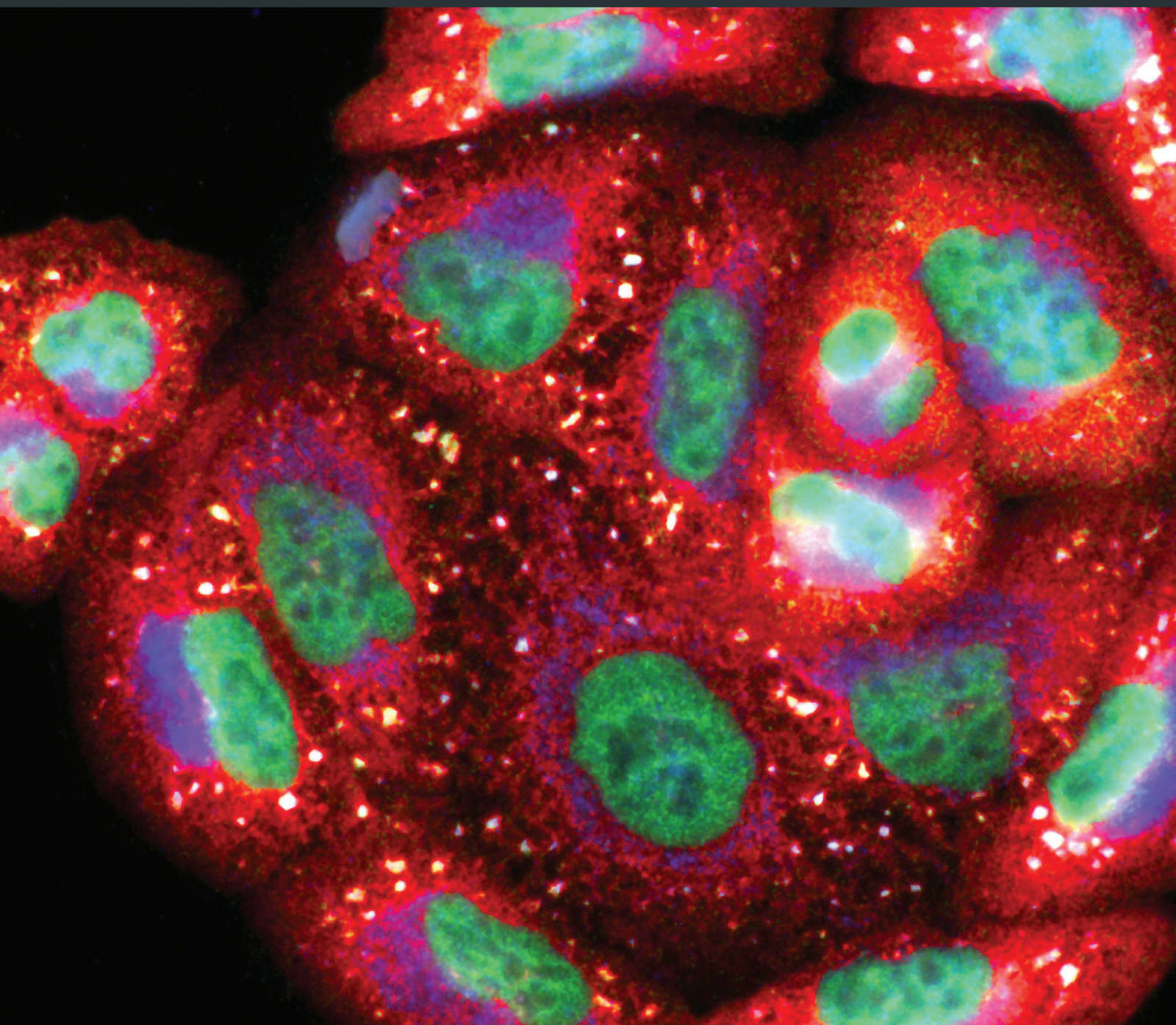
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Disease and Aging: Mechanisms and Therapies

Guest Editors: Claudio Cabello-Verrugio, Marta Ruiz-Ortega, Matias Mosqueira, and Felipe Simon



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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8786564, 2 pages
http://dx.doi.org/10.1155/2016/8786564

Editorial

Oxidative Stress in Disease and Aging: Mechanisms and Therapies

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Received 9 September 2015; Accepted 9 September 2015

There is an increase in the population affected by chronic disease and aging during the last decades in which the oxidative stress is common factor in its development. Cellular oxidative stress is defined as an imbalance between the formation of reactive oxygen species (ROS) and antioxidant defense mechanisms. Due to the broad and profound biological effects of ROS, in the last years numerous experimental and clinical studies have focused their attention on the participation of oxidative stress as a key regulator in chronic pathological status and aging.

This special issue of OSDA is devoted to the new and relevant findings about the mechanisms by which the altered balance between ROS and cellular antioxidant machinery induce oxidative damage during chronic disease or aging. Among other manuscripts in this special issue that are equally recommended by the editors, it is interesting to comment on the following manuscripts.

M. A. Gómez-Marcos et al. tested the hypothesis whether superoxide dismutase (SOD) serum levels are correlated with vascular structure and function in hypertensive and type 2 diabetic patients. They showed negative correlations between SOD and pressure wave velocity, peripheral and central augmentation index, ambulatory arterial stiffness index, pulse pressure, and plasma HDL-cholesterol and positive correlations between SOD and plasma uric and triglycerides, suggesting that SOD serum levels turn in a marker for cardiovascular alterations in hypertensive and diabetic patients.

Y. Ting Lam discusses the roles of ROS in regulating stem and progenitor cell function, highlighting the impact of unbalanced ROS levels on endothelial progenitor cells dysfunction and the association with age-related impairment in ischemia-induced neovascularization. Furthermore, it discusses strategies that modulate the oxidative levels of stem and progenitor cells to enhance the therapeutic potential for elderly patients with cardiovascular disease.

O. Kost et al. examine a nanoscale therapeutic modality for the eye on the base of antioxidant enzyme SOD1, termed "nanozyme." They show that the nanozyme was much more effective compared to the free enzyme in decreasing uveitis manifestations, considering SOD1-containing nanozyme in a potentially useful therapeutic agent for the treatment of ocular inflammatory disorders.

H. Liu et al. tested the antioxidant and anti-inflammatory effects of polydatin, an active compound isolated from *Polygonum cuspidatum* Sieb. et Zucc. roots on renal ischemia

reperfusion model. The authors reported that polydatin significantly improved renal function after dose dependent increased expression of Akt phosphorylation and strong suppression of tumor necrosis factor-α, interleukin-1β, cyclooxygenase-2, inducible nitric oxide (NO) synthase and NO levels, and prostaglandin E-2. These results together open a strategy for the prevention and treatment of acute renal ischemia and reperfusion.

Y. Yang and S. Li investigated the protective roles of dandelion extracts used in the traditional Chinese and traditional medicine in the treatment of skin diseases. They found that dandelion extracts, especially leaf and flower extracts, are potent protective agents against UVB damage and H_2O_2-induced cellular senescence in HDFs by suppressing ROS generation and metalloproteinase activities and improving UVB absorption.

These manuscripts mentioned together with others contained in this special edition show that diverse antioxidant strategies have significant improvement on the pathological status reported. We hope that the readers of this special issue appreciate the progress and the new strategies developed in the field oxidative stress associated with disease and aging.

Acknowledgments

The editors thank all the authors who submitted their research to this special issue. They also thank all the reviewers for their contribution to this special issue. The lead guest editor thanks all the guest editors for the critical and exhaustive process of review which was critical in handling the paper.

Claudio Cabello-Verrugio
Marta Ruiz-Ortega
Matias Mosqueira
Felipe Simon

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5194239, 13 pages
http://dx.doi.org/10.1155/2016/5194239

Research Article

Superoxide Dismutase 1 Nanozyme for Treatment of Eye Inflammation

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Received 9 April 2015; Revised 6 August 2015; Accepted 12 August 2015

Academic Editor: Felipe Simon

Use of antioxidants to mitigate oxidative stress during ocular inflammatory diseases has shown therapeutic potential. This work examines a nanoscale therapeutic modality for the eye on the base of antioxidant enzyme, superoxide dismutase 1 (SOD1), termed "nanozyme." The nanozyme is produced by electrostatic coupling of the SOD1 with a cationic block copolymer, poly(L-lysine)-poly(ethyleneglycol), followed by covalent cross-linking of the complexes with 3,3′-dithiobis(sulfosuccinimidylpropionate) sodium salt. The ability of SOD1 nanozyme as well as the native SOD1 to reduce inflammatory processes in the eye was examined *in vivo* in rabbits with immunogenic uveitis. Results suggested that topical instillations of both enzyme forms demonstrated anti-inflammatory activity; however, the nanozyme was much more effective compared to the free enzyme in decreasing uveitis manifestations. In particular, we noted statistically significant differences in such inflammatory signs in the eye as the intensities of corneal and iris edema, hyperemia of conjunctiva, lens opacity, fibrin clots, and the protein content in aqueous humor. Clinical findings were confirmed by histological data. Thus, SOD1-containing nanozyme is potentially useful therapeutic agent for the treatment of ocular inflammatory disorders.

1. Introduction

Uveitis is an inflammatory disease of the uvea, a section of the eye which consists of the middle pigmented vascular structures of the eye and includes the iris, ciliary body, and choroid. Common causes of uveitis include infections, multisystem disorders such as sarcoidosis and Behçet's disease and autoimmune disorders such as rheumatoid arthritis or ankylosing spondylitis [1–4]. Uveitis is a severe sight threatening disease, frequently leading to vision loss and blindness with retinal vasculitis, retinal detachment, and glaucoma. Uveitis accounts for 5–20% of legal blindness in United States and in Europe, and perhaps as much as 25% of blindness in the developing world [1]. Severe cases of uveitis need to be treated aggressively to prevent damage caused by chronic inflammation. Corticosteroids constitute the first line of therapy for patients with noninfectious ocular inflammatory disease. However, as the use of corticosteroids became more prevalent in treating ocular inflammation, the side effects of this treatment became more prevalent as well. Another class of compounds, known as "immunosuppressive drugs," such as cyclosporine A, was found to be successful in treating uveitis. However, such treatment is also complicated by side effects associated with immunosuppression [5–7]. Patients who cannot take medications because of the side effects or patients who are not responsive to the existing medications

experience unavoidable impaired visual function. Thus, it is important to investigate alternative approaches for the treatment of uveitis.

Inflammatory diseases, including ocular ones, are accompanied by excessive production of reactive oxygen species (ROS) and by depletion of endogenous antioxidants. Antioxidant enzymes, superoxide dismutase 1 (SOD1, also known as Cu/Zn SOD), catalase, and glutathione peroxidase are known to be very effective scavengers of ROS. These enzymes were shown to be effective in the treatment of various eye diseases associated with oxidative stress. Thus, SOD1 was used for the treatment of lens-induced and bovine albumin-induced uveitis in rabbits [8, 9], as well as for the treatment of acute corneal inflammation in animals induced by sodium hydroxide [10, 11]. Both SOD1 and glutathione peroxidase were employed for the treatment of severe experimental allergic uveitis induced by retinal S antigen in rats [12], while poly(ethylene glycol)- (PEG-) modified catalase and PEG-SOD were employed for the treatment of the same type of uveitis in guinea pigs [13]. We have shown recently [14] that SOD1 instillations may help to reduce clinical presentations of immunogenic uveitis in rabbits.

Eye diseases are most commonly and preferably treated by topical instillations of eye drops. These formulations face technical and clinical problems, such as solubility of the components and instability of drug solutions, limited efficacy and limited corneal/sclera permeability, and local and systemic toxicity. Moreover, 2 min after instillation the major part of the topical drug solution is eliminated via the nasolacrimal drainage system limiting ocular penetration of the drug to less than 5% of the administered dose [15].

Nanoparticles are colloidal drug carrier systems that can improve the efficacy of drug delivery into the eye by overcoming corneal/sclera diffusion barrier. Drug loaded polymeric nanoparticles offer several favorable biological properties, such as biocompatibility and mucoadhesiveness, enhancing bioavailability without blurring the vision. The use of drug-containing nanoparticles can decrease the dose of the drug and diminish side effects. So, nanoparticles are a promising drug delivery system, which fulfills the requirements for ophthalmic application (for reviews, see [16, 17]).

Recently, new formulations of antioxidant enzymes, SOD1 and catalase, were prepared by electrostatic coupling of these negatively charged enzymes (pI values are 4.95 and 5.8 for SOD1 and catalase, resp.) with cationic block copolymers, such as methoxy-PEG-*block*-poly(L-lysine hydrochloride) block copolymer (PEG-pLL$_{50}$), followed by covalent cross-linking to stabilize nanoparticles. Catalytic nanoparticles based on polyion complexes of enzymes with block copolymers of opposite charge were termed "nanozymes" [18–22]. Spontaneous self-assembly of oppositely charged proteins and polymers results in stoichiometric complexes with 100% loading efficiency. These nanozymes were shown to be prospective agents for the treatment of various diseases of the central nervous system due to prolonged ability to scavenge experimentally induced ROS in cultured brain microvessel endothelial cells and central neurons, increased stability in both blood and brain, enhanced penetration through the blood-brain barrier, and, therefore, increased accumulation in brain tissues, in comparison with non-cross-linked complexes and native enzyme [18, 21, 22].

In the current study, we demonstrate the advantages of topical instillations of superoxide dismutase 1 in the form of "nanozyme" in the treatment of ocular inflammation in a rabbit model of immunogenic uveitis.

2. Methods

2.1. Preparation of and Characteristics of Nanozyme. SOD1 nanozyme was synthesized by self-assembly of recombinant SOD1 ("Enzyme Technologies", St. Petersburg, Russia) with cationic block copolymer, PEG-pLL$_{50}$ (MW 13 kDa, polydispersity index 1.09, Alamanda Polymers, Huntsville, AL) in aqueous solution followed by cross-linking with 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) as in [21]. Unreacted cross-linker was desalted using NAP-25 column, and cross-linked nanozymes were purified using a 100 kDa MWCO filter. Purified particles were then lyophilized from 0.05 M Hepes-buffer, pH 7.5, containing 0.15 M NaCl, and stored at −20°C. For further experiments, precalculated quantity of lyophilized nanozyme was dissolved in deionized water and gently vortexed for 2 min until sample dissolved completely. Intensity-mean z-averaged particle diameter (effective diameter), polydispersity index (PDI), and ζ-potential were measured after filtration via a 0.2 μm filter using a Zetasizer Nano ZS (Malvern Instruments Ltd., MA). Aliquots of nanozyme solution required for daily experiments were then frozen and kept at −20°C.

2.2. Enzyme Activity. SOD1 activity was determined using SOD1 ability to inhibit autooxidation of quercetin as in [23] with detection kit (Belarusian State University, Belarus). The experimental sample in phosphate buffer, pH 7.8, containing 0.08 M EDTA, 0.125% (v/v) TEMED, was mixed with quercetin solution in DMSO. The absorbance was measured at 406 nm immediately after addition of quercetin (D_0) and after 20 min. (D_{20}). In control, phosphate buffer was used instead of the sample, and the absorbances ${}^cD_0$ and ${}^cD_{20}$ were measured, correspondingly. The percent of inhibition of quercetin autoxidation by SOD1 in experimental samples was calculated by the formula $[({}^cD_0 - {}^cD_{20}) - (D_0 - D_{20})]/({}^cD_0 - {}^cD_{20}) \times 100$. One unit of SOD1 activity was defined as the amount of SOD1, which inhibits the quercetin autoxidation by 50%. The protein content was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

2.3. In Vitro Drug Release Study. One mg of SOD1 nanozyme was dispersed in 0.5 mL of PBS, transferred to 100 kDa MWCO membrane, and centrifuged for 5 min at 1200 ×g. Supernatant (about 0.05 mL) was diluted by PBS to the initial volume and centrifuged again. The "filtration-dilution" procedure was repeated 5 times. SOD1 activity and protein concentration were measured in the initial nanozyme solution, in each filtrate, and in the final supernatant. In another series of experiments, equal amounts of freshly dissolved nanozyme in PBS were incubated at room temperature for different time periods. Then, the solutions were filtered

through 100 kDa membrane, and SOD1 activity in the filtrates was determined.

2.4. Animals. A randomized and double-blinded study was conducted using adult Chinchilla rabbits weighing 2.0–2.5 kg. All experiments with live rabbits were carried out in strict accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Helmholtz Institute for Eye Disease (Permit number 22/2). All efforts were made to minimize rabbits suffering. After the end of experiments the rabbits were sacrificed by lethal pentobarbital injection.

2.5. In Vivo Studies. Immunogenic uveitis was induced as described in [24]. Briefly, rabbits were initially injected subcutaneously with 5 mL of normal horse serum for sensitization. Ten days later, 5% anesthetic Alcain (Alcon, Belgium) was instilled into each eye before the intravitreal injections of 70 μL of the same serum in the eyes to induce acute uveitis.

Rabbits received 30 μL of the drug solutions as eye drops topically in each eye three times a day for 14 days. Three independent series of experiments for clinical estimation of uveitis were performed. In each series, animals were randomly divided into 4 groups ($n = 5$ per each group, i.e., 10 eyes) and treated as follows: (1) *control* (healthy) group without uveitis and (2) *placebo* group with uveitis received 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl; (3) *SOD1* group with uveitis received 1 mg/mL SOD1 solution in the same buffer; (4) *treatment (nanozyme)* group with uveitis received 8–10 mg/mL SOD1 nanozyme solution in the same buffer. Hepes buffer did not cause any irritation of the eye. SOD1 dose was chosen in accordance with what was recommended in [25]. The dose of nanozyme solution was calculated based on enzymatic activity of nanozyme (units per mg) so that the activities of SOD1 and nanozyme solutions were equal; that is, 8–10 mg nanozyme corresponded to 1 mg SOD1 by specific activity. Eyes were examined in a double-blinded trial by indirect ophthalmoscopy using a slit lamp (Zeiss slit lamp 30SL, USA). Clinical symptoms of uveitis, including eyelid and conjunctival edema and hyperemia, corneal edema and neovascularization, iris edema and hyperemia, fibrin clots and precipitates on the iris and on the lens, lens opacity, presence of synechiae (cohesions between the pupillary margin of iris and anterior part of the lens), which lead to immobilization of the pupil, and presence of purulent exudate (hypopyon) and blood (hyphema) in the anterior chamber of the eye, were estimated. Evaluation of inflammation scores was performed using a conventional scale: (0) no symptom; (1) low degree of manifestation; (2) medium; (3) strong.

2.6. Analyses of Aqueous Humor. Aqueous humor (intraocular fluid) from anterior chamber of the eye was collected by paracentesis in limb area under topical anesthesia on day 8 of uveitis (that is, 16 h after last instillations of the drugs) and on day 4 in the separate experiment on two rabbits (4 eyes) in each group. The samples were centrifuged at 21,000 ×g for 10 min, and the supernatant was stored at −20°C. The amount of leukocytes was determined microscopically. The α_2-macroglobulin content (in arbitrary units) was estimated indirectly as previously described [26, 27] based on the ability of the complex of α_2-macroglobulin with trypsin to react with benzoyl-L-arginine-p-nitroanilide as a substrate. Antioxidant activity was determined by chemiluminescence kinetics in hemoglobin-H_2O_2-luminol system as described in [28] with Trolox, a water-soluble analog of vitamin E, as a standard antioxidant. Antioxidant activity of the sample was expressed as trolox-equivalents calculated on the basis of a trolox standard curve.

2.7. Histopathology. Histopathological analysis was performed in a double-blinded fashion. For this experiment, we used 10 rabbits (20 eyes), 9 rabbits with uveitis and 1 healthy rabbit. For the topical treatment, rabbits with uveitis were randomly divided into three groups. The 1st group of 3 rabbits received placebo as described above, the 2nd group of 3 rabbits received native SOD1, and the 3rd group of 3 rabbits received SOD1 nanozyme with the same SOD1 activity. On day 4 rabbits were sacrificed by sodium pentobarbital injection (100 mg/kg), and the eyes were enucleated. Samples were fixed in 10% neutral buffered formalin, dehydrated in a graded series of alcohol, embedded in paraffin, and cut into 4-5 μm serial sections. The sections were stained with haematoxylin and eosin (H&E) and examined in upright light microscope (Olympus BX51) using dry-air (4./NA0.10; 10./NA0.25; 20./NA0.40) and oil-immersion (100./NA1.25 oil) objectives (Olympus Optical, Tokyo, Japan). Histology images were recorded in a single-frame mode using a digital video camera SDU-252 (2048 × 1536, "Spetsteletechnika", Russia) integrated into the microscope optical path.

2.8. Statistical Analysis. All data are means ± SEM. Significance was analyzed using the Mann-Whitney U test with STATISTICA 6 (StatSoft, Inc., OK).

3. Results

3.1. Synthesis and Characterization of Nanozyme. SOD1 nanozyme was synthesized as described earlier [21] by mixing of aqueous solutions of SOD1 and block copolymer, PEG-pLL_{50} at pH 7.4 followed by cross-linking and purification. SOD1 retained 100% its catalytic activity in polyion complex before cross-linking consistent with previous report [21, 22] but partly lost activity as a result of cross-linking with DTSSP and filtering through 100 kDa membrane. Altogether, the lyophilized dry nanozyme samples displayed the specific activity about 30 kU/mg, while the activity of the unmodified pure recombinant SOD1 was ca. 250 kU/mg. The observed decrease in the specific activity was mainly due to the presence of the bulk of polymer in nanozyme as well as to the presence of buffer substance and salt in the final lyophilized preparation. The DLS analysis revealed that the particles of SOD1 nanozyme had an effective diameter of 35 nm (compared to about 5 nm for native SOD1, as reported in [21]), narrow particle size distribution (PDI ca. 0.1), and nearly neutral (zero) ζ-potential.

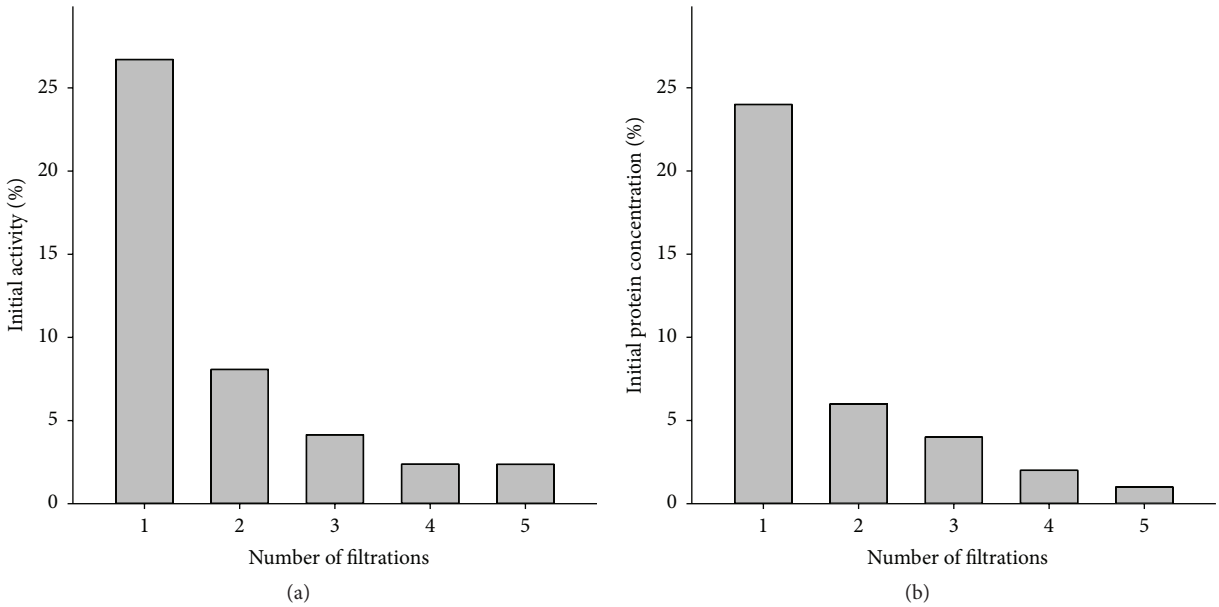

Figure 1: The release of SOD1 activity and protein upon subsequent dilutions and filtrations of nanozyme. Nanozyme was dissolved in deionized water and immediately filtered through 100 kDa MWCO filter, supernatant was diluted using PBS to the initial volume and filtered again. "Dilution-filtration" steps were repeated, and SOD activity (a) and protein content (b) were determined in each filtrate. The experiment was performed in duplicate.

To examine whether SOD1 can be released from the nanozyme we determined the activity of SOD1 in the filtrates and supernatant (1) after repeated centrifugal filtration of the nanozyme solution using 100 kDa MWCO filters and (2) after centrifugal filtration of nanozyme aqueous solution incubated for different periods of time. In the first experiment, the lyophilized nanozyme was dissolved in deionized water so that the final concentration of NaCl was 0.15 M and immediately filtered through 100 kDa membrane for 5 min. After this first filtration step, about 24% of the protein and about 27% of SOD1 activity (Figure 1) were found in the filtrate. Further dilutions of supernatant to the initial volume and subsequent filtration resulted in additional release of SOD1 activity and protein from the nanozyme, albeit to a lesser extent than the initial filtration step, from 2 to 7% of the initial amount. After 5 subsequent dilutions and filtrations, the nanozyme retained about 55–65% of both SOD1 activity and protein. It is interesting that the release of SOD1 from the nanozyme occurs not only after the "dilution-filtration-dilution" procedures but also upon incubation of its aqueous solution for various time periods after preparation. Specifically, freshly prepared solution of 2 mg nanozyme in 0.5 mL 0.15 M NaCl contained about 25 to 30% free SOD1, while after 2 hr incubation the same solution contained 40% of the free SOD1, and 4 hr 40 to 50% of the free SOD1. Further incubation of the nanozyme solution did not result in the further release of the free SOD1. These data suggest that the nanozyme synthesis process and specific chemistries used in this work produce nanozymes encapsulating significant portion of SOD1 that is not chemically coupled to the block copolymer and can be released in the surrounding media as it was observed previously [22].

3.2. Effects of Topical Instillations of SOD1 and SOD1 Nanozyme on Clinical Manifestations of Immunogenic Uveitis in Rabbits. We induced immunogenic uveitis in rabbits in three independent series of experiments. In one series, the uveitis appeared to show severe manifestations of inflammation in the outer part of the eye (eyelid, cornea, and conjunctiva), while, in the other two, inflammation of these tissues was rather moderate. Manifestations of inflammation in the inner part of the anterior segment of the eye, however, were significant in all three series of experiments. It is noteworthy that, in all series, uveitis developed similarly, with the most acute phase on days 3–5 and fading till the end of second week.

3.2.1. Rabbits without Treatment (Placebo). Three days past intravitreal injection of horse serum, the eyes of animals showed classical clinical symptoms of anterior uveitis which intensified on day 4. Edema of the eyelid, cornea, and conjunctiva were observed. Hyperemia of conjunctiva was significant. Iris had both edema and hyperemia; its structure was changed. There was a lot of fibrin clots in the anterior part of the eye, which, in several cases, formed massive clouds. In most eyes, there were multiple synechiae, which resulted in pupil immobilization, improper pupil form, and the lack of reaction of pupil to light. Fibrin clots were also found on the surface of lens of all eyes; lenses itself were characterized by significant opacity, which thwarted microscopic investigation of the vitreous body. Many animals (about half) had massive purulent exudates (so-called hypopyon) in the anterior camera of the eye, which was formed by leukocytes and detritus. Neovascularization of the cornea, which is known to be a result of oxidative stress [29], was observed in half

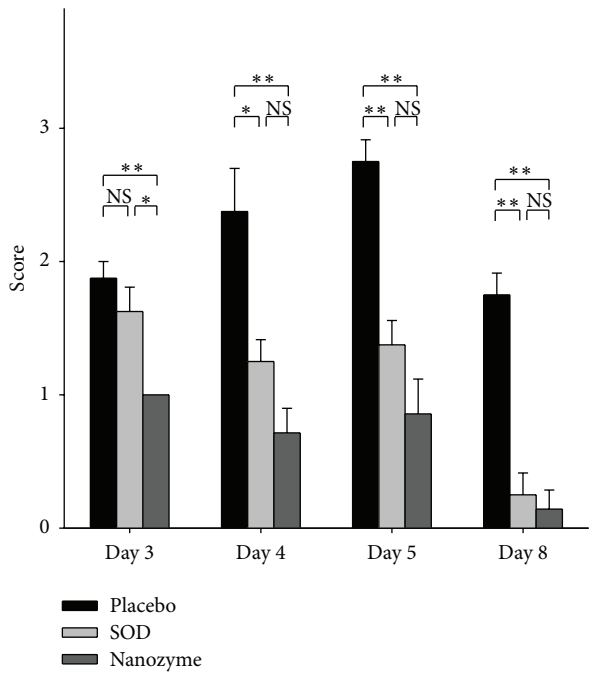

Figure 2: The effect of instillations of nanozyme and SOD solutions in the rabbit eye on conjunctival hyperemia at uveitis. The data from three series of experiments were analyzed, each experiment including 5 animals (10 eyes) in each group: control, placebo, SOD1-treated, and SOD1 nanozyme-treated group. Thus, $n = 30$ for each group. The scores were estimated as a degree of manifestation of hyperemia of conjunctiva: 0: no symptom; 1: low degree of manifestation; 2: medium degree; 3: strong degree. Symbols: $*$: the level of significance of differences by the Mann-Whitney U test $p < 0.05$; $**$: the level of significance of differences by the Mann-Whitney U test $p < 0.01$; NS: not significant differences.

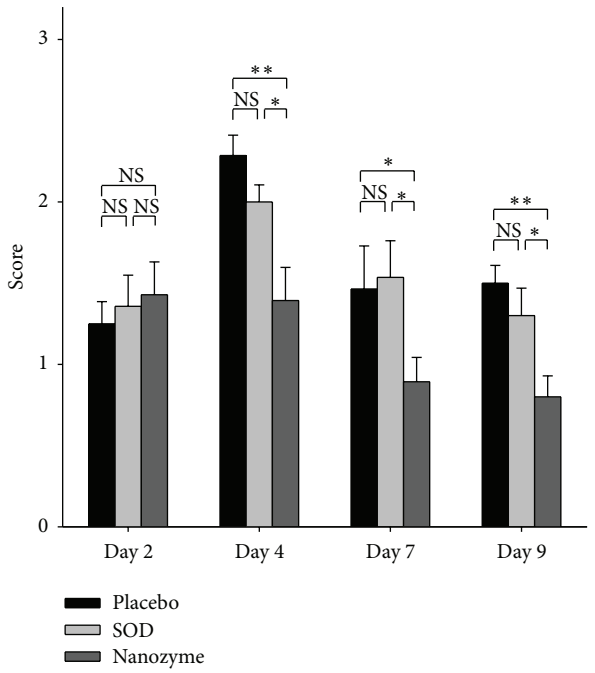

Figure 3: The effect of instillations of nanozyme and SOD solutions in the rabbit eye on the formation of fibrin clots at uveitis. The data from three series of experiments were analyzed, each experiment including 5 animals (10 eyes) in each group: control, placebo, SOD1-treated, and SOD1 nanozyme-treated group. Thus, $n = 30$ for each group. The scores were estimated as degree of fibrin clots formation: 0: no clots; 1: low degree of clot formation; 2: medium degree; 3: strong degree. Symbols: $*$: the level of significance of differences by the Mann-Whitney U test $p < 0.05$; $**$: the level of significance of differences by the Mann-Whitney U test $p < 0.01$; NS: not significant differences.

of the eyes. Some animals exhibited symptoms of elevated intraocular blood pressure (from 8th day) which indicated the development of common uveitis complication, secondary glaucoma.

3.2.2. SOD1-Treated Rabbits. The development of uveitis in this group remarkably differed from that in placebo group. Eyelid edema was much less pronounced, and hypopyon was absent at all times during the disease. Corneal and iris edema were only local and diminished in time. Conjunctival edema and hyperemia were less pronounced as well. Neovascularization of the cornea in the acute phase of uveitis was observed in 20–30% of eyes. During treatment, we observed regress of synechiae formation (from day 4 to day 8) and partial restoration of the reaction of the pupil to light. At the end of the treatment, however, lens opacity decreased insignificantly. Many eyes retained precipitates on the lens. Figures 2 and 3 are representative examples of the comparative effects of SOD1 and placebo instillations in the rabbit eye on conjunctival hyperemia and formation of fibrin clots at different times during uveitis. It was seen that while there was no statistical difference between the extents of hyperemia of conjunctiva in the eyes of SOD-treated and placebo-treated eyes on day 3, later (on days 4 and 5) this difference became statistically significant and on day 8 this difference is remarkable (Figure 2). On the contrary, there was no difference between the amount of fibrin clots observed in SOD-treated and placebo-treated rabbits (Figure 3).

3.2.3. SOD1 Nanozyme-Treated Rabbits. Clinical manifestations of uveitis in this group were less pronounced and appeared later than that in placebo- and SOD1-treated groups. Most importantly, hyperemia of conjunctiva, corneal edema, iris edema, and lens opacity were significantly less pronounced than in the two other groups. There were no eyes with neovascularization of the cornea in this group. Synechiae were lower by 20–25%, which improved the pupil reaction to light. Fibrin clots were less intense as well. Figure 2 demonstrates the effect of SOD1 nanozyme instillations on the conjunctival hyperemia in comparison with the effects of instillations of placebo and native SOD1. There was a clear statistical difference ($p < 0.01$) between SOD1 nanozyme and placebo groups at all times of the disease. Moreover, nanozyme was statistically more effective than native SOD1 on day 3 (in acute phase of uveitis). The formation of fibrin clots in the case of nanozyme-treated groups was statistically

Table 1: Biochemical parameters of aqueous humor on day 8 of uveitis in rabbits.

Biochemical parameter	Treatment			
	Control	Placebo	SOD1	Nanozyme
Total protein concentration, mg/mL	2.2 ± 0.3	19.3 ± 3.2	15.6 ± 2.3	10.5 ± 1.1**
α_2-Macroglobulin, arb. U/mL	0.6 ± 0.1	10.8 ± 1.0	8.7 ± 1.5	7.2 ± 1.6*
Antioxidant activity, U/mL	14.4 ± 1.0	2.3 ± 0.9	3.3 ± 0.6	3.7 ± 0.2
SOD1 activity, trolox-equivalents/mL	280 ± 40	460 ± 100	370 ± 80	330 ± 50*

*Significant difference between nanozyme and placebo by the Mann-Whitney U test, $p < 0.05$; **Significant difference between nanozyme and placebo, and nanozyme and native SOD1 by the Mann-Whitney U test, $p < 0.01$.

less pronounced (Figure 3) than in the placebo- and SOD1-treated groups at every time point of uveitis, starting from day 4. Thus, we demonstrated that nanozyme treatment resulted in the considerable improvement of uveitis condition in rabbits compared not only with the untreated animals but with the native SOD1-treated group as well.

3.3. Effects of Topical Instillations of SOD1 and SOD1 Nanozyme on Clinical Symptoms of Uveitis in the Acute Phase of the Disease. Most clearly, the differences in the effects of topical instillations of SOD1 nanozyme, SOD1, and placebo are seen in the acute phase of the disease, that is, on days 3-4. We compared the efficacy of these treatments using the sums of the scores for the manifestations of inflammation in the outer and inner parts of the anterior eye segment. This is a common approach in ophthalmology to test drug efficacy [30]. The manifestations of inflammation were assessed by (1) the eyelid edema, corneal edema, and hyperemia of conjunctiva in the outer part and (2) the iris edema, lens opacity, and fibrin clots in the inner part. The results are shown in Figures 4 and 5. While native SOD1 seems to have a healing effect on inflammation in the outer part of the eye, this effect was not statistically significant. In contrast, SOD1 nanozyme showed statistically significant healing effect (Figure 4) in comparison with both placebo ($p < 0.01$) and native SOD1 ($p < 0.05$). The difference between SOD1 formats was even more pronounced when we compared their effect on the inflammation in the inner part of the anterior segment of the eye (Figure 5). While the healing effect of native SOD1 was not statistically different from that of placebo, the SOD1 nanozyme showed remarkable healing effect, which was significantly distinct from the effects of native SOD1 and placebo ($p < 0.01$ in both cases).

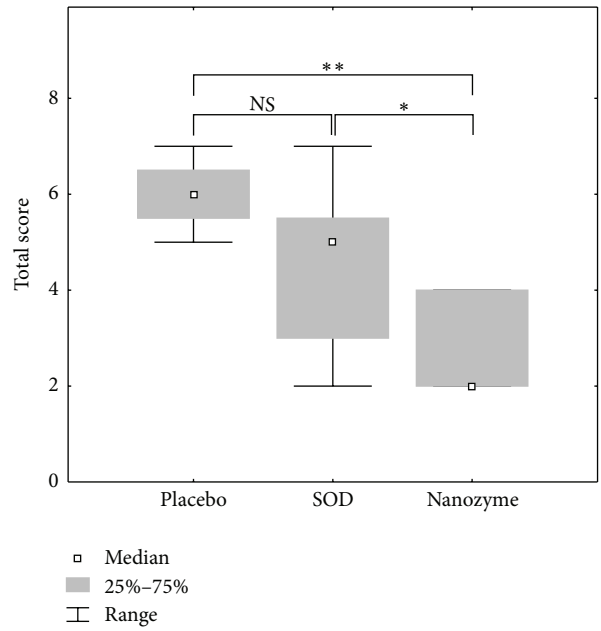

Figure 4: Comparison of the clinical symptoms of uveitis in external rabbit eye structures as a sum of the scores for eyelid edema, conjunctival hyperemia, and corneal edema in the acute phase of the uveitis. The data from three series of experiments were analyzed, each experiment including 5 animals (10 eyes) in each group: control, placebo, SOD1-treated, and SOD1 nanozyme-treated group. Thus, $n = 30$ for each group. The scores were estimated as degrees of manifestations of clinical symptoms of the disease: 0: no symptom; 1: low degree of manifestation; 2: medium degree; 3: strong degree. Symbols: $*$: the level of significance of differences by the Mann-Whitney U test $p < 0.05$; $**$: the level of significance of differences by the Mann-Whitney U test $p < 0.01$; NS: not significant differences.

3.4. Effect of Treatments on the Leukocyte Counts and Biochemical Parameter of the Aqueous Humor of the Eye. ROS metabolites are predominantly produced by polymorphonuclear leukocytes, which migrate to inflamed tissues and can serve as an indication of the inflammation. The aqueous humors from the eyes of all rabbits with uveitis contained considerable amount of leukocytes both in the acute (day 4) and later (day 8, e.g., after 16 h after the last instillations of the drugs) phases of the disease. On day 4, the SOD1-treated group displayed approximately the same leukocyte counts as placebo group, while SOD1 nanozyme-treated group exhibited decrease in leukocyte counts although statistically insignificant. On day 8, both SOD-treated groups showed the decrease in leukocyte counts, SOD1 nanozyme-treated group exhibiting statistically significant effect compared with placebo (Figure 6).

Tissue inflammation is characterized by elevated total protein concentration in biological fluids along with the increase in proteinase inhibitor α_2-macroglobulin and decrease of overall antioxidant activity, as well as increase in endogenous SOD1 [31–34]. Therefore, we determined these biochemical parameters in aqueous humor on day 8 after the disease onset (Table 1). The protein concentration

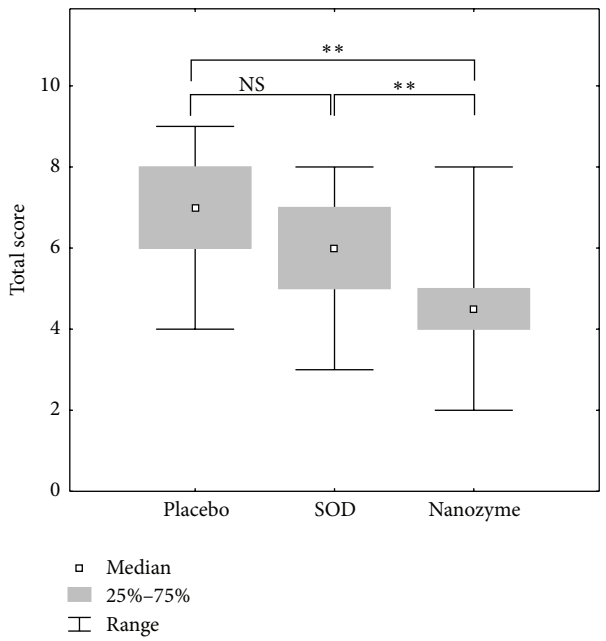

Figure 5: Comparison of the clinical symptoms of uveitis in internal rabbit eye structures as a sum of the scores for iris edema, fibrin clots, and lens opacity in the acute phase of the uveitis. The data from three series of experiments were analyzed, each experiment including 5 animals (10 eyes) in each group: control, placebo, SOD1-treated, and SOD1 nanozyme-treated group. Thus, $n = 30$ for each group. The scores were estimated as in the legend of Figure 3. Symbols: **: the level of significance of differences by the Mann-Whitney U test $p < 0.01$; NS: not significant differences.

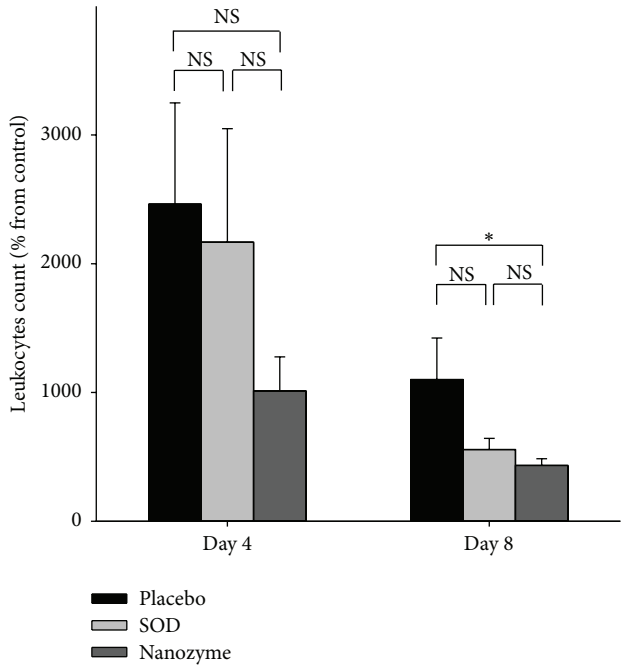

Figure 6: Leukocyte numbers in aqueous humor of the eye of uveitis rabbits as percentage from the value for control (healthy) rabbits on different times of the disease. $n = 4$ in each group on day 8; $n = 10$ in each group on day 8.

in nontreated group increased 8-fold compared to healthy animals and it was only slightly affected after the native SOD1 treatment. In contrast, SOD1 nanozyme treatment resulted in nearly 2-fold decrease in this parameter. The effect of nanozyme was significant compared to both placebo and native SOD1 ($p < 0.01$). The development of uveitis also resulted in drastic increase of α_2-macroglobulin activity in aqueous humor; however it was mitigated after both the native SOD1 and, especially, SOD1 nanozyme treatments (Table 1). Antioxidant activity increase in both SOD1 and nanozyme-treated groups was rather small and not significant (Table 1). The endogeneous SOD1 activity in aqueous humor of placebo-treated eyes with uveitis was increased by more than in 1.5 times compared to control group suggesting a compensatory reaction of the eye to the uveitis-induced oxidative stress. The native SOD1 treatments decreased the enzyme activity in aqueous humor, albeit nonsignificant, while SOD1 nanozyme treatment decreased this parameter significantly (Table 1). Notably, in the healthy and placebo-treated rabbits, SOD1 activity in aqueous humor represents only the endogenous enzyme, while in SOD1- and nanozyme-treated rabbits it may contain contributions of exogenous SOD1 as well. Still the decrease in the measured SOD1 activity in treated eyes clearly shows that SOD1 and, especially, nanozyme treatments decrease inflammation during experimental uveitis.

3.5. Histology Examination of the Disease Manifestation. The eyes of the control, healthy rabbits were unchanged. The cornea displayed its common structure with thin multilayer epithelium on the outer side (cells form 2 or 3 layers) and single-layer endothelium on the inner side (Figure 7(a)). The epithelium and endothelium cells had normal structure; the main part of the stroma could be clearly seen. The conjunctival tissue was loose, moderately full-blooded. The vessels in the region of conjunction of cornea, conjunctiva, and sclera were wide and full-blooded as well. The sclera and ciliary body (Figure 8(a)) possessed their normal structure as well. The retina in the eyes of normal rabbits was also unchanged.

3.5.1. Rabbits without Treatment (Placebo). In the placebo group having uveitis, one eye contained white, thick, non-transparent expanding mass. This mass is known to represent a purulent exudate, consisting of leukocytes (some of them in a stage of disintegration) and small amount of fibrin. The vitreous body in this eye was in the state of destruction and cell infiltration. Another eye of the same animal, as well as the eyes of other rabbits in this group, maintained the vitreous body but the inner part of choroid contained white precipitates. Choroid in these eyes was thickened, known to be due to infiltration of neutrophils, macrophages, and lymphocytes. Some destruction of the pigment cells layer was also observed. The vessels within choroid were full-blooded but with some extent of erythrocyte aggregation. The cornea in this group exhibited edema, swelling, and loosening of collagen fibers, as well as partial desquamation of endothelium and partial destruction of Descemet membrane

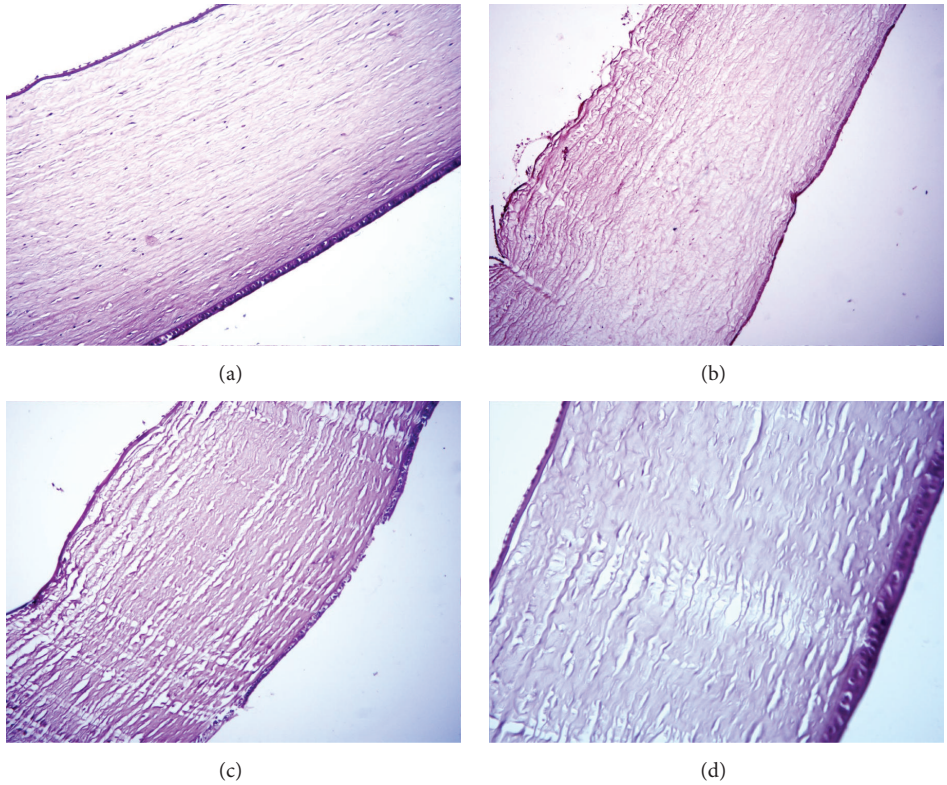

Figure 7: Histology picture of the cornea, stained with hematoxylin and eosin, magnification ×200, on day 4 of uveitis. (a) Control ($n = 2$): cornea is lined with epithelium and endothelium; collagen fibers and keratocytes (specialized corneal fibroblasts) are visible; (b) placebo ($n = 6$): swelling and loosening of corneal collagen fibers, endothelial desquamation, partial destruction of Descemet's membrane; epithelium is not changed; (c) SOD1 ($n = 6$): moderate loosening of the corneal collagen fibers, partial endothelial desquamation, cell infiltration is absent; (d) SOD1 nanozyme ($n = 6$): normal structure of the cornea.

(Figure 7(b)). Epithelium of the cornea was not changed. The sclera in almost all eyes exhibited pronounced edema and contained sporadic neutrophil-macrophage infiltrates. The bundles of collagen fibers within sclera were loosened. There were also significant edema and inflammatory infiltration in ciliary body (Figure 8(b)), while loosened stroma of ciliary body exhibited signs of cellular dystrophy, characterized by the formation of cytoplasm vacuoles. Partial destruction and desquamation of epithelium of ciliary body and its processes with deposits of purulent exudates was also observed. Retina in the eyes of rabbits from this group had regions of destruction and exhibited signs of dystrophy of cells elements.

3.5.2. SOD1-Treated Rabbits. The eyes of rabbits in the SOD1 treatment group had only moderate inflammation manifestation in uveal tract. The cornea was lined by unchanged epithelium. The Descemet membrane did not have defects; however, some regions of the cornea contained loosened collagen fibrils, and the endothelium was partially desquamated (Figure 7(c)). The sclera exhibited moderate edema and looseness with some inflammatory infiltration. The choroid was relatively thin, without purulent exudates but with slight infiltration by neutrophils, lymphocytes, and macrophages. Some eyes, however, contained thickened regions of choroid with more pronounced infiltrates, especially in the posterior segment of the eye. The ciliary body retained its ordinary structure, but exhibited some regions of edema and inflammatory cell infiltration (Figure 8(c)).

3.5.3. SOD1 Nanozyme-Treated Rabbits. In the SOD1 nanozyme-treated group, 2 eyes (from 6 eyes examined) hardly showed any inflammatory symptoms. These eyes appeared to be unaffected by the disease and were indistinguishable of the eyes of the healthy rabbits. In the remaining eyes the cornea was laid by epithelium and endothelium without desquamation; the collagen fibers and keratocytes of stroma showed no changes as well (Figure 7(d)). The sclera in the eyes of this group was of common thickness without inflammation and loosening. The choroid was thin without destruction and cell infiltration. The retina was also unchanged. The ciliary body also displayed its natural structure, without signs of infiltration (Figure 8(d)).

Thus, while native SOD1 showed a pronounced therapeutic effect in the treatment of experimental immunogenic uveitis in rabbits, the nanoformulated form, SOD1 nanozyme, provided much more remarkable effect as revealed by the histopathology analysis.

4. Discussion

ROS are excessively produced in many disease states and contribute to tissue degeneration and pathogenesis of

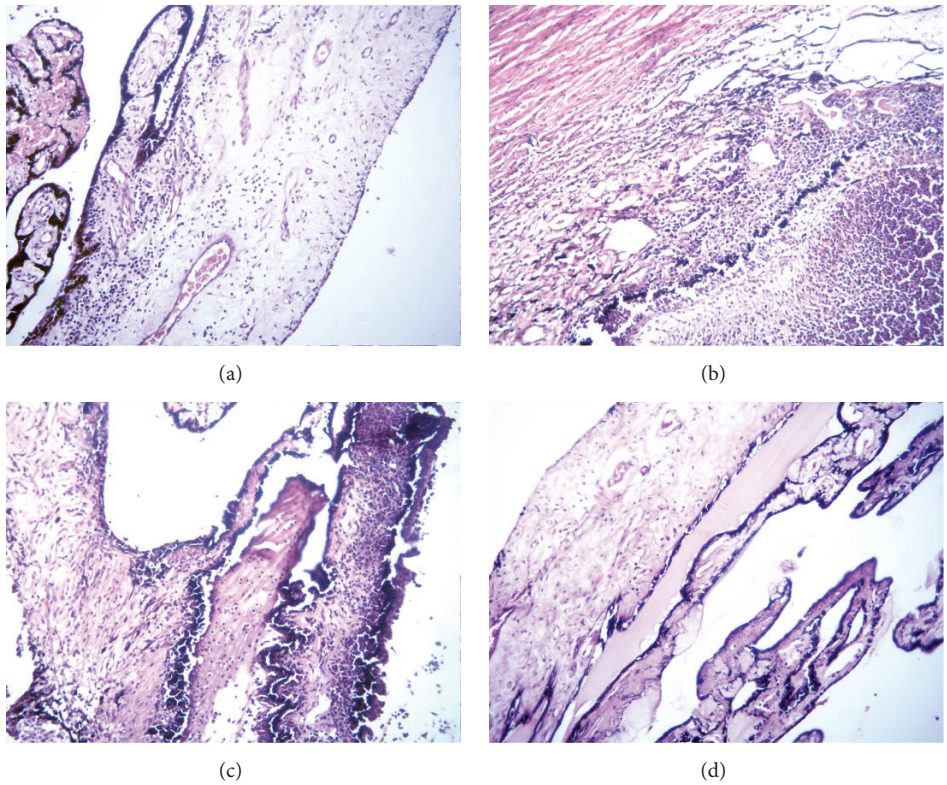

Figure 8: Histological picture of the ciliary body, stained with hematoxylin and eosin, magnification ×200, on day 4 of uveitis. (a) Control ($n = 2$): ciliary body with processes, moderate plethora; (b) placebo ($n = 6$): edema and infiltration of the ciliary body, deposition of purulent exudates; (c) SOD1 ($n = 6$): edema and inflammatory infiltration of the ciliary body; (d) SOD1 nanozyme ($n = 6$): ciliary body without cellular infiltration.

many clinical conditions including atherosclerosis, stroke, ischemia/reperfusion injury, myocardial infarction, central nervous system disorders, and wounds. In particular, ROS metabolites may be important factors in the early tissue damage that develops from immunopathologic inflammations [35–37]. Uncontrolled ROS production in acute inflammation can lead to destruction of structural and functional proteins as well as lipids in cell membranes. Because of the nonspecific nature of ROS-induced tissue injury, excessive release of these agents can cause substantial damage not only to the tissue in an inflamed state but also to the surrounding normal tissue. In particular, this is very important for the eye, as the transparency of the cornea and lens, as well as the functioning of photoreceptor apparatus, relies on their highly ordered structures, and excessive tissue damage will compromise visual function.

Antioxidants, SOD1 in particular, are known to be beneficial in the treatment of the various diseases connected with oxidative stress. Thus, SOD1 was reported to reduce inflammation [38], accelerate the healing of skin lesions caused by burns, systemic lupus erythematosus, and herpes [39–41], protect cultured human neurons under oxidative stress [42], reduce ischemia-reperfusion injury [22, 43, 44], inhibit angiotensin II (AngII) intraneuronal signaling [19], prolong viability of β-cells [45], be effective in the treatment of rat adjuvant arthritis [46], and so forth. Most relevant to this study, antioxidants, including SOD, are also thought to be beneficial in the treatment of eye diseases. The eye is rather isolated organ, and the pathological processes within it are preferably treated not *via* systemic but by local drug intake. SOD1 was found in the corneas of mammals [47], suggesting that the enzyme plays an important role in maintaining homeostasis of the ocular surface. The use of topical, subconjunctival, parabulbar, or intraocular injections of SOD1 could, therefore, provide a supplement for intrinsic antioxidants in eye tissues, which may be depleted during inflammation.

According to official statistics, inflammatory eye diseases are the most common eye pathologies which lead to partial disability and, sometimes, to the complete loss of vision. Among these diseases, the most severe one is uveitis, inflammation of uveal tract involving both outer and inner structures of the eye. Both noninfectious and infectious uveitis are accompanied by the enhancement of free radicals formation and by the increase of the content of the products of lipid oxidation in eye tissues. The major role in ROS formation in the eye belongs to polymorphonuclear leukocytes, which initiate as well as perpetuate the membrane oxidative processes at uveitis. Thus uveitis is believed to be strongly associated with overproduction of ROS in the eye tissues during inflammation, and antioxidants can play beneficial role in the treatment of this disease [48]. It was

shown [9] that superoxide production by the leukocytes of Behçet patients (with uveal inflammation) was significantly higher in the attack phase than in the remission phase. Leukocyte superoxide generation was also enhanced in guinea pigs with S-antigen-induced experimental autoimmune uveitis. These observations indicate the perspectives of the use of antioxidants, SOD1 in particular, as potential drugs in the treatment of uveitis.

Previous studies have shown beneficial effects of SOD1 in the treatment of eye inflammation, including uveitis and eye burns. Thus, animals with phacoanaphylactic endophthalmitis (lens-induced uveitis) were treated with SOD1 [8]. This treatment resulted in strong reduction of choroid inflammation, retinal edema, and vasculitis. In another study aqueous humor cell quantity and infiltration of the inflammatory cells in the anterior retina were markedly reduced in SOD1-treated animals with both S-antigen induced and bovine serum albumin-induced passive Arthus type uveitis [9]. Positive effect of SOD1 was also demonstrated on the rabbit model of immunogenic uveitis [14]. Moreover, topical antioxidant therapy by SOD1 in acute corneal inflammation (induced by alkali burn) was shown to be efficient in reduction of corneal ulcers [11], while subconjunctival injections of SOD1 were reported to prevent tissue destruction after alkali burns of the eye and prevented corneal perforation [10].

However, the methods of the treatment of the diseases, which include inner structures of the eye, are relatively less effective due to the poor transport of proteins and other drugs into the eye. In recent years, there has been significant interest in the developing nanosized drug delivery systems to overcome the limitations of drug therapy. Such nanosystems can improve the therapeutic efficacy of the drugs by overcoming diffusion barrier, by increasing their stability in biological tissues and fluids and enhancing cellular/tissue uptake. These nanosystems are attractive for the treatment of various eye diseases, including both acute and chronic conditions [17, 49]. So far, SOD1 entrapped in liposomes was previously shown to be effective in the treatment of noninfectious corneal ulcers [50].

Recently, a cross-linked polyion complex of SOD1 with a cationic block copolymer PEG-pLL_{50}, termed "SOD1 nanozyme," was developed [21, 22]. This SOD1 nanoformat is characterized by high dispersion stability, small particle size, particle uniformity, decreased cellular toxicity, and efficient transport into cells. SOD1-nanozyme was shown to be able to effectively scavenge ROS and decrease ischemia/reperfusion-induced tissue injury and improve sensorimotor functions in a rat middle cerebral artery occlusion model [22]. In this study, the therapeutic efficacy of SOD1 nanozyme for the treatment of ophthalmic inflammatory diseases was demonstrated in a rabbit model of immunogenic uveitis.

Immunogenic uveitis is an animal model of acute ocular inflammation induced by the intraocular injection of serum from a foreign animal after presensitization. This type of uveitis usually includes inflammation in the anterior, intermediate, and posterior segments of the eye, thus representing panuveitis. We hypothesized that antioxidant agent SOD1 in the form of nanozyme can attenuate oxidative stress and produce a significant therapeutic effect. For the treatment, we have chosen the most simple and convenient drug formulation, aqueous solution of nanozyme as eye drops.

In this study, we mostly followed clinical and biochemical parameters in the anterior segment of the eye in uveitis, while further histological study allowed us to estimate uveitis manifestations in the posterior segment of the eye as well. Clinical manifestations of uveitis in the anterior segment of the eye could be divided into two groups. The first are manifestations in the outer part of the segment including eyelid edema, eyelid hyperemia, conjunctival edema, conjunctival hyperemia, corneal edema, and neovascularization of the cornea. The second are manifestations in the inner part of the segment including iris edema, iris hyperemia, fibrin clots and precipitates on the iris and on the lens, lens opacity, the existence of synechiae, and the existence of exudates in the anterior chamber of the eye.

The major result of this study is a clear demonstration that topical instillations of SOD1 nanozyme solution into the eye exhibit remarkable effect on the clinical manifestation of the disease, improve biochemical characteristics of the aqueous humor, and help to maintain the cells of various eye tissues in normal condition. In the eyes of rabbits not receiving any treatment, but receiving placebo instead, we observed acute panuveitis. Inflammation included entire uveal tract, which, in turn, caused inflammation in other eye tissues. Deformation of almost every eye tissue was observed, retina (defects of photoreception apparatus), ciliary body (distortion in the aqueous humor formation and in accommodation), iris (changes in the structure and immobilization of the pupil), lens (opacity, cataract), and cornea (decrease of the transparency). In the second group of rabbits receiving native SOD1 solution, the clinical manifestations of uveitis were less severe. However, edema, inflammatory infiltration, and endothelium desquamation were still observed. In the third group of rabbits receiving SOD1 nanozyme solution the therapeutic effect of antioxidant agent was much more pronounced. We observed statistically significant differences in those clinical manifestations of uveitis, such as corneal and iris edema, hyperemia of conjunctiva, lens opacity, and amount of fibrin clots between this group and native SOD1-treated group. The biochemical characteristics of the aqueous humor were also improved. Moreover, histological study demonstrated almost normal structure of eye tissues from this group. Remarkably, the proposed therapy appears to be beneficial for treatment of not only the surface but also inner areas of the eye.

The current study did not allow precise delineating of the mechanism by which the nanozyme formulation improves the therapeutic effect of SOD1. The effect of the topical application of common drugs is greatly impeded by the protective physiological barriers of the eye, which effectively decrease the concentration of the drug in the site of the action [15]. Previous works have shown that incorporation of SOD1 in nanozyme format increases the efficacy of nanozyme delivery in cells [19, 51]. Moreover, the stability of the enzyme taken up into the cells within the nanozyme format is greatly increased, presumably, due to stabilization of the enzyme molecule against metabolic degradation and/or lysosomal escape [22]. Studies have also shown that nanozymes can

be taken up in macrophages, when they can reside for considerable periods of time [18, 20]. Nanozymes can be also transported with macrophages to distal disease sites, where they are released into the extracellular media as well as within other tissue cells and exerted the protective effect by scavenging the ROS [52, 53]. All these effects could in principle contribute to improved therapeutic effect of the SOD1 nanozyme during uveitis observed in this work.

Altogether the results obtained demonstrate high potential therapeutic efficacy of topical administration of SOD1 nanozyme for the treatment of inflammatory eye diseases. Most current therapies of uveitis are predominantly based on steroids and immunosuppressants [54, 55]. However, steroids have systemic side effects such as cataract, glaucoma, and secondary ocular hypertension [56, 57], while immunosuppressive drugs are teratogenic and contraindicated during pregnancy [55]. Intraocular or periocular injections can deliver relatively high doses of drug to the eye with fewer side effects [54, 55]; however, each such injection is in essence a minor surgical procedure that could be quite disruptive and inconvenient to a patient. Recently, several sustained-release drug delivery implants have been developed to treat noninfectious uveitis, but such implantation requires surgical operation and the cost of this invasive treatment is high to the patients and insurance companies [55].

Therefore a noninvasive topical SOD1 nanoformat that can be conveniently applied as eye drops by a patient if shown successful as a therapeutic modality could be a major breakthrough in treatment of uveitis and possibly other inflammatory conditions of the eye.

5. Conclusions

In summary our work demonstrates that the nanozyme formed by self-assembly of the SOD1 with PEG-pLL_{50} block copolymer and stabilized by cross-linking can be used as a carrier for sustained delivery of SOD1 into ocular tissues for the treatment of inflammation processes in the eye. Topical instillations of SOD1-nanozyme significantly decreased inflammation both in the outer and inner parts of the eye as determined using scores of the clinical manifestations of uveitis, multiple biochemical parameters, and histological analysis. These results may have broad clinical implications in the treatment of other disorders of the eye where oxidative stress contributes to pathology.

Conflict of Interests

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

Acknowledgments

This work was supported by the Government of Russian Federation Grant no. 11G.34.31.0004, Russian Scientific Foundation Grant 14-13-00731, an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under Grant P20GM103480, and *The Carolina Partnership*, a strategic partnership between the UNC Eshelman School of Pharmacy and The University Cancer Research Fund through the Lineberger Comprehensive Cancer Center.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 7095901, 14 pages
http://dx.doi.org/10.1155/2016/7095901

Review Article

Critical Roles of Reactive Oxygen Species in Age-Related Impairment in Ischemia-Induced Neovascularization by Regulating Stem and Progenitor Cell Function

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Received 9 April 2015; Accepted 12 August 2015

Academic Editor: Felipe Simon

Reactive oxygen species (ROS) regulate bone marrow microenvironment for stem and progenitor cells functions including self-renewal, differentiation, and cell senescence. In response to ischemia, ROS also play a critical role in mediating the mobilization of endothelial progenitor cells (EPCs) from the bone marrow to the sites of ischemic injury, which contributes to postnatal neovascularization. Aging is an unavoidable biological deteriorative process with a progressive decline in physiological functions. It is associated with increased oxidative stress and impaired ischemia-induced neovascularization. This review discusses the roles of ROS in regulating stem and progenitor cell function, highlighting the impact of unbalanced ROS levels on EPC dysfunction and the association with age-related impairment in ischemia-induced neovascularization. Furthermore, it discusses strategies that modulate the oxidative levels of stem and progenitor cells to enhance the therapeutic potential for elderly patients with cardiovascular disease.

1. Introduction

Reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), are generated as electrons "leak" and react with oxygen molecule (O_2) during mitochondrial oxidative phosphorylation. Alternatively, the formation of intracellular ROS can be catalyzed by an enzymatic reaction, where NADPH oxidase (Nox) transfers an electron to O_2 and generates $O_2^{\bullet-}$. Aging is associated with increased oxidative stress that is characterized by an unbalanced redox homeostasis when the rate of ROS formation exceeds the capacity of endogenous antioxidative system to remove ROS. "Free Radical Theory of Aging" proposes that the production of ROS causes an accumulation of cellular damage, including DNA, proteins, and lipids, leading to a decline in mitochondrial integrity. This, in turn, drives a vicious cycle of ROS formation and exacerbates cellular damage, contributing to cellular senescence and premature aging [1]. This theory is supported by numerous studies using a wide range of model organisms, such as *Saccharomyces cerevisiae, Drosophila melanogaster, Caenorhabditis elegans,* and rodents, demonstrating a strong correlation between increased levels of ROS and oxidatively damaged molecules as cells aged [2–6]. An abnormal elevation of intracellular ROS also has an implication in pathogenesis of various diseases, such as ataxia telangiectasia and Fanconi anemia [7]. Nevertheless, ROS are important for cell signaling and homeostasis. "Redox window" hypothesizes that while excessive ROS contribute to the pathological conditions, appropriate ROS production from mitochondrial oxidative phosphorylation and NADPH oxidase is required for normal physiological responses [8].

Cardiovascular disease is a major cause of world-wide mortality. Aging alone, without any other clinical manifest conditions, is a risk factor for coronary and peripheral artery diseases [9]. The majority of cardiovascular disease-related deaths are elderly individuals aged 75 and older. Following ischemia, vascular system is capable of repair and regeneration. The formation of new blood vessels (postnatal neovascularization) relies on two processes: (i) angiogenesis, the sprouting of mature endothelial cells from the preexisting vessels, and (ii) vasculogenesis, the mobilization of bone

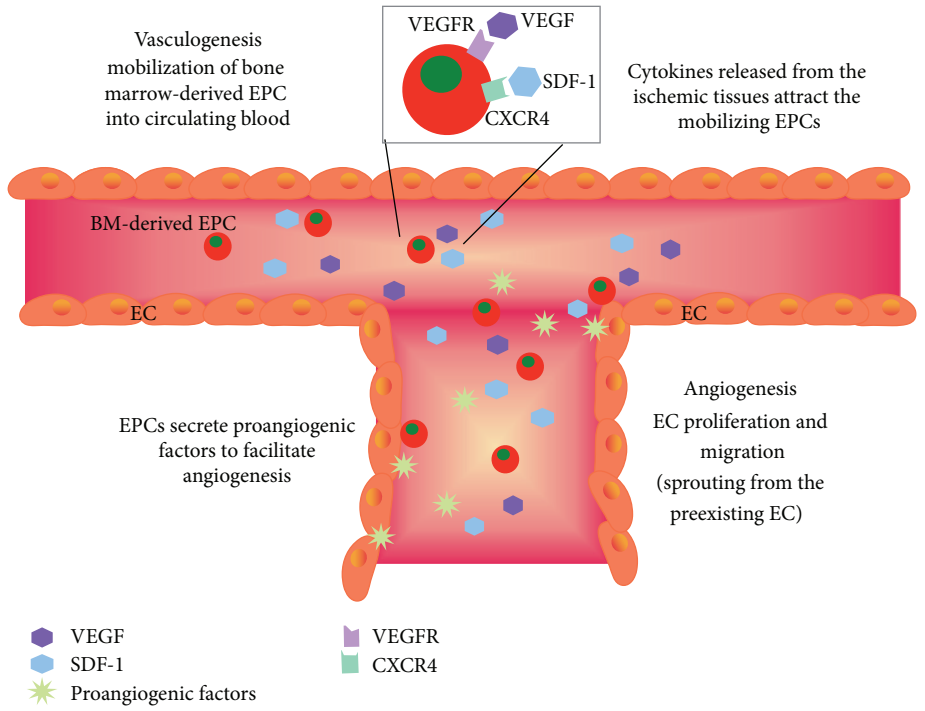

Figure 1: Schematic diagram of mechanisms involved in ischemia-induced neovascularization. Ischemia induces angiogenesis, the sprouting of new blood vessels from the preexisting ones. It involves the proliferation and migration of endothelial cells (ECs) at the local ischemic tissues. Cytokines, such as vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1), are released from the ischemic tissues to facilitate the recruitment of mobilizing endothelial progenitor cells (EPCs) through the binding of receptors. Vasculogenesis involves the proliferation and mobilization of EPCs from bone marrow to the circulating blood. EPCs express various surface receptors, such as VEGF receptor (VEGFR) and C-X-C motif receptor 4 (CXCR4). Once home to the ischemic sites, EPCs are capable of integrating with ECs and promote angiogenesis by secreting proangiogenic factors.

marrow-derived endothelial progenitor cells (EPCs) to the circulation (Figure 1). Aging is associated with impaired ischemia-induced angiogenesis and vasculogenesis *in vivo* [10–12]. EPCs are a subpopulation of progenitor cells originating from stem cells that differentiate into various lineage-committed cells. Although studies have used different markers to identify EPCs or referred to different nomenclatures, such as bone marrow-derived angiogenic cells, circulating progenitor cells, or proangiogenic myeloid cells [13–15], it is acknowledged that there is an age-dependent exhaustion of EPC numbers and/or impairment in EPC functions (Table 1).

This review summarizes current understanding of the involvement of (i) redox regulation in self-renewal, differentiation, and senescence of stem and progenitor cells; (ii) ROS as signaling molecules to mobilize progenitor cells from bone marrow to the circulation in response to ischemia; and (iii) how oxidative stress plays a role in age-dependent impairment in ischemia-induced neovascularization. With an increase in global aging population, a major concern is to understand the mechanistic role of age-related impairment in neovascularization in an attempt to develop better cell-based therapeutic strategies for elderly patients with vascular diseases.

2. The Role of ROS in Maintaining Stem Cell in Bone Marrow Microenvironment

Stem cells reside in a specialized bone marrow microenvironment (niche) [54]. Hematopoietic stem cells (HSCs) are one of the most characterized adult stem cells, which differentiate into all types of immune cells and maintain blood production. HSCs are predominantly located in hypoxic endosteal niche of the bone marrow with low-oxygen tension where a protection from ROS-related oxidative stress is provided [55, 56]. Jang and Sharkis 2007 have demonstrated that lineage depleted, CD45+ viable cell population (Lin−/CD45+/AnV−) could be separated into two fractions based on intracellular ROS levels, indicated by a fluorescence probe 2′-7′ dichlorofluorescein diacetate (DCF-DA). The levels of intracellular ROS correlate with stem cell capacities in self-renewal and differentiation. The isolated ROS^{low} population displays self-renewal ability by expressing higher levels of telomerase compared to ROS^{high} population [57]. Telomerase activity has been reported to be associated with the self-renewal potential of HSCs in mice [58]. On the other hand, the expression of a cyclin-dependent kinase inhibitor, p16InK4a, is upregulated in ROS^{high} population. As a biomarker of aging, p16Ink4a

Table 1: Examples of age-dependent exhaustion of EPC numbers and reduction of EPC functions.

Study	Subjects	Source of cells	EPC markers	Effect of aging	Reference
Rauscher et al. 2003	6-month-old *versus* 1-month-old ApoE−/− mice	BM	CD31+/CD45−	Reduced EPC numbers; progressive development of atherosclerosis	[16]
Zhang et al. 2006	12-month-old *versus* 3-month-old BALB/C mice	BM	CD117+/CD34+/Flk1+	Decrease in numbers; reduced EPC proliferation, migration, and phagocytic functions	[17]
Sugihara et al. 2007	18-month-old *versus* 2-month-old C57Bl/6J mice	BM	AC133+/CD34+ and CD34+/VEGFR2+	No difference in numbers of EPCs; impaired VEGF production and EPC migration	[18]
Shimada et al. 2004	*Klotho* mutant* *versus* wild type mice	BM and peripheral blood	c-kit+/CD31+ and CD34+/CD31+	Decrease in EPC numbers postischemia	[19]
Chang et al. 2007	18–24-month-old *versus* 4–6-month-old C57Bl/6J	Peripheral blood and BM	CD11b−/Flk1+ and Sca1+/c-kit+/Lin−	Decrease in CD11b−/Flk1+ numbers in blood, but not Sca1+/c-kit+/Lin− cells in BM postischemia	[11]
	68–95-year-old *versus* 18–35-year-old human	Peripheral blood	AC133+	No difference in EPC numbers at baseline	
Zhuo et al. 2010	15-16-month-old *versus* 2-month-old rat	Peripheral blood and spleen	CD34+/KDR+	Decreased numbers in response to ischemia, but not at baseline (prior to ischemia)	[20]
Shao et al. 2011	24–26-month-old *versus* 2-month-old C57Bl/6J mice	BM	Lin−/Sca1+ and Lin−/Sca1+/CXCR4+	Decrease in Lin−/Sca1+/CXCR4+, but not Lin−/Sca1+ subpopulation	[21]
Boon et al. 2011	16–18-month-old *versus* 1-month-old C57Bl/6J mice	Peripheral blood	Lin−/Sca1+/c-kit+, Sca1+/c-kit, and Sca1+/Flk1+	Decrease in all 3 populations	[22]
Scheubel et al. 2003	Patients with CAD; 69 years old *versus* younger patients	Peripheral blood	AC133+/CD34+	Reduced basal circulating EPC levels	[23]
Heiss et al. 2005	Healthy elderly (average 61 years old) *versus* healthy young subjects (average 25 years old)	Peripheral blood	CD133+/KDR+ and CD34+/KDR+	Comparable levels of EPCs	[24]

BM, bone marrow.
CAD, coronary artery disease.
* *Klotho* mutant mice, an animal model of typical aging, display accelerated arteriosclerosis.

expression is found increased in most of rodent tissues with advancing age [59]. The accumulation of p16Ink4a levels is also associated with decreased repopulating activity and self-renewal abilities of HSCs in the older mice [60]. Furthermore, ROS^{high} population exhibits an increase in p38/mitogen-activated protein kinase (MAPK) activation. Elevation of ROS induces phosphorylation of p38/MAPK, which has been reported to limit self-renewal function in HSCs [61]. The reduction of self-renewal ability in ROS^{high} population can be restored by suppressing ROS production or ROS-induced p38/MAPK activation with antioxidant N-acetyl-L-cysteine (NAC) or p38 specific inhibitor [57].

While low-oxygen niche that limits ROS production is required to maintain HSCs at quiescent state in the bone marrow, the more oxygenic vascular niche (due to the proximity to the blood circulation) is essential for the proliferation and differentiation of stem cells to become progenitor cells (Figure 2). Increased intracellular ROS levels are found during the early stages of embryonic stem cell differentiation. Low levels of H_2O_2 induce cardiomyogenesis of embryonic stem (ES) cell, stimulating the proliferation of ES cell-derived cardiomyocytes. Several antioxidative genes and stress resistance genes are downregulated during embryonic stem cell differentiation into embryoid bodies [62]. NADPH oxidase isoforms, Nox1, Nox2, and Nox4, are upregulated; as a result, there is a feed-forward regulation of ROS generation during ES differentiation. Inhibition of Nox-derived ROS abolishes ES cardiomyogenesis [63].

3. Ischemia-Induced ROS Mediate Stem/Progenitor Cell Proliferation and Mobilization

During early stages of hypoxia, there is a transient elevation of intracellular ROS formation, as detected in various isolated tissues, such as skeletal muscle [64], systemic vessels [65], and myocardium [66]. Hypoxia-induced ROS may be a part of normal physiological response to the imbalance in oxygen

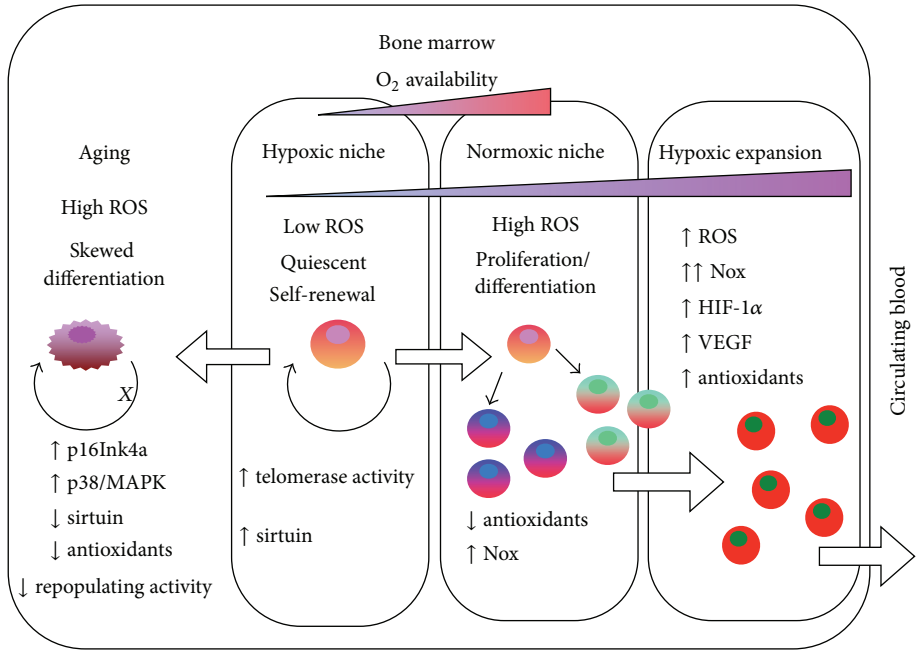

Figure 2: Schematic diagram of bone marrow microenvironment (Niche). Stem cells with self-renewal capacity reside in the hypoxic niche where the levels of reactive oxygen species (ROS) are low. Proliferation and differentiation of stem cells occur at the oxygenic niche where higher levels of ROS promote cell differentiation. During ischemia, hypoxic expansion upregulates transcription factor and hypoxia inducible factor-1α (HIF-1α), increasing the levels of vascular endothelial growth factor (VEGF) expression in the bone marrow. Meanwhile, there is an increase in NADPH oxidase- (Nox-) mediated ROS production. With aging, stem cells loss their self-renewal ability and displayed a skewed differentiation pattern (for details please see text).

supply and demand. Demonstrated by *in vivo* injection of $O_2^{\bullet-}$ reactive dye, dihydroethidium (DHE), Urao et al. 2012 show that hindlimb ischemia induces ROS production in both the endosteal and central regions of the entire bone marrow *in situ*. In conjunction with the increase in ROS levels, hindlimb ischemia also induces hypoxic expansion in bone marrow microenvironment [67]. The spatial distribution of hypoxia in bone marrow is visualized by an *in vivo* injection of hypoxic bioprobe, pimonidazole, that detects area less than 1.3% O_2 by cross-linking protein adducts at oxygen tension below 10 mmHg [68]. These changes in the bone marrow microenvironment lead to upregulation of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) throughout the bone marrow (Figure 2). The mechanisms of how a distal ischemia in the hindlimb is capable of inducing an increase in ROS and hypoxic expansion in the bone marrow are yet fully understood. Nevertheless, Nox2 deficiency abolishes ischemia-induced hypoxic expansion and HIF-1α expression in the bone marrow microenvironment. Moreover, the levels of circulating EPC-like c-kit+/Flk1+ cells or c-kit+/Lin− progenitor cells are decreased in Nox2−/− mice following hindlimb ischemia [67, 69]. Therefore, Nox2-derived ROS induces hypoxic expansion and HIF-1α expression in the bone marrow microenvironment, which plays a role in progenitor cell expansion and mobilization into the circulating blood following ischemia.

The components of NADPH oxidase are expressed in various stem and progenitor cells including human bone marrow-derived CD34+ cells [70, 71], mouse embryonic stem cells [63], skeletal muscle precursor cells [71], and rat mesenchymal stem cells [72]. The constitutively active NADPH oxidase generates low levels of H_2O_2 in HSCs, which in turn stabilizes HIF-1α expression by inhibiting prolyl hydroxylases- (PHD-) mediated degradation of HIF-1α under normoxic conditions. An increase in HIF-1α expression is found in granulocyte colony stimulating factor- (G-CSF-) mobilized CD133+ and CD34+ HSCs from the peripheral blood of healthy donors [71, 73]. ROS-mediated HIF-1α stabilization may offer an advantage of enhancing the proangiogenic and antioxidative potential of the mobilizing bone marrow HSCs prior to homing to the hypoxic tissues, thereby facilitating neovascularization and tissue repair.

Interestingly, populations of more committed progenitor cells are intrinsically less sensitive to the elevation of intracellular ROS levels compared to HSCs. Serial transplantation of human Lin−/CD34+/CD38− HSCs into immunodeficient mice triggers replicative stress-induced elevation of intracellular ROS and leads to HSC premature senescence due to persistent DNA damage. However, Lin−/CD34+/CD38+ progenitor cells are more resistance to oxidative DNA damage [74]. Human EPCs isolated from peripheral blood followed by a short term *ex vivo* culture (4 days) exhibit low intracellular levels of H_2O_2 and $O_2^{\bullet-}$. The expressions of antioxidative enzymes, such as manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase, are higher in EPCs compared to mature endothelial cells (ECs). Furthermore, the intracellular levels of ROS remain stable when

Table 2: Summary of distinctive features of stem and progenitor cells.

