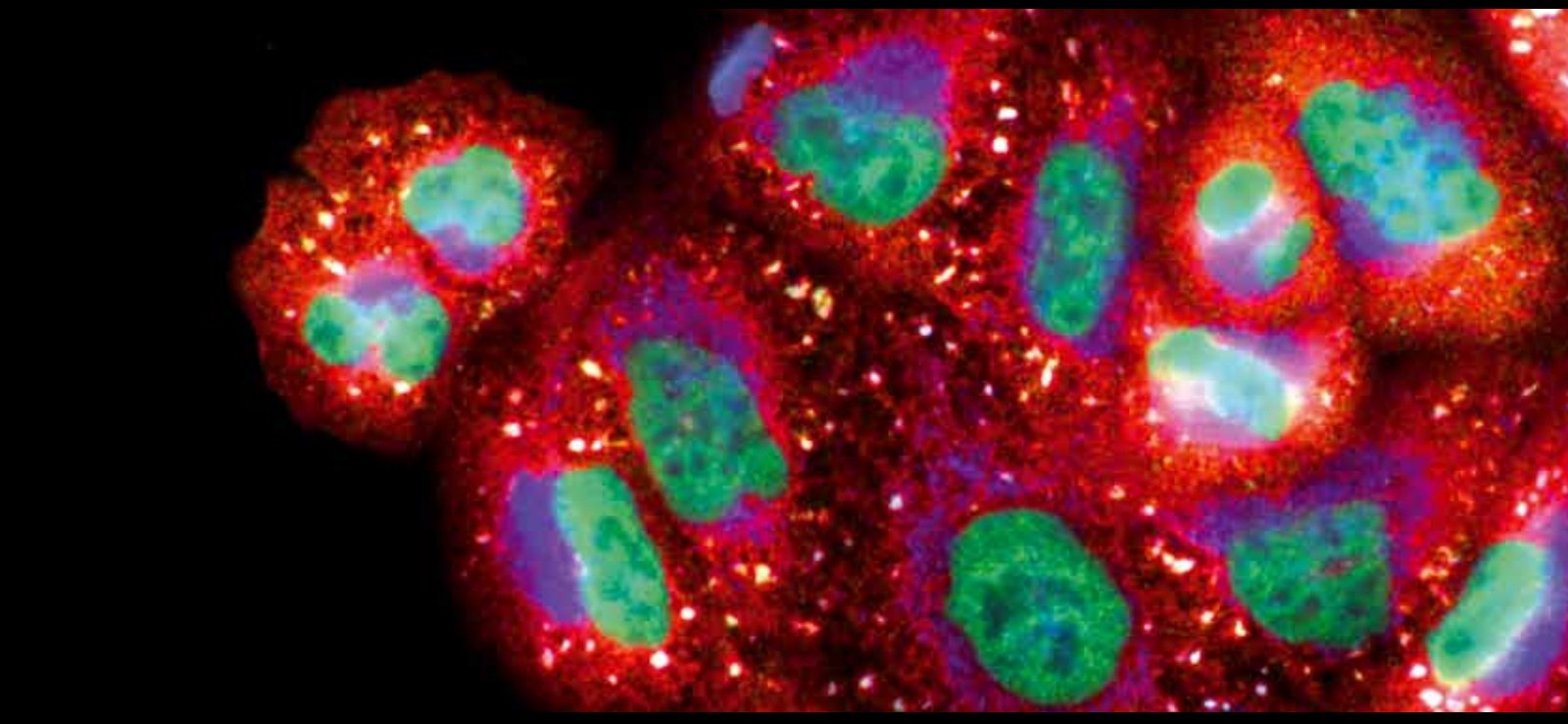
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Aging

Guest Editors: Mohammad Abdollahi, Majid Y. Moridani, Okezie I. Aruoma, and Sara Mostafalou



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Editorial
Oxidative Stress in Aging

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Constant formation of free radicals mainly reactive oxygen species (ROS) is the main characteristic of all living systems which use oxygen for their basal metabolism. Primarily, ROS are considered an integral component of basic cell regulation and signaling pathways certainly, suppressing tumor progression which forms the basis of the most chemotherapeutic and radiotherapeutic agents. The most common ROS are superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) which can stimulate consecutive reactions leading to further production of free radicals and related oxidative damage to cellular compartments. On the other side, there are several antioxidant complexes which defend cellular integrity against free radical-induced damages by neutralizing oxidative elements. Superoxide dismutase is the main antioxidant enzyme which converts $O_2^{\cdot -}$ to H_2O_2 and then the other enzyme located in peroxisomes, catalase, disposes of H_2O_2 by converting it to O_2 and H_2O. Keeping the balance of such cell homeostasis in a level at which ROS are not able to damage cells is vital. Excessive ROS not only cause damage toward cellular lipids and DNA but also participate in posttranslational dysfunction of proteins or enzymes involved in keeping viability and function of the cells [1, 2]. Accordingly, oxidative stress and the accompanying pathway, inflammation, are known to be involved in the pathology of numerous human diseases including cancer, neurodegenerative diseases, rheumatoid arthritis, and diabetes. As an example, oxidative and inflammatory mechanisms were considered for neurodegenerative diseases primarily when it was observed that using high amounts of anti-inflammatory drugs in rheumatoid arthritis patients for long times was associated with less incidence of Alzheimer's disease (AD). However, there are many factors affecting the rate of ROS formation in the body among which lifestyle related habits like diet, cigarette smoking, alcohol consumption, environmental exposures, and stress are the most important. Apart from all these factors, it is evident that ROS and the other free radicals are more produced and accumulated in the body over time. This has led to a hypothesis on the implication of oxidative stress in aging and inspired researchers to find new therapies for battling age-related diseases through exploiting agents which modulate oxidant/antioxidant homeostasis [1, 2]. This special issue is composed of articles focused on describing the seminal research and viewpoints pertinent to current understanding of the role of oxidative stress in aging and perspectives on treatment of age-related diseases.

In this special issue, the comparative effects of biodynes, tocotrienol-rich fraction, and tocopherol in enhancing collagen synthesis and inhibiting collagen degradation in stress-induced premature senescence model of human diploid fibroblasts have been described. Skin aging can be intrinsic or extrinsic, mediated genetically or due to environmental exposures. Biodynes, tocotrienol-rich fraction, and tocopherol upregulate collagen genes and increase the synthesis of procollagen proteins.

The AD is the outcome of a complex interaction among several factors which are not fully understood yet; nevertheless, it is clear that oxidative stress and inflammatory pathways are among these factors. In this special issue, lower plasmatic levels of α-tocopherol and mild systemic oxidative stress in the subclinical stage of Alzheimer's disease have been described.

Also it has been described that sublethal oxidative stress could induce premature senescence of human mesenchymal stem cells derived from endometrium. Thus induction of premature senescence might be a common physiological response to sublethal oxidative stress in human mesenchymal stem cells of any origin.

Carbon monoxide (CO), an endogenous small gaseous mediator, may exert important roles in physiological and pathophysiological states through regulation of cellular signaling pathways. It has been noted that CO when applied at low concentration can confer anti-inflammatory effects in macrophages and protect endothelial cells and hepatocytes against cytotoxic agents.

The relationship between oxidative stress and aging by addressing cellular expression profile analysis through proteomics studies using two-dimensional electrophoresis and mass spectrometry has been used as an integral approach to study the aging process. Emphasis is placed on postmitotic tissues, such as neuronal, muscular, and red blood cells, which appear to be those most frequently studied with respect to aging.

Ovarian aging-like phenotype in the hyperandrogenism-induced murine model of polycystic ovary (PCO) is a new topic. Exposure to some environmental factors and chemicals during life may accelerate progression towards the end of functional reproductive period. Hyperandrogenism-induced PCO in rats is associated with ovarian aging-like phenotypes. Meanwhile, hyperandrogenism triggers ovarian senescence in rats with PCO.

Also in this special issue it was noted that increased oxidative stress response in granulocytes from older patients with a hip fracture may account for slow regeneration. Aged granulocytes seem more sensitive towards damage induced by oxidative stress rather than young granulocytes, declaring why aged granulocytes cannot cope with the additional oxidative stress stimuli of the fracture.

The production of ROS is not limited to a specific tissue or organ and oxidative based damages to the cells can be detected from the most superficial layer, skin, to different internal compartments like nervous system, bone marrows and hematopoietic system, liver, and the other parts of endocrine system. Protective effects of antioxidative agents like α-tocopherol and ascorbic acid in decreasing the rate of senescence have been well studied, of course, mostly in experimental models. However, effectiveness of modulators of oxidative homeostasis in treatment of age-related diseases still needs more studies along with opening more windows from the interactions of oxidative elements in aging [3]. The collection of data brought in this special issue is anticipated to help researchers to resolve key questions about the role of oxidative stress in aging and find promising perspectives on the efficacy of oxidative modulating agents in treatment or controlling age-related diseases.

Mohammad Abdollahi
Majid Y. Moridani
Okezie I. Aruoma
Sara Mostafalou

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Research Article

Increased Oxidative Stress Response in Granulocytes from Older Patients with a Hip Fracture May Account for Slow Regeneration

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Proximal femur fracture, a typical fracture of the elderly, is often associated with morbidity, reduced quality of life, impaired physical function and increased mortality. There exists evidence that responses of the hematopoietic microenvironment to fractures change with age. Therefore, we investigated oxidative stress markers and oxidative stress-related MAPK activation in granulocytes from the young and the elderly with and without fractured long bones. Lipid peroxidation levels were increased in the elderly controls and patients. Aged granulocytes were more sensitive towards oxidative stress induced damage than young granulocytes. This might be due to the basally increased expression of SOD-1 in the elderly, which was not further induced by fractures, as observed in young patients. This might be caused by an altered MAPK activation. In aged granulocytes basal p38 and JNK activities were increased and basal ERK1/2 activity was decreased. Following fracture, JNK activity decreased, while ERK1/2 and p38 activities increased in both age groups. Control experiments with HL60 cells revealed that the observed p38 activation depends strongly on age. Summarizing, we observed age-dependent changes in the oxidative stress response system of granulocytes after fractures, for example, altered MAPK activation and SOD-1 expression. This makes aged granulocytes vulnerable to the stress stimuli of the fracture and following surgery.

1. Introduction

Proximal femur fracture is a typical fracture of the elderly who frequently exhibit a lower bone mineral density, like, for example, osteopenia and osteoporosis, as well as muscle atrophy [1]. All over the world, approximately 2.6 million patients suffer from a hip fracture each year. Due to the anticipated demographic changes, this number is expected to be doubled in 40 years [2, 3]. Despite the major progresses regarding surgical procedures, hip fractures are still a major public health concern. High postoperative complication rates negatively affect the general outcome as well as the quality

Table 1: Information about donors.

	Groups			
	YH	YF	OH	OF
Number of individuals	22	12	23	19
Age range (a)	24 to 38	22 to 47	71 to 90	69 to 99
Age median (a)	29.5	31	78	86
Number of male individuals	11	9	9	7
Number of female individuals	11	3	14	12

YH: young controls; YF: young patients with a fracture of the long bones; OH: elderly controls; OF: elderly patients with a fracture of the long bones.

of life and cause high costs for the health care system [2, 4]. However, little is known about the mechanisms influencing the recovery of the patients.

Changes in the microarchitecture and loss of bone substance may render the surgical treatment more complicated and delay fracture healing due to a disbalance of the function of osteoblasts and osteoclasts. However, these negative effects of bone remodeling do not provide sufficient explanation for the high rate of postoperative complications after hip fracture particularly in older patients [5]. The increased morbidity and mortality, for example, due to infections, in this subgroup of patients is more likely due to systemic changes which have negative effects on recovery [6]. Circulating cells of the immune system are directly exposed to the stress stimuli caused by the fracture and the following surgery. Thus, normally they should be able to respond to the external stress signal directly. In elderly patients, these responses are possibly impaired due to alterations of the immune system.

Granulocytes and monocyte derived macrophages constitute the first lines of defense against bacterial infections. After a trauma, neutrophils migrate to the site of injury within minutes, where mitogen-activated protein kinase (MAPK) signaling occurs in granulocytes as a concomitant circumstance to extracellular stimuli that affect the functions of the cell, like, for example, proliferation, differentiation, or apoptosis [7, 8].

MAPKs that have been reported to be involved in these processes are JNK, ERK1/2, and p38 [9–16]. These MAPKs are all directly affected by oxidative stress stimuli [7, 9]. Their activation may induce cellular defense mechanisms, for example, the expression of the antioxidative enzyme superoxide dismutase (SOD-1) which directly eliminates reactive oxygen species (ROS) by catalyzing the dismutation of superoxide to hydrogen peroxide and molecular oxygen [17].

Thus, the aim of the present study consisted in investigating oxidative stress levels, oxidative stress induced toxicity, and the related MAPK activation in granulocytes from young (<50 years of age) and elderly (>65 years of age) patients with fractures of the long bones and from healthy controls. This comparison should enable us to draw conclusions about alterations of the response of granulocytes to the stress stimuli of a fracture and following surgery in young and old patients.

2. Materials and Methods

Phosphate buffered saline, fetal calf serum, and RPMI1640 were purchased from PAA Laboratories GmbH (Pasching, Austria). Complete protease inhibitor was obtained from Roche (Mannheim, Germany). All other chemicals were purchased from Sigma (Munich, Germany).

2.1. Ethics Statement. Blood sampling was conducted in accordance with the Declaration of Helsinki (1964) and its amendments. The study protocol was approved by the hospital's Ethics Committee and informed consent was obtained from all subjects. Patients with chronic diseases were not included in the study.

2.2. Sample Population. 45 mL venous blood (36 mL EDTA (ethylenediaminetetraacetic acid) blood and 9 mL serum blood) was collected from each patient. Two different age groups were studied: young patients aged less than 50 years and elderly patients aged over 65 years. In both age groups, we distinguished between healthy controls and patients with a fracture of the long bones. The study population consisted of 22 young healthy controls (YH), 23 old healthy controls (OH), 12 young patients (YF), and 19 old patients (OF). In both groups of patients, the blood sampling was performed within 6 h after the operative reposition of the fracture of one of the long bones of the lower extremity. Demographic data is summarized in Table 1.

2.3. Granulocyte Isolation. The granulocytes were isolated by MACS (magnetic cell separation) using CD15 magnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec, Cologne, Germany) or by density gradient centrifugation as described in [18]. Immediately after their isolation, the granulocytes were lysed for Western blot analysis. Cell purity was over 95% as assessed by flow cytometry.

2.4. HL60 Cell Culture. The HL60 granulocyte cells were expanded in RMPI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2. For maturation culture medium was supplemented with 1% dimethylsulfoxide. Prior to the experiment, the cells were serum-starved overnight. After 1 h stimulation with culture medium containing 10% sera from young and old patients and controls, cells were lysed for Western blot analysis.

2.5. Quantification of Lipid Peroxidation to Assess Oxidative Stress Levels. In order to measure the oxidative stress, lipid peroxidation was determined through the malondialdehyde

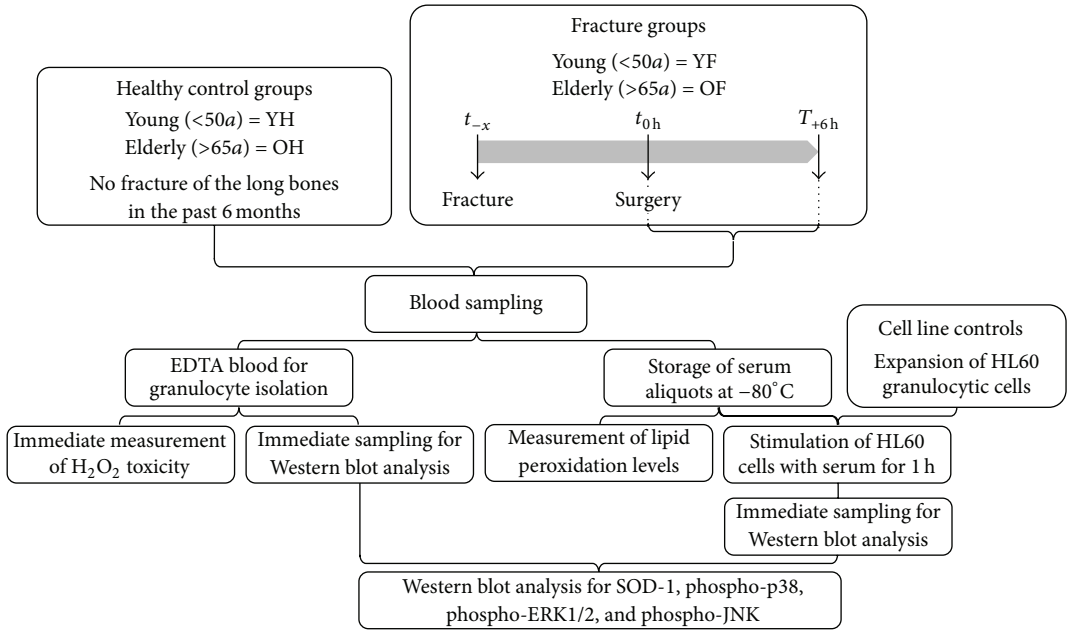

Figure 1: Overview on the experimental setup.

assay. Briefly, 15 μL serum was incubated with 45 μL thiobarbituric acid solution (0.33% thiobarbituric acid in 11.1% acetic acid) for 1 h at 100°C. The samples were diluted with distilled water and their fluorescence was measured immediately at an excitation/emission wavelength of 544/590 nm. Known concentrations of 1,1,3,3-tetraethoxypropane were used as a standard control.

2.6. Viability Measurement. The cells' viability was determined by resazurin conversion. Briefly, cells ($\sim 7 * 10^5$ cells/well) were stimulated with various concentrations of H_2O_2 (0, 0.002, 0.004, 0.008, 0.016, 0.03, 0.06, 0.125, 0.25, 0.5, and 1%). 1/10 volume of a 0.025% (w/v) resazurin solution (in phosphate buffered saline) was added to the cells. After 1 h incubation at 37°C fluorescence was measured (ex/em = 544/590 nm) and corrected to background control (no cells). Viability is given as % of untreated control.

2.7. Western Blot Analysis. Cells were lysed in freshly prepared ice-cold RIPA buffer (50 mM tris(hydroxymethyl)-aminomethan, 250 mM NaCl, 2% Nonidet-P40, 2.5 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, protease and phosphatase inhibitors, pH = 7.2). Protein concentration was determined by micro-Lowry [19]. 30 to 50 μg total protein was separated by SDS-PAGE and transferred to nitrocellulose membranes (Roth, Karlsruhe, Germany). Unspecific binding sites were blocked with 5% bovine serum albumin in TBST solution (25 mM tris(hydroxymethyl)-aminomethan, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, pH = 7.4) for 1 h at RT. After overnight incubation with primary antibodies (Cell Signaling, Beverly, USA) diluted 1 : 1,000 in TBST at 4°C, membranes were incubated with the corresponding horseradish peroxidase-labeled secondary antibodies (1 : 10,000 in TBST) for 2 h at RT. Chemiluminescent signals were detected with X-ray films or a charge-coupled device camera (CCD camera). In order to compare specific signals on different blots, a uniform positive control (HEK293T cells) was included for each membrane. Signal intensities were determined by densitometry using the ImageJ software (NIH, Bethesda, USA) and normalized to the corresponding GAPDH signals.

2.8. Statistics. A flow diagram on the experimental setup is given in Figure 1. Results are expressed as mean ± SEM. The amount of donors (N) and replicates (n) is given separately for each experiment. Datasets were compared by one-way analysis of variance or by the Student's t-test (GraphPad Prism Software, LaJolla, CA, USA). $P < 0.05$ was taken as the minimum level of significance.

3. Results

3.1. The Elderly Show Increased Lipid Peroxidation Levels Representing Elevated Oxidative Stress Levels. In order to determine the levels of oxidative stress in the donors, the lipid peroxidation in the serum ($N = 10$/group, $n = 2$) was measured. The levels of basal lipid peroxidation in the older controls were approximately twice as high as those of young controls ($P < 0.001$). Interestingly, after the occurrence of a fracture a significant increase of about 25% of the lipid peroxidation was detected only in the older patients. Young patients with a fracture of the long bone showed no statistic increase in lipid peroxidation (Figure 2(a)).

3.2. Viability Measurement Indicates That Aged Granulocytes Are More Sensitive Towards H_2O_2 Induced Toxicity as Compared to Young Granulocytes. In order to investigate whether the increased oxidative stress affects the viability of

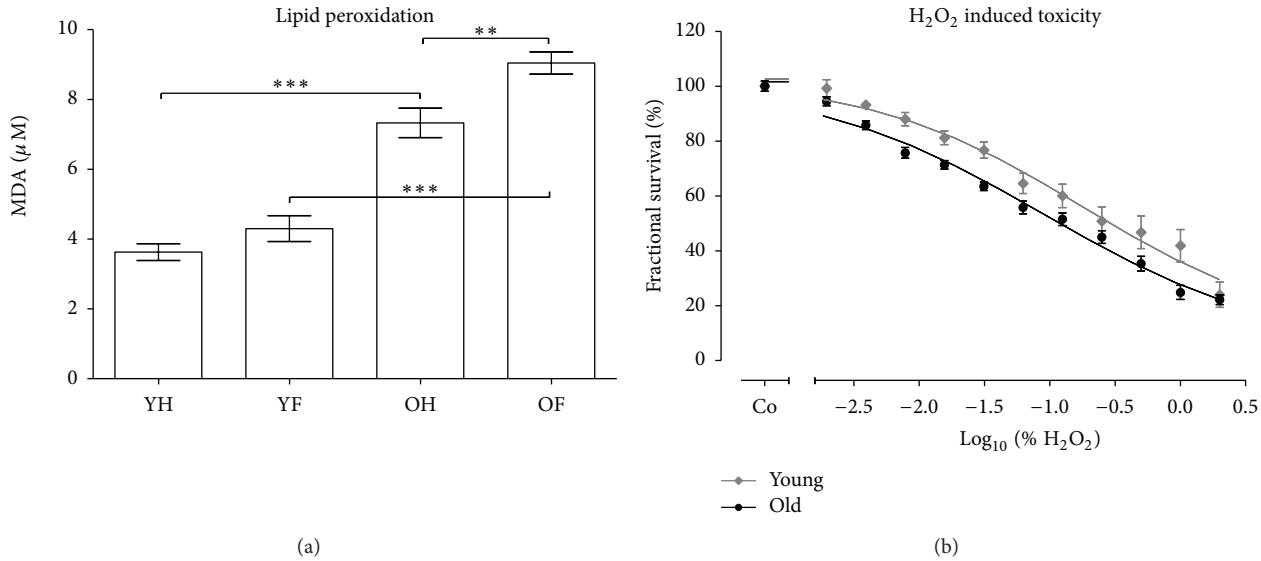

FIGURE 2: Aged granulocytes are more sensitive towards H_2O_2 induced toxicity as young granulocytes. (a) Levels of oxidative stress were determined by measuring lipid peroxidation in sera of young controls (YH), young patients with a fracture of the long bones (YF), elderly controls (OH), and elderly patients with a fracture of the long bones (OF). $N = 10$ and $n = 2$ per group. $^{**}P > 0.01$ and $^{***}P < 0.001$ as determined by one-way ANOVA. (b) Granulocytes from young controls ($N = 5$, $n = 4$) and elderly controls ($N = 5$, $n = 4$) were stimulated with various concentrations of H_2O_2 (0, 0.002, 0.004, 0.008, 0.016, 0.03, 0.06, 0.125, 0.25, 0.5, and 1%). After 1 h viability was assessed by resazurin conversion. $EC_{50/young} = 0.18\%$ and $EC_{50/old} = 0.09\%$ ($P < 0.05$ as determined by Student's t-test).

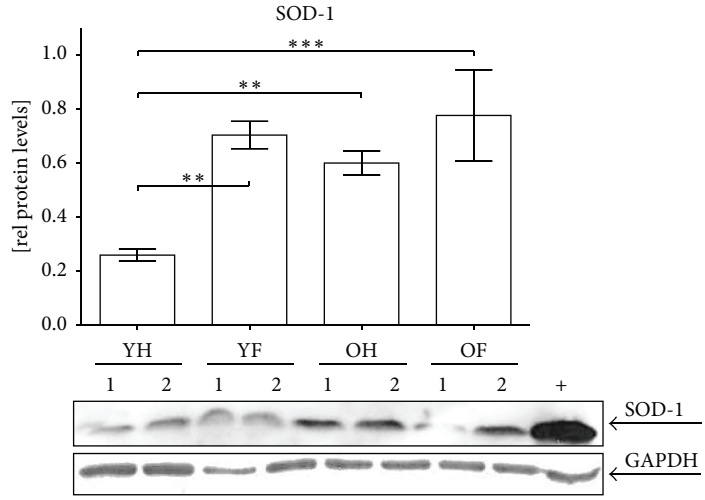

FIGURE 3: Altered SOD-1 expression in aged granulocytes. Representative Western blot for SOD-1. GAPDH was used as a loading control and for normalization. For intermembrane comparison HEK293T cells (+) were used. Densitometric analysis of all donors (YH: $N = 22$/YF: $N = 12$/OH: $N = 23$/OF: $N = 19$) was performed to determine the relative expression levels of SOD-1. $^{**}P > 0.01$ and $^{***}P < 0.001$ as determined by one-way ANOVA.

granulocytes, we stimulated granulocytes from young and elderly controls with various concentrations of H_2O_2 (0, 0.002, 0.004, 0.008, 0.016, 0.03, 0.06, 0.125, 0.25, 0.5, and 1%). After 1 h viability was assessed by resazurin conversion. Granulocytes from elderly controls ($N = 5, n = 4$) were more sensitive towards H_2O_2 induced toxicity than granulocytes from young controls ($N = 5$, $n = 4$), represented by an EC_{50} of 0.09% and 0.18%, respectively ($P < 0.05$) (Figure 2(b)).

3.3. Altered SOD-1 Expression in Aged Granulocytes Is Detected by Western Blot Analysis. In order to investigate why oxidative stress strongly affects aged granulocytes, we investigated the expression levels of SOD-1 by Western blot (Figure 3). Densitometric analysis revealed that the basal expression levels of SOD-1 were significantly elevated (2.2-fold) in the older controls in comparison to the young controls. After trauma and following surgery, the SOD-1 levels significantly

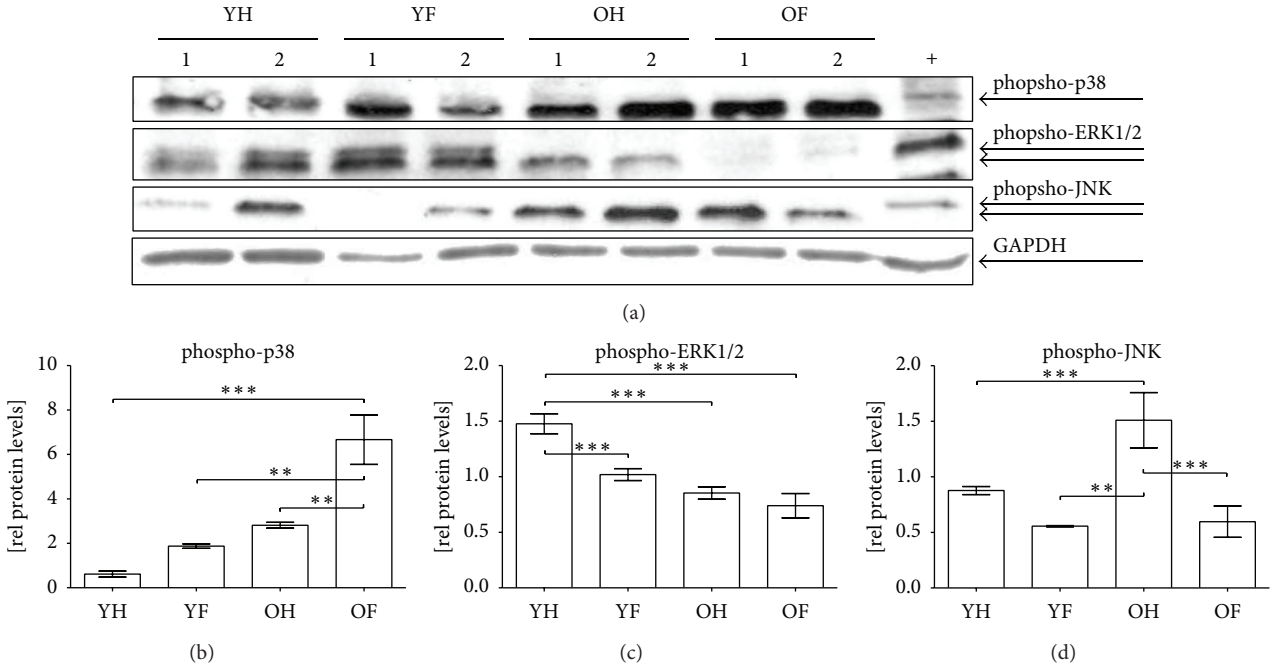

Figure 4: Altered activation of MAPKs in granulocytes of elderly patients and controls. (a) Representative Western blot for phospho-p38, phospho-ERK1/2, and phospho-JNK in granulocytes of young controls (YH), young patients with a fracture of the long bones (YF), elderly controls (OH), and elderly patients with a fracture of the long bones (OF). GAPDH was used as a loading control and for normalization. For intermembrane comparison HEK293T cells (+) were used. Densitometric analysis of all donors (YH: $N = 22$/YF: $N = 12$/OH: $N = 23$/OF: $N = 19$) was performed to determine the relative expression levels of (b) phospho-p38, (c) phospho-ERK1/2, and (d) phospho-JNK. $^{**}P > 0.01$ and $^{***}P < 0.001$ as determined by one-way ANOVA.

increased in the young patients (2.7-fold). Interestingly, in older patients, the basally elevated expression levels of the SOD-1 did not further increase after trauma and following surgery.

3.4. Altered MAPKs Activation in Granulocytes of the Elderly Is Detected by Western Blot Analysis. In order to investigate whether the increased oxidative stress may affect the stress response of granulocytes, we investigated activation (phosphorylation) of the MAPKs p38, ERK1/2, and JNK by Western blot (Figure 4(a)). Basal activation of p38 was increased 4.5-fold in elderly controls as compared to young controls. After the occurrence of a fracture, a significant increase (2.5-fold) in the p38 activation was observed only in elderly patients. In young patients, however, the increase in p38 activity was not significant and did not reach the basal levels of older controls (Figure 4(b)). On the contrary, basal activation of ERK1/2 signaling was significantly decreased (about 40%) in elderly controls. After a fracture, ERK1/2 activation was further decreased only in young patients by about 30% (Figure 4(c)). Similar to p38 activation, basal JNK activation was significantly increased (1.9-fold) in elderly controls in comparison to young controls. After a fracture of the long bones, phospho-JNK levels decreased in both groups with the decrease being more pronounced in the group of the elderly (Figure 4(d)).

3.5. Western Blot Analysis Shows an Inverse p38 Activation in Serum Stimulated HL60 Cells in Comparison to Granulocytes. In order to analyze whether the activation of MAPKs that has been observed in granulocytes is age-dependent or caused by the observed changes in the cellular microenvironment (serum), the activation of the three MAPKs in matured HL60 cells (considered to have the same age) cultured in the presence of the patients' sera was investigated (Figure 5(a)). Contrary to the granulocytes, basal activation of p38 was decreased (about 30%) in the HL60 cells stimulated with sera of the elderly controls in comparison to the HL60 cells stimulated with sera of the young controls. HL60 cells stimulated with patients' sera showed a significant decrease (2.5-fold) in p38 activation only in the young group (Figure 5(b)). Similar to the granulocytes, basal activation of ERK1/2 signaling was significantly decreased, by about 60%, in HL60 cells stimulated with sera of the elderly controls. In both age groups activation of ERK1/2 was reduced in HL60 cells stimulated with patients' sera as compared to stimulation with control sera (Figure 5(c)). On the contrary to granulocytes, basal JNK activation was significantly decreased, by 50%, in HL60 cells stimulated with sera of the elderly controls as compared to stimulation with control sera. The phospho-JNK levels further decreased in HL60 cells stimulated with patients' sera, with the decrease being more pronounced in the young group (Figure 5(d)).

4. Discussion

Hip fracture is a typical fracture of the elderly and its risk directly correlates with bone mineral density and bone

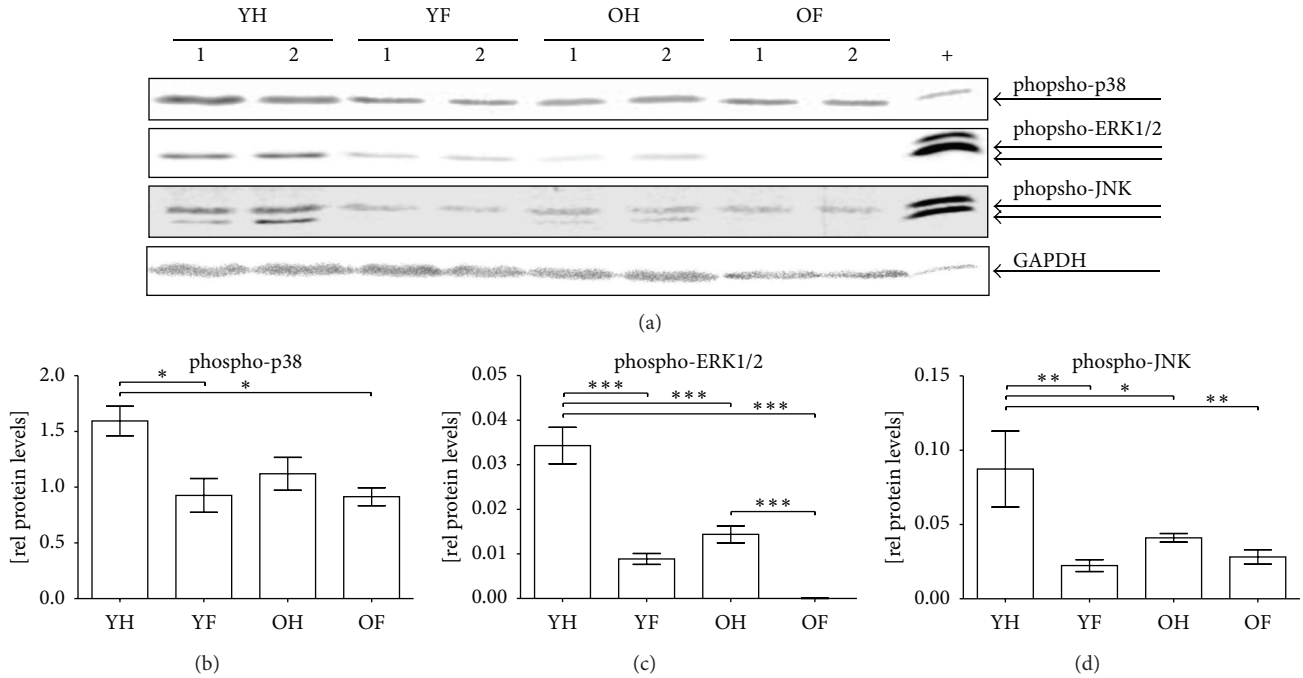

Figure 5: Inverted p38 activation in serum-conditioned HL60 cells in comparison to granulocytes. (a) Representative Western blot for phospho-p38, phospho-ERK1/2, and phospho-JNK in HL60 cells conditioned with serum of young controls (YH), young patients with a fracture of the long bones (YF), elderly controls (OH), and elderly patients with a fracture of the long bones (OF). GAPDH was used as a loading control and for normalization. For intermembrane comparison HEK293T cells (+) were used. Densitometric analysis of all samples ($N = 10$, $n = 2$/group) was performed to determine relative expression levels of (b) phospho-p38, (c) phospho-ERK1/2, and (d) phospho-JNK. $^{*}P > 0.05$, $^{**}P > 0.01$ and $^{***}P < 0.001$ as determined by one-way ANOVA.

strength [20, 21]. Thus, facing the current demographic changes with an increasing number of older people, the treatment of hip fractures represents an important problem for the public health systems [3, 4]. Despite all surgical advances over the past years, hip fractures are still accompanied by high complication rates, and therefore by an increased disability, morbidity, and mortality. While one-third of the patients require a higher level of long-term care, the in-hospital mortality accounts to almost 10% and the one-year mortality accounts for around 27% [22]. This high complication rate may be due to alterations in the immune system in the elderly, which render these patients prone to infections. Granulocytes are among the first lines of defense against bacterial infections. They are circulating immune cells and therefore they are directly exposed to the stress stimuli generated by the fracture and by the operative fracture fixation. Therefore, the aim of this project consisted in investigating the stress response of granulocytes obtained from young (<50 years of age) and elderly (>65 years of age) patients with a fracture of one of the long bones. Healthy individuals of both age groups served as control groups.

