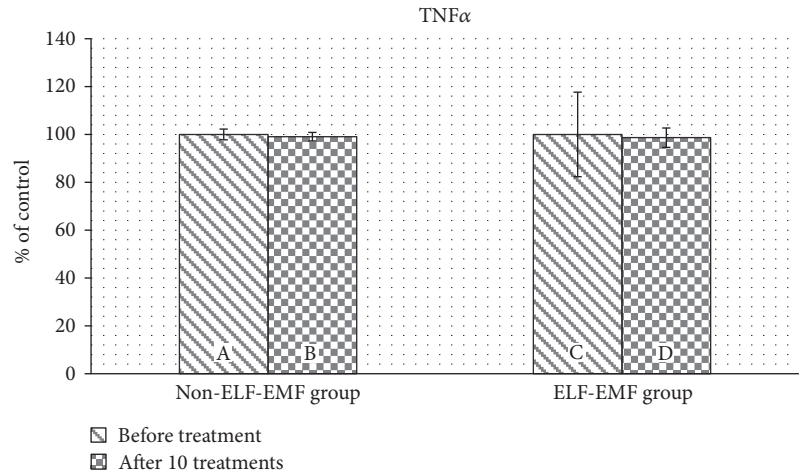


Figure 5: The comparison of TNFα levels in plasma proteins obtained from the ELF-EMF group versus those from the non-ELF-EMF group.

Table 2: Clinical parameters: ADL, MMSE, and GDS measured in the ELF-EMF and non-ELF-EMF groups. Data presented as the delta of a clinimetric scale before and after the standard series of treatments (ΔADL = the increase of ADL; ΔMMSE = the increase of MMSE; and ΔGDS = the decrease of GDS).

		Non-ELF-EMF group	ELF-EMF group	p
ADL	Before treatment	8.99	9.95	0.378
	After treatment	14.3	16.12	0.429
	Δ	5.31	6.17	0.194
	p	<0.001	<0.001	
MMSE	Before treatment	22.75	23.00	0.873
	After treatment	23.94	26.28	0.047
	Δ	1.19	3.28	0.036
	p	0.204	0.002	
GDS	Before treatment	11.38	13.00	0.054
	After treatment	9.13	6.72	0.049
	Δ	2.25	6.28	0.018
	p	0.009	0.001	

mice. They demonstrated that simvastatin causes an influx of calcium ions through the TRPV1-TRPA1 (transient receptor potential ankyrin 1) pathway, which then causes activation of CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II). This then enhances the formation of the TRPV1-eNOS complex, which also includes CaMKII, AMPK (5′AMP-activated protein kinase), and Akt (protein kinase B), which leads to activation of eNOS, production of NO, and thus the promotion of endothelial angiogenesis [24]. There have been numerous reports of the protective effects of NO against inflammation and oxidative stress [25]. Transgenic eNOS-deficient mice demonstrated a more extensive infarct of the middle cerebral artery (MCA), compared to controls [26]. NO effects on the regulation of endothelial integrity, anti-inflammatory and anti-apoptotic effects, as well as maintenance of cerebral blood flow, inhibition of platelet aggregation, and reduction of leukocyte adhesion [25, 27]. Khan et al. studied structurally different NO donors as agents of cerebrovascular protection in experimentally induced stroke in rats. They showed that NO donors promote cerebral blood flow through S-nitrosylation and may be an effective drug for acute stroke [28, 29].

Furthermore, Greco et al. proved the protective effect of nitroglycerin (donors of NO) on cerebral damage induced by MCA occlusion in Wistar rats. They observed a significant reduction in stroke volume in preinjected rats compared to their control group, which confirms the protective effect of nitroglycerin *in vivo*. They speculated that the mechanism of action is associated with the generation of a complex chain of phenomena, triggering activation of apoptosis and subsequent activation of antiapoptotic responses [30].

The biological action of ELF-EMF is still being investigated. It is suggested that ELF-EMF has an impact on the physicochemical properties of water, the liquid crystal structure generated by cholesterol, and its derivatives [31, 32]. Changes in ion balance caused by ELF-EMF appeal to the structure of tissue with piezoelectric and magnetostrictive properties, free radicals, diamagnetic molecules, and uncompensated magnetic spins of paramagnetic elements [33]. Therefore, ELF-EMF causes depolarization of cells having the ability to spontaneously depolarize, predominantly through Ca^{2+} influx [34]. In our previous study, we investigated the effect of ELF-EMF on oxidative stress in patients after ischemic stroke. We demonstrated that ELF-EMF causes activation of antioxidant enzymes [35], which leads to reduction of the oxidative modification of plasma protein (this is detailed in an article published in *Advances in Clinical and Experimental Medicine*). As a highly reactive molecule, NO can also regulate the level of oxidative stress. Through the covalent interaction, NO influences the activity of various enzymes. Mechanisms of this modulation can be varied: NO reacts with coenzymes and active centers containing metal ions and interacts with cysteine residues of proteins [36].

In the current study, we observed that in the ELF-EMF group, the level of plasma 3-NT was increased (Figure 2). The formation of 3-NT in protein molecules occurs *in vivo* by the action of nitrating agents on the polypeptide chain. The formation of 3-NT is mainly attributed to NO and superoxide anions ($O_2^{-\cdot}$), which react rapidly to form peroxynitrite ($ONOO^-$). This is one of the major oxidizing and nitrating agents produced *in vivo* in acute and chronic inflammation, as well as in ischemia/reperfusion. Endothelial cells, macrophages, and neutrophils release large amounts of NO and O_2^-. Thus, increased amounts of NO contribute to the creation of 3-NT [37].

To investigate the effect of ELF-EMF on NO metabolism, we determined nitrate/nitrite concentrations in plasma. We showed that in the ELF-EMF group, the level of nitrate/nitrite compounds in plasma increased after treatment (Figure 3), and these results correspond with the data presented by Chung et al. [38]. The authors investigated the effects of ELF-EMF (60 Hz, 2 mT) on the level of NO, biogenic amines, and amino acid neurotransmitters in the hippocampus, cortex, thalamus, cerebellum, and striatum in rats. They found a significant increase in NO concentration in the hippocampus, thalamus, and striatum. Moreover, ELF-EMF also caused a change in the level of biogenic amines and amino acid neurotransmitters in the brain. However, the observed effect and range were different, depending on the brain area. Balind et al. determined the effect of ELF-EMF (50 Hz, 0.5 mT) on oxidative stress in gerbils with induced cerebral ischemia. They measured the level of NO using the Griess reagent and showed an increased level of NO, provoked by electromagnetic fields. Moreover, ELF-EMF reduces oxidative stress generated during cerebral ischemia, thus leading to a decrease in the damaged brain tissue [39].

NO is produced from L-arginine with the involvement of nitric oxide synthase. Three NOS isoforms are expressed in different tissues. Although, in the blood, only NOS2 is expressed, in 35% of the subjects in both the ELF-EMF and non-ELF-EMF groups, mRNA expression of *NOS2* was under detection. In the remaining patients, the expression of *NOS2* had not significantly changed after treatment. The NOS2 gene in fact encodes for iNOS, which is primarily activated during inflammation. In order to exclude deeper inflammation, we measured the concentration of TNFα, one of the main proinflammatory cytokines. TNFα is a pleiotropic cytokine that is involved in nearly all phenomena of inflammatory responses: initiating chemokine synthesis, promoting the expression of adhesion molecules, promoting the maturation of dendritic cells, and inducing the production of inflammatory mediators and other proinflammatory cytokines [40]. TNFα stimulates collagenase synthesis in synovial fibroblasts and synovial cartilage chondrocytes and activates osteoclasts, leading to joint cartilage damage, hypertrophy, bone resorption and erosion, and angiogenesis. It also activates monocytes and macrophages, enhancing their cytotoxicity and stimulating cytokine production. Chemokines and growth factors are responsible for T cell proliferation, proliferation and differentiation of B lymphocytes, and the release of inflammatory cytokines by the lymphocytes. Moreover, in the hypothalamus, TNFα stimulates prostaglandin E and IL-1 synthesis [41]. Pena-Philippides et al. investigated the effect of pulsed electromagnetic fields on injury size and neuroinflammation in mice after middle cerebral artery occlusion (MCAO). They found, using magnetic resonance imaging (MRI), that EMF reduced infarct size, as well as changed expression of genes encoding pro- and anti-inflammatory cytokines in the hemisphere with ischemic injury. After EMF exposure, genes encoding IL-1α and TNF superfamily were downregulated, while *IL-10* expression was upregulated. Thus, the authors suggested that application of EMF to poststroke patients could have been beneficial through anti-inflammatory effect and reduction of injury size [42].

On the basis of our results, we suggest that the observed increase in NO level is associated with nNOS and/or eNOS activities, but not with iNOS expression. Our research is consistent with evidence shown by Cho et al., who established that ELF-EMF (60 Hz, 2 mT) increased the expression and activation of nNOS in rat brains [43].

The activities of nNOS and eNOS depend on calcium ions. There are many reports that the biological effect of ELF-EMF is related to the control of calcium channels [44–48]. In view of these findings, the observed mechanism of increased NO generation and metabolism may be associated with calcium-ion flux.

Additionally, we noticed that ELF-EMF treatment enhances the effectiveness of poststroke rehabilitation (Table 2). Some researchers suggest that electromagnetic fields have a beneficial effect on ischemic/reperfusion injury, and in some places, therapeutic programs using ELF-EMF are considered to be standard therapy for poststroke patients [49, 50]. The beneficial effects of ELF-EMF include the following: improvement in the transport of cellular and mitochondrial membranes; normalization of blood rheological values; counteraction of tissue oxidation; intensification of regenerative processes; stimulation of axon growth in undamaged neurons; intensification of neuronal dissociation and differentiation; reduction of stress-induced emotional reactions and free radicals; acceleration of the return of fibre function in functional disorders; reduction of periapical scarring; and increase of the level of energetic substances in the brain tissue and erythrocytes [48–53]. Grant et al. estimated the impact of low-frequency pulsed electromagnetic field on cerebral ischemia in rabbit. They observed using MRI that exposure to electromagnetic field caused extenuation of cortical ischemia oedema and reduction of neuronal injury in cortical area [54].

In conclusion, ELF-EMF therapy increases the metabolism and generation of NO, which has both neuroprotective and cytotoxic properties. An increase in NO level is probably associated with nNOS and/or eNOS activities, but not with iNOS expression, which increases mainly during inflammation. We suggested that in poststroke patients, NO demonstrated a protective effect due to significant improvement in patient functional status. Thus, our studies promote the validity of this method in poststroke rehabilitation therapy.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This study was supported by the Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz (no. 506/1136), and Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz (no. B161100000004601), and Grants for Young Scientists and PhD Students, Faculty of Biology and Environmental Protection, University of Lodz (B1611000001145.02).

References

[1] N. Townsend, L. Wilson, P. Bhatnagar, K. Wickramasinghe, M. Rayner, and M. Nichols, "Cardiovascular disease in Europe: epidemiological update 2016," *European Heart Journal*, vol. 37, no. 42, pp. 3232–3245, 2016.

[2] X. Li, L. Su, X. Zhang et al., "Ulinastatin downregulates TLR4 and NF-kB expression and protects mouse brains against ischemia/reperfusion injury," *Neurological Research*, vol. 13, pp. 1–7, 2017.

[3] T. Klarner, T. S. Barss, Y. Sun, C. Kaupp, P. M. Loadman, and E. P. Zehr, "Long-term plasticity in reflex excitability induced by five weeks of arm and leg cycling training after stroke," *Brain Sciences*, vol. 6, no. 4, 2016.

[4] Y. Cheng, Y. Dai, X. Zhu et al., "Extremely low-frequency electromagnetic fields enhance the proliferation and differentiation of neural progenitor cells cultured from ischemic brains," *Neuroreport*, vol. 26, no. 15, pp. 896–902, 2015.

[5] N. Cichon, A. K. Olejnik, E. Miller, and J. Saluk, "The multipotent action of magnetic fields," *Biologia*, vol. 71, no. 10, pp. 1103–1110, 2016.

[6] J. A. Robertson, A. W. Thomas, Y. Bureau, and F. S. Prato, "The influence of extremely low frequency magnetic fields on cytoprotection and repair," *Bioelectromagnetics*, vol. 28, no. 1, pp. 16–30, 2007.

[7] S. Kumar, R. K. Singh, and T. R. Bhardwaj, "Therapeutic role of nitric oxide as emerging molecule," *Biomedicine & Pharmacotherapy*, vol. 85, pp. 182–201, 2017.

[8] W. K. Alderton, C. E. Cooper, and R. G. Knowles, "Nitric oxide synthases: structure, function and inhibition," *The Biochemical Journal*, vol. 357, Part 3, pp. 593–615, 2001.

[9] H. Li and T. L. Poulos, "Structure-function studies on nitric oxide synthases," *Journal of Inorganic Biochemistry*, vol. 99, pp. 293–305, 2005.

[10] J. Lei, Y. Vodovotz, E. Tzeng, and T. R. Billiar, "Nitric oxide, a protective molecule in the cardiovascular system," *Nitric Oxide*, vol. 35, pp. 175–185, 2013.

[11] M. Rasool, M. A. Ashraf, A. Malik et al., "Comparative study of extrapolative factors linked with oxidative injury and anti-inflammatory status in chronic kidney disease patients experiencing cardiovascular distress," *PLoS One*, vol. 12, no. 2, article e0171561, 2017.

[12] A. V. Kozlov, S. Bahrami, H. Redl, and C. Szabo, "Alterations in nitric oxide homeostasis during traumatic brain injury," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 2017, In press.

[13] T. Mika, *Metodyka Magnetoterapii*, T. Mika and W. Kasprzak, Eds., pp. 337–339, Fizykoterapia, Wydawnictwo Lekarskie PZWL. Warszawa, 2013.

[14] J. Khan, D. M. Brennan, N. Bradley, B. Gao, R. Bruckdorfer, and M. Jacobs, Part 2, "3-Nitrotyrosine in the proteins of human plasma determined by an ELISA method," *The Biochemical Journal*, vol. 330, pp. 795–801, 1998.

[15] J. Kolodziejczyk, J. Saluk-Juszczak, and B. Wachowicz, "L-Carnitine protects plasma components against oxidative alterations," *Nutrition*, vol. 27, no. 6, pp. 693–699, 2011.

[16] A. É. Declèves, I. Jadot, V. Colombaro et al., "Protective effect of nitric oxide in aristolochic acid-induced toxic acute kidney injury: an old friend with new assets," *Experimental Physiology*, vol. 101, no. 1, pp. 193–206, 2016.

[17] K. Bobińska, J. Szemraj, P. Czarny, and P. Gałecki, "Expression and activity of metalloproteinases in depression," *Medical Science Monitor*, vol. 22, pp. 1334–1341, 2016.

[18] O. Désy, D. Carignan, M. Caruso, and P. O. de Campos-Lima, "Methanol induces a discrete transcriptional dysregulation that leads to cytokine overproduction in activated lymphocytes," *Toxicological Sciences*, vol. 117, no. 2, pp. 303–313, 2010.

[19] C. Allman, U. Amadi, A. M. Winkler et al., "Ipsilesional anodal tDCS enhances the functional benefits of rehabilitation in patients after stroke," *Science Translational Medicine*, vol. 8, no. 330, p. 330re1, 2016.

[20] H. G. Wang, F. M. Lu, I. Jin et al., "Presynaptic and postsynaptic roles of NO, cGK, and RhoA in long-lasting potentiation and aggregation of synaptic proteins," *Neuron*, vol. 45, pp. 389–403, 2005.

[21] Y. R. Yang, J. H. Jung, S. J. Kim et al., "Forebrain-specific ablation of phospholipase Cγ1 causes manic-like behavior," *Molecular Psychiatry*, 2017.

[22] C. D. Fekete, R. U. Goz, S. Dinallo et al., "In vivo transgenic expression of collybistin in neurons of the rat cerebral cortex," *The Journal of Comparative Neurology*, vol. 525, no. 5, pp. 1291–1311, 2017.

[23] Z. Ungvari, S. Tarantini, P. Hertelendy et al., "Cerebromicrovascular dysfunction predicts cognitive decline and gait abnormalities in a mouse model of whole brain irradiation-induced accelerated brain senescence," *Geroscience*, vol. 39, no. 1, pp. 33–42, 2017.

[24] K. H. Su, S. J. Lin, J. Wei et al., "The essential role of transient receptor potential vanilloid 1 in simvastatin-induced activation of endothelial nitric oxide synthase and angiogenesis," *Acta Physiologica (Oxford, England)*, vol. 212, no. 3, pp. 191–204, 2014.

[25] G. Cirino, S. Fiorucci, and W. C. Sessa, "Endothelial nitric oxide synthase: the Cinderella of inflammation?," *Trends in Pharmacological Sciences*, vol. 24, pp. 91–95, 2003.

[26] Z. Huang, P. L. Huang, J. Ma et al., "Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine," *Journal of Cerebral Blood Flow and Metabolism*, vol. 16, pp. 981–987, 1996.

[27] H. Li and U. Forstermann, "Nitric oxide in the pathogenesis of vascular disease," *The Journal of Pathology*, vol. 190, pp. 244–254, 2000.

[28] M. Khan, M. Jatana, C. Elango, A. S. Paintlia, A. K. Singh, and I. Singh, "Cerebrovascular protection by various nitric oxide

donors in rats after experimental stroke," *Nitric Oxide*, vol. 15, no. 2, pp. 114–124, 2006.

[29] M. Khan, B. Sekhon, S. Giri et al., "S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke," *Journal of Cerebral Blood Flow and Metabolism*, vol. 25, pp. 177–192, 2005.

[30] R. Greco, D. Amantea, F. Blandini et al., "Neuroprotective effect of nitroglycerin in a rodent model of ischemic stroke: evaluation of Bcl-2 expression," *International Review of Neurobiology*, vol. 82, pp. 423–435, 2007.

[31] M. Sulpizio, S. Falone, F. Amicarelli et al., "Molecular basis underlying the biological effects elicited by extremely low-frequency magnetic field (ELF-MF) on neuroblastoma cells," *Journal of Cellular Biochemistry*, vol. 112, pp. 3797–3806, 2011.

[32] G. Yi, J. Wang, X. Wei et al., "Effects of extremely low-frequency magnetic fields on the response of a conductance-based neuron model," *International Journal of Neural Systems*, vol. 24, no. 1, article 1450007, 2014.

[33] F. Brisdelli, F. Bennato, A. Bozzi, B. Cinque, F. Mancini, and R. Iorio, "ELF-MF attenuates quercetin-induced apoptosis in K562 cells through modulating the expression of Bcl-2 family proteins," *Molecular and Cellular Biochemistry*, vol. 397, no. 1-2, pp. 33–43, 2014.

[34] C. Morgado-Valle, L. Verdugo-Díaz, D. E. García, C. Morales-Orozco, and R. Drucker-Colín, "The role of voltage-gated Ca^{2+} channels in neurite growth of cultured chromaffin cells induced by extremely low frequency (ELF) magnetic field stimulation," *Cell and Tissue Research*, vol. 291, no. 2, pp. 217–230, 1998.

[35] N. Cichon, M. Bijak, E. Miller, and J. Saluk, "Extremely low-frequency electromagnetic field (ELF-EMF) reduces oxidative stress and improves functional and psychological status in ischemic stroke patients," *Bioeletromagtetics*, vol. 38, no. 5, pp. 386–396, 2017.

[36] D. A. Wink, K. M. Miranda, M. G. Espey et al., "Mechanisms of the antioxidant effects of nitric oxide," *Antioxidants & Redox Signaling*, vol. 3, no. 2, pp. 203–213, 2001.

[37] R. S. Ronson, M. Nakamura, and J. Vinten-Johansen, "The cardiovascular effects and implications of peroxynitrite," *Cardiovascular Research*, vol. 44, pp. 47–59, 1999.

[38] Y. H. Chung, Y. J. Lee, H. S. Lee et al., "Extremely low frequency magnetic field modulates the level of neurotransmitters," *The Korean Journal of Physiology and Pharmacology*, vol. 19, no. 1, pp. 15–20, 2015.

[39] B. S. Rauš, V. Selaković, L. Radenović, Z. Prolić, and B. Janać, "Extremely low frequency magnetic field (50 Hz, 0.5 mT) reduces oxidative stress in the brain of gerbils submitted to global cerebral ischemia," *PLoS One*, vol. 9, no. 2, article e88921, 2014.

[40] P. Wu, F. Jia, B. Zhang, and P. Zhang, "Risk of cardiovascular disease in inflammatory bowel disease," *Experimental and Therapeutic Medicine*, vol. 13, no. 2, pp. 395–400, 2017.

[41] J. Godos, A. Biondi, F. Galvano et al., "Markers of systemic inflammation and colorectal adenoma risk: meta-analysis of observational studies," *World Journal of Gastroenterology*, vol. 23, no. 10, pp. 1909–1919, 2017.

[42] J. C. Pena-Philippides, Y. Yang, O. Bragina, S. Hagberg, E. Nemoto, and T. Roitbak, "Effect of pulsed electromagnetic field (PEMF) on infarct size and inflammation after cerebral ischemia in mice," *Translational Stroke Research*, vol. 5, no. 4, pp. 491–500, 2014.

[43] S. I. Cho, Y. S. Nam, L. Y. Chu et al., "Extremely low-frequency magnetic fields modulate nitric oxide signaling in rat brain," *Bioelectromagnetics*, vol. 33, no. 7, pp. 568–574, 2012.

[44] J. Walleczek, "Electromagnetic field effects on cells of the immune system: the role of calcium signaling," *The FASEB Journal*, vol. 6, pp. 3177–3185, 1992.

[45] C. Grassi, M. D'Ascenzo, A. Torsello et al., "Effects of 50 Hz electromagnetic fields on voltage-gated Ca^{2+} channels and their role in modulation of neuroendocrine cell proliferation and death," *Cell Calcium*, vol. 35, pp. 307–315, 2004.

[46] R. Piacentini, C. Ripoli, D. Mezzogori, G. B. Azzena, and C. Grassi, "Extremely low-frequency electromagnetic fields promote in vitro neurogenesis via upregulation of Ca_V1-channel activity," *Journal of Cellular Physiology*, vol. 215, pp. 129–139, 2008.

[47] F. Gobba, D. Malagoli, and E. Ottaviani, "Effects of 50 Hz magnetic fields on fMLP-induced shape changes in invertebrate immunocytes: the role of calcium ion channels," *Bioelectromagnetics*, vol. 24, pp. 277–282, 2003.

[48] G. L. Craviso, S. Choe, P. Chatterjee, I. Chatterjee, and P. T. Vernier, "Nanosecond electric pulses: a novel stimulus for triggering Ca^{2+} influx into chromaffin cells via voltage-gated Ca^{2+} channels," *Cellular and Molecular Neurobiology*, vol. 30, pp. 1259–1265, 2010.

[49] A. Sieroń and G. Cieslar, "Use of magnetic fields in medicine - 15 years of personal experience," *Wiadomości Lekarskie*, vol. 56, pp. 434–441, 2003.

[50] M. Woldańska-Okońska and J. Czernicki, "Effect of low frequency magnetic fields used in magnetotherapy and magnetostimulation on the rehabilitation results of patients after ischemic stroke," *Przegląd Lekarski*, vol. 64, no. 2, pp. 74–77, 2007.

[51] F. Capone, M. Dileone, P. Profice et al., "Does exposure to extremely low frequency magnetic fields produce functional changes in human brain?," *Journal of Neural Transmission (Vienna)*, vol. 116, no. 3, pp. 257–265, 2009.

[52] A. Miecznik, J. Czernicki, and J. Krukowska, "Influence of magnetic field of different characteristics on blood pressure in patients with back pain syndromes and hypertensive disease," *Acta Bio-Optica et Informatica Medica*, vol. 7, no. 1-2, pp. 9–13, 2001.

[53] V. Di Lazzaro, F. Capone, F. Apollonio et al., "A consensus panel review of central nervous system effects of the exposure to low-intensity extremely low-frequency magnetic fields," *Brain Stimulation*, vol. 6, no. 4, pp. 469–476, 2013.

[54] G. Grant, R. Cadossi, and G. Steinberg, "Protection against focal cerebral ischemia following exposure to a pulsed electromagnetic field," *Bioelectromagnetics*, vol. 15, no. 3, pp. 205–216, 1994.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8475125, 11 pages
https://doi.org/10.1155/2017/8475125

Review Article

Cell Signaling with Extracellular Thioredoxin and Thioredoxin-Like Proteins: Insight into Their Mechanisms of Action

Thierry Léveillard[1,2,3] and Najate Aït-Ali[1,2,3]

[1]*INSERM, U968, Sorbonne Universités, 75012 Paris, France*
[2]*UPMC Univ Paris 06, UMR_S 968, Institut de la Vision, 75012 Paris, France*
[3]*CNRS, UMR_7210, 75012 Paris, France*

Correspondence should be addressed to Thierry Léveillard; thierry.leveillard@inserm.fr

Received 24 May 2017; Revised 6 July 2017; Accepted 17 July 2017; Published 12 September 2017

Academic Editor: Sergio Di Meo

Thioredoxins are small thiol-oxidoreductase enzymes that control cellular redox homeostasis. Paradoxically, human thioredoxin (TXN1) was first identified as the adult T cell leukemia-derived factor (ADF), a secreted protein. ADF has been implicated in a wide variety of cell-to-cell communication systems acting as a cytokine or a chemokine. TRX80 is a truncated TXN1 protein with cytokine activity. The unconventional secretion mechanism of these extracellular thioredoxins is unknown. The thioredoxin system is relying on glucose metabolism through the pentose phosphate pathway that provides reducing power in the form of NADPH, the cofactor of thioredoxin reductase (TXNRD). While a complete extracellular TXN system is present in the blood in the form of circulating TXN1 and TXNDR1, the source of extracellular NADPH remains a mystery. In the absence of redox regenerating capacity, extracellular thioredoxins may rather be prooxidant agents. Rod-derived cone viability factor (RdCVF) is the product of intron retention of the nucleoredoxin-like 1 (*NXNL1*) gene, a secreted truncated thioredoxin-like protein. The other product encoded by the gene, RdCVFL, is an enzymatically active thioredoxin. This is a very singular example of positive feedback of a superthioredoxin system encoded by a single gene likely emerging during evolution from metabolic constraints on redox signaling.

1. Introduction

The inverse correlation between the life-span and the rate of oxygen consumption of mammals has directed research on aging into the field of oxygen metabolism. Reactive oxygen species (ROS), which are too reactive to exist in biological systems, are formed *in situ* by leakage from the mitochondrial respiratory chain generating cumulative cellular dysfunctions. ROS are continuously produced in the cell as a product of aerobic life. In order to avoid or to reverse the damage to macromolecules by ROS, proper redox conditions must be maintained within the intracellular environment. Therefore, aerobic organisms have several antioxidant systems including superoxide dismutase, catalase, and thioredoxin (TXN) systems to compensate for this inherent fragility. The prototype of the thioredoxin proteins, TXN1, is a 12 kDa protein with the redox-active disulfide/dithiol group within the conserved active-site sequence CGPC. Reduced TXN1 catalyses the reduction of disulfide bonds in many cytoplasmic and nuclear proteins, and oxidized TXN1 is reversibly reduced by the action of the thioredoxin reductase using electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH) [1]. The TXN system is compartmentalized into distinct steady-state redox potentials within each cellular organelle.

Cysteine is a rarely used amino acid that accounts for about 2% of the amino acids in eukaryotic proteins. ROS, as well as reactive nitrogen species (RNS), can induce redox signals by means of oxidative modifications of cysteine residues in targeted proteins. ROS and RNS, including hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), and hydroxyl radical ($OH^{\bullet}$), can be produced *in vivo* from a wide range of cellular processes. The large, polarizable sulfur atom in a thiol group is electron rich and highly

nucleophilic; hence, cysteines can undergo a broad range of chemical reactions [2]. C-SH is in equilibrium with $C\text{-}S^-$ and to the disulfide S-S, which can be oxidized by ROS to C-SOH, $-SO_2H$, and SO_3H, or S-nitrosylated by RNS to C-SNO and finally, in the presence of glutathione (GSH), S-thiolated to -S-SG. Cysteines differ in their reactivity properties depending of the protein microenvironment [3]. As many of these modifications are reversible through reduction catalyzed by oxidoreductases, such as thioredoxins (TXNs), glutaredoxins (GRXs), sulfiredoxin (SRX), and sestrins (SESs), protein thiol redox state can respond to the redox environment.

Originally, human TXN1 was identified as an extracellular protein which is extending the field of redox biology to the functions of thioredoxins in a compartment that may not be under the control of the cellular TXN reducing system [4]. In order to develop this question in a comprehensive manner, we have chronologically reviewed key findings on extracellular thioredoxins.

2. Adult T Cell Leukemia-Derived Factor at the Origin of Extracellular Thioredoxins

Adult T cell leukemia (ATL) is the first human cancer found to be caused by a retrovirus [5]. ATL arises in ~1% of human T cell lymphotropic virus type 1- (HTLV1-) infected individuals after a latent period of 10 to 20 years [6]. ATL cell lines produce a soluble factor that stimulates the expression of interleukin-2 receptor alpha chain (IL2RA) by HTLV1-positive/ATL-negative cells, the ATL-derived factor (ADF) [7]. The activity was purified to homogeneity by four successive chromatographic steps from the conditioned medium of an ATL cell line (ATL-2). The N-terminal sequence obtained by Edman degradation was used to isolate a cDNA encoding a 105 amino acid protein by screening a cDNA library constructed with ATL-2 cells [4]. ADF is homologous to the bacterial thioredoxin TXN1 and identical to human TXN1. The same extracellular thioredoxin was identified in the conditioned medium of 3B6 cells from an Epstein-Barr virus- (EBV-) infected B-lymphoblastoid cell line [8]. Recombinant ADF/TXN1 enhances the cytokine effect of interleukin (IL) 1 and IL2, suggesting that ADF might sensitize these cells to a specific subset of interleukins. The potentiation of ADF/TXN1 biological activity by 2-mercaptoethanol, a reducing agent, suggests that cell signaling involves the reduction of an unknown ADF/TXN1 cell surface protein. The authors proposed at that time (1989) that using radiolabeled recombinant ADF/TXN1 could clarify the nature of the cell surface-binding molecules. Twenty-eight years later, the result of such experiment is still awaited [9]. Protein purification, while conducted to homogeneity, is not a guarantee that the identified peptide is necessarily carrying the biological activity. Current proteomic methods might have revealed the existence of many other proteins in the most purified ADF/TXN1 fraction. This remark emphasizes that even experts in protein purification are not immune to overinterpretations. The conditioned medium of COS cells transfected with ADF/TXN1 cDNA replicates the growth-promoting activity of that of ATL-2 cells, but COS cells might also secrete IL1, IL2, or other cytokines whose action may be enhanced by oxidoreduction of their cysteines. In fact, following the purification of ADF/TXN1 by four chromatographic steps, two peptides A and B were identified. Peptide A was used to identify ADF/TXN1, and later on, peptide B led to the identification of macrophage migration inhibitory factor (MIF) [10]. MIF binds to ADF/TXN1, and both form a complex in the conditioned medium of ATL-2 cells [11]. Internalization of extracellular MIF in ATL-2 cells is facilitated by ADF/TXN1. The activity of ADF/TXN1 could theoretically be relayed by CD74, C-X-C chemokine receptor type 2 (CXCR2), or CXCR4, one of the cell surface receptors of MIF [12, 13]. MIF is a cytokine with two distinct catalytic activities, tautomerase/isomerase and thiol-oxidoreductase activities [14]. The cytokine activity of MIF requires the formation of a homotrimeric complex through interdisulfide bond formation [15]. Ebselen, a MIF inhibitor, forms a covalent bond with MIF cysteine 80, which led to dissociation of trimers to monomers and the loss of cytokine activity [16]. Overall, these results suggest an alternative mechanism for which, besides the existence of a TXN1 cell surface receptor, TXN1 might regulate enzymatically the trimerisation of MIF and thus its biological activity through one of the MIF cell surface receptors (Figure 1).

Notwithstanding, *a bona fide* TXN1 cell surface receptor, the tumor necrosis factor receptor superfamily member 8 (TNFRSF8/CD30), was identified [17]. A mutant TXN1 protein lacking the resolving cysteine of the catalytic site (CXXC > CXXS), resulting in the formation of a stable intermediate between mutant TXN1 and its substrate, was used as a probe. This mutant trapped cell surface proteins from the EBV-transformed lymphoblastoid B-cell line, a previously identified target of ADF/TXN1 [8]. Competition analysis with TNFRSF8 antibodies suggested that ADF/TXN1 induces a redox-dependent conformational change within the extracellular domain of TNFRSF8. Only a partial conformation rearrangement was observed when reduced TXN1/ADF was applied in the absence of thioredoxin reductase and NADPH confirming that the enzymatic reaction requires the regeneration of reduced ADF/TXN1, oxidized after TNFRSF8 thiol-oxidoreductase reaction [18]. Surprisingly, proliferation assays mediated by TNFRSF8 of the EBV-transformed lymphoblastoid B-cell line have not been reported since 2007 [8]. Recombinant ADF/TXN1 also activates the short transient receptor potential channel 5 (TRPC5) by means of a similar thiol-oxidoreductase mechanism [19].

3. The Paradox of the Redox Power of Extracellular Thioredoxins

Deoxyribonucleotides are all made from a ribonucleotide precursor by the action of ribonucleotide reductase (RNR), a tetramer of two subunits, R1 and R2 (Figure 2(a)). During ribonucleotide reductase catalysis, a cysteinyl radical is formed. After the completion of one turnover cycle, a disulfide bond is formed between the conserved cysteine pairs at the R1 active site which is transferred at a C-

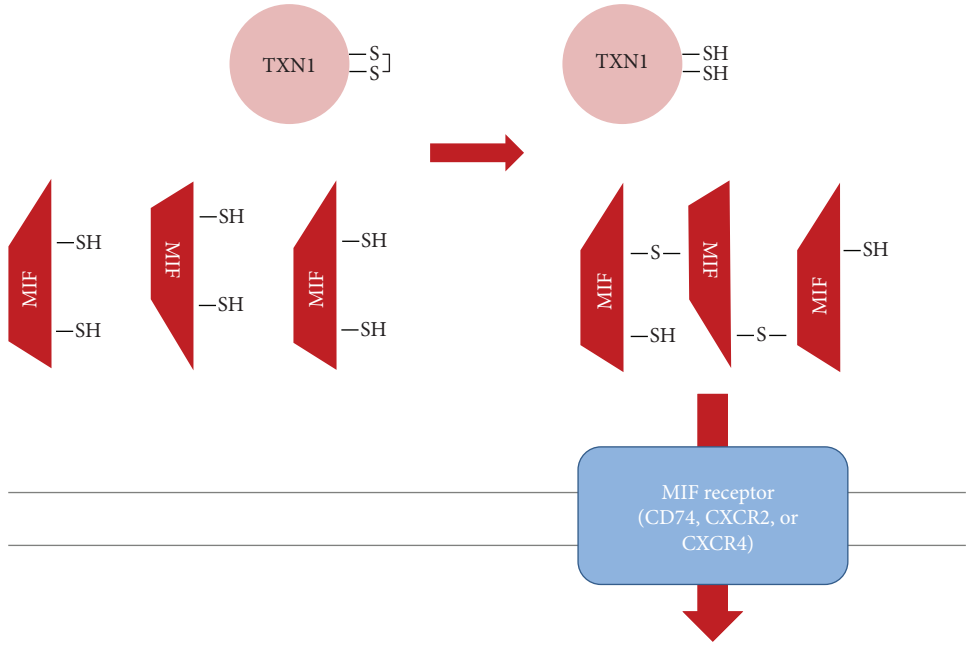


Figure 1: Hypothetical model for ADF/TXN1 extracellular signaling based on MIF trimerisation. The cytokine effect of ADF/TXN1 mediated through MIF cell surface receptors.

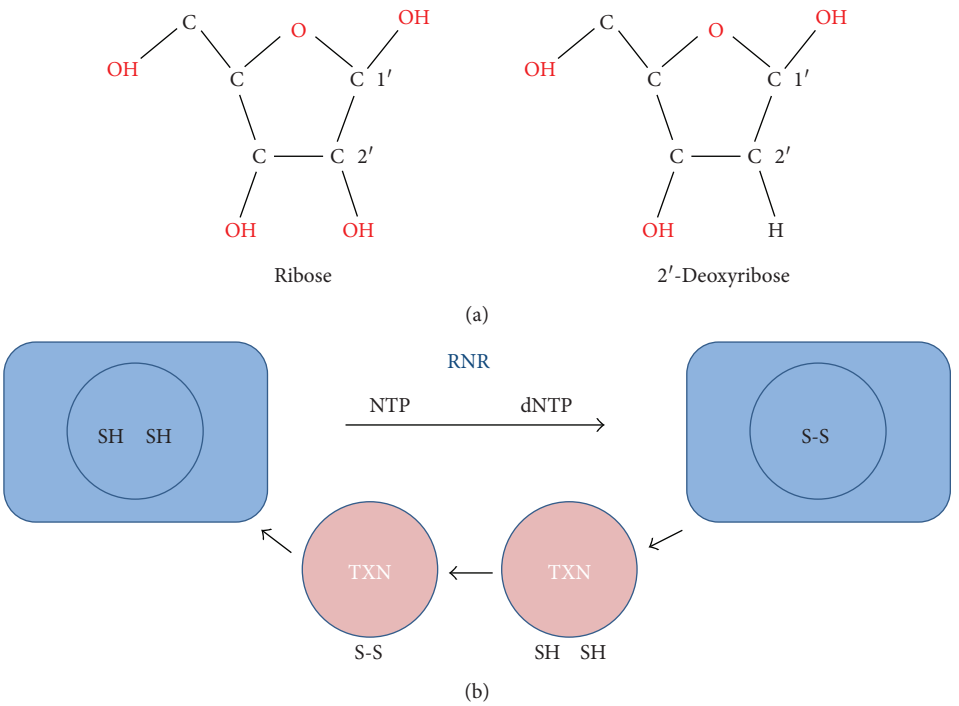


Figure 2: Deoxyribonucleotide by ribonucleotide reductase. (a) Chemical structure of ribose and 2′deoxyribose. (b) Ribonucleotide reductase catalysis and recycling. RNR: ribonucleotide reductase, NTP: ribonucleotide triphosphate, dNTP: deoxyribonucleotide triphosphate, TXN: thioredoxin.

terminal dithiol through disulfide exchange. Then, the resulting disulfide bond at the C-terminal tail is reduced by the thioredoxin (TXN) system reactivating R1 for the next cycle of RNR catalysis [20] (Figure 2(b)). So the reduction of ribonucleotides, the rate-limiting step of the DNA synthesis, depends on the reducing power provided by NADPH through thioredoxin enzymes. TXN is ubiquitously distributed from archaea, bacteria, plants, and

animals [21]. *E. coli* thioredoxin 1 (TRXA) was originally identified in 1964 by the group of Laurent et al. as a protein required for ribonucleotide reduction by ribonucleotide reductase [22]. The term thioredoxin refers to the biological function of a protein that depends on the cyclic reduction-oxidation of a single S-S group of the compound. Holmgren then disclosed the 109-amino acid sequence of TRXA and characterized the two cysteine residues (C32 and C35) of the catalytic site [23]. Years of intense research in this field revealed the dependence of the thioredoxin system on glucose metabolism through the pentose phosphate pathway that provides reducing power in the form of NADPH, the cofactor of thioredoxin reductase (TXNRD) [24]. How are oxidized extracellular thioredoxins regenerated in the rather oxidized extracellular compartment? [25]. A complete extracellular TXN system is present in the blood in the form of circulating TXN1 and TXNDR1. The procoagulant activity of cell surface tissue factor (F3), a member of the cytokine receptor family involved in the onset of coagulation, is negatively regulated by the TXN1 system that reduces F3 cysteines 186 and 209 [26]. Interestingly, the process is sensitive to change in the ratio NADPH/$NADP^+$ which dictates the redox states and activity of TXN1/TXNRD1. The origin of extracellular NADPH was not revealed by that study and remains mysterious since NADPH is not transported across intracellular membranes [27]. In the absence of NADPH and TXNRD, extracellular thioredoxins will not act as enzymes [28]. The catalytic site of reduced thioredoxins could become oxidized (SH, SH > S-S) while oxidized thioredoxins could become reduced (S-S > SH, SH), but those reactions are irreversible. The exchanged electrons would play here the role of a one-shot biological signal.

4. TRX80, a Proteolytic Product of Thioredoxin 1 Involved in Cell-to-Cell Communication

In addition to its cytokine activity, TXN1 is a chemoattractant for granulocytes, monocytes, and T-lymphocytes with a range of activities similar to other classical chemokines [29]. TXN1/ADF released from HTLV-1-infected cells carries this chemotactic activity. The mutant TXN1/ADF protein lacking the resolving cysteine of the catalytic site (CXXC > CXXS) is not active as a chemokine, showing that the redox function of TXN1/ADF is involved. It is presently not yet known if the chemokine and the cytokine activities of TXN1/ADF rely broadly or partly on the same downstream signaling molecules [9]. Through an independent research direction, a heterogeneous cytotoxic activity for the subclass of eosinophilic granulocytes was isolated from the conditioned medium of monocytes. It comprises two distinct forms of TXN1, a 14 and a 10 kDa peptide [30]. TXN1 (10 kDa) is 20-fold more active than TXN1 (14 kDa) in biological assays. The 10 kDa protein corresponds to the 80–84 N-terminal amino acids of TXN1 and was then named TRX80 accordingly [31]. TRX80 cannot be reduced by TXNRD and NADPH most likely because the missing C-terminal sequence is required for the interaction with TXNRD1 or alternatively because the truncation within the thioredoxin fold in TRX80, removing one strand (84–92) and one alpha helix (93–105), impairs with the overall 3D structure involved in the interaction [32]. Without the regenerative action of the TXNRD and NADPH, TRX80 cannot sustain thiol-oxidoreductase enzymatic activity. Interestingly, TRX80 catalytic cysteines can be reduced by TXN1 in a TXNRD1/NADPH-dependent manner. In that configuration, TRX80 is a target of TXN1. TRX80 is a truncated thioredoxin that shares similarities with another truncated thioredoxin, the rod-derived cone viability factor (RdCVF) [33] (Figure 3). The correlation between the induction of expression of TRX80 and that of metalloproteinases ADAM10 and ADAM17 by phorbol 12-myristate 13-acetate (PMA) [30, 34] led to the discovery that TRX80 is produced from TXN1 by cleavage in monocytes by the alpha-secretases ADAM10 and ADAM17 [35]. The biological functions of extracellular TRX80 is quite diverse; originally cytotoxic, it was shown to act as a cytokine for monocytes, to promote proinflammatory macrophage phenotype, to inhibit beta-amyloid peptide aggregation, and to activate the classical and alternative pathways of complement activation [35–38]. Importantly, here, the mitogenic cytokine effect on peripheral blood mononuclear cells does not involve any thiol-oxidoreductase activity provided by TRX80 [39]. It seems that the TXN1 sequence corresponding to TRX80 was recruited by the immune system to exert cell communication functions that lost their relation to redox biology.

5. Thioredoxin Secretion, a Long Lasting Mystery

Thioredoxins are secreted but have no N-terminal signal sequence and consequently are not secreted through the endoplasmic reticulum and Golgi pathway but by mechanisms that are referred to as unconventional protein secretion pathways [40]. The mechanism of secretion of thioredoxins has remained a mystery for more than 25 years after its first observation and is even currently ignored [41]. The four principal types of unconventional protein secretion can be divided into nonvesicular and vesicular pathways. The nonvesicular pathways encompass self-sustained protein translocation across plasma membranes (type I, FGF2) and ABC-transporter-based secretion (type II, yeast pheromone a-factor). Vesicular pathways are characterized by autophagy-based secretion and secretory lysosomes (type III, IL1β or IL1B) and proteins that bypass the Golgi complex for trafficking to the plasma membrane (type IV, CFTR). The type of secretory mechanism of thioredoxins remains unknown [4, 40]. TXN1 secretion *in vitro* is temperature sensitive and inhibited by unknown factors present in serum [42]. Its secretion appears to be mediated by a pathway distinct from IL1B as TXN1 could be detected neither in intracellular vesicles nor in its secretion blocked by ABC transporter inhibitors. However, even if controversial, secretion of thioredoxin is inhibited by methylamine, a lysosome inhibitor, as for that of IL1B [42]. Surprisingly,

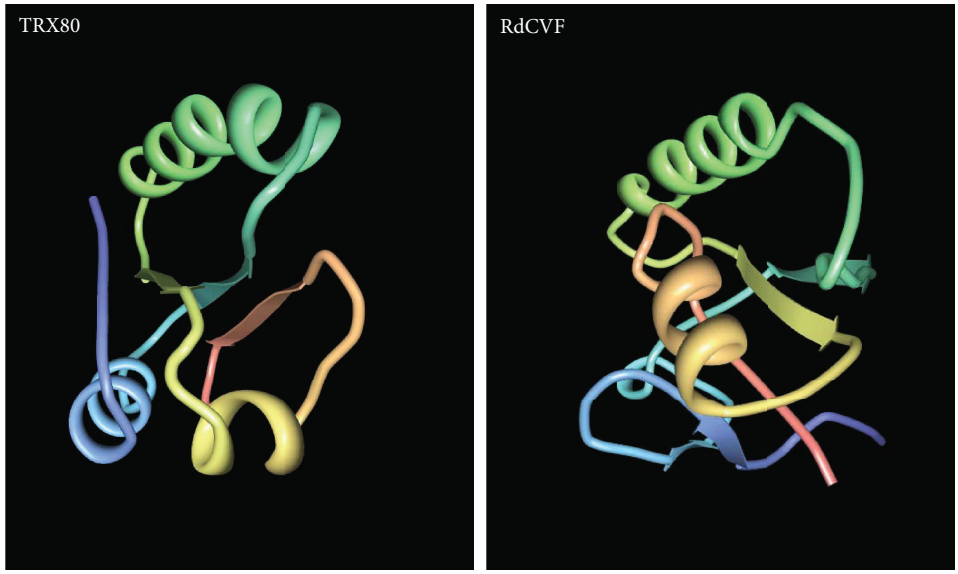


Figure 3: Structure models of TRX80 and RdCVF based on the X-ray structure of human TXN1 for TRX80 and on that of the tryparedoxin TRYX of *Crithidia fasciculata*.

the redox state of TXN1 does not influence its unconventional export [42].