Cell type	Features and functions
	Hematopoietic stem cells (HSCs)
Lin−/CD34+/AnV−	Lineage depleted, viable (annexin negative), undifferentiated, and primitive multipotential hematopoietic stem cells ROS^{low}: self-renewal, ↑telomerase ROS^{high}: limited self-renewal, ↑p16Ink4a, ↑p38/MAPK
Lin−/CD34+/CD38−	Lineage depleted, undifferentiated, and primitive multipotential hematopoietic stem cells ROS sensitive Serial transplantation of Lin−/CD34+/CD38− leads to DNA damage and premature senescence
Lin−/CD34+/CD38+	Lineage depleted, primitive hematopoietic/lymphoid stem cells Less sensitive to ROS elevation and resistant to oxidative DNA damage
Scal+/c-kit+/Lin−	Primitive stem cells Mobilized into circulation via increasing ROS in response to ischemia
	Embryonic stem cells (ES)
	Active in proliferation and differentiation Require low levels of H_2O_2 to trigger cardiomyogenesis ↑Nox1, ↑Nox2, and ↑Nox4 ↓Antioxidative and stress resistance genes
	Progenitor cells
Endothelial progenitor cells (EPCs)	*Ex vivo* cultured for 4 days ↑MnSOD, ↑catalase, and ↑glutathione peroxides Stable intracellular ROS levels Resistance to ROS-induced apoptosis
Late-outgrowth endothelial cells (OECs)	*Ex vivo* cultured for 2-3 weeks ↑MnSOD Similar levels of CuZnSOD and catalase compared to mature endothelial cells

EPCs are exposed to a redox cycler, napthoquinolinedione, that generates H_2O_2 and $O_2^{\bullet-}$. EPCs are also less sensitive to ROS-induced apoptosis compared to mature endothelial cells [75]. In another study, He et al. 2004 reported that catalase and CuZnSOD enzymatic activities are similar between EPCs and mature ECs and that mitochondrial MnSOD is the most likely of the three antioxidants to be responsible for EPC resistance to oxidative stress [76]. The discrepancy in antioxidant upregulation between the two studies may be due to the methodology of EPC culture and the definition of EPCs. In Dernbach et al. study, EPCs were cultured for 4 days and are characterized by an uptake of acetylated low-density lipoprotein (Dil-Ac-LDL) and lectin [77]. On the other hand, EPCs were cultured for an extended period, 2-3 weeks, and displayed cobblestone phenotype in He et al. study. In fact, the term "EPCs" in He et al. study may be better described as late-outgrowth endothelial cells (OECs). Demonstrated by Sieveking et al. 2008, EPCs and OECs have distinctive differences in angiogenic properties [78]. Therefore, it is speculated that the levels of antioxidative defense may be fine-tuned depending on functions and status of progenitor cells. OECs may require a higher tolerance toward mitochondrial oxidative stress and maintain mitochondrial structure integrity, which lessens the need for cytosolic antioxidants compared to EPCs. Features of stem and progenitor cells are summarized in Table 2.

ROS generation from the ischemic tissues also plays a role in promoting stem and progenitor cell mobilization. For example, ischemic skeletal muscles increase the production of hematopoietic cytokines, such as interleukin-3 and erythropoietin, which induce a rapid and transient ROS production. Subsequently, these cytokines promote bone marrow progenitor cells exiting quiescence through G1 to S cell cycle progression [79]. Stromal cell-derived factor-1 (SDF-1) released in ischemic tissue promotes stem and progenitor cell mobilization into the circulation by binding to C-X-C motif receptor 4 (CXCR4) [80]. SDF-1-induced chemotaxis is regulated by c-Met activation [81], which is known to control complex biological program of "invasive growth" and tumor spreading [82]. Activation of c-Met induces mTOR signaling

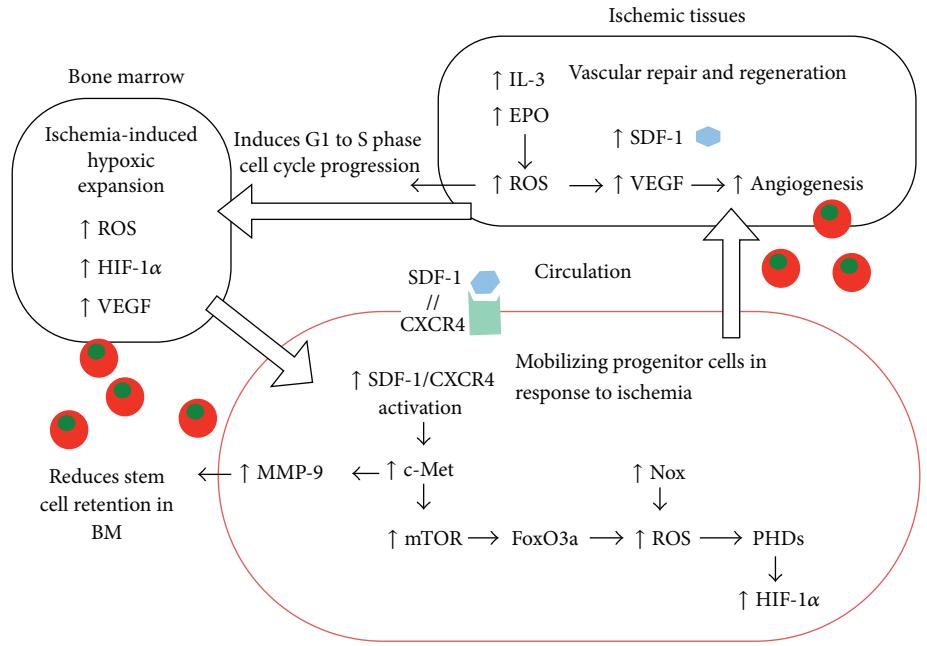

Figure 3: Schematic diagram of mechanisms involved in ischemia-induced progenitor cell mobilization. Under ischemic conditions, stromal cell-derived factor-1 (SDF-1) and hematopoietic cytokines, such as interleukin-3 (IL-3) and erythropoietin (EPO), are released from the tissues. Hematopoietic cytokines increase ROS and induce G1 to S phase cell cycle progression. Meanwhile, ischemia induces hypoxic expansion in the bone marrow to promote cell proliferation and differentiation (see Figure 2). In the circulation, SDF-1 binds to progenitor cells (outlined as red) expressing its receptor, CXCR4. SDF-1/CXCR4 activation induces c-Met and mTOR leads to downregulation of FoxO3a and increase in ROS production. Activation of c-Met upregulates the expression of matrix metalloproteinase-9 (MMP-9), which inhibits the adhesive interaction of progenitor cells to bone marrow (BM). In addition, NADPH oxidase (Nox) promotes ROS formation, which in turn stabilizes the levels of hypoxia inducible factor-1α (HIF-1α) by inhibiting prolyl hydroxylases (PHD). The mobilized progenitor cells facilitate the vascular repair and regeneration at the ischemic tissues.

and downregulates the levels of FoxO3a, which belong to the Forkhead Box, class O (FoxO) family of transcription factors that regulates oxidative stress. As a result, c-Met activation promotes the G-CSF-induced mobilization of bone marrow primitive progenitor cells (Sca1+/c-Kit+/Lin−) via increasing levels of ROS and upregulates the expression of proteolytic enzyme matrix metalloproteinase-9 (MMP-9) [81]. MMP-9 enhances progenitor cell mobilization into the circulation by inhibiting adhesive interaction necessary to retain stem and progenitor cells within the bone marrow [83] (Figure 3). Although it is beyond the scope of this review, low levels of ROS generated in the tissues during ischemic injury also promote local angiogenesis. The expressions of many angiogenic genes, such as VEGF, fibroblast growth factor, platelet-derived growth factor, and receptors are likely to be regulated by redox signaling [84]. Nox2-derived ROS is involved in VEGF signaling, which plays an important role in EC migration and proliferation, as well as reparative angiogenesis in response to hindlimb ischemia *in vivo* [85, 86].

4. Stem and Progenitor Cell Aging

With aging, stem cells lose self-renewal activity and terminally differentiated, thereby exiting the stem cell pool. On the other hand, they may undergo apoptosis or senescence induced by higher levels of ROS (Figure 2). Age-related depletion of stem cell pool may be driven by an imbalance of intracellular ROS that regulates stem cell quiescence and proliferation. Aged mice have a decrease in ROS^{low} population of Lin−/CD34+/AnV− cells, indicating a reduction of HSC populations that are capable of more durable long-term self-renewal [57]. HSCs from elderly individuals also exhibit higher levels of ROS and have reduced ability to reconstitute hematopoiesis of murine host compared with HSCs from middle-aged individuals [74]. Aged HSCs display a skewed differentiation potential, in which these cells overproduce myeloid lineage cells rather than a multilineage population, consisting of both myeloid and lymphoid lineage cells [57, 60, 87].

The sirtuin family of NAD-dependent deacetylases, a key regulator of organismal longevity, has been shown to modulate stem cell aging. Deletion of SIRT1 in young HSCs displays an aging phenotype with a skewed differentiation toward myeloid lineage that is associated with a decline in lymphoid compartment [88]. Lentiviral shRNA knockdown of SIRT1 in human bone marrow-derived mesenchymal stem cell accelerates cellular senescence [89]. Recently, a link between oxidative metabolism and sirtuin in modulating stem cell homeostasis has been reported. Brown et al. 2013 show that

SIRT3 is downregulated with age and is accompanied with a reduction of mitochondrial MnSOD activity which in turn contributes to increased ROS levels in aged HSCs. Demonstrated by an *in vivo* competitive transplantation assay, it is showed that SIRT3 is required to maintain HSC pool size and regenerative capacity under oxidative stress conditions, such as aging and serial transplantation [90]. SIRT3 preserves HSC functions by enhancing mitochondrial MnSOD antioxidative activity via posttranslational deacetylation of critical lysine residues [90, 91]. Furthermore, SIRT3 has a critical role in bone marrow cell-mediated cardiac repair. It is shown that intramyocardial injection of bone marrow cells from SIRT3 knockout mice results in reduced numbers of Sca1+/c-kit+ progenitor cells mobilized to the ischemic area following myocardial infarction. The loss of SIRT3 increases ROS formation and cellular apoptosis, reducing the proangiogenic capability in EPCs *in vitro* [92].

Bioactive peptides in the vascular system also play a role in progenitor cell aging. Angiotensin II (Ang II) is a key effector of the renin-angiotensin system. Ang II not only regulates blood pressure as a potent vasoconstrictor, but also promotes inflammation, hypertrophy, and fibrosis. Ang II plays a role in vascular damage and remodeling in cardiovascular diseases [93–95]. It has been demonstrated that inhibition of angiotensin-converting enzyme by Ramipril augments circulating EPCs with enhanced functional activity in patients with stable coronary artery disease [96]. In human EPCs, Ang II increases the expression of Nox2 component, gp91phox, and accelerates the onset EPC senescence through an induction of oxidative stress, as evidenced by peroxynitrite formation *in vitro*. Pretreatment of EPC with SOD prevents Ang II-induced telomerase inactivation [97]. Hepatocyte growth factor (HGF) also attenuates Ang II-induced EPC senescence by reducing gp91phox expression and limiting the production of $O_2^{\bullet-}$ in EPCs [98].

Excessive production of ROS in pathological conditions has been associated with HSC exhaustion. "Ataxia telangiectasia mutated" (Atm) gene is responsible for genomic stability in response to DNA damage and oxidative stress. Mice with Atm deficiency (Atm−/−) exhibit progressive bone marrow failure in association with increased ROS levels [99]. The elevation of ROS induces p38/MAPK phosphorylation in HSCs and is accompanied by a defect in maintaining HSC quiescence. Treatment of Atm−/− mice with antioxidant NAC restores the HSC reconstitutive capacity and prevents bone marrow failure [61]. Loss of FoxO3a in HSCs also results in elevated oxidative stress, increased p38/MAPK phosphorylation, and defective maintenance of quiescence. The ability of HSCs to support long-term reconstitution of HSC pool in a competitive transplantation assay is impaired in FoxO3a−/− mice [100]. Conditional deletion of FoxO1, FoxO3a, and FoxO4 in mouse hematopoietic system leads to increased ROS levels, myeloid lineage expansion, and a reduction in Lin−/Sca1+/c-kit+ HSC population [101]. Indisputably, excessive ROS production is associated with the disruption of HSC quiescence and impairment in hematopoietic repopulating ability of HSCs in the bone marrow.

5. Unbalanced ROS Levels and Age-Related Impairment in Ischemia-Induced Neovascularization

The importance of balanced ROS levels in mediating ischemia response and neovascularization has been demonstrated in animal models manipulated to have impaired cellular antioxidant mechanisms. Heterozygous knockout of mitochondrial MnSOD results in an increased lipid peroxidation and a defect in myocardial contractile function followed by ischemia-reperfusion injury in isolated heart, while deletion of a single copy of cytosolic CuZnSOD does not show any significant impairment [102]. This suggests that there is a tissue-specific requirement of antioxidative enzyme in the tolerance to oxidative stress that cannot be compensated by the other SOD isoforms. Studies have also demonstrated a differential effect of aging on ROS-mediated ischemic response. Carotid arteries from young heterozygote CuZnSOD (+/−) knockout mice show no significant alternation in endothelial relaxation in response to acetylcholine. However, aged CuZnSOD (+/−) mice are impaired in endothelial-dependent vasodilation in response to acetylcholine, which can be restored by the presence of superoxide scavenger, tempol [103]. The effect of a single deletion of CuZnSOD is minimal in young age. ROS seem to be well tolerated in young cells with highly proficient antioxidative defense, which may be compensated by the remaining copy of CuZnSOD and other antioxidative enzymes. With aging, cells with less efficient antioxidative defense become sensitive to oxidative stress, which aggravates ROS formation. Eventually, aged cells are inadequate to maintain proper vascular functions. On the other hand, young homozygous CuZnSOD (−/−) knockout mice exhibit an accelerated vascular aging and impaired ischemia-induced neovascularization. Young CuZnSOD−/− mice have similar oxidative stress levels in the ischemic tissues as those observed in older wild type littermate, examined by immunofluorescence staining of DHE and nitrotyrosine (an indicator of protein nitration by ROS). Aged CuZnSOD−/− mice have the highest level of oxidative stress and display severe necrosis and autoamputation in the second weeks after surgically induced hindlimb ischemia [104]. CuZnSOD deficiency leads to increased ROS levels and is associated with reduced proangiogenic functions of EPCs, such as migratory ability and integration into endothelial cell tubules *in vitro* [105]. The peripheral EPC levels in the spleen are lower in both young and aged CuZnDOS−/− mice [104]. Authors suggest that CuZnSOD deficiency may cause a depletion of EPC reserve in the bone marrow and result in the impaired EPC mobilization observed in the spleen of CuZnSOD−/− mice. However, EPC levels in the bone marrow of CuZnSOD−/− mice and whether CuZnSOD deficiency attenuates EPC mobilization from the bone marrow to the circulating blood remained to be determined. Nevertheless, aging exacerbates oxidative stress-associated EPC dysfunction in the absence of CuZnSOD. CuZnSOD has a critical role in limiting excessive ROS accumulation and preserves EPC angiogenic activities with aging. The role of CuZnSOD in modulating EPC numbers and functional

Table 3: Selected cell-based preclinical studies.

Cell treatment	Ischemic model	Outcomes	References
Ex vivo culture expanded human EPCs from healthy young individuals	Myocardial ischemia in athymic nude mice	Increased neovascularization; increased capillary density; reduced infarct size; improved LV function after myocardial ischemia	[25]
Human peripheral blood MNC-derived CD14+ or CD14− EPCs	Hindlimb ischemia in athymic nude mice	Increased blood perfusion; increased capillary density	[26]
Human blood-derived CD34+ cells	Hindlimb ischemia in diabetic mice	Increased blood flow perfusion in diabetic mice, but not in nondiabetic mice	[27]
Human blood cord-derived CD34+ EPCs	Cerebral ischemia in mice	Accelerated neovascularization of infarct neuronal tissue; increased cortical expansion; increased neuronal regeneration; improved recovery of motor deficits	[28]
Ex vivo expanded human EPCs from peripheral blood followed by VEGF transduction	Hindlimb ischemia in athymic nude mice	Reduced limb loss; increased blood flow recovery after ischemia; increased EPC incorporation *in vivo*	[29]
Autologous EPCs from peripheral blood	Pulmonary hypertension in dogs	Improved pulmonary artery pressure, cardiac output, and pulmonary vascular resistance	[30]
Autologous EPCs from peripheral blood	Carotid denudation in rabbits	Accelerated reendothelialization; improved endothelial function	[31]
Autologous CD34+ EPCs from bone marrow	Acute myocardial infarction in macaques	Improved regional blood flow; increased capillary density in the peri-infarct region; improved cardiac function; increased VEGF and bFGF levels in peri-infarct region	[32]

MNC, mononuclear cell.
VEGF, vascular endothelial growth factor.
bFGF, basic fibroblast growth factor.

activities may also have important clinical implications. Patients with chronic heart failure and coronary artery disease have lower levels of antioxidant enzymes, including CuZnSOD [106, 107], which may explain reduced EPC numbers and functions in patients [23, 108]. Alternatively, protection against age-dependent impairment in ischemia-induced neovascularization in association with excessive ROS formation has been demonstrated in Nox2 deficient mice. Nox2 deficiency ameliorates age-related increase in ROS levels and enhances bone marrow-derived EPC proangiogenic functions *in vitro*. As a result, aged Nox2−/− mice exhibit enhanced blood flow recovery following ischemia [109]. As the tolerance of oxidative stress decreases with age, it is essential to preserve stem and progenitor cell vasculogenic functions by maintaining ROS balance.

6. Therapeutic Potential of Cell-Based Therapy for Age-Related Impairment in Neovascularization by Modulating Redox Regulation

Implantation of autologous bone marrow-derived stem and progenitor cells is a potential treatment for ischemic diseases. Cell-based therapies have been safely conducted and demonstrated beneficial effects in augmenting neovascularization in preclinical animal studies (Table 3). Despite the promise of preclinical studies, human clinical trials involving an administration of autologous bone marrow cells or progenitor cells from bone marrow or peripheral blood have, to date, yielded neutral or underwhelming outcomes (Table 4). In experimental settings, stem and progenitor cells are often isolated from healthy young animals as donors and transplanted into young healthy recipients. The isolated stem cells are often highly regenerative and may contain less cumulative ROS-related damage. However, patients who would undergo cell-based therapy are elderly who have increased ROS levels and reduced numbers of stem and progenitor cells with impaired regenerative potential.

Studies have investigated different approaches to enhance the therapeutic potential of stem and progenitor cells by modulating their redox regulation. The first strategy involved suppressing excessive oxidative stress by promoting the antioxidative potential. For example, transgenic expression of MnSOD or administration of SOD mimic rescues impaired postischemia neovascularization and tissue survival in diabetic mice [110, 111]. Mesenchymal stem cell engraftment in the infarct heart is enhanced by coinjection of antioxidant NAC which mitigates ROS-induced inhibition of cell-matrix adhesion [112]. Intraperitoneal injection of SOD mimic reduces ROS formation and facilitates CD34+ progenitor cell recruitment to the infarct heart following coronary ligation in mice [113]. Preconditioning stem cells, with either a brief period of ischemia/anoxia or repeated cycles of intermittent hypoxia/reoxygenation, increase postengraftment cell survival or neovascular potential through oxidative stress resistance mechanism [114]. Electrical stimulation also provides preconditioning effect on the survival of cardiac stem cells and protects against oxidative stress-induced apoptosis via AKT activation by downregulating miR-378 [115]. Another

Table 4: Selected human cell-based clinical studies.

Conditions	Cell type	Therapy	Delivery methods	Outcome	Reference
Acute myocardial infarction	BMC	BOOST (randomized controlled)	Intracoronary injection	Improvement in LVEF at 6-month follow-up, but it failed to sustain the functional enhancement at 18-month and 5-year follow-ups	[33]
	BMC	REPAIR-AMI (randomized controlled)	Intracoronary infusion	Improved LVEF at 4-month follow-up; improvement of LV function sustained at 12-month follow-up and reduced major adverse CV events	[34–36]
	BMC	STEMI (randomized controlled)	Intracoronary infusion within 24 h administration	Reduced infarct size, but no significant improvement in LV function at 4-month follow-up	[37]
	BMC	ASTAMI (randomized controlled)	Intracoronary injection	No changes in LV end-diastolic volume or infarct size at 6-month follow-up	[38]
	BMC	BALANCE (controlled but nonrandomized)	Intracoronary infusion	Improved LV function, contractility, infarct size, haemodynamics, and exercise capacity at 12- and 60-month follow-up	[39]
	CD133+ progenitor cells	Small scale; nonrandomized	Intracoronary infusion	Improved LVEF at 4-month follow-up but increased incident of coronary events	[40, 41]
	CD133+ progenitor cells	Small scale; nonrandomized	Transplantation to peri-infract zone during CABG surgery	Improvements in myocardial viability and local perfusion; no adverse events at 6-month follow-up	[42]
	BMC and BM-derived CD34+/CXCR4+ progenitor cells	REGENT	Intracoronary infusion	Increased LVEF; no significant differences in absolute changes of LVEF between groups at 6-month follow-up	[43]
	BM-derived MSCs	Randomized controlled	Intravenous injection	Increased LVEF; improved global symptom at 6-month follow-up; MSCs traps in pulmonary passage in animal model	[44, 45]
	BMC and circulating blood-derived CD34+ progenitor cells	TOPCARE-AMI (randomized controlled)	Intracoronary infusion	Improvement in LVEF at 3-month follow-up; effect of BMC transplantation is greater than CPC; functional improvements sustained for 2 years	[46, 47]
Ischemic cardiomyopathy	Autologous skeletal myoblasts	MAGIC (randomized controlled)	Injection around the scar tissues	No significant improvement in global and regional LV function; an increase in arrhythmic events in treated patients	[48]
Chronic heart failure	Bone marrow cells	STAR	Intracoronary infusion	Improvements in LV function, exercise capacity, and oxygen uptake over a 5-year follow-up	[49]
Refractory myocardial ischemia	Bone marrow cells	(Randomized controlled)	Intramyocardial injection	Improvement of myocardial perfusion, angina severity, and quality of life at 3-month follow-up	[50]
	CD34+ progenitor cells	ACT34-CMI (randomized controlled)	Intramyocardial, transendocardial injection	Improvement in angina frequency and exercise tolerance	[51, 52]
Severe coronary artery diseases	Bone marrow cells	PROTECT-CAD (randomized controlled)	Endomyocardial injection	Improved LV function, exercise time, and NYHA functional class at 6-month follow-up	[53]

BMC, bone marrow cells.
LVEF, LV ejection fraction.
MI, myocardial infarction.
NYHA, New York Heart Association.
CABG, coronary artery bypass grafting.
MSC, mesenchymal stem cell.

approach involved stimulating stem cells with low-dose of prooxidants. Short-term treatment of mouse bone marrow cells with 5 μM H_2O_2 for 30 min enhances their angiogenic potency by promoting VEGF production and endothelial differentiation [116]. *In vitro* treatment of adipose-derived stroma cells (ADSCs) with pharmacological inhibitors to generate mitochondrial ROS increases the secretion of proangiogenic factors and protects ADSCs against ROS-induced apoptosis. Furthermore, *in vivo* injection of the treated ASCs promotes neovascularization in hindlimb ischemia [117]. Although most of these enhancements are demonstrated in cells from young donors and recipients, some have been shown effective in aged cells and old recipients. For example, preconditioning of bone marrow cells from aged mice (20–22 months old), by culturing cells at 2% O_2 for 24 hours, shows enhanced adhesion, survival, and proangiogenic potential *in vitro.* The preconditioned cells augment ischemia-induced neovascularization in aged mice following intramuscular injection [118]. Hypoxic preconditioning of human ADSCs from donor over 50 years old at 0.5% O_2 for 24 hours increases redox metabolism and promotes paracrine secretion [119]. Treatment of bone marrow-derived angiogenic cells from aged mice (17 months old) with dimethyloxalylglycine (DMOG), an α-ketoglutarate antagonist, induces HIF-1α that leads to metabolic reprogramming and decreases ROS formation in these aged cells. In combination with HIF-1α gene therapy in the ischemic muscle tissues, intravenous injection of DMOG-treated cells prevents limb necrosis and autoamputation in old recipients mice following ischemia [120]. While there are many strategies that modulate the redox regulation of stem and progenitor cells, it is essential for *in vitro* and preclinical studies to consider the clinical scenario, where elderly patients often suffer from comorbidities that affect neovascularization, such as diabetes, hypercholesterolemia, and advanced atherosclerosis. Therefore, it may be beneficial to develop cell-based therapies targeting combined pathophysiological conditions such as aging, metabolic disorders, and inflammatory diseases.

7. Conclusion

ROS production and aging are intertwined biological events that play a critical role in vascular repair and regeneration. ROS are intrinsic regulators that are involved in maintaining the abilities of self-renewal of stem cells and their differentiation into "lineage-committed" progenitor cells. The levels of ROS are attuned by the balance between ROS generation and antioxidative defense systems, depending on the cellular functions at different stages of stem and progenitor cells. On the other hand, ROS are extrinsic mediators that modulate bone marrow microenvironment in low-oxygen tension and induce hypoxic expansion in response to ischemic injury. Aging is associated with an increase in oxidative stress, in which the unbalanced ROS levels further contribute to cell aging. The age-dependent impairment in ischemia-induced neovascularization is, partly, due to oxidative stress-related dysfunction of stem and progenitor cells. Understanding the molecular targets of ROS and distinct redox signaling pathways in stem and progenitor cell function as well as how aging alters the redox balance will enable us to improve the efficacy of cell-based therapies and to better accommodate cardiovascular disease in aging populations.

Abbreviations

Ang II:	Angiotensin II
CuZnSOD:	Copper-zinc superoxide dismutase
CXCR4:	C-X-C motif receptor 4
DHE:	Dihydroethidium
EPC:	Endothelial progenitor cell
FoxO:	Forkhead Box class O
G-CSF:	Granulocyte colony stimulating factor
HIF-1α:	Hypoxia-inducible factor-1α
HSC:	Hematopoietic stem cell
MAPK:	Mitogen-activated protein kinase
MnSOD:	Manganese superoxide dismutase
NAC:	N-Acetyl-L-cysteine
Nox:	NADPH oxidase
SDF-1:	Stromal cell-derived factor-1
SIRT:	Sirtuin
VEGF:	Vascular endothelial growth factor.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This work was supported in part by University of Sydney Early Career Research Grant awarded to Yuen Ting Lam.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3286365, 7 pages
http://dx.doi.org/10.1155/2016/3286365

Research Article

Bioavailability Study of an Innovative Orobuccal Formulation of Glutathione

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Received 10 April 2015; Revised 19 June 2015; Accepted 12 July 2015

Academic Editor: Matías Mosqueira

Alteration of the ubiquitous thiol tripeptide glutathione (GSH) is involved in oxidative stress, which plays a role in ageing; consequently, GSH is closely related to this process characterized by progressive decline in the efficiency of physiological function and increased susceptibility to disease. When circulating GSH decreases, oral administration might be considered a therapeutic benefit. Unfortunately, due to the hydrolysis of the tripeptide by intestinal γ-glutamyltransferase, dietary glutathione is not a major determinant for its increase. Aim of this work was to evaluate improvement of GSH systemic availability testing, *in vitro* and *in vivo*, an optimized orobuccal fast-slow release formulation tablet containing pure stabilized GSH. *In vitro* evaluation of the penetration capability of the innovative GSH-release formulation showed that GSH was well absorbed by the reconstructed oral epithelium and its absorption has features of time-dependence. In addition, *in vivo* results, obtained from 15 healthy volunteers, were in favor of GSH level improvement in blood showing fast (after 30 and 60 minutes) absorption through oral mucosa. In conclusion, the intake of GSH formulated through optimized orobuccal fast-slow release tablets gave positive results in raising GSH blood concentration.

1. Introduction

The intracellular production of oxidant species, for example, reactive oxygen species (ROS), is elevated in many neurodegenerative diseases related to inflammation and mitochondrial dysfunction [1–3]. The related process well known as oxidative stress (OS) is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [4]. OS has been implicated also in normal ageing, particularly in the first phase of ageing [5], and many neurodegenerative diseases including Parkinson's (PD) and Alzheimer's (AD) diseases [6]. In both these diseases, multifactorial processes are involved, including OS, inhibition of mitochondrial complex I, ubiquitin-proteasome dysfunction, and inflammation. Furthermore, a number of events may occur simultaneously after OS including alteration of glutathione metabolism and GSH related enzymes, such as glutathione transferases (GSTs) [7].

Glutathione and Its Implication in Ageing. Glutathione (GSH) is a ubiquitous thiol tripeptide (gamma-glutamyl-cysteinyl-glycine), which plays an essential role in many cellular processes. It is believed to be the primary intracellular antioxidant for higher organisms. When oxidized, it forms a dimer (GSSG), which may be recycled in organs having glutathione reductase (GR). GSH is involved in many important biological phenomena, including the role of the brain's capacity to scavenge ROS, free radicals or not (e.g., superoxide radicals, hydroxyl radicals, and peroxynitrites).

In particular, protection against oxidative stress is related to the oxidation of GSH in mitochondria [8, 9]. Liver produces daily and distributes through the blood stream to the other tissues about 80% of the 8–10 grams of glutathione. GSH depletion is considered as one of the most important and early biochemical changes in PD insurgence, suggesting that not only is it a consequence of OS but it could also play an active role in PD pathogenesis [10]. Glutathione depletion can inhibit complex I, E1 ubiquitin ligase (E1), and proteasome activity. It can also exacerbate OS and activate the Jun kinase (JNK) pathway, leading to an inflammatory response [10]. Decreased concentrations of glutathione were reported in the ageing rat brain [11–13]. Also in humans, low concentrations of glutathione have been observed in plasma, erythrocytes, lymphocytes, and gastric mucosa in older compared with younger adults [14, 15].

Glutathione Availability. The intracellular level of glutathione in nonnervous system mammalian cells is in the range of 0.5–10 mM, normally over 99% in the reduced form (GSH); particularly in humans, it is typically around 1-2 mM, several hundred times greater than the level seen in plasma blood, which is about 2 μM in normal condition [16]. In the brain, however, GSH levels are often found at concentrations of 1–3 mM [7]. In particular, when the plasma circulating GSH decreases, due to different factors and provoking its lower availability into different districts and cells, oral administration might be considered a therapeutic benefit. Unfortunately, due to the hydrolysis of the tripeptide by intestinal γ-glutamyltransferase, dietary glutathione is not a major determinant for its increase; in fact results, obtained in a study of GSH systemic availability, showed that it is not possible to enhance GSH level to a clinically beneficial extent even by the oral administration of a high single dose of 3 g of GSH [17]. Therefore, there is the need to increase the systemic bioavailability of GSH when it is orally administered, to make it more suitable for therapeutic benefit, for example, those regarding the central nervous system.

Based on the above-mentioned considerations, the aim of this study was to evaluate the improvement of GSH absorption and systemic availability by testing an optimized orobuccal fast-slow release formulation tablet containing pure stabilized GSH. For this purpose, *in vitro* and *in vivo* evaluations were carried out. The term orobuccal refers to a composition capable of immediately dissolving and releasing the active ingredient contained therein upon contact with the oral mucosa. In this manner, the active compounds may be directly absorbed in the oral mucosa, which is well supplied with both vascular and lymphatic drainage, thus bypassing the intestinal degradation [18]. The above is only applicable to the molecules that can pass through the oral mucosa; all the other components are swallowed and go into the stomach, where they can be degraded by the gastric acid. Fast-slow release formulation reduces the problems of absorption and bioavailability of these components without losing the advantages of orobuccal absorption. The term "fast-slow release" means the differentiated release of selected ingredients: some components are delivered in orobuccal fast release and others in enteric slow release, by the use of new technology. This technology is applied to the selected molecules that are coated with a food grade fat matrix: when swallowed, the gastroprotected micro granules of the coated ingredient are not destroyed by the gastric acid and reach the intestine undamaged, where they are assimilated following the physiological lipid absorption pathway. The term "stabilized" refers to glutathione, which is maintained in a reduced form without substantial cyclization. This stabilization may be affected by the addition of one or more agents providing a formulation together with GSH, which is capable of delivering native reduced glutathione.

2. Materials and Methods

2.1. Sigma-Aldrich Chemicals Supplied All Chemicals and Solvents Used in This Study. SkinEthic supplied Reconstituted Epithelium HOE/S/5 (batch number 122 011J-M 037) and maintenance medium (batch number 12 022B 1103). Arbor Assays supplied hemoglobin (Hb) assay kit.

2.2. In Vitro Evaluation of the Penetration Capability of Optimized Orobuccal Fast-Slow GSH Release Formulation through Reconstructed Oral Tissue. The aim of the *in vitro* study was to investigate the penetration capability of GSH integrated in an orobuccal fast-slow release formulation through reconstructed human oral epithelium (SkinEthic HOE/S/5, batch number 12 022B 1103). This specific tissue was made up of 0.5 cm^2 epithelium reconstituted by air lift culture of transformed human keratinocytes TR146 (from a squamous cell carcinoma of the buccal mucosa) for 5 days in chemically defined medium (Maintenance Medium, batch number 122 011J-M 037) on inert polycarbonate filters. These transformed keratinocyte cells form an epithelial tissue devoid of *stratum corneum*, resembling histologically the mucosa of the oral cavity [18].

The tested sample was powder for tablets, each 220 mg of powder containing 50 mg of pure GSH. A quantity of powder just equal to the amount in a tablet (220 mg containing 50 mg of GSH, 5 mg/mL) was preliminarily dissolved in 10 mL artificial saliva (KCl 20 mM, KSCN 5.3 mM, NaH_2PO_4 1.4 mM, $NaHCO_3$ 15 mM, and $C_3H_6O_3$ 10 mM) at 37°C for 1 hour. According to Chotaliya (2013) [19] absorbing buccal surface is about 200 cm^2, with a great vascularization network. About the saliva volume, there is a nonhomogeneous saliva production of about 1500 mL during the day, with a mean saliva production in normal condition of about 10 mL/h until 250 mL/h under stimulation or 0 mL/h during sleeping [19]. Specific volumes of the above-described solution, corresponding to 10 mL of saliva per 200 cm^2 of surface, were applied onto 2 tissue units (0.5 cm^2) in replicate following a chronological order of experimental times T10 (10 minutes after product application); T20 (20 minutes after product application); T30 (30 minutes after product application). We evaluated the dose of the reduced glutathione absorbed and released by the tissue into the underlying medium during the time. Furthermore, at the end of the experimental period, treated tissue was used to determine its viability in order to evaluate any possibility of irritation of oral mucosa by MTT

Table 1: Orobuccal fast-slow release tablet.

Nutritional information		
Substance with nutritional or physiological effect	Daily dose (1 tablet 1.1 g)	%RDI (∗)
GSH	250 mg	
L-Cystine	50 mg	
Vitamin C	40 mg	50%
Selenium	55 mcg	100%

(∗) Recommended daily intake according to Dir.100/2008/EC.

(methylthiazolyldiphenyl-tetrazolium bromide) colorimetric assay [20]. At the end of the experimental period, we also evaluated the amount of glutathione absorbed in the tissue structure that was not released. In order to evaluate the endogenous production of glutathione during the treatment and correctly determine the absorption grade, a tissue series was treated with N-acetyl cysteine, precursor of glutathione. Glutathione dosage, in all treated models, was carried out by high-performance liquid chromatography (HPLC Jasco LC900) using C18 column Zorbax ODS column 250 mm 4.6 mm, 5 mm (Agilent Technologies).

2.3. In Vivo Evaluation of GSH Systemic Bioavailability Using an Optimized Orobuccal Fast-Slow Release Formulation Tablet. The aim of the *in vivo* study was to evaluate GSH bioavailability using an optimized orobuccal fast-slow release tablet on a number of 15 healthy volunteers (females and males), weight 60 ± 5 Kg, aged between 20 and 40 years, belonging to the European population (white); they did not practice exhaustive exercise and they are not smokers; they declared that they did not get any other food supplements or drugs. Criteria for the definition of the number and the expected number are 95% confidence level; estimates of the prevalence level of GSH/GSSG 0.2; estimate of the desired precision 0.2 (20%); CI 1.96; expected number 15. Volunteers were earlier informed on the procedures to be followed in the study, which was conducted with their understanding and consent. Each procedure was drawn up in agreement with the Helsinki declaration adopted at the Eighteenth General Assembly of the World Medical Association (WMA), held in 1964 on ethical principles for medical research involving human subjects. An Independent Ethical Committee of the University of Pavia approved the work procedures.

Supplementation Protocol. Subjects were provided with an orobuccal fast-slow release tablet of pure GSH taken through an oral absorption way. The principal ingredients of the tablet, with nutritional or physiological effect, are reported in Table 1. Pure GSH forms a flaky powder, which retains a static electrical charge, due to triboelectric effects, which makes processing difficult. The powder may also have an electrostatic polarization, which is akin to an electret (elektr- from "electricity" and -et from "magnet"). Glutathione is a strong reducing agent, so that autooxidation occurs in the presence of oxygen or other oxidizing agents. Demopouolus and Ross (1993) [21] provided a method of manufacturing glutathione tablets and capsules by the use of crystalline ascorbic acid as an additive to reduce triboelectric effects, which interfere with high-speed equipment and maintaining glutathione in a reduced state. Ascorbic acid has the advantage that it is well tolerated and antioxidant and reduces the net static charge on the glutathione [21].

A preferred formulation includes 250 mg or more of reduced glutathione with at least equimolar ascorbic acid, to fulfill three functions: acting as a sacrificial nonspecific antioxidant during preparation and storage and after ingestion; reducing or neutralizing static electrical charge of glutathione powder, allowing dense packing of capsules; and acting as a lubricant for the encapsulation device. The ascorbic acid also maintains an acidic and reducing environment, which pharmaceutically stabilizes the glutathione molecule. Ascorbic acid is believed to form a charge couple with glutathione, which enhances penetration through cell membranes and reduces the tendency for the gamma-glutamyl and glycinyl residues to assume a cyclic conformation or to form an internal cyclic amide. The ascorbate thus complexes with the glutathione in solution to maintain a linear conformation. This linear conformation, in turn, sterically hinders the free cysteinyl thiol group. This steric hindrance stabilizes a free radical, which may be formed, and thus maintains the biological activity of glutathione.

The oral mucosa has been found to allow rapid and efficient uptake of glutathione into the blood. In contrast to the digestive tract, the significance of facilitated or active transport mechanisms in the oral mucosa is believed to be low; rather, a high concentration of glutathione in the oral mucosa is believed to permit passive transport of the glutathione through the cells or around the cells into the capillary circulation. Therefore, compositions intended for absorption through the oral mucosa are preferably of high purity, as contaminants may be absorbed similarly to glutathione, and as relatively small, uncharged molecules [21].

To assess the GSH systemic bioavailability each volunteer had one tablet of orobuccal fast-slow GSH release melting it in the oral cavity in about 1 minute. The *in vivo* study was developed in acute, otherwise evaluations carried out in short experimental times. A draw blood was carried out before absorption (basal time) and after 30 minutes (T30) and 60 minutes (T60) starting from complete tablet absorption. Analysis of GSH levels was executed on whole blood employing enzymology GSH recycling method [22], on the basis of conversion of GSSG to GSH by GR and NADPH and reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). We analyzed whole blood because we considered that the endogenous disappearance rate (utilization rate) of GSH was about 25 μmol/(kg∗h) in healthy adult humans and that this rate accounted for 65% of whole body cysteine flux [38.3 μmol/(kg∗h)]. Among extrahepatic cells, the erythrocyte had a relatively high turnover rate for GSH. For example, the whole-blood fractional synthesis rate of GSH in healthy adult subjects was 65% per day, which meant that all the GSH was completely replaced in 1.5 days; this value was equivalent to 3 μmol/(kg∗h) [23]. We also took into consideration that whole blood (mainly erythrocytes) contributed up to 10% of whole body GSH synthesis in humans and that extracellular

Table 2: GSH absorption.

	GSH (mg)		GSH (%)		
Applied amount	0.125				
	Mean value	SD	Partial absorption	Total absorption	SD
T10 (medium)	0.069	0.002	55.04%	55.04%	1.9%
T20 (medium)	0.015	0.0008	12.32%	67.36%	0.63%
T30 (medium)	0.003	0.0002	2.56%	69.92%	0.14%
Tissue	0.001	0.0001	1%	1%	0.17%
Total absorption	0.088	0.0031	70.92%	70.92%	0.17%

In vitro data obtained from GSH dosage in the experimental model are reported. The applied and measured GSH amounts are expressed as mg (mean ± standard deviation SD) in the collected medium at different times and homogenized tissue at the end of experimental period. The (%) absorption values, calculated on GSH applied amount, both partial absorption related to each single monitored experimental time and total absorption (the sum of the % in the consecutive times), are calculated and reported in the table too.

concentrations of GSH were relatively low (e.g., 2 μmol/L in plasma), due to the cysteine residue of GSH, which was readily oxidized in a nonenzymatic way to GSSG by electrophilic substances (e.g., free radicals and reactive oxygen/nitrogen species). Another important reason for using whole blood was that the acids that are commonly used in protein unfolding and precipitation induce an overestimation of the amount of GSSG in blood samples because GSH is oxidized to GSSG (5–15%). We prevented this error by applying an innovative derivatization process that added the thiol-alkylating agent N-ethylmaleimide (NEM) to whole blood [24]. Therefore, we considered it to be accurate to measure glutathione in whole blood and to normalize and express it per g of Hb.

Results are expressed as nmol of GSH per gram of Hb. Evaluation of Hb concentration was implemented with a colorimetric kit assay (*Arbor Assays*, Michigan, US).

2.4. Data Analysis. Descriptive statistics were expressed as means and standard deviation of the mean (SD). In the *in vivo* study, a repeated measure analysis of variance was used to test the within-subjects variation in GSH absorption over time. We tested the sphericity assumption (through Mauchly's test) and since it was violated, we used the multivariate approach (MANOVA, Wilk's test) to test the within-subjects effects. The level of statistical significance was set at a p value of <0.05 ($^{*}p$ value < 0.05; $^{**}p$ value < 0.01). NS means not statistically significant.

3. Results and Discussion

3.1. In Vitro Study. We evaluated the dose of the reduced glutathione absorbed and released by the tissue into the underlying medium following a chronological order of experimental times (10, 20, and 30 minutes) and starting with applying 0.125 mg of GSH onto units of tissue. At the end of the experimental period, we also evaluated the amount of glutathione absorbed in the tissue structure that was not released (Table 2, Figure 1).

The amount of GSH released into the medium decreased over time: 0.069 mg after 10 minutes, 0.015 mg after 20 minutes, and almost null (0.003 mg) after 60 minutes. These

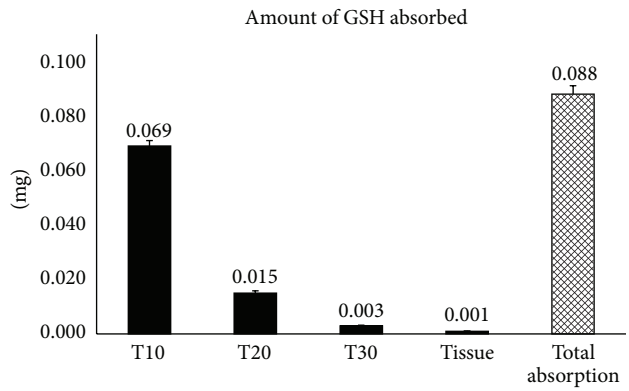

Figure 1: *In vitro* data obtained from GSH dosage as mg in the experimental model are reported. The measured glutathione amounts are expressed as mg (mean ± standard deviation SD) in the collected medium at different experimental times and in homogenized tissue at the end of experimental period. Total absorption at the end of the experimental period is also reported considering GSH amounts in the medium at different times and in the tissue.

results showed that GSH within an orobuccal fast-slow release formulation was able to penetrate reconstructed human oral epithelium. Moreover, obtained data showed a very small capacity of the tissue to hold the absorbed glutathione, contributing to its bioavailability (Table 2, Figures 1 and 2). We found only 0.001 mg of GSH (100 parts less than the applied amount of 0.125 mg) in the homogenized tissue at the end of the experimental time.

Furthermore, the analysis of the obtained results showed not only a penetration capability but also a fast absorption of the glutathione through the *in vitro* reconstructed oral epithelium, from 55% after 10 minutes to about 70% after 30 minutes (Table 2, Figure 2). The kinetics of the passage through the epithelium mimic a progressive absorption gradient for glutathione, saturated over time.

According to the product classification reported on validated methods with EpiSkin or Reconstructed Human Epithelium (RHE), performed MTT test showed no sign of irritation for the oral epithelium after the application of the sample solved in artificial saliva (Table 3). The relative viability percentage (%) was calculated for test substance

Table 3: MTT toxicity test.

	OD 540	Tissue viability	Classification
Untreated tissue	1.486	100%	
Treated tissue	1.476	99.33%	NI

In vitro data obtained from the MTT irritation test performed on the HOE tissue for the evaluation of tissue viability after tested product treatment.

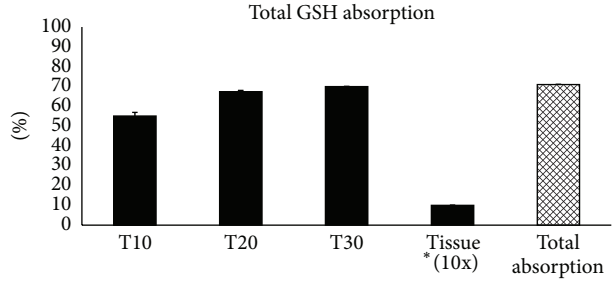

Figure 2: *In vitro* data obtained from the GSH dosage as % in the experimental model are reported. The percentage (%) of GSH absorption values are calculated on the applied amount of GSH (0.125 mg) and reported on the bases of single monitored experimental time as total % of absorption (mean ± standard deviation SD). Tissue *(%) value of GSH evaluated in the homogenized tissue is resized (10x).

(treated tissue) compared to negative control (nontreated tissue) and compared to the following criteria: mean tissue viability is ≤50%: irritant (I); mean tissue viability is >50%: nonirritant (NI).

Collected experimental data showed that the GSH insight orobuccal fast-slow release formulation was absorbed by the *in vitro* reconstructed oral mucosa and its absorption had features of time-dependence. According to the *in vitro* data and to the considered experimental protocol, optimized orobuccal fast-slow GSH release formulation was capable of carrying the GSH for the absorption through the oral mucosa.

3.2. In Vivo Study. The analysis of the obtained results in acute study showed a fast absorption of the glutathione through the *in vivo* oral mucosa. GSH blood concentration was increased both 30 minutes and 60 minutes after taking the orobuccal fast-slow GSH release formulation compared to the basal time before starting absorption. This increase was statistically significant when comparing the baseline concentration levels and those of concentration after 30 and 60 minutes. It could therefore be inferred that GSH was actually absorbed through mucous membrane, in a rapid manner, going to increase the quantity of reduced glutathione present in the blood (as it was expressed in Table 4 and represented in Figure 3). Finally, obtained *in vivo* results suggested that glutathione, taken by tablets with orobuccal fast-slow release, showed good bioavailability. Optimized orobuccal fast-slow release tablet was able to increase GSH blood level.

In vivo study confirmed results obtained *in vitro*; in other words orobuccal fast-slow GSH release tablet showed to have a good and rapid absorption through oral mucous membrane.

Table 4: GSH level.

	Absorption times		
	Basal (nmol/g Hb)	T30 (nmol/g Hb)	T60 (nmol/g Hb)
Mean	7358	8502	8913
SD	1590	1303	1309
p value		0.014 (∗)	

In vivo data obtained from the GSH dosage in total blood are reported. Results are expressed as mean ± SD and expressed in nmol GSH per gram of Hb at different experimental times T30 and T60 versus basal (before starting orobuccal fast-slow release tablet absorption). Statistical analysis was carried out using repeated measures analysis of variance in order to compare the mean values at subsequent times. The GSH level increased significantly ($^{*}p = 0.014$) with absorption time.

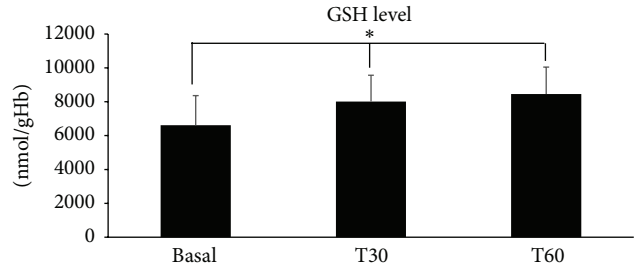

Figure 3: *In vivo* data obtained from the glutathione (GSH) dosage in whole blood are reported. Results are expressed as mean ± SD in nmol GSH per gram of Hb at different experimental times T30 and T60 versus basal (before starting orobuccal fast-slow release tablet absorption). Statistical analysis was carried out using repeated measures analysis of variance in order to compare the mean values at subsequent times. The GSH level increased significantly ($^{*}p = 0.014$) with absorption time.

Furthermore, derivatization process with alkylating agent NEM [24] demonstrated to be efficient; in fact obtained GSH levels found correspondence with data presented in literature (e.g., considering Hb mean value of 0.5 g/mL, GSH concentration in cells has a range: 1000–20000 nmol/gHb).

Through orobuccal formulations, GSH is administered and absorbed directly in the oral cavity generating a much quicker absorption and a considerably higher bioavailability with respect to the current method of administration by means of gastroresistant tablets and considering the same dose of active ingredient. The orobuccal compositions may be formulated in form of tablet, capsule, and/or granules, preferably in form of orobuccal tablet. Due to high permeability, the oral absorption route can produce rapid onset of action so the "drug" with short delivery period can be delivered and dose regimen is frequent [18].

The interest in the studies of the mechanisms, which correlate and interfere with ageing, is ever growing. On one hand, oxygen is essential for aerobic organisms, because it is the final electron acceptor in the mitochondria; on the other hand, oxygen is harmful because it can generate ROS continuously, which is believed to be factors closely related to ageing. Aerobic organisms possess antioxidant defense systems, able to remove ROS in the cells. These systems

consist of a series of enzymes and molecules of which the most effective is glutathione. Therefore, GSH plays a central role in the maintenance of cellular redox homeostasis and protection against ROS. Given the involvement of decreased GSH levels and alterations in glutathione-related enzyme activities in OS and related disease and regression states, maintaining or restoring GSH levels represents a promising "therapeutic" strategy. The apparently most straightforward approach for increasing GSH levels, through its direct administration, failed due to the solubility, absorption, and stability constraints that limit its bioavailability [25]. The direct delivery of the precursor amino acid cysteine was also a failure, due to its toxic effects [26]. Hence, research efforts turned to develop innovative GSH formulation to strengthen its cellular redox protective effects.

4. Conclusions

In conclusion, the intake of GSH, formulated through optimized orobuccal fast-slow release tablets, gave positive results in raising GSH blood concentration, which is probably going to strengthen all *in vivo* by-products and processes that involve this important tripeptide.

We can conclude that orobuccal fast-slow GSH release tablet is a new, innovative, efficient, and functional dosage form.

Abbreviations

AD:	Alzheimer's diseases
CI:	Confidence interval
GR:	Glutathione reductase
GSH:	Glutathione reduced form
GSSG:	Glutathione disulfide oxidized form
GSTs:	Glutathione transferases
Hb:	Hemoglobin
HOE:	Human oral epithelium
HPLC:	High-performance liquid chromatography
I:	Irritant classification
JNK:	Jun kinase
MTT:	Methylthiazolyldiphenyl-tetrazolium bromide
NADPH:	β-Nicotinamide adenine dinucleotide phosphate
NEM:	N-ethylmaleimide
NI:	Nonirritant classification
OS:	Oxidative stress
PD:	Parkinson's disease
RDI:	Recommended dietary intake
RHE:	Reconstructed Human Epithelium
ROS:	Reactive oxygen species
SD:	Standard deviation of the mean
WMA:	Assembly of the World Medical Association.

In Vitro Times

T10: 10 minutes after product application
T20: 20 minutes after product application
T30: 30 minutes after product application.

In Vivo Times

T30: 30 minutes starting from tablet absorption
T60: 60 minutes starting from tablet absorption.

Conflict of Interests

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

Acknowledgments

This work was supported by FAR (Progetto di Ricerca Finalizzata/Finalize Research Project) grants. The authors thank the human volunteers for their substantial contribution to this study, Carlotta Galeone for statistical support, and Charlotte Buckmaster for English support.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 5719291, 12 pages
http://dx.doi.org/10.1155/2016/5719291

Research Article

Curcumin Supplementation Decreases Intestinal Adiposity Accumulation, Serum Cholesterol Alterations, and Oxidative Stress in Ovariectomized Rats

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Received 22 October 2014; Revised 21 January 2015; Accepted 29 January 2015

Academic Editor: Claudio Cabello-Verrugio

The aim of this study was to investigate the potential of curcumin oral supplementation (50 and 100 mg/Kg/day, for 30 days) in circumventing menopause-associated oxidative stress and lipid profile dysfunctions in a rat ovariectomy (OVX) model. Female Wistar rats were operated and randomly divided into either sham-operated or OVX groups. Sham-operated group ($n = 8$) and one OVX group ($n = 11$) were treated with vehicle (refined olive oil), and the other two OVX groups received curcumin at 50 or 100 mg/Kg/day doses ($n = 8$/group). OVX vehicle-treated animals presented a higher deposition of intestinal adipose tissue as well as increased serum levels of IL-6, LDL, and total cholesterol when compared to sham-operated rats. In addition, several oxidative stress markers in serum, blood, and liver (such as TBARS, carbonyl, reduced-sulphydryl, and nonenzymatic antioxidant defenses) were altered toward a prooxidant status by OVX. Interestingly, curcumin supplementation attenuated most of these parameters to sham comparable values. Thus, the herein presented results show that curcumin may be useful to ameliorate lipid metabolism alterations and oxidative damage associated with hormone deprivation in menopause.

1. Introduction

Ageing is accompanied by changes in the activity of several genes involved in the control of metabolism, antioxidant systems, DNA repair, cellular senescence, and death [1]. Even though human lifespan in the 21th century has increased all over the world, especially in developed countries, the age when women enter their major age-related hormonal change (i.e., menopause) has remained constant, at around 50 years [2]. It means this phase could take part in almost one-third of women's life. In fact, menopause is characterized by loss of ovarian function and subsequent decrease in serum levels of estrogen and progesterone, which are associated with the development/acceleration of arteriosclerosis, skin aging, and immune dysfunctions among others [2].

Oxidative stress has been defined as an unbalance between increased reactive oxygen species (ROS) production and a noncorrespondent enzymatic and nonenzymatic antioxidant activity [3, 4]. Several evidences have described menopause as a prooxidant and inflammatory state, which directly impact the development of several ageing and oxidative stress-associated diseases [2]. For example, menopause is a risk factor associated with the onset or/and progression of cardiovascular diseases [5, 6], and much of this correlation is attributed to the benefic effects of female sexual hormones in protecting the cardiovascular system, by either acting as inducers of antioxidant genes or functioning as endogenous free-radicals scavengers *per se* [7, 8]. Because the oxidative stress consequent of the lack sexual hormones has been

a major concern in the menopause and postmenopausal landscapes [9, 10], hormone replacement therapy (HRT) has been chosen as the standard approach to alleviate menopause-associated symptoms, thus preventing the clinical consequences of an estrogen-deficient state. However, because of the possible negative effects associated with long-term HRT, especially the increased risk of thromboembolic accidents, stroke, and breast cancer [2, 11], HRT has lost ground among women, and a growing interest in alternative strategies has been established. In this regard, there is a particular interest in validating the antimenopausal properties of natural herbs with antioxidant/anti-inflammatory potential as well as few or even none significant side effects.

Curcumin has been described to regulate signaling and metabolic pathways by modulating diverse molecular events, including transcription factors activity, cytokines production, and antioxidant status, as well as cell proliferation and apoptosis genes [12–14]. As an antioxidant, for example, curcumin is able to prevent the drop in hepatic glutathione and decreases lipid peroxidation in the hepatocarcinogenesis promoted by N-nitrosodiethylamine in male Wistar rats [15]. In ovariectomized (OVX) rodent, which is a classical animal model of chronic progressive bone loss/osteoporosis, curcumin supplementation exhibited bone sparing effects [16–18]. Besides osteoporosis, curcumin could also act beneficially on other symptoms caused by menopause such as arteriosclerosis, obesity, and other pathologies associated with oxidative stress progression along this period [19]. In this context, even though not only have studies on the efficacy and safety of curcumin been claimed, but also ambiguous results and a complete lack of reports from human clinical trials show that the potential use of curcumin for prophylaxis or treatment of postmenopausal remains underestimated [11].

Considering the aforementioned, the aim of this study was to investigate the effects of curcumin supplementation (50 and 100 mg/Kg/day, during 30 days) on adiposity accumulation and serum biochemical and oxidative stress parameters in a menopause model of ovariectomized (OVX) Wistar rats, aiming to determine its potential to attenuate menopause-associated metabolic and oxidative alterations caused by hormone deprivation.

2. Material and Methods

All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication Number 80–23 revised 1996) and were carried out according to the determinations of the Brazilian Council for the Control of Animal Experimentation (CONCEA). Experiments were approved by the University Animal Research Ethic Committee (Register Number 25320).

2.1. Animals and Reagents. Female Wistar rats (200–250 g) in regular estrous cycles were obtained from our breeding colony. The animals were maintained in groups of five individuals with access to standard pellet food and water *ad libitum*. The animals were maintained under a 12-hour light–dark cycle (7 am–7 pm) in a temperature-controlled room (23 ± 1°C). Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ketamine hydrochloride was from Virbac Ltda (Jurubatuba, SP, Brazil) and xylazine hydrochloride was from Vetbrands Ltda (Goiania, GO, Brazil). All other chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Surgical Procedures. The rats were allowed 2 weeks to acclimatize before the beginning of the experimental protocol. Afterwards, seventy-day-old female rats were randomly divided into either sham-operated group ($n = 8$) or OVX group ($n = 27$). Rats were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) plus xylazine (15 mg/kg), and ovariectomy was performed under aseptic conditions as previously described [20].

2.3. Treatment. Two months after surgery, the animals were treated with curcumin or vehicle every other day for a total of 30 days. All treatments were carried out at night. Sham-operated ($n = 8$) and one OVX group ($n = 11$) were treated with vehicle (refined olive oil) and the other two OVX groups ($n = 8$ each) received curcumin at 50 and 100 mg/Kg/day doses, respectively. Treatment was performed via intragastric gavage in a maximum volume of 0.4 mL. The animals were weighted weekly.

2.4. Sample Acquisition. After 30 d treatment (90 d after ovariectomy), the rats were decapitated and blood, serum, liver, visceral adipose tissue, and uterus samples were collected for analysis. The uterus was cut above the cervical junction, the visible fat was removed, and the cleaned uterus was weighed. Intestinal adipose tissue (IAT) was carefully removed from small intestine and weighted. The ratios of the uterus and IAT weight relative to animal weight were calculated. Whole blood was collected and serum was separated. Blood, serum, and liver samples were stored at −80°C for subsequent analyses. Blood samples were frozen and thawed (−80°C/25°C) twice and centrifuged (900 g, 5 min) to remove debris. The liver samples were dissected and frozen at −80°C·s.

2.5. Serum Markers Profiling. Serum samples were used to determine the levels of high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), total cholesterol, and total triglycerides, as well as the hepatotoxicity markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. All these parameters were assayed using commercial kits (Labtest Diagnóstica SA; Lagoa Santa, MG, Brazil). Serum interleukin-6 (IL-6) levels were determined by ELISA following manufacturer's instructions (Catalog Number RAB0311, Sigma Aldrich, USA).

Table 1: Body weight gain, individual uterus, and visceral adipose tissue weight (g) in relation to respective animal body weight (g).

	Sham	OVX	Curcumin (mg/Kg/day)	
			OVX 50	OVX 100
Weight gain (g)	22.62 ± 2.618	61.818 ± 3.60[d]	59 ± 3.37[c]	61.625 ± 2.77[d]
Uterus (g)/body mass (g) ratio	1 ± 0.07	0.17 ± 0.01[d]	0.18 ± 0.01[d]	0.19 ± 0.02[d]
Visceral adipose tissue (g)/body mass (g) ratio	1 ± 0.05	1.41 ± 0.1[c]	0.99 ± 0.04[#]	1.08 ± 0.09[*]

Fresh uterine and visceral adipose tissues weight from Sham and OVX rats. Data are shown as mean ± SEM (sham $n = 8$, OVX $n = 11$, OVX50 $n = 8$, and OVX100 $n = 8$).
[c]$P < 0.01$: different from sham group; [d]$P < 0.001$: different from sham group; [*]$P < 0.05$: different from OVX group; [#]$P < 0.01$: different from OVX group (one-way ANOVA followed by Tukey's test).
OVX: ovariectomized.

2.6. Redox Profile in Blood, Serum, and Liver Samples

2.6.1. Antioxidant Enzymes Activity Quantification. Superoxide dismutase activity was estimated from the inhibition of superoxide anion-dependent adrenaline autooxidation in a spectrophotometer at 480 nm as previously described [21]. Results were expressed as units of SOD/mg protein. Catalase activity was assayed by measuring the ratio of decrease in hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer at 240 nm [22]. To determine GPx activity, the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione, *tert*-butyl hydroperoxide, and glutathione reductase as previously described [23].

2.6.2. Nonenzymatic Antioxidant Potential (TRAP Assay). We used the total reactive antioxidant potential test (TRAP) as an index of tissue nonenzymatic antioxidant potential. This assay is based on the decrease of chemiluminescence produced from the reaction of 2,20-azobis[2-amidinopropane] (AAPH) derived peroxyl radical with luminol due to free radical quenching by the antioxidant compounds present in the sample [24]. Briefly, we prepared AAPH solutions and added luminol ("System" solution, 100% chemiluminescence); thereafter, we waited 2 h for the system to stabilize before performing the first reading. After the addition of the samples, the chemiluminescence was monitored over a 40 min period, the results were transformed to percentages, and the area under curve (AUC) was calculated as previously described [25]. The samples displaying lower AUC will be those with higher antioxidant capacity.

2.6.3. Oxidative Damage Markers. The formation of thiobarbituric acid reactive species (TBARS) was quantified as an index of lipid peroxidation as previously described [26]. The samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 30 min. TBARS were determined at 532 nm in a spectrophotometer reader. Results are expressed as ηMol of TBARS/mg protein.

Oxidative damage to proteins was measured by quantification of carbonyl groups as previously described [27]. This method is based on the reaction of dinitrophenylhydrazine (DNPH) with protein carbonyl groups, and the absorbance was read in a spectrophotometer at 370 nm. Results are expressed in μmol carbonyls/mg protein.

In order to measure the levels of reduced thiol (-SH) groups in protein and nonprotein fractions from rat tissues, we used the Ellman's reagent based assay [28]. For total SH content measurement, a 50–100 μg sample aliquot was diluted in PBS and reacted with 10 mM 5,5-dithionitrobis 2-nitrobenzoic acid. After 60 min incubation at room temperature, the absorbance was read in a spectrophotometer set at 412 nm. To assess the nonprotein SH content (which includes glutathione and other small peptides), 1 mg protein aliquot was reacted with trichloroacetic acid (10% v/v) for deproteinization and centrifuged (10,000 g/10 min), and the supernatants were used to measure the level of SH in the protein-free fraction. Results are expressed as "mmol SH groups/mg protein" or "μmol SH groups/mg protein" for protein and nonprotein SH, respectively.

2.7. Statistical Analysis. Data were expressed as average ± standard error (SEM). Differences were compared by one-way ANOVA, followed by Tukey's test. $P < 0.05$ were considered significant.

3. Results

3.1. Body Weight Gain, Uterine Tissue, and Intestinal Adipose Tissue Ratio. Table 1 shows the body weight gain at the end of a 30-day treatment period. Irrespective of the curcumin supplementation, weight gain (g) was significantly higher in all the OVX groups when compared to sham. We also collected, weighted, and analyzed the uterine morphology at the end of the treatments (Table 1). We observed that sham animals presented different uterine morphology according to the estrous cycle phase. Two sham rats presented characteristic proestrus (high fluid content and thick tissue) whereas five others displayed nonproestrus, estrus, or diestrus morphologies. All OVX rats, independent of the treatment, showed a significant reduction in uterine tissue weight when compared to sham groups. Uterus from OVX rats was found highly atrophied, confirming the absence of ovarian hormones secretion and ovulation along this period. Intestinal adipose tissue (IAT) was also removed and weighted. OVX vehicle-treated animals presented a higher IAT/body weight ratio when compared to sham rats and supplementation with curcumin decreased IAT accumulation (Table 1).

Table 2: Serum parameters and lipid profile.

	Sham	OVX	Curcumin (mg/Kg/day)	
			OVX 50	OVX 100
IL-6 (pg/mL)	74.73 ± 1.08	78.80 ± 0.65[c]	75.96 ± 0.56[*]	75.39 ± 0.72[*]
AST activity (U/dL)	18.937 ± 1.72	26.888 ± 1.37[c]	25.507 ± 1.22[b]	26.975 ± 0.98[c]
ALT activity (U/dL)	16.974 ± 0.67	16.753 ± 0.68	16.739 ± 0.48	16.979 ± 0.91
HDL (mg/dL)	30.714 ± 1.99	31.666 ± 1.55	31.285 ± 1.12	29.857 ± 1.47
LDL (mg/dL)	17.285 ± 2.74	26.933 ± 3.40[b]	26.171 ± 1.36	23.742 ± 1.46
VLDL (mg/dL)	9.428 ± 1.19	8.222 ± 0.75	5.714 ± 0.42[b]	6.285 ± 0.86[b]
Total cholesterol (mg/dL)	54.750 ± 3.56	70.888 ± 4.41[b]	63.857 ± 2.67	59.714 ± 3.04
Total triglycerides (mg/dL)	47.142 ± 5.88	40.888 ± 2.27	27.714 ± 2.07[d]	30.571 ± 4.41[d]

IL-6 levels, AST/ALT activities, and lipid profile were measured in serum samples. Sham and OVX groups were treated once a day for 30 days with refined olive oil containing or not curcumin. Data are expressed as mean ± SEM (sham $n = 8$, OVX $n = 11$, OVX50 $n = 8$, OVX100 $n = 8$).
Statistically different from Sham group: [b]$P < 0.05$, [c]$P < 0.01$ [d]$P < 0.001$; [*]$P < 0.05$: different from OVX group (one-way ANOVA followed by Tukey's test).
ALT: alanine aminotransferase; AST: aspartate aminotransferase; HDL: high-density lipoprotein; IL-6: interleukin-6; LDL: low-density lipoprotein; OVX: ovariectomized; VLDL: very low-density lipoprotein.

3.2. Effects of Curcumin on IL-6, Serum Lipid Profile, and Tissue Damage Biomarkers in OVX Rats. It has been established that the abdominal/visceral adiposity accumulation in menopause sets out predisposition to a proinflammatory status due to macrophages recruitment and activation with the adipose tissue and/or imbalance between leptin and adiponectin productions by adipocytes [29, 30]. In our model, OVX promoted slight but significant increases in serum levels of IL-6, which were restored to control levels by curcumin at both concentrations tested (Table 2).

Taking into account the positive effect of curcumin on IAT accumulation, we sought to investigate whether curcumin could alter serum lipid profiles in the OVX model (Table 2). We detected that basal levels of LDL and total cholesterol increased in OVX compared to sham-operated rats. Animals that received curcumin showed LDL and total cholesterol levels comparable to to sham values. Even though the OVX model did not change HDL, VLDL, and triglycerides concentrations, at least at the end of the 90 days studied, curcumin supplementation decreased the basal levels of these circulating lipids. Besides lipid markers, AST and ALT serum activities were quantified in order to address the impact of OVX and curcumin upon systemic tissue damage. In all OVX groups, AST activity presented a modest but significant increase when compared to sham, and curcumin was not able to restore AST to sham levels. ALT activities did not differ among all groups investigated (Table 2).

3.3. Effects of Curcumin on the Antioxidant Profile in OVX Rats. Switching cellular metabolism toward a prooxidant environment is a hallmark of menopause. Substances such as reduced-sulphydryl groups (R-SH), vitamins A and E, albumin, GSH, and polyphenols among others possess chemical characteristics that directly affect the antioxidant balance in serum and tissues. In OVX rats, we observed a decrease in the nonenzymatic free radical scavenger potential, which was detected in both serum (OVX = 244456 ± 6572, sham = 197410 ± 7209 AUC units) and liver (OVX = 161070 ± 6808, sham = 132628 ± 5132 AUC units) as assessed by TRAP assay (Figure 1). Curcumin treatment made OVX animals enriched in nonenzymatic antioxidants as determined from AUC in serum (OVX 50 = 233252 ± 11902, OVX 100 = 208913 ± 12835) and liver (OVX 50 = 144215±5854, OVX 100 = 138784 ± 6299) and also clearly observed from the determination of "time for 50% induction" of chemiluminescence (Table 3).

Table 3

Treatments	Time of induction 50% (seconds)	
	Serum	Liver
Sham	1380	715
OVX	780	450
OVX + 50 mg/kg	961	603
OVX + 100 mg/kg	1260	665

Besides the aforementioned nonenzymatic antioxidants, we quantified the activity of the main antioxidant enzymes (Figure 2). Different from the effect on serum nonenzymatic defenses, neither OVX nor curcumin treatment caused modifications in antioxidant enzymes activities (as units/mg protein) in red blood cells homogenates. All OVX groups presented similar activities of SOD (OVX = 44.9 ± 2.0, OVX 50 = 43.2 ± 1.6, and OVX 100 = 50.5 ± 4.7), CAT (OVX = 116.5 ± 11.4, OVX 50 = 115.2 ± 12.5, and OVX 100 = 99.0 ± 11.7), and GPx (OVX = 5.1 ± 0.3, OVX 50 = 4.3 ± 0.17, and OVX 100 = 5.0 ± 0.25) when compared to sham (sham_{SOD} = 48.0 ± 1.6, sham_{CAT} = 102.3 ± 11.2, and sham_{GPx} = 4.5 ± 0.18).

In liver, GPx activity decreased in OVX samples when compared to sham-operated group (OVX = 50.0 ± 1.6, sham = 59.3 ± 3.3; $P < 0.05$), and curcumin was able to prevent the decrease in GPx at both of the doses tested herein (OVX 50 = 50.5 ± 3.4, OVX 100 = 55.8 ± 2.7) (Figure 2(b)). SOD (sham = 62.3 ± 4.5, OVX = 53.9 ± 4.9, OVX 50 = 58.9 ± 4.1, and OVX 100 = 63.6 ± 6.1) and CAT (sham = 129.4 ± 23.3, OVX = 138.0 ± 15.3, OVX 50 = 143.3 ± 11.9, and OVX 100 = 141.8 ± 23.4) activities across OVX groups also did not alter.

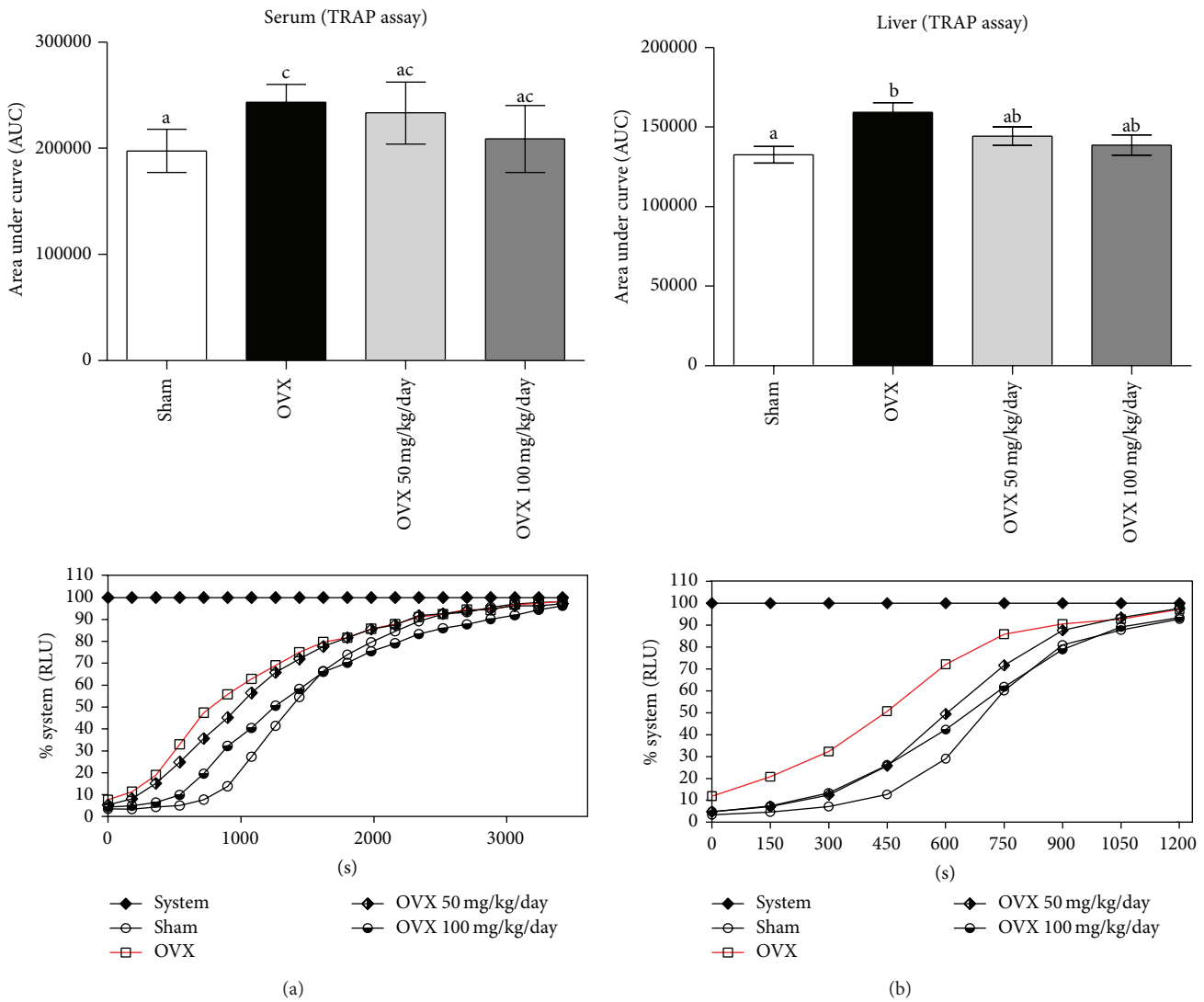

Figure 1: Effects of ovariectomy and curcumin supplementation on serum (a) and liver (b) nonenzymatic antioxidant potential. An experiment's representative graphic and the area under curve of total reactive antioxidant potential were analyzed on both samples. Data are expressed by mean ± SEM (sham $n = 8$, OVX $n = 11$, OVX 50 $n = 8$, and OVX 100 $n = 8$) and the experiments were performed in triplicate. Statistical difference from sham group: $^{b}P < 0.05$, $^{c}P < 0.01$ (one-way ANOVA followed by the post hoc Tukey's test).

3.4. Oxidative Damage Markers and SH Status in Blood, Liver, and Serum. Damage to biomolecules is a major consequence of a prooxidant imbalance. We observed that OVX, and its consequent menopause-like changes, promoted a significant effect on the oxidative stress markers. TBARS (lipid peroxidation), carbonylated proteins (carbonyl index), and nonproteic and protein SH contents were altered by OVX in different tissues (Figure 3). TBARS levels (ρmol/mg protein) and carbonylated proteins (μmol/mg protein) increased in erythrocytes of OVX vehicle-treated animals (OVX = 0.48 ± 0.06, 2.3 ± 0.25, sham = 0.28 ± 0.02, 0.76 ± 0.32, resp.) while both nonproteic (μmol/mg protein) and proteic (mmol/mg protein) SH contents decreased (OVX = 0.41 ± 0.02, 1.70 ± 0.03, sham = 0.50 ± 0.03, 1.95 ± 0.08; $P < 0.05$) when compared to sham (Figure 3(b)). These changes suggest a typical prooxidant status damaging both proteins and lipids in the blood of OVX animals. Curcumin supplementation attenuated the OVX-induced damage by decreasing lipoperoxidation (TBARS) and restoring of nonprotein and protein sulfhydryl homeostasis to levels comparable to sham. Higher doses (100 mg/Kg/day) of curcumin were also able to restore carbonylated proteins to sham levels in erythrocytes (Figure 3(b)).

As observed in red blood cells, oxidative damage profiling in liver samples revealed that OVX increased lipoperoxidation (TBARS) and proteins carbonylation (OVX =1.12 ± 0.09, 4.26 ± 0.44, sham = 0.79±0.06, 1.92±0.27, resp.; $P < 0.05$) and 50 and/or 100 mg/Kg/day curcumin was able to prevent the oxidative damage to these biomolecules (Figure 3(c)). Protein SH groups did not change across the experimental groups.

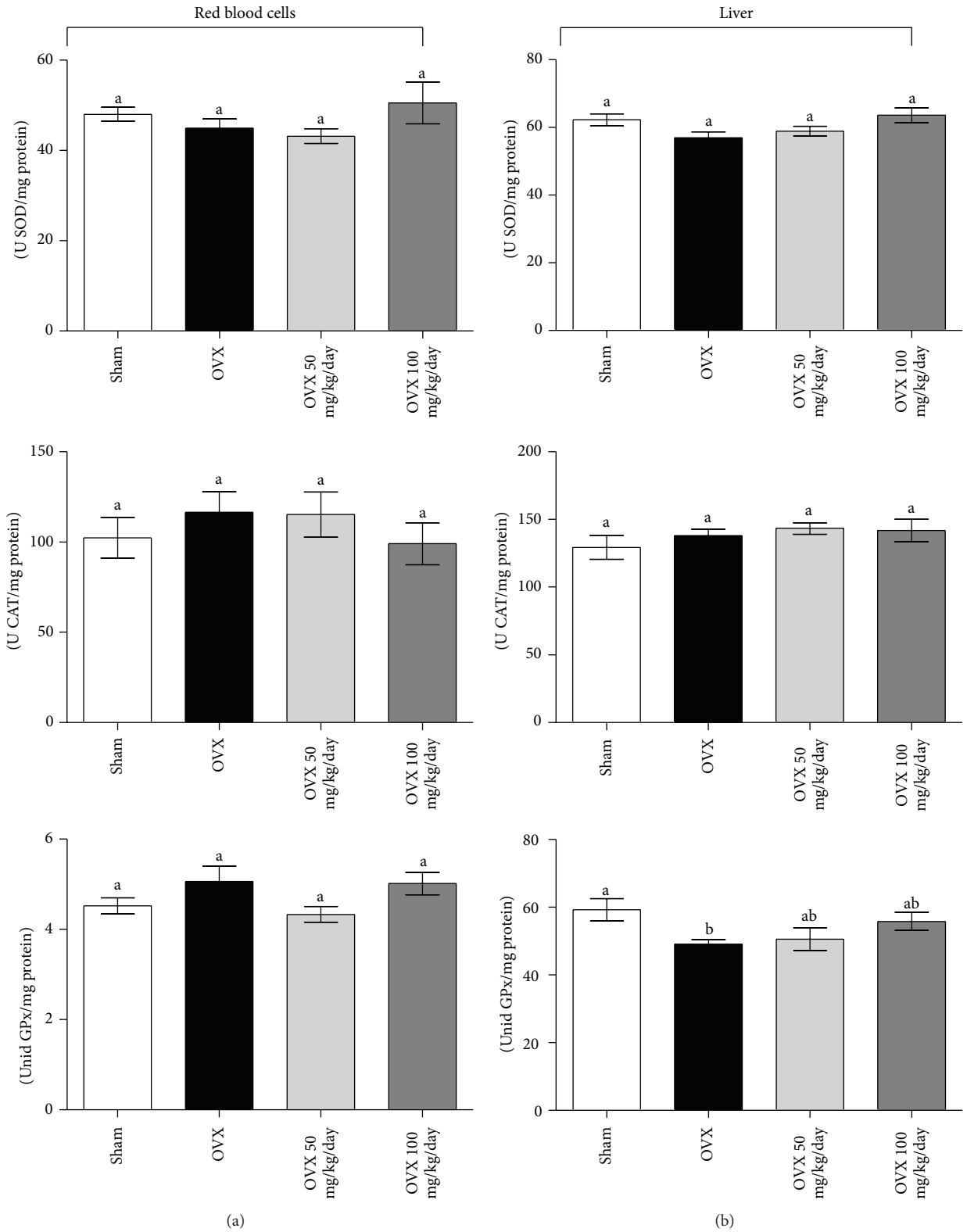

Figure 2: Effects of ovariectomy and curcumin supplementation on blood and liver antioxidant enzyme activities. Total superoxide dismutase (SOD) activity, catalase (CAT) activity, and glutathione peroxidase (GPx) activity were measured in blood (a) and liver (b) samples. Data are mean ± SEM (sham $n = 8$, OVX $n = 11$, OVX 50 $n = 8$, and OVX 100 $n = 8$) and the experiments were performed in triplicate. Statistical difference from sham group: $^{b}P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

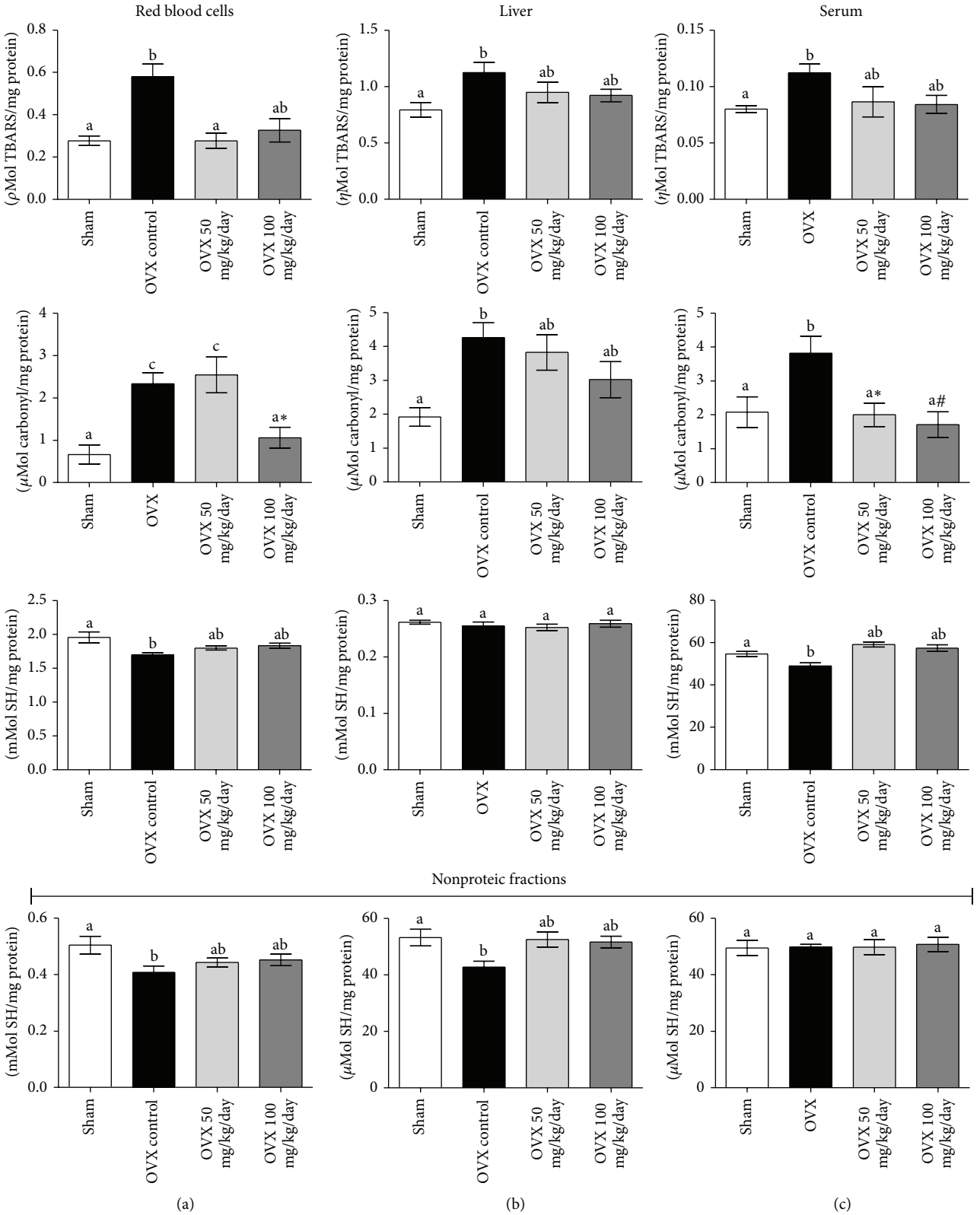

Figure 3: Effects of ovariectomy and curcumin supplementation on blood, liver, and serum thiol reduced content and oxidative damage markers. Proteic and nonproteic SH content, protein carbonyls groups, and lipid peroxidation were analyzed in blood (a), liver (b), and serum (c) samples. Data are expressed by mean ± SEM (sham $n = 8$, OVX $n = 11$, OVX 50 $n = 8$, and OVX 100 $n = 8$) and the experiments were performed in triplicate. Statistical difference from sham group: $^{b}P < 0.05$, $^{c}P < 0.01$. Statistical difference from OVX group: $^{*}P < 0.05$, $^{\#}P < 0.01$ (one-way ANOVA followed by the post hoc Tukey's test).