In the serum of older controls an increase in oxidative stress, represented by increased lipid peroxidation levels, was observed. ROS, particularly superoxide and its derivatives, induce an accumulation of oxidative damage to macromolecules in the cell, including proteins, lipids, and DNA, which in turn causes aging and eventually cell death [23, 24]. After the occurrence of a fracture and the following surgical reposition of the bone, the lipid peroxidation levels increased in the elderly group. Interestingly, in young patients, the levels of oxidative stress did not significantly increase after the occurrence of a fracture. This observation suggests that the young patients are able to actively reduce excessive amounts of ROS and reactive oxygen and nitrogen intermediated (ROI and RNI), for example, by expressing antioxidative enzymes. This is supported by our finding that aged granulocytes were more sensitive towards H_2O_2 induced cellular damage than granulocytes from young controls. SOD-1 is a free radical scavenger which is believed to act as the first line of defense against oxidative damage by catalyzing the conversion of superoxide radicals to hydrogen peroxide, which can then be reduced to water [25, 26]. In granulocytes of elderly controls, we observed increased basal SOD-1 levels. The increased basal SOD-1 levels are probably at maximum and cannot be further elevated after fractures. After the occurrence of a fracture, SOD-1 levels significantly increased only in the young patients. These findings confirm the oxidative stress theory of aging by Harman, in which he predicts that the imbalance between the formation of oxidative stress and antioxidant defense mechanisms results in a steady accumulation of oxidative damage in a variety of macromolecules [27].

The increase in oxidative stress in turn affects circulating immune cells that are directly exposed to the stimuli.

Granulocytes migrate to the site of injury within minutes. In particular, the MAPKs JNK, p38, and ERK1/2 are directly affected by oxidative stress stimuli in these cells [7, 9].

Basal p38 signaling was increased in the granulocytes of the older controls. After the occurrence of a fracture, a considerable induction of p38 signaling was observed only in the older patients. After its activation, p38 initiates a signaling cascade that regulates the synthesis of a variety of proinflammatory mediators, like tumor necrosis factor α (TNF-α) [28], which was increased in the serum of older patients and controls. The basal JNK signaling was elevated in the elderly controls in a similar fashion to that of the p38 signaling. However, after the occurrence of a fracture, the activation of JNK dropped in young and older patients. Acute and transient activation of JNK is reported to induce cell proliferation and survival, while a prolonged and sustained activation of JNK may promote cell apoptosis [29] which is triggered by TNF-α and ROS [30] that are both elevated in the elderly patients and controls. Contrary to the p38 and JNK signaling, the basal ERK1/2 signaling was significantly lower in the elderly than in the young controls. After the occurrence of a fracture, ERK1/2 signaling was significantly lower in the young patients than in the young controls. However, in the elderly, this difference between the patients and the controls in the ERK1/2 signaling was much smaller. These findings are supported by the work of Schieven, in which it is shown that active p38 signaling may inhibit ERK1/2 signaling [28]. This is especially important as Larbi and coworkers were able to prove that the functional ERK1/2 signaling was able to inhibit granulocyte apoptosis which had been induced by Granulocyte-macrophage colony-stimulating factor (GM-CSF) [31]. Thus, granulocytes of older people have a higher susceptibility to apoptosis in the presence of elevated GM-CSF levels due to their impaired ERK1/2 signaling. Due to their increased rate of apoptosis in their granulocytes, elderly patients are more sensitive to infections following a trauma than young patients.

In order to investigate whether the observed alterations in the MAPK signaling are age-dependent or due to the observed changes in the microenvironment (cytokines, chemokines, and oxidative stress stimuli), we stimulated HL60 granulocytes (considered to have the same age) with patients' sera and investigated the activation of the MAPKs. ERK1/2 and JNK signaling were decreased in the elderly patients and controls and further decreased after a fracture analogously to the number of controls. However, contrary to the number of granulocytes, basal p38 signaling was decreased in the elderly controls and even further decreased after fracture. This observation suggests that p38 signaling is particularly prone to age-related changes. The finding of elevated p38 signaling in the granulocytes of the elderly patients is especially interesting as recent studies have demonstrated that it plays a key role in the persistent pain sensitization via neuronal and glial mechanisms [32–34]. Pain relief, before and after surgery, is one of the first priorities for patients with hip fractures, as adequate pain control may reduce complications, like, for example, cardiovascular events and restoring ambulation [6].

5. Summary

In summary, our data show an increase in oxidative stress levels in the elderly patients and controls. The strong upregulation of SOD-1 in granulocytes after fracture suggests that young patients are able to actively reduce ROS, ROI, and RNI, whereas in elderly patients, SOD-1 expression seems to have reached its limits already in a normal state. This might explain that aged granulocytes react more sensitively towards oxidative stress induced damage than the young granulocytes. The activation of MAPKs in the granulocytes indicates that young and old granulocytes react differently to the oxidative stress stimuli. p38 is especially age dependent. The shift in ERK1/2 and JNK signaling may sensitize the granulocytes of the elderly towards damage caused by oxidative stress stimuli.

6. Conclusion

Our data suggest that a balanced antioxidative response in granulocytes of elderly patients might protect them from additional stress stimuli, for example, fracture and following operation. A flavonoid rich diet is reported to induce the expression of antioxidative enzymes in various cell types [35–38]. However, this induction of the expression of antioxidative enzymes can only be reached, when flavonoids are consumed at high doses over a long period of time. This might even improve bone quality and thus reduce fracture risk, as the rat study with retinoic acid-induced bone loss from Orsolic and coworkers suggests [38]. However, in patients suffering from a fracture the first stress stimuli already occurred. Therefore, the clinic treatment can only be preventive against the second stress stimuli from the following operation, for example, by using drugs with strong antioxidative properties that can be applied in defined doses [35]. There have been first positive reports applying this strategy to prevent organ damage after abdominal surgery [39, 40]. However, if this treatment may also protect the immune cells has to be further evaluated in a controlled clinical trial.

Abbreviations

ERK1/2: Extracellular signal-regulated kinases 1 and 2
GM-CSF: Granulocyte-macrophage colony-stimulating factor
JNK: c-Jun N-terminal kinase
MACS: Magnetic cell separation
MAPK: Mitogen-activated protein kinase
RNI: Reactive nitrogen intermediates
ROI: Reactive oxygen intermediates
ROS: Reactive oxygen species
SOD-1: Superoxide dismutase 1
TNF-α: Tumor necrosis factor α.

Conflict of Interests

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

Authors' Contribution

Zhiyong Wang, Sabrina Ehnert, Florian Gebhard, Helen Vester, and Andreas K. Nussler contributed equally to this paper

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Research Article

Ovarian Aging-Like Phenotype in the Hyperandrogenism-Induced Murine Model of Polycystic Ovary

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There are prominently similar symptoms, effectors, and commonalities in the majority of characteristics between ovarian aging and polycystic ovarian syndrome (PCOS). Despite the approved role of oxidative stress in the pathogenesis of PCOS and aging, to our knowledge, the link between the PCO(S) and aging has not been investigated yet. In this study we investigated the possible exhibition of ovarian aging phenotype in murine model of PCO induced by daily oral administration of letrozole (1 mg/kg body weight) for 21 consecutive days in the female Wistar rats. Hyperandrogenization showed irregular cycles and histopathological characteristics of PCO which was associated with a significant increase in lipid peroxidation (LPO) and reactive oxygen species (ROS) and decrease in total antioxidant capacity (TAC) in serum and ovary. Moreover, serum testosterone, insulin and tumor necrosis factor-alpha (TNF-α) levels, and ovarian matrix metalloproteinase-2 (MMP-2) were increased in PCO rats compared with healthy controls, while estradiol and progesterone diminished. Almost all of these findings are interestingly found to be common with the characteristics identified with (ovarian) aging showing that hyperandrogenism-induced PCO in rat is associated with ovarian aging-like phenotypes. To our knowledge, this is the first report that provides evidence regarding the phenomenon of aging in PCO.

1. Introduction

Nowadays, couples considerably postpone childbearing to their late reproductive-age period due to increasing socioeconomic demands. Besides, industrialization of communities has added a wide variety of environmental risk factors that may lead to disorders, diseases, and cancers in various organs.

It has been shown that exposure to some environmental factors and chemicals during life may accelerate progression towards the end of functional reproductive period [1]. Accordingly, several environmental factors have been confirmed to contribute to reproductive aging [2]. Female reproductive system is unique in the fact that it displays physiologically much faster rate of aging and when it reaches a senescent state, other organs in the body remain generally healthy.

Ovary is one of the most important organs in the female reproductive system as well as an endocrine organ that undergoes aging by a continuous decreasing in the number of follicles, diminished quality of oocytes, menstrual irregularities, ovarian hormonal deficiency, anovulation, and subfertility leading to menopause which is the final step in this process [3].

Several mechanisms seem involved in aging which oxidative stress (OS) is considered as one of the most important ones [4]. OS occurs due to excessive formation of oxygen-derived and/or nitrogen-derived toxic products in the presence of minimal antioxidant activity [5].

At moderate concentrations, reactive oxygen species (ROS) regulate physiologic functions in female reproduction such as folliculogenesis, oocyte maturation, steroidogenesis, corpus luteal function, and luteolysis [5]. However, increased OS during ovarian aging may contribute to follicular atresia and diminishing quantity and quality of oocytes [6]. Substantial theories explain that a long lasting status of overproduced toxic free radicals, insufficient antioxidants, and exposure to endocrine disrupting chemicals may be involved in reproductive disorders which eventually confer acceleration of gonads senescence and premature reproductive aging.

Polycystic ovarian syndrome (PCOS) as an inflammatory condition is the leading cause of anovulatory infertility in reproductive-aged women. It is characterized by increasing androgen secretion, menstrual irregularity, oligo-ovulation/anovulation, polycystic ovaries (PCO), infertility, and pregnancy complications [7]. An ovarian cyst is developed as a result of hormonal imbalance and has an atretic fluid-filled follicular structure with thin granulosa cell walls [8]. Not only PCOS patients display reproductive features, but also they develop several metabolic risks of type 2 diabetes and cardiovascular diseases including abdominal obesity, insulin resistance (IR), hyperinsulinemia, glucose intolerance, hypertension, and metabolic syndrome [9]. There are several different diagnostic criteria for PCOS. Hence, regardless of ethnicity, PCOS has different prevalence in women at their functional reproductive period. According to Rotterdam criteria, it is estimated that the average prevalence of PCOS is approximately 18% that is too high [10].

Because of its complex pathogenesis and unrecognized etiology, no preventive measure has been implemented for PCOS to date. The roles of OS and chronic low-grade inflammation in the pathogenesis of PCOS and the potential benefits of antioxidants have been the subjects of recent studies [11, 12]. High levels of androgen have been proposed as an initial step in the majority of PCOS cases. Hyperandrogenism impairs maturation of developing follicles in ovaries and consequently leads to developing cystogenesis [13]. Extensive animal experimentations have indicated that prepubertal or pubertal exposure of low doses of androgens via aromatization to estrogen results in long-term reproductive consequences including constant estrous cycles, hyposteroidogenesis, anovulation, and development of cystic follicles at adulthood [14]. Moreover, exposure to exogenous estrogen in adulthood has been determined by having deleterious effects on the ovarian physiology and endocrinology which may ultimately lead to cystogenesis, loss of follicle pool, and early senescence [15]. There are some evidences implying that there might be an association between aging and PCO. For instance, it has been recently shown that accelerated aging in mouse induced by D-galactose was concurrent with ovarian cystogenesis [16]. However, the causal link between hyperandrogenism and accelerated ovarian aging is not definitely recognized. It is therefore essential to perceive what hormonal, cellular, molecular, metabolic, environmental, and/or genetic factors contribute to this condition and how they affect ovarian follicle development. We have recently documented the potential role of oxidative/nitrosative stress and inflammatory responses in the pathogenesis of hyperandrogenism-induced PCO in rat [17, 18]. However, negligible information exists on the hyperandrogenization effects on ovarian aging and the molecular basis of disturbed folliculogenesis and cystogenesis.

Overall, it should be of considerable interest to investigate possible development of ovarian aging in hyperandrogenic PCO rats. Therefore, the main objective of this study was to investigate the association of ovarian aging with hyperandrogenism-induced PCO through cellular, molecular, histopathological, biochemical, and endocrinal evaluations.

2. Materials and Methods

2.1. Reagents. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (USA). Krebs-ringer-bicarbonate (KRB) and ethyl acetate, from Fluka (USA), rat TNF-α ELISA kit from Bender Med System (Austria), rat MMP-2 ELISA kitfrom Cusabio Biotech Co., Ltd, (China), steroid hormone radioimmunoassay kits from Neogen (USA), and letrozole from SOHA Pharmaceutical Co. (Iran) were used in this study.

2.2. Rats. Adult female albino *Wistar* rats (180–200 g) with normal estrous cycle were used in the study. Animals were randomly divided into two groups containing 10 rats each. All rats had *ad libitum* access to pelleted food and tap water and housed under controlled temperature (22–25°C) with a relative humidity of 40–55% and 12 h lights and dark cycle. Only females with at least three consecutive 4-5 d regular estrous cycles were used in the experiment. Throughout the entire treatment, animals were weighed and a vaginal smear was done daily (to determine the stage of the reproductive cycle) up to the day of autopsy. All rats received care in accordance with the national health guidelines and the study protocol was approved by Tehran University of Medical Sciences (TUMS) review board.

2.3. Sexual Cycle. To study whether treatments altered the estrous cycle, smears were obtained daily by vaginal washing and evaluated microscopically during the treatment period. As determined in previous studies [17, 18] the observation of cornified cells in the smears during a minimum of 10 consecutive days was defined as persistent estrous, indicating anovulation and development of follicular cysts. At the beginning of the experiments, all rats had regular cycles.

2.4. Treatments. The Control group of ten rats received only vehicle (0.9% NaCl solution) orally, once daily. The treatment group of ten rats was gavaged with letrozole once daily at a concentration of 1 mg/kg orally dissolved in 0.9% NaCl. Effective dose of letrozole (SOHA Pharmaceutical Co. Tehran, Iran) were selected upon previous experiments. The treatment period was 21 days [17, 18].

2.5. Sampling. After the last treatment, all rats weighed and anesthetized with ether and blood samples were directly taken from the heart. Blood samples were centrifuged at 1000 ×g for 15 min and collected sera stored at −70°C until assayed for sex steroids (estradiol, progesterone, and testosterone), as well as insulin, TNF-α, and OS markers. All rats were killed after anesthesia and freshly dissected ovaries were weighed and divided as follows: five of each group was immediately fixed in 4% (w/v) paraformaldehyde for histopathology, whereas the remaining tissues were immediately frozen at −70°C until used for determination of matrix metalloproteinase 2 (MMP-2) and oxidant-antioxidant markers.

2.6. Histopathological Studies. Five ovaries from each group were processed step by step through formalin fixation, paraffin embedding, and longitudinally and serially sectioned at 4 μm with a rotary microtome placed on a glass slide, stained with H&E (Hematoxylin and Eosin), and assessed microscopically by two persons blinded to the origin of the sections. Only the follicles containing an oocyte nucleus were counted in every ovarian section. Follicles were characteristically divided to primordial follicles (an oocyte surrounded by a single layer of granulosa cells and a diameter <100 μm), growing follicles (an oocyte surrounded by several layers of granulosa cells, without an antrum, and diameter of 100 to 300 μm) or graafian follicles (a peripheral oocyte surrounded by cumulus cells and several layers of granulosa cells, an antrum, and diameter >300 μm). Atretic follicles were characterized by scattered pyknotic nuclei in the granulosa cell layer, detachment of the granulosa cell layers, loss of oocyte-granulosa cell communication, fragmentation and malformation of the oocyte, disruption of the zona pellucida (ZP), and presence of cellular debris in the antrum of the follicle. Histopathological changes were also categorized from (−) to (+++) according to their severity, where (−) was not a prominent or obvious pathological finding, and scores of (+), (++), and (+++) represented pathological findings of <33%, <33–66%, and >66% of the atretic follicles, respectively. The average value of histopathological findings was taken into consideration and when there was disagreement between the two observers as to the interpretation of some histologic characteristics, the case was reexamined and conclusion was made by consensus.

2.7. Evaluation of Oxidative Stress Biomarkers. To assess the effect of hyperandrogenization on ovarian oxidant-antioxidant balance, the LPO index, ROS, and level of TAC were analyzed.

2.7.1. Determination of Cellular Lipid Peroxidation (LPO). The cellular LPO was assessed in blood and ovaries using thiobarbituric acid reactive substances (TBARS) assay as described in our previous work [19]. The LPO levels were expressed as the extent of malondialdehyde (MDA) production during an acid-heating reaction. Data were reported as μg/mg of protein.

2.7.2. Determination of Reactive Oxygen Species (ROS). The isolated ovaries were placed in a mitochondrial isolation buffer containing 0.25 M sucrose, 20 mM KCl, 1.0 mM EDTA, and 5.0 mM HEPES (pH 7.4) at a weight: volume ratio of 1 : 10. Tissues were minced thoroughly and homogenized with a manual glass homogenizer at 0–4°C. A portion of the homogenate and blood serum were used to determine ROS production which was measured by use of fluorescence DCFH with some modifications as set up in our lab. The assay buffer contained 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris-HCl, 0.1 mM $FeCl_3$, 1.7 mM ADP, and 0.1 mM NADPH and 30 mM glucose (pH 7.4) with a total volume of 200 μL. Assay buffer contained 5 μM DCFH-diacetate (DCFH-DA) dissolved in 1.25 mM methanol with 5 μM final methanol concentration. For homogenates, 50 μL (~1 mg protein) was included in either assay medium and incubated at 37°C for 15 min. This permitted DCFH-DA to be broken by intracellular esterase to derive free DCFH. The rate of oxidation from DCFH to dichlorofluorescein (DCF) indicative of oxidant production was followed at the excitation wavelength of 488 nm and emission wavelength of 525 nm and measured every 6 min for 60 min using an ELISA F-2000 fluorescence spectrometer. The rate was linear for at least 60 min at various concentrations of protein present, corrected for the autooxidation rate of DCFH [20].

2.7.3. Total Ferric Reducing Antioxidant Power Assay. Total antioxidant power of the ovaries and sera was evaluated by measuring the ability to reduce Fe^{3+} to Fe^{2+}. To do that we used TPTZ which its interaction with Fe^{2+} results in the formation of a blue color, with a maximum absorbance at 593 nm. Data were expressed as mmol/L ferric ions reduced to ferrous per mg of protein, as described in our previous work [21].

2.8. Evaluation of Serum Insulin as a Metabolic Biomarker. After 8 h fasting, serum insulin concentration was analyzed by enzyme-linked immunoassay (ELISA) technique as described previously [20].

2.9. Evaluation of Inflammatory Biomarkers. Concentrations of TNF-α were assessed using a rat sandwich ELISA kit and expressed as pg/mg protein. According to the procedure, a color product is formed in proportion to the amount of cytokine present in the sample. After adding stop solution to terminate the reaction, absorbance was measured at 450 nm as the primary wavelength and 620 nm as the reference wavelength [22]. To determine the concentrations of TNF-α per unit of protein, the Bradford method was used to measure protein content using concentrated Comassie blue as reagent and BSA as the standard.

2.10. Evaluation of Matrix Metalloproteinase 2/Gelatinase A (MMP-2). Ovarian samples, which had been preserved at −80°C, were warmed to −20°C and weighed. A phosphate buffer (pH 7.4), which was prepared in weight-appropriate quantities, was diluted 10 times. The tissue samples were

homogenized, and then centrifuged in a refrigerated centrifuge at 2000 ×g for 15 minutes, and their supernatants were transferred into microtubes. The MMP-2 was analyzed using commercial quantitative immunoassay kit; (Cusabio Biotech Co., Newark, NJ, USA). Assay was conducted according to manufacturer's guidelines.

2.11. Evaluation of Sex Steroids. Ovarian steroidogenesis function after induction of ovarian cystogenesis was determined by competitive radioimmunoassay, using commercial RIA kits (Neogen, USA) as described in our previous studies [18, 19].

2.12. Statistical Analysis. Analysis of variance was used when several parameters of the two groups were compared. Differences between control and PCO group were calculated by Student's *t*-test. All analyses were conducted using StatsDirect 3.0.97. A $P < 0.05$ was considered as statistically significant. Results are presented as means ± SD.

3. Results

3.1. Body and Ovaries Weight (g). The results showed that the hyperandrogenized rats gained significantly ($P < 0.001$) more body and ovarian weights in comparison to the controls (Table 1).

3.2. Sexual Cycle. With respect to the sexual cycle, all control rats showed regular estrous cycles with an expected time of 4-5 days between two consecutive cycles. However, all hyperandrogenized rats were completely acyclic and exhibited constant estrous.

3.3. Morphological and Histopathological Findings. Histological characteristics of the ovaries are shown in Tables 1 and 2. All ovaries of control females had corpora lutea (CL) confirming normal ovulation and contained follicles in various stages of development, including primary and secondary follicles, graafian follicles, and recently formed CL (Figures 1(a) and 1(b)). In the hyperandrogenized females, a clear absence of both large secondary and tertiary follicles as well as CL was evident and multiple follicular cysts were visible as fluid-filled sacs on the ovarian surface forming PCO (Tables 1 and 2; Figure 1(c)). Furthermore, the number of atretic preantral and antral follicles increased and ovarian theca-interstitial tissues are hyperplastic in hyperandrogenized ovary (Table 2 and Figure 1(d)). In addition, granulosa cells in the majority of atretic antral follicles were found luteinized (Figure 1(e)) and many of oocytes were malformed with the signs of zona pellucida (ZP) fragmentation in the atretic follicles (Figures 1(e) and 1(f)).

3.4. OS Biomarkers. In ovarian tissues and serum, LPO and ROS were significantly higher in the PCO group than in the controls (Table 3). Moreover, the ovarian and serum antioxidants activities were decreased significantly ($P < 0.001$) in the PCO rats compared to the controls.

3.5. Inflammatory and Metabolic Biomarkers. The hyperandrogenized PCO rats showed a considerable increase ($P < 0.001$) in serum TNF-α concentrations compared to the controls (68.2 ± 2.57 versus 41.9 ± 0.42, resp.). The serum insulin concentration was notably ($P < 0.001$) increased after hyperandrogenization in PCO rats as compared with the controls (1.1 ± 0.14 versus 0.32 ± 0.047; Table 3).

3.6. MMP-2 Activity. The mean tissue levels of MMP-2 in the ovaries are presented in Table 3. Statistically significant difference ($P < 0.001$) was found between the MMP-2 values of control and hyperandrogenized rats (23.9 ± 6 versus 65.5 ± 15.57, resp.).

3.7. Sex Steroid Concentrations. As seen in Table 4, hyperandrogenization significantly increased serum testosterone concentrations compared with the controls ($P < 0.001$, 80.22 ± 11.02 versus 21.58 ± 2.23, resp.). Meanwhile, serum concentrations of estradiol ($P < 0.001$, 12.42±1.14 versus 60± 3.76) and progesterone ($P < 0.001$, 11.86±1.36 versus 29.34± 3.13) were significantly reduced in the hyperandrogenized rats in comparison with the controls.

4. Discussion

Aging and reproduction biology are two rapidly growing fields of modern biomedical researches. Aging process is of interest not only to scientists but also to public health; hence, it has been the research question of many studies, including those in the field of reproductive biology. However, the association of aging with reproductive disorders has not been well studied and documented. In the present study we investigated the evidences of the ovarian aging characteristics with PCO as one of the most prevalent female reproductive disorder.

In this study we showed that treatment with letrozole-induced hyperandrogenism due to blocking the conversion of androgens to estradiol which leads to higher serum testosterone concentrations (hyperandrogenemia). Hyperandrogenemia was associated with ovarian cystogenesis and significantly increased LPO and total ROS (markers of OS) and decreased TAC (marker of antioxidant potential) in PCO rats. In addition, synthesis or release of inflammatory mediators like TNF-α and concentrations of insulin and MMP-2 activity were significantly increased by hyperandrogenization. These molecular and biochemical alterations were consistent with histological evidence of significant disruption in microscopic characters of folliculogenesis when compared to the control group. Histopathology revealed a large increase in the number of atretic and cystic follicles, lack of CL formation, anovulation, and sexual acyclicity in hyperandrogenized rats which was due to aberrant folliculogenesis. Increased ovarian weight seemed to be a result of increased number of fluid-filled cysts and large atretic follicles within ovary of PCO cases. Almost all of these findings were interestingly found to be common with the characteristics identified with (ovarian) aging as discussed. Interestingly, we observed that there was a close association between identified symptoms and effectors regarding ovarian aging in hyperandrogenized PCO rats so

Table 1: Comparative assessment of weight and histopathological changes of ovary in experimental and control groups.

	Control	PCO
Body weight (g)	231.2 ± 1.11	249.8 ± 0.81***
Ovary weight (g)	0.044 ± 0.002	0.06 ± 0.002***
Luteinization of follicular wall and granulosa cells	—	+++
Vascularization of follicular wall	—	+++
Degenerated and deformed oocyte	—	+++
Pyknosis and chromatinization of granulosa cells	—	+++
Disintegration and dispersion of granulosa cells	—	+++
Hyperplasia of theca cells	—	+++
Breakdown and fragmentation of ZP	—	++

*represents a significant difference between control and PCO groups.
The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.
Intensity of the histopathological changes: (—) no change; (+) slight change; (++); marked change; (+++) sever change.

Table 2: Comparison of mean number of atretic and cystic primordial, growing, and graafian follicles and corpus luteum in PCO and control groups.

	Control	PCO
Mean number of atretic primordial follicles	35.81 ± 1.34	52.27 ± 1.01***
Mean number of atretic growing follicles	52.45 ± 0.57	81.18 ± 0.91***
Mean number of atretic graafian follicles	8.72 ± 0.33	27.36 ± 0.38***
Mean number of cystic primordial follicles	0	0
Mean number of cystic growing follicles	0.18 ± 0.12	7.72 ± 0.38***
Mean number of cystic graafian follicles	0.82 ± 0.18	10.9 ± 0.56***
Mean number of corpus luteum (CL)	5.27 ± 0.19	0.36 ± 0.15***

*represents a significant difference between control and PCO group.
The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 3: Comparative assessment of OS and inflammation parameters in blood and ovary of PCO and control groups.

	Control	PCO
Blood LPO (μmol/mL)	4.09 ± 0.32	12.72 ± 0.27***
Ovary LPO (μmol/mL)	3.45 ± 0.28	14.21 ± 0.56***
Blood ROS (nmol/min/mg protein)	0.78 ± 0.18	2.76 ± 0.32***
Ovary ROS (nmol/min/mg protein)	0.04 ± 0.00	0.14 ± 0.01***
Blood TAC (nmol/L per mg protein)	209.52 ± 4.66	116.60 ± 4.21***
Ovary TAC (u/mg)	7.68 ± 0.49	2.91 ± 0.16***
Serum TNF-α (pg/mg protein)	41.90 ± 0.42	68.20 ± 2.57***
MMP-2 (ng/mg protein)	23.90 ± 6.00	65.50 ± 15.57***
Insulin (ng/mL)	0.32 ± 0.05	1.10 ± 0.14***

*represents a significant difference between control and PCO group.
The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 4: Sex hormone levels in PCO and control groups.

	Control	PCO
Testosterone (ng/dL)	21.58 ± 2.23	80.22 ± 11.02***
Progesterone (ng/mL)	29.34 ± 3.13	11.86 ± 1.36***
Estradiol (pg/mL)	60.00 ± 3.76	12.42 ± 1.14***

*represents a significant difference between control and PCO group.
The symbols represent statistical significance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

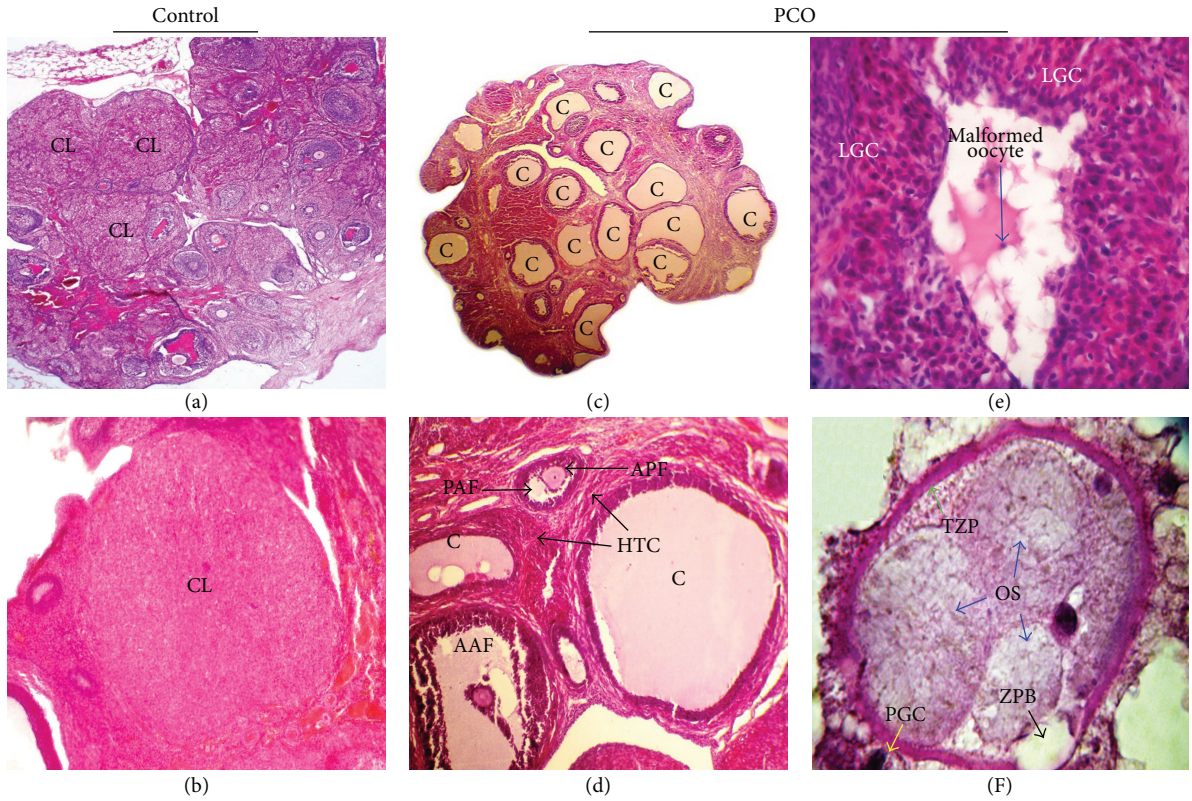

Figure 1: (a) Lower magnification (40x) of ovarian section showing normal folliculogenesis as well as several CL from the control rat. (b) Higher magnification (400x) of control ovary showing fresh CL. (c) Section of ovary from PCO rats showing multiple fluid-filled subcapsular cysts (40x). (d) Another section of ovary from PCO rats with two preantral and antral cystic follicles having hyperplastic theca cells along with thin granulosa cell layers. Two preantral and antral degenerating atretic follicles are also seen in this section (100x). (e) The completely luteinized granulosa cells are clear in an atretic follicle with a malformed oocyte (400x). (f) Higher magnification of a segmented oocyte in an atretic follicle with typical ZP thickness and also ZP breakdown and fragmentation (1000x). AAF: atretic antral follicle; APF: atretic preantral follicle; PAF: precocious antrum formation; C: cystic follicle; CL: corpora lutea; DGC: degenerated granulosa cells; HTC: hyperplasia of theca cells; LGC: luteinized granulosa cells; MO: malformed oocyte; OS: oocyte segmentation; PAF: precocious antrum formation; TZP: thicken zona pellucida; ZPB: zona pellucida breakdown.

that all of these characteristics have been related to (ovarian) aging in several recent studies [3, 4, 6, 7, 16].

4.1. Low Estradiol Levels and High Concentrations of Androgen (Hyperandrogenemia) Leading to Accelerated Follicular Atresia. Letrozole blocks aromatization of testosterone to estradiol leading to a significant reduction in serum estradiol concentration and consequently accumulation of nonaromatizable androgens [17, 18]. Furthermore, due to the developing of CL or decreased number of mature CL as a consequence of anovulation, decreased concentration of serum progesterone was predictable in PCO cases. These hormonal disturbances are involved in constant estrous manifestation. Estradiol is known to play important roles in preventing OS so that lower estradiol concentrations are associated with an increase in follicular OS [23]. A meaningful depletion in estradiol levels causes apoptosis and oxidative DNA damage. As well, elevated OS resulting from reduced estradiol concentrations would predispose granulosa cells to apoptosis in preovulatory follicles [24]. Therefore, it is likely that the decreased levels of estradiol, at least in part, stimulates progressive follicular atresia and predisposes the ovary to be senescent prematurely.

4.2. High Concentrations of Insulin (Hyperinsulinemia) Leading to Hyperandrogenemia and Accelerated Follicular Atresia. PCOS is associated with hyperinsulinemia. Hyperinsulinemia has been determined to stimulate the ovarian cystogenesis causing an increase in the size of the cystic follicles and in the size of the ovary [25]. Insulin excess stimulates androgen production by theca cells and elevates serum free testosterone levels, thereby perpetuating ovarian hyperandrogenism [26]. In the present study, ovarian theca-interstitial cell growth was consistent with high concentrations of testosterone and insulin in comparison with controls. Ovarian mesenchyma (theca-interstitial tissues) are a major source of androgens and normal ovarian function, including folliculogenesis and steroidogenesis, which requires effective mechanisms regulating theca-interstitial growth and function. However, in PCOS, ovarian theca-interstitial tissues are hyperplastic due to increased cellular proliferation and/or reduced apoptosis which is concomitant with androgens oversynthesis [27].

In this regard, androgens might enhance apoptosis of granulosa cells leading to increased rate of follicular atresia [28].

4.3. Increased TNF-α Activity Leading to Sustained Inflammatory Status, Progressive Ovarian Cystogenesis, and Follicular Atresia. In the present study, PCO rats had higher serum TNF-α concentrations than controls. There is a positive relationship between hyperandrogenism and serum TNF-α excessive concentration as hallmarks in patients with PCOS [11, 12]. TNF-α is considered as an initiator of the inflammatory cascade. Furthermore, higher TNF-α production is involved in OS [14, 29] which arrests follicular development [30] and correlates with poor oocyte quality [31]. A similar situation has been determined in female rats, where the levels of TNF-α and LPO were elevated in old ovariectomized animals as an experimental model of aging [32]. Therefore, in hyperandrogenism condition the crosstalk between OS and proinflammatory cytokines, particularly, TNF-α seem to play a pivotal role in the progression of ovarian cystogenesis, follicular atresia and premature ovarian dysfunction as was evident in the present study.

With respect to aging and TNF-α, age-dependent increase in TNF-α was found in senescence-accelerated mice [33]. It has been shown that aging is mechanistically associated with the gene expression of pro-inflammatory cytokines such as TNF-α, leading to inflammation and apoptosis [34]. Moreover, it has been well determined that TNF-α is able to markedly increase cellular senescence through stimulation of prolonged inflammation [35].

4.4. Overproduction of Intraovarian Toxic Free Radicals along with a Depletion of TAC. The role of ROS in the pathogenesis of PCOS has been the subject of numerous studies and emerging evidences that show that both ROS and the activation of inflammatory pathways play a central role in PCOS [5, 11, 12]. In the present study, we showed that hyperandrogenization significantly increased LPO and total ROS (markers of OS) and decreased TAC (marker of antioxidant potential) in PCO rats. Aging has been shown to be mechanistically associated with impaired mitochondrial function which is related to toxic free radicals overproduction and decreased antioxidant activity [36].

4.5. The Alternation in Extracellular Matrix (ECM). One of the main findings reported in this study is that hyperandrogenization increased MMP-2 activation in rats with respect to controls. Matrix metalloproteinases (MMPs) which are a group of matrix-degrading enzymes are involved in the breakdown of all kinds of ECM proteins and so play an important role in various physiological processes as well as pathological states of disease. It has been exhibited that follicular rupture requires focal degradation of the apical ECM that is controlled, in part, by ovarian MMPs. The MMP-2 has been proposed to play an important role in separation of the granulosa cells from the theca cells [37]. Increased level of MMP-2 in our experiment was in agreement with a previous report showing elevated MMP-2 levels in obese women with PCOS [38]. The imbalances in circulating MMPs are associated with excess circulating androgens in PCOS women [39]. In this regard, MMP-2 expression has been shown to be stimulated by androgen via androgen receptor transactivation [40]. There is also a correlation between MMPs and the quality of the developing follicles [41]. Moreover, luteinized granulosa cells (as an atresia characteristic) from women with PCOS have been approved to exhibit greater MMP-2 activity [41]. In terms of aging, the overexpression of several metalloproteinases in senescent cells has been reported [42]. Therefore, it is reasonable to speculate that MMP-2 may be associated with inappropriate follicular atresia in PCOS cases which eventually leads ovary to be senescent faster than the normal condition.