6. The Truncated Thioredoxin Rod-Derived Cone Viability Factor Links Redox Homeostasis to Glucose Metabolism

The retina of vertebrates is dual with rod photoreceptors for dim-light vision and cone photoreceptor for color and daylight vision [43]. They differentiate by the morphology of the outer segment made of stacks of membranal disks or invaginations containing the light-sensitive molecule as originally observed by Schultze in 1866 [44]. The truncated thioredoxin rod-derived cone viability factor (RdCVF) was identified by high content screening. A retinal cDNA library from a wild-type mouse was used as pools of 100 plasmids to transfect COS-1 cells. Conditioned media harvested from these cells were incubating with a cone-enriched culture prepared from the retina of chicken embryos in the absence of serum [45]. In this culture, progenitor cells isolated from chicken embryos at embryonic day 6 [stage 29] were platted at low density and differentiated into cones. The viability of cells after 7 days *in vitro* was used as the readout of the assay. The set-up of the screening implies that the active polypeptide would be secreted by COS-1 cells. One pool out of the 2100 screened was diluted and led to a unique clone encoding for an open reading frame of a putative polypeptide of 109 amino acids, the RdCVF protein [46]. RdCVF was further shown to protect cones from the mouse. Retrospectively, the thought that the RdCVF factor could not have been identified if we had realized the cone-enriched cultures in the presence of serum gives vertigo. RdCVF, an alternative splicing product of nucleoredoxin-like 1 (*NXNL1)* gene, is secreted by rods that also encodes for an active thioredoxin enzyme RdCVFL with an entire thioredoxin fold and a CXXC catalytic domain [33]. RdCVFL protects photoreceptors, rods, and cones, against oxidative damage [47–49]. Since RdCVF is truncated within the thioredoxin fold, like TRX80, it does not have enzymatic activity and consequently must signal through a cell surface receptor on cones. A far-western blotting approach using the cone-dominated retina of chicken embryos was used to identify cell surface proteins interacting with GST-RdCVF after the transfer of the proteins resolved by electrophoresis on a nitrocellulose membrane. Proteins migrating along with the specific signal that was detected were identified by mass spectrometry [50]. Among the identified proteins, basigin 1 (BSG1) is a transmembrane protein representing a splice variant with an additional third extracellular immunoglobulin domain (Ig0) of the basigin gene (*Bsg*) expressed specifically by photoreceptors. This interaction was validated in a cellular context using transiently transfected BSG1 cDNA with RdCVF-alkaline phosphatase fusion protein and a colorimetric assay. Silencing *Bsg* in chicken cone-enriched cultures reduced cell survival mediated by RdCVF indicating that the BSG1 is the RdCVF transducing cell surface receptor. Unfortunately, BSG1 was not previously known as a cell surface receptor but rather as a protein involved in cell adhesion [51]. Its intracellular domain is short and does not carry any informative motifs such as a tyrosine phosphorylation site. Therefore, we used a BSG1 antibody to coimmunoprecipitate BSG1-interacting proteins from membrane fraction of the retina of chicken embryos and identified these proteins by mass spectrometry [50]. BSG1 interacts with the glucose transporter GLUT1/SLC2A1. The formation of the complex RdCVF/BSG1/GLUT1 on cone surface stimulates glucose entry into the cones. The mechanism that leads to an accelerated glucose entry into cones via GLUT1/SLC2A1 after RdCVF binding to BSG1 is presently unknown. Considering that the catalytic site of extracellular thioredoxins could become oxidized (SH, SH > S-S), RdCVF could act as a

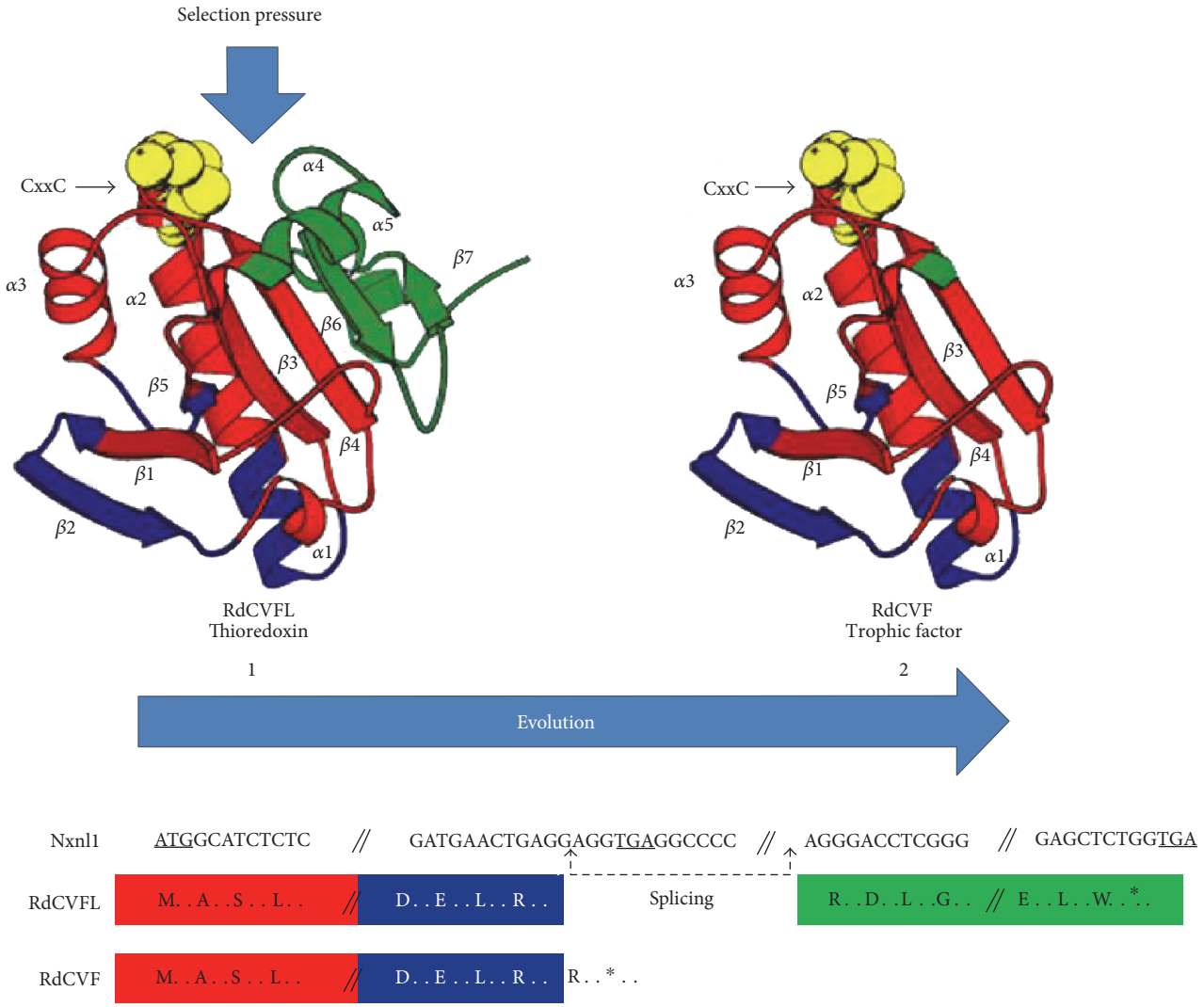


Figure 4: Theoretical consideration on the evolutionary history of the nucleoredoxin-like 1 gene.

prooxidant that triggers the conversion of homodimeric and reduced form of GLUT1/SLC2A1 to its homotetrameric and oxidized form that is known to transport glucose more efficiently [52]. RdCVF would be a positive allosteric heterotrophic effector of the glucose transporter GLUT1/SLC2A1 [53]. Notice that TXN1/ADF is also acting as a prooxidant for MIF in the hypothetical model proposed above (Figure 1). Glucose is used by cones to produce lactate by aerobic glycolysis. The idea that cones use this particular metabolism of glucose where the pyruvate produced by glycolysis is not transported to the mitochondria, but rather metabolized to lactate by lactate dehydrogenase, is usually restricted to cancer cells and named the Warburg effect [54, 55]. This peculiar use of glucose by cones was demonstrated by measuring the oxygen consumption and extracellular acidification resulting from lactate secretion. Inhibition of pyruvate transport to the mitochondria or pentose phosphate pathway does not affect the activity of RdCVF, while, oxamate, a lactate dehydrogenase inhibitor, abolishes RdCVF effects on cones. Quite interestingly, in 1924, Otto Warburg established that aerobic glycolysis is specific of cancer cells, he also reported that avian retina metabolizes glucose by aerobic glycolysis, but he suspected at this time that this was an artifact. So 91 years have been necessary to resolve the enigma of aerobic glycolysis in the retina of cone-dominated species such as the chicken and pigeon [56]. Glucose is partially metabolized to glycerol-3-phosphate, a molecule entering the composition of phospholipids forming the membranes of cone outer segments. Phospholipids are composed of two fatty acids, a glycerol unit and a phosphate group. Fatty acids derived from food are activated in the form of acyl-CoA intermediates and esterified with glycerol-3-phosphate into phospholipids through the Kennedy pathway [57]. Glycerol-3-phosphate is made from a glycolytic metabolite, dihydroxyacetone phosphate (DHAP), the breakdown of one molecule of fructose-1,6-bisphosphate (C6) by aldolase A. Aldolase produces one molecule of glyceraldehyde-3-phosphate (C3) and one molecule of DHAP (C3). Phosphatidic acid is synthesized through multiple enzymatic steps from glycerol-3-phosphate and fatty acyl-CoA. Phospholipid synthesis is taking place on the cytoplasmic face of the endoplasmic reticulum where the membrane-bound choline/

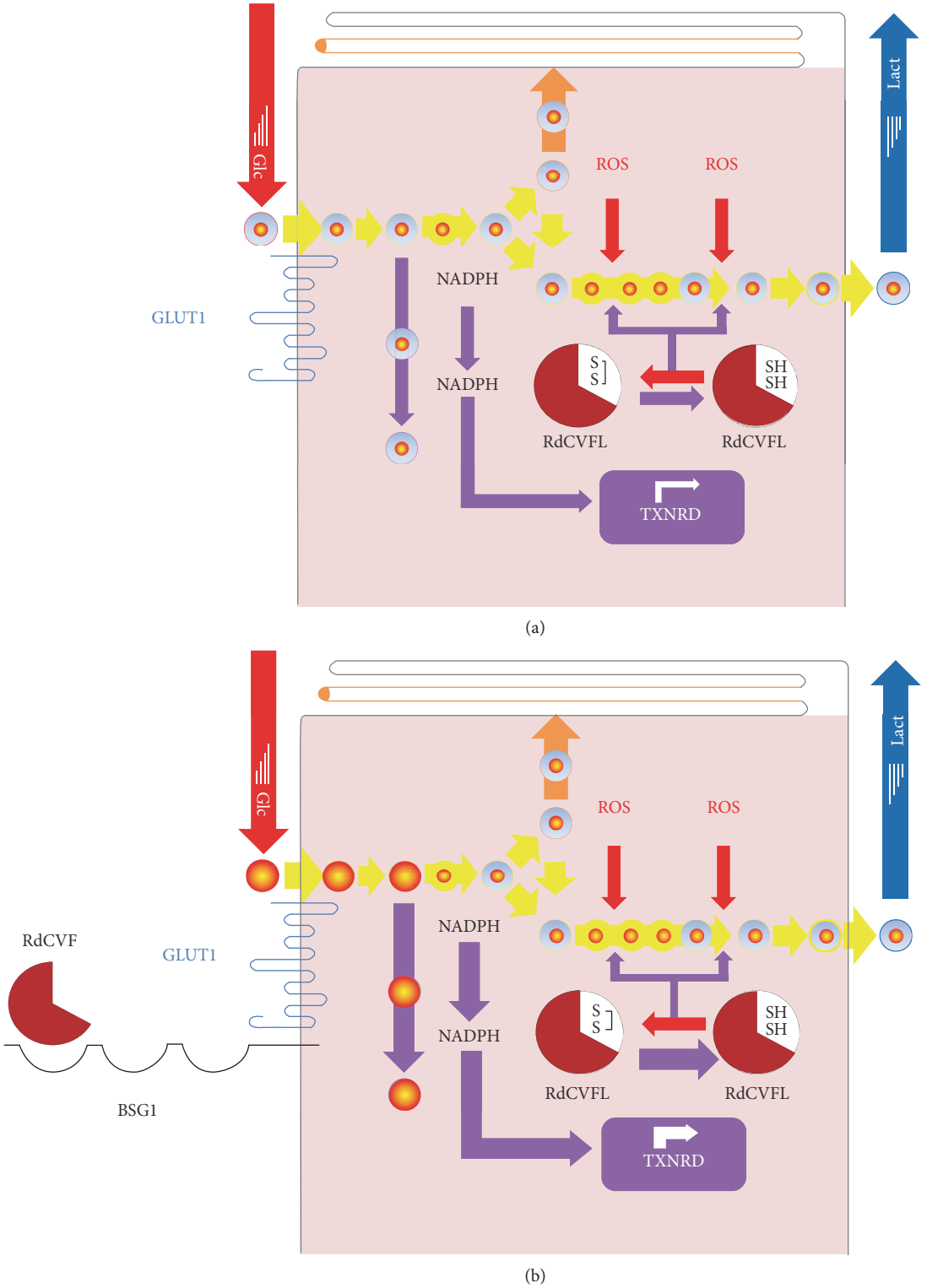


Figure 5: The nucleoredoxin-like 1 gene encodes for a superthioredoxin system. (a) In the absence of RdCVF signaling mediated by the complex BSG1/GLUT1, the reduction of oxidized RdCVFL in cones by thioredoxin reductase (TXNRD) and NADPH is limited by glucose uptake rate. (b) The extracellular truncated thioredoxin RdCVF accelerates glucose uptake by its interaction with the BSG1/GLUT1 complex at the surface of cones. The concentration of NADPH produced by the pentose phosphate pathway is increased. Consequently, the reduction of RdCVFL is ameliorated so that the combination of extracellular RdCVF and intracellular RdCVFL constitutes a superthioredoxin system. Glc: glucose, lact: lactate, and ROS: reactive oxygen species. Circles represent metabolites and their diameters and their concentrations. Yellow: glycolysis, Orange: triglyceride synthesis, and purple: pentose phosphate pathway.

ethanolamine phosphotransferase enzyme (CEPT1) is located. This reaction produces the phospholipids, phosphatidylcholine, or phosphatidylethanolamine.

7. The Nucleoredoxin-Like 1 Gene Encodes for a Superthioredoxin System

The retina of the mouse with a disruption of the *Nxnl1* gene shows signs of oxidative and photooxidative damages [47]. Using adeno-associated viral vector delivery through a ubiquitous promoter, we found that RdCVF (109 residues) protects the cones of the mouse retina but not RdCVFL (217 residues) [58]. The 109 residues of RdCVF are identical to the N-terminal region of the 217 amino acid-long RdCVFL protein since RdCVF is truncated within the thioredoxin fold. The fact that the full-length RdCVF sequence does not protect cones when delivered through a ubiquitous promoter suggests that the structure of the thioredoxin fold of RdCVF is altered by the truncation. The bifunctional gene *NXNL1*, whose expression is restricted to the retina, is composed by two coding exons framing one intron. RdCVF is produced by alternative splicing. When the intron is spliced, the gene encodes for a long product coding for the thioredoxin enzyme RdCVFL, whereas when the intron is retained and due to the presence of a conserved stop codon in the reading frame, the gene encodes for a short product coding for the truncated thioredoxin RdCVF [33]. Alternative splicing is cell specific as it occurs only in rods but not in cones of the mouse retina that express only RdCVFL [49]. When using adeno-associated viral vector delivery through a cone-specific promoter, RdCVFL (217 residues) protects the cones of the mouse retina against oxidative damage. RdCVF expressed by rods stimulates cone outer segment growth. Downstream of that metabolic signaling, RdCVFL is involved in redox homeostasis. Hence, in that sense, both products of the *NXNL1* are acting toward the same goal and have a complementary activity: protecting cones [59].

In an evolutionary perspective, the selection pressure acts first on the thioredoxin fold of RdCVFL before acting on the truncated thioredoxin RdCVF (Figure 4). This is correlated to the fact that cones precede rods [60]. What could have been the driving force leading to the emergence of the trophic factor RdCVF? An accidental splicing leading to an extracellular truncated thioredoxin as RdCVF would not work without the expression of BSG in the form of the splicing variant having a third extracellular domain Ig0, produced by photoreceptors by alternative splicing. Are there temporal links between the appearance of the inhibition of the *NXNL1* gene's splicing leading to its intron retention and the rod appearance during evolution? Regardless of the evolution history of RdCVF signaling, it is quite reasonable to propose that the thioredoxin activity of RdCVFL precedes that of RdCVF but that conjunction of both proteins is acting as a positive feedback loop for redox homeostasis in the cones and defines a superthioredoxin system (Figure 5). Meaning that the ancestral *NXNL1* gene originally encodes for a thioredoxin enzyme and then after for another protein RdCVF boosting the activity of the first one. Whatever the details, the identification of the molecular mechanisms leading to intron retention will certainly be very informative.

8. A Truncated Extracellular Thioredoxin as a Therapeutic Agent for Blinding Diseases

Opsin activation by photon capture requires the sensing molecule to be embedded in a lipid bilayer of optimal fluidity made of phospholipids rich in polyunsaturated fatty acids (PUFA) [61]. PUFA have the tendency to oxidize and must be removed daily for maintenance of the visual system [62]. Consequently, since cones are postmitotic neurons, 10% of their outer segment is removed daily by phagocytosis of the retinal pigment epithelium and renewed from the inner segment where glucose metabolism is very active. With a prevalence of 1/5000, retinitis pigmentosa is the most common form of inherited retinal degeneration. In patients suffering from retinitis pigmentosa, the vision loss develops in two successive steps. The first clinical sign of this disease is night blindness, which is the consequence of rod degeneration due to the direct action of mutations in any of the 60 distinct genes presently known to cause the disease. This is felt as a minor handicap, and these people retain an almost normal way of life [63]. Rod degeneration leads to the loss of expression of RdCVF [64]. The subsequent loss of function of cones at the centre of the retina results in reduced central vision leading ultimately to untreatable blindness. As patients usually visit ophthalmologists when their daylight vision decreases which corresponds to the moment when cones start to be affected, preventing the death of rods will not be medically effective [65]. Treating patients by restoring the expression of the superthioredoxin system made of RdCVF and RdCVFL, while not correcting the causative gene defect, should maintain cone-mediated central vision, potentially benefiting an estimated 1.5 million people worldwide [66, 67].

9. Conclusion

Extracellular thioredoxins have been implicated in intercellular communication early on from the discovery of TXN1/ADF. Meanwhile, in a detailed description of its mode of action and that of the truncated form of TXN1, TRX80 remains incomplete. The extracellular truncated thioredoxin RdCVF encoded by the *NXNL1* gene is to our knowledge a unique example of a complete extracellular thioredoxin signaling system.

Conflicts of Interest

Thierry Léveillard holds a patent on the use of *NXNL1* gene products to treat inherited retinal blindness.

Acknowledgments

The authors gratefully thank Frédéric Blond for his help in drawing the figures and for reading the manuscript. The authors would like to thank Erika Camacho for her contribution to the correction of the manuscript. This work was

supported by UPMC, Inserm, CNRS, ANR, and the Foundation Fighting Blindness.

References

[1] A. Holmgren, "Thioredoxin," *Annual Review of Biochemistry*, vol. 54, pp. 237–271, 1985.

[2] Y. M. Go, J. D. Chandler, and D. P. Jones, "The cysteine proteome," *Free Radical Biology and Medicine*, vol. 84, pp. 227–245, 2015.

[3] C. E. Paulsen and K. S. Carroll, "Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery," *Chemical Reviews*, vol. 113, no. 7, pp. 4633–4679, 2013.

[4] Y. Tagaya, Y. Maeda, A. Mitsui et al., "ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction," *The EMBO Journal*, vol. 8, no. 3, pp. 757–764, 1989.

[5] E. McNeill, M. J. Crabtree, N. Sahgal et al., "Regulation of iNOS function and cellular redox state by macrophage *Gch1* reveals specific requirements for tetrahydrobiopterin in NRF2 activation," *Free Radical Biology and Medicine*, vol. 79, pp. 206–216, 2015.

[6] J. M. Coffin, S. H. Hughes, and H. E. Varmus, "The interactions of retroviruses and their hosts," in *Retroviruses*, J. M. Coffin, S. H. Hughes and H. E. Varmus, Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY), USA, 1997.

[7] K. Teshigawara, M. Maeda, K. Nishino et al., "Adult T leukemia cells produce a lymphokine that augments interleukin 2 receptor expression," *The Journal of Molecular and Cellular Immunology*, vol. 2, no. 1, pp. 17–26, 1985.

[8] N. Wakasugi, Y. Tagaya, H. Wakasugi et al., "Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 21, pp. 8282–8286, 1990.

[9] Y. Matsuo and J. Yodoi, "Extracellular thioredoxin: a therapeutic tool to combat inflammation," *Cytokine & Growth Factor Reviews*, vol. 24, no. 4, pp. 345–353, 2013.

[10] J. Yodoi and M. Maeda, "Discovery of ATL: an odyssey in restrospect," *International Journal of Hematology*, vol. 94, no. 5, pp. 423–428, 2011.

[11] A. Son, N. Kato, T. Horibe et al., "Direct association of thioredoxin-1 (TRX) with macrophage migration inhibitory factor (MIF): regulatory role of TRX on MIF internalization and signaling," *Antioxidants & Redox Signaling*, vol. 11, no. 10, pp. 2595–2605, 2009.

[12] J. Bernhagen, R. Krohn, H. Lue et al., "MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment," *Nature Medicine*, vol. 13, no. 5, pp. 587–596, 2007.

[13] L. Leng, C. N. Metz, Y. Fang et al., "MIF signal transduction initiated by binding to CD74," *The Journal of Experimental Medicine*, vol. 197, no. 11, pp. 1467–1476, 2003.

[14] C. C. G. Nobre, J. M. G. de Araujo, T. A. A. D. Fernandes et al., "Macrophage migration inhibitory factor (MIF): biological activities and relation with cancer," *Pathology & Oncology Research*, vol. 23, no. 2, pp. 235–244, 2017.

[15] C. P. Fan, D. Rajasekaran, M. A. Syed et al., "MIF intersubunit disulfide mutant antagonist supports activation of CD74 by endogenous MIF trimer at physiologic concentrations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 27, pp. 10994–10999, 2013.

[16] H. Ouertatani-Sakouhi, F. El-Turk, B. Fauvet et al., "Identification and characterization of novel classes of macrophage migration inhibitory factor (MIF) inhibitors with distinct mechanisms of action," *Journal of Biological Chemistry*, vol. 285, no. 34, pp. 26581–26598, 2010.

[17] U. Schwertassek, Y. Balmer, M. Gutscher et al., "Selective redox regulation of cytokine receptor signaling by extracellular thioredoxin-1," *The EMBO Journal*, vol. 26, no. 13, pp. 3086–3097, 2007.

[18] A. Holmgren, "Antioxidant function of thioredoxin and glutaredoxin systems," *Antioxidants & Redox Signaling*, vol. 2, no. 4, pp. 811–820, 2000.

[19] S. Z. Xu, P. Sukumar, F. Zeng et al., "TRPC channel activation by extracellular thioredoxin," *Nature*, vol. 451, no. 7174, pp. 69–72, 2008.

[20] R. Sengupta and A. Holmgren, "Thioredoxin and glutaredoxin-mediated redox regulation of ribonucleotide reductase," *World Journal of Biological Chemistry*, vol. 5, no. 1, pp. 68–74, 2014.

[21] B. B. Buchanan, A. Holmgren, J. P. Jacquot, and R. Scheibe, "Fifty years in the thioredoxin field and a bountiful harvest," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1820, no. 11, pp. 1822–1829, 2012.

[22] T. C. Laurent, E. C. Moore, and P. Reichard, "Enzymatic synthesis of deoxyribonucleotides IV. Isolation and characterization of thioredoxin, the hydrogen donor from Escherichia coli B," *The Journal of Biological Chemistry*, vol. 239, pp. 3436–3444, 1964.

[23] A. Holmgren, "Thioredoxin. 6. The amino acid sequence of the protein from escherichia coli B," *European Journal of Biochemistry/FEBS*, vol. 6, no. 4, pp. 475–484, 1968.

[24] J. M. Berg, J. L. Tymoczko, and L. Stryer, *Biochemistry*, W.H. Freeman, New York, 5th edition, 2002.

[25] D. P. Jones and Y. M. Go, "Redox compartmentalization and cellular stress," *Diabetes, Obesity & Metabolism*, vol. 12, Supplement 2, pp. 116–125, 2010.

[26] P. Wang, Y. Wu, X. Li, X. Ma, and L. Zhong, "Thioredoxin and thioredoxin reductase control tissue factor activity by thiol redox-dependent mechanism," *The Journal of Biological Chemistry*, vol. 288, no. 5, pp. 3346–3358, 2013.

[27] C. A. Lewis, S. J. Parker, B. P. Fiske et al., "Tracing compartmentalized NADPH metabolism in the cytosol and mitochondria of mammalian cells," *Molecular Cell*, vol. 55, no. 2, pp. 253–263, 2014.

[28] E. S. Arner and A. Holmgren, "Physiological functions of thioredoxin and thioredoxin reductase," *European Journal of Biochemistry/FEBS*, vol. 267, no. 20, pp. 6102–6109, 2000.

[29] R. Bertini, O. M. Z. Howard, H. F. Dong et al., "Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells," *Journal of Experimental Medicine*, vol. 189, no. 11, pp. 1783–1789, 1999.

[30] M. K. Balcewicz-Sablinska, E. E. Wollman, R. Gorti, and D. S. Silberstein, "Human eosinophil cytotoxicity enhancing factor. II. Multiple forms synthesized by U937 cells and

their relationship to thioredoxin/adult T cell leukemia-derived factor," *Journal of Immunology*, vol. 147, no. 7, pp. 2170–2174, 1991.

[31] K. Pekkari, R. Gurunath, E. S. Arner, and A. Holmgren, "Truncated thioredoxin is a mitogenic cytokine for resting human peripheral blood mononuclear cells and is present in human plasma," *The Journal of Biological Chemistry*, vol. 275, no. 48, pp. 37474–37480, 2000.

[32] K. Fritz-Wolf, S. Kehr, M. Stumpf, S. Rahlfs, and K. Becker, "Crystal structure of the human thioredoxin reductase-thioredoxin complex," *Nature Communications*, vol. 2, p. 383, 2011.

[33] F. Chalmel, T. Leveillard, C. Jaillard et al., "Rod-derived cone viability factor-2 is a novel bifunctionalthioredoxin- like protein with therapeutic potential," *BMC Molecular Biology*, vol. 8, p. 74, 2007.

[34] J. D. Buxbaum, S. E. Gandy, P. Cicchetti et al., "Processing of Alzheimer beta-A4 amyloid precursor protein - modulation by agents that regulate protein-phosphorylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 15, pp. 6003–6006, 1990.

[35] F. Gil-Bea, S. Akterin, T. Persson et al., "Thioredoxin-80 is a product of alpha-secretase cleavage that inhibits amyloid-beta aggregation and is decreased in Alzheimer's disease brain," *EMBO Molecular Medicine*, vol. 4, no. 10, pp. 1097–1111, 2012.

[36] K. Pekkari and A. Holmgren, "Truncated thioredoxin: physiological functions and mechanism," *Antioxidants & Redox Signaling*, vol. 6, no. 1, pp. 53–61, 2004.

[37] D. F. D. Mahmood, A. Abderrazak, D. Couchie et al., "Truncated thioredoxin (Trx-80) promotes proinflammatory macrophages of the M1 phenotype and enhances atherosclerosis," *Journal of Cellular Physiology*, vol. 228, no. 7, pp. 1577–1583, 2013.

[38] B. C. King, J. Nowakowska, C. M. Karsten, J. Kohl, E. Renstrom, and A. M. Blom, "Truncated and full- length thioredoxin-1 have opposing activating and inhibitory properties for human complement with relevance to endothelial surfaces," *Journal of Immunology*, vol. 188, no. 8, pp. 4103–4112, 2012.

[39] K. Pekkari, J. Avila-Carino, R. Gurunath, A. Bengtsson, A. Scheynius, and A. Holmgren, "Truncated thioredoxin (Trx80) exerts unique mitogenic cytokine effects via a mechanism independent of thiol oxido-reductase activity," *FEBS Letters*, vol. 539, no. 1–3, pp. 143–148, 2003.

[40] A. Rubartelli, A. Bajetto, G. Allavena, E. Wollman, and R. Sitia, "Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway," *The Journal of Biological Chemistry*, vol. 267, no. 34, pp. 24161–24164, 1992.

[41] F. Ng and B. L. Tang, "Unconventional protein secretion in animal cells," *Methods in Molecular Biology*, vol. 1459, pp. 31–46, 2016.

[42] M. Tanudji, S. Hevi, and S. L. Chuck, "The nonclassic secretion of thioredoxin is not sensitive to redox state," *American Journal of Physiology-Cell Physiology*, vol. 284, no. 5, pp. C1272–C1279, 2003.

[43] S. J. Ryan, *Retina*, Elsevier Mosby, Philadelphia, 4th edition, 2006.

[44] M. Schultze, "Zur Anatomie und Physiologie der Retina," *Archiv für Mikroskopische Anatomie*, vol. 2, no. 1, pp. 175–286, 1866.

[45] R. Adler and M. Hatlee, "Plasticity and differentiation of embryonic retinal cells after terminal mitosis," *Science*, vol. 243, no. 4889, pp. 391–393, 1989.

[46] T. Leveillard, S. Mohand-Said, O. Lorentz et al., "Identification and characterization of rod-derived cone viability factor," *Nature Genetics*, vol. 36, no. 7, pp. 755–759, 2004.

[47] T. Cronin, W. Raffelsberger, I. Lee-Rivera et al., "The disruption of the rod-derived cone viability gene leads to photoreceptor dysfunction and susceptibility to oxidative stress," *Cell Death and Differentiation*, vol. 17, no. 7, pp. 1199–1210, 2010.

[48] G. Elachouri, I. Lee-Rivera, E. Clerin et al., "Thioredoxin rod-derived cone viability factor protects against photooxidative retinal damage," *Free Radical Biology and Medicine*, vol. 81, pp. 22–29, 2015.

[49] X. Mei, A. Chaffiol, C. Kole et al., "The Thioredoxin encoded by the rod-derived cone viability factor gene protects cone photoreceptors against oxidative stress," *Antioxidants & Redox Signaling*, vol. 24, no. 16, pp. 909–923, 2016.

[50] N. Ait-Ali, R. Fridlich, G. Millet-Puel et al., "Rod-derived cone viability factor promotes cone survival by stimulating aerobic glycolysis," *Cell*, vol. 161, no. 4, pp. 817–832, 2015.

[51] J. D. Ochrietor, T. P. Moroz, L. van Ekeris et al., "Retina-specific expression of 5A11/Basigin-2, a member of the immunoglobulin gene superfamily," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 9, pp. 4086–4096, 2003.

[52] D. N. Hebert and A. Carruthers, "Glucose transporter oligomeric structure determines transporter function. Reversible redox-dependent interconversions of tetrameric and dimeric GLUT1," *The Journal of Biological Chemistry*, vol. 267, no. 33, pp. 23829–23838, 1992.

[53] E. T. Camacho, T. Leveillard, J. A. Sahel, and S. Wirkus, "Mathematical model of the role of RdCVF in the coexistence of rods and cones in a healthy eye," *Bulletin of Mathematical Biology*, vol. 78, no. 7, pp. 1394–1409, 2016.

[54] M. G. Vander Heiden, L. C. Cantley, and C. B. Thompson, "Understanding the Warburg effect: the metabolic requirements of cell proliferation," *Science*, vol. 324, no. 5930, pp. 1029–1033, 2009.

[55] T. Leveillard, "Cancer metabolism of cone photoreceptors," *Oncotarget*, vol. 6, no. 32, pp. 32285-32286, 2015.

[56] H. A. Krebs, "The Pasteur effect and the relations between respiration and fermentation," *Essays in Biochemistry*, vol. 8, pp. 1–34, 1972.

[57] N. Chauhan, L. Farine, K. Pandey, A. K. Menon, and P. Bütikofer, "Lipid topogenesis—35 years on," *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, vol. 1861, 2016.

[58] L. C. Byrne, D. Dalkara, G. Luna et al., "Viral mediated RdCVF and RdCVFL expression protects cone and rod photoreceptors in retinal degeneration," *The Journal of Clinical Investigation*, vol. 125, no. 1, pp. 105–116, 2015.

[59] T. Leveillard and J. A. Sahel, "Metabolic and redox signaling in the retina," *Cellular and Molecular Life Sciences*, 2016.

[60] G. L. Fain, R. Hardie, and S. B. Laughlin, "Phototransduction and the evolution of photoreceptors," *Current Biology*, vol. 20, no. 3, pp. R114–R124, 2010.

[61] A. Watts, I. D. Volotovski, and D. Marsh, "Rhodopsin-lipid associations in bovine rod outer segment membranes. Identification of immobilized lipid by spin-labels," *Biochemistry*, vol. 18, no. 22, pp. 5006–5013, 1979.

[62] R. S. Molday and O. L. Moritz, "Photoreceptors at a glance," *Journal of Cell Science*, vol. 128, no. 22, pp. 4039–4045, 2015.

[63] A. M. Geller and P. A. Sieving, "Assessment of foveal cone photoreceptors in Stargardt's macular dystrophy using a small dot detection task," *Vision Research*, vol. 33, no. 11, pp. 1509–1524, 1993.

[64] T. Leveillard and J. A. Sahel, "Rod-derived cone viability factor for treating blinding diseases: from clinic to redox signaling," *Science Translational Medicine*, vol. 2, no. 26, article 26ps16, 2010.

[65] J. Krol and B. Roska, "Rods feed cones to keep them alive," *Cell*, vol. 161, no. 4, pp. 706–708, 2015.

[66] A. F. Wright, "A searchlight through the fog," *Nature Genetics*, vol. 17, no. 2, pp. 132–134, 1997.

[67] C. Cepko and C. Punzo, "Cell metabolism: sugar for sight," *Nature*, vol. 522, no. 7557, pp. 428-429, 2015.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7313090, 10 pages
https://doi.org/10.1155/2017/7313090

Research Article

Chronic Variable Stress Is Responsible for Lipid and DNA Oxidative Disorders and Activation of Oxidative Stress Response Genes in the Brain of Rats

Mariola Herbet,[1] Agnieszka Korga,[1] Monika Gawrońska-Grzywacz,[1] Magdalena Izdebska,[1] Iwona Piątkowska-Chmiel,[1] Ewa Poleszak,[2] Andrzej Wróbel,[3] Włodzimierz Matysiak,[4] Barbara Jodłowska-Jędrych,[4] and Jarosław Dudka[1]

[1]*Chair and Department of Toxicology, Medical University of Lublin, Chodźki 8, 20-093 Lublin, Poland*
[2]*Department of Applied Pharmacy, Medical University of Lublin, Chodźki 1, 20-093 Lublin, Poland*
[3]*Second Department of Gynecology, Medical University of Lublin, Jaczewskiego 8, 20-090 Lublin, Poland*
[4]*Department of Histology and Embryology, Medical University of Lublin, Radziwiłłowska 11, 20-080 Lublin, Poland*

Correspondence should be addressed to Mariola Herbet; mariola.herbet@umlub.pl

Received 26 May 2017; Revised 22 July 2017; Accepted 14 August 2017; Published 11 September 2017

Academic Editor: Tanea T. Reed

Chronic environmental stress is associated with reactive oxygen species (ROS) overproduction and the pathogenesis of depression. The purpose of this study was to evaluate biochemical and molecular changes associated with ROS generation in the brains of rats submitted to chronic variable stress. Male Wistar rats (50–55 days old, weighing 200–250 g) were divided in two groups ($n = 10$): control and stressed. Rats in the stressed group were exposed to stress conditions for 40 days. The animals were decapitated and the brain samples were collected. In prefrontal cortex, we measured the following biochemical parameters: lipid peroxidation and concentration of glutathione—GSH, GSSG, GSH/GSSG ratio, glutathione peroxidase, and glutathione reductase activities. In the hippocampus marker of DNA, oxidative damage and expression of DNA-repairing genes (*Ogg1*, *MsrA*) and gene-encoding antioxidative transcriptional factor (*Nrf2*) were determined. The results demonstrate indirect evidence of ROS overproduction and presence of oxidative stress. They also reveal disruption of oxidative defense systems (decreased GR activity, diminished GSH/GSSG ratio, and decreased *Nrf2* expression) and activation of the oxidative DNA repair system (increased *Ogg1* and *MsrA* expression). Together, the presented data suggest that independent activation of oxidative stress response genes occurs in chronic variable stress conditions.

1. Introduction

Depression is currently the most common affective disorder. It is estimated to affect over 120 million people worldwide and the number of cases is steadily increasing [1]. Forecasts indicate that by 2020, it will rank second in lifestyle diseases that reduce the capacity to work [2]. Known causes of depression do not provide sufficient explanation of pathophysiology, despite extensive research in this area. It is believed that the process is multifactorial and has many subtypes with more than one etiology. Studies have shown that chronic stress is directly implicated in the pathogenesis of depression [3, 4]. Stressful events may induce multiple behavioral, neurochemical, and biological alterations, presumably as an adaptive response to meet environmental demands. It has been described that a prolonged and sustained stimulation, caused by stress exceeding the body's capacity to maintain homeostasis, can result in psychopathological events [5].

It is postulated that chronic environmental stressors may have a significant impact on reactive oxygen species (ROS) generation in the brain [6, 7]. Studies have consistently reported an increase of ROS in the blood of patients with major depression [8]. The mechanism underlying the ROS

increase remains unclear and is likely mediated in part by stress-related hormones such as cortisol [9]. A stress-induced increase in cortisol levels has been reported to accelerate glucose metabolism and the production of ROS [10]. ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO•), are extremely reactive, due to unpaired electrons. Hydrogen peroxide is particularly damaging the DNA as it is less reactive than other radicals and able to travel into the cell nucleus, subsequently reacting with macromolecules such as DNA. In well-functioning central nervous system (CNS) cells, there is a balance between the formation of free radicals and their scavenging. The shift in this equilibrium toward the formation of ROS generates oxidative stress, which is defined as an increase in oxidation potential to the level leading to macromolecular oxidation (e.g., lipids, DNA, and protein), despite adaptive activation of antioxidative defense system [11, 12]. Oxidative stress leads to the formation of lipid peroxidation products, resulting in the loss of cell membrane fluidity, reduced membrane potential and possible rupture. Upon rupture, cell and organelle contents are released into the extracellular space, including neurotransmitters (serotonin and noradrenaline) related to major depression [13]. Subjecting cells to oxidative stress can result in cell membrane modifications and leads to disruption of receptor functions and enzyme and gene activity [14]. Oxidation-modified compounds and molecules that disturb cellular homeostasis can lead to apoptosis or necrosis [15, 16]. These events are related to various neurodegenerative diseases [3, 14, 17]. Neuronal cells in the brain are highly sensitive to oxidative stress due to a large dependence on oxidative phosphorylation for energy compared to other cell types. Normal brain function is dependent on a continuous and efficient use of oxygen. Although the brain represents only 2% of human body weight, it consumes 20% of the total body oxygen with 1-2% of the oxygen being converted into superoxide anion radicals and hydrogen peroxide. Increased O_2^{*-} production in the mitochondria inhibits the Krebs cycle through temporary aconitase inactivation. The Krebs cycle is inhibited by lipid peroxidation products—MDA and 4HNE [18, 19]. Moreover, MDA and 4HNE can cause DNA damage [20]. It is worth mentioning that the brain as an organ is a major metabolizer of oxygen (20% of the body consumption) and yet has relatively feeble protective antioxidant mechanisms [21].

One of the most important consequences of ROS overproduction and chronic oxidative damage is DNA modification, which can become permanent via the formation of mutations and other types of genomic instabilities [22–25]. Studies have shown that modulation of gene expression under oxidative stress conditions is an important mechanism in depression [24–27]. Among various types of DNA base modification induced by ROS, 7, 8-dihydro-8-oxoguanine (8-oxoG) is the most widely studied and is considered to be a key biomarker of oxidative DNA damage [14, 27, 28]. Studies have revealed significantly elevated levels of 8-OxoG in urine of depressed patients in comparison to healthy controls [29]. The 8-oxoguanine glycosylase1 (OGG1) gene is a key component of the base excision repair pathway, because OGG1 encodes the enzyme responsible for the excision of 8-oxoguanine, a mutagenic base byproduct that occurs as a result of exposure to reactive oxygen [22]. Methionine sulfoxide reductase A (MSRA) also plays an important role in the repair of oxidatively damaged proteins toward restoration of biological activity [23]. MSRA reduces methionine sulfoxide (MetO) to methionine. Because methionine residues are particularly susceptible to oxidation by ROS, MSRA has important functions in cellular metabolism: as an antioxidant enzyme that scavenges ROS by facilitating the cyclic interconversion of methionine between oxidized and reduced forms and as a repair enzyme by keeping critical methionine residues in their reduced form [30, 31]. Several studies have demonstrated high levels of *MsrA* expression in brain neurons and confirmed that *MsrA* overexpression results in an extension of lifespan in mice and in human T cells under conditions of oxidative stress [23, 31]. An emerging regulator of cellular resistance to oxidants is nuclear factor erythroid 2-related factor 2 (NRF2). NRF2, beyond its regulatory role in antioxidant enzyme expression, has been recognized as a key factor in the regulation of an array of genes that defend cells against the deleterious effects of environmental insults [32]. An important mechanism of cellular defense against oxidative or electrophilic stress is achieved through activation of the Nrf2-antioxidant response element signaling pathway. The pathway controls the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants through enhancing cellular antioxidant capacity [33–35]. By regulating oxidant levels and oxidant signaling, NRF2 participates in the control of mitochondrial biogenesis [36]. Moreover, NRF2 likely also affects mitochondrial ROS production [37]. Increased expression of *Nrf2* is an important preventative component in neurodegenerative diseases [38]. Lowering NRF2 activation may reduce antioxidative responses [39].

The role of oxidative stress in depression appears to be associated with neurogenesis and cell survival, but whether oxidative stress is a cause or merely a downstream consequence of the neurodegenerative process still remains unexplained [23]. It has been hypothesized that chronic environmental stress-induced oxidative stress may contribute to changes at the molecular level. The molecular studies seem to be crucial in this regard. Chronic oxidative damage can be indirectly assessed by measuring changes in the expression of genes involved in protection and repair systems.

In consideration of these findings, the main objective of our study was the assessment of oxidative DNA damage in the brain of rats submitted to chronic variable stress. The extent of AP sites (one of the major types of damage generated by ROS) and *Ogg1*, *MsrA*, and *Nrf2* genes was measured. In the present study, we also investigated the effect of chronic environmental stress on lipid peroxidation, glutathione redox status, and antioxidant enzyme activities (glutathione peroxidase and reductase). We used the prefrontal cortex to determine biochemical parameters and hippocampus for molecular tests because depressed patients present alterations in these cerebral structures and relevant research most often relates to these regions of the brain [40–42]. The chronic variable stress (CVS) paradigm is a well-validated

animal model of depression, as recent publications have confirmed that CVS can induce behavioral and neurochemical changes in animals that are similar to the symptoms and presumed neurochemical changes accompanying depression in humans [43–45]. Similarly, clinical evidence describes that stressful life events, which significantly increase the risk of depressive episodes, are generally of a chronic nature [46, 47].

2. Materials and Methods

2.1. Animals. Male Wistar rats, weighing 200–250 g, approximately 50–55 days old at the time of arrival, purchased from a licensed breeder (Brwinów, Poland) were used as test subjects. The animals were housed in standard rectangular polypropylene cages with standard diet and water available ad libitum. The colony room was maintained at a constant temperature (20 ± 2°C) under a 12 h day/12 h night cycle in constant environmental conditions (humidity, noise). All experimental procedures were approved by the Local Ethics Committee on Animal Experimentation of the Medical University of Lublin (number 12/2015) and were performed in accordance with obligatory European standards related to the experimental studies on animal models.

2.2. Chronic Variable Stress Procedure. A chronic variable stress protocol was carried out as described by Gamaro et al. with slight modifications [5, 43]. The animals were divided in two groups: control (CTL) and stressed (CVS); each group consisted of 10 animals. Rats in the control group were kept undisturbed in their home cages (5 rats in each cage of dimensions 65 × 25 cm, 18 cm high), while rats in the stressed group were exposed to various stress conditions for 40 days. In the experiment, the following stressors were used: 24 h of food deprivation, 24 h of water deprivation, 1–3 h of restraint, 1.5–2 h of restraint at 4°C, forced swimming for 10 or 15 min, flashing light for 120–210 min, and isolation (2-3 days). Specific stressors and length of time applied each day are listed in Table 1. To avoid predictability, rats were exposed to these stressors at different times each day.

The rat was placed inside a 26 × 6 cm plastic tube in order to restrain it, followed by plaster tape adjustments on the outside to prevent it from moving. Breathing was enabled through a 1 cm hole at the far end of the tube. Forced swimming was performed by placing the rat in a round glass tank (50 cm radius) filled with 23°C water. In order to expose the animal to the flashing light, we put the rat inside a 50 cm-high open-field container (40 × 60 cm) made of brown plywood with a frontal glass wall. A flashing light was delivered from a 40 W lamp, set at 60 flashes/min.

After 40 days of stress procedures and 24 h after the last stressor, the animals were decapitated and individual brain samples were washed with 20 mL of saline and stored at −75°C until the time of analysis.

2.3. Determination of Biochemical Parameters. Homogenates of the prefrontal cortex were prepared from frozen brain samples, using extraction buffer. All biochemical measurements were conducted from these homogenates. The experimental procedures were performed according to the instructions supplied with each respective kit. In this experiment, the following analysis was performed: lipid peroxidation (LPO), which was based on malondialdehyde and 4-hydroxyalkenals concentration (MDA + 4HAE) (OxisResearch, USA) and concentration of glutathione—GSH, GSSG, GSH/GSSG ratio (Calbiochem, Germany), glutathione peroxidase (GPX), and glutathione reductase (GR)

Table 1: Stressor agents used during the chronic variable stress.

Day of treatment	Stressor	Duration	Start time
1	Water deprivation	24 h	8:00 a.m.
2	Food deprivation	24 h	9:00 a.m.
3	Isolation	24 h	10:00 a.m.
4	Isolation	24 h	10:00 a.m.
5	Isolation	24 h	10:00 a.m.
6	Flashing light	3 h	12:00 a.m.
7	Food deprivation	24 h	8:00 a.m.
8	Forced swimming	10 min	9:00 a.m.
9	Restraint	1 h	11:00 a.m.
10	Water deprivation	24 h	9:00 a.m.
11	No stressor	—	—
12	No stressor	—	—
13	Restraint and cold	2 h	10:00 a.m.
14	Flashing light	2.5 h	9:00 a.m.
15	Food deprivation	24 h	8:00 a.m.
16	Forced swimming	15 min	12:00 a.m.
17	Isolation	24 h	8:00 a.m.
18	Isolation	24 h	8:00 a.m.
19	Isolation	24 h	8:00 a.m.
20	Water deprivation	24 h	10:00 a.m.
21	Food deprivation	24 h	9:00 a.m.
22	Flashing light	3 h	13:00 a.m.
23	Restraint	2 h	12:00 a.m.
24	Isolation	24 h	8:00 a.m.
25	Isolation	24 h	8:00 a.m.
26	Restraint and cold	1.5 h	12:00 a.m.
27	Forced swimming	10 min	10:00 a.m.
28	Flashing light	3.5 h	12:00 a.m.
29	No stressor	—	—
30	Food deprivation	24 h	8:00 a.m.
31	Restraint	3 h	9:00 a.m.
32	Flashing light	2 h	10:00 a.m.
33	Water deprivation	24 h	8:00 a.m.
34	Restraint and cold	2 h	10:00 a.m.
35	Forced swimming	15 min	11:00 a.m.
36	Isolation	24 h	8:00 a.m.
37	Isolation	24 h	8:00 a.m.
38	No stressor	—	—
39	Flashing light	3 h	13:00 a.m.
40	Forced swimming	10 min	8:00 a.m.

activities (Cayman Chemical, USA). In short, the principle underlying lipid peroxidation assessment was based on the reaction of a chromogenic reagent R1 (N-methyl-2-phenylindole) with malondialdehyde (MDA) and 4-hydroxyalkenals (4HAE) at 45°C. Two molecules of R1 react with one molecule of MDA or 4-hydroxyalkenals to form a chromophore with an absorbance maximum at 586 nm. Measuring the concentration of MDA in combination with 4-hydroxyalkenals in methane sulfonic acid was used as an indicator of lipid peroxidation. GSH and GSSG concentrations were determined by an enzymatic reaction using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) and reagent M2VP (1-methyl-2-vinyl-pyridine-trifluoro-methanesulfonamide sulfonate). In this method, GSH reacts with Ellman's reagent to form a product identified spectrophotometrically at a wavelength of $\lambda = 412$ nm. Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPX, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. GPX activity was measured by the kinetic method, using the aforementioned kit. In this method, GPX catalyzes the oxidation of glutathione by cumene hydroperoxide, and in the presence of GR and NADPH, the oxidized glutathione (GSSG) is immediately converted to a reduced form with a concomitant oxidation of NADPH to $NADP^+$. The GR activity assay is based on the reduction of GSSG catalyzed by GR in the presence of NADPH, which is oxidized to $NADP^+$. The reduction in absorbance was measured at 340 nm.

2.4. Determination of DNA Oxidative Damage. DNA was isolated with a Syngen DNA Mini Kit (Syngen, Poland) according to the manufacturer's protocol. The concentration and purity of the genomic DNA were measured using a NanoDrop MaestroNano Micro-Volume Spectrophotometer (Maestrogen Inc., Taiwan) and adjusted to 100 μg/mL in TE buffer. Oxidative DNA damage was evaluated by measuring the amount of basic sites (the so-called AP) with a DNA Damage Quantification Kit (Dojindo, Japan). Oxidative attacks by ROS on the deoxyribose moiety lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites (AP sites). Aldehyde-reactive probe (ARP; N′-aminooxymethylcarbonylhydrazin-D-biotin) reacts specifically with an aldehyde group present on the open ring form of AP sites, making it possible to detect DNA modifications that result in the formation of an aldehyde group. Biotin-avidin-specific connection and horseradish peroxidase were used for colorimetric detection at 650 nm. AP sites were measured in DNA isolated from the hippocampus of the rats.

2.5. mRNA Expression Analysis. The isolated hippocampus was rinsed with 20 μL of the solution used for injections and stored at −75°C until isolation of RNA was carried out. A quantitative real-time PCR (qPCR) method was used to evaluate expression of selected genes (*Ogg1*, *MsrA*, and *Nrf2*). RNA was isolated from 30 mg of tissue according to the manufacturer's instructions using Syngen Tissue RNA Mini Kit (Syngen Biotech, Poland). Reverse transcription was performed using NG dART RT-PCR kit (EURx, Poland) according to the manufacturer's instructions. The relative expression of genes was measured with the ΔΔCt method, using Hprt (Mn00446968_m1) as an endogenous control. The reaction was carried out in octuplicate by qPCR using the SmartChip Real-Time PCR System (WaferGen Biosystems) and TaqMan Fast Universal PCR Master Mix (2x) (Applied BioSystems, USA) according to manufacturer's instructions. Sample quality screening based on amplification, Tm, and Ct values was performed to remove any outlier data points before ΔΔCt calculation and to determine fold change in mRNA levels. The data were presented as RQ value ($RQ = 2 - \Delta\Delta Ct$).

2.6. Statistical Analysis. The results were analysed statistically in the STATISTICA versus 10 application (StaftSoft, Cracow, Poland). Data was calculated as mean ± SEM and expressed as percentage of control group. The statistical significance among the groups was determined by a Student's *t*-test. All parameters were considered statistically significantly different if p values were less than 0.05.

3. Results

3.1. Lipid Peroxidation. In this experiment, we evaluated lipid peroxidation by measuring malondialdehyde (MDA) and 4-hydroxyalkenals (4HAE) concentration in the prefrontal cortex of rats. MDA + 4HAE levels were significantly higher in the group of rats exposed to stress in comparison to the control group (Figure 1).

3.2. GSH and GSSG Levels. We noticed that the levels of GSH and GSSG in rats exposed to CVS did not change substantially. However, we also evaluated the GSH/GSSG ratio, since it is considered to be a sensitive indicator of the cellular redox state. Data obtained from the prefrontal cortex of rats submitted to chronic stress did show a statistically significant decrease in the GSH/GSSG ratio (Figures 2 and 3).

3.3. Glutathione Peroxidase and Glutathione Reductase Activity. No statistically significant changes were observed in glutathione peroxidase activity in rats subjected to CVS in comparison to the control group (Figure 4). However, the results indicate that stressors caused a decrease in glutathione reductase activity in the brain of rats exposed to CVS compared to the brain of control rats (Figure 5).

3.4. Oxidative DNA Damage. Assessment of oxidative DNA damage showed a threefold increase of AP site accumulation in DNA isolated from the hippocampus of rats subjected to CVS in comparison to the control group. This result demonstrates that the amount of oxidative damage in stressed rats rose significantly compared to controls (Figure 6).