On the other hand, OVX depleted nonprotein sulfhydryl levels (OVX = 42.7 ± 2.1, sham = 53.2 ± 2.9 μmol/mg protein; $P < 0.05$), which were restored by curcumin supplementation.

In serum, the antioxidant effects of curcumin were much similar to those observed in red blood cells and liver samples. OVX increased serum carbonyl groups when compared to sham (sham = 2.08 ± 0.45, OVX = 3.99 ± 0.61; $P < 0.05$), and treatment with curcumin rescued protein damage to sham levels (OVX + 50 = 2.00 ± 0.35, OVX + 100 = 1.71 ± 0.38) (Figure 3(a)). Protein sulphydryl content decreased in OVX animals (sham = 54.7 ± 1.2, OVX = 49.0 ± 1.4; $P < 0.05$), and curcumin supplementation restored it to sham levels (OVX 50 = 59.1 ± 1.1, OVX 100 = 57.4 ± 1.5; $P < 0.05$). On the other hand, neither OVX nor curcumin altered nonprotein SH content in the serum. Serum TBARS were increased by OVX, but curcumin was not able to statistically block this phenomenon. Hence, the aforementioned results show that curcumin intake optimizes or/and maintains the antioxidant status in OVX animals, thus protecting from oxidative stress-associated damage to biomolecules in different tissues. This effect seems to be more likely attributed to increments in the nonenzymatic potential than changes in the antioxidant enzymes activity, agreeing with previous studies from other models of curcumin supplementation [15, 31, 32].

4. Discussion

Menopause is characterized by a complete failure to produce progesterone and estrogen and, thus, represents a situation of premature aging. Sex hormone insufficiency is related to deep physiological alterations, which include increase in oxidative stress, bone loss, weight gain, and cardiovascular dysfunction among other complications associated with this period in animals and humans [33].

Menopause experimental models are widely used to reproduce the main aspects and changes associated with female hormone deprivation. The state-of-art procedure to mimic menopause in rodents is the bilateral ovariectomy. Not surprisingly, along the last years, there has been a great increase in the number of publications focusing on the metabolic alterations and menopause-related symptoms in OVX models, such as early aging of nervous and immune system [34], weight gain [35], behavioral changes [23], cardiovascular dysfunction [36], insulin resistance [37, 38], and alterations in cytokines levels (e.g., TNF-α, IL-1, IL-6, and IL-10) [39]. OVX rats also present increased oxidative stress levels and, consequently, an accelerated aging process in different tissues [7, 40, 41]. In this study, we observed that oral curcumin was able to prevent a number of biological impairments associated with hormone deprivation. Alterations in the levels of some lipid markers, IAT deposition, and, mainly, improvements in the antioxidant potential in blood and liver were observed after a 30-day supplementation, which is a noteworthy result given the well-recognized clinical safety of curcumin [42]. Given that most women are still reluctant to take the risks associated with HRT [2], diets based on antioxidants may help to protect menopausal and postmenopausal women against the high levels of oxygen stress implied in the acceleration of the arteriosclerotic process and skin aging, among others, that take place during middle age. However, taking into account that many menopausal and postmenopausal women actually do not consume the recommended five daily rations of such a healthy diet [43], they might obtain some benefit from dietary supplements. Here, we provided evidences that curcumin could enter as a possible alternative supplementation to alleviate the oxidative damage and lipid metabolism imbalances caused by sexual female hormone deprivation in menopause.

In fact, postmenopausal women showed higher levels of the prooxidant biomarkers MDA, 4-hydroxynonenal (4-HNE), and oxidized LDL, when compared to premenopausal subjects [44]. Much of the prooxidant condition associated with menopause is owed to the absence of estrogen, which, for example, has been reported to protect vascular smooth muscle cell membrane phospholipids against peroxidation [45] beyond preventing oxidative stress- induced endothelial cell apoptosis in rat models [46]. Previous studies underlie our results, since OVX rats display impaired total antioxidant capacity in serum and liver, which was accompanied by an increase in different oxidative damage markers [35, 47]. Our study revealed that neither ovariectomy nor curcumin had major effects upon the antioxidant enzymatic machinery. In the flip side, OVX depleted nonenzymatic antioxidants, which were restored by curcumin supplementation. Compounds like vitamins A, C, and E, uric acid, and sulphydryl reduced molecules as GSH accounts for most of the nonenzymatic antioxidants in biological systems. It is already shown by Dilek et al., 2010, [48] that OVX in rats decreases plasma concentrations of vitamins A, C, and E compared to healthy animals. We herein showed that depletion of nonenzymatic antioxidant by OVX was enough to result in free-radicals insult, which damaged different molecules in OVX rats. We demonstrated that curcumin supplementation could restore the nonenzymatic antioxidant buffer potential to protect biomolecules from damage in the context of female sexual hormone deprivation by OVX. Curcumin decreased the levels of carbonylated proteins and lipoperoxides in blood and liver compartments and also restored protein and nonprotein thiol homeostasis, without, overall, affecting enzymatic defenses in OVX rats. In a major perspective, it seems that the idea of supplementing antioxidants in menopause could be extended out of the curcumin context. For example, Abbas and Elsamanoudy [49] have observed increases in MDA levels and decreases in both enzymatic and nonenzymatic (GSH levels, GPx, CAT, and SOD activities) defenses in liver of OVX Sprague-Dawley rats, which were improved by vitamin E administration.

Obesity and increase in food intake are very common factors associated with menopause, leading to weight gain, gradual reduction in lean body mass, and progressive fat accumulation in different body regions. Abdominal fat induces oxidative stress, which in turn has been shown to cause low-level chronic systemic inflammation [50, 51].

In OVX rats, weight gain and excess of visceral adipose tissue are well-described phenotypic changes caused by this model [35, 52, 53]. In contrast to the previously described effects of curcumin in inhibiting weight gain in high-fat diet models [54, 55], our results showed that it did not reverse the weight gain caused by OVX. Perhaps the 30-day period of treatment with curcumin has been sufficient to only exert direct effects on the intestinal accumulated fat but not in overweight, since the studies above mentioned used curcumin at larger amounts for longer periods (12 and 24 weeks) [55, 56]. In addition, these studies looked at the effects in male rodents, which could be a considerable difference, given that the presence/absence of female sex hormones may be decisive for the imbalance in weight gain [57]. Indeed, more studies are claimed to determine the possible intervention of curcumin on the overweight caused by OVX in rats.

Estrogen is a key modulator of lipid homeostasis; consequently postmenopausal women usually exhibit increased levels of LDL and total cholesterol and decreased HDL, compared to premenopausal [58]. In OVX models, some alterations in lipid profile were already described such as increases in LDL or non-HDL and total cholesterol levels [28, 53]. In our model, OVX *per se* increased both LDL and total cholesterol, but no alterations in HDL fraction were found. Otherwise, curcumin intake seemed to improve lipid profiles of OVX rats by keeping the level of these lipids similar to sham group, or even by decreasing TG and VLDL levels irrespective of the noneffect of OVX upon these parameters. In experimental models of atherosclerosis in rabbits, curcumin showed antioxidant effects on LDL [59]. In addition, curcumin was also able to decrease the concentrations of oxidized HDL and LDL in the serum of women ranging from 40 to 90 years old without any toxic effect on the hepatic and renal tissues [60]. It has been proposed that curcumin and its metabolites function as peroxisome proliferator-activated receptor (PPARγ) ligands, thus explaining its action as a hypolipidemic agent [61].

Increased secretion of inflammatory cytokines is thought to be involved in pathogenesis of numerous aging diseases, and clinical trials strongly support a link between increased levels of TNF-α and IL-6 with menopause-related bone loss and cardiovascular diseases [62–64]. Furthermore, excess of adiposity is associated with greater systemic inflammation [51]. Using the OVX model, Wang et al. 2012 [65] did observe that estrogen would be protective against hepatocellular metastasis by reducing IL-6 levels. In addition, Kireev et al. 2010 showed that ovariectomy is associated with an increase in proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and reduction of the anti-inflammatory IL-10, leading to increases in lipoperoxidation in liver tissues [39]. It has been described that curcumin may exert inhibitory actions on the levels of the proinflammatory cytokines TNF-α and IL-6, thus reducing inflammation and oxidative damage induced by cadmium intoxication [66]. In our model, IL-6 levels were slightly increased in serum samples of OVX rats and curcumin treatment could restore the IL-6 levels to sham values at higher doses. In fact, curcumin is a well-known inhibitor of transcription factors involved in cytokine production as NFkappaB and STAT-3 [67, 68], which could be underlying its anti-inflammatory action in different disease models. Last, OVX rats are described to present increases in ALT and AST levels with 21 days after surgery [69]. In our model (90 days after surgery), although ALT was not altered, the serum levels of aspartate aminotransferase (AST) not surprisingly were slightly increased by experimental menopause, suggesting the presence of chronic low-level tissue damage; curcumin was unable to rescue the normal patterns. Based on all aforementioned effects of curcumin upon oxidative, lipid, and inflammatory parameters herein and previously described in different preclinical and clinical models, one therefore could conclude that curcumin harbors some potential for decreasing the predisposition to cardiovascular disease and other complications associated with menopause and ageing.

Taken together, we showed that curcumin was able to minimize the alterations in IAT accumulation, IL-6 serum levels, lipid profile, and oxidative stress caused by sexual female hormone deprivation in OVX rats. We also observed that the nonenzymatic antioxidant potential of curcumin seems to be a key component of this response, at least regarding the oxidative stress parameters. Our results and previous work from other groups [2, 17, 19, 70, 71] collectively support a more in-depth clinical trial investigation on the curcumin potential and safety to treat menopausal patients aiming to ameliorate aging-related processes frequently accelerated by menopausal status.

Abbreviations

AAPH: 2,2-Azobis[2-amidinopropane]
ANOVA: Analysis of variance
AUC: Area under curve
CAT: Catalase
DNPH: Dinitrophenylhydrazine
DTNB: 5,5-Dithionitrobis 2-nitrobenzoic acid
GPx: Glutathione peroxidase
HDL: High-density lipoprotein
HRT: Hormone replacement therapy
IAT: Intestinal adiposity accumulation
LDL: Low-density lipoprotein
OVX: Ovariectomized
ROS: Reactive oxygen species
TRAP: Total reactive antioxidant potential
SOD: Superoxide dismutase
TBARS: Thiobarbituric acid reactive species
VLDL: Very low-density lipoprotein.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

The Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD 11/1893-7, and PRONEX 1000274), CAPES (PROCAD 066/2007), CNPq, and PROPESQ UFRGS supported this study. Maurilio da Silva Morrone was the recipient of a CNPq fellowship and Alfeu

Zanotto-Filho was the recipient of DOCFIX (CAPES/FAPERGS no. 09/2012CNPq) and CNPq (Universal 485758/2013-0) Grants.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3571614, 10 pages
http://dx.doi.org/10.1155/2016/3571614

Review Article

Sinapic Acid and Its Derivatives as Medicine in Oxidative Stress-Induced Diseases and Aging

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Received 29 October 2014; Revised 2 January 2015; Accepted 12 January 2015

Academic Editor: Matías Mosqueira

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) is an orally bioavailable phytochemical, extensively found in spices, citrus and berry fruits, vegetables, cereals, and oilseed crops and is known to exhibit antioxidant, anti-inflammatory, anticancer, antimutagenic, antiglycemic, neuroprotective, and antibacterial activities. The literature reveals that sinapic acid is a bioactive phenolic acid and has the potential to attenuate various chemically induced toxicities. This minireview is an effort to summarize the available literature about pharmacokinetic, therapeutic, and protective potential of this versatile molecule in health related areas.

1. Introduction

As a result of metabolic processes, there is continuous production of reactive oxygen species (ROS), such as hydroxyl radicals [1], in human body. Various biological functions like antimicrobial activity depend on ROS [2]. In normal physiological state, ROS production in body is balanced by scavengers "antioxidants." This equilibrium is disturbed in pathological conditions owing to overproduction of ROS, but comparatively low concentration of endogenous antioxidants in body. It results in the reaction between ROS and intra- and extracellular species leading to emergence of oxidative stress which causes various ailments like aging, cancer, and necrosis [3]. To tackle the oxidative stress, it is needed to restore balance between ROS and antioxidants by administering exogenous antioxidants, for example, hydroxycinnamic acids.

Hydroxycinnamic acids belong to the class of phenolic acids with bioactive carboxylic acids; the class mainly includes caffeic acid, ferulic acid, and sinapic acid [4, 5]. According to literature, these compounds are capable of donating their phenoxyl hydrogen atom for neutralization of free radical species leading to production of corresponding phenoxyl radicals. These radicals are weekly reactive due to delocalization of unpaired electrons. Resultantly, the inhibition of dangerous radicals is useful for human health owing to antiaging potential of these phenolic acids [6, 7].

Sinapic acid exists in both free and ester form; some esters are sinapoyl esters, sinapine (sinapoylcholine), and sinapoyl malate [8, 9]. Sinapic acid is a phytochemical found in various edible plants such as spices, citrus and berry fruits, vegetables [10–12], cereals, and oilseed crops [13, 14]. Sinapic acid has been tested and reported against various pathological conditions such as infections [15], oxidative stress [16], inflammation [17, 18], cancer [19], diabetes [20], neurodegeneration [21], and anxiety [22]. Some derivatives of sinapic acid, such as sinapine, 4-vinylsyringol, and syringaldehyde, have also been studied for acetylcholinesterase inhibition [23, 24], antimutagenicity [25], and antioxidant activity [26], respectively. 4-Vinylsyringol, a decarboxylated sinapic acid, is also termed as canolol. The term "canolol" was coined by Wakamatsu et al. due to its source, canola oil [25]. The structural formulas of sinapic acid and its derivatives are shown in Figure 1 [23, 27, 28]. The literature search does not show any extensive research on the biological features of sinapic acid and its derivatives. Those studies have been summarized in this brief review article so that the scientific community may pay more attention to the biological aspects of sinapic acid and its derivatives.

2. Pharmacokinetics of Sinapic Acid

Fruit and vegetable consumption can potentially decrease the risk of degenerative diseases which mainly attributed to

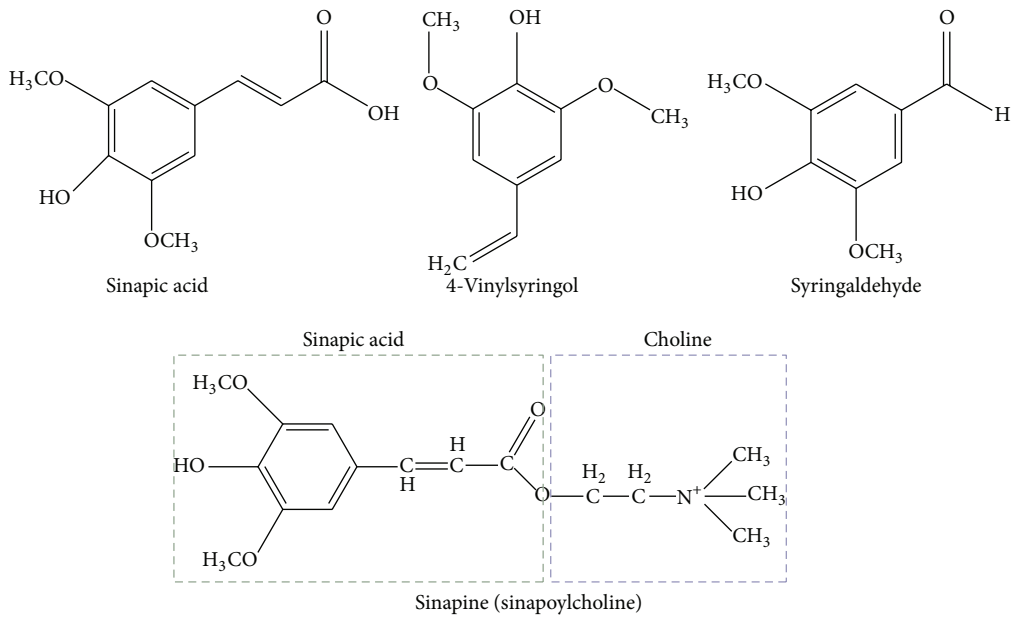

Figure 1: The structural formulas of sinapic acid and its derivatives (syringaldehyde, sinapine, and 4-vinylsyringol).

the phenolics present in them. Pharmacokinetic study helps to understand the role of these phenolics in human body. Serum albumin has been reported to be responsible for the transport of sinapic acid in blood due to its ability to bind with serum albumin through hydrophobic interaction and hydrogen-bonding [29, 30]. Maximum plasma-sinapic acid level has been described as 40 nM with a bioavailability of 3% of the total phenolics present in the nonprocessed cereal meal [31, 32]. Moreover, the small intestine was reported as the best place for absorption of orally administered sinapic acid through active Na^+ gradient-driven transport [33]. Plasma-sinapic acid level has also been quantified (1.5 μg/mL) after intake of cranberry juice in human by using GC-MS [34]. However, metabolism of sinapic acid takes place in the epithelium of the small intestine [35]; urine analysis, after sinapic acid ingestion in rats, showed the presence of sinapic acid, 3-hydroxy-5-methoxyphenylpropionic acid, methyl sinapate-sulfate, methyl sinapate-glucuronide, dihydrosinapic acid, 3-hydroxy-5-methoxycinnamic acid, and their acid-labile conjugates [35] and these are generated by the esterase activity of the intestinal microflora [32, 36]. Nature of these metabolites also indicates the possible metabolism of free and ester form of sinapic acid through phase I and II reactions in human small intestinal epithelium [37].

3. Antioxidant Activity

Reactive oxygen species (ROS) are continuously generated and are used in normal physiologically based activities [38]. Simultaneously, they are captured by different scavengers, known as antioxidants, to maintain their equilibrium in human body [39]. However, the overproduction of ROS destroys this equilibrium resulting in oxidative stress which is responsible for various pathological conditions, such as cancer, neurodegenerative disorders, and aging [40–42]. Polyphenols consist of four major classes of phytochemicals, that is, phenolic acids, flavonoids, stilbenes, and lignans [43], and behave as antioxidants, useful as anticancer, antiaging, and antimicrobial agents and scavengers of ROS produced in the body [44, 45]. Presence of methoxy- and hydroxyl-groups in the structure of polyphenols also improves their antioxidant ability [45, 46]. Sinapic acid belongs to this family of phenolics with remarkable antioxidant potential. Various modes of antioxidant activity of sinapic acid have been documented in the literature as described below.

3.1. DPPH$^\bullet$ Scavenging Potential. Sinapic acid is also known to show free radical scavenging ability against paramagnetic stable radical of *2,2*-diphenyl-*1*-picrylhydrazyl (DPPH$^\bullet$). According to the literature, the DPPH$^\bullet$ inhibition by 0.02 mM, 0.5 mM, and 0.3 mM of sinapic acid is 33.2% [8], 88.4% [47], and 50% [48], respectively. Moreover, 8-8′-bis-lactone-dimer of sinapic acid also shows DPPH$^\bullet$ scavenging activity but at concentrations higher than 200 μM [48].

Additionally, sinapic acid derivatives like sinapoyl glycosides are also reported for DPPH$^\bullet$ scavenging activity [49, 50]. However, these studies report the higher DPPH$^\bullet$ radical scavenging activity of sinapic acid as compared to its glycosides including sinapoyl glucose, sinapine, and 6-O-sinapoyl sucrose except methyl 2-O-sinapoyl-α-D-glucose and methyl 6-O-sinapoyl-α-D-glucose which showed a little higher activity than that of sinapic acid.

Synergism in DPPH$^\bullet$ scavenging activity of sinapic acid is also observed; however, comparatively higher antioxidant potential of rapeseed meal and oil extracts has been reported which contains 4-vinylsyringol (87% w/w) and sinapine (13% w/w) along with sinapic acid, in comparison with pure sinapic acid alone [54]. In addition, the DPPH$^\bullet$ scavenging activity of sinapic acid is also compared with its derivatives, for example, 4-vinylsyringol; however, DPPH$^\bullet$ scavenging activity of

sinapic acid (90.8%) was described to be higher than that of 4-vinylsyringol (78.7%) at a concentration of 1 mg/mL [54–56]. Moreover, another derivative syringaldehyde is also reported to show strong DPPH$^{\bullet}$ scavenging activity [26, 57].

3.2. $O_2^{\bullet-}$ Scavenging Potential. Superoxide anion radical ($O_2^{\bullet-}$) can suppress [4Fe-4S]-containing dehydratases and oxidize some compounds including leukoflavins, tetrahydropterins, and catecholamines. However, $O_2^{\bullet-}$ scavenging activity of sinapic acid has been found similar to that of 4-vinylsyringol (decarboxylated product of sinapic acid), which shows that the decarboxylation of sinapic acid does not modify its $O_2^{\bullet-}$ scavenging activity [56]. Moreover, an excellent $O_2^{\bullet-}$ scavenging activity of sinapic acid (IC50 = 17.98 mM) has been reported in comparison with Trolox used as an antioxidant (IC50 = 7.24 mM) [17]. In another study, $O_2^{\bullet-}$ inhibition was presented 35.52% by using 0.05 mM of sinapic acid [58], in both enzymatic (IC50 = 70.7 μM) and nonenzymatic (IC50 = 979.2 μM) $O_2^{\bullet-}$ generating systems. Moreover, the $O_2^{\bullet-}$ scavenging activity of sinapoyl glycosides is also reported; however, this study reports the lower $O_2^{\bullet-}$ radical scavenging activity of sinapic acid (IC50 = 90 mM) as compared to its glycoside, 6-O-sinapoyl sucrose (IC50 = 65 mM) [59].

3.3. $^{\bullet}$OH Scavenging Potential. Highly reactive hydroxyl radicals ($^{\bullet}$OH) have potential to damage their surroundings in living system [60, 61]. Sinapic acid has been reported as a good scavenger for $^{\bullet}$OH with an IC50 = 3.80 mM where ascorbic acid was used as standard showing IC50 = 5.56 mM [62]. Moreover, three ester derivatives of sinapic acid, methyl sinapate, β-D-(3,4-disinapoyl)fructofuranosyl-α-D-(6-sinapoyl)glucopyranoside, and 1,2-disinapoyl-β-D-glucopyranoside, have also shown comparable $^{\bullet}$OH scavenging activity [63].

3.4. Scavenging Potential against Other Free Radicals. Sinapic acid has been known for hydroperoxyl radical ($^{\bullet}$OOH) scavenging activity [64, 65]; however, 4-vinylsyringol, a derivative of sinapic acid, scavenges the $^{\bullet}$OOH more quickly than sinapic acid [65, 66].

Sinapic acid also possesses better ClO^- scavenging potential as compared to other hydroxycinnamic acids, that is, ferulic acid, chlorogenic acid, and *p*-coumaric acid. Sinapic acid has also been reported to be efficient nitric oxide radical ($^{\bullet}$NO) scavenger compared to the reference compound, that is, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt [17].

Peroxynitrite ($ONOO^-$) can potentially initiate apoptosis [65]. Sinapic acid has been described to perform better $ONOO^-$ scavenging activity by inhibiting 3-nitrotyrosine formation in protein (bovine serum albumin) through an electron donation mechanism as compared to standard antioxidants, that is, ascorbic acid, penicillamine, and tocopherol [65]; however, sinapic acid scavenging activity against $ONOO^-$ further increases in the presence of 25 mM Na_2CO_3, which contribute CO_2 for simulation of physiological environment [17, 67]. In addition, 4-vinylsyringol can also scavenge $ONOO^-$ [11].

3.5. Suppression of Lipid Peroxidation. Lipid peroxidation generates lipid hydroperoxides, which act as a source of lipid peroxyl (LOO$^{\bullet}$) and lipid alkoxyl (LO$^{\bullet}$) radicals [68]. In a comparative study, sinapic acid was compared with α-tocopherol and ferulic acid on the formation of hydroperoxides, and results showed that sinapic acid acts more efficiently to suppress the hydroperoxide formation by preventing the lipid oxidation in bulk methyl linoleate [16, 69]. Moreover, in another comparative study, the antioxidant potential of sinapic acid was compared with other antioxidants, that is, Trolox and butylated hydroxyanisole [70, 71]. Sinapic acid at a concentration of 500 μmol/kg has been found comparable in lipid peroxidation inhibition against Trolox and butylated hydroxyanisole; the results are even better than α-tocopherol. Similarly, the concentration-dependent inhibition of hydroperoxide formation by sinapic acid and sinapine was observed in purified rapeseed oil stored at 40°C in darkness; however, sinapine was found to be noneffective on hydroperoxide synthesis inhibition alone [49].

In another study, the prooxidant behavior of sinapine in rapeseed oil was reported and is attributed to its low solubility in oil [71]. An inverse relationship has been explained between the antioxidant property of sinapic acid and the concentration of tocopherols because sinapic acid may lose its function due to reaction with tocopherol radicals whose concentration got increased in elevated tocopherol level. Furthermore, an increased amount of sinapic acid is reported to produce less quantity of propanal (secondary oxidation product) at low tocopherol concentration and larger quantity at high levels. Concisely, sinapic acid can potentially play a role in the stability of oils containing small quantities of endogenous tocopherols [71].

Lipid peroxidation can be affected by sinapic acid derivatives. In a comparative study, 15% more antioxidant activity of 4-vinylsyringol has been observed against sinapic acid in a nonpolar system; however, a diminished activity of 4-vinylsyringol is reported in polar environment [60]. In another study, 4-vinylsyringol was found to be a more potent RCOO$^{\bullet}$ scavenger than vitamin C and α-tocopherol [25]. Moreover, a promising peroxyl radical scavenging activity of syringaldehyde has been reported in crocin method, involving a competition between antioxidant and crocin to bind with the peroxyl radical; a similar antioxidant activity of syringaldehyde has been published in bulk oil and lecithin liposome [29]. Similarly, in another study, liposome (lipid membrane model) was used to assess lipid peroxidation capacity of sinapic acid and was found to be an excellent protective agent for the membrane, especially when added at the liposome synthesis stage [72]. Furthermore, linoleic acid-based lipidic model was used and the diferential scanning calorimetric analysis of sinapic acid, its alkyl esters (methyl, ethyl, propyl, and butyl sinapates), and reference antioxidant (Trolox) was conducted to compare their peroxyl radical scavenging activity. The results revealed that the test substances had reducing abilities comparable to that of reference compound suggesting sinapic acid and its alkyl esters as promising antioxidants [73].

(1) Inhibition of Oxidation of Low-Density Lipoprotein (LDL). Low-density lipoprotein (LDL) oxidation has been found responsible for atherosclerosis development [74]. In a comparative study, sinapic acid showed higher (28%) antioxidant activity than 4-vinylsyringol (7.5%), in a LDL model system at a concentration of 10 μM [54]. Moreover, peroxyl radicals produced through Cu^{+2}-mediated oxidation of human LDL has been studied *in vitro*, and in terms of Trolox equivalent (TE) the following order has been observed with decreasing lipid peroxidation inhibition capacity: sinapic acid > caffeic acid > ferulic acid [75]. Additionally, concentration-dependent inhibition of LDL oxidation by sinapic acid has also been reported which can be attributed to its chelating power with Cu^{+2} [76–78]. Similarly, Cu^{+2}-mediated peroxidation of human LDL and peroxyl radical can attack on erythrocyte membranes resulting in AAPH-(2,2′-azobis(2-amidinopropane) dihydrochloride-) induced hemolysis; however, ethyl sinapate at a concentration of 10 μM was found to act more effectively (76%) and suppressed the LDL oxidation than sinapic acid (59%). Moreover, in terms of IC50 values, for 50% AAPH-induced hemolysis inhibition capacity, the studied hydroxycinnamates can be configured in the following decreasing order: sinapic acid (IC50 = 4.5 μM) > ethyl sinapate (IC50 = 5.0 μM) > caffeic acid (IC50 = 7.2 μM) > ferulic acid (IC50 = 6.8) [79].

3.6. Anti-Inflammatory and Anticarcinogenic Properties. Nitric oxide synthase, tumor necrosis factor-α (TNF-α), cyclooxygenase-2, and interleukin-1β are proinflammatory mediators and their expression by ROS and activated nuclear factor-kappa B (NF-κB) in macrophages cause inflammation [19]. Inflammation produced by incorrect regulation of NF-κB disturbs immunity and can produce autoimmune diseases, that is, cancer [80]; however, a suppressive action of sinapic acid on NF-κB has been reported in the literature [18, 81]. Moreover, sinapic acid has been described to have time-dependent and dose-dependent suppressive effect on colon and breast cancer cells (human breast cancer T47D cell line) and this inhibitory action is attributed to its antiproliferative feature [19, 82]. Furthermore, proinflammatory mediators are reported to be suppressed by 4-vinylsyringol [83]. In another study, sinapic acid and its alkyl esters were evaluated for anti-inflammatory activity in carrageenan-induced rat paw oedema model and an excellent anti-inflammatory activity of isopentyl sinapate was reported in comparison to other esters [84].

The ROS are generated due to *Helicobacter pylori* (*H. pylori*) infection, which attack and damage macromolecules, including DNA, fats, and proteins. Therefore, damaged DNA produces 8-hydroxy-2′-deoxyguanosine (8-OHdG); however, its level can be reduced by 4-vinylsyringol treatment [85]. In Mongolian gerbils infected with *H. pylori*, oral administration of 4-vinylsyringol (0.1% in the diet) has been described to efficiently suppress the gastric malignancy [83]. In an *in vivo* study, the protective effect of canolol against inflammatory bowel disease and colitis associated carcinogenesis via inhibition of inflammatory cytokines and oxidation stress was observed [86]. Same effect of canolol has also been reported in human retinal pigment epithelium (ARPE-19) cell line through an extracellular signal regulated kinase-mediated antioxidative pathway [87]. Additionally, canolol has also been found capable of inhibiting bacterial (*H. pylori*) mutation by protecting DNA damage from $ONOO^-$, a highly oxidative chemical [88]. Peroxynitrite radicals ($ONOO^-$) can cause DNA cleavage resulting in mutation [80]. Sinapic acid and 4-vinylsyringol have been studied for their antimutagenic characteristics and it was reported that both hydroxycinnamic acids have potential and dose-dependent antimutagenicity character, possibly through $ONOO^-$ scavenging action [25].

3.7. Anxiolytic Property. Elevated plus-maze (EPM) and hole-board test are generally used for anxiolytic studies in mice [89]. These tests were employed to study the behavior of sinapic acid and it was found that it increases the time spent in open arms significantly and also increases percentage entry in open arms [22]. Moreover, due to no side effects of sinapic acid even after its prolonged use and its selective anxiolytic features in comparison to existing anxiolytic agents [90, 91] a targeted research is required to use sinapic acid preferably in anxiety conditions.

3.8. Neuroprotective Property. Few studies are available in the literature, which elaborate the neuroprotective function of sinapic acid and its derivatives. Sinapine, a derivative of sinapic acid, during *in vitro* studies has been found to have dose-dependent acetylcholine (ACh) esterase inhibitory activity; moreover, sinapine and ACh both contain quaternary nitrogen to bind reversibly to specific region on AChE in a competitive mode [23, 24]. Furthermore, activity of sinapine is more effective in the cerebral homogenate than in blood serum of rats with IC50 values of 3.66 μM and 22.1 μM, respectively [23].

3.9. Antimicrobial Activity. Emergence of drug resistance in microbes is a fast growing issue in health sciences. Drugs available in market are constantly facing the problem of drug resistance, and therefore new drug molecules are required to counter this threat [92–94]. In *in vivo* studies, conducted on various Gram-positive and Gram-negative bacteria, 97–99% eradiation of various microorganisms was observed indicating significant antibacterial potential of sinapic acid [95]. Table 1 carries minimum inhibitory concentrations (MIC) of sinapic acid against various bacterial strains observed during *in vitro* studies. In another study, sinapic acid was reported to have the potential to selectively kill the pathogenic bacteria leaving beneficial lactic acid bacteria alive that can resist and metabolize the sinapic acid [14]. Moreover, syringaldehyde has been described for its antifungal potential against *Candida guilliermondii* [96].

3.10. Antihyperglycemic Activity. Antihyperglycemic activity of sinapic acid was reported using induced-hyperglycemic *in vivo* model [97, 98] by intraperitoneal administration (45 mg/kg body weight) of streptozocin (STZ, a compound which destroys the insulin secreting pancreatic-cells). Subsequently, both normal and hyperglycemic rats were studied for certain biochemical markers (blood urea, serum

Table 1: Minimum inhibitory concentrations (MIC) of sinapic acid against bacteria strains.

Number	Reference	Bacterial strain	Minimum inhibitory concentrations (MIC)
1	Barber et al., 2000 [51]	*Bacillus subtilis*	0.45 g/L
		E. coli	0.89 g/L
		Pseudomonas syringae	1.79 g/L
2	Tesaki et al., 1998 [52]	*E. coli*	0.49 g/L
		Salmonella enteritidis	0.45 g/L
		Staphylococcus aureus	0.43 g/L
3	Engels et al., 2012 [14]	*Bacillus subtilis*	0.3 g/L
		E. coli	0.7 g/L
		Staphylococcus aureus	0.3 g/L
		Listeria innocua	0.3 g/L
		Listeria monocytogenes	0.2 g/L
		Pseudomonas fluorescens	0.6 g/L
4	Johnson et al., 2008 [53]	*Salmonella enteric*	Not mentioned

creatinine, uric acid, total protein, albumin, and A/G ratio) and hepato- and nephron-histopathology; however, altered values of the studied biochemical markers and pathological features came to normal state after treating the rats with sinapic acid (15 mg/kg and 30 mg/kg) for 35 days; therefore, sinapic acid may have dose-dependent hepato- and nephron-protective effects in STZ-induced-hyperglycemic rats. In addition, sinapic acid can be further studied for applications in diabetic states.

3.11. Antilipidemic Activity. One of the causative agents of cardiovascular diseases, such as myocardial infarction, is abnormal lipid profile of a subject [99]. In this context, a study involving antilipidemic activity of sinapic acid has been proposed by Roy and Prince [100]. They administered isoproterenol (100 mg/kg body weight) to rats for inducing myocardial infarction, and then the myocardial infarcted rats (rats with raised levels of cardiac troponin-T, cholesterol, triglycerides, and free fatty acids in serum and higher ST-segments in electrocardiogram) were studied to evaluate the shielding effects of sinapic acid [100, 101]. Recently, during *in vivo* studies performed on rats, an orally administered sinapic acid dose (12 mg/kg body weight) showed shielding effects on hypertrophy of heart, abnormal lipid levels, and electrocardiogram; furthermore, pre- and cotreatment with sinapic acid standardized the levels of myocardial infarction parameters which further elaborate antioxidant potential as well as antilipidemic activity of sinapic acid. Moreover, lysosomal dysfunction in isoproterenol-induced myocardial infarcted rats can also be cured by sinapic acid [102, 103]. These evidences elaborate the antilipidemic activity of sinapic acid.

3.12. Toxicities and Sinapic Acid

3.12.1. Isoproterenol-Induced Myocardial Infarction. Isoproterenol (ISO), a synthetic catecholamine, can cause the lysosomal lipid peroxidation [103] followed by the production of various lysosomal enzymes, such as lysosomal hydrolases [104], which produce myocardial infarction (MI) [105]. The ISO-mediated lysosomal dysfunction in rats suffering from MI can be overcome by oral administration of sinapic acid in rats at a concentration of 12 mg/kg body weight. This effect is evident from the changes in lysosomal lipid peroxidation, serum lysosomal enzymes, heart homogenate, lysosomal fraction, and myocardial infarct size calculated before and after simultaneous intake of sinapic acid. The treatment with sinapic acid notably suppressed the ISO-provoked release of lysosomal enzyme activity, normalized all the biochemical parameters, and diminished myocardial infarct size [102]. The membrane stabilizing features and free radical scavenging potential of sinapic acid can be the possible mode for the above-mentioned activities [104]. Thus, sinapic acid may be employed as a protective agent in MI [102].

3.12.2. Kainic Acid-Induced Hippocampal Neuronal Damage. Neuron depolarization and extreme calcium influx by kainic acid (KA, a nonselective agonist of AMPA and kainate receptors) generate the free radicals, activate the nitric oxide synthase (NOS), and initiate the mitochondrial dysfunctioning [106, 107]; it results in glutamatergic activation- and oxidative stress-mediated inflammation and neurodegeneration [108, 109]. Sinapic acid has been evaluated due to its GABA receptor agonistic feature and free radical scavenging potential, during *in vivo* study in rats, for new glutamate receptors blockers and radical scavengers for neuroprotection. An oral administration of sinapic acid at a concentration of 10 mg/kg body weight was reported to efficiently treat the KA-induced brain damage. However, the neuroprotective effect of sinapic acid was attributed to its radical scavenging potential and anticonvulsive activity through GABA receptor activation [110, 111].

3.12.3. Amyloid β $(A\beta)_{1-42}$ Protein-Induced Alzheimer's Disease. Neuroprotective effect has been studied in mouse suffering from Alzheimer's disease, a neurological disease involving cognitive impairment [112, 113], and was induced in mouse by amyloid β $(A\beta)_{1-42}$ protein injected into the hippocampus. Simultaneously, after injecting $A\beta_{1-42}$ protein; an oral administration of sinapic acid was started with a dose of 10 mg/kg body weight per day. $A\beta_{1-42}$ protein-induced effects were reported to be abolished by the use of sinapic acid, including elevated expression of iNOS, glial cells, and nitrotyrosine. Similarly, in rats suffering from cognitive impairment induced by scopolamine, sinapic acid shows better results [21]. Moreover, promising neuroprotective effects were reported in rodents, where sinapic acid suppressed potassium cyanide-induced hypoxia and scopolamine-induced memory impairment [114].

3.12.4. Carbon Tetrachloride and Dimethylnitrosamine-Induced Acute Hepatic Injury. Carbon tetrachloride (CCl_4) can produce the proinflammatory mediators causing an acute hepatic inflammation and its associated pathologies [115]. Sinapic acid has been described for its potential to revert the CCl_4 intoxication of liver by oral administration of 10 or 20 mg/kg body weight in rats. Moreover, the sinapic acid treatment notably suppressed the CCl_4-provoked release of proinflammatory mediators by scavenging the free radicals [116]. Sinapic acid has the potential to be used as a remedial approach for inhibiting hepatic inflammation [117–119]. Moreover, sinapic acid has also effectively treated dimethylnitrosamine-induced hepatotoxicity [120].

3.12.5. Corticosterone-Induced Toxicity. Corticosterone administration in broiler chickens can produce oxidative stress, which retards the animal growth. Corticosterone-induced toxicity can be countered by the use of 4-vinylsyringol to preserve the tissue α-tocopherol level and to reduce the lipid peroxidation in the animal. Therefore, 4-vinylsyringol can also be added to broiler chicken feed to exert effective antioxidant effect [121].

3.12.6. tert-Butyl Hydroxide-Induced Toxicity. Antioxidant potential of 4-vinylsyringol against *t*-BH- (*tert*-butyl hydroxide-) mediated production of ROS, which induce the human retinal epithelial cell death, has been studied and compared to a standard antioxidant, *N*-acetyl cysteine; however, it has been reported that 4-vinylsyringol at a concentration of 200 μM exerts more protective effect than the reference compound [122].

3.12.7. Arsenic-Induced Toxicity. Arsenic can cause pathological conditions like cancer and diabetes on long-term exposure [123, 124] by disturbing various enzymatic reactions in liver resulting in generation of ROS (superoxide, peroxyl radicals, and hydrogen peroxide) which produce hepatotoxicity. During *in vivo* study, arsenic-induced toxicity can be shielded by the use of sinapic acid and is mainly attributed to its metal-chelating potential [62]. Therefore, sinapic acid administration can help in avoiding arsenic-induced toxicity [125].

3.13. Toxicity Study of Sinapic Acid. The toxicity profile of sinapic acid has been reported to be considerably low in broiler chickens; no effect on the serum activity of creatine kinase and lactate dehydrogenase has been reported and observed. Therefore, it is not harmful to various body organs of the animal [126].

4. Conclusion

Sinapic acid and its derivatives, particularly 4-vinylsyringol, are interesting natural compounds that has potential to express various health benefits, that is, antioxidant, anti-inflammatory, anticancer, antimutagenic, antiglycemic, neuroprotective, and antibacterial activities. Moreover, further extensive and targeted studies are required to explain relationship between the plasma concentrations of sinapic acid, in therapeutic dose, and the therapeutic outcomes.

Conflict of Interests

There is no conflict of interests over the contents of this paper.

Acknowledgments

This work was supported by the Key Laboratory of Biorheological Science and Technology (Chongqing University) and the General Program of National Natural Science Foundation of China [Grant no. 81202202].

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 6038798, 8 pages
http://dx.doi.org/10.1155/2016/6038798

Research Article

Higher Urinary Levels of 8-Hydroxy-2′-deoxyguanosine Are Associated with a Worse RANKL/OPG Ratio in Postmenopausal Women with Osteopenia

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Received 5 March 2015; Revised 12 June 2015; Accepted 21 June 2015

Academic Editor: Marta Ruiz-Ortega

Postmenopausal osteoporosis (PO) is a major public health issue which affects a large fraction of elderly women. Emerging *in vitro* evidence suggests a central role of oxidative stress (OxS) in postmenopausal osteoporosis (PO) development. Contrariwise, the human studies on this topic are still scarce and inconclusive. In the attempt to address this issue, we sought to determine if OxS, as assessed by 8-hydroxy-2-deoxyguanosine (8-OHdG), may influence the level of receptor activator of nuclear factor-κb ligand (RANKL)/osteoprotegerin (OPG) ratio (a central regulator of bone metabolism) in a sample ($n = 124$), including postmenopausal women with osteoporosis, osteopenia and normal bone mass density (BMD). The most striking result that emerged in our study was the independent and positive (beta = 0.449, $p = 0.004$, and $R^2 = 0.185$) association between the OxS marker and RANKL/OPG ratio which was found in osteopenic but not in the other 2 sample groups. If confirmed by longitudinal studies, our findings would suggest that OxS is implicated in the derangement of bone homeostasis which precedes PO development. In line with these considerations, antioxidant treatment of postmenopausal women with moderately low BMD might contribute to preventing PO and related complications.

1. Introduction

Postmenopausal osteoporosis (PO) is a disease characterized by gradual thickening of bone which leads to a reduced bone mass and an increased risk of fragility fractures [1]. PO occurs mostly because of the decline of oestrogens (especially 17β-estradiol, E2) levels produced by cessation of ovarian sex steroid secretion [2]. This endocrine change has major effects on bone remodelling, leading to derangement of the balance between resorption and formation activities of osteoclasts and osteoblasts, respectively [3]. A vast body of evidence suggests that the effects of E2 on bone are mediated by the mutual interaction of receptor activator of nuclear factor-κb (RANK), its ligand (RANKL), and osteoprotegerin (OPG) [4–6].

RANKL exists in both soluble and membrane-bound forms and is expressed by many cell types in bone and bone marrow, including osteoblasts, osteocytes, and activated

lymphocytes [7]. Both forms of this protein promote, although with different effectiveness, bone resorption by binding to RANK localized in both precursors and mature osteoclasts, inducing their formation and activation [8]. Osteoblasts are also one of the main sources of OPG which, acting as a decoy receptor that competes with RANKL for RANK, is able to inhibit osteoclastic proliferation and differentiation. The key-role of RANK/RANKL/OPG axis in the pathogenesis of PO has been largely confirmed in preclinical as well as clinical studies which showed that an increase in RANKL-to-OPG ratio can stimulate excessive bone resorption, whereas its decrease can favor bone neoformation [5, 8, 9].

Given the centrality of RANK/RANKL/OPG system in bone metabolism, the systemic factors able to regulate the concentration of these cytokines have acquired great scientific and clinical relevance in recent years. Besides E2 (and calciotropic hormones), there are also a series of inflammatory interleukins (e.g., IL-1 and IL-6) that can alter both RANKL and OPG secretion and activity [5, 6]. Notably, it is now well recognized that the events characterized by burst of these interleukins (i.e., inflammation), but also by physiological decline of E2 (i.e., menopause), are associated with systemic oxidative stress (OxS) [10–12]. This condition can potentially cause the damage against all types of biological molecules and is widely believed to be deeply implicated in the onset and progression of aging-related diseases, including PO [13–16]. More specifically, OxS seems to be a prodromic feature of PO, as suggested by several lines of evidence showing that E2-withdrawal might weaken bone defense against injury induced by reactive oxygen species (ROS) [15, 17, 18]. ROS are, indeed, generated in activated osteoclasts via nicotinamide adenine dinucleotide phosphate oxidase (NOX) and are thought to actively contribute to bone homeostasis "short-circuit" leading to osteoporotic damage [19].

Given these considerations, it was tempting to hypothesize that OxS could play a role in the modulation of RANKL/RANK/OPG triad. On these bases, the aim of the present population-based study was to investigate the potential association between systemic OxS, as assessed by a reliable marker of oxidative damage (urinary 8-hydroxy-2′-deoxyguanosine, 8-OHdG), and serum level of RANKL/OPG ratio in a population sample including healthy, osteopenic, and osteoporotic postmenopausal women.

2. Materials and Methods

2.1. Subjects. The subjects examined in this study were enrolled among women undergoing bone densitometry evaluation at the Menopause and Osteoporosis Centre of University of Ferrara (Ferrara, Italy), as described elsewhere [20]. The present population-based study was conducted in accordance with the Declaration of Helsinki (World Medical Association, http://www.wma.net) and it was approved by the human research ethics committee of the university. The women were included in the study sample if they were in postmenopausal status, defined as amenorrhea for at least 1 year [21].

Exclusion criteria were use of exogenous sexual hormones (including vaginal estrogens), supplementation with nutritional antioxidants (such as vitamins E, C, and A, beta-carotene, and selenium), vegetarian and vegan diet, chronic diseases (such as diabetes, malabsorption, and cardiovascular disease), or being not diagnosed with a chronic disease, but taking medications (antiobesity medications, thyroid hormones, diuretics, antihypertensive, anticholesterol drugs, etc.).

One hundred twenty-four subjects were found to be eligible and were enrolled in the study after signing an informed consent. Body weight, height and waist circumference were assessed in each enrolled subjects by trained personnel.

2.2. Biochemical Assays. Fresh blood samples were obtained from antecubital vein from all subjects between 8.30 and 10.00 am, after fasting for at least 8 h. After 30 minutes of incubation at room temperature (RT), blood samples were centrifuged (3000 g for 10 min), and the obtained serum was stored at −80°C until analysis. Commercially available Enzyme-Linked Immunosorbent Assays (ELISAs) kits were performed, according to the manufacturer's instructions.

Serum level of total (free plus bound) soluble RANKL was assayed by Human sRANKL (total) ELISA (catalog number RD193004200R, purchased from BioVendor Research and Diagnostic Products, Modrice, Czech Republic). In brief, standards, quality controls, or samples (100 μL each) were incubated in microplate wells precoated with monoclonal anti-human sRANKL antibody. After a 16–20-hour incubation (at 2–8°C), the plate was washed and incubated for 60 minutes at RT with biotin labelled polyclonal anti-human sRANKL antibody. After a further washing step, streptavidin-HRP conjugate was added and incubated for 60 minutes (RT). Then the plate was rewashed and the remaining conjugate was allowed to react with the substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB). After stopping the reaction, the plate was read at 450 nm. The concentration of RANKL in the serum samples was estimated from the standard curve and expressed as pmol/L (detection limit: 0.4 pmol/L). The intra-assay CV was 9.3%, whereas the interassay CV was 11.0%.

Serum concentration of OPG was detected by OPG ELISA kit (catalog number EK0480, Boster Biological Technology Co., Ltd., China). One hundred μL of either standards or properly diluted serum samples was added into anti-human OPG antibody precoated wells. After incubation (90 minutes at 37°C), biotinylated anti-human OPG antibody was added into each well and the plate was reincubated at the same temperature for a shorter time interval (60 minutes). Following the first washing step, a solution containing avidin, biotin, and peroxidase was added into each well of the plate which was then incubated for further 30 minutes (37°C) and thoroughly washed one more time. Afterwards, TMB color developing agent was added and, eventually, the absorbance was read at 450 nm. The serum concentration of OPG was estimated from the standard curve and expressed as pmol/L (detection limit: 1 pmol/L). The intra-assay CV was 5.3%, whereas the interassay CV was 7.0%.

Serum concentration of bone-specific alkaline phosphatase (BAP) was detected by OCTEIA Ostase BAP immunoenzymometric assay (catalog number AC-20F1, purchased by Immunodiagnostic Systems Ltd., Boldon, UK). Fifty μL of either standards, controls, or serum specimens was pipetted into streptavidin precoated wells and subsequently mixed with a biotin-labelled BAP-specific monoclonal antibody. After incubation (1 hour at RT), substrate reagent solution (i.e., p-nitrophenyl phosphate) was added into each well. A further incubation (15 minutes at RT) was followed by the addition of stop solution into each well. The absorbance was finally read at 405 nm (subtracting blank reading at 650 nm). The serum concentration of BAP was estimated from the standard curve and expressed as μg/L (detection limit: 0.7 μg/L). The intra-assay CV was 4.1%, whereas the interassay CV was 5.5%.

Serum concentration of C-terminal telopeptides of Type I (CTX-1) was measured by serum Cross-Laps ELISA kit (catalog number AC-02F1, purchased by Immunodiagnostic Systems Ltd., Boldon, UK). Briefly, 50 μL of either standards, control, or serum samples was pipetted into streptavidin precoated wells followed by the addition of the antibody solution (containing biotinylated monoclonal murine antibody plus monoclonal murine antibody conjugated with peroxidase). After 2 hours of incubation at RT, wells were washed and then chromogenic substrate (TMB) solution was added. Measurement of the absorbance at 450 nm with 650 nm as reference was made within two hours after the addition of the stop solution. The concentration of CTX-1 in the serum samples was obtained by standard curve and was expressed as ng/mL (detection limit: 0.020 ng/mL). The intra-assay CV was 2.2%, whereas the interassay CV was 7.7%.

High sensitivity C-reactive protein (Hs-CRP) serum concentration was assessed by commercial kit Hs-CRP the EiAsyTM Way (catalog number CAN-CRP-4360, purchased from Diagnostics Biochem Canada Inc., Dorchester, CAN). Twenty μL of either each calibrator, control, or properly diluted serum sample was pipetted into mouse anti-CRP precoated wells. After 30 minutes of incubation (RT) followed by a washing step, anti-CRP monoclonal conjugated with horseradish peroxidase was added into each well. After 15 minutes at RT, followed by a washing step, TMB substrate solution was added and, eventually, the absorbance was read at 450 nm within 20 minutes after the addition of the stop solution. The concentration of Hs-CRP in the serum samples was estimated from the standard curve and expressed as ng/mL (detection limit: 10 ng/mL). The intra-assay CV was 9.5%, whereas the interassay CV was 9%.

Urine concentration of 8-OHdG was detected by competitive 8-OHdG EIA kit (catalog number SKT-120-96, purchased from StressMarq Biosciences Inc., Victoria, BC, Canada). Fifty μL of either standard or properly diluted urine specimens was added into wells precoated with goat anti-mouse IgG. Afterwards, 2 equal volume aliquots of 8-OHdG-acetylcholinesterase conjugate and 8-OHdG monoclonal antibody were added to each well. After an overnight incubation (4°C) Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] was added to each well. The absorbance was finally read at 405 nm. The concentration of 8-OHdG in the urine samples was obtained by standard curve and was expressed as ng/mL (detection limit: 0.033 ng/mL). The intra-assay CV was 7.8%, whereas the interassay CV was 6.4%. The 8-OHdG concentration was normalized to urinary creatinine concentration and expressed as ng/mg creatinine.

Urinary creatinine determination was performed by a picric acid method [22]. Briefly, 50 μL of either standard or properly dilute urine samples was added into microplate well and mixed with 200 μL of a working solution containing 25 mM picric acid (purchased form Sigma-Aldrich, St. Louis, MO, USA) and 130 mM NaOH. The 490 nm absorbance was read after 30 minutes of incubation at RT and the obtained concentration was expressed as mg/dL (detection limit: 0.1 mg/dL). The intra-assay CV was 5.3%, whereas the interassay CV was 7.6%.

All the above ELISAs were assayed by a Tecan infinite (M200 Tecan Group Ltd., Männedorf, Switzerland) microplate spectrophotometer.

2.3. Bone Densitometry Assessment. Areal bone density was assessed at lumbar spine, hip, and total body by Discovery dual energy X-ray absorptiometry scanner (Hologic Inc., Bedford, MA). PO was diagnosed when BMD T-score (the number of standard deviations below the average for a young adult at peak bone density) was lower than 2.5 standard deviations from BMD peak at either femoral neck or lumbar spine, according to WHO guidelines [23]. In accordance with these criteria, women with T-score at either skeleton area between −2.5 and −1.0 were classified as osteopenic and those with a value higher than −1.0 as normal.

2.4. Statistical Analysis. SPSS 18.0 for Windows (IBM, Chicago, IL, USA) was used for statistical analysis. All variables were first analyzed for the normal distribution by the Kolmogorov-Smirnov and the Shapiro-Wilkinson test. Differences between groups were checked by one way analysis of variance (ANOVA) and Kruskal-Wallis for normally and non-normally distributed variables, respectively. Univariate analysis (by Pearson's or Spearman's test, depending on the distribution of the variable) was performed to check the associations between selected variables. Simple and multiple linear regression analysis were performed using base-10 logarithm transformed values of RANKL, OPG, RANKL/OPG, and 8-OHdG. We used log-transformed variables for these analyses to meet the assumption of normality of regression residuals. A two-tailed probability value <0.05 was considered statistically significant.

3. Results

The main characteristics of the 124 postmenopausal women enrolled in the present study are shown in Table 1. Osteoporotic and osteopenic women were older ($p = 0.009$) and presented lower BMI ($p = 0.04$) and waist circumference ($p = 0.03$) compared to those with BMD values within normal range. In accordance with the diagnostic criteria, total hip, neck, and lumbar spine BMD, as well as the correspondent T-score values, were significantly ($p < 0.01$)

Table 1: Principal characteristics of normal, osteopenic, and osteoporotic postmenopausal women.

	Normal BMD ($n = 25$)	Osteopenia ($n = 59$)	Osteoporosis ($n = 40$)	Statistics p
Age, yr	54.0 ± 4.2	56.2 ± 4.5	57.7 ± 4.7	0.01*
Years since menopause, yr	3 (1–9)	5 (2–10)	7 (4–12)	0.09#
BMI, kg/m^2	24.2 (23.0–27.4)	23.1 (21.5–27.3)	23.2 (22.1–26.7)	0.16#
Waist circumference, cm	85.9 ± 10.9	83.2 ± 8.4	82.5 ± 8.9	0.05*
DXA parameters				
L. spine BMD, g/cm^2	1.10 ± 0.91	0.92 ± 0.11	0.83 ± 0.09	<0.01*
L. spine T-score	0.1 (−0.6 to 0.3)	−1.8 (−2.1 to −1.2)	−2.5 (−2.8 to −1.8)	<0.01#
F. neck BMD, g/cm^2	0.81 (0.79–0.86)	0.67 (0.63–0.71)	0.62 (0.57–0.64)	<0.01#
F. neck T-score	−0.3 (−0.6 to 0.0)	−1.6 (−2.0 to −1.2)	−2.1 (−2.5 to −1.8)	<0.01#
Total hip BMD, g/cm^2	0.93 ± 0.08	0.81 ± 0.07	0.74 ± 0.07	<0.01*
Total hip T-score	−0.1 (−0.4 to 0.4)	−1.1 (−1.5 to −0.8)	−1.5 (−2.0 to −1.2)	<0.01#
Biochemical markers				
Hs-CRP, mg/L	1.5 (0.6–3.7)	1.1 (0.6–2.1)	1.2 (0.4–2.6)	0.54
CTX-1, ng/mL	0.47 ± 0.21	0.46 ± 0.39	0.52 ± 0.30	0.47*
BAP, μg/L	30.3 ± 1.3	31.0 ± 1.0	25.1 ± 1.3	0.24*
RANKL, pmol/L	270 (201–362)	255 (157–347)	281 (166–365)	0.18#
OPG, pmol/L	8.1 (7.3–10.6)	11.0 (6.9–16.6)	12.0 (5.7–18.6)	0.78#
RANKL/OPG	31.0 (18.2–64.1)	20.1 (11.5–60.1)	21.2 (11.4–34.2)	0.31#
8-OHdG, ng/mg creatinine	143 (109–189)	154 (109–208)	159 (112–212)	0.35#

Data presented are expressed as mean ± standard deviation for normally distributed variables; median (interquartile range) for not normally distributed variables.

* p value by Kruskal-Wallis; # p value by ANOVA.

BMI: body mass index; BMD: bone mass density; L.: lumbar; F.: femoral; Hs-CRP: high reactivity C-reactive protein; CTX-1: C-terminal telopeptide of type I collagen; BAP: bone-specific alkaline phosphatase; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa-B ligand; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

higher in controls with respect to osteopenic and osteoporotic women. In contrast, serum level of Hs-CRP, RANKL, OPG, RANKL/OPG ratio, CTX-1, BAP, and urinary level of 8-OHdG did not significantly vary among the three sample groups.

The possible association of 8-OHdG with the other biochemical markers and BMD values was initially checked by simple correlation analysis (Table 2). From this test it emerged that the DNA damage marker was significantly correlated only with RANKL ($p = 0.003$) and RANKL/OPG ratio ($p = 0.002$).

Afterwards, we checked the association between 8-OHdG and the two cytokines within each sample group (Table 3). As displayed in the table and in Figure 1, the OxS marker resulted to be significantly and positively correlated with RANKL ($p = 0.005$) and RANK/OPG ($p = 0.004$) merely in the osteopenic group, with a percentage of variance explained equal to 18.0 and 18.2%, respectively. Of note, the linear standardized coefficient for the association between 8-OHdG and OPG was negative (beta = −0.196) and, although not statistically significant ($p = 0.098$), markedly higher than those obtained among controls (beta = −0.037) and osteoporotic (beta = −0.089) women.

Finally, in order to unveil if the correlations found to be significant in the osteopenic group were independent of potential confounding factors, we performed two multiple regression models including age, years since menopause, BMI, waist circumference, and Hs-CRP as covariates (Table 4). These analyses showed that both associations, 8-OHdG versus RANKL and 8-OHdG versus RANKL/OPG, retained their significance even after multiple adjustments.

Table 2: Simple correlation between 8-OHdG and RANKL, OPG and RANKL/OPG, BMD values, and bone resorption/formation markers (total sample, $n = 124$).

	8-OHdG
L. spine BMD	−0.13
F. neck BMD	0.04
Total hip BMD	0.03
CTX-1	0.07
BAP	0.06
RANKL	0.263*
OPG	−0.116
RANKL/OPG	0.277*

* $p < 0.01$ by Pearson's analysis of base-10 logarithm transformed values of the 2 variables.

BMI: body mass index; BMD: bone mass density; L.: lumbar; F.: femoral; CTX-1: C-terminal telopeptide of type I collagen; BAP: bone-specific alkaline phosphatase; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa-B ligand; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

Table 3: Simple linear regression analysis for the relationship between urinary level of 8-OHdG and serum levels of RANKL, OPG, and RANKL/OPG ratio in normal, osteopenic, and osteoporotic postmenopausal women.

		Normal BMD	Osteopenia	Osteoporosis
RANKL	B (DS)	0.325 (0.389)	0.554 (0.164)	0.018 (0.212)
	Beta	0.188	0.423*	0.014
	R^2	0.035	0.180	0.001
OPG	B (DS)	−0.048 (0.288)	−0.256 (0.175)	−0.093 (0.256)
	Beta	−0.037	−0.196	−0.089
	R^2	0.001	0.038	0.004
RANKL/OPG	B (DS)	0.368 (0.549)	0.879 (0.262)	0.161 (315)
	Beta	0.160	0.429*	0.086
	R^2	0.026	0.184	0.007

* $p < 0.001$.
Beta: standardized regression coefficient; B: nonstandardized regression coefficient; BMD: bone mass density; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa-B ligand; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

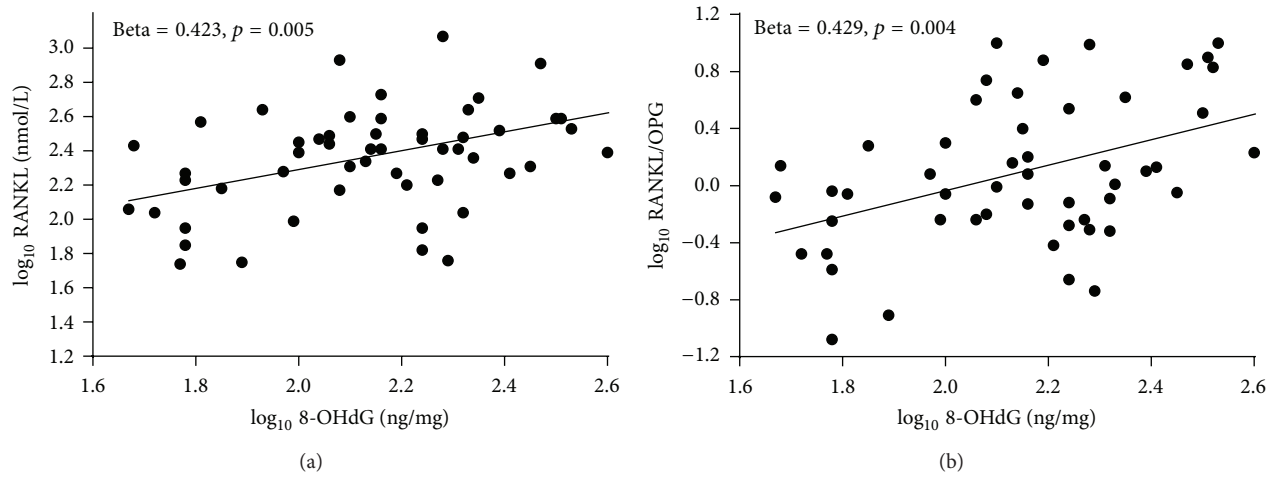

Figure 1: Box plots of the correlations: $\log_{10}$ 8-OHdG versus $\log_{10}$ RANKL (right); $\log_{10}$ 8-OHdG versus $\log_{10}$ RANKL/OPG.

4. Discussion

In the present study, we evaluated the possible link between PO and OxS, as assessed by 8-OHdG, by various angles. The analyses of the data obtained revealed that this OxS marker was not significantly associated with diagnosis of osteopenia/osteoporosis; BMD of different skeleton area; resorption/formation bone markers. In contrast, we found that higher level of 8-OHdG was strongly and independently related to increased serum concentration of RANKL and RANKL/OPG among postmenopausal women with osteopenia but not among those with normal BMD or osteoporosis.

In line with our results, several previous works [15, 16, 24, 25] did not find any significant difference in the peripheral level of OxS between osteoporotic and healthy postmenopausal women. In contrast, some of these studies [15, 16], along with others [14, 26, 27], found an inverse, although statistically weak, correlation between OxS and femoral neck and/or lumbar spine BMD. In our point of view, the reasons of these discrepancies mostly lay in the different indicators employed for peripheral OxS determination. Indeed, almost all the aforementioned studies dealt with markers (such as malondialdehyde, hydroperoxides, and F2-isoprostanes) which are all derived by lipid peroxidation. This cascade reaction is markedly different from the DNA repair process yielding 8-OHdG. Differently from the former, lipoperoxidation leads to the formation of several by-products, which are very reactive and can markedly amplify the initial ROS-induced oxidative spark. Furthermore, DNA oxidative injury merely occurs inside the cells, whereas the targets of lipoperoxidation are disseminated both in and out of the cytosol (e.g., membranes of cell and organelles and lipid moiety of circulating lipoproteins). Consistent with these observations, the precious systematic work by Kadiiska and colleagues suggests that there could be different forms of OxS, and each might bring about the rise of a different series of peripheral markers [28].

Our finding of the absence of a detectable increase in systemic OxS in relation to PO occurrence does not rule out that reactive species can play a role in the development of this bone disease. The gathered results also showed, indeed, that OxS might be an effective influencing factor of RANK/RANKL/OPG triad, which plays a paramount role in the pathogenesis of PO and other metabolic bone

Table 4: Multiple regression analysis for the relationship between 8-OHdG and RANKL and RANKL/OPG, among osteopenic postmenopausal women ($n = 59$).

Predictors	Dependent variable RANKL		Dependent variable RANKL/OPG	
	Beta	p value	Beta	p value
8-OHdG	0.469	0.002	0.449	0.004
Age	0.071	0.694	0.119	0.644
Years since menopause	0.031	0.849	0.015	0.930
BMI	0.016	0.939	0.059	0.274
Waist circumference	0.001	0.998	−0.049	0.812
Hs-CRP	−0.115	0.411	−0.079	0.523
	$R^2 = 0.202$		$R^2 = 0.185$	

Beta: standardized regression coefficient; BMI: body mass index; BMD: bone mass density; Hs-CRP: high reactivity C-reactive protein; RANKL: receptor activator of nuclear factor kappa-B ligand; 8-OHdG: 8-hydroxy-2′-deoxyguanosine.

diseases [5, 6, 29, 30]. This discovery adds to the current literature, because, to the best of our knowledge, it is the first time that such interaction is found in human subjects. Conversely, there is abundant supportive evidence from *in vitro* experiments on various cell lineages such as mouse osteoblasts, human MG63, and primary bone marrow cell cultures [14, 31]. More in detail, Baek and coworkers showed that oxygen peroxide can promote the number and activity of osteoclasts and RANKL expression, but not OPG. Of interest, these effects were abolished upon adding catalase, a potent H_2O_2-scavenger [14]. Increase in endogen ROS burden leading to enhancement of RANKL production of osteoclast precursor cells can be derived by NOX activation or by a reduced expression of nuclear factor (erythroid-derived 2-) like 2 (Nrf2), as shown in a recent work on Nrf-2 knockout mice [13]. Noteworthily, the partial activation of this redox-sensitive transcription factor, which regulates the expression of several genes encoding essential antioxidant enzymes, resulted in inhibition of osteoclast differentiation [13].

The clinical importance of the present study derives from the fact that RANK/RANKL/OPG axis is now widely regarded as one of the most promising molecular targets for novel therapeutic approaches in the management of bone diseases [4, 30]. Accordingly, the inhibition of RANKL by denosumab (a fully human antibody against RANKL) was more effective at reducing the occurrence of vertebral fractures than the traditional drugs [9]. In spite of these encouraging outcomes, there is still an intense demand for alternative, nonpharmaceutical (and, at least hopefully, safer) interventions on this high-incidence disease. In this context the *in vitro* and animal data highlighting the protective effects on bone elicited by various antioxidants such as lycopene [32], resveratrol [33], and tocotrienol [34] are promising.

Unfortunately, the human observational studies set out to examine the effects of antioxidants on bone health are still sparse and controversial [35] and do not allow translating the preclinical evidence in an effective antiosteoporotic treatment. Moreover, the interpretation of the epidemiological is difficult because most of these studies are affected by important limitations such as cross-sectional design [24, 36, 37] and lack of measurement of circulatory antioxidants concentration which should accompany the evaluation of nutrients intake by dedicated questionnaires [24, 38]. The latter point is of primary importance, because the bioavailability of these compounds depends on the food matrix consumed and on genetic variability and physiological condition of the subjects [35]. Besides, the findings may be biased by the interference of other nutrients as suggested by the authors of one of the few longitudinal studies on this field [38]. Indeed, examining a sample of 891 women, Macdonald et al. observed significant negative correlations between BMD and nutrients, in particular vitamin E (from diet alone) and polyunsaturated fatty acids (PUFAs). The researchers suggest that the strong association that was also found between PUFAs and vitamin E intake ($r = 0.822$, $p < 0.001$) could account for this unexpected result and, as consequence, this vitamin could simply represent a surrogate marker for fat intake [38]. Disappointing data were also obtained in a cross-sectional study by Wolf et al. [36], where dietary and total intake, or serum concentration of vitamin E, β-carotene, lycopene, and other antioxidants, failed to be associated with BMD in women ($n = 11068$, aged 50–79 years). Contrariwise, a clear beneficial effect of α-tocopherol was found in a recent longitudinal study which showed that low intake and serum concentration of the vitamin were both associated with an increased rate of bone fracture in both elderly women ($n = 61422$) and men ($n = 1138$) [39]. Finally, similar bone protective effects of carotenoids [40] or vitamin C [41] emerged from data collected in Framingham Osteoporosis Study.

Overall, the published epidemiological studies, although presenting some controversies and design issues, appear to support the commonly held belief that antioxidant-rich fruits improve bone health and are strongly suggestive of a beneficial role of these bioactive molecules [31]. However, one must be aware that it is not yet completely clear if these osteoprotective effects are merely exerted by an antioxidant pathway or by the simple restoring of mineral balance and/or vitamin K bioavailability [35, 36].

Thus, well-designed, randomized, controlled studies are warranted to confirm the findings from the animal studies on bone loss and subsequent development of osteoporosis. In our view, however, the concept that has to be borne in mind is the following: PO is a multifactor and multifaceted disease, and, thus, OxS should not be considered as the unique enemy to defeat. Owing to these considerations, it is conceivable to assume that antioxidants alone could not represent the definitive treatment for PO but more likely as therapeutic adjuvant of well-established antiosteoporotic drugs. The indication that emerged from our investigation was that this type of supplements might benefit postmenopausal women with osteopenia. Indeed only in those subjects, where bone remodeling cycle is altered, but not still completely compromised, the RANK/RANK/OPG system appeared to be sensitive to the elevation of 8-OHdG. Therefore, OxS, during this condition defined as prelude of PO, could contribute to uncoupling the balance between bone resorption and

formation, thus guiding the process of bone degeneration to the "definitive" osteoporotic damage. Arresting this process, before BMD is not too low (T-score < 2.5), is very important to prevent from onset of PO and related fragility fractures [31].

Finally, some important limitations of the study must be acknowledged. First, the design of the study was cross-sectional, thereby precluding our ability to establish any temporal relationship between the markers examined. Therefore, longitudinal investigations are mandatory to draw a definitive appreciation of causal nature of OxS with respect to alteration in circulatory level of RANKL and RANKL/OPG. Second, the lack of a full nutritional assessment of the sample subjects makes it difficult to rule out the fact that dietary antioxidant intake might interfere with the assessed level of 8-OHdG and, hence, with the reliability of study outcomes. However, to attenuate the influence of this factor, all the individuals reporting the use of antioxidant supplements or to follow a vegetarian diet were excluded a priori from the study. Third, the serum level of RANKL and OPG might not reflect the levels and activity of these cytokines in bone microenvironment, and a portion of them could originate from nonskeletal sources, in particular inflammation [4, 5]. In this regard, in the attempt to limit the potential interference of this factor on our statistical outcomes, we included Hs-CRP in the multivariate analysis. Finally, a limitation of the fact that RANKL/OPG is not a suitable marker for PO diagnosis could appear. On the contrary, it may represent a potential strength of the mounting consensus around the use of this peripheral index for monitoring bone health and antiosteoporotic therapy response of patients with bone diseases [42].

We would like to also underline some other strengths of the present work. To the best of our knowledge, this is the first study that provides *in vivo* data in support of the interaction between RANKL/OPG and OxS among women with high risk of osteoporosis. Noteworthily this correlation resulted to be independent by potential confounders such as age and measures of body fat. Moreover, we consider as further study strength the use of a widely recognized reliable marker of DNA oxidative damage such as urinary 8-OHdG [28, 43].

5. Conclusion

In conclusion, our findings demonstrate the existence of a positive association between systemic OxS and serum level RANKL/OPG ratio in osteopenic but not in normal and osteoporotic postmenopausal women. Thus, the data obtained, although warranted confirmation by longitudinal studies, suggest that women with moderately low BMD could be the target population for antioxidant-based interventions aimed at preventing osteoporosis-related bone loss and fracture.

Conflict of Interests

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

Acknowledgments

The authors thank Cristina Castaldini and Stefania Ferrazzini for their skilled organization of clinical protocol. They also want to thank Manuela Spagnolo and Ilaria Crivellari for their meaningful contributions in data collection and processing. The study was supported by "Local Research Project" Grant from University of Ferrara, Italy.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9124676, 8 pages
http://dx.doi.org/10.1155/2016/9124676

Research Article

Serum Superoxide Dismutase Is Associated with Vascular Structure and Function in Hypertensive and Diabetic Patients

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Received 17 February 2015; Revised 9 June 2015; Accepted 14 June 2015

Academic Editor: Matías Mosqueira

Oxidative stress is associated with cardiac and vascular defects leading to hypertension and atherosclerosis, being superoxide dismutase (SOD) one of the main intracellular antioxidant defence mechanisms. Although several parameters of vascular function and structure have a predictive value for cardiovascular morbidity-mortality in hypertensive patients, there are no studies on the involvement of SOD serum levels with these vascular parameters. Thus, we assessed if SOD serum levels are correlated with parameters of vascular function and structure and with cardiovascular risk in hypertensive and type 2 diabetic patients. We enrolled 255 consecutive hypertensive and diabetic patients and 52 nondiabetic and nonhypertensive controls. SOD levels were measured with an enzyme-linked immunosorbent assay kit. Vascular function and structure were evaluated by pulse wave velocity, augmentation index, ambulatory arterial stiffness index, and carotid intima-media thickness. We detected negative correlations between SOD and pressure wave velocity, peripheral and central augmentation index and ambulatory arterial stiffness index, pulse pressure, and plasma HDL-cholesterol, as well as positive correlations between SOD and plasma uric acid and triglycerides. Our study shows that SOD is a marker of cardiovascular alterations in hypertensive and diabetic patients, since changes in its serum levels are correlated with alterations in vascular structure and function.

1. Introduction

Hypertension is quantitatively the most important risk factor for premature cardiovascular disease; essential hypertension and diabetes are characterized by endothelial dysfunction mediated by an impaired NO availability secondary to oxidative stress production [1]. Vascular disease is one of the main causes for disability and death in patients with diabetes mellitus [2], which invariably show endothelial dysfunction as well as associated cardiovascular risk factors as hypertension, obesity, and dyslipidemia [3]. Either dyslipidemia, hyperinsulinemia, insulin resistance, or hyperglycemia contributes to the development of endothelial dysfunction [4].

Arterial stiffness, estimated by pulse wave velocity (PWV) determination, has an independent predictive value for cardiovascular events [5], is associated with the severity of coronary artery disease, and is impaired in coronary atherosclerosis [6]. The ambulatory arterial stiffness index (AASI) is related to cardiovascular morbidity-mortality [7] and to the associated target organ damage in hypertensive patients [8]. AASI is very useful for assessing arterial stiffness and is an independent predictor of cardiovascular mortality and morbidity in patients with cardiovascular disease and in healthy individuals. We have previously shown that AASI is positively correlated with carotid intima-media thickness (IMT) and PWV and negatively correlated with glomerular filtration [9]. Another parameter to measure wave reflection

and arterial stiffness is the augmentation index (AIx), which is a predictor of adverse cardiovascular events, and higher values are associated with target organ damage [10].

The role of reactive oxygen species (ROS) in the pathophysiology of cardiovascular diseases has been described, as oxidative stress is associated with cardiac and vascular defects leading to hypertension and atherosclerosis [11], but direct cause and effect relationships have not been clearly defined. Although ROS originate from different sources, the vascular NADPH seems to be one of the main sources in cardiovascular pathophysiology [12]. Elevated levels of superoxide anion have been detected in essential hypertension [13] and in the development of atherosclerosis [14]. The enzyme superoxide dismutase (SOD) is an intracellular antioxidant defence mechanism which catalyses the dismutation of superoxide radical into H_2O_2 and oxygen [11]. SOD has a protective role in atherogenesis [15] and improves hypertension modulating vasodilation, vasoconstriction, vascular remodelling, and cardiac hypertrophy, playing a relevant role in the development and the maintenance of chronic hypertension in various organs [16].

However, so far there have been no studies that evaluate the possible relationship between serum levels of this enzyme and different vascular parameters with a predictive value on cardiovascular risk. Thus, we have assessed the relationship between SOD serum levels and parameters of vascular function and structure (PWV, AASI, IMT, and AIx) as well as cardiovascular risk in hypertensive and type 2 diabetic patients.

2. Materials and Methods

This is a cross-sectional study performed in 307 consecutive patients (54 with type 2 diabetes and hypertension, 16 nonhypertensive diabetic, 185 hypertensive nondiabetic patients, and 52 nondiabetic and nonhypertensive controls), enrolled in the study over a period of 24 months (from January 2008 to January 2010) in the Primary Care Research Unit of La Alamedilla Health Centre (Castilla y León Health Service-SACYL), Salamanca, Spain, which complied with the inclusion/exclusion criteria.

Inclusion Criteria are as follows: patients aged 20–80 years, diagnosed with type 2 diabetes mellitus and/or hypertension. Exclusion criteria are as follows: patients with secondary hypertension, patients unable to comply with the protocol requirements (psychological and/or cognitive disorders, failure to cooperate, educational limitations and problems in understanding written language, and failure to sign the informed consent document), patients participating or who were going to participate in clinical trials during the study, and patients with serious comorbidities representing a threat to life (known coronary or cerebrovascular atherosclerotic disease, heart failure, moderate or severe chronic obstructive pulmonary disease, walking-limiting musculoskeletal disease, advanced respiratory, renal or hepatic disease, severe mental diseases, treated oncological disease diagnosed in the past 5 years, pregnant women, and terminal patients). Most of the patients with hypertension and diabetes received drug therapy (except those controlled by diet), which is described in Table 1.

Sample size calculation indicated that the 307 patients included in the study were sufficient to detect a coefficient correlation of 0.16 between superoxide dismutase with parameters of vascular function and structure in a two-sided test with an alpha risk of 0.05 and a power of 80%.

Hypertension was diagnosed as recommended by The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension and of the European Society of Cardiology [17]. Diabetes was diagnosed as recommended by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [18].

2.1. Ethical and Legal Issues. The experimental protocol was in accordance with the Declaration of Helsinki (2000) of the World Medical Association and approved by the Ethics Committee of the University Hospital of Salamanca (Spain) and complied with Spanish data protection law 15/1999 and its developed specifications (RD 1720/2007). Each patient signed a participation informed consent after full explanation of the study.

2.2. Sociodemographic and Cardiovascular Variables. We evaluated patient age and sex, hypertension, dyslipidemia, alcohol consumption, smoking, history of premature cardiovascular disease (before 55 years of age in males and before 65 in females), and patients on treatment with antihypertensive, antidiabetic, lipid lowering, and antiaggregant drugs.

2.3. Serum SOD and 8-Hydroxy-2-deoxyguanosine Determination. Serum concentrations of Cu/Zn SOD and 8-hydroxy-2-deoxyguanosine were determined with ELISA kits (Cu/Zn SOD: Northwest Life Sciences Inc., Vancouver, WA, USA; 8-hydroxy-2-deoxyguanosine: Abcam, Cambridge, UK), according to the instructions of the manufacturer. Absorbance was read on a spectrophotometer (Thermo Luminoskan Ascent, Waltham MA, USA) at 450 nm (Cu/Zn SOD) and 410 (8-hydroxy-2-deoxyguanosine).

2.4. Other Biochemical Determinations. Blood samples were collected after patient fasting for at least 8 hours. Determinations are as follows: creatinine, basal glucose, HbA1c, uric acid, HDL-cholesterol, LDL-cholesterol, total cholesterol, and triglycerides. The parameters were measured on a blind basis in a General Hospital Biochemistry laboratory using standard automatized techniques.

2.5. Blood Pressure Determination. Office blood pressure evaluation involved three systolic (SBP) and diastolic blood pressure (DBP) measurements, using the average of the last two measurements, with a validated OMRON model M10-IT sphygmomanometer (Omron Health Care, Kyoto, Japan), following the recommendations of the European Society of Hypertension [19]. Pulse pressure was estimated with the mean values of the second and third measurements.

Ambulatory blood pressure monitoring (ABPM) was performed on a day of standard activity using a control

Table 1: Characteristics of study population.

