Oxidative Medicine and Cellular Longevity

Role of Oxidative, Nitrative, and Chlorinative Protein Modifications in Aging and Age-Related Diseases

Lead Guest Editor: Izabela Sadowska-Bartosz
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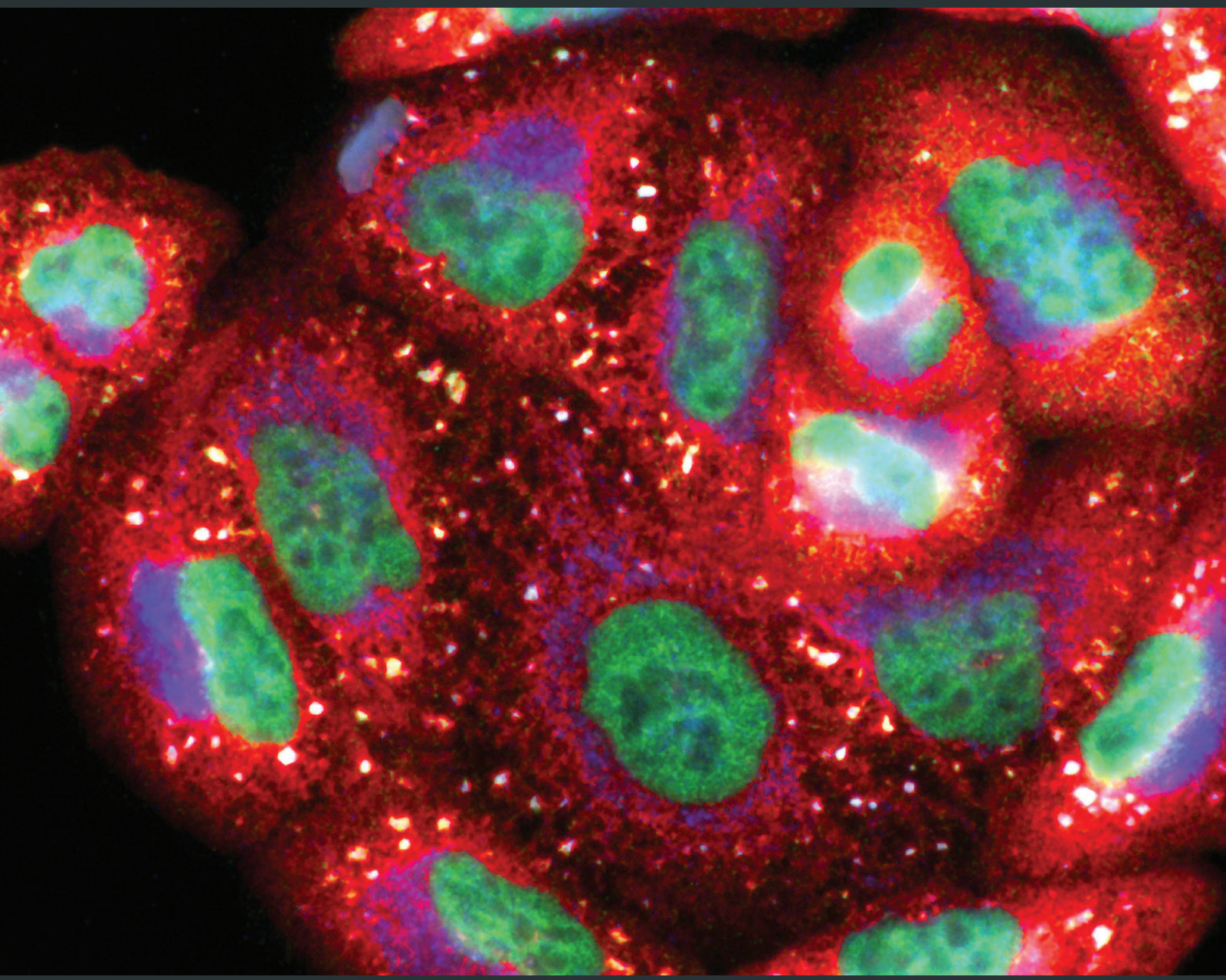

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Editorial Board

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Editorial

Role of Oxidative, Nitrative, and Chlorinative Protein Modifications in Aging and Age-Related Diseases

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Despite extensive studies, the molecular basis of physiological aging is still poorly understood. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as reactive halogen species (RXS) species are believed to play a key role in the aging process. They are generated during aerobic metabolism in living organisms. The term "*reactive oxygen species*" includes both free radicals (molecules having an odd electron, like superoxide radical anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$)) and species that are not free radicals, like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and ozone (O_3). The primary source of RNS is usually the nitric oxide radical ($^{\bullet}NO$). In consequence of ROS and RNS reactions, peroxynitrite $ONOO^-$, anion of peroxynitrous acid ONOOH, may be formed via the near diffusion-limited reaction of $^{\bullet}NO$ and $O_2^{\bullet-}$. The term "*reactive nitrogen species*" includes also nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), nitrosyl anion (NO^-), nitrosyl cation (NO^+), nitrogen dioxide radical ($^{\bullet}NO_2$), peroxynitrate ($ONOOO^-$), peroxynitric acid (ONOOOH), nitryl chloride (NO_2Cl), and nitronium cation (NO_2^+) [1, 2]. Reactive halogen species include HOCl, HOBr, HOI, chlorine, bromine, iodine, and so on. Hypohalogenous acids (HOX; X=F, Cl, Br, or I) are formed in the body mainly by oxidation of halogen ions by myeloperoxidase. The imbalance between ROS, RNS, and RXS production and the antioxidant defense, in favor of prooxidants, is called oxidative, nitr(os)ative, and halogenative stress (OS, NS, and XS), respectively. Although at physiological concentrations ROS, RNS, and RXS can function as signaling molecules regulating cell proliferation, growth, differentiation, and apoptosis [3, 4], they react with and damage all classes of endogenous macromolecules including proteins, nucleic acids, lipids, and carbohydrates [5]. Proteins are the main targets for such modifications as they are the most abundant cell components in the terms of mass content. The level of protein damage increases under stress conditions and can be in principle an integrative measure of the exposure to OS, NS, and XS. Another source of protein modification is glycoxidation leading to the formation of advanced glycation end products (AGEs).

In this issue, the comprehensive review by A. L. Santos and A. B. Lindner presents the interplay of nonenzymatic posttranslational protein modifications in aging-associated molecular processes underlying eukaryotic aging. Understanding of the roles played by posttranslational protein

modifications in aging and age-related diseases can facilitate targeted therapies or interventions in these diseases and the aging process itself. The review highlights also the potential of simple prokaryotic models to uncover complex aging-associated molecular processes in the emerging field of microbiogerontology. D. Weber et al. summarize the results of the European multicenter study MARK-AGE from 1559 participants. They demonstrate that, among others, protein carbonyls and 3-nitrotyrosine are biomarkers with the highest correlation with age.

Protein carbonyls are the most frequently assayed protein modification by ROS, RNS, and RXS. In this issue, M. Adamczyk-Sowa et al. demonstrate that blood serum proteins of multiple sclerosis patients suffer oxidative modifications, which are attenuated by interferon beta and further by coadministration of interferon beta and the antioxidant melatonin.

The pyrin domain-containing 3 (NLRP3) inflammasome, as a vital component of the innate immune system, is implicated in the pathogenesis of type 2 diabetes. X. Kong et al. show that the administration of AGEs (120 mg/kg for 6 weeks) in C57BL/6J mice induced an abnormal response to glucose, pancreatic β-cell ultrastructural lesion, and cell death. Ablation of the NLRP3 inflammasome or treatment with antioxidant N-acetyl-cysteine (NAC) clearly ameliorated these effects, suggesting AGE-induced NLRP3 inflammasome activation as a mechanism for pancreatic islet damage. J. Bai et al. provide evidence that ghrelin, a growth hormone-releasing peptide, protects against H_2O_2-induced oxidative stress in human lens epithelial cells and rat lenses. Their results suggest that ghrelin may prevent the progression of cataracts, a result that is potentially valuable for the clinical treatment of cataracts.

The articles contained in this special issue extend our knowledge on the role of nonenzymatic posttranslational protein modifications in age-related diseases and aging.

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Research Article

High Cholesterol Diet-Induced Changes in Oxysterol and Scavenger Receptor Levels in Heart Tissue

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Involvement of high cholesterol and oxidative stress in cardiovascular diseases is well studied, as it can be hypothesized that various products originated from lipid peroxidation, such as oxysterols, or affected protein expression might lead to cardiomyocyte damage followed by the pathological modifications. Although oxidation of excessive cholesterol to oxysterols in elevated stress conditions is identified by a number of studies, the role of a high cholesterol diet in regulating fatty acid and oxysterol accumulation, together with scavenger receptor mRNA levels, in the heart remains little investigated. Our study provides a detailed analysis of the changes in fatty acid, oxysterol, and scavenger receptor profiles and its relation with histological alterations in the heart tissue. We evaluated alterations of fatty acid composition, by the GC-MS method, while 4β-, 25-, and 27-hydroxycholesterol and 7-ketocholesterol levels by means of LC-MS/MS in high cholesterol diet-fed rabbits. Additionally, a number of proteins related to lipid metabolism and scavenger receptor mRNA expressions were evaluated by Western blotting and RT-PCR. According to our *in vivo* results, a high cholesterol diet enhances a number of unsaturated fatty acids, oxysterols, and LXRα, in addition to CD36, CD68, CD204, and SR-F1 expressions while α-tocopherol supplementation decreases LXRα and SR expressions together with an increase in 27-hydroxycholesterol and ABCA1 levels. Our results indicated that the high cholesterol diet modulates proteins related to lipid metabolism, which might result in the malfunction of the heart and α-tocopherol shows its beneficial effects. We believe that this work will lead the generation of different theories in the development of heart diseases.

1. Introduction

Cholesterol is a key component in regulating various cell functions, including the permeability and fluidity of membrane, steroid hormone synthesis, and bile acids. After the delivery of cholesterol into the cell via lipoprotein or scavenger receptors (SRs), it might be tranferred to (i) endoplasmic reticulum (ER), inducing the sequestration of sterol regulatory element-binding proteins (SREBPs) to reduce the synthesis and uptake of cholesterol, (ii) plasma membrane, enhancing the efflux of cholesterol (known as reverse cholesterol transport (RCT)) through ATP-binding cassette transporter A1 (ABCA1), and (iii) mitochondrial sterol 27-hydroxylase (CYP27A1), increasing endogenous production of 27-hydroxycholesterol followed by the activation of liver X receptors (LXRs) [1].

Increased production of reactive oxygen species (ROS), known as oxidative stress, is enhanced by the imbalance between antioxidant systems and cellular ROS production. Generation of oxygenated cholesterol products (oxysterols) inside the cell is majorly produced either as a result of the free radical attacks or enzymatic reactions and known as an essential reaction in cholesterol-mediated tissue damage [2]. For instance, while unoxidized cholesterol does not contain any inflammatory effect, endogenously originated oxysterols have shown to enhance inflammation in vascular remodeling [3]. Of the oxysterols, 7-ketocholesterol is one of the major oxysterols found increased in the plasma of patients with

coronary artery disease [4]. 7-Ketocholesterol also involves in atherosclerosis development either by inducing apoptosis [5] or by inhibiting reverse cholesterol transport [6].

Elevated levels of oxysterols have been also indicated as a regulator of gene transcription. LXRα and PPARα are two transcription factors that are highly expressed in macrophages and regulate the transcription of genes in modulating lipid metabolism, inflammation, and cholesterol efflux [7, 8]. Related studies have identified LXRα as a sterol receptor that modulates the expression of lipid metabolism-related genes, in addition to the reduction of cholesterol accumulation by upregulating ABCA1-related cholesterol efflux, following the binding of 25- and 27-hydroxycholesterol [8–10]. Oxysterols might also facilitate SREBPs that reduce the cholesterol uptake and synthesis [11].

SRs are identified as the members of the "membrane-bound receptor" family that can specifically bind to various ligands such as oxidized phospholipids/lipoproteins, modified lipid particles, and pathogens. Based on the current understanding of their structure and biological function, SRs have been classified into classes A–J [12]. Due to their variety on ligand binding and signal transduction, SRs are identified not only in atherosclerosis development but also in the immune response, inflammation, and neurodegenerative diseases. For instance, while oxLDL binding to class B scavenger receptor SR-B2 (CD36) activates signaling mechanisms including JNK, p38 MAPK, and tyrosine kinase Fyn [13], class D scavenger receptor SR-D1 (CD68) can recognize lectins, selectins, and OxLDL followed by the regulation of phagocytosis [14, 15].

However, the particular mechanisms of lipid and oxysterol metabolism, together with SR expressions, in heart tissue following a high cholesterol diet and α-tocopherol supplementation have not yet been fully clarified. The aim of our study is to investigate the alterations of lipid metabolism and SR expression in a hypercholesterolemic rabbit model and whether these alterations are affected by the supplementation of α-tocopherol, the most active form of vitamin E. To do that, intracellular accumulation of fatty acids and various oxysterols containing 4β-, 25-, and 27-hydroxycholesterol and 7-ketocholesterol, in addition to SREBP1c, LXRα and PPARα levels, was investigated in the heart tissue. Moreover, SR (CD36, CD68, CD204 (SR-A), SR-B1, SR-F1, and SR-G) and RCT system (ABCA1) were evaluated by measuring mRNA expressions. We observed high cholesterol diet-induced levels of a number of unsaturated fatty acids, oxysterols, and LXRα, in addition to CD36, CD68, CD204, and SR-F1 expressions. In this context, α-tocopherol supplementation showed its beneficial effect by decreasing LXRα and SR expressions while enhancing 27-hydroxycholesterol and ABCA1 levels.

2. Methods

2.1. Animals and Diets. All experimental procedures were approved by the Marmara University Ethics Committee, Istanbul (protocol number 872010). Twenty male albino rabbits (2-3 months old) were divided randomly into four groups which were fed with 100 g per day of vitamin E poor diet. The first group was only fed with an α-tocopherol poor diet. The second group was fed with the α-tocopherol poor diet containing 2% cholesterol. The third group was fed with the α-tocopherol poor diet containing 2% cholesterol with daily intramuscular injections of α-tocopherol (50 mg/kg), and the fourth group was fed with the α-tocopherol poor diet with daily intramuscular injections of α-tocopherol (50 mg/kg). α-Tocopherol concentration was in accordance with the previous literature [16, 17].

After 8 weeks of feeding, following overnight fasting, rabbits were anesthetised using 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride. The blood was taken for cholesterol and α-tocopherol measurements. The heart tissues of each animal were removed, rapid-frozen in liquid nitrogen, and stored at −80°C for GC-MS, LC-MS/MS, qPCR, and immunoblotting experiments.

2.2. Measurement of Cholesterol and α-Tocopherol Levels in Serum. Serum cholesterol levels were determined using an automated enzymatic technique by Hitachi Modular system P800 (Roche). The levels of alpha-tocopherol were determined in serum samples by using reversed-phase high-performance liquid chromatography (HPLC) according to Nierenberg and Nann [18]. Briefly, samples were dissolved in ethanol and applied to a C18 column (5 μm, 4.6 × 250 mm). MeOH : dH_2O (95 : 5, *v*/*v*) was used as a mobile phase, and detections were performed by a UV detector (Thermo) at 294 nm. The relative standard peak areas of α-tocopherol were determined and compared with peak areas of samples to calculate concentration as μg/mL.

2.3. Measurement of α-Tocopherol in Heart Tissue. Two mL of hexane was added into a clean test tube with 100 mg of heart tissue and incubated at 70 C for 10 min followed by the homogenization using an IKA Ultraturrax homogenizator at 15.000 rpm for 30 seconds. During the incubation, the test tube was vortexed every 5 min. The hexane layer was transferred to a clean tube after it was separated by the field of hypercholesterolemia mediated heart disease aging at 1000 ×g for 10 min Another 2 mL of hexane was added and vortexed for 5 min, with the residual aqueous layer. The test tube was centrifuged again. The separated hexane layer was combined with the previous one. Following the evaporation under nitrogen, ethanol was used to dissolve the sample and α-tocopherol was analyzed by LC-MS/MS.

LC-MS/MS analyses were performed by reversed phase HPLC on an Inertsil ODS-3 column (10 cm × 3 mm i.d., 3 μm particle size) using a Shimadzu UPLC system. The mobile phase was as follows: solvent A, distilled water containing 0.1% formic acid, and solvent B, methanol containing 0.1% formic acid. A constant flow rate of 0.5 mL/min and a gradient profile from 80% to 100% of solvent B were employed. Detection was done with an Applied Biosystems Sciex API 4000 QTrap mass spectrometer (Applied Biosystems) equipped with a TurboIonSpray ionization source. Data were acquired in the selective positive multiple reaction monitoring mode (MRM) alternating the following transitions: $[M + H] +$ =431.5 to 165.0 and 431.5 to 137.0.

2.4. Measurement of MDA in Heart Tissue. To prepare a MDA standard solution, 20 mL of 0.1 M HCl was added to 34 μL of 1,1,3,3-tetramethoxypropane (TMP) followed by incubation at 40°C for 1 h to hydrolyse TMP into MDA. The concentration of MDA in the standard solution was determined by measuring its absorbance at 245 nm ($\varepsilon = 13{,}700$) and freshly diluted with deionized water to establish a calibration curve.

Tissue samples (100 mg) were homogenized in 0.1 M phosphate buffer (pH 7.4) followed by an addition of 1 M KOH and 0.02 M BHT. After then, they were left to incubation at 60°C for 1 h with continuous shaking at dark. Following acidification with concentrated HCl to pH 2, they were centrifuged at 15000 ×g for 5 min at 4°C. The resulting supernatants were then derivatized with an equal volume of DNPH (1.2 mM) at 50°C for 60 min and protected from light. After derivatization, the sample was allowed to cool down and centrifuged at 15,000 ×g for 7 min at 4°C. The supernatant was transferred to a clean vial, filtered by 45 μm filter, and 20 μL of a resulting solution was injected onto the LC-MS/MS instrument (Shimadzu UPLC, AB-Sciex 4000 QTrap) for chromatographic analysis. The relative standard peak areas of MDA-DNPH were determined and compared with the peak areas of samples to calculate concentration as ng/mL.

2.5. Protein Carbonyl Analysis in Heart Tissue. Following the electroblotting step, the membranes were kept in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) containing 20% methanol for 5 min, washed in 2 N HCl for 5 min, incubated with 10 mM DNPH solution for 5 min, washed 3 × 5 min in 2 N HCl, and washed 5 × 5 min in 50% methanol. A DNPH-treated membrane was blocked with 5% nonfat dry milk in TBST for 1 h at room temperature followed by the blocking step. A blocked membrane was incubated with anti-DNP antibody (Sigma) in 5% nonfat dry milk/TBST for 1 h at room temperature with constant agitation. A blotted membrane was washed 3 × 5 min with TBST and incubated with HRP-conjugated secondary antibody followed by the washing step for 5 × 5 min with TBST. The membrane was developed using a chemiluminescence kit (Cell Signaling), and blots were quantified/normalized with β-actin by densitometry using Image J software.

2.6. Light Microscopy Examination of Heart Tissue. Heart tissue samples were fixed in 10% buffered formaldehyde for 4 hours, dehydrated, and incubated in xylol for 1 hour twice, embedded in paraffin, and sectioned in 5 μm thickness onto glass slides. Hematoxylin-eosin and Masson's trichrome stainings were performed and examined under light microscopy (Leica) at 200x magnification to evaluate the pathological features.

2.7. Determination of Fatty Acid Profile by GC-MS in Heart Tissue. Heart tissue fatty acids were extracted according to the Bligh and Dyer method [19]. To determine the fatty acid composition of extracted heart tissue, the fatty acids were converted into fatty acid methyl esters (FAME) by the method of methanolic HCl. The FAME was separated and analyzed using a gas chromatography-mass spectrometry (GC-MS QP2010; Shimadzu Scientific Instruments) equipped with a 30 m fused-silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Restek). The GC conditions were as follows: initial temperature of 130°C, 3°C min^{-1} to 240°C, injector and detector temperatures were 250°C, the column flow was 3.0 mL·min^{-1}, the split ratio was 1 : 100, and 1 μL injection volume was used. Fatty acid (FA) peaks were identified by using FAME standards (The Food Industry 37 FAME mix, 35077 Restek) and expressed as the percentage of total fatty acids. An overview of the identified monounsaturated, polyunsaturated, and saturated fatty acids is listed in Supplementary Table 1.

2.8. Determination of Oxysterols by LC-MS/MS in Heart Tissue. Oxysterols were isolated from the lipid extracts, according to the Bligh and Dyer method [19], by the solid-phase extraction (SPE) [20]. Following the isolation, oxysterols were then analyzed by LC-MS/MS after the derivatization with picolinic acid.

The MS method for the individual picolinic acid-oxysterol standard was analyzed in the "scan mode" of the single quadrupole mass spectrometer to determine if the oxysterols were derivatized to the compounds and fragmentation patterns described by Honda et al. [21]. The chromatographic separation of the picolinyl derivatives of oxysterols was carried out on the Shimadzu LC system, and the samples were analyzed and detected using the ESI-MS/MS detector in a positive mode. Hypersil GOLD C18 column (15 cm × 2.1 mm, 3 μm particle size Thermo Electron) was used. Column oven temperature was maintained at 40°C, and the pump flow rate was maintained at 0.3 mL/min. The mobile phase was implemented in a gradient with 100% acetonitrile with 0.1% acetic acid (mobile phase A) in pump A and 100% water with 0.1% acetic acid in pump B (mobile phase B). The gradient program began with 80% mobile phase B for 2 min and was thereafter ramped to % 90 mobile phase B over a 28 min period and hold for 2 min, then returned to initial conditions.

The LC-MS/MS system consisted of an API 4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems) through a TurboVTM ESI source and a Shimadzu UFLC system in which oxysterols 4β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol were analyzed.

2.9. Immunoblot Analysis. 100 mg of heart tissues was homogenized in RIPA buffer (Cell Signaling) by using an Ultraturrax homogenizator at 15.000 rpm for 30 seconds and centrifuged at 15.000g for 20 minutes. The protein concentrations of the supernatants were determined by the Lowry method. 30 μg of protein samples was separated with 10–12% SDS-PAGE gels and transferred to nitrocellulose or PVDF membranes. Membranes were probed with primary antibodies against SREBP1c and LXRα (Abcam). Following the use of HRP-conjugated secondary antibodies and chemiluminescence kit (Cell Signaling), blots were quantified and normalized with β-actin by densitometry using Image J software.

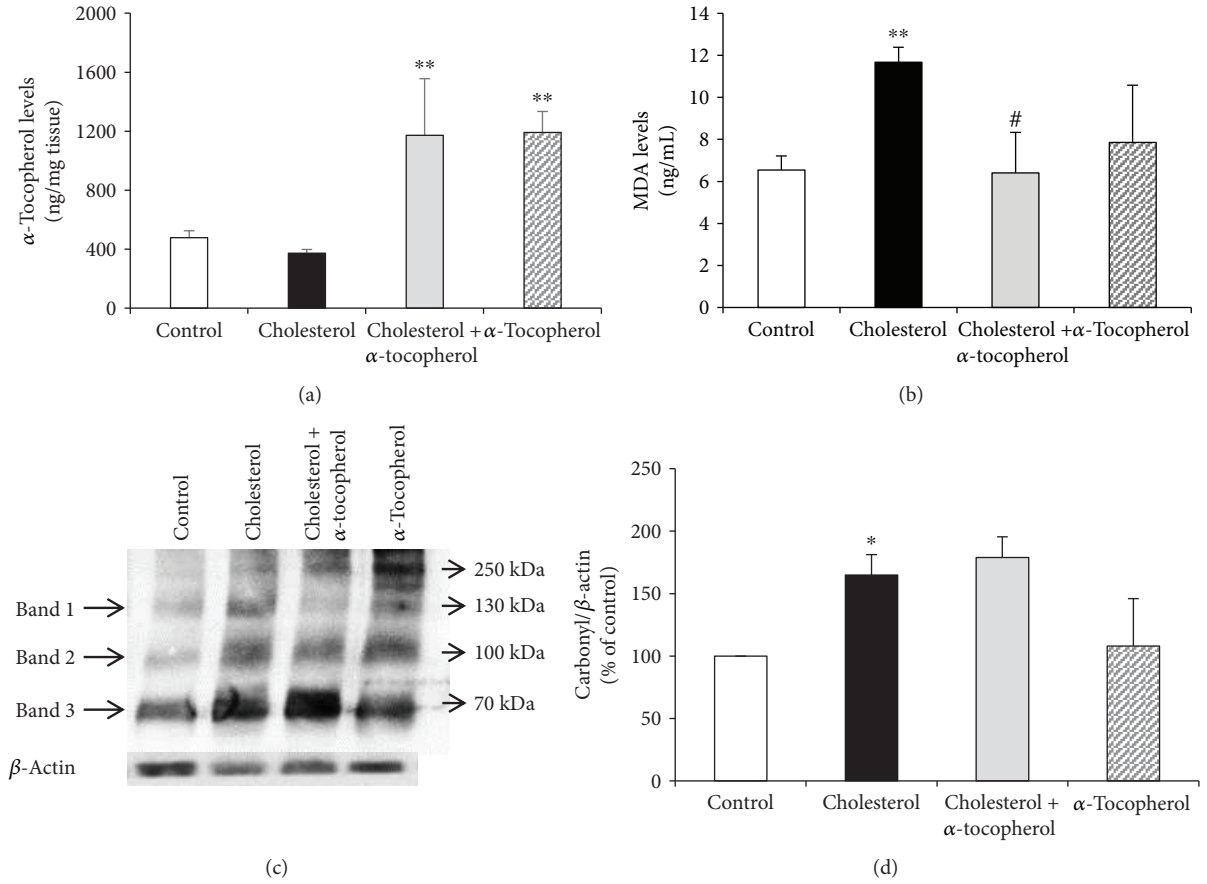

Figure 1: Alpha-tocopherol levels and oxidative status in heart tissue. Left ventricles of heart tissue were harvested, and α-tocopherol (a) and MDA levels (b) were quantified by LC-MS/MS. Protein carbonyl content was analyzed by Oxyblot following densitometric analysis of three different protein bands. Relative ratios were quantified and normalized using β-actin (c). Data are expressed as the mean ± SD. $^{*,**}p < 0.05$ and 0.01 versus the control group; $^{\#}p < 0.05$ versus the cholesterol group ($n = 3$).

2.10. Gene Expression Analysis. 100 mg of heart tissues was homogenized, and total RNAs were isolated with the RNA Midi Kit (QIAGEN) followed by reverse transcription using the Transcriptor High Fidelity cDNA Synthesis kit (ROCHE). Quantitative reverse transcriptase PCR was applied to cDNA with using the QuantiTect PCR Sybr Green kit (QIAGEN) and Rotor Gene Q-RT PCR system (QIAGEN). The threshold cycle (CT) was determined, and the relative gene expression subsequently was calculated as follows: fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT - CT$ target housekeeping (β-actin) and $\Delta(\Delta CT) = \Delta CT - CT$-treated control. The sequences of primers used to detect the expression of rabbit transcripts are listed in Supplementary Table 2.

2.11. Statistical Analysis. Statistical analysis was performed using Prism 4 (Graph-Pad) software. For the determination of statistical significances of differences, one-way ANOVA was performed followed by multiple comparisons using Student's *t*-test. A *P* value less than 0.05 has been accepted to be statistically significant.

3. Results

3.1. Serum Cholesterol and α-Tocopherol Levels. To reveal the effect of a 2% cholesterol diet for 8 weeks on lipid and oxysterol metabolism in the heart tissue, we have established a well-known hypercholesterolemic rabbit model. In order to ensure our *in vivo* hypercholesterolemic model and supplementation of α-tocopherol, firstly, we measured cholesterol and α-tocopherol levels in serum. We have found an approximately 40-fold increase in serum cholesterol of 2% cholesterol-fed rabbits and a 10-fold increase of α-tocopherol in serum by its supplementation (Table 1) which supports our previous results [17, 22, 23].

Table 1: Effect of 2% cholesterol diet and α-tocopherol supplementation on serum cholesterol and α-tocopherol levels following eight weeks.

	Serum cholesterol levels (mg/dL)	Serum α-tocopherol levels (μg/mL)
Control	52.0 ± 9.8	2.8 ± 1.3
Cholesterol	2027.2 ± 860.4***	24.5 ± 3.4***
Cholesterol + α-tocopherol	2341.4 ± 552.5***	21.1 ± 4.7***
α-Tocopherol	60.2 ± 23.9	17.3 ± 10.3***

Data are expressed as the mean ± SD. $^{***}p < 0.001$ versus the control group ($n = 5$).

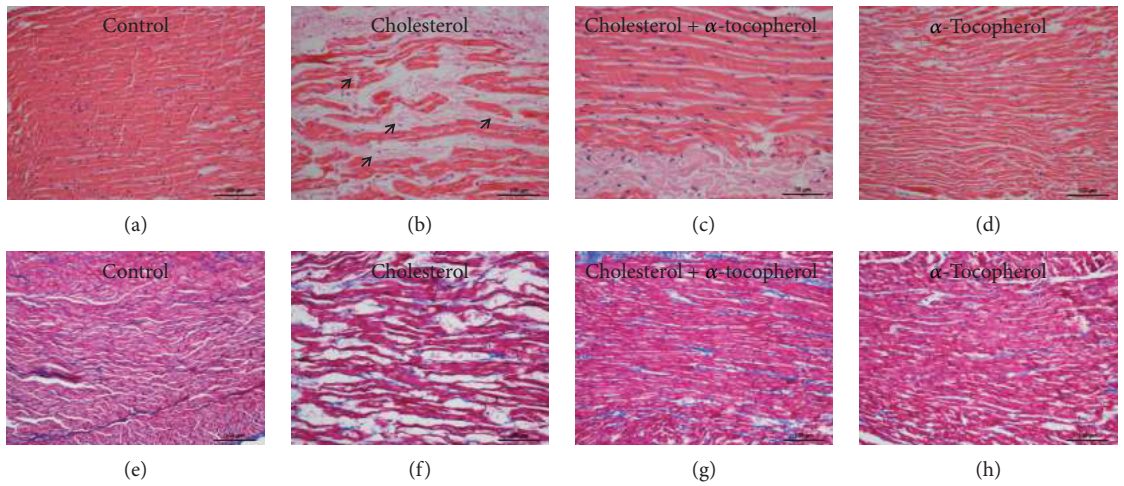

Figure 2: Light microscopic images of the myocardium from experimental animals. Fixed left ventricle of heart tissues in 10% buffered formaldehyde for 4 h dehydrated and incubated in xylol for 1 h twice, embedded in paraffin. 5 μm thick sections were stained with hemotoxylene eosin (a–d) and Masson's trichrome (e–h) before microscopic examination. Representative H&E-stained sections from the control (a), cholesterol (b), cholesterol + α-tocopherol (c), and α-tocopherol (d) groups. A significant number of cardiomyocytes exhibited myofibrillar loss (arrows) in cholesterol group rabbits (b). Masson's trichrome-stained sections from the control (e), cholesterol (f), cholesterol + α-tocopherol (g), and α-tocopherol (h) groups show fibrotic remodeling of the left ventricle. No difference of fibrosis levels was observed between all groups of rabbits. Photographs taken at 200x magnification (scale bar = 100 μm).

3.2. Alpha-Tocopherol Levels and Oxidative Status in Heart Tissue. To determine the α-tocopherol content and the oxidation status of lipids and proteins, we examined α-tocopherol and MDA levels, together with the protein carbonyl formation, by LC-MS/MS and Oxyblot, respectively. As expected, increased levels of α-tocopherol were observed in heart tissues of cholesterol + α-tocopherol and α-tocopherol rabbits compared to control (Figure 1(a)). In the scope of lipid oxidation, the cholesterol group demonstrated an increase of MDA levels which was decreased to control levels by α-tocopherol supplementation (Figure 1(b)). Additionally, as shown in Figure 1(c), we found that a high cholesterol diet also induces protein carbonyl formation compared to control followed by no significant change in the cholesterol + α-tocopherol group compared to cholesterol.

3.3. Light Microscopy Examination of Heart Tissue. To evaluate the morphological features of the heart, hematoxylin-eosin (Figures 1(a)–1(d)) and Masson's trichrome (Figures 2(e)–2(h)) stainings were performed. While the control and α-tocopherol groups had normal myocardium morphology (Figures 2(a) and 2(d)), cholesterol-fed rabbits were indicated with heart tissue damage as increased myofibrillar loss which was reduced in animals supplemented α-tocopherol (Figures 2(b) and 2(c)). Additionally, Masson's trichrome-stained sections visualized with light microscopy showed no difference in fibrosis levels between the control (Figure 2(e)), cholesterol (Figure 2(f)), cholesterol + α-tocopherol (Figure 2(g)), and α-tocopherol (Figure 2(h)) groups of rabbits.

3.4. Free Fatty Acid Profiling following 2% Cholesterol Diet and α-Tocopherol Supplementation in Heart Tissue. Following the confirmation of our *in vivo* model, we have identified the alterations of free fatty acid composition in heart tissue by the GC-MS method. As shown in Figure 3(a), neither the 2% cholesterol diet nor α-tocopherol supplementation had any significant effect on total levels of saturated fatty acid (SFA) and unsaturated fatty acid (UFA). However, by evaluating the distribution of the different SFAs in the heart, we found a significant decrease of palmitate levels (16 : 0) in both the cholesterol and the α-tocopherol groups compared to control while no significant effect of α-tocopherol was observed in hypercholesterolemic rabbits (Figure 3(b)).

Moreover, at the scope of UFA distribution, increased levels of eicosadienoate (20:2n6), eicosatrienoate (20:3n6), and arachidonate (20:4n6) were found in the cholesterol group compared to control. Accumulation of oleate (18:1n9) and α-linolenate (18:3n3) was also observed in the α-tocopherol group compared to control. Interestingly, we found that α-tocopherol supplementation, in cholesterol-fed rabbits, showed its effect by increasing γ-linolenate (18:3n6) levels in the heart tissue (Figure 3(c)).

3.5. Oxysterol Profiling in Heart following High Cholesterol Diet and α-Tocopherol Supplementation. Oxysterols occur enzymatically by side chain hydroxylation of cholesterol and were nonenzymatically formed by the attack of reactive oxygen species to the C-7 sterol ring of cholesterol [24]. Under induced inflammatory and oxidative stress conditions, excessive free cholesterol levels are prone to autoxidation to oxysterols which were identified as a major risk factor for cardiovascular disease development [25]. As shown in Figure 4, our LC-MS/MS findings from the heart tissue revealed that both 4β-, 25-, and 27-hydroxycholesterol and 7-ketocholesterol levels were increased significantly in the cholesterol group compared to control. Levels of 27-hydroxycholesterol also continued to increase significantly in the cholesterol + α-tocopherol group compared to

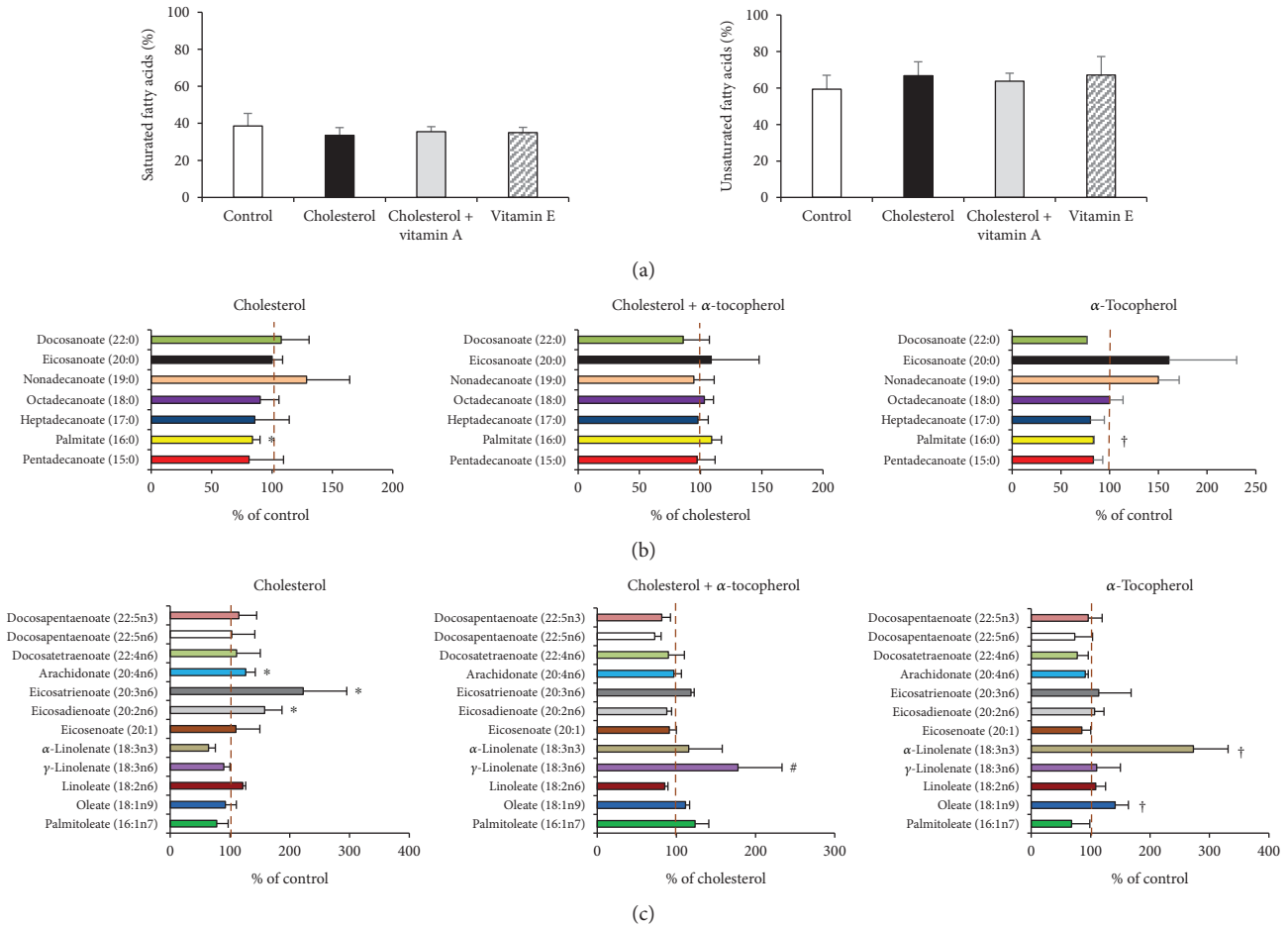

Figure 3: Fatty acid profile following 2% cholesterol diet and α-tocopherol supplementation. Left ventricles of heart tissue were harvested, and fatty acid content was analyzed by GC-MS. Levels of saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) (a). Complete profiles of SFAs (b) and UFAs (c). Data are expressed as the mean ± SD. $^{*}p < 0.05$ and $^{\dagger}p < 0.05$ versus the control group; $^{\#}p < 0.05$ versus the cholesterol group ($n = 3$).

cholesterol. This pattern was not observed in other oxysterols, such as 4β-hydroxycholesterol, 25-hydroxycholesterol, and 7-ketocholesterol. Additionally, α-tocopherol supplementation in normocholesterolemic rabbits showed its effect by increasing 7-ketocholesterol levels compared to control.