3.5. The Level of mRNA Expression for Ogg1, MsrA, and Nrf2. The expression levels of genes involved in oxidative stress were examined. Sample variation was accounted for by comparison to the expression levels of Hprt, which is a housekeeping gene responsible for nucleotide metabolism. Expression of mRNA was measured in reference to the

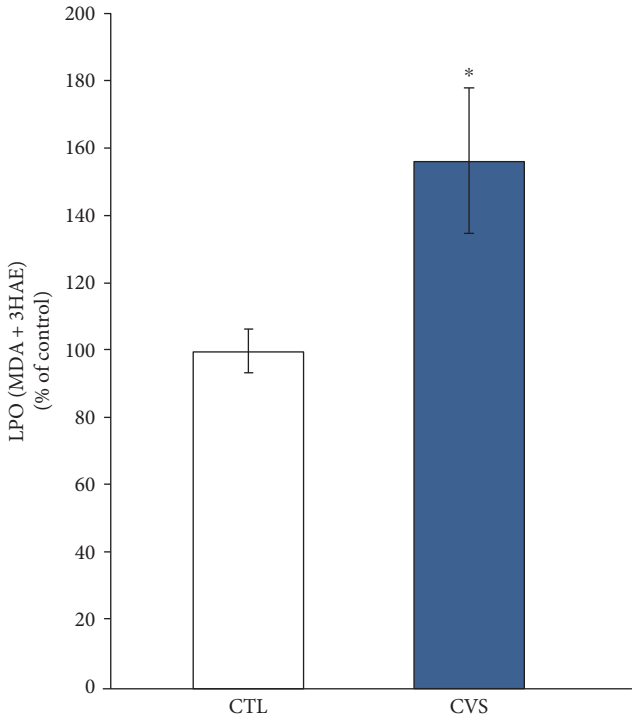


Figure 1: The effect of chronic variable stress on LPO (MDA + 4 HAE concentration) in the prefrontal cortex of rats. The method is based on the measurement of the following products of lipid peroxidation: malondialdehyde (MDA) and 4-hydroxyalkenals (4HAE), which react with a chromogenic reagent N-methyl-2-phenylindole. Data is displayed as mean ± SEM and expressed as percentage of control group. Significance: $^{*}p < 0.05$ by Student's t-test; $p = 0.0222$; $t = 2.503$ with 18 degrees of freedom.

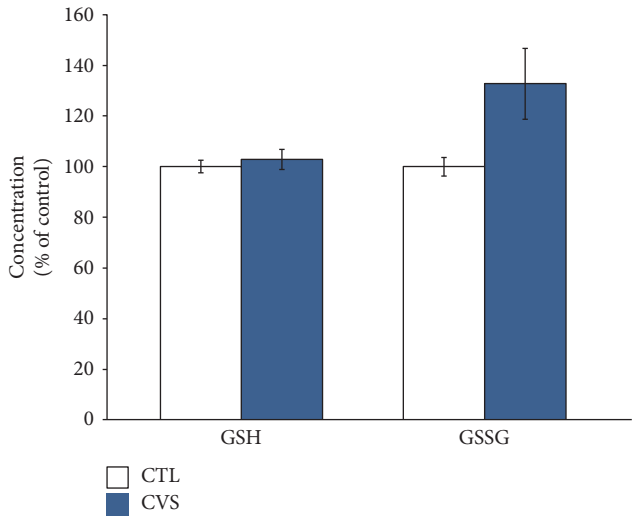


Figure 2: The effect of chronic variable stress on GSH and GSSG concentrations in the prefrontal cortex of rats. The concentrations were determined spectrophotometrically in an enzymatic reaction using the following reagents: 5,5′-dithiobis-2-nitrobenzoic acid and 1-methyl-2-vinyl-pyridine-trifluoro-methanesulfonamide sulfonate. Data is displayed as mean ± SEM and expressed as percentage of control group.

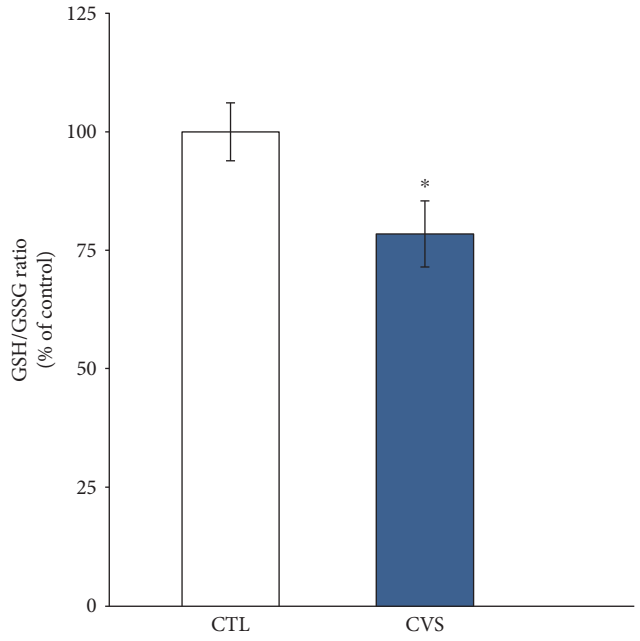


Figure 3: The effect of chronic variable stress on the GSH/GSSH ratio in the prefrontal cortex of rats. Data is displayed as mean ± SEM and expressed as percentage of control group. Significance: $^{*}p < 0.05$ by Student's t-test; $p = 0.0489$; $t = 2.320$ with 8 degrees of freedom.

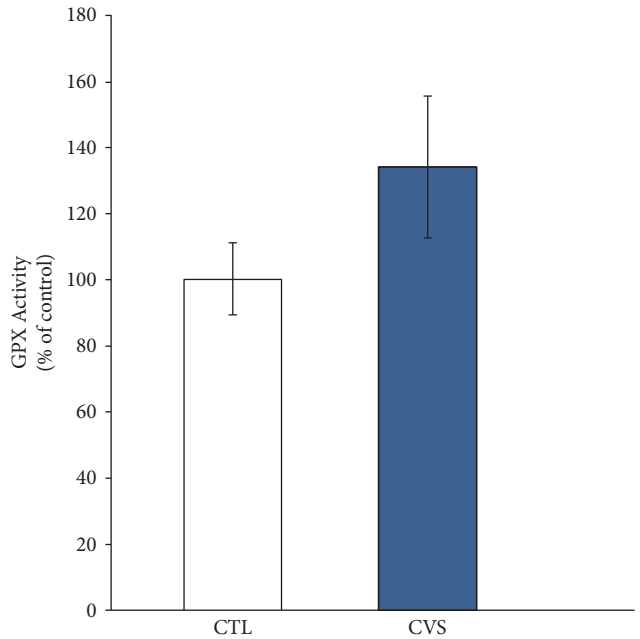


Figure 4: The effect of chronic variable stress on GPX activity in the prefrontal cortex of rats. The GPX activity was measured by a kinetic method based on the oxidation of NADPH to $NADP^{+}$ which is accompanied by a decrease in absorbance at 340 nm. Data is displayed as mean ± SEM and expressed as percentage of control group.

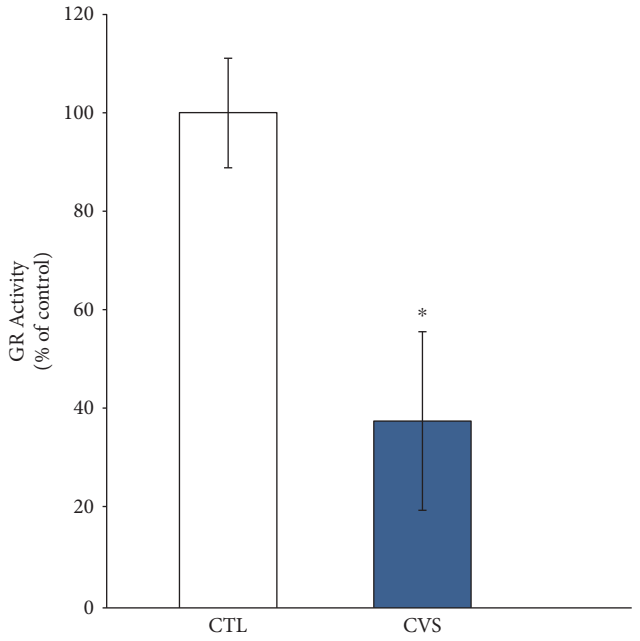


Figure 5: The effect of chronic variable stress on GR activity in the prefrontal cortex of rats. The GR activity was measured by kinetic method based on the oxidation of NADPH to $NADP^+$ which is accompanied by a decrease in absorbance at 340 nm. Data is displayed as mean ± SEM and expressed as percentage of control group. Significance: $^*p < 0.05$ by Student's t-test; $p = 0.0189$; $t = 2.932$ with 8 degrees of freedom.

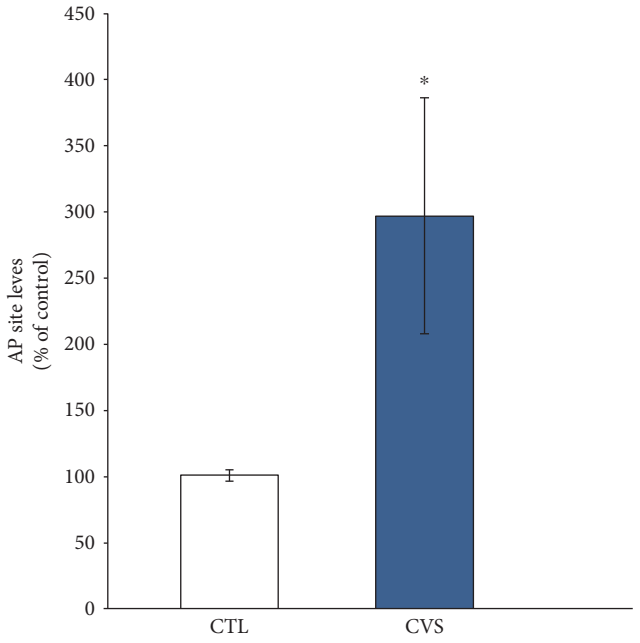


Figure 6: The effect of chronic variable stress on oxidative damages of DNA in the hippocampus of rats. The method is based on the measurement of simple abasic sites (AP sites) in DNA. Data is displayed as mean ± SEM and expressed as percentage of control group. Significance: $^*p < 0.05$ by Student's t-test; $p = 0.0391$; $t = 2.194$ with 22 degrees of freedom.

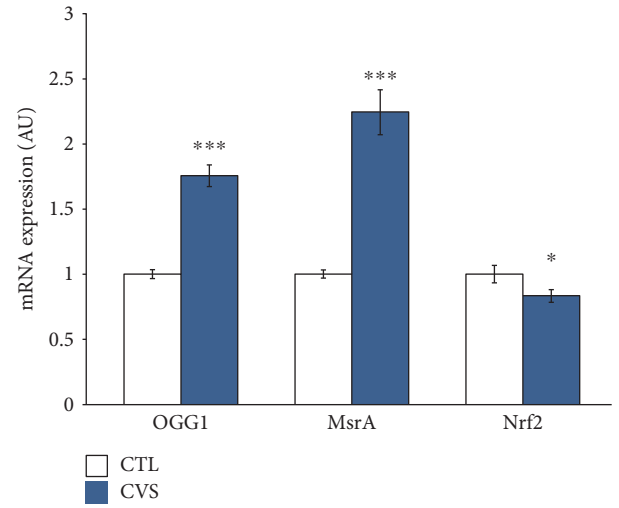


Figure 7: The effect of chronic variable stress on the level of mRNA expression for OGG1, MsrA, and Nrf2 genes in the hippocampus of rats. The mRNA's expression was assayed in regard to the control group, where the expression level is estimated at 1. Data is displayed as mean ± SEM. Significance: $^*p < 0.05$; $^{***}p < 0.001$ by Student's t-test; OGG1: $p < 0.0001$ and $t = 8.408$ with 18 degrees of freedom; MsrA: $p < 0.0001$ and $t = 7.155$ with 18 degrees of freedom; Nrf2: $p = 0.0422$ and $t = 2.091$ with 45 degrees of freedom.

control group, where the expression level is estimated as RQ = 1. In the hippocampus of rats submitted to CVS, the mRNA levels of *Ogg1* and *MsrA* were significantly increased, while a decrease in the expression of the *Nrf2* gene was noticed in comparison to the control. The results of qRT-PCR experiments are shown in Figure 7.

4. Discussion

It is postulated that environmental stress, at least partially, is related to oxidative stress. The main source of ROS formation is mitochondrial failure, that is, associated with neurodegeneration [48]. In stressogenic-related depressive disorders, the rate of conversion of the oxygen to ROS may increase and can result in severe metabolic dysfunction and oxidative damages to subcellular and cellular membrane lipids and enzymes [8, 12–14]. However, one of the most important consequences of ROS overproduction is modification of DNA. For these reasons, the objective of our study was to evaluate oxidative DNA damage in the brain of rats submitted to chronic variable stress. We also measured the biochemical changes associated with ROS generation, which can confirm the occurrence of oxidative stress.

The current study revealed an increase in lipid peroxidation in the prefrontal cortex of rats exposed to stress, which indicates oxidative stress. It is coherent with the results of the clinical tests, which indicate that the pathological stress causing a huge depression is accompanied by an increase of lipid peroxidation in the brain [8, 11, 17]. Highly reactive oxygen metabolites act on unsaturated fatty acids of phospholipid components of membranes to produce malondialdehyde, a lipid peroxidation product. Reactivity of MDA and 4-HNE may cause damage to DNA [20]. In the brain,

there is a significant amount of unsaturated fatty acids, which are susceptible to peroxidation. Moreover, in the brain, there is a relatively poor antioxidant defense system. This creates a risk of DNA damage and disturbances in secondary electron transport and cellular damage [49]. Lipid peroxidation decreases the life span of neurons, affects neurotransmitter release, and was reported as a major contributor to the loss of cell function under oxidative stress conditions in depression [26, 50].

In our study, exposure to stress caused a decrease of the GSH/GSSG ratio in the prefrontal cortex of rats in comparison to control. Glutathione plays an important role in a multitude of cellular processes, including cell differentiation, proliferation, and apoptosis. GSH is critical for protecting the brain from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation. The GSH/GSSG ratio is reduced in neurodegenerative diseases [51]. The brain is particularly susceptible to alterations in GSH homeostasis, probably due to the fact that GSH may be a neuromodulator or neurotransmitter and may thus be essential for central nervous system activities [48]. The oxidation of glutathione is considered to be one of the first and most important events leading to a change in the overall cellular redox state. The resulting damage is thought to be involved in neurodegenerative diseases [48, 52]. Thus, the GSH/GSSG ratio is considered to be a sensitive indicator of the cellular redox state [53, 54]. Taking this into account, the increase in LPO accompanied by the decrease in the GSH/GSSG ratio may suggest that the antioxidative adaptation is not sufficient. A decrease in the GSH/GSSG ratio manifests itself largely through an increased susceptibility to oxidative stress, and the resulting damage is thought to be involved in neurodegenerative diseases [48].

In addition to the GSH/GSSG ratio, the relative activities of the enzymes responsible for glutathione metabolism are an important factor for assessing the redox potential of tissues and cells. For this reason, glutathione peroxidase and glutathione reductase activities were measured. They are enzymatic antioxidant system chains and provide protection against the damaging effects of free radicals [52, 55]. In this system, glutathione peroxidase provides detoxification of organic and inorganic peroxides by using reduced glutathione. Glutathione reductase regenerates GSH and protects cell from death caused by oxidative stress, probably through maintaining a high GSH/GSSG ratio [54, 56]. This experiment showed that GR activity is decreased in the prefrontal cortex of stressed rats. The decrease in GR activity, observed in our work, may result in impairment of GSH regeneration. This statement is confirmed by the decrease in the GSH/GSSG ratio. Perhaps, regulation of GR is associated with cortisol, which is secreted in response to stress. Becerril-Chavez et al. recently showed that the activity levels of glutathione-related enzymes (GPX, GST) are disrupted in the prefrontal cortex of rats subjected to chronic stress. Their research proved that downregulation of glutathione S-transferase (GST) occurred [57]. This enzyme catalyzes deactivation of many harmful substances and requires reduced glutathione as a cofactor. In turn, glutathione reductase is responsible for the restoration of GSH. The decrease in GR activity may result in insufficient GSH regeneration and can adversely affect GST activity. The GST dysfunction was accompanied by a high cortisol level, which increases in response to an acutely stressful event [57]. Hence, chronic stress mounts a stronger corresponding cortisol response [9]. Importantly, the elevated cortisol level was decreased by application of an antioxidant, which also attenuated the GST activity level. However, further studies are required to confirm these correlations. So far, the above results are indirect evidence of ROS overproduction and presence of disrupted oxidative defense systems.

In stress-induced depressive disorders affecting the brain, DNA damage induced by oxidative stress is a major factor leading to neuronal dysfunction and cell death. Studies have shown a relationship between DNA damage and cortisol, especially during prolonged exposure to high levels of cortisol, in brain structures such as the hippocampus and frontal cortex [10]. Oxidant-induced DNA damage may be a useful biomarker for chronic oxidative stress determination [58]. Oxidative damage of DNA results from an interaction of DNA with reactive oxygen species, in particular the hydroxyl radical. Oxidative damage is believed to contribute substantially to the decline in cellular functions that are associated with nervous system diseases [59]. Moreover, studies have shown that oxidative DNA damage is linked to the onset of specific human diseases such as neuronal degeneration [58–60]. One of the major types of damage generated by ROS is the AP site, the most common DNA damage resulting from loss of a DNA base [61]. The current study revealed a statistically significant increase of AP site accumulation in DNA isolated from the hippocampus of rats subjected to CVS compared to that of the control group, an indicator of considerable oxidative DNA damage. An important role in the repair of oxidatively damaged proteins is to restore biological activity through specific gene activation. The *Ogg1* gene is induced by oxidative stress and its mRNA levels are correlated with base excision capacity. OGG1 is ubiquitously expressed in the brain and is considered a cellular marker for both oxidative stress and oxidative DNA damage [23]. Protein-bound methionine residues are the most susceptible to oxidation by ROS. However, this modification can be repaired by *MsrA*, which catalyzes the thioredoxin-dependent reduction of free and protein-bound MetO to methionine [31].

The current study revealed that, in the hippocampus of rats subjected to CVS, the mRNA levels of *Ogg1* and *MsrA* were significantly increased. Taking into consideration the results of related biochemical research, we can assume that overexpression of DNA repair enzymes, important for maintenance of mitochondrial functions, may result out of necessity to rescue cells from ROS. Disruption of antioxidant and DNA repair mechanisms in the cell by ROS may result in oxidative stress and oxidative damage to the cell. Moskovitz et al. showed that loss of antioxidant capacity is associated with a decrease in total OGG1 and MSRA activities [31]; thus, the significant increase in expression of these genes shown in our study suggests that they play an important role in the protection against ROS-related oxidative DNA damage. The results presented above confirm that chronic

environmental stress-induced oxidative stress causes DNA oxidative damage. At the same time, upregulation of *Ogg1* and *MsrA* increases efficiency of DNA repair.

Studies have determined that NRF2 is a regulator of antioxidant response and is a factor that regulates the transcription of oxidative DNA damage repair genes [62]. Oxidative stress can lead to NRF2 activation, which in turn acts as an autoregulatory feed-forward loop to dampen the increased ROS levels, thereby maintaining homeostasis following tissue or cellular injury. NRF2 activation would be expected in the presence of an increase in ROS. However, in our study, a decrease in *Nrf2* gene expression was observed in the brain of rats submitted to chronic stress in comparison to the control group. One explanation for these findings may be that NRF2 is not properly responding to oxidative stress under conditions of chronic stress. Disturbed NRF2 signaling in the brain may contribute to neurodegeneration via decreased antioxidative defense as documented in depressed patients [32, 63]. This phenomenon has also been confirmed by studies that reported decreased *Nrf2* expression in the hippocampus of rats submitted to chronic stressors [64, 65]. The increase in *Ogg1* and *MsrA* expression observed in our work seems to be independent from *Nrf2* expression levels. The obtained data suggest that oxidative stress directly activates the oxidative stress response genes but not the NRF2 pathway. These results support the theory that oxidative stress responses do not always involve a coordinated regulation of genes and their activities are regulated by different factors. The second explanation for the decrease in activity of Nrf2 gene is its exceptional sensitivity for oxidative products (ROS) and byproducts (MDA and 4-HNE), but this assumption should be supported by future studies.

The redox-sensitive transcription factor NRF2 also regulates the rate of GSH synthesis [66]. GSH-metabolizing enzymes are induced at the transcriptional level by mild oxidative stress, which involves binding of the NRF2 transcription factor to the antioxidant response element [67]. Taking into account the above results, downregulation of *Nrf2* may be correlated with decreased GR activity and, consequently, could lead to a decrease in the GSH/GSSG ratio, as recorded in our study. The decrease of *Nrf2* is corresponding with the data from biochemical studies and points on disorders in defense of oxidative system.

5. Conclusions

The current study confirmed that chronic variable stress causes oxidative stress in part of the brain involved in depression development, that is, the prefrontal cortex and hippocampus. In the hippocampus, an increase in oxidative damages of DNA was noticed. Our finding of *Ogg1* and *MsrA* upregulation indicates that the oxidative DNA repair system has been activated. The decrease of *Nrf2* may suggest an independent activation of oxidative stress response genes. Future studies are required to explain if oxidative damage of DNA originates from mitochondria or nucleus and if the decrease in important antioxidative *Nrf2* gene activity is related to special susceptibility for oxidative stress byproducts. This may help in clarifying whether oxidative stress is the cause or a downstream consequence of depression.

Additional Points

Compliance with ethical standards. All procedures were conducted in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU), and Polish legislation acts concerning animal experimentations. The experimental procedures and protocols were approved by the First Local Ethics Committee at the Medical University of Lublin.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by Funds for Statutory Activity of Medical University of Lublin, Poland.

References

[1] K. Hashimoto, "The role of glutamate on the action of antidepressants," *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, vol. 7, no. 35, pp. 1558–1568, 2011.

[2] R. C. Kessler, P. Berglund, O. Demler et al., "The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R)," *JAMA*, vol. 23, no. 289, pp. 3095–3105, 2003.

[3] J. Du, M. Zhu, H. Bao et al., "The role of nutrients in protecting mitochondrial function and neurotransmitter signaling: implications for the treatment of depression, PTSD, and suicidal behaviors," *Critical Reviews in Food Science and Nutrition*, vol. 15, no. 56, pp. 2560–2578, 2016.

[4] M. E. Robinson, D. S. Teyhen, S. S. Wu et al., "Mental health symptoms in combat medic training: a longitudinal examination," *Military Medicine*, vol. 6, no. 174, pp. 572–577, 2009.

[5] B. Tagliari, C. G. Noschang, A. G. Ferreira et al., "Chronic variable stress impairs energy metabolism in prefrontal cortex and hippocampus of rats: prevention by chronic antioxidant treatment," *Metabolic Brain Disease*, vol. 25, no. 2, pp. 169–176, 2010.

[6] F. U. Fontella, R. I. Siqueira, A. P. S. Vasconcellos, A. S. Tabajara, C. A. Netto, and C. Dalmaz, "Repeated restraint stress induces oxidative damage in rat hippocampus," *Neurochemical Research*, vol. 1, no. 30, pp. 105–111, 2005.

[7] L. P. Manoli, G. D. Gamaro, P. P. Silveira, and C. Dalmaz, "Effect of chronic variate stress on thiobarbituric-acid reactive species and on total radical-trapping potential in distinct regions of rat brain," *Neurochemical Research*, vol. 25, pp. 915–921, 2000.

[8] M. Blici, H. Efe, M. A. Köroğlu, H. A. Uydu, M. Bekaroğlu, and O. Değer, "Antioxidative enzyme activities and lipid peroxidation in major depression: alterations by antidepressant treatments," *Journal of Affective Disorders*, vol. 64, no. 1, pp. 43–51, 2001.

[9] K. Aschbacher, A. O'Donovan, O. M. Wolkowitz, F. S. Dhabhar, Y. Su, and E. Epel, "Good stress, bad stress and oxidative stress:

insights from anticipatory cortisol reactivity," *Psychoneuroendocrinology*, vol. 38, no. 9, pp. 1698–1708, 2013.

[10] Ş. Şimşek, T. Yüksel, İ. Kaplan, C. Uysal, and H. Aktaş, "The levels of cortisol and oxidative stress and DNA damage in child and adolescent victims of sexual abuse with or without post-traumatic stress disorder," *Psychiatry Investigation*, vol. 13, no. 6, pp. 616–621, 2016.

[11] T. M. Michel, D. Pulschen, and J. Thome, "The role of oxidative stress in depressive disorders," *Current Pharmaceutical Design*, vol. 18, no. 36, pp. 5890–5899, 2012.

[12] B. Roediger and P. J. Armati, "Oxidative stress induces axonal beading in cultured human brain tissue," *Neurobiology of Disease*, vol. 13, no. 3, pp. 222–229, 2003.

[13] G. Lucca, C. M. Comim, S. S. Valvassori et al., "Increased oxidative stress in submitochondrial particles into the brain of rats submitted to the chronic mild stress paradigm," *Journal of Psychiatric Research*, vol. 9, no. 43, pp. 864–869, 2009.

[14] A. Valavanidis, T. Vlachogianni, K. Fiotakis, and S. Loridas, "Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms," *International Journal of Environmental Research and Public Health*, vol. 9, no. 10, pp. 3886–3907, 2013.

[15] R. L. Torres, I. L. S. Torres, G. D. Gamaro et al., "Lipid peroxidation and total radical-trapping potential of the lungs of rats submitted to chronic and sub-chronic stress," *Brazilian Journal of Medical and Biological Research*, vol. 37, no. 2, pp. 185–192, 2004.

[16] H. Brenner-Lavie, E. Klein, and D. Ben-Shachar, "Mitochondrial complex I as a novel target for intraneuronal DA: modulation of respiration in intact cells," *Biochemical Pharmacology*, vol. 1, no. 78, pp. 85–95, 2009.

[17] A. Bajpai, A. K. Verma, M. Srivastava, and R. Srivastava, "Oxidative stress and major depression," *Journal of Clinical and Diagnostic Research*, vol. 12, no. 8, pp. CC04–CC07, 2014.

[18] A. Hausladen and I. Fridovich, "Superoxide and peroxynitrite inactivate aconitases, but nitric oxide does not," *The Journal of Biological Chemistry*, vol. 269, no. 47, pp. 29405–29408, 1994.

[19] J. J. Lemasters and A. L. Nieminen, *Mitochondria in Pathogenesis*, Kluwer Academic/Plenum, New York, USA, 2001.

[20] A. Ayala, M. F. Muñoz, and S. Argüelles, "Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal," *Oxidative Medicine and Cellular Longevity*, vol. 2014, Article ID 360438, 31 pages, 2014.

[21] A. Popa-Wagner, S. Mitran, S. Sivanesan, E. Chang, and A. M. Buga, "ROS and brain diseases: the good, the bad, and the ugly," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 963520, 14 pages, 2013.

[22] F. Zhou, W. Zhang, Y. Wei et al., "The changes of oxidative stress and human 8-hydroxyguanine glycosylase1 gene expression in depressive patients with acute leukemia," *Leukemia Research Reports*, vol. 31, no. 3, pp. 387–393, 2007.

[23] J. R. Teyssier, S. Ragot, J. C. Chauvet-Gélinier, B. Trojak, and B. Bonin, "Expression of oxidative stress-response genes is not activated in the prefrontal cortex of patients with depressive disorder," *Psychiatry Research*, vol. 186, no. 2-3, pp. 244–247, 2011.

[24] K. J. A. Davies, "Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems," *IUBMB Life*, vol. 50, no. 4-5, pp. 279–289, 2000.

[25] C. L. Powell, J. A. Swenberg, and I. Rusyn, "Expression of base excision DNA repair genes as a biomarker of oxidative DNA damage," *Cancer Letters*, vol. 229, no. 1, pp. 1–11, 2005.

[26] M. Vaváková, Z. Ďuračková, and J. Trebatická, "Markers of oxidative stress and neuroprogression in depression disorder," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 898393, 12 pages, 2015.

[27] J. Fukae, M. Takanashi, S. Kubo et al., "Expression of 8-oxoguanine DNA glycosylase (OGG1) in Parkinson's disease and related neurodegenerative disorders," *Acta Neuropathologica*, vol. 109, no. 3, pp. 256–262, 2005.

[28] M. Dizdarouglu, "Chemical determination of free radical-induced damage to DNA," *Free Radical Biology and Medicine*, vol. 10, no. 3-4, pp. 225–242, 1991.

[29] M. Maes, I. Mihaylova, M. Kubera, M. Uytterhoeven, N. Vrydags, and E. Bosmans, "Increased 8-hydroxy-deoxyguanosine, a marker of oxidative damage to DNA, in major depression and myalgic encephalomyelitis/chronic fatigue syndrome," *Neuroendocrinology Letters*, vol. 30, no. 6, pp. 715–722, 2009.

[30] J. Moskovitz, E. Flescher, B. S. Berlett, J. Azare, J. M. Poston, and E. R. Stadtman, "Overexpression of peptide-methionine sulfoxide reductase in *Saccharomyces cerevisiae* and human T cells provides them with high resistance to oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 24, pp. 14071–14075, 1998.

[31] J. Moskovitz, S. Bar-Noy, W. M. Williams, J. Requena, B. S. Berlett, and E. R. Stadtman, "Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 23, pp. 12920–12925, 2001.

[32] H. E. de Vries, M. Witte, D. Hondius et al., "Nrf2-induced antioxidant protection: a promising target to counteract ROS-mediated damage in neurodegenerative disease?," *Free Radical Biology and Medicine*, vol. 45, no. 10, pp. 1375–1383, 2008.

[33] T. Nguyen, P. Nioi, and C. B. Pickett, "The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress," *The Journal of Biological Chemistry*, vol. 284, no. 20, pp. 13291–13295, 2009.

[34] M. D. Martín-de-Saavedra, J. Budni, M. P. Cunha et al., "Nrf2 participates in depressive disorders through an anti-inflammatory mechanism," *Psychoneuroendocrinology*, vol. 38, no. 10, pp. 2010–2022, 2013.

[35] I. Mendez-David, L. Tritschler, Z. E. Ali et al., "Nrf2-signaling and BDNF: a new target for the antidepressant-like activity of chronic fluoxetine treatment in a mouse model of anxiety/depression," *Neuroscience Letters*, vol. 597, pp. 121–126, 2015.

[36] Q. Ma, "Role of Nrf2 in oxidative stress and toxicity," *Annual Review of Pharmacology and Toxicology*, vol. 53, pp. 401–426, 2013.

[37] 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*, vol. 1850, no. 4, pp. 794–801, 2015.

[38] M. Kobayashi and M. Yamamoto, "Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation," *Antioxidants and Redox Signaling*, vol. 7, no. 3-4, pp. 385–394, 2005.

[39] K. Tomobe, T. Shinozuka, M. Kuroiwa, and Y. Nomura, "Age-related changes of Nrf2 and phosphorylated GSK-3β in a

mouse model of accelerated aging (SAMP8)," *Archives of Gerontology and Geriatrics*, vol. 54, no. 2, pp. e1–e7, 2012.

[40] V. Lorenzetti, N. B. Allen, A. Fornito, and M. Yücel, "Structural brain abnormalities in major depressive disorder: a selective review of recent MRI studies," *Journal of Affective Disorders*, vol. 117, no. 1-2, pp. 1–17, 2009.

[41] J. W. Gawryluk, J. F. Wang, A. C. Andreazza, L. Shao, and L. T. Young, "Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders," *International Journal of Neuropsychopharmacology*, vol. 14, no. 1, pp. 123–113, 2011.

[42] B. M. Andrus, K. Blizinsky, P. T. Vedell et al., "Gene expression patterns in the hippocampus and amygdala of endogenous depression and chronic stress models," *Molecular Psychiatry*, vol. 17, no. 1, pp. 49–61, 2012.

[43] G. D. Gamaro, L. P. Manoli, I. L. S. Torres, R. Silveira, and C. Dalmaz, "Effects of chronic variate stress on feeding behavior and on monoamine levels in different rat brain structures," *Neurochemistry International*, vol. 42, no. 2, pp. 107–114, 2003.

[44] B. Tagliari, T. M. dos Santos, A. A. Cunha et al., "Chronic variable stress induces oxidative stress and decreases butyrylcholinesterase activity in blood of rats," *Journal of Neural Transmission*, vol. 117, no. 9, pp. 1067–1076, 2010.

[45] Z. Zhao, W. Wang, H. Guo, and D. Zhou, "Antidepressant-like effect of liquiritin from *Glycyrrhiza uralensis* in chronic variable stress induced depression model rats," *Behavioral Brain Research*, vol. 194, no. 1, pp. 108–113, 2008.

[46] V. Krishnan and E. J. Nestler, "Animal models of depression: molecular perspectives," *Current Topics in Behavioral Neurosciences*, vol. 7, pp. 121–147, 2011.

[47] K. Raudkivi, T. Mällo, and J. Harro, "Effect of chronic variable stress on corticosterone levels and hippocampal extracellular 5-HT in rats with persistent differences in positive affectivity," *Acta Neuropsychiatrica*, vol. 24, no. 4, pp. 208–214, 2012.

[48] N. Ballatori, S. M. Krance, S. Notenboom, S. Shi, K. Tieu, and C. L. Hammond, "Glutathione dysregulation and the etiology and progression of human diseases," *Biological Chemistry*, vol. 390, no. 3, pp. 191–214, 2009.

[49] M. Ott, V. Gogvadze, S. Orrenius, and B. Zhivotovsky, "Mitochondria, oxidative stress and cell death," *Apoptosis*, vol. 12, no. 5, pp. 913–922, 2007.

[50] K. B. Storey, "Oxidative stress: animal adaptations in nature," *Brazilian Journal of Medical and Biological Research*, vol. 29, no. 12, pp. 1715–1733, 1996.

[51] J. B. Owen and D. A. Butterfield, "Measurement of oxidized/reduced glutathione ratio," *Methods in Molecular Biology*, vol. 648, pp. 269–277, 2010.

[52] S. Kaushik and J. Kaur, "Chronic cold exposure affects the antioxidant defense system in various rat tissues," *Clinica Chimica Acta*, vol. 333, no. 1, pp. 69–77, 2003.

[53] R. Faria, M. M. Santana, C. A. Aveleira et al., "Alterations in phospholipidomic profile in the brain of mouse model of depression induced by chronic unpredictable stress," *Neuroscience*, vol. 273, pp. 1–11, 2014.

[54] D. P. Jones, "Redox potential of GSH/GSSG couple: assay and biological significance," *Methods in Enzymology*, vol. 348, pp. 93–112, 2002.

[55] D. E. Paglia and W. N. Valentine, "Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase," *Journal of Laboratory and Clinical Medicine*, vol. 70, no. 1, pp. 158–169, 1967.

[56] M. S. Yang, H. W. Chan, and L. C. Yu, "Glutathione peroxidase and glutathione reductase activities are partially responsible for determining the susceptibility of cells to oxidative stress," *Toxicology*, vol. 226, no. 2-3, pp. 126–130, 2006.

[57] H. Becerril-Chavez, A. L. Colin-Gonzalez, J. Villeda-Hernandez et al., "Protective effects of S-allyl cysteine on behavioral, morphological and biochemical alterations in rats subjected to chronic restraint stress: antioxidant and anxiolytic effects," *Journal of Functional Foods*, vol. 35, pp. 105–114, 2017.

[58] F. M. Yakes and B. Van Houten, "Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 2, pp. 514–519, 1997.

[59] E. W. Englander, "Brain capacity for repair of oxidatively damaged DNA and preservation of neuronal function," *Mechanisms of Ageing and Development*, vol. 129, no. 7–8, pp. 475–482, 2008.

[60] D. Huang, A. Shenoy, J. Cui, W. Huang, and P. K. Lui, "In situ detection of AP sites and DNA strand breaks bearing 3'-phosphate termini in ischemic mouse brain," *FASEB Journal*, vol. 14, no. 2, pp. 407–417, 2000.

[61] K. Filler, D. Lyon, J. Bennett et al., "Association of mitochondrial dysfunction and fatigue: A review of the literature," *Biochimica et Biophysica Acta Clinical*, no. 1, pp. 12–23, 2014.

[62] B. Singh, A. Chatterjee, A. M. Ronghe, N. K. Bhat, and H. K. Bhat, "Antioxidant-mediated up-regulation of OGG1 via NRF2 induction is associated with inhibition of oxidative DNA damage in estrogen-induced breast cancer," *BioMed Central Cancer*, vol. 13, no. 253, 2014.

[63] M. Maes, P. Galecki, Y. S. Chang, and M. Berk, "A review on the oxidative and nitrosative stress (O&NS) pathways in major depression and their possible contribution to the (neuro)degenerative processes in that illness," *Progress in Neuropsychopharmacology and Biological Psychiatry*, vol. 35, no. 3, pp. 676–692, 2011.

[64] J. Djordjevic, A. Djordjevic, M. Adzic, and M. B. Radojcic, "Chronic social isolation compromises the activity of both glutathione peroxidase and catalase in hippocampus of male wistar rats," *Cellular and Molecular Neurobiology*, vol. 30, no. 5, pp. 693–700, 2010.

[65] J. Djordjevic, A. Djordjevic, M. Adzic, M. Mitic, I. Lukic, and M. B. Radojcic, "Alterations in the Nrf2-Keap1 signaling pathway and its downstream target genes in rat brain under stress," *Brain Research*, no. 1602, pp. 20–31, 2015.

[66] M. L. Steele, S. Fuller, M. Patel, C. Kersaitis, L. Ooi, and G. Münch, "Effect of Nrf2 activators on release of glutathione, cysteinylglycine and homocysteine by human U373 astroglial cells," *Redox Biology*, vol. 1, no. 1, pp. 441–445, 2013.

[67] V. I. Lushchak, "Glutathione homeostasis and functions: potential targets for medical interventions," *Journal of Amino Acids*, vol. 2012, Article ID 736837, 26 pages, 2012.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8765972, 14 pages
https://doi.org/10.1155/2017/8765972

Review Article

The Contribution of Singlet Oxygen to Insulin Resistance

Arnold N. Onyango

Department of Food Science and Technology, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000, Nairobi 00200, Kenya

Correspondence should be addressed to Arnold N. Onyango; arnold.onyango@jkuat.ac.ke

Received 24 April 2017; Accepted 7 August 2017; Published 7 September 2017

Academic Editor: Victor M. Victor

Insulin resistance contributes to the development of diabetes and cardiovascular dysfunctions. Recent studies showed that elevated singlet oxygen-mediated lipid peroxidation precedes and predicts diet-induced insulin resistance (IR), and neutrophils were suggested to be responsible for such singlet oxygen production. This review highlights literature suggesting that insulin-responsive cells such as endothelial cells, hepatocytes, adipocytes, and myocytes also produce singlet oxygen, which contributes to insulin resistance, for example, by generating bioactive aldehydes, inducing endoplasmic reticulum (ER) stress, and modifying mitochondrial DNA. In these cells, nutrient overload leads to the activation of Toll-like receptor 4 and other receptors, leading to the production of both peroxynitrite and hydrogen peroxide, which react to produce singlet oxygen. Cytochrome P450 2E1 and cytochrome c also contribute to singlet oxygen formation in the ER and mitochondria, respectively. Endothelial cell-derived singlet oxygen is suggested to mediate the formation of oxidized low-density lipoprotein which perpetuates IR, partly through neutrophil recruitment to adipose tissue. New singlet oxygen-involving pathways for the formation of IR-inducing bioactive aldehydes such as 4-hydroperoxy-(or hydroxy or oxo)-2-nonenal, malondialdehyde, and cholesterol secosterol A are proposed. Strategies against IR should target the singlet oxygen-producing pathways, singlet oxygen quenching, and singlet oxygen-induced cellular responses.

1. Introduction

Insulin resistance is a condition in which a given concentration of insulin produces a less than expected effect on target cells, and this may lead to impaired glucose tolerance ahead of overt type II diabetes mellitus [1]. It was recently reported that elevated plasma levels of products formed by singlet oxygen-mediated lipid oxidation precede and predicts the development of insulin resistance and diabetes in both humans and mice [2, 3]. Neutrophils recruited to adipose tissue as a result of high-fat feeding were speculated to be responsible for generating the singlet oxygen-modified lipids [2, 3]. On the contrary, the present article highlights literature consistent with (i) a primary role of insulin-responsive cells such as endothelial cells, adipocytes, hepatocytes, and skeletal muscle cells in singlet oxygen formation even prior to the activation of neutrophils and (ii) an important role of singlet oxygen in decreased insulin signaling by the insulin-responsive cells. Key insulin resistance-associated singlet oxygen-producing pathways in these cells are proposed, as well as mechanisms by which this ROS induces insulin resistance. New pathways are proposed for the singlet oxygen-mediated formation of bioactive aldehydes, including cholesterol secosterol aldehyde A, which was previously considered to be exclusively generated by cholesterol ozonolysis and to be a key piece of evidence for endogenous ozone formation.

2. Insulin Signaling

As reviewed by Siddle [4], insulin signaling begins with insulin binding to its receptor, a receptor tyrosine kinase, and this results in the sequential activation of (i) an insulin receptor substrate (IRS), typically IRS-1 or IRS-2, (ii) phosphatidyl inositol 3 kinase (PI3K), (iii) protein kinase B (PKB/Akt), and (iv) various Akt substrates such as the Akt substrate of 160 kDa, whose phosphorylation facilitates translocation of glucose transporter 4 (GLUT 4) from cytoplasmic storage vesicles to the plasma membrane of adipocytes and skeletal muscle cells. Akt-mediated phosphorylation of glycogen synthase kinase 3 results in the activation of glycogen synthase

and enhanced glycogen synthesis, while Akt-mediated phosphorylation of the forkhead transcription factor (FOXO 1) prevents translocation of the latter to the nucleus and inhibits expression of enzymes responsible for hepatocyte gluconeogenesis and glycogenolysis [4]. In endothelial cells, Akt phosphorylates and activates endothelial nitric oxide synthase, leading to nitric oxide synthesis [5].

3. Obesity, Adipose-Derived Inflammation, and Insulin Resistance

Chronic adipose tissue inflammation during obesity promotes both adipose tissue and systemic insulin resistance, mainly through the release of proinflammatory compounds by various adipose tissue cells including adipocytes and macrophages, as well as neutrophils that were shown to infiltrate adipose tissue at an early stage of diet-induced obesity in mice [6, 7]. Nevertheless, there is evidence that such adipose tissue-derived inflammation is not essential for the initiation of systemic insulin resistance [8]. In a mouse model of diet-induced obesity, cellular inflammation and insulin resistance occurred first in arterial tissue, within the first week, followed by skeletal muscle and liver between weeks 4 and 8, and these changes were not detected in adipose tissue until week 14 [9]. Likewise, there was absence of systemic inflammation in grade 1 obese women, who displayed IR in skeletal muscle but not in adipose tissue [10]. At the cellular level, cultured endothelial cells, hepatocytes, or skeletal muscle cells treated with palmitate developed insulin resistance in the absence of neutrophils or macrophages [5, 11–14]. On the other hand, palmitate does not directly activate neutrophils, and it even reduces hydrogen peroxide production by these cells [15].

4. Toll-Like Receptor 4 or 2 (TLR4 or TLR2) Signaling in Response to High Fat, High Sugar, and Lipopolysaccharide Promotes Insulin Resistance through Oxidative Stress and the Activation of Serine Kinases

Oxidative stress refers to an imbalance between cellular reactive oxygen species (ROS) and antioxidants, in favor of the former [16]. Examples of ROS include superoxide anion ($\bullet -O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), singlet oxygen ($^1O_2$), and ozone (O_3). There is mounting evidence that oxidative stress has a causative role in insulin resistance. For example, attenuating mitochondrial hydrogen peroxide emission by treating rats with a mitochondrial-targeted antioxidant or by overexpressing catalase in mouse skeletal muscle mitochondria preserved insulin sensitivity [17]. Excessive caloric intake by healthy men for 1 week acutely induced weight gain, oxidative stress, and insulin resistance in adipocytes prior to the onset of inflammatory stress [18].

Toll-like receptor 4 (TLR4) signaling is responsible for much of the proinflammatory cytokine production by innate cells [19]. It is also expressed in other cells including endothelial cells, skeletal muscle cells, hepatocytes, and adipocytes, where it contributes to insulin resistance by promoting the formation of ROS and reactive nitrogen species such as nitric oxide (NO), as well as the activation of serine kinases which catalyze serine rather than tyrosine phosphorylation of IRS (Figure 1) [19, 20]. Such serine kinases include protein kinase C (PKC) isoforms, IκB kinase (IKK) complex, and mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK) and p38 MAPK [19]. p38 MAPK also activates phosphatase and tensin homolog (PTEN) which reduces phosphorylation of P13K and Akt [11].

Enterobacterial lipopolysaccharide (LPS), whose plasma levels are increased by a high-fat diet, is a direct ligand and activator of TLR4 through its lipid A component which partly consists of palmitic, lauric, or myristic acids [19, 20]. On the other hand, fatty acids and sugars may interact indirectly with TLR via diacylglycerol (DAG) synthesis and subsequent PKC and NADPH oxidase (Nox) activation (Figure 1), as discussed shortly. TLR signaling requires adaptors such as myeloid differentiation primary response protein 88 (myD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) [19, 21]. Both TLR2 and TLR4 signal via the myD88 pathway whereby myD88 leads to activation of TAK 1, a member of the MAPK kinase family, which activates both the IKK complex-nuclear factor kappa B (NF-kB) pathway and MAPKs such as ERK1/2, JNK, and p38 [21]. IKK activation leads to phosphorylation of the NF-kB inhibitor, IkBα, whose subsequent proteosomal degradation frees NF-kB to translocate to the nucleus and activate the expression of proinflammatory cytokines as well as inducible nitric oxide synthase (iNOS) and Nox components such as p91phox and p22phox [19–22].

An increase in intracellular diacylglycerol (DAG) was suggested as a unifying hypothesis that could explain most forms of insulin resistance [8]. During hyperglycemia, de novo DAG synthesis occurs via the polyol pathway [23]. During fatty acid overload, DAGs are synthesized as intermediates in triacylglycerol synthesis [24]. DAG is an essential cofactor and activator of PKC isoforms such as PKC-α, PKC-δ, PKC-ε, PKC-ζ, and PKC-θ which catalyze serine phosphorylation of IRS-1 [25]. These PKC isoforms also interact with TLR and other components of the TLR4 pathway such as myD88 to promote NF-kB activation [26]. PKCs also activate Nox by promoting p47phox translocation to the membrane [27]. Nox isoforms such as Nox2, Nox3, and Nox4 have a critical role in hepatocyte, endothelial cell, skeletal muscle cell, and adipocyte insulin resistance [11, 12, 28–30], and Nox-derived ROS contribute to TLR4 activation and signaling [31].

iNOS plays a key role in skeletal muscle, adipose tissue, and hepatic insulin resistance [32–34]. Nox-derived superoxide anion ($\bullet -O_2$) and iNOS-derived nitric oxide (NO) undergo a diffusion-controlled reaction to form the peroxynitrite anion (ONOO−) (Figure 1), and this is the only reaction that occurs at comparable or even higher rate than superoxide dismutase- (SOD-) catalyzed conversion of superoxide anion to hydrogen peroxide [35]. Peroxynitrite is a major contributor to insulin resistance. For example, treatment of cultured adipocytes with hypochlorous acid (HOCl) resulted in adipocyte peroxynitrite production,

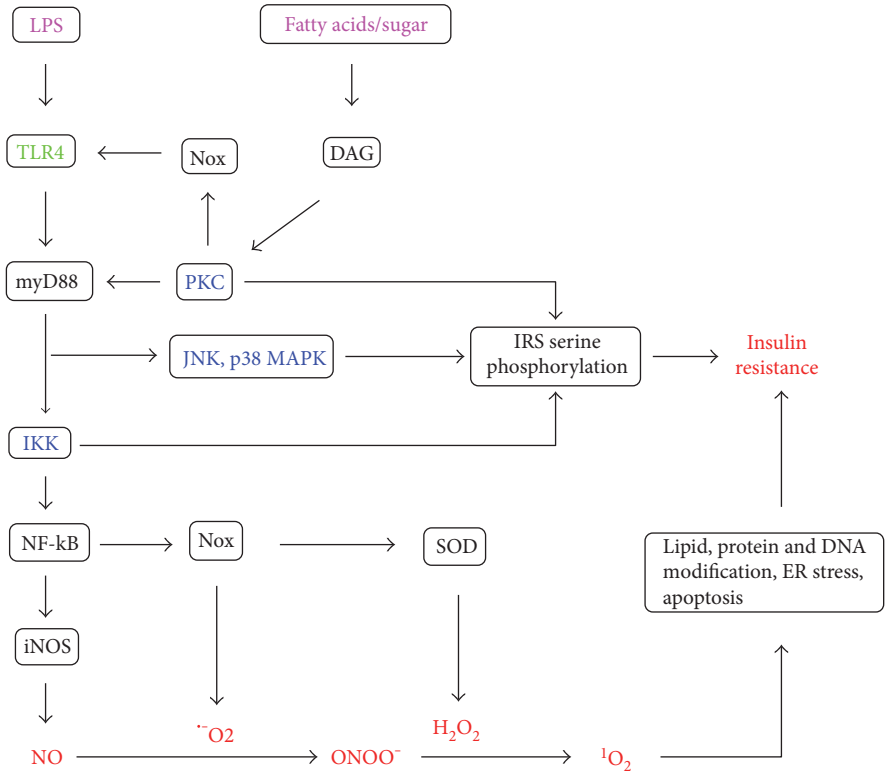


Figure 1: TLR4-dependent pathways for the development of insulin resistance via (i) the activation of serine kinases such as PKC isoforms, IKK, JNK, and p38 MAPK that induce serine phosphorylation of insulin receptor substrate (IRS) and (ii) the formation of reactive oxygen and nitrogen species such as hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite (ONOO−), and singlet oxygen ($^1O_2$). Enteric lipopolysaccharide (LPS) is a direct ligand of TLR4, while fatty acids and sugars such as fructose activate this pathway via DAG, PKC, and Nox-derived ROS.