4.6. Ovarian Aging-Like Phenotype in the Hyperandrogenism-Induced PCO. Ovarian aging process is characterized by the disappearance of the preovulatory follicle pool in the ovary and ceases in the female fertility due to lack of CL formation, anovulation, and discontinued activity of sexual cycling [43]. It has been proved that progressive follicular atresia by apoptosis at any stage could accelerate follicular depletion from the ovary and result in premature reproductive senescence [44]. Furthermore, hyperandrogenization as one of the common models of PCO seems to exhibit some aging-specific characteristics. Supporting this subject, postnatal androgenization has been shown to induce accelerated aging of ovarian interstitial glands and transformation of large antral into cystic follicles as common similarities between hyperandrogenized and aging rats [45]. It is believed that menopause associated with various diseases is mediated through depression of estradiol levels [46]. Interestingly, condition of chronic estradiol deficiency and higher androgen levels (as seen in our results) would lead to many age-related changes like obesity, metabolic syndrome, diabetes, skeletal abnormalities, and cardiovascular disturbances [47] where all of these diseases and disorders can be observed in PCOS women [7]. Considering the facts and results of this study, it would not be surprising to consider that the PCO(S) and ovarian aging notably have similar symptoms, effectors, and commonality in majority of their characteristics. Although not investigated in this study, there are several findings which support this idea. For instance, recently in mouse model of accelerated aging, irregular estrous cycles and ovarian cysts, as common signs of ovarian failure, were identified [16]. Another good example is advanced glycation end products (AGEs). Accumulation of AGEs is well known among many causes of aging [48]. AGEs contribute directly to protein damage, toxic stress, and increasing the inflammatory reactions [49]. Recent studies have shown a clear connection between PCOS and increased AGEs in the body so that AGEs and its receptor were highly expressed in granulosa cells of the PCOS ovary [50]. The AGEs seem associated with anovulation and hyperandrogenism [51].

Altogether, according to above discussion, the first concept coming to mind is that during PCOS condition, constant exposure of oocytes and other ovarian cells to OS, and hormonal alternations predispose the ovarian follicles to progressive rate of atresia leading to exhausted ovaries

with reduced number of intact follicles. Therefore, ovarian oocyte reserved will be washed up earlier in hyperandrogenism condition, in accordance with premature onset of acyclicity and constant estrous. Moreover, supraphysiologic long-lasting androgenic microenvironment may eventually affect process of follicular recruitment, growth and ovulation leading to early ovarian senescence [15] via disruption of intraovarian milieu. This is a phenomena resembling premature/accelerated ovarian aging. According to recent studies including our reports and based on current data, we give the idea that hyperandrogenism triggers ovarian senescence in PCOS cases through the following mechanisms: (1) pivotal decrease in estradiol levels and high concentrations of androgen leading to accelerated follicular atresia; (2) increased TNF-α activity leading to OS and sustained inflammatory status; (3) overproduction of intraovarian toxic free radicals along with a depletion of TAC.

Although, a close association with the symptoms and effectors of hyperandrogenemia-induced PCO and (ovarian) aging was observed in our study, a number of important questions may be raised: What is the mechanistically precise association of PCOS with ovarian aging? Is it logical to consider PCOS as one of the contributors of accelerated development of ovarian senescence in risky individuals? And in general, is PCOS a unique and sophisticated type of accelerated ovarian aging phenomenon? This is an idea/hypothesis which will be well scrutinized and discussed appropriately as a research question in our upcoming project.

5. Conclusion

To our knowledge, this is the first report that provides evidence on phenomenon of aging in PCO. Our results showed that PCO(S) which is coupled with premature onset of constant estrous, OS, and proinflammatory condition may lead to premature transition to reproductive senescence via endocrine disruption and develops as one of the features of accelerated aging at the level of the ovary. Further studies are required to clarify precise mechanisms explaining similarities of hyperandrogenism-induced PCO and ovarian aging.

Conflict of Interests

The authors have no conflict of interests to disclose.

Authors' Contribution

The work presented here was carried out in collaboration between all authors. Mohammad Amin Rezvanfar defined the research theme, designed methods and, experiments and drafted the paper. Habib A. Shojaei Saadi participated in the idea and editing the manuscript. Maziar Gooshe and Amir Hosein Abdolghaffari cocarried out experiments. Maryam Baeeri carried out the laboratory experiments and analysis of data. Mohammad Abdollahi gave the idea, conceived the study, and edited the manuscript.

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Review Article

Oxidative Stress in Aging: Advances in Proteomic Approaches

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Aging is a gradual, complex process in which cells, tissues, organs, and the whole organism itself deteriorate in a progressive and irreversible manner that, in the majority of cases, implies pathological conditions that affect the individual's Quality of Life (QOL). Although extensive research efforts in recent years have been made, the anticipation of aging and prophylactic or treatment strategies continue to experience major limitations. In this review, the focus is essentially on the compilation of the advances generated by cellular expression profile analysis through proteomics studies (two-dimensional [2D] electrophoresis and mass spectrometry [MS]), which are currently used as an integral approach to study the aging process. Additionally, the relevance of the oxidative stress factors is discussed. Emphasis is placed on postmitotic tissues, such as neuronal, muscular, and red blood cells, which appear to be those most frequently studied with respect to aging. Additionally, models for the study of aging are discussed in a number of organisms, such as *Caenorhabditis elegans*, senescence-accelerated probe-8 mice (SAMP8), naked mole-rat (*Heterocephalus glaber*), and the beagle canine. Proteomic studies in specific tissues and organisms have revealed the extensive involvement of reactive oxygen species (ROS) and oxidative stress in aging.

1. Introduction

Despite the great research efforts performed since molecular techniques have emerged, it is unquestionable that the step-by-step approach of studying one gene or one protein at a time, even if their partners could eventually be unveiled, is a parsimonious endeavor. Therefore, a more integral and holistic approach is required. Within this context, genomic and proteomic studies, mainly by microarray for messenger RNA (mRNA) and two-dimensional (2D) electrophoresis for protein expression profiles, would eventually elicit the comprehension of the entire process of cell function, and also that at tissue and organ levels, which in turn will provide us with a wider panorama and lead us to a more comprehensive understanding of the aging mechanisms and the intrinsic role of reactive oxygen species (ROS) at a molecular level.

Both approaches yield a large amount of information from every group of cells, tissue, or organ condition, both *in vivo* and *in vitro*, under certain circumstances, and at a particular developmental time. In this review, we present a compilation of advances of the influence of oxidative stress during aging, obtained by means of a proteomic approach in different cellular types, tissue types, or animal models. The second major global approach for expression analysis, by gene expression profile by microarrays, will be reviewed elsewhere.

The existence of free radicals, such as chemical entities, was inferred 100 years ago, but their importance in biological systems was not recognized until the mid-1950s; nonetheless,

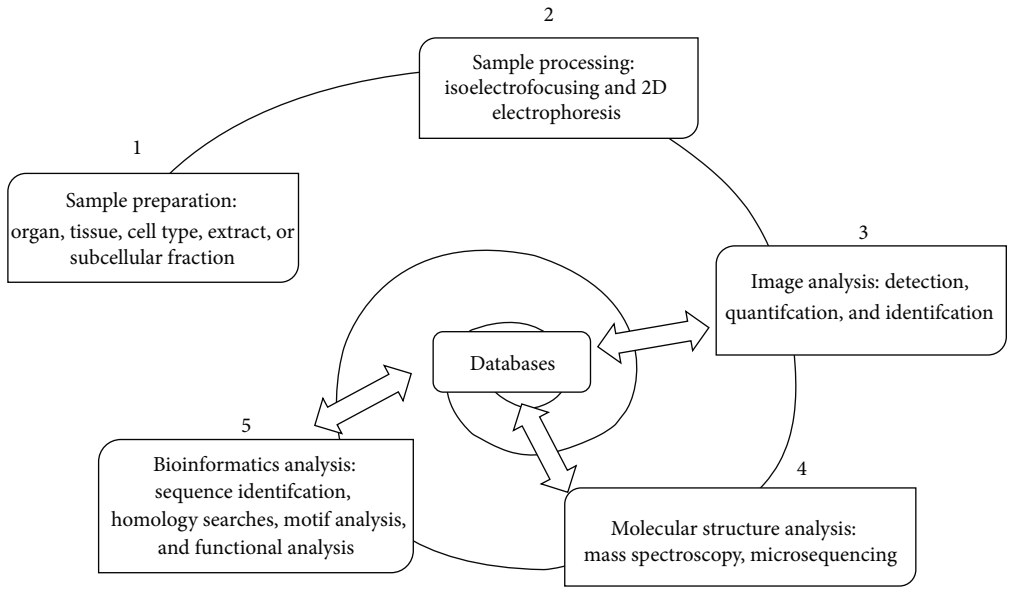

Figure 1: General stages of the proteomic analysis.

for the majority of the remaining 20th century, ROS were considered "*a type of biochemical rusting agent that caused stochastic tissue damage and disease*" [1]. Now, in the 21st century, reactive oxygen biochemistry is quite relevant among the biomedical sciences, and it is currently recognized that nearly every disease involves some degree of oxidative stress. Additionally, it is recognized that ROS are produced in a well-regulated manner to help maintain homeostasis at the cellular level in normal, healthy tissue [1]; but, when ROS concentrations exceed the cell's antioxidant capacity, severe damage can occur and the organism experiences oxidative stress [2]. The damaging effects of these concentrations have been implicated in aging [3, 4], and also in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, and in other chronic and degenerative diseases such as cancer, atherosclerosis, diabetes, and heart disease [5].

As early as 1956, Denham Harman proposed the "Free Radical Theory of Aging" [6]; additionally, other studies from Gilbert, Chance, and Commoner can be considered as the "founders" of reactive oxygen biochemistry. However, by 1980, oxyradicals were accepted biological entities, though their significance for aging and disease remained generally unappreciated [1]. Around 1980, the Stadtman group began to investigate the nature and consequences of protein oxidation *in vitro* and *in vivo*. These authors measured protein carbonyl groups as indices of oxidative damage and applied these techniques to the study of protein oxidation in aging tissue, describing the first, detailed determinations of protein oxidation in models of human aging [7, 8] and giving rise to serious consideration of oxidative stress as a pathological factor.

The application of genomics, proteomics, and even metabolomics to the research on aging, as well as the study of epigenetic influences that are able to induce histone and DNA modifications and influence enzyme activity, would increase our understanding of the origin and development of the different process that contributes to this unavoidable life consequence, senescence. Epigenetic mechanisms are typically associated with the aging process and age-related diseases and may have significant roles to play in the presence of oxidative stress during aging, thereby enabling the establishment of specific diagnostic profiles and therapeutic templates that could aid in improving Quality of Life (QOL) at advanced ages (for a striking revision regarding the relationship between epigenetic factors and aging, see [9]).

2. Global Approaches to the Study of Oxidative Stress in Aging at the Molecular Level

One of the major goals of Gerontology is to understand the complex mechanisms involved in aging at the molecular, cellular, and organ levels that would also make the understanding of age-related diseases possible. Because of practical limitations in studying the aging process in humans *in vivo*, animal models are frequently utilized [10]; however, differences in longevity between the majority of experimental animal models and humans make analysis somewhat difficult. Despite these limitations, research in this area has accelerated with the application of high-throughput technologies such as microarrays and mass spectrometry (MS). Once applied, the information generated must be analyzed accurately; thus, the use of bioinformatics is highly relevant [11]. One of the great advantages of the study of the proteomic is that the huge amount of information generated by a particular set of experiments, as occurs in genomics studies also, can be deposited in large databases, which in turn would accelerate, in the near future, experimental work and improve the results obtained, particularly in the study of aging as a comprehensive biological phenomenon (**Figure 1**). While aging has been considered a stochastic process, widespread

Table 1: Major posttranslational protein oxidative modifications during oxidative stress mediated aging.

Type of modification	Consists of
Irreversible	
Carbonylation	Covalent adduction of lipid aldehydes, often six, nine, or 12 carbons, to the side chains of lysine, histidine, and cysteine residues
3-Nitrotyrosilation	Formed between reactive nitrogen species and a protein's tyrosine residue
Reversible	
S-Sulfenation	Generation of sulfur-hydroxylation product (P-SOH) may be a prelude to sulfination, sulfonation, disulfide bond formation, and sulfenyl-amide bond formation
S-Nitrosylation	Covalent incorporation of a nitric oxide moiety into thiol groups to form S-nitrosothiol (SNO)
S-Glutathionylation	Covalent attachment of glutathione (GSH) to protein thiol groups
Disulfide formation	Disulfide bonds are usually formed from the oxidation of sulfhydryl (–SH) groups
4-Hydroxy-2-nonenal (HNE) modification	Is a major lipid peroxidation product formed during oxidative stress

opinion at present takes into account the existence of a strictly regulated system that fine-tunes the lifespan of an organism through modulation of its responses to oxidative stress [12].

Research on ROS and oxidative stress has become a rapidly growing and evolving subject of study. Specifically in the field of aging studies, a review of the PubMed database (http://www.ncbi.nlm.nih.gov/) under the terms ("oxidative stress" OR "reactive oxygen species") AND aging AND microarray or ("oxidative stress" OR "reactive oxygen species") AND aging AND proteomic (both restricted to title/abstract search fields) shows the increasing interest in these integral molecular approaches during the last decade.

It is relevant to bear in mind the relevance of posttranslational modifications that regulate the activity of proteins inside the cell. Thus, the number of distinct protein functionalities exceeds the number of protein sequences and concentrations; therefore, one must establish the relative concentration, location, and posttranslational modification of each isoform in order to completely characterize protein function.

3. Accumulation of Altered Posttranslational Modifications and Lack of Adequate Protein Degradation Are Involved in Aging

During the normal aging process and also in age-related diseases such as atherosclerosis, cataracts, type 2 diabetes, and neurodegenerative diseases, proteins are the targets of several posttranslational deleterious modifications that alter their biological functions (Table 1). ROS increase oxidative stress [13–15] and reactive nitrogen species (RNS) yield nitrosative stress [15]. Together, as well as in conjunction with other toxic compounds, such as dicarbonyl and reactive aldehydes, which are mainly responsible for this damage [16, 17], which in several cases translates into clinical pathology.

Additionally, protein degradation is integral for maintaining a healthy and functional proteome, particularly for turning over misfolded and damaged proteins. Removal of oxidized proteins first involves selective recognition of the modification and afterward, either their repair or their degradation [18]. Protein concentration increases with both decreased degradation and increased synthesis; yet, while decreased degradation results in the accumulation of "old" proteins, increased synthesis does not [19]. Thus, the age-related accumulation of damaged proteins is thought to result from both the increased occurrence of damage, which is due, at least in part, to alterations in the detoxification of the damaging agents, and from the decreased efficiency of the different systems involved in the elimination of damaged proteins [20]. This relationship is illustrated in Figure 2.

Protein quality, but not necessarily quantity, is altered in a disease state and can be reversed by appropriate treatment. This has been demonstrated, for example, in the case of apolipoprotein A1 (ApoA1) in type 1 diabetes, in which ApoA-1 proteins acutely acquired damage during insulin deprivation, providing a feasible mechanism for the association between chronically poor glycemic control and higher levels of protein oxidation in diabetes [21]. In fact, it may be that this rapid aging of ApoA-1, a key protein in lipoprotein metabolism, causes a higher risk of macrovascular disease in persons with type 1 diabetes [22]. Therefore, it has been proposed that aging, as well as certain pathologies such as diabetes, insulin resistance, and metabolic syndrome, are caused in part by the disproportionate accumulation of damaged and dysfunctional proteins, rather than by their increased concentration per se, by the impairing of cellular degradation systems, whereby oxidized proteins that would normally be targeted for degradation accumulate due to age-related slowing of degradation pathways, which are easily overwhelmed by an excess of posttranslational stress [19, 20, 23–28]. Accordingly, observational studies in animals suggest that enhancing protein degradation by caloric restriction and aerobic exercise may retard aging and reverse age-associated pathologies, and it has been hypothesized that caloric restriction helps to modulate the inflammatory process, subsequently leading to the reduction of chronic diseases known to compromise the functional longevity of humans [29]. Notwithstanding this, whether these strategies can promote similar improvements in humans remains to be shown [19].

Oxidation of proteins targets them for degradation; however, extensive oxidation acts against their recycling because

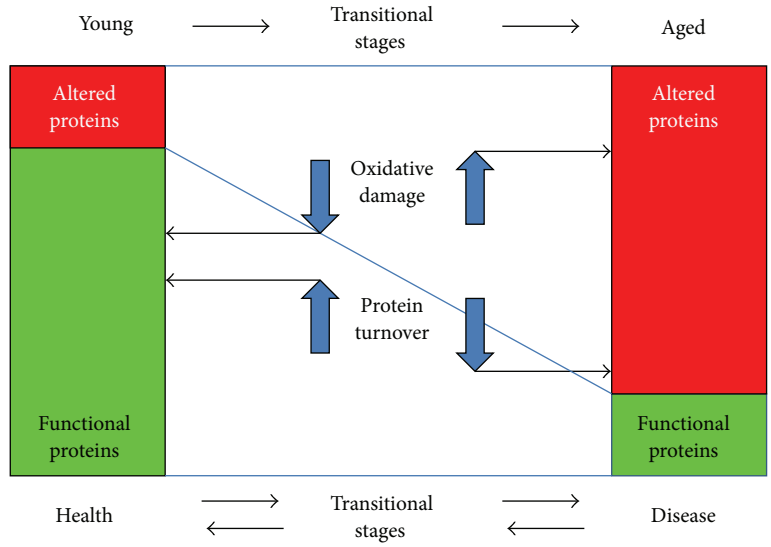

Figure 2: Alteration of proteins by oxidative damage and their turnover. The balance between functional proteins, present in young or healthy organisms, and detrimental or altered proteins, present in a large proportion of aged or diseased organisms, depends mainly on their modification and turnover. If proteins are affected by an increase in oxidative damage or by a low protein turnover, altered proteins accumulate, in contrast to when oxidative damage diminishes and protein turnover increases, when functional proteins increase their proportion and the organism transits to a healthy stage.

it inhibits proteolysis, leading to pathological accumulation and deposition [28, 30]. Modification of proteins also can modify an active site, block a phosphorylation site, or disrupt a binding site for substrates, cofactors, or partner proteins. Furthermore, it can create new epitopes for antibody recognition and induce autoimmune disorders [31, 32].

Although it is widely recognized that cellular aging causes changes in the proteome, the nature and targets of these changes and their consequences have not yet been completely identified. It is noteworthy that accumulative oxidative post-translational modifications are relevant only if these detected modifications are connected to functional consequences [33]. But in addition, it is relevant to consider that a slight modification in low abundance proteins may be of physiological importance; therefore, many proteomic studies have been undertaken to identify modified proteins. Distinguishing between inconsequential modifications and functionally significant ones requires careful biochemical and biophysical analysis of target proteins. Thus, proteomic approaches represent powerful tools to address these questions by identifying the targeted proteins and the extent of their modifications.

4. Oxidation of Proteins Directly Affects Energetic and Metabolic Pathways and Is Related to Longevity

Oxidation-reduction (Redox) regulatory control and oxidative stress are two sides of the same coin: oxidation of proteins can modify proteins under reversible Redox regulatory control or, alternatively, can result in reversible or irreversible oxidative damage. Proteins are very sensitive to the action of ROS [16] and represent nearly 70% of their targeted entities [34]; in addition, ROS can oxidize membrane lipids, generating intermediary compounds that have a longer lifespan than ROS and that can diffuse into the cell, acting as a "toxic second messenger," amplifying the damage of free radicals [35]. Recently, systemic Redox regulation has been recognized as a highly important element for longevity in a group of Japanese semisuper centenarians (>105 years of age), who probably escape from many serious chronic and age-related diseases by their ability to deal with a variety of stresses, including oxidative stress [36].

Therefore, recent proposals tend to prevent, rather than counteract, ROS production, particularly at the mitochondrial level, during oxidative metabolism (the mitochondrial free radical theory of aging) [37]. Although there is evidence that ROS production in the human skeletal muscle is lower in mitochondria from older subjects [38], adenosine triphosphate (ATP) synthesis was significantly decreased, supporting the concept that aging is associated with a decrease in mitochondrial function, although in this case, ROS production appears to be reduced. In the same study, when older subjects were under a regimen of physical exercise, an improvement was found in their mitochondrial function, as well as a concomitant increase in ROS production [38], which apparently counteracted the effect of aging at the molecular level with respect to mitochondrial function.

Therefore, these apparently contradictory results can be conciliated on the basis of equilibrium between the amount of ROS generated, which can be beneficial or defective, and the amount of oxidized proteins, depending on the tissue and its metabolic status. As a result of the analysis of cumulative evidences, two general characteristics responsible for the degree of high maintenance of long-lived animals emerge: a low generation rate of endogenous damage and

the possession of macromolecules that are highly resistant to oxidative modification [37].

To detect protein oxidation, carbonyls are the most commonly employed marker, and the use of 2D gel electrophoresis has provided very useful results for the study of specific carbonylated protein spots during oxidative stress and replicative senescence [39, 40]. Although MS is currently the most versatile technology in proteomics for the identification of proteins and their carbonylated residues, some limitations have led to the development of alternative strategies, such as fluorescent probes, which are able to detect lower abundance carbonylated proteins [20]. Additionally, cysteine oxidation became important because these lie precisely at the interface between Redox-sensitive and oxidative damage by ROS or nitrogen species during stress [41]. Interestingly, a highly significant inverse correlation between long lifespan and the percentage of mitochondrial cysteine is found in metaexaminations of genomic sequences (mitochondrial DNA) from 218 animal species (chordates and arthropods) [42], supporting the previously mentioned free radical theory of aging and point out the relevance of the vulnerability of the proteins in the organism to oxidative stress in terms of its lifespan.

Taking into account the alterations of metabolism in humans, the Cornelia de Lange syndrome is a rare multisystem disorder characterized by distinctive craniofacial dysmorphia, upper limb malformations, hirsutism, microcephaly, cardiac defects, gastroesophageal dysfunction, growth retardation, and neurodevelopment impairment ranging from moderate to severe, with a wide range of variability [43]. Patients present a premature aging process, and it is likely that a reduction in energy and the downregulation of proteins involved in antioxidant and detoxification pathways could lead to premature physiological aging and genome instability [44].

On the other hand, a possible model of longevity has been described; it is based on the knocking out of type 5 adenylyl cyclase (AC5) [45]. Adenylyl cyclase (AC) is a key enzyme that catalyzes the synthesis of cyclic adenosine monophosphate (cAMP) from ATP and it plays a pivotal role in β-adrenergic receptor signaling. In these AC5 KO mice, activation of the Raf/Mitogen-activated protein kinase/MAP kinase kinase (Raf/MEK/ERK) signaling pathway is present, which in turn promotes the upregulation of Mn-superoxide dismutase (Mn-SOD) and results in protection from oxidative stress and apoptosis, retarding aging phenotypes in the heart and bone and increasing resistance to stress, which leads to longevity in the lifespan, suggesting that retarding aging in an individual organ could be a fundamental therapy to prevent age-related diseases [46].

Delving deeper into the identification of proteins involved or affected by aging, postmitotic tissues have received more attention. Neurons, myocytes, and red blood cells have been studied during the aging process, and certain particular and some common factors have been discovered. In the following sections, the main advances in proteomics studies of these post-mitotic cells are presented.

5. Proteomic Studies Further Support Parallelism between Aging and Neurodegenerative Diseases

An increase in oxidative stress in the brain is part of normal aging and is related directly to decreased neurological activities and inversely to lifespan [47]. Common pathological pathways that are implicated both in aging and in the development of neurodegenerative disease include free radical damage and decreased energy production as characteristic hallmarks [48]. Consistent with this idea, proteins that increase with aging in the mice hippocampus are mainly enzymes that mediate energy production and oxidative stress [49]. In addition, one of the cellular processes that are altered mainly in the aging hippocampus is that of oxidative stress, as well as that of protein processing [50].

Consistent with previous ideas, wide proteomic analysis of brains during aging in mice identifies 40 proteins that exhibit changes in their natural pattern during the mouse lifespan (from 4 days to 15 months of age), showing that six proteins increased and 27 decreased in various ways. When analyzed together, the biological processes in which those proteins are involved correspond mainly to the following: protein metabolic processes (more than one third); transport (one third); nucleotide and nucleic acid metabolic process (one fourth); intracellular signal cascade (nearly one fifth), and response to stress proteins (more than one sixth). Additionally, about one fifth of the identified proteins can be localized in the mitochondria and the majority of these are related to energy metabolism [51], providing a broad panorama of proteomic changes in the brain during aging.

In addition, there is increasing evidence that protein oxidation is involved in the pathogenesis of Alzheimer's disease (AD), a neurodegenerative disorder associated with cognitive decline, oxidative stress, and aging [52–56]. Two initial studies using the proteomic approach have identified proteins that are specifically oxidized in AD [54, 55]. In the first study, three key enzymes in cellular metabolism, creatine kinase BB (CK BB), glutamine synthase (GS), and ubiquitin carboxy-terminal hydrolase L-1 (UCH L1), resulted as specific targets of protein oxidation in the brain of AD patients. In the second, a couple of additional targets were detected: dihydropyridine-related protein-2 (DRP-2), involved in axonal growth, and α-enolase, involved in glycolysis for energy metabolism and therefore related with the cerebral decrease of energy metabolism. These results strongly suggest that the process of free radical-mediated protein modification may be a crucial event in AD and that protein oxidation is a relevant part of the mechanism of neurodegeneration in AD brain.

Although in a pilot study of abundant carbonylated proteins in the cerebrospinal fluid of probable AD patients the extent of carbonylation did not vary in general, two proteins were detected that were highly carbonylated when compared to controls: immunoglobulin λ light chains and one unidentified protein [57], which suggests that further studies could be performed focusing on less abundant proteins, modified by oxidative stress, to detect some probable markers of early

pathological stages. Therefore, the establishment of differences in protein oxidation state may provide a diagnostic tool for neurodegenerative diseases.

Additionally, Weinreb et al. found a significant parallelism in the protein profile affected between aging and neurodegenerative diseases in the hippocampus of rats [58]. They found that in the aged hippocampus, oxidative stress and mitochondrial dysfunction are important and that in treatment with an anti-AD drug, Ladostigil, or with an anti-Parkinson drug, Rasagiline, both drugs reversed the effect of aging on various mitochondrial and key regulator genes involved in neurodegeneration, cell survival, synaptogenesis, oxidation, and metabolism. Another consequence of oxidative stress in the brain includes the generation of RNS. The cerebellum is especially vulnerable to oxidative stress and exhibits an age-dependent increase of total 3-nitrotyrosine (3-NT) [59, 60], and some proteins have been identified as targets for nitration [59].

Additionally, in cultured neurons exposed to amyloid beta (Aβ) (1–42), two proteins are significantly oxidized: 14-3-3ξ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and pretreatment with γ-glutamylcysteine ethyl ester, a compound that supplies the limiting substrate for antioxidant glutathione synthesis, protects both proteins from oxidation by Aβ 1–42 [61], which is consistent with the notion that antioxidant therapies may potentially be effective in slowing or ameliorating the neurodegenerative disease process [56, 62].

Conversely, the other cell type that is more abundant in the central nervous system, the glial cells, are more resistant than neurons to oxidative stress and are able to respond to protect them [63–66]. Surprisingly, proteomic studies that focus on the role of glial cells during aging, or in neurodegenerative diseases, in response to oxidative stress are very scarce. Miura et al. proposed that aging does not suppress the astrocytic capability to respond to oxidative stress. The authors found that α-tubulin was subjected to tyrosyl phosphorylation by H_2O_2-exposure and that aging enhanced this phosphorylation and prevented the formation of microtubules, but aging does not suppress the responses aimed at cell protection against severe oxidative stress [67]. Therefore, proteomic studies on the response of glial cells to oxidative stress during aging and in neurodegenerative diseases, focusing on their helping role for neuron metabolism, represent a promising avenue that is yet to be explored.

6. Aging in Cardiac and Skeletal Muscles Altered Energy Metabolism and Mitochondria

Cardiac performance declines with age [68] in a clear association with oxidative stress [3]. Accordingly, aging is a factor for cardiovascular disease. It appears that the protein signature or proteomic phenotype that changes during aging in the rodent heart renders the tissue more sensitive to oxidative stress damage with age by modifying the energy metabolism, particularly carbohydrate metabolism, fatty acid oxidation, cellular respiration, and energy production and their capacity to respond to oxidative stress [69].

In the aged rat heart, several proteins have been identified as differentially expressed when compared with those of young hearts [69–71], although there are many differences among studies, probably derived from methodologically different approaches and species particularities (mouse or rat), also due to the fact that many proteins change consistently during aging. Additionally, a study of a heart failure model, transverse aortic constriction in mice, demonstrated a differentially expressed protein expression of structural, signaling, and redox proteins [72]. Thus, it is possible to establish parallelism between aging heart dynamics and heart failure, both altering structural proteins as well as the oxidative metabolic profile and affecting the heart's capacity to respond adequately to oxidative stress during aging or under pathological distress.

Among the functional consequences of oxidative stress induced by ROS and RNS on cardiac and skeletal muscle tissue during aging, we find protein nitration [73], which may affect protein structure, function, and turnover. Thus, the accumulation of nitrated proteins in cardiac and skeletal muscle tissue [74, 75] may define the progress of biological aging or of any pathology.

7. During Red Blood Cell Aging, Hemoglobin-Generated Oxidants That Affect Cellular Membrane and Cytoskeleton

Many of the cell processes associated with the physiological removal of red blood cells (RBC) involve oxidative stress, which is generated by both endogenous hemoglobin (Hb) auto-oxidation and exogenous oxidants, which can result in functional impairment and in cellular aging [76]. The predominant factor that determines oxidative stress in RBC is Hb. The superoxide, H_2O_2, hydroxyl radicals, ferrylHb, oxoferrylHb, and peroxynitrite generated by redox reactions near the membrane can damage RBC membrane proteins, lipids, and the cytoskeleton, which are responsible for maintaining the RBC shape and deformability, thus being able to damage and promote cellular aging [77]. Consequently, instead of required large concentrations of antioxidants to neutralize the ROS species formed, blocking Hb interaction with the membrane will make it possible to eliminate Hb-generated oxidants, preventing oxidative stress in RBC [76]. In RBC, and due to their extensive use for blood transfusions, their oxidative stress is also highly relevant during long-term storage [78]. Consequently, the proteomic approach has been useful to identify molecular markers, such as Prx2, as a candidate biomarker for RBC oxidative injuries under blood bank conditions [79], possibly to be utilized in future blood component programs to improve the quality of stored RBC and to limit or avoid the risk of posttransfusional complications.

In brief, and beyond the cell type affected, on the basis of extensive experimental results, the main cellular dysfunctions directly caused by oxidative stress imbalance and by uncontrolled generation of ROS can be summarized as follows

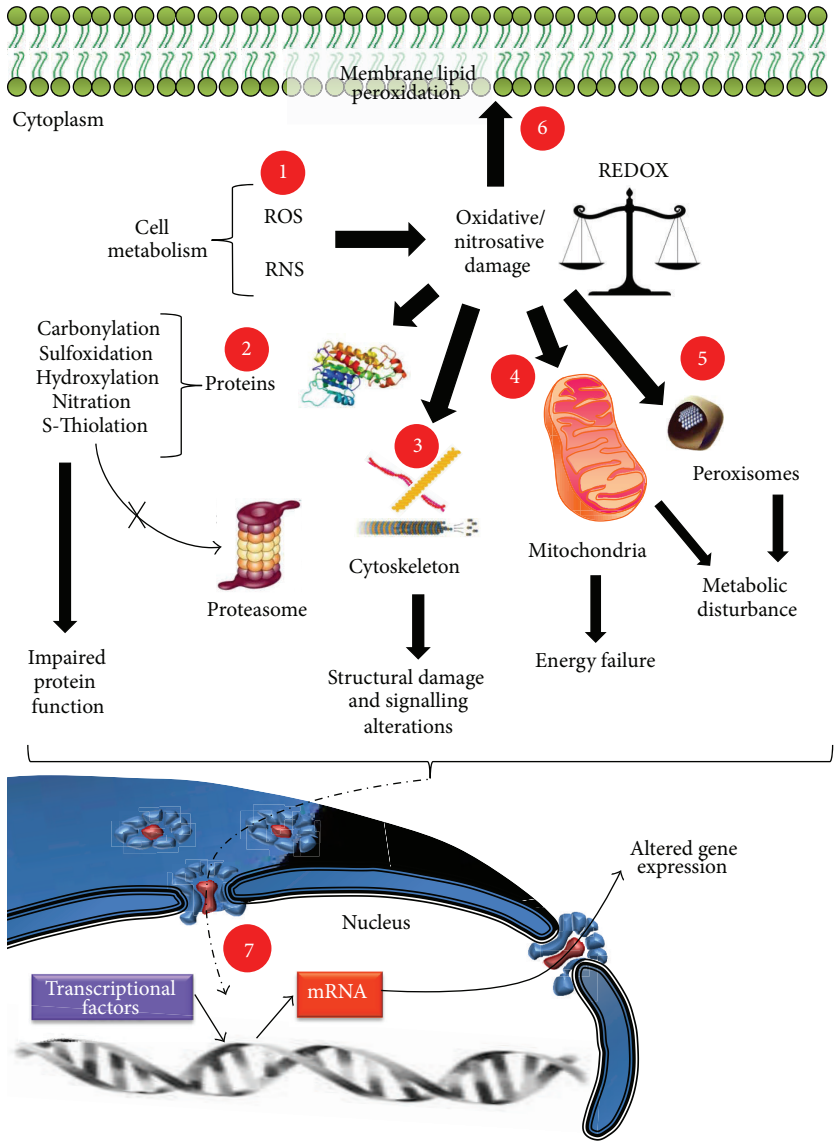

Figure 3: Cellular dysfunctions in aging or in age-related diseases by oxidative stress imbalance. (1) Cell metabolism generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), which in turn causes oxidative/nitrosative damage. (2) Proteins are the most affected macromolecules by oxidative stress, undergoing several modifications that avoid their being correctly degraded and recycled by the proteasome, thus generating impaired protein function. (3) Oxidative stress also directly affects cytoskeletal proteins, causing structural damage and signaling alterations. (4) On affecting the mitochondria, oxidative stress alters energy production and (5) on affecting peroxisomes, oxidative stress alters correct metabolic functioning. (6) Oxidative stress also affects the cellular membrane. (7) Finally, all of the previously mentioned affections cause an alteration in the transcriptional activity of the cell, leading to an altered gene expression that in turn leads the cell to the aging process or to degenerative disease.

(Figure 3). Whether from self-cell metabolism or from extracellular sources generating an uncontrolled increase of ROS and RNS, both of these produce cell damage by modifying proteins (mainly by carbonylation, sulfoxidation, hydroxylation, nitration, and S-thiolation) and avoiding the recycling of these by the proteasome, yielding impaired protein function. Additionally, oxidative stress can directly affect the cellular membrane, causing lipid peroxidation, thus instability, and can also modify cytoskeletal proteins, causing structural damage and moreover altering signaling pathways. Furthermore, oxidative stress directly affects mitochondria and peroxisomes, thereby altering cell metabolism and energy production. Taken together, these alterations would be able, in some manner, to distress nuclear transcription and generate altered gene expression during normal aging, or leading to disease. Although this is a general panorama, there are more specific alterations depending on the cellular type affected or on the tissue or organ altered. Therefore, it is a priority to establish more models to study the whole effect of oxidative stress during aging and disease.

8. Particular Organisms as Alternative Models for Studying the Proteomics of Oxidative Stress during Aging

In addition to the more widely used models employed to study aging (such as rodents like mice or rats, cultured line cells, or even tissue fragments, blood, or serum), other particular organisms represent suitable alternatives for approaching the problem. In Table 2, we summarize some of the main works that, based on their wide proteomic approach, deal with the aging process, selecting those that have obtained results that are clearly related with the involvement of oxidative stress and ROS in the aging process. The following is a brief summary of some particular models and their contribution to elucidating the participation of oxidative stress during aging.

