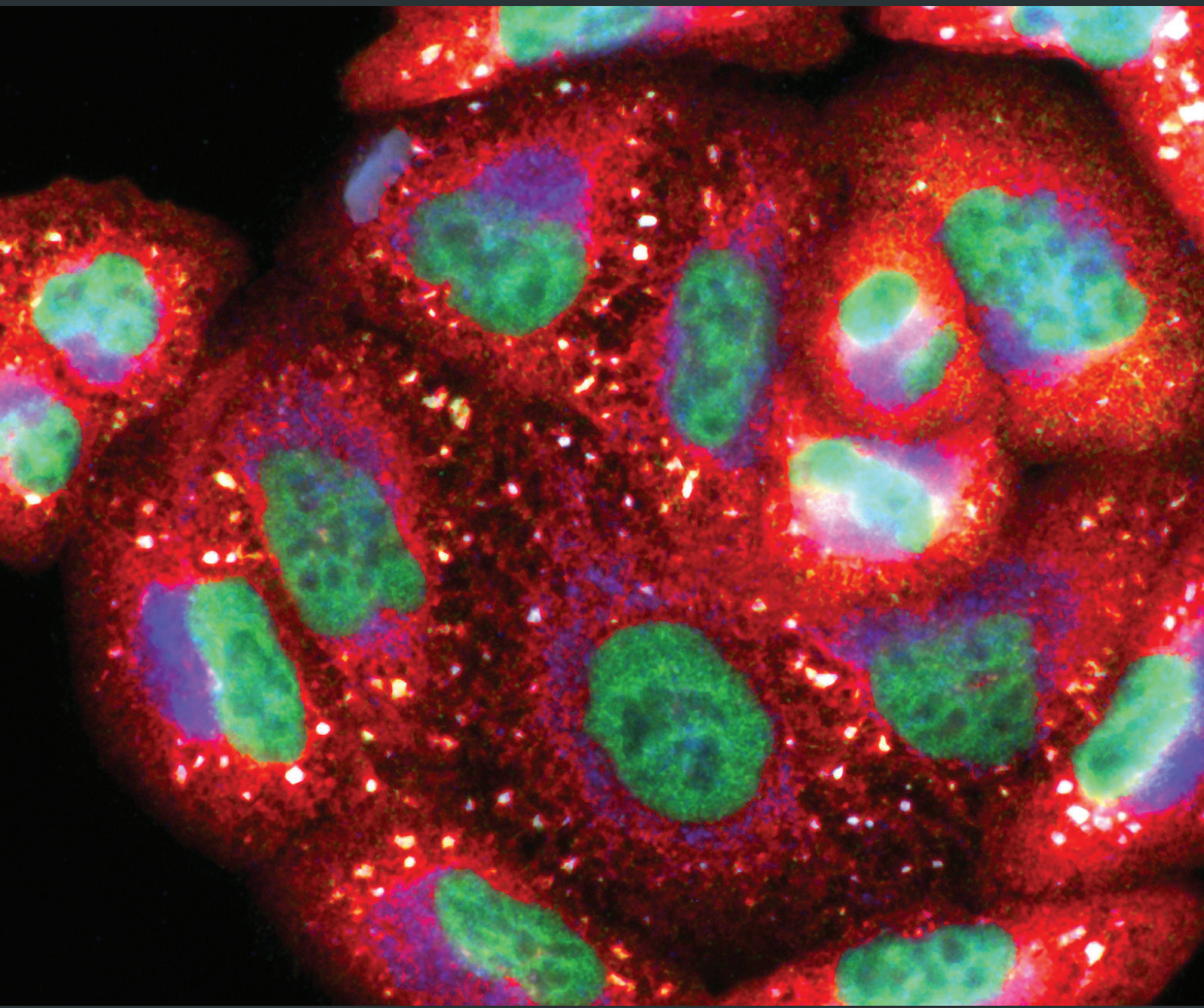
Oxidative Medicine and Cellular Longevity

Modulation of Oxidative Stress: Pharmaceutical and Pharmacological Aspects

Guest Editors: Liudmila Korkina, Tomris Ozben, and Luciano Saso



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Editorial Board

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Editorial

Modulation of Oxidative Stress: Pharmaceutical and Pharmacological Aspects

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Notwithstanding the fact that the multiple roles of oxidative stress in human biology and pathology have been intensely discussed over the last half century, the problem is still far beyond our full comprehension. Thus, in a comparatively short history of oxidative medicine, the roles of two major heroes, free radicals and antioxidants, have been entirely redefined. Free radicals and other reactive oxygen and nitrogen species, widely recognized two-three decades ago as absolute evils leading to and/or accompanying damage to biologically important molecules and structures, have been recently transformed into positive actors, in the appreciation of their essential impact in the intracellular signaling on the organism's defense against biotic and abiotic stresses. Several original research papers published in this special issue have been focused on this subject.

The evidence for detrimental cross interaction of reactive oxygen and nitrogen species was shown in a single clinical case report of rare human disease (Leber's Hereditary Optic Neuropathy (M. Falabella et al.)). The chronic change in the NO homeostasis and enhanced superoxide availability contributed to dysfunction of peripheral blood mononuclear cells derived from a patient due to a cooperative action of nitrosative and oxidative stresses in driving on the genetically determined pathology.

Relations between endothelial nitric oxide synthase genotypes and oxidative stress markers in patients with larynx cancer were studied in an original research (K. Yanar et al.). The results indicated a potential relationship among G894T polymorphism of NOS3 and impaired redox homeostasis that may influence the risk of laryngeal cancer.

An interesting mechanism of adaptation to oxidative stress evolved in enterohemorrhagic *Escherichia coli*, consisting of upregulation of Shiga toxin production by prophages of the bacteria in response to H_2O_2 excretion by infected human neutrophils, was described by K. Licznerska and coauthors. The intestinal haemorrhage induced by this type of bacteria depends mainly on Shiga toxin cytotoxicity. This way enterohemorrhagic *Escherichia coli* become tolerant to natural redox-based antibacterial defense of the host organism. One could assume that the modulation of hydrogen peroxide production by human neutrophils could be a promising strategy against this type of bacterial virulence/toxicity. The role of the *exo-xis* region of the bacterial genome in the induction of Shiga toxin-converting prophages is further elucidated by the same group of authors (K. Licznerska and coauthors).

Unfortunately, the great hope that direct antioxidants could be the panacea resolving practically all health problems has vanished, due to the growing number of inconclusive or negative data from epidemiological and clinical studies. The current state of uncertainty regarding feasibility of antioxidant therapy is partly due to pitfalls of biologically relevant and reliable methods of determination of free radical scavenging and antioxidant capacities of isolated substances and compositions. The paper by M. Colmán-Martínez and

coauthors suggests a simple and accurate method for simultaneous quantification of carotenes, xanthophylls, and retinol in human plasma based on reversed phase high-performance liquid chromatography coupled with diode array detector (HPLC-DAD).

An innovative approach to modulate and maintain normal redox profile in order to achieve desirable therapeutic effects has recently become a leading one in the management of a variety of redox-dependent pathologies with unmet as yet therapeutic needs. Thus, comprehensive review by V. Chiurchiù and coauthors attempted to answer the question whether modulation of oxidative stress could be therapeutically feasible in the treatment of severe neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, multiple sclerosis, and hereditary spastic paraplegia. Scrupulously analyzing redox-connected mechanisms underlying the pathogenesis and multiple redox fingerprints characteristic for these diseases, authors came to the conclusion that antioxidants seem to limit the oxidative tissue damage through inhibition of inflammatory events or exerting neuroprotective properties. They also emphasize that the main problem is embodied by the necessity to develop antioxidants that are able to cross the blood-brain barrier. The authors strongly consider that targeted specific redox modulators like dimethyl fumarate rather than conventional dietary antioxidants represent a novel avenue in the management of these human diseases.

Within the same line, the effects of various pharmaceuticals, nutraceuticals, some novel potential pharmacological approaches, and physical exercise on hypercholesterolemia-induced oxidative/nitrative stress and subsequent cardiac dysfunction are discussed in the review (C. Csonka et al.). The authors are focused on 3 different approaches: (i) cholesterol-lowering therapies which attenuate oxidative/nitrosative stress; (ii) combined cholesterol-lowering and antioxidant protocols; and (iii) inducers of endogenous antioxidants or inhibitors of prooxidant enzymes as promising drugs for the prevention and/or treatment of cholesterol-induced cardiac dysfunction. Among pharmaceuticals, statins, Ezetimibe, niacin, fibrates, and an antidiabetic drug Rosiglitazone are discussed in detail. As potent nutraceuticals, antioxidant vitamins, coenzyme Q10, flavonoids (rutin, quercetin, naringin, etc.), green tea catechins, and resveratrol are listed. Novel modulators of miRNAs leading to attenuation of hypercholesterolemia-induced oxidative/nitrosative stress are under development and investigation.

The review article from the Chinese group (Z.-W. Zhang et al.) highlights the potential of mitochondrion-permeable antioxidants in the management of oxidative burst-mediated acute inflammatory conditions. The authors compare and evaluate well-known mitochondrion-permeable antioxidants, such as edaravone, idebenone, alpha-lipoic acid, carotenoids, vitamin E, and coenzyme Q10. In addition, they focus on mitochondria-targeted MitoQ and SkQ antioxidants and propose astaxanthin, a carotenoid, for acute inflammatory conditions characterized by pathologically increased oxidative burst, for example, avian influenza with acute respiratory distress syndrome and ischemia-reperfusion. The limitations of the antioxidant use including adverse health effects under such acute conditions are discussed.

Negative conclusions were drawn (M. Graziani and coauthors) on feasibility of adjuvant therapy of cardiovascular and hepatic toxicity of cocaine with modulators of oxidative stress (N-acetylcysteine, SOD mimetics, nitroxides and nitrones, mitochondria targeting antioxidants, and NADPH oxidase and xanthine oxidase inhibitors,) notwithstanding multiple redox fingerprints in the toxicity of drug abuse. The discrepancy was ascribed to pitfalls in the clinical studies design, to the use of single instead of several targeted oxidative stress modulators, and to the lack of specific, reliable, and reproducible markers of oxidative stress.

An outstanding comprehensive review by N. Sallam and I. Laher concentrates on recent data and modern hypotheses on how physical exercise modulates oxidative stress and inflammation in ageing and cardiovascular diseases. A necessity for strictly individualized programs of age and cardiovascular pathology preventive physical exercises is based on the individual sensitivity to the type, intensity, frequency, and duration of physical challenge. As target organs for protective redox balancing and anti-inflammatory exercises, there are adipose tissue, skeletal muscles, immune system, and cardiovascular system components. The following molecular pathways mediate therapeutic and/or prophylactic effects of physical exercise: redox-sensitive transcription factors, pro- and anti-inflammatory cytokines, antioxidant and prooxidant enzymes, and repair proteins.

Another review (A. Cort et al.) describes in detail a complex redox pattern underlying huge multiple drug resistance (MDR) problem in antitumour therapies as well as the development and clinical use of oxidative stress modulators to combat MDR, thus enhancing efficacy of anticancer protocols. In the authors' opinion, redox active drugs (pro- or antioxidants) targeting an axis consisting of drug transporters, aryl hydrocarbon receptor, phase I/II metabolic enzymes, and the inducible Nrf2-linked pathway could provide a valid and promising way to overcome a dreadful obstacle of MDR in cancer therapies.

Reflecting the general state of the art in the development of novel biologically available antioxidants/redox modulators, a majority of original research papers dedicated to the testing of their pharmacological properties have been presented in the *in vitro* systems or *in vivo* animal experiments. The effects of the clinically used copper chelator D-penicillamine in the catecholamine model of acute myocardial injury were tested in cardiomyoblast cell line H9c2 and in Wistar Han rats (M. Říha et al.). D-Penicillamine had a protective effect against catecholamine-induced injury both *in vitro* and *in vivo*. The classical NOX inhibitors apocynin and diphenyleneiodonium impaired proliferation of cultivated mouse embryonic stem cells (J. Kučera et al.) that clearly reflected essential role(s) of NOX-produced reactive oxygen species as signalling agents for embryonic cell growth and differentiation. Preexposure of UV-B-irradiated human immortalised keratinocyte cell line (HaCaT) to complexed lycopene (A. Ascenso et al.), a skin located antioxidant, led to a shift of the ratio dead : apoptotic : viable subpopulations towards normal values. The authors concluded that the complexed

lycopene might have protective or cytotoxic effects in photodamaged and preneoplastic keratinocytes, while allowing other keratinocytes to accelerate repairing mechanisms and remain viable. A broad group of 1,4-dihydropyridine derivatives as long time known compounds with antioxidant potential have been extensively evaluated during last three decades. A. Velena and coauthors attempt to explain the innovative interest in these agents for biomedical applications. Examples of protective and antioxidant properties of 1,4-dihydropyridine derivatives have been provided in a number of recent *in vitro* studies on low density lipoproteins, mitochondria, microsomes, isolated cells, and cell cultures.

As expected, submissions of clinical data were scarce. Only one paper (C. De Luca and coauthors) depicted results of the clinical-laboratory study on the skin antiageing and systemic redox effects of supplementation with the composition of marine collagen peptides and plant-derived skin-targeting antioxidants (coenzyme Q_{10} + grape skin extract + luteolin + selenium). The data obtained clearly show the improvement of skin properties (elasticity, sebum production, biological age, and dermal ultrasound markers) due to enhanced collagen synthesis without risk of oxidative stress that is usually connected with the synthesis. Moreover, hormesis-like action of the supplementation allowed suggesting this mechanism to be responsible for its antiageing and energizing effects.

Due to the limited space of the special issue, we were not able to concentrate on the issues of extreme importance for discovery and development of safe and clinically efficient redox modulators, such as optimization of delivery systems, pharmacokinetics and metabolism, redox activity of metabolites, and biological markers for the assessment of *in vivo* prooxidant/antioxidant action correlating with clinical outcomes.

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Clinical Study

Skin Antiageing and Systemic Redox Effects of Supplementation with Marine Collagen Peptides and Plant-Derived Antioxidants: A Single-Blind Case-Control Clinical Study

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Recently, development and research of nutraceuticals based on marine collagen peptides (MCPs) have been growing due to their high homology with human collagens, safety, bioavailability through gut, and numerous bioactivities. The major concern regarding safety of MCPs intake relates to increased risk of oxidative stress connected with collagen synthesis (likewise in fibrosis) and to ROS production by MCPs-stimulated phagocytes. In this clinical-laboratory study, fish skin MCPs combined with plant-derived skin-targeting antioxidants (AO) (coenzyme Q_{10} + grape-skin extract + luteolin + selenium) were administered to volunteers ($n = 41$). Skin properties (moisture, elasticity, sebum production, and biological age) and ultrasonic markers (epidermal/dermal thickness and acoustic density) were measured thrice (2 months before treatment and before and after cessation of 2-month oral intake). The supplementation remarkably improved skin elasticity, sebum production, and dermal ultrasonic markers. Metabolic data showed significant increase of plasma hydroxyproline and ATP storage in erythrocytes. Redox parameters, GSH/coenzyme Q_{10} content, and GPx/GST activities were unchanged, while NO and MDA were moderately increased within, however, normal range of values. *Conclusions*. A combination of MCPs with skin-targeting AOs could be effective and safe supplement to improve skin properties without risk of oxidative damage.

1. Introduction

Dietary vitamins, plant-derived polyphenols, fatty acids, proteins, essential amino acids, and trace elements have demonstrated beneficial effects on skin health and appearance [1–3]; hence, the use of nutraceuticals targeting skin is steadily growing. For example, photoprotection by intake of dietary antioxidants has been a subject of numerous *in vitro*, animal, and human studies [4, 5]. Within this direction, the search for reliable and effective antiageing remedies for both topical and systemic administration has become a "hot spot" for cosmetic, food, and biomedical companies. One of the strongest limitations for skin-targeting plant-derived dietary substances with potential antiageing efficacy is their low bioavailability due to limited and selective penetration through the intestinal barrier, destruction by the intestinal microorganisms, high rate of metabolism, and preferential distribution between tissues and organs [6, 7].

Marine fish collagen and collagen peptides have been widely used as functional foods or dietary supplements due to their homology to human collagen structure [8], safety profile [9], stability, biocompatibility, high bioavailability through gastrointestinal barrier [10], and potent bioactivities [11]. Marine collagen peptides (MCPs) obtained by enzymatic digestion of fish skin have been shown to exert several health effects mainly in two directions: metabolic disorders and

skin/bone repair. Thus, they positively affected glucose and lipid metabolism in patients with type II diabetes mellitus [12], improved lipid metabolism in obese people [13] and genetically modified mice [14], ameliorated early alcoholic liver injury [15], and possessed hypotensive and lipid normalising action in patients with primary hypertension [16]. A great majority of publications demonstrated significant wound healing efficacy of orally administered MCPs in animal models of excision and full-thickness skin wounds [10, 17, 18]. Recently, collagen peptides isolated by enzymatic digestion from fish, bovine, and porcine skin as well as from chicken and bovine cartilage have drawn particular interest for the treatment of patients with osteoarthritis. Several clinical trials showed that MCPs were safe and provided an improvement in terms of pain and functions in such patients [19]. From mechanistic point of view, the oral intake of MCPs stimulated the synthesis of extracellular matrix (ECM) macromolecules such as endogenous collagen, by upregulating gene expression of several collagen-modifying enzymes involved in posttranslational collagen modification and cross-linking [20]. Several *in vitro* studies have shown antioxidant properties of very-low-molecular-weight (1–20 Da) MCPs [21, 22] containing proline, which is a scavenger of hydroxyl radicals. Of importance for the present study, MCPs are considered antiageing compounds because they seem to increase life span in rats by inhibiting spontaneous tumour incidence [9], possess photoprotective and immunomodulating properties [23–25], and improve/eliminate signs of premature senescence of human skin [26, 27].

The major concern regarding safety and clinical feasibility of regular intake of MCPs has been raised from the well established fact that the induction of collagen synthesis, mainly assessed by the increased hydroxyproline levels, is often associated with oxidative stress [28–30]. Moreover, MCPs of different origin have been shown to activate innate immune response of macrophages and neutrophils through Toll-like receptor 4, which leads to NADPH-oxidase (NOX4) activation and reactive oxygen species overproduction [31, 32]. A newly developed composition of MCPs with a complex of essential skin-targeting antioxidants, that is, coenzyme Q_{10} + selenium + luteolin + grape-skin extract, demonstrated UVA-protective effects in the preliminary *in vitro* experiments on human skin biopsies [25]. However, the composition under commercial name of CELERGEN® has never been evaluated clinically when administered as a food supplement.

The goal of the present clinical-laboratory study was to elucidate the effects of the oral administration of CELERGEN on skin physiology and dermal collagen deposition in the group of healthy middle-aged subjects with clinical signs of skin ageing. The cutaneous clinical-instrumental data were compared with the systemic metabolic parameters of collagen synthesis, redox balance, and energy storage. For the first time, we demonstrated (i) remarkable improvement of ageing skin physiology and structure, which corresponded to enhanced systemic markers of collagen synthesis; (ii) systemic redox balance, sustained by the antioxidant complex; and (iii) increased systemic energy storage. We also hypothesised that moderately increased plasmatic levels of nitric oxide (NO) and malonyl dialdehyde (MDA) may play positive roles of mediators in the MCPs-induced collagen and ATP synthesis/storage, as well as in sebum production. On these grounds, we suggested that selected antioxidants targeting the distinct organs/tissues should be essential components of MCPs-containing nutraceuticals for more effective, individualised, and safe supplementation.

2. Materials and Methods

2.1. Patients. The study enrolled a group of 41 adult healthy Caucasian volunteers of both sexes recruited from the Beauty Institute on Arbat (Moscow, Russia) staff (age 37–72 years; mean age 50.6 ± 10.4 years; 5 males and 36 females) following the exclusion and inclusion criteria for an open single-blind clinical study. The inclusion criteria were as follows: (i) healthy white adult subjects of both sexes, 35–75 years of age, (ii) subjects with visible symptoms of aged facial skin, (iii) subjects who agreed to interrupt any intake of antioxidant nutraceuticals/drugs for at least 1 week before and during the entire duration of the trial, and (iv) subjects without any difficulty to understand and follow the clinical investigator instructions. Pregnant and breastfeeding women, subjects with allergic/intolerance reactions to any component of the tested product, subjects on any other nutraceutical interventions or/and therapies, and subjects simultaneously engaged in other clinical trials were excluded from the study. The participants were informed that they could interrupt clinical trial at any moment, without any explanation of causative reason for their action, or in case they noticed any adverse reaction to the tested product or had any sensation that the product intake affected their appearance negatively.

The protocol of the clinical trial was duly analysed and approved by the Ethical Committee of the Beauty Institute on Arbat, Moscow, Russia (number 11/EK-2014). All recruited subjects gave their informed consent to personal and anamnestic data collection and biological material sampling. The guidelines of Helsinki Declaration for human experimentation were strictly followed during the conduct of the clinical trial.

2.2. Food Supplement under Investigation. Food supplement containing marine collagen peptides derived from skin of deep sea fish (MCPs, 570 mg), grape-skin extract (10 mg), coenzyme Q_{10} of plant origin (10 mg), luteolin (10 mg), and selenium (0.05 mg) of plant origin was formulated in soft gelatine capsules. As inactive solvents, refined and partly hydrogenated soybean oil as well as small admixture of pure soybean lecithin were used. The product, under the commercial name of CELERGEN (manufacturer: Laboratories-Dom, Carouge, Switzerland), was kindly provided by Suisse Ueli Corporation. According to the manufacturer's information, the deep sea fish sources, that is, *Pollachius virens*, *Hippoglossus hippoglossus*, and *Pleuronectes platessa*, originated from the French coast of the North Sea.

Fish skin was homogenised in distilled water, with addition of complex proteases. The enzymatic proteolytic process was carried out at 40°C and pH 8.0 for 3 h, after which the proteases were inactivated by short-term heating (56°C for 10 min). The liquid was sterilised by Millipore

filtration (pore size 0.02 mm) and spray-dried to prepare MCP powder, as described in detail previously [17, 33]. Chemical analysis by Kjeldahl assay of the powder confirmed a >90% content of collagen peptides, with moisture and ash content <10%. According to previous publications [10, 33, 34], the molecular weight distribution of MCPs after the described enzymatic digestion process was within the range of 10–60 Da, and MCPs were enriched in glycine, glutamine, proline, hydroxyproline, asparagine, alanine, and arginine.

The aqueous extract of grape skin was obtained from *Vitis vinifera* Linn. fruit and contained at least 70% of polyphenols and 20% of procyanidins as per UV/Vis spectrophotometry data. The coenzyme Q_{10} component of plant origin was of highest purity (100 ± 3%), confirmed by both IR spectrophotometry and high performance liquid chromatography (HPLC) methods. Food-quality luteolin was extracted from Marigold plant petals, and the extract contained 20% of luteolin and 1% of zeaxanthin evaluated by HPLC analysis. Selenium in the form of selenite (according to gravimetric method) was extracted from plant bulbs and leaves. Acute and chronic toxicity data and documents of Certificates of Analyses, Security, and Registration in Switzerland were duly provided by the manufacturer.

2.3. Clinical Study Design. The entire trial duration was 4 months (May–December 2014), that is, 2 months of pretreatment period, followed by 2 months of treatment with the test nutraceutical administration. The facial skin parameters of recruited volunteers were analysed three times: at the first visit (enrollment), at the second visit 2 months after the pretreatment period, and at the third visit immediately after the treatment period. Each assessment session comprised instrumental methods for measuring skin physiology parameters and ultrasound properties of the skin layers. This design allowed us to use the same subject as a control and an experiment. During the treatment period, the volunteers were recommended to take 2 capsules of CELERGEN a day (at breakfast and dinner time) for 60 consecutive days. At the second and third visits, the participants donated 20 mL of venous blood after overnight fasting and test tubes were coded by the principal clinical investigator. Blood samples were routinely processed for general haematology (haemoglobin content, differential cell count, and the rate of erythrocyte sedimentation) and biochemistry (glucose levels, plasma protein and lipid profiles, transaminases activities, and C-reactive protein content). Laboratory operators carried out analytical determinations blindly, and statistician was not informed which set of analyses was done in the control or experimental periods, hence ranking this study of clinical efficacy of the nutraceutical as "a single-blind" clinical investigation.

2.4. Assessment of Skin Physiology Parameters. Several physiological parameters, mainly barrier properties, of the facial skin were assessed by appropriate SOFT PLUS TOP probes, with microcamera visual analysis and patented computerised programs (Callegari, Parma, Italy). Skin elasticity was determined by the elastometric approach used in the SOFT PLUS technique. The transepidermal water loss (TEWL), an index of skin moisture, was assessed with Tewameter, which measures the water evaporation through cutaneous levels. When the skin is aged or damaged, the barrier properties of the skin are affected, with increased water evaporation and reduced skin hydration. Sebum content was measured by the SOFT PLUS sebometric probe.

2.5. Assessment of Ultrasound Properties of the Skin. Assessment of ultrasound properties of the skin was performed by a digital ultrasound imaging system DUB CUTIS (Digital Ultraschall Bildsystem, Germany), which allowed determining four parameters simultaneously: epidermal and dermal thickness and epidermal and dermal ultrasonic density. The first two parameters are indirect markers of collagen (dermis) and lipid (epidermis) synthesis and retention while the second pair of parameters characterises the evenness and order in the epidermal and dermal structures, respectively. The elastic properties of the skin were additionally analysed by a TPM system containing elastometric sensor (22 MHz) which combines digital ultrasound examination with an imaging record (DUB CUTIS, Germany). A computerised multifunctional diagnostic tool integrating different morphometric parameters (epidermal thickness, tone, wrinkles, and elasticity) for face skin biological age determination was used (SOFT PLUS TOP, Callegari, Parma, Italy).

2.6. Reagents and Assay Kits. The majority of chemical reagents, HPLC standards, mediums, solvents, and luciferin-luciferase for ATP assay were from Sigma Chemical Co. (St. Louis, MO, USA); kits for enzyme activity assays and Griess reagent for nitrites/nitrates determination were from Cayman Chemical Company (Ann Arbor, MI, USA). Manufacturers of other reagents are mentioned within the respective methods.

2.7. Redox and Oxidation Markers' Studies. Complete differential blood cell counts and metabolic analyses were performed on fresh ethylenediaminetetraacetic acid- (EDTA-) anticoagulated venous blood of 12 hrs fasting subjects. Biochemical assays were performed on peripheral blood plasma or red blood cells (RBC), either immediately (ATP, glutathione, and coenzyme Q_{10}) or within 72 hrs, on sample aliquots stored at −80°C under argon. Plasma levels of nitrites/nitrates (NO_2^-/NO_3^-, expressed as μmoles/L) were measured spectrophotometrically by Griess reagent [35]. Protein content was measured according to Bradford [36], using a microplate assay kit (Bio-Rad, Hercules, CA, USA). Total glutathione (reduced + oxidized glutathione, GSH + GSSG, mg/g Hb) levels in erythrocytes were measured by HPLC (Shimadzu Scientific Instruments, Columbia, MD, USA) according to Reed et al. [37]. Total coenzyme Q_{10} ($CoQ_{10}H_2$ + CoQ_{10}, mg/L) levels in plasma were quantified by HPLC as described previously [38]. In brief, 1 mL plasma sample, with adequate amount of coenzyme Q_9 (internal standard) and 500 μL acetic acid (50% solution), was extracted twice, first with 3.5 mL and then with 2.5 mL of ethanol/hexane mixture (2 : 5 vol/vol), with homogenisation and subsequent

Table 1: Subjective evaluation of the 2-month food supplement administration effects, by participants ($n = 41$).

Parameter	Number (%) of participants		
	Improvement	No effect	Aggravation
General health conditions	21 (51%)	20 (49%)	0 (0%)
Stamina/muscle strength/joint motility	15 (36%)	26 (64%)	0 (0%)
Digestive system	0 (0%)	41 (100%)	0 (0%)
Skin conditions	25 (61%)	16 (39%)	0 (0%)

centrifugation. The upper phase containing hexane extract was evaporated under nitrogen flux and then resuspended in an adjusted amount of a methanol/isopropanol (3 : 2 vol/vol) mixture for HPLC analysis. Reduced and oxidized forms of coenzyme Q_{10} ($CoQ_{10}H_2$ and CoQ_{10}) were quantified simultaneously with HPLC equipped with analytical Supelcosil LP-18 column (24 cm × 4.6 mm, 5 μm, Supelco, Bellefonte, PA, USA) plus its guard column, and in line photodiode array and electrochemical detector (ESA CoulArray, Bedford, MA, USA) in accord with previously published methods [39, 40]. The clinical normality range was extrapolated from the above publications.

Plasmatic Cu,Zn-superoxide dismutase 3 (Cu,Zn-SOD3, U/g protein) activity was measured spectrophotometrically at 505 nm using appropriate kit from Cayman Chemical Company (Ann Arbor, MI, USA) [41, 42]. RBC were lysed in hypotonic solution and the postspin cell lysates were analysed. Total RBC glutathione-S-transferase (GST, U/mg Hb) activity was measured spectrophotometrically by the methods described previously, using chloro-2,3-dinitrobenzene as substrate [43]. RBC glutathione peroxidase (GPx, U/g Hb) activity was determined using Cayman Chemical kit, according to the method [44].

Plasma levels of MDA were determined by slightly modified spectrophotometric analysis of thiobarbituric acid-reactive substances (TBARS) described elsewhere [45]. After a 15 min treatment of plasma (200 μL) with trichloroacetic (1.22 M) and hydrochloric (0.6 M) acids, alkaline solution of TBA was added and the mixture was boiled for 30 min. TBARS were extracted with butanol and analysed spectrophotometrically at 535 nm. The results were expressed in μM of MDA using the appropriate calibration curve.

2.8. ATP Measurement in Erythrocytes. 100 μL of erythrocyte pellet was stored on ice until analysis. Ice-cold water (990 μL) was added to 10 μL of the erythrocytes pellet and mixed and the lysed erythrocytes were kept on ice. The principle of ATP assay is based on the quantitative bioluminescent determination of *adenosine 5′-12 triphosphate (ATP), assessed by the Bioluminescence Assay Kit*. In the assay, ATP is consumed when firefly luciferase catalyses the oxidation of D-luciferin to adenyl-luciferin which, in the presence of oxygen, is converted to oxyluciferin with light emission. This second reaction is essentially irreversible. When ATP is the limiting reagent, the light emitted is proportional to the ATP present. The measurements of luciferin-luciferase chemiluminescence were performed on a Victor2 1420 multilabel counter, equipped with Wallac 1420 Software (Perkin Elmer, MA, USA). Results were expressed as mmoles/L.

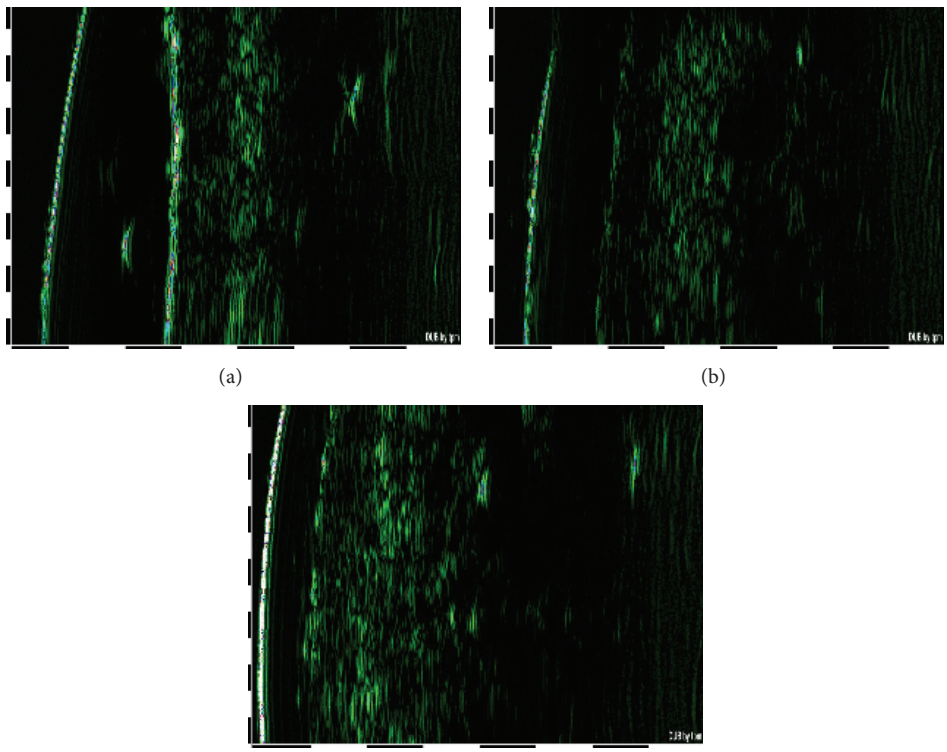
2.9. Hydroxyproline Assay. The plasma levels of free hydroxyproline (Hyp) and hydroxyproline in the form of oligopeptides, mainly proline-hydroxyproline, were determined by a chemical colorimetric method using a commercial kit (Hydroxyproline Detection Kit) in accord with the manufacturer's instructions. Hyp concentrations were quantified in the linear range of its calibration curve using an array reader (Bio-Rad, Hercules, CA, USA) and expressed in μg/mL of plasma.

2.10. Statistical Analysis. Statistical analysis of clinical data was carried out using WINSTAT programs for personal computers (Statistics for Windows 2007, Microsoft, USA). All biochemical and molecular measurements were done in triplicate and data were statistically evaluated. Values were presented as mean, standard error of the mean, and 1.96 × standard error of triplicate analyses. When several datasets were compared, data were analysed by Student's t-test for unpaired data. Differences between initial/final data for a single participant were analysed by paired t-test and by Mann-Whitney test for changes from baseline. All reported p values are from two-tailed tests, and p values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Subjective Evaluation by Participants and Clinical Investigators. All healthy volunteers ($n = 41$) recruited in the trial duly completed it. There were no drop-offs due to low compliance or adverse effects of the supplementation. Routine haematological and biochemical analyses, which were carried out after blood donation in the beginning and after the cessation of the study, did not show statistically significant changes possibly reflecting adverse consequences of the test nutraceutical in the prescribed dosages (data not shown). The subjective evaluation of the product effects on selected general health parameters is shown in Table 1. The participants were predominantly satisfied with the effects obtained on general health conditions and skin properties and partly also by enhanced muscle strength and stamina. No effect whatsoever on digestion was registered.

3.2. Effects on Facial Skin Properties. Comparison of digital photos taken before and after clinical trial showed visible



(a) (b) (c)

Figure 1: Digital images of facial skin ultrasound examinations (patient number 23, e.g.), made 2 months before the beginning of the trial (a), at the day of the trial beginning (b), and immediately after the trial cessation (c).

qualitative improvement of aesthetic aspect of face with pronounced lifting effect (*data not shown*).

Characteristic digital images of ultrasound examinations made at trial beginning (2 months before the beginning of supplementation), at the first day of nutraceutical administration, and immediately after the trial cessation are shown, respectively, in Figures 1(a), 1(b), and 1(c). The individual ultrasonic characteristics were rather stable and were not subjected to statistically significant changes during the 2 months of pretreatment period (Tables 2 and 3; compare columns 1 and 2). The analysis of individual data showed that highly enhanced dermal thickness and homogenous distribution of collagen fibers in dermis were detectable in 23% ($n = 11$) of the participants after the trial cessation. Statistical evaluation of dermal thickness and acoustic density revealed significant changes exclusively at the third visit (Table 2), while the ultrasonic properties of epidermis remained unchanged (Table 3).

Analyses of the main physiological parameters of the skin relevant to ageing, such as elasticity, moisture, and sebum content, demonstrated their comparative stability in the pretreatment period, as there were no significant changes between the first and the second sets of measurements (Table 4, columns 1 and 2). *Conversely*, CELERGEN administration statistically significantly enhanced skin elasticity and sebum production ($p < 0.0001$), whilst not influencing cutaneous moisture (Table 4, columns 2 and 3). Biological age, calculated on the basis of ultrasound and cutaneous physiology measurements, tended to decrease after the trial; however, the difference did not reach statistical significance.

It should be noticed that all tested parameters of skin physiology and structure were not subjected to temporal fluctuations during the 2-month pretreatment period, and

Table 2: Effects of the 2-month food supplement administration on the ultrasonic properties of the dermis ($n = 41$).

Parameter	Dermis		
	Pretreatment period	Before treatment	After treatment
Thickness, μm	3884 ± 30	3900 ± 31	4133 ± 28*
Acoustic density	5.2 ± 0.2	5.1 ± 0.2	6.3 ± 0.1*

* $p < 0.05$ *versus* "before treatment."

Table 3: Effects of the 2-month food supplement administration on the ultrasonic properties of the epidermis ($n = 41$).

Parameter	Epidermis		
	Pretreatment period	Before treatment	After treatment
Thickness, μm	76.9 ± 1.0	77.0 ± 0.8	77.6 ± 0.9
Acoustic density	35.6 ± 2.4	35.2 ± 2.2	35.4 ± 2.0

Table 4: Effects of the 2-month food supplement administration on the parameters of skin physiology ($n = 41$).

Parameter	Pretreatment period	Before treatment	After treatment
Elasticity	34.06 ± 1.54	33.66 ± 1.21	40.26 ± 0.87***
Moisture	48.83 ± 3.02	49.03 ± 3.52	46.54 ± 3.02
Sebum	29.89 ± 4.16	29.37 ± 4.76	56.86 ± 4.04***
Skin biological age	50.11 ± 1.91	49.51 ± 1.68	48.09 ± 1.74

*** $p < 0.0001$ *versus* "before treatment."

therefore changes observed can be viewed as a result of CELERGEN administration.

3.3. Plasmatic Oxidation Markers and Antioxidants. Surprisingly, CELERGEN administration did not affect several markers of glutathione metabolism such as total glutathione levels (normality range: 0.5–1.6 mg/g Hb) and glutathione-S-transferase and glutathione peroxidase activities (normality ranges: 0.2–0.7 U/mg Hb and 18.0–54.0 U/g Hb, resp.) (Figures 2(a), 2(b), and 2(c)). At the same time, nitrite/nitrate and MDA levels in plasma (normality ranges: 70.3–221.0 μM and 1.0–2.2 μM, resp.) were statistically significantly increased ($p < 0.05$ and $p < 0.0001$, resp.), although they remained within normal physiological range established in our laboratory (Figures 3(a) and 3(b)). Extracellular Cu,Zn-SOD3 activity was slightly suppressed ($p < 0.001$) but did not drop below the normality border (5.0–20.1 U/mL) (Figure 3(c)).

3.4. Parameters of Collagen and ATP Metabolism. Plasma content of hydroxyproline was found highly elevated ($p < 0.01$) (Figure 4(a)), the same with ATP content in erythrocytes ($p < 0.001$) (Figure 4(c)), although total content of coenzyme Q_{10} was not changed after supplementation with the coenzyme Q_{10}-containing nutraceutical (Figure 4(b)).

4. Discussion

In a preliminary *ex vivo* study of CELERGEN components against UVA-induced damage in human skin biopsies and fibroblasts [25], marine collagen peptides but not the complex of plant-derived antioxidants inhibited transcriptional and posttranscriptional matrix metalloproteinase-1 and elastase upregulation, leading the authors to hypothesise clinical feasibility for the prevention of skin photoaging. In contrast, another publication demonstrated that the bioflavonoid luteolin, a component of the CELERGEN antioxidant complex, effectively attenuated UVB-induced DNA damage, inflammation, and ROS overproduction in skin cells *in vitro* and *in vivo* [46].

In the present study, we obtained convincing clinical data on the efficacy of the marine collagen peptide and plant antioxidant formulation CELERGEN in improving dermal collagen deposition and structure (Table 2), as well as skin elasticity (Table 4). These effects were consistent with enhanced plasma levels of hydroxyproline, a systemic metabolic marker of collagen synthesis (Figure 4(a)). Nearly 100% of human Hyp is in fact found in collagen [47]. Hyp being an oxidative derivative of proline, both amino acids are essential for collagen biosynthesis, maturation, mode of deposition, and collagen fiber structure. Dietary proline intake promotes tissue repair in humans and animals [48]. Recently, Wang et al. [17] reported the experimental evidence that MCPs might improve collagen synthesis and maturation by inducing the expression of transforming growth factor beta-1 (TGF-β1) and basic fibroblast growth factor (bFGF). Our data (Figure 4(a)) are consistent with previously published ones on rats fed with MCPs from salmon or trout skin [49], showing that plasma levels of free and dipeptide (Pro-Hyp) forms of hydroxyproline were highly increased after single intake of MCPs in soybean oil. Similar data on the blood levels of Hyp and Hyp-containing peptides were obtained on healthy human volunteers [50].

Numerous animal studies on the effects of oral administration of natural or synthetic antioxidants towards collagen deposition, reactive species levels, and antioxidant defences generated highly conflicting data, depending on the experimental system. Thus, with various wound healing models, it was repeatedly demonstrated that either complex plant extracts containing active secondary metabolites (triterpenes, polyphenols, alkaloids, etc.) [18, 51] or a composition of collagen inducing polysaccharides like chitosan and antioxidants such as curcumin [52] or resveratrol [53] ameliorated wound healing increasing skin collagen deposition, while suppressing proinflammatory iNOS and myeloperoxidase, decreasing pathologically elevated levels of MDA and hydrogen peroxide, and improving enzymatic antioxidant defence. Recent studies showed that collagen peptides from fish skin remarkably promoted both wound healing and angiogenesis in different experimental settings [10, 17]. Of importance, excessive NO produced during the inflammatory phase of wound healing process impaired collagen accumulation [54], while moderate NO levels accelerated the granulation phase of wound closure [18, 55]. Moreover, wound healing acceleration by moderate levels of H_2O_2 through induction of vascular endothelial growth factor in keratinocytes and macrophages was proved in a number of experimental and clinical studies [56, 57]. Here, we found that, along with Hyp accumulation, plasma levels of nitrites and nitrates, related to NO production in the bloodstream, were moderately increased after CELERGEN treatment, though remaining within the range of normal values (Figure 3(a)). Similar results were obtained with plasmatic MDA (Figure 3(b)). This allowed us to suggest that redox regulation of cutaneous collagen synthesis process or/and fibroblast proliferation activation could have occurred due to physiologically relevant NO and/or MDA amounts generated following supplement

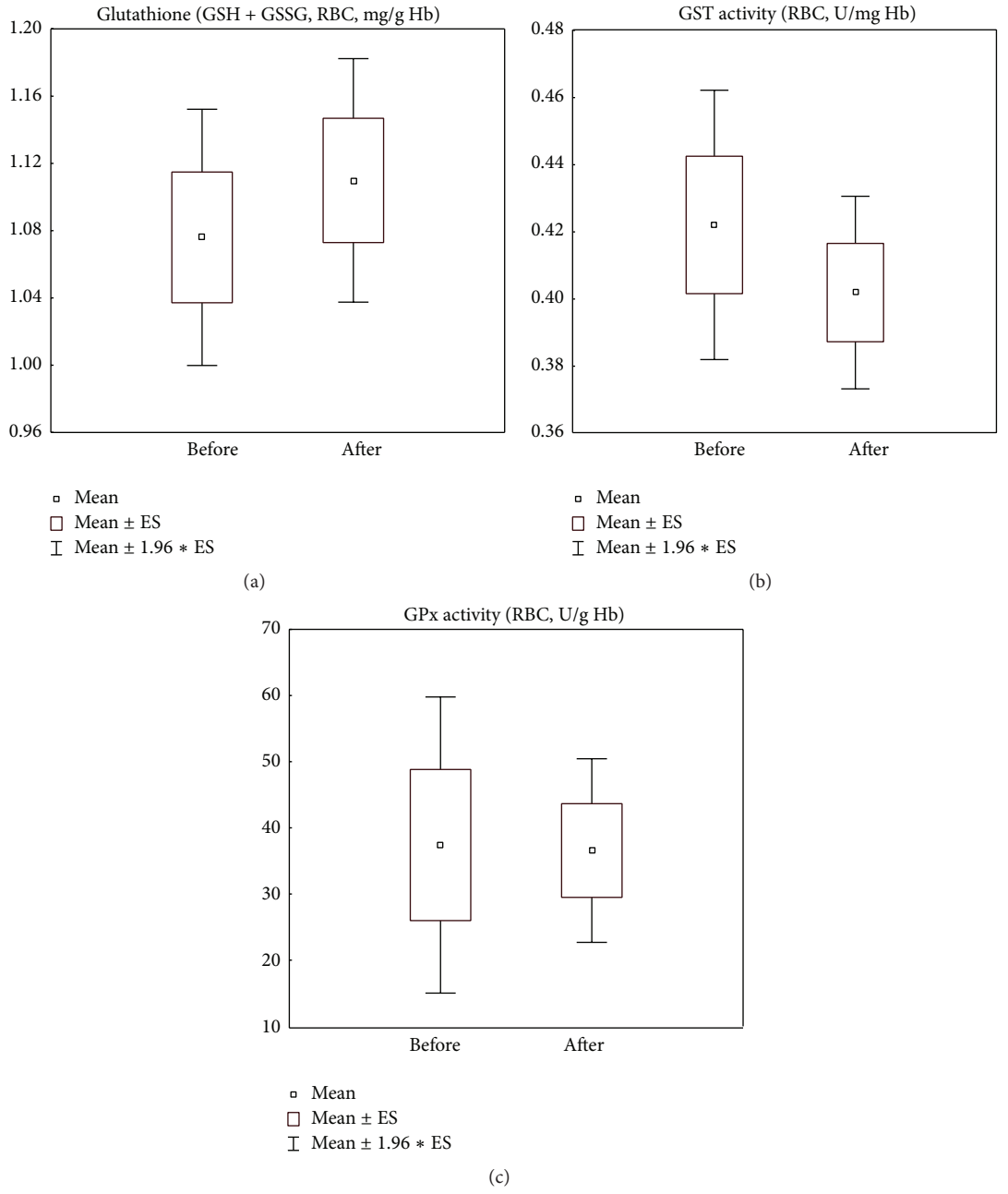


Figure 2: Glutathione cycle parameters: erythrocyte levels of total glutathione (reduced and oxidized forms, GSH + GSSG) (a) and erythrocyte enzymatic activities of glutathione-S-transferase (b) and of glutathione peroxidase (c) in the study group of patients ($n = 41$), before and after food supplement administration period. Values are represented as mean (□), standard error of the mean (upper and lower limits of the box), and 1.96 × standard error (upper and lower whiskers). GSH: reduced glutathione; GSSG: oxidized glutathione; RBC: red blood cells; Hb: haemoglobin; GST: glutathione S-transferase; GPx: glutathione peroxidase. Reference normality range: RBC total glutathione (0.5–1.6 mg/g Hb); RBC GST activity (0.2–0.7 U/mg Hb); RBC GPx activity (18.0–54.0 U/g Hb).

intake. However, the suggestion deserves further mechanistic *in vitro* and clinical research.

On the other hand, in the models of cardiac fibrosis [58, 59], the significant decrease of the model-related oxidative stress obtained by the use of *Momordica charantia* fruit extract [58] or *Fructose Chorpondiatis* total flavonoids was indeed associated with simultaneous attenuation of collagen deposition, as assessed by Hyp levels. Similar results were obtained in other tissue models of fibrosis [28, 60–62], including skin fibrosis [29]. It seems that complex mixtures of fruit extracts contained both collagen synthesis affecting agents and antioxidants.

UV irradiation could cause skin photodamage causing the symptoms of premature photoageing. Evaluating the photoprotective effects of dietary MCPs isolated from jellyfish umbrella [24] or from fish scale [63] in the model

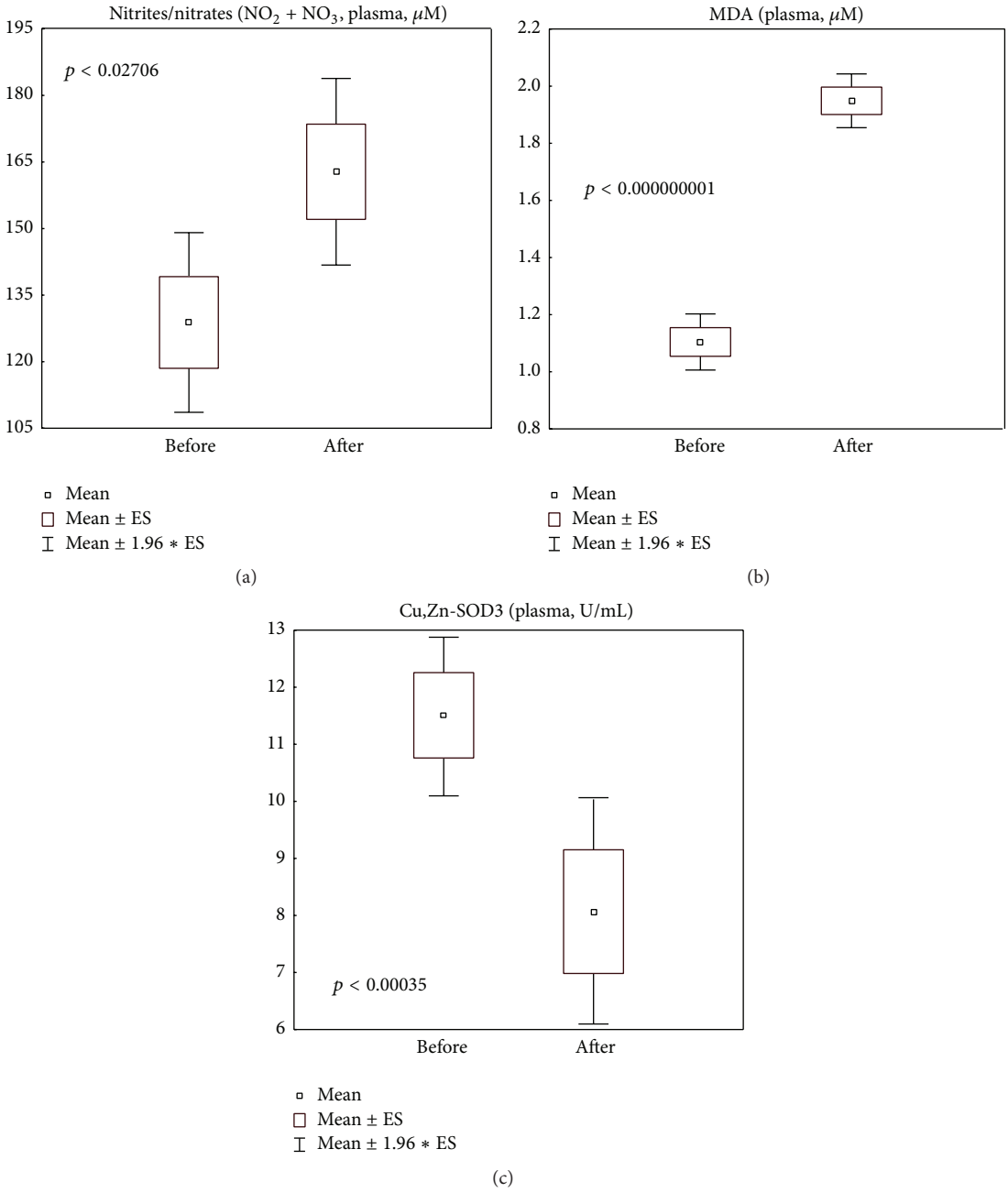


Figure 3: Systemic oxidative stress markers: plasma levels of nitrites/nitrates (NO_2 + NO_3) (a), of malonyl dialdehyde (MDA) (b), and of Cu,Zn-superoxide dismutase 3 (Cu,Zn-SOD3) (c) in the study group of patients (n = 41), before and after food supplement administration period. Values are represented as mean (□), standard error of the mean (upper and lower limits of the box), and 1.96 × standard error (upper and lower whiskers). Intergroup significant differences (p) are indicated in the relative panels. NO_2 + NO_3: nitrites + nitrates; MDA: malonyl dialdehyde. Reference normality range: plasma NO_2 + NO_3 (70.3–221.0 μM); plasma MDA (1.0–2.2 μM); plasma Cu,Zn-SOD3 (5.0–20.1 U/mL).

of chronic UVA + UVB irradiation of mice, the authors concluded that MCPs enhanced skin immunity, reduced water loss, restored cutaneous collagen and elastin levels and structure, and maintained type III to I collagen ratio. Under similar experimental design, Zhuang et al. [64] showed the protective action of MCPs on antioxidant enzymes activities and glutathione, lipid, and Hyp contents of murine skin. In this connection, we found a significant reduction (within the range of normality) of plasmatic SOD3 activity following CELERGEN supplementation (Figure 3(c)). Extracellular plasmatic Cu,Zn-SOD3, a glycoprotein with a heparin-binding domain, is predominantly expressed in tissue ECM, where it is bound to heparin sulfate proteoglycan [65]. Physiologically, SOD3 maintains redox balance and tissue

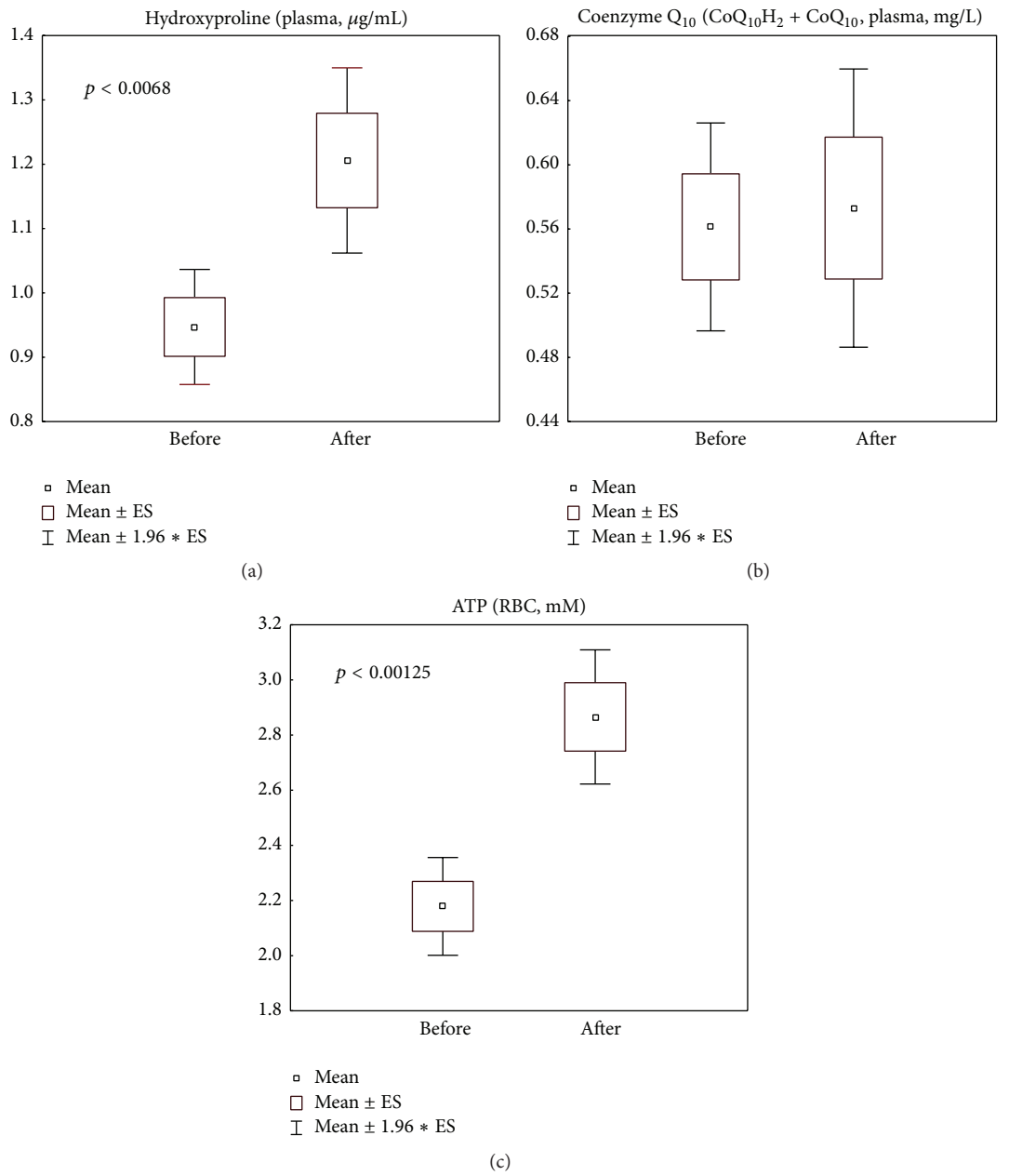


Figure 4: Metabolic parameters related to collagen and ATP synthesis: levels of plasma hydroxyproline (a) and of the lipophilic antioxidant total coenzyme Q_{10} (reduced and oxidized forms, $CoQ_{10}H_2$ + CoQ_{10}) (b) and erythrocyte ATP (c) in the study group of patients (n = 41), before and after food supplement administration period. Values are represented as mean (□), standard error of the mean (upper and lower limits of the box), and 1.96 × standard error (upper and lower whiskers). Intergroup significant differences (p) are indicated in the relative panels. $CoQ_{10}H_2$: reduced coenzyme Q_{10}; CoQ_{10}: oxidized coenzyme Q_{10}; ATP: adenosine triphosphate; RBC: red blood cells. Reference normality range: total coenzyme Q_{10} (0.4–1.6 mg/L); ATP (1.0–4.0 mM).

homeostasis and modulates innate and adaptive immune responses. Cutaneous homeostasis strongly depends on the ECM microenvironment; therefore, an elevated SOD3 activity may be a marker of adaptive response against intrinsic age-associated and external hazardous factors inducing immune suppression in the skin [66].

Since the supplementation of compounds with a direct antioxidant effect has failed so far to show clinical efficacy and sometimes even aggravated clinical picture [67], the search for drugs/therapeutic strategies to modulate oxidative stress has been drastically redirected nowadays towards (1) indirect AOs inducing endogenous enzymatic system of antioxidative defence, mainly, through Nrf2-connected pathway; (2) selective inhibitors of ROS/RNS-producing enzymes, for example, different isoforms of NADPH-oxidase, having shown definite clinical effects; (3) recognising essential and multiple physiological roles of redox balancing agents rather than mere inhibitors of free radical processes. Plant-derived

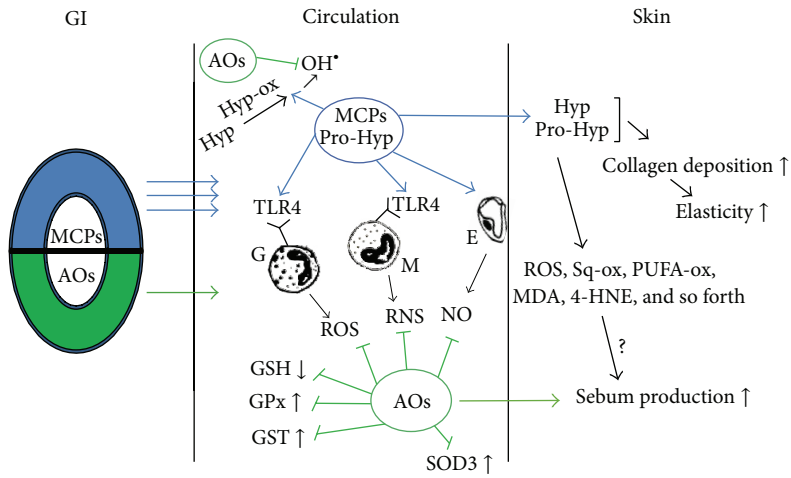


Figure 5: Scheme of the hypothesised redox-dependent mechanisms of CELERGEN physiological effects. Marine collagen peptides (MCPs) easily penetrate gastrointestinal wall (GI, three arrows) and through blood circulation are mainly deposited in the skin. Antioxidant component of the nutraceutical is partly metabolised in GI thus possessing low bioavailability (one arrow); however, skin-targeting antioxidants and their metabolites reach different skin layers. While in the circulation, MCPs stimulate blood phagocytes (granulocytes and monocytes) and endotheliocytes (E) to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) by activating Toll-like receptors 4 (TLR4). Hydroxyproline (HYP) and prolyl-hydroxyproline (Pro-HYP) dipeptides as major components of MCPs are metabolised by corresponding oxidases and hydroxyl radicals are formed as by-products. Antioxidants prevent systemic oxidative stress blocking GSH oxidation, GPx, GST, and SOD3 activation. In the skin, collagen synthesis and deposition as well as elasticity are increased while (hypothetically) low levels of oxidised forms of skin lipids such as unsaturated fatty acids (PUFA-ox), squalene (Sq-ox), malonyl dialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE) may facilitate cell signalling for ATP synthesis and sebum production.

polyphenols, quercetin, resveratrol, luteolin, and many others appear to possess all these multipotent capabilities [6]. The presence of quercetin and resveratrol from grape-skin extract and of luteolin in the antioxidant combination of CELERGEN may then well account for the observed redox balancing effects during the upregulation of MCPs-induced collagen synthesis (Figures 2(a), 2(b), and 2(c)). The majority of publications have in fact demonstrated a drop of GSH content and an increase of protective GPx activity when Hyp content was raised [28, 64, 68]. Of great importance, the presence of antioxidants in the tested formulation, whilst possibly protecting the redox balance from harmful side effects of collagen metabolism, did not negatively affect the desired process of dermal collagen synthesis/deposition. In fact, the observed elevation of plasmatic Hyp was comparable with that found previously [27], with pure fish MCPs in much higher dosages.

In the last decade, endogenously produced systemic and cutaneous redox-active substances (superoxide, hydrogen peroxide, NO, lipid peroxides, stable end products of lipid peroxidation, oxidative metabolites of cholesterol and squalene, etc.), previously recognised exclusively as undesirable metabolic by-products and markers of oxidative damage, have shown essential functions in cellular signalling and regulation of cell proliferation, differentiation, migration, innate immunity, energy production, ECM dynamics, vascular tone, stress responses and adaptation, and inflammation [55, 69–71]. In this frame, the moderate plasmatic elevation of MDA (Figure 3(b)) and NO (Figure 3(a)) observed in this study may reflect the regulatory functions of these mediators both in MCPs-induced collagen synthesis (Figure 4(a)) [69] and in the process of mitochondrial ATP production (Figure 4(c)). Only excessive amounts are damaging as they initiate cell senescence and death. It seems that, notwithstanding coenzyme Q_{10} supplementation during CELERGEN course, its plasmatic levels were not increased (Figure 4(b)), due to elevated consumption of the coenzyme in the mitochondrial cycle of energy production enhancing ATP storage in erythrocytes (Figure 4(c)) and in cell redox balance control (Figures 2(a)–2(c)).

The antiageing effects of CELERGEN supplementation were evidenced also by the highly increased sebum production (Table 4). It is established that the production of sebaceous lipids is strongly age dependent, being low in the prepubertal period, rising with sexual maturation, and gradually declining in the aging populations (starting from 46–55 years) [72] or in UV-induced premature skin ageing [70, 73]. Cutaneous lipid-soluble antioxidants such as vitamin E and squalene decay accordingly [74]. Since skin surface lipids (SSL) play multiple essential roles in skin barrier properties, skin smoothness, elasticity, and moisture, they are regarded as natural guards of normal cutaneous ecology. Moreover, moderate concentrations of specific SSL unsaturated components (squalene, cholesterol, and free fatty acids) are able to generate oxidised lipid by-products (MDA, 4-hydroxynonenal, oxidised cholesterol, and others), since being long recognised as key signalling molecules for skin immune and metabolic responses to environmental insults and microbial invaders [70, 75, 76]. On the other hand, excessive levels of microbially or photooxidised derivatives of unsaturated fatty acids and other sebum lipids could induce a vicious cycle of sebum overproduction followed by

oxidation, thus maintaining inflammation characteristic for acne disease [76]. As shown by our clinical data (Table 1), no complaints about skin conditions were registered during the trial. Conversely, the marked improvement of skin elasticity could be attributed not only to collagen deposition in derma, but also to a moderate physiological increase of SSL content.

On the grounds of the results obtained and existing literature data, we hypothesised redox-dependent pathways (Figure 5) which may lead to clinical and generalised health effects of CELERGEN supplementation. Obviously, more profound basic research and further clinical studies are needed to prove this hypothesis and to evaluate the underlying mechanisms.

5. Conclusions

The addition of dietary plant-derived antioxidants with known skin tropism and health effects towards human skin did not impair definite induction of collagen synthesis and its deposition as compact organised fibres in the dermal layer by marine fish skin-derived collagen peptides. Additional beneficial effects of antioxidants were observed systemically, as normal balance of systemic endogenous antioxidant defence was maintained, and protection of energy storage occurred.

Abbreviations

MCPs:	Marine collagen peptides
ECM:	Extracellular matrix
NOX4:	NADPH-oxidase
NO:	Nitric oxide
MDA:	Malonyl dialdehyde
HPLC:	High performance liquid chromatography
TEWL:	Transepidermal water loss
EDTA:	Ethylenediaminetetraacetic acid
RBC:	Red blood cells
Cu,Zn-SOD3:	Cu,Zn-superoxide dismutase 3
GST:	Glutathione-S-transferase
GPx:	Glutathione peroxidase
TBARS:	Thiobarbituric acid-reactive substances
Hyp:	Hydroxyproline
TGF-β1:	Transforming growth factor beta-1
bFGF:	Basic fibroblast growth factor
ROS:	Reactive oxygen species
RNS:	Reactive nitrogen species.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

1,4-Dihydropyridine Derivatives: Dihydronicotinamide Analogues—Model Compounds Targeting Oxidative Stress

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Many 1,4-dihydropyridines (DHPs) possess redox properties. In this review DHPs are surveyed as protectors against oxidative stress (OS) and related disorders, considering the DHPs as specific group of potential antioxidants with bioprotective capacities. They have several peculiarities related to antioxidant activity (AOA). Several commercially available calcium antagonist, 1,4-DHP drugs, their metabolites, and calcium agonists were shown to express AOA. Synthesis, hydrogen donor properties, AOA, and methods and approaches used to reveal biological activities of various groups of 1,4-DHPs are presented. Examples of DHPs antioxidant activities and protective effects of DHPs against OS induced damage in low density lipoproteins (LDL), mitochondria, microsomes, isolated cells, and cell cultures are highlighted. Comparison of the AOA of different DHPs and other antioxidants is also given. According to the data presented, the DHPs might be considered as bellwether among synthetic compounds targeting OS and potential pharmacological model compounds targeting oxidative stress important for medicinal chemistry.

1. Introduction

1,4-Dihydropyridines (DHPs) [1], including Ca^{2+} antagonist (CA) drugs [2], are large group of structurally diverse compounds. Functionally, they are similar to dihydronicotinamide redox-active synthetic compounds with radical scavenging and antioxidant (AO) properties and may be considered as protectors against oxidative stress (OS) and associated disorders [3].

Oxidative stress is extremely important for molecular pathogenesis, especially influencing the redox regulation of cellular signaling pathways [4–7]. Oxidative stress closely relates to presence of oxygen and nitrogen free radicals, known as reactive oxygen species and reactive nitrogen species (ROS and RNS, resp.). They cumulatively increase upon cellular exposure to various endogenous and/or exogenous insults. ROS and RNS have the "two-faced" character and play a dual role as both deleterious and beneficial species [8, 9]. Although explored in many diseases, various phenomena related to OS have been probably best studied in cancer cells in which, depending on various factors, OS may have anticancer-like effects. Its protumorigenic effects are primarily related to induction of oxidative DNA lesions (8-OH-G) and consequential increase of DNA mutations that may, if not repaired, lead to genome instability and an increased rate of cellular proliferation [10]. On the other hand, antitumorigenic actions of OS have been closely linked to cellular processes of senescence and apoptosis, two major molecular mechanisms that counteract tumor development. Which of these two actions will dominate depends on many factors including the metabolic status of the cell, as recently reviewed by Kujundžić et al., 2014 [11].

Antioxidants (AOs) are defined as substances that, even when present in low concentrations compared to those of an oxidizable substrate, prevent or significantly delay the oxidation process (Halliwell and Gutteridge, 1995 [12]). Their activity depends on complex factors including the nature of the antioxidants, the condition of oxidation, the properties of substrate oxidized, and the level of oxidation (reviewed in Kancheva and Kasaikina, 2013 [13]). Accordingly, an

antioxidative effect may be direct, resulting from direct ROS scavenging, or indirect from the influence on various signaling pathways related to cellular defense, that is, stress responses. In relation to human physiology, antioxidants are traditionally classified as exogenous (supplied mostly through food) and endogenous and are further subclassified as enzymatic (i.e., superoxide dismutase (SOD) and catalase (CAT)) and nonenzymatic (i.e., glutathione, vitamins A, C, and E, etc.) [3].

DHPs could be classified as the separate group of synthetic nonenzymatic, however, biomimetic AOs.

2. Oxidative Stress and Its Prevention: Wavy Scientific Process Development—*Pro et Contra*

There are opposite views both towards the role of oxidative stress and about potential applications of exogenous antioxidants in onset of OS [14–16].

Herewith, we need to mention that antioxidants have been studied for decades (starting from 1970s) as the tools for the treatment of various disorders. The role of native and synthetic antioxidants (acting on lipid peroxidation (LP) in biological membranes) in radiation damage and malignant growth was seriously evaluated [17]. The overall conclusions point out antioxidants role in decreasing the damage of cells by reducing oxidants before the occurrence of cellular damage [14]. It was elicited and accented (Burlakova et al. [15]) that

(i) antioxidants, nontoxic inhibitors of free radical processes, exhibit a wide gamut (pleiotropy) of biological activity (as further will be reported, this phenomenon is also characteristic for the DHP antioxidants group);

(ii) the biological effectiveness of AOs correlates with their antioxidant activity (AOA);

(iii) depending on dose, AOs may either increase or decrease the AOA;

(iv) the efficacy of AO depends on the time of introduction in the course of medical treatment because the development of the disease may be accompanied by stages of changing the AOA.

In relation to dose-effect dependence, Burlakova et al. [15] have found the nonlinear pattern: after addition of an AO, there is an initial increase of AOA, followed by returning to normal and finally decreasing drastically below the normal value. Therefore, antioxidants may produce a specific effect by decreasing (at low doses) or increasing (at high doses) the rate of free radical reactions. Hence, the compound may be efficient AO only if it is introduced in a low dose at the stage of reduced AOA or in a high dose at the stage of AOA elevation. The widespread opinion of opponents was that the antioxidant function, even that of tocopherol, was a side effect of its activity and important only for *in vitro* processes and without any role in bioobjects life. This opinion was supported by the fact that the deficiency of natural AO tocopherol (E-avitaminosis) cannot be cured completely by applying synthetic AO. Eventually, it was not certain also that detected lipid peroxides have been generated *in vivo* in the intact organs and were not artificially formed during the isolation [15]. All these objections and skepticism were rejected in due time.

However, some other research directions were suggested.

Fang et al. [18] reported two different therapeutic strategies for modulating OS in cancer and inflammation, including (1) antioxidant therapy and (2) "oxidation therapy."

For (1), polymeric superoxide dismutase (e.g., pyran copolymer-SOD), xanthine oxidase (XO) inhibitor, developed water-soluble form of 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP), heme oxygenase-1 (HO-1) inducers (e.g., hemin and its polymeric form), and other antioxidants or radical scavengers (e.g., phenolic compound canolol, 4-vinyl-2,6-dimethoxyphenol) were used.

About (2), besides neurodegenerative diseases, cancer may represent yet another very interesting field for exploring antioxidants and prooxidants as therapeutic substances due to their cytotoxic effects (including overproduction of ROS) that, if achieving proper selectivity, may be used for cancer cells destruction (Fang et al. [18]). To achieve this goal, a unique therapeutic strategy was developed, named as "oxidation therapy," by delivering cytotoxic ROS directly to the solid tumor or alternatively inhibiting the antioxidative enzyme system, such as HO-1 in tumor. This anticancer strategy was examined by use of $O_2^{\bullet -}$ or H_2O_2-generating enzymes (i.e., XO and d-amino acid oxidase [DAO], resp.) and by discovering the inhibitor of HO-1 (i.e., zinc protoporphyrin [ZnPP] and its polymeric derivatives).

While deleterious when present at high concentrations, low concentrations of ROS exhibit beneficial properties needed for controlling physiological cellular processes (reviewed in Valko et al., 2007 [19]).

Jimenez-Del-Rio and Velez-Pardo [20] have discussed oxidative stress as an important etiopathogenic factor for occurrence and development of neurodegenerative diseases (notably Alzheimer's disease and Parkinson's disease) and cancer. As an extension, possible preventive and therapeutic values of antioxidants were also discussed. Indeed, if considered within a narrow context of oxidative homeostasis, antioxidants may seem to be ideal weapon in preventing and fighting these diseases. However, the context of human pathology is very broad and, so far, there was little benefit of exogenous antioxidants in human intervention studies or clinical trials. There are numerous reasons for these failures. Maybe, the most important one is the design of the preclinical studies, especially related to concentration of the antioxidant used and time parameters relevant to the clinical setting (Kamat et al., 2008 [21]). The imbalance between uncritical acceptance of antioxidants as powerful "drugs" for various pathological conditions and disappointing results obtained in clinical studies has made a sort of confusion. This issue was addressed by Bast and Haenen [16] through listing ten misconceptions related to commercialized applications of antioxidants: (a) "pros": (1) antioxidants can cure any disease; (2) the more the better; (3) any AO will do (the trick); (4) AO status measures the level of health; (5) natural AOs are superior (over synthesized ones) and (b) "contras": (1) AOs

increase mortality; (2) when present at high doses, antioxidants become prooxidant; (3) theoretically, antioxidants cannot behave as such; (4) once used, antioxidants are inactive; (5) antioxidant drugs do not work.

The first three "pros" clearly cross the line of realistic way of thinking and cannot be considered seriously. The "pro" #4 was very informatively discussed by Pompella et al. [22] who comprehensively presented current problems with the methods (ORAC, oxygen radical absorbance capacity; ferric-reducing ability of plasma; and TEAC, Trolox equivalent antioxidant capacity) routinely used for measurement of total antioxidant capacity (TAC) in plasma (Pompella et al., 2014 [22]). These include lack of needed specificity, especially relevant for ORAC related measurements. Instead, precise measurement of specific compounds is recommended. Regarding the "pro" #5, the situation does not seem entirely clear, as some published metastudies related to protective role of vitamin C in coronary heart disease showed some contradictions (better protection with dietary vitamin C versus synthetic vitamin C) (Ye and Song [23]; Knekt et al. [24]). In any event, this kind of research is anything but simple, as observed health effects of fruit and vegetable ingestion are certainly related not only to the content of vitamin C but also to other macro- and micronutrients and phytochemicals, proven to confer additional health benefits (Carr and Vissers [25]). Similar to "pros," stated "contras" seem to be a common misconcept related to the design of the study (this is especially relevant for epidemiological studies), relevance of a specific pathological condition and measurement of its outcomes, and, finally, complexity of a living organism. For all these reasons, there is the realistic need for well-designed epidemiological, clinical, and molecular studies that would offer firm evidence and undoubtful conclusions on the role of antioxidants on human health (see also Sections 3.8 and 3.9).

There are still unanswered questions related to oxidative stress and its mediators in pathogenesis of OS-associated diseases. However, it is clear that overproduction of ROS has harmful cellular effects. For that reason, small synthetic antioxidants, molecular scavengers, have been developed to be used in various pathological conditions. The first one, implemented in the clinic for acute brain infarction, was 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186, Edaravone, Radicut, norphenazone), approved until now only in Japan (Tabrizchi, 2000 [26]). So far, its free radical scavenging properties were revealed by various biological effects (antioxidant, attenuation of cytokine production, antiapoptotic, antinecrotic, and some other effects), as recently reviewed (Kikuchi et al. [27]).

3. 1,4-Dihydropyridines: A Separate Group of Bioantioxidants

1,4-Dihydropyridines could be used as model compounds for studying molecular mechanisms of action modulated by cellular enzymes NADH and NAD(P)H due to their structural analogy to 1,4-dihydronicotinamide [28]. This structure represents the active part of these reduced coenzymes, which are important modulators of various enzymatic redox reactions and are involved in electron transfer.

Chemically, 1,4-dihydropyridines are synthetic hydrogenated N-heteroaromatic compounds. They may have various substituents at positions 2,6-, 3,5-, and 1,4- (Figures 1–3). Their derivatives can be obtained synthetically in the Hantzsch type cyclic condensation reactions.

Bossert and Vater [29] postulated DHPs as a basis for development of new cardiovascular drugs. Today there are many marketed drugs which contain 1,4-DHP ring as basic scaffold [30–32] (Figures 1 and 2).

Grover et al. [33] classified dihydropyridine nucleus (skeleton) as a novel pharmacophore and offered some examples related to DHPs pleiotropy. So far, AOAs have been revealed for several groups of DHP compounds and DHP-based drugs [34–36], contributing to their well-known pleiotropic ways of action (antiaging, neuroprotective, anticancer, antibacterial, [37] and many more). These features are promising for development of novel drugs in the future [32, 38].

It is well known that hydrogen donors such as amines, thiols (aminothiols), or phenols (plant phenols and polyphenols as well as synthetic hindered phenols) act as antioxidants, primarily through inhibition of oxidation reactions of various chemical targets/substrates. Similarly, depending on their particular chemical structure, 1,4-dihydropyridines have significant hydrogen donor ability (see further in Section 3.2). This feature allows them to act as direct inhibitors of free radical reactions. It further classifies them as specific group of dihydropyridine type of antioxidants. However, under certain conditions, primarily dependent on individual structure and applied dose, DHPs can act as prooxidants (see further in Section 3.8).

On the other hand, some DHPs may exert synergistic effects when applied together with other types of AOs [39]. They can also be involved in the redox regulation of Ca^{2+} ion channels [40]. Namely, oxidative stress, characterized by significant increase of ROS, closely relates to cellular imbalance of Ca^{2+} ions. Such a CA activity of DHPs can also result in the indirect OS modulation as an additional positive side effect. Accordingly, DHPs, acting as CA and as antioxidants, may modify various OS-associated pathological processes by influencing cellular redox signaling potential. Additionally, multiple biological effects of DHPs attenuating OS could be important at drug-drug interactions by combination therapy using DHPs and other CA and/or antioxidants.

It should be mentioned that the studies on the possible AOA of 1,4-DHPs have begun due to the assumption that these substances could be useful for the design for novel antioxidants intended to be used primarily in the food technology, notably as animal chow stabilizers [41–43]. The AOAs of 1,4-dihydropyridine derivatives, 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine (Hantzsch ester (HEH), diludine) and its close analogues, 4-unsubstituted 1,4-DHPs, were discovered by Latvian scientists that intended to use them for the termination of the lipid peroxidation (LPO) in various chemical lipid substrates/mixtures target (solutions, emulsions, and liposomes) [44, 45]. Afterwards, antioxidant properties of several calcium antagonists DHPs were discovered [31, 46–53]. Interestingly, research on the AOA of DHPs on LPO continues nowadays, including several

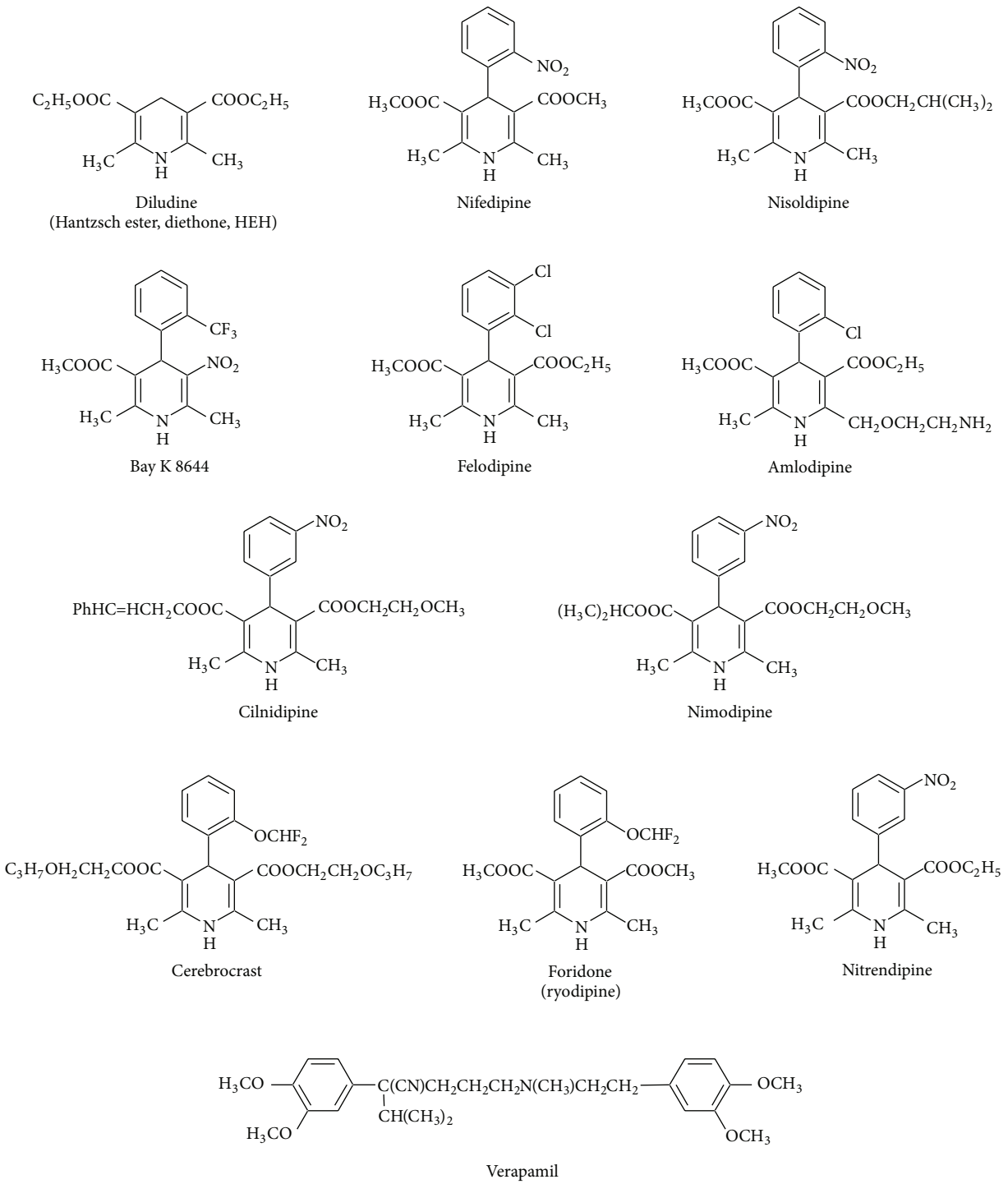


Figure 1: Structures of the most known 1,4-dihydropyridine derivatives and some non-DHP Ca^{2+} antagonists.

interdisciplinary projects funded by EU, in particular the COST B35 action [51, 52].

3.1. Synthesis of 1,4-Dihydropyridines: Routes and Approaches. Classical 3-component Hantzsch synthesis of DHP compounds [54–57] is usually performed in solutions (including ionic liquids) by heating. Discoveries related to this process and published between 1986 and 1990 are summarized in the review of Sausins and Duburs [57]. In 1993, Kazda [58] has reviewed "twenty years of dihydropyridines," including their synthesis, chemistry, progress in pharmacology, and therapy, and some other applications. Since then, there were many important discoveries in this field and there is a time for a review on "another twenty years of DHPs." It has to be

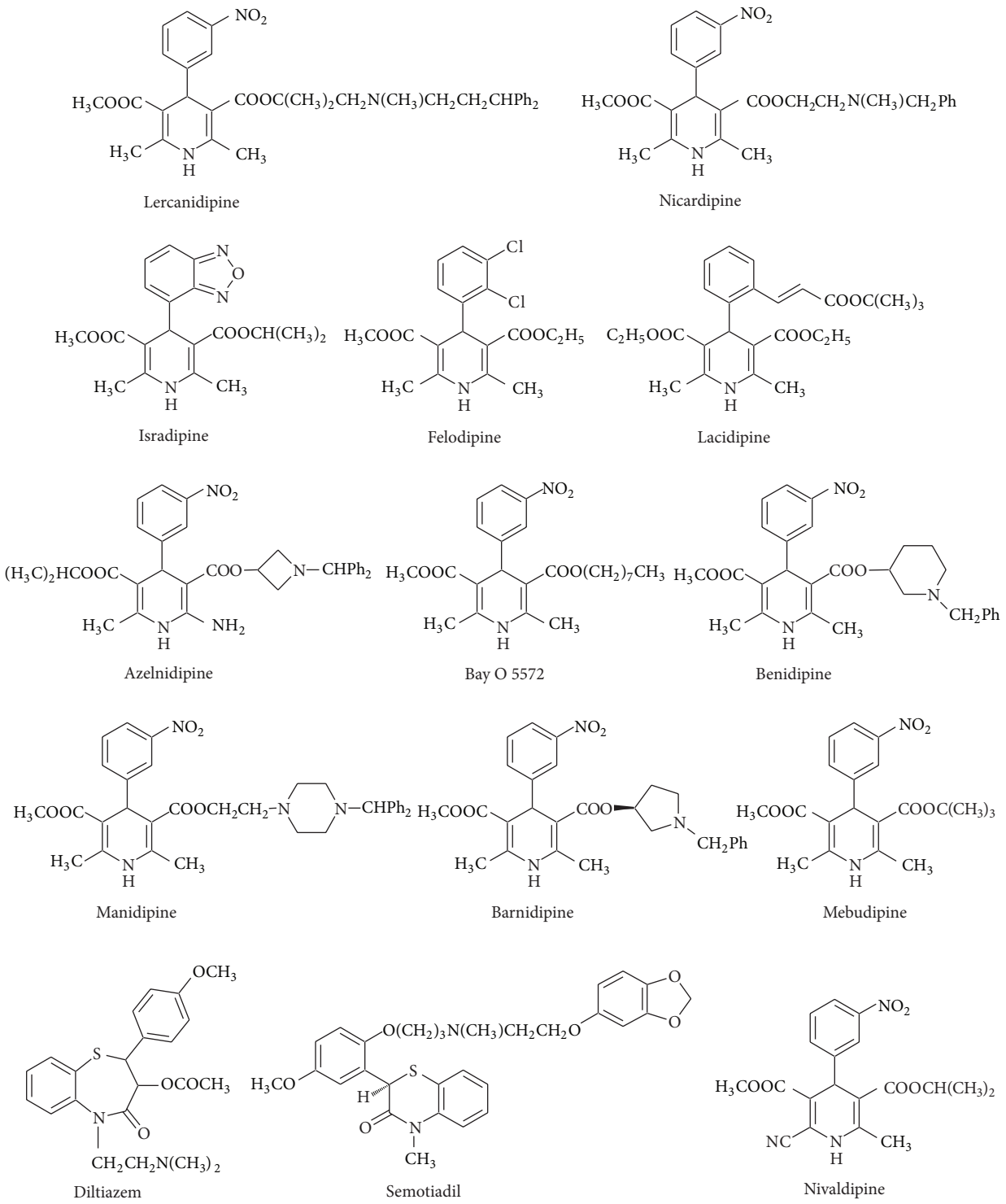


Figure 2: Structures of the most known 1,4-dihydropyridine derivatives and some non-DHP Ca^{2+} antagonists.

mentioned that nearby this classical multicomponent synthesis also a process to obtain structurally diversified 1,4-dihydropyridines at sophisticated conditions was recently reviewed by Wan and Liu [59].

Many discoveries relevant for novel routes in DHP designing and synthesis were published and deposited in various databases (see http://www.organic-chemistry.org/namedreactions/hantzsch-dihydropyridine-synthesis.shtm [60]). For example, http://www.scifinder.com/ [1] database lists approximately 1000 citations on the simple DHP compound, diludine. *Reaxys* database [61] contains data related to variations in starting materials, intermediates as building

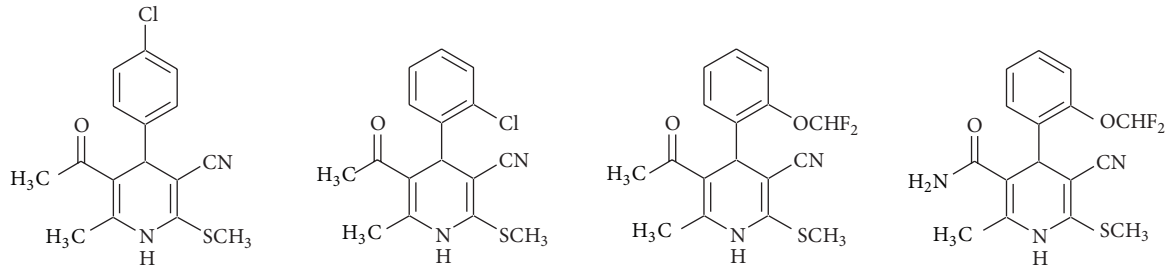


Figure 3: Molecular structures of OSI-1146, OSI-3701, OSI-3761, and OSI-9642 (according to [146]).

blocks, media, and reactions routes. Water and ionic liquids as reaction media, microwave and infrared irradiation, new catalysts, solid phase synthesis, and biotechnology based and green chemistry approaches were also proposed as attractive options for syntheses of DHPs [62–66].

Furthermore, several new dihydropyrimidin-(2H)-ones (DHPMs), close analogues of DHPs, were prepared in the Biginelli reaction under ultrasound irradiation and in the presence of NH_4Cl. Some of these compounds, when tested *in vitro* at concentrations higher than 100 μM [67], showed AOAs, manifested as inhibition of LPO induced by complex Fe + EDTA and reduction of ROS levels.

Recently, Sun et al. [68] reported about the synthesis and antioxidant activity of a series of novel 3-chalcone-substituted 1,4-dihydropyridine derivatives, based on dimethyl or diethyl 2,6-dimethyl-4-phenyl-1,4-DHP-3,5-dicarboxylate.

3.2. 1,4-Dihydropyridines as Hydrogen Donors. Steric, electrostatic, and hydrophobic descriptors in DHP molecule could serve as its potential pharmacophores [2]. In case of Hantzsch ester this implies partly hydrogenated N-heteroaromatic DHP nucleus itself or its fragments, that is, NH group or C-4 H- atom, as hydrogen donors necessary for the AO activity and/or carboxylic ester side groups (its C=O group and O atom as hydrogen bond acceptors) in positions 3- and 5- and alkyl side groups in positions 2- and 6- (as hydrophobic features) (Grover et al. [33] and Tikhonov and Zhorov [69]). The presence of labile hydrogen atoms (mainly in positions 1,4-) in DHPs molecule assigns significant hydrogen donating ability to these compounds.

DHPs (2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid esters) can be oxidized in chemical (Dubur and Uldrikis [70]), electrochemical, enzymatic (Duburs et al. [71]), and biological (including metabolism and biotransformation) systems. As already stated, dihydropyridines (especially unsubstituted in position 4) may transfer the hydrogen, similar to the reduced diphosphopyridine nucleotides, NADH and NADPH (Scheme 1) (Mauzerall and Westheimer [28]), while HEH hydrogen transfer studies and search for novel NADH model compounds are continuously developing (Xie et al. [72]).

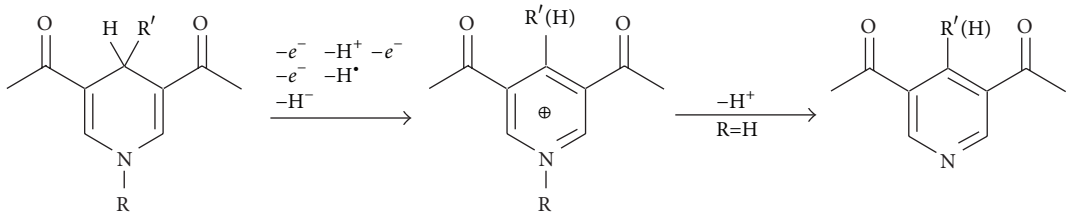
Tamagaki et al. [73] observed metal-ion-facilitated oxidations of DHPs with molecular oxygen and hydrogen peroxide. On the other side, Tirzite et al. [74] studied some 1,4-DHP derivatives as reductants in relation to trivalent iron. Hantzsch esters have been extensively utilized as stoichiometric biomimetic reducing agents. Recent summarized literature about DHPs as reducing agents, including references on diludine, may be found on specialized websites: http://www.organic-chemistry.org/chemicals/reductions/ [75].

DHPs form free radicals in chemical, electrochemical, and biological oxidation processes. The kinetic parameters and pathways of decay of the cationic radicals formed as primary products in the course of electrooxidation of the esters of 1,2- and 1,4-dihydropyridine have been extensively studied [76].

The regenerative system of nicotinamide cofactors may involve oxidizing or reducing reagents, regulating enzymes, and photochemical reactions. Thus, *in situ* regeneration of the consumed cofactors was observed in the biosystems engineering, which create superior biocatalysts by the reduction of $NAD(P)^+$, which can lead to the 1,4-DHP product (which is the only active form) and to the 1,6-DHP compound [77]. The NADPH models of HEHs can be regenerated *in situ* as biomimetic hydrogen sources by means of transition metal/Brønsted acid catalyzed relay asymmetric hydrogenation [78]. General regeneration strategies were reviewed by Chenault and Whitesides [79]. Based on these strategies, particularly related to methods of preparation and practical use of esters of 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid as antioxidants that might be probably applicable for radioprotection and adjuvant treatment against metastases, several patents were prepared [80].

Sambongi et al. [81] have found that the novel water-soluble Hantzsch 1,4-dihydropyridine compound (the potassium salt of 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid monomethyl ester) functions in biological processes through regeneration of NADH. Various parameters related to nicotinamide coenzymes regeneration, especially in a light of chiral compounds, have been published recently [82], while Okamura et al. [83] reported the use of the oxidative conversion of dihydropyridine to pyridinium ion and the metabolic trapping principle as an approach for measuring *in vivo* cerebral redox states.

3.3. Antioxidant Activity (AOA) and Antiradical Activity (ARA) of 1,4-Dihydropyridines. Antioxidative activity of 1,4-DHPs was first evaluated and studied in the Latvian IOS (Tirzit and Duburs [39], Zilber et al. [44], and Dubur et al. [45]).



Scheme 1: Reactions of 1,4-dihydropyridines leading to the formation of pyridine derivatives.

In the field of ARA, pioneering work was made by Schellenberg and Westheimer [84] in 1965. In 1979, Schellenberg [85] revealed the free radical oxidation of a dihydropyridine following Huyser et al. [86] who reported hydroxyl radical quenching by DHPs, especially Hantzsch ester, studying free radical oxidation of DHPs *in vitro*, in the Fenton system.

AOA and ARA of various 1,4-DHPs were further studied by several different methods in both *in vitro* and *ex vivo/in vivo* systems [3, 31, 44–53]. CA nisoldipine, nimodipine, nitrendipine, nifedipine, and nicardipine have AOA that correlates with their lipophilicity (modified Buege and Aust's method of TBA determination, applied in model of rat brain cortex ischemia/reperfusion) [87].

N-Aryl-DHPs, designed as sirtuin activators, were further reported as suitable agents for neuroprotection due to their radical avoidance properties (Hardeland [88]).

1,4-DHPs inhibit free radicals and, consequentially, the cascade of events related to lipid peroxidation. They may influence several stages (initiation and/or propagation) of the lipid peroxidation cascade, which consist of ~10 reactions [89] (detailed discussion in Section 3.3.1 (2)-(b), Scheme 2).

However, considering the great number of AO compounds (including DHPs) and the diversity in their action mechanisms [90], *in vivo* studies are not always convincing and conclusive. Therefore, concise *in vitro* models are necessary to screen each compound with antioxidative properties. Antioxidants are designed to react readily with oxidizing species and are often extensively oxidized already during incubations at atmospheric oxygen tension (oxidation of some water-soluble DHPs in water (buffer) solutions is very fast, especially in the presence of light). Even during a relatively short incubation period, the concentration can drop drastically, and the real potency of the compound could be underestimated [90].

3.3.1. Common AOA and ARA Features of Some DHPs

(1) In Vitro (in Solutions, Emulsions, and Liposomes). Basic molecular principles related to antioxidative and antiradical activity of various antioxidants, including DHPs, were published recently [91]. These data show that DHPs react with various types of free radical species, stable free radicals (DPPH a.o.) and alkyl radicals and with oxygen and nitrogen free radicals. Some derivatives of DHPs may quench a singlet oxygen and may react with peroxynitrite anion [92–95].

Reactivity of DHPs toward alkyl radicals was studied electrochemically [96].

The activity against DPPH radical was found for the 5-acetyl-2-alkylthio-4-aryl-6-methyl-1,4-dihydropyridine-3-carboxylic acid nitriles [97], structural analogues of the 5-acetyl(carbamoyl)-6-methylsulfanyl-1,4-DHP-carbonitrile (studied as mitochondriotropic compounds; see further in the text, Section 3.3.1 (2)-(b)). The highest antiradical activity occurred for a compound which contains two hydroxyl groups in the 4-phenyl substituent.

DHPs were proved to decrease oxygen uptake (2-3-fold) in the heme (methemoglobin, hematin, hemin, and cytochrome C) catalysis by oxidation of emulsions of esters of unsaturated fatty acids and liposomes of phospholipid phosphatidylcholine (Zilber et al. [44] and Dubur et al. [45]).

Reactivity of nitrosoaryl and nitroaryl derivatives of 1,4-DHPs toward alkyl, alkylperoxyl radicals, and ABTS radical cation was found in various LP modeling systems suitable for determination of DHPs AOA and ARE features [98–104]. Diludine and foridone and its analogues were shown to inhibit lipid peroxidation through inhibitory effect on lipoxygenase, in emulsions and in reversed micelles (Tsetlin et al. [105] and Panek et al. [106]). In addition to inhibition of thermally initiated oxidation of methyloleate in the solution [107] (where AOA of 4-unsubstituted 3,5-dicarbonylderivatives of 2,6-dimethyl-1,4-DHPs is not linearly dependent on the inhibitor concentration), DHPs derivatives containing hydroxy, alkoxy, or dimethylaminophenyl substituents in position 4 were shown to prevent loss of β-carotene in the disperse system of β-carotene and methyllinoleate (Plotniece et al. [108]).

The AOA of DHPs has been detected using different methods in various systems where lipid free radical generation (nonenzymatic, Fe^{2+}-dependent, and/or enzymatic, NADPH-dependent) took the place [109–112]. This activity was further confirmed *in vivo*, through prevention of damage caused by renal ischemia and reperfusion, as shown for diludine [113].

Some redox properties of calcium antagonist dihydropyridines were revealed through electroanalytical studies [114]. Competitive kinetic procedure was used for exploring the AO capacity of five (four 1,4-DHPs: lacidipine, felodipine, nifedipine, and amlodipine, and one 1,2-DHP compound GR44966) CA and one calcium ion agonist (Bay K 8644). All but one (amlodipine) antagonist displayed an unambiguous AO capacity (crocin test). The calcium agonist DHP revealed no reaction with peroxyl radicals. Lacidipine was the most effective. A calcium agonist Bay K 8644 is quite resistant to oxidation and does not bind H^+. This could be important

fact in the interaction with the target proteins (it should be mentioned that there are no studies on LP with other Ca^{2+} agonists).

The decreased oxidation potential correlates with AO capacity and increased basic character. These findings suggest the relevance of the electron density on the DHP ring.

For all the DHP compounds investigated, the overall oxidation process proceeds through two consecutive one-electron releases: a primary one-electron step accompanied by a fast proton release and the formation of a neutral radical ($PyH^{\bullet}$) undergoing a second, much easier one-electron step [114].

The final product is the protonated form of the parent pyridine derivative. This pattern is relevant for the antioxidative activity, since the radical intermediate is far less prone to be reduced than oxidized.

In the case of CA DHPs, the release of protons complicates the overall oxidation process by introducing a "parasitic" side reaction where a coupling between protons and the starting species takes place.

This DHP self-protonation subtracts part of the original species from the electrode process because the parent cationic species are no longer electroactive.

Conversely, the calcium agonist DHP, which is less prone to be oxidized, turned out to be so weak base to be even unable to undergo the self-protonation reaction.

Thus, the combined effect of oxidation potentials and proton binding capacity of DHPs is a key element for the redox transition, relevant for their AO activity. Yet, opposing effects (antagonistic *versus* agonistic) on protein targets as calcium ion channels connected with protein thiol oxidation to disulfide should be also considered [114].

Kouřimská et al. [115] found AO effect of diludine (HEH) in edible oil. Reactivity of 1,4-DHPs toward SIN-1-derived peroxynitrite was shown by López-Alarcón et al. [116]. Olek et al. [117] discovered antioxidative activity of NADH and its analogue *in vitro*.

Further see, as referred in several subparts below, Sections 3.4, 3.5, and 3.7.

(2) Ex Vivo (on Lipid Peroxidation in LDL, Mitochondria, Microsomes, and Cells). Main chemical structures of DHPs examined in numerous studies and reviewed in this paper are presented in Figures 1 and 2.

(a) Various DHPs: Calcium Antagonists as Inhibitors of LDL Peroxidation. Free radicals induce peroxidation of LDL. This process proceeds by a chain mechanism which reveals phosphatidylcholine hydroperoxides and cholesteryl ester hydroperoxides as the major primary products [118]. Calcium antagonist DHPs could act as antioxidants on LDL at least in three ways: (1) as inhibitors of isolated LDL peroxidation, caused by various inducers (Cu^{2+} ions, UV light, and xanthine/xanthine oxidase system); (2) if preincubated with cells, preventing against intracellular LDL oxidation; (3) preventing against the harmful effect of oxidized LDL on cells and decreasing cytotoxicity [119–131].

Combined application of ascorbic acid and CA DHPs (amlodipine and felodipine) has an additive (cytoprotective and LDL antioxidant activity) effect [120]. It includes a combination of peroxide-degrading and peroxyl radical scavenging reactions, thus demonstrating the importance of LP during LDL oxidation and cytotoxicity induced by oxidized LDL. Cytoprotection is associated with inhibition of oxidant-induced increases in intracellular free calcium.

Similar to the other model systems, the recorded values of the tested DHPs related to AO activity on LDL LP and related events [119–131] depend on the prooxidant model system and methods used for activity measuring (see Tables 1–5).

Commercial Ca^{2+} antagonists (including 1,4-DHP derivatives), as well as some other 1,4-DHPs with less CA activity, were shown to decrease the rate of oxidation (detected as TBARS) of low-density lipoprotein (LDL) induced by Cu^{2+} ions ($CuSO_4$) in two different cell lines: U937 human monocyte-like and J774A.1 murine monocyte-macrophage cell line (Rojstaczer and Triggle [119]). The strongest effect was recorded for vitamin E, followed by felodipine, 2-Cl analogue of nifedipine, nifedipine, amlodipine, nitrendipine, verapamil, and diltiazem.

Rojstaczer and Triggle [119] found that CA from different chemical groups had a concentration-dependent effect as antioxidants against LDL oxidation (see Table 1). However, the order of potency (activity rank order, ARO) of the drug(s) again depends on the oxidation system and the antioxidant assay. Both CA and antioxidative effects relate to the 2- (or *o*-, *orto*-) substituent of the 4-phenyl ring in the same potency order $o > m \gg p$ [119]. On the other hand, the requirement for the 1,4-DHP ring is essential for both AOA and Ca^{2+} channel antagonism. A charged substituent at the position C-2 of the 1,4-DHP ring influences the AO activity (analogous to [46–53]). However, some other factors should not be neglected: for example, although amlodipine has a positively charged amine at this position, this modification makes it less lipophilic and, indirectly, less potent antioxidant.

Similar results were obtained when testing antioxidant effect of CA on LDL peroxidation in bovine aortic endothelial cells (BAECs) (Cominacini et al. [123]; see Tables 2 and 3) as well as in HUVECs (Lupo et al. [129]) (see Table 4).

Cominacini et al. [123] observed antioxidant effect of CCBs and α-tocopherol in BAECs. The order of potency (see Tables 2 and 3) [123] was however different than in U937 human monocyte-like and J774A.1 murine monocyte-macrophage cells (see Rojstaczer and Triggle [119], Table 1). The tested DHPs were lacidipine, amlodipine, lercanidipine, nimodipine, and nifedipine (in two different intracellular concentrations: 2 and 4 fmol). ROS production was significantly lowered only by lacidipine (which is the compound with the highest lipophilicity) and lercanidipine; the effect of lacidipine was much more evident than lercanidipine. Surprisingly, amlodipine, nimodipine, and nifedipine had no effect on ROS formation suggesting that the positive effects on the earliest events of atherosclerosis are a peculiarity of lacidipine molecule through its antioxidant activity.

The strong AO action of lacidipine may be related to the lipophilic cinnamic acid side chain, which favors a drug partitioning in the membrane due to favorable physicochemical (hydrophobic) interactions of drug hydrophobic residues

Table 1: Relative structure-function relationships of calcium antagonists (DHPs, verapamil, and diltiazem) and vitamin E. Effect on oxidative modification of isolated *ex vivo* human low-density lipoprotein using two various oxidation systems (copper (II) ions induced and monocyte induced). Compiled according to data presented by Rojstaczer and Triggle [119].