PKC-θ, IKK, JNK phosphorylation, IRS-1 serine 307 phosphorylation, and insulin resistance, and peroxynitrite inhibitors abolished the rest of these events [36]. The biological effects of peroxynitrite have largely been attributed to its direct oxidation of thiol groups, or its involvement in the formation of radicals that participate in reactions such as lipid oxidation and protein tyrosine nitration [33, 35]. However, the mechanism by which peroxynitrite induces PKC-θ, IKK, JNK, and IRS-1 serine 307 phosphorylation may at least partly involve singlet oxygen-mediated ER stress because (i) peroxynitrite is involved in singlet oxygen formation (Sections 7 and 8), (ii) singlet oxygen induces ER stress (Section 10), and (iii) ER stress activates PKC-θ, IKK, and JNK [34, 37, 38].

5. The Receptor for Advanced Glycation End Products (RAGE) Mediates Similar Effects as TLR4

Serum advanced glycation end products (AGEs) are an independent determinant of insulin resistance as determined by the homeostatic model assessment method (HOMA-IR) in both males and females [39]. AGEs signal via the RAGE receptor, which, like TLR4, induces the recruitment of myD88 and TIRAP, and downstream signaling via NF-kB upon phosphorylation of the cytoplasmic domain of RAGE by PKC-ζ [40]. RAGE signaling is associated with a positive autoregulatory loop that perpetuates NF-kB activation, since NF-kB increases the expression of RAGE [41] and the RAGE-ligand, high mobility box protein [42]. Thus, AGE-RAGE signaling is a key player in endothelial cell dysfunction and adipocyte insulin resistance [40, 41]. Apart from AGEs, a high concentration of uric acid also induces endothelial dysfunction through the RAGE receptor [42].

6. Elevated Singlet Oxygen Production Precedes Insulin Resistance and Diabetes

According to recent reports, plasma levels of two hydroxy-octadecadienes (HODES) specifically derived from singlet oxygen-mediated linoleic acid (LA) oxidation, namely, 10-hydroxy-8(E), 12(Z)-octadecadienoic acid and 12-hydroxy-9(Z),13(E)-octadecadienoic acid, rather than two HODES specifically derived from free radical LA oxidation, namely, 13-hydroxy-9(E),11(E)-octadecadienoic acid and 9-hydroxy-10(E),12(E)-octadecadienoic acid, are suitable biomarkers for predicting insulin resistance and type 2 diabetes in humans [2, 43]. Further, Tsumura Suzuki obese diabetes (TSOD) mice on a high-fat diet had significantly higher singlet oxygen-associated fatty acid oxidation products than control mice at week 5, ahead of significant

differences in free radical oxidation-derived products and insulin resistance at week 8 [3]. Thus, it was suggested that excessive singlet-oxygen formation occurs as an early event in the pathogenesis of insulin resistance and type 2 diabetes and that singlet oxygen may be directly or indirectly involved in initiating these disorders [3].

7. Diverse Cell Types, Including Insulin-Responsive Cells, Produce Singlet Oxygen

Murotomi et al. [3] suggested that activated neutrophils were responsible for the elevated plasma levels of singlet oxygen-associated LPO products in TSOD mice, through the myeloperoxidase-HOCl-H_2O_2 system. However, as recently reviewed, singlet oxygen can be generated through many types of reactions involving molecules that are found in virtually all cell types [44], which is not consistent with this ROS being produced just by leukocytes. Interestingly, singlet oxygen is now recognized as an important signaling molecule in plant cells, as a result not only of its photodynamic formation in chloroplasts but also by dark reactions in nonphotosynthetic cells, even in roots, in response to wounding and other stresses [45]. Peroxisomes, mitochondria, and the nucleus are major intracellular regions of such plant cell singlet oxygen formation in the dark [45]. Although the mechanism of formation of singlet oxygen under such conditions is unknown, this may at least partly involve the reaction of peroxynitrite and hydrogen peroxide, because this reaction produces singlet oxygen [46] and peroxisomes are a site for both hydrogen peroxide and peroxynitrite formation in plant cells [47]. Singlet oxygen was also reported to be generated not only by cultured tumor cells but also by the cell-free culture medium upon the addition of hydrogen peroxide, by a process involving the formation of excited carbonyls [48]. Singlet oxygen formation has been demonstrated in enterocytes [49], endothelial cells [50], and hepatocytes [49, 51]. During liver ischemia-reperfusion injury, acute hepatocyte oxidative stress can occur independently of Kupffer cells, the resident macrophages [52], and hepatocyte oxidative stress produces cytokines and chemokines that activate the latter [53], which is a major source of singlet oxygen prior to neutrophil activation [54]. Such a sequence of hepatocyte-Kupffer cell-neutrophil oxidative stress and singlet oxygen formation might occur during nutrient overload and the development of hepatic insulin resistance. Cytochrome P450 2E1, whose protein levels are 10 times higher in hepatocytes than in Kupffer cells [55] and whose expression is induced by xenobiotics as well lipid overload [55, 56], is an important contributor to singlet oxygen formation by liver microsomes [57].

8. The Importance of Peroxynitrite Anion in Singlet Oxygen Formation during the Pathogenesis of Insulin Resistance

The reaction between peroxynitrite and hydrogen peroxide may produce more singlet oxygen than the neutrophil-associated reaction between hypochlorous acid and hydrogen peroxide in vivo because hypochlorous acid is very reactive with other biomolecules [58]. The related reaction between nitric oxide and hydrogen peroxide was also found to release large amounts of chemiluminescence due to singlet oxygen, and this reaction was suggested to be involved in nitric oxide-mediated cell killing [59]. Since insulin-responsive cells upregulate Nox and iNOS expression and the resultant formation of nitric oxide, peroxynitrite, and hydrogen peroxide under conditions relevant to insulin resistance (Sections 4 and 5), singlet oxygen should be generated under such circumstances. Peroxynitrite also reacts with various other molecules to generate singlet oxygen, as recently reviewed [44]. For example, as illustrated in Figure 2, the reaction of peroxynitrite (1) with CO_2 forms nitrosoperoxycarbonate (2) which decomposes to reactive carbonate and nitrogen dioxide radicals ((3) and (4), resp.) that readily convert glutathione (5) to glutathyl radical (6) [35, 60]. Glutathyl radicals reacts with oxygen to generate peroxysulphenyl radical (7) which, via tetroxide species (8) and peroxide (9), generates $^1O_2$ and glutathione disulfide (10) [61]. Since glutathione is one of the major peroxynitrite sinks [60], such reactions limit peroxynitrite-dependent free radical reactions while promoting singlet oxygen production.

9. Singlet Oxygen Generated near the Plasma Membrane May Induce Peroxynitrite Formation and Further Singlet Oxygen Formation in Insulin-Responsive Cells via the Death Receptor Fas

When tumor cells are exposed to a low dose of extracellular photodynamically generated singlet oxygen, the latter activates the death receptor Fas, which signals to upregulate Nox and NOS, resulting in peroxynitrite and H_2O_2 formation, and a "massive increase in secondary singlet oxygen" [62]. A similar phenomenon may contribute to endothelial cell dysfunction, because (i) endothelial cells express the Fas receptor [63]; (ii) endothelial cells may generate extracellular singlet oxygen because hydrogen peroxide and peroxynitrite formed in them can cross the plasma membrane [35]; (iii) treatment of endothelial cells with 3-morpholinosydnonimine (SIN-1), a peroxynitrite donor, increased iNOS expression via NF-kB and thus established a positive feedback loop for peroxynitrite formation [64]; (iv) high glucose-induced, Nox-mediated endothelial cell dysfunction is exacerbated by myeloperoxidase, which utilizes endothelial cell-derived hydrogen peroxide to generate hypochlorous acid [65], and should thus produce extracellular singlet oxygen by the reaction of the latter with hydrogen peroxide; and (v) apart from a direct effect of singlet oxygen on the endothelial cell Fas receptor, singlet oxygen may oxidize LDL to produce oxidized LDL, which signals via the Fas receptor to induce endothelial cell apoptosis accompanied by activation of MAP and Jun kinases [63]. In fact, the already mentioned (Section 4) hypochlorous acid-mediated, peroxynitrite-dependent induction of insulin resistance in cultured adipocytes [36] may involve a similar mechanism, whereby adipocyte-derived H_2O_2 reacts with HOCl to generate singlet oxygen, which then activates Fas.

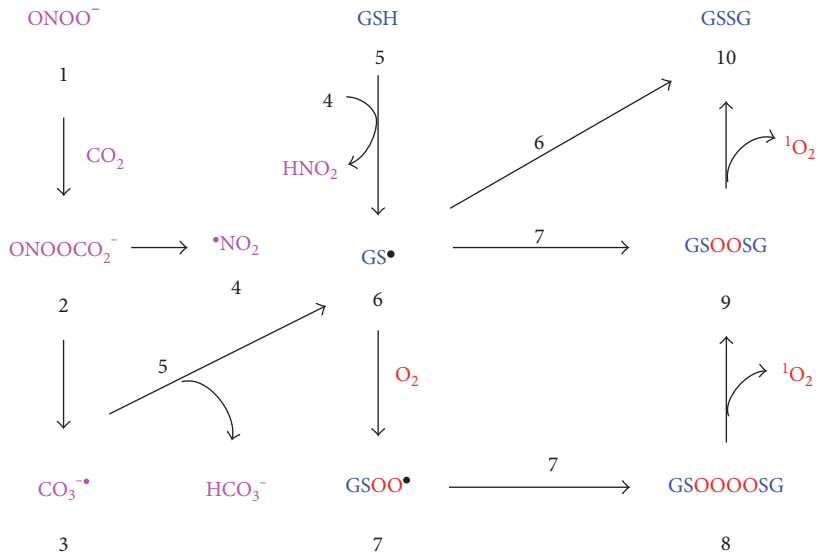


Figure 2: Formation of singlet oxygen ($^1O_2$) as a result of the facile conversion of peroxynitrite (1) to nitrosoperoxycarbonate (2), decomposition of the latter into carbonate and nitrogen dioxide radicals ((3) and (4), resp.) that convert glutathione (5) to glutathyl radical (6) [35, 60], reaction of (6) with oxygen to form peroxysulphenyl radical (7), and further reactions of the latter by a Russel-type mechanism to afford tetroxide (8), peroxide (9), oxidized glutathione (10), and $^1O_2$ [44, 61]. Oxidized glutathione (10) can also be formed by the direct combination of two glutathyl radicals (6).

The latter, which has been shown to induce adipocyte insulin resistance [66], will then induce iNOS and Nox. The induction of iNOS activity in response to Fas activation in hepatocytes was shown to be a mechanism to reduce apoptosis and enhance survival [67]. Although the foregoing examples assume the activation of Fas by extracellular singlet oxygen, the same effects might generally result from singlet oxygen generated on either side of the plasma membrane, since singlet oxygen photodynamically generated near the plasma membrane was found to induce endothelial cell apoptosis [68]. In hepatocytes, Fas activation also promotes CYP2E1 activity [69], which is an important source of singlet oxygen [57].

10. Singlet Oxygen Formation in the Endoplasmic Reticulum Induces ER Stress

Endoplasmic reticulum stress is an important contributor to adipose tissue and hepatic insulin resistance, through multiple mechanisms including (i) activating JNK and p38, (ii) inducing the pseudokinase tribble 3 (TRB3), which prevents insulin-induced Akt phosphorylation, and (iii) upregulating protein tyrosine phosphatase B (PTPB), a negative regulator of the insulin receptor [34, 70, 71]. The ER stress-inhibiting chaperone tauroursodeoxycholic acid (TUDCA) has been shown to improve insulin signaling in both mice and humans [72, 73].

Singlet oxygen photodynamically generated within the ER induces calcium efflux and ER stress [74]. Cytochrome P450 2E1 (CYP2E1), which is mainly located in the ER, is a strong producer of superoxide [56] and also produces peroxynitrite even in the absence of iNOS [75]. Since the ER constantly produces H_2O_2 during protein folding [76], the reaction of CYP2E1-derived peroxynitrite with H_2O_2 to generate singlet oxygen and thereby induce ER stress is highly likely. CYP2E1 also generates singlet oxygen by a mechanism independent of peroxynitrite [57]. This protein also promotes lipid peroxidation, and its expression correlates with lipid peroxidation in obese patients [56, 77]. CYP2E1 expression is induced by JNK [78], which may be activated by pathways such as TLR4 or Fas [Sections 4 and 9], and CYP2E1 in turn strongly activates JNK [78]. Interestingly, adipocytes and hepatocytes strongly express CYP2E1 [78] and are prone to ER stress [34], while skeletal muscle cells only weakly express this protein [79, 80] and are less prone to ER stress [34]. There are both iNOS-dependent and iNOS-independent pathways for ER stress in the adipose tissue and liver, and silencing iNOS and abolishing the residual ER stress completely abolishes insulin resistance in these organs [34]. The iNOS-independent ER stress can be well explained by CYP2EI in hepatocytes and adipocytes. Whole body CYP2E1 knockout protected mice from HFD-induced obesity and insulin resistance, and it especially improved insulin sensitivity in hepatic and adipose tissues but not skeletal muscle tissue [81].

11. Mitochondrial Singlet Oxygen Formation Damages Mitochondrial DNA

Mitochondria are a key site for peroxynitrite formation due to NO easily diffusing into them and reacting with superoxide formed as a result of electron leakage from the electron transport chain [35]. Peroxynitrite further promotes such electron leakage, resulting in the elevation of mitochondrial H_2O_2 [35, 82], thus setting the right conditions for singlet oxygen formation by these two reactive species. Hence, mitochondria should be a major site for peroxynitrite-dependent

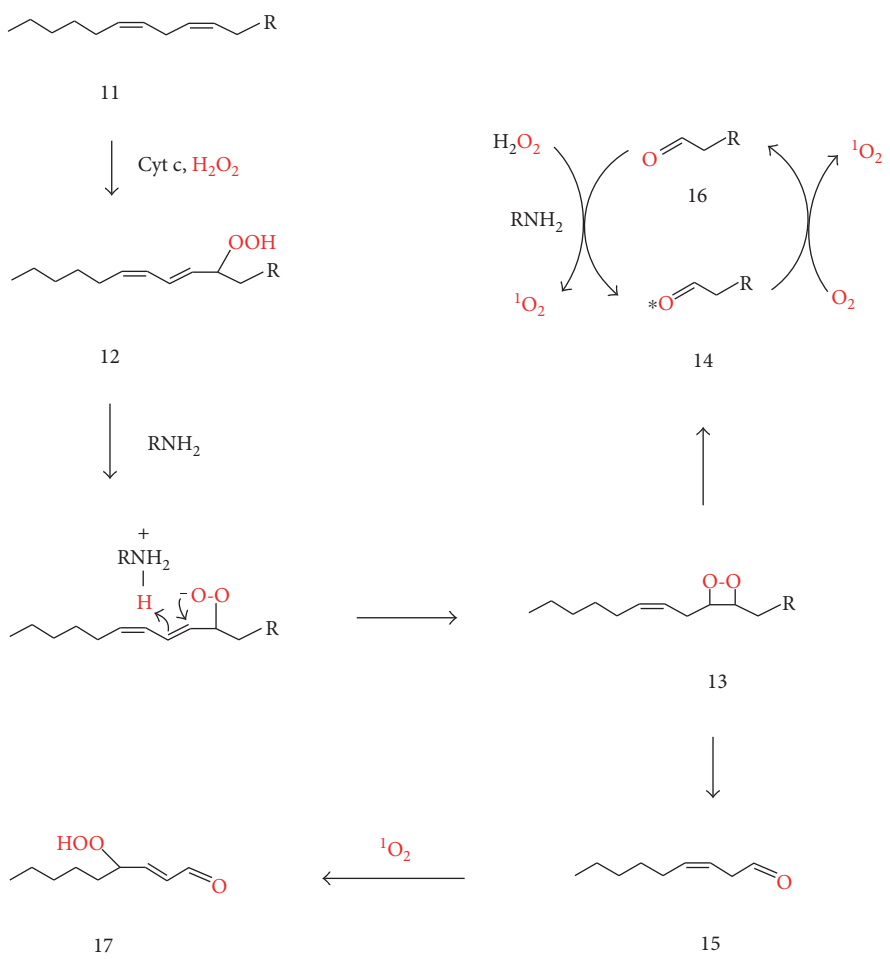


Figure 3: Proposed mechanisms for the mitochondrial formation of $^1O_2$ and 4-hydroperoxy-2-nonenal (17) during the oxidation of a cardiolipin (11) (which bears an n-6 fatty acid). In the presence of H_2O_2, cytochrome c converts the latter to cardiolipin hydroperoxide (12) [85], followed by lysine-catalyzed rearrangement of the latter into dioxetane (13) [44] which decomposes into aldehyde (14) (oxononanoyl-cardiolipin in case the fatty acid moiety being oxidized is linoleic acid) and 3-Z-nonenal (15). The asterisk on carbonyl (14) indicates the excited (triplet) state, since dioxetane decomposition produces such carbonyls. Energy transfer from (14) to triplet oxygen converts the latter to $^1O_2$ and the former to its ground state form (16), whose reaction with hydrogen peroxide regenerates (14) and forms singlet oxygen [44, 88, 90].

singlet oxygen formation. This is consistent with the findings that, in skeletal muscle cells, mitochondrial ROS and the resultant DNA damage and apoptosis contribute to insulin resistance [13], attenuating mitochondrial hydrogen peroxide emission prevents skeletal muscle insulin resistance [17], deletion of iNOS or addition of a peroxynitrite inhibitor prevents such mitochondrial DNA damage and insulin resistance [14, 34], and skeletal muscles from rats injected with LPS produced singlet oxygen, which was associated with increased Nox and iNOS activity, hydrogen peroxide and peroxynitrite formation, and enhanced mitochondrial lipid oxidation [83].

ER calcium efflux is another major contributor to mitochondrial ROS, since it causes mitochondrial calcium influx, which greatly enhances mitochondrial superoxide production, for example, in cardiomyocytes [76]. In hepatocytes, uric acid-induced activation of Nox preceded ER stress, which further induced mitochondrial ROS [84].

Increased mitochondrial H_2O_2 either as a result of peroxynitrite production or calcium influx may promote singlet oxygen formation by an additional pathway involving cardiolipin oxidation. In the presence of H_2O_2, cytochrome c acts as a cardiolipin-specific oxygenase, converting cardiolipin (11) to a cardiolipin hydroperoxide such as (12) (Figure 3) [85]. The decomposition of cardiolipin hydroperoxide generates triplet carbonyls that transfer energy to triplet oxygen and thus form singlet oxygen [86]. Formation of such triplet carbonyls from cardiolipin hydroperoxide (12) may partly involve the amine- (RNH_2-) catalyzed conversion of the latter to dioxetane (13), whose decomposition affords an aldehydic cardiolipin (14) and 3(Z)-nonenal (15), either of which could be in the excited triplet state (Figure 3). The amine (RNH_2) could be a lysine residue or the amino group of phosphatidylethanolamine or phosphatidylserine. Such amine-catalyzed conversion of the 13-hydroperoxide of linoleic acid (13-hydroperoxy-9Z, 11E-octadecadienoic acid,

HPODE) to a dioxetane that yields hexanal was recently suggested [44] to explain the known reaction of lysine with HPODE to form Nε-(hexanoyl)lysine (HEL), which does not form by reaction of preformed hexanal with lysine in the absence of HPODE [87]. The Schiff base between hexanal and lysine was suggested to react with a second HPODE molecule to form HEL [44]. Formyl-lysine, a product analogous to HEL, is formed in a system containing formaldehyde, lysine, and H_2O_2 [88], where H_2O_2 (rather than a lipid hydroperoxide) reacts with the corresponding Schiff base. The fact that plasma HEL levels were significantly and positively correlated with fasting plasma glucose, serum insulin, and HOMA-IR in obese males [89] indicates that this kind of reaction is important in vivo.

Amines also catalyze a reaction between hydrogen peroxide and carbonyls (including sugars) to form singlet oxygen and excited carbonyls [44, 88, 90]. Such reactions might be responsible for the already-mentioned formation of singlet oxygen upon addition of hydrogen peroxide to a cell-free culture medium (Section 7) [48]. Accordingly, aldehyde (16), the nonexcited form of (14), may react with H_2O_2 to form singlet oxygen and to regenerate (14), thus amplifying singlet oxygen formation. In this way, any other aldehydes formed in the mitochondrion may participate in singlet oxygen formation. This aldehyde-amine-hydrogen peroxide-dependent mechanism of singlet oxygen formation may be equally important in the ER since it has been demonstrated in liver microsomes [88, 90], where CYP2E1 induces lipid oxidation in an environment favoring H_2O_2 formation [77].

12. Singlet Oxygen Oxidizes Low-Density Lipoprotein

Oxidized low-density lipoproteins (oxLDLs) were positively associated with HOMA-IR in young human adults independently of obesity in a longitudinal study [91]. Similar strong association between oxLDL levels and insulin resistance was obtained in a weight reduction study [92]. Endothelial cells mediate oxLDL formation by a peroxynitrite-dependent mechanism [93], indicating the potential involvement of singlet oxygen, since both singlet oxygen and oxLDL are strong precursors of insulin resistance [3, 92]. oxLDL can perpetuate the effects of HFD on endothelial cells, because its signaling via the lectin-like oxidized low-density lipoprotein receptor-1 (Lox-1 receptor) and TLR4 receptors initiates a positive autoregulatory loop for NF-kB activation and upregulation of Lox-1 receptor expression [94]. This also induces the expression of vascular cell adhesion molecule 1 (VCAM 1) and monocyte chemoattractant protein (MCP-1), which promote the recruitment of immune cells [94]. OxLDL signaling in adipocytes induces adipocyte insulin resistance through the activation of IKK, JNK, and NF-kB, even independently of further ROS formation [95], and this may involve the interaction of oxLDL receptor with CD36, resulting in CD36 association with the Src family tyrosine kinases Fyn and Lyn upstream of JNK [96]. oxLDL also reduces adiponectin secretion [97], and this affects systemic insulin resistance because adiponectin improves insulin sensitivity in endothelial cells, hepatocytes, and skeletal muscle cells [98].

As already discussed (Section 3), palmitate induces insulin resistance in insulin-responsive cells independently of neutrophils, but the latter are recruited to adipose tissue and promote diet-induced insulin resistance by producing myeloperoxidase and other proinflammatory substances. While palmitate lowers ROS formation by neutrophils [15], oxLDL induces neutrophil transmigration across microvascular endothelial cell monolayers and their subsequent degranulation especially after endothelial cell activation [99]. Extracellular hydrogen peroxide mediates the paracrine recruitment of neutrophils to wounded tissue [100]. Therefore, H_2O_2 produced by adipocytes, together with endothelial cell-derived oxLDL, may contribute to neutrophil infiltration and activation in adipose tissue.

13. Singlet Oxygen Increases Intracellular Ceramide Levels

Ceramide is a key contributor to insulin resistance by several mechanisms including, but not limited to, (i) activation of protein phosphatase 2A (PP2A) which dephosphorylates Akt, (ii) activation of the atypical PKC isoform ζ which inhibits Akt, and (iii) JNK activation [101].

Singlet oxygen induces the nonenzymatic conversion of sphingomyelin to ceramide in tumor cells, and there is an autocrine loop linking such ceramide increase to de novo ceramide biosynthesis [102]. There is a possibility that, at least the nonenzymatic mechanism, may be involved in ceramide formation in insulin-responsive cells such as skeletal muscle cells and adipocytes. Singlet oxygen may also contribute indirectly to ceramide accumulation via oxLDL-mediated decrease in adiponectin, since the latter has ceramidase activity [101], or by oxLDL-mediated sphingomyelinase activation [103]. Besides, ER stress and ceramide accumulation induce each other in a vicious cycle [101].

14. Singlet Oxygen Contributes to the Formation of Insulin Resistance-Promoting Reactive Carbonyl Species

The decomposition of lipid hydroperoxides affords highly reactive aldehydic products such as malondialdehyde (MDA), glyoxal, acrolein, 4-hydroperoxy-2-nonenal (HPNE), 4-hydroxy-2-nonenal (HNE), and 4-oxo-2-nonenal (ONE), which contribute to insulin resistance in various ways. Plasma MDA concentration positively correlates with insulin resistance [104]. ONE induces primary hepatocyte apoptosis through increased xanthine oxidase (XO) activity [105]. By promoting XO activity, ONE potentially contributes to the formation of uric acid, which induces endothelial dysfunction as well as hepatocyte ER stress [42, 84]. ONE reacts with lysine to form Nε-(4-oxononanoyl)lysine, an important ligand of Lox-1 receptor [106] and may thus make a major contribution to oxLDL-mediated insulin resistance. Protein-HNE adducts but not protein carbonyl levels were found to be related to intramyocellular lipid content and the severity of insulin resistance in humans [107].

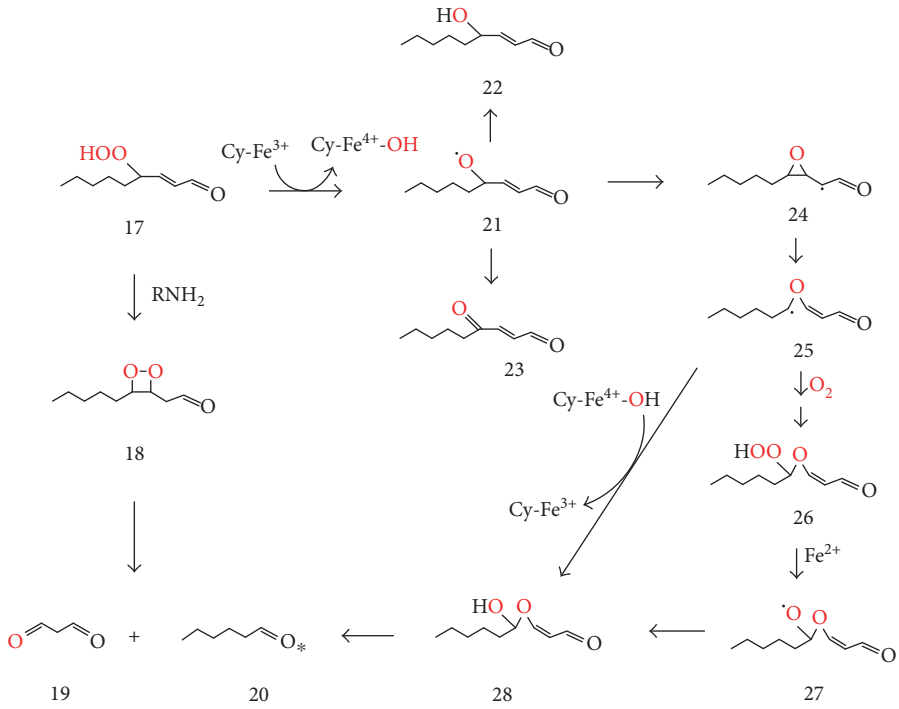


Figure 4: Suggested conversion of 4-hydroperoxy-2-nonenal (HPNE, (17)) to malondialdehyde (MDA, (19)), hexanal (20), 4-hydroxy-2-nonenal (HNE, (22)), and 4-oxo-2-nonenal (ONE (23)) through amine or metal ion-mediated processes.

Various mechanisms for the formation of some of the above-named aldehydes during free radical lipid oxidation have been proposed [108–110]. However, such purely free radical mechanisms do not adequately explain the generation of some lipid oxidation products in vivo. Notably, according to the free radical-dependent reactions, linoleic acid is not expected to be an important precursor of MDA [109], while, on the contrary, plasma linoleic acid was found to be an important precursor of this aldehyde [111]. Furthermore, the reaction of lysine with HPNE, a derivative of linoleic acid, was found to generate MDA by an unknown mechanism [112].

HPNE (17) may be formed by the reaction of 3(Z)-nonenal (15) with singlet oxygen (Figure 3). Similarly to the conversion of hydroperoxide (12) to dioxetane (13) (Figure 3), HPNE (17) may be converted by an amine (RNH_2) via dioxetane (18) to MDA (19) and hexanal (20) (Figure 4), thus explaining the previously reported, lysine-mediated conversion of HPNE to MDA [112]. Inorganic ferrous ion (Fe^{2+}) or organic ferric ions such as in cytochrome c (Cy-Fe^{3+}) may alternatively convert HPNE (17) to the corresponding alkoxyl radical (21), which can abstract or lose a hydrogen to form HNE (22) or ONE (23), respectively, or cyclize to form epoxyalkyl radical (24), which rearranges to oxygen-stabilized vinyl ether radical (25) [113, 114]. The latter may react with oxygen and be converted via hydroperoxy-ether (26) and oxygen-centered radical (27) to hemiacetal (28). Vinyl ether radicals such as (25) also undergo direct conversion to hemiacetals such as (28) by oxygen rebound from the Cy-Fe^{4+} –OH (or Fe^{3+} –OH) pair, and the hemiacetals easily cleave to aldehydes such as MDA (19) and hexanal (20) [113–114].

Cholesterol oxidation generates two main aldehydic products, namely, cholesterol secosterol A and secosterol B [115]. Secosterol A potently inhibits endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS), but not iNOS [116], and should therefore make an important contribution to endothelial dysfunction and insulin resistance, since eNOS promotes insulin sensitivity while iNOS promotes insulin resistance in humans [117].

Singlet oxygen but not free radical oxidation readily converts cholesterol to cholesterol-5-hydroperoxide (29), which, under acidic conditions, undergoes Hock cleavage to form cholesterol secosterol aldehyde A (30), followed by rapid acid-catalyzed aldolization of the latter to form secosterol B (31) (Figure 5), so that secosterol A is only detected as a minor product under such conditions [115, 118]. On the other hand, ozonolysis of cholesterol affords secosterol A as the main product [115, 118, 119]. Both secosterol aldehydes A and B have been isolated in significant quantities from various human tissues and LDL, confirming the importance of singlet oxygen, ozone, and/or an ozone-like oxidant in in vivo lipid oxidation [115, 118, 119]. These aldehydes were also detected in the plasma and other tissues of normal mice, further supporting the formation of singlet oxygen in endothelial cells and other cell types independently of leukocyte activation and inflammation [120]. The fact that secosterol A is a major product in vivo was interpreted as evidence for endogenous ozone formation [115, 118, 119]. Potential mechanisms for the antibody- or amino acid-catalyzed endogenous ozone formation in the presence of singlet oxygen have been proposed [119, 121]. However, Tomono et al. [122] reported that almost equal amounts of

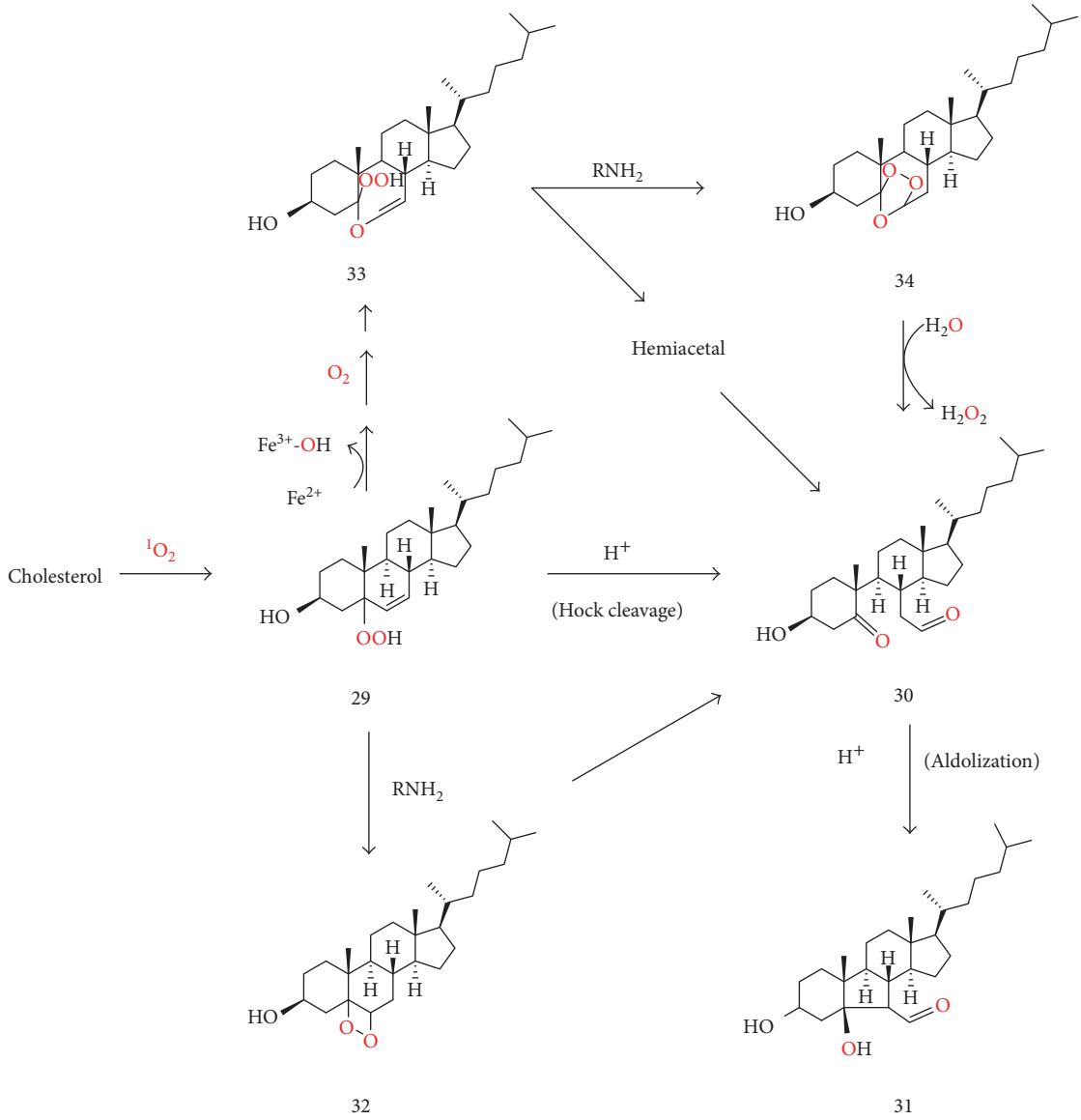


Figure 5: Suggested RNH_2 and Fe^{2+} or cytochrome c-mediated conversion of cholesterol 5-hydroperoxide (29) to secosterol aldehyde A (30) via dioxetane (32), hydroperoxyvinyl ether (33) and the corresponding vinyl hemiacetal, or secondary ozonide (34). Although Hock cleavage of (29) also yields (30), the acidic conditions for Hock cleavage facilitate very rapid aldolization of (30) to form secosterol B (31).

secosterols A and -B were formed in vitro from cholesterol oxidation by human myeloperoxidase (MPO) independently of antibody involvement and concluded that, in this case, singlet oxygen and possibly another oxidant, but not ozone, mediated the formation of both secosterols A and B.

As suggested in Figure 5, the amine-catalyzed decomposition of cholesterol 5-hydroperoxide (29) affords dioxetane (32) whose decomposition generates secosterol A (30) under nonacidic conditions. Moreover, cytochrome c-mediated conversion of cholesterol 5-hydroperoxide (29) to the corresponding alkoxyl radical and epoxyalkyl radicals was recently reported [123]. The said epoxyalkyl radical (not shown) may be converted to secosterol A (30) analogously to the conversion of epoxylakyl radical (24) to aldehydes (19) and (20) in Figure 4. It would not be unreasonable to postulate that hydroperoxyvinyl ether (33) (formed analogously to (26) in Figure 4) may undergo amine-catalyzed conversion to secondary ozonide (34), analogously to the amine-catalyzed formation of dioxetanes such as (32). Hydrolysis of secondary ozonide (34) then affords secosterol A (30) [124]. Thus, a combination of singlet oxygen, a metal ion, triplet oxygen, and an amine might act as an ozone-like oxidant.

15. Conclusion

Insulin resistance is a major precursor of diabetes and cardiovascular diseases, whose etiological pathways deserve attention. This review has highlighted the pathways of formation of singlet oxygen in insulin-responsive cells and how this ROS contributes to insulin resistance through ER stress, mitochondrial DNA damage, and the formation of oxLDL and bioactive aldehydes such as MDA, HNE, ONE,

and cholesterol secosterol aldehydes A and B. Strategies for the prevention or management of insulin resistance may need to include dietary singlet oxygen quenching antioxidants but only as part of a multipronged approach that also targets events both upstream and downstream of singlet oxygen formation, such as inhibiting TLR4 signaling, detoxification of bioactive aldehydes, and inhibiting ER stress. Nevertheless, there is a need for more studies to directly determine the relative contribution of singlet oxygen to insulin resistance vis a vis other reactive oxygen and nitrogen species.

Conflicts of Interest

The author declares that there is no conflict of interests regarding the publication of this paper.

References

[1] M. H. Shanik, Y. Xu, J. Skrh, R. Dankner, Y. Zick, and J. Roth, "Insulin resistance and hyperinsulinemia," *Diabetes Care*, vol. 31, pp. S262–S268, 2008.

[2] 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, article e63542, 2013.

[3] 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, pp. 133–138, 2015.

[4] K. Siddle, "Signaling by insulin and IGF receptors: supporting acts and new players," *Journal of Molecular Endocrinology*, vol. 47, pp. R1–R10, 2011.

[5] Q. Wang, X.-L. Cheng, D.-Y. Zhang, X.-J. Gao, and L. Zhou, "Tectorigenin attenuates palmitate-induced endothelial insulin resistance via targeting ROS-associated inflammation and IRS-1 pathway," *PLoS One*, vol. 8, article e66417, 2013.

[6] H. Kwon and J. E. Pessin, "Adipokines mediate inflammation and insulin resistance," *Frontiers in Endocrinology (Lausanne)*, vol. 4, p. 71, 2013.

[7] S. Talukdar, D. Y. Oh, G. Bandyopadhyay et al., "Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase," *Nature Medicine*, vol. 18, pp. 1407–1412, 2012.

[8] D. M. Erion and G. I. Shulman, "Diacylglycerol-mediated insulin resistance," *Nature Medicine*, vol. 16, pp. 400–402, 2010.

[9] F. Kim, M. Pham, E. Maloney et al., "Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance," *Arteriosclerosis, Thrombosis and Vascular Biology*, vol. 28, pp. 1982–1988, 2008.

[10] C. Amouzou, C. Breuker, O. Fabre et al., "Skeletal muscle insulin resistance and absence of inflammation characterize insulin resistant grade I obese women," *PLoS One*, vol. 11, article e0154119, 2016.

[11] D. Gao, S. Nong, X. Huang et al., "The effects of palmitate on hepatic insulin resistance are mediated by NADPH oxidase 3-derived reactive oxygen species through JNK and p38MAPK pathways," *Journal of Biological Chemistry*, vol. 285, no. 39, pp. 29965–29973, 2010.

[12] S. P. de Figueiredo, A. B. Salmon, F. Bruno et al., "Nox2 mediates skeletal muscle insulin resistance induced by a high fat diet," *Journal of Biological Chemistry*, vol. 290, pp. 13427–13439, 2015.

[13] L. Yuzefovych, G. Wilson, and L. Rachek, "Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 299, pp. E1096–E1105, 2010.

[14] L. Rachek, S. Musiyenko, S. P. LeDoux, and G. L. Wilson, "Palmitate induced mitochondrial deoxyribonucleic acid damage and apoptosis in l6 rat skeletal muscle cells," *Endocrinology*, vol. 148, pp. 293–299, 2007.

[15] H. Akamatsu, Y. Niwa, and K. Matsunaga, "Effect of palmitic acid on neutrophil functions in vitro," *International Journal of Dermatology*, vol. 40, pp. 640–643, 2001.

[16] J. Lugrin, N. Rosenblatt-Velin, R. Parapanov, and L. Liaudet, "The role of oxidative stress during inflammatory processes," *Biological Chemistry*, vol. 395, pp. 203–230, 2014.

[17] E. J. Anderson, M. E. Lustig, K. E. Boyle et al., "Mitochondrial H_2O_2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans," *The Journal of Clinical Investigation*, vol. 119, pp. 573–581, 2009.

[18] G. Boden, C. Homko, C. A. Barrero et al., "Excessive caloric intake acutely causes oxidative stress, GLUT4 carbonylation, and insulin resistance in healthy men," *Science Translational Medicine*, vol. 7, article 304re7, 2015.

[19] J. J. Kim and D. D. Sears, "TLR4 and insulin resistance," *Gastroenterology Research and Practice*, vol. 2010, Article ID 212563, 11 pages, 2010.

[20] L. A. Velloso, F. Folli, and M. J. Saad, "TLR4 at the crossroads of nutrients, gut microbiota, and metabolic inflammation," *Endocrine Reviews*, vol. 36, pp. 245–271, 2015.

[21] T. Kawasaki and T. Kawai, "Toll-like receptor signaling pathways," *Frontiers in Immunology*, vol. 5, p. 461, 2014.

[22] M. J. Morgan and Z. Liu, "Crosstalk of reactive oxygen species and NF-κB signaling," *Cell Research*, vol. 21, pp. 103–115, 2011.

[23] W. H. Tang, K. A. Martin, and J. Hwa, "Aldose reductase, oxidative stress, and diabetic mellitus," *Frontiers in Pharmacology*, vol. 3, p. 87, 2012.

[24] L. Liu, X. Shi, C. S. Choi et al., "Paradoxical coupling of triglyceride synthesis and fatty acid oxidation in skeletal muscle overexpressing DGAT1," *Diabetes*, vol. 58, pp. 2516–2524, 2009.

[25] S. Turban and E. Hajduch, "Protein kinase C isoforms: mediators of reactive lipid metabolites in the development of insulin resistance," *FEBS Letters*, vol. 585, pp. 269–274, 2011.

[26] D. J. Loegering and M. R. Lennartz, "Protein kinase C and toll-like receptor signaling," *Enzyme Research*, vol. 2011, Article ID 537821, 7 pages, 2011.

[27] D. Cosentino-Gomes, N. Rocco-Machado, and J. Roberto Meyer-Fernandes, "Cell signaling through protein kinase C oxidation and activation," *International Journal of Molecular Sciences*, vol. 13, pp. 10697–10721, 2012.

[28] S. Pereira, E. Park, Y. Mori et al., "FFA-induced hepatic insulin resistance in vivo is mediated by PKCδ, NADPH oxidase, and oxidative stress," *American Journal of Physiology - Endocrinology and Metabolism*, vol. 307, pp. E34–E46, 2014.

[29] P. Sukumar, H. Viswambharan, H. Imrie et al., "Nox2 NADPH oxidase has a critical role in insulin resistance-related endothelial cell dysfunction," *Diabetes*, vol. 62, no. 6, pp. 2130–2134, 2013.

[30] L. J. D. Hartigh, M. Omer, L. Goodspeed et al., "Adipocyte-specific deficiency of NADPH oxidase 4 delays the onset of insulin resistance and attenuates adipose tissue inflammation in obesity," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 37, no. 3, pp. 466–475, 2017.

[31] S. W. Wong, M. J. Kwon, A. M. Choi, H. P. Kim, K. Nakahira, and D. H. Hwang, "Fatty acids modulate toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner," *Journal of Biological Chemistry*, vol. 284, pp. 27384–27392, 2009.

[32] H.-N. Cha, S. E. Song, Y.-W. Kim, J.-Y. Kim, K.-C. Won, and S.-Y. Park, "Lack of inducible nitric oxide synthase prevents lipid-induced skeletal muscle insulin resistance without attenuating cytokine level," *Journal of Pharmacological Sciences*, vol. 117, pp. 77–86, 2011.

[33] A. Charbonneau and A. Marette, "Inducible nitric oxide synthase induction underlies lipid-induced hepatic insulin resistance in mice," *Diabetes*, vol. 59, pp. 861–871, 2010.

[34] T. M. Zanotto, P. G. F. Quaresma, D. Guadagnini et al., "Blocking iNOS and endoplasmic reticulum stress synergistically improves insulin resistance in mice," *Molecular Metabolism*, vol. 6, pp. 206–218, 2017.

[35] R. Radi, "Protein tyrosine nitration: biochemical mechanisms and structural basis of its functional effects," *Accounts of Chemical Research*, vol. 46, pp. 550–559, 2013.

[36] J. Zhou, Q. Wang, Y. Ding, and M. H. Zou, "Hypochlorous acid via peroxynitrite activates protein kinase Cθ and insulin resistance in adipocytes," *Journal of Molecular Endocrinology*, vol. 54, pp. 25–37, 2015.

[37] K. Sakaki and R. J. Kaufman, "Regulation of ER stress-induced macroautophagy by protein kinase C," *Autophagy*, vol. 4, pp. 841–843, 2008.

[38] Y. Chen, Z. Wu, S. Zhao, and R. Xiang, "Chemical chaperones reduce ER stress and adipose tissue inflammation in high fat diet-induced mouse model of obesity," *Scientific Reports*, vol. 6, article 27486, 2016.

[39] C. B. Tan, S. W. M. Shiu, Y. Wong, and X. Tam, "Serum advanced glycation end products (AGEs) are associated with insulin resistance," *Diabetes/Metabolism Research and Reviews*, vol. 27, pp. 488–492, 2011.

[40] M. Sakaguchi, H. Murata, K. Yamamoto et al., "TIRAP, an adaptor protein for TLR2/4, transduces a signal from RAGE phosphorylated upon ligand binding," *PLoS One*, vol. 6, no. 8, article e23132, 2011.

[41] K. H. J. Gaens, G. H. Goossens, P. M. Niessen et al., "Nε-(carboxymethyl) lysine-receptor for advanced glycation end product axis is a key modulator of obesity-induced dysregulation of adipokine expression and insulin resistance," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 34, pp. 1199–1208, 2014.

[42] W. Cai, X.-M. Duan, Y. Liu et al., "Uric acid induces endothelial dysfunction by activating the HMGB1/RAGE signaling pathway," *BioMed Research International*, vol. 2017, Article ID 4391920, 11 pages, 2017.

[43] A. Umeno, K. Yoshino, Y. Hashimoto, M. Shichiri, M. Kataoka, and Y. Yoshida, "Multi-biomarkers for early detection of type 2 diabetes, including10-and 12-(Z,E)-hydroxyoctadecadienoic acids, insulin, leptin, and adiponectin," *PLoS One*, vol. 10, article e0130971, 2015.

[44] A. N. Onyango, "Endogenous generation of singlet oxygen and ozone in human and animal tissues: mechanisms, biological significance, and influence of dietary components," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 2398573, 22 pages, 2016.

[45] E. Mor, L. Koh, S. Weiner, H. Rosenwasser, H. Sibony-Benyamini, and R. Fluhr, "Singlet oxygen signatures are detected independent of light or chloroplasts in response to multiple stresses," *Plant Physiology*, vol. 165, pp. 249–261, 2014.

[46] P. D. 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, pp. 287–289, 1994.

[47] F. J. Corpas, J. B. Barroso, J. M. Palma, and M. Rodriguez-Ruiza, "Plant peroxisomes: a nitro-oxidative cocktail," *Redox Biology*, vol. 11, pp. 535–542, 2017.

[48] M. Rác, M. Sedlářová, and P. Pospíšila, "The formation of electronically excited species in the human multiple myeloma cell suspension," *Scientific Reports*, vol. 5, p. 8882, 2015.

[49] E. D. Kerver, I. M. Vogels, K. S. Bosch, H. Vreeling-Sindelárová, R. J. 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," *Histochemical Journal*, vol. 29, pp. 229–237, 1997.

[50] W. Rosenblum and G. H. Nelson, "Singlet oxygen scavengers affect laser-dye impairment of endothelium-dependent responses of brain arterioles," *American Journal of Physiology*, vol. 270, pp. H1258–H1263, 1996.

[51] E. Cadenas, H. Wefers, and H. Sies, "Low-level chemiluminescence of isolated hepatocytes," *European Journal of Biochemistry*, vol. 119, pp. 531–536, 1981.

[52] Y. Kumamoto, M. Suematsu, M. Shimazu, and Y. Kato, "Kupffer cell-independent acute hepatocellular oxidative stress and decreased bile formation in post-cold-ischemic rat liver," *Hepatology*, vol. 30, pp. 1454–1463, 1999.

[53] H. S. Younis, A. R. Parrish, and I. G. Sipes, "The role of hepatocellular oxidative stress in Kupffer cell activation during 1,2-dichlorobenzene-induced hepatotoxicity," *Toxicological Sciences*, vol. 76, pp. 201–211, 2003.

[54] J. C. Cutrìn, A. Boveris, B. Zingaro, G. Corvetti, and G. Poli, "In situ determination by surface chemiluminescence of temporal relationships between evolving warm ischemia-reperfusion injury in rat liver and phagocyte activation and recruitment," *Hepatology*, vol. 31, pp. 622–632, 2000.

[55] T. Koivisto, V. M. Mishin, K. M. Mak, P. A. Cohen, and C. S. Lieber, "Induction of cytochrome P-450 2E1 by ethanol in rat Kupffer cells," *Alcoholism: Clinical and Experimental Research*, vol. 20, pp. 207–212, 1996.

[56] T.-M. Leung and N. Nieto, "CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease," *Journal of Hepatology*, vol. 58, pp. 395–398, 2013.

[57] S. Hayashi, H. Yasui, and H. Sakurai, "Essential role of singlet oxygen in cytochrome P450-dependent substrate oxygenation in rat liver microsomes," *Drug Metabolism and Pharmacokinetics*, vol. 20, pp. 14–23, 2005.

[58] J. K. Hurst, "What really happens in the neutrophil phagosome?," *Free Radical Biology and Medicine*, vol. 53, pp. 508–520, 2012.