3.6. Expression of Fatty Acid and Cholesterol Metabolism-Related Parameters. Increased levels of certain fatty acids and oxysterols direct us to evaluate gene/protein expressions of fatty acid and cholesterol metabolism parameters. LXRα and PPARα are two important transcription factors that are highly expressed in the heart and promote the genes responsible for lipid metabolism, inflammation, and cholesterol efflux [7, 8]. In the view of our results and literature, we have investigated SREBP1c and LXRα protein expressions together with LXRα and PPARα mRNA levels. In a translational manner, we have identified that both SREBP1c and LXRα expressions were significantly induced which were reduced to control levels by α-tocopherol supplementation (Figures 5(a) and 5(b)), although no band formation of cleaved SREBP1c was observed that was correlated with decreased palmitate levels by a high cholesterol diet. Similar to protein expression, the high cholesterol diet also induced the mRNA level of LXRα whereas α-tocopherol has no effect (Figure 5(c)). However, neither the high cholesterol diet nor α-tocopherol supplementation had any significant change on PPAR-α mRNA expression (Figure 5(d)). Taken together, LC-MS/MS, protein, and mRNA findings lead us to conclude that increased oxysterol levels might lead to the induction of LXRα signaling.

3.7. Alterations in Oxysterol Metabolism Effect Scavenger Receptor Expression and Reverse Cholesterol Transport. Although the involvement of scavenger receptors and reverse cholesterol transport is well documented in CVDs, the mechanistic link between oxysterols and SRs, following a high cholesterol diet, has not yet been fully identified. As shown in Figure 6, mRNA expressions of various scavenger receptors, such as CD36, CD68, CD204 (SR-A), and SR-F1, were induced by the high cholesterol diet while α-tocopherol supplementation significantly inhibited this induction. We also observed that α-tocopherol supplementation, in normocholesterolemic rabbits, showed its effect by inducing the mRNA levels of class F scavenger receptor (SR-F1).

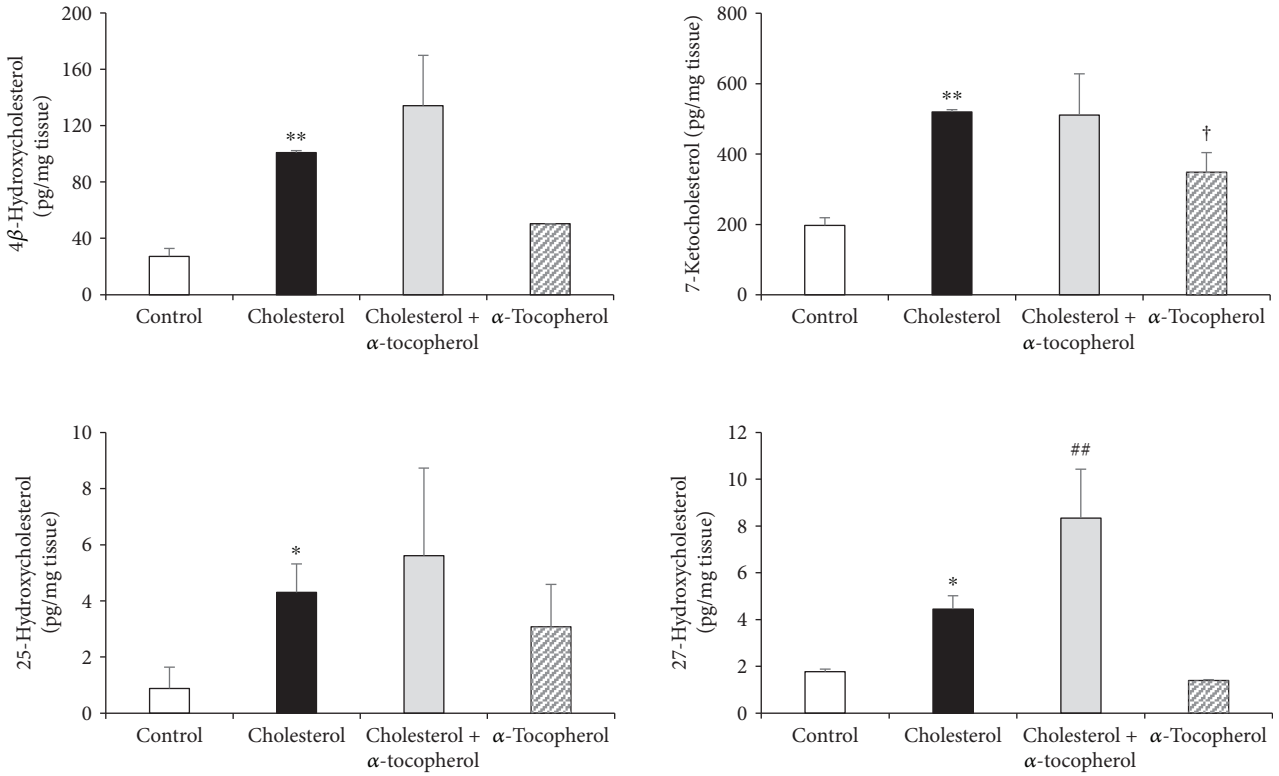

Figure 4: Effect of 2% of cholesterol diet and α-tocopherol supplementation on heart 4β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol concentrations. Oxysterol levels were quantified by LC-MS/MS in left ventricles of heart tissue. Data are expressed as the mean ± S.D. $^{*,**}p < 0.05$ and 0.01 and $^{\dagger}p < 0.05$ versus the control group; $^{\#\#}p < 0.01$ versus the cholesterol group ($n = 3$).

In order to evaluate the modulations in the reverse cholesterol transport system, we evaluated the mRNA expression of ABCA1 by qPCR. Our findings revealed an approximately 5-fold increase in mRNA expression of ABCA1 in the cholesterol-fed group which was continued to increase significantly by α-tocopherol supplementation in the cholesterol + α-tocopherol group (Figure 6). These results were also parallel to 27-hydroxycholesterol levels in our *in vivo* model and led us to hypothesize a boosting effect of α-tocopherol in a reverse cholesterol transport through 27-hydroxycholesterol induction.

4. Discussion

With the highest morbidity and mortality rates, CVDs are the leading death cause, globally. According to the WHO report, approximately 17.5 million people, which equals to 31% of all deaths, died from CVDs in 2012 [26]. Epidemiological studies identified elevated LDL cholesterol levels, even the absence of other risk factors might enhance the progression of CVD [27]. However, there is little information in the literature about the alterations of fatty acid and oxysterol profiles and scavenger receptors following a high cholesterol diet and α-tocopherol supplementation. Based on our hypercholesterolemic rabbit model, we showed that the 2% cholesterol diet and α-tocopherol supplementation for 8 weeks might be associated with changed free fatty acid and oxysterol compositions, proteins related to lipid metabolism, and scavenger receptors in parallel to histological alterations.

Our high cholesterol-fed rabbits showed significantly increased serum cholesterol levels compared to controls. Serum α-tocopherol levels were also enhanced in α-tocopherol-supplemented rabbits. α-Tocopherol is a beneficial nutrient transported in plasma lipoproteins because of its hydrophobic nature. The observation of increased serum α-tocopherol levels in the cholesterol group, compared to control, is mostly based on that α-tocopherol is a fat-soluble vitamin carried by LDL in the blood and a higher α-tocopherol uptake caused by the increased lipid uptake produced by the high cholesterol diet. Despite the increase of α-tocopherol levels in serum samples of the cholesterol group, we did not observe any change in heart tissue samples of the same animals. These results prove that tissue levels of α-tocopherol increased mostly by daily injections and α-tocopherol-mediated regulations can only be hypothesized in the cholesterol + α-tocopherol and α-tocopherol groups.

The use of MDA and protein carbonyls as a parameter of lipid and protein oxidation is accepted in patients during coronary heart surgery [28]. In the present study, detection of high MDA levels and protein carbonyl formation in the heart tissue reflects the effect of the high cholesterol diet on oxidized lipid and protein accumulation *in vivo*. We also demonstrated that α-tocopherol supplementation had the capacity of decreasing high cholesterol

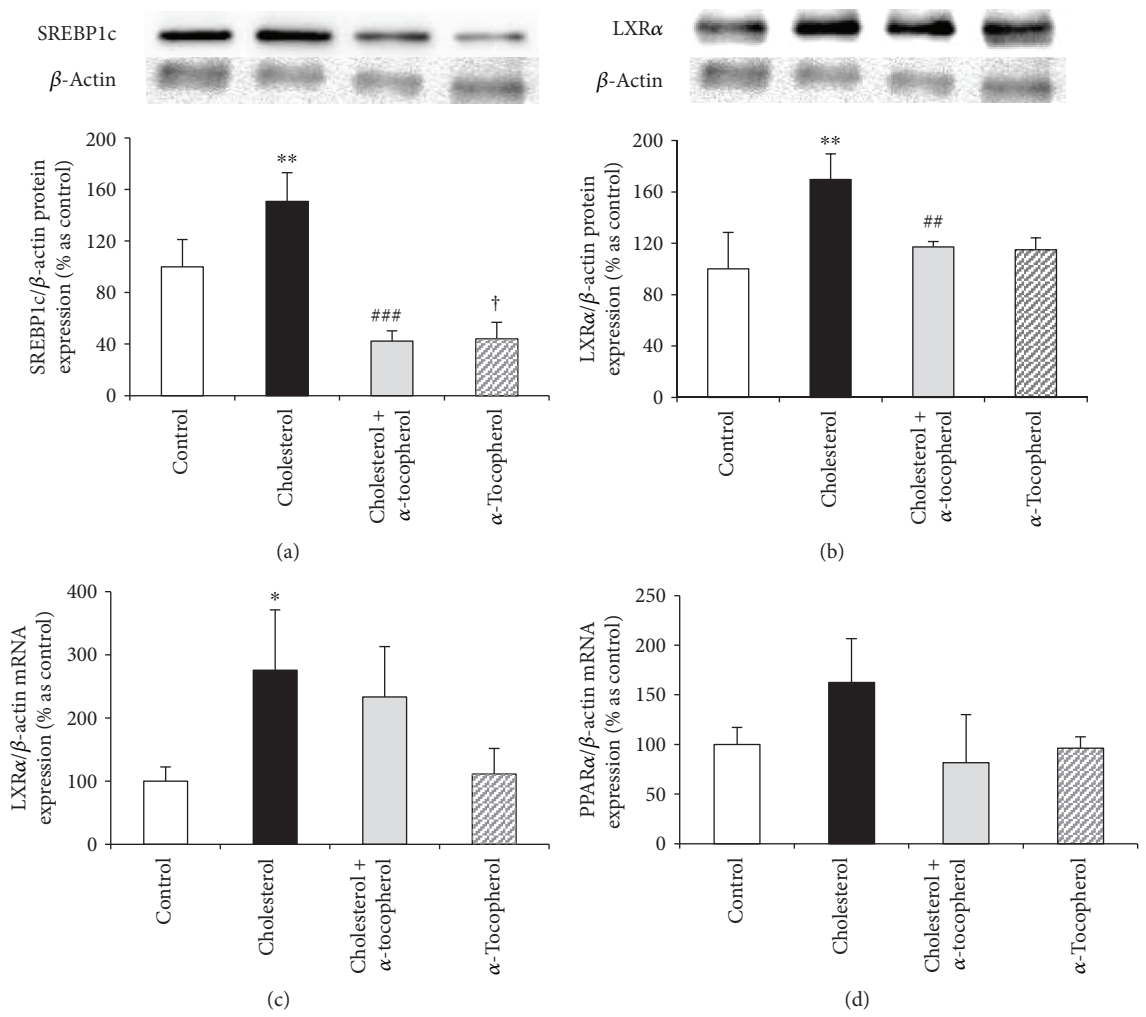

Figure 5: Expression of fatty acid and cholesterol metabolism-related parameters. Protein expressions in heart tissue of each animal were measured by Western blotting with densitometric analysis of protein bands, and relative ratios were quantified and normalized relative to β-actin against SREBP1c (a) and LXRα (b) antibodies. mRNA expressions of each animal were measured by RT-PCR and normalized to β-actin. Relative mRNA expressions of LXRα (c) and PPARα (d). Data are expressed as mean ± S.D. $^{*,**}p < 0.05$ and 0.01 and $^{\dagger}p < 0.05$ versus the control group; $^{\#\#,\#\#\#}p < 0.01$ and 0.001 versus the cholesterol group ($n = 3$).

diet-induced MDA levels with no influence on protein carbonylation. Altogether, cholesterol, α-tocopherol, and oxidative stress results indicate a positive correlation between hypercholesterolemia and oxidative stress, which supports our previous results [17, 22, 23] and also is in agreement with the literature [29, 30].

Recent studies have classified the progression of cardiomyopathy into four classes on the basis of pathology. While class I is characterized by cardiomyocyte alterations, class II contains fibrotic remodeling, class III exhibits fibrosis and cardiomyocyte damage, and class IV consists the accumulation of noncollagenous material or inflammatory response with or without cardiomyocyte alterations [31]. Our light microscopy findings from the cholesterol-fed group exhibited the loss of myofibrils, without effecting fibrosis, which suggests the presence of cardiomyopathy with class I characteristics. Moreover, cholesterol-fed rabbits undergoing α-tocopherol supplementation showed reduced alteration in the morphology of the myocardium.

Although the oxidation of fatty acids is the major energy source for contractility, in regard to limited de novo synthesis capacity, cardiomyocytes rely heavily on the uptake of fatty acids in the form of nonesterified (free) or lipoproteins [32]. To date, three major transport proteins are identified for the uptake of circulating fatty acids: CD36 (also known as fatty acid translocase (FAT)), fatty acid-binding protein (FABP), and fatty acid transport protein (FATP) [33]. To examine the effect of SFAs versus UFAs in primary cardiomyocytes, Vries et al. have identified C16:0 (palmitate) or C18:0 (stearate) has a capacity to induce apoptosis, while C16:1 (palmitoleate) or cis-C18:1 (oleate) has no effect on cell viability [34]. Our GC-MS findings lead us to conclude, despite the unchanged level of total UFAs, eicosadienoate (20:2n6), eicosatrienoate (20:3n6), and arachidonate (20:4n6) levels were increased in the cholesterol group. γ-Linolenate (18:3n6) levels were also increased in the cholesterol + α-tocopherol group compared to cholesterol (Figure 3). Since the accumulation

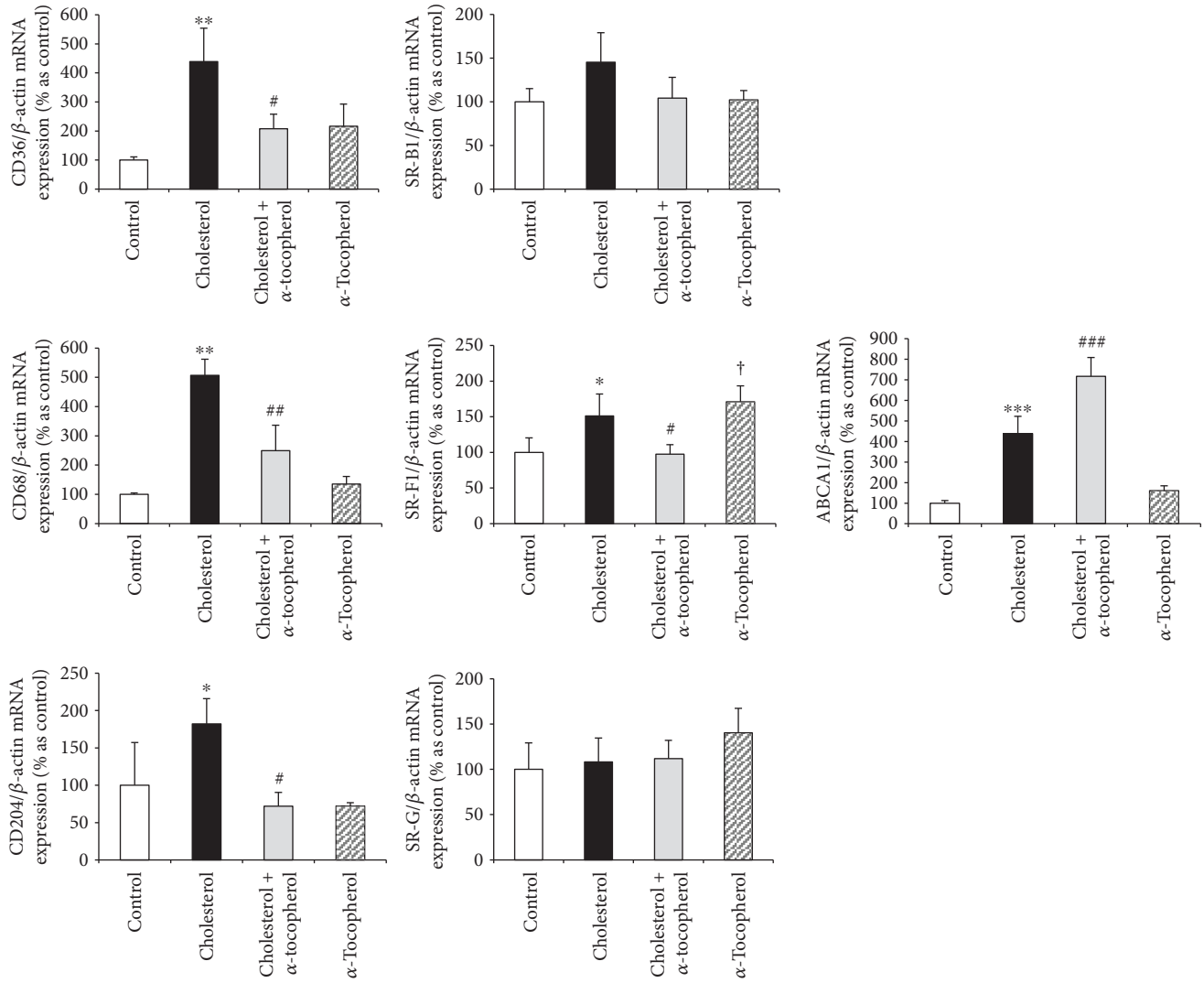

Figure 6: Alterations in oxysterol metabolism effect scavenger receptor expression and reverse cholesterol transport. mRNA expressions in heart tissue of each animal were measured by RT-PCR and normalized to β-actin. Relative mRNA expressions of CD36, CD68, CD204, SR-B1, SR-F1, SR-G, and ABCA1. Data are expressed as the mean ± SD. $^{*,**,***}p < 0.05$, 0.01, and 0.001 and $^{\dagger}p < 0.05$ versus the control group; $^{\#,\#\#,\#\#\#}p < 0.05$, 0.01, and 0.001 versus the cholesterol group ($n = 3$).

of eicosatrienoate (20:3n6) and arachidonate (20:4n6) can induce inflammation [35, 36], this might enlighten our previous results (using same tissue samples) which determines an induction of inflammatory cytokines (despite the absence of inflammatory infiltration histologically) in cholesterol-fed rabbits (unpublished data). However, the decrease of palmitate levels in the cholesterol and α-tocopherol groups, without effecting oxysterol and scavenger receptor expressions, might be based on the increased energy consumption. Further studies are needed to reveal this hypothesis.

Oxygenated derivatives of cholesterol (oxysterols) are biologically active molecules and, due to their lipophilic composition, easily penetrate from macrophages into the surrounding cells, including cardiac cells. Increased ROS levels in the macrophage are implied as the major source of oxysterol production in all tissues [2]. Association of cardiomyocyte oxysterols with CVD development is reported as an important factor through the regulation of cell hypertrophy and death [25]. Additionally, the involvement of oxysterols has been identified either in the plasma samples of coronary artery bypass grafted patients [37, 38] or in those of cardiac catheterized patients [39]. A variety of oxysterols, including 4β-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol is also investigated by LC-MS/MS experiments. As shown in Figure 4, the high cholesterol diet for 8 weeks had the capacity to enhance all four oxysterol levels. In contrast to the other oxysterols, 27-hydroxycholesterol continued to increase significantly in hypercholesterolemic animals supplemented with α-tocopherol. The effect of α-tocopherol supplementation in normocholesterolemic animals was also tested, and only 7-ketocholesterol was found to increase in the left ventricle of the heart tissue. Thus far, our results show that the boosting role of α-tocopherol on 7-ketocholesterol in normocholesterolemic animals has no significant effect on scavenger receptor levels in the heart tissue.

Following the delivery of cholesterol into the cell, it is transported either to ER, facilitating the sequestration of SREBPs that reduces cholesterol synthesis and uptake, or to the plasma membrane, enhancing cholesterol efflux by ABCA1. LXRα is a nuclear receptor which is highly expressed in macrophages. Following the activation by a number of oxysterols, such as 25- and 27-hydroxycholesterol, LXRα acts as a transcription factor and enhances the expression of genes involved in cholesterol homeostasis, fatty acid metabolism, and inflammation. It is clear that 27-hydroxycholesterol-mediated LXRα activation enhances cholesterol efflux via ATP-binding cassette transporters, such as ABCA1 and ABCG1, and reduces the expression of proinflammatory cytokines [1]. In this regard, a number of studies have also determined oxysterol-mediated LXRα activation as a crucial step in reverse-cholesterol transport and foam cell formation by inducing ABCA1 levels [40, 41]. Oxysterol-mediated LXRα activation might also enhance the transcription of sterol regulatory element-binding protein 1c (SREBP1c) that stimulates lipogenesis [8]. Besides the LXRα, oxysterols have been shown to accelerate SREBP sequestration by interacting with Insig-1/2 [1]. Similar to the protein level, mRNA expression of LXRα was induced by the high cholesterol diet, without any change in PPARα mRNA. Moreover, despite the significant increase of the uncleaved form of SREBP1c, no band formation of cleaved SREBP1c was observed, which is correlated with a decrease in palmitate levels and led us to hypothesize the downregulation of lipogenesis by the high cholesterol diet (Figure 5).

Our oxysterol and LXR data suggested to us a theory involving SRs. SRs are crucial transporters, together with lipoprotein receptors, in the delivery of exogenous cholesterol to endocytic pathways. CD36 is one of the most studied SRs, which binds a number of ligands including long-chain fatty acids, apoptotic cells, and OxLDL, in a high-fat diet, inflammation, or oxidative stress. [42]. Leonarduzzi et al. have observed a marked increase in both synthesis and expression of CD36 on human U937 promonocytic cells by using an oxysterol mixture, which contains major oxysterols of dietary origin but not 27-hydroxycholesterol [43]. In another study, the oxysterol mixture (contains high rate of 27-hydroxycholesterol compared to Leonarduzzi et al.) reduced the expression of CD36 and CD204 SRs, while upregulating LXRα and ABCA1 levels in the stimulation of macrophage polarization toward the M2 phenotype [44]. Moreover, in contrast to 25-hydroxycholesterol, 7-ketocholesterol treatment has been described to promote THP-1 differentiation, by increasing CD11b, CD36, and CD68 expressions, and leads to foam cell formation following the exposure of oxLDL [45]. In our study, the marked increase of various scavenger receptors, including CD36, CD68, CD204, and SR-F1, was observed in heart tissues of rabbits fed with the high cholesterol diet and these increases were completely inhibited by α-tocopherol supplementation. A similar increase in cholesterol rabbits was also obtained in ABCA1 levels which was differently continued to increase with α-tocopherol, similar to 27-hydroxycholesterol.

PPARα is another lipid-activated transcription factor and known to modulate lipid metabolism in the progression of atherosclerosis. Scavenger receptor class B type I (SR-BI), responsible for HDL-mediated cholesterol efflux, is shown to regulate by both PPAR-α ligands in human macrophages and atherosclerotic lesions of apoE knockout mice [46]. In our study, we did not observe any significant effect of the high cholesterol diet and α-tocopherol supplementation either in PPARα or in SR-B1 mRNA levels which proved ABCA1 was more effective in RCT in the heart tissue of our model.

Further studies have shown that 7-ketocholesterol-related changes in various gene-involved different pathways, such as inflammation and lipid homeostasis, might be regulated by vitamin E [47]. In a related study, 7-ketocholesterol-mediated activation of oxiapoptophagy, a mixed form of cell death that contains the features of oxidation, apoptosis, and autophagy, has been reported to be downregulated by α-tocopherol [48]. Additionally, 7-ketocholesterol-induced autophagic death of human smooth muscle cells has also been reported [49]. In our unpublished results, we have determined that excessively induced ER stress triggers autophagic activity in the same tissue samples of cholesterol-fed rabbits, which results in the loss of cardiomyocytes by autophagic cell death.

This study shows that the high cholesterol diet did not change total saturated/unsaturated fatty acid levels but increased oxysterols, particularly 7-ketocholesterol, stimulated various SR expressions, such as CD36, CD68, CD204, and SR-F1. At the same time, 25-hydroxycholesterol and 27-hydroxycholesterol-mediated LXR activation upregulated ABCA1 levels. α-Tocopherol supplementation, together with the high cholesterol diet, showed its effect by decreasing SR expressions in a transcriptional manner, while continuing to boost reverse transport of excess cholesterol through 27-hydroxycholesterol and ABCA1 levels without effecting LXRα expression. α-Tocopherol shows its beneficial effects on various diseases such as atherosclerosis, coronary heart diseases, neurodegenerative diseases, and cancer [50, 51]. According to Poirier et al., α-tocopherol has the capacity to increase 27-hydroxycholesterol synthesis following the induction in oxysterol-generating enzyme, hepatic 27-hydroxylase (CYP27A1), expression [52]. Based on the literature and our previous results in aorta tissue [17], we hypothesized that α-tocopherol supplementation might show its beneficial effect by increasing ABCA1 expression through 27-hydroxycholesterol in hypercholesterolemic conditions.

In conclusion, this study presents a comprehensive data of the alterations in fatty acids, oxysterols, and SR composition and their implication with histological modifications of the heart tissue in cholesterol-fed rabbits. Future studies are also needed to identify the biological association of the identified changes in the lipid profile, as well as the effect of α-tocopherol supplementation. In addition to the present study, new findings in the progression of lipid metabolism and SR alterations will not only increase our understanding but also lead the development of further theories and therapeutic strategies in the field of hypercholesterolemia-mediated heart diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Erdi Sozen, Burak Yazgan, and Nesrin Kartal Ozer generated the initial idea and experimental design. Erdi Sozen, Burak Yazgan, and Ali Sahin performed the experiments and analyzed the data. Umit Ince conducted the light microscopy experiments and analyzed the data. All authors performed the critical revision of the manuscript and gave final approval of the submitted version.

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Supplementary Materials

Supplementary material contains an overview of the identified fatty acids and the sequences of primers to detect the expression of rabbit transcripts. Supplementary Table 1: identified fatty acid profile which contains trivial and the IUPAC names together with short-hand nomenclature. Supplementary Table 2: primer sequences used for the quantitative real-time PCR experiments. *(Supplementary Materials)*

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Research Article

Work Intensity, Low-Grade Inflammation, and Oxidative Status: A Comparison between Office and Slaughterhouse Workers

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Limited knowledge exists about the impact of physical workload on oxidative stress in different occupational categories. Thus, we aimed to investigate the oxidative and inflammatory status in employees with different physical workloads. We enrolled a total of 79 male subjects, 27 office workers (mean age 38.8 ± 9.1 years) and 52 heavy workers, in a slaughterhouse (mean age 40.8 ± 8.2 years). Fasting blood was drawn from an antecubital vein in the morning of the midweek before an 8-hour or 12-hour work shift. The antioxidative capacity was assessed measuring total antioxidant capacity (TAC), uric acid, total polyphenols (PPm), and endogenous peroxidase activity (EPA). Total peroxides (TOC), malondialdehyde (MDA), and myeloperoxidase (MPO) were analyzed as prooxidative biomarkers, and an oxidative stress index (OSI) was calculated. In addition, hsCRP, interleukin-6 (IL-6), MDA-LDL IgM antibodies, galectin-3, adrenocorticotropic hormone (ACTH), and the brain-derived neurotrophic factor (BDNF) were measured as biomarkers of chronic systemic inflammation and emotional stress. TOC ($p = 0.032$), TAC ($p < 0.001$), ACTH ($p < 0.001$), OSI ($p = 0.011$), and hsCRP ($p = 0.019$) were significantly increased in the heavy workers group, while EPA, BDNF ($p < 0.001$), and polyphenols ($p = 0.004$) were significantly higher in office workers. Comparison between 8 and 12 h shifts showed a worse psychological condition in heavy workers with increased levels for hsCRP ($p = 0.001$) and reduced concentration of BDNF ($p = 0.012$) compared to office workers. Oxidative stress and inflammation are induced in heavy workers and are particularly pronounced during long working hours, that is, 12-hour versus 8-hour shifts.

1. Introduction

Modern life-style, that is, physical inactivity and fast food as well as occupational and environmental conditions, may induce oxidative stress. Different types of stress can be distinguished at the cellular and tissue level—namely, photooxidative stress, drug-dependent oxidative stress, metabolic oxidative stress, environmental oxidative stress, and nitrosative stress [1].

Reactive oxygen species (ROS) are endogenously generated, among others, in the respiratory chain. Hence, metabolic activity increases ROS production. These species react with biological molecules like lipids, carbohydrates, proteins, and even DNA, which are associated with the pathogenesis of degenerative diseases. Oxidative stress (OS) is associated with chronic inflammation, with a potential impact on diabetes mellitus, atherosclerosis, and cardiovascular and neurodegenerative diseases [2–5]. An increased consumption of oxygen during physical exercise also increases ROS production leading to oxidative stress and lipid peroxidation in athletes [6–8]. Nevertheless, increased ROS production during sports is also beneficial because it stimulates the antioxidative system [9]. Moreover, OS is a key factor during aging [10] together with other factors like deregulated autophagy, mitochondrial dysfunction, and telomere shortening [11]. Besides its involvement in the physiologic process of aging, OS appears to play an important role in the pathophysiology of several occupational diseases [12, 13]. Common problems in night and shift workers, such as fatigue, sleep problems, anxiety, difficulties in maintaining regular life-styles, and reduced recovery times, represent an increased health risk due to physiological exhaustion and a decreased capacity for regulation [14].

Nevertheless, there is limited knowledge about the impact of physical workload on OS in different occupational groups. Heavy workers often suffer from excessive workload and lack of social support. Shift work with extended working hours might negatively affect the psychological status of employees and reduce their motivation. It can be hypothesized that high physical and emotional stress in heavy workers is associated with increased OS and inflammation.

The present study aimed at comparing the oxidative and inflammatory status between office workers and heavy workers with a particular focus on the biochemical effect of extended working hours (8- to 12-hour shifts).

2. Materials and Methods

2.1. Study Population. We enrolled 79 healthy male volunteers between 18 and 65 years at their workplace. Thereof, 27 employees were office workers (age 38.8 ± 9.1 years) and 52 heavy workers in a slaughterhouse (age 40.8 ± 8.2 years). Exclusion criteria were infections, for example, flu-like infection, chronic diseases, and certified reduced work capacity due to illness. The study was approved by the ethics committee of the Medical University of Graz (EK number 26-488 ex 13/14) and conducted in compliance with guidelines for human studies as described in the Helsinki Declaration of 1975, revised in 1996. Written inform consent was obtained from all study participants.

2.2. Laboratory Analysis

2.2.1. Blood Sampling. Blood was drawn from an antecubital vein between 6:00 a.m. and 6:30 a.m., before an 8-hour work shift from 79 workers (27 office and 52 heavy workers). In a subgroup of 26 office workers and 8 heavy workers, we investigated the effects of twelve hours of work. Blood sampling was performed in the midweek, Wednesdays or Thursdays. Samples were immediately transferred on ice to the Lab within two hours, centrifuged, and stored at −80°C until use (6 to 13 months).

2.2.2. Inflammatory Parameters. High-sensitivity C-reactive protein (hsCRP) and interleukin 6 (IL-6) were determined on a COBAS® 8000 analyzer with turbidimetric and electrochemiluminescence immunoassays (ECLIA), respectively, from Roche Diagnostics (Rotkreuz, Switzerland). All measurements were batched into a single run. The total imprecision of both assays were below 5%. Galectin-3 was measured using the Human Galectin-3 Quantikine ELISA Kit from R&D (Minneapolis, USA).

2.2.3. Oxidative Stress Biomarkers. Malondialdehyde (MDA) was determined by GC-MS from Thermo Fisher Scientific (CA, USA). After addition of MDA-d 2 as internal standard, derivatization with 2,4-dinitrophenylhydrazine, and chemical ionization in negative mode, the representative ions m/z 204 (for MDA) and m/z 206 (for MDA-d 2) were recorded [15].

Colorimetric methods were used to determine total peroxides (TOC), endogenous peroxidase activity (EPA), and the total antioxidant capacity (TAC) purchased from LDN (Labor Diagnostika Nord, Nordhorn, Germany). These assays are based on the reaction between hydrogen peroxide, horseradish peroxidase, and tetramethylbenzidine to give a blue-green colour. After the addition of the stop solution, the colour changes to yellow, which can be measured at 450 nm (reference wavelength 620 nm). A linear standard curve was used for quantification. The intra- and interassay coefficients of variance were less than 5% for all assays [16]. MDA-LDL IgM was measured with the MDA-LDL-IgM ELISA from Omnignostica Ltd. (Höflein/D., Austria), which is standardized on a human monoclonal antibody as described previously [17]. Serum myeloperoxidase (MPO) concentrations were measured with the MPO enzyme-linked immunosorbent assay (ELISA) Kit (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions. The total imprecision of both ELISA assays was below 7%. Uric acid was determined with the enzymatic colorimetric test from Roche on a COBAS 8000 analyzer.

In the case of the brain-derived neurotrophic factor (BDNF), we used the Quantikine human BDNF immunoassay from R&D systems (Minneapolis, USA). Adrenocorticotropic hormone (ACTH) was determined with the ACTH ELISA from Hölzel Diagnostica (Köln, Germany), and total polyphenols (PPm) were determined according to the manufacturer's instructions with an adapted Folin-Ciocalteu microtitre method from Omnignostica Ltd. (Höflein/D., Austria). In short, the principle of this method is based on the reaction of polyphenols with transition metals. This leads

Table 1: Baseline characteristics of study participants and results of measurements after an 8-hour work shift.

	Office workers ($n = 27$)	Heavy workers ($n = 52$)	
	Mean (95% confidence interval)		p
Age, yrs	38.2 (35.2–41.1)	40.8 (38.5–43.0)	0.175
Body mass index, kg/m^2	26.1 (24.5–27.6)	28.3 (27.1–29.5)	0.026
hsCRP, mg/L	1.0 (0.7–1.5)	1.7 (1.4–2.2)	0.019
IL-6, pg/mL	1.9 (1.6–2.2)	2.0 (1.7–2.2)	0.220
Uric acid, mg/dL	5.3 (4.8–5.7)	5.6 (5.3–5.9)	0.245
Total antioxidant capacity, mmol/L	1.06 (0.91–1.21)	1.41 (1.29–1.53)	<0.001
Total oxidant capacity, mmol/L	0.08 (0.06–0.11)	0.12 (0.10–0.15)	0.032
Oxidative stress index (OSI), %	5.7 (3.5–8.4)	10.4 (8.2–12.8)	0.011
Endogenous peroxidase activity, U/L	7.2 (6.2–8.4)	3.7 (3.3-4.1)	<0.001
Polyphenols, mmol/L	9.9 (9.7–10.1)	9.5 (9.4–9.7)	0.004
Malondialdehyde, μmol/L	0.74 (0.68–0.80)	0.69 (0.65–0.73)	0.144
Myeloperoxidase, μg/L	61.7 (56.4–67.4)	68.3 (63.7–73.2)	0.078
MDA-LDL-IgM, U/L	184 (149–228)	150 (127–177)	0.141
ST2, ng/mL	14.5 (12.3–16.6)	15.3 (13.7–17.0)	0.521
ACTH, pg/mL	12.0 (9.1–16.0)	27.6 (22.2–34.4)	<0.001
Galectin-3, ng/mL	5.9 (5.0–6.9)	4.8 (4.2–5.4)	0.052
BDNF, pg/mL	22880 (16051–32616)	7417 (5651–9735)	<0.001

p values from the general linear model with body mass index included as covariate. hsCRP = high-sensitivity C-reactive protein; IL-6 = interleukin-6; OSI = oxidative stress index; ACTH = adrenocorticotropic hormone; ST2 = suppression of tumorigenicity 2; BDNF = brain-derived neurotrophic factor.

to a dark-coloured complex, which can be measured at 766 nm. Samples are quantified by the use of a standard curve with serial dilutions of a polyphenol standard. The intra- and interassay coefficients of variance were less than 5%.

2.3. Statistical Analysis. Statistical analyses were carried out using SPSS 23.0 for Windows 10 (IBM Corp., USA) and Stata 12 (StataCorp, TX, USA). Comparisons between groups were done by the use of the general linear model including body mass index (BMI) as a covariate because heavy workers had significantly higher BMI which itself could be related to oxidative stress and inflammation, as reported previously [18]. Residuals of analyses were stored and tested for deviations from a normal distribution by Kolmogorov-Smirnov tests with Lilliefors-corrected p values. In case of a significant deviation, distribution of residuals was inspected, and in case of a skewed distribution, a logarithmic transformation was applied. In all such cases, normality of residuals was obtained after transformation. Homogeneity of variance was tested by Levene's tests. Data are summarized as means within groups and 95% confidence intervals (back-transformed if necessary to the original scale). A similar approach was applied for comparison of 8 h versus 12 h shifts. In this case, the within-subject factor (8 h/12 h shift length) and between-subject factor groups (office versus heavy workers) and their interaction were tested by analysis of variance. Comparisons of 8 h and 12 h shifts within groups were done by linear contrasts. Variables were log-transformed in accordance with the analysis of baseline data. Based on the ratio between ROS and serum antioxidant capacity, the oxidative stress index (OSI) was calculated using the formula (TOC[mmol/L]/TAC[mmol/L] × 100). For all statistical tests, $p < 0.05$ was considered significant.

3. Results

An overview about the anthropometric data of the study cohort is given in Table 1. Due to the fact that the BMI was significantly increased in heavy workers versus office workers, all further analyses were corrected with respect to this biometric parameter, because BMI itself was shown to be associated with OS [18].

Heavy workers had significantly increased TAC ($p < 0.001$), TOC ($p = 0.032$), hsCRP ($p = 0.019$), and ACTH ($p < 0.001$) (for details, see Figures 1 and 2 and Table 1) and OSI levels ($p = 0.011$; Table 1). In contrast, EPA ($p < 0.001$), polyphenols ($p = 0.004$), and BDNF ($p < 0.001$) levels were significantly higher in office workers (Figures 3 and 4). Uric acid, MDA, MPO, IL-6, MDA-LDL IgM, and galectin-3 did not differ between the groups (Table 1).