The invertebrate *Caenorhabditis elegans* has been successfully used to study the aging process. It has been described that some proteins are involved in both cellular senescence and the ROS-induced condition. By using interfering RNA (iRNA) against some genes, a substantial reduction can be caused in adult lifespan, and the defensive mechanism against external oxidative stress is also disturbed [80]. Therefore, some proteins whose expression is increased with cellular senescence and oxidative stress play a protective role against these processes. Additionally, when *C. elegans* is submitted to oxidative stress through sublethal short-treatment of peroxide (H_2O_2) stress, the majority of worms experience severe, yet fully reversible, behavioral changes that are highly reminiscent of well-known age-related changes [83], such as declines in body movement, pharyngeal pumping, and reproduction, as well as morphological changes and reduced metabolic activity [83], which supports the Harman free radical theory of aging, but would also include the potentially beneficial aspects of ROS as modulatory second messengers that affect stress resistance and longevity early in life [83]. In another example, *C. elegans xpa-1* mutants, which are ultraviolet-light (UV)-sensitive and that have reduced capacity to repair UV-induced DNA damage [112], exhibit oxidative stress and its antioxidant defenses are induced, and they also show polyubiquitinated protein accumulation [84]. Obviously, there are differences between nematode (invertebrate) and mammalian (vertebrate) systems, but the fundamental mechanism of cellular senescence may be evolutionarily conserved.

Another very attractive model for studying the effect of oxidative stress during aging is the senescence-accelerated probe-8 (SAMP8) mouse, which exhibits age-related deterioration in memory and learning, along with an increase in oxidative markers and which is considered a useful model for the study of AD [113]. In AD, it has been demonstrated that treatment with α-lipoic acid, a coenzyme involved in the production of ATP in mitochondria and a potent antioxidant [114, 115], is able to reduce oxidative modification and increase the protein level of α-enolase, suggesting the possibility that the reduced glucose metabolism and neurochemical alterations in SAMP8 mouse brains can be reversed [116]. Additionally, it has been demonstrated that carbonyl modification of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) in liver and hippocampal cholinergic neurostimulating peptide-precursor protein (HCNP-pp) in the brain were higher in SAMP8 compared with the control, SAMR1. Therefore, progressive accumulation of oxidative damage to Cu, Zn-SOD may cause dysfunction of the defense systems against oxidative stress in SAMP8 with higher oxidative states, leading to the acceleration of aging [85].

A different and very interesting organism for studying the effect of oxidative stress during aging is the naked mole-rat (*H. glaber*) because it has very low metabolic and respiratory rates and its protein structure and function is not apparently affected by either oxidative stress or carbonylation during aging, probably due to a particular characteristic of the cellular environment that maintains the functional structure of proteins [104]. As an example, activation of the Nuclear factor [(erythroid-derived 2)-like 2] (Nrf2) antioxidant response pathway, which increases the transcription of antioxidant response-element genes, proteasomes, and antioxidants and which affects the efficient maintenance of protein homeostasis, can protect proteins from misfolding or aggregation by oxidative stress [117, 118], being part of a protective cellular environment that efficiently maintains protein homeostasis as part of a potential plausible mechanism that explains the exceptional longevity of the naked mole-rat [104].

Finally, an attractive model for the aging human brain is the aging beagle (canine) brain, especially also as a model of AD [119–122]. In the aging canine brain, a proteomics study reveals that a combined treatment of antioxidant-fortified food and an enriched environment reduces the levels of oxidative damage, improves the antioxidant reserve systems, increases the activity and expression of key endogenous antioxidant enzymes, and may contribute to improvements in learning and memory [106].

9. Concluding Remarks

One limitation of some of the reports presented here is that details regarding animal ages, care, and behavior assessments/measures are limited, which impedes cross-study comparison and meta-analyses. Also, cell types and their particular characteristics rendered comparison of the effect of ROS on RBC or neurons difficult, for example, as well as under *in vitro* or *in vivo* conditions. Thus, therefore more and wider studies are needed.

In humans, it is difficult to compare among proteomic studies because of insufficient characterization of the study material, the small number of patients involved in studies, and variations in experimental designs. At present, basic aging research has arrived at a pharmaceutical phase, with the testing of novel drugs designed to extend a healthy life by targeting specific biochemical pathways, perhaps in specific organs [123]. In this respect, the National Institute on Aging Interventions Testing Program (ITP) experimentally evaluates chemical compounds with potential senescence-retarding effects that can be administered to mice in food or water [124]. While initial results are far from surprising, the experimental design is robust; therefore, it will be useful in

Table 2: Comprehensive summary of proteomic studies focused on aging that involves oxidative stress-related proteins.

Animal model (specie) and tissue	Sample and age	Results related to oxidative stress or ROS influence on aging	Main proteins altered in aging*	References
Invertebrate *Caenorhabditis elegans* (worm)	*In vitro* knocked down by RNAi	Ten iRNA tested caused substantial reduction in adult lifespan. When these genes are disturbed defensive mechanisms against oxidative stress become altered.	UBH-1, UBH-3, PRDX2, PRDX3, AMPK-β1, AMPK-β2, LBP-4, LBP-6, LBP-9, RHI-1	[80]
	In vitro knocked down by RNAi for K10C2.4	K10C2.4 RNAi activates oxidative stress and endoplasmic reticulum stress response in the worm intestine by accumulation of tyrosine metabolites.	Enzymes in the tyrosine degradation pathway	[81]
	Long-lived *daf-2*(e1370) strain	Components of the enhanced longevity system identified in *daf-2* deficient mutant include the alpha-crystallin family of small heat shock proteins, anti-ROS defense systems, and cellular phase II detoxification. GSTP were significantly URg, which detoxify and/or bind short-chain aldehydic natural toxic products of lipid peroxidation and long-chained fatty-acids at physiologically relevant concentrations, indicating a role in longevity.	GPX, SOD1, NME, RPS12, STK, LBP-6, HSP-12.6, HSP-12.3	[82]
	Exposure of prdx-2 defective worms under H_2O_2-induced OS	Identified oxidation-sensitive cysteins in 40 different proteins involved in mobility (muscle contraction), feeding, protein translation, homeostasis, and ATP regeneration.	MYO-2, LET-75, EFT-1, HSP1, NME	[83]
	xpa-1 mutant UV-sensitive with shortened lifespan	Proteome changes in xpa-1 mutants correspond to transcriptome modulation by suffering oxidative stress and inducing antioxidative defenses. Polyubiquinated proteins accumulate, cyclopurine levels are reduced, and lesion-detection enzymes play active roles to generate a genomic stress signal.	NTH-1, XPC-1, DDB-1	[84]
Rodents *Mus musculus* (mice)	KO mice for 5 adenylyl cyclase (AC5)	AC5 KO mice are protected from aging-induced cardiomyopathy and their fibroblasts exhibited ERK-dependent resistance to oxidative stress. AC5 KO leads to upregulation of the Raf/MEK/ERK signaling pathway, which in turn mediates upregulation of SOD, an important mechanism mediating lifespan extension and stress resistance.	Increased: RSK, p-Bad, Bcl-xl, XIAP, HSP70, p-ERK, p-Raf-1	[46]
SAMP8	Brain and liver from SAMP8	Progressive accumulation of oxidative damage to Cu, Zn-SOD may cause a dysfunction of defense systems against oxidative stress in SAMP8, with a higher oxidative stress and leading to the acceleration of aging.	SOD1, HCNP-pp	[85]
	Hippocampus and cortex from 5 to 15 month old SAMP8	7 protein are related to age rather than strain and might be associated with brain aging process. One protein might be specifically associated with pathologically accelerated aging in SAMP8 mice; HEBP1.	NDRG2, enolase 2, SOD1, myosin, two unnamed protein (gi|74214304; gi|74178239), HEBP1	[86]
Brain	Brain tissue from 3 weeks to 18 months old C57B mice	Carbonylated proteins increased with aging are involved in cytoskeletal organization, mitochondrial energy metabolism, redox regulation (oxidative damage), and signal transduction.	Approximately 100 carbonylated proteins	[87]
	Brain tissue from 3-, 6-, 12- to 15-month-old male Kunming mice	60 proteins vary their expression on aging; 27 of them decrease, may be responsible for brain aging. Related with decline of protein quality control, shortage of energy and reducing agent, increase of DNA damage and transcription detuning, and disturbance of synaptic transport and ion signals. 6 proteins increase, may be involved in antiaging processes.	PSMA6, PSMA3, CALR, UCHL3, VCP, GLUD1, IDH1, UQCRC2, UBE2N, CALB1, HNRPA2/B1, AMPH, TKT, CKMT1, MRPL37, TPI1	[49]

Table 2: Continued.

Animal model (specie) and tissue	Sample and age	Results related to oxidative stress or ROS influence on aging	Main proteins altered in aging*	References
Brain	Adult NPCs from brain of C57BL/6 mice from 3, 15–18 months of age	Aging is correlated with a loss of mitochondria and oxidative metabolism in NPCs. A coordinated shift in protein expression, subcellular structure, and metabolic physiology in aging NPCs, allowing resistance to hypoxia and mitochondrial inhibition. 124 proteins result as age-related.	Increased: PGK1, SEPT9. Decreased: ATP5 α and β	[88]
Liver	Livers from male C57BL6/J mice of 10-week-old and 18-month-old. Peroxisome enriched fraction	Most of the proteins identified are related to ROS production/breakdown; however, high biological variability between individuals is even more pronounced than changes induced by aging.	EPHX2, Acaa1, Pipox, Amy2a, Decr2, Phb2, COX6c, UQCRC2	[89]
Kidney	Male and female mice CD1-Swiss outbred strain of 28, 52, 76-week-old	Differential protein expression of 8 aging related proteins (both genders). Increase in oxidative and proteolytic proteins and decrease in glycolytic proteins, and antioxidant enzymes with aging.	ATP syntase, Transferrin, HSP9A, Hibadh, IDH1	[90]
Cardiac muscle	Hearts from male CB6F1 mice from 3, 15 to 23 months old	Detected age-related alterations in the levels of 73 proteins. Mithocondrial metabolism is affected and a net loss in antioxidants occurs with aging.	Mortalin, PRDX3, EPHX, SOD1, SOD2	[70]
Adipose tissue	Male mutant mice deficient in Zmpste24 metalloproteinase	Zmpste24 deficiency causes premature aging. It enhanced lipolysis, fatty acid biogenesis, and β-oxidation as well as decreased fatty acid reesterification. Also URg protein networks related to tricarboxylic acid cycle and oxidative phosphorylation and increased mitochondrial response to oxidative stress and cytoskeleton. 37 proteins URg and 9 DRg.	Increased: ME1, PRDX3, HMGB1, CPT1, UCP1; Decreased: PCK1, vimentin isoforms	[91]
Macrophages	Peritoneal macrophages from male Balb/c mice (3-4 and 14-15 months)	An age-dependent increase in the extent of recruitment of macrophages into the peritoneum, as well as *ex vivo* functional changes involving enhanced nitric oxide production under resting conditions. Identified age-dependent increases in levels of proteins linked to immune cell pathways under basal conditions and following LPS activation. Immune pathways URg in macrophages isolated from aged mice include proteins critical to the formation of the immunoproteasome.	Hundreds of proteins	[92]
Rattus novergicus (rat)	Male Wistar rats weighing 80–90 g, 6 and 24 months old	A beneficial role for virgin olive oil in modulating inflammation, homeostasis, oxidative stress, and cardiovascular risk during aging. Diet diminishes in general the changes that occurred with age.	Decreased: HPX, HP, AHSG, PRDX2, FGg, T-KNG, APO H, APO E, APO A-IV Increased: APO A-1	[93]
Brain	Serum from young and old Fischer 344 rats	16 of the modified proteins by peroxynitrite and 4-hydroxy-2-nonenal are involved in blood coagulation, lipid transport, blood pressure regulation, and protease inhibition.	16 modified proteins	[94]
	Hippocampus from 8 to 27-month-old Wistar rats, and also treated with the anti-Parkinson drug; rasagiline or the anti-Alzheimer's disease drug; ladostigil	Significant molecular changes related to neurodegeneration were identified in aged rat hippocampus. Both drugs reversed the effect of aging on the expression of various mitochondrial and key regulator genes involved in neurodegeneration, cell survival, synaptogenesis, oxidation, and metabolism. Changes in proteins related to the iron-mediated oxidative stress pathway, including reduction in antioxidant enzymes. Oxidative stress and mitochondrial dysfunction may play a pivotal role in aging and age-associated neurodegenerative diseases.	Aprox. 200 proteins showed differential expression. NEFL, FTH1, TUFM, PEA15, PEBP, PFN1, CCT2, IDH3A, COX5A, COX5B, PRDX2	[58]
	cerebellum from Fisher 344/Brown Norway rats from 5-, 22- and 34-month-old rats	Genes encoding proteins of stress response and inflammatory processes show a significantly higher age dependent upregulation in the cerebellum suggesting higher levels of oxidative stress. Identification of nitrated proteins.	Ryr3, Lrp2, Nrap, Cnp	[95]

Table 2: Continued.

Animal model (specie) and tissue	Sample and age	Results related to oxidative stress or ROS influence on aging	Main proteins altered in aging*	References
Brain	Brain from male Wistar rats from 12 to 28 months old (hippocampus, cortex, striatum, and cerebellum)	Senescent animals showed significantly higher levels of oxidation. 11 proteins carbonylated in hippocampus, 15 in cortex, 10 in striatum, 11 in cerebellum, associated with significant changes in both cytosolic and mitochondrial redox status in all brain regions analyzed.	Decreased: PK, ATP5a1, ALDOC, CKB, a-enolase. Activity of PK and GAPDH diminished	[48]
	Hypotalamus and hypophysis from male Wistar rats from 3, 12 to 24 months old treated with an antioxidant	Alterations of eEF-2 levels, secondary to lipid peroxidation and adduct formation with aldehydes could contribute to the suboptimal hormone production from these tissues during aging	eEF-2, ALDOA, GSTA, CKB, PPIA, PK, GADPH, INA, CFL1	[96]
Retina	MSC cultures from the tibial and femoral BM of 83-week- and 12-month-old Sprague-Dawley rats	Number of MSCs is reduced in aged animals. Aged MSCs are more susceptible toward senescence and display a lower migratory capacity. Aging affects MSCs antioxidant defense and cytoskeleton turnover.	Several proteins as members of the actin-binding protein family of calponins, galectin-3	[97]
	Fisher 344/Brown Norway F1 rats from 3-4 to 24-25 months old	Decrease of antioxidant enzymes was detected in the old F344BN retina sections and increased presence of ROS and oxidative stress.	Increased: CD46, GABA2, DJ-1, EBP50, Ezrin, Cathepsin D. Decreased: NG, DDAH1, DPPX	[98]
	Primary cell cultures from retinas of newborn (PD 1 or 2) Sprague-Dawley rats under H_2O_2-induced OS	Retinal pigmentary epithelium (RPE) and retina have higher O_2 tension and ROS concentration with aging; this environment may contribute to the pathogenesis and progression of eye diseases. Decreased prohibitin in H_2O_2 treated RPE cells may indicate an antioxidative role.	Prohibitin	[99]
Adipose tissue	White adipose tissue from male Wistar rats from 6 and 24-month-old under caloric restriction (CR)	Caloric restriction (CR) improves oxidative stress and prevents age-associated changes in several antioxidant enzymes. Metabolic enzymes involved in energy metabolism and transduction (glucose and lipid), oxidative stress response, cytoskeleton, and iron homeostasis were also modulated by age and/or CR. Several enzymes involved in cell protection against oxidative stress are increased by CR, whereas these protein levels decrease or do not change with age.	133 differentially expressed spots, 57 of which were identified	[100]
Skeletal muscle	Skeletal muscles from Fisher 344/Brown Norway F1 rats, 34 months old	11 nitrated proteins were identified as age-related.	CKM, TPM1, GAPDH, MYL2, ALDOA, PKM, PYGM, NOTCH1, ACTN1, ACTC1, RYR3	[74]
	Gastrocnemius muscle from Lou/c/jall male rats from 7, 18, to 30 months old	Aging is associated with differential expression of myofibrillar regulatory proteins, up-regulation of cytoskeletal proteins, perturbations in the energy metabolism, and detoxification of cytotoxic products.	40 proteins differentially expressed	[101]
	Gastrocnemius muscle of 26-month-old Wistar rats	Mitochondria-enriched fraction revealed an age-related change in 39 protein species. An age-related increase in mitochondrial enzyme activity belonging to the inner membrane system, matrix, outer membrane, and intermembrane space, increasing aerobic-oxidative metabolism, involved in oxidative phosphorylation, ATP formation, and fatty acid oxidation.	Increased: NADH-DH, Immt mitofilin, PRDX3, F1-ATPase, SDH, Fis1, SUCLA2, ACAD, porin VDAC2, UQCRC1, prohibitin	[102]

Table 2: Continued.

Animal model (specie) and tissue	Sample and age	Results related to oxidative stress or ROS influence on aging	Main proteins altered in aging*	References
	Left ventricle from Fisher 344 rats from 4 to 24-month-old	117 proteins differentially expressed: 23 signalling proteins, 25 metabolic proteins, 7 fatty acid metabolism, 19 energy metabolism, 13 oxidative stress related (antioxidant proteins and chaperones). First network describing proteins affecting cellular organization and morphology is presented.	$\alpha\beta$-Crystallin, GST π isoform, GST μ type, GST Ω1, C1qbp, HSP90b1, GPX1, DJ-1, SOD2, PRDX5, PRDX3, HSP8	[69]
Cardiac muscle	Heart from Fisher 344/Brown Norway F1 rats, 5 and 26 months old	48 differentially nitrated proteins were identified that undergo an age-dependent protein tyrosine nitration.	α-Enolase, Aldolase, Desmin, ACO1, Aldh6a1, Acaa1a, GAPDH, MDH1, CKM, ETF, SOD2, F1-ATPase, VDAC	[103]
	Heart from Fisher 344/Brown Norway F1 rats, 5 and 34 months old	Abundance of 10 nitrated proteins identified in cardiac tissue increase with age.	N-RAP, neurofibromin, tropomyosin, MYO-HC	[73]
Heterocephalus glaber (Naked mole-rat)	Liver, heart, and kidney tissues from naked mole-rats (NMRs; 2 years) and wild-type C57BL/6 mice (0.3 year)	Global protein carbonylation in citosolic fraction was elevated in all three tissues. NMRs have a protective cellular environment which restores enzyme function and prevents formation of oligomers during oxidative stress, modulating structure and function of structural proteins and enzymes. Activation of NRF2 pathway, which increases the transcription of antioxidant response elements, proteasome, antioxidants, and autophagy, could be a potential mechanism for these processes.	TPI, PRDX1	[104]
Porcine *(Sus scrofa)*	Porcine oocyte and effect of caffeine	38 proteins were identified, 23 URg and 3 DRg by aging. Involved in metabolism, stress response, ROS, and cell cycle regulation.	CDK5, PCNA, AHCY, SLC25A6	[105]
Canine *(Canis domesticus)*	Brain from beagle dogs from 8.05 to 12.35 years old. Feeded with antioxidant-fortified food and growth in an enriched environment	Combined treatment (food and environment) significantly decreases protein cabonylation, nitrosylation, and lipid peroxidation, reducing the levels of oxidative damage and improving the antioxidant reserve systems in the brain. Propose a diagram of a functional interacteome of all parietal cortex proteins identified to be significantly less oxidatively modified following the combined treatment.	Decreased: GLUD1, GAPDH, a-Enolase, GST, FSCN1, NF-L. Increased: SOD1, ALDOC, CKB, GLUD1 (P), GAPDH (P)	[106]
Primate *(Homo sapiens)*	Human female of 20–39, 100, and 106–109 years old	Results suggest that systemic redox regulation is important for the longevity of supercentenarians in humans.	Decreased: PON1, APO E. Increased: Hp-b, AMBP, CLU	[36]
Brain	Inferior parietal lobule tissue samples from AD patients autopsy	Protein modification by ROS occurs to a greater extent in AD suggesting a possible role for oxidation-related decrease in protein function in the process of neurodegeneration. Oxidative damage to proteins, assessed by measuring the protein carbonyl content, is involved in several events such as loss in specific protein function, abnormal protein clearance, depletion of the cellular redox-balance and interference with the cell cycle, and, ultimately, neuronal death.	Increased in AD: DRP-2, α-Enolase, HSC-71	[54, 55]
CSF	Lumbar CSF samples from probable AD patients	Decreased concentrations of proteins in CSF may also be a secondary event to increased oxidative stress, since excessive carbonylation leads to an enhanced aggregation of proteins. Extent of protein carbonylation can vary between men and women, emphasizing the importance of sex-matched patients when studying carbonylation.	Decreased in AD: PTGDS, IgL, TTR. Increased carbonylation in AD: IgL and one unidentified protein	[57]

Table 2: Continued.

Animal model (specie) and tissue	Sample and age	Results related to oxidative stress or ROS influence on aging	Main proteins altered in aging*	References
Blood	Whole blood from healthy volunteer donors. Stored for various periods	A progressive linkage of typical cytosolic proteins to the membrane was detected, including both antioxidant and metabolic enzymes. This phenomenon was unequivocally related to oxidative stress, since storage under anaerobic conditions suppresses it.	Prx2	[79]
Skin	Fresh punch biopsies from the forearm of 21–30 and 75–92 years old donors	22 proteins were consistently deregulated. Support that aging is linked with increased oxidative stress that could lead to apoptosis *in vivo*.	Mx-A, SOD1, WARS, PIK3r2, proteasomal PA28-α and SSP 0107	[107]
Fibroblast	WI-38 human embryonic fibroblasts. Two stages PD < 25 and PD > 42	Oxidized proteins accumulate with aging *in vivo* and during replicative senescence *in vitro*. 37 proteins were modified related to protein quality control, energy metabolism, and cytoskeleton. Impairment of glyoxal- and aldehyde-detoxification mitochondrial systems.	Decrease activity of proteasomal CT-L, PGPH and detoxification GLO1	[39]
	HCA3 human dermal fibroblasts under H_2O_2-induced OS	H_2O_2-induced senescent like human diploid fibroblasts increase the production of IGFBP-6 protein.	Increased: Collagen 1(VI), collagen 2(I), fibronectin, lumican, MMP-2, IGFBP-6	[108]
	HCA3 human dermal fibroblasts under H_2O_2-induced OS	H_2O_2 treatment caused elevated levels of TXNRD1. Differences between mRNA versus proteins that vary under oxidative stress may be related to the regulatory mechanism of protein translation under oxidative stress.	Increased: TXNRD1, MMP-3, AURKA Decreased: Akap12, MDH1	[109]
Colon epithelial	Human normal colonic epithelial tissue from 25–30 to 60–65 years old	35 differentially expressed proteins, 16 URg and 19 DRg. Involved in metabolism, energy generation, chaperone, antioxidation, signal transduction, protein folding, and apoptosis.	Increased: ATPB, ETFA, catalase, GPX1, annexin A2, HSP7C; decreased: FUBP1, NDK B, ERp6C, VDAC-2	[110]
MSCs	Human BM-derived MSCs	Differentially expressed proteins under the low glucose condition may provide further information on the aging and differentiation of stem cells.	Increased: ALDH, neuropolypeptide h3, P4HA; Decreased: laminin-BP, actin, Sec 13, RPS12, PSMA1, SOD1, SNAP	[111]

*Proteins based on their human homologue. ROS: reactive oxygen species, URg: upregulated, DRg: downregulated, PD: postnatal day, SAMP8: senescence-accelerated mouse prone 8, NPCs: neural precursor cells, MSCs: mesenquimal stem cells, BM: bone marrow, CSF: cerebrospinal fluid, KO: knock out, AD: Alzheimer's disease.

order to develop a similar program in mouse genetics in aging.

It is evident that the sole fact of identifying the whole genome sequence of an organism, or to know the whole isoforms and modifications of its products (proteins), is not sufficient for complete elucidation of the aging process. It is necessary to integrate all of this information in a functional manner that reflects more precisely the real situation. Therefore, as important as the generation of all "omic" information is, the development of instruments to analyze and evaluate this efficiently is equally important. In this regard, bioinformatics and computational biology are devoted to performing these analyses, both based on systems biology, that is, the construction of gene, protein, and metabolic pathway networks that interact among them to constitute functional modules (Figure 1). In turn, they integrate design models for prediction from clinical phenotypes to diagnostic and therapeutic strategies after experimentation takes place. Albeit proteomics has already contributed relevant insights in the field of aging research and attempts have been made, in animal models such as mice to map aging-related brain proteins within the context of the biological processes involved [51]; a reference mapping of proteins in healthy aging human subjects has yet to be performed. Nonetheless, with the continued advances in proteomic technology, the study of the proteome during aging is entering a brand new phase of discovery.

Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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Research Article

Carbon Monoxide Protects against Hepatic Ischemia/Reperfusion Injury via ROS-Dependent Akt Signaling and Inhibition of Glycogen Synthase Kinase 3β

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Carbon monoxide (CO) may exert important roles in physiological and pathophysiological states through the regulation of cellular signaling pathways. CO can protect organ tissues from ischemia/reperfusion (I/R) injury by modulating intracellular redox status and by inhibiting inflammatory, apoptotic, and proliferative responses. However, the cellular mechanisms underlying the protective effects of CO in organ I/R injury remain incompletely understood. In this study, a murine model of hepatic warm I/R injury was employed to assess the role of glycogen synthase kinase-3 (GSK3) and phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathways in the protective effects of CO against inflammation and injury. Inhibition of GSK3 through the PI3K/Akt pathway played a crucial role in CO-mediated protection. CO treatment increased the phosphorylation of Akt and GSK3-beta (GSK3β) in the liver after I/R injury. Furthermore, administration of LY294002, an inhibitor of PI3K, compromised the protective effect of CO and decreased the level of phospho-GSK3β after I/R injury. These results suggest that CO protects against liver damage by maintaining GSK3β phosphorylation, which may be mediated by the PI3K/Akt signaling pathway. Our study provides additional support for the therapeutic potential of CO in organ injury and identifies GSK3β as a therapeutic target for CO in the amelioration of hepatic injury.

1. Introduction

Hepatic ischemia/reperfusion (I/R) injury is a cause of significant morbidity and mortality after liver transplantation, hemorrhagic shock, and extended liver resection for cancer. The pathophysiology of liver I/R injury includes both initial cellular damage due to ischemia as well as delayed liver dysfunction following reperfusion-initiated and inflammation-induced hepatocellular damage [1]. During I/R injury, Toll-like receptor 4 (TLR4) activation leads to neutrophil infiltration and may promote liver damage through the increase of proinflammatory cytokines. I/R injury can cause chronic inflammation and disease through TLR4 activation [2].

TLR4 activation by lipopolysaccharide (LPS) can be suppressed by the cytoprotective heme oxygenase-1/carbon monoxide (HO-1/CO) system [3]. CO, a reaction product of HO-1 activity, has been shown to have potent anti-inflammatory, antiproliferative, and antiapoptotic effects and thereby mimics the cytoprotective effects of HO-1 [4]. CO, when applied at low concentration, can confer anti-inflammatory effects in macrophages and protect endothelial cells and hepatocytes against cytotoxic agents [5, 6]. Likewise, the exogenous application of gaseous CO also protects against cold hepatic I/R injury in the *ex vivo* isolated liver perfusion model [7]. Although CO inhalation or pharmacological application using CO-releasing molecules (CORMs) has been reported

to ameliorate I/R injury in various animal models [7–9], the molecular mechanisms underlying the cytoprotective effects of the HO-1/CO system on hepatic I/R injury have not been well studied.

Recent studies have shown that inhibition of glycogen synthase kinase 3β (GSK3β) by Ser9 phosphorylation can confer cardioprotective effects during myocardial infarction [10, 11], and ameliorate liver I/R injury [12]. GSK3β activity has recently been identified in a number of studies as crucial in the regulation of the inflammatory response. Phosphatidylinositol-3-kinase (PI3 K)/Akt-dependent inhibition of GSK3β activity in monocytes can regulate TLR-dependent activation [13, 14]. Although hepatic I/R injury has been reported to be ameliorated by inhibition of GSK3β, the molecular mechanisms by which GSK3β confers cytoprotective effects in hepatic I/R injury through TRL4 modulation have not been well studied.

In this study, we establish a signaling pathway by which COz can confer anti-inflammatory protection for liver homeostasis. We demonstrate that CO inhibits GSK3β activity through a PI3 K/Akt-mediated pathway, leading to the downregulation of TLR4-dependent proinflammatory cytokines, and the upregulation of IL-10. Our results further validate the use of CO as a pharmacological cytoprotective agent against hepatic I/R injury and identify GSK3β as a major therapeutic target of CO action in the liver.

2. Materials and Methods

2.1. Animal. Male C57BL/6 wild type (WT) mice at 8–10 weeks of age were purchased from the Orient Bio (Seoul, Korea). Animals were maintained in a specific pathogen-free facility. Animal studies were approved by the University of Ulsan Animal Care and Use Committee.

2.2. Cell Culture. The human hepatocarcinoma cell line (HepG2) and the murine macrophage cell line, RAW 264.7, were cultured in DMEM (Gibco, Grand Island, NY). All media was supplemented with 10% fetal bovine serum and a 100 units/mL penicillin-streptomycin mixture (Gibco).

2.3. Carbon Monoxide Treatment. To evaluate the protective effect of inhaled CO, animals were randomly assigned to receive preconditioning with room air or room air supplemented with 250 parts per million (ppm) CO, for 12 hours in a sealed exposure chamber prior to the experiment. Mice were exposed to CO 250 ppm for 1 hour and 6 hours after reperfusion.

2.4. Mouse Liver I/R Injury Model. We used a well-established mouse model of warm hepatic ischemia followed by reperfusion [15]. An atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90 minutes the clip was removed; mice were sacrificed at various time points of reperfusion. Sham wild-type (WT) controls underwent the same procedure, but without vascular occlusion. Mice were exposed to compressed air or carbon monoxide (CO), at 250 parts per million (ppm). CO or room air was given to the mouse overnight prior to the liver ischemia and during reperfusion. In some experiments, the PI3 K inhibitor LY294002 (Sigma, St Louis, MO, 0.5 mg/kg, *i.p.*) or vehicle (10% DMSO in PBS *i.p.*) was given 30 min prior to the ischemic insult.

2.5. Hepatocellular Damage Assay. To detect serum alanine aminotransferase (sALT), serum was collected from peripheral blood. ALT activity, an indicator of hepatocellular injury, was measured using the EnzyChrom Alanine Transaminase Assay Kit (BioAssay System, Hayward, CA).

2.6. Liver Histology. For histopathological observations, portions of liver were fixed in 10% neutral-buffered formalin solution and then dehydrated in graded alcohol. The fixed tissue was embedded in paraffin and sliced into 4 μm thick sections. Tissue sections were mounted on regular glass slides, deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). Overall pathological changes, including immune cell infiltration and hepatic cell necrosis, were diagnosed according to previously described methods [16].

2.7. Immunohistochemistry. For the detection of p-GS (S641) and p-GSK3β (S9) by immunohistochemistry, the tyramide signal amplification (TSA) biotin system (Perkin-Elmer, Waltham, MA) was used according to the protocols recommended by the manufacturer. Briefly, after blocking, the sections were incubated first with either anti-p-GS (S641) or anti-p-GSK3β (S9) antibody (Cell Signaling Technologies, Danvers, MA). After overnight incubation, the sections were washed and then incubated with biotinylated anti-rabbit IgG antibody, next with streptavidin-horseradish peroxidase (HRP), and then with the biotinyl tyramide amplification reagent. Deposition of the biotin-tyramide on tissue sections was visualized with streptavidin-HRP and the substrate diaminobenzidine (DAB; Merck, Darmstadt, Germany); and then the sections were then counterstained with hematoxylin.

2.8. Immunoprecipitation. Preparation of nuclear extracts was carried out using the Nuclear/Cytosol Fractionation Kit (BioVision, Milpitas, CA). Proteins in the cell lysates were immunoprecipitated with anti-CBP antibodies for 3 h at 4°C, followed by incubation with Dynabeads protein G overnight at 4°C. Proteins in the immunoprecipitates were resolved using SDS-PAGE, followed by Western blotting with anti-phospho-NF-κB p65 and anti-phospho-CREB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

2.9. SDS-PAGE Analysis and Immunoblotting. Harvested liver tissues and cells were lysed with mammalian lysis buffer containing phosphatase and protease inhibitors. Equal amounts of cell lysates were measured with the BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). Lysates were boiled in sample buffer containing β-mercaptoethanol for 5 min. Proteins were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE healthcare, Piscataway, NJ). After blocking with 5% skim milk

in PBS, membranes were incubated with appropriate dilutions of antibodies at 4°C overnight as follows: polyclonal rabbit anti-phospho glycogen synthase kinase (Ser9), rabbit anti-phospho glycogen synthase (Ser641), mouse anti-glycogen synthase kinase, rabbit anti-glycogen synthase, rabbit anti-phospho CREB (Ser133), rabbit anti-phospho Akt (Ser473), rabbit anti-HMGB1 (Cell Signaling Technology, Danvers, MA), rabbit anti-CBP, rabbit anti-phospho-NF-κB-p65 (Ser276), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Membranes were then washed with 0.05% PBS-Tween 20 and incubated with a 1/5000 dilution of HRP-conjugated secondary Abs at room temperature for 1 h. Immunoreactivity was detected using the ECL detection system (GE Healthcare, Piscataway, NJ). Films were exposed at multiple time points to ensure that the images were not saturated.

2.10. Real-Time and Semiquantitative RT-PCR. Total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA). Three microgram of total RNA was used to synthesize the first-strand cDNA by using oligo-dT primers (QIAGEN, CA) and M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. The synthesized cDNA was subjected to the PCR-based amplification. Semiquantitative RT-PCR was performed using Taq polymerase (Solgent, Daejeon, Korea). Real-time PCR was performed using SYBR Green PCR Master Mix (Qiagen, Valencia, CA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island NY). PCR primer pairs were as follows: TNF: 5′-AGA CCC TCA CAC TCA GAT CAT CTT C-3′, 5′-TTG CTA CGA CGT GGG CTA CA-3′, IL-6: 5′-CGA TGA TGC ACT TGC AGA AA-3′, 5′-TGG AAA TTG GGG TAG GAA GG-3′ and IL-10: 5′-CAG TAC AGC CGG GAA GAC AA-3′, 5′-CAG CTT CTC ACC CAG GGA AT-3′.

2.11. Myeloperoxidase Assay. Neutrophil sequestration in liver was quantified by measuring tissue MPO activity. Tissue samples for MPO analysis were frozen in liquid nitrogen immediately after removal from the animal and were thawed and homogenized and centrifuged to remove insoluble materials. MPO activities were measured using a mouse myeloperoxidase DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. The supernatants were analyzed for MPO levels by sandwich ELISA. The levels of MPO in organ extracts were expressed as ng/mg of protein.

2.12. Statistical Analysis. All data were expressed as mean ± SD. Differences between experimental groups were compared using the Student's two-tailed unpaired t-test.

3. Results

3.1. Carbon Monoxide Inhalation Protects Liver Ischemia/Reperfusion Injury via AKT-GSK3β Activation in Mice. Carbon monoxide (CO) has been shown to exert protective effects in various tissue models of I/R injury [8, 9, 17]. We analyzed the effect of CO on hepatocellular function in mouse livers subjected to 90 min of warm ischemia followed by 6 h reperfusion. As shown in Figure 1(a), sALT levels in mice subjected to hepatic I/R were decreased in animals pretreated with CO gas, as compared with room air (3225 ± 891 U/L *versus* 1091 ± 230 U/L, respectively, $P < 0.01$). Figure 1(b) shows that hepatocellular necrosis (*panels* (A)–(C)) and immune cell infiltration (*panel* (D)–(F)) observed in the air-treated I/R group were markedly reduced with CO inhalation. Furthermore, myeloperoxidase (MPO) activity, reflecting liver neutrophil activity, was decreased in the CO inhalation group, compared with the air-treated group after hepatic I/R (5.371 ± 0.902 ng/mg *versus* 10.468 ± 1.700 ng/mg, resp., $P < 0.01$) (Figure 1(c)). Since HMGB1, an inflammatory cytokine, can promote liver damage following I/R injury [18, 19], we examined HMGB1 expression in our hepatic I/R model. As shown in Figure 1(d), I/R caused an increase in hepatic HMGB1 expression when compared with sham control mice. CO inhalation markedly attenuated the expression of HMGB1 during I/R injury compared to air-treated mice subjected to I/R (Figure 1(d)).

Previous reports have shown that HO-1 derived CO can activate the PI3 K-Akt pathway [20] and thereby confer protection against cardiac I/R injury [21]. To investigate whether CO activates Akt signaling in hepatic cell lines, we assessed the expression of phosphorylated (p)-Akt and of GSK3β, which is negatively regulated by Akt. As shown in Figure 1(e), CO increased the phosphorylation of both Akt and GSK3β in HepG2 cells. Conversely, CO decreased the phosphorylation of GS, the substrate of GSK3β.