Compound	Systems of LDL oxidation			
	Copper (II) ions induced system (comparison of three methods)			Monocyte induced cell oxidation system
	Methods			
	Reduction of TBARS level of LDL (relative efficacy)	Degradation of oxidized [^{125}I] LDL by J774 macrophages	Relative electrophoretic mobility of LDL on agarose gel	TBARS content of LDL (in %%)
	Relative efficacy (activity rank order (ARO); ARO = I for the most effective); effective inhibitor concentration [IC], in μM			
Amlodipine	+ + (ARO = IV)	+ + (ARO = II–V)	25 μM 50 μM	25 μM (ARO = III–V)
Felodipine	+ + + + + (ARO = I)	+ + + (ARO = I) 25 μM, 97 ± 2%	50 μM	25 μM, 65 ± 9% (ARO = II)
Nifedipine	+ + + (ARO = III)	+ + (ARO = II–V)	10 μM; 50 μM	25 μM, 96 ± 2% (ARO = I)
2-Chloro analog of nifedipine	+ + + + (ARO = II)	—	—	—
4-Nitro analog of nifedipine	—	+ + (ARO = II–V)	—	25 μM (ARO = III–V)
Nitrendipine	+ + (ARO = IV)	—	No effect	—
Verapamil	+ + (ARO = IV)	+ + (ARO = II–V)	—	25 μM (ARO = III–V)
Diltiazem	+ (ARO = V)	—	No effect	—
α-Tocopherol (vitamin E)	+ + + + + + (ARO = I)	—	1 μM; 5 μM; 10 μM; 50 μM	—

Table 2: Reduction of intracellular ROS in BAECs by CA DHPs. Compiled according to data reported by Cominacini et al. [123].

Compound	Cellular amounts of compounds (in fmol/cell) determining the 50% reduction (IC_{50}) in intracellular ROS concentrations
Lacidipine	4.6 ± 0.7
Lercanidipine	9.2 ± 0.7
Amlodipine	15.3 ± 0.8
Nifedipine	16.4 ± 0.7
Nimodipine	17.2 ± 0.9

Table 3: Modulation of ROS formation in BAECs by CA (DHPs and verapamil) and vitamin E. Compiled according to data presented by Cominacini et al. [123].

Compound	Method of flow cytometry (reduced 2′,7′-dichlorofluorescein diacetate (DCFH-DA) oxidation by ROS) Activity rank order (ARO = I for the highest activity; ARO = III for the mindest activity) (Effective [IC]: 1; 5; 10; 50 μM)
Lacidipine	+ + + (ARO = I)
Lercanidipine	+ + (ARO = II)
Amlodipine	No effect
Nifedipine	No effect
Nimodipine	No effect
Verapamil	+ (ARO = III)
α-Tocopherol	+ + + (ARO = I)

with polyunsaturated groups of membrane phospholipids. However, DHPs can also reduce the oxLDL-induced ROS concentration by affecting some intracellular ROS producers, such as NADPH oxidases, xanthine oxidase, and cyclooxygenase enzymes. The activity of these enzymes contributes to intracellular ROS elevation [125].

Preincubation of HUVECs with lacidipine inhibited an increase of intracellular ROS caused by oxidized LDL [124].

Lupo et al. [129] have studied the dose-dependent (1, 5, 10, and 50 μM) AOA of various CA (verapamil, diltiazem, and DHPs: nifedipine, amlodipine, isradipine, or lacidipine) against normolipidemic human blood LDL oxidation compared with α-tocopherol by measuring the content of TBARS and the diene formation (see Table 4).

Table 4: Normolipidemic human blood LDL (0.25 mg/mL) *in vitro* oxidation in the presence of 5 μM $CuSO_4$ and CA of 3 types (DHPs, verapamil, and diltiazem) and vitamin E. Compiled according to Lupo et al. [129].

Compound	Methods			
	TBARS method (fluorimetry at 515 nm/533 nm, 4 hours preincubation of LDL with compounds and copper (II) ions; 320% TBARS increase in control during 4 h period)		Inhibition of conjugated diene formation (at 234 nm) expressed as prolongation of induction period (in %% of control). t_{contr} = 36.8 min.	
	Effective [IC] (in μM): 1 μM; 10 μM; 50 μM	Activity rank order (ARO = I for the highest activity; ARO = VII for the mindest activity)	Effective [IC] (in μM): 1 μM; 5 μM; 10 μM; 50 μM	Activity rank order (ARO = I for the highest activity; ARO = VII for the mindest activity)
Nifedipine	10 μM; 50 μM	ARO = III	5 μM; 10 μM, 150%; 50 μM, 213%	ARO = III
Amlodipine	50 μM	ARO = IV	5 μM; 10 μM, 122%; 50 μM, 138%	ARO = IV–VI
Isradipine	50 μM	ARO = VI	10 μM, 150%; 50 μM, 183%	ARO = IV–VI
Lacidipine	1 μM; 10 μM; 50 μM	ARO = II	5 μM; 10 μM, 192%; 50 μM, 283%	ARO = II
Verapamil	50 μM	ARO = V	10 μM, 150%; 50 μM, 178%	ARO = IV–VI
Diltiazem	No effect	No effect (ARO = VII)	No effect	No effect (ARO = VII)
Vitamin E	1 μM; 10 μM (IC_{50}); 50 μM (20% of control)	ARO = I	5 μM; 10 μM, 230%; 50 μM, 370%	ARO = I

Table 5: Antiproliferative effect (oxLDL-induced HUVSMCs proliferation) of CA DHPs and simultaneous oxLDL-induced ROS production scavenging. Comparison with N-acetyl-L-cysteine, NAC (intracellular ROS scavenger). Compiled according to data presented by Zou et al., 2012 [130].

DHP compound	Methods			
	Antiproliferative effect against proproliferative effect induced by oxLDL (50 μg/mL) (UV detection of formazan production from tetrazolium salt)		oxLDL-induced ROS production (fluorescent DCF (2′,7′-dichlorofluorescein) production)	
	Effective [IC] in μM and I in %			
Amlodipine	3 μM	I = 18%	3 μM; 10 μM	No effect I = 20%
S(−)-Amlodipine	No effect		No effect	
Lacidipine	10 μM; 30 μM	I = 21% I = 27%	10 μM	I~2/3 of control
N-Acetyl-L-cysteine, NAC	—		5000 μM (5 mM)	I = 28%

As presented (Table 4, according to [129]), for diltiazem (poor lipid solubility), no AO was detected, whereas the other CA and α-tocopherol have demonstrated AOA at least at concentrations of 10 and 50 μM: α-tocopherol > lacidipine > nifedipine > isradipine, verapamil, and amlodipine. Additionally, α-tocopherol and lacidipine were able to significantly attenuate *in vitro* LDL oxidation at 1 and 5 μM. These results have confirmed the highest activity for the strongly lipophilic DHP type CA compound lacidipine. This might be a possible antiatherogenic mechanism of CA, since oxidative modification enhances the atherogenic potential of LDL.

The lipid peroxidation of LDL, promoted either by UV radiation or by copper ions, was inhibited (antioxidant effect) by nisoldipine in a dose-dependent manner (IC_{50} values were evaluated at around 10 μM), nimodipine was less potent (IC_{50} around 50–100 μM) and nicardipine almost inactive. In addition to this indirect protective effect, CA DHPs nisoldipine and nimodipine exerted direct protective effect on lymphoid

cells, against toxicity of previously oxidized LDL. The IC_{50} values were 6 ± 2 and $80 \pm 20\,\mu$M, respectively [122]. The inhibition of the cytotoxic effect of LDL oxidized in the presence of DHP type Ca^{2+} channel blockers correlated well with protection from oxidation by these compounds. Complete protection cannot be obtained because the DHPs are cytotoxic themselves. The potential relevance to the prevention of atherogenesis is envisaged.

DHP type CCB nifedipine was the most effective inhibitor of oxidation promoted either by UV radiation or by copper ions in experiments with cultured lymphoid cells LDL (2 mg apoB/mL); CCBs from other two CCB classes, diltiazem and verapamil, were only poorly active or completely ineffective [121]. The protective effect of nifedipine occurs at two levels: besides its direct antioxidant effect by inhibition of LDL oxidation, it also exhibits a direct cytoprotective effect against cytotoxicity of oxidized LDL by yet unknown mechanisms. The protective effect of CCBs was not due to an inhibition of LDL uptake. This effect seems to be independent of the inhibition of LDL oxidation *per se* since LDL was oxidized in the absence of the drug before the incubation with cells. Moreover, this direct protective effect was observed at lower concentrations (IC_{50} of $1 \pm 0.2\,\mu$M) compared to the antioxidant effect (IC_{50} of TBARS inhibition is around $10 \pm 2\,\mu$M at UV promoted and $4 \pm 0.5\,\mu$M by Cu^{2+} ions initiated). The AO effect of nifedipine is also correlated with the protection of endogenous tocopherols ($IC_{50} = 50\,\mu$M). It was suggested that the AO effect of CCBs protected cells indirectly from the cytotoxic effect of oxidized LDL [121].

A recent study has reported that beneficial vascular effects of lercanidipine in diabetic rats depend on its antioxidant activity related to attenuating the increase in oxidative stress and in vascular matrix metalloproteinase-2 (MMP-2) (Martinez et al. [126]). Lesnik et al. [127] studied the impact of a combination of this calcium antagonist and a β-blocker atenolol on cell- and copper-mediated oxidation of LDL and on the accumulation and efflux of cholesterol in human macrophages and murine J774 cells. They realized that lercanidipine reduced the oxidative modification of LDL rather than diminished cholesterol accumulation in human foam cells.

Comparing the antioxidative action of CA (DHPs, amlodipine, lacidipine, nifedipine, and isradipine, as well as diltiazem and semotiadil) in the copper-catalyzed oxidation of low-density lipoprotein (LDL) with that of glycated (g)/glycoxidated (go) LDL demonstrated that the strongest AO effects during long-term LDL glycation are seen for isradipine, lacidipine, nifedipine, and semotiadil [128]. Inhibitory effects were in the range 10^{-5}–10^{-3} M. Authors suggested that, due to the increased generation of ROS by glucose-modified LDL, the chain-breaking capacity of CA may be overridden. The AOA of CA depends on their lipophilicity and their ability to incorporate into the LDL particle, that is, to reach the site of peroxidation. CA, like other AOs, significantly retards advanced glycation end products (AGE) formation, whereas initial glycation reactions, such as Amadori product formation, are only weakly inhibited. The observation that both oxidative changes and at least long-term glycation effects are indeed drastically reduced by CA is corroborated by fluorescence analysis, AGE-ELISA, quantitation of lipid peroxidation, and TBARS measurement of long-term g/go LDL.

The effects of lipophilic DHP calcium channel blockers on oxidized LDL-induced proliferation and oxidative stress of vascular smooth muscle cells were also studied [130] (see Table 5).

Lacidipine and amlodipine reduced carotid intima-media thickness by decreasing proliferative effect of oxLDL, whereas (*S*-)-amlodipine had no antiproliferative effect. ROS-MAPKs (mitogen-activated protein kinases) pathway might be involved in the mechanism.

Both 1,4-DHP CCBs lacidipine and nifedipine reduce plasma and LDL oxidation and formation of oxidation-specific epitopes. Their application may also relate to prolonged survival of rats, independently of blood pressure modifications (in the SPSHR model, 1 mg/kg per day lacidipine and 80 mg/kg per day nifedipine). These results suggested that the protective effect of these two 1,4-DHP drugs *in vivo*, as shown in cerebral ischemia and stroke, may in part result from inhibition of LDL oxidative process, although these two drugs possess different lipophilic properties [131]. Both lacidipine (0.3 and 1.0 mg/kg) and nifedipine (80 mg/kg) prolonged lag time of the conjugated diene formation in LDL isolated from arterial wall, and t_{max}. These drugs significantly reduced electrophoretic mobility of oxLDL from SPSHR subjected to X/XO oxidation system. 1,4-DHP CCBs also protected apolipoprotein B, which is important for the binding with macrophage LDL receptor lysine residues. The doses used ($>10^{-6}$ mol/L for SPSHR and normotensive WKY rats), however, are 2 to 3 orders of magnitude higher than those inhibiting vascular smooth muscle contraction *in vitro* and *in vivo*. They also exceed values that are commonly used in clinical practice. The daily dose of lacidipine for hypertensive patients is 0.07 mg/kg, ≈4- to 14-fold lower than the 2 doses used in SPSHR. The maximum daily dose of nifedipine given to hypertensive patients is 2.0 mg/kg, ≈40-fold lower than what were used [131]. These discrepancies may be related to differences in bioavailability of CA between rats and humans [131].

Accordingly, in routine clinical use, 1,4-DHP CCBs do not reach the concentrations required for antioxidant activity *in vitro* [131].

Another data concerning the effect of CA DHPs on OS related to LDL is presented under Section 3.5.

(b) Effect of DHPs on Isolated Rat Liver and Heart Mitochondria. As a major cellular source of oxygen radicals (Cadenas [4, 5]), mitochondria are promising targets for pharmacological and toxicological actions of various membrane-active compounds, including several 1,4-DHP derivatives. Zernig et al. [132] have discovered CA binding sites associated with an inner mitochondrial membrane anion channel.

More than 40-year long research on mitochondrial effects of the DHPs (on their bioenergetics, chemiosmotic properties, and ion fluxes) clearly points them out as mitochondriotropic compounds.

The activity of the first 35 synthesized compounds (derivatives of 1,4-DHP, their heteroaromatic analogues,

NAD-H^+ and butylated hydroxytoluene (BHT, BOT)) originally was examined in rat liver mitochondrial LP system, in the presence of Fe^{2+} ions and using the ultraweak chemiluminescence method (Dubur et al. [89]).

Several 1,4-DHP derivatives, Hantzsch ester diludine and its analogues, were found to be effective antioxidants in this experimental system, changing the kinetics of LP, lengthening the time of the appearance of the maximum of the slow burst of the chemiluminescence (latency, latent period), and diminishing the reaction rate (the tangent of the slope angle during the time in which the amplitude of the slow burst characterizing LP rate increases) and its peak value. Their presence has influenced the reaction constant K_6, in relation to a very significant reduction of lipid hydroperoxides and/or inactivation of free radicals, as follows:

$$\mathrm{ROO}^{\bullet} + \mathrm{ROO}^{\bullet} \longrightarrow \mathrm{P} + \mathrm{h}\nu^{*} \text{ termination} \quad (1)$$

(P = molecular products) or

$$\mathrm{ROO}^{\bullet} + \mathrm{ROO}^{\bullet} + \mathrm{H_2O} \longrightarrow \mathrm{ROH} + \mathrm{RO}^{\bullet} + {}^{1}\mathrm{O_2}^{*} \quad (2)$$

In this study, diludine was one of the most active compounds. DHPs had activity similar to the standard synthetic AO-BHT (ionol). However, when plotted against applied concentration and time window, diludine's activity profile differed from that of BHT.

There were also similar studies (using different LP rate experimental detection system and method, Hunter et al. [133]), based on exploring a group of 26 2,6-dimethyl-3,5-disubstituted- and 2,6-dimethyl-3,4,5-trisubstituted-1,4-dihydropyridines (1,4-H_2Py=1,4-DHPs) and five related pyridines as inhibitors of rat liver Mit swelling ($\Delta A_{520}/t$) and O_2 uptake by ascorbic acid- (AsA-) dependent lipid peroxidation and as modulators of Mit swelling induced by Na^+-linoleate or Na^+-pyrophosphate (Velēna et al. [112]).

Some of tested 4-DHPs (4-unsubstituted 3,5-dialkoxycarbonyl-2,6-dimethyl-1,4-DHPs and 3,5-diamido-2,6-dimethyl-1,4-DHPs, both 4-unsubstituted, or those possessing lipophilic 4-aryl- groups) have shown significant AO and membrane stabilizing activity. These studies further revealed that 1,4-DHPs preferably act as AO during the stages of initiation and prolongation of LP chain reactions, at low concentrations. The studied 1,4-DHPs had IC_{50} (when V_0/V or $\tau/\tau_0 = 2$) 0.1 μM to 100 μM and the minimal activity was scored for oxidized (heteroaromatized) derivatives.

At the concentration of 100 μM, 3,5-di-*n*-butyloxycarbonyl-2,6-dimethyl-1,4-DHP entirely stops mitochondrial swelling in the presence of 0.8 mM Na^+-pyrophosphate. At the same concentration, the following compounds alter the mitochondrial swelling rate in the presence of natural protonophore, Na^+-linoleate: 3,5-di-*p*-hydroxyphenoxycarbonyl- and 3,5-di-*p*-tolyloxycarbonyl-2,6-dimethyl-1,4-DHPs, 3,5-diethoxycarbonyl-2,6-dimethyl-pyridine (oxidized form of Hantzsch ester), and more lipophilic 3,5-diamyloxycarbonyl-2,6-dimethyl-pyridine. The alteration of swelling may be scored as prolonged, promoted, accelerated, or inhibited. The type of alteration depends on the structure and concentration of 1,4-DHPs, the type of initiators of the swelling process, and the medium composition.

In accord with previously published Janero's results (lack of AO for Ca^{2+} antagonists, nifedipine and nicardipine, even at 500 μM concentration in LP tests performed on heart membrane [134]), no antioxidative activity for 4-phenyl substituted derivatives of 3,5-dialkoxycarbonyl 1,4-DHP (close analogues of Ca^{2+} antagonists) was found, contrary to various 4-nitrophenyl 1,4-DHP derivatives, calcium antagonists, for which the significant antioxidant activity was reported [31, 46–53].

Studies made on phosphatidylcholine liposomes (our unpublished data) suggest approximately three and two times more antioxidative activity for 100 μM 4-unsubstituted DHP compound diludine, when compared to 4-substituted DHPs riodipine/nifedipine and nicardipine, respectively, at methemoglobin-induced LP (oxygraphy).

Inhibition of mitochondrial AsA-dependent LP and stabilization of mitochondria were shown to be characteristic for a large group of 1,4-DHP compounds [112], showing to possess the AOA in simplest *in vitro* systems (Tirzit and Duburs [39], Zilber et al. [44], and Dubur et al. [45]) based on reactions with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), LP of fatty acid ester (linethole and methyloleate) emulsions, and phospholipid (phosphatidylcholine) liposomes. Generally, these properties did not coincide with Ca^{2+} antagonism. Depending on DHP structure, it seems that AOA properties are less specific than Ca^{2+} antagonist properties. Both properties may be interrelated but not interdependent.

These data show that the presence and the nature of a substituent in position 4, as well as 3,5-substituents, are important factors for 1,4-DHP antioxidant effects in various systems, that is, AsA-dependent nonenzymatic as well as enzymatic NADPH-dependent lipid peroxidation. Sometimes, the efficacy of inhibition of nonenzymatic LP by 1,4-DHPs is higher than the inhibition of the enzymatic LP. However, the action may be opposite, stimulation of the LP. Hantzsch ester (HEH, diludine) and its close analogues exhibited significant AOA and membrane stabilizing properties in both AsA-dependent nonenzymatic peroxidation of mitochondria and NADPH-dependent enzymatic LP of microsomes, usually at similar 10 to 100 μM concentrations [112].

The order of AO potency (IC_{50} values) *in vitro* depends on drug structure as well as on the experimental conditions and specificity of the biological system. Each method for determination of AOA and ARA has advantages and disadvantages (Karadag et al. [135]).

Accordingly, as reported by Gubskiĭ et al. [136], IC_{50} for the AsA-dependent LP was 0.25 μM and 2.0 μM for 1,4-DHP Ca^{2+} antagonists nitrepine (nitrendipine) and nifedipine, respectively. Takei et al.'s [137, 138] studies on mitochondrial swelling induced by LP or arachidonic acid in the rat brain determined the IC_{50} values of 12.7, 10.5, 156.8, and 38.4 μM for efonidipine, nicardipine, nifedipine, and nimodipine, respectively. For LDL in the copper-induced oxidation system the order of potency was vitamin E > felodipine > 2-chlorophenyl analogue of nifedipine > nifedipine > amlodipine, nitrendipine, verapamil, and diltiazem (Rojstaczer and Triggle [119]).

It was interesting to compare the AOA of DHPs with their susceptibility to oxidation, that is, electron and hydrogen donating properties.

It has been estimated that electron donor substituents in positions 2 and 6 of 1,4-DHP cycle usually promote oxidation, while electron acceptor substituents promote quench oxidation. Stronger electron acceptors in positions 3 and 5 also significantly quench oxidation. These estimations are based on studies including chemical, enzymatic, and electrochemical oxidation of 1,4-DHP derivatives (Dubur and Uldrikis [70], Duburs et al. [71], and Stradin et al. [139]).

On the other hand, diminished AOA of 1,4-DHP relates to presence of substituents in position 4 (both electron donor and electron acceptor) (Velēna et al. [112]).

3,5-Dicarbamoyl substituents possess minimal quenching feature and are followed by benzoyl-, acetyl-, and alkoxycarbonyl- groups. Maximal decrease was obtained with condensed substituents (i.e., oxoindeno- or oxocyclohexeno- groups) and a CN-group. 4-Unsubstituted 3,5-dicarbamoyl derivatives can be easily oxidized and consequentially inactivated, whereas 4-substituted 3,5-dicarbamoyl-1,4-DHPs possess an oxidation potential, analogous to the 4-unsubstituted 3,5-COOR derivatives. Therefore, they have adequate electron donor properties and are considerably stable. This may be the reason for significant membrane stabilization upon exposure to 4-substituted derivatives. Of importance, their AOA was usually more pronounced in comparison to 4-unsubstituted derivatives.

Among them, 2,6-dimethyl-3,5-difurfuryloxycarbonyl-1,4-DHP showed the highest antioxidative activity. In the group of 3,5-dialkoxycarbonyl derivatives, the strongest activity was attributed to compounds with medium length alkyl chains (*i*-butyl-, *t*-butyl-, and *i*-amyl- substituents), high level of lipophilicity, minimal electron acceptor properties, and moderate steric hindrance, as contrasted to short or long alkyl chain ester derivatives (3,5-dimethoxycarbonyl-, 3,5-diethoxycarbonyl derivatives and 3,5-didodecyloxycarbonyl derivative). These data demonstrate the bell-shaped dependence of AOA on alkyl chain length [112] and are in accord with results obtained in liposomes. However, these data differ from those obtained in emulsions, where diludine was the most active compound. Finally, oxidized heteroaromatic derivatives showed only minimal activity.

In both LP systems studied (AsA-dependent in mitochondria and NADPH-dependent in microsomes), some of 1,4-DHPs showed activity similar to classical antioxidant, butylated hydroxytoluene (ionol, BHT) (Velēna et al. [112]). However, there was a significant difference related to concentration and incubation time. It allowed us to postulate that 1,4-dihydropyridines (InH), acting as antioxidants-reductants and scavengers of reactive oxygen species and lipid free radicals, preferably influence initiation and propagation (prolongation) of lipid peroxidation chain reactions (1)–(5), according to Scheme 2. The phenomenon is particularly prominent in the presence of Fe^{2+} and other ions of variable valency.

Chain break and termination reactions (6)–(10) of the LP reaction cascade [89] were influenced by 1,4-DHPs in a lesser degree than were initiation and propagation steps. This may be important for their therapeutic effects even in the advanced stages of LP.

Scheme 2 (stages of initiation, propagation, and termination of lipid peroxidation chain reactions (1–10)). Initiation and propagation reactions are as follows:

(1) $HOO^{\bullet} + RH \rightarrow R^{\bullet} + H_2O_2$ (RH = membrane lipid)
$HO^{\bullet} + RH \rightarrow R^{\bullet} + H_2O$
$HOO^{\bullet} + InH \rightarrow In^{\bullet} + H_2O_2$ (InH = 1,4-DHP)
$HO^{\bullet} + InH \rightarrow In^{\bullet} + H_2O$

(2) $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$ ($R^{\bullet}$; $RO^{\bullet}$; $ROO^{\bullet}$ = lipid radicals)

(3) $ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

(4) $ROOH + Fe^{2+} \rightarrow RO^{\bullet} + Fe^{3+} + HO^{-}$

(5) $RO^{\bullet} + RH \rightarrow ROH + R^{\bullet}$; $R^{\bullet} + InH \rightarrow RH + In^{\bullet}$

Chain break and termination reactions are as follows:

(6) $ROO^{\bullet} + ROO^{\bullet} \rightarrow P + h\nu^{*}$ (P = molecular products) or $ROO^{\bullet} + ROO^{\bullet} + H_2O \rightarrow ROH + RO^{\bullet} + {}^{1}O_2{}^{*}$

(7) $ROO^{\bullet} + InH \rightarrow ROOH + In^{\bullet}$ (ROOH = membrane lipid peroxides)

(8) $RO^{\bullet} + In^{\bullet -} \rightarrow Y$ (Y = molecular products)

(9) $ROO^{\bullet} + Fe^{2+} \rightarrow Fe^{3+} + X$ (X = molecular products)

(10) $RO^{\bullet} + RO^{\bullet -} \rightarrow Y$ (Y = molecular products)

In the reversible swelling of mitochondria accompanying LP (initiated by mixture of 5 mM GSSG/1 mM GSH), several 1,4-DHPs showed low or no activity, manifested only as a decrease of the swelling amplitude, without a rate decrease. An addition of GSH (4 mM) or ATP to swollen mitochondria caused their contraction in both control and tested system. It may be suggested that 1,4-DHPs, acting as antioxidants in mitochondria, preferably influence LP reactions initiated by ions with variable valency or their complexes with heme type compounds: methemoglobin, hemin, hematin, and so forth (Velēna et al. [112]). If the peroxidation process has a maximal velocity and 50 percent of initial O_2 were consumed, 1,4-DHPs cannot completely break the chain reactions and prevent subsequent membrane damage: by addition of DHP substance at 10 μM concentration at the moment of 50 percent oxygen consumption, the subsequent oxygen uptake proceeded unchanged. This observation is important for the application of DHPs as inhibitors of initiation and, to a lesser degree, propagation stages of LP chain reactions.

The influence of 1,4-DHPs on Mit swelling is not strictly associated with their own oxidation. There is the possibility that the labilizing (or stabilizing) effect relates to surface activity (connected with substituent lipophilicity) or may be the consequence of complexation with some -OH (or $-CH_3$) group sensitive receptors at the mitochondrial membrane. Namely, a bathochromic shift of the absorption band maximum (about 10 nm) was observed in the visible region before swelling. However, after swelling in the presence of Na^{+} linoleate, the spectrum returns to its initial value [112].

Some 1,4-DHPs not only protect mitochondria against swelling caused by AsA-dependent LP, salts of fatty acids

in vitro [112], but also have beneficial effects on repairing their integrity *in vivo*, after exposure to irradiation, hepatotoxins, ischemia, hypoxia, or hypothermia. Some of them were shown to normalize the process of intracellular reparation and physiological regeneration of ultrastructures. They were also shown to stimulate reparative processes. If pretreated with 1,4-DHPs, irradiated mitochondria will not swell (Ivanov et al. [140, 141]).

Diludine, ionol, and some other AOs, mitochondria protectors, act as anti-ischemic agents. If applied prophylactically *in vivo*, they may prevent ischemic and reperfusion lesions in heart, kidney, and other organs (Bilenko et al. [113]). The effect is dependent on applied dose, timing, and way of application. When added onto the cryoconservation medium for mitochondria preservation, 1,4-DHPs prevented decrease of membrane potential, normalized facilitated respiration, and prevented loss of mitochondrial Na^+ and Ca^{2+} ions, after thawing ([112], see citation number 36 (Subbota et al., Kharkov, 1984) therein). Diludine was stronger protector, when compared to ionol.

CA drug foridone (riodipine) was shown to possess cardioprotective features, primarily due to is protective effect on mitochondria exposed to OS [142, 143].

Similarly, the DHP water-soluble antiarrhythmic compound glutapyrone inhibits initiation of LP by free radicals in erythrocytes and heart mitochondria. Its cardioprotective effect has been experimentally shown in heart mitochondrial membranes, especially during deep hypothermia (Utno et al. [144]).

Cerebrocrast was effective in several translation models mimicking pathological situations, known to be associated with cellular OS. The potential protective action of 1,4-DHP derivatives (4-substituted compounds: cerebrocrast, gammapyrone, glutapyrone, and 4-unsubstituted drug diethone) has been studied in rat liver, in experimental models relevant for oxidative stress and mitochondrial bioenergetics (Fernandes et al. [145]). When succinate was used as the respiratory substrate, higher concentrations (>25 μM) of cerebrocrast depressed respiratory control ratio (RCR), ADP to oxygen ratio (ADP/O), state 3, and uncoupled respiration rates, transmembrane potential (deltapsi), and the phosphate carrier rate. At the same time, state 4 respiration rate was three times increased. At concentrations lower than 25 μM, cerebrocrast inhibited mitochondrial IMAC and partially prevented Ca^{2+}-induced opening of the mitochondrial PTP. Gammapyrone, glutapyrone, and diethone did not induce these phenomena. When applied at concentrations up to 100 μM, cerebrocrast, gammapyrone, and glutapyrone did not affect ADP/Fe^{2+}-induced LP of mitochondria in rat liver (as measured by oxygen consumption and TBARS formation). On the other hand, low diethone concentrations (up to 5 μM) inhibited it in a dose-dependent manner. Diethone also prevented against deltapsi dissipation induced by LP initiated by ADP/Fe^{2+}. Based on these data, it may be speculated that cerebrocrast (inhibition of the IMAC) and diethone (acting as an AO) may provide effective protection of mitochondria during OS. Cerebrocrast has shown some therapeutic potential for treatment of several pathological conditions related to cellular OS [145].

5-Acetyl(carbamoyl)-6-methylsulfanyl-1,4-DHP-carbonitriles (Figure 3) with minor differences in their molecular structure, displaying antioxidant and antiradical activities *in vitro*, show different biological activities. Namely, 4-*p*-chlorophenyl derivative OSI-1146 displays AO and antiradical activities in cardiovascular OS models, whereas OSI-3701 and OSI-3761 display hepatoprotective activity. Thus, these compounds may be potentially useful for treating several pathological processes, including those associated with OS (Fernandes et al. [146]). However, besides mitochondria, the cellular targets for their pharmacological actions have not been fully investigated [146]. All these compounds increase the susceptibility of Mit to MPT. The most potent is OSI-3701, although it does not affect bioenergetic parameters.

Although all these compounds protected mitochondria against LP induced by the oxidant pair ADP/Fe^{2+}, OSI-1146 was shown to be the most potent. Current data point out mitochondria as potential targets for protective and toxic actions of DHPs, suggesting that the potential for their use as therapeutic agents should also take into consideration their toxic effects on mitochondria (Fernandes et al. [146]).

Several structurally different DHP derivatives (antioxidant diludine (diethone), as a 4-unsubstituted DHP, 4-substituted DHPs: CA foridone (bicyclic compound), and the 4-phenyldiethone compound where phenyl group is joined to the DHP in position 4) inhibited the 1-methyl-4-phenylpyridinium iodide (MPP^+) induced ROS production in cerebellar granule cells (CGC) with a distinct potency order: foridone (2,6-dimethyl-3,5-dimethoxycarbonyl-4-(*o*-difluoromethoxyphenyl)-1,4-dihydropyridine) > 2,6-dimethyl-3,5-diethoxycarbonyl-4-phenyl-1,4-dihydropyridine > diludine. They also reversed the MPP^+-induced decrease of the mitochondrial membrane potential in the same order (Klimaviciusa et al. [147]). Accordingly, it was postulated that the classical two-ring (bicyclic) structure of DHP derivatives represents an advantage in relation to neuroprotection and ROS defense and is independent on compound's properties related to calcium ions.

Novel adamantane-containing 1,4-DHP compounds (Klimaviciusa et al. [148]) were also found to improve mitochondrial functions (MPP^+ model) (Klimaviciusa et al. [148]). Klusa et al. [149] have discovered antineurotoxic effects of 1,4-DHP taurine derivative, tauropyrone, recorded as Mit function improvement.

Many 1,4-DHPs, including Ca^{2+} antagonists and AO, modify LP processes and influence mitochondrial function in various organs (liver, heart, kidney, and brain) in a different way and degree. Their beneficial action, oxygen or lipid free radical scavenging, antioxidative effects, binding with or intercalating into phospholipid bilayer, regulation of ion gating, and regulation of mitochondrial permeability transition pores (Tirzit and Duburs [39], Zilber et al. [44], and Dubur et al. [45]), separately or in combination with each other, depends on two strong elements: (1) their individual structure including nature of substituents and their positions and (2) the nature of the biological system. For example, the direction of LP (inhibition of promotion) was shown to depend on structure and concentration of applied

1,4-DHPs as well as stages of chain reactions. Accordingly, mitochondrial swelling may be prolonged (retarded), accelerated (promoted), or inhibited (Velēna et al. [112]).

Therefore, there is a ground for 1,4-dihydropyridines, either Ca^{2+} antagonists or antioxidants, to be nominated as useful tools in development of "mitochondrial drugs" related to the control of OS.

(c) DHPs as AOs in Endoplasmic Reticulum (Inhibition of NADPH-Dependent LP System): Inhibition of NADPH Oxidase by DHPs. Elevated level of NADPH oxidase 4- (NOX4-) derived hydrogen peroxide (H_2O_2) joined with concomitant decrease of nitric oxide (NO) mediated signaling and reactive oxygen species scavengers are considered to be central factor in molecular pathogenesis of fibrosis (Sampson et al. [150]). Inhibition of microsomal NADPH-dependent LP, with particular focus on NADPH oxidases (NOX1–5 and DUOX1), may be very important for neuro-, cardio-, and hepatoprotection (Velēna et al. [112], Leto and Geiszt [151], Griendling et al. [152], and Chen et al. [153]). Endoplasmic reticulum may be an important target, as this is where 1,4-DHPs could display their antioxidative properties (Velēna et al. [112], Leto and Geiszt [151], Griendling et al. [152], and Chen et al. [153]).

However, the initiation of LP in the NADPH-dependent microsomal system does not appear to involve either superoxide or hydrogen peroxide, since neither SOD nor catalase can inhibit it. On the other hand, reduced iron plays an important role in both the initiation and propagation of NADPH-dependent microsomal lipid peroxidation (Hochstein and Ernster [154] and Repetto et al. [111]).

Many DHPs possess inhibitory activity not only towards AsA-dependent LP in mitochondria but also towards NADPH-dependent LP, as shown in isolated rat liver microsomes (Velēna et al. [112]). This means that these compounds interact with the shared parts (nonenzymatic and enzymatic) of LP pathways.

Microcalorimetry and fluorescent probes procedures were used for studying the interaction of alpha-tocopherol and 1,4-DHPs with endoplasmic reticulum membranes and model systems, human serum albumin, and phospholipid bilayers [155]. Modification of microviscosity of the endoplasmatic reticular membranes depends on localization of antioxidants within the protein structures or phospholipid phase. Increase of membrane structuralization under the influence of 1,4-DHPs blocked their antioxidant action in spontaneous and induced lipid peroxidation.

Inhibition of rat heart and liver microsomal lipid peroxidation by nifedipine was observed [156], while Goncalves et al. [157] found antioxidant effect of calcium antagonists on microsomal membranes isolated from different brain areas.

Nitroaryl-1,4-DHPs are both calcium channel antagonists and antioxidant agents (Letelier et al. [158, 159]), commonly used for treatment of cardiovascular diseases. These drugs must be metabolized through cytochrome P450 oxidative system (NADPH-cytochrome P450 reductase), mainly localized in the hepatic endoplasmic reticulum. Several lipophilic drugs generate OS while being metabolized by this cellular system. Thus, DHP antioxidant properties may prevent the OS associated with hepatic biotransformation of drugs. Various commercial and new nitro-phenyl-DHPs were studied against LP using rat liver microsomes under oxidative stress [159].

Incubation of rat liver microsomes with the 4′-nitro-4-phenyl-1,4-DHP compounds (2,6-dimethyl-4-(4′-nitrophenyl)-1,4-dihydropyridin-3,5-diethyl-dicarboxylate and N-ethyl-2,6-dimethyl-4-(4′-nitrophenyl)-1,4-dihydropyridin-3,5-dimethyl-dicarboxylate) results in an inhibition of LP, the UDPGT (UDP-glucuronyltransferase) oxidative activation, and the microsomal thiol oxidation induced by Fe^{3+}/ascorbate, a generator system of ROS. This effect was also produced by nitrofurantoin and naphthalene in the presence of NADPH.

Interestingly, IC_{50} of DHPs obtained from microsomal LP assays decreased to the same extent as the microsomal thiols oxidation provoked by Fe^{3+}/ascorbate [159]. Nevertheless, the AO effects of a nitrophenyl-DHP compound, in which hydrogen at position one of the DHP ring was replaced by the ethyl group, were significantly weaker. Authors speculated that DHPs can resemble NADH, transferring one hydrogen atom of 4-position (H^-) to anion superoxide and another of the 1-position (H^+) by way of a cationic radical intermediate to generate pyridine derivatives and water [159].

The AO effects of various tested DHP derivatives (*m*- and *p*-NO_2 phenyl as well as methyl or ethyl and isopropyl-DHP 3,5-dicarboxylate derivatives) were not significantly different. The authors assumed that the -NH- group of the dihydropyridine ring could contribute both to the development of the calcium channel antagonism and to the antioxidative properties of DHPs [159].

Prevention of the membrane LP seemingly depends on the concentration of potential antioxidants, such as vitamin E or even 1,4-DHP in lipids. However, only the differences in synthetic DHPs lipophilicity cannot explain significant variations of DHPs concentration in microsomal membrane and cannot clarify the strength of their antioxidative activity. This work [159] has further demonstrated that 1,4-DHPs may prevent the OS induced by biotransformation of some drugs, for example, antibiotic nitrofurantoin. Simultaneous administration of DHPs and nitrofurantoin may be beneficial in reducing nitrofurantoin side effects.

While most of Ca antagonist 1,4-DHPs are metabolized by CYP3A4 (Guengerich et al. [160]), not all of them are good inhibitors of its activity. Thus, nicardipine, but not nifedipine and nitrendipine, inhibits CYP3A4 *in vitro* [53]. Interaction of different DHPs with various types of cytochrome P450 was described by Carosati et al. [53]. It was also reported that DHP class calcium channel blockers reduce the antiplatelet effect of clopidogrel (Park et al. [161]). This implies the mutual interactions of both drugs with CYP3A4.

(3) In Vivo. Evaluation of nifedipine effects on *Saccharomyces cerevisiae* was recently published (Asma and Reda [162]). Surprisingly, nifedipine exercised a toxic effect on *Saccharomyces cerevisiae* shown through measuring cellular proliferation, respiratory activity, and the level of some biomarkers (CAT and MDA).

However, majority of data obtained on various animal cells and tissues by other authors show the protective role of DHPs against both LP and oxidative stress [113, 163, 164].

The AOA attributed to many 1,4-DHPs, Ca^{2+} antagonists and other compounds, reflecting on catalytic LDL peroxidation (see Section 3.3.1 (2) and Section 3.5), should encourage their testing for treating cardiovascular diseases and/or alterations of lipid metabolism.

The possibility that 1,4-DHP-based calcium antagonists exert an antiatherosclerotic action (*via* inhibition of LDL oxidation and other mechanisms) has been proved by many experimental data [165] and several clinical trials. Besides antihypertensive effect, nicardipine was shown to possess antioxidative and antielastase activity [165, 166]. These properties may be useful for prevention of inflammatory reaction which is relevant for hypertension pathogenesis.

1,4-DHPs administration inhibits LDL oxidation mediated by oxygen radicals, leading to decreased carotid intimal media thickness and reduced progression of coronary atherosclerosis [130]. It additionally preserves Apo B-100 integrity against ROS. Of importance, antiatherogenic mechanisms differ between animals and humans (primarily in the stage of conversion of aldehydes to carboxylic acids) (Parthasarathy et al. [167]).

For example, furyl-DHP compound (FDP-1, diethyl 2,6-dimethyl-4-(furyl)-1,4-dihydropyridine-3,5-dicarboxylate) was shown to act as an antioxidant (decreasing MDA, GOT, and FFA release of ischemic myocardium and inhibiting Ca-ATPase of erythrocyte membranes), preventing against heart myocardium ischemia-reperfusion injury and arrhythmia, when applied (in rats) at 10 mg/kg (Liu et al. [168]).

Similarly, antioxidative effects of azelnidipine and amlodipine prevented neuronal damage by CCBs, after transient focal ischemia in rats (Lukic-Panin et al. [169]).

Allanore et al. [170] found that both nifedipine and nicardipine significantly decrease the mean level of plasma markers for oxidative stress in patients suffering from systemic sclerosis.

Antioxidants may be considered as promising neuroprotective compounds. Still, while experimental data demonstrate neuroprotective effect *in vitro* and in animal models, clinical evidence is still unsatisfactory and insufficient [171].

(a) Role of Metabolism of DHPs in Their AOA. Metabolic pathways and "bioavailability" of the probable AOA compound determine antioxidant activity *in vivo*. Antioxidant metabolites may vary in stability and activity leading to two opposite scenarios: lack or presence of activity, substantially contributing to the overall AOA [172]. Metabolic biotransformation of DHPs includes oxidation (heteroaromatization), side chain ester group cleavage (deesterification), and 4-substituent abstraction a.o. [160]. None of the DHPs metabolites was shown to be more toxic than original, reduced form of the compound. The commonly detected metabolites of the DHPs do not seem to possess the AO activity (with some exceptions as in the case of metabolites of nifedipine and its analogues, including nitrosonifedipine [173, 174]) (see further in Section 3.5). Due to DHPs intrinsic instability, achieving and maintaining an adequate concentration may be problematic both *in vitro* and *in vivo*.

(b) Role of Concentration and Lipophilicity (Membrane/Water or Lipid/Water Partition Coefficients) of DHPs in Their Action as AOs and Antiradical Compounds. Antioxidative effects of any antioxidant depend on its concentration at the site of action. This parameter is hardly measurable, especially in two-phase systems, representing one of obstacles in comparison to AOA upon applying various compounds [172]. It is often incorrectly assumed that the concentrations in the aqueous solution and at the site of action are the same. However, even when the concentration in the aqueous phase may be well controlled, the concentration at the site of action in the lipid matrix of the membranes might fluctuate between different test compounds, depending on a difference in lipophilicity [175]. The prevention of the membrane LP also seems to be dependent on the DHP concentration in the lipid matrix (Mason and Trumbore [46]) and its amphiphilicity. For example, AOA of diludine is associated with its lipophilicity and consequential ability to be incorporated into liposomes (Panasenko et al. [176]). It was also found that diludine easily incorporates into the outer monolayer of erythrocyte membranes [176].

Membrane/buffer partition coefficients (lambda) were directly measured in the sarcolemma and sarcoplasmic reticulum membranes for three CA DHPs. The obtained values were in a broad range between 5000 and 150000 (Herbette et al. [177]). These drugs interact primarily with the membrane bilayer component but may also bind to proteins, both nonreceptors and receptors. The intrinsic forward rate constants for DHP binding to sarcolemmal calcium channel receptors were apparently not strongly dependent on their membrane partition coefficients. For example, nimodipine (lambda = 6300) had a forward rate constant of $6.8 \pm 0.6 \times 10^6$/M/s, whereas the forward rate constant for Bay P 8857 (lambda = 149000) was $1.4 \pm 0.8 \times 10^7$/M/s. Since these DHPs are highly liposoluble, model calculations for this binding reaction demonstrated that these rates on lipid solubility would probably not be reflected in the experimental forward rate constants. In addition, the intrinsic forward rate constant for nimodipine binding to sarcolemmal calcium channel receptors was found not to be linearly dependent on the viscosity of the buffer medium over a fivefold range. The rate of drug nonspecific binding to nonreceptor protein present in highly purified sarcoplasmic reticulum membranes appears to be extremely fast, at least 10^3 times faster than specific drug binding to the receptor in the sarcolemma. Authors concluded that partitioning into the lipid bilayer matrix of the sarcolemma could be a general property of CA DHPs and may be a prerequisite for their binding to sarcolemmal membrane receptors (Herbette et al. [177]).

The binding of DHP calcium channel agonists and antagonists (including those with AO properties) to receptors in cardiac sarcolemmal membranes is a complex reaction that may involve an interaction with the lipid bilayer matrix of the sarcolemma (Herbette et al. [178]). Belevitch et al. [179] studied the binding of DHP CCBs (riodipine and nifedipine) and verapamil to model and biological membranes by

fluorescence analysis. The consistent location of Ca agonist Bay K 8644 was determined to be within the region of the first few methylene segments of the fatty acyl chains of the membranes (Mason et al. [180]). This position is near to that observed for the DHP calcium channel antagonists nimodipine and Bay P 8857.

The majority of studies on OS were performed with DHPs with various lipophilicity, but only a few studies reported amphiphilicity of DHP derivatives. Amphiphilic DHP derivative K-2-11 reduced the cellular generation of ROS. It also revealed complete reversal of multidrug resistance (MDR) of the resistant cells. K-2-11 was more efficient than well-known MDR inhibitor verapamil. Cytotoxic effects of anticancer drug doxorubicin were enhanced by K-2-11 in both MDR and parental, nonresistant cell line (Cindric et al. [181]). K-2-11 suppresses increase of ROS and consequentially prevents NF-κB activation leading to decreased expression of MDR1 and increased expression of antiapoptotic genes. This signaling switch is necessary for restoring the chemosensitivity of cancer cells. This phenomenon is characteristic both for 1,4-DHPs [182] (18 novel asymmetrical DHPs bearing 3-pyridyl methyl carboxylate and alkyl carboxylate moieties at C3 and C5 positions, resp., as well as nitrophenyl or heteroaromatic rings at C4) and for their oxidized forms, pyridine compounds (Zhou et al. [183]).

3.4. Dependence of AOA of DHPs on the Experimental System. AO effect of DHPs depends on their structure and the experimental system used (*in vitro* model system, subcellular organelle, and cells, *ex vivo* and *in vivo*). Ideally, for the evaluation of the profile and value of DHPs AO properties, each compound should be tested in as many systems as possible (Dubur et al. [45]).

Lipidomics studies have been traditionally explored for studying AOA of DHPs. Proteomics methods are less represented and are mostly focused on the properties of DHPs related to scavenging of protein free radicals. So far, there are no studies on the role of DHPs in scavenging nitrosoperoxycarbonate, the reactive species formed out of peroxynitrite, in the presence of carbon dioxide. Although it was shown that albumin binds diludine, no studies revealed the relevance of this effect for the AOA of diludine.

There are findings showing that dihydropyridine calcium antagonists (DHPs CA) could indirectly play a beneficial, protective role during development of atherosclerosis. Namely, Berkels et al. [184] have studied antioxidative properties of four substances: the DHP prototype CA, nifedipine, the long-acting CA, lacidipine, the DHP calcium channel agonist, Bay K 8644, and the bulky DHP derivate, Bay O 5572, in three different models: (1) in an *in vitro* superoxide anion generating system (hypoxanthine/xanthine oxidase) for testing the "pure" antioxidative effect, (2) in an artificial membrane preparation (dimyristoylphosphatidylcholine) for mimicking a more physiological environment, and (3) under conditions of stimulated ROS release (hyperglycemia) from native endothelial cells derived from porcine coronary arteries.

The study also revealed the potential correlation between lipophilic and AO properties of DHPs. In the first model, Bay K 8644 was significantly more effective in scavenging superoxide anions than lacidipine, Bay O 5572, or nifedipine (micro- to millimolar concentration range). Addition of an artificial membrane preparation resulted in an enhanced AO effect, with lacidipine being the most effective DHP in quenching radicals (low micromolar concentration range). In the third model, mimicking hyperglycemia (30 mmol/L), nifedipine was significantly more potent antioxidant (therapeutical nanomolar concentration range) than the other DHPs. Calculated lipophilicity of these four substances (lacidipine > Bay O 5572 > Bay K 8644 > nifedipine) was positively correlated with antioxidative potential only in the second experimental model. It has been concluded that AO properties of DHP substances need to be tested in various models for demonstrating that nifedipine exhibits ROS-quenching properties in a therapeutic concentration range [184].

3.4.1. AOA of DHPs in Isolated Cells and Cell Cultures (Comparison with Other Simplest Systems). Although DHPs possess neuromodulatory and/or antimutagenic properties, the mechanisms of action related to these phenomena are not entirely elucidated. Borovic et al. [185] have studied 1,4-dihydroisonicotinic acid (1,4-DHINA) derivatives of 1,4-DHP, water-soluble analogues of a well-known AO diludine (diethone): 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydroisonicotinic acid, sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)glutamate, glutapyrone and sodium 2-(2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropyridine-4-carboxamido)ethane-sulphate, tauropyrone as AO and bioprotectors (Figure 4).

1,4-DHINA's activities were studied in comparison to Trolox by N,N-diphenyl-N′-picrylhydrazyl (DPPH$^{\bullet}$), deoxyribose degradation, ABTS$^{\bullet}$ radical scavenging, and AOA (antioxidative capacity method) assays; copper-induced LP of cultured rat liver cells (MDA determination by HPLC and 4-hydroxynonenal-protein conjugates by dot-blot); ^{3}H-thymidine incorporation and trypan blue assay for liver cells growth and viability. Ic decreased the amount of 4-HNE-protein adducts. In all assays, Ia was the most potent AO, able to completely abolish copper induced LP of liver cells, while Ic only slightly decreased it. Thus, AOA is important activity principle of Ia, which was even superior to Trolox in treated cell cultures. Ia (and its analogues) are easily oxidized in the Fenton system (Rubene et al. [186]), exerting ARA too.

3.5. Peculiarities Related to Antioxidative and Antiradical Activity of Some 1,4-DHPs: Ca Antagonists. Nine commercialized, structurally and functionally different DHPs, CA, will be discussed further. Their common feature is ability to prevent OS. This also counts for some of their metabolites, as already discussed. The comparative effects of some DHPs, CA, on oxidative stress-induced modification of LDL were already reviewed in Section 3.3.1 (2)-(a). AOA of CA DHPs was discussed in Sections 3.3.1 (2)-(b) and 3.3.1 (2)-(c).

3.5.1. Nifedipine and Its Close Analogues. Nifedipine, verapamil, and antiarrhythmic-antihypoxic drug, stobadin, were shown to depress lipid peroxidation of phosphatidylcholine

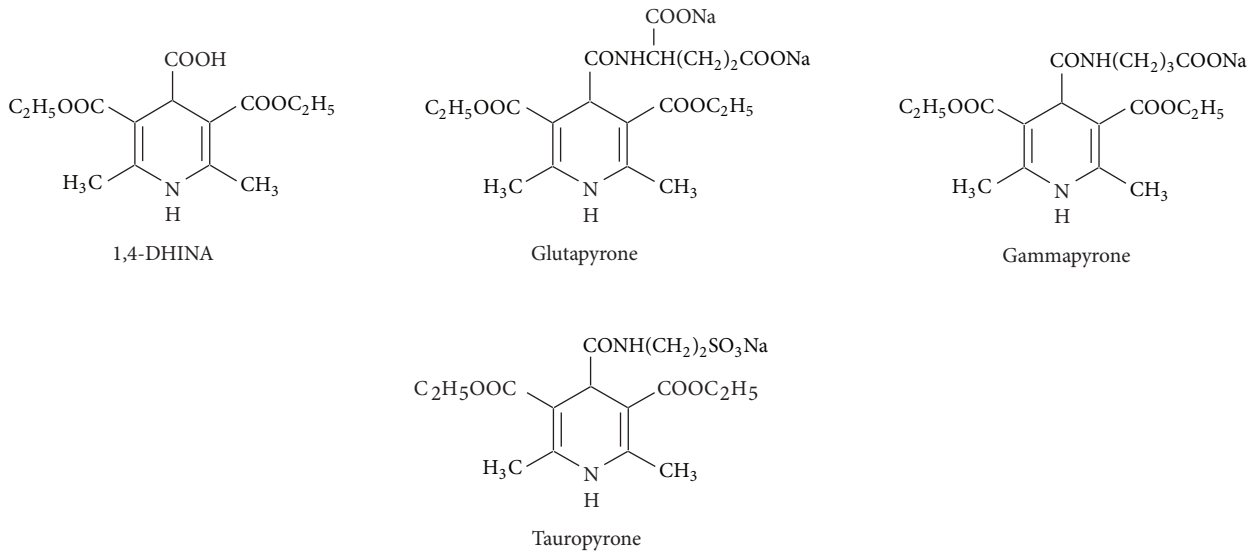


Figure 4: Derivatives of 1,4-dihydroisonicotinic acid (1,4-DHINA).

liposomes (Ondriaš et al. [187]). However, data obtained in some other experimental systems are conflicting.

In an *in vitro* model of sarcolemmal membrane lipid peroxidation, three calcium blockers (nifedipine, verapamil, and diltiazem) exhibited concentration-dependent (10–400 μM) inhibitory effects [188, 189]. Nifedipine, the most effective calcium blocker, was more than two-fold potent compared to propranolol, achieving significant effect at 10 μM. Nifedipine protective role on LP using reduced glutathione as model marker was recently described (Ray et al. [190]). Antiperoxidative properties of CA nifedipine and its analogues were explored in different systems/pathogenic processes: atherogenesis (Henry [165]), brain focal ischemia (Yamato et al. [191]), nephroprotection related to cyclosporine intake (Chander and Chopra [192]), and hepatoprotection related to intake of diethyldithiocarbamate (Gaafa et al. [193]). Recent data suggest that nifedipine action as protector for endothelial cells proceeds independently from its CA properties.

The absence of antioxidant effects of nifedipine and diltiazem on myocardial membrane lipid peroxidation, opposite to nisoldipine and propranolol, was also described [194]. Nisoldipine and propranolol were shown to have a concentration-dependent antiperoxidant effect, with IC_{50} values of 28.2 and 50.1 μM, respectively. Finally, nisoldipine appeared to possess dual antiperoxidant mechanisms, involving both preventive and chain-breaking properties.

These findings were confirmed in some other studies, including reports on the lack of antioxidative activity of nifedipine and nicardipine, even at 500 μM concentration in heart membrane lipid peroxidation tests [134]. Similarly, ROS formation in bovine aorta smooth muscle cells was not affected by addition of amlodipine, nimodipine, and nifedipine [123].

(1) Metabolites of Nifedipine and Its Analogues as Antioxidants and Regulators of OS. Antioxidant activity of nifedipine, 3,5-dimethoxycarbonyl-2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine, was originally studied *in vitro* by Kirule et al. [195] and Tirzit et al. [196]. According to the kinetic data of peroxide accumulation and the ESR spectra (inhibition of the autoxidation of methyl oleate in presence of nifedipine) AO action was exerted by the formation of nitroso analogue of the oxidized nifedipine, nitroso nifedipine: 2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridine dicarboxylate (NO-NIF). This nitroso aromatic derivative can form nitroxyl radicals exhibiting remarkable AOA in the presence of unsaturated fatty acids and lipids [196].

The primary species of free radicals that have been obtained and identified were ion radicals of the nitrophenyl type (Ogle et al. [76]). Such a mechanism coincides with mechanisms proposed afterwards by Núñez-Vergara et al. [96], López-Alarcón et al. [103], Valenzuela et al. [104], Fukuhara et al. [174], and Yáñez et al. [197].

There are also data showing that nitroso compounds may inhibit LP by direct radical trapping and subsequent formation of stable nitroxide radicals. It was further found that the reactivity between the synthesized 1,4-DHP derivatives with alkylperoxyl radicals involves electron transfer reactions. This is documented by the presence of pyridine as a final product of the reaction and complete oxidation of the nitroso group in the case of the nitrosoaryl 1,4-dihydropyridine derivatives (Valenzuela et al. [104]). Tested compounds reacted faster toward alkylperoxyl radicals and ABTS radical cation than alkyl ones (López-Alarcón et al. [103]).

Nitrosonifedipine, a photodegradation product of nifedipine, significantly recovers cellular damage induced by tumor necrosis factor-alpha. It also prevents toxic effects of cumene peroxide which hampers integrity of cell membranes through oxidative stress. Its positive effects are equal to Trolox-C.

As a result, nitrosonifedipine was already a long time ago claimed as a candidate for a new class of antioxidative drugs (Kirule et al. [195]), cellular protectors against oxidative stress in glomerular endothelial cells [174].

Moreover, Misik et al. [198], Ondriaš et al. [199], and Staško et al. [200] studied AOA of nifedipine and its oxidized nitroso analogue. NO-NIF prevents the progression of type 2 diabetic nephropathy associated with endothelial dysfunction through selective AO effects (Ishizawa et al. [201]). NO-NIF administration reduces albuminuria and proteinuria as well as glomerular expansion without affecting glucose metabolism or systolic blood pressure. NO-NIF also suppresses renal and systemic OS and decreases the expression of intercellular adhesion molecule-1 (ICAM-1), a marker of endothelial cell injury, in the glomeruli of the KKAy mice. Similar effects were achieved in endothelial nitric oxide synthase (eNOS) knockout mice. Moreover, NO-NIF suppresses urinary angiotensinogen (AGT) excretion and intrarenal AGT protein expression in proximal tubular cells in the KKAy mice. On the other hand, hyperglycemia-induced mitochondrial superoxide production was not attenuated by NO-NIF in cultured endothelial cells.

Fujii and Berliner found EPR evidence for free radical adducts of nifedipine *in vivo* [202]. The nature of these radicals was surmised by comparing the reaction of illuminated nitrosonifedipine with polyunsaturated fatty acids. Surprisingly, identical radical spectra were detected from excised liver doped with nonilluminated nifedipine, suggesting that this drug can be enzymatically converted *in vivo* to its nitroso analogue without the requirement for illumination. This is one of the first reports of *in vivo* EPR evidence for a class of unsaturated fatty acid radical conjugates resulting from the normal metabolism of a common drug.

Díaz-Araya et al. [173] studied some 4-nitrophenyl-DHPs on Fe^{3+} initiated LP in rat brain slices. LP, as measured by MDA formation, was inhibited by all the tested nitroaryl derivatives of 1,4-DHP over a wide range of concentrations. On the basis of both time course and IC_{50} experiments the tentative order of AOA on rat brain slices was nicardipine > nisoldipine > (R,S/S,R)-furnidipine > (R,R/S,S)-furnidipine > nitrendipine > nimodipine > nifedipine. 1,4-DHP derivatives that lack a nitro group in the molecule (isradipine and amlodipine) also inhibited LP in rat brain slices but at higher concentrations than that of nitro-substituted derivatives. All tested compounds reduced and oxidized nitrosoaryl derivatives (2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (photooxidation product of nifedipine – NTP) a.o.) and were more potent inhibitors of LP than their parent molecules (Valenzuela et al. [104]).

The electrooxidation process of 4-nitrosoaromatic DHPs is a strongly pH-dependent (two-electron two-proton mechanism): ECEC type of mechanism, that is, the sequence: $e^-/H^+/e^-/H^+$ at pH > 8.5; ECCE mechanism ($e^-/H^+/H^+/e^-$) at pH < 8.5 dominates. Reduction reaction of nitroso group is as follows: R-NO + $2e^-$ + $2H^+$ → RNHOH (Bollo et al. [203]).

3.5.2. Lacidipine. It is a generic DHP type antihypertensive CA, 3,5-diethyl 4-{2-[(1*E*)-3-(*tert*-butoxy)-3-oxoprop-1-en-1-yl]phenyl}-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate.

Ursini [204] described redox behaviour of lacidipine and showed its tissue protective features. Cristofori et al. studied antiatherosclerotic activity, in addition to lacidipine's CA and AO properties [205]. Lacidipine reduced the extent of atherosclerotic area in hypercholesterolemic apoE-deficient mice (these mice show widespread vascular lesions which closely resemble the inflammatory fibrous plaques seen in humans in atherosclerosis). The reduction may be associated with the capacity of the drug to maintain endothelial NO levels at concentrations useful to protect against vascular damage. This work suggested that DHPs modulate vascular relaxation *via* increased release of NO.

Herbette et al. [178] remarked optimal hydrophobicity of lacidipine due to cinnamic acid substituent, so membrane interactions and facilitation of the treatment of atherosclerosis could proceed (see also Section 3.3.1 (2)-(a)).

3.5.3. Amlodipine. Amlodipine (*Norvasc*), (*RS*)-3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (AML), has an antioxidant effect on vessels *in vitro* and is a 3rd generation of charged dihydropyridine CCB that is widely used for the treatment of hypertensive patients.

Amlodipine was shown to have the highest affinity (amlodipine > verapamil ≫ diltiazem) for the membrane bilayer ($K_p = 10^4$). It produced the significant changes in membrane thermodynamic properties, including a reduction in the thermal phase transition temperature (−11%), enthalpy (−14%), and cooperative unit size (−59%), relative to the control, phosphatidylcholine liposomes (Mason et al. [49]).

Amlodipine AOA is related to its reductant nature or hydrogen donor properties, respectively. Its ability for donating protons and electrons to the lipid peroxide molecules blocks the LP process.

Amlodipine and even its enantiomers (Zhang et al. [206]) act as ROS and NOS effectors in several model systems of OS. Antioxidant properties of amlodipine were recently reviewed by Vitolina et al. [32]. Both *in vitro* and *in vivo* studies of amlodipine AO properties revealed inhibition of lipids oxidative damage, primarily those associated with cellular membranes and lipoprotein particles (LDL) (Mason et al. [50]).

Under controlled experimental conditions *in vitro* amlodipine showed AOA and ARA, by inhibition of lipid peroxide formation and trapping ROS. Its scavenging activity for hydroxyl and peroxyl radicals at concentrations as low as 10.0 nmol/L (which is remarkably less compared to the classical antioxidants, GSH, uric acid, and Trolox) was shown to be independent of the calcium channel modulation (Franzoni et al. [207]).

AML showed efficiency as scavenger of peroxyl radicals (TOSC assay: 5945 ± 544 units/mg), significantly stronger (>50%, $P < 0.001$) than GSH (2733 ± 636 units/mg) and 70% weaker ($P < 0.0001$) than uric acid (18144 ± 696 units/mg) and Trolox (17522 ± 734 units/mg).

Of interest, the scavenging capacity of AML towards hydroxyl radicals (1455 ± 154 units/mg) was 320% higher ($P < 0.00001$) than that of GSH (358 ± 112 units/mg), 20% higher than that of uric acid (1198 ± 121 units/mg), and 100% higher than that of Trolox (759 ± 143 units/mg).

Amlodipine was shown to increase enzyme activity of paraoxonase (PON) and glutathione peroxidase (GSH-Px). However, it also decreases glutathione reductase (GSSG-R) activity and diminishes the concentration of the endogenous antioxidant α-tocopherol (vitamin E). Moreover, AML in a concentration of 2 ng/mL decreased the content of malonic dialdehyde and activity of superoxide dismutase in the blood (Gatsura [208]).

Verapamil and amlodipine produced a potent anti-ischemic effect and reduced area of myocardial infarction in rats. The observed changes were accompanied by inhibition of LP. In contrast to verapamil, *in vitro* application of AML in a dose of 50 ng/mL decreased hemoglobin affinity for oxygen. When present in a concentration of 2 ng/mL, AMD decreased the content of MDA and activity of SOD in the blood.

On the other hand, amlodipine shows no activity related to inhibition of macrophage superoxide release and cell migration, which occurs as a consequence of decreased TNFα induced $O_2^{\bullet}$ release.

Amlodipine-induced reduction of OS in the brain is associated with sympathoinhibitory effects in stroke-prone spontaneously hypertensive rats (SHRSP) (Hirooka et al. [209]). Antihypertensive treatment with amlodipine reduced OS in all examined areas of the brain and decreased blood pressure without a reflex increase in sympathetic nerve activity. Nicardipine, another CA DHP, surprisingly, was significantly less active than amlodipine.

3.5.4. Lercanidipine. Tomlinson and Benzie reported AO effect of lercanidipine [210], which is well known as antihypertensive drug Zanidip, 2[(3,3-diphenylpropyl)(methyl)-amino]-1,1-dimethylethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate. Comparative data about this drug AOA were presented in parts of this paper about other individual CA DHPs and in the part about the *ex vivo* DHPs effects on LDL.

3.5.5. Nimodipine. Nimodipine (ND), commercially known as Nimotop, is 3-(2-methoxyethyl) 5-propan-2-yl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate. It is centrally active CA.

Treatment with glutathione blocked and with nimodipine attenuated neuronal cell death, caused by prolonged exposure of cell culture to 4-HNE (Faraqui [211]).

Nascimento et al. [212] found AO effect of nimodipine in young rats after pilocarpine- (PIL-) induced (in 400 mg/kg) seizures. The PIL administration increased the striatal catalase (CAT) activity. The administration of ND, 30 mg/kg, 30 min before PIL, preserved normal value of CAT activity. On the other hand, no difference was detected in the animals treated with lower dose, 10 mg/kg. These results confirm the neuroprotective/antiepileptic effect of ND in young rats, suggesting that this drug acts positively on lipid peroxidation (in both doses). Nimodipine cannot induce these effects *via* blockade of Ca^{2+} channel.

Ismailoglu et al. [213] studied the therapeutic effects of melatonin and nimodipine in rats after cerebral cortical injury. These beneficial effects in rats after cerebral cortical injury seemed to be related to AOA of nimodipine.

3.5.6. Benidipine. Licensed in Japan and South Asia as CA (CCB) benidipine possesses AO properties. Chemically, it is 5-methyl 3-[(3*R*)-1-(phenylmethyl)piperidin-3-yl] 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (or its hydrochloride, (4R)-rel-3,5-pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-, 3-methyl 5-[(3R)-1-(phenylmethyl)-3-piperidinyl] ester, hydrochloride (1 : 1)).

Benidipine influences processes connected with OS in several ways. It prevents lysophosphatidylcholine- (lysoPC)-induced injury and ROS production in human aortic endothelial cells (HAECs) (Matsubara and Hasegawa [214]). Matsubara et al. [215] explained this effect, based on stimulation of nitric oxide release.

LysoPC is a component of oxidized low-density lipoproteins (oxLDLs), which plays an important role in the pathogenesis of atherosclerosis. Pretreatment with benidipine (0.3–3 μmol/L) for 24 h protected against lysoPC-induced cytotoxicity in the HAECs through inhibition of both lysoPC-stimulated ROS production and caspase-3/7-like activation, with a similar potency. Since caspase-3/7 is involved in executing the apoptotic process, the reduction of the activity of this enzyme by benidipine may explain the antiapoptotic effect of the drug. However, benidipine did not suppress lysoPC-induced phosphorylation of mitogen-activated protein kinases and Ca^{2+} influx in HAECs. These results suggest that the antioxidant properties of benidipine may be responsible for its ability to inhibit ROS production, a possible reason for reduced activation of caspase-3/7. In conclusion, benidipine suppresses lysoPC-induced endothelial dysfunction through inhibition of ROS production, which is due at least in part to its antioxidant effect, and not through the inhibition of L-type voltage-dependent calcium channels.

Matsubara and Hasegawa [216] examined the effects of benidipine on cytokine-induced expression of adhesion molecules and chemokines (chemoattractants), which are important for the adhesion of monocytes to endothelium. Pretreatment of HAECs with benidipine (0.3–10 μmol/L) for 24 h significantly suppressed cytokine-induced vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) mRNA and protein expression, resulting in reduced adhesion of THP-1 monocytes. Benidipine also suppressed induction of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8. Benidipine also inhibited redox-sensitive transcriptional nuclear factor-κB (NF-κB) pathway, as determined by Western blotting of inhibitory κB (IκB) phosphorylation and luciferase reporter assay. Results of analysis using optical isomers of benidipine and antioxidants suggest that these inhibitory effects were dependent on pharmacological effects other than Ca^{2+} antagonism. Benidipine may thus have anti-inflammatory properties and benefits for the treatment of atherosclerosis.

Benidipine was also shown to inhibit ROS production in polymorphonuclear leukocytes and oxidative stress in salt-loaded stroke-prone spontaneously hypertensive rats (Matsubara et al. [217]).

It should be mentioned that other DHPs also have endothelial AO actions [218].

3.5.7. Azelnidipine (AZL). Azelnidipine, 3-[1-[di(phenyl)methyl]azetidin-3-yl] 5-propan-2-yl 2-amino-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (AZL), CAS number: 123524-52-7, is commercially available 4-nitroaryl-DHP type calcium antagonist with long-acting antihypertensive action (long-acting CA (CCB)) and a low reported incidence of tachycardia. It additionally possesses beneficial effects in OS and diabetic conditions.

Azelnidipine prevents cardiac dysfunction in streptozotocin-diabetic rats by reducing intracellular calcium accumulation (altering intracellular Ca^{2+} handling proteins), OS, and apoptosis (Kain et al. [219]). AZL can reduce the superoxide production. It exerts its protective effects by targeting the NADPH oxidase and mitochondrial redox enzymes. AZL-treated diabetic rats express enhanced level of bcl-2 in the lysates of heart muscle indicating that AZL plays protective role in cardiac apoptosis.

It has been previously observed that azelnidipine inhibits tumor necrosis factor-alpha-induced endothelial cell (EC) oxidative stress through its AO properties (Nakamura et al. [220]). Azelnidipine, but not nitrendipine, completely inhibits the Ang II-induced ROS generation in ECs (Matsui et al. [221]).

Furthermore, azelnidipine, but not nitrendipine, was found to partially restore decreased pigment epithelium-derived factor (PEDF) mRNA levels in Ang II-exposed ECs. This study suggests that AZL influence depends on its antioxidative properties. Authors concluded that upregulation of PEDF by azelnidipine may become a therapeutic target for the treatment of diabetic retinopathy associated with hypertension.

Antihypertensive agents with AO effects are potentially useful for diabetic patients with hypertension. While DHP type CA are among the most frequently used antihypertensive drugs, azelnidipine has been reported to have a unique AO effect *in vitro* and *in vivo*, in experimental animals (Ohmura et al. [222]). In hypertensive diabetic patients, azelnidipine treatment for 12 weeks induced a more significant decrease in erythrocyte LOOH level than amlodipine, although the values related to blood pressure during each treatment remained comparable. These data confirm the usefulness of LOOH level in erythrocyte membrane as a marker of OS *in vivo* and indicate that azelnidipine has a unique antioxidative property in humans.

Daikuhara et al. [223] reported the results of the OLCA study, based on combination of (1) olmesartan and a calcium channel blocker (azelnidipine) or (2) candesartan and a CCB amlodipine in two groups of diabetic hypertensive patients. Patients treated with the first combination presented highly persistent early morning antihypertensive effect and stronger decrease in heart rate, fasting blood glucose and HbA1c levels, and microalbuminuria, when compared to patients treated with the combination (2). Because diabetes is associated with severe chronic OS the observed results might be at least in a part due to the AOA of azelnidipine.

In favor of this are findings of Abe et al. [224] who found additive antioxidative effects of azelnidipine on angiotensin receptor blocker olmesartan treatment for type 2 diabetic patients with albuminuria.

Similarly, the AOA of thiazolidinediones (insulin sensitizer) and their effect on cardiovascular function in type 2 diabetic model rats and also those of some DHPs (nifedipine, amlodipine, or AZL, commonly used antianginal and antihypertensive agents) in cultured human endothelial cells LP were examined (Mizushige [225]). The AOA was evaluated by measuring 8-iso-prostaglandine $F_{2\alpha}$ concentration and azelnidipine exhibited potent AOA.

Insulin (INS) resistance combined with hyperinsulinemia is involved in the generation of OS. A relationship exists between increased production of ROS and the diverse pathogenic mechanisms involved in diabetic vascular complications, including nephropathy. Manabe et al. [226] revealed that high doses of INS augmented mesangial cell proliferation through generation of intracellular ROS and activation of redox signaling pathways. Cell proliferation was increased in a dose-dependent manner by high doses of INS (0.1–10 μM) but was inhibited by 0.1 μM AZL. Namely, the INS-increased phosphorylation of mitogen activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/ERK 1/2) was inhibited by 0.1 μM AZL. The same AZL concentration blocked intracellular ROS production more effectively than 0.1 μM nifedipine. The NADPH oxidase inhibitor, apocynin (0.01–0.1 μM), prevented INS-induced mesangial cell proliferation. So, azelnidipine inhibits insulin-induced mesangial cell proliferation by inhibiting the production of ROS. Therefore azelnidipine may have the potential to protect against the onset of diabetic nephropathy and slow its progression.

Azelnidipine inhibited H_2O_2-induced cell death in neonatal rat cardiomyocytes (Koyama et al. [227]). Azelnidipine and nifedipine did not affect the H_2O_2-induced activation of extracellular signal-regulated protein kinases (ERK) and p38 MAPK (mitogen-activated protein kinase). In contrast, azelnidipine, but not nifedipine, inhibited H_2O_2-induced c-Jun NH_2-terminal kinases (JNK) activation. Authors concluded that azelnidipine has inhibited the H_2O_2-induced JNK activation and cardiac cell death. Therefore azelnidipine may have cardioprotective effects against OS.

A specific atheroprotection activity of azelnidipine relates to inhibition of TNF-α-induced activator protein-1 activation and interleukin-8 expression in human umbilical vein endothelial cells (HUVEC), through suppression of NADPH oxidase-mediated reactive oxygen species generation (Nakamura et al. [220]). TNF-α could play a central role in pathogenesis of insulin resistance and accelerated atherosclerosis in the metabolic syndrome. The concentration of AZL found to be effective in these *in vitro* experiments is within therapeutic range. As EC do not possess voltage-operated L-type calcium channels, it is suggested that the beneficial effects of azelnidipine are not likely due to CA property but to its unique AO

ability. Furthermore, it has been recently found that serum levels of monocyte chemoattractant protein-1, a biomarker for subclinical atherosclerosis, were significantly decreased by the AZL treatment in patients with essential hypertension. In this paper [220], authors hypothesize that, due to its unique TNF-α signal modulatory activity and antioxidative property, azelnidipine may be a promising DHP for targeting diabetes and cardiovascular diseases in hypertensive patients with metabolic syndrome.

Shinomiya et al. [228] evaluated its AOA in cultured human arterial EC, under OS. Azelnidipine has shown a potent antioxidative effect that could be of significant clinical benefit when combined with its long-lasting antihypertensive action and low incidence of tachycardia.

Azelnidipine inhibited TGF-β1 and angiotensin II- (Ang II-) activated α1(I) collagen mRNA expression in hepatic stellate cells (HSCs) (Ohyama et al. [229]). Furthermore, TGF-β1- and Ang II-induced OS and TGF-β1-induced p38 and JNK phosphorylation were reduced in HSCs treated with AZL. Azelnidipine significantly decreased inflammatory cell infiltration, profibrotic genes expression, HSC activation, LP, oxidative DNA damage, and fibrosis in liver of CCl_4- or TAA-treated mice. Finally, AZL prevented decrease of the expression of some AO enzymes and accelerated regression of liver fibrosis in CCl_4-treated mice. Hence, the antifibrotic mechanism of AZL against CCl_4-induced liver fibrosis in mice may have been due to an increased level of AO defense. As azelnidipine is widely used in clinical practice without serious adverse effects, it may provide an effective new strategy for antifibrotic therapy.

3.5.8. Manidipine. Manidipine, (2-[4-(diphenylmethyl)piperazin-1-yl]ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate), is a DHP CCB with reported nephroprotective properties. Calò et al. [230] studied effect of manidipine on gene expression and protein level of OS related proteins: p22(phox) (human neutrophil cytochrome b light chain (CYBA)) and heme oxygenase-1, HO-1. Relevance for antihypertensive effects was revealed. The study assessed the effect of manidipine on normal subjects' monocyte gene and protein expression of OS related proteins such as p22phox, a NADPH oxidase system subunit, critical in generating $O_2^{\bullet-}$, and HO-1, induced by and protective against OS. Manidipine was compared with the ACE inhibitor captopril and the CCB nifedipine, in the presence and in the absence of sodium arsenite ($NaAsO_2$) as an inducer of OS. Monocyte p22phox (CYBA) mRNA production was reduced by both manidipine and captopril, while no changes were induced by nifedipine. Manidipine increased monocyte HO-1 mRNA production, while nifedipine and captopril showed no effect. The effects of manidipine on p22phox and HO-1 gene expression in the presence of OS were also confirmed at the protein level. Thus, manidipine seems to suppress p22phox and to increase the HO-1 mRNA production and protein level. The manidipine-induced increase of HO-1 gene and protein expression seems to be a peculiar effect of this drug since it is not observed with captopril and nifedipine. This effect, together with the reduction of p22phox mRNA production, could play a role in its protective mechanism against OS.

3.5.9. Mebudipine. The protective effect of mebudipine (1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 3-methyl-5-tert-butyl ester; BAY-n-6391) was revealed on OS and LP (MDA decrease, SOD, GPX, and catalase increase) in myocardial ischemic-reperfusion injury in male rats (Ghyasi et al. [231]).

There are articles about other commercial and experimental DHPs on OS, but we have reviewed only the most commonly studied compounds. Effects of other commercial CA DHPs on OS are also mentioned in several parts of this review.

3.6. 1,4-DHPs: Ca Agonists and Their AOA and ARA. For the most popular calcium agonist DHP Bay K 8644 no reaction with peroxyl radicals was registered (Toniolo et al. [114]). However, interaction with other compounds possessing AOA and ARA (quercetin) was found.

Opposite to that, AO N-acetylcysteine (NAC) diminished increase in Ca^{2+} transient amplitude and cell shortening induced by ISO and forskolin, whereas NAC had no effect on the (S)-(−)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate—(−)-Bay K 8644-induced increases (Andersson et al. [232]).

Increased vasoconstriction responses to Bay K 8644 (3×10^{-7}–3×10^{-5} M) were significantly decreased by pyridoindole antioxidant stobadine treatment in diabetes (Ceylan-Isik et al. [233]).

The functional interaction between two L-type Ca^{2+} channel activators, quercetin and Bay K 8644, has been investigated in vascular smooth muscle cells. Biological ARA compound quercetin at nutritionally meaningful concentrations limited the responsiveness of vascular L-type Ca^{2+} channels to the pharmacological stimulation operated by Bay K 8644. These data contribute to a better understanding of quercetin effects on experimental *in vivo* cardioprotection (Saponara et al. [234]). Thus, these findings indicated that although Bay K 8644 does not exert potent and direct AOA, yet acting as calcium agonist it may affect efficiency of AO substances and *vice versa*.

Interaction of grapefruit juice (containing quercetin and its analogues) with DHPs CA diminished effectiveness of CA action of DHPs (Sica [235]).

3.7. Interpretations of the Mechanism(s) of Radical Scavenging and AOA by DHPs

3.7.1. Molecular Mechanism(s) of Radical Scavenging and AOA of DHPs in the Model Systems. 3,5-Dicarbonyl-1,4-dihydropyridine derivatives possess powerful bis-β-carbonylvinyl-amino conjugation and for that reason cannot be considered as ordinary amino antioxidants. The electron and/or H donation from DHPs ensures their reductant role and results in AOA and ARA. Oxidation reaction from DHPs results in production of corresponding heteroaromatic pyridine derivatives.

Detailed studies were made about substituent in DHP ring positions: 1,4-, namely, 4-unsubstituted-; 4-substituted: 4-alkyl-; 4-aryl-; 4-alkylaryl- a.o.; 2,6-; 3,5- (diacetyl or dialkoxycarbonyl chain a.o.) electronic and steric effects on AOA and ARA of DHPs [44, 45, 51], see Sections 3.3.1 and 3.5. The bell-shaped dependence of DHPs AOA on the 3,5-dialkoxycarbonyl- chain length was observed [44, 45, 107, 109, 112], with the maximum activity at C_2H_5–C_4H_9. Decrease of AOA and incorporation into liposomes for DHPs with alkyl chains longer than R > C_4H_9 further were clarified as probable tendency to self-aggregation of these compounds ([51] and citation number 245 therein). Electron acceptor/electron donor properties are relevant for expression of AOA or ARA of 3,5-disubstituted DHPs. 3,5-Diacyl-substituted and 3,5-dicarbanilido- and 3,5-dipyridylamido-substituted DHPs are more active as AOs as their 3,5-dicyano-substituted analogues, which have electron acceptor properties [186].

Dubur et al. [89] observed overwhelming steric influence of substituents in position 4 of the DHP ring. Gaviraghi et al. [163, 164] proposed that AO activity of DHPs is partly dependent on capacity of the 1,4-DHP ring to donate electrons to the propagating radical ($ROO^{\bullet}$ or $RO^{\bullet}$) and to reduce it to a less reactive form. The abstraction (donation) of electron and/or H in the oxidation and LP reactions takes place from all 3,5-dicarbonyl-1,4-DHP systems and results in the formation of corresponding pyridine derivatives (Augustyniak et al. [51]). The physicochemical mechanism of ARA and AOA of 1,4-DHP has been extensively studied and discussed (Mulder et al. [236]), but precise mechanisms of their activity need further clarification.

The reactivity of C-4 substituted 1,4-DHPs possessing either secondary or tertiary nitrogen atom in the DHP ring toward alkyl, alkylperoxyl radicals, and ABTS radical cation was determined in aqueous media at pH 7.4 [103]. These compounds reacted faster toward alkylperoxyl radicals and ABTS radical cation than alkyl ones. N-Ethyl-substituted DHPs showed the lowest reactivity.

The 4-methyl substituted DHP was the most reactive compound in previously mentioned reactions (López-Alarcón et al. [103]). However, it was less active (0.68 versus 1.0) than Trolox-C. DHPs having electron-donating substituents (4-Me-DHP and *p*-MeO-Phe-DHP) showed the highest kinetic rate constants toward ABTS radical cation; *p*-nitro-Phe-DHP, a compound with an electron-withdrawing substituent, showed a lower kinetic rate constant; and N-alkyl-DHP compounds show kinetic rate constants lower than the -NH-DHP.

Hydrogen at the 1-position of the DHP ring was revealed, according to the deuterium kinetic isotope effect studies, to be involved in the proposed ARA mechanism. This fact is mostly noticeable in the case of alkyl radicals. N-Ethyl-substituted DHPs show the lowest reactivity when compared to Trolox or nisoldipine. In all cases, the respective pyridine derivative was detected as the main product of the reaction (López-Alarcón et al. [103]). Authors indicate that the kinetic rate constants toward alkyl, alkylperoxyl, and ABTS radical cation depend on the nature of the substituents in the C-4 position of DHP and the presence of the secondary amine group in the dihydropyridine ring, that is, the presence of the hydrogen in 1-position.

Yáñez et al. [197] have studied the reactivity of 11 derivatives of 1,4-DHPs (including commercial CA) with alkylperoxyl radicals and ABTS radical cation. The tested 1,4-DHPs were 8.3-fold more reactive towards alkylperoxyl radicals than to the ABTS cation radical. All commercial 1,4-DHP type CCBs were weaker than Trolox-C. The participation of the hydrogen atom in the 1-position appears to be very relevant for exhibited reactivity. Hantzsch ester (diludine) was again found to be the most active compound in the reaction with alkylperoxyl radicals, 2.3-fold more active than Trolox. The photodegradation product of nifedipine (nitrosophenyl derivative of pyridine) also showed a high activity. Kinetic rate constants for the reaction between 1,4-DHP compounds and alkylperoxyl radicals exhibited a fairly good linear correlation with the oxidation peak potential of DHP derivatives. However, the activity of tested 1,4-DHPs towards ABTS radical cation showed an independence between kinetic rate constants and oxidation peak potentials.

Kirule et al. [195] and Tirzit et al. [196] studied mechanism of AOA of 4-nitrophenyl-1,4-DHPs, nifedipine and its analogues, involving formation of 4-(2′-nitrosophenyl)-pyridine derivative (as active principle) as a result of intramolecular redox reaction, using chemical, electrochemical, and biochemical approaches (see Sections 3.3.1 and 3.5.1).

Núñez-Vergara et al. [237] reported the electrochemical oxidation of C4-hydroxyphenyl-substituted 1,4-DHP derivatives. The oxidation proceeds *via* formation of the unstable dihydropyridyl radical, as confirmed by controlled-potential electrolysis (CPE) and ESR experiments. This type of 1,4-DHPs has significant activity towards the radicals even when compared with commercial 1,4-DHP drugs with well-known antioxidant ability.

It was observed that nicardipine preferentially targets $RO^{\bullet}$ radicals and is inactive against $ROO^{\bullet}$. Lacidipine, on the other hand, is equally active towards both types of radicals (Gaviraghi et al. [164]). The cytoprotective effect against exposure to H_2O_2 was more significant for lacidipine (ID_{50} = 14 nM, its log P = 5.4, membrane partition = 136000, assumes position located 7 Å near to the membrane center; other less lipophilic DHPs located 12–16 Å far from the center) as compared to amlodipine, nifedipine, and nicardipine, in smooth muscle cell culture (Gaviraghi et al. [164]). Oxidative effect of H_2O_2 shifts the Ca channel toward an open state. Thus, the redox property of CCBs DHPs may augment their CCB properties.

Oxidation of pharmacologically active Hantzsch 1,4-dihydropyridines was found by electrogenerated superoxide, using a voltammetric approach in DMSO solutions (Ortiz et al. [238] and Ortiz et al. [239]). Raghuvanshi and Singh [240] have also reported oxidative aromatization of these DHPs, induced by superoxide.

Chemiluminescence (CL) was used in the studies analyzing the antioxidant activity of 12 various 4-flavonil-1,4-dihydropyridine derivatives (Kruk et al. [241]) on a chemical system involving a superoxide radical anion. These derivatives showed structural similarity to flavonoids, with respect to the presence of rings A, B, and C. The results obtained in

this study indicate that the tested derivatives may catalyze conversion of superoxide radicals, through mimicking the activity of superoxide dismutase by delivering H^+ for reaction:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + {}^1O_2 \quad (3)$$

The enhanced emission of the light in the presence of tested compounds was significant and related to stimulated production of H_2O_2 and $^1O_2$ from $O_2^{\bullet}$. The latter species were removed from the reaction mixture by the following sequence of reactions:

$$\begin{gathered} H^+ \\ O_2^{-\bullet} \longrightarrow HO_2^{\bullet} \\ HO_2^{\bullet} + O_2^{\bullet -} \longrightarrow HO_2^{-} + {}^1O_2 \\ 2HO_2^{\bullet} \longrightarrow H_2O_2 + {}^1O_2 \\ 2\left({}^1O_2\right) \longrightarrow (O_2)_2 + h\nu^* \end{gathered} \quad (4)$$

or to take part in spontaneous dismutation of H_2O_2:

$$2H_2O_2 \longrightarrow 2H_2O + {}^1O_2 \quad (5)$$

The authors have offered an original concept of action for 4-flavonil-1,4-dihydropyridine derivatives unrelated to that of $O_2^{\bullet}$ radical-trapping, chain-breaking antioxidants. Instead, they showed that these compounds act similar to superoxide dismutases, converting $O_2^{\bullet}$ to H_2O_2. Hydrogen peroxide is less toxic for cells than $O_2^{\bullet}$ because it is predominantly removed by peroxidases and catalases. Finally, AO effect of these DHPs differed from those mediated by flavonoids with a catechol structure of ring B, which are well-known $^1O_2$ quenchers.

Mulder and collaborators came to similar conclusions, especially related to molecular mechanisms of antioxidative activity of DHPs [236]. The AO properties of Hantzsch 1,4-dihydropyridine esters and two dibenzo-1,4-dihydropyridines, 9,10-dihydroacridine (DHAC) and N-methyl-9,10-dihydroacridine (N-Me-DHAC), have been explored by determining the autoxidation of styrene or cumene at 30°C. These experiments showed that Hantzsch esters are virtually inactive as chain-breaking antioxidants (CB-AOs), contrary to the findings observed by López-Alarcón et al. [103] who used CB-AOA in aqueous media at pH 7.4. Their reactivity toward peroxyl radicals was shown to be some 5 orders of magnitude lower than that of the excellent CB-AO, 2,2,5,7,8-pentamethyl-6-hydroxy-chroman (PMHC).

DHAC was found to be ~10 times less reactive than PMHC kinetic measurements using DHAC, N-deuterio-DHAC, and N-Me-DHAC, pointing out the abstraction of N-H hydrogen in DHAC by peroxyl radicals, despite the fact that the calculated C-H bond dissociation enthalpy (BDE) in DHAC is about 11 kcal/mol lower than the N-H BDE. The rates of hydrogen atom abstraction by the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) have also been determined for the same series of compounds. The trends in the peroxyl$^{\bullet}$ and $DPPH^{\bullet}$ rate constants were found to be similar [236].

Tirzit et al. [242] have observed quenching of singlet oxygen by DHPs. This observation paved the ground for further research related to reactions of DHPs with hydroxyl radicals (Tirzit et al. [243]), singlet oxygen (Kazush et al. [94]), and mechanisms of action. A series of 1,4-DHP derivatives in NAD-H-Cu^{2+}-H_2O_2 system inhibited forming of the hydroxyl radical ($HO^{\bullet}$), while 1,4-DHP derivatives with electron donor substituents in the molecule were shown to be capable themselves of generating $HO^{\bullet}$ in the presence of Cu^{2+} and H_2O_2. Rubene et al. [186] also described interaction of 1,4-DHP derivatives with Fenton's reagent, which produces hydroxyl radical ($HO^{\bullet}$). Rate constants of the DHPs reaction (1st order) with $HO^{\bullet}$ radical were high: in the range 10^9 L × mol × sec^{-1}, close to that of NADH, cysteine and thiourea. 3,5-Diacetyl- derivatives reacted faster compared to 3,5-dialkoxycarbonyl- ones. The reaction rate decrease was observed in the case of substitution at position 4 as compared to 4-unsubstituted DHPs. Some DHPs having electron donor -COO^- groups in the 3,5- or 2,6- positions of DHP ring reacted immediately (having rate constants higher as 10^9 L × mol × sec^{-1}). Rate constants with $HO_2^{\bullet}$ and $O_2^{\bullet -}$ radicals were of lower degree. Thus DHPs acting as oxygen radical scavengers could effectively inhibit ROS related reactions of LP initiation stage.

Nifedipine and nitrendipine reactivity toward singlet oxygen was also studied [244]. Nifedipine was shown to be a good scavenger of excited oxygen, mainly *via* physical deactivation with values of the total rate constant ranging from $20.8 \times 10^5\ M^{-1}\ s^{-1}$ (in dioxane) to $93.0 \times 10^5\ M^{-1}\ s^{-1}$ (in propylene carbonate). The less reactive pathway generated a photooxidation product. For that reason, a mechanism involving a perepoxide-like encounter complex in the first step of the reaction path was proposed (see [244], Figures 8 and 9 therein). The dependence was observed on solvent microscopic parameters of the total rate constant for the reaction between singlet oxygen and 1,4-DHPs. These findings show that nifedipine possesses stronger protective activity in biological systems than nitrendipine.

Density-functional-theory (DFT) calculations made by Wang et al. [245] confirmed the former experimental observations that Hantzsch ester, diludine, is potent antioxidant with high H atom-donating ability and relatively low prooxidant activity. Possible reaction pathways for radicals derived from 1,4-dihydropyridine and the resonance modes for radical species were given [245].

Moreover, two ethoxycarbonyl (EtOCO) substituents at C(2) and C(6) should further enhance Hantzsch ester, diludine H-atom-donating ability due to resonance effects. However, DHPs should be used in nonpolar rather than in polar solvents since, in the latter, not H-atom but electron transfer is preferred in the radical scavenging process [245].

Mulder et al. [246] proposed that quantum-thermochemical calculations must be used with caution to indicate "a promising lead antioxidant," as they criticized the density-functional-theory (DFT) calculations made by Wang et al. [245].

3.7.2. Possible Mechanisms of DHPs ARA and AOA in the Biological Systems: Interaction with Other OS Modifiers. Some of these mechanisms were already described (Sections 3.3.1 (2)-(b); 3.3.1 (2)-(c); 3.3.1 (3)-(b); 3.5).

Enzymatic sources of ROS with confirmed functional role in hypertension are NADPH oxidase, NO synthase (NOS), xanthine oxidase, and cyclooxygenase. Godfraind [3] has reviewed AO effects and protective action of calcium channel blockers (CCBs). Yao et al. [247] observed antioxidant effects (as inhibition of LP) for cardio- and neuroprotective CCBs (3–300 μmol/L), including 7 DHPs, in homogenates of rat brain. IC_{50} values (μM) were as follows: nifedipine (51.5) > barnidipine (58.6) > benidipine (71.2) > nicardipine (129.3) > amlodipine (135.5) > nilvadipine (167.3) > nitrendipine (252.1) > diltiazem (>300) = verapamil (>300). There are also research articles describing the AO properties of CCBs through direct scavenging effect or through preservation of the endogenous SOD activity. These findings indicate that CCBs may also act by reducing the production of vasoconstrictors, angiotensin, and endothelin.

When present in concentrations that can be achieved in plasma, CCBs may inhibit LP formation [3]. This AO activity seems to be typical for high lipophilic CCBs because their chemical structure facilitates proton-donating and resonance-stabilization mechanisms that quench the free radical reaction. Their insertion in the membrane, near polyunsaturated fatty acids at relatively high concentrations, potentiates proton donation (or atomary H) to lipid peroxide molecules, thereby blocking the peroxidation process. The remaining unpaired free electron associated with the CCB molecule can be stabilized in well-defined resonance structures associated with the DHP ring (Mason et al. [48]).

The radical reaction (according to Godfraind [3]) that describes the AO effects of a DHP CCBs is LOO^{*} + DHP $\rightarrow$ LOOH + DHP^{*} (where LOO^{*} is lipid peroxide radical), which in general is reaction (7) of the LP reaction cascade consisting of ~10 reactions (Scheme 2) [89].

As the rate constants of *in vitro* interaction of 4-substituted DHPs with peroxyl radicals are three orders of magnitude lower than that of the vitamin E derivative, these DHPs must be considered as weak AO (Ursini [204]). However, due to partition coefficient of DHPs in membranes and in case of specific binding, high local concentration of DHPs may be obtained.

DHPs without CCB properties, for instance Bay w 9798, although structurally related to nifedipine, inhibit TNF-α-induced vascular cell adhesion molecule-1 expression in endothelial cells by suppressing reactive oxygen species generation [248].

Mitrega et al. [249] have discovered that antiarrhythmic and hemodynamic effects of oxidized heteroaromatic DHPs, oxy nifedipine, oxy nimodipine, oxy nitrendipine, and oxy nisoldipine, suggest that CCB DHPs and their metabolites could act at least in two ways: targeting OS related events as reductants (see Section 3.5.1 (1)) and/or bypassing OS related metabolic routes. Authors postulated that, contrary to current belief, NIF metabolites are pharmacologically active. ATP sensitive potassium channels were mentioned as a target.

3.8. DHPs: Anti- or Prooxidants? Several substances (ascorbic acid being the most commonly studied) can serve either as antioxidants or as prooxidants, depending on given conditions (Herbert [250]). Therefore, Halliwell [251] has reported dilemma related to polyphenols as possible antioxidants and prooxidants, causing experimental artifacts (about 25) by oxidation of antioxidant compounds in the cell culture media. Nevertheless, it is generally accepted opinion that polyphenols act as antioxidants *in vivo*. Studies on DHPs also face such a dilemma. The exact roles (anti- or prooxidative) of any specific DHP depend on its structure, applied/achieved concentration, and specificity of the target/experimental testing system.

This situation resembles the case of antioxidative effects of tocopherol, which depends on the fate of the secondary radical as proposed by Winterbourn [252]. The question was "Vitamin E - Pro- or Antioxidant?":

Antioxidant:

$$\begin{aligned} &LOO^{\bullet} + Toc \\ &\quad \longrightarrow LOOH + Toc^{\bullet} \left(Toc^{\bullet} = \alpha\text{-tocopheryl radical}\right) \\ &Toc^{\bullet} + LOO^{\bullet} \\ &\quad \longrightarrow \text{chain termination of lipid peroxidation} \end{aligned} \tag{6}$$

Prooxidant:

$$Toc^{\bullet} + \text{Lipid-H} \longrightarrow \text{Lipid}^{\bullet} \left(\text{in LDL particles}\right) \tag{7}$$

This example shows that, generally speaking, any AO must fulfil several criteria to be considered as an effective compound physiologically:

(i) It must be able to functionally interact with endogenous scavengers, even at low concentrations.

(ii) It must affect endogenous pathways of OS.

(iii) It should not have undesirable adverse effect.

(iv) It should manifest the antioxidant efficacy dependent on the oxidant.

(v) It must discriminate among different strategies needed for 1-electron and 2-electron processes.

(vi) Radical scavengers can be prooxidant unless linked to a radical sink (Winterbourn [252]).

According to these statements, DHPs could be effective as AO under physiological conditions *in vivo* (Godfraind [3] and others [30, 31, 38]) and *in vitro* in various experimental systems (cell and tissue) (see Sections 3.3; 3.4; 3.5).

Hence, calcium antagonists appeared to disrupt the fine balance between the production and scavenging of ROS. Nifedipine, verapamil, and diltiazem were shown to induce significant oxidative stress in the epididymal sperm (increased MDA and decreased catalase and superoxide dismutase activity). This may be the reason for the induction of male infertility [253].

The dualism of 1,4-DHP effects has been recorded as inhibition or promotion of LP, as quenching or initiating

oxygen and nitrogen free radical chain reactions, radioprotecting or radiosensitizing, antagonizing or synergizing Ca^{2+} in electromechanical coupling, as well as in the membrane stabilization or labilization.

3.9. Could DHPs Be Involved in Antioxidative Stress? Before being applied *in vivo*, the optimal dose and optimal time intervals for DHPs application must be known. Namely, while ROS have been traditionally considered as toxic byproducts of aerobic metabolism, we know nowadays that ROS may act as essential signaling molecules, needed for the control of many different physiological processes. Whether the role of ROS will be damaging, protective, or signaling depends on the delicate equilibrium between time- and location-specific ROS production and scavenging. Accordingly, the imbalance of the increased AO potential, so-called antioxidative stress, could be dangerous similar to chronic OS, in particular in case of extended exposure. Inappropriate interventions in the oxidative homeostasis by excessive antioxidants especially in case of chronic exposure to antioxidants might have very negative effects as was published in the ATBC study, showing an increased cancer incidence in smokers treated by excessive beta-carotene [254]. Therefore, overconsumption of any natural or synthetic AO, including DHPs, as dietary supplements or drugs, must be avoided in order to suppress oxidative damage and must not disrupt the well-integrated antioxidant defense network (Poljsak [255] and Poljsak and Milisav [256]). This is especially important when administrating lipid-soluble antioxidants that can be stored in biomembranes, thus not only attenuating or preventing LP but also affecting physiological effects of the natural antioxidants, in particular tocopherol. The interrelationship with the status of endogenous antioxidants/prooxidants should be followed.

DHPs primarily suppress the initiation stages of LP process. They do not entirely stop the LP propagation. Acting synergistically with tocopherol, diludine may prevent pathological excess of ROS production within the lipid moiety of the cellular membranes and LDL. However, due to its low solubility and fast metabolism, its concentration in the cells is low. For that reason, it cannot cause antioxidative stress, even if used for an extended period of time. Thus diludine (diethone) could be only a mild antioxidant; it has potential for restoring the pool of natural antioxidants (as synergist of α-tocopherol and polyphenols) in the cells.

Moreover, DHPs CA used for cardioprotection and vasodilatation as commercial drugs in low concentrations are fast metabolized *via* CYP3A4, and for that reason their application does not induce cellular AO stress [53, 160]. However Godfraind and Salomone [257] have postulated no evidence that allows recommending dietary supplementation with antioxidants for the primary or secondary prevention of cardiovascular disease.

So far, there are no reports on antioxidative stress caused by some DHPs, diludine and its analogues. Diludine and its analogues therefore could act as adaptogens supporting hormetic effects of mild oxidative stress. These compounds may act as potential multisided modulators of Yin-Yang cycles of redox and cell functions (the electroplasmic cycle) (Wagner et al. [258]).

4. Conclusions

1,4-Dihydropyridines (1,4-DHPs) have broad spectrum of OS modulating activities. DHPs have reducing and lipid peroxidation inhibitor properties, act as reductants in simple chemical systems, and stabilize various biological systems (LDL, mitochondria, microsomes, cells, and tissues) against OS. Examples and peculiarities and mechanisms of antioxidant activity (AOA) and antiradical activity (ARA) as well as stress-protective effect of DHPs including commercial calcium antagonists (CA) were highlighted. These activities depend on various structural parameters related to DHPs (presence and character of substituents), lipophilicity, and depth of the incorporation in the biological membranes. They also depend on the experimental model system for exploring the lipid peroxidation or oxidative stress. Stress-protective effect of some metabolites of CA (nifedipine) is reviewed. Although some DHPs, including CA, have prooxidant properties (on epididymal sperm cells), they can generally be considered as potent antioxidants. Therefore, comparison of the AOA and ARA of different DHPs (mutually and with other AOs) was described in detail. According to the data presented, the DHPs might be considered as bellwether among synthetic compounds targeting OS and as a kind of pharmacological measure for respective field of organic and medicinal chemistry.

Abbreviations

AD: Alzheimer disease
AO(s): Antioxidant(s)
AOA: Antioxidant activity
ARA: Antiradical activity
CA: Calcium antagonist(s)
CCB: Calcium channel blocker
DHP(s): Dihydropyridine(s)
DNA: Deoxyribonucleic acid
HEH: Hantzsch ester
4-HNE: 4-Hydroxy-2-nonenal
IMAC: Inner membrane anion channel
Lox: Lipoxygenase
LP: Lipid peroxidation
MDA: Malonyl dialdehyde
Mit: Mitochondria
NADH: Reduced nicotinamide adenine dinucleotide
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
NO: Nitrogen oxide
OS: Oxidative stress
PD: Parkinson's disease
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid reactive substances
TG: Triglycerides.

Conflict of Interests

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

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Review Article

Redox Control of Multidrug Resistance and Its Possible Modulation by Antioxidants

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Clinical efficacy of anticancer chemotherapies is dramatically hampered by multidrug resistance (MDR) dependent on inherited traits, acquired defence against toxins, and adaptive mechanisms mounting in tumours. There is overwhelming evidence that molecular events leading to MDR are regulated by redox mechanisms. For example, chemotherapeutics which overrun the first obstacle of redox-regulated cellular uptake channels (MDR1, MDR2, and MDR3) induce a concerted action of phase I/II metabolic enzymes with a temporal redox-regulated axis. This results in rapid metabolic transformation and elimination of a toxin. This metabolic axis is tightly interconnected with the inducible Nrf2-linked pathway, a key switch-on mechanism for upregulation of endogenous antioxidant enzymes and detoxifying systems. As a result, chemotherapeutics and cytotoxic by-products of their metabolism (ROS, hydroperoxides, and aldehydes) are inactivated and MDR occurs. On the other hand, tumour cells are capable of mounting an adaptive antioxidant response against ROS produced by chemotherapeutics and host immune cells. The multiple redox-dependent mechanisms involved in MDR prompted suggesting redox-active drugs (antioxidants and prooxidants) or inhibitors of inducible antioxidant defence as a novel approach to diminish MDR. Pitfalls and progress in this direction are discussed.

1. Introduction

It is common knowledge that multiple drug resistance (MDR) has crucial negative impact on the clinical outcomes of conventional cytotoxic anticancer therapies and of those based on specific drugs targeting molecular pathways implicated in cancer cell functions and survival strategies. Since the discovery of the first ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp), ABC drug transporters have become targets for improving anticancer chemotherapy. Up to now, more than 49 different ABC transporters have been found and cloned [1]. A majority of MDR modulators or reversals are themselves substrates of the transporters that compete with anticancer agents for the efflux from tumour cells [2]. Frustrating the great expectations raised, ABC transporter/modulators/reversals proved to have insufficient clinical efficacy and very high toxicity. Novel "biological" approaches have been recently developed in laboratory to modulate ABC transporter-mediated MDR, including a monoclonal antibody that binds specifically to P-gp, thus suppressing drug transport, small interfering RNA technology to decrease the expression of ABCB1, antisense oligonucleotides, and agents attenuating P-gp transcription [3]. Though very promising, these "biologicals" are still lacking clinical proof-of-concept data.

In any case, the evident and numerous adverse effects of MDR modulators stimulated additional studies on physiological role(s) of MDR in the human organism. It has been

reported that MDR relies not exclusively on transporting systems for drug uptake and efflux, but also on intracellular drug metabolism and DNA damage [4]. Transporting and metabolic systems defining MDR are expressed in the majority of normal cells, are essential for nutrients uptake and metabolites efflux, and play a vital role in protecting cells against xenobiotics. Hence, harsh inhibition of a functionally essential mechanism results in general intoxication.

To gain protection against foreign invasions and maintain homeostasis, the human organism employs several types of physical, chemical, and biological defence systems. For example, skin and other lining epithelia mechanically prevent invasion of relatively large organic and inorganic particles. The immune system has been evolved to fight cellular invaders and high-molecular-weight compounds of biological origin.