	All patients	DIA + HYP	DIA	HYP	Control	p
Number	307	54	16	185	52	
Age (years)	54.76 ± 11.69	60.75 ± 8.35	55.27 ± 12.82	55.26 ± 10.99	46.63 ± 12.54	<0.001
Male sex (N, %)	191 (62.21%)	39 (72.22%)	11 (68.75%)	108 (58.38%)	33 (63.46%)	0.286
Superoxide dismutase (ng/mL)	123.48 ± 57.20	134.76 ± 63.60	112.00 ± 35.23	117.63 ± 47.69	137.91 ± 82.68	0.079
8-Hydroxy-2-deoxyguanosine (ng/mL)	6.97 ± 5.07	7.97 ± 5.52	6.24 ± 4.17	6.35 ± 5.13	7.40 ± 4.76	0.377
Systolic blood pressure (mmHg)	138.26 ± 17.15	142.59 ± 18.20	119.31 ± 9.61	143.66 ± 16.09	126.26 ± 8.92	<0.001
Diastolic blood pressure (mmHg)	86.89 ± 10.92	84.94 ± 12.07	74.13 ± 5.19	90.62 ± 9.88	79.56 ± 6.66	<0.001
Pulse pressure (mmHg)	52.49 ± 12.37	57.37 ± 12.92	45.39 ± 10.15	53.16 ± 12.23	46.91 ± 10.00	<0.001
IMT medium average (mm)	0.73 ± 0.11	0.80 ± 0.11	0.73 ± 0.12	0.73 ± 0.10	0.68 ± 0.14	<0.001
IMT maximum average (mm)	0.90 ± 0.14	0.98 ± 0.12	0.90 ± 0.15	0.90 ± 0.12	0.84 ± 0.17	<0.001
Pulse wave velocity (m/s)	8.98 ± 2.22	10.39 ± 2.35	8.65 ± 2.27	9.00 ± 2.09	7.59 ± 1.61	<0.001
Peripheral augmentation index	92.76 ± 21.43	95.85 ± 20.87	88.94 ± 22.34	95.15 ± 21.45	82.22 ± 18.63	0.001
Central augmentation index	30.31 ± 11.66	31.35 ± 10.67	30.44 ± 12.10	31.81 ± 10.94	23.85 ± 13.04	<0.001
AASI	38.25 ± 6.05	41.39 ± 5.96	38.27 ± 5.44	37.71 ± 5.89	36.92 ± 5.96	<0.001
D'Agostino cardiovascular risk	19.73 ± 16.27	36.59 ± 18.22	17.34 ± 11.88	18.00 ± 13.72	8.59 ± 8.24	<0.001
Smokers (N, %)	73 (23.77%)	13 (24.07%)	4 (25%)	38 (20.54%)	18 (34.62%)	0.202
HDL (mg/dL)	51.98 ± 12.45	47.48 ± 9.73	50.75 ± 12.68	52.86 ± 12.63	53.96 ± 13.42	0.023
LDL (mg/dL)	125.91 ± 32.76	108.13 ± 27.96	108.50 ± 22.07	132.71 ± 34.03	125.75 ± 26.68	<0.001
Total cholesterol (mg/dL)	204.40 ± 36.98	187.46 ± 34.70	183.81 ± 23.66	211.44 ± 37.58	203.25 ± 32.77	<0.001
Alcohol consumption (units/week)	10.98 ± 20.28	14.94 ± 21.35	3.69 ± 4.87	9.63 ± 15.81	13.87 ± 32.15	0.111
HbA1c (%)	5.41 ± 1.05	6.84 ± 1.40	6.68 ± 0.80	5.05 ± 0.46	4.83 ± 0.40	<0.001
Glycemia (mg/dL)	98.44 ± 30.44	136.61 ± 46.27	132.63 ± 32.63	88.63 ± 11.14	83.19 ± 8.75	<0.001
Antihypertensive drugs (N, %)	143 (46.58%)	48 (88.89%)	0 (0.00%)	95 (51.35%)	0 (0.00%)	<0.001
Antidiabetic drugs (N, %)	66 (21.50%)	51 (94.44%)	15 (93.75%)	0 (0.00%)	0 (0.00%)	<0.001
Lipid-lowering drugs (N, %)	84 (27.36%)	32 (59.26%)	6 (37.50%)	43 (23.24%)	3 (5.77%)	<0.001
Antiaggregants (N, %)	61 (19.87%)	29 (53.70%)	6 (37.50%)	23 (12.43%)	3 (5.77%)	<0.001

Demographic, physical, and medical characteristics and drug therapies of patients included in the study. Data are expressed as mean ± standard deviation or percentage. AASI: ambulatory arterial stiffness index; DIA: diabetic nonhypertensive patients; DIA + HYP: diabetic hypertensive patients; HbA1c: glycosylated haemoglobin; HDL: cholesterol associated with high density lipoproteins; HYP: hypertensive patients; IMT: intima-media thickness; LDL: cholesterol associated with low density lipoproteins; p: p value, statistically significant differences (ANOVA).

system (Spacelabs 90207, Healthcare, Issaquah, Washington, USA). We obtained blood pressure measurements every 20 min (waking period) and every 30 min (resting period). Valid records of readings were 80% of the total.

2.6. Determination of Pulse Wave Velocity (PWV) and Peripheral (PAIx) and Central (CAIx) Augmentation Index. These parameters were estimated using the SphygmoCor System (AtCor Medical Pty Ltd., Head Office, West Ryde, Australia). Pulse wave analysis was performed with a sensor in the radial artery, using mathematical transformations to estimate the aortic pulse wave. CAIx was estimated from the morphology of the aortic wave using the following formula: increase in central pressure × 100/pulse pressure. PAIx was calculated as follows: (second peak SBP [SBP2] − [DBP])/(first peak SBP − DBP) × 100 (%). Measurements reliability was evaluated using the CAIx intraclass correlation coefficient, which showed values of 0.974 (95% CI: 0.936–0.989) for intraobserver agreement on repeated measurements in 22 subjects. According to the Bland-Altman analysis, the limit of intraobserver agreement was 0.454 (95% CI: −9.876–10.785). The carotid-femoral pulse wave was analysed estimating the delay with respect to the ECG wave and calculating the PWV. Distance measurements were taken with a measuring tape from the sternal notch to the carotid and femoral arteries at the sensor location.

2.7. Ambulatory Arterial Stiffness Index (AASI). Arterial stiffness was evaluated with AASI, defined as one minus the regression slope of DBP over SBP readings obtained from individual 24-hour blood pressure recordings. The stiffer the arterial tree, the closer the regression slope and AASI to 0 and 1, respectively.

2.8. Assessment of Carotid Intimamedia Thickness (C-IMT). Carotid ultrasound to assess carotid IMT was performed by two investigators trained for this purpose before starting the study. The reliability of such recordings was evaluated before the study, using the intraclass correlation coefficient, which showed values of 0.97 (95% CI: 0.94 to 0.99) for intraobserver agreement on repeated measurements in 20 subjects, and 0.90 (95% CI: 0.74 to 0.96) for interobserver agreement. According to the Bland-Altman analysis, the limit of interobserver agreement was 0.02 (95% limits of agreement: −0.05–0.10),

Table 2: Pearson correlations between serum superoxide dismutase and parameters of vascular structure and function and cardiovascular risk.

	Superoxide dismutase				
	All patients	DIA + HYP	DIA	HYP	Control
Intima-media thickness medium average	−0.08	0.06	0.45	−0.12	−0.22
Intima-media thickness maximum average	−0.06	0.06	0.41	−0.07	−0.22
Pulse wave velocity	−0.15*	−0.06	−0.03	−0.16*	0.25
Peripheral augmentation index	−0.16**	−0.08	−0.09	−0.13	−0.37*
Central augmentation index	−0.16**	−0.01	−0.18	−0.10	−0.35*
AASI	−0.19**	−0.32*	0.20	−0.24**	−0.15
Pulse pressure	−0.17**	−0.20	−0.07	−0.18*	−0.19
D'Agostino cardiovascular risk	−0.03	−0.09	0.46	−0.06	−0.10
8-Hydroxy-2-deoxyguanosine	−0.17	−0.29	0.21	−0.21	−0.22
Uric acid	0.19**	0.19	0.25	0.20**	0.28
HDL	−0.18**	−0.10	−0.22	−0.18*	−0.28
LDL	0.02	−0.06	0.40	0.13	−0.10
Triglycerides	0.19**	0.19	0.44	0.20**	0.25

AASI: ambulatory arterial stiffness index; DIA: diabetic nonhypertensive patients; DIA + HYP: diabetic hypertensive patients; HDL: cholesterol associated with high density lipoproteins; HYP: hypertensive patients; LDL: cholesterol associated with low density lipoproteins. Statistical significant differences: * $p < 0.05$; ** $p < 0.01$.

and the limit of intraobserver agreement was 0.01 (95%: −0.03–0.06). A Sonosite Micromax ultrasound (Sonosite Inc., Bothell, Washington, USA) device paired with a 5–10 MHz multifrequency high-resolution linear transducer with Sonocal software was used for automatic measurements of IMT to optimize reproducibility. Measurements were made of the common carotid after the examination of a longitudinal section of 10 mm at a distance of 1 cm from the bifurcation, performing measurements in the proximal wall and in the distal wall in the lateral, anterior, and posterior projections, following an axis perpendicular to the artery to discriminate two lines: one for the intima-blood interface and the other for the media-adventitious interface. 6 measurements were obtained in both the right and the left carotid, using average values (average C-IMT) and maximum values (maximum C-IMT) automatically calculated by the software [20]. The average IMT was considered abnormal if it measured 0.90 mm, or if there were atherosclerotic plaques with a diameter of 1.5 mm or a focal increase of 0.5 mm or 50% of the adjacent IMT [17].

2.9. Cardiovascular Risk Assessment. Risk of cardiovascular morbidity and mortality was estimated using the risk equation (D'Agostino scale) based on the Framingham study [21]. The individuals performing the different tests were blinded to the clinical data of the patient. All organ damage assessment measures were made within a period of 10 days.

2.10. Statistical Analysis. Data input was made using the Teleform system (Autonomy Cardiff, Vista, CA, USA), exporting the data to the PASW version 18.0 statistical package (SPSS Inc., Chicago IL, USA). Data was presented as mean ± standard deviation or percentage. One-way analysis of variance (ANOVA) for independent samples was used to compare quantitative variables among SOD quartiles. Pearson correlation test was used to analyze associations between quantitative variables. Using the general linear model procedure, we have conducted two multivariate analyses in which we have considered AASI, PAIx, PWV, and IMT as dependent variables, and SOD quartiles as independent variables. We have performed a first model without adjustments and a second model adjusted for age in each of the independent variables (represented in Figure 1). Hypothesis contrasting established an alpha risk factor of 0.05 as the limit of statistical significance.

3. Results

General and medical characteristics of the patients are presented in Table 1. The average age of the patients was 55 years, and 62 percent of them were male. 143 subjects (46.6%) have antihypertensive treatment, 66 (21.5%) with antidiabetic treatment, and 84 (27.4%) with lipid-lowering therapy, of whom 74 (24%) are taking statins, 7 (2.3%) are taking fibrates, and 6 (2%) other lipid-lowering drugs. The values of the different parameters of vascular structure and function (IMT, PWV, PAIx, CAIx, and AASI) are lower in the control group and higher in the group with diabetes and hypertension associated ($p < 0.001$) (Table 1). Plasma levels of 8-hydroxy-2-deoxyguanosine, one of the predominant forms of free radical-induced oxidative lesions, were similar in the different groups of patients.

In our study population, serum SOD was inversely correlated with PWV, PAIx, CAIx, AASI, and pulse pressure. We also found a negative correlation with plasma HDL-cholesterol and a positive correlation with uric acid levels and triglycerides only in hypertensive patients, correlations that remain after adjusting the data for the intake of antihypertensive and lipid-lowering drugs (Table 2).

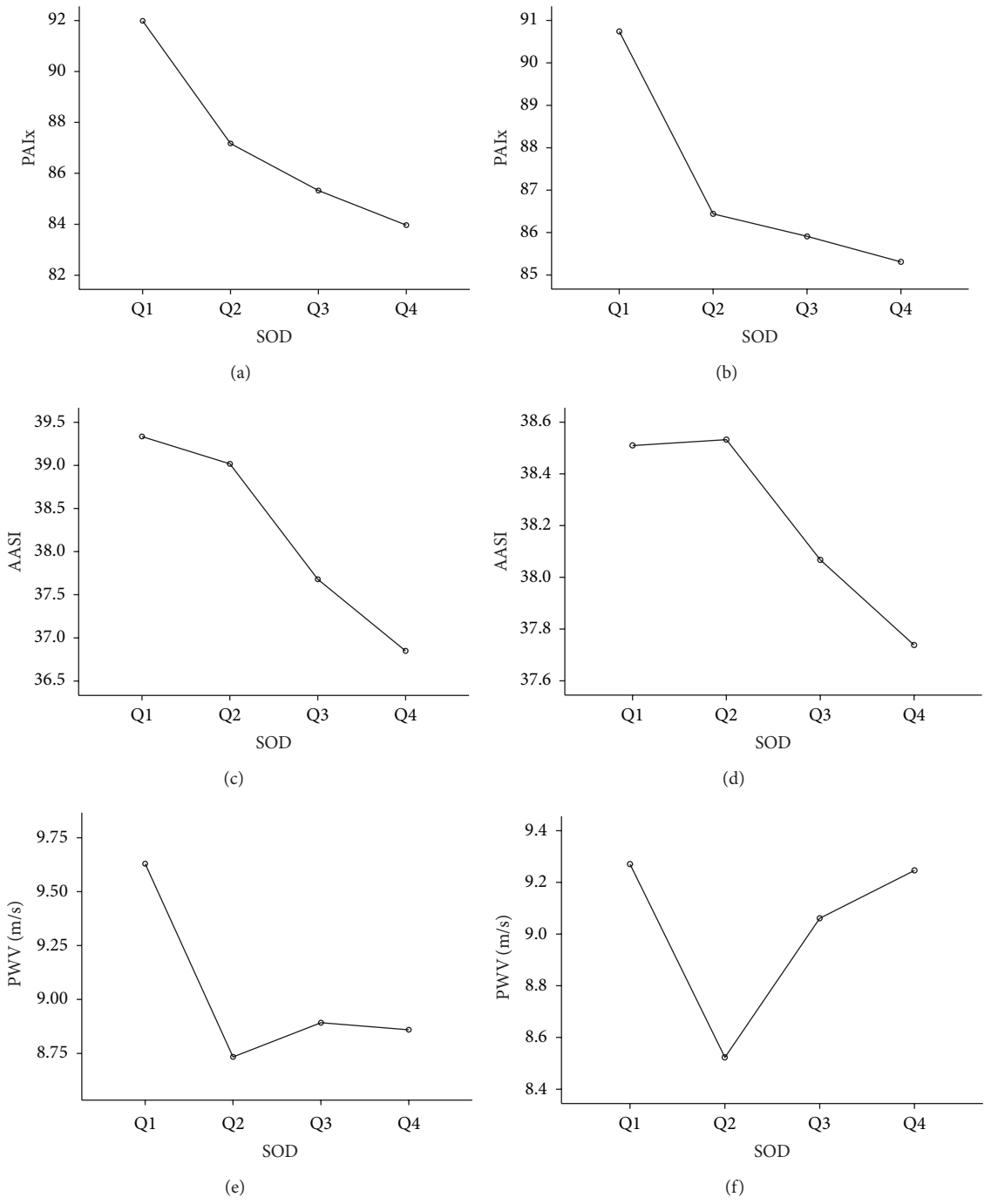

Figure 1: Peripheral augmentation index, ambulatory arterial stiffness index, and pulse wave velocity values divided into 4 quartiles according to SOD serum levels. Multivariate analysis: AASI, PAIx, PWV, and IMT are dependent variables, and SOD is the independent variable. (b), (d), and (f) show values after adjusting for age the independent variable. AASI: ambulatory arterial stiffness index; PAIx: peripheral augmentation index; PWV: pulse wave velocity. p values: (a): 0.166; (b): 0.469; (c): 0.076; (d): 0.825; (e): 0.102; (f): 0.090.

After dividing the sample into quartiles according to SOD serum levels, the values of PAIx, AASI, PWV, and cardiovascular risk estimated with D'Agostino index are higher in the first quartile, although only AASI shows statistically significant differences ($p = 0.019$) (Table 3). This trend was maintained after adjusting for age in the case of PAIx and AASI (Figure 1).

4. Discussion

This is the first study linking serum SOD levels with PWV, AIx, AASI, and pulse pressure, thus suggesting that oxidative stress significantly affects the vascular structure and function in hypertensive and diabetic patients and showing the role of SOD as an indicator of hypertension and diabetes-induced

Table 3: Parameters of vascular structure and function divided into 4 quartiles according to superoxide dismutase serum levels.

	Q1 (SOD: <87.3 ng/mL)	Q2 (SOD: 87.3–112.8 ng/mL)	Q3 (SOD: 112.8–142.6 ng/mL)	Q4 (SOD: >142.6 ng/mL)	p
IMT medium average (mm)	0.73 ± 0.11	0.75 ± 0.13	0.73 ± 0.10	0.72 ± 0.12	0.436
IMT maximum average (mm)	0.90 ± 0.12	0.92 ± 0.16	0.90 ± 0.12	0.89 ± 0.14	0.620
PWV (m/s)	9.64 ± 2.47	8.83 ± 1.94	8.83 ± 2.46	8.88 ± 1.81	0.088
Central AIx	29.41 ± 9.87	30.72 ± 9.50	27.04 ± 10.67	26.77 ± 10.94	0.076
Peripheral AIx	91.92 ± 22.84	87.72 ± 14.71	85.32 ± 22.48	84.05 ± 22.82	0.167
AASI	39.62 ± 6.40	39.29 ± 6.69	37.64 ± 5.84	36.74 ± 5.34	0.019
D'Agostino cardiovascular risk	21.75 ± 19.30	18.30 ± 13.79	19.92 ± 17.11	19.91 ± 14.79	0.705

Data are expressed as mean ± standard deviation. AASI: ambulatory arterial stiffness index; AIx: augmentation index; CV cardiovascular; IMT: intima-media thickness; p: p value, statistically significant differences (ANOVA); PWV: pulse wave velocity.

impairment of cardiovascular function, cardiovascular risk, and target organ damage. After adjusting for age, we still detect correlations between SOD and PAIx, AASI, and IMT, confirming the role of this serum marker as predictor of alterations in vascular structure and function.

Serum levels of SOD are negatively correlated with PWV, PAIX, CAIx, and AASI in our study population (hypertensive and diabetic patients recruited from primary care centers), thus suggesting that oxidative stress promotes or encourages the development of endothelial dysfunction, as it has been previously suggested by other authors [22]. Although the role of CAIx in the clinical setting remains unclear, it seems to be a predictor of adverse cardiovascular events in several patient populations, and higher AIx is associated with target organ damage [10]. According to our data, it has been previously observed that SOD improves endothelial function [23] and Mn-SOD protects against oxidative stress and endothelial dysfunction in ApoE-deficient mice [24].

We detected negative correlations between serum SOD and most of the parameters of vascular structure and function analysed. Our data suggest that serum SOD is not increased in response to vascular injury in hypertensive and diabetics patients; its serum levels are not increased in these patients with vascular disorders, but its decline indicates a deficit in antioxidant defence mechanisms, since hypertensive and diabetic patients are unable to remove the circulating superoxide anion and therefore suffer an increase in vascular damage induced by reactive oxygen species. Thus, a lower level of serum SOD is associated with increased vascular damage.

We observed a negative correlation between SOD and HDL-cholesterol and positive correlation between SOD and triglycerides. HDL-cholesterol levels are inversely related to the risk of clinical events due to atherosclerosis [23]. On the other hand, a univariate association between triglycerides and cardiovascular risk has been described [25]. However, although the relationship between SOD serum levels with HDL-cholesterol and triglycerides has not been previously described, our results suggest that these molecules are related but further experiments are needed to assess the nature and characteristics of this relationship.

In our study population, there are positive correlations between SOD and uric acid. In agreement with our finding, Brand et al. [26] described a strong correlation between hyperuricemia and elevated cardiovascular risk in the general population, and it has been observed that serum uric acid correlates with extracellular SOD activity in patients with chronic heart failure [27]. As we said previously regarding HDL-cholesterol and triglycerides, additional experiments are needed to elucidate the pathophysiological significance of this correlation.

Limitations of our study are its cross-sectional design, which precludes longitudinal analysis between SOD, vascular structure, and function. We have analysed SOD expression level, but not its activity. Sampling of the study was performed consecutively, including hypertensive patients with a short course of hypertension, or with diabetes and hyperlipidemia, and many patients receiving drug therapy, which may modify blood pressure levels. However, the heterogeneity of the sample is similar to the distribution of the real population of short-course hypertensive patients with some risk factors and without previous cardiovascular disease.

5. Conclusions

We describe for the first time that SOD serum levels are correlated with alterations in vascular structure and function, being an indicator of cardiovascular alterations, cardiovascular risk, and target organ damage in hypertensive and diabetic patients, thus confirming that oxidative stress negatively contributes to the proper functioning of blood vessels. These encouraging data have to be followed by prospective studies to establish the relative strength of the prediction of cardiovascular risk and the appearance of target organ damage according to the SOD levels presented by the patient.

Abbreviations

AASI: Ambulatory arterial stiffness index
ACEi: Angiotensin converting enzyme inhibitors
AIx: Augmentation index

ARB: Angiotensin receptor blockers
CV: Cardiovascular
DIA: Diabetic nonhypertensive patients
DIA + HYP: Diabetic hypertensive patients
HbA1c: Glycosylated haemoglobin
HDL: Cholesterol associated with high density lipoproteins
HYP: Hypertensive patients
IMT: Intima-media thickness
LDL: Cholesterol associated with low density lipoproteins
PWV: Pulse wave velocity
SOD: Superoxide dismutase.

Conflict of Interests

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

Authors' Contribution

Manuel A. Gómez-Marcos, Luis García-Ortiz, and Carlos Martínez-Salgado designed the study; Manuel A. Gómez-Marcos, Ana M. Blázquez-Medela, Luis Gamella-Pozuelo, and José I. Recio-Rodriguez performed the study and collected data; Ana M. Blázquez-Medela, José I. Recio-Rodriguez, Luis Gamella-Pozuelo, Luis García-Ortiz, and Carlos Martínez-Salgado analysed data; Luis García-Ortiz and Carlos Martínez-Salgado wrote the paper. Luis García-Ortiz and Carlos Martínez-Salgado contributed equally to this paper.

Acknowledgments

This work was supported by grants from Instituto de Salud Carlos III (Ministerio de Sanidad y Consumo, PS09/01067, PI12/00959, and Retic RD06/0018, Rediapp), Junta de Castilla y León (Excellence Group GR100, IES095U14), and Obra Social Caja de Burgos (Founding for Clinical Research 2009, 2010). Ana M. Blázquez-Medela is supported by Fondo de Investigaciones Sanitarias (FIS) from the Institute Carlos III, Ministerio de Ciencia e Innovación. The authors thank the collaboration of the members of the *Vaso-risk group*: Luis García-Ortiz, Manuel A. Gómez-Marcos, José I. Recio-Rodriguez, Cristina Agudo-Conde, Maria C Patino-Alonso, Emiliano Rodriguez-Sanchez, Emilio Ramos-Delgado, Luis J González-Elena, Ángel García-García, Javier Parra-Sánchez, Isabel Sánchez Herrero, Carmela Rodríguez-Martín, Carmen Castaño-Sánchez, Ángela de Cabo-Laso, Benigna Sánchez-Salgado, Carlos Martínez-Salgado, and Ana M. Blázquez-Medela.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 219569, 6 pages
http://dx.doi.org/10.1155/2015/219569

Research Article

An Increase of Plasma Advanced Oxidation Protein Products Levels Is Associated with Cardiovascular Risk in Incident Peritoneal Dialysis Patients: A Pilot Study

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Received 4 April 2015; Revised 27 May 2015; Accepted 28 May 2015

Academic Editor: Claudio Cabello-Verrugio

Advanced oxidation protein products (AOPPs) are considered as markers and even mediators of the proinflammatory effect of oxidative stress in uremia. We hypothesized that an increase of oxidative stress associated with peritoneal dialysis (PD), estimated by the variation of plasma AOPPs over time, might be associated with cardiovascular (CV) risk and overall prognosis. In 48 PD patients, blood samples were collected on two occasions: the first one in the first six months after starting PD therapy and the second one, one year after. The plasma AOPPs level variation over the first year on PD was significantly associated with CV antecedents and also with CV prognosis. In those patients in whom the AOPPs levels increased more than 50% above the baseline value, a significant association with past and future CV disease was confirmed. These patients had 4.7 times greater risk of suffering later CV disease than those with a smaller increase, even after adjusting for previous CV history. Our data suggest that the increase of AOPPs plasma level over the first year on PD is conditioned by CV antecedents but also independently predicts CV prognosis. AOPPs plasma levels seem to represent the CV status of PD patients with sufficient sensitivity to identify those with a clearly sustained higher CV risk.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in end-stage renal disease patients [1]. Besides the traditional risk factors for CV events such as hypertension, diabetes mellitus, and hyperlipidemia, "non-traditional" factors, such as oxidative stress, abdominal fat deposition, and endothelial dysfunction, have also been proposed [2, 3]. Oxidative stress, defined as the tissue damage resulting from an imbalance between an excessive generation of oxidant compounds and insufficient antioxidant defense mechanisms, probably contributes to endothelial dysfunction and atherosclerosis and, therefore, CV complications [4].

Witko-Sarsat et al. [5] found that, due to the oxidative damage, proteins can modify their spectroscopic characteristics. These altered proteins, named advanced oxidation protein products (AOPPs), have a molecular weight of 600 kDa and are highly elevated in hemodialysis (HD) patients. The same authors demonstrated that AOPPs act to trigger the oxidative burst and the synthesis of inflammatory cytokines in neutrophils and monocytes [6]. Since glycation-modified proteins also induced protein cross-linking and are elevated in uremic patients, AOPPS and advanced glycation end products (AGEs) are highly correlated [6].

Oxidative stress causes damage to important biological structures and may enhance the inflammatory response. New

compounds, such as AOPPs, but also AGEs and advanced lipoperoxidation end products (ALEs) may constitute a new molecular basis for the deleterious activity of oxidants, and they could be considered to be true mediators of the proinflammatory effect of oxidative stress in uremia [7, 8].

Moreover, these authors have also evaluated the relationships between plasma AOPPs and markers of monocyte activation in uremia and demonstrated a high correlation between AOPPs and renal creatinine clearance and inflammatory cytokine levels such as tumor necrosis factor alpha (TNF-α) [5, 9–12].

Residual renal function (RRF) affects the survival rate and the development of CVD in peritoneal dialysis (PD) patients. In incident PD patients, lower RRF has also been associated with increased inflammation. Loss of RRF is associated with increased AOPP and AGEs plasma levels, suggesting that preservation of RRF has a beneficial effect on reducing the oxidative stress in PD patients [11].

Endothelial dysfunction is an early initiating event in atherosclerosis and a risk factor for future CV events. Oxidative stress and uremia-related CV risk factors probably play a role in the pathogenesis of endothelial dysfunction [11, 12]. In a previous article [13], our group demonstrated that peritoneal protein clearance (PrC) and 24 h effluent peritoneal protein losses (PPL) on initiating PD are directly and independently related to peripheral arterial disease (PAD), as an expression of the highest CV disease grade. A greater rate of peritoneal transported protein might be the result of peritoneal endothelial dysfunction, reflecting systemic endothelium damage.

We hypothesized that an increase of oxidative stress associated with PD and estimated by AOPPs plasma level variation over time might be associated with CV risk factors development and overall PD patient prognosis.

Therefore, our primary objective was to evaluate, in this pilot study, the association of dynamic plasma AOPPs levels with CV background and outcome in a cohort of incident PD patients. Our secondary objective was to explore the biological variation of AOPPs plasma levels throughout a year in this high risk population and to study the influence of renal and peritoneal functions on this variation.

2. Patients and Methods

2.1. Patients. We studied 48 patients who remained at least one year in the PD program of the Hospital Universitario La Paz, Madrid, Spain. Patients comprised 37 men and 11 women. Their mean age was 54.0 ± 15.9 years and at baseline mean duration of preceding time on PD at inclusion was 6 months. There were 11 (22.9%) patients with diabetes, 42 (87.5%) with hypertension, and 27 (56.3%) with previous CVD. Plasma samples were obtained from each patient on two occasions: the first one in the first six months after starting the PD therapy (period between 2000 and 2009) and the second one, one year after. The following information was collected from patient records: demographic data (including age, sex, height, weight, and body mass index); prevalence of CV risk factors (hypertension, diabetes mellitus, hyperlipidemia, CV disease at the beginning of dialysis, smoking habits, and secondary hyperparathyroidism); laboratory tests (blood glucose levels, serum levels of cholesterol, albumin, triglycerides, albumin, and high-sensitivity C-reactive protein (hs-CRP)); and PD-related parameters: type of dialysis (continuous peritoneal ambulatory dialysis (CAPD) or automated peritoneal dialysis (APD)), RRF, and urea (U-MTAC) and creatinine (Cr-MTAC) mass transfer area coefficients. All patients gave their consent to give blood samples in order to participate in the study.

Patients were followed up until death or end of follow-up (January, 2014). Causes of deaths and CV events were determined by clinicians based on clinical presentation and examination of patients. CV events included electrocardiographically documented angina, myocardial infarction, heart failure, atrial fibrillation, stroke, and peripheral vascular disease.

2.2. Methods. AOPPs classic determination was based on spectrophotometric detection according to Witko-Sarsat et al. [5]. In order to minimize the impact of storage time and the influence of triglyceride, we decided to use the modified AOPP assay developed by Anderstam et al. [14]. The modified AOPP assay included, in addition to the Witko original AOPP methodology, a sample preparation procedure to precipitate lipoproteins (very low density lipoproteins (VLDL) and low density lipoprotein (LDL)) in the plasma (Konelab HDL-cholesterol precipitating reagent, Thermo Electron Corporation, Vantaa, Finland). This reagent is normally used as a preparation step before determination of HDL-cholesterol on Konelab analyzers. Fifty μL of reconstituted precipitating reagent (dextran sulphate and magnesium ions) was mixed with 500 μL of EDTA plasma, centrifuged at 1000 ×g for 20 min, upon which the supernatant was carefully removed. AOPPs were immediately measured in the supernatant at 340 nm on a microplate spectrophotometer under acidic conditions and expressed as chloramine-T equivalents (μmol/L). Glomerular filtration rate (GFR) was expressed calculating the mean between creatinine and urea kidney clearances. Peritoneal protein clearance was assumed to be peritoneal albumin clearance as almost all protein in peritoneal effluent is albumin (the plasma measurement for the calculation was albumin).

All patients were subjected to a baseline peritoneal kinetic study (within 4 weeks after the start of dialysis) and one year after. This study was performed using a standard protocol of four-hour dwell period with 3.86% glucose concentration 2 L volume exchange. During the peritoneal function study, the patients fasted and were given no medication except for low doses of subcutaneous insulin as necessary. To measure the diffusive capacity, six samples of the peritoneal effluent were collected (at time 0, 30, 60, 120, 180, and 240 minutes) and a blood sample was also taken. Based on these determinations, D/P Cr was calculated as described by Twardoski et al. [15], and mass transfer coefficients of urea (urea-MTAC) and creatinine (cr-MTAC) were calculated based on a mathematical model described previously by our group [16].

Table 1: Clinical and biochemistry values and kidney and peritoneal function parameters in 48 PD patients at inclusion and at one year after starting PD.

	PD patients (n = 48)	
	Baseline	One year after
BMI, kg/m^2	25 ± 3.3	25.4 ± 3.2
Cholesterol, mg/dL	175 (149–199)	164 (145–187)
Triglycerides, mg/dL	121 (97–188)	107 (85–123)
Albumin, g/dL	3.7 ± 0.4	3.7 ± 0.4
hs-CRP, mg/L	1.5 (0.7–4.7)	1.9 (0.7–3.2)
AOPPs, μmol/L	76.6 (61.4–92.3)	95.2 (75.3–126.3)
Residual diuresis, mL/24 h	1713 ± 1125	1302 ± 945
Residual GFR, mL/min/1.73 m^2	5.9 ± 3.6	4.3 ± 3.4
Creatinine MTAC, mL/min	8.7 ± 2.7	8.7 ± 4.3
Urea MTAC, mL/min	22.4 ± 4.5	22 ± 6.9
Ultrafiltration, mL/4 h (dwell time 3.86% glucose)	708 ± 241	693 ± 265
Peritoneal protein clearance, mL/day	4.7 ± 3.5	4.9 ± 3.8

Values are presented as mean ± SD for normally distributed variables, or median (interquartile range) for nonnormal data.
BMI: body mass index; hs-CRP: high-sensitivity C-reactive protein; AOPPs: advanced oxidative protein products; GFR: glomerular filtration rate; MTAC: mass transport area coefficient.

2.3. Statistical Analysis. Results are expressed as mean ± standard deviation (SD) for normally distributed continuous variables, or median (interquartile (IQ) range) for nonnormal data, or percentage of total, as appropriate, for categorical variables. Participant data were compared by using a chi-square test, Fisher test, Student's t-test, Wilcoxon signed-rank test, or Mann-Whitney U-test, as appropriate. Spearman correlation analysis was used to examine the significance of associations between variables. Two-tailed 95% confidence intervals (CI) and p values are presented with $p < 0.05$ regarded as significant. All statistical analyses were performed using statistical software SPSS for Windows, version 15.0 (Chicago, SPSS Inc., USA).

3. Results

We studied 37 men and 11 women. Mean age was 54.0 ± 15.9 years and mean duration of preceding time on PD at inclusion was 6 months. There were 11 (22.9%) patients with diabetes, 42 (87.5%) with hypertension, and 27 (56.3%) with previous CVD. The main clinical, analytical data and kidney and PD function parameters throughout the study period are reported in Table 1.

In univariate analysis, plasma AOPPS levels were neither associated with demographic, clinical, or kidney variables nor associated with peritoneal function parameters. Baseline AOPPs levels were positively correlated (Spearman Rho 0.69, $p < 0.01$) with AOPPs levels at 1 year.

Mean time of follow-up after the second AOPPs determination was 71.4 ± 38 months (median 67 [5–151 months]). During that period, 28 patients (58.3%) had undergone renal transplantation, 9 (18.8%) had been transferred to hemodialysis, and 9 (18.8%) had died during PD therapy. Thirteen patients died during the total follow-up (*census date*: January, 2014); CV disease was the most common cause (7 patients), followed by infection (4 patients). In the univariate Cox proportional hazards model, age, presence of diabetes or CV disease, serum albumin concentration, CRP level, PD modality, peritoneal parameters, or residual renal function was not associated with mortality.

Since there were individuals who increased their AOPPS plasma levels during the study period while others decreased them, we assumed that the analysis using the median value would not differentiate differences among patients. For this reason, and due to the lack of literature to recommend a clear cut-off value for the analysis, we decided to compare the groups according to the increase or decrease of the baseline AOPPs level and its magnitude (percentage) of change (median value 29.6%, with a range from −63.3 to +998.4%). Forty patients (83.3%) showed an increase of plasma AOPPs level at one year (in 14 of them this increase was > 50% from baseline value). The AOPPS levels decreased only in 8 (16.7%) patients. In those patients in whom the AOPPS levels increased more than 50% of baseline value, an association with past and future CV disease was found. A direct relationship between the percentage increment in AOPPs level at 12 months and CV antecedents was found (effect size; phi = 0.605, $p < 0.001$). In fact, patients with a CV history had 8.4 times higher risk (95% CI [2.09, 33.48]) to present a percentage of AOPPs increase greater of 50% at month 12 of PD treatment than those patients with no CV disease (Figure 1).

The percentage of increase in AOPPs at month 12 was also significantly associated with the development of new CV disease (effect size; phi = 0.612, $p < 0.001$). Among the patients who developed a CV event, the percentage of patients showing an increase of AOPPs greater than 50% at month 12 was significantly higher than those patients with an increase lower than 50% (64.3% versus 7.7%, $p < 0.01$). The first group of patients had 4.7 times greater risk (95% CI [2.04, 11.05]) to suffer later CV disease than those with the smaller increase (Figure 2), even after adjustment for prior CV disease history

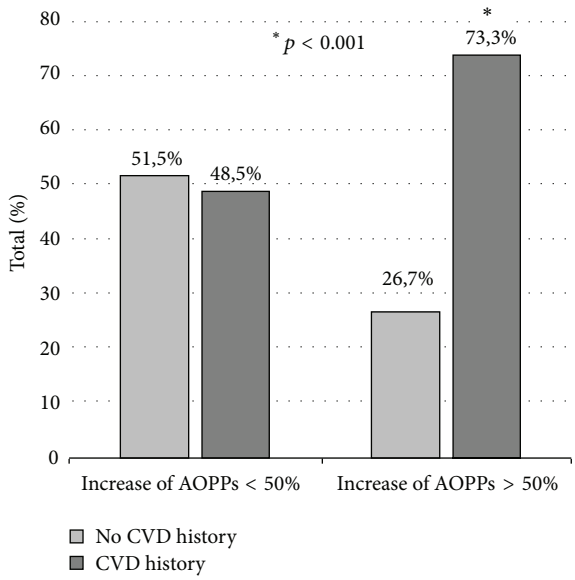

Figure 1: Prevalence of cardiovascular disease (CVD) according to the change of plasma AOPPs levels after one year on PD. Among patients with an increase lower than 50% ($n = 33$), there was no difference in prior CV disease prevalence whereas, among those with AOPPs increase higher than 50% ($n = 15$), there was a higher prevalence of prior CV disease. $^{*}p < 0.001$ (significant CVD history in patients with an increase of plasma AOPPs levels greater than 50% versus lower than 50%).

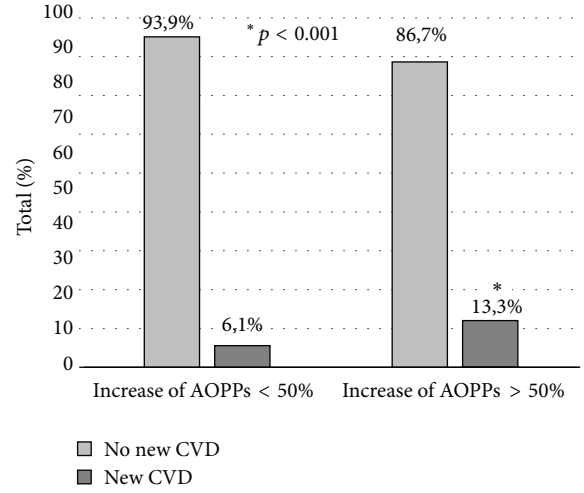

Figure 2: Incidence of new cardiovascular (CV) disease according to changes of plasma AOPPs level during one year on PD. Patients with an increase of AOPP higher than 50% ($n = 15$) had 4.7 times greater risk of developing a new CV event than those with a smaller increase of AOPP ($n = 33$). $^{*}p < 0.001$ (significant CV disease in patients with an increase of plasma AOPPs levels greater than 50% versus lower than 50%).

(we first ruled out that the AOPPs levels were a modifier factor between the risk to develop a new CV event in patients with a CV history (Breslow-Day test, $p = 0.23$) and also that it was a confusion factor (Mantel-Haenszel test, $p < 0.05$)).

4. Discussion

Our interest has focused on the estimation of the oxidative stress of PD patients by measuring circulating AOPPs over a period of time on risk and to relate their behavior to CV status. The main finding of our study was that plasma AOPPs levels increase over time mostly among patients with CV history and also in patients with subsequent CV events suggesting that the change in AOPPs level represents a marker of a permanent CV risk status.

The median AOPP value in our study is in agreement with previous literature [17, 18], which uses the same methodology. To our knowledge, ours is the first study in which the AOPPs values were measured in the same patients at two different time points. Alike other markers such as CRP, it seems that AOPPs could be more valuable when being periodically monitored in clinic rather than when assessed as an isolated value. A prooxidant status defined by continuous increase in AOPPs levels would thus reflect a CV prone-event status. Probably, the prooxidant status will maintain a permanent endothelial dysfunction and promote new CV events. The opportunity of reducing this process and to estimate this reduction by AOPPs plasma levels give to our data potential clinical usefulness.

Dialysis patients have an increased risk of CV morbidity and mortality. Nonclassical CV risk factors such as inflammation, malnutrition, endothelial dysfunction, and oxidative stress have been suggested to be responsible for this risk [1, 2]. Witko-Sarsat et al. [5] first described the presence of high levels of plasma oxidized proteins in hemodialysed patients and named them AOPPs. They found that these altered proteins seem to act not only as true inflammatory mediators, but also as uremic toxins with proinflammatory effects [6]. Besides, CKD is a low grade inflammatory process due to several mechanisms such as a failure of reactive oxygen species (ROS) clearance or a low level of antioxidant vitamins due to dietary restrictions (fruits and vegetables).

Oxidative stress is also the unifying mechanism for many CV risk factors, which additionally supports its central role in CV disease. The majority of CV disease results from complications of atherosclerosis [19–22]. An important initiating event for atherosclerosis may be the transport of oxidized-LDL (Ox-LDL) across the endothelium into the artery wall. Endothelial cells, smooth muscle cells, and macrophages are the sources of oxidants in the cells, which induce the expression of adhesion molecules and chemotactic factors. These processes lead to the activation, attachment of T lymphocytes and monocytes to the endothelial cells, and the generation of reactive oxygen species (ROS), which convert Ox-LDL into highly oxidized LDL, which, in turn, will form foam cells.

Although the concept of atherosclerosis as an inflammatory disease is now well established, evidence suggests that chronic inflammation may be considered a common pathogenic step in the pathogenesis of insulin resistance, diabetes, atherosclerosis, and CV disease [23]. Inflammation is one manifestation of oxidative stress, caused by

a mitochondrial overgeneration of free radicals, and the pathways that generate the mediators of inflammation, such as adhesion molecules and interleukins, are all induced by oxidative stress.

Our data did not show a relationship between plasma AOPPs and RRF, which disagrees with previous studies performed on PD patients [24]; although our small sample size could mask a real association, it may also be related to the greater RRF and diuresis of our patients as compared to previous literature, even at one year after starting PD [24]. The suggestion of AOPPs as an endogenous nephrotoxic agent cannot be confirmed by our data.

However, to our knowledge, this is the first study analyzing the relationship between AOPPs and CV disease in PD patients. The difficulty in performing the AOPPs determination encourages conducting a pilot study before performing larger studies or introducing them into clinical practice. Due to the relatively low number of patients in this study, the results should be interpreted with caution; on the other hand, we were able to identify statistically significant associations related to AOPPs levels. Nevertheless, the small sample size and the difficulty of measuring these markers are the main limitations of our study. Furthermore, we cannot dismiss the possibility that RRF may have interfered with the results. Nevertheless, the possibility to include AOPPs as a new marker to predict or measure "non-classical" CV risk in patients at high risk, such as those on PD, should be considered when designing further clinical studies to confirm and further exploit these results.

In conclusion, the results of the current pilot study suggest that the increment of plasma AOPPs levels over the first year on PD associates with CV antecedents and also relates to the risk of developing new CV events. The plasma AOPP levels may be a useful marker that can represent the CV status of PD patients with sensitivity to reflect changes in those patients at a clear higher permanent CV risk.

Conflict of Interests

Baxter Healthcare Corporation employs Bengt Lindholm. None of the other authors have reported any conflict of interests.

Acknowledgments

This study was partially funded with the Rio Hortega Grant Program from the ISCIII, the Extramural Grant Program of Baxter, FIS 12/00241, and REDinREN program from the ISCIII-FEDER Funds, IRSIN-FRIAT. Baxter Novum is a result of a grant from Baxter Healthcare to Karolinska Institutet.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 732596, 15 pages
http://dx.doi.org/10.1155/2015/732596

Review Article

Nrf2 Signaling and the Slowed Aging Phenotype: Evidence from Long-Lived Models

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Received 8 April 2015; Revised 28 May 2015; Accepted 4 June 2015

Academic Editor: Claudio Cabello-Verrugio

Studying long-lived animals provides novel insight into shared characteristics of aging and represents a unique model to elucidate approaches to prevent chronic disease. Oxidant stress underlies many chronic diseases and resistance to stress is a potential mechanism governing slowed aging. The transcription factor nuclear factor (erythroid-derived 2)-like 2 is the "master regulator" of cellular antioxidant defenses. Nrf2 is upregulated by some longevity promoting interventions and may play a role in regulating species longevity. However, Nrf2 expression and activity in long-lived models have not been well described. Here, we review evidence for altered Nrf2 signaling in a variety of slowed aging models that accomplish lifespan extension via pharmacological, nutritional, evolutionary, genetic, and presumably epigenetic means.

1. Introduction

The incidence of chronic disease increases with age. Understanding the relationships between the processes of aging and age-related diseases is an important initiative of the National Institutes of Health to improve the health of the aging population [1]. Slowing the aging process limits the burden of age-related chronic disease [2]. Identifying characteristics that slow aging may also provide approaches for preventing chronic diseases. Animals with increased lifespan aid in understanding the aging process by allowing the study of physiological and biochemical adaptations associated with slowed aging. Further, studying characteristics shared among long-lived models provides insight into pathways that are key to slowing the aging process and age-associated chronic diseases.

Lifespan can be extended by genetic, dietary, and pharmacological interventions. Additionally, multiple species have independently evolved long lifespan, including humans and naked mole rats, both of which live more than four times longer than predicted by body size [3]. Some of the earliest discoveries of lifespan extension were single-gene mutations associated with the insulin-like growth factor I (IGF-1) and growth hormone (GH) pathways. These mice, including the Snell dwarf [4], are smaller than their heterozygote counterparts and significantly longer-lived, some by 40% or more compared with controls. Long-term caloric restriction is the most consistent dietary manipulation to extend lifespan and recent evidence suggests that short-term transient nutrition restriction prior to weaning, accomplished by litter enlargement, also increases mean and maximal lifespan in mice [5]. Pharmaceutical manipulation of lifespan is in its infancy, with evidence that rapamycin can extend lifespan in mice [6].

It is well established that oxidant stress increases with age across a variety of tissues, including cardiac [7] and skeletal muscle [8], liver [9], and brain [10], and is associated with a wide variety of chronic age-related diseases including cancer, neurodegeneration, sarcopenia, and cardiovascular disease. Although the oxidative stress theory of aging has received criticism [11], it remains true that oxidant stress is associated with the aging process. In response to oxidative stress, cells upregulate antioxidant pathways, including activation of the transcription factor nuclear factor (erythroid-derived 2)-like

2 (Nrf2), the master regulator of antioxidant defenses and the proposed "master regulator" of the aging process [3]. Further, the therapeutic potential of Nrf2 is well supported in neurodegeneration and cancer (reviewed in [12, 13]), highlighting a role for Nrf2 in attenuating age-related chronic disease. Below, we will review what is known about Nrf2 in four models of lifespan extension: caloric restriction, rapamycin feeding, short-term nutrition restriction, and the Snell dwarf mouse. Further, we will discuss what is known about Nrf2 in the exceptionally long-lived naked mole rat and in humans who show enhanced longevity, with the overall goal of describing Nrf2 signaling in longevity interventions and in naturally occurring models of long life.

2. Nrf2 Signaling Basics

A member of the basic leucine zipper transcription factor family, Nrf2, controls both basal and inducible expression of over 200 target genes. When cellular stress is low, Nrf2 is sequestered in the cytoplasm by its involvement in an inactive complex with the actin-binding protein Kelch-like ECH-associated protein 1 (Keap1). Under these conditions, Keap1 targets Nrf2 for ubiquitination and degradation by the 26S proteasome system, resulting in basal low-level expression of Nrf2 [14]. However, when activated, Nrf2 translocates to the nucleus and transcriptionally upregulates its cytoprotective transcriptional program through binding to the antioxidant response element (ARE) in the promoter region of its target genes. Activation by reactive oxygen species (ROS) is the best understood mechanism of Nrf2 activation. Oxidant exposure modifies cysteine residues on Keap1 resulting in conformational changes that protect Nrf2 from targeting for ubiquitination and degradation [15], thus resulting in Nrf2 accumulation and activation. In addition to ROS and electrophilic species, Nrf2 can also be activated by phytochemicals [16–18], as well as various pharmaceuticals (reviewed in [19]) via overlapping and distinct mechanisms.

Nrf2 target genes exhibit antioxidant properties and facilitate cellular responses against xenobiotics. Antioxidant enzymes include NAD(P)H dehydrogenase quinone 1 (NQO1), heme oxygenase-1 (HO-1), peroxiredoxin 1 (Prdx1), superoxide dismutase-1 (SOD-1), and many enzymes involved in glutathione synthesis such as glutathione S-transferases (GSTs) and glutamate-cysteine ligase modifier (GCLM), the rate-limiting step in glutathione synthesis (reviewed in [20]). In addition, the cytoprotective properties of Nrf2 activation extend beyond these classic target genes, as other ARE-containing genes exhibit anti-inflammatory activity [21] and autophagic properties [22] and aid in proteasomal removal of oxidative damaged proteins [23]. Nrf2 also regulates its own expression. Two ARE-like motifs in the 5′ flanking region of the Nrf2 promoter are responsible for the induction of Nrf2 upon activation [24], ensuring a feed-forward process with Nrf2 activation promoting its own expression and thus facilitating a profound cellular response to stress.

Studies of Nrf2 knockout animals highlight the importance of Nrf2 in cytoprotection. These animals display a diminished ability to activate prosurvival genes [30] and are more susceptible to a wide range of stressors including hyperoxia, lipopolysaccharide, cigarette smoke, and UV irradiation, as well as various chemical insults [31]. Nrf2 knockout animals display diminished basal activity of Nrf2-regulated antioxidant enzymes [30], as well as inducible targets. In a transcriptional analysis of Nrf2 target genes, treatment with the Nrf2 activator 3H-1,2-dithiole-3-thione (D3T) induced 292 genes in wild type animals, compared to only 15 in Nrf2 knockout mice [32]. Together, investigations of Nrf2 knockout animals demonstrate diminished basal and inducible gene programs, as well as increased sensitivity to cell stresses, and support Nrf2 as an important transcriptional regulator of the cytoprotective program.

3. Nrf2 and Aging

The role of Nrf2 in responding to cytotoxic stressors is well defined. However, only within the last few years have studies elucidated how Nrf2 function changes with age and how changes in Nrf2 activity contribute to the aging phenotype. Aging is sufficient to diminish cardiac Nrf2-ARE binding activity [7], and aged Nrf2 knockout mice exhibit decreased expression of antioxidant target genes [8]. Disruptions in Nrf2-Keap1 signaling have been reported in skeletal muscle from sedentary older humans [33] and cardiac muscle from aging rats [7]. Impaired liver Nrf2 transcriptional activity in old rats results in glutathione depletion and significant downregulation of Nrf2-regulated glutathione biosynthetic enzymes [34]. Aged mice show similar losses in cellular redox capacity to those observed in Nrf2 knockout mice [34, 35], suggesting that Nrf2 dysregulation with age may be responsible for the loss of cellular redox status. Diminished Nrf2 target gene expression with age is accompanied by increased muscle ROS production, glutathione depletion, and increased oxidant damage to proteins, DNA, and lipids in both humans [33] and rodents [34]. Therefore, given that Nrf2 activity decreases with age alongside increased oxidant stress, interventions that activate Nrf2 may impact the aging process and longevity.

Support for the role of Nrf2 in regulation of lifespan comes from Nrf2 gain of function and loss of function studies. For example, experimental deletion of the anti-electrophilic gene glutathione transferase (*gGsta4*) activated Nrf2 and significantly extended lifespan in mice [36]. This mutation increased electrophilic lipid peroxidation products and increased nuclear Nrf2 activity by 43% and 38% in liver and skeletal muscle, respectively. The authors propose that deletion of this glutathione transferase gene resulted in chronic moderate Nrf2 activation and presumably elevated downstream Nrf2 signaling throughout the mouse lifespan. Studies of the Nrf2 homolog SKN-1 in *Caenorhabditis elegans (C. elegans)* and the *Drosophila* homolog CncC further suggest that Nrf2 may be implicated in longevity processes. Upon activation, SKN-1 and CncC upregulate genes involved in the oxidative stress response, including many orthologs to those regulated by mammalian Nrf2 [37, 38]. Similar to mouse Nrf2 knockouts, SKN-1 mutants show diminished resistance to oxidative stress and shortened lifespan. On the other hand,

moderate overexpression of a constitutively active SKN-1 increases lifespan, alongside increased resistance to oxidative stress [37]. Similarly, Keap1 loss-of-function mutations extend the lifespan of male *Drosophila* [38]. Various lifespan extending genetic manipulations in *C. elegans* require SKN-1. Dietary restriction activates SKN-1, and expression of the transcription factor in *C. elegans* neuronal cells is required for longevity to be extended by dietary restriction [39]. The long-lived *daf*-2 (nematode homologue of FOXO) mutant increases lifespan in part through resultant activation of SKN-1 [40]. Together, what is known about Nrf2 and aging, alongside preliminary studies of the role of Nrf2 and SKN-1 in cytoprotection, suggests that loss of Nrf2 is important in age-associated declines in oxidant stress resistance, and perhaps in the aging pathology itself.

4. Nrf2 in Long-Lived Models

4.1. Naked Mole Rat. The naked mole rat is an exceptionally long-lived species, with a lifespan four times longer than similarly sized rodents, thus making the naked mole rat an important model for longevity studies [41]. Naked mole rats do not have typical lifespan curves in which mortality rates increase with age, but rather they experience few of the biological changes typically associated with aging such as decreased metabolic rate, body composition changes, and declines in genomic and proteomic integrity [42]. Naked mole rats are resistant to age-associated diseases such as cancer [43], cardiac diastolic dysfunction [44], and neurodegenerative diseases [45]. Thus, the naked mole rat represents a naturally occurring, unique model of healthy aging.

Surprisingly, early studies of naked mole rats revealed that they have higher levels of oxidative damage, including lipid peroxidation products, protein carbonyls, and oxidative DNA modification in the liver, compared to shorter-lived mouse controls [46]. Subsequent comparisons of young and old naked mole rats revealed a striking difference between age-associated changes in oxidative stress markers [47]. While macromolecular oxidant damage increases with age in mice, as it does in humans, naked mole rats maintain high levels of oxidative damage throughout their lifespan, similar to those observed in old mice [47]. In fact, few genes show differential expression between young and old animals, as assessed by transcriptome analyses in the brain, liver, and kidney from 4- and 20-month-old naked mole rats [26]. Therefore, while naked mole rats from a young age contain higher levels of oxidative damage than shorter-lived control mice, the typical age-associated increase in damage is blunted in this species, suggesting maintenance of oxidant stress defenses over time.

Naked mole rats also have significantly elevated proteasome quality control mechanisms [25]. The high breakdown and clearance of damaged proteins is suspected to be largely due to increased Nrf2 expression, as Nrf2 regulates the transcription of α and β subunits of the 26S proteasome, as well as the selective autophagy cargo protein p62 [48–50]. In support of the hypothesized role of Nrf2 in naked mole rat longevity, under nonstressed conditions, naked mole rats have greater protein levels of Nrf2 including nuclear Nrf2, elevated Nrf2-ARE binding activity, and greater expression of Nrf2-regulated enzymes in fibroblasts and liver [51, 52]. These data suggest Nrf2 may be responsible for the heightened quality control mechanisms in naked mole rats and may be associated with their exceptional longevity.

4.2. Caloric Restriction. Caloric restriction (CR), a decrease in caloric intake without malnutrition, is the most consistent and robust means to increase lifespan across species, from flies to rodents [53], as well as nonhuman primate models [54]. Additionally, CR imparts slowed aging effects and delays the incidence of age-related disease [55]. Although the mechanisms underlying the effects of CR on longevity remain largely unknown, protection against carcinogenesis [56], reduced insulin/insulin-like growth factor (IGF) signaling [57], and prolonged survival [53] are documented. In addition, CR improves cellular adaptation to stress [58], as evidenced by liver mitochondria isolated from CR rats which show delayed opening of the mitochondrial transition pore upon oxidative challenge [58]. In an elegant study using sera collected from humans practicing long-term CR compared to sera collected from age- and sex-matched individuals following a typical western diet, treatment of cultured human primary fibroblasts with CR sera significantly upregulated gene expression of stress-response genes and enhanced tolerance to oxidants [59]. CR decreases ROS production, enhances the plasma membrane redox system, improves insulin signaling, and attenuates inflammation [27], all of which have been associated with improved age-related disease outcomes.

Many of the positive outcomes of CR have been associated with Nrf2 activation. For example, a variety of carcinogens activate Nrf2 and the ARE, protecting against carcinogenesis [60]. Changes in insulin levels, such as those elicited by fasting, elicit a small acute oxidant stress and subsequent activation of Nrf2 and its targets [61]. CR prevents the age-induced loss of cellular antioxidant capacity, in part due to increased levels of Nrf2 targets NQO1 and GSTs in brain and liver [62, 63]. In cerebral vascular endothelial cells, CR prevents the age-related decline in Nrf2 activity. Further, CR upregulates Nrf2 expression in old mice to a level that surpasses that of young ad libitum (AL) fed animals [64], highlighting the importance of Nrf2 with aging and its potential activation by longevity promoting interventions. Several proposed CR mimetics, such as resveratrol, quercetin, and curcumin [65], act to increase lifespan and slow aging at least in part through activation of Nrf2 [28, 66–68]. We assessed Nrf2 signaling in cardiac muscle from 7-month-old animals that underwent lifelong 40% reduction in caloric intake compared to mice fed AL. We found Nrf2 protein expression to be unchanged by CR. However, NQO1 and SOD-1 were significantly upregulated (Figure 1) in CR, suggesting that a lifelong reduction in caloric intake may activate Nrf2 and be implicated in longevity in these animals.

Despite the positive associations between CR and Nrf2, the seminal study of Nrf2 and CR-induced lifespan extension is conflicting [69]. At 20 weeks of age, male and female Nrf2 knockout and wild type mice initiated a 40% reduction in caloric intake. As anticipated, the wild type mice

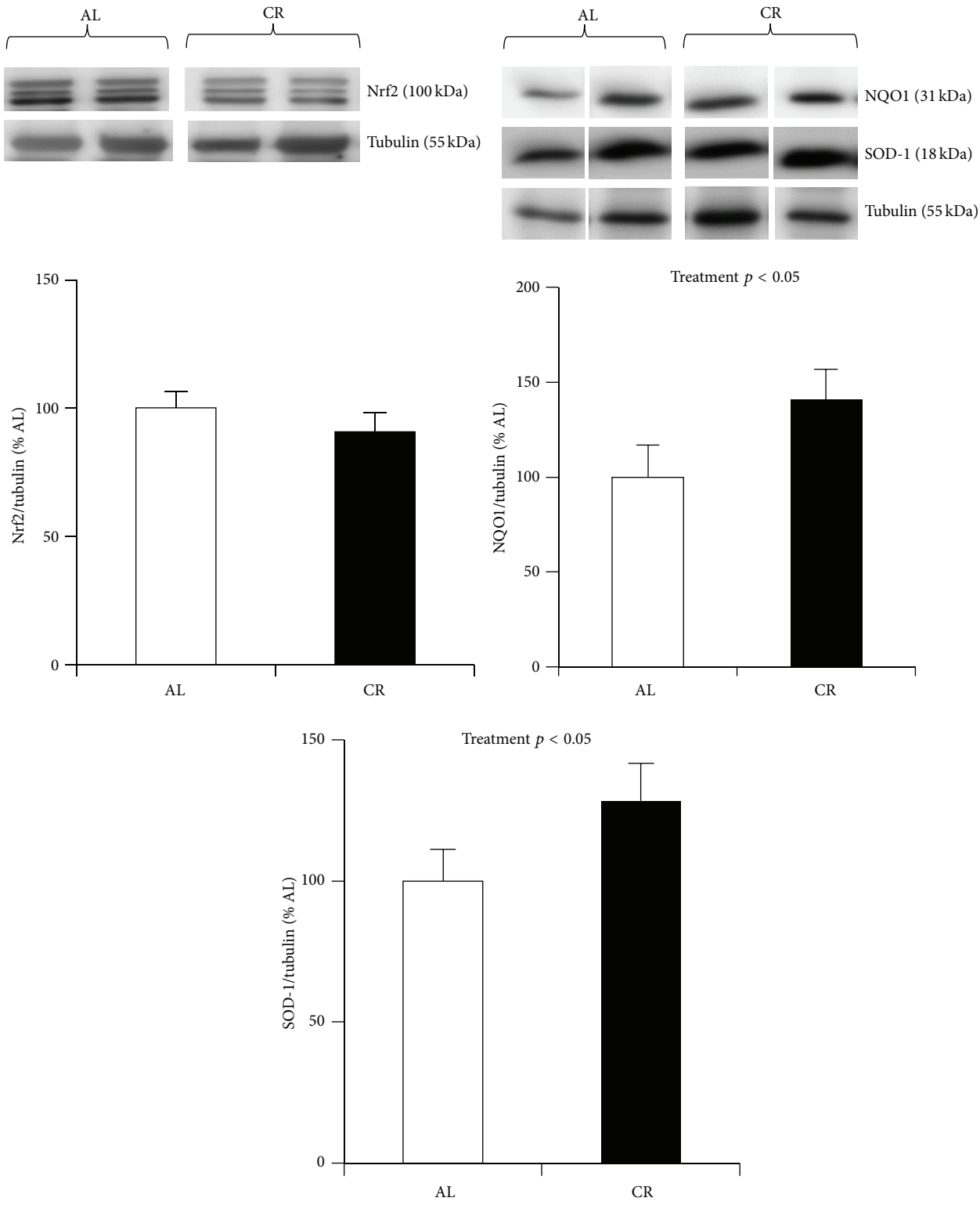


Figure 1: Nrf2 regulated protein expression in cardiac muscle from lifelong calorically restricted mice. Nrf2 was not different compared to ad libitum (AL) mice, while NQO1 and SOD-1 were significantly higher in cardiac muscle from CR mice compared to AL. Nrf2, NQO1, and SOD-1 were analyzed by western blotting and normalized to tubulin, shown below the proteins from each blot. Data are expressed as a ratio of target protein to tubulin (mean ± SEM). $n = 6$ males in each condition. Lifelong B6D2F1 CR mice were maintained at the NIA colony at 40% food restriction compared with AL. Male mice were purchased at 6 months of age and divided into AL and CR groups. CR animals were maintained on NIH-31/NIA Fortified Diet, whereas AL animals were maintained on NIH-31 diet.

were significantly protected against carcinogenesis on the CR diet compared to Nrf2 KO animals. However, survival curves showed that the CR and AL Nrf2 knockout mice overlapped until week 80, when they began to deviate for the remainder of the lifespan study, with CR Nrf2 knockout mice significantly outliving AL Nrf2 knockout mice. Therefore, while CR protected against carcinogenesis in a manner that was dependent on Nrf2, genetic ablation of Nrf2 did not attenuate lifespan extension by CR. This study suggests that lifespan extension in mice by CR is not entirely dependent on upregulation of the Nrf2 pathway, despite its promising role as a chemoprevention target. However, this conclusion is not without criticism, and a role of the antioxidant response in CR-mediated longevity cannot be completely ruled out [29]. Future investigations into whether or not other age-related diseases are slowed or reversed by Nrf2 activation and whether the attenuation of these diseases by CR requires Nrf2 are warranted.

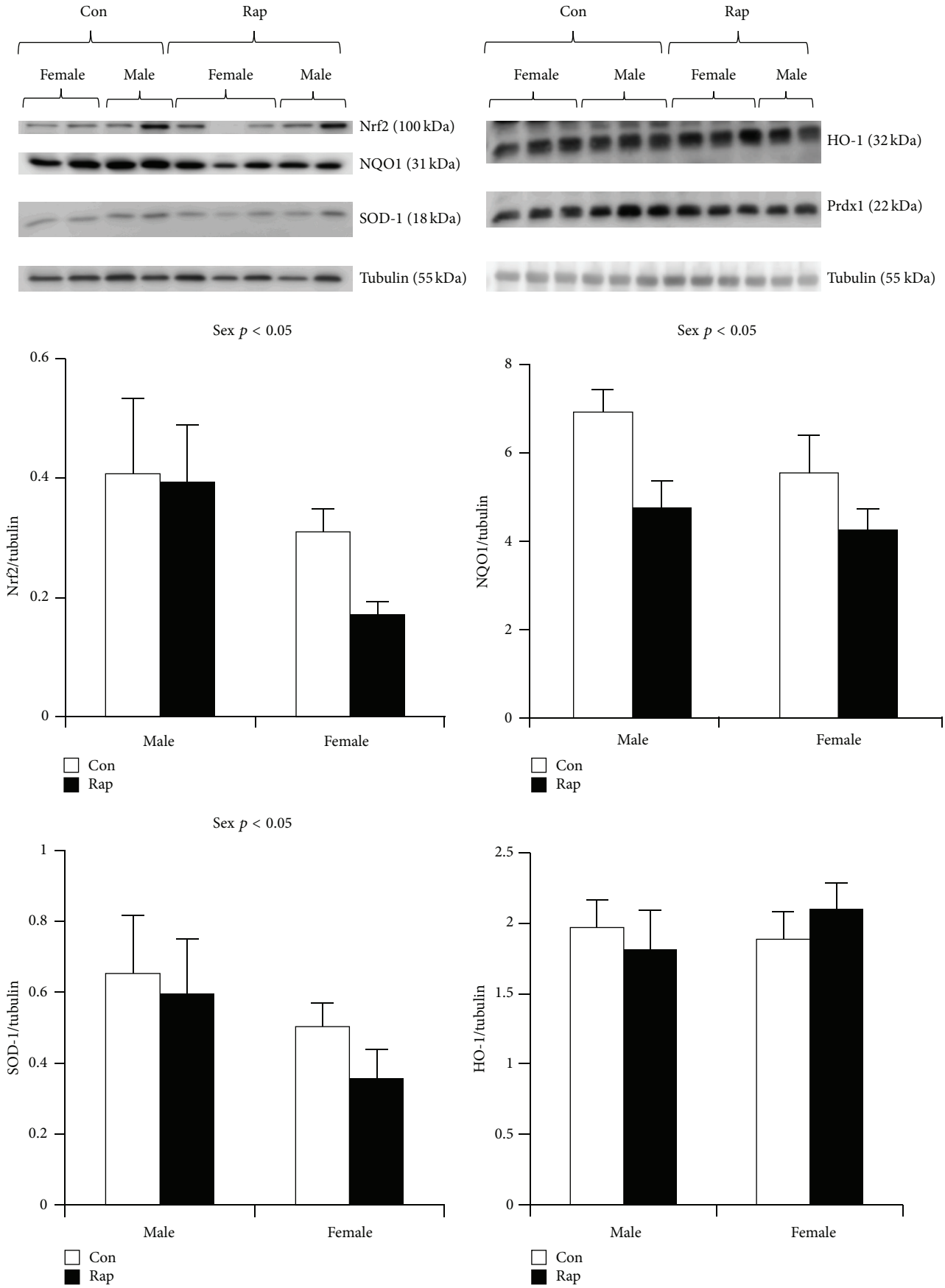
4.3. Rapamycin. Rapamycin, a well-defined inhibitor of the mechanistic target of rapamycin (mTOR), interacts with the Nrf2 signaling pathway, and Nrf2 may be implicated in rapamycin-mediated longevity. Exposure of adult *C. elegans* to rapamycin activates SKN-1 and downstream gene targets and significantly increases oxidative stress resistance in SKN-1 dependent manner [70]. Further, rapamycin treatment increased nematode lifespan through SKN-1, as evidenced by an abolished effect of rapamycin-mediated lifespan extension when SKN-1 was silenced by RNA interference [70]. Treatment with rapamycin also extends lifespan [71, 72] and slows the aging phenotype in mice, including decreased liver degeneration, attenuated cataract severity, and blunting of the age-associated decline in physical activity [73]. In vitro, chronic treatment with rapamycin significantly upregulates Nrf2 protein and transcript expression Nrf2 in fibroblasts, protects cultures from exogenous ROS exposure, and increases replicative lifespan of fibroblasts in culture [74]. We recently assessed skeletal muscle Nrf2 expression, as well as the expression of Nrf2 target proteins NQO1, HO-1, Prdx1, and SOD-1 in mice treated with rapamycin for 12 weeks. We were surprised to find no differences in Nrf2 and Nrf2 target protein expression between the long-lived rapamycin fed mice and controls. Prdx1 expression in skeletal muscle was significantly lower than control, but no other differences were observed in Nrf2 target protein expression between long-lived and control animals (Figure 2). Interestingly, we further assessed sex differences and found NQO1, Nrf2, and SOD-1 to be significantly greater in skeletal muscle from male mice compared to females (Figure 2). Given the disparate lifespan between males and females, and dissimilar effects of longevity interventions on male versus female mice, it is important to understand sex-specific signaling of the Nrf2 cytoprotective pathway, as elucidation of Nrf2 signaling in long-lived males and females may provide insight into the mechanisms behind sexual dimorphic longevity.

4.4. Snell Dwarf Mice. Snell dwarf mice are homozygous for a single-gene mutation at the *Pit1* locus. This mutation results in an underdeveloped anterior pituitary and decreases in growth hormone (GH) and insulin-like growth factor-1 (IGF-1) signaling. These mice display a 40% increase in mean and maximal longevity in both male and female mice compared to mice on similar backgrounds [4] and show delay in many age-related pathologies including attenuation of age-dependent collagen cross-linking and age-sensitive indices of immune system status [4]. This single gene mutation thus controls both maximal lifespan and the timing of senescence and age-related pathology, supporting the role of the IGF-1 and GH pathways in regulating mammalian longevity.

Snell mice display heightened Nrf2 signaling, as evidenced by increased skin-derived fibroblast expression of total cell Nrf2 protein compared to controls, in addition to upregulated expression of Nrf2 targets HO-1, thioredoxin, and GCLM [75]. In addition, Snell-derived fibroblasts show enhanced resistance to various forms of cytotoxic stress, like paraquat, peroxide, cadmium, and others that kill cells in part via ROS [76]. We recently assessed Nrf2 and targets in skeletal and cardiac muscle from 7-month-old Snell mice and found no differences in Nrf2 and target protein expression compared to control mice (Figure 3) in either males or females. Part of the discordance in Nrf2 signaling in primary fibroblasts compared to skeletal and cardiac muscle may be due to tissue-specific differences. Previous work suggests that Nrf2 activators induce the Nrf2 transcriptional program in some cell types, but not others [19, 77], demonstrating that cell and tissue types respond quite differently to Nrf2 activators. Varying metabolic demands between tissues may be responsible for tissue-specific Nrf2 activity [77], or inherent properties of the tissue, such as expression of Nrf2 activators/inhibitors. An assessment of tissue-specific differences in cellular stress responses is lacking and may play an important role in determining the response of Nrf2 to different longevity interventions.

4.5. Crowded Litter. Recent evidence shows that very-short-term nutrient restriction limited only to the preweaning phase by litter crowding extends lifespan by 16% in female mice and 7% in males [5]. In this model termed crowded litter (CL), increasing litter size from eight pups per mother to 12 imposes a transient energy restriction thought to represent an epigenetic means of improving lifespan [78], as the energy stress imposed only in the first three weeks of life extends lifespan and healthspan [79]. At weaning and through adult life, CL mice are leaner and consume more oxygen relative to body mass compared to control mice and have improved lifelong alterations in metabolic status [79]. CL mice have less body fat, lower leptin levels, and higher glucose tolerance and are more insulin sensitive than control mice [79]. Despite few studies interrogating mechanisms by which CL intervention imparts improved healthspan and lifespan, it seems clear that long-lasting endocrine and metabolic effects result from the early-life nutrient restriction.

As studies of CL mice are in their infancy, minimal data thus far directly link Nrf2 and CL. However, investigations of xenobiotic metabolism and resistance to cytotoxic insult suggest that CL mice maintain elevated levels of xenobiotic



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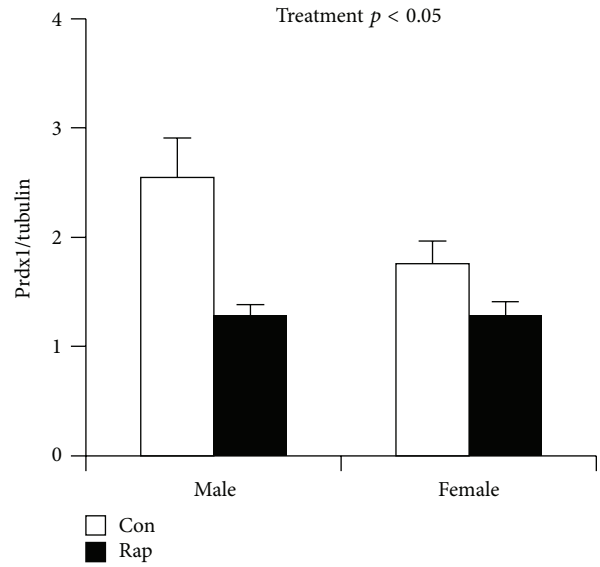
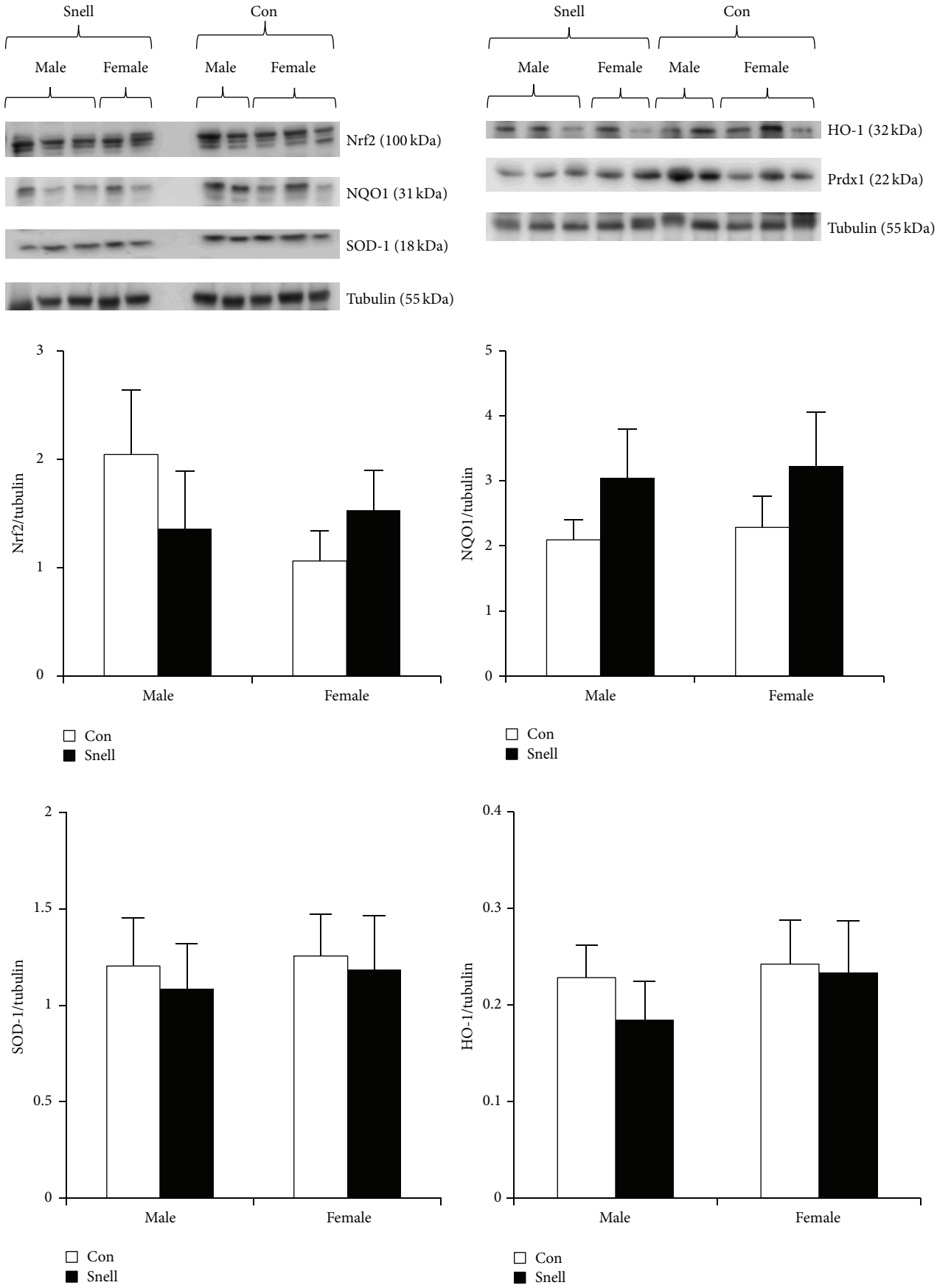


Figure 2: Nrf2 regulated protein expression in skeletal muscle from mice chronically fed with rapamycin. Nrf2, NQO1, and SOD-1 were significantly greater in skeletal muscle from male rapamycin (Rap.) treated mice. Chronic rapamycin feeding suppressed Prdx1 expression compared to controls. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting and normalized to tubulin, shown below the proteins from each blot. Data are expressed as a ratio of target protein to tubulin (mean ± SEM). $n = 6$ males and $n = 6$ females in each condition. UM-HET3 mice were generated by the offspring of crosses between (BALB/cByJ x C57BL/6J) F1 females and (C3H/HeJ x DBA/2J) F1 males. Mice were fed with chow mixed with encapsulated rapamycin at 14 mg/kg food (equivalent to 2.24 mg of rapamycin/kg body weight/day) or normal chow for 12 weeks in accordance with the original study describing lifespan extension [6].

phase I metabolizing enzymes in the liver, alongside heightened resistance against *tert*-butylhydroquinone [78], an oxidant known to activate Nrf2 [80]. Based on these data showing enhanced stress resistance in CL, we assessed Nrf2 and targets in skeletal muscle from 3-4-month-old CL mice. In this young cohort, we failed to find differences in Nrf2 targets between CL and control (Figure 4) in either male or female mice. As metabolic and hormonal status change throughout development in the CL mice, corresponding alterations in Nrf2 activation may occur. Given the early stages of this longevity intervention, few investigations have been conducted into developmental changes in CL mice and how metabolism and Nrf2 may interface to regulate longevity in this model. Future investigations are warranted to more completely assess whether Nrf2 mediates lifespan extension and what role Nrf2 may play throughout the lifespan of CL mice.

4.6. Humans. Humans are amongst the longest-lived mammals, with a maximum species lifespan potential (MLSP) of over 100 years and a predicted lifespan four times longer than estimated by body mass [42]. Exceptionally long-lived humans, centenarians, may have constitutively upregulated Nrf2, allowing them to better respond to cell stresses and minimize cell damage with age [81]. Investigations of centenarians who seem to reside in areas with high nutrient density and low caloric density diets support this hypothesis, as these diets may be characterized as a prolonged mild form of CR. Physical activity is one of the most effective interventions to prevent chronic disease and delay the detrimental cellular changes with age (reviewed in [82]). Acute exercise stress activates Nrf2 [83] and is associated with enhanced antioxidant capacity. Therefore, it is plausible that exercise, a longevity-promoting intervention in humans, may promote healthspan in part through activation of Nrf2. Nrf2 activators are currently undergoing phase II clinical trials for treatment of various human chronic diseases. Bardoxolone methyl therapy, a therapeutic pulmonary hypertension intervention [84], and dimethyl fumarate (BG-12), a currently approved therapy for relapsing-remitting multiple sclerosis, promote cytoprotective properties, in part through activation of Nrf2 [85]. A sulforaphane-based pharmaceutical is slated to begin trials for prostate cancer [86] following preliminary studies showing diminished Nrf2 signaling in prostate cancer that is restored by sulforaphane treatment [87]. Thus it appears that some of the human diseases associated with aging may be treated with Nrf2 activators. However, whether Nrf2 expression is higher in long-lived centenarians remains unknown. Further, whether Nrf2 activation can increase human lifespan or delay human aging is still unexplored.

4.7. Nrf2 Activity and Maximal Lifespan Potential. The simultaneous study of species with varying MLSP facilitates the identification of associations between species longevity and specific mediators of lifespan, such as Nrf2. A comparison of eight rodent species with widely divergent longevities ranging from 4 to 31 years yielded a positive association between Nrf2-ARE binding activity and MLSP [52]. Quantitatively, for a 10-year increase in lifespan, Nrf2 activity increased 1.4-fold. Surprisingly, the authors of this study found that Nrf2 protein



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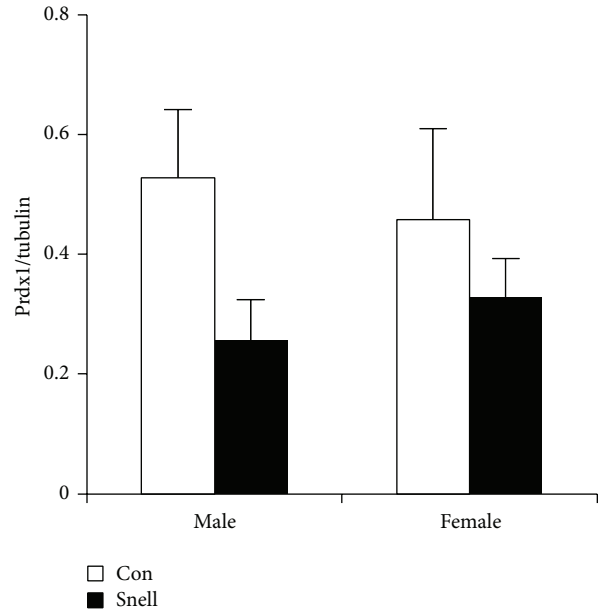


Figure 3: Nrf2 regulated protein expression in skeletal muscle from Snell dwarf mice. The *Pit1* mutation does not influence Nrf2 or Nrf2 target protein expression in skeletal muscle. Nrf2, NQO1, HO-1, SOD-1, and Prdx1 were analyzed by western blotting and normalized to tubulin, shown below the proteins from each blot. Densitometric analyses were conducted on the multiple band of Nrf2. Data are expressed as a ratio of target protein to tubulin (mean ± SEM). $n = 10$ males and 10 females of each genotype. Snell dwarf (*dw*/*dw*) and heterozygote (*dw*/+) control mice were bred as the progeny of (DW/J x C3H/HeJ) F1 *dw*/+ females and (DW/J x C3H/HeJ) F1 *dw*/*dw* males [4].

