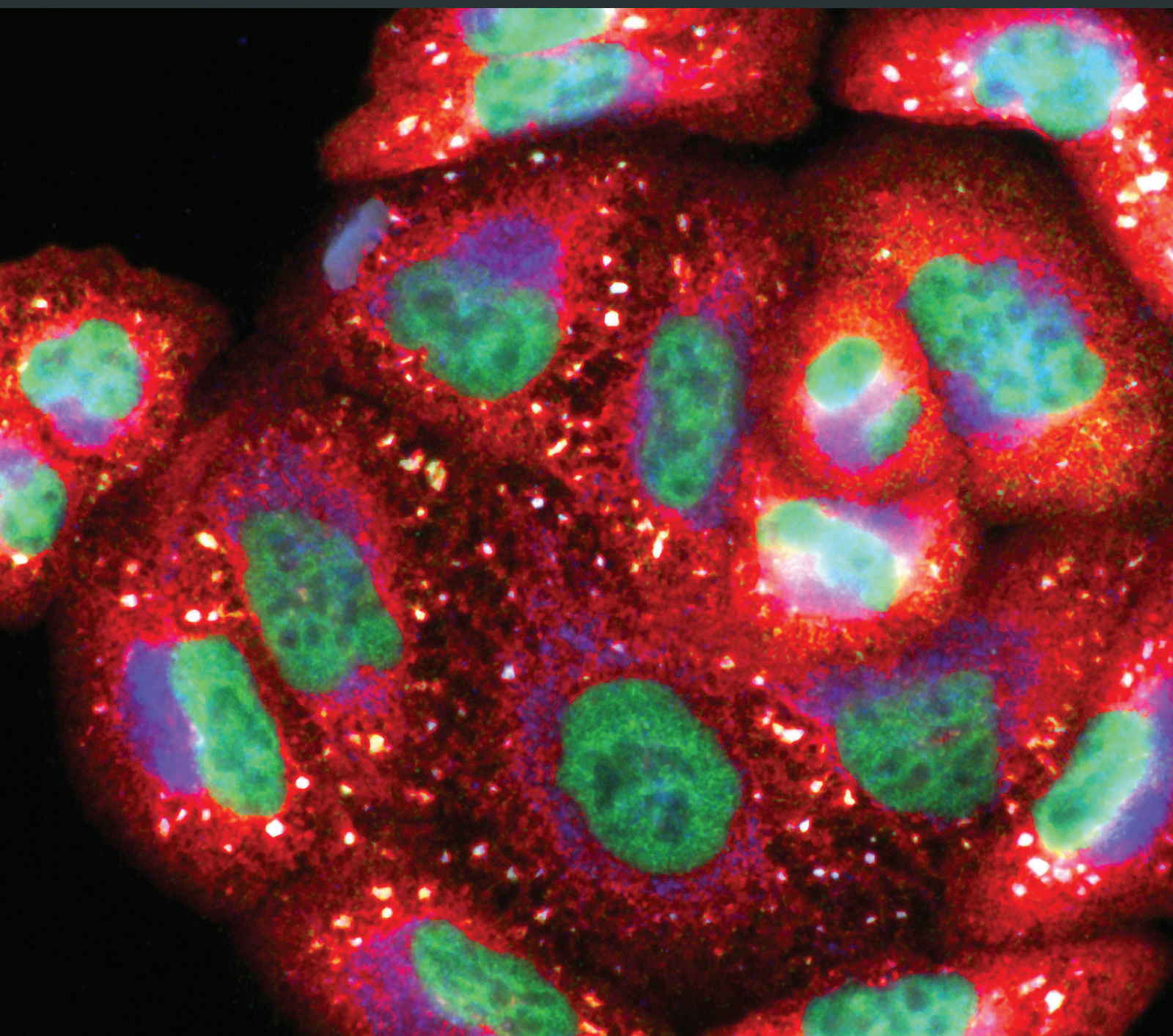
Oxidative Medicine and Cellular Longevity

Oxidative Stress and Diseases: Clinical Trials and Approaches

Guest Editors: Eiichiro Ichiishi, Xiao-kang Li, and Eugenio L. Iorio



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Editorial Board

Contents

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Editorial

Oxidative Stress and Diseases: Clinical Trials and Approaches

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Due to the fact that oxidative stress may play important roles in human disease development, great many basic and experimental studies have been conducted to clarify the mechanisms that regulate the imbalance between prooxidant and antioxidant systems. Knowledge and understanding of these mechanisms have led to the development of clinical trials and approaches, which have resulted in successful diagnosis and therapies, as well as novel tools to characterize these clinical mechanisms and provide better care to patients.

As to evidence-based clinical applications in humans, significant progress is expected in future medicine. Identifying oxidative stress markers in humans is important, as well as developing strong tools in health promotion and disease prevention for outpatient clinics, bedside monitoring, and home medical care.

In recent years, emerging evidence suggests oxidative stress may play an important role in many skin diseases and skin aging, possibly including atopic dermatitis. The paper by H. Ji and X.-K. Li entitled "Oxidative Stress in Atopic Dermatitis" provides an overview of an update on scientific progress linking oxidative stress to AD and discusses future treatment strategies for better disease control and improved quality of life for atopic dermatitis patients.

There have been intense interest and active researches in the area of dietary antioxidants to develop functional food products. The work by W. Huang et al. entitled "Effect of Blueberry Anthocyanins Malvidin and Glycosides on the Antioxidant Properties in Endothelial Cells" reports that blueberries are a good resource of anthocyanins, which can protect cells from oxidative deterioration, and used as a potential functional food to prevent diseases related to oxidative stress.

Peritonitis, which is the inflammation of the peritoneal tissue, can cause systemic inflammatory response and sepsis. Etanercept is the competitive inhibitor of TNF-α which inhibits the binding of TNF-α to cell surface receptors and limits its biological activity. In the work by Y. Yildirim et al. entitled "Effect of Intraperitoneal Etanercept on Oxidative Stress in Rats with Peritonitis" the authors showed that the TNF-α inhibitor, etanercept, in addition to antibiotics given in the early treatment of peritonitis results in more significant improvement of histopathological and oxidative parameters as compared to antibiotics alone.

The pathogenesis of chronic spontaneous urticaria (CSU) has not been fully understood; nevertheless, significant progress has been achieved in recent years. In the work by F. Dilek et al. entitled "Oxidative Stress in Children with Chronic Spontaneous Urticaria" the authors showed that plasma oxidative stress is increased in children with chronic spontaneous urticaria when compared to healthy subjects, and plasma oxidative stress markers are positively correlated with disease activity.

High levels of circulating low-density lipoprotein (LDL) are a primary initiating event in the development of atherosclerosis. Recently, the antiatherogenic effect of polyphenols has been shown to be exerted via a mechanism unrelated to their antioxidant capacity and to stem from their interaction with specific intracellular or plasma proteins. In their paper "Punicalagin Induces Serum Low-Density Lipoprotein Influx to Macrophages," D. Atrahimovich et al. demonstrate that, upon binding, punicalagin, the

main polyphenol in pomegranate, stimulates LDL influx to macrophages, thus reducing circulating cholesterol levels.

Schizophrenia is a complex, heterogeneous, and severe psychiatric illness that affects about 1% of the population. Exact molecular mechanisms underlying the pathogenesis of this disorder remain to be elucidated. Preclinical and clinical studies of the last decade have highlighted a number of data demonstrating the involvement of oxidative stress in the pathophysiology of psychiatric diseases. In the paper by K. Kriisa et al. entitled "Antipsychotic Treatment Reduces Indices of Oxidative Stress in First-Episode Psychosis Patients," the authors evaluated the markers of total antioxidative capacity, lipid peroxidation, and protein oxidation and revealed no high-grade oxidative stress in first-episode psychosis patients. Nevertheless, antipsychotic treatment induced a considerable anti-inflammatory effect. Oxidative stress levels were also significantly decreased if compared in first-episode psychosis patients before and after antipsychotic treatment.

Renal transplantation has been considered the best therapeutic option for end stage renal disease. To determine the effect of renal transplantation on the evolution of oxidative DNA status, the work by J. I. Cerrillos-Gutiérrez et al. entitled "The Beneficial Effects of Renal Transplantation on Altered Oxidative Status of ESRD Patients" reports that patients with end stage renal disease have important oxidative damage before RT. The RT significantly reduces oxidative damage and partially regulates the antioxidant enzymes.

Xeroderma pigmentosum group A (XPA) is a genetic disorder in DNA nucleotide excision repair (NER) with severe neurological disorders, in which oxidative stress and disturbed melatonin metabolism may be involved. In the work by R. Miyata et al. entitled "Circadian Rhythms of Oxidative Stress Markers and Melatonin Metabolite in Patients with Xeroderma Pigmentosum Group A" the authors showed that the administration of melatonin has the possibility of ameliorating the augmented oxidative stress in neurodegeneration, especially in the older XPA patients, modulating the melatonin metabolism and the circadian rhythm.

Albumin supplementation of culture media induces sperm capacitation in assisted reproduction technique cycles. Synthetic serum supplementation is clinically used to replace albumin for preventing transmission of infectious agents. However, the effects of synthetic serum supplementation on sperm capacitation have rarely been investigated. In their paper "Effects of Synthetic Serum Supplementation in Sperm Preparation Media on Sperm Capacitation and Function Test Results," Y.-F. Shih et al. demonstrate that the effects of synthetic serum supplementation on sperm capacitation varied according to the combination of media. These differences may lead to variations in spermatozoon ROS levels, thus affecting sperm function test results.

Molecular hydrogen therapy has been widely concerned and researched. Many animal experiments were carried out in a variety of disease fields. The contribution by Y. Zheng and D. Zhu entitled "Molecular Hydrogen Therapy Ameliorates Organ Damage Induced by Sepsis" provides an overview of molecular hydrogen therapy that can reduce damage of various organ functions from sepsis and improve survival rate. Molecular hydrogen therapy is a prospective method against sepsis.

The positive health benefits stemming from physical activity are well established. In their paper "Effects of Moderate Aerobic Exercise on Cognitive Abilities and Redox State Biomarkers in Older Adults," A. H. Alghadir et al. demonstrate that moderate aerobic training for 24 weeks has a positive significant effect in improving cognitive functions via modulating redox and inflammatory status of older adults.

Oxidative stress is one of the key mechanisms affecting the outcome throughout the course of organ transplantation. S. Shi and F. Xue in the paper entitled "Current Antioxidant Treatments in Organ Transplantation" provide an overview of emerging antioxidant treatments, targeting donor, graft preservation, and recipient as well.

Kidney ischemia/reperfusion injury emerges in various clinical settings as a great problem complicating the course and outcome. The paper by A. Kezic et al. entitled "Mitochondria-Targeted Antioxidants: Future Perspectives in Kidney Ischemia Reperfusion Injury" provides an overview of the current status of results achieved in numerous studies investigating these novel compounds in ischemia/reperfusion injury which specifically target mitochondria such as MitoQ, Szeto-Schiller (SS) peptides (Bendavia), SkQ1 and SkQR1, and superoxide dismutase mimics.

Cigarette smoking is the most important risk factor for the development or deterioration of Graves' ophthalmopathy. Smoke-induced increased generation of reactive oxygen species may be involved. In the paper by H.-C. Kau et al. entitled "Cigarette Smoke Extract-Induced Oxidative Stress and Fibrosis-Related Genes Expression in Orbital Fibroblasts from Patients with Graves' Ophthalmopathy," the authors evaluated some clues for the impact of cigarette smoking on Graves' ophthalmopathy and offered a theoretical basis for the potential and rational use of antioxidants in treating Graves' ophthalmopathy.

The molecular mechanisms for hypoxic environment causing the injury of intestinal mucosal barrier (IMB) are widely unknown. To address the issue, Han Chinese from 100 m altitude and Tibetans from high altitude (more than 3650 m) were recruited. The work by K. Li et al. entitled "Genome-Wide Transcriptional Analysis Reveals the Protection against Hypoxia-Induced Oxidative Injury in the Intestine of Tibetans via the Inhibition of GRB2/EGFR/PTPN11 Pathways" reports that the transcriptome analysis showed the protecting functions of IMB patients against hypoxia-induced oxidative injury in the intestine of Tibetans via affecting GRB2/EGFR/PTPN11 pathways.

Mitochondrial superoxide dismutase 2 (SOD2) converts superoxide anions to hydrogen peroxide and oxygen. Human data on SOD2 protein content in chronic kidney disease (CKD) are sparse and mortality data are lacking. In the work by K. Krueger et al. entitled "Lower Superoxide Dismutase 2 (SOD2) Protein Content in Mononuclear Cells Is Associated with Better Survival in Patients with Hemodialysis Therapy" the authors showed that SOD2 protein content declined in CKD until stage 4 while SOD2 gene expression did not. Increased cellular superoxide anion production might affect SOD2 protein content. In advanced CKD (stage 5), SOD2

protein content increased again, but higher than median SOD2 protein content in these patients did not confer a survival benefit.

Overall, several new findings have been presented in this special issue which have further advanced our knowledge in this area. We hope that the readers of this special issue appreciate the progress and the new strategies developed in the field.

Acknowledgments

We would like to express our appreciation to all the authors for their informative contributions and the reviewers for their support and constructive critiques in making this special issue possible.

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Research Article

Lower Superoxide Dismutase 2 (SOD2) Protein Content in Mononuclear Cells Is Associated with Better Survival in Patients with Hemodialysis Therapy

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Mitochondrial superoxide dismutase 2 (SOD2) converts superoxide anions to hydrogen peroxide and oxygen. Human data on SOD2 protein content in chronic kidney disease (CKD) are sparse and mortality data are lacking. We investigated SOD2 protein content in monocytes from patients with hemodialysis therapy ($n = 81$), CKD stage 1–5 ($n = 120$), and healthy controls ($n = 13$) using in-cell Western assays. SOD2 protein decreased from CKD stage 1 until stage 4 whereas it increased again in stage 5 with and without hemodialysis. SOD2 gene expression, analyzed by quantitative real-time PCR, was not significantly different between the groups. Elevating cellular superoxide production reduced SOD2 protein content. This effect was abolished by the superoxide dismutase mimetic Tempol. Using gelelectrophoresis and Western blot we did not detect nitrotyrosine modifications of SOD2 in CKD. Finally, in patients with CKD stage 5 with hemodialysis therapy higher than median SOD2 protein content was associated with higher all-cause mortality. In conclusion, SOD2 protein content declined in CKD until stage 4 while SOD2 gene expression did not. Increased cellular superoxide anion production might affect SOD2 protein content. In advanced CKD (stage 5) SOD2 protein content increased again, but higher than median SOD2 protein content in these patients did not confer a survival benefit.

1. Introduction

In chronic kidney disease (CKD) oxidative stress occurs frequently and has been proposed to be a central mechanism in the pathogenesis of CKD progression and CKD associated complications and mortality [1]. In particular dialysis patients show a higher mortality, and the cardiovascular mortality in patients with dialysis treatment is 10 to 20 times higher compared to the general population [2]. Oxidative stress describes the imbalance between formation of reactive oxygen species, like superoxide anions and hydrogen peroxides, and antioxidant defense systems [3]. Increased production of reactive oxygen species due to inflammation or decreased antioxidant capacity leads to lipid peroxidation and oxidation of proteins, carbohydrates, and amino acids [4]. Reactive oxygen species are normal by-products of cellular metabolism [5]. It has been estimated that 1 to 3% of the oxygen consumed by mitochondria is reduced to reactive oxygen species such as superoxide anion which is generated by one-electron-reduction of molecular oxygen [6, 7]. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anions to oxygen and hydrogen peroxide and thus represents a major antioxidant defense mechanism [8]. In mammals, different superoxide dismutase isoforms exist: superoxide

dismutase 1 (SOD1, cytosolic copper-zinc superoxide dismutase), superoxide dismutase 2, and superoxide dismutase 3 (SOD3, extracellular copper-zinc superoxide dismutase) (for review see Fukai and Ushio-Fukai [9]). Superoxide dismutase 2 (SOD2, manganese superoxide dismutase) is one of the three isoforms and is located in mitochondria [10].

Early studies with mutant mouse models showed the importance of SOD2 in mitochondria [11, 12]. In the homozygous mutant mouse (Sod−/−) a complete loss of SOD2 protein results in impairment of further mitochondrial enzymes followed by death of animals in 1 to 20 days after birth. Heterozygous Sod2+/− mice have shown increased oxidative stress [12].

In chronic kidney disease, contrasting results about SOD2 enzyme were reported. While in a uremic rat model Lim et al. found reduced SOD2 protein content in the liver Finch et al. found increased SOD2 protein in the kidney [13, 14]. In human CKD SOD2 studies are so far limited to gene expression analyses in peripheral white blood cells. Akiyama et al. did not find a significant difference in SOD2 gene expression between healthy subjects and patients with CKD or CKD and hemodialysis treatment while Zaza et al. reported in 15 peritoneal dialysis patients a significantly higher SOD2 gene expression compared to healthy controls [15, 16]. SOD2 protein analyses in human CKD as well as mortality analyses in relation to SOD2 protein content are lacking.

We therefore investigated SOD2 protein content and gene expression in patients with CKD and analyzed survival rates in CKD stage 5 patients with hemodialysis therapy in relation to SOD2 protein.

2. Materials and Methods

A total of 81 consecutive patients with chronic kidney disease stage 5 undergoing maintenance hemodialysis, 120 patients with chronic kidney disease stages 1–5 without hemodialysis treatment, and 13 healthy control subjects were investigated. Written informed consent was obtained from each subject and ethical approval was given by the local ethics committee. Hemodialysis patients were dialyzed 4-5 hours three times per week using biocompatible membranes. Blood samples from hemodialysis patients were taken before the start of the hemodialysis session.

We investigated monocytes from peripheral blood as these cells are of special interest in CKD patients. The monocyte-macrophage lineage is involved in different pathogenic processes in CKD: systemic inflammation [17], vascular disease and atherosclerosis [18–21], kidney injury [22], and impaired immune function [23]. Monocytes were isolated from heparinized blood using superparamagnetic polystyrene beads coated with a primary monoclonal antibody specific for the CD14 membrane antigen (Invitrogen DYNAL, Norway) and washed several times in Hanks balanced salt solution (HBSS).

For protein analyses by Western blotting monocytes were lysed in homogenization buffer (containing 50 mmol/L Tris-HCl, pH 8; 100 mmol/L NaCl, 100 mmol/L β-mercaptoethanol, 50 mmol/L NaF, 2 mmol/L ethylenediaminetetraacetic acid, and complete mini protease inhibitor cocktail (Roche Diagnostics; Germany)); proteins were separated by 12.5% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 150 V for 90 minutes and transferred to pure nitrocellulose membranes (Biorad; USA). Membranes were blocked with Odyssey blocking buffer (Licor biosciences; USA) and incubated with primary antibodies against superoxide dismutase 2 (SOD2) and beta-actin (Abcam). After washing with HBSS, the membranes were incubated with Alexa Fluor680-allophycocyanin-fluorescence-labelled (MoBiTec, USA) or IRDye800CW-infrared fluorescent dye-labelled (biomol, Germany) secondary antibodies. Imaging was performed using the Odyssey infrared imaging system (Licor biosciences; USA) at 700 nm or 800 nm emission with an excitation wavelength of 680 nm or 780 nm, respectively. Our experiments confirmed the specificity of the used SOD2 and beta-actin antibodies in human monocytes.

For the detection of nitrotyrosine the membranes were blocked with Odyssey blocking buffer (Licor biosciences; USA) and incubated with primary antibodies against nitrotyrosine (Chemicon, Millipore; USA). After washing with HBSS, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Dako-Cytomation; Denmark). Visualization was performed using the ECL chemiluminescence system (Amersham, USA).

For the quantification of SOD2 protein content we performed in-cell Western assays of monocytes as recently described by our group [24]. The method provides a sum measurement for different posttranslationally modified and nonmodified forms of a protein (protein species) that can be detected with the primary antibody. We have proven this concept in a publication about superoxide dismutase 1 (SOD1) protein species, where we investigated SOD1 protein content in monocytes of CKD patients in parallel with two-dimensional gel electrophoresis. The latter revealed that our SOD1 sum analysis of protein content in in-cell Western assay covered at least 6 SOD1 protein species [25].

Human monocytes in 96 well plates were permeabilized with Triton X100 and coincubated with the primary antibodies against SOD2 and beta-actin (Abcam) for 2 hours. After washing steps, monocytes were coincubated with corresponding secondary antibodies (see below) for 1 hour. Imaging was performed at 700 nm or 800 nm emission with an excitation wavelength of 680 nm or 780 nm. The protein content of SOD2 was always normalized to the beta-actin protein content of the same cells. Control experiments were performed without incubation of primary antibodies.

For activation and inhibition experiments phorbol-myristate-acetate (PMA, Sigma-Aldrich, final concentration 100 ng/mL) and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol, Sigma-Aldrich, final concentration 100 μM) were used (incubation time 3 hours).

RNA isolation, transcription, and quantitative real-time PCR were performed according to manufacturer's descriptions (High Pure RNA Isolation Kit, Transcriptor First Strand cDNA Synthesis Kit, LightCycler®FastStart DNA MasterPlus

Table 1: Population characteristics of subjects for superoxide dismutase 2 protein comparisons. Values are given as median (interquartile range) or number (percent).

	n	Age (years)	BMI (kg/m^2)	Gender male (%)
Healthy controls	13	46 (41–52)	23.6 (20.6–25.2)	7 (54)
CKD stage 1	32	56 (44–67)	24.6 (22.7–28.5)	23 (72)
CKD stage 2	22	65 (57–70)	27.5 (22.7–32.3)	12 (55)
CKD stage 3	28	71 (62–80)	26.1 (22.0–28.4)	18 (64)
CKD stage 4	29	67 (60–74)	26.7 (22.6–31.0)	22 (76)
CKD stage 5	9	71 (56–75)	26.2 (24.6–30.7)	3 (33)
CKD stage 5 HD*	81	66 (57–73)	24.4 (22.2–28.5)	47 (58)

*HD = hemodialysis therapy.

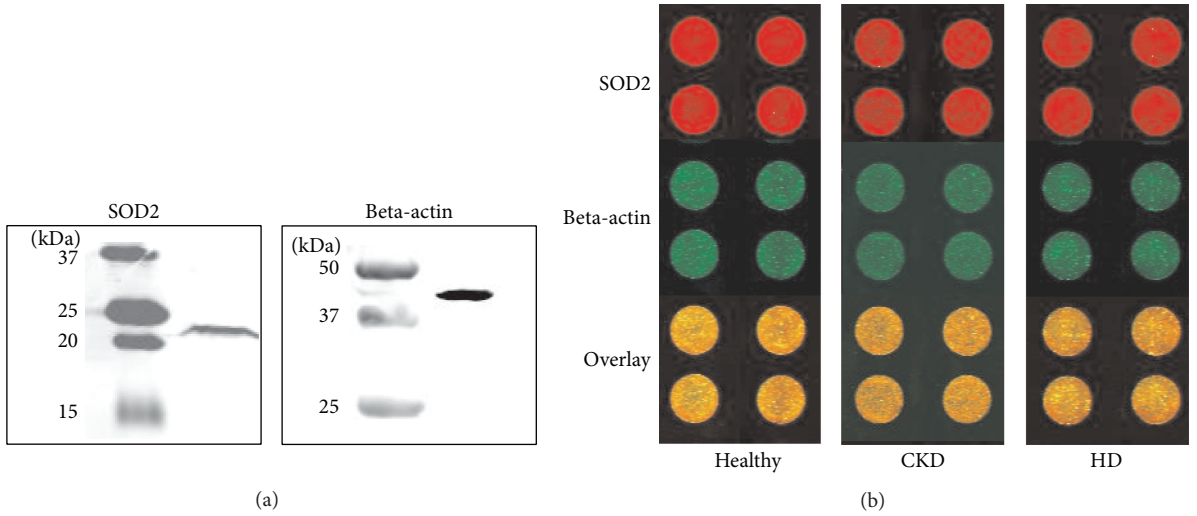

Figure 1: (a) Detection of proteins SOD2 and reference protein beta-actin in monocytes by immunoblotting. (b) Representative in-cell Western assay for quantification of SOD2 protein content in monocytes from a healthy control subject (Healthy), a patient with chronic kidney disease (CKD), and a hemodialysis patient (HD). Upper panels show SOD2 protein content (red fluorescence); middle panels of beta-actin (green fluorescence) and lower panels show overlay. Fluorescence intensities were analyzed in quadruplicate for each sample.

SYBR Green I Kit; Roche; Germany). The following primers for real-time PCR were used:

SOD2/NM_000636:

F-5′- ggt ggt cat atc aat cat ag -3′;

R-5′- agt gga ata agg ttt gtt gt -3′ (260 bp);

Beta-actin/NM_001101:

F-5′- aac tgc tta gca ccc ctg gc -3′;

R-5′- atg acc ttg ccc aca gcc tt -3′ (200 bp);

The PCR conditions using a LightCycler 2.0 Instrument (Roche Diagnostics, Germany) were as follows: 95°C for 10 min and 40 cycles of 95°C for 10 s; 55°C (for SOD2) or 60°C (for beta-actin) for 10 s; and 72°C for 10 s. Normalized ratios of SOD2 mRNA expression were calculated relative to housekeeping gene beta-actin mRNA expression including efficiency correction and calibrator normalization. PCR products were also size-fractionated on 1.0% agarose gels and visualized by ethidium bromide staining.

2.1. Statistics. Data are given as median and interquartile range. Data between groups were compared using Mann-Whitney test or Kruskal-Wallis test and Dunn's multiple comparison posttest, as appropriate. Kaplan Meier survival curves were compared using log-rank test (GraphPad prism software, version 5.0, GraphPad Software, San Diego, CA). All statistical tests were two-sided. A two-sided value of p less than 0.05 was considered statistically significant.

3. Results

We investigated superoxide dismutase 2 (SOD2) protein in monocytes from patients with chronic kidney disease (CKD) and healthy controls using in-cell Western assays (subject characteristics given in Table 1). First, the specificity of the SOD2 and beta-actin antibodies used in our experiments was shown by Western blotting (Figure 1(a)). We demonstrated SOD2 and beta-actin protein detection in monocytes with molecular weights of 21 kDa and 41 kDa. Figure 1(b) shows

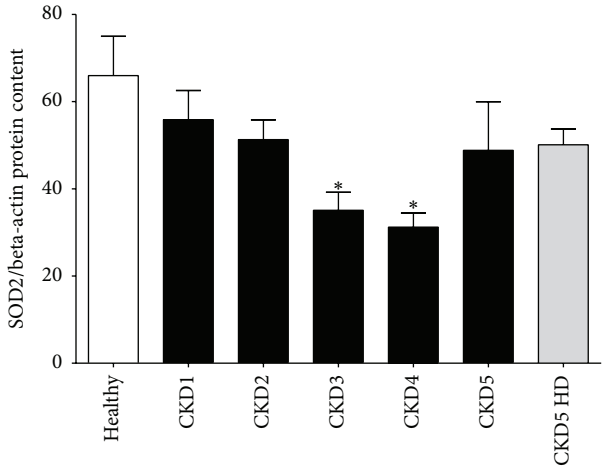

Figure 2: J-shaped pattern of SOD2 protein content. Quantification of SOD2 protein content relative to housekeeping protein beta-actin by in-cell Western assays. The figure shows the comparison between healthy control subjects, patients with CKD stage 1 through 5, and hemodialysis patients (CKD5 HD); $^{*}p < 0.05$ compared to healthy controls by Dunn's multiple comparison posttest.

representative in-cell Western assays of SOD2 and beta-actin protein in monocytes from healthy control subject, a patient with CKD, and a hemodialysis patient (quadruplicate determination for each subject).

Next, SOD2 protein content was analyzed for patients with all CKD stages, patients with hemodialysis therapy, and healthy controls. Figure 2 shows the summary data for SOD2 protein content which is decreased with declining glomerular filtration rate until CKD stage 4. In contrast, patients with CKD stage 5 without and also with hemodialysis therapy then show again higher SOD2 protein content so that a J-shaped pattern results. Kruskal-Wallis analysis showed a significantly different distribution of SOD2 protein content between all groups ($p < 0.002$).

In addition to quantification of the protein content, we investigated the SOD2 gene expression in monocytes from hemodialysis patients, patients with CKD, and healthy control subjects. Figures 3(a) and 3(b) present typical amplification and melting curves for SOD2 mRNA and the housekeeping gene beta-actin mRNA from a hemodialysis patient, a patient with CKD, and a control subject. PCR products were also size fractionated on 1.0% agarose gels and stained by ethidium bromide (Figure 3(c)). The SOD2 mRNA expression levels from patients with CKD and healthy control subjects are shown in Figure 4. The relative SOD2 to beta-actin gene expression in monocytes is expressed as ratio. Although there seemed to be a trend to increased SOD2 gene expression from healthy to CKD stage 4 patient the Kruskal-Wallis analysis did not show a significant difference in the distribution of SOD2 gene expression between the groups ($p = 0.10$).

Since we did not detect changes in SOD2 gene expression that explained the differences which we found in SOD2 protein content we investigated the effect of an increased cellular superoxide production on SOD2 protein content in mononuclear cells. To stimulate cellular superoxide production mononuclear cells were treated with PMA in the presence and absence of the SOD-mimetic Tempol. Figure 5 shows changes in SOD2 protein after cell incubation with PMA with and without Tempol. The protein content of SOD2 in the cells at baseline (1.00 (0.98–1.02)) was reduced after incubation with PMA (0.82 (0.77–0.86); $n = 8$) whereas the addition of Tempol significantly increased SOD2 protein content (1.22 (1.02–1.44; $n = 6$); each $p < 0.01$).

We also performed SDS-PAGE and Western blot analyses to search for nitrotyrosine modifications on SOD2 proteins in healthy subjects (H) and CKD stage 4 (CKD) and CKD stage 5 HD (HD) patients. No protein staining at the expected site of SOD2 bands was detected by the anti-nitrotyrosine antibody (Supplementary Figure 1(C) in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7423249).

Finally, we were interested in the relation between SOD2 protein content in monocytes of CKD stage 5 patients with hemodialysis therapy and all-cause mortality. We divided the patients in a group with SOD2 protein below and a group with SOD2 protein above the median (SOD2/beta actin protein = 42.54). Table 2 shows a comparison of the clinical parameter of these two patient groups. As indicated in Figure 6 survival was significantly better in hemodialysis patients with lower SOD2 protein content in peripheral blood monocytes (Chi square, 6.25; $p < 0.05$ by log-rank test).

4. Discussion

We investigated SOD2 gene expression and protein content in monocytes of CKD patients. Also, in hemodialysis patients, a population with reportedly high all-cause and cardiovascular mortality, we analyzed the relation between SOD2 protein content and mortality. The major findings in our study are the following: (1) SOD2 protein content showed a J-curve pattern with significantly lower values compared to healthy controls and a progressive reduction until CKD stage 4 followed again by higher SOD2 protein content in CKD stage 5 and patients with hemodialysis treatment. (2) The SOD2 gene expression was not significantly different between the groups. (3) Higher than median SOD2 protein content was associated with higher mortality in patients with hemodialysis therapy.

Both, the results on protein content and gene expression of SOD2 in our study, are interesting. In an earlier study our group investigated gene expression and protein content of the cytosolic superoxide dismutase isoform (SOD1) in monocytes of patients with CKD. In contrast to SOD2, the SOD1 protein was reduced in CKD showing the lowest protein content in patients with hemodialysis therapy; and SOD1 gene expression was increased in CKD, with a high significance in patients with hemodialysis [25]. These results are supported by the literature. In peripheral blood mononuclear cells of hemodialysis patients a significant increase of SOD1 mRNA compared to healthy controls was reported while SOD2 mRNA did not differ significantly from control [15]. The different gene expression of SODs in CKD could be explained by the multiple differences in gene regulation between SOD1 and SOD2 (for review see Miao and St. Clair

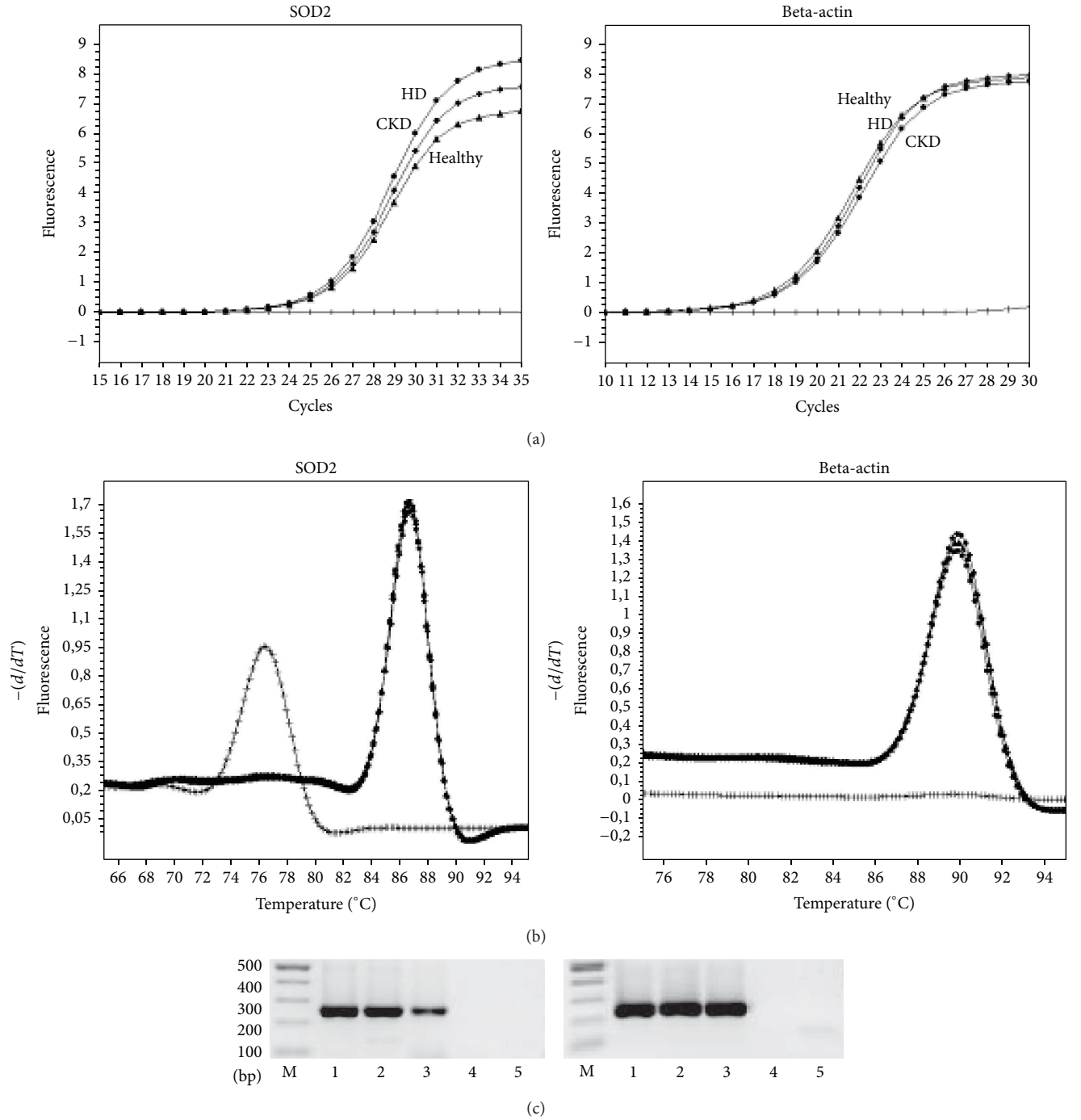

Figure 3: (a) Representative amplification curves of the quantitative real-time PCR for SOD2 and the housekeeping gene beta-actin in hemodialysis patients (HD, circles), patients with chronic kidney disease (CKD, squares), and control subjects (healthy; triangles). Curves with x-symbols indicate the no-template control in the PCR. (b) Melting analysis after amplification of SOD2 and the housekeeping gene beta-actin. The melting curve analysis confirmed the presence of one single peak in hemodialysis patients (HD, circles), patients with chronic kidney disease (CKD, squares), and healthy control subjects (healthy; triangles). Curves with x-symbols indicate the no-template control in the PCR and show a peak at lower temperature representing primer-dimers in the no-template reaction. (c) Example of size fractionation of PCR products on 1.0% agarose gels. M denotes the base pair DNA marker, lane 1 hemodialysis patient, lane 2 CKD patient, lane 3 healthy control, lane 4 negative control (no reverse transcriptase), and lane 5 negative control (no template). Left side shows PCR products for SOD2; right side shows PCR products for beta-actin.

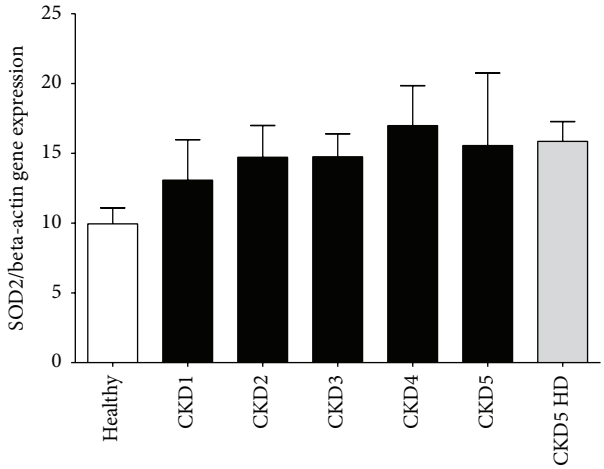

Figure 4: SOD2 mRNA expression analyzed by quantitative real-time PCR normalized to beta-actin in monocytes from healthy subjects (healthy), patients with chronic kidney disease (CKD1–5), and hemodialysis patients (CKD5 HD); $p = 0.10$ in the Kruskal-Wallis analysis.

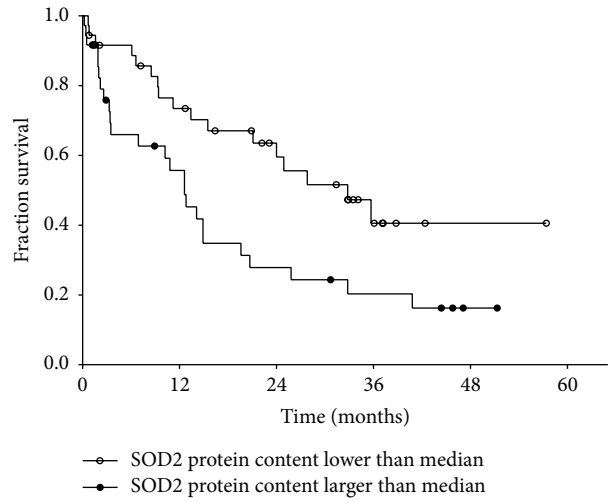

Figure 6: Kaplan Meier survival curves of hemodialysis (CKD5 HD; $n = 81$) patients according to the SOD2 protein content in peripheral blood monocytes (Chi square, 6.25; $p < 0.05$ by log-rank test).

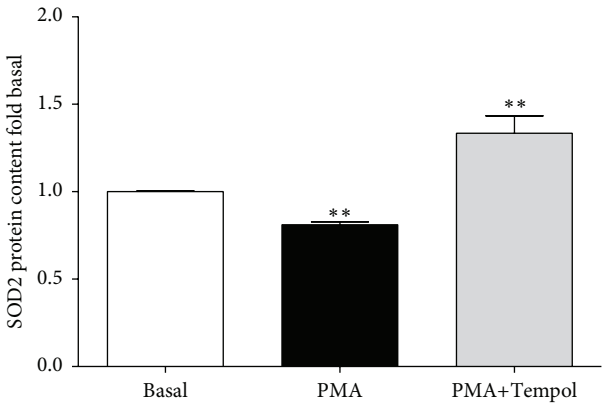

Figure 5: SOD2 protein content in cells under baseline conditions (control) and after incubation with PMA to stimulate cellular superoxide production with ($n = 6$) and without ($n = 8$) the SOD-mimetic Tempol. $^{**}p < 0.01$ versus basal.

[26]). With respect to the reduced SOD2 protein content found in CKD stages 3 and 4 in our present study different explanations are suggested by the literature. Two factors in CKD can contribute to an enhanced degradation of proteins: oxidative stress and uremia. Oxidative modification of SOD2 proteins has been described. Our own Western blot analyses did not suggest tyrosine nitration of SOD2 in CKD although refined analysis requires mass-spectrometric analyses of SOD2 protein from CKD patients and healthy subjects. While some authors using recombinant SOD2 protein showed tyrosine nitration and tyrosine oxidation upon treatment with peroxynitrite [27], protein biochemical analyses of SOD2 protein species from medulloblastoma cells showed tryptophan oxidation and histidine oxidations but no tyrosine nitration [28]. Therefore, it will be necessary to analyze oxidative SOD2 modifications for each pathogenic condition individually. Further insights could be revealed by adjacent analyses of SOD2 protein degradation and SOD2 enzymatic activity.

Our own results in the current study point to an involvement of reactive oxygen species in the regulation of SOD2 protein content since we observed a PMA-induced decrease of SOD2 protein in mononuclear cells that could be abolished by treatment with the SOD-mimetic Tempol. Furthermore, increased SOD2 protein degradation in the uremic environment may be involved. SOD2 protein was shown to be degraded by the ubiquitin-proteasomal pathway [29]. In CKD protein degradation via the ubiquitin-proteasomal pathway is enhanced [30, 31]. Generally, posttranslational protein modifications known for SOD2 from other cell types should also be considered for monocytes. These are tyrosine nitration and tyrosine oxidation [27], histidine and tryptophan oxidation [28], acetylation (for review see Zou et al. [32]), and ubiquitination [29].

Numerous studies have shown that the correlation between gene expression, quantified by messenger RNA (mRNA) analysis, and quantification of protein content is often limited (for review see [33, 34]). Specifically, for changes in protein content but unaltered mRNA levels a regulation of translation or regulatory changes of protein degradation can be underlying mechanisms.

The following conditions for instance have been described: reduced mRNA levels but constant protein content [35], constant mRNA levels but reduced protein content due to increased protein degradation [36], and constant mRNA levels but increased protein content due to decreased protein degradation [37]. Furthermore, our own group showed for superoxide dismutase 1 in monocytes from chronic kidney disease patients an increased mRNA level together with reduced superoxide dismutase 1 protein content [25].

The mechanisms underlying the increase of SOD2 protein in advanced CKD (CKD5 with and without hemodialysis)

Table 2: Clinical and biochemical characteristics of CKD patients with hemodialysis therapy. Values are median (interquartile range).

Characteristics	Lower than median SOD2 protein	Higher than median SOD2 protein
Age (years)	66 (58–73)	66 (56–74)
Gender (male/female)	24/16	22/18
Body mass index (kg/m^2)	24.4 (22.7–28.6)	24.3 (22.1–27.8)
Time since initiation of dialysis treatment (days)	244 (30–481)	295 (39–1193)
Hemoglobin (g/dL)	10.1 (9.3–11.4)	10.1 (8.6–12.0)
C-reactive protein (mg/dL)	2.7 (0.1–6.2)	2.6 (0.9–6.9)

compared to CKD 3 and 4 that we observed and that resulted in a J-shape of SOD2 protein content are not known. In this respect it is of interest that an upregulation of SOD2 protein that was reported in uremic rats could be reversed by vitamin D receptor agonists [14]. Since advanced uremia is a state of 1,25-dihydroxyvitamin D deficiency but also of vitamin D receptor deficiency and dysfunction, vitamin D receptor-related uremic effects could be involved in the J-shape of SOD2 protein that we observed [38].

Finally, we observed a better survival in hemodialysis patients with a SOD2 protein content below the median of this patient group compared to those with SOD above the median. The underlying mechanism for this observation is not known but considering the above discussed connection to vitamin D metabolism an association of higher content of SOD2 protein with more severe uremic disturbance of vitamin D receptor signal transduction could be involved.

In future experiments it will be necessary to identify underlying SOD2 protein modifications using 2 DE, mass-spectrometry and immunoblotting.

Taken together, our study shows significant changes of SOD2 protein content with increasing degree of renal function impairment. We also proof, in line with our own previous research but also with many other groups, that in clinical-experimental research a parallel investigation of gene expression and protein content is indispensable. And finally, we provide the first report of SOD2 protein content together with its association with survival in CKD patients with hemodialysis therapy.

Competing Interests

The authors have declared that no conflict of interests exists.

Authors' Contributions

Katharina Krueger and Jianlin Shen contributed equally to this work.

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Research Article

Genome-Wide Transcriptional Analysis Reveals the Protection against Hypoxia-Induced Oxidative Injury in the Intestine of Tibetans via the Inhibition of GRB2/EGFR/PTPN11 Pathways

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The molecular mechanisms for hypoxic environment causing the injury of intestinal mucosal barrier (IMB) are widely unknown. To address the issue, Han Chinese from 100 m altitude and Tibetans from high altitude (more than 3650 m) were recruited. Histological and transcriptome analyses were performed. The results showed intestinal villi were reduced and appeared irregular, and glandular epithelium was destroyed in the IMB of Tibetans when compared with Han Chinese. Transcriptome analysis revealed 2573 genes with altered expression. The levels of 1137 genes increased and 1436 genes decreased in Tibetans when compared with Han Chinese. Gene ontology (GO) analysis indicated most immunological responses were reduced in the IMB of Tibetans when compared with Han Chinese. Gene microarray showed that there were 25-, 22-, and 18-fold downregulation for growth factor receptor-bound protein 2 (GRB2), epidermal growth factor receptor (EGFR), and tyrosine-protein phosphatase nonreceptor type 11 (PTPN11) in the IMB of Tibetans when compared with Han Chinese. The downregulation of EGFR, GRB2, and PTPN11 will reduce the production of reactive oxygen species and protect against oxidative stress-induced injury for intestine. Thus, the transcriptome analysis showed the protecting functions of IMB patients against hypoxia-induced oxidative injury in the intestine of Tibetans via affecting GRB2/EGFR/PTPN11 pathways.

1. Introduction

Intestinal mucosa is more likely to be damaged if the person is living in an altitude above 3000 meters. Animal experiment showed that high-altitude hypoxia induced impaired intestinal mucosal barrier (IMB) [1, 2]. The incidence of digestive system disease has been reported to be increased in the residents who live at high-altitude environments [3]. Impaired IMB threaten the residents living at high places. The therapy for impaired IMB complicated. The main problem is that the mechanism for high-altitude inducing IMB injury remains widely unknown and no practical therapeutic method can be used yet.

The intestinal tract is an important barrier for preventing bacterial translocation and endotoxin entering human organs. It is well known that intestinal mucosal injury may decrease the function of IMB. The most significant changes in oxygen level in living environments, such as from normobaric normoxia to hypobaric hypoxia (3450 m terrestrial altitude), will result in the increase of reactive oxygen species (ROS) when the balance between prooxidant and antioxidant activity is impaired following exposure to terrestrial hypobaric hypoxia [4]. ROS play an important role in chronic intestinal inflammatory diseases production by increasing the permeability of the endothelium and the mucosa and allowing infiltration of inflammatory leukocytes into intestinal area. Scavenging of ROS is beneficial for intestinal disease [5]. Most Tibetans live at high-altitude plateaus with hypoxic environments [6]. In the environment, many tissues produce ROS, which may arise under the conditions of hypoxia [7]. On the other hand, it has been reported that high-level ROS induces intestinal cell apoptosis [8]. Thus, it almost seems

like that ROS levels are aberrant in the intestinal mucosal barrier of Tibetans. ROS has been well known to be associated with oxidative stress [9–12] by damaging lipids, proteins, and DNA [13]. Oxidative stress is an important contributor to the damage of vascular cells [14] and the pathogenesis of hypoxia/reoxygenation injury [15]. Intestinal oxidative stress also is a main factor contributing to intestinal injury, resulting in endotoxin translocation [16]. Dysfunction of the intestinal barrier has been reported to be associated with high-level intestinal oxidative stress [17]. High altitude often induces oxidative stress by affecting biochemical metabolisms, such as lipid metabolism dysfunction [10]. Thus, impaired IMB in the people living at high altitude may be linked to altered control of oxidative stress.

Oxidative stress causes the injury of intestinal tissues via multiple signaling pathways. For example, increased oxidative stress induces the damage in the small intestine of male Sprague-Dawley rats by activating the pathway of p38 mitogen-activated protein kinase [18]. Another example, ischemia/reperfusion results in oxidative injury in animal's intestine. Chinese jujube polysaccharides showed good enzyme activities and ameliorated the injury of the small intestine in rabbits with ischemia/reperfusion [19]. However, most experiments on oxidative stress-induced injury for intestine have been performed in animals and the molecular mechanisms remain unclear for hypobaric hypoxia promotes intestinal barrier dysfunction in the residents at high altitude. Additionally, the molecular mechanisms for hypobaric hypoxia causing the dysfunction of intestinal barrier and development of impaired IMB remain unknown.

The oxidative stress-induced intestinal injury may be associated with many signaling pathways. It is impossible to resolve such complex issues using a single signaling pathway. Transcriptome has been widely used to explore the related pathways in human various diseases [20–22]. To comprehensively understand the effects of hypoxia on IMB in the residents at high altitude, transcriptome experiment was performed here. These gene expression differences were analyzed using DNA microarray. The work will provide important information for hypoxia inducing impaired IMB of the patients at high altitude and basic knowledge for causing IMB injury. The gene expressing profiles of intestinal mucosa from the Tibetans at high altitude more than 3650 m and Han Chinese at 100 m altitude were analyzed to investigate the potential molecules involved in the pathophysiology of IMB injury caused by hypoxic environments. The database for gene ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) was referred to predict the genes involving important functions and signaling transduction. Meanwhile, we observed the microstructure of intestine mucosa of Tibetans at high altitude and Han Chinese from plain area with a normal and an electron microscope.

2. Material and Methods

2.1. Materials. The Trizol reagent was purchased from Life Technologies (Carlsbad, CA, USA). All primers were synthesized by Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). The cDNA reverse transcription kit and Takara Bio SYBR Premix Ex Taq were from Takara too. Hematoxylin and eosin (H&E) dyes were purchased from Sigma (St. Louis, MO, USA).

2.2. Participants. All the protocols in present study were specially approved by Human Research Ethical Committee from the People's Hospital from the Tibet (Tibet, China). All experiments were in compliance with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects. From June 2013 and August 2013, 3 Han Chinese from plain area were recruited at Guangzhou First People's Hospital (Guangzhou, China), and 3 Tibetans at high altitude more than 3650 m were recruited at People's Hospital from Tibet (Tibet, China). Each patient had the similar parameter to a healthy participant on gender, birthplace, work intensity, and so on. Research objects were native Tibetans at Lhasa with the age of 40–45. Each participant would sign a consent form before his intestinal mucosa could be taken.

2.3. Sample Extraction. The biopsies of mucosa were taken at People's Hospital of the Tibet (Tibet, China) and the Guangzhou First People's Hospital (Guangzhou, China), respectively. Six intestinal biopsies were obtained from the IMB of 10 Tibetans at high altitude more than 3650 m as an experimental group and 10 Han Chinese at 100 m altitude as a control group. All the participants were with underlining normal mucosa. The samples were frozen using liquid nitrogen and kept at −80°C.

2.4. The Observation of Intestinal Mucosa by Scanning Electron Microscope. Specimens of sigmoid colon mucosa were obtained at colonoscopy examination from all participants. Twenty samples were used in present work. The samples were fixed with formalin. Ten mm pieces of samples were washed and fixed using osmium tetroxide. All the blocks were made for subsequent histological analysis. Thirty sections were made from one block, dewaxed, dried, and coated using gold palladium by a vacuum evaporator. The microstructure of final samples was observed by a FEI Quanta 400 scanning electron microscope (FEI Company, Hillsboro, OR, USA).

2.5. Histological Analysis. All intestinal samples were rinsed with saline solution, fixed in ten percent formaldehyde at 4°C for one day, and washed with PBS. The treated samples were made as four μm species and dyed using H&E stain (hematoxylin and eosin). The microstructure of samples was observed under a microscope. The amounts of villi were calculated within one visual place. IMB was assessed in a double-blind way. The mucosae were injured if intestinal surface was discontinuous, gland was dilated, or superficial cells were damaged [12].

2.6. RNA Extraction. Intestinal samples from 10 Tibetans and 10 Han Chinese were collected. All samples were digested in three mL Trizol and ground using a homomixer. Chloroform was added, and RNAs were collected by addition of ethanol. Final RNA samples were resuspended in a buffer with ten mM

tris hydrochloride, pH 8.0, one mM EDTA. The quantity of RNA verified by a NanoDrop 1000 Spectrophotometer V3.7 (NanoDrop Technologies, Inc. Wilmington, DE).

2.7. RNA Microarray. Genome Oligo Microarray represents the genes and transcripts, which are determined by genome sequencing. RNA microarrays are often regarded as cDNA database after the reverse transcription. All the sequences were obtained from six participants (3 Han Chinese and 3 Tibetans) and verified by aligning these sequences from all known mRNA sequences.

2.8. RNA Amplified, Labeled, and Hybridized with Agilent Microarrays. Sample labeling and hybridization was conducted based on the protocols for Microarray-Based Gene Expression. All RNAs were increased and marked by Cy3-UTP. The amount and cRNAs activity were identified using NanoDrop 1000 Spectrophotometer V3.7 (NanoDrop Technologies, Inc., Wilmington, DE). One μg labeled cRNAs were disrupted and then incubated at 60°C within half an hour. Subsequently, cRNA was diluted by GE Hybridization buffer. RNA microarray was assembled by adding 100 μL hybridization solution to the slide. The final sample was heated at 65°C and measured by using Agilent Microarray Scanner.

We used the software for Agilent Feature Extraction to assess all the final data. DEGs (differently expressed genes) were confirmed by a Volcano Plot. Hierarchical Cluster analysis was conducted by the software Agilent GeneSpring GX. Signaling transduction was analyzed and the enrichment in the microarray was calculated.

2.9. Enrichment Analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The GO work offers control vocabularies to indicate the DEGs functions. GO has 3 parts: biology processing (BP), cell components (CC), and molecules functions (MF). P values showed the richness of DEGs. There were significantly statistical differences if $P < 0.05$. EASE scores, P values of Fisher, or hypergeometer presents the significance for the correlated pathways.

2.10. Real-Time Quantitative PCR (qRT-PCR). To further confirm above RNA microarray data, qRT-PCR was used to analyze the top DEGs in 10 Han Chinese at 100 m altitude and 10 Tibetans at high altitude more than 3480 m, including epidermal growth factor receptor (EGFR), growth factor receptor-bound protein 2 (GRB2), and tyrosine-protein phosphatase nonreceptor type 11 (PTPN11). The RNAs were extracted using above intestinal mucosa by Trizol. Five μg RNA was reversely transcribed using reverse transcription kits. All the primers were given as Table 2 showed, and qRT-PCR was conducted by SYBR® Green RT-PCR Kit on the real-time PCR system. The amplification situation was given as follows: 94°C for 5 min, 45 cycles of 95°C for 20 s, 65°C for 30 s, and 65°C for 40 s.

2.11. Western Blot Analysis. Rabbit anti-human polyclonal EGFR antibody (Cat. number ab2430), rabbit anti-human polyclonal GRB2 antibody (ab32037), rabbit anti-human monoclonal PTPN11 antibody (Cat. number ab32083), and rabbit anti-human beta-actin antibody (Cat. number ab8227) and goat anti-rabbit HRP (IgG H&L) (Cat. number ab6721) were purchased from Abcam Shanghai office launch (Shanghai, China). The intestinal samples were taken from all participants by a noninvasive method using endoscopic techniques. All tissues were ground by using a sterile mortar and pestle. Sample proteins were collected by centrifugation and separated by SDS-PAGE and then electrophoretically transferred onto PVNF membranes. After blocking the membranes with free-fat milk, they were then incubated with primary antibody. The members were washed three times and incubated with HRP-linked secondary antibody. The protein expression level was normalized by beta-actin expression. The immunoreactive result was visualized by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Stockholm, Sweden).

2.12. The Locations of DEGs on Human Chromosomes. The locations of DEGs on human chromosomes were marked on the human chromosomes using the data from RNA microarray results. Messenger RNA expression profiles were analyzed at genome level using the above results. About 400 significantly differently expressed genes were marked on 24 human chromosomes. The fold changes were marked with different colors.

2.13. Data Analysis. All data were showed using average values ± S.D. An ANOVA analysis was performed to compare the difference between different groups and the statistical significance was verified. There were significantly statistical differences if $P < 0.05$.

2.14. Construction of Gene Networks Based on Microarray Data. The significantly differentially expressed genes from microarray data were used. String software was used to retrieve the interacting genes (http://string-db.org/). Up- or downregulated genes from the microarray were visualized on this network. According to experimental results and computational prediction, a confidence score was used to confirm the interaction between miRNA and DEGs. The confidence score > 0.5 is regarded as statistically significant.

3. Results

3.1. Baseline Characteristics of Participants. The baseline and physical characteristics of the study population were listed in Table 1. There were significant differences for the distribution of living altitude ($P < 0.01$) but no difference for years at their locations ($P > 0.05$) between Tibetans and Han Chinese. In contrast, no significant difference was found for other parameters including age, cigarette smoking, alcohol drinking, and BMI ($P > 0.05$, Table 1). In contrast, there were statistically significant differences for Hb and diastolic pressures ($P < 0.05$, Table 1). The levels of oxygen saturation were lower in the residents from high altitude than at low

Table 1: The baseline characters of all participants.

Characteristic	Han Chinese		Tibetans		F-ratio	P value
	Group I	Group II	Group I	Group II		
Age (years)	41–45	40–49	40–45	39–48	0.38	0.77
Smoking (no/yes)	1/2	3/4	1/2	2/5	1.77	0.29
Drinking (no/yes)	1/2	3/4	1/2	2/5	1.77	0.29
Gender (male/female)	2/1	5/2	2/1	4/3	1.77	0.29
BMI	29–34	28–35	30–33	27–36	0.26	0.29
Food calorie intake (kcal/d)	2545.4–2089.3	2435.6–2132.7	2533.8–2134.7	2510.6–2184.1	0.85	0.36
Frequency of food (per day)	3 times	3 times	3 times	3 times	0	1
Habit	Rural	Rural	Rural	Rural	—	—
Marital status	Married	Married	Married	Married	—	—
Physical activity	Routine work	Routine work	Routine work	Routine work	—	—
Emotional makeup	Normal	Normal	Normal	Normal	—	—
Sleep habits	Day	Day	Day	Day	—	—
Mental stress	Social	Social	Social	Social	—	—
Water intake	During meal	During meal	During meal	During meal	—	—
Diet and sleep timings	Regular	Regular	Regular	Regular	—	—
Living at altitude (meters)	100	100	3650–3690	3650–3690	297.3160	<0.0001
Time at plain area or high altitude (years)	41–45	40–46	40–45	42–44	1.54	0.28
Hb (g/L)	144–156	140–160	166–175	165–180	2.56	0.04
Systolic pressure (mmHg)	112–124	108–129	125–130	122–138	2.20	0.03
Diastolic pressure (mmHg)	68–78	65–80	82–89	80–92	3.62	0.01
Blood oxygen saturation (%)	98-99	98-99	82–85	80–86	5.36	0.01
Heart rate (time/mini)	64–78	60–80	78–87	78–90	2.04	0.06

Note: BMI, body mass index; Hb, hemoglobin. There is a significant difference if $P < 0.05$.
Group I, the participants underwent genome-wide transcriptional analysis, real-time PCR, and Western Blot analysis. Group II, the participants underwent real-time PCR and Western Blot analysis.

Table 2: The primers used for real-time quantitative PCR.

Genes	GenBank accession number		Primers (5′-3′)	Size (bp)
EGFR	BC094761.1	Forward	accatccaggaggtggctgg	440
		Reverse	ggatcacacttttgtccctg	
GBR2	JX512444.1	Forward	aagacggcttcattcccaag	134
		Reverse	ctctctcggataagaaaggc	
PTPN11	NM_002834.3	Forward	ttcacactttccgttagaag	162
		Reverse	attgcccgtgatgttccatg	

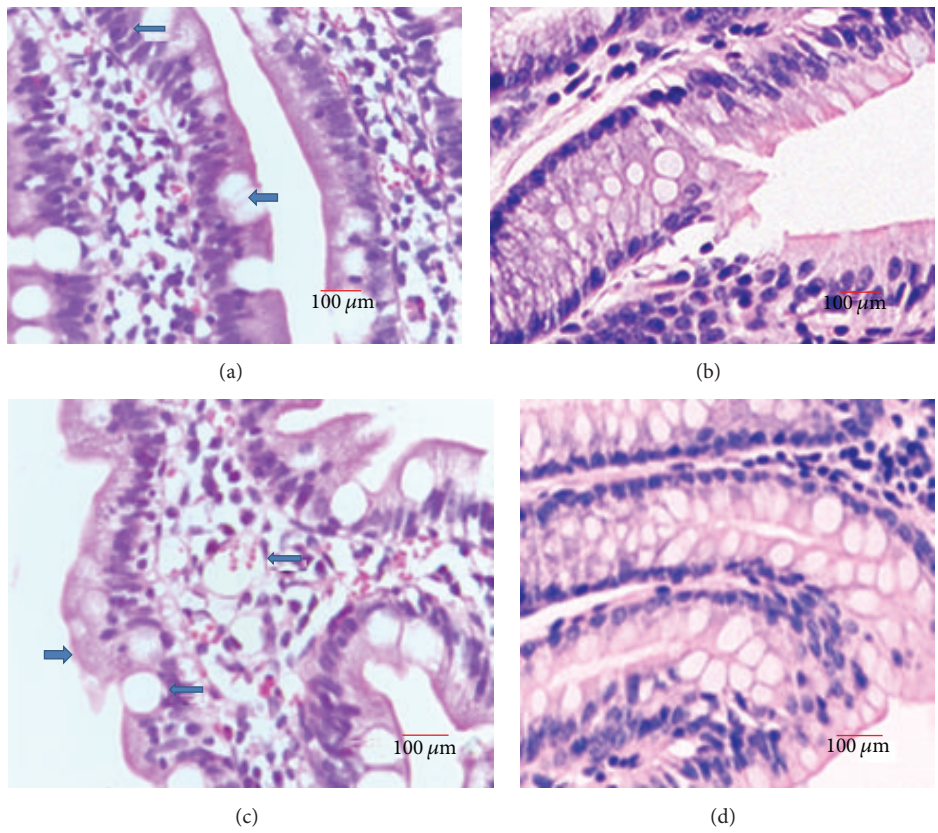
Note: epidermal growth factor receptor (EGFR), growth factor receptor-bound protein 2 (GRB2), and tyrosine-protein phosphatase nonreceptor type 11 (PTPN11).

altitude ($P < 0.01$), although heart rates in Tibetans were faster than in Han Chinese.

3.2. Hematoxylin-Eosin Staining Analysis of Intestinal Tissues. Hematoxylin-eosin stained results showed cylindric and cup cells were mostly destroyed as arrow indicated in Tibetans (Figure 1(a)) while cylindric and cup cells had normal structures in Han Chinese (Figure 1(b)). There were more capillary microvessels in the intestinal mucosa in antrum region of the Tibetans than in Han Chinese (Figure 1(c)) while there were no more capillary microvessels in the intestinal mucosa in antrum region of Han Chinese (Figure 1(d)).