A previous study has shown that both endogenous and exogenous CO can increase cellular ROS generation [22]. ROS can exert a critical role in maintaining homeostasis by protecting the host against excessive inflammatory responses [23]. Because activation of the phosphatidylinositol 3-kinase (PI3 K)/Akt pathway by ROS signaling can function in cellular adaptation [24, 25], we sought to determine whether CO-mediated ROS generation can increase LPS-induced phosphorylation of Akt and GSK3. As shown in Figure 1(f), we used the ROS scavenger N-acetyl-cysteine (NAC) to determine whether phosphorylation of Akt and GSK3β was modulated by ROS generation. Treatment with CO dramatically increased LPS-induced phosphorylation of Akt and GSK3β. Inhibition of ROS generation with NAC attenuated LPS/CO-induced phosphorylation of Akt and GSK3β. These results suggest that CO stimulates LPS-dependent PI3 K/Akt-GSK3β phosphorylation *via* enhanced ROS production.

Therefore, to examine whether CO activates PI3 K/Akt signaling during hepatic I/R injury, we examined the expression of p-Akt in mice subjected to hepatic I/R. There was a significant increase in p-Akt in the CO-treated mice subjected to hepatic I/R injury at 1 hour of reperfusion relative to air-treated controls, which was sustained until 6 hours of reperfusion (Figure 1(g)). These findings suggested that the Akt-GSK3β axis may be involved in CO-mediated protection in the liver I/R model.

3.2. The Protective Effect of CO in Liver Ischemia/Reperfusion Injury Is Dependent on the Inactivation of GSK3 Activity. I/R stimulation has been previously shown to trigger GSK3β

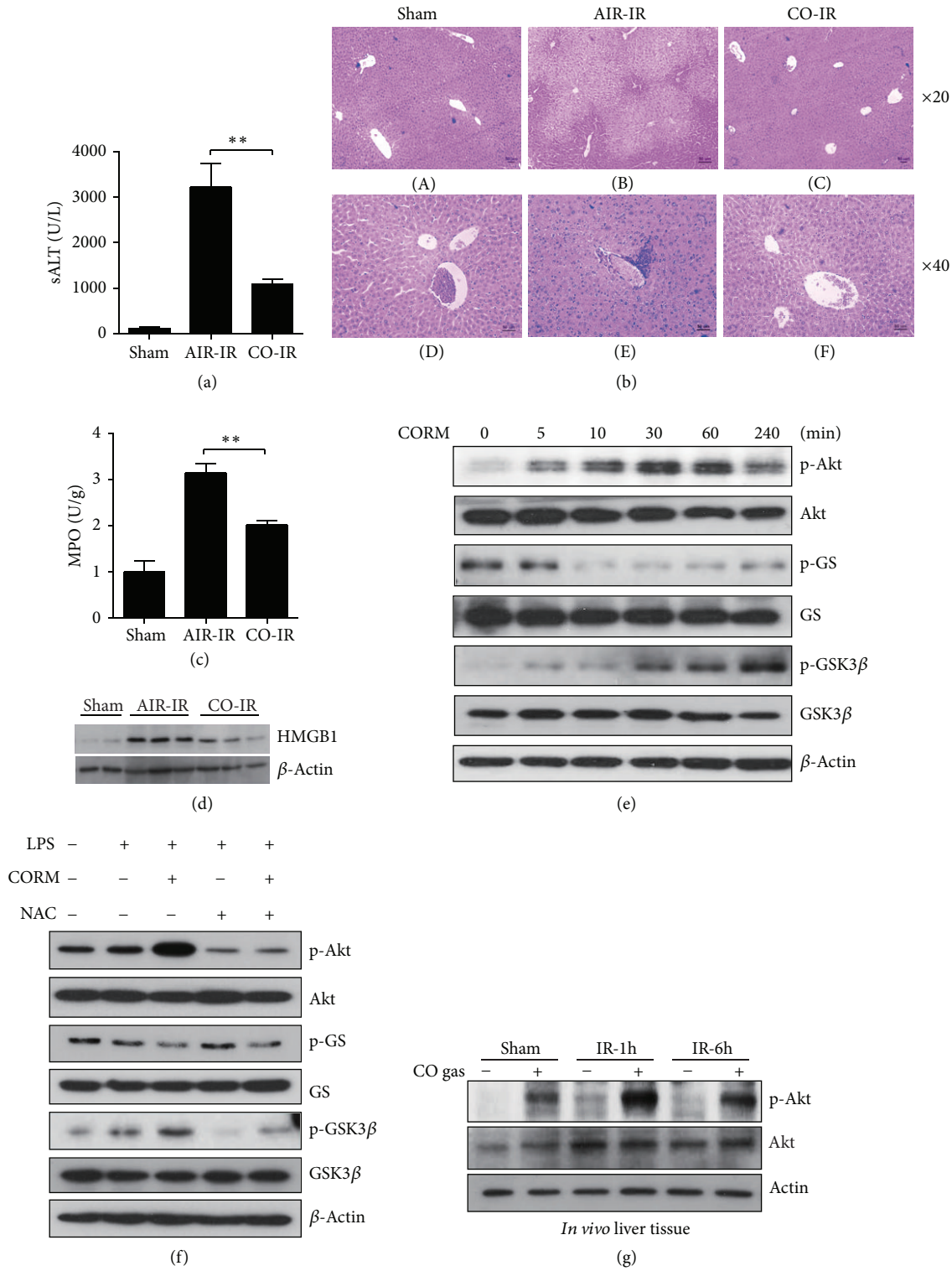

Figure 1: Pretreatment of mice with CO gas inhalation ameliorates liver I/R injury via AKT-GSK3β activation. Mice were subjected to 90 minutes liver warm ischemia, followed by 6 h reperfusion. (a) Hepatocellular function was evaluated by sALT (IU/L). (b) Representative liver histology of ischemic liver lobes. (c) Liver neutrophil accumulation, assessed by MPO activity. Data represent the mean ± standard deviation (SD) (N = 4–6 samples/group). $^{**}P < 0.01$. (d) Hepatic HMGB1 expression in liver tissue was assessed by Western blot analysis at 1 h and 6 h of reperfusion. Total cell lysates were analyzed for HMGB1 and β-actin protein levels by Western blot analysis. (e) Western-blot analysis of phospho (p)-GSK3β (Ser 9), p-GS (Ser641), and p-Akt in HepG2 cells after treatment with CORM2 (50 μM) at the indicated times. (f) RAW264.7 cells were stimulated with 10 ng/mL of LPS for 30 minutes in the absence or presence of CORM2 and the ROS scavenger, N-acetyl-cysteine (NAC). Total cell lysates were analyzed for phosphorylated GS, GSK3β, and Akt as well as total GS, GSK3β, Akt, and β-actin protein levels by Western immunoblot analysis. (g) Mice were subjected to 90 minutes of liver warm ischemia, followed by 1 h or 6 h reperfusion. Liver tissue was analyzed by Western blotting of p-Akt and total Akt. β-Actin served as the standard.

phosphorylation in mouse liver as a self-regulatory mechanism [12]. Given that inhibition of GSK3β is involved in amelioration of liver pathology, we hypothesized that GSK3β inhibition may represent the underlying mechanism for CO-mediated liver protection. To evaluate the potential role of GSK3β in CO-mediated liver protection, we analyzed the effects of CO on GSK3β phosphorylation at Ser-9 and glycogen synthase (GS) phosphorylation at Ser-641, the substrate of GSK3β. As shown in Figure 2(a), mice exposed to CO inhalation displayed marked increases in the phosphorylation of GSK3β in the ischemic livers, compared with the sham control and air-inhaled groups. Conversely, the level of GS phosphorylation was decreased in ischemic mice given CO inhalation, compared with the air inhalation group. According to a previous report (26), the decrease of GS phosphorylation that occurs in response to I/R stimulation alone is due to GS phosphatase activation by I/R. Thus, transient ischemia induces GS activation and glycogen synthesis *in vivo*. These observations suggest that this occurs through a different mechanism than through the increase of pGSK3-β, as observed with CO treatment.

Previous studies have demonstrated that the cytoprotective effects of CO involve p38 MAPK signaling [7]. Consistently, we found that phosphorylated p38 MAPK was increased by CO inhalation in the livers of mice subjected to hepatic I/R, compared with air-treated control mice (Figure 2(a)). However, JNK phosphorylation was not altered by CO.

We used immunohistochemistry to examine whether CO-mediated liver protection was associated with GSK3 inhibition. As shown in Figure 2(b), inhalation with CO gas increased GSK3β phosphorylation in ischemic liver and reduced GS phosphorylation, as compared with the air-inhalation group. Consistent with immunohistochemistry data, decreased mRNA levels of TNF-α, IL-6, and CXCL10 were consistently found in the livers of mice subjected to CO-inhalation, compared with those of the air-inhalation group (Figure 2(c)). TNF-α and IL-6 proteins production was also inhibited by CO (Figure 2(d)). The anti-inflammatory cytokine IL-10 was significantly increased by CO inhalation in mice subjected to hepatic I/R injury (Figures 2(c) and 2(d)).

3.3. CO Dependent Inhibition of GSK3 and Attenuation of Liver Injury Involve PI3 K Signaling in Mice. The PI3 K-Akt pathway has been shown *in vitro* to regulate GSK3β phosphorylation through the activation of TLR4 [26]. We used LY294002, an irreversible PI3 K-specific inhibitor, to address the functional role of PI3 K/Akt signaling in CO-mediated liver cytoprotection. Indeed, LY294002-treated mice displayed significantly lower levels of phosphorylated GSK3β in the liver after I/R (Figure 3(a)). Increased sALT levels were consistently found in mice treated with the PI3 K inhibitor with or without CO inhalation (Figure 3(b), 11791 ± 940 U/L and 9012 ± 3657 U/L, resp.), compared with CO inhalation alone (2423 ± 1145 U/L, $P < 0.01$) (Figure 3(b)). Unlike mice pretreated with CO, which displayed minimal liver damage (Figure 3(c), *panel* (D)), mice given the PI3 K inhibitor revealed significant hepatocellular necrosis, cytoplasmic vacuolization, and sinusoidal congestion (*panel* (C)). Livers of animals treated with the PI3 K inhibitor after CO inhalation showed moderate to severe hepatocellular changes (*panel* (E)). As shown in Figure 3(d), MPO levels were elevated in PI3 K inhibitor-treated mice (12.458 ± 1.947 ng/mg), compared with DMSO controls (7.864 ± 0.891 ng/mg, $P < 0.01$). In contrast, livers from the CO inhalation group showed decreased MPO activity (3.738 ± 1.203 ng/mg), compared with the group subjected to PI3 K inhibitor treatment after CO inhalation (9.964 ± 3.099, $P < 0.01$). Thus, PI3 K/Akt-dependent GSK3β phosphorylation, a therapeutic target of CO, serves as a self-regulatory mechanism of liver homeostasis to limit the excessive I/R-induced tissue damage.

3.4. CO-Mediated GSK3 Inhibition via PI3 K/AKT Signaling Regulates the LPS-Mediated Inflammatory Response In Vitro. Previous reports have shown that LPS stimulation in monocytes can result in Ser9 phosphorylation of GSK3β in a PI3 K/Akt-dependent pathway [27] and that this pathway downregulates TLRs-dependent inflammatory responses. To investigate the cellular mechanisms underlying our *in vivo* findings, we analyzed whether CO-dependent activation of the PI3 K/Akt pathway may regulate TLR4-dependent inflammatory responses through GSK3β inhibition *in vitro*. LPS-stimulated macrophages were first pretreated with the PI3 K inhibitor (LY294002) and then treated with a CO releasing molecule (CORM2). Western blot analysis showed that treatment with CORM2 dramatically increased LPS-induced phosphorylation of Akt, GSK3β, and its downstream target, the cAMP response element-binding protein (CREB). However, pretreatment with PI3 K inhibitor (LY294002) inhibited phosphorylation of GSK3β, Akt, and CREB in LPS-stimulated macrophages treated with CORM2 (Figure 4(a)). In addition, GSK3β inactivation by CORM2 significantly reduced TNF-α and increased IL-10 gene expression in response to LPS (Figure 4(b)). The modulatory effects of CORM2 on TNF-α and IL-10 expression were inhibited by LY294002. These results demonstrate that CO-induced GSK3β inactivation is mediated by the PI3 K/Akt signaling pathway and consequently modulates the TLR4-driven inflammatory response.

3.5. Negative Regulation of GSK3 Activity by CO Affects the Associations of NF-κB p65 and CREB with CBP That Regulates the Production of IL-10. We next sought to validate the cellular mechanism by which CORM2-induced phosphorylation of GSK3β leads to the upregulation of the anti-inflammatory cytokine IL-10. Previous studies have identified CREB, a target of GSK3β, as an important transcription factor regulating IL-10 production in monocytes [28]. GSK3β can negatively regulate the activation and DNA-binding activity of CREB [28]. GSK3β increases the binding of NF-κB p65 to the coactivator CREB-binding protein (CBP), leading to pro-inflammatory gene activation, which competes against the binding of CREB to CBP, the latter regulating IL-10 expression [27].

We sought to determine whether GSK3β inactivation by CORM2 influenced the ability of CREB and NF-κB p65 to associate with CBP. As shown in Figures 4(a) and 4(b),

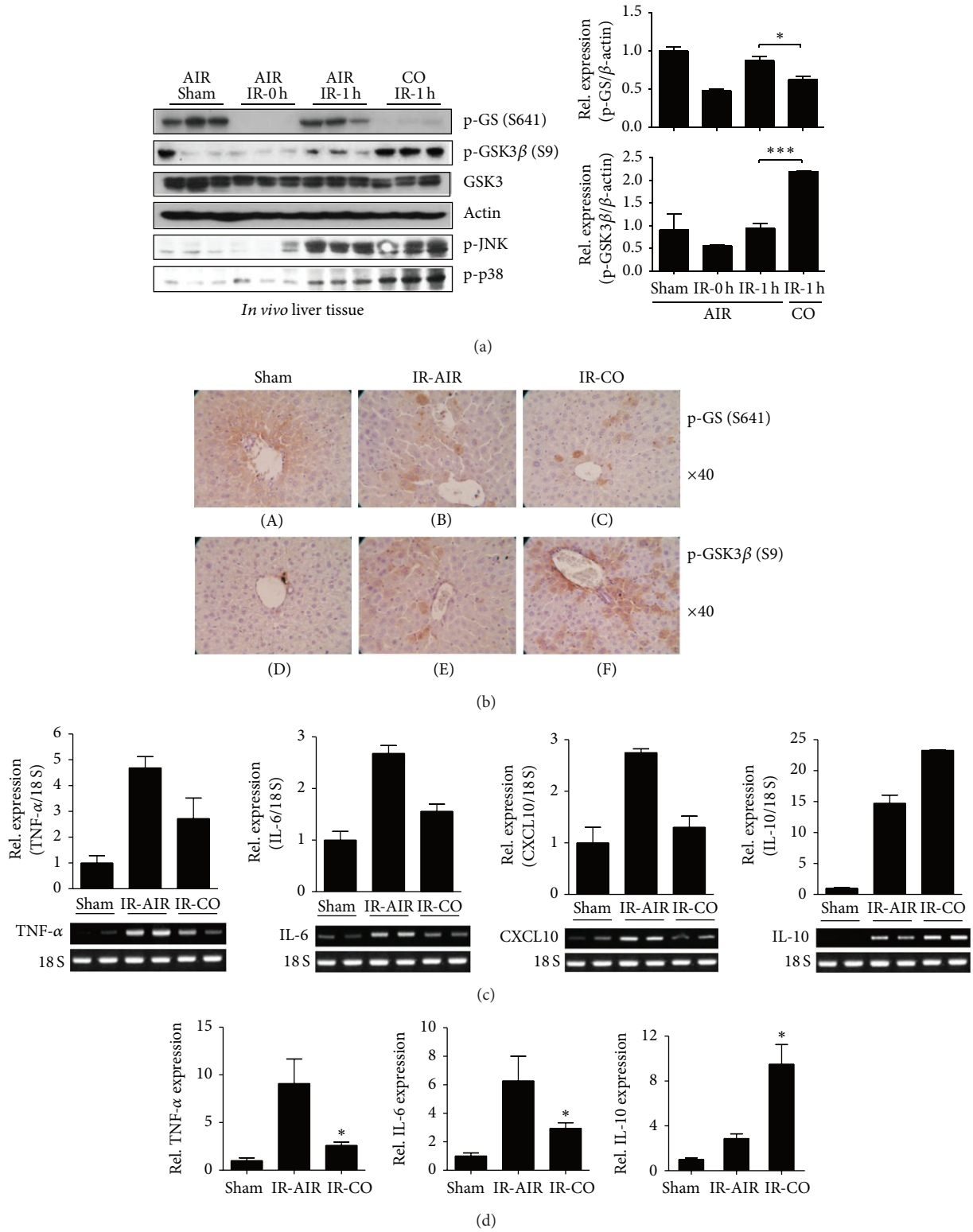

Figure 2: Inhibition of GSK3β by CO inhalation ameliorates liver I/R injury. Mice were sham-operated or subjected to 90 minutes hepatic warm ischemia followed by 1 hour reperfusion. Recipients were treated with air or CO gas (250 ppm) inhalation. (a) Liver samples, harvested 1 hour later, were subjected to Western blot analysis of phospho (p)-GS (GS 641), p-GSK3β (S9), p-JNK, and p-p38. β-Actin was used as an internal control. $^{*}P < 0.05$, $^{***}P < 0.001$. (b) Immunohistochemical staining of GSK3 inhibition. Mice were sacrificed, liver tissues were harvested, and the tissue slices were processed for formalin-fixed paraffin embedding. Hematoxylin counterstaining after 3-amino-9-ethylcarbazole-based immunohistochemical staining was used to detect GS 641 ((A)–(C), red/brown) and phosphorylation of GSK3β S9 ((D)–(F), red/brown). ((c) and (d)) Quantitative RT-PCR-assisted detection of TNF-α, IL-6, CXCL10, and IL-10 gene expression at 1 hour or 6 hours in liver tissue. Data were normalized to 18S gene expression. Data shown represent the mean ± S.D. (N = 4-5/group), $^{*}P < 0.05$.

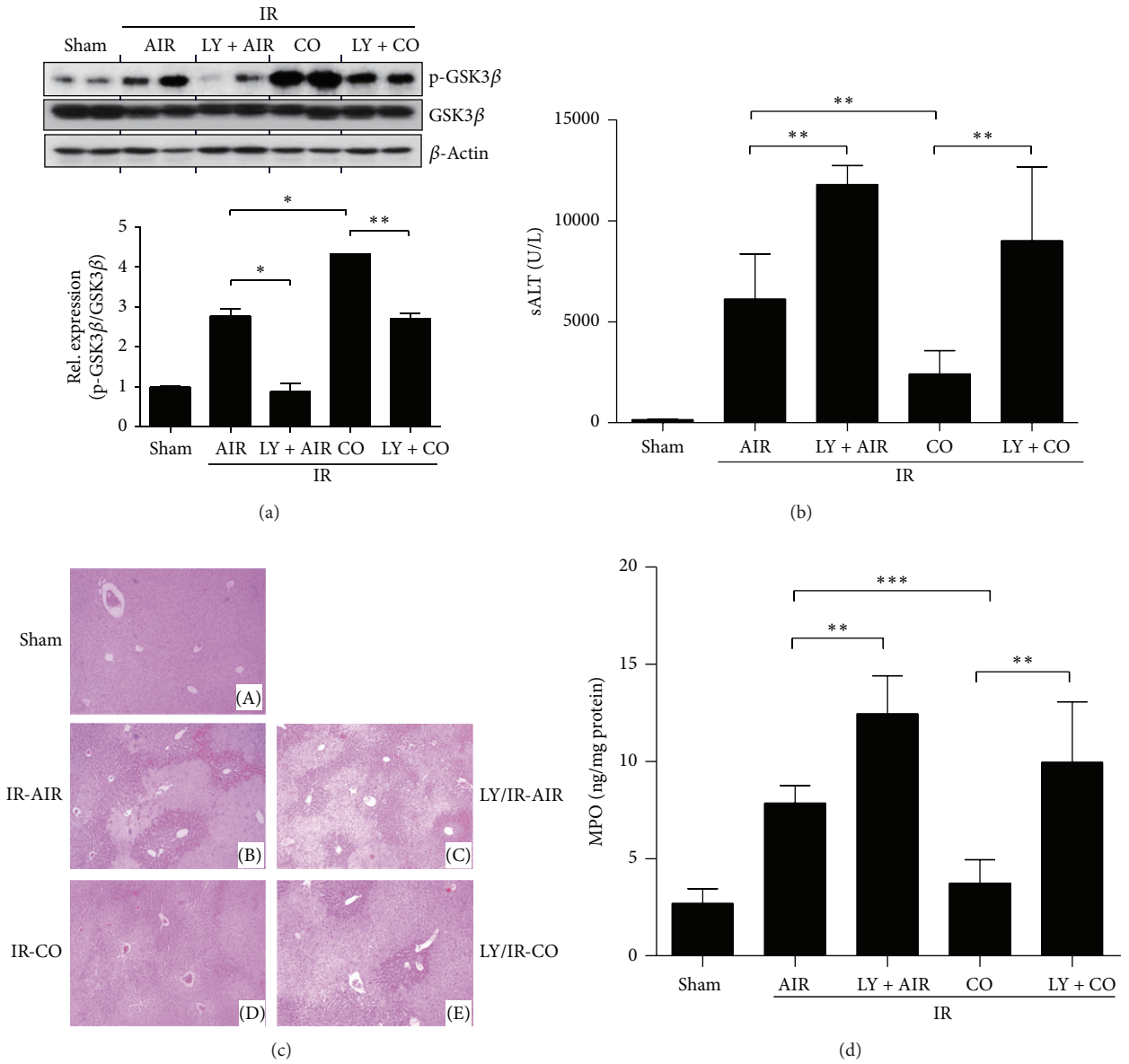

Figure 3: PI3 K blockade restores liver I/R injury in mice pretreated with CO inhalation. Mice were treated with CO gas (CO), LY294002 (LY), or both or vehicle at 30 minutes prior to the liver ischemia insult, as described in Materials and Methods. Liver samples were harvested at 6 hours after-reperfusion. (a) Proteins were analyzed by Western blotting with Abs against phosphorylated or total GSK3β and β-actin. Sixty minutes ischemia time was used to show the effect of PI3 K inhibition in liver I/R injury. $^{*}P < 0.05$, $^{**}P < 0.01$. (b) Average sALT levels in different experimental groups were measured. sALT levels were measured at 6 hours of reperfusion. (c) Representative liver histology (H&E staining) is shown. To establish the functional relationship between PI3 K and GSK3β, CO inhalation was administered 12 hr and LY 30 minutes prior to the ischemic insult and 6 hr after reperfusion. (d) Liver neutrophil accumulation, assessed by MPO activity. Data represent mean ± S.D. (N = 4–6 samples/group). $^{**}P < 0.01$, $^{***}P < 0.001$.

LPS-stimulated RAW 264.7 cells had increased association of p65 with CBP relative to unstimulated control cells. However, LPS-stimulated cells that were pretreated with CORM2 showed a considerable decrease in the association of p65 with CBP (Figure 4(c)), whereas the binding of CREB to CBP was potently augmented (Figure 4(d)), consistent with GSK3β inhibition.

4. Discussion

In the current study, we identify the GSK3β pathway as a novel therapeutic target of the anti-inflammatory effects of CO in a hepatic I/R injury model. The GSK3β pathway has previously been identified as an important target of inflammatory regulation. GSK3β is a proline-directed Ser/Thr

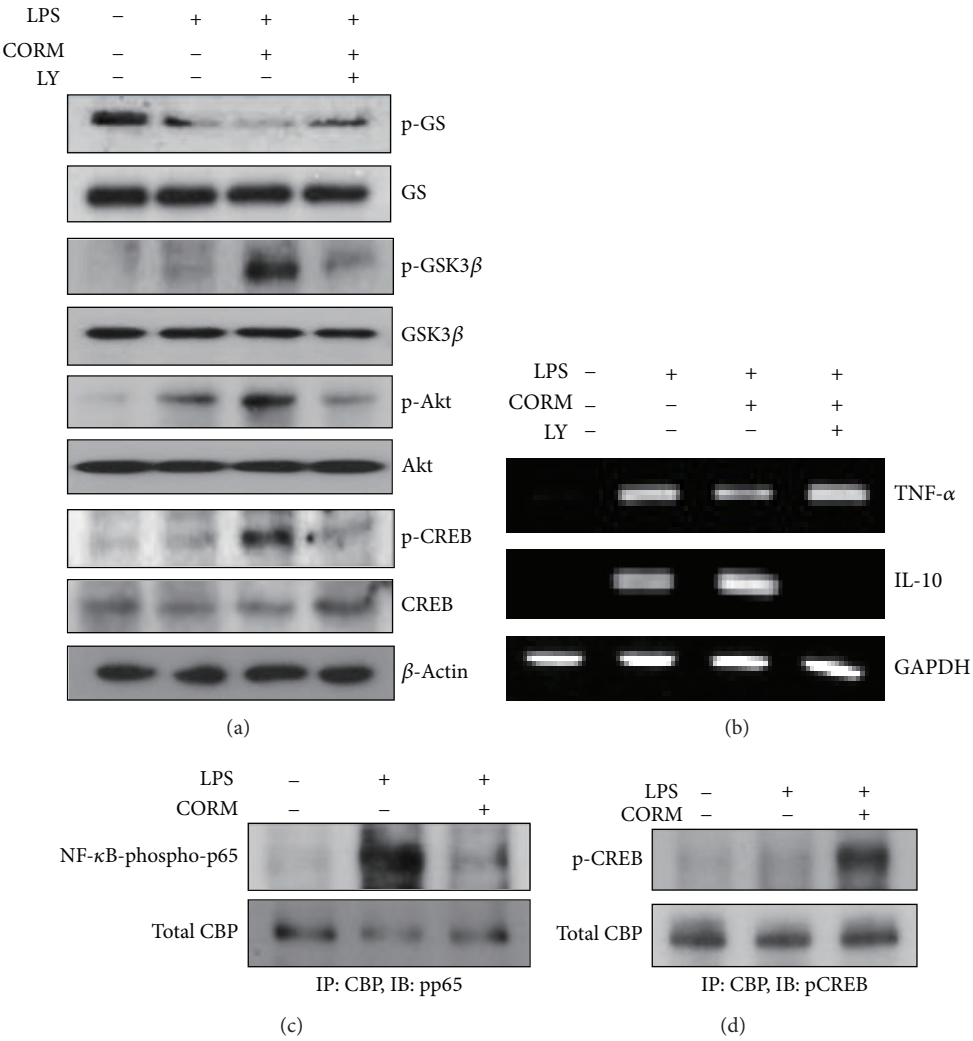

Figure 4: CO-induced PI3 K/Akt-dependent GSK3 inactivation regulates TLR4 responses and affects the ability of CREB and NF-κB-p65 to associate with CBP. RAW264.7 cells were stimulated with 100 ng/mL of LPS for 1 hour in the absence or presence of CORM2 and the PI3 K inhibitor (LY294002). (a) Total cell lysates were analyzed for phosphorylated GS, GSK3β, Akt, and CREB as well as total GS, GSK3β, Akt, CREB, and β-actin protein levels by Western immunoblot analysis. (b) The mRNA expression of TNF-α and IL-10 was determined by semiquantitative RT-PCR. GAPDH was used as an internal control. ((c) and (d)) RAW264.7 cells were stimulated with 0.1 μg/mL of LPS for 1 hour in the absence or presence of CORM2 (50 μM) and nuclear extracts were obtained. Interaction of CBP with NF-κB p65 and CREB was assessed by immunoprecipitation of CBP followed by immunoblotting for NF-κB or CREB. Total CBP served as the input standard.

kinase that phosphorylates a number of substrates including glycogen synthase (GS) as well as constituents of numerous intracellular signaling pathways including SMAD3, β-catenin, NOTCH2, CREB, and others [29–31]. Recent studies show that downregulation of GSK3β can negatively regulate the inflammatory response and protect mice from endotoxin shock [26]. In this model, GSK3β inhibition was associated with increased cAMP-response element binding (CREB) protein DNA binding activity, resulting in the increased production of the anti-inflammatory cytokine IL-10 and the decreased NF-κB-dependent production of proinflammatory genes [26]. Recent studies have shown that inhibition of GSK3β ameliorates liver I/R injury through an IL-10 mediated immune regulatory mechanism [12]. During myocardial infarction, GSK3 inhibition with pharmacological inhibitors has been shown to exert a cardioprotective effect, potentially related to inhibition of mitochondrial permeability transition pore opening [10].

Carbon monoxide (CO), which can be applied by inhalation or by pharmacological delivery with CORMs, continues to show promise as an anti-inflammatory therapeutics in several models of organ I/R injury. For example, inhaled CO conferred tissue protection in rodents subjected to lung I/R injury, as evidenced by reduced markers of apoptosis, which depended on activation of the MKK3/p38 MAPK pathway [8, 9]. Additional mechanisms for CO-mediated protection during lung I/R include the derepression of the fibrinolytic axis and downregulation of the proinflammatory factor Egr-1

[32]. Furthermore, several studies demonstrated that pretreatment with CO donor compounds can ameliorate lung transplant-associated I/R injury with increased hepatic HSP70 expression [33] and can cause suppression of inflammatory responses *via* downregulation of the MEK/ERK1/2 signaling pathway [7].

In rodent models, CO has been shown to protect against acute liver injury caused by TNFα challenge [6] and can confer anti-inflammatory protection in hepatic I/R injury models [7, 17, 34]. CO preserved hepatic function *ex vivo* in an isolated perfused liver model subjected to cold ischemia injury, in part by upregulating the p38 MAPK pathway [34]. CO has been shown to protect against I/R injury during orthotropic rat liver transplantation by downregulating proinflammatory mediators, including TNFα and iNOS expression [17]. Furthermore, the protection afforded by CO in this model was also associated with the modulation of STAT1/STAT3 and inhibition of the MEK/ERK1/2 signaling pathway [7].

ROS have been shown to be involved in the counter-regulation of inflammation in response to LPS treatment through modulation of macrophage production of IL-10 [23]. Additionally, CO can act *via* inhibition of cytochrome *c* oxidase leading to the generation of low levels of reactive oxygen species (ROS) that mediate adaptive signaling pathways [35]. Our data demonstrate that CO inactivates GSK3β through a mechanism that involves increased ROS-induced Akt phosphorylation. These results provide evidence that the protective effects of CO are mediated through redox mechanisms that can lead to the activation of adaptive pathways.

The phosphatidylinositol-3-kinase (PI3 K)/Akt pathway represents another multifunctional signaling pathway that promotes cell survival under adverse conditions. Previous studies have implicated the PI3 K/Akt pathway in the cytoprotective effects of CO. For example, during anoxia/reoxygenation of pulmonary endothelial cells, CO treatment protected against apoptosis by upregulating the p38 MAPK and PI3 K/Akt-dependent STAT3 pathway. The protection afforded by CO in a cardiac I/R injury model *in vivo* has also been shown to be dependent on the activation of the p38 MAPK and PI3 K/Akt pathways.

The PI3 K/Akt pathway has been identified as an important regulator of GSK3 signaling. PI3 K/Akt-dependent inhibition of GSK3β activity in monocytes regulates TLR-dependent activation of inflammatory responses [13, 14]. Activation of the PI3 K/Akt pathway resulting in Ser9 dependent phosphorylation of GSK3β signaling pathway and inhibition of NF-κB nuclear translocation were shown to contribute to cardioprotection during myocardial I/R injury [11]. Consistent with these observations, our *in vivo* data demonstrate that CO activates PI3 K/Akt signaling to promote GSK3β inhibition through phosphorylation at Ser9 during hepatic I/R injury. Furthermore, we have shown that activation of this pathway by CO therapy is a crucial mediator of the protection afforded by CO against hepatic injury and inflammation during I/R injury in mice.

GSK3β can differentially regulate TLR4-dependent signaling leading to modulation of anti-/proinflammatory cytokine balance. LPS stimulation in monocytes has been shown to result in Ser9 phosphorylation of GSK3β in a PI3 K/Akt-dependent pathway [26], which downregulates TLRs-dependent inflammatory responses. Hepatic warm ischemia and reperfusion (I/R) injury and inflammation are largely TLR4-dependent, whereas TLR4 appears to have marginal role in the early liver inflammatory response [36]. GSK3β inhibition was associated with hepatoprotection through the augmentation of the expression of the anti-inflammatory cytokine IL-10. Consistently, our results demonstrate that GSK3β inactivation by CO results in IL-10 upregulation in macrophages through a mechanism involving the augmentation of the binding of CREB to the nuclear co-activator CBP, leading and the suppression of the binding of NF-κB p65 to the nuclear coactivator CBP.

In summary, our results establish a signaling pathway by which CO can confer anti-inflammatory protection in the liver: CO activates PI3 K/Akt which results in inhibition of GSK3β through Ser9 phosphorylation, leading to the downregulation of TLR4-dependent proinflammatory cytokines, and the upregulation of IL-10. The latter effect is mediated through activation of CREB and disruption of the p65/CBP interaction. Our results further validate the use of CO as a pharmacological cytoprotective agent against hepatic I/R injury and identify GSK3β as a major therapeutic target of CO action in the liver.

Conflict of Interests

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

Acknowledgments

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Research Article

Mild Systemic Oxidative Stress in the Subclinical Stage of Alzheimer's Disease

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Alzheimer's disease (AD) is a late-onset, progressive degenerative disorder that affects mainly the judgment, emotional stability, and memory domains. AD is the outcome of a complex interaction among several factors which are not fully understood yet; nevertheless, it is clear that oxidative stress and inflammatory pathways are among these factors. 65 elderly subjects (42 cognitively intact and 23 with probable Alzheimer's disease) were selected for this study. We evaluated erythrocyte activities of superoxide dismutase, catalase, and glutathione peroxidase as well as plasma levels of total glutathione, α-tocopherol, β-carotene, lycopene, and coenzyme Q10. These antioxidant parameters were confronted with plasmatic levels of protein and lipid oxidation products. Additionally, we measured basal expression of monocyte HLA-DR and CD-11b, as well as monocyte production of cytokines IL1-α, IL-6, and TNF-α. AD patients presented lower plasmatic levels of α-tocopherol when compared to control ones and also higher basal monocyte HLA-DR expression associated with higher IL-1α production when stimulated by LPS. These findings support the inflammatory theory of AD and point out that this disease is associated with a higher basal activation of circulating monocytes that may be a result of α-tocopherol stock depletion.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder resulting in progressive neuronal death and memory loss [1]. Histopathologically, the disease is characterized by intraneuronal neurofibrillary tangles (NFT) (aggregations of a hyperphosphorylated form of the microtubule-associated protein tau) and extracellular deposits of neuritic plaques composed of amyloid-β (Aβ) protein, a 40–43 amino acid proteolytic fragment derived from the amyloid precursor protein [2].

Although AD is considered a neurodegenerative disease, both inflammation and oxidative stress are present early and throughout its pathogenesis. One of the early clues as to AD as an inflammatory process was the finding that rheumatoid arthritis patients taking high levels of anti-inflammatory drugs had a low incidence of AD [3]. Inflammatory mechanisms may significantly contribute to disease progression and chronicity, with proinflammatory cytokines (notably IL-1α and IL-1β) being found throughout the brain of individuals with AD when analyzed at autopsy [1]. Many neuroinflammatory mediators including complement activators and inhibitors, chemokines, cytokines, reactive species, and inflammatory enzymes are generated and released by microglia, astrocytes, and neurons [4].

However, the precise implications of the inflammatory response for neurodegeneration have not been elucidated. A current hypothesis considers that an extracellular insult to neurons could trigger the production of inflammatory cytokines by astrocytes and microglia. The cytokines, namely, IL-1β, TNF-α, and IL-6, could affect the normal behavior of neuronal cells. Therefore, dysfunction at this core level may lead to abnormalities such as neurofibrillary degeneration in AD [5].

It is well characterized that there is oxidative stress in the AD brain. Lower plasma antioxidant levels and alterations in antioxidant enzyme activities were reported in mild cognitive impairment (MCI) patients and patients at early AD stages, suggesting an imbalance between reactive species production and antioxidant defense systems in the plasma of AD patients. This was substantiated by an increase in DNA, lipid, and protein oxidation products found in blood and cerebrospinal fluid (CSF) obtained from AD patients in comparison with controls [6].