Comparison between 8-hour and 12-hour shifts revealed significant differences exclusively after a 12-hour shift in heavy workers, that is, a significant increased ACTH level ($p = 0.001$), while BDNF was significantly decreased at overtime work ($p = 0.012$) (Table 2). Correlation analysis between oxidative stress biomarkers revealed a significant negative correlation between TAC and EPA in both working groups whereas a positive correlation was found for TAC and uric acid (Table 3). TOC correlated positively with hsCRP in both working groups ($r = 0.612$ and 0.493 in office and heavy workers, resp.). In contrast, IL-6 was correlated to TOC merely in office workers ($r = 0.462$), while the

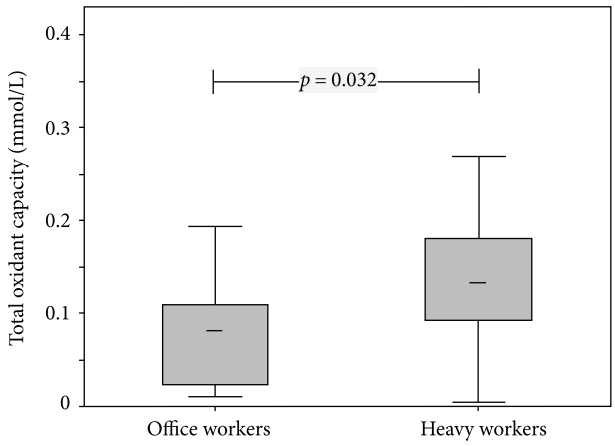

Figure 1: Box plots (medians, interquartile, and nonoutlier ranges) of total oxidant capacity by groups of workers.

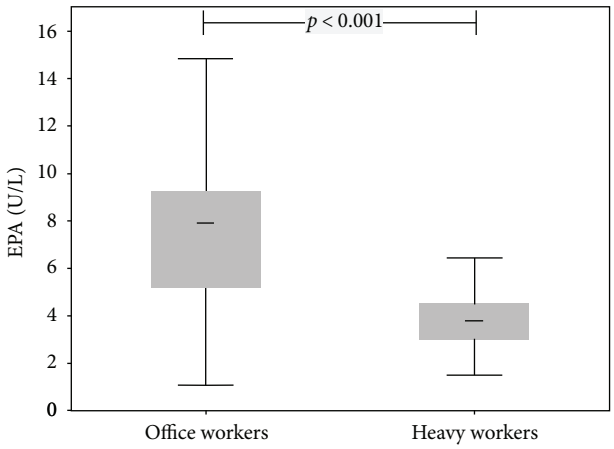

Figure 3: Box plots (medians, interquartile, and nonoutlier ranges) of endogenous peroxidase activity by groups of workers.

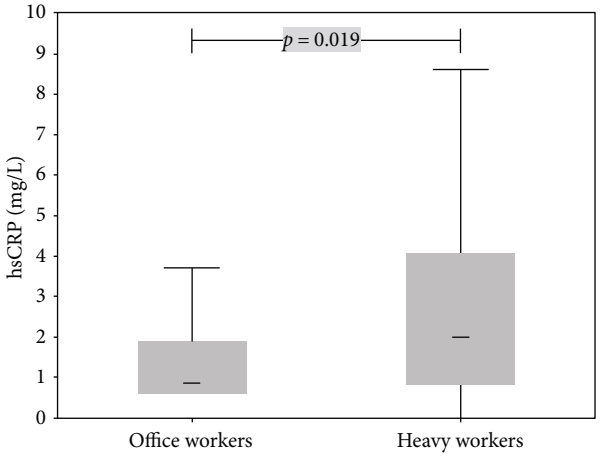

Figure 2: Box plots (medians, interquartile, and nonoutlier ranges) of hsCRP by groups of workers.

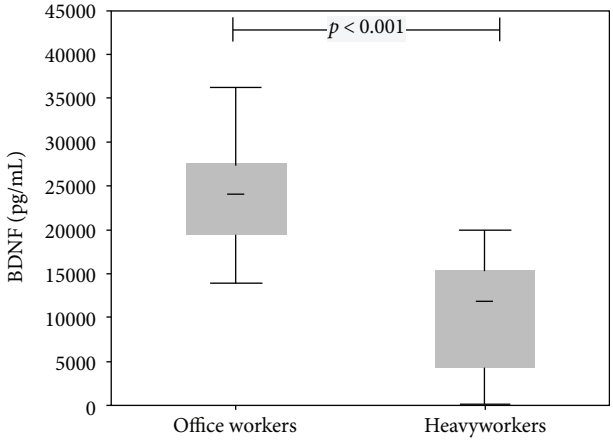

Figure 4: Box plots (medians, interquartile, and nonoutlier ranges) of brain-derived neurotrophic factor by groups of workers.

correlation with polyphenols was only significant in heavy workers ($r = 0.565$). Furthermore, TAC showed a highly significant negative correlation with TOC in office workers ($r = -0.526$) (Table 3).

4. Discussion

In the present study, we indicated an increased inflammation through raised hsCRP levels at baseline in heavy workers compared to office workers. This was associated with oxidative stress, that is, increased total peroxides and a concomitant decrease of peroxidase activity. In addition, we observed a decrease in polyphenols, although the total antioxidant capacity was increased (Table 1). OSI, which reflects the redox balance between prooxidants and antioxidants, showed significant differences between these two working groups.

This was further related to psychological stress, due to an increase in ACTH and a very low level of BDNF indicating emotional stress (Table 1). In spite of significant differences in several biomarkers between office workers and heavy workers in a slaughterhouse, it must be emphasized that this might even be an underestimation due to the working environment of the latter; that is, low temperatures were previously associated with reduced OS [19, 20].

A stressful working environment may affect the health of employees. Night shifts disrupt the circadian rhythm and increase OS [21]. There can be no doubt that a better understanding of the main stressors in the workplace would be effective in preventing disease and that determination of oxidative stress biomarkers could be helpful in this context [7]. Since reduction of sickness-related absenteeism implies economic benefits, individual health care at the workplace should be given priority. Increased disease risks in workers with demanding jobs have frequently been reported, among others by Ramey et al. [22]. Release of catecholamines and increased blood pressure, along with chronic work-related stress, may lead to cardiovascular diseases. A combination of psychological and physical stress could induce chronic inflammation and subsequent disease [23]. For such reasons, a dietary regimen including antioxidants was suggested

Table 2: Comparison of stress and inflammatory biomarkers between 8 and 12 h shifts in office and heavy workers.

	Office workers ($n = 26$)			Heavy workers ($n = 8$)		
	After 8 hours Mean (95% CI)	After 12 hours Mean (95% CI)	p	After 8 hour Mean (95% CI)	After 12 hour Mean (95% CI)	p
hsCRP, mg/L	1.23 (0.89–1.71)	1.08 (0.82–1.43)	0.311	0.98 (0.54–1.79)	0.84 (0.50–1.39)	0.482
IL-6, pg/mL	1.89 (1.57–2.28)	1.71 (1.51–1.93)	0.350	1.79 (1.27–2.53)	1.97 (1.57–2.46)	0.641
Total antioxidant capacity, mmol/L	1.03 (0.81–1.25)	1.20 (0.94–1.46)	0.236	1.26 (0.91–1.61)	0.98 (0.56–1.40)	0.220
Total oxidant capacity, mmol/L	0.10 (0.07–0.16)	0.10 (0.07–0.15)	0.786	0.05 (0.02–0.10)	0.07 (0.03–0.14)	0.110
Endogenous peroxidase activity, U/L	6.54 (5.12–8.36)	7.23 (6.21–8.43)	0.434	8.00 (5.40–11.86)	8.00 (6.26–10.23)	0.998
Malondialdehyde, μmol/L	0.72 (0.64–0.80)	0.75 (0.65–0.86)	0.610	0.79 (0.64–0.96)	0.76 (0.59–0.98)	0.857
Myeloperoxidase, μmol/L	65.9 (55.0–78.8)	67.5 (59.0–77.4)	0.812	64.5 (46.4–89.7)	56.3 (43.9–72.2)	0.488
Paraoxonase, ng/mL	14.6 (13.1–16.2)	14.2 (12.9–15.5)	0.441	12.7 (10.4–15.4)	11.5 (9.8–13.6)	0.171
ACTH, pg/mL	12.2 (7.7–19.4)	13.6 (10.3–18.0)	0.510	8.8 (3.8–20.5)	24.9 (15.0–41.4)	0.001
BDNF, pg/mL	24030 (21661–26658)	22941 (20777–25330)	0.479	24634 (20370–29790)	17921 (14947–21485)	0.012

p values from linear contrasts after analysis of variance with body mass index included as a covariate.

Table 3: Spearman correlation coefficients between stress and inflammation biomarkers.

	TAC		TOC		EPA		Polyphenols	
	Office workers ($n = 27$)	Heavy workers ($n = 52$)	Office workers ($n = 27$)	Heavy workers ($n = 52$)	Office workers ($n = 27$)	Heavy workers ($n = 52$)	Office workers ($n = 27$)	Heavy workers ($n = 52$)
hsCRP	−0.181	0.217	0.612***	0.493***	0.172	−0.074	−0.132	0.290*
IL-6	−0.219	0.167	0.462*	0.168	0.228	−0.069	−0.098	0.033
Uric acid	0.506**	0.516***	−0.105	−0.001	−0.343	−0.069	−0.083	0.218
TAC			−0.526**	−0.058	−0.648***	−0.633***	−0.164	0.075
TOC	−0.526**	−0.058			0.184	−0.196	0.123	0.565***
EPA	−0.648***	−0.633***	0.184	−0.196			−0.160	−0.292*
Polyphenols	−0.164	0.075	0.123	0.565***	−0.160	−0.292*		
MPO	0.177	0.152	0.075	0.010	−0.093	−0.097	−0.050	−0.112
Paraoxonase	0.206	0.075	0.106	−0.154	−0.249	−0.221	0.040	−0.016
ACTH	−0.042	0.059	−0.026	−0.096	−0.014	−0.031	0.129	−0.069
BDNF	−0.001	0.048	0.121	−0.003	−0.014	−0.072	0.296	0.007

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. hsCRP = high-sensitivity C-reactive protein; IL-6 = interleukin-6; TAC = total antioxidant capacity; TOC = total oxidant capacity; EPA = endogenous peroxidase activity; MPO = myeloperoxidase; ACTH = adrenocorticotropic hormone; BDNF = brain-derived neurotrophic factor.

[24, 25], but it is not clear if such a strategy is of much help [26], especially if the working conditions otherwise remain unchanged.

Notably, sensitive biomarkers identified these effects, pointing to early development of an imbalance in the redox system. Nevertheless, there were no changes in MDA, one of the end products of lipid peroxidation, MDA-LDL IgM, a biomarker for immune activation, MPO, uric acid, and galectin-3. Although the significant differences seen between occupational groups were fluctuations within "normal" ranges, it must be kept in mind that individuals may be exposed to these changes throughout their working lives. Such mild chronic stress responses over prolonged time periods are in line with our results and were also reported in an animal experiment with increased oxidative stress and consumption of antioxidants, especially in the pancreas. This led to systemic inflammation and contributed to degenerative diseases [27].

It was striking that overtime was accompanied by an almost threefold increase of ACTH and a significant decrease in BDNF in laborers only (Table 2), pointing to a combined impact of a heavy workload and 12 h shift.

Overtime, shift work [23] and extended exposure to occupational and environmental stressors diminish antioxidative capacity, which may elevate the impact of increased production of OS due to a heavy workload [28, 29]. Walker et al. [30] reported that inflammation and alterations of the immune system were associated with altered mood and reduced well-being, thus highlighting the need for improved risk management in the workplace.

We observed a significant correlation between the total antioxidant capacity and uric acid, as has been reported previously [18]. There is also a strong inverse correlation between endogenous peroxidase activity and total antioxidant capacity. The correlation between (hsCRP) and oxidative stress (TOC) underlines the link between inflammation and cellular stress responses (Table 3).

Monitoring with sensitive biomarkers may be advisable, particularly in cases of smoking, obesity, and older age, to counteract an accumulation of stress-related biological changes that could have adverse health effects. Research of oxidative stress under real-life working conditions is a win-win situation for both employers and employees. It could help to tailor health care and counseling for workers, minimizing sickness absenteeism and reducing fluctuation in the workforce.

The small number of manual laborers doing a 12-hour work shift could be a limitation for this study due to insufficient compliance. In addition, the lack of female subjects is a constraint of this work. Therefore, further research in these working groups with a larger collective, including female workers, should be performed.

In conclusion, we found increased oxidative stress and inflammation in manual laborers as compared to office workers. Indications of psychological stress were observed for overtime work in combination with hard physical work. The relationship between antioxidant consumption, oxidative stress, and inflammation was clearly shown in the correlation analysis. These data provide a solid basis for further research on this important subject with a larger collective.

Abbreviations

ROS: Reactive oxygen species
OS: Oxidative stress
OSI: Oxidative stress index
TAC: Total antioxidant capacity
TOC: Total oxidant capacity
EPA: Endogenous peroxidase activity
MDA: Malondialdehyde
MPO: Myeloperoxidase
PPm: Total polyphenols
ACTH: Adrenocorticotropic hormone
hsCRP: High-sensitivity c-reactive protein
IL-6: Interleukin-6
BDNF: Brain-derived neurotrophic factor
SD: Standard deviation
IQR: Interquartile range
95% CI: 95% confidence interval
IQRs: Interquartile ranges
CV: Coefficient of variation
ICC: Intraclass correlation coefficient
BMI: Body mass index.

Conflicts of Interest

The authors declare they have no competing interests that might be perceived to influence the results and discussion reported in this article—except W. Wonisch, who is a member of Omnignostica Ltd.

Authors' Contributions

Sieglinde Zelzer and Franz Tatzber contributed equally to this study.

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Research Article

Activation of NLRP3 Inflammasome by Advanced Glycation End Products Promotes Pancreatic Islet Damage

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Accumulation of advanced glycation end products (AGEs) contributes to ageing and age-related diseases, especially type 2 diabetes. The NLRP3 inflammasome, as a vital component of the innate immune system, is implicated in the pathogenesis of type 2 diabetes. However, the role of the NLRP3 inflammasome in AGE-induced pancreatic islet damage remains largely unclear. Results showed that administration of AGEs (120 mg/kg for 6 weeks) in C57BL/6J mice induced an abnormal response to glucose (as measured by glucose tolerance and insulin release), pancreatic β-cell ultrastructural lesion, and cell death. These effects were associated with an excessive superoxide anion level, significant increased protein expression levels for NADPH oxidase 2 (NOX2), thioredoxin-interacting protein (TXNIP), NLRP3, and cleaved IL-1β, enhanced caspase-1 activity, and a significant increase in the levels of TXNIP–NLRP3 protein interaction. Ablation of the NLRP3 inflammasome or treatment with antioxidant *N*-acetyl-cysteine (NAC) clearly ameliorated these effects. In conclusion, our results reveal a possible mechanism for AGE-induced pancreatic islet damage upon NLRP3 inflammasome activation.

1. Introduction

Advanced glycation end products (AGEs) are generated nonenzymatically, and the formation of AGEs is greatly accelerated by prolonged hyperglycemia in patients with diabetes mellitus (DM) [1, 2]. AGE accumulation is one of the main factors that contribute to ageing and is an important element of etiopathology of age-related diseases, especially type 2 DM and its complications [3–5]. Recent studies have pointed out that AGE accumulation directly causes insulin-producing β-cell dysfunction and apoptosis *in vivo* [6–9]. These effects are attributed, at least in part, to an increase in cellular reactive oxygen species (ROS) production. Nonetheless, the precise role of AGE-mediated ROS release in β-cell damage remains unclear.

Inflammation is a critical mechanism leading to β-cell dysfunction and death, wherein the inflammatory cytokines play an important role [10, 11]. Among the various proinflammatory cytokines, interleukin-1β (IL-1β) plays a major role in DM. Clinical studies reported that inhibition of IL-1β by either IL-1 receptor antagonist or IL-1β antibody in patients with type 2 DM leads to improvement in glycemia and β-cell function [12, 13]. It is known that IL-1β secretion is predominantly mediated by the cysteine protease caspase-1, which is mainly activated by inflammasomes, especially the nucleotide-binding domain leucine-rich repeat containing receptor, the pyrin domain-containing 3 (NLRP3) inflammasome [14].

Thioredoxin-interacting protein (TXNIP), a member of the arrestin protein superfamily, regulates diabetic islet β-cell

apoptosis and dysfunction [15–17]. TXNIP is the endogenous inhibitor and regulator of thioredoxin, which maintains the balance of both the cellular antioxidant and antiapoptotic system [18]. Recent research demonstrates that the dissociation of TXNIP from thioredoxin in a ROS-sensitive manner could activate the NLRP3 inflammasome and induce IL-1β release, namely, oxidative stress links TXNIP to inflammasome activation [19–21].

Increasing evidence suggests that metabolic stress (glucose, free fatty acids, and human amyloid polypeptide) appears to activate the IL-1 system through the NLRP3 inflammasome in pancreatic islet [19, 22, 23]. However, it remains unclear whether the NLRP3 inflammasome participates in the AGE-induced β-cell damage, although the latter process is companied by ROS production. As a result, in the current study, we test the hypothesis that the ROS/TXNIP pathway contributes to AGE-induced activation of NLRP3 inflammasome, which results in the release of active IL-1β, leading to pancreatic islet β-cell damage *in vivo*.

2. Materials and Methods

2.1. Animals and Reagents. Ten-week-old male NLRP3 knockout (NLRP3 KO) mice were used in our study as we described previously [24]. Age-matched C57BL/6J mice were obtained from the Jackson Laboratory and used as the wild-type (WT) control group. All of the mice were maintained in specific pathogen-free facilities at the Experimental Animal Centre of Xinhua Hospital. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Xinhua Hospital, Shanghai Jiaotong University School of Medicine.

All of the reagents were purchased from Sigma unless stated otherwise. Monoclonal mouse anti-NLRP3 antibody was obtained from Adipogen Inc. Polyclonal rabbit anti-TXNIP and insulin antibodies were purchased from Abcam Inc. Polyclonal rabbit anti-glucagon, NADPH oxidase 2 (NOX2), apoptotic speck-like protein (ASC), and IL-1β antibodies were purchased from Santa Cruz Inc. Dihydroethidium (DHE) probes, caspase-1 activity, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kits were purchased from Beyotime Biotechnology Inc. IL-1β ELISA kit was purchased from R&D Systems. Mouse insulin ELISA kit was purchased from Shibayagi Inc.

2.2. Preparation of AGEs. The AGEs used in this study were described previously [8, 9] and prepared as in Makita et al. [25]. In brief, bovine serum albumin (BSA, 50 mg/mL) was incubated under sterile condition with glyceraldehyde (0.1 M) in phosphate buffer (pH 7.4, 0.2 M) for seven days. The unincorporated sugar was removed by dialysis. Nonglycated BSA, incubated in the absence of glyceraldehyde, was used as a negative control. The AGE preparation was tested for the presence of endotoxins using a limulus amebocyte lysate (LAL) reagent yielding an endotoxin level of less than 15 EU/L.

2.3. Laboratory Rodent Studies. In the first part of the experiment, mice were separated into the following groups ($n = 10$ each group): WT mice with BSA, NLRP3 KO mice with BSA, WT mice with AGEs, and NLRP3 KO mice with AGEs. BSA (used as a control) or AGEs were daily administered intraperitoneally at the dosage of 120 mg/kg of body weight for 6 weeks according to our previous report [9].

In the second part of this study, groups of C57BL/6J mice ($n = 6$ each group) were given daily intraperitoneal injections of either AGEs or BSA for 6 weeks as mentioned above. An additional subgroup of mice injected with AGEs ($n = 6$) received treatment with the antioxidant *N*-acetyl-cysteine (NAC, 40 mM in drinking water), at a concentration that provided sufficient *in vivo* antioxidant capacity [26, 27]. We did not include a group of C57BL/6J mice treated with NAC alone in this study because NAC does not influence the NLRP3 inflammasome activity in unstimulated human peripheral blood monocytes [28].

2.4. Intraperitoneal Glucose Tolerance Test (GTT), Insulin Releasing Test (IRT), and Intraperitoneal Insulin Tolerance Test (ITT). Overnight-fasted mice were intraperitoneally injected with a 10% glucose solution (1.5 mg/g body weight). For intraperitoneal glucose tolerance test (GTT), glucose levels were determined at different time points with a glucometer. For insulin releasing test (IRT), blood was collected at different time points after glucose loading, and insulin levels were determined with an ELISA kit.

For intraperitoneal insulin tolerance test (ITT), mice were fasted 6 hours and then intraperitoneally injected with human regular insulin (0.75 U/kg). Glucose levels were measured at 0, 15, 30, 45, and 60 min with a glucometer.

2.5. Determination of Physiological Indices. At the end of the present study, overnight-fasted mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital. Blood samples were collected, centrifuged to obtain serum, and kept at −80°C until assayed. Fasting blood glucose levels were determined by the glucose oxidase method. Fasting insulin concentrations were measured with an ELISA kit.

2.6. Immunofluorescent Staining. A portion of the pancreas was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. For TUNEL staining, the pancreatic sections were incubated with TUNEL reagent for 1 h in the dark. Nuclear staining was achieved by incubating with DAPI. In addition, a positive control was prepared from permeabilized pancreatic sections preincubated with deoxyribonuclease 1 to induce DNA strand breaks. For immunofluorescent staining, fixed pancreatic sections were heated for 15 min in boiling 10 mM citrate buffer (pH = 6.0) for antigen retrieval. Then, sections were probed with anti-insulin (1 : 200), glucagon (1 : 150), or IL-1β (1 : 100) antibodies, followed by incubation with specific secondary antibodies. Negative controls were prepared, in which the antibody probing of the pancreatic sections was substituted by PBS buffer addition, at the same concentration of nonimmune rabbit immunoglobulin G. Sections were photographed by

fluorescent microscopy and analyzed using ImageJ software as described in our previous reports [9, 29].

2.7. Detection of Superoxide Anion in Mice Pancreatic Islet. As in our previous report [9], a portion of the pancreas was embedded in O.C.T. embedding medium. Sections (10 μm) were incubated for 30 minutes with DHE (10 μM) to evaluate pancreatic superoxide anion levels in situ. DHE is oxidized by superoxide anion to yield ethidium, which is trapped intracellularly by intercalation into the DNA. Ethidium fluorescence was quantified using ImageJ software [9].

2.8. Transmission Electron Microscopy. Pancreatic tissues from the tail of the pancreas were harvested and fixated in 2.5% glutaraldehyde. Thereafter, fixed samples were treated with 1% osmium tetroxide, dehydrated, and embedded. After localization of islets under a light microscope, the tissues were cut into ultrathin sections. The sections were put on uranyl acetate and lead citrate before examined in a transmission electron microscope.

2.9. Caspase-1 Activity Assay. The caspase-1 activity of pancreatic tissue lysates was measured using a colourimetric assay. This assay is based on the ability of caspase-1 to change acetyl-Tyr-Val-Ala-Asp p-nitroaniline (Ac-YVAD-pNA) into the yellow formazan product p-nitroaniline (pNA). Production of pNA per minute in tested samples was used as a measure of the level of caspase-1 activity and inflammasome activation. Results are expressed as fold increase in caspase-1 activity.

2.10. Western Blot Analysis and Immunoprecipitation. Equal protein of pancreatic tissue lysates (40 μg) was separated by electrophoresis on a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred electrophoretically to PVDF membranes and then incubated with the primary antibodies against NLRP3 (1 : 500), ASC (1 : 800), IL-1β (1 : 200), TXNIP (1 : 500), and tubulin (1 : 1000) overnight and with the correspondent secondary peroxidase-conjugated anti-rabbit or mouse antibodies. Immunoprecipitation (IP) was performed as described previously. Total protein (100 mg) was immunoprecipitated with anti-TXNIP antibody (5 μg/mL) and incubated with A/G agarose beads overnight. Precipitated proteins were analysed by SDS-PAGE and blotted with primary antibodies (anti-TXNIP and anti-NLRP3). Antibody-bound proteins were detected with an enhanced chemiluminescence (ECL) kit (Millipore). Blots were quantified by densitometry using Image J software. The intensity of the bands was normalized to that of tubulin or TXNIP.

2.11. Statistical Analysis. Data were expressed as the mean ± standard deviation (S.D.). The differences among groups were determined by the use of one-way analysis of variance followed by Newman–Keuls test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. NLRP3 Knockout Improves the Abnormal Response to Glucose in Mice Administration of AGEs. NLRP3 KO mice were used to understand the role of the NLRP3 inflammasome in AGE-induced pancreatic islet damage. WT and NLRP3 KO mice were injected intraperitoneally with AGEs or BSA (control) daily for 6 weeks. We first performed insulin and glucagon double immunofluorescence staining of pancreatic sections for insulin and glucagon to evaluate the islet morphology in mice. As shown in Figure 1(a), the 6-week AGE treatment caused few morphological changes in the pancreatic islet. Total β-cell and α-cell mass were not significantly different among the four groups (Figures 1(b) and 1(c)).

There were no significant differences in fasting blood glucose level (Figure 1(d)) and insulin concentration (Figure 1(e)) among the four groups. Whole-body insulin tolerance, as assessed by the intraperitoneal ITT, was not significantly different among groups (Figure 1(f)). Then, an intraperitoneal GTT was performed to assess metabolic alterations, namely, glucose tolerance and glucose-stimulated insulin release. Blood glucose level after glucose loading was significantly raised in WT mice treated with AGEs compared with WT mice treated with BSA (Figure 1(g)), which correlated with a lower insulin level in AGE-treated WT mice as compared to BSA-treated WT mice (Figure 1(h)). These effects were not owed to the development of insulin resistance because the AGE treatment did not affect fasting glucose and insulin levels or whole-body insulin sensitivity, which compared to those of BSA-treated WT mice. Therefore, these results demonstrate that administration of AGEs directly impaired β-cell function *in vivo*. Interestingly, AGE-dependent effects such as higher level of blood glucose and impaired insulin secretion after glucose loading were significantly ameliorated by the ablation of NLRP3 (Figures 1(g) and 1(h)).

3.2. NLRP3 Knockout Decreases Islet β-Cell Apoptosis in Mice Administration of AGEs. Previous studies have clarified that impaired *in vivo* insulin secretion can result from a decrease in β-cell survival or function or a combination of both [30]. As shown in Figure 2, the number of apoptotic cells identified by positive TUNEL staining was much higher in AGE-treated WT mice compared to the number of apoptotic cells in AGE-treated NLRP3 KO mice. These data suggest that deletion of NLRP3 gene protects islet β-cell from AGE-induced death *in vivo*.

3.3. NLRP3 Knockout Reverts Ultrastructural Lesion of Islet β-Cell in Mice Administration of AGEs. We used transmission electron microscopy to better characterize the protective effect of the NLRP3 deletion at single cell level. As shown in Figure 3, the electron micrograph of β-cells within islet displayed the swollen mitochondria in AGE-treated WT mice compared with those in BSA-treated WT mice, although the insulin-secretory granules were almost unaltered among groups. Ablation of NLRP3 led to a

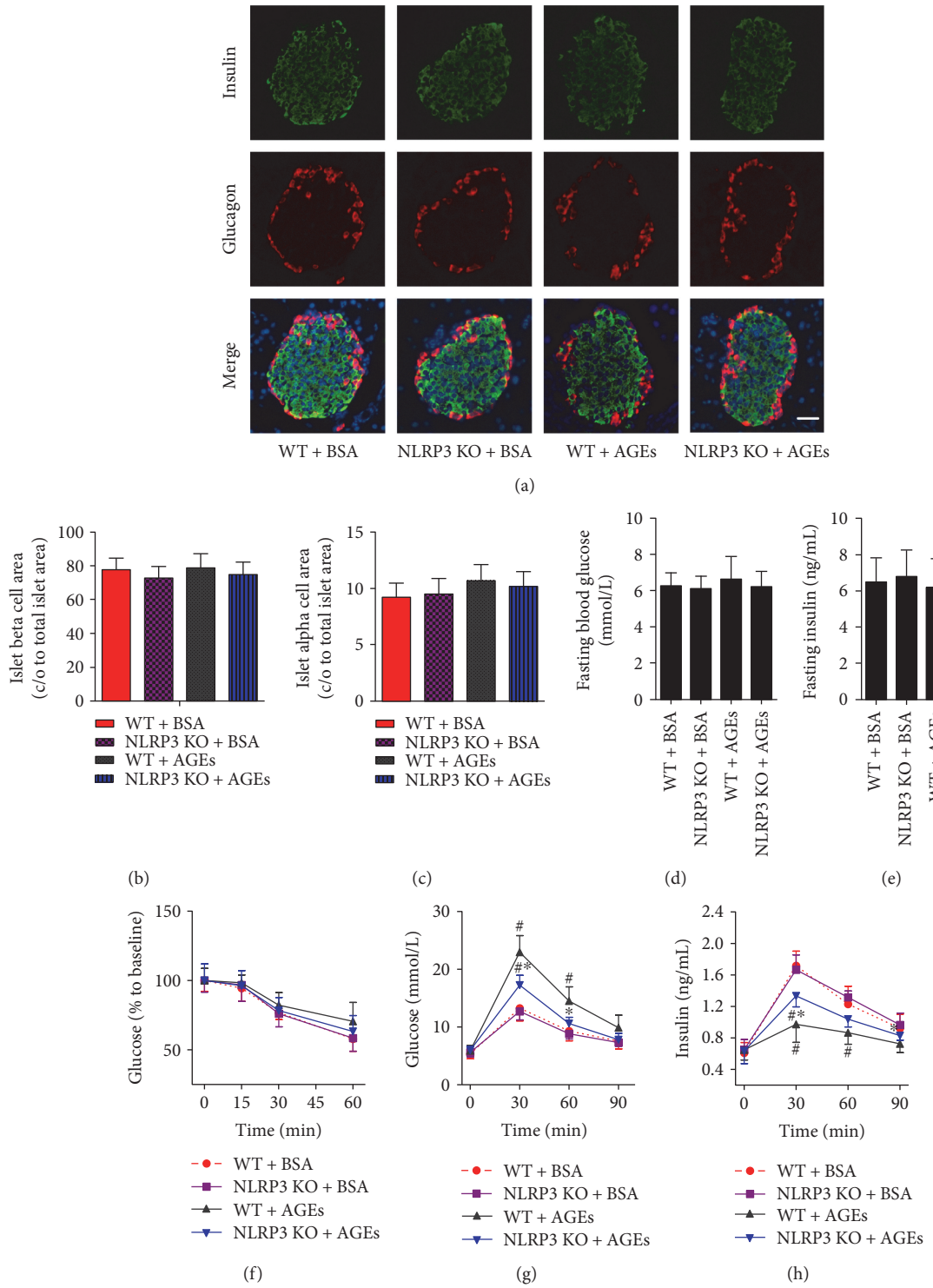

Figure 1: NLRP3 knockout improves the abnormal response to glucose in mice administration of AGEs. C57BL/6J (WT) and NLRP3 knockout (NLRP3 KO) mice were injected intraperitoneally with 120 mg/kg AGEs or BSA for 6 weeks. (a) Pancreatic sections were stained by insulin (green), glucagon (red), and DAPI (blue), with representative islets shown (scale bar = 50 μm). Histogram represents quantitative analysis of insulin-positive beta cell area (b) and glucagon-positive alpha cell area (c) in each experimental group. $n = 5 - 6$ per group. Fasting glucose (d) and insulin (e) as well as insulin tolerance (f) were not obviously changed in each group. GTT (d) and IRT (e) were performed after intraperitoneal injection of glucose (1.5 mg/g). $n = 5$ per group. Ablation of NLRP3 improved the abnormal glucose metabolism induced by AGE injection. Values are expressed as mean ± SD, $^{\#}P < 0.05$ versus WT + BSA group. $^{*}P < 0.05$ versus WT + AGEs group.

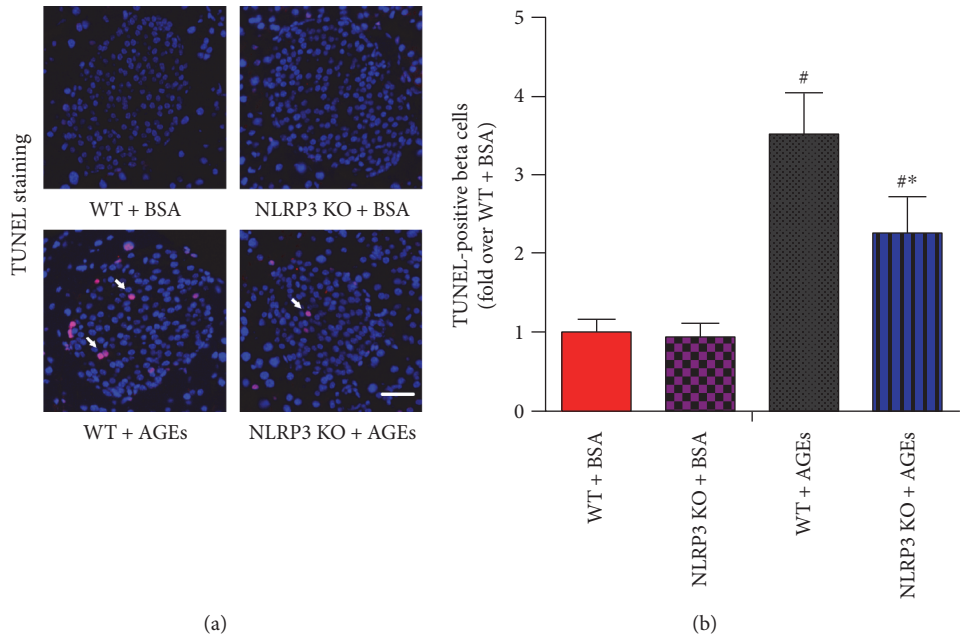

Figure 2: NLRP3 knockout decreases islet β-cell apoptosis in mice administration of AGEs. WT and NLRP3 KO mice were injected intraperitoneally with 120 mg/kg AGEs or BSA for 6 weeks. (a) Representative photomicrographs of TUNEL-positive cells in mice pancreatic sections. Scale bar = 50 μm. (b) Histogram represents quantitative analysis of TUNEL-positive β-cells per islet in each experimental group. $n = 5$ per group. Values are expressed as mean ± SD, $^{\#}P < 0.05$ versus WT + BSA group. $^{*}P < 0.05$ versus WT + AGEs group.

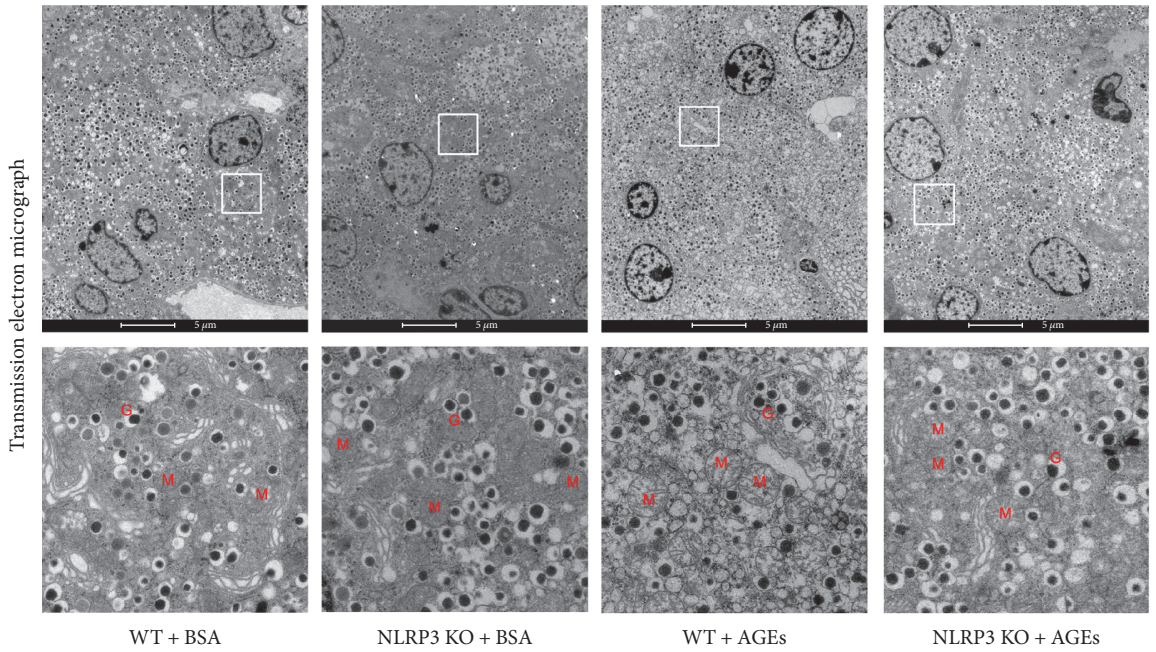

Figure 3: Ultrastructural changes in WT and NLRP3 KO mice after administrated with AGEs. The insulin-secretory granules were almost unaltered in each group. The swollen mitochondria in AGE-treated WT mice were protected by the NLRP3 ablation. M: mitochondria; G: granules.

significant amelioration of AGE-induced mitochondrial damage in the β-cells within islets.