3.3. Scanning Electron Microscope (SEM) Analysis of Intestinal Mucosa. SEM of the intestinal mucosa from jejunum was shown in Figure 2, revealing the basic characteristics of the intestinal tissues. The features of intestinal symptoms were seen in these specimens. Electromicroscopy showed that Tibetans had intestinal mucosa injury while Han Chinese had normal intestinal mucosa. Intestinal villi were usually reduced and appeared irregular in the IMB of Tibetans (Figure 2(a)) while intestinal villi were usually rich and in regular form in the IMB of Han Chinese (Figure 2(b)). Glandular epithelium was destroyed in the IMB of Tibetans (Figure 2(c)) while the glandular epithelium was in a fine situation in the IMB of Han Chinese (Figure 2(d)).

3.4. Screening of Differentially Expressed Genes. Hierarchical cluster analysis was conducted as Figure 3 showed. Three Han Chinese and three high-altitude Tibetans showed different gene expressing patterns. The levels of 1237 genes increased and 1336 genes decreased in the intestinal tissues from



(a) (b) (c) (d)

Figure 1: Histopathological examination of intestinal mucosa sections. (a) Cylindric and cup cells were mostly destroyed as arrow indicated in the IMB of Tibetans. (b) Cylindric and cup cells had normal structures in the IMB of Han Chinese. (c) There were more capillary microvessels as arrow indicated in the intestinal mucosa in antrum region of the Tibetans. (d) There were no more capillary microvessels as arrow indicated in the intestinal mucosa in antrum region of Han Chinese.

Tibetans when compared with Han Chinese. There were 25-, 22-, and 18-fold downregulation for GRB2, EGFR, and PTPN11 in the IMB from Tibetans when compared with Han Chinese.

3.5. qRT-PCR. We measured the levels of three significantly changed genes using real-time PCR. GRB2, EGFR, and PTPN11 had similar expressing profiles with those obtained from the RNA transcriptomes analysis. GRB2, EGFR, and PTPN11 were more than 20-fold downregulated in Tibetans when comparing to Han Chinese ($P < 0.05$) (Figure 4). The results showed the similar changing trend with that from microarray analysis while their baseline characters were also similar with patients analyzed by microarray method (Table 1).

3.6. Protein Expression of GRB2, EGFR, and PTPN11. The protein levels of GRB2, EGFR, and PTPN11 had similar changing trends with those obtained from qRT-PCR analysis. GRB2, EGFR, and PTPN11 were significantly downregulated in Tibetans when comparing to Han Chinese ($P < 0.05$) (Figure 5). The results showed the similar changing trend with those from microarray analysis.

3.7. Comprehensive Peptidome Profiling of DEGs. All DEGs were explained using GO terms by peptidome profiling analysis. All the events were elucidated in intestinal mucosa from the IMB of Tibetans when compared to those from Han Chinese. In the GO, the upregulated DE genes were involved in the reactive oxygen species activity, oxygen ions and peroxides, lipid peroxidation and so on; the downregulated DE genes were involved in the decrease of immunological ability. All the changes can be caused by oxidative stress, such as cancer suppressor [23], cell normal functions [24], special responses for pathogens [25], maximal actions, and antigen presentation [26] (Figure 6). Pathway analysis revealed that, in intestinal mucosa tissues of participants from high altitude, many pathway genes had aberrant expression and may be also related with oxidative stress, especially in inflammatory bowel diseases [27, 28], myeloperoxidase [29], ROS production [10], apoptotic cell death [30], tissue damage [31], interleukin-1 [32], oxidative modification [33], cystic fibrosis [34], natural killer cells activity [35], lymphocytes [36], fibrinolysis [37] and so on (Figure 7).

3.8. Clusters of Differently Expressed Genes on Human Chromosomes. Just as Figure 8 showed, four hundred DEGs are mapped on using human chromosomes. The differently

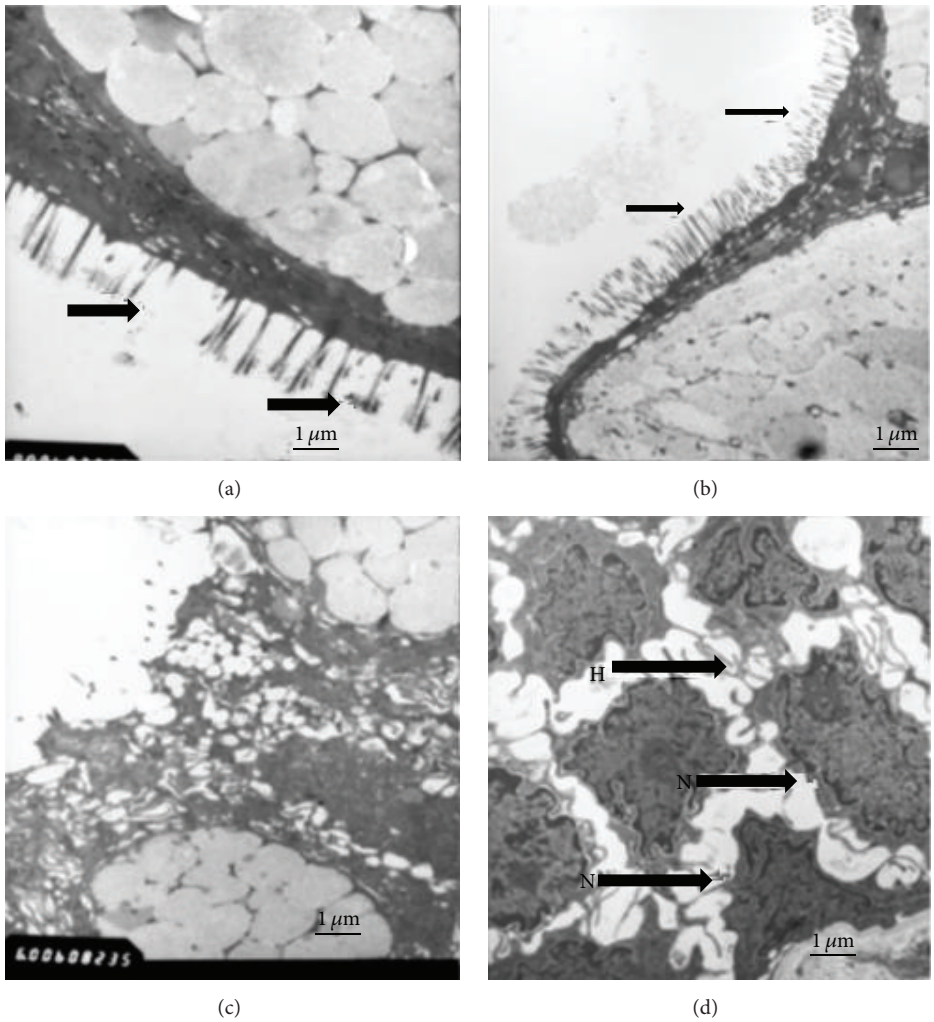


Figure 2: Electroscopic studies of the digestive tract in the IMB of Tibetans and Han Chinese at high altitude. (a) Intestinal villi are usually reduced and appear irregular in the IMB of Tibetans. (b) Intestinal villi are usually rich in the Han Chinese. (c) Glandular epithelium is destroyed in the IMB of Tibetans. (d) Glandular epithelium is in a fine situation in Han Chinese.

expressed genes on chromosomes thirteen, fourteen, fifteen, eighteen, twenty, twenty-one, twenty-two, and Y had fewer DEGs locations involved with reactive oxygen species activities. The high density of DEGs gathered on chromosomes one, six, seven, eleven, fourteen, seventeen, and nineteen. The most significantly expressed genes with more than 10-fold were all located on three different chromosomes: epidermal growth factor receptor (EGFR), chromosome 7p12.3-p12.1; growth factor receptor-bound protein 2 (GRB2), chromosome 17q24-q25; and tyrosine-protein phosphatase nonreceptor type 11 (PTPN11), chromosome 12q22-qter (Figure 8).

3.9. Visualization of Microarray Data by Using DEGs Networks. The microarray data mainly showed the up- and downregulated genes and were visualized on the network (Figure 9), which was created around mainly interesting proteins relating to the protecting functions for oxidative stresses and significantly differently expressed between Tibetans and Han Chinese. On the network, genes that were downregulated were shown as green circles and genes that upregulated were shown as red circles (Figure 9). In Tibetans, three main signaling pathways associated with GRB2, EGFR, and PTPN11 were shown on a network to be significantly downregulated when compared with Han Chinese (Figure 9).

4. Discussion

Many factors can result in the injury of intestinal mucosa and hypoxia is an important risk for causing the injury of intestinal tissues [1, 38–42]. Firstly, hypoxic pressure affects basic metabolic processes [43], resulting in the changes for many biological functions. Secondly, hypoxia-caused carbonic anhydrase, which constitutes an acidic microenvironment [44], is harmful to the residents at high altitude. Thirdly, hypoxia environment disturbs the gut flora imbalance in the residents at high altitude causing the intestinal injury [45]. Hypobaric hypoxia will inhibit the secretion of IgG, which is an important immune-related molecule in intestine

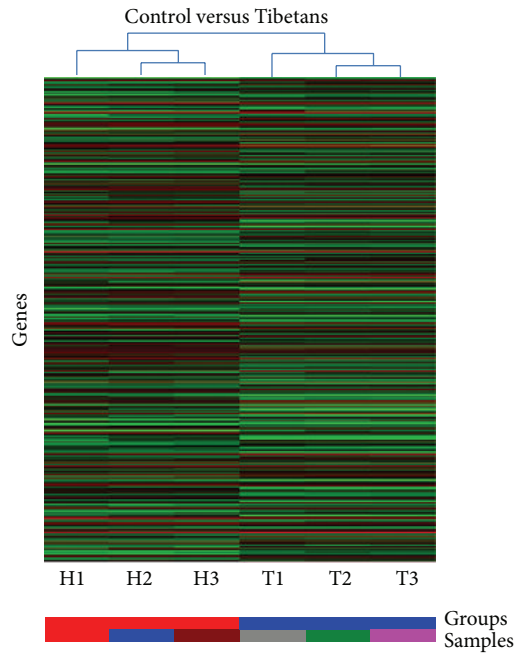


Figure 3: Hierarchical cluster analysis of the altered genes in the intestinal mucosa of IMB of Tibetans and Han Chinese. The color code in each heat map has been lineared with green as the lowest level for mRNA and red as the highest level for mRNA. The increased genes expression was shown in green to red, whereas the decreased genes expression was shown from red to green.

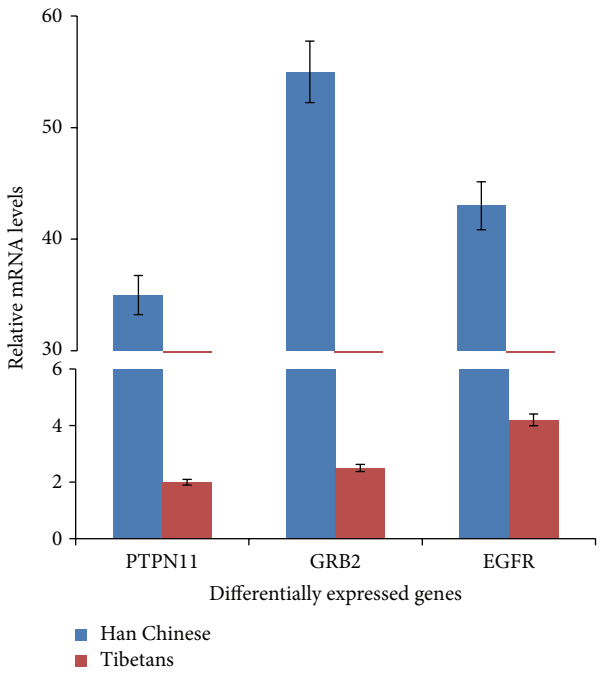


Figure 4: Validation of microarray results (the top 3 up- and down-regulated DEGs) by qRT-PCR. The results represented quantification of mRNA levels relative to beta-actin. Normalized expression values were obtained by qRT-PCR ($n = 10$). C = Han Chinese at 100 m altitude and **P** = Tibetans at high altitude more than 3480 m. All the data were present as average value ± SD. $P < 0.05$ via IMB of Tibetans.

[46, 47]. Furthermore, hypobaric hypoxia also inhibits bile secretion and decreases enterohepatic circulation, resulting in intestinal dysfunction and bacterial overproliferation and increasing the damage of intestinal biological barriers [48]. Especially for the first point, hypoxic pressure affects basic metabolic processes and produces high-level ROS, which lead to the increase of oxidative stress [49, 50]. Oxidative stress is an important contributor for tissue injuries, including intestine injury [18, 51].

Homo sapiens Genome Oligo Microarray has most well-known genes for human being. All data can be compared with the resourceful sequences with clear functions [52–54]. We use the data to explore the expressing profiles of intestinal tissues from Tibetans at high altitude. Meanwhile, the data compared with those from Han Chinese at plain area were analyzed using genome microarrays, in which 1137 DEGs increased while 1436 DEGs decreased with more than two-fold changes in the Tibetans. Based on those data, we then studied differentially expressed genes function. Our results indicated that IMB involves the oxidant responses.

Present findings indicated that GRB2/EGFR/PTPN11-associated pathways were significantly downregulated (fold change = 25, 22, and 18). As reported in previous studies, GRB2 was related with formation of reactive and oxidative products [55, 56]. ROS are directly involved in gastrointestinal injury. High concentration of ROS in intestinal mucosa possibly decreases mucosal organ-protective efficacy. Many factors, such as intestinal food, are more likely to destroy the mucosal structure when intestinal mucosa is in a fragile condition [57]. Meanwhile, ROS increase the permeability of small intestinal epithelial cells and lead to intestinal mucosal injury at an early stage [58].

The inhibition of GRB2 can significantly reduce fat accumulation, improve glucose metabolism, ameliorate oxidative stress [55], and activate mitogen-activated protein kinase pathways [59]. Additionally, GRB2 deficiency reduces cell apoptosis by inactivating caspase-3. The decrease in GRB2 improves hepatic steatosis and glucose metabolism and reduces oxidative stress. All these activities will improve the intestinal injury induced by hypoxia-induced oxidative stress.

Despite the important role of EGFR in intestinal epithelial cells [60], the study on the effects of EGFR on the intestinal injury is very limited. According to a previous report, the overexpression of EGFR increases the levels of ROS, accumulates DNA strand damage, and makes genome unstable. The levels of EGFR activation are associated with oxidative stress [61]. Therefore, inactivation of EGFR pathway will decrease the level of ROS and reduce the oxidative stress. The downregulation EGFR pathway will improve the protecting functions for intestinal injury.

The role of PTPN11 pathway is seldom reported in intestinal tissues. From the network, PTPN11 is closely associated with the JAK and STAT signaling pathways (Figure 9), which are activated by protein tyrosine kinases and phosphatases, and is necessary in regulating cellular activities responding various cytokines. Dysregulation of the JAK and STAT pathways will lead to hematopoietic and immune diseases. PTPN11 plays an important regulatory role in JAK and

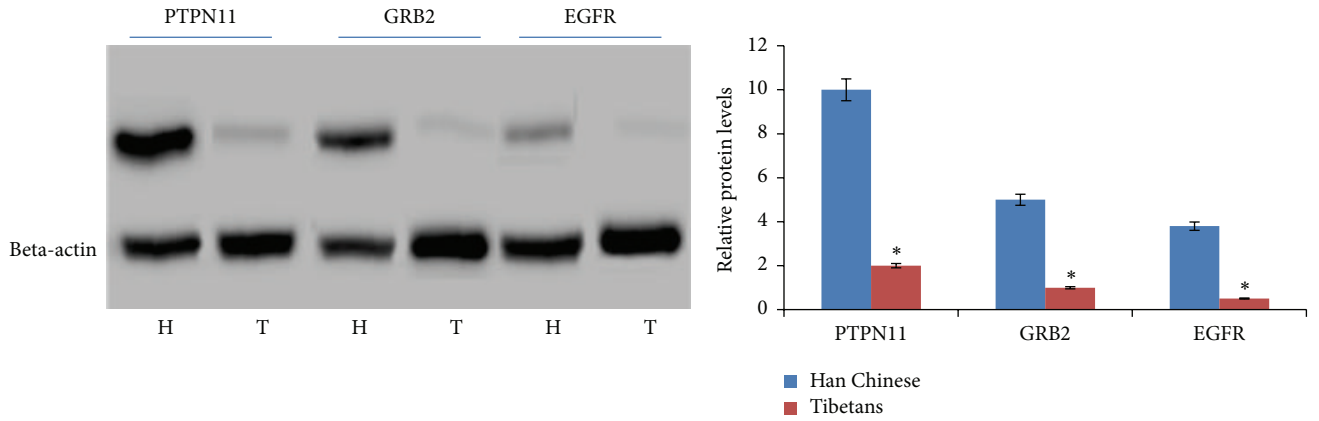
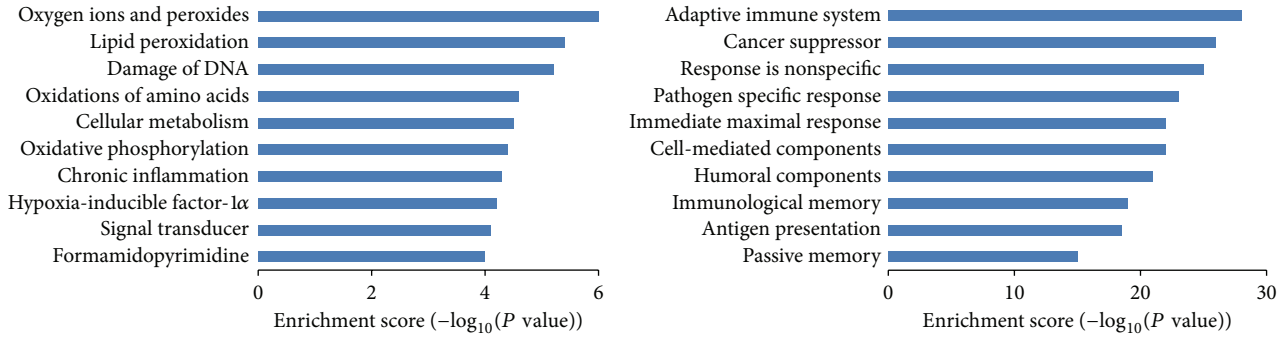


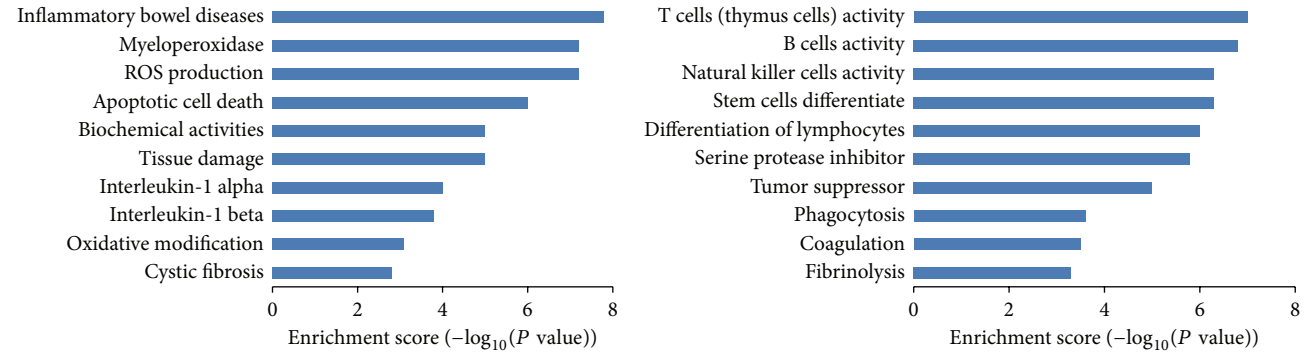
FIGURE 5: Validation of microarray (the top 3 up- and downregulated DEGs) results by Western Blot. The results represented quantification of protein levels relative to beta-actin. Normalized expression values were obtained by Western Blot ($n = 10$). C = Han Chinese at 100 m altitude and **P** = Tibetans at high altitude more than 3480 m. All the data were present as average value ± SD. $^{*}P < 0.05$ via IMB of Tibetans.



(a) Sig GO terms of upregulated DE gene-BP

(b) Sig Go terms of downregulated DE gene-BP

FIGURE 6: Gene ontology (GO) analysis used for analysis of the altered genes. (a) The bar plot shows the top ten upregulated Enrichment Score values of the significant enrichment. (b) The bar plot shows the top ten downregulated Enrichment Score values of the significant enrichment BP.



(a) Sig pathway of upregulated genes

(b) Sig pathway of downregulated genes

FIGURE 7: Pathway analysis of DEG. (a) The bar plot shows the top ten upregulated Enrichment Score values of the significant enrichment pathway. (b) The bar plot shows the top ten downregulated Enrichment Score value of the significant enrichment pathway.

STAT signaling pathways [62]. The JAK2 and STAT pathways have been reported in cell protection and injury. The JAK2 inhibitor and overexpression of its dominant negative JAK2 protein improve endothelial cells against peroxide and superoxide anion. Inactivation of JAK2 has been proved to be a potential method for endothelial cells against oxidative stress-induced death [63]. Parthenolide has been reported to inhibit JAK1 and STAT3 activity. ROS product will inhibit STAT3 signaling pathway by targeting JAK1 [64]. From the network, PTPN11 can regulate JAK and STAT pathways, and

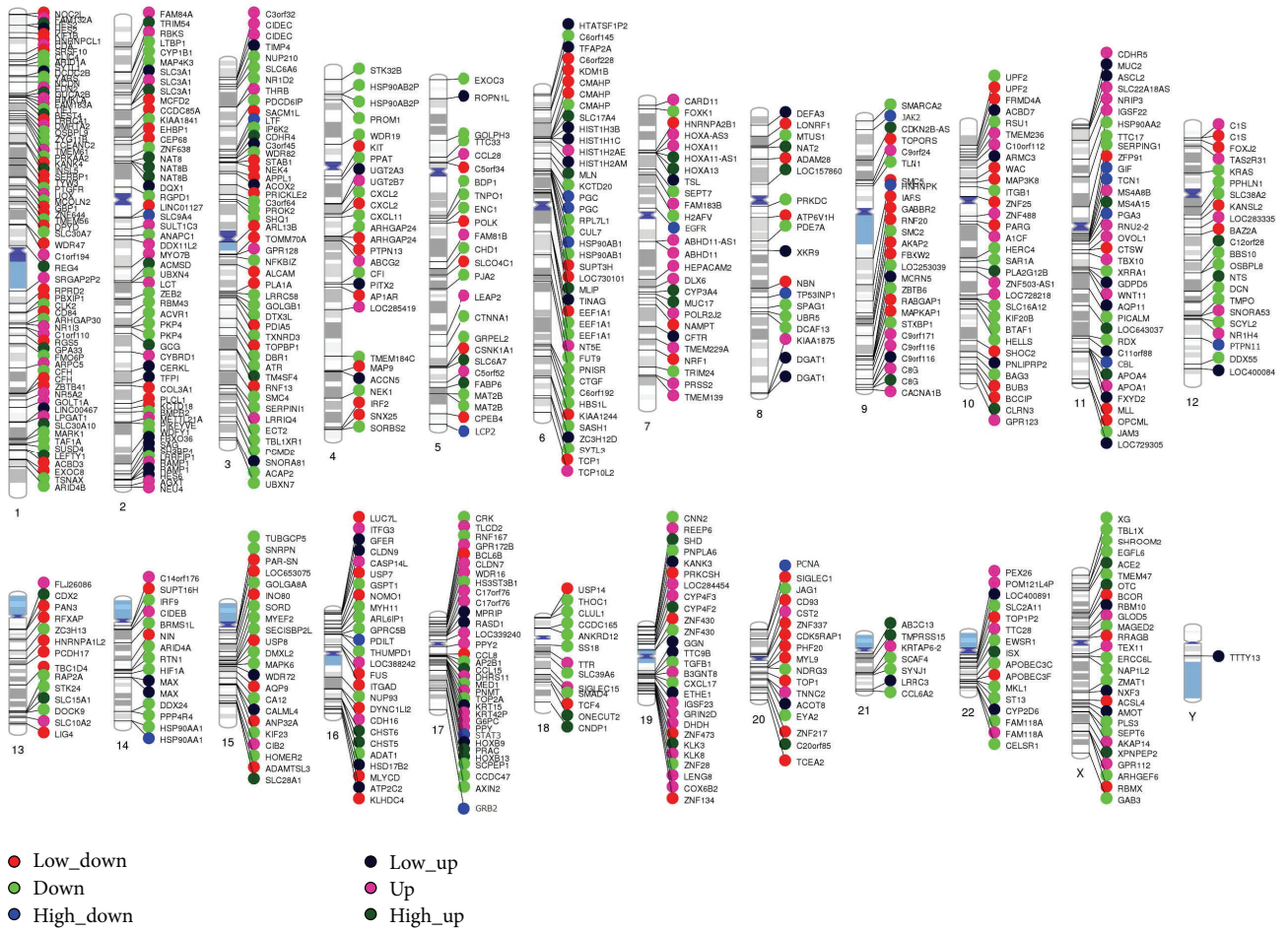


Figure 8: Whole-chromosome bird-view of expression levels of the 400 top DEGs located to 23 chromosomes. The color of each circle stands for the relative level of one DEG. Expression levels are normalized to six grades (low-up/4–6-fold, up/7–10-fold, and high up/more than 10-fold; low-down/4–6-fold, down/7–10-fold, and high down/more than 10-fold).

its inhibition will contribute to prevent oxidative-induced injury for the intestine of Tibetans. On the other hand, there is also different report for PTPN11. Gain of function mutations of PTPN11 in hematopoietic cells caused cytokine hypersensitivity by enhancing the levels of ROS. PTPN11 mutations will improve mitochondrial aerobic metabolism via the interaction with a new molecule. The mutation of PTPN11 has a therapeutic benefit by improving antioxidant activities [65].

One question should be paid here. There were 1336 downregulated genes but only three downregulated genes GRB2/EGFR/PTPN11 were selected. Three top-changed genes were analyzed because all of them were more than 20-fold downregulated while the left is less than 10-fold downregulated. Furthermore, the three genes were closed associated with the ROS production. The generation of ROS is tightly regulated by GRB2 in colorectal tumorigenesis [56]. ROS production will be beneficial to EGFR activation [66]. A conditionally deleted allele of PTPN11 will result in lower ROS levels [67]. Thus, the three genes were analyzed in the work.

From above results, it is easy to find that the three pathways have similar functions for controlling ROS levels and inhibiting oxidative-induced injury for human tissues or cells. Furthermore, our network also shows the close relationship among GRB2, EGFR, and PTPN11 pathways (Figure 9), which is accordant with previous reports [68, 69] except of PTPN11 pathway. Further work is needed to confirm the detailed relation among the three pathways.

There are some limitations for present study: (1) we only recruited a few participants from each group (living at altitude versus not). This is not remotely representative of the larger human population living within this region. To avoid the values bias caused by small sample size, the results were confirmed by using qRT-PCR in 10 Han Chinese at 100 m altitude and 10 Tibetans at high altitude more than 3480 m and were stable when compared with those of microarray analysis (Figure 4). (2) Present results only reflect one aspect for the differences noted in the study. There are still other molecular mechanisms existed, such as phenotype differences between Tibetans and Han Chinese or fundamentally different lifestyles. It would have been more relevant to study the same individuals moving from the plains into Tibet and vice versa. To address this issue, we tried such work for many times and always failed finally. The main

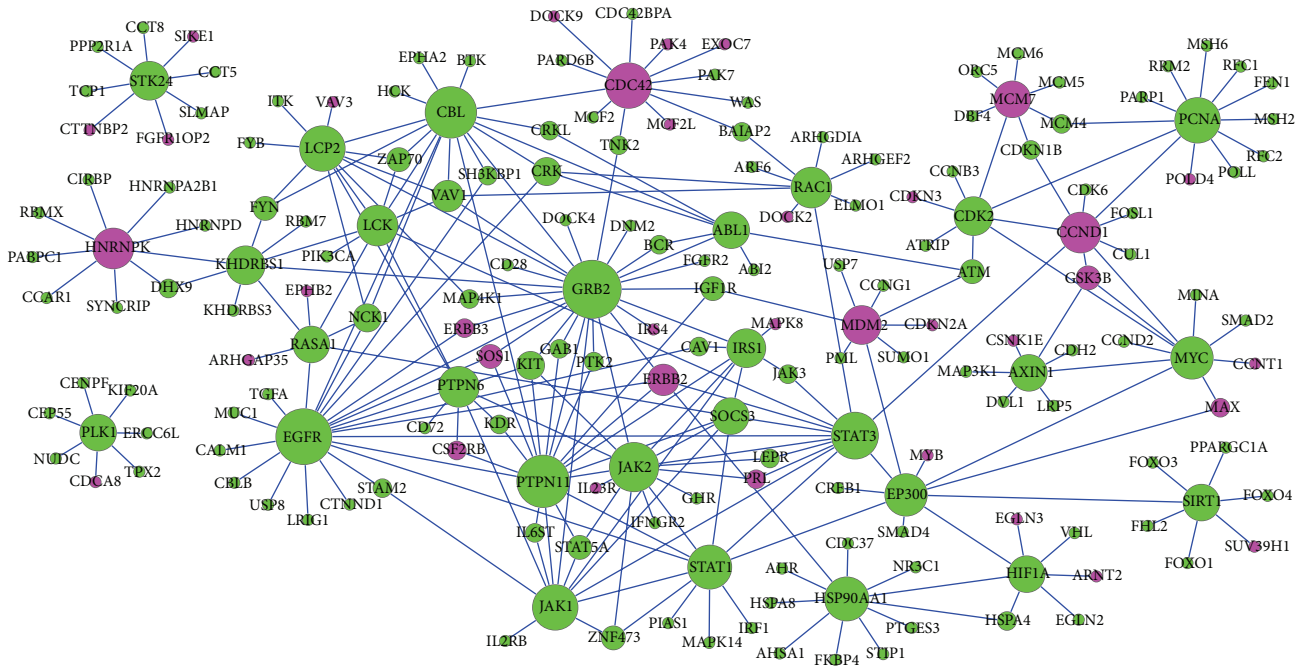


Figure 9: Gene network of top downregulated and upregulated DEGs from PCR microarray data. Significantly regulated genes were shown as purple and green, respectively. The size of circle represented the expression level.

reason was caused by the fact that most persons from plain cannot stay longer at high-altitude places. Furthermore, to reduce the disturbance, the lifestyle (similar daily activity, food calorie intake, and so on) and occupation (office workers) are similar between groups. Actually, Tibetan and Han Chinese populations diverged less than 3,000 years [70], suggesting that most genes are stable.

5. Conclusions

Present findings are obtained by comparing the gene expressing profiles of the participants from high altitude and plain area, providing clues to the molecular pathogenesis of this condition. Genome-wide transcriptional analysis suggests that hypoxia-induced oxidative stress leads to the intestinal injury of Tibetans via the inhibition of GRB2/EGFR/PTPN11 pathways. The study provides important information for the molecular mechanism causing IMB injury at high altitude and lays a foundation for subsequent gene validation and functional researches.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors designed and interpreted this study and analyzed the data and reviewed the full text. Kang Li performed the experiments and wrote the paper. Kang Li and Luobu Gesang equally contributed to the work.

Acknowledgments

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Research Article

The Beneficial Effects of Renal Transplantation on Altered Oxidative Status of ESRD Patients

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Renal transplantation (RT), has been considered the best therapeutic option for end stage renal disease (ESRD). *Objective*. To determine the effect of RT on the evolution of oxidative DNA status. *Methods*. Prospective cohort ($N = 50$ receptors of RT); genotoxic damage, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and DNA repair enzyme, human 8-oxoguanine-DNA-N-glycosylase-1 (hOGG1); and antioxidants, superoxide dismutase (SOD) and glutathione peroxidase (GPx), were evaluated. *Results*. Before RT, 8-OHdG were significantly elevated (11.04 ± 0.90 versus 4.73 ± 0.34 ng/mL) compared to healthy controls ($p = 0.001$), with normalization after 6 months of 4.78 ± 0.34 ng/mL ($p < 0.001$). The same phenomenon was observed with hOGG1 enzyme before RT with 2.14 ± 0.36 ng/mL ($p = 0.01$) and decreased significantly at the end of the study to 1.20 ng/mL ($p < 0.001$) but was higher than controls, 0.51 ± 0.07 ng/mL ($p < 0.03$). Antioxidant SOD was elevated at 24.09 ± 1.6 IU/mL versus healthy controls ($p = 0.001$) before RT; however, 6 months after RT it decreased significantly to 16.9 ± 1.6 IU/mL ($p = 0.002$), without achieving the levels of healthy controls ($p = 0.01$). The GPx, before RT, was significantly diminished with 24.09 ± 1.6 IU/mL versus healthy controls (39.0 ± 1.58) ($p = 0.01$), while, in the final results, levels increased significantly to 30.38 ± 3.16 IU/mL ($p = 0.001$). *Discussion*. Patients with ESRD have important oxidative damage before RT. The RT significantly reduces oxidative damage and partially regulates the antioxidant enzymes (SOD and GPx).

1. Introduction

End stage renal disease (ESRD) is a global, public health problem that causes great economic burden and increases morbidity and mortality [1]. ESRD is considered the last stage in chronic kidney disease (CKD), where uremia is the most serious complication. Renal replacement therapies (RRT) are needed to manage ESRD patients, among which are peritoneal dialysis (PD), hemodialysis (HD), and renal transplantation (RT) [2]. The RT is considered the treatment of choice and the best option for ESRD patients.

Oxidative stress (OS) results from imbalance between production of oxidants and the mechanisms of antioxidant defense [3]. In ESRD there is dysregulation of the oxidative state and the antioxidant systems due to the presence of inflammation, anemia, high levels of homocysteine, and treatment conditions as parenteral iron use, dialysate nonbiocompatible membranes, and significant reduction in endogenous antioxidants levels [4].

In addition, HD is considered an important source of oxidative stress in ESRD, due to the production of interleukins and anaphylatoxins (powerful activators of the

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase) during HD sessions. The enzyme NAPDH oxidase is responsible for the overproduction of reactive oxygen species (ROS) and constitutes the link between the activation of different cells types as leukocytes and organic toxicity. The HD can induce ROS production by many paths, one the bioincompatibility of dialysis system, the reactivity of dialysis membrane, and the production of endotoxins in the dialysate, and can induce deterioration of the antioxidant mechanisms. Superoxide anion (O_2^-) levels significantly increase after the HD session. Also, they produce high levels of homocysteine in plasma in the early phase of CKD promoting a prooxidative state by the interaction with hydrogen peroxide (H_2O_2) [5].

Antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, are the main defense system against ROS and oxidative stress. During the oxidative phosphorylation of the mitochondria an electron is transferred to oxygen which results in formation of the O_2^- that converts into hydrogen peroxide (H_2O_2) by the SOD, or it transforms into the hydroxyl radical. The O_2^- is a highly reactive species capable to react with proteins, lipids, and nucleic acids [6]. There are three isoforms of SOD, SOD1 (CuZnSOD) which is present in red blood cells, SOD2 (MnSOD) in the mitochondria, and SOD3 in the extracellular media [7]. GPx is responsible for the conversion of H_2O_2 and other organic peroxides to water and oxygen [8]. Five isoforms of GPx have been identified; two are present in human red blood cells: GPx 1 [9] and GPx 3 (eGPx), which are produced by the kidney, in plasma [10].

Peritoneal dialysis uses peritoneum as "natural" dialysis membrane to eliminate the waste products from blood flow. The success and efficacy of PD depend on the integrity of the peritoneal membrane; PD itself produces chronic inflammatory conditions in the peritoneal cavity that coincide with increased levels of proinflammatory cytokines, which alter the integrity of the tissue (peritoneal membrane). The high concentrations of glucose and glucose metabolites in PD solutions promotes structural and functional reorganization of the peritoneal tissue in the long term [11]; this condition is associated with an important increase in malondialdehyde production and a decrease of antioxidants compared to healthy controls [12].

Renal transplant seems to induce less oxidative stress compared to patients routinely dialysed. However, in RT other factors can induce OS, for example, immune response to allograft, ischemia/reperfusion injury, infections, and immunosuppressive therapy [13]. Inflammation and OS can produce graft tissue damage due to the formation of fibrosis and nephrons loss by necrosis or apoptosis [14].

When the O_2^- levels increase, can be converted to H_2O_2 by the SOD enzyme, or transform into the hydroxyl radical (OH^-) [15], this is highly reactive and capable of reacting with the nitrogen-base guanine in the DNA [16]. The 8-hydroxy-2′-deoxyguanosine (8-OHdG) is the product of oxidative DNA damage, and it is a sensitive and specific marker with higher mutagenic effect [17, 18]. In human cells oxidative damage is primarily repaired by the endonuclease enzyme, 8-oxoguanine-DNA-N-glycosylase-1 (hOGG1), through mechanisms of base excision [19].

Renal transplant is considered the best therapeutic option in patients with ESRD, with higher survival and quality of life compared to HD and PD patients; this RRT is associated with higher OS [20]; however, there is scarce information regarding OS after RT. The aim of this study was to evaluate the beneficial effect of RT on the evolution of oxidative *status*.

2. Material and Methods

A prospective cohort, with 50 patients subjected to RT with a six-month follow-up, was performed; all patients were from the Division of Nephrology/Organ Transplants at Unidad Médica de Alta Especialidad, UMAE, of Mexican Social Security Institute, in Guadalajara, Jalisco, Mexico. Receptors of a first RT from living donor (related or unrelated) were included, between 16 and 50 years of age, who agreed to participate in the study (signing an informed consent form). Patients with diabetes mellitus and end stage renal disease due to inflammatory disease (vasculitis, systemic lupus erythematous, or other connective tissue diseases or intestinal inflammatory illnesses) were excluded. Preemptive RT or multiple organ recipients were also excluded. All HD patients (18) receive 3 h/3 sessions per week with a similar conventional HD treatment; of them, 28% had fistulae and the remaining 72% had permanent HD catheter; they use polysulphone hemofilter (no re-used); all HD prescriptions were individualized by their own clinicians, according to their clinical conditions as ultrafiltration rate, *Kt/V*, and so forth.

Exclusion criteria were as follows: loss of renal graft function within the follow-up period, presence of severe systemic infection or infection due to cytomegalovirus at the time of the follow-up evaluations, and severe acute rejection treated with high doses of steroids or thymoglobulin. All patients receive the same triple immunosuppressive scheme based on the following: prednisone, mofetil mycophenolate, and tacrolimus; only tacrolimus had to be adjusted according to serum levels by their clinicians.

All oxidative markers were evaluated baseline and follow-up (6 months), using ELISA to determine DNA damage with 8-OHdG and the repairing enzyme hOGG1; and endogenous antioxidative markers SOD and GPx were evaluated with colorimetric techniques.

As a control of normal OS levels, 20 healthy subjects of similar age (36 years old) and gender (8 females and 12 males) were included as a control group, to standardize the normal values of reagents (blood donors from the blood bank who agreed to donate 10 mL extra blood apart from the amount donated).

Twenty-four h before RT 10 mL of venous blood was collected in a tube with 0.1% ethylenediaminetetraacetic acid (EDTA). The plasma was separated by centrifuge at 3000 revolutions per minute (rmp) for 10 minutes at room temperature, and the samples were stored at −80°C until processing.

2.1. 8-Hydroxy-2′-Deoxyguanosine. The manufacturer's suggested method for the ELISA kit was followed (8-hydroxy-2′-deoxyguanosine number ab10124 Abcam®, Cambridge,

United Kingdom). The plasma sample, EIA buffer, the standards, and the 8-OHdG-AChE tracer were added to all the wells except the blank. Then the monoclonal antibody 8-OHdG was added and the plate was incubated for 18 h at 4°C. The plate was washed with the buffer for the recommended times and 200 μL of Ellman's reagent was added to each well. The optical density was read at 405 nm.

2.2. 8-Oxoguanine-DNA-N-Glycosylase-1. Repair of the oxidative damage to DNA was determined through the use of a commercial kit (human 8-oxoguanine-DNA-N-glycosylase MBS702793, MyBiosource®, San Diego, CA). The manufacturer's instructions were followed, and the reactive species and samples were prepared for the indicated dilutions. 100 μL of plasma and standards were added to the wells and the plate was incubated at 37°C. Then, the biotinylated antibody was added and incubated under the same conditions. The corresponding washings were done and the HRP-avidin was added, followed by the substrate and then the stop solution at the corresponding times. The optical density was read at 450 nm.

2.3. Superoxide Dismutase. The instructions of the kit manufacturer were followed (SOD number 706002, Cayman Chemical Company®, USA) for the detection of O_2^- generated by the xanthine oxidase and hypoxanthine enzymes through the reaction of tetrazolium salts. The serum samples were diluted 1 : 5 in sample buffer: 200 μL of the radicals' detector, diluted 1 : 400, was placed, and 10 μL of the sample was then added. After slow agitation, 20 μL of xanthine oxidase was added to the wells. The microplate was incubated for 20 minutes at room temperature and the absorbency was read at a wavelength of 440 nm. The levels are reported in IU/mL.

2.4. Glutathione Peroxidase. Measurement of GPx activity was performed according to the manufacturer's instructions (Bioxytech GPx-340, cat, 21017, OXIS Int, CA, USA). The reagent was based on the oxidation of reduced glutathione in the presence of tert-butyl hydroperoxide, glutathione reductase, and NADPH. The decrease in absorption at 340 nm following the substrate addition was recorded and a decreased rate of absorption is directly proportional to GPx activity.

For all of the technical readings of optical density the Synergy HT (BIOTEK) microplate reader was used.

2.5. Statistical Analysis. Results are expressed as mean ± SD to evaluate differences within the group, Wilcoxon's Signed Rank test was used; and to evaluate differences between groups Mann-Whitney U test was done. A value of $p \leq 0.05$ was considered significant, with a confidence interval of 95%.

2.6. Ethics. The study was evaluated and approved by the Local Ethics and Research Committee at the *Unidad Medica de Alta Especialidad, Centro Médico Nacional de Occidente, IMSS* (R-2015-1301-91).

3. Results

Clinical and metabolic results are shown in Table 1. Seventy percent (35) were males; the average age was 31 years; height was 1.66 metres, and weight was 64 kg; two-thirds had PD as RRT; there were metabolic findings in hemoglobin, lipids creatinine, and albumin according to an ESRD patient; inflammation (determine by CRP) was present in at least 25%. At the end of the follow-up the low density cholesterol decreased significantly (LDL–$p = 0.008$) and the high density cholesterol increased significantly (HDL–$p < 0.001$). As expected, renal function improved significantly; and CRP decreases too.

3.1. 8-Hydroxy-2′-Deoxyguanosine. Normal plasma levels of the 8-OHdG marker were 4.7 ± 0.3 ng/mL. Evaluations made 24 h before RT in patients with ESRD were significantly elevated with 11.04 ± 0.9 ng/mL versus healthy controls ($p = 0.001$). However, the significant decrease between the measurement prior to RT and the measurement six months later is noteworthy: 4.7 ± 0.3 ng/mL, reaching normal limits ($p < 0.001$). This finding suggests RT low oxidative damage to DNA in patients with ESRD who undergo RT (Table 2).

3.2. 8-Oxoguanine-DNA-N-Glycosylase-1. Normal levels of the hOGG1 were 0.51 ± 0.07 ng/mL. However, measurements 24 h before patients underwent RT were importantly elevated at 2.14 ± 0.36 ng/mL ($p = 0.01$) compared to healthy controls, possibly in an attempt to counteract the effect of 8-OHdG. It is noteworthy that the final results of the enzyme decreased significantly to 1.20 ng/mL ($p < 0.001$) without reaching normal limits ($p < 0.03$) (Table 2).

3.3. Antioxidants. The SOD enzyme and the GPx are among the endogenous antioxidant enzymes that protect from oxidative damage. Normal levels of SOD found in healthy controls were 10.2 ± 0.09 IU/mL. Twenty-four h before RT the SOD was significantly increased with 24.09 ± 1.6 IU/mL versus healthy controls ($p = 0.001$). In the final results, the enzyme demonstrated a significant difference compared to the levels obtained in healthy controls, although it did not reach the normal limits ($p = 0.01$). On the other hand, the enzymatic activity of the GPx behaved differently. Levels in healthy controls were 39.0±1.58 U/minute/mg of protein, and the initial levels in patients were significantly diminished with 24.14 ± 2.53 U/minute/mg of protein compared to the healthy controls. Six months after RT an increase was observed in the enzyme's activity with 30.38 ± 3.16 U/minute/mg of protein ($p = 0.001$) (Table 2).

4. Discussion

Patients with CKD frequently had inflammation and OS and can contribute to deterioration of renal function [21, 22]. The RRT could also increase inflammation and OS in different ways. In PD the human peritoneal mesothelial cells, a critical component of the peritoneal membrane, play an important role in the suitability of PD. The loss of these

Table 1: Clinical and metabolic characteristics.

	24 h before RT	6 months after RT	p
Clinical characteristic			
Gender			
Male n (%)	35 (70)	—	—
Female n (%)	15 (30)	—	—
Age (years)	30.9 ± 12.0	—	—
Weight (kg)	63.9 ± 11.2	63.6 ± 10.3	0.329
PD n (%)	32 (64)	—	—
HD n (%)	18 (36)		
Catheter n (%)	13 (72)	—	—
Fistula n (%)	5 (28)	—	—
Metabolic characteristics			
Glucose (mg/dL)	92 ± 26	88 ± 16	0.366
Hemoglobin (g/dL)	10.8 ± 2.0	13.8 ± 1.9	**<0.0001**
Creatinine (mg/dL)	12.4 ± 4.2	1.1 ± 0.3	**<0.0001**
LDL (mg/dL)	91 ± 36	71 ± 23	**0.008**
HDL (mg/dL)	43 ± 14	52 ± 19	**<0.001**
CT (mg/dL)	162 ± 43	159 ± 31	0.439
TAG (mg/dL)	141 ± 63	180 ± 126	0.184
Albumin (mg/dL)	4 (3.3–4.4)	4.1 (4.0–4.8)	**0.008**
CRP (mg/L)	3.3 (3.0–7.4)	3.0 (3.0–4.0)	**0.003**

PD: peritoneal dialysis; HD: hemodialysis; LDL: low density lipoprotein; HDL: high density lipoprotein; TC: total cholesterol; TAG: triglycerides. CRP: C-reactive protein. The values are presented as mean and SD; albumin and CRP results are shown in median (interquartile rank).

Table 2: Markers of DNA status 24 h before and 6 months after RT.

	Healthy control	SEM	24 h before RT	SEM	6 months after RT	SEM	p WCX	p U-M	p U-M
Oxidative DNA status									
8-OHdG (ng/mL)	4.73	0.34	11.04	0.91	4.78	0.34	<0.001	<0.001	0.26
hOGG1 (ng/mL)	0.51	0.07	2.14	0.37	1.20	0.24	0.01	<0.001	0.03
Endogenous antioxidants									
SOD (UI/mL)	10.22	0.93	24.09	1.58	16.96	1.55	0.15	0.001	0.01
GPx (U/min/mg protein)	39.01	1.58	24.14	2.53	30.38	3.16	<0.001		

WCX: wilcoxon test, U-M: Mann-Whitney U test.

cells can contribute to the appearance of complications from PD due to the high levels of glucose dialysate, a key factor that favors the appearance of functional alterations and death of the human peritoneal mesothelial cells [23]. Patients subjected to HD show an increase oxidative damage to DNA and lower antioxidant activity [24]. In a study reported in 2006 performed in 7 patients with RT, ELISA was used to determine serum levels of 8-OHdG before reperfusion of the transplant, without an existing association between the serum levels of 8-OHdG just before the reperfusion and in the postoperative course. In all patients, serum 8-OHdG levels increased after reperfusion and decreased 2 h later; however, in 6 patients the same levels as the preoperative evaluation were maintained [25]. It has been reported that an increase in 8-OHdG (a product of oxidized DNA) is associated with resistance to erythropoietin and is found to be elevated in HD patients [26]. In the present study, a higher oxidative damage to the DNA was found, determined by higher levels of 8-OHdG (oxidant of the deoxyguanosine, base component of the DNA) 24 h before RT. However, the decrease to normal levels of 8-OHdG at the end of follow-up was evident, suggesting that RT is effective to reduce oxidative damage to the DNA.

Evaluation of specific mechanisms of DNA base excision can be done through determination of the glycosylase enzymes (hOGG1) that have specificity for 8-OHdG [27]. The hOGG1 can repair oxidative damage to the DNA, and it is well known that the OGG1 gene alteration increases susceptibility of the human cells to toxic compounds related to some toxins such as tobacco smoke, alcohol, and uremia, with a strong relationship between deregulation of the hOGG1 enzyme and the risk of suffering different kinds of cancers (e.g., laryngeal),

due to consumption of tobacco and alcohol. This is the first study that evaluates the hOGG1 associated with uremia in ESRD patients and the evolution after RT [28].

On the other hand, we found dysregulation of the endogenous antioxidant enzymes (SOD and GPx). The baseline SOD levels were significantly elevated and normalized at the 6-month follow-up. This can be explained as a compensatory response to the important OS in which these patients were before RT, as previously reported in other chronic degenerative pathologies [29]. Recently, it was published that the plasma SOD was significantly elevated in patients subjected to HD before RT compared to healthy controls, with subsequent decrease in the endogenous concentrations after RT [30], similar to the findings of our study. The GPx activity was importantly diminished compared to healthy controls and significantly increases 6 months after RT. This finding could be explained by the increase in SOD, since the SOD is the first antioxidant enzyme to act in the presence of oxidative damage [31]. In general we found an improvement of other markers of oxidative stress also, in agreement with other studies that measure isoprostanes (markers of damage to the cellular membranes) that decreased 2 months after RT [32]. In the case of OS in PD and HD patients still has many challenges; one of them could be focused in improving hemocompatibility of the dialysis systems; the other could be supplementation with antioxidants and modulation of the NADPH oxidase through pharmacological treatments.

In conclusion, this is the first study that evaluates the beneficial effect of RT on glycosylase after RT and shows the recovery in the natural DNA repairing capability after RT and of the antioxidants SOD and GPx measured 24 h before RT and 6 months later; these findings suggest that RT improves the conditions of altered oxidative status in ESRD patients.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Antipsychotic Treatment Reduces Indices of Oxidative Stress in First-Episode Psychosis Patients

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38 first-episode psychosis (FEP) patients and 37 control subjects were recruited for the study of indices of oxidative stress (OxS). The main purpose of the study was to compare the OxS statuses (serum total antioxidant capacity (TAC), total level of peroxides (TPX), oxidative stress index (OSI), and ratio oxidized methionine (Met-SO) to methionine (Met)) between antipsychotic-naïve FEP patients and individuals without a history of psychiatric disorders. Subsequently, the impact of 7-month antipsychotic treatment was evaluated on the OxS status in FEP patients. An attempt was made to assess links between OxS signature and inflammation markers. The oxidative stress indices remained generally unchanged in antipsychotic-naïve FEP patients compared to control subjects. Despite that, there was a significant correlation between the levels of TPX and EGF (endothelial growth factor) in FEP patients. This correlation disappeared after antipsychotic treatment of FEP patients. Moreover, antipsychotic treatment was associated with a significant reduction in OxS indices, including TPX, OSI, and ratio between Met-SO and Met. By contrast, in chronic SCZ patients we established a significant high-grade OxS. In conclusion, the markers of total antioxidative capacity, lipid peroxidation, and protein oxidation revealed no high-grade OxS in FEP patients. Nevertheless, antipsychotic treatment induced a considerable anti-inflammatory effect. OxS levels were also significantly decreased if compared in FEP patients before and after antipsychotic treatment.

1. Introduction

Schizophrenia (SCZ) is a complex, heterogeneous, and severe psychiatric illness that affects about 1% of the population [1]. Exact molecular mechanisms underlying the pathogenesis of this disorder remain to be elucidated. Preclinical and clinical studies of the last decade have highlighted a number of data demonstrating the involvement of OxS in the pathophysiology of psychiatric diseases [2, 3]. Recent studies support the understanding that both susceptibility to OxS and the status (level) of OxS may underlay the pathogenesis of SCZ via different mechanisms [4, 5].

There is a growing interest in the research of the development and course of first-episode psychosis (FEP) [6]. FEP can be seen as an intermediate state which has important implications for further clinical course, treatment, and management. Several studies [7, 8] suggest that OxS-driven injury occurs at the onset of psychosis. Recent evidence suggests that OxS plays a role in the etiopathogenesis of mental diseases usually starting with FEP [9]. Growing evidence shows that FEP is associated with some features of OxS [10, 11]. Considering the potential role of OxS in the course of FEP, the OxS signature of FEP patients should be studied by measuring OxS-related parameters in comparison with carefully selected mentally healthy subjects. To describe the blood overall status (level) of OxS, it is preferable to use markers that are clinically easy to apply.

Several studies show links between inflammation and OxS [12, 13]. Recently we demonstrated [14] that antipsychotic-naïve FEP patients display increased indices of elevated low-grade inflammation. Antipsychotic therapy resulted in significant clinical improvement of psychotic symptoms and decline in inflammatory status. The main purpose of this study was to compare the OxS statuses (serum total antioxidant capacity (TAC), total level of peroxides (TPX), oxidative stress index (OSI), and ratio oxidized methionine (Met-SO) to methionine (Met)) between antipsychotic-naïve FEP patients and individuals without a history of psychiatric disorders. Subsequently, the impact of 7-month antipsychotic treatment was evaluated on the OxS status in FEP patients. An attempt was made to assess the link between OxS and inflammation markers.

2. Patients and Methods

2.1. Participants. 38 FEP patients (21 males, 17 females; mean age 25.4 ± 0.89 years) were recruited from the Psychiatric Clinic, Tartu University Hospital, Estonia. They fulfilled the following inclusion criteria: age between 18 and 45; experience of the first psychotic episode; duration of untreated psychosis less than 3 years; no antipsychotic treatment received before the first contact with medical services for psychosis. Patients were excluded from the study when they had psychotic disorders due to a general medical condition or a substance-induced psychosis. FEP diagnoses were based on clinical interview according to ICD-10 [15] criteria. 36 FEP patients completed the follow-up study. Two patients refused to take antipsychotic medications and they were excluded from the follow-up analysis. Antipsychotic history was collected according to the medical chart review. Patients were treated with various antipsychotic medications as clinically indicated. During the follow-up period, patients were receiving antipsychotic medications of either atypical ($n = 24$), typical ($n = 1$), or mixed manner ($n = 11$) and the mean theoretical chlorpromazine dose equivalent was 396 ± 154 mg/day (range 80–640). 28 patients were treated with only antipsychotics, 5 patients additionally needed mood stabilizers, and 6 patients received antidepressants or hypnotics in addition to antipsychotic drugs. Using the information obtained from the participants, we determined that 10 patients and one control subject had used cannabis in their lifetime.

37 healthy subjects participated in the study as control subjects (CS). The sample of CS was recruited by an advertisement from the same geographical area as FEP patients. Both patients and controls were interviewed by experienced psychiatrists in order to avoid the inclusion of subjects with apparent mental disorders as controls. Exclusion criteria for the control group also included psychotic disorder among close relatives. Participants were enrolled between September 2009 and December 2013. The study was approved by the Ethics Committee of University of Tartu, Estonia, and written informed consent was obtained from all participants. The sample of this study contains the same participants as our previous study by Haring et al. [14].

2.2. Procedures. For the FEP patients the following activities were conducted at admission and after the follow-up (mean duration 7.18 ± 0.73 months) period: venous blood sampling after a 12-hour overnight fast, application of the PANSS (a rating instrument to evaluate the presence and severity of positive, negative, and general psychopathology, consisting of 30 items, each scored from 1 (absent) to 7 (severe)) [16] for the assessment and clinical monitoring of the disease course and antipsychotic treatment response, and physical examination including evaluating of blood pressure and body mass index (BMI) (weight (kg)/height $(m)^2$) data. Blood samples, blood pressure, BMI, and demographic data from CS were collected cross-sectionally.

2.3. Blood Collection and Clinical Laboratory Measurements. Blood samples of the participants were collected between 09:00 and 11:00 a.m. Blood (5 mL) was sampled in anticoagulant-free tubes and kept for 1 hour at 4°C (for platelet activation) before serum was isolated (centrifugation at 2000 × 15 minutes at 4°C). Serum was kept at −70°C before testing and all following procedures were performed similarly to our previous studies [17]. The clinical levels of triglycerides, total cholesterol, low-density lipoproteins (LDL cholesterol), high-density lipoproteins (HDL cholesterol), C-reactive protein (CRP), and glycated hemoglobin (HbA1c) were determined by standard laboratory methods using certified assays in the local clinical laboratory. The results of these measurements were presented in our previous study [14].

2.4. Oxidative Stress Markers Assay. TPX concentration and TAC of samples were assayed as described previously [18]. TPX concentration of samples was determined using OXYSTAT Assay Kit Cat. Number BI-5007 (Biomedica Gruppe, Biomedica Medizinprodukte GmbH & Co KG, Wien). The kit detects peroxide concentrations based on reaction of the biological peroxides with peroxidase and a subsequent color-reaction using tetramethylbenzidine (TMB) as substrate. After addition of a stop solution, the colored liquid is measured photometrically at 450 nm, using ELISA plate reader Photometer Sunrise (Tecan Austria GmbH, Salzburg). For the assay, a calibrator is used to calculate the concentration of biological peroxides in the sample. The concentration is stated as H_2O_2-equivalents (μmol/L). To measure TAC a new more stable coloured 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical ($ABTS^{*+}$) was employed. The basic principle of the method is that a colorless molecule, reduced ABTS, is oxidized to a characteristic blue-green $ABTS^{*+}$, using hydrogen peroxide in acidic medium (the acetate buffer 30 mml/L pH 3.6). When the colored $ABTS^{*+}$ is mixed with any substance, which can be oxidized, it is reduced to its original colorless ABTS form again. The $ABTS^{*+}$ is decolorized by antioxidants according to their concentrations and antioxidant capacities. The bleaching rate is inversely related with the TAC of sample. This change in color is measured as a change in absorbance at 660 nm. The reaction rate was calibrated with Trolox, which is used as a traditional standard for TAC measurement assays. The results are expressed

in mmol Trolox equivalent/L. Within- and between-batch precision data obtained by TAC method were 2.5% and 2.9%, respectively. Percent ratio of the TPX to the TAC is used as OxS index (OSI), an indicator of the degree of OxS [17]. Thus, OSI was calculated as follows: OSI = [(TPX, μmol/L) : ((TAC, μmol Trolox/L) × 100)].

Serum level of oxidized methionine (methionine sulfoxide, Met-SO) and methionine (Met) was determined with the AbsoluteIDQ™ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) using the flow injection analysis tandem mass spectrometry (FIA-MS/MS) as well as liquid chromatography ((LC)-MS/MS) technique. All measurements were performed as described in the manufacturer's manual UM-P180. Identification and quantification of the metabolites were achieved using multiple reaction monitoring along with internal standards. Calculation of metabolite concentrations was automatically performed by MetIDQ™ software (BIOCRATES Life Sciences AG).

2.5. Cytokine and Growth Factors Assay. The levels of the pro- and anti-inflammatory cytokines of the interleukin (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10) family, tumour necrosis factor-alpha (TNF-α), and growth factors, vascular endothelial growth factor (VEGF) and endothelial growth factor (EGF), were measured according to the manufacturer's protocol and the results of these measurements were presented in detail in our previous study [14].

2.6. Statistics. Demographic and clinical variables of the FEP patients and CS were compared using analysis of variance or t-test for continuous variables and chi-squared test for categorical variables.

The application of Shapiro-Wilk tests indicated that values of oxidative stress markers were not normally distributed ($p < 0.05$). A Mann-Whitney U test was applied to compare the raw data of two independent samples (FEP patients before treatment and CS) and a Wilcoxon signed rank test to compare two dependent samples (FEP patients before and after treatment condition). For establishing the effect of treatment, patients were paired one by one.

Spearman's rank correlation analysis was applied to establish the correlations between OxS and low-grade inflammation and metabolic markers, in FEP patients' group, before and after 7-month antipsychotic treatment.

General linear model (GLM) was used to demonstrate the differences in OxS markers levels between antipsychotic-naïve FEP patient and CS. In order to establish treatment effects to OxS serum levels between the groups (FEP patients after 7 months of treatment with antipsychotics) versus CS as well as between subjects (GLM: repeated measures) GLM was utilised. Categorical (disease, gender, and smoking status) and continuous (age) covariates were used in the GLM to compare OxS levels (dependent variables). To study within-subjects' differences in OxS, difference between pre- and posttreatment condition was used as an independent variable. Because GLM analyses required normally distributed data, biomarkers values were $\log_{10}$-transformed to approximate normality.

The statistical analyses were performed using Statistica software (StatSoft Inc., 12th Edition) for Windows. All statistical tests were two-sided, and p value < 0.05 was considered to be statistically significant.

3. Results

3.1. General Description of the Study Groups. There were no statistically significant differences between FEP patients (n = 38) and CS (n = 37) in terms of age ($t(73) = 0.49$, $p = 0.62$) and gender ($\chi^2(1) = 1.08$, $p = 0.30$). In addition, the differences in tobacco use (8 patients [21.1%] versus 7 controls [18.9%]) were not statistically significant ($\chi^2(1) = 0.05$, $p = 0.82$). As expected, there was a statistically significant effect after seven months of treatment (Wilcoxon signed rank test, $Z = 5.23$, $p < 0.000001$) on the total symptom score measured by PANSS (median = 112.5, range 80–155 during the recruitment, and median = 62, range 34–100, at the follow-up period). No differences were observed between the 2 samples regarding BMI (Mann-Whitney U test, $Z = -1.01$, $p = 0.31$). After 7 months of treatment with antipsychotics, patients BMI was significantly increased (Wilcoxon signed rank test, $Z = 4.13$, $p < 0.00004$).

3.2. Differences in Oxidative Stress Markers Levels among Antipsychotic-Naïve FEP Patients and Control Subjects. Regarding OxS-related parameters (TPX, TAC, and OSI) we did not find any difference in FEP patients before treatment compared to CS (Table 1 and Figure 1(a)). Similarly, the levels of Met-SO and Met and the ratio between Met-SO and Met in FEP patients before treatment did not differ from that in CS (Table 1 and Figure 1(b)).

To test the potential effect of the presence of FEP on the combination of OxS markers, we conducted a multivariate GLM analysis. The overall difference between the groups is shown in Table 2. The main effect of the disease emerged on OSI level ($t_{(5,64)} = 2.26$, $p = 0.03$, and $R_{\text{adj}}^2 = 0.17$). This effect was accompanied by the main effect of male gender ($t_{(5,64)} = -3.32$, $p = 0.001$). However, interaction between group status and gender regarding OSI level was not statistically significant. At the whole model level, the effect size (partial η^2) of the disease (before the treatment) on the OxS markers was 0.13 ($F_{(5,64)} = 1.9$, $p = 0.11$).