At the same time microglial cells activation is prominent during AD. Microglia are bone marrow-derived cells of the monocyte lineages that, like peripheral tissue macrophages, become phagocytic and produce reactive oxygen species [3]. However, the evaluation of microglia activity during the development of AD in human patients is impossible due to its localization, thus justifying demand for models that use parameters of easy access and reflecting the state of activation of microglia. However, the studies with monocytes could contribute to the understanding of inflammatory component in AD, establishing, at the same time, the possibility of using a minimally invasive technique, such as blood collection.

As exposed, AD has an important component that involves inflammatory reactions associated with reactive species release and lower antioxidant levels. Thus, this paper aims at verifying blood oxidative markers and monocyte inflammatory parameters in elderly subjects who present with very mild cognitive impairment.

2. Methods

2.1. Subjects. Patients participating in this study were part of the last wave of Epidoso Project receiving monitoring on aging by a multidisciplinary team [7].

65 elderly subjects (mean age 82 years) were selected for this study. All patients had their functional capacity and laboratory profile evaluated. These seniors were initially subjected to a clinical survey conducted by a geriatrician, consisting of history taking, physical examination, and evaluation of mental autonomy, through the degree of cognitive function. Patients with a history of cardiovascular disease, cancer, or chronic inflammatory diseases as well as individuals with current inflammatory alterations, assessed by erythrocyte sedimentation rate or plasma levels of C-reactive protein, were not included in this study.

The study protocol was submitted to the Ethics Committee of UNIFESP and was approved under number 0859/03. All patients or their guardians signed informed consent.

2.2. Assessment of Patients' Cognitive Ability. Minimental state examination was used for screening cognitive deficits among patients [8]. The maximum score is 30 and scores lower than 24 were considered indicative of some kind of cognitive dysfunction.

Patients who scored less than 24 points in MMSE were then tested with CDR (Clinical Dementia Rating) [9, 10].

Patients were considered cognitively intact (INT group; $n = 42$) when CDR was <1, while others were included in the group of patients with probable Alzheimer's disease (AD group; $n = 23$) if CDR was ≥1.

2.3. Measurements of Erythrocytes Parameters. Blood samples from participants were obtained after 12 hours of fasting. Part of whole blood was used for flow cytometry. After plasma separation, erythrocytes were used to measure total glutathione content (TGSH) [11] and the remaining hemolysate to assess the activities of antioxidant enzymes [7], Cu-Zn superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), all adjusted by hemoglobin concentration.

2.4. Measurements of Plasma Parameters. Plasma samples were stored at −80°C for later determination of α-tocopherol, β-carotene, lycopene, coenzyme Q10, and vitamin C [7]. For extraction, 20 μL plasma aliquots were mixed with 100 μL extraction solvent (50% ethanol proanalysis (pa), 50% 1-butanol pa, containing 5 mg butylhydroxytoluene/mL, and 8 μmol Tocol/L as an internal standard) to extract the vitamins and to denature plasma proteins. The sample was then mixed by vortex for 30 s and centrifuged at 21 000 ×g for 10 min at 4°C. The supernatant was transferred to a polypropylene vial. This method uses precolumn and reverse phase C18 column and UV-VIS detector with deuterium lamp, with a mobile phase consisting of 80% acetonitrile, 3% methanol (100 mM ammonium acetate, 0.1% triethylamine), and 15% dioxan. The mobile phase is pumped through the system by a flow of 1.5 mL/min with maximum pressure of 350 Kgf and the minimum of 0. The wavelength was established by the method for each parameter and run time for it was 4 to 6 minutes.

Plasma was also used to determine the concentration of thiobarbituric acid reactive substances (TBARS) [7] and level of oxidized proteins, expressed as plasma carbonyls [12].

2.5. Monocyte Activation Parameters. In order to evaluate basal activation of circulating monocytes, cell surface HLA-DR and CD-11b levels were assessed by flow cytometry, as described elsewhere [13]. Monocytes were identified by monoclonal antibodies anti-CD14-pernidin chlorophyll protein (PerCP); neutrophils were excluded with CD66b-fluorescein isothiocyanate (FITC) labeling. Cell surface molecules were marked with anti-HLA-DR-phycoerythrin (PE) and anti-CD11b-allophycocyanin (APC); controls were incubated with anti-HLA-DR-PE and CD11b-APC isotypes.

Intracellular production of IL-1α, IL-6, and TNF-α was quantified by flow cytometry in monocytes stimulated with LPS and treated with monensin in order to prevent

Table 1: Oxidative stress parameters in erythrocyte (RBC). Antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and total glutathione level (TGSH). INT (cognitively intact patients; $n = 42$) and AD (patients with probable Alzheimer's disease; $n = 23$). Values shown correspond to the means ± SEM. Comparison between average results of the groups was performed using Student's t-test for independent samples.

Parameter	INT	AD
RBC SOD (U/mg Hb)	7.82 ± 0.24	8.2 ± 0.31
RBC CAT (U/mg Hb)	187 ± 7	184 ± 8
RBC GPx (U/mg Hb)	12.8 ± 0.4	12.6 ± 0.7
RBC TGSH (mmol/Hb)	7.67 ± 0.21	7.45 ± 0.36

$P < 0.05$ was considered significant.

cytokine release [13]. Monocytes were identified by monoclonal antibodies for surface staining (anti-CD66b-FITC and anti-CD14-PerCP) and cytokine production was detected using monoclonal antibodies anti-IL-1α-PE, anti-IL-6-PE, and anti-TNFα-APC or their respective isotype controls.

Data were acquired using a FACSCalibur flow cytometer and CellQuest software (both from BD Immunocytometry Systems).

2.6. Reactive Oxygen Species Assay. Whole blood samples were first stained with anti-CD14 PerCP for monocyte identification; samples were subsequently incubated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA), a stable compound, nonfluorescent, which diffuses into the cells and is hydrolyzed to 2′,7′-dichlorofluorescein (DCFH). Upon cell stimulation with LPS, DCFH was oxidized to DCF by reactive species, mostly H_2O_2, emitting high levels of green fluorescence, which was detected by flow cytometry [14] using a FACSCalibur flow cytometer and CellQuest software (both from BD Immunocytometry Systems).

2.7. Statistical Analysis. Normality of data distribution was assessed by Shapiro-Wilk test. Comparison between average results of the groups was performed using Student t test for independent samples. Correlation analysis between data was performed using Spearman correlation test. All data were processed and analyzed by appropriate statistical tests using SPSS 17 software.

3. Results

No changes were observed on plasma activity of the major erythrocyte antioxidant enzymes SOD, catalase, and glutathione peroxidase (Table 1). Table 1 also shows that total glutathione levels were similar in both cognitively intact (INT) and in group of patients with probable AD.

Lipid peroxidation (measured as TBARS) and plasmatic protein oxidation measured as the amount of circulating protein carbonyls did not significantly differ between the groups INT and AD (Table 2).

Plasma vitamin C levels were equivalent in both groups, as illustrated in Table 2. Values of circulating α-tocopherol were about 20% lower in patients with probable Alzheimer's disease compared with cognitively intact patients. This difference was statistically significant. It was observed that the two groups studied had similar plasma levels of β-carotene, lycopene, and coenzyme Q10 (Table 2).

Table 2: Oxidative stress parameters in plasma. INT (cognitively intact patients; $n = 42$) and AD (patients with probable Alzheimer's disease; $n = 23$). Values shown correspond to the means ± SEM. Comparison between average results of the groups was performed using Student's t-test for independent samples.

Parameter	INT	AD
Vitamin C (μM)	48 ± 3	52 ± 5
α-Tocopherol (μM)	21 ± 1	17 ± 1*
β-Carotene (μM)	0.79 ± 0.9	0.76 ± 1
Lycopene (μM)	0.73 ± 0.09	0.67 ± 0.13
Coenzyme Q10 (μM)	0.15 ± 0.02	0.13 ± 0.03
TBARS (thiobarbituric acid reactive substances) (nmol/mL)	5.82 ± 0.51	5.83 ± 0.62
Oxidized proteins (nmol/mg prot)	0.14 ± 0.01	0.16 ± 0.02

*$P < 0.05$ was considered significant.

Table 3 shows that Cu, Zn-SOD activity had a positive correlation with catalase and glutathione peroxidase activities, as well as total glutathione content. Catalase activity, in turn, also had positive correlation with glutathione peroxidase activity and total glutathione content in erythrocytes. Glutathione peroxidase activity and total glutathione content in red blood cells also had a significant positive correlation. The same two parameters negatively correlated with lipid peroxidation products in plasma.

Monocytes of patients with probable Alzheimer's disease expressed approximately 70% more HLA-DR than the cognitively intact patients. On the other hand, there were no differences on the expression of CD11b by monocytes obtained from the two groups (Figure 1 and Table 4).

The proportion of cells producing IL-1α was approximately 25% higher in patients with probable diagnosis of the disease as compared to those cognitively intact (Table 4). In the case of IL-6 and TNF-α, there were no statistically significant differences in the proportion of cells that produced those interleukins (Figure 2 and Table 4).

The oxidative metabolism of monocytes studied with flow cytometry techniques showed no differences between groups (Table 4).

4. Discussion

The aim of this study was to investigate the interrelationship between AD, blood parameters of oxidative stress, and indicators of inflammatory activity in monocytes. It follows the reasoning by which monocytes are circulating cells that resemble the brain microglia and may even be recruited from blood to brain tissue [15] in AD. Microglia are considered the main element for the localized inflammatory events that accompany this disease [16]. This would lead to localized inflammation and increased production of reactive oxygen

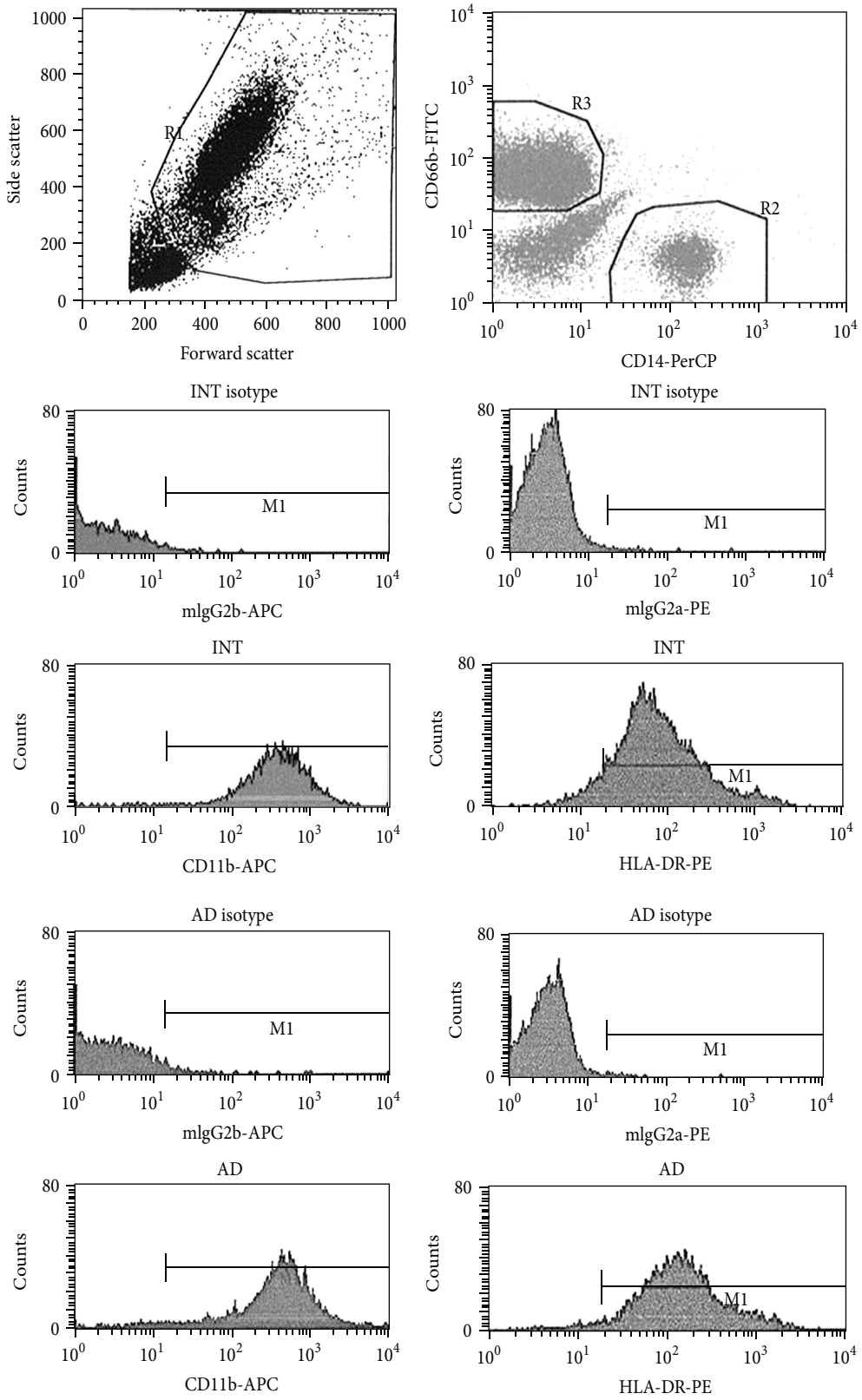

Figure 1: Representative flow cytometric analysis of whole blood samples stained with anti-CD11b-APC (right column) and anti-HLA-DR-PE (left column) monoclonal antibodies. From all events acquired in gate R1 (upper left graph), CD14-positive monocytes (gate R2, separated from CD66b-positive neutrophils in gate R3; upper right graph) were used for histogram calculation of the geometric mean fluorescence intensity (GMFI). A similar analysis was performed for the detection of fluorescent DCF in the ROS assay (histogram not shown). INT (sample from cognitively intact patient) and AD (sample from patient with probable Alzheimer's disease).

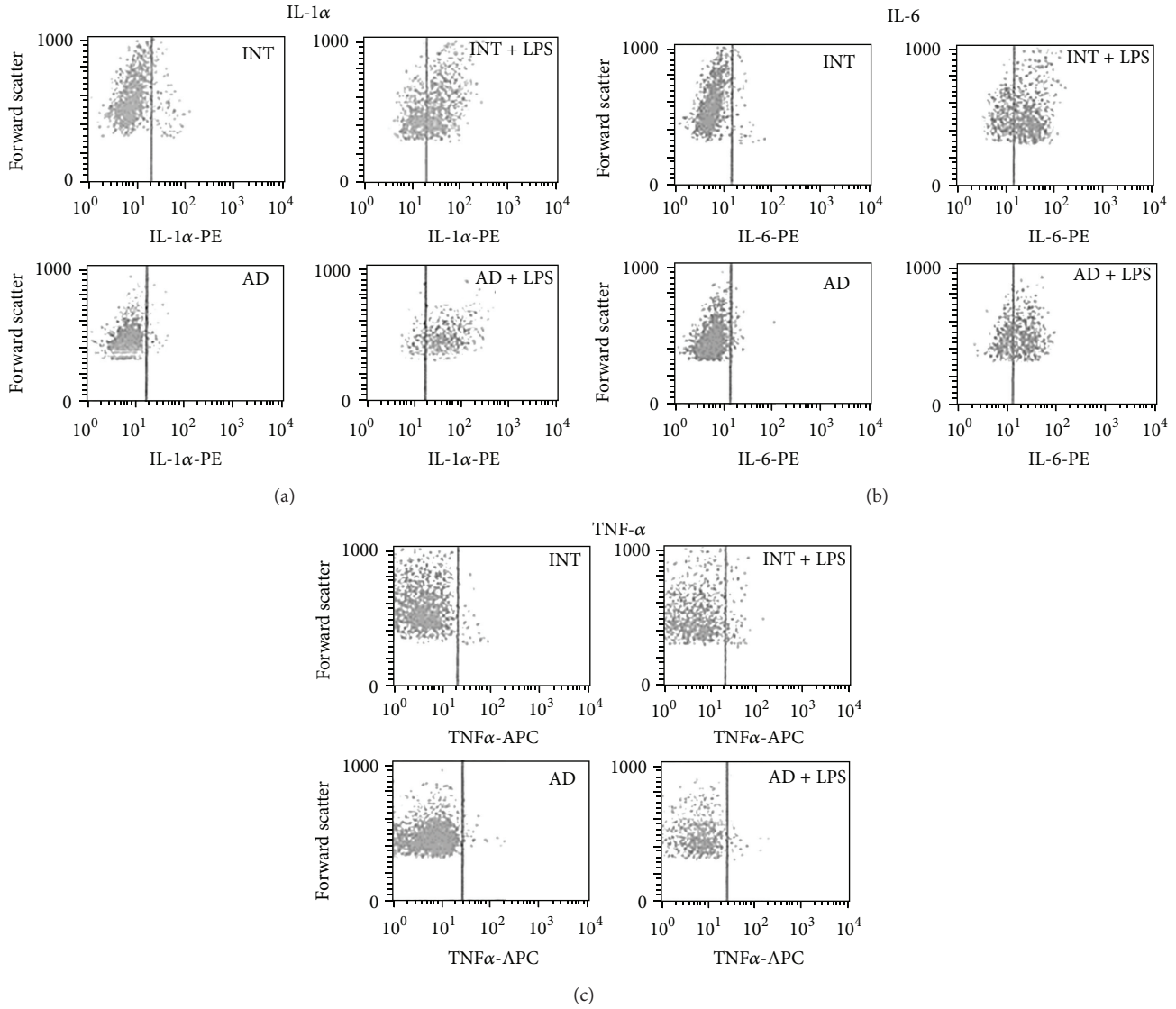

Figure 2: Representative flow cytometric detection of monocyte intracellular production of IL-1α, IL-6, and TNF-α. Monocytes were selected in a similar manner as described in Figure 1. LPS-induced production of each cytokine was determined by the percentage of events in the upper right quadrant (%URQ). Only upper quadrants are shown. INT (sample from cognitively intact patient) and AD (sample from patient with probable Alzheimer's disease).

species, causing oxidative stress, which could be reflected by circulating parameters [17].

In the conditions of this work, AD is not associated with any evidence of systemic oxidative stress. Although the activities of erythrocyte antioxidant enzymes do not show changes in patients with AD, Spearman rank correlation test reveals a positive correlation between the mutual activities of erythrocyte superoxide dismutase, catalase, and glutathione peroxidase and erythrocyte total glutathione levels. These data confirm the validity of the postulate that says that the body's antioxidant systems act together in an integrated manner. Spearman test has also identified a negative correlation between the levels of lipid peroxidation products, plasma glutathione peroxidase activity, and total glutathione levels in erythrocytes. This fact indicates that lipid peroxidation is influenced by the activity of antioxidant glutathione peroxidase-glutathione system in this experimental model. Indeed, glutathione-glutathione peroxidase system is an important component of antioxidant protection network to cells [18]. As a counterpoint to the measurement of erythrocyte antioxidant activities, circulating levels of oxidation products of macromolecules in the body have been evaluated. To this end, we analyzed levels of plasma protein carbonyls and plasma levels of lipid peroxidation products. Plasma level of protein oxidation products is not altered in AD compared with cognitively intact group. This lack of change is found in some studies [18]. On the other hand, some authors have found an increase of oxidized proteins in plasma from AD patients [19, 20]. This difference may be due to difference of sensitivity of the methods employed

Table 3: Correlation between antioxidant erythrocytes parameters and plasmatic oxidation products. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), total glutathione level (TGSH), and thiobarbituric acid reactive substances (TBARS). Analysis was performed using the Spearman correlation test.

Spearman test	SOD	CAT	GPX	GSH	TBARS
SOD					
Coefficient of correlation	1.000	0.415*	0.316*	0.406**	0.097
Significance		0.001	0.011	0.001	0.244
CAT					
Coefficient of correlation	0.415*	1.000	0.368**	0.317*	0.158
Significance	0.001		0.003	0.010	0.129
GPX					
Coefficient of correlation	0.316*	0.368**	1.000	0.416**	0.323**
Significance	0.011	0.003		0.001	0.009
TGSH					
Coefficient of correlation	0.406**	0.317*	0.416**	1.000	0.253*
Significance	0.001	0.010	0.001		0.034
TBARS					
Coefficient of correlation	0.097	0.158	0.323**	0.253*	1.000
Significance	0.244	0.129	0.009	0.034	

** $P < 0.01$, * $P < 0.05$.

Table 4: Monocyte activation parameters. INT (cognitively intact patients; $n = 42$) and AD (patients with probable Alzheimer's disease; $n = 23$); GMFI: geometric mean fluorescence intensity; % URQ: percentage of events in the upper right quadrant. Values shown correspond to the means ± SEM. Comparison between average results of the groups was performed using Student t test for independent samples.

Parameter	INT	AD
HLADR (GMFI)	109.93 ± 10.59	177.74 ± 30.64*
CD11B (GMFI)	406.74 ± 39.24	412.92 ± 48.97
IL-1α (%URQ)	65.78 ± 2.46	82.82 ± 6.52*
IL-6 (%URQ)	52.89 ± 4.31	49.71 ± 6.57
TNF-α (%URQ)	6.85 ± 1.28	8.26 ± 2.47
Oxidative metabolism (GMFI)	266.32 ± 38.99	257.77 ± 63.07

* $P < 0.05$ was considered significant.

in the mentioned studies (two-dimensional electrophoresis, mass spectrometry) in relation to enzyme-immunoassay method chosen for this work and the possible differences in quality of care and feeding among the target populations of each study. Concentration of circulating lipid peroxidation products is equivalent in both groups, results that agree with those commonly found in the literature [18]. In general, the population studied seems to enjoy a privileged status in relation to nutritional populations examined in similar studies in other locations. In addition to the antioxidant profile outlined above, measurements of plasma levels of nonenzymatic antioxidants of low molecular weight were also carried out.

Values of vitamin C, β-carotene, and α-tocopherol, obtained in this study are within the range previously described for a healthy population group in that age [7, 21]. A review of several published papers, performed by Stocker and Frei [22], shows populational values for plasma concentrations of vitamin C, α-tocopherol, and β-carotene ranging from 30 to 150 μM, 15 to 40 μM, and 0.3 to 0.6 μM, respectively. Other authors report that, for cardiovascular disease and cancer prevention, concentrations above 30 μM α-tocopherol in combination with concentrations greater than 50 μM of vitamin C, 0.4 μM β-carotene, and 0.5 μM lycopene are desired [23]. Thus, the two groups of elderly patients studied have positioned themselves within the limits found in several other studies to molecular plasma levels of antioxidants. These parameters reflect part of the antioxidant defense system of the body of patients, evaluating components that can be absorbed directly from the diet, so further promoted by the antioxidant defense mentioned above. Of all low molecular weight antioxidants studied, only vitamin E significantly differs between groups. The INT group has levels of plasma vitamin E higher than those in AD patients.

This apparent homogeneity in antioxidant levels between the various groups opposed to other studies in the literature [24–27]. This finding, as previously mentioned, reinforces the notion that the oxidative processes that are triggered during the course of AD are not always reflected in circulating blood, and even when that occurs, it may be only an indication of other underlying mechanisms. Of special interest is the fact that the establishment of AD, expressed by the comparison between the INT and AD groups in our study, is accompanied by the decrease of circulating vitamin E, which possesses antioxidant capabilities as well as recognized anti-inflammatory activities due to its action on signal transduction mechanisms in phagocytes [28, 29]. Not surprisingly, studies that have obtained the best results in preventing or treating AD in recent years used α-tocopherol as an agent [30].

To complete the picture outlined up to this point, circulating monocytes were analyzed by flow cytometry as indicators

of inflammatory activity involved in AD. To this end, we quantified the level of expression of surface molecules HLA-DR, as an indicator of activation of circulating monocytes, and CD-11b, an adhesion molecule involved in the process of marginalization and migration of monocytes. The results show that AD patients have circulating monocytes that express higher amounts of HLA-DR on the surface even without external stimulus, indicating a higher basal activation state of these cells, confirming the data available in the literature [31]. Markers of increased activation of monocytes in the bloodstream are found in patients from a number of neurodegenerative diseases [32]. As previously mentioned, these circulating monocytes can cross the blood-brain barrier and migrate to the brain tissue, differentiating into microglia. Since circulating monocytes of AD patients are more active at baseline than those of INT, it can be postulated that microglia derived from these cells react more intensely to inflammatory stimuli, producing greater injury to brain. Level of expression of surface protein CD-11b is the same in both groups indicating that the potential for adhesion and migration of these cells is not altered in this pathology.

Also by flow cytometry, monocytes production of IL-1α, IL-6, and TNF-α was measured. This measurement was intended to evaluate the capacity of circulating monocytes to stimulate the inflammatory process by attracting new monocytes to the inflammatory site. The production of IL-1α is higher in monocytes from AD patients than in INT group. Since IL-1α is an important proinflammatory cytokine, it is plausible to assume that migration and differentiation of these monocytes produce more active microglia. This increased production of IL-1α was shown *in vitro* in human monocytes lineage, when stimulated by PβA, by means of mechanisms of signal transduction mediated by tyrosine kinases, which reinforces the above hypothesis [33].

Levels of IL-6 produced by stimulated peripheral monocytes are similar in both groups, as well as of TNF-α, agreeing with Beloosesky et al. [34]. Other authors detected an increase in IL-6 production by monocytes of AD patients [35] using, however, a different stimulus (phytohemagglutinin) than that used in this present study (LPS).

From the data shown here, it is reasonable to conclude that AD is accompanied by the activation of circulating monocytes and a decrease in circulating levels of vitamin E. Several studies show α-tocopherol modulating inflammation activity [28, 36]. In our study, the group of patients, where plasma α-tocopherol is decreased, also showed more activated monocytes that respond to stimuli with an increased production of proinflammatory cytokine IL-1α. This study points out that AD is associated with a higher basal activation of circulating monocytes that may be a result of α-tocopherol stock depletion.

Abbreviations

AD: Alzheimer's disease
APC: Allophycocyanin
CDR: Clinical dementia rating
FITC: Fluorescein isothiocyanate
INT: Cognitively intact group
GMFI: Geometric mean fluorescence intensity
LPS: Lipopolysaccharide
MMSE: Minimental state examination
PE: Phycoerythrin
PerCP: Periidinin chlorophyll protein
ROS: Reactive oxygen species
URQ: Upper right quadrant.

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Research Article

Comparative Effects of Biodynes, Tocotrienol-Rich Fraction, and Tocopherol in Enhancing Collagen Synthesis and Inhibiting Collagen Degradation in Stress-Induced Premature Senescence Model of Human Diploid Fibroblasts

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Biodynes, tocotrienol-rich fraction (TRF), and tocopherol have shown antiaging properties. However, the combined effects of these compounds on skin aging are yet to be investigated. This study aimed to elucidate the skin aging effects of biodynes, TRF, and tocopherol on stress-induced premature senescence (SIPS) model of human diploid fibroblasts (HDFs) by determining the expression of collagen and MMPs at gene and protein levels. Primary HDFs were treated with biodynes, TRF, and tocopherol prior to hydrogen peroxide (H_2O_2) exposure. The expression of *COL1A1, COL3A1, MMP1, MMP2, MMP3,* and *MMP9* genes was determined by qRT-PCR. Type I and type III procollagen proteins were measured by Western blotting while the activities of MMPs were quantified by fluorometric Sensolyte MMP Kit. Our results showed that biodynes, TRF, and tocopherol upregulated collagen genes and downregulated *MMP* genes ($P < 0.05$). Type I procollagen and type III procollagen protein levels were significantly increased in response to biodynes, TRF, and tocopherol treatment ($P < 0.05$) with reduction in MMP-1, MMP-2, MMP-3, and MMP-9 activities ($P < 0.05$). These findings indicated that biodynes, TRF, and tocopherol effectively enhanced collagen synthesis and inhibited collagen degradation and therefore may protect the skin from aging.

1. Introduction

Human skin which consists of epidermis, dermis, and subcutaneous tissues provides a shielding layer for internal organs. During chronological aging, increased wrinkling, sagging, pigmentation, fragility, and lack of moisture plus elasticity are the universal manifestations observed on the skin. Skin aging can be intrinsic, which is genetically determined and extrinsic, which is caused by environmental exposure such as UV light. Oxidative stress is one of the factors that contribute to skin aging [1, 2].

Fibroblasts which are the crucial collagen-producing cells provide flatten appearance and elasticity to the skin in cooperation with collagen. However, fibroblasts have collapsed appearance with little cytoplasm when they aged [3, 4]. Therefore evaluating the loss of collagen, either decreased synthesis or increased degradation, is important in analyzing the factors that may contribute to skin aging [5]. Matrix metalloproteinases (MMPs) play an important role in regulating the turnover of collagen. In aged skin, the elevated level of MMPs caused increased collagen degradation and deterioration of skin structure [6]. Previous study which used stress induced premature senescence (SIPS) model of human diploid fibroblasts has shown the role of MMPs in regulating collagen degradation [7, 8].

Collagen fibers comprised approximately 75% of the dry weight of the dermis [9]. Total collagen in skin will decrease with age. Previous study showed that collagen markers such

as type I C-terminal propeptide (PICP) did not show any detectable increase during adolescence but decreased towards adult concentrations after the age of puberty while cross-linked C-terminal telopeptide of type I collagen (ICTP) and procollagen type III N-terminal propeptide (p3NP) increased in pubertal-aged children before decreasing towards adults concentrations [10]. Aged individuals have been reported to have lower collagen levels in skin as compared to young individuals while the amount of elastic materials and associated fibro-hexis or fiber breakdown can be large and is probably responsible for wrinkle formation seen in photoaged skin [2, 8, 11, 12].

MMPs are a family of zinc containing proteases with various substrate specificities, cellular sources, and inducers [4]. They degrade the stable components in extracellular matrix (ECM) such as collagens, gelatin, elastin, laminin, and basement membranes. MMPs levels in skin increase with age [6]. It has been suggested that the presence of damaged collagen may act in some manner to downregulate collagen synthesis. Study has shown that damage to type I collagen in three-dimensional *in vitro* culture following MMP-1 treatment has similar ultrastructural appearance to the damage seen *in vivo* in aged skin [13].

Development of aging is associated with oxidative stress as postulated in the free radical theory of aging [14]. Free radicals such as reactive oxygen species (ROS), which can be produced intrinsically through normal metabolic processes or from exogenous agents, attack cellular structures like DNA and protein causing to continuous accumulation of cellular damage. *In vitro*, the oxidative stress condition can be manipulated in order to study the aging process. For instance, studies have shown that exposure to ultraviolet or hydrogen peroxide (H_2O_2) was able to elevate ROS content and induce premature senescence in young fibroblasts [15, 16]. H_2O_2 is the oxidant species that can induce oxidative damage in human fibroblasts and produce similar characteristics as senescent cells [17].

Since oxidative stress is vital in aging, compounds with antioxidant properties might be beneficial in preventing aging. In this study we evaluated the combined effects of biodynes, tocotrienol-rich fraction (TRF), and tocopherol in promoting skin regeneration prior to oxidative stress exposure. TRF is comprised of all forms of tocotrienols and α-tocopherol. Tocopherol and tocotrienol are isomers of vitamin E. The difference in their chemical structure contributes to the different efficacy and potential as antioxidant [18]. Biodynes is an active compound which is derived from *Saccharomyces cerevisiae*. Recent studies showed that it acts as an antiaging compound due to its collagen synthesis promoting effect. When combined with other active compounds, it may work synergistically [19].

In this study we aimed to elucidate the molecular mechanism of biodynes, tocotrienol-rich fraction, and tocopherol in preventing skin aging. We would like to determine whether single treatment is giving thrilling outcomes or the synergic effects of combined compounds provide a better impact in preventing skin aging by determining collagen biosynthesis and degradation in HDFs.

2. Materials and Methods

2.1. Cell Culture and Treatment Protocols. This research has been approved by Ethics Committee of Universiti Kebangsaan Malaysia (Approval Project Code: FF-328-2009). Primary human diploid fibroblasts (HDFs) were derived from the circumcision foreskins of three young male subjects, aged between 9 and 12 years old. Written informed consents were obtained from parents of all subjects. The samples were aseptically collected and washed several times with 75% alcohol and phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (PAA, Austria). After removing the epidermis, the pure dermis was cut into small pieces and transferred into a falcon tube containing 0.03% collagenase type I solution (Worthington Biochemical Corporation, USA). Pure dermis was digested in the incubator shaker at 37°C for 6 to 12 h. With PBS, the derived HDFs were washed and maintained in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (PAA, Austria) and 1% antibiotic-antimycotic solution at 37°C in 5% CO_2 humidified incubator. After 5 or 6 days, the HDFs were trypsinized and further expanded with expansion degree of 1 : 4 into a new T25 culture flask (Nunc, Denmark). When cell confluency reached 80 to 90%, serial passaging was performed until passage 4. The population doublings (PDs) were monitored throughout the experiment. For subsequent experiments, HDFs were treated with biodynes, TRF, or tocopherol and combination of biodynes, TRF, and tocopherol (BTT) at passage 4. After that, the cells were exposed to 20 μM H_2O_2 for two weeks (prolong exposure to low concentration of H_2O_2) at passage 6. Immediately after two weeks of H_2O_2 exposure, the cells were harvested for further analysis.

Biodynes TRF (Arch Chemicals Inc. NJ, USA), tocotrienol-rich fraction (TRF) (Sime Darby Plantation Sdn. Bhd, Malaysia), and Copherol F 1300 C (Cognis Care Chemicals) were used to treat the HDFs prior to H_2O_2 exposure at a concentration of 1% (v/v), 500 μg/mL, and 100 μg/mL, respectively, either single or in combination.

2.2. Total RNA Extraction. Total RNA of HDFs in different groups was extracted by using TRI Reagent (Molecular Research Center, USA), according to manufacturer's instructions. Polyacrylcarrier (Molecular Research Center, USA) was added in each extraction to precipitate the total RNA. Extracted total RNA pellet was then washed with 75% ethanol and air-dried before being dissolved in RNase DNase free distilled water (Gibco, USA). Total RNA was stored at −80°C immediately after extraction. The yield and purity of the extracted total RNA were determined by using Nanodrop ND-1000 (Thermo Fisher Scientific, USA).

2.3. Primer Design and qRT-PCR. All primers were designed by using Primer 3 software (http://frodo.wi.mit.edu/primer3), with reference of Genbank (http://www.ncbi.nlm.nih.gov) database. The targets amplified by the primer pairs were characterized by BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov). Table 1 shows the primers sequence for targeted genes in this study. Specificity of all

Table 1: Primers sequence for genes expression analysis.

Genes	Forward primer sequence	Reverse primer sequence
GADPH	CTT TGG TAT CGT GGA AGG ACT C	GTA GAG GCA GGG ATG ATG TTC T
COL1A1	GTG CTA AAG GTG CCA ATG GT	ACC AGG TTC ACC GCT GTT AC
COL3A1	CCA GGA GCT AAC GGT CTC AG	CAG GGT TTC CAT CTC TTC CA
MMP1	ACA GCT TCC CAG CGA CTC TA	CAG GGT TTC AGC ATC TGG TT
MMP2	AAC CCA GAT GTG GCC AAC TA	TGA TGT CTG CCT CTC CAT CA
MMP3	GGC CAG GGA TTA ATG GAG AT	GGA ACC GAG TCA GGT CTG TG
MMP9	CCA TTT CGA ACG ATG ACG AGT	CCT CGA AGA TGA AGG GGA AG

primers was determined by using iScript One-Step RT-PCR Kit with SYBR Green (Biorad, USA). The size of the PCR products was then checked by running on 1.8% agarose gel prestained with ethidium bromide along with a 100 bp DNA step ladder (Promega, USA). Optimization of the qRT-PCR procedures was established by performing the standard curve. Four serial dilutions of total RNA were used: 0, 2, 4, 8 and 16. By using Bio-Rad iCycler and programmed protocol, each primer pair was optimized and the expression of all targeted genes was determined. The amplification protocol was as follows: cDNA synthesis at 50°C for 30 min, iScript reverse transcriptase inactivation at 94°C for 2 min, followed by 38 amplification cycles of denaturation at 94°C for 30 sec and 60°C (primer annealing and extension) for 30 sec. After the end of the last cycle, the melting curve was generated at 95°C for 1 min, 55°C for 1 min, and 60°C for 10 sec (70 cycles, increase set point temperature after cycle 2 by 0.5°C). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as reference gene that acts as an internal reference to normalize the mRNA expression [20].