3.4. NLRP3 Inflammasome Activation Is Involved in AGE-Induced Mice Pancreatic Islet Damage. To directly address the involvement of the NLRP3 inflammasome in AGE-induced islet lesions, we investigated the local processing of IL-1β, which is the ultimate step of inflammasome activation. Immunofluorescence results suggested that, although IL-1β expression was relatively low in normal WT mice as

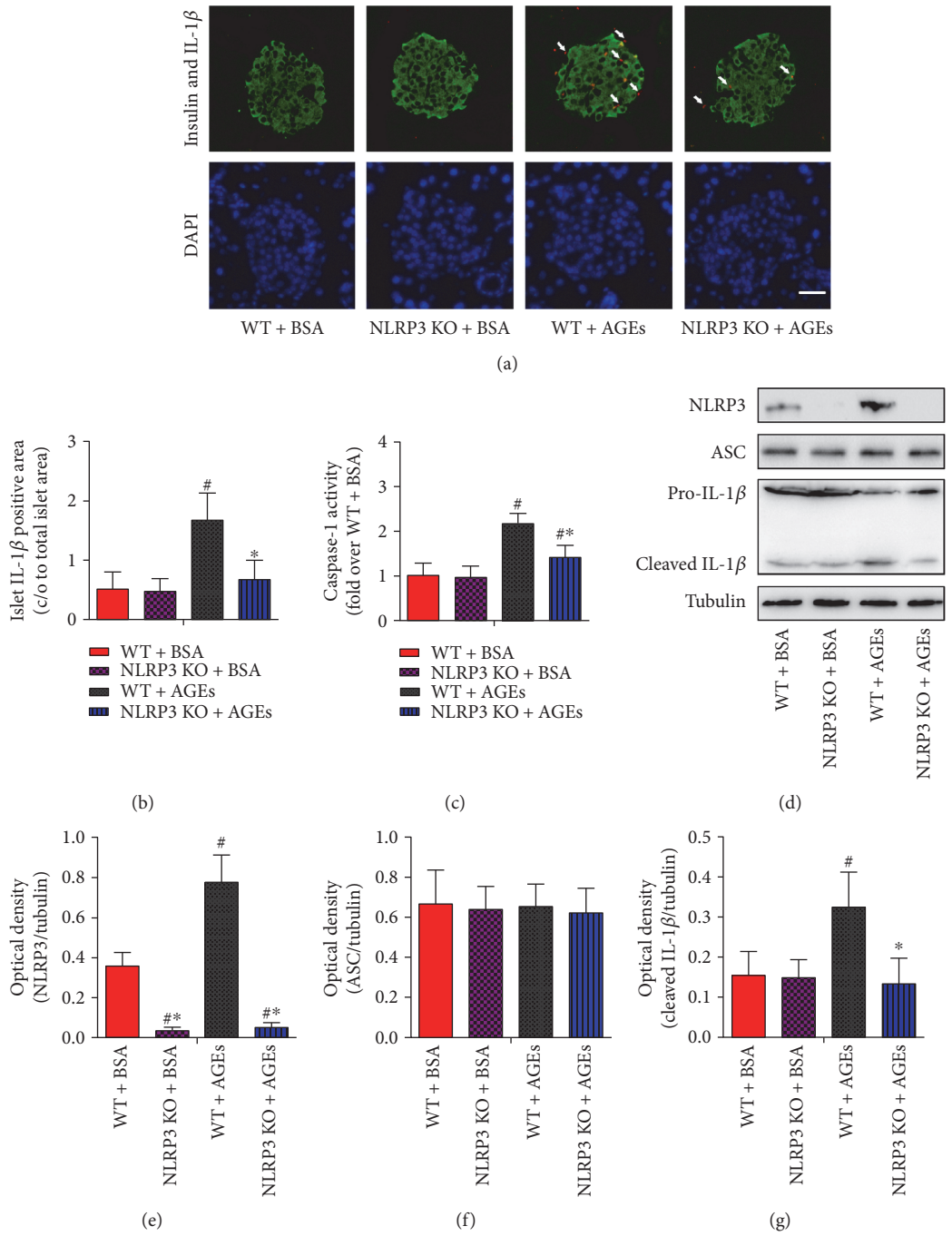

Figure 4: NLRP3 inflammasome activation is involved in AGE-induced mice pancreatic islet damage. WT and NLRP3 KO mice were injected intraperitoneally with 120 mg/kg AGEs or BSA for 6 weeks. (a) Pancreatic sections were stained by insulin (green), IL-1β (red), and DAPI (blue), with representative islets shown (scale bar = 50 μm). (b) Histogram represents quantitative analysis of IL-1β-positive cell area in each experimental group. $n = 5$ per group. (c) The caspase-1 activity in pancreas tissue lysates was assessed using Ac-YVAD-pNA. $n = 5 - 6$ per group. (d) Immunoblotting analysis was performed for NLRP3, ASC, and active IL-1β (p17) in pancreas tissue lysates of mice. Histograms represent optical density values of NLRP3 (e), ASC (f), and active IL-1β (g) normalized to the corresponding tubulin. $n = 3 - 4$ per group. Values are expressed as mean ± SD, $^{\#}P < 0.05$ versus WT + BSA group. $^{*}P < 0.05$ versus WT + AGEs group.

consistent with a previous report [31], IL-1β staining could still be detected in the islets. Nevertheless, daily AGE injection induced a significant elevated immunoexpression level of IL-1β in WT mice, and this change was reversed by the NLRP3 deletion (Figures 4(a) and 4(b)).

Since the immunostaining of IL-1β reflects the total IL-1β protein expression including precursor and mature forms, we performed immunoblotting to analyze IL-1β partitioning into the two forms. Caspase-1 assays were also performed to detect the enzymatic activity of caspase-1. As shown in

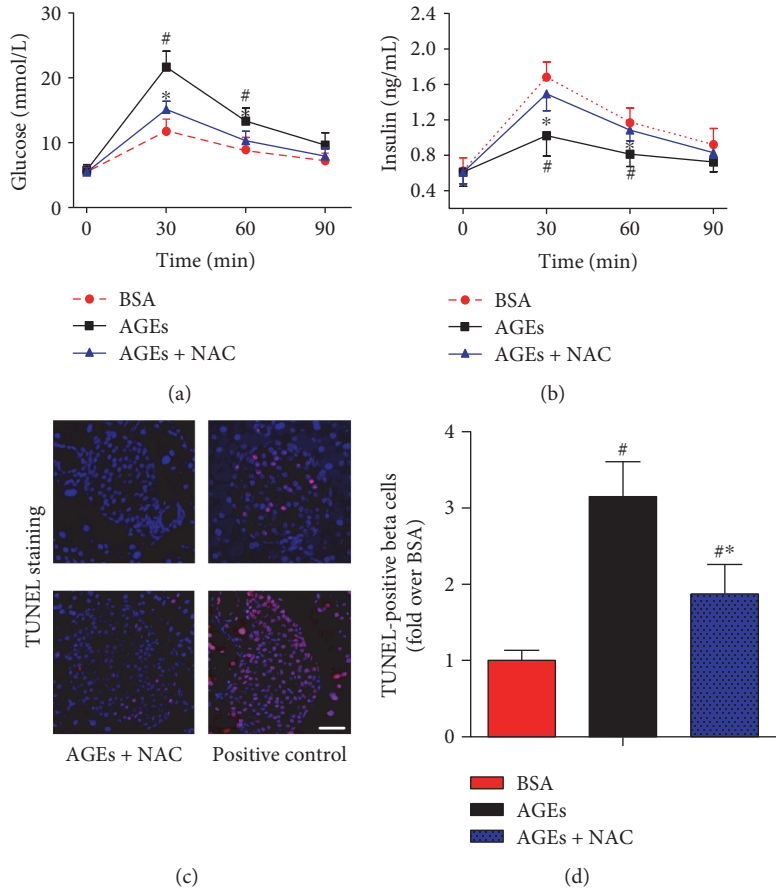

Figure 5: Effects of NAC treatment on the abnormal response to glucose and islet β-cell apoptosis in mice administration of AGEs. C57BL/6J mice were injected intraperitoneally with BSA, AGEs, or AGEs plus the antioxidant NAC (40 mM in drinking water) for 6 weeks. GTT (a) and IRT (b) were performed after intraperitoneal injection of glucose (1.5 mg/g). $n = 6$ per group. (c) Representative photomicrographs of TUNEL-positive cells in mice pancreatic sections. The positive control includes permeabilization of sections with deoxyribonuclease 1 to induce DNA strand breaks. Scale bar = 50 μm. (d) Histogram represents quantitative analysis of TUNEL-positive β cells per islet in each experimental group. $n = 6$ per group. Values are expressed as mean ± SD, $^{\#}P < 0.05$ versus BSA group. $^{*}P < 0.05$ versus AGEs group.

Figures 4(c), 4(d), 4(e), 4(f), and 4(g), AGEs caused a substantial increase in the expression of NLRP3 protein, activation of caspase-1, and maturation of IL-1β in WT mice, whereas ASC expression levels remained constant. Deletion of NLRP3 prevented the occurrence of AGE-induced effects of enhanced caspase-1 activity and increased activation of IL-1β (17 kDa subunit) (Figures 4(c) and 4(d)).

3.5. Effects of NAC on AGE-Induced Mice Pancreatic NLRP3 Inflammasome Activation. We next explored whether the ROS/TXNIP pathway contributes to AGE-induced NLRP3 inflammasome activation. To this end, C57BL/6J mice were administered daily with BSA, AGEs, or AGEs plus NAC (a well-known ROS inhibitor) for 6 weeks. Our data illustrated that NAC treatment significantly improved islet β-cell function (Figures 5(a) and 5(b)) and inhibited β-cell death (Figures 5(c) and 5(d)). As shown in Figures 6(a) and 6(b), the superoxide anion production and NOX2 protein level were obviously enhanced in AGE-treated mice. These effects were associated with the significantly increased protein expression levels of TXNIP, NLRP3, and cleaved IL-1β, the enhanced caspase-1 activity, and an obvious increase in TXNIP-NLRP3 protein interaction, as assessed by coimmunoprecipitation (Figure 6(e)). Moreover, treatment with NAC significantly reduced the level of superoxide anion, inhibited the AGE-induced expression level of TXNIP, NLRP3, and cleaved IL-1β, decreased the caspase-1 activity, and reversed the phenotype for increased protein interaction levels between TXNIP and NLRP3 (Figure 6).

4. Discussion

Among the various AGE subtypes, the glyceraldehyde-derived AGEs (the predominant components of toxic AGEs) have been shown to play an important role in the development of inflammation and angiopathy in patients with DM [32–34]. In preliminary experiments, glyceraldehyde or glucose-derived AGEs were daily administered intraperitoneally to C57BL/6J mice at the dosage of 120 mg/kg of body weight for 6 weeks. Exposure to glyceraldehyde-derived AGEs caused significantly impaired glucose tolerance in C57BL/6J mice, which was not observed in mice treated with glucose-derived AGEs (data not shown). Therefore, the

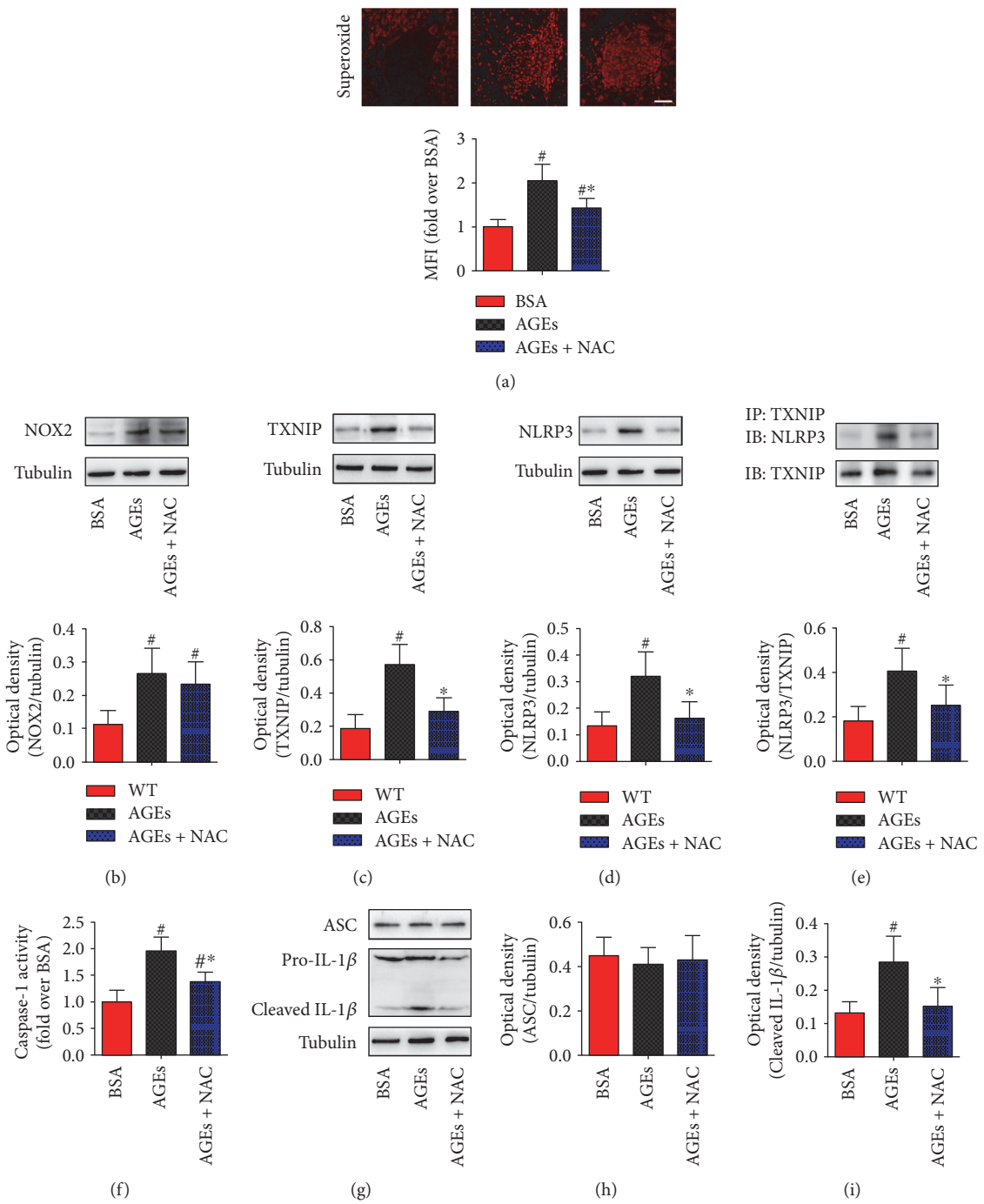

Figure 6: AGE-induced mice pancreatic NLRP3 inflammasome activation through ROS/TXNIP pathway. C57BL/6J mice were injected intraperitoneally with BSA, AGEs, or AGEs plus the antioxidant NAC (40 mM in drinking water) for 6 weeks. (a) Representative photomicrographs of mice pancreatic sections stained with DHE. Scale bar = 50 μm. Histogram represents quantitative analysis of superoxide anion generation in each experimental group. $n = 6$ per group. Representative blots and Western blot analyses of NOX2 (b), TXNIP (c), and NLRP3 (d) protein expression in mice pancreas tissue lysates. $n = 3 - 4$ per group. (e) Representative blot and quantification of immunoprecipitation (IP) with TXNIP and blotting (IB) with NLRP3 showed higher association of TXNIP with NLRP3 ($n = 3$ per group), which was associated with increased caspase-1 activity ((f), $n = 6$ per group) and enhanced cleaved IL-1β expression ((g, h, i), $n = 3$ per group). Values are expressed as mean ± SD, $^{\#}P < 0.05$ versus BSA group. $^{*}P < 0.05$ versus AGEs group. MFI: mean fluorescence intensity.

glyceraldehyde-derived AGEs were selected for subsequent studies and referred to as AGEs.

Proinflammatory cytokine overproduction is widely known to lead to DM. Several evidences suggest that the local IL-1β generation in pancreatic islets causes β-cell death and impairs their ability to produce insulin [35, 36]. There is growing support for the concept that AGEs play a pathological role due to their induction of proinflammatory cytokines,

such as IL-1β, tumor necrosis factor-α, and so on [37–40]. AGEs directly increase IL-1β secretion without lipopolysaccharide priming in human peritoneal macrophages [40] and in adipose tissue of women with gestational diabetes [41]. The NLRP3 inflammasome appears as the sentinel, sensing metabolic stress and alarming the immune defense in pancreatic islets. NLRP3 activation leads to recruitment of the adaptor protein ASC and the effector protein caspase-1 to form the NLRP3 inflammasome complex, which ultimately is responsible for the cleavage and secretion of IL-1β [14]. Here, we showed that the AGE-induced increase in the maturation of pancreatic IL-1β was dependent on the NLRP3 inflammasome activation. Knockout of NLRP3 ameliorated the abnormal response to glucose (glucose tolerance and insulin release) in mice injected with AGEs. In addition, deletion of NLRP3 inflammasome protected the pancreatic β-cells from ultrastructural lesion and cell death caused by long-term AGE administration. Together, our results indicated that NLRP3 inflammasome activation is a key mechanism that participates in AGE-induced pancreatic damage. High concentrations of AGEs may trigger the NLRP3 inflammasome complex and result in the activation IL-1β. Subsequently, secreted IL-1β in the microenvironment exacerbates the chronic inflammatory response in pancreatic islets [42].

In line with our results, AGEs were reported to upregulate protein expression levels of the pattern recognition receptor for AGE (RAGE), increase ROS production, stimulate the activation of the NLRP3 inflammasome, and spark the development of renal injury in mice [43]. Treatment with RAGE antagonist obviously inhibits AGE/RAGE-induced ROS production and attenuates NLRP3 activation, consequently reducing the levels of IL-1β and attenuating abnormal kidney function in mice [43]. In addition, AGE treatment directly activates the NLRP3 inflammasome and stimulates mature IL-1β secretion in human placental tissues [44]. AGEs could also induce an inflammatory response in nucleus pulposus cells in a NLRP3 inflammasome-dependent manner related to the RAGE/NF-κB pathway [45]. Other endogenous non-AGE ligands of RAGE, including S100A8 and S100A9, have been revealed to activate the NLRP3 inflammasome by stimulating the production of ROS [28]. Nevertheless, Kang et al. recently demonstrated that RAGE activates the melanoma 2 (but not NLRP3) inflammasome in acute pancreatitis [46]. A possible involvement of RAGE/NLRP3 inflammasome cannot be completely ruled out because macrophages used in their experiment were stimulated with histone and DNA, but not with AGEs. In fact, the components of the inflammasome, ASC, caspase-1, and NLRP3, are required for the development of inflammation in acute pancreatitis [47–49]. Knockout of NLRP3 gene protects against experimental acute pancreatitis [47] and chronic obesity-induced pancreatic damage [30]. Contrary to the results mentioned above, a recently published abstract has reported that pretreatment with AGEs attenuates diverse NLRP3 stimuli-triggered caspase-1 activation and IL-1β secretion in bone marrow-derived macrophages [50]. The direct effect of AGEs on immune cells requires further study.

Interestingly, little colocalization was observed between IL-1β and the β-cell marker insulin in the present study (Figure 4(a)), which suggests that islet resident and/or infiltrating macrophages may be the major source of proinflammatory cytokine in islets *in vivo*. NLRP3 inflammasome-mediated IL-1β production from infiltrating macrophages within the pancreas can contribute to the death of pancreatic β-cell and subsequent diabetes [51]. Further investigation to clarify the key cell types involved in AGE-stimulated IL-1β secretion in pancreatic islets is necessary.

Remarkably, ROS/TXNIP pathway has an essential role in triggering NLRP3 inflammasome activation and IL-1β secretion [19–21]. Both *in vivo* animal studies and *in vitro* cell culture studies have clarified that NADPH oxidase-related excessive ROS induces the separation of thioredoxin and TXNIP, resulting in the activation of NLRP3 inflammasome [42, 52, 53]. In the present study, we found that the administration of AGEs significantly increased the superoxide anion level through upregulation of NOX2 protein, which in turn contributed to an increase in expression levels of TXNIP and NLRP3 inflammasome components and an increase in protein interaction levels between TXNIP and NLRP3. Furthermore, treatment with NAC decreased the oxidative stress as indicated by a decrease in superoxide anion, led to the inhibition of the subsequent TXNIP-NLRP3 protein interaction levels and IL-1β secretion, and attenuated pancreatic islet injury. These results suggested that the NLRP3 expression and inflammasome activation in response to AGE stimulation are associated with ROS/TXNIP pathway *in vivo*.

5. Conclusion

Our research demonstrates that NLRP3 inflammasome activation is a key signalling mechanism in AGE-induced pancreatic islet damage. This study also provides direct *in vivo* evidence that NLRP3-deficient mice have suppressed pancreatic islet inflammatory response and damage upon AGE challenge. Thus, our work suggests that regulating the NLRP3 signalling might help control AGE-induced pancreatic islet damage in diabetic patients.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xiang Kong and Ai-Ling Lu contributed equally to this work.

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Research Article

Oxidative Modification of Blood Serum Proteins in Multiple Sclerosis after Interferon Beta and Melatonin Treatment

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Multiple sclerosis (MS) is a disease involving oxidative stress (OS). This study was aimed at examination of the effect of melatonin supplementation on OS parameters, especially oxidative protein modifications of blood serum proteins, in MS patients. The study included 11 control subjects, 14 de novo diagnosed MS patients with the relapsing-remitting form of MS (RRMS), 36 patients with RRMS receiving interferon beta-1b (250 μg every other day), and 25 RRMS patients receiving interferon beta-1b plus melatonin (5 mg daily). The levels of N′-formylkynurenine, kynurenine, dityrosine, carbonyl groups, advanced glycation products (AGEs), advanced oxidation protein products (AOPP), and malondialdehyde were elevated in nontreated RRSM patients. N′-Formylkynurenine, kynurenine, AGEs, and carbonyl contents were decreased only in the group treated with interferon beta plus melatonin, while dityrosine and AOPP contents were decreased both in the group of patients treated with interferon beta and in the group treated with interferon beta-1b plus melatonin. These results demonstrate that melatonin ameliorates OS in MS patients supporting the view that combined administration of interferon beta-1b and melatonin can be more effective in reducing OS in MS patients than interferon beta-1b alone.

1. Introduction

Multiple sclerosis (MS) is one of the most widespread chronic inflammatory, demyelinating diseases of the central nervous system (CNS), which leads to damage of myelin and axons. Although the exact cause of MS is unknown, it is considered that genetic predisposition, environmental factors, and abnormal immune response, consisting of delivery of cytokines from lymphocytes including Th1 and Th17 cells, contribute to the pathogenesis of this disease [1, 2]. In recent years, the factors involved in the etiology of the disease have also included oxidative stress (OS), which is defined as an imbalance between the generation of reactive oxygen species (ROS) and the mechanisms that are responsible for their elimination. It is suggested that the increased generation of ROS and reactive forms of nitrogen (RNS) leads to oxidative and nitrosative stress causing damage to mitochondria and myelin, oligodendrocyte apoptosis, and astrocyte dysfunction [3]. Elevated OS markers were observed in the blood, plasma, and cerebrospinal fluid of patients with relapsing-remitting (RRMS) form of MS [4–7] and in the plasma of patients with secondary progressive (SPMS) form of MS

Table 1: Demographic data of the subjects studied.

Group	Control	RRMS INF-beta + MEL	RRMS INF-beta	RRMS untreated
Subject number (n) (total $n = 86$)	11	25	36	14
Age (years) mean ± SD	34.54 ± 9.6	38.16 ± 8.29	39.49 ± 10.16	40.65 ± 10.01
Female/male number (n)	6/5	18/7	26/10	7/7
EDSS mean ± SD	NA	1.85 ± 0.75	2.52 ± 1.14	2.68 ± 1.11
Disease duration (years) mean ± SD	NA	4.89 ± 1.41	6.07 ± 3.97	0.88 ± 0.65
Treatment duration (months) mean ± SD	NA	29.51 ± 5.03	28.18 ± 7.13	NA
Number of T2 brain MRI lesions (mean ± SD)	NA	8.70 ± 7.54	9.18 ± 6.56	11.11 ± 10.94
Number of T1 Gd(+) brain MRI lesions (mean ± SD)	NA	0.52 ± 0.17	0.66 ± 0.65	0.86 ± 0.85

NA: nonapplicable. Data are presented as mean ± SD.

[7], which confirms that OS plays a significant role in MS and implies that oxidative damage to blood serum proteins correlates with the severity of disease.

Melatonin (N-acetyl-5-methoxytryptamine; MEL) is a natural hormone derivative of tryptophan. In animals, it is synthesized primarily by pinealocytes of the pineal gland and regulates sleep and wakefulness. Melatonin is known as a scavenger of reactive oxygen and nitrogen species and an agent decreasing ROS generation as well as increasing the activity of antioxidant enzymes and glutathione content [8]. More and more studies have reported that MEL can improve memory impairment as a result of its antioxidant properties, which indicates that MEL may have a beneficial role in the treatment of neurodegenerative disorders involving enhanced OS [9–12].

Furthermore, MEL reduced clinical scores as well as ROS generation and delayed manifestation of motor symptoms in experimental autoimmune encephalomyelitis, which is an often used animal MS model. MEL also restored increased levels of microglia and CD4+ T cells in untreated animals to the control level and decreased the loss of oligodendrocytes, demyelination, and axonal injury [13].

In view of the occurrence and postulated role of OS in MS, examination of the effects of antioxidants in combination with standard treatment seems noteworthy. This study was aimed at examining the effect of MEL on the OS markers in MS patients treated with interferon beta-1b, basing mainly on the oxidative modifications of serum proteins as sensitive markers of OS [14, 15].

2. Patients and Methods

2.1. Patients. The study was approved by the local Ethics Committee of the Medical University of Silesia (KNW/0022/KB1/130/12). Informed consent was obtained from all individual participants included in the study. After obtaining informed consent, demographic data, Kurtzke's Expanded Disability Status Scale (EDSS) [16], and MRI examinations were performed in all MS patients at the beginning of the study, in accordance with standard clinical protocols. Neurological examination was performed by a qualified neurologist using the EDSS before the therapy and after its completion.

We excluded patients with the following chronic disorders: diabetes, obesity (BMI over 30), hormonal, urinary or liver abnormalities, infectious or inflammatory diseases, dyslipidemia, and smoking. We also excluded patients taking antioxidant substances, vitamins, or anti-inflammatory medications, as well as those who received hormonal treatment within the last 3 months before the study and those who took sleeping medication in the last 2 weeks before the study.

The patients were divided into the following groups:

(i) Control group consisted of 11 healthy controls observed in the Department of Neurology in Zabrze, Medical University of Silesia, Poland, due to undiagnosed headaches. Controls were matched for age and sex with the study group.

(ii) RRMS untreated group was composed of 14 de novo diagnosed patients, with the relapsing-remitting form of MS (RRMS), according to the McDonald criteria (2005) [17], with immunomodifying pretreatment, but without any immunomodifying MS treatment.

(iii) RRMS INF-beta group was composed of 36 patients with RRMS, diagnosed according to the McDonald criteria. All of them received interferon beta-1b [Betaferon (250 μg injected subcutaneously every other day)].

(iv) RRMS INF-beta + MEL group consisted of 25 RRMS patients receiving interferon beta-1b injected subcutaneously every other day supplemented orally with MEL, 5 mg per day, over a period of 90 days.

Demographic characteristics of the studied groups are presented in Table 1.

2.2. Materials. All basic reagents were from Sigma-Aldrich (Poznań, Poland), unless indicated otherwise. Fluorimetric and absorptiometric measurements were done in a Tecan Infinite 200 PRO multimode reader (Tecan Group Ltd., Männedorf, Switzerland) or in an EnVision Multilabel Plate Reader (Perkin-Elmer, Überlingen, Germany). All measurements were performed in triplicate and repeated a minimum of three times.

2.3. MRI Examination. Head magnetic resonance imaging (MRI) was performed in all MS patients at the beginning of the study. The 1.5T scanner imaging (General Electric HDx, USA) and standard head protocol for MS patients (multiple planes, slice thickness 5 mm, contrast media: Gadovist [Gd]) and additional postcontrast 3DT1 sequences (1 mm slice thickness) were used. Supratentorial, infratentorial, and number of enhancing T1 plaques were evaluated.

2.4. Blood Sampling. Samples of venous blood (10 ml) from MS patients and controls were collected into serum-separating tubes and immediately centrifuged to isolate serum. Collected serum samples were stored at −80°C until biochemical analysis, for not more than 2 months. They were thawed at room temperature only once at the time of analysis.

2.5. Estimation of Protein Carbonyls. The content of protein carbonyls was estimated using OxiSelect™ Protein Carbonyl Fluorometric Assay Kit (Cell Biolabs Inc.) according to the protocol supplied by the manufacturer.

2.6. Estimation of Protein Oxidative Modifications. Products of oxidative modifications of proteins were estimated on the basis of their characteristic fluorescence. Fluorescence measurements were done by applying 150 μl of the serum diluted 1 : 50 with phosphate-buffered saline (PBS; 1 tablet of PBS/100 ml H_2O) to wells of a 96-well plate. Fluorescence was measured at wavelengths of 325/440 nm (AGEs), 330/415 nm (dityrosine), 325/434 nm (N′-formylkynurenine), 365/480 nm (kynurenine), and 295/340 nm (tryptophan) [14, 15].

2.7. Estimation of Thiol Groups. Thiol groups were estimated using a modification of the Ellman's method [18]. Samples (20 μl) were pipetted to wells of a 96-well plate containing 100 μl of 0.1 M phosphate buffer, pH 8.0. Afterwards, 2 μl of 10 mg/ml Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid); DTNB] was added. Absorbance was measured after 1 h incubation in the dark at 37°C at the wavelength of 412 nm against a reagent blank. The thiol group content was calculated on the basis of a standard curve using glutathione as a standard.

2.8. Estimation of Protein. The protein concentration was estimated using the method of Lowry et al. [19]. Serum diluted 200 times with PBS (100 μl) was mixed with 500 μl of the Lowry reagent (formed by mixing 30 ml of 2% Na_2CO_3 in 0.1 M NaOH, 0.6 ml of 5% $C_4H_4O_6KNa{\cdot}4H_2O$, and 0.6 ml of 2% Cu_2SO_4) and incubated at room temperature for 10 min. Afterwards, 50 μl of the Folin–Ciocalteu reagent was added; the plate was shaken and incubated at room temperature for 30 min. The absorbance was measured at 750 nm. Standard curve was prepared with human serum albumin (0–300 μg/ml).

2.9. Estimation of Malondialdehyde (MDA). The serum samples (50 μl serum plus 50 μl PBS or 100 μl PBS blank) were mixed with ice-cold 200 μl of mixture (1 : 1) of 0.37% thiobarbituric acid (TBA) and 15% trichloroacetic acid (TCA) in 0.25 M HCl to precipitate protein. The reaction was performed at pH 2-3 at 100°C for 40 min. The precipitate was pelleted by centrifugation at 3000 ×g at 4°C for 10 min. Absorbance of supernatants was read at a wavelength of 532 nm.

The majority of TBA-reactive substances (TBARS) is malondialdehyde; thus, the concentration of MDA in blood serum was expressed as μM MDA. The results were calculated using an absorption coefficient for MDA of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.10. Estimation of AOPP. Advanced oxidation protein products (AOPP) were estimated using the method of Witko-Sarsat et al. [20]. 200 μl of serum diluted 1 : 5 with PBS was applied to a 96-well plate, and 20 μl of acetic acid was added to each well. Absorbance was measured at 340 nm against a blank containing 200 μl of PBS, 20 μl of acetic acid, and 10 μl of 1.16 M potassium iodide. Calibration curve was prepared using chloramine-T at concentrations of 0–100 μM by applying 200 μl chloramine-T, 20 μl acetic acid, and 10 μl of 1.16 M potassium iodide to the plate. AOPP concentration is expressed in nmol chloramine-T-equivalents/mg protein.

2.11. Estimation of Total Antioxidant Capacity of Blood Serum as FRAP. Total antioxidant status was measured in serum using the ferric reducing antioxidant power assay (FRAP). The ferric reducing antioxidant potential assay measures the ability of antioxidants to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions [21]. 0.3 M acetate buffer (pH = 3.6), 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl, and 0.02 M $FeCl_3{}^*\ 6H_2O$ mixed in 10 : 1 : 1 and 180 μl of this mixture were added to wells of a 96-well plate containing 10 μl of sample and 10 μl of PBS. The reduction of Fe^{3+}-2,4,6-tripyridyl-s-triazine complex to the ferrous form at low pH was monitored by measuring the absorption change after 20 min incubation at room temperature at 593 nm. The value was calculated relevant to the activity of Trolox and expressed as μmoles Trolox equivalents/l (μM).

2.12. Estimation of Total Antioxidant Capacity with ABTS.* Antiradical activity is a measure of the ability of a given compound to react with free radicals. One stable free radical employed in such reactions is the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS*). Standard antioxidants react rapidly with ABTS* (within seconds; "fast antioxidants") while some react at a lower rate ("slow antioxidants") [22]. Briefly, 2 μl of sample and 18 μl of PBS were added to a solution of ABTS*, diluted such that 200 μl of the solution had absorbance of 1.0 in a microplate well. The decrease in ABTS* absorbance was measured after 1 min ("fast" scavenging) and between 10 and 30 min ("slow" scavenging) of incubation at ambient temperature (21 ± 1°C) at 414 nm. ABTS* scavenging activity was calculated relevant to the activity of Trolox and expressed as μmoles Trolox equivalents/l (μM).

2.13. Statistical Analysis. All experiments were performed in triplicate. Data are shown in the form of arithmetic mean values and standard deviations. Statistical analysis was done using one-way analysis of variance (ANOVA/Dunnett's test) for multiple samples and Student's *t*-test for comparing

paired sample sets. p values less than 0.05 were considered statistically significant. The statistical analysis of the data was performed using STATISTICA (version 12.5, StatSoft Inc. 2XXX, Tulsa, OK, USA, http://www.statsoft.com).

3. Results and Discussion

There are several amino acid residues in proteins which are most sensitive to oxidative insult: first of all, cysteine, tryptophan, and tyrosine residues. The decrease in the level of these residues and increase in the level of their modification products are useful biomarkers of OS *in vitro* and *in vivo*.

The level of thiol groups in blood serum reflects predominantly the cysteine thiol groups of serum proteins. The level of thiol groups in the serum, expressed both as thiol concentration and the thiol content of serum proteins, decreased in nontreated patients (from 0.58 ± 0.05 to 0.52 ± 0.09 mM and from 0.52 ± 0.09 to 6.84 ± 0.95 nmol/mg protein, resp.), and the magnitude of this decrease was attenuated in patients treated with INF-beta and INF-beta plus MEL. However, these changes were devoid of statistical significance (not shown).

As a result of OS, the level of tryptophan fluorescence decreases and the levels of products of oxidative destruction of tryptophan such as kynurenine and N′-formylkynurenine, easily detectable by fluorescence, increased.

The values of tryptophan fluorescence decreased in the nontreated patients to $94.9 \pm 8.4\%$ of the control value. This decrease was attenuated in patients treated with INF-beta-1b and INF-beta plus MEL, but all these changes lacked statistical significance (not shown).

The content of N′-formylkynurenine in blood serum proteins of nontreated MS patients was significantly increased with respect to control ($p < 0.001$). Treatment with INF-beta plus MEL prevented this increase ($p < 0.001$), while treatment with INF-beta alone was ineffective (Figure 1).

The content of kynurenine in blood serum proteins of nontreated MS patients was significantly increased with respect to control ($p < 0.05$). Treatment with INF-beta plus MEL prevented this increase ($p < 0.01$), while treatment with INF-beta alone was again ineffective (Figure 2).

Tyrosine residues are other residues in proteins sensitive to oxidation and also to nitration. Free radical oxidation of tyrosine creates tyrosyl radicals; dimerization of tyrosyl radicals forms dityrosine, which can also be estimated on the basis of its characteristic fluorescence.

The level of dityrosine in blood serum proteins of nontreated MS patients was significantly elevated with respect to control ($p < 0.001$). Treatment with INF-beta ($p < 0.01$) and especially with INF-beta plus MEL ($p < 0.001$) eliminated this elevation (Figure 3).

Protein carbonylation is perhaps the most commonly studied oxidative protein modification induced by ROS. It usually refers to a process that produces reactive ketone or aldehyde residues on proteins that can react with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Protein carbonylation can occur via two ways, as "primary protein carbonylation" by direct oxidation of side chains of some amino acid residues, initiated by ROS and usually metal-catalyzed, and by "secondary protein carbonylation" via addition of aldehydes, generated mainly from lipid peroxidation, such as 4-hydroxynonenal (4-HNE), 2-propenal (acrolein), and malondialdehyde, as well as carbonyl-bearing products of sugar glycoxidation [23, 24].

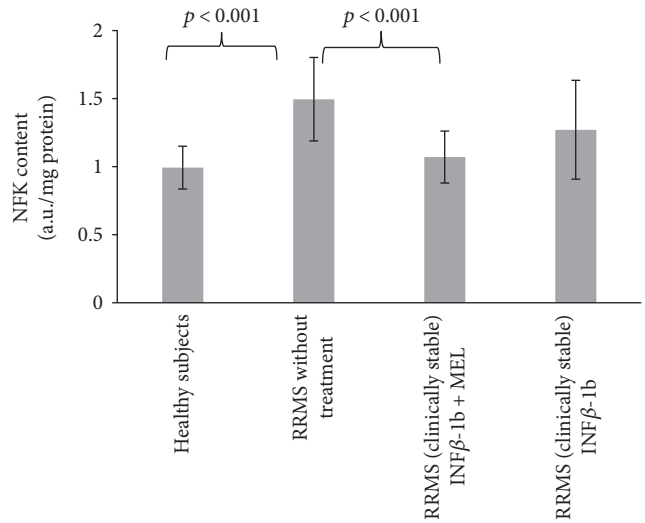

Figure 1: Comparison of N′-formylkynurenine content of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta plus MEL. If not indicated, differences are not statistically significant.

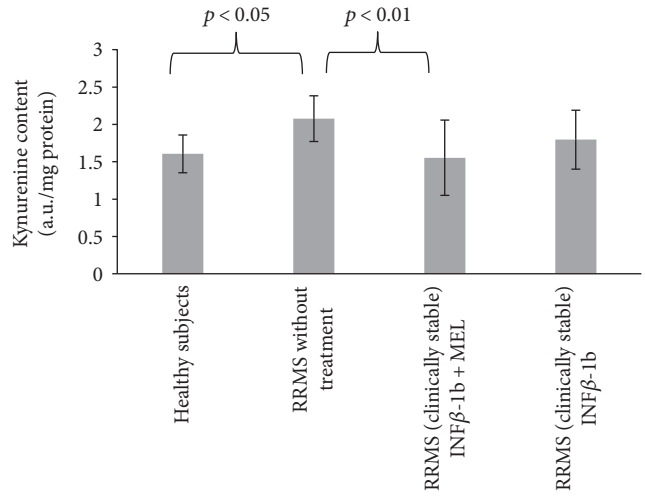

Figure 2: Comparison of kynurenine content of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta plus MEL.

The level of protein carbonyls increased in nontreated patients with respect to control ($p < 0.001$); this increase was significantly attenuated ($p < 0.05$) in patients treated with INF-beta plus MEL. The level of protein carbonyls was also decreased in patients treated with only INF-beta, but the decrease was not statically significant with respect to nontreated patients (Figure 4).

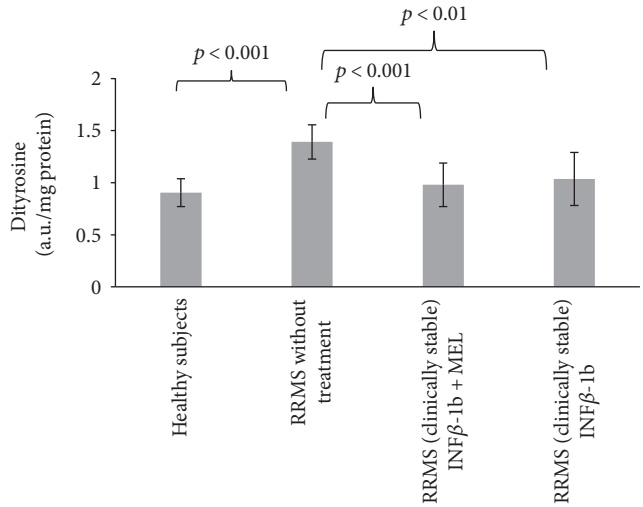

Figure 3: Comparison of dityrosine content of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta plus MEL.

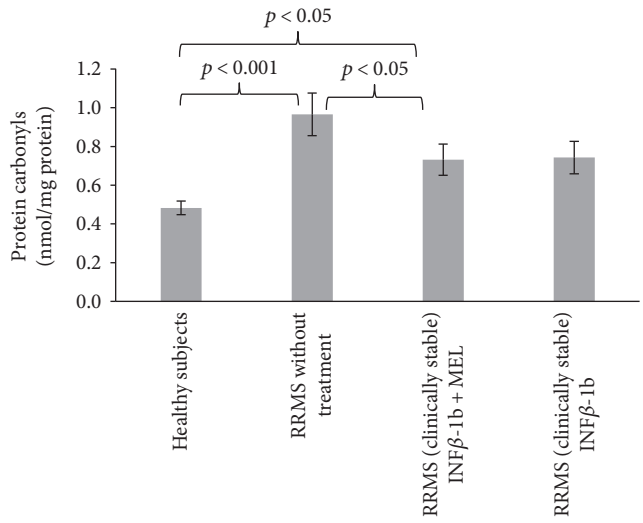

Figure 4: Comparison of carbonyl content of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta plus MEL.