[59] A. A. Noronha-Dutra, M. E. Epperlein, and N. Woolf, "Reaction of nitric oxide and hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing," *FEBS Letters*, vol. 321, pp. 59–62, 1993.

[60] E. Ford, M. N. Hughes, and P. Wardman, "Kinetics of the reactions of nitrogen dioxide with glutathione, cysteine, and uric acid at physiological pH," *Free Radical Biology and Medicine*, vol. 32, pp. 1314–1323, 2002.

[61] 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, pp. 29–36, 1983.

[62] G. Bauer, "The antitumor effect of singlet oxygen," *Anticancer Research*, vol. 36, pp. 5649–5663, 2016.

[63] C. Napoli, O. Quehenberger, F. de Nigris, P. Abete, C. K. Glass, and W. Palinski, "Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells," *FASEB Journal*, vol. 14, pp. 1996–2007, 2000.

[64] C.-L. M. Cooke and S. T. Davidge, "Peroxynitrite increases iNOS through NF-κB and decreases prostacyclin synthase in endothelial cells," *American Journal of Physiology-Cell Physiology*, vol. 282, no. 2, pp. C395–C402, 2002.

[65] R. Tian, Y. Ding, Y.-Y. Peng, and N. Lu, "Myeloperoxidase amplified high glucose-induced endothelial dysfunction in vasculature: role of NADPH oxidase and hypochlorous acid," *Biochemical and Biophysical Research Communications*, vol. 484, pp. 572–578, 2017.

[66] S. Wueest, R. A. Rapold, E. J. Schoenle, and D. Konrad, "Fas activation in adipocytes impairs insulin-stimulated glucose uptake by reducing Akt," *FEBS Letters*, vol. 584, pp. 4187–4192, 2010.

[67] E. Hatano, B. L. Bennett, A. M. Manning, T. Qian, J. J. Lemasters, and D. A. Brenner, "NF-κB stimulates inducible nitric oxide synthase to protect mouse hepatocytes from TNF-α– and Fas-mediated apoptosis," *Gastroenterology*, vol. 120, pp. 1251–1262, 2001.

[68] C. P. Lin, M. C. Lynch, and I. E. Kochevar, "Reactive oxidizing species produced near the plasma membrane induce apoptosis in bovine aorta endothelial cells," *Experimental Cell Research*, vol. 259, pp. 351–359, 2000.

[69] X. Wang, Y. Lu, and A. I. Cederbaum, "Induction of cytochrome P450 2E1 increases hepatotoxicity caused by Fas agonistic Jo2 antibody in mice," *Hepatology*, vol. 42, pp. 400–410, 2005.

[70] E. Panzhinskiy, J. Ren, and S. Nair, "Protein tyrosine phosphatase 1B and insulin resistance: role of endoplasmic reticulum stress/reactive oxygen species/nuclear factor kappa B axis," *PLoS One*, vol. 8, no. 10, article e77228, 2013.

[71] W. Zhang, V. Hietakangas, S. Wee, S. C. Lim, J. Gunaratne, and S. M. Cohen, "ER stress potentiates insulin resistance through PERK-mediated FOXO phosphorylation," *Genes & Development*, vol. 27, pp. 441–449, 2013.

[72] U. Ozcan, E. Yilmaz, L. Ozcan et al., "Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes," *Science*, vol. 313, pp. 1137–1140, 2006.

[73] R.-Q. Sun, H. Wang, X.-Y. Zeng et al., "IRE1 impairs insulin signaling transduction of fructose-fed mice via JNK independent of excess lipid," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1852, pp. 156–165, 2015.

[74] I. Moserova and J. Kralova, "Role of ER stress response in photodynamic therapy: ROS generated in different subcellular compartments trigger diverse cell death pathways," *PLoS One*, vol. 7, no. 3, article e32972, 2012.

[75] M. A. Abdelmegeed, K.-H. Moon, C. Chen, F. J. Gonzalez, and B.-J. Song, "Role of cytochrome P450 2E1 in protein nitration and ubiquitin-mediated degradation during acetaminophen toxicity," *Biochemistry and Pharmacology*, vol. 79, pp. 57–66, 2010.

[76] R. Chaube and G. H. Werstuck, "Mitochondrial ROS versus ER ROS: which comes first in myocardial dysregulation?," *Frontiers in Cardiovascular Medicine*, vol. 3, p. 36, 2016.

[77] I. A. Leclercq, G. C. Farrell, J. Field, D. R. Bell, F. J. Gonzalez, and G. R. Robertson, "CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis," *Journal of Clinical Investigation*, vol. 105, pp. 1067–1075, 2000.

[78] J. M. Schattenberg and M. J. Czaja, "Regulation of the effects of CYP2E1-induced oxidative stress by JNK signaling," *Redox Biology*, vol. 3, pp. 7–15, 2014.

[79] D. Molina-Ortiz, J. F. González-Zamora, R. Camacho-Carranza et al., "Xenobiotic-metabolizing enzymes in skeletal muscle of children and adolescents," *Pharmacology and Pharmacy*, vol. 4, article 29888, 2013.

[80] C. Smith, S. C. Stamm, J. E. Riggs et al., "Ethanol-mediated CYP1A1/2 induction in rat skeletal muscle tissue," *Experimental and Molecular Pathology*, vol. 69, pp. 223–232, 2000.

[81] H. Zong, M. Armoni, C. Harel, E. Karnieli, and J. E. Pessin, "Cytochrome P-450 CYP2E1 knockout mice are protected against high-fat diet-induced obesity and insulin resistance," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 302, pp. E532–E539, 2012.

[82] P. Tommasini, A. Sestili, A. Guidarelli, and O. Cantoni, "Hydrogen peroxide generated at the level of mitochondria in response to peroxynitrite promotes U937 cell death via inhibition of the cytoprotective signalling mediated by cytosolic phospholipase A2," *Cell Death and Differentiation*, vol. 11, pp. 974–984, 2004.

[83] V. Vanasco, P. Evelson, A. Boveris, and S. Alvarez, "In situ and real time muscle chemiluminescence determines singlet oxygen involvement in oxidative damage during endotoxemia," *Chemico-Biological Interactions*, vol. 184, pp. 313–318, 2010.

[84] Y.-J. Choi, H.-S. Shin, H. S. Choi et al., "Uric acid induces fat accumulation via generation of endoplasmic reticulum stress and SREBP-1c activation in hepatocytes," *Laboratory Investigation*, vol. 94, pp. 1114–1125, 2014.

[85] N. A. Belikova, Y. A. Vladimirov, A. N. Osipov et al., "Peroxidase activity and structural transitions of cytochrome c bound to cardiolipin-containing membranes," *Biochemistry*, vol. 45, pp. 4998–5009, 2006.

[86] S. Miyamoto, I. L. Nantes, P. A. Faria et al., "Cytochrome c-promoted cardiolipin oxidation generates singlet molecular oxygen," *Photochemical & Photobiological Sciences*, vol. 11, pp. 1536–1546, 2012.

[87] Y. Kato, Y. Mori, Y. Makino et al., "Formation of Nε-(hexanonyl)lysine in protein exposed to lipid hydroperoxide.

A plausible marker for lipid hydroperoxide-derived protein modification," *Journal of Biological Chemistry*, vol. 274, pp. 20406–20414, 1999.

[88] L. Trezl, G. Torok, G. Vasvari, J. Pipek, and L. Hullan, "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.

[89] F. Tokuda, Y. Sando, H. Matsui, and T. Yokoyama, "N epsilon-(hexanoyl) lysine, a new oxidative stress marker, is increased in metabolic syndrome, but not in obstructive sleep apnea," *American Journal of Medical Science*, vol. 338, pp. 127–133, 2009.

[90] 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, pp. 437–447, 1998.

[91] K. Park, M. Gross, D.-H. Lee et al., "Oxidative stress and insulin resistance," *Diabetes Care*, vol. 32, pp. 1302–1307, 2009.

[92] M. S. Linna, M. Ahotupa, K. Kukkonen-Harjula, M. Fogelholm, and T. J. Vasankari, "Co-existence of insulin resistance and high concentrations of circulating oxidized LDL lipids," *Annals of Medicine*, vol. 47, pp. 394–398, 2015.

[93] J. Tanaka, L. Qiang, A. S. Banks et al., "Foxo1 links hyperglycemia to LDL oxidation and endothelial nitric oxide synthase dysfunction in vascular endothelial cells," *Diabetes*, vol. 58, pp. 2344–2354, 2009.

[94] Y. Feng, Z. R. Cai, Y. Tang et al., "TLR4/NF-κB signaling pathway-mediated and oxLDL-induced up-regulation of LOX-1, MCP-1, and VCAM-1 expressions in human umbilical vein endothelial cells," *Genetics and Molecular Research*, vol. 13, pp. 680–695, 2014.

[95] B. Scazzocchio, R. Varì, M. D'Archivio et al., "Oxidized LDL impair adipocyte response to insulin by activating serine/threonine kinases," *Journal of Lipid Research*, vol. 50, pp. 832–845, 2009.

[96] D. J. Kennedy, S. Kuchibhotla, K. M. Westfall, R. L. Silverstein, R. E. Morton, and M. Febbraio, "A CD36-dependent pathway enhances macrophage and adipose tissue inflammation and impairs insulin signaling," *Cardiovascular Research*, vol. 89, pp. 604–613, 2011.

[97] B. Scazzochio, R. Vari, C. Filesi et al., "Cyanidin-3-*O*-β-glucoside and protocatechuic acid exert insulin-like effects by upregulating PPARγ activity in human omental adipocytes," *Diabetes*, vol. 60, pp. 2234–2244, 2011.

[98] R. Ye and P. E. Scherer, "Adiponectin, driver or passenger on the road to insulin sensitivity?," *Molecular Metabolism*, vol. 2, pp. 133–141, 2013.

[99] J. B. Sedgwick, Y. S. Hwang, H. A. Gerbyshak, H. Kita, and W. W. Busse, "Oxidized low-density lipoprotein activates migration and degranulation of human granulocytes," *American Journal of Respiratory Cell and Molecular Biology*, vol. 29, pp. 702–709, 2003.

[100] 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, pp. 996–999, 2009.

[101] J. A. Chavez and S. A. Summers, "A ceramide-centric view of insulin resistance," *Cell Metabolism*, vol. 15, pp. 585–594, 2012.

[102] S. Grether-Beck, A. Timmer, I. Felsner, H. Brenden, D. Brammertz, and J. Krutmann, "Ultraviolet A-induced signaling involves a ceramide-mediated autocrine loop leading to ceramide de novo synthesis," *Journal of Investigative Dermatology*, vol. 125, pp. 545–553, 2005.

[103] L. Liao, Q. Zhou, Y. Song et al., "Ceramide mediates Ox-LDL-induced human vascular smooth muscle cell calcification via p38 mitogen-activated protein kinase signaling," *PLoS One*, vol. 8, no. 12, article e82379, 2013.

[104] A. Özer, M. Bakacak, H. Kıran et al., "Increased oxidative stress is associated with insulin resistance and infertility in polycystic ovary syndrome," *Ginekologia Polska*, vol. 2016, no. 87, pp. 733–738, 2016.

[105] S. Sakuma, M. Negoro, T. Kitamura, and Y. Fujimoto, "Xanthine oxidase-derived reactive oxygen species mediate 4-oxo-2-nonenal-induced hepatocyte cell death," *Toxicology and Applied Pharmacology*, vol. 249, pp. 127–131, 2010.

[106] T. Shibata, Y. Shimozu, C. Wakita et al., "Lipid peroxidation modification of protein generates Nepsilon-(4-oxononanoyl)-lysine as a pro-inflammatory ligand," *Journal of Biological Chemistry*, vol. 286, pp. 19943–19957, 2011.

[107] K. H. Ingram, H. Hill, D. R. Moellering et al., "Skeletal muscle lipid peroxidation and insulin resistance in humans," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, no. 7, pp. E1182–E1186, 2012.

[108] C. Schneider, N. A. Porter, and A. R. Brash, "Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation," *Journal of Biological Chemistry*, vol. 283, pp. 15539–15543, 2008.

[109] A. N. Onyango and N. Baba, "New hypotheses on the pathways of formation of malondialdehyde and isofurans," *Free Radical Biology and Medicine*, vol. 49, pp. 1594–1600, 2010.

[110] A. N. Onyango, "Small reactive carbonyl compounds as tissue lipid oxidation products; and the mechanisms of their formation thereby," *Chemistry and Physics of Lipids*, vol. 165, pp. 777–786, 2012.

[111] M. Öhrvall, S. Tengblad, B. Ekstrand, A. Siegbahn, and B. Vessby, "Malondialdehyde concentration in plasma is inversely correlated to the proportion of linoleic acid in serum lipoprotein lipids," *Atherosclerosis*, vol. 108, pp. 103–110, 1994.

[112] Y. Shimozu, K. Hirano, T. Shibata, N. Shibata, and K. Uchida, "4-Hydroperoxy-2-nonenal is not just an intermediate but a reactive molecule that covalently modifies proteins to generate unique intramolecular oxidation products," *Journal of Biological Chemistry*, vol. 286, pp. 29313–29324, 2011.

[113] A. N. Grechkin, F. Brühlmann, L. S. Mukhtarova, Y. V. Gogolev, and M. Hamberg, "Hydroperoxide lyases (CYP74C and CYP74B) catalyze the homolytic isomerization of fatty acid hydroperoxides into hemiacetals," *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, vol. 1761, pp. 1419–1428, 2006.

[114] A. N. Onyango, "Formation of aldehydic phosphatidylcholines during the anaerobic decomposition of a phosphatidylcholine bearing the 9-hydroperoxide of linoleic acid," *BioMed Research International*, vol. 2016, Article ID 8218439, 10 pages, 2016.

[115] A. D. Wentworth, B.-D. Song, J. Nieva, A. Shafton, T. Sangeetha, 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.

[116] Y. L. Lai, S. Tomono, N. Miyoshi, and H. Ohshima, "Inhibition of endothelial- and neuronal-type, but not inducible-type, nitric oxide synthase by the oxidized cholesterol metabolite secosterol aldehyde: implications for vascular and neurodegenerative diseases," *Journal of Clinical Biochemistry and Nutrition*, vol. 50, pp. 84–89, 2011.

[117] S. Kashiwagi, D. N. Atochin, Q. Li et al., "eNOS phosphorylation on serine 1176 affects insulin sensitivity and adiposity," *Biochemical and Biophysical Research Communications*, vol. 431, pp. 284–290, 2013.

[118] N. Miyoshi, "Chemical alterations and regulations of biomolecules in lifestyle-related diseases," *Bioscience, Biotechnology and Biochemistry*, vol. 80, pp. 1046–1053, 2016.

[119] P. Wentworth Jr., J. Nieva, C. Takeuchi et al., "Evidence for ozone formation in human atherosclerotic arteries," *Science*, vol. 302, pp. 1053–1056, 2003.

[120] S. Tomono, N. Miyoshi, M. Ito, T. Higashi, and H. Ohshima, "A highly sensitive LC–ESI-MS/MS method for the quantification of cholesterol ozonolysis products secosterol-A and secosterol-B after derivatization with 2-hydrazino-1-methylpyridine," *Journal of Chromatography B*, vol. 879, pp. 2802–2808, 2011.

[121] A. N. Onyango, "Alternatives to the 'water oxidation pathway' of biological ozone formation," *Journal of Chemical Biology*, vol. 9, pp. 1–8, 2016.

[122] S. Tomono, N. Miyoshi, K. Sato, Y. Ohba, and H. Ohshima, "Formation of cholesterol ozonolysis products through an ozone-free mechanism mediated by the myeloperoxidase-H2O2-chloride system," *Biochemical and Biophysical Research Communications*, vol. 383, pp. 222–227, 2009.

[123] T. C. Genaro-Mattos, R. F. Queiroz, D. Cunha et al., "Cytochrome c reacts with cholesterol hydroperoxides to produce lipid-and protein-derived radicals," *Biochemistry*, vol. 54, pp. 2841–2850, 2015.

[124] R. C. Murphy and K. M. Johnson, "Cholesterol, reactive oxygen species, and the formation of biologically active mediators," *Journal of Biological Chemistry*, vol. 283, pp. 15521–15525, 2008.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 5621989, 6 pages
https://doi.org/10.1155/2017/5621989

Research Article

Total Oxidant and Antioxidant Status in Prepubertal Children with Obesity

Grażyna Rowicka,[1] Hanna Dyląg,[1] Jadwiga Ambroszkiewicz,[2] Agnieszka Riahi,[1] Halina Weker,[1] and Magdalena Chełchowska[2]

[1]*Nutrition Department, Institute of Mother and Child, Warsaw, Poland*
[2]*Screening Department, Institute of Mother and Child, Warsaw, Poland*

Correspondence should be addressed to Grażyna Rowicka; grazyna.rowicka@imid.med.pl

Received 25 May 2017; Accepted 24 July 2017; Published 20 August 2017

Academic Editor: Sergio Di Meo

Aims. Obesity is accompanied by the formation of oxygen free radicals, whose intensified activity without effective defense mechanisms can lead to oxidative stress and related complications. We evaluated the presence of oxidative stress in obese prepubertal children. *Methods*. The study included 83 healthy children aged 2–10 years (62 with obesity and 21 nonobese controls). Total oxidant capacity (TOC), total antioxidant capacity (TAC), oxidized low-density lipoprotein (ox-LDL), lipid parameters, glucose, and C-reactive protein (CRP) were measured in serum. Oxidative stress index (OSI) was calculated. *Results*. Serum TOC concentration was significantly higher ($p < 0.05$) and TAC concentration was lower ($p < 0.05$) in obese children. OSI was higher ($p < 0.01$) in obese subjects compared with controls. CRP levels were normal in all children, but median CRP value was higher ($p < 0.01$) and HDL cholesterol levels were lower ($p < 0.05$) in the obese group. We found a significant negative correlation between TAC and ox-LDL concentrations ($r = -0.27$, $p < 0.05$) in obese children. Furthermore, obesity duration was positively correlated with TOC level ($r = 0.32$, $p < 0.05$) in this group. *Conclusions*. Obesity-related oxidative stress already occurs in prepubescence. Early obesity diagnosis and the necessary therapeutic activity implementation is a vital strategy for the prophylaxis of free radical damage and related multiorgan complications.

1. Introduction

Over the last few decades, we have observed a steady growth in the frequency of obesity occurrence, not only among adults but also in children and adolescents. The global incidence of childhood obesity varies depending on the country and the year; however, there is an overall increase [1–4]. Studies show that approximately 40% of overweight children will continue to have increased weight during adolescence, and 75–80% of obese adolescents will become obese adults. A child with a high BMI has a high risk of being overweight or obese at 35 years old, and this risk increases with age [5, 6]. The majority of obesity in adulthood has its origins in childhood, which makes obesity a pediatric concern and the period when interventions should be undertaken [4].

Energy imbalances lead to the storage of excess energy in adipocytes, resulting in both hypertrophy and hyperplasia. These processes are associated with abnormalities in adipocyte functioning. Obesity alters the metabolic and endocrine functions of adipose tissue and leads to increased release of hormones, fatty acids, and proinflammatory molecules that contribute to obesity-related complications [7]. Proinflammatory mediators released from adipose tissue cannot only cause direct endothelial damage but also generate excess free radical formation. Such activities are exhibited by, for example, TNF-α (tumor necrosis factor alpha), Il-6 (interleukin 6), CRP (C-reactive protein), leptin, and resistin [8].

The formation of reactive oxygen (ROS) and nitric oxide (NOS) species is an intrinsic phenomenon accompanying biochemical changes occurring in the human body, which has developed mechanisms to protect biomolecules from the deleterious effects of free radicals [9]. These include the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and water and lipid-soluble

antioxidants, such as glutathione, ascorbate (vitamin C), α-tocopherol (vitamin E), and β-carotene, and also endogenous antioxidant, for example, albumin, bilirubin, and uric acid [10, 11]. Much evidence indicates that white adipose tissue mitochondria, particularly in people with obesity, are the main site of ROS generation, accompanied by augmented expression of NDPH (nicotinamide adenine dinucleotide phosphate) oxidase and decreased expression of antioxidative enzymes [12].

An imbalance between the formation of ROS in cells and antioxidant defense causes oxidative stress, which is responsible for oxidative damage to lipids, proteins, and nucleic acids, and modifies their structure as well as functioning [13].

Oxidative damage to important cellular structures is one of the factors responsible for the development of obesity-related complications, such as atherosclerosis, hypertension, ischemic heart disease, insulin resistance, and type 2 diabetes [9, 14]. Obesity significantly increases the risk of developing these diseases and early death as a result [5, 15].

Many markers are currently used to assess oxidant and antioxidant status. They include total oxidant capacity (TOC), total antioxidant capacity (TAC), oxidative stress index (OSI), which expresses the TOC/TAC ratio, and oxidized low-density lipoproteins (ox-LDL), which are lipid peroxidation metabolites [16–19]. There are many studies on oxidative stress in adults with obesity, whereas knowledge about the body's reaction to oxidative stress caused by obesity in children, particularly the younger ones, is limited.

The aim of our study was to assess the severity of oxidative processes (TOC, ox-LDL, and OSI) as well as total antioxidant capacity (TAC) in children with obesity aged 2–10 years.

2. Materials and Methods

The study was performed in accordance with the Helsinki Declaration for Human Research, and the study protocol was approved by the Ethics Committee of the Institute of Mother and Child in Warsaw, Poland. All parents of the participating children were informed of the study's objectives, and written consent was obtained for blood sample analysis before participation in the study.

2.1. Subjects. The study was conducted at the Institute of Mother and Child in Warsaw between January 2014 and June 2016. The study included 83 healthy children aged 2–10 years. Group I ($n = 62$) consisted of children with obesity, wherein the criterion for obesity diagnosis in children up to 5 years old was BMI z-score $\geq$ 3SD, and in children over 5 years old BMI z-score $\geq$ 2SD [20]. Group II ($n = 21$) included nonobese children whose BMI z-score was <−1 + 1>. Exclusion criteria included infections of various etiologies and localizations as well as intake of prescription medications and food supplements with antioxidant properties.

2.2. Anthropometric Measurements. Height and weight were assessed using a standard stadiometer and electronic scale, respectively. Anthropometric measurements were taken using calibrated instruments. The same team examined all the study participants. Weight (kg) and height (m) were used to calculate BMI (body mass index). Body mass index (BMI) was calculated as body weight (kg) divided by height squared (m^2). BMI values were compared with BMI norms for age and sex according to WHO criteria, thus obtaining a BMI z-score, which is a normalized relative weight indicator independent of age and sex.

2.3. Blood Sampling and Biochemical Analysis. For biochemical measurements, peripheral blood (3 mL) was taken in the morning after an overnight fast. Serum samples were obtained after centrifugation (2,500g at 4°C for 10 min) and were used for lipid profile, glucose, and C-reactive protein determination. Residual serum was stored in small portions at −70°C until analyses of TOC, TAC (max 4 weeks), and ox-LDL (max 6 months) were performed.

Total oxidant capacity (TOC) and total antioxidant capacity (TAC) values were measured by colorimetric assay (Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany). The method is based on the enzymatic reaction of peroxides and peroxidases. Oxygen produced by this reaction oxidizes the chromogenic substrate tetramethylbenzidine (TMB), which changes its colour from colourless to blue. By addition of sulfuric acid, the reaction cascade is stopped and the colour of the mixture changed to yellow and can be detected at 450 nm. Serum peroxide levels were calculated as the difference of the absorbance readings relating to the hydrogen peroxide standard curve. Antioxidants inhibit this reaction and can be detected analogously on the basis of the indirect proportionality of this inhibition reaction. The limit of detection was 0.06 mmol/L for TOC and 0.08 mmol/L for TAC. The intra- and interassay coefficients of variation (CV) were less than 4.9% and 7.33% for TOC and 2.5% and 3.33% for TAC, respectively. Oxidative stress index (OSI) was defined as the percentage ratio of TOC levels to TAC levels [21].

Oxidized-LDL (Ox-LDL) levels were determined by enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik AG, Bensheim, Germany). The intra- and interassay coefficients of variability were found less than 5.7% and 9.0%, respectively. The detection limit was 4.13 ng/mL.

Total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and triglyceride (TG) concentrations as well as glucose and C-reactive protein (CRP) were determined by standard methods on Integra Cobas 400 plus analyzer (Roche Diagnostics, Basel, Switzerland).

2.4. Statistical Analysis. All of the statistical analyses were performed using Statistica 12 software. The Shapiro-Wilk test was used to evaluate the normality of variable distribution. The results are presented as means and standard deviations (SD) for normally distributed variables or medians and interquartile ranges for nonnormally distributed variables. Differences in baseline characteristics and biochemical parameters of obese and nonobese children were assessed using the Student t-test for normally distributed data and the Mann–Whitney U test for nonnormally distributed data. Pearson's correlation coefficients (r) were calculated to evaluate correlations between biochemical parameters, age, and

Table 1: Characteristics of the studied children.

Variable	Obese children ($n = 62$)	Nonobese children ($n = 21$)	p value
Age (years)$^{+}$	7.5 (6.3–8.8)	6.4 (5.5–8.6)	0.130
Weight (kg)$^{++}$	40.2 ± 9.3	22.2 ± 6.6	<0.001*
Height (cm)$^{+}$	130.5 (121.0–141.0)	116.0 (102.5–129.0)	0.001*
BMI (kg/m^2)$^{+}$	23.5 (21.9–24.6)	15.5 (15.2–16.3)	<0.001*
BMI z-score^{+}	3.0 (2.5–3.5)	−0.03 (−0.5–0.7)	<0.001*

$^{+}$Data are presented as median value and interquartile ranges (1Q–3Q); $^{++}$data are presented as mean value and standard deviation (SD); *$p < 0.05$; BMI: body mass index; BMI z-score: a normalized relative weight indicator independent of age and sex.

Table 2: Biochemical parameters in obese and nonobese children.

Variable	Obese children ($n = 62$)	Nonobese children ($n = 21$)	p value
TOC (mmol/L)$^{+}$	0.22 (0.15–0.28)	0.15 (0.14–0.23)	0.03*
TAC (mmol/L)$^{++}$	1.22 ± 0.44	1.47 ± 0.52	0.03*
Ox-LDL (ng/mL)$^{+}$	283.8 (114.9–633.9)	363.8 (133.9–611.6)	0.60
OSI^{+}	0.18 (0.12–0.27)	0.11 (0.08–0.18)	0.006*
CRP (mg/L)$^{+}$	0.66 (0.43–1.66)	0.24 (0.10–0.59)	0.008*
Glucose (mg/dL)$^{+}$	86.0 (83.0–90.0)	91.0 (83.0–92.0)	0.50
Cholesterol total (mg/dL)$^{+}$	164.0 (150.0–194.5)	164.5 (157.0–186.0)	0.90
Cholesterol HDL (mg/dL)$^{+}$	51.0 (43.5–59.5)	63.0 (58.0–73.0)	0.04*
Cholesterol LDL (mg/dL)$^{+}$	109.0 (94.0–129.0)	116.0 (88.0–120.0)	0.90
Triglycerides (mg/dL)$^{+}$	72.5 (58.5–86.0)	55.0 (48.0–91.0)	0.40

$^{+}$Data are presented as median value and interquartile ranges (1Q–3Q); $^{++}$data are presented as mean value and standard deviation (SD); *$p < 0.05$; TOC: total oxidant capacity; TAC: total antioxidant capacity; ox-LDL: oxidized low-density lipoprotein; OSI: oxidative stress index; CRP: C-reactive protein.

BMI z-score was used for statistical analysis. A p value < 0.05 was considered statistically significant.

3. Results

Children in both studied groups did not differ in terms of age. The percentage of boys and girls was 35.5% ($n = 22$) and 64.5% ($n = 40$) in the obese group, and 47.6% ($n = 10$) and 52.4% ($n = 11$) in the nonobese group, respectively. BMI and BMI z-score were significantly higher ($p < 0.001$) in children with obesity compared with control peers. Average obesity duration was 3.5 (2.6–4.5) years. Characteristics of the studied children are presented in Table 1.

Serum TOC concentration was significantly ($p < 0.05$) higher, and TAC concentration was lower ($p < 0.05$) in children with obesity than in nonobese ones. The ox-LDL serum value was lower in children with obesity, but this difference was not statistically significant. Additionally, we observed significantly higher ($p < 0.01$) OSI in subjects with obesity compared with nonobese ones. Concentrations of glucose, total cholesterol, LDL cholesterol, and triglycerides did not differ statistically between both groups of children; however, serum HDL cholesterol levels were lower ($p < 0.05$) in children with obesity. CRP concentrations were low in all children, but the median value of this parameter was higher ($p < 0.01$) in the obese group. Serum concentrations of biochemical parameters in the studied children are presented in Table 2.

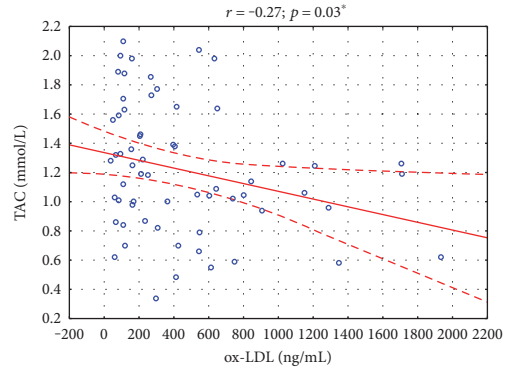


Figure 1: Correlations between serum TAC and ox-LDL concentrations in children with obesity ($N = 62$). *$p < 0.05$.

We found a significant negative correlation between serum TAC and ox-LDL concentrations ($r = -0.27$, $p < 0.05$) (Figure 1) and positive correlation between CRP and TAC concentrations ($r = 0.36$, $p < 0.05$) in children with obesity. We also observed that obesity duration was positively correlated with TOC level ($r = 0.32$, $p < 0.05$) in the obese group (Table 3).

4. Discussion

In people with obesity, the source of reactive oxygen species (ROS) responsible for oxidative stress could be the obesity

Table 3: Correlations between serum concentrations of oxidative status markers and clinical/biochemical parameters in children with obesity ($N = 62$).

	TOC		TAC		OSI		ox-LDL	
	r	p value	r	p value	r	p value	r	p value
Duration of obesity	0.32	0.01*	0.15	0.2	0.17	0.2	−0.16	0.2
Age	0.12	0.4	0.09	0.5	0.03	0.8	−0.09	0.5
BMI z-score	0.03	0.8	0.15	0.2	−0.09	0.4	−0.15	0.2
CRP	0.20	0.2	0.36	0.009*	−0.04	0.8	0.05	0.7
Glucose	0.06	0.7	0.10	0.5	0.06	0.6	−0.14	0.3
Cholesterol total	−0.08	0.6	−0.06	0.6	−0.01	0.9	0.01	0.9
HDL cholesterol	0.01	0.9	−0.04	0.7	−0.04	0.8	0.10	0.5
LDL cholesterol	−0.12	0.4	−0.03	0.8	−0.06	0.7	0.01	0.9
Triglycerides	−0.03	0.8	−0.07	0.6	0.09	0.5	0.07	0.6

r: Pearson's correlation coefficient; *$p < 0.05$; TOC: total oxidant capacity; TAC: total antioxidant capacity; ox-LDL: oxidized low-density lipoprotein; OSI: oxidative stress index; CRP: C-reactive protein.

itself and obesity-related accumulation of fat in the body as an independent factor of ROS formation, the generated chronic low-grade inflammatory state by obesity, and complications resulting from obesity [17, 18, 22].

Our study indicates that prepubertal children with obesity already have a greater intensification of oxidative processes measured by TOC concentrations and OSI values, while simultaneously lowered antioxidant defense measured by TAC concentrations compared with nonobese children. For total oxidant capacity determination, we used the method described by Tatzber et al. [23] based on oxidation of TMB by horseradish peroxidase (HRP)/H_2O_2. This type of reaction is the basic mechanism for detecting both peroxide levels and peroxidase activity in blood serum. In the case of measurement of total peroxides, HRP was added, while excess hydrogen peroxide was added for determination of enzyme activity [23]. Most previous studies on oxidative stress in children with obesity were conducted in children over 6 years old and adolescents. They provide evidence of the existence of a dependence between obesity and oxidative stress. Codoñer-Franch et al. [24] studying a group of children with obesity (6–14 years) with SDS-BMI ≥ 3 found significantly higher concentrations of free radical damage markers, such as malondialdehyde (MDA) and plasma carbonyl groups (CG) compared in nonobese children. Similarly, Albuali [25] showed that children with noncomplicated obesity aged 6–12 years with BMI > 35 kg/m^2 had higher concentrations of lipid oxidation products, such as malonodialdehyde (MDA), ox-LDL, or advanced oxidation protein products (AOPPs), and lower concentrations of enzymes with antioxidative activities, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSSG-R), and glutathione (GSH), than the nonobese. Simultaneously, the authors found increased antioxidant enzyme activity in overweight children, which suggests that this may be a response to increased oxygen free radical production. This is concordant with previous findings, on the basis of which it was determined that in the early stages of obesity development, there is initially increased antioxidant enzyme activity, the aim of which is to prevent the effects of oxidative stress [26, 27].

This also pertains to TAC concentrations, which in the study of Kilic et al. [28] were significantly higher in the group of children with obesity compared with normal weight children. However, the authors demonstrated that increased TAC was the result of elevated TOC levels in an attempt to balance oxidation. They also suggest that the cause of greater TAC concentrations could be more active antioxidant systems in young people. However, reduced antioxidative activities in adults as well as children with obesity could be caused by considerable ROS production, which could be responsible for the depletion of antioxidant enzymes and a secondary reduction in the body's antioxidative capability. This seems to explain the observed increased as well as decreased TAC concentrations in people with obesity.

These discrepancies, according to Brown et al. [29], could be related to obesity duration. The positive correlation we found between obesity duration and TOC concentration may confirm the intensification of oxidative processes along with the duration of obesity occurrence not only in adults but also in children. Taking into account that our study included children up to 10 years old, with a relatively short obesity duration—median obesity duration in this group of children was 3.5 (2.6–4.5) years—one should expect elevated TAC levels in accordance with the concept of Brown et al. as well as other authors' observations. All the more so that CRP concentration as a marker of low-grade inflammation was significantly higher in the group of children with obesity and HDL cholesterol, a fraction that shows anti-inflammatory and antioxidative activity was lower in this group; however, both parameters were in the reference value range. Furthermore, CRP showed a positive correlation with TAC, and ox-LDL concentrations were similar in both groups. All this could mean the onset of an elevated response of antioxidant mechanisms of the body to oxidative stress.

We observed not only lower TAC concentrations in children with obesity but also elevated OSI levels compared

with nonobese ones. Additionally, TAC exhibited a negative correlation with ox-LDL. This is all the more interesting because the studied children with obesity did not have markers of metabolic syndrome, which would explain not only the increased TOC but also decreased TAC and higher OSI in such small children.

Vehapoglu et al. [30], in a study of similarly-aged children (2–11 years) to our studied groups, also found significantly lowered TAC concentrations but did not demonstrate a difference in TOC or OSI levels in children with obesity compared with children of normal weight or underweight. On the other hand, Eren et al. [31] found that increased TOC and OSI levels can be accompanied by elevated TAC concentration in children with obesity with metabolic syndrome compared with children with obesity without metabolic syndrome and children of normal weight. With so many different observations and dependencies established between oxidative stress markers and BMI, it can be assumed that excess adipose tissue is the main factor responsible for increased oxidative stress [32]. This is supported by an intervention study (a 4-week diet and exercise regimen) conducted among teenagers with obesity, which showed that a reduction in body mass positively affected oxidative stress parameters [33]. This claim could also be supported by the observation of Santoro et al. who demonstrated that in obese adolescents, free oxygen radicals are responsible for oxidation of not only LDL but also fatty acids derived from the linoleic acid (LA). This results in generation of oxidized derivatives of LA (oxidized LA metabolites: OXLAMs), which are deleterious for the hepatocyte and may cause liver injury, while the diet with a lower intake of $n = 6$ PUFAs may help reduce the damage, as observed, for example, in people suffering from NAFLD (nonalcoholic fatty liver disease) [34].

Although obesity increases oxidative stress even during childhood, it is difficult to identify a typical oxidative stress-related response, because most studies include different oxidative stress markers. ROS are produced during metabolic reactions, but it is largely unknown which factors modulate their production [22].

Due to the fact that our study is one of a few concerning prepubertal children, we are treating it as a pilot investigation. It is possible that including a larger group of younger children with obesity or use of other methods to assess oxidative stress would provide more important information on the oxidant and antioxidant status in this group.

The present study had several limitations. First, our sample size was relatively small, particularly the control group. However, both studied groups were similar in age, which may be an important factor for oxidative stress marker levels. Second, both groups differed in terms of sex distribution. There is, however, no unequivocal evidence that sex affects the intensification of oxidative stress in children with obesity. Although Sobieska et al. [35] suggest that girls are more resistant to the negative effects of increased adiposity status compared with boys, Kilic et al. [28] did not confirm such a dependence. It should also be taken into account that these observations concerned children over 6 years old and teenagers, while all children included in our study were prepubertal.

In summary, obesity-related oxidative stress already occurs in early childhood; elevated TOC and OSI levels as well as lowered TAC in the blood serum of prepubertal children may be evidence of this. Early obesity diagnosis and the necessary therapeutic activity implementation is a vital strategy for the prophylaxis of free radical damage and related multiorgan complications.

Conflicts of Interest

The authors have declared that no competing interests exist.

Authors' Contributions

All authors are aware of and agree to the content of the manuscript, approved the final submitted version, and agreed to being listed as an author of the manuscript. All authors of this manuscript have directly participated in the execution and analysis of the study.

References

[1] World Health Organization, "School policy framework: implementation of the WHO global strategy on diet, physical activity and health," 2009, http://whqlibdoc.who.int/publications/2009/9789241597418_eng.pdf.

[2] W. P. James, "The epidemiology of obesity: the size of the problem," *Journal of International Medicine*, vol. 263, no. 4, pp. 336–352, 2008.

[3] WHO, "Obesity and overweight. Fact sheet no. 311," September 2006, http://www.who.int/mediacentre/factsheets/fs311/en/.

[4] F. Lifshitz, "Obesity in children," *Journal of Clinical Research in Pediatric Endocrinology*, vol. 1, no. 2, pp. 53–60, 2008.

[5] D. S. Freedman, L. K. Khan, M. K. Serdula, W. H. Dietz, S. R. Srinivasan, and G. S. Berenson, "The relation of childhood BMI to adult adiposity: the Bogalusa heart study," *Pediatrics*, vol. 115, no. 1, pp. 22–27, 2005.

[6] T. D. Brisbois, A. P. Farmer, and L. J. McCargar, "Early markers of adult obesity: a review," *Obesity Reviews*, vol. 13, no. 4, pp. 347–367, 2012.

[7] S. P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante Jr., "Obesity is associated with macrophage accumulation in adipose tissue," *The Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1796–1808, 2003.

[8] S. E. Shoelson, J. Lee, and A. B. Goldfine, "Inflammation and insulin resistance," *The Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1793–1801, 2006.

[9] S. Di Meo, T. T. Reed, P. Venditti, and V. M. Victor, "Harmful and beneficial role of ROS," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 7909186, 3 pages, 2016.

[10] J. Gutiérrez-Salinas, L. García-Ortíz, J. A. Morales González et al., "In vitro effect of sodium fluoride on malondialdehyde concentration and on superoxide dismutase, catalase, and glutathione peroxidase in human erythrocytes," *The Scientific World Journal*, vol. 2013, Article ID 864718, 7 pages, 2013.

[11] I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini, and A. Milzani, "Biomarkers of oxidative damage in human disease," *Clinical Chemistry*, vol. 52, no. 4, pp. 601–623, 2006.

[12] C. K. Roberts, R. J. Barnard, R. K. Sindhu, M. Jurczak, A. Ehdaie, and N. D. Vaziri, "Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome," *Metabolism*, vol. 55, no. 7, pp. 928–934, 2006.

[13] P. Martín-Gallán, A. Carrascosa, M. Gussinyé, and C. Domínguez, "Changes in oxidant-antioxidant status in young diabetic patients from clinical onset onwards," *Journal of Cellular and Molecular Medicine*, vol. 11, no. 6, pp. 1352–1366, 2007.

[14] I. Janssen, P. T. Katzmarzyk, and R. Ross, "Body mass index, waist circumference, and health risk: evidence in support of current National Institutes of Health guidelines," *Archives of Internal Medicine*, vol. 162, no. 18, pp. 2074–2079, 2002.

[15] N. Santoro, "Childhood obesity and type 2 diabetes: the frightening epidemic," *World Journal of Pediatrics*, vol. 9, no. 2, pp. 101-102, 2013.

[16] M. F. Faienza, R. Francavilla, R. Goffredo et al., "Oxidative stress in obesity and metabolic syndrome in children and adolescents," *Hormone Research in Pediatrics*, vol. 78, no. 3, pp. 158–164, 2012.

[17] B. R. Clapp, A. D. Hingorani, R. K. Kharbanda et al., "Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress," *Cardiovascular Research*, vol. 64, no. 1, pp. 172–178, 2004.

[18] D. Bonnefont-Rousselot, J. P. Bastard, M. C. Jaudon, and J. Delattre, "Consequences of the diabetic status on the oxidant/antioxidant balance," *Diabetes & Metabolism*, vol. 26, no. 3, pp. 163–176, 2000.

[19] M. Morita, N. Ishida, K. Uchiyama et al., "Fatty liver induced by free radicals and lipid peroxidation," *Free Radical Research*, vol. 46, no. 6, pp. 758–765, 2012.

[20] M. De Onis, "World Health Organization reference curves," *The ECOG's eBook on Child and Adolescent Obesity*, M. L. Frelut, Ed., 2015, Retrieved from http://ebook.ecog-obesity.eu.

[21] O. Erel, "A new automated colorimetric method for measuring total oxidant status," *Clinical Biochemistry*, vol. 38, no. 12, pp. 1103–1111, 2005.

[22] S. Sfar, R. Boussoffara, M. T. Sfar, and A. Kerkeni, "Antioxidant enzymes activities in obese Tunisian children," *Nutrition Journal*, vol. 12, p. 18, 2013.

[23] F. Tatzber, S. Griebenow, W. Wonisch, and R. Winkler, "Dual method for the determination of peroxidase activity and total peroxides-iodide leads to a significant increase of peroxidase activity in human sera," *Analytical Biochemistry*, vol. 316, pp. 147–153, 2003.

[24] P. Codoñer-Franch, L. Boix-García, R. Simó-Jordá, C. Del Castillo-Villaescusa, J. Maset-Maldonado, and V. Valls-Bellés, "Is obesity associated with oxidative stress in children?," *International Journal of Pediatric Obesity*, vol. 5, no. 1, pp. 56–63, 2010.

[25] W. H. Albuali, "Evaluation of oxidant-antioxidant status in overweight and morbidly obese Saudi children," *World Journal of Clinical Pediatrics*, vol. 3, no. 1, pp. 6–13, 2014.

[26] J. Woo, K. O. Shin, J. H. Yoo, S. Park, and S. Kang, "The effects of detraining on blood adipokines and antioxidant enzyme in Korean overweight children," *European Journal of Pediatrics*, vol. 171, no. 2, pp. 235–243, 2012.

[27] E. Torun, S. Gökçe, L. T. Ozgen, S. Aydın, and Y. Cesur, "Serum paraoxonase activity and oxidative stress and their relationship with obesity-related metabolic syndrome and non-alcoholic fatty liver disease in obese children and adolescents," *Journal of Pediatric Endocrinology and Metabolism*, vol. 27, no. 7-8, pp. 667–675, 2014.

[28] E. Kilic, O. F. Ozer, T. A. Erek et al., "Oxidative stress status in childhood obesity: a potential risk predictor," *Medical Science Monitor Basic Research*, vol. 22, pp. 3673–3679, 2016.

[29] L. A. Brown, C. J. Kerr, P. Whiting, N. Finer, J. McEneny, and T. Ashton, "Oxidant stress in healthy normal-weight, overweight, and obese individuals," *Obesity (Silver Spring)*, vol. 17, no. 3, pp. 460–466, 2009.

[30] A. Vehapoglu, S. Turkmen, N. Goknar, and Ö. F. Özer, "Reduced antioxidant capacity and increased inflammation markers in prepubescent obese children and their relationship with nutritional markers and metabolic parameters," *Redox Report*, vol. 21, no. 6, pp. 271–280, 2016.

[31] E. Eren, M. Abuhandan, A. Solmaz, and A. Taşkın, "Serum paraoxonase/arylesterase activity and oxidative stress status in children with metabolic syndrome," *Journal of Clinical Research in Pediatric Endocrinology*, vol. 6, no. 3, pp. 163–168, 2014.

[32] J. F. Keaney Jr., M. G. Larson, R. S. Vasan et al., "Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 3, pp. 434–439, 2003.

[33] Z. Gao, M. Novick, M. D. Muller et al., "Exercise and diet-induced weight loss attenuates oxidative stress related-coronary vasoconstriction in obese adolescents," *European Journal of Applied Physiology*, vol. 113, no. 2, pp. 519–528, 2013.

[34] N. Santoro, S. Caprio, and A. E. Feldstein, "Oxidized metabolites of linoleic acid as a biomarkers of liver injury in nonalcoholic steatohepatitis," *Journal of Clinical Lipidology*, vol. 8, no. 4, pp. 411–418, 2013.

[35] M. Sobieska, E. Gajewska, G. Kalmus, and W. Samborski, "Obesity, physical fitness and inflammatory markers in Polish children," *Medical Science Monitor*, vol. 19, pp. 493–500, 2013.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8379539, 15 pages
https://doi.org/10.1155/2017/8379539

Research Article

Resveratrol-Enriched Rice Attenuates UVB-ROS-Induced Skin Aging via Downregulation of Inflammatory Cascades

Lalita Subedi,[1] Taek Hwan Lee,[2] Hussain Mustatab Wahedi,[1,3] So-Hyeon Baek,[4] and Sun Yeou Kim[1]

[1]*Laboratory of Pharmacognosy, College of Pharmacy and Gachon Institute of Pharmaceutical Sciences, Gachon University, Incheon 21936, Republic of Korea*
[2]*College of Pharmacy, Yonsei University, No. 162-1, Songdo-dong, Yeonsu-gu, Incheon 406-840, Republic of Korea*
[3]*Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan*
[4]*National Institute of Crop Science, Rural Development Administration, Iksan 570-080, Republic of Korea*

Correspondence should be addressed to Sun Yeou Kim; sunnykim@gachon.ac.kr

Received 4 May 2017; Accepted 12 July 2017; Published 16 August 2017

Academic Editor: Sergio Di Meo

The skin is the outermost protective barrier between the internal and external environments in humans. Chronic exposure to ultraviolet (UV) radiation is a major cause of skin aging. UVB radiation penetrates the skin and induces ROS production that activates three major skin aging cascades: matrix metalloproteinase- (MMP-) 1-mediated aging; MAPK-AP-1/NF-κB-TNF-α/IL-6, iNOS, and COX-2-mediated inflammation-induced aging; and p53-Bax-cleaved caspase-3-cytochrome C-mediated apoptosis-induced aging. These mechanisms are collectively responsible for the wrinkling and photoaging characteristic of UVB-induced skin aging. There is an urgent requirement for a treatment that not only controls these pathways to prevent skin aging but also avoids the adverse effects often encountered when applying bioactive compounds in concentrated doses. In this study, we investigated the efficacy of genetically modified normal edible rice (NR) that produces the antiaging compound resveratrol (R) as a treatment for skin aging. This resveratrol-enriched rice (RR) overcomes the drawbacks of R and enhances its antiaging potential by controlling the abovementioned three major pathways of skin aging. RR does not exhibit the toxicity of R alone and promisingly downregulates the pathways underlying UVB-ROS-induced skin aging. These findings advocate the use of RR as a nutraceutical for antiaging purposes.

1. Introduction

In humans, the skin is the outermost barrier between the internal and external environments [1]. Internal factors, such as genetic changes, can cause intrinsic aging while external factors, such as UVB and environmental toxins, can result in extrinsic aging [2]. Long-term exposure to ultraviolet (UV) radiation is a major cause of skin aging [3]. Histologically, wrinkled skin is characterized by the accumulation of altered elastic fibers and degradation or degeneration of collagen bundles in the dermis [4, 5]. UVB-induced ROS production activates mitogen-activated protein kinase (MAPK) signaling and the transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), which further induce the inflammaging and apoptosis in cells and cause skin aging.

UVB can induce an imbalance in mitochondrial fusion and fission that itself causes mitochondrial dysfunction, oxidative stress, prolonged inflammation, and increased apoptosis, which are the major hallmarks of skin aging [6]. Hence, the mechanisms underlying skin photoaging and wrinkling are closely associated with the inflammaging, apoptosis, and ROS-induced damage that occurs as part of the normal homeostatic processes in the skin [7, 8–11]. Aging-associated inflammation, otherwise known as inflammaging, is a major consequence of immunosenescence. Most aging-associated diseases share inflammation-related characteristics, such as upregulated tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels, which further complicate such conditions [12]. Inflammaging is responsible for the activation of transcription factors such as NF-κB and

sirtuins, which propagate inflammation-induced signals and aggravate skin aging by inducing apoptosis and increased ROS production [13]. The production of ROS and matrix metalloproteinases is common in both intrinsic and extrinsic aging. It has been reported that the accumulation of ROS induces the activation of MAPK pathways. The activation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs induces the activation of the transcription factors AP-1 and NF-κB. Additionally, there is upregulated transcription of inflammatory mediators such as NO, iNOS, COX-2, and proinflammatory cytokines (including TNF-α and IL-6) [14]. Such inflammatory mediators will further induce collagen degradation by promoting apoptosis in dermal fibroblasts, enhancing the expression of the matrix metalloproteinases MMP-1, MMP-3, and MMP-9, and preventing the expression of procollagen [15]. In particular, UVB- or ROS-induced MMP-1, known as interstitial collagenase, initiates the degradation of TGF-β, elastin, and collagen types I, II, and III, especially procollagen type I (PIP-1) [16, 17]. These cascades have also been shown to induce inflammation and apoptosis in cultured cells, further hastening the skin aging process [18]. Apoptosis can result from direct DNA damage (intrinsic), the clustering of death receptors on the cell surface (extrinsic), and the generation of ROS and activation of tumor suppressor gene p53-mediated modulation of Bcl2 family proteins [19].