3.3. Antipsychotic Treatment Effect on Biomarkers Levels among FEP Group. 7-month treatment of FEP patients with antipsychotic drugs was associated with a significant decrease in TPX and OSI if compared in FEP patients before therapy and after (Table 1 and Figure 2(a)). Treatment was also associated with a significant increase in Met level and decrease in Met-SO and Met-SO/Met (Table 1 and Figure 2(b)).

On further analyses we evaluated the simultaneous impact of treatment on OxS levels. Repeated measures GLM was performed, to compare the main effects of the 7-month antipsychotic treatment on the serum biomarkers concentrations. The effect of the treatment is summarized in Table 3. TAC, Met, Met-SO, and Met-SO/Met levels were similar between pre- and posttreatment condition while all measured

Table 1: Comparisons of oxidative stress markers (OxS) between the first-episode psychosis patients at baseline (FEP_b) and control subjects (CS) as well as FEP patients at baseline (FEP_b) and after 7 months of treatment (FEP_f) with antipsychotics.

OxS markers	CS Median (min–max)	FEP_b Median (min–max)	FEP_f Median (min–max)	Z-value[a]	p value[a]	Z-value[b]	p value[b]
TAC	1.43 (0.92–2.08)	1.42 (0.79–2.19)	1.47 (1.06–2.43)	−1.15	0.25	1.56	0.12
TPX	332.50 (101.00–737.00)	380.00 (166.76–1357.53)	289.00 (88.00–915.55)	1.03	0.30	2.20	0.03
OSI	21.87 (6.16–53.80)	24.38 (11.36–92.02)	19.34 (6.03–67.82)	1.32	0.19	2.56	0.01
Met	9.08 (4.43–35.20)	7.75 (4.46–26.30)	12.50 (4.53–33.50)	0.80	0.43	2.50	0.01
Met-SO	10.80 (3.04–23.10)	10.35 (2.11–24.90)	8.72 (1.69–20.30)	−0.98	0.33	2.05	0.04
Met-SO/Met	1.44 (0.16–4.29)	1.35 (0.11–4.39)	0.66 (0.05–3.54)	−0.86	0.39	2.15	0.03

Z-adjusted values[a] according to Mann-Whitney U test (FEP_b compared to CS).
Z-values[b] according to Wilcoxon matched pairs test (FEP_b compared to FEP_f).
TAC: total antioxidant capacity; TPX: total level of peroxides; OSI: oxidative stress index; Met: methionine; Met-SO: methionine sulfoxide; Met-SO/Met: ratio of oxidized methionine to methionine.

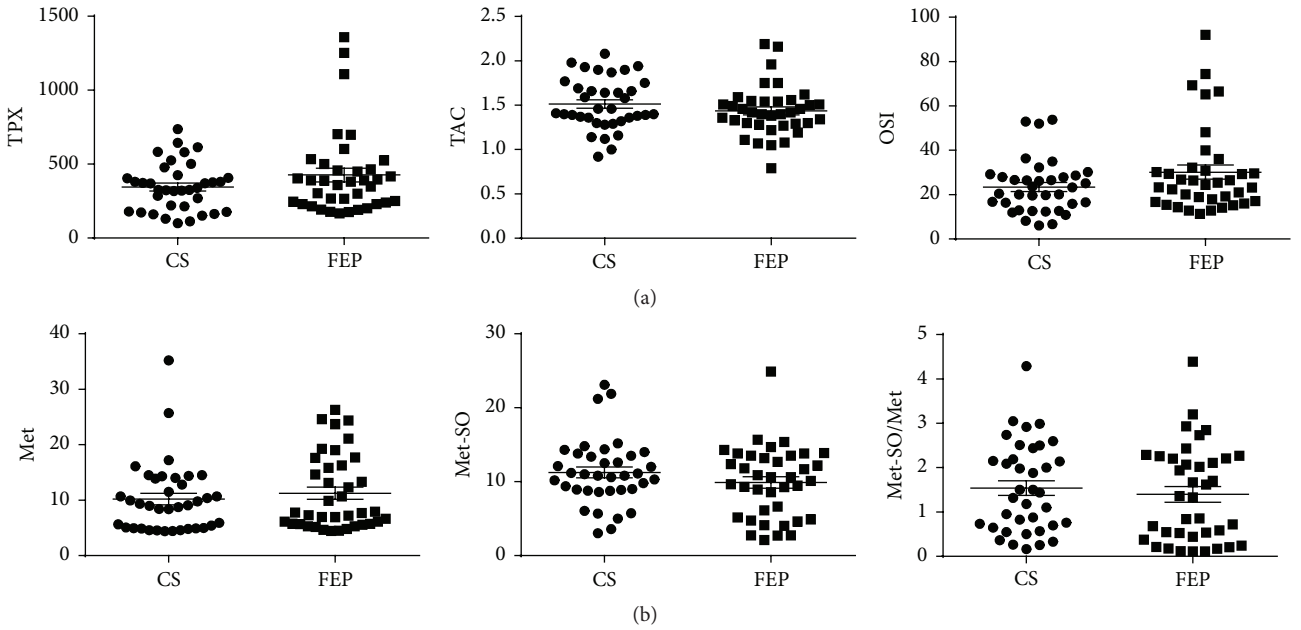

Figure 1: The changes in TPX, TAC, and OSI (a) and Met, Met-SO, and Met-SO/Met (b) due to FEP (Wilcoxon signed rank test). FEP: first-episode psychosis; CS: control subjects.

Table 2: Regression coefficients (β) and significance values of $\log_{10}$-transformed oxidative stress (OxS) markers levels with disease.

Biomarkers	β	β (95% CI)	t-value	p value
OxS markers				
TAC	−0.13	−0.37–0.11	−1.10	0.28
TPX	0.21	−0.02–0.43	1.82	0.07
OSI	0.25	0.03–0.47	2.26	0.03
Met	0.08	−0.15–0.31	0.70	0.49
Met-SO	−0.18	−0.41–0.05	−1.58	0.12
Met-SO/Met	−0.15	−0.37–0.08	−1.28	0.21

CI: confidence intervals; TAC: total antioxidant capacity; TPX: total level of peroxides; OSI: oxidative stress index; Met: methionine; Met-SO: methionine sulfoxide; Met-SO/Met: ratio of oxidized methionine to methionine.

OxS markers were taken into account and a decrease over time was detected in the serum levels of TPX ($p = 0.04$) and OSI ($p = 0.01$). Thus, our results confirm that treatment with antipsychotics has a positive impact on the OxS markers levels during the early phase of the psychotic disease. The effect size (partial η^2) of the treatment on the OxS markers was 0.17 ($F_{(5,64)} = 2.00$, $p = 0.08$).

Furthermore, to evaluate the FEP patients' posttreatment status regarding OxS markers levels compared to CS (adjusted for age, gender, and smoking status), GLM was performed. As seen from Table 4 levels of TAC, TPX, and OSI in FEP patients group were comparable with CS.

In addition, the 7-month treatment with antipsychotics caused a significant increase in Met ($p = 0.03$) as well as a decrease in Met-SO ($p = 0.02$) and Met-SO/Met ($p = 0.006$) levels in FEP patients' group compared to CS. These changes were associated with effects of age. Treatment effect was significantly associated with higher Met levels in younger

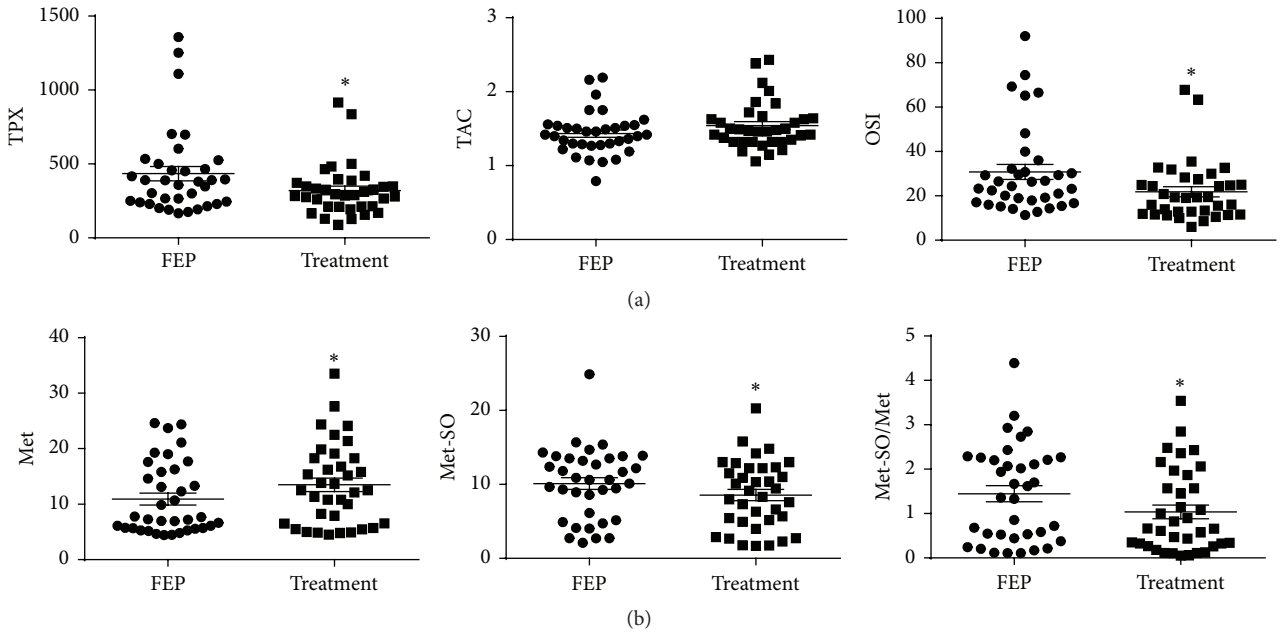

Figure 2: The changes in TPX, TAC, and OSI (a) and Met, Met-SO, and Met-SO/Met (b) due to FEP and antipsychotic treatment. $^{*}p < 0.05$ (Wilcoxon signed rank test). FEP: first-episode psychosis.

Table 3: Regression coefficients (β) and significance values of $\log_{10}$-transformed oxidative stress (OxS) markers' levels in first-episode patients' group before treatment compared to $\log_{10}$-transformed markers' values measured after 7 months of treatment with antipsychotics.

Biomarkers	β	β (95% CI)	t-value	p value
OxS markers				
TAC	−0.19	−0.42–0.05	−1.56	0.12
TPX	0.25	0.02–0.48	2.12	0.04
OSI	0.30	0.07–0.53	2.61	0.01
Met	−0.19	−0.43–0.05	−1.62	0.11
Met-SO	0.15	−0.10–0.38	1.21	0.23
Met-SO/Met	0.18	−0.06–0.42	1.51	0.14

CI: confidence intervals; TAC: total antioxidant capacity; TPX: total level of peroxides; OSI: oxidative stress index; Met: methionine; Met-SO: methionine sulfoxide; Met-SO/Met: ratio of oxidized methionine to methionine.

Table 4: Regression coefficients (β) and significance values of $\log_{10}$-transformed oxidative stress (OxS) markers' levels in first-episode patients' group after 7 months of treatment with antipsychotics compared to control subjects.

Biomarkers	β	β (95% CI)	t-value	p value
OxS markers				
TAC	0.02	−0.22–0.26	0.17	0.87
TPX	−0.01	−0.24–0.21	−0.12	0.90
OSI	−0.02	−0.24–0.20	−0.19	0.85
Met	0.24	0.02–0.47	2.21	0.03
Met-SO	−0.32	−0.54–(−0.09)	−2.77	0.007
Met-SO/Met	−0.31	−0.53–(−0.10)	−2.86	0.006

CI: confidence intervals; TAC: total antioxidant capacity; TPX: total level of peroxides; OSI: oxidative stress index; Met: methionine; Met-SO: methionine sulfoxide; Met-SO/Met: ratio of oxidized methionine to methionine.

patients ($t_{(5,63)} = -3.06$, $p = 0.003$) as well as lower levels of serum Met-SO and Met-SO/Met in older patients ($t_{(5,63)} = 2.45$, $p = 0.02$ and $t_{(5,63)} = 3.14$, $p = 0.003$, resp.).

Spearman rank correlation established a significant positive correlation of TPX with IL-1β ($\rho = 0.26$, $p = 0.03$) and EGF ($\rho = 0.30$, $p < 0.01$) in FEP patients' group (Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9616593). A similar correlation was established for OSI and EGF ($\rho = 0.24$, $p = 0.04$) and for TAC and IL-1β ($\rho = 0.25$, $p = 0.04$) (Supplementary Tables 2 and 3). After treatment, we found a significant negative correlation between treatment and OSI ($\rho = -0.28$, $p = 0.02$) and positive correlation between BMI and TAC ($\rho = 0.31$, $p < 0.01$) (Supplementary Tables 4 and 5).

4. Discussion

First-episode psychosis can be seen as an intermediate state which has crucial implications for further clinical course, treatment, and management of chronic psychotic disorder. The patients have to receive the best comprehensive science-based management. Therefore, all helpful information (e.g., clinical inspection of actual OxS signature of a patient) for applying contemporary treatment should be taken into account.

Recent articles have shown that OxS plays a role in the etiopathogenesis of psychiatric disorders starting with FEP [9]. Several studies refer to associations between FEP and OxS [10, 11]. However, in order to support these potential associations, further research is needed for verifying such

association in FEP patients from different endemic populations and studying the relations between OxS and low-grade inflammation markers. Serum markers of OxS cannot directly reflect OxS status in the brain, but there is data available that changes in serum lipid peroxidation markers are associated with some changes in the brain [19]. TPX, measured in our study, is a well-accepted marker for lipid peroxidation.

Our recent study [14] focused on the characterization of inflammation-related signature of FEP patients before and after 7 months of antipsychotic treatment. Using a carefully selected CS group and the shortest possible time window between the appearance of FEP and collection of blood samples, we tried to capture OxS-related changes resulting from the FEP of schizophrenia. As systemic OxS is a part of the pathogenetic mechanism of chronic psychotic disorder and is causing changes in the entire organism [20], it is reasonable to assess systemic OxS level in the early phase of chronic psychotic disorder.

In this study we did not find significant differences in OxS-related parameters like TPX, TAC, OSI, and Met-SO/Met, when comparing FEP patients with CS (Figures 1(a) and 1(b)). Therefore, total antioxidative capacity, lipid peroxidation, and protein oxidation related indices did not differ in FEP patients before treatment in comparison with CS. Our data are consistent with several studies on FEP [21]. However, one has to note the existence of data showing increased plasma malondialdehyde level in FEP [11]. At the same time the latter study did not show any significant differences in serum TAC. Another recent study also revealed no significant changes in some OxS markers (lipid or protein oxidation or nitric oxide production) in FEP patients compared to CS [10].

Nevertheless, this study underlines that antipsychotic treatment has a double positive impact in FEP patients—simultaneous decrease in OxS-status (Figures 2(a) and 2(b)) with improvement of inflammatory status [14]. However, one has to note that such positive effects of antipsychotic treatment on low-grade inflammation and OxS-status of FEP patients did not continue in long-term chronic schizophrenia patients who developed significant high-grade OxS. In order to support and illustrate the importance of clinical inspection of OxS signature in different stadiums of chronic psychotic disorder, we would give a brief overview of the unpublished results of SCZ patients ($n = 99$) compared to CS ($n = 51$) gained by using the same methodology as in this current study. We found significantly decreased level of TAC (1.43 ± 0.03 and 1.72 ± 0.03, $p < 0.001$ (Mann-Whitney U test) in SCZ patients and CS, resp.) and increased levels of TPX (353 ± 21 and 220 ± 9, $p < 0.0001$ (Mann-Whitney U test) in chronic SCZ patients and CS, resp.) and OSI (24.4 ± 1.41 and 12.9 ± 0.56, $p < 0.0001$ (Mann-Whitney U test) in chronic SCZ patients and CS, resp.) in chronic SCZ patients.

Thus, based on our previous [14] and current data we believe that, already after the diagnosis of FEP, the following treatment strategy has to consider both inflammation and OxS as tightly interrelated counterparts. This statement is supported by the following facts. First, correlations exist between inflammatory and OxS markers in FEP patients [14, Supplementary tables]. Second, antipsychotic treatment, significantly improving the inflammatory signature in FEP patients [14], has a positive impact on the indices of OxS at the early stage of the chronic psychotic disorder. Third, recent reviews and meta-analyses [22–24] focusing on the interactions between inflammation and OxS highlight their tight interrelation in SCZ. Fourth, a recent review emphasized that the administration of EGF to rats causes an elevation of reactive oxygen species and induces behavioural impairments resembling SCZ in rodents. These impairments were significantly improved after applying antioxidative compounds [25]. The established findings are consistent with our previous data concerning the interaction between EGF and inflammation in FEP patients [14] as well as with the effects of antipsychotic treatment on the OxS markers in this study.

The current study had several limitations. First, the limited sample size may create generalizability problems. Although our results should be confirmed in a larger group of patients, they, nevertheless, suggest that there is a need to consider the changes caused by OxS at the early phase of chronic psychotic disorder. Second, we collected data from CS at one time point and did not control their health condition and OxS markers' levels after the same follow-up period, as was done for the FEP patients' group. Third, according to the naturalistic study design, we did not exclude participants who had lifetime exposure to factors such as cannabis misuse or patients who were treated additionally with other psychotropic drugs. Substance misuse [26] as well as the use of antidepressants and mood stabilisers [27, 28] has been associated with effects on OxS status.

5. Conclusions

We did not find changes in OxS signature in FEP patients. However, before treatment, TPX levels were significantly correlated with EGF, disappearing after 7 months of antipsychotic medication use. Moreover, the antipsychotic medication of FEP patients not only had a considerable anti-inflammatory effect but also reduced lipid peroxidation and protein oxidation related indices of OxS if compared in FEP patients before and after antipsychotic treatment. By contrast, chronic SCZ displays a signature of high-grade OxS and inflammation.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Punicalagin Induces Serum Low-Density Lipoprotein Influx to Macrophages

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High levels of circulating low-density lipoprotein (LDL) are a primary initiating event in the development of atherosclerosis. Recently, the antiatherogenic effect of polyphenols has been shown to be exerted via a mechanism unrelated to their antioxidant capacity and to stem from their interaction with specific intracellular or plasma proteins. In this study, we investigated the interaction of the main polyphenol in pomegranate, punicalagin, with apolipoprotein B-100 (ApoB100) that surrounds LDL. Punicalagin bound to ApoB100 at low concentrations (0.25–4 μM). Upon binding, it induced LDL influx to macrophages in a concentration-dependent manner, up to 2.5-fold. In contrast, another polyphenol which binds to ApoB100, glabridin, did not affect LDL influx. We further showed that LDL influx occurs specifically through the LDL receptor, with LDL then accumulating in the cell cytoplasm. Taken together with the findings of Aviram et al., 2000, that pomegranate juice and punicalagin induce plasma LDL removal and inhibit macrophage cholesterol synthesis and accumulation, our results suggest that, upon binding, punicalagin stimulates LDL influx to macrophages, thus reducing circulating cholesterol levels.

1. Introduction

Low-density lipoprotein (LDL) particles are the major peripheral tissues providing cholesterol in the human circulation, and they play a key role in the development of atherosclerosis [1]. LDL is surrounded by a single copy of apolipoprotein B-100 (ApoB100) [2] which binds the LDL receptor on the cell surface of target tissues [1].

Punicalagin is a soluble polyphenol isolated from pomegranate with potent antioxidative properties. Punicalagin protects macrophage cells from lipid accumulation and foam cell formation [3–5]. Similarly, coadministration of punicalagin with statin significantly protects against macrophage foam cell formation and inhibits macrophage cholesterol biosynthesis. The use of statins in combination with pomegranate juice in hypercholesterolemic patients enables lowering the dosage of the former, thereby preventing its side effects, such as increases in liver enzymes, muscle problems, cognitive loss, neuropathy, pancreatic and hepatic dysfunction, and sexual dysfunction [6, 7]. Pomegranate juice supplementation to atherosclerotic mice reduced macrophage lipid peroxidation, cellular cholesterol accumulation, and development of atherosclerosis [5]. The antiatherogenic effect of punicalagin is known to stem from its antioxidant capacity [8]. However, antioxidant activity cannot be the sole explanation for polyphenols' cellular effects *in vivo* since they are poorly absorbed through the gut into the bloodstream and extensively metabolized in the small intestine, liver, and colon; thus, their bioavailability is often poor [9, 10].

Another antiatherogenic polyphenol is glabridin, isolated from licorice root. Its antiatherogenic properties are assumed to derive from its strong antioxidant capacity [11]. Recently, we showed that, apart from it being an antioxidant, glabridin can protect plasma protein through specific binding [12].

In fact, alongside our research, accumulating evidence in the literature suggests that polyphenols interact directly with enzymes, membranes, receptors, and cell or plasma proteins and modulate the activity of key proteins involved in cell signaling [13–16]. Polyphenols' beneficial effect might thus be exerted via a mechanism that is not necessarily related to their antioxidant capacity.

Cells acquire cholesterol through uptake of lipoproteins and through *de novo* synthesis. Yet (with the exception of steroidogenic tissues), they are unable to catabolize it. Since excess unesterified cholesterol is toxic to cells, organisms have developed several ways to protect themselves from cholesterol accumulation [17]. Macrophages are the best example of this "self-protection"; they take up dead cells containing a large amount of cholesterol, modified lipoproteins, and other extracellular debris. Macrophages take up more cholesterol per cell than any other cell type and protect themselves from cholesterol toxicity by two pathways: one is the esterification of cholesterol to cholesteryl ester. However, accumulation of high levels of cholesteryl ester may lead to the formation of foam cells and, later, to atherogenesis. The second and major line of defense against cholesterol toxicity is high-density lipoprotein (HDL) cholesterol efflux. In addition, in comparison to other cells, macrophages have additional pathways of cholesterol efflux [17]. Excess "peripheral" cholesterol is returned to the liver where the whole-body steady-state level of cholesterol is maintained.

In this study, a possible interaction of punicalagin with ApoB100 and the biological consequences of this interaction were investigated. It was shown that punicalagin binds specifically to ApoB100 and that upon binding it induces LDL influx to macrophages via the LDL receptor; on the other hand, glabridin, which also binds to ApoB100, did not affect LDL influx. These results provide a new mechanism—different from the classical mechanism of "antioxidant activity"—by which punicalagin reduces cholesterol levels in the circulation and attenuates atherosclerosis.

2. Materials and Methods

2.1. J774A.1 Macrophage Cell Line. J774A.1 murine macrophage cells were purchased from the American Tissue Culture Collection (ATCC, Rockville, MD). The cells were grown at 37°C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose (4500 mg/L), 2 mM glutamine, 10% w/v fetal calf serum (FCS), 1% w/v pyruvate, and 0.5% w/v penicillin, streptomycin, and nystatin (all chemicals purchased from Sigma-Aldrich).

2.2. Human LDL Isolation. LDL was prepared from human plasma taken from fasting normolipidemic volunteers (approved for research by Helsinki Committee regulations). It was separated from the plasma by discontinuous density gradient ultracentrifugation [18] and dialyzed against saline with disodium ethylenediaminetetraacetate (EDTA) (1 mM, pH 7.4). LDL was diluted in phosphate buffered saline (PBS) to 1 mg protein/mL and dialyzed twice, for 1 h each time, and once more overnight against PBS at 4°C to remove EDTA (PBS and EDTA were purchased from Sigma-Aldrich).

2.3. LDL Oxidation. LDL (100 mg protein/L) was incubated with 10 μmol $CuSO_4$/L (Sigma-Aldrich) under gentle shaking for 3 h at 37°C. The formation of conjugated dienes was monitored by measuring the increase in absorbance at 234 nm. Measurements were carried out using a SpectraMax M2 Reader [8].

2.4. Fluorescence-Quenching Measurements. Measurements were performed using a previously reported procedure [19]. Briefly, the solution was prepared in a 96-well black enzyme-linked immunosorbent assay (ELISA) plate (Greiner Bio-One, Germany). To each well, 2 μL polyphenol [glabridin, catechin, or quercetin (in ethanol) or punicalagin in double distilled water (DDW)] was added to 5 μL LDL or apolipoprotein B-100 (ApoB100) diluted in PBS buffer, to give a final polyphenol concentration in the range of 0.25 to 4 μM and a final LDL or ApoB100 concentration of 25 or 10 μg protein/mL, respectively. ApoB100 was purchased from Abcam, USA, quercetin and catechin were purchased from Sigma-Aldrich, glabridin was isolated from licorice root extract [11], and punicalagin was a generous gift from Professor Michael Aviram of the Lipid Research Laboratory, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa.

Fluorescence emission intensity was measured within 30 min of adding the polyphenol to the LDL or ApoB100 solution (25 or 37°C, resp.).

Fluorescence measurements were performed with an Infinite M200 PRO fluorescence spectrophotometer (Tecan) with emission spectra recorded from 320 to 450 nm at an excitation wavelength of 290 nm. If needed, the inner filter effect, which can decrease the fluorescence intensity, was corrected by using the following relationship:

$$F_{\text{corr}} = F_{\text{obs}} \times e^{(A_{\text{ex}}+A_{\text{em}})/2} = F_{\text{obs}} \times e^{(\varepsilon_{\text{ex}}CL+\varepsilon_{\text{em}}CL)/2}, \quad (1)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively, ε_{ex} is 0.0165 μM^{-1} cm^{-1} and ε_{em} is 0.0034 μM^{-1} cm^{-1}, and L is the well path length [20, 21]. Fluorescence quenching can occur via two different major mechanisms: static and dynamic. Both quenching pathways are described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{\text{sv}}[Q], \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, K_{sv} is the Stern-Volmer quenching constant, and $[Q]$ is the quencher concentration. For dynamic quenching, K_{sv} can be written as $K_q\tau_0$:

$$K_{\text{sv}} = K_q\tau_0, \quad (3)$$

where K_q is the quenching rate constant of the bimolecule and τ_0 is the lifetime of the fluorophore in the absence of quencher, which is approximately 10^{-8} s for a Trp residue [22]. Binding parameters were calculated as described previously [12]. For static quenching, the equilibrium between free and bound molecules can be described by

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_a + n \log[Q], \quad (4)$$

where K_a is the binding constant, reflecting the degree of interaction between ApoB100/LDL and the polyphenols, and n is the number of binding sites specifying the number of polyphenol molecules bound to the macromolecule. Thermodynamic parameters were calculated as described previously [12]. To characterize the ApoB100-punicalagin interaction, the thermodynamic parameters enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) were calculated. ΔH can be estimated indirectly by examining the temperature dependence of K_a and using (5). ΔG was estimated from (6) based on the binding constants at different temperatures, and ΔS was estimated from their relationship (see (7)):

$$\ln \frac{K_{a2}}{K_{a1}} = \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \frac{\Delta H}{R}, \quad (5)$$

$$\Delta G = -RT \ln K_a, \quad (6)$$

$$\Delta G = \Delta H - T\Delta S, \quad (7)$$

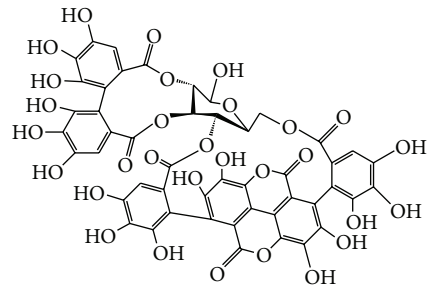
where K_{a1} and K_{a2} are the binding constants at temperatures T_1 and T_2, respectively, and R is the gas constant.

2.5. LDL Influx by J774A.1 Macrophages. LDL (1 mg protein/mL) was incubated with 10 μg/mL fluoroisothiocyanate (FITC) (purchased from Pierce, USA) for 1 h at room temperature in the dark and then dialyzed twice, for 1 h each time, and once more overnight against carbonate buffer (pH 9) at 4°C to remove excess FITC. FITC-conjugated LDL (LDL-FITC) was used for cellular uptake studies. J774A.1 macrophages were incubated at 37°C for 3 h with LDL-FITC at a final concentration of 25 μg protein/mL in DMEM enriched with 20% (w/v) BSA instead of FCS. LDL uptake was determined by flow cytometry [23]. Measurements of cellular fluorescence were determined by fluorescence-activated cell sorting (FACS) (FACSCalibur 4CA) at 510–540 nm after excitation at 488 nm with an argon ion laser. To determine the effect of the polyphenol (glabridin, catechin, quercetin, or punicalagin) on LDL influx, LDL-FITC was incubated with the polyphenol for 15 min before adding it to DMEM for 3 h.

To confirm that the influx occurs through the LDL receptor, macrophages were incubated simultaneously with LDL-FITC (25 μg protein/mL) and unlabeled LDL (12, 25, or 50 μg protein/mL) to create competitive inhibition.

2.6. Confocal Microscopy Analysis. Macrophages were incubated at 37°C for 3 h with LDL-FITC at a final concentration of 25 μg protein/mL in the presence or absence of 2 μM punicalagin and observed using a Zeiss LSM 700 confocal laser scanning microscope at 63x magnification. A vertical stack through the z-axis of the cells was created with the 488 nm laser and images were collected at 1 μm intervals. Axio Observer.Z1 was used to process the images.

2.7. Statistical Analysis. Statistical analysis was carried out using GraphPad Prism 5.01. Student's paired t-test was used to compare the means of two groups. Each experiment was repeated separately at least three times and was always performed in triplicate. Results are presented as mean fluorescence intensity (MFI) with significance determined at $p < 0.01$ (∗) or $p < 0.001$ (∗∗).



Scheme 1: Punicalagin.

3. Results

3.1. Tryptophan- (Trp-) Fluorescence Quenching. Punicalagin (Scheme 1) and glabridin were assayed for possible binding with the LDL particle and its ApoB100 protein. Figure 1 shows the emission spectra of ApoB100 in the presence of various concentrations of punicalagin (Figure 1(a)) and glabridin (Figure 1(b)) and of LDL in the presence of various concentrations of punicalagin (Figure 1(c)) and glabridin (Figure 1(d)) in the 320–415 nm range with excitation at 290 nm (T = 298 K, pH 7). Both glabridin and punicalagin quenched the Trp-fluorescence of ApoB100 and LDL in a concentration-dependent manner. Other polyphenol antioxidants that were examined, such as catechin and quercetin, did not quench the Trp-fluorescence of ApoB100 or LDL (data not shown). The Stern-Volmer curve (F_0/F versus polyphenol concentration), shown in Figure 2, was only linear for the interaction of punicalagin with ApoB100 at the tested concentrations, indicating static or dynamic single-type quenching [24].

Quenching constant (K_q) of ApoB100 initiated by punicalagin was calculated, using (3), to be $3.895 \times 10^{13}\ M^{-1}\ s^{-1}$, which is much greater than the maximum diffusion collision quenching rate constant of various drugs with proteins ($2 \times 10^{10}\ M^{-1}\ s^{-1}$). This indicated that ApoB100 quenching by punicalagin is not initiated by dynamic collision but via stable complex formation [21, 24]. The ApoB100-glabridin interactions and the interactions with LDL particles, however, were not stable but diffusion dependent and binding parameters of the interaction cannot be determined (Figure 2).

3.2. Binding Constant and Binding Sites. Static quenching was demonstrated for the interaction of ApoB100 with punicalagin by the fact that the Stern-Volmer plot did not show any significant deviation from linearity toward the y- or x-axis at the reported punicalagin concentrations (Figure 2) and by the quenching constant (K_q) value (see (3), Section 2). These results suggest a specific interaction between ApoB100 and punicalagin and that the binding parameters can be determined. However, the quenching of ApoB100 and LDL fluorescence by glabridin involves both static and dynamic quenching, as demonstrated by the fact that the Stern-Volmer

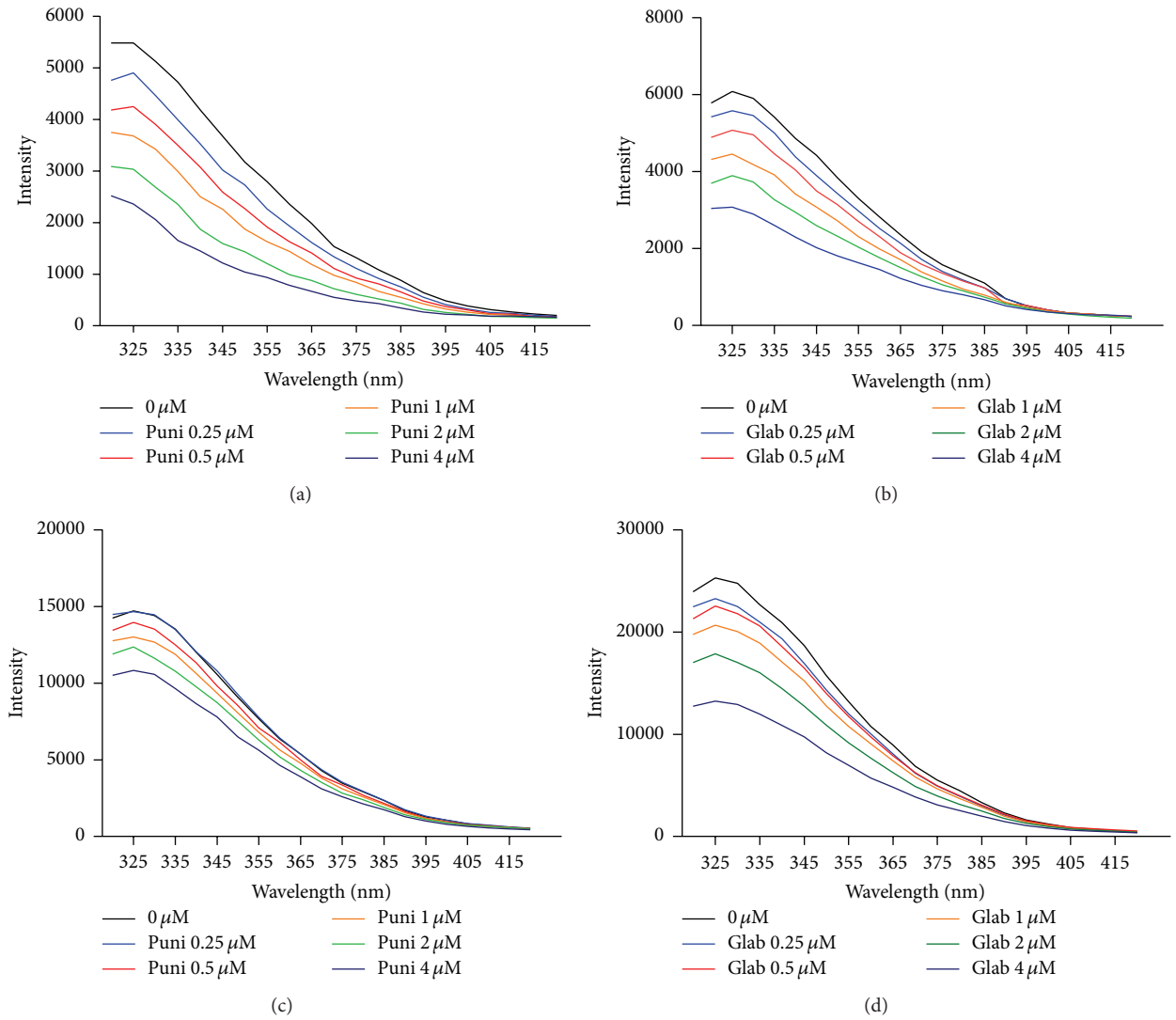


Figure 1: Fluorescence spectra of ApoB100 and LDL in the presence of punicalagin (puni) or glabridin (glab) at various concentrations. Fluorescence spectra of ApoB100 in the presence of various concentrations of punicalagin (a) and glabridin (b) and of LDL in the presence of various concentrations of punicalagin (c) and glabridin (d). In all solutions, the total concentration of ApoB100 and LDL was 0.01 and 0.025 mg/mL, respectively, and the polyphenol concentration was 0.25, 0.5, 1, 2, and 4 μM (λ_{ex} = 290 nm, pH 7.4, and T = 298 K). Each experiment was repeated separately at least three times and was always performed in triplicate.

plot deviates from linearity toward the x-axis, which indicates some site inaccessibility [20]. Thus, for glabridin, binding parameters of the interaction cannot be determined.

For static quenching and complex formation the binding parameters between punicalagin and ApoB100 can be determined using (4), Section 2. A plot of $\log(F_0 - F)/F$ versus $\log[Q]$, where Q is the polyphenol concentration, was used to determine the binding constant (K_a), 3.78×10^6 M^{-1}, and the number of binding sites (n) which was close to 1 and not significantly affected by pH or temperature. These values indicate a single binding site for punicalagin in ApoB100 with a high affinity interaction (Table 1).

3.3. Thermodynamic Parameters and Nature of the Binding Forces. Thermodynamic parameters and nature of the binding forces were calculated for the interaction between punicalagin and ApoB100 using (5), (6), and (7), in Section 2. Table 1 shows negative values for ΔG and positive values for ΔH and ΔS. Such thermodynamic results indicate that the interaction is spontaneous and mainly entropy-driven [21].

3.4. LDL Influx to J774A.1 Macrophages. The effect of various polyphenols on LDL influx into macrophages is shown in Figure 3. Figure 3(a) shows that, upon macrophage incubation with 2 μM punicalagin, LDL-FITC influx (cell fluorescence intensity) increased from 36% to 88%. On the other hand, the same concentration of glabridin had no effect on LDL influx (from 36.18% to 36.09%). Interestingly, only punicalagin (in purple) affected LDL influx, as displayed by the curve shift compared to the control curves (in red, black, and brown). No shift was observed for the blue, green, and light-blue curves, representing glabridin, quercetin, and catechin, respectively.

Table 1: Binding constant (K_a), number of binding sites (n), and thermodynamic parameters for the ApoB100-punicalagin interaction (see Scheme 1).

T (K)	K_a (1/M)	n	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol K)
310	$3.78 \cdot 10^6$	0.95 ± 0.04	51.2	−39.05	291
298	$1.7 \cdot 10^6$	0.7 ± 0.05		−35.54	

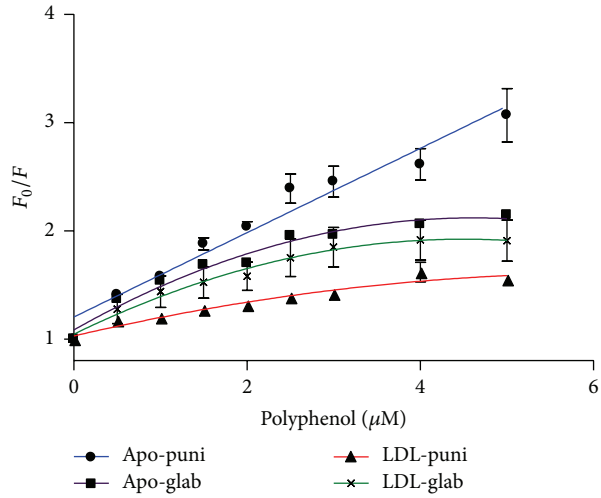


Figure 2: Stern-Volmer plots of the fluorescence quenching of ApoB100 and LDL by glabridin (glab) and punicalagin (puni). Each experiment was repeated separately at least three times. Results are presented as mean ± SD. $R^2 = 0.9$ and $p < 0.0001$ for the linear plot (ApoB100-punicalagin interaction).

Remarkably, LDL influx into the macrophage increased by up to 2.5-fold, mean fluorescence intensity (MFI) from 13 to 35 (Figure 3(b)), only in those cells that were incubated with 2 μM punicalagin but not in the cells incubated with glabridin. This finding was unexpected, since both glabridin and punicalagin bind the LDL particle at the same concentrations. It should be noted that, under incubation of oxidized LDL (oxLDL) with macrophages, influx to macrophages is not affected by punicalagin (MFI values were 15.06 and 16.66 in the absence and presence of 2 μM punicalagin, resp.). This is because oxLDL penetrates the macrophages through a different receptor, termed "scavenger" receptor (Figure 3(c)). The ability of punicalagin to increase LDL influx to macrophages was concentration-dependent. Figure 4(a) demonstrates that while 2 μM punicalagin bound to LDL increased cell MFI by 60%, 4 μM punicalagin increased the LDL influx by 80%.

Next, we attempted to determine whether LDL influx occurs specifically through the LDL receptor. First, the cells were simultaneously incubated with LDL and LDL-FITC in various ratios to create competition. When cells were incubated with LDL (12 μg protein/mL) + LDL-FITC (25 μg protein/mL), macrophage MFI decreased by 30%; when cells were incubated with an LDL concentration that was twice that of LDL-FITC, MFI decreased by ≈45% (Figure 4(b)). Finally, images of a vertical z stack of two macrophage cells that were treated with LDL-FITC upon 2 μM punicalagin incubation were taken. This image confirms that LDL particles indeed penetrate and accumulate in the cell cytoplasm. In Figure 4(c), a central image of the z stack shows LDL-FITC particles accumulated in the cell cytoplasm around the nucleus. It should be noted that, under incubation of oxidized LDL (oxLDL) with macrophages, influx to macrophages is not affected by punicalagin (MFI values were 15.06 and 16.66 in the absence and presence of 2 μM punicalagin, resp.). This is because oxLDL penetrates the macrophages through a different receptor, termed "scavenger" receptor.

4. Discussion

Dietary polyphenols are found in fruit, vegetables, nuts, and teas [25]. The benefits of consuming polyphenols are commonly assumed to stem from their antioxidant activity, which may contribute to preventing diseases such as cancer, cardiovascular disease, and neurodegenerative disorders [26, 27]. In favor of its antioxidant capacity, punicalagin is antiatherogenic. However, much uncertainty surrounds its mechanism of action as punicalagin concentration in the blood hardly reaches the level needed for effective antioxidant activity (10–100 μM) [15]. Recent studies suggest that the cellular effects of polyphenols are mediated by their interaction with specific intracellular or plasma proteins [28]. For example, punicalagin interacts with BSA [14]. The present study aimed to investigate a possible interaction of punicalagin with ApoB100 and the biological consequences of such an interaction.

Trp-fluorescence technique has been widely applied to the study of protein-drug interactions, as changes in the emission spectra of Trp can be seen in response to ligand binding or denaturation [24]. ApoB100 (and LDL) has 37 Trp residues [29] and natural fluorescence quenching can be used to measure its binding affinities. Both glabridin and punicalagin bound LDL or ApoB100 (with no shift in λ_{em}), while catechin and quercetin did not (Figure 1).

The type of interaction between ApoB100 and punicalagin was interpreted from their fluorescence-quenching spectra [24] and was found to be the only strong and stable one. The values obtained for n indicated a single binding site for punicalagin in ApoB100 (Table 1). Thermodynamic parameters indicated that hydrophobic forces play a major role in the punicalagin-ApoB100 interaction [21].

Macrophages are central to the initiation and progression of atherosclerosis and can be highly appropriate targets for therapy. To examine the biological consequences of polyphenol's interaction with the LDL particle, macrophage cells were incubated with LDL-FITC solution after the latter had been incubated with each polyphenol, to examine the effect of each polyphenol on LDL influx. Similarly, as a negative control, cells were incubated first with the polyphenol without any

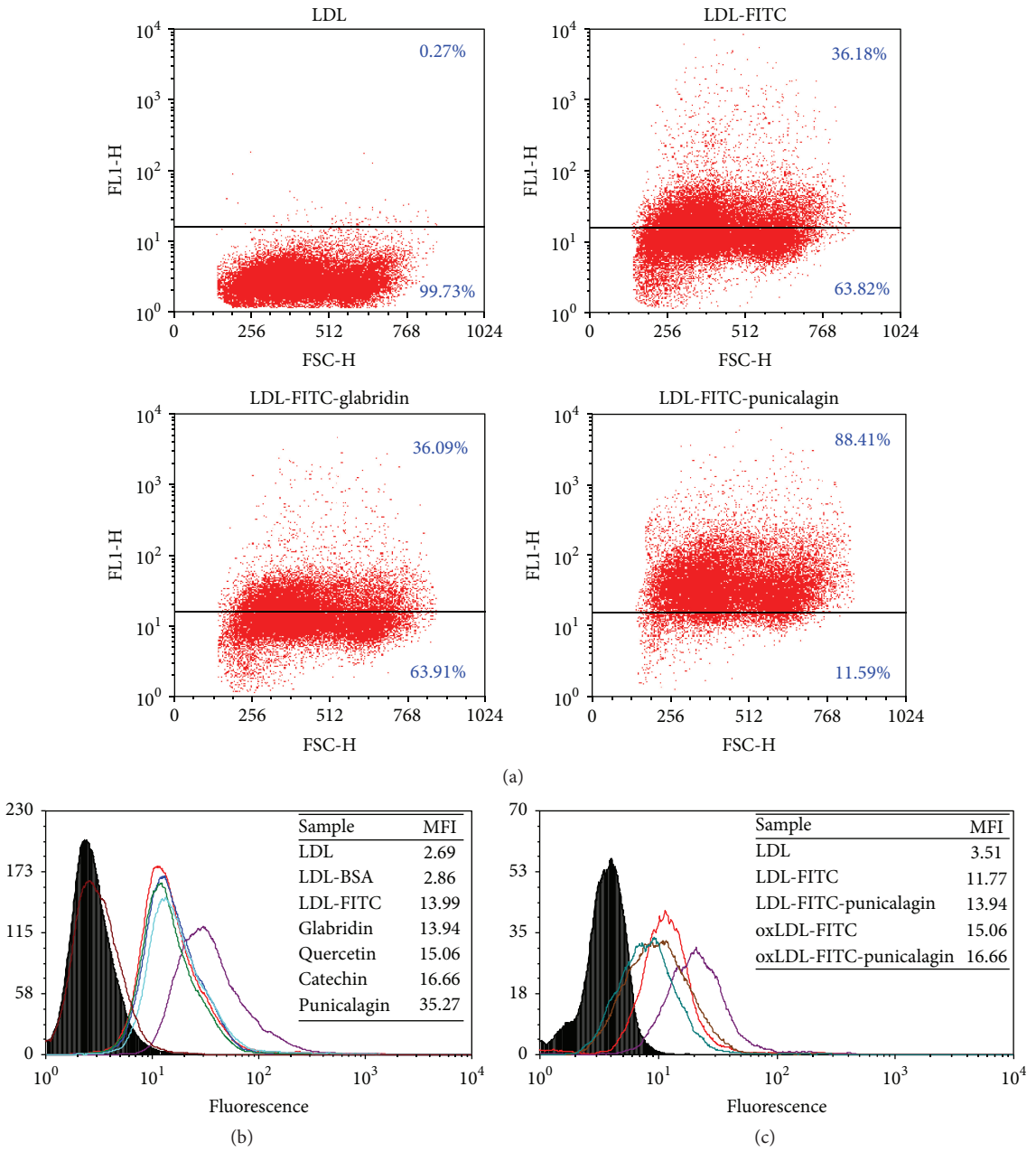


Figure 3: LDL or oxLDL influx into macrophages. (a) The ability of glabridin or punicalagin (2 μM) to induce LDL influx to macrophages. (b) FACS histogram of macrophages incubated with LDL-FITC upon addition of 2 μM punicalagin (purple), glabridin (blue), quercetin (green), or catechin (light-blue). Note that only punicalagin affects LDL influx as displayed by the curve shift compared to the control curves (red, brown, and black) representing macrophages incubated with LDL-FITC (positive control), LDL (negative control), and FITC-conjugated BSA (negative control), respectively. (c) FACS histogram of macrophages incubated with LDL-FITC (red) upon addition of 2 μM punicalagin (purple) or oxLDL-FITC (brown) upon addition of 2 μM punicalagin (green). LDL cellular fluorescence was measured in mean fluorescence intensity (MFI) or percent fluorescent cells.

LDL, washed to remove free excess polyphenol, and then incubated with LDL-FITC solution to examine a possible effect of the polyphenol alone on the cell (and in particular on cell LDL receptor). No effects on cells or LDL influx were observed. Supplementary Figure 1 (in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7124251) shows that, upon incubation of the studied cells with LDL/LDL-FITC for 3 hours, no foam cell formation was evident. The cells did not change their morphology, even after incubation with LDL/LDL-FITC for 16 hours. We may conclude that, under the present experimental conditions, the interaction of punicalagin with LDL leads specifically to LDL influx to the macrophages without their conversion into foam cells.

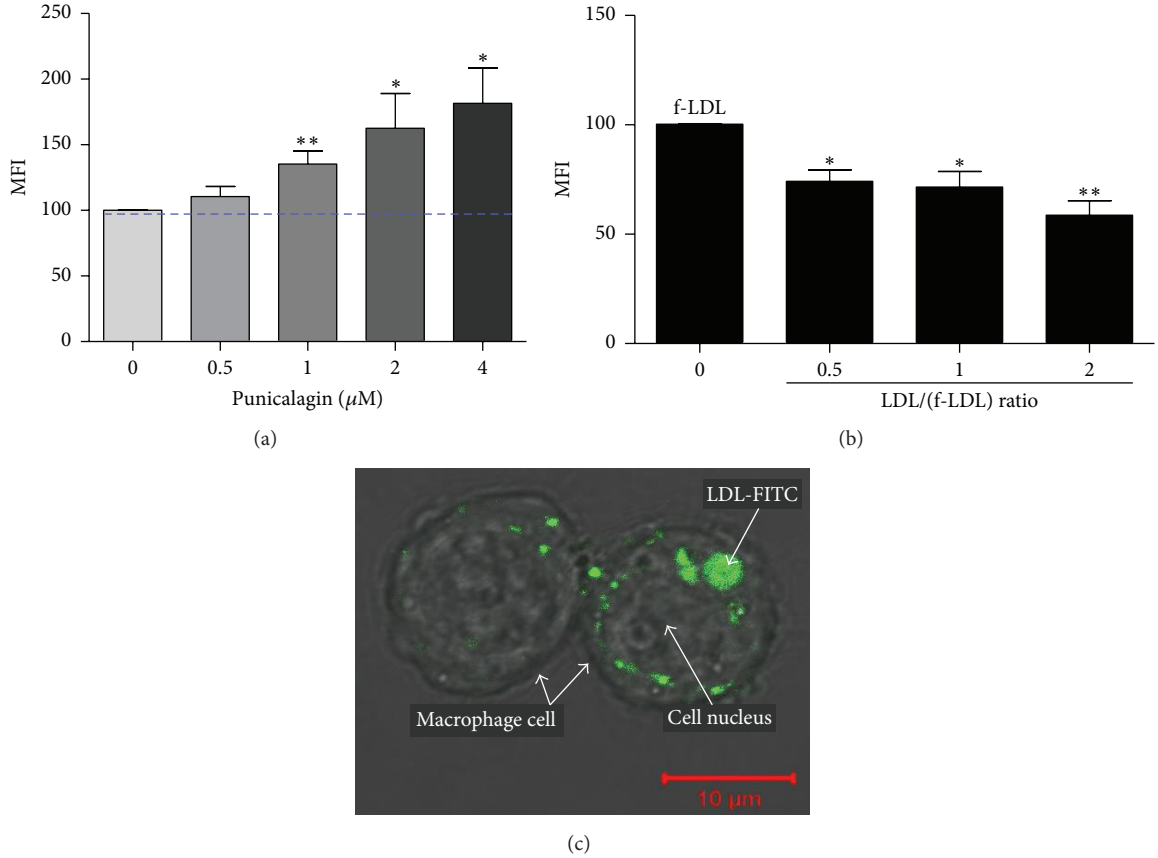


Figure 4: Punicalagin induces LDL influx. (a) Dose-response effect of 0.5–4 μM punicalagin on LDL influx. (b) Competitive macrophage influx upon adding LDL and LDL-FITC (f-LDL) at various concentrations simultaneously. (c) LDL-FITC particles, upon punicalagin incubation, accumulate in the cell cytoplasm around the nucleus. Each experiment was repeated separately at least three times. LDL cellular fluorescence was measured in MFI with significance determined at $p < 0.01$ ($*$) or $p < 0.001$ ($**$).

This result highlighted the specificity of the consequences of such interaction between polyphenol and protein (upon macrophages incubation with 2 μM punicalagin, LDL influx increased up to 2.5-fold while the same concentration of glabridin did not affect LDL influx (Figure 3(b))) and that punicalagin induction of LDL influx is in a concentration-dependent manner (Figure 3(c)).

Macrophages are phagocytes that engulf cellular debris and pathogens. We were interested in corroborating the concept that LDL influx occurs specifically through LDL receptor and that macrophages do not take up these particles non-specifically by endocytosis as part of their defensive activity. FITC reagent was bound to BSA (for which LDL has no known receptor) in the same procedure as LDL-FITC to show that, upon incubation with punicalagin, there is no BSA influx into the cells (Figure 3(b)). In another experiment, competition for LDL receptor was generated by incubating the macrophages with LDL and LDL-FITC simultaneously. Adding LDL in a 1/1 ratio with LDL-FITC led to a 30% reduction in LDL-FITC influx. Increasing the ratio of LDL to LDL-FITC to 2/1 led to a 45% reduction in LDL-FITC influx (Figure 4(b)). These results validate the assumption that LDL influx occurs through the LDL receptor.

We postulate that punicalagin binds to ApoB100 in close proximity to the LDL receptor-binding site. Upon binding, punicalagin changes the protein's conformation and might increase LDL's affinity for LDL receptor. Similarly, the conformation of ApoB100 on the surface of the LDL particle is likely to depend on the composition of the core lipids, the surface phospholipid content, and the diameter of the LDL particle [30]. Thus, punicalagin probably interacts with both the lipid part of the LDL particle and the protein, which induces LDL influx into the macrophage. Finally, a vertical z stack of macrophage cells confirmed LDL penetration and accumulation in the cells (Figure 4(c)).

As LDL influx into hepatic cells may contribute to fatty liver disease [31], LDL absorption into hepatic cells in the presence or absence of 2 μM punicalagin was also examined (data not shown). Unlike macrophage cells, LDL influx into hepatic cells (hepG2) was not affected by punicalagin when cells were exposed to similar LDL concentrations.

It is important to note that punicalagin from ingestion of pomegranate juice or extract does not reach high concentrations in the blood [15]. It is largely metabolized to ellagic acid through hydrolysis in the small intestine and over time by the gut bacteria to circulating urolithins [32].

Therefore, therapeutic administration of punicalagin preferentially should not be oral but rather intravenous. The results presented in this paper are collected from *in vitro* experiments. We are now examining the *in vivo* effect of punicalagin, using subcutaneously implanted osmotic minipumps. Serum lipoprotein parameters of mice will be determined after 28 days' exposure to punicalagin.

This study shows that punicalagin binds to a hydrophobic site of ApoB100 and to LDL, which may change the conformation of LDL's bound protein, ApoB100, and enhance its affinity for LDL receptor. LDL influx is induced and cholesterol accumulates in the macrophage cell without foam cell formation. In a future study, the effect of punicalagin on HDL's ability to remove excess cholesterol from these cells to the liver will be explored to determine the mechanism by which punicalagin lowers cholesterol blood concentration as reported in the literature [4, 5, 8] and attenuates the development of atherosclerosis.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Molecular Hydrogen Therapy Ameliorates Organ Damage Induced by Sepsis

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Since it was proposed in 2007, molecular hydrogen therapy has been widely concerned and researched. Many animal experiments were carried out in a variety of disease fields, such as cerebral infarction, ischemia reperfusion injury, Parkinson syndrome, type 2 diabetes mellitus, metabolic syndrome, chronic kidney disease, radiation injury, chronic hepatitis, rheumatoid arthritis, stress ulcer, acute sports injuries, mitochondrial and inflammatory disease, and acute erythema skin disease and other pathological processes or diseases. Molecular hydrogen therapy is pointed out as there is protective effect for sepsis patients, too. The impact of molecular hydrogen therapy against sepsis is shown from the aspects of basic vital signs, organ functions (brain, lung, liver, kidney, small intestine, etc.), survival rate, and so forth. Molecular hydrogen therapy is able to significantly reduce the release of inflammatory factors and oxidative stress injury. Thereby it can reduce damage of various organ functions from sepsis and improve survival rate. Molecular hydrogen therapy is a prospective method against sepsis.

1. Introduction

Sepsis is a systematic inflammatory response to infection. It is one of the most serious diseases in ICU, which is a worldwide challenge. Although comprehensive therapy has been developed for it, sepsis is still associated with high morbidity and mortality and costs a lot for hospitalization. In the United States, severe sepsis affects 750,000 people per year, which costs $16.7 billion annually and increases in its incidence over time of 8.7% [1, 2].

Sepsis leads to abnormal blood pressure, heart rate, and PaO_2. It also influences different organs and even leads to multiple organ dysfunction syndromes (MODS). The main clinical characteristics of brain include delirium, coma, disorientation, the slowing of mental processes, and cognitive dysfunction. Sepsis leads to acute lung injury and acute respiratory distress syndrome (ARDS), whose mortality rate is as high as 30% to 50% in critically ill patients. In liver, disruption of protein synthetic function manifests as progressive disruption of blood clotting and disruption of metabolic functions leads to impaired bilirubin metabolism. The incidence of acute kidney injury is nearly 65% in critically ill patients and is able to aggravate the condition of patients with septic shock. Sepsis also decreases the blood flow of the gastrointestinal tract, which might induce severe ischemia, hypoxia, and reperfusion injury.

Recent research suggests that molecular hydrogen works as a therapeutic antioxidant activity by selectively reducing hydroxyl radicals and protects against organ damage effectively. In 2007, inhalation of hydrogen gas was found to suppress brain injury by buffering the effects of oxidative stress in an acute focal ischemia and reperfusion rat mode [3]. In 2008, hydrogen therapy was found to inhibit the inflammatory reaction in the rat model of small intestinal transplantation. Contemporarily, hydrogen therapy was proved to protect against acute pancreatitis [4]. In 2010, hydrogen's protective effects on sepsis and sepsis-associated organ damage were found, which mainly relied on its antioxidative property [5].

There are 3 main methods of molecular hydrogen therapy: inhalation of hydrogen (H_2), oral intake of hydrogen-rich water (HRS), and injection of hydrogen-saturated saline (HRS). Molecular hydrogen therapy also can be combined with other therapy such as resuscitation and oxygen therapy.

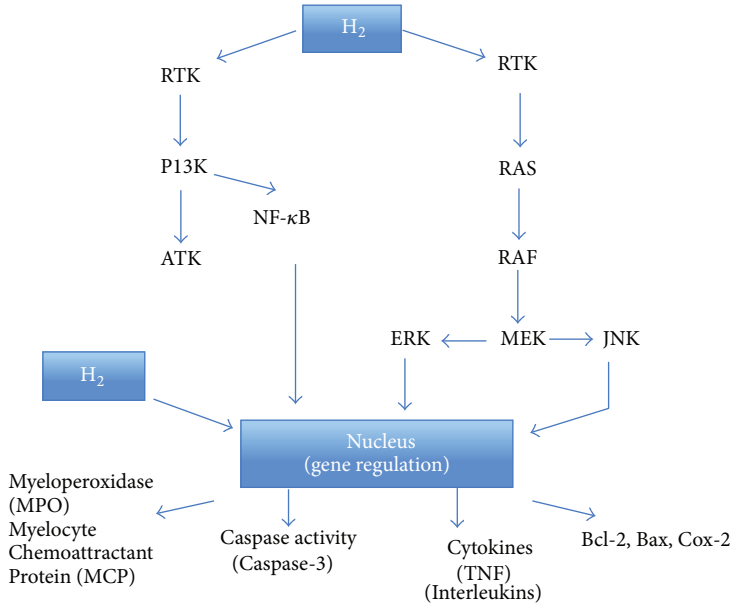

Figure 1: Possible mechanisms of molecular hydrogen. Possible pathways for molecular hydrogen. It has been proposed that molecular hydrogen has the capabilities to affect the pathways mentioned and to directly or indirectly assist in the gene regulation or protein expression of the following: MPO, MCP, Caspase-3, Caspase-12, TNF, interleukins, Bcl-2, Bax, and Cox-2.

2. Oxidative Stress in Sepsis

Autoimmune injury occurs in sepsis, and the pathogenesis is very complicated, in which oxidative stress plays an important role.

The immunocyte is activated and the respiratory burst creates amount of reactive oxygen species (ROS). Oxidative stress induced by ROS can change the permeability of epithelial cells by destroying the cell membrane. The imbalance of antioxidant defense systems against oxidative stress also can damage the epithelial cells [6].

3. Mechanism of Molecular Hydrogen

Molecular hydrogen is a scavenger of the hydroxyl radical. H_2 can selectively reduce ROS in vitro; it will react with only the strongest oxidants, which means the use of H_2 is mild enough having no serious side effects [3].

Molecular hydrogen can suppress the release of cell adhesion molecules, as well as proinflammatory cytokines. H_2 could elevate anti-inflammatory cytokine levels. H_2 enhanced HO-1 expression and activity, which suggest that H_2 could suppress excessive inflammatory responses and endothelial injury via an Nrf2 (nuclear factor erythroid 2 p45 related factor 2)/HO-1 pathway [14]. In addition, it has been proposed that molecular hydrogen has the capabilities to affect several pathways and assist in the gene regulation or protein expression of MPO (myeloperoxidase), MCP, Caspase-3, Caspase-12, TNF (tumor necrosis factor), interleukins, Bcl-2, Bax, and Cox-2 (as shown in Figure 1 [15]).

4. The Impact of Molecular Hydrogen on General Condition

In animal experiments, sepsis alters general condition of mice, such as mean artery pressure (MAP) decreasing and PaO_2 declining.

In Liu et al.'s study, MAP decreased in 20 minutes after LPS (Lipopolysaccharide) injection. There was no significant difference in resuscitation group and resuscitation+H_2 group, while the fluid volume and usage of norepinephrine were less used in resuscitation+H_2 group [6]. In another study, resuscitation group needs more fluid and norepinephrine than H_2 group although these two groups get to similar MAP [13].

Many studies showed that sepsis makes PaO_2 and PaO_2/FiO_2 decline, and molecular hydrogen therapy can alleviate this change. Xie et al. stated that PaO_2/FiO_2 ratio declined significantly in cecal ligation and puncture (CLP) group. Inhalation of H_2 can remit the change [10]. PaO_2 declined in Liu et al.'s study of septic mice. Resuscitation improved PaO_2 to 62.34 ± 2.46 mmHg ($p < 0.05$), but resuscitation+H_2 inhalation showed more effectivity by improving PaO_2 to 88.98 ± 3.17 mmHg [6]. When molecular hydrogen therapy is used alone, it is also valid. Li et al. stated that HRS increased PaO_2 of 59 ± 6 mmHg to 67 ± 8 mmHg ($p < 0.05$) in CLP mice [11]. Xie et al. showed PaO_2/FiO_2 significantly decreased in LPS-challenged mice, which improved by H_2 inhalation [12].

Generally, sepsis decreased MAP, PaO_2, and PaO_2/FiO_2. Traditional resuscitation can alleviate these changes. It works better while combined with molecular hydrogen therapy. Molecular hydrogen therapy makes it possible for using less fluid and the vasoactive agent to reach the target MAP level.

Table 1: The impact of changes of the biochemistry indicator level.