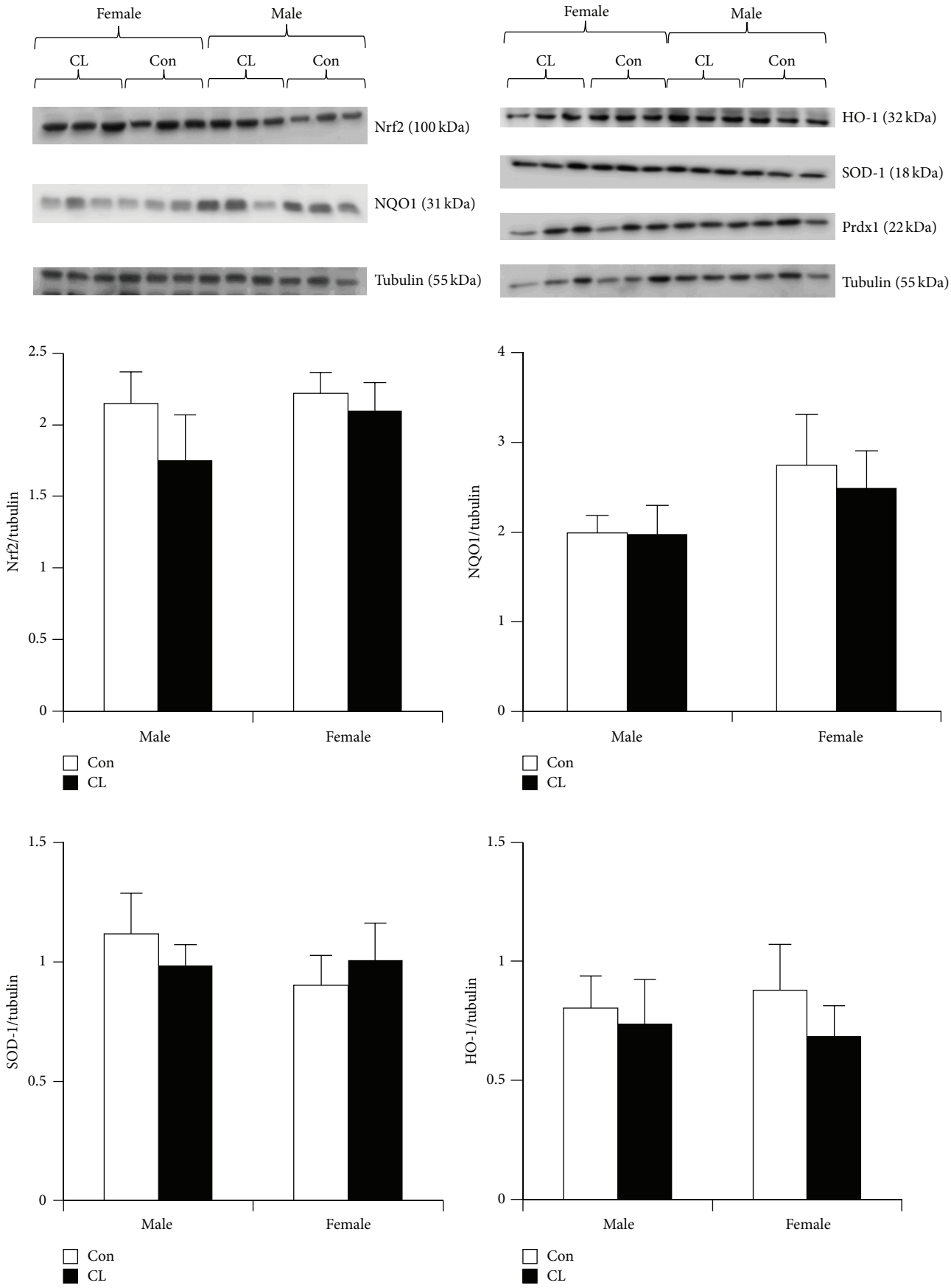
levels did not correlate with MLSP. Rather, mechanisms that control Nrf2 activity, such as Keap1 expression, significantly correlated with MLSP. Therefore, the authors posited that long-lived species are poised to effectively respond to cellular stresses associated with age and chronic disease due to the natural variation in Nrf2 activity. Together, these data support the notion that Nrf2 may be the "guardian of healthspan and the gatekeeper of species longevity" [3] and suggest that future investigations should elucidate whether activation of Nrf2 facilitates increases in healthspan and lifespan.

5. Mechanisms of Nrf2 Activation: Basal versus Inducible Activity

Our results of Nrf2 in long-lived mice presented here were obtained from young (3-4 and 7 months of age) mice that were not exposed to acute or chronic stresses. Young, unstressed animals generally do not display elevated Nrf2 signaling, as supported by investigations of young Nrf2 knockout mice, which do not have evidence of redox imbalance as assessed by either expression of Nrf2 target genes, overall cellular antioxidant status, or overt macromolecule oxidant damage [83]. However, when these Nrf2 knockout mice undergo an acute stress or when mice age, the ability to respond to and recover from the stress is attenuated. Thus, we suggest that the young mice in the long-lived cohorts reviewed here did not display enhanced Nrf2 signaling because there was no stressful stimulus from which to necessitate enhanced Nrf2 activation. Further, we assessed Nrf2 protein expression, as well as downstream Nrf2 target expression. Previous correlations between Nrf2 protein expression and MSLP, however, show no association between these two measurements [52]. Instead, Nrf2 activity, as assessed by Nrf2-ARE binding activity, along with proteins that influence Nrf2 activity, such as Keap1 expression [38], predicted MLSP. Therefore, we suggest that future investigations of Nrf2-mediated longevity should assess Nrf2 activity and the key proteins that regulate Nrf2 activation and nuclear localization. Further, we propose that these investigations be conducted in aged animals or younger animals administered a stressful stimulus. It is under these conditions that differences in Nrf2 signaling between long-lived and control animals should be most apparent.

6. Conclusions and Future Directions

Chronic disease incidence increases with age. Slowing the aging process limits the burden of chronic disease [2]. The transcription factor Nrf2, a proposed "master regulator of the aging process," regulates a wide battery of cytoprotective responses and helps attenuate age-related disease, and its activity is positively associated with species lifespan potential. Previous work of Nrf2 in long-lived models shows promise for slowed aging interventions (summarized in Table 1), with naked mole rats, an exceptionally long-lived species, showing enhanced Nrf2 signaling compared to shorter-lived mouse species. Caloric restriction and many of its pharmaceutical mimetics activate Nrf2 and protect against age-associated carcinogenesis, despite contradicting evidence showing no effect of Nrf2 expression in CR-induced longevity. Studies



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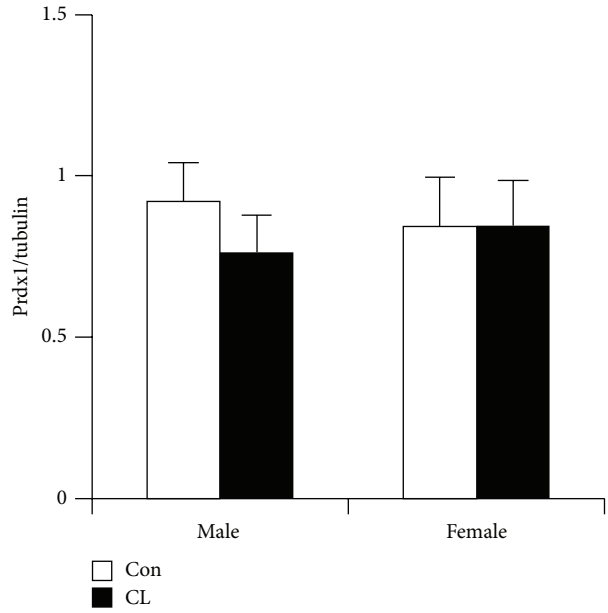

Figure 4: Nrf2 regulated protein expression in skeletal muscle from crowded litter mice. No significant sex or model differences were observed in skeletal muscle from crowded litter (CL) animals. Nrf2, NQO1, SOD-1, and Prdx1 were analyzed by western blotting and normalized to tubulin, shown below the proteins from each blot. Data are expressed as a ratio of target protein to tubulin (mean ± SEM). $n = 8$ males and $n = 8$ females in each condition. CL mice, 3-4 months old, were generated following the previously described procedure [5]. UM-HET3 litters were culled to eight pups (control) or supplemented by transfer of newborn mice to produce litters of 12 mice (CL). Litters were weaned at three weeks of age, after which both groups were fed ad libitum.

Table 1: Summary of Nrf2 expression, Nrf2 target expression, and Nrf2 activity in long-lived models.

Model	Tissue/cell type	Nrf2 expression	Nrf2 target expression (NQOl, HO-1, SOD-1, etc.)	Nrf2 activity (Nrf2-ARE binding)	Reference
Naked mole rat	Liver	↑	↑	↑	[25]
	Fibroblast	↑	↑	↑	[26]
Caloric restriction	Heart	↔	↑	N.R.	This manuscript
	CMVEC	↑	N.R.	↑	[27]
Rapamycin	SkM	↔	↔	N.R.	This manuscript
	C. elegans	↑	↑	↑	[28]
Snell	SkM	↔	↔	N.R.	This manuscript
	Heart	↔	↔	N.R.	This manuscript
	Fibroblast	↑	↑	N.R.	[29]
Crowded litter	SkM	↔	↔	N.R.	This manuscript
Humans	—	—	—	—	

Arrows indicate difference in the long-lived model compared to corresponding control, with ↑ indicating "greater in the long-lived model compared to control," ↔ indicating "no difference between long-lived model and control," and N.R. indicating "data were not reported in the investigation." To date, no experimental data for Nrf2 expression or activity have been reported in aged humans. CMVEC: cerebromicrovascular endothelial cells; SkM: skeletal muscle (mixed skeletal muscle: gastrocnemius, soleus, and plantaris).

of rapamycin treated, Snell dwarf, and CL mice suggest that primary cells from these animals are more resistant to cytotoxic stress, which may be linked to elevated Nrf2 signaling. Future investigations should identify whether aged animals from long-lived cohorts have enhanced Nrf2 signaling that may explain their stress resistance. We hypothesize that pharmaceutical, genetic, epigenetic, and dietary manipulations that extend lifespan will enhance Nrf2 activation at advanced ages and under stressful cellular conditions, contributing to stress resistance and extended healthspan. Further, future investigations of Nrf2 signaling in humans, and the ability of Nrf2 activation to prevent chronic disease associated with aging, will lend further insight into the role of Nrf2 activation as a possible longevity-promoting intervention.

Abbreviations

Nrf2: NFE2L2, nuclear factor (erythroid-derived 2)-like 2
SOD-1: Superoxide dismutase-1
NQO1: NAD(P)H hydroquinone oxidoreductase-1
HO-1: Heme oxygenase-1
Prdx1: Peroxiredoxin 1
CL: Crowded litter
CR: Caloric restriction
Rap: Rapamycin
MLSP: Maximum species lifespan potential.

Disclosure

Benjamin F. Miller and Karyn L. Hamilton are co-principal investigators.

Conflict of Interests

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

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 964321, 14 pages
http://dx.doi.org/10.1155/2015/964321

Review Article

Key Roles of Glutamine Pathways in Reprogramming the Cancer Metabolism

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Received 20 March 2015; Revised 7 April 2015; Accepted 8 April 2015

Academic Editor: Claudio Cabello-Verrugio

Glutamine (GLN) is commonly known as an important metabolite used for the growth of cancer cells but the effects of its intake in cancer patients are still not clear. However, GLN is the main substrate for DNA and fatty acid synthesis. On the other hand, it reduces the oxidative stress by glutathione synthesis stimulation, stops the process of cancer cachexia, and nourishes the immunological system and the intestine epithelium, as well. The current paper deals with possible positive effects of GLN supplementation and conditions that should be fulfilled to obtain these effects. The analysis of GLN metabolism suggests that the separation of GLN and carbohydrates in the diet can minimize simultaneous supply of ATP (from glucose) and $NADPH_2$ (from glutamine) to cancer cells. It should support to a larger extent the organism to fight against the cancer rather than the cancer cells. GLN cannot be considered the effective source of ATP for cancers with the impaired oxidative phosphorylation and pyruvate dehydrogenase inhibition. GLN intake restores decreased levels of glutathione in the case of chemotherapy and radiotherapy; thus, it facilitates regeneration processes of the intestine epithelium and immunological system.

1. Introduction

The development of cancer therapy is the urgent aim for science today. Growing knowledge about the metabolism of cancer cells provides new interesting hints concerning the metabolic targeting of the treatment and searching for new drugs inhibiting the growth of cancer [1]. The current paper presents the review of recently developed biochemical aspects of cancer metabolism and possible use of this knowledge for targeting the therapy into mainstream metabolic enzymes. Main emphasis is placed on the possible effect of glutamine (GLN) supplementation as a nutrient supporting both the cancer growth and organism to fight against the cancer. The individual kinds of tumors are characterized by different metabolic alterations which determine possible positive or negative effect of GLN supplementation. In the current paper, different aspects of cancer metabolism are discussed and analyzed in this context.

The aim of this paper is to point especially to these aspects of GLN metabolism that could be positively used while planning the treatment of the cancer patient and to point to conditions that should be fulfilled in order to make the positive effects of GLN supplementation surpass the negative ones.

2. Metabolism of Glutamine

2.1. Metabolism of Glutamine in Healthy Cells. GLN is one of 20 amino acids, commonly existing in every protein. The

mammal organism is able to synthesize it. GLN is a central point in the metabolism of majority of amino acids [2].

The first stage of amino acids metabolism consists in its change inside muscles tissue into the GLN and to lower degree into the alanine. The amino acids are metabolized to different tricarboxylic acid (TCA) cycle metabolites, enter TCA cycle, and go away as α-ketoglutarate (αKT) or alanine. αKT is next metabolized to GLN (Figure 1). Then, GLN is carried to other tissues like hepar, immunological system, intestinal tract, and fibroblasts where it is used up as the priority fuel [3, 4]. For this reason GLN comprises 60% of the total free amino acid pool in the blood plasma [5]. GLN synthesis rapidly decreases in the deficiency of energy in the cell. It occurs due to the fact that the energy from ATP and $NADH_2$ has to be supplied to the synthesis of glutamine (Figure 1).

First, in Figure 1 let us analyze two reactions that metabolize reciprocal reactions between GLN and glutamate (GLU). These reactions are catalyzed by two different enzymes. It results from the relatively high free Gibbs energy between both substances. The decay of GLN occurs easily and with relatively high loss of free energy. In the opposite, the ATP is necessary to synthesize GLN. Thus, in the case of low ATP level in the cell, the equilibrium of this reaction is strongly shifted to the left (GLU creation).

The relation between αKT and GLU is more complicated. Three enzymes are capable of catalyzing this reaction [4]. Transaminase (TA^{19}) carries the amine group from other amino acids into the αKT and it makes it possible to transfer the nitrogen into the urine cycle. The l-amino acid oxidase (AAO^{20}) is the enzyme that acts only in one direction. It catalyses the deamination of GLU and produces the ammonia for urine cycle. The hydrogen peroxide that is generated in this reaction is quickly decayed by catalase and it makes this reaction irreversible. This reaction is characterized by the significant loss of free energy, as well. The third, crucial enzyme is the glutamate dehydrogenase (GDH^{21}). This reaction is characterized with $K_{eq} \approx 1$. It means that the direction of the reaction depends on the metabolic state of the organism. It should be noted that the decay of GLU is combined with production of $NADH_2$ or $NADPH_2$. Both these molecules are high energy compounds and its free energy corresponds approximately to the energy of 3 ATP molecules. The activity of GDH^{21} is regulated by allosteric inhibitors, ATP, $NADH_2$ and by the activator, ADP, as well. Thus, this enzyme is active in the state of the lack of energy in the cell. It makes the biological sense. In the case of high energy level in the cell (high concentration of ATP, $NAD(P)H_2$, and αKT), the synthesis of GLU should dominate. This would open the minicycle between GLU and αKT catalyzed by GDH^{21} and AAO^{20} and would lead to the dissipation of energy stored in $NAD(P)H_2$. Thus, GDH^{21} is turned off. In the case of insufficient energy level in the cell the quick energy pathway from GLN and GLU is open. The result is the increase of GLU and GLN decay and the decrease in GLN levels in the blood. It causes the activation of muscle decay in order to maintain the GLN levels in the blood and provide the GLN to the cells that need it. The tissues that especially need the energy for the fight against the tumor and for repairing damages after chemo- and radiotherapy are the intestine epithelium and the immune system [6–21].

The gluconeogenesis is the second reason of quicker GLN metabolism in cancer patients, especially in the advanced state of the illness. It is known that cancers use the glucose as the main source of ATP, according to the so-called Warburg effect [22]. In the case of high glucose intake by the cancer, the glucose is restored in the liver from amino acids and GLN is the main source of amino acids in this process [23].

Thus, GLN is released from muscles in the periods of increased metabolic stress and the concentration of the intracellular GLN decreases by more than 50% [24, 25]. The resynthesis of GLN cannot be sustained on the exact level in these periods.

The suggested glutamine requirement after uncomplicated major operations, major injury, gastrointestinal malfunctions, and during cachexia is ~0.15–0.20 g glutamine per kg body weight. In patients with serious immune deficiency, after bone marrow transplantation, during episodes of sepsis, systemic inflammatory response syndrome, or multiorgan failure, the requirement is increased to ~0.3–0.5 g glutamine per kg body weight [5, 26–28].

It is difficult to provide this amount of GLN to the diet because generally the intake of natural GLN does not exceed 10 g. Thus, although the organism is capable of synthesizing the GLN, it should be treated as deficient nutrient in the periods of increased metabolic stress.

2.2. Metabolism of Glutamine in Cancer Cells. The cancer cells differ significantly from the normal cells in the intensity of the main metabolic pathways. The most important differences consist in the following:

(i) higher level of the oxidative stress accompanying the metabolic malfunctions [25, 29–36],

(ii) increased aerobic glycolysis and production of lactic acid (Warburg effect) [22],

(iii) production of ATP mainly in the aerobic glycolysis process [22, 34, 37, 38],

(iv) reduction of the activity of pyruvate dehydrogenase complex $PDHC^6$ that converts pyruvate to mitochondrial Acetyl-CoA [34, 37, 39] *(the upper indices at the enzyme abbreviation point to the reaction numbers in Figures 1 and 2)*,

(v) reduction of intensity of TCA cycle due to reduced activity of some TCA cycle enzymes [29–31, 33, 40, 41],

(vi) reduction of activity of the oxidative phosphorylation (OXPH) in the cytochrome chain [25, 29–31, 33],

(vii) reduced activity of the pentose phosphate cycle and production of $NADPH_2$ for fatty and nucleic acid synthesis mainly by the malic enzyme (ME^{16}),

(viii) high utilization of glutamine mainly for the production of $NAPDH_2$ that is used mainly for fatty and nucleic acid synthesis and for restoration of the

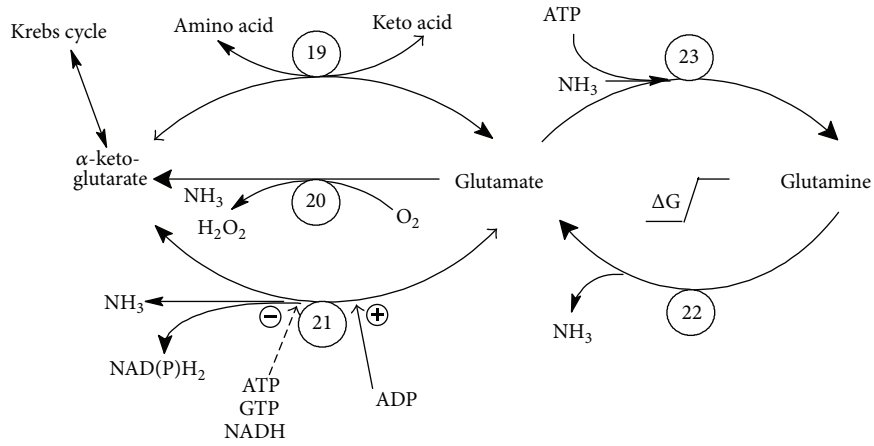

Figure 1: The connection between glutamine and tricarboxylic acid cycle: glutamine is metabolized to glutamate and next to α-ketoglutarate. Quick energy pathway from glutamine is open if glutamate dehydrogenase is activated by the low ATP and high ADP levels (as in majority of cancers). In the opposite, ATP and $NAD(P)H_2$ are necessary for the synthesis of glutamine which takes place especially in muscles. Reactions 20, 21, and 22 are the source of ammonia and ammonia induced autophagy. *(Indices at enzyme abbreviations point to reaction numbers in Figures* 1 *and* 2.*)*

molecules of TCA cycle that leave mitochondria for different anabolic purposes of the intensively dividing cells [42, 43],

(ix) high activity of ATP citrate lyase (ACL^{18}) in the cancer cells that produce cytoplasmatic Acetyl-CoA due to higher demand of the cancer cells for the fatty acid synthesis [44, 45],

(x) increased activity of hypoxia inducible factor (HIF-1α) in cancer cells both in the hypoxic [46–48] and normoxic conditions [49].

Glutamine is the controversial diet supplement that can modify the metabolism in both cancer and normal cells. Different aspects of GLN point either to the potential disadvantages or to potential benefits of its supplementation in cancer patients. GLN is recently presented to be one of the main nutrients for cancer growth [42, 50]. The most important role consists in supplying the reduced hydrogen in the form of $NADPH_2$. $NADPH_2$ is next utilized by cancer cells mainly for the fatty and nucleic acids synthesis. GLN can be also the significant source for gluconeogenesis in the liver, being the source of glucose for the cancer.

On the opposite, many papers point to the positive role of GLN supplementation. Let us summarize the potential beneficial effects of GLN supplementation [23]:

(1) stimulation of NK lymphocytes metabolism and intestine mucosa regeneration [12, 51–54];

(2) reduction of the side effects of chemo- and radiotherapy and especially the reduction of intestinal mucosa injures [13–21, 55];

(3) increase in some therapeutic effect of chemotherapy, among other things by the increased concentration of some drugs inside the tumor cells [15, 18].

In the next step, let us deal with these aspects that exhibit the potential slowing down effect on tumor growth, however, accompanied by increase in the resistance to starvation and/or oxidative stress:

(4) activation of the autophagy in the cancer cells due to the increased ammonia production. Autophagy is the process of self-digestion which makes it possible to recycle the cellular proteins and lipids into its metabolic precursors. This process promotes cell survival in the case of starvation or other metabolic stresses [42, 56];

(5) stimulation of the glutathione synthesis which inhibits the oxidative stress in healthy cells and contributes to the cancer cell growth inhibition [57–59]. It can make, however, the cancer cells more resistant to chemo- and radiotherapy [60].

In analyzing GLN intake as a possible positive or negative factor supporting the cancer growth and/or cancer treatment, one must take into consideration the differences between the metabolism of healthy and cancer cells [2]. The main problem of this analysis is the variety of metabolic changes characterizing different cancer types. Individual types of cancer metabolism should be analyzed with regard to the possible positive or negative effect of glutamine supplementation. Some methods of metabolic analysis of cancer cells are available [39, 61, 62].

At first, the division into two main groups of cancer types should be analyzed: cancers with the (a) normal and (b) reduced activity of pyruvate dehydrogenase complex ($PDHC^6$). The activity of this enzyme is deteriorated in the majority of cancers [34, 37, 39]. Theoretical analysis of GLN degradation presented in this paper shows that the GLN supplementation may be beneficial especially in cancers with reduced $PDHC^6$ activity.

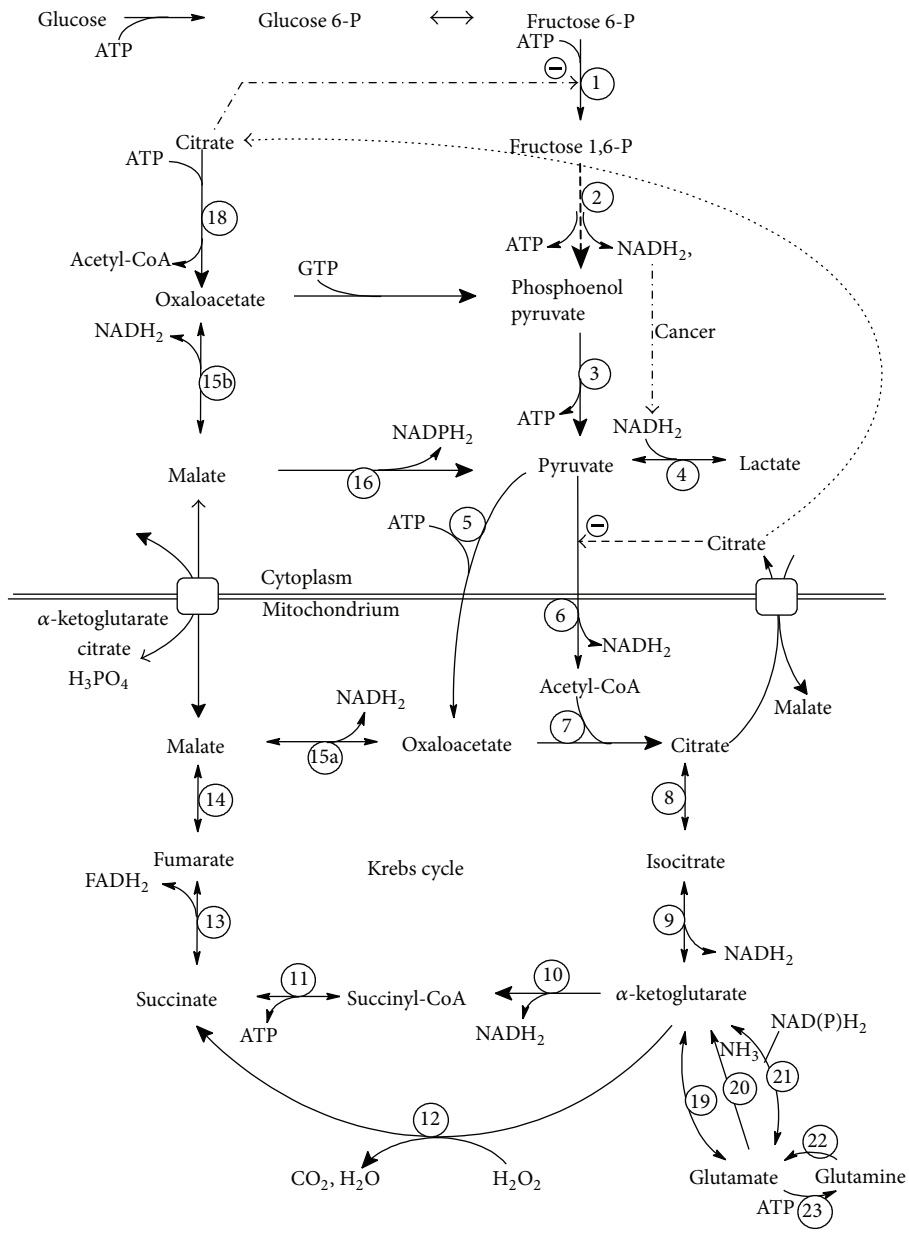

Figure 2: General scheme of glutamine (GLN) utilization in main metabolic pathways with details concerning ATP, $NADH_2$, and $NADPH_2$ production/utilization. PDHC[6] catalyzing the reaction pyruvate → Acetyl-CoA is the only reaction that is able to reduce the total number of TCA molecules in the cell; thus, every GLN degradation pathway goes to pyruvate. Two pathways can be defined (see also Figure 3): (1) glutaminolysis (GLL): GLN → αKT → SC → malate → (to cytoplasm) → pyruvate and (2) reverse-TCA (R-TCA): GLN → citrate → (*to cytoplasm*) → oxaloacetate → malate → pyruvate. The balance of GLL pathway is +1 $FADH_2$, +1 $NADPH_2$, and +1 ATP (alternatively 0 ATP in the case of reaction 12). The balance of R-TCA pathway is −2 $NADH_2$, −1 ATP, and +1 $NADPH_2$. The alternative $NAD(P)H_2$ from the reaction 21 is omitted. The conversion of Acetyl-CoA to malonyl-CoA in the fatty acid synthesis utilizes 1 additional ATP. Only one ATP molecule can be created directly during degradation of GLN in the GLL pathway (reaction 11). R-TCA utilizes rather ATP (for enzyme names, see enzyme abbreviation list).

3. Glutamine and Glutathione

Glutamine is significantly involved in the synthesis of glutathione (GSH)—the tripeptide that comprises three amino acids: glutamic acid, cysteine and glycine. This compound serves as a very important intracellular antioxidant and detoxication factor. Besides working as a scavenger of reactive oxygen species (ROS), GSH is involved in a variety of other metabolic functions such as DNA repair, activation of transcription factors, cell cycle regulation, modulation of calcium homeostasis, and regulation of enzyme activity. Most of these functions of GSH are related to its ability to maintain

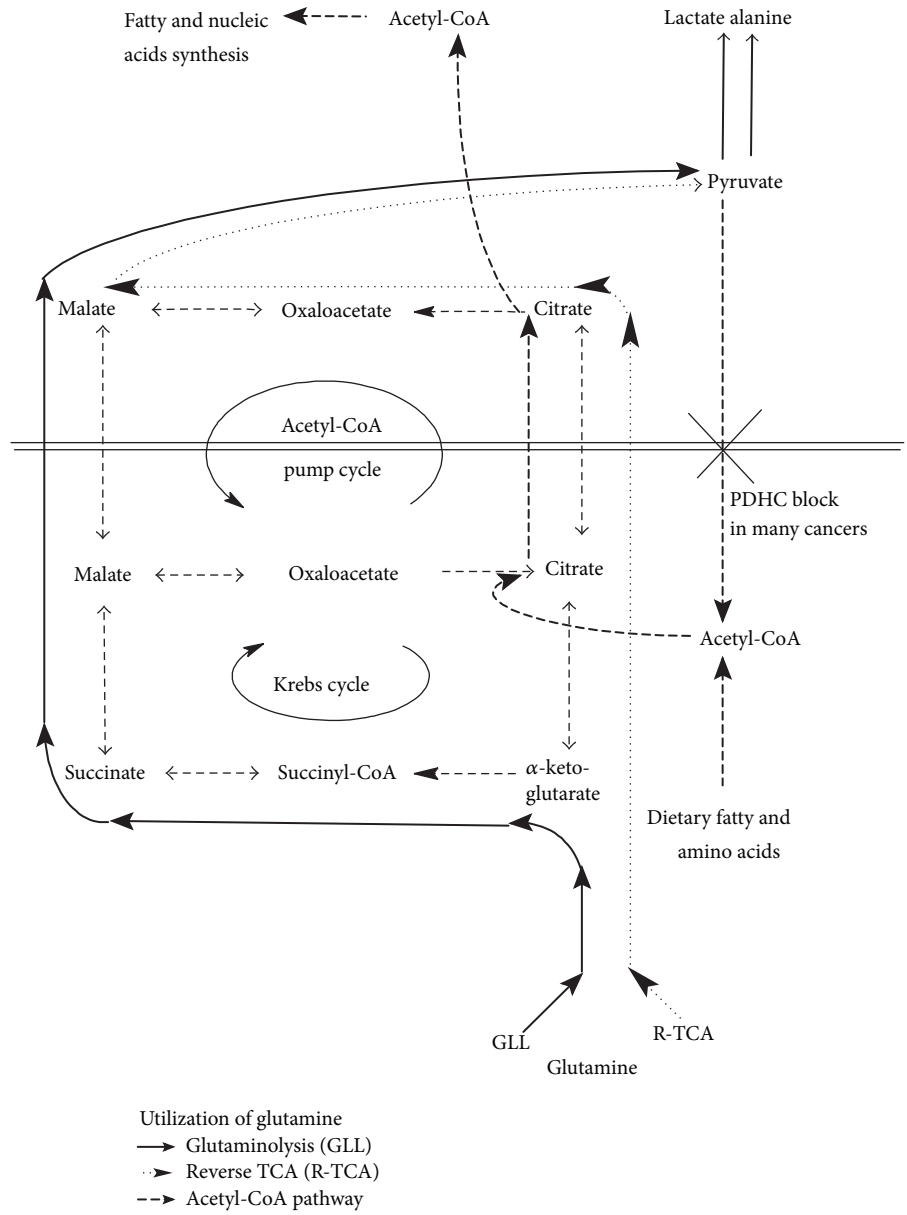

Figure 3: The schematic presentation of two glutamine metabolism pathways: glutaminolysis and reverse TCA and its connection with the Acetyl-CoA pump that transports Acetyl-CoA from mitochondria to cytoplasm. In the case of deteriorated PDHC[6] activity as in majority of cancers, the carbon skeleton of GLN can be metabolized only to lactate or alanine. The other pathways are rather negligible.

reduced cellular environment [60, 63]. Malignant diseases are accompanied by GLN deficiency and reduction of GSH in the host organism, which can be reversed by dietary GLN [58]. In addition, several reports suggested that the GSH constituent amino acids, including GLN, inhibit tumor promotion, at least in part, by their interference with GSH metabolism [57, 59].

The benefits of glutamine supplementation in cancers through the influence on GSH metabolism were broadly presented by Todorova et al. [60]. Tumor cells are shown to have higher concentration of reduced (active) form of GSH than the surrounding normal cells, which contributes to higher rate of cell proliferation and resistance to chemo- and radiotherapy. Therefore, selective tumor depletion of GSH presents a promising strategy in cancer treatment. Todorova et al. [60] have examined the effects of GLN on GSH levels in 7,12-dimethylbenz[α]anthracene- (DMBA-) induced mammary tumors and correlated the results with protein and mRNA expression of apoptosis-related proteins Bcl-2, Bax, and caspase-3 in tumor cells. The results have shown that GLN supplementation caused a significant decrease by 57% in tumor GSH levels and similar ratio GSH/oxidized GSH (GSSG) accompanied by upregulation of Bax and caspase-3 (apoptosis induced factors) and downregulation of Bcl-2

(apoptosis inhibiting factor). Bcl-2 is known to play a role in promoting cell survival and inhibition of apoptosis, while Bax, a member of the Bcl-2 family can induce apoptosis. Caspase-3 is the main apoptosis-induced enzyme. In the GLN supplemented group Bax mRNA has increased by 19% and caspase-3 mRNA by 30% and Bcl-2 mRNA decreased by 33%.

The importance of GSH depletion and reduction of GSH/GSSG ratio for stimulation of apoptosis has also been demonstrated in several *in vitro* models [59, 64–66]. GSH depletion was found to be necessary and sufficient to induce cytochrome c release, which is the key event in the apoptotic mitochondrial signaling pathway [67].

The next possible effect of GLN supplementation on the growth of cancer cells is the modulation of IGF-I and TGF-β1 concentrations in both cancer and normal cells. The proteins of insulin-like growth factor system (IGF) are known to play an important role in tumor genesis and inhibition of apoptosis. Transforming growth factor (TGF-β) is a cytokine involved in the process of cell migration, tumor vascularisation, and inhibition of cell proliferation. It is not explained if the observed effect of GLN supplementation on Bax, Bcl-2, capsace-3, IGF-I, and TGF-β1 levels is the direct effect of GLN or the intermediate effect of the altered GSH metabolism.

4. Glutamine and ATP Production in Cancer

Alterations in the cancer cell metabolism consist as a rule in the increased glycolysis [22], decreased TCA cycle activity [40, 41], and decreased oxidative phosphorylation (OXPH) [25] in mitochondria. The TCA produces $NADH_2$/$FADH_2$ and OXPH uses them for ATP production. Thus, ATP production in mitochondria is often deteriorated in cancer cells and the main source of ATP remains glycolysis [22]. The common feature in cancer is the overproduction of reactive oxygen species (ROS) in the OXPH chain that leads to the downregulation of the ATP production in mitochondria and to the oxidative stress [25, 29–33].

The analysis of GLN metabolism presented below takes into account its lack of ability to produce ATP, especially in the context of the deteriorated ATP production via TCA cycle and OXPH chain [68].

4.1. Relation between Glutamine and Krebs Cycle. In order to investigate the effects of GLN supplementation, let us analyze the decay paths for GLN. In Figure 1, let us analyze two reactions that metabolize reciprocal reactions between GLN and GLU. As discussed earlier, in the case of low ATP level in the cell, which takes place in the majority of cancers, one can expect that the equilibrium between these both reactions is strongly shifted to the left (GLU creation).

The activity of GA^{22} in the individual types of cancers is one of important features of cancer metabolism concerning GLN [69]. This enzyme is encoded by GLS and GLS2 gene [70]. Expression of GLS2 is necessary for cells to maintain GSH levels and silencing GLS2 increased ROS and oxidative damage of DNA. It is reported that gene GLS2 encoding "liver-type" isozyme of GA^{22} is highly expressed in normal adult liver but silenced in hepatocellular carcinomas [71, 72]. Thus, in the case of cancers having reduced activity of GA^{22}, the increased oxidative stress in the cancer cells is expected. GLN supplementation is, however, not expected to reduce this oxidative stress and support the growth of the cancer cells. The benefits of GLN supplementation may potentially surpass disadvantages in this case.

Now, let us analyze relations between αKT and GLU. The crucial enzyme here is glutamate dehydrogenase (GDH^{21}). As mentioned, this reaction relies on the metabolic state of the organism and the direction GLU $\rightarrow$ αKT dominates, due to the allosteric regulation. The activity of GDH^{21} is inhibited by ATP and $NADH_2$ and activated by ADP. It means that this enzyme is active in the state of energy shortage in the cell opening the quick energy pathway from GLN. It results in the GLN decay and decrease in GLN levels in the blood. The low GLN level activates the muscle proteins decay in order to maintain the GLN levels in the blood. In the case of high GLN utilization by the advanced cancer, this process causes the cancer cachexia. The decay of GLN takes place, however, also in healthy cells. In particular, the intestine epithelium and immune system need GLN as a primary fuel and both these systems are crucial against tumor and repair damages after chemo- and radiotherapy [10, 12, 14, 15, 73–75].

The reactions catalyzed by AAO^{20}, GDH^{21}, and GA^{22} produce ammonia. Ammonia produced in these reactions inside the tumor plays a crucial role in autophagy regulation [42, 56]. GLN supplementation is expected to increase the ammonia production, especially in the case of cancers with normal activity of GA^{22}. Stimulation of autophagy denotes potentially slower growth of the tumor and, at the same time, possibly higher resistance to chemo-/radiotherapy, starvation, and/or oxidative stress.

It is reported that in the case of glucose (GLC) deprivation, oxidation of GLN supports cell viability rather than its growth [76]. Thus, supplementation of GLN, especially accompanied by GLC withdrawal or glycolysis-inhibition therapy, may be treated as a potentially beneficial approach. An inverse process (αKT $\rightarrow$ GLU $\rightarrow$ GLN) occurs especially in muscles after the protein consumption. The reaction αKT $\rightarrow$ GLU is catalyzed by the transaminase (TA^{19}) and it is connected with the shift of $-NH_2$ pool from other amino acids into GLN. Thus, this pathway can take place especially in the abundance of other amino acids that can shift its $-NH_2$ group to αKT, as it is carried out in muscles.

4.2. Glutamine versus Pyruvate Dehydrogenase Complex ($PDHC^6$). TCA cycle and oxidative phosphorylation (OXPH) are two factors that are essential for the effective aerobic ATP production. Figure 2 shows the TCA cycle reactions, the final part of the glycolysis pathway, and additional reactions in the cytoplasm that are involved in the glucose and amino acids metabolism. One of the main changes in cancer metabolism observed in majority of cancers is the deterioration of the activity of pyruvate dehydrogenase complex ($PDHC^6$) that catalyses the reaction: pyruvate $\rightarrow$ Acetyl-CoA. This inhibition causes the reduced ATP creation [22, 34, 37–40]. Cancers with reduced $PDHC^6$

activity produce ATP mainly using glycolysis that is a significantly worse source of ATP than TCA + OXPH pathway. But also metabolic blocks of other TCA cycle enzymes or OXPH chain can lead to the deteriorated ATP production. Thus, the ATP level can be treated in many cases as an essential factor determining the velocity of tumor growth. Due to this observation, metabolism of the given nutrient should focus especially on its ability to support or inhibit ATP production.

In the analysis of cancer metabolism, it must be observed that one of the crucial points to be analyzed is the total number of TCA molecules in the cell (N_{TCA}), counted both in mitochondria and in cytoplasm. N_{TCA} is supplied by the stream of GLC and amino acids, but only one reaction is able to reduce N_{TCA}, namely, reaction catalyzed by PDHC[6]. Every other reaction presented in Figure 2 converts only the molecules of TCA without changing its total quantity in the cell. Thus, every considered pathway of GLN degradation must go to the pyruvate.

The alternative ways of pyruvate degradation are the reactions pyruvate → lactate and pyruvate → alanine and its successive excreting outside the cell. This process takes place in many cancer types and particularly in those which are characterized by the decreased PDHC[6] activity. Thus, the remaining PDHC[6] activity, LDH[4] activity, and the ability to remove lactate outside the cell are crucial points determining the velocity of all metabolic paths lying before these reactions.

GLN is a molecule that must be metabolized to pyruvate. It means that, in the case of cancers with the deteriorated PDHC[6], its degradation will not, in theory, occur quicker after supplementation, because GLN will "wait in line" to be metabolized by PDHC[6] or LDH[4]. The GLN-"waiting in line" is, however, not expected in healthy cells which are characterized by normal PDHC[6] activity.

In theory, another possibility for N_{TCA} reduction is entering the pentose phosphate pathway by conversion to glucose-6P. This process is, however, inhibited by high level of AMP being connected with low level of ATP. Those cancers with low PDHC[6] activity are characterized by low ATP level since the effective energy production in mitochondria is not efficiently supplied with Acetyl-CoA. Thus, one can expect that the reduction of N_{TCA} by pentose phosphate pathway is nondominating.

The other possibility for N_{TCA} reduction is the conversion to some nonessential amino acid and its use in the protein synthesis. One should, however, remember that the limitation for the protein synthesis in the cancer cells depends mainly on the essential amino acids pool. The standard diet supplies both the essential and nonessential ones. GLN may support only the pool of nonessential ones. The conclusion may be drawn that this way is also not a dominating one. The possible negative influence of GLN supplementation may be expected and, however, is the case of simultaneous supplementation of GLN and essential amino acids only. Taking the essential amino acid pool into account, one can assume that the influence of GLN supplementation should be more effective if the essential amino acid pool would be simultaneously reduced in the diet.

5. Utilization of GLN

The analysis of GLN utilization in cancer cells must be considered separately for the cancers possessing the reduced and normal activity of PDHC[6] and it must be performed especially with respect to ATP and reduced hydrogen production. Two pathways of conversion GLN → pyruvate can be considered depending on the activity of different enzymes and physiological state of the cell. They are presented in Figure 3 where they are called *glutaminolysis* (GLL) and *reverse TCA* (R-TCA). The basal way is GLL. Malate is the molecule that leaves mitochondria and it is next converted to pyruvate. This pathway is, however, reduced in the case of decreased activity of TCA enzymes catalyzing the reactions between αKT and malate that are described in some types of cancer [48, 77, 78].

The alternative pathway is R-TCA that consists in the conversion of αKT to citrate. Citrate leaves mitochondria and it is converted in the cytoplasm through oxaloacetate to pyruvate. This pathway is combined with one cycle of Acetyl-CoA pump that transports Acetyl-CoA from the mitochondria to the cytoplasm. Acetyl-CoA can be defined as the third most important (together with ATP and $NADPH_2$) crucial nutrient for the tumor growth. This pathway is, however, from N_{TCA} point of view, independent of Acetyl-CoA pump cycle which transports Acetyl-CoA to the cytoplasm and $NADH_2$ from cytoplasm to mitochondria.

The source for Acetyl-CoA can be the pyruvate, dietary fatty acids, and amino acids. Pyruvate can be effectively converted to Acetyl-CoA only if the activity of PDHC[6] is maintained. The process of Acetyl-CoA transport to the cytoplasm for the purpose of fatty and nucleic acid synthesis was analyzed in the subject literature [44, 45]. The current paper focuses rather on the importance of proportion $ATP/NADPH_2$ in cancer cell because GLN supplementation seems not to influence the Acetyl-CoA pump. The amount of Acetyl-CoA appears important only in the case of cancers having deteriorated both PDHC[6] activity and fatty/amino acid transport and/or their metabolism to Acetyl-CoA in mitochondria. On the other hand, it is possible that the Acetyl-CoA pump can be also deteriorated in some cases, making Acetyl-CoA the vital nutrient for the cancer growth. The crucial enzyme of Acetyl-CoA pump is cytoplasmatic ATP citrate lyase (ACL[18]). Its activity is often increased in cancer cells and its inhibition is proposed to be the target for cancer treatment [44, 45]. GLN can be the source of Acetyl-CoA only in the case of normal or close to normal activity of PDHC[6] [43].

5.1. Glutaminolysis. Now, let us analyze in details, two GLN → pyruvate pathways. GLN enters TCA cycle before the α-ketoglutarate dehydrogenase complex (KGDHC[10]). It means that the proper activity of both KGDHC[10], succinyl-CoA synthetase (SCS[11]), succinate dehydrogenase (SDH[13]), and fumarate hydratase (FH[14]) is necessary for GLN utilization inside the TCA cycle. This process (*glutaminolysis,* GLL; see Figures 2 and 3) is the basal pathway of GLN metabolism in the normal cells with the only exception that

the pyruvate is metabolized to Acetyl-CoA and next enters the TCA cycle. Enzymes KGDHC[10] and SDH[13] are present only in mitochondria. Thus, the proper metabolism of GLN in the GLL pathway requires proper functioning of this part of TCA cycle in mitochondria.

GLN is able to provide directly in the GLL pathway only 1 molecule of ATP in the reaction of succinyl-CoA to succinate (SDH[11]). It is twice less than the amount of ATP produced from glucose (GLC) in the anaerobic glycolysis (2 ATP/1 GLC). The remaining reactions of GLN degradation produce energy through $NADH_2$ (GDH[19], KGDHC[10], and MDH[15ab]), $FADH_2$ (SDH[13]), or $NADPH_2$ (GDH[19], ME[16]). Moreover, the only molecule of ATP may not be created in the existence of the oxidative stress in cancer cell. Fedotcheva et al. [79] show that the nonenzymatic decarboxylation of αKT (reaction 12), pyruvate, and oxaloacetate induced by H_2O_2 results in the formation of succinate, acetate, and malonate, respectively. The only molecule of ATP may not be created in this situation. It is proved that many cancer cells (lung, breast, kidney, prostate, colon, liver, skin, thyroid, and bladder) present the existence of oxidative stress according to mtDNA mutations followed by impaired TCA cycles enzyme synthesis and/or OXPH chain [29–31, 33]. Thus, one can assume that the nonenzymatic decarboxylation without synthesis of the only ATP molecule may occur common in cancers.

Many experiments point to the decreased activity of both different TCA enzymes and OXPH in cancer cells [29–31, 33]. Blocking or reducing some of these enzymes makes it difficult to metabolize GLN in the GLL pathway.

The decreased activity of KGDHC[10] catalyzing the reaction αKT → succinyl-CoA can be expected in tumors having deteriorated activity of PDHC[6] since PDHC[6] and KGDHC[10] are twin enzymatic complexes that can undergo similar regulatory processes [80].

The additional effect can be connected with the nonenzymatic conversion of oxaloacetate to malonate. Malonate is known to be mitochondrial toxin [81] considered as the competitive inhibitor of SDH[13] and trigger of superoxide radicals [82]. The malonate degradation in the cell depends on the concentration of ATP and Mg^{2+} [83]. The concentration of these two compounds is decreased in the majority of cancers causing possible further decrease in activity of this part of TCA, due to possible increased malonate concentration in the cancer cell.

It can be observed that the presented pathway of GLN decay does not produce significant ATP amounts in the cancer cells. $NADH_2$ and $FADH_2$ can be the source of ATP only after they enter the OXPH chain. The decrease in OXPH protein contents, respiratory chain activities, and mitochondrial DNA amounts in cancer are well evidenced, particularly in CCRCs [78, 84–86] but also in other types of cancers [87–90]. Thus, the ability of GLN to support ATP production is expected to be strongly reduced in the cancers having lowered OXPH activity.

However, in the case of cancers with OXPH chain and GLL pathway working properly, GLN supplementation may support ATP production which is generated from $NADH_2$ and $FADH_2$ in the OXPH chain. It can take place, however, if the twin enzyme complexes (PDHC[6] and KGDHC[10]) have different activities: PDHC[6] is inactive and KGDHC[10]—active.

Concluding, mainly cancers having deteriorated OXPH activity or αKT → malate part of TCA cycle can be treated as potentially surpassing the benefits over the disadvantages while supplementing GLN.

5.2. Reverse TCA. The GLN metabolism can take place in the case of decreased activity of KGDHC[10], SCS-A[11], SDH[13], or FH[14]. GLN can be metabolized to citrate in the R-TCA reactions (IDH[9] and Aconitase[8]). Next, citrate is transported to the cytoplasm (see Figures 2 and 3). Here, citrate inhibits two enzymes: phosphofructokinase (PFK[1]) and pyruvate dehydrogenase complex (PDHC[6])—the key enzymes of glycolysis. Thus, in the case of cancers possessing deteriorated GLL pathway, GLN supplementation can slow down the glycolysis (the main source of ATP) in some types of tumor by the agency of citrate [91]. This phenomenon is mainly expected in the case of tumors possessing deteriorated GLL pathway which forces conversion of GLN to citrate. Next, the citrate is metabolized in the cytoplasm by ATP citrate-lyase (ACL[18]) to Acetyl-CoA and oxaloacetate. This reaction uses 1 molecule of ATP. Oxaloacetate can be metabolized to malate (MDH[15b]) and next by using the NADP-malic enzyme (ME[16]) to pyruvate. Both products of ME[16] ($NADPH_2$ and Acetyl-CoA) can be the substrates to fatty and nucleic acid synthesis. The use of Acetyl-CoA for this process utilizes one additional molecule of ATP for its activation to malonyl-CoA [92]. There is, however, also a disadvantage of this pathway. The reactions catalyzed by cytoplasmatic MDH[15b] and ME[16] convert cytoplasmatic $NADH_2$ to $NADPH_2$, which leads to the additional glycolysis reaction producing $NADH_2$ without accompanying lactate creation that produces extra 2 ATP molecules. Summing up, the ATP production balance of R-TCA pathway is close to zero. The described process occurs particularly in the case of deteriorated activity of KGDHC[10], SCS-A[11], SDH[13], or FH[14]. The normal pathway of TCA cycle cannot follow in that case.

GLL and R-TCA pathways are the only two fundamental pathways for GLN conversion to pyruvate. The other pathways could be considered as the connections of one of the above defined pathways and of another cycle of reaction. Namely, if GLL pathway was joined with Acetyl-CoA pump cycle (see Figure 3), then the following pathway could be obtained: GLN → *αKT* → *SC* → *malate* → *oxaloac* → *citrate* → (*transport to cytoplasm*) → *oxaloac* → *malate* → *pyruvate*.

The analysis of GLN metabolism presented above leads to the main conclusion that GLN cannot be, in practice, the significant source of ATP. In the case of lowered ATP level in the cancer cell, the supplementation of GLN does not supply the cell significantly in ATP and the supplementation of GLN may be beneficial rather than disadvantageous. It should be, however, stressed that the beneficial effect is expected particularly if the ATP reduction is supported by accompanying glucose withdrawal in the diet and/or

glycolysis inhibition therapy that decreases ATP being a crucial metabolic substance for tumor growth.

6. Glioblastoma

All considerations presented in the former section concerned mainly cancers with the deteriorated PDHC[6]. Some cancers possess, however, the normal or close to normal activities of PDHC[6] and OXPH chain. The example is the glioblastoma multiforme (GM) [32, 43] that shows normal activity of the TCA cycle and only partially deteriorated ability to produce the ATP from fatty acids in the OXPH chain [2]. The activity of pyruvate kinase PC[5] in place of PDHC[6] is deteriorated in these cells. The escape of citric acid from mitochondria for fatty acid synthesis (Acetyl-CoA pump) is also observed. The oxaloacetate used for citric acid synthesis in the mitochondria is derived to a greater extent from the GLN and the Acetyl-CoA from glucose in this case. It must be observed that, due to proper activity of PDHC[6], pyruvate is able to enter TCA cycle and to be the source for ATP production [93]. Thus, it can be concluded that the supplementation of GLN is rather not recommended in this case because GLN can be the source of both ATP, $NADPH_2$, and Acetyl-CoA. DeBerardinis et al. [43] showed GM by using the ^{13}C NMR spectroscopy that in the case of abundance of both GLN and GLC about 1/3 of Acetyl-CoA for fatty acid synthesis comes from GLN and about 2/3 from GLC. This is possible due to maintained PDHC[6] activity.

Since the number of TCA molecules in the cell is reduced by PHDC[6] and not increased by PC[5], GM cells need to be continuously supplied with TCA molecules for maintaining the metabolic pathways. In this case, the main source is GLN as the most important amino acid in blood that can be converted to some TCA molecule.

Many cancers lower ATP production in the case of GLC withdrawal [39, 94] but not the GM [32]. In the case of glucose abundance, about 84% of GLC is metabolized to lactic acid, 9% is metabolized to alanine, and 5% is metabolized in the OXPH chain [43]. This proportion reflects the typical Warburg effect. In the case of GLC withdrawal, GM stops the ATP production in the glycolysis pathway but follows the OXPH chain and the total ATP level does not decrease in the cancer cell. This process is combined with high ROS production in the OXPH chain and the GM cells start to die out due to the increased oxidative stress [32]. Thus, the supplementation of GLN as the source of $NADH_2/FADH_2$ for the impaired OXPH chain producing many ROS can be potentially beneficial in this case. It should be, however, accompanied with the strong carbohydrate reduction in the diet and/or with the glycolysis inhibition therapy and/or with the increase in amino- and fatty acids in the diet that supports TCA cycle and in this way the oxidative stress in the cell. The detailed "*in vivo*" analysis of this approach must be, however, performed to answer if the destroying effect of the oxidative stress overcomes the tumor growth stimulation.

7. Glutamine and HIF-1α

It is proved that many tumors show the overexpression of hypoxia inducible factor (HIF-1α). It is reported that HIF-1α can be activated by a number of other oncogenes even under normoxic conditions [49]. Thus, the increased activity of HIF-1α is probably a feature of many tumors.

HIF-1α is degraded by one of three different HIF prolyl hydroxylases. They are members of a superfamily of iron and α-ketoglutarate-dependent dioxygenases [95, 96]. The overexpression of HIF-1α occurs due to the inhibiting activity of accumulated succinate (SC) on the HIF prolyl hydroxylases. In other words, HIF-1α activation is stimulated by lowered αKT/SC ratio.

The overexpression of HIF-1α leads to the increased transcription of genes encoding glycolysis enzymes like aldolase[2], pyruvate kinase[3], and LDH-A[4] [46–48]. The target is also the pyruvate dehydrogenase kinase that inactivates the PDHC[6]. The inhibition of PDHC[6] by HIF-1α causes the accumulation of pyruvate and lactate. If the removal of lactate from the cancer cell is not sufficient then the other probable consequence of this inhibition is the accumulation of preceding metabolites like malate, oxaloacetate, fumarate, and alternatively the succinate. In the case of GLN deficiency (and successive deficiency of αKT), this situation can lead to the decreased ratio of αKT/SC and further stabilization of HIF-1α.

GLN is a quick source of αKT in the cancer cell. The inhibiting effect of αKT supplementation on HIF-1α activity is described by Matsumoto et al. [97]. The *in vitro* antiproliferative effect of αKT on some kinds of tumors was also presented by Brzana et al. [98]. Thus, one of the possible positive effects of GLN supplementation can be making αKT/SC ratio increased in cancer cells which can reduce the overexpression of HIF-1α.

Many other beneficial effects of αKT supplementation are described by Harrison and Pierzynowski [99]. It suggests that some of the benefits of GLN supplementation are probably obtained by the agency of αKT. The reduction of HIF-1α should lead to the reduction of overexpressed glycolysis and to restoration to some degree of the PDHC[6] activity. It is recently shown that restoration of PDHC[6] activity through dichloroacetate in cancer cells can promote the apoptosis of cancer cells [37]. On the other hand, activation of PDHC[6] can support the energy production in the cancer cells and the effect of GLN could be negative if the apoptosis caused by HIF-1α inhibition did not occur. However, the detailed analysis of this problem was not found.

8. Gluconeogenesis

Main problem related to the GLN intake by cancer patients is gluconeogenesis. Gluconeogenesis is one of the reasons for quicker GLN metabolism in cancer patients, especially in severe stages of the illness. It is known that cancers use the glucose as the main source for ATP production [22]. In the case of high glucose intake by the cancer, glucose is restored in the liver from amino acids and GLN is the main source

for this process. Thus, there is a potential danger of GLN supplementation to produce glucose for ATP production in the tumor. The main aim of GLN supplementation in cancer patients is to support the immunological system, intestine tract, and glutathione synthesis and inhibit the cancer cachexia. This problem can be probably solved by experiments that will monitor the concentration of GLC and GLN in the plasma in different dietary and/or therapy conditions. Most effective doses and application time of GLN should be found in order to substantially support the organism rather than gluconeogenesis and cancer. The optimal effect of GLN supplementation is expected when it is accompanied by gluconeogenesis and/or glycolysis inhibition to slow down ATP production. Supplementation of GLN seems to make sense if the concentration of both GLN and GLC in plasma is low. GLN supports, in this case, the regeneration of the organism without significant support of tumor growth. Support for the intestine tract (e.g., after chemo- or radiotherapy) should be made orally, but supporting the immunological system and other healthy tissues should be made rather intravenously to omit the effect of first GLN passage through the liver. The elementary doses of GLN should be probably small enough to be consumed by healthy tissue in particular rather than converted by liver to GLC.

9. Summary

Based on the analysis of GLN metabolism, it can be concluded that if the OXPH chain is deteriorated, GLN cannot be an effective source of ATP for the cancer cell regardless of the metabolic pathway. The benefits of GLN supplementation should be probably more significant if they are accompanied by significant carbohydrate restrictions in the diet and by glycolysis and/or gluconeogenesis inhibition therapy, which will reduce the ATP level in the cancer cells that have the deteriorated mitochondria, but not in the normal cells with correctly functioning mitochondria. GLC and GLN monitoring in plasma is recommended in order to find the optimal doses and intervals of GLN that minimize gluconeogenesis effects in the liver. On the other hand, the apoptosis induction effect of GLN supplementation via HIF-1α inhibition is also possible. Supporting the intestine tract, immunological system and glutathione synthesis by GLN are especially expected to be beneficial for patients undergoing chemo- and radiotherapy. The supplementation may be highly effective after the chemo-/radiotherapy to avoid any therapy resistance effects. The other problem of GLN supplementation is its inhibition of cancer cachexia. GLN can be considered to be applied as a part of palliative care.

The dose dependent effect is expected to be strongly nonlinear. In the case of small doses, the positive supplementation effect seems to depend on the reduction of GLN deficiency in healthy cells and supporting the intestine epithelium and immunological system. Medium doses are expected to support cancer metabolism rather than the organism itself. Big doses may be considered to induce HIF-1α inhibition that could activate PDHC[6] and, next, apoptosis. αKT supplementation can be considered as an alternative approach that is expected to exhibit all effects of GLN with the exceptions concerning ammonia induced autophagy and promotion of GSH synthesis [100].

The following individual cancer metabolism features are very important in analyzing the effect of GLN supplementation: PHDC[6] activity, individual TCA enzymes activity profile, malonate concentration, functioning of OXPH chain, oxidative stress, and HIF-1α activity.

Abbreviations

Enzymes (The Upper Indices Point to the Reaction Numbers in Figures 1 and 2)

AAO[20]: l-Amino acid oxidase
ACL[18]: Citrate-lyase
FH[14]: Fumarate hydratase
GDH[21]: Glutamate dehydrogenase
GA[22]: Glutaminase
GS[23]: Glutamine synthase
IDH[9]: Isocitrate dehydrogenase
KGDHC[10]: Alfa-ketoglutarate dehydrogenase complex
LDH[4]: Lactate dehydrogenase
MDH[15]: Malate dehydrogenase
ME[16]: Malic enzyme
PC[5]: Pyruvate carboxylase
PDHC[6]: Pyruvate dehydrogenase complex
PFK[1]: Phosphofructokinase
SCS-A[11]: Succinyl-CoA synthase
SDH[13]: Succinate dehydrogenase
TA[19]: Transaminase.

Other Terms

αKT: Alfa-ketoglutarate
GLC: Glucose
GLN: Glutamine
GLU: Glutamate
GM: Glioblastoma multiforme
GSH: Glutathione
HIF-1α: Hypoxia inducible factor
OXPH: Oxidative phosphorylation
SC: Succinate
TCA: Tricarboxylic acid cycle.

Conflict of Interests

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

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 416738, 13 pages
http://dx.doi.org/10.1155/2015/416738

Research Article

Glucose Oxidase Induces Cellular Senescence in Immortal Renal Cells through ILK by Downregulating *Klotho* Gene Expression

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Received 8 April 2015; Accepted 17 May 2015

Academic Editor: Claudio Cabello-Verrugio

Cellular senescence can be prematurely induced by oxidative stress involved in aging. In this work, we were searching for novel intermediaries in oxidative stress-induced senescence, focusing our interest on integrin-linked kinase (ILK), a scaffold protein at cell-extracellular matrix (ECM) adhesion sites, and on the *Klotho* gene. Cultured renal cells were treated with glucose oxidase (GOx) for long time periods. GOx induced senescence, increasing senescence associated β-galactosidase activity and the expression of p16. In parallel, GOx increased ILK protein expression and activity. Ectopic overexpression of ILK in cells increased p16 expression, even in the absence of GOx, whereas downregulation of ILK inhibited the increase in p16 due to oxidative stress. Additionally, GOx reduced *Klotho* gene expression and cells overexpressing Klotho protein did not undergo senescence after GOx addition. We demonstrated a direct link between ILK and *Klotho* since silencing ILK expression in cells and mice increases *Klotho* expression and reduces p53 and p16 expression in renal cortex. In conclusion, oxidative stress induces cellular senescence in kidney cells by increasing ILK protein expression and activity, which in turn reduces *Klotho* expression. We hereby present ILK as a novel downregulator of *Klotho* gene expression.

1. Introduction

Cellular senescence is a permanent cell cycle arrest accompanied by the alteration of the cell structure and functions. Senescence can be promoted in response to stress stimuli that result in DNA damage or by the replicative life of cells. Senescence induction can have beneficial effects in pathologies such as cancer or wound healing; however, the permanent presence of senescent cells in several tissues can induce or increase some pathologies [1]. Cellular senescence was discovered by Hayflick and Moorhead [2], whose experiments showed the limited number of cell divisions in cultured cells. This limited proliferation of cells is promoted by a shortening of telomeres in a chromosome [3], which, if too short, can induce cell senescence [4]. Moreover, cell senescence can be induced by other telomere-independent mechanisms, such as oncogene activation [5], DNA damage [6], or stressful stimuli called stress-induced premature senescence [7, 8]. Recent studies from our group have demonstrated that hyperosmolar stress induced by high glucose concentration and Amadori products promoted premature senescence in kidney cells [9, 10].

Senescent cells are not able to proliferate and present some morphological and biochemical changes, such as increased activity of β-galactosidase (SA-β-GAL) due to an increase in lysosomal content [11] and in the expression of cell cycle inhibitors such as p16 or p53 tumor suppressor genes.

Oxidative stress is involved in many diseases, such as cancer and inflammation, and it plays a relevant role in aging [12–14]. The evidence that reactive oxygen species (ROS) are involved in the senescence process has been provided by the use of antioxidant compounds that can prevent or delay cellular senescence [15]. ROS such as H_2O_2 or superoxide anions produce acute damage to proteins, lipids, and DNA. In addition, some works have shown that they can induce aging by the activation of senescence genes through MAPK cascade and their downstream kinase effector p38 [16, 17] and replicative senescence [14]. Thus, it has been shown that ROS can downregulate Klotho [18, 19], an aging-related kidney-secreted hormone with antioxidant properties [20]. Klotho is a glucuronidase activity protein which acts as a coreceptor for fibroblast growth factor 23 (FGF23) and regulates phosphate homeostasis and IGF-1 signaling [21–23]. Klotho is downregulated in chronic kidney diseases (CKD) where aging features are accelerated. In fact, Klotho-deficient mice show multiple age-related pathologies and a short lifespan.

In the last years, the integrin-linked kinase protein (ILK) has emerged with a relevant role in kidney and vascular physiology and physiopathology, mediating a relationship between extracellular matrix (ECM) and intracellular processes. ILK is a protein which is part of a cytoplasmic multiprotein complex and mediates the interaction between ECM proteins and intracellular pathways by a serine-threonine kinase activity [24]. This interaction between ECM proteins and the integrin receptor activates ILK, which regulates cellular processes such as proliferation, survival, migration, and fibrosis [25, 26]. However, only few works have established a relationship between ILK and the senescence process [27, 28] and its role in the aging process is still unknown.

In the present work, we test the hypothesis that ROS induce premature cellular senescence in renal cells through the increase in ILK expression and activity and the down-regulation of Klotho protein expression, which modulate the expression of senescence genes such as p16.

2. Material and Methods

2.1. Reagents. Culture plates, culture media, blueStar-prestained protein marker, BCA protein assay reagent, CL-Xposure films, and SuperSignal West Pico detection system were from Cultek (Thermo Fisher Scientific, Madrid, Spain). The secondary horseradish peroxidase-conjugated goat anti-mouse IgG was from Dako Cytomation (Glostrup, Denmark). The secondary horseradish peroxidase-conjugated goat anti-rabbit IgG was from Merck Millipore (Darmstadt, Germany). Acrylamide-bisacrylamide was from Hispanlab-Pronadisa (Madrid, Spain). Electrophoresis equipment and PVDF membrane were from Bio-Rad Laboratories (Richmond, CA, USA). Protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). The β-galactosidase substrate (C12FDG, 5-dodecanoyl-aminofluorescein di-β-D-galactopyranoside), the mounting medium ProLong Gold antifade reagent with DAPI, Trizol reagent, OptiMEM medium, Lipofectamine, ILK siRNA, and negative control used as a nonsilencing control were from Life Technologies (Paisley, UK). Polyclonal rabbit anti-p53 and monoclonal rabbit anti-p16 were from Abcam (Cambridge, UK). Polyclonal rabbit anti-ILK1, monoclonal rabbit antibodies for anti-phospho-GSK-3β (Ser9) and anti-GSK-3β, and the buffer kinase 10X, ATP, and GSK-3β fusion protein were from Cell Signaling Technology Inc. (Boston, MA, USA). Polyclonal rabbit anti-4-hydroxy-2-nonenal adducts were from Alexis (Farmingdale, NY, USA). Glucose oxidase, bovine serum albumin (BSA), polyclonal rabbit anti-actin, and monoclonal mouse anti-GAPDH were from Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO, USA).

2.2. Culture Cells. Immortal mouse cortical tubule (MCT) cells are a cultured line of proximal tubular cells harvested originally from the renal cortex of SJL mice [29]. The cells were maintained in culture in DMEM supplemented with penicillin 100 U/mL and streptomycin 100 μg/mL and 10% heat-inactivated fetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO_2 at 37°C, as previously described [29, 30]. Embryonic kidney epithelial human cells, HEK293T cells, were purchased from American Type Culture Collection (Rockville, MD, USA) and they were grown in Eagle's Minimum Essential Medium supplemented with 10% FBS. Culture media were changed every 2 days. For the experiments, both types of cells were treated with 2.5 mU/mL glucose oxidase (GOx) at different times.

2.3. Protein Extraction and Immunoblot Analysis. Total protein extracts from cultured cells were obtained by using the Lysis Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100, and 10 mM sodium pyrophosphate) containing a protease inhibitor cocktail. The resulting solution was spun at 13,000 rpm for 30 min at 4°C. The protein concentration was determined by BCA protein assay. Equal amounts of protein (30 μg protein/lane) from each sample were separated on SDS-polyacrylamide gels under reducing conditions and transferred onto PVDF membranes. Membranes were blocked with 5% nonfat dry milk or 5% BSA in Tween Tris buffered saline (TTBS) (20 mM Tris–HCl pH 7.5, 0.9% NaCl, and 0.05% Tween 20) for 1 h at room temperature and then incubated overnight at 4°C with different specific antibodies for mouse (anti-p53, anti-p16, anti-ILK1, anti-phospho-GSK-3β (Ser9), and anti-GSK-3β). Phospho-GSK-3β and GSK-3β were used to measure ILK activity. After washing in TTBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG as secondary antibodies. The immunoreactive bands were visualized with the SuperSignal West Pico detection system after 30 sec of exposure to CL-Xposure films. Then blots were reblotted with a rabbit anti-actin antibody in order to normalize p53, p16, or ILK1 levels.

Proteins from kidney cortex portions were obtained using the Lysis Buffer and, after incubation on ice for 30 min, tissues were homogenized and spun at 13,000 rpm for 30 min at 4°C.

2.4. Detection of Senescence Associated β-Galactosidase Activity by Fluorescence Confocal Microscopy. To determine

cellular senescence, SA-β-GAL activity was measured by fluorescence confocal microscopy, using the fluorogenic substrate C12FDG [11, 31]. MCT cells grown in microscope cover glasses were treated with 33 μM C12FDG for 4 h. At the end of incubation, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min. Subsequently, cells were washed again and mounted in ProLong Gold antifade reagent with DAPI overnight. Samples were analyzed using LEICA TCS-SP5 confocal microscope (Leica Microsystems; Wetzlar, Germany) at 488 nm argon laser to detect the fluorescence of SA-β-GAL activity and at 405 nm to detect DAPI. Pictures were obtained and fluorescence intensity was measured by densitometry by Image J software (http://rsbweb.nih.gov/ij/).

2.5. Transfection of ILK and Klotho. Subconfluent MCT cells (60% to 80% confluent) cultured in 6-well plate were transfected with plasmids containing ILK or Klotho.

For transient transfection of ILK [32], cells were transfected with different doses of a plasmid containing ILK wild type (0, 1, and 2 μg) (ILK-WT which was provided by Dr. S. Dedhar, University of British Columbia, Canada). Then, each one was mixed with Lipofectamine (5 μL/well) and incubated for 15 minutes at room temperature for 24 hours prior to addition to the cells.

For stable transfection of Klotho [10, 33], cells were transfected with 1 μg of a plasmid containing the transmembrane form of mouse Klotho cloned into pEF1/Myc-His vector (pEF1-Klotho) or with the empty pEF1/Myc-His vector as control (pEF1-Empty). Both plasmids were kindly provided by Dr. M. Kuro-O from the University of Texas Southwestern Medical Center, Texas. For transfection, Opti-MEM media and Lipofectamine were used. To select the transfected MCT cells, cells were treated with 200 μg/mL of G418 (Invitrogen) for 14 days. Transfection of Klotho in HEK293T cells was transient as these cells have resistance to Geneticin and they cannot be selected with G418.

At the end of transfection, media were removed and replaced with complete media for 24 h, after which MCT cells were lysed. Protein concentration was measured to analyze p16 protein levels by western blot, and RNA was isolated to test *Klotho* mRNA levels.

2.6. Measurement of mRNA Expression. The total RNA from MCT cells or kidney cortex from mice was isolated using Trizol reagents according to the manufacturer's protocol. The RNA integrity was checked using agarose-formaldehyde gels, and the RNA concentration was measured using a Vis-UV spectrophotometer (Nanodrop). cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems Inc., Foster City, CA, USA), and *Klotho*, ILK-1, and GAPDH expression was measured by quantitative RT-PCR (qPCR) (ABI Prism 7000), using Taqman genes and double delta Ct method. Klotho (Mm00473122_m1), ILK-1 (Mm01274251_g1), and the endogenous control GAPDH (Mm99999915_g1) were used (Applied Biosystems Inc., Foster City, CA, USA).

2.7. ILK siRNA Transfection. ILK was silenced in MCT cells by transfecting a specific small interfering RNA against ILK (siILK). An unspecific scrambled RNA was used as transfection control (scRNA). Both siILK and scRNA were transfected using Lipofectamine 2000 for 24 h. Cells were incubated with complete RPMI for 24 h. Cells were processed in duplicate to evaluate p16 expression by western blot after adding GOx for 48 h using serum-free RPMI and to check ILK and *Klotho* mRNA expression by RT-qPCR, as described above.

2.8. Conditional ILK Knockout Mouse Model. All animal procedures were in accordance with the EU Directive 2010/63/EU and they were previously approved by the Institutional Animal Care and Use Committee at the University of Alcala. Animals were housed in a pathogen-free and temperature-controlled room (22 ± 2°C). Food and water were available ad libitum. Conditional inactivation of the ILK gene was accomplished by crossing C57Bl/6 mice homozygous for the floxed ILK allele, flanked by loxP sites (ILKfl/fl), with homozygous mice carrying a tamoxifen-inducible CreER (T) recombinase gene ($CRE^{+/+}$) which express Cre under the control of the cytomegalovirus promoter [34, 35]. Eight-week-old male mice were injected intraperitoneally with 1.5 mg of tamoxifen (TX) (Sigma Co., St. Louis, MO, USA) (dissolved in a 10 : 1 volumetric mix of corn oil and ethanol) or Vehicle alone (VH), once a day for 5 consecutive days to induce ILK deletion. After 5, 15, and 30 days following VH or TX injections, routine genotyping of tail DNA samples was performed to monitor the Cre-driven ILK deletion [36, 37]. TX-treated CRE-LOX mice displaying successful deletion of ILK are named conditional KO-ILK (cKO-ILK) mice and their control VH-treated CRE-LOX are named wild type (WT). Animals were euthanized 20 days after the last injection.

The p16 and p53 expression in renal cortex from WT or cKO-ILK mice was assessed by western blot as well as ILK and *Klotho* mRNA levels by RT-qPCR in these samples, as described above.

2.9. Statistical Analysis. Results are shown as mean ± standard error of the mean (s.e.m.) of a variable number of experiments, detailed in figure captions. Most experiments are presented as fold increase in basal or control values. To compare the different experimental situations, 1-way or 2-way ANOVA was used, depending on the experiments. Pairwise comparisons were performed using Fisher's least significant difference method. The Dunnett test was used to analyze the changes in respect to basal values. All tests were two-tailed, and a value of $p < 0.5$ was considered statistically significant.

3. Results

3.1. Glucose Oxidase Induced Cellular Senescence in Renal Mouse Cortical Tubule (MCT) Cells Increasing the ILK Expression and Activity. MCT cells were treated with 2.5 mU/mL GOx for 24, 48, and 72 h, to induce oxidant stress. GOx induced a time-dependent increase in oxidative damage, which was evaluated quantifying the formation of 4-hydroxynonenal protein adducts by western blot (Figure 1(a)). Protein expression of senescence genes

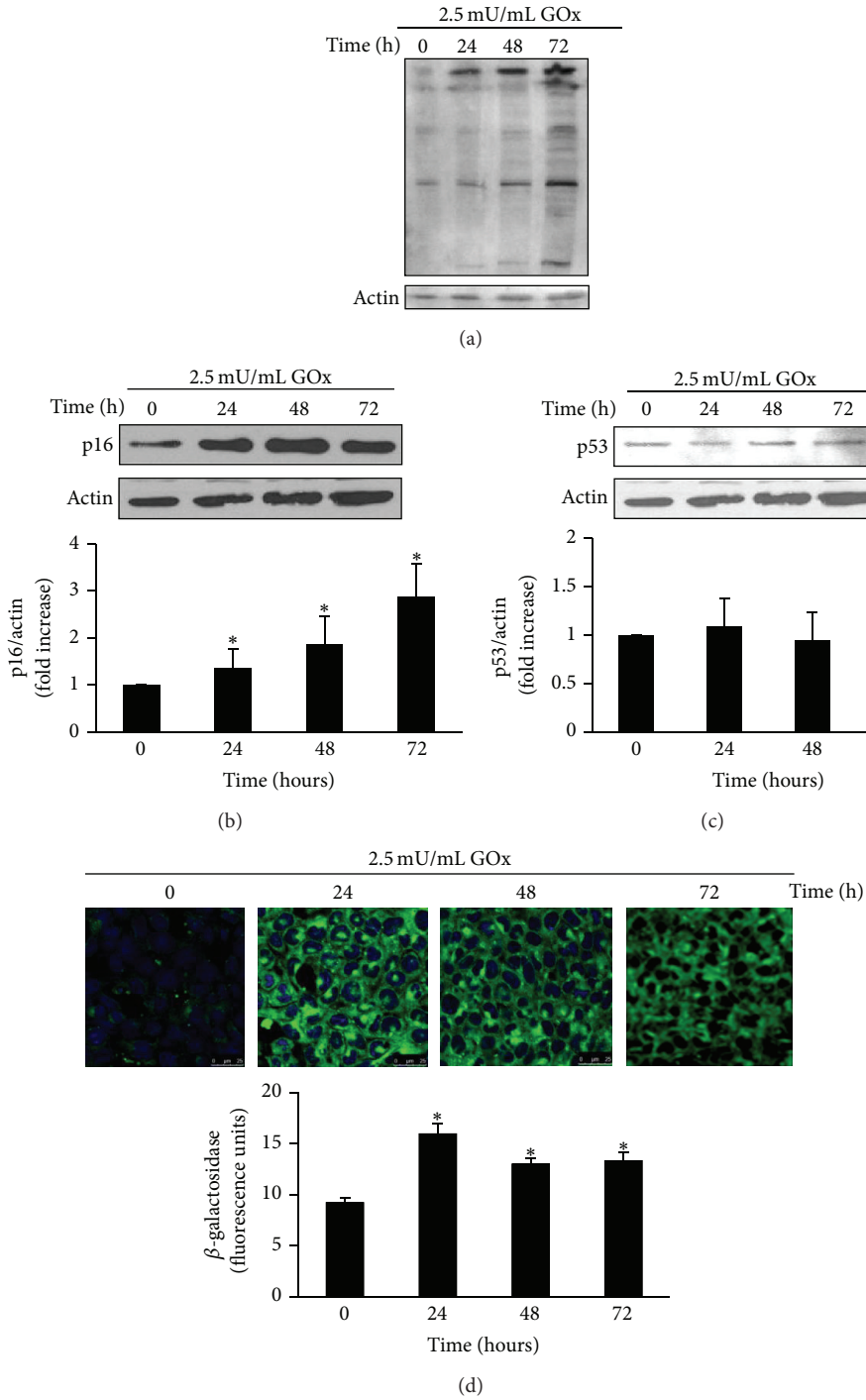

Figure 1: *GOx induces senescence in wild-type mouse cortical tubule (MCT) cells*. MCT cells were treated with 2.5 mU/mL GOx for 24, 48, or 72 h. (a) 4-Hydroxy-2-nonenal proteins adducts were analyzed by western blot. (b) p16 and (c) p53 protein expressions were analyzed by western blot. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands. The results are expressed as densitometric units and are the mean ± s.e.m. from six different experiments. (d) SA-β-GAL activity was analyzed by confocal microscopy using the fluorescent substrate C12FDG. A representative experiment is shown. Bar graph represents the analysis of the fluorescence from five different experiments and the mean ± s.e.m. is expressed as fluorescence units. $^{*}p < 0.05$ versus control (time 0).

was evaluated by western blot. Results showed a time-dependent increase in p16 expression (Figure 1(b)), whereas no significant changes were found in p53 expression (Figure 1(c)). GOx also increased SA-β-GAL activity detected with confocal microscopy, indicating an increase in the percentage of senescent cells 24 h after GOx treatment (Figure 1(d)).

Next, to analyze whether there was a link between the increase in ILK and the induction of senescence, we over-expressed ILK on MCT cells by transfection with a plasmid

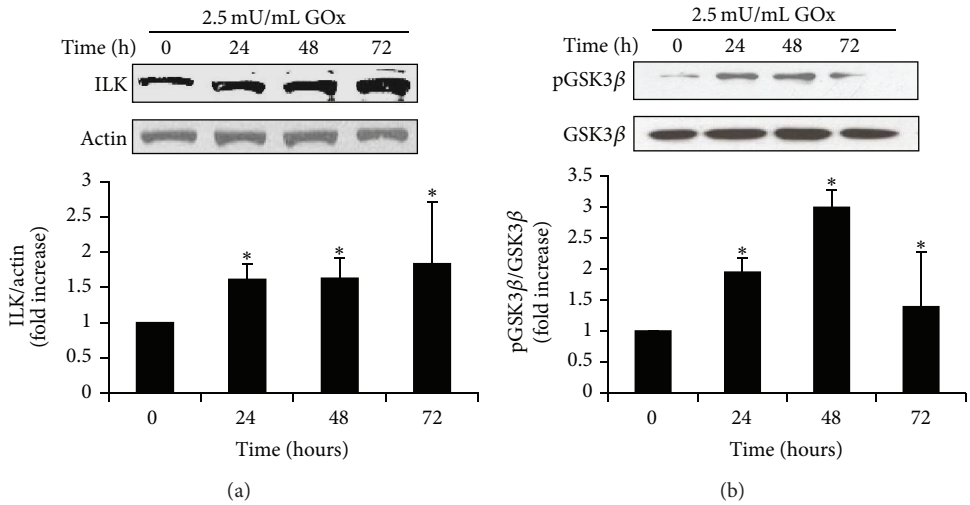

Figure 2: *GOx induces an increase in ILK protein expression and activity*. MCT cells were treated with 2.5 mU/mL GOx for 24, 48, or 72 h. (a) ILK and (b) phospho-GSK3β and GSK3-β protein expression was analyzed by western blot. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands. The results are expressed as densitometric units and are the mean ± s.e.m. from four different experiments. $^{*}p < 0.05$ versus control (time 0).

containing a wild-type ILK protein (WT-ILK). Figure 3(a) shows how the expression of ILK increases in MCT cells transfected with WT-ILK in a dose-dependent way. MCT cells overexpressing ILK also had a strong expression of senescence gene p16, even in the absence of oxidant stress (Figure 3(a)). Moreover, ILK expression was knocked down by transfecting cells with specific siILK and then treated with 2.5 mU/mL GOx for 48 h. Transfection significantly reduced ILK mRNA expression, measured by RT-qPCR (Figure 3(b)), and also the protein content, measured by western blot (Figure 3(c)). Cells transfected with siILK did not show the expected increase in p16 expression after GOx treatment compared to cells transfected with scRNA (Figure 3(c)). Both results allow establishing a direct link between the increase in ILK protein content and increased senescence gene p16 expression.

In parallel, we analyzed the effect of oxidant stress on ILK expression and activity and found that GOx induced a significant increase in the protein expression at all times examined (Figure 2(a)). GOx also induced an increase in the ILK activity evaluated as the phosphorylation of its substrate GSK-3β, as shown in Figure 2(b), without changes in the protein content of total GSK-3β.

3.2. Glucose Oxidase Reduced the Expression of the Gene Klotho in MCT Cells. MCT cells were treated with 2.5 mU/mL GOx for 24, 48, and 72 h, and *Klotho* mRNA expression was analyzed by RT-real time PCR. We found that oxidative stress significantly reduced the mRNA expression of *Klotho* gene 24 h after GOx addition (Figure 4(a)). To analyze whether the reduction in *Klotho* expression could be related to the increase in senescence gene p16, MCT cells were transfected with a plasmid containing Klotho protein. The transfection of pEF1-Klotho induced a strong increment in *Klotho* mRNA expression compared with transfection of pEF1-Empty (Figure 4(b)). The highest expression of Klotho remained until 72 h after transfection (data not shown).

After transfection, cells were treated with 2.5 mU/mL GOx and p16 expression was evaluated by western blot. Cells transfected with the empty vector (pEF1-Empty) responded to GOx treatment increasing p16 expression, as expected, whereas cells overexpressing Klotho protein (pEF-1-Klotho) did not increase the expression of senescence gene p16 (Figure 4(c)).

3.3. Reduced Klotho Expression Depended on the Increase in ILK Expression in Response to Oxidative Stress. To analyze whether there was a relationship between the reduction of *Klotho* and the increase in ILK expression, we analyzed *Klotho* expression in MCT cells transfected with specific siRNA against ILK. Cells transfected with siILK showed a stronger reduction in ILK mRNA expression (Figure 5(a)) and a stronger increase in *Klotho* mRNA expression (Figure 5(b)) than cells transfected with the unspecific siRNA (scRNA), which were evaluated by RT-qPCR.