Natural products are often used in the cosmetics industry because of the consumers' growing preference for environment-friendly items [20]. Resveratrol (R) is a trihydroxy derivative of stilbene (3,5,4′-trihydroxystilbene) that is present in grapes, berries, peanuts, and red wine [21]. It has been widely used in the cosmetics and pharmaceutical industries for its antitumor, anti-inflammatory, antiaging, and antimelanogenic effects [21–23]. R is particularly well suited to addressing inflammatory processes in the skin, because its antioxidant properties work well against the high levels of oxidative stress frequently encountered by skin cells. However, there are several impediments to applying this promising agent as a treatment, such as its poor bioavailability and fast metabolism [24]. Most of the adverse effects associated with R occur at higher doses and relate primarily to nephrotoxicity. Such limitations have attracted attention from researchers seeking to design a derivative, nanoparticle, or genetically engineered vehicle for R, so that its therapeutic effect can be elicited in a safer, more effective, and more promising way. In the case of designing a genetically engineered vehicle for R, it is necessary to select a foodstuff that can be easily consumed or applied. These conditions led us to consider using rice (*Oryza sativa* L. var. *japonica*) as the vehicle for a genetically engineered R product. Rice has been used in folk medicine and the cosmetics industry in Korea, China, and Japan for many years [25]. It has been used for the treatment of various allergic disorders, such as dermatitis and bronchitis, as well as skin aging and other conditions [26–28]. Because of the demonstrated biological efficacy of R and normal rice (NR) in various skin disorders, we hypothesized that resveratrol-enriched rice (RR) may exhibit synergistic or additive effects. The transgenic cereal crop, called RR, was designed to overexpress the stilbene synthase gene isolated from the peanut (*Arachis hypogaea* var. Palkwang) and therefore contains high levels of R. The excellent antiobesity and antimelanogenic effects of RR have previously been demonstrated [29]. We have previously reported that the effects of NR and R combine synergistically in RR, when used to treat obesity in mice fed on a high-fat diet or to control metabolic syndrome and its related disorders [29, 30]. However, the efficacy of RR in a UVB-induced skin aging model has not been reported thus far. Hence, we investigated the antiwrinkle properties of RR relative to those of R or NR. In order to evaluate the antiwrinkle properties of RR, we used a cellular model of photoaging (UVB-induced damage to dermal fibroblasts) and determined the effects of RR, R, and NR on aging-related parameters.

The search for improved cosmetic products has prompted the development of multifunctional cosmetic formulations. Those formulations that harness the synergistic effects of different active substances and maintain integrity against UVB-induced toxicity could be better candidates for the prevention and treatment of UVB-induced skin aging and skin disorders. Three of the major molecular pathways that can be downregulated to reduce skin aging (characterized by skin wrinkles and photoaging) include MMP-mediated aging, inflammaging, and apoptosis-induced aging. This study demonstrated that NR, R, and RR have good potential for protecting the skin against UVB-induced toxicity. The additive effect elicited by RR renders it a potential candidate for the preparation of safe and effective cosmeceuticals in the future. This study found that genetically engineered natural products can not only be better for skin protection but also safer and of greater potential utility as a cosmetic preparation.

2. Methods

2.1. Materials and Chemicals. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). An enzyme-linked immunosorbent assay (ELISA) kit for PIP-1 was obtained from Takara (Procollagen Type I C-Peptide enzyme immunoassay (EIA) Kit; Takara, Shiga, Japan). The ELISA kit for MMP-1, TNF-α, and IL-6 was purchased from R&D Systems (Human Total MMP-1, TNF-α, and IL-6 kit R&D Systems Inc., Minneapolis, MN, USA). Transfer membrane was purchased from the Millipore Corporation (Bedford, MA, USA). Materials for the enhanced chemiluminescence (ECL) detection and lysis buffer for skin cells and tissues were purchased from Intron (Sungnam, Korea). The antibodies against α-tubulin, MMP-1, type I procollagen, iNOS, COX-2, ERK, JNK, p38, Bax, Bcl2, cleaved caspase-3, p53, TGF-β, and elastin were purchased from Santa Cruz (Dallas, TX, USA), Cell Science (Canton, MA, USA), and Cell Signaling (Beverly, MA, USA). Secondary

antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz. Resveratrol was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Rice (NR) (*Oryza sativa* var. japonica) and resveratrol-enriched rice (RR) were supplied by the Rural Development Administration (RDA) of South Korea.

2.2. Extract Preparation. NR and RR obtained from RDA were undergone for extraction in methanol. Firstly, each sample weighed 10 g. 100 mL MeOH was added in both crude drugs and then placed in an ultrasonic bath for 60 min with sonication. After 60 min incubation for extraction, the mixture was filtered and evaporated using rotary evaporator followed by freeze drying for complete evaporation. The obtained yield was dissolved in MeOH in order to make a stock of 10 mg/mL concentration. This stock was diluted and used for the treatment of cells as well as reconstructed skin tissue during experiment.

2.3. Cell Culturing. Normal human dermal fibroblast cells (NHDFs) were obtained by skin biopsy from a healthy young male donor (MCTT Core Inc., Seoul, Korea). The cells were plated in 100 mm tissue culture dishes and cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were cultured in 100 mm culture dishes and seeded in 60 mm culture dishes (1.2×10^5 cells/well) when they reached more than 80% confluence. All experiments were performed using cells between passages 6 and 10.

2.4. UVB Irradiation and Sample Treatments. UVB irradiation and treatment with the samples were performed according to a method previously reported by Hwang et al. [5]. When NHDFs seeded in 60 mm culture dishes covered more than 80% of the dish, the cells were washed twice with phosphate-buffered saline (PBS). The cells were suspended in a small amount of PBS and exposed to UVB (144 mJ/cm^2) using a UVB irradiation machine (Bio-Link BLX-312; Vilber Lourmat GmbH, Marne-la-Vallée, France). After UVB irradiation, the cells were washed with warm PBS three times. The cells were immediately treated with the samples NR, RR, and R (10 and 100 μg/mL) under serum-free medium conditions. Nonirradiated control cells were maintained under the same culture conditions without UVB exposure.

2.5. Measurement of Cell Viability (MTT Assay). The MTT assay measures cell viability by monitoring color change during the reduction of MTT to formazan dye, which is purple in color. MTT assay was performed as described previously [31] with slight modification. NHDF cells were treated with UVB followed by sample treatment and incubated for total 72 h. After 72 h of incubation, the volume of the medium was reduced to 1 mL, and 100 μL of 1 mg/mL MTT was added to each well. Next, the cells were incubated in the presence of 5% CO_2 at 37°C for 2 h. The substrate-containing medium was removed, and 800 μL of DMSO was added to each well to dissolve the formazan crystals. The plates were shaken on an orbital shaker for 10 min at room temperature. The absorbance of 100 μL aliquots of formazan dissolved in DMSO was quantified by measuring the optical density (OD) at 570 nm using an ELISA reader (Molecular Devices E09090; San Francisco, CA, USA).

2.6. Measurement of ROS Production. After 24 h of UVB irradiation (144 mJ/cm^2) and sample treatment, NHDFs were stained with 30 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich) for 30 min at 37°C in a CO_2 incubator. The cells were then analyzed using flow cytometry (FACSCalibur™; Becton-Dickinson, San Jose, CA, USA).

2.7. Measurement of MMP-1, Type I Procollagen, TNF-α, and IL-6. After 72 h of incubation, cell medium was collected from each well. The concentrations of MMP-1, type I procollagen, TNF-α, and IL-6 were analyzed from conditioned medium using commercially available ELISA kits (Human Total MMP-1, TNF-α, and IL-6 kit; R&D Systems Inc.; Procollagen Type I C-Peptide EIA Kit, Takara) in accordance with the manufacturers' instructions. Each sample was analyzed in triplicate.

2.8. Western Blot Analysis. For the Western blot analysis, cells were lysed with lysis buffer (50 mM Tris-Cl, pH 8.0, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and phosphatase inhibitor) and centrifuged at 12,000 ×g for 20 min at 4°C temperature. Cell and skin lysates were then homogenized to yield equivalent amounts of protein based on protein concentration measurements carried out with Bradford reagent (Bio-Rad, Hercules, CA, USA). Homogenized proteins were resolved using 6% or 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were then blocked with 5% nonfat milk in Tris-buffered saline with tween (TBST) (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20) for 1 h at room temperature to block nonspecific interactions. The membranes were incubated in primary antibodies overnight at 4°C, washed with TBST three times, and incubated with secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Protein levels were determined using ECL reagents (Fujifilm, LAS-4000, Tokyo, Japan) and Image Master TM 17 2D Elite software, version 3.1 (Amersham Pharmacia Biotech, NJ, USA).

2.9. Reconstructed Human Skin Tissue Model. Reconstructed human skin (Keraskin™ FT) was purchased from Modern Cell & Tissue Technologies Inc. (Seoul, Korea). The reconstructed skin model is composed of multilayered keratinocytes and fibroblasts. To evaluate the effects of NR, RR, and R on UVB-induced photoaging, the reconstructed skin was topically treated with the samples. The samples were dissolved at a concentration of 1% (*w*/*v*) in 10% propylene glycol with phosphate-buffered saline (PBS) to form the treatment solution, 20 μL of which was applied to the reconstructed skin. After 24 h, the skin tissue was exposed to 100 mJ/cm^2 UVB radiation. The UV source, which generated radiation at a wavelength of 310 nm, was supplied by Sankyo Denki sunlamps (Kanagawa, Japan). After 24 h, the skin

tissue was collected and fixed in 10% formalin and processed for histological analysis. Paraffin sections (4 μm) were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT) and immunohistochemically analyzed. To carry out the immunohistochemical analysis, the sections were incubated in 0.1% protease in PBS for antigen retrieval and were then incubated in 3% H_2O_2 in PBS for 10–15 min. The sections were incubated with 2% normal horse serum in PBS. After 1 h, the sections were incubated with primary antibody procollagen type I (Santa Cruz Biotechnology Inc.) and MMP-1 (Abcam, Cambridge, MA, USA). After washing with PBS, the slides were incubated in Vectastain ABC reagent (Vector Laboratory, Piscataway, NJ, USA) for 1 h. The color was developed with 3,3′-diaminobenzidine (DAB).

2.10. Statistical Analysis. The results were evaluated using the Statistical Analysis System (GraphPad Prism 5, La Jolla, CA, USA). The results are presented as mean ± standard error of the mean (SEM), and all results are the mean of at least three independent experiments. A statistical comparison of different treatment groups was determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. RR Protects against UVB-Induced Toxicity in NHDF Cells. UVB exposure induces cell death in dermal fibroblasts, as well as various inflammatory cascades, resulting in skin aging, skin wrinkling, and skin pigmentation. In order to evaluate the cytotoxicity of the samples, after 72 h of UVB (144 mJ/cm^2) and sample treatment, the viability of NHDF cells was measured using an MTT assay (as described in the Methods). We photographed the cells to show their morphology with or without UVB and sample treatment. In the UVB-exposed cells, NR and RR were not toxic and did not affect the normal morphology of NHDF cells until the concentration reached 100 μg/mL. However, R alone showed significant toxicity, causing cell death at the higher concentration of 100 μg/mL and a completely altered, shrunken cell morphology. Using a lower concentration (10 μg/mL) of R induced the level of cell death, but its significant toxicity was still revealed at this dose by comparing the morphology of the treated cells with that of the cells in the UVB-treated control group (Figure 1).

3.2. RR Downregulated UVB-Induced ROS Production in NHDF Cells. ROS are the major toxic substances generated by UVB exposure in the skin and dermal fibroblast cells. To measure ROS production in NHDFs, we treated cells with UVB and the samples for 24 h. The change in intracellular ROS compared with the nonirradiated controls was determined using 2′,7′-dichlorofluorescein diacetate (DCF-DA), which is oxidized by ROS in cells to DCF. The cells were stained with 30 μM of DCF-DA and incubated for 30 min, after which the fluorescence level was measured. The UVB-induced ROS production in dermal fibroblast cells was significantly reduced following treatment with NR, RR, and R. RR demonstrated a greater reduction in ROS production at concentrations of 10 and 100 μg/mL than NR or R alone. While all of the samples were capable of reducing UVB-induced ROS production, RR was found to be the most effective one (Figure 2).

3.3. NR, RR, and R Control the Level of MMP-1, TGF-β, and PIP-1 in NHDF Cells. To evaluate the effects of the samples on MMP-1 and PIP production in UVB-exposed NHDF cells, the levels of protein expression and MMP-1 and PIP-1 secretion were measured by using Western blotting and an ELISA, respectively (Figures 3(a), 3(b), 3(c), 3(d), and 3(e)). According to both the protein expression measurements and secreted protein assay, NR, RR, and R all reduced UVB-induced MMP-1 production and increased PIP production. The RR-mediated downregulation of MMP-1 and upregulation of PIP appear to have been caused by the additive effect of NR and R. This is because, despite R showing the most potent activity in reducing MMP-1 and increasing PIP levels (even at only 10 μg/mL), the activity of RR seems to be better than that of NR and R alone in terms of the levels of secreted MMP-1 and PIP when tested using the ELISA kit. NR, RR, and R play significant roles in reducing MMP-1, but RR exhibits significantly greater (and concentration-dependent) activity against UVB-induced MMP-1 production. Only RR demonstrated the ability to increase PIP production almost two- and threefold at concentrations of 10 and 100 μg/mL, respectively, in comparison with the UVB-treated control group, whereas NR and R were unable to affect a significant increase. This result stimulated our interest in elucidating the mechanism by which RR increases PIP levels to this extent. We therefore evaluated the protein expression of TGF-β, as the TGF-β/Smad pathway is a major pathway controlling PIP production. We found that RR significantly induced TGF-β protein expression in UVB-irradiated NHDF cells and thereby stimulated PIP production. This has the concomitant effect of preventing UVB-induced skin wrinkle formation, as upregulating TGF-β and PIP levels also results in increased elastin production. Similar result was obtained here in the RR and UVB-treated group. RR increased elastin production to a greater extent than NR and R alone (Figures 3(f), 3(g), and 3(h)). This result suggests that RR can protect NHDF cells against UVB-ROS-MMP-1-induced skin aging, particularly skin wrinkle formation.

3.4. RR Protects Human Reconstructed Skin Tissue against UVB-Induced Toxicity. To investigate the histological effects of RR on photoaging, UVB-exposed reconstructed human skin tissue was stained with H&E and MT. According to the H&E staining results, NR, RR, and R had no toxicity, although NR showed a mildly toxic effect in the epidermal layer. Staining with MT revealed the disruption and decomposition of collagen fibers in skin tissues exposed to UVB and that RR treatment in UVB-exposed skin tissue increased the abundance and density of collagen fibers (Figure 4). This indicates that RR protects skin tissue against UVB-induced collagen degradation in reconstructed human skin tissue. Furthermore, to investigate the effects of NR, RR, and R on

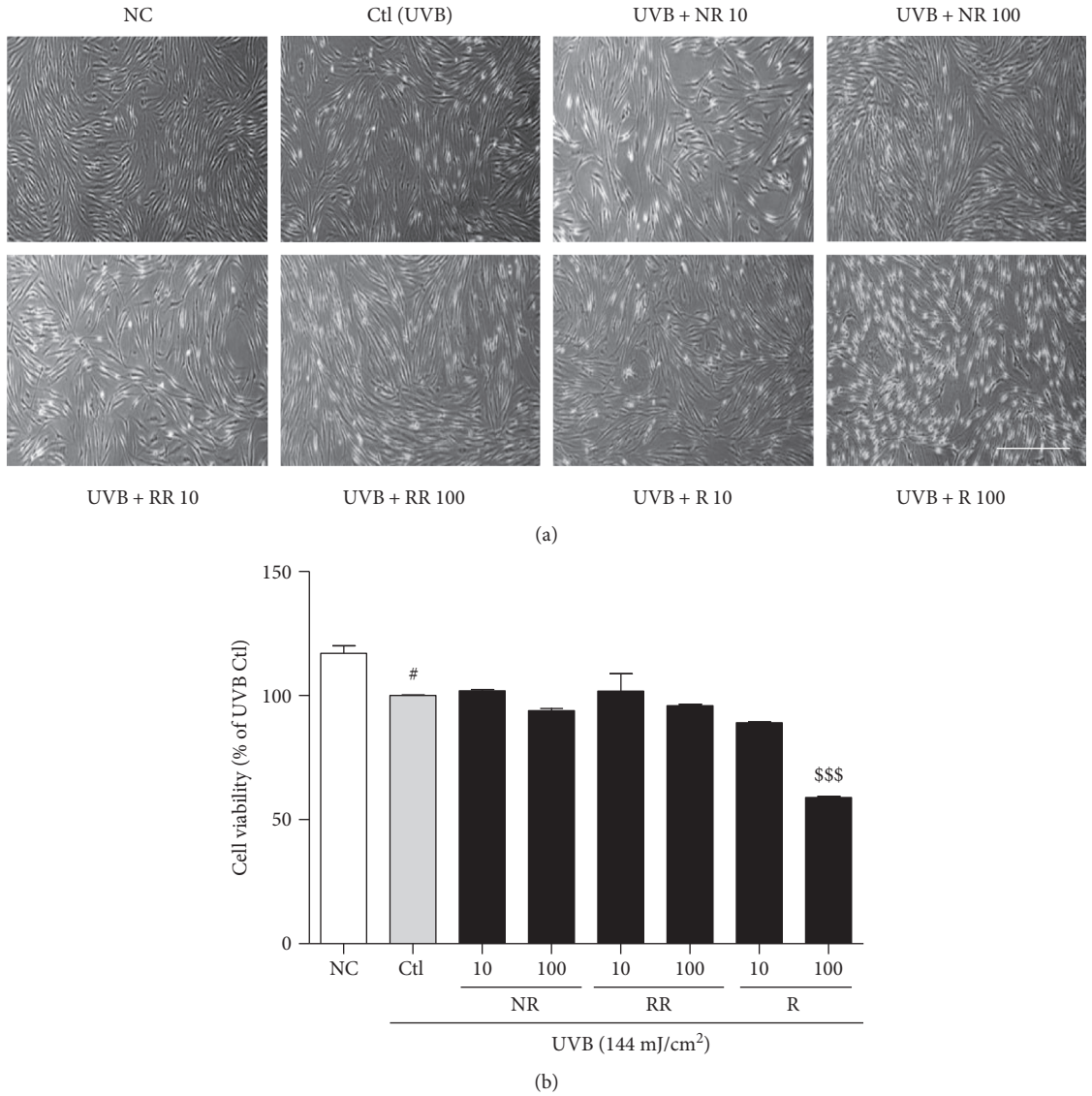


Figure 1: Normal human dermal fibroblast (NHDF). (a) Cell morphology and (b) cell viability after 72 h of treatment with or without 144 mJ/cm^2 ultraviolet B (UVB) and 10 or 100 μg/mL of the following samples: normal rice (NR), resveratrol-enriched rice (RR), and resveratrol (R). All data are presented as the mean ± SEM of three independent experiments. $^{\#}p < 0.05$ versus the normal control. $^{\$\$\$}p < 0.001$ indicates the significant toxicity versus a UVB-irradiated control. NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. NR and RR were treated in μg/mL, and R was treated in μM.

type I procollagen and MMP-1 expression in reconstructed human skin tissue, we carried out immunohistochemical analysis on the sections. After UVB irradiation, the expression of MMP-1 increased and that of PIP-1 decreased. This effect was reversed with NR, RR, and R treatments especially for 10 μg/mL for all samples and 10 μg/mL for NR and RR samples, without any toxicity. Among these treatments, RR demonstrated the greatest efficacy (Figure 4). Hence, RR increased the expression of type I procollagen and decreased that of MMP-1 in reconstructed human skin, with the effect of protecting against UVB-induced skin aging or wrinkle formation.

3.5. RR Regulates the MAPK and AP-1-Mediated Signaling and Transcription in UVB-Irradiated NHDF Cells. Inflammation-mediated skin aging is a major factor in UVB-induced skin aging and therefore a good target for controlling photoaging in dermal fibroblasts and skin more generally. Inflammation-mediated skin aging is initiated by the huge production of ROS that arises from UVB-induced oxidative stress in the skin. The excessive ROS production brought about by UVB activates MAPK signaling proteins to induce the AP-1- and NF-κB-mediated transcription and translation of inflammatory proteins. To reproduce these conditions, we treated NHDF cells with UVB and the samples for 3 h, and the MAPK signaling proteins were evaluated. RR exhibited additive effects in this case, with NR, RR, and R downregulating the protein expression of pERK and pJNK but upregulating p38 expression (Figures 5(a), 5(b), 5(c), and 5(d)). Additionally, NR, RR,

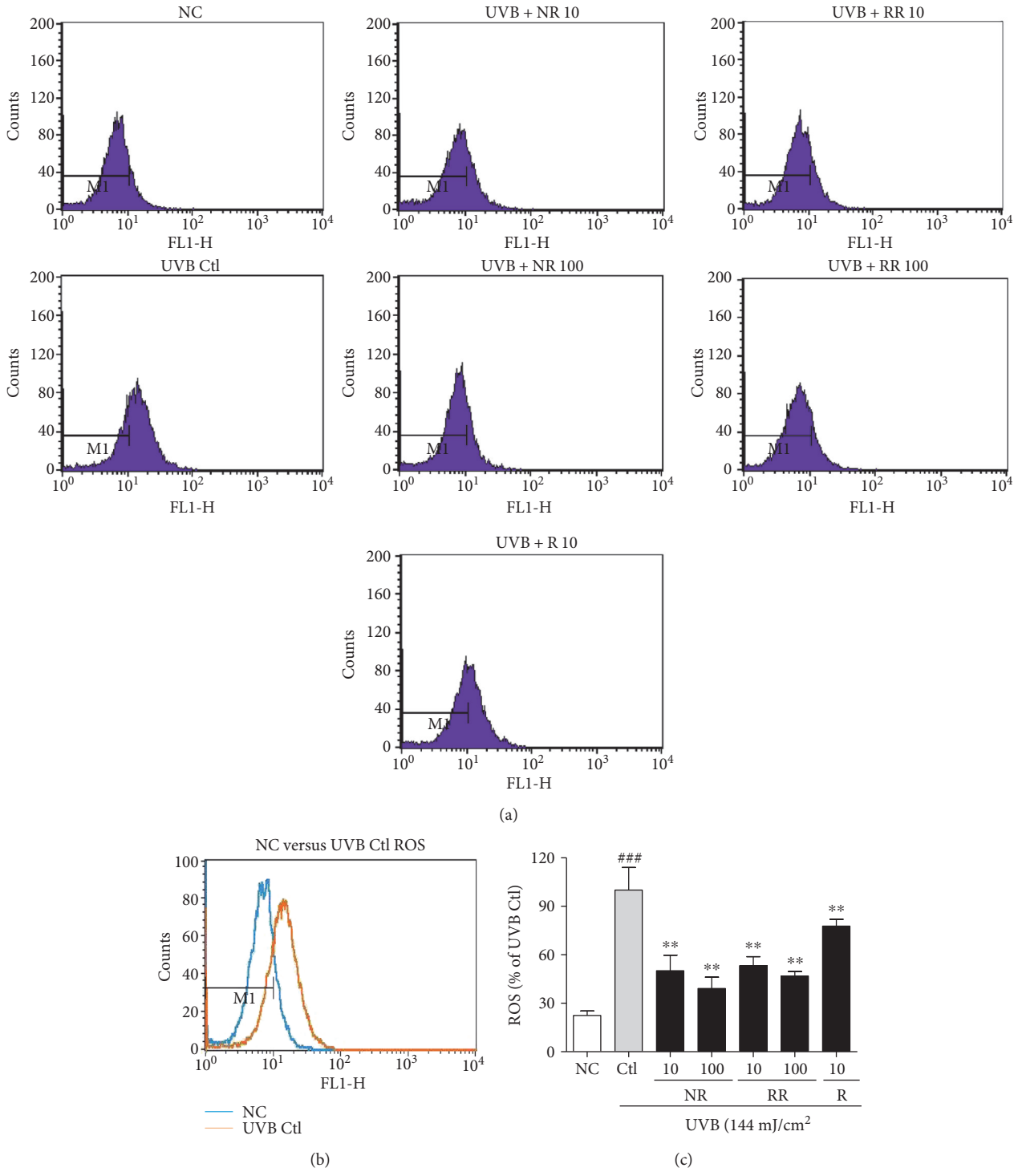


Figure 2: The levels of ROS in NHDFs treated as indicated for 24 h were measured using flow cytometry with DCFH-DA dye. The number of cells is plotted against the dichlorofluorescein fluorescence detected by the FL-1 channel (a). The relative ROS production of the cells is shown in each histogram (b). Values are mean ± SEM. The labels # and ∗ indicate significant differences ($p < 0.05$) when compared with the normal control and UV (+) control, respectively. $^{###}p < 0.001$ versus the normal control, $^{**}p < 0.01$ versus the UVB-irradiated control. NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. NR and RR were treated in μg/mL, and R was treated in μM.

and R significantly downregulated p-c-Fos and p-c-Jun, which indicates that they inhibited the AP-1-mediated transcription of the inflammatory proteins responsible for inflammaging (Figures 5(e), 5(f), and 5(g)).

3.6. RR Inhibits the Inflammatory Cascades in UVB-Treated Dermal Fibroblast Cells Preventing Inflammaging. AP-1, activated by MAPK signaling, induces the transcription and translation of inflammatory proteins, which further

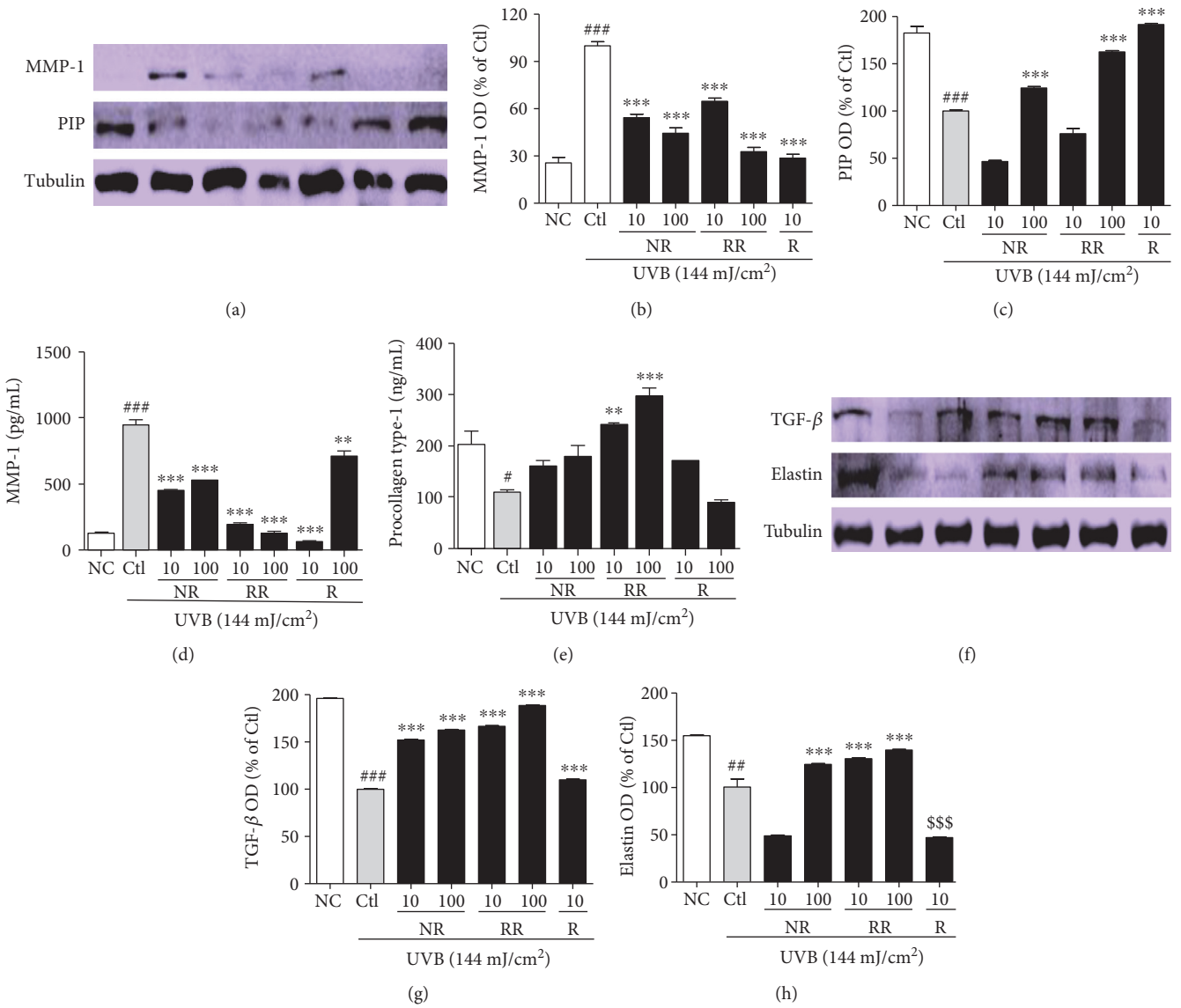


Figure 3: UVB-MMP1-mediated protein expression and the secretion of MMP-1 and PIP were measured using Western blot analysis and an ELISA kit, respectively. NHDF cells were incubated for 72 h with or without UVB exposure and treated with or without NR, RR, and R. (a) MMP-1 and PIP expression, accompanied by the corresponding (b, c) densitometric analysis results (d, e) and the quantities MMP-1 and PIP secreted into the treated medium supernatant. (f) TGF-β and elastin expression, accompanied by the corresponding (g, h) densitometric analysis results, taking the UVB-treated control as 100%. All data are presented as the mean ± SEM of three independent experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the NC and $^{**}p < 0.01$, $^{***}p < 0.001$ versus the UVB control. NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. $^{\$\$\$}p < 0.001$ versus UVB control represents the inhibition of elastin expression by resveratrol treatment. NR and RR were treated in μg/mL, and R was treated in μM.

aggravates inflammation and makes the skin and cells more prone to aging, as well as cancer. To evaluate the production of proinflammatory cytokines, we performed an ELISA using the cell supernatants, following treatment with or without UVB and the samples, and the iNOS and COX-2 protein expression in those cells was evaluated. NR, RR, and R affected the concentration-dependent downregulation of TNF-α and IL-6 production in the conditioned medium with or without UVB treatment (Figures 6(a), 6(b), 6(c), and 6(d)). NR, RR, and R appear equally capable of reducing TNF-α secretion in the cells without UVB treatment. However, RR is more potent in this regard than NR and R alone when UVB treatment is applied. R elicited a marked reduction in IL-6 in both cases, but RR seems to be equally capable of reducing IL-6 levels in the cells treated with UVB. As R is a pure compound and NR and RR are merely extracts, we can clearly infer that the similarity in the efficacy of RR and R indicates the higher potency of RR in protecting cells against UVB-induced skin aging. Additionally, NR, RR, and R significantly downregulated the expression of iNOS and COX-2 in the dermal fibroblast cells (Figures 6(e), 6(f), and 6(g)). When applied at the higher concentration of

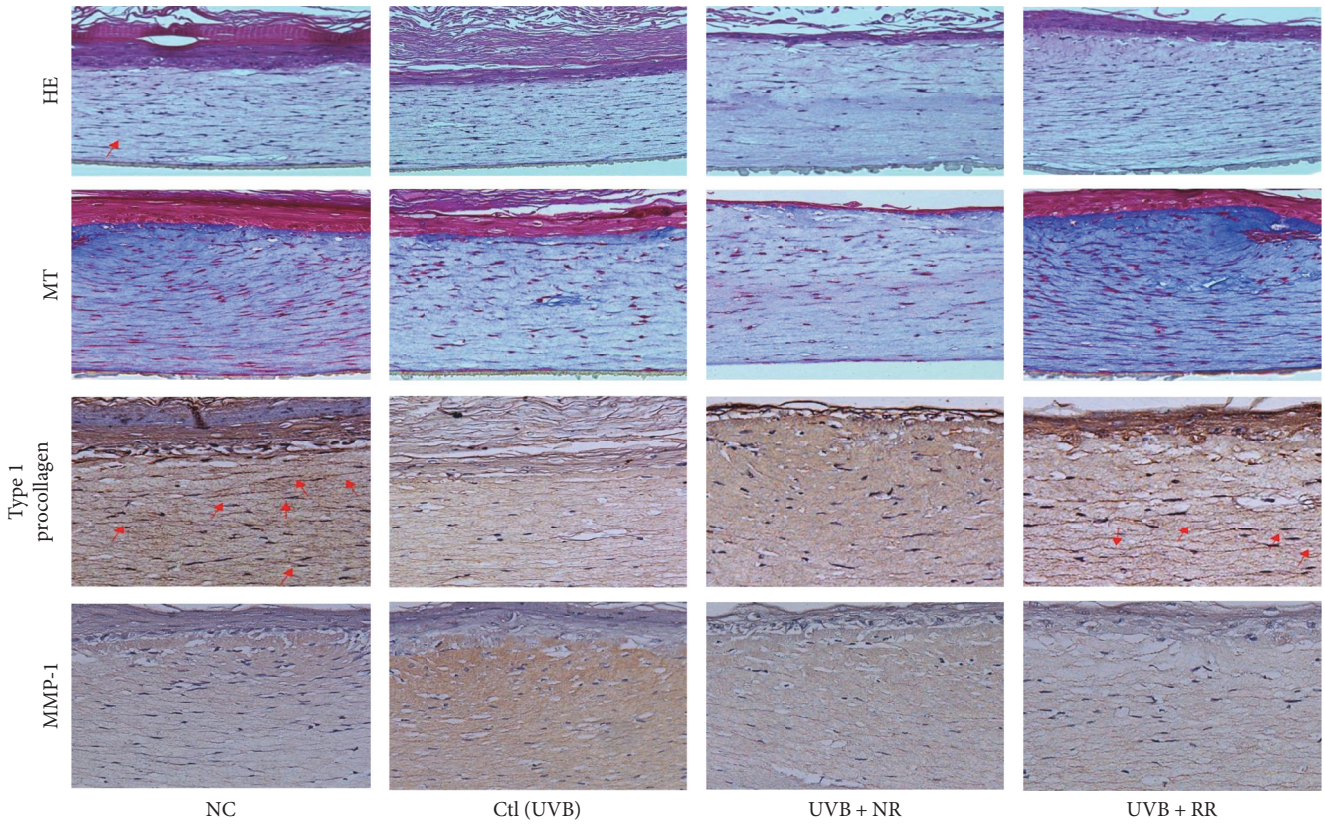


Figure 4: Photomicrographs of Masson's trichrome-stained sections and PIP-1 and MMP-1 production in reconstructed skin after treatment with UVB and samples (NR, RR, and R) at a concentration of 1% (*w*/*v*). NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. NR and RR were treated in μg/mL, and R was treated in μM.

100 μg/mL, the samples reduced the iNOS and COX-2 expression levels to below those observed in the normal control. The samples also caused the significant downregulation of iNOS and COX-2 when applied at 10 μg/mL; RR exhibited greater potency than NR, but 10 μg/mL of R was more potent than 100 μg/mL of NR or RR.

3.7. RR Ameliorates UVB-Induced Apoptosis in Dermal Fibroblast Cells. Apoptosis-induced skin aging can be induced by various aging processes, such as the excessive production of proinflammatory cytokines and inflammatory mediators, or the UVB-induced production of ROS. We observed that UVB-mediated ROS induced the expression of p53, Bax, Cytochrome C, and cleaved caspase-3 while reducing the expression of the antiapoptotic protein Bcl2 in dermal fibroblast. NR, RR, and R significantly reduced the production of proapoptotic proteins, such as p53, Bax, cleaved caspase-3, and cytochrome C, in UVB-treated dermal fibroblasts (Figure 7). RR and R were equally effective in downregulating the expression of p53, but RR reduced the expression of Bax, cleaved caspase-3, and cytochrome C more effectively. On the other hand, RR and R did not alter the expression of antiapoptotic protein Bcl2. In summary, RR can reduce the apoptosis arising from UVB-induced ROS production and inflammation but does not play a role in increasing antiapoptotic protein expression (Figure 7). Hence, RR promisingly inhibit the UVB-ROS mediated skin aging via various pathways such as MMP-1-mediated collagen degradation, inflammaging, and apoptosis-mediated aging as shown in (Figure 8).

4. Discussion

The changes in physical appearance brought on by aging, such as the development of wrinkles, can detrimentally affect the quality of life by impairing personal interactions, occupational functioning, and self-esteem. The prevention of skin aging and improvement of fine and coarse wrinkling in adults with minimal adverse effects are the main goals of skin care treatments [32, 33]. In this study, we examined the anti-aging potential of normal rice (NR), resveratrol-enriched rice (RR), and resveratrol (R). Genetically engineered RR provided a similar biological effect to R, while avoiding its cellular toxicity even at 100 μg/mL. RR was shown to control UVB-induced aging through downstream mechanisms in all the major pathways involved in skin aging and wrinkle formation. NR, RR, and R were found to control UVB-induced skin aging via downregulating oxidative stress-mediated aging, inflammation-induced skin aging, and apoptosis-mediated skin aging and wrinkle formation.

Photoaging is the hallmark of prolonged UV exposure. Exposing the skin to UVB activates oxidative stress in the normal rheostat of the dermis and epidermis or the respective cells that induce cell death, as well as altering cellular

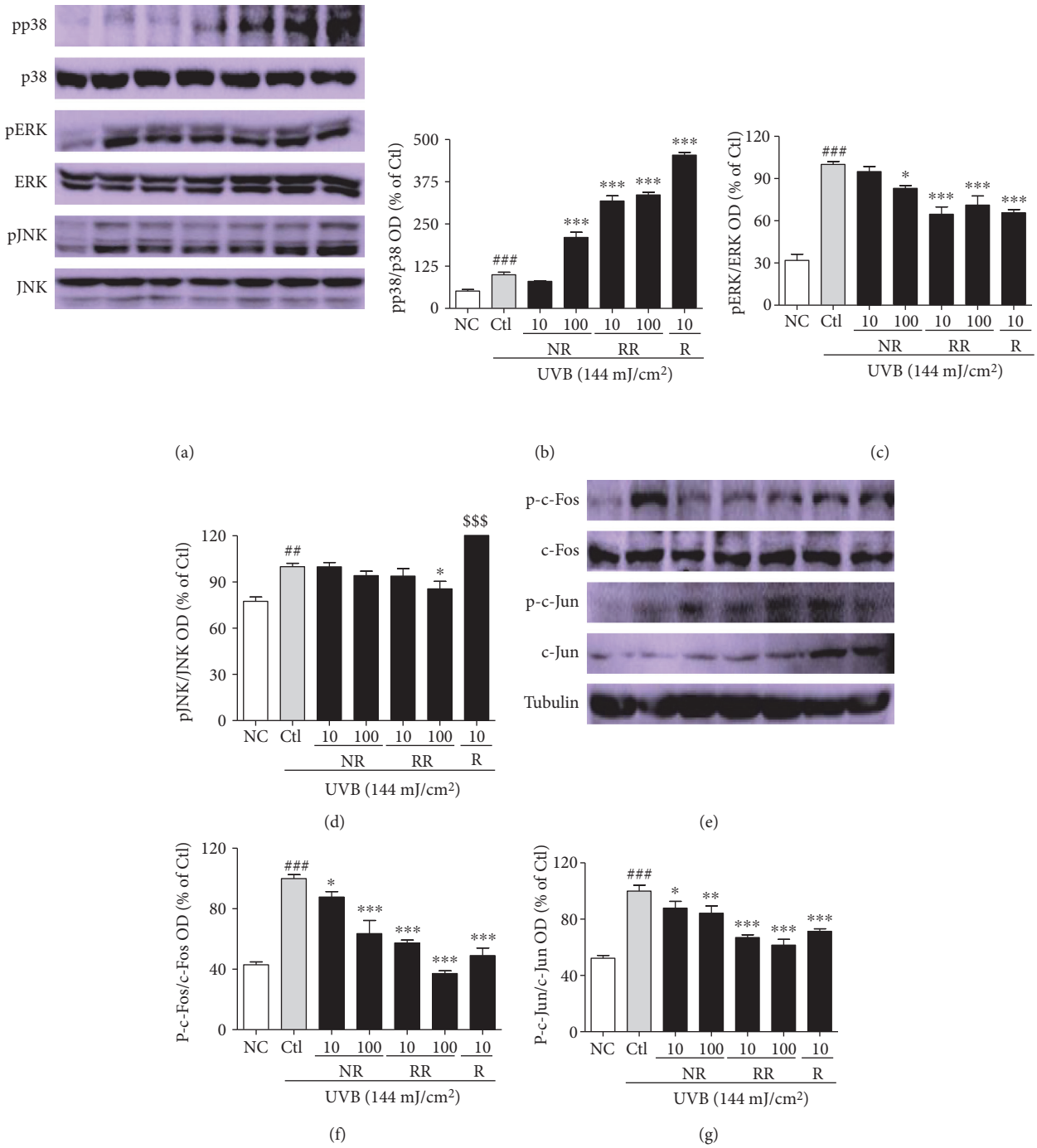


Figure 5: Protein expression levels of MAPKs and AP-1. (a) MAPK signal intensities from multiple experiments. (b, c, d) Bar graphs represent quantitative densitometric results of upper bands. (e) AP-1 protein intensities from multiple experiments. (f, g) Bar graphs represent quantitative densitometric results of upper bands. All data are presented as the mean ± SEM of three independent experiments. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the NC and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the UVB-irradiated control. NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. $^{\$\$\$}p < 0.001$ versus UVB control represents the resveratrol-induced pJNK expression. NR and RR were treated in μg/mL, and R was treated in μM.

morphology [34–36]. In this study, we found that NR and RR do not have cellular toxicity, while 100 μg/mL of R was toxic to NHDF cells. Although 10 μg/mL of R was found not to be toxic in terms of cell viability, measured using an MTT assay, its toxicity was still evident through its adverse effects on cell morphology. These results demonstrate that, although it has promising biological activity, R has various toxic effects on the cells *in vitro*, which manifest as changes to NHDF cell morphology at the lower concentration (leading to shrunken cells, but not cell death) and cell death at the higher concentration. Furthermore, we found that NR, RR, and R promisingly reduced the UVB-induced production of reactive

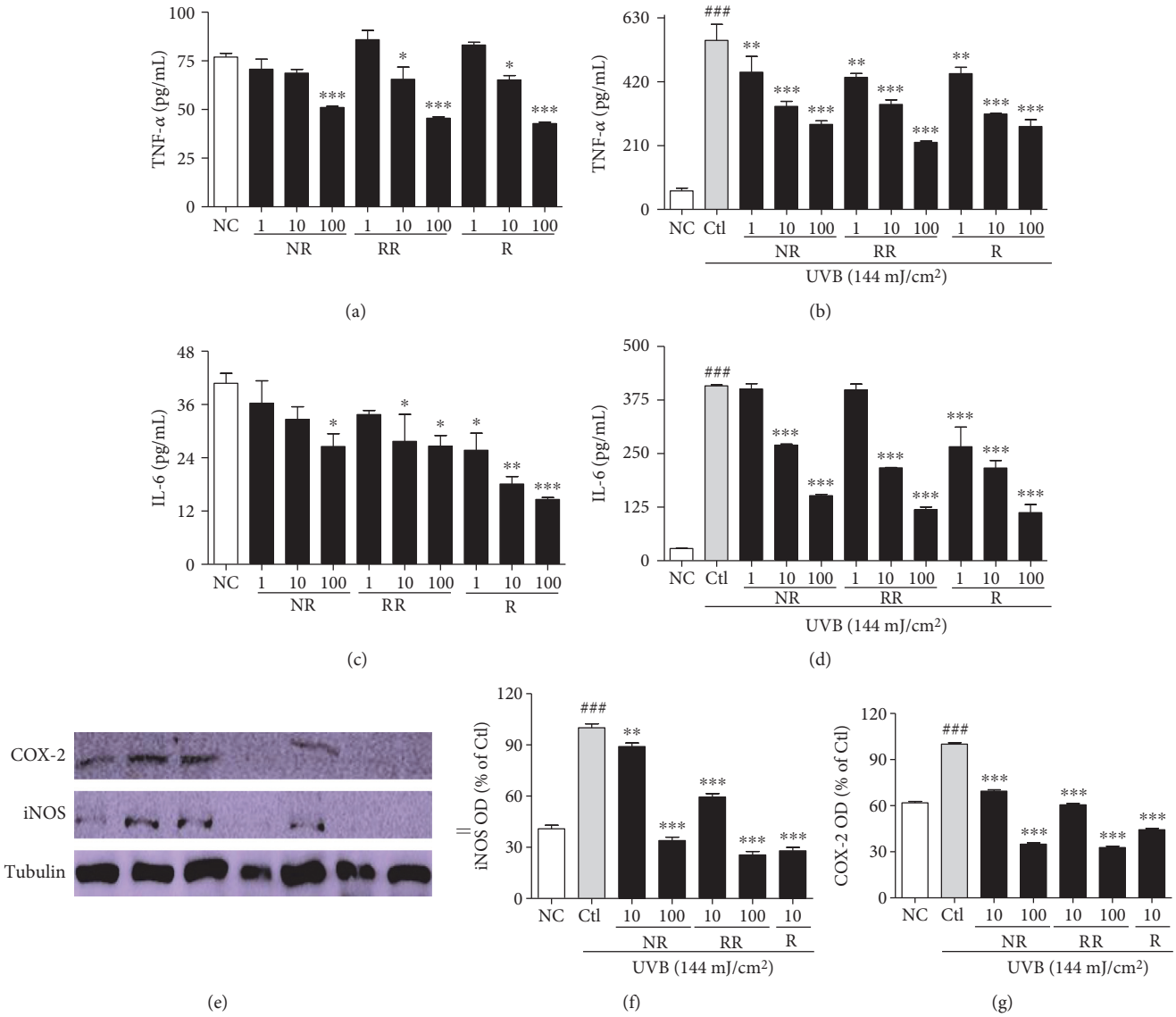


Figure 6: Proinflammatory cytokines (TNF-α and IL-6) secretion and inflammatory markers (iNOS and COX-2) expression. NHDF cells were treated with or without UVB (144 mJ/cm^2) and with NR, RR, and R for 72 h. Proinflammatory cytokines were measured in cell supernatant using an ELISA kit, and protein expression of iNOS and COX-2 was measured using Western blot analysis. (a, b) TNF-α secretion in NHDF cells without UVB-conditioned medium and with UVB-conditioned medium. (c, d) IL-6 secretion in NHDF cells without UVB-conditioned medium and with UVB-conditioned medium. (e) iNOS and COX-2 protein expression from multiple experiments. (f, g) Bar graphs represent quantitative densitometric results of upper bands. All data are presented as the mean ± SEM of three independent experiments. $^{###}p < 0.001$ versus the NC and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the UVB-irradiated control. NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. NR and RR were treated in μg/mL, and R was treated in μM.

oxygen species (ROS) in normal human dermal fibroblasts. The UVB-induced production of ROS and free radicals upregulates the production of MMPs, particularly MMP-1, which is responsible for the degradation of the collagen network in tissue. This will result in the reduced secretion of TGF-β and subsequently procollagen, especially procollagen type II (PIP-II) [37, 38]. Furthermore, the downregulation of elastin and TGF-β in UVB-irradiated dermal fibroblast cells has been shown to further worsen the complications of the oxidative stress-induced aging process in the skin [39]. As elastin also plays an important role in the ECM of the dermis, its degradation leads to line and wrinkle formation in the skin. Therefore, agents that inhibit elastase activity are ideal candidates for the treatment or prevention of skin photoaging [40]. All of the samples described herein downregulated MMP-1 significantly, but the activity of RR appeared to be more potent than that of NR or R alone. Interestingly, a higher concentration of R (100 μg/mL) resulted in increased MMP-1 production, whereas a lower concentration of R (10 μg/mL) downregulated MMP-1 production more effectively than 100 μg/mL of RR. This indicates that R is better able to reduce the level of MMP-1 than RR, even at a lower