	Brain	Lung	Liver	Kidney	Intestine
Capase-3	HRS ↓	HRS ↓	/	/	/
SOD	HRS ↑,H_2 ↑	HRS ↑	H_2 ↑	H_2 ↑	H_2 ↑
ROS	H_2 ↓, HRS ↓	/	/	/	/
MPA	H_2 ↓, HRS ↓	HRS ↓	/	H_2 ↓	H_2 ↓
CAT	HRS ↑, H_2 ↑	HRS ↑	H_2 ↑	H_2 ↑	/
8-iso-PGF2α	H_2 ↓	HRS ↓	H_2 ↓	H_2 ↓	/
Nrf2	H_2 ↑	/	/	/	/
TNF-α	H_2 ↓	HRS ↓ or →	H_2 ↓	H_2 ↓	H_2 ↓
IL-1β	H_2 ↓	HRS ↓	/	/	/
IL-6	/	HRS ↓	/	H_2 ↓	/
IL-8	/	HRS ↓	/	H_2 ↓	/
HMGB1	H_2 ↓	HRS ↓	H_2 ↓	/	H_2 ↓
IL-10	H_2 ↑	HRS ↑ or →	H_2 ↑	H_2 ↓ or →	/
MPO	/	HRS ↑ or →	/	/	/
Others	Cognitive impairment: HRS is less than sepsis group; HRS is similar to sham group	(1) W/D weight ratio: H_2 ↓, HRS ↓ (2) BALF cell counts: H_2 ↓	ALT, ACT: H_2 ↓, HRS ↓ H_2 group is similar to sham group	BUN, Cr: H_2 ↓	DAO: H_2 ↓
References	[7–9]	[5, 6, 10–12]	[5, 10, 11]	[5, 10, 11, 13]	[6]

HRS: hydrogen-rich water injection or oral-taken group.
H_2: H_2 inhalation group.
SOD: superoxide dismutase.
CAT: catalase.
HMGB1: high mobility group box 1.
IL: interleukin.
W/D: wet/dry.
BALF: bronchoalveolar lavage fluid.
ALT: alanine aminotransferase.

Molecular hydrogen therapy also significantly improves PaO_2 and PaO_2/FiO_2 in sepsis.

5. The Impact of Molecular Hydrogen on Different Organs

The impact of molecular hydrogen on changes of the biochemistry indicator level in different organs was summarized in Table 1.

5.1. Brain. Brain is one of the organs to be affected during early sepsis. It is strongly associated with higher mortality and lower quality of life.

Morphologic changes can be detected by pathologic examination. Brain sections were stained with H&E (hematoxylin-eosin staining). In normal condition, the hippocampal CA1 region shows tightly arranged nerve cell bodies with clear structures; cytoplasm in cells is plentiful. However, animal experiments found that most neurons in CLP-challenged mice were shrunken and stained dark; the intracellular space was enlarged. With HRS injection, the cells with eumorphism were significantly preserved. Total normal cell count in sham group was 295.50 ± 12.91, while the number in CLP group was significantly decreased. Compared with CLP group, the numbers of normal cells were much higher in HRS treatment group. This data revealed the dose-response relationship of HRS treatment [7]. Inhalation of H_2 stated similar consequence with HRS treatment. In Liu et al.'s study, the pyramidal neurons in hippocampal CA1 region were arranged in disorder in CLP group, containing the dissolved Nissl bodies. This disorder was slighter in H_2 inhalation group. A mass of apoptotic cells in hippocampal CA1 region was found in CLP group, and there were fewer apoptotic cells found in H_2 inhalation group [8]. Zhou et al.'s study also confirmed the result [7].

Both immunohistochemical staining of cleaved Caspase-3 and western blot of cleaved Caspase-3 expression in hippocampus indicate a great increase in CLP group. With HRS therapy Caspase-3 was dramatically reduced after the CLP event. The number of cleaved Caspase-3-positive cells was 223.62 ± 25.71 in CLP group, which was significantly greater than the sham group. Nevertheless, the numbers in 2.5 mg/kg and 10 mg/kg HRS treatment group were 142.26 ± 9.89 and 84.13 ± 12.48, respectively. Significant differences were found in those groups [7].

H_2 treatment can attenuate blood-brain barrier disruption. Evans blue (EB) is a dye binding to serum albumin, which can seldom go through the Blood Brain Barrier (BBB). But in CLP group, obvious rise of EB quantification was observed compared with sham group; H_2 treatment group

showed less EB quantification compared with CLP group ($p < 0.001$). H_2 treatment can also reduce brain water content. The brain water content was 74.85 ± 0.75 in sham group, which increased to 78.34 ± 0.82 (%) in CLP group ($p < 0.001$) and to 76.57 ± 0.87 (%) in H_2 treatment group [8].

HRS treatment and H_2 treatment prevented the abnormal changes of oxidation and antioxidation. Septic mice had lower levels of SOD and higher levels of ROS and MDA (Malondialdehyde); both HRS treatment and H_2 treatment can prevent those changes. There was also a dose-response relationship showed in studies [7]. Moreover, activities of antioxidant enzymes (SOD and CAT) in both serum and hippocampus were significantly diminished in CLP group. On the contrary, the levels of oxidative products (MDA and 8-iso-PGF2α) were markedly increased. H_2 treatment could upregulate the expression of Nrf2, which is an important transcription factor of antioxidant stress to lighten those abnormal changes. This finding may explain the antioxidant effects of molecular hydrogen therapy [8, 9].

H_2 inhalation can significantly decrease the levels of proinflammatory cytokines (TNF-α, IL-1β, and HMGB1) and increase the level of anti-inflammatory cytokines (IL-10) ($p < 0.001$), in both serum and hippocampus [8].

Researchers measured the cognitive function of CLP-challenged mice with several methods. In Y-maze test and Fear conditioning test, H_2 group showed higher cognitive function at days 3 to 14 after CLP operation [8]. The Morris water maze test results in cognitive impairment in CLP group; HRS injection could alleviate cognitive impairment. When the dosage of HRS boosts into 10 mL/kg, no significant difference was found between sham group and HRS treatment group. Interestingly, cognitive dysfunction recovered 10 days after the CLP event. It indicated that forced exercise may influence learning and memory.

In conclusion, sepsis can destroy the structure of brain especially hippocampus CA1 region through stimulating oxidative stress reaction and inflammatory response, which lead to impairment of cognition. However, molecular hydrogen therapy was proved to attenuate the disruption.

5.2. Lung. Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) are common syndromes in sepsis. When ALI occurs, the oxygenation index, lung MPO activity, lung W/D weight ratio, BAL (bronchoalveolar lavage) and total protein, lung's histology, antioxidant enzymatic activity, and inflammatory cytokines are all different from normal conditions.

The normal lung structure has no hyperemia, neutrophil infiltration. But in sepsis, there can be found disordered alveolar structures, collapse of alveoli, incomplete alveolar wall, severe neutrophil infiltration, alveolar capillary congestion, and thickened alveolar wall by edema. Resuscitation only can reduce neutrophil accumulation and the alveolar-capillary exudate but cannot alleviate alveolar edema. When combined with H_2 inhalation, the therapy significantly decreased in alveolar damage and alveolar edema as well [6]. In addition, HRS administration individually could also decrease infiltration of neutrophils, interstitial edema, and atelectasis [11]. H_2 inhalation is confirmed to be effective as well to attenuate sepsis-induced lung injury in mice. 2% H_2 treatment resulted in reduction of inflammatory cells infiltration and improvement in lung structure [5, 12].

Moreover, effects of H_2 treatment on pulmonary cell apoptosis were investigated. Numerous lung cells were positive for TUNEL (TdT-mediated dUTP Nick-End Labeling) staining which identified apoptotic cells in LPS group. In the samples of H_2 treatment group, a few of positive cells were observed. Caspase-3 detection showed the same tendency in those groups. Those data revealed LPS-induced septic stimulated pulmonary cell apoptosis and H_2 therapy would prevent this process [12].

Lung W/D weight ratio is an indicator of the magnitude of pulmonary edema. Septic lung showed higher W/D ratios in all studies. Resuscitation+H_2 group showed a significant decrease in the lung W/D value compared with resuscitation group, which indicates that H_2 inhalation was benefit to relieve edema [6]. HRS administration also decreased pulmonary W/D weight ratio [11]. H_2 inhalation alone decreased W/D ratio as well [5, 12].

Examination of cell counts and protein concentration in BALF is a particular technique to evaluate lung effusion and its character. Animals studies mentioned that CLP or LPS increased the cell counts and protein in BALF, which could be remitted with H_2 inhalation [5, 10]. Xie et al. proved that H_2 inhalation and HRS injection were both effective to reduce the cell counts, PMNs (Polymorphonuclears), and total protein increased by LPS [12].

Antioxidant enzymatic activity in lung (SOD and CAT) was suppressed and level of oxidative products (MDA and 8-iso-PGF2α) was increased in sepsis. H_2 inhalation and HRS injection both could restrain oxidative stress [5, 6, 10, 11]. Some study presented that increased MPO in the lung of septic mice could be lightened by molecular hydrogen therapy [5, 6, 10, 12] but others presented that HRS injection had no effect of decreasing MPO level [11].

Inflammatory cytokines (TNF-α, HMGB1, IL-1β, IL-6, and IL-8) were increased while anti-inflammatory cytokines (IL-10) were decreased, in serum and lung in sepsis patients. Molecular hydrogen therapy could reduce the level of inflammatory cytokines [5, 6, 10] and increase the level of anti-inflammatory cytokines [10] in septic mice. There are also some researchers who considered molecular hydrogen therapy had no significant effect on the level of TNF-α and IL-10 [11, 12].

Liu et al. [16] combined H_2 therapy with NO therapy in LPS-challenged mice and found that the combination therapy had significant interaction between the two and had more beneficial effect than H_2 inhalation alone.

Generally speaking, lung structure was damaged by sepsis. Lung W/D ratios, cell counts and protein concentration in BALF, level of oxidative products, and inflammatory cytokines were found increased, while antioxidative enzyme activity and anti-inflammatory cytokines were found decreased. Although there still were controversies [11, 12], most researchers regarded molecular hydrogen therapy as a valid technique to alleviate all those pathologic changes.

5.3. Liver. Liver is one of the most important organs, but also one of the first organs to be affected during sepsis. Except for degree of oxidative stress reaction and inflammatory reaction, ALT and AST (aspartate aminotransferase) can also reveal hepatic function.

Histopathological changes in liver were shown in sepsis. Animal study shows liver histologic scores significantly increased in CLP group; O_2 inhalation group and H_2 inhalation group both showed much lower scores, which even had no difference to sham group [5, 10].

In addition, CLP mice developed significant liver injury, which was assessed by ALT and AST increase. H_2 inhalation and HRS injection both could attenuate these abnormal changes [5, 10, 11]. Especially in Xie et al.'s study, H_2 inhalation group even had no significant difference with sham group, indicating the dramatic effect of H_2 therapy [5].

Oxidative stress reaction and inflammatory reaction of liver were similar with lung. Inflammatory cytokines like TNF-α and HMGB1 were increased, while anti-inflammatory cytokines like IL-10 were decreased in sepsis. Antioxidant enzyme activities (SOD and CAT) were decreased and oxidative products (8-iso-PGF2α) were increased. H_2 inhalation alleviated those changes [5, 10].

Studies about liver damage and hepatic function in sepsis with molecular hydrogen therapy were in a small number. Even so, these results revealed a dramatic effect of molecular hydrogen therapy. It may indicate that molecular hydrogen therapy is much more efficacious in liver protection. More research is needed in this area.

5.4. Kidney. Acute kidney injury (AKI) is a common disease in septic patients and can aggravate the condition of septic shock patients, resulting in higher mortality. Except for degree of oxidative stress reaction and inflammatory reaction, blood urea nitrogen (BUN) and creatinine (Cr) can also reveal hepatic function.

H&E staining of kidney tissues exhibited edema in renal tubular epithelial, damaged brush border, and interstitial edema with hemorrhage in septic mice. Tubular epithelial cell damage was ameliorated in H_2 inhalation group. The similar result showed in transmission electron microscopic analysis of glomerular filtration membrane [13]. Kidney histologic scores increased significantly in CLP group; it is marvelously alleviated in H_2 inhalation group which even had no difference with sham group [5, 10].

Serum BUN and Cr were much higher in LPS or CLP group than in sham group. H_2 inhalation group had significant reductions of serum BUN and Cr [5, 10, 11, 13]. But one study revealed there was no significant difference of BUN/Cr ratio in all the groups. As BUN/Cr ratio is used to analyze whether prerenal azotemia or tubular ischemia exists in AKI, molecular hydrogen therapy may not be as effective as we thought [13].

Oxidative stress reaction and inflammatory reaction of kidney were similar with lung and liver. Inflammatory cytokines (TNF-α, IL-6, and HMGB1) were increased, while anti-inflammatory cytokines (IL-10) were decreased in sepsis. Antioxidant enzyme activities (SOD and CAT) were decreased and oxidative products (MDA and 8-iso-PGF2α) were increased. H_2 inhalation alleviated those changes [5, 10, 13]. However, in study of Liu et al., level of IL-10 had no change between all groups [13].

In spite of some dispute, molecular hydrogen therapy was considered as a useful method to alleviate structure damage of kidney, protect renal function, and resist inflammatory reaction and oxidative reaction.

5.5. Intestine. Sepsis leads to significant decrease in blood flow of the gastrointestinal tract. Hyperperfusion induces severe ischemia, hypoxia, and reperfusion injury. Researchers also work at molecular hydrogen therapy alleviating septic damage in intestine.

In animal study, after LPS manipulating, the structure of the small intestinal mucosa was damaged. Glands of the small intestine were destroyed. Edema of mucosal villi, neutrophil infiltration, and even intestinal ulceration was also commonly observed in sepsis. Resuscitation therapy worsened the damage mentioned above while H_2 inhalation reduced the damage. The histologic score of LPS group was significantly higher than sham group, but the score of H_2 inhalation group was significantly decreased compared with LPS group [6].

The serum diamine oxidase (DAO) activity reflects degree of intestinal mucosa epithelium cell impaired. Levels of DAO in sham group, LPS group, and H_2 group were 4.32 ± 0.33 kU/L, 6.54 ± 0.68 kU/L, and 5.14 kU/L ($p < 0.05$), respectively [6]. The result demonstrated that H_2 inhalation could protect epithelium cell of intestine from septic damage.

Oxidative stress reaction and inflammatory reaction of intestine were similar with lung, liver, and kidney. Inflammatory cytokines (TNF-α, IL-6, IL-8) were increased in LPS group. Antioxidant enzyme activity (SOD) was decreased and oxidative products (MDA) were increased in sepsis. H_2 inhalation alleviated those changes.

The effect of molecular hydrogen therapy of intestinal damage in sepsis curing with molecular hydrogen therapy needs more research. According to the only literature of study, molecular hydrogen therapy protected intestine from sepsis.

6. The Impact of Molecular Hydrogen on Outcomes

All the research indicated molecular hydrogen therapy can improve survival rate of septic animal whatever the method of drug administration and sepsis inducing.

Zhang et al. compared 3 different ways to induce sepsis and the survival rate, respectively. The survival rates of LPS-induced septic mice at 24, 48, and 72 hr were 88.89%, 66.67%, and 66.67%. With HRS treatment, the survival rate increased to 100% ($p < 0.05$), 75%, and 75%, respectively. When challenged by feces injection, survival rates of mice at 24, 48, and 72 hr were 100%, 75%, and 75%. With HRS treatment, the survival rate increased to 85.71%, 85.71% ($p < 0.05$), and 85.71% ($p < 0.05$), respectively. Survival rates of CLP-induced septic mice at 24, 48, and 72 hr were 76.47%, 47.06%, and 35.23%. With HRS treatment, the survival rate increased to 72.73%, 72.73% ($p < 0.01$), and 54.54% ($p < 0.01$),

respectively. The 3-day mortality rates after modeling were 33.33% (LPS model), 25% (feces model), and 64.7% (CLP model), while HRS treatment reduced them to 25%, 14.29% ($p < 0.05$), and 45.45% ($p < 0.01$), respectively [4]. There was still no denied data yet.

7. Safety Concerns

As mentioned before, there are 3 methods of molecular hydrogen therapy: inhalation of hydrogen (H_2), oral intake of hydrogen-rich water, and injection of hydrogen-saturated saline. In low concentration (4.1% in pure oxygen or 4.6% in the air), hydrogen is neither explosive nor dangerous. Others thought it is safer to dissolve hydrogen into water and administrate the HRS by oral or by injection. However, the best way of molecular hydrogen therapy, the appropriated dosage, and the safety concerns are still to be discussed.

8. Conclusion

Molecular hydrogen therapy has a protective effect on sepsis, which has been proved by pathological biopsy, level of inflammatory factors/anti-inflammatory factors, oxidative stress reaction, behavioral experiment, and other related indicators of organ function. Although there is a dispute of affections of molecular hydrogen therapy in liver and kidney, the mainstream view shows molecular hydrogen therapy is benefit to organs, such as brain, lung, liver, kidney, and small intestine.

Molecular hydrogen therapy combining with oxygen therapy or fluid resuscitation can reduce oxygen free radical damage, the amount of fluid and vasoactive drugs, and the overload of liquid. As a result, molecular hydrogen therapy may reduce the complications of oxygen therapy and fluid resuscitation.

However, most of the study conclusion came from animal experiment while reports of clinical research were rare. Much more clinical evidence is still demanded.

In conclusion, molecular hydrogen therapy is a promising method to alleviate organ damage, improve outcome, and reduce mortality rate in sepsis.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Effects of Synthetic Serum Supplementation in Sperm Preparation Media on Sperm Capacitation and Function Test Results

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Albumin supplementation of culture media induces sperm capacitation in assisted reproduction technique cycles. Synthetic serum supplementation is clinically used to replace albumin for preventing transmission of infectious agents. However, the effects of synthetic serum supplementation on sperm capacitation have rarely been investigated. Spermatozoa from 30 men with normal basic semen analysis results were collected, divided into five aliquots, and cultured in capacitating conditions in four combinations of two synthetic serum supplements, serum substitute supplement (SSS) and serum protein substitute (SPS), and two fertilization media, Quinns Advantage™ Fertilization (QF) and human tubular fluid (HTF) media. Reactive oxygen species (ROS) levels in spermatozoa were measured through chemiluminescence. Furthermore, acrosome reaction and western blotting for tyrosine phosphorylation were used to evaluate sperm capacitation. HTF+SSS had significantly higher ROS levels than QF+SPS did (11,725 ± 1,172 versus 6,278 ± 864 relative light units). In addition, the spermatozoa cultured in QF+SPS had lower motility, acrosome reaction rates, and tyrosine phosphorylation levels compared with those cultured in HTF+SSS. In conclusion, the effects of synthetic serum supplementation on sperm capacitation varied according to the combination of media. These differences may lead to variations in spermatozoon ROS levels, thus affecting sperm function test results.

1. Introduction

When passing through the cervix, uterus, and oviducts, human spermatozoa undergo a physiological process called capacitation to become capable of fertilizing oocytes [1]. During capacitation, various cellular changes occur, including generation of a limited amount of reactive oxygen species (ROS) and protein phosphorylation at tyrosine residues [2]. After these alterations, spermatozoa undergo acrosome reaction, in which hydrolytic enzymes enabling spermatozoa to fertilize oocytes are released [3, 4].

In clinical practice, basic semen analysis—which focuses on the concentration, motility, and morphology of spermatozoa, according to the World Health Organization (WHO) guidelines [5]—is used to determine the fertilization potential of human spermatozoa. However, the criteria or requirements for spermatozoa differ for natural fertilization, intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) [6]. For the management of infertile couples without evident female or male factors, IUI is initially considered. If IUI fails more than three times, IVF cycles are recommended for these patients because the accurate fertilization potential of spermatozoa under these conditions is uncertain.

For infertile couples with normozoospermia, failure of IUI necessitates advanced sperm function tests for determining the fertilization potential of spermatozoa. These tests can

be used to generate a standard IVF or ICSI treatment plan [7–9]. Most sperm function tests analyze parts of the capacitation process, such as hyperactivation, sperm-zona binding, and acrosome reaction [7]. The hemizona assay and induced acrosome reaction test are valuable predictors of IVF outcome [7, 9, 10].

ROS is a positive trigger for capacitation-related modifications [11–13]. Donà et al. reported that spermatozoon ROS content directly influences the levels and locations of tyrosine phosphorylation and then enables the spermatozoa to undergo acrosome reaction [14]. Nevertheless, spermatozoa are sensitive to oxidative stress because they have a limited amount of antioxidant enzymes, but they have abundant unsaturated fatty acid on their cell membrane as well as abundant DNA, both of which are targets of free radical attack. Oxidative stress-mediated damage to spermatozoa is a major pathology contributing to male infertility [15–17]. High ROS levels in the seminal fluid impair the sperm DNA integrity and thus inhibit spermatozoon function [18]. Furthermore, infertile men have lower nonenzymatic antioxidant activity in the seminal plasma than fertile men do [19, 20].

Before IUI, IVF, or ICSI, spermatozoa are processed through in vitro preparation, which induces certain levels of sperm hyperactivation. Serum albumin and sodium bicarbonate can induce sperm capacitation during in vitro culturing of spermatozoa [1, 13]. In andrology laboratory settings, synthetic serum supplements for fertilization media are used, rather than albumin, to prevent transmission of infectious agents. However, the effects of synthetic serum supplementation on sperm capacitation during the preparation and insemination period have rarely been investigated.

A recent meta-analysis suggested that, compared with overnight coincubation, a short period of spermatozoon and oocyte coincubation provides more satisfactory IVF outcomes [21]. Therefore, the capacitation process in the IVF settings may need to be more effectively completed within a shorter incubation period. This study was specifically focused on the exact status of functions of spermatozoa (e.g., capacitation events and DNA damage) cultured in synthetic serum-supplemented sperm preparation media for infertile couples with normozoospermia.

2. Materials and Methods

2.1. Patient Selection and Semen Collection. All experimental procedures were approved by the Institutional Review Board of Chung Shan Medical University Hospital, Taichung, Taiwan (CS07162 and CS14066). To prevent the interference of an infertile etiology (e.g., male, tubal, and ovarian factors), only infertile couples with secondary infertility and an unexplained etiology (UI) were recruited in this study. Semen samples were obtained from 30 male partners in the UI couples. Informed consent was obtained from all participating couples from July 2013 to December 2014.

Basic semen analyses were performed according to the fourth edition of the WHO guidelines after 3–5 days of sexual abstinence. All semen samples showed normal results in the basic semen analysis (sperm count $> 20 \times 10^6$/mL, motility > 50%, and morphology > 14%) and Endtz test ($<1.0 \times 10^6$/mL).

For liquefaction before analyses, the semen samples were kept at room temperature for an average of 1 h (range: 0.5–1.5 h). One liquefied neat semen aliquot was used for sperm motion analysis and ROS measurement. Each liquefied semen sample from one man was separated into four aliquots and then cultured in the following four semen preparation media: (1) modified human tubal fluid media (HTF) with 5% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA) in the control (HTS) group; (2) HTF with 15% Quinns Advantage Serum Protein Substitute (SPS; SAGE In Vitro Fertilization Inc., Trumbull, CT, USA) in the HTP group; (3) Quinns Advantage Fertilization Medium (QF; SAGE In Vitro Fertilization Inc.) with 5% SSS in the QFS group; and (4) QF with 15% SPS in the QFP group. The protein supplements SSS and SPS are used as a replacement for human serum albumin, a recognized sperm capacitating agent.

To determine the effect of antioxidant supplementation in sperm preparation media, we collected semen samples again from these UI couples. Then, all sperm samples were separated into two aliquots and incubated in the two following sperm preparation media: (1) the HTS group and (2) HTS with 5 mM glutathione in the GSH group.

The liquefied semen was prepared through density gradient centrifugation (DGC) at 300 ×g with PureSperm (Nicadon, Gothenburg, Sweden; 90/45%) for 15 min. The sperm motion and ROS levels of the washed spermatozoa were also analyzed. The spermatozoa were incubated in the aforementioned preparation media at 37°C under 5% CO_2 for 3 h. After the incubation, acrosome reaction rates and tyrosine phosphorylation levels of the spermatozoa were evaluated.

2.2. ROS Level Measurements. ROS levels were measured using a chemiluminescence assay with luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, St. Louis, MO, USA) as a probe. Samples were prepared as 100 μL aliquots of sperm at 10×10^6/mL with 2.5 μL of luminol, prepared as a 5 mM stock solution in dimethyl sulfoxide (Sigma). Each sample was scanned using a luminometer (FlexStation 3 Benchtop Multi-Mode Microplate Reader; Molecular Devices, LLC, USA). All samples were measured in duplicate. We then scanned the washed spermatozoon samples for 180 min for detecting the dynamic changes in ROS levels. Here, ROS levels are expressed as relative light units (RLU).

2.3. Sperm Motion Analysis. Sperm motion characteristics were analyzed using computer-assisted sperm analysis (CASA; Hamilton Thorne, Inc., Beverly, MA, USA), as per the 1998 guidelines of the European Society of Human Reproduction and Embryology [22]. In brief, the parameters settings for analysis included the following: image acquisition rate 80 Hz; number of spermatozoa sampled ≥200; and number of microscopic fields sampled at 200x magnification ≥1. Chambers used for sperm analysis measured 0.01 mm^2 in surface area, with a 0.02 mm depth.

CASA was used to determine various sperm parameters, including concentration, motility, average path velocity (VAP), straight line velocity (VSL), straightness of sperm

motion (STR), and lateral displacement amplitude of head (ALH). Other measured parameters included the percentage of progressive motile spermatozoa exhibiting a VAP > 25 μm/s and STR > 80%.

2.4. Hemizona Assay. After incubation of sperm samples for 2 h, sperm-zona interactions were assessed using our hemizona assay, as described previously [9] but with modifications. In brief, fresh unfertilized oocytes from our assisted reproduction program were used as the source of zona pellucida. After sperm preparation in the various culture media, 20,000 sperm in total were added to a droplet of the media. A pair of hemizona was coincubated at 37°C under 5% CO_2 in air for 2 h, with spermatozoa either from the QFP group (test) or from the HTS group (control). The number of spermatozoa tightly bound to the zona was counted; the results of the hemizona assay were expressed as the hemizona assay index: the ratio of the number of spermatozoa bound to the test droplet to that of spermatozoa bound to the control droplet.

2.5. Acrosome Reaction Evaluation. After incubation of sperm samples for 2 h, the acrosome status was assessed through FITC-PNA staining (Sigma), as described in our previous report [23]. In brief, 20 μL of sperm suspension was spread over a clean microscopy slide, air-dried, fixed in 95% ethanol for 5 min, and again air-dried. The fixed slides were stained using FITC-PNA (600 μL of FITC-PNA in 15.4 μL reagent water in a foil-covered Coplin jar) for 15 min at ambient temperature. The slides were rinsed by dipping them in phosphate-buffered saline (PBS) two times before fixing them for 15 min in paraformaldehyde at ambient temperature. The slides were then air-dried, mounted, and stored in the dark until scoring. Between 100 and 250 spermatozoa were counted per slide and scored. Labeling of only the equatorial segment of the acrosome indicates a normally acrosome-reacted spermatozoon that has lost the outer acrosomal membrane present over the anterior acrosomal cap but has an intact equatorial segment.

2.6. Western Blot Analysis of Tyrosine Phosphorylation. After incubation in the capacitating condition, proteins extracted from spermatozoa were analyzed through sodium dodecyl sulphate- (SDS-) polyacrylamide gel electrophoresis (PAGE) and western blot analysis. In brief, samples were resuspended in the Laemmli sample buffer (2% SDS, 10% glycerol, 5% b-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8) and heated at 100°C for 5 min. Proteins were then separated through 8% SDS-PAGE and transferred onto a nitrocellulose membrane [24]. Nonspecific binding sites on the membrane were blocked using 5% (w : v) nonfat milk in Tris-buffered saline (20 mM Tris and 137 mM NaCl, pH 7.6). The nitrocellulose membrane (0.22 mm pore size; Micron Separations Inc., Westboro, MA, USA) was incubated overnight at 4°C with an antiphosphotyrosine monoclonal antibody (clone 4G10, 1/1000; Upstate Technology Inc., Lake Placid, NY, USA). The blots were then incubated with a horseradish peroxidase goat antimouse IgG (Kirkegaard and Perry Lab., Gaithersburg, MD, USA) for 1 h. The signals were then detected using an enhanced chemiluminescence (ECL) commercial kit (Amersham Biosciences, Piscataway, NJ, USA), and the relative photographic density was quantified by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

2.7. Sperm DNA Damage Assessment. The Calbiochem OxyDNA Kit (Merck KGaA, Darmstadt, Germany), which entails employing an in vitro fluorescent protein binding method, was used to detect oxidative injury to DNA in spermatozoa. One aliquot of a semen sample containing 3×10^6 spermatozoa was pelleted, washed using PBS, and fixed; spermatozoa were permeabilized by incubating them in ice-cold 70% ethanol at −20°C for 1 h. Fixed cells were centrifuged at 1,600 rpm for 5 min, washed with PBS two times, resuspended in 1 mL of wash solution (Tris-buffered saline/Tween 20 containing thimerosal), and pelleted at 1,600 rpm for 5 min. Next, 100 μL of 1x FITC conjugate was added to the cell pellet, which was then incubated in the dark for 60 min at room temperature. The cells were then washed with a wash solution. The fluorescence was read using a flow cytometer at a 495 nm excitation wavelength and 515 nm barrier filter.

DNA fragmentation was evaluated using our terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) assay (Boehringer Mannheim, Mannheim, Germany), as reported previously [23]. In brief, the sperm samples were washed in PBS and then centrifuged for collecting spermatozoa at 200 ×g. The spermatozoa were then treated with a solution containing 0.1% Triton X-100 (Sigma). A 30 mL TUNEL mixture was added to the same volume of each sample. The samples were then incubated for 60 min at 37°C in a moist chamber in the dark, washed three times with PBS, and then analyzed through FACS. At least 10,000 cells were counted. The presence of green fluorescent signals indicated positivity.

2.8. Statistical Analysis. Sperm motion characteristics, acrosome reaction rates, the intensity of tyrosine phosphorylation on the western blot, oxidative injury rates, and DNA fragmentation rates were subjected to the Wilcoxon signed-rank test to evaluate the differences among the four groups (HTS, HTP, QFS, and QFP) or the difference between two groups (HTS and GSH). A confidence level of $p < 0.05$ was considered the limit of statistical significance.

3. Results

We collected 30 semen samples from the male partners of the UI couples in our andrology laboratory. The basic semen analysis results and demographic data of these samples are presented in Table 1. After DGC, each semen sample was divided into four groups according to the sperm preparation media used—QFS, HTS, QFP, and HTP—and cultured for a short incubation period of 2-3 h. Figure 1 presents the dynamic changes observed in the ROS levels of representative samples during the incubation period. Transient elevation in the ROS levels was noted in all sperm samples. After 3 h of incubation, spermatozoa in the QFS and HTS groups showed

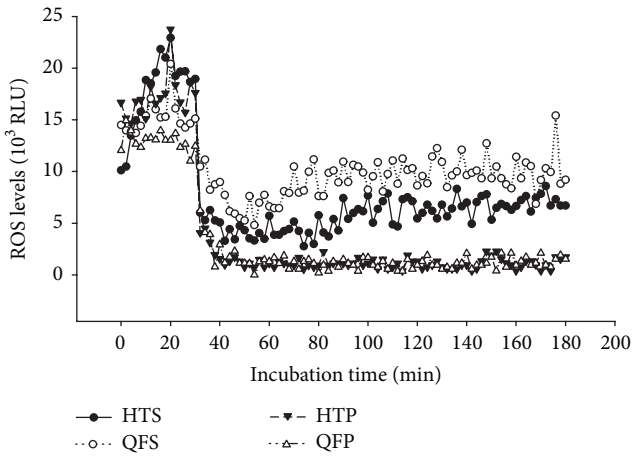

Figure 1: Dynamic patterns of reactive oxygen species (ROS) levels in washed spermatozoa cultured in various sperm preparation media.

Table 1: Demographic data and sperm motion characteristics in the semen samples from 30 male partners of couples with unexplained secondary infertility. The data are presented as the median (interquartile range).

Data (n = 30)	Median	25%~75% range
Age (years)	33	29~38
Concentration (M/mL)	86.8	49.2~172.9
Morphology (%)	18.3	15~32
Motility (%)	77.3	66.1~83.5
Progressive motility (%)	37.8	28.4~58.7
VAP(μm/s)	28.4	25.5~35.5
VSL (μm/s)	19.5	16.1~22.4
ALH (μm)	1.7	1.0~3.0

consistently higher ROS levels than those in the QFP and HTP groups did.

We selected various combinations of sperm preparation media and serum supplements for ROS measurement and sperm function tests. Sperm preparation media (HTF or QF) with SSS had significantly higher ROS levels than those with SPS did (Figure 2(a)). HTF media with SSS demonstrated the highest ROS levels (11,948 ± 2,162 RLU) after only 2 h of incubation.

For the spermatozoa incubated for 2 h in PBS solution, ROS levels were associated with the sperm concentration (Figure 2(b)). By contrast, the ROS levels were relatively constant for the spermatozoa cultured in HTF+SSS at 0.5–2.5 × 10^6/mL (Figure 2(b)). All sperm function tests were performed using a sperm concentration of 1 × 10^6/mL after a 2 h incubation period, unless otherwise specified in Section 2.

We performed two sperm function tests: CASA and the zona binding assay. The motility (median (interquartile range): 65.7% (61.4%–88.8%) versus 51.5% (45.6%–79.7%), p = 0.047, by Wilcoxon signed-rank test) and progressive motility (31.2% (27.0%–51.4%) versus 24.3% (15.5%–48.0%), p = 0.047, by Wilcoxon signed-rank test) decreased significantly in the QFP group compared with those in the HTS group (Table 2). Similar findings were observed in the zona binding assay. More spermatozoa from the HTS group were bound to the hemizona than those from the QFP group (33.3% (22.7%–46.5%) versus 1%, p = 0.021, by Wilcoxon signed-rank test).

Acrosome reaction test and western blotting for tyrosine phosphorylation levels (proteins of 105 and 81 kDa) were performed after 2 h of incubation in the four types of media. We demonstrated the protein of 105 kDa as an example. The addition of SPS to QF significantly reduced tyrosine phosphorylation levels (0.46 (0.23–0.67) versus 1, p = 0.008; Figure 3(a)) and acrosome reaction rates (39.0% (27.0–69.2) versus 52.1% (34.0–74.2), p = 0.024; Figure 3(b)) in the cultured spermatozoa compared with those in the HTS media.

To evaluate the sperm DNA damage caused by ROS or oxidative stress, 8-OHdG and TUNEL assays were performed. Although the ROS levels of spermatozoa cultured in preparation media with SSS supplementation were elevated, the 8-OHdG and TUNEL results did not differ significantly among the spermatozoa cultured in the four preparation media (Figures 3(c) and 3(d)).

To test the effect of antioxidants on the capacitation of spermatozoa cultured in the sperm preparation media, 5 mM reduced glutathione was added to the HTS medium (the GSH group). CASA revealed that motility (median (interquartile range): 76.1% (58.9%–89.2%) versus 68.7% (38.2%–88.4%), p = 0.014, by Wilcoxon signed-rank test) and ALH (5.0 μM (3.6–6.4 μM) versus 3.8 μM (0–5.4 μM), p = 0.018, by Wilcoxon signed-rank test) decreased with the addition of glutathione in the capacitating condition (addition of SSS; Figure 4(a)).

The results of western blotting for tyrosine phosphorylation levels (proteins of 105 and 81 kDa) and immunostaining for acrosome reaction were similar to those of sperm motion analysis. Spermatozoa in the GSH group have significantly decreased tyrosine phosphorylation levels of protein of 105 kDa (0.78 (0.60–0.89) versus 1, p = 0.001, by Wilcoxon signed-rank test; Figure 4(b)) and acrosome reaction rates

Table 2: Sperm motion characteristics observed through computer-assisted semen analysis and hemizona assay results of the washed spermatozoa after 2 h of incubation. The data are presented as the median (interquartile range).

	HTS (HTF+SSS)	QFS (QF+SSS)	HTP (HTF+SPS)	QFP (QF+SPS)
Motility (%)	65.7 (61.4~88.8)[a]	60.1 (47.7~94.1)	59.0 (42.6~73.1)	51.5 (45.6~79.7)[a]
Progressive motility (%)	31.2 (27.0~51.4)[b]	28.2 (22.2~54.4)	27.4 (21.3~37.6)	24.3 (15.5~48.0)[b]
VAP(μm/s)	29.0 (27.0~33.6)	39.8 (29.5~46.6)	27.9 (32.4~38.4)	29.8 (27.0~37.0)
VSL (μm/s)	15.4 (13.4~17.0)	20.6 (16.7~27.3)	16.6 (15.7~21.6)	14.8 (12.1~18.3)
ALH (μm)	4.2 (3.2~5.2)	4.6 (1.7~6.5)	5.0 (2.9~5.6)	4.6 (0~5.8)
Hemizona assay (%)	100	—	—	33.3 (22.7~46.5)[c]

[a] $p = 0.047$, [b] $p = 0.047$, and [c] $p = 0.021$ by Wilcoxon signed-rank test.

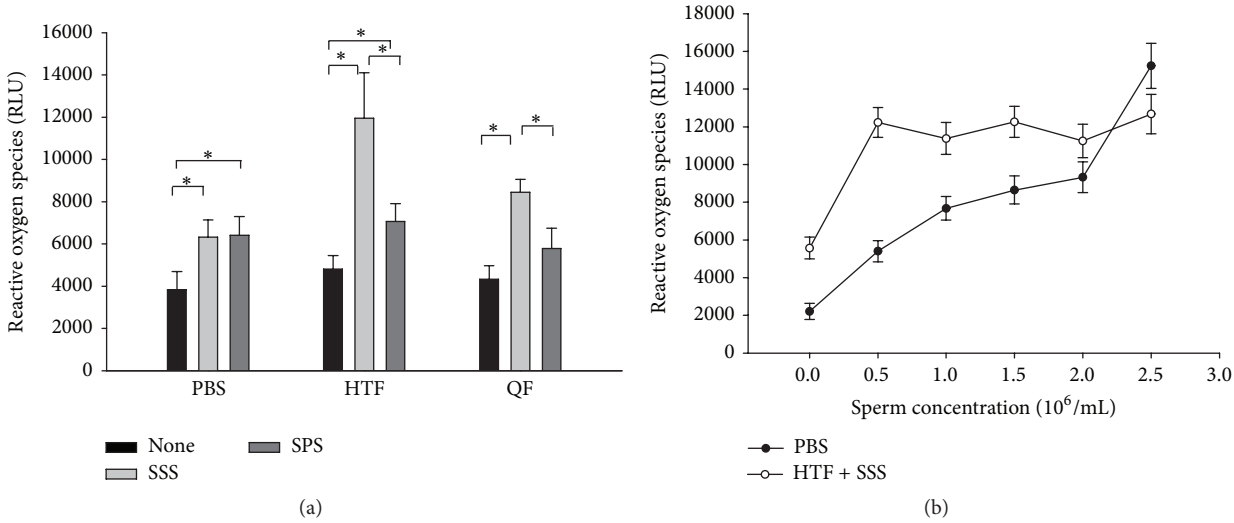

Figure 2: Reactive oxygen species (ROS) levels in the sperm preparation medium components. The data are presented as the mean (SD). RLU denotes relative light units, and $*$ indicates significantly different ROS levels between the two groups according to the Mann-Whitney U test.

(34.4% (26.5%–54.45) versus 43.7% (29.3%–66.0%), $p = 0.002$, by Wilcoxon signed-rank test; Figure 4(c)) compared with those in the HTS group.

4. Discussion

Our results indicated that various commercial synthetic serum supplements could induce sperm capacitation at different levels. The varied sperm capacitation levels and sperm function test results were positively associated with the ROS levels in the preparation media. Furthermore, the addition of glutathione (an antioxidant) reduced the capacitation levels. However, the transient elevations in ROS levels during the sperm preparation process are not directly associated with DNA damage of spermatozoa.

The present study results are consistent with those of previous studies indicating that a limited amount of ROS can trigger the sperm capacitation process [11–13]. When we analyzed each preparation medium component separately, the media supplemented with SSS showed higher ROS levels and a higher proportion of capacitated spermatozoa than those supplemented with SPS did. Taken together, these findings further confirm that some preparation medium components, such as SSS in the present study, can modify the ROS levels of spermatozoa and simultaneously sperm function test results.

Albumin is considered an antioxidant because its molecules contain cysteine-34, which has free sulfhydryl (SH) groups that capture radicals [25]. However, the presence of albumin in culture media facilitates the transfer of free radicals from one molecule to another [26]. Compared with SPS, SSS may have more free SH groups, which facilitate the transfer of ROS during sperm capacitation. This transfer is critical for sperm activation [13].

The strength of the present study is that we used divided spermatozoa from individual patients and cultured them in four sperm preparation media. In theory, they should have demonstrated similar sperm function test results. However, spermatozoa from a single man exhibited significantly different sperm function test results in the various preparation media. The tyrosine phosphorylation levels confirmed that the preparation media, specifically the synthetic serum supplement, induced the varying capabilities of sperm capacitation within a short period (2-3 h) of in vitro incubation. The different sperm function test results induced by the various

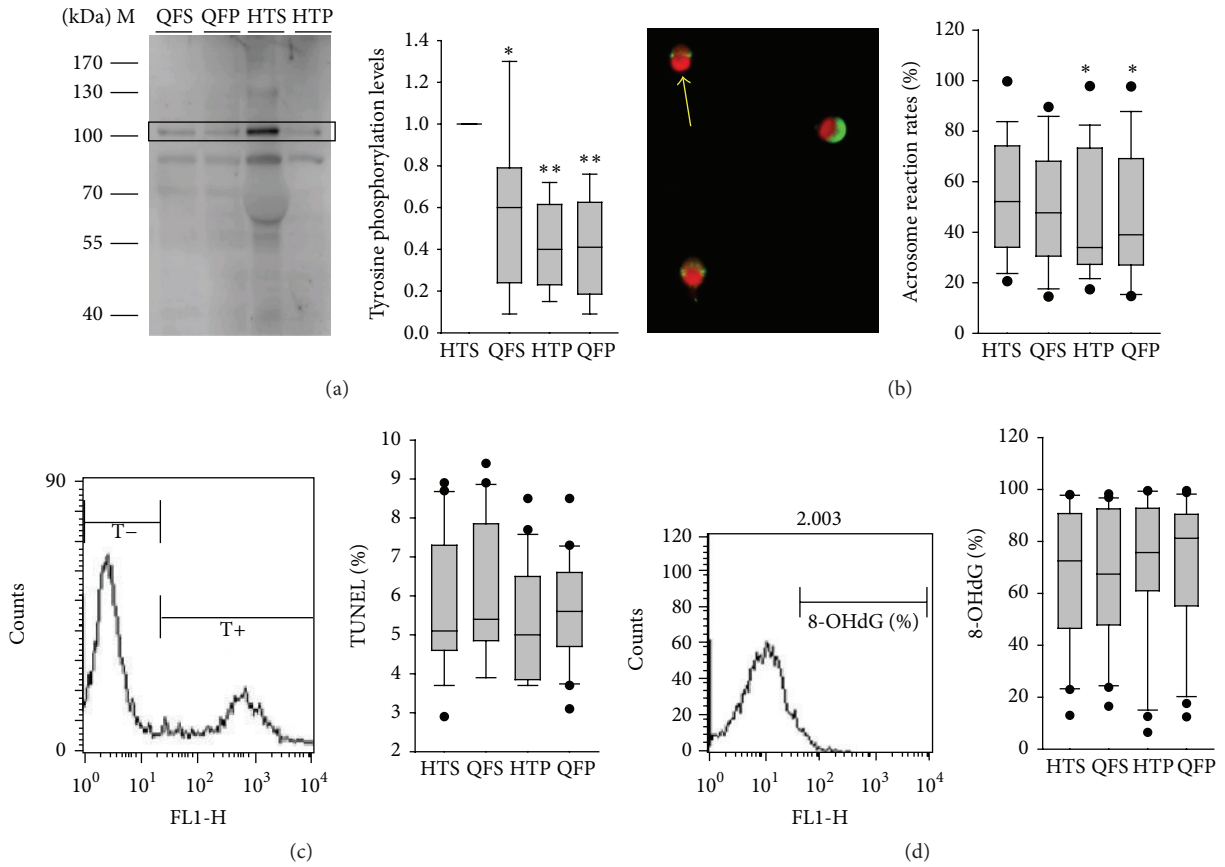

Figure 3: Tyrosine phosphorylation levels of protein of 105 kDa according to western blotting (a), acrosome reaction rates (b), DNA fragmentation according to the TUNEL test (c), and oxidative injury according to the 8-OHdG test (d) in spermatozoa cultured in various sperm preparation media. $*$ and $**$ denote $p < 0.05$ and $p < 0.01$, respectively, compared with spermatozoa in the HTS capacitating condition according to the Wilcoxon signed-rank test. The arrow in (b) indicates a spermatozoon with a reacted acrosome. The black circles denote all data points that lie outside the 10th and 90th percentiles.

sperm preparation media may lead to incorrect interpretation regarding the fertilization potential of spermatozoa and consequently overuse or underuse of IVF and ICSI.

The elevated ROS levels in sperm preparation media were not associated with higher oxidative DNA injury or spermatozoon DNA fragmentation. We offer two possible explanations for this observation. First, the elevation of ROS levels was transient (within <30 min) and the increasing DNA injury or fragmentation was not evident in such a short period. A recent study focusing on sperm preparation by using the DGC method for ICSI demonstrated that DNA fragmentation levels decreased after DGC but gradually and nonsignificantly increased during a short incubation period of 2 h [27]. We used DGC as the sperm preparation method, which increases the ROS levels and reduces DNA fragmentation in the spermatozoa [28]. However, the present data indicated that elevated ROS levels do not aggravate DNA fragmentation in a short period of in vitro incubation. Second, our patients featured normal basic semen analysis results. All sperm preparation media could induce substantial capacitation of the spermatozoa, probably sufficient for fertilizing relatively few oocytes. Nevertheless, it remains unknown whether the differences in ROS and capacitation levels after serum supplementation affect the fertilization potential of spermatozoa from patients with inadequate sperm parameters, such as oligozoospermia, asthenozoospermia, and teratozoospermia.

5. Conclusion

Serum supplementation of sperm preparation media may alter the ROS levels and modify the function test results of spermatozoa. In IVF settings, the transient elevation in ROS levels does not lead to sperm DNA fragmentation and oxidative injury. However, inaccurate sperm function test results because of elevated ROS levels may lead to overuse or underuse of ICSI. To establish generally applicable criteria for sperm function tests, further investigation is warranted.

Competing Interests

The authors have no competing interests to declare.

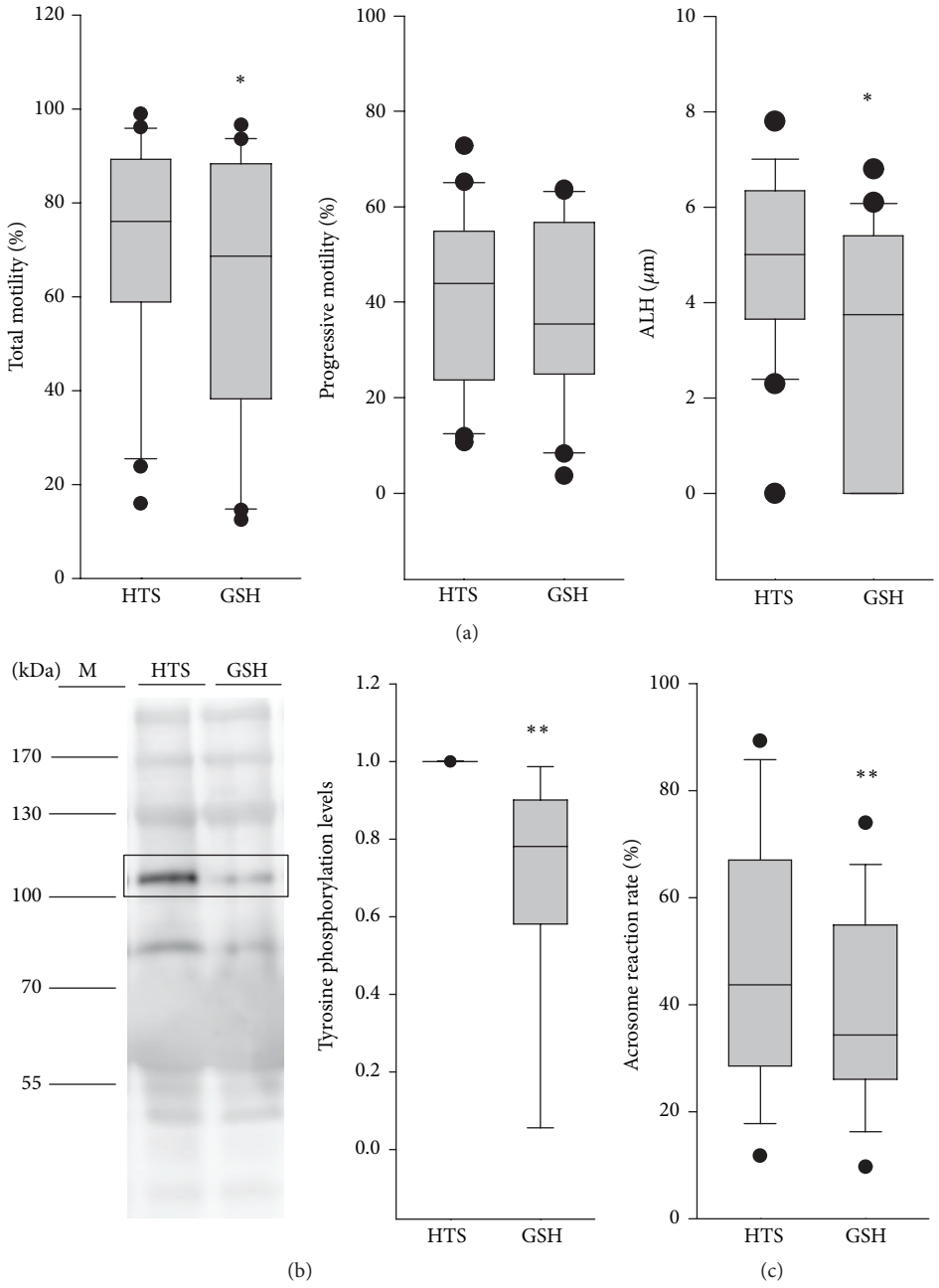

Figure 4: Motion characteristics (a), western blotting for tyrosine phosphorylation levels of protein of 105 kDa (b), and acrosome reaction rates (c) in spermatozoa cultured in various sperm preparation media. $*$ and $**$ denote $p < 0.05$ and $p < 0.01$, respectively, compared with spermatozoa in the HTS capacitating condition according to the Wilcoxon signed-rank test. GSH denotes 5 mM glutathione added to the HTS medium.

Authors' Contributions

Y. F. Shih, T. H. Lee, and M. S. Lee contributed to the conception and design. Y. F. Shih, S. L. Tzeng, W. J. Chen, H. H. Chen, and C. C. Huang acquired, analyzed, and interpreted the data. Y. F. Shih and T. H. Lee drafted the paper. S. L. Tzeng revised the paper critically for important intellectual content. All authors have approved the final version of this paper. Tsung-Hsien Lee and Maw-Sheng Lee contributed equally to this work.

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Review Article

Current Antioxidant Treatments in Organ Transplantation

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Oxidative stress is one of the key mechanisms affecting the outcome throughout the course of organ transplantation. It is widely believed that the redox balance is dysregulated during ischemia and reperfusion (I/R) and causes subsequent oxidative injury, resulting from the formation of reactive oxygen species (ROS). Moreover, in order to alleviate organ shortage, increasing number of grafts is retrieved from fatty, older, and even non-heart-beating donors that are particularly vulnerable to the accumulation of ROS. To improve the viability of grafts and reduce the risk of posttransplant dysfunction, a large number of studies have been done focusing on the antioxidant treatments for the purpose of maintaining the redox balance and thereby protecting the grafts. This review provides an overview of these emerging antioxidant treatments, targeting donor, graft preservation, and recipient as well.

1. Introduction

Ischemia/reperfusion injury (IRI) is present in many medical situations, specifically in organ transplantation. This event can lead to immediate and long-term graft dysfunction, such as allograft rejection, delayed graft function (DGF), and even primary nonfunction (PNF) [1]. It is directly associated with endothelial and parenchymal cell injury, increased vascular permeability, inflammatory response, and generation of reactive oxygen species (ROS).

Oxidative stress, known as an imbalance between the generation of ROS and antioxidant defense system, is the disease mechanism most commonly involved in IRI. It has been proven that ischemia initiates the noxious generation of ROS, while the reoxygenation process during reperfusion is responsible for most ROS production, activation of complement system, and inflammatory response [2]. Evidence supports the fact that occlusion of the vascular supply during transplantation results in serious hypoxia among the endothelial cells, which turns into an important source and target of ROS. Mitochondrial dysfunction, neutrophil priming, xanthine oxidase, and NADPH oxidases play a pivotal role in this process [3]. Subsequently, excessive oxidants cause tissue damage and cell death by inducing the peroxidation of DNA, protein, and lipids.

The ROS-induced injurious effect on graft and recipient is related to various posttransplant complications. Besides, marginal grafts are significantly vulnerable to the oxidative stress and thus restrict graft pool, which aggravates organ shortage. To alleviate such adverse outcomes, a volume of preclinical and clinical studies against oxidative stress are under investigation, targeting consecutive process throughout the transplantation, including donor, graft preservation, and recipient as well. Despite some new discoveries in the mechanism against ROS, the clinical results remain controversial. Therefore, we summarize some advanced development of antioxidant treatments in organ transplantation and their corresponding mechanisms.

2. Antioxidant Treatment for Donor

2.1. Local Ischemic Preconditioning. Local ischemic preconditioning (LIPC), a widely accepted antioxidant approach, is a brief period of ischemia/reperfusion that leads to tolerance of subsequent ischemia/reperfusion injury (IRI). In animal studies, LIPC has been proven to be an efficient tool to protect most organs (e.g., liver, kidney, heart, and intestine), particularly via antioxidant pathway [4]. Concerning mechanism, a recent study demonstrates that LIPC can enhance a series of antioxidant genes' expression via activating cytoplasmic

redox-sensitive transcription factors, effecting regulating cell redox status, inhibiting oxidative injury to cell components, altering the disturbance of Ca^{2+}, and preventing nucleus injury and DNA fragmentation [5]. From bench to bedside, a randomized clinical trial (RCT) involves 60 liver donors by 10 min inflow occlusion and 10 min reperfusion to improve the graft tolerance to IRI, and the results indicate that LIPC approach significantly improves liver biochemical markers of hepatocyte function in deceased donor liver transplantation (LT) [6]. However, another similar clinical study with smaller sample size has not shown any difference between LIPC and control group [7] nor has any beneficial result of LIPC been seen in liver resection surgery [8]. These controversial results may arise from invasive procedure to induce LIPC, in which direct vessel clamping may cause uncontrollable degree of graft injury and thus contribute to varied clinical results. Ischemia duration is another key point. An RCT investigating the safety and efficiency of 5 min LIPC displays that, different from 10 min occlusion, 5 min inflow occlusion does not protect liver grafts from subsequent IRI [9]. To further investigate the effects of time-dependent LIPC, an LIPC model with 5/8/10/15 min inflow occlusion and 10 min reperfusion is performed on rat fatty livers, followed by index ischemia. The result indicates that 5/8 min inflow occlusion is an optimal regimen for protection of fatty liver, with a dramatically lower serous malondialdehyde (MDA) concentration [10]. Moreover, ischemia tolerance differs from organ to organ and clinical studies focusing on other solid organs should also be explored to verify LIPC application, and patient-related factors such as age, gender, or comorbidities should also be taken into consideration.

2.2. Hydrogen Preconditioning. Hydrogen is a reducing gas that displays antioxidant properties and exhibits protective effects against graft IRI and dysfunctions. Though application of mechanical ventilation (MV) in intensive care unit supports donor's life, it can also provoke oxidative stress and inflammatory response, causing ventilator-induced lung injury (VILI) and reducing graft viability [11]. To prevent VILI occurrence, inhaled hydrogen, an ROS scavenger, has been applied in a recent study and effectively reduced VILI-associated inflammatory responses, at both local and systemic levels [12]. In lung transplantation, preloading hydrogen in lung tissue decreases toxic ROS during reperfusion [13]. Moreover, hydrogen inhalation at 2% concentration 1 h prior to liver procurement can also protect the liver from IRI by activation of NF-κB signaling pathway [14]. Therefore, hydrogen preconditioning might be a practical way for the treatment of transplant donors, especially for those subjected to MV supporting before graft procurement. There is no consensus on what the most optimal concentration of inhaled hydrogen should be yet. It is reported that concentration of inhaled hydrogen ought to be monitored and kept less than 4.6% when mixed with air and less than 4.1% when mixed with oxygen [15], though the safety of hydrogen inhalation is still needed to be testified clinically. Besides, hydrogen-rich water (HRW) has been developed recently and applied for various clinical purposes. To investigate HRW effects on patients with either type 2 diabetic mellitus or impaired glucose tolerance, an RCT has been performed and shows that hydrogen reduces the concentrations of oxidized low density lipoprotein and free fatty acids, while it increases plasma levels of extracellular-superoxide dismutase (SOD) in diabetics [16]. Another human study shows that HRW remarkably attenuates oxidative stress, improves liver function, and reduces HBV DNA in patients with chronic hepatitis B [17]. Nowadays, some groups are dedicated in developing practical techniques to stabilize hydrogen-rich solution for graft storage during transportation.

2.3. Antioxidant Carriers Therapy. High levels of ROS play a pivotal role in transplant-associated IRI, especially in cadaveric donors. Antioxidant enzymes (AOEs), such as catalase and SOD that detoxify H_2O_2 and superoxide, are highly potent and specific agents to the ROS-induced injury and not consumed in reaction with ROS. However, due to inability to cross cell membrane barriers and fast elimination, a significant hurdle in the clinical translation lies in the insufficient delivery of these enzymes to targeted sites, especially the vascular endothelium suffering oxidative injury [18]. It is also believed that megadoses of nonenzymatic antioxidants (e.g., N-acetylcysteine (NAC) and curcumin) can only alleviate subtle chronic oxidative stress, whereas their protective effects in acute conditions including IRI and inflammation are extremely limited [19]. To enhance the bioavailability and efficacy of AOEs and nonenzymatic antioxidants, various antioxidant carriers have been developed to protect antioxidant cargoes from inactivation and improve intracellular delivery.

Application of vascular immune targeting AOEs to specific endothelial epitopes has been proven to be an effective donor preconditioning method in lung transplantation model. The nanosized conjugates, consisting of AOEs and specific antibodies, can be directed against the endothelial determinant accumulation in vascular endothelium after intravenous administration and eventually delivered into endothelial cell, thus alleviating oxidative stress. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) is such an endothelial epitope and the specific nanosized particles (anti-PECAM/catalase conjugates) have been examined in a porcine lung transplantation model, in which the immune targeting treatment allows immediate reconstitution of pulmonary gas exchange and microcirculation, and improve both graft and recipient outcomes [20]. Moreover, anti-PECAM/SOD conjugates have also been conformed to specifically downgrade pulmonary endothelial ROS flux [21]. Likewise, angiotensin-converting enzyme (ACE) is another ideal endothelial epitope for immune targeting therapy. Conjugates of ACE monoclonal antibody 9B9 with catalase (9B9-catalase) have been proven to augment antioxidant defenses of pulmonary endothelium in a rat lung IRI model [22]. Despite strong antioxidant effects, the adverse effects induced by immune targeting conjugates, including disturbance of vasoreactivity and pulmonary arteriolar constriction, cannot be neglected in clinical translation process. It should also be considered that immune targeting therapy along with other novel approaches, such as supplementation of NO, targeting

of antithrombotic drugs, and donor preconditioning, may be a promising alternative.

Liposomes are artificial vesicles consisting of one or more phospholipid layers, with an aqueous core enclosed; lipid-soluble antioxidants can be incorporated into the lipid bilayer, while water-soluble antioxidants (e.g., NAC and curcumin) can be encapsulated in the aqueous space. NAC is a well-known antioxidant which can function both as a ROS scavenger and as a precursor of reduced glutathione, thus modulating the redox status. To improve the bioavailability, NAC is encapsulated in the aqueous space of liposome, gaining a higher protective potency than the free drug. Liposomal NAC has been administered separately in rodents to protect lung [23] and liver [24] injury and provided a protective effect through antioxidant pathway. Similarly, liposome can be incorporated with a potent inhibitor of the NF-κB pathway, curcumin, to target delivery to renal tubular epithelial and antigen-presenting cells. In a renal IR model, liposomal curcumin provided cytoprotection effect through multiple antioxidant mechanisms following renal IR injury [25].

Conclusively, immune targeting therapy and liposome formulation are promising approaches to facilitate the delivery of AOEs and nonenzymatic antioxidants before ischemia process. Though there is no associated animal transplantation study performed, we hold that the carriers can help the antioxidants administered to a donor quickly bind to endothelium and subsequently protect graft from IRI. However, these carriers must be carefully tested in terms of toxicity, activation of defense systems, inflammation or thrombosis, and aggregation in circulation and embolism of the microvasculature.

3. Antioxidant Treatment for Grafts

Ex vivo graft preservation is necessary for allocating and transporting the graft to its recipient. It has been widely accepted that primary occurrence of ROS-induced injury arises from the cellular alteration during the ischemia process, especially in cold preservation period. Several approaches including machine perfusion and modified preservation solutions have been developed to reduce the injury.

3.1. Machine Perfusion. Machine perfusion (MP) is increasingly used as an alternative method to overcome the present shortage of donors by expanding the graft pool and prolong the storage time. It is a dynamic technique using a continuous flow of solutions to perfuse and maintain residual metabolism of the graft [26]. In recent years, the interest in MP preservation has been revived, especially in hypothermic machine perfusion (HMP). HMP is able to maintain the viability of non-heart-beating donors (NHBD) grafts effectively, though the HMP-induced cold damage, such as ROS injury, should not be neglected. MP temperature is a key factor affecting antioxidant potential. The ROS production in steatotic liver submitted to MP at 20°C, known as subnormothermic machine perfusion (SNMP), is significantly lower than that of HMP at 8°C or 4°C [27]. Similarly, various clinical studies focusing on normothermic machine perfusion (NMP) have also been carried out. To examine the feasibility of transplanting high-risk donor lungs that has undergone NMP, a prospective clinical trial subjects lungs of high-risk donors to 4 hours of NMP and demonstrates that NMP group shows similar physiological stability to those in control group [28]. In addition, red cell-based perfusate and a short period of oxygen supply during MP are designed to reduce the likelihood of inflammation and oxidative injury. However, few studies have been performed to explore the oxidative stress mechanism in NMP and the pragmatic antioxidant potential of NMP needs to be examined. To further improve the viability of perfused grafts, perfusion mediums supplemented with various antioxidant agents can also alleviate ROS-induced injury effectively [29].