The chemical defence system, consisting of biosensoring, transmitting, and responsive elements, has been evolved, starting from primitive eukaryotes and lower plants [5], to protect multicellular organisms against environmental chemical insults (xenobiotics) and to maintain homeostasis of endogenous low-molecular-weight metabolites (endobiotics) [6]. Being exposed to xenobiotic (drug) stress, an organism is challenged to rapid and appropriate adaptation by activating constitutive and expressing inducible systems, thus attenuating negative biological consequences. For this purpose, an array of gene families and molecular pathways have been developed during evolution to prevent cellular access, to detoxify and eliminate toxins, and to repair chemical damage. The active efflux proteins, for example, P-glycoproteins (P-gp) [7], multidrug resistance (MDR) proteins [8], and multixenobiotic resistance (MXR) proteins [9], directly eliminate slightly lipophilic organic xenobiotics from cells serving as the first line of chemical defence. Escaping the first-line guardians, once in the cytoplasm, toxic nucleophilic compounds undergo biotransformation by the oxidative phase I enzymes (cytochrome P450 (CYP), flavoprotein monooxygenase, hemeoxygenase, amine oxidases, xanthine oxidase, and others) to become electrophilic. The electrophile is subjected to reductive or conjugative modification by phase II enzymes (glutathione-S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), catechol-*O*-methyl transferases (COMT), N-acetyl transferases (NATs), and many others).

Reactive oxygen species (ROS) generated as by-products of phase I reactions are rapidly reduced to nontoxic "physiological" levels by antioxidant enzymes (superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPx), peroxiredoxins (PRx), and nonenzymatic antioxidants, such as reduced glutathione (GSH), uric acid, ascorbic acid, and ceruloplasmin, among others). All these constitutive protective systems are sufficient to cope with low levels of xenobiotics or endobiotics. The inducible chemical defence relies on the array of stress responsive genes. In this case, chemical stressors like anticancer chemotherapeutics should first be recognised by specific sensors which, in turn, transmit alarm signals to activate or express *de novo* transporting, biotransforming, and detoxifying enzymes.

The primary member of mammalian proteins-sensors of organic chemicals is the aryl hydrocarbon receptor (AhR), activated by planar aromatic hydrocarbons of natural or synthetic origin [10–12]. A second group of chemical sensors comprises nuclear receptors, such as pregnane X, constitutive androstane, peroxisome proliferators-activated, liver-X, and farnesoid-X receptors, recognising a wide variety of xeno- and endobiotics [12–14]. Nuclear factor erythroid-derived 2-related factors (Nrf1 and Nrf2) and related cap'n'collar- (CNC-) basic leucine zipper proteins belong to another family of sensors activated by oxidants and electrophiles [15, 16]. Activation of such recognition elements after ligand binding may result in alterations of ion channel conductivity, kinase machinery, and cytoplasmic and nuclear transcription factors, inducing cell response (signal transduction).

Signal transduction is often mediated by redox substances (superoxide anion radical, hydrogen peroxide, lipid peroxides, aldehydes, and others) [17–21]. At moderate concentrations, they are signals to start gene transcription via activation of transcription factors (nuclear factor κB (NF-κB), activator protein 1 (AP-1), and antioxidant response element- (ARE-) binding proteins) or initiating the protein kinase cascade [5, 16, 19]. The latter pathway leads to the interaction with specific ARE of DNA motifs on promoters of antioxidant defence enzymes such as GST, Mn-superoxide dismutase (MnSOD), and glutamyl-cysteine ligase, among others [15].

Inherited or acquired alterations at any key point of the chemical defence system might lead to chronic intoxication and numerous human pathologies (chronic inflammation, degeneration, carcinogenesis, multiple chemical sensitivity syndrome, etc.) in the inherited or acquired MDR, respectively (Figure 1). Usually, the course of anticancer chemotherapy induces the overexpression of drug transporters MDR/MXR/P-gp [3, 4], activation of sensoring receptors, electrophile/oxidant sensors, transcription factors, and overexpression/activation of detoxifying/antioxidant enzymes [1]. Collectively, it causes rapid metabolism and elimination of both the anticancer drugs and cytotoxic by-products targeting tumour cells. Since the chemical defence system is ubiquitous for all human organs and tissues and central to organism functions, the attempts for its pharmacological suppression in order to diminish MDR potentially bear the risk of a multitude of undesired side effects. Upon the pharmacological interaction with components of universal chemical defence system, the "good guy" evolved on purpose to protect multicellular organisms from low-molecular-weight chemicals could become a "bad guy" blocking desired therapeutical effects of anticancer drugs (MDR).

On the grounds of our current knowledge, redox regulation of multiple molecular pathways essential for human chemical defence system can be implicated differentially in normal host and in tumour cells. Owing to the fact that redox balance in tumour cells is greatly altered as compared to that of normal host cells [22, 23], selective redox inhibitors targeting tumour-associated chemical defence as a cause of MDR should be developed. Regarding potential health effects of redox modulators on tumours, they are mainly attributed to cancer chemoprevention, direct anticancer action (for comprehensive review, see [23]), cancer sensitisation to conventional chemotherapeutics, preferentially through MDR suppression/reversal, cancer sensitisation to radio- and

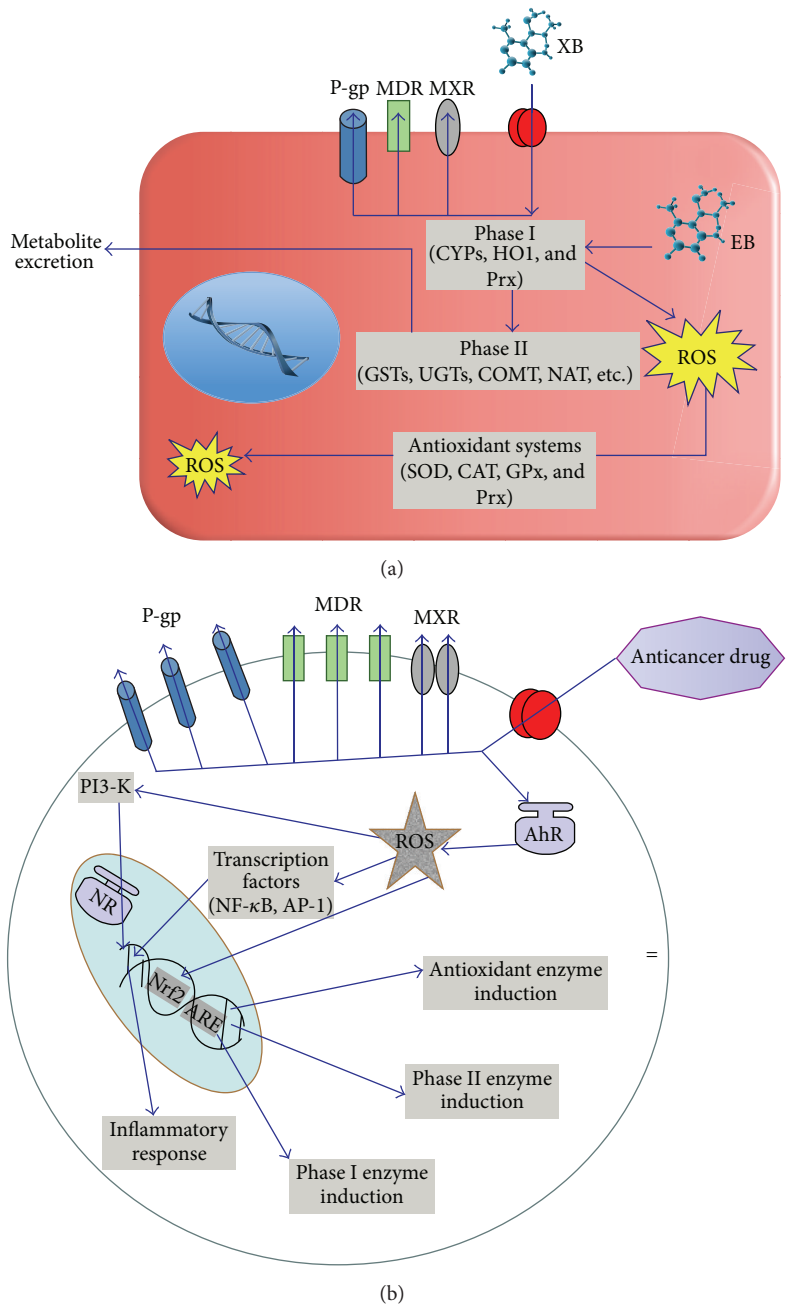

Figure 1: Inherited and acquired multiple drug resistance. (a) In the inherited multiple drug resistance (MDR), chronic exposure of normal cells to low levels of unknown xenobiotics (XB) or/and endobiotics (EB) takes place. It causes upregulation of ATP-binding cassette transporters such as P-glycoprotein (P-gp), MDR proteins (MDRs), and multiple xenobiotic resistance (MXR) without induction by anticancer drugs. Single nucleotide polymorphisms of phase I and II metabolic enzymes and efflux transporters often accompany inherited MDR and they could also be a causative reason for the resistance. Reactive oxygen species-mediated modulation of xenobiotics/drug metabolism is similar to that in the acquired drug resistance. This cellular pattern seems to be associated with high risk of tumour transformation. ROS: reactive oxygen species; MDR: multiple drug resistance transporters; MXR: multiple xenobiotic resistance transporters; P-gp: P-glycoprotein; CYP: cytochrome P450; HO1: hemeoxygenase-1; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; PI3K: phosphatidylinositol-3 kinase; AhR: aromatic hydrocarbon receptor; NF-κB: nuclear factor kappa B; AP-1: activator protein 1; NR: nuclear receptor; Nrf2: nuclear factor erythroid-derived 2-related factor 2; ARE: antioxidant responsive elements. (b) In the acquired MDR, chemotherapeutics induce redox-dependent MDR expression and activity in tumour cells. Chemotherapeutics activate also aromatic hydrocarbon receptor- (AhR-) driven and ROS-regulated expression of transcriptional factors (nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1)) which initiate inflammatory response. Reactive oxygen species (ROS) mediate activation of phosphoinositol-3 kinase upstream of inflammatory cytokine transcription and synthesis. ROS and AhR-associated stimulation of Nrf2 followed by antioxidant responsive element of DNA motif causes upregulation of protective, antioxidant, and detoxifying systems, such as antioxidant phase I and II enzymes.

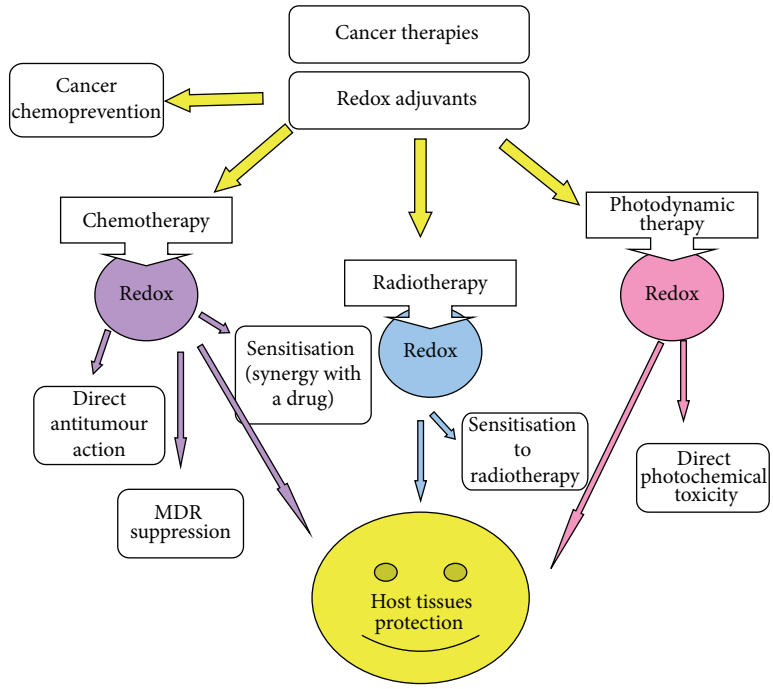

Figure 2: Redox-active substances and cancer. A variety of redox-active substances (direct or indirect antioxidants) are known to exhibit cancer chemopreventive properties. In the pharmacological anticancer protocols, redox-active agents could be used as direct anticancer chemotherapeutics or synergies with cytotoxic effects of conventional anticancer drugs. Here, we discuss the feasibility of such substances in suppression/reversal of acquired MDR. The redox agents are often used for the protection of normal tissues/organs against toxic effects of chemotherapy and radiotherapy.

photodynamic therapies, and protection of normal host organs/tissues against damage by chemo- and radiotherapy (Figure 2).

This review will discuss existing and perspective possibilities of differential targeted modulation of redox-dependent components/pathways of intrinsic and induced chemical defence as an emerging strategy for combinatory anticancer therapies to overcome MDR. Molecular pathways-targets for MDR attenuation or even reversal by redox-active substances will be described in detail.

2. Intrinsic Multidrug Resistance (MDR): Is It Possible to Overcome It by Redox Modulation?

2.1. Inherited Overexpression of Drug Transporters Accelerates Drug Efflux from Target Cells. Some individuals possess the so-called intrinsic MDR having never been exposed to chemotherapy. Genetical predisposition to resist xenobiotic stress could, in principle, be explained in terms of single nucleotide polymorphisms (SNPs) of complex MDR system components, starting from drug transporters, censoring receptors, and xenobiotic/drug metabolising enzymes (see several examples below). On the other hand, a leading hypothesis indicates intrinsic MDR as a result of chronic ("silent") exposure to low-level xenobiotic stressors or endogenous disturbances of lipid, glucose, and/or hormone metabolism. Therefore, the molecular pathways of its regulations are similar to those characteristic for acquired/chemotherapy-induced MDR. In this line, the development of intrinsic MDR correlates with increased risk of carcinogenesis, and the process is under network-like redox control (for comprehensive review, see [1]). It appears that the classical paradigm of cancer chemoprevention with redox-active nontoxic substances could be interpreted in terms of intrinsic MDR prevention. Furthermore, intrinsic MDR is a hallmark of stem cells, both normal and tumour, because high resistance to any toxin would guarantee survival and maintenance of stem cell populations.

2.2. Gene Polymorphisms Influencing Drug Metabolising Enzymes May Result in Ultrafast Drug Elimination or Extremely Slow Formation of Cytotoxic Redox By-Products. The cytochrome P450 (CYP) system is a superfamily of isozymes, located in the smooth endoplasmic reticulum, mainly in the liver, but also in extrahepatic tissues (e.g., intestinal mucosa, lung, kidney, brain, lymphocytes, placenta, and skin), involved in the biotransformation of numerous lipophilic xenobiotics into more hydrophilic, less toxic, and more easily excreted metabolites [11, 24, 25]. The major CYP enzymes involved in human drug metabolism belong to families 1, 2, and 3, the specific drug metabolising isoforms being *Cyp1A2*, *Cyp2C9*, *Cyp2C19*, *Cyp2D6*, and *Cyp3A4/3A5*.

Each CYP isoform is a product of specific gene. For some isoforms, the existence of genetic polymorphisms has been demonstrated. The allelic variants may be due to the deletion

of the entire gene, SNPs, deletion or insertion of fragments of DNA within the gene, or multiplication of gene copies, leading to absent, deficient, or enhanced enzyme activity. Thus, the population can be classified into Extensive Metabolisers (EM, individuals with normal capacity), Poor Metabolisers (PM, individuals with reduced/null metabolic activity), and Ultrarapid Metabolisers (UM, individuals with a higher-than-normal metabolic activity). It seems that opposite populations of PM and UM could be at risk of constitutive MDR, because UM would rapidly metabolise/excrete parent molecules of anticancer drugs, while PM would not produce ROS as by-products of anticancer drug metabolism. These by-products possess strong cytotoxicity against cancer cells. Hence, the routine clinical diagnostics based on determination of CYP SNPs produce a reliable prediction of individual chemosensitivity/chemoresistance/MDR to anticancer therapies. Several polymorphisms have been connected with the inducibility or enzymatic activity of the abovementioned drug metabolising CYP isoforms [6].

2.3. Redox-Active Inhibitors of Drug Transporters and Receptors Associated with Drug Detoxifying Enzymes: Hopes and Reality. Notwithstanding the growing interest and great hopes for natural nontoxic redox agents to prevent/inhibit/reverse MDR ([26]; in this review), drug development remains rather complicated due to low bioavailability, defined by restricted absorption through intestine, lining epithelia, and skin [24, 25], rapid metabolism, and excretion. Absorbed MDR inhibitors become themselves targets for the classical pathways of xenobiotic detoxification/drug metabolism [27–29]. In phase I, they are predominantly metabolised by microsomal CYPs. Then, phase II glucuronidation by UGT [30], sulfation by phenol and catecholamine specific sulfotransferases (SULT1A1 and SULT1A3) [11], methylation by COMT [31], and binding with glutathione through GST occur [12, 28].

To improve candidate MDR inhibitor bioavailability and attenuate its metabolic disruption, several approaches have been implied such as injectable forms, other sophisticated drug delivery systems, combination with adjuvants like piperine and caffeine to diminish glucuronidation, and chemical modification of parent molecules to bypass efficient metabolic guardians [6, 24, 27].

A very high probability of drug-drug interactions between the adjuvant therapeutics for MDR inhibition and anticancer therapies themselves, as inducers of MDR, should also be taken into consideration [32]. It has been reported that potential MDR suppressors of herbal origin may easily interact with the same efflux (P-gp) and metabolic (*Cyp3A4*) pathways as anticancer agents do, resulting in opposite outcomes: inhibition or expression of MDR components, depending on timing, dosages, posology, and route of drug administration [33]. Recent findings have shown that this kind of drug-drug interaction is highly influenced by genetic polymorphisms of efflux proteins (MDR1) and metabolic enzymes (*Cyp3A5*) [34].

Emerging evidence shows that MDR could be an evolutionary defined mechanism to preserve normal and cancer stem cell populations. In this direction, redox signalling becomes a probable candidate to maintain cell stemness [1]. Therefore, the development of clinically efficient redox modulators of MDR should selectively target cancer stem cells, while leaving normal stem cells intact.

3. Redox Dependence of Acquired Multidrug Resistance: Modulation by Direct and Indirect Antioxidants

Most chemotherapeutic agents generate ROS, which bind to specific structures within the cancer cells and promote cell death. Chemotherapeutic agents disturb the redox homeostasis in cells and change their ability to cope with excessive ROS levels through the production of protective direct antioxidants [35]. Direct antioxidants are modified in this process and need to be resynthesised [36]. Glutathione (GSH) is considered as the main redox buffer in a cell because it supplies large amounts (millimolar concentrations) of reducing equivalents [37]. The intracellular thiol redox *status* is described as the ratio of reduced to oxidised forms of thiols (GSH/GSSG), which decreases under oxidative stress conditions, and GSH reversibly forms mixed disulfide bonds between protein thiols (S-glutathionylation) to prevent protein oxidation [38]. Besides GSH, thioredoxin (Trx), another important endogenous antioxidant, provides protection against oxidative stress [39, 40]. Nrf2 regulates Trx and sulfiredoxin enzymes, which are involved in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and synthesis of GSH [41]. Among the potential mediators of chemoresistance, Trx plays critical roles in the regulation of cellular redox homeostasis and redox-regulated chemoresistance [42–44].

In a recent study, Zhang et al. have shown that inhibiting Nrf2 expression through the transfection of shRNA plasmids in non-small-cell lung cancer cells significantly inhibited the expressions of glutathione pathway genes, antioxidants, and multidrug resistance proteins and induced the generation of ROS, decreased the level of GSH, and inhibited cell proliferation [45]. It has been reported that diffuse large B lymphoma cells expressed higher-than-normal basal levels of Trx, which was associated with decreased survival. Suppressed Trx inhibited cell growth and clonogenicity and sensitised the lymphoma cells to doxorubicin [44]. In a recent study, Raninga et al. [46] have reported the cytoprotective role of tTrx1 and thioredoxin reductase 1 (TrxR1) enzyme in multiple myeloma. Trx inhibitors were utilised in a variety of human cancers including acute myeloid leukemia [47], colorectal cancer [48], and lung cancer [49] to inhibit tumour growth and to stimulate ROS-induced apoptosis. Auranofin, a TrxR1 inhibitor, caused oxidative stress-induced cytotoxicity and apoptosis in cancers including chronic myeloid leukemia [50], chronic lymphocytic leukemia [51], prostate cancer [52], and breast cancer [53]. Signal transducer and activator of transcription 3 (STAT3) activation is commonly observed in multiple myeloma, chronic lymphocytic leukemia, gastric cancer, lung cancer, and laryngeal carcinoma. Dietary gamma-tocotrienol inhibited both induced and constitutive activation of STAT3 in multiple myeloma and prostate cancer

cell lines [54]. High-dose intravenous ascorbate inhibited NADPH-oxidase and was selectively toxic to tumours with low CAT activity [55]. Recent study has reported that a naphthoquinone derivative induced cell death depending on Bax deficiency. In conclusion, it has been suggested that naphthoquinone might be clinically feasible to overcome chemoresistance [56].

Plant-origin polyphenols or their synthetic derivatives have been recognised as redox-active molecules with relatively low toxicity. Some of them, for example, luteolin, apigenin, and chrysin, exert both direct and indirect antioxidant effects by scavenging ROS and increasing Nrf2 activity, followed by the induction of its target antioxidant genes [57]. The natural polyphenols are also substrates for ABC transporters as they bind to the active sites of the transporters and reduce drug efflux [58]. The prooxidant capacity of some polyphenols (quercetin, epigallocatechin gallate) allowed their identification as chemotherapeutic adjuvants since they selectively enhanced cytotoxic effects of chemotherapeutics [59]. Shin et al. have reported that some specific polyphenols triggered cell cycle arrest and apoptotic cell death in cisplatin-resistant A2780/Cis human ovarian cancer cells [60].

3.1. Antioxidant-Associated Modification of Drug Transporting Systems. Elevated GSH levels trigger chemoresistance by different pathways: direct interaction with drugs and ROS, prevention of protein and DNA damage, and induction of DNA repair. For example, MRP1 causes efflux of some xenobiotics (e.g., vincristine, daunorubicin) through a cotransport mechanism with GSH [26, 61–63]. Oxidative stress was more cytotoxic towards B16 melanoma cells with low GSH concentrations [64]. Tumour cells overexpressing γ-glutamyl-transpeptidase were more resistant to H_2O_2 and chemotherapeutics, such as doxorubicin, cisplatin, and 5-fluorouracil [65]. GST-related chemoresistance modulated protein-protein interactions with members of the mitogen-activated protein (MAP) kinases including c-Jun N-terminal kinase 1 and apoptosis signal-regulating kinase 1 and altered balance of kinases during drug treatment [66]. This complex mechanism involved the interaction of promoter regions for *GST* and *GGT* with NF-κB and Nrf2 followed by upregulation of several detoxification genes, such as ferritin, GSH-*S*-reductase, and hemeoxygenase-1. Hypoxia induced breast cancer resistance protein (BCRP) expression in tissues by interacting with heme and porphyrins thus increasing levels of cytoprotective protoporphyrins [67]. Overexpression of BCRP is known to induce resistance to various chemotherapeutic drugs, such as topotecan and methotrexate [68].

3.1.1. MDR Induction and Possibility to Inhibit It by Redox-Active Substances Affecting Glutathione Metabolism. Definite redox-active compounds, such as quinones, polyphenols, oligomeric proanthocyanidins, ergothioneine, ovothiols, tannins, or terpenes, behave as redox modulators and trigger redox-related events, such as ROS increase and GSH depletion, causing apoptosis of cancer cells [69]. In general, redox modulations in cancer cells could initiate cell differentiation or could induce apoptosis [70]. Acetaminophen, a widely used drug to combat pregnancy-connected toxicity [71], induced ROS production in human choriocarcinoma cells by reducing BCRP and GSH content and activating Nrf2-targeted genes: NAD(P)H dehydrogenase, quinone 1 (NQO1), and hemeoxygenase-1. On the other hand, genetic knockout of *TrxR1* gene resulted in liver insensitivity to acetaminophen due to drastic disruption of the link between redox homeostasis and drug metabolism in the liver [72]. Glyoxalase 1, a key enzyme converting α-oxoaldehydes into corresponding α-hydroxy acids, has been found to be amplified in many primary tumours and cancer cell lines [73]. In this regard, Young et al. [73] have reported that overexpression of both enzymes glyoxalase 1 and transglutaminase 2, an enzyme catalysing polyamine conjugation/deamidation, led to increased tumour cell survival, drug resistance, and metastasis [74]. The use of photodynamic anticancer therapy is particularly attractive because of its specificity and selectivity [75]. Hypericin, a naphthodianthrone, is a promising photosensitizer, which is feasible for photodynamic therapy, for fluorescence diagnosis, and for topical applications [76]. Mikešová et al. [77] have shown that hypericin content in cells, GSH levels, and redox *status* correlated with hypericin-induced photocytotoxicity. In contrast, resveratrol attenuated cisplatin toxicity by maintaining GSH levels [78, 79]. It has also been demonstrated that buthionine sulfoximine, an inhibitor of GSH biosynthesis, increased the sensitivity of the cells to chemotherapeutics, while N-acetyl cysteine exhibited the reverse effect, particularly in drug-resistant cells [61, 62, 80]. Malabaricone-A, a diarylnonanoid with a potency of MDR reversal, induced depletion of GSH, inhibited GPx activity, and caused redox imbalance [81]. Collectively, molecular pathways-targets for MDR modulation by GSH controlling agents are schematically presented in Figure 3.

3.1.2. Overexpression of ABC Transporters: Effects of NADPH-Oxidase and CYP Inhibitors. NADPH-oxidase (NOX) is an oxidoreductase and plays crucial roles in cell growth, proliferation, and regulation of phosphatases and transcription factors via redox-sensitive cysteine residues [82]. Elevated NOX expression has been shown in breast cancer [83], colon cancer [84], and neuroblastoma cells [85]. Barth et al. have demonstrated that pharmacological block of glucosylceramide synthase, a stimulator of NOX activity, substantially improved cytotoxicity of chemotherapeutics in glioblastoma cells [86]. The molecular mechanism of anticancer effects of cisplatin involves activation of Akt/mTOR pathway regulated by NOX-generated ROS, and NOX inhibition by diphenyl iodonium was critical for cisplatin cytotoxicity [87].

Overexpression of the drug and xenobiotic metabolising cytochrome P450 enzymes for a long time has been considered as one of the major mechanisms of chemoresistance in solid tumours [88, 89]. Types 1 and 2 CYPs have been proven to activate procarcinogens into ultimate carcinogens [90]. CYPs have become therapeutic targets in anticancer protocols amid their involvement in the activation and/or inactivation of chemotherapeutic drugs [91]. Molina-Ortiz et al. reported that altered CYP expression played a crucial role in the therapy of Rhabdomyosarcoma patients [92]. The *in vitro* antitumour action of natural product austocystin D

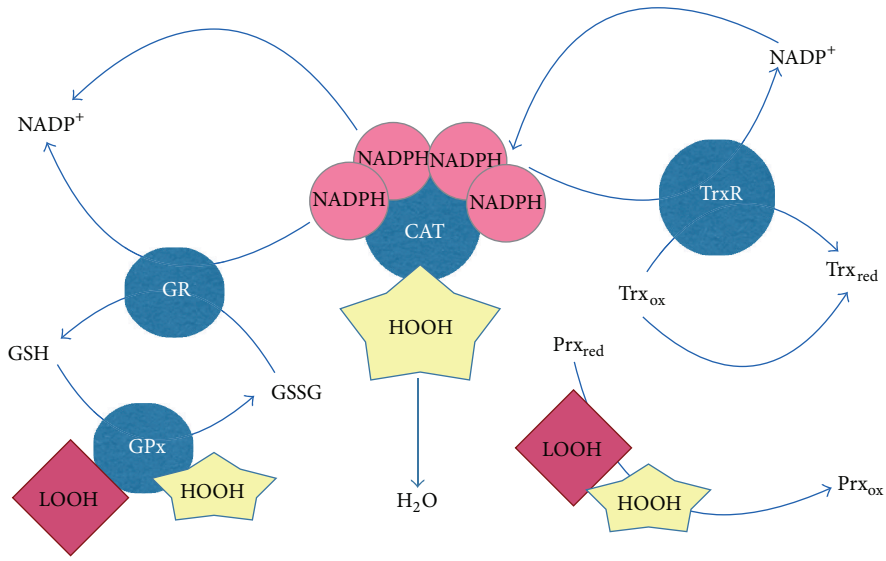

FIGURE 3: Antioxidant and prooxidant systems as molecular targets for redox-active MDR modulators. Glutathione and enzymes involved in the glutathione metabolism such as glutathione reductase (GR) and glutathione peroxidase (Gpx) as well as gamma-glutamyl cysteine ligase, catalase, NADPH-oxidase, thioredoxin (Trx), and peroxiredoxins (Prx) are potential molecular targets for future MDR modulators.

has been explained by selective activation of CYP enzymes leading to DNA damage [93].

3.2. Chemotherapy-Induced Inflammatory Responses May Cause Redox-Regulated Multidrug Chemoresistance. To increase clinical efficacy of chemotherapy and combat MDR, molecular and cellular processes promoting inflammation have been targeted due to the common knowledge that (i) inflammatory cells are present within tumours and (ii) tumours arise at sites of chronic inflammation [94, 95]. Cancer promotion and progression stages are accelerated by ROS generated by immune cells, mediators of inflammation [96]. The induction of antioxidant defence enzymes in tumours as an adaptation to oxidative attacks from host immune cells might contribute to chemoresistance. Thus, hydrogen peroxide-resistant thymic lymphoma cells with increased catalase and total SOD activities, altered GSSG/GSH redox potential, and oxidised $NADP^+$/NADPH pool exhibited resistance to conventional chemotherapeutics, such as cyclophosphamide, doxorubicin, vincristine, and glucocorticoids [97]. Chemotherapeutic agents caused release of ATP into the extracellular space as they induced tumour cell death [98]. Following accumulation of adenosine in tumours through CD39 and CD73, immune responses were suppressed [99]. Clayton et al. showed that exosome-expressing CD39 and CD73 suppressed T cells through adenosine production [100]. Oxidative stress has putative impact on the activation and regulation of protein kinase C (PKC) with redox-sensitive regions in both N-terminal regulatory domain and C-terminal catalytic domain. Rimessi et al. [101] have demonstrated that PKCζ induced resistance to apoptotic agents following its translocation into the nucleus as a result of oxidative stress. Nuclear PKCζ inhibitor restored the apoptotic susceptibility of doxorubicin-resistant cells by forming a complex with the proinflammatory transcription factor NF-κB and promoting IL-6 synthesis, thus favouring tumorigenesis and MDR [102].

3.2.1. Activation of NF-κB-Dependent Pathways and Their Inhibition by Antioxidants. NF-κB is the key transcription factor involved in the inflammatory pathway. NF-κB is constitutively active in many of the signalling pathways implicated in cancer. Hyperactivation of NF-κB in cancer cells promotes cancer cell survival by inducing the upregulation of antiapoptotic proteins such as MnSOD and Bcl-2 family members and the inhibition of proapoptotic proteins and is linked directly to the inflammation-induced chemoresistance. NF-κB protects against oxidative stress and activates transcription factor c-myc, *MMP* gene expression, and tumour angiogenesis and remodels extracellular matrix, while NF-κB inhibition blocks cell proliferation [95, 103–106]. NF-κB is associated with aberrant growth, resistance to apoptosis, and overexpression of the genes involved in cell cycle promotion in cancer cells. In a recent study, it has been shown that isorhamnetin, a metabolite of quercetin, enhanced antitumour effects of chemotherapeutic drug capecitabine through negative regulation NF-κB [107]. Singh et al. have reported that tea polyphenols inhibited cisplatin enhanced activity of NF-κB [108]. FADD-like IL-1beta-converting enzyme inhibitory protein (FLIP) is a potent inhibitor of caspase-8-mediated apoptosis involved in NF-κB activation. Talbott et al. revealed that FLIP regulates NF-κB through protein S-nitrosylation, a key posttranslational mechanism controlling cell death and survival strategies [109]. Overexpression of cyclin D1 in cancer cells was reported in cisplatin chemoresistance. In contrast, reduction of cyclin D1 expression resulted in the increased sensitivity to cisplatin due to reduced NF-κB activity and apoptosis [110]. It was shown that vitamin E compounds, such as δ- and γ-tocotrienol, inhibited NF-κB activity, cell growth, cell survival, and tumour growth.

In parallel, δ-tocotrienol augmented sensitivity of pancreatic cancer to gemcitabine [111].

Curcumin, a nontoxic food additive extensively used for food flavouring [112], has been found to suppress human hepatoma through inhibition of tumour cell proliferation, cell cycle arrest in G2/M phase, and induction of apoptosis [113]. Numerous publications have reported curcumin as a sensitiser for a number of anticancer drugs [114], first of all, cisplatin [115]. Results of recent studies have also suggested that curcumin was a reversal of induced MDR by multiple mechanisms such as the inhibition of ABC transporter expression and function, activation of ATPase, and modulation of NF-κB activity during anticancer therapy [116]. In contrast, caffeic acid, a natural phenolic, prevented antiproliferative and proapoptotic effects induced by paclitaxel in lung cancer cells by the activation of NF-κB-survivin-Bcl-2 axis, thus contributing to acquired MDR [117].

3.2.2. Activation of Phosphoinositol-3 Pathway and Its Inhibition in a Redox Fashion. The phosphatidylinositol-3 kinase (PI3K) pathway has been widely considered to be associated with oncogenesis, cancer progression, and multiple hallmarks of malignancy [118]. Consistently, PI3K pathway is a common mechanism of resistance to antineoplastic agents [119]. Of note, resistance to PI3K inhibitors may also develop due to aberrant compensatory signalling through other pathways [120]. The three main molecules in this pathway are PI3K, Akt, and mammalian target of rapamycin (mTOR). Recently, it has been reported that PI3K-mTOR inhibitor enhanced the cytotoxicity of temozolomide, an advanced chemotherapy for malignant gliomas [121].

Since activation of integrins, proteins expressed on the cytoplasmic membrane of malignant cells, is controlled directly by a redox site by disulfide exchange in their extracellular domain, redox modifications of thiols could alter essential functions of integrins [122]. It was suggested that inactivation of VLA-4 integrin by nontoxic tellurium compound was due to its binding to the thiol groups of cysteines that decreased PI3K/Akt/Bcl-2 signalling while enhancing drug sensitivity [123]. Gao et al. demonstrated that the natural bioflavonoid apigenin reversed drug-resistant phenotype by its suppressor effect on PI3K/Akt/Nrf2 pathway in doxorubicin-resistant Nrf2 overexpressing cells [124].

3.2.3. Activation of Toll-Like Receptors (TLRs) by Chemotherapeutics and Inhibitory Effects of Redox Modulators. Recent studies implicate bacterial, parasitic, and viral infections as a possible link between inflammation and carcinogenesis [125]. One possible redox-sensitive signalling pathway connecting infection-associated inflammation and carcinogenesis is mediated by Toll-like receptors (TLRs). The hypothesis is that bacterial products, such as lipopolysaccharide, could activate TLR4-MyD88 axis in tumour cells followed by the production of proinflammatory cytokines, overexpression of antiapoptotic signals (XIAP and pAkt), and, finally, acquisition of chemoresistance by ovarian cancer cells [126]. Both *in vivo* and *in vitro* experiments have shown that anticancer drug paclitaxel exerted two opposite modes of action: killing of breast cancer cells and enhancement of their survival through activation of TLR4 pathway [127]. The authors suggested that simultaneous TLR4 block could reverse MDR to paclitaxel and improve efficacy of the anticancer therapy.

Activation of TLRs in cancer cells seems to contribute to the tumour growth, cancer cell survival, and MDR via a signalling cascade involving cytokine/chemokine production [128]. It was shown that ligation to the TLR2 in lung cancer cells induced activation of mitogen-activated protein kinases (MAPK) and NF-κB, a classical pathway of survival strategy [129]. Stimulation of TLR7/TLR8 in pancreas cancer cells resulted in elevated NF-κB and COX-2 expression, increased cancer cell proliferation, and reduced chemosensitivity [130]. Active TLR-4/MyD88 signalling was also found in epithelial ovarian cancer cells and influenced the drug response [131]. The relationships between the expressions of TLR-4, MyD88, and NF-κB have been examined in epithelial ovarian cancer patients. Increased MyD88 expression was found to be associated with poor survival rate [132].

3.3. Chemotherapy-Induced Redox-Dependent Stress Responses Leading to Adaptation. Along with killing cancer cells, chemotherapeutic agents induce their stress and adaptive responses. Signalling pathways and gene expression in response to chemotherapeutics play pivotal roles in the development of acquired MDR [133]. Key functional aspects of cellular stress response include damage to membrane lipids, proteins, and DNA and alterations in the redox *status*, energy metabolism, cell cycle, and proliferation [134]. Thus, there is clear-cut evidence that upregulation of nonenzymatic and enzymatic antioxidant defence, molecular chaperones, and stress responsive proteins are responsible for acquired MDR [135]. These molecular pathways are potential targets to enhance the cytotoxic effects of chemotherapeutics and to overcome drug resistance.

3.3.1. Nrf2 as a Perspective Target to Overcome MDR in Tumours. Nrf2, a redox-sensitive transcription factor, plays a crucial role in redox homeostasis during oxidative stress. Nrf2 is sequestered in cytosol by an inhibitory protein Keap1 causing its proteosomal degradation [136]. In response to oxidative stress, Nrf2 translocates to nucleus and binds to ARE that increases the expression of antioxidant genes such as hemeoxygenase-1, NAD(P)H: quinone oxidoreductase 1, aldo-keto reductases, and several ATP-dependent drug efflux pumps [137]. Many genes involved in phase II metabolism are also induced by Nrf2, including GSTs, UGT, and UDP-glucuronic acid synthesis enzymes [138]. While Nrf2 upregulation causes chemoresistance, its blockade sensitises a variety of cancer cells, including neuroblastoma, breast, ovarian, prostate, lung, and pancreatic cancer cells, to chemotherapeutic drugs [139]. Several flavonoid compounds have been reported to be potent Nrf2 inhibitors, such as epigallocatechin 3-gallate, luteolin, and brusatol [140, 141]. Nrf2 was upregulated in hepatocellular carcinoma and positive correlation was found between Nrf2 expression and antiapoptotic Bcl-xL and MMP-9 [142]. Quercetin treatment increased the total cellular amount and nuclear accumulation of Nrf2 protein in malignant mesothelioma cells [143]. *In vitro*

suppression of Keap1 in human prostate and non-small-cell lung carcinoma cell lines elevated Nrf2 activity and increased sensitisation to various chemotherapeutic agents and radiotherapy [144, 145]. These results demonstrated that Nrf2 inhibitors are effective adjuvants of chemotherapeutic drugs.

3.4. Chemotherapy-Induced Prosurvival and Antiapoptotic Cellular Strategies: Roles for Anti- and Prooxidants. Cellular redox homeostasis is maintained by the balance between endogenous antioxidant defence system, including antioxidant enzymes such as SOD, catalase (CAT), GPX, GSH, proteins, and low-molecular-weight scavengers, such as uric acid, coenzyme Q, and lipoic acid, and the prooxidant molecules, leading to the formation of several highly oxidising derivatives.

3.4.1. p53 Proapoptotic Protein. p53 is considered as the guardian of the genome, and several gene mutations encoding p53 have been detected in several tumour cells. Under physiological conditions, activated p53 plays a key role in tumour prevention by promoting synthesis of antioxidant enzymes. ROS-induced DNA damage activates p53, leading to apoptosis via the mitochondrial intrinsic pathway and increasing the synthesis of prooxidant enzymes. Since p53 is a redox-sensitive factor, ROS negatively modulates its activity via oxidative modification of the cysteine residues at the DNA-binding site. It has been proposed that the loss of p53 function in cancer cells is associated with their ability to avoid apoptosis [146]. Mutations of p53 are involved in resistance to chemotherapy [147]. It was demonstrated that p53 regulated Nrf2 negatively and interfered with the ability of Nrf2 to bind to DNA. A low level of p53 favoured binding of Nrf2 to DNA. In a recent study, the treatment with bortezomib, which is a selective proteasome inhibitor, induced Myelocytomatosis Viral Oncogene Neuroblastoma (MYCN) downregulated p53 expression, leading to cell survival in neuroblastoma [148].

3.4.2. Bcl-2 Antiapoptotic Protein. Bcl-2 protein is a member of a family of apoptosis-modulating proteins which protects against a variety of apoptotic stimuli, mainly acting at the mitochondrial level. In addition to its antiapoptotic action, Bcl-2 has been shown to exert potent antioxidant effects [149] such as protection of lipid membranes against peroxidation reactions, maintenance of cellular redox status (i.e., NADH/NAD^+ and GSH/GSSG in a reduced state) in response to oxidative stress [150], and elevation of cellular levels of glutathione and reducing equivalents [151]. Bcl-2 upregulates antioxidant defence systems, and its mitochondrial localisation contributes to achieving this effect. Bcl-2 is capable of forming ion channels as a regulator of mitochondrial permeability transition [152]. Mitochondrial permeability transition blockade prevents release of cytochrome c, an apoptosis initiating factor. Herrmann et al. reported that Bcl-2 selectively regulates nuclear localisation of cell death regulators such as p53 and NF-κB [153]. Cellular redox state via thiols plays a major role in regulating mitochondria-mediated events during apoptosis, and oxidation of mitochondrial thiols is an apoptotic sensor. GSH is involved in detoxifying reactions and it has a high intracellular concentration. It serves as the major reducing peptide within all of the cells, due to its sulfhydryl group buffering and removing free radicals generated during metabolic processes such as respiration. GSH is an important factor for Bcl-2 ability to suppress apoptosis. Chaiswing et al. observed that thiol redox *status* and activities and expression of several antioxidant enzymes exhibited distinct patterns in two prostate cancer cell lines at different growth phases, suggesting that modulation of thiol redox status might be useful as a therapeutic tool to modify cancer cell proliferation and tumour aggressiveness [154]. Mitochondrial metabolism is altered in malignant cells so that, in contrast to normal or benign cells, malignant cells accumulate citrate due to low activity of mitochondrial aconitase, become citrate-oxidising cells, and exhibit low amounts of citrate. The higher rate of mitochondrial substrate metabolism explains the increased levels of ROS associated with malignancy and metastasis. The foregoing discussion illustrates the importance of GSH in mitochondrial function and redox *status*, in determining the metastatic aggressiveness and sensitivity of cancer cells to chemotherapeutic agents, and provides the rationale for mitochondrial GSH as a potential therapeutic target in cancer [155].

3.5. Chemotherapy-Induced Transcription and Activity of Aromatic Hydrocarbons (Ah) Receptor. AhR is a basic helix-loop-helix transcription factor that, prior to ligand binding, is stabilised in the cytoplasm by direct interaction with several proteins such as heat shock protein 90, its cochaperon, and X-associated protein 2. Upon binding to aromatic ligands, toxins, drugs, phytochemicals, and sterols, the AhR-ligand complex shuttles from cytoplasm to the nucleus, where it heteromerises with the AhR nuclear translocator (Arnt) to form the transcription complex able to bind to xenobiotic responsive elements (XRE) DNA-binding motifs located in the promoter region of the target drug metabolising genes, such as phase I (mainly, *Cyp1* subfamily) and II metabolising enzymes and Nrf2 [156–158]. One of the downstream targets for AhR is BCRP encoded by *ABCC3* gene [159–161]. The coordinate AhR- and Nrf2-dependent transcriptional regulation of human UGTs by utilising both XRE- and ARE-binding motifs takes place to protect cells from xenobiotic and oxidative stresses [162]. The elegant study with genetically modified mice has clearly demonstrated that Nrf2 is required for ligand-associated induction of classical "AhR battery" genes NQO1, GST isoforms, and UGTs [163]. Apart from metabolic enzymes, a number of growth factors, cytokines, chemokines, and their receptors are downstream gene targets for activated AhR [164, 165]. AhR is also functionally connected with epidermal growth factor receptor, presumably, through NF-κB-regulated pathway [166], thus influencing the epithelial cell proliferation. AhR can also cross-talk and directly interact with proteins involved in major redox-regulated signalling pathways such as NF-κB and various kinases such as Src, JNK, p38, and MAPK [167] and with oestrogen receptors to mediate oestrogen metabolism [168, 169]. Recent studies have unraveled unsuspected physiological roles and novel alternative ligand-specific pathways for this receptor that allowed hypothesising numerous pharmacological roles of

AhR ligands useful for the development of a new generation of anti-inflammatory and anticancer drugs [159, 170].

The AhR-mediated regulation of aromatic hydrocarbons metabolism has been implicated in a variety of cancers [164, 171] affecting different stages of carcinogenesis. If metabolic activation of the organic molecules increased the levels of their adducts with DNA thus promoting cancer initiation, anticancer drug- or toxin-induced AhR activation played a pivotal role in cancer promotion and progression [172, 173]. Elevated AhR expression associated with constitutive nonligand activation has been found in several cancers as evidenced by the nuclear localisation of AhR and induced downstream gene *Cyp1A1* [174, 175]. Stable knockdown of AhR decreased the tumorigenic and metastatic properties of breast cancer cell line *in vitro* and *in vivo*. On the other hand, AhR overexpression in nontumour human mammary epithelial cells transformed them in cells with malignant phenotype [176]. Of importance, AhR knockdown downregulated the expression of *ABCC3*; overexpression of this gene in breast cancer has been strongly associated with acquired MDR [177] and resistance to paclitaxel, a drug widely used in the treatment of metastatic breast cancer [178]. Inherited polymorphisms in AhR, for example, substitution Arg554Lys, and its machinery [179, 180] or presence of endogenous ligands-stimulators for the receptor (cAMP, bilirubin, prostaglandins, oxidative lipids, etc.) could be implicated into inherited MDR.

To suppress AhR transcription pharmacologically several approaches have been proposed, including the modulation of protein-protein interaction between transcription factor, coactivators, and corepressors [160, 167, 181]. A number of dietary polyphenols with redox properties (resveratrol, quercetin, curcumin, etc.), indoles, tryptophane metabolites, bilirubin, and oxidised products of lipid metabolism have been suggested as nontoxic ligands-activators or ligands-inhibitors of AhR expression by competitive and noncompetitive pathways [181, 182]. If ligands-activators of AhR are regarded as potential anti-inflammatory agents [181–184], redox-active ligands able to suppress AhR expression/functions could be candidates for MDR reversals. For example, 7-ketocholesterol, a major dietary oxysterol, may actually strongly inhibit AhR activation [185]. Alpha-naphthoflavone is considered as a classical AhR antagonist blocking activation of XRE-containing reporter gene and *Cyp1* upregulation in hepatoma cells [186]. However, alpha-naphthoflavone is also a partial agonist of AhR and acts as a competitive inhibitor exclusively in the presence of another agonist. Recently, more selective pure ligands-antagonists of AhR have been developed such as 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide, 3′-methoxy-4′-nitroflavone, 3′,4′-dimethoxyflavone, and 6,2′, 4′-trimethoxyflavone. These were able to block the induction of *Cyp1A1*-dependent ethoxyresorufin *O*-deethylase (EROD) activity [187–190]. They all belong to redox-active flavones and after proper clinical studies on safety and efficacy could be feasible for combinatory anticancer therapy to combat MDR. Among a number of plant polyphenols used for topical application, exclusively the phenylpropanoid verbascoside and flavonoid quercetin proved to be strong inhibitors of UV- or FICZ-upregulated AhR-*Cyp1A1*-*Cyp1B1* axis in human keratinocytes [184], suggesting their potency as topical MDR suppressors/reversals.

4. Conclusions

The multiple pleiotropic interactions of redox-active molecules so far demonstrated on the molecular pathways controlling cellular MDR, from xenobiotic cellular uptake inhibition to the modulation of phase I/II enzyme detoxification, to the inhibition of Toll-like receptor activation and/or AhR expression and function, provide a consistent rationale for the necessity of more intense and systematic research efforts in the field of anti-/prooxidant adjuvants for anticancer chemotherapy, to attempt clinically effective MDR inhibition with tolerable toxicity.

The design and implementation of more selected and targeted clinical studies centred on redox-active candidate MDR-interfering molecules will possibly contribute to overcoming the presently dominating clinical practice, which confers a constantly growing interest in redox modulators and antioxidants as a mere palliative against the potent cytotoxicity of conventional and biologic anticancer drugs.

Abbreviations

ABC: ATP-binding cassette
AhR: Aryl hydrocarbon receptor
Akt: Protein kinase B
AP-1: Activator protein 1
ARE: Antioxidant response element
Bcl2: Apoptosis regulator Bcl-2
BCRP: Breast cancer resistance protein
CAT: Catalase
COMT: Catechol-O-methyl transferase
CYP: Cytochrome P450
EM: Extensive Metabolisers
FLIP: FADD-like IL-1beta-converting enzyme inhibitory protein
GPx: Glutathione peroxidase
GSH/GSSG: Reduced glutathione/oxidised glutathione
GST: Glutathione-S-transferase
JNK1: c-Jun N-terminal kinase 1
Keap 1: Kelch-like ECH-associated protein 1
MDR: Multiple drug resistance
MMP: Matrix metalloproteinase
mTOR: Mammalian target of rapamycin
MXR: Multiple xenobiotic resistance
NADPH: Nicotinamide dinucleotide phosphate, reduced form
NAT: N-Acetyl transferase
NF-κB: Nuclear factor kappa B
NQO1: NAD(P)H: quinone oxidoreductase 1
NOX: NADPH-oxidase
Nrf1/2: Nuclear factor E2-related factor 1/2
P-gp: P-glycoprotein
PPI3K: Phosphoinositol-3-kinase
PKC: Protein kinase C
PM: Poor Metabolisers

Prx: Peroxiredoxins
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SNP: Single nuclear polymorphism
SOD: Superoxide dismutase
STAT3: Signal transducer and activator of transcription 3
SULT1A1/A3: Sulfotransferases A1/A3
TNFα: Tumour necrosis factor alpha
TLR: Toll-like receptor
Trx: Thioredoxin
TrxR: Thioredoxin reductase
UGT: UDP-glucuronosyltransferase
UM: Ultrarapid Metabolisers
XRE: Xenobiotic responsive elements.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Is Modulation of Oxidative Stress an Answer? The State of the Art of Redox Therapeutic Actions in Neurodegenerative Diseases

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The central nervous system is particularly sensitive to oxidative stress due to many reasons, including its high oxygen consumption even under basal conditions, high production of reactive oxygen and nitrogen species from specific neurochemical reactions, and the increased deposition of metal ions in the brain with aging. For this reason, along with inflammation, oxidative stress seems to be one of the main inducers of neurodegeneration, causing excitotoxicity, neuronal loss, and axonal damage, ultimately being now considered a key element in the onset and progression of several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and hereditary spastic paraplegia. Thus, the present paper reviews the role of oxidative stress and of its mechanistic insights underlying the pathogenesis of these neurodegenerative diseases, with particular focus on current studies on its modulation as a potential and promising therapeutic strategy.

1. The Role of Oxidative Stress in Neurodegeneration

Although molecular oxygen (O_2) is crucial for life of most organisms, it is not totally innocuous. The deleterious effects of O_2 are thought to result from its univalent metabolic reduction that leads to the formation of chemically reactive and toxic species, known as reactive oxygen species (ROS). These include molecules that contain oxygen-centered free radicals, such as the superoxide radical anion ($^{\bullet}O_2^-$), the hydroxyl radical ($HO^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), and peroxyl radicals ($ROO^{\bullet}$), as well as nonradical derivatives of O_2 like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) [1–4]. Several sources, either exogenous or endogenous, contribute to intracellular ROS formation. Exogenous sources include radiation, atmospheric pollutants, and chemicals [5]. Endogenously, ROS originate mainly from mitochondria, when $^{\bullet}O_2^-$ is formed by electrons leaking between complexes I and III of the electron-transport chain [6] and through NADPH oxidase, an enzyme that uses NADPH to reduce O_2, thus generating large amounts of $^{\bullet}O_2^-$ on the membrane surface as a toxic agent during elimination of pathogens [7]. The reactivity of $NO^{\bullet}$ with ROS leads to the formation of many other reactive species, termed reactive nitrogen species (RNS), which include the key effector molecule $ONOO^-$, but also other species such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and dinitrogen tetroxide (N_2O_4) [8]. In mammals, $NO^{\bullet}$ is an essential biological molecule mostly generated by a family of specific NO synthase (NOS) isozymes: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isozymes [9]. However, $NO^{\bullet}$ can be produced also by other redox enzymes such as xanthine oxidase or nonenzymatically by guanidine-substitute L-arginine analogs in the presence of NADPH [10, 11]. Not surprisingly, the major producers of ROS and RNS are indeed immune cells and specifically phagocytic cells, either resident cells in the brain (i.e., microglia) or infiltrated leukocytes, due to their elevated expression of NADPH oxidase, iNOS, and

xanthine oxidase [12, 13]. However, cells are equipped with enzymatic and nonenzymatic antioxidant systems to eliminate ROS and RNS, thus maintaining redox homeostasis. Antioxidants include naturally occurring molecules of high or low molecular weight, as well as nutritional antioxidants, whose action is strictly linked to their bioavailability. Naturally occurring antioxidants are mainly enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase/reductase (GPx/GR), and peroxiredoxin or molecules like glutathione (GSH), uric acid, pyruvate, amino acids, transferrin, ferritin, and caeruloplasmin. On the other hand, nutritional antioxidants include lipid-soluble antioxidants (α-tocopherol, carotenoids, quinones, and some polyphenols) and water-soluble antioxidants (ascorbic acid and some other polyphenols) [2]. Oxidative stress is strictly dependent on the balance between the rate of radicals production and that of their clearance. This overall balance seems to be under the regulation of transcription factor nuclear factor-E2-related factor (Nrf2), which is indeed a central component of cellular defense against oxidative stress [14]. Intriguingly, a very recent discovery reported the presence of a specific protein, termed negative regulator of ROS, that is, NRROS, which is capable of regulating the production of ROS by modulating their generation from phagocytes during inflammatory responses [15]. In particular, this regulator, which is localized in the endoplasmic reticulum, directly interacts with the membrane-bound subunit gp91(phox) of the NADPH oxidase complex and facilitates the degradation of NOX1 and NOX2 proteins, thereby modulating ROS production.

The central nervous system (CNS) is particularly sensitive to oxidative stress and this is due to several reasons. One reason is its high consumption of O_2 (the brain can metabolize up to $\sim 4 \times 10^{21}$ molecules of glucose per minute.) A second reason is the high production of ROS and RNS, which originate from specific neurochemical reactions (e.g., dopamine oxidation), in addition to the sources discussed previously. A third reason is the increasing deposition of metal ions in the brain with aging, catalyzing the production of increasing levels of ROS and RNS [2]. Another reason is the relatively high abundance of lipids within the CNS (i.e., myelin), which are particularly sensitive to oxidation. For instance, $HO_2^{\bullet}$ is particularly relevant for the *in vivo* lipid peroxidation and it acts via two different pathways: one that is lipid peroxides independent and the other one that is lipid peroxides dependent [4], leading to the formation of several secondary breakdown products including epoxides and saturated and unsaturated aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), cyclopentenones (i.e., cyclopentenone isoprostanes), and nitro-fatty acids (NO_2-FAs). Accumulated evidence indicates that oxidative stress plays a major role in the pathogenesis of several neurodegenerative diseases, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), multiple sclerosis (MS), and hereditary spastic paraplegia (HSP). High levels of ROS and RNS are consistently generated by infiltrating monocytes/macrophages and activated microglia and have been implicated as mediators of neurodegeneration and axonal damage typical of these disorders [16] (Figure 1). Mitochondrial dysfunction in these cells is likely to be one of the causes of such alteration of oxidative metabolism. Along with strong and persistent production of these reactive species, also associated with significant upregulation of their producing enzymes (myeloperoxidase, xanthine, and NADPH oxidases), increases in lipid and DNA oxidation products (i.e., OH8dG, 8-hydroxydeoxyguanosine) have been also reported. Free radicals can also activate certain transcription factors, like NF-κB, which upregulate the expression of many genes involved in neurodegenerative diseases, including proinflammatory cytokines and vascular adhesion molecules. Additionally, redox reactions are involved in the activity of matrix metalloproteinases (MMPs), which are important to cell trafficking into the CNS [17]. Interestingly, along with increased ROS and RNS, direct examination of brain tissues from patients affected by neurodegenerative diseases also revealed a weakened cellular antioxidant defense, especially due to impairment and/or decrease of relevant antioxidants such as superoxide dismutase, catalase, glutathione/glutathione peroxidase, α-tocopherol, and uric acid [18]. Indeed, lower levels of antioxidants may promote increased activity of lipoxygenase (that catalyzes one branch of the arachidonate cascade), thereby increasing the immunoinflammatory processes within the brain. In line with this, excessive ROS can stimulate T-cell activity through the arachidonate cascade or can produce direct/indirect damage to the blood brain barrier (BBB) or to neurons [19].

2. Alzheimer's Disease

Alzheimer's disease (AD) is probably the most common neurodegenerative disease, accounting for 60% to 70% of cases of dementia with nearly 44 million affected people worldwide, and although its etiology is still unclear, it is characterized by the presence of brain amyloid plaques and neurofibrillary tangles whose accumulation ultimately leads to extensive neuronal loss and progressive decline of cognitive function [2, 39, 40]. They are deposits of proteins distributed throughout the brain of AD patients, particularly in the entorhinal cortex, hippocampus, and temporal, frontal, and inferior parietal lobes. Amyloid plaques are primarily composed of aggregates of β-amyloid (Aβ), as well as other protein aggregates (e.g., hyperphosphorylated Tau, ubiquitin, and presenilins 1 and 2), whereas neurofibrillary tangles are aggregates of hyperphosphorylated Tau protein [41]. The production of ROS and its involvement in AD pathogenesis are supported by the significant amount of lipid peroxidation detected in the brain of AD patients, as well as by the increased levels of HNE found postmortem in their cerebrospinal fluid (CSF) [42]. Further, β-amyloid-induced damage promotes the generation of ROS, contributing to cell death and neurodegeneration, and induces also glial recruitment and activation, thus triggering local inflammation. Further, oxidative stress promotes abortive cell cycle reentry and hence apoptosis of nerve cells of the adult brain and gene duplication without cell division, leading to aneuploidy and DNA damage [39]. In addition, oxidative stress can damage DNA, leading to strand breaks and large deletions, and can affect various enzymatic and mitogenic pathways. Interestingly, oxidative stress has been shown to decrease neurogenesis in the adult brain,

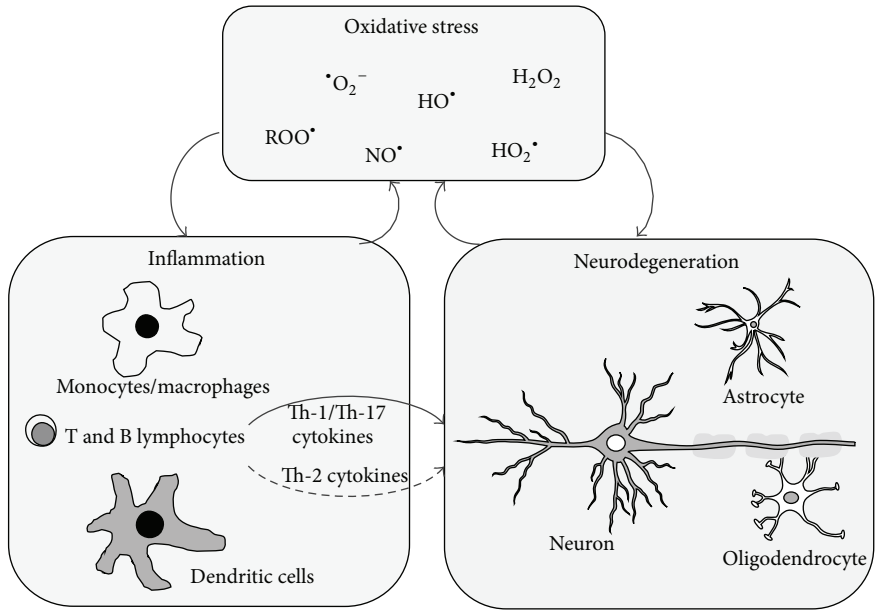

Figure 1: Cross talk between oxidative stress, inflammation, and neurodegeneration. The different reactive species are produced by several cell types, either by resident brain cells (i.e., glia) or by infiltrated leukocytes (i.e., monocytes/macrophages and dendritic cells), and affect both inflammatory processes, by enhancing cytokine release from proinflammatory T cells, and neurodegeneration, by inducing neuronal cell death and axonal loss. $^{\bullet}O_2^-$: superoxide radical anion; HO$^{\bullet}$: hydroxyl radical; ROO$^{\bullet}$: peroxyl radical; $HO_2^{\bullet}$: hydroperoxyl radical; H_2O_2: hydrogen peroxide; NO$^{\bullet}$: nitric oxide; Th: T-helper.

thus limiting its neurodegenerative capacity [43–45]. For the treatment of AD, the current therapy involves drugs that are only able to reduce symptoms or delay disease progression such as acetylcholinesterase inhibitors and those targeting the glutamatergic system. Concerning redox homeostasis, several antioxidant strategies are under study and aim not only at reducing the deleterious activities of ROS, but also at promoting the regenerative capacity of the adult brain [46] (Table 1). These drugs have been experimented in rodent models of AD and include garlic extracts, curcumin, melatonin, resveratrol, *Gingko biloba* extracts, green tea, and vitamin C. Although the clinical value of these antioxidants for the prevention of AD is often elusive, some of these compounds can be recommended based upon epidemiological evidence and already known benefits for prevention of other pathologies [47, 48]. Yet, further long-term studies can be recommended to better understand their mode of action. Vitamin E supplementation in moderately severe AD is to date the most promising approach, although its efficacy is fairly limited and does not apply to all AD patients. The beneficial effect of vitamin E is mainly exerted against peroxidation of membrane lipids of neurons. Several drugs with vitamin E are in use (e.g., *Sursum*, *Ephynal,* and *Rigentex*) and are orally administered twice a day. Yet, their therapeutic efficacy has not been thoroughly investigated and lack of data reveals the limitations of general antioxidant therapies, whereby they simply lower oxidative stress rather than interfering with the molecular mechanisms underlying disease pathogenesis [47]. In order to design more effective antioxidant therapies against AD, the multiple contributing factors that foster the clinical manifestations of this neurodegenerative disease should be unraveled, particularly in relation to their effects on adult neurogenesis and on synaptic communication. A more sophisticated redox approach involves the interaction between heavy metals and Aβ. For instance, ionic zinc and copper are able to accelerate the aggregation of Aβ and to promote its neurotoxic redox activity by induction of oxidative cross-linking of the peptide into stable oligomers [49]. Therefore, small molecules targeting these interactions are currently under clinical trials and hold promise as disease-modifying agents for AD. These novel drugs are referred to as "metal protein attenuating compounds" (MPAC) [50]. These are different from classical metal chelators, inasmuch as they bear a relatively low affinity for metals and are able to cross the BBB. Furthermore, MPAC stabilize metal homeostasis and interaction with proteins, rather than binding and eliminating metals from tissues. For instance, clioquinol is a zinc/copper ionophore that facilitates the clearance of Aβ aggregates in the cortex of animal models of AD. Its ionophoric properties liberate copper and zinc ions trapped within amyloid plaques, facilitating the reuptake of these essential metal ions into cells and hence promoting memory functions such as long-term potentiation [20]. Oral treatment with this MPAC has been shown to have striking effects in transgenic mouse models of AD, markedly improving learning and memory within days, paralleled by a significant reduction of Aβ content [2]. Other MPAC molecules are being tested in preclinical murine models of AD with the aim of assessing their effect on

Table 1: Current redox clinical investigations for the treatment of neurodegenerative diseases.

Redox therapy	Clinical application
Vitamin E	Alzheimer's disease [20, 21] Parkinson's disease [22, 23] Multiple sclerosis [24, 25]
Polyphenols	Parkinson's disease [22, 23] Multiple sclerosis [24, 25] Hereditary spastic paraplegia [ClinicalTrials.gov: NCT02314208]
Coenzyme Q10	Parkinson's disease [26–29] Multiple sclerosis
MPAC (i.e., clioquinol)	Alzheimer's disease [30–32] Parkinson's disease [33, 34]
ω-3 PUFAs	Multiple sclerosis [35, 36]
Metalloporphyrins	Amyotrophic lateral sclerosis [37]
Pramipexole (i.e., KNS-760704)	Amyotrophic lateral sclerosis [38]

MPAC: metal protein attenuating compounds; PUFAs: polyunsaturated fatty acids.

memory loss and evaluating therapeutic efficacy and toxicity *in vivo*.

3. Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting an estimated 10 million people worldwide, which produces muscular rigidity, bradykinesia, tremor of resting limbs, and loss of postural balance. The basic neuropathology of PD involves degeneration of pigmented neurons in substantia nigra, resulting in depletion of striatal dopamine (DA) and its metabolites. The pathological hallmarks of PD are large cytoplasmic inclusions called Lewy bodies, which occur predominantly in the melanin-containing neurons of substantia nigra pars compacta (SNpc), and contain aggregates of α-synuclein. Another gene encoding a protein termed parkin is involved in autosomal recessive Parkinsonism. Parkin is one member of the family of ubiquitin ligases and may be involved in normal turnover of α-synuclein. Although the exact cause of PD is still obscure, both environmental and genetic factors have been implicated in its pathogenesis [21]. Recent evidence points toward a putative role of mitochondrial dysfunction and oxidative stress as well as prooxidant environmental toxicants in the pathogenesis of PD [30, 31], as demonstrated in postmortem brains from PD patients. Apparently, there is a specific chemical fingerprint indicative of the damaging oxidative events, that is, higher levels of cholesterol hydroperoxide, MDA, HNE, and OH8dG. One of the suggested causes of oxidative stress in the SNpc is the production of ROS during normal DA metabolism. In human SNpc, the oxidation products of DA (mainly 6-hydroxydopamine) may polymerize to form neuromelanin, which may also be toxic by inducing apoptosis [32]. Furthermore, postmortem studies revealed reduced levels of GSH and increased levels of GSSG in the SNpc. This could be a critical primary event that weakens or abrogates the natural antioxidant defense of the cell, thereby triggering degeneration of the nigral neurons and causing PD [51]. Since dysregulation of metal ion homeostasis is a potential catalyst to further production of reactive species, the highly oxidative environment for DA interaction with α-synuclein, and the resulting oxidant-mediated toxicity and protein aggregation, is one of the most likely underlying mechanisms for PD. Thus, the destruction of neuronal cells occurs as a result of self-propagating reactions that involve DA, α-synuclein, and redox-active metals [52]. As for AD, also for PD no cures are available yet. However, pharmacological treatment and surgery could help with symptom relief. The most commonly used drugs to treat motor symptoms are *L*-DOPA, a precursor of dopamine, which is usually used in combination with a DOPA decarboxylase inhibitor and a catechol-*O*-methyltransferase inhibitor; dopamine agonists; and monoamine oxidase inhibitors. These enzymes are all involved in the chemical inactivation of several neurotransmitters, including dopamine. In the early stages of the disease, the treatment aims at controlling both symptoms and side effects caused by dopaminergic enhanced activity, but when the disease gets more severe surgery can be useful. However, in the last stages of PD, palliative care seems to be the only alternative to improve the quality of life [53]. Over the last decade, neuroprotective approaches for PD have been tried with the aim of slowing the rate of disease progression by decreasing oxidative stress (Table 1). There has been much interest in the use of supplemental vitamin E, which seems to inhibit cell death of neuronal cells of SNpc. Regular consumption of vitamin E-rich foods may have the potential to decrease the risk or delay the onset of PD. Even β-carotene seems to reduce the risk of PD onset, although no studies to prove its efficacy are available yet. On the other hand, intake of vitamin C and flavonoids did not show any significant beneficial effect in either prevention or treatment of PD. Overall, high intake of dietary antioxidant supplements (mainly vitamin E) might protect against the occurrence of PD rather than treating its symptoms [54, 55]. The use of melatonin and α-lipoic acid has also been investigated for PD treatment, but their effects, though promising, have not been fully characterized [56]. The antioxidant with the most efficacious therapeutic potential is coenzyme Q10. Indeed, several clinical trials based on coenzyme Q10 have been undertaken, showing significant beneficial effects on motor functions [22, 23, 57]. Currently, a phase III clinical trial is ongoing and is based on combinations of coenzyme Q10 and vitamin E or creatine, with the aim of evaluating the effective dosage according to the disease stage. Initial results seem to document additive neuroprotective effects in terms of significant reduction of DA depletion in the striatum and loss of tyrosine hydroxylase neurons in the SNpc, as well as reduction in lipid peroxidation and pathologic accumulation of α-synuclein in the same SNpc neurons [26]. Further, a double blind phase I/IIa clinical study proved the safety and tolerability of intranasal GSH administration to early and untreated patients, although pharmacokinetic and dose-finding investigations still need to be verified [27]. As already

described for AD, another potential therapeutic strategy lies in the modulation of heavy metals, whereby the control of their bioavailability could prevent not only the increase of oxidative stress through metalloredox reactions, but also their interaction with other proteins like α-synuclein. Thus, MPAC have been also tested in PD [28] and clioquinol has been reported to reduce cell death of substantia nigra neurons by 50% [29]. Of note, the inhibition of monoamine oxidase isoforms reduces the formation of dopamine-derived peroxides and the subsequent generation of reactive species and of the overall oxidative stress of substantia nigra.

4. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of both upper and lower motor neurons of the brain, brain stem, and spinal cord, overall leading to progressive weakness and atrophy of skeletal muscles [2, 33]. Approximately 10% of the cases are inherited in an autosomal dominant manner, and 1/5 of these familial ALS patients carries mutations in the Cu/Zn-SOD (SOD-1) gene, suggesting involvement of ROS in disease pathogenesis [34]. The toxicity of mutant SOD-1 seems to be due to gain of function of this enzyme, whereby its catalytic activity is enhanced with abnormal substrates like $ONOO^-$, thus sustaining nitration of tyrosine and subsequent oxidative stress. This may also be related to impaired ability of mutant SOD-1 to bind zinc, because *in vivo* the mutant enzyme is likely to denature more quickly than the normal form, releasing zinc and copper ions. Oxidative stress may also be involved in misfolding of mutant SOD-1, to yield abnormal protein aggregates that can be found as early as in 1-month-old SOD-1-mutant mice [58]. Also, the disorganization of intermediate filaments could be due to mutant SOD-1-induced toxicity, as these cytoskeletal proteins are vulnerable to oxidative damage [59]. In addition, protein carbonyl and nitrotyrosine modifications, which are indexes of protein oxidation, were found to be elevated in the majority of patients with sporadic ALS, suggesting that oxidative stress may indeed be involved in all types of ALS. Other mechanisms that have been implied in ALS, such as excitotoxicity and defective axonal transport, may be consequences of oxidative stress [60]. What remains as yet unclear is whether this increased redox stress is a primary defect or a secondary consequence of the disease. An interesting study has demonstrated that SOD-1-mutant ALS transgenic mice activate cellular Nox2 activity and subsequent $^{\bullet}O_2^-$ production in spinal cord microglia, through disruption of the redox-sensitive regulation of Rac1-dependent Nox activation [61]. Of note, Nox enzymes control relevant proinflammatory signaling pathways involved in the progression of ALS, such as those mediated by IL-1β and TNF-α via redox-dependent activation of NF-κB. Finally, marked elevation of 2-thiobarbituric reactive substances in plasma (i.e., MDA) was found both in mutant SOD-1 mice and in patients with sporadic ALS [62, 63]. However, plasma concentrations of antioxidants like α-tocopherol, β-carotene, ubiquinol-10, and GSH, as well as SOD activity in red blood cells, were not significantly different between ALS patients and healthy subjects [63]. Even though we still lack a definitive cure for ALS, the Food and Drug Administration (FDA) has already approved the first molecule to treat the disease, that is, riluzole. This drug is thought to reduce the motoneuron-associated damage by affecting the release of glutamate. Treatment with riluzole of ALS patients in clinical trials only elicited a three-month improvement of survival rate, with the subjects needing constant monitoring of liver damage and other side effects [64]. Nonetheless, this first attempt of a specifically aimed therapy nurtures the hope that the clinical course of ALS might be managed by new kinds of treatments or combinations of new drugs. Moreover, several antioxidant molecules have been tested as putative therapeutic agents in the treatment of ALS (Table 1). Such compounds include NAC and N-acetylmethionine (NAM), vitamins C and E, resveratrol, dithiotreitol (or any of its isomers), and dithioeritrol. However, although such antioxidant agents never caused noxious effects, all of them failed in eliciting any kind of significant effect on patients' survival rate [65]. To date, two antioxidant compounds have been used in clinical trials. The first one is manganese-metalloporphyrin, which was able to extend survival rate in murine models [66]. Metalloporphyrins are compounds made by a tetrapyrrole ring that coordinates a central metal atom. Two phase I trials, conducted to assess possible drug-associated toxicity, showed very good tolerance of this compound even at fairly high doses (up to 2 mg/kg/day), with ALS patients showing an excellent pharmacokinetic profile. The second antioxidant drug under investigation is KNS-760704, a pramipexole enantiomer, usually used for treatment of PD patients, which acts as a dopamine receptor agonist [67]. This compound possesses ROS-scavenging activity and has been proven to extend the lifespan of ALS animal models. Moreover, a recent phase II clinical trial also reported that KNS-76074 is able to exert protective action against oxidative stress-associated neurotoxic damage, which led this compound to be tested in phase III trials in order to evaluate its efficacy and tolerability. New therapeutic approaches are currently being developed with the aim of blocking the production of SOD-1 mutated forms, a strategy that could likely ameliorate the clinical course of patients affected by the familial form of ALS. Curiously, a recent study pointed out that the mutant SOD-1 transgenic mice model of ALS is not representative of the human sporadic form, accounting for most failures in experimental or clinical research.

5. Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory, progressive, and degenerative disorder of autoimmune origin characterized by intermittent episodes of demyelination and axonal loss or damage in the CNS. Although its etiology has not been fully elucidated yet, it is likely that both genetic and environmental components play a crucial role in disease onset and progression, and it is now well recognized that immunological mechanisms are the initial trigger [2, 16, 37]. MS is classified into four independent subtypes or forms: relapsing-remitting (RR), primary progressive (PP), secondary progressive (SP), and progressive relapsing (PR); the former is the most prevalent form and accounts for

approximately 85% of all cases [38]. Interestingly, its pathogenesis and pathophysiology have been extensively studied, especially on experimental autoimmune encephalomyelitis (EAE) mouse model, and are thought to be due to disruption either of the immune system or of the myelin-producing cells. As a matter of fact, the hallmarks of MS are inflammation and neurodegeneration, where, upon damage of the BBB, massive infiltration of highly proinflammatory and autoreactive leukocytes occurs (especially T-helper 1 and T-helper 17 cells), causing demyelination as well as oligodendrocyte death, axon damage, and even neuronal loss [68]. These autoimmune processes are paralleled by continuous activation of resident macrophages/microglia that potentiate the inflammatory response by producing proinflammatory cytokines and chemokines, as well as reactive oxidants [69]. The autoimmune and inflammatory hypothesis dominated the MS research field for almost 50 years until the early 2000s; however, whether inflammatory demyelination is primary or secondary in the disease process is yet unclear. Indeed, over the last decade, the concept of a neurodegenerative and microglia-centered view has been gaining increasing attention. In this view, MS might be primarily a neurodegenerative disease with secondary inflammatory demyelination, whose trigger starts in the brain and the progress of which is modified and amplified by inflammation [70, 71]. Accumulated evidence indicates that oxidative stress plays a major role in the pathogenesis of MS. ROS and RNS are mainly generated in excess by activated microglia and have been implicated as mediators of demyelination and axonal damage typical of MS [72]. In addition, free radicals can activate certain transcription factors, like NF-κB, which upregulate the expression of many genes involved in human MS and EAE, including TNF-a, iNOS, ICAM-1, and VCAM-1 [73]. Additionally, redox reactions are involved in the activity of matrix metalloproteinases (MMPs), which are important to T-cell trafficking into the CNS [74]. Several studies have found evidence of lipid peroxidation in the CSF and plasma of MS patients, with higher concentration of isoprostanes and MDA. Further, a weakened cellular antioxidant defense, especially due to impairment of SOD, GR, and GPx, as well as elevated levels of GSSG and reduced vitamin E : lipid ratio, was found in red blood cells of these subjects [75]. Moreover, direct examination of MS plaques revealed an increase in free radical activity and decreased levels of relevant antioxidants like GSH, a-tocopherol, and uric acid [18]. Furthermore, activated mononuclear cells of MS patients produce high amounts of ROS and $NO^{\bullet}$, and oxidative damage to DNA (mitochondrial DNA included) develops in association with inflammation in chronic active plaques [76]. Another study documented that oxidative damage of CNS was provoked by the release of iron from injured cells and by low levels of enzymatic and nonenzymatic antioxidants (particularly ubiquinone and vitamin E) in plasma and lymphocytes of MS patients [77]. The CNS damage induced by low levels of antioxidants or high levels of ROS might be caused by the fact that lower levels of antioxidants may promote increased activity of lipoxygenase (that catalyzes one branch of the arachidonate cascade) [78], thereby increasing the immunoinflammatory processes within the brain, and by the evidence that excessive ROS can stimulate T-cell activity via the arachidonate cascade, or they can produce direct/indirect damage to the BBB or to myelin [79]. As yet, a final treatment for MS has not been found, and no therapy has been developed to date for its progressive forms, even though RR-MS can count on several disease-modifying treatments (DMTs). Besides methylprednisolone, a corticosteroid used immediately after the diagnosis of MS and before any other more specific therapeutic approaches, FDA has approved seven other DMTs which are already in commerce: Interferon-β-1a (IFN-β-1a), IFN-β-1b, Glatiramer acetate, Mitoxantrone, Teriflunomide, Fingolimod, and Natalizumab [16]. Such drugs are all immunomodulatory and aim at halting the pathological immune responses by directly inhibiting cell activation and the release of proinflammatory mediators or by limiting cell transmigration into the CNS; yet they still do not represent a definitive solution to the problem, especially for the 15–20% of MS patients affected by the progressive forms of MS. Given the involvement of ROS and RNS in MS pathogenesis, it is possible that antioxidant compounds could play a pivotal role in the prevention of the free radical-mediated tissue damage as well as inhibiting the early proinflammatory events, such as T-cell activation and CNS infiltration, which would ultimately lead to brain inflammation and neuronal death [16]. Treatment with antioxidant could theoretically prevent the spreading of tissue damage promoting cellular survival, thus ameliorating the disease outcome (Table 1). In this context, the antioxidant compound tirilazad mesylate, a member of lazaroid family known for its peroxyl-radical-scavenger properties and for its ability to reduce iron-catalysed lipid peroxidation, has been proven useful in preventing the onset of acute EAE (the animal model of MS), as well as reducing its severity. Another study showed that the administration of NAC, a molecule particularly efficient in boosting intracellular levels of GSH, is able to hinder the induction of acute EAE [80]. On the other hand, Euk-8, a synthetic salen-manganese complex, could emerge as a key compound in the development of a brand new class of molecules possessing scavenging properties along with superoxide dismutase and catalase abilities. As a matter of fact, repeated injections of Euk-8 upon encephalomyelitis induction in mice were able to delay the onset of EAE symptomatic phase as well as reduce the severity of the clinical phenotype, giving hope to the hypothesis of its possible application in human MS [81]. α-Lipoic acid, an antioxidant molecule capable of crossing the BBB, has also been recently reported to suppress inflammation, demyelination, and axonal damage in EAE mice. Its effects are mediated by the reduction of T-cell traffic in the spinal cord, possibly through the inhibition of MMPs activity. Uric acid too has been proven to affect EAE clinical outcome, especially ameliorating neurologic deficits in treated mice, in a mechanism involving its ability to inhibit iNOS along with $NO^{\bullet}$ and $ONOO^{-}$ scavenging properties [82]. However, other authors reported that inhibition of $NO^{\bullet}$ production is instead deleterious in EAE, leaving a wide-open debate on the true efficacy of this compound. Some other encouraging data obtained from EAE mice led the scientific community to theorize that the dietary income of antioxidant, such as vitamin

E or selenium, could somehow hinder the progression of MS. Of note, despite the relative abundance of reports describing the ameliorative effects of restrictive dietary regimens on the clinical outcome of affected patients, we still lack a true and solid body of evidence supporting the actual action of antioxidant in slowing the progression of MS [83]. Natural occurring molecules currently being investigated in phase I and II trials include polyphenols (especially *Gingko biloba* extracts), essential fatty acids/α-lipoic acid, and vitamin E/selenium [84]. The results of these promising studies will lay the foundation for phase III trials, which will be pivotal in establishing the long-term efficacy of antioxidant therapies on MS. An ongoing phase I-II trial is recruiting patients in order to assay the effects of idebenone, a synthetic compound chemically related to coenzyme Q10. Currently gathered data suggest that idebenone could be able to stop demyelination and neuronal death. Another study is also investigating the efficacy of ω-3 PUFAs included in fish oil such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as anti-inflammatory, antioxidant, and neuroprotective agents [24]. MS patients treated with fish oil (4 g/die) showed a significant reduction of the levels of proinflammatory cytokines and $NO^{\bullet}$ catabolites, but no variation in the serum levels of lipoperoxides or the number of relapses per year [25]. The results coming from such studies are laying the foundations for a phase III clinical trial that should give information about the long-term efficacy of this strategy on the number of relapses. The use of antioxidants, even in combination with conventional immunomodulatory therapies, could have synergistic effects on the disease, resulting in a more powerful therapy. Indeed, in order to evaluate the real benefit of antioxidant therapies on MS patients, adequately designed clinical studies will be needed in conjunction with observational investigations that will evaluate on sufficiently large cohorts of patients the long-term effectiveness of this potential treatment. The pivotal role of oxidative stress in MS pathogenesis and the idea that a therapeutic strategy could reside in the control of such a phenomenon is highly supported by the recent approval of the first redox-modulating drug in the treatment of multiple sclerosis. This compound, dimethyl fumarate (DMF), which is a methyl ester of fumaric acid, is the only oral administration DMT to be approved by both FDA and the European Medicines Agency (EMA), with the trade name Tecfidera. This compound was initially used in the treatment of psoriasis, though administered as a different preparation; however, it was later proposed as therapeutic drug for MS due to the immunopathogenic features these two diseases share. Right after administration, the small intestine esterases readily hydrolyze DMF into mono-methyl fumarate (MMF), which is more stable and possesses a 12-hour *in vivo* half-life, higher than its precursor. The mechanism of action of DMF is based on its ability to interfere with the redox-regulating cellular systems and the consequent modulation of intracellular thiols, which in turn boosts GSH levels. In detail, DMF interacts with Kelch-like erythroid cell-derived protein with cap "n" collar homology-associated protein 1 Keap-1 at the level of its critical cysteine residue Cys151 which is covalently adducted, leading to cleavage of this protein and the subsequent translocation and activation of Nrf-2, which in turn triggers several cellular antioxidant pathways, resulting in anti-inflammatory and neuroprotective responses [35]. The detailed action is based on the Nrf-2-induced expression of proteins that regulate intracellular antioxidant systems such as NQO1, heme-oxygenase-1 (HO-1), glutathione S-transferase Mu-1 (GSTM1), Prx1, and Trx, along with a vast number of heat shock proteins (HSPs), thus promoting immune cell survival even in the presence of high ROS and RNS concentrations [36]. Thus, Nrf-2 orchestrates a complex machinery that protects neurons and glial cells against the oxidative stress-induced cell damage. Moreover, a great deal of evidence suggests that DMF is also able to modulate immune responses either through the Nrf-2-dependent inhibition of the redox signals governed by NF-κB and/or skewing Th1/Th2 balance towards Th2 by directly inducing T-cell apoptosis, without however resulting in immunosuppression. DMF has been also shown to protect neural stem/progenitor cells and neurons from oxidative damage through Nrf2-ERK1/2 MAPK pathway [85, 86]. As a consequence, BG-12, a specific oral preparation of DMF, has already been showed in two phase III trials as being able to reduce relapse episodes as well as to delay disease progression in patients affected by RR-MS [87–89]. In these trials, BG-12 was well tolerated, the most common side effects being characterized by redness, gastrointestinal symptoms, and headaches. Another phase III clinic trial is currently investigating BG-12 long-term safety profile with the main aim of using it in the future as a first-line DMT.

6. Hereditary Spastic Paraplegia

Hereditary spastic paraplegia (HSP) includes a large and diverse group of genetic disorders whose main feature is progressive spasticity and weakness in the lower limbs, as a result of continuous distal axonopathy caused by defects in the mechanisms that transport proteins and substances along the axons [90, 91]. At least four autosomal dominant HSPs are caused by mutations in genes encoding proteins that are involved in ER morphogenesis and that bear an intramembrane hairpin loop responsible for the curvature of ER membranes and for their reciprocal interactions. These include spastin, the most commonly mutated protein in HSP, atlastin-1, REEP1, and RTN2 [91–93]. To date, 72 different spastic gait disease loci have been identified, and 55 spastic paraplegia genes (SPGs) have already been cloned, most of which play a role in intracellular trafficking [94]. The products of these genes are all implicated in disease onset of many forms of HSP and can be grouped into "functional modules" in which they are part of specific molecular pathways or perform similar functions, including dysfunctional axonal transport, axon development, dysregulation of myelination, and abnormal cellular signaling in protein morphogenesis. Oxidative stress is, in fact, one of these functional modules inasmuch as many of these genes affect mitochondrial function and thus determine increase in ROS and RNS production, [95]. Indeed, several reports suggest that oxidative stress could be strictly involved in the pathogenesis of many forms of HSP. In this context, one of the most studied genes is paraplegin (SPG7), a mitochondrial metalloprotease belonging to the family of

ATPases associated with diverse cellular activities (AAA) [96, 97], whose mutations result in mitochondrial dysfunction of muscle tissue and mitochondrial-dependent impairment of axonal transport [98, 99]. HSP fibroblasts of patients affected by SPG7 show reduced complex I activity in mitochondria and an increased sensitivity to oxidative stress [100]. Such dysfunction could directly contribute to neurodegeneration via free radical mechanism by direct ROS production and by a decreased ATP synthesis leading to energy failure. Of note, HSP cells are more sensitive to DNA damage induced by H_2O_2 treatment [101], also because abnormal DNA repair is another functional module associated with HSP. Also another form of HSP, that is, SPG13, is caused by mutations in the HSPD1 gene that encodes heat shock protein 60, which is crucial for the folding of several mitochondrial proteins, once again affecting mitochondrial function [102]. Further, abnormal lipid metabolism is another key functional module and is also associated with oxidative stress. For instance, mutations in *DDHD1* and *CYP2U1* genes, which code for two enzymes involved in fatty-acid metabolism, cause alteration of mitochondrial architecture and bioenergetics with increased oxidative stress, accounting for lipid metabolism as a critical HSP pathway with a deleterious impact on mitochondrial bioenergetic function [103]. Overall, these lines of evidence suggest that oxidative stress is likely a crucial biomarker and a novel pathogenic mechanism for these neurodegenerative disorders. No specific treatments are yet available to prevent, slow, or reverse HSP. The available therapies mainly deal with management of symptoms and physical and emotional promotion and include drugs for muscle tone and spasms as well as antidepressants for patients experiencing clinical depression. Physical therapy is also used to restore and maintain the ability to move. The probable implication of mitochondrial dysfunction and oxidative stress in the pathophysiology of HSP set the basis for the development of novel therapeutic strategies focused on organelle dynamics and bioenergetics, as well as ROS scavenging. To date, only one clinical trial is investigating the therapeutic potential of targeting oxidative stress in HSP and is investigating the efficacy of resveratrol in order to decrease the production of oxysterols by reducing the synthesis of cholesterol and/or regulating the production of bile acids and/or enabling neuroprotective action within the motor neuron [ClinicalTrials.gov: NCT02314208]. Of note, one of the challenges to accurately define the HSP pathways and to design efficacious therapies is that many genes have multiple functions and are involved in more than one pathway.

7. Conclusions

Due to the harmful role of ROS and RNS in the pathology of neurodegenerative diseases, antioxidants seem to limit the tissue damage induced by these reactive species through inhibition of early or late proinflammatory events or exerting neuroprotective properties. Accordingly, several lines of research considered nutritional interventions with vitamins, micronutrients, and antioxidants to complement conventional treatments; yet only few of them were translated into clinical practice. This is due to multiple reasons, including the large number of reactive species and the multiple routes for their metabolism that simultaneously occur and that interact with each other, the overlapping of common signaling redox pathways in health and disease, and the scarce knowledge of their mechanistic insights *in vivo*. Furthermore, it must be considered that the main problem regarding the treatment of neurodegenerative diseases is embodied by the necessity of developing compounds that are able to cross the BBB, that is, the main obstacle between the CNS milieu and the peripheral bloodstream. The BBB is able to reduce the efficacy of antioxidant drugs as well as several other compounds, which may exert therapeutic action. Consequently, future development of antioxidant therapeutics will undoubtedly depend on a wider knowledge of the BBB-associated transport mechanisms. Yet, targeting specific oxidative stress pathways, rather than the use of dietary antioxidants, seems to represent a novel avenue of research in the management of neurodegenerative diseases, especially after the recent approval of dimethyl fumarate by both the FDA and EMA which acts by enhancing the antioxidant responses, ultimately promoting cytoprotection of neurons and glial cells.

Conflict of Interests

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

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Research Article

Evidence for Detrimental Cross Interactions between Reactive Oxygen and Nitrogen Species in Leber's Hereditary Optic Neuropathy Cells

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Here we have collected evidence suggesting that chronic changes in the NO homeostasis and the rise of reactive oxygen species bioavailability can contribute to cell dysfunction in Leber's hereditary optic neuropathy (LHON) patients. We report that peripheral blood mononuclear cells (PBMCs), derived from a female LHON patient with bilateral reduced vision and carrying the pathogenic mutation 11778/ND4, display increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), as revealed by flow cytometry, fluorometric measurements of nitrite/nitrate, and 3-nitrotyrosine immunodetection. Moreover, viability assays with the tetrazolium dye MTT showed that lymphoblasts from the same patient are more sensitive to prolonged NO exposure, leading to cell death. Taken together these findings suggest that oxidative and nitrosative stress cooperatively play an important role in driving LHON pathology when excess NO remains available over time in the cell environment.

1. Introduction

Leber's hereditary optic neuropathy (LHON) is a mitochondrial disorder leading to severe visual impairment, due to retinal ganglion cells (RGCs) death and atrophy with demyelination of the optic nerve [1]. The primary cause of the disease is a mitochondrial genome (mtDNA) mutation leading to a single amino acid substitution in one of the mitochondrially encoded subunits of NADH:ubiquinone oxidoreductase, complex I of the electron transport chain (ETC). The most common mutations are at positions 11778/ND4, 3460/ND1, and 14484/ND6 [2, 3].

LHON pathology generally occurs in the second or third decade of life and affects predominantly males [4]. The only clinically relevant phenotype in most of the patients is RGCs loss, seldom accompanied by other complicating neurological disorders, such as dystonia, multiple sclerosis- (MS-) like illness parkinsonism, cerebellar ataxia, and myoclonus [5], pointing to a diffused mitochondrial energetic failure. Interestingly, a higher risk of developing MS in women with clinically established LHON has been reported [6–8]. Rarely other phenotypes have been described, such as chronic renal failure [9, 10], involving as common feature tissues/cells that are exquisitely energy dependent and require adequate supply of reducing substrates and O_2 to sustain mitochondrial adenosine-5′-triphosphate (ATP) production.

All LHON mutations induce an impairment of mitochondrial function. A decline in complex I-sustained cell

respiration and ATP production has been reported in assays performed on isolated mitochondria derived from muscle, Epstein-Barr Virus- (EBV-) transformed leukocytes, peripheral blood mononuclear cells (PBMCs), and cybrids [11–15]. Based on these specific defects, experiments carried out on patient's tissues and cell models of the disease showed an overproduction of reactive oxygen species (ROS) and an increased propensity to apoptotic cell death [16]. Nevertheless, LHON cells seem to be able to cope with the ETC dysfunction, maintaining apparently a normal growth and total cellular ATP level, yet being more vulnerable to metabolic/oxidative stress or other stressful conditions [2, 17].

Consistently, a recently developed mouse model revealed, together with some of the key histopathological features typically observed in LHON patients, the decrease of complex I activity, respiratory defects, and the increase in ROS levels, but no reduction in ATP synthesis, pointing to oxidative stress as the major driver of the pathology [18]. On these bases, most of therapeutic approaches to LHON, and generally to mitochondrial disorders, currently rely on the use of mitochondrial substrates, together with redox active effectors and free radical scavengers. Idebenone, curcumin, and vitamins C and E as well as other antioxidant compounds have been used separately or combined in "cocktails" customized for the individual patients [19, 20], all treatments unfortunately with limited success.

Under severe pathological conditions, such as inflammation or sepsis, a disruption of the homeostatic control of oxygen supply and utilization has been observed, accompanied by ROS formation, together with an imbalance of nitric oxide (NO) production and breakdown. Nitric oxide produced either enzymatically by nitric oxide synthases (NOSs) [21] or directly via the reduction of bulk nitrite at low pH [22] is a fundamental second messenger involved in a number of pathophysiological processes [23], inducing detrimental or cytoprotective effects depending on its concentration and localization [24]. Importantly, NO regulates mitochondrial respiration by reversibly binding to cytochrome *c* oxidase and limiting O_2 consumption, while extending O_2 gradients in tissues [25, 26]. Such inhibition is more effective in actively respiring cells and at low oxygen concentration [27–29]. Under conditions associated with increased ROS levels, NO participates in reactions with other reactive species to generate secondary products that can impair mitochondrial function. Particularly, the reaction of NO and superoxide anion ($O_2^{-\bullet}$), leading to the formation of toxic peroxynitrite ($ONOO^-$), is very rapid (diffusion limited) and known to induce macromolecular damage, including nitration and inactivation of mitochondrial proteins [30].

Although many biochemical aspects of LHON have been elucidated, the role of NO in the LHON disease has not been investigated yet. Interestingly, an increased immunoreactivity for inducible nitric oxide synthase (iNOS) has been detected in macrophages and in the microglia of demyelinated lesions in the brain white matter of a LHON female patient, suggesting an early immunological mechanism in addition to the primary degeneration of the optic nerve [31]. In this patient, administration of corticosteroids improved visual and neurological function. This observation suggests that increased NO levels, as those produced by iNOS, in combination with ROS overproduction can result into mitochondrial defects, eventually triggering severe cell dysfunctions.

Here, we tested this hypothesis on PBMCs and lymphoblasts derived from a female LHON patient with bilateral reduced vision and immunological disorders. We found that the cells, although carrying the pathogenic mutation 11778/ND4, are still endowed with a suitable bioenergetic apparatus, producing ATP, thus well compensating complex I mutation. However, lymphoblasts proved to be more susceptible to NO toxicity, suggesting that the mitochondrial genetic defect, via the enhancement of the basal ROS levels, potentiates the formation of secondary reactive nitrogen species (RNS), particularly $ONOO^-$, leading to reduced cell viability.

2. Materials and Methods

2.1. Chemicals. RPMI-1640 medium, fetal bovine serum (FBS), and nonessential amino acid solution were from Sigma-Aldrich and Invitrogen Life Technologies (GIBCO). Thiazolyl blue tetrazolium bromide (MTT) was from Sigma-Aldrich and (Z)-1-[-2-(aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate) was purchased from Cayman Chemicals.

2.2. Case Report. The proband is 34-year-old woman with a previous diagnosis of autoimmune thyroiditis. When she was 28 years old, she suffered a sudden and painful loss of vision in OS and after two weeks in OD, accompanied by xerophthalmia. Visual field showed retinal sensibility reduction, especially at superior areas, whereas fluoroangiography was normal. She was treated with high doses of corticosteroids and intravenous immunoglobulins without benefits. Brain MRI scan was unremarkable. After 4 months, plasmapheresis was performed with little improvement, so it was decided to start cyclophosphamide treatment. This treatment was carried on for 6 months without any benefit. In the meantime, after a femur fracture due to an accidental fall, a severe osteoporosis was diagnosed. After one year from the visual loss, a workout for autoimmune disorders gave positive ANA (speckled pattern, 1 : 160) and the presence of anti-Ro/SSA. Cytochemical, bacteriological, viral, and immunoelectrophoretic analyses on cerebrospinal fluid were normal.

Brainstem auditory evoked responses, along with motor and somatosensory evoked potentials, were normal, while visual evoked potentials could not be performed due to bilateral visual loss. A new brain and orbital and spinal cord MRI scan showed bilateral callosal, periventricular, and paratrigonal white matter symmetric hyperintensities on T2-weighted images without contrast enhancement on T1. No alterations were found at the level of the spinal cord and optic nerves. On admission to our neurological division, bilateral visual acuity was 1/10. We excluded neoplastic or paraneoplastic processes by full-body CT scan and screening for antibodies associated with paraneoplastic syndromes. The infectious aetiology was also discarded after negative hepatitis B and hepatitis C virus, HIV, EBV, cytomegalovirus

serology, and tuberculin skin test. Neuromyelitis optica, carential, metabolic, and endocrinological disorders were also excluded. The standard genetic screening for LHON disease showed the presence of 11778/ND4 mutation. She began ubidecarenone therapy (600 mg/day) without any subjective or measurable improvement of visual acuity. After two months she developed *Escherichia coli* pyelonephritis and chronic tubulointerstitial nephritis was diagnosed. After one year and a half from the visual loss, the patient presented a bilateral visual acuity worsening with a severe headache and hypoesthesia at four limbs. A brain MRI showed a massive extension of bilateral paratrigonal white matter lesions with an involvement of bilateral occipital subcortical white matter and increased signal intensity on T2-weighted images of optical radiations and retrochiasmatic optic tracts. After one month, brain MRI with contrast and diffusion weighted imaging was repeated showing an additional enlargement of bilateral occipital lesions with positivity in DWI sequence and a circled enhancement. Therefore she was admitted again to our division and a hemogasanalysis proved a severe metabolic acidosis (pH 7.14, pCO_2 = 13 mmHg, pO_2 = 145 mmHg, sO_2 = 98.5%, HCO_3^- = 4.40 mmol/L, BE = −22.40 mmol/L, and BE_{ecf} = −24.60). Blood lactate measurements showed an abnormal increase during exercise (at rest 13.69 mg/dL; after 5-minute exercise 48.13 mg/dL; after 10-minute exercise 65.53 mg/dL; after 15-minute exercise 85.57 mg/dL; 15 minutes after the end of exercise 59.28 mg/dL; and normal value 4.5–19.8). Blood analysis showed a renal injury associated with nephrocalcinosis diagnosed by ultrasonography and CT scan. During the hospitalization the medical care focused on the improvement of electrolyte balance and renal injury until values normalization. Simultaneously, by brain MRI scan, we appreciated a clear reduction of white matter lesions and contrast enhancement absence.

2.3. Peripheral Blood Mononuclear Cells (PBMCs) Isolation. Blood samples were obtained from the case report and from three matched (age, sex, and geographical origin) healthy controls. The local Institutional Review Board approved the study and all participating subjects gave written informed consent.

PBMCs were obtained by density centrifugation over Ficoll-Hypaque according to standard procedures. Genomic DNA was extracted from PBMCs using a commercial kit (QIAamp DNA Mini Kit, Qiagen). Purified PBMCs (5×10^4 cells/well) were seeded in 96-flat well plates and cultured in 200 μL/well RPMI-1640 medium, supplemented with 20% FBS (HyClone), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.4. Preparation and Cultures of Lymphoblast Cell Lines. Lymphoblast cell lines were established from peripheral blood PBMCs of the patient and the three healthy controls by EBV infection. The mutational analysis of the patient mtDNA was performed by PCR, restriction analysis, and electrophoresis and confirmed by sequencing (Sanger method). Both PBMCs and lymphoblast cell lines derived from the LHON patient and controls were grown in RPMI-1640 medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1X nonessential amino acids. Cells were cultured in 25 cm^2 or 75 cm^2 flasks or in multiwell plates and incubated at 37°C under standard conditions [5% CO_2; 95% relative humidity]. Viable-cell counting was carried out using the Trypan blue dye exclusion test (Sigma-Aldrich).

2.5. Cell Respiration. Oxygen consumption in intact PBMCs derived from the LHON patient and controls was measured using a high resolution respirometer (2k-Oxygraph, OROBOROS Instruments). Data acquisition and analysis were carried out using the software Datalab (OROBOROS Instruments). Control and LHON PBMCs were collected, washed with sterile phosphate buffered saline (PBS), counted, and suspended in DMEM containing 1 g/L glucose. Cells, at a final density ranging from 7 to 9×10^6 cells/mL, were incubated in the 2k-Oxygraph chambers at 37°C for 30 min to allow temperature and pH equilibration. Respiration was evaluated under basal metabolic conditions, thus sustained by endogenous substrates. After recording an oxygen consumption rate (OCR) baseline, 4 μM antimycin A (AA) was added to the chamber in order to inhibit cytochrome *c* reductase (complex III) and stop mitochondrial respiration. Data were recorded at sampling intervals of 2 s.

2.6. Determination of ATP Levels. Cellular concentration of ATP was measured by chemiluminescence, under stationary conditions. Cells were incubated overnight in antibiotic/FBS-free DMEM medium. Steady-state ATP levels were measured in LHON and control PBMCs (3×10^5 cells/mL) 4 h after resuspending the cells in PBS containing L-glutamine (2 mM), in the presence of glucose (2 g/L) or its absence to stimulate OXPHOS; when necessary, oligomycin (2.5 μg/mL) was added over the last 1.5 h of incubation. Synthesized ATP was measured using the ATPlite 1step kit (PerkinElmer) in a luminometer (VICTOR Multilabel Counter, PerkinElmer, USA) equipped with 96-well plates.

2.7. Reactive Oxygen Species (ROS) Quantification by Flow Cytometry. To measure the intracellular ROS concentration, the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich) was used. PBMCs from the LHON patient and controls were incubated for 30 min at 37°C in the dark in RPMI-1640 medium containing 10 μM DCFDA. Afterwards, cells were washed twice with Hank's Buffered Salt Solution (HBSS) supplemented with calcium and magnesium, as suggested by the manufacturer, collected, and analysed immediately by flow cytometry (BD Accuri C6); ROS levels were estimated from the mean fluorescence intensity. The green fluorescence was measured using the FL-1 setting (log mode) after having gated out cell debris. In each experiment 10.000 events were recorded.

2.8. 3-Nitrotyrosine Level Detection. The content in 3-nitrotyrosine (3-NT) modified proteins was used as marker of protein damage by $ONOO^-$. The intracellular 3-NT levels were assessed colorimetrically using a competitive ELISA kit (Abcam). For each independent experiment a standard curve

was generated with the provided 3-NT standard and the 3-NT content quantified.

2.9. Nitrate/Nitrite (NO_x) Determination. NO_x concentration in cell supernatants was determined fluorimetrically using Fluorimetric Assay Kit (Cayman Chemical), 48 h after cells seeding.

2.10. Cell Viability Assay. After 48 h incubation in presence or absence of the NO donor DETA-NONOate, the viability of lymphoblast cells was assessed using the MTT [-3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay as described in [32]. Briefly, cells (2×10^5/mL) were seeded in a 96-well plate and incubated for 48 h at 37°C in the presence of increasing DETA-NONOate concentrations (0.01–0.5 mM) in a final volume of 100 μL/well. Having a half-life of 20 h at 37°C, the NO donor was readded after 24 h. At the end of the 48 h incubation, 10 μL of MTT solution (5 mg/mL) was added to each well, followed by 4 h incubation at 37°C. Afterwards, in order to dissolve the dark-coloured formazan crystals produced by reduction of the MTT tetrazolium salt, cells were incubated at 37°C overnight with 100 μL of 10% sodium dodecyl sulphate (SDS) in 0.01 M HCl. The optical density of reduced MTT was measured at 570 nm with a reference wavelength at 690 nm using Appliskan Microplate Reader (Thermo Scientific). The experiments were carried out in triplicate.

2.11. Statistical Analysis. Data are reported as mean ± SEM of at least three independent experiments and significance (P) was determined using Student's t-test. P values ≤ 0.05 were considered significant.

3. Results

3.1. Mitochondrial Function in LHON Patient PBMCs. RGCs, brain, and kidneys, the most affected sites in the LHON patient, are all high-energy demanding, so they rely more on OXPHOS ATP. Importantly, PBMCs have a metabolism mainly sustained by OXPHOS [33, 34] and therefore represent a good model to be investigated in the present study. Figure 1(a) shows a typical oxygen consumption trace acquired with intact PBMCs. The oxygen consumption was sustained by endogenous substrates and relied almost completely on mitochondria, as it was almost completely inhibited by antimycin A, a specific inhibitor of cytochrome *c* reductase. After normalization to the protein content, we found the O_2 consumption rate (≈50 pmol O_2 s^{-1} mg^{-1}) to be very similar in both LHON and control cells.