Reaction of glycoxidation end products (AGEs) with proteins leads to formation of products with characteristic fluorescence. The fluorescence of AGEs in blood serum proteins of nontreated MS patients was significantly elevated with respect to control ($p < 0.01$). Treatment with INF-beta and MEL prevented this elevation ($p < 0.001$), while the effect of INF-beta alone was devoid of statistical significance (Figure 5).

The reasons for increase in the AGE fluorescence seem to be unclear as the glucose level is not increased in the patients. It may be perhaps attributed to the acceleration of protein glycation by OS demonstrated to occur *in vitro* [25]. The majority of AGEs *in vivo* are mainly formed in a fast reaction

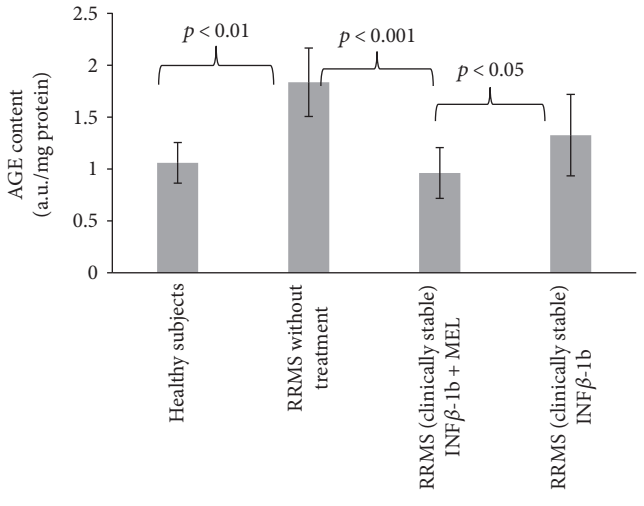

Figure 5: Comparison of AGE content of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta and MEL.

of dicarbonyl compounds, such as methylglyoxal (MGO) and glyoxal, with proteins. Furthermore, the main detoxification system of dicarbonyl compounds, the glyoxalase system, seems to be affected in MS patients, which may contribute to high MGO-derived AGE levels [26]. Advanced glycation end products are increased in inflammatory diseases such as atherosclerosis, obesity, and diabetes and also in neuroinflammatory diseases such as Alzheimer's disease and Parkinson's disease. It was reported that AGEs are increased in the plasma and CNS of MS patients [26, 27]. Wetzels et al. [26] suggested that the accumulation of AGEs in the plasma and central nervous system of MS patients compared to healthy controls may contribute to neuroinflammation and the progression of MS.

Advanced oxidation protein products (AOPP) consist of oxidized, dityrosine-containing, crosslinked proteins formed mainly by reactions of reactive chlorine species with plasma proteins [28]. The level of AOPP was increased in the nontreated patients ($p < 0.05$); this increase was attenuated in patients treated with INF-beta and INF-beta plus MEL (Figure 6).

Oxidative stress in MS patients was also assessed on the basis of standard parameters such as the level of malondialdehyde (MDA) and total antioxidant capacity of blood serum.

The MDA concentration in blood serum was elevated in the nontreated patients, but treatment with either INF-beta or INF-beta plus melatonin did not attenuate this increase (Figure 7).

Blood serum FRAP was decreased in nontreated MS patients from 368.6 ± 77.7 to $318.0 \pm 77.8\,\mu$M Trolox equivalents; this decrease was attenuated especially in patients treated with INF-beta plus MEL ($378.0 \pm 112.8\,\mu$M Trolox equivalents), but all these changes lacked statistical significance.

"Fast" ABTS* scavenging activity of blood serum was decreased in the patients with respect to control subjects,

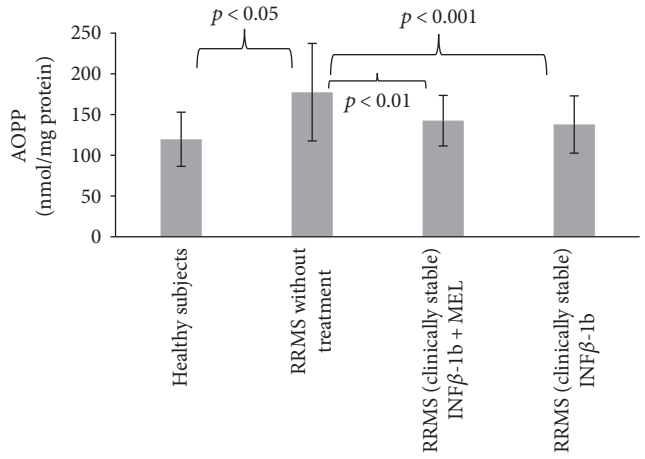

Figure 6: Comparison of AOPP content of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta and MEL.

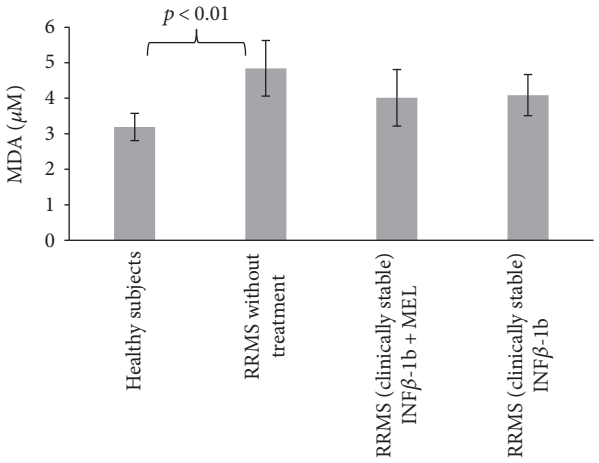

Figure 7: Comparison of malondialdehyde concentration in blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta and MEL.

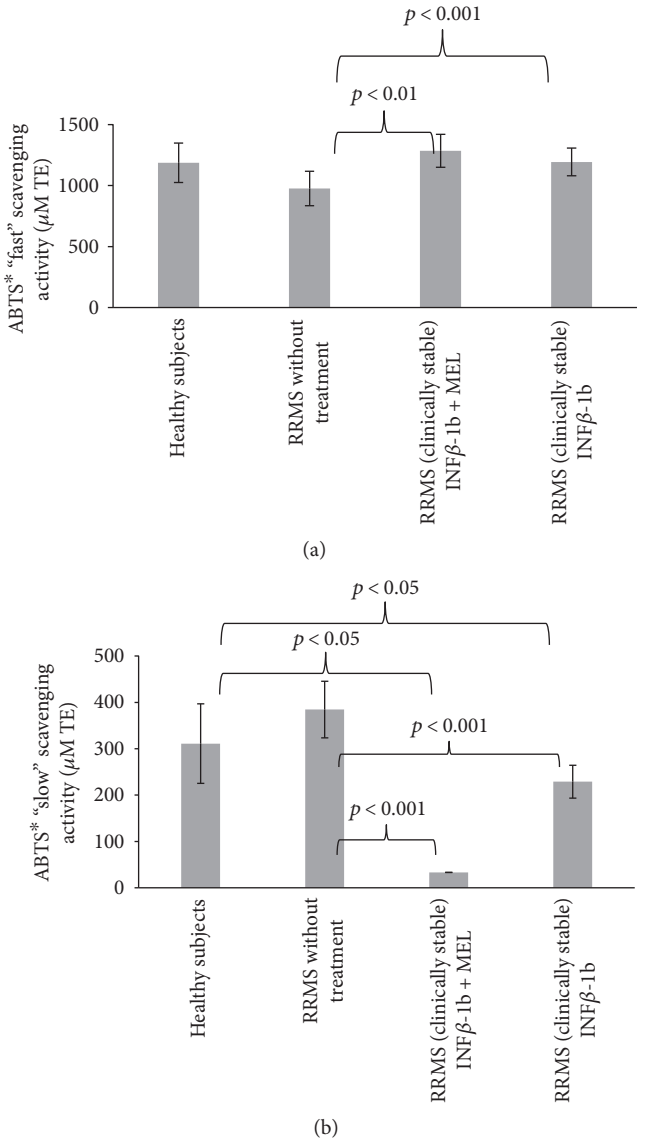

Figure 8: Comparison of "fast" (a) and "slow" (b) ABTS* scavenging activity of blood serum proteins in healthy controls, in RRMS patients without treatment, in RRMS patients treated with INF-beta, and in RRMS patients treated with INF-beta and MEL.

but this decrease was devoid of statistical activity. However, treatment with INF-beta and with INF-beta plus MEL caused significant elevation of this activity ($p < 0.001$ and $p > 0.01$, resp.). MEL alone could not contribute significantly to the ABTS* scavenging activity of blood serum as its submicromolar concentrations attainable *in vivo* are insignificant with respect to other contributing compounds [29]. "Slow" ABTS* scavenging activity was increased in the nontreated patients (devoid of statistical significance); treatment with either INF-beta or INF-beta plus MEL decreased "slow" ABTS-scavenging activity ($p < 0.001$ in both cases; Figure 8).

It is not obvious what determines the "slow" ABTS* scavenging activity of blood serum. Amino acids such as tryptophan and tyrosine show this type of reactivity [22]. It is possible that the increase in the "slow" ABTS* scavenging activity may be due to leakage of some intracellular slowly reacting antioxidants.

In summary, the study corroborates the usefulness of protein oxidative modifications as sensitive markers of OS in MS. Our results confirm also previous findings on the occurrence of OS treatment of MS and attenuation of OS by INF-beta treatment, judging from the levels of oxidative modifications of blood serum proteins [14, 15]. The antioxidant effect of INF-beta is due to its immunomodulatory and anti-inflammatory action [1]. Combined treatment with INF-beta and melatonin was more effective in attenuating oxidative stress, being effective in diminishing the increase in the levels of N′-formylkynurenine, kynurenine, carbonyl, and AGE content where treatment with INF-beta alone was ineffective. These results confirm our previous finding on the attenuation of OS in MS patients treated with INF-beta and MEL, on the basis of reduction of lipid plasma hydroperoxide level [30].

It does not seem probable that the attenuation of OS by MEL in MS patients is due to the direct antioxidant activity of this compound, as discussed above. However, MEL is also an indirect antioxidant, acting via stimulation of biosynthesis of antioxidants and other protective proteins, which may be the major mechanisms of its action. MEL was shown to increase the level of expression of genes coding for catalase, MnSOD, and sirtuin 1 [31] and is known to regulate the mitochondrial bioenergetic function [32]. Moreover, MEL directly interferes with the differentiation of T cells, inducing the expression of the repressor transcription factor Nfil3, blocking the differentiation of pathogenic Th17 cells and boosting the generation of protective Tr1 cells via Erk1/2 and the transactivation of the IL-10 promoter by ROR-α [33]; MEL inhibits also demyelination and increases remyelination [34]. These multiple effects of MEL may, apart from its antioxidant effects, contribute to its positive effects in MS [1, 30, 33, 35].

4. Conclusion

This study demonstrates that MEL administration to MS patients undergoing therapy with INF-beta ameliorates oxidative stress, decreasing the extent of the majority of protein oxidative modifications examined. These results support the view that combined administration of INF-beta and MEL can be more effective in reducing oxidative stress in MS patients than INF-beta alone.

Conflicts of Interest

The authors have no conflicts to disclose.

Authors' Contributions

Izabela Sadowska-Bartosz was responsible for the concept and design of the experiments and supervision of experimental work, performed part of the experiments, and had a leading role in the analysis of the results and preparation of the manuscript. Monika Adamczyk-Sowa and Paweł Sowa participated in the design of the experiments and were responsible for patient recruitment, clinical characterization of the patients, and collection of blood samples. Sabina Galiniak performed part of the experiments and their statistical evaluation. Ewa Żyracka, Michalina Grzesik, and Katarzyna Naparło performed part of the experiments. Grzegorz Bartosz participated in the preparation of the manuscript. All authors have approved the final manuscript.

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Research Article

Ghrelin Protects Human Lens Epithelial Cells against Oxidative Stress-Induced Damage

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Oxidative stress has been recognized as an important mediator in the pathogenesis of age-related cataracts; using antioxidant supplements is one plausible strategy to protect the antioxidative defense system against oxidative stress. Ghrelin administration is expected to reduce ROS, preventing the onset of different diseases. The role of ghrelin, if any, in protecting against oxidative stress in HLECs has never been examined. In the present study, we investigated the effects of ghrelin against H_2O_2-induced oxidative stress and the associated molecular mechanisms in HLECs and rat lenses. The results showed that pretreatment with ghrelin reduced H_2O_2-induced cellular apoptosis and ROS accumulation, increased the expression levels of SOD and CAT, and decreased the expression level of MDA. The morphological examination showed that the ghrelin-treated lens organ culture maintained transparency. This is the first report to show that ghrelin can protect HLECs from H_2O_2-induced oxidative stress. Our findings suggest that ghrelin may prevent the progression of cataracts, which has treatment value for ophthalmologists.

1. Introduction

Oxidative stress has been recognized as an important mediator in the pathogenesis of age-related cataracts; it is widely recognized as a state of imbalance between prooxidants and antioxidants [1, 2]. Excessive production of reactive oxygen species (ROS) plays an important role in the destruction of normal lens epithelial cell function [3, 4]. Hydrogen peroxide (H_2O_2), a nonradical member of the active oxygen family, is a major intracellular ROS and accumulates in substantial amounts in the lens [5]. Several studies have shown that free radicals and ROS can affect the growth and function of human lens epithelial cells (HLECs) [1, 2, 5]. Our previous studies have found that ROS produced by H_2O_2 causes protein degradation and epithelial cell damage—the damage similar to the damage found in human cataracts [6, 7].

Cataracts, which are the leading cause of visual disability globally, are a protein conformational disease characterized by the aggregation of oxidatively damaged proteins [8]. Oxidation of lens proteins is a major risk factor in cataract formation—any external insult or insufficient degradation of damaged proteins may affect the antioxidant status of the lens and cause opacification [1, 2, 7].

Using antioxidant supplements is one plausible strategy to protect the antioxidative defense system against oxidative stress [9]. Although surgery can remedy cataracts, there are numerous postoperative complications; further, some regions lack the necessary surgical instruments and face shortages of doctors who can meet the medical needs of large numbers of patients. These factors limit the availability of cataract surgery. There are no effective therapeutic agents to halt the formation of a cataractous lens; therefore, it is necessary to develop a pharmacological

intervention to improve lens transparency and delay the progression of cataracts.

The growth hormone-releasing peptide ghrelin, a 28-amino-acid endogenous peptide, is secreted primarily from the gastric mucosa [10]. Its transcripts have also been found in the intestine, pancreas, liver, placenta, heart, lungs, central nervous system (CNS), and kidneys, suggesting its extraendocrine as well as endocrine action. Studies have illustrated that ghrelin exhibits favorable cytoprotective effects against oxidative stress [11]. It can remove ROS and reactive nitrogen species (RNSs) by increasing the expression of antioxidant enzymes and directly scavenging free radicals [12]. Ghrelin administration is expected to reduce ROS, preventing the onset of various diseases. The role of ghrelin, if any, in protecting against oxidative stress in HLECs has never been examined. In the present study, we investigated the effects of ghrelin against H_2O_2-induced oxidative stress and the associated molecular mechanisms in HLECs and rat lenses.

2. Material and Methods

2.1. Reagents and Antibodies. Ghrelin was purchased from Sigma Chemical (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY, USA). Medium 199 was obtained from Sigma-Aldrich (St. Louis, MO). Annexin V-FITC and propidium iodide (PI) were obtained from Becton Dickinson (Mountain View, CA, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and H_2DCFDA were obtained from Beyotime (Beyotime Institute of Biotechnology, Shanghai, China). Anti-SOD, anti-CAT, and anti-MDA antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SOD, CAT, and MDA colorimetric kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Cell Culture and Treatment. HLECs (ATCC, America) were cultured in DMEM with heat-inactivated (56°C, 0.5 h) FBS (15%), 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified 5% CO_2 at 37°C. The cells were routinely subcultured every 2-3 days. When grown to 70% confluence, they were treated with the indicated concentration of H_2O_2 for 24 h or pretreated with different concentrations of ghrelin for 12 h before the H_2O_2 treatment.

2.3. Cell Viability Assay. The cells were plated at a density of 2×10^4 cells/well in a 96-well culture plate and incubated with 0, 50, 100, 200, 400, and 800 μM H_2O_2 for 24 h alone or after pretreatment with different concentrations of ghrelin (10^{-9}–10^{-6} M) for 12 h. The culture medium was removed, and cell viability was measured using the MTT method as previously described [13].

The morphological changes of the cells were observed under an inverted microscope (Olympus CK-30, Tokyo, Japan).

2.4. Cell Apoptosis Assay. Annexin V-FITC/PI staining was used to quantify the amount of cell apoptosis. Cells were plated and incubated on a six-well plate at 1×10^6 cells/well and pretreated with or without different concentrations of ghrelin for 12 h, after which they were treated with 100 μM H_2O_2 for 24 h. The cells were collected and stained with annexin V-FITC/PI in binding buffer at room temperature in the dark for 20 min. The stained cells were then analyzed using a flow cytometry system.

2.5. Fluorescent Staining of Cells with H_2DCFDA. The levels of intracellular ROS were monitored using H_2DCFDA. The cells were incubated in 10 μM H_2DCFDA for 20 min at 37°C and then washed twice with PBS. The fluorescence intensity of DCF was detected using a fluorescence microscope (Leica DMI 4000, Germany).

2.6. Lens Organ Culture. Rat eyes (Harbin Medical University, Harbin, China) were removed and placed in mammalian physiological saline prewarmed to 37°C. Freshly extracted transparent lenses were incubated in Medium 199 containing 50 mg/mL gentamicin and 0.1% BSA with 5% CO_2 at 37°C. The lenses were treated with ghrelin at concentrations of 10^{-9}–10^{-6} M for 24 h at 37°C. The medium was changed, and 100 μM H_2O_2 was added to the medium for 6 h. The lenses were observed under a stereomicroscope and photographed against a background of black gridlines to record the development of opacity.

2.7. Antioxidant Enzyme Content Assay. Whole lenses were removed from the eyes and sonicated in extract buffer. After centrifugation, the supernatants were used for testing according to the assay manufacturer's instructions. Total SOD content was determined spectrophotometrically at 550 nm, and the results were expressed as U·mg^{-1} protein [14]. MDA was measured with reference to the MDA assay kit using the thiobarbituric acid method [15]. The results were expressed as U·mg^{-1}, and the intensity of the resulting pink color was read at 532 nm. CAT content was assayed using the ammonium molybdate method according to the instructions of the CAT assay kit [16]. The results were expressed as U·mg^{-1}, and the faint yellow complexes were detected at 405 nm.

2.8. Western Blot Analysis. Lenses from each group were washed with cold saline, dried with filter paper, cut with Vannas scissors, and then ground on ice. The tissue was spun down in a refrigerated centrifuge at 1000 r/min for 10 min, and the supernatants were lysed on ice for 20 min in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and centrifuged at 12000g for 20 min. The total protein (30 μg) was subjected to 10–15% SDS-PAGE and transferred into a polyvinylidene difluoride membrane. The blot was incubated with antibodies against SOD, CAT, and MDA. The enzyme was used with a horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence was used to detect the immunoreactive bands, and ImageJ software (image processing and analysis in Java) was used to quantify the results. After normalizing to the individual actine levels,

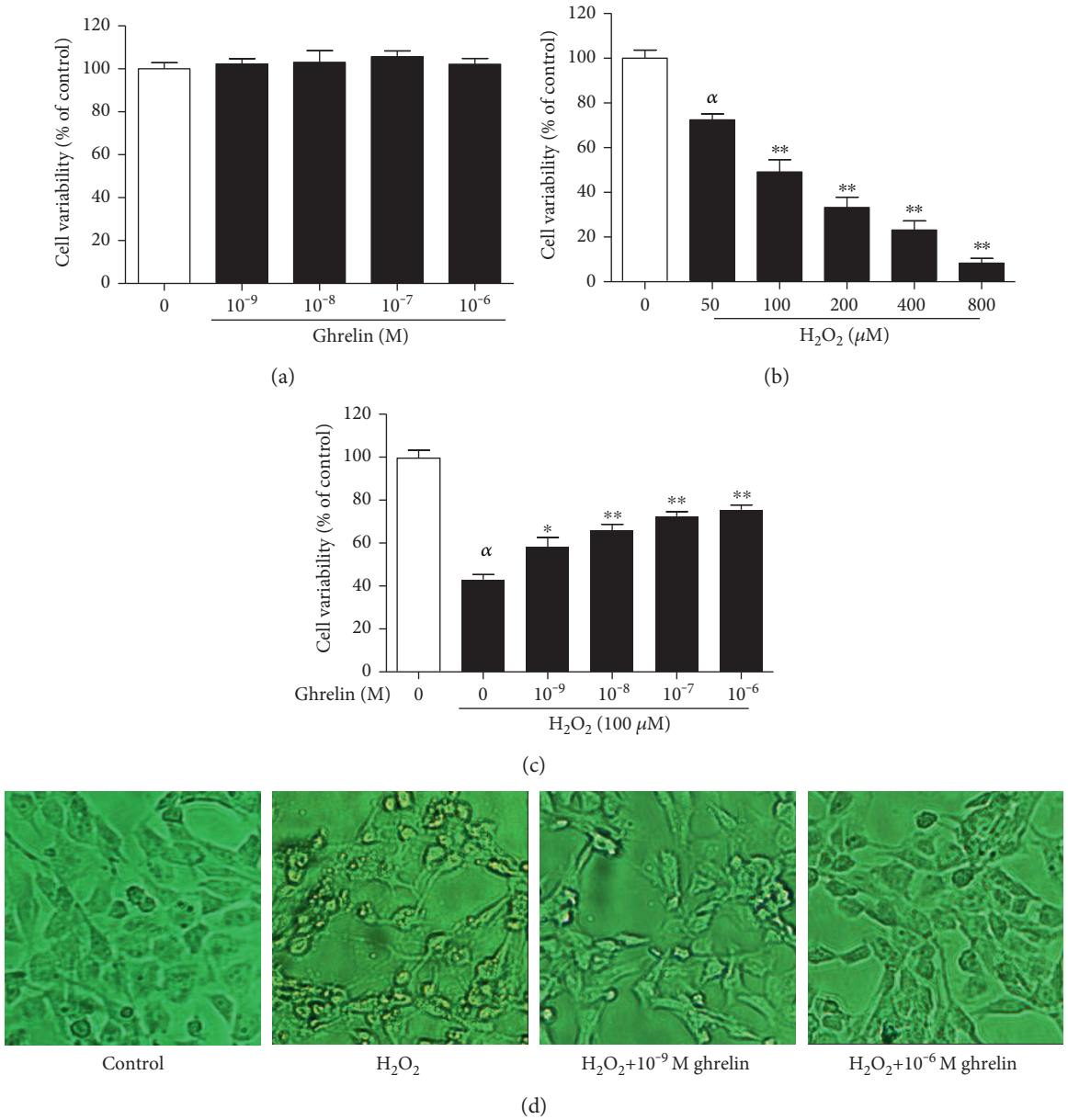

Figure 1: The effects of ghrelin on the cell viability of H_2O_2-treated HLECs. (a) HLECs were incubated with different concentrations of ghrelin (10^{-9}–10^{-6} M) for 24 h. (b) HLECs were incubated with different concentrations of H_2O_2 (50–800 μM) for 24 h. (c) HLECs were preincubated with ghrelin (10^{-9}–10^{-6} M) for 12 h before being treated with 100 μM H_2O_2 for 24 h. Cell viability was assessed via MTT assay. (d) HLECs were detected by microscopy. The results were represented as the mean ± SEM ($n = 3$) from three independent experiments. $^{\alpha}P < 0.01$, compared with the untreated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the H_2O_2-treated group.

the ratio of the expression of target proteins was determined. Each experiment was repeated three times.

2.9. Statistical Analysis. Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All values are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. The significance of pairwise group was evaluated using Student's *t*-test. For comparison of more than two groups, one-way ANOVA was used; $P < 0.05$ was considered to be significant.

3. Results

3.1. Effects of H_2O_2 and Ghrelin on HLECs' Viability. As shown in Figure 1(a), ghrelin did not exhibit any cytotoxic effects on HLECs. H_2O_2 impaired cell viability in a dose-dependent manner (Figure 1(b)). Treatment with a concentration of 100 μM H_2O_2 for 24 h was selected for subsequent experiments because it reduced cell viability to approximately 49.12% compared with the control group (cells not treated with H_2O_2). The pretreatment of HLECs with ghrelin showed a dose-dependent protective effect against H_2O_2 damage (Figure 1(c)). Figure 1(d) shows the

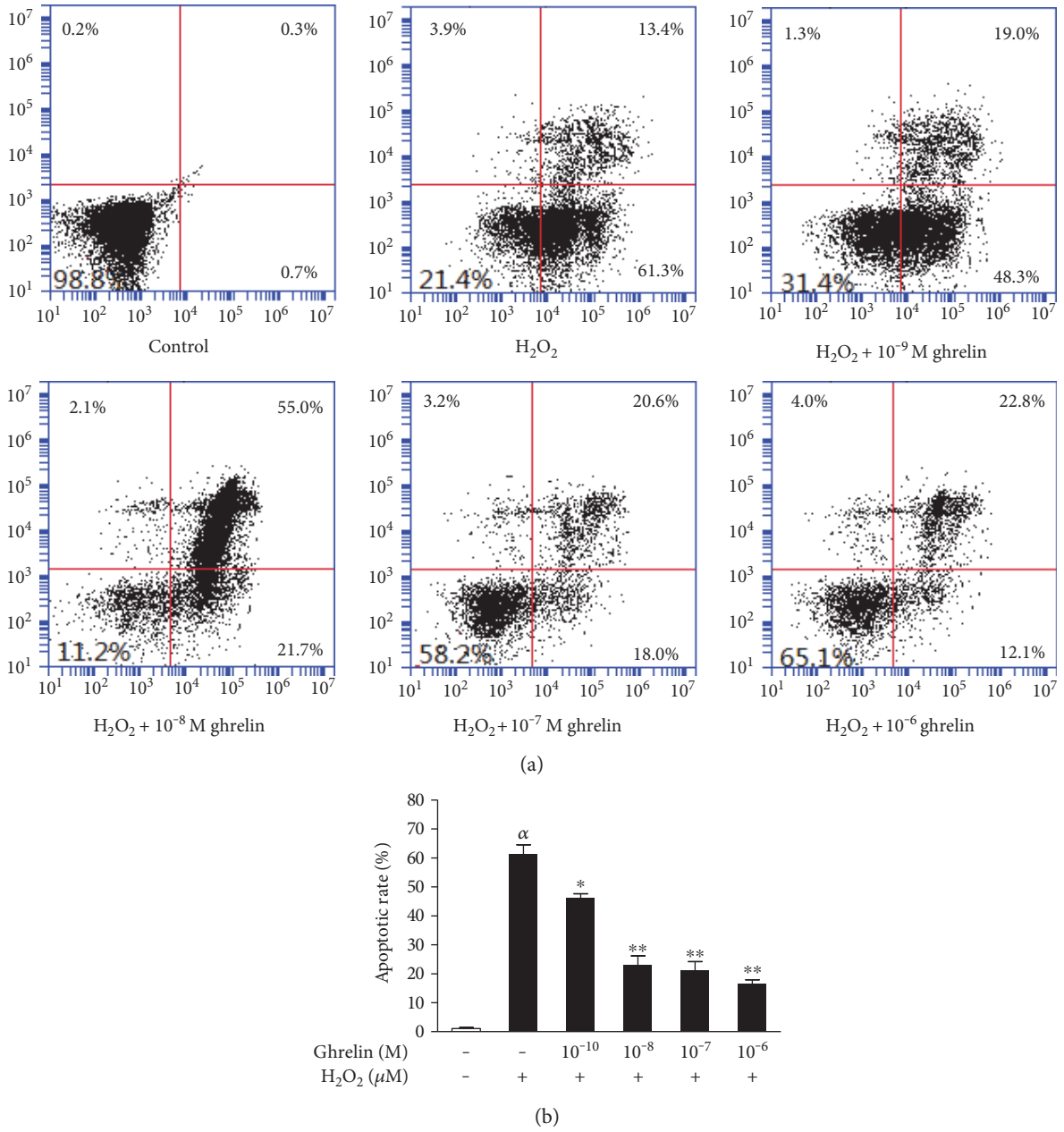

Figure 2: Ghrelin prevented H_2O_2-induced cellular apoptosis in HLECs. HLECs were preincubated with ghrelin (10^{-9}–10^{-6} M) for 12 h before being treated with 100 μM H_2O_2 for 24 h. (a) Apoptosis of HLECs as detected by flow cytometry. The results were represented as the mean ± SEM ($n = 3$) from three independent experiments. (b) Ghrelin significantly decreased the apoptosis rate of HLECs. $^{\alpha}P < 0.01$, compared with the untreated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the H_2O_2-treated group.

morphological changes of HLECs. The control group showed normal cell morphology and a regular range of cells with intact junctions. The H_2O_2-treated group showed shrinkage of the cells with abnormal shapes, and the distance between cells increased; however, pretreatment with ghrelin inhibited H_2O_2 damage to cell morphology.

3.2. Ghrelin Inhibits Apoptosis Induced by H_2O_2. The rate of cell apoptosis was quantified using flow cytometric analysis by double staining with annexin V-FITC and PI. An increase of apoptotic cells was observed in the H_2O_2-treated group, while ghrelin pretreatment decreased the apoptosis rate of the cells exposed to H_2O_2 (Figure 2(a)). This positive effect of ghrelin was observed in a concentration-dependent manner (Figure 2(b)).

3.3. Ghrelin Reduced the Generation of ROS in HLECs. As expected, there was a lack of staining in the H_2O_2-free control group (Figure 3). HLECs that were only exposed to H_2O_2 had a light green color, indicating that there was a

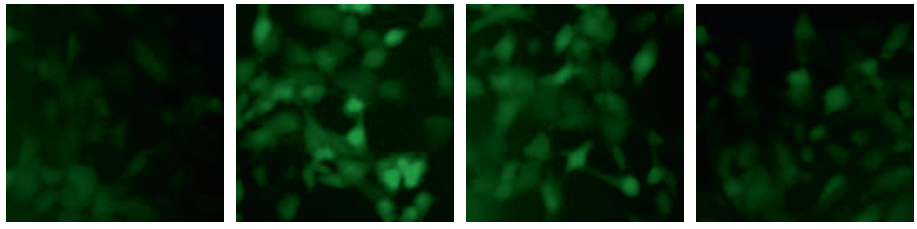

Figure 3: Effect of ghrelin on H_2O_2-induced generation of ROS in HLECs. Cells were treated with 100 μM H_2O_2 for 24 h after incubation in the absence or presence of ghrelin for 12 h. Then, the production of ROS was determined using 10 μM H_2DCFDA. The morphological features of the cells were observed using fluorescence microscopy.

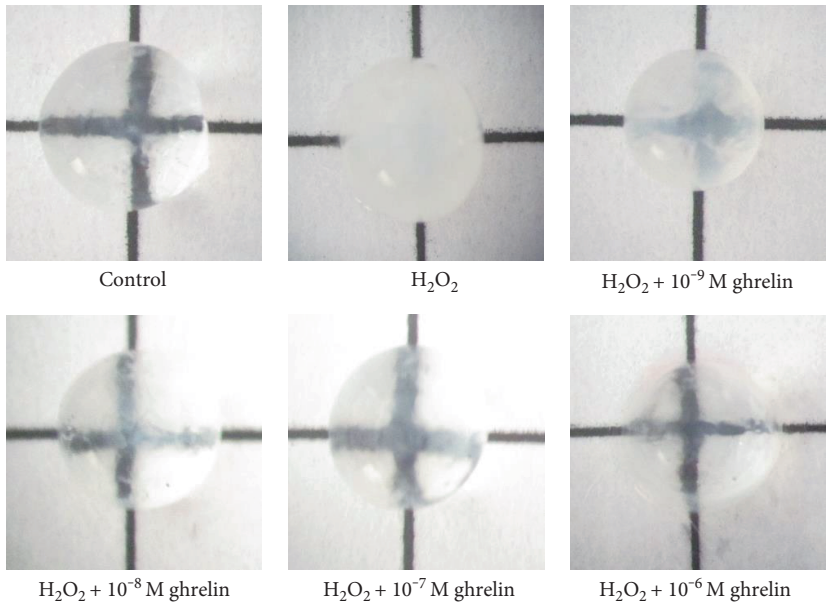

Figure 4: Morphological observations confirmed the reduction in opacity when the lenses were incubated in ghrelin. Whole lenses were pretreated with ghrelin at concentrations of 10^{-9}–10^{-6} M for 24 h and then cultured for 6 h with 100 μM H_2O_2. The control group received no treatment, and the H_2O_2 group was treated only with 100 μM H_2O. Photographs were taken against a background of black gridlines. The higher the concentrations of ghrelin, the less opacity was observed in the lenses.

marked increase in ROS levels. This increase in intracellular ROS was prevented in a dose-dependent manner by pretreatment with ghrelin. This finding indicates that ghrelin can prevent the generation of intracellular ROS in HLECs challenged with H_2O_2.

3.4. Grading the Lenses. We developed an organ culture experiment to examine the effects of ghrelin on the lenses. Morphological observations confirmed that the lenses incubated in ghrelin showed a reduction in opacity (Figure 4). Lenses in the control group had an absence of opacification, and the gridlines were clearly visible; however, lens opacity measurements showed that 100 μM H_2O_2 induced obvious cataract formation in the lenses. Ghrelin blocked the effect of H_2O_2 in a dose-dependent manner.

3.5. Effects of Ghrelin on the Protein Expression of SOD, CAT, and MDA in Lenses. Western blot was used to measure the levels of SOD, CAT, and MDA in H_2O_2-stimulated HLECs to detect the antioxidative capability of ghrelin. Exposure to H_2O_2 markedly decreased the activity of SOD and CAT and increased the MDA content compared with the control group. Pretreatment with ghrelin significantly increased the expression of SOD and CAT, and decreased MDA compared with the H_2O_2 group (Figure 5).

3.6. Effect of Ghrelin on SOD, CAT Activity, and MDA Content in Lenses. The biological activity assay showed that 100 mM H_2O_2 significantly decreased SOD and CAT activity in the lenses, and pretreatment with ghrelin blocked the effect of H_2O_2. As shown in Table 1, 100 mM H_2O_2 significantly increased MDA content in the lenses. Finally, ghrelin blocked the effect of H_2O_2 in a dose-dependent manner.

4. Discussion and Conclusion

Cataracts are the leading cause of legal blindness worldwide. Until now, there has not been an effective pharmacological agent that can inhibit or reverse the progression of cataracts,

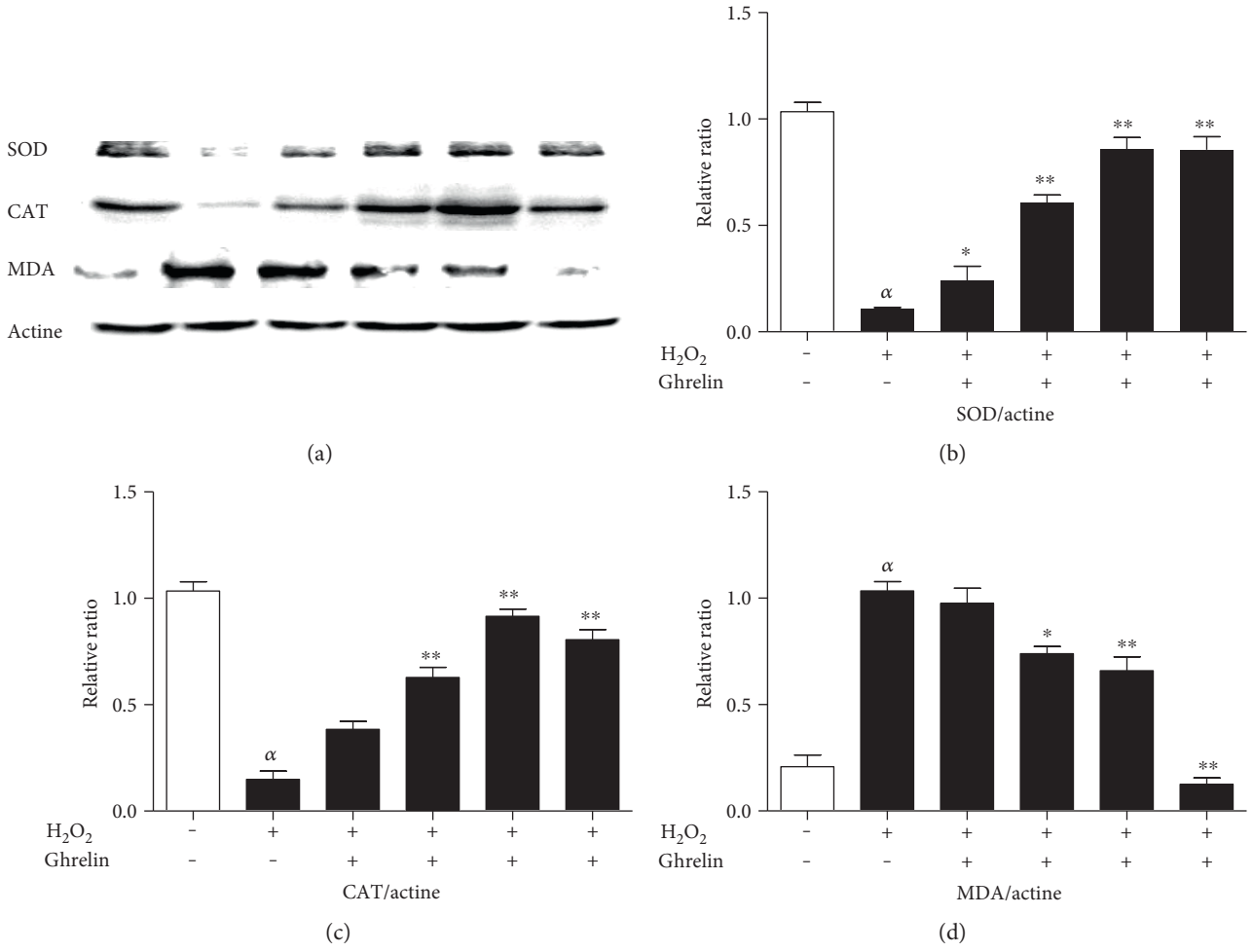

Figure 5: Effects of ghrelin on expression of SOD, CAT, and MDA in lenses. (a) The protein levels of SOD, CAT, and MDA were measured using western blot analyses. (b, c, d) The relative protein expression levels of SOD, CAT, and MDA. $^{\alpha}P < 0.01$, compared with the untreated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the H_2O_2-treated group.

Table 1: Effect of ghrelin on SOD, CAT activity, and MDA content in lenses.