2.4. Western Blotting. To determine the amount of type I procollagen and type III procollagen protein, cells were lysated by using lysis buffer which was prepared by mixing complete protease inhibitor cocktail tablet (Roche, German) and RIPA buffer (Sigma-Aldrich, USA). Approximately 30 μg of cell lysate was heated at 70°C for 10 min in the sample buffer. The samples were then separated on 4–12% bis-tris gel (Invitrogen, USA) by gel electrophoresis. After that, proteins were transferred onto nitrocellulose membrane and incubation of primary antibody was performed. Two primary antibodies were used which were anti-mouse monoclonal type I procollagen (Santa Cruz, USA) at 1 : 500 dilution and type III procollagen (Santa Cruz, USA) at 1 : 200 dilution. After incubation of secondary antibody, the proteins expression was detected by gel documentation and analysis MultiImage Light Cabinet 504551 (Alpha Innotech, USA). The band intensities were measured by using Image Master Total Lab software (Amersham Bioscience, Buckinghamshire, UK).

2.5. Determination of MMP-1, MMP-2, MMP-3, and MMP-9 Activities. The activity of MMP-1, 2, 3, and 9 was quantified by using the fluorometric SensoLyte 520 MMP assay kit (Anaspec, USA) according to the manufacturer's instructions. Briefly, cells were treated for 24 h. The supernatant of conditioned medium was collected and centrifuged for 15 min at 4°C, 10,000 g. Samples containing MMP were then incubated with 4-aminophenylmercuric acetate (APMA) to activate pro-MMP followed by initiating the enzyme reaction. The activity of MMP was detected by fluorescence microplate reader (Bio-Tek Instruments, USA) at excitation/emission wavelengths of 360 nm/460 nm.

2.6. Statistical Analysis. Experiments were performed in triplicates and data was analyzed by one-way analysis of variance (ANOVA) using SPSS statistic software. Significance was accepted at $P < 0.05$.

3. Results

3.1. Effect of Biodynes, Tocotrienol-Rich Fraction, and Tocopherol on Collagen Synthesis. Biodynes, TRF, tocopherol and combined biodynes, TRF, and tocopherol (BTT) significantly increased the expression of *COL1A1* gene as compared to SIPS at 5.07-fold, 2.92-fold, 3.10-fold, and 2.13-fold, respectively (Figure 1(a)) ($P < 0.05$). However, the significant elevation was only found in HDFs treated with TRF, tocopherol, and BTT ($P < 0.05$) but not in biodynes-treated cells when the levels of type I procollagen protein were analyzed (Figure 1(b)).

For *COL3A1* expression, a significant upregulation by 9.63 and 1.22 fold over SIPS was observed in cells treated with biodynes and TRF respectively (Figure 2(a)) ($P < 0.05$). Expression of type III procollagen was significantly increased in all treated cells as compared to SIPS (Figure 2(b)) ($P < 0.05$).

3.2. Effect of Biodynes, Tocotrienol-Rich Fraction, and Tocopherol on Collagen Degradation. Treatment with TRF, tocopherol and BTT significantly down regulated *MMP1* gene as compared to SIPS at 0.10-fold, 0.23-fold and 0.03-fold respectively (Figure 3(a)) ($P < 0.05$). Analysis on MMP activities showed that only BTT significantly reduced MMP-1 activity (Figure 3(b)). For *MMP2* gene expression, the result was similar to the expression of *MMPI* whereby TRF, tocopherol and BTT significantly decreased *MMP2* gene expression by 0.09-fold, 0.12, and 0.10, respectively, but not in biodynes-treated cells (Figure 4(a)). However, all treatment groups showed significant reduction in MMP-2 activity (Figure 4(b)) ($P < 0.05$).

Similar findings were observed in the expression of *MMP3* gene and MMP-3 activity (Figures 5(a) and 5(b)),

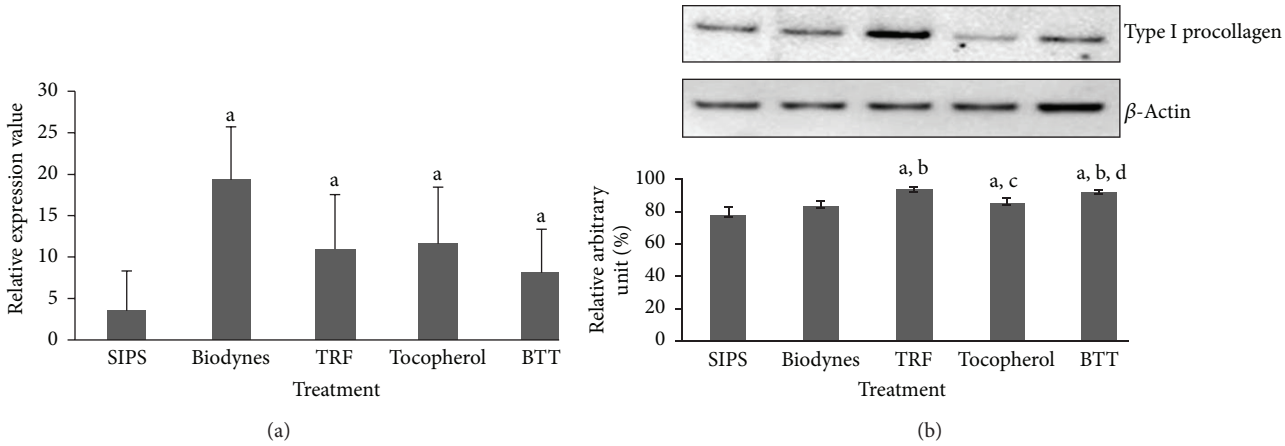

Figure 1: Effects of biodynes, TRF, and tocopherol on *COL1A1* gene and procollagen type I protein expression in HDFs. *COL1A1* expression (a). Biodynes, TRF, tocopherol, and BTT significantly increased *COL1A1* expression compared to SIPS. Procollagen type I protein expression (b). TRF, tocopherol, and BTT significantly increased the expression of procollagen type I. [a]Denotes $P < 0.05$ compared to SIPS, [b]denotes $P < 0.05$ compared to biodynes, [c]denotes $P < 0.05$ compared to TRF, and [d]denotes $P < 0.05$ compared to tocopherol. Data are presented as the mean of three experiments ±S.D, $n = 6$.

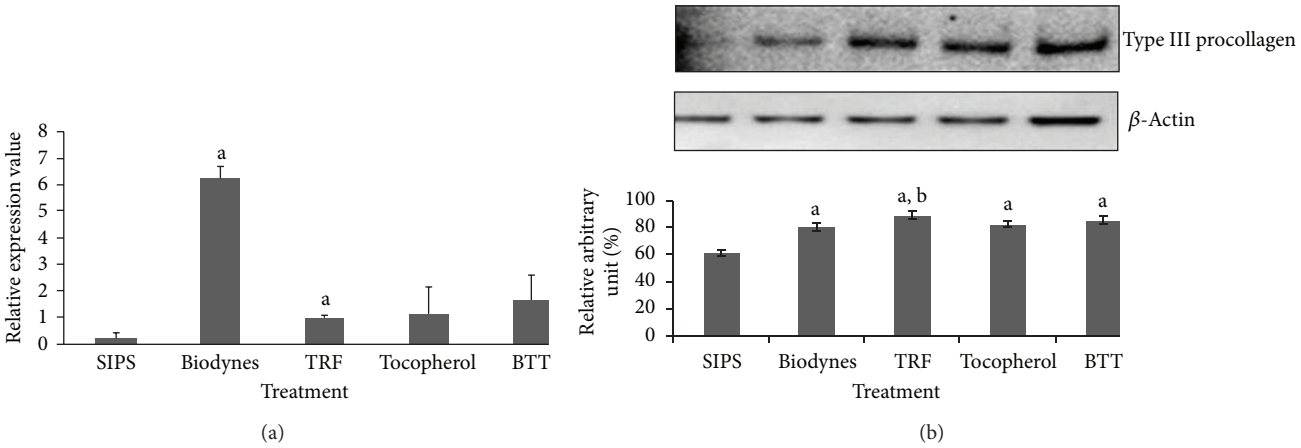

Figure 2: Effects of biodynes, TRF, and tocopherol on *COL3A1* gene and procollagen type III protein expression in HDFs. *COL3A1* expression (a). Biodynes and TRF significantly increased *COL3A1* expression compared to SIPS. Procollagen type III protein expression (b). Biodynes, TRF, tocopherol, and BTT significantly increased the expression of procollagen type III. [a]Denotes $P < 0.05$ compared to SIPS, [b]denotes $P < 0.05$ compared to biodynes. Data are presented as the mean of three experiments ±S.D, $n = 6$.

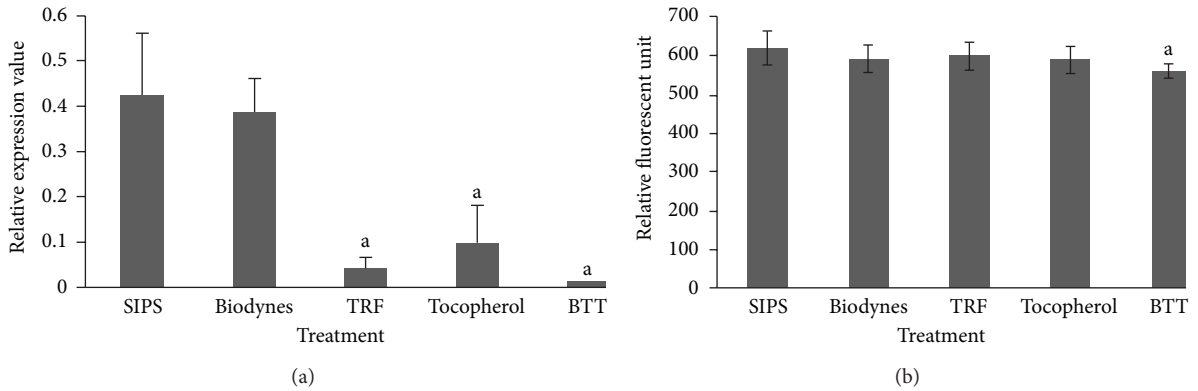

Figure 3: Effects of biodynes, TRF, and tocopherol on *MMP1* gene expression and MMP-1 activity in HDFs. *MMP1* expression (a). TRF, tocopherol, and BTT significantly downregulated *MMP1* compared to SIPS. MMP-1 activity (b). BTT decreased the MMP-1 activity compared to SIPS. [a]Denotes $P < 0.05$ compared to SIPS. Data are presented as the mean of three experiments ±S.D, $n = 6$.

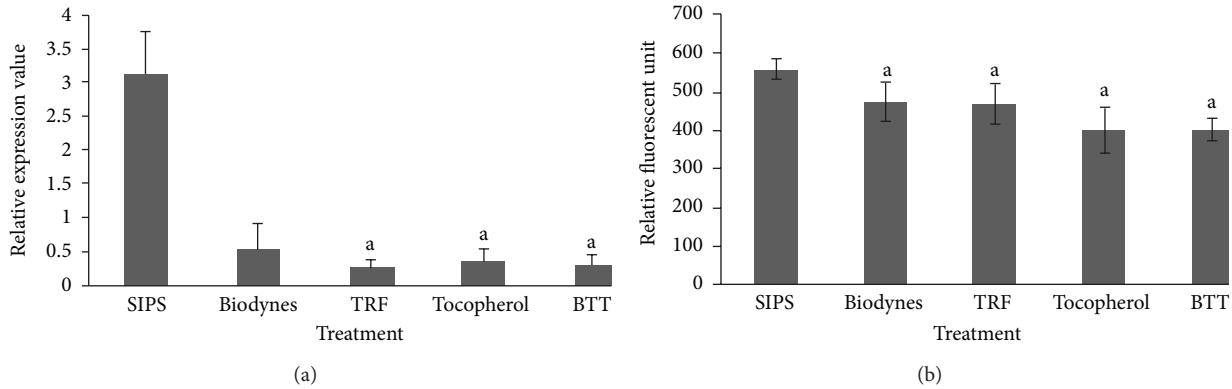

Figure 4: Effects of biodynes, TRF, and tocopherol on *MMP2* gene expression and MMP-2 activity in HDFs. *MMP2* expression (a). TRF, tocopherol and BTT downregulated *MMP2* compared to SIPS. MMP-2 activity (b). Biodynes, TRF, tocopherol, and BTT significantly decreased, the MMP-2 activity compared to SIPS. [a]Denotes $P < 0.05$ compared to SIPS. Data are presented as the mean of three experiments ±S.D, $n = 6$.

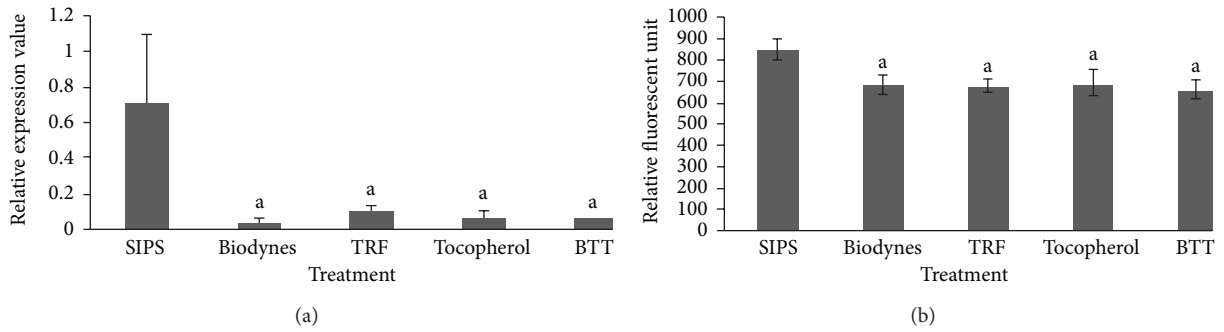

Figure 5: Effects of biodynes, TRF, and tocopherol on *MMP3* gene expression and MMP-3 activity in HDFs. *MMP3* expression (a). Biodynes, TRF, tocopherol, and BTT significantly downregulated *MMP3* compared to SIPS. MMP-3 activity (b). Biodynes, TRF, tocopherol, and BTT significantly decreased MMP-3 activity. [a]Denotes $P < 0.05$ compared to SIPS. Data are presented as the mean of three experiments ±S.D, $n = 6$.

whereby biodynes, TRF, tocopherol, and BTT showed significant downregulation of *MMP3* gene at 0.05-fold, 0.15-fold, 0.09-fold, and 0.08-fold, respectively ($P < 0.05$). Biodynes, TRF, tocopherol, and BTT also significantly downregulated *MMP9* gene as compared to SIPS at 0.71-fold, 0.35-fold, 0.14-fold, and 0.12-fold, respectively (Figure 6(a)). Although biodynes, tocopherol, and BTT caused significant decrease in MMP-9 activity compared to SIPS, TRF-treated group showed higher MMP-9 activity as compared to biodynes (Figure 6(b)) ($P < 0.05$).

4. Discussion

This study evaluated the effects of biodynes, tocotrienol-rich fraction, and tocopherol in modulating collagen synthesis and degradation, in order to elucidate their underlying mechanism in preventing skin aging. HDFs were exposed to prolonged low dose of H_2O_2 to induce premature senescence. H_2O_2 has been used in various studies as the senescence induction agent that may produce similar characteristics to chronological aging on induced cells [15, 17, 21].

Progressive loss of skin tissue due to deterioration of cellular and extracellular matrix components of dermis is vital in skin aging. The dermis layer of the skin is mainly comprised of collagen fibers, consisting of several types of collagens such as types I, III, and V [5]. The turnover of collagen is crucially important for maintaining skin structure and function in which impaired production can lead to skin thinning and increase skin vulnerability. Thus, collagen synthesis and degradation were the focus of our study. Our results showed that exposure to prolonged low dose of hydrogen peroxide downregulated *COL1A1* and *COL3A1* genes with concomitant reduction in type I procollagen and type III procollagen synthesis. These findings are supported by report from previous studies which showed that type I collagen as the main component of dermis decreases during aging or photoaging [22, 23].

In addition to decreased collagen production, increased collagen degradation may result to collapsed fibroblasts during aging which contributed to the shift of aged skin appearance [13]. In this study, *MMP1*, *MMP2, MMP3,* and *MMP9* genes were upregulated and MMP-1, MMP-2, and

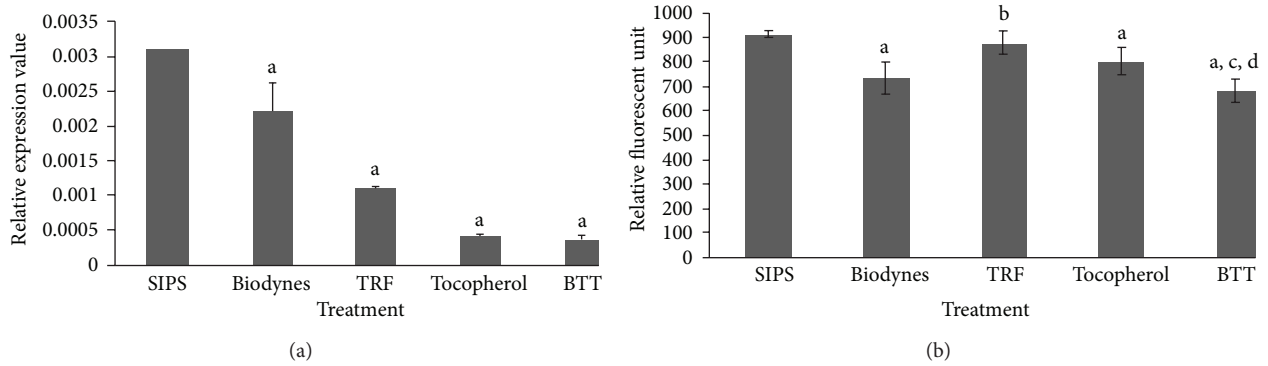

Figure 6: Effects of biodynes, TRF and tocopherol on *MMP9* gene expression and MMP-9 activity in HDFs. *MMP9* expression (a). Biodynes, TRF, tocopherol and BTT significantly down regulated *MMP9* expression compared to SIPS. MMP-9 activity (b). Biodynes, tocopherol and BTT significantly decreased MMP-9 activity. [a]Denotes $P < 0.05$ compared to SIPS, [a]denotes $P < 0.05$ compared to biodynes, [c]denotes $P < 0.05$ compared to TRF, [d]denotes $P < 0.05$ compared to tocopherol. Data are presented as the mean of three experiments ±S.D, $n = 6$.

MMP-3 activities were increased in oxidative stress-induced fibroblasts. Oxidative stress caused by UV irradiation, ozone, H_2O_2, and free radicals may lead to activation of AP-1, accordingly increased MMPs expression and collagen degradation [7]. In addition, association of oxidative stress and the level of MMP-1 was reported by Fisher et al. [8] which is in line with our findings. Over expression of MMPs during aging observed in this study may results into collagen loss in the skin which has been reported to be prominent and was proposed as the hallmark to designate matrix-degrading phenotype in senescent cells [23–25].

In this study, we focused on the molecular mechanism of combined biodynes, TRF, and tocopherol in preventing skin aging. The findings from this study demonstrated that these compounds regulate collagen synthesis and degradation in cultured HDFs by upregulating collagen synthesis and downregulating MMPs expression at gene and protein levels. These effects could be attributed to the properties of these compounds. Biodynes is a collagen production promoting compound that enhances integrin synthesis in fibroblast cells and prevents or reduces wrinkle formation [26]. It also stimulates collagen synthesis by increasing oxygen uptake in human fibroblasts [19] and shows synergistic effect when combined with other active compounds which enhances products benefit [19].

Vitamin E, a potent antioxidant that scavenges ROS, has been used in various studies for the past few decades. In previous studies, vitamin E has been reported as an effective antiaging agent attributable to its antioxidant property. It exerted other beneficial effects such as in modulating signal transduction pathways [27–29]. Most of the studies used α-tocopherol as a representative of vitamin E [30, 31]. However, the lesser known form of vitamin E, tocotrienols, has been considered as greater options compared to tocopherols [32]. The tocotrienols have slightly higher antioxidant activity than the tocopherols, possess neuroprotective properties, and exhibit anti-cancer and cholesterol lowering properties that are often not exhibited by the tocopherols [13].

Our data showed that biodynes, TRF, and tocopherol upregulated collagen genes and increased the synthesis of procollagen. Interestingly, combined compounds gave a better effect in stimulating collagen synthesis as compared to treatment with single compound. Hence, combination of biodynes, TRF, and tocopherol is effective due to the contribution of each active compound. The effectiveness of vitamin E when employed in combination with biodynes can be postulated due to their antioxidant properties. However the molecular mechanism is not well understood, even though it may be related to the antioxidant properties of these active compounds and/or their effects in modulating genes expression and signaling pathways.

Several studies indicated that the MMPs are crucial for initiating the degradation of collagen. Collagenase is secreted into the extracellular spaces as a proenzyme and is later activated. It has been shown that MMP-2, stromelysin which is secreted together with collagenase from connective tissue cells in culture, is believed to play a role as an activator for collagenase. The presence of stromelysin is important for the expression of full collagenase activity [33]. Results obtained from this study showed that biodynes, TRF, and tocopherol decreased *MMPs* genes expression and reduced the activity of MMPs enzymes. Combined treatment with biodynes, TRF, and tocopherol exerted better effects in inhibiting collagen degradation as compared to single treatment. This effect may be explained by the report from recent findings on antiaging properties of tocotrienol [34]. Besides, biodynes have been reported to stimulate collagen synthesis by increasing oxygen uptake in human fibroblasts [19]. The findings from this study showed that biodynes, TRF, and tocopherol works synergistically and exerted better effect in modulating collagen synthesis and degradation.

5. Conclusion

Biodynes, TRF, and tocopherol effectively enhanced collagen synthesis and inhibited collagen degradation indicated by upregulation of collagen genes, type I and type III procollagen synthesis and down regulation of MMPs gene and reduced MMPs activity. These properties may indicate their potential in protecting the skin from aging.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Sublethal Oxidative Stress Induces the Premature Senescence of Human Mesenchymal Stem Cells Derived from Endometrium

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The specific responses of mesenchymal stem cells to oxidative stress may play a crucial role in regulation of tissue homeostasis as well as regeneration of organs after oxidative injury. The responses of human endometrium-derived mesenchymal stem cells (hMESCs) to oxidative stress remain still unknown. Herein, we examined the impact of H_2O_2 on cell viability, induction of premature senescence, and apoptosis. hMESCs were highly resistant to H_2O_2 compared with human diploid fibroblasts. To test a hypothesis whether hMESCs may undergo oxidative stress-induced premature senescence, cells were briefly exposed to the sublethal H_2O_2 doses. H_2O_2-treated cells were permanently arrested, lost Ki67 proliferation marker, and exhibited a senescent phenotype including cell hypertrophy and increased SA-β-Gal activity. Additionally, in stressed cells the expression levels of p21Cip1, SOD1, SOD2, and GPX1 were elevated. hMESCs survived under stress were not able to resume proliferation, indicating the irreversible loss of proliferative potential. While the low H_2O_2 doses promoted senescence in hMESCs, the higher H_2O_2 doses induced also apoptosis in a part of the cell population. Of note, senescent hMESCs exhibited high resistance to apoptosis. Thus, we have demonstrated for the first time that hMESCs may enter a state of premature senescence in response to sublethal oxidative stress.

1. Introduction

Stress responses of human embryonic and adult stem cells to γ-radiation, oxidative stress, heat shock, and so forth are widely researched to establish cell-based strategies of tissue repair, tissue engineering, and transplantation [1]. Human mesenchymal stem cells are adult multipotent stem cells with the capacity of self-renewal and undergoing adipogenic, osteogenic, chondrogenic, myogenic differentiation [2, 3]. They contribute to the homeostatic maintenance of many organs and tissues [4, 5]. Unlike some other adult stem cells (e.g., hematopoietic stem cells) human mesenchymal stem cells are not immortal. These cells exhibit ex vivo growth characteristics typical of the Hayflick model of cellular senescence with a limited life span [6]. Recently, it has been reported that the mesenchymal stem cells subjected to oxidative stress [7–9] or ionizing radiation [10–12] may undergo stress-induced premature senescence in vitro. Many types of normal and tumor cells also enter a state of premature senescence after exposure to radiation [13–15], H_2O_2 [16–19], or treatment with histone deacetylase inhibitors [20, 21]. Prematurely senescent cells exhibit some of the characteristics inherent in replicatively senescent cells, including a large flat morphology, increased senescence-associated β-galactosidase (SA-β-Gal) activity, and permanent cell cycle arrest [14, 17]. Besides, cellular overactivation and hyperfunction, feedback signal resistance, and loss of regenerative potential are considered hallmarks of senescence [22]. Progress in understanding the causes and mechanisms of cellular senescence and significance of senescence for ageing and suppressing cancer has been reviewed [23–25].

In the current study, oxidative stress responses of human mesenchymal stem cells derived from endometrium (hMESCs) were investigated. Our knowledge of specific responses of these cells to stress is very limited, though they prove to be useful in the treatment of pathologies in which tissue damage is linked to oxidative stress. Unlike most of the human mesenchymal stem cells, the isolation of which as a rule is complicated by invasive procedures, the mesenchymal stem cells produced from desquamated endometrium in

menstrual blood by a simple noninvasive way provide a good opportunity to explore the stress responses of hMESCs. Regarding hMESCs, phenomenon of premature senescence induced by oxidative stress remains still unknown. This study aimed to test a hypothesis whether hMESCs after exposure with sublethal doses of H_2O_2 may undergo the stress-induced premature senescence. In parallel, the impact of H_2O_2 on cell viability and development of apoptosis has been evaluated.

2. Materials and Methods

2.1. Cell Culture and Cell Treatment. Human mesenchymal stem cells isolated from desquamated endometrium in menstrual blood (hMESCs, line 2304), as described previously [26], as well as human embryonic lung-derived diploid fibroblasts (HDF, line FRL-9505) were cultured in complete medium (DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS (HyClone, Waltham, MA, USA), 1% gentamycin, and 1% glutamax (Gibco BRL, Gaithersburg, MD, USA)) at 37°C in humidified incubator, containing 5% CO_2. hMESCs have a positive expression of CD73, CD90, CD105, CD13, CD29, and CD44 markers and absence of expression of the hematopoietic cell surface antigens CD19, CD34, CD45, CD117, CD130, and HLA-DR (class II). Multipotency of isolated hMESCs is confirmed by their ability to differentiate into other mesodermal cell types, such as osteocytes and adipocytes. Besides, the isolated hMESCs partially (over 50%) express the pluripotency marker SSEA-4 but do not express Oct-4. Immunofluorescent analysis of the derived cells revealed the expression of the neural precursor markers nestin and beta-III-tubulin. This suggests a neural predisposition of the established hMESCs. These cells are characterized by high rate of cell proliferation (doubling time 22-23 h) and high cloning efficiency (about 60%). Cells at early passages (between 6 and 9 passages for hMESCs and between 16 and 21 passages for HDF) were used in all experiments to avoid complications of replicative senescence. Cells were harvested by trypsinization and plated at a density of 15×10^3 cells per cm^2. For microscopy experiments, cells were grown on glass coverslips. H_2O_2 treatments were performed on subconfluent cells to avoid variability of H_2O_2 toxicity. H_2O_2 stock solution in serum-free medium was prepared from 30% H_2O_2 (Sigma, St. Louis, MO, USA) just before adding. Cells were treated with H_2O_2 in the range of concentrations from 200 μM to 2,000 μM for 1 h for MTT assay. Based on LD_{50} values 200 μM H_2O_2 was chosen as a sublethal concentration for the induction of premature senescence of hMESCs, whereas apoptosis was tested under higher concentrations of H_2O_2 (900 and 3,000 μM). The cells were washed twice with serum-free medium to remove H_2O_2 and then recultured in fresh complete medium for various durations as specified in individual experiments.

2.2. Assessment of Cell Viability. The cell viability after exposure to H_2O_2 for 1 h was evaluated by the enzymatic conversion of MTT (AppliChem, Darmstadt, Germany, number A2231) to formazan in live cells. The culture medium from the cells grown in plates was removed, and 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT; 0,715 mg/mL) in serum-containing growth medium was added to each well. In 2 h the solution was changed to DMSO to solve formazan produced. The plates were shaken for 15 min at room temperature; thereafter the absorbance was measured at 570 nm using microplate reader (Fluorofot "Charity," Russia). All points were read as parallels of 8 similar samples. The average absorbance at a given time point was normalized to the start time point.

2.3. Flow Cytometry. Adherent cells were rinsed twice with PBS and harvested by trypsinization. Detached cells were collected with supernatants, pelleted by centrifugation. Detached and adherent cells were finally pooled and resuspended in PBS. One part of each sample was used for propidium iodide (PI) staining to evaluate cell viability and another part for cell cycle phase distribution analysis that was performed as described previously [27]. 50 μg/mL PI was added to each sample just before analysis and mixed gently. Samples were analyzed on a Coulter EPICS XL Flow Cytometer (Backman Coulter, Brea, CA, USA). For cell cycle analysis, each cell sample was suspended in 300 μL PBS containing 200 μg/mL of saponin (Fluka, NY, USA), used for cell permeabilization, 250 μg/mL RNase A (Sigma, St. Louis, MO, USA, number R4642), and 50 μg/mL PI, incubated for 30 min at room temperature and subjected to FACS analysis. At least 10,000 cells were measured per sample. Cell cycle analysis was performed using Win MDI program version 2.8 and ModFit LT software (Verity Software House, Topsham, ME, USA).

2.4. FACS Analysis of Cell Enlargement. The same procedure of sample preparation as described previously was done for light-scattering cytometry. As after H_2O_2 treatment live and dead cells were very close to each other on one-parameter histogram, two-parameter histogram was used (FL4LOG versus FSLOG) to discriminate live and dead cells. Analysis of each sample was performed for 100 sec with high sample delivery. Cell size of control and H_2O_2-treated cells was measured by means of cytometric light scattering of PI-stained cells by using Win MDI program version 2.8.

2.5. SA-β-Gal Activity. Cells expressing senescent-associated β-galactosidase were detected with senescence β-galactosidase staining kit (Cell Signaling Technology, Beverly, MA, USA, number 9860) according to manufacturer's instructions. The kit detects β-galactosidase activity at pH 6 in cultured cells which is present only in senescent cells and is not found in presenescent, quiescent, or immortal cells. The percent of SA-β-Gal-positive cells was calculated by counting not less than 500 cells.

2.6. Immunofluorescence Staining. Cells cultured on coverslips were fixed with PBS/4% formalin for 15 min and then permeabilized with 0.1% Triton X-100. After blocking with 1% bovine serum albumin, they were incubated with a rabbit polyclonal antibody against Ki67 (Abcam, Cambridge, UK, number 15580) (1 : 1000) overnight at 4°C and then with Alexa Fluor 568 donkey anti-rabbit antibody (Invitrogen, Carlsbad,

USA, number A10042) (1 : 500) at room temperature for 1 h after extensive washing with PBS/0.1% Tween 20 between each step. The slides were counterstained with 1 μg/mL DAPI (Sigma, St. Louis, MO, USA, number D9564) and mounted using 2% propyl gallate. A Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Germany) equipped with a digital camera DFC 420C (Leica, Germany) utilizing Adobe Photoshop software was used to view and acquire images.

2.7. Apoptosis Detection. Apoptosis detection was performed by Annexin V/PI staining according to standard manufacture protocols (BD Pharmingen). H_2O_2-treated and control cells were harvested as described previously. Then cells were washed twice with cold PBS, resuspended in 1X binding buffer at concentration 10^6 cells/mL. 100 μL of this suspension was transferred to 5 mL tube, and 5 μL Annexin V/FITC (Annexin V/FITC Apoptosis Detection Kit II, BD Biosciences, San Diego, CA, USA) and 10 μL PI were added; suspension was gently mixed and incubated for 15 min at room temperature in the dark. Just before flow cytometric analysis 400 μL of 1X binding buffer was added to each sample.

2.8. Western Blotting. Total cell lysates were prepared as described previously [27]. Protein content was determined by the method of Bradford. The cell lysates were dissolved in SDS sample buffer and separated on 8% or 12% SDS gel. SDS-PAGE electrophoresis, transfer to nitrocellulose membrane, and immunoblotting with ECL (Thermo Scientific, USA) detection were performed according to standard manufacturer's protocols (Bio-Rad Laboratories, USA). The following antibodies were used: rabbit monoclonal antibodies against $p21^{Waf1/Cip1}$ (12D1, number 2947S) (1 : 1000) and against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, clone 14C10, number 2118S) (1 : 1000), as well as horseradish peroxidase-conjugated goat anti-rabbit IG (number 7074S) (1 : 10000). All antibodies were purchased from Cell Signaling, USA. Hyperfilm (CEA) was from Amersham (Sweden). Equal protein loading was confirmed by Ponceau S (Sigma, St. Louis, MO, USA, number P7170) staining.

2.9. RT-PCR Assay. To analyze gene expression, total RNA from cells was isolated with RNesy Micro Kit (Qiagen, USA) according to manufacturer's instructions. cDNA synthesis was performed with 1 μg of total RNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to manufacturer's instructions. Specific genes were amplified by Taq DNA polymerase (Fermentas, Lithuania) with C1000 Touch Thermal Cycler amplifier (Bio-Rad Laboratories, USA). The program was as follows: hot start-denaturation at 93°C for 3 min, primer annealing at 51–70°C for 2 min, and then elongation at 72°C for 1 min 30 sec; thereafter, 25–27 cycles of denaturation at 93°C for 45 sec, primer annealing at 51–70°C for 1 min, elongation at 72°C for 1 min 30 sec, and then final elongation at 72°C for 10 min; primers $p21^{Waf1/Cip1}$, sense 5′ CCA CAT GGT CTT CCT CTG CTG 3′, antisense 5′ GAT GTC CGT CAG AAC CCA TG 3′, annealing temperature 55°C (316 bp); *SOD1*, sense 5′ GGT CCT CAC TTT AAT CCT CTA T 3′, antisense 5′ CAT CTT TGT CAG CAG TCA CAT T 3′, annealing temperature 62°C (96 bp); *SOD2*, sense 5′ TGA CAA GTT TAA GGA GAA GC 3′, antisense 5′ GAA TAA GGC CTG TTG TTC C 3′, annealing temperature 56°C (148 bp); *GPX1*, sense 5′ CGC CAC CGC GCT TAT GAC CG 3′, antisense 5′ GCA GCA CTG CAA CTG CCA AGC AG 3′, annealing temperature 68°C (238 bp); *β-actin* gene was used for RNA quantitative control and DNA contamination monitoring: sense primer 5′-GCC GAG CGG GAA ATC GTG CGT-3′, antisense 5′-CGG TGG ACG ATG GAG GGG CCG-3′, annealing temperature 70°C (507 bp). All primers were obtained from SYNTOL (Russia). The electrophoresis of amplified products was performed in 2% agarose gel with TAE buffer and ethidium bromide. 100 kb DNA ladder (Fermentas, Lithuania) was used as molecular weight markers. Amplified products were visualized in UV light (302 nm) with transilluminator and registered with a digital Canon camera.

2.10. Statistics. All data are presented as the mean and standard error of the mean from at least three separate experiments performed. Statistical differences were calculated using the Student's t-test and considered significant at $^*P < 0.05$.