In agreement with these data, no significant differences in the steady-state concentration of ATP were observed in control and patient PBMCs. ATP levels were evaluated either in the absence or in the presence of glucose to sustain glycolysis and using oligomycin to inhibit OXPHOS activity. When glucose was present (Figure 1(b)), both LHON and control cells displayed a similar ATP content (ATP_{TOTAL} ≈ 6 μg ATP/mg total protein), and oligomycin inhibition of OXPHOS was found poorly effective in control and LHON PBMCs, suggesting that both cell types possess an efficient glucose-dependent glycolytic compensation (Warburg effect). Without glucose (Figure 1(c)), both LHON and control cells showed ~20% decrease in the ATP content, whose concentration dropped dramatically in the presence of oligomycin. Under the latter conditions, the glycolytic contribution is minimal and the difference between the ATP measured in the presence and absence of oligomycin (ΔATP) is indicative of the ATP generated by OXPHOS (ATP_{OXPHOS}). The ATP_{OXPHOS}/ATP_{TOTAL} ratio (Figure 1(d)) is very similar in LHON and control cells, indicating that the mutation does not affect OXPHOS efficiency.

Taken together, these results suggest that, over and above a bioenergetic deficit imputable to complex I mutation, other molecular mechanisms contribute to the clinical onset of LHON and its progress that we hypothesize to be also related to increased cell levels of reactive oxygen and nitrogen species.

3.2. Increased Level of ROS, 3-Nitrotyrosine, and Nitrite in PBMCs. In order to investigate whether the PBMCs derived from the LHON patient displayed increased oxidative/nitrosative stress levels, we measured the amount of ROS and 3-NT under basal conditions. The latter is an important marker of RNS, including $ONOO^-$. We found that LHON PBMCs display a significant 1.4-fold increase in ROS production compared to controls (Figure 2(a)). Consistently, LHON PBMCs displayed a higher concentration of 3-NT, suggesting a role of NO chemistry in cellular stress (Figure 2(b)). To assess the basal NO level in LHON, we measured the concentration of nitrite/nitrate, the oxidation products of NO. Interestingly we found that the nitrite/nitrate levels (Figure 2(c)) tend to increase compared to controls, although the reported variation did not reach statistical significance.

Owing to a limited availability of biological samples (cells from patient) and in order to obtain larger amounts of starting material, EBV-transformed lymphoblasts were used for further cell biochemical analysis. The bioenergetic behaviour of primary and transformed cells was very similar, with the two cell types displaying superimposable mitochondrial parameters (data not shown).

3.3. DETA-NONOate Decreases LHON Lymphoblasts Viability. A chronic increase in NO levels was artificially mimicked using the NO donor DETA-NONOate (DETA-NO). Cell viability was examined in both LHON and control lymphoblasts after 48 h incubation with different amounts of the NO releaser. As a regulator of cell proliferation, NO can either enhance or inhibit cell growth depending on its concentration. Figure 3 shows that at 10 μM DETA-NO no cytotoxicity was associated with NO exposure. Higher concentrations of DETA-NO affected cell viability proportionally to the amount added. However, the decline in cell viability was significantly larger in LHON than in control cells: at 0.1 and 0.5 mM DETA-NO, the residual viability of LHON cells was 35% and 15%, respectively, while in control cells it was, respectively, 75% and 35%. Trypan blue exclusion assays, carried out under comparable experimental conditions, indicated a similar cellular behaviour (data not

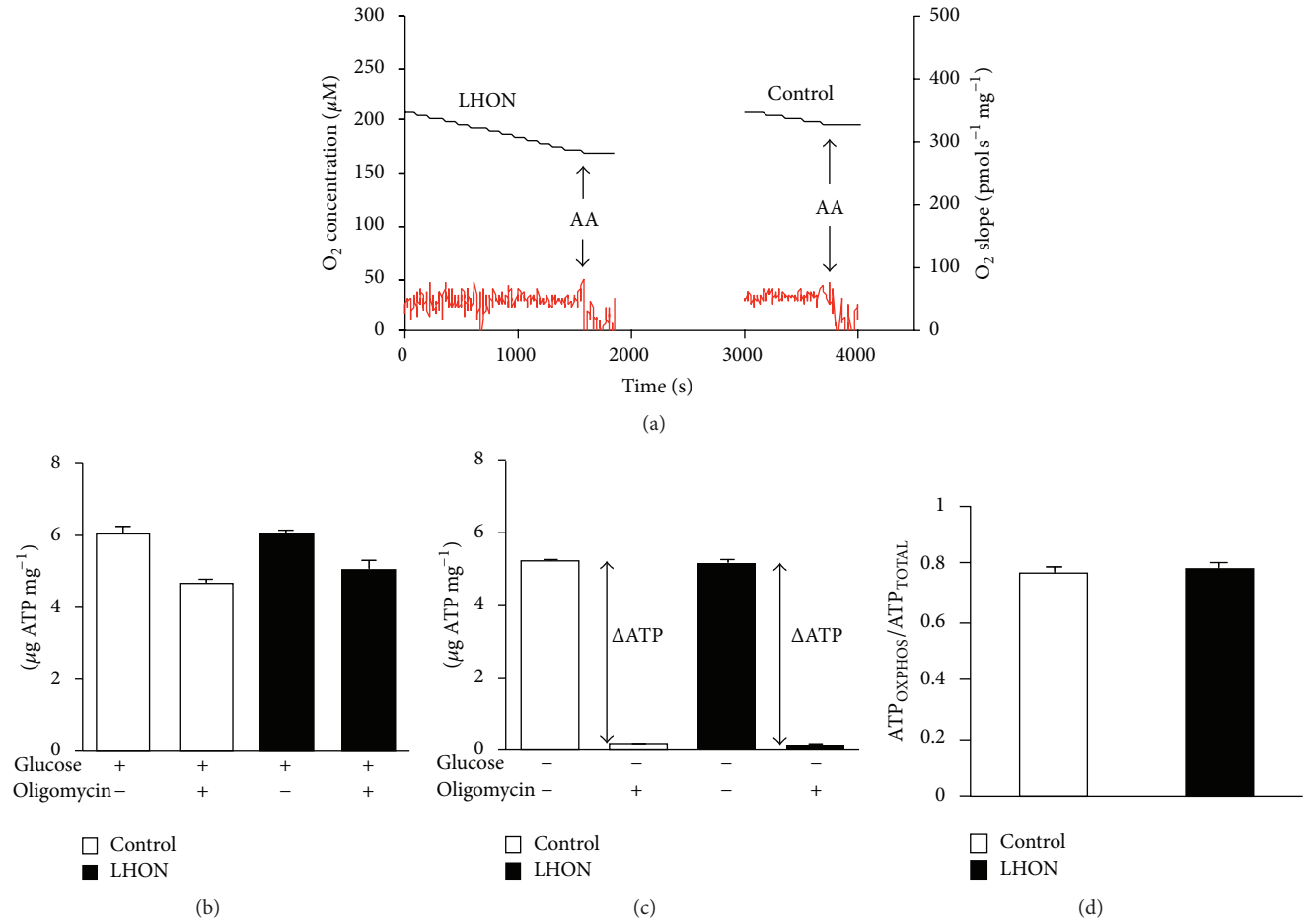

Figure 1: Bioenergetic properties of 11778/ND4 mutated PBMCs. (a) Representative cell O_2 consumption measurements. Black line: O_2 concentration trace; red line: O_2 consumption rate. After recording basal respiration, 4 μM antimycin A (AA) was added. (b) Cellular ATP levels measured in the presence of glucose with and without oligomycin. (c) Cellular ATP levels measured in the absence of glucose with and without oligomycin. (d) Fractional ATP expressed as the ratio between ATP_{OXPHOS} and ATP_{TOTAL}. ATP_{OXPHOS} was obtained from ΔATP (c) and ATP_{TOTAL} was measured in the presence of glucose without oligomycin (b). Data (mean ± SEM) were collected in three triplicate experiments on cells derived from the LHON patient and the three healthy controls.

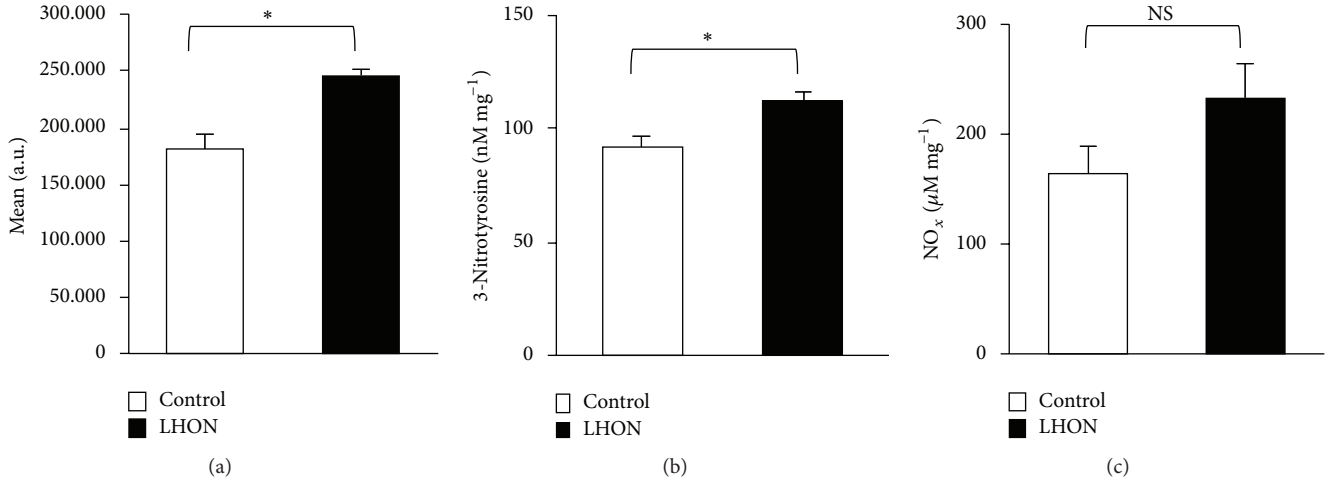

Figure 2: Oxidative and nitrosative stress is increased in 11778/ND4 mutated PBMCs. (a) Intracellular ROS levels were measured using the DCFDA dye. (b) The 3-NT content was determined by competitive ELISA and normalized to total protein. (c) Concentration of NO_x (nitrite and nitrate) in the cell supernatant as normalized to total protein. Data (mean ± SEM) collected in three duplicate experiments on cells derived from the LHON patient and the three healthy controls. Values are considered significant when $P < 0.05$: $^*P \leq 0.05$; Ns: not significant.

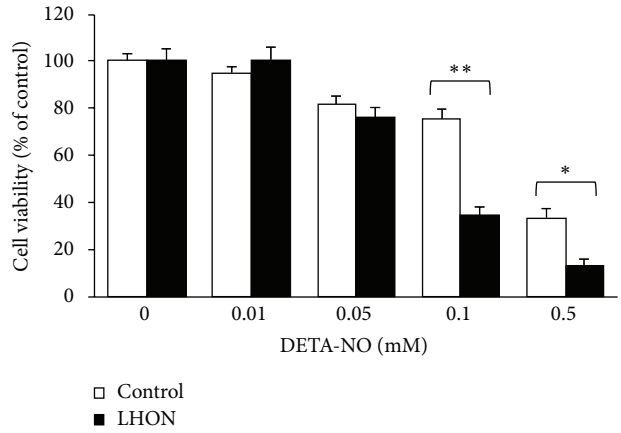

Figure 3: NO impairs the viability of 11778/ND4 mutated lymphoblasts. The cells derived from the LHON patient and the three healthy controls were treated with various concentrations of DETA-NONOate for 48 h and their viability was assessed using the MTT assay. Data are shown as the percentage of cell viability measured in the absence of the NO releaser. Data acquired in three triplicate experiments are expressed as mean ± SEM. Values are considered significant when $P < 0.05$: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

shown), showing that LHON cells are more susceptible to nitrosative stress than control cells.

4. Discussion

LHON is characterized by a low penetrance, as most individuals carrying mtDNA mutations remain asymptomatic, with a limited subset of them expressing the disease. Age and gender are the most important risk factors, with approximately 30–50% of males and 10–20% of female LHON mutation carriers becoming symptomatic at a median age of 19 years (range 5–56 years) [35]. Indeed, genetic, epigenetic, and environmental factors all contribute to the onset and evolution of the disease [36]. Complex I mutation, the primary etiologic cause of LHON, despite being a necessary determinant of the pathology, is not sufficient to induce the clinical expression of the disease so that a convincing explanation for the pathogenesis of this disorder is still under debate. The impairment of bioenergetics parameters, such as cell respiration and ATP production, has been found absolutely modest both in patient's tissues and in cell models of the disease. On the contrary, a marked overproduction of ROS has been reported, together with a low capability of the cellular antioxidant machinery to maintain the redox homeostasis.

LHON cybrid models have shown lower levels of glutathione peroxidase, glutathione reductase, and Mn-superoxide dismutase (MnSOD) or total glutathione, compared to controls [37, 38], particularly after cell treatment to enhance their OXPHOS dependency. These observations strongly support the idea that the genetic alteration of complex I, over and above the simple action on primary mitochondrial parameters, induces an alteration of the cell redox-signalling and homeostasis. Interestingly, in a cybrid model carrying the G11778A/ND4 mutation, it has been observed that mitochondrial overexpression of MnSOD induces a decrease in superoxide levels and enhances cell survival [39]. While chronic oxidative stress has been shown to play an important role in the onset of the disease, the role of NO and related reactive species remained unexplored. Together with CO and H_2S, NO forms the gasotransmitter triad acting as a redox-signalling regulator of several physiological functions, the mitochondrial one included [40]. NO, particularly at low concentrations, protects against cell death [41–44], whereas in the presence of superoxide it becomes toxic by forming $ONOO^-$ [30]. It should be kept in mind, however, that, even in the absence of $ONOO^-$, depending on the electron flux level through the respiratory chain, that is, on the concentration of mitochondrial ferrocytochrome *c*, and particularly under low O_2 tension (hypoxia), inhibition of mitochondrial complex IV by NO can be severe and persistent [29, 45, 46].

According to our results, LHON cells are apparently more prone to such NO-dependent detrimental chemistry. We present here the case of a female patient with a point mutation at nucleotide position G11778A, who suffered from both eyes' visual loss and subsequently displayed markers for autoimmune disorders, as well as white matter alterations outside the visual system.

We show that PBMCs and lymphoblasts derived from our patient are more susceptible not only to oxidative but also to nitrosative stress. In these cells we found that, under metabolic basal conditions and in the absence of exogenous NO, the 3-NT levels are enhanced. This finding is in agreement with prior studies carried out on optic nerve and retinal histological specimens obtained from LHON patients and on synaptosomes from a mouse model of the disease that have evidenced increased levels of 3-NT [18, 47]. The higher basal 3-NT concentration suggests that LHON cells produce larger amounts of nitrosating agents, such as $ONOO^-$. Consistently, we found that the concentration of nitrite/nitrate, the oxidation products of NO, tends to increase in patient cells, compared to controls. Increase in nitrosative stress might be particularly likely when NO overproduction takes place.

The current study reveals that chronic exposure to NO in proband lymphoblasts carrying the 11778/ND4 mutation determines a significant decrease in cell viability compared to controls, suggesting that in this pathological state the NO chemistry is more active. Interestingly, our patient has shown an abnormal increase in lactate serum levels after exercise, suggesting a limited mitochondrial reserve capacity. In terms of energy metabolism, under basal conditions the patient PBMCs are able to fully compensate complex I mutation allowing an efficient mitochondrial function. We observed that the ATP levels in LHON cells were not significantly different from those ones of control cells, even when the glycolytic system was restricted by glucose deprivation. Both cell types were equally able to efficiently compensate OXPHOS defects with glycolysis, as shown by abolishing the contribution of ATP_{OXPHOS} with oligomycin.

Mitochondrial ATP generation is crucial for cell function; therefore mitochondria have evolved several different strategies to maintain it to face dysfunctions. Switching to glycolysis is fundamental for cell survival during acute

stress, although this pathway represents a much less efficient mode of ATP production, resulting in an energy deficit and acidosis over long periods, especially in those cells relying on high-energy demand. Cells derived from LHON carriers, having the mitochondrial mutation but not expressing the disease, were shown to adopt different responses that allow the maintenance of the respiratory function and ATP levels, such as increasing the activity of the succinate-dependent pathway or the mitochondrial mass [13, 48]. The bioenergetic compensation observed in individuals affected by LHON can be disrupted by various factors, such as the frequently suggested nuclear modifying genes and environmental triggers. Smoking and alcohol are both known to decrease the mitochondrial reserve capacity [49–51] and have been reported to play a role in the onset of LHON disease [36] also contributing to its incomplete penetrance. A loss of mitochondrial reserve capacity, as obtained by the chronic exposure to NO, decreases the ability to respond to endogenous secondary energetic stressors, such as ROS and RNS, which we observed to be increased in the patient PBMCs. All together these findings point to a novel role played by RNS, particularly $ONOO^-$, whose accumulation in LHON cells appears to have a role in the pathology and its pharmacological control may be important in the patients [52].

5. Conclusions

In conclusion, we show that lymphoblasts derived from a LHON patient carrying the 11788/ND4 mutation are more susceptible to NO, suggesting that the exposure to high NO concentrations could impair *in vivo* the ability to cope with the oxidative stress caused by the genetic defect, thereby driving the pathology. This unprecedented observation is consistent with both LHON cell and animal models pointing to oxidative stress and impairment of redox homeostasis as important factors contributing to the onset and progression of the disease. Further work is needed to extend these studies to other LHON cells or tissues in order to better understand the reciprocal role of ROS and RNS in this pathology and to design new drugs and appropriate therapeutic approaches.

Conflict of Interests

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

Authors' Contribution

Micol Falabella and Elena Forte contributed equally.

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Review Article

Exercise Modulates Oxidative Stress and Inflammation in Aging and Cardiovascular Diseases

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Despite the wealth of epidemiological and experimental studies indicating the protective role of regular physical activity/exercise training against the sequels of aging and cardiovascular diseases, the molecular transducers of exercise/physical activity benefits are not fully identified but should be further investigated in more integrative and innovative approaches, as they bear the potential for transformative discoveries of novel therapeutic targets. As aging and cardiovascular diseases are associated with a chronic state of oxidative stress and inflammation mediated via complex and interconnected pathways, we will focus in this review on the antioxidant and anti-inflammatory actions of exercise, mainly exerted on adipose tissue, skeletal muscles, immune system, and cardiovascular system by modulating anti-inflammatory/proinflammatory cytokines profile, redox-sensitive transcription factors such as nuclear factor kappa B, activator protein-1, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha, antioxidant and prooxidant enzymes, and repair proteins such as heat shock proteins, proteasome complex, oxoguanine DNA glycosylase, uracil DNA glycosylase, and telomerase. It is important to note that the effects of exercise vary depending on the type, intensity, frequency, and duration of exercise as well as on the individual's characteristics; therefore, the development of personalized exercise programs is essential.

1. Exercise Training and Aging

There is mounting evidence based on epidemiologic and experimental studies that physical activity and exercise training combat the sequels of aging. Physical activity is defined as any bodily movement coordinated by skeletal muscles, which increases energy expenditure over resting condition [1], whereas exercise training is a more regular and structured form of physical activity. Higher levels of physical activity and regular exercise are associated with reduced risks of all-cause mortality [2–20] and also with increased longevity [18, 21–23]. In fact, the World Health Organization has identified physical inactivity as the fourth leading risk factor for global mortality [24]. Furthermore, physical activity and exercise training reduce the risk of age-associated diseases, namely, cardiovascular diseases [4, 10, 25–31], type 2 diabetes [32], metabolic syndrome [33], colon cancer [34], obesity [35], osteoporosis [36], sarcopenia [37], anxiety [38], and cognitive impairment [39–41]. Most importantly, exercise improves the quality of life of elderly people [42, 43].

2. Exercise Training and Cardiovascular Diseases

Age is a major risk factor for cardiovascular diseases (CVDs) [44, 45]. Numerous studies, confirmed by meta-analyses, indicate that exercise training reduces cardiovascular mortality [7, 20, 26, 46–54] and cardiovascular events [4, 10, 25–28, 30, 31], particularly stroke [55–58], coronary heart disease [25, 59–61], heart failure [60, 62, 63], atherosclerosis [64–66], and preeclampsia [67–69]. Accordingly, physical inactivity is now regarded as one of the most prevalent cardiovascular risk factors [70, 71]. Moreover, exercise training is an effective therapeutic strategy for patients with peripheral arterial

diseases [72–74], coronary heart disease [75–81], heart failure [82–84], atherosclerosis [64], and hypertension [85–88].

The cardiovascular benefits of exercise have been frequently attributed to the reduction of many classical cardiovascular risk factors including blood lipids [20, 28, 50, 89–95], high blood pressure [20, 28, 50, 95], obesity [50, 95–97], glucose, and type 2 diabetes [98, 99] as well as novel risk factors such as inflammation [28, 100–103] and oxidative stress [95]. However, the mechanisms underlying the protective and therapeutic effects of exercise go beyond reducing cardiovascular risk factors [104] to modulating angiogenesis [105], endothelial progenitor cells [106–109], basal heart rate [110], endothelial function [111–115], autonomic control [116], arterial stiffness [41, 117–120], and arterial remodeling [121]. In this review we will focus on the molecular transducer of the antioxidant and anti-inflammatory effects of exercise.

3. Oxidative Stress and Inflammation in Aging and Cardiovascular Diseases

Aging is associated with oxidative stress that is mainly attributed to defective mitochondria, resulting from reduction in cytochrome C oxidase (complex IV) activity [23, 122, 123] and peroxidative damage of mitochondrial membrane [124]. Hence, a greater number of electrons are generated that can escape from the mitochondria to create a long trail of reactive oxygen species (ROS) [125, 126], leading to further mitochondrial dysfunction and ROS generation and creating a vicious cycle of oxidative damage [127]. Age-associated increases in ROS production occur in skeletal muscles [128] and other organs such as the heart, liver, brain, and kidney [23, 126, 129, 130].

Reduced protein synthesis limits antioxidant defense mechanisms and repair capacity in aged individuals, which further contributes to the state of oxidative stress. The free radical theory of aging hypothesizes that oxidative stress damages macromolecules, including lipids, proteins, and nucleic acids, overwhelming cellular antioxidant defense and repair mechanisms, leading to progressive deleterious changes over time [131, 132]. Indeed, oxidatively damaged proteins [133], nucleic acids [134, 135], and lipids [113, 136–138] are abundant in various organs and tissues such as kidney, liver, heart, arteries, skeletal muscles, and plasma in aged subjects.

Aging is also accompanied with a state of chronic inflammation that is mainly attributed to sarcopenia and adiposity. Sarcopenia, defined as age-associated progressive loss of muscle mass and strength [139, 140], increases the incidence of muscle injury [18], which increases the infiltration of immune cells into injured muscles. Activated immune cells and injured muscles release proinflammatory mediators and reactive oxygen and nitrogen species (RONS) via lipoxygenase, NADPH oxidase, xanthine oxidase, and inducible nitric oxide synthase (iNOS) [141–147] leading to oxidative stress.

Sarcopenia can also lead to reduced physical activity and increased adiposity. Adiposity induces a state of low-grade but chronic inflammation through the release of a multitude of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) [148–150]. Indeed, aging is associated with increased levels of TNF-α, IL-6, and interleukin-1 receptor agonist (IL-1ra) and systemic inflammatory biomarkers such as C-reactive protein (CRP) as well as higher count of inflammatory cells such as neutrophil and monocytes [151–153]. Hence, aging is associated with oxidative stress and inflammation.

Cardiovascular diseases are also associated with high level of inflammation and oxidative stress [154–157].

4. Oxidative Stress and Inflammation Overlapping Signaling Pathways

Oxidative stress and inflammation share common and overlapping signaling pathways. By damaging macromolecules, ROS can initiate inflammation [158]; ROS are also products of the inflammatory process. During the respiratory burst, immune cells generate RONS via NADPH oxidase and iNOS and release proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 [141, 159, 160]. Similarly, injured tissues can also release proinflammatory cytokines that activate specific ROS-generating enzymes such as lipoxygenase, NADPH oxidase, myeloperoxidase, and xanthine oxidase [142–147] and specific reactive nitrogen species generating pathways such as NOS, protein kinase B (Akt), and Sph1P (sphingosine-1-phosphate) [161–163].

ROS overproduction activates redox-sensitive transcription factors including nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) via stress kinases such as extracellular signal regulated kinases (ERKs), c-jun N-terminal kinases (JNKs), mitogen activated protein kinase p38 (MAPK p38), protein kinase C (PKC), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt, and Src family kinases (SFKs). This leads to increased expression of inflammatory target proteins such as matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), iNOS, cyclooxygenase-2 (COX-2), and cytosolic phospholipase A2 (cPLA2) (Lee and Yang) [164–171] and proinflammatory mediators such as TNF-α gene [172], IL-1, and IL-8 [169]. Many of these inflammatory proteins or their products such as NOS, COX, and PGE_2 are prominent sources of RONS [173]; this creates an autoactivating loop which feeds the vicious cycle of inflammation and oxidative stress.

There are also other proteins such as thioredoxin-interacting protein (TXNIP) linking oxidative stress and inflammation. Under resting conditions, TXNIP is bound to thioredoxin (TRX) via a disulphide bound, keeping it in an inactive form. Increased levels of ROS generation cause the dissociation of TXNIP from TRX, leaving it free to scavenge ROS and allowing TXNIP to stimulate the inflammatory cytokine IL-1β [174, 175]. In agreement with this is the observation that antioxidant supplementation blocked the anti-inflammatory effect of exercise by reducing IL-6 production [176, 177].

In short, proinflammatory mediators such as TNF-α, IL-1, and IL-6 generate RONS which activate redox-sensitive

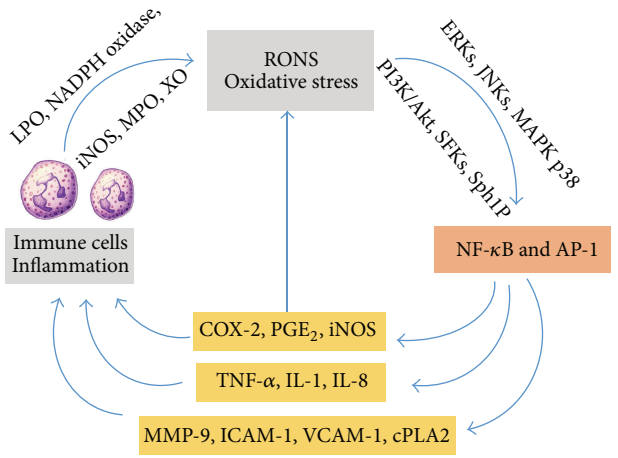

Figure 1: Oxidative stress and inflammation overlapping signaling pathways in aging. AP-1 = activator protein-1, COX-2 = cyclooxygenase-2, cPLA2 = cytosolic phospholipase A2, ERKs = extracellular signal regulated kinases, ICAM-1 = intercellular adhesion molecule-1, IL-1 = interleukin-1, IL-8 = interleukin-8, iNOS = inducible nitric oxide synthase, JNKs = c-jun N-terminal kinases, LPO = lipoxygenase, MAPK p38 = mitogen activated protein kinase p38, PI3K = phosphatidylinositol-4,5-bisphosphate 3-kinase, MMP-9 = matrix metalloproteinase-9, MPO = myeloperoxidase, NF-κB = nuclear factor kappa B, PGE_2 = prostaglandin E_2, PKC = protein kinase C, RONS = reactive oxygen nitrogen species, Sph1P = sphingosine-1-phosphate, TNF-α = tumor necrosis factor-alpha, VCAM-1 = vascular cell adhesion molecule-1, and XO = xanthine oxidase.

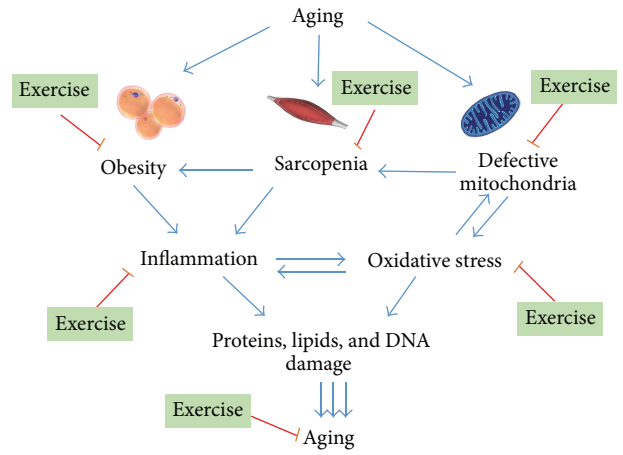

Figure 2: Modulation of oxidative stress and inflammation in aging by exercise.

transcription factors such as NF-κB and AP-1 resulting in the generation of large quantities of these proinflammatory mediators and ROS (Figure 1). Indeed, aging is associated with adverse health conditions such as atherosclerosis, metabolic syndrome, sarcopenia, arthritis, and chronic obstructive pulmonary disease that are characterized by elevated levels of both oxidative stress and inflammatory markers [178].

Not surprisingly, ROS can also induce proteins such as heat shock proteins (HSPs), HSP70 in particular [179], and heme oxygenase 1 (HO-1) [180] that can protect cells and tissues from the deleterious effects of inflammation. However, the balance of antioxidant/anti-inflammatory to oxidant/inflammatory proteins is tilted towards the latter during the aging process.

5. Exercise Training: Modulation of Oxidative Stress and Inflammation

Exercise and regular physical activity counteract the deleterious effects of aging, not only by combating sarcopenia, obesity, and mitochondrial dysfunction, the major triggers of oxidative stress and inflammation in aging, but also by exerting additional antioxidant and anti-inflammatory actions as illustrated in Figure 2.

5.1. Effect of Exercise Training on Adiposity. Adipose tissue, particularly visceral fat depots, and the macrophages trapped within fat depots are able to release proinflammatory cytokines such as IL-6 and TNF-α [148–150, 181, 182]. Physical activity and exercise training increase energy expenditure and reduce body fat, particularly visceral fat, with/without weight loss [35, 183, 184], and therefore reduced production and release of IL-6 and TNF-α [185–189]. Exercise training increased gene expression of PGC-1 alpha, a master regulator of mitochondrial biogenesis, in rat adipose tissue [190], leading to increased energy expenditure particularly in the visceral area. Exercise training inhibited the infiltration of the inflammatory phenotype M1 macrophages into adipose tissue, while also favoring the switch of macrophages to the less inflammatory phenotype M2 in obese mice [191]. Exercise training/physical activity also induces the release of adiponectin from adipose tissues [192–197]; adiponectin exerts antiapoptotic, anti-inflammatory, and antioxidative activities [198, 199].

5.2. Effect of Exercise Training on Skeletal Muscles. Physical activity/exercise increases nutritive blood supply to and removes waste from skeletal muscles, while also upregulating the expression of the anabolic myokine IL-15 [195–197, 200, 201]. Most importantly, physical activity/exercise stimulates mitochondrial biogenesis [202] and oxidative capacity [203] that provide energy for the synthesis of new proteins. Thus, physical activity/exercise improves muscle mass and strength and renders them less vulnerable to acute injury [204], therefore suppressing triggers of inflammation and oxidative damage [205–209].

Physical activity/exercise also induces the release of several myokines from skeletal muscle such as IL-6 [210–212], which suppresses IL-1 and TNF-α [213] and triggers the release of many anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1ra) and IL-10, in addition to cortisol [214, 215]. In turn, IL-10 inhibits the synthesis of some proinflammatory cytokines such as TNF-α and IL-1β [200]. Exercise also reduces TNF-α and IL-1β production in skeletal muscles [212, 216, 217].

Heat shock proteins (HSPs) are also generated in skeletal muscles in response to physical activity/exercise; they exert vital anti-inflammatory action as will be explained later [218–222].

5.3. Effect of Exercise Training on Mitochondrial Aging. Exercise mitigates mitochondrial aging and interrupts the vicious cycle of oxidative damage by stimulating mitochondrial biogenesis [202] and enhancing mitochondrial oxidative capacity [203, 223, 224]. Excellent reviews on this topic are available [225, 226].

5.4. Anti-Inflammatory Effects of Exercise Training. Acute bouts of exercise cause transient damage to contracting skeletal muscles, triggering an inflammatory response that increases the levels of proinflammatory cytokines and acute-phase reactants in the blood [227–230]. However, regular exercise reduces levels of systemic inflammatory markers such as CRP, IL-6 TNF-α, soluble TNF-α receptor 1 (sTNF-R1), and soluble TNF-α receptor 2 (sTNF-R2) in young and middle aged adults [231–243] and also more importantly in the elderly [195, 244–250]. Additionally, higher levels of the anti-inflammatory cytokines interleukin-10 (IL-10) [246] and adiponectin [195] are associated with increased physical activity in the elderly. Several interventional studies report that exercise reduces inflammatory markers, particularly CRP, TNF-α, interferon-gamma (INF-γ), monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), interleukin-18 (IL-18), sTNFR2, sTNF-R1, and soluble IL-6 receptor (sIL-6R), and increases levels of anti-inflammatory factors such as IL-10, interleukin-12 (IL-12), interleukin-4 (IL-4), and transforming growth factor beta 1 (TGFβ1) [74, 188, 197, 217, 251–265]. These benefits of exercise also occur in the elderly [100, 196, 250, 263, 266–271] as summarized in Table 1. However, only a few randomized controlled trials confirm the anti-inflammatory effect of exercise [74, 261, 262, 269]. Exercise training can exert anti-inflammatory effects with/without accompanied weight loss; however, the most substantial anti-inflammatory effects occur in patients with high baseline inflammatory biomarkers, particularly when associated with weight loss [260].

It is worth noting that some interventional and randomized controlled trials studies did not detect a significant effect of regular exercise on systemic inflammatory biomarkers in adults [243, 272–274] or in aged adults [275–278] as shown in Table 1. A meta-analysis found only five randomized controlled trials that examined the effects of regular aerobic exercise (of at least 4-week duration) in adults and concluded that aerobic exercise did not reduce CRP levels [279]. It is likely that these discrepancies may be attributed to the smaller sample size used in the clinical trials examined.

On the other hand, the effects of resistance exercise on inflammatory mediators are mostly negative [280–282], although Brooks et al. [196] reported that 16 weeks of resistance training reduced CRP and increased adiponectin levels in older diabetic patients. The effects of physical activity and different exercise programs on inflammatory mediators in the elderly are detailed in Table 1. Clearly, the effects of exercise depend on the type (aerobic/resistance), intensity (mild/moderate/intense/exhaustive), and frequency (sessions per day/week/month) of exercise and also on the subject's characteristic (age, sex, endurance capacity, and health condition).

5.4.1. Molecular Transducer of the Anti-Inflammatory Effects of Exercise Training. The signaling pathways underlying the anti-inflammatory effects of exercise are complex and not completely understood. In addition to the effects of exercise on adipose tissue, skeletal muscles, and mitochondrial biogenesis mentioned above, exercise exerts additional anti-inflammatory actions on the immune system, repair mechanisms, and vasculature.

Effects of Exercise on the Immune System. Regular exercise downregulates the innate immune response and activates the adaptive immune system with consequent suppression of inflammation. Exercise modulates the immune system by reducing the number of inflammatory CD14+CD16+ monocytes [250], increasing the number of CD4CD25 regulatory T cells [264, 283], shifting blood macrophages towards the less inflammatory phenotype M2 [284], increasing the dominance of the anti-inflammatory Type 2 helper T cell over proinflammatory Type 1 helper T cell [265, 284–286], and reducing monocyte chemoattractant protein-1 (MCP-1) [188] and toll-like receptor-4 (TLR4) expression on monocyte surfaces [239, 263, 275]. On the other hand, ET increases the production of transforming growth factor beta (TGFβ1) [254, 264] from regulatory T cells.

Exercise also stimulates the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis to increase serum glucocorticoid levels [287] with subsequent inhibition of the immune system [288].

Effects of Exercise on Repair Mechanisms. Heat shock proteins are highly conserved chaperone proteins that regulate the folding and processing of damaged proteins and therefore exert significant anti-inflammatory action. Numerous studies have shown that exercise is capable of upregulating the expression of HSP72 [219, 222, 289–293], HSP70 [137, 218, 220, 221, 294–297], HSP60 [294, 298], HSP27 [294, 295], and HSP25 [222] in skeletal muscles, blood cells, hearts, and arteries of humans as well as young and aged experimental animals (see Table 2). However, Hägg et al. [299] reported that voluntary wheel running of spontaneously hypertensive rats for 5 weeks reduced aortic gene expression of HSP70 and HSP60. The effect of exercise on HSPs depends on age [296], sex [220], time course [221, 291], and HSP subtype [222, 293, 294, 298].

Effects of Exercise on Vascular Endothelial Cells. By increasing shear stress on vascular endothelial cells, exercise modulates key players in the inflammatory process such as ICAM-1, NF-κB, MAPK, and COX-2 [300, 301]. Voluntary wheel running of aged mice for 10–14 weeks reduced the activation of NF-κB in the aorta [302]. Subjecting aortic endothelial cells to *in vitro* shear stress for 4 h reduced the expression of vascular cell adhesion molecule-1 (VCAM-1) [303]. Treadmill training for 1–3 weeks reduced the expression of intercellular

Table 1: Effect of physical activity/exercise on inflammatory mediators in the elderly.

Study	Mediator	Subjects	Tissue	Physical activity/exercise	Effect of physical activity/exercise	Reference
Observational	TNF-α	≥65 years $n = 1004$	Plasma	Self-reported physical activity	Inverse association between log TNF-α and physical activity	[249]
		65–80 years $n = 30$	Serum	Regular exercise	Lower percentage in the physically active subgroup	[250]
	CRP	≥65 years $n = 5,888$	Blood	Self-reported physical activity	Inverse association between physical activity and CRP	[241]
		Men, 58 years $n = 391$	Blood	Self-reported leisure time physical activity	Inverse association between physical activity and CRP	[242]
		70 to 79 years $n = 870$	Blood	Self-reported physical activity	Inverse association between CRP and physical activity	[244]
		70 to 79 years $n = 3,075$	Blood	Previous week exercise and physical activities	Inverse association between physical activity and CRP	[245]
		60 to 79 years $n = 3810$	Plasma	Self-reported physical activity	Inverse association between CRP and physical activity	[247]
		70 to 79 years $n = 880$	Plasma	Physical function measures included handgrip strength, signature time, chair stands, and 6-minute walk time	Inverse association between CRP and higher walking speed and grip strength	[248]
		≥65 years $n = 1004$	Plasma	Self-reported physical activity	Inverse association between and log CRP and physical activity	[249]
		65–80 years $n = 30$	Serum	Regular exercise	Lower level in the physically active subgroup	[250]
		50 to 70 years $n = 3289$	Plasma	Self-reported physical activity	Inverse association between CRP and physical activity	[195]
	IL-6	Men, 65–74 years $n = 12$	Serum	Self-reported physical activity	Lower levels of IL-6 in the physically active group	[246]
		70 to 79 years $n = 3,075$	Blood	Previous week exercise and physical activities	Lower level associated with higher level of physical activity	[245]
		70 to 79 years $n = 870$	Blood	Self-reported physical activities	Inverse association between IL-6 and physical activity	[244]
		≥65 years $n = 1004$	Plasma	Self-reported physical activity	Inverse association between log IL-6 and physical activity	[249]
		70 to 79 years $n = 880$	Plasma	Physical function measures included handgrip strength, signature time, chair stands, and 6-minute walk time	Inverse association between IL-6 and higher walking speed	[248]
	IL-10	Men, 65–74 years $n = 12$	Serum	Self-reported physical activity	Higher levels of IL-10 in the physically active group	[246]
	CD14+CD16+	65–80 years $n = 30$	Serum	Regular exercise	Lower percentage in the physically active subgroup	[250]
	Adiponectin	50 to 70 years $n = 3289$	Plasma	Self-reported physical activity	Direct association between adiponectin and physical activity	[195]

Table 1: Continued.

Study	Mediator	Subjects	Tissue	Physical activity/exercise	Effect of physical activity/exercise	Reference
Interventional	TNF-α	≥64 years $n = 105$	Blood	Aerobic or flexibility/strength exercise for 10 months	Reduced level by aerobic and strength exercise	[266]
		Men, 67 ± 8 years with congestive heart failure $n = 28$	Plasma	Exercise training for 3 months	Level reduced after training	[267]
		81 ± 1 years $n = 13$	Skeletal muscle	Exercise training for 3 months	Reduced mRNA and protein levels after training	[268]
		65–80 years, physically inactive $n = 15$	Blood	3 days/week endurance and resistance exercise training for 12 weeks	Reduced level compared with pretraining values	[250]
		Postmenopausal women, 65–80 years $n = 20$	Blood	Regular exercise for previous 6 months	No change in protein or mRNA	[275]
		65–80 years $n = 8$	Serum	Progressive resistance strength training for 12 weeks	No change	[280]
	sTNF-R1	Overweight/obese sedentary with knee osteoarthritis ≥60 years $n = 316$	Serum	Combined weight training and walking for 1 h, 3 times/week for 18 months	No change	[277]
	CRP	Type 2 diabetic patients, >55 years $n = 62$	Serum	Strength training for 16 weeks	Reduced level after training	[196]
		>64 years $n = 105$	Blood	Aerobic or flexibility/strength exercise for 10 months	Reduced level by aerobic but not strength exercise	[266]
		Postmenopausal overweight or obese, sedentary women, 50–75 years $n = 115$	Serum	Moderate-intensity aerobic exercise for 12 months	Level reduced after training	[269]
		Women with the metabolic syndrome, 68.7 ± 3.4 years $N = 32$	Blood	Four sessions of high-intensity aerobic and resistance exercise per week for 12 months	Level reduced after training	[271]
		Patients with CHD, 66.7 ± 11 years $n = 235$ Controls 63.9 ± 11.1 years $n = 42$	Blood	Cardiac rehabilitation and exercise training for 3 months	Level reduced after training	[100]
		60 to 85 years $n = 30$	Serum	Exercise training for 6 months	No change	[276]
		Overweight/obese sedentary with knee osteoarthritis ≥60 years $N = 316$	Serum	Combined weight training and walking for 1 h, 3 times/week for 18 months	No change	[277]

Table 1: Continued.

Study	Mediator	Subjects	Tissue	Physical activity/exercise	Effect of physical activity/exercise	Reference
		Postmenopausal breast cancer survivors, 50 to 69 years $n = 52$	Serum	Cycling 3 times/week for 15 weeks	No change	[278]
	IL-6	>64 years $n = 105$	Blood	Aerobic or flexibility/strength exercise for 10 months	Reduced level by aerobic but not strength exercise	[266]
		70–89 years $n = 424$	Plasma	Moderate-intensity combination of aerobic, strength, balance, and flexibility exercises for 12 months	Reduced IL-6 level but not CRP	[270]
		Young (20–30 years) and aged (66–76 years) $n = 60$	Blood	Endurance (20 min) and resistance exercise 3 days/week for 12 weeks	Stimulated level was reduced in young and old subjects	[263]
		Postmenopausal women, 65–80 years $n = 20$	Blood	Regular exercise for 6 months	No change in protein or mRNA	[275]
		Overweight/obese sedentary with knee osteoarthritis ≥60 years $N = 316$	Serum	Combined weight training and walking for 1 h, 3 times/week for 18 months	No change	[277]
		65–80 years $n = 8$	Serum	Progressive resistance strength training for 12 weeks	No change	[280]
	IL-1β	65–80 years $n = 8$	Serum	Progressive resistance strength training for 12 weeks	No change	[280]
	IL-18	>64 years $n = 105$	Blood	Aerobic or flexibility/strength exercise for 10 months	Reduced level by aerobic but not strength exercise	[266]
	TLR4	Postmenopausal women, 65–80 years	Blood	Regular exercise for 6 months	Lower level in trained versus untrained	[275]
		Young (20–30 years) and aged (66–76 years) $n = 60$	CD14+ cell	Endurance (20 min) and resistance exercise 3 days/week for 12 weeks	Level reduced in young and old subjects	[263]
	CD14+CD16+	65–80 years sedentary $n = 15$	Blood	Endurance and resistance exercise training for 12 weeks (3 days/week)	Reduced level compared with pretraining values	[250]
	Adiponectin	Type 2 diabetic patients >55 years $n = 62$	Serum	Strength training for 16 weeks	Increased level after training	[196]

Table 2: Effects of exercise training on HSPs in humans and experimental animals.

HSP	Species		Tissue	Physical activity/exercise mode	Effect of physical activity/exercise	References
HSP72	Human	Men, 22.1 ± 3.8 years $n = 7$	Plasma	Semirecumbent cycling for 120 min	Levels increased after exercise	[289]
		Men and women, 22–30 years $n = 6$	Serum	Acute bout of treadmill running for 60 min	Protein expression increased during and after exercise	[219]
		Men and women, 22–30 years $n = 6$	Skeletal muscle	Acute bout of treadmill running for 60 min	mRNA level increased after exercise	[219]
	Rats	Young (3 months) and aged (30 months)	Skeletal muscle	4.5 weeks of resistance exercise	Protein expression increased in young and old rats	[222]
		Adult females	Heart	1 or 3 consecutive days for 100 min at a speed of 20 m/min	Increased expression	[290]
		Adult males	Heart	Treadmill running for 1 or 3 days	Increased levels after 3 but not 1 day	[291]
		Adult males	Heart	24-week but not 12-week treadmill training	Increased expression	[292]
		Females, 4 months	Heart	Endurance exercise for 10 weeks	Increased expression	[293]
		Females, 4 months	Ventricle	3–5 consecutive days of treadmill exercise [60 min/day at 60–70% maximal O_2 uptake]	Increased levels	[368]
HSP70	Human	Male athletes, 32.3 ± 9.3 years $N = 12$	Leukocytes	Half marathon run	Protein expression increased	[294]
		Men and women, 26 ± 4 years $N = 5$	Skeletal muscle	30 min on a treadmill	mRNA level but not protein level increased at 4 min, 30 min, and 3 h after exercise	[221]
		Women, 22 ± 2.2 years $n = 10$	Skeletal muscles	Acute bout of eccentric contractions	Protein expression increased after exercise	[218]
	Rats	Aged 24 months old	Hearts	Treadmill training for 30 m/min, 45 min/day, 5 days/week for 6 weeks	Expression increased	[295]
		Young (6 months) and aged 27 months	Left ventricle	Treadmill for 60 min/day, 5 days/week for a total of 12 weeks	Protein increased in the young group compared with sedentary control	[296]
		Males, young (4 months) and aged (21 months)	Heart	Acute exercise for 60 min at 70–75% of maximum oxygen consumption	Expression increased in young and old rats	[297]
		Males, 2 months	Heart	Treadmill for 3 days/week for 14 weeks	Increased protein level	[137]
		Spontaneously hypertensive females (9 weeks)	Aorta	Voluntary wheel running for 5 weeks	Reduced gene expression	[299]
		Males and females, 11 weeks	Skeletal muscle	Acute treadmill running for 30 min	Protein and mRNA expression increased in males but not females	[220]
	Mice	Males (6–8 weeks)	Cardiac ventricles	Swimming training for 14 weeks	No change	[298]

Table 2: Continued.

HSP	Species		Tissue	Physical activity/exercise mode	Effect of physical activity/exercise	References
HSP60	Human	Male athletes, 32.3 ± 9.3 years $N = 12$	Leukocytes	Half marathon run	Expression increased	[294]
	Rats	Males, 2 months	Heart	Treadmill for 3 days/week for 14 weeks	Decreased mRNA	[137]
		Spontaneously hypertensive females (9 weeks)	Aorta	Voluntary wheel running for 5 weeks	Reduced gene expression	[299]
	Mice	Males, 6–8 weeks	Ventricles	Swimming training for 14 weeks	Increased level	[298]
HSP32	Rats	Females, 4 months	Heart	Endurance exercise for 10 weeks	No change	[293]
HSP27	Human	Male athletes, 32.3 ± 9.3 years $N = 12$	Leukocytes	Half marathon run	Expression increased	[294]
	Rats	Males, aged (24 months)	Hearts	Treadmill training for 30 m/min, 45 min/day, 5 days/week for 6 weeks	Expression increased	[295]
HSP25	Rats	Males, young (3 months) and aged (30 months)	Skeletal muscles	4.5 weeks of resistance exercise	Protein expression increased in young and old rats	[222]
HSC70	Human	Male athletes, 32.3 ± 9.3 years $N = 12$	Leukocytes	Half marathon run	No change	[294]
	Rats	Males, young (3 months) and aged (30 months)	Skeletal muscles	Resistance exercise for 4.5 weeks	No change in protein expression	[222]

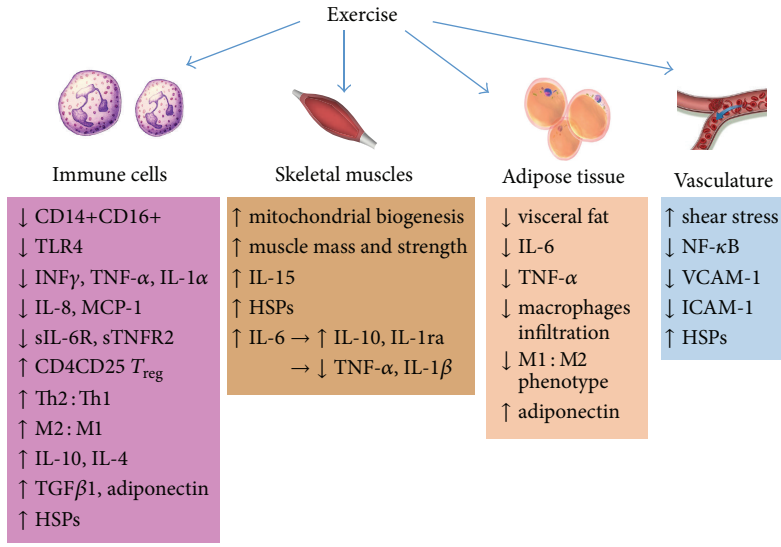

Figure 3: Signaling pathways underlying the anti-inflammatory actions of exercise. HSPs = heat shock proteins, IL-1α = interleukin-1-alpha, IL-1ra = interleukin-1 receptor antagonist, IL-1β = interleukin-1 beta, IL-6 = interleukin-6, IL-8 = interleukin-8, IL-10 = interleukin-10, IL-15 = interlukin-15, INFγ = interferon gamma, M1 = macrophage phenotype 1, M2 = macrophage phenotype 2, ROS = reactive oxygen species, sTNFR2 = soluble TNF-α receptor 2, sIL-6R = soluble IL-6 receptor, TLR4 = toll-like receptor-4, TGFβ1 = transforming growth factor beta 1, TNF-α = tumor necrosis factor-alpha, Th1 = Type 1 helper T cell, and Th2 = Type 2 helper T cell.

cell adhesion molecule-1 (ICAM-1) in response to cerebral ischemia in rats [304]. The mechanisms underlying the anti-inflammatory actions of exercise are summarized in Figure 3.

5.5. Antioxidant Effects of Exercise Training. Generation of ROS is transiently increased during exercise; however, the incidence of diseases associated with oxidative stress is reduced by regular exercise. Regular exercise attenuates oxidative damage in the brain [23, 305–307], liver [23, 130, 308–310], kidney [23, 136], skeletal muscle [311], blood [113, 136], and heart [23, 297]. However, Goto et al. [135] found that high-intensity exercise for 12 weeks increased the indices of oxidative stress in young men.

Importantly, regular exercise ameliorates age-associated oxidative stress in the heart [297, 312], liver [130], plasma [113], arteries [138], and skeletal muscles [313, 314]. In the study of Navarro et al. [23], exercise reduced age-associated mitochondrial oxidative damage and upregulated mitochondrial NADH-cytochrome C reductase and cytochrome oxidase activities in brain, heart, liver, and kidney of 52-week-old but not older rats. However, exercise caused an increase in oxidative damage in skeletal muscles [315] and hearts of aged rats [316].

In elderly people, regular exercise reduced serum/plasma levels of myeloperoxidase, a marker of inflammation and oxidative stress [205], and thiobarbituric-reactive acid substances, a marker of lipid peroxidation [317]. Lower levels of nitrotyrosine [133] and thiobarbituric-reactive acid substances [318] were found in the more physically active elderly people. However, de Gonzalo-Calvo et al. [319] reported that although regular exercise increased protein carbonyl content and lipid peroxidation levels in the plasma and erythrocytes of long term trained elderly men, their overall health condition was markedly improved. Another clinical study showed that 8 weeks of walking exercise did not significantly change low density lipoprotein (LDL) oxidation or nitration in the elderly [320].

5.5.1. Molecular Transducer for the Antioxidant Effects of Exercise Training. As discussed above, exercise exerts prominent anti-inflammatory actions, thus suppresses major sources of ROS and RNS generation, and produces indirect antioxidant effects. Exercise also upregulates the antioxidant defense mechanisms and repair proteins in the body via redox-sensitive transcription factors, mainly NF-κB, activator protein-1 (AP-1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and by increasing laminar shear stress on vascular endothelial cells.

The metabolic demands of skeletal muscles increase during exercise; the body responds by increasing oxygen uptake and blood flow to the muscles and other body organs. The increased metabolic rate results in greater ROS production in skeletal muscles [128, 321] and in other organs as well [129, 322]. Sources other than the electron transport chain enzymes in the mitochondria, such as xanthine oxidase [323–325] and NADPH oxidase [128, 326], contribute to ROS generation during exercise. This transient increase in ROS levels activates NF-κB, AP-1, and PGC-1α signaling.

Effects of Exercise on NF-κB and AP-1 Signaling. Exercise-induced increase in ROS levels triggers an adaptive antioxidant response that is mediated via mitogen activated protein kinases (MAPK p38, ERK 1, and ERK 2) [323, 327–329], cAMP-response-element binding (CREB) [330, 331], and synapsin [330, 331], to activate redox-sensitive transcription factors such as NF-κB [323, 332, 333] and AP-1 [327, 333],

resulting in increased expression of antioxidant enzymes [334] such as superoxide dismutase (SOD) [323, 333] and catalase [333], repair proteins such as hear shock proteins HSP25, HSP60, HSP72, HSP70, and heat shock cognate 70 HSC70 [315, 333–336], proteasomes complex, and nitric oxide synthase (NOS) [308, 323]. These signaling cascades were demonstrated in skeletal muscles [323, 332], brain [330, 331], leukocytes [337], and hearts [327] of experimental animals as well as in humans [328, 337] and in aged animals [333, 335, 338] and elderly people [337]. However, other studies report that exercise-induced activation of NF-κB and AP-1 [333] and upregulation of HSP70 were attenuated in fast skeletal muscles of old rats [339]. Interestingly, aging also increased ROS production and NF-κB activity in the livers of aged rats; these effects were attenuated by exercise [130, 308].

Effects of Exercise on PGC-1α Signaling. Exercise stimulates mitochondrial biogenesis [202] and ameliorates the age-associated decline in mitochondrial oxidative capacity in skeletal muscles [223] and other organs [23, 190, 340] via PGC-1α signaling [190, 341, 342]. PGC-1α is a redox-sensitive transcription factor that is activated by 5′-AMP-activated protein kinase (AMPK) [329, 343–345] to trigger the transcription of nuclear respiratory factor 1 (NRF-1) and expression of mitochondrial transcription factor A (mtTFA), a key regulator of mitochondrial DNA replication [346]. PGC-1α also increases the expression of antioxidant proteins such as glutathione peroxidase (GPX) and SOD-2 [347]. Safdar et al. [348] report that exercise reversed most of the multisystem pathology and premature mortality in mice which were genetically modified to accumulate mitochondrial mutations. The effects of exercise on AMPK and PGC-1α were preserved in the hippocampus of aging rats. However, results from Derbré et al. [341] suggest a blunted effect of exercise response in PGC-1α and NRF-1 in skeletal muscles of aged rats.

Effects of Exercise on Vascular Endothelial Cells. To meet the increasing metabolic demands of the body during exercise, perfusion of skeletal muscles and other tissues increases, subjecting vascular endothelial cells to higher levels of laminar shear stress. Increased laminar shear stress modulates gene expression and activity of SOD [349–351] possibly via NF-κB and MAPK signaling [300, 301].

Effects of Exercise on Antioxidant and Prooxidant Enzymes Expression and Activity. The NF-κB, AP-1, PGC-1α, and shear stress signaling cascades converge to upregulate antioxidant defense mechanisms to counteract and interrupt the vicious cycle of inflammation and oxidative stress associated with aging and cardiovascular diseases. The most intensely studied antioxidant enzymes in laboratory animals and in humans are SOD, catalase, GPx, glutathione transferase (GST), and glutathione reductase (GSR). The effects of exercise on antioxidant enzymes are summarized in Table 3.

Athletes' erythrocytes had higher SOD activity compared with untrained individuals [352, 353]. Regular and acute exercise increased SOD activity in erythrocytes of men and women [354, 355] and heart [23, 136, 292, 293, 316, 356–358], lung [23, 356], kidney [23, 136], brain [23], skeletal muscles [136, 311, 358], and arteries [359, 360] of experimental animals, particularly of aged animals [311, 316, 356, 358, 360].

(1) SOD-1. Just increasing *in vitro* shear stress is sufficient to upregulate the gene and protein expression of SOD-1 in human endothelial cells [349–351]. A study by Ennezat et al. [361] showed that regular exercise for 12 weeks increased gene expression of SOD-1 in skeletal muscles of congestive heart failure patients. Increases in protein expression of SOD-1 were observed after exercise in skeletal muscles [222, 362], heart [295, 363], brain [307], and arteries [194, 364, 365] of several experimental animals, importantly of aged animals [295]. However, other studies reported decreased or no change in expression of SOD-1 following long term exercise in aged animals [222, 366] and adult animals [367].

(2) SOD-2. Increases in activity have been observed in heart [230, 292, 296, 297, 368–370], skeletal muscles [371], plasma [137], and liver [372] of experimental animals following exercise. Increased protein expression has been reported in plasma and vascular endothelial cells of humans [133, 373] and in heart [230, 295, 296, 363, 374], skeletal muscles [362, 371, 375], liver [372], and arteries [138, 376] of experimental animals, notably in aged animals [138, 295, 296, 375].

(3) SOD-3. Higher activity was found in more physically active older men [133] and increased protein expression was observed in men after a bout of acute exercise but not endurance training [373]. However, increased protein levels were detected in experimental animals after endurance exercise [366, 367].

(4) Catalase. Winter swimmers had higher catalase activity in their erythrocytes than untrained subjects [353], while sprint-trained athletes exhibited lower activity than controls [352]. Exercise increased catalase activity in brain [23, 377, 378], heart [23, 136, 297, 316, 370], lung [377], skeletal muscles [136, 371, 377], liver [23, 126, 136, 356, 377], kidney [23], and cardiac mitochondria [379] of experimental animals and importantly in aged animals [126, 297, 316, 356, 377, 378]. However, other studies reported reduced [292, 311, 380] or no change in catalase activity after endurance exercise [293, 365].

(5) GPx. Athletes had higher activity than untrained subjects [352]. Also, physically active elders had higher activity than less active individuals in their erythrocytes [381]. Long term endurance exercise increased GPx activity in healthy adults [354, 355] and upregulated gene expression in congestive heart failure patients [361]. In experimental animals, increased activity was observed in heart [136, 356, 382, 383], liver [126, 136, 356, 372, 377, 382], lung [356, 377], kidney [136], brain [377, 378], testes [377], and skeletal muscles [311, 313, 371, 382–384], particularly in aged animals [126, 313, 356, 377, 378, 384]. Other investigators reported reduction [380] and no change [292, 293, 298] in GPx activity following endurance exercise. Increased GPx gene and protein expression following long term endurance exercise were also reported [307, 372].

Table 3: Effects of exercise training on expression and activity of antioxidant and prooxidant enzymes.

Enzyme	Species		Tissue	Exercise mode	Effect of physical activity/exercise	Reference
SOD	Human	Untrained males $n = 9$	Erythrocytes	High-intensity endurance training for 12 weeks	Activity increased after training	[354]
		Healthy young men and women $n = 17$	Erythrocytes	16 weeks of training then an acute bout of aerobic exercise for 30 min	Transient increase in activity after acute exercise	[355]
		Athletes $n = 18$ and sedentary control $n = 6$	Erythrocyte	Marathon or sprint training	Higher activity in sprint-trained athletes and marathon runners	[352]
		Winter swimmers $n = 40$ and controls $n = 36$	Erythrocyte	Regular winter swimming	Higher activity in winter swimmers	[353]
	Rats	Males, young and aged (17 months)	Lung, heart, and liver	Regular swimming exercise for 1 year	Increased activity in lung and heart of old rats relative to sedentary controls	[356]
		Males, young and aged	Heart	Treadmill endurance exercise for 2 months	Increased activity in young and aged rats	[316]
		Male, young, adult, and aged	Skeletal muscles	Exercise training for 10 weeks	Increased activity in deep vastus lateralis muscle of young rats only	[311]
		Males, 16-17 weeks	Heart and skeletal muscle	Sprint training on a treadmill for 6 weeks	Unchanged activity	[383]
		Females, 4 months	Ventricles	Endurance exercise training for 10 weeks	Increased activity	[293]
		Females, 17 weeks	Ventricles	High-intensity exercise treadmill for 10 weeks	Increased activity	[357]
		Males, adults	Heart	Treadmill training for 12 or 24 weeks	Increased activity after 24 but not 12 weeks	[292]
		Males, myocardial infarcted	Aorta	Treadmill training 5 times per week, 60 min/day for 11 weeks	Increased activity but not expression	[359]
	Mice	Males, aged 29–32 months	Aorta	Voluntary wheel running for 10–14 weeks	Increased activity	[360]
		Males and females, aged 28, 52, and 78 weeks	Bain, heart, liver, and kidney	Long term moderate-intensity treadmill exercise	Increased activity in all tissues at 52 but not 78 weeks old	[23]
		Females, 3 months	Kidney, heart, and skeletal muscle	Treadmill exercise for 8 weeks	Increased activity	[136]
		Males (6–8 weeks)	Ventricles	Swimming training for 14 weeks	No change in activity	[298]
	Microtus	Short-tailed field vole *Microtus agrestis*	Skeletal muscle and heart	Voluntary running over 1 or 7 days	Reduced activity in the heart	[401]

Table 3: Continued.

Enzyme	Species		Tissue	Exercise mode	Effect of physical activity/exercise	Reference
SOD-1	Human		Umbilical vein endothelial cells	Laminar fluid shear stress	mRNA and protein levels increased after 24 hours	[350]
			Endothelial progenitor cells	Shear stress	Increased mRNA expression and activity	[349]
			Aortic endothelial cells	Fluid shear stress	Increased protein expression	[351]
		Patients with congestive heart failure $n = 14$	Skeletal muscle	12 weeks of training	Increased gene expression	[361]
	Rats	Young (3 months) and aged (30 months)	Skeletal muscles	4.5 weeks of resistance exercise	Protein expression increased in young rats but decreased in old rats	[222]
		Males, young (3–5 months) and aged (24–27 months)	Skeletal muscles and heart	Exhausting treadmill running	Increased activity in skeletal muscles and heart of young rats and hearts of old rats	[358]
		Males, aged 24 months	Hearts	Treadmill training 30 m/min, 45 min/day, 5 days/week for 6 weeks	Protein expression increased	[295]
		Males, young (2 months) and old (22 months)	Soleus muscle feed arteries	Exercise training for 10–12 weeks	No change in protein expression	[366]
		Females, 12 months old	Hippocampus	Treadmill training for 15 weeks	Protein expression increased	[307]
		Females	Skeletal muscle	Acute bout of exhaustive treadmill exercise	Increased protein level but not mRNA or activity	[362]
		Males, adults	Heart	Acute session of treadmill running for 25–30 min	No change	[230]
		Females, adults	Ventricles	20 weeks of training	Increased protein expression	[363]
		Males, high caloric fed	Aorta and mesenteric artery	Running 60 min, 5 days/week for 12 weeks	Increased expression relative to sedentary controls	[364]
	Mice		Aorta	Treadmill running 15 m/min, 30 min/day, 5 days/week for 3 weeks	No change in protein expression	[367]
		Males, diabetic young	Aorta	Treadmill exercise for 10 weeks	Increased protein expression	[194]
	Pigs	Females	Aortic endothelial cells	Chronic exercise training for 16–19 weeks	Protein and activity increased	[365]

Table 3: Continued.

Enzyme	Species		Tissue	Exercise mode	Effect of physical activity/exercise	Reference
SOD-2	Human	Men $n = 18$	Plasma	Swimming or running for 3 months then a bout of acute exercise	Protein level increased by acute exercise	[373]
		Men 62 ± 3 years Physically active $n = 13$ and sedentary $n = 26$	Vascular endothelial cells from the brachial artery	Habitual aerobic exercise	Higher protein expression than sedentary men	[133]
	Rats	Males, 10-11 weeks	Cardiac mitochondria	Long term voluntary wheel running	Reduced activity	[402]
		Males, adults	Heart	Treadmill training for 12 or 24 weeks	Increased activity after 24 but not 12 weeks	[292]
		Males, adults	Heart	Acute session of treadmill running for 25–30 min	Activity increased at 0.5 and 48 h, and protein content increased at 48 h after exercise	[230]
		Females, 4 months	Heart	3–5 consecutive days of treadmill exercise [60 min/day at 60–70% maximal O_2 uptake]	Increased activity	[368]
		Females, 4 months	Ventricles	Treadmill exercise (60 min/day) at 25 degrees for 3 days	Increased activity	[369]
		Females, adults	Ventricles	20 weeks of training	Increased protein expression	[363]
		Males subjected to IR	Ventricles	3 consecutive days of intensive treadmill exercise 60 min/day, at 30 m/min	Increased activity of SOD-2 but not SOD-1	[370]
		Males, aged 24 months	Heart	Treadmill training 30 m/min, 45 min/day, 5 days/week for 6 weeks	Protein expression increased	[295]
		Males, young (4 months) and aged (21 months)	Heart	Acute exercise 60 min at 70–75% of maximum oxygen consumption	Activity increased in old rats	[297]
		Young (6 months) and aged 27 months	Left ventricle	Treadmill for 60 min/day, 5 days/week for a total of 12 weeks	Protein expression and activity increased in the aged group compared with sedentary control	[296]
		Females	Skeletal muscle	Treadmill running for 10 weeks	Increased activity and protein expression	[371]
		Females	Skeletal muscles	Acute bout of exhaustive treadmill exercise	Increased mRNA level in deep vastus lateralis muscle. Increased protein level in superficial vastus lateralis	[362]
		Male Zucker diabetic fatty rats (18 weeks)	Skeletal muscles	Swimming training for 6 weeks	Protein expression increased	[375]
		Males, 2 months	Plasma	Treadmill training 3 days/week for 14 weeks	Increased activity	[137]
		Males, obese Zucker	Liver	Treadmill running at 20 m/min for 1 h/day, 7 days/week, for 8 weeks	mRNA and protein levels and activity increased	[372]
		Male, young (3 months) and aged (23 months)	Aorta	Treadmill training for 12 weeks	Increased protein expression in aged rats	[138]
	Mice	Male, diabetic and young	Heart	Motorized exercise-wheel for 1 h/day, 5 days/week for 8 weeks	Increased protein expression	[374]
		Male, diabetic and young	Aorta	Motorized exercise-wheel for 1 h/day, 5 days/week for 8 weeks	Increased protein expression	[376]
	Pigs	Females	Aortic endothelial cells	Chronic exercise training for 16–19 weeks	No change in protein levels	[365]

Table 3: Continued.

Enzyme	Species		Tissue	Exercise mode	Effect of physical activity/exercise	Reference
SOD-3	Human	Men 62 ± 3 years Physically active $n = 13$ and sedentary $n = 26$	Vascular endothelial cells from the brachial artery	Habitual aerobic exercise	Higher activity than sedentary men	[133]
		Men $n = 18$	Plasma	Swimming or running for 3 months then a bout of acute exercise	Reduced protein level after endurance training but increased by acute exercise	[373]
	Rats	Males, young (2 months) and old (22 months)	Soleus muscle feed arteries	Exercise training for 10–12 weeks	Increased protein expression in old rats	[366]
	Mice		Aorta	Treadmill running 15 m/min, 30 min/day, 5 days/ week for 3 weeks	Increased protein expression	[367]
CAT	Human	Athletes $n = 18$ and sedentary control $n = 6$	Erythrocytes	Marathon or sprint training	Lower activity than controls in sprint-trained athletes	[352]
		Winter swimmers $n = 40$ and controls $n = 36$	Erythrocytes	Regular winter swimming	Higher activity in winter swimmers	[353]
	Rats	Males, young and aged (17 months)	Lung, heart and liver	Regular swimming exercise for 1 year	Increased activity in liver of old rats relative to sedentary controls	[356]
		Aged	Brain, liver, lung, muscle, and testes	Regular exercise	Increased activity in all tissues	[377]
		Males, young (4 months) and aged (21 months)	Heart	Acute exercise 60 min at 70–75% of maximum oxygen consumption	Activity increased in young and old rats	[297]
		Male, young and aged	Heart	Treadmill exercise for 2 months	Increased activity in young and aged rats	[316]
		Males, young (9 months) and aged (20 months)	Liver	Regular exercise	Increased activity	[126]
		Male, young (8 months) and aged (22 months)	Brain	Swimming 30 min/day, 5 days/week for 12 weeks	Increased activity in hippocampus in young and old rats	[378]
		Male, young (8 weeks), adult (12 months), and old (24 months)	Skeletal muscles	Exercise training for 10 weeks	Decreased activity in soleus muscle of adult and old rats	[311]
		Males (4 months)	Cardiac mitochondria	Treadmill for 16 weeks (5 days/week, 60 min/day, 25 m/min)	Increased activity	[379]
		Males, adults	Heart	Treadmill training for 12 or 24 weeks	Reduced activity after 24 but not 12 weeks	[292]
		Females, 4 months	Ventricles	Endurance exercise training for 10 weeks	No change in activity	[293]
		Males subjected to IR	Ventricles	3 consecutive days of intensive treadmill exercise 60 min/day, at 30 m/min	Increased activity	[370]
		Females	Skeletal muscle	Treadmill running for 10 weeks	Increased activity in deep vastus lateralis muscle	[371]
		Male, normotensive and hypertensive (11-12 weeks)	Liver, kidney, skeletal muscles, and heart	Treadmill running for 10 weeks	Reduced activity in all tissues in hypertensive and normotensive rats	[380]
	Mice	Males and females, aged 28, 52, and 78 weeks	Brain, heart, liver, and kidney	Long term moderate-intensity treadmill exercise	Increased activity in all issues at 52- but not 78-week-old mice	[23]
		Females, 3 months	Liver, heart, skeletal muscle, and salivary gland	Treadmill for a total of 8 weeks	Increased activity	[136]
	Pigs	Females	Aortic endothelial cells	Exercise training for 16–19 weeks	No change in protein level	[365]

Table 3: Continued.

Enzyme	Species		Tissue	Exercise mode	Effect of physical activity/exercise	Reference
GPX	Human	Untrained males $n = 9$	Erythrocytes	High-intensity endurance training for 12 weeks	Increased activity after training	[354]
		Healthy young $n = 17$	Blood	16 weeks of training then an acute bout of aerobic exercise for 30 min	Activity increased after regular training and transiently reduced following acute exercise	[355]
		Exercising and sedentary, young (21–38 years) and old (65–75 years) $n = 50$	Erythrocyte	Regular exercise	Higher activity in the exercising elderly compared to the sedentary elderly	[381]
		Athletes $n = 18$ and sedentary control $n = 6$	Erythrocyte	Marathon or sprint training	Higher activity in sprint-trained athletes	[352]
		Patients with CHF $n = 14$	Skeletal muscle	Moderate-intensity semirecumbent bicycle training for 12 weeks	Increased gene expression	[361]
	Rats	Males, young and aged (17 months)	Lung, heart, and liver	Regular swimming for 1 year	Activity increased in liver, lung, and heart of old rats relative to sedentary controls	[356]
		Aged	Brain liver, lung, muscle, and testes	Regular exercise	Increased activity in brain, liver, lung, and testes	[377]
		Male, young (8 months) and aged (22 months)	Brain	Swimming 30 min/day, 5 days/week for 12 weeks	Increased activity in hippocampus in young and aged	[378]
		Females, 12 months old	Hippocampus	Treadmill for a period of 15 weeks	Protein expression increased	[307]
		Females (24 months)	Skeletal muscles	Treadmill training (60 min, 5 days/week for 10 weeks)	Increased activity	[313]
		Male, young (8 weeks), adult (12 months), and old (24 months)	Skeletal muscles	Exercise training for 10 weeks	Increased activity in deep vastus lateralis muscle of young rats only	[311]
		Young (5 months) and aged (27.5 months)	Skeletal muscles	Treadmill training for 10 weeks	Increased activity in deep vastus lateralis muscle of old rats only	[384]
		Males, young (9 months) and aged (20 months)	liver	Regular exercise	Increased activity	[126]
		Males, obese Zucker	Liver	Treadmill running at 20 m/min for 1 h/day, 7 days/week for 8 weeks	mRNA and protein levels and activity increased	[372]
		Males, young	Liver, heart, and muscle	Swim training for 10 weeks	Increased activity in all tissues	[382]
		Females, 4 months	Ventricles	Endurance training for 10 weeks	No change in activity	[293]
		Males, adult	Heart	Treadmill training for 12 or 24 weeks	No change in activity	[292]
		Males, 16-17 weeks	Skeletal muscle and heart	Sprint training on a treadmill for 6 weeks	Increased activity in heart and some skeletal muscle fibres	[383]
		Females	Skeletal muscle	Treadmill running for 10 weeks	Increased activity in deep vastus lateralis muscle	[371]
		Male, normotensive and hypertensive (11-12 weeks)	Liver, kidney, skeletal muscles, and heart	Treadmill running for 10 weeks	Reduced activity in all tissues in hypertensive and normotensive rats	[380]
	Mice	Females, 3 months	Liver, kidney, and heart	Treadmill for a total of 8 weeks	Increased activity	[136]
		Males (6–8 weeks)	Ventricles	Swimming training for 14 weeks	No change in activity	[298]

Table 3: Continued.

Enzyme	Species		Tissue	Exercise mode	Effect of physical activity/exercise	Reference
GSR	Human	Healthy young $n = 17$	Plasma	16 weeks of training then an acute bout of aerobic exercise for 30 min	Activity increased after regular training	[355]
	Mice	Males (6–8 weeks)	Ventricles	Swimming training for 14 weeks	No change in activity	[298]
	Rats	Males, adults	Heart	Treadmill training for 12 or 24 weeks	No change in activity	[292]
		Males, 16-17 weeks	Skeletal muscle and heart	Sprint training on a treadmill for 6 weeks	Increased activity in heart and some skeletal muscle fibres	[383]
		Males, young	Liver, heart, and muscle	Swim training for 10 weeks	Increased activity in all tissues	[382]
		Aged	Brain liver, lung, muscle, and testes	Regular exercise	Increased activity in testes	[377]
		Males, young (8 weeks), adult (12 months), and old (24 months)	Skeletal muscles	Exercise training for 10 weeks	Activity increased in deep vastus lateralis muscle of young rats and decreased in soleus muscle of adult rats only	[311]
GST	Mice	Females, 3 months	Liver and salivary gland	Treadmill for a total of 8 weeks	Increased activity	[136]
NAD(P)H oxidase	Human	Patients with symptomatic coronary artery disease $n = 45$	Internal mammary artery	Aerobic training for 4 weeks	Reduced protein and gene expression and activity	[386]
		Men, 62 ± 3 years, physically active $n = 13$ and sedentary $n = 26$	Vascular endothelial cells from the brachial artery	Habitual aerobic exercise	Lower level of p47(phox) compared with sedentary men	[133]
	Rats	Males, young and myocardial infarcted	Aorta	Treadmill training 5 times per week, 60 min/day for 11 weeks	Reduced activity	[359]
		Male, adult (6 months) and aged (24 months)	Aorta	Swim training (60 min/day, 5 days/week for 10 weeks)	Decreased expression of gp91(phox)	[387]
	Pigs	Females	Aortic endothelial cells	Chronic exercise training for 16–19 weeks	Reduced protein expression of p67(phox)	[365]
	Mice	Young (6–8 months) and aged (29–32 months)	Aorta	Voluntary wheel running for 10–14 weeks	Reduced expression and activity	[360]
		Males, diabetic and young	Aorta	Treadmill exercise for 10 weeks	Decreased protein expression of gp91(phox)	[194]

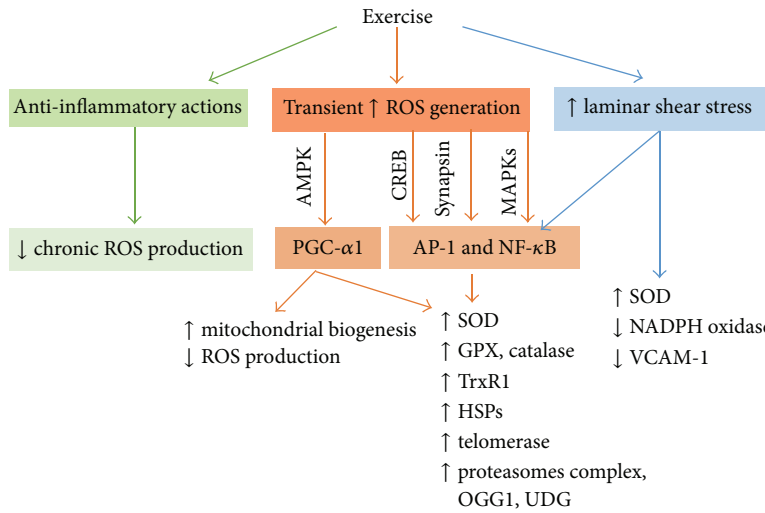

Figure 4: Signaling pathways underlying the antioxidant actions of exercise. AMPK = AMP-activated protein kinase, AP-1 = activator protein-1, CREB = cAMP-response-element binding, HSPs = heat shock proteins, GPX = glutathione peroxidase, MAPKs = mitogen activated protein kinases, NF-κB = nuclear factor kappa B, OGG1 = oxoguanine DNA glycosylase, PGC-1α = peroxisome proliferator-activated receptor gamma, coactivator 1-alpha, SOD = superoxide dismutase, ROS = reactive oxygen species, TrxR1 = thioredoxin reductase 1, and UDG = uracil DNA glycosylase.

(6) GSR. Increased activity was reported in humans [355] as well as in rats' brain, liver, lung, muscles, heart, muscle, and testes [311, 377, 382, 383]. Other studies reported that exercise training produced no change in GPx activity [292, 298].

(7) GST. Increased activity was reported in liver and salivary glands of mice after 8 weeks of treadmill training [136].

(8) Thioredoxin Reductase 1. Exercise increased thioredoxin reductase 1 (TrxR1), one of the thioredoxin system enzymes with direct and indirect antioxidant effects, in peripheral blood mononuclear cells in humans [205, 385].

(9) NAD(P)H Oxidase. Reduced gene [386], protein expression [194, 360, 365, 386, 387], and activity [359, 360] have been detected in humans [386] and experimental animals following endurance exercise. Also, the physically active elderly had lower NAD(P)H oxidase activity in their vascular endothelial cells compared with less active subjects [133].

Exercise-induced adaptation of antioxidant and prooxidant enzymes is highly isoform [296, 307, 370, 372, 373], tissue [311, 362, 371, 377, 378, 383], age [23, 138, 222, 297, 311, 366, 384], time course [23, 230, 292, 362], and exercise mode specific [352, 373, 388]. Exercise modulates the three SOD isoforms differently [296, 362, 365, 372, 373, 389] as the promoter region of SOD-2 contains more ROS-sensitive binding sites [390]. Exercise-induced protein expression of SOD is time dependent; SOD-1 protein expression was increased in rat skeletal muscles 48 hours after exercise, whereas SOD-2 protein content was increased after 10 and 24 hours but not 48 hours [362].

Effects of Exercise on Repair Mechanisms. Exercise can also stimulate the proteasome complex, which is responsible for the degradation of oxidatively damaged proteins [308, 391–393], and therefore enhances the cellular repair processes. Exercise modulates the activity of DNA repair enzymes, particularly oxoguanine DNA glycosylase (OGG1) and uracil DNA glycosylase (UDG), and thus reduces the accumulation of nuclear 8-hydroxydeoxyguanosine (8-OHdG) and mutations in skeletal muscles [314, 394, 395] but not brains of aged rats [396].

Effects of Exercise on Telomeres. Telomeres are often regarded as "the guardians of the genome." Telomere dysfunction activates p53, leading to suppression of PGC-1α and PGC-1β promoters with consequent metabolic and organ failure [397]. Ten cross-sectional and longitudinal studies described a positive association of physical activity with telomere length in immune cells and skeletal muscles [398]. The leukocyte telomere was 200 nucleotides longer in people who exercise regularly, which roughly corresponds to a ten-year increase in longevity [399]. Exercise increases the activity of telomerase and induces the expression of telomere repeat-binding factor 2 and Ku70 in thoracic aorta and leukocytes from mice and humans [400]. However, other studies showed no association or inverted U relationship of physical activity with telomere length [398] warranting further investigation. The signaling pathways underlying the antioxidant actions of exercise are summarized in Figure 4.

Exercise training confers a myriad of physiological benefits in aging and cardiovascular diseases through its antioxidant and anti-inflammatory actions. The inflammatory actions of exercise are mainly exerted on adipose tissue (by reducing its mass and inflammatory environment), on the immune system (by shifting immune cells towards the less inflammatory phenotype, modulating the cytokines profile, and stimulating glucocorticoids), on skeletal muscles

(by stimulating mitochondrial biogenesis, upregulating the anabolic myokine IL-15, anti-inflammatory cytokines, and repair proteins, improving muscle mass and strength, and reducing proinflammatory cytokines), and on the vasculature (by increasing laminar shear stress). It is likely that regular exercise exerts the most substantial anti-inflammatory effects in patients having high baseline inflammatory biomarkers, particularly when associated with visceral fat loss.

Exercise exerts antioxidant effects by suppressing inflammatory pathways and therefore inhibiting prominent sources of RONS generation. Importantly, exercise also activates redox-sensitive transcription factors, mainly NF-κB and AP-1 and PGC-1α, leading to the enhancement of the antioxidant defense mechanisms by enhancing the expression and activities of SOD, catalase, GPx, GSR, GST, and TrxR1, while downregulating NADPH oxidase. Exercise also upregulates repair proteins such as HSPs, proteasome complex, OGG1, UDG, and telomerase. It is clear that the effects of exercise vary depending on the type, intensity, frequency, and duration of exercise, and also on the individual's age, sex, fitness level, health status, and endurance capacity. More integrative and innovative research approaches such as proteomics and metabolomics should be utilized to reveal the whole map of the molecular transducers of exercise benefits and risks not only at tissue/organ level but also at the whole organism level. This will allow the development of personalized exercise program and hold the promise for transformative discoveries of novel therapeutic targets.

Conflict of Interests

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

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Review Article

Cardiovascular and Hepatic Toxicity of Cocaine: Potential Beneficial Effects of Modulators of Oxidative Stress

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Oxidative stress (OS) is thought to play an important role in the pharmacological and toxic effects of various drugs of abuse. Herein we review the literature on the mechanisms responsible for the cardiovascular and hepatic toxicity of cocaine with special focus on OS-related mechanisms. We also review the preclinical and clinical literature concerning the putative therapeutic effects of OS modulators (such as N-acetylcysteine, superoxide dismutase mimetics, nitroxides and nitrones, NADPH oxidase inhibitors, xanthine oxidase inhibitors, and mitochondriotropic antioxidants) for the treatment of cocaine toxicity. We conclude that available OS modulators do not appear to have clinical efficacy.

1. Introduction

Oxidative stress (OS) can be defined as an unbalance between the production of reactive oxygen and nitrogen species (ROS and RNS) and the compensatory response of physiological antioxidant mechanisms. The main ROS are superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl ($^{\bullet}OH$), hydroperoxyl ($HO_2^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), and ozone (O_3) while the main RNS are nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), peroxynitrite ($ONOO^-$), nitrous acid (HNO_2), dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3), and nitronium cation (NO^{2+}). The most important sources of ROS and RNS are represented by enzymatic reactions localized in the mitochondria, the microsomes (cytochrome P450 enzymes), the cytosol such as xanthine oxidase (XO), and the membrane-associated protein complex with its cytosolic subunits NADPH oxidase (Nox). The production of ROS in the phagocytes depends on the activity of peroxidases such as myeloperoxidase and eosinophil peroxidase.

It has been suggested that OS plays an important role in the physiopathology of various apparatuses and organs including the cardiovascular system (ischemia and reperfusion injury, heart failure, atherosclerosis, hypertension, etc.) and the liver (acute and chronic damage) [1, 2]. There is also evidence of significant involvement of OS in the pharmacological and toxic effects of drugs of abuse and particularly of psychostimulants such as cocaine and methamphetamine [3].

The level of OS in the aforementioned conditions can be measured by a number of biomarkers, including H_2O_2, NO derivatives (nitrite, nitrate, and S-nitrosothiols), isoprostanes (deriving from the peroxidation of arachidonic acid), MDA and other thiobarbituric acid reactive substances (TBARS), 4-hydroxynonenal (4-HNE), acrolein, thiol/disulfide ratio, oxidation products of DNA (8-hydroxy-2-deoxyguanosine, 8-OH-G) and RNA (8-hydroxyguanosine, 8-OHD), and nitrotyrosine. It is of note that, in several studies, cocaine-induced OS was evaluated by the measurement of TBARS [4–9] which is considered inferior to other methods for lipid peroxidation like the evaluation of F2-isoprostanes [10].

In the present paper, we review the literature concerning the cardiovascular and hepatic toxicity of cocaine with special attention to the role of OS and the evidences about the possible modulators of OS which could have beneficial effects in cocaine users.

2. Cardiovascular Toxicity of Cocaine

The earliest case reports of cardiovascular toxicity attributed to cocaine date from the 1980s [11–13]. Cocaine abuse is associated with both acute and chronic cardiovascular toxicity [14–16], including myocardial ischemia [13, 17] and infarction [18], arrhythmias [19], and cardiomyopathy [20–22]. Recent epidemiological data indicate that cocaine is responsible for a sizeable proportion of emergency department visits and of sudden deaths [23, 24]. Data from 19 European countries indicated more than 500 cocaine-related deaths in 2012 [25]. Approximately 5% to 10% of emergency department visits in the United States have been attributed to cocaine-acute toxicity, chest pain being the most common symptom [15]. The upward trend in cocaine-related chest pain and myocardial infarction cases has induced the America Heart Association to draft diagnostic and therapeutic guidelines [26]. Data from the relative National Cardiovascular Data Registry was recently published [27]. Histopathological studies have shown that cocaine can precipitate myocardial ischemia in the presence of coronary artery occlusion [28] as well as of normal coronary arteries [29]. A recent review [23] of 49 cocaine-related deaths identified coronary atherosclerosis, ventricular hypertrophy, cardiomegaly, myocarditis, and contraction band necrosis in almost a third of cases.

The pathogenesis bases of cocaine-induced cardiovascular toxicity [14, 30, 31] have been studied in detail [32, 33]. Cardiovascular cocaine toxicity can be related to its pathophysiological effects on the sinoatrial node, myocardium, and vasculature, including the coronary district.

2.1. Pathogenetic Mechanisms of the Cardiac Toxicity of Cocaine. Cocaine can damage the heart through a variety of mechanisms that have been elucidated only in part. In the first place, cocaine has a direct cardiotoxic effect, due its ability to block voltage-dependent K+ and Na++ channels in the sinoatrial node and the myocardium, leading to reduced contractility and to prolongation of the QT interval and the QRS complex. It has been proposed that these two effects may produce acute myocardial ischemia and infarction also in absence of long-term cocaine abuse, of abnormalities in the coronary arteries, and of other risk factors [34, 35].

Cocaine can exert its toxic effect on the heart also indirectly, through the actions of catecholamines, and in particular of norepinephrine. Indeed, cocaine is known to block the reuptake of catecholamines by binding the transporters for dopamine (DAT) and norepinephrine (NET) [36]. Increased norepinephrine levels in the terminals of the sympathetic nervous system lead to activation of adrenergic receptors. Activation of β1 adrenergic receptors located on the cells of the sinoatrial node and of the myocardium results in increased heart rate and contractility. Activation of α1 receptors located on the smooth muscle cells of blood vessels leads to vasoconstriction and increased blood pressure. Increased heart rate, heart contractility, and blood pressure may lead to an acute imbalance in oxygen supply/demand [37, 38]. Oxygen deficiency can be further exacerbated by the reduction in blood supply to myocardium produced by α1-mediated constriction of coronary arteries [13]. In turn, oxygen deficiency may lead to myocardial infarction. Furthermore, the combination of the direct toxic effect of cocaine and those of norepinephrine may lead to complex arrhythmia [39].

In addition to producing oxygen imbalance, catecholamines may damage the myocardium via at least three additional pathogenetic mechanisms [30, 40]. First catecholamines can promote cation translocation from the vascular space to intracellular compartment [41] thus reducing serum K+ [42] and Mg 2+ [43]. Concurrent hypokalaemia and hypomagnesaemia may lead, given the pivotal role of these cations in activity of the Na+/K+-ATPase pump [44], to further cation dyshomeostasis, which in turn may contribute to apoptosis and necrosis of cardiomyocytes. Moreover, this mechanism [45], adding to the direct arrhythmogenic effects of cocaine described above, may facilitate the development of arrhythmias such as atrial fibrillation and ventricular tachycardia.

A second pathway responsible for cardiotoxicity is related, as first hypothesized by Fleckenstein and Coworkers in 1974 [46], to catecholamines-induced calcium overload in cytosol and mitochondria of cardiomyocytes [41]. Indeed, stimulation of β-adrenergic receptors leads to activation of protein kinase A (PKA) and increased Ca^{2+} levels in the cytosol. This leads to phosphorylation of Ca^{2+}-protein substrates, including phospholamban, L-type calcium channel, ryanodine receptor, cardiac troponin I, and myosin-binding protein C [47, 48]. Increased cytosolic Ca^{2+} triggers the release of Ca^{2+} in the mitochondria. The mitochondrial role in physiological intracellular Ca^{2+} homeostasis as well as in necrosis and apoptosis signaling is well demonstrated [49]. Mitochondrial Ca^{2+} overload impairs respiration and ATP production and produces change in permeability of the mitochondrial membrane (eventually leading to its rupture), which represents critical events for the further structural degeneration of cardiomyocytes [50].

Moreover Ca^{2+} overload (as well as ROS mitochondrial production, see below) is responsible for the massive opening of mitochondria Permeability Transition Pores (mPTP) [51] resulting in further dysfunctional and structural degeneration of these organelles.

An indirect cardiotoxic effect of catecholamines may derive from action of their oxidation products, the aminochromes. Notably, excessive level of circulating catecholamines (and consequent saturation of the monoaminooxidase and catechol-o-methyl transferase systems) may cause the increased formation of adrenochrome (obtained by oxidation of adrenaline) [40, 52] of 5,6-dihydroxy-1-methylindole and of adrenochrome alkaline rearrangement product adrenolutin. In the heart the enzyme cytochrome c oxidase has been associated with adrenochrome formation [52].

Experimental studies investigating the relationship between aminochromes and cardiotoxicity [52, 53] demonstrated a direct toxic effect on cardiomyocytes: disturbances in cellular Ca^{2+} homeostasis and a perturbation of oxidative phosphorylation have been reported. Accordingly, increased levels of adrenolutin were observed in death-associated heart failure [54]. Moreover, aminochromes are known to induce redox cycling with consequent generation of ROS (see below).

More recently, OS and generation of ROS have been identified as one of the most important mechanisms of cocaine-induced cardiomyocyte toxicity [6, 30, 31, 55, 56]. Increased expression iNOS and decreased levels of myocardial SOD and catalase were found in the cardiomyocytes of patients with dilated cardiomyopathy related to chronic cocaine abuse [20]. Indeed, ROS formation has been thought to be related to cocaine-induced catecholamine release [56]: α_1 and β receptors stimulation, as well as enzymatic and nonenzymatic degradation of catecholamines, lead to intracellular ROS formation.

2.1.1. α_1-Adrenoceptors Stimulation and Production of ROS. In the plasma membranes of cardiac cells, α_1-adrenoceptors stimulation increases the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [57], an electron donor which in turn produces the superoxide anion radical $O_2^{\bullet-}$ formation [56, 58]. The role of NADPH-driven superoxide production in cocaine induced cardiac dysfunction is well recognized [59]. Indeed, the family of Nox enzymes is considered the major sources of ROS in the cardiovascular system [58, 60]: the proteins of the Nox family produce $O_2^{\bullet-}$ by transferring an electron from NADPH (or NADH) to O_2. It is believed that Nox1, Nox2, and Nox4 are expressed significantly in the vascular system [61, 62] while experimental data in cardiomyocytes had demonstrated that the Nox subunit Nox1, but not Nox2 or Nox4, is implicated in norepinephrine-induced Nox-generated ROS [63]. Inhibition of Nox activity by apocynin prevented the increase in ROS production and cardiac dysfunction induced by chronic administration of cocaine *in vivo* in rats [64]. Moreover, Nox activity is associated with other ROS-inducing enzymatic sources such as xanthine oxidoreductase (XOR): a fundamental role of XO was confirmed in an *in vivo* experimental model of cocaine-induced diastolic dysfunction [65], in which treatment with XO-inhibitor allopurinol prevented the cocaine-increase in mitochondrial ROS levels.

2.1.2. β-Adrenergic Receptors Stimulation and Production of ROS. As discussed above, β-adrenergic receptors (β-AR) stimulation activates a GTP-binding protein S, which stimulates adenylyl cyclase (AC) to produce cAMP, which in turn activates PKA. It has been demonstrated that β-AR stimulation may contribute to mitochondrial ROS production in cardiomyocytes: indeed the β-adrenergic agonist isoproterenol (ISO) increased, in a concentration- and cAMP-protein kinase A-dependent manner, mitochondrial $O_2^{\bullet-}$ production in freshly isolated mouse cardiomyocytes and induced a twofold increase of MDA protein adducts relative to controls in perfused hearts [66]. Accordingly, *in vivo* administration of mitochondria-targeted antioxidant MitoQ had shown to prevent left ventricular (LV) diastolic dysfunction (characterized by an increase in the index of LV relaxation, in LV end-diastolic pressure-volume relation, and in LV end-diastolic pressure) induced in rats treated with cocaine for 7 days [67]. Beta-AR stimulation induced both cAMP [68] and PKA increase both in bovine [69] and in rat [68, 70] cardiomyocytes mitochondria.

Experimental data in mouse cardiomyocytes [66] have demonstrated that β-AR exposure to agonist ISO leads to an increase in ROS, suggesting ROS production as a direct consequence of activation of the cAMP-PKA signaling pathway in mitochondria. Importantly, the increase in mitochondrial ROS production appeared to be Ca^{2+}-independent, since a selective increase of the amplitude of Ca^{2+} transient did not increase ROS production [66].

β-AR stimulation is also implicated in the impairment of antioxidant system. Indeed, significant reduction in CuZn-SOD enzyme activity has been found in hearts from ISO-treated rats as well as in ISO-stimulated isolated cardiomyocytes [71]. Moreover, a reduction in manganese-SOD (Mn-SOD) expression induced by chronic ISO was more recently found in type 5 AC (1 of 2 major AC isoforms in heart) transgenic mice [72].

2.1.3. OS from Catecholamines Metabolites (Aminochromanes). As mentioned above, when the enzymatic catabolism of catecholamines is not sufficient, they can undergo chemical oxidation causing additional OS [40]. The implication of aminochromanes in the pathogenesis of cardiac diseases [53, 73] is also well recognized.

Formation of superoxide anion $O_2^{\bullet-}$ (due to an oxidation pathway that involves the formation of highly reactive intermediaries *o*-semiquinones and *o*-quinones) [74] has been observed in both *in vivo* [75] and *in vitro* models [74, 76]. The superoxide anion $O_2^{\bullet-}$ can cause the oxidation of epinephrine [52, 77, 78] and of metal ions copper [79] and iron [77], enhancing the oxidation of catecholamines. Thus, iron chelation can protect against the cardiotoxicity induced by the metabolites of catecholamines: an *in vitro* study in rat ventricular cardiomyoblast assessing the potential cardioprotective effects of some chelating agents had suggested further investigation *in vivo* animal models [80].

Catecholamines metabolites can also reduce antioxidant defences by decreasing the levels of reduced glutathione (GSH) and increasing the oxidized glutathione (GSSG) content as observed in adrenaline-treated isolated rat cardiomyocytes [74, 79].

2.2. Pathogenetic Mechanisms of Cocaine Vascular Toxicity

2.2.1. Cocaine Effect on Vascular Smooth Muscle Cells. Cocaine sympathomimetic activity and consequent agonist action at α_1 adrenergic receptors (α_1-AR) in vascular smooth muscle cells cause contraction in the vascular system. The activation of these receptors leads in fact to the formation of inositol triphosphate and diacylglycerol (via phospholipase

C), which in turn cause an increase in Ca^{2+} entry and in release from Ca^{2+} stores in smooth muscle cells [81]. Despite the different contribution of each α_1-AR subtype [82, 83] in the regulation of contraction in different vascular districts, the net result of α_1-AR stimulation is represented by an acute increase in blood pressure.

2.2.2. Cocaine Effect on Endothelial Cells. A further contribution in the pathogenesis of cocaine-induced vasoconstriction is due to its acute and chronic effect on endothelial cells function [84]. An impairment in endothelium-dependent vasorelaxation, assessed as a decrease in forearm blood flow in response to intraarterial acetylcholine and nitroprusside, was found in long-term users of cocaine [85]. Accordingly experimental studies [86, 87] demonstrated a cocaine-induced endothelial dysfunction with a decrease in NO release and in the constitutive enzyme NO-synthase (eNOS) content, as well as an increase in endothelin-1 (ET-1) production and ET-1 receptor type-A (ET_AR) protein expression [84]. Increased number of circulating endothelial cells (CECs) indicating endothelial dysfunction was recently demonstrated in cocaine abusers [88]. Furthermore, enhancement of cocaine-induced vasoconstriction was observed after N(G)-nitro-L-arginine methyl ester-induced inhibition of NO synthesis *in vitro* [89].

Concomitant decrease in NO-induced vasodilatation and ET-1-induced vasoconstriction may enhance α_1-AR-mediated vasoconstriction. It has been suggested that inhibition of eNOS expression may partially derive from high levels of ET-1 through a PKC-mediated pathway in endothelial cells [90] and/or through ET-1 receptor type-A (ET_AR) stimulation, which in turn can increase ROS production [91, 92].

Chronic cocaine exposure and consequent endothelial dysfunction may precipitate early atherosclerosis [93] and the persistence of endothelial cell damage beyond its acute effect on the blood vessels can further increase cardiovascular risk in cocaine abusers [88, 94]. Since that ET-1 increase was significantly associated with atherosclerotic lesion [95], it may be argued that it plays a fundamental role in the cocaine-induced vasoconstriction at sites of significant stenosis [96].

Although discrepant results were also reported [97], probably due to methodological differences, there is some evidence indicating that cocaine may exert an inhibitory effect on the production of prostacyclin (PGI_2) by endothelial cells [98, 99]. Since the release of both vasodilatatory PGI_2 and NO may result from stimulation of ET-1 receptor type-B_1 (ETB_1) receptors on endothelial cells, it is possible that the inhibitory effect of cocaine on NO release also extends to PGI_2 release [100].

Another important factor that can contribute to cocaine prothrombotic action is its direct action on endothelial cells secretion of von Willebrand factor (VWF). Indeed, a recent *in vitro* study [101] demonstrated that cocaine and its metabolites induced VWF secretion in a concentration-dependent manner from three endothelial cell types (human umbilical vein, brain microvasculature coronary artery endothelial cells).

2.2.3. Cocaine Effect on Platelet Function. Whereas both experimental and clinical data agree on the fact that cocaine can affect endothelial function, leading to vasoconstriction, there is conflicting evidence about the direct effect of cocaine on platelets. Increased platelet activation was observed *in vitro* in rabbit platelet-rich plasma (PRP), preincubated with cocaine hydrochloride [102] as well as *in vivo* in dogs treated with intravenous cocaine [103]. The findings obtained with human platelets *in vitro* are less consistent. At concentrations comparable to the systemic concentrations produced by lethal doses, cocaine decreased platelet aggregation induced by agonists in PRP obtained from healthy human subjects [104]. In contrast, two *in vitro* studies conducted with cocaine concentrations comparable to the systemic concentrations associated with the "high" showed increased platelet activation in whole blood preparations [105, 106], whereas other studies found no effect of cocaine [107]. Finally, increased platelet expression of surface P-selectin was found in blood samples from chronic cocaine users [107]. It is possible that these discrepancies were due to differences in substrate (animal versus human platelets), in model (*in vitro* versus *in vivo*), in methodology (whole blood versus PRP), and in the concentrations of cocaine used.

In vivo studies in healthy subjects [108] and in chronic cocaine user [109], although not univocally [110], gave evidence of activation of platelets, assessed by increase of P-selectin expression [109] and of soluble CD40L, a transmembrane molecule mainly expressed by activated platelets [111]. Due to physiological interaction of platelets with vascular endothelium and circulating blood cells such leukocytes, it may be argued that an indirect, rather than a direct, mechanism of action is involved in the cocaine platelets activation. Indeed, the above mentioned cocaine-induced vasospasm, consequent increase in shear stress, and endothelial dysfunction may induce a platelet activation, as demonstrated by release of constituents of their α-granules [106] and of thromboxane A2 [99]. A further contribution may derive from VWF interaction with the platelet receptor GPIb and their subsequent activation [108].

2.2.4. OS and Cocaine-Induced Endothelial Toxicity. Besides cardiomyocytes, also endothelial cells (ECs) may release ROS, mainly due to its presence of enzymes such as XO, NOS, mitochondrial MAO, and NAPDH oxidase. Some data in literature indicate a direct or an indirect cocaine action on endothelial cells, both on enzymes expression and on mitochondrial function.

2.2.5. XO and Production of ROS. In bovine aortic endothelial cells, it has been observed that shear stress induced an enhancement of xanthine-dependent $O_2^{\bullet-}$ production [112], associated with a decrease in xanthine dehydrogenase (XDH) protein. Furthermore, inhibition of the Nox decreased XO levels and prevented the increase of $O_2^{\bullet-}$, highlighting the central role of Nox in modulating endothelial production of ROS. It may be suggested that vasoconstriction effect of cocaine and consequent blood shear stress may trigger endothelial ROS production via Nox/XO enzyme.

In agreement with this hypothesis, cocaine-induced cardiac dysfunction (alteration of cardiac output and stroke volume) was found to be associated with increased Nox and XOR activity *in vivo* study in rats [64]. Furthermore, apocynin or allopurinol treatment inhibited the cocaine-induced cardiac alteration and the myocardial production of $O_2^{\bullet-}$ confirming the role that Nox-derived ROS play in modulating ROS production by XO [64].

2.2.6. Nitric Oxide Synthase and Production of ROS. As mentioned above, cocaine-induced decrease in endothelial NO release and in the constitutive enzyme eNOS content has been observed [84]. A contribution to this cocaine endothelial toxic effect may derive from its increasing action in Nox, which in turn (besides the increase in $O_2^{\bullet-}$) may induce oxidation of tetrahydrobiopterin, a cofactor of NO synthase: as a consequence eNOS uncoupling leads to the observed reduction in NO synthesis and to an enhancement in $O_2^{\bullet-}$ [113].

2.2.7. ROS in Endothelial Mitochondria. Besides the well-recognized energy-producing activity, notably mitochondria are the major source of cellular reactive oxygen [114] both in cardiomyocytes and in EC [40, 115]. An important role of endothelial mitochondria in pathogenesis of endothelial dysfunction is due to their role in signaling cellular responses, among which the production of ROS [116].

2.2.8. Mitochondrial Monoaminooxidase and Production of ROS. Among the sources of mitochondrial ROS, the monoamine oxidase (MAO) family, located to the outer mitochondrial membrane, causing the oxidative deamination of catecholamines, results in hydrogen peroxide (H_2O_2) formation [117]. Accordingly, experimental data in literature indicate an increase in H_2O_2 cardiac production after the sympathomimetic drug amphetamine administration [118]. In this regard a recent *in vitro* study has demonstrated in human pulmonary EC [119] exposed to cocaine a significant increase in H_2O_2 production; due to the modulating action of H_2O_2 on endothelium functions such as endothelium-dependent vasorelaxation, apoptosis, and remodeling [120] it may be argued that cocaine-induced increase in catecholamines and in the consequent MAO-mediated H_2O_2 production in endothelial mitochondria could further enhance endothelial dysfunction, contributing to cocaine toxic effects on vascular district.