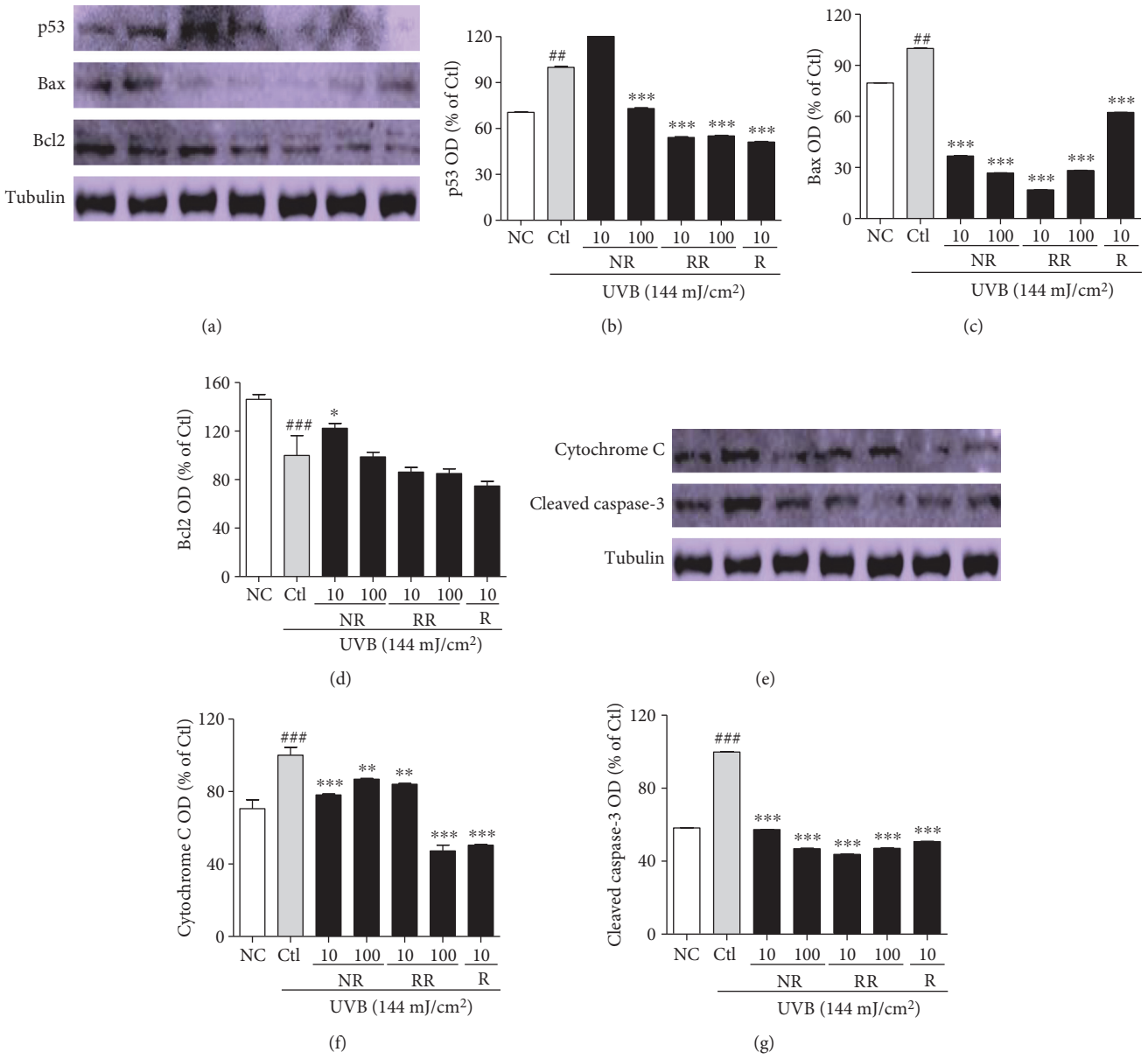


Figure 7: Apoptosis-induced skin aging and related protein expression in NHDF cells. NHDF cells were treated with or without UVB (144 mJ/cm^2) and with NR, RR, and R for 72 h. Protein expression was measured using Western blot analysis. (a) p53, Bax, and Bcl2 protein expression from multiple experiments. (b, c, d) Bar graphs represent quantitative densitometric results of upper bands. (e) Cytosolic cytochrome C and cleaved caspase-3 protein expression from multiple experiments. (f, g) Bar graphs represent quantitative densitometric results of upper bands. Tubulin was used as a loading control. All data are presented as the mean ± SEM of three independent experiments. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus the NC and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the UVB-irradiated control. NC is normal control, Ctl is UVB control, NR is normal rice, RR is resveratrol-enriched rice, and R is resveratrol. NR and RR were treated in μg/mL, and R was treated in μM.

concentration. Additionally, a reduction in the protein expression for MMP-1 and increase in that for PIP were observed in the cells, as well as the treated cell supernatants. The effect of RR was found to be more promising than that of NR or R alone. UVB induced MMP-1 production and reduced the level of collagen in the dermis of the skin, but treatment with RR significantly decreased this toxicity and maintained homeostasis. Hence, the additive effect of NR and R, in the form of RR, was successfully demonstrated in NHDFs and reconstructed tissue. Only RR demonstrated an ability to increase PIP production almost two- and three-fold at doses of 10 and 100 μg/mL, respectively, in comparison with the UVB-treated control group, whereas NR and R were unable to affect a significant increase. This result led us to investigate the mechanism by which RR so greatly increases PIP levels. We therefore measured the protein expression of TGF-β, as the TGF-β/Smad pathway is a major pathway controlling PIP production. We found that RR significantly induced TGF-β protein expression in UVB-irradiated NHDF cells and thereby stimulated PIP

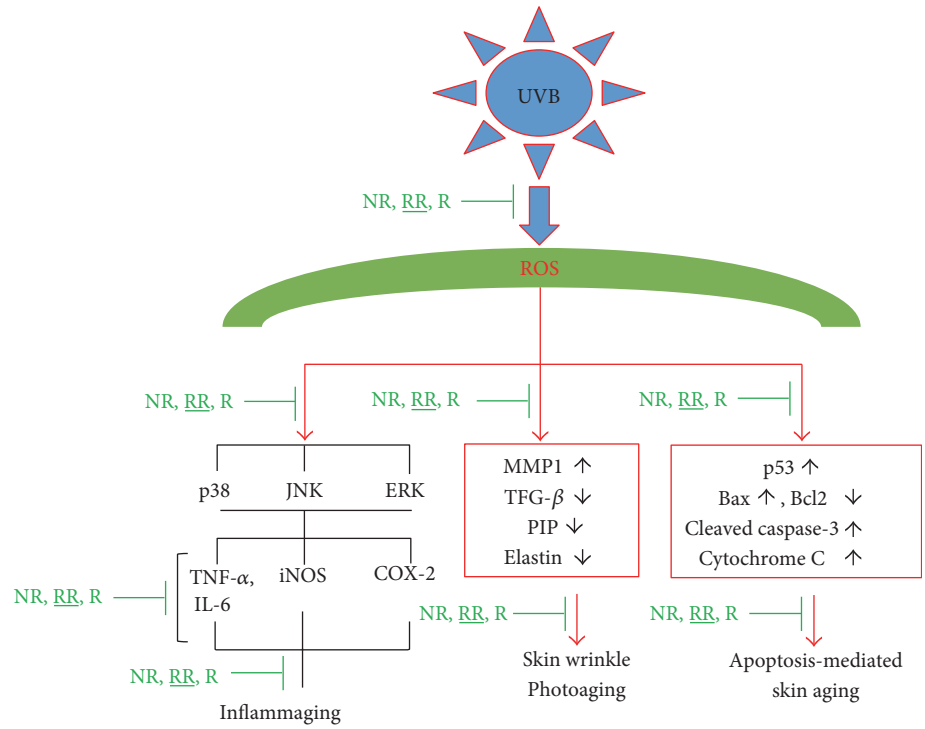


Figure 8: Scheme of the UVB-ROS-mediated skin aging and the protective role of resveratrol rice against its toxicity to prevent skin aging.

production. This can simultaneously prevent UVB-induced skin wrinkle formation, as upregulating TGF-β and PIP levels also results in increased elastin production. RR was shown to increase elastin production to a greater extent than NR and R alone. To confirm this *in vitro* finding, we conducted the same experiment in reconstructed tissue and obtained very similar results. Even in the reconstructed tissue, NR, RR, and R had no toxicity, although NR showed a mildly toxic effect in the epidermal layer. The disruption and decomposition of collagen fibers were observed in skin tissues exposed to UVB using Masson's trichrome (MT) staining, hematoxylin and eosin (H&E) staining, and staining for the determination of PIP and MMP-1. RR treatment increased the abundance and density of collagen fibers in UVB-exposed skin tissue. NR and RR treatment reduced the level of MMP-1 and increased that of type I procollagen in cells exposed to UVB. Of all the tested samples, RR most effectively increased the expression of type I procollagen and decreased that of MMP-1 in reconstructed human skin, thereby protecting against UVB-induced skin aging or wrinkle formation. These data collectively indicate that RR downregulates UVB-induced oxidative stress more potently than NR or R alone and therefore reduces its contribution to skin aging.

UVB irradiation induces ROS production. ROS-mediated oxidative stress activates MAPK signaling by increasing the phosphorylation of p38, JNK, and ERK (pp38, pJNK, and pERK) to induce inflammaging. Inflammaging is closely associated with many aging-associated diseases, such as Alzheimer's disease, as well as atherosclerosis, heart disease, type II diabetes, and cancer. One factor that exacerbates UVB-induced ROS-mediated inflammaging is immunosenescence [41]. Inflammaging is initiated after the activation of MAPK signaling, AP-1 (c-Fos and c-Jun) activation and the increased transcription of inflammatory mediators. Through the MAPK signaling pathways, AP-1 controls the expression of MMPs, especially MMP-1, MMP-2, and MMP-9, in inflammation-induced skin aging. Heterodimer complexes made between c-Jun and c-Fos, with various growth factors, cytokines, and UV exposure, can cause aggressive inflammation and skin aging [42]. In this study, we found that irradiating NHDF cells with UVB activates MAPK signaling, which activates the AP-1- and NF-κB-mediated transcription of MMPs, proinflammatory cytokines, inflammatory mediators, and so forth, while simultaneously downregulating PIP, TGF-β, and elastin production [43]. Inflammatory mediators, such as iNOS, COX-2, and cytokines, as well as IL-6, IL-1β, and TNF-α produced by innate immune cells, will cause chronic inflammation and thus initiate inflammaging [44]. In this study, we demonstrated that treatment with NR, RR, and R can significantly modulate MAPK and AP-1 signaling, by inhibiting NF-κB-mediated transcription. This was evidenced by the activation of PIP and the inhibition of TNF-α, IL-6, and MMP-1 in the treated dermal fibroblast cells. RR most effectively reduced TNF-α and IL-6 production in UVB-irradiated NHDF cells, but its ability to reduce iNOS and COX-2 levels, while still superior to that of NR, was inferior to that of R alone. This result further demonstrates the anti-inflammatory properties of these treatments and their great potential for protecting against UVB-induced inflammaging.

Oxidative stress and inflammaging together induce apoptosis, which is another key factor in skin aging, photoaging, wrinkling, and related disorders [45]. UVB-induced ROS cause further oxidative stress in the cellular environment, with the MAPK- and NF-κB-mediated transcription of proinflammatory cytokines, particularly TNF-α, being the major cause of cell apoptosis and aging by apoptosis in the skin [46]. UVB, ROS, proinflammatory cytokines, and other toxins produced by UVB will induce the expression of apoptotic protein p53. This subsequently activates the expression of Bax, Bad, PUMA, and cleaved caspase-3, while simultaneously downregulating the expression of antiapoptotic proteins such as Bcl2 [47]. Activated cleaved caspase-3 translocates mitochondrial cytochrome C to the cytosol, which further induces apoptosis and contributes to skin aging by further activating factors that aggravate skin aging and wrinkle formation [19]. In this study, we confirmed that NR, RR, and R promisingly downregulate the levels of p53, Bax, cleaved caspase-3, and cytochrome C and that RR and R did not simultaneously alter the expression of the antiapoptotic protein Bcl2. RR downregulated the expression of Bax and cleaved caspase-3 more effectively than the other treatments and was as potent as R for the inhibition of p53 in the UVB-treated fibroblast cells. The RR-mediated reduction in p53 lowered the transcription of Bax and cleaved caspase-3. Bax and cleaved caspase-3 were consequently unable to translocate mitochondrial cytochrome C to the cytosol, thereby protecting fibroblasts from mitochondrial apoptosis and apoptosis-induced skin aging. In this way, and considering that RR (an extract) is generally at least as effective as R (a pure compound) or NR alone, we conclude that the pure compounds or standard compound in RR might have better synergistic biological activity than R alone for every considered aging pathway.

In conclusion, NR, R, and particularly RR have been shown to control MMP-1-mediated UVB-induced skin aging, apoptosis-induced skin aging, and inflammation-mediated complications called inflammaging in dermal fibroblasts. A schematic explanation for the UVB-ROS-mediated aging and the role of NR, RR, and R has been shown in Figure 8. In this way, our study has demonstrated the potential of RR as an antiaging product for the prevention of UVB-induced complications *in vitro* and ex vivo.

Conflicts of Interest

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

Acknowledgments

This work was supported by a grant from the Next-Generation Bio-Green 21 Program (no. PJ01118803), Rural Development Administration, Republic of Korea and National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) (no. NRF-2017R1A4A1015594) and also supported by the High Value-added Food Technology Development Program, the Ministry of Agriculture, Food and Rural Affairs (114006041HD020).

References

[1] C. J. Pendegrass, A. E. Goodship, J. S. Price, and G. W. Blunn, "Nature's answer to breaching the skin barrier: an innovative development for amputees," *Journal of Anatomy*, vol. 209, no. 1, pp. 59–67, 2006.

[2] M. Wlaschek, I. Tantcheva-Poor, L. Naderi et al., "Solar UV irradiation and dermal photoaging," *Journal of Photochemistry and Photobiology B: Biology*, vol. 63, no. 1–3, pp. 41–51, 2001.

[3] L. Rittie and G. J. Fisher, "Natural and sun-induced aging of human skin," *Cold Spring Harbor Perspectives in Medicine*, vol. 5, no. 1, article a015370, 2015.

[4] M. J. Sherratt, "Tissue elasticity and the ageing elastic fibre," *Age (Dordrecht, Netherlands)*, vol. 31, no. 4, pp. 305–325, 2009.

[5] K. A. Hwang, B. R. Yi, and K. C. Choi, "Molecular mechanisms and in vivo mouse models of skin aging associated with dermal matrix alterations," *Laboratory Animal Research*, vol. 27, no. 1, pp. 1–8, 2011.

[6] M. R. Rippo, F. Olivieri, V. Monsurro, F. Prattichizzo, M. C. Albertini, and A. D. Procopio, "MitomiRs in human inflamm-aging: a hypothesis involving miR-181a, miR-34a and miR-146a," *Experimental Gerontology*, vol. 56, pp. 154–163, 2014.

[7] T. G. Lim, J. E. Kim, S. Y. Lee et al., "The daidzein metabolite, 6,7,4′-trihydroxyisoflavone, is a novel inhibitor of PKC alpha in suppressing solar UV-induced matrix metalloproteinase 1," *International Journal of Molecular Sciences*, vol. 15, no. 11, pp. 21419–21432, 2014.

[8] E. Hwang, S. Y. Park, H. J. Lee et al., "Vigna angularis water extracts protect against ultraviolet b-exposed skin aging in vitro and in vivo," *Journal of Medicinal Food*, vol. 17, no. 12, pp. 1339–1349, 2014.

[9] Q. Yu, H. M. Zou, S. Wang, Y. M. Xu, J. M. Li, and N. Zhang, "Regulative effect of bakuchiol on ESF-1 cells anti-aging gene," *Zhong Yao Cai*, vol. 37, no. 4, pp. 632–635, 2014.

[10] E. Hwang, T. H. Lee, S. Y. Park, T. H. Yi, and S. Y. Kim, "Enzyme-modified Panax ginseng inhibits UVB-induced skin aging through the regulation of procollagen type I and MMP-1 expression," *Food & Function*, vol. 5, no. 2, pp. 265–274, 2014.

[11] J. E. Oh, M. S. Kim, W. K. Jeon et al., "A nuclear factor kappa B-derived inhibitor tripeptide inhibits UVB-induced photoaging process," *Journal of Dermatological Science*, vol. 76, no. 3, pp. 196–205, 2014.

[12] S. Salvioli, D. Monti, C. Lanzarini et al., "Immune system, cell senescence, aging and longevity—inflamm-aging reappraised," *Current Pharmaceutical Design*, vol. 19, no. 9, pp. 1675–1679, 2013.

[13] A. P. Navarrete-Reyes and M. Montana-Alvarez, "Inflammaging. Aging inflammatory origin," *Revista de Investigación Clínica*, vol. 61, no. 4, pp. 327–336, 2009.

[14] S. J. Cooper and G. T. Bowden, "Ultraviolet B regulation of transcription factor families: roles of nuclear factor-kappa B (NF-kappaB) and activator protein-1 (AP-1) in UVB-induced skin carcinogenesis," *Current Cancer Drug Targets*, vol. 7, no. 4, pp. 325–334, 2007.

[15] F. Debacq-Chainiaux, C. Leduc, A. Verbeke, and O. Toussaint, "UV, stress and aging," *Dermato-endocrinology*, vol. 4, no. 3, pp. 236–240, 2012.

[16] E. Hwang, S. Y. Park, H. J. Lee, T. Y. Lee, Z. W. Sun, and T. H. Yi, "Gallic acid regulates skin photoaging in UVB-exposed fibroblast and hairless mice," *Phytotherapy Research*, vol. 28, no. 12, pp. 1778–1788, 2014.

[17] E. Hwang, D. G. Lee, S. H. Park, M. S. Oh, and S. Y. Kim, "Coriander leaf extract exerts antioxidant activity and protects against UVB-induced photoaging of skin by regulation of procollagen type I and MMP-1 expression," *Journal of Medicinal Food*, vol. 17, no. 9, pp. 985–995, 2014.

[18] D. Kulms and T. Schwarz, "Molecular mechanisms of UV-induced apoptosis," *Photodermatology, Photoimmunology & Photomedicine*, vol. 16, no. 5, pp. 195–201, 2000.

[19] C. H. Lee, S. B. Wu, C. H. Hong, H. S. Yu, and Y. H. Wei, "Molecular mechanisms of UV-induced apoptosis and its effects on skin residential cells: the implication in UV-based phototherapy," *International Journal of Molecular Sciences*, vol. 14, no. 3, pp. 6414–6435, 2013.

[20] F. Rodrigues, A. Palmeira-de-Oliveira, J. das Neves, B. Sarmento, M. H. Amaral, and M. B. Oliveira, "Coffee silverskin: a possible valuable cosmetic ingredient," *Pharmaceutical Biology*, vol. 53, no. 3, pp. 386–394, 2015.

[21] M. Ndiaye, C. Philippe, H. Mukhtar, and N. Ahmad, "The grape antioxidant resveratrol for skin disorders: promise, prospects, and challenges," *Archives of Biochemistry and Biophysics*, vol. 508, no. 2, pp. 164–170, 2011.

[22] J. Dun, X. Chen, H. Gao, Y. Zhang, H. Zhang, and Y. Zhang, "Resveratrol synergistically augments anti-tumor effect of 5-FU in vitro and in vivo by increasing S-phase arrest and tumor apoptosis," *Experimental Biology and Medicine (Maywood, New Jersey)*, vol. 240, no. 12, pp. 1672–1681, 2015.

[23] J. Wittenauer, S. Mackle, D. Sussmann, U. Schweiggert-Weisz, and R. Carle, "Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity," *Fitoterapia*, vol. 101, pp. 179–187, 2015.

[24] C. H. Cottart, V. Nivet-Antoine, C. Laguillier-Morizot, and J. L. Beaudeux, "Resveratrol bioavailability and toxicity in humans," *Molecular Nutrition & Food Research*, vol. 54, no. 1, pp. 7–16, 2010.

[25] G. S. Sim, D. H. Lee, J. H. Kim et al., "Black rice (Oryza sativa L. var. japonica) hydrolyzed peptides induce expression of hyaluronan synthase 2 gene in HaCaT keratinocytes," *Journal of Microbiology and Biotechnology*, vol. 17, no. 2, pp. 271–279, 2007.

[26] T. H. Lee, J. O. Seo, M. H. Do, E. Ji, S. H. Baek, and S. Y. Kim, "Resveratrol-enriched rice down-regulates melanin synthesis in UVB-induced Guinea pigs epidermal skin tissue," *Biomolecules & Therapeutics (Seoul)*, vol. 22, no. 5, pp. 431–437, 2014.

[27] H. Utsunomiya, A. Takaguri, A. M. Bourne et al., "An extract from brown rice inhibits signal transduction of angiotensin II in vascular smooth muscle cells," *American Journal of Hypertension*, vol. 24, no. 5, pp. 530–533, 2011.

[28] S. H. Lee, Y. S. Sohn, K. K. Kang, J. W. Kwon, and M. Yoo, "Inhibitory effect of DA-9201, an extract of Oryza sativa L., on airway inflammation and bronchial hyperresponsiveness in mouse asthma model," *Biological & Pharmaceutical Bulletin*, vol. 29, no. 6, pp. 1148–1153, 2006.

[29] S. H. Baek, H. J. Chung, H. K. Lee et al., "Treatment of obesity with the resveratrol-enriched rice DJ-526," *Scientific Reports*, vol. 4, p. 3879, 2014.

[30] S. H. Baek, W. C. Shin, H. S. Ryu et al., "Creation of resveratrol-enriched rice for the treatment of metabolic syndrome and related diseases," *PLoS One*, vol. 8, no. 3, article e57930, 2013.

[31] E. Hwang, S. H. Kim, S. Lee et al., "A comparative study of baby immature and adult shoots of Aloe vera on UVB-induced skin photoaging in vitro," *Phytotherapy Research*, vol. 27, no. 12, pp. 1874–1882, 2013.

[32] L. Hsin-Ti, L. Wen-Sheng, W. Yi-Chia et al., "The effect in topical use of Lycogen via sonophoresis for anti-aging on facial skin," *Current Pharmaceutical Biotechnology*, vol. 16, no. 12, pp. 1063–1069, 2015.

[33] Y. Tamatsu, K. Tsukahara, Y. Sugawara, and K. Shimada, "New finding that might explain why the skin wrinkles more on various parts of the face," *Clinical Anatomy*, vol. 28, no. 6, pp. 745–752, 2015.

[34] J. Y. Lim, O. K. Kim, J. Lee, M. J. Lee, N. Kang, and J. K. Hwang, "Protective effect of the standardized green tea seed extract on UVB-induced skin photoaging in hairless mice," *Nutrition Research and Practice*, vol. 8, no. 4, pp. 398–403, 2014.

[35] M. Rinnerthaler, J. Bischof, M. K. Streubel, A. Trost, and K. Richter, "Oxidative stress in aging human skin," *Biomolecules*, vol. 5, no. 2, pp. 545–589, 2015.

[36] J. D'Orazio, S. Jarrett, A. Amaro-Ortiz, and T. Scott, "UV radiation and the skin," *International Journal of Molecular Sciences*, vol. 14, no. 6, pp. 12222–12248, 2013.

[37] Y. F. Hong, H. Lee, B. J. Jung, S. Jang, D. K. Chung, and H. Kim, "Lipoteichoic acid isolated from Lactobacillus plantarum down-regulates UV-induced MMP-1 expression and up-regulates type I procollagen through the inhibition of reactive oxygen species generation," *Molecular Immunology*, vol. 67, no. 2, Part B, pp. 248–255, 2015.

[38] T. G. Lim, S. K. Jung, J. E. Kim et al., "NADPH oxidase is a novel target of delphinidin for the inhibition of UVB-induced MMP-1 expression in human dermal fibroblasts," *Experimental Dermatology*, vol. 22, no. 6, pp. 428–430, 2013.

[39] Z. W. Sun, E. Hwang, H. J. Lee et al., "Effects of Galla chinensis extracts on UVB-irradiated MMP-1 production in hairless mice," *Journal of Natural Medicines*, vol. 69, no. 1, pp. 22–34, 2015.

[40] R. Ganceviciene, A. I. Liakou, A. Theodoridis, E. Makrantonaki, and C. C. Zouboulis, "Skin anti-aging strategies," *Dermato-endocrinology*, vol. 4, no. 3, pp. 308–319, 2012.

[41] S. Xia, X. Zhang, S. Zheng et al., "An update on inflamm-aging: mechanisms, prevention, and treatment," *Journal of Immunology Research*, vol. 2016, Article ID 8426874, 12 pages, 2016.

[42] D. R. Bickers and M. Athar, "Oxidative stress in the pathogenesis of skin disease," *The Journal of Investigative Dermatology*, vol. 126, no. 12, pp. 2565–2575, 2006.

[43] Y. Jeon, Y. Jung, J. K. Youm, K. S. Kang, Y. K. Kim, and S. N. Kim, "Abietic acid inhibits UVB-induced MMP-1 expression in human dermal fibroblast cells through PPARalpha/gamma dual activation," *Experimental Dermatology*, vol. 24, no. 2, pp. 140–145, 2015.

[44] S. Ponnappan and U. Ponnappan, "Aging and immune function: molecular mechanisms to interventions," *Antioxidants & Redox Signaling*, vol. 14, no. 8, pp. 1551–1585, 2011.

[45] Y. Zhuang and J. Lyga, "Inflammaging in skin and other tissues - the roles of complement system and macrophage," *Inflammation & Allergy Drug Targets*, vol. 13, no. 3, pp. 153–161, 2014.

[46] S. Reuter, S. C. Gupta, M. M. Chaturvedi, and B. B. Aggarwal, "Oxidative stress, inflammation, and cancer: how are they linked?," *Free Radical Biology & Medicine*, vol. 49, no. 11, pp. 1603–1616, 2010.

[47] S. Elmore, "Apoptosis: a review of programmed cell death," *Toxicologic Pathology*, vol. 35, no. 4, pp. 495–516, 2007.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8416763, 13 pages
https://doi.org/10.1155/2017/8416763

Review Article

Oxidative Stress: Harms and Benefits for Human Health

Gabriele Pizzino,[1] Natasha Irrera,[1] Mariapaola Cucinotta,[2] Giovanni Pallio,[1] Federica Mannino,[1] Vincenzo Arcoraci,[1] Francesco Squadrito,[1] Domenica Altavilla,[2] and Alessandra Bitto[1]

[1]*Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy*
[2]*Department of Biomedical Sciences, Dentistry and Morphological and Functional Images, University of Messina, Messina, Italy*

Correspondence should be addressed to Gabriele Pizzino; cgpizzino@unime.it

Received 26 May 2017; Accepted 5 July 2017; Published 27 July 2017

Academic Editor: Victor M. Victor

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products. ROS can play, and in fact they do it, several physiological roles (i.e., cell signaling), and they are normally generated as by-products of oxygen metabolism; despite this, environmental stressors (i.e., UV, ionizing radiations, pollutants, and heavy metals) and xenobiotics (i.e., antiblastic drugs) contribute to greatly increase ROS production, therefore causing the imbalance that leads to cell and tissue damage (oxidative stress). Several antioxidants have been exploited in recent years for their actual or supposed beneficial effect against oxidative stress, such as vitamin E, flavonoids, and polyphenols. While we tend to describe oxidative stress just as harmful for human body, it is true as well that it is exploited as a therapeutic approach to treat clinical conditions such as cancer, with a certain degree of clinical success. In this review, we will describe the most recent findings in the oxidative stress field, highlighting both its bad and good sides for human health.

1. Introduction

Superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) are commonly defined reactive oxygen species (ROS); they are generated as metabolic by-products by biological systems [1, 2]. Processes, like protein phosphorylation, activation of several transcriptional factors, apoptosis, immunity, and differentiation, are all dependent on a proper ROS production and presence inside cells that need to be kept at a low level [3]. When ROS production increases, they start showing harmful effects on important cellular structures like proteins, lipids, and nucleic acids [4]. A large body of evidences shows that oxidative stress can be responsible, with different degrees of importance, in the onset and/or progression of several diseases (i.e., cancer, diabetes, metabolic disorders, atherosclerosis, and cardiovascular diseases) [5].

ROS are mainly produced by mitochondria, during both physiological and pathological conditions, that is, $O_2^{\bullet-}$ can be formed by cellular respiration, by lipoxygenases (LOX) and cyclooxygenases (COX) during the arachidonic acid metabolism, and by endothelial and inflammatory cells [6]. Despite the fact that these organelles have an intrinsic ROS scavenging capacity [7], it is worth to note that this is not enough to address the cellular need to clear the amount of ROS produced by mitochondria [8].

Cells deploy an antioxidant defensive system based mainly on enzymatic components, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to protect themselves from ROS-induced cellular damage [9].

2. Oxidants and Free Radical Production

ROS production basically relies on enzymatic and nonenzymatic reactions. Enzymatic reactions able to generate ROS are those involved in respiratory chain, prostaglandin synthesis, phagocytosis, and cytochrome P450 system [10–20].

Superoxide radical ($O_2^{\bullet-}$) is generated by NADPH oxidase, xanthine oxidase, and peroxidases. Once formed, it is involved in several reactions that in turn generate hydrogen peroxide, hydroxyl radical (OH•), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and so on. H_2O_2 (a nonradical) is produced by multiple oxidase enzymes, that is, amino acid oxidase and xanthine oxidase. Hydroxyl radical (OH•), the most reactive among all the free radical species in vivo, is generated by reaction of $O_2^{\bullet-}$ with H_2O_2, with Fe^{2+} or Cu^+ as a reaction catalyst (Fenton reaction) [12–19]. Nitric oxide radical (NO•), which plays some important physiological roles, is synthesized from arginine-to-citrulline oxidation by nitric oxide synthase (NOS) [12–19].

Even nonenzymatic reactions can be responsible for free radical production, that is, when oxygen reacts with organic compounds or when cells are exposed to ionizing radiations. Nonenzymatic free radical production can occur as well during mitochondrial respiration [15, 16, 19].

Free radicals are generated from both endogenous and exogenous sources. Immune cell activation, inflammation, ischemia, infection, cancer, excessive exercise, mental stress, and aging are all responsible for endogenous free radical production. Exogenous free radical production can occur as a result from exposure to environmental pollutants, heavy metals (Cd, Hg, Pb, Fe, and As), certain drugs (cyclosporine, tacrolimus, gentamycin, and bleomycin), chemical solvents, cooking (smoked meat, used oil, and fat), cigarette smoke, alcohol, and radiations [15–25]. When these exogenous compounds penetrate the body, they are degraded or metabolized, and free radicals are generated as by-products.

3. Physiological Activities of Free Radicals

When maintained at low or moderate concentrations, free radicals play several beneficial roles for the organism. For example, they are needed to synthesize some cellular structures and to be used by the host defense system to fight pathogens. In fact, phagocytes synthesize and store free radicals, in order to be able to release them when invading pathogenic microbes have to be destroyed [16, 21]. The pivotal role of ROS for the immune system is well exemplified by patients with granulomatous disease. These individuals are unable to produce $O_2^{\bullet-}$ because of a defective NADPH oxidase system, so they are prone to multiple and in most of the cases persistent infections [15, 16]. Free radicals are also involved in a number of cellular signaling pathways [18–20]. They can be produced by nonphagocytic NADPH oxidase isoforms; in this case, free radicals play a key regulatory role in intracellular signaling cascades, in several cell types such as fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue. Probably, the most well-known free radical acting as a signaling molecule is nitric oxide (NO). It is an important cell-to-cell messenger required for a proper blood flow modulation, involved in thrombosis, and is crucial for the normal neural activity [18]. NO is also involved in nonspecific host defense, required to eliminate intracellular pathogens and tumor cells. Another physiological activity of free radicals is the induction of a mitogenic response [18, 19]. Summarizing, free radicals, when maintained at low or moderate levels, are of crucial importance to human health.

4. Detrimental Effects of Free Radicals on Human Health

As stated before, if in excess, free radicals and oxidants give rise to a phenomenon known as oxidative stress; this is a harmful process that can negatively affect several cellular structures, such as membranes, lipids, proteins, lipoproteins, and deoxyribonucleic acid (DNA) [16–21]. Oxidative stress emerges when an imbalance exists between free radical formation and the capability of cells to clear them. For instance, an excess of hydroxyl radical and peroxynitrite can cause lipid peroxidation, thus damaging cell membranes and lipoproteins. This in turn will lead to malondialdehyde (MDA) and conjugated diene compound formation, which are known to be cytotoxic as well as mutagenic. Being a radical chain reaction, lipid peroxidation spreads very quickly affecting a large amount of lipidic molecules [25]. Proteins may as well being damaged by oxidative stress, undergoing to conformational modifications that could determine a loss, or an impairment, of their enzymatic activity [20, 25].

Even DNA is prone to oxidative stress-related lesions, the most representative of which is the 8-oxo-2′-deoxyguanosine (8-OHdG) formation; this is a particularly pernicious DNA lesion, which can be responsible for both mutagenesis, as pointed out by Nishida et al. [26]. It can also cause a loss in the epigenetic information, probably due to an impairment in CpG island methylation asset in gene promoters [27]. It is worth to note that Valavanidis and colleagues [28] have already proposed 8-OHdG levels in a tissue as biomarker of oxidative stress. Of course cells can put in place several mechanisms, such as the base excision repair (BER) or antioxidants, as defense response against DNA lesions [17–20].

If not strictly controlled, oxidative stress can be responsible for the induction of several diseases, both chronic and degenerative, as well as speeding up body aging process and cause acute pathologies (i.e., trauma and stroke).

4.1. Cancer and Oxidative Stress. Cancer onset in humans is a complex process, which requires both cellular and molecular alterations mediated by endogenous and/or exogenous triggers. It is already well known that oxidative DNA damage is one of those stimuli responsible for cancer development [14, 15, 22]. Cancer can be driven and/or promoted by chromosomal abnormalities and oncogene activation determined by oxidative stress. Hydrolyzed DNA bases are common by-products of DNA oxidation and are considered one of the most relevant events in chemical carcinogenesis [14, 22]. The formation of such kind of adducts impairs normal cell growth by altering the physiological transcriptomic profile and causing gene mutations. Oxidative stress can also cause a variegated amount of modifications against DNA structure, for example, base and sugar lesions, DNA-protein cross-links, strand breaks, and base-free sites. For instance, tobacco smoking, environmental pollutants, and chronic inflammation are sources of oxidative DNA damage that could contribute to tumor onset [14, 17, 29]. Oxidative stress

resulting from lifestyle reasons can also play an important role in cancer development, as suggested by the strong correlation between dietary fat consumption (a factor that exposes the organism at greater risk of lipid peroxidation) and death rates from different types of cancer [16, 21].

4.2. Cardiovascular Disease and Oxidative Stress. Cardiovascular diseases (CVDs) are clinical entities with a multifactorial etiology, generally associated with a very large amount of risk factors, the most broadly recognized of which are hypercholesterolaemia, hypertension, smoking habit, diabetes, unbalanced diet, stress, and sedentary life [11, 30, 31]. During the last years, research data pointed out that oxidative stress should be considered either a primary or a secondary cause for many CVDs [18]. Oxidative stress acts mainly as a trigger of atherosclerosis. It is well known that atheromatous plaque formation results from an early endothelial inflammation, which in turn leads to ROS generation by macrophages recruited in situ. Circulating LDL are then oxidized by reactive oxygen species, thus leading to foam cell formation and lipid accumulation. The result of these events is the formation of an atherosclerotic plaque. Both in vivo and ex vivo studies provided evidences supporting the role of oxidative stress in atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure [11, 16, 30, 31].

4.3. Neurological Disease and Oxidative Stress. Oxidative stress has been linked to several neurological diseases (i.e., Parkinson's disease, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis, depression, and memory loss) [32–35]. In AD, several experimental and clinical researches showed that oxidative damage plays a pivotal role in neuron loss and progression to dementia [34]. β-amyloid, a toxic peptide often found present in AD patients' brain, is produced by free radical action and it is known to be at least in part responsible for neurodegeneration observed during AD onset and progression [35].

4.4. Respiratory Disease and Oxidative Stress. Several researches pointed out that lung diseases such as asthma and chronic obstructive pulmonary disease (COPD), determined by systemic and local chronic inflammation, are linked to oxidative stress [36–39]. Oxidants are known to enhance inflammation via the activation of different kinases involving pathways and transcription factors like NF-kappa B and AP-1 [38, 39].

4.5. Rheumatoid Arthritis and Oxidative Stress. Rheumatoid arthritis is a chronic inflammatory disorder affecting the joints and surrounding tissues, characterized by macrophages and activated T cell infiltration [15, 40, 41]. Free radicals at the site of inflammation play a relevant role in both initiation and progression of this syndrome, as demonstrated by the increased isoprostane and prostaglandin levels in synovial fluid of affected patients [41].

4.6. Kidney Diseases and Oxidative Stress. Oxidative stress is involved in a plethora of diseases affecting renal apparatus such as glomerulo- and tubule-interstitial nephritis, renal failure, proteinuria, and uremia [16, 42]. The kidneys are negatively affected by oxidative stress mainly because of the fact that ROS production induces the recruitment of inflammatory cells and proinflammatory cytokine production, leading to an initial inflammatory stage. In this early phase, a predominant role is played by TNF-alpha and IL-1b, as proinflammatory mediators, as well as by NF-κB as transcriptional factor required to sustain the inflammatory process. The latter stage is characterized by an increase in TGF-beta production, which orchestrates the extracellular matrix synthesis. So, when the oxidative stress stimuli act chronically on kidney tissues, the results will be an initial stage of inflammation and later the formation of abundant fibrotic tissue that impairs organ function potentially leading to renal failure. Certain drugs, such as cyclosporine, tacrolimus, gentamycin, and bleomycin, are known to be nephrotoxics mainly because of the fact that they increase free radical levels and oxidative stress via lipid peroxidation [42–45]. Heavy (Cd, Hg, Pb, and As) and transition metals (Fe, Cu, Co, and Cr), acting as powerful oxidative stress inducers, are responsible for various forms of nephropathy, as well as for some types of cancers [22, 23].

4.7. Sexual Maturation and Oxidative Stress. Several authors pointed out that oxidative stress could be responsible for a delayed sexual maturation and puberty onset [46, 47]. This seems to be true when children in prepubertal age are exposed to Cd, a well known responsible for an increase in free radicals and oxidative stress, as well as when pregnant women are exposed to the same metallic element.

Summarizing, we can affirm that oxidative stress and free radicals are confirmed to be responsible for several pathological conditions affecting different tissues and systems, thus being one of the most important and pervasive harms to human health.

5. Exogenous Antioxidants and Human Health

Human body put in place several strategies to counteract the effects of free radicals and oxidative stress, based on enzymatic (e.g., SOD, CAT, and GPx) and nonenzymatic (e.g., lipoic acid, glutathione, L-arginine, and coenzyme Q10) antioxidant molecules, all of them being endogenous antioxidants. Beside these, there are several exogenous antioxidant molecules of animal or vegetal origin, mainly introduced by diet or by nutritional supplementation.

Here, we will discuss the most relevant nutritional antioxidants and their protective effects for human health.

5.1. Vitamin E. The term vitamin E encompasses a constellation of lipophilic molecules (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) synthesized by vegetal organisms [48] and contained in edible oils and seeds, as well as in food artificially enriched in α-tocopherol [49, 50].

The most active form of vitamin E, RRR-α-tocopherol, showed in vitro an antiproliferative activity against vascular smooth muscle cells via PKC modulation [51], even when under stimulation from low-density lipoproteins (LDL)

[52]. These results were confirmed in vivo, both in mouse and rabbit models of atherosclerosis [53–55].

Macrophage transition to foam cells is one of the earlier and important steps in atherosclerotic lesion formation; CD36 receptor is one of the key players involved, being a scavenger receptor responsible for oxidized-LDL (oxLDL) uptake from bloodstream [56, 57].

Several studies described that vitamin E is able to prevent CD36 mRNA expression induced by cholesterol, thus playing a beneficial role in preventing foam cell formation. This was true in vivo, as well as in vitro on human macrophages and vascular smooth muscle cells [58, 59]; vitamin E supplementation was also useful to upregulate PPARγ, LXRα, and ABCA1, in ApoE knockout mice, ameliorating early (but not advanced) atherosclerotic lesions [60].

Vitamin E modulates the oxidative stress-induced NF-κB pathway and oxLDL-induced foam cell formation, decreases c-Jun phosphorylation (thus inhibiting inflammation and monocyte invasion), and matrix metalloprotease (MMP) expression [61–65].

A degree of CD36 mRNA reduction was also observed in animals undergoing to vitamin E supplementation under a regimen of high-fat diet [66–68].

RRR-γ-tocopherol (the most abundant after RRR-α-tocopherol) showed a potent proinflammatory function during allergic inflammation [69–77].

Each form of vitamin E seems to have different regulatory effects when it comes to recruit leukocytes to allergic inflammation site, which is however strictly dependent on vascular cell adhesion molecule-1 (VCAM-1) [78]. VCAM-1 is responsible also for the activation of several signals in endothelial cells which are causative of ROS generation, such as NOX2 complex activation that generates ROS which lead to PKCα activation [79]. This rapid and transient PKCα activation is consistent with a leukocyte migration across endothelia required in a timeframe of minutes, consistent with the rapid migration of leukocytes across endothelial cells in minutes at sites of migration.

Endothelial cells pretreated with α-tocopherol are less prone to let the lymphocytes and eosinophils migrate, while the opposite is true when pretreated with γ-tocopherol, this is due to the fact that the first strategy decreases VCAM-1 expression, while the latter increases it [80]. This phenomenon was observed even in vivo, in a mouse model of allergic lung inflammation [80, 81].

Interestingly, a research found a correlation between the prevalence of asthma and the average plasma tocopherol in several countries, based on nutritional consumption of foods and oils rich in tocopherol. Briefly, countries with an average plasma γ-tocopherol concentration of 2–7 μmol/L had the highest asthma prevalence compared to those with a concentration of 1-2 μmol/L, independently from α-tocopherol plasma levels [74].

Olive and sunflower oils, which have little or not at all γ-tocopherol [74], seem to have to be preferred to soybean oil, because the latter one seems responsible for an increase in plasma γ-tocopherol [82].

A large prospective study, covering 4500 individuals and spanning 20 years, demonstrated an association between α- or γ-tocopherol serum concentrations and lung function [72]. The results highlighted as in those individuals with higher γ-tocopherol serum levels (>10 μmol/L) demonstrated significantly lower FEV1/FVC (10–17%); it is relevant to point out that similar degrees of lung function impairments were observed in individuals exposed to other respiratory stressors (e.g., particulate matter) [83–87]. These observations suggest that γ-tocopherol could negatively affect pulmonary function.

It has been also observed, from in vivo experiments, that α- and γ-tocopherol supplementation of allergic and nonallergic pregnant mice can alter the allergic responsiveness development in offspring of mice.

It is known that (i) proper dendritic cell development and responsiveness are crucial for an optimal allergen sensitization, (ii) it relies on PKC isoforms activity, and (iii) all of the PKCs include a C1A regulatory domain, which is targeted by both α- and γ-tocopherol [88–107].

In mice prone to allergic disease, supplementing allergic mothers (at the time of mating) with α-tocopherol was enough to inhibit the pup allergic responses [67], while γ-tocopherol supplementation amplified pup responses to allergens [70].

These differences in allergic response development exerted by α- or γ-tocopherol supplementation are dependent from their modulation of eosinophils and $CD11c^+$ $CD11b^+$ dendritic cell numbers in the lungs; α-tocopherol reduced both the cellular species, without affecting the number of $CD11c^+$ $CD11b^-$ regulatory dendritic cells, while γ-tocopherol increased both eosinophils and $CD11c^+$ $CD11b^+$ dendritic cells [69, 70].

Summarizing, α-and γ-tocopherol forms of vitamin E exert a differential set of biological effects, which cannot be always regarded as positive to human health; this is something that needs to be taken in account when considering to enrich the content of vitamin E into a diet with antioxidant purposes.

5.2. Flavonoids. Flavonoids are a class of polyphenolic compounds with a benzo-γ-pyrone structure largely represented in plants, responsible for several pharmacological activities [108, 109]. These substances have been investigated because of their potential health benefits as antioxidants, action mediated by their functional hydroxyl groups, which are able to scavenge free radicals and/or chelate metal ions [109–116].

Their antioxidant activity relies on the conformational disposition of functional groups; configuration, substitution, and total number of hydroxyl groups are important factors in determining mechanisms of antioxidant activity like ROS/RNS scavenging and metal chelation [111, 117].

Flavonoid determines (i) ROS synthesis suppression, inhibition of enzymes, or chelation of trace elements responsible for free radical generation; (ii) scavenging ROS; and (iii) improvement of antioxidant defenses [118, 119].

Genistein is a soy isoflavone that is probably the most interesting and well-studied flavonoid compound, due to its broad pharmacological activities.

Genistein has been extensively employed as antioxidant in a plethora of studies, showing the potential to scavenge

ROS and RNS with a high degree of efficacy. This flavonoid compound is able to improve the antioxidant defenses of a cell, thus prevents apoptotic process through the modulation of several genes and proteins [120]. In nonhuman primates and rabbits [121, 122], dietary-supplemented genistein delayed atherogenesis. An additional study observed an increase in antioxidant protection of LDL and an atheroprotective effect [123]. In general, soy isoflavones confer protection against lipoprotein oxidation [124–126], as well as against oxidative DNA damage in postmenopausal women [127], but the point is still debated [128–130]. There are other mechanism that genistein can be used to suppress oxidative stress and related inflammation in the vascular intima layer. Genistein inhibits NF-κB activation (inducible by oxidative stress) and regulates the expression of genes relevant to immune and inflammatory processes [131]. Genistein increases the expression of antioxidant enzymes in human prostate cancer cells conferring protection against oxidative DNA damage [132, 133].

Briefly, flavonoids are a class of natural compounds extensively present in foods of vegetal origin (fruits, oils, seeds, etc.) showing a good potential in terms of usefulness for human health, as antioxidant molecules but also because of some ancillary yet pharmacologically interesting properties. Nonetheless, they need to be managed carefully, and their supplementation into the diet (as diet enrichment or as nutraceuticals) have to take in account also some potential drawback concerning human health and wellness.

6. Prooxidant Agents in Therapy

Prooxidant agents, beside their well-known detrimental effects on human health, have been investigated and, in some cases, actually used, as therapeutic agents mainly against cancer diseases.

Here, we will briefly discuss two emerging prooxidant compounds showing interesting pharmacological activities, such as ascorbic acid (AA) and polyphenols, and the most well-known and employed prooxidant in therapy, ionizing radiation.

6.1. Ascorbic Acid. Ascorbic acid (vitamin C) is a water-soluble compound classified under the group of natural antioxidants. Ascorbate reacts with ROS, quenching them and promoting the conversion into semihydroascorbate radical, which is a poorly reactive chemical species, thus efficiently reducing the risk of cancer by suppressing free radicals and oxidative stress [134].

Apart from this, ascorbate also reduces metal ions like Fe^{3+} and Cu^{3+}, thus promoting a reaction that gives rise to highly reactive free radical (by the so-called Fenton reaction) [135, 136]; these radicals have been reported to be able to induce cytotoxicity by causing DNA backbone breaks and base modifications [134].

This effect seems to be more relevant on cancer cells, in fact while normal cells take advantage from redundant mechanisms for H_2O_2 clearing and/or repair of H_2O_2-induced damage, to counteract the effects of pro-oxidant concentrations of AA; cancer cells lacking of these compensatory mechanisms (e.g., catalase deficiency, mutated DNA repair, and tumor suppressor genes) are more susceptible to pharmacologic ascorbate concentrations [132]. The authors reported that 10 mM AA induces apoptosis in leukemia cell lines; the authors proposed that AA-induced $O_2^{\bullet-}/H_2O_2$ production led to NF-κB-p53-caspase 3 signaling axis, by which this proapoptotic effect is exerted [137–139]. Another study pointed out that AA was able to inhibit Raji cell proliferation, apparently by downregulating the set of genes needed for S-phase progression in actively proliferating cells [140]. In an in vivo study, guinea pigs supplemented with AA at various doses showed a complete regression of fibrosarcoma and liposarcoma tumors [141]. In general, there have been several studies assessing the antiblastic activities of AA, mostly in vitro on different cell lines [142–156].

Despite these somehow surprising but still very interesting results, there is the urge of conducting more researches, both in vitro and in vivo, to definitely assess the mode of action and efficacy of AA as prooxidative anticancer agent.

6.2. Polyphenols. Under conditions like high concentrations, high pH, and the presence of redox-active metals, phenolic compounds can acquire a prooxidant behavior [157, 158], mainly based on the generation of an aroxyl radical or a labile complex with a metal cation exerting redox activity. Aroxyl radical can lead the formation of $O_2^{\bullet-}$ or of ternary compound between DNA, copper, and flavonoids [159]. Polyphenols, like caffeic acid, ferulic acid, and apigenin, can exert a prooxidant effect through the increased intracellular production of ROS by NOX [160, 161].

Polyphenols can as well induce oxidative stress via transition metals, promoting the generation of hydroxyl radicals through Fenton and Fenton-like reactions; it is important to note that transition metal ions are more represented into cancer than into normal cells [162].

Prooxidant polyphenols seem to exert their cytotoxic activity by inducing apoptosis and cell cycle arrest via several pathways. Anthocyanins, pigments present in red wine and berry (*Aronia melanocarpa*, Rosaceae, *Vaccinium myrtillus*, and Ericaceae) fruits, cause apoptosis in cancer cells by increasing intracellular ROS formation [162–164].

Esculetin, a coumarin derivative present in plants such as chicory (*Cichorium intybus* and Asteraceae), showed both in vivo and in vitro antiproliferative activity against hepatocellular carcinoma. Esculetin delay Hepa 1–6 cell growth inoculated subcutaneously in C57BL/6J mice in a time- and dose-dependent manner [165]. Human hepatocellular carcinoma SMMC-7721 cells incubated with esculetin undergo to mitochondrial membrane potential collapse, with Bcl-2, caspase-9-, and caspase-3-mediated apoptosis [165]. In addition, esculetin also exerted a cytotoxic effect on HeLa cells inducing redox-dependent apoptosis, even in this case by causing the disruption of mitochondrial membrane potential, cytochrome C release, and caspase activation [166].

Curcumin, a compound extracted from *Curcuma longa*, induced ROS-mediated apoptosis in human gastric BGC-823 cells by activating the apoptosis signal-regulating kinase 1 (ASK1) signaling cascade (ASK1/MKK4/JNK) [167].

During the last years, a very large amount of in vitro studies investigated the prooxidative effects of polyphenols against cancer cell proliferation and survival, all of them presenting interesting results that nonetheless need to be confirmed by more in-depth researches [168–190].

Although polyphenols showed the pharmacological potential to inhibit tumorigenesis and arrest cancer cell proliferation in animal models, the role of ROS generation is still poorly understood, mainly because a large majority of the in vivo studies are limited to cancer growth arrest and apoptosis evaluation, and rarely or not at all they go deeper in the mechanistic explanation of a potential prooxidant action in vivo [191, 192].