In addition, this effect was confirmed by using a conditional ILK knockout mouse model (cKO-ILK). After treatment with TX for several days, ILK deletion was confirmed by PCR (Figure 5(c)). ILK expression was reduced in renal cortex from cKO-ILK versus WT mice (Figure 5(d)). The reduction in ILK gene expression was parallel with a higher *Klotho* gene expression in cKO-ILK mice versus WT (Figure 5(e)), as we found in MCT cells.

Additionally, the protein content of senescence genes p53 and p16 was analyzed in the renal cortex isolated from cKO-ILK mice, finding lower expression than in WT mice (Figure 5(f)).

3.4. HEK293T Cells Showed the Same Response to GOx Treatment of ILK and Senescence. To assess whether oxidative

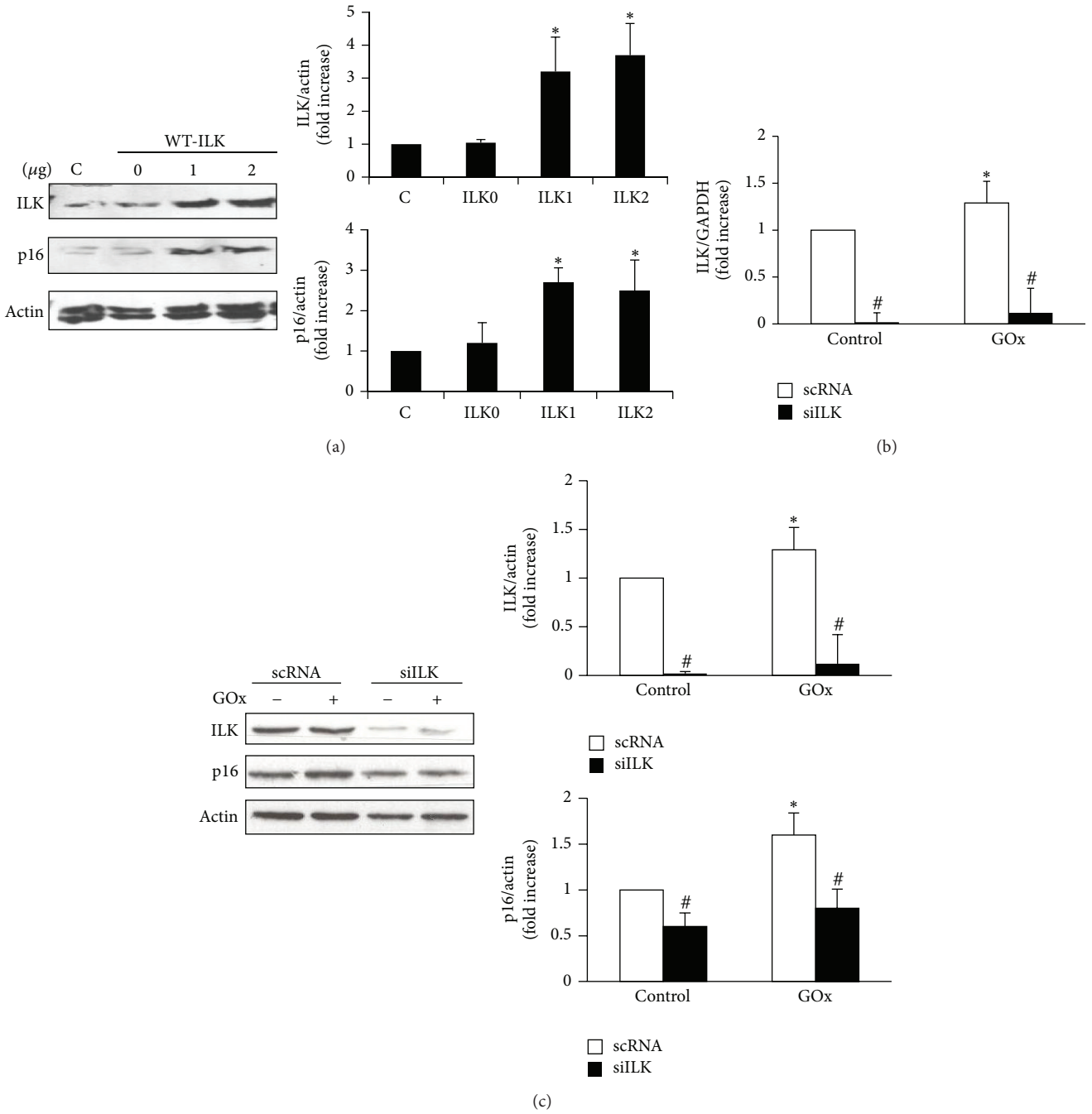

Figure 3: *ILK mediates cellular senescence induced by GOx*. (a) MCT cells were transfected with a plasmid coding for wild-type ILK protein (WT-ILK) at several doses (0, 1, or 2 μg). ILK and p16 proteins were evaluated by western blot. (b, c) MCT were transfected with the specific siRNA for ILK (siILK) or unspecific scramble siRNA (scRNA) as control and then treated with 2.5 mU/mL of GOx for 48 h. In those conditions, mRNA ILK was evaluated by RT and real time PCR (b) and ILK and p16 protein expression was evaluated by western blot (c). A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands. The results are expressed as densitometric units and are the mean ± s.e.m. of the mean from five different experiments. $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus scRNA.

stress induces senescence in other cultured cells proceeding from kidney and whether the mechanisms involved were similar to those of MCT cells, HEK293T cells were treated with 2.5 mU/mL of GOx for 24, 48, and 72 h. Then, ILK and p16 expressions were analyzed by western blot. Figure 6(a) shows that GOx induced an increased expression of both ILK and p16 with a similar pattern to the expression found in MCT cells. In addition, HEK293T cells were transfected with *Klotho* expression vector to analyze whether Klotho overexpression was able to inhibit the increase in p16 expression after GOx

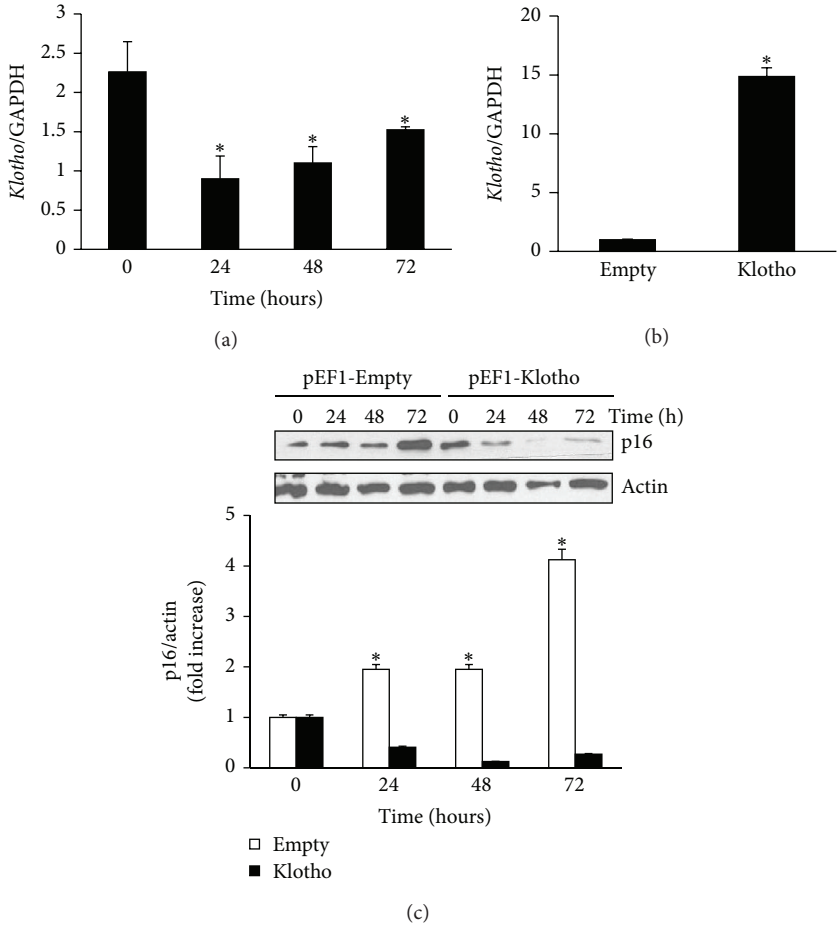

Figure 4: *Antiaging gene Klotho is modulated by GOx and mediates cellular senescence induced by GOx*. (a) MCT cells were treated with 2.5 mU/mL of GOx for 24, 48, and 72 h and mRNA *Klotho* expression was analyzed by RT and real time PCR. Bar graph represents the mean ± s.e.m. from three different experiments. MCT cells were transfected with a plasmid containing Klotho protein (pEF1-Klotho) or with an empty vector (pEF1-Empty) as control, and (b) mRNA *Klotho* expression was analyzed by RT and real time PCR. Bar graph represents the mean ± s.e.m. of three different experiments. (c) MTC cells transfected with Klotho protein were treated with 2.5 mU/mL of GOx for 24, 48, and 72 h and p16 protein expression was evaluated by western blot. A representative blot is shown. Bar graphs represent the densitometric analysis of the bands. The results are expressed as densitometric units and are the mean ± s.e.m. from five different experiments. $^{*}p < 0.05$ versus control (time 0).

addition. Results shown in Figure 6(b) demonstrated that cells overexpressing Klotho did not present the expected increase in the senescence gene p16.

4. Discussion

The free-radical theory of aging is one of the most popular theories in aging research and the role of oxidative stress in cellular senescence has been extensively studied. Many stressor conditions, such as hyperosmolar stress [9] and advanced glycation end products [10], are able to induce premature senescence in renal cells by disrupting the balance between reactive oxygen species (ROS) generation and the activity of antioxidant systems. However, the mechanisms activated after the manifestation of oxidative stress, which lead to cellular senescence, are less known. For this reason, in this work, we explored some of the mechanisms which could be activated by oxidative stress to lead cells to senescence. To induce a chronic oxidative stress, we used glucose oxidase, as previously described [38, 39]. GOx is an enzyme, absent in mammalian cells [40], which converts oxygen to hydrogen peroxide, in a stoichiometrically simple 1:1 relationship, using glucose as substrate. Its product, D-gluconolactone, is metabolically inert [41, 42]. The activity of GOx is stable and remains fully active over 24 h. For this reason, we replaced the medium with fresh medium and GOx every 24 h [40]. In our experiment, GOx addition increases the oxidative damage of proteins in a time-dependent way. Hydrogen peroxide is well known as a tissue damage mediator in several pathological conditions. In the kidney, hydrogen peroxide is known to play an important role in the pathogenesis of ischemia-reperfusion events in tubular cells [43], in CKD [44], and in several inflammatory kidney diseases [45, 46]. Depending on the kidney environmental conditions, oxidative stress generated by hydrogen peroxide induces cell proliferation, apoptosis, and fibrosis [43, 47–49], leading to tissue damage.

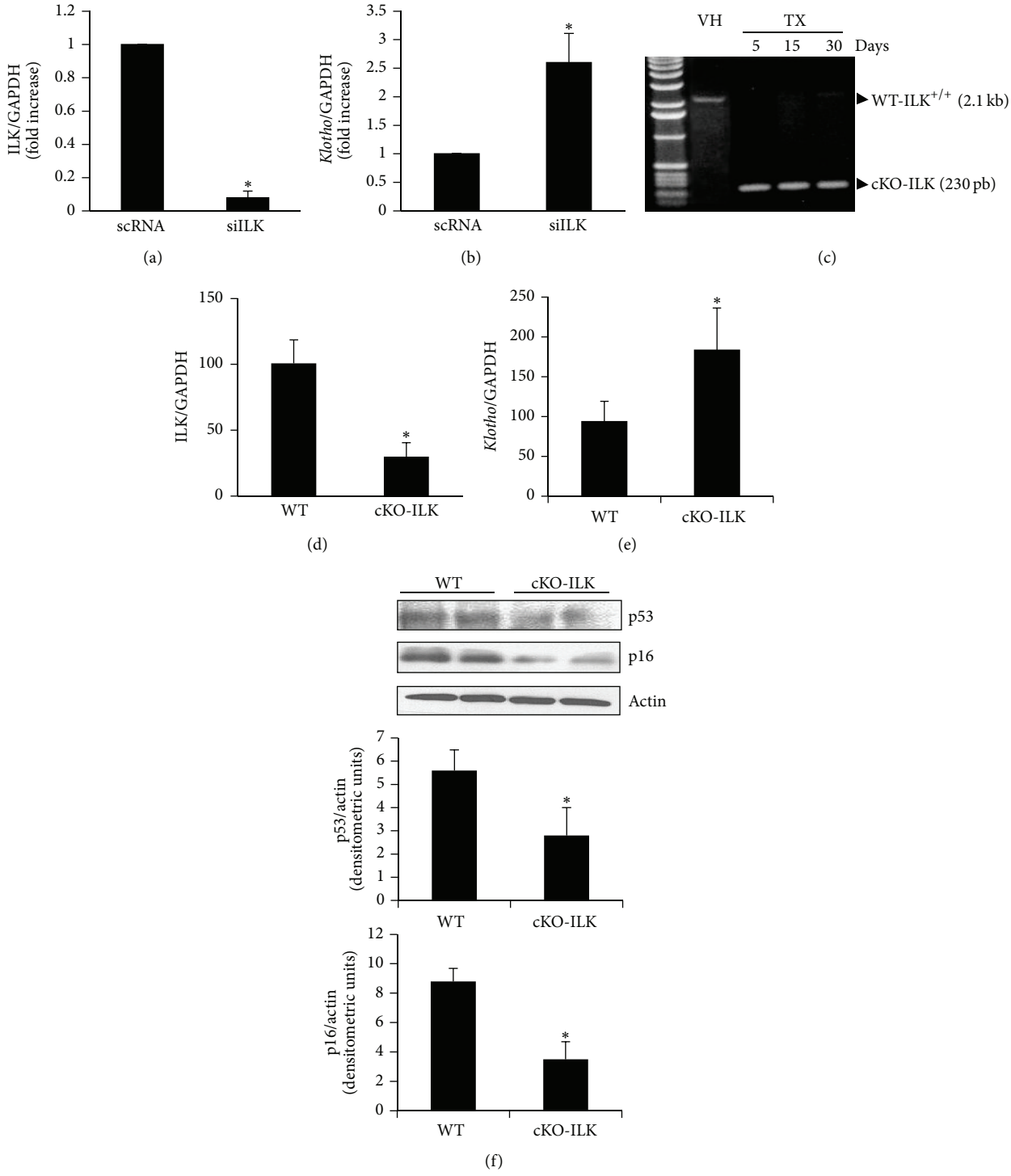

Figure 5: *Increased ILK expression by GOx induced decrease in Klotho expression*. (a, b) MCT cells were transfected with specific siRNA for ILK (siILK) or unspecific scramble siRNA (scRNA) as control. ILK (a) and *Klotho* (b) mRNA expression was evaluated by RT and real time PCR. Bar graph represents the mean ± s.e.m. of three different experiments, and the results are expressed as densitometric units. (c) A conditional ILK knockout mouse model (cKO-ILK) was used and, after treatment with TX for several days, ILK deletion was confirmed versus VH treatment in those mice by PCR of genomic DNA isolated from kidney. ILK (d) and *Klotho* (e) mRNA expression was analyzed in kidney from WT and cKO-ILK mice by RT and real time PCR. Bar graph represents the mean ± s.e.m. of 10 animals per group. (f) p53 and p16 protein expression was analyzed in kidney from WT and cKO-ILK mice by western blot. A representative blot is shown. Bar graphs represent the densitometric analysis of the bands. The results are expressed as densitometric units and are the mean ± s.e.m. of 10 animals per group. $^{*}p < 0.05$ versus control (scRNA in panels (a)-(b) and WT in the rest panels).

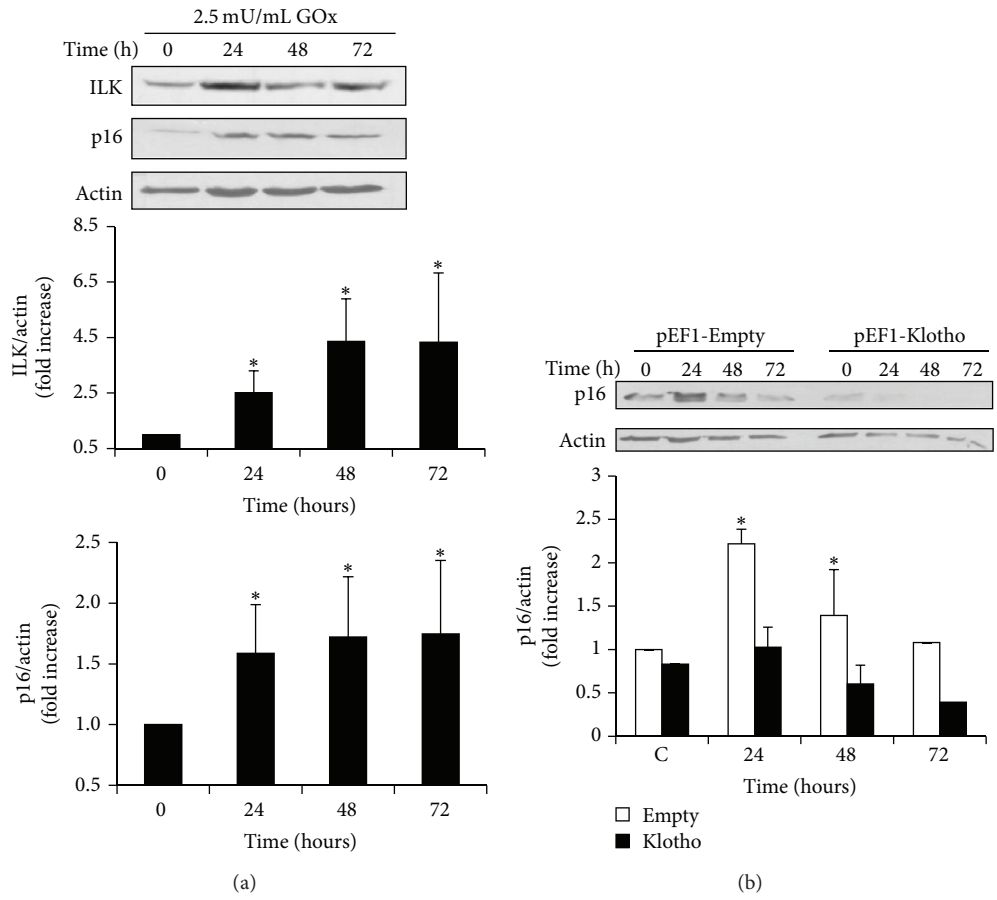

Figure 6: *GOx induced senescence through ILK in other renal cells.* HEK293T cells were treated with 2.5 mU/mL GOx for 24, 48, or 72 h. (a) ILK and p16 protein expressions were analyzed by western blot. A representative blot is shown in each case. Bar graphs represent the densitometric analysis of the bands. The results are expressed as densitometric units and are the mean ± s.e.m. from six different experiments. (b) HEK293T cells were transfected with Klotho protein (pEF1-Klotho, closed bars) or empty vector (pEF1-Empty, open bars) and then treated with 2.5 mU/mL of GOx for 24, 48, and 72 hs. p16 protein expression was evaluated by western blot. A representative blot is shown. Bar graphs represent the densitometric analysis of the bands. The results are expressed as a percentage of control and are the mean ± s.e.m. from five different experiments. $^{*}p < 0.05$ versus control or time 0.

We hereby propose that a possible mechanism for tissue damage caused by hydrogen peroxide could be the induction of cellular senescence in renal cells. The accumulation of senescent cells in tissues has been identified as a mechanism involved in tissue dysfunction. In this regard, hydrogen peroxide is described to cause senescence in HK-2 tubular cells in a CKD experimental model [50], inducing cell loss. We found that oxidative stress induced by GOx increased senescence in MCT cells in culture 24 h after addition, which were identified to express SA-β-GAL activity. Cellular senescence is defined as an irreversible cell cycle arrest characterized by the expression of the cell cycle inhibitor p16 or/and the tumor suppressor p53 [51]. Immortal MCT cells under GOx treatment show an increase in p16 but not in p53 gene expression. The increase in the expression of p16 has been described as a robust biomarker for cellular senescence [52] and has been associated with many age-related diseases [53]. Both increased expression of p16 protein and increased SA-β-GAL activity allow us to confirm that prolonged exposition to GOx induced cellular aging in MCT cells.

Next step was to elucidate how oxidative stress leads to senescence. Recently, it has been reported that hydrogen peroxide increases TGF-β1 expression through ILK in glomerular mesangial cells [39]. ILK is a component of the intracellular complex that links transmembrane integrins with the actin cytoskeleton and other intracellular signaling pathways [54]. ILK regulates proliferation, differentiation, and motility in many cell types and has recently been involved in cellular senescence [55, 56]. For this reason, we analyzed whether oxidative stress due to hydrogen peroxide also increases ILK expression in MCT cells and found a significant increase after GOx treatment. ILK has a pseudokinase activity [57], which can be measured by the phosphorylation of one of its substrates, GSK-3β [58]. Besides, an increase in phospho-GSK-3β was found in MCT cells. In order to assess a direct relationship between the increase in ILK expression and activity and cellular senescence, ILK protein was overexpressed by transfecting MCT cells with a plasmid containing the wild-type ILK gene. ILK and p16 expression levels increased 3-fold in transfected cells as compared to

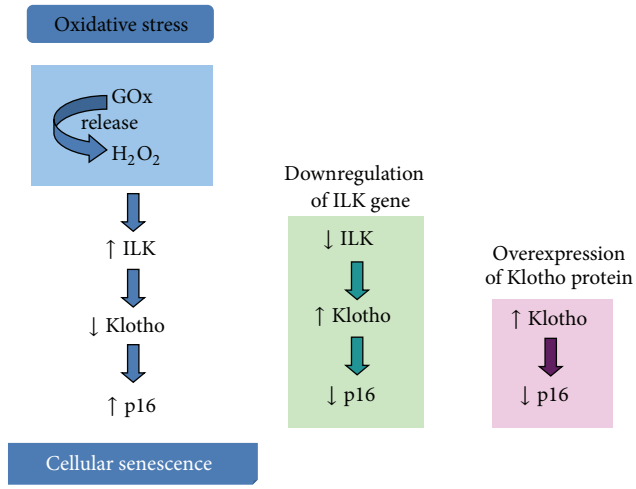

Figure 7: *Proposed integrative mechanisms involved in cellular senescence induced by chronic oxidative stress in renal cells.* Oxidative stress increases ILK activity and expression, which in turn reduces *Klotho* gene expression. Cells with reduced levels of Klotho are less protected from oxidative stress and undergo senescence.

nontransfected ones, even in the absence of oxidative stress. Moreover, MCT cells transfected with small interfering RNA against ILK to silence ILK expression did not undergo senescence despite the GOx addition. These results clearly indicate that ILK overexpression has a critical role in cellular senescence induced by oxidative stress. In accordance with this result, it has been described that genetic reduction of ILK in both *C. elegans* and *Drosophila* led to lifespan extension [59].

One of the antiaging factors most studied is the *Klotho* gene, which is expressed mainly in renal tubular cells; Klotho protein protects cells from apoptosis and senescence [60, 61] and from oxidative damage [62]. We analyzed the effect of oxidative damage on the expression of *Klotho* gene in MCT cells and found a reduction in *Klotho* mRNA expression. *Klotho* expression is downregulated in multiple pathological conditions, such as hyperlipidemia, hypertension, and CKD [63, 64]. A significant correlation was found between Klotho levels and oxidative damage in the serum of CKD patients [65]. Our results show that chronic oxidative stress induces a significant reduction in *Klotho* expression, which could lead to senescence, since cells with an ectopic overexpression of Klotho protein did not undergo senescence after GOx addition.

Therefore, we explored whether the reduction of Klotho protein was linked to the increase in ILK under oxidative stressing conditions. For this purpose, we performed two different experimental approaches. First, we downregulated ILK expression by transfection with siILK and found an increase in the transcription of *Klotho* gene. Second, we analyzed the *Klotho* expression in a conditional KO of ILK animal model, as previously reported [37]. Conditional KO-ILK mice showed a strong reduction in renal ILK expression, which was accompanied with an increase in the *Klotho* expression as compared with WT mice. Finally, p16 and p53 senescence gene expression was analyzed in the renal cortex from these animals. Results showed a significant reduction in the expression of both genes.

In accordance with these results, a work performed in *C. elegans* has recently reported that genetic reduction in ILK induced stress response genes related to antioxidant response and heat shock proteins [60]. We hereby propose that the reduction of ILK expression induces *Klotho* gene as a mechanism of stress response. Klotho protein decreases with age [66] and in many age-related diseases [67], but there are few works about the transcriptional regulation of the *Klotho* gene. Among these, it has been described that the hypermethylation of *Klotho* gene promoter increases with age, reducing its expression [68]. Similar results were found in HEK 293T cells, suggesting that ILK plays an important role in renal aging.

5. Conclusion

We conclude that chronic oxidative stress induces senescence in renal cells by increasing ILK activity and expression, which in turn reduces *Klotho* gene expression. Cells with reduced levels of Klotho are less protected from oxidative stress and undergo senescence (Figure 7).

Evidence suggesting ILK as a novel downregulator of *Klotho* gene expression is presented here. Further experiments will be necessary to analyze the potential therapeutic implications of the inhibition of ILK in age-related renal pathologies.

Disclosure

All the authors are part of Programa Redes REDinREN from the FIS (RD12/0021/0006) and Fibroteam, Community of Madrid, Spain.

Conflict of Interests

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

Authors' Contribution

Gemma Olmos and María Piedad Ruíz-Torres have the same contribution.

Acknowledgments

The authors would like to thank Dr. Makoto Kuro-O from the University of Texas Southwestern Medical Center (Dallas, TX, USA) for providing them with Klotho transfection vectors; Dr. Shoukat Dedhar, University of British Columbia (Vancouver, Canada, USA) for the cKO-ILK mouse model and the WT-ILK plasmid; and Dr. Ricardo J. Bosch from the University of Alcalá of Madrid (Madrid, Spain) for wild-type mouse cortical tubule (MCT) cells. This work was supported by grants from the Fondo de Investigaciones Sanitarias (FIS) (Grants: PI13/02270, PI13/00336), Networks Program REDinREN from the FIS (Grant REDinREN: RD12/0021/0006), and the Community of Madrid (FIBROTEAM). Nuria Troyano-Suárez (BES-2009-029413) and María del Nogal-Avila (SAF 2007-623471) were supported by a Fellowship from the Spanish Ministry of Education and Science. Susana López-Ongil holds a contract from the Research Stabilization Program of FIS (CES07/032).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 682861, 9 pages
http://dx.doi.org/10.1155/2015/682861

Research Article

Regulation of MUTYH, a DNA Repair Enzyme, in Renal Proximal Tubular Epithelial Cells

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Received 12 November 2014; Revised 10 January 2015; Accepted 17 January 2015

Academic Editor: Claudio Cabello-Verrugio

MUTYH is a DNA repair enzyme that initiates a base excision repair (BER) by recognizing and removing 8-Oxoguanine (8-oxoG) and its paired adenine. We demonstrated that both TGF-β1 and H_2O_2 treatment led to an increased 8-oxoG in cultured human proximal tubule epithelial (HK-2) cells, while the former induced epithelial-mesenchymal transition and the latter caused cell apoptosis. Without stimulation, HK-2 cells showed MUTYH expression in mitochondria. TGF-β1 triggered a transient upregulation of mitochondrial MUTYH and induced the expression of nuclear isoforms, while H_2O_2 showed no role on MUTYH expression. Ureteral obstruction (UUO) mice exhibited high 8-oxoG reactivity with tubulointerstitial lesions. After obstruction, the MUTYH expression was increased only in tubules at day 3 and decreased with obvious tubular atrophy at day 10. Particularly, MUTYH was primarily located in normal tubular cytoplasm with a dominant mitochondrial form. A few cells with nuclear MUTYH expression were observed in the fibrotic interstitium. We confirmed that increased MUTYH expression was upregulated and positively correlated with the severity of kidney fibrosis. Thus, renal fibrosis caused a cell-type-specific and time-dependent response of oxidative DNA repairs, even within the same tissues. It suggests that intervention of MUTYH might be effective for therapies.

1. Introduction

Renal fibrosis occurs in many forms of chronic kidney disease progressing to end-stage renal disorders (ESRD) [1]. Oxidative stress in kidneys is often suggested to contribute to interstitial fibrosis [2]. DNA is one of the most important biological targets of oxidative stress, and oxidative DNA lesions are a major type of endogenous damage leading to human disorders. The most stable product of oxidative DNA damage is 8-Oxoguanine (8-oxoG) [3, 4], and 8-oxoG has been observed in kidneys in several conditions.

Adenine can pair with 8-oxoG in double-stranded DNA, leading to mispairing during genome replication [5]. If the mismatch is not repaired, the G: C to T: A mutation will be inherited in future cell cycles. Mammalian cells are equipped with elaborate means to minimize 8-oxoG accumulation in DNA [6, 7]. The human MutY homolog (*MUTYH*) initiates a base excision repair (BER) by recognizing and removing 8-oxoG and its paired adenine. Mutated BER proteins are reported to increase the risk of accumulating 8-oxoG in mitochondrial DNA (mtDNA) as well as nuclear DNA (nDNA). Mutant mice lacking MUTYH exhibit increased spontaneous mutation rates and susceptibility to carcinogenesis, with increased accumulation of 8-oxoG in DNA.

Oxidative stress is important in the etiology of several renal disorders, and 8-oxoG levels are increased in the kidneys of patients with several diseases, including diabetic nephropathy (DN) [8]. We therefore explored the possible

association between MUTYH and ESRD and showed an increased risk of germline MUTYH polymorphisms during ESRD development [9]. This implied that MUTYH might be implicated in the pathogenesis of renal fibrosis. However, how this enzyme influences fibrosis is poorly understood. In addition, there are more than ten splice variants in mammalian cells [10], which localize to either nuclei or mitochondria. The 52 or 53 kDa MUTYH variant is generally localized in nuclei, whereas the 57 kDa MUTYH form localizes in mitochondria. The two forms are involved in distinct signaling pathways. The exact MUTYH form involved in renal fibrosis is not yet known.

Unilateral ureteral obstruction (UUO) is a widely used *in vivo* model of renal fibrosis [11]. It is a good example of the role of epithelial-mesenchymal transition (EMT) and renal oxidative stress in chronic progressive renal disease. The tubular epithelial cells play a key role in this process, and the immortalized HK-2 cell line is often used to study the mechanism of renal fibrosis *in vitro* [12]. Transforming growth factor-β1 (TGF-β1) induced EMT and hydrogen peroxide induced renal tubular cell apoptosis were used to study the association with MUTYH. The *in vivo* and *in vitro* responses of MUTYH (mitochondrial and/or nuclear forms) in renal fibrosis were investigated in the present study.

2. Materials and Methods

2.1. Reagents. The primary antibodies used in this study were mouse monoclonal anti-8-oxoG (JaICA, Shizuoka, Japan); rabbit anti-MUTYH (BS2535, Bioworld Technology); mouse monoclonal anti-alpha smooth muscle actin (ab119952, Abcam); and anti-GAPDH (AP0066, Bioworld Technology). Recombinant human transforming growth factor beta-1 (TGF-β1) was purchased from R&D (240-B-010, R&D Systems).

2.2. Patients and Materials. Needle biopsies from renal tissues were analyzed in the present study. The patients with diabetic nephrology (DN) were randomly chosen from the Biobank of the National Clinical Research Center of Kidney Diseases, Jinling Hospital. The biopsy specimens were processed according to standard procedures and divided into two groups according to the tubular atrophy (IFTA) scores [13]. The mild renal fibrosis group ($n = 5$) was defined as Grades 0~1 and the severe group ($n = 5$) as Grades 2~3. The study protocol was approved by the Ethics Committee of Jinling Hospital, Nanjing University School of Medicine, Nanjing, China.

For the UUO models, C57BL/6 mice (8 weeks old, male) were purchased from the Experimental Laboratory of Animal Models (Nanjing, China). The test group was anesthetized and the left ureter was ligated (UUO group, $n = 8$), while controls were anesthetized and manipulated without ligation (sham group, $n = 8$). The study was approved by the Institutional Animal Care and Use Committee of Nanjing University School of Medicine.

Injured tubular cells were further investigated using two *in vitro* models. Immortalized proximal tubular epithelial cells from the normal adult human kidney (HK-2) were cultured in DMEM/F12 medium (1 : 1, Gibco) supplemented with 10% FBS. In one model, HK-2 cells were treated with 5 ng/mL of recombinant TGF-β1 for 24 h, 48 h, and 72 h, as previously described [14]. In the other model, HK-2 cells were incubated with 0.5 mM hydrogen peroxide (H_2O_2) for 1 h or 3 h [15]. Control cells were treated with vehicle medium for the same times. All experiments were performed in triplicate.

2.3. Histological Analysis. The kidney samples were fixed in 10% formaldehyde, embedded in paraffin, cut into 4 μm sections, and subjected to periodic acid-Schiff (PAS) and Masson Trichrome staining. The pathological changes were observed under a light microscope. Photographs were obtained, and morphology was quantitatively analyzed using the Image-Pro Plus system (Media Cybernetics). The percentage of interstitial collagen deposition was calculated in renal fibrosis [16], and an average of 20 visual fields of each sample was evaluated.

Other kidney sections were used for immunohistochemical analysis. After heat-induced antigen unmasking, sections were incubated with anti-MUTYH antibody or anti-alpha smooth muscle actin antibody for 16 h at 4°C, followed by incubation with secondary antibodies labeled for detection. Other free-floating sections were pretreated as described previously [17] and subjected to 8-oxoG immunodetection in nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). The immunofluorescence images were photographed using a laser scanning confocal microscopy (Zeiss) and the immunohistochemistry slides were recorded using a Nikon light microscope (Nikon Inc). Semiquantitative analysis of MUYTH or 8-oxoG expression was evaluated using the Image-Pro Plus system. The integrated optical density (IOD) in tubules was examined for at least 20 consecutive microscopic fields each section. The percentage of tubules with MUTYH immunoreactivity was also evaluated in a mean (+SE) of 281 (+11) tubules.

2.4. Immunofluorescent Analysis In Vitro. The cultured HK-2 cells were first washed with PBS and fixed in 4% paraformaldehyde for 20 min at 4°C. Following three washes with PBS (5 min each), cells were permeabilized with 0.5% Triton X-100 for 10 min, blocked in 5% BSA for 30 min, and incubated overnight at 4°C with and anti-MUTYH or anti-8-oxoG antibody in PBS in a dark chamber, followed by secondary antibody incubation. The special pretreatment for 8-oxoG staining was conducted as previously described [17]. Before the addition of a coverslip, the cell slides were incubated with 2 mM DAPI (D1306, Molecular Probes). Fluorescent images were examined by laser scanning confocal microscopy.

2.5. TUNEL Analysis. The terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) staining was analyzed with a DNA fragmentation detection kit (11684817910, Roche Corp) according to the manufacturer's instructions. The positive apoptotic cells were detected, and the apoptotic index was calculated as the ratio of positive

apoptotic cardiocytes to total number of HK-2 cells. A mean (+SE) of 961 (+147) cells was counted for analysis.

2.6. Western Blot Analysis. The kidney tissue and cells were lysed on ice for 30 min using RIPA lysis buffer (P002A, AURAGENE). After boiling with loading buffer, 30 μg of extracted protein was subjected to SDS-PAGE and transferred to PVDF (polyvinyl difluoride) membranes (IPVH00010, Millipore). After blocking the nonspecific binding sites, the primary antibodies rabbit anti-MutY and mouse monoclonal anti-alpha smooth muscle actin were incubated at 4°C overnight, followed by incubation with an HRP-labeled secondary antibody. The bands were detected with an enhanced chemiluminescent reagent (Millipore), and specific ~57 kDa and ~53 kDa bands for MUTYH were observed. Relevant bands were quantified by densitometry using Image J, background corrected and normalized to GAPDH levels.

2.7. Statistical Analysis. Data were shown as the mean ± SD and analyzed using SPSS version 13.0. Comparisons were performed using Student's t-test for two groups or ANOVA (one-way analysis of variance) for three groups, and a nonparametric test was used when necessary. Two-tailed P values less than 0.05 were considered statistically significant.

3. Results

3.1. Regulation of MUTYH in Two Models In Vitro. To explore the response of MUTYH in renal fibrosis *in vitro*, we analyzed HK-2 cells treated by H_2O_2 and TGF-β1 [2]. With both H_2O_2 and TGF-β1 treatments, HK-2 cells showed higher 8-oxoG expression compared with vehicle controls (Figure 1(a)). The immunoreactivity of 8-oxoG was mainly located in HK-2 cell mitochondria and only partly in nuclei.

Different cell responses were observed after H_2O_2 or TGF-β1 treatment. H_2O_2 leads to cell apoptosis, and the percentage of apoptotic cells increased significantly after incubation with 0.5 mM H_2O_2 for 1 h and 3 h (Figure 1(b)). TGF-β1 only slightly increased cell apoptosis after treatment for 24 h or 48 h ($P > 0.05$). However, α-SMA expression increased in HK-2 cells after 24 to 72 h of TGF-β1 treatment (Figure 1(c)). The expression of α-SMA was unchanged by H_2O_2 treatment. These findings suggested that HK-2 cells respond to H_2O_2 by cell apoptosis and to TGF-β1 by EMT.

H_2O_2 and TGF-β1 also showed diverse regulation of MUTYH in HK-2 cells (Figure 2). In the vehicle group, HK-2 cells had high mitochondrial MUTYH expression (Figure 2(a), top line) and ~57 kDa bands in Western blotting (Figures 2(b) and 2(c)). After H_2O_2 treatment, similar mitochondrial expression was detected in HK-2 cells (Figure 2(a), second line), and semiquantitative analysis demonstrated equal MUTYH expression compared with controls (Figure 2(b)). TGF-β1 treatment induced HK-2 cells from a cobblestone-like morphology to a spindle-like morphology, and MUTYH immunoreactivity was located in nuclei (Figure 2(a)). After TGF-β1 treatment for 72 h, almost all of the cells demonstrated nuclear MUTYH (Figure 2(a), last line). Western blotting analysis confirmed increased 53 kDa nuclear MUTYH and decreased 57 kDa mitochondrial MUTYH in HK-2 cells after TGF-β1 treatment. The concentration of both mitochondrial and nuclear MUTYH in HK-2 cells was also upregulated by TGF-β1 in 24 hours ($P < 0.001$, Figure 2(c)).

3.2. MUTYH Expression In Vivo Kidney of UUO Mice. The UUO mice at 3 and 10 days after the operation showed proximal tubule dilation, atrophy, and extracellular matrix (ECM) accumulation as a result of collagen deposition. The typical lesions of obstructed kidneys are demonstrated in Figures 3(a) and 3(b). The interstitial collagen deposition was significantly higher in UUO mice than sham-operated kidneys ($P < 0.001$, ANOVA; Figure 3(c)). The 8-oxoG staining was undetectable in sham-operated kidneys, while oxidative DNA lesions were observed in UUO kidneys (Figure 3(d)). The intensity of 8-oxoG staining significantly increased by Day 3 in obstructed kidneys and was even greater at Day 10 ($P < 0.001$, ANOVA; Figure 3(e)) when compared with the shams.

MUTYH immunoreactivity can be observed in sham-operated kidneys, and this increased in some tubules in UUO kidneys but did not in interstitium (Figure 4(a)). The MUTYH density in positive tubules was increased significantly in UUO kidney at Day 3 when compared with sham controls, and percentage of positive tubules was also increased (Figure 4(b)). However, the density of MUTYH and the percentage of positive tubules declined in UUO kidneys between Day 3 and Day 10. Particularly, the great MUTYH staining was observed in the residual obstructed tubules at Day 10, but the number of the residual tubules was decreased compared with sham or Day 3 groups (Figure 4(a), the right panel). It suggested that injured tubules presented increased MUTYH expression, while atrophic ones lost its expression. As injured and atrophic tubules were observed at same time, Western blotting only showed slightly increased MUTYH expression in UUO kidneys at Day 3 and then followed by a decrease at Day 10 (Figure 4(c)).

Moreover, the MUTYH bands were mainly at ~57 kDa (Figure 4(c)), suggesting that the mitochondrial form of MUTYH was mainly expressed in kidney. Correspondingly, the immunofluorescent images showed that MUTYH was mainly located in the cytoplasm of renal tubular epithelial cells (Figure 4(d), upper line), where α-SMA was not highly expressed. In fibrotic UUO kidneys, the expression of α-SMA increased in the interstitium, and expression of nuclear MUTYH in a few cells was also observed (Figure 4(d), lower line).

3.3. MUTYH Immunoreactivity in Human Kidneys. Diabetic nephropathy (DN) is a common kidney disease, presenting with glomerular lesions and tubulointerstitial fibrosis. Kidney samples were divided into two groups: mild renal fibrosis (Figure 5(a)) and severe renal fibrosis (Figure 5(b)). In subjects with mild fibrosis, MUTYH immunoreactivity was observed in renal tubule epithelium (Figure 5(c)), but not in the glomeruli or the interstitium (Figure 5(d)). In kidneys with severe fibrosis, the tubule epithelium showed intense MUTYH immunostaining (Figure 5(e)), while cells

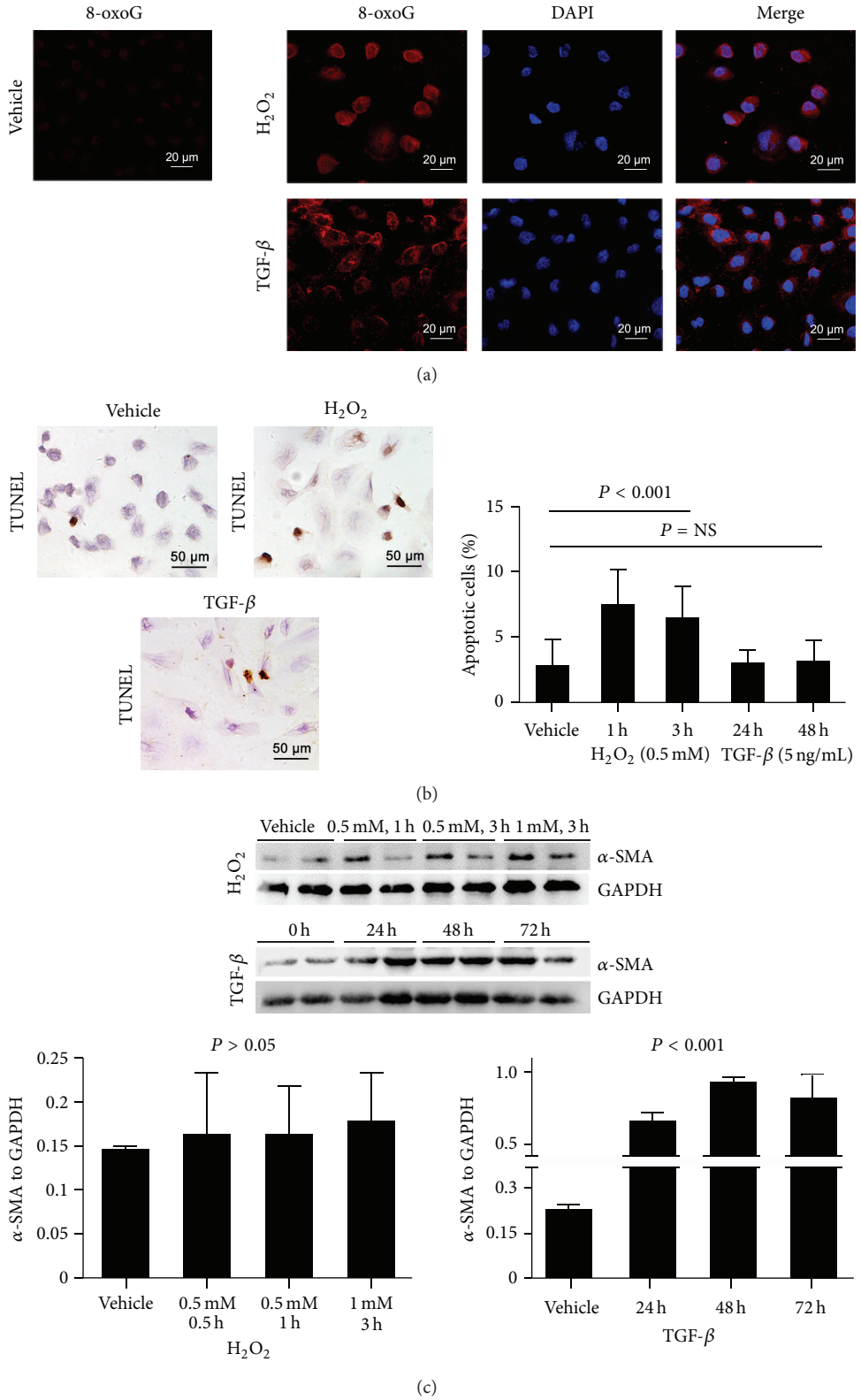

Figure 1: Induced response to H_2O_2 and TGF-β1 in HK-2. Increased 8-oxoG staining was observed in HK-2 cells induced by H_2O_2 and TGF-β1 (a). Apoptotic cells with positive TUNEL staining increased after H_2O_2 treatment, but not after TGF-β1 treatment (b). Increased α-SMA expression and EMT in HK-2 cells was induced by TGF-β1, but not H_2O_2 (c).

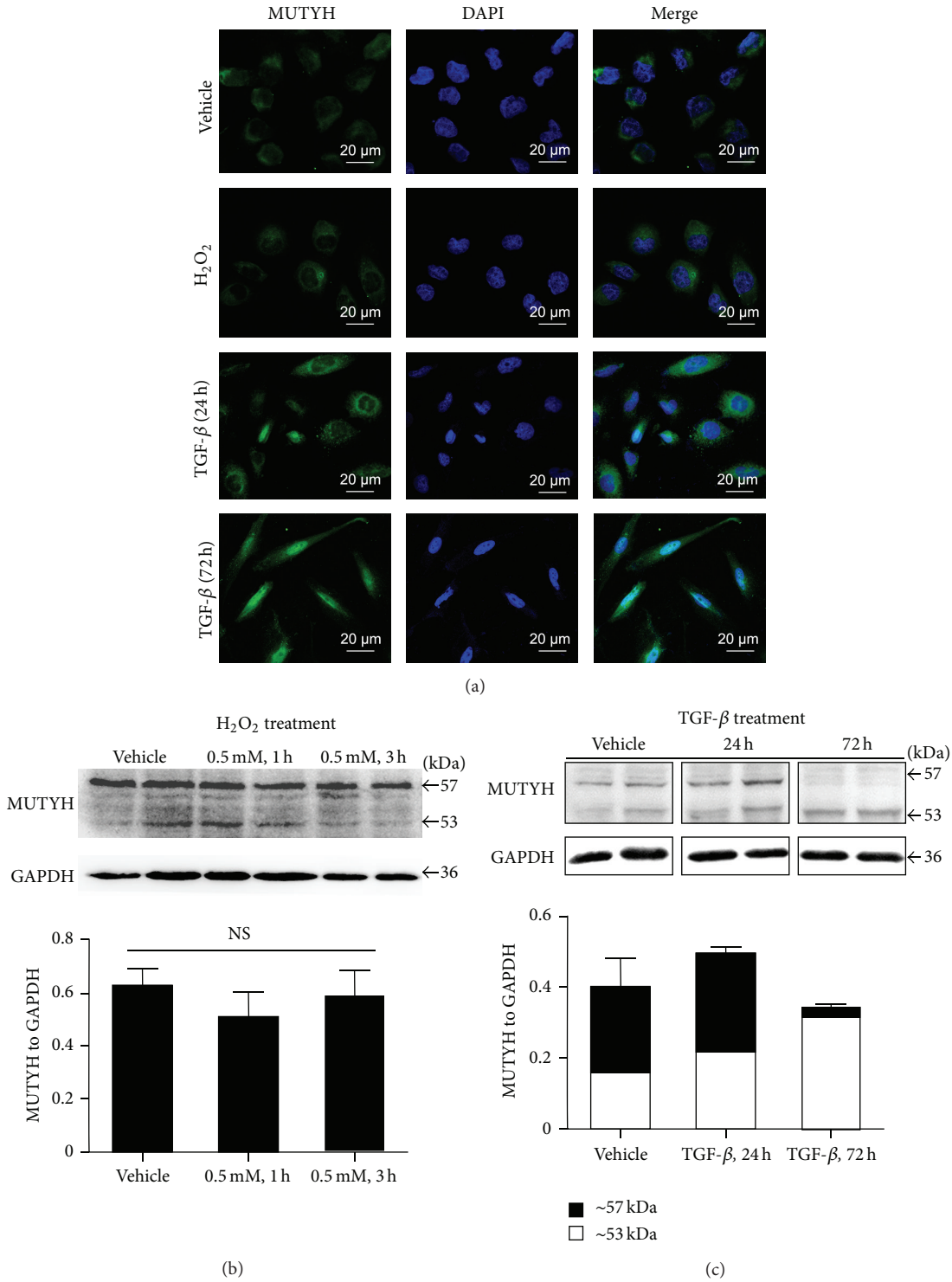

Figure 2: *In vitro* regulation of MUTYH in HK-2 tubular cells treated with H_2O_2 and TGF-β1. Immunofluorescent images demonstrated the location of MUTYH (in green) compared to DAPI (in blue) staining (a). Immunoblot analysis showed similar expression of MUTYH after H_2O_2 treatment (b) and distinct regulation of MUTYH after TGF-β1 treatment (c).

in glomeruli and interstitial spaces were rarely observed (not shown). The MUTYH intensity in tubules significantly increased with the degree of renal fibrosis ($P < 0.001$, Figure 5(f)).

4. Discussion

In kidneys, accumulating evidence has demonstrated that kidney fibrosis induced by renal diseases, including diabetic

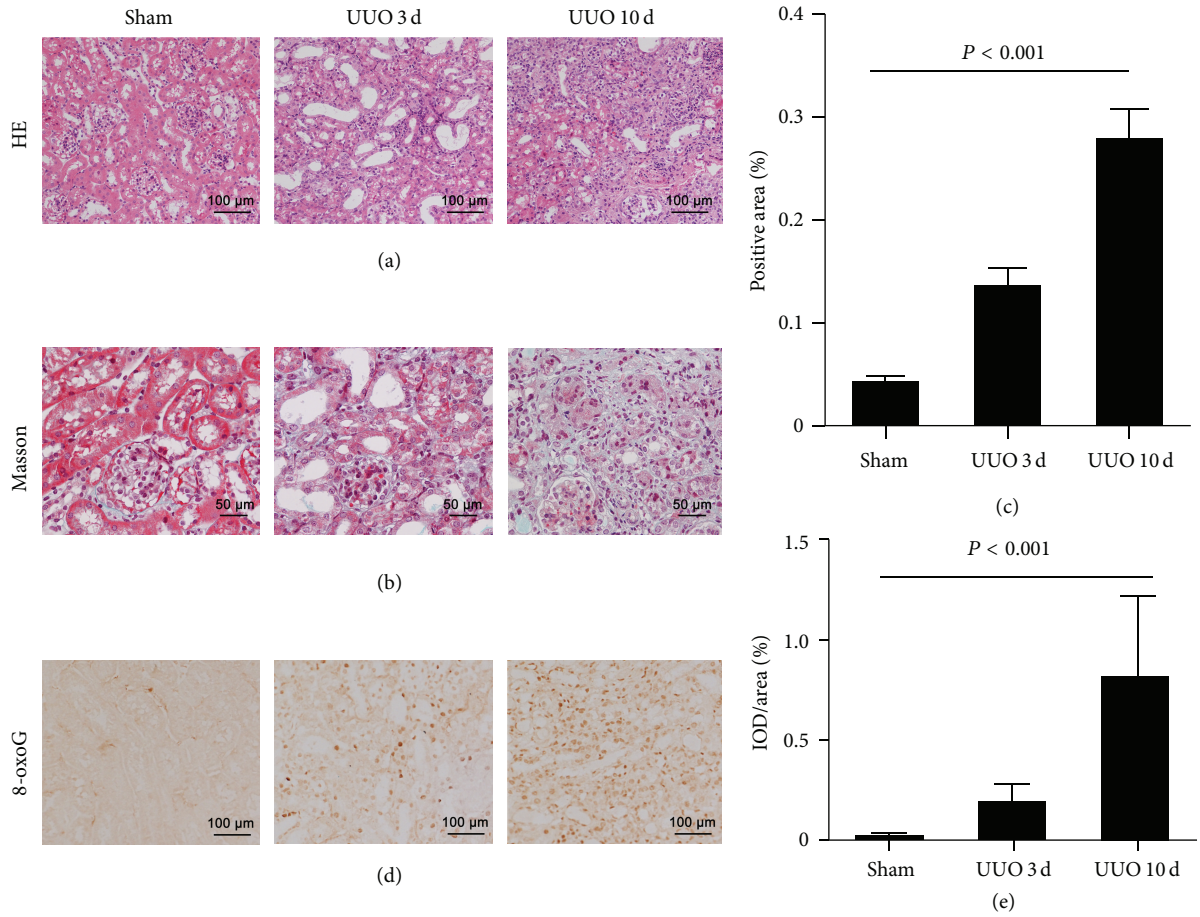

Figure 3: Histological analysis in kidneys from UUO mice. Representative microscopy images of sham-operated kidneys and UUO kidneys for 3 days and 10 days with HE staining (a) and Masson staining (b). Collagen deposition was significantly higher in UUO mice than sham-operated kidneys ((c), $P < 0.001$). The immunoreactivity of 8-oxoG was shown in UUO kidneys (d) and 8-oxoG intensity increased in UUO kidneys compared to sham controls ((e), $P < 0.001$).

nephropathy, is mediated by ROS/oxidative stress [2, 18, 19]. MUTYH is a well-known oxidative DNA repair enzyme which initiates DNA oxidative damage repair by recognizing A:8-oxoG mismatches and removing the mispaired A. This study demonstrated that expression of MUTYH is regulated in renal tubular epithelial cells from renal fibrosis, suggesting that oxidative DNA repair is involved in this process. The mitochondrial isoform of MUTYH was dominant in kidney and acted in a cell-type-specific manner, even within the same tissue type. Additionally, our data demonstrated that upregulation of MUTYH began at an earlier stage after obstruction injury and was sustained with tubular atrophy. This implied that oxidative DNA repair pathway was involved in the renal fibrosis.

MUTYH expression in kidney tubules was shown in patients with DN. Increased MUTYH expression was associated with the severity of renal fibrosis. Detailed information was obtained from UUO models *in vivo*. Murine UUO is a frequently used model of progressive renal injury and fibrosis [20]. In obstructed kidneys, increased MUTYH expression was detected in tubule cells compared with sham-operated kidneys.

We observed an interesting pattern of MUTYH staining with immunohistochemistry analysis. At early stage of lesions, MUTYH expression was increased in tubular cells, while it was decreased with tubular atrophy. The MUTYH expression in interstitial cells seemed not to respond to obstruction injury. It suggested that MUTYH expression in UUO performed a cell-type-specific and stage-dependent response under obstruction injury. Such inconsistence with the same tissues and timely response of expression would result in light change in whole kidney at a specific time, when analyzed by Western blotting analysis. Kim et al. [21] also demonstrated that ROS was harmful, playing a role in proliferation and death in a cell-type-specific way in tubule cells, but not in interstitial cells.

The 8-oxoG lesion is a major marker of oxidative damage and is associated with the progression of renal fibrosis. Antioxidative treatment or blockage of specific pathways will therefore attenuate the fibrotic progress [22]. In the present study, we confirmed that 8-oxoG levels increased in UUO kidneys, related with the severity of renal fibrosis. However, oxidative DNA damage did not parallel MUTYH regulation in the kidney. The 8-oxoG staining increased in both tubule

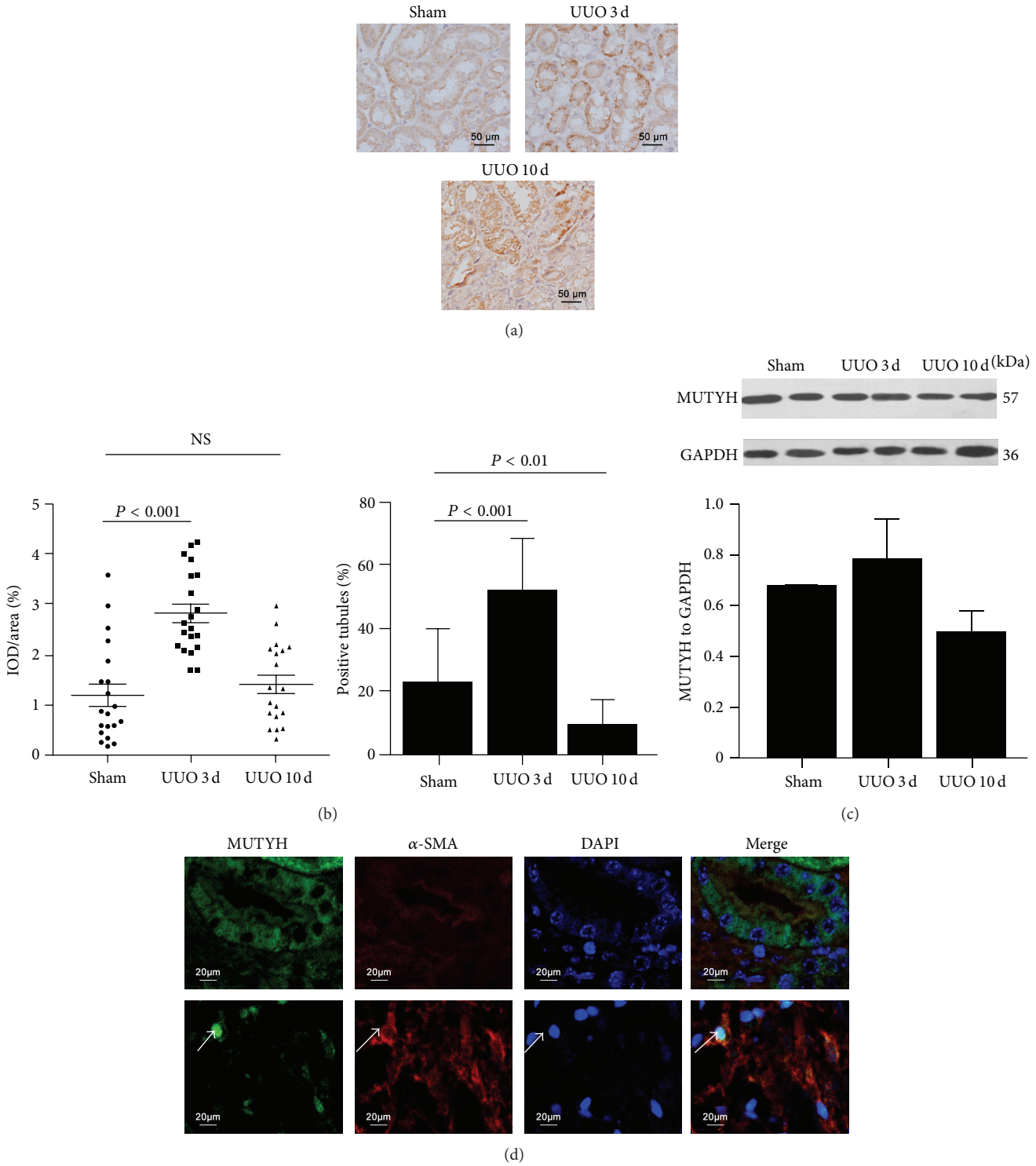

Figure 4: Regulation of MUTYH in renal fibrosis *in vivo*, as shown in UUO kidneys. Immunohistochemical staining of MUTYH showed granular accumulation in tubules of UUO kidneys (a). Both intensity (left panel) and the number of positive tubules (right panel) increased in obstructed kidneys for 3 days and then declined by 10 days ((b), $P < 0.001$). Western blotting showed MUTYH expression in kidneys with dominant mitochondrial bands of ~57 kDa (c). When α-SMA (red) was observed ((d), upper panel), MUTYH staining (in green) localized to the cytoplasm, without overlapping DAPI (in blue). Otherwise, when α-SMA was highly expressed, a few cells with nuclear MUTYH staining (white arrows) were detected in fibrotic interstitial cells ((d), low panel).

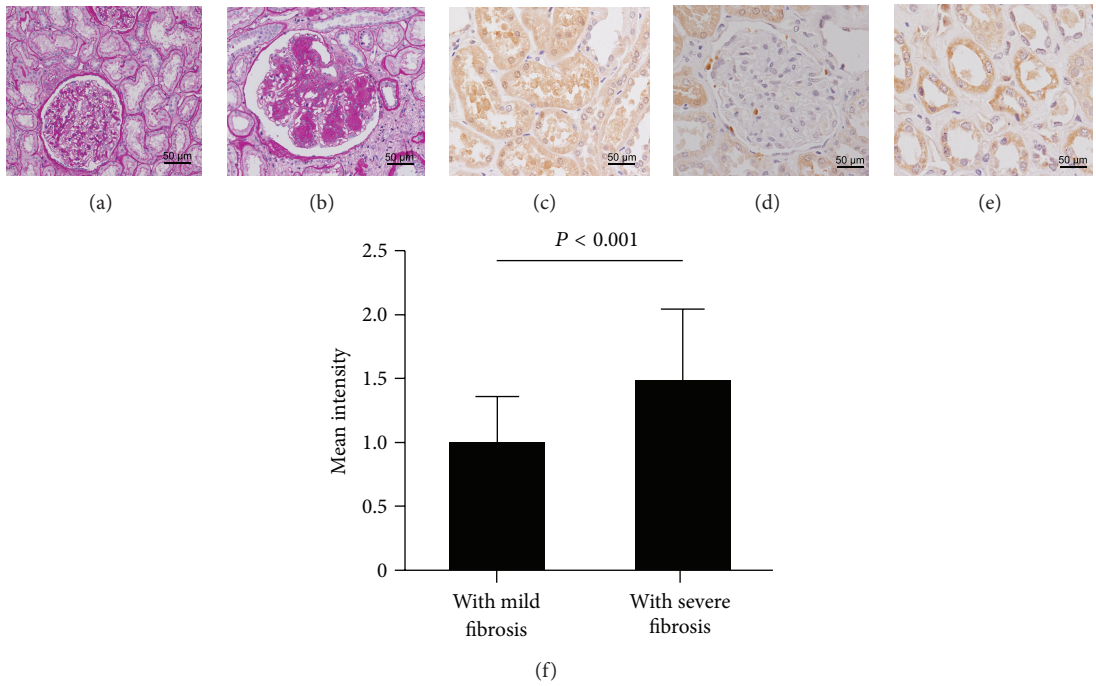

Figure 5: MUTYH immunoreactivity in kidneys from patients with diabetic nephropathy. Representative images from two groups of patients: mild fibrosis (a) and severe fibrosis (b). MUTYH staining was observed in renal tubules (c) but was minimal in glomeruli and interstitium (d). Intense MUTYH staining was visible in tubules in kidneys with severe fibrosis (e) and the IOD (integral optical density) for MUTYH was statistically higher in the severe fibrosis group than in the mild group ((f), $P < 0.001$).

epithelial cells and interstitial cells, whereas MUTYH upregulation was only detected in tubules. Epithelial-mesenchymal transition (EMT) and apoptosis of tubule epithelial cells are two major contributors to renal fibrosis [23]. EMT and apoptosis were induced *in vitro* by TGF-β1 and H_2O_2, respectively. Both H_2O_2 stimulation and TGF-β1 stimulation induced increased 8-oxoG in HK-2 cells. However, only TGF-β1 treatment leads to MUTYH upregulation. H_2O_2, a powerful source of ROS, had no effect on MUTYH expression. These inconsistencies implied that mechanisms in addition to the oxidative DNA damage underlying renal fibrosis were involved in MUTYH regulation.

Moreover, MUTYH has different forms that localize to mitochondria and nuclei, with relative molecular masses of ~57 kDa and ~53 kDa, respectively [10]. During kidney fibrosis, 8-oxoG concentrations increased in both nuclei and mitochondria, and the mitochondrial 8-oxoG was dominant. Similarly, MUYTH was detected in tubule epithelial cell cytoplasm, and increased mitochondrial MUTYH was related to renal fibrosis. Induced expression of nuclear MUTYH was also observed in the fibrotic area labeled by a-SMA staining, but only a few cells were detected *in vivo*. Interestingly, when TGF-β1 induced EMT in HK-2 cells, the dominant MUTYH form changed from mitochondrial to nuclear. This finding suggested that both mitochondrial and nuclear forms of MUTYH were involved in renal fibrosis. They seemed to play distinct roles in tubule cell injury, but more evidence is needed.

An accumulation of 8-oxoG and upregulation of MUTYH were observed in tumor tissues [24]. Dysfunction of MUTYH leading to accumulative 8-oxoG and subsequently mutation was previously considered to perform role of disease. Recently, MUTYH triggered single-strand breaks (SSBs) in DNA were suggested to aggravate brain damage and promote neurodegeneration [25]. Suppression of MUTYH may even be protective under oxidative stress. To understand the association of MUTYH polymorphisms with renal fibrosis and ESRD [9], the present study demonstrated MUTYH upregulation in kidneys, which might be involved in EMT in renal fibrosis. Additionally, the oxidative DNA damage might not be the only trigger for MUTYH regulation. Although the results in the present study could not demonstrate the role of MUTYH on renal fibrosis, the significance of this study is by the first time to illustrate the association between MUTYH regulation and renal obstruction. The present study is fundamental for further studies, and our ongoing studies showed that MUTYH deficiency protects mice from renal fibrosis (data not shown). The underlying mechanism concerning SSBs and EMT is being investigated.

Taken together, our results demonstrated the regulation of MUTYH in tubule cells in renal fibrosis. Renal fibrosis caused a cell-type-specific and time-dependent response of oxidative DNA repairs, even within the same tissues. There are several reports on MUTYH in cancer research, but few on renal diseases. To our knowledge, this is the first report to describe MUTYH in the kidneys, and it provides a new insight into renal fibrosis. More studies will focus on the regulatory role of MUTYH in the kidney and provide target validation for therapeutic strategies.

Disclosure

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

Conflict of Interests

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

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81370788, 81100490), the "Six Major Talent Peaks" of Jiangsu Province (WSN-071-2012), and the Returned Overseas Chinese Scholars, State Education Ministry (to Chen Huimei).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 619560, 10 pages
http://dx.doi.org/10.1155/2015/619560

Research Article

Dandelion Extracts Protect Human Skin Fibroblasts from UVB Damage and Cellular Senescence

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Received 2 December 2014; Accepted 17 February 2015

Academic Editor: Felipe Simon

Ultraviolet (UV) irradiation causes damage in skin by generating excessive reactive oxygen species (ROS) and induction of matrix metalloproteinases (MMPs), leading to skin photoageing. Dandelion extracts have long been used for traditional Chinese medicine and native American medicine to treat cancers, hepatitis, and digestive diseases; however, less is known on the effects of dandelion extracts in skin photoageing. Here we found that dandelion leaf and flower extracts significantly protect UVB irradiation-inhibited cell viability when added before UVB irradiation or promptly after irradiation. Dandelion leaf and flower extracts inhibited UVB irradiation-stimulated MMP activity and ROS generation. Dandelion root extracts showed less action on protecting HDFs from UVB irradiation-induced MMP activity, ROS generation, and cell death. Furthermore, dandelion leaf and flower but not root extracts stimulated glutathione generation and glutathione reductase mRNA expression in the presence or absence of UVB irradiation. We also found that dandelion leaf and flower extracts help absorb UVB irradiation. In addition, dandelion extracts significantly protected HDFs from H_2O_2-induced cellular senescence. In conclusion, dandelion extracts especially leaf and flower extracts are potent protective agents against UVB damage and H_2O_2-induced cellular senescence in HDFs by suppressing ROS generation and MMP activities and helping UVB absorption.

1. Introduction

When skin is aged, skin is more transparent, loose, and fragile. One of the most damaging actions on skin is from solar radiation, especially from its ultraviolet (UV) component, leading to both clinical and histologic damage on human skin [1, 2]. UV irradiation causes distinct alterations of the connective tissues by generating excessive reactive oxygen species (ROS) and induction of matrix metalloproteinases (MMPs). MMPs-mediated degradation of the collagenous extracellular matrix (ECM) accounts for most of the connective tissue damage that occurs in photodamaged skin [3, 4]. Dermal fibroblasts are responsible for generating ECM and allowing the skin to recover from injury [5].

UV irradiation consists of three components, UVA, UVB, and UVC. Whereas UVA and UVB reach the earth in sufficient amounts to damage the skin, UVC is almost completely absorbed by the ozone layer [2, 6]. UVB is particularly damaging, as it penetrates the epidermis and the upper part of the dermis, where it damages fibroblast cells and leads to sunburn, photoageing, and skin cancer [7, 8]. In Canada, sunlight is strong enough to cause skin cancer and premature ageing of the skin. People often use sunscreen to protect the skin from UV damaging from the sun, and sunscreen absorbs UV rays and prevents them from penetrating the skin. However some sunscreen ingredients, including oxybenzone, benzophenone, and octocrylene, have been shown to be potentially skin carcinogenic or penetrate into our body and have other health risks [9]. It is necessary to find effective, safer, and environmentally friendly nature products for antiageing and UV protection.

Taraxacum officinale, commonly known as dandelion, is widely distributed in the warmer temperate zone of the Northern Hemisphere [10]. Dandelion extracts have been used for centuries for traditional Chinese medicine and native American medicine to treat cancers, hepatitis, and digestive diseases [11–14]. Dandelion extracts are shown to have anti-inflammatory, antioxidant, and anticarcinogenic activities;

however, there are few scientific studies to investigate the effects of dandelion extracts in the treatment of skin diseases, especially photoageing [10, 15].

The purpose of this study was to determine whether dandelion leaf, flower, or root extracts can protect human dermal fibroblasts (HDFs) from UVB damage and cellular senescence and the underlying mechanisms. We found that dandelion leaf and flower extracts but not root extracts are potent protective agent against UVB damage and H_2O_2-induced cellular senescence in HDFs by suppressing ROS generation and MMP activities.

2. Materials and Methods

2.1. Preparation of Dandelion Extract. Dandelions were collected at flowering stage in June-July 2013 from the house yards in Thunder Bay (latitude 48°22′56″N; longitude 89°14′46″W), Ontario, Canada. Leaf, flower, and root were separated, cleaned, and air-dried before extraction through 4 hours of boiling in distilled water [13, 16]. The water extracts were filtered through grade 1 Whatman filter paper under vacuum, concentrated, and then dissolved in distilled water following filtration through a 0.2 μm filter to avoid cell contamination, and 300 mg/mL stock aqueous extract was prepared and stored at 4°C for experimental use. The identity of plant was confirmed by Dr. Wei Cao (Lakehead University), and the herbarium specimens were deposited in Cardiovascular and Metabolic Research Unit, Lakehead University for future reference.

2.2. Cell Culture. HDFs were obtained from American Type Culture Collection (Manassas, VA) and cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and grown in a CO_2 incubator at 37°C in 5% CO_2 [17, 18]. The experiments were performed when the cells reached 70–80% confluence between passages 4 and 8. In all studies, cells were incubated in the serum-free medium for 12 h, and then 10% serum added together with different treatments. The media were changed every three days.

2.3. Cellular Viability Assays. Cell viabilities were measured based on conversion of yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to dark blue formazan by viable cells [19, 20]. Briefly, cells at equal number were plated onto each well of 96-well plates for 24 h. After treatment, 100 μL medium containing 5 mg/mL MTT was added to each well, and the cells were then cultured at 37°C for 4 h. The medium was then discarded and the crystals were dissolved in dimethyl sulfoxide and absorbance of formazan products at 570 nm was measured in a Multiskan spectrum microplate spectrophotometer (Thermo Labsystems, Franklin, MA). The cells incubated with control medium were considered 100% viable.

2.4. UVB Irradiation. HDFs were rinsed with phosphate buffered saline (PBS) and irradiated using a UVB lamp (UVB-18, Claremont, CA) with a wavelength range of 280–315 nm [3, 21]. UVB was administered for 60 seconds (200 mJ/cm^2) and the cells were cultured for another 24 hours following detection of cell viability. Dandelion extracts were added 30 minutes before or immediately after UVB radiation. The control cells were untreated with UVB irradiation.

2.5. Measurement of ROS and Glutathione (GSH) Contents. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA) was used to detect ROS [13, 22]. After treatments, HDFs cultured in 35 mm plates were washed with PBS once and then incubated with 1 μg/mL H_2DCFDA in medium for 30 minutes at 37°C. Then the cells were rinsed twice in PBS, and the fluorescence signals were detected using a fluorescent microscope. ImageJ software was used to quantify fluorescent intensity. Total GSH contents were measured using a commercial GSH assay kit (Cayman Chemical, Ann Arbor, MI), as previously described.

2.6. UV Absorption. UV absorption ability of dandelion extracts in medium (300 μg/mL) under 280, 290, 300, and 310 nm was measured with a UV-visible spectrophotometer [23].

2.7. Cell Staining for MMPs. After treatments, HDFs cultured in 35 mm plates were incubated with 1 μg/mL DQ gelatin (Invitrogen) for 30 minutes to analyze MMP activity under a fluorescent microscope. ImageJ software was used to quantify fluorescent intensity. DQ gelatin is a fluorogenic substrate used to detect MMP activity [4, 20]. Upon digestion, its green fluorescence is revealed to be used to measure MMP activity.

2.8. Determination of mRNA Level. Total RNA of HDFs was isolated using TriReagent (Invitrogen) [24]. First strand cDNA was prepared by reverse transcription using M-MuLV reverse transcriptase and random hexamer primers according to manufacturer's protocol (New England Biolabs, Pickering, ON). Real-time PCR was performed in an iCycler iQ^5 apparatus (Bio-Rad, Mississauga, ON) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix, as described previously. The primers of glutathione reductase (GR) were 5′-AGGGCCGCCGTGGTGGAGAGC-3′ (forward, position 265–285) and 5′-ATGGGACTTGGTGAGATTGTTTTG-3′ (reverse, position 475–498). These primers produced a product of 234 bp. The primers of β-actin were purchased from Ambion (Streetsville, ON), which produce a product of 295 bp. A standard curve was constructed using a series of dilution of total RNA (Ambion) transcribed to cDNA using the same protocol outlined above to confirm the same amplifying efficiency in the PCR. A standard melting curve analysis was performed using the following thermal cycling profile: 95°C for 10 s, 55°C for 15 s, and ramping to 95°C at 1° increments to confirm the absence of primer dimers. Relative mRNA quantification was calculated by using the arithmetic formula $2^{-\Delta\Delta CT}$, where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference β-actin cDNA.

2.9. Cell Ageing. Cell ageing was evaluated with senescence-associated β-galactosidase (SA-β-gal) staining [18, 24]. HDFs

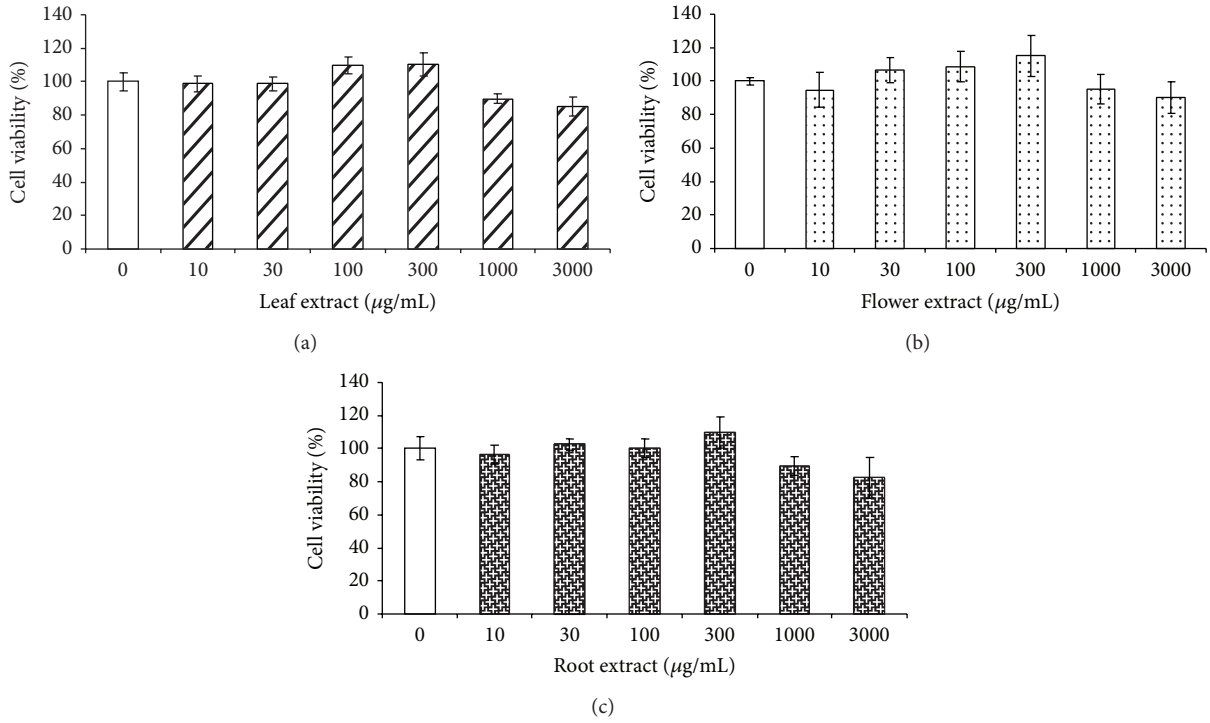

Figure 1: Dandelion extracts have no effect on HDFs cell viability. HDFs were treated with the extracts of dandelion leaf (a), flower (b), and root (c) at 10–3000 μg/mL for 24 hours, and cell viability was then measured with MTT method. $n = 4$.

cultured in 35 mm plates were treated with H_2O_2 (100 μM) with or without dandelion extracts (300 μg/mL) for 72 hours. After that, the cells were fixed and stained with X-gal at pH 6.0 overnight. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) for cell counting. The percentage of ageing cells was calculated as the ratio of blue-stained cells to total cells counted. More than 600 cells were counted from each group.

2.10. Materials and Data Analysis. All chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise. Student's t-test in Microsoft excel was used to analyze data between control and treatment group, and the data were presented as mean ± standard error of the mean. Experiments were repeated a minimum of 3 times. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Dandelion Leaf and Extracts Protect UVB Irradiation-Induced Cell Death. The dandelion extracts were prepared from dandelion leaves, flowers, and root, separately, and the extract rate was 10.6%, 11.2%, and 12.7%, respectively. Dandelion extracts alone at 10–3000 μg/mL had no effect on HDFs cell viability (Figure 1). When dandelion extracts (30, 100, and 300 μg/mL) were added into HDFs for 30 minutes before UVB irradiation (Figures 2(a) and 2(b)) or when added promptly after irradiation (Figures 2(a) and 2(c)), leaf and flower extracts significantly protected UVB irradiation-inhibited cell viability. Dandelion root extracts had less effect on UVB-induced cell death when added into HDFs 30 minutes before UVB irradiation. Although supplement of dandelion root extract at 300 μg/mL immediately after irradiation significantly protected HDFs from UVB-induced cell damage, it was quite inefficient compared with the same dose of leaf and flower extracts (Figure 2(b)).

3.2. Dandelion Leaf and Flower Extracts Inhibit UVB Irradiation-Stimulated MMP Activity and Oxidative Stress. UVB irradiation strengthened the fluorescent intensity of DQ gelatin (Figure 3) and H_2-DCFDA (Figure 4), reflecting the higher activities of MMPs and higher level of ROS, which leads to degradation of ECM and induction of cell death [4, 13]. Dandelion leaf and flower extracts (300 μg/mL) significantly reversed UVB irradiation-induced MMP activity and ROS generation when added either before or after UVB irradiation (Figures 3 and 4). Dandelion root extracts showed less action on protecting HDFs from UVB irradiation-induced MMP activity and ROS generation.

3.3. Dandelion Leaf and Flower Extracts Stimulate GSH Generation and GR mRNA Expression. GSH is master antioxidant in our body [24]. We observed that GSH level is significantly lower in UVB-irradiated HDFs (0.075 mg/mg protein), which is completely reversed by dandelion leaf and flower extracts but not dandelion root extracts. Dandelion leaf and flower extracts alone also significantly increased GSH level (0.45 mg/mg protein and 0.46 mg/mg protein, resp.) compared with the control cells (0.3 mg/mg protein) (Figure 5(a)). GR reduces glutathione disulfide to form GSH

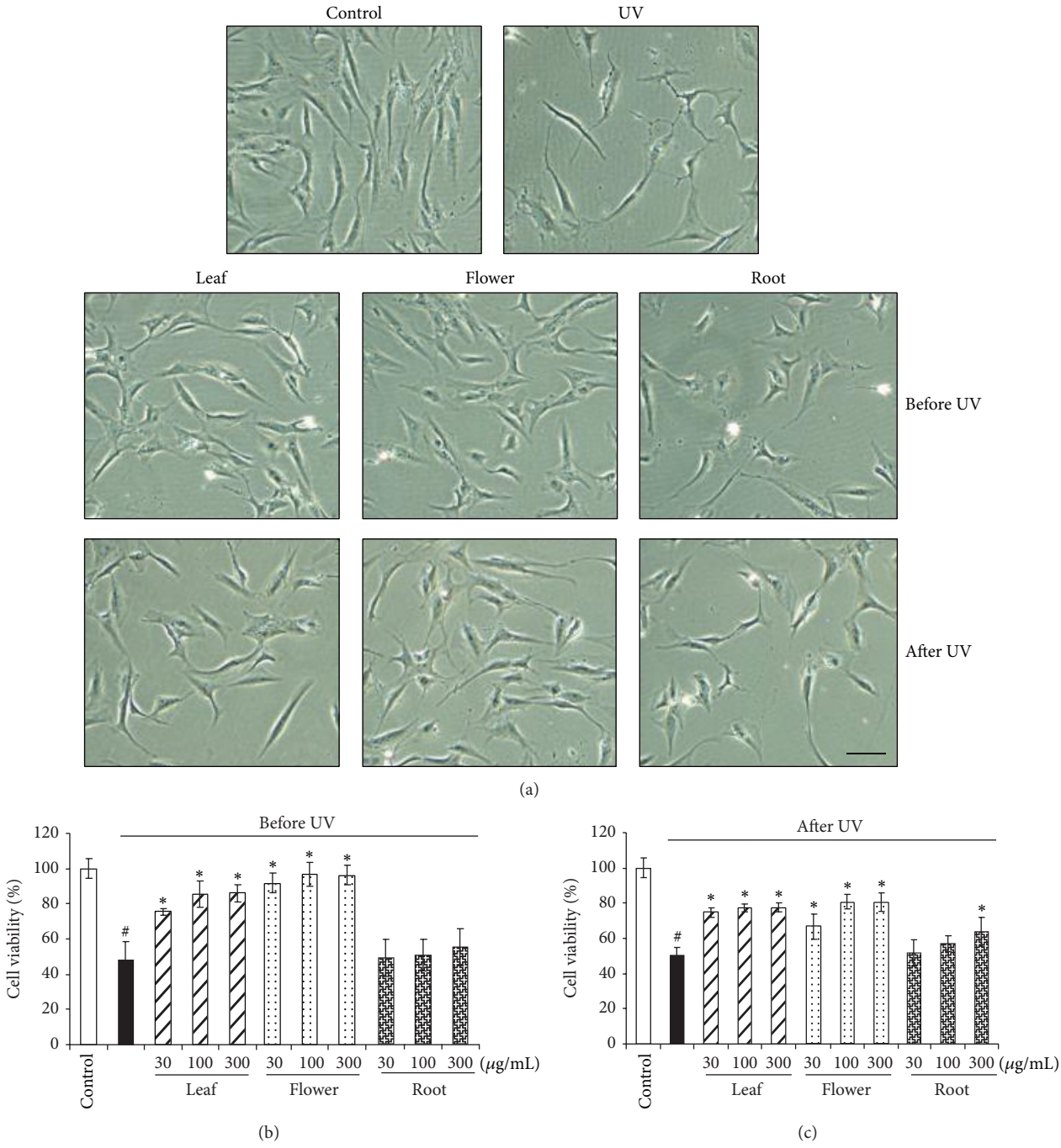

Figure 2: Dandelion leaf and flower extracts protect HDFs from UVB irradiation-induced damage. Dandelion extracts at the indicated concentrations were added 30 minutes before UVB (200 mJ/cm^2) (b) or promptly after UVB radiation (c). Cell viability was measured by MTT 24 hours after culture. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus UVB only. Images in (a) were taken under microscope showing UVB irradiation damages cells, but dandelion leaf and flower extracts (300 μg/mL) protected the cells added from either before or after UVB radiation. Under UVB irradiation, the cells became smaller and less in number. Scale bar: 20 μm. $n = 3$.

and is an important GSH-maintaining gene [25]. The mRNA expression of GR was decreased by 62.0% in UVB-irradiated HDFs in comparison with the control cells (Figure 5(b)). Consistent with the GSH data, incubation of HDFs with dandelion leaf and flower extracts significantly induced GR mRNA expression by 29.1% and 37.2% compared with the control HDFs even in the presence of UVB irradiation. In addition, dandelion leaf and flower extracts alone stimulated GR mRNA expression by 89.4% and 116.2%, respectively. Dandelion root extract had no effect on GR mRNA expression in the presence or absence of UVB irradiation (Figure 5(b)).