An important contribution to OS may derive from the process, namely, ROS-induced ROS release (RIRR) [120]: this process makes a significant amplification to ROS production [121]. Briefly, OS in the mitochondria may trigger the opening of mitochondrial transition pore that leads to a further increase in ROS generation. RIRR phenomena have been recognized in both physiological (promoting an elevation in the cell tolerance to OS, until the destruction of impaired-function mitochondria) [122] and pathological conditions such as cardiac ischemia-reperfusion [123] associated with acute myocardial infarction.

2.2.9. ROS Production by Mitochondrial Nox4. Another possible source of ROS in endothelial mitochondria is Nox4 [124], generating a higher hydrogen peroxide to superoxide ratio than Nox1 and Nox2. Nox4 involvement in endothelial cells process and in responses to hypoxia and OS is well recognized [125]; moreover it has been demonstrated that the expression or activity of Nox4 is increased in response to the proinflammatory mediators TNF-α [126]. While a cocaine-induced increase in Nox has been demonstrated in cardiac tissue [64], to date no data in literature are present on the effects of cocaine on Nox at endothelial level. An indirect effect of cocaine activation of Nox may derive from the cocaine induction of the TNF-α expression, observed in bovine aortic endothelial cells [127]. Moreover a Nox increase and the consequent endothelial superoxide production were also found in bovine aortic endothelial cells exposed to oscillatory shear stress [112].

3. Cocaine Hepatotoxicity

Cocaine abuse is known to induce acute [128–132] and chronic [130, 131, 133] liver toxicity. Clinical manifestations of cocaine-induced hepatic damage range from elevation of liver enzyme levels in chronic users [131, 133, 134] to acute liver failure associated with hepatitis [128], to fulminant liver failure associated with acute rhabdomyolysis [132] or thrombotic microangiopathy [128]. Histopathological examination has shown midzonal [132] and periportal [130] necrosis, as well as steatosis in the surviving hepatocytes [131]. The involvement of OS in cocaine liver toxicity has been reviewed recently [135, 136].

In vivo animal studies suggest the involvement of cocaine metabolites in the genesis of hepatotoxicity. Experiments conducted in rats [137] and mice [138, 139] have shown that cocaine-induced hepatotoxicity is at least partly dependent on cocaine N-demethylated metabolites norcocaine (NCOC), and N-hydroxynorcocaine (N-OH-NCOC) and norcocaine nitroxide (NCOC-NO$^{\bullet}$) [139, 140]. Inhibition of cytochrome P-450 (CYP450) mediated activation of cocaine metabolism produced a significant inhibition of hepatotoxicity in mice *in vivo* [138] and *ex vivo* in liver microsomes [141], while *in vivo* induction of CYP450 activity enhanced the cocaine hepatotoxicity [142]. Studies in mice demonstrated that cocaine reduces GSH levels in the liver [143, 144] and that depletion of intracellular GSH concentrations exacerbated cocaine hepatotoxicity [144]. Finally, it has been shown that pretreatment with the GSH precursor NAC exerted a protective effect against cocaine hepatotoxicity in mice [145], while pretreatment with endotoxin lipopolysaccharide (LPS) led in Kuppfer cells to the formation of NO which exacerbated cocaine toxicity [146].

Given that the synthesis of both NCOC-NO$^{\bullet}$ and GSH requires the presence of the cofactor NADPH, it has been hypothesized that a decrease in hepatocytes NADPH content and consequent impairment of antioxidant system may contribute to enhance OS [147].

The ability of cocaine to deplete intracellular GSH may also depend on the effects of its N-oxidative metabolites on the mitochondrial function of the hepatocytes. *In vivo* studies

in rats [148, 149] have shown that these metabolites can decrease GSH levels and membrane potential and increase ROS production in the mitochondria [9]. Mitochondrial generation of ROS has been shown also in isolated mouse liver mitochondria treated with NCOC, N-OH-NCOC, and NCOC-NO$^{\bullet}$ but not with cocaine [150], confirming the fundamental role of the N-oxidative metabolites of cocaine in OS-mediated hepatotoxicity.

Ex vivo and *in vitro* studies have confirmed the *in vivo* data. Cocaine-induced GSH depletion and GSSG production were observed in cultures of mouse and, to a lesser extent, in rat cocaine-treated hepatocytes [151, 152] with mouse being more sensitive to cocaine hepatotoxicity, as observed in cultured liver slices from different species [153]. In primary cultures of rat hepatocytes treated with the CYP-450 inducer phenobarbital, it has been observed that cocaine-induced alteration in the thiol redox equilibrium may be crucial for the development of hepatocyte toxicity and consequent LDH release [154]. Moreover cocaine-treated hepatocytes had been associated with decrease in catalase and Mn-SOD [155]. Further confirmation of implication of OS is derived from rat hepatocyte model of cocaine cytotoxicity in which a partial prevention of cytotoxicity by NAC [156] and by deferoxamine (a ferric iron chelator) was observed [152, 156].

In summary, human and experimental data provide evidence of direct toxic effects of cocaine on the hepatocytes. This hepatotoxicity appears to be dependent mainly on N-oxidative metabolites of cocaine. Impairment of the antioxidant system as depletion of intracellular and mitochondrial GSH also contributes to cocaine hepatotoxicity.

4. Potential Therapeutic Effects of OS Modulators in Cocaine Abuse

Antioxidants or modulators of OS as they are often referred to due to their possible prooxidant activity can be classified in low (LMW) and high (HMW) molecular weight and further subdivided into endogenous or exogenous. The exogenous ones can be natural or synthetic [2]. Several evidences indicate that some of these compounds could have beneficial effects on cocaine-induced toxic effects.

4.1. NAC. NAC has been used for many years in the treatment of acute paracetamol intoxication, as a mucolytic for chronic obstructive pulmonary disease and as a protectant in contrast-induced nephropathy. NAC can scavenge directly ROS and particularly the hydroxyl radical $^{\bullet}$OH and hypochlorous acid but due to its high first pass metabolism and low bioavailability acts mainly as a precursor of GSH in many organs, including the liver [145] and the brain [157]. As mentioned before, mice pretreatment with NAC had a protective effect against cocaine-mediated hepatotoxicity [146]. Importantly, NAC treatments appear to be safe and tolerable both in preclinical and in clinical studies [158, 159]. GSH is a very important endogenous antioxidant in the brain and it has been known for a long time that its level decreases in neuropsychiatric disorders such as depression, schizophrenia, and bipolar disorder [160] and is currently considered a promising drug for these pathological conditions [158, 160]. A recent double-blind placebo-controlled trial failed to demonstrate that NAC reduces cocaine use in cocaine-dependent subjects but showed some evidence that it can prevent relapse in individuals who had already achieved abstinence from cocaine [161]. Thus NAC may be useful as a relapse prevention agent in abstinent cocaine-dependent subjects [161].

The mechanism of action of NAC in the aforementioned conditions is complex and not completely elucidated. The main activities contributing to its efficacy seem to be related to (i) its capability of providing cysteine which is the limiting amino acid in GSH production; (ii) the exchange of extracellular cysteine provided by NAC with intracellular glutamate by the cysteine/glutamate transporter causing the activation of presynaptic mGluR2/3 receptors which inhibit glutamatergic neurotransmission and excitotoxicity [162, 163]; (iii) direct antioxidant activity related to its capability of scavenging radicals such as hydroxyl and peroxynitrite [164]; (iv) induction of the expression of antioxidant enzymes through the nuclear E2-related factor (Nrf2)/ARE system [165]. It is of note that the Nrf2 pathway represents a promising therapeutic approach to restore the redox balance in the CNS and in other organs and that only a few Nrf2-activating compounds have been tested in a clinical setting until now [166].

4.2. SOD Mimetics. SOD is a very important antioxidant enzyme in mammals existing in three different physiological forms: MnSOD in the mitochondrial matrix, CuZnSOD in the cytoplasm, and CuZnSOD in extracellular fluids [167]. Given the central role of the superoxide radical in oxidative pathological phenomena, different drugs with SOD activity were developed [168].

An infusible form of human manganese SOD (rMn-SOD) as new therapeutic option capable of crossing cell membranes was recently proposed for dilated cardiomyopathy caused by cocaine [20].

4.3. Nitroxides and Nitrones. Most antioxidants acting as spin traps have a nitroxide or nitrone nucleus.

Nitroxides are nonmetal catalytic antioxidants. One of the most common is Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) with SOD and catalase enzymatic activities which can be administered orally and can cross biological membranes and react with ROS intracellularly and within mitochondria [169].

Nitrones are potent antioxidants capable of forming stable nitroxyl radicals when reacting with oxygen radicals. Several nitrones, including α-phenyl-tert-butylnitrone (PBN), have been shown to have neuroprotective properties in experimental animal models of stroke, Parkinson's and Alzheimer's disease [170], as well as in preclinical models of CNS injury [171]. Treatment with Tempol can attenuate OS in the prefrontal cortex and in the nucleus accumbens [172] and inhibited cocaine self-administration *in vivo* in rats, suggesting that reinforcing effects of cocaine are mediated, at least in part, by ROS [173].

To the best of our knowledge, no data are present in literature on efficacy of nitroxides and nitrones on cocaine-induced cardiac or hepatic injury.

4.4. Nox Inhibitors. As mentioned above, Nox are among the best characterized sources of superoxide and the predominant ROS producing enzymes in the vascular smooth muscle cells, in the myocardium, and in blood vessels [174]. Hypertension, atherosclerosis, hyperlipidemia, and diabetes are associated with endothelial dysfunction probably mediated by Nox driven production of ROS [175]. Compared to radical scavengers, Nox inhibitors could be more effective because they are able to block the formation of ROS at the source [176].

Several Nox inhibitors are currently available, including apocynin, which is an orally active natural compound obtained from the roots of *Picrorhiza kurroa* capable of inhibiting Nox activation probably by preventing the assembly of Nox2 with p47phox [177, 178].

Importantly it was reported that apocynin can prevent cocaine-induced myocardial damage in rats [64] providing a good evidence for the therapeutic potential of Nox inhibitors in the treatment of cocaine cardiotoxicity [179].

4.5. XO Inhibitors. Allopurinol is a well-known inhibitor of XO used for decades for the treatment of gout [180]. It can prevent OS by inhibiting XO and also by scavenging directly hydroxyl free radicals.

Clinical studies showed that allopurinol has beneficial effects in chronic kidney disease patients [181]. XO inhibition by allopurinol (or the active metabolite oxypurinol) could be beneficial in hyperuricemic patients with congestive heart failure [182]. Unfortunately, allopurinol is not devoid of side effects such as severe skin reactions and renal impairment [182].

Allopurinol and 1,3-dimethylthiourea, a scavenger of hydroxyl radical, produced a potent inhibition of hepatotoxicity induced by cocaine in adult male mice [183].

Apocynin or allopurinol prevented cocaine-induced cardiac alteration by restoration of cardiac output, stroke volume, and fractional shortening, associated with a reduction of the myocardial production of superoxide anions and an enhancement of catalase activity [55] suggesting that NADPH and XO can act synergically to form myocardial ROS and that their inhibition could prevent the onset and progression of cocaine-induced left ventricular dysfunction [55].

4.6. Mitochondriotropic Antioxidants. Cationic triphenylphosphonium (TPP) derivatives such as MitoQ have high affinity for the inner mitochondrial membrane that is negatively charged. They can cross the blood-brain barrier even if the uptake in the brain appears less than for other organs [184]; TPP compounds can be administered orally and did not raise safety concerns so far [184]. MitoQ was protective in a large number of cell models of mitochondrial OS and has been shown to be well tolerated, orally active, and safe in humans [185].

Cocaine can be toxic for mitochondria, causing formation of ROS, impaired electron transfer, suppression of ATP production, membrane permeabilization with release of cytochrome C, and subsequent cell death: MitoQ can prevent these abnormalities protecting against cardiac dysfunction [67].

Preparation of small cell-permeable peptides (SS-peptides) which accumulate in the mitochondria and bind to the inner membrane thanks to the presence of basic amino acids positively charged at physiological pH had shown to protect mitochondria against oxidative damage [185, 186]. The use of these peptides to deliver antioxidant molecules into mitochondria and provide protection against cocaine induced OS has been proposed [187].

4.7. Deferoxamine. NAC and deferoxamine (DFO, an iron chelating agent that prevents ROS generation by inhibiting the Fenton reaction) protected rat hepatocytes in culture against the OS induced by cocaine, confirming the involvement of oxygen radicals in cocaine-induced necrosis/apoptosis [188]: hepatocytes were preincubated for 24 h either in the presence or in the absence of NAC or DFO and exposed for another 24 h to increasing concentrations of cocaine together with NAC or DFO. Pretreatment with deferoxamine complex with iron ion (III), aminoguanidine, and N-methyl-d-glucamine dithiocarbamate complex with iron ion (II) produced a marked inhibition of the hepatotoxicity induced by a single administration of cocaine, as indicated by histopathological examination, alanine aminotransferase activity, and nitrite/nitrate levels [183].

4.8. Selenium. In rats, pretreatment for 4 weeks with selenium reversed both the OS and the contractile dysfunction induced by repeated (7 days) cocaine administration in rats [189]: cardiac function was evaluated by cardiac index and left ventricular fractional shortening measured by echocardiography.

4.9. Minocycline. Minocycline, a second generation tetracycline, has been found in rats to prevent cardiac myocyte death (associated with an increase in OS evidenced by low GSH/GSSG ratio and increased levels of 4-hydroxy-2-nonenal) induced by prenatal cocaine exposure [190].

4.10. Auranofin. Pretreatment of mice with auranofin (a well-known disease-modifying gold compound used for rheumatoid arthritis recently proposed also for other therapeutic applications) [191] showed an interesting protective effect against hepatic injury caused by cocaine by inducing overexpression of heme oxygenase-1 (HO-1), an important OS marker responsible for heme degradation [192]. Unfortunately, auranofin is not devoid of toxic effects [193] but this result indicates that it could be worthwhile to search for less toxic inhibitors of HO-1 to reduce hepatic injury induced by cocaine.

4.11. Amiodarone. Amiodarone is a class III antiarrhythmic drug used in heart failure and postischemic heart capable of protecting cardiac myocytes against OS [194]. However, amiodarone pretreatment did not affect the seizure incidence

or mortality in mice treated with high doses of cocaine [195]. The efficacy of amiodarone in the treatment of cocaine-induced dysrhythmias is still undefined [196] and further studies should be performed on this and other antiarrhythmic drugs.

4.12. Trolox. Trolox, a hydrophilic analog of vitamin E with well-known antioxidant properties [197], was found to reduce the production of ROS induced by exposure to cocaine and the neurotoxic effect of the HIV-1 transactivating protein Tat [198].

5. Conclusion

In the present paper, several studies were reported demonstrating the important role of OS in the activity and toxicity of cocaine with special attention to its cardiovascular and hepatic effects. Several evidences were reported showing the potential beneficial effects of antioxidants or modulators of OS (as they are referred to considering their possible prooxidant effects in certain conditions) even if it should be kept in mind that, like in the case of NAC, their mechanism of action, which is often complex and not always fully elucidated, is not only related to their antioxidant activity. Unfortunately, for most of these modulators of OS the preclinical and clinical studies are limited and do not allow drawing definitive conclusions on their efficacy in relation to cocaine toxicity. Even in the case of NAC limited clinical trials were performed in cocaine users and the biomarkers of OS were not considered with sufficient attention.

Other studies should then be performed also considering the difficulty to demonstrate the clinical efficacy of modulators of OS. In fact, several clinical trials on antioxidants in pathological events such as neurodegenerative, cardiovascular, or pulmonary diseases, conditions associated with ischemia and reperfusion injury, chronic kidney disease, or psychiatric disorders failed in the last few years for different possible reasons including [1, 2]:

(1) In physiological conditions, OS is controlled by a complex mixture of endogenous and exogenous antioxidants (*lipophylic* or hydrophilic, enzymatic or nonenzymatic) and when used therapeutically one single antioxidant may not be sufficient. The challenge is then to design trials with the right mixtures of substances at the right doses.

(2) Certain conditions probably require the delivery of higher amounts of antioxidants to specific organs or tissues or organelles such as the mitochondria.

(3) The dose and/or duration and/or regimen and/or timing of the treatment should be appropriate.

(4) Some antioxidants may have prophylactic activity but not be very effective after the onset of the condition. If the stage of the disease is too advanced, patients will not probably receive benefit from the therapy.

(5) Most clinical trials were performed without a proper selection of the patients based on validated biomarkers of oxidative stress. Furthermore, whole body OS markers may not be appropriate for specific diseases in which OS involves specific tissues or organs or organelles.

(6) Some antioxidants can act as prooxidants in certain conditions or have adverse side effects.

In conclusion the effect of modulators of OS on the activity and toxicity of cocaine should be further studied in order to develop new drugs useful for the treatment of OS-mediated cocaine toxic effects.

Abbreviations

CNS: Central nervous system
GSH: Glutathione
HO: Heme oxygenase
MDA: Malondialdehyde
NAC: N-Acetylcysteine
Nox: NADPH oxidase
NO: Nitric oxide
OS: Oxidative stress
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SOD: Superoxide dismutase
XO: Xanthine oxidase.

Conflict of Interests

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

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Review Article

Oxidative Stress in Shiga Toxin Production by Enterohemorrhagic *Escherichia coli*

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Virulence of enterohemorrhagic *Escherichia coli* (EHEC) strains depends on production of Shiga toxins. These toxins are encoded in genomes of lambdoid bacteriophages (Shiga toxin-converting phages), present in EHEC cells as prophages. The genes coding for Shiga toxins are silent in lysogenic bacteria, and prophage induction is necessary for their efficient expression and toxin production. Under laboratory conditions, treatment with UV light or antibiotics interfering with DNA replication are commonly used to induce lambdoid prophages. Since such conditions are unlikely to occur in human intestine, various research groups searched for other factors or agents that might induce Shiga toxin-converting prophages. Among other conditions, it was reported that treatment with H_2O_2 caused induction of these prophages, though with efficiency significantly lower relative to UV-irradiation or mitomycin C treatment. A molecular mechanism of this phenomenon has been proposed. It appears that the oxidative stress represents natural conditions provoking induction of Shiga toxin-converting prophages as a consequence of H_2O_2 excretion by either neutrophils in infected humans or protist predators outside human body. Finally, the recently proposed biological role of Shiga toxin production is described in this paper, and the "bacterial altruism" and "Trojan Horse" hypotheses, which are connected to the oxidative stress, are discussed.

1. Introduction: Enterohemorrhagic *Escherichia coli* Strains and Shiga Toxin-Converting Phages

Escherichia coli is a bacterial species commonly known as a commensal occurring in the mammalian intestine [1]. This is true in most cases; however, some *E. coli* strains are capable of causing disease in humans. One example of pathogenic *E. coli* is a series of strains called Shiga toxin-producing *E. coli* (STEC) [2, 3].

Among STEC strains (defined as *E. coli* producing Shiga toxins), the most dangerous for humans is the subset classified as enterohemorrhagic *Escherichia coli* (EHEC, defined as *E. coli* causing bloody diarrhea) [2, 3]. Infection of humans by EHEC strains causes hemorrhagic colitis (HC) and in some patients it may result in various complications, including the most severe of them, the hemolytic-uremic syndrome (HUS) [2]. The most common symptoms of this syndrome are acute renal failure, anemia, and thrombocytopenia; however, other organs such as lung, pancreas, and heart may also be affected [4]. Furthermore, some patients suffer from the disorders of the central nervous system [4].

The main virulence factors causing EHEC-mediated HUS are Shiga toxins, produced by the infecting bacteria. These toxins are hexameric proteins, composed of a single A-subunit and five identical B subunits [5]. The main receptor, called Gb3 and occurring on the surface of many types of eukaryotic cells, is recognized by the B-subunits. The toxin enters cells by endocytosis, which is followed by its retrograde transport from the early endosome through the Golgi-apparatus and to the endoplasmic reticulum. The specific proteolytic cleavage of the A-subunit results in the release of

the A1 polypeptide from the A2 fragment attached to the B pentamer. A1 is the actual toxin that is translocated from the ER to the cytoplasm [6, 7]. The Shiga toxin A1 polypeptide is an N-glycosidase that depurinates a single adenine residue (A4324) within the α-sarcin/ricin loop of the 28S rRNA [8, 9]. This modification results in an inhibition of amino-acyl-tRNA binding to the ribosome and cessation of protein synthesis, which leads to cell death [5].

Since cattle are resistant to Shiga toxins, due to the lack of the Gb3 receptor, they serve as a natural reservoir of STEC strains. However, any cattle-derived products contaminated by STEC, and particularly EHEC, may cause severe human infections, occurring usually as outbreaks. A few years ago (in 2011) such an outbreak took place in Germany, where over 4,000 patients developed severe symptoms and 54 of them died [10–15]. Contaminated fenugreek and lentil sprouts were recognized as the source of the infection [13], indicating that unwashed or improperly washed vegetables, especially those coming from the so-called "ecological farming" where only natural fertilizers (including those coming from cattle) are used, may be a significant source of such outbreaks. In that case, the strain of the O104:H4 serotype, which caused the outbreak, had a combination of virulence factors characteristic of enteroaggregative *E. coli* (EAEC) and EHEC [12, 14]. The high virulence of this particular strain could be ascribed to enhanced adhesion, survival adjustment, antibiotic resistance, and Shiga toxin production [12].

Interestingly, genes coding for Shiga toxins (*stx* genes) are located in genomes of prophages rather than in actual bacterial genome [16, 17]. Bacteriophages bearing the *stx* genes, called Shiga toxin-converting phages or Stx phages, can lysogenize *E. coli* strains making them STEC. All Stx phages described to date belong to the family of lambdoid phages, viruses having genomes organized in a manner similar to that found in bacteriophage λ [17]. The genome of a lambdoid phage consists of blocks of genes coding for proteins responsible for specific functions. This makes recombination and exchange of genes between various phages relatively easy and leads to mosaicism of genomes of lambdoid phages [18]. In genomes of Shiga toxin-converting phages, the *stx* genes are present between the *Q* antiterminator gene and the genes coding for proteins causing cell lysis (Figure 1(a)).

As long as the Stx bacteriophage is present in the *E. coli* host as a prophage, vast majority of its genes, including *stx* genes, are silent due to the repression caused by the phage-encoded cI protein [19–21]. Under such conditions, Shiga toxin is not produced. Effective expression of *stx* genes, together with all genes required for lytic development of the bacteriophage, occurs only after prophage induction, though Shiga toxin 1 may also be produced under conditions of low iron levels due to the presence of the Fe-sensitive promoter upstream of the *stx1* locus [17, 20]. The prophage induction occurs generally due to activation of the bacterial SOS response which is a defensive mechanism provoked by any conditions causing appearance of single-stranded DNA fragments. The RecA protein recognizes such fragments and is activated to stimulate the self-cleavage of the LexA repressor (bearing the peptidase domain in its structure), which under normal conditions inhibits expression of the SOS regulon (Figure 1(b)). However, the phage cI repressor resembles LexA (Figure 2) and it is also degraded under the SOS stress response, causing derepression of bacteriophage promoters, excision of the prophage, and subsequent lytic development of the virus. Importantly, in the case of Stx phages, expression of the *stx* genes proceeds together with other phage genes [17, 20, 21] (Figure 1(a)). It is worth mentioning that RecA-independent induction of Shiga toxin-converting prophages by chelating agents, like EDTA, has also been reported [22]. In conclusion, production of Shiga toxins requires induction of Stx prophages, caused by either any stress conditions provoking the SOS response or by chelating agents.

Under laboratory conditions, induction of lambdoid prophages is relatively easy, and standard methods for the efficient induction include UV-irradiation and treatment with antibiotics that interfere with bacterial DNA replication, like mitomycin C [20, 21]. Such treatments lead to prophage excision in a large fraction, if not most, of lysogenic cells in a bacterial population. Nevertheless, when infection of humans by EHEC is analyzed, one should consider prophage induction conditions which can naturally occur in human intestine. Obviously UV-irradiation is very unlikely there, and high concentrations of antibiotics may be administered only to patients subjected to intensive therapy, while symptoms of EHEC infection appear also in nontreated persons. Moreover, other inducers of prophage excision, like EDTA [22], irradiation with ^{60}Co [27], or high hydrostatic pressure [28], are also unlikely to occur in the human gut. Therefore, an important question arose: what are factors or agents that can induce Shiga toxin-converting prophages in EHEC-infected human intestine? Understanding the mechanism of stimulation of Shiga toxin production might lead to development of novel methods for prevention or treatment of EHEC-caused diseases, as well as deciphering a biological role for maintaining the Stx prophages in bacterial genomes.

2. Hydrogen Peroxide as an Inducer of Shiga Toxin-Converting Prophages

There were various attempts to find conditions which both induce Stx prophages and are likely to occur in the human gut. Different conditions, factors, and agents (including high and low temperatures, high salt concentrations, chelators, ^{60}Co, high hydrostatic pressure, nitric oxide, and starvation) were tested, and the results of these studies have been summarized [17]. Most of the tested conditions either did not induce lambdoid prophages or were unlikely to occur in human intestine.

Under conditions of bacterial infection, including infection of the human gut, neutrophils are the first cells of the immune system which attack the pathogens. Among other bactericidal mediators, neutrophils excrete hydrogen peroxide to weaken bacterial cells. This oxidative stress-inducing agent is dangerous for bacteria that are much more sensitive to it than eukaryotic cells. However, it was demonstrated that such an action of neutrophils enhances production of Shiga toxins by EHEC strains [29]. Subsequent studies indicated

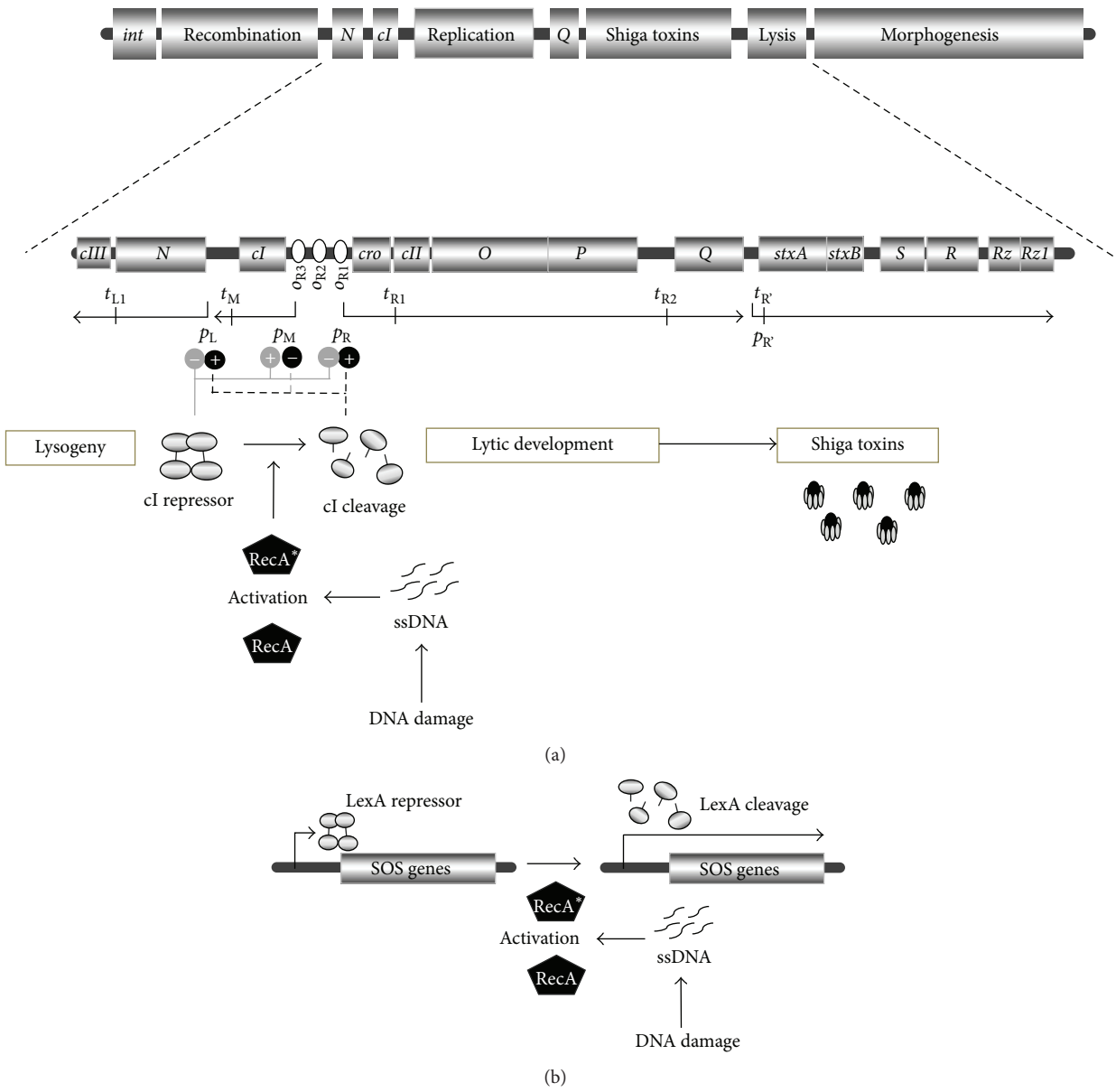


FIGURE 1: Schematic map of a Shiga toxin-converting phage genome. At the top of (a), regions bearing genes for particular phage functions are shown (as they appear in a prophage). The region containing genes involved in regulation of phage development, DNA replication, Shiga toxin production, and cell lysis is enlarged and shown in more detail. Major transcripts are shown by arrows, with arrowheads demonstrating directionality of transcription, and promoters marked by short vertical lines at the beginning of transcripts. Terminators are marked by vertical lines crossing the transcript lines. The cI repressor binds to o_{R1}, o_{R2}, and o_{R3} operator sites, repressing p_L and p_R promoters and stimulating its own promoter p_M. When DNA is damaged, single stranded DNA (ssDNA) fragments appear which are recognized by RecA protein. This activates RecA to switch to the RecA* form, able to stimulate self-cleavage by the cI repressor. Inactivated cI can no longer repress p_L and p_R and p_M is not activated. This leads to effective transcription from p_L and p_R, prophage excision, and expression of vast majority of phage genes, including those coding for Shiga toxin. (b) represents a similar mechanism leading expression of the SOS regulon which under normal growth conditions is repressed by the LexA protein. Phage cI repressor resembles LexA; thus under conditions of the SOS response, induction of the prophage occurs.

that hydrogen peroxide, when added to cultures of bacteria lysogenic for various Shiga toxin-converting phages, is a potent inducer of the prophages [30]. This was true for bacteriophage λ as well as for different Stx phages. Moreover, the prophage induction was accompanied by synthesis of considerable amounts of the fusion protein, encoded by a gene located in the place of the natural *stx* locus [30]. Very similar results were obtained when natural isolate of EHEC was tested instead of laboratory strains. Again, hydrogen peroxide-mediated induction of the Stx prophage and efficient production of Shiga toxin were observed [31]. Therefore, the oxidative stress, mediated by hydrogen peroxide, leads to

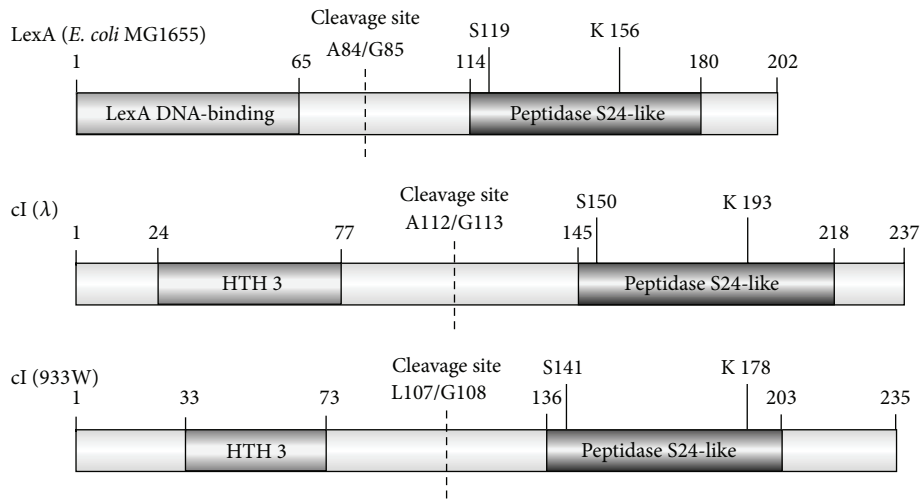


FIGURE 2: Comparison of domain structures of *E. coli* LexA protein and cI repressors of bacteriophage λ [19] and Shiga toxin-converting bacteriophage 933W [23, 24]. Two domains of these proteins are shown, and crucial amino acid residues are marked. Upon stimulation by the activated form of RecA (RecA*) both LexA and cI cleave their own molecules (at indicated positions) by the peptidase S24-like domains. The models were prepared using the DOG 1.0: Illustrator of Protein Domain Structures software [25].

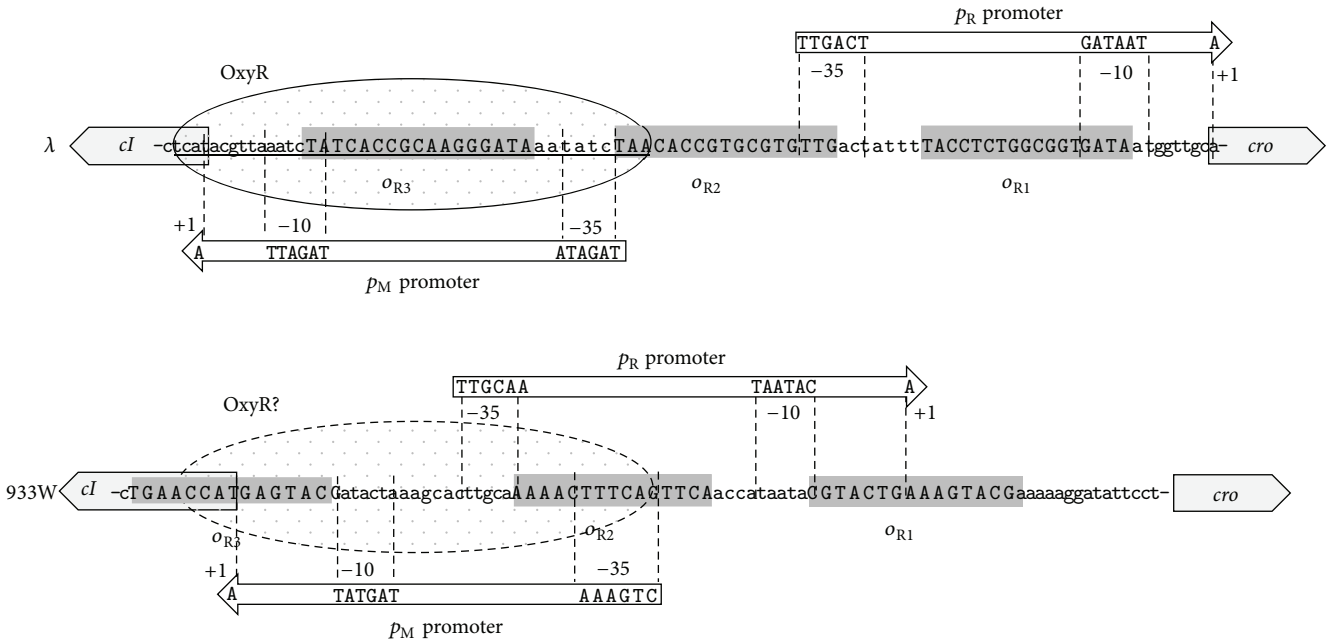
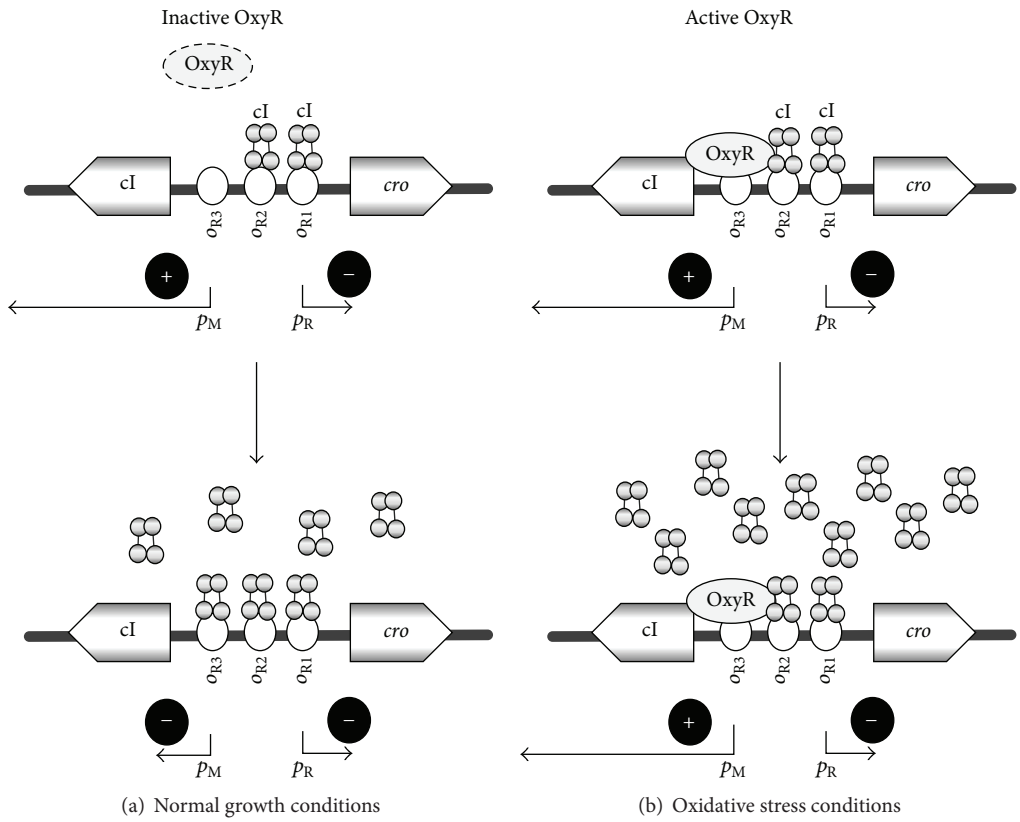


FIGURE 3: The p_R-p_M regions of phage λ and Shiga toxin-converting phage 933W. Structural elements of each promoter are indicated, and o_{R1}, o_{R2}, and o_{R3} operator sequences are marked. The OxyR binding to the λp_R-p_M region (demonstrated experimentally [26]) is shown as a solid oval, and a putative (not verified experimentally) OxyR binding to the 933W p_R-p_M region is suggested by a dashed oval.

stimulation of expression of *stx* genes. Since such conditions can occur in human intestine, the oxidative stress is a likely candidate for a natural inducer of Shiga toxin-converting prophages.

An interesting observation in studies on both laboratory Stx lysogens and natural isolates of EHEC was that the maximal efficiency of prophage induction occurred at a final H_2O_2 concentration of 3 mM, and further increases in H_2O_2 concentrations caused a decrease in induction efficiency [30, 31]. Moreover, while prophage induction by UV-irradiation or mitomycin C caused a lysis of bacterial cultures in a few hours, no such phenomenon could be detected after treatment with hydrogen peroxide [30]. Subsequent calculations of the efficiency of prophage induction have shown that while low concentration (1 μg/mL) of mitomycin C caused initiation of the lytic phage development in about 10–30% of cells (depending on the kind of the Stx phage), the value of this parameter was as low as 0.03–1.6% at the



(a) Normal growth conditions (b) Oxidative stress conditions

Figure 4: A model for OxyR-mediated modulation of lambdoid prophage maintenance under normal growth conditions and oxidative stress. Under normal growth conditions (a), the OxyR protein is inactive. The cI protein binds to o_{R1} and o_{R2} operators, repressing p_R and stimulating p_M. At high concentrations, cI binds also to o_{R3} which caused repression of p_M. Under oxidative stress conditions (b), OxyR is activated and binds to the o_{R3} region. This stimulates binding of cI to o_{R1} and o_{R2}, enhancing p_R repression and p_M stimulation, and when cI concentration increases, p_M repression is prevented by competitive binding to o_{R3} by OxyR. This results in higher activity of p_M than that under normal growth conditions, increased levels of cI, and more efficient maintenance of the prophage.

optimal (for prophage induction, i.e., 3 mM) concentration of hydrogen peroxide [32]. Therefore, in H_2O_2-treated lysogenic bacteria, only a very small fraction of cells (usually less than 1%) is induced for prophage excision and subsequent lytic development. Since the rest of bacterial population in the culture can grow and propagate due to resistance to infection by the same phage as the prophage present inside the cell, it is not possible to observe culture lysis.

Another question was what is the mechanism causing the low efficiency of prophage induction under conditions of oxidative stress? Studies on bacteriophage λ, the best known representative of lambdoid phages, indicated the factor responsible for such a phenomenon. Since DNA sequences of the regulatory regions of λ and Stx phages are very similar [33], one can suppose that the processes occurring in these phages are generally the same.

It was demonstrated that the prophage induction by hydrogen peroxide is over 100 times more effective in cells with deletion of the *oxyR* gene than in wild-type control [26]. The OxyR protein is a transcription factor acting as a major regulator of the oxidative stress [34]. In the phage DNA region responsible for the control of maintenance of the prophage, there are 3 sites for binding of the cI repressor, called o_{R1}, o_{R2}, and o_{R3} (Figure 3). Binding of the cI protein to o_{R1} and o_{R2} represses p_R, the major promoter for expression of genes required during the lytic development, but at the same time stimulates transcription of the *cI* gene from the p_M promoter. At high concentrations of cI, this protein can bind also to o_{R3} which causes a repression of its own promoter p_M [20] (Figure 4).

Detailed molecular studies indicated that OxyR can bind specifically to the region of the p_M-p_R promoters (introduction of mutations to the putative OxyR-binding site abolished interactions of this protein with DNA), though with a weaker affinity than to its own promoter [26]. Interestingly, in the presence of OxyR, the cI protein interactions with o_{R1}, o_{R2} were enhanced, while binding of this repressor to o_{R3} was impaired. These results suggested that OxyR might stimulate repression of p_R and activation of p_M but at the same time downregulate repression of p_M [26]. This would lead to a considerably more efficient maintenance of the prophage due to more efficient blocking of the p_R promoter by abundant cI. Indeed, studies with gene fusions showed that while under normal growth conditions (when OxyR is inactive) the activity of the p_M promoter was similar in both *oxyR*$^+$ and Δ*oxyR* strains, the oxidative stress conditions (treatment

of cells with H_2O_2 which activates OxyR) caused enhanced transcription from p_M in wild-type bacteria and decreased in the Δ*oxyR* mutant [26]. Therefore, it appears that the OxyR protein is responsible for the low efficiency of prophage induction caused under conditions of the oxidative stress (Figure 4).

3. The Oxidative Stress and Biological Role of Shiga Toxin Production by STEC

There is an intriguing question regarding the biological role of Shiga toxin production by STEC strains. As described in preceding sections, expression of *stx* genes is effective only after Shiga toxin-converting prophage induction. However, this also results in subsequent lytic development of the bacteriophage and eventual death of the host cell. Thus, what can be a benefit for the bacterium from production of Shiga toxin while it is linked to its death? On the other hand, if toxin production was not beneficial for *E. coli*, one should expect a positive selection of lysogenic bacteria with mutations causing deficiency in prophage induction and thus elimination of STEC cells from the bacterial population. Since this is not the case, it should be beneficial for STEC to produce Shiga toxin.

It was suggested that STEC virulence in humans may be coincidental with the biological role for Shiga toxin being unrelated to human infection [35]. This hypothesis assumed that synthesis of Shiga toxins by STEC may enhance survival of bacteria in food vacuoles of protozoan predators. Interestingly, such a phenomenon was demonstrated experimentally [36]. Moreover, a bacterivorous, protozoan predator, *Tetrahymena thermophila*, was shown to be killed when cocultured with bacteria lysogenic with Stx bacteriophage [37]. However, this killing did not occur in the presence of catalase, an enzyme responsible for hydrogen peroxide breakdown [37]. In fact, *Tetrahymena* produces H_2O_2 to damage bacterial cells during attack by this predator [37]. This may be a successful predatory strategy in the case of the vast majority of bacteria; however, if STEC cells are being attacked, Shiga toxin-converting prophages are induced due to action of hydrogen peroxide (as demonstrated experimentally [30, 31]), Shiga toxin is produced, and after toxin release from *E. coli* due to phage-mediated cell lysis, it kills the predator. The crucial point of such a defensive bacterial strategy is a low effective prophage induction by H_2O_2 which has also been shown [30, 31]. Therefore, of the total STEC population, only 1% or less is lost for production of Shiga toxin (which is enough to produce relatively large amounts of the toxin, sufficient to kill the predator) while the rest of bacteria are saved. When STEC infects human intestine, neutrophils' action is similar to that of protist predators, and H_2O_2 is produced to kill bacteria [38], but the effects are analogous to the *Tetrahymena*-STEC interplay. The hypothesis on such an "bacterial altruism" has been proposed independently by two groups [17, 39], and detailed analyses of the literature indicated that the predicted scenario may be true [32]. Moreover, the hypothesis has been further confirmed by recent discoveries that STEC strains are more resistant to the impact of grazing protists than *E. coli* devoid of the *stx* genes [40] and that bacteriophage-mediated lysis of STEC is necessary for killing of protist cells by Shiga toxin, since the toxin released as a consequence of digestion of bacteria by *Tetrahymena* is harmless to it [41]. The latter finding was the argument to call the STEC cells a "Trojan Horse," carrying genes encoding the toxin into target organisms [42].

4. Concluding Remarks

The oxidative stress plays a pivotal role in the production of Shiga toxins in cells of enterohemorrhagic *Escherichia coli* (EHEC) infecting human intestine, as well as in response to the attack of predator protists. In both cases, hydrogen peroxide is excreted by eukaryotic cells (either protist predators or neutrophils in an infected organism) to weaken bacteria which is a successful strategy against most prokaryotes; however, EHEC strains are lysogenic for Shiga toxin-converting prophages, and H_2O_2 stimulates their induction. This leads to the switch to lytic development and production of the toxin. It appears that Shiga toxin-producing bacteria use the specific strategy of "bacterial altruism," based on the OxyR-mediated low efficiency of prophage induction during the oxidative stress. As a consequence, only a small fraction of bacterial cells is destroyed due to prophage induction, which is nevertheless sufficient to produce relatively large amounts of Shiga toxins able to kill eukaryotic cells. In this way the rest of the *E. coli* population can survive the attack of the predator or neutrophils.

Conflict of Interests

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

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Research Article

The Role of the *Exo-Xis* Region in Oxidative Stress-Mediated Induction of Shiga Toxin-Converting Prophages

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Previous studies indicated that these genetic elements could be involved in the regulation of lysogenization and prophage induction processes. The effects were dramatic in Shiga toxin-converting phage $\Phi24_B$ after treatment with oxidative stress-inducing agent, hydrogen peroxide, while they were less pronounced in bacteriophage λ and in both phages irradiated with UV. The hydrogen peroxide-caused prophage induction was found to be RecA-dependent. Importantly, in hydrogen peroxide-treated *E. coli* cells lysogenic for either λ or $\Phi24_B$, deletion of the *exo-xis* region resulted in a significant decrease in the levels of expression of the S.O.S. regulon genes. Moreover, under these conditions, a dramatic decrease in the levels of expression of phage genes crucial for lytic development (particularly *xis, exo, N, cro, O, Q*, and *R*) could be observed in $\Phi24_B$-, but not in λ-bearing cells. We conclude that genes located in the *exo-xis* region are necessary for efficient expression of both host S.O.S regulon in lysogenic bacteria and regulatory genes of Shiga toxin-converting bacteriophage $\Phi24_B$.

1. Introduction

Infection of humans by enterohemorrhagic *Escherichia coli* (EHEC) strains causes hemorrhagic colitis, and in some patients it may result in various complications, including, the most severe of them, the hemolytic-uremic syndrome and neurological dysfunctions [1–3]. The main causes of EHEC-mediated complications are Shiga toxins, produced by the infecting bacteria [4]. The severity of EHEC infections and significance of the medical problem related to them are exemplified by local outbreaks, occurring in various geographical regions around the world. One of the most famous of them took place in 2011 in Germany, where over 4,000 patients developed severe symptoms, and 54 died [5–10].

In EHEC strains, Shiga toxins are encoded by genes (called *stx* genes) located in genomes of prophages [11, 12]. The phages bearing *stx* genes are referred to as Shiga toxin-converting bacteriophages, and all of them belong to the family of lambdoid phages (with phage λ serving as a paradigm) [12]. *stx* genes are present between *Q* antiterminator gene and the genes coding for proteins causing cell lysis; thus, in the lysogenic state, these genes are not transcribed [13–15] and Shiga toxins are not produced. Their expression is possible only after prophage induction [11, 12] which usually requires activation of the bacterial S.O.S. response, mediated by RecA protein, though RecA-independent induction of Shiga toxin-converting prophages by EDTA has also been reported [16].

During infection of human intestine by EHEC, the oxidative stress appears to be the most likely condition causing the bacterial S.O.S. response and subsequent induction of Shiga toxin-converting prophages [17]. In fact, it was demonstrated that hydrogen peroxide (which is produced by neutrophils as a response to infection) enhanced production of Shiga toxins by EHEC [18] due to oxidative stress-mediated induction of Shiga toxin-converting prophages [19, 20].

Since many antibiotics not only kill bacteria and inhibit their growth but also induce prophage lytic development, their use is not recommended when EHEC infection is

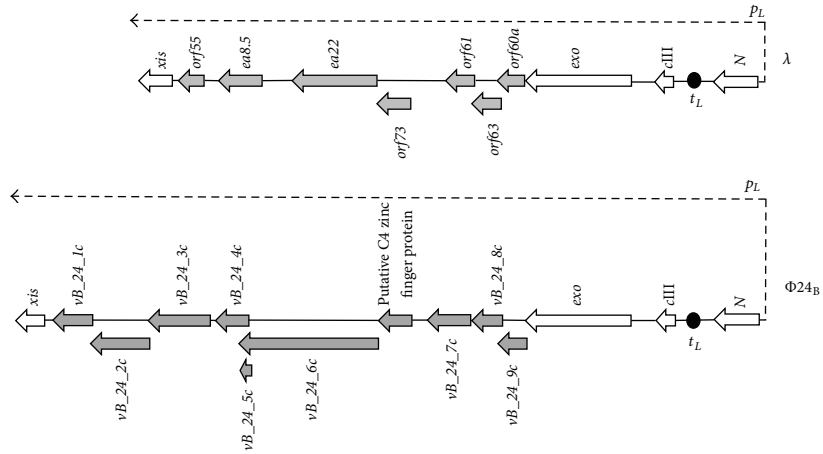

Figure 1: Schematic maps of the *exo-xis* regions of bacteriophages λ and $\Phi24_B$. Genes from the *exo-xis* region are marked as thick grey arrows, and other genes are shown as thick open arrows. Directionality of transcription from p_L promoter is indicated as thin dashed arrow. t_{L1} terminator is marked as black oval.

confirmed or even suspected (reviewed in [12]). Therefore, there are serious problems with treatment of patients, indicating that searching for new targets of potential therapies against Shiga toxin-producing bacteria is important. One might consider that such therapies should be focused on inhibition of Shiga toxin-converting prophage induction which would impair production of the EHEC virulence factor. All lambdoid phages, including Shiga toxin-converting bacteriophages, contain the *b* region in their genomes which is dispensable for the development under standard laboratory conditions [14, 15]. Inside this part of the phage genome, there is an evolutionarily conserved fragment, located between *exo* and *xis* genes and transcribed from p_L promoter, called the *exo-xis* region (Figure 1). This region encompasses several genes and open reading frames whose functions in phage development are largely unknown, and only a few articles are available in the literature that focused on them. Nevertheless, some interesting observations have been reported. Namely, induction of expression of genes from the *exo-xis* region resulted in synchronization of the host cell cycle [21] and inhibition of host DNA replication [22]. Moreover, overexpression of these genes impaired lysogenization of *E. coli* by bacteriophage λ [23] and enhanced induction of prophages λ and $\Phi24_B$ (one of Shiga toxin-converting phages) [24]. Ea8.5 protein, encoded by a gene located in the *exo-xis* region, contains a fused homeodomain/zinc finger fold [25] which suggests a regulatory role for this protein. Interestingly, prophage induction with mitomycin C or hydrogen peroxide caused different expression patterns of genes from the *exo-xis* region; such differences were observed in both phages, λ and $\Phi24_B$ [26]. In this work, we used the deletion mutants to investigate the role of the *exo-xis* region in induction of λ and $\Phi24_B$ prophages under oxidative stress conditions.

2. Materials and Methods

2.1. Bacteria and Bacteriophages. *E. coli* MG1655 strain [27] and its derivatives, used in this work, are listed in Table 1. Bacteria were routinely cultured in the Luria-Bertani (LB) medium at 30°C (most experiments) or 37°C (lysogenization and recombination procedures during construction of strains and SOS ChromoTest, according to the instructions of kits' manufacturers), under aerobic conditions. Where appropriate, the following antibiotics were added: chloramphenicol up to 20 μg/mL, kanamycin up to 50 μg/mL, and/or tetracycline up to 12.5 μg/mL.

Bacteriophages λ papa (from our collection) [26] and $\Phi24_B$ ($\Delta stx2::cat$) [28] were employed in this study. Phage suspensions were stored in the TM buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.2) at 4°C.

The deletion mutants were constructed as described previously [29], by using the Quick and Easy *E. coli* Gene Deletion Kit (from Gene Bridges). The deletion of the indicated region was performed according to the manufacturer's protocol using primers listed in Table 2. In the first step, the targeted sequence has been replaced with the FRT-flanked kanamycin resistance cassette, and the selection marker was subsequently removed in the FLP-recombinase step, leaving only 87 nucleotides of the cassette in the place of the original sequence. Each deletion was confirmed by DNA sequencing.

Lysogenic strains were constructed according to the procedure described previously [24], with slight modifications. Briefly, host bacteria were cultured to A_{600} of 0.5 in LB medium supplemented with $MgSO_4$ and $CaCl_2$ (to final concentrations of 10 mM each) at 37°C with shaking. At this point, one milliliter of the culture was withdrawn and centrifuged (10 min, 2000 ×g). Pellet was washed twice with TCM buffer (10 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 10 mM $CaCl_2$) and then suspended in 1 mL of the same buffer. Next, bacteria were incubated for 30 min at 30°C and mixed with phage suspensions at multiplicity of infection (m.o.i.) = 5. Mixtures of bacteria and phages were incubated in TMC buffer for 30 min at 30°C; then serial dilutions were prepared in TM buffer (10 mM Tris-HCl, 10 mM $MgSO_4$; pH 7.2), and the mixture was plated onto LB agar. Plates were incubated at 37°C overnight. Lysogens were verified by sensitivity to UV irradiation and confirmed by PCR with primers designed to amplify phage sequence (Table 3).

Table 1: *Escherichia coli* strains.

Strain	Genotype or relevant characteristics	Reference
E. coli MG1655	F^- λ^- *ilvG rfb-50 rph-1*	[27]
E. coli MG1655 (λ)	MG1655 bearing λ prophage	[24]
E. coli MG1655 ($\lambda\Delta$*exo-xis*)	MG1655 bearing λ prophage with deletion of all orfs and genes localized between genes *exo* and *xis*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*orfs*)	MG1655 bearing λ prophage with deletion of *orf60a, orf63, orf61*, and *orf73*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*orf60a*)	MG1655 bearing λ prophage with deletion of *orf60a*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*orf63*)	MG1655 bearing λ prophage with deletion of *orf63*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*orf61*)	MG1655 bearing λ prophage with deletion of *orf61*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*orf73*)	MG1655 bearing λ prophage with deletion of *orf73*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*ea22*)	MG1655 bearing λ prophage with deletion of *ea22*	This study, by recombination
E. coli MG1655 ($\lambda\Delta$*ea8.5*)	MG1655 bearing λ prophage with deletion of *ea8.5*	This study, by recombination
E. coli MG1655 ($\Phi24_B$)	MG1655 bearing $\Phi24_B$ prophage	[24]
E. coli MG1655 ($\Phi24_B\Delta$*exo-xis*)	MG1655 bearing $\Phi24_B$ prophage with deletion of all orfs and genes localized between genes *exo* and *xis*	This study, by recombination
E. coli MG1655 ($\Phi24_B\Delta$*orfs*)	MG1655 bearing $\Phi24_B$ prophage with deletion of 4 orfs being homologues of λ*orf60a, λorf63, λorf61*, and λ*orf73*	This study, by recombination
E. coli MG1655 ($\Phi24_B\Delta$*orf60a*)	MG1655 bearing $\Phi24_B$ prophage with deletion of vb_24_B_9c, the homologue of λ*orf60a*	This study, by recombination
E. coli MG1655 ($\Phi24_B\Delta$*orf63*)	MG1655 bearing $\Phi24_B$ prophage with deletion of vb_24_B_8c, the homologue of λ*orf63*	This study, by recombination
E. coli MG1655 ($\Phi24_B\Delta$*orf61*)	MG1655 bearing $\Phi24_B$ prophage with deletion of vb_24_B_7c, the homologue of λ*orf61*	This study, by recombination
E. coli MG1655 ($\Phi24_B\Delta$*orf73*)	MG1655 bearing $\Phi24_B$ prophage with deletion of the sequence of putative C4 zinc finger protein, the homologue of λ*orf73*	This study, by recombination
E. coli MG1655 ($\Phi24_B\Delta$*ea22*)	MG1655 bearing $\Phi24_B$ prophage with deletion of vb_24_B_6c, the analogue of λ*ea22*	This study, by recombination
E. coli MG1655*recA13*	MG1655 but *recA13*	[33]
E. coli MG1655*recA13* (λ)	MG1655*recA13* bearing λ prophage	This study, by lysogenization
E. coli MG1655*recA13* ($\lambda\Delta$*exo-xis*)	MG1655*recA13* bearing λ prophage with deletion of all orfs and genes localized between genes *exo* and *xis*	This study, by lysogenization
E. coli MG1655*recA13* ($\Phi24_B$)	MG1655*recA13* bearing $\Phi24_B$ prophage	This study, by lysogenization
E. coli MG1655*recA13* ($\Phi24_B\Delta$*exo-xis*)	MG1655*recA13* bearing $\Phi24_B$ prophage with deletion of all orfs and genes localized between genes *exo* and *xis*	This study, by lysogenization
E. coli PQ37	*sfi*A::Mud(Ap *lac*) *cts*, *lac*ΔU169, *mal*$^+$, *gal*E, *gal*Y, PhoC, *rfa*, F^-, *thr*, *leu*, *his*, *pyrD*, *thi*, *trp*::*MUC*$^+$, *srl300*::Tn*10*, *rpoB*, *uvrA*$^+$	[31]
E. coli PQ37 (λ)	PQ37 bearing λ prophage	This study, by lysogenization
E. coli PQ37 ($\lambda\Delta$*exo-xis*)	PQ37 bearing λ prophage with deletion of all orfs and genes localized between genes *exo* and *xis*	This study, by lysogenization
E. coli PQ37 ($\Phi24_B$)	PQ37 bearing $\Phi24_B$ prophage	This study, by lysogenization
E. coli PQ37 ($\Phi24_B\Delta$*exo-xis*)	PQ37 bearing $\Phi24_B$ prophage with deletion of all orfs and genes localized between genes *exo* and *xis*	This study, by lysogenization

2.2. Phage Lytic Development after Prophage Induction. Bacteria lysogenic with tested phages were cultured in LB medium at 30°C to A_{600} of 0.1. Two induction agents were tested: H_2O_2 (1 mM) and UV irradiation (50 J/m^2; this dose was achieved by 20 sec incubation of the bacterial suspensions in Petri dishes under UV lamp hanged 17 cm above the laboratory table). At indicated times after induction, samples of bacterial cultures were harvested, and 30 μL of chloroform was added to 0.5 mL of each sample. The mixture was vortexed and centrifuged for 5 min in a microcentrifuge. Then, serial dilutions were prepared in TM buffer, and phage titer (number of phages per mL) was determined by spotting 2.5 μL of each dilution of the phage lysate on a freshly prepared LB agar (1.5%) or LB agar with 2.5 μg/mL chloramphenicol (according to a procedure described previously [30]), with a poured mixture of 1 mL of the indicator *E. coli* MG1655 strain culture and 2 mL of 0.7% nutrient agar (prewarmed to 45°C), supplemented with $MgSO_4$ and $CaCl_2$ (to a final concentration of 10 mM each). When full-plate titration was used, 0.1 mL of phage lysate dilutions was

Table 2: Primers used for construction of *E. coli* strains.

Primer name	Sequence ($5' \rightarrow 3'$)
pF_λ_exo-xis = pF_λ_orf60a	ATATCCGGGTAGGCGCAATCACTTTCGTCTACTCCGTTACAAAGCGAGGAATTAACCCTCACTAAAGGGCG
pR_λ_exo-xis	AGGCGGCTTGGTGTTCTTTCAGTTCTTCAATTCGAATATTGGTTACGTCTTAATACGACTCACTATAGGGCTC
pF_λ_orfs = pF_λ_orf60a	ATATCCGGGTAGGCGCAATCACTTTCGTCTACTCCGTTACAAAGCGAGGAATTAACCCTCACTAAAGGGCG
pR_λ_orfs = pR_λ_orf73	ACCTCTCTGTTTACTGATAAGTTCCAGATCCTCCTGGCAACTTGCACAAGTAATACGACTCACTATAGGGCTC
pF_λ_orf60a	ATATCCGGGTAGGCGCAATCACTTTCGTCTACTCCGTTACAAAGCGAGGAATTAACCCTCACTAAAGGGCG
pR_λ_orf60a	AGATGCTTTGTGCATACAGCCCCTCGTTTATTATTTATCTCCTCAGCCAGTAATACGACTCACTATAGGGCTC
pF_λ_orf63	CACAAAGCATCTTCTGTTGAGTTAAGAACGAGTATCGAGATGGCACATAGAATTAACCCTCACTAAAGGGCG
pR_λ_orf63	TCATACCTGGTTTCTCTCATCTGCTTCTGCTTTCGCCACCATCATTTCCATAATACGACTCACTATAGGGCTC
pF_λ_orf61	AGAGAAACCAGGTATGACAACCACGGAATGCATTTTTCTGGCAGCGGGCTAATTAACCCTCACTAAAGGGCG
pR_λ_orf61	TTATCCGGAAACTGCTGTCTGGCTTTTTTTGATTTCAGAATTAGCCTGACTAATACGACTCACTATAGGGCTC
pF_λ_orf73	ACATCATTGATTCAGCATCAGAAATAGAAGAATTACAGCGCAACACAGCAAATTAACCCTCACTAAAGGGCG
pR_λ_orf73	ACCTCTCTGTTTACTGATAAGTTCCAGATCCTCCTGGCAACTTGCACAAGTAATACGACTCACTATAGGGCTC
pF_λ_ea22	GAAATTAACTCTCAGGCACTGCGTGAAGCGGCAGAGCAGGCAATGCATGAAATTAACCCTCACTAAAGGGCG
pR_λ_ea22	GTCAGACATCATATGCAGATACTCACCTGCATCCTGAACCCATTGACCTCCTAATACGACTCACTATAGGGCTC
pF_λ_ea8.5	ATGAGTATCAATGAGTTAGAGTCTGAGCAAAAAGATTGGGCGTTATCAATAATTAACCCTCACTAAAGGGCG
pR_λ_ea8.5	TAATCATCTATATGTTTTGTACAGAGAGGGCAAGTATCGTTTCCACCGTATAATACGACTCACTATAGGGCTC
pF_Φ24$_B$_exo-xis = pF_24$_B$_orf60a	TGGTAATGAAGCATCCTCACGATAATATCCGGGTAGGCACGATCACTTTCAATTACCTCACTAAAGGGCG
pR_Φ24$_B$_exo-xis	TGGCAATATGCTTTTCCTTCTCAATTTCCGCTTTAATCATATGCAGTTCGTAATACGACTCACTATAGGGCTC
pF_Φ24$_B$_orfs = pF_Φ24$_B$_orf60a	TGGTAATGAAGCATCCTCACGATAATATCCGGGTAGGCACGATCACTTTCAATTACCTCACTAAAGGGCG
pR_Φ24$_B$_orfs = pR_Φ24$_B$_orf73	CTTCGAACCTCTCTGTTTACTGATAAGCTCCAGATCCTCCTGGCAACTTGTAATACGACTCACTATAGGGCTC
pF_Φ24$_B$_orf60a	TGGTAATGAAGCATCCTCACGATAATATCCGGGTAGGCACGATCACTTTCAATTACCTCACTAAAGGGCG
pR_Φ24$_B$_orf60a	GGAGATGCTTTGTGCATACAGCCCCTCGTTATTATTTATCTCTTCAGCCTAATACGACTCACTATAGGGCTC
pF_Φ24$_B$_orf63	TGCACAAAGCATCTCCTGTTGAATTAAGAACGAGTATCGGGATGGCACATAATTAACCCTCACTAAAGGGCG
pR_Φ24$_B$_orf63	CATCATTTCCAGCTTTTGTGAAAGGGATGTGGCTAACGTATGAAATTCTTTAATACGACTCACTATAGGGCTC
pF_Φ24$_B$_orf61	TTCTGGCAGCAGGCTTCATATTCTGTGTGCTTATGCTTGCCGACATGGGAAATTAACCCTCACTAAAGGGCG
pR_Φ24$_B$_orf61	CACCGTTCCTTAAAGACGCCGTTTAACATGCCGATCGCCAGACTTAAATGTACGACTCACTATAGGGCTC
pF_Φ24$_B$_orf73	TGGCAGACCTCATTGATTCAGCATCAGAAATTGAAGAATTACAGCGCAACAATTAACCCTCACTAAAGGGCGG
pR_Φ24$_B$_orf73	CTTCGAACCTCTCTGTTTACTGATAAGCTCCAGATCCTCCTGGCAACTTGTAATACGACTCACTATAGGGCTC
pF_Φ24$_B$_ea22	ATCAGGCACTGCGTGAAAAGGCAGAGAAAGCAACTAAAGGAAGCTACATCAATTAACCCTCACTAAAGGGCG
pR_Φ24$_B$_ea22	ATCCAGTGTGACGGTTTCCACGACGCACCAGGAATTATCCACCCATCATTATACGACTCACTATAGGGCTC

Table 3: Primers used for PCR.

Primers for bacterial sequences		Primers for phage sequences	
Name	Sequence (5′ → 3′)	Name	Sequence (5′ → 3′)
pF_recA	AGATTTCGACGATACGGCCC	pF_λ_int	TTTGATTTCAATTTTGTCCCACT
pR_recA	AACCATCTCTACCGGTTCGC	pR_λ_int	ACCATGGCATCACAGTATCG
pF_lexA	ATGGATGGTGACTTGCTGGC	pF_λ_xis	TACCGCTGATTCGTGGAACA
pR_lexA	TTCGTCATCAATACGTGCGAC	pR_λ_xis	GGGTTCGGGAATGCAGGATA
pF_ssb	ATCGAAGGTCAGCTGCGTAC	pF_λ_exo	TGCCGTCACTGCATAAACC
pR_ssb	CGACTTCTGTGGTGTAGCGA	pR_λ_exo	TCTATCGCGACGAAAGTATGC
pF_recF	CGATACCGGCGCTATACTCC	pF_λ_cIII	ATTCTTTGGGACTCCTGGCTG
pR_recF	TTACGAACAGCTACGCCCG	pR_λ_cIII	GTAAATTACGTGACGGATGGAAAC
pF_rpoD	GAATCTGAAATCGGGCGCAC	pF_λ_N	CTCGTGATTTCGGTTTGCGA
pR_rpoD	GTCAACAGTTCAACGGTGCC	pR_λ_N	AAGCAGCAAATCCCCTGTTG
pF_rpoH	GCTTTGGTGGTCGCAACTTT	pF_λ_cI	ACCTCAAGCCAGAATGCAGA
pR_rpoH	TCGCCGTTCACTGGATCAAA	pR_λ_cI	CCAAAGGTGATGCGGAGAGA
pF_rpoS	TTGCTCTGCGATCTCTTCCG	pF_λ_cro	ATGCGGAAGAGGTAAAGCCC
pR_rpoS	GAACGTTTACCTGCGAACCG	pR_λ_cro	TGGAATGTGTAAGAGCGGGG
pF_uvrA	GTCCATATCCGCCACTACCG	pF_λ_cII	TCGCAATGCTTGGAACTGAGA
pR_uvrA	TTACCCAACGTCTTGCCGAG	pR_λ_cII	CCCTCTTCCACCTGCTGATC
pF_ftsK	ACAAACCGTTTATCTGCGCG	pF_λ_O	AATTCTGGCGAATCCTCTGA
pR_ftsK	ATCTTTACCCAGCACCACGG	pR_λ_O	GAATTGCATCCGGTTT
pF_16SrRNA	CCTTACGACCAGGGCTACAC	pF_λ_Q	TTCTGCGGTAAGCACGAAC
pR_16SrRNA	TTATGAGGTCCGCTTGCTCT	pR_λ_Q	TGCATCAGATAGTTGATAGCCTTT
		pF_λ_R	ATCGACCGTTGCAGCAATA
		pR_λ_R	GCTCGAACTGACCATAACCAG
		pF_Φ24$_B$_int	CAGTTGCCGGTATCCCTGT
		pR_Φ24$_B$_int	TGAGGCTTTCTTGCTTGTCA
		pF_Φ24$_B$_xis	TATCGCGCCGGATGAGTAAG
		pR_Φ24$_B$_xis	CGCACAGCTTTGTATAATTTGCG
		pF_Φ24$_B$_exo	TGCCGTCACTGCATAAACC
		pR_Φ24$_B$_exo	TCTATCGCGACGAAAGTATGC
		pF_Φ24$_B$_cIII	ATTCTTTGGGACTCCTGGCTG
		pR_Φ24$_B$_cIII	GTAAATTACGTGACGGATGGAAAC
		pF_Φ24$_B$_N	AGGCGTTTCGTGAGTACCTT
		pR_Φ24$_B$_N	TTACACCGCCCTACTCTAAGC
		pF_Φ24$_B$_cI	TGCTGTCTCCTTTCACACGA
		pR_Φ24$_B$_cI	GCGATGGGTGGCTCAAAATT
		pF_Φ24$_B$_cro	CGAAGGCTTGTGGAGTTAGC
		pR_Φ24$_B$_cro	GTCTTAGGGAGGAAGCCGTT
		pF_Φ24$_B$_cII	TGATCGCGCAGAAACTGATTTAC
		pR_Φ24$_B$_cII	GACAGCCAATCATCTTTGCCA
		pF_Φ24$_B$_O	AAGCGAGTTTGCCACGAT
		pR_Φ24$_B$_O	GAACCCGAACTGCTTACCG
		pF_Φ24$_B$_Q	GGGAGTGAGGCTTGAGATGG
		pR_Φ24$_B$_Q	TACAGAGGTTCTCCCTCCCG
		pF_Φ24$_B$_R	GGGTGGATGGTAAGCCTGT
		pR_Φ24$_B$_R	TAACCCGGTCGCATTTTTC

plated onto LB agar. Plates were incubated at 37°C overnight. Analogous experiment but without induction agents (control experiments), which allows estimation of effects of spontaneous prophage induction, was performed with each lysogenic strain. The relative phage titer, expressed as plaque forming units (pfu)/mL, was calculated by subtracting the values obtained in the control experiment from the values determined in the main experiment, and as a consequence it represents the ratio of phage titers in induced and noninduced cultures. Each experiment was repeated three times.

2.3. The S.O.S. Assay. The S.O.S. assay was performed using the SOS-ChromoTest Kit (Environmental Bio-Detection Products Inc.), following the manufacturer's protocol and using provided 4-nitro-quinoline oxide (4-NQO) as a positive reference standard, and 1 mM H_2O_2 and UV irradiation (50 J/m^2) as tested inducers of the S.O.S. response [31, 32]. In the case of UV light irradiation, the production of β-galactosidase was evaluated immediately after the exposure, without 2 h incubation at 37°C (recommended by the manufacturer), as, without this modification, the visual detection of the blue color was not possible due to rapid S.O.S. response after UV irradiation. Before use, the SOS-ChromoTest bacterial strain (*E. coli* PQ37, provided with the kit) was lysogenized by following phages: λ, $\lambda\Delta$*exo-xis*, $\Phi24_B$, or $\Phi24_B\Delta$*exo-xis*, according to procedure described above.

2.4. Preparation of RNA and cDNA from Bacteria. For the isolation of total RNA, the previously described [26] procedure was employed. Briefly, the prophage induction was performed with 1 mM H_2O_2 or UV irradiation (at the dose of 50 J/m^2). Following induction, the samples were withdrawn at indicated times and the growth of bacteria was inhibited by the addition of NaN_3 (Sigma-Aldrich) to a final concentration of 10 mM. Total RNA was isolated from 10^9 bacterial cells by using the High Pure RNA Isolation Kit (Roche Applied Science). Bacterial genomic DNA carryover was removed by incubation with TURBO DNase from TURBO DNA-*free* Kit (Life Technologies) for 60 min at 37°C, according to the manufacturer's guidelines. To evaluate the quality and quantity of the isolated RNA, a NanoDrop spectrophotometer was employed, considering the absorbance ratio (which should be $1.8 \leq A260/A280 \leq 2.0$). Moreover, band patterns of total RNA were visualized by electrophoresis. The absence of DNA from RNA samples was controlled by PCR amplification and by real-time PCR amplification (all analyzed genes were tested). RNA preparations were stored at −80°C. cDNA was obtained with Transcriptor Reverse Transcriptase and random hexamer primers (Roche Applied Science), using total RNA samples (1.25 μg) as templates. cDNA reaction mixtures were diluted 10-fold for use in real-time PCR.

2.5. Real-Time PCR Assay and Data Analysis. The patterns of genes' expression were determined by quantitative real-time reverse transcription-PCR (qRT-PCR), using the LightCycler 480 Real-Time PCR System (Roche Applied Science) and cDNA samples from lysogenic bacteria. Transcripts of tested phage and bacterial genes were compared in parallel to *16S rRNA* housekeeping gene (according to a procedure described previously [34]), whose expression was found to be constant. Primers were developed by Primer3web version 4.0.0 and produced by Sigma-Aldrich or GENOMED. The transcriptional analysis of phage and bacterial genes from lysogenic strains was performed with primers presented in Table 3. Real-time PCR amplifications were carried out for 55 cycles in 20 μL reaction volume, using LightCycler 480 SYBR Green I Master (Roche Applied Science) as a fluorescent detection dye. Reactions were performed in Roche 96-well plates containing 10 μL 2x SYBR Green I Master Mix, 6.25 ng/μL cDNA, and 200 nM of each gene-specific primer (Table 3). Relative quantification assays were performed with cDNA of 16S rRNA and phage/bacterial genes multiplex assay. All templates were amplified using the following program: incubation at 95°C for 5 min, followed by 55 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. No template control was included with each run. The specificity of amplified products was examined by melting curve analysis immediately after the final PCR cycle and confirmed by gel electrophoresis. Each experiment was conducted in triplicate.

The relative changes in gene expression revealed by quantitative real-time PCR experiments were analyzed using the calibrator, normalizing relative quantification method with efficiency correction (called the E-Method). This method has been used and described in detail previously [26, 29, 35]. The values obtained at time 0 (representing the conditions of spontaneous prophage induction) were used as calibrators. Thus, the following equation was employed to calculate the final results:

$$\text{Normalized relative ratio} = \frac{E_t^{\text{CT}(t)\ \text{calibrator}-\text{CT}(t)\ \text{sample}}}{E_r^{\text{CT}(r)\ \text{calibrator}-\text{CT}(r)\ \text{sample}}}, \quad (1)$$

where t is target and r is reference.

2.6. In Silico Analyses. The multiple sequence alignment was performed using the ClustalW algorithm available at the website http://www.genome.jp/tools/clustalw/. The Pfam protein families database [36], available at the website http://pfam.xfam.org/, was used to identify protein domains.

3. Results and Discussion

3.1. Deletion of the Exo-Xis Region Impairs $\Phi24_B$ but Not λ Prophage Induction after Treatment with Hydrogen Peroxide. Until now, all *in vivo* studies on effects of the *exo-xis* region on host or phage development were performed with the use of strains overexpressing genes from this region [21–24, 26]. In this work, we have constructed a series of bacteriophage λ and $\Phi24_B$ mutants with deletions of either the whole *exo-xis* region or individual genes or open reading frames (Table 1). When wild-type λ and $\Phi24_B$ prophages were induced by UV irradiation (employed in this work as positive control conditions causing effective prophage induction) or hydrogen peroxide treatment of the lysogenic cells, efficiencies of induction and further phage lytic development were comparable in both phages, though some differences were observed in the duration of the lag phase of the phage development (Figure 2). Induction of $\lambda\Delta$*exo-xis* mutant with UV irradiation was similar to that observed for the wild-type λ, and treatment with hydrogen peroxide caused only a slight delay in the mutant phage development. The decrease in the phage titer at later times of the experiments is characteristic for λ and most probably arises from adsorption of the progeny virions on fragments of disrupted cell envelopes [15, 24]. However, induction of $\Phi24_B\Delta$*exo-xis* prophage by UV irradiation was less efficient than that of the wild-type $\Phi24_B$, and induction

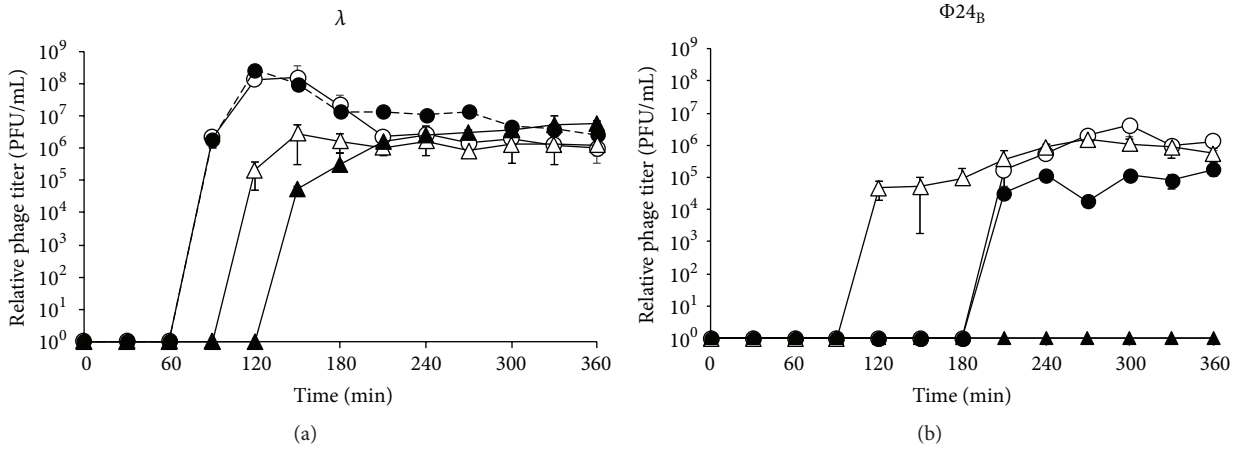

FIGURE 2: Lytic development of bacteriophages λ (a) and $\Phi24_B$ (b), either wild-type (open symbols) or Δ*exo-xis* (closed symbols), after induction of lysogenic *E. coli* MG1655 with UV irradiation (50 J/m^2, circles) or hydrogen peroxide (1 mM, triangles). The presented results are mean values from 3 independent experiments (biological samples), with error bars indicating SD. Statistically significant differences ($p < 0.05$ in *t*-test) between wild-type and Δ*exo-xis* phages were found at times 270, 300, and 360 min of experiments with hydrogen peroxide and at 270, 300, 330, and 360 min of experiments with UV for λ and at times 120, 240, 270, 300, 330, and 360 min of experiments with hydrogen peroxide and at 270, 300, 330, and 360 min of experiments with UV for $\Phi24_B$.

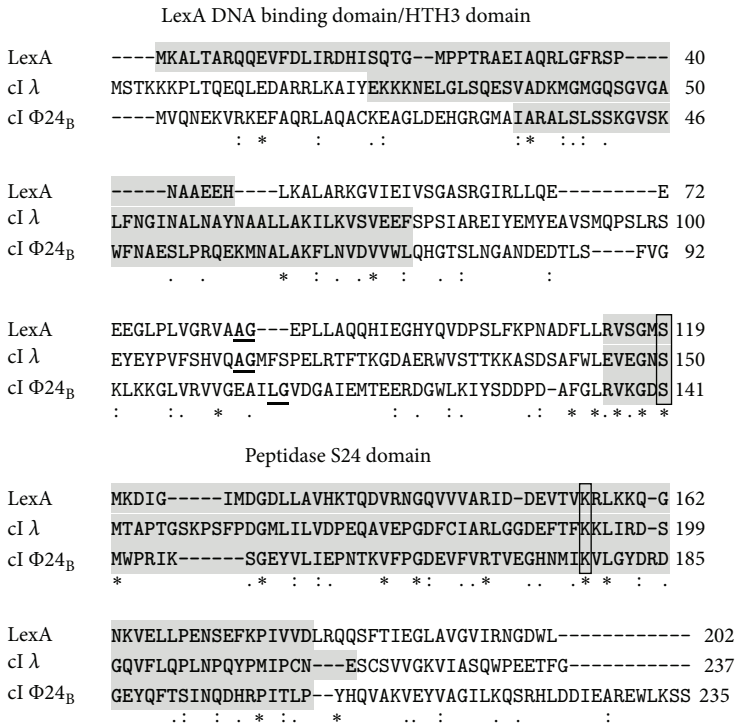

FIGURE 3: Alignment of amino acid sequences of *E. coli* LexA protein and cI repressors of bacteriophages λ and $\Phi24_B$. Specific protein domains are indicated by grey background. Self-cleavage sites are underlined (two amino acid residues between which the cleavage occurs). The active sites of the peptidase domains are framed. Symbols under the sequence alignment indicate conserved sequence (∗), conservative mutations (:), semiconservative mutations (.), and nonconservative mutations ().

of the mutant by hydrogen peroxide was severely impaired (Figure 2). More detailed analyses, based on the full-plate phage titration method, allowing detection of 10 pfu/mL (see Section 2 for details), indicated that the number of pfu per mL of $\Phi24_B\Delta$*exo-xis* phage after induction with hydrogen peroxide was at the same range (10^3/mL) as that measured without specific induction (i.e., representing efficiency of spontaneous prophage induction). Nevertheless, the titer of $\Phi24_B\Delta$*exo-xis* measured at 240 and 360 min after induction was $9.0 \pm 0.2 \times 10^3$ and $7.9 \pm 0.9 \times 10^3$, respectively, that

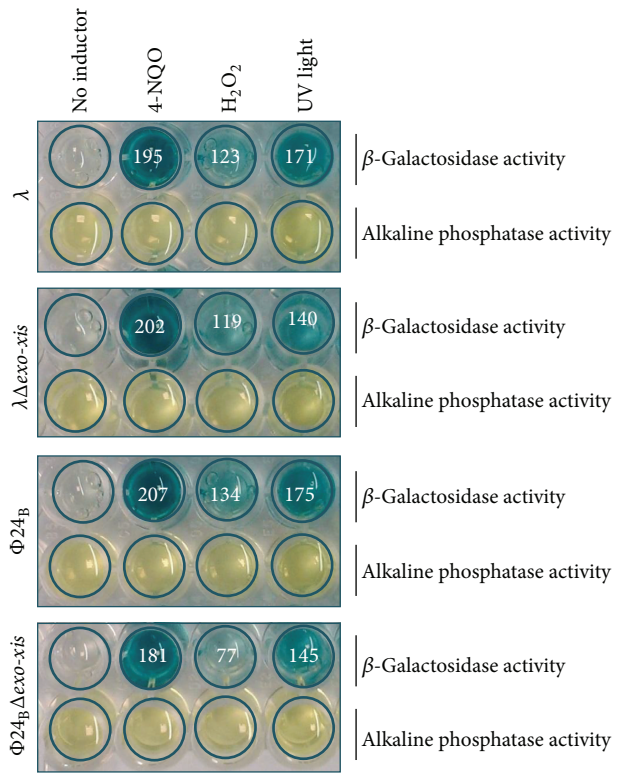

Figure 4: Induction of the S.O.S. response in *E. coli* PQ37 lysogenic with λ, λΔ*exo-xis*, $\Phi24_B$, or $\Phi24_B\Delta$*exo-xis*, treated with 4-NQO (4-nitro-quinoline oxide, positive control), H_2O_2 (1 mM), or UV (50 J/m^2), using the SOS ChromoTest. β-Galactosidase activity (identified by the blue spots) represents induction of the S.O.S. regulon. Alkaline phosphatase activity (identified by yellow spots) evaluates viability of tested bacteria. Quantification of β-galactosidase activity was performed by densitometry, using the ImageJ software (available at http://imagej.nih.gov/ij/index.html). The results (in arbitrary units reflecting value = 1 ascribed to samples with no inductor), presented as numbers inside the corresponding spots, are mean values from three measurements (with SD < 10% in each case). All these values were significantly ($p < 0.001$ in *t*-test) higher than that in the control experiments with no inductor. When Δ*exo-xis* mutants were compared to wild-type phages, the only significant difference ($p < 0.05$ in *t*-test) occurred between $\Phi24_B$ and $\Phi24_B\Delta$*exo-xis* lysogens induced with hydrogen peroxide.

is, still 3-4 times higher than that without induction, which was $2.1 \pm 0.3 \times 10^3$ and $2.6 \pm 0.4 \times 10^3$, respectively (note that the titer of the wild-type $\Phi24_B$ after prophage induction was several orders of magnitude higher than that without induction, Figure 2).

Deletions of individual genes and open reading frames from the *exo-xis* region in $\Phi24_B$ did not affect significantly the phage titer. However, such deletions resulted in delays in prophage induction by hydrogen peroxide (Table 4). Interestingly, when prophage induction was stimulated by UV irradiation, such effect was not observed, and in some cases even more rapid induction of the mutant prophages occurred. In bacteriophage λ, only slight effects of deletions of individual genes and open reading frames were detected (Table 4).

We conclude that the genes and open reading frames from the *exo-xis* region play important roles in the regulation of lambdoid prophage induction, as deletions of the whole region or single *loci* caused significant changes in efficiency and timing of this process. The effects of mutations are more pronounced in Shiga toxin-converting phage $\Phi24_B$ than in λ and in lysogenic *E. coli* cells treated with hydrogen peroxide than in UV-irradiated ones. Thus, the *exo-xis* region seems to be particularly important for $\Phi24_B$ phage under conditions of the oxidative stress, the most likely conditions causing Shiga toxin-converting prophage induction during infection with EHEC.

3.2. Hydrogen Peroxide-Mediated Prophage Induction Is a RecA-Dependent Process. Efficient induction of lambdoid prophages is a RecA-dependent process due to a molecular mimicry between the phage cI repressor and the host-encoded LexA repressor which is self-cleaved after stimulation by the activated form of RecA protein under the S.O.S. response conditions [12–15]. Such a mimicry is well known for bacteriophage λ cI protein and LexA [12, 13], and we found that both domain structure and amino acid residues crucial for the self-cleavage are also conserved in cI repressor of phage 24_B (Figure 3) (note that cI sequence of 24_B is identical to that of another Shiga toxin-converting bacteriophage, 933 W [37]). Nevertheless, since RecA-independent induction of Shiga toxin-converting prophages has also been reported [16], we asked whether hydrogen peroxide-caused prophage induction depends on the activation of the S.O.S. response.

When testing H_2O_2- or UV-dependent induction of prophages λ, λΔ*exo-xis*, $\Phi24_B$, and $\Phi24_B\Delta$*exo-xis* in *recA13*

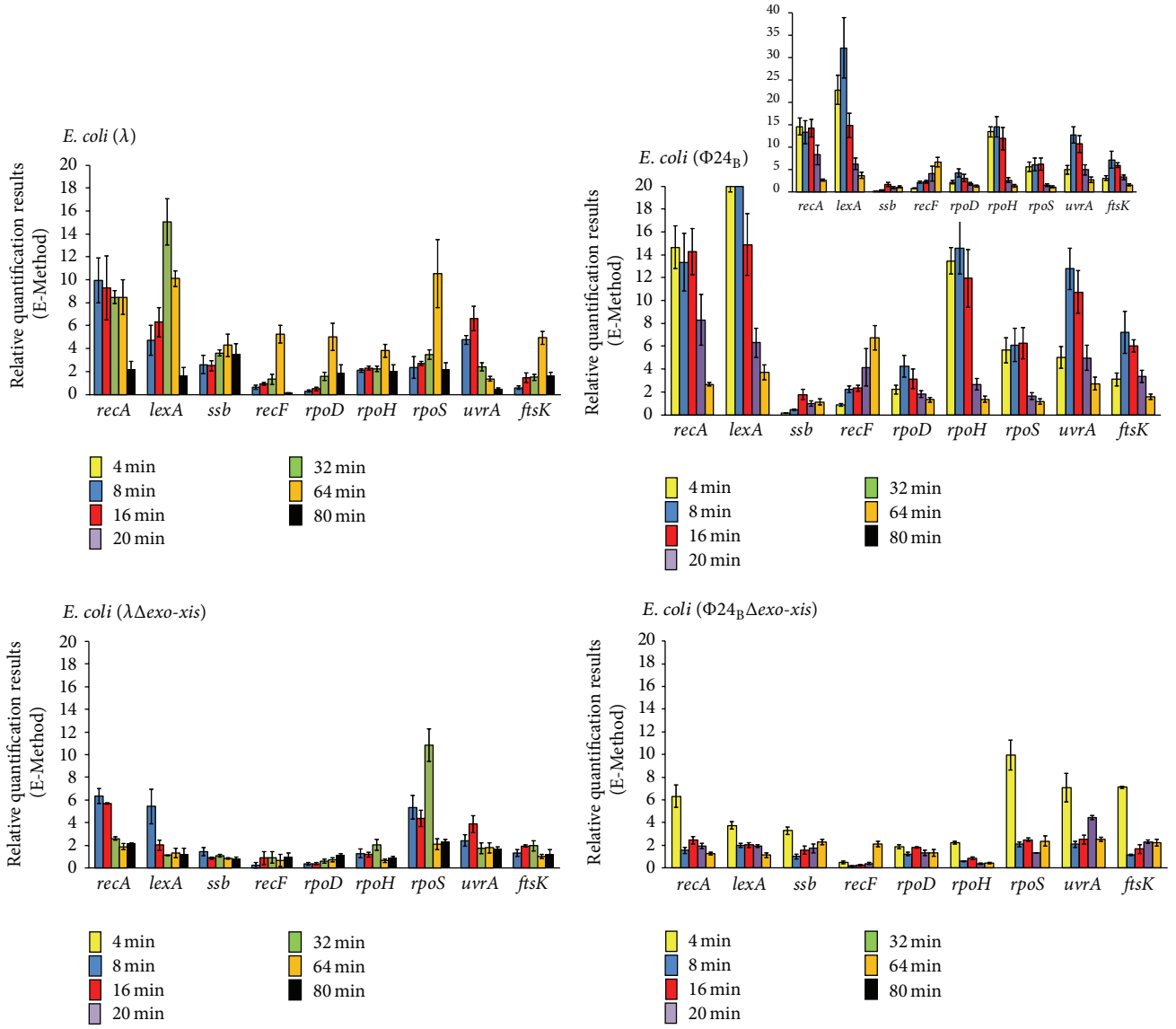

Figure 5: Expression of genes from the S.O.S. regulon in *E. coli* MG1655 lysogenic with λ, λΔ*exo-xis*, $\Phi24_B$, or $\Phi24_B$Δ*exo-xis*, at indicated times after treatment with 1 mM H_2O_2, as estimated by reverse transcription quantitative real-time PCR. The values obtained with untreated cells were used as calibrators and were subtracted from the values determined at particular time points; thus, the presented values indicate the induction of expression of tested genes. The presented results are mean values from 3 independent experiments (biological samples), with error bars indicating SD. The additional panel for *E. coli* (24_B) represents the results with different scale.

mutant host, in the assays analogous to those presented in Figure 2, pfu/mL values were at the levels of those estimated for the uninduced controls ($1.8 \pm 0.1 \times 10^2$, $4.1 \pm 1.4 \times 10^2$, $1.3 \pm 0.1 \times 10^3$, and $2.8 \pm 0.3 \times 10^3$ pfu/mL for λ, λΔ*exo-xis*, $\Phi24_B$, and $\Phi24_B$Δ*exo-xis*, resp.). Therefore, we conclude that induction of the investigated prophages under conditions of the oxidative stress (treatment with hydrogen peroxide) strongly depends on RecA function. Indeed, in cells lysogenic for λ or $\Phi24_B$ and treated with UV light or hydrogen peroxide, efficient induction of the S.O.S. response was evident, as estimated with the SOS ChromoTest (Figure 4). Intriguingly, while induction of the S.O.S. response by hydrogen peroxide in λΔ*exo-xis* lysogen was comparable to that in λ lysogen, the signal in the SOS ChromoTest in $\Phi24_B$Δ*exo-xis* lysogen was considerably weaker than in the analogous experiment with $\Phi24_B$ lysogen (Figure 4). No such difference could be observed in UV-irradiated bacteria (Figure 4).

3.3. Deletion of the Exo-Xis Region Negatively Influences Expression of Genes from the S.O.S. Regulon in Hydrogen Peroxide-Treated Lysogenic Bacteria. Since unexpected results were obtained in experiments with hydrogen peroxide-treated $\Phi24_B$Δ*exo-xis* lysogenic cells (Figure 4), we aimed to investigate the phenomenon of a less efficient induction of the S.O.S. response in more detail. Thus, expression of genes from the S.O.S. regulon was tested by reverse transcription quantitative real-time PCR in *E. coli* cells lysogenic for λ, λΔ*exo-xis*, $\Phi24_B$, and $\Phi24_B$Δ*exo-xis*

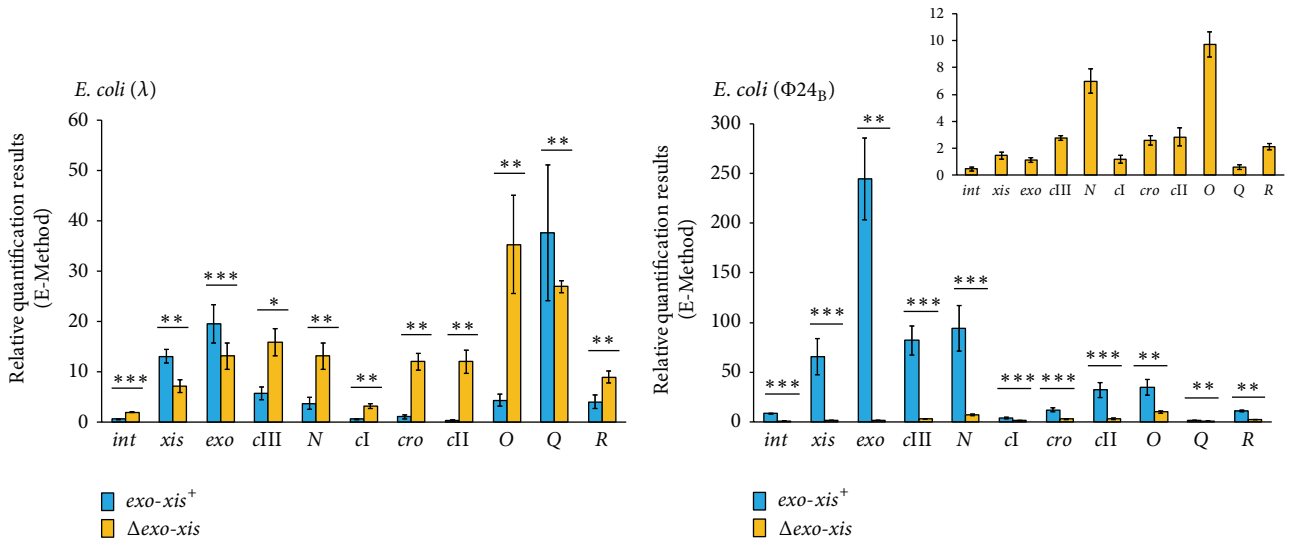

Figure 6: Expression of selected bacteriophage genes in *E. coli* MG1655 lysogenic with λ or $\Phi24_B$, either wild-type (blue columns) or Δ*exo-xis* (yellow columns) at 160 min after treatment with 1 mM H_2O_2, as estimated by reverse transcription quantitative real-time PCR. The values obtained with untreated cells were used as calibrators and were subtracted from the values determined at particular time points; thus, the presented values indicate the induction of expression of tested genes. The presented results are mean values from 3 independent experiments (biological samples), with error bars indicating SD. The additional panel for *E. coli* (24_B) represents the results of Δ*exo-xis* variant with different scale, due to very small values measured. Statistically significant differences (in *t*-test) are marked as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Table 4: Duration of the lag phase of the phage lytic development after prophage induction with either hydrogen peroxide (1 mM) or UV irradiation (50 J/m^2).

Strain	The time range of the switch from lag to log phase	
	H_2O_2 (1 mM)	UV (50 J/m^2)
MG1655 (λ)	60–90 min	60 min
MG1655 (λΔ*exo-xis*)	90–120 min	30–60 min
MG1655 (λΔ*orfs*)	60–90 min	30–60 min
MG1655 (λΔ*orf60a*)	60–90 min	30–60 min
MG1655 (λΔ*orf63*)	60–90 min	30–90 min
MG1655 (λΔ*orf61*)	60–90 min	30–60 min
MG1655 (λΔ*orf73*)	30–60 min	0–30 min
MG1655 (λΔ*ea22*)	60–90 min	30–60 min
MG1655 (λΔ*ea8.5*)	60–90 min	30–60 min
MG1655 ($\Phi24_B$)	60–90 min	150–180 min
MG1655 ($\Phi24_B$Δ*exo-xis*)	a	150–180 min
MG1655 ($\Phi24_B$Δ*orfs*)	150–180 min	150–180 min
MG1655 ($\Phi24_B$Δ*orf60a*)	120–150 min	90–120 min
MG1655 ($\Phi24_B$Δ*orf63*)	120–150 min	30–60 min
MG1655 ($\Phi24_B$Δ*orf61*)	90–120 min	30–60 min
MG1655 ($\Phi24_B$Δ*orf73*)	90–120 min	120–150 min
MG1655 ($\Phi24_B$Δ*ea22*)	120–150 min	30–60 min

[a]The value was not determined due to a very low efficiency of prophage induction under these conditions (as shown in Figure 2).

and treated with hydrogen peroxide. In both λ and $\Phi24_B$, deletion of the *exo-xis* region caused a significant reduction in the mRNA levels of most of the S.O.S. regulon genes relative to wild-type prophages, with exceptions of *rpoS* gene in both phages and *ssb*, *uvrA*, and *ftsK* genes in $\Phi24_B$, especially at later times after the treatment (Figure 5). Interestingly, in the case of wild-type $\Phi24_B$ lysogenic cells, the enhanced expression of particular genes from the S.O.S. regulon persisted longer, in most cases until 16 min after induction, whereas in the deletion mutant it decreases after 4 min (Figure 5). The impairment in expression of genes from the S.O.S. regulon (in particular *recA* and *lexA* genes, encoding the main regulators of the S.O.S. response) in the absence of the *exo-xis* region was more pronounced in $\Phi24_B$ than in λ. Moreover, induction of the S.O.S. regulon occurred significantly earlier in $\Phi24_B$ and $\Phi24_B$Δ*exo-xis* lysogens than in cells bearing λ and λΔ*exo-xis* prophages (Figure 5). These results might explain, at least partially, effects of deletions of *exo-xis* genes on prophage induction, demonstrated in Figure 2 and Table 4, particularly delayed induction of $\Phi24_B$ prophage devoid of certain genes and open reading frames, and less pronounced effects of their lack in λ than in $\Phi24_B$.

Indications that overexpression of some genes from the *exo-xis* region of λ can influence host cell cycle and DNA replication have been reported previously [21, 22]. Suggestions that some genes of $\Phi24_B$ prophage may affect host growth were also published [38]. However, the results described in this subsection demonstrate for the first time that the *exo-xis* region can significantly modulate one of global cellular responses, the S.O.S. response, after treatment with hydrogen peroxide.

3.4. Expression of Crucial Phage Genes Is Dramatically Decreased after Treatment of Lysogenic Cells with Hydrogen Peroxide in the Absence of the Exo-Xis Region in $\Phi24_B$ Prophage. Expression of phage genes, crucial for the

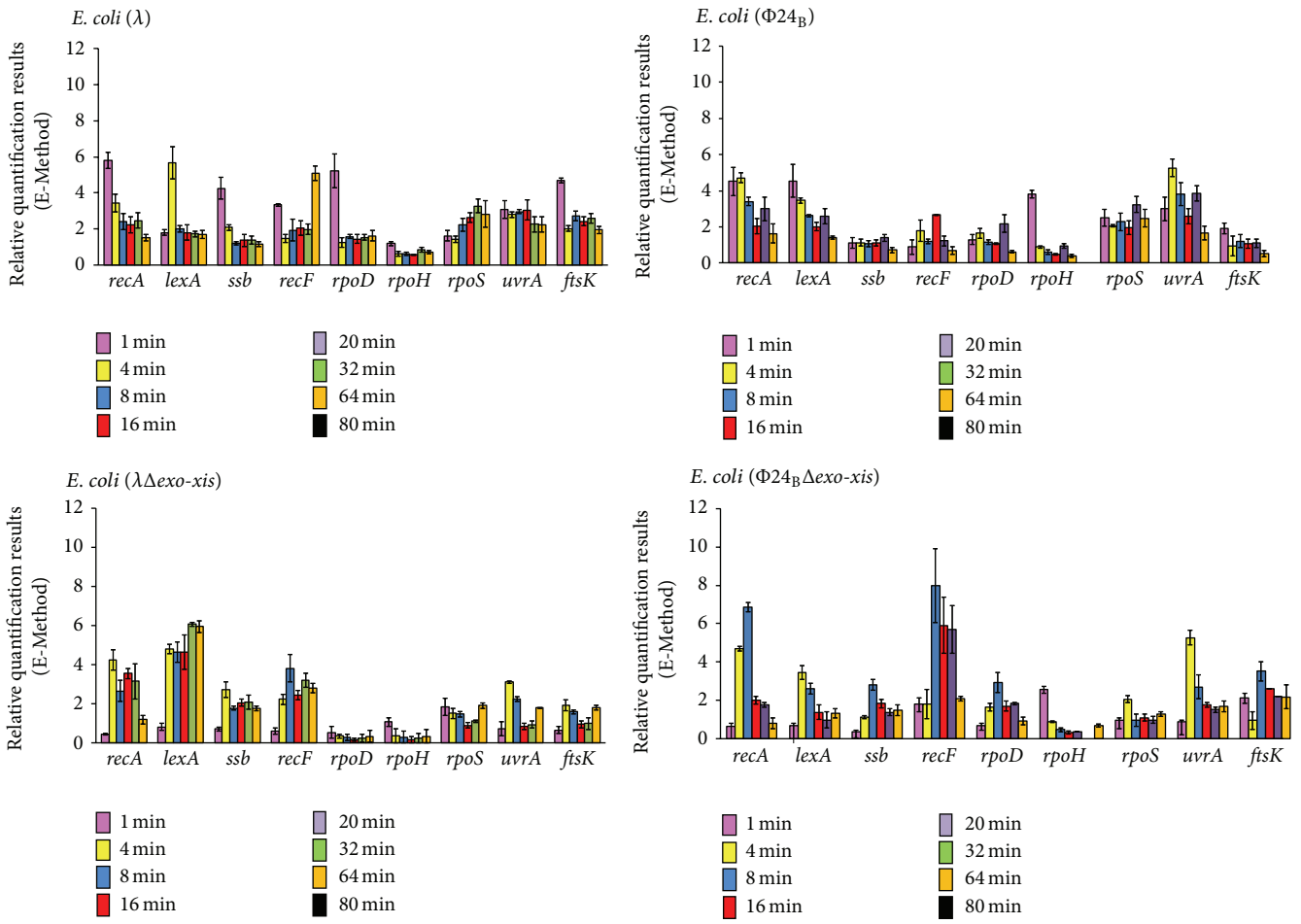

Figure 7: Expression of genes from the S.O.S. regulon in *E. coli* MG1655 lysogenic with λ, $\lambda\Delta exo\text{-}xis$, $\Phi 24_B$, or $\Phi 24_B\Delta exo\text{-}xis$, at indicated times after UV irradiation (50 J/m^2), as estimated by reverse transcription quantitative real-time PCR. The values obtained with untreated cells were used as calibrators and were subtracted from the values determined at particular time points; thus, the presented values indicate the induction of expression of tested genes. The presented results are mean values from 3 independent experiments (biological samples), with error bars indicating SD.

regulatory processes and lytic development, has been tested under the same conditions as described in the preceding subsection. The specific conditions and time after addition of hydrogen peroxide into the cell culture at which samples were withdrawn were chosen on the basis of similar experiments reported previously [26]. Interestingly, different effects of the deletion of the *exo-xis* region were observed for phages λ and 24_B. In λ, deletion of genes and open reading frames located between *exo* and *xis* genes did not cause considerable effects on mRNA levels for *xis, exo*, and *Q*, whereas expression of *int, c*III, *N, c*I, *cro, c*II, *O*, and *R* was enhanced upon treatment with hydrogen peroxide (Figure 6). Completely different results were obtained when $\Phi 24_B$ and $\Phi 24_B\Delta exo\text{-}xis$ lysogens were studied; namely, expression of all tested genes was drastically impaired in hydrogen peroxide-treated bacteria in the absence of the *exo-xis* region on the prophage (Figure 6).