6.3. Radiation Therapy. The ability of ionizing radiation to counteract proliferation of cancer cells is well explained [193–195] and widely used in clinical practice. In the last decades, there has been an extensive effort to understand the physical and molecular cellular response that follow the exposure to ionizing radiation. It is well recognized that damage to DNA operated by generation of radicals that indirectly cause DNA double-strand beaks (DSBs) is the most severe kind of damage induced by this prooxidant physical agent [196, 197]. These lesions are promptly repaired, as the results of the rapid activation of DSB damage repair mechanism, most importantly nonhomologous end joining or homologous recombination and the execution of a complex and finely tuned sequelae of those cellular signaling pathways belonging to the DNA damage response (DDR) [194, 198]. These responses span from posttranslational modifications and/or differential gene expression of proteins to start cell cycle reprogramming (e.g., radiation-induced arrest) or to execute cell death by mitotic catastrophe, apoptosis, autophagy, or induction of senescence [194, 195, 198].

Radiotherapy plays a key role in cancer treatment, so that almost 40% of cancer patients have been treated with this approach at least once [199]. In the last 2 decades, several technological advancements, like intensity-modulated radiotherapy (IMRT), image-guided radiotherapy (IGRT), and stereotactic radiotherapy (SRT), were put in place to address the need to reach that level of precision required to take advantage from radiation prooxidant activity avoiding, as much as possible, the side effects in terms of oxidative stress-induced cellular damage on healthy cells and tissues.

7. Conclusions

Oxidative stress and free radicals are generally known to be detrimental to human health. A large amount of studies demonstrates that in fact free radicals contribute to initiation and progression of several pathologies, ranging from CVD to cancer.

Antioxidants, as class of compounds able to counteract oxidative stress and mitigate its effects on individuals' health, gained enormous attention from the biomedical research community, because these compounds not only showed a good degree of efficacy in terms of disease prevention and/or treatment but also because of the general perception that they are free from important side effects. If it is true that antioxidants can be very useful in preventing, managing, or treating human pathologies, it is true as well that they are not immune to generating adverse effects. On the other hand, some prooxidant compounds or agents can be as well useful to human health, particularly regarding cancer treatment.

We can reach to the conclusion that oxidative stress, as phenomenon, although being one of the major harms to individuals' wellness and health, it can also be exploited as a treatment tool when and if we will be able to operate a fine tuning of this process inside human organism.

Conflicts of Interest

The authors state no conflict of interest.

Authors' Contributions

Gabriele Pizzino and Natasha Irrera equally contributed to this paper.

Acknowledgments

The authors are thankful to all members of the Squadrito laboratory for the technical support.

References

[1] H. Sato, H. Shibata, T. Shimizu, S. Shibata, H. Toriumi, and T. Ebine, "Differential cellular localization of antioxidant enzymes in the trigeminal ganglion," *Neuroscience*, vol. 248, pp. 345–358, 2013.

[2] J. Navarro-Yepes, L. Zavala-Flores, A. Anandhan, F. Wang, M. Skotak, and N. Chandra, "Antioxidant gene therapy against neuronal cell death," *Pharmacology & Therapeutics*, vol. 142, pp. 206–230, 2014.

[3] P. Rajendran, N. Nandakumar, T. Rengarajan, R. Palaniswami, E. N. Gnanadhas, and U. Lakshminarasaiah, "Antioxidants and human diseases," *Clinica Chimica Acta*, vol. 436, pp. 332–347, 2014.

[4] J. Q. Wu, T. R. Kosten, and X. Y. Zhang, "Free radicals, antioxidant defense system, and schizophrenia," *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, vol. 46, pp. 200–206, 2013.

[5] Y. Taniyama and K. K. Griendling, "Reactive oxygen species in the vasculature," *Hypertension*, vol. 42, pp. 1075–1081, 2003.

[6] K. H. Al-Gubory, C. Garrel, P. Faure, and N. Sugino, "Roles of antioxidant enzymes in corpus luteum rescue from reactive oxygen species-induced oxidative stress," *Reproductive Biomedicine Online*, vol. 25, pp. 551–560, 2012.

[7] J. M. Hansen, Y. M. Go, and D. P. Jones, "Nuclear and mitochondrial compartmentation of oxidative stress and redox signalling," *Annual Review of Pharmacology and Toxicology*, vol. 46, pp. 215–234, 2006.

[8] A. Glasauer and N. S. Chandel, "Targeting antioxidants for cancer therapy," *Biochemical Pharmacology*, vol. 92, pp. 90–101, 2014.

[9] M. Deponte, "Glutathione catalysis and the reaction mechanism of glutathione-dependent enzymes," *Biochimica et Biophysica Acta*, vol. 2013, pp. 3217–3266, 1830.

[10] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, UK, 4th edition, 2007.

[11] T. Bahorun, M. A. Soobrattee, V. Luximon-Ramma, and O. I. Aruoma, "Free radicals and antioxidants in cardiovascular health and disease," *Internet Journal of Medical Update*, vol. 1, pp. 1–17, 2006.

[12] S. Kumar and A. K. Pandey, "Free radicals: health implications and their mitigation by herbals," *British Journal of Medicine and Medical Research*, vol. 7, pp. 438–457, 2015.

[13] S. Kumar and A. K. Pandey, "Chemistry and biological activities of flavonoids: an overview," *The Scientific World Journal*, vol. 2013, Article ID 162750, 16 pages, 2013.

[14] M. Valko, M. Izakovic, M. Mazur, C. J. Rhodes, and J. Telser, "Role of oxygen radicals in DNA damage and cancer incidence," *Molecular and Cellular Biochemistry*, vol. 266, pp. 37–56, 2004.

[15] M. Valko, D. Leibfritz, J. Moncola, M. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *The International Journal of Biochemistry & Cell Biology*, vol. 39, pp. 44–84, 2007.

[16] W. Droge, "Free radicals in the physiological control of cell function," *Physiological Reviews*, vol. 82, pp. 47–95, 2002.

[17] J. K. Willcox, S. L. Ash, and G. L. Catignani, "Antioxidants and prevention of chronic disease," *Critical Reviews in Food Science and Nutrition*, vol. 44, pp. 275–295, 2004.

[18] P. Pacher, J. S. Beckman, and L. Liaudet, "Nitric oxide and peroxynitrite in health and disease," *Physiological Reviews*, vol. 87, pp. 315–424, 2007.

[19] M. Genestra, "Oxyl radicals, redox-sensitive signalling cascades and antioxidants," *Cellular Signalling*, vol. 19, pp. 1807–1819, 2007.

[20] B. Halliwell, "Biochemistry of oxidative stress," *Biochemical Society Transactions*, vol. 35, pp. 1147–1150, 2007.

[21] I. Young and J. Woodside, "Antioxidants in health and disease," *Journal of Clinical Pathology*, vol. 54, pp. 176–186, 2001.

[22] 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, pp. 1–40, 2006.

[23] M. Valko, H. Morris, and M. T. D. Cronin, "Metals, toxicity and oxidative stress," *Current Medicinal Chemistry*, vol. 12, pp. 1161–1208, 2005.

[24] S. Parthasarathy, N. Santanam, S. Ramachandran, and O. Meilhac, "Oxidants and antioxidants in atherogenesis: an appraisal," *Journal of Lipid Research*, vol. 40, pp. 2143–2157, 1999.

[25] B. Frei, *Reactive Oxygen Species and Antioxidant Vitamins*, Linus Pauling Institute, Oregon State University, 1997, http://lpi.oregonstate.edu/f-w97/reactive.html.

[26] N. Nishida, T. Arizumi, M. Takita et al., "Reactive oxygen species induce epigenetic instability through the formation of 8-hydroxydeoxyguanosine in human hepatocarcinogenesis," *Digestive Diseases*, vol. 31, no. 5-6, pp. 459–466, 2013.

[27] M. Yasui, Y. Kanemaru, N. Kamoshita, T. Suzuki, T. Arakawa, and M. Honma, "Tracing the fates of site-specifically introduced DNA adducts in the human genome," *DNA Repair (Amst)*, vol. 15, pp. 11–20, 2014.

[28] A. Valavanidis, T. Vlachogianni, K. Fiotakis, and S. Loridas, "Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms," *International Journal of Environmental Research and Public Health*, vol. 10, no. 9, pp. 3886–3907, 2013.

[29] G. Pizzino, A. Bitto, M. Interdonato et al., "Oxidative stress and DNA repair and detoxification gene expression in adolescents exposed to heavy metals living in the Milazzo-Valle del Mela area (Sicily, Italy)," *Redox Biology*, vol. 2, pp. 686–693, 2014.

[30] M. Chatterjee, R. Saluja, S. Kanneganti, S. Chinta, and M. Dikshit, "Biochemical and molecular evaluation of neutrophil NOS in spontaneously hypertensive rats," *Cellular and Molecular Biology*, vol. 53, pp. 84–93, 2007.

[31] A. Ceriello, "Possible role of oxidative stress in the pathogenesis of hypertension," *Diabetes Care*, vol. 31, Supplement 2, pp. S181–S184, 2008.

[32] B. Halliwell, "Role of free radicals in neurodegenerative diseases: therapeutic implications for antioxidant treatment," *Drugs & Aging*, vol. 18, pp. 685–716, 2001.

[33] R. P. Singh, S. Sharad, and S. Kapur, "Free radicals and oxidative stress in neurodegenerative diseases: relevance of dietary antioxidants," *Journal, Indian Academy of Clinical Medicine*, vol. 5, pp. 218–225, 2004.

[34] Y. Christen, "Oxidative stress and Alzheimer disease," *The American Journal of Clinical Nutrition*, vol. 71, pp. 621S–629S, 2000.

[35] D. A. Butterfield, "Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. A review," *Free Radical Research*, vol. 36, pp. 1307–1313, 2002.

[36] G. Caramori and A. Papi, "Oxidants and asthma," *Thorax*, vol. 59, pp. 170–173, 2004.

[37] R. F. Guo and P. A. Ward, "Role of oxidants in lung injury during sepsis," *Antioxidants & Redox Signaling*, vol. 9, pp. 1991–2002, 2007.

[38] Y. Hoshino and M. Mishima, "Antioxidants & redox signaling redox-based therapeutics for lung diseases," *Antioxidants & Redox Signaling*, vol. 10, pp. 701–704, 2008.

[39] W. MacNee, "Oxidative stress and lung inflammation in airways disease," *European Journal of Pharmacology*, vol. 429, pp. 195–207, 2001.

[40] J. Walston, Q. Xue, R. D. Semba et al., "Serum antioxidants, inflammation, and total mortality in older women," *American Journal of Epidemiology*, vol. 163, pp. 18–26, 2006.

[41] A. Mahajan and V. R. Tandon, "Antioxidants and rheumatoid arthritis," *Journal of Indian Rheumatology Association*, vol. 12, pp. 139–142, 2004.

[42] J. Galle, "Oxidative stress in chronic renal failure," *Nephrology, Dialysis, Transplantation*, vol. 16, pp. 2135–2142, 2001.

[43] N. Sadeg, C. Pham-Huy, C. Martin, J. M. Warnet, and J. R. Claude, "Effect of cyclosporin A and its metabolites and analogs on lipid peroxidation in rabbit renal microsomes," *Drug and Chemical Toxicology*, vol. 16, pp. 165–174, 1993.

[44] F. Massicot, C. Martin, H. Dutertre-Catella et al., "Modulation of energy status and cytotoxicity induced by FK506 and cyclosporin A in a renal epithelial cell line," *Archives of Toxicology*, vol. 71, pp. 529–531, 1997.

[45] F. Massicot, A. Lamouri, C. Martin et al., "Preventive effects of two PAF-antagonists, PMS 536 and PMS 549, on cyclosporin-induced LLC-PK1 oxidative injury," *Journal of Lipid Mediators and Cell Signalling*, vol. 15, pp. 203–214, 1997.

[46] J. B. Samuel, J. A. Stanley, R. A. Princess, P. Shanthi, and M. S. Sebastian, "Gestational cadmium exposure-induced ovotoxicity delays puberty through oxidative stress and impaired steroid hormone levels," *Journal of Medical Toxicology*, vol. 7, no. 3, pp. 195–204, 2011.

[47] M. Interdonato, G. Pizzino, A. Bitto et al., "Cadmium delays puberty onset and testis growth in adolescents," *Clinical Endocrinology*, vol. 83, no. 3, pp. 357–362, 2015.

[48] L. Mene-Saffrane and D. DellaPenna, "Biosynthesis, regulation and functions of tocochromanols in plants," *Plant Physiology and Biochemistry*, vol. 48, pp. 301–309, 2010.

[49] A. Sheppard, J. A. T. Pennington, and J. L. Weihrauch, "Analysis and distribution of vitamin E in vegetable oils and foods," in *Vitamin E in Health and Disease*, F. J. Packer, Ed., pp. 9–31, Marcel Dekker Inc, New York, 1993.

[50] I. Sundl, M. Murkovic, D. Bandoniene, and B. M. Winklhofer-Roob, "Vitamin E content of foods: comparison of results obtained from food composition tables and HPLC analysis," *Clinical Nutrition*, vol. 26, pp. 145–153, 2007.

[51] D. Boscoboinik, A. Szewczyk, C. Hensey, and A. Azzi, "Inhibition of cell proliferation by alpha-tocopherol. Role of protein kinase C," *The Journal of Biological Chemistry*, vol. 266, pp. 6188–6194, 1991.

[52] N. K. Özer, P. Palozza, D. Boscoboinik, and A. Azzi, "D-Alpha-tocopherol inhibits low density lipoprotein induced proliferation and protein kinase C activity in vascular smooth muscle cells," *FEBS Letters*, vol. 322, pp. 307–310, 1993.

[53] Ö. Sirikci, N. K. Özer, and A. Azzi, "Dietary cholesterol-induced changes of protein kinase C and the effect of vitamin E in rabbit aortic smooth muscle cells," *Atherosclerosis*, vol. 126, pp. 253–263, 1996.

[54] N. K. Özer, O. Sirikci, S. Taha, T. San, U. Moser, and A. Azzi, "Effect of vitamin E and probucol on dietary cholesterol-induced atherosclerosis in rabbits," *Free Radical Biology & Medicine*, vol. 24, pp. 226–233, 1998.

[55] M. Meydani, P. Kwan, M. Band et al., "Long-term vitamin E supplementation reduces atherosclerosis and mortality in Ldlr−/− mice, but not when fed Western style diet," *Atherosclerosis*, vol. 233, pp. 196–205, 2014.

[56] J. F. Keaney Jr., D. I. Simon, and J. E. Freedman, "Vitamin E and vascular homeostasis: implications for atherosclerosis," *The FASEB Journal*, vol. 13, pp. 965–975, 1999.

[57] M. Febbraio, E. Podrez, J. Smith et al., "Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice," *Journal of Clinical Investigation*, vol. 105, pp. 1049–1056, 2000.

[58] N. K. Ozer, Y. Negis, N. Aytan et al., "Vitamin E inhibits CD36 scavenger receptor expression in hypercholesterolemic rabbits," *Atherosclerosis*, vol. 184, pp. 15–20, 2006.

[59] R. Ricciarelli, J. M. Zingg, and A. Azzi, "Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells," *Circulation*, vol. 102, pp. 82–87, 2000.

[60] F. Tang, M. Lu, S. Zhang et al., "Vitamin E conditionally inhibits atherosclerosis in ApoE knockout mice by anti-oxidation and regulation of vasculature gene expressions," *Lipids*, vol. 49, pp. 1215–1223, 2014.

[61] B. Catalgol, I. Ziaja, N. Breusing et al., "The proteasome is an integral part of solar ultraviolet a radiation-induced gene expression," *The Journal of Biological Chemistry*, vol. 284, pp. 30076–30086, 2009.

[62] A. Hershko and A. Ciechanover, "The ubiquitin system," *Annual Review of Biochemistry*, vol. 67, pp. 425–479, 1998.

[63] E. Sozen, B. Karademir, B. Yazgan, P. Bozaykut, and N. K. Ozer, "Potential role of proteasome on c-Jun related signaling in hypercholesterolemia induced atherosclerosis," *Redox Biology*, vol. 2, pp. 732–738, 2014.

[64] P. Otero, B. Bonet, E. Herrera, and A. Rabano, "Development of atherosclerosis in the diabetic BALB/c mice. Prevention with vitamin E administration," *Atherosclerosis*, vol. 182, pp. 259–265, 2005.

[65] Z. G. Huang, C. Liang, S. F. Han, and Z. G. Wu, "Vitamin E ameliorates ox-LDL-induced foam cells formation through modulating the activities of oxidative stress-induced NF-kappaB pathway," *Molecular and Cellular Biochemistry*, vol. 363, pp. 11–19, 2012.

[66] S. Gaedicke, X. Zhang, C. Schmelzer et al., "Vitamin E dependent microRNA regulation in rat liver," *FEBS Letters*, vol. 582, pp. 3542–3546, 2008.

[67] L. Barella, P. Y. Muller, M. Schlachter et al., "Identification of hepatic molecular mechanisms of action of alpha-tocopherol using global gene expression profile analysis in rats," *Biochimica et Biophysica Acta*, vol. 1689, pp. 66–74, 2004.

[68] M. C. Podszun, N. Grebenstein, A. Spruss et al., "Dietary alpha-tocopherol and atorvastatin reduce high-fat-induced lipid accumulation and down-regulate CD36 protein in the liver of guinea pigs," *The Journal of Nutritional Biochemistry*, vol. 25, pp. 573–579, 2014.

[69] H. Abdala-Valencia, S. Berdnikovs, F. Soveg, and J. M. Cook-Mills, "Alpha-tocopherol supplementation of allergic female mice inhibits development of CD11c+CD11b+ dendritic cells in utero and allergic inflammation in neonates," *American Journal of Physiology - Lung Cellular and Molecular Physiology*, vol. 307, pp. L482–L496, 2014.

[70] H. Abdala-Valencia, F. Soveg, and J. M. Cook-Mills, "γ-Tocopherol supplementation of allergic female mice augments development of CD11c+CD11b+ dendritic cells in utero and allergic inflammation in neonates," *American Journal of Physiology - Lung Cellular and Molecular Physiology*, vol. 310, pp. L759–L771, 2016.

[71] J. M. Cook-Mills and P. C. Avila, "Vitamin E and D regulation of allergic asthma immunopathogenesis," *International Immunopharmacology*, vol. 23, pp. 364–372, 2014.

[72] M. E. Marchese, R. Kumar, L. A. Colangelo et al., "The vitamin E isoforms alpha-tocopherol and gamma-tocopherol have opposite associations with spirometric parameters: the CARDIA study," *Respiratory Research*, vol. 15, p. 31, 2014.

[73] J. M. Cook-Mills, "Isoforms of vitamin E differentially regulate PKC alpha and inflammation: a review," *Journal of Clinical & Cellular Immunology*, vol. 4, no. 137, 2013.

[74] J. M. Cook-Mills, H. Abdala-Valencia, and T. Hartert, "Two faces of vitamin e in the lung," *American Journal of Respiratory and Critical Care Medicine*, vol. 188, pp. 279–284, 2013.

[75] H. Abdala-Valencia, S. Berdnikovs, and J. M. Cook-Mills, "Vitamin E isoforms differentially regulate intercellular

adhesion molecule-1 activation of PKCalpha in human microvascular endothelial cells," *PLoS One*, vol. 7, article e41054, 2012.

[76] C. A. McCary, H. Abdala-Valencia, S. Berdnikovs, and J. M. Cook-Mills, "Supplemental and highly elevated tocopherol doses differentially regulate allergic inflammation: reversibility of alpha-tocopherol and gamma-tocopherol's effects," *Journal of Immunology*, vol. 186, pp. 3674–3685, 2011.

[77] J. M. Cook-Mills and C. A. McCary, "Isoforms of vitamin E differentially regulate inflammation," *Endocrine, Metabolic & Immune Disorders Drug Targets*, vol. 10, pp. 348–366, 2010.

[78] J. M. Cook-Mills, M. E. Marchese, and H. Abdala-Valencia, "Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants," *Antioxidants & Redox Signaling*, vol. 15, pp. 1607–1638, 2011.

[79] H. Abdala-Valencia and J. M. Cook-Mills, "VCAM-1 signals activate endothelial cell protein kinase Cα via oxidation," *Journal of Immunology*, vol. 177, pp. 6379–6387, 2006.

[80] S. Berdnikovs, H. Abdala-Valencia, C. McCary et al., "Isoforms of vitamin E have opposing immunoregulatory funcitons during inflammation by regulating leukocyte recruitment," *Journal of Immunology*, vol. 182, pp. 4395–4405, 2009.

[81] J. M. Cook-Mills, T. Gebretsadik, H. Abdala-Valencia et al., "Brief research report: interaction of vitamin E isoforms on asthma and allergic airway disease," *Thorax*, vol. 71, pp. 954–956, 2016.

[82] D. Wu, S. N. Han, M. Meydani, and S. N. Meydani, "Effect of concomitant consumption of fish oil and vitamin E on T cell mediated function in the elderly: a randomized double-blind trial," *Journal of the American College of Nutrition*, vol. 25, pp. 300–306, 2006.

[83] D. C. Christiani, T. T. Ye, D. H. Wegman, E. A. Eisen, H. L. Dai, and P. L. Lu, "Pulmonary function among cotton textile workers. A study of variability in symptom reporting, across-shift drop in FEV1, and longitudinal change," *Chest*, vol. 105, pp. 1713–1721, 1994.

[84] R. R. Jacobs, B. Boehlecke, M. van Hage-Hamsten, and R. Rylander, "Bronchial reactivity, atopy, and airway response to cotton dust," *The American Review of Respiratory Disease*, vol. 148, pp. 19–24, 1993.

[85] R. J. Delfino, P. J. Quintana, J. Floro et al., "Association of FEV1 in asthmatic children with personal and microenvironmental exposure to airborne particulate matter," *Environmental Health Perspectives*, vol. 112, pp. 932–941, 2004.

[86] H. Koskela, H. Tukiainen, A. Kononoff, and H. Pekkarinen, "Effect of whole-body exposure to cold and wind on lung function in asthmatic patients," *Chest*, vol. 105, pp. 1728–1731, 1994.

[87] P. D. Blanc, M. D. Eisner, P. P. Katz et al., "Impact of the home indoor environment on adult asthma and rhinitis," *Journal of Occupational and Environmental Medicine*, vol. 47, pp. 362–372, 2005.

[88] A. V. Fedulov and L. Kobzik, "Allergy risk is mediated by dendritic cells with congenital epigenetic changes," *American Journal of Respiratory Cell and Molecular Biology*, vol. 44, pp. 285–292, 2011.

[89] R. H. Lim and L. Kobzik, "Maternal transmission of asthma risk," *American Journal of Reproductive Immunology*, vol. 61, pp. 1–10, 2009.

[90] C. Langlet, C. Springael, J. Johnson et al., "PKC-alpha controls MYD88-dependent TLR/IL-1R signaling and cytokine production in mouse and human dendritic cells," *European Journal of Immunology*, vol. 40, pp. 505–515, 2010.

[91] P. J. Cejas, L. M. Carlson, J. Zhang et al., "Protein kinase C betaII plays an essential role in dendritic cell differentiation and autoregulates its own expression," *The Journal of Biological Chemistry*, vol. 280, pp. 28412–28423, 2005.

[92] Y. F. Lin, H. M. Lee, S. J. Leu, and Y. H. Tsai, "The essentiality of PKCalpha and PKCbetaI translocation for CD14+monocyte differentiation towards macrophages and dendritic cells, respectively," *Journal of Cellular Biochemistry*, vol. 102, pp. 429–441, 2007.

[93] Y. F. Lin, S. J. Leu, H. M. Huang, and Y. H. Tsai, "Selective activation of specific PKC isoforms dictating the fate of CD14(+) monocytes towards differentiation or apoptosis," *Journal of Cellular Physiology*, vol. 226, pp. 122–131, 2011.

[94] K. Asehnoune, D. Strassheim, S. Mitra, J. Yeol Kim, and E. Abraham, "Involvement of PKCalpha/beta in TLR4 and TLR2 dependent activation of NF-kappaB," *Cellular Signalling*, vol. 17, pp. 385–394, 2005.

[95] G. Ramadan, R. E. Schmidt, and J. Schubert, "In vitro generation of human CD86+ dendritic cells from CD34+ haematopoietic progenitors by PMA and in serum-free medium," *Clinical and Experimental Immunology*, vol. 125, pp. 237–244, 2001.

[96] T. A. Davis, A. A. Saini, P. J. Blair et al., "Phorbol esters induce differentiation of human CD34+ hemopoietic progenitors to dendritic cells: evidence for protein kinase C-mediated signaling," *Journal of Immunology*, vol. 160, pp. 3689–3697, 1998.

[97] D. Rajotte, P. Haddad, A. Haman, E. J. Cragoe Jr., and T. Hoang, "Role of protein kinase C and the Na+/H+ antiporter in suppression of apoptosis by granulocyte macrophage colony-stimulating factor and interleukin-3," *The Journal of Biological Chemistry*, vol. 267, pp. 9980–9987, 1992.

[98] B. Salh, K. Hoeflick, W. Kwan, and S. Pelech, "Granulocyte-macrophage colony-stimulating factor and interleukin-3 potentiate interferon-gamma-mediated endothelin production by human monocytes: role of protein kinase C," *Immunology*, vol. 95, pp. 473–479, 1998.

[99] D. C. St Louis, J. B. Woodcock, G. Franzoso et al., "Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model," *Journal of Immunology*, vol. 162, pp. 3237–3248, 1999.

[100] P. J. Cejas, L. M. Carlson, D. Kolonias et al., "Regulation of RelB expression during the initiation of dendritic cell differentiation," *Molecular and Cellular Biology*, vol. 25, pp. 7900–7916, 2005.

[101] M. R. Farren, L. M. Carlson, and K. P. Lee, "Tumor-mediated inhibition of dendritic cell differentiation is mediated by down regulation of protein kinase C beta II expression," *Immunologic Research*, vol. 46, pp. 165–176, 2010.

[102] N. Geijsen, M. Spaargaren, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, and P. J. Coffer, "Association of RACK1 and PKCbeta with the common beta-chain of the IL-5/IL-3/GM-CSF receptor," *Oncogene*, vol. 18, pp. 5126–5130, 1999.

[103] D. Verdelli, L. Nobili, K. Todoerti et al., "Molecular targeting of the PKC-beta inhibitor enzastaurin (LY317615) in multiple myeloma involves a coordinated downregulation of

MYC and IRF4 expression," *Hematological Oncology*, vol. 27, pp. 23–30, 2009.

[104] M. Hamdorf, A. Berger, S. Schule, J. Reinhardt, and E. Flory, "PKCdelta-induced PU.1 phosphorylation promotes hematopoietic stem cell differentiation to dendritic cells," *Stem Cells*, vol. 29, pp. 297–306, 2011.

[105] J. S. Lee, I. S. Kim, J. S. Ryu, and C. Y. Yun, "House dust mite, Dermatophagoides pteronissinus increases expression of MCP-1, IL-6, and IL-8 in human monocytic THP-1 cells," *Cytokine*, vol. 42, pp. 365–371, 2008.

[106] R. Guler, M. Afshar, B. Arendse et al., "PKCdelta regulates IL-12p40/p70 production by macrophages and dendritic cells, driving a type 1 healer phenotype in cutaneous leishmaniasis," *European Journal of Immunology*, vol. 41, pp. 706–715, 2011.

[107] C. A. McCary, Y. Yoon, C. Panagabko, W. Cho, J. Atkinson, and J. M. Cook-Mills, "Vitamin E isoforms directly bind PKCalpha and differentially regulate activation of PKCalpha," *The Biochemical Journal*, vol. 441, pp. 189–198, 2012.

[108] M. F. Mahomoodally, A. Gurib-Fakim, and A. H. Subratty, "Antimicrobial activities and phytochemical profiles of endemic medicinal plants of Mauritius," *Pharmaceutical Biology*, vol. 43, no. 3, pp. 237–242, 2005.

[109] A. K. Pandey, "Anti-staphylococcal activity of a pan-tropical aggressive and obnoxious weed Parihenium histerophorus: an in vitro study," *National Academy Science Letters*, vol. 30, no. 11-12, pp. 383–386, 2007.

[110] K. E. Heim, A. R. Tagliaferro, and D. J. Bobilya, "Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships," *Journal of Nutritional Biochemistry*, vol. 13, no. 10, pp. 572–584, 2002.

[111] S. Kumar, A. Mishra, and A. K. Pandey, "Antioxidant mediated protective effect of Parthenium hysterophorus against oxidative damage using in vitro models," *BMC Complementary and Alternative Medicine*, vol. 13, article 120, 2013.

[112] S. Kumar and A. K. Pandey, "Phenolic content, reducing power and membrane protective activities of Solanum xanthocarpum root extracts," *Vegetos-An International Journal of Plant Research*, vol. 26, pp. 301–307, 2013.

[113] M. Leopoldini, N. Russo, S. Chiodo, and M. Toscano, "Iron chelation by the powerful antioxidant flavonoid quercetin," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 17, pp. 6343–6351, 2006.

[114] S. Kumar, A. Gupta, and A. K. Pandey, "Calotropis procera root extract has capability to combat free radical mediated damage," *ISRN Pharmacology*, vol. 2013, Article ID 691372, 8 pages, 2013.

[115] N. C. Cook and S. Samman, "Review: flavonoids-chemistry, metabolism, cardioprotective effects and dietary sources," *Journal of Nutritional Biochemistry*, vol. 7, no. 2, pp. 66–76, 1996.

[116] C. A. Rice-Evans, N. J. Miller, P. G. Bolwell, P. M. Broamley, and J. B. Pridham, "The relative antioxidant activities of plant-derived polyphenolic flavonoids," *Free Radical Research*, vol. 22, no. 4, pp. 375–383, 1995.

[117] A. K. Pandey, A. K. Mishra, and A. Mishra, "Antifungal and antioxidative potential of oil and extracts derived from leaves of Indian spice plant Cinnamomum tamala," *Cellular and Molecular Biology*, vol. 58, pp. 142–147, 2012.

[118] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, UK, 1998.

[119] A. Mishra, S. Kumar, and A. K. Pandey, "Scientific validation of the medicinal efficacy of Tinospora cordifolia," *The Scientific World Journal*, vol. 2013, Article ID 292934, 8 pages, 2013.

[120] A. A. Ganai, A. A. Khan, Z. A. Malik, and H. Farooqi, "Genistein modulates the expression of NF-κB and MAPK (p-38 and ERK1/2), thereby attenuating d-galactosamine induced fulminant hepatic failure in Wistar rats," *Toxicology and Applied Pharmacology*, vol. 283, pp. 139–146, 2015.

[121] T. B. Clarkson, M. S. Anthony, and T. M. Morgan, "Inhibition of postmenopausal atherosclerosis progression: a comparison of the effects of conjugated equine estrogens and soy phytoestrogens," *The Journal of Clinical Endocrinology and Metabolism*, vol. 86, pp. 41–47, 2001.

[122] M. R. Adams, D. L. Golden, J. K. Williams, A. A. Franke, T. C. Register, and J. R. Kaplan, "Soy protein containing isoflavones reduces the size of atherosclerotic plaques without affecting coronary artery reactivity in adult male monkeys," *The Journal of Nutrition*, vol. 135, pp. 2852–2856, 2005.

[123] J. Yamakoshi, M. K. Piskula, T. Izumi et al., "Isoflavone aglycone-rich extract without soy protein attenuates atherosclerosis development in cholesterol-fed rabbits," *The Journal of Nutrition*, vol. 130, pp. 1887–1893, 2000.

[124] T. Kanazawa, T. Osanai, X. S. Zhang et al., "Protective effects of soy protein on the peroxidizability of lipoproteins in cerebrovascular diseases," *The Journal of Nutrition*, vol. 125, pp. 639S–646S, 1995.

[125] M. J. Tikkanen, K. Wahala, S. Ojala, V. Vihma, and H. Adlercreutz, "Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, pp. 3106–3110, 1998.

[126] H. Wiseman, J. D. O'Reilly, H. Adlercreutz et al., "Isoflavone phytoestrogens consumed in soy decrease F(2)-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans," *The American Journal of Clinical Nutrition*, vol. 72, pp. 395–400, 2000.

[127] T. A. Ryan-Borchers, J. S. Park, B. P. Chew, M. K. McGuire, L. R. Fournier, and K. A. Beerman, "Soy isoflavones modulate immune function in healthy postmenopausal women," *The American Journal of Clinical Nutrition*, vol. 83, pp. 1118–1125, 2006.

[128] J. M. Hodgson, I. B. Puddey, K. D. Croft, T. A. Mori, J. Rivera, and L. J. Beilin, "Isoflavonoids do not inhibit in vivo lipid peroxidation in subjects with high-normal blood pressure," *Atherosclerosis*, vol. 145, pp. 167–172, 1999.

[129] S. Samman, P. M. Lyons Wall, G. S. Chan, S. J. Smith, and P. Petocz, "The effect of supplementation with isoflavones on plasma lipids and oxidisability of low density lipoprotein in premenopausal women," *Atherosclerosis*, vol. 147, pp. 277–283, 1999.

[130] S. Vega-Lopez, K. J. Yeum, J. L. Lecker et al., "Plasma antioxidant capacity in response to diets high in soy or animal protein with or without isoflavones," *The American Journal of Clinical Nutrition*, vol. 81, pp. 43–49, 2005.

[131] C. Choi, H. Cho, J. Park, C. Cho, and Y. Song, "Suppressive effects of genistein on oxidative stress and NFkappaB activation in RAW 264.7 macrophages," *Bioscience, Biotechnology, and Biochemistry*, vol. 67, pp. 1916–1922, 2003.

[132] K. A. Naidu, "Vitamin C in human health and disease is still a mystery? An overview," *Nutrition Journal*, vol. 2, p. 7, 2003.

[133] J. W. Crott and M. Fenech, "Effect of vitamin C supplementation on chromosome damage, apoptosis and necrosis ex vivo," *Carcinogenesis*, vol. 20, no. 6, pp. 1035–1041, 1999.

[134] A. C. Carr and B. Frei, "Does vitamin C act as pro-oxidant under physiological conditions?," *FASEB Journal*, vol. 13, pp. 1007–1024, 1999.

[135] K. Suzuki, H. Koike, H. Matsui et al., "Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3," *International Journal of Cancer*, vol. 99, pp. 846–852, 2002.

[136] M. Raschke, I. R. Rowland, P. J. Magee, and B. L. Pool-Zobel, "Genistein protects prostate cells against hydrogen peroxide-induced DNA damage and induces expression of genes involved in the defence against oxidative stress," *Carcinogenesis*, vol. 27, pp. 2322–2330, 2006.

[137] Y. Takada, A. Mukhopadhyay, G. C. Kundu, G. H. Mahabeleshwar, S. Singh, and B. B. Aggarwal, "Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein tyrosine kinase," *Journal of Biological Chemistry*, vol. 278, no. 26, pp. 24233–24241, 2003.

[138] S. Harakeh, M. Diab-Assaf, J. C. Khalife et al., "Ascorbic acid induces apoptosis in adult T-cell leukemia," *Anticancer Research*, vol. 27, no. 1A, pp. 289–298, 2007.

[139] H. Nakano, A. Nakajima, S. Sakon-Komazawa, J. H. Piao, X. Xue, and K. Okumura, "Reactive oxygen species mediate crosstalk between NF-kappaB and JNK," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 730–777, 2006.

[140] S. Belin, F. Kaya, G. Duisit, S. Giacometti, J. Ciccolini, and M. Fontés, "Antiproliferative effect of ascorbic acid is associated with the inhibition of genes necessary to cell cycle progression," *PLoS One*, vol. 4, no. 2, 2009.

[141] J. A. Migliozzi, "Effect of ascorbic acid on tumour growth," *British Journal of Cancer*, vol. 35, p. 448, 1977.

[142] K. Kishino, K. Hashimoto, O. Amano, M. Kochi, W. Liu, and H. Sakagami, "Tumor-specific cytotoxicity and type of cell death induced by sodium 5,6-benzylidene-l-ascorbate," *Anticancer Research*, vol. 28, pp. 2577–2584, 2008.

[143] Q. Chen, M. G. Espey, A. Y. Sun et al., "Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice," *Proceedings of the National Academy of Science*, vol. 105, no. 32, pp. 11105–11109, 2008.

[144] Q. Chen, M. G. Espey, A. Y. Sun et al., "Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo," *Proceedings of the National Academy of Science*, vol. 104, no. 21, pp. 8749–8754, 2007.

[145] D. R. Richardson and P. Ponka, "The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells," *Biochimica et Biophysica Acta*, vol. 1331, no. 1, pp. 1–40, 1997.

[146] H. W. Hann, A. E. Evans, S. E. Siegel et al., "Prognostic importance of serum ferritin in patients with stages III and IV neuroblastoma: the Children's Cancer Study Group experience," *Cancer Research*, vol. 45, no. 6, pp. 2843–2848, 1985.

[147] L. Shen, H. Y. Zhao, J. Du, and F. Wang, "Anti-tumor activities of four chelating agents against human neuroblastoma cells," *In Vivo*, vol. 19, no. 1, pp. 233–236, 2005.

[148] Q. Chen, M. G. Espey, M. C. Krishna et al., "Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues," *Proceedings of the National Academy of Science*, vol. 102, no. 38, pp. 13604–13609, 2005.

[149] S. H. Bhat, A. S. Azmi, S. Hanif, and S. M. Hadi, "Ascorbic acid mobilizes endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties," *International Journal of Biochemistry and Cell Biology*, vol. 38, pp. 2074–2081, 2006.

[150] N. Kinoshita, T. Yamamura, H. Teranuma et al., "Interaction between dental metals and antioxidants assessed by cytotoxicity assay and ESR spectroscopy," *Anticancer Research*, vol. 22, pp. 4017–4022, 2002.

[151] H. Sakagami, H. Arakawa, M. Haeda et al., "Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants," *Anticancer Research*, vol. 21, pp. 2633–2642, 2001.

[152] A. Vojdani, M. Bazargan, E. Vojdani, and J. Wright, "New evidence for antioxidant properties of vitamin C," *Cancer Detection and Prevention*, vol. 24, no. 6, pp. 508–523, 2000.

[153] E. E. Kelley, F. E. Domann, G. R. Buettner, L. W. Oberley, and C. Patrick Burns, "Increased efficiency of in vitro Photofrin photosensitization of human oral squamous cell carcinoma by iron and ascorbate," *Journal of Photochemistry and Photobiology B: Biology*, vol. 40, pp. 273–277, 1997.

[154] V. Noto, H. S. Taper, Y.-H. Jiang, J. Janssens, J. Bonte, and W. De Loecker, "Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K3) treatment on human tumor cell growth in vitro. 1. Synergism of combined vitamin C and K3 action," *Cancer*, vol. 63, pp. 901–906, 1989.

[155] C. R. Leveille and E. R. Schwartz, "Effect of ascorbate on lysosomal enzyme activities in guinea pig cartilage and adrenals," *International Journal for Vitamin and Nutrition Research*, vol. 52, pp. 436–441, 1982.

[156] T. Harada, A. Enomoto, T. Kitazawa, K. Maita, and Y. Shirasu, "Oral leukoplakia and costochondral hyperplasia induced by diethylnitrosamine in hamsters exposed to cigarette smoke with or without dietary vitamin C," *Veterinary Pathology*, vol. 24, p. 257, 1987.

[157] D. Prochazkova, I. Bousova, and N. Wilhelmova, "Antioxidant and prooxidant properties of flavonoids," *Fitoterapia*, vol. 82, pp. 513–523, 2011.

[158] E. J. Park and J. M. Pezzuto, "Flavonoids in cancer prevention," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 12, pp. 836–851, 2012.

[159] W. F. Hodnick, E. B. Milosavljevic, J. H. Nelson, and R. S. Pardini, "Electrochemistry of flavonoids. Relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids," *Biochemical Pharmacology*, vol. 37, pp. 2607–2611, 1988.

[160] S. I. Choi, C. S. Jeong, S. Y. Cho, and Y. S. Lee, "Mechanism of apoptosis induced by apigenin in HepG2 human hepatoma cells: involvement of reactive oxygen species generated by NADPH oxidase," *Archives of Pharmacal Research*, vol. 30, pp. 1328–1335, 2007.

[161] Y. S. Lee, "Role of NADPH oxidase-mediated generation of reactive oxygen species in the mechanism of apoptosis induced by phenolic acids in HepG2 human hepatoma cells," *Archives of Pharmacal Research*, vol. 28, pp. 1183–1189, 2005.

[162] M. Alhosin, A. J. Leon-Gonzalez, I. Dandache et al., "Bilberry extract (Antho 50) selectively induces redox-sensitive caspase 3-related apoptosis in chronic lymphocytic leukemia cells by targeting the Bcl-2/Bad pathway," *Scientific Reports*, vol. 5, p. 8996, 2015.

[163] J. H. Kim, C. Auger, I. Kurita et al., "Aronia melanocarpa juice, a rich source of polyphenols, induces endothelium-dependent relaxations in porcine coronary arteries via the redox-sensitive activation of endothelial nitric oxide synthase," *Nitric Oxide: Biology and Chemistry*, vol. 35, pp. 54–64, 2013.

[164] T. Sharif, M. Stambouli, B. Burrus et al., "The polyphenolic-rich Aronia melanocarpa juice kills teratocarcinomal cancer stern-like cells, but not their differentiated counterparts," *Journal of Functional Foods*, vol. 5, pp. 1244–1252, 2013.

[165] J. Wang, M. L. Lu, H. L. Dai, S. P. Zhang, H. X. Wang, and N. Wei, "Esculetin, a coumarin derivative, exerts in vitro and in vivo antiproliferative activity against hepatoular carcinoma by initiating a mitochondrial-dependent apoptosis pathway," *Brazilian Journal of Medical and Biological Research*, vol. 48, pp. 245–253, 2015.

[166] J. Yang, Y. L. Xiao, X. R. He, G. F. Qiu, and X. M. Hu, "Aesculetin-induced apoptosis through a ROS-mediated mitochondrial dysfunction pathway in human cervical cancer cells," *Journal of Asian Natural Products Research*, vol. 12, pp. 185–193, 2010.

[167] T. Liang, X. Zhang, W. Xue, S. Zhao, X. Zhang, and J. Pei, "Curcumin induced human gastric cancer BGC-823 s apoptosis by ROS-mediated ASK1-MKK4-JNK stress signaling pathway," *International Journal of Molecular Sciences*, vol. 15, pp. 15754–15765, 2014.

[168] J. D. Lambert and R. J. Elias, "The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention," *Archives of Biochemistry and Biophysics*, vol. 501, pp. 65–72, 2010.

[169] J. T. Hwang, J. Ha, I. J. Park et al., "Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway," *Cancer Letters*, vol. 247, pp. 115–121, 2007.

[170] S. Oikawa, A. Furukawaa, H. Asada, K. Hirakawa, and S. Kawanishi, "Catechins induce oxidative damage to ular and isolated DNA through the generation of reactive oxygen species," *Free Radical Research*, vol. 37, pp. 881–890, 2003.

[171] S. Palit, S. Kar, G. Sharma, and P. K. Das, "Hesperetin induces apoptosis in breast carcinoma by triggering accumulation of ROS and activation of ASK1/JNK pathway," *Journal of Cellular Physiology*, vol. 230, pp. 1729–1739, 2015.

[172] Q. Zhang, G. Cheng, H. Qiu et al., "The p53-inducible gene 3 involved in flavonoid-induced cytotoxicity through the reactive oxygen species-mediated mitochondrial apoptotic pathway in human hepatoma cells," *Food & Function*, vol. 6, pp. 1518–1525, 2015.

[173] G. T. Kim, S. H. Lee, and Y. M. Kim, "Quercetin regulates sestrin 2-AMPK-mTOR signaling pathway and induces apoptosis via increased intracellular ROS in HCT116 Colon cancer cells," *Journal of Cancer Prevention*, vol. 18, pp. 264–270, 2013.

[174] M. Iwasaki, M. Inoue, T. Otani et al., "Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case-control study from the Japan Public Health Center-based prospective study group," *Journal of Clinical Oncology*, vol. 26, pp. 1677–1683, 2008.

[175] S. Jin, Q. Y. Zhang, X. M. Kang, J. X. Wang, and W. H. Zhao, "Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway," *Annals of Oncology*, vol. 21, pp. 263–268, 2010.

[176] Y.-L. Lo, W. Wang, and C. T. Ho, "7,3′,4′-Trihydroxyisoflavone modulates multidrug resistance transporters and induces apoptosis via production of reactive oxygen species," *Toxicology*, vol. 302, pp. 221–232, 2012.

[177] X. J. Yang, A. Belosay, J. A. Hartman et al., "Dietary soy isoflavones increase metastasis to lungs in an experimental model of breast cancer with bone micro-tumors," *Clinical & Experimental Metastasis*, vol. 32, pp. 323–333, 2015.

[178] S. Rakshit, L. Mandal, B. C. Pal et al., "Involvement of ROS in chlorogenic acid-induced apoptosis of Bcr-Abl+ CML cells," *Biochemical Pharmacology*, vol. 80, pp. 1662–1675, 2010.

[179] K. K. Kim, A. P. Singh, R. K. Singh et al., "Anti-angiogenic activity of cranberry proanthocyanidins and cytotoxic properties in ovarian cancer cells," *International Journal of Oncology*, vol. 40, pp. 227–235, 2012.

[180] C. Luo, Y. Li, H. Wang et al., "Hydroxytyrosol promotes superoxide production and defects in autophagy leading to anti-proliferation and apoptosis on human prostate cancer cells," *Current Cancer Drug Targets*, vol. 13, pp. 625–639, 2013.

[181] L. J. Sun, C. Luo, and J. K. Liu, "Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation," *Food & Function*, vol. 5, pp. 1909–1914, 2014.

[182] P. Guha, A. Dey, R. Sen, M. Chatterjee, S. Chattopadhyay, and S. K. Bandyopadhyay, "Intracellular GSH depletion triggered mitochondrial Bax translocation to accomplish resveratrol-induced apoptosis in the U937 cell line," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 336, pp. 206–214, 2011.

[183] M. Alhosin, T. Sharif, M. Mousli et al., "Down-regulation of UHRF1, associated with re-expression of tumor suppressor genes, is a common feature of natural compounds exhibiting anti-cancer properties," *Journal of Experimental & Clinical Cancer Research*, vol. 30, 2011.

[184] M. Achour, M. Mousli, M. Alhosin et al., "Epigallocatechin-3-gallate up-regulates tumor suppressor gene expression via a reactive oxygen species-dependent down-regulation of UHRF1," *Biochemical and Biophysical Research Communications*, vol. 430, pp. 208–212, 2013.

[185] J. Kang, J. Chen, Y. Shi, J. Jia, and Y. Zhang, "Curcumin-induced histone hypoacetylation: the role of reactive oxygen species," *Biochemical Pharmacology*, vol. 69, pp. 1205–1213, 2005.

[186] P. Rajendran, E. Ho, D. E. Williams, and R. H. Dashwood, "Dietary phytochemicals, HDAC inhibition, and DNA damage/repair defects in cancer cells," *Clinical Epigenetics*, vol. 3, p. 4, 2011.

[187] M. Remely, L. Lovrecic, A. L. de la Garza et al., "Therapeutic perspectives of epigenetically active nutrients," *British Journal of Pharmacology*, vol. 172, pp. 2756–2768, 2015.

[188] W. Vanden Berghe, "Epigenetic impact of dietary polyphenols in chemoprevention: lifelong remodeling of our epigenomes," *Pharmacological Research*, vol. 65, pp. 565–576, 2012.

[189] S. Malireddy, S. R. Kotha, J. D. Secor et al., "Phytochemical antioxidants modulate mammalian ular epigenome: implications in health and disease," *Antioxidants & Redox Signaling*, vol. 17, pp. 327–339, 2012.

[190] T. P. Ong, F. S. Moreno, and S. A. Ross, "Targeting the epigenome with bioactive food components for cancer prevention," *Journal of Nutrigenetics and Nutrigenomics*, vol. 4, pp. 275–292, 2011.

[191] T. Nakazato, K. Ito, Y. Miyakawa et al., "Catechin, a green tea component, rapidly induces apoptosis of myeloid leukemic cells via modulation of reactive oxygen species production in vitro and inhibits tumor growth in vivo," *Haematologica*, vol. 90, pp. 317–325, 2005.

[192] J. C. Jeong, S. W. Jang, T. H. Kim, C. H. Kwon, and Y. K. Kim, "Mulberry fruit (Moris fructus) extracts induce human glioma cell death in vitro through ROS-dependent mitochondrial pathway and inhibits glioma tumor growth in vivo," *Nutrition and Cancer*, vol. 62, pp. 402–412, 2010.

[193] P. Dent, A. Yacoub, J. Contessa et al., "Stress and radiation-induced activation of multiple intracellular signaling pathways," *Radiation Research*, vol. 159, no. 3, pp. 283–300, 2003.

[194] E. Mladenov, S. Magin, A. Soni, and G. Iliakis, "DNA double-strand-break repair in higher eukaryotes and its role in genomic instability and cancer: cell cycle and proliferation-dependent regulation," *Seminars in Cancer Biology*, vol. 37-38, pp. 51–64, 2016.

[195] W. P. Roos, A. D. Thomas, and B. Kaina, "DNA damage and the balance between survival and death in cancer biology," *Nature Reviews Cancer*, vol. 16, no. 1, pp. 20–33, 2016.

[196] J. F. Ward, "DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 35, pp. 95–125, 1988.

[197] M. O'Driscoll and P. A. Jeggo, "The role of double-strand break repair—insights from human genetics," *Nature Reviews Genetics*, vol. 7, no. 1, pp. 45–54, 2006.

[198] S. P. Jackson and J. Bartek, "The DNA-damage response in human biology and disease," *Nature*, vol. 461, no. 7267, pp. 1071–1078, 2009.

[199] M. Tubiana, "The role of local treatment in the cure of cancer," *European Journal of Cancer*, vol. 28A, pp. 2061–2069, 1992.