Group	SOD ($U \cdot mg^{-1}$)	CAT ($U \cdot mg^{-1}$)	MDA ($nmol \cdot mg^{-1}$)
Control	27.87 ± 1.57	7.47 ± 1.43	0.44 ± 0.02
H_2O_2	10.27 ± 1.03^{a}	3.34 ± 1.15^{a}	1.83 ± 0.09^{a}
$H_2O_2 + 10^{-9}$ M ghrelin	15.29 ± 2.11^{b}	4.99 ± 1.23^{b}	0.93 ± 0.04^{b}
$H_2O_2 + 10^{-8}$ M ghrelin	15.43 ± 2.00^{b}	4.87 ± 1.01^{b}	0.86 ± 0.05^{b}
$H_2O_2 + 10^{-7}$ M ghrelin	17.03 ± 1.59^{b}	5.34 ± 0.74^{b}	0.77 ± 0.04^{b}
$H_2O_2 + 10^{-6}$ M ghrelin	19.03 ± 2.08^{b}	5.65 ± 0.81^{b}	0.67 ± 0.07^{b}

Compared with control group $^{a}P < 0.05$; compared with the H_2O_2-treated group $^{b}P < 0.05$.

and the search for affordable and nonsurgical pharmacological treatment is necessary to delay the progression of lens opacification [17, 18]. The effects of ghrelin on HLECs and the regulatory mechanism of those effects have never been reported. Therefore, the present study is the first to investigate the effect of ghrelin on H_2O_2-induced cell injury in HLECs.

Accumulating evidence shows that oxidative stress is a major contributor to the initiation and progression of cataracts. Oxidative stress refers to a state of elevated levels of ROS, with the latter being affected by an intracellular oxidative and antioxidant defense mechanism [19–21]. An increasing level of ROS is a crucial determinant of oxidative damage and impaired cellular function. During oxidative stress, the activity of antioxidant enzymes is inhibited, while the concentration of reactive nitrogen species (RNSs) or ROS increases dramatically. Oxidative stress is involved in the pathogenesis of eye diseases, including age-related cataracts and macular degeneration [19, 22]. Considering that oxidative stress plays an important role in the pathogenesis of cataracts, reducing oxidative stress is a plausible potential therapeutic target for cataracts.

Ghrelin has several biological actions, including regulating cell survival and proliferation, inhibiting inflammation, and exerting antioxidative effects [23, 24]. Previous studies have indicated that the antioxidative effect of ghrelin is based on increasing the activity of endogenous antioxidant enzymes [25]. For example, ghrelin has been reported to alleviate SAH-induced oxidative brain damage by maintaining a

balance in oxidant-antioxidant status [26]. Ghrelin has also been found to significantly reduce the protein expression of iNOS and increase the expression of CuZnSOD, MnSOD, CAT, and GPx in the liver [27]. Ghrelin is an amino acid peptide—it is safe for humans, as has been shown in many studies [28]. Our study also showed that ghrelin exhibited no obvious cytotoxicity in HLECs and pretreatment with ghrelin inhibited H_2O_2 damage to cell morphology. Considering its lack of toxicity and its excellent antioxidative effect, we propose that ghrelin should be considered as a prophylaxis for preserving visual function and could be used to treat age-related cataracts.

HLECs are a single layer of epithelial cells on the lens' anterior surface. The normal construction and function of HLECs is crucial for the maintenance of the transparency and metabolic homeostasis of the entire lens. Once damaged, they cannot self-renew, and they become permanently impaired. Oxidative stress could increase the permeability of HLECs, causing dysfunction. Studies have indicated that oxidants, especially H_2O_2, could trigger lens epithelial cell apoptosis and initiate early cataract formation [6, 7, 29]. Once the lens' defense system is weakened, cataracts begin to form.

The antioxidant systems protect cells against oxidative damage during normal metabolism and after an oxidative insult; they contain numerous antioxidant enzymes such as SOD, CAT, and glutathione peroxidase (GPx). These antioxidative enzymes can protect the lens from oxidative stress and maintain lens clarity [30]. H_2O_2-induced cataracts were associated with decreases in SOD and CAT activity and increase in MDA activity in the lens. These three enzymes are essential in oxidative stress protection and normal lens metabolism (SOD, CAT, and MDA). The antioxidant enzymes are able to catalytically remove free radicals and other reactive species. SOD can prevent lipid peroxidation, scavenge ROS, and protect cells from the damaging effects of toxic oxygen radicals [31]. CAT reduces H_2O_2 to water; a decline in the level of CAT weakens the antioxidant capacity of the lens epithelial cells and induces their apoptosis, which causes cataracts [32]. MDA is one of the metabolic products of lipid peroxides (LPO) and is well known as a widely used marker for oxidative stress [33].

Our in vitro test has verified the effect of ghrelin in preventing oxidative stress-induced cell dysfunction; however, can ghrelin also be effective for lens tissue? To answer this, we present the results of an ex vivo study to assess the anticataract potential of ghrelin in H_2O_2-induced isolated rat lenses through observation of lens transparency and estimation of some biochemical parameters such as SOD, CAT, and MDA contents.

Western blot results showed increased protein expression of SOD and CAT and a decreased expression of MDA under ghrelin treatment. Antioxidant enzyme content assays showed similar results; the mean content of SOD and CAT significantly decreased in the H_2O_2-treated lenses compared with the control lenses. In the ghrelin-treated group, the mean content of antioxidant enzymes was restored compared with the lenses in the H_2O_2-treated group. The elevated content of MDA in the H_2O_2-treated lenses may account for the disruption of membrane lipids. In addition, the reduction of the MDA level in ghrelin-treated group suggests that ghrelin may have prevented the disruption of lenticular membrane lipids, thereby impeding opacification of the lens.

In conclusion, the data from our experiments demonstrated that ghrelin effectively retarded H_2O_2-induced cataract formation. As an antioxidant agent, ghrelin increased the levels of SOD and CAT and decreased the level of MDA, thus sustaining lens transparency. Furthermore, ghrelin is an amino acid and should therefore be safe for use in humans. Due to its safety and efficacy, ghrelin has potentially important implications for the prevention of cataractogenesis.

Abbreviations

ROS: Reactive oxygen species
HLECs: Human lens epithelial cells
H_2O_2: Hydrogen peroxide
SOD: Superoxide dismutase
CAT: Catalase
MDA: Malondialdehyde
MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

Conflicts of Interest

The authors declare that they have no financial competing interests.

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Review Article

Protein Posttranslational Modifications: Roles in Aging and Age-Related Disease

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Aging is characterized by the progressive decline of biochemical and physiological function in an individual. Consequently, aging is a major risk factor for diseases like cancer, obesity, and type 2 diabetes. The cellular and molecular mechanisms of aging are not well understood, nor is the relationship between aging and the onset of diseases. One of the hallmarks of aging is a decrease in cellular proteome homeostasis, allowing abnormal proteins to accumulate. This phenomenon is observed in both eukaryotes and prokaryotes, suggesting that the underlying molecular processes are evolutionarily conserved. Similar protein aggregation occurs in the pathogenesis of diseases like Alzheimer's and Parkinson's. Further, protein posttranslational modifications (PTMs), either spontaneous or physiological/pathological, are emerging as important markers of aging and aging-related diseases, though clear causality has not yet been firmly established. This review presents an overview of the interplay of PTMs in aging-associated molecular processes in eukaryotic aging models. Understanding PTM roles in aging could facilitate targeted therapies or interventions for age-related diseases. In addition, the study of PTMs in prokaryotes is highlighted, revealing the potential of simple prokaryotic models to uncover complex aging-associated molecular processes in the emerging field of microbiogerontology.

1. Introduction

In recent years, aging research has shifted its focus to the concept of healthspan, the extension of the period of life during which an individual remains healthy, rather than focusing only on ways to extend lifespan [1]. Worldwide, the average life expectancy at birth is now over sixty years as a result of improved healthcare access, decreased child mortality rates, reduced maternal mortality, improved lifestyle, and higher standards of living, among other factors [2]. This increase in life expectancy has led to a shift in population structure; between 2000 and 2050, the number of people aged 60 and over is expected to increase from 605 million to 2 billion worldwide [3]. A growing aged population can have a profound impact on society, both socially and economically [4]. Although a complete arrest of the aging process may be impossible, progress in developing pharmacological, dietary, and genetic interventions that lead to healthy aging might allow individuals to live longer while being less burdened by physical and/or mental decline. This issue is highlighted in the recent World Health Organization (WHO) Global Strategy and Action Plan on Ageing and Health (GSAP), which envisions a world in which everyone experiences healthy aging by maintaining the functional ability that enables well-being in old age [5].

From a biodemographic point of view, aging is defined as an exponential increase in mortality with time [6, 7], sometimes accompanied by a deceleration or plateau at later ages [6, 8–10]. Although the changes that underlie aging are complex [11], it is characterized by the gradual accumulation of a wide variety of molecular and cellular damage throughout the lifespan [12]. The nine proposed hallmarks of aging in mammals are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [13]. However, the connections between these hallmarks, their contributions to aging, and their links with

Table 1: Example of posttranslational modifications, their target amino acid residue(s), and the enzyme(s) or proteins involved [20, 77, 145].

Posttranslational modification	Target amino acid residue(s)	Enzyme(s) or proteins involved
Phosphorylation	Tyrosine, serine, threonine	Kinases, phosphatases
Glycosylation N-linked	Asparagine	Glycosyltransferases, deglycosylases
Glycosylation O-linked	Serine/threonine	Glycosyltransferases, deglycosylases
Acetylation	Lysine	Acetyltransferases, deacetylases
Methylation	Lysine, arginine	Methyltransferases, demethylases
Ubiquitination	Lysine	Ubiquitin-activating enzymes, ubiquitin-conjugating enzymes, ubiquitin ligases, deubiquitinases
Sumoylation	Lysine	Ubiquitin-activating enzymes, ubiquitin-conjugating enzymes, ubiquitin ligases, deubiquitinases
Myristoylation	Glycine	N-Myristoyltransferases
Prenylation	Cysteine	Farnesyltransferases, geranyl geranyltransferases
Palmitoylation	Cysteine	DHHC protein acyltransferases, acyl-protein thioesterases
Sulfation	Tyrosine	Sulfatases, desulfatases
S-Nitrosylation	Cysteine, methionine	
Glycation	Lysine	
Nitration	Tyrosine	Denitrases
Chlorination	Tyrosine	Myeloperoxidases
Oxidation/reduction	Cysteine	Peroxidases, oxidases, glutathione, thioredoxin
Carbonylation	Lysine, proline, arginine, threonine	

frailty and disease remain incompletely understood [13]. In fact, uncovering the biological basis of aging is one of the greatest contemporary challenges in science [14].

Evidence suggests that it is possible to intervene at the level of the putative mechanisms underlying aging, subsequently leading to a slower rate of age-associated damage accumulation [12–14]. In this context, applying the multidisciplinary approaches of systems biology to probe the complex mechanisms of aging and various age-related disorders could generate the necessary evidence that leads to effective regulation of aging mechanisms [15]. Indeed, the use of model organisms has promoted rapid advances in the field of aging research through the identification of gene mutations that extend lifespan [16]. The major genes and pathways regulating lifespan are well conserved across eukaryotes such as yeast, worms, flies, and mammals [12].

Interestingly, epigenetics also plays a crucial role in aging [16–21]. While there are several different types of epigenetic mechanisms, protein posttranslational modifications (PTM) are intriguing contributors in regulating aging [22–27]. In this review, we discuss the involvement of PTMs in aging with a focus on PTM types, mechanisms of action, and detection methods. An overview of recent progress in PTMs and aging research across different model organisms is also included. Understanding PTMs and their contributions to aging provides a foundation for the development of interventions or targeted approaches to aging and age-related diseases.

2. Protein Posttranslational Modifications

Proteins are the basis of cellular and physiological functioning in living organisms, and the physical and chemical properties of proteins dictate their activities and functions. The primary sequence of a protein is a main determinant of protein folding and final conformation as well as biochemical activity, stability, and half-life [19]. However, at any given moment in the life of an individual, its proteome is up to two or three orders of magnitude more complex than the encoding genomes would predict [20]. One of the main routes of proteome expansion is through posttranslational modifications (PTM) of proteins. PTMs are present in both eukaryotes and prokaryotes, but it is estimated that PTMs are more common in eukaryotic cells, in which about 5% of the genome is dedicated to enzymes that carry out posttranslational modifications of proteins [20].

Protein PTM results from enzymatic or nonenzymatic attachment of specific chemical groups to amino acid side chains [20]. Such modifications occur either following protein translation or concomitant with translation. PTM influences both protein structure and physiological and cellular functions. Examples of enzymatic PTMs include phosphorylation, glycosylation, acetylation, methylation, sumoylation, palmitoylation, biotinylation, ubiquitination, nitration, chlorination, and oxidation/reduction [21]. Nonenzymatic PTMs include glycation, nitrosylation, oxidation/reduction, acetylation, and succination [22–26]. Some rare and unconventional PTMs, such as glypiation, neddylation, siderophorylation, AMPylation, and cholesteroylation, are also known to influence protein structure and function [27]. The major PTMs in eukaryotes, their target amino acid residue(s), and the enzyme(s) or protein(s) involved are shown in Table 1.

Alterations in the rate and extent of protein synthesis, accuracy, PTMs, and protein turnover are among the major molecular characteristics of aging. A decline in the cellular

capacity to recognize and preferentially degrade the damaged proteins through proteasomal and lysosomal pathways ultimately leads to the accumulation of abnormal proteins during aging [28]. The consequent increase in molecular heterogeneity and impaired functioning of proteins is the basis of several age-related pathologies, including cataracts, sarcopenia, and various neurodegenerative diseases [29]. Therefore, understanding the spectrum of PTMs and their functional implications in aging will facilitate the development of effective intervention, prevention, and therapy for aging and age-related diseases.

Research on PTMs in prokaryotes started with the assumption that bacteria lack many features regularly found in more complex organisms. However, ongoing investigation continues to reveal new types of PTMs of bacterial proteins and their importance in bacterial adaptability and cell cycle control. Most bacterial PTMs are dynamic and reversible. This allows the cell to exploit them as regulatory devices. Among different bacterial PTMs, protein phosphorylation is the most extensively studied [30] and seems to be particularly relevant among important bacterial pathogens [31, 32]. Bacterial pathogens have developed diverse strategies to interact with host cells, manipulate their behaviors, and thus to survive and propagate. During pathogenesis, phosphorylation of proteins on hydroxyl amino acids (serine, threonine, and tyrosine) occurs at different stages, including cell-cell interaction and adherence, translocation of bacterial effectors into host cells, and changes in host cellular structure and function induced by infection. Among the various virulence factors involved in bacterial pathogenesis, special attention has been recently paid to the cell wall components, exopolysaccharides. A major breakthrough demonstrated the existence of a biological link between the activity of certain protein-tyrosine kinases/phosphatases and the production and/or transport of surface polysaccharides. From a general standpoint, the demonstration of a direct relationship between protein phosphorylation on serine/threonine/tyrosine and bacterial pathogenicity represents a novel concept with significance for deciphering the molecular and cellular mechanisms that underlie pathogenesis [33].

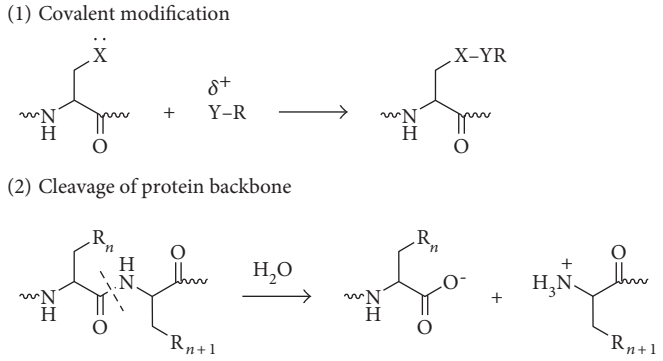
Moreover, several studies have begun uncovering the broad spectrum of PTMs involved in key bacterial cellular processes, including redox regulation via reversible S-thiolation [34], posttranslational hydroxylation [35], and the role of citrullination in the interaction between bacteria and human mucosal surfaces [36]. Several experimental studies have been done on the posttranslationally modified antimicrobial peptides known as lantibiotics [37]. Furthermore, studies have also pointed out an important role of PTMs on PII proteins, which are the key signal transduction proteins involved in the control of nitrogen metabolism in bacteria and archaea [38, 39]. More recently, Stannek et al. [40] have studied the regulatory mechanisms involving arginine phosphorylation and regulated proteolysis in *Bacillus subtilis* and proposed a mechanism whereby protein phosphorylation plays a role in quality control of bacterial proteins, targeting unstable and aggregation-prone proteins for degradation.

2.1. Methods to Detect Protein Posttranslational Modifications. Specific amino acid residues are subjected to PTMs depending on the chemistry of the reaction and the sequence specificity of the enzyme involved [20]. Initially, the detection of PTMs was carried out by various analytical methods, such as radiolabeling of the proteins, thin-layer chromatography, column chromatography, and/or polyacrylamide gel electrophoresis [31]. Other methods, such as protein sequencing by Edman degradation and Western blotting using protein-specific antibodies, have since been developed. Currently, antibody-based detection methods and mass spectrometry-based proteomic analysis are predominant methods used to detect and analyze PTMs. However, mass spectrometric methods are the only available tool to perform global or large-scale PTM analysis [32].

Antibody-based methods mostly rely on the availability of antibodies that can specifically recognize a modified amino acid residue within a protein or peptide. Such antibodies can be polyclonal or monoclonal and are developed against either the modified peptide/protein or against the modified amino acid. Moreover, antibody-based detection and quantification of PTMs on protein/peptide samples can be performed by two methods: chemiluminescence-based Western blotting and absorbance/fluorescence-based ELISA. However, the detection of PTMs depends entirely on the recognition site of the antibody used [41]. If the antibody detects only the modified amino acid, additional analysis—for instance, protein/peptide isolation and sequencing—should be performed to detect the sequence context of the modification. However, if the antibody detects the PTM within a specific sequence context, the presence of PTM at other sites will remain undetected.

Mass spectrometric detection of specific PTMs is based on mass changes [42]. Depending on the type of modification, a specific change in mass of the modified amino acid or peptide occurs. Subsequently, the change in mass is detected by the mass spectrometer to identify the presence of a PTM in a peptide sample. Using tandem mass spectrometric methods, identification of the specific site of PTM can be achieved by subsequent fragmentation and sequencing of the relevant peptide [43]. Yet, technical challenges hamper MS-based investigation of biologically important PTMs, such as ADP-ribosylation, one of the key signaling molecules that regulates DNA repair, a critical process in maintaining genome stability that is compromised in cancer and aging [38, 39].

Data increasingly implicate PTMs not only during aging and/or under pathological conditions but also for the normal functioning of the cell [39, 44]. In turn, PTMs are increasingly studied for their role in health and disease. For example, the precise and accurate measurement of distinct PTM-containing moieties offers potential biomarker utility to aid early diagnosis, prognosis, monitoring response to therapy and decisions regarding inclusion in clinical trials as new medicines are developed [45]. However, technical difficulties limit these studies, leaving many unanswered questions. The identification of unknown/unexpected PTMs by proteomic data reanalysis is an emerging subfield of proteomics recently boosted by the increased availability of raw data shared in



Scheme 1: Two categories of posttranslational modifications of proteins: (1) covalent modification of a nucleophilic amino acid side chain by an electrophilic fragment of a cosubstrate and (2) cleavage of a protein backbone at a specific peptide bond. Reproduced with permission from Walsh et al. [20].

public repositories. Notably, though, a sampling of the proteome in a given organism or cell provides only a snapshot of a highly dynamic process, confounding the analytical problem and ultimately arguing for time-resolved inventories [20]. Thus, while many tools are currently available for the study of PTMs, new methods are needed to further advance the study of these modifications.

3. PTMs in Aging

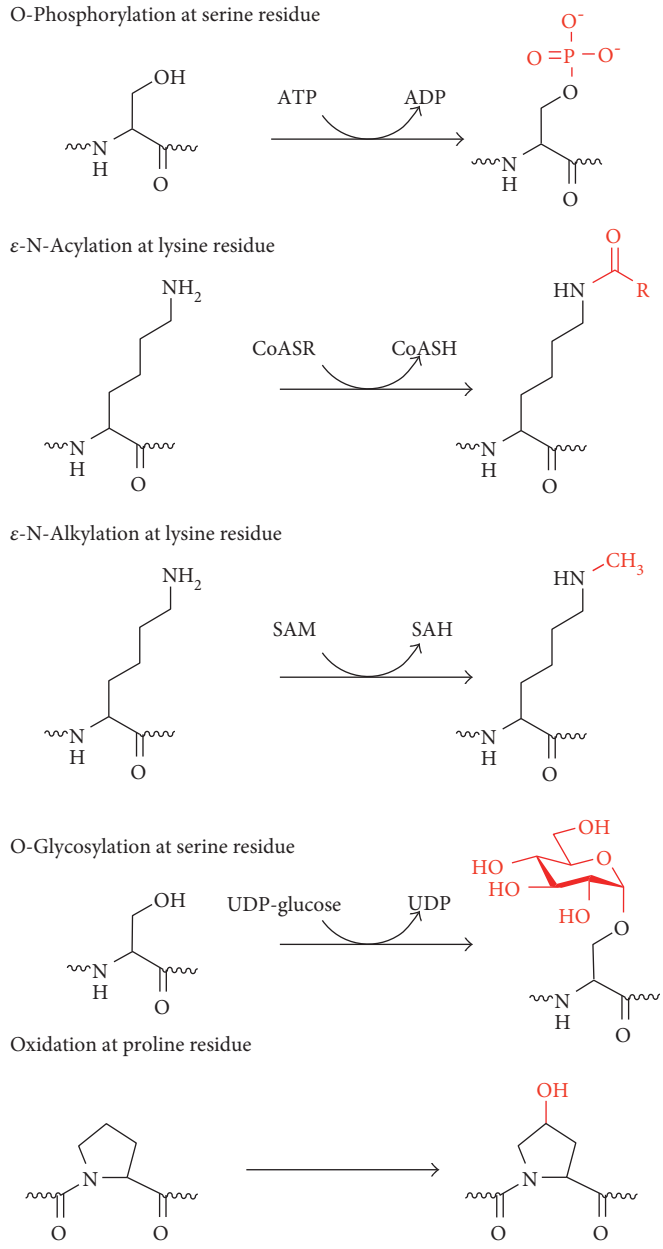
Generally, protein PTMs occur as a result of either modifying enzymes related to posttranslational processing (such as glycosylation) or signaling pathway activation (such as phosphorylation). Moreover, PTM patterns are known to be affected by disease conditions [46]. Similarly, the dysregulation of PTM is associated with the aging process [18, 47–49]. In this context, both enzymatic and nonenzymatic PTMs can undergo age-related alterations. Alteration in the pattern of nonenzymatic PTMs depends mainly on the nature of the modifying substances, such as metabolites and free radicals. For instance, reactive oxygen species can lead to oxidation of amino acid side chains (oxidation of thiols to different forms, oxidation of methionine, formation of carbonyl groups, etc.), modification by-products of glycoxidation and lipoxidation, and formation of protein-protein cross-links as well as oxidation of the protein backbone, resulting in protein fragmentation [50]. In contrast, changes in the nature of enzymatic PTMs rely primarily on the activities of modifying enzymes. In this review, we provide an overview of some of the most well-characterized PTMs implicated in aging and aging-associated pathologies across different levels of biological complexity.

3.1. A Brief Overview of Types of PTMs. Protein PTMs fall under two broad categories (Scheme 1). The first category encompasses covalent additions of some chemical group by enzymatic catalysis. Typically, an electrophilic fragment of a cosubstrate is added to an electron-rich protein side chain, which acts as a nucleophile in the transfer. The other category of PTMs encompasses covalent cleavage of peptide backbones. This cleavage occurs by one of two mechanisms: proteases or, less commonly, autocatalysis. Common covalent protein PTMs include phosphorylation, acylation, alkylation, glycosylation, and oxidation. These PTMs, catalyzed by dedicated mechanisms (Scheme 2), play roles in aging and age-related diseases. A brief description of the main types of PTMs associated with aging and age-related diseases is provided below.

3.1.1. Protein Phosphorylation. The most common posttranslational modification, protein phosphorylation, is the reversible addition of a phosphoryl group from adenosine triphosphate (ATP) principally to serine, threonine, or tyrosine residues. This modification causes conformational changes that either (1) affect the catalytic activity to activate or inactive the protein and/or the tendency of a protein to misfold and aggregate [51] or (2) recruit other proteins to bind; both result in altered protein function and cell signaling [52]. Phosphorylated proteins have critical and well-known functions in diverse cellular processes across eukaryotes, but phosphorylation also occurs in prokaryotic cells. In humans, about one-third of proteins are estimated to be substrates for phosphorylation [53]. Indeed, phosphorylated proteins are now identified and characterized by high-throughput phosphoproteomics studies.

The reversibility of protein phosphorylation is attributed to the actions of kinases and phosphatases, which phosphorylate and dephosphorylate substrates, respectively. The temporal and spatial balance of kinase and phosphatase concentrations within a cell mediates the size of its phosphoproteome [54]. Accordingly, phosphatases have recently been proposed as potential next-generation therapeutic targets for age-related diseases, such as α-synucleinopathies like Parkinson's disease [55].

3.1.2. Protein N-Acetylation. N-Acetylation is the reversible or irreversible transfer of an acetyl group to a nitrogen molecule through the actions of cleavage of methionine by methionine aminopeptidase (MAP) and the addition of an acetyl group from acetyl-CoA by N-acetyltransferase (NAT). Interestingly, 80–90% of eukaryotic proteins are acetylated, yet the underlying biological significance remains unclear [56]. In the case of histone proteins, which make up chromatin, lysine acetylation regulates gene transcription,



Scheme 2: Five major types of covalent additions to protein side chains: phosphorylation, acylation, alkylation, glycosylation, and oxidation. Reproduced with permission from Walsh et al. [20].

thereby affecting the cell's transcriptome. Histone acetylation typically results in transcriptional activation; deacetylation typically results in transcriptional suppression. Acetylation occurs via histone acetyltransferases (HATs) and is reversible via the action of histone deacetylases (HDACs). One group of histone deacetylases are the sirtuins (silent information regulator), which maintain gene silencing via hypoacetylation. Sirtuins have been reported to aid in maintaining genomic stability [57].

Although first described in histones, acetylation is also observed in cytoplasmic proteins. Acetylated proteins can also be modulated by the cross-talk with other posttranslational modifications, including phosphorylation, ubiquitination, and methylation [58]. Therefore, acetylation may contribute to cell biology beyond transcriptional regulation [59].

3.1.3. Protein Glycosylation. Protein glycosylation involves the addition of a diverse set of sugar moieties. This major type of PTM has significant implications for protein folding, conformation, distribution, stability, and activity. Glycosylated proteins can have additions of simple monosaccharides (e.g., nuclear transcription factors) or highly complex branched polysaccharides (e.g., cell surface receptors).

More than half of all mammalian proteins are believed to be glycosylated [60]. However, glycoprotein functions, at both molecular and cellular levels, remain unclear. While proteins exhibit improved stability and trafficking after

glycosylation in vivo, glycan structures can alter protein functions or activities. These structures often result from the activities of glycan-processing enzymes working within a cell at any given time. However, the structures are sometimes protein-specific, depending on protein trafficking properties and interactions with other cellular factors [61].

There are three types of protein glycosylation in higher eukaryotes: N-linked, O-linked, and C-linked. These types reflect their glycosidic linkages to amino acid side chains [62]. In N-linked glycosylation, β-N-acetylglucosamine (GlcNAc) is attached through an amide linkage to the side chain of Asn in an AsnXaaSer/Thr group [63]. N-linked glycans have multiple functions. While they act as ligands for glycan-binding proteins in cell-cell communication, they also can regulate glycoprotein aggregation in the plasma membrane and affect the half-life of antibodies, cytokines, and hormones in serum [64].

O-linked glycosylation in higher eukaryotes occurs through several different mechanisms. The most abundant type of O-linked glycosylation is mucin-type, involved attachment of an α-N-acetylgalactosamine (GalNAc) to the hydroxyl group of Ser/Thr side chains [65, 66]. Aberrant expression of mucin-type O-linked glycans occurs in cancer cells [65] and may provide targets for anticancer vaccines [67].

O-linked glycosylation occurring with the addition of α-O-mannose is the only form of O-linked glycosylation in yeast but also occurs in the brains of higher eukaryotes [68, 69]. Higher eukaryotes also have an α-O-fucose modification of Ser/Thr residues that occur within the consensus sequon $\mathrm{CysXaa}_{(3\text{-}5)}\mathrm{Ser} = \mathrm{ThrCys}$ [70]. This glycosylation modulates Notch signaling during eukaryotic development [71, 72]. Another modification, β-O-galactosylation, may contribute to rheumatoid arthritis [73–75].

Finally, C-linked glycosylation involves the addition of α-mannose (Man) to the 2-position of the indole side chain of tryptophan residues [76, 77]. While first identified on ribonuclease 2, it also occurs on other proteins, including MUC5AC and MUC5B [78], thrombospondin [79], and the Ebola virus soluble glycoprotein [80].

3.1.4. Protein Ubiquitination and Sumoylation. Ubiquitination is the addition of an 8 kDa polypeptide to the N-terminus of target proteins via the C-terminal glycine of ubiquitin. The addition of one ubiquitin is followed by the formation of a ubiquitin polymer. The resultant polyubiquitinated proteins are recognized by the 26S proteasome in the protein degradation pathway [81].

Protein sumoylation is a reversible posttranslational modification whereby a small ubiquitin-like modifier (SUMO) is covalently attached to proteins [82]. Accordingly, protein sumoylation is mediated by a reversible enzymatic cascade in a manner similar to protein ubiquitination [83]. Like ubiquitin, SUMO is conjugated to the lysine side chains of target proteins via a cascade of activating, conjugating, and ligating enzymes, and it is removed by SUMO-specific isopeptidases [82]. Over the last few decades, it has been well established that sumoylation controls many aspects of nuclear function [84]. However, recent research has started to unveil a determinant role of protein sumoylation in many extranuclear neuronal processes and potentially in a wide range of neuropathological conditions [85].

3.1.5. Protein S-Nitrosylation. Nitrosylation is a reversible addition of a nitric oxide (NO) to cysteine residues, forming S-nitrosothiols (SNOs), via redox-mediated reactions. S-Nitrosylation is used by cells to stabilize proteins, regulate gene expression, and provide NO donors. SNO generation, localization, activation, and catabolism are tightly regulated, and S-nitrosylation reactions depend on catalytic amounts of transition metals, O_2, O_2^-, and pH, among other factors [86, 87]. Indeed, these molecules have a short half-life because of the action of enzymes like glutathione (GSH) and thioredoxin that denitrosylate proteins [88].

S-Nitrosylation is increasingly recognized as a ubiquitous regulatory reaction comparable to phosphorylation. SNOs may play an important role in many processes ranging from signal transduction, DNA repair, host defense, and blood pressure control to ion channel regulation and neurotransmission [89].

S-Nitrosylation specificity can mainly be achieved by two strategies. The existence of a consensus nitrosylation acid-based motif has been postulated [90]. S-Nitrosylation specificity may also be achieved through the subcellular localization of the NOSs, which may be in proximity to potential targets. The effect of NO on cells depends on its local concentration, the redox status of its immediate environment, and the susceptibility of target sites for modification [91]. Different degrees of accessibility to NO (RSNO) or different reaction rates with NO, as well as important functional differences in the -SH group being modified by NO, might explain why and how specific S-nitrosylation of precise cysteine residues induces protein modulation [92].

A classical example of SNOs is caspases, which mediate apoptosis. Stored in the mitochondrial intermembrane space as SNOs, caspases are then released into the cytoplasm and denitrosylated. The activated caspase then induces apoptosis [93].

3.1.6. Protein Methylation. Alkyl substituents are attached to specific regions of proteins by PTM enzymes. The introduction of such alkyl groups results in the alteration of the hydrophobicity of the modified protein [94].

The most common type of protein alkylation is protein methylation. Methylation is a well-known PTM mediated by methyltransferases. One-carbon methyl groups are added to nitrogen or oxygen (N- and O-methylation, resp.) on amino acid side chains, increasing protein hydrophobicity or neutralizing a negative charge when bound to carboxylic acids. While N-methylation is irreversible, O-methylation is potentially reversible. Methylation occurs so often that its primary methyl donor, S-adenosyl methionine (SAM), is suggested as the most-used enzymatic substrate after ATP [56].

A common theme with methylated proteins, as is also the case with phosphorylated proteins, is the role this modification plays in the regulation of protein-protein interactions. For instance, the arginine methylation of proteins can either

inhibit or promote protein-protein interactions depending on the type of methylation [95, 96].

Protein methylation has been most studied in the histones. The transfer of methyl groups from S-adenosyl methionine to histones is catalyzed by enzymes known as histone methyltransferases. The N-terminal tails of histones H3 and H4 receive methyl groups on specific lysines. Methylation then determines if gene transcription is activated or repressed, thus leading to different biological outcomes [97].

Histone methylation was traditionally thought to be irreversible. However, histone demethylases demonstrate the reversibility of this PTM [98]. Indeed, chromatin modification dynamic changes were imposed by an ability or inability to maintain equilibrium in the opposing effects of methylases and demethylases. The simultaneous removal of one histone methylation mark and an addition of another enable transcriptional tuning [99, 100].

Nonhistone proteins also exhibit methylation as a common PTM that regulates signal transduction via MAPK, WNT, BMP, Hippo, and JAK-STAT signaling pathways. Further, methylation works in concert with other types of PTMs, as well as with histone and nonhistone proteins, to exert influence on not only chromatin remodeling but also gene transcription, protein synthesis, and DNA repair [101].

3.1.7. Protein Oxidation. The reaction of proteins with a variety of free radicals and reactive oxygen species (ROS) leads to oxidative protein modifications such as formation of protein hydroperoxides, hydroxylation of aromatic groups and aliphatic amino acid side chains, oxidation of sulfhydryl groups, oxidation of methionine residues, conversion of some amino acid residues into carbonyl groups, cleavage of the polypeptide chain, and formation of cross-linking bonds. Aromatic and sulfur-containing residues are particularly susceptible to oxidative modification [66–68].

Unless repaired or removed from cells, oxidized proteins are often toxic and can impair cellular viability [102], since oxidatively modified proteins can form large aggregates [103]. Oxidatively damaged proteins undergo selective proteolysis, primarily by the 20S proteasome in an ubiquitin- and ATP-independent way. Ultimately, upon extensive protein oxidation, these aggregates can become progressively resistant to proteolytic digestion and actually bind the 20S proteasome and irreversibly inhibit its activity [70–72].

Protein carbonylation is defined as an irreversible post-translational modification (PTM) whereby a reactive carbonyl moiety, such as an aldehyde, ketone, or lactam, is introduced into a protein. The first identified source of protein-bound carbonyls was metal-catalyzed oxidation (MCO) [104]. MCO results from the Fenton reaction when transition metal ions are reduced in the presence of hydrogen peroxide, generating the highly reactive hydroxyl radicals in the process [105]. These hydroxyl radicals can oxidize amino acid side chains or cleave the protein backbone, leading to numerous modifications including reactive carbonyls [106]. For example, oxidation of proline and arginine results in the production of glutamic semialdehyde, while lysine is oxidized to aminoadipic semialdehyde and threonine to 2-amino-3-ketobutyric acid [107]. Direct oxidation of other amino acid residues can also lead to protein-bound carbonyls. Tryptophan oxidation by ROS produces at least seven oxidation products. Among them are kynurenine and N-formyl kynurenine, as well as their hydroxylated analogs, which contain aldehyde or keto groups formed by oxidative cleavage of the indole ring [108].

Another important source of protein-bound carbonyls is reactive lipid peroxidation products, which are produced during oxidation of polyunsaturated fatty acids [78–81]. Protein carbonylation can also occur via glycoxidation. Reactive α-carbonyls formed during glycoxidation, such as glyoxal, methylglyoxal, and 3-deoxyglucosone, can then modify the basic residues Lys and Arg to generate, for example, pyrralines and imidazolones [82, 83]. Glycation (i.e., the reaction of reducing sugars such as glucose or fructose with the side chains of lysine and arginine residues) forms Amadori and/or Hynes products. These glycated residues can be further decomposed by ROS into advanced glycation end products (AGE) carrying carbonylated moieties that can also contribute for protein carbonylation [109].

4. PTMs in Aging and Aging-Associated Diseases

Loss of cellular homeostasis during aging alters tissue functions, which leads to a general decline in physical/mental well-being and, ultimately, death. As individuals age, control of gene expression, which is orchestrated by multiple epigenetic factors, deteriorates. Epigenetic control of chromatin remodeling, through histone acetylation, is associated with cellular metabolism [110, 111]. Changes in metabolism with aging affect the concentration of acetyl-CoA and of citrate; this, in turn, alters the cytosolic level of acetyl-CoA. Altered acetyl-CoA levels, then, affect other metabolic processes such as the synthesis of fatty acids, exerting downstream effects on other physiological functions. Moreover, altered acetyl-CoA levels affect histone acetylation, thereby dysregulating transcription [110, 111]. These transcriptional changes occur with aging or with the progression of aging-related diseases. Acetylases and deacetylases likely exhibit different affinities for their acetyl-CoA and NAD^+, respectively, which affects their responses to age-associated alterations in cofactor concentrations [112]. Thus, chromatin may act to sense changes in cellular metabolism [113]. In fact, lifespan can be extended by several manipulations that reverse age-dependent changes in chromatin structure, indicating the pivotal role of chromatin structure during aging [114]. Accordingly, mutations in genes that link metabolism and chromatin, such as lysine acetyltransferases (KATs), lysine deacetylases (KDACs) (sirtuins), and ATP citrate lyase (ACLY/ATPCL), can influence lifespan and the development of age-associated diseases [113].

Protein acetylation has been suggested to play a key role in the process of aging by enhancing the function of certain genes, most notably the AMPK regulatory subunit, which can promote longevity [115]. Likewise, it is widely accepted that sirtuins, a class of proteins that modulate stress responses and metabolism by removing the acetyl groups from target proteins, have an impact on lifespan and the aging process [116, 117]. Most notably, sirtuin SIRT3 plays

a critical role in deacetylating many proteins in the mitochondria, suggesting that acetylation/deacetylation may be involved in the regulation of mitochondrial function [118]. More recently, it has been found that caloric restriction (CR), an intervention known to extend the lifespan in many organisms ranging from budding yeast to mammals, is associated with dramatic changes in mitochondrial acetylation. Many proteins are altered by acetylation in response to CR [119, 120]. These changes may contribute to mitochondrial adaptation to reduced caloric intake and may help to promote longevity. Likewise, regular exercise has been found to reduce oxidatively modified proteins in the brain with improved cognitive functions [121], through processes involving PTMs in histone tails controlled by HATs, HDACs, and histone demethylases [122].

Many pathways and processes appear to regulate the rate of aging and organismal susceptibility to age-related diseases such as neurodegeneration, atherosclerosis, and cancer. One process that is increasingly implicated is autophagy. First described in yeast, autophagy is a tightly regulated process stimulated by stressful conditions, such as starvation. Once activated, autophagy involves the recycling of old and damaged proteins and organelles to provide building blocks for new cellular components. Accordingly, disruption of this process results in diseased phenotypes and decreased lifespan, as revealed by studies using mouse models [96–98], *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* [90–93].