3. Results and Discussion

3.1. Cell Viability under Oxidative Stress. H_2O_2 treatment of cultured cells is a commonly used model to test oxidative stress susceptibility in different cell types. Accumulating evidence pointed to a high resistance of mesenchymal stem cells to oxidative stress caused by H_2O_2 [28, 29]. On the other hand, it has been recently reported that some types of human mesenchymal stem cells are very sensitive to H_2O_2 exposure [9]. Susceptibility of hMESCs to oxidative stress remains unexplored up to date. We earlier reported that hMESCs subjected to prolonged treatment (for 24 h) with H_2O_2 demonstrated a higher resistance compared with human diploid fibroblasts [30]. In this study, a pulse cell treatment with H_2O_2 in varying concentrations from 200 μM to 2 mM for 1 h was applied. Human diploid fibroblasts (HDF) were used as a H_2O_2 sensitive cell model to compare effect of H_2O_2 cytotoxicity on hMESCs. Firstly, it was necessary to assess hMESCs viability under oxidative stress to examine a sublethal H_2O_2 concentration required for further experiments. Previously we have found out that, at a fixed H_2O_2 concentration, both cytotoxicity and rate of degradation were dependent on the volume of H_2O_2 solution added to the culture medium; therefore the volume used was adjusted proportionally according to the surface area in order to obtain a consistent H_2O_2 cytotoxicity. In addition, it was very important to control plating cell density because the H_2O_2 effect (at equal volume and concentration) on cells was inversely related to cell density; that is, confluent cell cultures were more resistant to H_2O_2 than subconfluent ones. Cell viability was evaluated by MTT assay as a broad indicator of cellular activity that allows estimating a number of viable cells via monitoring mitochondrial dehydrogenase activity. As shown in Figure 1, H_2O_2 affected the cell viability of both cell

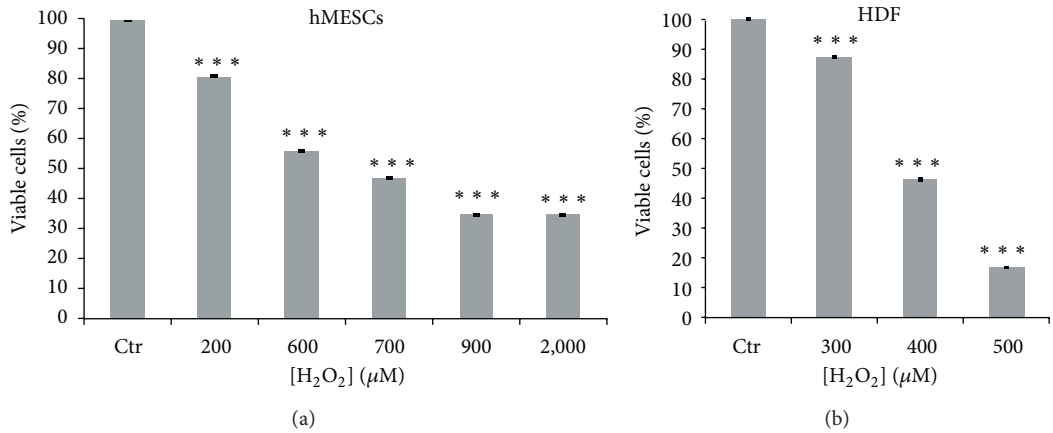

Figure 1: The viability of hMESCs and HDF under oxidative stress. Cells were either treated or not with H_2O_2 at indicated concentrations for 1 h. The percentage of viable cells was evaluated in 24 h after treatment using MTT assay as described in Section 2. Results are shown as a percent of control. Data represent mean ± SEM of at least three independent experiments. $^{***}P < 0.001$ significantly different from the untreated control cells. LD values were 600–700 and 370–400 μM for hMESCs and HDF, respectively. Control (Ctr): untreated cells.

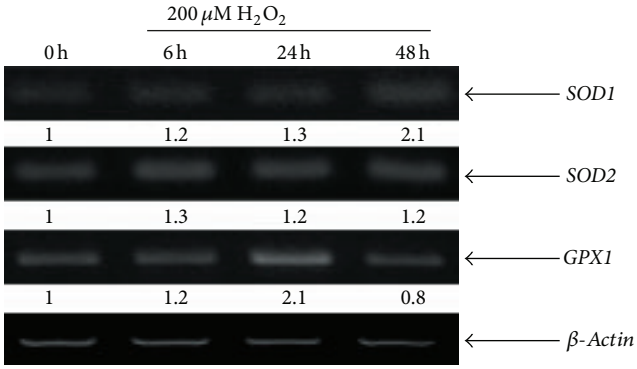

Figure 2: Gene expression of *SOD1*, *SOD2,* and *GPX1* is enhanced in hMESCs in response to H_2O_2 treatment. Exponentially growing cells were treated with the sublethal dose (200 μM) of H_2O_2 for 1 h with following H_2O_2 replacement and then cultured under normal conditions for the indicated time. Total RNA isolated from hMESCs was amplified with specific primers for referred genes. *β-Actin* was used as a loading control.

lines in a dose-dependent manner. LD_{50} values characterizing cell viability corresponded to 600–700 μM (15–17.5 pmol/cell) for hMESCs and 370–400 μM (3.5–4.0 pmol/cell) for HDF. Thus, hMESCs were found to be very resistant to H_2O_2 compared to HDF.

3.2. Expression of Antioxidant Enzymes. To examine whether this high resistance to effect of H_2O_2 correlates with the ability of hMESCs to effectively scavenge reactive oxygen species (ROS), expression of the genes coding for enzymes involved in the elimination of ROS such as SOD1, SOD2, and GPX1 was tested. Previously, we have observed a rapid increase of the intracellular ROS levels in hMESCs after exposure to H_2O_2 (data not presented). Here, it was shown that, in untreated cells, the basal levels of mRNA expression of all of the antioxidant enzymes were elevated, while after treatment with 200 μM H_2O_2 an expression level of each enzyme was upregulated to different extent (Figure 2). Consequently, the significant insensitivity to H_2O_2 was consistent with the enhanced expression levels of the antioxidant enzymes. These findings are in agreement with previous report demonstrating a high resistance of human bone marrow-derived mesenchymal stem cells to oxidative stress [29]. The conflicting findings demonstrating a particular sensitivity of human umbilical cord blood-derived mesenchymal stem cells to H_2O_2 have correlated with the low levels of antioxidant enzyme activity [9].

3.3. A Sublethal Oxidative Stress Induces a Premature Senescence Phenotype in hMESCs. In hMESCs, phenomenon of H_2O_2-induced premature senescence was not so far described. In all experiments, early-passage cells were used to avoid undesirable replicative senescence of cells, because the major features of both replicative and stress-induced senescence are known to be alike [14, 17, 18]. To test whether hMESCs after treatment with a sublethal H_2O_2 concentration (200 μM) could undergo SIPS, we assessed a variety of senescent-associated biomarkers: change of cell morphology, SA-β-Gal staining, increasing of cell size, loss of proliferative potential, cell cycle arrest, and $p21^{Waf1/Cip1}$ (hereafter p21) status. H_2O_2 treatment was found to lead to development of senescent-like morphology: cells become enlarged, flattened, and heterogeneous. It should be noted that, in a part of cell population, we could see such morphological changes within 24 h after H_2O_2 treatment. In addition, senescent cells demonstrated SA-β-Gal staining which increased gradually, and the most remarkable effect was reached at 7 days after treatment: more 95% H_2O_2-treated cells were SA-β-Gal positive (Figures 3(a) and 3(b)). Exponentially growing control cells displayed very weak, if any, increase of SA-β-Gal staining. Importantly, a premature senescence phenotype was maintained during the follow-up period of 21 days (data not shown).

The increased heterogeneity of the cellular size of H_2O_2-treated hMESCs was further confirmed and quantified by

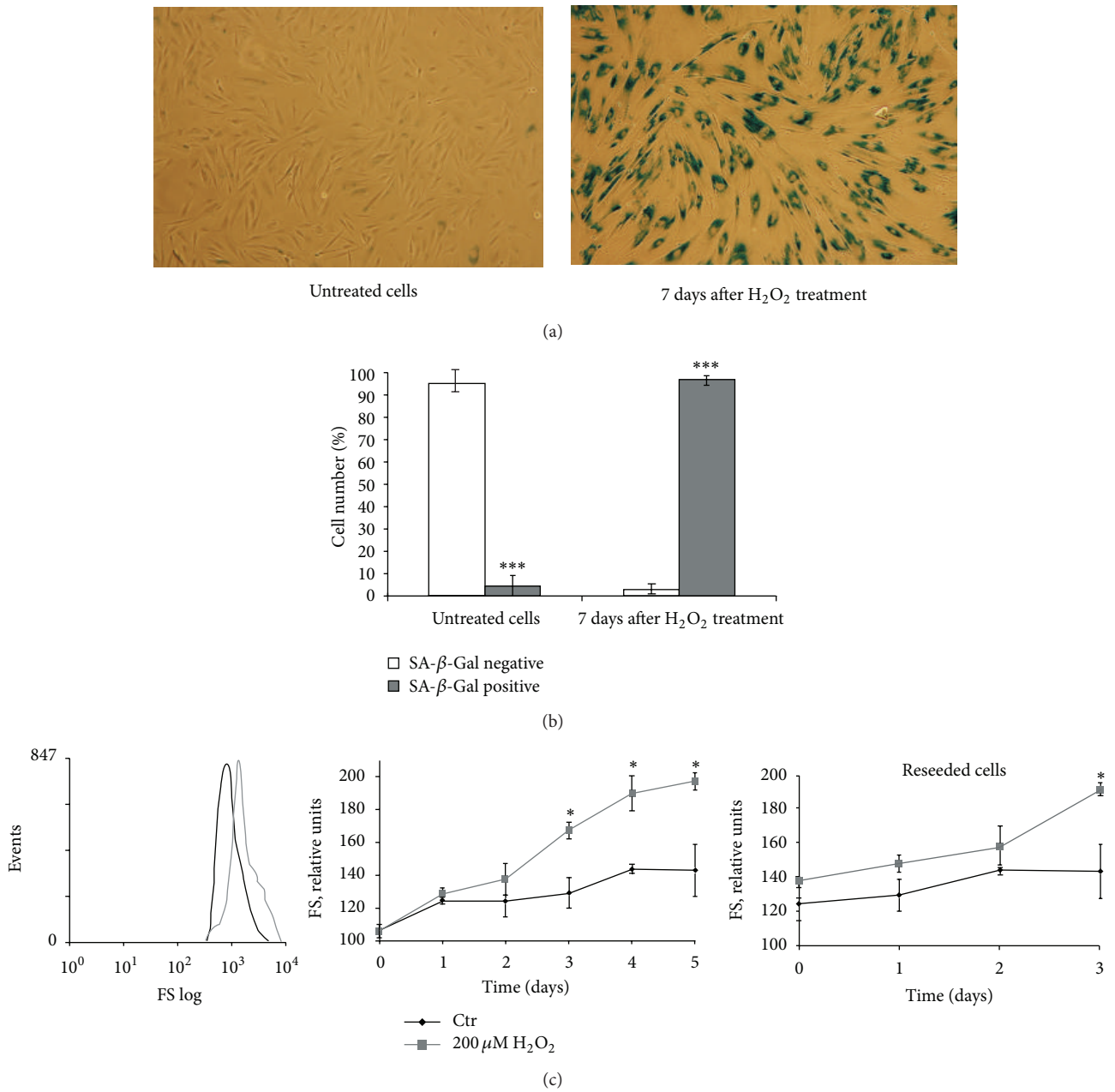

FIGURE 3: The sublethal dose of H_2O_2 induces senescent phenotype in hMESCs. Cells were treated as indicated in the legend of Figure 2. (a) SA-β-Gal staining. (b) Quantitative assay of SA-β-Gal-positive cells. (c) H_2O_2-induced cell size increase. Typical presentation of forward scatter (FS), reflecting the average cell size (left). Cell size was determined daily: H_2O_2-treated cells were either cultured for 5 days under standard conditions (middle) or were reseeded in 2 days and additionally cultivated for 3 days (right). Data were obtained by light-scattering cytometry with using Win MDI program version 2.8. Data represent mean ± SEM of at least three independent experiments. Significant difference was based on the Student's t-test ($^{*}P < 0.05$, $^{***}P < 0.001$). Control (Ctr): untreated cells.

light-scattering cytometry of PI-stained cells. As indicated in Figure 3(c), H_2O_2 induced a 2-fold increase of cell size after 5 days compared with control, as measured by the shift in the mean value of the forward scatter. The elevated size of treated cells was sustained constant, at least, for 5 days, whereas the size of control cells was almost not changed. To test whether treated hMESCs retain their increased size being reseeded, after H_2O_2 treatment cells were cultured for 2 days and then reseeded and additionally cultured under normal cell culture conditions for 3 days. As a result, we observed the similar increase of cell size (1.8-fold) in both reseeded and cultured cells for 5 days without reseeding cells (Figure 3(c)).

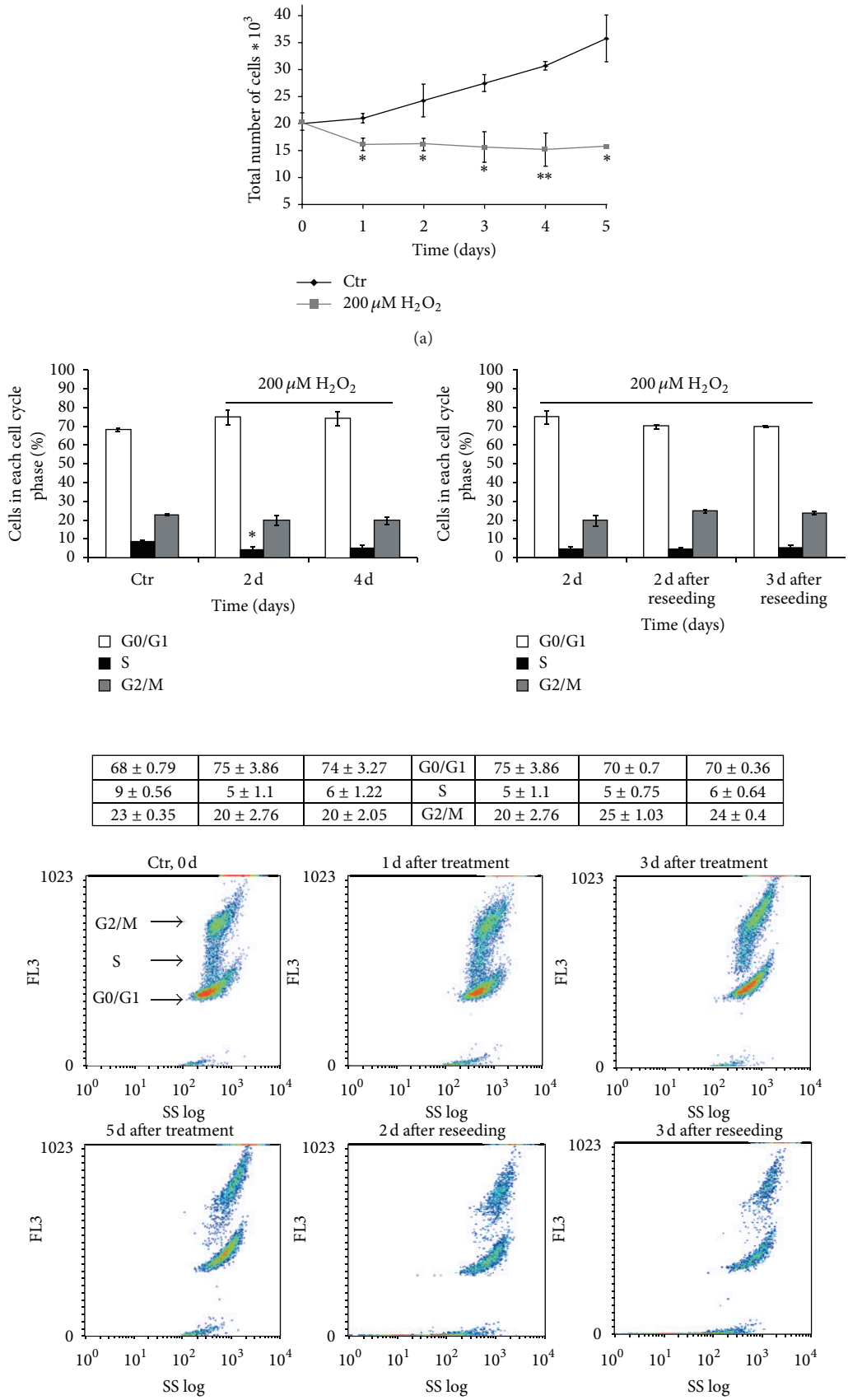

Figure 4: Continued.

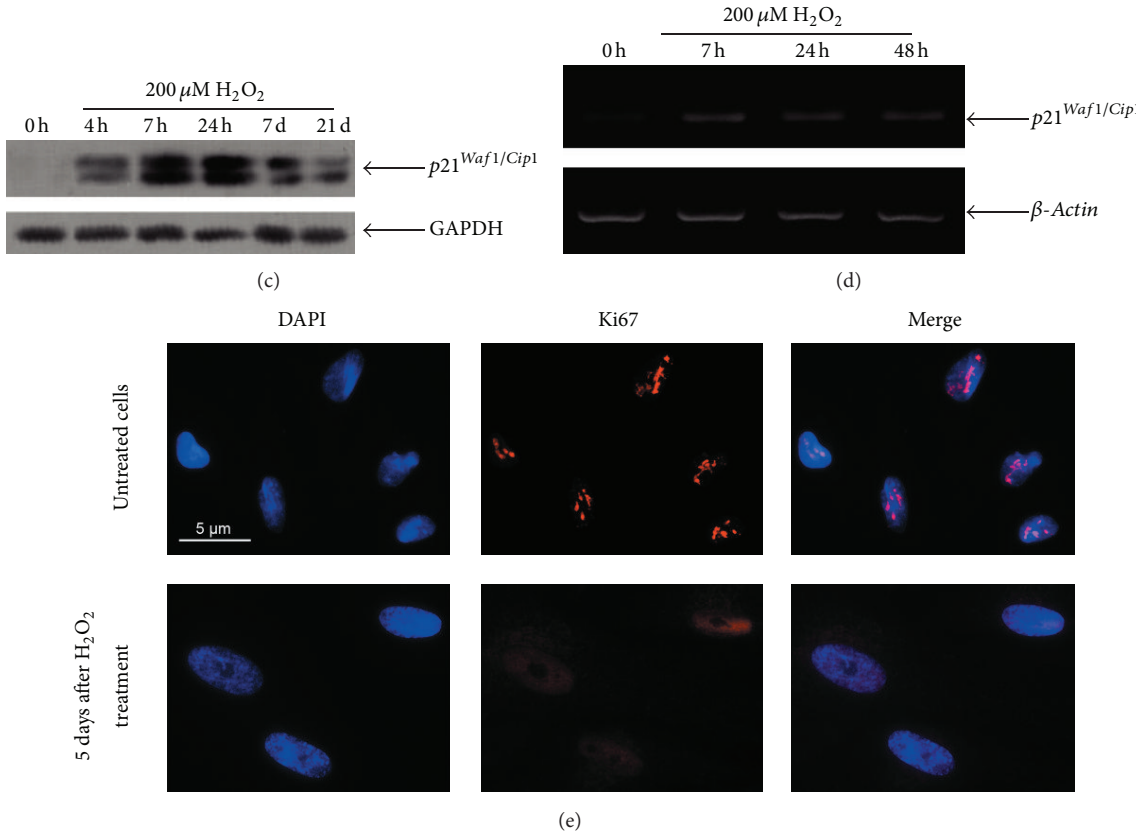

Figure 4: Induction of the premature senescence in hMESCs under oxidative stress leads to the permanent arrest of cell cycle and irreversible loss of proliferative potential. Cell treatment was done as described in Figure 2. (a) Growth curve of both H_2O_2-treated and untreated cells. Cell number was determined daily after cell exposure to H_2O_2 by FACS analysis (M ± SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). (b) H_2O_2-treated cells were either cultured for 5 days or were reseeded in 2 days and additionally cultured for 3 days. Flow cytometry analysis of cell cycle phase distribution: the percentage of cells in the G0/G1, S, and G2/M phases (upper panel) ($^{*}P < 0.05$); visualization of phase distribution based on light-scattering analysis (lower panel); SS: side scattering, FL3: PI fluorescence. (c) The expression levels of p21 protein. Representative results of the three experiments are shown in the figure. (d) The levels of *p21* mRNA expression. GAPDH and *β-actin* were used as loading controls. (e) The nuclear localization of Ki67 was tested in control or H_2O_2-treated cells by immunofluorescence and DAPI staining. Representative photomicrographs of the staining are shown. Images were taken at magnification 100x. Control (Ctr): untreated cells.

Importantly, cell size increase was accompanied with protein content elevation, suggesting protein synthesis in H_2O_2-treated cells. Together, the results obtained demonstrate cellular hypertrophy within hMESCs population in response to H_2O_2.

3.4. The Permanent Cell Cycle Arrest and Loss of the Proliferative Potential in hMESCs Subjected to Sublethal Oxidative Stress. In order to further characterize H_2O_2-induced senescent-like state of hMESCs, we analyzed their proliferative potential. Cell number in both untreated and H_2O_2-treated cell cultures was counted during 5 days. As seen in Figure 4(a), the pattern of growth curves indicates a significant increase (more than two times in 5 days) in the number of proliferating control cells compared with H_2O_2-treated cells. Consequently, 200 μM H_2O_2 caused a permanent growth arrest, that is, a permanent loss of the proliferative potential. Additionally, a proliferative status of cells was examined by staining with antibodies against proliferation marker Ki67. As seen in Figure 4(e), in 5 days after H_2O_2 treatment, there were no Ki67-positive cells in the cell culture, while the proliferating control cells had a pronounced staining. As viability of hMESCs in response to 200 μM H_2O_2 did not decrease appreciably (Figure 1), we suggested that H_2O_2-induced growth inhibition of hMESCs could be associated with rather the cell cycle arrest than promotion of cell death.

The analysis of the cell cycle phase distribution in hMESCs showed that a pulse H_2O_2 treatment led to the arrest in all of the cycle phases (Figure 4(b), upper panel). Treated cells demonstrated the prolonged arrest, at least, for 5 days. The phase distribution of treated cells in each time point tested was characterized with a minor accumulation of cells in G0/G1 phase compared with control cells. The distribution analysis with using light scattering confirmed these findings (Figure 4(b), lower panel). To test whether arrested cells could recover their proliferative potentials, in 2 days after treatment cells were reseeded and cultivated for 3 more days. As expected, reseeded cells also displayed the cell cycle arrest. Moreover, cell cycle phase distributions of both reseeded

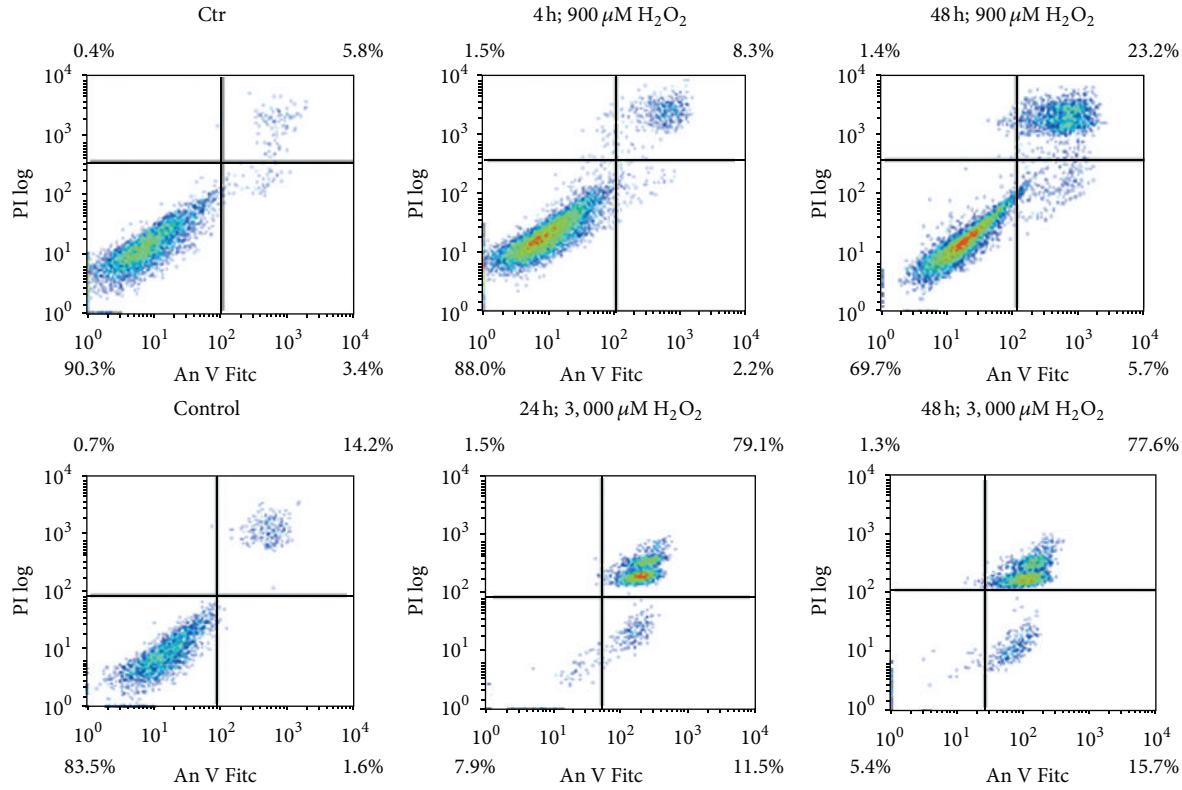

Figure 5: Dot plots of FITC-annexin V/PI flow cytometry. hMESCs were subjected to 900 μM or 3,000 μM H_2O_2 for 1 h with following H_2O_2 replacement and cell cultivation under normal conditions. Apoptosis was detected at indicated time points. Control (Ctr): untreated cells.

and taken-before-reseeding cells were identical (Figure 4(b)). Consequently, the senescent cells were not able to resume proliferation even after being reseeded, indicating the irreversible growth arrest. These observations were confirmed by proliferation assay (Figure 4(a)), which demonstrated that H_2O_2-treated cells were not able to proliferate normally for 5 days. Overall, these results suggest that cellular senescence in hMESCs was induced through growth arrest by H_2O_2.

3.5. A Permanent Loss of the Proliferative Potential in hMESCs Is Accompanied with Elevated Levels of p21. In human mesenchymal stem cells, cyclin-dependent kinase inhibitor p21 was recently shown to be upregulated during H_2O_2-induced premature senescence [7, 8]. Increased levels of p21 may mediate the initiation of H_2O_2-induced cell cycle arrest by inhibiting various cyclin-dependent kinases that contribute cell cycle phase progression [14, 16]. To find out whether p21 could be involved in the regulation of H_2O_2-induced senescence of hMESCs, protein and mRNA expression levels of p21 were determined. 200 μM H_2O_2 promoted a significant elevation in protein (Figure 4(c)) and mRNA (Figure 4(d)) expression of p21 in 7 h after treatment. An inducible expression of p21 was upregulated during 1-2 days with a following decline to insignificant, but not control, levels and was accompanied with the cell cycle arrest at the same time (Figure 4(b)). Importantly, the arrested cells thereafter could acquire a senescent morphology (Figure 3(a)) but could not resume proliferation (Figure 4(a)). We assume that the elevated p21 expression is essential to drive H_2O_2-induced premature senescence in hMESCs. In support of our findings, it has been reported that, in bone marrow-derived mesenchymal stem cells exposed to sublethal doses of H_2O_2, a rapid decrease of proliferation rate was detected within 3 days and correlated with G1 phase arrest of the cell cycle when p21 was accumulated at the same time [8].

In summary, our findings strongly indicate that hMESCs under a sublethal oxidative stress are able to undergo premature senescence.

3.6. Effect of High H_2O_2 Doses on hMESCs. H_2O_2 is well documented to cause apoptosis by a dose-dependent manner in various cell types. According to our data, at 200 μM H_2O_2, no apoptosis was detected in the cell population up to day 21; therefore we tested here whether H_2O_2 at a high concentration causes apoptosis in hMESCs. In order to detect apoptosis, annexin V/PI staining was performed. As presented in Figure 5, upper panel, 900 μM H_2O_2 reduced the number of viable cells from 90.3% to 69.7% in 48 h after treatment by increasing the number of AnV/PI+ cells. Remarkably, no significant changes in the number of early apoptotic cells (AnV+) compared with control were observed for 48 h. By contrast, H_2O_2 at higher concentration (3,000 μM) caused apoptosis with no evidence of necrosis in a similar pattern but with much stronger effect (Figure 5, lower panel). Thus, the apoptotic levels in H_2O_2-treated hMESCs were regulated by a dose-dependent manner. According to our

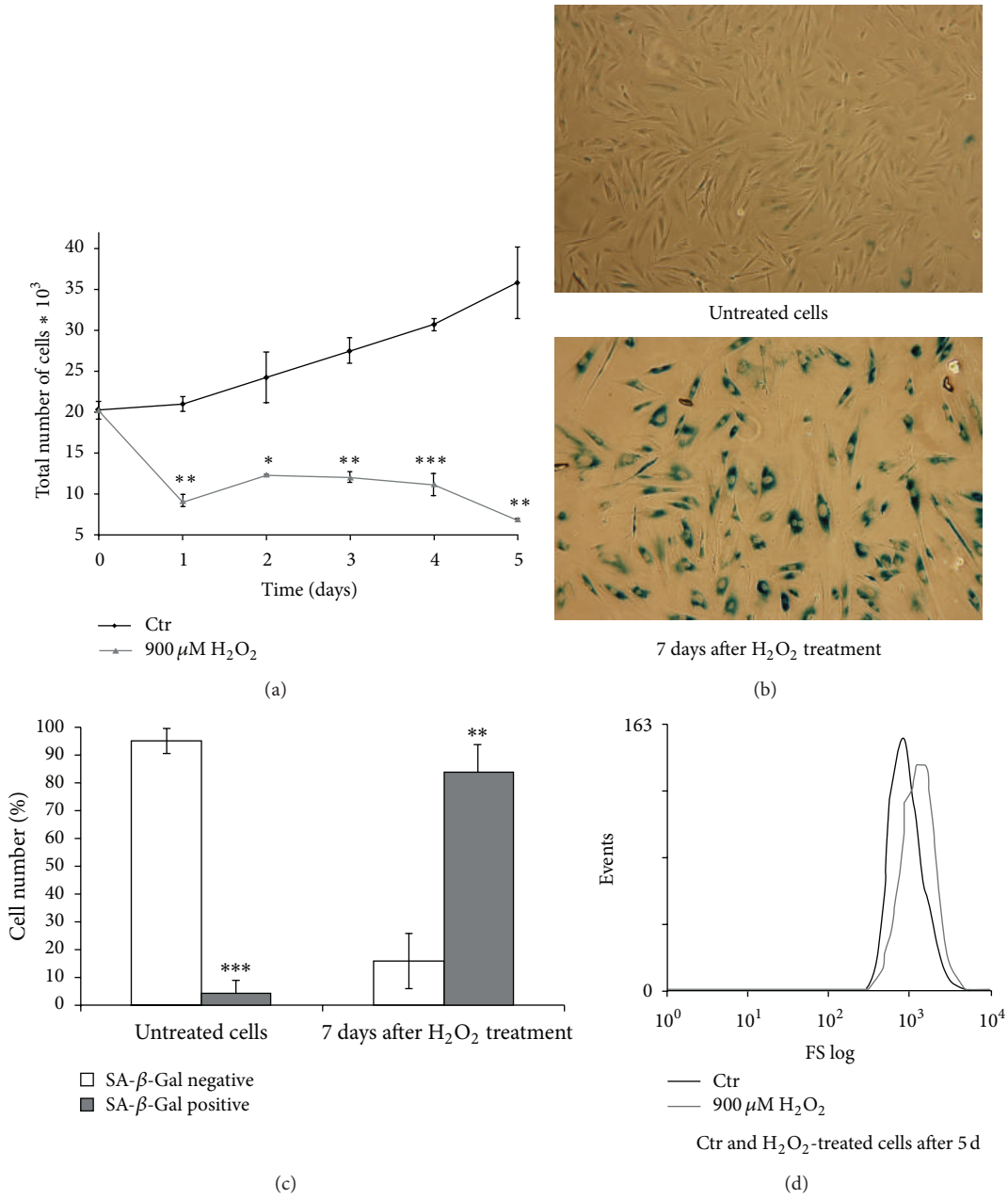

FIGURE 6: The senescent phenotype in hMESCs treated with 900 μM H_2O_2. (a) H_2O_2-induced inhibition of cell proliferation (M ± SEM, $n = 3$). (b) SA-β-Gal staining. (c) Quantitative assay of SA-β-Gal-positive cells. (d) Cell hypertrophy detected by light-scattering cytometry. Significant difference was based on the Student's t-test ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). Control (Ctr): untreated cells.

preliminary results, apoptosis was mediated by both caspase-8 and activated caspase-3; however, the exact mechanism of H_2O_2-induced apoptosis in hMESCs remains to be further elucidated.

Interestingly, 900 μM H_2O_2 triggered not only delayed apoptosis but also led to the emergence of enlarged and flattened cells in the same cell cultures. Cell changes in the presence of both 200 μM and 900 μM H_2O_2 were similar in appearance. These observations prompted us to test if cell hypertrophy could be connected with premature senescence. As shown in Figure 6, 900 μM H_2O_2 actually promoted the senescent morphology and SA-β-Gal staining, permanent growth arrest, and approximately 2-fold increase of cell size, pointing to premature senescence of the main part of cell population. Notably, the major senescence features induced by both 200 μM and 900 μM H_2O_2 were found to be alike. Together, these findings demonstrate that hMESCs exhibit high apoptosis resistance compared with human mesenchymal stem cells derived from both umbilical cord blood [9] and bone marrow [7], in which H_2O_2 above 200 μM triggered apoptosis, whereas 100–150 μM H_2O_2 induced senescence. Moreover, after exposure

to sublethal doses of H_2O_2, senescent hMESCs acquired the increased stability in culture and displayed enhanced resistance to H_2O_2-induced apoptosis (data not shown). Many cell types acquire resistance to some apoptotic signals when they become senescent. So, senescent human fibroblasts resist apoptosis induced by oxidative stress or growth factor deprivation but do not resist Fas-mediated apoptosis [31, 32]. Resistance to apoptosis might in part explain the enhanced stability of senescent cells in culture. The mechanisms by which senescent cells resist apoptosis are poorly investigated. The senescence and apoptosis regulatory systems are supposed to communicate probably through their common regulator, p53 tumor suppressor protein [33].

In this study, we have provided the reliable evidence for our hypothesis that hMESCs are able to undergo the premature senescence in response to oxidative stress induced by H_2O_2 in a wide range of concentrations from 200 to 900 μM. According to data obtained, entering senescence was accompanied with a rapid initiation of the cellular events, such as the changes of cell phenotype, the increase of SOD1, SOD2, and GPX1 expression, and upregulation of p21 without increase over time, leading to the irreversible cell cycle arrest and loss of proliferative potential. Since 2009, when phenomenon of stress-induced premature senescence in human mesenchymal stem cells was described for the first time, there were only a few publications concerning the oxidative stress-induced premature senescence of human mesenchymal cells derived from bone marrow [7, 8] and umbilical cord blood [9]. Even though both stem cell lines under sublethal stress respond with senescence, the major features of this process, in particular, dynamics of p21 accumulation and decline of cell proliferation rate, were extremely different, depending on the cell context. The precise molecular mechanism required to regulate the oxidative stress-induced premature senescence of human mesenchymal stem cells is far from understanding. Senescence program seems to develop in mesenchymal stem cells as a result of DNA damage response, leading to functional activation of either the p53/p21 or the p16INK4a (p16)/retinoblastoma protein (pRb) pathway, both of which can establish and maintain the growth arrest that is typical of senescence [23, 24]. The cyclin-dependent kinase inhibitors p16 and p21 may maintain pRb in active hypophosphorylated state [34, 35]. In turn, pRb halts cell proliferation by suppressing the activity of transcription factor E2F that regulates cell cycle progression. Our preliminary data, indicating a time- and dose-dependent formation of foci that contain phosphorylated histone H2AX (γH2AX), activation of both ATM and p53, and upregulation of p21 expression, suggest that in hMESCs subjected to sublethal doses of H_2O_2 the senescence process may be controlled by the p53 pathway. In parallel, by monitoring the kinetics of p38 mitogen-activated protein kinase (MAPK) activation in H_2O_2-induced senescence of hMESCs, we have revealed a rapid and continued phosphorylation of p38 MAPK, indicating its possible role in the regulation of the premature senescence [36]. On the other hand, pRb was reported to induce growth arrest as a downstream molecule of p38 MAPK [37]. Taking into consideration these results, we cannot exclude the possibility that p16/pRb signaling cascade is also implicated in hMESCs senescence promotion.

Understanding the mechanisms of senescence process will be of great importance in developing applications of hMESCs in regenerative medicine to provide new strategies in autologous transplant and bioengineering. Primarily, hMESCs may be applied for cell therapy of infertility associated with decidualization insufficiency. Decidualization of endometrium is known to be an essential process for embryo implantation, placenta forming, and maintenance of pregnancy [38]. A noninvasive and easily available source for isolation of hMESCs, high proliferation activity during long-term cultivation, genetic stability, lack of tumorigenicity [39], and low immunogenicity make hMESCs a promising source of stem cells for clinical applications, including reproduction technology.

In summary, we have displayed for the first time that hMESCs in oxidative stress conditions undergo a premature senescence. Data obtained broaden a conception of mesenchymal stem cell senescence under oxidative stress. Taken together, the findings presented here and the data published allow us to assume that induction of premature senescence might be a common physiological response to sublethal oxidative stress in human mesenchymal stem cells of any origin.

Abbreviations

hMESCs: Human endometrium-derived mesenchymal stem cells
HDF: Human diploid fibroblasts
SIPS: Stress-induced premature senescence
H_2O_2: Hydrogen peroxide
FACS: Flow-activated cell sorting
SA-β-Gal: Senescence-associated β-galactosidase
SOD1: Cytosolic superoxide dismutase
SOD2: Mitochondrial superoxide dismutase
GPX1: Glutathione peroxidase.

Acknowledgments

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