3.2. Polymer Solutions. Polyethylene glycol (PEG), synthesized as liner or branched polymers in different sizes, functions as an alternation of hydroxyethyl starch (HES) contained in UW solution due to its low viscosity. As an "immunocamouflage" agent, PEG binds covalently to various biological surfaces and forms complexes with cell membrane lipids, membrane proteins, or carbohydrates, preventing osmotic swelling as well as lipid peroxidation (LPO) in graft cold storage. PEG is also an effective free radical scavenger and can modulate oxidative stress during preservation. Owing to these protective effects, several PEG-based preservation solutions, including Polysol, IGL-1 solution, and SCOT, have been developed for organ preservation.

Polysol solution is a colloid-based low-viscosity organ preservation solution containing vitamins, amino acid, and a variety of ROS scavengers (including allopurinol, glutathione, alpha-tocopherol, and ascorbic acid), which possess strong antioxidant capacity. Polysol solution is applied on a steatotic rat liver perfusion model and significantly attenuates LPO to nearly one fourth of that in HTK control [30]. In rat partial liver transplantation, Polysol solution also brings a protective effect on overall quality of partial liver graft, evidenced by improved microcirculation, higher graft compliance, less hepatocyte damage, reduced apoptosis, and improved regeneration [31]. However, to assess the safety of Polysol solution for clinical application, a human study engages nine donor-recipient couples in adult living kidney transplantation and uses Polysol or UW solution for washout and cold storage of kidney grafts, respectively. The result demonstrates a high incidence of acute rejection and antibody-mediated rejection episodes in the recipients of Polysol solution group [32]. It is obvious that the complex composition of Polysol solution does not permit an accurate elucidation of the mechanisms in terms of both protective and detrimental effects.

Institute Georges Lopez-1 (IGL-1) solution is characterized by lower viscosity (1.250 mm^2/s), higher sodium, and lower potassium compared with UW solution. The application of IGL-1 solution has been reported in the SCS of pancreas [33], kidney [34], intestine [35], and liver [36]. IGL-1 solution can inhibit endothelial dysfunction and protect graft against oxidative stress through activation of eNOS by

both AMPK and AKT pathway. Livers preserved in IGL-1 solution are better protected from IRI than those in Celsior solution, with reduced liver injury, improved function, and less oxidative stress [37]. A recent human study randomly assigns deceased donor liver grafts to IGL-1 or UW solution for preservation and subsequent implantation. Until postoperative day 30, the incidence of hepatic artery thrombosis, PNF, and biliary nonanastomotic strictures are similar in both groups, while the costs of preservation solution for one liver procurement are 992.0 Euros for IGL-1 solution versus 1609.0 Euros for UW solution [36]. Obviously, IGL-1 solution exhibits comparable efficacy and safety to those of the reference preservation solutions, with a lower cost as well.

The Solution de Conservation des Organes et des Tissus (SCOT) has shown its protective potential in a pancreatic islet transplantation model, reducing IRI and ameliorating the long-term outcome of recipients' immune response [38]. To examine the safety of SCOT, a clinical trial performs 29 kidney transplantations (25 cadaveric donors and 4 living related donors) and applies SCOT for in situ washout and SCS. In the first 3 months after surgery, kidney function of SCOT group is comparable to that of controlled UW solution group, which verifies preliminary safety and efficacy of SCOT [39]. However, the long-term outcome of recipients should be monitored further. In addition, to facilitate clinical application, the concentration and chain length of PEG in SCOT is examined on an islet transplantation model. The SCOT containing PEG 20 kDa 15 g/L significantly prolongs allograft survival and induces no PNF and DGF, and thus it may be the most optimal concentration and chain length of PEG for islet graft preservation [38].

3.3. Gaseous Supplements. Hydrogen-rich preservation solution has been proven to have high antioxidant potential and tested in liver, kidney, pancreas, bone marrow, lung, and intestinal cold storage [40]. The antioxidant property of hydrogen-rich preservation solution might arise from inhibition of high mobility group box 1 (HMGB1) release and ROS scavenging effect [41]. A novel hydrogen-rich UW solution (HRUW) has been tested for cold preservation and subsequent renal transplantation in rats, showing that HRUW solution can improve renal function and prolong rat survival rate by protecting tubular epithelial cells from inflammation and apoptosis [42]. In rat intestinal transplantation, HRUW can reduce graft damage and protect the recipient from the systemic effects of transplantation via alleviating graft oxidative stress, ultimately facilitating recipient survival [43]. Moreover, the combination of hydrogen inhalation for donor and the hydrogen-rich preservation solution for graft is a prospective way to prolong the graft preservation time and the survival of recipient, which requires further basic and clinical studies.

NO is a kind of free radical diatomic gas and gaseous signaling molecule. The protective potential of NO is associated with the reduction of superoxide anion-induced tissue toxicity and the inflammatory response. Furthermore, NO can modulate mitochondrial energy generation and thus decrease ROS formation during I/R period. Kageyama et al. [44] investigated the effect of venous systemic oxygen persufflation (VSOP) supplemented with NO gas during cold storage of liver grafts and demonstrated that NO combined with VSOP could recondition warm ischemia-damaged grafts, presumably by decreasing ET-1 upregulation and oxidative damage. Similarly, ventilation of NHBD lung grafts with NO during warm ischemia, ex vivo perfusion, and posttransplantation can also reduce IRI and ameliorate lung injury [45]. Although NO has been used as a clinical therapy for pediatric acute respiratory distress syndrome at present, the application of NO in clinical organ preservation is still under investigation, possibly owing to the lack of the safety studies.

Carbon monoxide (CO) is also a gaseous signaling molecule and possesses a high affinity for heme prosthetic group. CO supplemented to preservation solution has been proven to improve the graft function in experimental studies [46, 47]. A preclinical study performs ex vivo delivery of CO to rat kidney graft and suggests that CO remarkably reduces oxidative injury and improves recipients' survival compared to the control group. The combination of CO and cytochrome P450 (CYPs) may interpret the potential mechanism, which reduces ROS production via blocking CYPs degradation and harmful heme/iron release [48].

Hydrogen sulfide (H_2S) is considered as the third gaseous signaling molecule with properties to help relax vascular smooth muscle, inhibit apoptosis, modulate inflammatory response, and alleviate oxidative stress [49]. NaHS (a source H_2S) has been proven to possess better antioxidant potential in vitro when compared to that of L-arginine (a source of endogenous NO). The effective doses of both compounds in human body are believed to be 56 μM for H_2S and 1.2 g/mL for L-arginine, respectively, which provides essential information for subsequent human study [50].

3.4. Pharmacologic Approaches. One of the major sources of ROS is the mitochondria that are particularly vulnerable to oxidative injury. Mitochondrial damage may impair the electron flow and promote the formation of superoxide. Mitochondrial permeability transition (MPT) plays an essential role in cell death during IRI, induced by ROS and reversely aggravating the oxidative stress [51]. To protect mitochondrial integrity during ischemia, various antioxidant agents have been developed and applied to preclinical studies. Melatonin, an essential ROS scavenger, is an inducible nitric oxide synthase (iNOS) inhibitor and well-known antioxidant substance secreted from pineal gland, which can reduce mitochondrial swelling before I/R occurrence. Treated by melatonin supplemented IGL-1 solution, the liver grafts with/without steatosis are protected from IRI, possibly owing to increased NO generation (via constitutive e-NOS activation) and reduction of oxidative stress [52].

Ascorbic acid (AA) is a potent physiological extracellular scavenger of ROS. AA has been supplemented into HTK and Polysol solution to prevent ROS. Noticeably, high-concentrated AA has been reported to aggravate hepatic IRI owing to its excess reduction of iron [53]. Alpha-tocopherol (Vit E), an exogenous antioxidant, prevents the process of LPO in both cell membranes and plasma lipoproteins. Trolox is a water-soluble analogue of Vit E and provides

similar antioxidant property to that of Vit E. In a porcine heart transplantation model, trolox-UW perfusion has shown remarkable antioxidant effect against IRI [54]. Matrix metalloproteinases (MMPs) are associated with oxidative stress in cardiovascular diseases. Doxycycline (DOX), an antibiotic of tetracycline family, has been found to inhibit MMP-2 expression and thus protects cardiac function from IRI [55]. The cardioplegia solution containing DOX shows protective effects in heart preservation, despite its potent role in modulating cellular redox status during SCS [29].

4. Antioxidant Treatment on Recipients

4.1. Ischemic Postconditioning. Remote ischemic postconditioning (RIPoC) is induced by several cycles of I/R on a remote tissue (arm or leg) to produce systemic protection against IRI in distant organs, without direct access to the vessels of the organ of interest. RIPoC can increase antioxidant capacity of liver and kidney temporarily by reducing NF-κBp65 and MDA expression, as well as ameliorating the destructive capacity of oxygen free radicals. From bench to bedside, induced by three cycles of brief 5 min repetitive ischemia and reperfusion via clamping the exposed external iliac artery, Wu et al. [56] demonstrated that RIPoC could enhance the early renal function recovery in renal recipients after transplantation. However, Kim et al. [57, 58] performed two RCTs including living and kidney transplantation separately. The results display that RIPoC did not improve graft function after operation, possibly due to different RIPoC protocols with an arterial tourniquet cuff placed around the patients' thigh or direct occlusion of the macrovessel, whereas RIPoC appeared to be feasible and safe [59]. Although the potential role of RIPoC in transplantation remains to be determined, this approach provides a simple intervention to protect all the grafts against IRI, particularly via the antioxidant pathway. Noticeably, this approach requires predictable timing of organ donation and may not be applied for DCD donors. In addition, a meta-analysis has pointed out that RIPoC stimulus should be delivered 24 h before the index ischemia [60]. Nevertheless, there is no consensus on how many ischemic stimuli should be performed and what the duration of the transient I/R periods should be, which leads to varying results in basic and clinical studies. Formulating a well-accepted protocol for specific organ, as well as clarifying the potential mechanism, is needed in the translating process.

Similar to LIPC, local ischemic postconditioning (LIPoC) is defined as rapid and intermittent interruptions of blood flow in the early phase of reperfusion after a prolonged period of ischemia. In a canine autotransplantation model, after flushing and static preservation of the kidney for 24 hours, LIPoC was performed with six cycles of 10 or 30 seconds or three cycles of 1-minute I/R before final reperfusion. The result indicates enhanced level of expression of SOD and decreased levels of MDA, implying that LIPoC may protect the graft via an antioxidant pathway [61], whereas another clinical study displays that LIPoC is feasible and appeared safe in human DCD renal transplantation, though without any better renal function observed, which requires further investigation [62].

5. Conclusion

Oxidative stress is a common cause of PNF, DGF, and allograft rejection, especially in marginal donors. This review summarizes the innovative antioxidant treatments for the donor, graft preservation, or recipient designed to improve the graft viability and long-term outcome (Tables 1(a), 1(b), and 1(c)). However, the lack of intensive studies concerning the mechanism of oxidative stress hampers the development of these approaches. Ischemia conditioning (LIPC, RIPoC, and LIPoC) is believed to be a series of prospective approaches regardless of its controversial benefit and complex procedure. Considering the safety and effectiveness, the consensus of the protocol should be made on the basis of experimental and clinical trials.

Although SCS has been effective for decades on optimal organs preservation, the preservation protocol is not adapted to the increasing marginal grafts which is able to extend the graft pool. Thus, the novel antioxidant preservation solution, as well as various supplements, requires more mechanism researches and pragmatic RCT. Combined utilization of antioxidant approaches may be more promising than attempts to reinforce the antioxidant capacity of organs by a single agent functioning as ROS scavenger. Even though the antioxidant potential of NMP is still unclear, this approach is worth more intensive researches. To this end, though a combination of antioxidant treatments seem to provide the best outcome, accurate models for preclinical studies and unified protocols for clinical trials are needed before the treatments can be translated into clinical practical. Moreover, the potential adverse effect of local and systemic antioxidant interventions to donors, grafts, and recipients, such as host defense hazard and prooxidant effects, should not be neglected. Antioxidant administration should also be controlled at a gradual, controlled rate, thus avoiding burst release and comprising effect.

Abbreviations

AA: Ascorbic acid
ACE: Angiotensin-converting enzyme
AOE: Antioxidant enzymes
CABG: Coronary artery bypass grafting
CO: Carbon monoxide
CYPs: Cytochrome P450
DGF: Delayed graft function
DOX: Doxycycline
HES: Hydroxyethyl starch
HMP: Hypothermic machine perfusion
HO-1: Hemeoxygenase-1
HRUW: Hydrogen-rich UW solution
HRW: Hydrogen-rich water
H_2S: Hydrogen sulfide
IGL-1: Institute Georges Lopez-1
iNOS: Inducible nitric oxide synthase
I/R: Ischemia and reperfusion
IRI: Ischemia/reperfusion injury
LIPC: Local ischemic preconditioning
LIPoC: Local ischemic postconditioning

Table 1: (a) Characteristics of reviewed studies concerning antioxidant treatment for donor. (b) Characteristics of reviewed studies concerning antioxidant treatment for graft. (c) Characteristics of reviewed studies concerning antioxidant treatment for recipient.

(a)

Treatment	Subject	Organ	Model or disease	Effects
Local ischemic preconditioning	Rat [5]	Liver	I/R	NF-κB ↓, MDA ↓, MPO ↓, AST ↓, ALT ↓, Proinflammatory cytokines ↓
	Human [6]	Liver	LiT	Apoptosis ↓, PNF ↓, AST ↑, HIF-1α ↓
	Human [7]	Liver	LiT	No beneficial effect
	Human [8]	Liver	LiR	No beneficial effect
	Human [9]	Liver	LiT	10 min occlusion is optimal
	Rat [10]	Liver	I/R	5/8 min occlusion is optimal
Hydrogen preconditioning	Mice [12]	Lung	MV	W/D ratio ↓, MDA ↓, Egr-1 ↓, TNF-α ↓, IL-1β ↓, CCL2 ↓, apoptosis ↓
	Rat [13]	Lung	LuT	PO_2 ↑, PCO_2 ↓, ICAM-1 ↓, IL-1β ↓, IL-6 ↓, MDA ↓
	Rat [14]	Liver	I/R	NF-κB ↑, HO-1 ↑, Bcl-2 ↑
	Human [16]	Diabetic	T2DM	LDL ↓, SOD ↑
	Human [17]	Liver	HBV	HBV DNA ↓, ALT ↓, TBiL ↓, SOD ↑, GST ↑
Antioxidant carriers therapy				
Immune targeting therapy	Pig [20]	Lung	LuT	Gas exchange ↑, W/D ratio ↓, Edema ↓, MDA ↓
	Human [21]	Cell	HUVECs	VCAM ↑, TNF ↓, IL-1β ↓, LP S ↓
Liposome	Rat [22]	Lung	I/R	PO_2 ↑, endothelin-1 ↓, iNOS ↓
	Rat [23]	Liver	LPS-LiI	NPSH ↓, MDA ↓, 4-HNE ↓, ALT ↓, AST ↓, TNF-α ↓
	Rat [24]	Lung	LPS-LuI	NPSH ↓, MDA ↓, 4-HNE ↓, MPO ↓, TNF-α ↓

(b)

Treatment	Subject	Organ	Model or disease	Effects
Machine perfusion	Rat [27]	Liver	HMP	MP at 20°C is optimal, AST ↓, LDH ↓, ATP/ADP ↑, bile production ↑, TNF-α ↓
	Human [28]	Lung	LuT	Subtle beneficial effect
	Rat [29]	Heart	HMP	Apoptosis ↓, MMP-2 ↓, H_2O_2 ↓, pAkt/Akt ↑
Polymer solutions				
Polysol solution	Rat [30]	Liver	SCS	AST ↓, GLDH ↓, PVP ↓, ATP ↑, O_2 consumption ↑, bile production ↑, MDA ↓, W/D ratio ↓
	Rat [31]	Liver	PLiT	PVF ↑, ALT ↓, LDH ↓, MDA ↓, VEGF ↑
	Human [32]	Kidney	KT	Acute rejection rate ↑
IGL-1 solution	Pig [33]	Pancreas	PT	Same degree of safety and effectiveness with UW solution
	Human [34]	Kidney	KT	DGF ↓, Cr ↓, apoptosis ↓, Ccr ↑
	Pig [35]	Intestine	IAT	Acute cellular rejection ↓, iNOS ↑, necrosis ↓, apoptosis ↑,
	Human [36]	Liver	LiT	Same degree of safety and effectiveness with UW solution
SCOT solution	Mice [38]	Pancreas	PT	PNF + DGF + allograft survival time ↑
	Human [39]	Kidney	KT	Same degree of safety and effectiveness with UW solution
Gaseous supplements				
Hydrogen	Rat [41]	Liver	I/R	ALT ↓, HMGB1 ↓, MDA ↓, TNF-α ↓, IL-6 ↓
	Rat [42]	Kidney	KT	Recipient survival rate ↑, Cr ↓, Ccr ↑, MDA ↓, 8-OHdG ↓
	Rat [43]	Intestine	IAT	MDA ↓, LDH ↓, EGR-1 ↓, IL-6 ↓, iNOS ↓, IL-1β ↓
Nitric oxide	Rat [44]	Liver	LiT	ALT ↓, HA ↓, MDA ↓, eNOS ↑, iNOS ↓, ET-1 ↓, 8-OHdG ↓
	Rat [45]	Lung	LuT	W/D ratio ↓, vascular resistance ↓, cGMP ↑, iNOS ↓, TNF-α ↓
Carbon monoxide	Rat [46]	Kidney	KT	Recipient survival ↑, IL-6 ↓, TNF-α ↓, iNOS ↓, PARP ↑
	Rat [48]	Kidney	KT	ALAS-1 ↑, MDA ↓, IL-6 ↓, TNF-α ↓, Egr-1 ↓, Cox-2 ↓, Ccr ↑
Pharmacologic approaches				
Melatonin	Rat [52]	Liver	SCS	AST ↓, ALT ↓, BSP clearance ↑, vascular resistance ↓, eNOS ↑, TNF-α ↓, MDA ↓, HO-1 ↑
Trolox	Pig [54]	Heart	HT	ET-1 ↓, MDA ↓, SOD ↑, TA ↓, LDH ↓, CK ↓, calcium ↓
Doxycycline	Rat [29]	Heart	HMP	Apoptosis ↓, MMP-2 ↓, H_2O_2 ↓, pAkt/Akt ↑

(c)

Treatment	Subject	Organ	Model or disease	Effects
Ischemic postconditioning				
RIPoC	Human [56]	Kidney	KT	Cr ↓, pathology (—), GFR ↑, uNGAL ↓
	Human [57]	Liver	LiT	No beneficial effect
	Human [58]	Kidney	KT	No beneficial effect
LIPoC	Canine [61]	Kidney	KT	MDA ↓, MPO ↓, SOD ↑, apoptosis indices ↓, Cr ↓, BUN ↓, Ccr ↑
	Human [62]	Kidney	KT	Safe but no beneficial effect

ACR: acute cellular rejection; ALAS-1: 5-aminolevulinate synthase; ALT: alanine aminotransferase; AST: aminotransferase; ATP: adenosine triphosphate; BSP: bromosulfophthalein; BUN: blood urea nitrogen; Ccr: creatinine clearance; cGMP: cyclic guanosine monophosphate; Cox-2: cyclooxygenase-2; Cr: creatinine; DGF: delayed graft function; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; GFR: glomerular filtration rate; GLDH: glutamate dehydrogenase; GST: glutathione S transferase; HA: hyaluronic acid; HBV: hepatitis B virus; HMP: hypothermic machine perfusion; HSP: heat shock protein; HT: heart transplant; HUVECs: human umbilical endothelial cells; IAT: intestinal allotransplantation; iNOS: inducible nitric oxide synthase; I/R: ischemia and reperfusion; KT: kidney transplantation; LDH: lactate dehydrogenase; LDL: low density lipoprotein; LIPoC: local ischemic postconditioning; LiR: liver resection; LiT: liver transplantation; LPS: lipopolysaccharide; LuT: lung transplantation; MDA: malondialdehyde; MPO: myeloperoxidase; MV: mechanical ventilation; NOS: nitric oxide synthase; NPSH: nonprotein thiols; pAkt: phosphorylated Akt; PARP: poly(ADP-ribose) polymerase; PLiT: partial liver transplantation; PNF: primary nonfunction; PT: pancreas transplantation; PVF: portal venous flow; PVP: portal venous pressure; RIPoC: remote ischemic postconditioning; TA: total antioxidants; TBiL: total bilirubin; TNF: tumor necrosis factor; TLR-4: toll-like receptor-4; T2DM: type 2 diabetes mellitus; SCS: static cold storage; SOD: superoxide dismutase; uNGAL: urine neutrophil gelatinase-associated lipocalin; VEGF: vascular endothelial growth factor; W/D: wet-to-dry; 4-HNE: 4-hydroxyalkenals; 8-OHdG: 8-hydroxy-2-deoxyguanosine.

LPO: Lipid peroxidation
LT: Liver transplantation
MDA: Malondialdehyde
MMPs: Matrix metalloproteinases
MP: Machine perfusion
MPT: Mitochondrial permeability transition
MV: Mechanical ventilation
NHBD: Non-heart-beating donors
NMP: Normothermic machine perfusion
NO: Nitric oxide
PEG: Polyethylene glycol
PNF: Primary nonfunction
PECAM-1: Platelet/endothelial cell adhesion molecule-1
RCT: Randomized clinical trial
RIPoC: Remote ischemic postconditioning
SCOT: Solution de Conservation des Organes et des Tissus
SCS: Static cold storage
SNMP: Subnormothermic machine perfusion
SOD: Superoxide dismutase
VILI: Ventilator-induced lung injury
VSOP: Venous systemic oxygen persufflation.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Cigarette Smoke Extract-Induced Oxidative Stress and Fibrosis-Related Genes Expression in Orbital Fibroblasts from Patients with Graves' Ophthalmopathy

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Cigarette smoking is the most important risk factor for the development or deterioration of Graves' ophthalmopathy. Smoke-induced increased generation of reactive oxygen species may be involved. However, it remains to be clarified how orbital fibroblasts are affected by cigarette smoking. Our study demonstrated that Graves' orbital fibroblasts have exaggerated response to cigarette smoke extract challenge along with increased oxidative stress, fibrosis-related genes expression, especially connective tissue growth factor, and intracellular levels of transforming growth factor-β1 and interleukin-1β. The findings obtained in this study provide some clues for the impact of cigarette smoking on Graves' ophthalmopathy and offer a theoretical basis for the potential and rational use of antioxidants in treating Graves' ophthalmopathy.

1. Introduction

Graves' ophthalmopathy (GO), also called Graves' orbitopathy, thyroid-associated orbitopathy, or thyroid eye disease, is a cosmetically disfiguring and potentially vision-threatening disease. Although the pathophysiology of GO is still not fully clarified, it is known as a complex interplay process between multiple endogenous and environmental factors [1–4]. Cigarette smoking is the most important environmental and risk factor for the development or deterioration of GO, and the risk increases in parallel with the current number of cigarettes smoked per day [5–7]. Furthermore, cigarette smoking is associated with poor response to treatment for GO [8–10], and quitting smoking currently is the only method of GO prevention [11, 12]. In the study by Planck et al., some adipocyte-related immediate early genes, interleukin- (IL-) 1β, and IL-6 were overexpressed in smokers with severe active GO compared to nonsmokers, indicating that smoking activates pathways associated with adipogenesis and inflammation [13]. However, the exact mechanisms underlying the deleterious effect of smoking in GO remain to be identified. It has been proposed that smoke may induce the generation of reactive oxygen species (ROS) in the orbital socket, either through direct contact with the surrounding sinuses or indirectly through the blood circulation [14]. Cigarette smoke extract (CSE) has been reported to stimulate adipocyte differentiation in cultured orbital fibroblasts by synergizing with either IL-1 or ROS [12, 14]. In our previous study, we demonstrated that ROS could induce the protein expression of connective tissue growth factor (CTGF), an important fibrogenic factor, in cultured GO orbital fibroblasts [15]. The aim of the present study is to investigate the change of oxidative stress, fibrotic-related genes expression, and *intracellular cytokines* in the primary cultures of orbital fibroblasts in response to CSE. We also assessed whether or not CSE-induced oxidative stress, fibrotic-related genes

expression, and *intracellular cytokines* in the GO orbital fibroblasts could be reduced by pretreatment of the cells with antioxidants.

2. Materials and Methods

2.1. Patients and Tissues Acquisition. The surgical specimens of 5 patients with GO (GO1–GO5) during orbital decompression surgery (one man and four women; mean age: 37 years) and the specimens of 5 age- and sex-matched patients (N1–N5) (one man and four women; mean age: 36 years) who received oculoplastic surgery for noninflammatory conditions were used in this study. All specimens were collected in accordance with the Declaration of Helsinki and with informed consent of the patients. All GO patients achieved stable euthyroidism with antithyroid medications for at least 6 months before surgery and are maintained in the inactive stage of GO. In addition, all study subjects had not received specific treatment (systemic steroids or radiotherapy) for GO. Exclusion criteria include ocular diseases other than GO, alcohol drinking, regular ingestion of antioxidants, and pregnancy. In addition, the patients suffering from chronic or acute diseases, such as diabetes mellitus, hyperlipidemia, diseases of the lung, liver, or kidney, cancer, other endocrine dysfunctions, and immunological or inflammatory disorders, were also excluded.

2.2. Primary Cultures of Orbital Fibroblasts. The primary cultures of orbital fibroblasts were established according to our previous study [16]. Briefly, the orbital tissues were minced aseptically in phosphate-buffered saline (PBS, pH 7.3) and then incubated with a sterile solution containing 0.5% collagenase and dispase (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 24 hours in an incubator filled with an atmosphere of 5% CO_2 and kept at 37°C. The mixture of digested orbital tissues was pelleted by centrifugation at 1,000 ×g and then resuspended in Dulbecco's Modified Eagle's Medium (DMEM, purchased from Gibco Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) and a cocktail of antibiotics (Biological Industries, Kibbutz Beit Haemek, Israel), which was composed of 100 U/mL penicillin G and 100 μg/mL streptomycin sulfate (Biological Industries, Kibbutz Beit Haemek, Israel). The cultured orbital fibroblasts were used between the 3rd and 5th passages and the cell cultures at the same passage number were used for the same set of experiments.

2.3. Preparation of Cigarette Smoke Extract (CSE). CSE was prepared according to prior studies with minor modification [12, 14]. The commercial cigarettes (The Longlife tobacco package purchased from Taipei, Taiwan) were used in this study, and each cigarette contained 10 mg tar and 0.8 mg nicotine. Ten pieces of cigarettes were smoked continuously by a pump-smoke machine, and this smoke was used to generate 200 mL of prewarmed CSE-PBS solution. Each cigarette was smoked for 3 min, and the pH of CSE-PBS solution was adjusted to 7.4 and then passed through a 0.22-μm pore size filter (Millipore Corporation, Billerica, MA, USA) to remove large particulates and bacteria. The CSE-PBS solution is defined as 100% CSE, and this CSE will be diluted with DMEM in the following experiments. CSE preparation is standardized by measuring the absorbance at a wavelength of 320 nm (optical density = 2.0–2.2), and the pattern of absorbance observed at a wavelength of 320 nm shows insignificant variation between different preparations of CSE. CSE concentrations in the current study are ranged from 0 to 15%.

2.4. Treatment of Orbital Fibroblasts with CSE and Antioxidants. After washing with PBS buffer (pH 7.4) twice, the orbital fibroblasts were treated with various concentrations of CSE ranging from 1% to 15% for 24 hours. To investigate whether the effect of CSE could be blocked by antioxidants, we pretreated the orbital fibroblasts with 1 mM N-acetylcysteine (NAC) or 2 mM vitamin C (VitC) for 1 hour, followed by the induction of 5% CSE treatment for another 24 hours, respectively.

2.5. Determination of Cell Viability. Cell viability was measured by the Trypan blue exclusion assay and was counted by using a hemocytometer. The number of viable cells was determined on the basis of their exclusion of 0.4% Trypan blue (Sigma-Aldrich, St. Louis, MO, USA). The relative cell viability was normalized by the value of cells without CSE treatment and was expressed as mean ± SD of the results from three independent experiments.

2.6. Measurement of ROS Content. The probes from 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) will be used to evaluate the intracellular H_2O_2 contents [17]. After incubation of orbital fibroblasts with 20 μM DCFH-DA at 37°C for 20 min, cells were trypsinized and then resuspended in 0.5 mL of PBS buffer (pH 7.4) and analyzed with a flow cytometer (Model EPICS XL-MCL, Beckman-Coulter, Miami, FL, USA). The excitation wavelength is set at 488 nm and the intensity of emitted fluorescence of a total of 10,000 cells at 525 nm is recorded on channel FL1 for the DCFH-DA probe. Data were analyzed by the EXPO32™ software (Beckman-Coulter, Miami, FL, USA). The intracellular H_2O_2 contents in the treated cells were presented as relative values compared with that of the cells without H_2O_2 or CSE treatment.

2.7. Determination of Lipid Peroxidation. The lipid peroxidation product, malondialdehyde (MDA), in cultured orbital fibroblasts was measured by the spectrophotometric assay kit (MDA-586; OxisResearch Inc., Portland, OR, USA) according to the manufacturer's instructions [18]. The MDA is quantified in the reaction with a chromogenic regent N-methyl-2-phenylindole to form an intensely colored carbocyanine dye with a maximum absorbance at 586 nm. The method is specific for MDA instead of other lipid peroxidation products such as 4-hydroxyalkenal because they cannot produce significant absorbance at 586 nm under the experimental conditions. An MDA standard curve was established by using the MDA samples at the concentration range of 0–50 μM, and the MDA levels in orbital fibroblasts were normalized to cell

numbers (10^6 cells). The results were expressed as mean ± SD of the results from three independent experiments.

2.8. Western Blot Analysis. An aliquot of 50 μg proteins was separated on 10% SDS-PAGE and blotted onto a piece of the PVDF membrane (Amersham-Pharmacia Biotech Inc., Buckinghamshire, UK). After blocking by 5% skim milk in the TBST buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4) at room temperature for 1 hour, the membrane was incubated for another 1 hour with the primary antibody at room temperature. After washing three times with the TBST buffer, the membrane was incubated with a horseradish peroxidase- (HRP-) conjugated secondary antibody for another 1 hour at room temperature. An enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech Inc., Buckinghamshire, UK) was used to detect the protein signals with a Fuji X-ray film (Fuji Film Corp., Tokyo, Japan), and the intensities of signals were quantified by ImageScanner III with LabScan 6.0 software (GE Healthcare BioSciences Corp., Piscataway, NJ, USA). The antibodies of HO-1 (SC-10789) and CTGF (SC-14939) were purchased from Santa Cruse Biotechnology Inc. (CA, USA), and β-actin (#A1978) was purchased from Sigma-Aldrich Chemical Co. (MO, USA), respectively. All data were expressed as mean ± SD of the results obtained from three independent experiments.

2.9. Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The expression levels of fibrosis-related genes were determined by SYBR green-based real-time quantitative PCR. Briefly, the total cellular RNA from orbital fibroblasts lysates was extracted with a chloroform solution after adding the TRIZol reagent (MO, USA). The extracted RNA was precipitated with isopropanol solution, dried on ice, and dissolved in DEPC-H_2O. An aliquot of 5 μg RNA was reverse-transcribed to cDNA with the Ready-to-Go RT-PCR kit (Amersham Biosciences, Uppsala, Sweden) at 42°C overnight. Quantitative RT-PCR was performed using the SYBR Green Master kit (Sigma-Aldrich) according to the manufacturer's instructions [18]. The primer pairs were 5′-CTCAACACGGGAAACCTCAC-3′ and 5′-CGCTCCACC-AACTAAGAACG-3′ for 18S rRNA, 5′-CTGCAGGCTAGA-GAAGCAGAG-3′ and 5′-GATGCACTTTTTGCCCTTCT-3′ for CTGF, 5′-CTGGCCGAAAATACATTGTAA-3′ and 5′-CCACAGTCGGGTCAGGAG-3′ for fibronectin, and 5′-GGACATCCACTTCCACAGC-3′ and 5′-GGTCATCGT-CGCCTTCTC-3′ for apolipoprotein J, respectively. The mRNA expression level of each gene in the orbital fibroblasts was normalized with the mRNA level of the 18S rRNA gene, respectively.

2.10. Measurement of the Intracellular Cytokine Content. The human transforming growth factor-beta 1 (TGF-β1; catalog #DB100B) and interleukin-1β (IL-1β; catalog #DLB50) levels in cell culture supernatant were quantified with enzyme-linked immunosorbent assay kits purchased from R&D Systems, Inc. (Minneapolis, MN). Briefly, about 10^5 orbital fibroblasts were seeded in a 3.5-cm culture dish and incubated for 48 hours at 37°C in a cell incubator with an atmosphere of 5% CO_2 followed by treatment of 5% CSE for another 24 hours, or the cells were pretreated with 1 mM NAC or 2 mM VitC for 1 hour followed by the induction of CSE treatment for another 24 hours, respectively. According to the manufacturer's recommendation and our previous study [19], cell culture supernatant was centrifuged at 12,000 ×g at 4°C, and the aliquots were immediately assayed. The standards for TGF-β1 and IL-1β were used in a range of 0–200 pg/mL, and the results were normalized by the cell number and expressed as pg/10^4 cells.

2.11. Statistical Analysis. The Microsoft Excel 2010 statistical package and SigmaPlot software version 12.3 (Systat Software Inc., San Jose, CA, USA) were used to analyze the results, and data were presented as means ± standard deviation (SD) of the results obtained from three independent experiments. The significance level of the difference between the control and the experimental groups was determined by Student's t-test. A difference was considered statistically significant when the $^{*}p$ value < 0.05 and $^{**}p$ value < 0.01, respectively.

3. Results

3.1. CSE-Induced Cytotoxicity and Oxidative Stress in the Orbital Fibroblasts. In order to investigate the cytotoxic effect of smoke extracts in the orbital fibroblasts, we treated the orbital fibroblasts with various concentrations of CSE for 24 hours. The effect of CSE ranging from 0 to 15% on the viability of the orbital fibroblasts from patients with GO (GO1–GO5) and normal subjects (N1–N5), as determined with the Trypan blue exclusion assay, is illustrated in Figure 1. The data show that both normal and GO fibroblasts were reduced in a dose-dependent manner, respectively (Figure 1(a)). The difference in cell viability between normal and GO orbital fibroblasts was statistically significant upon treatment with 5% CSE (Figure 1(b), 85% versus 62%, $p = 0.0374$). On the other hand, we also observed the CSE-induced oxidative stress and oxidative response in the GO orbital fibroblasts. After treatment of GO orbital fibroblasts with various concentrations of CSE (0–15%) for 24 hours, the intracellular ROS measured by DCF staining with a flow cytometer and the heme oxygenase-1 (HO-1) protein expression with Western blot were both increased in a dose-dependent manner (Figure 2).

3.2. Susceptible to 5% CSE-Induced Oxidative Stress and Oxidative Damage in the GO Orbital Fibroblasts as Compared to Those of Normal Controls and the Protective Role of Antioxidants. Due to the fact that the exposure of 5% CSE could significantly reduce the cell viability in the GO orbital fibroblasts as compared to those in the normal controls, we decided to treat normal and GO orbital fibroblasts with 5% CSE in the following experiments. After treatment of orbital fibroblasts with 5% CSE for 24 hours, the intracellular ROS measured by DCF staining was significantly increased in both normal and GO orbital fibroblasts, respectively (Figure 3(a), $p = 0.0347$ and $p = 0.0021$). In addition, the lipid peroxidation marker, malondialdehyde (MDA), was also significantly increased in both normal and GO orbital fibroblasts, respectively, after the addition of 5% CSE for

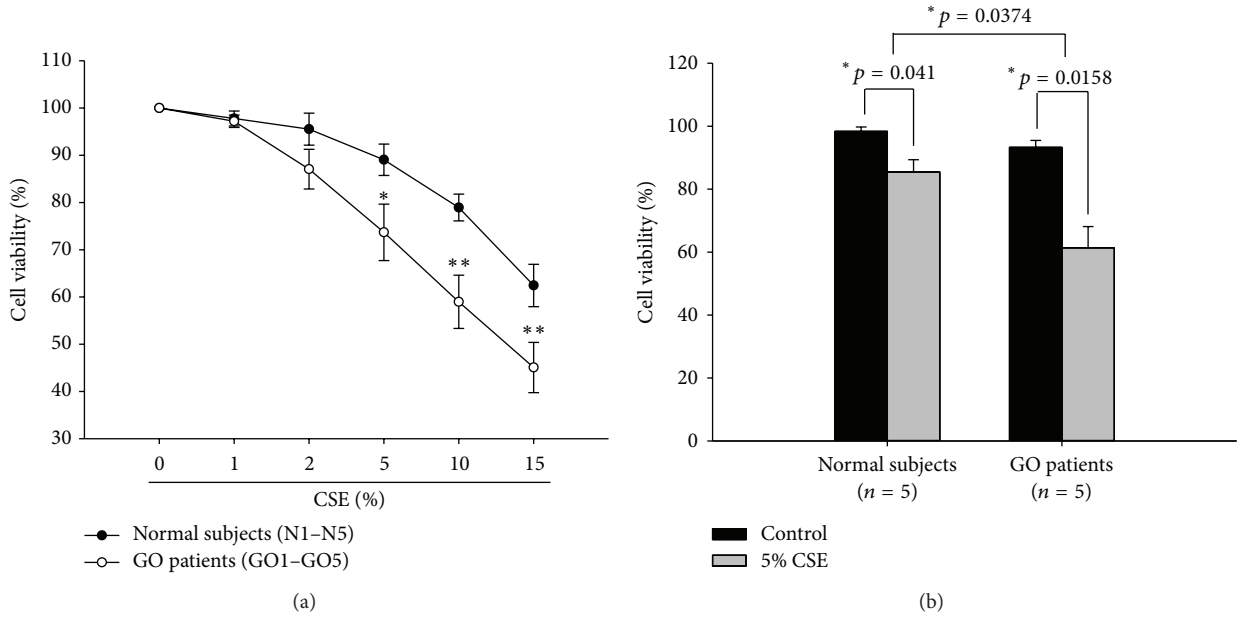


Figure 1: Susceptible to cigarette smoke extract- (CSE-) caused cytotoxicity in the GO orbital fibroblasts as compared to those of normal controls. (a) After treatment of orbital fibroblasts from GO patients (GO1–GO5) and age-matched normal subjects (N1–N5) with various concentrations of CSE ranging from 0 to 15% for 24 hours, the cell viability was determined by the Trypan blue exclusion assay. (b) The mean values of cell viability by 5% CSE treatment were shown in the histogram, and data were presented as means ± SD of the results from three independent experiments ($^{*}p < 0.05$; $^{**}p < 0.01$ versus the indicated group).

24 hours (Figure 3(b), $p = 0.0186$ and $p = 0.0032$). Moreover, we noted that the induction ratio of intracellular ROS and MDA levels after treatment with 5% CSE was more pronounced in the GO orbital fibroblasts than those in the normal controls, respectively (Figures 3(a) and 3(b), $p = 0.0273$ and $p = 0.0075$). On the other hand, we also observed the protective effects of NAC and VitC on CSE-induced oxidative stress and oxidative damage in the GO orbital fibroblasts, respectively. Preincubation with 1 mM NAC or 2 mM VitC for 1 hour, respectively, significantly decreased 5% CSE-induced elevations of intracellular ROS measured by DCF staining in the GO orbital fibroblasts (Figure 3(c), $p = 0.0451$ and $p = 0.0071$). A significant reduction in 5% CSE-induced elevations of MDA contents was also obtained after the GO orbital fibroblasts were preincubated with 1 mM NAC or 2 mM VitC, respectively (Figure 3(d), $p = 0.0382$ and $p = 0.0064$).

3.3. Susceptible to 5% CSE-Induced Changes of Fibrosis-Related Genes Expression in the GO Orbital Fibroblasts as Compared to Those of Normal Controls. Previously, we have shown that the elevated intracellular oxidative stress was associated with the increase of fibrosis-related genes expression in the GO orbital fibroblasts [15]. In this study, we further investigated whether 5% CSE-induced ROS could lead to inducing the fibrosis-related genes expression in the orbital fibroblasts. By a SYBR green-based RT-PCR, we observed the significant elevation in the levels of fibrosis-related genes expression including apolipoprotein J, fibronectin, and CTGF in both normal and GO orbital fibroblasts after treatment of 5% CSE for 24 hours (Table 1, $p = 0.0431$ versus $p = 0.0085$, $p = 0.0318$ versus $p = 0.0033$, and $p = 0.0441$ versus $p = 0064$, resp.). In addition, the induction ratio of apolipoprotein J, fibronectin, and CTGF by 5% CSE was more pronounced in the GO orbital fibroblasts than those in the normal controls (Table 1, $p = 0.0086$, $p = 0.0031$, and $p = 0.0054$, resp.).

3.4. Inhibition of 5% CSE-Induced Fibrosis-Related Genes Expression by Antioxidants in GO Orbital Fibroblasts. To investigate whether 5% CSE-induced fibrosis-related genes expression in GO orbital fibroblasts could be blocked by antioxidants, we pretreated the orbital fibroblasts with 1 mM NAC or 2 mM vitamin C, respectively, for 1 hour followed by the 5% CSE treatment. The results showed that preincubation of cells with 1 mM NAC or 2 mM VitC could significantly inhibit the 5% CSE-induced fibrosis-related genes expression including apolipoprotein J, fibronectin, and CTGF in the GO orbital fibroblasts by a SYBR green-based RT-PCR (Table 2). The inhibition ratio by 1 mM NAC treatment for apolipoprotein J, fibronectin, and CTGF is 14%, 17%, and 13%, respectively ($p = 0.0437$, $p = 0.0251$, and $p = 0.0470$, resp.). The inhibition ratio in the GO orbital fibroblasts by 2 mM vitamin C treatment for apolipoprotein J, fibronectin, and CTGF is 24%, 28%, and 27%, respectively ($p = 0.0294$, $p = 0.0085$, and $p = 0.0224$, resp.). Accordingly, we also examined CSE-induced expression levels of CTGF protein by Western blot. The result showed that the protein expression of CTGF was significantly increased in the GO orbital fibroblasts after the addition of 5% CSE for 24 hours (Figure 4, $p = 0.0041$). Besides, the pretreatment of GO fibroblasts with 2 mM VitC could also inhibit 43% of the elevations in the CTGF protein expression (Figure 4, $p = 0.0371$).

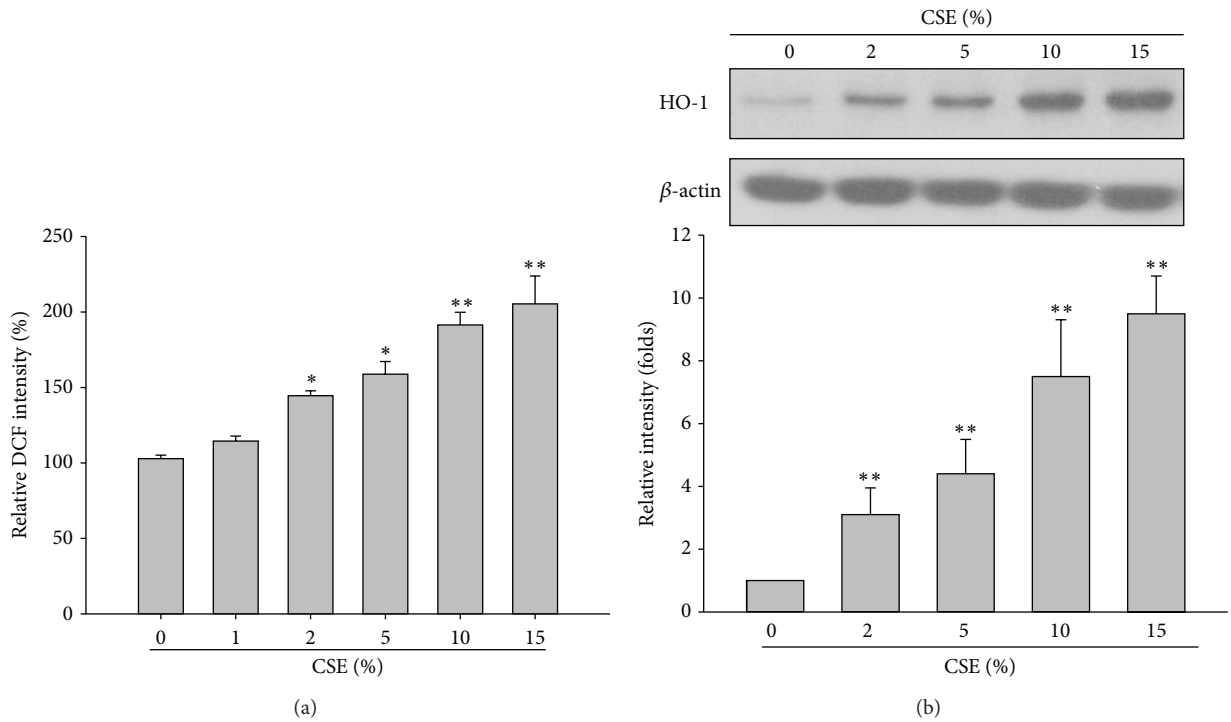


Figure 2: CSE-induced oxidative stress and oxidative response in a dose-dependent manner in the GO orbital fibroblasts. (a) After treatment of GO orbital fibroblasts with various concentrations of CSE ranging from 0 to 15% for 24 hours, the intracellular ROS was measured by DCF staining with a flow cytometer, and (b) the HO-1 protein expression was determined by Western blot. The representative histogram was constructed on the basis of the results from three independent experiments, and data were presented as means ± SD ($^{*}p < 0.05$; $^{**}p < 0.01$ versus the control group without CSE treatment).

Table 1: The induction ratio of fibrotic-related genes expression in the orbital fibroblasts from normal subjects and GO patients before and after 5% CSE treatment.

Fibrosis-related genes	Basal levels (mean ± SD)%[a]	5% CSE-treated (mean ± SD)%[a]	Induction ratio (%) (mean ± SD)%	p value
Apolipoprotein J				
Normal ($n = 5$)	108.36 ± 5.63	135.49 ± 9.74	125.83 ± 11.74	0.0431
GO ($n = 5$)	173.54 ± 6.67	301.67 ± 12.73	168.96 ± 15.47	0.0085
	$p = 0.0381$		$p = 0.0086$	
Fibronectin				
Normal ($n = 5$)	105.38 ± 5.83	147.37 ± 9.47	139.84 ± 14.37	0.0318
GO ($n = 5$)	263.45 ± 10.37	484.21 ± 15.87	180.47 ± 16.71	0.0033
	$p = 0.0046$		$p = 0.0031$	
CTGF				
Normal ($n = 5$)	117.38 ± 4.22	158.57 ± 8.42	135.09 ± 12.88	0.0441
GO ($n = 5$)	223.07 ± 14.17	379.22 ± 14.85	169.74 ± 11.53	0.0064
	$p = 0.0074$		$p = 0.0054$	

[a]The expression levels from 5 normal subjects and 5 GO patients were normalized to each individual 18S rRNA gene expression followed by adjusting to N1 whose expression was defined as 100%.

3.5. Susceptible to 5% CSE-Induced Changes of Intracellular Cytokines in the GO Orbital Fibroblasts as Compared to Those of Normal Controls and the Protective Role of Antioxidants. The changes in the intracellular cytokines after stimulation of orbital fibroblasts with 5% CSE are shown in Table 3. Basal levels of TGF-β1 and IL-1β were significantly higher in the GO orbital fibroblasts as compared to those of the normal controls ($p = 0.004$ and $p = 0.008$, resp.). In addition, CSE induced significant increase in TGF-β1 and IL-1β levels in GO orbital fibroblasts as compared to the respective controls ($p = 0.006$ and $p = 0.005$, resp.). Moreover, the induction ratio of TGF-β1 and IL-1β after stimulation with 5% CSE

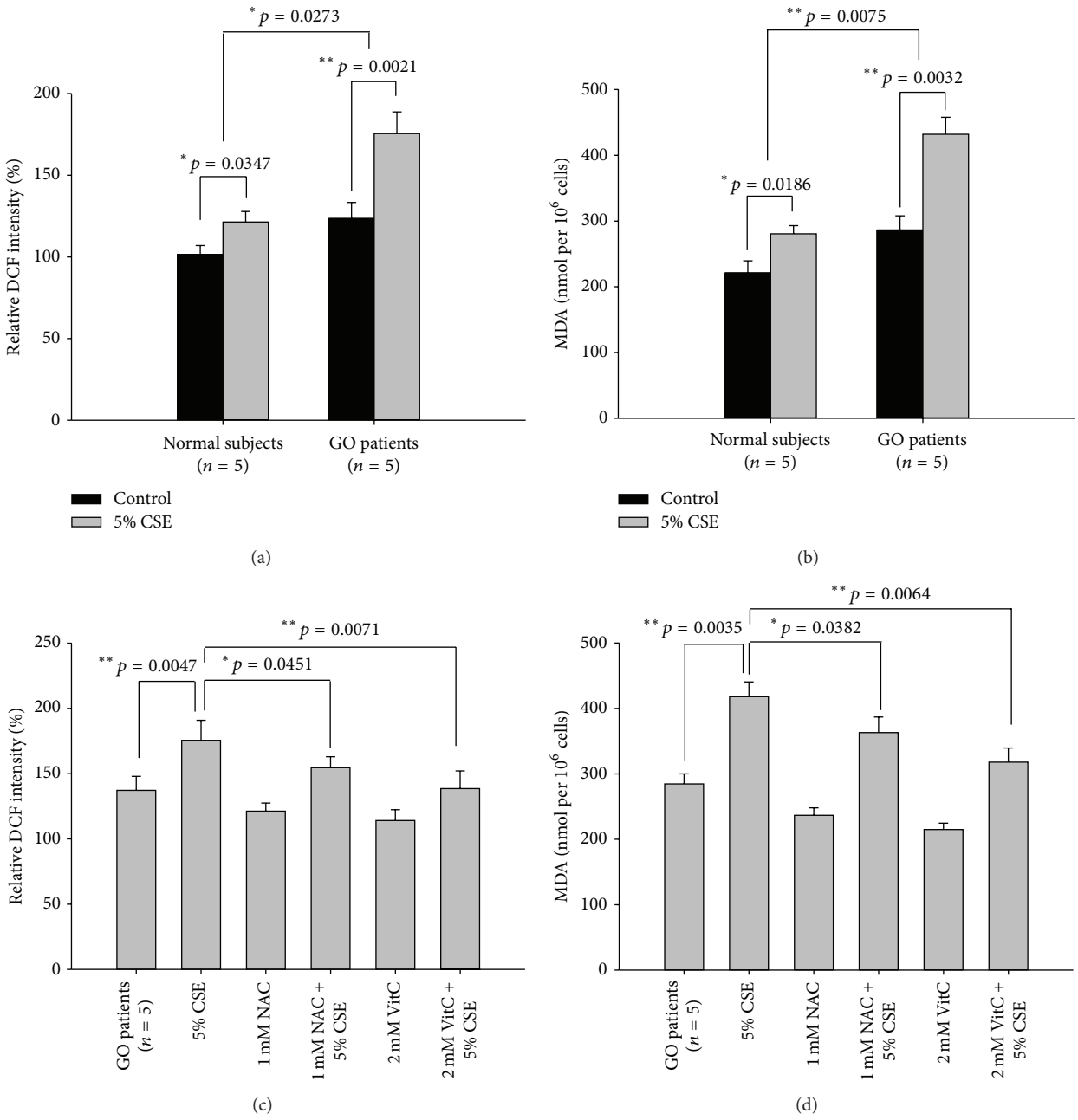


Figure 3: Susceptible to CSE-induced oxidative stress and oxidative damage in the GO orbital fibroblasts as compared to those of normal controls and the role of antioxidants. (a) After treatment of orbital fibroblasts from GO patients (GO1–GO5) and normal subjects (N1–N5) with 5% CSE for 24 hours, the intracellular ROS and (b) MDA levels were determined as described in Materials and Methods. (c) After pretreatment of GO orbital fibroblasts with 1 mM NAC or 2 mM vitamin C (VitC) for 1 hour followed by the addition of 5% CSE for another 24 hours, the intracellular ROS and (d) MDA levels were determined. The results were from three independent experiments, and data were presented as means ± SD ($^{*}p < 0.05$; $^{**}p < 0.01$ versus the indicated group).

was more pronounced in GO orbital fibroblasts than those in the normal controls ($p = 0.008$ and $p = 0.003$, resp.). Table 4 showed a significant reduction in 5% CSE-induced elevations of intracellular TGF-β1 and IL-1β after the GO orbital fibroblasts were pretreated with 1 mM NAC ($p = 0.037$ and $p = 0.028$, resp.) or 2 mM VitC ($p = 0.008$ and $p = 0.003$, resp.).

4. Discussion

Evidence is mounting that oxidative stress plays an important role in the development of GO [17, 20–22]. We demonstrated in this study that CSE elicited more pronounced response of oxidative stress in GO orbital fibroblasts. More importantly, this is the first study to reveal that CSE could induce

Table 2: The inhibition ratio of 5% CSE-induced fibrotic-related genes expression by antioxidants in the orbital fibroblasts from GO patients.

Fibrosis-related genes	5% CSE-treated (mean ± SD)%[a]	1 mM NAC + 5% CSE-treated (mean ± SD)%[a]	Inhibition ratio (%) (mean ± SD)	2 mM VitC + 5% CSE-treated (mean ± SD)%[a]	Inhibition ratio (%) (mean ± SD)
Apolipoprotein J					
GO ($n = 5$)	285.17 ± 22.47	245.18 ± 20.33	14.02 ± 3.27	217.32 ± 18.53	23.79 ± 8.47
			$p = 0.0437$		$p = 0.0294$
Fibronectin					
GO ($n = 5$)	496.35 ± 28.64	412.11 ± 26.72	16.96 ± 4.48	355.38 ± 23.29	28.40 ± 5.05
			$p = 0.0251$		$p = 0.0085$
CTGF					
GO ($n = 5$)	350.32 ± 25.73	305.32 ± 23.37	12.82 ± 5.67	254.29 ± 21.77	27.41 ± 9.67
			$p = 0.0470$		$p = 0.0224$

[a]The expression levels from 5 normal subjects and 5 GO patients were normalized to each individual 18S rRNA gene expression followed by adjusting to N1 whose expression was defined as 100%.

Table 3: The induction ratio of intracellular cytokine in the orbital fibroblasts from normal subjects and GO patients before and after 5% CSE treatment.

Cytokines species	Basal levels (mean ± SD)	5% CSE-treated (mean ± SD)	Induction ratio (%) (mean ± SD)	p value
TGF-β1 (pg per 10^4 cells)				
Normal ($n = 5$)	87.46 ± 13.88	117.05 ± 18.11	133.83 ± 10.61	0.041
GO ($n = 5$)	148.67 ± 18.77	282.37 ± 16.29	189.67 ± 12.55	0.006
	$p = 0.004$		$p = 0.008$	
IL-1β (pg per 10^4 cells)				
Normal ($n = 5$)	57.05 ± 9.73	68.19 ± 8.33	119.07 ± 7.22	0.033
GO ($n = 5$)	75.83 ± 7.52	122.38 ± 11.37	161.58 ± 9.73	0.005
	$p = 0.008$		$p = 0.003$	

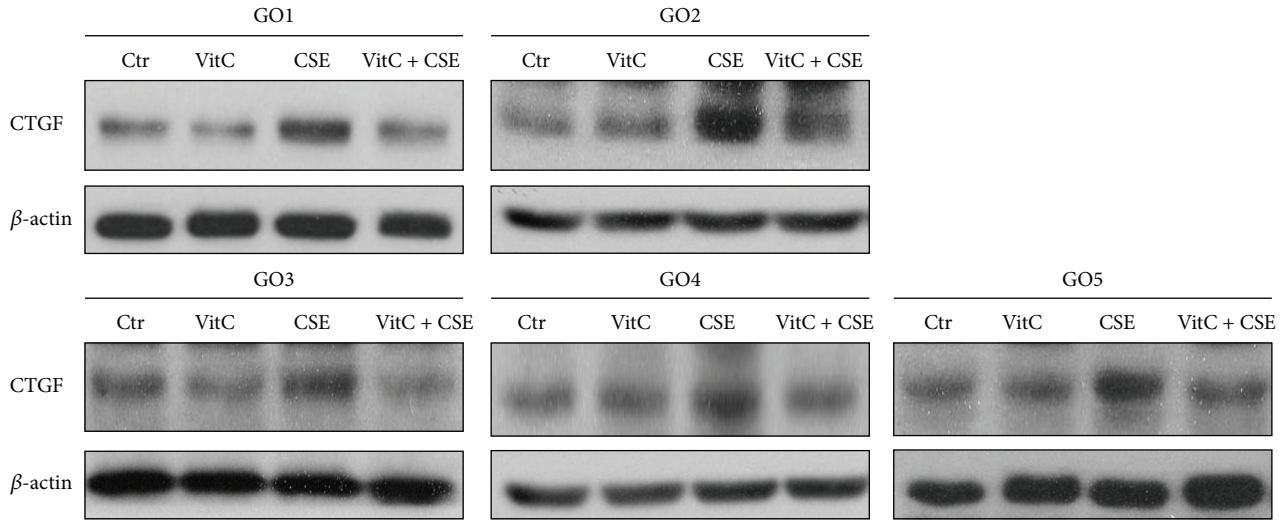
fibrosis-related genes expression, especially CTGF, in the GO orbital fibroblasts as compared with those of normal controls. In addition, pretreatment with antioxidants such as NAC and vitamin C could confer significant protection against the influence of CSE on oxidative damage, fibrosis-related genes expression, and induction of TGF-β1 and IL-1β.

Orbital fibroblasts, one of the major target cells in GO, are associated with many GO-related pathologic conditions, including oxidative stress [14, 15, 23]. Oxidative stress also has been suggested to play a role on the deleterious impact of smoking in GO [14]. The present study provided evidence that the GO orbital fibroblasts were more susceptible to CSE-induced cytotoxicity and oxidative stress than those of normal controls. Accumulation of CSE-induced ROS may cause more oxidative damage including oxidative DNA damage and lipid peroxidation, which could explain in part our previous observation that smokers had significant higher urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) than did never smokers in GO patients [24]. In addition, increased generation of ROS, especially the superoxide anions and hydrogen peroxide, can stimulate proliferation of GO orbital fibroblasts [19] and induce the production of proinflammatory cytokines [25], which all are key pathological features in GO. Moreover, cigarette smoke-mediated oxidative stress could recruit inflammatory and immune cells such as lymphocytes, macrophages, and neutrophils and activate some proinflammatory mediators [26], which may exacerbate the inflammation and tissue remodeling processes of GO.

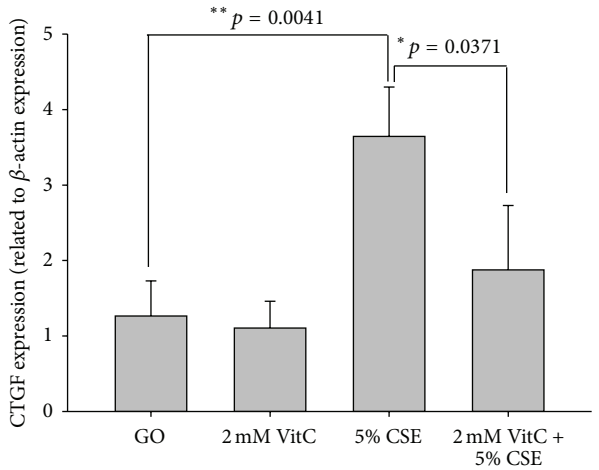
The disease course of GO is characterized not only by early inflammatory process but also by tissue remodeling and/or fibrosis. Although fibrosis represents a quiescent stage in GO, it may cause much of the substantial morbidity, which is often unresponsive to conventional medical treatment and requires surgical intervention. Oxidative stress is known as a factor that can induce various pathological fibrosis [27, 28]. In current study, we also noted that cigarette smoke-mediated oxidative stress could induce fibrotic-related genes expression including apolipoprotein J, fibronectin, and CTGF in the GO orbital fibroblasts, and these effects could be inhibited by pretreatment with antioxidants. Apolipoprotein J, fibronectin, and CTGF are commonly known as important fibrogenic factors. Although it remains to be clarified whether apolipoprotein J plays as a fibrosis biomarker or adaptive response in the development of fibrotic process of GO, CTGF has been shown to be substantially involved in the pathogenesis of various fibrotic disorders such as liver, heart, kidney, and ocular fibrosis [29–32]. CTGF can exhibit diverse cellular functions, including extracellular matrix production, cell migration, proliferation, and differentiation.

Table 4: The inhibition ratio of 5% CSE-induced intracellular cytokine by antioxidants in the GO orbital fibroblasts.

Cytokines species	5% CSE-treated (mean ± SD)	1 mM NAC + 5% CSE-treated (mean ± SD)	Inhibition ratio (%) (mean ± SD)	2 mM VitC + 5% CSE-treated (mean ± SD)	Inhibition ratio (%) (mean ± SD)
TGF-β1 (pg per 10^4 cells)					
GO ($n = 5$)	265.91 ± 13.67	177.08 ± 14.05	33.41 ± 12.39	153.39 ± 9.22	42.32 ± 11.25
			$p = 0.037$		$p = 0.008$
IL-1β (pg per 10^4 cells)					
GO ($n = 5$)	139.77 ± 8.20	92.83 ± 9.37	33.09 ± 7.33	81.27 ± 7.26	41.85 ± 5.28
			$p = 0.028$		$p = 0.003$



(a)



(b)

Figure 4: Inhibition of CSE-induced fibrotic markers by pretreatment of the GO orbital fibroblasts with antioxidants. (a) After pretreatment of orbital fibroblasts from GO patients (GO1–GO5) with 2 mM VitC for 1 hour followed by the addition of 5% CSE for another 24 hours, the CTGF protein expression was determined by Western blot, respectively. (b) The levels of the CTGF protein expression were normalized to each of corresponding β-actin expression levels and were adjusted to GO1 without CSE and/or vitamin C (VitC) treatment, whose CTGF expression was defined as 1.00. The representative histogram was constructed on the basis of the results from three independent experiments, and data were presented as means ± SD ($^*p < 0.05$; $^{**}p < 0.01$ versus the group without CSE treatment). Ctr: without CSE treatment.

Importantly, CTGF is critical for TGF-β-mediated fibroblast-myofibroblast transdifferentiation and subsequent deposition of extracellular matrix [33], which may contribute to the tissue remodeling and fibrosis process in GO. It has also been reported that periodontal fibrosis can be promoted by nicotine from smoking via effects on CTGF [34]. Taken together, previous reports and our findings in this study may explain in part why smoking is associated with severe GO and poor response to immunosuppressive therapy in GO.