While the core components that regulate autophagy have been widely studied (e.g., [123]), less is known about the inputs that specifically alter this process, particularly how posttranslational modifications can influence autophagy flux and/or autophagic turnover. Three types of PTM, dubbed "The Three Musketeers of Autophagy"—phosphorylation, ubiquitylation, and acetylation—are crucial for autophagy induction, regulation, and fine-tuning and are influenced by a variety of stimuli. Understanding the mechanisms of autophagy regulation will provide biogerontologists deeper insight into the process and point to new therapeutic avenues [124].

4.1. Protein Oxidation and Aggregation. One of the earliest mentions of the effects of oxidative stress in cells can be found in a description of the chemical nature of prooxidant and antioxidant molecules [125]. A balance between oxidative and antioxidative effects maintains cellular health, whereas an imbalance is associated with diseases and aging. ROS are hallmarks of oxidative damage. The effects of an imbalanced redox status of cells primarily involve the modification of redox-sensitive molecules, such as the oxidation of cysteine and methionine in proteins, the peroxidation of lipids, and the oxidation of DNA bases [13]. The consequences of these modifications include direct effects on disease-causing proteins and indirect effects on enzymes and/or cofactors that in turn influence the function of disease-causing proteins [116, 117].

Several studies have identified proteins involved in mediating or countering reactive oxygen species production and action. A recent review [126] focusing on aging-related oxidative damage in the context of the damage accumulation theory of aging has stated that chronic oxidative damage is the primary cause of age-related diseases. Cellular senescence, defined as a loss of cell division, motility, and protein turnover, occurs as a result of damage accumulation over time and is considered an important feature of aging [13]. Morphological changes due to the accumulation of protein aggregates in the cells are also considered as a feature of cellular senescence induced by oxidative protein damage.

A wide range of aging-related diseases is at least in part associated with protein oxidative damage. These include eye diseases, metabolic disorders such as diabetes and obesity, inflammatory conditions such as arthritis, cardiovascular complications such as atherosclerosis, kidney disorders, respiratory disease, cancer, and neurodegenerative disorders such as Alzheimer's [119] and Parkinson's [120] diseases. Accordingly, Radman [127] recently proposed that aging and age-related diseases could be phenotypic consequences of proteome damage patterns.

Eye lens cataracts are a common affliction of aging populations that result in the progressive worsening of vision. One of the primary underlying changes during cataract formation is protein aggregation in the eye lens. While environmental factors like smoke, UV radiation, and chemical fumes contribute to the formation of cataracts, protein PTMs also play a significant role in the structure and stability of lens proteins, resulting in their aggregation within the lens [128]. Protein oxidation plays a particularly important role in lens protein aggregation, and antioxidants are often prescribed in the clinical management of cataracts [129]. Experimental studies using both human and mouse models have identified cysteine oxidation at the critical sites of several enzymes in human and mouse lens, including several metabolic enzymes, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glutathione synthase, aldehyde dehydrogenase, and sorbitol dehydrogenase, as well as protein deglycase DJ-1 (Parkinson disease protein 7 or PARK7) [130]. Extensive oxidation of intermediate filament proteins such as BFSP1 and BFSP12, vimentin, and cytokeratins, as well as the microfilament and microtubule filament proteins such as tubulin and actin, has also been reported [130].

Alzheimer's disease (AD) is one of the major aging-related disorders that severely impact the quality of life of elderly individuals [131]. The clinical symptoms of AD include a decline in cognitive function and memory and a state of confusion. At the cellular level, AD is associated primarily with two proteins: tau and amyloid-β. Dissociation of the microtubule-associated protein, tau, from the cytoskeleton in neuronal cells leads to its subsequent intracellular aggregation into paired helical filaments known as neurofibrillary tangles. Extracellular accumulation of amyloid-β peptide in the brain is another major factor driving the pathology of AD. The formation of amyloid-β peptide occurs due to the degradation of amyloid precursor protein (APP). Under normal circumstances, the peptide is degraded by proteases, including zinc proteases called neprilysins, endothelin-converting enzymes, and insulin-degrading enzyme [132]. Oxidative stress during aging may contribute to the inhibition of amyloid-degrading enzymes,

which subsequently results in an aberrant extracellular accumulation of amyloid-β peptide in the brain [133].

The progression of AD is accompanied by hyperphosphorylation of tau. Hyperphosphorylated tau protein is found in degradation-resistant helical filament cores of neurofibrillary tangles [134]. Intriguingly, a recent report has shown that hydrogen peroxide-mediated oxidative stress can cause a temporary reduction in tau phosphorylation [135]. Further, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), a second messenger of the nitric oxide (NO) signaling pathway causing the oxidative S-guanylation of cysteine residues, results in a reduction of tau aggregation [136]. AD pathology involves posttranslationally modified forms of Aβ and tau, as well as other proteins. The study of these PTMs is key to the understanding of the molecular mechanisms associated with disease onset and also provides new opportunities for therapeutic strategies and drug development.

Parkinson's disease (PD) is another neurodegenerative disorder of unknown origin that affects approximately 6.3 million people worldwide [137]. The pathological hallmark lesions of PD are Lewy bodies (LBs), intraneuronal proteinaceous inclusions mainly comprising of misfolded α-synuclein [138]. LBs containing aggregated α-synuclein are found not only in PD but also in other neurodegenerative diseases, such as multiple system atrophy, dementia with LB, or AD [139]. Posttranslational modifications of α-synuclein, such as phosphorylation, ubiquitination, or nitration, are involved in the α-synuclein aggregation process and have different impacts on its cellular neurotoxicity [140–144].

The molecular mechanism involved in the clearance of α-synuclein aggregates is a central question for elucidating α-synuclein-related toxicity. However, clues to deciphering protein aggregation, which may eventually contribute to progress in understanding α-synucleinopathies, may emerge through the use of unicellular model organisms. Heterologous expression of α-synuclein in *Saccharomyces cerevisiae* also leads to protein aggregation and cellular toxicity characteristic of LB-containing human cells. In *S. cerevisiae*, cellular clearance mechanisms include ubiquitin-mediated 26S proteasome function as well as lysosome/vacuole-associated degradative pathways (i.e., autophagy). Various posttranslational modifications were found to change the cytotoxicity of α-synuclein and its distribution to different clearance pathways in *S. cerevisiae*. Several of the identified modification sites appear to be conserved from yeast to humans [145].

Aging is also a risk factor for cardiovascular diseases, such as hypertension, coronary heart disease, stroke, and heart failure. Several experimental and clinical observations support the hypothesis that excessive oxidative stress or reactive oxygen species (ROS) production plays a role in the pathogenesis of these diseases [146]. For instance, oxidative damage in cardiovascular disease is primarily related to low-density lipoproteins (LDL), which produce lipid peroxidation products such as lipid peroxides, isoprostanes, oxysterols, hydroxyl fatty acids, and aldehydes [147]. Likewise, recent studies on BMAL1 (brain and muscle ARNT-like protein-1) have shown that *Bmal1* null mice age prematurely because of increased ROS production. These mice also showed an aging-related decline in cardiac function, characterized by changes in ventricular diameter and ejection fraction [148]. Treatment with the antioxidant 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) prevented compromised cardiac function in these mice. Protection of cardiac telomeres from the oxidation by TEMPOL in BMAL1-deficient mice was also observed, further supporting the therapeutic relevance of targeting protein oxidation in aging [148].

Studies of acute kidney injury and chronic kidney disease during aging have also highlighted the role of oxidatively damaged proteins and protein aggregates [149]. Proteins that are subjected to oxidative damage in the kidney include NADPH oxidase (NOX), heme oxygenase-1 (HO-1), thioredoxin 1 (TRX1), and the transient receptor potential cation channel, subfamily M, member 2 (TRPM2). In this context, a balance between oxidative stress and autophagy has been recognized as an important factor controlling inflammation and cell death in kidney disorders [150].

Various metabolic disorders, such as obesity, insulin resistance, and diabetes are characterized by increased body weight, high glucose levels, and reduced energy levels. Environmental and nutritional stresses are considered to be the main drivers of such metabolic disorders, potentially involving oxidative damage. The accumulation of reactive oxygen species mediates oxidative modification of metabolic enzymes and proteins, as does the consumption of high-carbohydrate or high-fat diets [151]. The enzyme methionine sulfoxide reductase A (MsrA) is an antioxidant enzyme in cells that is involved in countering the effects of oxidative stress and has been implicated significantly in developing protection against oxidative stress and protein maintenance, two crucial factors in the aging process [152]. A recent study [153] using transgenic mice has found that MsrA affects lifespan and ameliorates some of the effects of age-associated metabolic disorders, such as insulin resistance.

Taken together, these results highlight the role of protein oxidative damage in the process of aging and aging-related pathologies. Thus, pharmacological and nonpharmacological strategies that influence the oxidative stress balance of the cell are important as proximal strategies in the road towards extending healthspan.

4.2. Protein Chlorination in Aging. Reactive chlorine species are considered a primary source of enzymatically catalyzed protein chlorination [154]. The free-hydroxyl-containing tyrosine is the primary amino acid target for halogen modification. The enzyme myeloperoxidase catalyzes the formation of 3-chlorotyrosine [155]. An early study on protein chlorination [156] found that a tyrosine residue in apolipoprotein A-I (apoA-I) serves as a site for either chlorination or nitration depending on the action of either myeloperoxidase or peroxynitrite, respectively. Interestingly, chlorination but not nitration affected apoA-I function and markedly reduced its cholesterol efflux activity.

Elevated levels of myeloperoxidase are associated with chronic heart failure, and its expression increases in cardiac

endothelial cells following exposure to hydrogen peroxide [157]. A recent study found that inhibition of myeloperoxidase using 2-thioxanthines resulted in a reduction of protein chlorination in a mouse model of peritonitis [158].

Skin aging is typically used as a physiological parameter to assess age-related changes in the body. A recent report on photoaging of the skin [159] proposed a link between inflammation-induced protein denitration and light-induced skin aging. The authors found elevated levels of halogenated tyrosine and inflammatory cells in skin samples both exposed to and protected from light, indicating that halogenation is likely a part of the normal aging process.

Neurodegeneration is another major consequence of aging that occurs due to a combination of factors, including oxidative stress. A recent report found that serotonin acts as a scavenger of hypochlorous acid (HOCl) in the brain [160] and prevents HOCl-induced oxidation of 2-thio-5-nitrobenzoate, loss of cellular α-ketoglutarate dehydrogenase activity, and cell death. Intriguingly, the biphasic removal of HOCl and subsequent prevention of 2-thio-5-nitrobenzoate oxidation involves HOCl-induced chlorination of serotonin as well as the formation of inactive aggregates of chlorinated serotonin, implicating a feedback process. Furthermore, selective serotonin reuptake inhibitors, such as fluoxetine, reduce protein chlorination in the brain, suggesting a potential therapeutic approach against age-related protein chlorination effects [160].

4.3. Protein Nitration. Nitration is an oxidative protein modification that occurs on tyrosine residues. Excess levels of reactive nitrogen species (RNS) are the primary source of nitrating reactions [154]. The excessive presence of ROS, along with RNS, leads to the formation of additional nitrating entities, namely peroxynitrite. One common example of RNS-induced protein nitration is the formation of 3-nitrotyrosine, which is associated with increased nitroxidative stress during the aging process [161]. Tyrosine nitration modifies the biochemical properties of the amino acid, including its pKa, redox potential, hydrophobicity, and size, subsequently leading to significant changes in the structure and function of affected proteins. Alterations in protein biochemistry provoke the cellular and physiological manifestations of nitration in aging. Additionally, protein tyrosine nitration is mediated by nonenzymatic free radical reactions involving the formation of an intermediate tyrosyl radical. Studies using fast reaction kinetics and bioanalytical methods as well as structural assessments using electron paramagnetic resonance have enabled the comprehensive characterization of tyrosine nitration [162]. Recent studies have shown that membrane-associated protein tyrosine nitration involves oxidation by lipid peroxyl radicals, a by-product of membrane lipid peroxidation, which is also associated with aging [163]. Moreover, several studies have revealed that protein tyrosine nitration occurs site-specifically to a few tyrosine residues within the target proteins and, thereby, is restricted to a fraction of the proteome [162]. The spatial and temporal localization of nitrating entities plays an important role in selecting the tyrosine residue within a target protein. Studies on mitochondrial proteins that are homogenously nitrated have further supported the site-specific selectivity as well as the overall effects of protein tyrosine nitration in aging and age-associated diseases [58, 59]. While protein tyrosine nitration was initially thought to be irreversible, recent studies have identified a denitrase enzyme [164]. Denitrase activity is found in a range of tissues and cells but not in smooth muscle cells. One recent study has demonstrated that denitrase utilizes nitrated cyclooxygease-1 (COX-1) as a substrate to facilitate the denitration reaction, suggesting that the reversible cycle of nitration and denitration may play a role in regulating cellular oxidative/nitrosative burdens, which subsequently modulate aging [164].

Another recent study [165] regarding the effect of protein nitration in age-related systemic inflammation (systemic inflammatory response syndrome or SIRS) has shown that toxemia-induced lung injury increases the level of protein tyrosine nitration and reduces the activity of superoxide dismutase in mouse lung. Additionally, aged mice showed higher protein nitration in the vascular endothelia compared to younger mice. The specific proteins that maintain pulmonary vascular permeability also showed higher tyrosine nitration, including profilin-1, transgelin-2, LASP 1, tropomyosin, and myosin [165].

5. Bacteria as Potential Simple Tools to Study PTMs in Aging and Age-Associated Pathologies

The observations of senescence in unicellular organisms in the absence of genetic or environmental variability opened the door to suggestions that such organisms could be used as simple quantitative experimental systems to address molecular mechanisms underlying aging [79, 110]. Bacterial aging seems to share some common features with the process of eukaryotic aging, namely, the role of oxidative damage, and the effect of protein quality control systems to trigger senescence [166]. For instance, as in eukaryotes, bacterial aging is associated with the accumulation of oxidized proteins in the form of aggregates in the older poles of cells [76, 78] (Figure 1). This accumulation resembles many known age-related eukaryotic protein folding diseases [130, 131], and at least in eukaryotes, increased protein aggregation and altered cell proteostasis have been associated with oxidative stress-related posttranslational modifications [167]. Whether this process also plays a role in the accumulation of protein aggregates in bacteria remains unclear. The patterns of oxidative protein damage and aggregation accompanying aging in *E. coli* seem to be similar to those induced by UVA radiation, suggesting that the same type of ROS may be involved in determining cellular damage under both processes [81, 82]. In fact, the similarity between the biological effects of radiation and aging is easily observed in survival curves plotted as a function of radiation dose or time: both display a "shoulder" indicative of negligible mortality followed by an exponential decay in survival with increasing radiation dose or age (Figure 2) [127]. Thus, bacteria are now being considered as useful model organisms in aging studies,

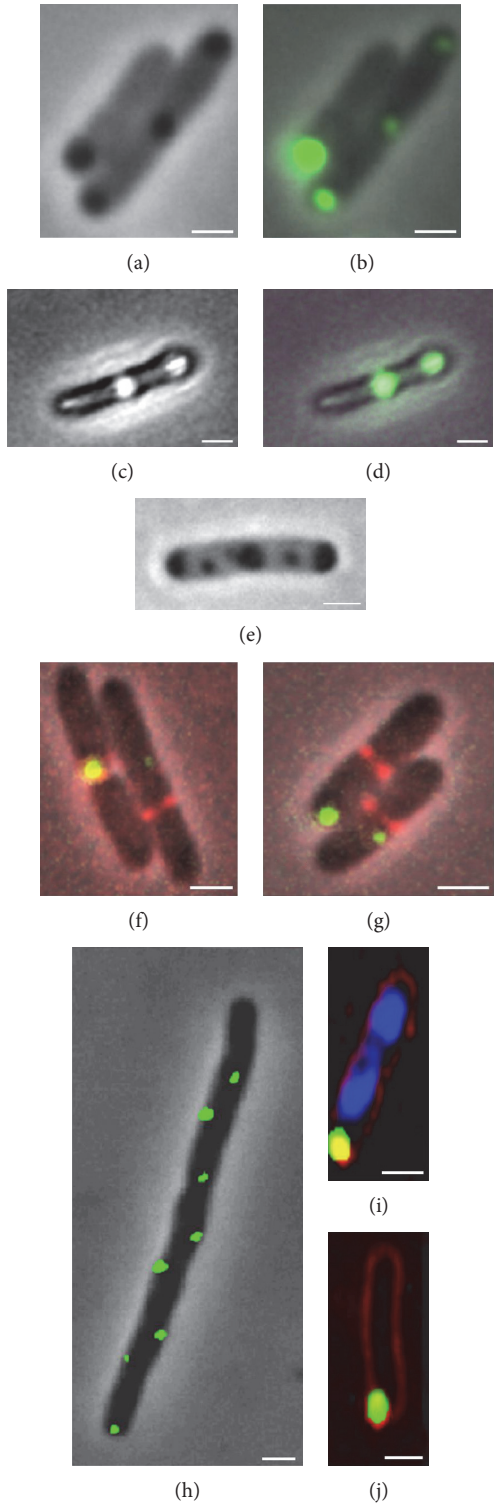


Figure 1: Accumulation of protein aggregates and inclusion bodies in *E. coli*. Reproduced with permission from Lindner et al. [180].

particularly in understanding the effects of aging and aging-related stress on protein stability and function [168].

In the case of *E. coli*, a significant portion of the age-related fitness loss has been accounted for by the presence of protein aggregates that accumulate in the older bacterial

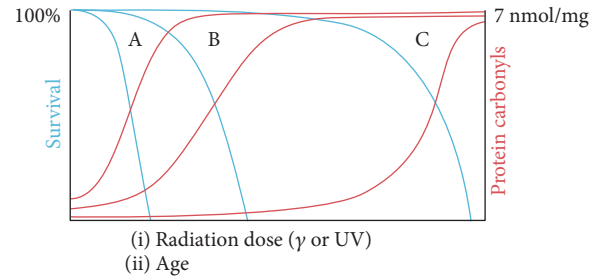


Figure 2: Schematic relationship between survival and protein carbonylation for different species (A, B, and C) as a function of radiation dose or age. Blue lines depict survival. Red lines depict protein carbonyl levels. Reproduced with permission from Radman [127].

poles (Figures 3 and 4). Misfolded proteins can passively and spontaneously aggregate at the cell poles in *E. coli* as a result of decreased diffusion and nucleoid occlusion [114, 118]. Thus, misfolded proteins freely diffuse in the cytoplasm and tend to stick to each other owing to the exposure of hydrophobic patches on their surface. As the amorphous aggregates grow by the addition of more misfolded peptides, they are excluded from the nucleoid and accumulate at the cell poles where they can expand further. Supporting this model, in silico simulations have demonstrated that the passive diffusion of a particle, its intrinsic ability to multimerize, and the absence of nucleoids at the poles are sufficient to obtain a polar localization pattern by entropy alone [169].

Additionally, a variety of posttranslational modifications, such as changes in phosphorylation state or nucleotide binding, can control the complex intracellular distribution of several proteins that are involved in cell cycle regulation, signal transduction, polarized motility, and adhesion [122, 170] (Figure 5(a)). Although most of the examples known to date are related to proteins that are at some point recruited to the poles through protein-protein interactions, similar modifications could also influence the ability of some proteins to multimerize, thereby impacting their spontaneous polar accumulation. If the presence and the activity of cognate kinases, such as phosphatases and GTPase-activating proteins (GAPs), is under the temporal regulation, this can provide a way to regulate an otherwise spontaneous polar localization in time (Figure 5(b)), as reported in the case of *Streptomyces coelicolor* [171–173]. Protein cleavage by specific proteases might also represent a strategy to modulate polar localization in space and time, as proposed for the polar beacon PodJ. PodJ is converted from a long form (PodJL) to a shorter form (PodJS) by a cell-cycle-regulated proteolytic sequence that eventually degrades PodJS, ensuring its proper localization and subsequently its function [123, 124] (Figure 5(c)). However, the precise mechanisms whereby both forms of PodJ differentially localize at the poles remain to be determined.

N^{ε}-Lysine acetylation has been recognized as a ubiquitous regulatory posttranslational modification that influences a variety of important biological processes in eukaryotic cells. Recently, acetylation has also been found to be prevalent

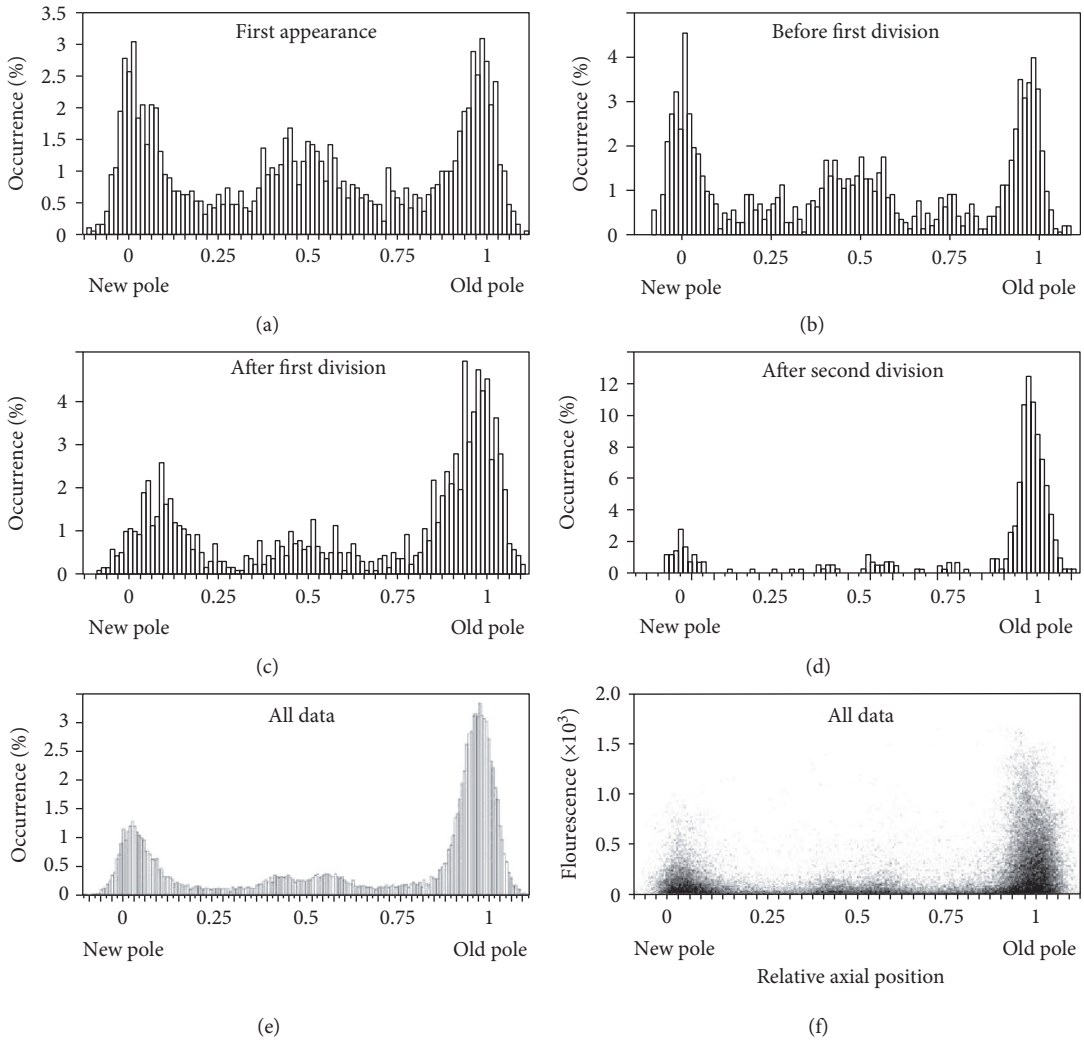


Figure 3: Aggregate distribution and associated fluorescence levels along the cell axis in *E. coli* throughout successive divisions. Reproduced with permission from Lindner et al. [180].

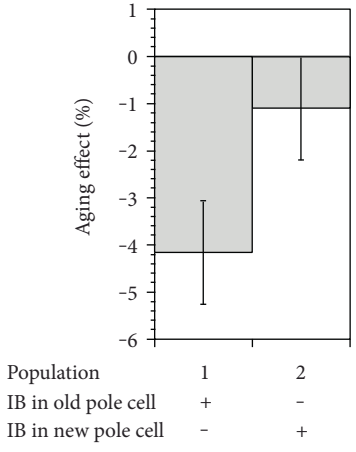


Figure 4: Aging correlation with the presence of protein aggregation. The aging effect was calculated from the relative growth rate difference between old-pole and new-pole offspring of newborn mother cells where inclusion bodies are inherited by the old pole cell (population 1) or the new pole cell (population 2). Reproduced with permission from Lindner et al. [180].

among bacteria. Bacteria contain hundreds of acetylated proteins that affect diverse cellular pathways. Still, little is known about the regulation or biological relevance of nearly all of these modifications. To uncover the potential regulatory roles of acetylation, a recent study analyzed how acetylation patterns and abundances change between growth phases in *B. subtilis*. The authors discovered a subset of critical acetylation events that are temporally regulated during cell growth. Furthermore, they demonstrated a stationary-phase-enriched acetylation on the essential shape-determining protein MreB, which led them to propose a role for MreB acetylation in controlling cell width by restricting cell wall growth [174]. Lysine acetylation also coordinates carbon source utilization and metabolic flux in *Salmonella* in a reversible manner, so that cells are able to respond to environmental changes by promptly sensing cellular energy status and flexibly altering reaction rates or directions [175]. Thus, lysine acetylation may represent a metabolic regulatory mechanism that is conserved from bacteria to mammals. As evidence supporting the conservation of at least some of the hallmarks of aging in bacteria

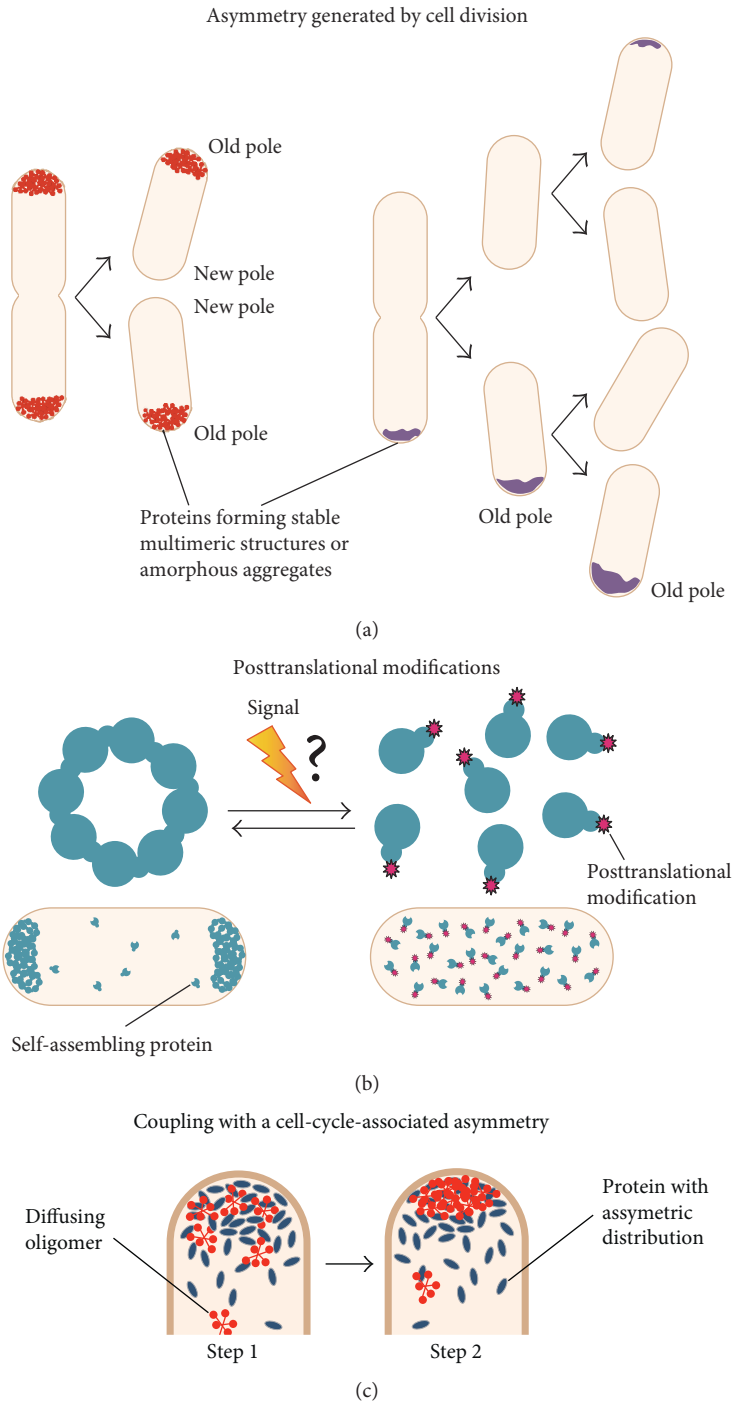


Figure 5: Possible strategies for spatial and temporal regulation of polar localization. (a) Asymmetric polar patterns can be naturally produced by a cell division event. Left: bipolar to old-pole localization; right: propagation of an old pole accumulation. Misfolded proteins produced in the progeny accumulate onto the existing polar aggregate. Eventually, de novo polar accretions can appear in progeny that did not acquire a polar focus (top cell), for example, after new protein synthesis. (b) The ability of some proteins to self-assemble and thereby to localize at the poles could be influenced by modifications such as phosphorylation upon a specific signal. The question mark indicates a hypothetical step. (c) The concentration of a self-assembling protein or oligomer and, thereby, its propensity to multimerize can be modified locally through protein-protein interaction with a partner whose subcellular distribution is asymmetric. In step 1, the protein (blue) has an asymmetric distribution inherent to a cell cycle event. The concentration of the diffusing protein oligomer (red) increases locally owing to interaction with the asymmetric protein. In step 2, the self-assembly of a protein or oligomer leads to the formation of a large structure at the pole. This provides spatial and temporal regulation to a multimerization-dependent polar localization. Reproduced with permission from Laloux and Jacobs-Wagner [169].

continues to emerge [74, 129, 176, 177], it will be interesting to investigate the role of PTMs in regulating bacterial aging.

6. Conclusions and Future Perspectives

As awareness of the role of PTMs in aging and aging-related diseases grows, there is an urgent need for the development of methods to detect protein PTMs more rapidly and accurately. Furthermore, the recent finding of rare and unconventional modifications in age-related pathologies calls for the development of more specific and sensitive methods to detect such modifications [27]. The recent rapid progress in large-scale genomics and proteomics technologies is likely to be a catalyzing factor for such studies. Drugs that target PTMs, such as phosphorylation, acetylation, methylation, and ubiquitination, will serve as useful tools in exploring the basic mechanism of PTM modulation and provide a pharmacological platform to combat the detrimental effects of aging [178].

From a nonpharmacological perspective, exercise interventions are known to be an effective means of delaying the negative effects of aging at the physical and metabolic level. Several lines of evidence have shown that exercise can bring about benefits for elderly people through the modulation of both inflammatory and redox status, with impacts on proteostasis, insulin sensitivity, body composition (e.g., adipose tissue, skeletal muscle) and hormonal profile, among others. Likewise, caloric restriction is another nongenetic and almost universal process known to delay the onset of aging and extend maximum lifespan [179]. However, the influence of exercise and diet on protein PTMs remains relatively underexplored. Studies covering this particular area have the potential to develop widely accessible and affordable intervention strategies to fight aging-related diseases.

Finally, the utility of prokaryotic models in understanding the biology of aging is noteworthy, given the possibility of the conservation of aging-associated molecular mechanisms throughout evolution. As research progresses in the field of microbiogerontology, it will be interesting to discover to what extent such molecular mechanisms are conserved. This might open a completely new window of opportunities to search for ways to slow aging and extend healthy lifespan.

Disclosure

The funders had no role in study design, data collection, and analysis; decision to publish; or preparation of the manuscript.

Conflicts of Interest

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

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Research Article

Associations between Specific Redox Biomarkers and Age in a Large European Cohort: The MARK-AGE Project

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Oxidative stress and antioxidants play a role in age-related diseases and in the aging process. We here present data on protein carbonyls, 3-nitrotyrosine, malondialdehyde, and cellular and plasma antioxidants (glutathione, cysteine, ascorbic acid, uric acid, α-tocopherol, and lycopene) and their relation with age in the European multicenter study MARK-AGE. To avoid confounding, only data from countries which recruited subjects from all three study groups (five of eight centers) and only participants aged ≥55 years were selected resulting in data from 1559 participants. These included subjects from (1) the general population, (2) members from long-living families, and (3) their spouses. In addition, 683 middle-aged reference participants (35–54 years) served as a control. After adjustment for age, BMI, smoking status, gender, and country, there were differences in protein carbonyls, malondialdehyde, 3-nitrotyrosine, α-tocopherol, cysteine, and glutathione between the 3 study groups. Protein carbonyls and 3-nitrotyrosine as well as cysteine, uric acid, and lycopene were identified as independent biomarkers with the highest correlation with age. Interestingly, from all antioxidants measured, only lycopene was lower in all aged groups and from the oxidative stress biomarkers, only 3-nitrotyrosine was increased in the descendants from long-living families compared to the middle-aged control group. We conclude that both lifestyle and genetics may be important contributors to redox biomarkers in an aging population.

1. Introduction

Oxidative stress and antioxidants have been in the focus of research for decades due to their association with numerous age-related diseases [1–4]. In humans, elevated levels of oxidative stress have been reported in several medical conditions, including neurodegenerative diseases [2], obesity, diabetes mellitus, and the aging process itself [3–5]. There is some epidemiological evidence on the role of oxidative stress in aging, age-related diseases, and mortality [6–10]. However, these studies focused mostly on single biomarkers or on biomarkers which are not widely used, thus making it difficult to compare result.

When attempting to assess redox biomarkers, it is important to note that there is not one single biomarker which is considered a "gold standard." In fact, it is recommended to measure a set of different biomarkers [11]. Thus, our attempt was to analyze markers of protein oxidation, nitration, lipid peroxidation, and cellular and plasma antioxidants and study their relation with age in participants of the MARK-AGE project. The MARK-AGE project was a European multicenter study, supported by the European Commission, aiming to identify biomarkers of human aging and to model a robust set of markers of biological age and healthy aging [12].

For this purpose, women and men were recruited from the general population from eight European countries as age-stratified subgroups, as well as subjects belonging to a family with long-living members together with their spouses. In different biological matrices (whole blood, serum, plasma, urine, buccal mucosa cells, and peripheral blood mononuclear cells), a large number of candidate biomarkers of aging, including DNA-based markers, markers based on proteins and their modifications, immunological markers, clinical chemistry, hormones and markers of metabolism, oxidative stress markers, and antioxidant micronutrients were assessed. The project design including details of the study population and standard operating procedures have been published recently [12–15].

The objectives of the present work were to assess and to compare levels of redox biomarkers within the three different study groups of the MARK-AGE project. Our hypotheses were (1) that oxidative stress is elevated in higher age groups as it has been shown in different rather small population-based studies in different biological matrices and (2) that subjects from families with long-living members would be genetically better equipped to handle oxidative stress than the general population. The results of the spouses might show whether (3) a shared lifestyle may be able to influence biomarker concentrations.

2. Materials and Methods

This study was conducted in accordance with the Declaration of Helsinki (1964) and with informed written consent of each participant. Ethical clearance had been given by the ethics committee of each of the recruiting centers. This study has been registered retrospectively at the German Clinical Trials Register (DRKS00007713).

2.1. Study Population and Sample Collection. For the whole MARK-AGE project, 3158 participants were recruited in 8 recruiting centers and the details for the three study groups will be described briefly below.

The first study group was recruited through various public platforms such as radio and newspaper advertising. These participants were included in the RASIG group (recruited from the age-stratified general population). For this group, the main inclusion criteria were the ability to give informed consent and being in the age-range from 35 to 75 years (both genders).

The second study group consisted of 537 descendants from long-living subjects (nonagenarians; persons who reached the age of 90) who had been recruited as a follow-up from the GEHA study (Genetics of Healthy Aging; 2004–2009, for details, see [16]); this group is abbreviated as GO (GEHA offspring). The third study group consisted of the spouses of the GEHA offspring ($n = 311$) and served as a lifestyle control group, the so-called SGO (spouses of GO). The GO and SGO participants were between the age of 55 and 75 years [12] and were recruited in Belgium, Finland, Greece, Italy, The Netherlands, and Poland.

Collection of anthropometric data, questionnaire data, and data on cognitive function was carried out by trained nurses or physicians between November 2008 and June 2012 at the following recruiting centers: Hall in Tirol/Innsbruck (Austria), Namur (Belgium), Esslingen (Germany), Athens, and other nearby regions (Greece), Bologna (Italy), Warsaw (Poland), Tampere (Finland), and Leiden (The Netherlands). Furthermore, participants were asked to complete questionnaires on lifestyle characteristic (nutrition, smoking habits), family history, and living environment.

To avoid possible confounding, we used only data from those countries which recruited subjects from all three study groups (RASIG, GO, and SGO). This was the case in five of the eight centers (Belgium, Greece, Italy, Poland, and Finland). Germany and Austria did not provide GO or SGO data while The Netherlands did not recruit RASIG participants; therefore, data from these three countries were excluded in the present analyses.

Since the age ranges of participants in the GO and SGO groups were different to the RASIG group in general (55–75 versus 35–75 years, resp.), only participants who were ≥55 years of age were selected. This resulted in a total of 1559 participants in all three study groups to be included in the present work. Additionally, to compare these study groups to a younger (middle-aged) reference group, we selected all RASIG participants aged 35–54 ("middle aged") from Belgium, Greece, Italy, Poland, and Finland, which resulted in a total of 683 reference participants.

2.2. Determination of Total Glutathione and Total Free Cysteine in Whole Blood. Total glutathione and total free cysteine in whole blood were measured as previously described by Chen et al. [17] by using Ellman's reagent (5,5′-dithiobis-[2-nitrobenzoic acid]) after the reduction of any disulfides present. The modifications regarding the reduction agent DTT (1,4-dithiothreitol), the adaption for whole blood samples, and HPLC conditions were as

follows: Whole blood (100 μL) was vortex-mixed with DTT (12.5 mM, 100 μL) and incubated for 3 min; cold trichloroacetic acid solution (10% *w*/*v*, 200 μL) was added; and samples were thoroughly mixed and centrifuged at 19,500 ×g for 5 min at 4°C. The clear supernatant (200 μL) was then added to Ellman's reagent (30 mM, 50 μL) together with di-potassium hydrogen phosphate buffer (2 M, 100 μL), and vortex-mixed. Twenty μL was analyzed on a Shimadzu Prominence HPLC (LC-20A) equipped with an UV-Vis detector (SPD-20AV set at 326 nm). The separation of cysteine and glutathione was achieved by using a Reprosil-Pur 120 C18 AQ column (5 μm, 250 mm × 4.6 mm; Dr. Maisch, Germany) set to 40°C and a mobile phase consisting of methanol (15% *v*/*v*) and acetate buffer (0.05 M, pH 5) at a flow rate of 1 mL/min. Standards diluted to physiological concentrations (62.5–250 μM for cysteine and 500–2000 μM for glutathione) and treated as a sample were used for quantification.