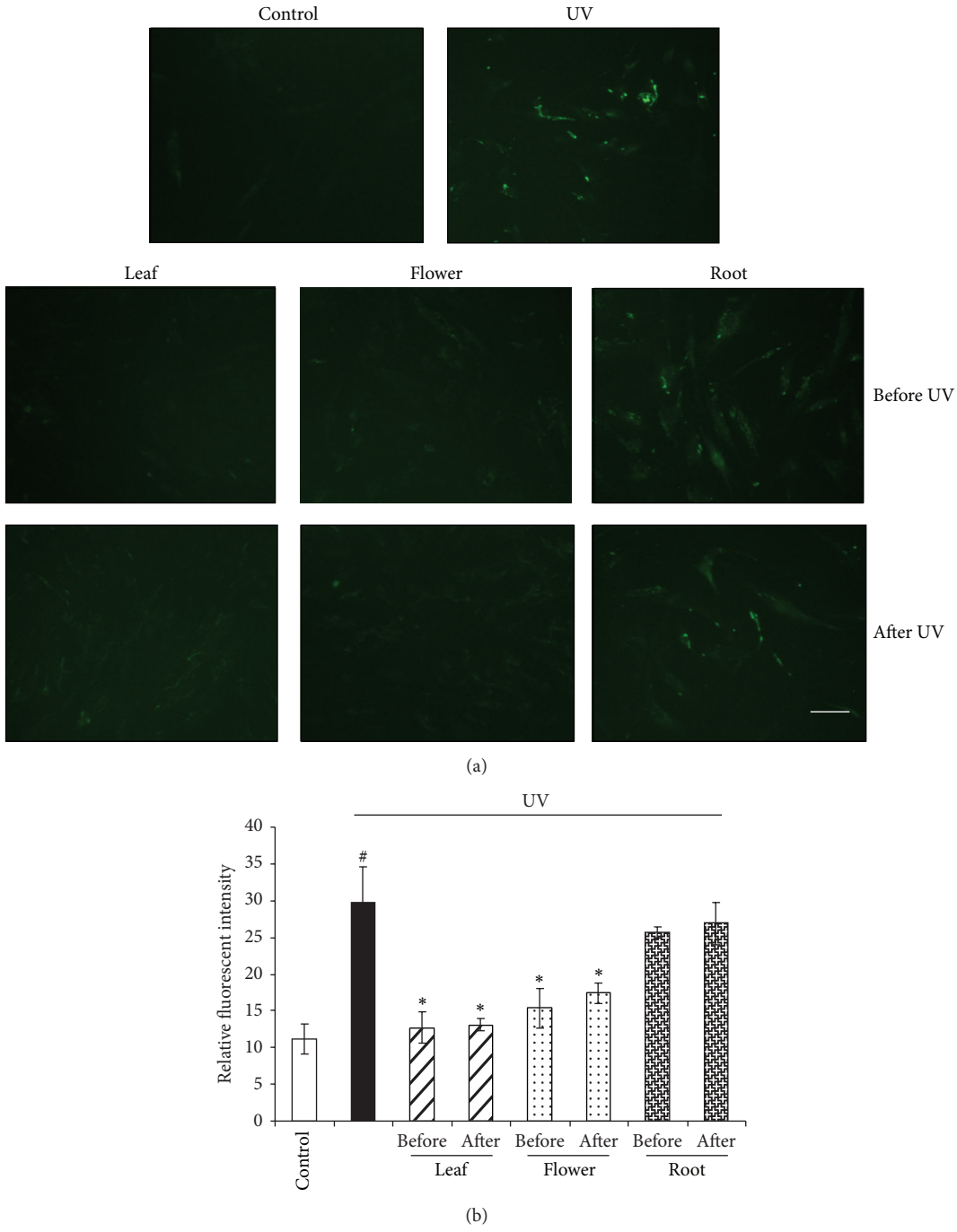

Figure 3: Dandelion leaf and flower extracts inhibit UVB irradiation-stimulated MMP activity. UVB irradiation strengthened the fluorescent intensity of DQ gelatin, reflecting higher MMP activity; however, dandelion leaf and flower extracts (300 μg/mL) significantly decreased MMP activity when added both before and after UVB irradiation. Scale bar: 20 μm. (b) was the statistical analysis from (a) by imageJ software. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus UVB only. $n = 3$. Scale bar: 20 μm.

3.4. Dandelion Leaf and Flower Extracts Help UV Absorption. As shown in Figure 6, dandelion extracts could help absorb UV irradiation. At the wavelengths of 300 nm and 310 nm, flower and leaf extracts had the highest UV absorption capacity. At 290 nm, dandelion root extracts displayed higher UV absorption ability compared with dandelion leaf extract but significantly lower than dandelion flower extract. These data suggest the protective role of dandelion extracts against UV irradiation is partially through UV absorption.

3.5. Dandelion Leaf and Flower Extracts Protect H_2O_2-Induced Cell Ageing. Oxidative stress has been considered one of crucial factors associated with cellular senescence. To

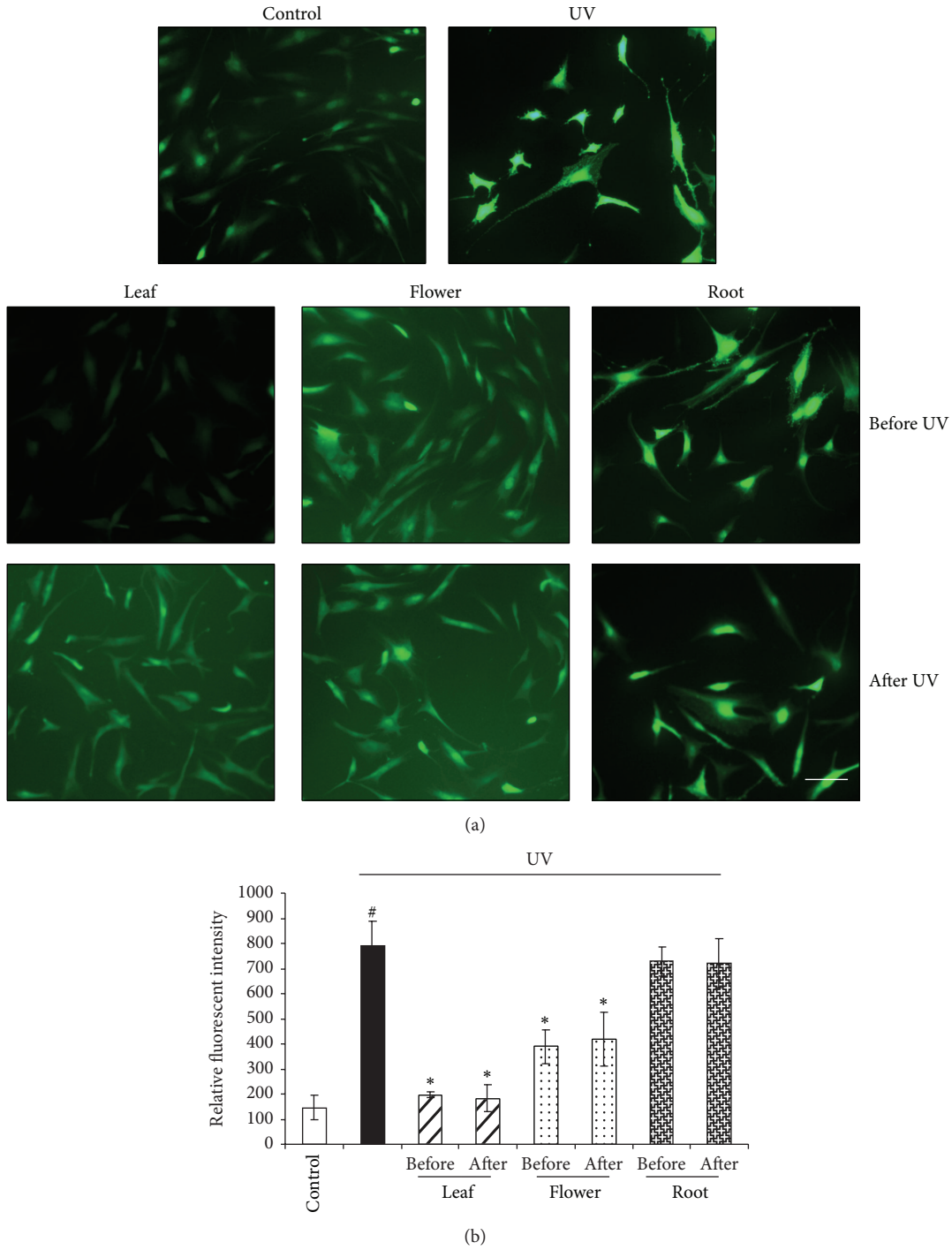

Figure 4: Dandelion leaf and flower extracts inhibit UVB irradiation-induced ROS production. UVB irradiation caused significant increases in the fluorescent intensity of H_2-DCFDA, reflecting the higher level of ROS; however, dandelion leaf and flower extracts (300 μg/mL) significantly decreased UVB irradiation-induced ROS generation when added both before and after UVB irradiation. (b) was the statistical analysis from (a) by imageJ software. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus UVB only. $n = 3$. Scale bar: 20 μm.

study the protective roles of dandelion extracts on oxidative stress-induced premature senescence, we treated HDFs with H_2O_2, one main source of oxidative stress. HDFs became enlarged and flattened with a decreased nucleus-to-cytoplasm ratio after H_2O_2 (100 μM) treatment for 72 hours (Figure 7(a)), showing the typical characteristics of senescent cells [18, 21]. The cells were then stained for SA-β-gal, and the data showed that 78.6% of cells in the H_2O_2-treated group become aged (stained with blue); however, dandelion leaf, flower, and root extracts (300 μg/mL) significantly protect H_2O_2-induced cell ageing by 61.8%, 73.3%, and 40.0%, respectively (Figure 7(b)).

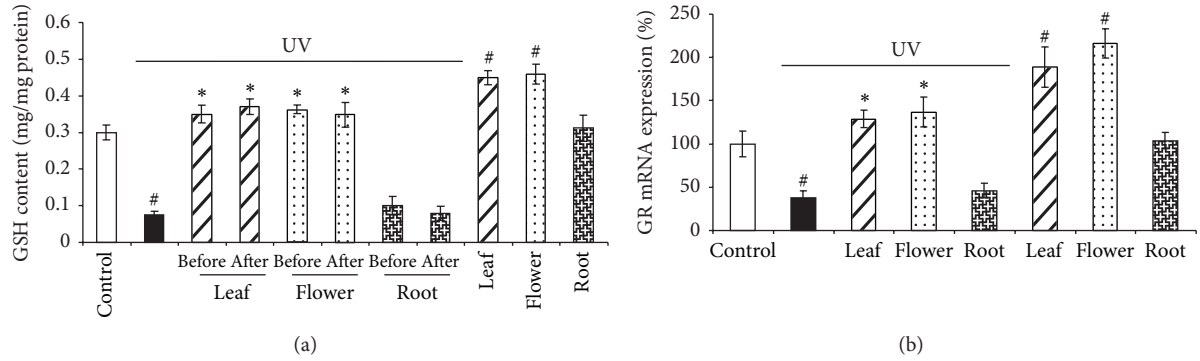

Figure 5: Dandelion leaf and flower extracts stimulate GSH generation and the expression of GR. (a) Dandelion leaf and flower extracts protected UVB irradiation-decreased GSH level. Dandelion extracts (300 μg/mL) were added 30 minutes before UVB or promptly after UVB radiation, and GSH was measured after additional 24-hour culture. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus UVB only. $n = 3$. (b) Dandelion leaf and flower extracts stimulated GR mRNA expression. Dandelion extracts (300 μg/mL) were added 30 minutes before UVB, and GR mRNA was measured after additional 24-hour culture with real-time PCR. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus UVB only. $n = 3$.

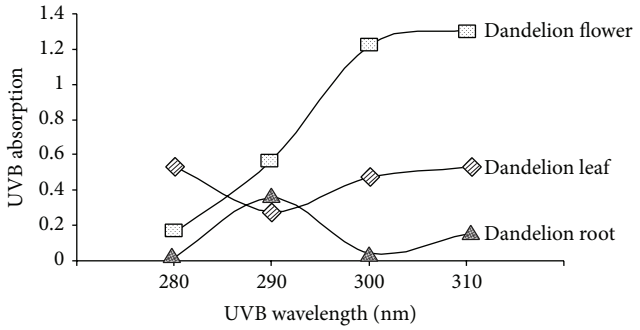

Figure 6: Dandelion extracts help absorb UV irradiation. At wavelengths of 300 nm and 310 nm, flower and leaf extracts had the highest UV absorption capacity. UV absorption of dandelion extracts (300 μg/mL) was measured with a UV spectrometer at 280, 290, 300, and 310 nm, respectively. $n = 3$.

4. Discussion

In recent years, interest on the research of herb medicine has increased all over the world. Many herb extracts have shown therapeutic properties as reported elsewhere [2, 6, 19, 20, 23]. Dandelion is one of the most common and recognizable herbs and is found in almost every part of the world. Many studies have shown that dandelion extracts have a wide range of pharmacological activities, including anticarcinogenic, antioxidant, anti-inflammatory, and anti-heart burn activities [10, 11, 16]; however, there are limited scientific studies investigating the antiageing and anti-UV activity of dandelion extracts and very little is known about the mechanisms of action.

Skin ageing is mainly attributed to extrinsic (photoageing) and intrinsic (chronological ageing) processes that are often manifested by increased wrinkles, loss of tensions and elasticity, and altered pigmentation, and so forth [1, 8]. Dermal fibroblasts are within the dermis layer of skin and are responsible for generating connective tissues, including laminin and fibronectin which comprise the ECM [5]. In this study, we found that exposure of HDFs to UVB leads to reduced cell viability. Without dermal fibroblasts, the skin cannot properly recover from injury. Recognition of supplementing natural antioxidant components in skin care is growing important among dermatologists and other medical professionals. Sunscreens are considered to be the gold standard for protecting skin from UV damage. It is recently noted that sunscreen ingredients may become free radicals themselves when activated by UV radiation. In addition, sunscreen chemicals may be absorbed into the skin, causing harmful effect [9]. Here we demonstrated that supplement of dandelion leaf and flower extracts at as lower as 30 μg/mL rescued the cells from UVB-induced cell death, suggesting dandelion may be useful for preventing and treating skin photoageing. More importantly, dandelion extracts produce protective action when added at both before and after UVB irradiation, pointing to the convenient use of this new product. The dandelion extracts at the concentration as higher as 3 mg/mL did not affect cell growth, so dandelion leaf and flower water extracts would be a green choice for development of novel sunscreens.

It is well established that cutaneous exposure to UV irradiation causes higher activities of MMPs and excessive production of ROS [4, 8, 26]. Our studies further validated that UVB irradiation in HDFs induces significantly higher MMP activities and ROS level. Increased activation of MMPs causes the degradation or disorganization of skin connective tissues, which would lead to increased skin wrinkles and loss of skin tone [5]. Oxidative stress is one of the primary causes of skin ageing, and it is believed that oxidative stress is caused by an imbalance between the production of ROS and skin cell's ability to clean up the reactive intermediates [24]. This is really the case, and the present study found that UVB irradiation markedly decreased the content of GSH, a master antioxidant inside the cells. The reduced expression of GR, a GSH producing gene, would be attributed to the lower level of GSH by UVB irradiation [25]. With less GSH generation, the cells will have less ability to remove overproduced ROS. Excitedly, dandelion leaf and flower extracts attenuated

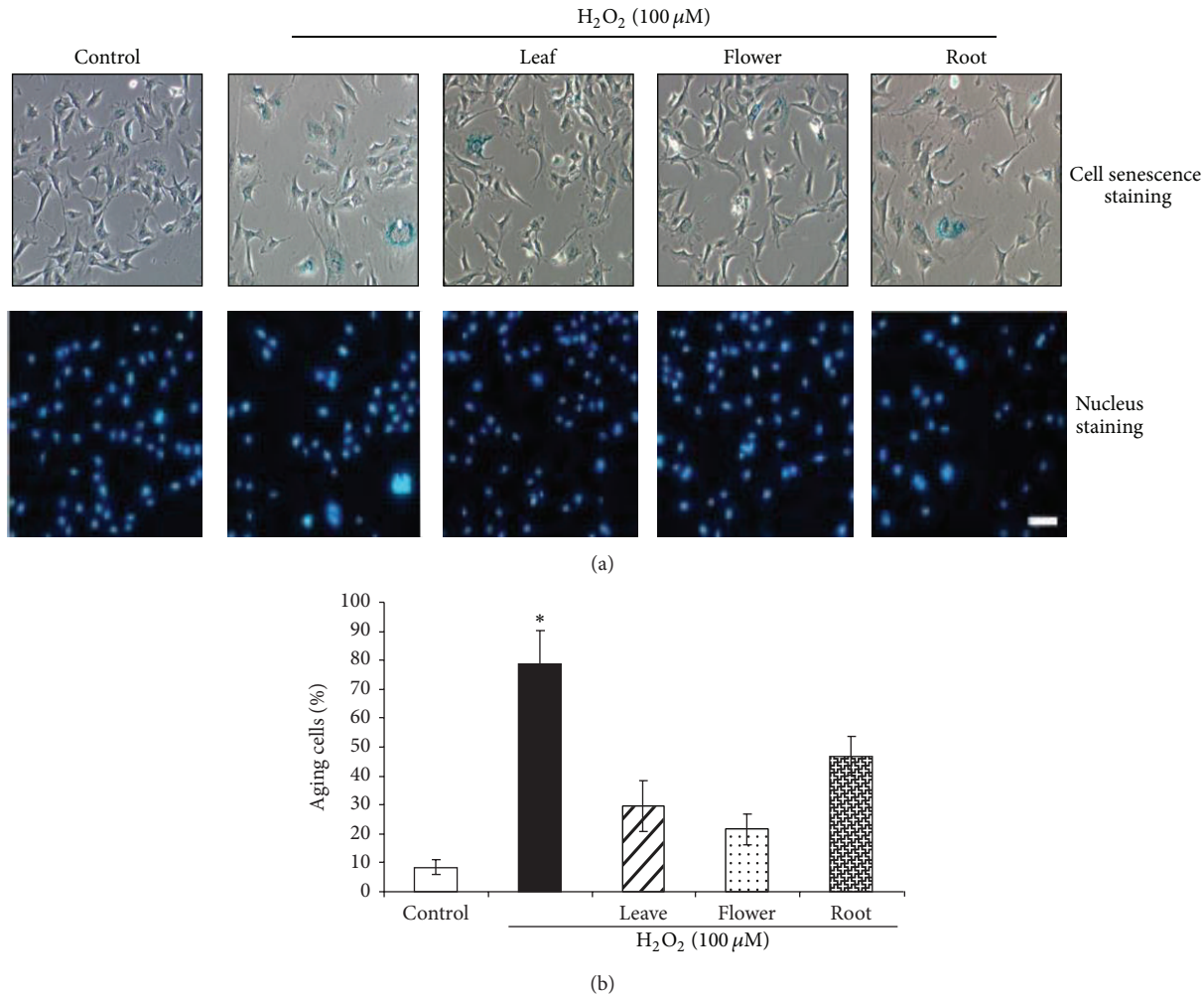

FIGURE 7: Dandelion leaf and flower extracts protect H_2O_2-induced cell ageing. (a) The images showed that H_2O_2 provokes cell ageing, which is protected by dandelion extracts. The ageing cells had higher gal activity and stained with blue color. HDFs were also incubated with DAPI for nucleus staining for cell counting. Scale bar: 20 μm. (b) was the statistical analysis from (a). HDFs were treated with H_2O_2 (100 μM) with or without dandelion extracts (300 μg/mL) for 72 hours. The percentage of aged cells was calculated from the number of blue-stained cells to total cells counted. $^*P < 0.05$ compared all other groups.

UVB-induced MMP activation and ROS generation, with leaf extracts having higher efficiency. Incubation of HDFs with either dandelion leaf or flower extracts alone significantly increased GR mRNA expression and GSH generation. Even in the presence of UVB irradiation, dandelion leaf and flower extracts maintained intracellular GSH level unchanged. All these data indicate that dandelion leaf and flower extracts can protect HDFs from UVB-induced cell death by increasing GSH generation, inhibiting MMP activities and ROS generation.

Ageing at the cellular level is known as cellular senescence [18, 24]. Oxidative stress has been considered one of crucial factors associated with cellular senescence. Most of the senescent cells have flattened cell morphology, promiscuous gene expression, a proinflammatory secretory response, and positive SA-β-gal staining at pH 6.0 [18]. Exposure of HDFs to lower doses of H_2O_2 (100 μM) for 72 hours induced premature senescence, as evidenced by enlarged and flattened cell morphology with a decreased nucleus-to-cytoplasm ratio, and most of the cells were stained blue for SA-β-gal, the typical characteristics of senescent cells. All these changes by H_2O_2 in HDFs were significantly reversed by dandelion extracts. Dandelion extracts improve cellular ageing possibly by directly altering the expressions of senescence-related genes, such as p53, or indirectly lowering oxidative stress.

Unlike dandelion leaf and flower extracts, dandelion root extracts had less effect on UV protection. Compared to dandelion roots, dandelion leaves and flowers are characterized by higher polyphenol contents [10]. So it is highly possible that polyphenols but not other components contribute to the protective role of dandelion leaf and flower extracts against UV damage. Most of the components of dandelion leaf and flower extract have been isolated and identified, and some of the important components of the extract, including sesquiterpene lactones and phenylpropanoids, are believed to have anti-inflammatory, antioxidative, and anticancer properties. Whether these components are also playing the critical roles in protecting HDFs from UVB damage needs to be tested.

Other components which have not been fully characterized also need to be explored.

In conclusion, dandelion water extracts are able to protect HDFs against UVB damage, before irradiation and also when added promptly after irradiation, via increased UV absorption and reduced MMP activity and oxidative stress. The extracts are also able to prevent oxidative stress-induced premature senescence; however, with both UV protection and antiageing, the root extracts have the smallest effect compared with leaf and flower extracts. These findings can lead to more effective, safer, and environmentally friendly antiageing and UV protection. Future studies, both preclinical and clinical, will be necessary to test the efficacy of dandelion extracts for photoageing.

Abbreviations

ECM: Extracellular matrix
HDF: Human dermal fibroblasts
GR: Glutathione reductase
GSH: Glutathione
MMP: Matrix metalloproteinase
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS: Phosphate buffered saline
ROS: Reactive oxygen species
SA-β-gal: Senescence-associated β-galactosidase
UV: Ultraviolet.

Conflict of Interests

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

Acknowledgments

The authors would like to thank Drs. Yaoge Huang and Wei Cao in Lakehead University for suggestion and comments on this project as well as the identification of the plants, and they are also very grateful for the generous support and guidance from all the members in the Cardiovascular and Metabolic Research Unit, Lakehead University, Thunder Bay, Ontario.

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 314560, 14 pages
http://dx.doi.org/10.1155/2015/314560

Review Article

Protective Mechanisms of Flavonoids in Parkinson's Disease

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Received 22 October 2014; Accepted 29 January 2015

Academic Editor: Claudio Cabello-Verrugio

Parkinson's disease is a chronic, debilitating neurodegenerative movement disorder characterized by progressive degeneration of dopaminergic neurons in the *substantia nigra pars compacta* region in human midbrain. To date, oxidative stress is the well accepted concept in the etiology and progression of Parkinson's disease. Hence, the therapeutic agent is targeted against suppressing and alleviating the oxidative stress-induced cellular damage. Within the past decades, an explosion of research discoveries has reported on the protective mechanisms of flavonoids, which are plant-based polyphenols, in the treatment of neurodegenerative disease using both *in vitro* and *in vivo* models. In this paper, we have reviewed the literature on the neuroprotective mechanisms of flavonoids in protecting the dopaminergic neurons hence reducing the symptoms of this movement disorder. The mechanism reviewed includes effect of flavonoids in activation of endogenous antioxidant enzymes, suppressing the lipid peroxidation, inhibition of inflammatory mediators, flavonoids as a mitochondrial target therapy, and modulation of gene expression in neuronal cells.

1. Introduction

James Parkinson (1817), in his paper entitled "Essay on the Shaking Palsy," described Parkinson's disease (PD) as a progressive neurodegenerative disease, characterized by selective loss of dopaminergic neurons in the human midbrain region known as the substantia nigra pars compacta (SNpc) [1]. Degeneration of dopaminergic neurons results in the depletion of the dopamine neurotransmitter production, which manifests clinically as motor dysfunctions such as tremors of hands, bradykinesia, postural instability, and rigidity [2]. Parkinson's disease is also associated with the presence of α-synuclein inclusions known as the Lewy bodies in the *substantia nigra* [3]. However, the basis of selective neuronal loss is still elusive since the disease is only diagnosed at the advanced stage.

The etiology of PD is not clearly defined as the disease does not present any clinical symptoms at the early stage [4]. In most instances, by the time the patient experiences the first clinical symptoms, about 50–70% of the dopaminergic neurons have been damaged or degenerated [5]. Up to now, the factors that trigger the onset of this disease still remain unknown [6]. Although, it has been proposed that PD may be caused by a genetic predisposition or environmental toxins, there is no direct evidence to substantiate these claims [6]. However, researchers have delineated the accumulation of abnormal or "toxic" protein in the neuronal cells to be one of the major causes of neuronal death [7]. There is also evidence to support the role of oxidative stress and imbalance in the natural antioxidant defense system, which could be contributing factors that support the formation and/or accumulation of abnormal or toxic proteins in the neurons [8]. Other factors associated with the pathogenesis of PD include living in rural areas [9], farming activity [10], and drinking well-water [11], as these factors can cause exposure to neurotoxic agents that are usually found in pesticides and environmental toxins [12]. On the contrary, some factors like regular consumption of caffeine and tea [13] as well as

smoking [14] have been found to exert some protective effects against the onset of PD. A few prospective cohort studies as well as animal studies have suggested and proven that moderate to vigorous physical exercise can be a prophylaxis measure against PD [15, 16].

Flavonoids are water-soluble, broad polyphenol family found ubiquitously in plants, which contribute to the orange, blue, and purple color of fruits, flowers, and leaves [17]. Currently, more than 8000 flavonoid compounds have been identified and these are distributed in various kinds of food such as fruits, grains, nuts, green and black tea, and vegetables [18]. Primarily these flavonoids are synthesized by plants via the photosynthesis process and functions in protecting plants against reactive oxygen species (ROS) and are consumed by herbivores [19]. As shown in Table 1, flavonoids can be classified into six main subgroups: flavonones, flavones, isoflavones, flavanols, flavanones, and anthocyanidins [20].

Within the last 20 years, there has been an explosion of facts on the protective effects of flavonoids. However, the initial information on the health benefits of flavonoids relates to the profound antioxidant properties of these compounds [21]. To date, there is growing evidence that flavonoids do not only exhibit antioxidant effects, but also exhibit a variety of other protective effects such as antiapoptotic [22], anticancer [23], anti-inflammatory [24], antiviral [25], and antibacterial [26] effects. Interestingly, flavonoid compounds benefited humans in overcoming oxidative damage-related diseases such as cancer, atherosclerosis, asthma, neurodegenerative disease like PD and Alzheimer's disease (AD) [27]. In this review, the current literature on the protective mechanisms of flavonoids in delaying neuronal cell loss in Parkinson's disease is discussed in depth.

2. Activation of Intracellular Antioxidant Enzymes

Several studies have suggested that PD is a consequence of free radicals-induced oxidative stress [28–30]. In a normal state, free radicals are usually detoxified by various internal antioxidant enzymes to less toxic molecules, which are then removed by various ways [31]. However, the natural antioxidant defense systems may not be able to support overwhelming production of free radicals and this could result in a reduction in the activity of these enzymes [23]. Hence, increased free radicals and oxidative stress in cells can culminate in damaging biological molecules including DNA, proteins, and carbohydrates and cell death [32]. The antioxidant enzymes are including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) that facilitate reactions that help to catalyze the ROS to less toxic molecules [33] (Table 2), thereby playing a key role in preventing lipid peroxidation [23, 24].

Flavonoid compounds have been found to activate the endogenous antioxidant status in neuronal cells hence protecting them from undergoing neurodegeneration [31]. Polyphenols such as quercetin glycosides, rutin, and isoquercitrin (Table 3) have distinct features in upregulating the production of intracellular antioxidant enzymes such as SOD, GPx, CAT, and glutathione in a 6-hydroxydopamine-(6-OHDA-) induced in PC-12, rat pheochromocytoma cells [31, 34, 35]. Besides that, quercetin, fisetin, methyl gallate, and propyl gallate were also found to protect neuronal cells from oxidative stress through elevation of intracellular glutathione level [20]. Apart from that, the neuroprotective effects of polyphenols in protecting neuronal cells were further demonstrated in animal studies using naringin [36]. In this study, naringin was found to suppress the 3-nitropropionic acid-induced neuronal apoptosis via activation of SOD, GPX, CAT, and GR (glutathione reductase) in both striatum and plasma of Wistar rats [36]. Genistein [37] and naringenin [38] also have proved to elevate the antioxidant enzymes, namely, superoxide dismutase, and glutathione peroxidase. Although there are numerous findings on the activation of antioxidant enzymes by flavonoids polyphenols, the mechanisms of action is still unclear.

Several studies have investigated the correlation between free radicals and antioxidant enzymes and a vast number of findings have shown increase in free radical-induced oxidative damage in *in vitro* test systems to be indirectly proportional to the activation of internal antioxidant enzymes [31]. Intriguingly, flavonoids caused activation of these antioxidant enzymes in free radical-induced test systems [31]. Along with this, the activation of intracellular enzymes can be explained by two mechanisms; (i) flavonoids attenuated the free radicals-induced damage on antioxidant enzymes by scavenging the radicals [51] and (ii) flavonoids bound with the antioxidant enzymes and caused direct activation of these enzymes, where any of these mechanisms will result in increased activity of the antioxidant enzyme [31]. In one of the earlier discoveries, Nagata and coworkers (1999) have shown that the antioxidant effects of quercetin and catechin are mediated by direct interaction with the GPx enzyme [52]. These flavonoids cause modulation in the structure-activity of GPx and thereby enhanced its antioxidant activity [52, 53]. In a similar study, it was found that addition of rutin, quercitrin, myricetin, and kaempferol to catalase had a direct effect of activation of catalase and this effect was attributed to the binding of these polyphenols to heme moiety or protein region of this enzyme [54].

3. Suppression of Lipid Peroxidation in Parkinson's Disease

Free radicals and ROS are generated as by-products of several normal cellular functions such as the mitochondrial oxidative phosphorylation system, phagocytosis, and the arachidonic acid metabolism pathway [55]. Most ROS such as the superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), nitrogen species (NO), hydroxyl (OH^-), and alkoxyl radicals are hazardous to cells until it is well catabolized to its less toxic substance by the natural antioxidant enzyme systems. However, if the consistent build-up of ROS and free radicals cannot be supported by the various antioxidant enzyme systems, these conditions can result in oxidative stress and lipid peroxidation, which eventually lead to cellular damage. Several studies have suggested that the presence of neurotoxic

Table 1

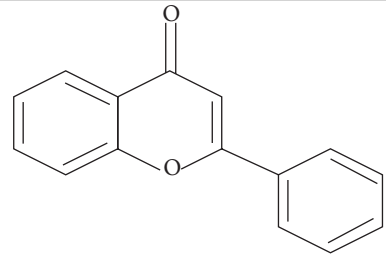
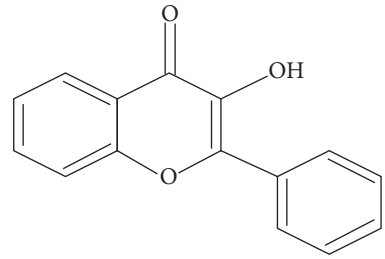
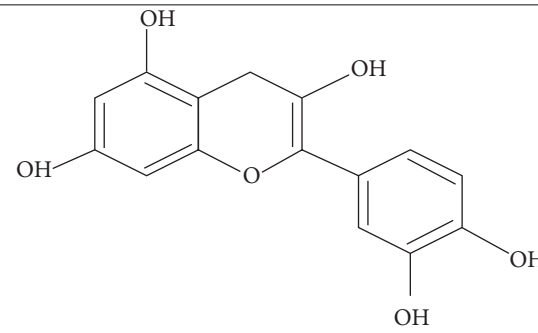
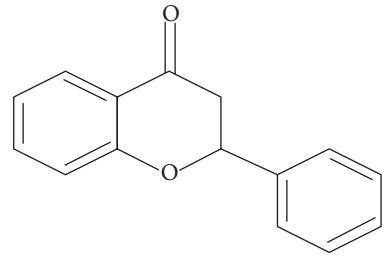
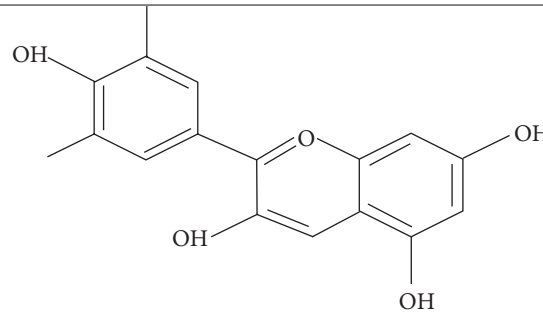
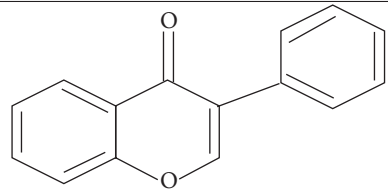
Subgroups	Types of flavonoids	Structures	Food sources
Flavone	Apigenin Luteolin		Apple skins Celery
Flavonol	Kaempferol Myricetin Quercetin Quercetin glycosides, rutin Quercetin glycosides, isoquercitrin		Broccoli Fruits peels Lettuce, olives, onions Buckwheat, citrus fruits Mango, apples, onion
Flavanol	(−)−Epicatechin (−)−Epicatechin 3-gallate (−)−Epigallocatechin (−)−Epigallocatechin 3-gallate (+)−Gallocatechin		Berries, blueberries, fava beans, mature seeds, broccoli, Brussels sprouts
Flavanone	Hesperetin Fisetin Naringin Naringenin		Citrus peel Citrus fruit
Anthocyanidin	Cyanidin Delphinidin Malvidin Pelargonidin Petunidin		Berries Cherries Grapes Raspberries Red wines, strawberries, tea
Isoflavone	Daidzein Genistein Glycitein		Soy bean

Table 2

Antioxidant enzyme	Function	Chemical reaction
Superoxide dismutase	Catalysing superoxide anion to oxygen and hydrogen peroxide	$2O_2^{\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	Detoxifying hydrogen peroxide to water and oxygen molecule	$2H_2O_2 \rightarrow O_2 + 2H_2O$
Glutathione (GSH)	Electron donor to GPx in reducing hydroperoxides to water molecules	$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$
Glutathione peroxidase	Reducing hydroperoxides to water molecules	$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$
Glutathione reductase	Catalyzing the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH)	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

$O_2^{\bullet}$ (superoxide anion); **H_2O_2** (hydrogen peroxide); **O_2** (oxygen); **H_2O** (water molecule); **GSSG** (reduced glutathione); **NADPH** (nicotinamide adenine dinucleotide phosphate).

substances in the human brain may augment the ROS-induced oxidative damage [56–58]. For instance, in PD, prolonged exposure to neurotoxins such as paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to increased generation of ROS in brain neurons as these toxic substances could not be effectively removed by the natural antioxidant enzymes in the brain. This, in turn, inhibited the mitochondrial complex I system, oxidation of polyunsaturated fatty acid (PUFA), protein aggregation, and DNA damage in the neuronal cells [59].

Pryor and Porter were the pioneers in suggesting that lipid peroxidation of certain polyunsaturated fatty acids (PUFA) produces 4-hydroxy-2-nonenal (HNE) as one of the many by-products [60]. HNE is an interesting by-product as it is cytotoxic and appears to be involved in various degenerative diseases, including diabetes [61], pulmonary diseases [62], and Parkinson's disease [63]. In Parkinson's disease, for instance, HNE is found to be an effective protein modifier that induces cross-linking of the monomeric α-synuclein molecules, thereby converting these proteins into high molecular weight β-sheet-rich oligomers [63, 64]. The α-synuclein is a soluble protein consisting of 140 amino acid molecules and is usually located at the presynaptic regions of neurons. Several studies have suggested that α-synuclein is involved in neurotransmitter secretion as well as in the regulation of synaptic vesicle pool and plasticity [65–67]. In the pathogenesis of PD, it has been proposed that oxidative stress triggers a vicious cycle by inducing lipid peroxidation and accumulation of α-synuclein aggregates, which forms Lewy bodies, which forms Lewy bodies, which are associated with neuronal dysfunction that triggers the onset of PD symptoms [68, 69].

There is a substantial body of evidence, which suggest that flavonoid-rich cocoa-derived foods possess free radicals scavenging property against the superoxide anions such as H_2O_2, HClO, and peroxynitrite [70, 71]. The flavan-3-ol compounds present in cocoa are the monomers catechin and epicatechin and the dimer procyanidin B2. These compounds were shown to inhibit lipid peroxidation in brain homogenates and human plasma via anon-enzymatic system [70, 72]. Inhibition of lipid peroxidation in neuronal cells could help to delay the ongoing neurodegeneration process in PD [73]. Besides that, quercetin glycoside derivatives, rutin, and isoquercitrin have shown potent antioxidant potential by attenuating lipid peroxidation induced by 6-OHDA on PC12 neuronal cells (Table 3) [31, 34, 35]. In addition, black tea extract, which contains epigallocatechin (EGCG) polyphenol, was also reported to suppress lipid peroxidation in a 6-OHDA induced rat model of PD [74, 75]. The black tea pretreated rats showed attenuation of lipid peroxidation by 59% compared to the rats in the control group, which were only treated with 6-OHDA [75]. The polyphenol theaflavin was reported to inhibit xanthine oxidase (XO), an enzyme involved in producing superoxides, hence protecting the neuronal cells from undergoing lipid peroxidation [76]. The common feature of most polyphenols, that is, their antioxidant property, is only evident during oxidative stress condition and is not usually demonstrable under normal condition [75]. These convincing evidences markedly support the ability of flavonoids to exert neuroprotective roles via scavenging ROS generated during oxidative stress and subsequently suppress lipid peroxidation in neuronal cells or in animal models of PD.

4. Inhibition of Proinflammatory and Proapoptotic Mediators

Several lines of evidence suggest that microglia activation has a close association with the pathogenesis of PD [77, 78]. This is in line with the discovery of high levels of microglia activation in the vicinity of degenerating dopaminergic neurons and other areas of the human brain such as hippocampus, cingulate cortex, and temporal cortex [79, 80]. Microglia

Table 3

Types of polyphenol	Studied model: cell or animal	Outcome	References
Apigenin	BV-2 murine microglia cell line and cerebral artery occlusion-induced focal ischemia in mice	(i) Inhibiting production of nitric oxide and prostaglandin E2 (ii) Suppressing p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) phosphorylation (iii) Protecting neuronal cells from injury in middle cerebral artery occlusion	Ha et al., 2008 [39]
Luteolin	Lipopolysaccharide (LPS) induced primary mesencephalic neuron-glia	(i) Attenuating the decrease in dopamine uptake and loss of tyrosine hydroxylase (ii) Inhibiting activation of microglia and excessive production of tumor necrosis factor-α, nitric oxide, and superoxide	Chen et al., 2008 [40]
Kaempferol	Rotenone-induced SH-SY5Y cells and primary neurons	(i) Enhancing mitochondrial turnover by autophagy	Filomeni et al., 2012 [41]
Myricetin	MPP^+-treated MES23.5 cells	(i) Attenuating cell loss and nuclear condensation (ii) Suppressing the production of intracellular reactive oxygen species (ROS) (iii) Restoring the mitochondrial transmembrane potential (iv) Increasing Bcl-2/Bax ratio and decreasing Caspase 3 activation (v) Decreasing phosphorylation of MAPK kinase 4 and JNK	Zhang et al., 2011 [42]
Quercetin	Rotenone-induced rats	(i) Reducing cell loss in striatal dopamine (ii) Scavenging hydroxyl radicals (iii) Upregulating mitochondrial complex-I activity	Karuppagounder et al., 2013 [43]
Rutin	6-OHDA induced PC-12 neuronal cells	(i) Activating antioxidant enzymes (SOD, CAT, GPx, GSH) (ii) Suppressing lipid peroxidation	Magalingam et al., 2013 [31]
Isoquercitrin	6-OHDA induced PC-12 neuronal cells	(i) Activating antioxidant enzymes (SOD, CAT, GPx, GSH) (ii) Suppressing lipid peroxidation	Magalingam et al., 2014 [34]
Catechin	6-OHDA-lesioned rats	(i) Attenuating the increase in rotational behavior (ii) Improving the locomotor activity (iii) Restoring GSH levels, increasing dopamine and DOPAC content	Teixeira et al., 2013 [44]
(−)−Epigallocatechin 3-gallate	Serum deprived human SH-SY5Y neuroblastoma cells	(i) Inducing the levels of beta tubulin IV and tropomyosin 3 (ii) Increasing the levels of the binding protein 14-3-3 gamma (iii) Decreasing protein levels and mRNA expression of the beta subunit of the enzyme prolyl 4-hydroxylase (iv) Decreasing protein levels of the immunoglobulin-heavy-chain binding protein and the heat shock protein 90 beta	Weinreb et al., 2007 [45]

Table 3: Continued.

Types of polyphenol	Studied model: cell or animal	Outcome	References
Hesperidin	6-OHDA induced aged mice	(i) Preventing memory impairment (ii) Attenuating reduction in GPx and CAT activity, total reactive antioxidant potential, and the DA and its metabolite levels in the striatum (iii) Attenuating reactive species levels and glutathione reductase	Antunes et al., 2014 [46]
Fisetin	lipopolysaccharide (LPS) stimulated BV-2 microglia cells	(i) Suppressing the production of TNF-α, nitric oxide, and PG E_2 (ii) Inhibiting the gene expression of TNF-α, interleukin (IL-1β), COX-2, and (iNOS) at both mRNA and protein levels. (iii) Suppressing IκB degradation, nuclear translocation of NF-κB, and phosphorylation of p38 MAPKs	Zheng et al., 2008 [47]
Naringenin	6-OHDA induced SH-SY5Y cells and mice	(i) Increasing in nuclear factor E2-related factor 2 (Nrf2) protein levels and activating of antioxidant response pathway genes (ii) Protecting nigrostriatal dopaminergic neurons against neurodegeneration and oxidative damage	Lou et al., 2014 [48]
Theaflavin	MPTP-induced mouse	(i) Reducing oxidative stress (ii) Improving motor behavior and expression of dopamine transporter and vesicular monoamine transporter 2 in striatum and substantia nigra.	Anandhan et al., 2012 [49]
Proanthocyanidin	Rotenone in a primary neuronal cell	(i) Protecting dopaminergic cell (ii) Rescuing mitochondrial respiration in a dopaminergic cell line	Strathearn et al., 2014 [50]

activation is initiated by the presence of extracellular stimuli including endotoxin, cytokines, misfolded or damaged proteins, and chemokines [81]. In the event of microglial activation, the redox-sensitive nuclear factor-kappa-B (NF-κB) found in the cytoplasm will translocate to the nuclear compartment of the cell and form adducts with the DNA. This results in the activation of various proinflammatory genes such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) as well as IL-6 (Figure 1) [82, 83].

Some studies have found neuroinflammation to be associated with the pathogenesis of PD. Hence, a host of anti-inflammatory therapies have been tested using cell-based and rat models of PD to test the ability of various neuroinflammatory mediators such as Dexamethasone [84], aspirin [85], interleukin-10 [86], and Minocycline [87] to suppress the onset of Parkinson-like symptoms. However, one potential approach to ameliorate the neuroinflammation process is by applying natural polyphenols as the therapeutic agent, since these compounds do not have any significant known adverse effects and they appear to promise almost similar outcomes as conventional drug therapies [88]. The potency of various polyphenols tested using both cell-based and animal model of PD is summarized in Table 3. Various flavonoids such as genistein [89], morin [90], kaempferol [91], and emodin [92] were reported to suppress secretion of TNF-α. A recent study found decreased expression of *NF-κB*, *iNOS,* and *COX-2* genes in naringenin pretreated Wistar rats in an experimental model of focal cerebral ischemia/reperfusion (I/R) induced inflammation [83]. In this study, naringenin upregulated the antioxidant status in the naringenin-treated rats as well as inhibiting the expression of *NF-κB* and activation of downstream genes that can trigger the inflammation cascade [83]. Apigenin has gained particular attention as an anti-inflammatory agent that inhibit the expression of nitric oxide (NO), iNOS, and COX-2 in lipopolysaccharide- (LPS-) induced RAW 264.7 cells [93].

Interestingly, there are also studies that show that flavonol polyphenol myricetin can increase the Bcl/Bax ratio as well as decreasing Caspase 3 expression in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP^+) induced cell model of PD (Figure 1) [94]. The Bcl-2 family is associated with mitochondrial function and plays a pivotal role in the activation of caspases and apoptosis [95]. The Bcl-2 protein is an antiapoptotic agent that binds to Bax, which decreases the proapoptotic effect of Bax by forming Bcl/Bax heterodimers, a preset ratio that determines the survival or death of cells following an apoptotic stimulus [96]. Hence, an increase in the Bcl/Bax

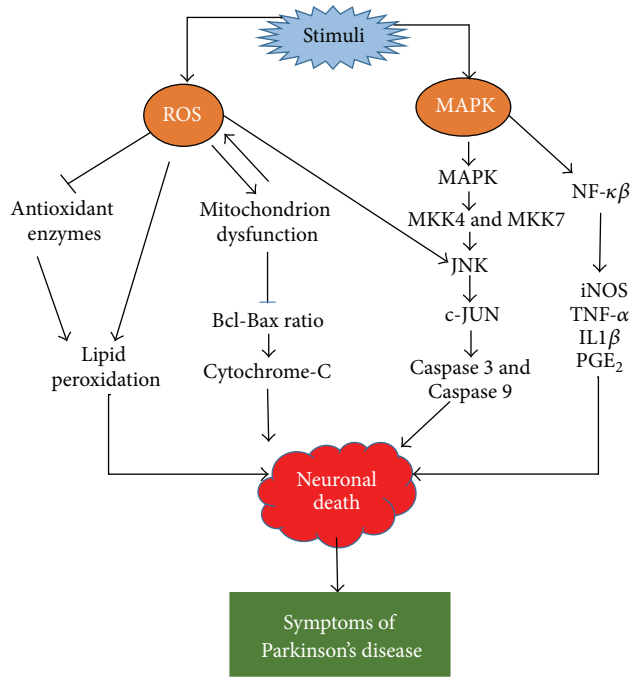


Figure 1: Simplified depiction of ROS and MAPK-induced cytotoxicity. External stimuli including neurotoxin or lipopolysaccharide could generate ROS that is able to suppress the endogenous antioxidant enzymes particularly superoxide dismutase, glutathione peroxidase, and catalase and leads to increase in lipid peroxidation and cell death. The ROS has the ability to directly cause lipid peroxidation and cellular damage as well affecting the mitochondria metabolism, which suppresses the Bcl-Bax ratio and result in leakage of cytochrome-c from mitochondria and eventually cell death. The presence of external stimuli activates MAPK-induced inflammatory mediators including JNK and c-JUN that cause activation of proapoptotic caspases, namely, Caspase 3 and Caspase 9; and the effect is cellular apoptosis. The MAPK family is also responsible in initiating the NF-κB induced expression of proinflammatory cytokine genes (iNOS, TNF-α, and ILIβ). The symptoms of Parkinson's disease occur as a result of neurodegeneration of dopamine producing neurons.

ratio by myricetin pretreatment increased the survival of the neuronal cells [42]. Genistein, an isoflavone, largely found in Soy bean has been shown to possess antiapoptotic effects in 6-OHDA induced SK-N-SH human neuroblastoma cells, where genistein was found to attenuate upregulation of Bax induced by exposure 6-OHDA as well as downregulating the expression of Bcl-2 mRNA and protein [97].

Flavonoids can also exert its protective mechanism via modulation of the mitogen-activated protein kinase (MAPK) signaling pathways [98]. During oxidative stress, the MAPK pathways are activated, ensuing phosphorylation of MAPK kinase 4 (MKK4), a unique protein among the MKK family that is widely distributed in rat and human brains, particularly in the cerebral cortex, hypothalamus, and hippocampus [99]. The stress-induced phosphorylation of the MKK4 results in activation of extracellular signal-regulated kinase 5 (ERK5), c-Jun N-terminal kinase (JNK), and p38 that activates downstream proinflammatory mediators (Figure 1) [100]. Apigenin was found to protect neuronal cells via suppressing the phosphorylation of p38, MAPK, and JNK but not the ERK pathway [93]. A similar finding was observed with theaflavins and thearubigins, the major polyphenols of black tea, whereby a sustained activation of the p38 MAPK and JNK were observed but not in the ERK pathway [101]. Moreover, cocoa procyanidin binds directly to MKK4, inhibiting its activity and also suppressing the JNK signaling pathway [102]. In most of these studies, polyphenols have been reported to attenuate the proinflammatory and proapoptotic mediators and protect the neuronal cells.

5. Mitochondria Targeted Flavonoid Therapy

Mitochondria, a defined cytoplasmic organelle, plays a pivotal in cellular aerobic respiration and regulation of Ca^{2+} homeostasis and is also involved in orchestration of cellular apoptosis and production of ROS [103, 104]. Dysfunctions in the physiological processes of mitochondria can lead to the onset of age-related diseases such as PD and AD [104]. In line with recent understanding, mitochondrial dysfunction can be caused by deficiency in Complex I, which plays a crucial role in mitochondria respiration chain [105]. Exposure of neuronal cells to neurotoxins such as MPP^+, 6-OHDA, and paraquat causes selective uptake of these toxins by the dopaminergic neurons where these toxins inhibit the

activity of Complex I [106]. The decrease in Complex I activity produces excess superoxide radicals that are capable of overwhelming the natural antioxidant systems and eventually cause oxidative stress and neurodegeneration [107]. Several studies have shown mitochondrial ROS production in cells to be the most important source of ROS despite other sources such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) [108], XO, cytochrome P450, and the mitochondrial electron transport chain (ETC) [109]. The main reason for mitochondrial ROS to garner mounting attention is its ability to directly activate mediators of proinflammatory cytokine and MAPK [110], which can lead to several pathological conditions such as cancers, cardiovascular, and neurodegenerative diseases.

Mitochondrial dysfunction can also be caused by the accumulation and aggregation of amyloid-beta (Aβ) peptides [111]. Occurrence of Aβ peptides in neuronal cell is a pathological hallmark in neurodegenerative diseases, particularly PD and AD [112]. A recent study has proposed that soluble amyloid aggregates that are formed in neuronal cells have the inherent capacity to penetrate the mitochondrial membrane and induce neuronal death [113]. The penetration of the Aβ peptides occurs through the mitochondria-associated endoplasmic reticulum membranes, which is a physical connection between the membrane of the endoplasmic reticulum and the mitochondrial outer membrane [113]. Hence, mitochondria targeted polyphenol therapy is an excellent approach in modulating mitochondrial dynamic, function, and biogenesis [114].

Quercetin polyphenol is one of the flavonoids compounds that is widely investigated and reported for its antioxidant [115], anticancer [116], anti-inflammatory [117], and antiviral [118] effects. Interestingly, a recent study has shown that quercetin has the ability to repair the mitochondrial electron transport defect in a rotenone-induced rat model of Parkinsonism (Table 2) [43]. This study also demonstrated a dose-dependent upregulation of Complex I activity in the mitochondria, which the authors attribute to the powerful hydroxyl radicals scavenging action of quercetin [43]. In another study, EGCG, a natural polyphenol derived from green tea, was reported to restore mitochondrial energy deficit in lymphoblasts and fibroblasts from Down syndrome patients [119]. The protective mechanism of EGCG is not clearly defined, but it is proposed that Complex I activity and ATP synthase catalytic activities have been activated beside promotion of cellular levels of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) dependent phosphorylation of Complex I. Treatment with EGCG effectively stimulated mitochondrial biogenesis in the lymphoblasts and fibroblasts Down syndrome patients via activation of the Sirtuin 1 (SIRT1) dependent Peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial DNA content [119]. There is also accumulating evidence that supports the protective effect of genistein on neuronal cells against oxidative damage [120] and glutamate and Aβ amyloid toxicity [121]. Furthermore, it has been reported that genistein exerts its protective mechanism via restoring mitochondrial membrane potential that was significantly decreased by 6-OHDA treatment in SK-N-SH neuroblastoma cells [97]. Naringin, a ubiquitously found flavanone glycoside, has been reported to exhibit several protective effects including antioxidant, ROS scavenging, and metal chelating activities [122–124]. Besides improving cognitive dysfunction and oxidative defense, it was reported that naringin can restore mitochondrial enzyme functions, specifically Complexes I and III activity in a murine model [38].

6. Modulation of Gene Expression Changes

Neuronal cells that undergo a programmed cell death or apoptosis are regulated by both "*protective*" and "*destructive*" genes. "*Protective genes*" are genes that execute protective mechanism by suppressing oxidative stress, thereby protecting cells, such as thioredoxin reductase-1, glutathione S-transferase, pi 2 (Gstp2), superoxide dismutase (SOD2), copper chaperone for SOD1 (CCS), glucose-6-phosphate dehydrogenase (G6PD), and Bcl-2 [125]. In contrast, increased expression of certain genes such as *neuronal cell death-inducible putative kinase* (NIPK), *ankyrin repeat domain-3 (Ankrd3), protein phosphatase 1G (Ppm1g)*, and *ubiquitin carboxyl-terminal hydrolase-20* results in cellular death in PC-12 cells following exposure to 6-OHDA [125]. Studies on PD model showed that treatment of dopaminergic cells (e.g., PC12 cells) with neurotoxins like 6-OHDA or MPTP upregulated proapoptotic genes and other "*destructive*" genes that promote cellular apoptosis [125]. Hence, therapeutic drug targeting these "*destructive*" genes may protect neurons from undergoing apoptotic process and neuronal death.

In our previous study, we have shown that quercetin glycosides, rutin, and isoquercitrin induced neuroprotection by changes in gene expression in 6-OHDA treated PC-12 rat pheochromocytoma cells [126]. Rutin pretreatment attenuated the expression of *Parkin 5 (Park 5), Parkin 7 (Park 7), Caspase 3, Caspase 7,* and *Ataxin 2* gene expression that were highly expressed by 6-OHDA. Moreover, rutin upregulated the protective genes, including *tyrosine hydroxylase (Th), neuron specific gene family member 1 (Nsg1), N-ethylmaleimide-sensitive factor (Nsf)*, and *optic atrophy 1 homolog (Opa1)* genes [126]. On the hand, isoquercitrin suppressed *Park 5* and *Park 7* genes and stimulated *Nsf* and *Nsg1* genes [126] A recent study found that quercetin inhibited NO production by suppressing inducible the transcription of the *iNOS* gene [127]. Quercetin reportedly exerted this effect by suppressing the signaling pathway that leads to the activation of NF-κB, activating protein-1 (AP-1) and signal transducer, and activator of transcription-1 (STAT1), which are the key intracellular agents that contribute to the neuroinflammatory process [128]. This study also found that quercetin upregulated the expression of the *heme oxygenase-1* gene in the BV-2 microglia cells [128]. The heme oxygenase-1 was recently named the "therapeutic funnel" as it was found to possess anti-inflammatory and antiapoptotic effects [129]. In addition, quercetin-induced *heme oxygenase 1* gene

expression was reported to be related to the activation of tyrosine kinase and MAPK [127].

Emerging evidence suggests that genistein, an isoflavone naturally present in Soy beans, reduced MPTP-induced neurotoxicity in a murine model of PD via activation of the Bcl-2 mRNA level in the midbrain [130]. Bcl-2 is an antiapoptotic regulatory protein that maintains mitochondrial integrity by inhibiting the release of cytochrome-c and the intrinsic cascade that leads to activation of Caspase 3 and apoptosis [131]. The neuroprotective mechanism of genistein was attributed to its increased affinity towards the estrogen receptor (ER) hence affecting estrogen-regulated *Bcl-2* gene expression [130]. Previous studies have shown that estradiol treatment could stimulate *Bcl-2* expression in the hypothalamus [132], cerebral cortex [133] as well as neuronal cell line [134]. Therefore, isoflavone genistein could be an effective neuroprotective therapy to reduce the neurotoxin-induced dopaminergic neuronal loss and increase cell survival in human midbrain. Amongst different flavonoids, epigallocatechin-3-gallate (ECGC) was found to modulate changes in gene expression in neuronal cell cultures [135]. Exposure to EGCG was reported to attenuate 6-OHDA induced neuronal loss by preventing the expression of proapoptotic genes such as *Bax*, *Bad,* and *Mdm2* and decrease the expression of antiapoptotic gene expression including *Bcl-2*, *Bcl-w*, and *Bcl-x(l)* [135].

7. Summary

Polyphenol flavonoids are found ubiquitously in a wide range of fruits and vegetables such as apple skin, celery, oranges, onion, mango, apples, and buckwheat, as well as food and beverages derived from plants including olive oil, black/green tea, and red wine. Over the last two decades, a significant amount of data pertaining to the antioxidant effects of different types of flavonoids has been documented. Studies to validate neuroprotective effects of flavonoids were performed induced neurotoxins with either pre- or posttreatment with flavonoid compounds based on the objective of the study. Almost all the published literature suggested that flavonoids can exert neuroprotective effects in pathological conditions, that is, in the presence of prooxidants or neurotoxins but not under normal physiological conditions. These findings clearly explain the antioxidant nature of flavonoids in arresting free radical-induced oxidative damage, which is known to be central to many degenerating diseases including PD. Various types of flavonoid were tested in many types of disease model in both *in vitro* and *in vivo* experimental set-ups. Some of the many protective effects of flavonoids reported included antiapoptosis, antibacterial, antiviral, antioxidant, anticancer, antidiabetic, and anti-inflammatory. However, in terms of neuroprotection, the antiapoptotic and anti-inflammatory ability of flavonoids appear to impede the progressive neuronal loss in neurodegenerative diseases particularly PD. Apart from that, flavonoids such as quercetin, rutin, isoquercitrin, and catechin were found to increase the levels of the natural antioxidant enzymes in the cellular compartment as a bid to suppress the free radical-induced lipid peroxidation. Besides that, flavonoids were also found to downregulate the neuroinflammation process by inhibiting the MAPK signaling pathways that can attenuate the activation of the ERK5, JNK, and p38 signalling pathways, which stimulate production of more downstream proinflammatory mediators. In addition, EGCG was found to modulate expression of proapoptotic genes like *Bax*, *Bad*, and *Mdm2* whilst genistein induced changes in the expression of the *Bcl-2* gene, thereby increasing survival of cells in neurodegenerative diseases. Although flavonoids have shed some light as neuroprotective agents, there are many barrels and barricades in this area of research. To date, the pathogenesis of PD is still poorly defined as the main trigger of the dopaminergic neuronal loss is still largely unknown, although some of the main contributing risk factors like genetic predisposition, environmental toxins, and lack of exercise have been identified. Elucidation of the pathogenesis of PD will further aid in the search to identify flavonoid compounds to stop the trigger point of the "domino" cascade of events involved in neuronal cell death.

8. Future Perspectives

The design of the "magic bullet" as a therapeutic approach to help either prevent or treat PD depends on our understanding of the mechanisms by which flavonoids counteract neuronal damage. Although some mechanisms have been described well, we are still far from getting the complete picture of protective mechanism of flavonoid polyphenol. There are many loop holes in the comprehension of the mechanism by which flavonoids protect neuronal cell; for instance, (i) studies evaluating flavonoids to promote neuronal function and neurite outgrowth in human dopaminergic neurons are limited; (ii) clinical trials of neuroprotection evidence of most promising flavonoid polyphenols in PD patient are scarce; and (iii) strategies to introduce flavonoids and therapeutic dosage as these molecules change compositions in *in vivo* system upon exposure to the acidic environment of gastric cavity and finally studies evaluating the ability of emerging flavonoids compounds to cross the blood brain barrier are needed as very few flavonoids have been tested for this ability in animal models.

Conflict of Interests

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

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 964518, 7 pages
http://dx.doi.org/10.1155/2015/964518

Review Article

Mechanisms of Neuronal Protection against Excitotoxicity, Endoplasmic Reticulum Stress, and Mitochondrial Dysfunction in Stroke and Neurodegenerative Diseases

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Received 7 January 2015; Revised 9 March 2015; Accepted 11 March 2015

Academic Editor: Claudio Cabello-Verrugio

In stroke and neurodegenerative disease, neuronal excitotoxicity, caused by increased extracellular glutamate levels, is known to result in calcium overload and mitochondrial dysfunction. Mitochondrial deficits may involve a deficiency in energy supply as well as generation of high levels of oxidants which are key contributors to neuronal cell death through necrotic and apoptotic mechanisms. Excessive glutamate receptor stimulation also results in increased nitric oxide generation which can be detrimental to cells as nitric oxide interacts with superoxide to form the toxic molecule peroxynitrite. High level oxidant production elicits neuronal apoptosis through the actions of proapoptotic Bcl-2 family members resulting in mitochondrial permeability transition pore opening. In addition to apoptotic responses to severe stress, accumulation of misfolded proteins and high levels of oxidants can elicit endoplasmic reticulum (ER) stress pathways which may also contribute to induction of apoptosis. Two categories of therapeutics are discussed that impact major pro-death events that include induction of oxidants, calcium overload, and ER stress. The first category of therapeutic agent includes the amino acid taurine which prevents calcium overload and is also capable of preventing ER stress by inhibiting specific ER stress pathways. The second category involves N-methyl-D-aspartate receptor (NMDA receptor) partial antagonists illustrated by S-Methyl-N, N-diethyldithiocarbamate sulfoxide (DETC-MeSO), and memantine. DETC-MeSO is protective through preventing excitotoxicity and calcium overload and by blocking specific ER stress pathways. Another NMDA receptor partial antagonist is memantine which prevents excessive glutamate excitation but also remarkably allows maintenance of physiological neurotransmission. Targeting of these major sites of neuronal damage using pharmacological agents is discussed in terms of potential therapeutic approaches for neurological disorders.

1. Introduction

Neuronal excitotoxicity that culminates in neuronal death is a hallmark of cellular responses to major stresses such as those that occur in hypoxia/ischemia injury and in neurodegenerative diseases including Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD). Excitotoxicity arises from a massive release of the neurotransmitter glutamate. Under conditions of cerebral hypoxia and/or ischemia that are characteristic of ischemic stroke, diminished oxygen and glucose availability elicit increased neuronal glutamate release which in turn causes overexcitation of neurons postsynaptically. This high level excitation is known to trigger a cascade of prodeath processes. Glutamate excitotoxicity is associated with a failure to maintain calcium homeostasis in the cell, mitochondrial dysfunction, high level generation of oxidants including reactive oxygen species (ROS) and reactive nitrogen species (RNS), and a loss of mitochondrial membrane potential. Decreased ATP levels, resulting from mitochondrial damage, can contribute to increased levels of

oxidants, as can the activation of NADPH oxidase and xanthine oxidase. With severe stress, collapse of the mitochondrial membrane potential may be irreversible, under which circumstances mitochondrial permeability transition pore (MPTP) opening may occur, resulting in apoptosis. In addition to necrosis, which is catastrophic cell death associated with energy loss, other key pathways of cell death signaling include apoptosis, initiated by Bcl-2 family members and MPTP opening, as well as another key prodeath process, namely, ER stress. In the current review article we will examine the major steps that contribute to the induction of cell death through stress from excitotoxicity and hypoxia/ischemia and excessive production of oxidants and we will highlight two categories of neuroprotective agent that are effective in impacting or interrupting important aspects of prodeath cascades. The first category involves the amino acid taurine which acts to restore calcium homeostasis and inhibits two out of the three primary ER stress pathways. The second category of agent is illustrated by two examples of NMDA receptor partial antagonists: (1) S-methyl-N,N-diethyldithiocarbamate sulfoxide (DETC-MeSO) which was shown to protect *in vivo* against infarction that results from transient brain ischemia through inhibiting a subset of endoplasmic reticulum stress (ER stress) pathways and (2) memantine that blocks glutamate receptor mediated calcium influx while in large part maintaining physiological glutamate neurotransmission.

2. Neuronal Excitotoxicity

Under conditions of hypoxia/ischemia and in neurodegenerative disorders such as Parkinson's disease or Alzheimer's disease, neuronal cells are subjected to overwhelming ionic and biochemical stresses that induce mitochondrial dysfunction as well as elicit cell death processes. Glutamate is the principal excitatory neurotransmitter in the mammalian nervous system and excessive release of glutamate is a key characteristic of these diseases. Importantly, the excessive quantities of extracellular glutamate are toxic and result in neuronal death. High extracellular glutamate results in activation of N-methyl-D-aspartate (NMDA) receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors as well as metabotropic glutamate receptors [1]. Glutamate receptor activation contributes to calcium overload, which in turn activates calcium dependent enzymes, increasing reactive oxygen species and reactive nitrogen species and triggering cascades of cell death. In glutamate excitotoxicity calcium overload and collapse of the mitochondrial membrane potential are two key steps in the commitment of cells to die [2].

Following cerebral ischemia and restoration of the blood supply, prooxidant enzymes and mitochondria generate large quantities of oxidants. Superoxide, a major damaging oxidant, is generated in the mitochondria. Prooxidant enzymes, including xanthine oxidase and NADPH oxidase (NOX), also contribute to generating superoxide. Recent studies on oxygen-glucose deprivation (OGD) treated neurons demonstrate three mechanisms for generating damaging ROS with specific stages of involvement during hypoxia and reoxygenation [3]. The mechanisms were the following: in hypoxia both mitochondria and xanthine oxidase were responsible for ROS generation but during reoxygenation the key source of ROS was NOX.

3. The Role of Oxidants in Signaling and Cell Damage

3.1. Reactive Oxygen Species. Neurons are vulnerable to toxicity from free radicals, in part because of the limited capabilities of their antioxidant mechanisms [4, 5]. Redox homeostasis in normal neurons involves sustaining intracellular signaling with low levels of ROS and a balanced compatible level of antioxidant mechanisms. Under these circumstances, ROS contribute to cell signaling through several mechanisms, such as modulation of activities of protein kinase pathways including receptor tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinases (MAP kinases) and through activation of key transcription factors including activator protein-1 (AP-1) and nuclear factor- (NF-) kappa-B [6, 7]. However, in stroke and neurodegenerative disease, high levels of ROS are damaging to macromolecules and elicit prodeath processes.

Dysfunctional mitochondria have been shown to act as an important source of free radicals in neurodegenerative diseases including stroke, AD, and PD. The mitochondrial defects may involve deficiency in energy metabolism and, depending on the disease, are related to defects in complexes I and III (stroke), complex I (Parkinson's disease), complex II (Huntington's disease) [8], or complex IV (Alzheimer's disease) [9] of the electron transport chain (ETC). Deficiencies in energy supply are particularly damaging to neurons, which have a high energy demand and hence mitochondrial dysfunction may contribute to neuronal cell death by either necrosis or apoptosis.

A second major source of ROS in neurons was shown to be NADPH oxidase and recent data on N-methyl-D-aspartate receptor (NMDA receptor) activation in neuronal cultures points to NADPH oxidase as a greater contributor to superoxide elevation following excessive glutamate exposure than the mitochondrion [10]. Importantly, it was recently demonstrated that an NMDA receptor activated production of superoxide can be inhibited by blocking Na/H exchange and eliciting mild acidosis, [11] therefore providing a metabolic mechanism for the link between NMDA receptor activation and superoxide production.

3.2. Reactive Nitrogen Species: RNS. Upon NMDA receptor activation, NO is generated from neuronal nitric oxide synthase (nNOS) which is linked to the NMDA receptor through shared interaction with postsynaptic density-95 (PSD-95) [12, 13]. Calcium influx through activated NMDA receptors will activate nNOS and produce NO [14].

Nitric oxide plays an important role in excitotoxicity and under conditions of excessive glutamate receptor activation NO interacts with O_2^- (superoxide) to form the toxic molecule peroxynitrite ($ONOO^-$) [6]. Peroxynitrite interferes with mitochondrial respiration and then, through

releasing zinc from intracellular stores, it elicits additional mitochondrial damage. NO has also been shown to elicit cell death responses through mechanisms that involve S-nitrosylation of protein targets, where a NO group forms a covalent interaction with a reactive cysteine. Targets of this process that are S-nitrosylated include caspases and metalloproteases [5, 15].

4. Calcium Overload and Excitotoxicity

Several channels and transporters known to be activated in ischemia are responsible for altering calcium levels in the cytoplasm and these have been shown to include Na^+/Calcium exchanger [16], acid sensing ion channels [17], volume regulated ion channels [18, 19], and TRP channels [5, 20]. An inhibition of calcium efflux through the Na^+/Calcium exchanger during ischemia results in increased calcium accumulation [21].

5. Endogenous Antioxidants and the Protective Effects of Ischemic Preconditioning

Oxidant levels in the cell are generally maintained at low levels by endogenous antioxidants including SOD, glutathione peroxidase, and catalase. Other cellular antioxidants, including glutathione, ascorbate, and vitamin E, also contribute to maintaining a low level of oxidants in the cell [6]. The mitochondria have been shown to contain a range of antioxidant systems for coping with elevated ROS and these include ascorbate, mnSOD, catalase, glutathione, glutathione peroxidase, glutathione reductase, and thioredoxin [22, 23].

Reactive oxygen species are known to have the potential to elicit either detrimental or protective effects in the cell. Whereas high levels of ROS tend to be toxic, low levels of ROS production may be important for signaling and these can serve a beneficial role, which is demonstrated in activation of protective pathways during ischemic preconditioning [23, 24]. An important mechanism of ischemic preconditioning in brain is through induction of cellular antioxidant defenses in part through transcriptional activation by nuclear factor erythroid-2-related factor 2 (Nrf-2) of a number of endogenous antioxidant genes [25]. Nrf-2 is a transcription factor that binds to the promoter domain of key genes that include NAD(P)H quinone oxidoreductases (NQO1 and NQO2), glutathione S-transferase, and heme-oxygenase. Nrf-2 activation is mediated by induction of MAPK signaling, through increased ROS as well as by NO induced S-nitrosylation of PKC [26, 27].

Further important pathways of antioxidant induction in preconditioning include those regulated by hypoxia-inducible factor-1 (HIF-1) [28] and by SIRT1 [29]. HIF-1 contributes to cellular survival through activating transcription of a range of protective molecules including heme oxygenase (HO-1) and Bcl-2/adenovirus E1B 19 kDa-interacting protein (BNIP3). BNIP3 has been recently shown to contribute to autophagy and to decrease mitochondrial ROS production [27, 30]. SIRT1 is known to contribute to regulation of expression of antioxidants, which include manganese superoxide dismutase (MnSOD), glutathione peroxidase 1, and catalase.

6. Two Major Prodeath Mechanisms: Apoptosis and ER Stress

Under conditions of hypoxia/ischemia, neuronal apoptotic processes are initiated through the actions of proapoptotic Bcl-2 family members including Bax and Bak, to open the mitochondrial permeability transition pore (MPTP) and enable the release of cytochrome C from the mitochondrion into the cytoplasm. Cytochrome C interacts with apoptotic protease activating factor-1 (Apaf-1) to form the apoptosome and caspase 9 becomes activated and initiates a downstream caspase cascade [31]. These caspases cleave several different substrates that include poly (ADP-ribose) polymerase-1 (PARP-1) leading to DNA damage. Furthermore, overactivation of PARP-1 will decrease NADH and ATP which results in energy failure and cellular necrosis [1, 3, 32].

A major site for folding and processing of newly synthesized proteins is the endoplasmic reticulum (ER), which plays a central role in cellular calcium storage and signaling [33]. The initiation and progression of protein processing functions in the ER have been shown to be strictly calcium-requiring processes [34, 35]. When these functions are impaired (a pathological state termed ER stress), unfolded proteins then accumulate in the ER. This protein accumulation represents a severe form of stress that can result in apoptosis if ER function cannot be restored.

Impairment of ER function can arise from depletion of ER calcium stores, oxidative stress, from blocking of the proteasome for degrading unfolded proteins, or from proteins that arise from genetic mutations and cannot be correctly folded. Perturbations in protein folding lead to accumulation of defective proteins in the ER lumen which then represent a signal that is detected by ER sensors eliciting downstream signaling events. ER stress activates the unfolded protein response (UPR), a complex signal-transduction pathway responsible for cellular adaptation to reestablish ER homeostasis. However, under conditions of chronic ER stress, the UPR will elicit cell apoptosis [36]. ER stress is known to play a crucial role in hypoxia/ischemia-induced cell damage [33, 37–39]. The accumulation of misfolded proteins in neurons is associated with excitotoxicity and is found in stroke in addition to a number of neurodegenerative diseases including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Parkinson's disease (PD).

7. Unfolded Protein Response (UPR) Pathways

The UPR serves primarily to restore ER function by decreasing the quantity of misfolded proteins that must be correctly processed in the ER and by enhancing ER protein processing capacity. The UPR is triggered by activation of the following three stress sensors on the ER membrane: double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring kinase 1a (IRE1a). The sensing mechanism is understood to involve the ER chaperone Grp78/Bip which recognizes unfolded proteins and then dissociates from each of the three sensing molecules, releasing them from inhibition [36].

In neurons, under conditions of physiological homeostasis, PERK, ATF6, and IRE1a interact with Grp78, but when the ER is dysfunctional, Grp78 dissociates resulting in phosphorylation of PERK and IRE1a and cleavage of ATF6 (P90) to ATF6 (P50) [40]. Activated PERK has been shown to phosphorylate eukaryotic initiation factor 2a (eIF2a) which induces suppression of global protein synthesis while also increasing translation of activating transcription factor 4 (ATF-4) [36]. Under activated conditions, the second sensor IRE1a signals to regulate the mRNA for the transcription factor X box binding protein (XBP1). XBP1 is responsible for regulating a specific subset of UPR target genes, involved in folding and ER-associated degradation (ERAD) [41]. A second function of IRE1a is to bind to adaptor proteins in the cytosol and activate signaling pathways known as alarm pathways (including JNK, Ask1, and NF-kappaB) resulting in activation of autophagy and apoptosis [36]. The third stress sensor, ATF6, a membrane spanning protein, dissociates from Grp78 and then becomes proteolytically cleaved before translocating to the nucleus to contribute to induction of protein quality control genes [33].

Under conditions of severe stress, following PERK activation and subsequent eIF2a phosphorylation, elevated ATF4 contributes to cell death processes by upregulating transcription of proapoptotic Bcl-2 family members in addition to the key transcription factor C/EBP homologous protein (CHOP), which controls transcription of genes encoding both pro- and antiapoptotic Bcl-2 family members. All of these three stress sensing pathways upregulate the transcription factor CHOP and are therefore capable of contributing to cell fate decisions, by altering levels of Bcl-2 family members to elicit apoptosis [42]. A further specialized caspase mechanism involves caspase-12, which is an ER membrane-associated caspase that is upregulated by glutamate excitotoxicity and then activates the caspase pathway cascade hence transducing prodeath signals and apoptosis [43].

8. Therapeutic Interventions Targeting Prodeath Mechanisms

8.1. Taurine Induced Protection through Mechanisms Involving Inhibition of Excitotoxicity, Calcium Overload, Oxidative Stress, and ER Stress. Taurine is the most abundant amino acid in brain, skeletal muscle, and cardiac muscle and has been investigated as a potential therapeutic agent in experimental models of several neurodegenerative diseases including stroke, Alzheimer's disease, and Huntington's disease. Taurine is responsible for contributing to several different cellular processes including neuromodulation, neurotransmission, regulation of calcium dependent functions, acting as an osmolyte, and maintaining the structural integrity of the membrane [44, 45]. Taurine can also serve as a neuroprotective agent to combat glutamate toxicity and H_2O_2 induced cell injury [45]. Previously, it was demonstrated that taurine protected against glutamate induced increases in intracellular free calcium. It was subsequently shown that taurine inhibited glutamate induced calcium influx through L-, P/Q, and N-type voltage gated calcium channels and the NMDA receptor channel [46].

Although taurine is known to be protective against oxidative stress, in a number of tissues it was demonstrated by Aruoma et al. (1988) [47] that taurine is not able to directly scavenge reactive oxygen species. Taurine may be able to restore endogenous antioxidant levels in stressed cells and this effect has been demonstrated in several cell types including neurons, vascular smooth muscle cells, and liver. Taurine has also been found to upregulate antioxidant defenses in normal cells, as shown in a study by Vohra and Hui (2001) [48], demonstrating increased superoxide dismutase and glutathione peroxidase in unstressed neurons. Taurine may block generation of free radicals, through inhibiting cytoplasmic calcium increases, and as a consequence preventing mitochondrial dysfunction [49]. Another recently described antioxidant effect of taurine involves a role of this key amino acid in a mechanism underlying the correct translation and expression of mitochondrial proteins. A deficiency in this function is found in the disease known as mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), which is caused by a specific mutation in a particular taurine conjugated tRNA. The need for taurine is central to the nature of this disorder because the mutated mitochondrial tRNA leu (UUR) fails to modify uridine to 5-taurinomethyluridine. Consequently, defective translation of mitochondrial encoded proteins ensues, eliciting ETC dysfunction and superoxide generation [50, 51]. MELAS presents as a cluster of clinical symptoms that include neuropathy, myopathy, cardiomyopathy, endocrine modifications and retinopathy.

In examining the effects of taurine on primary neuronal cultures, it was found that taurine protected against glutamate excitotoxicity and against cellular damage, following hypoxia/reoxygenation, through regulation of key ER stress pathways. Specifically taurine suppressed the upregulation of caspase-12 and CHOP, following reoxygenation, pointing to a significant involvement in combating ER stress [40]. In a detailed analysis of the three major ER stress pathways, it was found that taurine could downregulate the ratio of cleaved ATF-6 to full length ATF-6 and decrease expression of p-IRE1. Taurine protected against ER stress, following either hypoxia/reoxygenation or glutamate treatment, through suppressing ATF6 and IRE1 pathways but in these studies taurine had no detectable effect on PERK pathway activation [40].

8.2. Partial NMDA Receptor Antagonists

8.2.1. DETC-MeSO. Partial blocking of glutamate receptors shows considerable promise in strategies to combat stroke and neurodegenerative disease. S-Methyl-N,N-diethyldithiocarbamate sulfoxide (DETC-MeSO), a metabolite of disulfiram, which has been used to treat alcoholism for more than 5 decades, is known to be a partial NMDA receptor antagonist. DETC-MeSO selectively and specifically blocks NMDA receptors and was shown to be protective against glutamate excitotoxicity in primary rat neuronal cultures.

In *in vivo* mouse studies DETC-MeSO pretreatment prevented ethanol induced kindling seizures, as well as seizures induced by either NMDA or ammonium acetate, all of which are mediated by NMDA receptors [52]. Using a rat model

of transient focal cerebral ischemia, the effects of DETC-MeSO were examined on infarct size as well as on specific ER stress pathways. DETC-MeSO was found to provide potent neuroprotection, by reversing the ischemia induced activation of the PERK pathway components, in both the core and the penumbra [53]. The results also implicated inhibition of downstream components of the IRE-1 pathway in this neuroprotection. By contrast the ATF-6 pathway of ER stress was not activated in response to DETC-MeSO treatment.

8.2.2. Memantine. Studies on the partial NMDA receptor antagonist memantine indicate that this drug is capable of blocking pathological NMDA receptor pathways, while maintaining physiological functions. Memantine has a low affinity for the NMDA receptor although it has selectivity in terms of its action on the NMDA receptor. The "off-rate" of a drug is intrinsic to the nature of the drug-receptor complex and memantine's low affinity for the NMDA receptor arises, because of the drug's fast off-rate [54]. Memantine inhibits by blocking the NMDA receptor associated ion channel when excessive channel opening occurs. Memantine falls into the category of an uncompetitive antagonist in that its action is dependent upon previous activation of the receptor by the agonist. As a result, a set concentration of antagonist blocks a high concentration of agonist better than it blocks a low concentration of agonist [54]. In combination, the uncompetitive inhibitor mechanism, together with its fast off-rate, enables memantine to block excessively opened NMDA receptor channels, while mostly sparing physiological neurotransmission. In a rat stroke model memantine delivered after the ischemic insult substantially decreased the area of infarct [55, 56].