While negative regulation of transcription from cII-dependent promoters by overexpression of the *exo-xis* region has been reported previously in phage λ [23], this study demonstrated for the first time significant effects of this region on expression of a battery of phage genes under conditions of the oxidative stress. The results presented in Figure 6 for phage λ are compatible with those published previously (though obtained with different methods) [23], as overexpression of the *exo-xis* region had opposite effects to those observed in its absence. On the other hand, severely impaired expression of all tested phage genes in $\Phi 24_B\Delta exo\text{-}xis$ was unexpected. However, these results (Figure 6) can explain a strong defect in the induction of $\Phi 24_B\Delta exo\text{-}xis$ prophage (and perhaps further lytic development) by hydrogen peroxide (Figure 2). Similarly, drastic differences between effects of $\Delta exo\text{-}xis$ mutations on hydrogen peroxide-mediated prophage induction between λ and 24_B (Figure 2) can be ascribed to opposite regulation of expression of phage genes in the absence of the *exo-xis* region.

3.5. Effects of the Exo-Xis Region on Expression of Host and Phage Gene in UV-Irradiated Lysogenic Cells. Experiments analogous to those described in two preceding subsections were performed with lysogenic cells irradiated with UV.

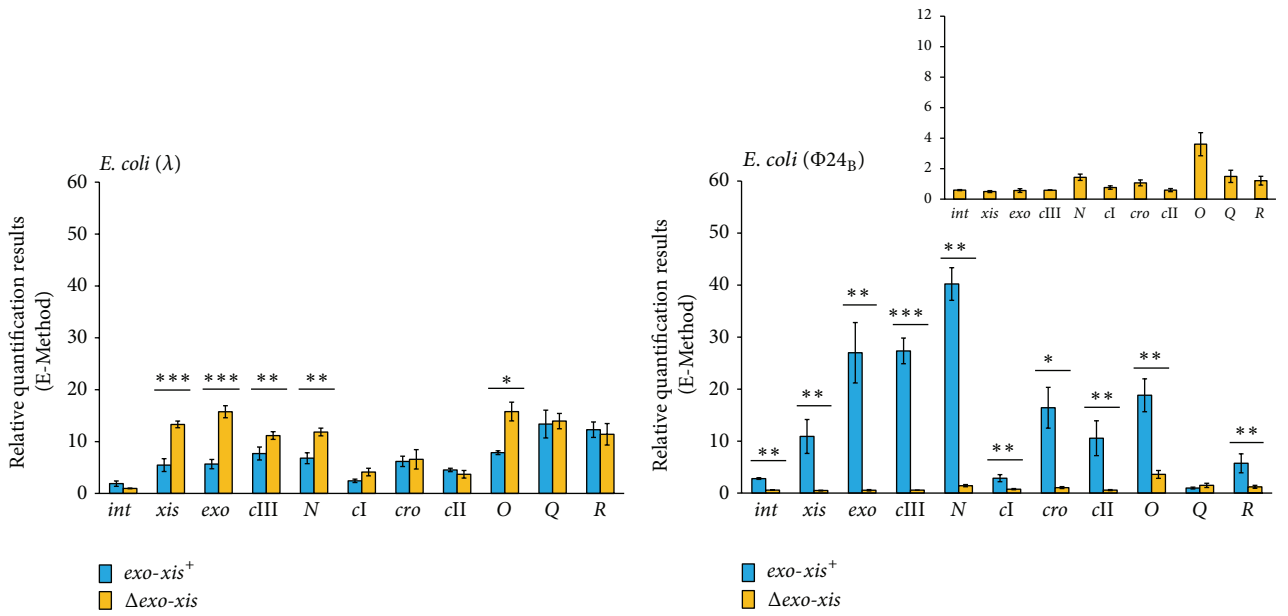

Figure 8: Expression of selected bacteriophage genes in *E. coli* MG1655 lysogenic with λ or $\Phi 24_B$, either wild-type (blue columns) or Δ*exo-xis* (yellow columns) at 160 min after UV irradiation (50 J/m^2), as estimated by reverse transcription quantitative real-time PCR. The values obtained with untreated cells were used as calibrators and were subtracted from the values determined at particular time points; thus, the presented values indicate the induction of expression of tested genes. The presented results are mean values from 3 independent experiments (biological samples), with error bars indicating SD. The additional panel for *E. coli* (24_B) represents the results of Δ*exo-xis* variant with different scale, due to very small values measured. Statistically significant differences (in *t*-test) are marked as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Interestingly, in both λ and 24_B phages, deletion of the *exo-xis* region caused only moderate effects on expression of most genes from the S.O.S. regulon (Figure 7), contrary to hydrogen peroxide-treated bacteria where the differences were significantly higher (compare Figures 5 and 7). The exceptions in UV-irradiated cells were *rpoD, rpoH*, and *rpoS* genes in λ and *rpoH* and *rpoS* in 24_B, whose expressions were at considerably lower level in the absence of the *exo-xis* region (Figure 7). One should also note that the induction of the S.O.S. regulon with UV irradiation was quicker than that with hydrogen peroxide. These results indicate that the influence of the *exo-xis* region on the S.O.S. response is particularly well pronounced under conditions of the oxidative stress.

Unlike the S.O.S. regulon expression, levels of mRNAs of bacteriophage genes in UV-irradiated cells were affected similarly to those in hydrogen peroxide-treated lysogenic bacteria by the absence of the *exo-xis* region (Figure 8). Again, although some differences were observed between λ and $\lambda\Delta$*exo-xis*, the differences between $\Phi 24_B$ and $\Phi 24_B\Delta$*exo-xis* were dramatic. This indicates that the influence of the *exo-xis* region on expression of phage genes after prophage induction does not depend on the induction agent.

4. Conclusions

The *exo-xis* region is necessary for effective, RecA-dependent induction of Shiga toxin-converting bacteriophage $\Phi 24_B$ under conditions of the oxidative stress. In hydrogen peroxide-treated *E. coli*, this region positively influences expression of the S.O.S. regulon in both $\Phi 24_B$ and λ lysogens and expression of phage genes crucial for lytic development (particularly *xis, exo, N, cro, O, Q,* and *R*) in $\Phi 24_B$, but not in λ. Since the oxidative stress appears to be the major cause of induction of Shiga toxin-converting prophages during infections of human intestine by enterohemorrhagic *E. coli* (EHEC), the *exo-xis* region and/or products of its expression might be considered as potential targets for anti-EHEC drugs.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Katarzyna Licznerska and Aleksandra Dydecka contributed equally to this work.

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Review Article

Modulation of Hypercholesterolemia-Induced Oxidative/Nitrative Stress in the Heart

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Hypercholesterolemia is a frequent metabolic disorder associated with increased risk for cardiovascular morbidity and mortality. In addition to its well-known proatherogenic effect, hypercholesterolemia may exert direct effects on the myocardium resulting in contractile dysfunction, aggravated ischemia/reperfusion injury, and diminished stress adaptation. Both preclinical and clinical studies suggested that elevated oxidative and/or nitrative stress plays a key role in cardiac complications induced by hypercholesterolemia. Therefore, modulation of hypercholesterolemia-induced myocardial oxidative/nitrative stress is a feasible approach to prevent or treat deleterious cardiac consequences. In this review, we discuss the effects of various pharmaceuticals, nutraceuticals, some novel potential pharmacological approaches, and physical exercise on hypercholesterolemia-induced oxidative/nitrative stress and subsequent cardiac dysfunction as well as impaired ischemic stress adaptation of the heart in hypercholesterolemia.

1. Introduction

Hypercholesterolemia, a frequent form of hyperlipidemia, is a metabolic disorder characterized by elevated levels of total cholesterol in the blood. Hypercholesterolemia may develop as a consequence of unbalanced diet, obesity, inherited (genetic) diseases (familial hypercholesterolemia), or other diseases (e.g., diabetes). According to large clinical studies, hypercholesterolemia affects a significant population of adults in developed countries [1]. For instance, approximately 100 million people (44.4%) suffered from hypercholesterolemia (>5.2 mmol/L) in the United States in 2008 [2]. The relationship between hypercholesterolemia and cardiovascular mortality has been known for decades [3]. Hypercholesterolemia, especially elevated low density lipoprotein (LDL) cholesterol, is a major risk factor for the development of atherosclerosis and subsequent ischemic heart disease [4], which is a leading cause of death worldwide [5]. Moreover, several experimental studies have demonstrated that, in addition to its well-known proatherogenic effect in the vasculature, hypercholesterolemia may directly affect the heart causing contractile dysfunction [6–8], aggravated ischemia/reperfusion injury [9], and attenuated responses to cardioprotective interventions including ischemic pre- and postconditioning [10, 11]. Although the pathoetiology of hypercholesterolemia has been studied extensively, the precise molecular mechanisms leading to cardiac complications are not entirely clear. Nevertheless, substantial evidence exists demonstrating that hypercholesterolemia induces oxidative and nitrative stress in the heart and that oxidative/nitrative stress plays a role in several cardiac pathologies. Therefore, modulation of oxidative stress in the hypercholesterolemic myocardium appears to be a rational approach. In this review we aim to discuss relevant literature related to potential modulation of hypercholesterolemia-induced oxidative stress and subsequent complications in the heart (Figure 1). Our attention is focused on certain pharmaceuticals, nutraceuticals, novel pharmacological approaches, and physical exercise as potential modulators.

2. Hypercholesterolemia and Oxidative/Nitrative Stress

Oxidative/nitrative stress can be defined as an excess formation or insufficient removal of highly reactive molecules such as reactive oxygen and/or nitrogen species (ROS and

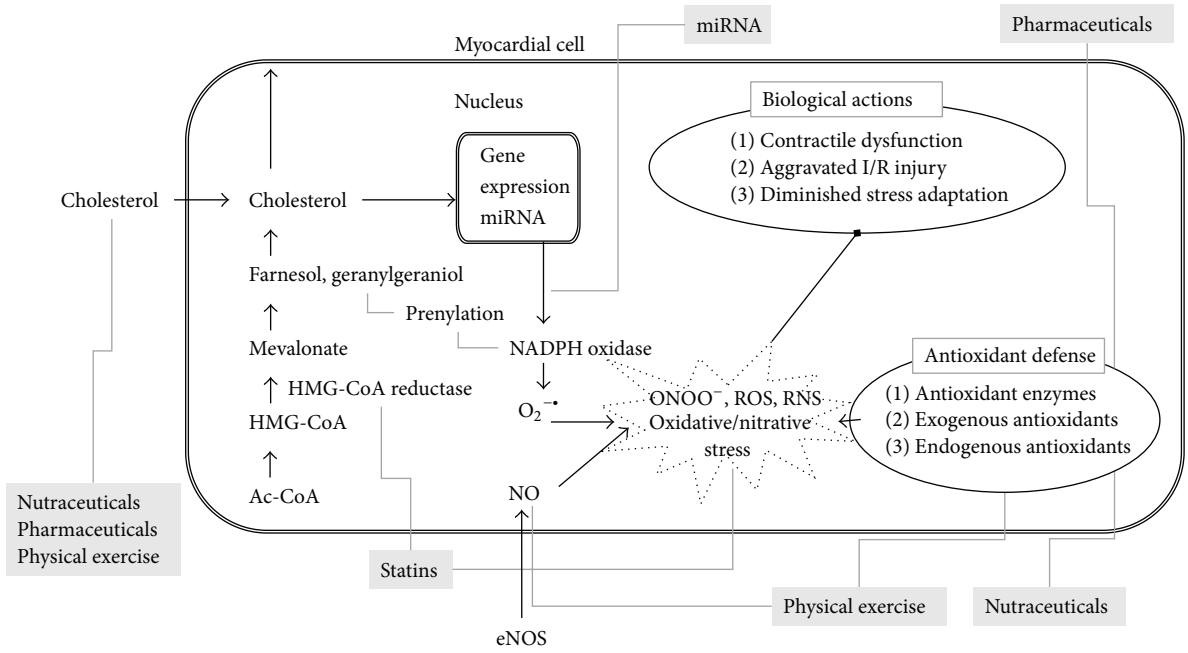

Figure 1: Hypercholesterolemia-induced myocardial oxidative/nitrative stress and its possible modulations (in grey boxes) to prevent or treat deleterious cardiac consequences. Ac-CoA: acetyl-coenzyme A; HMG-CoA: 3-hydroxy-3-methyl glutaryl coenzyme A; eNOS: endothelial nitric oxide synthase; $ONOO^-$: peroxynitrite; ROS: reactive oxygen species; RNS: reactive nitrogen species; miRNA: microRNA.

RNS, resp.) including, for instance, superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite [12]. Enzymatic sources for ROS formation include the mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase, cyclooxygenases, uncoupled nitric oxide synthase (NOS), and peroxidases, while antioxidant enzymatic systems include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, and heme oxygenase (HO) [13]. Although there is a consensus in the literature that hypercholesterolemia is associated with increased cardiac oxidative stress (Figure 1), the precise molecular mechanisms by which hypercholesterolemia induces oxidative stress in the heart are not entirely clear. Accumulating evidence shows increased superoxide production in the hearts of hypercholesterolemic animals, and one of the major sources of this superoxide appears to be increased NADPH oxidase activity in cholesterol-fed Wistar rats and apoB100 transgenic mice [6, 8]. In the hearts of cholesterol-fed CFLP mice and Wistar rats, transcript levels of NADPH oxidase 4 (NOX4) were significantly increased when compared to normal controls fed a standard chow [8, 14]. The increased NOX4 transcript levels in hypercholesterolemic hearts can be related to transcriptional, posttranscriptional, or epigenetic regulation of NOX4 expression. One study shows an association of cardiac NOX4 transcript and protein levels as well as NADPH oxidase activity with decreased myocardial level of the microRNA miR-25 in rats with diet-induced hypercholesterolemia [8]. MicroRNAs (miR or miRNA) are endogenous, small, noncoding RNAs that are responsible for posttranscriptional silencing of a wide variety of specific target genes. NOX4 was identified as a direct target of miR-25, suggesting that decreased miR-25 allows the upregulation of NOX4, which contributes to increased ROS production in the hypercholesterolemic heart [8]. Although alterations in cardiac gene expression have been demonstrated in animal models of diet-induced hypercholesterolemia [14, 15] and metabolic syndrome [16], to the best of our knowledge, the precise molecular link between hypercholesterolemia and altered cardiac gene expression has not yet been elucidated. Interestingly, two key transcriptional factors have been implicated to date in cholesterol-dependent transcriptional regulation of gene expression, that is, the sterol-regulatory element binding protein (SREBP) and the liver X receptor (LXR) [17]; however, direct evidence for their role in hypercholesterolemia-induced oxidative stress is still lacking.

Another possible explanation for increased oxidative stress could be diminished endogenous antioxidant capacity. Indeed, hypercholesterolemia has been reported to be associated with decreased cardiac expression and activity of antioxidant enzymes [18–21]. Nevertheless, the precise molecular mechanisms by which high cholesterol downregulates myocardial antioxidant enzymes remain to be elucidated. Besides enzymatic mechanisms, tissue level of the endogenous antioxidants is also an important determinant of oxidative/nitrative stress. Nitric oxide (NO) is a key antioxidant molecule that is synthesized by NO synthases in various cells including cardiomyocytes. In the hearts of hypercholesterolemic animals, cardiac NO level was decreased when compared to controls [10, 22, 23]; however, this effect was independent of the modulatory effect of cholesterol on the mevalonate pathway [23]. Moreover, transcript levels of NOS

3 were decreased, while NOS 1 and 2 remained unaffected in the hearts of cholesterol-fed mice [14].

In addition to the effects of cholesterol and its derivatives on gene expression, membrane-related effects of cholesterol are also plausible [39]; however, these effects have barely been investigated in the hypercholesterolemic heart.

3. Functional Effects of Hypercholesterolemia on the Heart

Hypercholesterolemia has been shown to exert direct myocardial effects independent of the development of atherosclerosis in both clinical [40, 41] and preclinical studies [7, 8]. These effects include impaired cardiac performance and contractile dysfunction [6, 7], aggravated ischemia/reperfusion injury [9], and diminished adaptation to ischemic stress [10, 11, 42] (Figure 1).

3.1. Cardiac Contractile Dysfunction. Observations in human clinical trials and animal models suggest a direct effect of cholesterol on myocardial contractile function leading to impaired diastolic and in some cases also systolic function [6–8, 43–45]. Although the symptoms of hypercholesterolemia are not pronounced initially and the disorder may be dormant for a long time, preclinical diastolic dysfunction shows a clear progression to heart failure [46]. Development of heart failure aggravates with advancing age and contributes to age-related morbidity and mortality [47].

The direct effects of cholesterol exposure on cardiomyocyte function were demonstrated in a cell culture model; that is, elevation of membrane cholesterol content in ventricular cardiomyocytes resulted in decreased cytosolic calcium levels and impaired cardiac myocyte contractility [48]. This has been confirmed in cholesterol-fed rabbits and rats, showing apparent contractile dysfunction characterized by decreased maximum rate of shortening, decreased rate of relaxation, and increased left ventricular end-diastolic pressure [7, 8, 10, 44]. Moreover, impairment of cardiac performance assessed by measuring aortic flow was also demonstrated in hearts isolated from hypercholesterolemic apoB100 transgenic mice [6]. Hypercholesterolemia-induced cardiac dysfunction was further confirmed by echocardiography in humans [43, 45, 49].

It has been shown previously that myocardial oxidative/nitrative stress induced by hypercholesterolemia significantly contributes to the development of cardiac dysfunction [6, 8, 50]. However, the exact underlying molecular mechanisms are still not entirely clear. One possible mechanism is the oxidation of contractile proteins [8, 51, 52]. In failing human heart samples, for instance, there is an obvious oxidation and nitrosylation of tropomyosin and actin, showing a positive correlation with diminished contractile function indicated as a decrease in ejection fraction [51]. Moreover, increased protein oxidation was confirmed in the hearts of hypercholesterolemic rats [8].

3.2. Aggravated Ischemia/Reperfusion Injury and Attenuated Stress Adaptation. Hypercholesterolemia facilitates the risk of atherosclerosis and subsequent myocardial infarction. The current treatment of myocardial infarction includes the attempt to reopen the occluded coronary artery (termed reperfusion) in a timely manner by coronary intervention procedures or thrombolytic therapies in order to reduce infarct size, which is one of the major determinants of long term complications and survival. Thus the majority of patients with acute myocardial infarction undergo ischemia/reperfusion injury. However, the myocardium is remarkably good at adapting to ischemic conditions by triggering endogenous adaptive mechanisms against ischemia/reperfusion injury. One of the most powerful endogenous adaptive cardioprotective mechanisms is ischemic preconditioning, that is, when brief exposure to repetitive ischemia/reperfusion cycles markedly enhances the ability of the heart to withstand a subsequent, potentially lethal ischemic attack [53]. Another possible intervention is termed ischemic postconditioning when the initial phase of reperfusion is interrupted with short periods of ischemia [54]. In addition, remote conditioning has also been introduced when cardioprotection is achieved by exposing an organ at a distance from the heart to ischemia/reperfusion insults [55]. To date, a very large number of preclinical and clinical studies are available suggesting that hypercholesterolemia enhances the severity of ischemia/reperfusion injury and interferes with the endogenous cardioprotective mechanisms. The mechanisms of ischemia/reperfusion injury and endogenous cardioprotection as well as the effect of hypercholesterolemia on these phenomena have been extensively reviewed elsewhere [56–58].

4. Modulation of Hypercholesterolemia-Induced Oxidative/Nitrative Stress

Modulation of oxidative/nitrative stress in hypercholesterolemia can be approached by at least 3 different ways (Figure 1). First, cholesterol-lowering therapies should be effective in attenuation of oxidative/nitrative stress due to their trigger-eliminating effect. Second, the mechanism of action of several drugs used for cholesterol-lowering is complex and may involve antioxidant properties. The second approach may be the most obvious, that is, the application of antioxidant molecules and especially natural products to reduce oxidative/nitrative stress. Third, support or induction of endogenous enzymatic antioxidant systems or inhibition of the prooxidant enzymes may be a feasible way to control cardiac oxidative/nitrative stress in hypercholesterolemia. These distinct mechanisms of action are often combined in the case of certain modulators. In this section we discuss various pharmaceuticals, nutraceuticals, some novel approaches, and even physical exercise as potential modulators of hypercholesterolemia-induced oxidative/nitrative stress.

4.1. Pharmaceuticals. To the best of our knowledge, there are no drugs on the market approved to specifically target cardiac oxidative/nitrative stress induced by hypercholesterolemia. Nevertheless, cholesterol-lowering drugs are excessively prescribed for patients presenting hypercholesterolemia and due to their cholesterol-lowering effect they should have

a secondary attenuating effect on hypercholesterolemia-induced oxidative/nitrative stress. Interestingly, several of these drugs have also been implicated in directly modulating oxidative/nitrative stress. Moreover, large clinical trials have shown that antihyperlipidemic agents, for example, statins [59], fibrates [60], and niacin [61], could reduce the incidence of cardiovascular events in hypercholesterolemic patients [59]. In addition, some drugs used for other indications than treatment of hypercholesterolemia (e.g., some antidiabetics and vasodilators) have also been demonstrated to attenuate hypercholesterolemia-induced oxidative/nitrative stress and deleterious cardiac consequences.

4.1.1. Statins. Statins are a class of cholesterol-lowering drugs that inhibit the key enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, of the endogenous cholesterol biosynthesis. Statins have therefore become established in the treatment of hypercholesterolemia and attained a central place in cardiovascular medicine [62]. However, there are potential side effects of statin therapy including skeletal muscle complaints and/or mild elevation of serum creatine kinase level and very rarely rhabdomyolysis [63]. The main members of statins on the market are lovastatin, rosuvastatin, simvastatin, atorvastatin, fluvastatin, pravastatin, and the newest addition, pitavastatin. By inhibition of the formation of mevalonate, the direct reaction product of HMG-CoA reductase and HMG-CoA, statins not only inhibit the formation of the end-product, cholesterol, but also they reduce the formation of other cholesterol pathway intermediers such as the 15-carbon isoprenoid farnesol or the 20-carbon isoprenoid geranylgeraniol which finally lead to reductions in protein prenylation, Coenzyme Q10 (see later), and dolichol synthesis. Besides lipid-lowering effects, statins have pleiotropic effects on different cell types. Some of these cholesterol-independent effects of statins involve improved endothelial function, stabilization of atherosclerotic plaques, and attenuation of oxidative stress and inflammation as well as inhibition of the thrombogenic response [64, 65]. Pleiotropic effects may play an important part in reducing cardiovascular mortality and morbidity and may act at multiple points in the complex cascade of events leading to atherosclerosis.

Regarding the antioxidative effect of statins, a recent study showed that, beside the cholesterol-lowering effect, simvastatin is able to ameliorate endothelial dysfunction through increasing NO bioavailability and through suppression of oxidative stress in a rat model of hypercholesterolemia [66]. Similar results were shown by Iliodromitis et al., [67] who found that a 3-week simvastatin treatment limits infarct size and attenuates oxidative and nitrosative stress both in normocholesterolemic and in hypercholesterolemic rabbits subjected to ischemia/reperfusion irrespective of the presence of postconditioning, while postconditioning was effective only in normocholesterolemic animals. According to another study [68], pravastatin, in contrast to same-dose simvastatin or postconditioning, was found to reduce infarction in hypercholesterolemic rabbits independently of lipid-lowering, potentially through eNOS activation and attenuation of oxidative/nitrative stress. The antioxidative effect of atorvastatin was also shown by several studies [69–72]. Moreover, in hyperlipidemic subjects with metabolic syndrome, atorvastatin is associated with a greater reduction in lipid markers of oxidation compared with pravastatin [73]. In contrast, Sodha et al. [74] found increased levels of myocardial biomarkers of oxidative stress in hypercholesterolemic swine treated with atorvastatin. Pitavastatin, the newest member of the HMG-CoA reductase inhibitor family, has shown improvements in both cardiovascular function and markers of oxidative stress [75], presumably via decreasing NADPH oxidase activation [64]. Fluvastatin was also shown to play a protective role against high-cholesterol-induced oxidative stress and DNA damage [76]. Taken together, the cardioprotective effect of almost every statin is associated with some pleiotropic effect which can decrease oxidative stress in the cardiovascular system.

4.1.2. Ezetimibe. Ezetimibe, another lipid-lowering drug, is used in monotherapy or in combination with statins and is responsible for lowering intestinal cholesterol absorption by inhibiting the Niemann-Pick C1-Like 1 (NPC1L1) sterol transporter. Clinical studies showed that ezetimibe attenuates the markers of oxidative stress in hyperlipidemic subjects [77–80]. The drug is suggested to exert both cholesterol-dependent and independent actions [78–80]. Reduction of serum cholesterol results in decreased cholesterol influx to the cells thereby attenuating cholesterol-induced oxidative stress. For instance, ezetimibe reduced hepatic cholesterol level in obese male mice which in turn attenuated oxidative stress via downregulation of NADPH oxidases, cytochrome P4502E1, and beta oxidation [81]. In addition to its cholesterol-lowering action, ezetimibe may exert a direct cholesterol-independent effect on cells [78–80, 82]. A feasible explanation is that NPC1L1 is widely expressed in many other tissues including liver, kidney, muscle, and heart [83]. Cell culture studies support the possible direct effect of ezetimibe on cellular oxidative/nitrative stress, for example, knockdown of NPC1L1 in hepatocytes attenuated ROS production [84]. Based on these findings it is plausible to speculate that ezetimibe has a direct impact on the heart and may decrease cholesterol-induced oxidative stress. Nevertheless, direct cardioprotective effect of ezetimibe has not yet been investigated in the literature; thus preclinical studies needed to address this issue.

4.1.3. Niacin. Although niacin (nicotinic acid or Vit B_3) is a potent lipid-lowering agent when used in pharmacologic doses, its clinical use is limited by side effects, so it can be applied particularly in combination to treat marked dyslipidemia with strict monitoring ([85], for review see [86]). Niacin administration was shown to reduce markers of serum oxidative stress and increase antioxidant paraoxonase-1 in patients with hypercholesterolemia [87]. A randomized controlled clinical trial reported that niacin improves both 6- and 15-year mortality of patients after myocardial infarction with or without metabolic syndrome thereby suggesting a cardioprotective effect for niacin [88]. Direct cardioprotective effect of niacin was reported in preclinical studies as well [89–92]. However, evidence is not available to clarify the

potential beneficial effect of niacin in cholesterol-induced cardiac stress.

4.1.4. Fibrates. Fibrates (amphipathic carboxylic acid derivatives) lower plasma triglyceride and small LDL level and increase high density lipoprotein (HDL) concentration; thus fibrates are particularly used to treat combined dyslipidemia related to diabetes or metabolic syndrome [93]. This class of drugs acts on nuclear peroxisome proliferator-activated receptor α (PPARα), leading to transcriptional changes [94]. Studies revealing the effect of fibrates on cholesterol-induced cardiac oxidative stress are not available in the literature. Nevertheless, some investigations have reported an antioxidant effect of fibrates. A subcohort clinical trial has demonstrated that 3-week fenofibrate treatment reduced ox-LDL and 8-isoprostane, systemic markers of oxidative stress in patients with hypertriglyceridemia [95]. In accordance with these results, fenofibrate decreased ox-LDL with a modest reduction in cholesterol level in healthy normolipidemic older adults [96]. Fibrates may exert antioxidant actions independently from their lipid-lowering effect. For instance, Sugga et al. have reported that *in vivo* administration of fenofibrate and clofibrate before *ex vivo* myocardial ischemia/reperfusion reduced infarct size and oxidative stress [97]. Others have shown antioxidant and cardioprotective effects of fibrates against development of ventricular hypertrophy associated with oxidative stress [98, 99]. Furthermore, fibrates prevent endothelial dysfunction by ameliorating oxidative stress, without affecting plasma lipid levels [100, 101]. It has been also published that knockout of PPARα, the ligand of fibrates, leads to oxidative stress associated with cardiac dysfunction [102]. In contrast, fenofibrate failed to improve lipotoxic cardiomyopathy [103], and high dose fibrate induced cardiac damage in healthy rats [104]. Taken together, the studies focusing on antioxidant action of fibrates suggest a potential beneficial effect on cholesterol-induced cardiac oxidative stress, although this needs to be confirmed in further studies.

4.1.5. Other Pharmaceuticals. There are some available drugs, which are basically used to treat other diseases than hypercholesterolemia and which have beneficial effects on high-cholesterol-induced cardiac consequences.

Rosiglitazone is an antidiabetic drug; however, it also improves blood cholesterol level in hypercholesterolemic models [105, 106]. It was reported that the drug prevents the development of high-cholesterol-induced cardiac hypertrophy [105] and ameliorates postischemic recovery and nitrative stress in the heart of high-cholesterol-fed rabbits [107]. The latter research group has also shown that rosiglitazone alleviates aggravated postischemic myocardial injury and myeloperoxidase upregulation caused by hypercholesterolemia, independently from the lipid-lowering action [108]. Rosiglitazone has a direct antioxidant effect on cardiomyocytes [109] and has been demonstrated to prevent the upregulation of cardiac NOX4 in a high-fat high-sugar diet, streptozotocin-induced diabetic model associated with dyslipidemia [110]. These findings make rosiglitazone a promising modulator of cholesterol-induced cardiac oxidative stress.

Increasing the level of glucagon-like peptide-1 by administration of analogues or by inhibiting dipeptidyl peptidases is used for the treatment of diabetes and has recently emerged as a potential cardioprotective approach [111]. Specifically, a glucagon-like peptide-1 agonist was shown to reverse cardiac dysfunction induced by high-fat diet [112] and a dipeptidyl peptidase inhibitor was found to improve cardiac dysfunction and oxidative stress in high-fat high-fructose-fed mice [113].

The vasodilator fasudil (a potent rho kinase inhibitor) was shown to lower serum cholesterol level and decrease cardiac oxidative stress by enhancing antioxidants in hypercholesterolemic rat hearts [114]. The group has reported that fasudil restores cardiac eNOS and NO level diminished by high-cholesterol diet [114]. Interestingly, even in the state of hypercholesterolemia, fasudil can induce both preconditioning and postconditioning [115]. Moreover, fasudil restores the cardioprotective effect of ischemic postconditioning in rats with hypercholesterolemia [116].

4.2. Nutraceuticals. In spite of the extensive research in cardiology, particularly in ischemic heart diseases, only very few new cardioprotective drugs have found their way into clinical practice [117]. Therefore, there is a growing interest in nutraceuticals to treat and also prevent certain cardiac diseases or restore the injured adaptation of the heart. Nutraceuticals is a term that refers to a wide range of products including but not limited to natural herbal products, dietary supplements, isolated nutrients, and special diets. Since there is also a growing desire among potential customers to consume natural dietary products instead of chemically synthesized compounds, the importance of these nutraceuticals is being further emphasized.

Natural food substances have the potential to alter biological functions of the cells by mechanisms enhancing the endogenous antioxidant systems or through altering the redox signaling status of the cell. This is often related to the unique composition of different antioxidant compounds in the various nutraceuticals. These products could be beneficial in pathological conditions where oxidative stress plays an important role.

4.2.1. Antioxidant Vitamins. A number of preclinical studies have demonstrated that classical antioxidant vitamins including beta carotene (Vit A precursor), folic acid (Vit B_9) vitamin C, and vitamin E administered alone or in combination with other drugs or in multivitamin preparations could improve serum lipid profile and myocardial oxidative/nitrative stress in hypercholesterolemia [18, 118–126]. Moreover, low dose beta carotene has been shown to improve cardiac function and reduce myocardial oxidative stress after ischemia/reperfusion injury in hearts of rats [63]. In contrast, vitamin C alone or combined with vitamin E failed to reduce myocardial infarct size in an open chest rabbit model [127]. Indeed, Trolox, a vitamin E analogue did not reduce infarct size but accelerated functional recovery after myocardial infarction in porcine hearts [128]. In addition, we have previously shown that a multivitamin preparation supplemented with phytosterols decreased serum cholesterol level in hypercholesterolemic rats; however, it did

not reduce infarct size either in normocholesterolemic or in hypercholesterolemic hearts of rats [118]. In contrast, preconditioning with vitamin E has been shown to improve postischemic contractile and vascular functions in hearts of rats after ischemia/reperfusion injury [129]. To the best of our knowledge, the effects of antioxidant vitamins have not been tested on cardioprotection conferred by ischemic pre- or postconditioning. However, decrease of oxidative/nitrative stress by antioxidant vitamins could be a potential therapeutic target in the restoration of cardioprotective adaptive mechanisms lost in hypercholesterolemia.

Despite a number of preclinical studies proving beneficial effects of antioxidant vitamins on cardiac pathologies in hypercholesterolemia, clinical trials investigating the effects of antioxidant vitamins on cardiovascular morbidity and mortality have been disappointing. The complex reasons that might explain the translational failures of preclinical results into clinical therapy are discussed in detail in recent reviews [130–132] and include the following: (i) oxidative/nitrative stress is just a late consequence of cardiac pathologies and not the primary cause; (ii) pathogenesis of cardiovascular diseases is complex and increased oxidative/nitrative stress is not the only cause of these disorders; (iii) antioxidant vitamin therapy is not able to reduce oxidative/nitrative stress due to inappropriate study design (inappropriate patient selection, failure in the administration route, suboptimal time and duration of antioxidant therapy, poor target specificity, and potential interaction with other drugs, etc.); (iv) a single antioxidant therapy is not enough to overcome increased oxidative/nitrative stress; (v) antioxidant molecules might have harmful effects on physiological processes or compensatory mechanisms in pathologic conditions induced by oxidative/nitrative stress.

The lack of beneficial effects of antioxidant vitamins on cardiovascular pathologies in clinical trials does not disprove that oxidative/nitrative stress plays a crucial role in cardiac pathologies in hypercholesterolemia. These clinical trials challenge us to develop better antioxidant approaches and better preclinical models as well as to design more appropriate clinical trials considering the aforementioned reasons of translational failure.

4.2.2. Coenzyme Q. Coenzyme Q10 (CoQ10), also called ubiquinone or ubiquinol, is an isoprene derivative and an alternative product of the mevalonate/cholesterol pathway. CoQ10 is an endogenous nonenzymatic lipophilic antioxidant and free radical scavenging molecule playing a role in the mitochondrial electron transport in the inner mitochondrial membrane [133]. Moreover, CoQ10 has been reported to play part in many levels of the redox control of cellular signaling; in fact the autoxidation of its semiquinone form, generated in various membranes during electron transport, can be a primary source for hydrogen peroxide production, which activates transcription factors [134]. Due to its localization in the mitochondria, it plays a key role in the cellular bioenergetics especially in tissues with high energy requirements such as the myocardium which is extremely sensitive to CoQ10 deficiency [133, 135]. CoQ10 has been shown to decrease the levels of proinflammatory cytokines and to reduce LDL oxidation and consequently the progression of atherosclerosis. It also decreased ischemia/reperfusion injury after myocardial infarction [135, 136]. Significant improvement has been demonstrated in clinical and hemodynamic parameters and in exercise tolerance in patients given adjunctive CoQ10 in various trials conducted in patients suffering from heart failure, hypertension, ischemic heart disease, and other cardiac illnesses [135–137]. Therefore, CoQ10 could be a potential therapeutic molecule in heart diseases.

Due to the common biosynthetic pathway of cholesterol and CoQ10, the HMG-CoA reductase inhibitor statins may potentially reduce the levels of CoQ10 in different tissues [138]. Indeed, it has been reported that CoQ10 levels in the plasma, platelets, and lymphocytes were decreased after statin treatment [138]. Some papers indicated that CoQ10 depletion during statin therapy might be associated with subclinical cardiomyopathy and this situation is reversed upon CoQ10 treatment [138]. In contrast, a preclinical study reported that coadministration of CoQ10 with simvastatin impaired mitophagy and cardioprotection after ischemia/reperfusion injury in mice and cardiomyocytes [139]. These results raise the concern that CoQ may interfere with the anti-ischemic benefit of statins mediated through stimulation of mitophagy. Therefore, patients treated with statins should be also monitored for their CoQ10 status and clinicians should be aware of the aforementioned interaction between statins and CoQ10 as well as the ability of statins to impair skeletal muscle and myocardial bioenergetics [138].

4.2.3. Flavonoids. Flavonoids are a large family of natural polyphenolic compounds in the human diet and their beneficial effect on cardiovascular diseases is widely studied. These compounds favorably affect a wide range of biological processes. Beside their antioxidant capacity, flavonoids improve lipid profile and have anti-inflammatory, antiplatelet, and antithrombotic effects as well.

Green Tea Catechins (GTC). Catechins are abundant polyphenols in green tea. GTC were shown to reduce blood cholesterol level in both animals [140–143] and humans [144–147]. In addition, GTC reduced accumulation of cholesterol in the rat myocardium in a state of diabetic dyslipidemia [141]. In a double-blind, placebo-controlled clinical study, obese, hypertensive patients had decreased LDL-cholesterol and increased HDL and total serum antioxidant levels after 3 months of GTC supplementation [148]. Mehra et al. demonstrated that catechin hydrate improved high sucrose, high-fat-induced cardiac lipid peroxidation and activity of antioxidant enzymes [149]. GTC were reported to beneficially affect high-fructose diet-associated hypercholesterolemia and cardiac signaling pathways related to lipid metabolism and inflammation [150]. Age-related cardiac oxidative stress was also improved by GTC [151]. The effect of GTC on hypercholesterolemia associated with cardiac dysfunction is not known, but it was reported that GTC ameliorates myocardial function after ischemia [152, 153] in pressure-induced chronic heart failure [154] and in autoimmune myocarditis as well [155].

Troxerutin. Troxerutin can be isolated from the Japanese pagoda tree (*Sophora japonica*). To the best of our knowledge, the antioxidant effect of troxerutin on the heart was first described in an ischemia/reperfusion model [156]. Geetha et al. have investigated the cardiac impact of troxerutin in high-fat high-fructose mice model. They have found that troxerutin reduces cholesterol content and oxidative stress markers in both the plasma and the heart and increases cardiac enzymatic and nonenzymatic antioxidant levels [157]. Moreover, the same group have shown in the same dyslipidemic model that troxerutin reverses fibrotic changes in the heart and improves cardiac function probably by reducing ROS production [158].

Quercetin. The cardioprotective effect of the dietary flavonoid quercetin (found in many fruits, vegetables, leaves, and grains) against ischemia/reperfusion injury is well studied [159–163]; however, few studies are available in the literature regarding the impact on hypercholesterolemia-induced oxidative stress in the heart. It was reported that quercetin ameliorates left ventricular function, collagen deposition, and inflammatory cell infiltration in rats with metabolic syndrome [164]. In that study, quercetin increased the expression of the transcription factor Nrf2 and the enzyme heme oxygenase as protective proteins against oxidative stress. Interestingly, quercetin exerted a cardioprotective effect without reducing plasma cholesterol level thereby suggesting a direct cardiac effect. In other studies, quercetin has been shown to reduce serum LDL and increase HDL cholesterol level in hypercholesterolemic rats [165] and lower total cholesterol in rabbits fed a high-cholesterol [166] and high-fat [167] diet. Ulasova et al. showed that quercetin improves lipid profile of ApoE knockout mice and prevents ventricular hypertrophy without affecting myocardial function [168]. In a cadmium-induced toxicity study, quercetin was found to reduce cardiotoxicity and dyslipidemia by attenuating cardiac oxidative stress and lipid parameters [169].

Rutin. Rutin (the glycoside between the quercetin and the disaccharide rutinose) improves cholesterol level in different hypercholesterolemic [170, 171] and dyslipidemic [172, 173] rodent models. Panchal et al. have shown that rutin supplementation ameliorates blood cholesterol level, cardiac structure, function, inflammation, and oxidative stress related to high carbohydrate high-fat-induced dyslipidemia [173]. In a streptozotocin-induced diabetes model associated with hyperlipidemia, rutin attenuated serum cholesterol level and myocardial necrosis and improved left ventricular dysfunction [174]. It was also reported that rutin exerts cardioprotection by attenuating oxidative stress and dyslipidemia induced by high fluoride administration in rats [172].

Silymarin. Silymarin is a mixture of three flavonolignans (silibinin, silydianin, and silychristin) extracted from milk thistle seeds (*Silybum marianum*). Krečman et al. demonstrated that silymarin prevented the development of hypercholesterolemia in high-cholesterol-fed rats [175]. They have also shown that silibinin itself was not as effective as silymarin. Others also reported that silymarin or its fraction decreased total and LDL cholesterol and attenuated oxidative stress in the plasma [176–178]. The effect of silymarin on hypercholesterolemia-induced myocardial oxidative stress is not yet investigated, but some studies indicate that silymarin exerts cardioprotection against high-cholesterol-mediated oxidative stress. Silymarin attenuates myocardial ischemia/reperfusion injury by modulating oxidative stress [179] and silibinin has a direct antioxidant effect on H9c2 cardiac cells against oxidative stress [180]. Moreover, silibinin was reported to improve both plasma and cardiac cholesterol content and attenuate oxidative markers and degenerative changes in the heart of animals exposed to arsenic [181]. In this study, silibinin prevented cardiac oxidative stress by inhibiting the induction of prooxidants (e.g., NOX2 and NOX4) and enhancing antioxidants.

Naringin and Hesperidin. Naringin and hesperidin are natural flavonglycosides in citrus fruits. Many studies reported hypocholesterolemic effect of naringin and hesperidin in animal models [182–188] and in a clinical study [189]. However, in moderately hypercholesterolemic subjects, naringin and hesperidin failed to lower serum cholesterol [190]. Alam et al. have published that naringin improves ventricular diastolic dysfunction, cardiac inflammatory cell infiltration, and plasma cholesterol in high carbohydrate, high-fat-fed rats [182]. Recently, naringin has been shown to protect against hypercholesterolemia-induced oxidative stress in the heart [191]. The study has demonstrated that naringin ameliorates cardiac lipid accumulation and cardiac oxidative stress markers by enhancing enzymatic and nonenzymatic antioxidants. Moreover, tissue and serum markers of cholesterol-induced cardiac damage were also attenuated by naringin supplementation [191]. Although hesperidin was reported to protect against cardiac injury induced by doxorubicin [192] or ischemia [193, 194], its role in cholesterol-induced cardiac oxidative stress is not known.

4.2.4. Resveratrol. Pleiotropic beneficial effects of the polyphenol resveratrol (food sources include the skin of grapes, blueberries, raspberries, and mulberries) have been extensively studied and many publications indicate its protective function on cholesterol-induced cardiac oxidative stress. A direct antioxidant effect of resveratrol was demonstrated on H9c2 cardiac cells [195]. Louis et al. have demonstrated that resveratrol ameliorates cardiac relaxation dysfunction in high-fat-fed rats with hypercholesterolemia [196]. They have also shown reduced oxidative stress and inflammatory markers in the serum as a result of resveratrol treatment [196]. In hypercholesterolemic rats with normal cardiac function, resveratrol improved postischemic recovery of the heart [197]. Large animal studies from the same research group reported that resveratrol improved high-cholesterol and chronic ischemia-induced cardiac dysfunction and oxidative damage of myocardial proteins [198–200]. The group also described that resveratrol improved high-cholesterol-induced myocardial dysfunction without decreasing protein oxidation in the absence of ischemia [198]. Chu et al. have shown that red wine ameliorates cardiac dysfunction and

oxidative stress in hypercholesterolemic swine subjected to chronic ischemia [50]. Resveratrol was reported to improve lipid profile and cardiac dysfunction related to hyperlipidemia in streptozotocin-induced diabetes [201]. Finally, these results may promote new studies focusing on cholesterol-induced cardiac oxidative stress.

4.2.5. Grape Seed. Grape seed and skin are rich in natural antioxidants, so these are possible supplements to alleviate hypercholesterolemia-induced oxidative stress. In a high-fat-induced obesity model, grape seed extract was shown to improve lipid profile and prevent postischemic heart dysfunction and cardiac lipid accumulation along with attenuated oxidative stress [202, 203]. Lee et al. reported that grape skin ameliorates total serum antioxidant capacity of rats with high-fat diet and low-fat diet [204]. Antioxidant effect of grape seed was tested on cardiomyoblast H9c2 cell culture [205], where it increased endogenous antioxidant systems and prevented ROS-induced apoptosis [205]. *In vivo* pretreatment with grape seed proanthocyanidins improved postischemic functional recovery and reduced ROS production in normocholesterolemic rats [206] and partially restored the harmful cardiac effects of hypercholesterolemia via their ability to reduce ROS in the myocardium [207].

4.2.6. Sour Cherry Seed Extract. Based on the observation that cherries contain bioactive phytochemicals, for example, phenolics and anthocyanins, which are reported to possess antioxidant, anti-inflammatory, anticancer, antidiabetic, and antiobesity properties, Tosaki and his group hypothesized that the seed kernel of sour cherry (*Prunus cerasus*) may contain different bioactive constituents [208]. They demonstrated that kernel extract obtained from sour cherry seed improves postischemic cardiac functional recovery and the incidence of ventricular fibrillation and tachycardia in isolated working rat hearts [209]. Moreover, sour cherry seed extract-induced improvement in cardiac function after ischemia/reperfusion along with decreased atherosclerotic plaque formation and infarct size was also observed in hypercholesterolemic New Zealand rabbits fed a 2% cholesterol-enriched diet for 16 weeks [210]. In this model, the authors demonstrated an increased HO-1 and cytochrome c oxidase III protein expression following administration of sour cherry seed extract as a possible mechanism of action [210, 211].

4.2.7. Spices. Aqueous extracts of certain spices including garlic (*Allium sativum*), ginger (*Zingiber officinale*), and cayenne pepper (*Capsicum frutescens*) as well as their mixture were shown to attenuate cardiac lipid peroxidation induced by high-cholesterol, high-fat diet in a rat model of hypercholesterolemia [20]. Moreover, in the same study, hypercholesterolemia-induced decrease in the myocardial activities of antioxidant enzymes (i.e., SOD, glutathione peroxidase, and glutathione reductase) was also markedly attenuated by administration of the individual spices as well as their combination [20].

4.2.8. Red Palm Oil. Red palm oil (RPO) is a product from the fruits of the oil palm tree (*Elaeis guineensis*). RPO depending on the producer consists of about 51% saturated fatty acids (SFAs), 38% monounsaturated fatty acids (MUFAs), 11% polyunsaturated fatty acids (PUFAs), and a spectrum of antioxidative carotenoids with tocopherols and tocotrienols as the major constituents [212]. Other minor components present in this oil are ubiquinones (mainly CoQ10) and phytosterols. Red palm oil is therefore a natural carotenoid rich oil that has the potential to act as a very potent antioxidant [213]. Red palm oil contains the highest concentration of tocotrienols compared with other vegetables or plants and Serbinova et al. showed that tocotrienols can be 40–60 times more potent as antioxidants than tocopherols [214].

Van Rooyen's research group investigated the effect of dietary administration of RPO on the heart. They showed that prolonged dietary feeding with RPO-supplemented diet (~7 g RPO/kg diet) protected the heart against ischemia/reperfusion injury in rats. They used isolated heart models, perfused both in Langendorff technique [215–218] and working mode [212, 219], showing the vascular-independent direct cardioprotective effects of RPO. RPO administration improved cardiac function during reperfusion [212] and decreased infarct size [215, 216]. The real advantage of RPO administration became apparent when RPO was given to hyperlipidemic rats. Hyperlipidemia was induced by feeding animals with 2% cholesterol-enriched diet for 5–9 weeks. This model was characterized by mild cholesterol elevation but a marked decrease of cardiac performance. RPO was able to markedly increase aortic output recovery after 25 min global ischemia [219, 220], or infarct size [215] after 30 min global ischemia, showing the protective effect of RPO in the presence of comorbidities.

The proposed mechanism by which RPO exerts its cardioprotective effect in animals fed high-cholesterol diet is not fully understood. Supplementation with RPO in the presence of potentially harmful cholesterol showed no significant difference in serum cholesterol level; therefore, its protection cannot be explained by the cholesterol-lowering effect of RPO. It is proposed that the protective effect of RPO in high-cholesterol diet may be associated with either the RPO antioxidant characteristics and/or changes in the fatty acid composition of the myocardium during ischemia/reperfusion.

4.3. Promising Novel Approaches to Modulate Hypercholesterolemia-Induced Oxidative Stress

4.3.1. miR Modulation. Only 3% of the human genome codes for proteins and the remaining part consist of noncoding RNAs including short microRNAs (miRNAs, miRs; approximately 18–25 nucleotides in length) [221, 222]. miRNAs can inhibit the translation or promote mRNA degradation by binding to specific mRNAs according to the complementarity of their seed sequences [223]. Individual miRNAs may simultaneously target multiple mRNAs. However, the expression of individual mRNAs can be regulated by multiple miRNAs. Therefore, miRNAs may act as fine tuners or as on/off switchers of gene expression [223, 224]. Dysregulation of miRNAs

Table 1: Regulation of miRNAs in hyperlipidemia.

miRNA	Regulation of miRNA	Organ	Target	Regulation of target	Role	References
let-7g	down	Aorta	ox-LDL receptor 1	up	ox-LDL cholesterol uptake	[24]
miR-25	down	Heart	NOX4	up	Oxidative stress	[8]
miR-33	up	Liver, macrophages	ABCA1	down	Reverse cholesterol transport	[25]
miR-33	up	Peripheral tissues	ABCG1	down	Reverse cholesterol transport	[25, 26]
miR-33	down	Liver	Fatty acid synthesis	down	VLDL synthesis	[27]
miR-33	down	Liver	Fatty acid oxidation	up	VLDL synthesis	[27]
miR-144	up	Liver	ABCA1	down	Reverse cholesterol transport	[28]
miR-223	up	Liver	HMG-CoA synthase 1	down	Cholesterol biosynthesis	[29]
miR-223	up	Liver	Scavenger receptor B1	down	HDL cholesterol uptake	[29]
miR-208a	up	Heart	MED13	down	Glucose tolerance	[30]
miR-378 and miR-378*	up	Liver	MED13	down	Glucose tolerance	[31]
miR-378 and miR-378*	up	Insulin dependent tissues	Fatty acid oxidation	down	Obesity	[31]

ABC: ATP-binding cassette transporter; HDL: high density lipoprotein; VLDL: very low density lipoprotein; MED: mediator complex subunit; NOX: NADPH oxidase.

in pathological conditions may alter gene networks; therefore miRNA replacement or antisense inhibition therapy offers a new approach to treating diseases by modulating gene pathways rather than single molecular targets [222].

In recent years, a growing body of evidence has demonstrated that miRNAs play a role in the development of numerous cardiovascular diseases. Several excellent reviews focus on miRNAs as diagnostic markers and potential therapeutic targets in cardiac pathologies including acute coronary syndrome [221, 225] and remodelling after myocardial infarction as well as heart failure [25, 221, 224, 226–228] and their risk factors including hypercholesterolemia [229], diabetes mellitus [230, 231], arterial hypertension [232, 233], atherosclerosis [234, 235], and aging [227].

Hypercholesterolemia is a well-known risk factor of cardiovascular diseases and it leads to increased oxidative/nitrative stress in the myocardium. Experimental data are very limited on the regulatory role of miRNAs in hypercholesterolemia-induced oxidative/nitrative stress in cardiac pathologies. We have previously shown that the myocardial downregulation of miR-25 results in the upregulation of NADPH oxidase 4 (NOX4) mediating oxidative/nitrative stress and subsequent myocardial dysfunction in male hypercholesterolemic rats [8] (Table 1). Moreover, in a recent study, decreased circulating miRNA-25 level has been related to the level of oxidative stress indicators in septic patients and the clinical accuracy of miRNA-25 for sepsis diagnosis has been reported to be better than C-reactive protein [236]. In addition, downregulation of miR-25 expression has been demonstrated in cardiac hypertrophy induced by transverse aortic constriction surgery in mice; however, there is no data published on increased oxidative/nitrative stress or cholesterol levels in that study [237]. Furthermore, *in vivo* inhibition of miR-25 by a specific antagomir resulted in the spontaneous development of cardiac dysfunction and sensitized the myocardium to develop heart failure in a Hand2-dependent manner [237]. In contrast, inhibition of overexpressed miR-25 was reported to ameliorate contractile dysfunction by improving sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)2a activity and Ca^{2+} handling in chronic heart failure induced by transverse aortic constriction surgery in mice as well as in failing human heart samples; however, data on the presence of hypercholesterolemia in humans were lacking in this study [238].

It is well known that hyperlipidemia, obesity, metabolic syndrome, and heart failure are associated with abnormal cardiac metabolism. In obese mice, a heart specific miRNA, miR-208a, has been reported to negatively regulate mediator complex subunit 13 (MED13), which controls transcription by thyroid hormone and other nuclear hormone receptors [30] (Table 1). Indeed, cardiac-specific overexpression of MED13 or pharmacologic inhibition of miR-208a in mice has been demonstrated to confer resistance to high-fat diet-induced obesity and improve systemic glucose tolerance [30] (Table 1). Moreover, mice genetically lacking miR-378 and miR-378* have been shown to be resistant to high-fat diet-induced obesity and exhibit enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity of

insulin-target tissues [31] (Table 1). Interestingly, MED13 and carnitine O-acetyltransferase, a mitochondrial enzyme involved in fatty acid metabolism, are among the many targets of miR-378 and miR-378* [31] (Table 1). Thus, these miRNAs provide potential therapeutic targets in hyperlipidemia and metabolic disorders, although their myocardial function needs to be further investigated.

High level of LDL cholesterol and low level of HDL cholesterol are both well known as independent risk factors of coronary artery diseases. miRNAs have been shown to regulate lipoprotein metabolism and their pro-/antiatherogenic effects at many levels in different tissues as reviewed by others [25, 222, 229]. Cholesterol efflux from cells is the first step in reverse cholesterol transport to the liver carried out by HDL. miR-33 has been reported to modulate cholesterol efflux by repressing the expression of ATP-binding cassette transporter (ABC) A1 in the liver and ABCA1 as well as ABCG1 in peripheral tissues [25] (Table 1). Moreover, anti-miR-33 therapy was shown to induce the expression of ABCA1 in macrophages in atherosclerotic plaques thereby reducing the plaque size and local inflammation in mice [26] (Table 1). In addition, anti-miR-33 therapy in nonhuman primates increased the expression of miR-33 target genes involved in fatty acid oxidation and reduced the expression of genes associated with fatty acid synthesis in the liver resulting in a marked suppression of plasma VLDL triglyceride levels [27] (Table 1). Another miRNA, miR-144, has been reported to decrease hepatic ABCA1 expression and plasma HDL cholesterol levels [28] (Table 1). Moreover, miR-223 has been demonstrated to reduce HDL cholesterol uptake by decreasing the expression of scavenger receptor B1 and to decrease cholesterol biosynthesis through the direct repression of HMG-CoA synthase 1 in the liver of ApoE$^{-/-}$ mice [29] (Table 1). Furthermore, genetic ablation of miR-223 resulted in elevated hepatic and plasma total cholesterol levels as well as increased HDL cholesterol levels and particle size [29] (Table 1). Interestingly, aortae of mice fed with high-fat diet for 6 weeks and human hypercholesterolemic sera showed decreased let-7g expression [24] (Table 1). In the same study a negative feedback regulation has been identified between oxidized LDL receptor 1 and let-7g in primary human aortic smooth muscle cells [24] (Table 1).

Certain microRNAs have been implicated in cellular responses to oxidative/nitrative stress in cardiovascular pathologies in preclinical studies [33, 238–241]. Many miRNAs, including miR-21 and miR-199a, have been reported to play a role in cardiomyocyte survival during ischemia [33, 242] (Table 1). Moreover, injection of AAV9 vectors expressing miR-199 and 590 into the peri-infarcted area of the myocardium could reduce infarct size and improve regeneration after myocardial infarction in mice [243]. We and others have shown that several miRNAs including miR-1, miR-21, miR-125b*, miR-139-3p, miR-139-5p, miR-181a, miR-188, miR-192, miR-212, miR-320, miR-487b, and miR-532 play a role in the mechanism of ischemic preconditioning conferring cardioprotection after ischemia/reperfusion injury [32, 34] (Table 2). These miRNAs also drive the synthesis of important cardioprotective proteins including heat shock protein- (HSP-) 70, endothelial and inducible NOS, HSP-20, NAD-dependent deacetylase sirtuin-1 (Sirt1), and hypoxia-inducible factor 1a [34]. miRNAs are also associated with the protective effect of ischemic postconditioning against myocardial ischemia/reperfusion injury. Heart specific miR-1 and miR-133a have been associated with playing a role in the cardioprotection conferred by ischemic postconditioning through the regulation of apoptosis-related genes [35, 37]; however, the regulation of miR-1 by ischemic postconditioning is controversial [32, 35, 37] (Table 2). Another miRNA, miR-21, has been demonstrated to be implicated in ischemic postconditioning, though its role in cardioprotection is controversial [35, 36] (Table 2). In addition, loss of the miR-144/451 cluster function has been shown to limit the cardioprotective effect of ischemic preconditioning by upregulating Rac-1-mediated oxidative stress signaling [38]. Therefore, further preclinical and clinical studies are needed to investigate the role of miRNAs in ischemia/reperfusion injury and myocardial stress adaptation in healthy and diseased conditions, including hypercholesterolemia.

miRNAs exert control over diverse metabolic pathways and are frequently dysregulated in cardiovascular diseases.

Table 2: miRNAs affected by ischemic pre- or postconditioning in the heart.

miRNA	I/R versus control	Ipre versus I/R	Ipost versus I/R
let-7b	down [32]	n.a. [32]	up [32]
miR-21	down [33]	up [34, 35]	n.a. [35], up [36]
miR-125b*	down [32]	up [32]	up [32]
miR-139-3p	down [32]	up [32]	up [32]
miR-181a	down [32]	down [32]	up [32]
miR-199a	down [33]	up [34]	no data
miR-328	down [32]	n.a. [32]	up [32]
miR-335	down [32]	n.a. [32]	up [32]
miR-503	down [32]	n.a. [32]	up [32]
let-7e	n.a. [32]	n.a. [32]	up [32]
let-7i	n.a. [32]	n.a. [32]	up [32]
miR-1	n.a. [32], down [37, 38]	up [34]	up [32, 37], down [35]
miR-139-5p	n.a. [32]	up [32]	n.a. [32]
miR-188	n.a. [32]	up [32]	up [32]
miR-192	n.a. [32]	up [32]	n.a. [32]
miR-212	n.a. [32]	up [32]	n.a. [32]
miR-532	n.a. [32]	up [32]	up [32]
miR-133a	up [34], down [37]	no data	up [37]
miR-208a	up [32, 34]	n.a. [32]	down [32]
miR-320	up [34], down [32]	down [32]	down [32]
miR-487b	up [32]	down [32]	n.a. [32]

I/R: ischemia/reperfusion; Ipre: ischemic preconditioning; Ipost: ischemic postconditioning; n.a.: not affected.

Thus, miRNAs have become a class of promising therapeutic targets. Until miRNA-based therapeutic interventions become a reality in clinical medicine many questions should be answered in preclinical and clinical studies [244]. Better technologies and more applicable *in vitro* and *in vivo* models of human diseases should be developed to identify and validate direct mRNA targets of miRNAs [244]. Furthermore, improved understanding of the mechanism of action in each tissue type is necessary. Moreover, development of organ specific delivery methods for miRNA mimics and anti-miR oligonucleotides are needed [244]. Nevertheless, assessment of the efficacy and safety including the analysis of the off-target effects of miRNA-based therapeutic tools and understanding of the long-term effects of miRNA modulation *in vivo* are of key importance in the future [244].

4.3.2. Peroxynitrite Scavenging. Peroxynitrite is formed by the rapid reaction of superoxide and NO and is responsible for a variety of deleterious effects in cardiovascular pathologies [245]. Therefore, development of peroxynitrite scavengers or compounds catalyzing the decomposition of peroxynitrite to nontoxic products has been an emerging field in the last decade [245]. Formation of peroxynitrite in the heart of hypercholesterolemic animals has been demonstrated in various experimental models [6, 8, 246]. The beneficial effect of decomposition of cardiac peroxynitrite leading to improved cardiac function in experimental hypercholesterolemia was also shown [6, 246]. In isolated working hearts from Wistar rats or apoB100 transgenic mice fed with cholesterol-enriched diet, deterioration of cardiac function characterized by increased left ventricular end-diastolic pressure (LVEDP) or decreased aortic flow was demonstrated, respectively [6, 246]. Pretreatment of the animals with the peroxynitrite decomposition catalyst FeTPPS (5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), chloride) before isolation of the hearts resulted in an improved LVEDP and aortic flow, respectively [6, 246]. Whether application of peroxynitrite scavengers would reverse impaired conditioning in hypercholesterolemic animals remains to be addressed in future studies.

4.3.3. Hydrogen. Recent advances in basic and clinical research have indicated that hydrogen gas is an important physiological regulatory factor with antioxidant, anti-inflammatory, and antiapoptotic protective effects, and thus the application of molecular hydrogen as a therapeutic medical gas in diverse disease conditions has become a feasible therapeutic strategy [247]. Hydrogen is suggested to be an efficient, nontoxic, highly bioavailable, and low-cost antioxidant supplement for patients with pathological conditions involving ROS-induced oxidative stress [248]. Therapeutic hydrogen can be applied by different delivery methods including inhalation of hydrogen gas, drinking hydrogen dissolved in water, and injection with hydrogen-saturated saline [247]. In the heart, hydrogen attenuated doxorubicin-induced heart failure in rats [249], cardiac dysfunction in streptozotocin-induced diabetic mice [250], rat cardiac cold ischemia/reperfusion injury [251], and left ventricular hypertrophy in spontaneous hypertensive rats [252] and exerted cardioprotective effects on isoproterenol-induced myocardial infarction in rats [253]. Moreover, inhalation of hydrogen attenuated increased serum cholesterol, cardiac superoxide production, and left ventricular remodeling induced by intermittent hypoxia in mice [254]. Similarly, consumption of hydrogen-rich water beneficially affected serum cholesterol status and oxidative stress markers in patients with potential metabolic syndrome or isolated hypercholesterolemia [255, 256]. These promising results should be confirmed.

4.3.4. Miscellaneous Examples. Diphenyl diselenide has been recently reported to attenuate hypercholesterolemia-associated cardiac oxidative stress and increase antioxidants without affecting plasma level of cholesterol in LDL receptor knockout mice [257].

Local infiltration of neuropeptide Y to hypercholesterolemic swine heart subjected to ischemia was shown to ameliorate cardiac diastolic dysfunction probably by decreasing oxidative stress and fibrosis and increasing cell survival in the heart [258, 259].

4.4. Physical Activity. A dramatic decrease in an individual's physical activity is the most obvious change accompanied by western-type lifestyle and technical development in the industrial countries. Physical inactivity is a risk factor and promotes development of civilization diseases. It is widely accepted that physical activity positively influences a variety of clinical diseases including obesity, metabolic syndrome, dys- and hyperlipidemias, diabetes, and cardiovascular diseases [260, 261].

Hypercholesterolemia is accepted as one of the most important risk factors in the development of different vascular and heart diseases. Physical activity and change in lifestyle are the first choices in normalization of patient's high blood cholesterol level. The mechanisms by which physical activity prevents development of metabolic diseases are rather diverse. The primary effect of physical activity can be seen in a metabolic level. Due to higher energy demand, physical activity intensively increases weight loss. A weight loss of 10 percent can significantly lower the risk of cardiovascular diseases by reversing hyperlipidemia. Moreover, by increasing the catabolic rate of the body, physical exercise positively influences carbohydrate and lipid metabolism and blood lipid profile. Thus, exercise training is associated with increased reliance on lipids as an energy substrate, has a systemic lipid-lowering effect, and results in remodeling of skeletal muscle lipid metabolism toward increased oxidation and neutral lipid storage and turnover [262]. Physical activity has substantial effects on the liver metabolism as well, modifying lipoprotein levels to a more healthy composition. These effects can be enhanced by using concurrent dietary restrictions; for example, a low-cholesterol diet definitely helps to regulate lipid profile in the body. Regular participation in physical activity as well as a single exercise session can positively alter cholesterol metabolism [263]. Exercise is involved in increasing the production and action of several enzymes that function to enhance the reverse cholesterol transport system [263].

Nevertheless, physical activity has secondary effects on the prevention of development of metabolic diseases by, for example, modifying oxidative stress. Physical activity is believed to be a protective modulator of oxidative stress in hyperlipidemia; however, high intensity physical exercise itself can definitely increase oxidative stress in patients [264–266]. Two key free radicals are the most important during physical activity, that is, superoxide and NO. The exact sources of these radicals are not fully known; however, mitochondria are often cited as the predominant source of ROS in muscle cells. Investigators have often assumed that the increased ROS generation that occurs in muscle fibers during contractile activity is directly related to the elevated oxygen consumption. However, growing evidence argues against mitochondria being the dominant source of ROS production in skeletal muscle during exercise [264]. Another possible source is NADPH oxidase, which is normally quiescent, but when it becomes activated, during muscle contraction or when recruited for antimicrobial and proinflammatory events, it can generate large amounts of superoxide [264, 267, 268]. Since the discovery that contracting skeletal muscles produce ROS, many investigators have assumed that skeletal muscle provides the major source of free radical and ROS generation during exercise. Nonetheless, other tissues such as the heart, lungs, or blood may also contribute to the total body generation of ROS during exercise [264]. Thus the increased catabolic process together with multiplied oxygen consumption in both skeletal and cardiac muscles during exercise exacerbates superoxide production.

The other major free radical which contributes to oxidative/nitrative stress is NO. NO is responsible for the relaxation of vessels [269] and plays an important role in matching tissue perfusion to demand [270]. The release of nitric oxide by the endothelial cell can be upregulated by exercise [261, 271]. However, hypercholesterolemia impairs endothelial function (e.g., the NO-cyclic GMP-phosphodiesterase 5 pathway), limits shear stress-induced vasodilation, and is therefore expected to reduce exercise-induced vasodilation [272].

In addition to the modulation of ROS and RNS production, physical exercise may also affect the antioxidant defense systems. McCommis et al. have demonstrated that familial hypercholesterolemia reduces mitochondrial antioxidants, increases mitochondrial oxidative stress, and enhances the mitochondrial permeability transition response in the porcine myocardium [19]. They also showed that exercise training can reverse these detrimental alterations without altering serum cholesterol level [19].

In spite of the extensive research, the cardioprotective effect of exercise which is mediated by redox changes is still a question of debate. Several studies showed that hyperlipidemia impairs exercise capacity itself [273] or the effect of physical activity [272, 274, 275]. However, a number of exercise programs have effectively reversed hypercholesterolemia-induced changes mainly within the vasculature by improving NO bioavailability in both animal studies and humans [276–281].

The exact explanation why physical activity, which similarly to hyperlipidemia leads to an increased oxidative stress, is able to protect the heart in hypercholesterolemia is rather difficult. One possible answer is that during exercise an intensive but only a temporary increase of oxidative stress occurs resulting in a possibility for cardiac adaptation by an improved enzymatic and nonenzymatic antioxidant capacity, cytoprotection, aerobic capacity, training-induced muscular adaptation, mitochondrial biogenesis, and so forth [268]. In hypercholesterolemia with no exercise, the continuously elevated oxidative/nitrative stress, however, does not allow the completion of cardioprotective mechanisms. End-effectors of cardioprotection involve the activation of ATP-sensitive K^+-channels (KATP). We have recently demonstrated that a cholesterol-enriched diet inhibited cardioprotection induced by KATP activators and that cholesterol diet may impair cardiac KATP channels [282]. It is well accepted that the opening of KATP channels generates ROS; however, an ambient oxidative state also modifies redox-sensitive KATP channels, as superoxide, hydrogen peroxide, and peroxynitrite open KATP channels in the heart. These results show that increased oxidative stress interferes with KATP channel function and therefore might explain why cardioprotection is lost in hyperlipidemia.

Increased physical activity can be also mimicked in experimental animal models by applying ventricular overdrive pacing of the heart, a method that was reported to induce both preconditioning and postconditioning by increasing the oxygen demand (relative hypoxia) instead of limiting oxygen supply (absolute hypoxia) in isolated heart models [283–285]. Peroxynitrite plays an important role in different conditionings induced by either ischemia or ventricular pacing [11, 286]. It is known that experimental hypercholesterolemia blocks the cardioprotective effect of postconditioning at least in part via deterioration of postconditioning-induced early increase in peroxynitrite formation during reperfusion [11]. Thus one can speculate that regular physical exercise is able to restore the protective effects of pre- or postconditioning in hypercholesterolemia via modifying cardiac oxidative stress and by beneficially altering lipid profile in the blood.

In addition to emphasizing the importance of regular exercise, novel future directions have been implicated to take advantage of exercise-induced benefits. Thus development of new "exercise mimetics" has a promising role in the future for treatment of patients [287].

5. Conclusions

Oxidative and nitrative stress has been implicated as a pathophysiological mechanism of cardiovascular diseases; however, there is still no breakthrough regarding the use of general antioxidant therapies in clinical practice. The possible reasons for these disappointing results and some promising aspects of potential antioxidant therapy have been discussed in detail recently [288]. Although hypercholesterolemia occurs frequently in the adult population, the number of publications investigating myocardial oxidative/nitrative stress and its cardiac consequences is relatively limited especially in humans. This is unfortunate, as patients with hypercholesterolemia are at an increased risk for severe pathological conditions such as myocardial infarction and heart failure and hypercholesterolemia has been shown

to interfere with endogenous cardioprotective mechanisms. Therefore, finding proper approaches to beneficially affect hypercholesterolemia and its myocardial consequences is crucial. These reasons warrant further preclinical and clinical studies to better understand the pathological events in the heart relating to hypercholesterolemic conditions and to find the best approaches to interact. Moreover, many of the potential modulators of oxidative/nitrative stress require further development to optimize their effects for application in hypercholesterolemia.

Conflict of Interests

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

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Research Article

Apocynin and Diphenyleneiodonium Induce Oxidative Stress and Modulate PI3K/Akt and MAPK/Erk Activity in Mouse Embryonic Stem Cells

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Reactive oxygen species (ROS) are important regulators of cellular functions. In embryonic stem cells, ROS are suggested to influence differentiation status. Regulated ROS formation is catalyzed primarily by NADPH-dependent oxidases (NOXs). Apocynin and diphenyleneiodonium are frequently used inhibitors of NOXs; however, both exhibit uncharacterized effects not related to NOXs inhibition. Interestingly, in our model of mouse embryonic stem cells we demonstrate low expression of NOXs. Therefore we aimed to clarify potential side effects of these drugs. Both apocynin and diphenyleneiodonium impaired proliferation of cells. Surprisingly, we observed prooxidant activity of these drugs determined by hydroethidine. Further, we revealed that apocynin inhibits PI3K/Akt pathway with its downstream transcriptional factor Nanog. Opposite to this, apocynin augmented activity of canonical Wnt signaling. On the contrary, diphenyleneiodonium activated both PI3K/Akt and Erk signaling pathways without affecting Wnt. Our data indicates limits and possible unexpected interactions of NOXs inhibitors with intracellular signaling pathways.

1. Introduction

Reactive oxygen species (ROS) play multiple roles in the biology of the cell [1]. NADPH oxidase (NOX) and oxidative reactions on the mitochondrial membrane are the main sources of ROS, although it can also be produced by other enzymatic and nonenzymatic sources [2, 3]. NOX is a membrane-bound protein complex generating superoxide anion ($O_2^{\bullet -}$) from molecular oxygen which initiates the cascade of free radical reactions in response to various stimuli. Production of ROS by NOX family has long been considered a unique property of phagocytic cells, which utilize this enzyme as a part of host defense immune system. Currently, the regulated ROS production in nonphagocytic cells by NOX was linked to regulation of different processes including proliferation, migration, differentiation, immunomodulation, and oxygen sensing, and therefore, its expression and activity are tissue specific and are tightly controlled [4, 5]. On the basis of the homology to the catalytic subunit of the original phagocytic NOX (gp91phox or preferably NOX2), other NOX isoforms—NOX1, NOX3, NOX4 and NOX5—have been identified in nonphagocytic cells. In parallel, two other members of the NOX family were discovered, namely, dual oxidases 1 and 2 (DUOX 1, 2), initially also referred to as thyroid oxidases [6].

Despite studies suggesting the importance of NOXs in general, the involvement of individual NOX family members in specific function is still not completely understood. One of the reasons is a lack of highly specific inhibitors that would

reliably block particular NOX. The most frequently used inhibitors employed in experiments are vanillin derivative 4-hydroxy-3-methoxyacetophenone (trivial names: apocynin or acetovanillone, APO) and diphenyleneiodonium chloride (DPI). Both of these drugs were applied in numerous *in vitro* and *in vivo* studies and although their effect was attributed primarily to NOX inhibition, specificity of APO and DPI remains questioned [7].

The proposed molecular mechanism of APO-mediated NOX inhibition is not fully understood but it involves impairment of NOX complex assembly and activation [8]. APO was also shown to act directly as ROS scavenger [9]. Contrarily to this finding, other studies suggest that APO is rather a prooxidant stimulating ROS production [10–12]. Further, APO was also shown to modulate the generation of arachidonic acid-derived inflammatory mediators [13].

DPI was reported not only to affect NOX, but also to interfere with other flavoenzymes, including nitric oxide synthase and xanthine oxidase [14, 15]. Inhibitory effects of DPI on mitochondrial ROS production were also shown [16]. On the other hand, DPI induces $O_2^{\bullet-}$ mediated apoptosis [17], inhibits cell redox metabolism, and promotes general oxidative stress [18]. DPI is also suggested as a nonselective blocker of ionic channels [19]. The above mentioned nonspecific effects are thought to be responsible for contradictory results obtained using these inhibitors. Further studies are needed to better understand the actions of APO and DPI not directly related to NOX inhibition.

Intracellular formation of ROS leading to overall redox status modulation is important for regulation of pluripotent cells differentiation [20]. In mouse embryonic stem cells (mES), pluripotent cells derived from inner cell mass of blastocyst, redox alterations are thought to play a role in the balance between self-renewal and differentiation. Undifferentiated mES have several times lower ROS level in comparison with differentiating mES [21]. It was shown that short term increase of the ROS favors differentiation into cardiomyocytes [22, 23] and into endoderm and mesoderm lineage [24].

Several signaling pathways are crucial for the regulation of self-renewal and differentiation of mES. Primarily, the mES pluripotency is controlled by Stat3 together with PI3K/Akt signaling pathways [25–27]. Importance of PI3K signaling was demonstrated in many studies where its inhibition negatively regulates the self-renewal in mES [28, 29]. Further, MAPK/Erk signaling pathway is rather important for mES differentiation as its inhibition improves maintenance of the pluripotent stem cell phenotype [30]. A growing body of evidence indicates that Wnt/β-catenin signaling pathway plays a vital role in the regulation of mES fate [31]. Signaling pathways including Stat3, PI3K/Akt, Erk, and Wnt are modified by ROS production [5, 32, 33] and therefore might be affected by NOX or other ROS modulating agents, although the importance of this phenomenon in mES remains elusive.

Our study demonstrates unexpected prooxidant activity of APO and DPI in undifferentiated mES together with impairment of cell proliferation. Further, we show that APO inhibits PI3K signaling accompanied by decrease in protein level of critical ES pluripotency regulator Nanog, whereas DPI enhances both PI3K and Erk activity. Interestingly, APO strengthens Wnt activity, pointing out another unknown mechanism of APO-mediated changes in signaling cascades regulating mES. In contrast to PI3K and Erk, we did not observe any effect of APO or DPI on Stat3 phosphorylation, which is considered to play the major role in mES maintenance.

2. Material and Methods

2.1. Cell Culture and Treatment. A feeder-free adapted mES line R1 was propagated as described previously [29] in an undifferentiated state by cell culturing on tissue culture plastic coated by gelatin (0.1% porcine gelatin solution in water) in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1x nonessential amino acid (all from Gibco-Invitrogen, UK), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, USA), supplemented with 5 ng/mL of leukemia inhibitory factor (LIF, Chemicon, USA) referred to here as the complete medium.

APO, DPI, hydrogen peroxide (H_2O_2), N-acetylcysteine (NAC), and LY294002 (LY) were provided from Sigma-Aldrich. Stock solutions of APO (0.4 M), DPI (10 mM), and LY (10 mM) were prepared by dissolving the compounds in dimethyl sulfoxide. Aliquots were stored at −20°C. NAC was prepared as a 0.5 M stock solution in serum-free DMEM medium, pH was adjusted to 7.4, and filter-sterilized aliquots were stored at −20°C. Drugs were added directly to the incubation medium or freshly prediluted in sterile phosphate-buffered saline (PBS) to desired concentration.

2.2. Cell Proliferation. The cell proliferation was determined by estimation of overall cellular protein mass in whole cell lysates that reflects the cell number as demonstrated previously [34]. ES cells were seeded to 24-well plate in complete media at density 5 000 cells per cm^2. Next day, the cells were treated with drugs for further 48 hours. Finally, the cells were washed twice with PBS and lysed in SDS buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 1% SDS, 1 mM EDTA). Protein concentration was determined using DC protein assay (Bio-Rad, USA) kit according to manufacturer's instructions.

2.3. Determination of NOX Expression. Total RNA was extracted using the UltraClean Tissue & Cells RNA Isolation Kit (MO BIO Laboratories, USA) for ESC and RNAzol RT (Molecular research center Inc., USA) for mouse tissues according to manufacturer's instructions. 1 μg of total RNA was used for cDNA synthesis with DyNAmo cDNA Synthesis Kit (Finnzymes, Finland) according to the manufacturer's instructions. qPCR was performed in LightCycler 480 instrument using LightCycler Probes Master with Universal Probe library probes (all from Roche, Germany) according to manufacturer's instructions. Ribosomal protein L13A (RPL13A) was used as reference gene; data are presented as

$2^{-(\mathrm{Cq(target)}-\mathrm{Cq(reference)})}$. The primers and probes used were as follows:

NOX1: 5′tggattttctaaactaccgtctcttc, 5′caaagtttaatgctgcatgacc3′, #20; NOX2: 5′tgccaacttcctcagctaca3′, 5′gtgcacagcaaagtgattgg3′, #20; NOX3: 5′tgaacaagggaaggctcatt3′, 5′catcccagtgtaaagctatgtga3′, #20; NOX4: 5′catttgaggagtcactgaactatga3′, 5′tgtatggtttccagtcatccag3′, #5; DUOX1: 5′acagatggggcagcaaag3′, 5′gctggtagacaccatctgcat3′, #20; DUOX2: 5′cagacagctttttgctcaggt3′, 5′cacttgctgggatgagtcc3′, #64; RPL13A: 5′catgaggtcgggtggaagta3′, 5′gcctgtttccgtaacctcaa3′, #25.

2.4. Analysis of ROS Production in Live Cells by Automated Time-Lapse Image (Live Imaging Fluorescent Microscopy). The cells were seeded to 96-well plate designed for live imaging fluorescence microscopy (Greiner Bio-one, Germany). After 24 hours, cells were pretreated with tested drugs for 15 minutes and loaded with hydroethidine (HE, 5 μM, Sigma-Aldrich). Live imaging of prepared samples was performed at 37°C and 5% CO_2 atmosphere using the high-content screening microscope ImageXpressMicroXL (Molecular Devices, USA). Seven images per well were acquired during 120 minutes with scanning interval of 10 minutes. By Gaussian thresholding of the HE fluorescence images, mask area of the viewfield covered with metabolizing cells was detected using the Otsu method of fitting individual objects. The 10640 images for every measured plate were analyzed with CellProfiler [35], running on processor cluster provided by MetaCentrum (The National Grid Infrastructure). Intensity of fitted regions was lowered by minimal intensity of an appropriate image to subtract the background and the mean intensity per individual well was then calculated and visualized with Knime [36], using HCS tools and R Statistics Integration extensions [37].

2.5. High-Performance Liquid Chromatography (HPLC) Analysis of ROS Production. The HPLC detection of $O_2^{\bullet-}$ was based on the detection of a specific product 2-hydroxyethidium 2-OH-E(+) which is formed in the reaction of $O_2^{\bullet-}$ with HE [38, 39]. Besides specific 2-OH-E(+), also a nonspecific product of hydride acceptors with HE – ethidium (E+) was detected. The cells were seeded to 6-well plate and before the treatment the medium was changed for DMEM (without phenol red and sodium pyruvate) with 1% FBS. The cells were treated with APO and DPI for 120 minutes in total; 30 minutes before the end of the experiment HE in final concentration 10 μM was added. The medium after centrifugation was stored for optional HPLC analysis. To extract the HE products, ice cold methanol was added to the cells [40] for 15 minutes at 4°C in dark, shaking. The supernatant was transferred to an Eppendorf tube and centrifuged. A 75 μL sample was injected into the HPLC system (Agilent series 1100) equipped with fluorescence and UV detectors (Agilent series 1260) to separate the 2-OH-E(+) product. Fluorescence was detected at 510 nm (excitation) and 595 nm (emission). The mobile phase consisted of H_2O/CH_3CN. Kromasil C18 (4.6 mm × 250 mm) column was used as the stationary phase. Elution conditions for the analysis of HE and its products were used from Nature Protocols [38].

2.6. Western Blot Analysis. Western blot analysis, cell sample harvesting, and preparation were performed by a standard procedure as presented previously [29]. We used the following primary antibodies against Nanog, β-actin (Abcam, USA), p-Akt (S473), Akt, p-Stat3 (Y705), Stat3, Erk1/2, p-Erk1/2 (T202/Y204), and p-GSK3β (S9) (all from Cell Signaling Technology, USA). Following immunodetection, each membrane was stained by amido black to confirm the transfer of the protein samples. The total level of β-actin was detected as loading control.

2.7. Cell Transfection and TOPflash Luciferase Reporter Assay. Cells were transiently transfected using polyethyleneimine in a stoichiometry of 4 μL per 1 μg of DNA. Super8X TOPflash construct, *Renilla* luciferase construct, and expression vector for mutant nondegradable β-catenin, and S33-β-catenin (codon 33 substitution of Y for S, generously provided by Professor Korswagen) were used in concentration of 0.5 μg per well in a 24-well plate 24 hours after seeding. 6 hours after transfection medium was changed and cells were treated with NOX inhibitors or LY for 24 hours. For cell stimulation Wnt3a or control conditioned medium [41] was added 8 hours before harvest. Dual-Luciferase assay kit (Promega, USA) was used according to the manufacturer's instructions for the evaluation of luciferase activity. Relative luciferase units were measured on a plate luminometer Chameleon V (Hidex, Finland) and normalized to the *Renilla* luciferase expression.

2.8. Statistical Analysis. Data are expressed as mean + standard error in the mean (SEM). Statistical analysis was assessed by t-test or by one-way analysis of variance ANOVA and Bonferroni's Multiple Comparison posttest. The values of $P < 0.05$ were considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

3. Results

3.1. Expression of NOXs in mES Is Relatively Low. To confirm the assumption of the negligible presence of NOX and DUOX homologues in the undifferentiated mES, the gene expression was compared to the selected mouse tissues. Particular organs were chosen based on the described presence of NOX homologues by various authors [4, 6]. In agreement with the literature, all determined NOX homologues NOX1, NOX2, NOX3, NOX4, DUOX1, and DUOX2 showed two to three orders lower expression in undifferentiated mES compared to selected tissues (Figures 1(a)–1(f)). The comparison of the NOXs relative expression in mES revealed the NOX4 expression to be the highest, approximately 10 times compared to other NOXs (Figure 1(g)), altogether, it can be concluded that the expression of all NOXs except NOX4 is very low.

3.2. APO and DPI Affect mES Proliferation. Both APO and DPI affected growth of mES in dose-dependent manner in the concentration range 0.1, 0.25, 0.5, 1.0, and 2.0 mM for APO (Figure 2(a)) and 10, 20, 40, 80, 160, and 320 nM for DPI (Figure 2(b)). Data showed decrease in cell proliferation

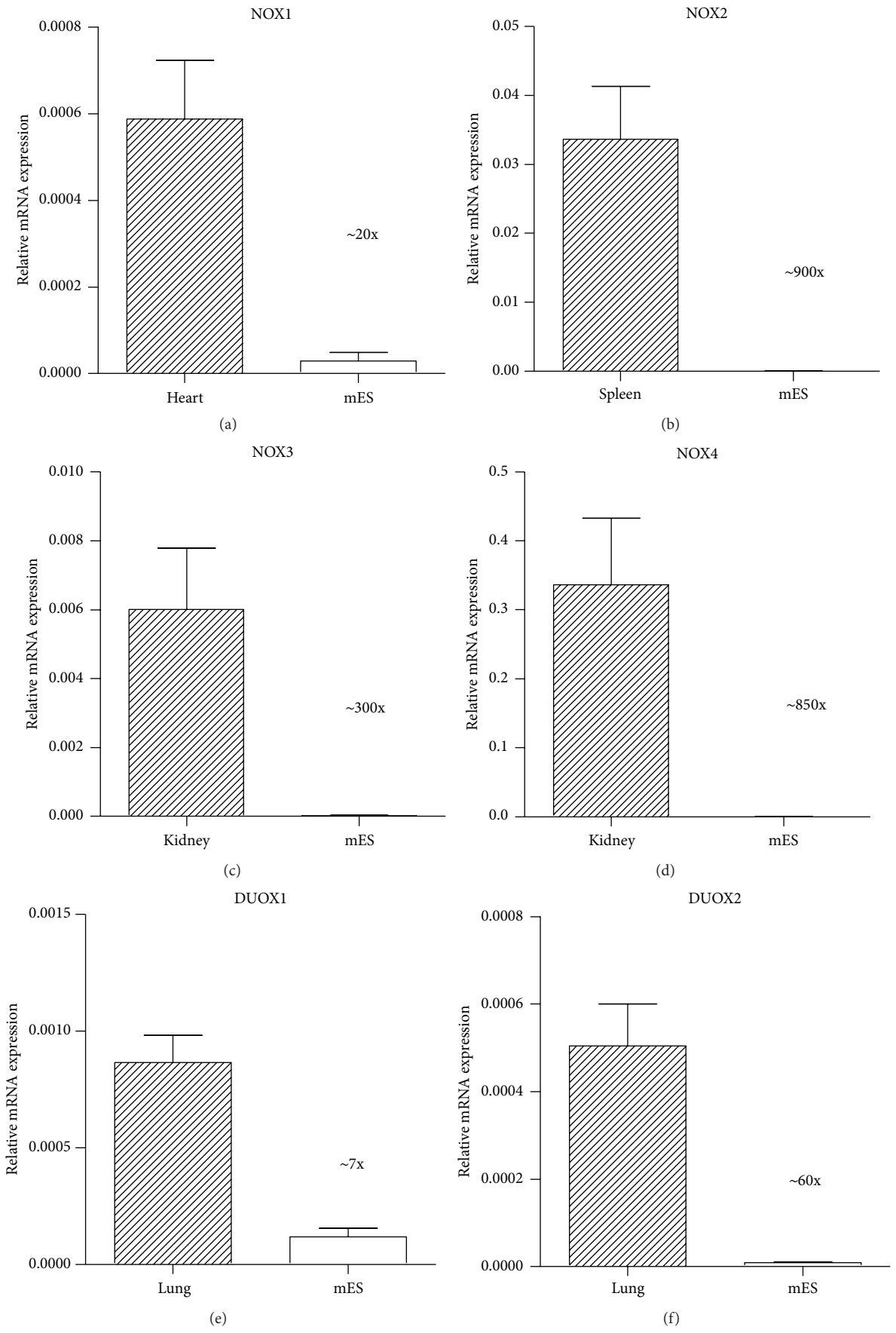

Figure 1: Continued.

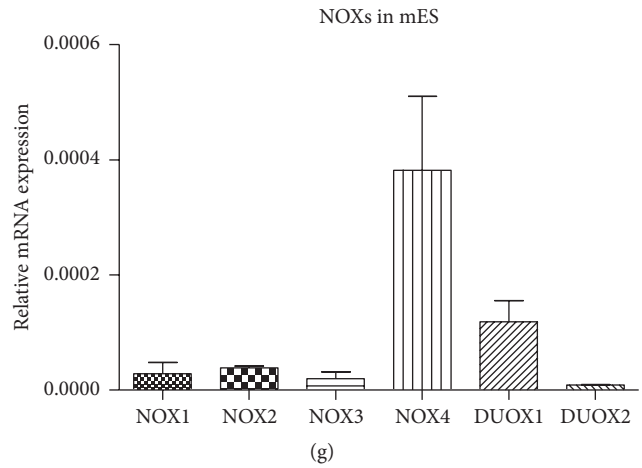

Figure 1: Relative expression of NOX1 (a), NOX2 (b), NOX3 (c), NOX4 (d), DUOX1 (e), and DUOX2 (f) mRNA in mES and selected tissues. Comparison of relative expression of individual NOXs and DUOXs mRNA within mES cells is also shown (g). Data are presented as mean + SEM from at least two independent experiments.

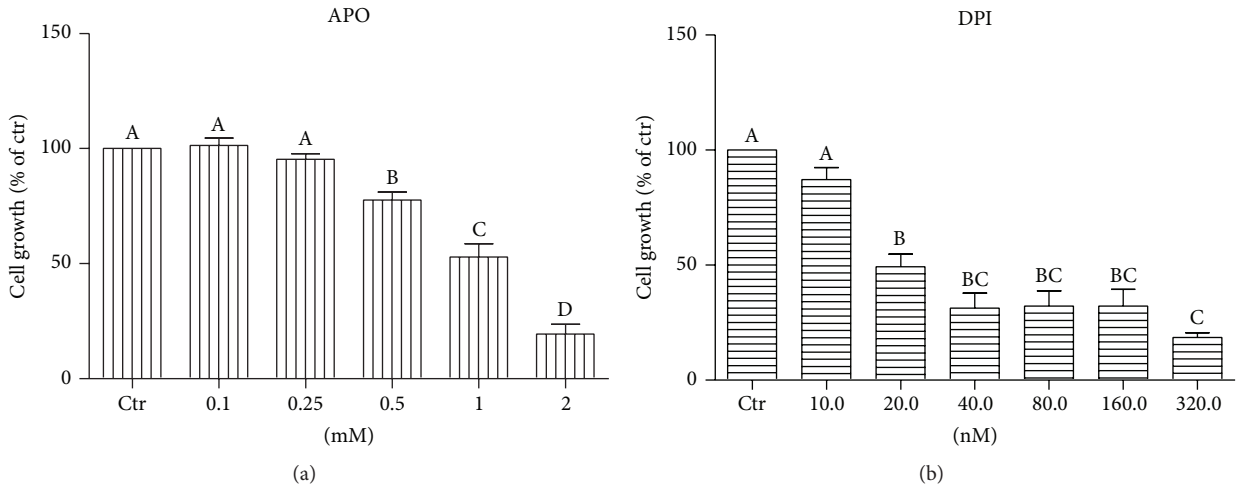

Figure 2: Effect of APO (a) and DPI (b) on mES proliferation after 48 h treatment based on total cellular protein mass. Data represent mean + SEM from four independent experiments. Statistical significance was determined by ANOVA post hoc Bonferroni's Multiple Comparison test, $P < 0.05$. The groups marked differently by symbol letters are statistically significantly different from each other.

using concentration from 0.5 mM (APO) and from 20 nM (DPI) after 48-hour treatment. IC50 of APO and DPI for mES determined from these data were 1.1 ± 0.2 mM and 18.5 ± 3.2 nM, respectively.

3.3. APO and DPI Do Not Inhibit but Potentiate Formation of ROS in mES. Despite the very low expression of NOXs enzymes in undifferentiated mES, the ROS production was detectable in our mES lines. Contrary to the expectations, treatment by APO or DPI significantly induced generation of ROS in mES cells detected by live imaging analysis of HE fluorescence, continuously for up to 120 minutes (Figures 3(a), 3(b), and 3(c)). To confirm the specificity of this determination, the formation of specific product of HE reaction with $O_2^{\bullet-}$, the 2-OH-E(+), and also nonspecific product, ethidium (E+), were determined by HPLC (Figures 3(d) and 3(e)). This analysis shows potentiation of nonspecific HE oxidation rather than $O_2^{\bullet-}$ formation in DPI treated cells, but not in APO treated cells.

3.4. Effect of APO and DPI on Stat3, Akt, and Erk Phosphorylation in mES. To investigate the effect of short term treatment, mES were serum and LIF starved for 12 hours and treated by APO and DPI for 20 minutes followed by 20-minute stimulation with FBS or LIF. APO treatment resulted in decrease of Akt phosphorylation in every condition without effect on Erk. DPI had no significant effect on Akt and Erk kinases signaling. Level of p-Stat3 remained unchanged by NOX inhibitors (Figure 4).

To further clarify the dose-dependent effect of tested drugs, we employed the same experimental design with serum starvation and FBS activation. Moreover, glutathione

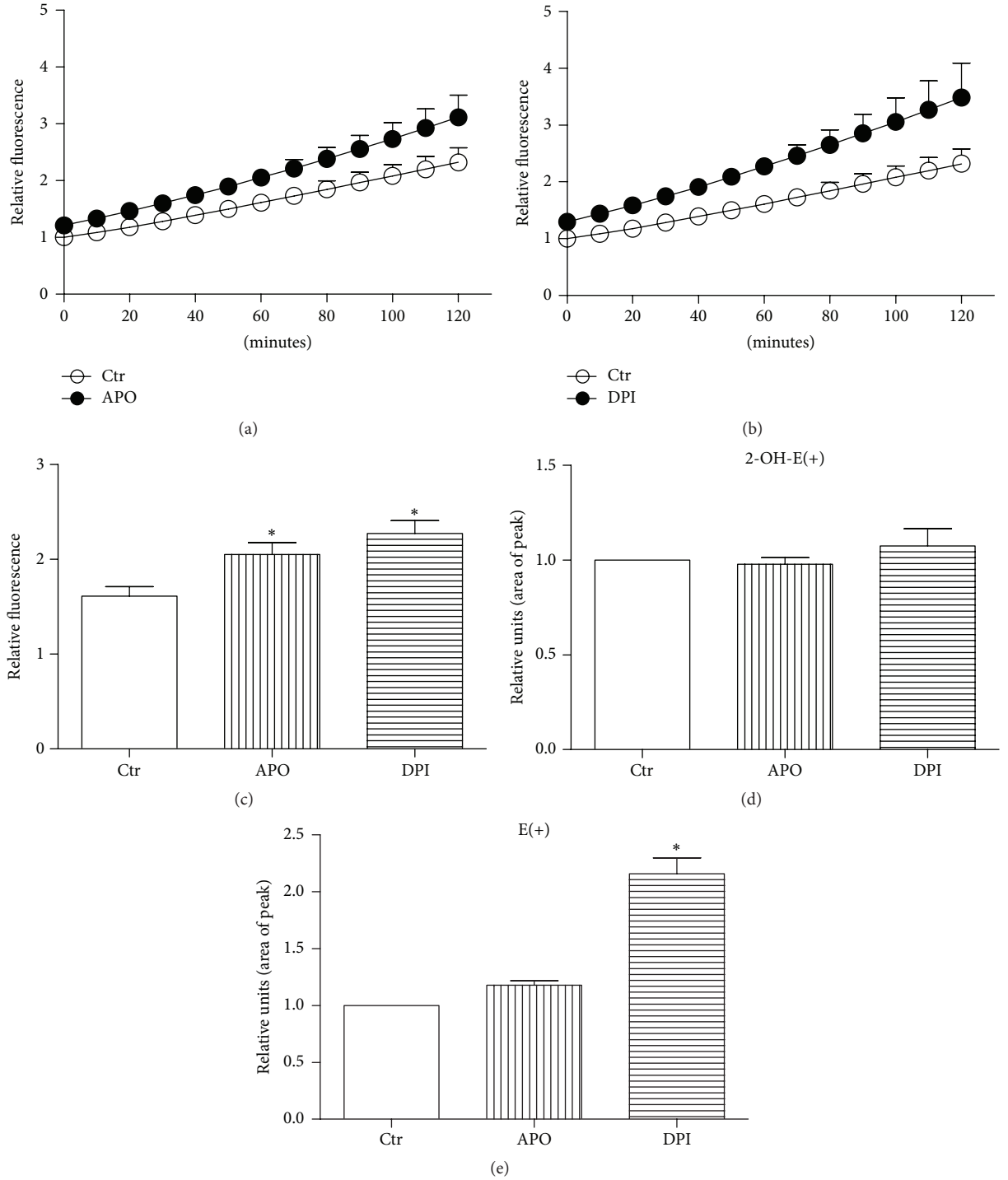

Figure 3: ROS production in mES cells treated by 1 mM APO and 100 nM DPI computed from the automated time-lapse image acquisition of HE fluorescence for 120 minutes (a, b); selected single time point 60 minutes (c). HPLC determination of specific 2-OH-E(+) and nonspecific product E(+) of HE oxidation in the presence of 1 mM APO and 100 nM DPI (d, e). Data are presented as mean + SEM from four independent experiments. Statistical significance was determined by ANOVA post hoc Bonferroni's Multiple Comparison test, $P < 0.05$. The groups marked by an asterisk are statistically significantly different from control.

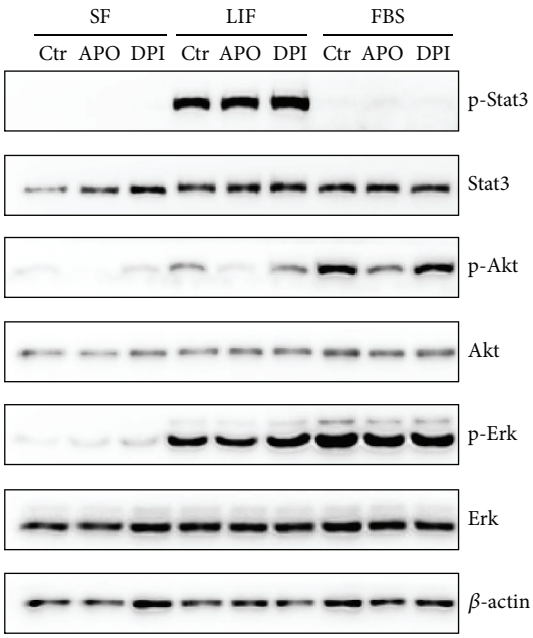

Figure 4: Effect of APO (2 mM) and DPI (100 nM) on the Stat3, Akt, and Erk phosphorylation in serum starved mES cells (SF) treated by drugs for 20 minutes followed by LIF (5 ng/mL) or FBS (15%) stimulation for 20 minutes. Total level of β-actin was used as a loading control. A typical representative western blot is shown.

precursor NAC and H_2O_2 were used as a *bona fide* antioxidant and a prooxidant, respectively, to distinguish between effects mediated via redox changes in cultivated mES and ROS independent actions of APO and DPI.

APO abolished phosphorylation of Akt in a dose-dependent manner with supportive effect on phosphorylation of Erk in higher concentrations. In contrast, DPI slightly increased phosphorylation of Akt and also upregulated phosphorylation of Erk in the highest concentration. Consistent with expectations, H_2O_2 increased both Akt and Erk phosphorylation. NAC had no effect on signaling in this setup (Figure 5(a)). To test whether the effect on signaling was also preserved during cultivation in complete medium, mES were treated by the same concentration of drugs for 1 hour. In this case, similarly to previous treatment, APO inhibited Akt phosphorylation in a dose-dependent manner. Erk phosphorylation was impaired in the presence of the highest concentration of APO. Notably, in this setup 100 μM concentration of DPI strongly induced both Akt and Erk phosphorylation. Contrary to the previous setup, NAC decreased phosphorylation of both pathways (Figure 5(b)).

Finally, we examined the impact of APO and DPI on Akt and Erk activity after 24 hours in absence or presence of NAC (Figure 6). Contrary to the effect observed after a short term treatment, Akt phosphorylation was upregulated when APO was employed. Phosphorylated form of Akt was also increased following the DPI treatment. This activation could be prevented by addition of NAC. Erk phosphorylation was slightly decreased by APO treatment and augmented by DPI even in the presence of NAC. APO strongly downregulated Nanog protein level in mES, independently of NAC supplementation (Figure 6). Level of Stat3 phosphorylation was modulated in the above mentioned experimental condition by neither APO nor DPI treatment (data not shown). We did not observe any effect of H_2O_2 on the evaluated signaling pathways after 24 hours (data not shown).

3.5. APO Augments Canonical Wnt Activity in mES. To assess the effects of APO and DPI supplementation on activity of Wnt pathway, we used TCF/LEF reporter gene assay (TOPflash) to determine the level of canonical Wnt activation mediated by β-catenin which specifically induces the transcriptional activity of TCF/LEF [42]. Firstly, we examined our system with addition of Wnt3a conditioned media or exogenous nondegradable β-catenin, both known agonists, to promote its activation. These interventions induced transcription activity of reporter gene 45 and 25 times, respectively (Figure 7(a)).

PI3K inhibitor LY294002 (LY) and both NOXs inhibitors APO and DPI did not change the spontaneous β-catenin mediated transcription activity of TCF/LEF (Figure 7(b)). In contrast, when the cells were treated by Wnt3a conditioned media, presence of LY and APO significantly augmented the TCF/LEF transcription activity (Figure 7(c)). On the other hand, DPI had no effect (Figure 7(c)). However, all tested drugs did not have any effect in the presence of exogenous nondegradable β-catenin (Figure 7(d)).

To further clarify the effects of LY, APO, and DPI on Wnt/β-catenin signaling, the GSK3β S9 phosphorylation, allowing accumulation of β-catenin, was evaluated [43]. Treatment with both APO and LY but not DPI decreased GSK3β S9 phosphorylation in this setup as shown by the western blot analysis (Figure 7(e)).

4. Discussion

APO and DPI are the most commonly used inhibitors of NOX, involved in numerous studies despite the increasing evidence questioning their specificity. We employed mES as a model to analyze effects of these compounds that might not be directly mediated through impairment of NOXs, because of their generally negligible expression in undifferentiated mES. We aimed to investigate modulation of ROS production by APO and DPI in mES as well as interactions with signaling pathways important for stem cell regulation.

Although NOXs were attracting attention as an important source of intracellular ROS production for a long time, their role in stem biology remains poorly understood. Previously, it was demonstrated that NOXs expression is precisely regulated during embryonic stem cell differentiation into cardiomyocytes [44, 45] and vascular smooth muscle lineage [46].

In our experiments, we observed generally low level of NOXs/DUOXs expression close to the limit of detection, which we concluded both from real Cq values of PCR amplification and from comparison of NOXs/DUOXs expression in several employed tissues. As such control sample, tissues with well described NOXs/DUOXs expression and activity were

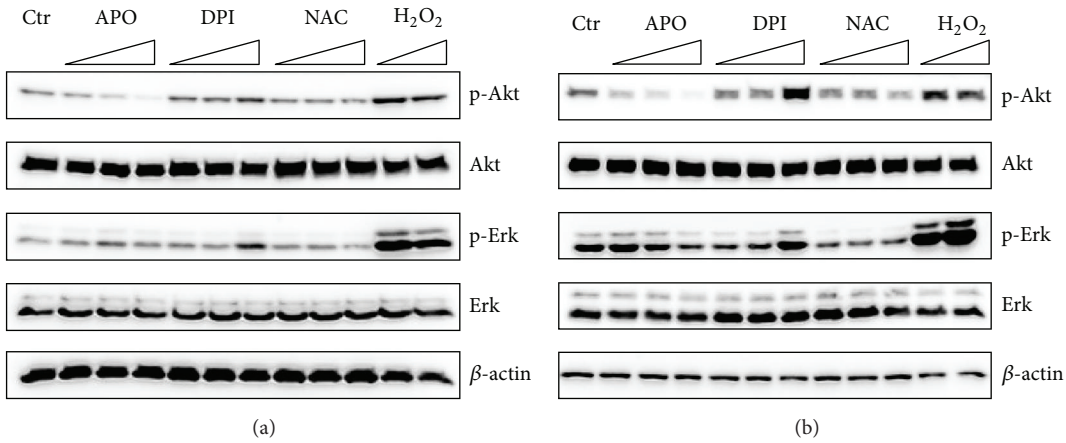

Figure 5: Effect of APO (1, 2, 4 mM), DPI (0.1, 1, 100 μM), NAC (5, 10, 20 mM), and H_2O_2 (0.5, 1 mM) on the Akt and Erk phosphorylation in serum starved mES cells treated by drugs for 20 minutes followed by 15% FBS stimulation for 20 minutes (a) and cells treated for 1 hour in complete medium (b). Total level of β-actin was used as a loading control. A typical representative western blot is shown.