We previously revealed that low concentrations of hydrogen peroxide can induce the production of proinflammatory cytokines such as TGF-β1 and IL-1β in GO orbital fibroblasts [19]. The observations in this study further show that 5% CSE induced higher intracellular levels of TGF-β1 and IL-1β in GO orbital fibroblasts than those in the normal controls. Moreover, 5% CSE-induced elevation of TGF-β1 and IL-1β in GO orbital fibroblasts was abolished by the antioxidant treatment. TGF-β1, a potent fibrogenic factor, has been reported to modulate the proliferation of fibroblasts and tissue fibrosis [35, 36]. IL-1β, an important proinflammatory cytokine in GO, has been shown to stimulate hyaluronan synthesis in orbital fibroblasts [37]. Fibroblast proliferation, tissue fibrosis, and hyaluronan accumulation are all important pathological features in the clinical expression of GO. Collectively, these findings suggest that oxidative stress plays an important role on the deteriorative effect of cigarette smoking on GO.

In conclusion, this study demonstrated that GO fibroblasts have exaggerated response to cigarette smoke extract challenge along with increased oxidative stress, fibrosis-related genes expression, and intracellular levels of TGF-β1 and IL-1β. The findings obtained in this study provide some clues for the impact of cigarette smoking on GO and offer a theoretical basis for the potential and rational use of antioxidants in treating GO.

Competing Interests

None of the authors has any commercial interest in the material mentioned herein.

Acknowledgments

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Review Article

Mitochondria-Targeted Antioxidants: Future Perspectives in Kidney Ischemia Reperfusion Injury

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Kidney ischemia/reperfusion injury emerges in various clinical settings as a great problem complicating the course and outcome. Ischemia/reperfusion injury is still an unsolved puzzle with a great diversity of investigational approaches, putting the focus on oxidative stress and mitochondria. Mitochondria are both sources and targets of ROS. They participate in initiation and progression of kidney ischemia/reperfusion injury linking oxidative stress, inflammation, and cell death. The dependence of kidney proximal tubule cells on oxidative mitochondrial metabolism makes them particularly prone to harmful effects of mitochondrial damage. The administration of antioxidants has been used as a way to prevent and treat kidney ischemia/reperfusion injury for a long time. Recently a new method based on mitochondria-targeted antioxidants has become the focus of interest. Here we review the current status of results achieved in numerous studies investigating these novel compounds in ischemia/reperfusion injury which specifically target mitochondria such as MitoQ, Szeto-Schiller (SS) peptides (Bendavia), SkQ1 and SkQR1, and superoxide dismutase mimics. Based on the favorable results obtained in the studies that have examined myocardial ischemia/reperfusion injury, ongoing clinical trials investigate the efficacy of some novel therapeutics in preventing myocardial infarct. This also implies future strategies in preventing kidney ischemia/reperfusion injury.

1. Introduction

Ischemia/reperfusion injury (IRI) is a major cause of acute kidney injury (AKI) formerly known as acute renal failure [1]. The incidence of AKI in hospitalized patients has been reported to be between 2% and 7% and even greater than 10% in intensive care unit (ICU) patients contributing to increased mortality rate [2]. Kidney IRI is of great importance occurring in various clinical settings including shock, vascular and cardiac surgery, sepsis, and kidney transplantation. During kidney transplantation, IRI causes delayed graft function (DGF) that has been associated with more frequent episodes of acute rejection and progression to chronic allograft nephropathy [2–4]. Complex interplay of pathophysiological processes linking inflammation, abnormal repair, and fibrosis makes AKI an important risk factor for progression of chronic kidney disease [5–7]. Basically, reperfusion phenomena consist of events which "paradoxically" continue to damage tissue in spite of established circulation and oxygen supply to the tissue that previously was under ischemia. Pathogenesis of IRI is rather complex and involves hypoxic injury, production of reactive oxygen species (ROS), inflammation, apoptosis, and necrosis [8]. Reactive oxygen species (ROS) include oxygen radicals such as superoxide radical anion ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$) and certain nonradicals that either are oxidizing agents

or are easily converted into radicals, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). ROS generation represents a cascade of reactions starting with the production of $O_2^{\bullet-}$ that can be further converted to H_2O_2 via superoxide dismutases (SOD), manganese (MnSOD) in mitochondria and copper-zinc (CuZnSOD) in the cytosol. The main sinks for H_2O_2 are catalase (CAT) and glutathione peroxidase (GPx). The latter uses glutathione (GSH) which is oxidized to GSSG and recycled by glutathione reductase. There are other enzymes that can remove H_2O_2, such as peroxiredoxin/thioredoxin/thioredoxin reductase (Prx/Trx/TrxR) system. However, CAT activity is about three orders of magnitude higher compared to Prx/Trx/TrxR system [9], which is essential under physiological settings for keeping low levels of mitochondrial H_2O_2 emission and for normal redox signaling via regulation of thiol redox switches on different proteins [10].

H_2O_2 can also react with transition metals, such as iron or copper, to produce $HO^{\bullet}$, the most reactive species in living systems [11]. The main reactive nitrogen species (RNS) are nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$). $ONOO^-$ is formed via reaction between NO and $O_2^{\bullet-}$ and can be further protonated and decomposed to nitrogen dioxide radical ($NO_2^{\bullet}$) and $HO^{\bullet}$ [12, 13]. These radicals are "caged" (i.e., generated close to each other), so they can recombine quickly, and much of $ONOO^-$ undergoes isomerisation to nitrate. Some amount of $ONOO^-$ *in vivo* reacts with CO_2 to form nitrosoperoxycarbonate ($ONOOCO_2^-$). About 35% of $ONOOCO_2^-$ is decomposed to $NO_2^{\bullet}$ and carbonate radical ($CO_3^{\bullet-}$) [14]. The latter is highly oxidizing species targeting NADPH and proteins [15, 16].

Mitochondria are the major site of ROS production, due to inevitable leakage of electrons from electron transport chain (ETC) onto oxygen [17]. Other major intracellular sites of ROS generation are enzymes, such as NADPH oxidase (NOX) and xanthine oxidase (XO).

Initially, in ischemic phase kidney tubular epithelial and endothelial cells are main producers of ROS and are later accompanied by activated leucocytes, that is, oxidative burst related to inflammation. These events reveal the role of ROS in exerting detrimental effects on cellular structure, linking oxidative stress, inflammation, and cell death. ROS through interactions with small metabolites as well as proteins, lipids, and nucleic acids might irreversibly destroy or alter the function of these target molecules and belonging organelles and cells. ROS can also serve as homeostatic signaling molecules which primarily depends on magnitude and duration of provoking stimuli for ROS production.

In recent years it has become clear that mitochondria have critical role in initiation and progression of renal IRI. They are early responders to the anoxia and then reoxygenation initiating responses that lead to changed metabolic and bioenergetic status, autophagy, inflammation, and induction of cell death pathways. Several approaches, mainly in experimental studies with a few human trials, have been used in investigating the options for preventing and treatment of IRI with special emphasis on modulation of inflammatory response, inhibition of apoptosis, and amelioration of oxidative stress, but currently there is no effective pharmacological treatment to address the main mechanisms of ischemic AKI [18–20]. Recently, novel therapeutic approach called ischemic preconditioning has become translated from animal models to humans [20, 21]. It is based on the observations that episodes of nonlethal ischemia can precondition the kidney to be protected in subsequent prolonged ischemia.

Nevertheless, oxidative stress is crucial for the cascade of processes participating in the pathogenesis of IRI. Since mitochondria as both sources and targets of ROS are initiators of complex mechanisms in IRI, it seems reasonable from therapeutic perspective to develop pharmacological method aiming to decrease mitochondrial oxidative damage.

In this review, we will summarize the mechanisms of mitochondrial ROS production and some options for potential treatment strategies.

2. The Role of Mitochondria in the Pathogenesis of IRI

By impairing electron transport and energy metabolism and by altering cellular redox potential via ROS production, mitochondria trigger events leading to apoptosis, a hallmark of IRI. Electron transport along ETC to O_2 is tightly coupled to oxidative phosphorylation for ATP synthesis. In normal, for instance, nonischemic cells, the main source of ROS is ETC [22, 23]. Most oxygen consumed is reduced to water through 4 steps of single electron reduction by cytochrome c oxidase. Electrons generated from reduced nicotinamide adenine dinucleotide (NADH) are accepted by NADH dehydrogenase (Complex I) and those from succinate are accepted by succinate dehydrogenase (Complex II). Electrons are then passed to cytochrome bc1 (Complex III) through coenzyme Q (CoQ) and to cytochrome c oxidase (Complex IV) using cytochrome c as a carrier. The last step is transfer of electron from cytochrome c oxidase to O_2 to form water (Figure 1). Electron flow mediated by the respiratory chain enzyme complex drives proton (H^+) translocation from the matrix side at the level of complexes I, III, and IV to the intermembrane space side, thereby establishing an electrochemical potential gradient or proton motive force across the inner membrane [23]. Since the inner mitochondrial membrane is almost impermeable, this electrochemical gradient is used to reintroduce protons back through the proton channel of complex V (ATP synthase). ATP synthesis from ADP and inorganic phosphate is then catalyzed by F_0F_1ATPase. Every decrease in the rate of mitochondrial phosphorylation increases electron leakage from the ETC and consequently increases the production of $O_2^{\bullet-}$. Mitochondria have defense mechanism to neutralize ROS. Superoxide radical anion is converted to H_2O_2 by MnSOD, and H_2O_2 is further degraded to H_2O. In mitochondria, about 70–80% of H_2O_2 removal has been attributed to GPx [9]. ROS may induce mild mitochondrial oxidative stress or diffuse to the cytosol playing an important role in cellular homeostasis, mitosis, and differentiation and serving as signaling molecules in different physiological responses [24, 25]. This constant production of small amounts of ROS is necessary to maintain the appropriate "redox state" of cell

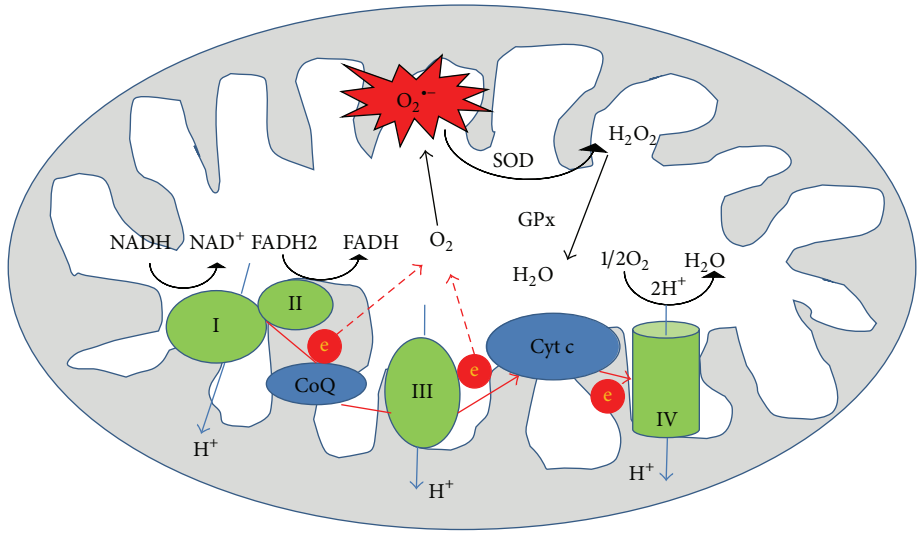


Figure 1: Formation of various reactive oxygen species from electron transport chain in mitochondria. $O_2^{\bullet-}$: superoxide anion; SOD: superoxide dismutase; GPx: glutathione peroxidase; CoQ: coenzyme Q; Cyt c: cytochrome c.

which is crucial for the activation of several genes and the function of numerous enzymes. On the other hand, H_2O_2 that escapes mitochondria is removed by CAT, GPx, and other H_2O_2 removing systems, but an excess can activate potentially detrimental cascades, for example, via NF-κB.

What happens to the mitochondria in ischemia? The effect depends on the duration of ischemia. Ischemia causes alterations to the mitochondrial ETC complexes. If ischemic episodes are of short duration the electronegativity of the ETC complexes and leakage of electrons is increased with consequently increased ROS formation. ROS can trigger signaling events that lead to the synthesis of proteins, including MnSOD and thereby providing beneficial role that is the part of ischemic preconditioning phenomena [26]. A prolonged period of ischemia results in decreased activity of the complexes I and IV of ETC and subsequent electron leak of that reduce O_2 to form superoxide radicals when O_2 is reintroduced following reperfusion [24, 27]. Also, impaired ETC results in decreased ATP production following reperfusion. During prolonged ischemia more of detrimental radicals are produced. Superoxide mainly attacks Fe-S centers in ETC proteins to provoke the reduction of Fe^{3+} and liberation of Fe^{2+}, leaving aconitase and other transporters of electrons dysfunctional [28]. These Fe^{2+} ions are important in Fenton chemical reaction whereby H_2O_2 can be converted to the highly reactive $HO^{\bullet}$ which is more detrimental for cell structure proteins and membrane lipids. It is important to note that $HO^{\bullet}$ has a drastically shorter diffusion radius in physiological milieu compared to H_2O_2, and it is too reactive to pass membranes. In short, $HO^{\bullet}$ is more dangerous for targets that are nearby the site of production. H_2O_2 generated in mitochondria can affect other organelles, nucleus, and surrounding cells [29]. The prolonged ischemia decreases the activity of antioxidant enzymes, such as MnSOD, and causes GSH depletion [30, 31].

The involvement/interference of ROS in signaling cascades might have both detrimental and beneficial effects. Cellular hypoxia appears to be the key signal for activation of HIF-1α, nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and mitogen activated protein kinases (MAPK). In addition, ROS have been directly implicated in programmed cell death [2, 32, 33]. Under hypoxia, transcriptional cell activity is directed to synthesis of proinflammatory and cytoprotective molecules [34]. So far the available data have implied a proinflammatory action of NF-κB as one of the key players in pathogenesis of IRI [35–38]. Besides chemokines and cytokines, NF-κB is implicated in the production of both ROS and HIF-1; that is, there is a positive feedback loop serving as an amplification mechanism [35, 39].

Massive production of ROS during reperfusion is secondary to electron leak mostly at complexes I and III [40]. The overproduction of $O_2^{\bullet-}$ may lead to formation of $ONOO^-$ which is a highly reactive molecule and leads to nitration of proteins, including complexes I and III, and further tissue injury [40, 41]. Renal content of 3-nitrotyrosine (the footprint of peroxynitrite) increases during ischemia. Pertinent to this, $^{\bullet}NO$ that is formed via activity of inducible NO synthase (iNOS) is also increased [42–44]. iNOS is induced in kidney IRI [45–48]. Studies using inhibition of expression and activity of iNOS or even absence of iNOS showed amelioration of kidney IRI suggesting that NO generated by iNOS had detrimental role and contributed to kidney IRI [45, 47, 49]. This, by oxidant-induced disruption of protein complexes I and III, potentiates electron leak and further $O_2^{\bullet-}$ generation. The whole process is driven by ROS-induced ROS release and may become a vicious cycle that induces mitochondrial permeability transition pore (mPTP) opening [50]. Because of mitochondrial GSH depletion during ischemia, conversion of H_2O_2 to water is insufficient in reperfusion favoring formation of $HO^{\bullet}$ via

Fenton reaction [24, 31]. As a consequence, membrane permeability is affected. "Redox state" is altered by the oxidation of pyridines and thiols with consequent modification of $NADH/NAD^+$ and GSH/GSSG ratio [44]. Excessive ROS formation, recovery of pH, and calcium overload facilitate the opening of mPTP with consequent loss of cytochrome c and pyridine nucleotides favoring further ROS generation and triggering cell death [50–52]. The opening of mPTP results in redistribution of NADH and calcium to the cytosol and an influx of water to mitochondria causing mitochondrial matrix swelling and outer mitochondrial membrane rupture with release of proapoptotic factors leading to cell death. Released calcium to cytosol activates proteases, nucleases, and phospholipases, which trigger apoptosis [52].

Additionally, mitochondria has other components besides ETC that contribute to ROS production including NOX4, monoamine oxidase, and growth factor adaptor protein, Shc ($p66^{Shc}$), but this contribution is rather low compared with the generation of ROS from ETC [22].

3. Mode of the Action of Mitochondria-Targeted Drugs

According to previously mentioned data, it seems reasonable to develop pharmacological method that decreases mitochondrial oxidative damage in order to decrease kidney IRI. The relatively unsatisfactory efficacy of conventional antioxidants may be the consequence of their low penetrance to the mitochondria interior, which not only is the main site of ROS production but also suffers from oxidative stress as other cellular compartments. The inner mitochondrial membrane is highly impermeable and rich in cardiolipin and maintains a strong negative internal potential of –180 mV that is required for the function of electron transport chain.

To overcome these limitations, mitochondria-targeted antioxidants have been developed to provide their delivery to the mitochondrion interior. Mitochondria-targeted antioxidants are usually chimeric molecules of a cation triphenylphosphonium (TPP) conjugated with an antioxidant moiety such as coenzyme Q_{10} or plastoquinone [53, 54]. The proton motive force in the inner mitochondrial membrane maintaining the large mitochondrial membrane potential and the positive charge of lipophilic cation drive a transport of these cationic antioxidants into mitochondria. The result of this mitochondrial uptake is a chimeric drug concentration 10,000 times higher in the mitochondrial matrix than in the cytosol [55]. Apart from the TPP, rhodamine 123 is another suitable lipophilic cation to be conjugated to mitochondria-selective molecules [56]. However, lipophilic cations have a disadvantage. Since the charge accumulation into the matrix leads to mitochondrial membrane depolarization, at concentrations greater than 10 μM, toxicity has been observed [56].

Recently, use of short peptide sequences with specific physicochemical properties for delivery of compounds to inner mitochondria has emerged [57]. The Szeto-Schiller SS peptides have exhibited marked antioxidant properties by scavenging ROS and inhibiting linoleic acid oxidation [58, 59]. They feature a common structural motif of alternating aromatic (Phe, Tyr, and Dmt (2′,6′-dimethyltyrosine)) and basic (Arg, Lys) residues. SS peptides freely penetrate membranes in a potential-independent manner due to their aromatic-cationic amino acid sequence. Tyr or Dmt residues are likely responsible for the ROS scavenging abilities of these peptides [60]. Another optional oligopeptide is conjugated to manganese metalloporphyrin and belongs to novel class of mitochondria-targeted SOD mimics named Mn-porphyrin-oligopeptide conjugates [61].

N-acetyl-L-cysteine (NAC) has been known for the efficiency in protecting cells against oxidants [62]. In order to deliver the tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine or GSH) and its analog NAC into mitochondria, choline esters (MitoGSH and MitoNAC) have been utilized [63]. Experiments with MitoNAC were performed using cultured cells, However, *in vivo* data are missing.

Among the other mitochondria-targeted compounds with different mode of penetrance and action that are worth mentioning is diazoxide, the opener of mitochondrial K_{ATP} channels. The precise mechanisms how active mitochondrial K_{ATP} channels lead to decreased ROS production when oxygen is delivered during reperfusion are still unclear, although they are in some ways similar to events elicited by ischemic preconditioning [64]. Opened K channels result in a lowered mitochondrial membrane potential and lowered redox state of NAD system leading to decreased ROS production by respiratory chain Complex I [65].

4. Mitochondria-Targeted Antioxidants in Renal IRI

Mitochondria-targeted antioxidants have already been used in other experimental pathology models and one of them, MitoQ, has been used in two Phase II trials in humans regarding treatment of Parkinson disease and chronic hepatitis C, showing long term safety and tolerance [66, 67]. Because of a positively charged lipophilic cation, MitoQ is accumulated in the negatively charged interior of mitochondria. The antioxidant component of MitoQ is the ubiquinone that is also found in coenzyme Q_{10} (Figure 2) [53]. By the action of the enzyme Complex II in the mitochondrial respiratory chain, ubiquinone part of MitoQ is rapidly activated to the active ubiquinol antioxidant [68]. After detoxifying ROS, the ubiquinol part of MitoQ is converted to ubiquinone, which is again subjected to Complex II to be recycled back to active antioxidant ubiquinol [68]. This process makes MitoQ an effective mitochondria-targeted antioxidant.

The reason for the use of MitoQ in kidney IRI came from the studies using MitoQ to decrease heart and hepatic IRI and to prevent kidney damage during cold storage [69–71]. Using this model, it was demonstrated that administration of MitoQ prior to the onset of ischemia reduced oxidative damage and severity of renal IRI, thereby providing functional protection to the kidney [72]. The group of Skulachev et al. synthesized plastoquinonyl-decyl-triphenylphosphonium. In this compound named SkQ1, ubiquinone was replaced

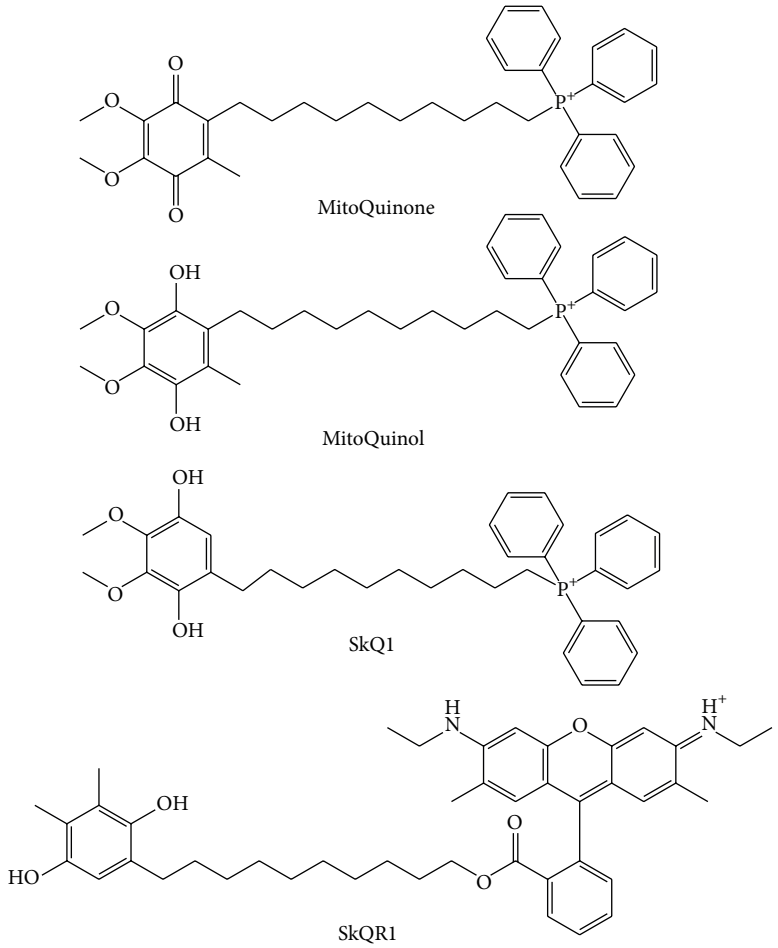
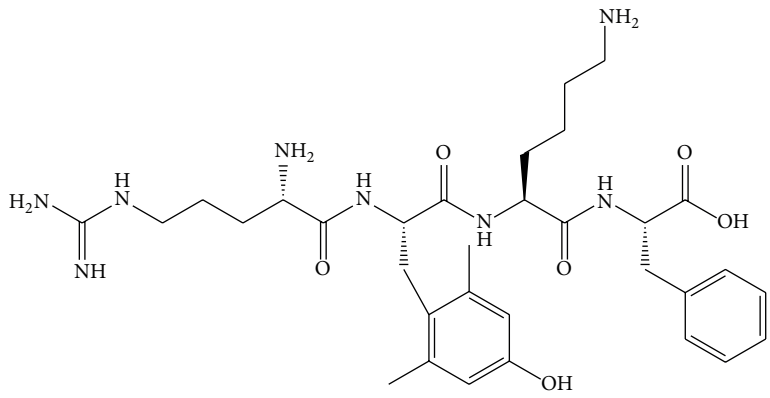


Figure 2: Mitochondria-targeted antioxidants with ubiquinone or plastoquinone antioxidant moiety, that are used in kidney ischemia/reperfusion injury.

by plastoquinone (Figure 2) [54, 73]. The effect of SkQ1 on kidney IRI using a culture of kidney epithelial cells has been studied. Preincubation with SkQ1 increased survival of these cells and diminished mitochondrial fission induced by the anoxia/reoxygenation procedure [74, 75]. In experimental model of a single-kidney ischemia (90 min) followed by reoxygenation, injection of SkQ1 to rats a day before kidney ischemia significantly improved survival compared to the experimental animals subjected to kidney IRI without any previous treatment [74].

In another experimental study, the authors used a mitochondria-targeted compound containing a charged rhodamine molecule conjugated with plastoquinone named SkQR1 (Figure 2) [76]. An intraperitoneal injection of SkQR1 to rats exposed to kidney IRI normalized the ROS level and lipid peroxidized products in kidney mitochondria, significantly decreased BUN and blood creatinine and lowered mortality compared to animals that were subjected to kidney IRI without any given drug [76]. Additionally, SkQR1 was found to provide the kidney with elements of ischemic tolerance signaling mechanisms. Administration of SkQR1 induced erythropoietin (EPO) and the phosphorylated form of GSK-3β in rat kidney [76]. EPO is known to afford some protection against ischemic damage [77, 78]. In a pilot clinical trial investigating the efficacy of EPO to prevent AKI after coronary artery bypass grafting (CABG), it was shown that EPO administration resulted in an incidence of AKI of 8% compared with an incidence of 29% in the placebo group [79]. Phosphorylated form of GSK-3β correlates with activity of prosurvival genes. Via inhibition of GSK-3β, protective signaling pathways act on the end effector mPTP; that is, they prevent the induction of the mitochondrial permeability transition, restore mitochondrial membrane potential, and decrease ROS production [80–82]. The authors concluded that treatment with SkQR1 provided linked synergy between antioxidative effects and ischemic tolerance signaling mechanisms due to the targeted delivery of this compound to mitochondria [76]. Beside the kidney IRI, MitoQ and SkR1 have been shown to have the role in amelioration of AKI induced by cisplatin and gentamycin [83, 84].

A number of reports demonstrate that open mitochondrial K_{ATP} channels, which are redox sensitive, effectively block the generation and release of ROS [85, 86]. Interestingly, agents opening the ATP-sensitive K channel (K_{ATP})



SS-31

Figure 3: Chemical structure of SS-31.

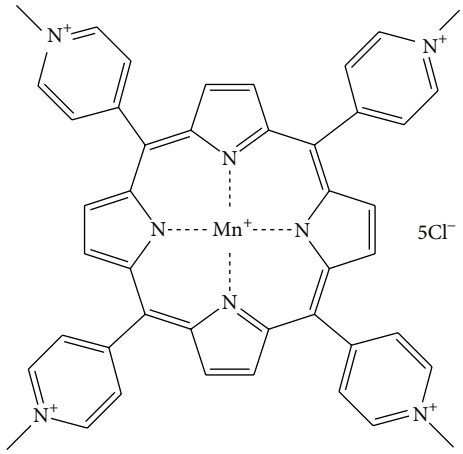
including diazoxide were effective in ameliorating cardiac and neural IRI [87, 88], but ineffective and even injurious in kidney IRI [89]. In a report of Sun et al., a large dose of diazoxide given before kidney ischemia prevented ROS accumulation in mitochondria, thereby reducing oxidative stress, consequent tubular cell apoptosis, and increase in serum creatinine [90]. Considering this effect dependence on widespread opening of the mitochondrial K_{ATP} channels, a large dose of diazoxide is needed, but this phenomenon raises the question of the role of mitochondrial K channels or associated respiratory chain components in the ROS production, depending on specific tissue.

mPTP as another potential target for pharmacological intervention in IRI was supported by preclinical and clinical studies [91, 92]. The substances that block opening of mPTP protect mitochondrial structure and respiration during early reperfusion and accelerate recovery of ATP. CypD is a component of mPTP. Animals with CypD gene ablation or treated with cyclosporin A (CsA), a CypD inhibitor, were protected from renal IRI [93–95]. These promising results were not translated into clinical nephrology compared to extensive investigation in cardiology since the nephrotoxic profile of CsA makes this drug unsuitable for clinical application in the treatment of renal IRI [96, 97].

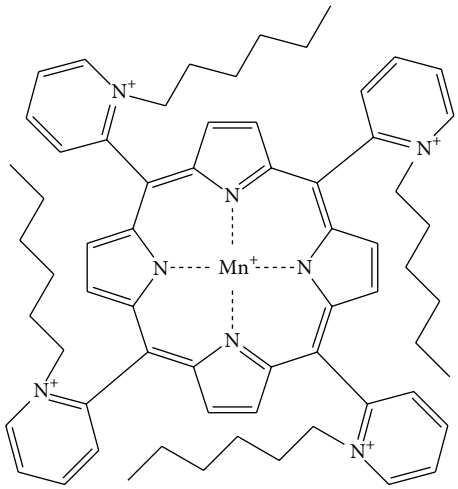
A growing number of publications speak in favor of compound Szeto-Schiller- (SS-) 31 peptide also known as Bendavia (Figure 3). Besides scavenging of ROS, Bendavia also inhibits mPTP and thereby prevents mitochondrial release of cytochrome c and consequently apoptosis [98]. However, recent study of Brown et al. did not confirm the role of Bendavia in direct inhibition of mPTP but rather emphasized its role in decreased ROS production and indirectly decreased mPTP opening. Bendavia may optimize the mitochondrial phospholipid cardiolipin microdomains, resulting in reduced electron leak from the inner membrane [99].

SS-31 peptide, that is, Bendavia, demonstrated efficacy in several animal models of different pathologic conditions by reducing oxidative stress [100–102]. Regarding IRI, the efficacy of Bendavia was mostly investigated in experimental models of myocardial ischemia. If Bendavia was given to animals in minutes prior to reperfusion or in the first 10 minutes of reperfusion, then the effect of reduced infarction and the extent of coronary no-reflow were achieved [103, 104]. These encouraging experimental results need confirmation in ongoing clinical trials for acute coronary syndromes [105]. Also, in experimental model of kidney, IRI Bendavia reduced oxidative stress, prevented tubular apoptosis and necrosis, and reduced inflammation [106]. Because of the rapid ATP recovery after Bendavia administration, microvascular endothelial cells are protected leading to reduced microvascular congestion that provides better reflow to the medulla [106]. The effect of Bendavia was explored in experimental model of percutaneous transluminal renal angioplasty (PTRA), a condition that may be associated with impairment of renal function. Infusion of Bendavia at the time of percutaneous transluminal renal angioplasty, decreases oxidative stress, apoptosis, and inflammation and improves renal function in animal experimental model of renal artery stenosis [107].

Since oxidative stress is dependent on both ROS production and removal by antioxidant enzymes and taking into consideration that IRI significantly reduces Mn SOD and Cu/Zn SOD mRNA expression, the use of antioxidative enzymes might alleviate kidney IRI. MnSOD knockout (KO) mice that exhibit low expression and activity of MnSOD in distal nephron, after ischemia/reperfusion, show similar levels of injury to the proximal tubule and distal nephron and equally altered renal function when compared with wild-type mice [108]. Additionally, these KO mice exhibit increased proliferating cell nuclear antigen- (PCNA-) positive nuclei in the distal nephrons, autophagy, and mitochondrial biogenesis indicating that chronic oxidative stress stimulates multiple survival signaling pathways to protect kidney against acute oxidative stress following ischemia/reperfusion [108]. The limitation of this study is that proximal tubule, especially its S3 fragment, damaged in IRI the most, in this specific mouse strain was not affected by lack of MnSOD and therefore failed to assess the role of abolished MnSOD activity within proximal tubules in IRI.



MnTMPyP



MnTnHex-2-PyP^{5+}

Figure 4: Chemical structures of Mn porphyrins investigated in experimental models of kidney ischemia/reperfusion injury.

There are several reports showing protective role of SOD mimics in experimental models of kidney IRI [109–113]. SOD mimics are a group of substances which catalyze the oxidation and reduction of $O_2^{\bullet-}$. They include cationic metalloporphyrins with Mn porphyrins (MnP), Mn(III) salens, Mn(II) cyclic polyamines, metal oxides, Mn(III) biliverdins, and metallocorroles (MCs) [114]. MnSOD mimics are stoichiometric scavengers of $O_2^{\bullet-}$ and accumulate in mitochondria depending on positive charge and lipophilicity [115]. MnPs are among the most potent MnSOD mimics designed and optimized to mimic the action of the enzyme catalytic site and to increase mitochondrial accumulation [115]. Manganese (III) tetrakis(1-methyl-4 pyridyl) porphyrin (MnTMPyP), one of the MnPs, acts as SOD mimic and peroxynitrite scavenger (Figure 4). MnTMPyP decreased lipid peroxidation, nitrotyrosine content in the proximal tubular region, caspase-3 activation, and tubular epithelial cell damage following ischemia/reperfusion [109]. In addition, MnTMPyP decreased the expression of the proapoptotic genes Bax and FasL. At first, the effects of SOD mimics were almost exclusively assigned to the removal of $O_2^{\bullet-}$ and $ONOO^-$, but recent data suggest the direct H_2O_2-driven oxidation of signaling proteins such as NF-κB [114, 116]. This was implied in experimental work of Dorai's group who used Mn(III) meso-tetrakis(N-n-hexylpyridinium-2-yl)porphyrin (MnTnHex-2-PyP^{5+}) (Figure 4) [113]. They showed that giving of renoprotective cocktail containing MnTnHex-2-PyP^{5+} to rats 24 h before, at the beginning, and 24 h after kidney IRI ameliorated AKI and induced adaptive response via mild prooxidative stress [113]. The improvement of renoprotective cocktail was achieved by adding N-acetylcysteine that couples with MnTnHex-2-PyP^{5+}. As a result, oxidative stress was enhanced via production of H_2O_2 [112, 116]. The prooxidative action of MnPs is manifested by oxidation of Cys-62 of p50 subunit of NF-κB, thereby preventing NF-κB activation [116, 117]. It has been proposed that, upon oxidation of cysteines of Kelch-like ECH-associated protein 1 (KEAP1) that is nuclear factor-E2-related factor (Nrf2) inhibitor, porphyrin-based SOD mimics, Mn(II) cyclic polyamines, and nitroxides activate Nrf2. Activation of Nrf2 results in upregulation of endogenous antioxidative defenses [117].

There are several other investigated MnSOD mimics such as MnSalens, mangafodipir (Mn complex with dipyridoxyl diphosphate), substances named tempone and oxazolidine-5-doxylstearate belonging to nitroxides, and MitoSOD, consisting of TTP cation conjugated with a $O_2^{\bullet-}$-selective pentaaza macrocyclic Mn(II) SOD mimic, but the data on the use of these compounds *in vivo* kidney ischemia/reperfusion models are still missing [115]. Mito-CP is a five-membered nitroxide CP, conjugated to a TTP cation. It has prevented cisplatin-induced renal dysfunction, renal cell inflammation, and tubular cell apoptosis [83]. Favorable effects in this model of AKI are promising for investigating the role of Mito-CP in renal IRI. Other potential compounds to be investigated in the prevention of kidney IRI include vitamin E [118], lipoic acid [119], and Ebselen [120] conjugated to the TPP cation and targeted to mitochondria. Although 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) is not SOD mimic, it is a free radical scavenger and has been used in experimental model of kidney IRI. Tempol significantly reduced the increase in creatinine after kidney IRI induction in rats [121, 122]. Also, Tempol significantly reduced kidney MDA level and nitrotyrosine staining.

Overall, these findings support the potential use some of SOD mimics and previously mentioned mitochondria-targeted antioxidants as therapeutic agents in renal IRI (Table 1).

5. Conclusion

Mitochondrial ROS generation participates in deleterious cascade of events provoked by ischemia/reperfusion leading to tubular cell death and AKI. So far, investigated treatment by antioxidants has not been successfully translated into clinical practice. Mitochondria-targeted antioxidants represent a novel approach especially in clinical settings in which kidney IRI could be prevented or ameliorated. Experimental data

Table 1: Mitochondria-targeted antioxidants in kidney ischemia/reperfusion injury.

Drug	Antioxidant moiety	Reference
MitoQ	Ubiquinone	[72]
SkQ1, SkQR1	Plastoquinone	[74–76]
SS-31 (Bendavia)	Tyr or Dmt (2′,6′-dimethyltyrosine) residues	[106, 107]
SOD mimic		
(i) MnTMPyP	Manganese metalloporphyrin	[109–113]
(ii) MnTnHex-2-PyP^{5+}		

are encouraging despite the fact that most of the data come from the studies exploring myocardial IRI. Those results justify development of this preventative strategy in kidney IRI for clinical use as some of them such as MitoQ and (SS)-31 are already being evaluated in humans for prevention of myocardial IRI.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Circadian Rhythms of Oxidative Stress Markers and Melatonin Metabolite in Patients with Xeroderma Pigmentosum Group A

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Xeroderma pigmentosum group A (XPA) is a genetic disorder in DNA nucleotide excision repair (NER) with severe neurological disorders, in which oxidative stress and disturbed melatonin metabolism may be involved. Herein we confirmed the diurnal variation of melatonin metabolites, oxidative stress markers, and antioxidant power in urine of patients with XPA and age-matched controls, using enzyme-linked immunosorbent assay (ELISA). The peak of 6-sulfatoxymelatonin, a metabolite of melatonin, was seen at 6:00 in both the XPA patients and controls, though the peak value is lower, specifically in the younger age group of XPA patients. The older XPA patients demonstrated an increase in the urinary levels of 8-hydroxy-2′-deoxyguanosine and hexanoyl-lysine, a marker of oxidative DNA damage and lipid peroxidation, having a robust peak at 6:00 and 18:00, respectively. In addition, the urinary level of total antioxidant power was decreased in the older XPA patients. Recently, it is speculated that oxidative stress and antioxidant properties may have a diurnal variation, and the circadian rhythm is likely to influence the NER itself. We believe that the administration of melatonin has the possibility of ameliorating the augmented oxidative stress in neurodegeneration, especially in the older XPA patients, modulating the melatonin metabolism and the circadian rhythm.

1. Introduction

Xeroderma pigmentosum (XP), a genetic disorder in DNA nucleotide excision repair (NER), is characterized by skin hypersensitivity to sunlight and progressive neurological impairment [1]. There are eight complementation subgroups of XP, and group A (XPA) is common in Japan, showing severe neurological disorders such as mental deterioration, cerebellar ataxia, extrapyramidal abnormalities, and neuronal deafness, but no effective treatment has been developed for neurological disorders [2]. Oxidative stress originates from an imbalance between the production of reactive oxygen species and reactive nitrogen species and the antioxidant capacities of cells and organs [3]. Oxidative stress has been confirmed to play a role in adult-onset neurodegenerative diseases, such as Alzheimer's disease [4], and we confirmed the involvement of oxidative neuronal damage in child-onset and adult neurodegenerative diseases, such as dentatorubral-pallidoluysian atrophy and superficial siderosis [5, 6]. We clarified the accumulation of oxidative stress markers in the basal ganglia in autopsy rains in XPA and Cockayne syndrome (CS), having a genetic defect in transcription-coupled repair, which results in multiple organ impairment and various neurological disorders [7]. In addition, we reported an increase in the urinary levels of oxidative stress markers in both XPA and CS in the preliminary analysis [8].

Melatonin is a functionally pleiotropic and neuroendocrine molecule and is produced mainly by the pineal gland under the control of the suprachiasmatic nucleus [9]. Melatonin regulates circadian rhythm and plays a role in the transduction of the chronobiological actions of multiple hormones. In addition, it also has antioxidant properties and anti-inflammatory abilities and is likely to be one of therapeutic tools for neurological disorders, such as multiple sclerosis and Huntington's disease [10]. Melatonin secretion has a 24-hour rhythm, in which the peak is during the midnight [11]. We preliminarily examined the urinary level of melatonin metabolite in XPA and CS, which was reduced

predominantly in CS patients, suffering from the disturbed circadian rhythms of sleep-wakefulness and body temperature regulation [12].

Recently, redox regulation and/or oxidative stress has been reported to have diurnal variation, and the circadian rhythms of oxidative stress markers and antioxidant enzymes have been examined in healthy subjects and patients with neurological disorders [13]. Nevertheless, the relationships in diurnal variation between melatonin and oxidative stress markers still remain to be investigated. Herein, we analyzed the circadian rhythms of oxidative stress markers and melatonin metabolites in urine of patients with XPA.

2. Materials and Methods

We analyzed the urine from 8 patients with genetically confirmed XPA and 8 normal controls, aged from 6 to 37 years. It is well known that melatonin secretion varies by age. The levels of plasma melatonin and its urinary metabolites are high in infancy and early children, decline dramatically around adolescence, and keep reducing gradually in adults and aged people [14].

Accordingly, we divided both XPA patients and controls into younger and older age groups, consisting of three subjects each less than 15 years and five ones each equal to and more than 15 years, respectively. Urine samples were collected four times a day (at 0:00, 06:00, 12:00, and 18:00). All specimens were stored at −80°C in a deep freezer avoiding lights. Consents were granted from all parents of patients and child controls and adult controls. This analysis was approved by the Ethical Committee of Tokyo Metropolitan Institute of Medical Science. We measured the urinary concentrations of 6-sulfatoxymelatonin (6-SM), a main metabolite of melatonin, a marker of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine (8-OHdG), an early stage marker of lipid peroxidation, hexanoyl-lysine adduct (HEL), and total antioxidant power (TAO), respectively, as previously reported [8, 12]. We used commercially available enzyme-linked immunosorbent assay (ELISA) kits for 6-SM (GenWay Biotech, CA, USA), 8-OHdG and HEL (Japan Institute for the Aging, Shizuoka, Japan), and TAO (Oxford Biomedical Research, MI, USA), respectively.

The results were normalized to urine concentration of creatinine (Cre), except TAO. Correlations among 6-SM, 8-OHdG, HEL, and TAO at each time point were confirmed by Spearman's rank correlation coefficient.

3. Results

The peak of urinary 6-SM was identified at 6:00 in the younger and older age group of both controls and all XPA patients, and the peak value was reduced by age (Figure 1). The XPA patients demonstrated a lower peak value, which was approximately one-third and half of each of those in controls. The urinary levels of 8-OHdG were low and lacked the diurnal variation in the younger age group in both controls and XPA patients (Figure 2(a)). In the older age group, the urinary levels of 8-OHdG were increased in the XPA patients but not in controls, in which the robust peak was identified at 6:00 (Figure 2(b)). The urinary levels of HEL were low and lacked the diurnal variation in the younger age group in both controls and XPA patients (Figure 3(a)). In the older age group, the urinary levels of 8-OHdG and HEL were increased in the XPA patients but not in controls, in which the robust peak was identified at 6:00 and 18:00, respectively (Figures 2(b) and 3(b)). There was no significant difference in the urinary levels of TAO between the controls and XPA patients, lacking the diurnal variation in the younger age group (Figure 4(a)). In the older age group, the urinary levels of TAO in the XPA patients were reduced to half of those in controls, showing the dim peak at 12:00 (Figure 4(b)). The correlation among biomarkers could not be demonstrated statistically.

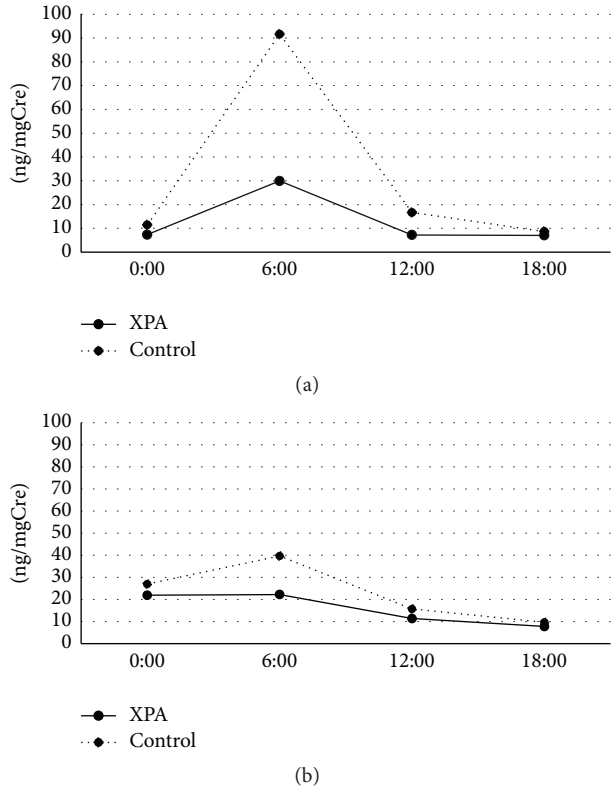

Figure 1: Circadian rhythm of urinary levels of 6-sulfatoxymelatonin (6-SM) (ng/mgCre) in younger age group (a) and older age group (b). Solid and dotted lines denote values of patients with xeroderma pigmentosum group A (XPA) and controls, respectively.

4. Discussion

Our preliminary study suggested the possible reduction in the urinary levels of 6-SM in XPA patients [12], which was confirmed in both the younger and older age groups in this analysis, and the presence of a dim peak is noteworthy (Figure 1). Generally XPA patients should avoid sun exposure in order to reduce the risk of ultraviolet hazard, and such lesser sunlight exposure may disturb the hypothalamic control in melatonin metabolism. Intriguingly, XPA patients rarely

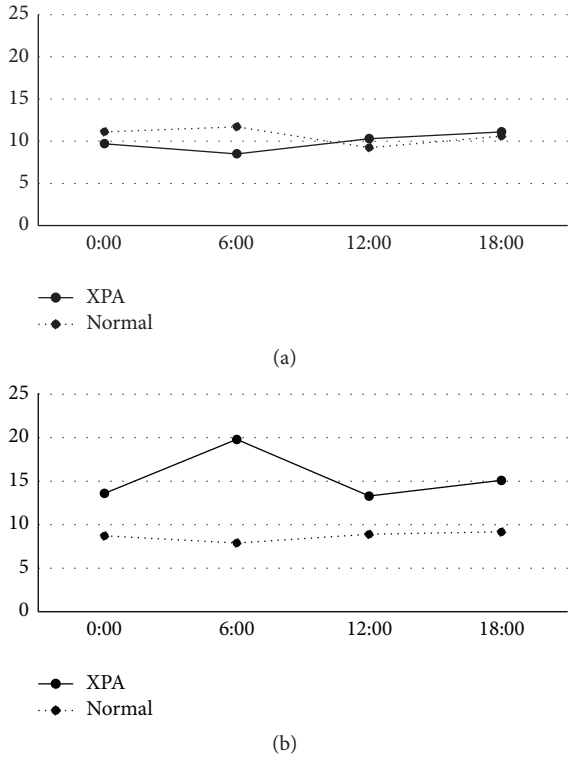

Figure 2: Circadian rhythm of urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) (ng/mgCre) in younger age group (a) and older age group (b). Solid and dotted lines denote values of patients with xeroderma pigmentosum group A (XPA) and controls, respectively.

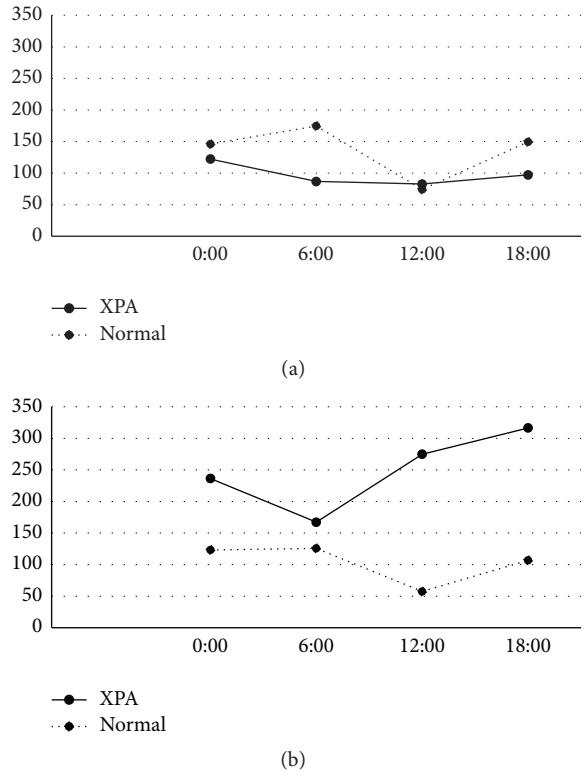

Figure 3: Circadian rhythm of urinary levels of hexanoyl-lysine (HEL) (pmol/mgCre) in younger age group (a) and older age group (b). Solid and dotted lines denote values of patients with xeroderma pigmentosum group A (XPA) and controls, respectively.

show circadian rhythm disturbances and/or sleep disorders except excessive daytime drowsiness [12]. As well as the reduced urinary excretion of 6-SM, the increase of urinary oxidative stress markers in aged XPA patients, which was suggested by our previous study in fewer subjects [8], was also confirmed in the older age group. Furthermore, the increased urinary secretions of 8-OHdG and HEL demonstrated the circadian rhythm with the robust peak (Figures 2(b) and 3(b)), in association with the reduced TAO (Figure 4(b)). As mentioned above, the XPA patients show various neurological disorders [1], and the aged ones tend to suffer from respiratory disturbance due to laryngeal dystonia, vocal cord paralysis, or sleep apnea [15–17]. Needless to say, the progression of neurodegeneration and/or respiratory failure can disturb the redox control in the whole body, but the reduction of melatonin secretion, occurring even in the younger age group (Figure 1(a)), may also precipitate the oxidative stress. Accordingly, we strongly speculate that the administration of exogenous melatonin may possibly correct its disturbed metabolism, alter or improve alertness, and/or prevent the exacerbation of oxidative stress.

Oxygen and circadian rhythmicity are essential in a myriad of physiological processes to maintain homeostasis, from blood pressure and sleep/wake cycles down to cellular signaling pathways that play critical roles in health and disease [13]. It is speculated, reviewing several papers, that the concentrations of many antioxidant enzymes, such as superoxide mutase, have a morning peak, while the occurrence of lipid peroxidation has an evening peak, like the peak at 18:00 in the urinary levels of HEL in the older age group of XPA patients. On the other hand, urinary markers of nucleic acid oxidation did not have diurnal variation in healthy subjects [18]. Similarly in this analysis, the controls in both the younger and older age groups did not show any circadian rhythms in the urinary levels of 8-OHdG, HEL, or TAO (Figures 2, 3, and 4). It is possible that the diurnal variation may be exaggerated in shift workers [19] and/or patients with neurological disorders like XPA. In addition, the circadian clock plays an important role in the determination of strengths of cellular responses to DNA damage and may subsequently influence the NER [20]. Accordingly, XPA patients have the potential of demonstrating a robust diurnal variation of biomarkers related to oxidative stress, because NER is genetically damaged, oxidative stress may be augmented in XPA [8], and melatonin metabolism was disturbed [12].

Unfortunately, long-lasting therapies have not been established for neurological disorders in the XPA patients. We tried the treatment with low-dose levodopa for laryngeal dystonia, providing the temporary improvement of dystonia and hand tremor [15]. Melatonin is remarkably functionally diverse with actions as a free radical scavenger and

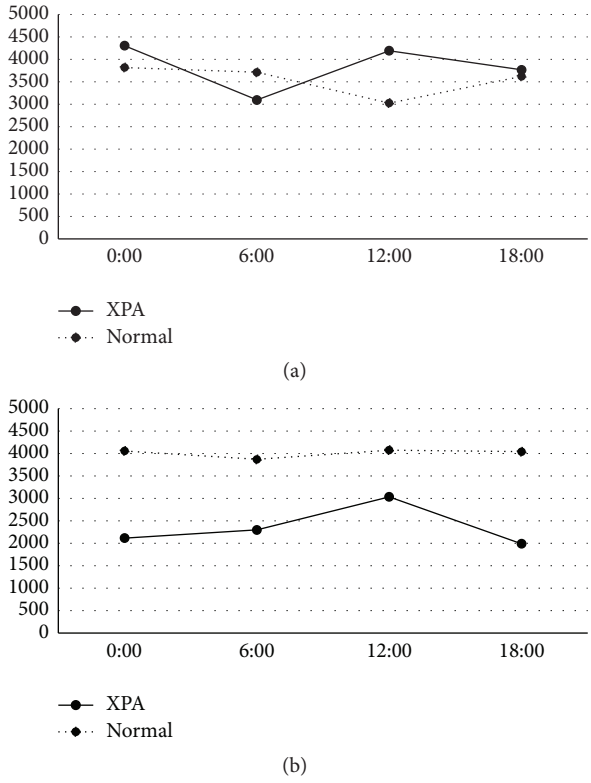

Figure 4: Circadian rhythm of urinary levels of total antioxidant power (TAO) (μM) in younger age group (a) and older age group (b). Solid and dotted lines denote values of patients with xeroderma pigmentosum group A (XPA) and controls, respectively.

antioxidant, circadian rhythm regulator, anti-inflammatory, and immunoregulating molecule [9]. The diseases, having both the circadian rhythm disturbance and oxidative stress, are likely to benefit from therapeutic agents with the combination of circadian rhythm-resynchronizing properties and antioxidant actions. Clinical trials of melatonin have been performed for sleep problems in children with neurodevelopmental disorders, resulting in the partial amelioration of problems without adverse events [21, 22]. In Japan, we are also proceeding with a clinical trial of melatonin for sleep problems in autism spectrum disorders, and other clinical trials, using melatonin, are feasible. We strongly believe that the clinical trial of melatonin should be considered in XPA patients, and we are preparing the trial.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Effects of Moderate Aerobic Exercise on Cognitive Abilities and Redox State Biomarkers in Older Adults

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We used a moderate aerobic exercise program for 24 weeks to measure the positive impact of physical activity on oxidative stress and inflammatory markers and its association with cognitive performance in healthy older adults. A total of 100 healthy subjects (65–95 Yrs) were randomly classified into two groups: control group ($n = 50$) and exercise group ($n = 50$). Cognitive functioning, physical activity score, MDA, 8-OHdG, TAC, and hs-CRP were assessed using LOTCA battery, prevalidated PA questionnaire, and immunoassay techniques. LOTCA 7-set scores of cognitive performance showed a significant correlation with physical activity status and the regulation of both oxidative stress free radicals and inflammatory markers in all older subjects following 24 weeks of moderate exercise. Physically active persons showed a higher cognitive performance along with reduction in the levels of MDA, 8-OHdG, and hs-CRP and increase in TAC activity compared with sedentary participants. Cognitive performance correlated positively with the increase in TAC activity and physical fitness scores and negatively with MDA, 8-OHdG, and hs-CRP, respectively. There was a significant improvement in motor praxis, vasomotor organization, thinking operations, and attention and concentration among older adults. In conclusion, moderate aerobic training for 24 weeks has a positive significant effect in improving cognitive functions via modulating redox and inflammatory status of older adults.

1. Introduction

Cognitive abilities refer to all essential mental skills that control the behavioral lifestyle of humans such as everyday routine work [1]. Decline in cognitive abilities was shown to produce more drastic problems for older adults in performing their daily life activities [2, 3]. However, more studies tried to maintain or enhance cognitive abilities in older adults via enhancing or delaying functional disabilities [4]. The results of these trials are not clear; this is maybe due to the fact that most of these trials concentrated on treatment schedules rather than prevention in older subjects with cognitive deficits or functional disabilities.

Previously it was reported that prevention or improvement of cognitive deficits among normal older adults is accessible but the treatment parameters did not include the outcome measures which may be related to the limited sample randomization and lack of it [5, 6].

The impairment in brain function in older age occurs via many pathological mechanisms [7–10], the most important of which are tissue damage and neural cell death which occurs via the interaction of complex pathophysiological processes [11].

It was reported in many studies that human aging is demarcated with an increase in serious age-related diseases, which related to chronic pathological processes such as inflammation. Whereas the incidence of inflammation along with immunosenescence results in a decline of multiple physiological systems and functional dependence among the elderly [12–14], the negative effects of the imbalance between pro- and anti-inflammatory cytokines on cognitive abilities such as memory and learning deficits were greatly reported among older people with Alzheimer's disease [15].

Many research studies reported that C-reactive protein (CRP) and high-sensitivity CRP (hs-CRP) markers of chronic inflammation have been associated with numerous clinical

conditions, including cognitive decline and depression in old age [16–21]; the causative effects of higher CRP or hs-CRP inflammatory markers on cognitive impairment speculatively occurred via promoting vascular disease or as a result of the inflammatory process linked with disorders in many lifestyle factors including obesity, physical inability, and smoking [22].

Also, many research studies supported the pathogenic role of oxidative stress in chronic diseases related to human aging like cognitive impairment. It is widely accepted that oxidative stress is characterized by disturbance in the hemostatic balance between oxidative stress free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) in human cells and the ability of these cells to conquer this change by their own antioxidant defense pathways [23, 24]. It was reported that older people with cognitive disorders showed lower activity in antioxidant pathways like antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase) and numerous nonenzymatic antioxidants (GSH, vitamins A, C, and E, and carotenoids) [10, 11, 25–27]. The increase in oxidative free radicals leads to their higher accumulation in human neural cells ultimately damaging lipids, proteins, and DNA [28, 29] and produces more biologically active molecules such as malondialdehyde (MDA) and 8-hydroxyguanine (8-OHdG) adducts which are liberated from oxidative cellular damage of the polyunsaturated fatty acids and DNA, respectively. These newly formed free radicals may be involved in further oxidation reactions, generating new oxidative damage [30, 31], and that may be recognized as a reason for brain disorders, cognitive decline, and dementia in old age [32].

Many studies focused on the importance of body physical activity and its positive effects upon cognitive abilities especially in older ages. Physical exercise was shown to play a protective role against hippocampal cell injury which produces brain memory loss [33–36]. Also, physical activity facilitates recovery from injury and improves cognitive function via increase of the expression of many neurotrophic and physiological factors involved in neural survival, differentiation, and improvement of memory function [37–41].

Recent studies reported the potential action of exercise as an antiapoptotic parameter against many brain diseases such as brain inflammatory conditions [42] and mice Parkinson's disease [43] and in the improvement of depressive symptoms [44] and traumatic brain injury [45, 46] and alleviation of memory impairment [47].

It was reported that physical exercise exerts good effects on cognitive abilities with different ways which argue its importance as nondrug and noninvasive essential targets for long term health programs for all ages [48, 49]. The marked improvement was manifested on both function and biomarker integrity as shown in recent studies [50, 51]. Previously, it was reported that antiapoptotic effect of exercise depends mainly on modulation of many physiological processes including DNA damage, oxidative stress, and hormonal changes which are involved in the regulation of apoptosis in various cell types [52]. This depends mainly upon the type of exercise, its intensity, frequency, and duration as the training endpoint [53, 54]. Thus, the benefits of regular physical exercise as a health-ensuring necessity over age, gender, occupation, and affective status cannot be overestimated [55, 56].

Table 1: General characteristics of subjects.

Parameters	Control group ($n = 50$)	Exercise group ($n = 50$)
Male/female	30/20	35/15
Age (years)	67.3 ± 2.8	66.8 ± 3.7
BMI (kg/m^2)	22.3 ± 2.7	23.5 ± 1.7*
Waist (cm)	75.3 ± 10.2	86.3 ± 11.7
Hips (cm)	88.5 ± 5.2	87.5 ± 18.3
WHR	0.82 ± 0.07	0.98 ± 0.10*
Systolic BP (mmHg)	122.2 ± 6.5	118.5 ± 10.8
Diastolic BP (mmHg)	78.5 ± 11.9	82.5 ± 10.3
Fasting blood sugar (mg/dL)	98.5 ± 6.3	105 ± 3.5
HbA1c (%)	6.2 ± 1.5	6.4 ± 1.9
VO_2 max (mL/kg∗min)	32.6 ± 3.7	35.4 ± 2.9
Mean LOTCA score (SD)	97.8 ± 7.91	86.6 ± 8.24**
LTPA (MET-H/week)	123.9 ± 15.6	96 ± 9.7**

Values are expressed as mean ± SD; $^*P < 0.05$ and $^{**}P < 0.01$. Significance at $P < 0.05$.

Therefore, the present study was designed to evaluate the effects of 24 weeks of moderate aerobic exercise on the levels of oxidative stress, MDA, 8-OHdG, TAC, and hs-CRP inflammatory markers and its association with cognitive performance in healthy older adults.

2. Material and Methods

2.1. Subjects. The participants involved in this study were subjected to randomized selection. A random selection of 200 subjects on electoral roll was informed for participation. Out of them, only 100 healthy subjects (70 males, 30 females) were randomized into this study. Their age ranged between 65 and 95 years and mean age was 69.7 ± 5.91 (Table 1). Subjects with physical disability and with endocrine, immune, and psychiatric illness and eating disorders and taking glucocorticoid medication that could interfere with apoptotic and cognitive ability measurements were excluded from this study. Based upon participation in exercise program, subjects were classified randomly into two groups: control group ($n = 50$) and exercise group ($n = 50$). Demographic and anthropometric data of participants were included in Table 1. This study was approved by the Ethical Committee of the Rehabilitation Research Chair of King Saud University (file ID: RRC-2012-08).

2.1.1. Training Procedure. Participants were involved in exercise program designed according to Karvonen's formula [57], three times per week for 24 weeks, whereas training intensity of each intervention was prepared according to maximum and resting heart rate of each participant. During warming the subject performed stretching exercises and walking for

5 to 10 minutes. During the active phase, the subject was allowed to reach his precalculated training heart rate (THR max: 60 to 70% for 45–60 min) in bouts form using treadmill, bicycle, and StairMaster [58, 59]. The exact calculated heart rate of each participant was monitored via a wearable automatic portable heart rate meter (Polar Electro, Kempele, Finland). The exercise test was performed to give the participants physical activities corresponding to 30–45% of VO_2 max uptake [60].

2.1.2. Leisure-Time Physical Activity (LTPA). A validated questionnaire was used to calculate physical activity in the form of a leisure-time physical activity (LTPA). The energy expenditure rates were calculated weekly in metabolic equivalents per hour/week (T-LPTA-MET/H/W) as previously reported [61].

Assessment of Cognitive Abilities

Instrument. Trained research assistants assessed the cognitive abilities of older adults before and after supervised aerobic exercise using the Loewenstein Occupational Therapy Cognitive Assessment (LOTCA) battery. Assessments required between 45 and 90 minutes. The LOTCA consists of seven major domains divided into 26 subtests, with each subtest scored on a four- or five-point Likert scale. The assessment of LOTCA test was performed according to instruction manuals as reported in literature [62].

Results are presented as a profile along all subtests. A composite score for each domain was calculated by summing the scores of the relevant subtests. The LOTCA score was calculated by summing the scores of all subtests. The maximum score on the test is 123, and the minimum score is 27. A higher score indicates better cognitive performance.

LOTCA Test Validity. The test has excellent intrarater reliability (100%) and good interrater reliability (86%) as well as criterion validity (78%) [63]. This LOTCA test was chosen because of its psychometric properties and primarily nonverbal nature, making it potentially more suitable for evaluating the cognitive abilities of individuals from non-Western and non-English-speaking cultures. Several studies have been conducted using this instrument in both Western [63] and Arab populations [1].

Assessment of Oxidative Stress and Inflammatory Parameters. All serum samples were taken from all participants in the morning following an overnight fast at pre- and postexercise training program estimation of the following parameters.

Analysis of hs-CRP. The acute-phase reactant highly sensitive CRP (hs-CRP) is analyzed using commercially available ELISA kits (IBL Inc., Cat. Number: IB59126, USA) according to manufacturers' instructions.