2.3. Determination of Ascorbic Acid and Uric Acid in Plasma. Plasma ascorbic acid and uric acid were analyzed by RP-HPLC and UV detection after reduction with tris-(2-carboxyethyl)-phosphine [18]. Briefly, plasma (100 μL) was mixed with tris-(2-carboxyethyl)-phosphine (20% *w*/*w*, 25 μL) and incubated for 5 min on ice; then, freshly prepared metaphosphoric acid solution (10% *w*/*w*, 75 μL) was added and vortex-mixed; and samples were centrifuged at 19,500 ×g and 4°C for 10 min. Twenty μL of clear supernatant was analyzed on a Shimadzu Prominence HPLC and using a 5 μm analytical column (Reprosil-Pur 120 C18 AQ, 250 mm × 4.6 mm; Dr. Maisch, Germany) set to 40°C, a mobile phase consisting of 0.05 M sodium phosphate buffer (pH 2.5) at a flow rate of 1 mL/min and UV-Vis detector (SPD-20AV) set to 245 nm. Pure standards diluted to physiological concentrations (2.5–20 mg/L for ascorbic acid and 20–100 mg/L for uric acid) and treated as a sample were used for quantification.

2.4. Malondialdehyde. Plasma malondialdehyde was determined by RP-HPLC coupled with fluorescence detection after derivatization with thiobarbituric acid as described by Wong et al. [19] with modifications [20].

2.5. Analysis of Protein Carbonyls and 3-Nitrotyrosine. The analyses of protein carbonyls [21] and 3-nitrotyrosine in plasma by in-house ELISA have been described elsewhere [20].

2.6. Analysis of α-Tocopherol and Lycopene. Plasma lycopene and α-tocopherol were analyzed by RP-HPLC coupled with UV-vis and fluorescence detection as previously described [22].

2.7. Statistical Analysis. Demographic characteristics are described by using means ± standard deviation (SD) for continuous variables (age, weight, and BMI) and frequencies (%) for categorical variables (gender, smoking status, age groups, and country). Differences in characteristics between age groups and study groups were compared by one-way ANOVA (continuous variables) with Tukey's post hoc test and Pearson's chi-squared test (prevalence; for categorical variables). Data of plasma biomarkers were transformed appropriately to achieve normal distribution using square root (SR) or logarithmic (LN) transformation and are described by geometric means with 95% confidence intervals (CI). Correlations among biomarkers and between biomarkers and age are shown as Pearson product-moment correlation for transformed data. Mean values of plasma biomarkers between study groups were compared using one-way ANOVA and general linear models with Fisher's least significant difference test. The models were adjusted for age, BMI, gender, smoking status (covariates), and country (factor). In addition, a multiple linear regression analysis with all biomarkers in the initial model and a forward stepwise approach was applied to identify independent plasma biomarkers with the highest correlation with age. Differences of concentrations in biomarkers between RASIG, GO, SGO, and age groups (5-year intervals) are presented as box plots. All statistical analyses were carried out using SPSS software (SPSS Inc., Chicago, IL; Version 19); statistical significance for all tests was considered at $P < 0.05$.

3. Results

Characteristics of the study groups are shown in Table 1. The mean age of participants was 64.3 ± 5.4 (55–75) years with no significant difference between the matched study groups. Men represented 47.7% of participants. The mean BMI was 27.3 ± 4.5 kg/m^2 with no statistical significant difference among the study groups. Only 14.8% of participants were current smokers. The prevalence of smoking was different among the three study groups being 9.9% in SGO, 12.6% in GO, and 17.9% in RASIG. The number of participants from each study center was significantly different as shown in Table 1.

A total of 1559 participants were distributed into the groups as follows: RASIG ($n = 794$), GO ($n = 493$), and SGO ($n = 272$).

Biomarker concentrations differed significantly between study groups (Table 2 and Figures 1, 2, and 3). It is noteworthy that GO and SGO differed only in uric acid with significantly higher concentrations in the SGO group. Cysteine was highest in GO, glutathione in SGO, α-tocopherol was highest in both GO and SGO while lycopene was lower in GO and SGO than in the RASIG group. Interestingly, GO differed in all biomarkers from the RASIG group except in uric acid and total glutathione (Table 2). In detail, GO had significantly higher concentrations of ascorbic acid, total free cysteine, α-tocopherol, and 3-nitrotyrosine and lower concentrations of lycopene, protein carbonyls, and malondialdehyde. Furthermore, SGO were different from RASIG in all biomarkers except for uric acid, 3-nitrotyrosine, and malondialdehyde with higher concentrations of ascorbic acid, total free cysteine, total glutathione, and α-tocopherol and lower concentrations of protein carbonyls and lycopene.

We performed a univariate general linear model adjusted for age, BMI, gender, smoking status, and country to assess whether these differences were still present after adjustment. This was true for glutathione and α-tocopherol, as well as for all three oxidative stress biomarkers (protein carbonyls, 3-

Table 1: Characteristics of the three study groups.

	All ($n = 1559$)	RASIG ($n = 794$)	GO ($n = 493$)	SGO ($n = 272$)	P
Age (years)	64.3 ± 5.4	64.5 ± 5.8	64.3 ± 4.9	63.9 ± 4.7	0.230
55–59 years (n, (%))	345 (22.1)	193 (24.3)	94 (19.1)	58 (21.3)	
60–64 years (n, (%))	448 (28.7)	210 (26.4)	149 (30.2)	89 (32.7)	**<0.001**
65–69 years (n, (%))	465 (29.8)	204 (25.7)	166 (33.7)	95 (34.9)	
70–75 years (n, (%))	301 (19.3)	187 (23.6)	84 (17.0)	30 (11.0)	
Gender, male (n, (%))	743 (47.7)	386 (48.6)	213 (43.2)	144 (52.9)	**0.027**
Smoker, current (n, (%))	231 (14.8)	142 (17.9)	63 (12.6)	27 (9.9)	**0.001**
BMI (kg/m^2)	27.3 ± 4.5	27.6 ± 4.7	26.6 ± 4.4	27.3 ± 4.2	0.692
<25 (n, (%))	516 (33.1)	249 (31.4)	188 (38.1)	79 (29.0)	
25 to <30 (n, (%))	673 (43.2)	343 (43.3)	199 (40.4)	131 (48.2)	**0.036**
≥30 (n, (%))	369 (23.7)	201 (25.3)	106 (21.5)	62 (22.8)	
Country					
Belgium (n, (%))	472 (30.3)	155 (19.5)	190 (38.5)	127 (46.7)	
Finland (n, (%))	253 (16.2)	69 (8.7)	132 (26.8)	52 (19.1)	
Greece (n, (%))	209 (13.4)	187 (23.6)	18 (3.7)	4 (1.5)	**<0.001**
Italy (n, (%))	323 (20.7)	187 (23.6)	87 (17.6)	49 (18.0)	
Poland (n, (%))	302 (19.4)	196 (24.7)	66 (13.4)	40 (14.7)	

Values are means ± SD; P value: one-way ANOVA (continuous variables) and Pearson's chi-squared test (prevalence).

Table 2: Biomarker concentrations in the three study populations[1].

	All ($n = 1559$)	RASIG ($n = 794$)	GO ($n = 493$)	SGO ($n = 272$)	P
Antioxidants					
Ascorbic acid (mg/L)	4.41 (4.26; 4.58)	4.13 (3.91; 4.36)	4.73 (4.44; 5.02)[‡]	4.70 (4.33; 5.08)[‡]	**0.002**
Adjusted GLM[2]		4.32 (4.13; 4.52)	4.17 (3.87; 4.49)	3.85 (3.32; 4.41)	0.258
Uric acid (mg/L)	45.9 (45.3; 46.5)	45.7 (44.9; 46.5)	45.3 (44.2; 46.3)	47.4 (46.0; 48.8)[◊]	**0.047**
Adjusted GLM[2]		45.4 (44.6; 46.1)	46.0 (44.7; 47.2)	47.0 (44.7; 49.3)	0.341
Total cysteine (μmol/L)	148.2 (146.6; 149.8)	144.8 (142.5; 147.1)	150.8 (147.9; 153.6)[‡]	153.3 (149.3; 157.3)[‡]	**<0.001**
Adjusted GLM[2]		144.2 (141.8; 146.6)	149.6 (145.7; 153.4)[‡]	145.5 (138.4; 152.6)	0.072
Total glutathione (μmol/L)	1100 (1091; 1110)	1088 (1074; 1102)	1110 (1093; 1127)	1120 (1099; 1141)[‡]	**0.024**
Adjusted GLM[2]		1092 (1077; 1106)	1112 (1089; 1135)	1149 (1106; 1193)[‡]	**0.025**
Lycopene (μmol/L)	0.574 (0.556; 0.592)	0.631 (0.604; 0.659)	0.525 (0.497; 0.554)[‡]	0.503 (0.464; 0.543)[‡]	**<0.001**
Adjusted GLM[2]		0.608 (0.583; 0.635)	0.566 (0.526; 0.606)	0.539 (0.470; 0.613)	0.069
α-Tocopherol (μmol/L)	28.8 (28.4; 29.2)	27.8 (27.3; 28.3)	30.1 (29.4; 30.8)[‡]	29.6 (28.6; 30.5)[‡]	**<0.001**
Adjusted GLM[2]		27.9 (27.4; 28.5)	29.3 (28.4; 30.3)[‡]	29.9 (28.2; 31.7)[‡]	**0.005**
Oxidative stress biomarkers					
Protein Carbonyls (nmol/mg)	0.577 (0.573; 0.582)	0.595 (0.590; 0.601)	0.558 (0.551; 0.566)[‡]	0.561 (0.552; 0.571)[‡]	**<0.001**
Adjusted GLM[2]		0.591 (0.585; 0.597)	0.566 (0.557; 0.575)[‡]	0.564 (0.547; 0.581)[‡]	**<0.001**
3-Nitrotyrosine (pmol/mg)	4.11 (3.97; 4.25)	3.94 (3.75; 4.13)	4.40 (4.14; 4.67)[‡]	4.10 (3.77; 4.44)	**0.018**
Adjusted GLM[2]		3.90 (3.70; 4.11)	4.38 (4.03; 4.74)[‡]	3.76 (3.18; 4.38)	**0.048**
Malondialdehyde (μmol/L)	0.316 (0.306; 0.326)	0.336 (0.322; 0.350)	0.284 (0.266; 0.302)[‡]	0.317 (0.290; 0.346)	**<0.001**
Adjusted GLM[2]		0.334 (0.319; 0.348)	0.288 (0.267; 0.311)[‡]	0.314 (0.274; 0.357)	**0.004**

[1]Geometric mean (95% CI). [2]Adjusted general linear model (GLM): univariate general linear model adjusted for age, BMI, smoking status, gender, and country (center). [‡]Statistically significant difference to RASIG. [◊]Statistically significant difference to GO. P values: statistically significant differences were determined by one-way ANOVA with Tukey's post hoc test and by Fisher's least significant difference post hoc test in the GLM.

nitrotyrosine, and malondialdehyde), although lycopene ($P < 0.069$) and cysteine ($P < 0.072$) also reached the borderline of significance.

The comparison of biomarker concentrations of our study groups with a middle-aged control group is shown in Table 3. In general, the older groups revealed significantly

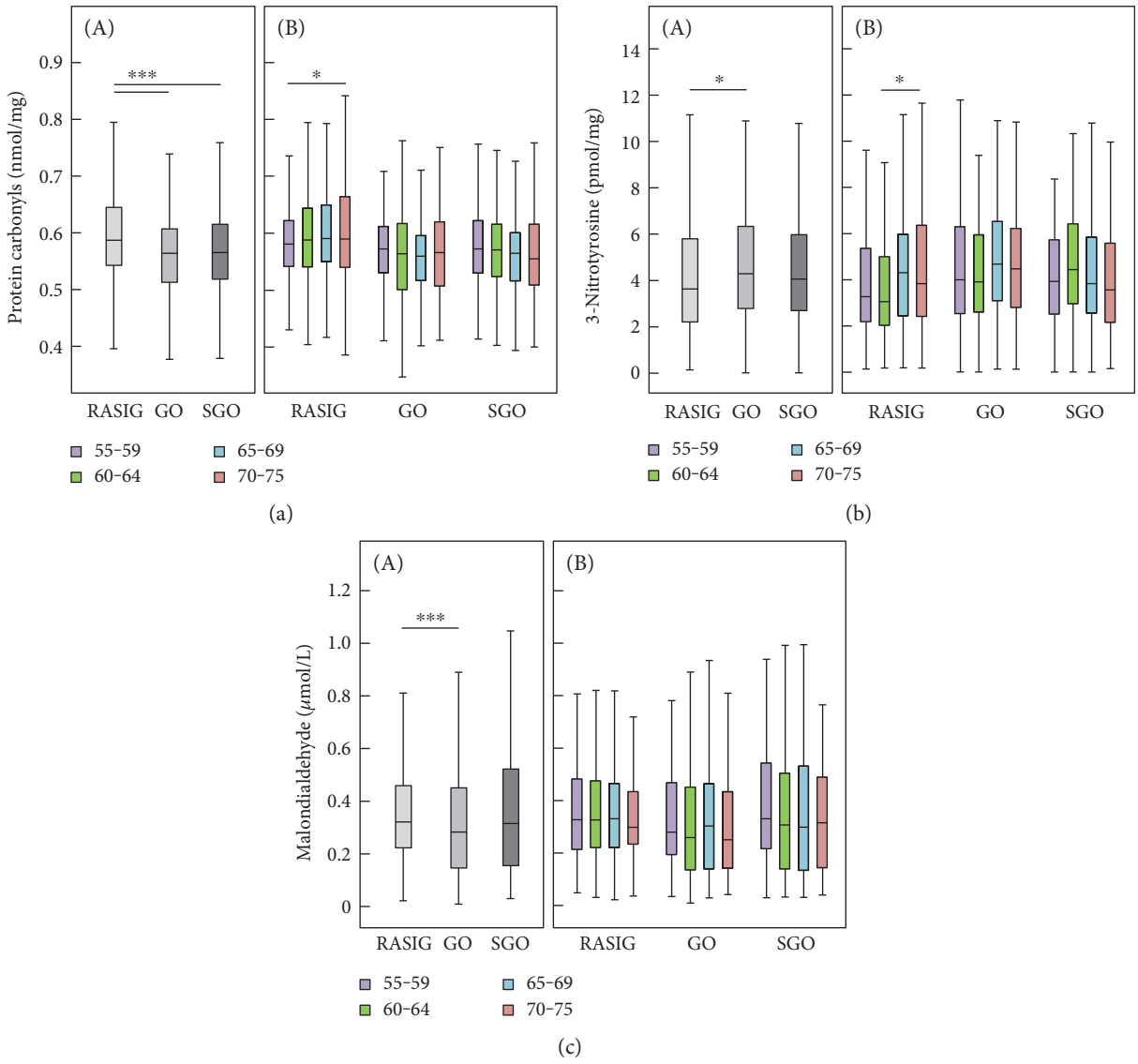

Figure 1: Classical biomarkers of oxidative stress: protein carbonyls (a), 3-nitrotyrosine (b), and malondialdehyde (c). Biomarker concentrations are displayed according to study groups (A) and age groups (B), respectively. RASIG ($n = 794$); GO ($n = 493$); SGO ($n = 272$). Outliers and extreme values are included in the analyses but not shown in the figure. Statistically significant differences are indicated by asterisks: $^{*}P < 0.05$ and $^{***}P < 0.001$.

different antioxidant concentrations compared to the reference group. The RASIG, GO, and SGO groups had significantly higher mean uric acid, cysteine, and α-tocopherol and lower lycopene compared to the reference group. There were no differences in terms of oxidative stress biomarkers between the reference group and RASIG aged 55–75 years. Interestingly, while the GO and SGO groups had significantly lower protein carbonyl concentrations compared to the reference group, only the GO group had higher 3-nitrotyrosine compared to the reference group.

Table 4 shows the correlation coefficients among the assessed biomarkers in all participants aged ≥55 years. The highest correlation coefficients were found between malondialdehyde and protein carbonyls ($r = 0.322$), malondialdehyde and ascorbic acid ($r = -0.240$), followed by protein carbonyls and α-tocopherol ($r = -0.193$). A positive correlation was observed between the antioxidants ascorbic acid and α-tocopherol ($r = 0.164$). When these correlations were performed only in the RASIG group, the direction and strength of correlations were similar (results not shown).

The correlations of the individual biomarkers with age are shown in Table 5. Significant positive correlation coefficients were observed for uric acid, cysteine, and 3-nitrotyrosine while a significant inverse association was only seen for lycopene. A weak significant positive correlation between protein carbonyls and age was only observed in the RASIG group ($r = 0.098$; $P < 0.01$, data not shown).

In a final multiple regression model with a forward approach, we aimed to identify independent biomarkers with the highest correlation with age (Table 6). Therefore, all biomarkers were assessed as covariates. Confirming the correlations from Table 5, uric acid, cysteine, 3-nitrotyrosine,

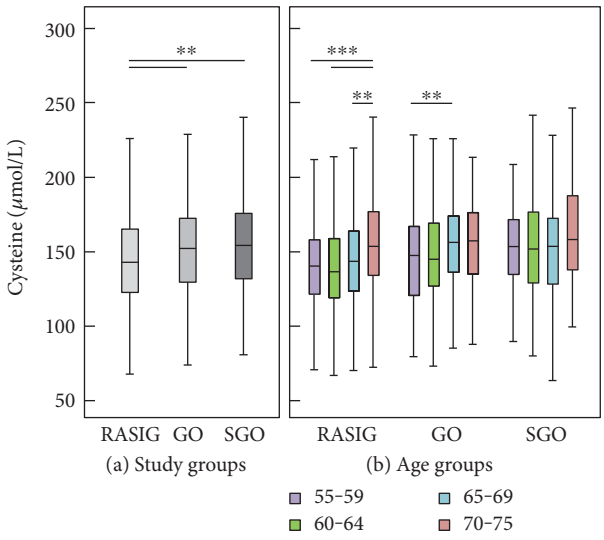

Figure 2: Cysteine concentration by study groups (a) and age groups (b). RASIG ($n = 794$); GO ($n = 493$); SGO ($n = 272$). Outliers and extreme values are included in the analyses but not shown in the figure. Statistically significant differences are indicated by asterisks: $^{**}P < 0.01$ and $^{***}P < 0.001$.

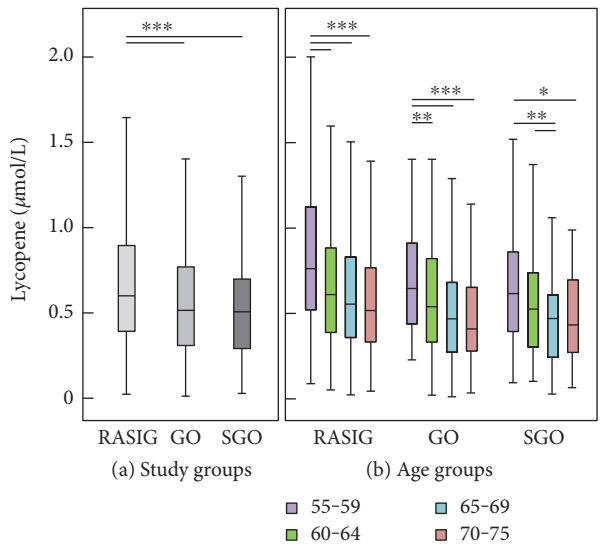

Figure 3: Lycopene concentration by study groups (a) and age groups (b). RASIG ($n = 794$); GO ($n = 493$); SGO ($n = 272$). Outliers and extreme values are included in the analyses but not shown in the figure. Statistically significant differences are indicated by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and lycopene were significantly associated with age, and also, protein carbonyls remained in the model with a positive association.

4. Discussion

In general, the older groups revealed higher antioxidant (uric acid, cysteine, and α-tocopherol) and lower lycopene concentrations compared to the reference group. Lycopene, total cysteine, uric acid, protein carbonyls, and 3-nitrotyrosine were significantly and independently associated with age in the multiple linear regression model among participants aged 55–75 years. Higher cysteine and α-tocopherol, but lower lycopene in both GO and SGO compared to RASIG seem to be associated with a "beneficial" lifestyle, while the significantly lower malondialdehyde and higher 3-nitrotyrosine only in the GO compared to the RASIG group may indicate that families with long-living members are genetically better equipped to handle oxidative stress, yet the cause and impact of the higher 3-nitrotyrosine levels remain unclear.

We assume that (1) if there are differences between RASIG and the other two groups but no differences between GO and SGO, the reason may be lifestyle-related and (2) differences between GO and SGO (irrespective of the RASIG results) indicate genetic reasons.

Applying these criteria to the biomarkers assessed in the present study leads to the conclusion that after adjustment for age, BMI, smoking status, gender, and country, the differences in protein carbonyls, malondialdehyde, 3-nitrotyrosine, α-tocopherol, cysteine, and glutathione between study groups seem to be lifestyle-related whereas genetics seem to play a minor role. For the case where both GO and SGO differ to the control group (protein carbonyls, lycopene, and α-tocopherol), this might indicate the influence of lifestyle. Thus, our results show that lifestyle is an important contributor to redox biomarkers.

One might hypothesize that when GO differ to the other two groups and the reference group, this difference may be attributed to genetics. This was the case for 3-nitrotyrosine, cysteine (both higher than the other groups), and malondialdehyde (lower than the other groups). In our study, there was no significant difference between GO and SGO groups for these three biomarkers, yet a clear tendency exists according to the data in Tables 2 and 3. Concentrations of malondialdehyde, 3-nitrotyrosine, and total cysteine were still different between the GO and the RASIG groups after the GLM adjustment, which may be an indication of a genetic contribution in age-associated handling of oxidative stress.

Direct comparison of the study groups revealed that the GO group had significantly lower concentrations of protein carbonyls and malondialdehyde accompanied by higher concentrations of cysteine, ascorbic acid, and α-tocopherol compared to RASIG. Contrarily, GO had lower lycopene and higher 3-nitrotyrosine than the RASIG group. These differences may be due to better metabolic profiles or due to a generally healthier nutrient intake, despite less processed tomato products which are especially rich in lycopene.

It is widely accepted that there is a relationship between the aging process and oxidative stress; however, most studies leading to this theory have been carried out in model systems and only few studies have analyzed different biomarkers of oxidative stress in healthy humans of various age groups [3, 6].

Protein carbonyls are considered to be relatively stable [23] and early markers [24] of oxidative stress. Measuring carbonylation of plasma proteins enables evaluating the global oxidation status in plasma. Protein carbonyls have been analyzed in a multitude of studies ranging from cell culture, animal, to human studies and are related to the aging

Table 3: Comparison of study groups with a reference group.

	Reference Group (RASIG; 35–54 years) ($n = 683$)	RASIG (55–75 years) ($n = 794$)	GO (55–75 years) ($n = 493$)	SGO (55–75 years) ($n = 272$)
Antioxidants				
Ascorbic acid (mg/L)	4.37 (4.12; 4.63)	4.13 (3.91; 4.36)	4.73 (4.44; 5.02)	4.70 (4.33; 5.08)
Uric acid (mg/L)	42.4 (41.6; 43.3)	**45.7 (44.9; 46.5)*****	**45.3 (44.2; 46.3)*****	**47.4 (46.0; 48.8)*****
Total cysteine (μmol/L)	130.3 (128.1; 132.5)	**143.0 (140.7; 145.3)*****	**149.0 (146.1; 151.9)*****	**151.4 (147.4; 155.5)*****
Total glutathione (μmol/L)	1107 (1092; 1121)	1088 (1074; 1101)	1110 (1093; 1127)	1120 (1099; 1142)
Lycopene (μmol/L)	0.83 (0.80; 0.86)	**0.63 (0.60; 0.66)*****	**0.53 (0.50; 0.55)*****	**0.50 (0.46; 0.54)*****
α-Tocopherol (μmol/L)	25.7 (10.4; 10.6)	**27.8 (10.9; 11.2)*****	**30.1 (11.4; 11.7)*****	**29.6 (11.2; 11.7)*****
Oxidative stress biomarkers				
Protein carbonyls (nmol/mg)	0.603 (0.598; 0.609)	0.595 (0.590; 0.601)	**0.558 (0.551; 0.566)*****	**0.561 (0.552; 0.571)*****
3-Nitrotyrosine (pmol/mg)	3.8 (3.6; 4.0)	3.9 (3.8; 4.1)	**4.4 (4.1; 4.7)****	4.1 (3.8; 4.4)
Malondialdehyde (μmol/L)	0.31 (0.30; 0.32)	0.34 (0.32; 0.35)	0.28 (0.27; 0.30)	0.32 (0.29; 0.35)

$^{***}P < 0.001$ and $^{**}P < 0.01$ by one-way ANOVA with Tukey's post hoc test.

Table 4: Correlations between biomarkers in all three study groups in participants aged ≥55 years[1].

	Ascorbic acid	Uric acid	Total cysteine	Total glutathione	Lycopene	α-Tocopherol	Protein carbonyls	3-Nitro-tyrosine	Malondialdehyde
Ascorbic acid		**−0.083*****	**0.140*****	0.030	**−0.103*****	**0.164*****	**−0.157*****	−0.027	**−0.240*****
Uric acid			**0.067****	−0.021	**−0.099*****	**0.059***	−0.020	**−0.054***	0.019
Total cysteine				0.047	**−0.053***	**0.103*****	**0.062***	0.043	−0.034
Total glutathione					0.032	−0.033	−0.007	−0.015	0.030
Lycopene						**0.073****	**0.088*****	**−0.092*****	**0.159*****
α-Tocopherol							**−0.193*****	**0.062***	**−0.132*****
Protein carbonyls								0.001	**0.322*****
3-Nitrotyrosine									0.019
Malondialdehyde									

[1]Pearson correlation coefficient r. Statistically significant correlations are marked by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. ($n = 1559$).

Table 5: Correlations between biomarkers and age among all participants (aged ≥55 years)[1].

	r	P
Ascorbic acid	0.026	0.297
Uric acid	**0.092**	**<0.001**
Total cysteine	**0.152**	**<0.001**
Total glutathione	−0.035	0.167
Lycopene	**−0.224**	**<0.001**
α-Tocopherol	−0.005	0.847
Protein carbonyls	0.036	0.158
3-Nitrotyrosine	**0.066**	**0.009**
Malondialdehyde	−0.037	0.146

[1]Pearson correlation coefficient r ($n = 1559$).

process. They have been shown to be a predictor of mortality in moderately to severely disabled older women [10]. Komosinska-Vassev et al. showed a positive correlation of protein carbonyls with age in a study of 56 men and women aged ≥55 years ($r = 0.52$) [25]. Other authors also found a strong positive correlation of protein carbonyls with age ($r = 0.786$) in 80 participants between the age of 18 and 85 [6], whereas in the present study, the correlation for the age range of 55–75 was very weak ($r = 0.098$) but still statistically significant. Cakatay et al. demonstrated that plasma protein carbonyl levels of elderly participants were significantly higher in comparison to those of middle-aged and young participants [26]. In the present study, protein carbonyls only correlated with age in the RASIG group ($r = 0.098$, $P < 0.01$; data not shown) but not when all three study groups were combined. Most interestingly, the RASIG group had statistically significantly higher protein carbonyl concentrations than the GO as well as the SGO while there was no difference between the GO and SGO. After adjusting for covariates (including smoking status), this difference remained.

For 3-nitrotyrosine, we observed a weak, positive association with age. In contrast to protein carbonyls, 3-nitrotyrosine was significantly higher in GO compared to RASIG. 3-Nitrotyrosine has been described to be a stable marker of oxidative/nitrative stress in some inflammatory diseases [27]. It arises from nitration involving reactive

Table 6: Associations of oxidative stress markers and antioxidants with age[1].

Compound	B	95% CI	r	r^2	P
(Constant)	59.13	56.79, 61.48			**<0.001**
Lycopene (μmol/L)	−2.783	−3.440, −2.126	−0.207	0.047	**<0.001**
Total cysteine (mol/L)	0.021	0.013, 0.029	0.133	0.020	**<0.001**
Uric acid (mg/L)	0.031	0.009, 0.054	0.071	0.004	**0.005**
Protein carbonyls (nmol/mg)	3.274	0.252, 6.296	0.054	0.003	**0.034**
3-Nitrotyrosine (pmol/mg)	0.094	0.004, 0.183	0.052	0.003	**0.040**

[1]Multiple linear regression analysis with a forward approach to identify independent blood biomarkers with highest correlation to age; all biomarkers including ascorbic acid, glutathione, malondialdehyde, and α-tocopherol were assessed as covariates in the initial model; partial r and r^2; $R = 0.277$, $R^2 = 0.077$ ($n = 1545$).

nitrogen species (RNS) and peroxidase-mediated nitrite oxidation. Today, myeloperoxidase is considered to be involved in this pathway. Thus, 3-nitrotyrosine may play a role in inflammation rather than the aging process itself [28]. Frijhoff et al. question whether 3-nitrotyrosine is clinically useful, in comparison to the already established markers of inflammatory processes such as C-reactive protein [29]. Plasma 3-nitrotyrosine levels in patients treated with anti-inflammatory drugs have been shown to decrease [30, 31]. This is one more hint why 3-nitrotyrosine may be a better marker for inflammation than for the aging process in general. Although the nitration of tyrosine residues on proteins can result in a loss of function [32], some authors have reported a gain of function [33]. Perhaps site-specific nitration may have a protective function or a role in longevity. In our study population, there might have been no dramatic difference of 3-nitrotyrosine in the different age groups because the participants were generally healthy. Only 4.0% of our participants had CRP concentrations ≥ 10 mg/L. Nevertheless, we checked the correlation between CRP and 3-nitrotyrosine but there was no correlation, neither in all participants nor in those with a high CRP level. Thus, the usefulness of 3-nitrotyrosine as a biomarker to evaluate oxidative stress remains to be elucidated.

Few large epidemiological studies have analyzed malondialdehyde. Block et al. suggest plasma malondialdehyde should be considered for most epidemiologic research on redox biomarkers [34] since they observed in a validation study that this biomarker had a good day-to-day stability. They propose malondialdehyde to be an effective marker of oxidative stress and state that the use of a single measure of malondialdehyde resulted in little attenuation [34]. In another study, the same authors found that malondialdehyde was not associated with age in 298 participants aged 18–78 years [35]. These results are supported by our finding that malondialdehyde was not associated with age in any of the three study groups. However, the GO group had significantly lower plasma malondialdehyde concentrations than RASIG. This difference remained even after adjusting for age, BMI, smoking status, gender, and country.

To counteract oxidative stress, there exist a network of antioxidant defense mechanisms. One of these antioxidants is the tripeptide glutathione (γ-glutamylcysteinylglycine). A decline of glutathione with age has previously been suggested in humans [36], and it has been demonstrated that the correlation of thiol groups in plasma with age was inverse ($r = -0.718$) [6].

Concentrations of glutathione and cysteine were lower in healthy old (mean age 70.3 years) than in middle-aged (mean age 39.8 years) participants ($n = 8$ each) [37].

We did not observe any correlation between glutathione and age. One explanation may be that we measured total glutathione instead of GSH/GSSH which is considered a better marker of the redox state. Giustarini et al. found an inverse correlation of glutathione with age but no correlation of cysteine with age in 41 participants [38]. Similarly, Jones et al. used plasma of 122 healthy individuals aged 19–85 years to analyze thiol-based redox changes [39]. They suggest that the capacity of the glutathione antioxidant system is maintained until 45 years and then declines rapidly. For the present analyses, participants were selected which were ≥55 years, yet we were unable to show a correlation between glutathione and age. In contrast to previous findings, cysteine is higher in the higher age groups in the RASIG and GO study groups and correlates positively with age among all participants ($r = 0.152$, $P < 0.001$).

It is assumed that lower glutathione concentrations occurring during aging and in different diseases may more likely result from low cysteine concentrations rather than due to oxidation since cysteine is the rate-limiting precursor of glutathione. Free cysteine is one of the main nonprotein thiols in plasma [40, 41] and considered a semiessential amino acid, since it must be taken up or synthesized from the essential amino acid methionine [42]. It is able to regulate nutrient metabolism, oxidative stress, physiologic signaling pathways, and associated diseases through the production of glutathione, hydrogen sulfide, and taurine [43]. An oral intervention with cysteine (as N-acetylcysteine) and glycine for 14 days resulted in significantly increased glutathione concentrations [37]. This was also true when only cysteine was supplemented, resulting in an increase in hepatic glutathione synthesis [43]. It is likely that the glutathione concentrations in our cross-sectional study were similar in the different age groups because cysteine may not have been limited. Previous results also suggest that the participants in our study had sufficient cysteine and thus glutathione levels [38]. Nevertheless, the requirement of cysteine may be elevated after oxidative events due to the consumption of glutathione. Some future research should clarify the role of cysteine in aging and as a dietary precursor of glutathione.

Furthermore, it should be noted that some authors measured glutathione/thiol groups in plasma [6]. Glutathione is known as the most powerful cellular antioxidant, that is, present in red blood cells, and consequently, we analyzed glutathione in whole blood.

Concerning uric acid, our results show that there was no difference between RASIG and GO but higher concentrations in the SGO group than in the GO. However, adjusting for age, BMI, gender, smoking status, and country, these differences did not remain. Uric acid is derived from the degradation of purine nucleotides which can be of dietary or endogenous origin. Therefore, an increased intake of animal products or legumes/pulses in the SGO group is possible. There is a controversial discussion whether the positive effects of uric acid as an antioxidant are outweighed by its adverse effects concerning gout, coronary artery disease, hypertension, and stroke (for review, see [44]).

Besides endogenous antioxidants such as glutathione, cysteine, and uric acid, exogenous antioxidants are also required to counteract oxidative stress. Some of the most powerful antioxidants are of dietary origin such as ascorbic acid, α-tocopherol, lycopene, and other carotenoids. It is known that a high intake of fruits and vegetables is associated with a high plasma concentration of ascorbic acid [45]. Simultaneously, a diet rich in fruits and vegetables is associated with a reduced risk of some diseases such as CVD and cancers [46]. α-Tocopherol and ascorbic acid act synergistically in counteracting free radicals. α-Tocopherol is able to quench free radicals in a hydrophobic environment, for example, to terminate lipid peroxidation and the resulting tocopherol radical is then recycled by ascorbic acid [47]. We found an inverse correlation between α-tocopherol and malondialdehyde, an inverse association between ascorbic acid and malondialdehyde (all $P < 0.001$), and a positive correlation between α-tocopherol and ascorbic acid. These results are in accordance with the assumption that these antioxidants act synergistically.

Since these are diet-derived antioxidants and we did not adjust our models for dietary intake of fruit and vegetables, we cannot exclude that the diet or the season had an effect on the plasma concentration of these micronutrients. In a recent study, we have shown over the whole age-range of the MARK-AGE project from 35 to 75 (RASIG group) that lycopene was lower in higher age groups and this effect was independent of season [22]. It remains unclear if these differences observed in the older age groups result from a reduced intake, reduced absorption, increased storage in adipose tissue, or elevated degradation of lycopene.

The strengths of the present study include the large sample size and that all biomarkers described here were measured in one single laboratory in blinded form (samples from study groups and countries were in mixed random order by the biobank). For lipid-soluble micronutrient analysis, we have recently published the interbatch coefficients of variation which were 7.6% for lycopene and 6.3% for α-tocopherol in the MARK-AGE cohort [22]. Concerning the validity of the methods used for 3-nitrotyrosine analysis, we have previously reported good specificity, reproducibility, and accuracy for this in-house ELISA [48]. The ELISA method used here to analyze protein carbonyls has been validated in a multicenter ring study [49]. It was shown that carbonyl concentrations from three out of four laboratories participating in the ring study fell within 95% confidence intervals. Additionally, protein concentration was measured before both ELISAs and plasma samples were diluted to the same protein concentration. Standards for protein carbonyl ELISA were prepared according to Buss et al. [21] and run on every 96-well plate.

In terms of comparability to other studies, there is an excellent review from Giustarini et al. [50]. As the authors demonstrate, there exist numerous analytical methods to analyze protein carbonyls, malondialdehyde, GSH, tocopherols, and lycopene, among others. An overview is given for different derivatization and detection methods, health conditions, and units, demonstrating huge variety. For instance for malondialdehyde, Giustarini et al. showed that authors using methods comparable to ours (TBA derivatization and HPLC separation) published concentrations between 0.44 and 6.8 μmol/L for plasma samples [50]. One reason for this variation may be that heparin plasma was used in some cases. Especially for malondialdehyde, it is important to measure this marker in EDTA plasma since lipid peroxidation can continue in serum and heparin plasma, thus artificially contributing to elevated malondialdehyde concentrations. EDTA and citrate can complex iron thus preventing Fenton reaction leading to lipid peroxidation.

For GSH and cysteine, the authors also report different methods (e.g., enzymatic, HPLC coupled with UV or fluorescence detection) and mean concentrations in whole blood, plasma, and erythrocytes for healthy and diseased individuals, which span over one to two orders of magnitude within the kind of used specimen; however, total GSH levels in the present study are in the upper range of previously reported mean whole blood values for healthy individuals measured by HPLC or recycling spectrophotometry (using Ellman's reagent).

This study has some limitations which must be mentioned. Since this study was observational, we cannot make statements on the changes of these biomarkers with age; therefore studies with repeated measurements and/or follow-up are needed. Furthermore our results may be specific for European/Caucasian subjects and thus not transferrable to other countries.

The inclusion of three different study groups with a large sample size ($n = 1559$) is one feature that distinguishes this study from others. Samples were collected and distributed in a blinded form to guarantee unbiased measurement and interpretation, and all analyses of biomarkers described here were carried out in one single laboratory, which significantly reduces interlaboratory variations. The assessment of different specific cellular and plasma biomarkers, that is, markers for oxidative damage together with antioxidants that are not analyzed by commercial kits but by in-house methods is certainly an important strength.

5. Conclusion

Here, we have provided an overview of the levels of the different redox biomarkers in human plasma and whole blood in

three different study groups of the MARK-AGE project and their correlation with age. Interestingly, from all antioxidants measured, only lycopene was lower in the three aged groups. In addition, from the oxidative stress biomarkers, only 3-nitrotyrosine was increased in the descendants from long-living families compared to the control group, while the aged general population did not exhibit any difference compared to the middle-aged controls. Higher cysteine and α-tocopherol, but lower lycopene in both GO and SGO, compared to the RASIG seem to be associated with a "beneficial" lifestyle, while the significantly lower malondialdehyde and higher 3-nitrotyrosine which were observed only in the GO compared to the RASIG group may indicate that families with long-living members are genetically better equipped to handle oxidative stress. Thus, our present study suggests that age, lifestyle, and genetics could contribute to an individual's oxidative stress status.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay
GEHA: Genetics of healthy aging
GLM: General linear model
GSH: Glutathione
GO: GEHA offspring
HPLC: High-performance liquid chromatography
RASIG: Recruited from the age-stratified general population
SGO: Spouses of GO.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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