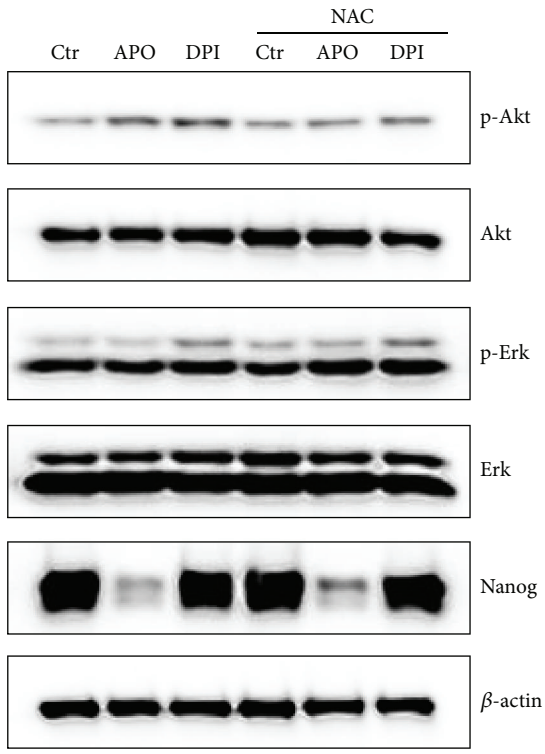

Figure 6: Effect of APO (1 mM) and DPI (10 nM) in absence and presence of antioxidant NAC (10 mM) on the Akt and Erk, phosphorylation and Nanog protein level in mES cells after 24 hours in complete medium. Total level of β-actin was used as a loading control. A typical representative western blot is shown.

used [4, 6]. Among NOXs and DUOXs, NOX4 expression was the highest in mES, which is in agreement with other authors [46, 47]. It may also correspond to relative abundant expression of this enzyme across other tissues [48]. However, it should be emphasized that level of NOX4 transcript in mES was still nearly three orders below expression in selected control tissue (kidney). NOX4 was shown to be constitutively active, and hence its activity is mostly regulated by the level of its expression [49]. Concerning other potential sources of intracellular ROS, it is also noteworthy that favored reliance on anaerobic glycolysis in undifferentiated mES leads to reduced mitochondrial biogenesis and activity, manifested by declined ROS production [50, 51]. Thus mES can be considered not only NOX-low, but also overall ROS-low model.

Next, we aimed to assess effect of APO and DPI on proliferation of undifferentiated mES. Selection of used concentrations was based on comprehensive literature search. APO as NOX inhibitor was used in range 30–1200 μM [9, 52] with the upper range of these concentrations corresponding to APO supplementation preferably employed in our experiments. Regarding DPI, many authors used concentrations ranging 1–100 μM {summarized in [53]} which is approximately one order of magnitude higher than doses used in our experiments, as we observed significant growth impairment even when concentrations as low as 20 nM were applied. Impact on cell proliferation after NOX inhibitors treatment was described earlier, for both normal and transformed cell lines. The observed effects of DPI and APO were suggested to be attributed to the various mechanisms including changes in NOX mediated ROS production, downregulation of integrin expression, cell cycle arrest, or modulation of mitogenic-signaling pathways [54–57]. Therefore, we can assume that direct modulation of ROS production could contribute to the observed decrease of mES proliferation. At the same time, the effects of inhibitors employed in this study can also be related to their direct effects on the other promitogen cell signaling pathways as discussed later. The potential of APO and DPI to inhibit cell proliferation could also be beneficial in the context of anticancer agents. A recent publication showed that APO suppressed prostate cancer and that the reduction of Rac1 and NFκB phosphorylation was involved [58].

Further, we assessed how NOX inhibitors affected ROS production in our model. Due to their natural short half-life and high reactivity, precise detection of ROS represents

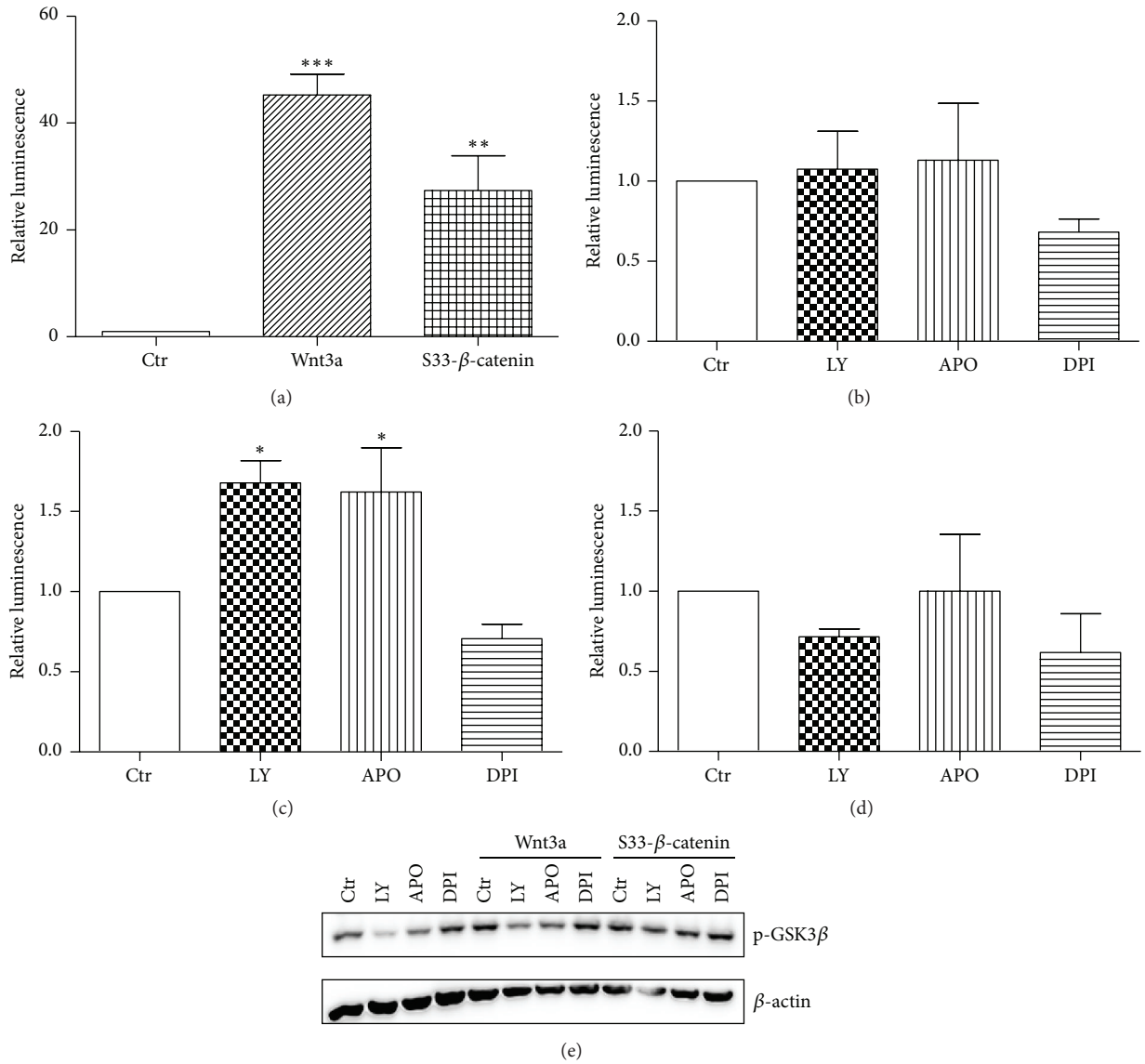

Figure 7: Effect of APO and DPI on Wnt pathway activity determined by TOPflash assay and GSK3 phosphorylation on S9. Effect of the Wnt3a conditioned media or exogenous nondegradable β-catenin on the transcriptional activation of a reporter gene (a). Effect of LY (10 μM), APO (1 mM), and DPI (10 nM) on the spontaneous (b), Wnt3a conditioned media induced (c), and exogenous nondegradable β-catenin-induced transcriptional activation (d). Data represent mean + SEM, from at least four independent experiments. Statistical significance was determined by ANOVA post hoc Bonferroni's Multiple Comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). Effect of LY, APO, and DPI on GSK3β (S9) phosphorylation (e). Total level of β-actin was used as a loading control. A typical representative western blot is shown.

a tremendous challenge, especially in nonphagocytic cells. We were aware of limits in ROS measurement with respect to specificity and generation of possible artefacts; therefore, we used two different assays, live imaging fluorescent microscopy and HPLC, both utilizing ROS-sensitive probe HE. The reaction between $O_2^{\bullet-}$ and HE generates a highly specific red fluorescent product 2-hydroxyethidium 2-OH-E(+). However, in the intracellular milieu, the presence of redox metal ions or hemeproteins with peroxidase activity or other one-electron oxidants can oxidize HE to several nonspecific products, including the ethidium E(+) and dimeric products [38, 59, 60]. Despite the expectations, the live imaging assay showed significant prooxidant activity of APO and DPI when continuous nonoscillating probe oxidation was determined by live imaging. On the other hand, HPLC analysis used for determination of specific HE derivatives did not confirm $O_2^{\bullet-}$ production after APO and DPI treatment. The only observed effect was significant DPI-mediated elevation of E(+). APO did not induce generation of HE oxidation products {both 2-OH-E(+) and E(+)}, which is partially in contrast with the results from live imaging fluorescent microscopy where all nonspecific HE oxidation

fluorescent products are summarized [60]. This suggests that other oxidants, rather than $O_2^{\bullet-}$, are responsible for increase in HE fluorescence.

Although NOX inhibitors should generally relieve oxidative stress, several lines of evidence for APO and DPI exist demonstrating the opposite. It was reported that DPI inhibits pentose phosphate pathway (PPP) responsible for the synthesis of NADPH, a redox cofactor important for many antioxidant enzymes, thus making the cells more prone to oxidative stress [18]. Suggested mechanism included direct inhibition of NADP-dependent enzymes such as glucose 6-phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, and lactate dehydrogenase. In agreement with our data, evidence from different group exists, showing that DPI is exerting prooxidative effects in given cell types and conditions [17]. Further, it was reported that APO, contrary to DPI, stimulates PPP which is known to be a subsequent step following oxidative stress exposure. This was prevented by addition of GSH into medium, further suggesting that APO-induced GSH oxidation might be involved in observed PPP activation [11]. However, in a different study APO opposingly increased the synthesis of the GSH through activation of transcription factor AP-1. Notably, levels of GSSG were not altered, implying that APO itself does not cause oxidative stress [61]. Many studies are highlighting that APO is a prodrug that must be first metabolized through oxidation to its oligomeric form; thus certain redox environment must be present in order to achieve full APO activation [62] narrowing its function preferably to cells with strong ROS production like stimulated endothelial cells [63] or professional phagocytes [64]. In compliance with these findings, it was demonstrated that APO can act both as an antioxidant and as a prooxidant, depending on the cell type and its oxidizing potential [9, 10]. This is in agreement with our experiments performed on nonphagocytic low-level ROS cells where APO might rather contribute to oxidative stress as demonstrated by live imaging. Interestingly, different modifications of APO are suggested for *in vivo* experiments [65], further revealing the complexity of this issue.

Critical features of mES, pluripotency, self-renewal, and unlimited proliferation, are predominantly exerted through actions of cytokine LIF, which is routinely added to the culture medium. Binding of LIF to its receptor triggers the activity of three major intracellular signaling cascades: JAK/Stat3, PI3K/Akt, and MAPK/Erk. These pathways converge to regulate the gene expression pattern typical for mES [27]. To clarify possible effects of APO and DPI on selected signaling pathways, the modulation of Stat3, Akt, and Erk activation status was analyzed in mES cells. Firstly, mES were serum and LIF depleted in order to increase cellular response, as cultivation in complete medium leads to a constant activation of those pathways. Stimulation by LIF and FBS was employed to reactivate signaling [25, 66]. As expected, LIF addition induced preferably Stat3 response while FBS, containing growth factors and cytokines, augmented Akt and Erk signaling. To further analyze effect of drugs on selected kinases, we examined their phosphorylation status also in complete medium after 1-hour and 24-hour treatment, to elucidate early changes and later cell response in signaling pathways. In agreement with other studies, both Akt and Erk kinases responded in ROS-sensitive manner [67, 68], which we demonstrated by H_2O_2-induced phosphorylation. Similarly, we can assume that DPI-mediated ROS production is responsible for the observed effects on Akt and Erk activation which is in contrast to the response to APO. Properties of general antioxidant NAC in sense of attenuation of kinase phosphorylation were more profound during treatment in complete medium. Notably, we did not observe changes of Stat3 phosphorylation in presence of any drugs tested in our study. The most striking effect was detected downstream of PI3K on the level of S473 Akt phosphorylation when APO treatment was employed. In serum starved cells, in FBS or LIF activated cells, and also in the presence of complete medium, APO decreased Akt phosphorylation when short term impact was studied. It was earlier reported that vanillin and some of its derivatives, including APO, readily inhibited PI3K in lung adenocarcinoma cell line [69]. These authors are also suggesting, in agreement with our data, that radical scavenging or other antioxidant properties of those compounds are not responsible for the observed effect. Thus, the mode and mechanism of inhibition needs to be further clarified.

Remarkably, critical role of PI3K was also described for the process of NOX activation [70]; thus it can be hypothesized that some of the APO inhibitory actions towards NOXs might be also mediated *via* PI3K inhibition. To further elucidate impact of APO-induced PI3K inhibition, we examined the level of homeodomain transcription factor Nanog, which was suggested as a downstream target of PI3K and is also recognized as an important intrinsic regulator of stem cell pluripotency [28]. After 24 hours of APO treatment, the level of Nanog was dramatically reduced. Interestingly Akt phosphorylation was upregulated by APO after 24 hours compared to untreated cells, and this increase could be reverted by addition of NAC, although Nanog levels remained diminished, suggesting that this impairment is perhaps not related to APO-induced modulation of ROS levels in the cell. Phosphorylation of both Akt and Erk was augmented by DPI treatment that may be attributed to its above described prooxidative effect, as it was reduced by NAC supplementation in the case of Akt.

Next, we examined the impact of NOX inhibitors on canonical Wnt signaling, as it also plays a distinctive role in regulation of embryonic stem cells [31, 71] and the modulator of this pathway, GSK3, is a known PI3K downstream [72]. GSK3 regulates Wnt pathway by phosphorylating β-catenin on multiple sites that enhances its subsequent degradation [43]. GSK3 is active in resting cells but is readily inhibited through the PI3K/Akt-mediated phosphorylation of N-terminal serine residues (S9 in GSK3β and S21 in GSK3α) [72]. Therefore, in the conventional view, it is assumed that activity of PI3K/Akt should promote canonical Wnt signaling. In contrast to this, experimental evidence argues against this simplified scenario, as Akt activation failed to promote Wnt/β-catenin signaling when insulin and constitutively active Akt were administrated [73]. Moreover, it was reported that Axin-associated GSK3, responsible for mediating β-catenin degradation, represents just a minor fraction of GSK3 cellular content and this complex is not accessible to Akt

phosphorylation, thus preventing PI3K/Akt/Wnt cross-talk [74]. In our experiments, we did not see any effect on basal Wnt transcription activity, when NOX inhibitors or LY was employed. Moreover, treatment of both APO and LY decreased GSK3β S9 phosphorylation, further questioning the simplified model of PI3K/Wnt cross-talk mediated solely by level of GSK3 inhibition. However, after Wnt3a induction, both LY and APO augmented Wnt transcription activity. Notably, PI3K inhibition was recently shown to increase the amount of active nuclear β-catenin and to promote induction in TOPflash assay in epithelial cell culture system [75]. In agreement with other studies [76, 77], authors were suggesting the pivotal role of bidirectional loop between receptor tyrosine kinase-driven MAPKs and Wnt/β-catenin signaling. Although Erk kinase was not dramatically affected by APO treatment in our system, we are not excluding the possibility of a different MAPK involvement in observed phenomena, as numerous convergence points were described between PI3K and MAPKs [78].

5. Conclusions

Altogether, our study suggests prooxidant activity of APO and DPI in mES. Moreover, treatment with those drugs results in different modulation of intracellular pathways critical for regulation of proliferation and differentiation. APO markedly downregulates activity of Akt and its downstream Nanog and augments Wnt signaling. DPI promotes Akt and Erk activation. Taking into account described negligible NOXs levels in mES we suggest that actions of drugs observed in our experiments are rather independent of their typical function as NOX inhibitors. Therefore, caution should be taken to potential applications of these NOX inhibitors and interpretation of obtained results, especially in studies focused on the stem cell biology and intracellular and redox signaling.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. The authors alone are responsible for the content and writing of the paper.

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Research Article

Protective Effects of D-Penicillamine on Catecholamine-Induced Myocardial Injury

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Iron and copper release participates in the myocardial injury under ischemic conditions and hence protection might be achieved by iron chelators. Data on copper chelation are, however, sparse. The effect of the clinically used copper chelator D-penicillamine in the catecholamine model of acute myocardial injury was tested in cardiomyoblast cell line H9c2 and in Wistar Han rats. D-Penicillamine had a protective effect against catecholamine-induced injury both *in vitro* and *in vivo*. It protected H9c2 cells against the catecholamine-induced viability loss in a dose-dependent manner. In animals, both intravenous D-penicillamine doses of 11 (low) and 44 mg/kg (high) decreased the mortality caused by s.c. isoprenaline (100 mg/kg) from 36% to 14% and 22%, respectively. However, whereas the low D-penicillamine dose decreased the release of cardiac troponin T (specific marker of myocardial injury), the high dose resulted in an increase. Interestingly, the high dose led to a marked elevation in plasma vitamin C. This might be related to potentiation of oxidative stress, as suggested by additional *in vitro* experiments with D-penicillamine (iron reduction and the Fenton reaction). In conclusion, D-penicillamine has protective potential against catecholamine-induced cardiotoxicity; however the optimal dose selection seems to be crucial for further application.

1. Introduction

Cardiovascular diseases remain the main cause of mortality in developed countries. The main culprit is atherosclerosis associated with coronary heart disease which can lead to its acute form, acute myocardial infarction [1]. The current treatment based mainly on the percutaneous coronary intervention has largely substituted other treatment modalities in developed countries. However, in some cases, due to contraindications or inaccessibility of adequate medical devices, particularly in developing countries, pharmacotherapy using fibrinolytics is still used. Irrespective of the procedure used, the recovery of blood flow of previously ischemic myocardium is associated with a phenomenon called reperfusion paradox [2]. This is based on a burst of reactive oxygen species (ROS) generated when previously ischemic tissue is

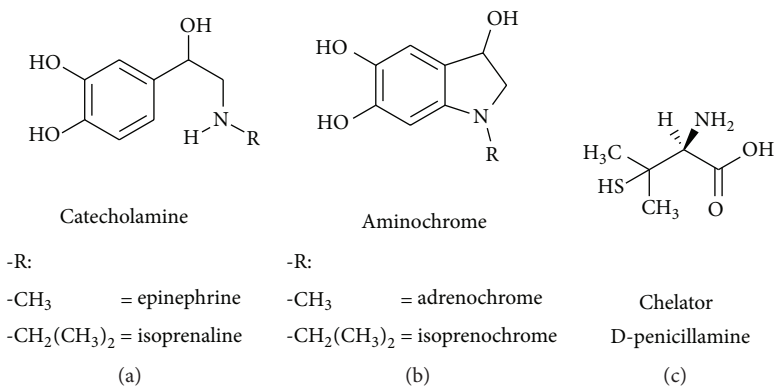

Figure 1: Chemical structures of compounds under investigation: (a) catecholamines epinephrine /(R)-4-(1-hydroxy-2-(methylamino)ethyl)benzene-1,2-diol/ and ISO /(RS)-4-(1-hydroxy-2-(isopropyl-amino)ethyl)benzene-1,2-diol/; (b) aminochromes, that is, catecholamine oxidation products, adrenochrome /3-hydroxy-1-methyl-2,3-dihydro-1H-indole-5,6-dione/ and isoprenochrome /3-hydro-xy-1-isopropyl-2,3-dihydro-1H-indole-5,6-dione/; and (c) copper chelator D-PA /(2S)-2-amino-3-methyl-3-sulfanyl-butanoic acid/.

reexposed to oxygenated blood flow. Release of free or loosely bound transition metals, namely, iron and copper, into the circulation plays an important role in this phenomenon, well documented in experimental animals [3, 4]. Our research group has previously shown that iron chelators might protect the myocardium from acute catecholamine-induced cardiac injury, which resembles the acute myocardial infarction in many aspects [5, 6]. Similarly, other groups have demonstrated some degree of protection by iron chelators in both experimental *in vivo* and clinical studies [7–9].

Much less attention has been paid to copper and its chelators and their potential effects in acute myocardial ischemic conditions. In human, copper elevation in patients with acute myocardial infarction has been well documented and a recent study has clearly demonstrated that higher serum copper is associated with higher cardiovascular mortality [10, 11]. To our knowledge, only one study tested the effect of a known copper chelator in an experimental model of myocardial ischemia. That study documented protective role of neocuproine against hydrogen peroxide induced cardiac damage and reperfusion arrhythmias in isolated perfused heart [12]. As far as we know, the effect of a copper chelator in a whole animal model of myocardial injury has never been tested. In the present study, we selected D-penicillamine (D-PA), a drug with a long history of clinical use in both copper-based and non-copper based pathologies. It is considered as the standard copper chelator and its rapid effect on copper excretion in urine is well documented [13, 14]. Although the drug is a close derivative of endogenous amino acid cysteine, it is known to have side effects (e.g., severe gastrointestinal disturbances, rash, proteinuria, and hematological adverse effects) when given in a long-term basis [15]. This is likely not true for its acute administration, where even the dose of 1.2 g/kg of D-PA did not produce toxic effects in rats [16]. For a possible clinical use of D-PA relevant to the acute myocardial injury, timely limited administration is suggested and therefore such therapy might be without significant adverse reactions. The main aim of this study was to assess if D-PA can protect cardiomyoblast derived cells and/or modify the acute myocardial injury caused by administration of synthetic catecholamine isoprenaline (ISO) in rats.

2. Materials and Methods

2.1. In Vitro Experiments

2.1.1. Reagents and Solutions. The catecholamines epinephrine (EPI, Figure 1(a)) and ISO (Figure 1(a)), copper chelator D-PA (Figure 1(c)), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid sodium salt (ferrozine), sodium acetate, acetic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), HEPES sodium salt, hydroxylamine, salicylic acid, and 2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids were purchased from Sigma-Aldrich (USA). Deferoxamine was purchased from Novartis (Switzerland), dimethyl sulfoxide (DMSO) was purchased from Avantor Performance Materials (USA), and methanol for HPLC was purchased from JT Baker (USA). The chemicals and solutions used for cellular cultivation (cultivation media, sera, etc.) were purchased from Sigma-Aldrich or Lonza Group (Switzerland).

2.1.2. Iron Chelation and Reduction Assay. Ferrozine is a specific indicator which forms a magenta colored complex with ferrous ions. In principle, ferric ions do not react with ferrozine. But the methodology can be extended for the assessment of total iron chelation after reduction of ferric ions by a suitable reductant like hydroxylamine. Similarly, iron reduction can be easily assessed. If a tested compound is able to reduce these ferric ions into ferrous ions, the indicator rapidly forms a complex with them which is thereafter measured spectrophotometrically [17].

Stock solutions of ferric ions and ferrous ions were prepared in distilled water (Milli-Q RG, Merck Millipore, USA). The corresponding fresh working solutions (0.25 mM) were

prepared by dilution with distilled water. Hydroxylamine hydrochloride and ferrozine were dissolved in distilled water; D-PA was dissolved in DMSO. Experiments were performed in 15 mM buffers, acetate (pH 4.5 and 5.5) and HEPES (pH 6.8 and 7.5). Metal chelation experiments were performed in 96-well microplates, at least in duplicates, at room temperature using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., USA) as described previously [17]. Briefly, for this assessment of iron chelation, various concentrations of D-PA were mixed in mentioned buffers with ferrous or ferric ions (at a final concentration of 25 μM) for 2 min. In case of the assessment of total iron chelation, hydroxylamine was then added for reduction of nonchelated iron. The absorbance was measured immediately after the addition of ferrozine and after 5 min at 562 nm.

For the determination of the degree of ferric ions reduction, various concentrations of the tested compounds were mixed for 2 min with ferric ions in acetate or HEPES buffers. Afterwards, ferrozine was added and absorbance was measured at 562 nm immediately and after 5 min. Hydroxylamine was used as a positive control (100% reduction).

2.1.3. Inhibition of Iron-Catalyzed Production of Hydroxyl Radicals. Ferrous ions react with hydrogen peroxide to produce hydroxyl radical (the Fenton reaction). The formed radical can be trapped by salicylic acid and its ensuing products (2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids) can be detected by HPLC [18]. Briefly, ferrous ions were mixed with the tested compounds dissolved in methanol in different concentration ratios for 2 min. Salicylic acid and hydrogen peroxide (both 7 mM) were added subsequently, and the mixture was then analysed by HPLC (Dionex Ultimate 3000, Dionex Corp., USA) with Eclipse Plus C18 column (4.6 × 100 mm, 3.5 μm, Agilent Inc., USA), using 40% methanol and 0.085% aqueous solution of phosphoric acid as a mobile phase. All experiments were checked by addition of "internal standard," that is, known amounts of 2,3-dihydroxybenzoic and 2,5-dihydroxybenzoic acids.

2.1.4. Cell Culture. The H9c2 cell line derived from embryonic BD1X rat heart tissue [19] was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Lonza), 1% penicillin/streptomycin solution (Lonza), and 10 mM HEPES buffer (pH 7.4; Sigma). Cell cultivation was performed in 75 cm^2 tissue culture flasks from Techno Plastic Products AG (TPP) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured twice in a week when they reached approximately 90% confluence (i.e., every 3rd-4th day).

For particular experiments, cells were seeded into appropriate microplates (TPP) at given cellular density. The medium was changed for serum-free cell-culture medium (pyruvate-free DMEM (Sigma) supplemented with 1% penicillin/streptomycin solution (Lonza) and 10 mM HEPES buffer (pH 7.4; Sigma)) 24 h prior to all cellular experiments. Serum deprivation was used to stop cellular proliferation

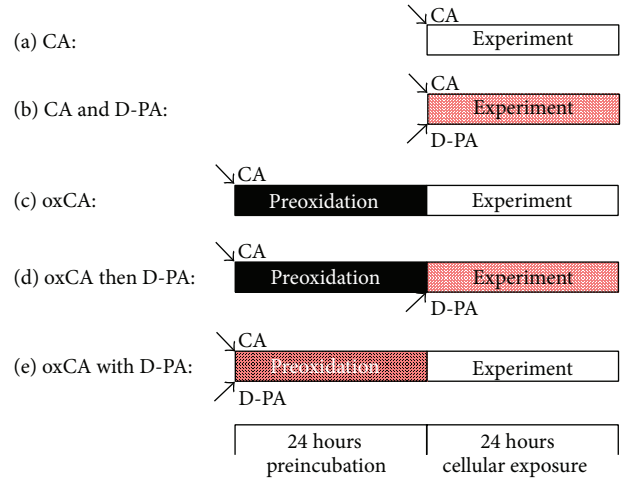

Figure 2: Overview of the protocols for cell experiments using the H9c2 cardiomyoblast cell line. Catecholamines (CA-ISO or epinephrine) were added to H9c2 cells either as freshly prepared solutions (a and b) or after 24 h preincubation in the cell-culture medium at 37 °C (oxidized oxCA) (c–e). D-PA was added either at the beginning of 24 h cellular experiments (b and d) or at the start of 24 h catecholamine preincubation before the 24 h cellular experiments (e).

to mimic the situation in postmitotic cardiomyocytes [20]. Pyruvate was omitted because it is an antioxidant and may interfere with ROS-related toxicity. The lipophilic compounds were dissolved in DMSO, yielding a final concentration of 0.1% in all experimental groups. At this concentration, DMSO had no effect on cellular viability.

2.1.5. Cell Experiments. All cell experiments were based on the ability of catecholamines to undergo spontaneous oxidation to a number of chemically related products [21]. For that reason, there were created five different protocols for the study of toxic and protective properties of compounds under investigation (Figure 2). In brief, work solutions of catecholamine (ISO or EPI) used in all experiments were either freshly prepared or 24 h preoxidized (i.e., left spontaneously to oxidize) at 37°C. The copper chelator D-PA was added into the solution with catecholamine either at the beginning of the cell experiment itself or at the beginning of 24 h preoxidaton of catecholamine.

2.1.6. Neutral Red Uptake Assay for Assessment of Compounds Cytotoxicity. Cellular viability was determined using the assay based on the ability of viable cells to incorporate neutral red (NR; Sigma). This well-established assay consists in the readily penetration of intact cell membranes by weak cationic dye, NR, and its accumulation in the lysosomes of viable cells [22]. H9c2 cells seeded in 96-well plates at a density of 10,000 cells per well were incubated with compounds under investigation (alone or in combinations) for 24 h. Half of the medium volume from each well was removed at the end of incubation, and the same volume of medium with NR was added, yielding a final concentration of 40 μg/mL.

After 3 h at 37°C, the supernatant was discarded, and the cells were fixed in 0.5% formaldehyde supplemented with 1% $CaCl_2$ for 15 min. The cells were then washed twice with phosphate buffered saline and solubilized with 1% acetic acid in 50% ethanol for 30 min of continuous agitation; thus the accumulated NR was released into the extracellular fluid. The light absorption (optical density) of released NR was measured using a microplate spectrophotometer Tecan Infinite 200 M (Tecan, Switzerland) at λ = 540 nm. The viability of experimental groups was expressed as a percentage of the untreated control (100%).

2.1.7. Epifluorescence Microscopy for Imaging of Cellular Morphology Changes. Changes in cellular morphology were observed and imaged using an inverted epifluorescence microscope Nikon Eclipse TS100 with 10–40x air objectives (Nikon, Japan) equipped with a digital camera 1300Q (VDS Vosskühler GmbH, Germany) and the software NIS-Elements AR 3.0 (Laboratory Imaging s.r.o., Czech Republic). The cellular viability was visualized using nuclei staining with Hoechst 33342 (Molecular Probes, USA) and propidium iodide (PI; Molecular Probes), which are well-established and sensitive probes to determine apoptosis and necrosis: Hoechst 33342 is a blue-fluorescent probe (λ_{ex} = 360 nm, λ_{em} = 460 nm) staining all nuclei. In apoptotic cells, chromatin condensation occurs and apoptotic cells can thus be identified as those with condensed and more intensely stained chromatin. The red-fluorescent (λ_{ex} = 560 nm, λ_{em} = 630 nm) DNA-binding dye, PI, is unable to cross the plasma membrane of living cells but readily enters necrotic (or late-stage apoptotic) cells and stains their nuclei red. H9c2 cells seeded in 12-well plates at a density of 75,000 cells per well were incubated with compounds under investigation (alone or in combinations) for 24 h. After that, cells were stained with 10 μg/mL Hoechst 33342 and 1 μg/mL PI for 10 min at 37°C.

2.2. In Vivo Experiments

2.2.1. Animals. Forty-six Wistar Han male rats were obtained from Meditox (Czech Republic) and housed in cages located in a special air-conditioned room with a periodic light-dark (12-12 h) cycle for 2 weeks. During this period, they were provided with free access to tap water and standard pellet diet for rodents. After the acclimatization period, healthy rats weighing approximately 390 g were used for the experiments.

The study was approved by the Experimental Animal Welfare Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication Number 85-23, revised 1996).

2.2.2. Experimental Design. The rats were randomly divided into six groups. Firstly, D-PA in the doses of 11 or 44 mg/kg or water for injection (B. Braun, Germany, 2 mL/kg) was administered into the tail vein. ISO (Sigma-Aldrich, 100 mg/kg, 50 mg/mL) or water for injection was given s.c. 5 min later. The groups in which rats received ISO are designated as ISO (positive control), D-PA11+ISO, and D-PA44+ISO, while the others are designated as C (negative control), D-PA11, and D-PA44.

2.2.3. Anaesthesia and Surgery. The animals had free access to water and diet during the first 12 h after drug administration. The rats were then fasted for the next 12 h before the surgery. Animals were anaesthetized with i.p. injection containing aqueous solution of urethane (Sigma-Aldrich) in a dose of 1.2 g/kg. Surgical and instrumental procedures were similar to our previous studies [6]. Briefly, the left common iliac artery was connected to a pressure transducer MLT0380/D (ADInstruments, Australia) *via* a polyethylene catheter (0.5/1.0 mm filled with heparinized saline 50 IU/mL) for arterial blood pressure measurement. A high-fidelity pressure-volume micromanometer catheter (Millar pressure-volume catheter SPR-838 2 F, 4E, 9 mm, Millar Instruments Inc., USA) was inserted into the left heart ventricle through the right common carotid artery. Both pressure transducer and Millar pressure-volume catheter together with subcutaneous electrodes for the ECG standard limb lead II MLA1215 (ADInstruments) were connected to PowerLab with LabChart 7 software (ADInstruments). Data were analyzed for 30 min, and necessary calibrations with hypertonic saline (2 × 20 μL of 25% w/w sodium chloride solution) were performed at the end of the experiment. A blood sample was collected from the abdominal aorta into a heparinized test tube (170 IU). Following the experiment, all surviving animals were killed painlessly in anaesthesia by intravenous administration of 1 mL of 1 M aqueous solution of potassium chloride (Sigma-Aldrich).

2.2.4. Biochemical Analyses. Cardiac troponin T (cTnT) was measured in serum; vitamin C and vitamin E were measured in plasma. cTnT was determined by high sensitive electrochemiluminescence immunoassay (Cobas e411, Roche) using two monoclonal antibodies specifically directed against cTnT. Vitamin E was measured with fluorometric detection after deproteinization in an HPLC system LC-10A (Shimadzu, Japan). Analogously, vitamin C was measured after deproteinization by electrophoresis using UV detection (PrinCE 750, Netherlands).

For lipid peroxidation measurement, 100–150 mg of heart tissue was diluted 1 : 9 (by weight) in ice-cold 0.1 M potassium phosphate buffer. The tissue was diced and then sonicated with ultrasonic cell disruptor (Model XL2000, Misonics, USA). 20 μL of heart sonicate was incubated for 30 min in 37°C with 100 μM ascorbate (Sigma-Aldrich) and 6 μM $FeSO_4$ as previously described [23]. The amount of CO produced into vial was quantified by gas chromatography with reduction gas detector serving as an index of lipid peroxidation.

2.3. Data Analysis. The amount of nonchelated or reduced iron was calculated from the difference of absorbance between the tested sample (with ferrozine) and its corresponding blank (without ferrozine) divided by the difference of the control sample (the known amount of iron without

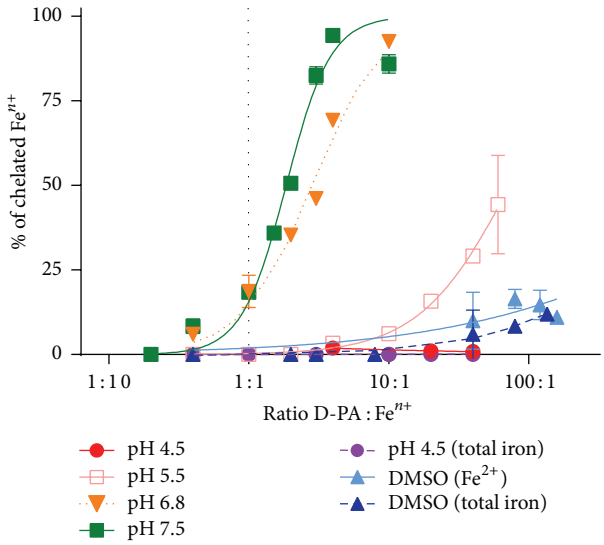

FIGURE 3: Iron-chelating activity of D-PA at (patho)physiologically relevant pH conditions and nonbuffered conditions (DMSO).

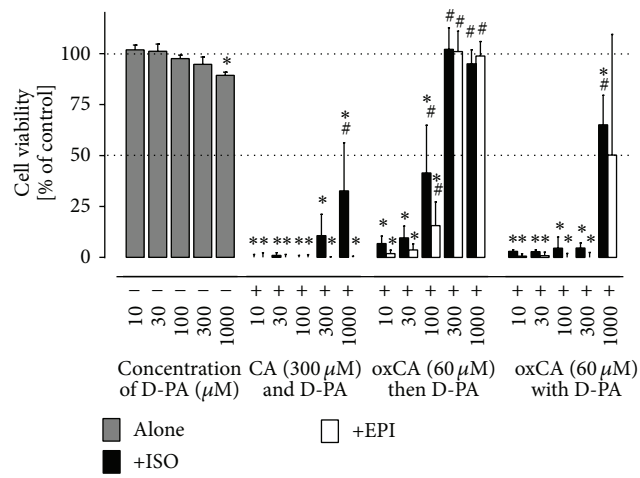

FIGURE 4: Cell viability studies using the H9c2 cardiomyoblast cell line. Figure shows dose-dependent own toxicity of D-PA (10–1000 μM, *left*) and its protective effects against toxicity induced by catecholamines (CA) ISO and EPI. D-PA was added to freshly prepared catecholamine solution before the start of 24 h cell experiments (CA and D-PA, *middle left*); D-PA was added immediately before cellular experiments to catecholamines preincubated for 24 h in cell-culture medium (oxCA then D-PA, *middle right*); or D-PA was preincubated for 24 h together with catecholamines and then added to cells (oxCA with D-PA, *right*). Schematic overview of experimental protocols is in Figure 2. Cell viability was determined by neutral red uptake assay and expressed as a percentage of the untreated control group. Data are presented as means ± SD; $n = 4$; statistical significance (ANOVA) $^{*}P < 0.05$ versus control and $^{\#}P < 0.05$ versus corresponding catecholamine group.

the tested substance) and its control blank. The concentration of hydroxyl radical was calculated as the mean of the samples mixed (1) with methanol and (2) with added internal standards. Calculations in animal study were performed as previously described [6].

Grubb's test was used for detection of outlier values in animal and cell culture studies. Data were expressed as mean ± SD. For multiple comparisons, one-way ANOVA followed by Tukey's multiple comparisons test (*in vivo* experiments) and Bonferroni *post hoc* analysis (cell culture study) were used. Differences between groups were considered significant at $P < 0.05$ unless indicated otherwise. All statistical analyses were performed by GraphPad Prism version 6.0 for Windows (GraphPad Software, USA) except for the cell culture experiments where the statistical software SigmaStat for Windows 3.5 (Systat Software, Inc., USA) was used.

3. Results

3.1. In Vitro Experiments. Since metal chelators are generally not very specific which seems to be true for D-PA as well [24, 25], we were firstly interested if D-PA can chelate iron. Our competitive spectrophotometric approach confirmed the ability of D-PA to chelate iron. However, the chelating capacity dropped with decreasing pH (Figure 3).

As we have previously shown, compounds with iron-chelating potential could reduce catecholamine cardiotoxicity both *in vitro* and *in vivo* [5]; we firstly assessed the effect of D-PA on cell damage caused by catecholamines EPI and ISO and their oxidation products (oxEPI and oxISO) in cardiomyoblast H9c2 cell line (Figure 4). D-PA alone did not significantly influence the cell viability in the tested concentration range (10–1000 μM) which is achievable in plasma by administration of D-PA [26]. However, D-PA was able to restore the viability after catecholamine treatment in a dose-dependent manner. Interestingly, D-PA mediated protection was observed especially in cells treated with preoxidated catecholamines. In this case, 300 and 1000 μM of D-PA completely prevented cell death.

Changes in cell morphology were observed and imaged using an inverted epifluorescence microscope in H9c2 cardiomyoblasts (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/5213532). While necrotic (or late-stage apoptotic) cells were found after 24 h preincubated catecholamines ISO and EPI (ox-ISO and ox-EPI, resp.), 300 μM D-PA markedly prevented the damage of cardiomyoblasts. D-PA alone did not apparently change cellular morphology.

3.2. In Vivo Study. With regard to the protective action of D-PA *in vitro*, its *in vivo* effects on rats were consecutively tested. The s.c. ISO dose of 100 mg/kg (after i.v. dose of solvent) caused 4 deaths of 11 rats (36% mortality) within 24 h. Intravenous premedication by two doses of D-PA (11 and 44 mg/kg) decreased the mortality in ISO-treated animals in both doses (Figure 5(a)). The lower dose of D-PA was more beneficial in comparison with the higher one (14% versus 22%, 1 death of 7 rats and 2 deaths of 9 rats, resp.). Four rats (1 in the ISO group, 2 in D-PA44+ISO, and 1 in D-PA44 group) died during the surgery. These deaths, however, were likely not to be linked to the type of treatment but were caused by problems during the surgical procedure. Except for

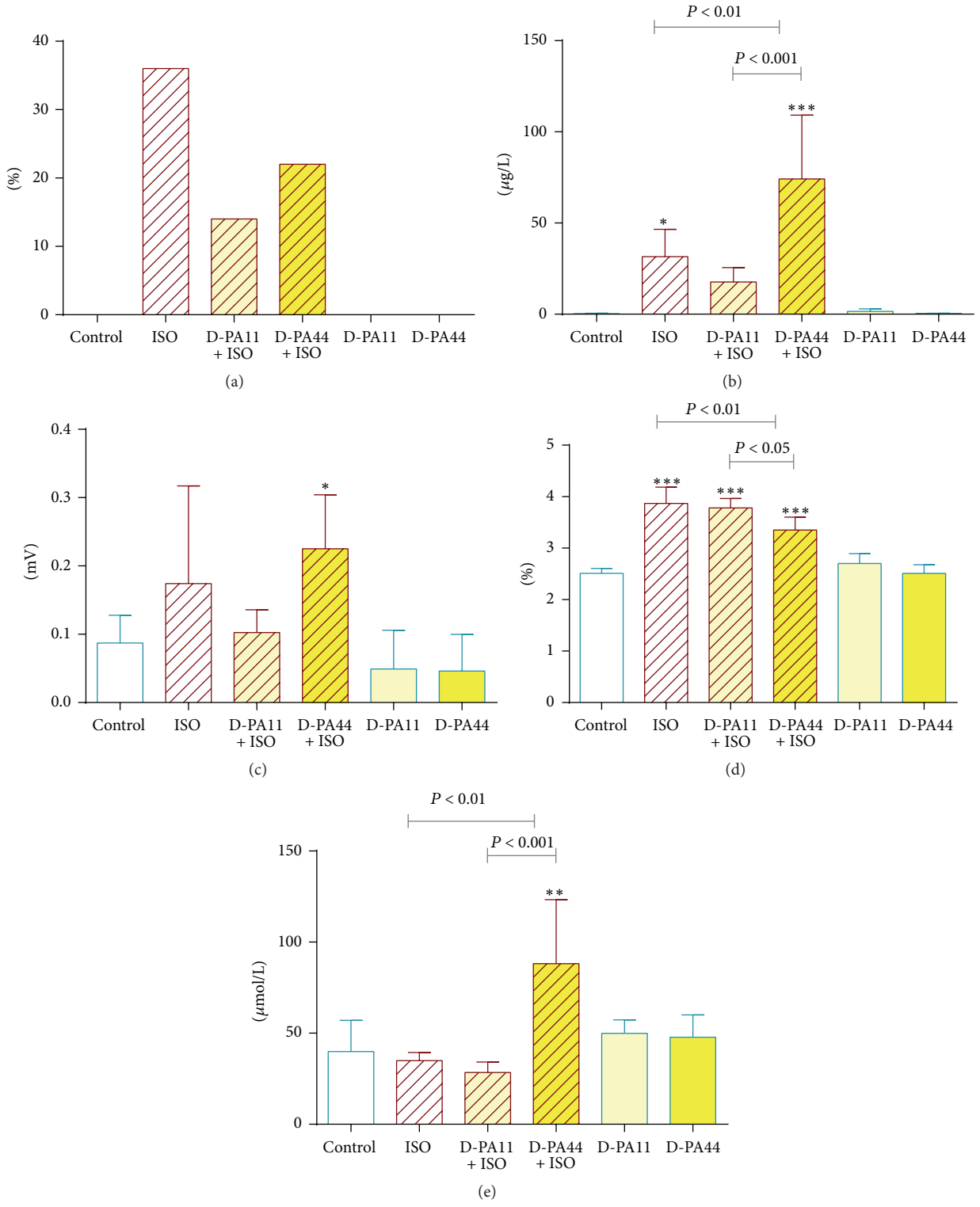

FIGURE 5: Mortality (a), changes in levels of cardiac troponin T (b), QRS-T junction (c), wet ventricles weight index (d), and levels of vitamin C in plasma (e). Statistical significance versus control: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

one animal in D-PA44 group, mentioned above, no deaths occurred in any control rat which did not receive ISO.

The lower mortality in the group with the lower D-PA dose in comparison to positive ISO group apparently corresponded to a lower release of cTnT (Figure 5(b)). There was not a significant difference in cTnT levels between D-PA11+ISO and ISO. However, there was also no difference between D-PA11+ISO and controls suggesting a partial protection by this lower dose. Unexpectedly, higher dose of D-PA with ISO increased significantly cTnT when compared to both the controls and the ISO group. Only very low serum levels of cTnT were found in all rats which did not receive ISO. Data on QRS-T junction (analogous to ST-segment elevation in human) corresponded to cTnT results (Figure 5(c)). An increase of wet ventricles weight index after administration of ISO indicates myocardial oedema in these acute experiments [27]. The effects of both lower and higher dose of D-PA on wet ventricles weight index (Figure 5(d)) were opposite to our above-mentioned results. The higher dose of D-PA improved myocardial oedema, while the lower dose only tended to improve it.

Hemodynamic Parameters. ISO treatment did not significantly modify blood pressure, but it accelerated heart rate and decreased stroke volume and ejection fraction 24 h after drug administration. D-PA alone in both doses did not affect the mentioned parameters in comparison to the solvent control group. Coadministration of ISO with D-PA did not lead to the normalization of the hemodynamic parameters with exception of the higher dose, where the drop in stroke volume was prevented. Representative hemodynamic parameters are depicted in Figure S2.

Nonsignificant changes were observed in other hemodynamic parameters, especially left-ventricular end-diastolic pressure, developed pressure, dp/dt_{max}, and dp/dt_{min}. The relaxation parameter (the time constant of left ventricular isovolumic pressure decay, tau) was likely due to high variability only insignificantly elevated in the ISO group in comparison to controls (data not shown).

Markers of Oxidative Stress. The level of vitamin C in plasma in both D-PA groups and solvent were almost identical and only insignificant drop was observed in ISO group which was analogous in the case of D-PA11+ISO group. However, the higher dose of D-PA markedly increased serum concentration of vitamin C in ISO-treated rats (Figure 5(e)). No changes in serum concentration of vitamin E and lipid peroxidation in the heart were observed (data not shown).

3.3. Additional In Vitro Experiments. Since the results of lower and higher dose of D-PA were divergent in the *in vivo* study and, in particular, the higher dose of D-PA markedly increased levels of vitamin C in plasma, additional *in vitro* experiments were performed in order to assess possible anti- or prooxidant action of D-PA. D-PA substantially reduced ferric ions and this reduction was dependent on the acidity of the environment, especially at pH 4.5 or at nonbuffered conditions; iron was completely reduced from the ratio 2 : 1 (D-PA : Fe^{3+}, Figure 6).

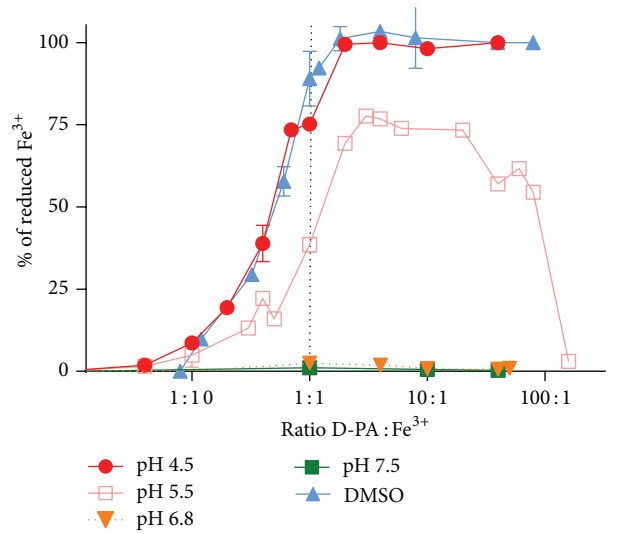

Figure 6: Reduction of ferric ions by D-PA at four pH conditions and at nonbuffered conditions (DMSO).

Since iron reduction may lead to potentiation of the Fenton chemistry, we also tested the influence of D-PA on the *in vitro* production of hydroxyl radical in the iron-catalyzed Fenton reaction using the HPLC system (Figure 7). In contrast to the standard iron chelator deferoxamine (DEF) which dose-dependently decreased the formation of hydroxyl radical, D-PA showed more complex, inverse bell-shaped behaviour. At very low ratios of D-PA to Fe^{2+}, D-PA efficiently blocked the Fenton reaction, while at higher ratios the effect of this amine was neutral.

4. Discussion

D-PA is a drug with complex mechanism of action as can be documented by its large therapeutic indication. D-PA represents the basic decoppering agent used for the treatment of Wilson's disease [28]. Other indications include especially rheumatoid arthritis, cystinuria, scleroderma, or heavy metal poisoning [29]. Our first idea was to test this drug mainly because of its known effect on copper homeostasis. Although its copper chelation effect may be limited [14, 30], it is well known for its ability to induce copper excretion in the urine [13, 26]. Therefore, the main hypothesis was that copper released from ischemic myocardium could be chelated or mobilized to urine by D-PA, which might protect myocardium from catecholamine-induced injury. Furthermore, since iron is also known to be released during myocardial ischemia [3, 4] and metal chelators including D-PA are generally not completely selective, we also evaluated D-PA chelating effect on iron ions. Interestingly, D-PA showed pronounced and stable iron chelation effects at neutral or slightly acidic pH in our experiments (Figure 3). While more than 80% of ferrous ions were chelated at the concentration ratio of 10 : 1 (D-PA : Fe^{2+}) at slightly acidic conditions or physiological pH, neither cuprous nor cupric ions were bound by D-PA at the same ratio [30]. This was

rather unexpected since a previous report has shown that the affinity of D-PA for iron is not particularly high and lower than that for other metals [31]. However, chelation and/or increased excretion of both iron and copper might be positive for the use of D-PA in the case of ROS-mediated myocardial injury. Moreover, before we started to test D-PA in rats, we firstly tested its effects on cardiomyoblast cells. D-PA had apparently very low toxicity and clear protection against catecholamines was seen at this level (Figures 4 and S1). Our previous experiments with a strong and lipophilic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) revealed that the protective action of chelation with SIH was associated with at least two distinct effects: (i) slowing down the progressive catecholamine oxidation to reactive toxic intermediates and (ii) reduction of toxicity of the already formed oxidation products [21]. The present results suggest that D-PA may act by similar mechanisms.

The injury caused by synthetic catecholamine ISO resembles in many aspects the acute myocardial infarction in human. For an *in vivo* model of myocardial infarction, we administered ISO in a dose of 100 mg/kg s.c. which is a generally used dosage to produce significant mortality with about 1/3 of deceased animals within 24 h [5, 6, 32, 33]. The mortality in our current study was analogous (36%). In agreement with *in vitro* findings, both doses of D-PA reduced the mortality but the effect was not dose-dependent (lower dose decreased the mortality to 14% while the higher to 22%). In our previous experiments, only two iron-chelating compounds had clearly protecting effects on the mentioned model in the terms of the most severe parameter, the mortality: 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone in the dose of 20 mg/kg completely prevented the mortality, while the lower dose of 10 mg/kg did not have an effect [5] and dexrazoxane in the dose of 20.4 mg/kg reduced the mortality to 12.5% [6]. Other drugs with iron-chelating properties, namely, deferoxamine and lactoferrin, were without any effect, while rutin deteriorated the mortality [5, 32, 33].

The results with D-PA were different since both lower and higher doses of D-PA evoked apparently diverse effects on our model. The lower dose, 11 mg/kg, is equimolar to 50 mg/kg of deferoxamine and was selected for reason of comparison on the molar basis with other chelators as in our previous studies [5]. Moreover, this dose fits in the dosage range in the treatment of Wilson's disease [28]. Higher dose was added to the study protocol because of partially protective effects of the lower dose. Both doses decreased the mortality, but the mechanism does not seem to be identical. The lower dose decreased the release of cTnT suggesting cardioprotection, while the higher dose had rather an opposite effect on cTnT but decreased the wet ventricles weight index and normalized the stroke volume. From the biochemical point of view, it is quite surprising that the higher dose of D-PA evoked the marked increase in plasma vitamin C (Figure 5(e)). It is not easy to explain this result which was found only in the combination of D-PA with isoprenaline but not with the solvent. It is therefore likely linked to the toxic effects of isoprenaline. Oxidative stress might play a role. We are not the first to suggest it, since (1) hydrogen peroxide production

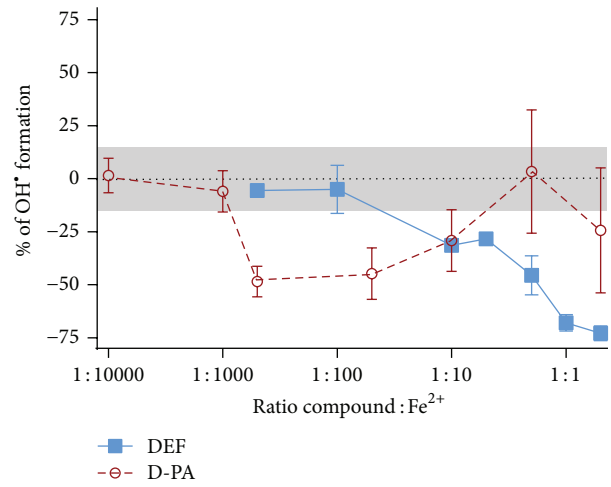

Figure 7: The impact of D-PA and the standard iron chelator deferoxamine (DEF) on hydroxyl radical formation. Grey area represents the error of the method.

from D-PA after the addition of copper was documented [34, 35], (2) D-PA in high doses produces intracellular oxidative stress in cell experiments but antioxidant enzymes might be both upregulated or downregulated [36], and (3) a recent clinical study showed that treatment with D-PA led to significant decrease in the activity of glutathione peroxidase, one of the most important antioxidant enzymes in blood, and a tendency to increase the total antioxidant capacity [37]. The latter is a similar finding to the significant plasma vitamin C increase reported in the present study. On the other hand, it should be mentioned that, in healthy animals, D-PA in these acute settings was very well tolerated in both doses and we did not observe any negative haemodynamic changes. Therefore, as suggested above, the negative effect of the higher dose of D-PA seems to be related to ischemia caused by administration of ISO or to direct effect of this catecholamine and/or its oxidation products. Ischemia leads to a decrease in pH [38] and one of the plausible mechanisms of D-PA prooxidation is iron reduction seen particularly at lower pH (Figure 6). In addition, copper reduction by D-PA was documented in our previous study [30]. It is well known that reduction of iron or copper intensifies the Fenton reaction due to redox-cycling of the catalyst [39] and therefore D-PA could increase the production of hydroxyl radical. D-PA behaviour in relation to the Fenton chemistry appears to be similar to some reducing antioxidants [40], since D-PA acted as an efficient antioxidant in low concentrations but its protective properties decreased or were even reversed at higher ratios (Figure 7). This may explain the unexpected effect of the higher dose of D-PA in the animal model but we are aware that it is not possible to directly transform *in vitro* data into *in vivo* situation. Although it is tempting to speculate that increased oxidative stress could explain why the higher dose was not more efficient than the lower one, more *in vivo* experiments will be needed to confirm it. On the other hand, the high dose of D-PA had some protective effects which were very different from those of

the lower dose and might be caused by other mechanisms apart from interaction with transition metals. Since D-PA had some effect in autoimmune disease, especially rheumatoid arthritis and scleroderma [29], one could speculate that D-PA can inhibit the activation of the immune system [35, 41], which is an important component of the myocardial injury [42]. Indeed, wet ventricle weight might increase due to myocardial oedema caused by activation of immune system [27] and this parameter was decreased by the higher dose of D-PA. However, the direct effect of D-PA on immune system in acute myocardial injury was not tested in this study.

5. Conclusion

In conclusion, this study has shown that D-PA has potential cardioprotective effects on acute myocardial injury caused by catecholamines likely due to its effect(s) on copper and/or iron homeostasis. However, in higher doses, despite positive effects on some cardiovascular parameters (normalization of stroke volume and decrease of wet ventricles weight index), the overall protective effect was attenuated, possibly due to the reduction of transition metals followed by prooxidation. The mentioned positive hemodynamic effects were presented only in the higher dose and do not seem to be based on interaction with transition metals.

Abbreviations

CA: Catecholamine
cTnT: Cardiac troponin T
D-PA: D-Penicillamine
EPI: Epinephrine
ISO: Isoprenaline
NR: Neutral red
PI: Propidium iodide
ROS: Reactive oxygen species
SIH: Salicylaldehyde isonicotinoyl hydrazone.

Conflict of Interests

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

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Research Article

A New Method to Simultaneously Quantify the Antioxidants: Carotenes, Xanthophylls, and Vitamin A in Human Plasma

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A simple and accurate reversed phase high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) method for simultaneously determining and quantifying the antioxidants carotenes, xanthophylls, and retinol in human plasma is presented in this paper. Compounds were extracted with hexane, a C30 column, and a mobile phase of methanol, methyl *tert*-butyl ether, and water were used for the separation of the compounds. A total of 8 carotenoids, 3 *Z*-β-carotene isomers, and 1 fat-soluble vitamin (retinol) were resolved within 72 min at a flow rate of 0.6 mL/min. Detection was achieved at 450 nm for carotenoids and 330 nm for retinol. To evaluate the effectiveness of themethod, it has been applied to an intervention study conducted on eight volunteers. *Results*. Limits of detection were between 0.1 μg/mL for lycopene and astaxanthin and 1.3 μg/mL for 15-*Z*-β-carotene. Recoveries were ranged between 89% and 113% for α-carotene and astaxanthin, respectively. Accuracy was between 90.7% and 112.2% and precision was between 1% and 15% RSD. In human plasma samples compounds studied were identified besides three lycopene isomers, demonstrated to be suitable for application in dietary intervention studies. *Conclusions*. Due to its accuracy, precision, selectivity, and reproducibility, this method is suitable to dietary habits and/or antioxidants status studies.

1. Introduction

Several epidemiologic studies have shown that oxidative stress plays an essential role in the pathogenesis of many degenerative diseases, such as cancer, diabetes, age-related eye diseases, and cardiovascular diseases [1–6] and it has been suggested that antioxidants may exert a protective role against these chronic diseases by defending against oxidative damage [7, 8]. There is an increasing interest in the analysis of carotenoids and some fat-soluble vitamins such as retinol (vitamin A) due to their antioxidant properties and their relationship with the prevention of chronic diseases [9–12]. The characterization and quantification of carotenoids and retinol in human plasma are essential for best interpretation of epidemiologic studies linking oxidative stress, diet, and health.

Carotenoids comprise a group of fat soluble phytochemicals widely distributed in nature, responsible for the colours of many fruits and vegetables, as well as certain animal tissues and leaf coloration after the degradation of chlorophyll [13]. Considering the number of double bonds in the molecules, carotenoids can be found with *cis* or *trans* configuration (or *E*/*Z* isomers). In general the all-*E* form is predominant in nature but numerous researches show that more than 50% of some carotenoids, as lycopene, present in human plasma and tissues are *Z*-isomers [14] and it is believed that geometrical configuration of carotenoids could have implications in the solubility, absorption, and transport in humans [15, 16] or even geometrical isomers of provitamin A carotenoids have different vitamin A activities [17]. The enhanced absorption of lycopene *Z*-isomers is hypothesised to result from higher

solubility in mixed micelles, the shorter length of the *Z*-isomers, and/or a lower tendency to aggregate [14, 18]. This hypothesis was supported by studies in both animals and humans [18, 19].

Among fat-soluble vitamins, retinol exerts an important antioxidant action via the inhibition of lipid peroxidation and has free-radical-scavenging properties [11, 20]; meanwhile, carotenoids are known to be effective quenchers of singlet oxygen, as well as strong scavengers of different reactive oxygen species (ROS) [21].

High-performance liquid chromatography (HPLC) is one of the most used techniques for the identification of carotenoids [22, 23] as well as vitamin A in human plasma [24–26]. The advantages of the use of this technique are speed, sensitivity, and accuracy for the determination of the compounds in addition to the economy of the solvents required and the simple coupling with other techniques.

In light of the importance of these compounds for health maintenance, an accurate determination and quantification in plasma is necessary. The aim of this study is to develop a simple HPLC method to identify the main carotenoids and their geometrical isomers as well as retinol, one of the major antioxidant fat-soluble vitamins, in human plasma.

2. Experimental Procedures

2.1. Materials and Methods

2.1.1. Reagents and Standards. Carotenoids and vitamins standards: retinol, astaxanthin, lutein, zeaxanthin, *E*-β-apo-8′-carotenal, cryptoxanthin, 15-*Z*-β-carotene, 13-*Z*-β-carotene, α-carotene, β-carotene, 9-*Z*-β-carotene, and lycopene were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methanol (MeOH) and methyl-*tert*-butyl ether (MTBE) of HPLC grade were obtained from Panreac Quimica SA (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore System (Bedford, MA, USA). Human plasma and butylated hydroxytoluene (BHT) were acquired from Sigma-Aldrich.

2.1.2. Preparation of Standard and Stock Solutions. Individual working standards of retinol, astaxanthin, lutein, zeaxanthin, *E*-β-apo-8′-carotenal, cryptoxanthin, 15-*Z*-β-carotene, 13-*Z*-β-carotene, α-carotene, β-carotene, 9-*Z*-β-carotene, and lycopene were prepared at a concentration of 1 mg/mL in MTBE. All working standards were manipulated under protection of light to minimise light-induced isomerisation, stored in eppendorf tubes, and kept at −20°C until use.

The stock solution used to spike plasma samples was prepared by mixing individual working standards at a concentration of 50 mg/mL in MTBE.

2.1.3. Extraction and Isolation of Carotenoids and Retinol. Plasma was subjected to a liquid-liquid extraction procedure previously described by our working group [27]. Briefly, 800 μL of plasma was mixed with 800 μL of ethanol and 2 mL of hexane/BHT (100 mg/L). Then, 300 μL of stock solution was added followed by a vortex-mixing for 1 minute and centrifuged at 2062 gr for 5 minutes at 4°C. The upper nonpolar layer was removed and the remaining aqueous plasma mixture was reextracted as described above. The two nonpolar extracts were combined in a glass vial and dried under nitrogen gas at <25°C followed by a reconstitution with 300 μL of MTBE. Then, the samples were stored into insert-amber vials for HPLC at −20°C until the day of analysis.

2.1.4. Instrumentation. Chromatographic analysis was carried out in an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany), consisting of a quaternary pump and an autosampler coupled to a diode array detector DAD G1315B. Chromatographic separation was performed on a reversed-phase column YMC Carotenoid S-5 μm, 250 mm × 4.6 mm (Waters, Milford, MA), maintained at 25°C, and connected to a precolumn YMC Guard Cartridge Carotenoid 20 × 4.0 mm i.d, S-5 μm. The guard column was replaced every 500 injections. The integration was performed with Agilent ChemStation Software. The DAD detector was adjusted at 450 nm for the carotenoids and 330 nm for retinol, respectively. All compounds were identified by retention time compared with pure standards as well as by the UV-Vis spectra of each compound.

2.1.5. Chromatographic Conditions. The chromatographic separation was performed using the following solvents: Milli-Q water (A), methanol (B), and MTBE (C). Solvent A was used isocratically at 4% while the following linear gradient was used for B (*t* (min), %B): (0.0, 90); (40.0, 40); (60.0, 6); (62.0, 90); (72.0, 90). Twenty microliter aliquots of the samples were injected in the HPLC-DAD system. Total run time was 72 minutes at a flow rate of 0.6 mL/min.

2.1.6. Quality Parameters. The method was fully validated based on the criteria of the AOAC International and the U.S. Department of Health and Human Services Food and Drug Administration (FDA) [28, 29]. The quality parameters established for the validation of the method were accuracy, intra- and interday precision, recovery, limit of detection (LoD), limit of quantification (LoQ), and linearity.

Three dilutions were prepared from the stock solution in MTBE to the final concentrations of low (1.5 μg/mL), medium (4 μg/mL), and high (9 μg/mL) for all analytes for precision and accuracy assays.

Accuracy consisted in the closeness of agreement between the measured value and the reference value and was established by repetitively spiking blank plasma with three known concentrations of analyte standards: low, medium, and high with respect to the calibration curves, in five replicates. The results were determined as the percentage of the ratio of the mean observed concentration and the known spiked concentration in the biological matrices. The mean value should be within ±15% of the nominal value.

Intraday precision and interday precision were considered using five determinations per three concentration levels: low, medium, and high in a single analytical run or in three different days, respectively. The precision of the method was assessed on the % RSD (percentage of relative standard deviation) of intra- and interday repeatability and the values determined at each concentration level should not exceed

15% of RSD, according to the regulation of the AOAC and FDA.

Recovery was accomplished by preparing seven-point calibration curves and seven-point external curves, spiked before and after extraction, respectively. The detector response obtained from the amount of analyte added to and extracted from the biological matrix was compared to the detector response obtained for the same concentration of the pure authentic standard. A linear regression between the ratio analyte concentration against the calculated concentration has been applied, and the slope multiplied by 100 corresponded to the analyte recovery.

Limit of detection (LoD) and limit of quantification (LoQ) were determined by comparing measured signals from samples with the low concentration of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected or quantified. A signal-to-noise ratio in the order of 3 : 1 and 10 : 1, for LoD and LoQ, respectively, was considered acceptable.

Linearity was tested by assessing signal responses of target analytes from plasma samples spiked in duplicate at seven different concentrations and by calculation of linear regression.

2.2. Method Application: Pilot Dietary Intervention Study

2.2.1. Biological Material. To assess the efficiency in the identification of carotenoids and retinol in human plasma, the present method was applied to a small-scale prospective, open and controlled, single-arm intervention study conducted in eight volunteers free of cardiovascular disease but with high risk of developing it, aged 69.9 ± 3.8 years with a mean body mass index of 32.3 ± 3.8 kg/m^2.

The volunteers were instructed to avoid the consumption of tomato and tomato-based products 3 days before the study. On the experimental day, after 12 hours of fasting, blood samples were collected early in the morning to quantify carotenoids and retinol as baseline. After that, all volunteers have followed a similar diet developed by a trained dietitian, which took into account their preferences and tastes, as well as the consideration that participants were diabetic, obese, hypertensive, and/or dyslipidemic. Each day during 4 weeks, participants consumed 250 mL of tomato juice before dinner and at the end of the study blood samples were taken for comparing with baseline. All samples were stored at −80°C until analysis.

The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (ISRCTN20409295). Written informed consent was obtained from all participants.

2.3. Statistical Analysis. Statistical analysis was performed using the SPSS Statistical Analysis System (version 22.0; SPSS Inc, Chicago, IL). Data are presented as means and standard deviation (SD). Statistical differences between the two interventions were analysed by the nonparametric statistical Wilcoxon test for paired comparisons. Statistical significance was set at $p < 0.05$.

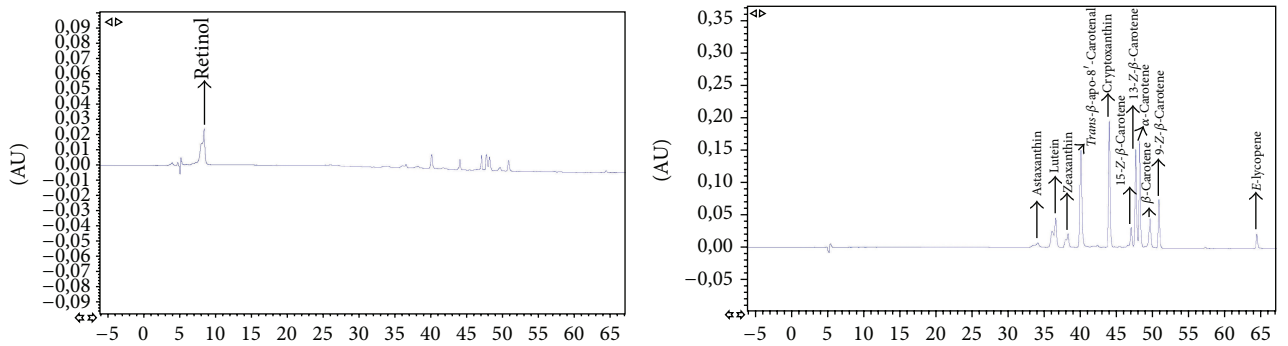
3. Results and Discussion

3.1. HPLC Method Development

3.1.1. Extraction. For the extraction of carotenoids and retinol, precipitation of proteins from plasma as a prior step is required. To achieve this issue, most methods use organic solvents such as methanol, ethanol, or acetonitrile [30]. Some studies use ethanol-BHT at different concentrations [31, 32] while others use ethanol-ascorbic acid [33] or ethanol-saline [34] for protection of carotenoids besides deproteinization. Different solvents and solvent combinations have been described in the literature for removing lipophilic analytes from biosamples. Hexane alone [35, 36] or combined with other solvents, such as hexane/acetone [37], hexane/ether [34], or hexane/ethanol/acetone/toluene [38], seems to be the most used solvents for the extraction of carotenoids and lipophilic compounds from plasma. There are few studies where hexane-BHT is used for protection of carotenoids during the extraction [39, 40] while other authors prefer to use hexane-saline [34]. Both mixtures, hexane-BHT and hexane-saline, were tested for extraction. Recoveries obtained with hexane-saline were between 57.4% and 86.9% corresponding to *E*-lycopene and 13-*Z*-β-carotene, respectively, versus recoveries range between 73.4% and 90.4% corresponding to *E*-lycopene and β-carotene, respectively, using hexane-BHT as extraction solvent. Due to its simplicity, speed, and good recoveries achieved, ethanol for deproteinization and repeated extraction with hexane-BHT were chosen for performing this study.

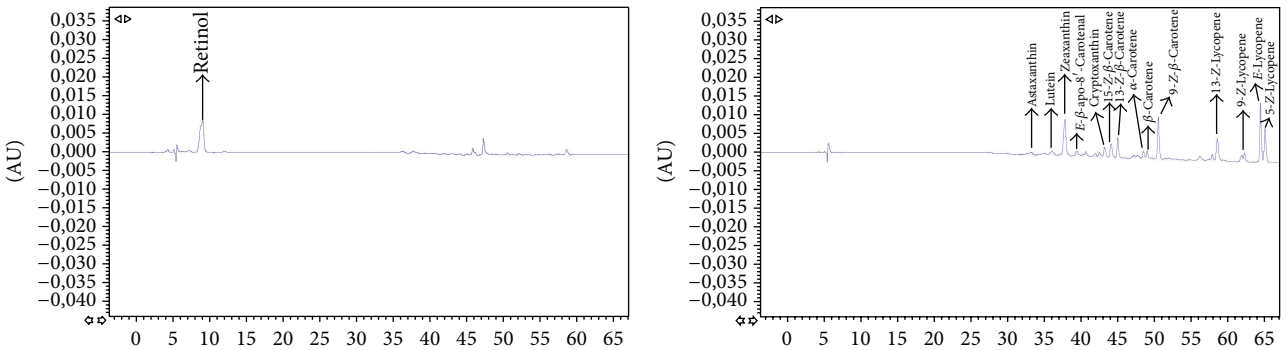
3.1.2. Separation and Identification. Numerous HPLC methods are reported for separation and identification of carotenoids and fat-soluble vitamins from diverse complex matrices such as food, food products, or plasma. The use of isocratic or gradient systems coupled to different types of columns and/or coupled to different detectors or even the use of different temperatures for the stability of the analytes, depending of the compounds studied is the best described technique for these purposes.

Reversed-phase HPLC with C_{18} columns and isocratic or gradient elution seems to be the modality most commonly used for identification and quantification of carotenoids and fat-soluble vitamins [41–44]. One of the major problems in the identification of carotenoids and lipophilic compounds using C_{18} columns seems to be the separation of geometrical isomers of carotenoids [41–47]. Tzeng et al. [33] have developed an isocratic method for the identification of carotenoids using a C_{18} column, but they could only separate three carotenoids: lutein, lycopene, and β-carotene. Olmedilla et al. [40] also have described a gradient method with a C_{18} column but could not identify isomers of the carotenoids. To solve this issue, polymeric C_{30} columns were developed for separation of *Z*-*E* isomers [22, 48, 49]. The use of this kind of column has allowed us the separation of the twelve compounds studied, including 3 *Z*-isomers of β-carotene and another 3 *Z*-isomers



(a) DAD Sig = 330 nm (b) DAD Sig = 450 nm

Figure 1: Representative HPLC chromatogram of carotenoids and retinol standards in MTBE.



(a) DAD Sig = 330 nm (b) DAD Sig = 450 nm

Figure 2: Representative HPLC chromatogram of carotenoids and retinol in human plasma corresponding to a volunteer after tomato juice intervention.

of lycopene. Figures 1 and 2 show a representative HPLC-DAD chromatogram of carotenoids and retinol in a standard mixture and a human plasma sample, respectively.

Likewise lutein and zeaxanthin are reported as two difficult compounds to separate on monomeric C_{18} columns [42, 43]. Gueguen et al. [44] found an inadequate resolution of lutein and zeaxanthin with the method proposed using a C_{18} column and an isocratic elution system; Thibeault et al. [31] were not able to differentiate lutein and zeaxanthin with these same conditions. A high resolution can be achieved by using polymeric C_{30} columns with gradient elution [22, 49, 50]. With the present method, all analytes, including lutein and zeaxanthin, have reached a resolution higher than 1.5, indicating a good separation of the compounds and a good symmetry of all peaks. Results are shown in Table 1.

Coupling of photodiode array detector (DAD) and fluorescence detector (FLD) is the technique chosen by Epler et al. [51] and Liu et al. [32] for identification of carotenoids and retinols from human serum and foods. Using more complex techniques, such as photoisomerization of some analytes as a prior step, Ferruzzi et al. [49] identified 13 lycopene isomers (*Z*/*E*) by using an electrochemical detector (ECD). Lyan et al. [46] and Lee et al. [47, 52] have identified various carotenoids by the coupling of two different detectors or columns. Other authors like Gleize et al. [53] have set up a gradient method for the identification of eleven carotenoids and fat-soluble vitamins from complex matrixes such as food samples, human plasma, and human adipose tissue using a single C_{30} column kept at 35°C. All these techniques allow the separation and identification of lipid compounds such as carotenoids and fat soluble vitamins from different matrices but are complex and require coupling of columns and use of temperature or combination of detectors, which is not always possible in all laboratories.

In this study, carotenoids and retinol were separated in a single run on a reversed-phase column using a gradient system of water, methanol, and MTBE. The mobile phase was optimized in order to obtain the best separation of the compounds in the shortest time possible and to achieve this, several gradients were assessed. The best results obtained for the conditions were described in the chromatographic conditions section.

3.2. Validation Parameters

3.2.1. Linearity. According to the maximal reported value in plasma for each analyte, plasma samples were spiked in duplicate at seven different concentrations ranged from LoQ of each compound to 10 μg/mL. The analytical procedure was linear over the concentration range tested with the correlation coefficient from 0.9952 to 0.9984 for all compounds in plasma samples, demonstrating a good linearity of the curves. Table 4

Table 1: Resolution of the analytes studied.

Analyte	Rt (min)	Wavelength (nm)	Width (min)	Resolution
Retinol	8.54	330	0.6	N.d.
Astaxanthin	34.07	450	0.38	2.3
Lutein	36.55	450	0.72	1.4
Zeaxanthin	38.24	450	0.53	2.2
E-β-apo-8′-carotenal	40.01	450	0.29	7.7
Cryptoxanthin	43.94	450	0.22	6.9
15-*Z*-β-carotene	46.96	450	0.22	1.4
13-*Z*-β-carotene	47.57	450	0.21	1.3
α-carotene	48.12	450	0.2	2.9
β-carotene	49.57	450	0.31	2.3
9-*Z*-β-carotene	50.76	450	0.2	33.0
E-lycopene	64.29	450	0.21	33.0

N.d.: not determined.
$R = 2[(\mathrm{Rt})B - (\mathrm{Rt})A]/(WA + WB)$.
R = resolution.
Rt = retention time.
W = width.

Table 2: Accuracy and recovery of the compounds studied.

Analyte	Accuracy (%)	Recoveries (%)
Retinol	105 ± 9	96 ± 3
Astaxanthin	99 ± 7	113 ± 6
Lutein	99 ± 13	112 ± 9
Zeaxanthin	101 ± 11	107 ± 5
E-β-apo-8′-carotenal	98 ± 10	94 ± 3
Cryptoxanthin	103 ± 12	96 ± 3
15-*Z*-β-carotene	90 ± 13	101 ± 2
13-*Z*-β-carotene	105 ± 12	92 ± 5
α-carotene	98 ± 12	89 ± 4
β-carotene	97 ± 13	96 ± 2
9-*Z*-β-carotene	112 ± 7	93 ± 3
E-lycopene	100 ± 12	91 ± 3

summarizes the correlation coefficients of the curves of all compounds.

3.2.2. Accuracy and Precision. Accuracy and intra and interday precision were studied. All compounds analysed met the acceptance criteria to not overcome 15% RSD in both intra- and interday precision and in the three concentration levels. The highest values were 15% belonging to astaxanthin, zeaxanthin, α-carotene, β-carotene, and 13-*Z*-β-carotene. Accuracy results obtained were between 90.7% and 112.2% being within limits of accuracy, 85–115%. The method proposed demonstrated good accuracy and precision in plasma samples, asserting that was feasible for the determination of carotenoids and retinol in human plasma. Results are expressed in Tables 2 and 3.

3.2.3. Recovery. Recovery for retinol was 96%, similar to the value achieved by Kand'ár et al. [54]. For carotenoids, the recoveries were between 89% and 113%, corresponding to α-carotene and astaxanthin, respectively. Comparing our results with those reported by Talwar et al. [43] we achieved a recovery 18% higher for lutein and 9% higher for β-carotene with the described method; comparing with the data presented by Tzeng et al. [33], we achieved a better recovery for lutein (20% higher), and comparing with Rajendran et al. [22] we obtained a better recovery for lutein (19% higher), zeaxanthin (13% higher), and cryptoxanthin (7% higher). Karppi et al. [39] have reported similar values of recovery except for lutein, zeaxanthin, and β-carotene that were 10%, 17%, and 16%, respectively, lower than in the present study.

The extraction procedure was really effective, since high recoveries can be observed in Table 2.

3.2.4. Limit of Detection (LoD) and Limit of Quantification (LoQ). The LoD found was 0.1 μg/mL for astaxanthin and lycopene; 0.2 μg/mL for retinol, zeaxanthin, *E*-β-apo-8′-carotenal, cryptoxanthin, β-carotene, and 9-*Z*-β-carotene; 0.4 μg/mL for lutein and 13-*Z*-β-carotene; 0.5 μg/mL for α-carotene, and 1.3 μg/mL for 15-*Z*-β-carotene. The LoQ was from 0.3 μg/mL to 4.4 μg/mL for astaxanthin and 15-*Z*-β-carotene, respectively. All LoD and LoQ values obtained are expressed in Table 4. In a study carried out by Mitrowska et al. [55] similar values were obtained for *E*-β-apo-8′-carotenal, astaxanthin, and lycopene. In another study developed by Talwar et al. [43] a similar value for retinol but not for the other compounds studied can be observed; nonetheless, with the presented method we have achieved the separation and identification of 11 carotenoids and 1 fat-soluble vitamin in a single run.

3.2.5. Plasma Levels of Carotenoids and Retinol. The method was used for measuring concentrations of carotenoids and retinol in human plasma samples. All compounds were determined following the procedure described above and the results are expressed in Table 5.

Nine of the 12 validated analytes were identified in plasma at baseline: retinol, astaxanthin, lutein, *E*-β-apo-8′-carotenal, cryptoxanthin, 13-*Z*-β-carotene, α-carotene, β-carotene, and lycopene. It is important to highlight that, besides the identification of the validated compounds, we also have searched for *cis* isomers of lycopene which have been suggested to be more bioavailable than *E*-lycopene, typically found in raw food [18]. In fact, in human plasma, total lycopene is an isomeric mixture containing 40% to 50% as *Z*-isomers [5] and according to the antioxidant properties of lycopene, from greatest to least are 5-*Z*, 9-*Z*, 7-*Z*, 13-*Z*, 15-*Z*, 11-*Z*, and all-*E* lycopene [56]. We have identified 5-*Z*, 9-*Z*, and 13-*Z* lycopene, all present in human plasma according to the study carried out by Arranz et al. [27]

After tomato juice intervention, an increase in retinol, astaxanthin, cryptoxanthin, 13-*Z*-β-carotene, β-carotene, 13-*Z*-lycopene, 9-*Z*-lycopene, *E*-lycopene, and 5-*Z*-lycopene was observed, with values between 5.194 and 0.140 μg/mL corresponding to *E*-lycopene and 13-*Z*-β-carotene, respectively.

Table 3: Intra- and interday precision.

Analyte	Precision											
	1,5 μg/mL ($n = 5$)				6 μg/mL ($n = 5$)				9 μg/mL ($n = 5$)			
	Day 1 (RSD %)	Day 2 (RSD %)	Day 3 (RSD %)	RSD Interday	Day 1 (RSD %)	Day 2 (RSD %)	Day 3 (RSD %)	RSD Interday	Day 1 (RSD %)	Day 2 (RSD %)	Day 3 (RSD %)	RSD Interday
Retinol	3	4	5	4.9	10	12	8	14.1	13	10	11	14.3
Astaxanthin	7	3	3	14.5	11	13	12	14.6	15	12	12	14.7
Lutein	8	9	4	14.2	12	14	8	14.3	10	9	12	13.3
Zeaxanthin	4	15	4	10.8	12	3	7	12.9	11	11	10	14.8
E-β-apo-8′-carotenal	11	10	4	14.3	11	12	8	14.7	13	9	12	13.8
Cryptoxanthin	5	13	3	12.1	11	6	9	14.3	11	10	8	13.9
15-*Z*-β-carotene	5	13	1	9.4	11	5	9	15.1	10	7	11	13.4
13-*Z*-β-carotene	6	14	3	14.9	12	15	12	14.0	11	10	10	14.9
α-carotene	4	15	2	9.4	11	15	11	13.7	11	9	10	14.8
β-carotene	11	10	8	11.2	11	15	14	14.9	12	6	10	12.8
9-*Z*-β-carotene	6	10	7	10.4	12	12	4	13.9	11	11	11	14.8
E-lycopene	5	5	2	11.6	10	13	9	13.8	10	15	8	14.8

N.d.: not determined.
RSD: relative standard deviation.

Table 4: Limit of detection (LoD), limit of quantification (LoQ), range of concentration, calibration curve, and correlation coefficient of the analytes in blank plasma spiked with standard solution.

Analytes	LoD (μg/mL)	LoQ (μg/mL)	Linearity range (μg/mL)	Calibration curve	Correlation coefficient (r)
Retinol	0.2	0.7	0.7–10	$y = 69.684x - 15.857$	0.9952
Astaxanthin	0.1	0.3	0.3–10	$y = 49.138x + 3.0197$	0.9964
Lutein	0.4	1.3	1.3–10	$y = 119.37x - 2.263$	0.9954
Zeaxanthin	0.2	0.7	0.7–10	$y = 69.14x + 1.53$	0.9955
Apo-8′-carotenal	0.2	0.7	0.7–10	$y = 249.4x - 20.908$	0.9957
Cryptoxanthin	0.2	0.7	0.7–10	$y = 200.48x - 15.759$	0.9952
15-Z-β-carotene	1.3	4.3	4.3–10	$y = 91.235x - 10.848$	0.9984
13-Z-β-carotene	0.4	1.3	1.3–10	$y = 90.591x + 8.7376$	0.9969
α-carotene	0.5	1.6	1.6–10	$y = 243.07x - 37.135$	0.9966
β-carotene	0.2	0.7	0.7–10	$y = 105.8x - 2.9437$	0.9983
9-Z-β-carotene	0.2	0.7	0.7–10	$y = 75.685x - 8.4189$	0.9959
E-lycopene	0.1	0.3	0.3–10	$y = 34.532x - 4.2861$	0.9952

Table 5: Carotenoids and retinol in human plasma before (baseline) and after the dietary intervention.

Analytes	Concentration			
	Baseline	After intervention	Baseline	After intervention
	Mean ± SD (μg/mL)	Mean ± SD (μg/mL)	Mean ± SD (μmol/L)	Mean ± SD (μmol/L)
Retinol	1.82 ± 0.37	1.90 ± 0.49	3.46 ± 0.70	3.62 ± 0.93
Astaxanthin	0.76 ± 0.44	0.88 ± 0.37	1.27 ± 0.74	1.48 ± 0.63
Lutein	0.07 ± 0.02	0.06 ± 0.06	0.13 ± 0.03	0.11 ± 0.11
Zeaxanthin	N.d.	N.d.	N.d.	N.d.
E-β-apo-8′-carotenal	0.57 ± 0.03	0.57 ± 0.03	1.38 ± 0.06	1.38 ± 0.08
Cryptoxanthin	0.18 ± 0.12	0.20 ± 0.07	0.32 ± 0.22	0.37 ± 0.12
15-*Z*-β-carotene	N.d.	N.d.	N.d.	N.d.
13-*Z*-β-carotene	0.13 ± 0.00	0.14 ± 0.02	0.23 ± 0.00	0.26 ± 0.04
α-carotene	0.22 ± 0.00	N.d.	0.41 ± 0.00	N.d.
β-carotene	0.95 ± 0.50	1.09 ± 0.53	1.77 ± 0.93	2.03 ± 0.98
9-Z-β-carotene	N.d.	N.d.	N.d.	N.d.
13-*Z*-lycopene	N.d.[a]	2.79 ± 1.44[a]	N.d.[a]	5.20 ± 2.69[a]
9-Z-lycopene	N.d.[a]	0.38 ± 1.42[a]	N.d.[a]	0.71 ± 2.64[a]
E-lycopene	1.15 ± 0.83[a]	5.19 ± 2.35[a]	2.14 ± 1.54[a]	9.67 ± 4.38[a]
5-*Z*-lycopene	0.75 ± 1.10[a]	3.07 ± 1.43[a]	1.41 ± 2.06[a]	5.72 ± 2.67[a]

N.d.: not determined.
SD: standard deviation.
[a]Values in a row with the same letter are significantly different ($p < 0.05$). Data analyzed by Wilcoxon test for repeated measures.

Among these compounds, lycopene and its isomers have presented a significant increase after tomato juice consumption ($p < 0.05$). Results are shown in Table 5.

In a study carried out by Pellegrini et al. [57], they found a concentration of 0.31 μg/mL of lycopene and 0.17 μg/mL of β-carotene in human plasma after a consumption of tomato purée. In another study performed by Porrini et al. [58], they have seen values in the order of 0.18, 0.21, 0.02, 0.13, 0.03, and 0.23 μg/mL for lycopene, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene, respectively, after a daily supplementation of tomato purée. Gärtner et al. [59] have determined values of 0.02, 0.0002, and 0.001 μg/mL for lycopene, α-carotene, and β-carotene, respectively, after a consumption of tomato paste. Regarding retinol, the results obtained with the present method are higher than those reported by Liu et al. [32] who have found a mean of 0.55 μg/mL and 0.56 μg/mL in plasma of women and men, respectively, participants of the Toronto Nutrigenomics and Health Study.

4. Conclusions

Based on previous reported work, the present method is simple, accurate, reliable, sensitive, and selective for the determination of the antioxidants carotenoids and retinol in human plasma samples. With this method, all analytes of interest were successfully resolved, including lutein, zeaxanthin, and *Z*-isomers of β-carotene which have previously

been reported as critical compounds to identify and separate. The sample preparation procedure in this method provides excellent recoveries for all analytes.

A total of 8 carotenoids, 3 *Z*-isomers of the β-carotene, 1 fat-soluble vitamin, and also 3 *Z*-isomers of the lycopene were simultaneously separated and identified in human plasma by the use of polymeric C_{30} chromatography column with a gradient elution.

Future approaches to enhance the analysis of the isomers should focus on the conclusive identification of the compounds that remain tentatively identified. For this purpose, the isolation of standards of most *cis*-isomers and the determination of their absorption coefficients are needed for their accurate quantification.

The HPLC method was completely validated and due to the good results obtained after the intake of tomato juice, this method may be applied to evaluate the liposoluble antioxidants carotenoids and vitamin A in clinical intervention antioxidants trials and epidemiological studies status investigations.

Conflict of Interests

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

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Research Article

Relation between Endothelial Nitric Oxide Synthase Genotypes and Oxidative Stress Markers in Larynx Cancer

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Nitric oxide synthase (eNOS/NOS3) is responsible for the endothelial synthesis of nitric oxide ($NO^{\bullet}$). G894T polymorphism leads to the amino acid substitution from Glu298Asp that causes lower NOS3 activity and basal $NO^{\bullet}$ production in NOS3 894T (298Asp) allele carriers compared with the GG homozygotes. $NO^{\bullet}$ acts as an antioxidant protecting against Fenton's reaction which generates highly reactive hydroxyl radicals. Allelic variation of NOS3 may influence an individual's risk of laryngeal cancer (LC). In the current study we have examined the possible relationship between NOS3 G894T genotypes and various systemic oxidative damage markers such as protein carbonyl, advanced oxidation protein products, Cu, Zn-superoxide dismutase, thiol group fractions, and lipid hydroperoxides in LC patients. Genotyping was carried out by PCR-RFLP. In LC patients with TT genotype, Cu, Zn-superoxide dismutase activities and nonprotein thiol levels were significantly higher than the controls. In patients with GT and GG genotype, high levels of lipid hydroperoxides showed statistical significance when compared to controls. Our results indicate a potential relationship among G894T polymorphism of NOS3, and impaired redox homeostasis. Further studies are required to determine the role of NOS3 gene polymorphism and impaired plasma redox homeostasis.

1. Introduction

Laryngeal cancer (LC) represents about 30% of the malignant tumors of head and neck cancers, and this corresponds to 8% of all cancer types worldwide [1]. Similar to other cancer types, LC is a multifactorial disease which can be induced by both genetic and environmental factors [2]. Among the various etiological factors sharing in the development of laryngeal tumors, increased production of reactive oxygen species (ROS) is playing an important role in the development of an impaired redox homeostasis [3]. In addition, increased ROS production may increase the mutation rate in a tissue, thereby increasing the rate of tumor recurrence. Thus, oxidative stress markers in LC have been a step-forwarding issue and have received attention by various investigators [4, 5].

Balance of the $NO^{\bullet}$ levels has a critical importance in cell fate. Nitric oxide synthase (NOS) is the main source of the $NO^{\bullet}$ production which produces $NO^{\bullet}$ while converting L-arginine to L-citrulline. Three functional classes of NOS have been described so far as endothelial-NOS (eNOS or NOS3), neuronal-NOS (nNOS), and inducible form of NOS (iNOS) [6, 7]. NOS3 was first defined in vascular endothelial cells; however, later studies showed that this isoform can also be found in other cell types such as airway epithelia, neurons, and certain types of tumors [6, 8]. Angiogenesis dependent

tumors have a significant genetic component, including major causes of morbidity and mortality in LC patients [9]. NO synthesis by endothelial cells plays an important role in regulating angiogenesis, killing neoplastic cells, and reducing tumor cell adhesion to endothelium [10].

Many of the other genetic polymorphisms have been reported in NOS3 gene [11–13], among them G894T polymorphism (rs1799983), which is located in exon 7 of the NOS3 gene and leads to the amino acid substitution from Glu298Asp that causes reduced NOS3 activity and basal NO production in NOS3 894T (298Asp) allele carriers compared with the GG homozygotes [12, 13]. $NO^{\bullet}$ acts as an antioxidant protecting against Fenton's reaction which generates highly reactive hydroxyl ($OH^{\bullet}$) radicals [14].

It has been proposed that the increased level of oxidized proteins is observed during carcinogenesis and progression of the cancer [15, 16].

Reactive $OH^{\bullet}$ radicals can cause oxidative damage to various plasma proteins, leading to the loss of enzymatic activity and/or transformation of amino acids into protein carbonyl groups (PCO) [15, 16]. Protein carbonylation includes reactive aldehydes and ketones formed via different molecular mechanisms: (i) direct oxidation of the polypeptide backbone by $OH^{\bullet}$ radicals leading to truncated peptides; (ii) side chains oxidation of lysine, arginine, proline, and threonine; (iii) reaction of histidine, cysteine, and lysine amino acid residues with reactive aldehydes and hydroperoxides (LHP); for example, PCO are produced by lipid peroxidation; and (iv) glycation (nonenzymatic glycosylation) of lysine residues forming Amadori rearrangement products (advanced glycated end products: AGE) [17]. The peroxynitrite ($ONOO^{-}$) is a short-lived reactive nitrogen species that is generated by the reaction of $NO^{\bullet}$ and superoxide ($O_2^{\bullet -}$) radicals [18]. Cellular proteins are oxidized and/or dimerized by peroxynitrite-derived radicals, including thiol groups and tyrosine residues. AOPPs are PCO and dityrosine-containing cross-linking protein products and considered a novel marker of oxidant mediated protein damage in systemic circulation [15, 19–21]. Advanced oxidation protein products (AOPPs) can be formed during increased oxidative stress by reaction of plasma proteins [19, 20]. Plasma thiols can be classified into two major groups: protein thiols and nonprotein thiols. Thiol groups can be oxidized by $ONOO^{-}$-derived radicals and initiate radical-dependent chain reactions to produce higher oxidation states of sulphur such as sulfinic and sulfonic acid derivatives [18].

The aim of the current study was to evaluate the possible relation of NOS3 G894T genotypes with the levels various protein and lipid oxidation markers and antioxidant status such as PCO, AOPP, LHP, total thiol protein thiol, nonprotein thiol, and Cu, Zn-superoxide dismutase (Cu, Zn-SOD) in patients with LC.

2. Methods

2.1. Subjects. Primary LC patients were enrolled in the current study. Mean duration of the complaints of the LC patients was 4 months. Oropharyngeal, nasal, laryngeal, neck, and systematic examination of all patients were made. All the patients were diagnosed as well-differentiated laryngeal squamous cell carcinoma by histopathology. This study consists of 3 women and 55 men with LC and 84 women and 63 men as healthy controls (HC). There were no significant differences between patients and HC in terms of age ($60.94 \pm 9.03/56.83 \pm 12.38$; $p = 0.062$). In addition, smoking status and alcohol consumption were significantly different between groups [$p = 0.0001$ for both (98.3% (LC)-6.1% (HC)/53.4% (LC)-2.7% (HC), resp.)]. All LC patients and HC had a Caucasian ethnic background.

Some of the patients and control group individuals were excluded from the study. The HC were selected from the volunteers. All LC patients and HC with previous chemotherapy, radiotherapy, and surgery history were excluded from the study. Both LC group and HC group individuals had no known chronic metabolic disease. LC patients and HC with any vitamin or antioxidant drug supplementation within 12 months before study entry were also not included in current study.

Fasting venous plasma samples were obtained from LC patients before operation and healthy case-control individuals.

2.2. Isolation of Genomic DNA. For genomic DNA extraction 300 μL of whole blood containing EDTA was used. DNA samples were isolated according to salting out technique [22] and quantified by UV spectrophotometry (Biotek US, Winooski, VT, USA). Isolated genomic DNA samples were stored at +4°C.

2.3. PCR Analysis of G894T Polymorphism. Extracted DNA was amplified with polymerase chain reaction (PCR). NOS3 Glu298Asp polymorphism was analyzed using primers (forward, 5′ AAG GCA GGA GAC AGT GGA TGG A-3′; reverse, 5′ CCC AGT CAA TCC CTT TGG TGC TCA 3′). PCR-restriction fragment-length polymorphism method was used for genotyping [23]. PCR products were digested with BanII and then visualized and analyzed with agarose-gel electrophoresis. Genotype analysis was carried out by two independent investigators who were unaware of clinical data.

PCR analysis was carried out using BIORADT-100 thermal cycler (BIORAD, Hercules, USA). Genomic DNA was incubated in a total reaction volume of 25 μL containing equal concentration of the forward primer 5′ AAG GCA GGA GAC AGT GGA TGG A-3′ and reverse primer 5′ CC AGT CAA TCC CTT TGG TGC TCA 3′ (İnvitrogen, Carlsbad, California, USA), 200 μM deoxynucleotide triphosphate, 10x PCR buffer pH 8.3 containing $MgCl_2$ 15 mM, and 1.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). All genotypes were read by two independent researchers. In case of any conflicts, the genotype was repeated.

2.4. Assay of Protein Carbonyl Groups. PCO groups were measured spectrophotometrically with Biotek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek US, Winooski, VT, USA).

We analyzed plasma PCO levels as previously described by Reznick and Packer [24] with some of the volumetric

modifications. 2,4-Dinitrophenylhydrazine (DNPH) reagent reacts with PCO groups to form chromophoric dinitrophenylhydrazones (100 μL plasma : 400 μL DNPH). DNPH reagent was prepared in hydrochloric acid. Proteins were precipitated with an equal amount of 20% (w/v) trichloroacetic acid upon the DNPH reaction completed. The resulting pellets were washed three times with 400 μL of an ethanol/ethyl acetate mixture (1 : 1). Washing procedure was performed by mechanical disruption of pellets in ethanol/ethyl acetate mixture and repelleting by centrifugation at 3000 ×g for 5 min. Finally, PCO precipitates were dissolved in a 200 μL 6 M guanidine-HCl solution and the related absorbance values were recorded at 360 nm. The molar extinction coefficient of DNPH (ε = 22,000 M^{-1} cm^{-1}) was used for the calculation of PCO concentration. The intra- and interassay CV% values for modified PCO assay were 4.1% (n = 8) and 8.1% (n = 8), respectively.

The untreated bovine serum albumin (BSA) and PCO-bovine serum albumin (BSA) positive control samples were both prepared according to the method of Lenarczyk et al. [25] and analyzed with the PCO assay procedure.

2.5. Assay of Advanced Oxidation Protein Products. Spectrophotometric determination of AOPP concentrations was determined by modification of Hanasand's method [26]. Samples were prepared in the following way: 10 μL of plasma, 40 μL of phosphate buffered saline (PBS), and 200 μL of citric acid solution (20 mmol/L) were mixed in microplate. One minute later, 10 μL of 1.16 M potassium iodide was added to the microplate well; the absorbance of the reaction mixture was read at 340 nm against reagent blank. The chloramine-T absorbance at 340 nm is linear within the range of 0 to 100 μmol/liter. AOPP values were given as micromoles per liter of chloramine-T equivalents. The coefficients of intra- and interassay variations were 1.5% (n = 8) and 2.2% (n = 8), respectively. The untreated BSA and AOPP-BSA positive control samples were both prepared in vitro and analyzed according to the AOPP assay protocol [27].

2.6. Assay of Thiol Fractions. Plasma total thiol, nonprotein thiol, and protein thiol concentrations were determined by using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Sedlak and Lindsay [28]. We realized some of the modifications for previously described total thiol method in order to apply small volumes of plasma samples. A portion (20 μL) of the plasma sample was mixed in 1.5 mL test tube with 400 μL of 0.2 M Tris buffer, pH 8.2, and 20 μL of 0.01 M DTNB for total thiol group analysis. Nonprotein thiol samples were assayed in the following way: 20 μL of plasma was mixed in 400 μL of 50% TCA. The test tubes were vortexed intermittently for 10 min and centrifuged for 15 min at 3000 ×g. Supernatant fractions were assayed as total thiol. The absorbance values of the resulting samples were read at 412 nm wavelength against reagent blank. The value of molar extinction coefficient of thiol (-SH) groups at wavelength 412 nm is approximately ε = 13.100 M^{-1} cm^{-1}. The PSH group concentrations were calculated by subtracting the nonprotein thiol from total thiol. The coefficients of intra- and interassay variations were 1.3% (n = 8) and 3.4% (n = 9), respectively.

2.7. Assay of Lipid Hydroperoxides. Plasma LHPs concentrations were analyzed spectrophotometrically with the method of FOX2 (ferrous oxidation with xylenol orange, version 2) [29]. LHPs groups oxidized ferrous ions to ferric ions in dilute acid solution, and the concentration of resultant ferric ions was determined by using ferric-sensitive dye, which was related to the concentration of LHPs. Xylenol orange binds to ferric ions with high selectivity to form a colored (blue-purple) complex. Fifth microliters aliquots of plasma sample were transferred into microcentrifuge reaction vials. FOX2 reagent (950 μL) was then added, and the samples were mixed on vortex. After incubation with FOX2 reagent at room temperature for 30 min, the final samples were centrifuged at 3.000 ×g at 20°C for 10 min. The resulting supernatant fractions were transferred into microplate wells, and absorbance values were read at 560 nm against reagent blank.

2.8. Assay of Cu, Zn-Superoxide Dismutase Activity. This assay involves the inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine oxidase (XO) used as a superoxide generator. Cu, Zn-SOD activity was determined by measuring the inhibition rate of substrate hydrolysis in the assay mixture containing 0.3 mmol/L xanthine, 0.6 mmol/L Na_2EDTA, 150 μmol/L NBT, 400 mmol/L sodium carbonate, and 1 g/L BSA. The pH value of the assay mixture needs to be adjusted to pH 10.2 [30]. Nine hundred seventy-two μL assay mixture and 13 μL XO (167 U/L) were added to 25 μL plasma. At the end of the 20 min incubation period, 250 μL, 0.8 mmol/L, $CuCl_2$ was added to the well in order to terminate reaction. The final absorbance was read at 560 nm against reagent blank. Percent inhibition rate was calculated according to the following equation: $A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}} \cdot 100$. One unit of Cu, Zn-SOD is defined as the amount of enzyme needed to exhibit a 50% dismutation of superoxide radical anion. The coefficients of intra- and interassay variations for modified Cu, Zn-SOD assay were 3.2% (n = 8) and 4.5% (n = 8), respectively.

2.9. Statistical Analyses. Descriptive statistics were expressed as mean ± SD. Statistical analyses were performed by using SPSS (Statistical Package for the Social Sciences) v16.0 software. The statistical analyses of the nonnormally distributed data of plasma oxidative stress parameters between patients and controls sharing the same genotypes were performed by using Mann-Whitney U test. Genetic frequencies were compared in patients and controls by chi-square (χ^2) test. A level of $p < 0.05$ was considered statistically significant.

3. Results

Restriction band pattern of G894T polymorphism in exon 7 of the NOS3 gene is shown in Figure 1.

Characteristics of patients with laryngeal carcinoma are given in Table 1.

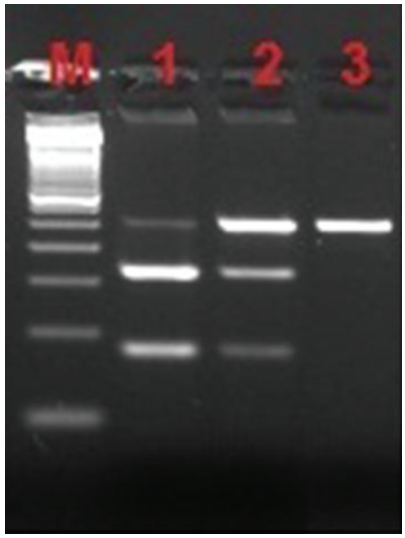

Figure 1: Restriction band pattern of G894T polymorphism in exon 7 of the NOS3 gene. Agarose gel electrophoresis of PCR products after endonuclease restriction with enzyme BannII. Lane 1, GG homozygote (163 bp and 85 bp); lane 2, GT heterozygote (248 bp, 163 bp, and 85 bp); and lane 3, TT homozygote (248 bp). M represents 50 bp ladder.

Genotypes and allele frequencies of NOS3 Glu298Asp in primary larynx cancer patients and their respective controls are shown in Table 2.

Variations in the levels of plasma oxidative stress parameters of patients and their healthy controls are given in Table 3.

The plasma levels of oxidative stress parameters were determined with manual colorimetric methods according to NOS3 genotypes in LC patients and their respective controls. The plasma levels of oxidative stress parameters were determined with manual colorimetric methods according to NOS3 genotypes in LC patients and HC (Table 4). In LC patients with TT genotype, plasma nonprotein thiol and Cu, Zn-SOD levels were significantly higher than those of the control group. On the other hand, plasma AOPP levels were not significantly different in any genotypes for LC patients and their corresponding HC. In patients with GG and GT genotype, elevated levels of LHP showed statistically high significance when compared to HC.