2.1.3. Total Antioxidant Capacity (TAC). Serum total antioxidant capacity (TAC) was measured by Colorimetric Assay Kit (Catalog #K274-100; BioVision Incorporated, CA 95035, USA). The antioxidant equivalent concentrations were measured at 570 nm as a function of Trolox concentration according to the manufacturer's instructions:

$$\frac{\text{Sa}}{\text{Sv}} = \text{nmol}/\mu\text{L or mM Trolox equivalent}, \quad (1)$$

where Sa is the sample amount (in nmol) read from the standard curve; Sv is the undiluted sample volume added to the wells.

Estimation of Malondialdehyde (MDA) and 8-Hydroxyguanine (8-OHdG). Lipid peroxidation was estimated quantitatively by analyzing the levels of malondialdehyde using high performance liquid chromatography as reported previously in the literature [64]. Immunoassay technique was performed to estimate serum 8-OHdG as a marker of DNA damage using a commercially available ELISA kit (DNA Damage ELISA Kit, Product #: EKS-350, Stressgen Co., USA).

2.2. Statistical Analysis. Statistical analysis was performed using SPSS version 17. The data were expressed as mean ± SD. The comparison and correlation of the studied parameters were investigated using both Student's t-test and Pearson's correlation coefficient, respectively. The data was deemed to be significant at P values <0.05.

3. Results

A total of 100 healthy subjects were involved in this study. Seventy percent of the sample was male ($n = 65$), and 60% of subjects were highly educated ($n = 60$). They are classified according to exercise program into control group ($n = 50$) and exercise group ($n = 50$). There was significant difference in WHR ($P = 0.05$), LPTA ($P = 0.001$), and average LOTCA scores ($P = 0.01$) of exercise participants compared to control group (Table 1).

In this study, there was a significant ($P = 0.01$) improvement in all LOTCA 7-subset variables among subjects following 12 weeks of moderate aerobic training compared to pretest and control group that showed slight improvement in LOTCA scores as shown in Table 2. However, significant increase ($P = 0.001$) in the improvement of motor praxis, vasomotor organization, thinking operations, and attention and concentration was reported among older adults following 24-week aerobic exercise. The data revealed positive significant correlations between the LOTCA scores of older subjects and their performance of cognitive abilities. Moreover, significant correlations were obtained between the older subjects in the motor praxis, vasomotor organization, thinking operations, and attention and concentration domains of the LOTCA scores and their performance of functional physical activity as shown in Table 5.

Oxidative stress and inflammatory makers TAC, MDA, 8-OHdG, and hs-CRP were greatly reported in this study. There was significant increase in the activity of TAC and decrease in the levels of MDA, 8-OHdG, and hs-CRP in participants of exercise group following 24 weeks of moderate aerobic training. Also, significant increase in LPTA activity was observed

Table 2: LOTCA scores in studied subjects following 12-week supervised aerobic training program (means ± SD).

Parameters	Control group		Exercise group	
	Pre	Post	Pre	Post
Orientation	12.8 ± 1.8	16.7 ± 2.5*	9.0 ± 2.3	21.8 ± 0.5**
Visual perception	18.2 ± 2.9	21 ± 0.98*	11.3 ± 2.5	18.1 ± 1.9**
Spatial perception	10.5 ± 0.4	13.5 ± 0.4*	9.5 ± 2.1	21.13 ± 0.91**
Motor praxis	8.8 ± 0.68	11.9 ± 0.52*	9.4 ± 3.8	25.7 ± 2.3**
Vasomotor organization	21.3 ± 2.6	25.1 ± 2.86*	11.9 ± 3.7	38.1 ± 2.9**
Thinking operations	23.7 ± 3.7	26.8 ± 2.95*	9.6 ± 2.65	315 ± 2.6**
Attention and concentration	3.7 ± 0.51	3.9 ± 0.18*	2.1 ± 0.31	5.3 ± 0.45**
Total LOTCA score	92.8 ± 6.3	98.9 ± 7.5*	88.7 ± 8.3	110.8 ± 5.6**

Values are expressed as mean ± SD; * $P < 0.05$ and ** $P < 0.01$. Significance at $P < 0.05$.

Table 3: Changes in the level of oxidative stress and inflammatory markers and leisure-time physical activity (LTPA) score of participants following 12-week supervised aerobic training program (means ± SD).

Parameters	Control group ($n = 50$)		Exercise group ($n = 100$)	
	Pre	Post	Pre	Post
TAC (nmol/μL)	16.5 ± 6.3	22.9 ± 11.7*	9.7 ± 2.3	31.2 ± 5.1**
MDA (μmol/L)	4.7 ± 3.5	2.7 ± 1.7*	15.5 ± 6.7	5.1 ± 1.8**
8-OHdG (ng/mL)	0.98 ± 0.05	0.65 ± 0.08*	25.7 ± 4.5	4.6 ± 2.9**
hs-CRP (mg/L)	2.6 ± 2.1	1.8 ± 0.98*	6.8 ± 2.5	2.8 ± 1.3**
LTPA (MET-H/week)	123.9 ± 15.6	145.3 ± 14.5*	96 ± 9.7	315 ± 17.6**

Values are expressed as mean ± SD; * $P < 0.05$ and ** $P < 0.01$. Significance at $P < 0.05$.

Table 4: Correlation coefficients analysis of oxidative damage and antioxidant biomarkers in relation to normal and abnormal hs-CRP levels.

Parameters	Normal (hs-CRP < 3.0) (R) ($n = 50$)	Abnormal (hs-CRP > 3.0) (R) ($n = 50$)
TAC (nmol/μL)	−0.215**	−0.157**
MDA (μmol/L)	0.245**	0.235**
8-OHdG (ng/mL)	0.512**	0.540**

Data presented as coefficient (R); ** significance at <0.001.

Table 5: Posttraining correlation analysis of oxidative stress and inflammatory markers and cognitive abilities (LOTCA scores) variables according to the level of leisure-time physical activity (LPTA-MET-H/week) after 12 weeks of exercise.

Parameters	Exercise group ($n = 100$) (R)	
	(Low LTPA)	(High LTPA)
Total LOTCA score	0.571*	0.561**
Orientation	0.25 *	0.45**
Visual perception	0.48*	0.88**
Spatial perception	0.651*	0.263**
Motor praxis	0.21*	0.13**
Vasomotor organization	0.421*	0.425**
Thinking operations	0.35*	0.22**
Attention and concentration	0.517*	0.275**
TAC (nmol/μL)	0.429*	0.312**
MDA (μmol/L)	−0.545*	−0.668**
8-OHdG (ng/mL)	−0.320*	−0.541**
hs-CRP (mg/L)	−0.350*	−0.515**

Data presented as coefficient (R); * significance at <0.01; ** significance at <0.001.

in exercise group compared to pretest and control group as shown in Table 3.

Also, the data showed significant correlation between oxidative stress markers and the levels of hs-CRP inflammatory markers. There was significant positive correlation between MDA and 8-OHdG along with negative correlation of TAC activity in both participants of normal and abnormal hs-CRP. The data obtained proposed significant interrelations between oxidative stress free radicals which may produce inflammation resulting in increase in hs-CRP levels (Table 4).

The changes in these parameters were shown to be significantly correlated with physical fitness score. In physically active participants, the physical fitness score correlated negatively with the reduction in the levels of MDA, 8-OHdG, and hs-CRP markers and positively with the increase in TAC activity compared to those of lower activity scores (Table 5). Physically active persons showed higher cognitive performance with lower expression in the levels of MDA, 8-OHdG, and hs-CRP and significant increase in TAC activity compared to less active ones. The improvements of cognitive performance of physically active persons correlated positively with the increase in TAC activity and negatively with other

Table 6: Posttraining correlation coefficients among factors involved in oxidative and inflammatory status and cognitive parameters (n = 100).

Parameters	TAC (nmol/μL)	MDA (μmol/L)	8-OHdG (ng/mL)	hs-CRP (mg/L)
Orientation	0.315^{**}	-0.450^{**}	-0.225^{**}	-0.610^{**}
Visual perception	0.215^{**}	-0.314^{**}	-0.250^{**}	-0.435^{**}
Spatial perception	0.198^{**}	-0.289^{**}	-0.365^{**}	-0.580^{**}
Motor praxis	0.311^{**}	-0.352^{**}	-0.465^{**}	-0.674^{**}
Vasomotor organization	0.250^{**}	-0.412^{**}	-0.512^{**}	-0.750^{**}
Thinking operations	0.413^{**}	-0.387^{**}	-0.535^{**}	-0.752^{**}
Attention and concentration	0.365^{**}	-0.478^{**}	-0.620^{**}	-0.588^{**}

Data presented as coefficient (R); **significance at <0.001.

stress and inflammatory markers, MDA, 8-OHdG, and hs-CRP, respectively (Table 6).

4. Discussions

Physical activity as a nondrug modulation is considered one of the most promising strategies to prevent or improve cognitive disabilities among elderly populations [65]. It was reported that physically active people across their entire life minimize the incidence rates of dementia and cognitive difficulties [66–68], whereas many studies reported lower rates of cognitive disorders among subjects who participated in higher levels of physical activity interventions than persons with lower scores of physical activity [69, 70]. Recently, many research works revealed that physical exercise with moderate intensity produces remarkable higher levels of improvement in skills, mobility, and mood in both younger [71] and older [72] adults. However little is known about the positive effect of moderate exercise on cognitive abilities which occurs via modulating oxidative stress and inflammation profile.

Therefore, the current research work aimed to investigate the probable correlation between antioxidative and anti-inflammatory mechanisms of exercise on cognitive abilities among 100 older adults that participated in supervised aerobic training program for 24 weeks.

In the present study, the cognitive abilities of 100 older adults of both control and exercise group were measured using LOTCA scores, a cognitive evaluation test formed of 7-subset variables. In older subjects that participated in moderate aerobic exercise for 24 weeks, there was significant improvement in cognitive performance via increase in all LOTCA 7-subset variables compared to nonexercised group. The data revealed positive significant correlations between the total LOTCA scores of older subjects and their performance of cognitive abilities. Thus, the accuracy and evaluation of LOTCA test support its use as a diagnostic tool for cognitive function as previously reported [1].

Moreover, significant positive correlation was obtained between the older subjects in the motor praxis, vasomotor organization, thinking operations, and attention and concentration domains of the LOTCA scores and their performance of functional physical activity. The data matched with others who suggested the strongest indication of physical exercise benefits on cognition function via enhancing academic performance and psychological well-being [40, 73].

Similarly, our study was in accordance with recent studies that reported improving in cognitive performance on a working memory task among younger and older adults following moderate intensity cycling [74]. Also, our study supported that positive effects of physical exercise intervention are relayed in enhancing psychological well-being, cognitive functioning, and quality of life especially in older subjects with mild cognitive impairment as reported recently in literature [39, 75].

In the present study, there was slight insignificant change in cognitive abilities scores and oxidative and inflammatory related markers among nonexercised control group. This change may be due to low-intensity physical activity such as routine daytime life which accounts for most activity energy expenditure (AEE) in people who do not regularly exercise [76]; these activities may be useful in health outcomes such as cognitive impairment. This is indicated with other research work that reported that older women identified a positive association between cognitive performance and total daytime movement which suggests that total activity may be important for cognitive outcomes [77].

Cognitive disabilities among older adults occurred as a result of brain dysfunction which occurs through many pathological mechanisms including brain tissue damage and neural cell death or apoptosis induced by oxidative free radical and inflammation mechanisms [7, 8, 27, 78].

In this current study, the anti-inflammatory activity of moderate exercise was evaluated in older adults following 24-week exercise interventions. There was decrease in the levels of hs-CRP in moderately exercised older adults compared to baseline and nonexercise control values. The change in the level of hs-CRP significantly correlated with physical activity status and the improvement of cognitive abilities especially motor praxis, vasomotor organization, thinking operations, and attention and concentration in all participants. Many research studies reported multiple positive effects such as lower levels of hs-CRP [79, 80] and a better quality of life [81] among older adults participating in physical exercise training. Previously, higher serum levels of hs-CRP were shown to be closely related to cognitive disorders among older ages of both genders [82]. The positive effects of exercise training on the levels of hs-CRP depend on the mechanisms and type of exercise training [83, 84], which exerts a reduction in serum hs-CRP concentrations via decreasing associated metabolic

risk factors [85], and changes in anthropometric parameters [86], of the participants.

Finally, the data obtained showed that physical activity status, inflammatory status, and oxidative stress played a pivotal role on cognitive performance of healthy older adults.

In conclusion, the data concluded that supervised moderate aerobic training for 24 weeks has a positive significant effect in improving cognitive functions via modulating redox and inflammatory status of older adults.

Competing Interests

The authors declare that they have no competing interests.

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Clinical Study

Oxidative Stress in Children with Chronic Spontaneous Urticaria

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The pathogenesis of chronic spontaneous urticaria (CSU) has not been fully understood; nevertheless, significant progress has been achieved in recent years. The aim of this study was to investigate the possible role of reactive oxygen species (ROS) in the pathogenesis of CSU. Sixty-two children with CSU and 41 healthy control subjects were enrolled in the study. An extensive evaluation of demographic and clinical features was done, and serum oxidative stress was evaluated by plasma total oxidant status (TOS) and total antioxidant status (TAS) measurements. The median value of plasma TOS was found to be 10.49 μmol H_2O_2 equiv./L (interquartile range, 7.29–17.65) in CSU patients and 7.68 μmol H_2O_2 equiv./L (5.95–10.39) in the control group. The difference between the groups was statistically significant ($p = 0.003$). Likewise, the median plasma TAS level in the CSU group was decreased significantly compared to that of the control group (2.64 [2.30–2.74] versus 2.76 [2.65–2.86] mmol Trolox equiv./L, resp., $p = 0{,}001$). Our results indicated that plasma oxidative stress is increased in children with CSU when compared to healthy subjects, and plasma oxidative stress markers are positively correlated with disease activity.

1. Introduction

Chronic urticaria (CU) is defined as urticaria that has been continuously or intermittently present for at least six weeks [1]. The lifetime prevalence of CU is 2-3% in the general population, and at any time 0.5–1% of the population suffers from the disease [1, 2]. The prevalence of CU in children is much lower than in adults and is reportedly as low as 0.1–0.3% of the child population [3]. The etiology of CU is quite heterogeneous; autoimmunity, physical stimuli, infections, vasculitis, and allergies (e.g., foods, drugs, latex, and food constituents) are major etiologic causes [1]. An underlying cause can be outlined in only 20–55% of children with CU [4, 5]. Physical stimuli are the most commonly identified etiological reasons, and approximately 15% of patients with CU have a physical trigger for the development of urticarial lesions; this subgroup of CU is termed as physical urticaria (PU) [6].

The exact pathogenesis of CU is not well delineated. The presence of persistent activation of dermal mast cells is a hallmark of CU pathogenesis, but the underlying mechanism(s) of mast cell activation is/are an enigma [1]. Autoimmune origin has become the most accepted hypothesis in recent years [7]. Functional autoantibodies in CU patients' sera have been demonstrated against IgE and FcεRIα by basophil and mast cell histamine release assays, by basophil activation assays, and by autologous serum skin test (ASST) [8]. This subgroup comprises roughly one-third of CU patients and is called autoimmune urticaria (AIU) [2, 9]. Chronic spontaneous urticaria (CSU) is by far the most common form of CU. In this

form, triggering factors cannot be revealed despite detailed clinical and laboratory investigations.

Reactive oxygen species (ROS) are defined as molecules containing oxygen with unpaired electrons capable of initiating oxidation [11]. Although ROS play roles in host defense (e.g., phagocytosis) and as messenger molecules of the autocrine and paracrine systems, overproduction of these molecules can cause tissue damage and inflammation and even cell death [12, 13]. Fortunately, our bodies are equipped with an effective antioxidant system that includes enzymes, proteins, and low-weight molecules [12]. All inflammatory cells produce a significant amount of ROS upon stimulation [11]. Eosinophils have elevated peroxidase levels compared to other inflammatory cells and play a unique role in generating oxidative stress [11]. Also, mast cell stimulation via FcεRI signaling causes intracellular and extracellular ROS generation [14–16]. Blockades of ROS generation in mast cells by a pharmacological inhibitor cause a decrease in the release of preformed granular mediators [17]. There is a growing body of literature indicating that ROS can cause endothelial dysfunction and increased vascular permeability [18, 19]. In the light of the above information we aimed to investigate possible role of ROS in the pathogenesis of CSU.

2. Materials and Methods

2.1. Participants. All patients (total of 96) who were admitted with an urticarial episode lasting more than six weeks between February and December 2014 to pediatric allergy outpatient clinics of two university hospitals (Istanbul University Istanbul Medical Faculty and Bezmialem Vakif University) were enrolled in the study. Detailed medical histories were taken and physical examinations were performed. Subjects who had acute infections, who had a history of maternal or paternal smoking, who were taking any medications except antihistamines or montelukast (including polyvitamins, mineral supplements, analgesics, and mucolytics), who were overweight or obese (body mass index [BMI] > 85th percentile), and who had concomitant diagnosed asthma, allergic rhinitis, or atopic dermatitis were excluded from the study [20–24]. Twelve children who were diagnosed with PU, seven patients in whom an underlying cause was identified [thyroid autoantibodies in three children, *Helicobacter pylori* (*H. pylori*) infection in two patients, food allergy in one, and urinary infection in one], one patient who was obese, two patients who had a history of paternal smoking, one patient whose family refused to participate in the study, six patients who had proven concomitant asthma, four patients who had concomitant allergic rhinitis, and one patient who had concomitant atopic dermatitis were excluded from the study (Figure 1). The control group was constituted by 41 healthy children who were periodically attending pediatric welfare clinics of the same hospitals for regular checkups. Children who were included in the control group had no history of acute or chronic urticaria, no history of any allergic disease, and no paternal or maternal smoking and were not taking any medications, including multivitamins, minerals, and analgesics. These subjects also had no signs and symptoms of infectious disease and were not overweight or obese. The study was performed in accordance with the tenets of the Declaration of Helsinki and good clinical practice and was approved by the Bezmialem Vakif University Ethical Committee (Number 71306642/050-99/88). Informed consent was obtained from the parents of all study participants.

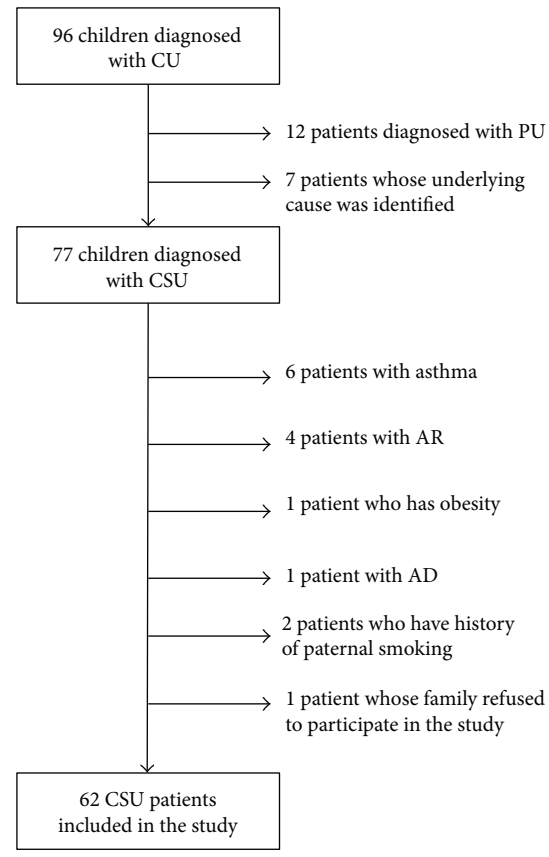

Figure 1: Flow diagram of patients with CU. Exclusion criteria are shown to the right. CU, chronic urticaria; CSU, chronic spontaneous urticaria; PU, physical urticaria; AR, allergic rhinitis; AD, atopic dermatitis.

2.2. Assessment of Disease Activity. Disease activity was monitored using the urticaria activity score (UAS7) according to EAACI/GA^{2}LEN/EDF/WAO Guidelines [10]. The UAS7 consisted of the sum of the wheal number score and the itch severity score, that is, the sum score of seven consecutive days, which can be from 0 to 42 (Table 1). The guidelines state that UAS7 is best measured by patients documenting 24-hour self-evaluation scores once daily for several days [10]. Therefore, we teach our patients and their families how to calculate the UAS7 and instruct them to bring us this information.

2.3. Blood Sample Collection. Antihistamines and montelukast were discontinued at least 24 hours before blood sampling. After overnight fasting, peripheral blood samples (total, 4 mL) were collected from an antecubital vein into heparinized tubes; thereafter, blood was centrifuged at 1500 ×g for 10 min to obtain the plasma. The separated plasma was

Table 1: The UAS7 for assessing disease activity in CSU [10].

Score	Wheals	Pruritus
0	None	None
1	Mild (<20 wheals/24 h)	Mild (present but not annoying or troublesome)
2	Moderate (20–50 wheals/24 h)	Moderate (troublesome but does not interfere with normal daily activity or sleep)
3	Intense (>50 wheals/24 h or large confluent areas of wheals)	Intense (severe pruritus, which is sufficiently troublesome to interfere with normal daily activity or sleep)

then stored at −80°C until further analysis of TAS and TOS levels.

2.4. Laboratory Investigations. Complete blood count, erythrocyte sedimentation rate, liver function tests, thyroid stimulating hormone, free thyroxin, serum total IgE, antithyroid peroxidase and anti-thyroglobulin antibodies, microscopic investigation of stool for parasites and stool enzyme immunoassay for *Helicobacter pylori* antigens, urinalysis with test strips, and urine cultures were carried out in all patients as per standard laboratory procedures.

Skin prick tests and ASST were also performed. Commercial allergen solutions manufactured by Stallergenes (Paris, France) were used for skin tests. Ten different aeroallergens consisting of house dust mites, grass, tree pollens, fungi, and animal dander and nine food allergens consisting of milk, egg, wheat, soy, cocoa, peanut, hazelnut, banana, and strawberry were tested. A positive skin prick test was one with at least a wheal of maximum diameter of 3 mm once the negative value had been subtracted. ASST was performed using the method described by Sabroe et al. [25]. We were not able to perform skin prick tests and ASST in 13 patients because their antihistaminic treatments could not be discontinued due to their intense complaints caused by the disease.

2.5. Measurement of Total Oxidant Status (TOS). Plasma TOS was measured using an automated method developed by Erel [26]. Oxidants present in a sample oxidize the ferrous ion of an o-dianisidine complex to ferric ions. Oxidation is enhanced by glycerol, which is abundant in the reaction medium, and the ferric ion forms a colored complex with xylenol orange under acidic conditions. Color intensity (which can be measured spectrophotometrically) is associated with the total level of oxidants present. Hydrogen peroxide is used to calibrate the assay, and results are expressed in terms of micromoles of hydrogen peroxide equivalent per liter (μmol H_2O_2 equiv./L).

2.6. Measurement of Total Antioxidant Stress (TAS). Plasma TAS was measured using another automated method developed by Erel [27]. This involves the production of the hydroxyl radical, which is a potent biological reactant. A ferrous ion solution (Reagent 1) was mixed with hydrogen peroxide (Reagent 2). Radicals produced by the hydroxyl radical, including the brown dianisidinyl radical cation, are also potent in biological terms. Thus, it is possible to measure the antioxidative capacity of a sample in terms of the inhibition of free radical reactions initiated by the production of the hydroxyl radical. Variation in assay data is very low (less than 3%), and results are expressed as mmol Trolox equiv./L.

2.7. Calculation of the Oxidative Stress Index (OSI). The OSI was the TOS-to-TAS ratio. It was calculated as follows: OSI (arbitrary units) = TOS (μmol H_2O_2/L)/TAS (mmol Trolox/L). Results are expressed as arbitrary units (AU).

2.8. Statistical Analyses. Statistical analysis was performed using IBM SPSS 19 (IBM, Armonk, NY, USA). The Shapiro-Wilk normality test was used to test the distribution of the data. Parametric data were expressed as the mean ± standard deviation (SD), and nonparametric data were expressed as the median, IQR (interquartile range). A Mann-Whitney U test was used to compare the two groups. The correlation between two variables was tested using Spearman's rho coefficient. Categorical data were evaluated using the chi square test, and a p value of less than 0.05 was accepted as statistically significant.

3. Results

The study group consisted of 37 boys and 25 girls, and the control group consisted of 22 boys and 19 girls. The mean ages of the CSU patients and those of the control group were 10.5± 4.1 and 9.2 ± 3.8 years, respectively. The mean values of BMI were 20.3±3.7 in CSU patients and 18.9±2.9 in control group. There were no significant differences between the groups with respect to age, gender, and BMI ($p > 0.05$). Forty percent of CSU patients were experiencing angioedema during their illness. The median duration of disease was 12 months (5–24) and the median value of UAS7 was 22.0 (13.0–30.5). Some clinical features of CSU patients are shown in Table 2.

The median values of plasma TOS were 10.49 μmol H_2O_2 equiv./L (7.29–17.65) in CSU patients and 7.68 μmol H_2O_2 equiv./L (5.95–10.39) in the control group. The difference between the groups was statistically significant ($p = 0.003$, Figure 2). Plasma TAS levels in the CSU group were significantly decreased compared to the control group (2.64 [2.30–2.74] versus 2.76 [2.65–2.86] mmol Trolox equiv./L, resp., $p = 0.001$; Figure 2). Additionally, there was a statistically significant difference between OSI levels in the CSU group and healthy controls (3.97 [3.01–6.47] versus 2.95 [2.22–3.76] AU, resp., $p < 0.001$; Figure 2). All of these oxidative stress parameters did not vary between the groups in terms of gender, presence or absence of angioedema, or ASST

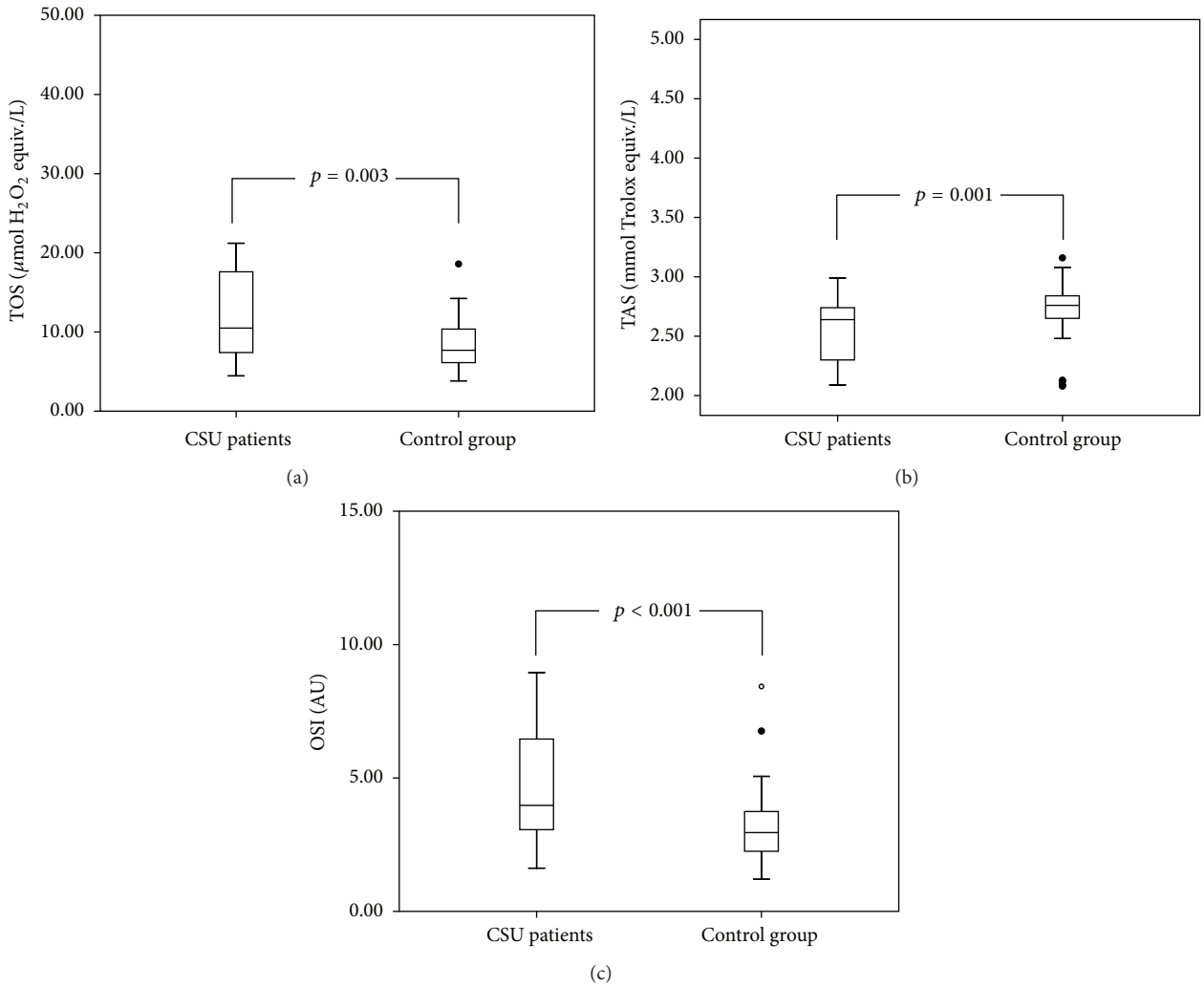

Figure 2: (a) Plasma total oxidant status (TOS); (b) total antioxidant status (TAS); and (c) oxidative stress index (OSI) levels in study and control groups.

positivity ($p > 0.05$). Also there was not a correlation between body weight or BMI and all these parameters ($p > 0.05$). TOS and OSI levels showed statistically significant positive correlation with UAS7 (rho = 0.381, $p = 0.002$, and rho = 0.337, $p = 0.008$, resp.; Figure 3). Moreover, TAS values showed statistically significant negative correlation with disease duration (rho = −0.407, $p = 0.001$).

4. Discussion

To the best of our knowledge, this is the first study showing that patients with CSU have elevated plasma TOS and OSI levels and reduced TAS levels compared to healthy controls. Also, this is the first study that focuses on the pediatric age group and demonstrates oxidative stress in CSU. This is especially important because CSU is a rare condition in children compared to adults, and recommendations for its management and treatment in the pediatric age group are based on data obtained from studies conducted on adults [28]. Even comprehensive guidelines that have been published on this issue contain little information related to children [1, 10]. We defined strict exclusion criteria to avoid confounding results. All proven diseases and factors associated with oxidative stress were eliminated as thoroughly as possible. Although only little evidence presents *H. pylori* as an etiological factor in CSU, we accepted *H. pylori* infection as an exclusion criterion for eliminating possible confusion [1, 10].

Although CSU is reportedly more prevalent in females [29, 30], there was male dominance (60%) in our study, which is compatible with studies by Volonakis et al. and Sahiner et al. [4, 31]. We did not find any difference in plasma TOS, TAS, OSI levels, and UAS7 score between boys and girls ($p > 0.05$). We excluded patients with concomitant asthma, allergic rhinitis, and atopic dermatitis from the study, because there are some studies showing that all these diseases are associated with elevated oxidative stress [22–24]. However, the frequency of concomitant allergic diseases in our CSU group was not significantly higher when compared to prevalence rates of allergic diseases in our city. The rates of elevated serum IgE, eosinophilia, and aeroallergen sensitization of our

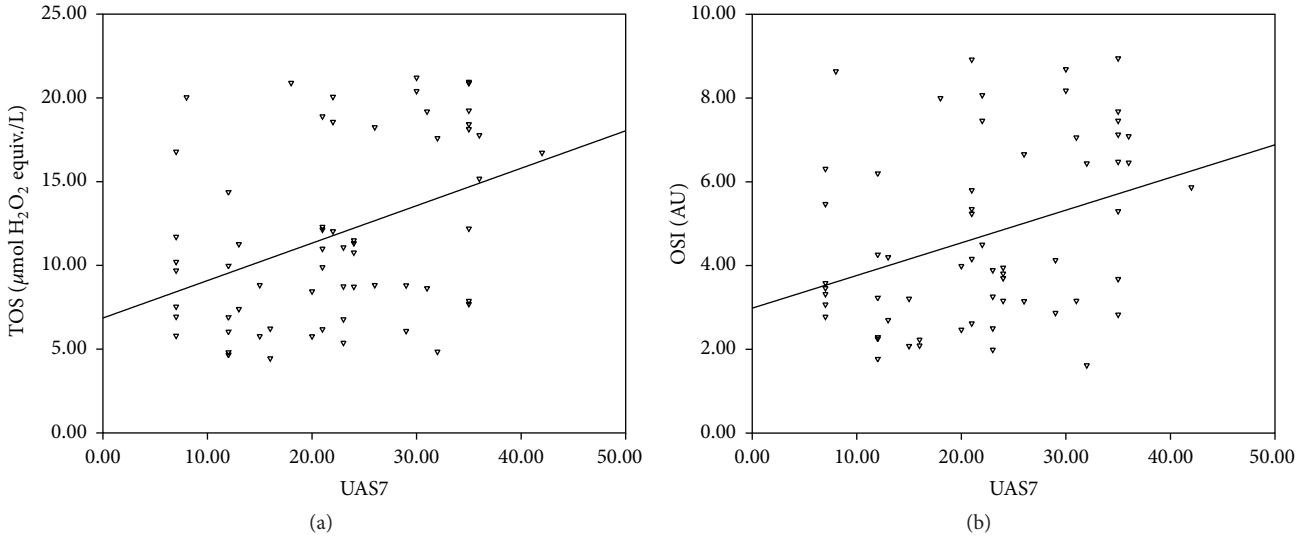

Figure 3: (a) Correlation graphs between total oxidant status (TOS) and urticaria activity score (UAS7) and between oxidative stress index (OSI) and UAS7 (b).

Table 2: Some demographic and clinical features of the study group.

	CSU patients (n = 62)
Age years (mean ± SD)	10.5 ± 4.1
Gender (M/F)	37/25
Disease duration months (median, [IQR])	12 (5–24)
Angioedema (n [%])	25/62 (40%)
BMI (mean ± SD)	20.3 ± 3.7
Total IgE > 100 Iμ/L (n [%])	22 (35%)
Eosinophils > 5% (n [%])	6 (10%)
Aeroallergen sensitization (n [%])	10/49 (20%)
ASST positivity (n [%])	
ASST-positive	14/49 (29%)
ASST-negative	35/49 (71%)
UAS7 (median, [IQR])	22.0 (13–30.5)

CSU, chronic spontaneous urticaria; SD, standard deviation; IQR, interquartile range; ASST, autologous serum skin test; UAS7, urticaria activity score; BMI, body mass index.

study group were compatible with literature data [31]. We concluded, like other researchers, that atopy and gender are not associated factors with CSU [31].

We detected ASST positivity in 29% of patients. Although this is consistent with the literature findings, including the results of our previous study [29, 30], higher rates of up to 46% have also been reported [31]. Sabroe et al. have suggested that the presence of a positive ASST is associated with more severe symptoms in adult patients [32], but studies conducted on children do not support this judgement and reported the same clinical severity or remission rates in children with negative ASST [30, 31, 33]. According to our results, UAS7, plasma TAS, TOS, and OSI levels were not different between ASST-positive and ASST-negative CSU patients ($p > 0.05$). Hence, AIU is not associated with more pronounced oxidative stress or more severe clinical courses compared to idiopathic forms of CSU.

There is a growing body of literature on the role of oxidative stress in allergic diseases, especially asthma, and to a lesser degree atopic dermatitis [11, 22–24]. There are only a few studies with conflicting results in urticaria [34–37]. There are many members of the oxidative and antioxidative systems, and the half-lives of ROS are usually short. Thus, the measurement of these molecules individually is time-consuming and costly and requires complicated techniques. Plasma TOS and TAS measurements are rapid, easy, reliable, sensitive, and inexpensive methods for assessing oxidative stress and have shown good correlation with other markers of oxidative stress and clinical parameters [23, 26, 27]. Although the normal values of TAS and TOS levels in healthy newborns have been determined, the normal values of other age groups including our study group are not yet known [38]. TAS and TOS values were not statistically different in male and female newborns in this study [38]. But it is well known that younger people have higher levels of oxidative stress biomarkers due to their high metabolic rates [12].

Cassano et al. reported elevated ROS production in platelets of adult CU patients compared to healthy controls, and, after treatment with desloratadine, they showed significant decreases in ROS production [34]. Nevertheless, even after treatment with desloratadine, ROS production was still high and not comparable to healthy controls [34]. In another study performed in adult females with CSU, plasma and erythrocyte manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/ZnSOD), glutathione peroxidase (GSH-PX), and catalase (CAT) activities were measured as indices of enzymatic antioxidant capacity, as well as malondialdehyde (MDA) levels as a marker of lipid peroxidation [35]. However, no difference was detected in the levels of those markers between the patients and healthy controls.

Nonenzymatic antioxidant capacity could not be evaluated in this study [35]. It is well known that the antioxidant system has numerous members, whose antioxidant effects are additive [12, 27]. Results might also be influenced by the study population, which consists of female patients only, because the expression of some antioxidant enzymes, including those that are evaluated in their study, and others, like paraoxonase-2, is influenced by gender and sex hormones [39, 40]. Sagdic et al. reported that patients with CU have similar erythrocyte MDA levels and GSH-PX activities and decreased Cu/Zn SOD activities compared to healthy controls [36]. However, only 25 patients are enrolled in this study; hence, the small sample size makes it difficult to demonstrate the difference between the groups statistically. On the other hand, in the study of Kalkan et al., patients with acute urticaria were found to have elevated Cu/Zn SOD activities and MDA levels and decreased plasma GSH-PX activities compared to healthy controls [37].

Although UAS7 is family-derived information, it is a simple, widely accepted, and validated scoring system [10]. CSU symptoms change frequently in intensity and physicians can assess symptoms only for a short period of time. Therefore, disease activity is best measured by self-evaluation scores for several days [10]. Our findings revealed that plasma TOS and OSI levels are positively correlated with UAS7 score ($p = 0.002$ and $p = 0.008$, resp.). Likewise, in the study of Rajappa et al., the authors reported that adult patients with CSU had significantly reduced platelet SOD and GSH-PX levels and elevated platelet MDA levels compared to healthy controls. Platelet MDA levels also showed a statistically significant positive correlation with urticaria severity score [41]. We also showed a statistically significant negative correlation between the duration of CSU and plasma TAS levels (rho: −0.407, $p = 0.001$). The reduction in the plasma levels of the antioxidant system components for balance of the oxidative stress over a prolonged period may be possible explanation of this situation. However, TOS and OSI levels were not correlated with disease duration ($p > 0.05$).

Wheals, flares, and angioedema, which are characteristics of CSU, develop as a result of increased vascular permeability and extravascular leakage of intravascular fluid and proteins [42]. Also, there is convincing evidence showing that ROS can directly lead to degranulation of mast cells, endothelial dysfunction, and increased vascular permeability [11, 18, 19, 43]. When our study results and the aforementioned literature data are analyzed together, it comes plausible that antioxidants may be useful complementary therapies for CSU. We think that despite some limitations vitamin C, vitamin E, carotenoids, selenium, and N-acetylcysteine may be promising molecules for this purpose [44–51].

5. Conclusion

Our study results showed that oxidative stress is increased in children with CSU and is positively correlated with the disease activity. This information is valuable for both understanding the pathogenesis of CSU and finding targeted, more effective, and less toxic treatment alternatives. Fortunately, many drugs currently available, such as vitamins and minerals, can modify oxidative stress. However, for a better understanding of this issue many more studies are needed.

Competing Interests

The authors declare no competing interests with respect to the research, authorship, and/or publication of this paper.

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Research Article

Effect of Intraperitoneal Etanercept on Oxidative Stress in Rats with Peritonitis

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Our aim was to evaluate effect of etanercept on oxidative stress parameters in rats with experimental peritonitis and investigate the availability of etanercept usage in the treatment of peritonitis in the future. Twenty-eight rats were divided into four groups as control (group 1), peritonitis (group 2), peritonitis + cefazolin sodium (group 3), and peritonitis + cefazolin sodium + etanercept (group 4). Peritoneal tissue and blood samples were taken from all of the rats for histopathological and biochemical examination. The oxidative stress parameters were examined in blood and tissue samples. It was observed that rats with peritonitis benefit from cefazolin sodium treatment. Evaluating the effectiveness of etanercept was our main objective for this study. In this perspective, we compared group 3 and group 4 and found statistically significant decreases in oxidative parameters and statistically significant increases in antioxidants in serum and tissue samples in group 4. It is observed that there was a significant contribution of etanercept on biochemical and also histopathological results. As a result, the TNF-α inhibitor, etanercept, in addition to antibiotics given in the early treatment of peritonitis results in more significant improvement of histopathological and oxidative parameters as compared to antibiotics alone.

1. Introduction

Peritonitis, which is the inflammation of the peritoneal tissue, can cause systemic inflammatory response and sepsis. This most likely occurs because infectious agents can pass rapidly from the peritoneal surface to circulation [1]. Peritoneal sepsis is a clinical entity with high rates of morbidity and mortality. Therefore, limiting peritonitis in early stages is of paramount importance. In fact, proinflammatory cytokines such as TNF-α and IL-1 are increased as a part of the inflammatory response in order to limit tissue damage [2]. However, overproduction of proinflammatory cytokines disrupts the normal immune response and causes a pathological response. As a result, capillary leakage, tissue damage, and multiorgan failure can occur. TNF-α plays an important role in this process and is considered as the main mediator in the early stage of inflammation [3]. Etanercept is the competitive inhibitor of TNF-α which inhibits the binding of TNF-α to cell surface receptors and limits its biological activity. Suppression of TNF-α by etanercept in the case of excessive immune response can play an important role in the limiting of inflammation [4]. Inflammation causes oxidative stress which often results in decreased antioxidant levels and increased production of oxidant. The presence of oxidative stress is an important parameter which can be measured to indicate tissue damage.

2. Material and Methods

Our experimental study was conducted in Dicle University by Professor Dr. Sabahattin Payzın at the Health Science Research and Application Center (DUSAM) with

Table 1: Histopathological scoring system for the tissue evaluation.

Absent	Minimal	Weak	Moderate	Strong
0	1	2	3	4

the approval of ethical committee for animal experiments. The project was supported by Dicle University Coordination of Scientific Research Projects (project number 13-TF-34). A total of 28 adult female Wistar Albino rats weighing between 200 and 250 g were used and fed with standard chow and water prior to the experiment.

Twenty-eight rats were divided into four groups ($n = 7$ per group) as follows:

Group 1: control group, in which rats did not receive any drugs.

Group 2: peritonitis group, in which intraperitoneal injection of 1.5 mL *Escherichia coli* suspension (107 CFU/mL) was made to cause experimental peritonitis.

Group 3: peritonitis + cephazolin sodium group, in which intraperitoneal cephazolin sodium injection (50 mg/kg) was made one hour after the injection of *E. coli* suspension.

Group 4: peritonitis + cephazolin sodium + etanercept group, in which intraperitoneal cephazolin sodium injection was made one hour after the injection of *E. coli* suspension (50 mg/kg). Also intraperitoneal etanercept injection was made one and four hours after the injection of *E. coli* suspension. The study was terminated 24 hours after the etanercept injection.

Ketamine hydrochloride (70 mg/kg) was administered intramuscularly (im) to rats before surgery. Rats under anesthesia were fixed at the supine position. Midline incision of the anterior abdominal wall was performed on all of the rats for histopathological and biochemical examination. Full thickness tissues except skin with 1 cm of length and 3 mm of thickness were taken from left half and right half of the midline abdominal wall, respectively, for biochemical and histopathological examination. Tissue samples for histopathological examination were fixed in 10.0% buffered formalin. Tissue samples for biochemical examination were taken into the aluminum foil. Then, sternotomy was made and 5 mL blood sample was taken from the heart. Also, with this method, killing of the animals was achieved.

2.1. Biochemical Analysis. Blood was centrifuged at 5,000 rpm and 4°C for 8 minutes and supernatants were collected for study. Tissue samples were placed in refrigerator at −20°C until homogenized. Tissue homogenization was performed using a homogenizer in the laboratory. After that, paraoxonase (PON), malondialdehyde (MDA), nitric oxide (NO), total antioxidant capacity (TAC), total oxidant stress (TOS), and tumor necrosis factor alpha (TNF-α) were studied from tissue and blood samples in the biochemical laboratory of Dicle University.

PON was studied in the Abbott Architect® c16000 autoanalyzer by using RL0031 Rel Assay® Diagnostics Paraoxonase (Gaziantep, turkey) kit. NWLSS (Northwest Life Science Specialties) Malondialdehyde Assay kit was used for malondialdehyde and reading was made at 450 nm by Dynex micro ELISA device. Cayman Chemical Company 780001 Nitrite Colorimetric Assay kit was used for NO and reading was made at between 540 nm and 550 nm by Dynex micro ELISA device. TAC was studied in in the Abbott Architect® c16000 autoanalyzer by using RL0017Rel Assay® Diagnostics TAS kit (Gaziantep, Turkey). TOS was studied in the Abbott Architect® c16000 autoanalyzer by using RL0024 Rel Assay® Diagnostics (Gaziantep, Turkey) TOS Assay kit. TAC AND TOS were studied by the method of Erel. Serum levels of TNF-α were measured by Biosource Rat TNF-α kit (lot number KRC3011), which is a solid phase sandwich enzyme linked immune sorbent assay (ELISA).

2.2. Histopathological Analysis. Paraffin sections of 4-5-micrometer thickness were taken from tissue samples by using rotary microtome. These sections were stained with hematoxylin and eosin (H&E) and examined by the Nikon Eclipse 400 digital camera (Nikon DSRi). Peritoneal epithelial shedding (desquamation), congestion in the lamina propria, neutrophil infiltration in the lamina propria, and edema in the lamina propria were evaluated histopathologically and scored from 0 to 4 (Table 1).

2.3. Statistical Analysis. Data analyses were performed using Statistical Package for Social Sciences (SPSS), Version 16.0 for Windows. All the data are presented as mean ± standard error. Kruskal-Wallis test was used to analyze multiple groups and Mann-Whitney U test was used for binary comparison of groups. $p < 0.05$ was considered statistically significant.

3. Results

We found statistically significant differences between group 1 and group 2 in terms of oxidative parameters and antioxidants in the serum and peritoneal tissue. There were an increase in oxidative parameters and a decrease in antioxidants in group 2 according to group 1. When we compared group 2 and group 3, there was a statistically significant decrease in the oxidative parameters (MDA, TOS, and TNF-α) in serum and tissue samples in group 2, whereas there was a statistically significant increase in the antioxidant parameters (TAC, PON) in serum and tissue sample in group 3 (Table 2). However, there was no statistically significant difference between groups in terms of NO in serum ($p = 0.090$) and the tissue samples ($p = 0.264$). Evaluating the effectiveness of etanercept was our main aim in this study. In this perspective, we compared group 3 and group 4 and found statistically

Table 2: Comparison of serum and tissue oxidant and antioxidant parameters.

Parameters	Group 1 ($n = 7$)	Group 2 ($n = 7$)	Group 3 ($n = 7$)	Group 4 ($n = 7$)
Serum				
MDA (mmol/mL)	0.73 ± 0.17	1.67 ± 0.32[d]	1.28 ± 0.21[b]	0.90 ± 0.26[c,e]
TOS (μmol/L)	33.59 ± 11.23	80.30 ± 11.26[d]	52.73 ± 12.48[c]	39.07 ± 7.07[c,e]
NO (μM/L)	4.85 ± 0.39	5.80 ± 0.46[a]	5.41 ± 0.32[c]	4.90 ± 0.32[b,c]
TNF-α (pg/mL)	2.72 ± 1.45	18.50 ± 10.01[a]	9.01 ± 2.06[b]	5.77 ± 2.34[b,c]
TAC (mmol/L)	1.32 ± 0.10	0.87 ± 0.30[a]	1.18 ± 0.10[b]	1.56 ± 0.11[e,f]
PON (U/L)	197.03 ± 37.63	124.61 ± 15.81[a]	145.67 ± 7.75[b]	157.70 ± 10.25[b,c]
Peritoneal tissue				
MDA (mmol/mL)	0.12 ± 0.25	1.31 ± 0.41[d]	0.59 ± 0.35[b]	0.12 ± 0.07[c,e]
TOS (μmol/L)	7.16 ± 3.53	45.90 ± 26.57[a]	14.18 ± 4.58[b]	8.77 ± 4.11[b,c]
NO (μM/L)	3.95 ± 0.87	6.65 ± 1.36[a]	6.00 ± 0.52	5.29 ± 0.22[b,c]
TNF-α (pg/mL)	116.39 ± 42.36	487.21 ± 238.44[a]	265.22 ± 52.74[b]	136.83 ± 24.26[b,f]
TAC (mmol/L)	0.80 ± 0.11	0.34 ± 0.08[d]	0.47 ± 0.064[b]	0.66 ± 0.14[c,e]
PON (U/L)	12.74 ± 3.32	1.83 ± 2.54[d]	4.82 ± 1.37[b]	8.34 ± 1.14[e,f]

MDA: malondialdehyde, NO: nitric oxide, TNF-α: tumor necrosis factor alpha, TAC: total antioxidant capacity, TOS: total oxidant stress, and PON: paraoxonase.
[a] $p < 0.005$ as compared to group 1, [b] $p < 0.005$ as compared to group 2, [c] $p < 0.005$ as compared to group 3, [d] $p < 0.001$ as compared to group 1, [e] $p < 0.001$ as compared to group 2, and [f] $p < 0.001$ as compared to group 3.

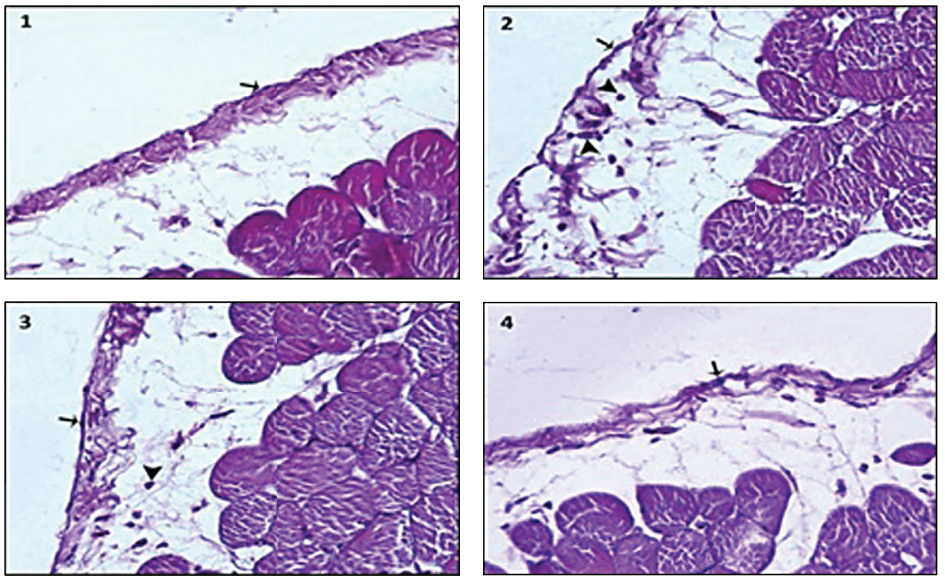

Figure 1: Microscopic findings of the parietal peritoneum in groups ((1) control group, (2) peritonitis group, (3) peritonitis + cephazolin sodium group, and (4) peritonitis + cephazolin sodium + etanercept group).

significant decreases in oxidative parameters (MDA, TNF-α, TOS, and NO) in serum and tissue samples and statistically significant increases in antioxidants (TAC, PON) in serum and tissue samples in group 4 (Table 2). These results were more prominent in comparison of group 2 and group 4.

Microscopic findings of parietal peritonitis in the groups were shown in Figure 1. In our study, we also found that there were no histopathological changes in peritoneal epithelium and lamina propria in group 1. In group 2, there were peritoneal epithelial shedding, diffuse neutrophil infiltration and edema in the lamina propria, and congestion in capillary vessels. We observed that there was a statistically significant reduction in peritoneal epithelium shedding ($p < 0.01$), edema ($p < 0.01$), congestion, and neutrophil infiltration in the lamina propria ($p < 0.01$) in group 3 compared to group 2 due to effect of cephazolin sodium (Table 3). In group 4, in which we used etanercept, there was a statistically significant reduction in the epithelial shedding, congestion, edema, and neutrophil infiltration in the lamina propria according to both group 2 (resp., $p < 0.01$, $p < 0.01$, and $p < 0.001$) and

Table 3: Histopathological results.

Histopathological results	Control group (group 1)	Peritonitis group (group 2)	Peritonitis + cephazolin sodium group (group 3)	Peritonitis + cephazolin sodium + etanercept group (group 4)
Peritoneal epithelial shedding (desquamation)	0.0 ± 0.0	3.71 ± 0.48[a]	2.71 ± 0.48[c]	1.0 ± 0.57[c.e]
Congestion	0.0 ± 0.0	4.0 ± 0.0[b]	2.42 ± 0.78[c]	1.28 ± 0.48[c.f]
Neutrophil infiltration	0.0 ± 0.0	4.0 ± 0.0[b]	2.28 ± 0.48[c]	1.14 ± 0.37[d.e]
Edema	0.0 ± 0.0	3.71 ± 0.48[a]	1.85 ± 0.69[c]	1.14 ± 0.37[c.f]

[a] $p < 0.01$ as compared to group 1, [b] $p < 0.001$ as compared to group 1, [c] $p < 0.01$ as compared to group 2, [d] $p < 0.001$ as compared to group 2, [e] $p < 0.01$ as compared to group 3, and [f] $p < 0.05$ as compared to group 3.

group 3 (resp., $p < 0.01$, $p < 0.05$, and $p < 0.01$). There were statistically significant differences between groups in terms of histopathological results (Table 3).

4. Discussion

Peritonitis is the inflammation of visceral or parietal peritoneum. It can cause systemic inflammatory response and sepsis if not treated properly. It is still one of the significant causes of morbidity and mortality worldwide despite improvements of treatment methods, intensive care unit conditions, and intensive care units devices. Peritonitis is not a simple infection process; inflammation and immunological dysregulation are activated by different mechanisms and can lead to multiple organ failure. Therefore, peritonitis is an important clinical table, which can cause multiple organ failure. Limiting this process in stage of peritonitis is crucial. It is thought that there are some unexplained pathologic mechanisms in this process and therefore experimental models are needed.

Peritoneal membrane is semipermeable and has an advanced ability of secretion and absorption. If urea, fluids with electrolytes, drugs, and infective material are given to peritoneal cavity, they can rapidly pass to systemic circulation. Therefore, peritonitis models in experimental animal studies are valuable for creating sepsis and they are closest designs to human sepsis.

Inflammation is an important part in the formation of sepsis. Proinflammatory cytokines such as TNF-α and IL-1 were secreted to limit tissue damage. However, overproduction of proinflammatory cytokines disturbs the normal order of immune response and can lead to a pathological inflammatory response. This situation results in capillary leakage, tissue damage, and multiple organ failure [3]. TNF-α plays an important role in the inflammation process and is considered a master mediator of early stage inflammation. Guo et al. found that TNF-α levels were highest in the early period after injection (30 min–1 h) in a sepsis model elicited lipopolysaccharide injection [5]. Etanercept is the competitive inhibitor of TNF-α, which inhibits the binding of TNF-α to cell surface receptors and limits its biological activity. Suppression of TNF-α by etanercept in the case of excessive immune response can play an important role in the limiting of inflammation. Karabacak and Yazar predicted clinical benefit [6] and surveillance may also improve [5].

Release of oxygen particles due to infectious and immunological causes can alter the lipid, protein, and DNA structure of cells and results in cell damage and death [7, 8]. In addition to this, antioxidants are also released to protect against harmful effects of oxidants. Stable and sufficient function of this system is important to deal with harmful effects of oxidative stress [9].

According to these pieces of information, we created an experimental peritonitis model in the rats, and we injected intraperitoneal etanercept in early stage to suppress inflammation. We aimed to show the effects of intraperitoneal etanercept on oxidant and antioxidant parameters. Therefore, we also indirectly investigated effect of etanercept on preventing oxidative damage in sepsis and peritonitis clinics. We created peritonitis in the rats by injection of 1.5 mL (107 CFU/mL) *E. coli* suspension, followed by dividing them into 4 groups: group 1, control group; group 2, peritonitis group; group 3, peritonitis + cephazolin sodium group; group 4, peritonitis + cephazolin sodium + etanercept group.

Antibiotics play a main role in the treatment of infectious peritonitis. Therefore, many researchers have compared the effectiveness of various antibiotics in peritonitis [10, 11]. In our study, the use of cephazolin sodium as effective antibiotic in the treatment of peritonitis was preferred [10]. Intraperitoneal cephazolin sodium in a dose of 50 mg/kg was given to groups 3 and 4 one hour after the injection of *E. coli* suspension. There are many clinical studies made by antibiotics or antibiotics and other drugs combinations to limit peritonitis such as antibiotic + vitamin E [12], antibiotics + normobaric oxygen therapy [13], and antibiotics + oxygen-free radicals scavengers [14]. It can be seen that etanercept has been used for suppression of inflammation in autoimmune diseases such as rheumatoid arthritis [15], ankylosing spondylitis [16], psoriatic arthritis [17], and psoriasis vulgaris [18]. We also observed that etanercept has been used experimentally in the nonautoimmune disease. It was shown that etanercept can improve neuroinflammation and myocardial ischemia reperfusion injury [19, 20].

It is known that TNF-α is responsible for early responses of inflammation, which was secreted minutes after the inflammatory stimulus, peaked at first hour, and stopped after

3-4 h [21]. Therefore, we injected etanercept at first hour and TNF-α peaked at the 4th hour when secretion of TNF-α stopped. We injected etanercept in a dose of 8 mg/kg which is not toxic but can block TNF-α [22, 23]. We basically compared these two treatment methods by looking to serum and tissue levels of MDA, NO, TNF-α, TOS, TAC, and PON. We evaluated the histopathological findings in all groups. An increase in levels of MDA, TNF-α, and TOS shows oxidative stress and an increase in levels of TAC and PON shows antioxidant activity. Upon a literature search, we found that NO has been shown to be cytoprotective at low levels and cytotoxic at high levels unlike the other parameters [24].

If we correlate the histopathological findings with the oxidant and antioxidant parameters variability in serum and tissue samples, we can conclude that addition of etanercept to cephazolin sodium treatment in early stages of peritonitis treatment can be useful for limiting the peritonitis. Increased suppression of TNF-α levels with the usage of etanercept seems to be useful for limiting peritonitis. In a study by Chen et al., they found decreased antioxidant levels in rats with subacute peritonitis compared with the control group [25]. Similar to this study, we also found decreased antioxidant levels. Di Paola et al. showed an increase in TNF-α levels in infected tissues of rats with periodontitis compared with control group [26]. We also found an increase in TNF-α levels in groups with peritonitis compared with control group (TNF-α levels in group 1: 116.39 ± 42.36 pg/mL and TNF-α levels in group 2: 487.21 ± 238.44 pg/mL, $p = 0.002$). A decrease in TNF-α levels by using etanercept in the same study was also seen in group 4 in our study (TNF-α levels in group 4: 136.83 ± 24.26). We also showed a statistically significant decrease in MDA levels in serum ($p < 0.001$) and tissue samples ($p < 0.001$) in group 4 compared with group 2 similar to decrease in MDA levels by using etanercept in the study [26]. A decrease in TNF-α and MDA by using etanercept was collocated with histopathological improvement. We found increased NO levels in peritonitis group similar to previous reports [27]. In our study, there were statistically significant differences between group 1 and group 2 in terms of NO levels in serum ($p = 0.001$) and tissue samples ($p = 0.001$). Unlike other parameters, there was a statistically nonsignificant decrease in NO levels in group 3. In group 4, there were statistically significant decreases in NO levels. PON, which is a powerful antioxidant, decreases in case of infection [28, 29]. There was a statistically significant difference between group 1 and group 2 in terms of serum ($p = 0.001$) and tissue ($p < 0.001$) for PON levels. PON levels were decreased in peritoneal tissue (1.83 ± 2.54 U/L) and serum (124.61 ± 15.81 U/L) in rats with peritonitis. With the addition of the treatment, there was an increase in PON levels in serum and tissue samples in group 3 and group 4. This increase was prominent in group 4 in terms of both serum ($p = 0.029$) and tissue samples ($p < 0.001$).

Consistent with the literature, there was a decrease in oxidative parameters and an increase in antioxidant levels by using etanercept in addition to cephazolin sodium in rats with experimental peritonitis. A decrease in the edema, congestion, and neutrophil infiltration in the lamina propria of the peritoneum was another important finding of this study. It appears that the suppression of TNF-α in early stage can reduce excessive immune response resulting in the destruction of the organism.

As a result, etanercept, in addition to antibiotics given in the early treatment of peritonitis, results in more significant improvement in histopathological and oxidative parameters according to antibiotics alone. We believe that etanercept can decrease sepsis incidence in peritonitis but there is a need for more comprehensive experimental studies for usage in the treatment of peritonitis.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Effect of Blueberry Anthocyanins Malvidin and Glycosides on the Antioxidant Properties in Endothelial Cells

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The objective of this research was to survey the antioxidant functional role of the main anthocyanins of blueberries in endothelial cells. Changes on the reactive oxygen species (ROS), xanthine oxidase-1 (XO-1), superoxide dismutase (SOD), and heme oxygenase-1 (HO-1) in cells of malvidin and the two glycosides were investigated. The results showed that these anthocyanins decreased the levels of ROS and XO-1 but increased the levels of SOD and HO-1. Glycosides improved the antioxidant capacity of malvidin to a great extent. The changes in the antioxidant properties of malvidin-3-glucoside were more pronounced than malvidin-3-galactoside. Variation in levels of malvidin-3-glucoside and malvidin-3-galactoside had a significant impact on antioxidant properties to different extents. It indicates that blueberries are a good resource of anthocyanins, which can protect cells from oxidative deterioration and use blueberry as a potential functional food to prevent diseases related to oxidative stress.

1. Introduction

There have been intense interest and active researches in the area of dietary antioxidants to develop functional food products [1]. Rabbiteye blueberries (*Vaccinium ashei*) are used as endogenous antioxidant defense ingredients in the modern health-conscious food industry, due to their significant levels of anthocyanins, phenolic acids, and vitamins [2, 3]. The health benefits of blueberries have been reported as reduction of coronary heart disease risk, visual improvement, and antimutagenic and anti-inflammatory effects [4]. The composition and molecular structure of anthocyanin determine the functional properties of blueberries [5]. Among fresh fruits and vegetables, blueberries contain greater amount of anthocyanin, especially malvidin-3-glucoside (Mv-3-glc) and malvidin-3-galactoside (Mv-3-gal) [6].

Malvidin possesses great antioxidant capacity with excellent free radical scavenging properties *in vitro* [7]. Malvidin exhibits antihypertensive activity by inhibiting angiotensin I-converting enzyme and anti-inflammatory effect by blocking NF-κB pathway [8, 9]. Gopu et al. reported that malvidin interrupted quorum sensing in *Klebsiella pneumoniae* by docking with LasR receptor protein and reducing the violacein, EPS production, and biofilm formation [10]. In addition, malvidin plays a role in controlling both short- and long-term cellular activities. Several studies have shown that malvidin could inhibit different tumor cell lines *in vitro* or *in vivo*, including human promyelocytic/monocytic leukemia cells, gastric adenocarcinoma cells, and HT-29 colon cancer cells [11–13]. Matsunaga et al. found that malvidin could counteract oxidative stress in neuronal cells [14].

Malvidin presents in nature principally as the glycosylated form with the sugar moiety attached at position 3 on the c-ring, that is, malvidin-3-glucoside and malvidin-3-galactoside. Bioavailability of anthocyanins was extensively studied due to the different mechanisms of the uptake and metabolism (deconjugation, glucuronidation, sulfation, and deglucuronidation) in various cell lines. Passamonti et al. found Mv-3-glc possessed better bioavailability than other anthocyanidins due to the greater efficacy of binding

to organic anion membrane carrier, bilitranslocase [15]. Rossetto et al. reported that Mv-3-glc showed synergistic antioxidant effect with catechin on free radical-initiated peroxidation of linoleic acid in micelles [16], and Grace et al. reported its significant hypoglycemic effect in diabetic C57b1/6J mice [17]. Additionally, Mv-3-glc is a potent anti-inflammatory agent *in vitro* and *in vivo*, without detectable toxicity on human peripheral blood mononuclear cells, which inhibits human macrophage-derived inflammatory mediators and decreases clinical scores in arthritic rats and inhibits ear oedema and leukocytes migration [18, 19]. Our previous study also found that Mv-3-glc and Mv-3-gal could inhibit TNF-α-induced inflammatory response in endothelial cells [20]. Quintieri et al. reported that Mv-3-glc could modulate mammalian myocardial and coronary performance and protect the heart against ischemia/reperfusion injury by activating the PI3K/NO/cGMP/PKG pathway and phosphorylating AKT and eNOS [21]. Paixão et al. confirmed the capacity of Mv-3-glc to increase NO bioavailability and to inhibit peroxynitrite-induced NF-κB activation, supporting its benefits in cardiovascular health [22]. Anthocyanins could be as a promising tool for development of nutraceuticals to improve endothelial function. However, the antioxidant mechanisms of these anthocyanins in endothelial cells are still not clear. The objective of this study was to investigate the antioxidant functional role of the main anthocyanins in blueberries, malvidin, and its two glycosides (malvidin-3-glucoside and malvidin-3-galactoside) in human umbilical vein endothelial cells.

2. Materials and Methods

2.1. Chemicals and Reagents. Human umbilical vein endothelial cells (HUVECs) were obtained from Zhongqiaoxinzhou Biological Technology Co., Ltd. (Shanghai, China). Dulbecco's phosphate buffer saline, malvidin, malvidin-3-glucoside, malvidin-3-galactoside, and trypsin were obtained from Sigma Chemical Co., Ltd. (Nanjing, China). Fetal bovine serum and DMEM medium were obtained from Gibco/Invitrogen (Shanghai, China). Streptomycin and penicillin were obtained from Life Technologies (Shanghai, China). ROS Assay Kit was obtained from Biyotime Institute of Biotechnology (Shanghai, China). XO-1, SOD, and HO-1 ELISA Kit were obtained from Boster Biotechnology Inc. (Wuhan, China). All chemicals and reagents are of analytical grade.

2.2. Antibodies. Rabbit monoclonal primary antibody against XDH, mouse polyclonal primary antibodies to the β-actin, and goat anti-rabbit/mouse IgG-HRP conjugated secondary antibody were obtained from Boster Biotechnology Inc. (Wuhan, China). Primary antibodies against XDH were used at 1 : 400 dilutions, and primary antibodies against β-actin were used at 1 : 1000 dilutions. Secondary antibodies were used at 1 : 2000 dilutions.

2.3. Endothelial Cell Culture and Treatment. HUVECs could be characterized as a model system for studying oxidative stress in the vasculature. The cells were cultured according to a laboratory protocol [20]. After quiescing in a reduced serum medium for 4 h, the cells were treated with 1, 5, and 10 μmol/L of Mv, Mv-glc, and Mv-gal, or mixture of Mv-glc and Mv-gal for 24 h, respectively. DMSO was instead in the control.

2.4. Reactive Oxygen Species (ROS) Assay. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay is a quantitative method for oxidative stress assessment in cells. In this study, DCFH-DA detection kit was used to assess the ROS level in endothelial cells. Briefly, the cells were seeded in 6-well plates, treated with different samples to incubate for 24 h. After washing cells with PBS, 10 μmol/L DCFH-DA was added to each well and reacted for 20 min at 37°C, and then the cells were washed thoroughly with PBS. A group of cells was visualized under an IX53 Inverted Fluorescent Microscope (Olympus, Tokyo, Japan) at 530 nm emission and 485 nm excitation filters immediately. All images presented are in ×200 magnification. Another one was collected in 1 mL PBS after dissociated, and fluorescence was recorded by LB 941 TriStar Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). The total fluorescence intensity of cells in each well was noted, and ROS generation was measured as fold increase over the untreated control.

2.5. ELISA Analysis and Western Blotting. The levels of XO-1, SOD, and HO-1 in the supernatants were detected using ELISA kits according to the kit protocol booklet instructions. The absorbance was measured at 450 nm on a StatFax-2100 Microplate Reader (Awareness Technology Inc., Plam, FL, USA) via Hyper Terminal Applet ELISA software.

XO-1 protein expression was also analyzed by western blotting performed on the HUVEC lysates. β-actin was used as a loading control. All data were expressed as fold increase over the untreated control.

2.6. Statistical Analysis. Data were expressed as mean value ± standard deviation (SD) of triplicate determinations. Figures were obtained using GraphPad Prism Version 5 (GraphPad Software, Inc., CA, USA). One-way ANOVA was used for the determination of statistical significance using SPSS 19.0 Software. The differences were considered statistically significant with a P value of 0.05.

3. Results

3.1. Effects of Malvidin and Its Glycosides on Reactive Oxygen Species in Cells. Addition of all of the tested malvidin, malvidin-3-glucoside, malvidin-3-galactoside, and the mixture of the two glycosides decreased ROS values in endothelial cells (Figure 1). Malvidin treated at 1, 5, and 10 μmol/L concentration inhibited 4%, 6%, and 11% ROS, respectively. When Mv-3-glc was present, the values for ROS production decreased 21%, 35%, and 28% at the concentration of 1, 5, and 10 μmol/L, respectively, whereas when Mv-3-gal was present, the values decreased 12%, 8%, and 9%, respectively. The mixture of Mv-3-glc and Mv-3-gal significantly decreased ROS level in cells. Both Mv-3-glc and the mixture of

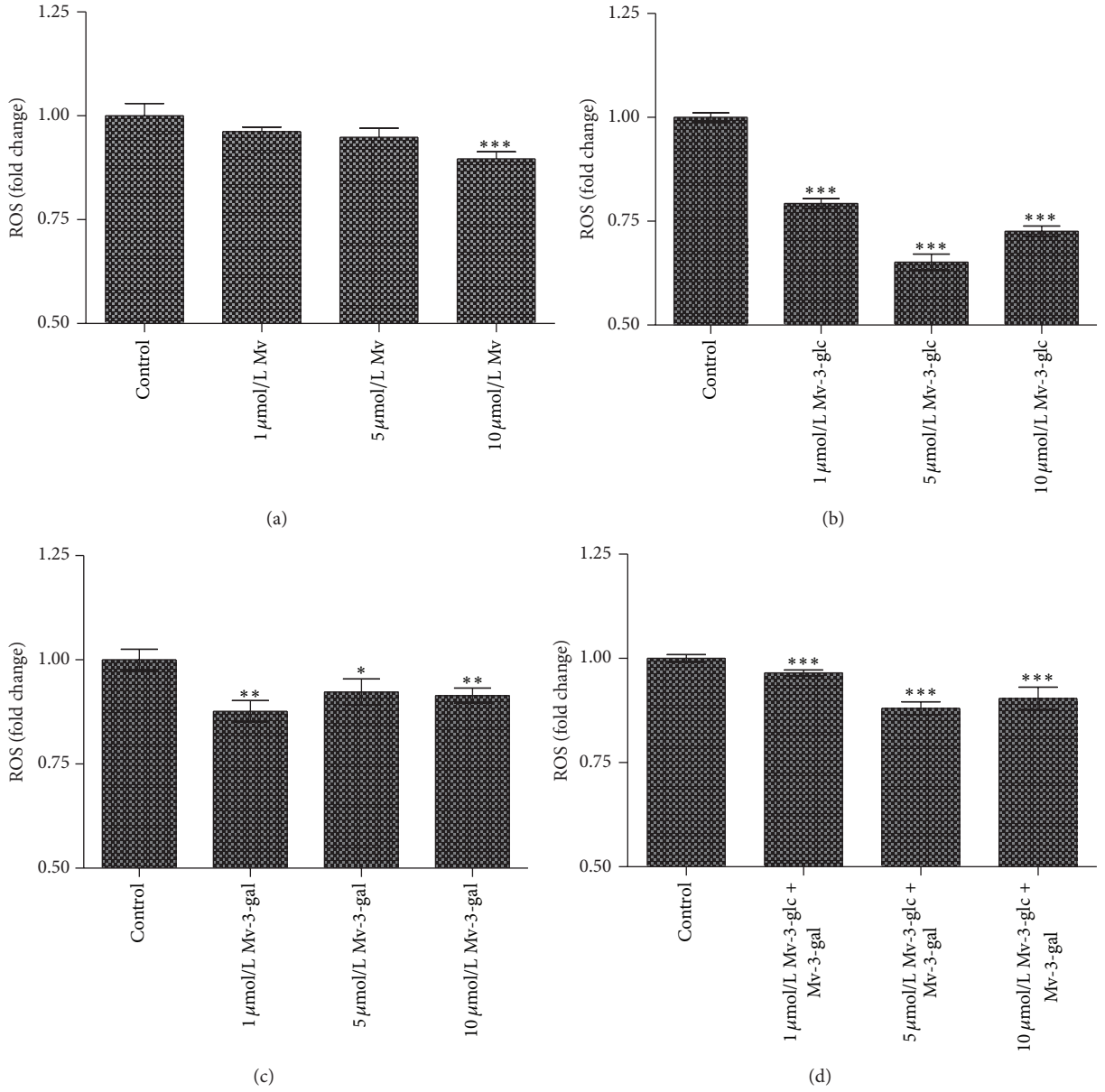

Figure 1: Effects of different concentrations Mv (a), Mv-3-glc (b), Mv-3-gal (c), and Mv-3-glc + Mv-3-gal (d) on the level of ROS in HUVECs. $*$, $**$, and $***$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to the control.

the two glycosides produced the greatest inhibition rate at the concentration of 5 μmol/L, whereas Mv-3-gal produced the largest inhibition rate at the concentration of 1 μmol/L. The extent of the decrease in ROS of Mv-3-glc was more pronounced than that of Mv-3-gal. However, there seemed to be an antagonism effect between Mv-3-glc and Mv-3-gal, since the mixture possessed lower ROS scavenging activity than Mv-3-glc. The fluorescence intensity levels of ROS showed similar effects (Figure 2).

3.2. Effects of Malvidin and Its Glycosides on XO-1 Production in Supernatant and Cells. Addition of all the tested Mv, Mv-3-glc, Mv-3-gal, and the mixture decreased XO-1 production in supernatant (Figure 3). When the values of XO-1 production in supernatant treated with 1, 5, and 10 μmol/L malvidin were compared to those of the control, they were found to be 0.68, 0.76, and 0.92 times, respectively. Interestingly, only Mv treated at lower concentration (1 μmol/L) had significant inhibitory effect on XO-1 protein. The cells treated with 1, 5, and 10 μmol/L Mv-3-glc, Mv-3-gal, and the mixture all significantly inhibited XO-1 level at the rate of 15%, 19%, 51%, and 13%, 23%, 31%, and 19%, 29%, 5%, respectively. The two malvidin glycosides exhibited greater inhibitory effect than malvidin, whereas Mv-3-glc showed stronger effect than Mv-3-gal in most cases. Mv-3-glc at the concentration of 10 μmol/L reached the greatest inhibition rate. Consistent with ROS results, Mv-3-glc had antagonistic effect with Mv-3-gal at high concentration (10 μmol/L).

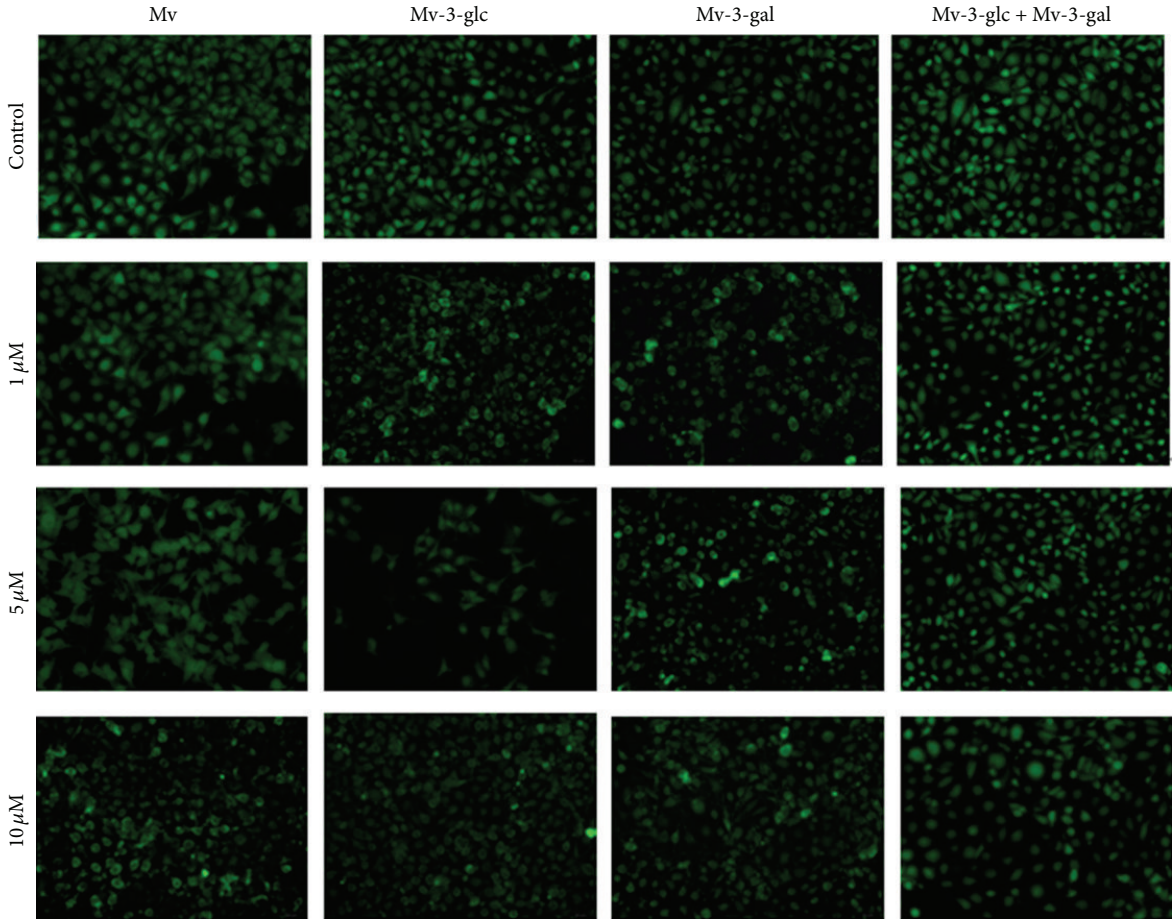

Figure 2: The fluorescence intensity of ROS in HUVECs treated with different concentrations Mv, Mv-3-glc, Mv-3-gal, and Mv-3-glc + Mv-3-gal. A representative set of images from three independent experiments is shown. All images presented are in ×200 magnification.

Addition of Mv, Mv-3-glc, Mv-3-gal, and the mixture also decreased the values of XO-1 production in cells to different extents (Figure 4). Low concentration of Mv (1 and 5 μmol/L) had significant inhibitory effects on endothelial XO-1 protein. The relative contents (XO-1/β-actin) were 0.88 and 0.86 times compared to those of the control, respectively. The XO-1 relative contents in the cells treated with 1, 5, and 10 μmol/L Mv-3-glc, Mv-3-gal, and the mixture were 0.91, 0.74, 0.87 and 0.95, 0.79, 0.81, and 0.83, 0.76, 0.93 times than those of the control, respectively. High concentration of the mixture (10 μmol/L) had no effect on the XO-1 protein expression in cells, whereas Mv-3-glc tended to produce greater inhibition rate.

3.3. Effects of Malvidin and Its Glycosides on SOD Production in Supernatant. Malvidin increased the level of SOD production as a function of concentration. When the values of SOD production of the cells treated with 1, 5, and 10 μmol/L malvidin were compared to those of the control, they were found to be 1.64, 2.07, and 2.27 times, respectively. Addition of Mv-3-glc and Mv-3-gal greatly increased SOD production in supernatant. When the SOD production for Mv-3-glc and Mv-3-gal treated at the concentration of 1, 5, and 10 μmol/L was compared to those of the control, they were found to be 3.26, 4.79, 2.59 and 3.06, 2.01, 1.90 times, respectively. However, all products of the mixture of Mv-3-glc and Mv-3-gal at different concentration showed less effect, with changes over the control at 1.55, 1.81, and 1.37 times, respectively (Figure 5).

3.4. Effects of Malvidin and Its Glycosides on HO-1 Production in Supernatant. The control contained 2.09 μg/L HO-1, while the cells treated with 1, 5, and 10 μmol/L malvidin contained 2.59 μg/L, 2.84 μg/L, and 2.24 μg/L HO-1, which were 1.24, 1.36, and 1.08 times than those of the control. The supernatant HO-1 levels of the cells treated with 1, 5, and 10 μmol/L Mv-3-glc, Mv-3-gal, and the mixture were 1.25, 1.38, 1.18, and 1.05, 1.38, 1.23, and 1.67, 2.03, 1.24 times than those of the control, respectively (Figure 6). Addition of malvidin and its glycosides produced a variety of effects on HO-1 production, but they all reached the greatest values at the concentration of 5 μmol/L. There were synergistic effects between Mv-3-glc and Mv-3-gal, since the mixtures at all the concentration showed greater increase rates than the others.

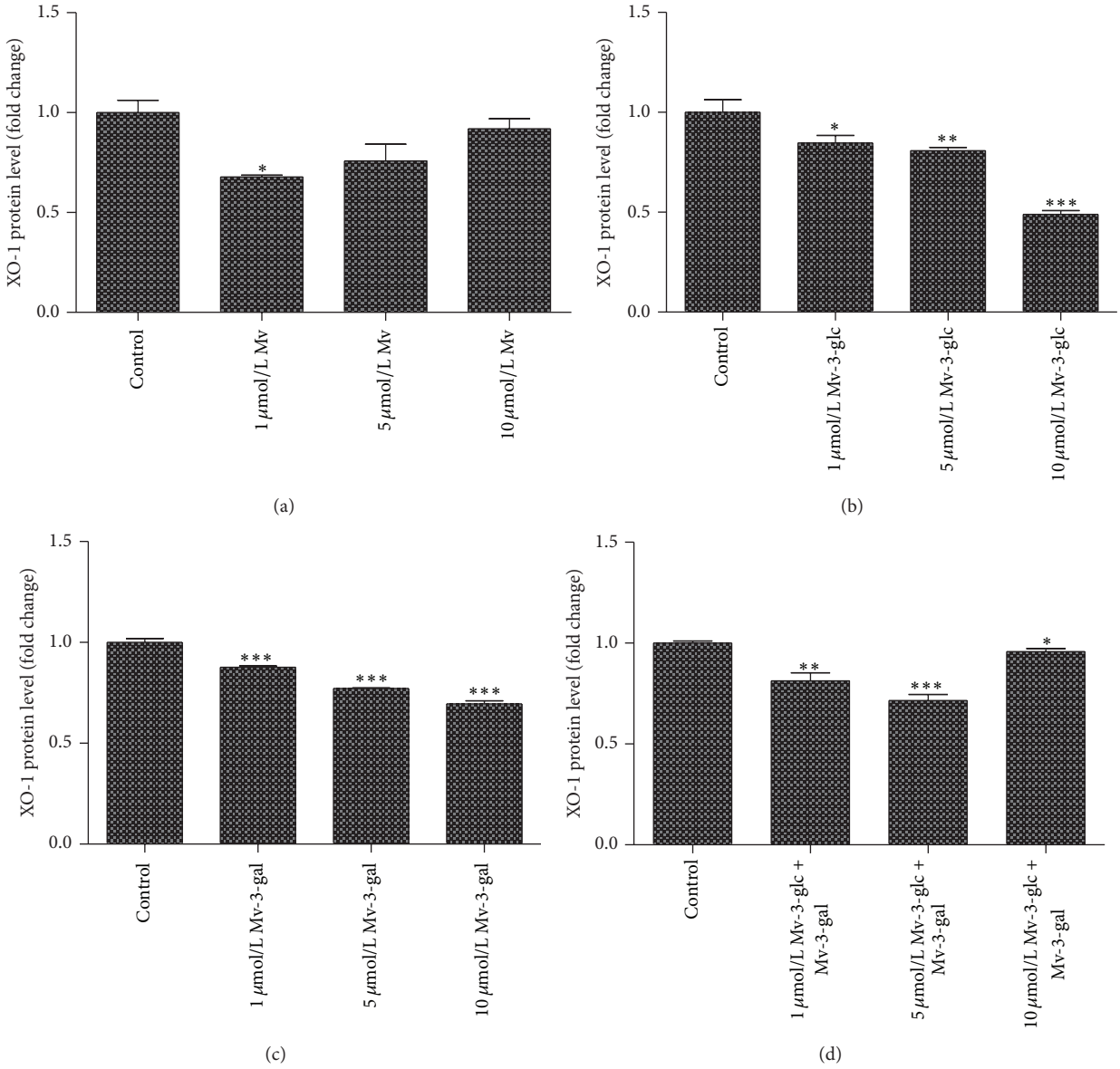

Figure 3: Effects of different concentrations Mv (a), Mv-3-glc (b), Mv-3-gal (c), and Mv-3-glc + Mv-3-gal (d) on XO-1 production released into the supernatant. ∗, ∗∗, and ∗ ∗ ∗ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to the control.

4. Discussion

The molecular structure and concentration of anthocyanins in berries determine their bioactivities. Anthocyanins have peculiar chemical structures deficient in electron [23]. The efficacy to scavenge diverse ROS differs from one to another. Generally the antioxidant capacity of anthocyanins is associated with the number of free hydroxyls around the pyrone ring, but this is not always true [24]. Different parameters, including bond dissociation enthalpy, electron transfer enthalpy, electrophilicity, frontier charge density, hardness, ionization potential, and proton affinity, should be calculated to evaluate anthocyanin antioxidant characteristics [7]. A density functional theory study found that antioxidant capacity of different anthocyanin structures was in the following order: cyanidin > malvidin > aurantinidin > delphinidin > peonidin > pelargonidin [25]. Blueberries are known to contain a significant level of anthocyanins, in which malvidin-3-galactoside and malvidin-3-glucoside are the most dominant [6]. Malvidin has four hydroxyls, leading to a good antioxidant capacity. Therefore, blueberries exhibited obvious superiority in the prevention of chronic diseases caused by oxidative damage.

Antioxidants have been shown to delay, inhibit, and prevent the oxidation by interacting with biological systems through many potential mechanisms, such as absorbing oxygen radicals, chelating of the metal ions, scavenging free radicals, regulating enzyme activity and protein levels, and blocking signaling pathways [26]. In the present study, blueberry anthocyanins, malvidin, and its glycosides could

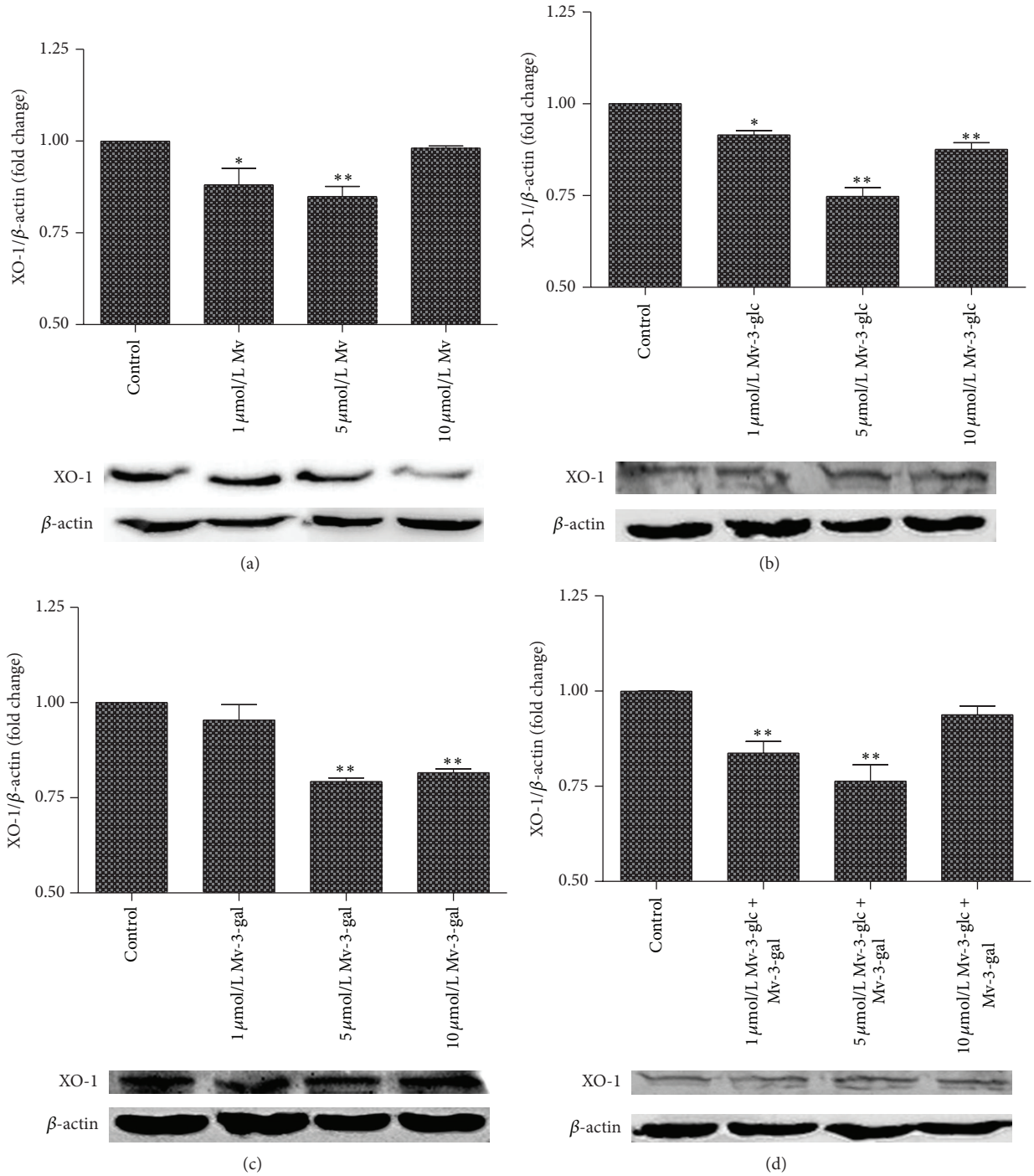

Figure 4: Effects of different concentrations Mv (a), Mv-3-glc (b), Mv-3-gal (c), and Mv-3-glc + Mv-3-gal (d) on XO-1 production in the cells. $*$ and $**$ indicate $P < 0.05$ and $P < 0.01$, respectively, compared to the control.

reduce oxidative stress and alleviate harmful effects by greatly decreasing the level of ROS in endothelial cells, as well as changing several key proteins' levels. Xanthine oxidase-1 (XO-1), a major source of superoxide, has been implicated in endothelial dysfunction partly due to the rapid inactivation of nitric oxide [27]. Superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) are both the important antioxidant defense against endothelial oxidative damage [28, 29]. The capacity of Mv, Mv-3-glc, Mv-3-gal, and the glycoside mixture on decreasing XO protein level and increasing SOD and HO-1 protein levels further confirmed that blueberry anthocyanins are a potential source of antioxidants.

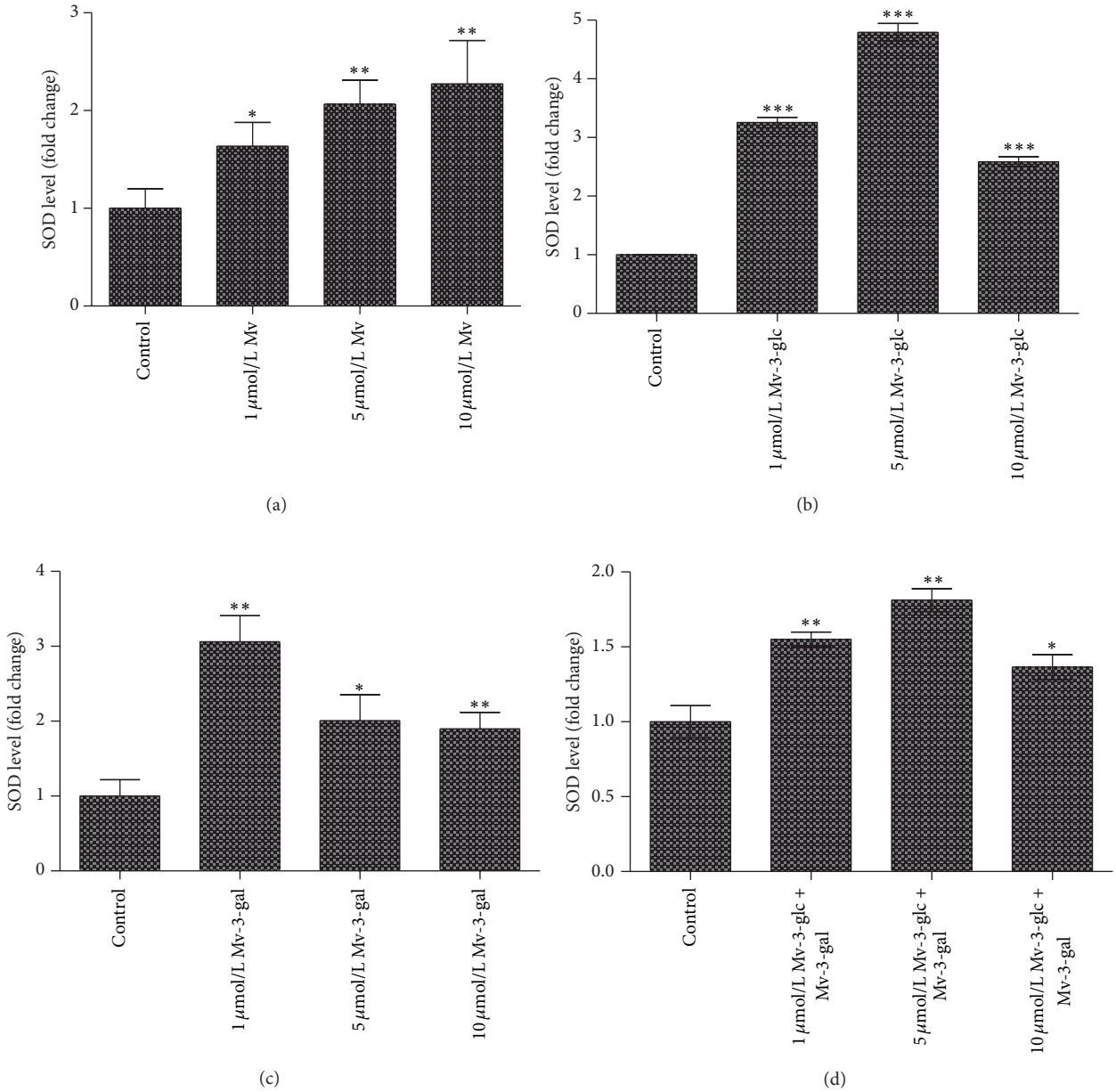

Figure 5: Effects of different concentrations Mv (a), Mv-3-glc (b), Mv-3-gal (c), and Mv-3-glc + Mv-3-gal (d) on SOD level in the supernatant. $*$, $**$, and $***$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to the control.

Generally glycosylation of an anthocyanin seems to decrease the antioxidant capacity compared with the aglycone because it reduces free hydroxyls and metal chelation sites [30]. However, Kähkönen and Heinonen found that different glycosylation patterns either enhanced or diminished the antioxidant power depending on the anthocyanidin and models used for antioxidant analysis because the *in vitro* effect of glycosylation on antioxidant activity depended on the environment when oxidations occurred [31]. Fukumoto and Mazza reported that antioxidant activity increased with hydroxyl groups but decreased with glycosylation of anthocyanidins. Interestingly, this study showed that malvidin-3-glucoside and malvidin-3-galactoside had better antioxidant capacity than malvidin in endothelial cells [32]. In addition, glucoside seemed to be more effective than galactoside on antioxidant improvement. It further confirmed that the molecular structure of glucosylation was the most important factor in determining antioxidant properties of anthocyanins [24]. The results showed that the typical major dietary anthocyanins in blueberries, Mv-3-glc and Mv-3-gal, had great antioxidant properties in endothelial cells. It indicated that they could protect cells from oxidative deterioration and used as a potential functional food ingredients to prevent diseases related to oxidative stress.

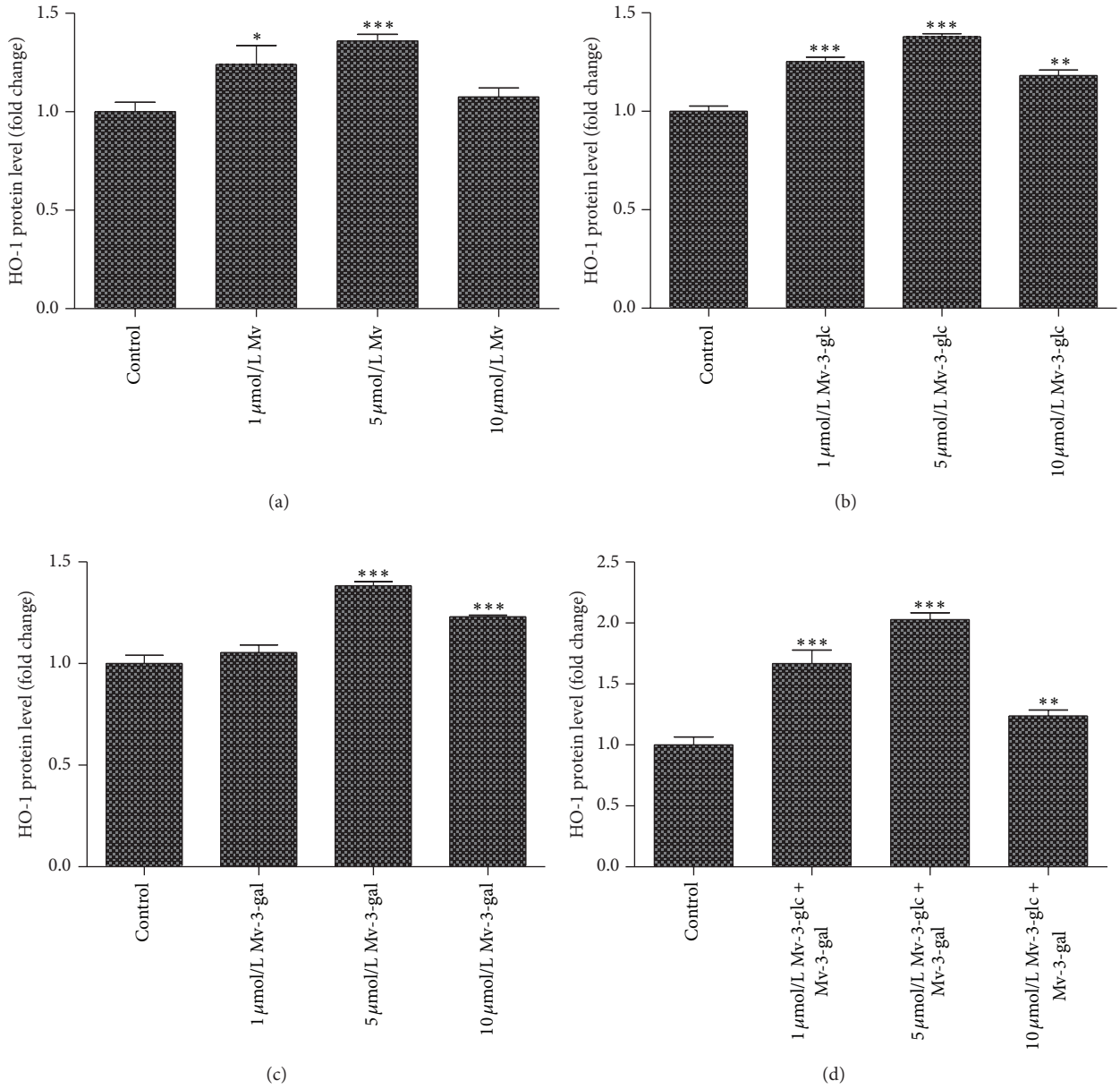

Figure 6: Effects of different concentrations Mv (a), Mv-3-glc (b), Mv-3-gal (c), and Mv-3-glc + Mv-3-gal (d) on HO-1 production released into the supernatant. $*$, $**$, and $***$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to the control.

5. Conclusions

In the present study, treatment with malvidin, malvidin-3-glucoside, malvidin-3-galactoside, and the mixture of the two glycosides significantly attenuated oxidative stress in human umbilical vein endothelial cells. Mv, Mv-3-glc, Mv-3-gal, and the mixture all showed good antioxidant capacity in cells by the mechanism of inhibiting ROS and XO-1 levels and increasing the SOD and HO-1 levels. In most cases, Mv-3-glc had better potential antioxidant effect than Mv-3-gal. This indicated that bioactive anthocyanins in blueberries, such as Mv-3-glc and Mv-3-gal, could be applied in the production of smart and innovative pharmaceutical or functional food ingredients to improve endothelial function and prevent the progression of diseases caused by oxidative stress.

Conflict of Interests

There is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Oxidative Stress in Atopic Dermatitis

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Atopic dermatitis (AD) is a chronic pruritic skin disorder affecting many people especially young children. It is a disease caused by the combination of genetic predisposition, immune dysregulation, and skin barrier defect. In recent years, emerging evidence suggests oxidative stress may play an important role in many skin diseases and skin aging, possibly including AD. In this review, we give an update on scientific progress linking oxidative stress to AD and discuss future treatment strategies for better disease control and improved quality of life for AD patients.

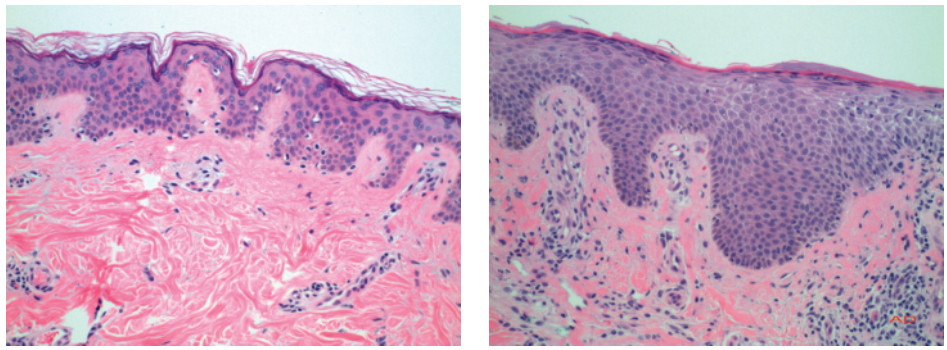
1. Introduction

Atopic dermatitis (AD) or atopic eczema is a chronic relapsing inflammatory skin disease. Its prevalence is continuously increasing, affecting up to 25% of children and 2-3% of adults [1]. It is clinically manifested by itching and scratching, dry skin, patchy eczema especially on flexural locations, exudation, and skin thickening and discoloration. AD has an early onset, usually in infancy or early childhood. It may regress spontaneously after puberty in some patients, but wax and wane for life in many others. The current standard treatment for AD includes moisturizing lotions and creams, topical corticosteroids, and calcineurin inhibitors [2]. For severe cases or in acute exacerbation phase, systematic agents are often efficacious, including oral corticosteroids, cyclosporine, methotrexate, mycophenolate, and azathioprine [1]. Several biologic drugs have become available in recent years, mainly monoclonal antibodies against interleukin 4-receptor, immunoglobulin (Ig) E, and activated T or B cells [3, 4].

The pathogenesis of AD is complex and still poorly understood. In addition to genetic predisposition attributed to immune dysregulation and hypersensitivity, development and maintenance of AD are thought to be associated with environmental and psychological triggers and skin barrier defects [1, 2]. Genetic predisposition is obvious in AD patients, who often have a personal or familial history of other allergic diseases, such as asthma and allergic rhinitis. Mutation of several genes has been implicated in the systemic "atopic" immune response, characterized by a Th2 dominance and elevated IgE levels, such as IL-4, IL-4 receptor, and IL-13, or altered cutaneous inflammation, such as mast cell chymase [5]. In addition, the mutations in the filaggrin gene and the SPINK5 (serine protease inhibitor kazal-type 5) gene are associated with defective epidermal differentiation and skin barrier formation [5].

Apart from genetic predisposition, the hallmark pathology of AD is an acute, subacute, or chronic dermatitis of nondistinctive type. The dermal layer contains perivascular or interstitial inflammatory infiltrate composed of many types of inflammatory cells, including plasma cells, mast cells, eosinophils, and B and T lymphocytes. Many types of proinflammatory cytokines are increased in AD patients, such as tumor necrosis factor (TNF) and interleukins (IL-4, IL-9, IL-22), for example [6]. The epidermis often shows edema with spongiosis and increased cell layers with parakeratosis, hyperkeratosis, and dyskeratosis. Stratum corneum, also called basket-wave keratin, the outmost layer of the epidermis normally functioning as the skin barrier, is lost in AD lesions (Figure 1).

A simplistic version of pathogenesis of AD is illustrated in Figure 2. It is well known that environmental and/or psychological triggers when applied to a genetically predisposed person can initiate skin inflammatory change and destroy intact skin barrier, resulting in clinical manifestations of AD



(a) (b)

Figure 1: Histology finding of the normal and AD patient's skin. (a) Histology of normal skin. Normal thickness of epidermis (top layer) composed of several layers of squamous cells with the delicate basket-wave keratin (stratum corneum) on the surface. The dermis (bottom part) is composed of sparse fibroblasts with abundant extracellular collagen bundles and embedded capillaries lined by a single layer of endothelial cells (magnification 200x). (b) Histology of subacute spongiotic dermatitis, typically seen in affected skin of AD patients. The epidermis is thickened with slit-like spaces between squamous cells, indicating edema/spongiosis. The overlying basket-wave keratin is replaced by abnormal hyperkeratosis and parakeratosis. The dermis shows increased cellularity composed of mixed inflammatory cells predominantly surrounding small vessels. The inflammatory cells are of predominantly lymphocytes with some mast cells, macrophages, and occasional eosinophils (magnification 200x).

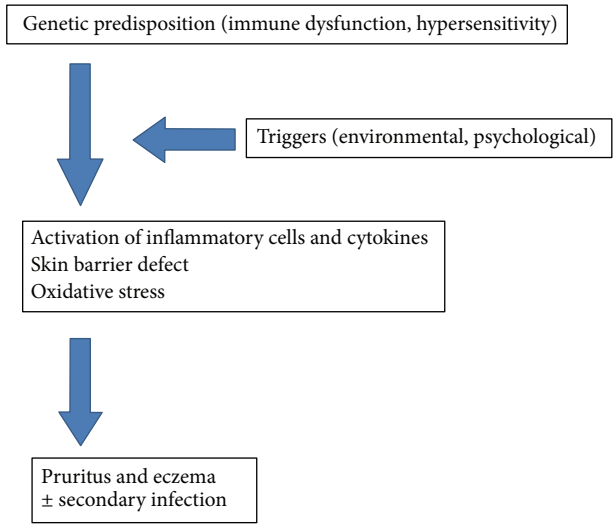


Figure 2: Development and maintenance of atopic dermatitis.

[7, 8]. In recent years, oxidative stress has also been implicated in the pathogenesis of AD.

Oxidative stress is defined as the formation of oxidants in the cells of the human body that acutely or chronically exceeds the antioxidant defense capacity. Oxidants, including free radicals (any species capable of independent existence which contains one or more unpaired electrons) [9], reactive oxygen species (ROS), and nitrogen oxygen species (NOS) and reactive metabolites are produced during normal metabolic activities. Biological antioxidant defense systems exist in cells, including enzyme-based systems (superoxide dismutase, glutathione peroxidase, and peroxiredoxins) and nonenzyme-based systems (vitamins A, C, and E, glutathione, polyphenols, and coenzyme Q10). In excess, the oxidants can react with all cellular macromolecules, including lipids, proteins, nucleic acids, and carbohydrates, particularly polyunsaturated fatty acids on the cell membranes. After the initial reaction with ROS, a chain reaction is started, proceeding to cell injury and, ultimately, cell death [10]. Oxidation metabolites can be quantitatively measured, such as urine or serum nitrate for nitric oxide, malondialdehyde (MDA) for lipid oxidation, and 8-hydroxydeoxyguanosine (8-OHdG) for DNA oxidation [11].

For several decades, there has been increasing evidence linking oxidative stress to several chronic diseases, including cardiovascular disease, diabetes, neurodegenerative disorders, inflammatory diseases, and cancer [12–14]. For example, excess free radicals created through hyperglycemia damage mitochondria, the energy-producing cellular organelle, and are largely responsible for the life-threating complications of type 2 diabetes [14]. It has also been shown that oxidative stress plays an important role in skin aging and development of skin cancer.

It is also well known that oxidative stress promotes tissue inflammation through upregulation of genes that code proinflammatory cytokines. Inflammatory cells in turn release free radicals when activated. Given its prominent inflammatory component, it is conceivable that oxidative stress may play a role in the pathogenesis of AD. The exploration of the association between inflammation and oxidative stress in AD will enhance our understanding of the development and maintenance of the disease, which can be incorporated into formulating new treatment strategies, such as combining anti-inflammatory drugs, immune regulatory agents, skin barrier enhancers, and antioxidants.

In this review, we will summarize available studies exploring the role of oxidative stress in AD and the relationship between oxidative stress and other crucial pathological factors associated with AD. Potential future treatment options will also be discussed.

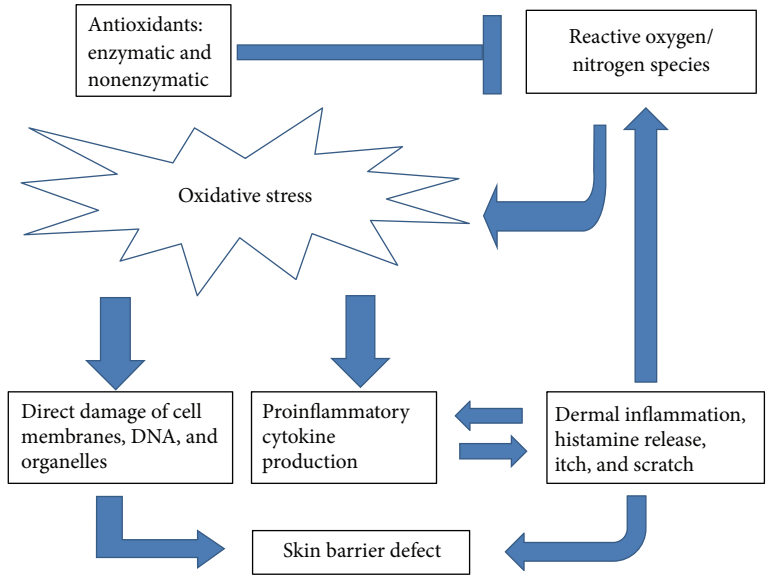


Figure 3: The interplay among oxidative stress, skin barrier defect, and inflammation in atopic dermatitis.

2. Oxidative Stress in Atopic Dermatitis, Involvement, and Possible Mechanisms

Skin is the largest organ in the human body. It protects the body from external insults, such as chemicals, environment pollutants, and allergens. The skin is therefore a major target of oxidative stress due to reactive species that are constantly generated in the keratinocytes in response to environmental and endogenous prooxidant agents. Physical activity and psychological stress can also create oxidative stress to the skin. Free radicals generated during normal metabolism are an integral part of normal skin function and are usually of little harm because intracellular mechanisms can reduce their damaging effects. However, increased or prolonged free radical action can overwhelm antioxidant defense mechanisms of the skin and contribute to the development of skin disorders, including skin cancer, skin aging, and dermatitis (Figure 3).

Oxidative stress has been implicated in atopic dermatitis for more than 15 years, mainly in the following three aspects: (1) the presence of oxidative stress; (2) increased oxidative stress during AD exacerbation; and (3) decreased antioxidant capability. It was demonstrated that urine markers of oxidative stress are altered in children with AD, including 8-OHdG, nitrite/nitrate and selenium [15]. Those marker levels are higher in children with AD than that in non-AD children. It was suggested that impaired homeostasis of oxygen/nitrogen radicals and increased oxidative stress are involved in the pathophysiology of childhood AD [15]. Chung et al. [16] also found blood antioxidant capacity was significantly less and MDA was higher in preschool children with AD compared to a control group. More recently, Amin et al. and Sivaranjani et al. conducted case-control studies on eczema patients with healthy individuals as controls. They found that, compared to the control group, patients with eczema have a significantly higher level of lipid peroxidation by measuring serum malondialdehyde (MDA), and lower levels of antioxidants including vitamins A, C, and E [17, 18]. Similar findings of the presence of oxidative stress and increased lipid peroxidation were reported in patients with alopecia areata, an inflammatory skin condition closely related to AD [19, 20]. Subsequently, Tsukahara et al. [21, 22] observed oxidative stress and altered antioxidant defenses in children with acute exacerbation of AD. They found that urinary glycosylation end products and bilirubin oxidative metabolites are significantly higher in AD children during hospitalization. Nakai et al. [11] also demonstrated urine nitrate and MDA levels correlate with the severity of AD. Later, Kirino et al. [23] found that heme oxygenase 1, an inducible antioxidant, attenuates the development of atopic dermatitis-like lesions in mice and AD patients. Chung et al. [16] also claimed an association of glutathione-S-transferase polymorphisms with AD risk in preschool age children, implying decreased antioxidant capability may play a role in the pathogenesis of AD. The source of oxidative stress for AD patients could be environmental, physical, and psychological. It is now known that a variety of air pollutants, such as tobacco smoke, volatile organic compounds, formaldehyde, toluene, nitrogen dioxide, and particulate matter act as risk factors and aggravators of AD. Those air pollutants probably induce oxidative stress in the skin, leading to skin barrier dysfunction or immune dysregulation [7]. Song et al. observed increased urine 8-OHdG, a DNA oxidation marker, in children with eczema exposed to short term ultrafine particles [24]. It has been recently shown that the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator (AhR/ARNT) signaling system plays an important role in keratinocytes. AhR ligation induces not only oxidative stress but also antioxidant response in a ligand-dependent manner. Environmental pollutants, such as cigarette smoke, bind to AhR and induce ROS production, DNA damage, and inflammatory cytokine production to cause skin inflammation. In contrast, certain flavonoids bind

to AhR, resulting in the activation of nuclear factor-erythroid 2-related factor-2 (Nrf2) to produce key molecules that protect cells from oxidative damage [25]. Another source of oxidative stress might be skin microbes. As early as 1970, it was noted that resident flora in AD patients are different from the rest of population. The normal as well as diseased skin of AD patients is markedly colonized with *Staphylococcus aureus*. This may be due to preferential expression of bacterial receptors in AD skin, which may predispose to increased carriage of staphylococci, or defective host defense mechanisms involved in the control of bacterial infection. It was recently shown that increased epidermal fatty acid binding protein is noted and associated with methicillin resistant *Staphylococcus aureus* [26]. The presence of the bacterial pathogen stimulates IL-4 and IgE synthesis to cause dermal inflammation and therefore itching and scratching [27].

Psychological stress, as a social pollutant, is a well-known cause of oxidative stress [8] and causes abnormal skin barrier function in humans [28] and is a frequent cause of AD flares. This may be because psychological stress induces an increase in endogenous corticosteroids, which in turn appears to disrupt not only barrier function but also stratum corneum cohesion as well as epidermal antimicrobial defense [8]. Given the poor sleep pattern, psychosocial burden, and poor quality of life in many AD patients, there has been significant association between AD and depression, namely, a 59% increased likelihood of depression in AD patients. This association could also be linked to neuroinflammatory pathways [29]. Furthermore, recent studies have shown that extraneous physical activity is associated with oxidative stress and increase of proinflammatory mediators [30].

The hallmark of AD is dermal inflammation in affected areas, which could be enhanced by oxidative stress. It is known that oxidative stress can activate nuclear factor kappa-B (NF-κB) pathways to activate gene expression and synthesis of antioxidant enzymes. But the NF-κB pathway activation also induces expression of proinflammatory cytokines, such as IL-6, IL-8, IL-9, and IL-33, which in turn enhances dermal inflammatory infiltrate and histamine release in the affected skin to worsen symptoms [30–33]. In animal experiments, oxidative stress in the skin seems to elicit itching and scratching, even in nonatopic animals. Repeated painting of formaldehyde on the skin of 8-week-old BABL/c mice caused ear swelling and infiltration of inflammatory cells. This was related to the increased expression of IL-4 [34]. But the IL-4 gene expression can be suppressed by antioxidant desferrioxamine treatment [35]. It has also been shown that intradermal hydrogen peroxide can provoke itching through a histamine-independent pathway [36]. These animal studies are suggestive of the possibility that oxidative stress and redox imbalance might develop or aggravate AD by trigging pruritus or enhancing Th2 polarization [7]. On the other hand, inflammation generates high levels of ROS/NOS and other oxidants by activation of several enzymes leading to oxidative stress and cellular damage [30].

Oxidative stress can directly cause damage to epidermal keratinocytes by DNA damage, damage of cellular enzymes, or damage to cell membrane structures through lipid oxidation. These intracellular changes will manifest histomorphologically as epidermal edema/spongiosis and disrupted stratum corneum. One of the most important lipids involved in maintaining an intact skin barrier is the ceramides. These molecules are composed of sphingosine and fatty acid and are produced during keratinization in the stratum corneum (the basket-wave keratin, see Figure 1(a)). The intact epidermal barrier has a key function in limiting the entry of allergens and infectious agents and preventing transdermal water loss. Comparative proteomic profiling has demonstrated that proteins related to skin barrier function (filaggrin-2, corneodesmosin, desmoglein-1, desmocollin-14, and transglutaminase-3) are expressed in significantly lower levels in lesion sites in AD patients [26]. Studies have also shown that the skin barrier is directly damaged by oxidative stress initiated by external pollutants. In a study of 75 adult patients with AD, skin biopsies were taken and dinitrophenylhydrazine (DNP) was measured for the content of carbonyl moieties, a marker of oxidative protein damage. It was noted that DNP formation is significantly increased in AD lesions and correlated with AD severity. It was also observed that DNP is more intense in the superficial layers of the stratum corneum than in the lower layers, indicating the oxidative damage might be attributed to exposure to environmental oxidants. The authors conclude that increased ROS generated from environmental pollutants and solar UV light can induce oxidative protein damage in the stratum corneum, resulting in skin barrier dysfunction and aggravation of AD [37]. It is also observed that exposure of keratinocytes to cigarette smoke will increase the production of hydrogen peroxide, which could induce modification, translocation, and degradation of scavenger receptor B1, a protein that plays an important role in cholesterol trafficking and thereby contributes to the permeability barrier [38]. Furthermore, dermal exposure to m-xylene can induce pathologic change and increase expression of IL-1 alpha and inducible nitric oxide synthase in a rat model [39]. On the other hand, studies have shown that retinoic acid, a vitamin A derivative, and an antioxidant, even at very low levels, can stimulate ceramide production in the epidermis in *in vitro* culture models. Recently, retinoic acid was also shown to be able to down-regulate proinflammatory cytokine IL-1 production induced by ultraviolet B radiation [40]. Furthermore, a disrupted skin barrier promotes skin colonization by microbes, and heavy microbial colonization facilitates skin penetration of microbial agents leading to subsequent IgE sensitization [41]. Monocytes from patients with AD are primed to generate ROS in response to zymogens produced by *Staphylococcus aureus* that is heavily colonized on skin of AD patients, leading to damage of the skin barrier by ROS production [42].

3. Managing Oxidative Stress in AD

Given the association of oxidative stress with other factors in development and maintenance of AD, it is worthwhile to consider incorporating strategies in reducing oxidative stress in managing AD. This can be accomplished in multiple ways, including reducing free radical production and

enhancing antioxidant capacity; diminishing the intensity of inflammation and proinflammatory cytokine production; avoiding environmental, physical, and psychological triggers to achieve prolonged remissions; and applying emollients to maintain the intact skin barrier. The practical approach would be to combine anti-inflammatory agents, immune modulatory drugs, skin emollients, and antioxidants. The antioxidant agents to be considered include melatonin; vitamins A, C, D, and E; oxytocin; and others.

Melatonin is an indolamine mainly produced by the pineal gland [43]. Human skin expresses melatonin receptors (MT1 and MT2). Melatonin has many roles in a variety of physiological functions, such as regulating circadian rhythms for its sleep-inducing activity as well as regulating visual, reproductive, cerebrovascular, and neuroendocrine systems. In addition, melatonin is a powerful endogenous free radical scavenger and functions as a potent anti-inflammatory agent as documented in both *in vitro* and *in vivo* studies [40, 43–45]. It also stimulates some important antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and glutathione reductase, to protect cell membranes from lipid peroxidation and neutralizing toxic radicals [43]. Furthermore, melatonin may have important neuroimmunological actions and immunomodulatory effects in allergic diseases. Melatonin has been successfully used in the treatment of cancer, sleep disorders, and aging [43]. In AD patients, melatonin can be used to facilitate a better night's sleep and to reduce skin inflammation. The potential use of melatonin in atopic dermatitis was reported [46]. There are no significant side effects after long-term use; it is safe to use in all ages, including newborns and infants [46].

Vitamin A. Vitamin A is a group of chemicals with the same basic bioactive structure. Humans are unable to synthesize vitamin A. The bioactive chemicals of vitamin A can only be obtained from the diet, retinol from animal food sources, and carotenoids from plant sources. They are important in several bodily processes, such as vision, immunity, and hair follicle development, as well as circadian rhythms and in oxidative stress. Vitamin A nuclear receptors are present in the regulatory regions of certain antioxidant enzyme genes in rat livers [47], which are important in the regulation of antioxidant capacity. Vitamin A also has effects on lipid oxidation and may be important in skin health, as lipids are extremely important in maintaining the barrier function of the epidermis [40].

Vitamin D. Vitamin D is a steroid hormone that can be produced in the body during a chemical reaction catalyzed by UVB radiation. When there is lack of UV exposure, vitamin D can only be obtained through the diet. The antioxidant capability of vitamin D in skin is not clear. It was shown that, after vitamin D exposure, several antioxidant genes were upregulated in the prostate, including SOD, thioredoxin reductase, and G6PD. Vitamin D was also shown to be able to protect prostate cells from H_2O_2-induced cell death [48]. Similar results may be expected in human skin cells. However, no clinical efficacy has been reported so far in AD patients.

Vitamin E. Plevnik Kapun et al. [49] found reduced vitamin E concentrations in canine atopic dermatitis. They then divided AD dogs to two groups: one receiving vitamin E supplementation and the other receiving mineral oil as a placebo. The levels of oxidative stress markers showed significant improvement in dogs receiving vitamin E. Similar studies are not currently available for humans, but vitamin E has been used to deter skin aging.

Oxytocin. The neuropeptide hormone oxytocin mediates a wide spectrum of tissue-specific actions, ranging from cell growth, cell differentiation, and sodium excretion to stress responses, reproduction, and complex social behaviors. Oxytocin and its receptors are detected in skin keratinocytes and dermal fibroblasts. It appears that it is a novel neuroendocrine mediator in human homeostasis and clinically relevant to stressed skin conditions such as AD. It is postulated that, in AD patients, the oxytocin system is deregulated in terms of cellular proliferation, inflammation, and response to oxidative stress. Oxytocin receptor reduction in dermal fibroblasts and keratinocytes leads to elevated levels of reactive oxygen species and reduced levels of glutathione. Those keratinocytes also exhibited an increased release of the proinflammatory cytokines, such as IL-6 [50].

Other methods used in treatment of AD also apply their antioxidant capabilities, one of which is coal tar. Coal tar has been around more than 200 years. It consists of more than 10,000 chemical compounds. Until recently, the molecular mode of action was obscure. A recent study demonstrated that coal tar induces AhR-dependent skin barrier repair by inducing epidermal gene and protein expression including filaggrin in AD patients [51]. Topical application of coal tar is an effective skin AD therapy for reducing inflammation and itch. Another example is hydrogen water. Yoon et al. fed mice with AD with hydrogen water, a potent and harmless antioxidant, and showed positive effect in relieving AD [52]. Wiegand et al. tested a zinc oxide- (ZnO-) functionalized textile for its skin-protective effects in AD patients [53]. In addition to possessing very good biocompatibility and being well tolerated by AD patients, rapid improvement of AD severity, pruritus, and subjective sleep quality were observed in AD patients wearing this type of textile. The authors attributed the success to the high antioxidative capacity of the ZnO textile and its strong antibacterial activity.

Although theoretically promising, dietary antioxidant supplement has not shown significant clinical benefit [54]. But most studies have been small in scale with low numbers of participants and poor quality control. Although most dietary supplements have no side effects to health, high dose vitamin D has been implicated in causing serious medical problems. The cost of long-term use of supplements is also a concern. Additional large scale and well-designed studies are needed to fully evaluate the efficacy in AD. A holistic approach would encompass assessment of the severity and impact on quality of life, assessment and management of environmental physical and psychological triggers, recognition and treatment of infection, and restoration of the skin barrier function [55].

4. Conclusion

Oxidative stress appears to be one of the important factors in the pathogenesis of atopic dermatitis. It not only directly damages the cellular structures of the skin but also enhances dermal inflammation and weakens the skin barrier function and enables infections by microbial pathogens. Given our current understanding of the pathogenesis of AD, strategies should be focused on multimodality and individualized therapy. Treatment goals should include (1) reducing environmental insults and psychological stress; (2) enhancing the skin barrier function by skin hydration and emollients; (3) exploring anti-inflammatory and immune modulatory agents as second-line therapy; and (4) using oral antioxidant supplements, such as appropriate amount of daily vitamins and melatonin. At this time, well-designed clinical studies are needed to fully evaluate those approaches for the ultimate goal of not only relieving symptoms but also improving the overall quality of life in AD patients.

Abbreviations

AD: Atopic dermatitis
Ig: Immunoglobulin
MDA: Malondialdehyde
ROS: Reactive oxygen species
NOS: Nitrogen oxygen species
8-OHdG: 8-Hydroxydeoxyguanosine.

Conflict of Interests

The authors declare no competing financial interests.

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