No significant differences were determined between genotype and clinical pathologies such as recurrence, lymph node, differentiation, reflux, and also oxidative stress biomarkers. Our results also show that the subjects with NOS3 homozygote variants may have a risk for reflux 1.74-fold compared to heterozygotes.

4. Discussion

It is well known that ROS can initiate oxidative damage in both plasma constituents and cells of systemic circulation such as proteins, lipids, and DNA [14, 15, 31]. Oxidative modifications of these macromolecules play an important role in carcinogenesis [14–16]. It has been previously concluded that systemic oxidant/antioxidant balance was impaired in favor of lipid peroxidation and oxidative DNA damage in LC patients [32].

Table 1: Characteristics of patients with laryngeal carcinoma.

Parameters	Larynx cancer patients	
	n =	%
Reflux		
Yes	26	44.8
No	32	55.2
Family history of any kind of cancer		
Yes	17	30.9
No	38	69.1
Tumor location		
Glottic	38	69.1
Supraglottic	17	30.9
Tumor grade		
T1	8	14.1
T2	10	17.5
T3	30	52.6
T4	9	15.8
Lymph node		
N0	36	62.1
N1	20	34.5
N2	2	3.4
Metastasis		
Yes	1	1.8
No	54	98.2
Differentiation		
Poor	8	14
Medium	38	66.7
Well	11	19.3
Tumor recurrence		
Yes	8	13.8
No	50	86.2

Table 2: Genotypes and allele frequencies of NOS3 Glu298Asp in primary larynx cancer patients and their controls. Hardy-Weinberg equilibrium analysis showed the genotype distribution for the NOS3 gene (G894T) in larynx cancer patients in accordance with Hardy-Weinberg equilibrium.

	Patients	Controls
	n (%)	n (%)
Genotype		
GG	18 (31)	31 (21.1)
GT	29 (50)	81 (55.1)
TT	11 (19)	35 (23.8)
	$p = 0.300$	
Alleles		
G	65 (56.03)	143 (48.64)
T	51 (43.97)	151 (51.36)
	$p = 0.177$	

Table 3: Variations in the levels of plasma oxidative stress parameters of larynx cancer patients and their controls.

	Patients	Controls	p value
PCO (nmol/mg pr)	1.19 ± 0.08	0.86 ± 0.08	0.008**
AOPP (μmol/L chloramine-T equivalent)	56.04 ± 5.61	41.04 ± 5.22	0.037*
Total thiol (nmol/mg pr)	15.54 ± 1.19	16.23 ± 1.55	0.843
Nonprotein thiol (nmol/mg pr)	3.35 ± 0.30	3.46 ± 0.19	0.703
Protein thiol (nmol/mg pr)	12.17 ± 1.19	12.86 ± 1.51	0.860
LHP (μmol/mg pr)	3.71 ± 0.42	1.20 ± 0.12	0.000*
Cu, Zn-SOD (U/mg pr)	7.27 ± 0.31	5.99 ± 0.34	0.004**

* $p < 0.05$; ** $p < 0.01$.

Table 4: Mean ± SD values of oxidative stress parameters according to NOS3 genotypes in larynx cancer patients and controls.

	GG			GT			TT		
	Patients	Controls	p value	Patients	Controls	p value	Patients	Controls	p value
PCO	1.3 ± 0.2	1.0 ± 0.3	0.517	1.2 ± 0.1	0.9 ± 0.1	0.125	1.1 ± 0.1	0.7 ± 0.1	0.088
AOPP	54.1 ± 9.4	43.4 ± 6.7	0.833	52.7 ± 6.4	34.6 ± 7.8	0.029*	66.4 ± 18.9	48.3 ± 10.1	0.562
Total thiol	16.8 ± 3.2	21.9 ± 3.0	0.315	15.5 ± 1.6	13.9 ± 2.0	0.371	14.1 ± 1.2	16.4 ± 3	0.731
Nonprotein thiol	3.4 ± 0.9	4.0 ± 0.5	0.524	2.9 ± 0.3	3.4 ± 0.3	0.363	4.4 ± 0.3	3.3 ± 0.3	0.037*
Protein thiol	13.0 ± 3.3	17.7 ± 2.6	0.315	12.8 ± 1.6	10.7 ± 2.1	0.285	9.7 ± 1.3	13.1 ± 2.9	0.628
LHP	4.2 ± 0.8	1.4 ± 0.3	0.006**	4.0 ± 0.6	1.3 ± 0.2	0.001***	2.4 ± 1.0	1.1 ± 0.2	1.22
Cu, Zn-SOD	6.9 ± 0.4	5.9 ± 0.6	0.279	7.2 ± 1.8	6.4 ± 0.6	<0.05	7.9 ± 0.8	5.5 ± 0.5	0.036*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

There are contradictory reports about the possible role of $NO^{\bullet}$ in carcinogenesis: some of the studies indicate a potential carcinogenic role of $NO^{\bullet}$ related to the promotion of tumor angiogenesis [15, 33]. However, Kong et al. suggest the possible protective role of NOS with a potential to reduce the tumor cell adhesion to endothelium [34]. Therefore, being the responsible enzyme of $NO^{\bullet}$ production, NOS3 is thought to be involved in this critical regulation of $NO^{\bullet}$ synthesis and thus in possible carcinogenic mechanisms [6]. Almost 400 NOS3 variants have been defined so far and some of them are known to be related to carcinogenic transformations [35]. A few of these polymorphisms have been reported to be significantly associated with the development of certain cancer types [36–38]. G894T polymorphisms of the NOS3 gene are very important in the angiogenesis pathway and have also been found to have functional and clinical significance in malignancies [39, 40]. The basis of our choosing G894T polymorphism among others can be explained as the product of NOS3 is constitutively expressed in endothelial cells and vascular epithelium of the cancer cells [40, 41]. All these experimental findings suggest that $NO^{\bullet}$ may play a significant role in angiogenesis and a prominent role in human carcinogenesis. The prevalence of this analyzed G894T polymorphism in general population represents heterogeneity. The source of heterogeneity may arise from many aspects, such as the ethnic region of study, the sample size, the case and the control group, clinical characteristics of different tumors, and the genotyping methodology [42]. Since ethnicity related genetic polymorphism plays an important role in cancer risk, further studies need to be focused to clarify mortality and morbidity rates for various ethnic groups.

It is well known that the extent of the intravascular oxidative stress is the main risk factor for the occurrence and progression of various types of cancers [14–16, 31]. Neoplastic transformations give rise to the generalized oxidative and nitrosative stress in plasma, and the secondary reactive products of oxidative and nitrosative damage tend to accumulate during the progression of cancer [15, 16]. Plasma nitrite and nitrate levels may not be sensitive biomarkers of systemic NO status, and they reflect not only NO levels but also other reactive nitrogen species in plasma [43, 44]. The accurate measurement of the nitrite/nitrate couple is analytically problematic due to interferences and other methodological restrictions [44]. Hence, we decided to estimate stable systemic oxidative stress parameters in LC patients with G894T polymorphism. Dissimilarities with respect to T allele are found when our results are compared to the results of Ritt et al. [45]. These investigators also found that in patients with diabetes who carry the T allele of the G894T the magnitude of oxidative stress tends to increase. Reduction in the production of $NO^{\bullet}$ may be related to increased oxidative stress and the presence of T allele.

Plasma proteins are also the direct target for ROS because of their high concentrations in systemic circulation. Plasma proteins can be oxidized by a variety of free radicals and oxidants. Oxidative modifications of plasma proteins, such as PCO and AOPP, usually result in a loss of protein function. Identifying the carbonylation of proteins is critical for the determination of intravascular redox homeostasis, and it could potentially provide important information concerning molecular mechanisms underlying the development and progression of cancer linked to oxidative damage [15, 16]. PCOs are early and reliable biomarkers of ongoing protein

oxidation [46]. AOPP can be formed during increased oxidative stress by reaction of plasma proteins such as albumin with chlorinated oxidants. Thus, AOPP has been considered a novel marker of oxidant-mediated protein damage [21]. AOPP plays an important role in advance phase of oxidative protein damage, which consists of different types of protein oxidation markers such as dityrosine, pentosidine, and PCO [26]. In our study, no statistically significant increase was seen in plasma AOPP levels in patients with TT genotype. No reports are available in current literature that investigates G894T polymorphism in exon 7 of the NOS3 gene and oxidative protein damage in laryngeal cancer. Disruption of redox regulation of plasma proteins may therefore be the result of genotype-related increase in the magnitude of oxidative stress and occurrence of carcinogenesis.

Nonprotein thiol groups such as glutathione are physiological free radical scavengers [28]. Glutathione may be a primary agent involved in redox regulation of protein thiols. Plasma Cu, Zn-SOD activity and nonprotein thiol levels were statistically increased in laryngeal cancer patients with TT genotype. The increased levels of aforementioned parameters may be related to their preventive role for the formation of the AOPP. On the other hand, plasma Cu, Zn-SOD activities were not different with G allele and their allele-matched controls. We attribute the increase in antioxidant activity of Cu, Zn-SOD and nonprotein thiol groups to a function of effective homeostatic redox regulation mechanism in patients with T allele. Phospholipids, cholesterol, cholesterol esters, and triglycerides are major lipids in the plasma. LHPs are the major primary product of lipid peroxidation and they can be measured with FOX2 method [29]. The formation of LHPs is accepted as an important initial event in the progression of lipid peroxidation. The possible pathophysiological role of increased lipid peroxidation for the aforementioned genotypes needs to be clarified in future studies.

5. Conclusions

Male gender, smoking, and alcohol consumption may induce laryngeal carcinoma. Since intervention in preclinical conditions would have the greatest public health impact, there is an important need to pay attention to the dysregulation of the redox balance of plasma proteins in high risk groups of laryngeal cancer. Plasma redox imbalance in patients with larynx cancer could be related to the occurrence of risk alleles. In order to help in early identification of the individuals harboring high risk for laryngeal cancer, there is also a need to develop new allele specific and redox-sensitive biomarkers for diagnosis. Further studies are required to provide cytological pattern of the distribution of NOS isoforms and should compare these results with other systemic oxidative stress markers.

Abbreviations

AGE: Advanced glycated end products
AOPP: Advanced oxidation protein products
Cu, Zn-SOD: Cu, Zn-superoxide dismutase
DNPH: 2,4-Dinitrophenylhydrazine
DTNB: 5,5-Dithiobis(2-nitrobenzoic acid)
FOX2: Ferrous oxidation with xylenol orange, version 2
LC: Laryngeal cancer
LHP: Lipid hydroperoxides
$NO^{\bullet}$: Nitric oxide
NOS: Nitric oxide synthase
NOS3: Endothelial-NOS
PBS: Phosphate buffer saline
PCO: Protein carbonyl group
PCR-RFLP: PCR-restriction fragment-length polymorphism
ROS: Reactive oxygen species.

Ethical Approval

The ethical protocol of the current research was approved by the Haydarpaşa Numune Education and Research Hospital, İstanbul, Turkey, Ethics Committee Issue Number: HNEAH-KAEK 2013/262.

Consent

All subjects provided written informed consent before they participated in the study.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

U. Çakatay, S. Aydın, and İ. Yaylım were the principal investigators and take primary responsibility for the paper. A. Verim, S. Turan, and G. Korkmaz recruited the patients. K. Yanar, K. Karatoprak, T. Cebe, C. Cacına, and O. Küçükhüseyin also made contributions to work at laboratory. E. Ozkök and N. E. Özkan performed the statistical analysis. U. Çakatay, K. Yanar, P. Atukeren, and İ. Yaylım wrote the paper. U. Çakatay, K. Yanar, and P. Atukeren made the revisions.

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Research Article

The Effect of Lycopene Preexposure on UV-B-Irradiated Human Keratinocytes

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Lycopene has been reported as the antioxidant most quickly depleted in skin upon UV irradiation, and thus it might play a protective role. Our goal was to investigate the effects of preexposure to lycopene on UV-B-irradiated skin cells. Cells were exposed for 24 h to 10 M lycopene, and subsequently irradiated and left to recover for another 24 h period. Thereafter, several parameters were analyzed by FCM and RT-PCR: genotoxicity/clastogenicity by assessing the cell cycle distribution; apoptosis by performing the Annexin-V assay and analyzing gene expression of apoptosis biomarkers; and oxidative stress by ROS quantification. Lycopene did not significantly affect the profile of apoptotic, necrotic and viable cells in nonirradiated cells neither showed cytostatic effects. However, irradiated cells previously treated with lycopene showed an increase in both dead and viable subpopulations compared to nonexposed irradiated cells. In irradiated cells, lycopene preexposure resulted in overexpression of *BAX* gene compared to nonexposed irradiated cells. This was accompanied by a cell cycle delay at S-phase transition and consequent decrease of cells in G0/G1 phase. Thus, lycopene seems to play a corrective role in irradiated cells depending on the level of photodamage. Thus, our findings may have implications for the management of skin cancer.

1. Introduction

Human skin is constantly exposed to the UV irradiation that may induce a number of pathobiological cellular changes. Through lipid peroxidation, protein cross-linking, and DNA damage, UV-A and UV-B radiation (UVR) can cause photoaging and photocarcinogenesis [1–3]. Skin has a variety of enzymatic and small molecular antioxidants that can inhibit oxidative damage. However, the excessive ROS production often exceeds the skin antioxidant ability [4]. In this regard, emphasis on developing novel preventive and therapeutic strategies based on phytocompounds capable of ameliorating the adverse effects of ROS has become an important area of research. Moreover, primary prevention approaches of skin cancer proved to be inadequate in lowering the incidence of this type of cancer, emphasizing the need to develop novel skin cancer chemopreventive agents. Among the vast number of photochemoprotective agents, botanical antioxidants have given promising results [4]. Two types of chemopreventive agents could be useful for the management of skin cancer. Primarily, the agents that could inhibit the damage caused by UVR may prevent the formation of initiated cells (cells with cancerous potential). Secondly, the agents that could eliminate the initiated cells may reduce the risk of skin cancer [5].

Lycopene is a powerful antioxidant both *in vitro* and *in vivo* against the oxidation of proteins, lipids, and DNA, and it has been identified as one of the most potent scavengers of

singlet species of oxygen free radicals—the highest among the carotenoids [6, 7]. At low oxygen tension, it can also scavenge peroxyl radicals, inhibiting the process of lipid peroxidation [8]. Lycopene was reported as the most quickly depleted antioxidant in skin upon exposure to solar radiation [9] and might play a role of protection against UVR. Recent research has been developed to assess if lycopene has potential for prevention of skin cancer. In fact, lycopene has been shown to inhibit proliferation of several types of cancer cells through different mechanisms in *in vitro* systems [10, 11]. Chemopreventive antioxidants are mostly studied for their role as radical scavengers, but this preventive role can be complemented by a corrective activity as selective inducers of apoptosis in transformed cells [12]. Moreover, Ribaya-Mercado et al. [9] suggested a role of lycopene in mitigating photooxidative damage in tissues.

Keratinocytes are the predominant cell type (95%) in the epidermis, the outermost layer of the skin [13]. Considering that the principal site of action of UV-B is the epidermis layer [14], keratinocytes might be more susceptible to UV-B-induced apoptosis than fibroblasts which are located in dermis layer (reached by UV-A) [15]. However, keratinocytes may be more UV-B resistant in terms of their proliferative ability as measured by colony survival assays and have greater ability for UV-DNA repair [15].

To date, most of the studies on the therapeutic potential of lycopene have been performed *in vivo* [16, 17]. These studies may be obscured by the complexity of biological system models. *In vitro* conditions may circumvent some of these contingencies and complement *in vivo* data within the 3Rs perspective (*Refine, Replace,* and *Reduce*). Despite the lower complexity of *in vitro* systems, the study of cellular photoprotection by antioxidants could be challenging because of the high chemical instability (especially to air and light) and strong lipophilicity of many antioxidant molecules such as lycopene. According to Zefferino et al. [11] *in vitro* experiments may occasionally produce inconsistent results due to lycopene's poor solubility in cell culture media [18]. In fact, lycopene is very hydrophobic ($\log P \approx 15$) and is usually solubilized in organic solvents such as tetrahydrofuran (THF). However, an uncontrolled precipitation process may occur upon addition to aqueous media, besides the high toxicity associated with these solvents. The solubility and uptake of these large crystals in the cells are quite limited and there is almost no protection against chemical degradation [19]. Alternative ways of delivering lipid-soluble compounds include micelles, microemulsions, nanoparticles, water-dispersible beadlets, artificial liposomes, enriched bovine serum, or other formulations, each of which has an influence on the cellular uptake and compounds stability [18, 20–23]. According to Palozza et al., niosomes provide a suitable, safe, and low-cost vehicle for β-carotene in cell culture [24]. Lipid-based delivery systems also show UV-blocking effects dependent on lipid composition and the particle size (the smaller the particle size, the higher the sunscreen activity). Lipid matrices can act as sunscreen carriers and increase the sun protection factor obtained after topical application of UV absorbers ($BaSO_4$, $SrCO_3$, and TiO_2) incorporated within these carriers because they provide a fixation medium for these pigments [25, 26]. However, the UV-blocking effect of the vehicle is not desired in this case, besides the difficulties of using these hydrophobic systems for cell culture studies.

The main limitations of different vehicles used for lycopene cell delivery are summarized on Table 1. Each vehicle provides specific advantages but also offers some limitations such as cytotoxicity, poor solubility, and crystallization in the cell medium [27].

In addition, the half-life of free lycopene in solution at 37°C is less than few hours. Thus, until an efficient method of solubilizing lycopene in aqueous buffers and cell culture media is developed, *in vitro* studies on the effects of lycopene on living cells will continue to show considerable variation between laboratories and cell lines and should be interpreted with caution [39].

Pfitzner et al. [18] have demonstrated that methyl-β-CD (M-β-CD) was an improved vehicle for the investigation of carotenoids and other lipophilic compounds in *in vitro* test systems, compared to organic solvents. Carotenoids-M-β-CD complex was superior concerning biological availability, missing cytotoxicity and presenting excellent stability when compared to other application forms such as organic solvents, mixed micelles, liposomes, or beadlets. At least, the solubilization with M-β-CD was easily and reproducibly achievable under routine laboratory conditions.

According to these literature references [18, 27] and preformulations studies, we decided to use another similar CD derivative, dimethyl-β-CD (DM-β-CD), to solubilize and stabilize lycopene for cell exposure experiments. Depending on the formulation and exposure conditions, lycopene has been shown to prevent cellular damage or otherwise to sensitize damaged cells leading to increased cell death. We aimed to study the effects of lycopene as sensitizer and inducer of cell death in UV-B damaged keratinocytes. Our hypothesis was that cells preexposed to lycopene would be more sensitized to death, in case of subsequent irreversible damage by UV-B. For this, the nontumorigenic keratinocyte cell line HaCaT [40] was used. HaCaT cells were preexposed to lycopene for sensitization and subsequently exposed to damaging UV-B irradiation. The effect of lycopene preexposure was analyzed by assays focused on cytotoxicity, genotoxicity, oxidative stress, and apoptosis.

2. Materials and Methods

2.1. Preparation of Lycopene Complex Solution. In order to avoid the use of organic solvents, lycopene (Extrasynthese, Genay, France, with a purity ≥ 98%, UV assay) was solubilized by complexation with dimethyl-beta-cyclodextrin (CD, degree of substitution: 1.8) which was a generous gift from Wacker (Stuttgart, Germany). Aqueous solutions of lycopene complexed with CD (1 : 4 molar ratio) were prepared under aseptic conditions within concentrations of 0, 5, 10, 15, and 20 μM from a concentrated lycopene solution previously stirred with CD during approximately 48 h and sonicated 30 min (before use), always protected from light and air. Lycopene solutions were always freshly prepared under light and air protection and stored at −20°C (except the pure

Table 1: Limitations of different vehicles used for lycopene cell delivery (adapted from Lin et al. [27]).

Vehicle	Limitations	References
Tetrahydrofuran (THF)	Rapid oxidation in media, leading to lycopene instability and cytotoxicity	[17, 20, 22, 28–32]
Dimethyl sulfoxide (DMSO)	Reduced solvent capacity for carotenoids (0.01 mg/mL for lycopene)	[20, 33–35]
Tween	Possible oxidation of carotenoids, after solvent drying and filtration	[31]
Micelles	Low carotenoid stabilization and increased cytotoxicity	[20, 27, 36]
Water-dispersible beadlets	Low toxicity, but also low cellular uptake, depends on chemicals that interfere in assays (e.g., hexane and chloroform)	[29, 37, 38]

lycopene standard, stored at −70°C). The osmolarity of the sample containing the highest lycopene concentration was determined using an automatic osmometer (Knauer, Berlin, Germany).

When nonspecified, all higher grade reagents were from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared using ultrapure water obtained in a MILLI-Q System from Millipore (Billerica, MA, USA).

2.2. Human Immortalized Keratinocytes (HaCaT) Cell Line. The HaCaT cell line was obtained from Cell Lines Services (CLS) (Eppelheim, Germany). Handling and culture of these cells were adapted to meet CLS protocol procedures. Cells were aseptically grown in Dulbecco's modified Eagle's medium (DMEM, no HEPES, no Pyruvate), high glucose, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin-streptomycin (10,000 U/mL), and 1% fungizone (250 U/mL) (Gibco, Life Technologies, Grand Island, NY, USA) at 37°C in a humidified atmosphere with 5% CO_2.

2.3. HaCaT Cell Growth and Confluence under Normal Culture Conditions. The standard cell growth conditions were established after the analysis of HaCaT growth curves. HaCaT cells were seeded in a 6-well cluster plate (300,000 cells/well). Cell confluence and morphology were daily observed under microscope. After 3 days, cells were harvested by trypsinization according to CLS procedure. Briefly, the culture was rinsed twice with phosphate-buffered saline solution (PBS) without Ca^{2+} or Mg^{2+} (Gibco, Life Technologies, Grand Island, NY, USA) and then with PBS containing 0.05% EDTA to remove desmosomes and incubated at 37°C about 5–10 min. After this, EDTA solution was replaced by a 1 : 1 mixture of EDTA/trypsin-solution (final concentrations of 0.025% and 0.05%, resp.). The trypsinization was achieved after incubating at 37°C for 15–20 min. At this time, a double volume of complete culture medium (with FBS) was added to stop the detaching process and cells were suspended with stronger shaking, followed by the use of a syringe (21 G) because of its mechanical resistance. All cell manipulation and growth and subsequent exposure conditions were performed under strict aseptic procedures.

Cell density was calculated by counting with a hemocytometer (Neubauer Improved) under a phase-contrast microscope Nikon Eclipse 80i (Coyoacán, Mexico).

2.4. Selection of Exposure Conditions

2.4.1. Selection of UV-Irradiation Dose. Ten UV-B lamps (Sankyo Denki G8T5E, Kanagawa, Japan) with a peak emission at 312 nm were used as the UV-B source. The UV-B irradiation was measured with a VLX 312 radiometer equipped with a UV-B sensor (Vilber Lourmat, Marne-la-Vallée Cedex, France). Around 7,000 cells/well were cultured in a 96-well cluster plate with complete medium. Twenty-four hours after, the cells were exposed to five different UV-B irradiation doses [based on related literature [41–45] of ~75, 150, 200, 225, and 325 mJ/cm^2 (moderate-high to very-high dose)]. In order to prevent UV quenching, prior to irradiation, the cell culture medium was replaced by the same volume of PBS after two washing steps with PBS. After UV-B irradiation, cells were fed with fresh growth medium and incubated for 24 h. Cell metabolic activity was assessed as described below by the MTT assay (often used as viability method) in order to choose just one UV-B dose. Nonirradiated samples were used as negative control.

2.4.2. Selection of Complexed Lycopene Dose. From previous cell viability results of HaCaT cells exposed to UV-B irradiation, new exposure conditions were tested with different Lyc-CD concentrations and a fixed UV-B irradiation dose. Briefly, cells were seeded and grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% fungizone, for 24 h, and then DMEM medium was replaced with α-MEM without nucleosides (Gibco, Life Technologies) with identical supplementation, containing complexed lycopene solutions (0, 5, 10, 15, and 20 μM). Cells were exposed for 24 h, and after UV-B irradiation (225 mJ/cm^2) HaCaT irradiated cells were incubated under standard culture conditions for a final 24 h period. The metabolic activity of cell culture was analyzed by MTT assay in order to choose just one lycopene dose for the subsequent analyses (Figure 1).

Briefly, for further experiments, HaCaT cells were seeded in a 6-well cluster plate (150,000–300,000 cells/2 mL well) and incubated 24 h under the same culture conditions, as mentioned above. After this period, the cells were exposed to complexed lycopene (10 μM) for 24 h in order to achieve its cellular internalization and a higher cell confluence, and after UV-B irradiation (225 mJ/cm^2) cells were allowed to grow under standard culture conditions for another 24 h to enable the occurrence of cellular repair mechanisms.

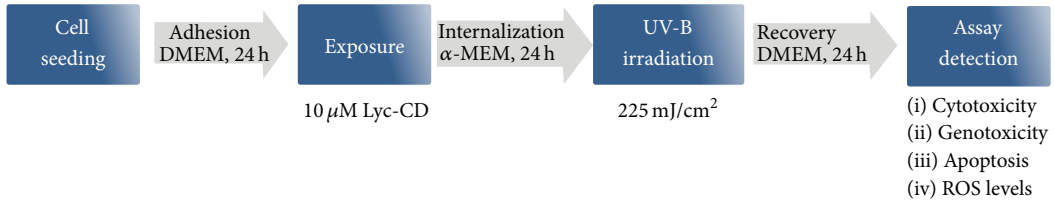

Figure 1: Culture conditions and experimental setting for the study of HaCaT cells exposed to complexed lycopene (Lyc-CD) and UV-B irradiation.

2.5. MTT Assay. Cell metabolic activity was assessed by the MTT (Sigma-Aldrich, St. Louis, MO, USA) assay, which is often used to roughly estimate culture's viability/proliferation characteristics. Cells were seeded at a density of 7,000 cells/well in a 96-well cluster plate. After complexed lycopene exposure, UV-B irradiation, and 24/h recovery the MTT assay was performed as previously described [46]. The optical density of reduced MTT was measured at 570 nm by spectroscopy on an automatic microtiter plate reader (Synergy HT Multi-Mode from BioTeK Instruments Inc., Winooski, VT, USA) and cell metabolic activity (MA ≈ viability or proliferation characteristics) was calculated according to

$$\text{Cell MA (\%)} = \frac{\left(\text{Abs } 570\,\text{nm}_{\text{sample}} - \text{Abs } 570\,\text{nm}_{\text{DMSO}}\right)}{\left(\text{Abs } 570\,\text{nm}_{\text{negative control}} - \text{Abs } 570\,\text{nm}_{\text{DMSO}}\right)} \times 100. \quad (1)$$

Besides the negative control (nonexposed and nonirradiated cells), the assays results were also compared to CD aqueous solutions (vehicle control) with the same dilution factors used for complexed lycopene samples.

2.6. Cell Cycle Analysis by Flow Cytometry. After complexed lycopene exposure and UV-B irradiation as described before, the cells were harvested with Accutase and centrifuged at 1157 g for 5 min at 4°C. The supernatant was removed and the cells were washed in PBS and suspended in 1 mL of 85% ethanol at 4°C and kept at −20°C until analysis. After that, the cells were centrifuged twice at 1157 g for 5 min at 4°C and suspended in 800 μL PBS and vortexed. Cell cycle analysis was done as previously described [46]. Briefly, samples were filtered on a nylon mesh (50 μm pore size) to the analysis tubes. Propidium iodide (PI), a DNA intercalating fluorochrome, and RNase were added to the samples (50 μL each one) and vortexed. The mixture was incubated 20 min at room temperature and then analyzed by flow cytometry (FCM) using a Coulter EPICS XL flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm for excitation. Data was acquired using the SYSTEM II (v. 2.5) software. Integral fluorescence together with fluorescence pulse height and width emitted from nuclei was collected through a 645 dichroic long-pass filter and a 620 band-pass filter and converted on 1024 ADC channels. Prior to analysis the amplification was adjusted so that the peak corresponding to G0/G1 was positioned at channel 200. This setting was kept constant. The results were obtained in the form of three graphics: linear fluorescence light intensity (FL), forward angle light scatter (FS) versus side angle light scatter (SS), and FL pulse integral versus FL pulse height. This last cytogram was used to eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets (these events have a higher pulse area but the same pulse height as single nuclei). Cell cycle analysis was performed using the FlowJo software (Tree Star Inc., Ashland, Oregon, USA) applying the Dean-Jett Model. Results were expressed as % of nuclei in G0/G1, S, and G2 phases of the cell cycle. The % of nuclei in Sub-G0/G1 indicative of DNA fragmentation was also computed.

2.7. Analysis of Apoptosis by Flow Cytometry. Early and late apoptosis were investigated by FCM using Annexin V-FITC Apoptosis Detection Kit from BD Pharmingen (Franklin Lakes, NJ, USA), as previously described [47]. Briefly, after complexed lycopene exposure and UV-B irradiation as described before, the cells were gently harvested with Accutase (PAA Laboratories, Pasching, Austria) and washed twice in cold PBS (1 mL). Finally, Annexin V-FITC and PI, 5 μL of each, were added to 100 μL of cell suspension (10^5 cells/mL) in binding buffer. Samples were left in the dark for 15 min and 400 μL of binding buffer was added. Samples were analyzed by FCM using a Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, Florida, USA). Data were acquired using the SYSTEM II (v. 2.5) software. The cytogram of FITC fluorescence in log scale versus PI fluorescence in log scale allows the identification of nonapoptotic cells (Annexin V-FITC negative, PI negative), exclusively early apoptotic cells (Annexin V-FITC positive, PI negative), predominantly late apoptotic cells (Annexin V-FITC positive, PI positive), and predominantly necrotic or dead cells (Annexin V-FITC negative, PI positive). FCM data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR).

2.8. RNA Extraction and qPCR Analysis of Apoptosis Regulating Genes

2.8.1. Total RNA Extraction. At least 1×10^5 HaCaT cells were rinsed twice with sterile PBS after removal of culture medium. After rinsing, cells were lysed in 1 mL TRIzol reagent (Life Technologies, Saint Louis, MO, USA) and after 5 min room temperature incubation, 200 μL chloroform was added to each sample, shaken on vortex for 10 s, and incubated at room temperature for 2 min. Phase separation was achieved by centrifugation at 12,000 g for 5 min at 4°C

in Phase-Lock Gel Heavy tubes (5 Prime 3 Prime, Inc., Boulder, CO, USA). The aqueous phase was mixed with 1 volume 70% ethanol and RNA was further purified using RNeasy Mini Kit columns (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The total RNA was quantified by spectrophotometry at 260–280 and 230–260 nm (Nanodrop Spectrophotometer ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA).

2.8.2. cDNA Synthesis. 1 μg total RNA was reverse-transcribed using the Omniscript RT Kit (Qiagen, Hilden, Germany) in a reaction mixture containing 1 μM Oligo(dT)18 primer, 5 mM dNTPs, reaction buffer, and RT enzyme according to the manufacturer's instructions. After the enzymatic reaction (incubation at 37°C for 1 h), cDNA samples were prediluted in milliQ water (1 : 20).

2.8.3. Quantitative RT-PCR (qPCR). For qPCR, primers were used complementary to the genes coding for Bax, Bcl-2, caspase 3, and TRAIL proteins, respectively, BAX, BCL2, CASP3, and TNFSF10. SDHA (succinate dehydrogenase) was used as the reference gene. The target genes and corresponding oligonucleotide primer sequences (5′ to 3′) were as follows: BAX-F: GACGGCCTCCTCTCCTACTT; BAX-R: CAGCCCATCTTCTTCCAGAT; BCL2-F: GGAGGATTGTGGCCTTCTTT; BCL2-R: GCCGGTTCAGGTACTCAGTC; CASP3-F: GAACTGGACTGTGGCATTGA; CASP3-R: TGTCGGCATACTGTTTCAGC; TNFSF10-F (TRAIL-F): CCTGCAGTCTCTCTGTGTGG; TNFSF10-R (TRAIL-R): ACGGAGTTGCCACTTGACTT; SDHA-F: CTGCAGAACCTGATGCTGTGT; SDHA-R: GGATGGGCTTGGAGTAATCG.

Primer design was performed using Primer3 [48] and primer specificity was confirmed using the In-Silico PCR UCSC Genome Browser [49]. The final individual qPCR reactions contained iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA), 150 nM of each primer, and 1 : 4 (v/v) prediluted cDNA (1 : 20). Two qPCR technical replicates were performed per sample in the iQ5 Bio-Rad thermal cycler. The qPCR program included 1 min denaturation at 95°C, followed by 60 cycles at 94°C for 5 s, 58°C for 15 s, and 72°C for 15 s. After qPCR, a melting temperature program was performed. Mean PCR efficiencies and cycle thresholds were determined from the fluorescence data using the algorithm Real-Time PCR Miner [50]. Relative gene expression of cell samples relative to SDHA was calculated using the Pfaffl method [51].

2.9. Reactive Oxygen Species (ROS) Quantification by Flow Cytometry. ROS ($O_2^{\bullet-}$ and $^{\bullet}OH$) generation was assayed by FCM using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA), as described previously [47], which upon acetate cleavage is oxidized to fluorescent dichlorofluorescein (DCF) by hydrogen peroxide. After complexed lycopene exposure and UV-B irradiation as described before, the medium was replaced by serum-free α-MEM containing 10 μM H_2DCF-DA for 30 min, at 37°C in dark. Cells were washed with PBS, trypsinized with Accutase collected, and analyzed in a Coulter EPICS XL flow cytometer and ROS formation was estimated by the median fluorescence intensity (MFI) parameter using the FlowJo software.

2.10. Statistical Analysis. The results are reported as mean ± SD of at least three replicates/treatment. In addition, at least three independent assays were performed for each analysis. For gene expression analysis, results are reported as mean ± SE of at least three replicates from 2 independent assays. The results of all these experiments were statistically analyzed by Analysis of Variance (ANOVA with All Pairwise/Nonpairwise Multiple Comparison Procedures) using SigmaPlot 11.0 software. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. HaCaT Cell Growth and Confluence under Normal Culture Conditions. HaCaT cell growth and confluence under normal culture conditions until 120 h are represented on Figure 2(a). As it can be observed, the exponential phase extends until approximately 72 h and the full confluence can be maintained more than 1 week. The selected confluence for complexed lycopene exposure and UV irradiation in this experiment was attained at 24 h and 48 h, respectively.

Under phase-contrast microscopy, the cells displayed typical intermediate phenotype of polygonal cells interspersed with giant often multinucleated cell and single morphology (Figure 2(b)).

3.2. Effect of UV-B Doses on Cell Metabolic Activity by MTT Assay. As theoretically expected, increasing UV-B dose resulted in decreased cell metabolic activity (Figure 3). UV-B irradiation resulted in distinct morphological changes in HaCaT cells, as irradiated cells became round and detached from the surface of the plate.

According to MTT results, the UV-B condition tested that was near but above 50% viability was 225 mJ/cm^2. This UV-B dose was chosen as high UV-exposure condition to analyze the maximum range of effects and response to UV exposure.

Complexed lycopene (Lyc-CD) up to 15 μM did not affect the metabolic activity of nonirradiated cells, and only 20 μM Lyc-CD led to significant decrease in metabolic activity in these cells (Figure 4). At doses equal to or higher than 15 μM, complexed lycopene decreases the MA of irradiated cells (225 mJ/cm^2), compared to cells not preexposed to lycopene (Figure 4).

According to preliminary MTT assays, it was also observed that concentrations of Lyc-CD higher than 20 μM induced a higher decrease in MA in irradiated cells (supplementary data in Supplementary Material available online at http://dx.doi.org/10.1155/2016/8214631).

According to these results, we decided to choose an intermediate complexed lycopene concentration (10 μM) whose effects have been previously established in other cell lines [52–54]. At this lycopene concentration, the cell metabolic activity was higher than 50% and was not significantly different from

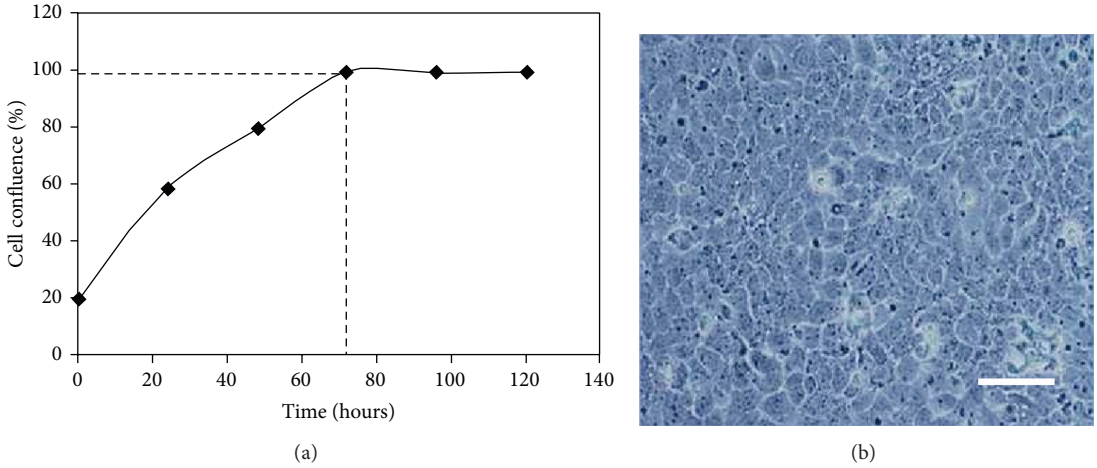

Figure 2: (a) HaCaT cells growth and confluence curves under normal culture conditions for 120 h. (b) HaCaT cells observed by phase-contrast microscope (100x magnification), scale bar: 20 μm.

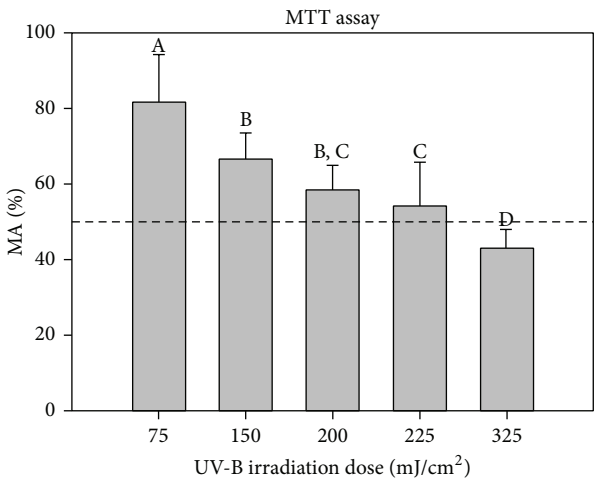

Figure 3: Effect of different UV-B doses on cell metabolic activity (MA) determined by MTT assay. Results are expressed as percentage (mean ± SD) of cell MA compared to nonirradiated cells. Statistical analysis: One-Way ANOVA with All Pairwise Multiple Comparisons by Holm-Sidak method: statistical differences between the samples are represented by different letters when $P < 0.05$.

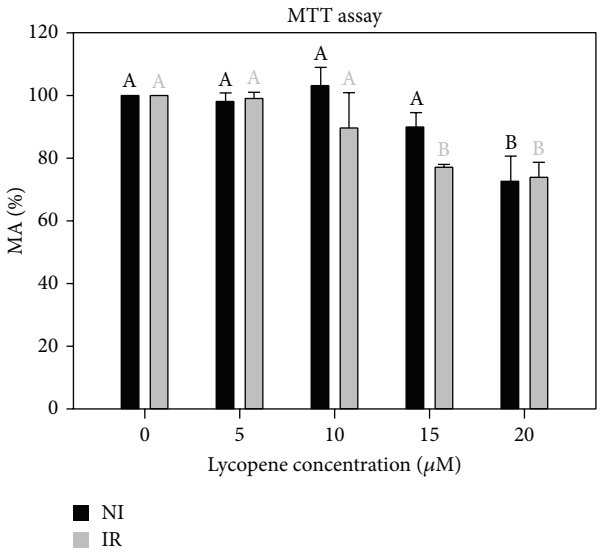

Figure 4: Effect of complexed lycopene preexposure on UV-B-irradiated (IR, 225 mJ/cm^2) and nonirradiated (NI) HaCaT cells on cell MA measured by MTT assay. Results are expressed as percentage (mean ± SD of 3 independent experiments with 6 replicates each one). Statistical analysis: One-Way ANOVA with Multiple Comparisons versus Control Group (Holm-Sidak method): statistical differences between the samples within nonirradiated and irradiated groups (in respect to cells not exposed to lycopene) are represented by different letters when $P < 0.05$.

the nonexposed cells. Higher lycopene concentrations could decrease the cell viability, for example, due to a prooxidant effect, as suggested by a decrease in viability compared to nonexposed, nonirradiated cells.

3.3. Cell Cycle Analysis. Figure 5 shows representative histograms of cell cycle of HaCaT cells after 10 μM complexed lycopene exposure and UV-B irradiation (225 mJ/cm^2). Cell cycle analysis shows that complexed lycopene exposure alone did not significantly ($P > 0.05$) affect the dynamic of cell cycle in comparison to control cells. Compared to nonirradiated and nonexposed cells, irradiation induced a decrease in the percentage of cells in the G0/G1 phase of cell cycle especially in complexed lycopene and CD exposed cells ($P = 0.011$ and 0.008, resp.) (Figure 5). Although the S and G2 phases were not significantly affected by any of the treatments, an increase in S-phase frequency can be observed.

As previously observed in Figure 5, the analysis of PI-stained nuclei by FCM cell cycle analysis showed an increase in sub-G1 subpopulations upon UVB irradiation. Exposure to CD vehicle induced a small decrease in % sub-G1 irradiated cells, compared to nonexposed, irradiated cells. Contrarily to cells exposed to CD, cell exposed to Lyc-CD did not present

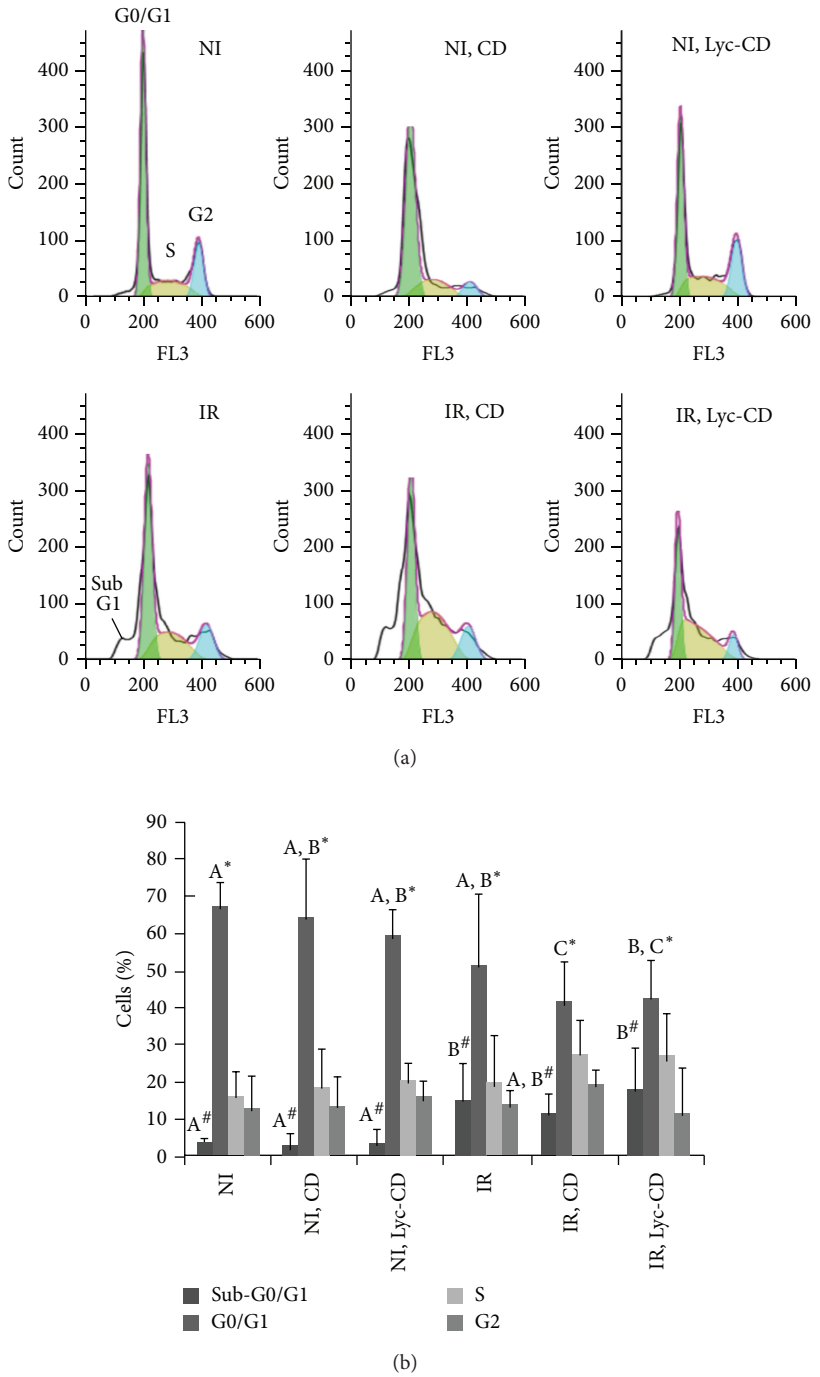

Figure 5: Effect of lycopene and UV-B on the cell cycle: (a) cell cycle histograms of UV-B (225 mJ/cm^2) irradiated (IR) and nonirradiated (NI) HaCaT cells exposed to 10 μM complexed lycopene (Lyc-CD) and to the respective controls; (b) cell cycle phase distribution of UV-B (225 mJ/cm^2) irradiated (IR) and nonirradiated (NI) HaCaT cells exposed to 10 μM complexed lycopene (Lyc-CD) and the respective controls, including cyclodextrin vehicle (NI, CD and IR, CD). Results are expressed as percentage (mean ± SD). Statistical analysis: One-Way ANOVA with All Pairwise Multiple Comparison Procedures: means with different letters (A, B, and C) are significantly different ($P < 0.05$). In this case, only the statistical differences between groups presenting differences (G0/G1 and sub-G1) were marked for simplification purposes. Comparison between % cells with sub-G1 amount of DNA (#) and % cells in G0/G1 phase (∗) is presented.

small decrease in % sub-G1, compared to nonexposed, irradiated cells.

3.4. Apoptotic Markers. Annexin V assay differentiates subpopulations that are necrotic, apoptotic, or viable. Comparative analysis of these subpopulations in irradiated (225 mJ/cm^2) and nonirradiated HaCaT cells, treated with 10 μM complexed lycopene, was performed using the Annexin V assay (Figure 6).

Within this study, a high UVB dose was used to induce cell alterations and apoptosis, but not excessive necrosis. For this study only the adherent cells were analyzed, which means that the necrotic cells in suspension were not considered in the assay results. Nevertheless, as shown in Figure 6, the main shift upon UVB irradiation was from viable to apoptotic cells, confirming that the high UVB dose used was not excessively detrimental for the HaCaT cells attached to plate and used in all the assays described here. In nonirradiated cells, complexed lycopene did not affect ($P > 0.05$) the percentage of necrotic, apoptotic, and viable cells compared to the control (nonexposed cells). Contrarily, UV-B irradiation alone increased the percentage of both early and late apoptotic cells ($P < 0.05$) and decreased the percentage of viable cells compared to the control. Contrarily to what was observed for nonirradiated cells, the Annexin V assay showed a decrease in the % cells with Annexin V positive staining (early and late apoptosis) preexposed to complexed lycopene in irradiated cells ($P < 0.05$), while the % necrotic cells increased.

Also complementary data showed that irradiated cells previously exposed to the vehicle (CD) presented, once again, results quite similar to those of nonirradiated cells (supplementary data).

3.5. Effect of Complexed Lycopene on the Expression of Anti- and Proapoptotic Genes. Under UVB irradiation, lycopene complex exerted proapoptotic effects compared to irradiated but not exposed cells (Figure 7). Compared to irradiated and nonexposed cells, in irradiated cells the exposure to vehicle CD inhibited antiapoptotic *BCL2* expression but did not increase proapoptotic *BAX* expression. Contrarily to this, in irradiated cells Lyc-CD increased proapoptotic *BAX* expression but did not inhibit antiapoptotic BCL2 expression. This observation points to a proapoptotic effect of Lyc-CD mediated by *BAX* upregulation. Moreover, in irradiated cells Lyc-CD showed lower caspase 3 gene (*CASP3*) gene expression compared to nonexposed, irradiated cells; however, it increased *CASP3* gene expression comparatively to CD vehicle. TRAIL is a proapoptotic cytokine secreted by many cell types; however, in this study, UVB light was found to decrease TRAIL expression.

3.6. Reactive Oxygen Species (ROS) Analysis. Analysis of oxidative stress induction in HaCaT cells after 10 μM complexed lycopene exposure and UV-B irradiation (225 mJ/cm^2) was performed by determining reactive oxygen species (ROS) formation by FCM.

The results of irradiated and nonirradiated cells are represented in Figure 8. UVB irradiation induced an increase in ROS intracellular production. In fact, regarding ROS production, two cell populations were observed in irradiated cells against one in nonirradiated cells. The Median Fluorescence Intensity (MFI) from irradiated samples was statistically higher than MFI from correspondent nonirradiated cells, as theoretically expected. Complexed lycopene did not increase ROS production in nonirradiated cells. However, in irradiated cells Lyc-CD induced a significant ROS increase compared to irradiated nonexposed cells.

4. Discussion

In this work, we used *HaCaT cells*, a "spontaneously transformed human epithelial cell line from adult skin" which maintains full epidermal differentiation capacity. This cell line is immortal (>140 passages) but remains nontumorigenic, and it is aneuploid (hypotetraploid) with unique stable marker chromosomes indicating monoclonal origin [40]. As performed in this experimental work, further investigation needs to include studies dealing with normal cells, their transformation into malignant cells, and the association between malignant cells and the surrounding normal cells in order to determine the cytotoxicity in both cell populations.

It is noteworthy that cell culture studies are usually carried out under abnormal conditions known as "*culture shock*," where cells are exposed to high oxygen tension and to free metal ions in the medium [27, 55]. Thus, it must always be taken into account if the study compound reacts with the cell medium. Different half-life values of lycopene under standard cell culture are reported in the literature, for example, 12–20 h [37]. Experiments were conducted with exposure time of 24 h which was sufficient to reveal the cellular effects of Lyc-CD. Lycopene chemical stability was conferred by CD complexation.

The antioxidant action of carotenoids is related to their ability to trap free radicals and quench singlet oxygen. However, depending on the redox potential of lycopene and the surrounding environment, its antioxidant activity may shift to prooxidant activity [56, 57]. In fact, the HaCaT cells medium (DMEM) contains ferric nitrate ($Fe(NO_3)_3{\cdot}9\,H_2O$) and Fe(III) can react with excess neutral carotenoids, such as lycopene. Ferric ions have been proposed to degrade carotenoids by the following mechanism [58, 59]: Fe^{3+} + carotenoid $\Rightarrow$ Fe^{2+} + carotenoid$^{\bullet+}$. Although there are many Fe chelators that inhibit the reactions of Fe, oxygen, and their metabolites [60], these chelating agents may also mediate toxicity by stimulating Fe-mediated oxygen radical generation [61]. The chelating agent used for HaCaT trypsinization was ethylenediaminetetraacetic acid (EDTA) which can induce Fenton-chemistry mediated radical damage. In fact, the autoxidation of Fe(II) enhanced by EDTA was observed by others [57, 58]. Therefore, in order to prevent lycopene oxidation by Fe (III), another cell culture medium (α-MEM) was used without this element during cells exposure to lycopene. A normal cellular growth was observed. Furthermore, it should be also noted that the solution with the highest lycopene dose had a suitable osmolarity lower than 320 mOsm/kg (~268 mOsm/kg).

During storage, *special precautions* were taken such as the protection of lycopene from temperature, light, and

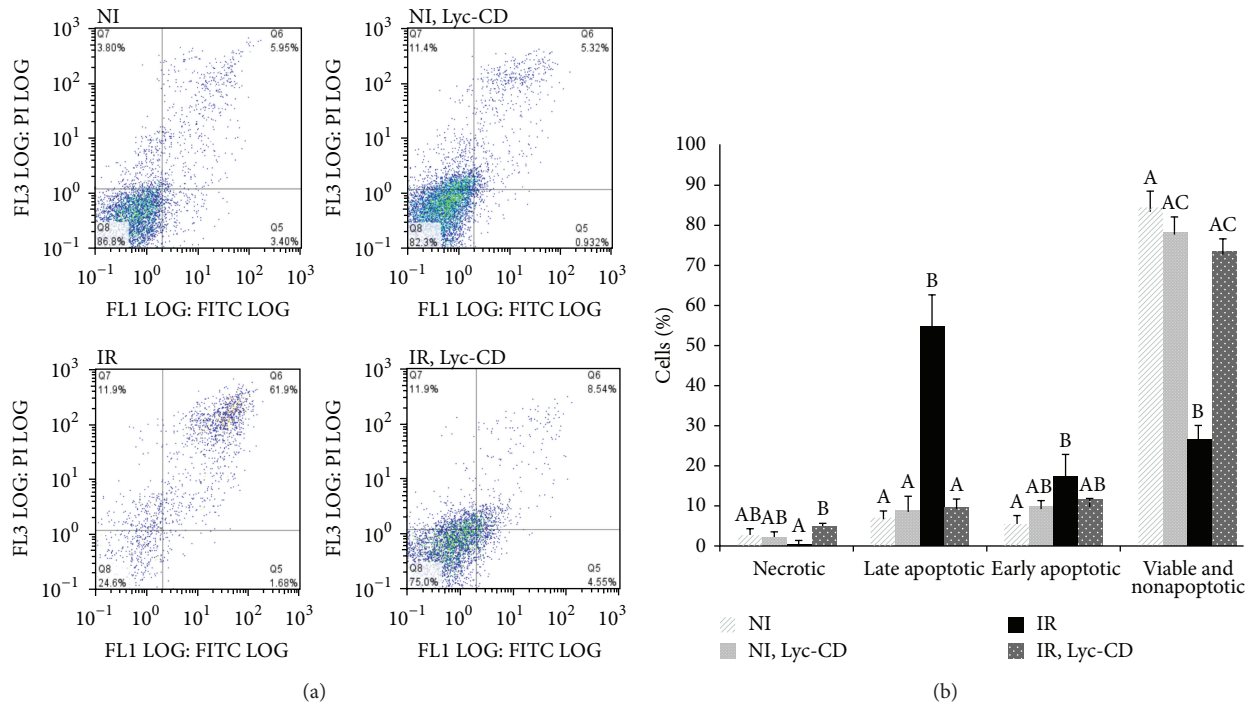

Figure 6: (a) Representative examples of Annexin V-FITC Dot-Plots Gating (FL1 LOG versus FITC LOG) of nonirradiated (NI) and UV-B (225 mJ/cm^2) irradiated (IR) HaCaT cells not previously exposed or exposed to 10 μM complexed lycopene (Lyc-CD); (b) results are expressed as percentage (mean ± SD, $n = 3$) of *nonapoptotic and viable cells*, Q8: Annexin-FTIC (−) and PI (−); *early apoptotic cells*, Q5: Annexin-FTIC (+) and PI (−); predominantly *late apoptotic or dead cells*, Q6: Annexin- FTIC (+) and PI (+); and predominantly *necrotic* and *dead cells*, Q7: Annexin-FTIC (−) and PI (+). Statistical analysis: One-Way ANOVA with All Pairwise Multiple Comparison Procedures (Holm-Sidak method): means with different letters are significantly different ($P < 0.05$).

air. However, during lycopene exposure, cell culture was maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. Even considering other experimental conditions reported in the literature [62] that comprise the addition of lycopene solutions to the cell culture medium under N_2 environment, we preferred to maintain our protocol to avoid perturbing the cell culture with other variables.

The level of lycopene normally observed in human plasma is on the order of 0.5 μM even with dietary supplementation [20, 52, 53]. Therefore, for therapeutic purposes (assuming topical application) we used a range above these normal plasma levels (5 up to 20 μM). According to *MTT results*, 20 μM is revealed to be toxic in *in vitro* studies (Figure 4). Thus, 10 μM lycopene nominal concentration was selected for further studies, supporting the selected dose used in other *in vitro* studies [52–54].

The option to use cyclodextrins for lycopene solubilization and photoprotection was based on data resulting from previous studies (Table 1) on parameters/conditions affecting the delivery of lycopene to cells, including the solubilization, stabilization, and cellular uptake by using other vehicles. In addition, similar MTT results were obtained with another vehicle (supplementary data).

HaCaT keratinocytes have a mean intermitotic time of 22–24 h *in vitro* [13] and their *cell cycle* is subjected to regulation by cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors.

UV-B irradiation activates diverse cellular responses in human cells, such as cell cycle arrest, DNA repair, and apoptosis through signal transduction pathways [15]. Previous studies have shown that UVR can cause cell cycle arrest both at G1 [63] and G2 phases [64] of cell cycle. However, in our study, irradiation did not affect significantly the percentage of cells on S and G2 phases (Figure 5). On the contrary, the percentage of cells in G1 phase decreased in irradiated cells, especially those previously exposed to complexed lycopene or the respective vehicle alone (cyclodextrins). Cyclin D regulates the transition from G0 to early G1 phase, while cyclin E regulates the transition of the cell from late G1 phase to S phase; p21 and p27 CDK inhibitors bind and inhibit the activity of cyclin E/CDK2 complex, blocking cell cycle progression in G1 phase. In fact, after UV irradiation, the half-life of the tumor suppressor (p53) appears to be extended which will induce the p21 CDK inhibitor leading to G1 phase arrest or cell death by apoptosis. G2-phase checkpoint control does not appear to be affected [65]. It has been reported that the growth inhibition of lycopene on MCF-7 breast cancer cells was also associated with decreased G1-S cell cycle progression, decreased cyclin D1 expression, and stabilization of p27 in the cyclin E-CDK complex [7, 66]. Cell cycle arrest increases the time available for DNA repair before its replication and mutation fixation processes [5]. Regarding the cell cycle results obtained with CD, it has been reported that methyl β-CD inhibits cell growth and induces cell cycle arrest

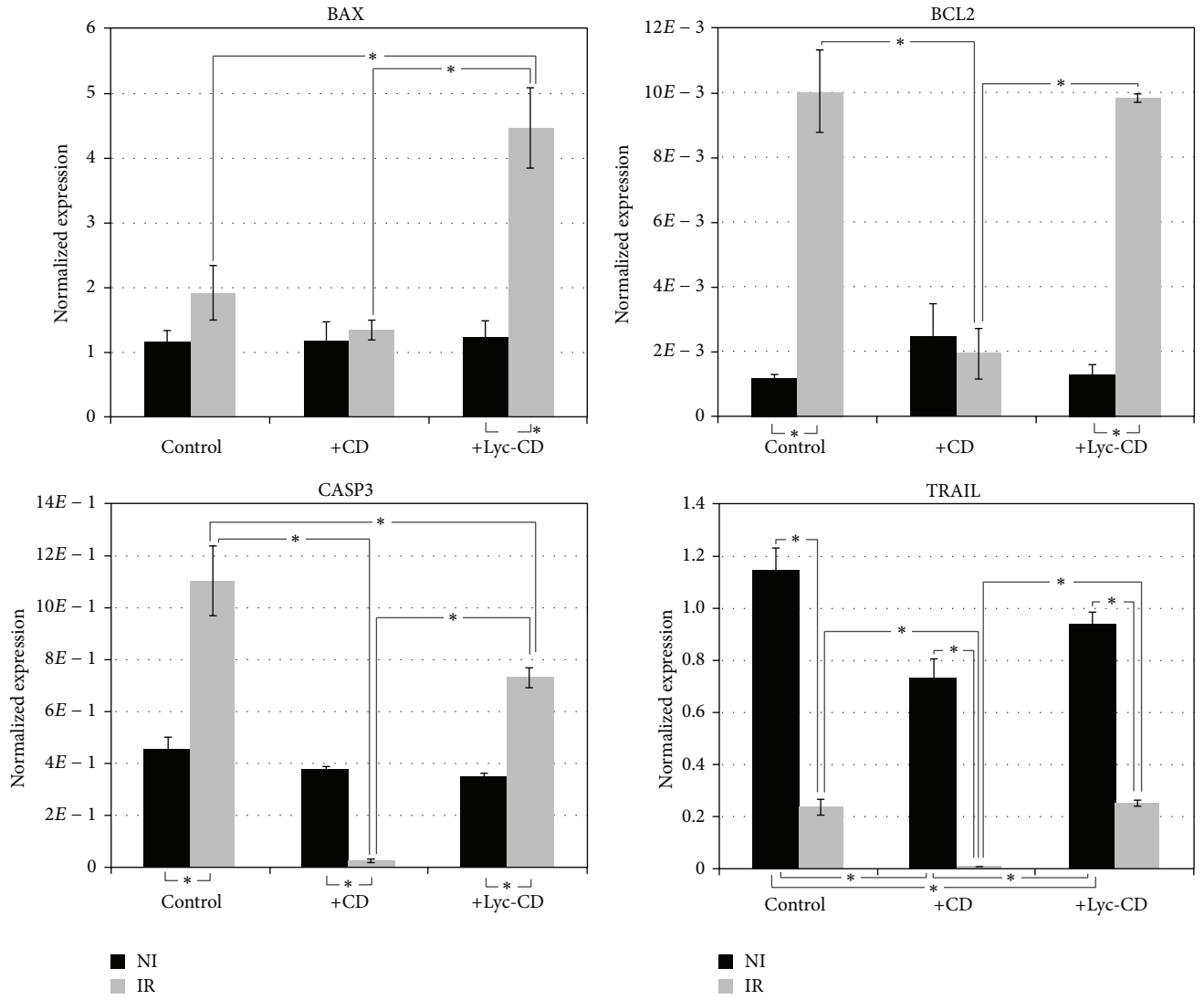

Figure 7: Representation of Bax, Bcl-2, caspase 3, and TRAIL gene expression values in HaCaT cells exposed to 10 μM complexed lycopene (Lyc-CD) normalized to the SDHA reference gene. Cells were irradiated with 225 mJ/cm^2 UV-B (IR) and nonirradiated (NI). Results are expressed as mean ± SEM of three technical replicates from two independent assays.

via a prostaglandin E2 independent pathway in Raw264.7 macrophage cells [67]. Thus, a control assay with CD alone is mandatory in these studies.

Apoptosis is the best-characterized type of programmed cell death because of its importance in development, homeostasis, and pathogenesis of different diseases, such as cancer. Cells respond to specific apoptotic signals by initiating intracellular processes that result in typical physiological changes. Among these changes are externalization of phosphatidylserine to the cell surface, depolarization of mitochondrial membranes, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, loss of cell membrane integrity, and cellular shrinkage. We studied one of the earliest apoptotic events, that is, the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Moreover, Bowen et al. [68] have found that HaCaT cell line (which harbors two mutant p53 alleles) are more susceptible to apoptosis than normal keratinocytes *in vitro*, possibly because of aberrant signaling pathways resulting from long-term culture [69, 70].

In this work, a decrease in % apoptotic cells was observed in irradiated cells preexposed to Lyc-CD (Figure 6). However, this result must be interpreted cautiously, as CD has been previously reported to deplete cholesterol from cell membranes [71, 72]. Cholesterol presence is essential for lipid raft formation and Fas-receptor activation, and this could inhibit UV-B induced apoptosis, as demonstrated by George et al. [73]. Depletion of cholesterol by methyl-β-cyclodextrin reduced Fas aggregation which was accompanied with a reduced apoptotic but increased nonapoptotic death of UV-B-irradiated HaCaT cells [73]. In preliminary experiments, we in fact observed that CD alone can decrease the % apoptotic cells (supplementary data). Moreover, since CD can form complexes with lipids from the plasma membrane, it

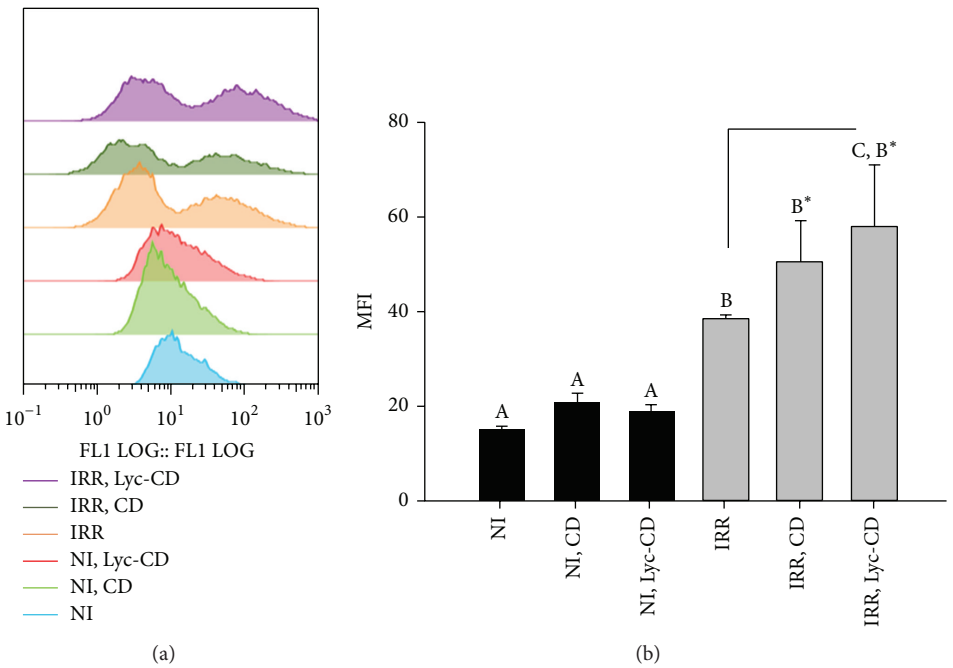

Figure 8: Intracellular ROS quantification: (a) FL1 histogram plots, showing the distribution of cell count versus DCF fluorescence, and (b) Mean Fluorescence Intensity (MFI) histograms results of HaCaT UV-B irradiated cells, IR (225 mJ/cm^2), and nonirradiated cells (NI) exposed to 10 μM complexed lycopene (Lyc-CD) and to the respective controls labelled with DCF-DA. Statistical analysis: One-Way ANOVA with Multiple Pairwise Comparisons: medians with different letters are significantly different ($P < 0.05$).

might be possible that PS was shielded by CD against binding to Annexin V, a requirement for the validity of Annexin V assay. Nevertheless, cells exposed to Lyc-CD showed an increase in the Bax/Bcl2 gene expression ratio. Moreover, Lyc-CD completely reverted the inhibitory effect of CD in TRAIL expression. These results suggest a proapoptotic effect of lycopene which is not found in CD. UV-B irradiation has been shown to increase levels of *apoptosis biomarkers*, especially the proapoptotic protein Bax, thereby inducing apoptosis in skin cells. Regarding the apoptosis biomarkers, Bcl-2 family proteins can modulate mitochondrial permeability through oxidative phosphorylation during apoptosis. Changes in the Bax/Bcl-2 ratio suggest a corresponding change in mitochondrial permeability to release apoptogenic molecules from the mitochondria to the cytosol [5]. In this work, irradiated cells showed significantly higher levels of apoptosis, confirmed by independent parameters (namely, increased sub-G1 population indicative of DNA fragmentation) and binding to Annexin V, indicative of increased phosphatidylserine present in the outer leaflet of the cell membrane. From the gene expression data obtained, this means that the most relevant parameters to take into account for UVB-induced apoptosis are *BAX* and *CASP3* expression. *BCL2*, a known antiapoptotic effector was found upregulated in this study, and *TRAIL*, a proapoptotic cytokine, was found downregulated under UVB irradiation. This suggests that under the experimental conditions used, these genes have a comparatively lower predictive value for apoptosis induction by UVB. It might be a response at transcription level which does not reflect the physiological level of the irradiated cells. A more close focus on *BAX* and *CASP3* expression reveals that lycopene induced increased *BAX* expression in cells treated with the high UVB dose used in this study and this might represent an important mechanism for its therapeutic action.

Besides apoptosis, necroptosis, and necrosis or senescence, damaged keratinocytes can also be physiologically eliminated by induction of terminal differentiation. This may be readily monitored by detection of, for example, "suprabasal" keratins or cytoplasmic involucrin for late steps in epidermal differentiation; however, this type of analysis would probably be most useful for very late time points. It should be noted that more biomarkers could be also analyzed considering the huge cascade events triggered by UVR, for example, protein kinase C family which sensitizes skin to UVR [74] and others regarding the influence on intracellular calcium levels or retinol signaling which are profoundly altered upon differentiation of keratinocytes [75].

UVR results in an increased generation of *ROS* that interacts with proteins, lipids, and DNA, overwhelming the antioxidant defense mechanisms of the cells.

The epidermis is composed mainly of keratinocytes, which are rich in ROS detoxifying enzymes such as superoxide dismutase, catalase, and glutathione peroxidase and in low-molecular-mass antioxidant molecules [76]. Although skin spontaneously responds to increased ROS levels, this response may not be sufficient to prevent the progression of skin cancer [4]. Despite the extensive evidence implicating ROS in oxidative DNA damage, little is known about its involvement in DNA damage of keratinocytes, which are the

most relevant cell type in nonmelanoma skin cancer. The singlet oxygen and hydroxyl radicals are the major damaging oxidative species which can be formed under normal aerobic metabolism and by certain processes including photosensitization [77]. The major DNA oxidation products include 8-oxo-7-hydro-deoxy-guanosine (8-oxodG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine [78].

Comparatively to nonirradiation, UV-B irradiation significantly altered the distribution of cells in terms of fluorescence (Figure 8). In irradiated cells two populations can be distinguished. The first peak might correspond to dead cells, which had a very weak signal of DCF (compatible with autofluorescence). In this case, the DCF might not be activated and metabolized within the cell (no fluorescence), and/or the cells might not retain the DCF because of the loss of membrane integrity. Complexed lycopene increased the ROS levels in irradiated cells. Reagan-Shaw et al. [5] have also obtained a significant decrease of SOD activity (MnSOD) after treatment with sanguinarine, enhancing UV-B-mediated oxidative stress in HaCaT cells. However, according to Onoue et al. [79], ROS data might not always provide a reliable indication for the capacity of a chemical to participant in a photogenotoxic cascade. This fact could be more realistic when irradiation is used in experiments. In fact, the photodegradation of lycopene may contribute to cytotoxicity, including oxidative damage. For example, apo-6′-lycopene and 2-methyl-hepte-6-one as well as further reaction products are formed during irradiation of lycopene [28, 80]. Nagao [81] found that oxidized metabolites of lycopene but not lycopene itself can inhibit cell growth and stimulate apoptosis in a different cell line (HL-60). In another study, lycopene in human prostate cancer cells inhibited cell growth, but the oxidized mixtures displayed markedly more potent growth inhibition [81, 82]. On the contrary, some recent studies suggested that lycopene or lycopene metabolites may, as β-carotene and its metabolites do, enhance carcinogenesis.

In general, similar results were obtained by Reagan-Shaw et al. [5] who suggested that sanguinarine (also a botanical antioxidant) may protect skin cells (also HaCaT cell line) from UVB-mediated damage via apoptotic elimination of damaged cells that escaped from the programmed cell death. These are clearly important observations since apoptosis is a mechanism of defense and acts by opposing the creation of damaged and preneoplastic cells and expansion to a clone. Once mutations arise, apoptosis also removes preneoplastic cells that are aberrantly proliferating due to genetic defects.

However, further investigation into the dose effect of lycopene and further understanding of the metabolism of apo-10′-lycopenoids on carcinogenesis are still needed [7]. On the other hand, studies on skin organotypic 3D-cocultures (long-lived 3D-cocultures, epiSC-markers, label-retaining cells, etc.) would be useful to have all picture considering the loss of complex interactions between the epithelium and stroma in 2D cell cultures [83]. An UV-irradiation protocol for a normal skin model or even the malignant model for both prevention and treatment approaches of skin cancer could be used [84, 85].

5. Conclusions

According to data obtained from all experimental assays, we demonstrate here that complexed lycopene up to 10 μM does not show metabolic toxicity (MTT assay) under standard cell culture conditions. On one hand, at nontoxic dose (10 μM) complexed lycopene does not affect the profile of apoptotic, necrotic, and viable cells or show cytostatic effects despite slightly increasing the ROS content. However, cells previously exposed to complexed lycopene when irradiated with metabolically damaging UV-B dose show a distinguishing switch in the dead : apoptotic : viable subpopulations compared to nonexposed irradiated cells. On the other hand, exposed irradiated cells showed a decrease in G0/G1 phase. In fact, the increased sub-G0/G1 phase and a trend for S-phase delay (even not statistically different) could contribute to this cell cycle change. In addition, an increased expression of different apoptosis biomarkers (*BAX* and caspase 3) was observed in exposed irradiated cells when compared to the respective controls. These two biomarkers were the most useful probably because those genes might be more involved in this process.

Therefore, complexed lycopene might play a corrective role or cytotoxic effect in photodamaged and preneoplastic keratinocytes, while allowing other keratinocytes to accelerate repairing mechanisms becoming viable. However, some results could be altered by the CD interference with some *in vitro* assays and also with the cells particularly after a high damage effect. Although CDs seemed to be a good candidate to vehicle lycopene in order to counterbalance the disadvantages of most solvents usually used, here we found that it was not suitable under this protocol conditions, especially UV irradiation. Nevertheless, these are interesting data regarding the CD effect on cell cultures and reveal the importance of the analysis of this control compared to cells exposed to active molecules, as it was here performed.

Future studies will continue with other exposure conditions, that is, another lycopene vehicle and a lower UV-B dose which could be set by other assays, such as TUNEL and/or detection of chromosomal breakage by Cytokinesis-Blocked Micronucleus Cytome Assay (CBMN) (besides MTT). Moreover, detection of Ki67 protein, a marker of proliferation, could be attempted. The analysis of other biomarkers and final studies using 3D skin models as previously suggested would resemble more closely the situation *in vivo*, which may give definite and clear answers.

Conflict of Interests

All authors have no conflict of interests to declare.

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Review Article

Mitochondrion-Permeable Antioxidants to Treat ROS-Burst-Mediated Acute Diseases

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Reactive oxygen species (ROS) play a crucial role in the inflammatory response and cytokine outbreak, such as during virus infections, diabetes, cancer, cardiovascular diseases, and neurodegenerative diseases. Therefore, antioxidant is an important medicine to ROS-related diseases. For example, ascorbic acid (vitamin C, VC) was suggested as the candidate antioxidant to treat multiple diseases. However, long-term use of high-dose VC causes many side effects. In this review, we compare and analyze all kinds of mitochondrion-permeable antioxidants, including edaravone, idebenone, α-Lipoic acid, carotenoids, vitamin E, and coenzyme Q10, and mitochondria-targeted antioxidants MitoQ and SkQ and propose astaxanthin (a special carotenoid) to be the best antioxidant for ROS-burst-mediated acute diseases, like avian influenza infection and ischemia-reperfusion. Nevertheless, astaxanthins are so unstable that most of them are inactivated after oral administration. Therefore, astaxanthin injection is suggested hypothetically. The drawbacks of the antioxidants are also reviewed, which limit the use of antioxidants as coadjuvants in the treatment of ROS-associated disorders.

1. Introduction

Endogenous reactive oxygen species (ROS) were produced in cells over time, causing oxidative-damage to nucleic acids, protein, lipids, and other cellular components. ROS are now considered as signalling molecules to change the expression of a large number of genes [1]. The relationship between some diseases and oxidative-damage has been well studied. A large number of reports showed that oxidative stress is correlated with the pathogenesis of multiple age-related diseases, like cancer and neurodegenerative diseases, and several other common diseases such as ischemia-reperfusion injury, stroke, hypertension, heart failure, atherosclerosis, diabetes, rheumatic diseases, and Alzheimer disease [2–10]. Therefore, a lot of antioxidants have been adopted to prevent and alleviate disease-accompanying oxidative-damage. However, some human clinical data of antioxidant therapeutics indicated negative or ambiguous results or insignificant benefits. Even some antioxidants showed apparent side effects [10, 11]. In this review, we compare representative mitochondrion-permeable antioxidants through analyzing their therapeutic mechanisms, the application ranges, and side effects.

2. ROS-Burst-Mediated Acute Diseases

2.1. ROS Burst in Ischemia-Reperfusion. When blood supply or oxygen supply returns to the ischemic tissue, the reperfusion injury occurs. In this condition, restoration of blood flow and oxygen supply does not restore cellular normal functions but induces inflammation and oxidative-damage [3, 12].

Reperfusion of ischemic tissues is usually accompanied with microvascular damage, which increases capillary and arteriole permeability and leads to fluid filtration and diffusion. These damaged endothelial cells generate more ROS but less nitric oxide after reperfusion, and the disequilibrium induces subsequently inflammatory responses [3, 12, 13]. At the same time, leukocytes, circulated with the newly returning blood, release interleukins, free radicals, and other

inflammatory factors, which damage the tissue further [3, 12, 13]. The reintroduced oxygen damages nucleic acids, enzymes, and the plasma membrane. Oxidative-damaged cellular membrane may release more ROS in turn. Then ROS may also trigger redox signalling indirectly and the subsequent cell death or apoptosis. Leukocytes may also bind to the small capillary endothelium, causing more ischemia [3, 12, 13].

2.2. ROS Burst in Avian Influenza Infections. The infection of avian influenza virus (AIV) results in multiple complications to the patient, causing multiorgan failures and may be associated with the excessive immune responses, which may be the main reason for its high pathogenicity and mortality [2, 10]. The AIV infection induces a cytokine storm, including chemokines, interferon-inducible protein IP-10, interferon β, and interleukin-6 (IL-6) and cell death presumably [14–17]. Investigations suggested that healthy young people with stronger immune system may become a main target of AIV attacks [2].

In our previous review of the drugs to avian influenza infection, a large drug combination (including antioxidants, protectant of mitochondrial membrane permeability, immunomodulators, protease inhibitors, and antiviral drugs) is proposed, which mainly focuses on cytokine control and may greatly reduce the mortality rate hypothetically [2]. For the drug combination, antioxidant is the most important medicine suggested, because of the fact that ROS play a crucial role in the inflammatory response and cytokine outbreak [18]. Neutrophil aggregation and oxidative-damage to alveolar epithelial membrane result in acute respiratory distress syndrome (ARDS) finally. The activated neutrophils induce a ROS burst (more than ten times explosion).

There are many similarities between pulmonary ischemia-reperfusion and AIV infection-induced ARDS. Both of the oxidative injuries include the following: (i) lipid peroxidation and oxidative-damage to cytomembrane and the organelle-membrane; (ii) enzyme activity inhibition; (iii) lysosomal protease releasing; and (iv) chemoattractant generation and aggregation of more neutrophils [19]. Thereby free radicals form a self-amplification feedback loop [18].

H1N1 infection also inhibits patient's catalase expression, therefore causing the hydrogen peroxide accumulation [20], while H5N1 triggers extracellular calcium influx, which induces apoptosis [21].

Major inflammatory response is the mitochondrial dysfunction. AIV infection or ischemia-reperfusion induces calcium overload and mitochondrial permeability transition (mPT). Apoptosis indicator cytochrome c is released from mitochondria. Cyclosporine A (CsA) can prevent this mitochondrial permeability transition and the subsequent apoptosis [22]. CsA-treated cells are protected from ischemia-reperfusion injury, but not from tumour necrosis factor α (TNFα) or Bax (Bcl-2 associated X protein) induced cell death [22]. Both ROS-mediated apoptotic pathway and NF-κB-mediated survival pathway are activated by the TNFα. ROS accumulation facilitates the cell-death pathway [23]. The ratio of proapoptotic protein Bax to antiapoptotic protein Bcl-2 is also regulated by the ROS level. Superoxide anion induces the survival pathways, while hydrogen peroxide triggers the cell-death pathways [24]. Thus antioxidants may block both TNFα and Bax-mediated apoptosis pathways [2].

2.3. Vitamin C May Be Potentially Used as the Candidate Antioxidant to Treat Avian Influenza Infections. In view of the advantages such as relatively effective, nontoxic, and easy to be absorbed, ascorbic acid (vitamin C, VC) was suggested as the candidate antioxidant for avian influenza infections [2]. VC scavenges free radicals through a nonenzymatic process. In the 19th century, VC was used to cure cold (influenza infection), encephalitis, hepatitis, and some other viral diseases for over a hundred years [25–27].

An investigation indicated that 50% of H5N1-infected patients in Vietnam did not die. Ely [28] found that the survivals may take large amounts of VC from their foods, which may alleviate the inflammatory responses.

Influenza patients need 4.4 g or higher levels of VC to control the virus or alleviate the symptom [25–28]. However, the common oral dosage of VC tablets is 100–300 mg a day, much lower than the influenza treatment requires. Oral intakes of VC that exceed 1 g may cause side effects, like vomiting, stomach cramps, diarrhea, and nausea [25–28]. Therefore the VC injection should be used for AIV infections. Nevertheless, high doses are still required. Additionally, long-term use of high level of VC (>2-3 g a day) may result in scurvy after VC administration is stopped [25–27]. These drawbacks should be considered before the clinical therapies.

2.4. Other ROS-Related Airway Disorders, Chronic Obstructive Pulmonary Disease, for Example. Chronic obstructive pulmonary disease (COPD) is a major and rapidly increasing health problem associated with a chronic inflammatory response, predominantly in small airways and lung parenchyma. Oxidative stress induced by reactive oxygen species and nitrogen species plays a central role in the pathophysiology of COPD [29]. At the subcellular level, mitochondrial dysfunction (accompanied with a decreased mitochondrial membrane potential) in patients with COPD is associated with excessive mitochondrial ROS levels, which contribute to enhanced inflammation and cell hyperproliferation. Thus, targeting mitochondrial ROS represents a promising therapeutic approach in patients with COPD, such as the mitochondria-targeted antioxidant MitoQ (see later discussion of MitoQ) [30].

3. Mitochondrion-Permeable Antioxidants

The most pivotal aspects of antioxidant therapies are the site concentration effects. Antioxidant efficiency is fully dependent on the locus concentration, since, as many other pharmaceutical compounds, antioxidants also have their "pharmacological windows." Therefore, these scavenging/quenching compounds should concentrate in the target-tissue (or subcellular site) in order to efficiently remove exceeding ROS without eliminating essential redox signalling molecules, such as nitric oxide, hydrogen peroxide, S-Nitrosoglutathione (GSNO), and nitro/nitrosyl-lipid peroxides [24, 31]. It is well known that cellular redox status defines

Table 1: Licensed antioxidants for alleviating disease-related oxidative-damage. Their evidenced clinical uses, drawbacks, and possible side effects are summarized.

Drug's name	Clinical uses	Drawbacks	Possible side effects
Edaravone	Ischemic stroke	Limited testing and sometimes ineffective	Nephrotoxicity [95]
Idebenone	Alzheimer disease	Limited testing and sometimes ineffective	Gastrointestinal complaints, neurotoxicity, and cardiotoxicity [95]
α-Lipoic acid	Diabetic neuropathy and eye-related disorders	Limited testing and sometimes ineffective	Headache, tingling, skin rash, or muscle cramps [95]
Carotenoids	Inflammation, cancer, and cardiovascular diseases	Sometimes ineffective	Damage to skeletal muscle integrity (high-dose) [44], canthaxanthin retinopathy [102], and lung cancer in heavy smokers [103]
Vitamin E	Inflammation, cancer, and cardiovascular diseases	Sometimes ineffective	Hemorrhage and vitamin K deficiency (high-dose) [45]
Coenzyme Q10	Heart failure, migraine, hypertension, and neurodegenerative diseases	Limited testing, insoluble in water, therefore in low bioavailability, and sometimes ineffective	Largely gastrointestinal complaints (very high-dose) [50]
MitoQ	Alzheimer's disease, Parkinson's disease, hypertension, diabetes, heart attack, sepsis, alcohol-induced steatohepatitis, and cocaine cardiotoxicity	Sometimes ineffective in human bodies	No side effect observed (even after a long-term oral administration) [56]
SkQ	Age-related diseases	Limited testing	No side effect observed [59]
Astaxanthin	Atherosclerosis, coronary heart disease and ischemic brain damage, age-related macular degeneration, acute pain, inflammation, cancer, and cardiovascular diseases	Insoluble in water and sometimes ineffective	No side effect observed [60–64]

the fate of one cell. Depending on the redox status, eukaryotic cells could proliferate, keep it in steady state (G0 phase), or enter into cell death, either by apoptosis (moderate oxidative condition; intrinsic mitochondrial pathway) or by necrosis (high oxidative insults) [24, 31]. More interestingly, the redox status sensibility varies obviously upon the cell type that hepatic cells are more plastic than neurons [32]. Therefore, the biggest challenge researchers have nowadays on prescribing antioxidant therapies is how to reach the proper antioxidant concentration *in situ* for a precise redox modulation against a ROS-mediated pathology.

As discussed above, ROS-burst-mediated mitochondrial dysfunction and mitochondrial-derived apoptosis play a crucial role in the inflammatory response during avian influenza infection or ischemia-reperfusion. Thus for these ROS-burst-mediated acute diseases, mitochondrion-permeable antioxidants should be much more effective than water-soluble antioxidants (like VC). Edaravone, idebenone, α-Lipoic acid, carotenoids (especially astaxanthin), vitamin E, coenzyme Q10, and mitochondria-targeted antioxidants MitoQ and SkQ are summarized as follows (Table 1 and Figure 1). Interestingly, most of them contain a six-membered carbon-ring with a long alkyl side chain and multiple hydroxyl groups and aldehyde groups (Figure 1). All of them are liposoluble. Therefore, they could traverse across the cell membrane and the mitochondrial membrane and accumulate in mitochondria. On the contrary, most water-soluble antioxidants are distributed in the cytosol (Figure 1).

3.1. Representative Mitochondrion-Permeable Antioxidants. Edaravone (3-methyl-l-phenyl-pyrazoline-5-one) has been approved in Japan since 2001. Edaravone can reduce ischemic-stroke-induced neuronal damage [33]. However, there are also studies that do not approve the effects. Even some cases of nephrotoxicity were reported for edaravone [34].

Idebenone (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinonenoben) is a short chain benzoquinone, structurally similar to coenzyme Q10. Idebenone functions as an antioxidant and electron carrier [35]. Although idebenone has some effects on Alzheimer's diseases [35, 36], the solid clinical evidences are still missing. Therefore, its clinical application is limited [37]. The most common side effects are gastrointestinal complaints and some level of neurotoxicity or cardiotoxicity [38].

α-Lipoic acid (LA) is a unique lipid and water-soluble antioxidant. It is a naturally occurring dithiol compound and essential for mitochondrial bioenergetic process [39].

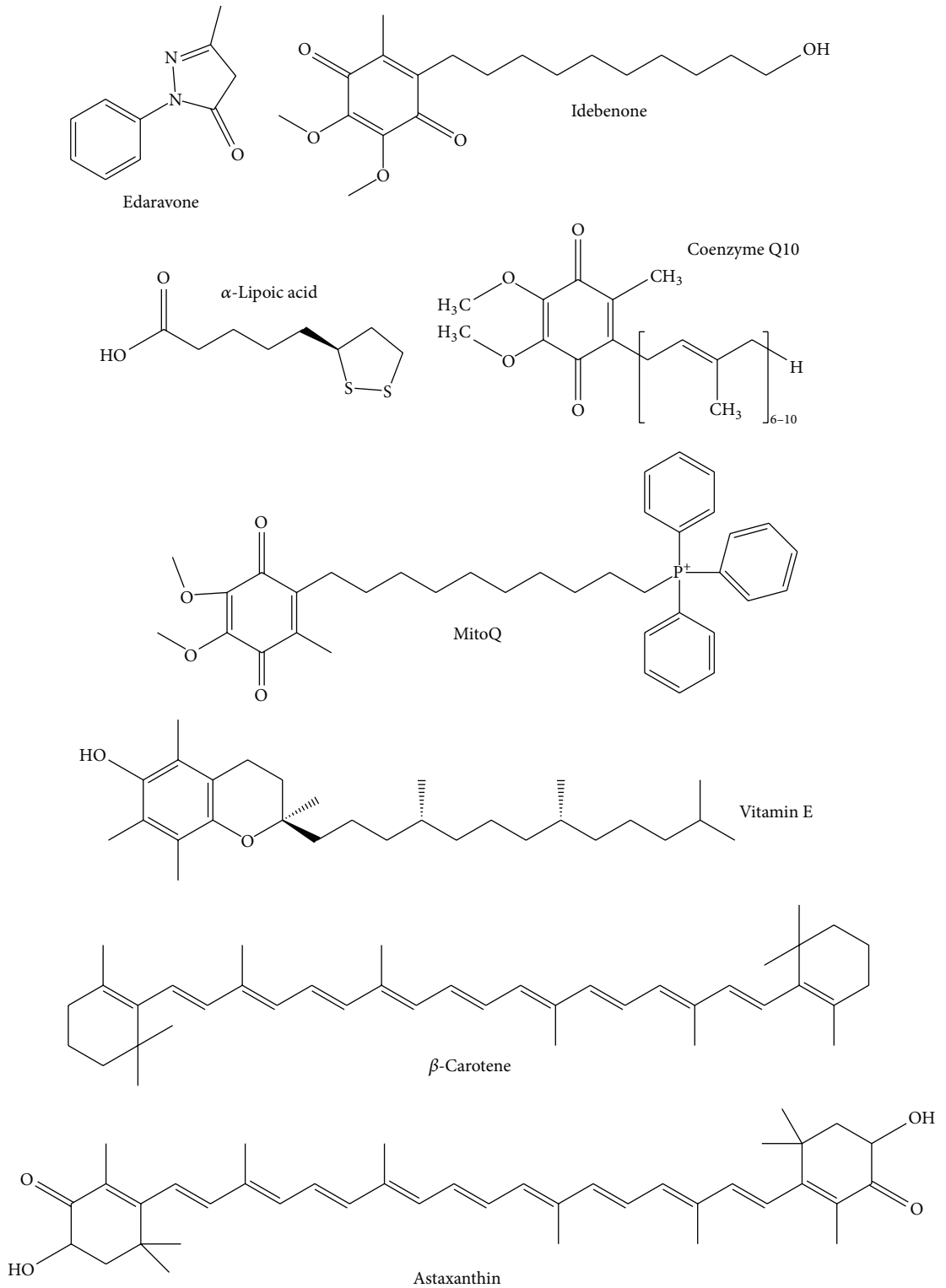

Figure 1: Chemical structures of representative mitochondrion-permeable antioxidants (edaravone, idebenone, α-Lipoic acid, coenzyme Q10, MitoQ, vitamin E, β-carotene, and astaxanthin).

LA and its reduced-form dihydrolipoic acid are important mitochondrion-permeable antioxidants. LA has been approved for diabetic neuropathy treatment [39, 40].

Carotenoids, consisting of over 600 lipid-soluble plant pigments and a few water-soluble carotenoids (such as crocin), are present in many fruits and vegetables. The common carotenoids include α-carotene, β-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin [41]. Among them, β-carotene, the vitamin A precursor, has been most well studied. They neutralize free radicals effectively [42]. However, there are inconsistent conclusions about the role of β-carotene in cardiovascular diseases (CVD) prevention [43]. Moreover, a study indicated that high intake of carotenoids resulted in a faster skeletal muscle breakdown (skeletal muscle integrity reducing) [44]. Astaxanthin is a peculiar carotenoid, which will be discussed in detail later.

Among the vitamin E family, α-tocopherol is the most predominant form. The hepatic α-tocopherol transfer protein binds and carries α-tocopherol to all body's cells [45]. Most α-tocopherol is associated with lipoproteins, scavenging $LCOO^{\bullet}$ results and inhibiting low-density lipoprotein (LDL) oxidation. Thus, α-tocopherol is thought to have a role in atherosclerosis prevention. The uptake process of oxidized LDL by the macrophage scavenger receptor and the foam cell formation are blocked by the α-tocopherol treatment [46]. However, some reports did not support the protective role of vitamin E in prostate cancer [47, 48].

Coenzyme Q10 (CoQ10), with its oxidized-form ubiquinone and reduced-form ubiquinol, is an endogenous lipid, which participates in the mitochondrial electron transport in the respiratory chain [49]. CoQ10 has been used to treat a variety of diseases, such as cardiovascular diseases [50], migraine [51], hypertension [52], and neurodegenerative diseases [53]. Although CoQ10 is considered a safe drug, further large-scale studies are still needed to show its clinical usefulness.

MitoQ was designed in the late 1990s as a mitochondria-targeted antioxidant by Kelso et al. [54]. Both MitoQ and coenzyme Q10 belong to the ubiquinone components. The ubiquinone structure of MitoQ can be activated in the mitochondrion (by the mitochondrial respiratory complex II) to form the ubiquinol antioxidant. Thus, MitoQ increases the mitochondrial antioxidant capacity *in situ* and thereby decreases mitochondrial oxidative-damage [54].

MitoQ is a lipophilic molecule bearing a cation moiety, which makes it pass directly through the mitochondrial membrane, because of the fact that the component is positively charged (a hydrophobic structure) [55]. Therefore, MitoQ is an effective mitochondria-targeted antioxidant.

The ability of MitoQ and the mitochondrial oxidative-damage after the treatments (oral or intraperitoneal administration) have been studied in the mouse model. The following diseases have been studied: Alzheimer's disease, hypertension, type I diabetes, heart attack, sepsis, fatty liver disease, alcohol-induced steatohepatitis, doxorubicin, and cocaine cardiotoxicity [56, 57]. These findings are consistent with the conclusion that mitochondrial oxidative-damage is the potential therapeutic target in multiple diseases and pathologies.

However, for Parkinson's disease trials, MitoQ did not show a benefit, maybe because of the irreversible neuronal damage in patient's brain cells [58]. Therefore, more studies of MitoQ in humans are much needed. Moreover, only successful phase II assessments of oral MitoQ tablets were reported. It is not a FDA-approved drug so far.

SkQ (10-(6′-plastoquinonyl)decyltriphenyl-phosphonium) also is organic molecules composed of a large number of organic cations attached with a plastoquinone. SkQ traverses across the cellular membranes and accumulates in mitochondria. The level of a penetrating cation in mitochondria can be more than 1000-fold higher than its extracellular level [59]. Therefore, it is another mitochondria-targeted antioxidant.

Several studies indicated that SkQ protects cells from age-related diseases efficiently, including cataract, retinopathy, glaucoma, balding, canities, osteoporosis, hypothermia, and torpor [59]. However, its safety and the clinical usefulness need further investigations. Like MitoQ, SkQ is also not a FDA-approved drug so far.

3.2. Astaxanthin Is a Promising Antioxidant. Better than above antioxidants, here we introduce another one, astaxanthin, to be a candidate drug for AIV infection cure and some other diseases (Table 1). Astaxanthin, a dietary carotenoid, is present in algae, shrimp, lobster, crab, salmon, and some other organisms [60–64]. Its antioxidant activity is far exceeding the existing antioxidants. The ROS-scavenging capacity is 6000 times that of VC, 800 times that of coenzyme Q10, 550 times that of VE, 200 times that of polyphenols, 150 times that of anthocyanins, and 75 times that of α-Lipoic acid [65]. Most importantly, no apparent side effects or negative results have been reported for astaxanthin [60–62]. In leukocyte cells, half of the total astaxanthin is distributed in the mitochondria. Astaxanthin is also distributed in microsomes and nuclei [66]. Therefore, it is a mitochondrion-permeable antioxidant.

Natural astaxanthin plays an important role in preventing atherosclerosis. Low-density lipoprotein (LDL) oxidation is the main reason of atherosclerosis. Astaxanthin treatment increased high-density lipoprotein (HDL) significantly and reduced LDL effectively, while β-carotene or canthaxanthin has no such effect. The main reason may be that only astaxanthin can reduce apolipoprotein oxidation, therefore being important for preventing arteriosclerosis, cardiovascular diseases, and ischemic brain damage [67, 68].

Astaxanthin also maintains the eyes and central nervous system healthy. Retina contains high levels of unsaturated fatty acids and oxygen supply. The singlet oxygen is generated in the retina upon high-energy light illumination. However, for mammals, carotenoids in diet are enough to maintain eye health and can quench these free radicals [69]. Recent study indicated that astaxanthin can pass through the blood-brain barrier and prevent retina cell oxidation [70]. Astaxanthin also has a good effect on preventing and treating macular degeneration [70].

Astaxanthin is an anti-inflammatory and pain reliever, blocking different biochemical factors that cause ouch and

pain [71]. More specifically, astaxanthin inhibits cyclooxygenase 2 (COX2) enzyme activities, which are related with many diseases, such as osteoarthritis, rheumatoid arthritis, dysmenorrhea, and acute pain [72]. Astaxanthin and Celebrex (another COX2 inhibitor) work cooperatively for some diseases, which therefore were suggested to be taken both together to alleviate oxidative-damage [72].

Astaxanthin affects not only the COX2 signalling pathway but also multiple cytokines, like nitric oxide, interleukin 1-β, prostaglandin E2, C-Reactive Protein (CRP), NF-κB, and TNFα [72]. A study also showed that astaxanthin is a useful antioxidant to treat insulin resistance by protecting cells from TNFα and palmitate-induced oxidative-damage [73]. Recent study suggested that astaxanthin also inhibits apoptosis in alveolar epithelial cells via mitochondrial ROS signalling pathways, also indicating its mitochondrial location [74].

Astaxanthin also activates T-cell and inhibits autoimmune reactions [75]. The risk of many different types of cancer can be significantly reduced by dietary intake of astaxanthin along with other carotenoids [76–78]. In the mouse breast cancer model, astaxanthin treatment caused higher levels of apoptotic cancer cells and protective interferons, inhibiting tumor growth [79]. (i) Astaxanthin prevents cancer initiation by alleviating DNA oxidative-damage [80, 81]. (ii) Astaxanthin promotes early check and elimination of cells undergoing malignant transformation by activating immune surveillance [82]. (iii) Astaxanthin prevents cancer cell growth in cells by boosting immune detection [83, 84]. (iv) Astaxanthin inhibits rapid tumor cell growth by blocking tumor cell reproductive cycle and inducing tumor cell apoptosis [85–87]. (v) Astaxanthin prevents tumor cell spreading by decreasing tumor cell's tissue-melting proteins [84].

McNulty et al. [88] studied membrane structures of carotenoids and the relationship to their biological activities. They found that the vertical orientation of astaxanthin in membranes may be crucial for its high efficiency on removing aggressive free radicals from membranes, especially in the presence of water-soluble antioxidants, such as glutathione and/or ascorbic acid [88].

4. Side Effects of Antioxidants

Most of the so-called antioxidant compounds also develop prooxidant properties under specific conditions, such as ascorbic acid (>1 mM) that induces Fe(III) reduction to Fe(II) [89]. Epigallocatechin 3-gallate (EGCG) produces hydrogen peroxide and hydroxyl radicals in the presence of Fe(III) [90].

Clinical trials of some antioxidants in humans showed negative or ambiguous results or insignificant benefits [10, 11, 91–95]. The reasons may be as follows. (i) Oxidative-damage is neither the primary cause nor the only cause of the disease. (ii) Patients do not benefit from the same antioxidant treatment equally. (iii) Some antioxidants by oral administration are of lower efficiencies. (iv) Some antioxidant molecules have toxic effects that mask their ROS-scavenging activities. (v) Certain antioxidants are not effective in well-nourished populations [11, 95].

On the other hand, ROS accumulation does not always correlate with disease onsets positively. Watson [96] postulates that diabetes (especially the type 2 diabetes), dementias, cardiovascular disease, and some cancers may develop, when oxidative redox potential in the endoplasmic reticulum is too low to form normal disulphide bonds [97–99]. Maintaining a certain level of ROS may be necessary for correct protein folding with disulphide bonds, which may be associated with type 2 diabetes and Alzheimer's disease or some other diseases [96, 100, 101]. Thus, the antioxidants may produce negative or ineffective impacts on some diseases.

Interactions of carotenoids (such as canthaxanthin) with the lipid membranes and the aggregation of this pigment may be the factors enhancing canthaxanthin toxicity towards the macula vascular system, which leads to the further development of canthaxanthin retinopathy [102]. And high and long-term beta-carotene supplementation may increase lung tumor rates in heavy smokers [103].

5. Hypothesis of Astaxanthin Injection

Side effects of antioxidants (antioxidant-induced stress) only present when antioxidants overwhelm the body's free radicals [11]. Thus, antioxidants should be used carefully for chronic diseases, such as diabetes and Alzheimer's disease, when cellular ROS levels are not particularly high (no ROS bursts occur). However, for ROS-burst-mediated acute diseases, such as avian influenza infection and ischemia-reperfusion, antioxidants should be used as early as possible to avoid or retard excessive immune responses. Mitochondrion-permeable or mitochondria-targeted antioxidants are preferred.

Astaxanthin is a good candidate drug for these acute diseases. However, so far, astaxanthin is not a clinical drug but merely a health care product. Most studies showed that its treatment effects are not as good as people expect, contrasting to its extraordinary high antioxidant activities [61]. One of the reasons may be that astaxanthin is usually applied by oral administration (such as astaxanthin soft capsule). All nourishments and oral drugs are digested in the gastrointestinal tract and then absorbed into gastric veins and intestinal veins and transported to the liver through the portal vein. Then, after the liver's process, they are transported throughout the body via heart and arteries. Astaxanthin is easy to be oxidized that most of them are inactivated during the digestion, absorption, and transportation. After avian influenza virus infection, for instance, severe oxidative-damage occurs at the lungs, where neither oral VC tablets nor oral astaxanthin capsules could reach effectively. For the same reason, active (reduced) astaxanthin could not reach atherosclerosis sites, retina, or brain arteries ideally too. Therefore the injection approach of astaxanthin may be adopted to these patients. It is well known that vitamin E injection (a mixture of oil for injection and VE) is better absorbed by the body since it goes directly into the blood stream [60, 62]. And recent studies indicated that VC injections have strong anticancer effects, especially when intravenous glutathione or vitamin K3 was applied synergistically [104, 105]. A similar astaxanthin injection could be easily developed. For AIV

infections, astaxanthin by injection can be quickly absorbed and go directly into the pulmonary alveoli, where inflammatory reactions occur, through the body's blood circulation system.

It is well known that water- and lipid-soluble antioxidants act in synergism to efficiently remove aggressive radicals from hydrophobic compartments and, thereby, inhibit lipid peroxidation, which is extremely harmful to most organelles [106]. The collaborative mechanism involving α-tocopherol through tocopheryl formation and ascorbic acid has been studied since the middle of the 90s [106]. In other words, by combining lipid-soluble antioxidants (such as astaxanthin) with water-soluble ones (such as ascorbic acid) in lower concentrations, higher efficiency on ROS removal may be expected. However, cellular ROS should not be removed entirely for retaining the essential redox signalling molecules. The precise dosages need further investigations.

The astaxanthin injection might be suitable for other kinds of disease-accompanying oxidative-damage and inflammations. However, the astaxanthin injection must be subjected to clinical trials and FDA approval. Nevertheless, they are time-consuming processes. Before FDA approval, oral astaxanthin capsules are still suggested for AIV-infected patients. Because when most patients are identified as having avian influenza infections, they have been sick for several days, near or after the time pulmonary symptom developed. Their alveolar cells may become damaged. Thus the risk to develop acute respiratory distress syndrome (ARDS) is very high. So, for the general public, timely oral administration of antioxidants before the diagnosis in the hospital is very important [2, 107]. No matter if infected with avian influenza or common influenza, the patient is recommended to take VC (800–1000 mg a day presumably) or/and astaxanthin (24–48 mg a day presumably) before the hospital examination.

6. Conclusions

Considering the adverse effects of antioxidants, antioxidant drugs should be used carefully for chronic diseases, especially for diabetes and Alzheimer's disease, when a certain level of ROS is required for normally cellular functions. However, for ROS-burst-mediated acute diseases, mitochondrion-permeable antioxidants should be used in the early stage.

To treat ROS-accompanying diseases, no matter chronic or acute, antioxidants should be used combined with other therapeutic drugs. However, drug combinations may have additive or possible antagonistic effects on the disease development. And the dosage of the single compound should be adjusted according to the combination. Thus, carefully pharmaceutic studies should be done before certain antioxidant (e.g., astaxanthin injection) can really enter the clinical trial to oxidative-damage-related illnesses.

Conflict of Interests

The authors confirm that this paper content has no conflict of interests.

Authors' Contribution

Zhong-Wei Zhang and Xiao-Chao Xu contribute equally to this work.

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