Memantine is currently an approved drug in Europe and the USA for treatment of moderate-to-severe Alzheimer's disease. Beta-amyloid as soluble oligomers is proposed as the major cause of synaptic dysfunction in Alzheimer's disease. Soluble oligomeric beta-amyloid is known to interact with proteins, which contribute to maintaining glutamate homeostasis, and notably the NMDA receptor. Recent evidence indicates that increased cytosolic calcium, induced by beta-amyloid in neuronal cultures, was only slightly decreased by ifenprodil, an antagonist to NMDA receptors containing the NRB2B subunit. The data suggested that beta-amyloid oligomers directly activate NR2A subunit containing NMDA receptors [57]. Interestingly, in addition to its antagonist function on NMDA receptors, memantine has been reported to decrease levels of secreted APP, A-Beta (1–40), and A-Beta (1–42) as well as decreasing secretion of A-Beta (1–42) in neuroblastoma cells and neuronal cultures [58, 59].

8.3. Concluding Remarks. Included in the initiating stimuli for neuronal prodeath pathways are glutamate excitotoxicity, calcium overload, and high level oxidant production. In this paper we have described classes of therapeutic agent, which may contribute towards preventing these early events, including taurine and the partial NMDA antagonists DETC-MeSO and memantine. Major sites of neuronal damage and potential therapeutic agents indicating their sites of action are presented in Table 1. Glutamate toxicity, calcium overload, and increased oxidants may activate downstream pathways such as apoptosis and the three distinct ER stress pathways. A number of drugs, including taurine and DETC-MeSO, have been shown to inhibit apoptosis cascades and specific subsets of the major ER stress pathways. Current and ongoing therapeutic strategies for stroke and neurodegenerative disease are likely to incorporate targeting of specific signaling mechanisms and to combat these, either in the early adaptations to severe stress just mentioned or during the downstream signaling events, such as in apoptotic caspase activation or in ER stress signaling (such as PERK, ATF6, IRE-1, or CHOP activation). An understanding of the specific prodeath components that can be successfully blocked should aid with finding both the optimal timing for the protective actions of therapeutic agents and the most appropriate choice of drugs for particular diseases, such as stroke, Alzheimer's disease, or other neurodegenerative disorders.

Table 1: Major sites of neuronal damage and potential therapeutic agents indicating their sites of neuroprotective action.

Important sites for protection in neuronal stress		
	Type of neuronal stress	Neuroprotective agent
1	Excitotoxicity	DETC-MeSO, memantine, or taurine
2	Calcium overload	Taurine or preconditioning mechanisms
3	Increases in oxidative stress	Taurine or preconditioning mechanisms
4	Apoptosis	Taurine, DETC-MeSO, or preconditioning mechanisms
5	Endoplasmic reticulum stress	Taurine or DETC-MeSO

Conflict of Interests

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

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 362158, 13 pages
http://dx.doi.org/10.1155/2015/362158

Research Article

Nephroprotective Effects of Polydatin against Ischemia/Reperfusion Injury: A Role for the PI3K/Akt Signal Pathway

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Received 22 November 2014; Accepted 30 January 2015

Academic Editor: Matías Mosqueira

Oxidative stress and inflammation are involved in the pathogenesis in renal ischemia/reperfusion (I/R) injury. It has been demonstrated that polydatin processed the antioxidative, anti-inflammatory, and nephroprotective properties. However, whether it has beneficial effects and the possible mechanisms on renal I/R injury remain unclear. In our present study I/R models were simulated both *in vitro* and *in vivo*. Compared with vehicle control, the administration of polydatin significantly improved the renal function, accelerated the mitogenic response and reduced cell apoptosis in renal I/R injury models, strongly suppressed the I/R-induced upregulation of the expression of tumor necrosis factor-α, interleukin-1β, cyclooxygenase-2, inducible nitric oxide synthase, prostaglandin E-2, and nitric oxide levels, and dramatically decreased contents of malondialdehyde, but it increased the activity of superoxide dismutase, glutathione transferase, glutathione peroxidase and catalase, and the level of glutathione. Further investigation showed that polydatin upregulated the phosphorylation of Akt in kidneys of I/R injury dose-dependently. However, all beneficial effects of polydatin mentioned above were counteracted when we inhibited PI3K/Akt pathway with its specific inhibitor, wortmannin. Taken together, the present findings provide the first evidence demonstrating that PD exhibited prominent nephroprotective effects against renal I/R injury by antioxidative stress and inflammation through PI3-K/Akt-dependent molecular mechanisms.

1. Introduction

Renal ischemia/reperfusion (I/R) injury still remains to be a major medical problem due to the lack of more effective treatment [1, 2]. Inflammatory response and oxidative stress are involved in the pathogenesis in I/R injury [3]. It is likely to be an important therapeutical strategy to implement antioxidant and anti-inflammatory agents to treat renal diseases after I/R injury.

Polydatin ($C_{20}H_{22}O_8$, resveratrol glucoside with a 3,4′,5-trihydroxystibene-3-β-mono-D-glucoside molecular structure, has also been named piceid (Figure 1(a)) is a natural stilbene compound extracted from the dried roots of the perennial herb *Polygonum cuspidatum* Sieb. et Zucc., which has been widely used in traditional Chinese medicine for its multiple pharmacological activities, including its strong antioxidative effects, anti-inflammatory reactions, and improvement of microcirculation [4]. Mounting studies thus far have focused on the beneficial effects of polydatin in prevention of I/R-induced oxidative stress and inflammation. Our previous studies have demonstrated that polydatin exerts cardioprotective effects by activating protein kinase C and mito K_{ATP}-dependent signaling and the direct antioxidative stress mechanisms in myocardial I/R rat models [5]. In recent years, polydatin has been suggested to have the properties of nephroprotective effects in diabetes and urate nephropathy [6–9]. However, little work has been done on its underlying possible mechanism as a drug in treating I/R-induced renal dysfunction.

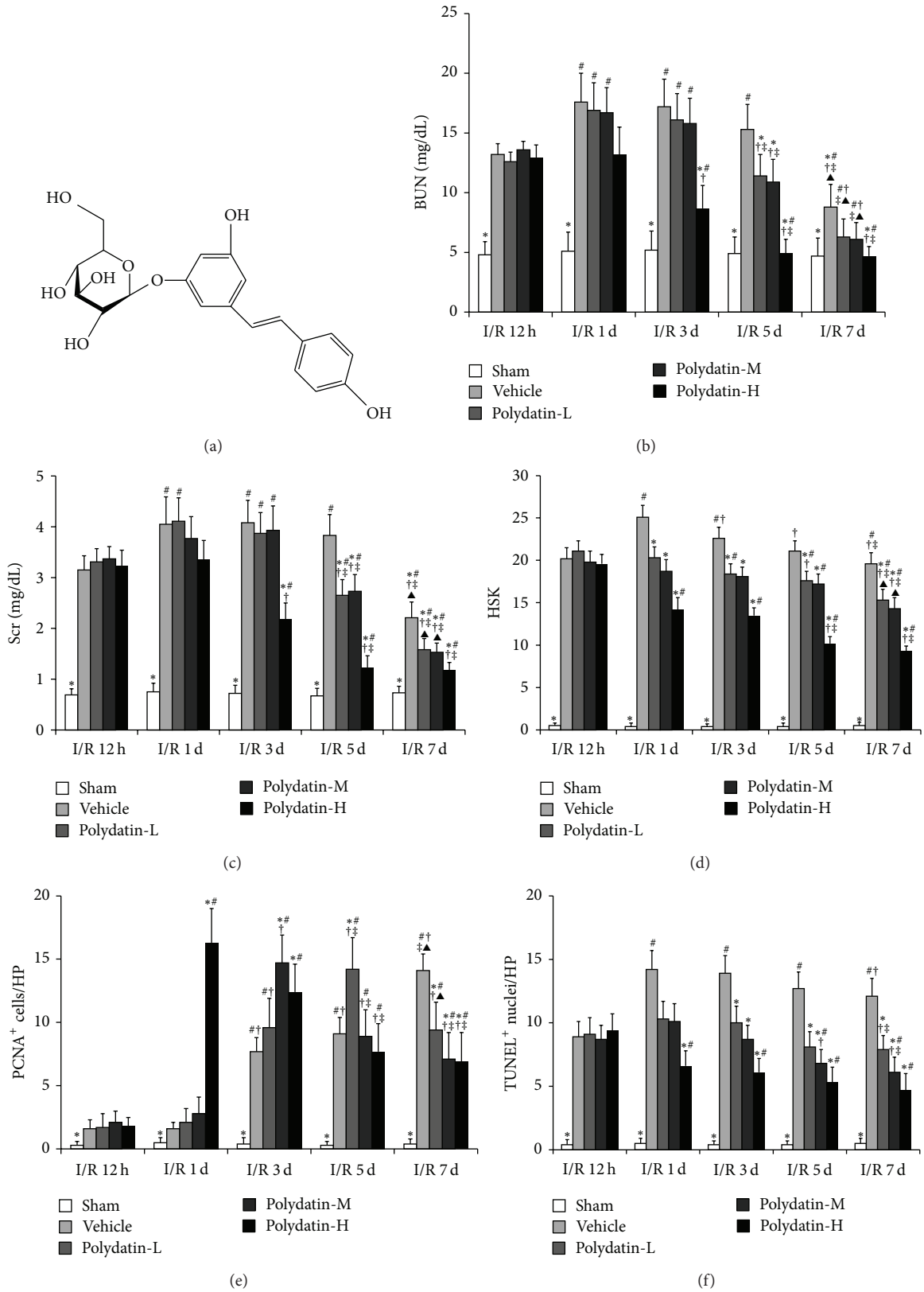


Figure 1: Therapeutical effects of polydatin on renal I/R injury. (a) Chemical structure of polydatin; (b) blood urea nitrogen (BUN); (c) serum creatinine (Scr) in renal I/R mice that received polydatin-L (10 mg/kg), polydatin-M (20 mg/kg), polydatin-H (40 mg/kg), or vehicle (saline with 1% DMSO); (d) histological score of kidney injuries (HSK); (e) immunohistochemical staining for PCNA; and (f) TUNEL in I/R mice that received polydatin-L, polydatin-M, polydatin-H, or vehicle. $^{*}P < 0.05$ versus vehicle; $^{\#}P < 0.05$ versus I/R 12 h respective group; $^{\dagger}P < 0.05$ versus I/R 1 d respective group; $^{\ddagger}P < 0.05$ versus I/R 3 d respective group. $^{\blacktriangle}P < 0.05$ versus I/R 5 d respective group.

The phosphatidylinositol 3-kinase (PI3K) family is a group of revolutionary conserved signal transduction molecule, which can activate its downstream signaling protein, serine/threonine kinase Akt (also known as protein kinase B, PKB), to participate in the regulation of cell proliferation, survival, apoptosis, and various biological responses including oxidative stress, inflammation, and chemotaxis [10, 11]. We [12] and other authors [13] reported that I/R can induce the activation of PI3K/Akt, which promotes the proliferation and viability of renal tubular epithelial cells. However, whether PI3K/Akt signaling pathways participate in the mechanism of actions of polydatin in I/R injury models is unclear.

Therefore, in this study we tested the potential protective effects of polydatin on renal I/R injury models both *in vitro* and *in vivo*. And by the intervention with wortmannin, the specific PI3K/Akt inhibitor, we explored the role of PI3K/Akt pathway in regulating the therapeutic effects of polydatin in acute renal I/R injury.

2. Materials and Methods

2.1. Induction of I/R-AKI. Male BALB/c mice (weighing 20~25 g, 7~9 weeks of age) were obtained from the Experimental Animal Center of the Forth Military Medical University (Xi'an, China) and bred in an experimental animal room of specific-pathogen-free (SPF) grade. The experiment procedures were in line with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1985) and with the European Communities Council directive of 24 November 1986 (86/608/EEC). All mice were provided with food and water *ad libitum* in a 12 : 12-h light/dark cycle (lights on at 6 am and off at 6 pm). They were allowed to adapt to new surroundings for at least 5 days prior to any experimentation. All efforts were made by us to minimize the suffering of animals in present study.

For the establishment of renal I/R injury models, operation was performed in BALB/c mice by clamping bilateral renal pedicles for renal ischemia of 30-minute time, followed by releasing clamps to allow blood reperfusion as described previously [14]. Briefly, the bilateral renal pedicles of mice were carefully bluntly dissected and then occluded using nontraumatic vascular clamps. The similar operation was performed on mice in sham group, with the pedicles also dissected but not clamped. Animals were ethically sacrificed at 12 h, 1 d, 3 d, 5 d, and 7 d after renal I/R injury, respectively, and whole blood and kidneys were harvested for further analysis.

The animals were randomly divided into five experimental groups as follows: (1) sham group; (2) vehicle group: I/R injury mice with saline vehicle (dimethyl sulfoxide, DMSO 1%) intraperitoneally injected; (3) polydatin low dose group (polydatin-L group): I/R injury mice with polydatin (Weijia Technology Company, Xi'an, China) of 10 mg/kg intraperitoneally injected; (4) polydatin middle dose group (polydatin-M group): I/R injury mice with polydatin of 20 mg/kg intraperitoneally injected; and (5) polydatin high dose group (polydatin-H group): I/R injury mice with polydatin of 40 mg/kg intraperitoneally injected. For groups 3 to 5, the initial dose of polydatin was given before the incision sutured after I/R completed and then continued with daily injections for 6 days. For evaluated PI3K/Akt pathway *in vivo*, 1 mg/kg of wortmannin (Sigma), the specific inhibitor of PI3K/Akt signaling, was given 30 min before operation, intraperitoneally, and then continued with daily injection for 2 days. In studies *in vivo* polydatin and wortmannin were first dissolved in DMSO (Sigma) and then diluted by physiological saline with a final concentration of 1% (vol/vol) DMSO for intraperitoneal application.

2.2. Blood Physiochemical Assays. The whole blood drawn from the heart or the retroocular vein plexus was centrifuged at 4°C, 3000 g, for 10 min to obtain the serum sample. The level of blood urea nitrogen (BUN) and serum creatinine (Scr) was measured by the automatic biochemistry analyzer (Beckman; Fullerton, CA).

2.3. Histological Score of Kidney Injuries. Kidney samples were fixed overnight in 10% phosphate-buffered formalin and then embedded in paraffin. Renal sections were next prepared and then subjected to hematoxylin and eosin (H&E) staining to assess the histological injury. Evaluation of histological score of kidney injuries (HSK) was performed by a renal pathologist under blinded conditions. HSK was graded using a 4-point quantitative scale as described previously [15]: 0 represented normal histology; 1 represented mild damage [less than 1/3 of nuclear loss (necrosis) in a tubular cross section]; 2 represented moderate damage [more than 1/3 and less than 2/3 of a tubular cross section shows nuclear loss (necrosis)]; 3 represented severe damage [more than 2/3 of nuclear loss (necrosis) per tubular cross section]. We calculated the total score of per kidney section by adding up all 10 scores with a possible maximum injury score of 30.

2.4. Immunohistochemical Staining. The tissue sections were subject to immunohistochemical staining for proliferating cell nuclear antigen (PCNA, a marker of mitogenesis) 12 h, 1 d, 3 d, 5 d, and 7 d after I/R injury. For immunohistochemical staining, we used the rabbit specific horseradish peroxidase-diaminobenzidine (HRP-DAB) detection immunohistochemical kit (ab64261, Abcam). After being deparaffinized, hydrated, and peroxidase-blocked, 4 μm sections of kidneys were incubated overnight at 4°C with a rabbit polyclonal FL-261 antibody (1 : 200, sc-7907, Santa Cruz Biotechnology, Santa Cruz, USA). Then sections were incubated with a biotinylated secondary antibody, goat anti-rabbit IgG (H + L), for 10 min. Control experiments were performed by omitting either the primary or secondary antibody. Then we developed the sections using an enzymatic conversion of the DAB, visualizing the color of specific antibody binding sites to change into brown. After all sections were counterstained with hematoxylin (Sigma), they were cleared and finally coverslipped for observation. We randomly selected 10 sections from the corticomedullary area per kidney, counting the number of positive nuclei in high-power fields (HPF, 620 magnification). Then we calculated the mean number of PCNA-positive cells of each kidney. The tubular cell apoptosis in kidneys after I/R was detected by terminal deoxynucleotidyl transferase dUTP nick

end labeling (TUNEL) assay following the manufacturer instructions (In Situ Cell Death Detection Kit; Roche China, Ltd.). Meanwhile, we stained all cell nuclei with DAPI (Sigma, USA). We examined TUNEL-stained sections for screening positive nuclei with a fluorescence microscope and selected 10 random fields in renal cortex and outer medullar area in every kidney and then counted them at 640 magnification.

2.5. Western Blot. As previously described [1], routinely, we carried out western blot analyses to detect target protein levels in kidney tissues at 3 days after I/R injury with or without polydatin (10, 20, and 40 mg/kg) and wortmannin (1 mg/kg) treatment and cells with or without OGD/R treatment in presence or absence of polydatin (10, 20, and 40 μM) and wortmannin (1 μM). The primary antibodies included rabbit polyclonal antibodies against phospho-Akt (P-Akt, Ser 473, 1 : 1000, Cell Signaling, Danvers, MA, USA), total Akt (T-Akt, 1 : 1000, Cell Signaling, Danvers, MA, USA), cyclooxygenase-2 (COX-2, 1 : 500, Abcom, USA), inducible nitric oxide synthase (iNOS, 1 : 1000, Abcom, USA), and β-actin (1 : 2000, Abcom, USA). The primary proteins were detected by using horseradish peroxidase-conjugated secondary antibodies (Abcam, USA), and the immune complexes were finally developed by using the enhanced chemiluminescence Plus kit (Amersham, Freiburg, Germany).

2.6. Real-Time PCR (RT-PCR) Analysis. Total RNA was extracted from renal tissues using Trizol according to the manufacturer's instructions (Takara, Japan). We obtained complementary DNA (cDNA) by reverse transcribing four micrograms of total RNA by using the PrimeScript RT Master Mix (Takara, Japan) as instructed. Real-time PCR amplifications were performed by using qPCR technique of SybrGreen assay on the ABI 7500 system (Applied Biosystems, USA). PCR primers (Takara, Japan) for all analyzed genes are as follows: tumor necrosis factor-α (TNF-α), amplicon size 122 bp, forward, 5′-GTG GAA CTG GCA GAA GAG GC-3′ and reverse, 5′-AGA CAG AAG AGC GTG GTG GC-3′; interleukin-1β (IL-1β), amplicon size 230 bp, forward, 5′-GCC CAT CCT CTG TGA CTC AT-3′ and reverse, 5′-AGG CCA CAG GTA TTT TGT CG-3′; COX-2, amplicon size 121 bp, sense: 5′-CCT GGT CTG ATG ATG TAT GC-3′; antisense: 5′-GTA TGA GTC TGC TGG TTT GG-3′; iNOS, amplicon size 108 bp, forward, 5′-TCC ATG ACT CCC AGC ACA-3′ and reverse, 5′-CCA TCT CCT GCA TTT CTT CC-3′; GAPDH, amplicon size 211 bp, forward, 5′-CAT CAA CGG GAA GCC CAT C-3′ and reverse, 5′-CTC GTG GTT CAC ACC CAT C-3′. PCR conditions were as follows: 94°C for 5 min; 35 cycles at 94°C for 40 s, 58°C for 40 s, and 72°C for 60 s; final elongation at 72°C for 10 min. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as reported.

2.7. Oxygen-Glucose Deprivation (OGD). The human proximal tubular epithelial cells, HK-2 (ATCC-CRL-2190, Manassas, VA), were seeded in high glucose DMEM (Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and incubated in humidified cell culture incubator containing gas mixture composed of 21% O_2, 74% N_2, and 5% CO_2 at 37°C for 48 h. OGD followed by reoxygenation (OGD/R) was used to simulate an *in vitro* model of I/R injury [16]. Specifically, the cells in OGD group were incubated in glucose-free DMEM (Gibco, USA) without serum and placed in a hypoxic chamber (Billups-Rothenberg, USA) filled with anoxic gas mixture (95% N_2/5% CO_2) for 6 h. Meanwhile, cells in normal control were incubated in high glucose DMEM supplemented with 10% FBS and placed in a normoxic incubator. At the end of OGD, the plates were taken out from the hypoxic chamber, cells were transferred to high glucose DMEM containing 10% FBS, and they continued to incubate for 24 h under normoxic conditions to generate reoxygenation. In some groups, polydatin (10, 20, and 40 μM) was continuously applied for 30 min before OGD to the end of reoxygenation. To determine the involvement of PI3K/Akt pathway, the PI3K/Akt inhibitor and wortmannin (1 μM) were continuously applied for 30 min before OGD to the end of reoxygenation. In studies *in vitro* polydatin and wortmannin were first dissolved in DMSO (Sigma) and then diluted by DMEM with a final concentration of 1‰ (vol/vol) DMSO for cell treatment.

2.8. Cell Apoptosis Assay. We performed apoptosis assays by using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (catalog number 556419; BD Pharmingen) in accordance with the manufacturer's instructions. Briefly, HK-2 cells in the dish (10^5 cells/well) were collected and then resuspended in binding buffer. Annexin V-FITC and PI were added into the single-cell suspension, which was then incubated in dark place for 15 min. Finally, cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, BD Biosciences, USA).

2.9. Enzyme Linked Immunosorbent Assay (ELISA). The levels of TNF-α, IL-1β, prostaglandin E-2 (PGE-2), and nitric oxide (NO) in renal tissue homogenate were measured by ELISA using a commercially available ELISA kit (R&D Systems, USA) referring to the manufacturer's recommendation. And the quantification of all these factors was implemented by using BCA protein assay reagent (Pierce, USA). Optical density values at 450 nm were measured with wavelength correction set to 570 nm. All standards and samples were measured in duplicate.

2.10. Measurement of Renal Oxidative Indexes. Renal tissue samples were weighed and homogenized (1 : 10, w/v) in 50 mmol/L phosphate buffer (PH 7.4) in an ice-bath and centrifuged at 1500 g for 20 min at 4°C. The supernatant was used to measure the activity of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione transferase (GST), glutathione peroxidase (GPx), catalase (CAT), and the content of glutathione (GSH) that followed the commercial kit instructions by using a spectrophotometer (Spectrophotometer DU640, Beckman Coulter, Fullerton, CA) with the associated detection kits (Jiancheng, Nanjing, China). All of the levels are expressed as U/mg protein or nmol/mg protein, respectively.

2.11. Statistical Analysis. All of the values in the present study were expressed as means ± SD. Differences between data means were compared by use of analysis of variance (ANOVA) or Student's t-test by the SPSS statistical software package (SPSS, Inc., Chicago, IL, USA). A threshold of statistical significance was set at $P < 0.05$ for all analyses.

3. Results

3.1. Polydatin Improved the Renal Function in Renal I/R Injury Mice. First, we detected whether polydatin can improve the renal function of mice after I/R injury. For this purpose, BUN and Scr levels were examined at 12 h, 1 d, 3 d, 5 d, and 7 d after I/R injury in mice with different doses of polydatin administration, respectively. Compared with sham group, the renal functions of mice in vehicle group and polydatin groups were all worsened significantly, which suggested that the renal I/R models were successfully established in the present study (Figures 1(b) and 1(c)). The impaired renal function in mice of vehicle group self-recovered significantly at 7 days after I/R operation. However, compared with vehicle (saline) group, the administration of all three doses (10, 20, and 40 mg/kg) of polydatin significantly improved the impaired renal function of mice after I/R injury, but, respectively, at day 5 and 3 after I/R. These results suggested that polydatin can accelerate the recovery of renal function in mice after I/R injury in dose dependent manner (Figures 1(b) and 1(c)).

Histological examinations including HSK (Figure 1(d)), PCNA (Figure 1(e)), and TUNEL staining (Figure 1(f)) were evaluated at 12 h, 1 d, 3 d, 5 d, and 7 d after I/R. As expected, compared with control kidneys from saline-treated mice, polydatin reduced HSK, increased number of PCNA-positive cells, and decreased number of apoptotic cells on TUNEL assay (Figures 1(d)–1(f)). In particular, at 24 h after I/R injury, the number of PCNA-positive cells in kidneys from mice in polydatin-H group was significantly increased compared with that in the groups of vehicle (+10.2-fold), polydatin-L (+7.8-fold), and polydatin-M (+5.8-fold). Meanwhile, the similar increasement was delayed and detected, respectively, at 3 d, 5 d, and 7 d after I/R in polydatin-M, polydatin-L, and vehicle groups (Figures 1(d)–1(f)). The increase in renal cell survival following I/R injury was confirmed by measure of apoptosis using TUNEL analysis, which showed that polydatin remarkably decreased cell apoptosis in kidneys of mice after I/R, especially the polydatin-H groups, compared with vehicle control at 1 d, 3 d, 5 d, and 7 d after I/R (Figures 1(d)–1(f)).

Given that the beneficial effect of polydatin-H was the most significant at 3 d after I/R in mice, so the subsequent experiments in this study were all performed following this treatment.

3.2. PI3K/Akt Pathway Participated in the Nephroprotective Effects of Polydatin. To validate the association between Akt signaling and nephroprotective effect of polydatin, we detected the activation of Akt. Compared with sham group, the level of p-Akt increased at 3 d after I/R; polydatin dose dependently further elevated the I/R-induced increase of p-Akt. Intraperitoneal injection of the inhibitor of PI3K/Akt, wortmannin, significantly blocked the polydatin-elevated phosphorylation of Akt (Figure 2(a)). In mice of sham group, the renal function after operation was not affected by wortmannin, suggesting that wortmannin had no apparent renal toxicity (data not shown). However, wortmannin significantly reversed the beneficial effect of polydatin in decreasing the levels of BUN and Scr in renal I/R injury mice (Figures 2(b) and 2(c)). Meanwhile, compared with polydatin-H group, kidneys from mice treated with both polydatin-H and wortmannin had significantly increased HSK and the percentage of apoptotic cells on TUNEL assay and reduced number of PCNA-positive cells (Figures 2(d)–2(f)).

To further confirm the results *in vivo*, *in vitro* renal I/R injury models were simulated. The results of western blot showed that the phosphorylation of Akt was activated by OGD/R and further elevated by polydatin dose dependently, but it was counteracted in the presence of wortmannin (Figure 2(g)). The results of apoptosis assays showed that, compared with normal cultured cells, OGD/R notably increased the apoptosis of HK-2 cells, which was obviously suppressed by 20 μM of polydatin (Figure 2(h)). Wortmannin did not increase the cell apoptosis under normoxic conditions which revealed that wortmannin had no apparent cytotoxicity. However, wortmannin not only further increased the OGD/R-induced apoptosis, but also obviously blocked the protective effects of polydatin on HK-2 cells (Figure 2(h)). In short, these results suggested that the nephroprotective effects of polydatin were associated with the PI3K/Akt signaling pathway.

3.3. PI3K/Akt Pathway Is Involved in Polydatin-Attenuated Expression of the Proinflammatory Factors in Renal I/R Injury. To evaluate the potential anti-inflammation effects of polydatin in renal I/R injury, we assessed the expression of TNF-α, IL-1β, COX-2, and iNOS in the kidneys from mice at 3 d after I/R. RT-PCR showed that these cytokines were extensively expressed in kidneys from mice after I/R injury, but only mildly expressed in kidneys from mice with polydatin treatment. However, wortmannin significantly increased the polydatin-attenuated expression of the proinflammatory factors induced by I/R (Figure 3(a)). ELISA analysis showed that the expression of TNF-α and IL-1β was increased at 72 h after I/R injury, which was decreased by polydatin in dose dependent manner (Figure 3(b)). Western blot analysis showed that the expression of COX-2 and iNOS was significantly increased in kidneys at 72 h after I/R injury, which was dose dependently decreased by polydatin (Figure 3(c)). These results suggested the beneficial effect of polydatin on ameliorating the inflammation in renal I/R injury mouse model.

Meanwhile, the intraperitoneal injection of wortmannin obviously abolished the polydatin-induced decreased expression of TNF-α, IL-1β, COX-2, and iNOS (Figures 3(b) and 3(d)). Additionally, we also detected PGE-2 and NO and the downstream factors of COX-2 and iNOS, respectively. Similarly, the levels of PGE-2 and NO were significantly increased by I/R, obviously suppressed by polydatin, and went up again when treating mice with wortmannin (Figure 3(e)). These

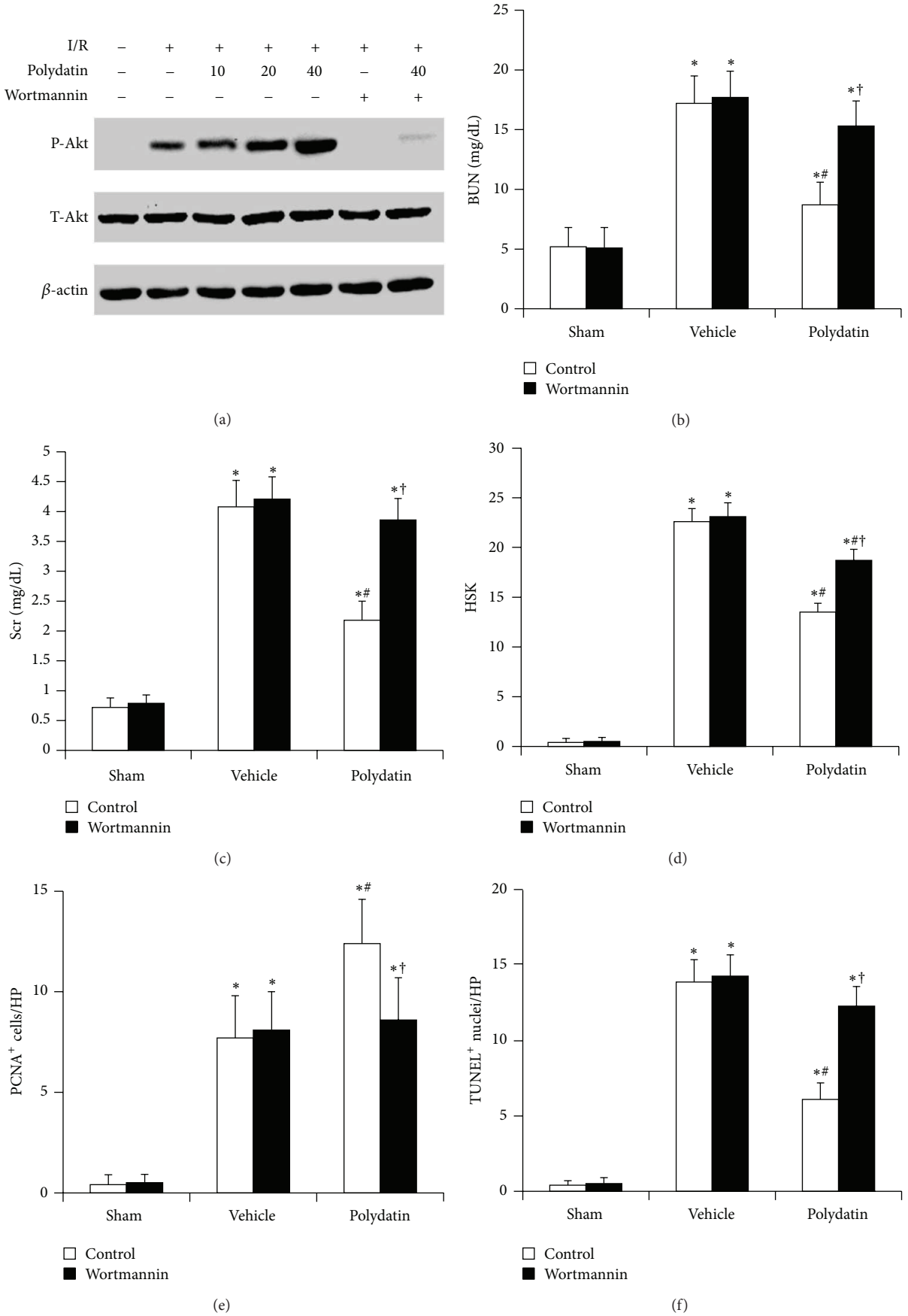


Figure 2: Continued.

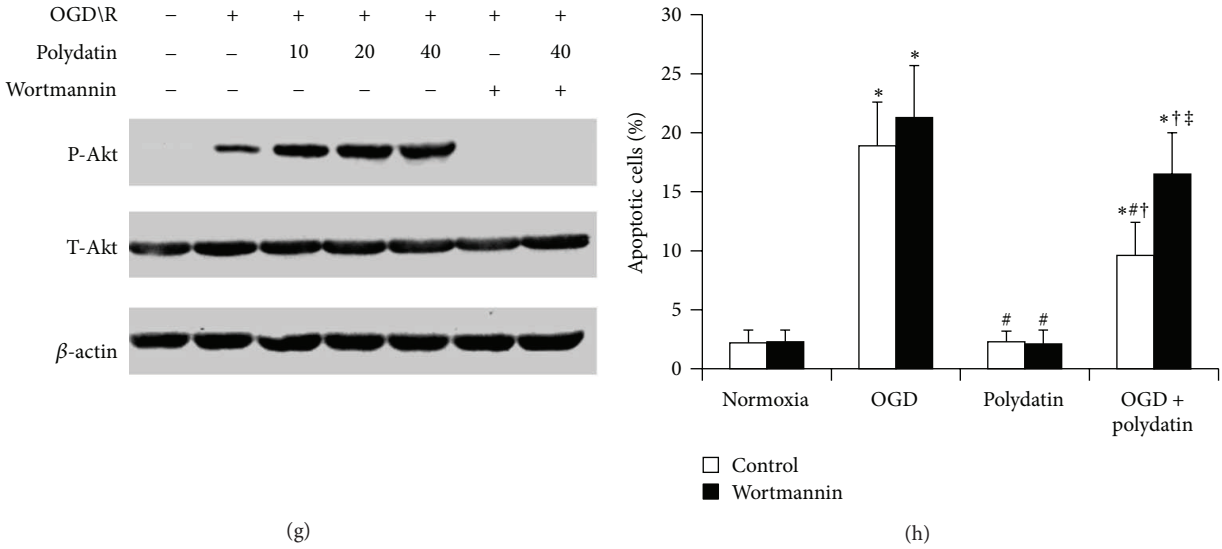


Figure 2: PI3K/Akt pathway participated in the nephroprotective effects of polydatin in renal I/R injury. (a) Western blot for P-Akt and T-Akt proteins in kidneys of mice at 3 d after I/R with or without polydatin and wortmannin treatment. β-actin was used as a control; (b) blood urea nitrogen (BUN), (c) serum creatinine (Scr), (d) histological score of kidney injuries (HSK), (e) renal PCNA expression, (f) renal TUNEL-apoptosis were detected in mice at 3 d after I/R with or without 40 mg/kg of polydatin and 1 mg/kg wortmannin treatment. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus vehicle; $^{\dagger}P < 0.05$ versus control; (g) P-Akt and T-Akt protein levels in cells with or without OGD/R, in presence or absence of polydatin (10, 20, and 40 μM) and wortmannin (1 μM). (h) *In vitro* survival analysis of human proximal tubular epithelial HK-2 cells treated with or without polydatin (20 μM) and wortmannin (1 μM) in basal conditions and after OGD/R. Bar graph described from the FACS-based Annexin V/propidium iodide apoptosis assay. $^{*}P < 0.05$ versus normoxia; $^{\#}P < 0.05$ versus OGD; $^{\dagger}P < 0.05$ versus polydatin; $^{\ddagger}P < 0.05$ versus control.

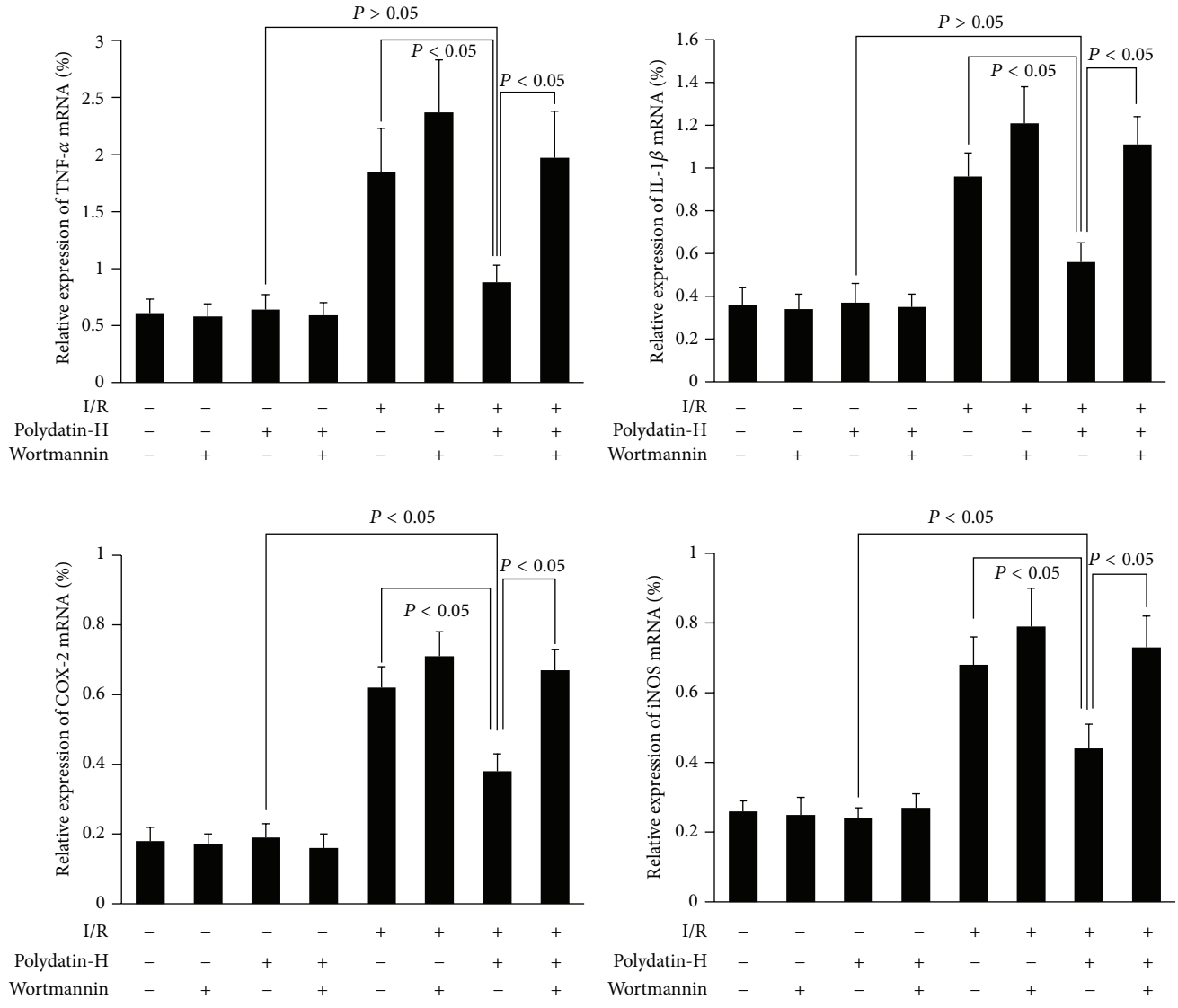
results indicated that PI3K/Akt pathway is involved in the anti-inflammation effect of polydatin in renal I/R injury mice.

3.4. PI3K/Akt Pathway Was Associated with Polydatin-Attenuated Oxidative Stress in Renal I/R Injury. To validate the potential effect of polydatin on antioxidative stress in renal I/R injury, we detected the contents of MDA and GSH and the activity of four antioxidases (SOD, GST, GPx, and CAT) in kidneys, respectively. Compared with sham group, the MDA content was significantly increased in I/R injury mice and was reversed in polydatin-M and polydatin-H groups but not in polydatin-L group (Figure 4(a)), while the activities of the four antioxidases were all significantly decreased in the kidneys of I/R injury mice, and polydatin elevated the activity that was decreased by I/R (Figures 4(b)–4(e)). Compared with vehicle group, the activities of SOD, GST, GPx, and CAT were all significantly increased in polydatin groups except polydatin-L group (Figures 4(b)–4(e)). Compared with sham group, the level of GSH decreased in I/R injury mice. Compared with vehicle group, the GSH content was elevated in polydatin-M and polydatin-H group, but not in polydatin-L group (Figure 4(f)). All measurements mentioned above had no significant statistic difference between sham group and polydatin-H group. These results suggested the antioxidative stress effect of polydatin in alleviating renal I/R injury. However, wortmannin significantly abolished the effect of polydatin on decreasing MDA content, increasing the activity of SOD, GST, GPx, and CAT, and elevating the level of GSH (Figure 4), which suggested that PI3K/Akt pathway was associated with polydatin-attenuated oxidative stress in renal I/R injury.

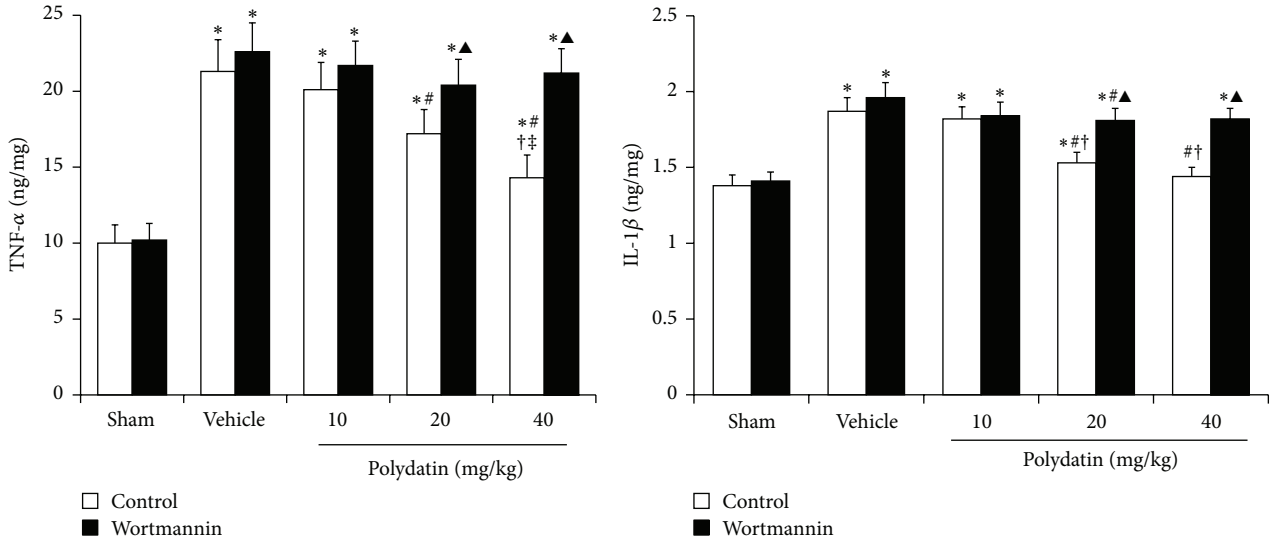
4. Discussion

Polydatin is an active stilbene compound isolated from the roots of *Polygonum cuspidatum* Sieb. et Zucc. and has been manifested to possess antioxidative and anti-inflammatory activities [5, 9, 17]. We and other authors have demonstrated the therapeutical effects of polydatin on I/R-induced injury in multiple organs including heart and brain [4, 5, 18–20]. It has been identified that polydatin also has nephroprotective effects in diabetes and urate nephropathy through prevention of oxidative stress and inflammation [6–9, 21]. In the present study, we further investigated the potential therapeutic effects and mechanism of polydatin on renal I/R injury. Our results showed that the administration of polydatin significantly improved the renal function, accelerated the mitogenic response, and reduced HSK and cell apoptosis in renal I/R injury models, suggesting the beneficial effect of polydatin against renal I/R injury.

In fact, renal I/R injury always induced the excessive generation of proinflammatory cytokines in kidneys, which resulted in leukocyte infiltration and tissue damage [22, 23]. TNF-α and IL-1β are two important proinflammatory mediators in renal I/R injury, which produce a number of injurious changes in proximal tubular epithelial cells [22–26]. Loss of TNF-α, either through using neutralization antibody of TNF-α blockade or knockout mice of TNF-α, resulted in



(a)



(b)

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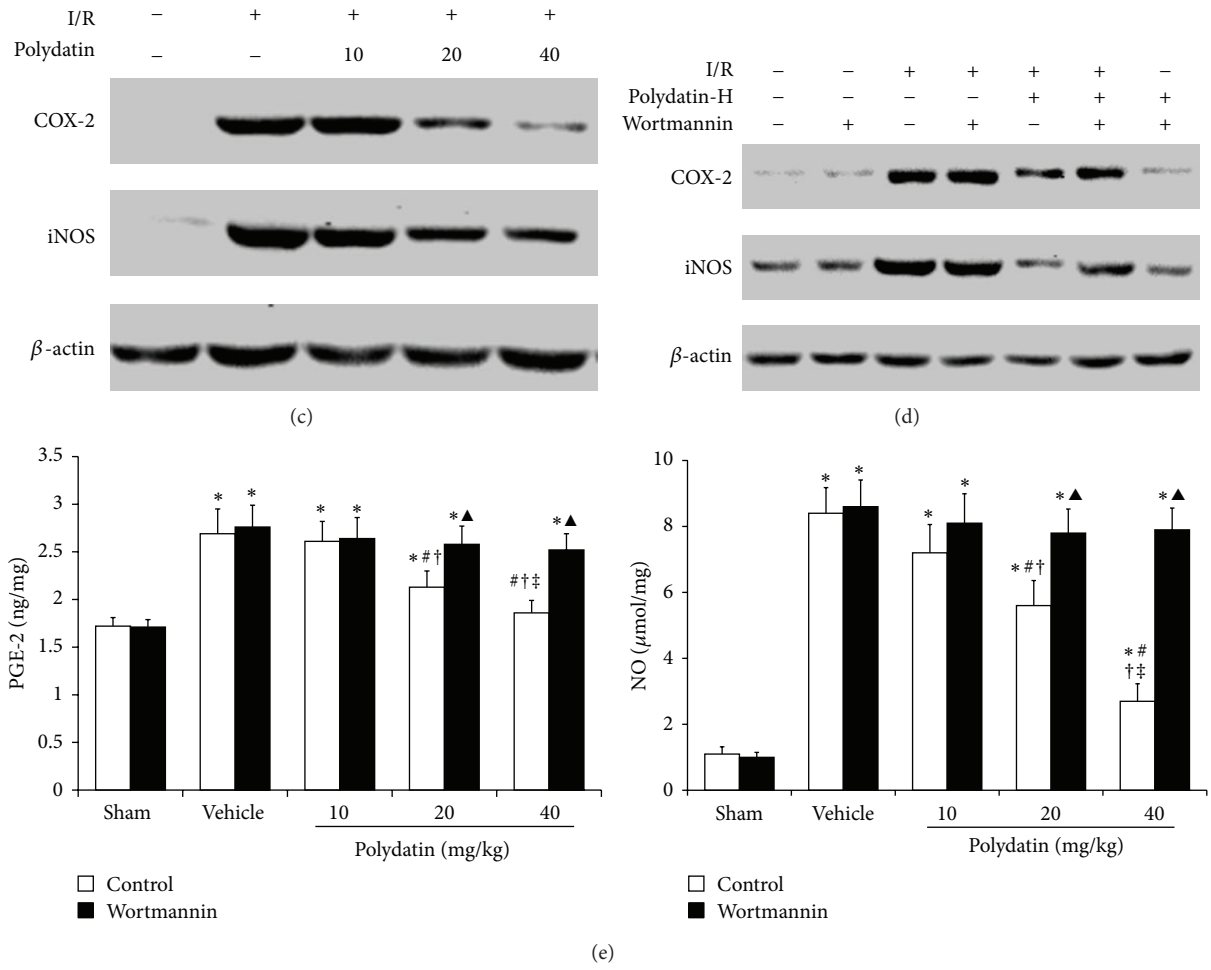

Figure 3: PI3K/Akt pathway was involved in polydatin-attenuated expression of the proinflammatory factors. (a) RT-PCR was used for the analysis of TNF-α, IL-1β, COX-2, and iNOS mRNA levels in kidneys of mice with or without polydatin-H (40 mg/kg) and wortmannin (1 mg/kg) at 3 d after I/R. GAPDH was used as a control. (b) ELISA was used to detect the protein levels of TNF-α and IL-1β in renal tissues in mice at 3 d after I/R administrated with different doses of polydatin, with or without wortmannin (1 mg/kg), intraperitoneally. (c) Western blot analysis assessed the expression of COX-2 and iNOS in kidneys of mice at 3 d after I/R with different doses of polydatin treatment. β-actin was used as a control. (d) Western blot analysis was used to detect the expression of COX-2 and iNOS in kidneys of mice at 3 d after I/R with polydatin-H treatment in presence or absence of wortmannin (1 mg/kg). β-actin was used as a control. (e) ELISA was used to detect the levels of PGE-2 and NO in renal tissues in mice at 3 d after I/R administrated with different doses of polydatin, with or without wortmannin (1 mg/kg), intraperitoneally. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus vehicle; $^{\dagger}P < 0.05$ versus polydatin (10 mg/kg); $^{\ddagger}P < 0.05$ versus polydatin (20 mg/kg). $^{\blacktriangle}P < 0.05$ versus control.

significantly alleviated tissue injury and elevated function in kidneys after renal ischemia, while the transgenic mice with TNF-α overexpression had more pronounced susceptibility to acute kidney injury induced by I/R than that in mice of wild type [27]. There were also enormous studies that have demonstrated that decreasing IL-1β was associated with improved renal function in renal I/R injury models [28–30]. Therefore, we examined the levels of TNF-α and IL-1β to determine whether they were associated with the mechanism of polydatin in treating renal I/R injury diseases. The results showed that polydatin significantly decreased I/R-induced TNF-α and IL-1β levels in kidneys. These data suggested that the positive effects of polydatin on improving renal function in mice after I/R injury might be achieved by inhibiting the proinflammatory cytokines of TNF-α and IL-1β.

With the exception of proinflammatory cytokines of TNF-α and IL-1β, COX-2 and iNOS, two enzymes associated with inflammation, were also correlated with the pathogenesis of I/R injury. COX-2, an inducible enzyme, plays crucial roles in regulating the inflammatory response and oxidative stress in I/R injury [31]. Selective or nonselective inhibition of COX-2 with either rofecoxib or indomethacin ameliorated renal tissue damage induced by I/R injury [32]. Another study has shown that continuous intrarenal infusion of parecoxib (40 mg per pig) improved renal function in pigs with the operation of suprarenal aortic cross clamping [33]. These results of previous studies suggested that inhibition of COX-2 has the potential to improve renal function in renal I/R injury. iNOS which was highly expressed after renal I/R injury mediated the generation of NO [34, 35]. High activity of

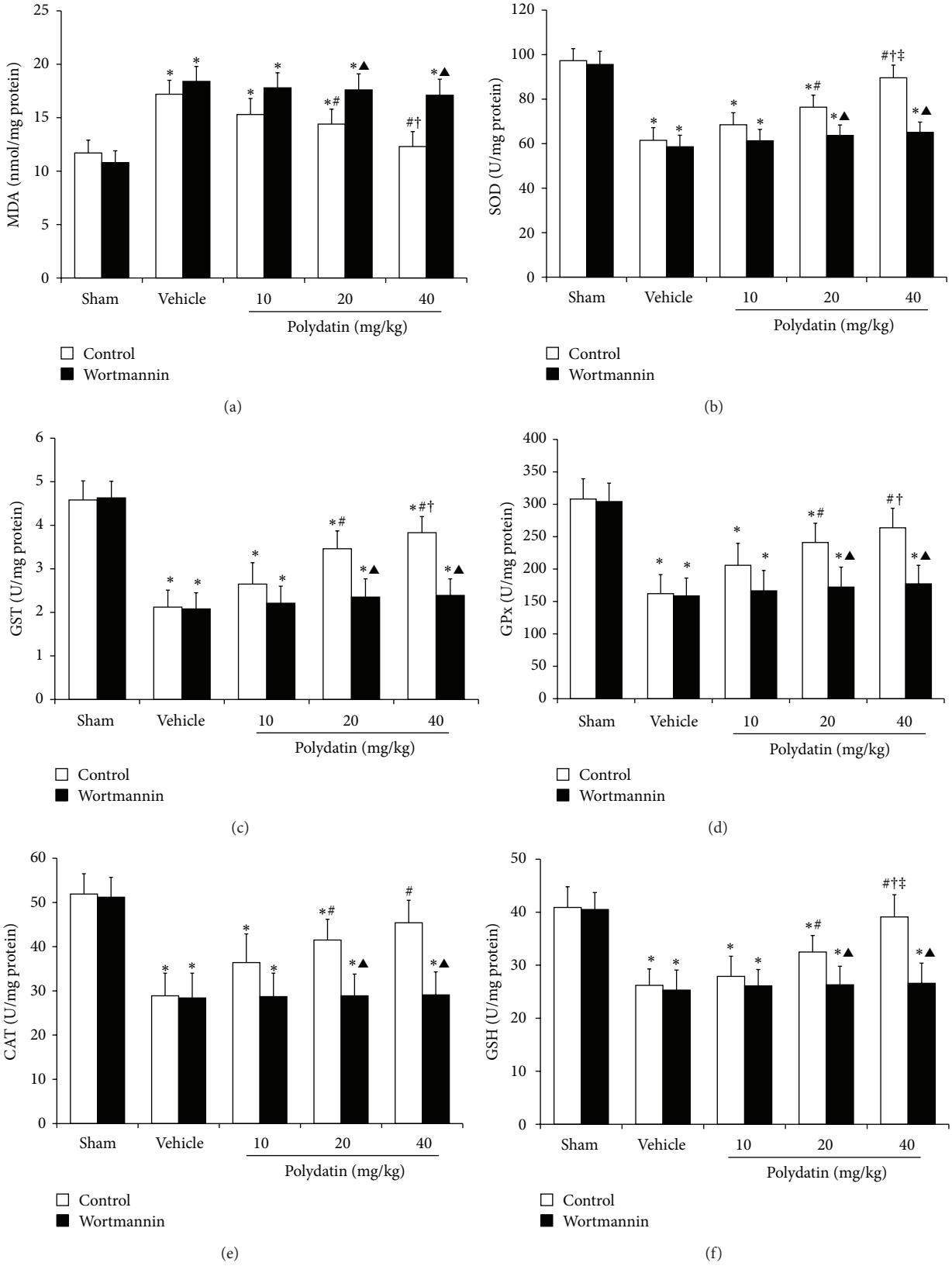

Figure 4: PI3K/Akt pathway was associated with polydatin-attenuated oxidative stress in renal I/R injury. Measurement of MDA (a), SOD (b), GST (c), GPx (d), CAT (e), and GSH (f) was performed on mice treated with different doses of polydatin and with or without wortmannin (1 mg/kg) at 3 d after I/R. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus vehicle; $^{\dagger}P < 0.05$ versus polydatin (10 mg/kg); $^{\ddagger}P < 0.05$ versus polydatin (20 mg/kg). $^{\blacktriangle}P < 0.05$ versus control.

iNOS aggravated the damage of kidneys in renal I/R injury, and selective inhibition of iNOS significantly improved the renal function in rats with I/R [36–38]. Based on the results of the studies above, we detected the expressions of COX-2 and iNOS protein and the contents of PEG_2 and NO, downstream factors of COX-2 and iNOS, in the kidneys after renal I/R injury. Our results showed that, compared with mice in vehicle group, polydatin notably inhibited the expressions of COX-2 and iNOS protein which were upregulated by I/R in renal I/R mice, dose dependently. The results were in line with the improved renal function induced by polydatin (Figure 1), indicating that the protective effects of polydatin on acute renal I/R injury might be by inhibiting the expression of the potential inflammatory mediators of COX-2 and iNOS and decreasing the generation of the downstream factors of them, PEG-2 and NO.

There are compelling evidences that oxidative stress is particularly involved in the pathogenesis of renal I/R injury [35]. Briefly, I/R has been proposed to have the potential to promote oxidative stress, which in turn can promote I/R injury [25]. The elevation of oxidative molecules and the reduction of antioxidant substance can aggravate I/R injury. It has been demonstrated that polydatin was a prominent antioxidant and exhibited cardioprotective effects in myocardial I/R injury rat models via increasing SOD activity and decreasing MDA content [4, 5]. The results in our experiment substantiated that polydatin significantly elevated the activity of SOD, GST, GPx, and CAT, increased GSH level, and decreased the MDA content in kidneys of I/R injury mice, which indicated the prominently antioxidative properties of polydatin and was in line with the data previously reported by Chen et al. in urate nephropathic mice [9].

The PI3K/Akt pathway was originally recognized to play a crucial role in regulating the growth and survival of cells, which nowadays has been rediscovered to be implicated in the protection of brain, myocardium, lung, liver, and kidney against I/R injury by regulating oxidative stress and inflammatory response [39–44]. Recently it has been demonstrated that polydatin exerted hepatoprotective effect in rats fed with high-fat diet [45] and regulated glucose and lipid metabolism in diabetic models [46] through upregulating the phosphorylation of Akt in liver, and polydatin also exhibited antitumor activity [47] through downregulating the phosphorylation of Akt in human nasopharyngeal carcinoma CNE cells. These results suggested that Akt signaling pathway was a potential therapeutic target of polydatin in treating various diseases. Therefore, in the present study, we assessed whether PI3K/Akt pathway was associated with the nephroprotective effects of polydatin in renal I/R injury models. First, we identified that the phosphorylation of Akt was activated in renal tissues by I/R and further increased by polydatin in dose dependent manner. However, the polydatin-induced increase of phosphorylation of Akt was significantly decreased by the specific PI3K/Akt inhibitor, wortmannin, suggesting the positive role of polydatin on the activation of Akt in renal I/R injury. Importantly, when blocking the phosphorylation of Akt by intraperitoneal injection of wortmannin, the beneficial effect of polydatin on the regeneration of renal tissues was also abolished. And wortmannin remarkably counteracted the polydatin-attenuated levels of proinflammatory factors and oxidative stress in kidneys of I/R injury. These results suggested that PI3K/Akt pathway, at least partly, was involved in the nephroprotective effects of polydatin in renal I/R injury. Of course, the potential downstream functional molecules mediated by PI3K/Akt signaling pathway to take part in polydatin's actions still remain to be further investigated.

5. Conclusions

In this study, we identified for the first time that, in acute renal I/R injury models, the administration of polydatin significantly improved the renal function, accelerated the mitogenic response, reduced cell apoptosis, strongly suppressed the I/R-induced upregulation of the expression of TNF-α, IL-1β, COX-2, PGE-2, iNOS, and NO, and dramatically decreased contents of MDA, but increased the activity of SOD, GPx, GST, CAT, and the level of GSH. However, all these beneficial effects of polydatin were counteracted when we inhibited PI3K/Akt pathway with its specific inhibitor, wortmannin. These findings taken together elucidated that polydatin exhibited prominent nephroprotective effects against renal I/R injury, at least in part, through PI3K/Akt-dependent phosphorylation. In conclusion, our data support that polydatin is promising to be a good drug for prevention and treatment of I/R-induced renal injury in the clinical practice.

Disclosure

Xiao-Wei Liu is the cocorresponding author.

Conflict of Interests

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

Authors' Contribution

Hong-Bao Liu, Qiu-Hong Meng, and Chen Huang are equal contributors.

Acknowledgments

This work was supported by Grants from the National Nature Science Foundation of China (nos. 81370016 and 81400677), Midwestern Excellent Young Scientist Foundation of Chinese Medical Doctor Association (2012), and the National Major Scientific and Technological Special Project for "Significant New Drugs Development" during the Twelfth Five-year Plan Period founded by Ministry of Science and Technology of China (no. 2012ZX09J12109-04C).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 520872, 9 pages
http://dx.doi.org/10.1155/2015/520872

Research Article

pCramoll and rCramoll as New Preventive Agents against the Oxidative Dysfunction Induced by Hydrogen Peroxide

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Received 26 August 2014; Accepted 2 March 2015

Academic Editor: Felipe Simon

Oxidative stress plays an important role in the induction of cell death and is associated with various pathologic disorders; therefore, the search for natural products that attenuate the effects produced by oxidant agents is greatly increased. Here, the protective effects of native lectin from *Cratylia mollis* seeds (pCramoll) and recombinant Cramoll 1 (rCramoll) against H_2O_2-induced oxidative stress in Vero cells were evaluated. Both lectins significantly attenuated the H_2O_2-induced cytotoxicity in a concentration-dependent way. The maximum protective effects were 96.85 ± 15.59% (rCramoll) and 59.48 ± 23.44% (pCramoll). The Live/Dead analysis showed a reduction in the percentage of dead cells from 65.04 ± 3.29% (H_2O_2) to 39.77 ± 2.93% (pCramoll) and 13.90 ± 9.01% (rCramoll). The deleterious effects of H_2O_2 on cell proliferation were reduced to 10.83% (pCramoll) and 24.17% (rCramoll). Lectins treatment attenuated the excessive superoxide production, the collapse of the mitochondrial membrane potential, and the lysosomal and DNA damage in H_2O_2-treated cells. In conclusion, our results suggest that pCramoll and rCramoll blocked H_2O_2-induced cytotoxicity through decreasing reactive oxygen species, restoring the mitochondrial potential, preventing the lysosomal damage and DNA fragmentation, and thus promoting cell survival and proliferation.

1. Introduction

Oxidative stress is characterized by an imbalance in the redox status of the cell and has been implicated in a range of age-associated and neurodegenerative diseases, such as aging, cancer, diabetes, Alzheimer's disease, and Parkinson's disease [1]. The reactive oxygen species (ROS) are oxygen-containing molecules that are constitutively produced in cells as a result

of normal metabolic processes. They include superoxide anions (O_2^-), hydroxyl radicals ($OH^\bullet$) and hydrogen peroxide (H_2O_2; nonradical derivative of oxygen). ROS are known to be responsible for cell toxicity when the generation of ROS exceeds the clearance capacity of the cellular antioxidant systems [2]. H_2O_2 is thought to be the major precursor of highly reactive free radicals, such as hydroxyl radicals via Fenton's reaction [3]. ROS may damage relevant classes of biological macromolecules in the cells through direct oxidation of lipids, proteins, and nucleic acids, thereby disrupting cellular function and integrity, which leads to cell death [1, 3]. Nowadays, the search for natural products that attenuate the effects produced by oxidant agents is greatly increased [4, 5].

Lectins are a heterogeneous group of nonimmune proteins and glycoproteins that specifically and reversibly bind with high affinity to carbohydrates without altering the covalent structure of any of their recognized ligands. Lectins can agglutinate cells through binding to cell surface glycoconjugates. They are distributed in plants, animals, and microorganisms [6, 7].

Cratylia mollis Mart (Fabaceae family) is a native leguminous forage from the semiarid region of the Northeast of Brazil (Caatinga biome), popularly known as Camaratu bean. Four multiple molecular forms of lectin have been purified from this plant: Cramoll-1, Cramoll-2, Cramoll-3, Cramoll-4; which exhibit different carbohydrate specificities. The isoforms 1, 2, and 4 are nonglycosylated and glucose/mannose specific proteins; and Cramoll 3 is a galactose specific glycoprotein [8, 9]. Cramoll 1,4 (preparation containing isolectins 1 and 4; pCramoll) is isolated in a similar way to concanavalin A (Con A), a well-known lectin from *Canavalia ensiformis* seeds [8]. This preparation has shown interesting biological activities such as immunomodulatory, antitumoral, antiparasitic, and healing agent [9]. Biotechnological applications of Cramoll also involve the characterization of human malignant tissues, affinity matrix for protein purification, and the development of sensors for microbial detection [9]. Cramoll 1 (major isolectin in this preparation) consists of 236 residues with 82% identity with Con A. Cramoll 1 tertiary structure was determined by X-ray crystallography at 1.77 Å and revealed three β-sheets connected by loops, known as the jellyroll domain (this topological architecture is essentially identical to Con A) [9]. Recently, the expression of soluble, functional recombinant Cramoll 1 in *Escherichia coli* was reported by our group: rCramoll, which shares the molecular mass, charge density, sugar recognition, and secondary and tertiary structures of pCramoll [10, 11].

In this study the cytoprotective effects of pCramoll and rCramoll against H_2O_2-induced oxidative damage in Vero cells were investigated. We found that the cytoprotective effects of these lectins are mediated by the decrease of superoxide species production that prevent the mitochondrial and lysosomal dysfunctions and the DNA damage.

2. Materials and Methods

2.1. Lectins Purification. pCramoll was purified from seeds of *C. mollis* using Sephadex G-75 column as previously reported [9]. The *E. coli* Rosetta (DE3) was used for the expression of rCramoll using expression vector pET-28a-Cramoll 1 and affinity chromatography (Sephadex G-75 column) [10, 11].

2.2. Cell Culture. The monkey kidney fibroblast line (Vero) was maintained at 37°C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured in RPMI medium containing 10% heat-inactivated fetal calf serum, penicillin, and streptomycin (100 U/mL), all from Sigma-Aldrich.

2.3. MTT Assay. Cell viability was evaluated using the MTT assay, which measures the metabolic conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) salt to the colored formazan dye. Vero cells (1×10^5/mL) were incubated in a 96-well plate in quadruplicate for 24 h (37°C and 5% CO_2), treated with lectins (0.625–10 μM) for 30 min and subsequently with H_2O_2 (1 mM) for 24 h. At the end of the incubation time, the medium was removed and a MTT solution (5 mg/mL in RPMI) was added to the culture and the cells were incubated for additional 3 h (37°C and 5% CO_2). Afterwards, the medium was removed again and the intracellular formed formazan product was dissolved in DMSO. The optical density (OD) was measured at 595 nm in a microplate reader (Benchmark Plus, Bio-Rad, California, EUA). Cell viability (CV) was calculated in comparison to the OD obtained by control cell, considered as 100%. The protective effect was calculated following this formula:

$$\text{Protective effect } (\%) = \frac{\left(\text{CV}_s - \text{CV}_{H_2O_2}\right)}{\text{CV}_{H_2O_2}} * 100, \quad (1)$$

where CV_s is the viability of cells treated with each lectin in the presence of H_2O_2; $\text{CV}_{H_2O_2}$ is the viability of cells treated only with H_2O_2.

2.4. Viability/Cytotoxicity Assay. To confirm the cytoprotective effect of the lectins the Live/Dead Viability/Cytotoxicity kit for mammalian cells (Molecular Probes) was used, following manufacturer's instructions. Briefly, the Vero cells (1×10^5/mL, cultured on a 24-well plate for 24 h at 37°C and 5% CO_2) were pretreated with both lectins (10 μM, for 30 min) followed by the addition of H_2O_2 (100 μL at 1 mM), as inductor of oxidative stress. After 24 h of incubation, the cells were trypsinized, centrifuged at 3000 g for 5 min, washed with PBS and resuspended in 500 μL of PBS containing 2 μL of calcein AM (50 μM) and 4 μL of ethidium homodimer, and then incubated for 20 minutes at room temperature, protected from light. Afterward, the samples were immediately analyzed using FACSCalibur-BD flow cytometer (Becton Dickinson Co., San Jose, CA). For each sample, 10.000 events were collected and the results were analyzed by using the software CELLQuestPro (Becton Dickinson Co., San Jose, CA).

2.5. Mitochondrial Superoxide Production. The production of superoxide anion by the mitochondria was measured using the MitoSOX Red mitochondrial superoxide indicator (Molecular Probes). The cells were pretreated with lectins (10 μM, for 30 min) and treated with H_2O_2 for 30 min. After the trypsinization and washing, the MitoSOX reagent

(1 mL/5 μM) was added and the samples were incubated for 10 minutes at 37°C, protected from light. The cells were washed with warm buffer (three times) and analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro (Becton Dickinson Co., San Jose, CA) for acquisition and analysis of data.

2.6. Lysosomal Membrane Stability. An experiment using the lysosomotropic base acridine orange was carried out to measure severe or late lysosomal membrane stability by assessing change in red fluorescence (AO uptake method). The cells were prepared as described above (2.5) and incubated at 37°C in the presence of 1 mM acridine orange (Sigma-Aldrich) and washed in PBS (2X) after 30 min. The pellet containing the cells was resuspended in PBS and analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA; FL3 channel) using the software CELLQuestPro (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.7. Determination of Mitochondrial Membrane Potential ($\Delta\Psi m$). The uptake and retention of the cationic fluorescent dye rhodamine123 was used to evaluate the mitochondrial membrane potential ($\Delta\Psi$m). Treated cells were trypsinized, washed twice with PBS, and centrifuged at 300 ×g for 10 min. The cell pellet was then resuspended in 1 mL of fresh medium containing 2 μM rhodamine123 and incubated at 37°C in a thermostatic bath for 20 min with gentle shaking. The stained Vero cells were washed and then resuspended in 1 mL of PBS. The variation index (VI) was calculated following this formula:

$$\text{Variation Index (VI)} = \frac{\text{FI}_c - \text{FI}_s}{\text{FI}_c}, \quad (2)$$

where FI_c is the mean of fluorescent intensity of control and FI_s the mean of treated cells.

2.8. CFSE Proliferation Assay. To evaluate the cytoprotective effect of Cramoll on Vero cells the CFSE (carboxyfluorescein diacetate succinimidyl ester) proliferation assay was used. The Vero cells (1×10^5/mL) were stained with CFSE (2.5 μM) and incubated at 37°C for 10 min. The reaction was stopped by the addition of cold RPMI. The cells were centrifuged (300 g for 10 min) and the cell pellet was washed with PBS in the same conditions. Afterwards, the cells were resuspended in RPMI and pretreated with lectins, following the addition of H_2O_2 as described (Section 2.5). The proliferation indexes were determined after 72 h by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro (BD Bioscience, San Jose, CA) for acquisition and the FlowJo software (Tree Star, Ashland OR) for analysis of data. The proliferation index of control cells was considered as 100%.

2.9. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Click-iT Assay. TUNEL assay was performed using the Click-iT TUNEL Alexa Fluor 488 (Invitrogen) following the manufacturer's protocol. Briefly, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized (0.25% Triton X-100 in PBS) for 20 minutes at room temperature. Then they were washed twice with deionized water. The TdT reaction cocktail was added and incubated for 1 h, followed by 30 min incubation with the Click-iT reaction solution. The stained Vero cells were washed and resuspended and the fluorescence intensity was analyzed by flow cytometry (FACSCalibur-BD, San Jose, CA) using the software CELLQuestPro (BD Bioscience, San Jose, CA) for acquisition and analysis of data.

2.10. Spectroscopic Measurements. The effects of H_2O_2 treatment on lectin tertiary structure were evaluated by intrinsic fluorescence using a Jasco FP-6300 spectrofluorometer (Jasco, Tokyo, Japan). Both lectins (10 μM) were incubated with H_2O_2 (1 mM). The fluorescence emission of tryptophan was measured at 25°C in a rectangular quartz cuvette with a 1 cm path length, the excitation was at 295 nm, and emission was recorded from 305 to 450 nm using 5 nm band pass filters (for both).

2.11. Statistical Analysis. Data is analyzed by one-way analysis of variance (ANOVA) and Turkey test to determine the statistical significance. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. pCramoll and rCramoll Attenuated the H_2O_2-Induced Cytotoxicity. The cytoprotective effects of lectins against H_2O_2-induced cell death were evaluated by MTT assay, which measures the loss of metabolic activity of cells and it is an early indicator for cell death. Both lectins inhibited the cytotoxicity in a concentration-dependent way (Figure 1). pCramoll showed maximum protective effects at 5 μM (48.36 ± 8.12%) and 10 μM (59.48 ± 23.44%), with no statistically significant differences ($P > 0.05$) between these concentrations. rCramoll induced higher/maximum protection at 10 μM (96.85 ± 15.59%) ($P < 0.05$). It is important to note that rCramoll was more effective than the pCramoll at 5 and 10 μM ($P < 0.05$). Because the best activity of both lectins was achieved at 10 μM we chose this concentration to further investigate the subcellular effects involved in the Cramoll-mediated cytoprotective effects.

The cytoprotective action of both lectins was confirmed by Live/Dead assay, a flow cytometry analysis that uses the hydrolysis of calcein AM by intracellular esterases of live cells and the ethidium heterodimer (EthD-1) that enter nucleus of cells with damage membranes to discriminate viable from unviable cell, respectively. Figure 2 shows an increase of cell death when Vero cells were treated with H_2O_2 alone (65.04 ± 3.29%) compared to control cells. On the other hand, both lectins reduced the rates of cell death to 39.77 ± 2.93% for pCramoll and 13.90 ± 9.01% for rCramoll.

3.2. pCramoll and rCramoll Inhibited the Deleterious Effects of H_2O_2 on Cell Proliferation. To evaluate the cell proliferation a FACS assay using CFSE staining followed by FlowJo analysis was performed. CFSE proliferation assay is based on the ability of CFSE probes to bind to lipids within the cell membrane. After each mitosis, the fluorescence intensity decreases to approximately the half. The H_2O_2-treated cells showed,

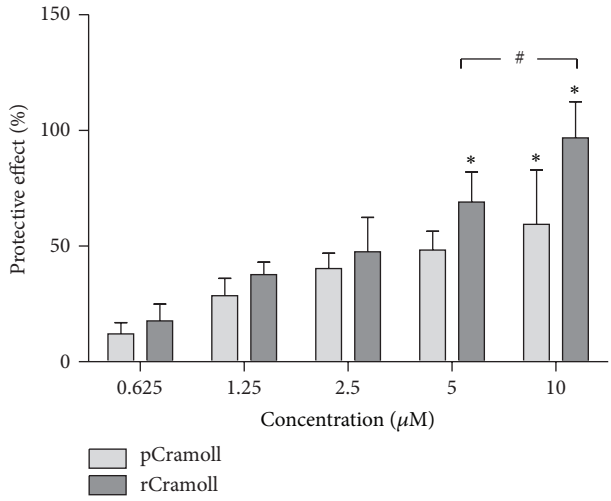

Figure 1: Cytoprotective effects of pCramoll and rCramoll determined by MTT assay. (∗) Significant differences between other concentrations. (#) Significant differences between lectins.

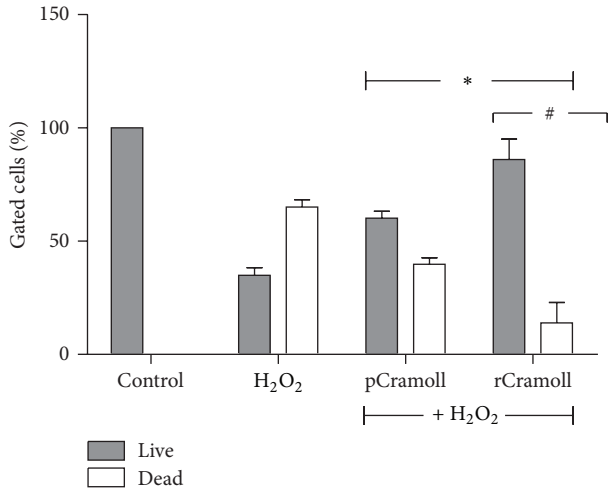

Figure 2: Cytoprotective effects of pCramoll and rCramoll determined by Live/Dead kit using flow cytometry. (∗) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

after 72 h, a decrease of proliferation index of 34.71% compared to the control cells. The lectins were able to enhance the proliferation index in the presence of H_2O_2 (78.84 ± 4.04% for pCramoll and 84.20 ± 1.16% for rCramoll) (Figure 3).

3.3. pCramoll and rCramoll Blocked the H_2O_2-Induced Mitochondrial ROS Generation. As shown in Figure 4, after H_2O_2 exposition ROS generation increased more than 52-fold as compared to control. Pretreatment of the cells with 10 μM of lectins for 30 minutes induced significantly the attenuation of mitochondrial ROS production. The reduction rates (in relation to H_2O_2) were 20.31 ± 7.82% and 39.84 ± 2.36%, for pCramoll and rCramoll, respectively ($P < 0.05$).

3.4. pCramoll and rCramoll Restore the Mitochondrial Membrane Potential ($\Delta\Psi m$). The alterations in mitochondrial functions were evaluated by the $\Delta\Psi$m variation index (VI) using rhodamine123. The H_2O_2-treated cell exhibited a loss of $\Delta\Psi$m in relation to the control cells (depolarization, VI: −1.47 ± 0.18). The lectin pretreatments were able to restore the loss of $\Delta\Psi$m induced by H_2O_2 treatment, inhibiting the depolarization (pCramoll, VI: −0.34 ± 0.03; and rCramoll, VI: −0.05 ± 0.06) ($P < 0.05$) (Figure 5).

3.5. pCramoll and rCramoll Protect the Lysosomal Damage Induced by H_2O_2. In order to evaluate the lysosomal function the acridine orange (AO) fluorescence was analyzed by FACS analysis. The control cells showed a strong AO fluorescence emission in the red channel, confirming that these cells had intact lysosomes, while H_2O_2 reduced in 85.66 ± 0.3% the fluorescence signal. The lectins showed great potential to inhibit the lysosomal membrane permeabilization induced by H_2O_2, showing the reestablishment of AO fluorescence signal in treated cells of 64.37 ± 7.98% (pCramoll) and 75.17 ± 7.91% (rCramoll) in relation to the control ($P < 0.05$) (Figure 6).

3.6. pCramoll and rCramoll Prevent the Fragmentation of Nuclear DNA. The formation of DNA ladders is a consequence of a specific nucleosomal-sized fragmentation and is a conventional event in apoptotic process. The TUNEL assay was carried out to detect the extension of DNA degradation in apoptotic cells. DNA damage was quantified in relation to control cells. As expected, no significant DNA injury could be detected in the control cells. On the other hand, H_2O_2-treated cells presented an increase in the TUNEL fluorescence intensity, indicative of intense DNA fragmentation (this fluorescence was 170.41% higher than control cell). Pretreatment with both lectins leads to decrease in TUNEL staining in about 2-fold (61.62 ± 1.44% for pCramoll and 55.53 ± 5.86% for rCramoll) (Figure 7).

3.7. H_2O_2 Decreased Tryptophan Fluorescence Emission of pCramoll and rCramoll but Did Not Alter Their Hemagglutination Ability. Conformal stability of pCramoll and rCramoll (10 μM) after H_2O_2 treatment (1 mM) for 24 hours was investigated. Both proteins showed the same behavior: the intrinsic fluorescence emission decreased after exposition to H_2O_2 without changing the λ_{max}, at approximately 330 nm for hydrophobic residues. However, the hemagglutination ability was not modified (data not shown).

4. Discussion

In this work we reported the protective effects of native and recombinant Cramoll against H_2O_2-induced cell death. The induction of cell death by H_2O_2 has been already reported in the literature using different cell types [5, 12–14]. The severe damage caused by H_2O_2 is related to its capacity to cross the cellular membranes and react with intracellular metal ions, yielding highly toxic hydroxyl radicals, which are able to cause serious damage to macromolecules, including DNA, proteins, and lipids, and ultimately lead to the cell death [2]. In fact, several diseases had the oxidative stress as the main

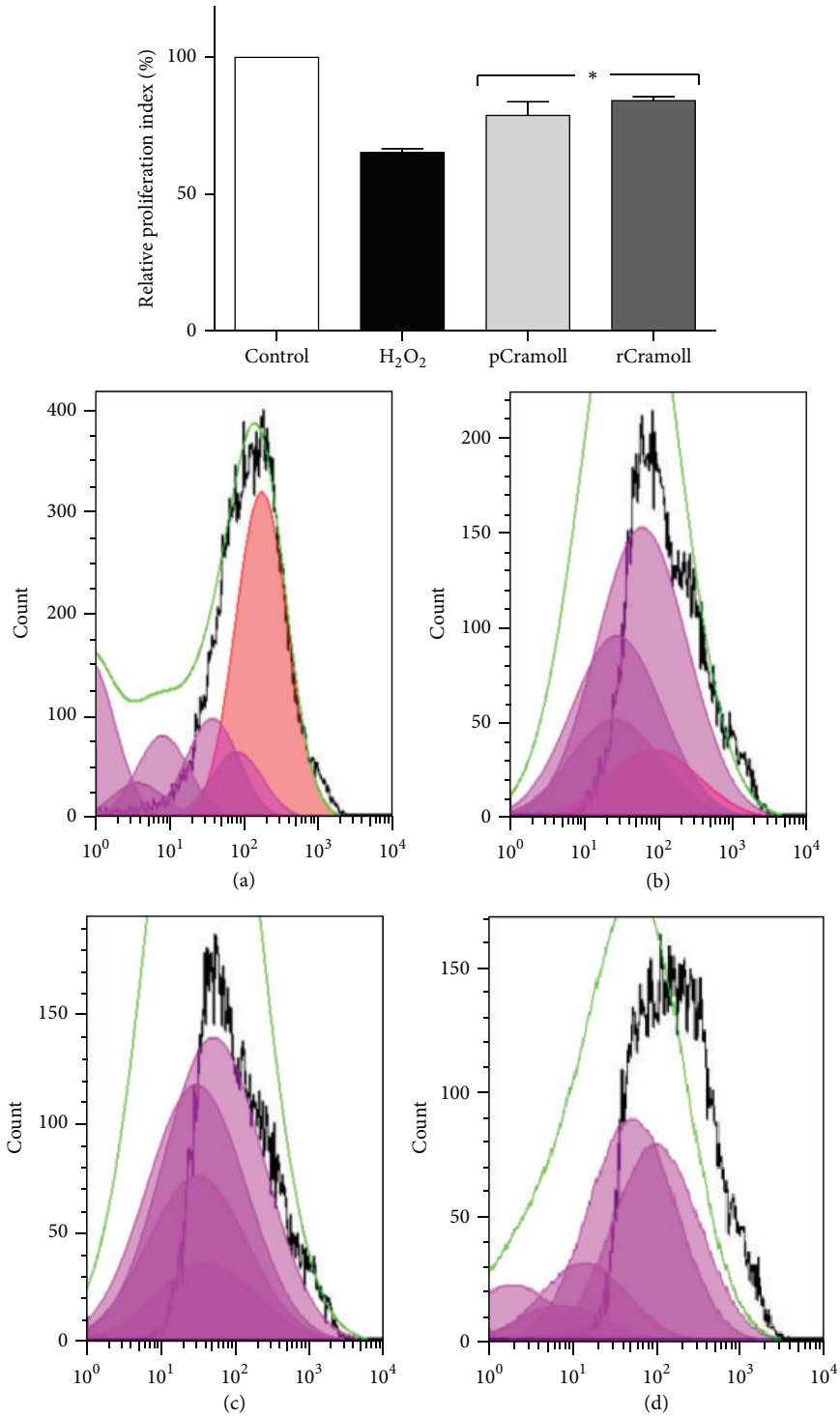

Figure 3: Effects of pCramoll and rCramoll on the deleterious effects of H_2O_2 on cell proliferation using CFSE probe, analysed by FlowJo software. (a) Control cells without H_2O_2 or lectin treatment. (b) Cells treated with H_2O_2. (c) Cells pretreated with pCramoll for 30 minutes prior to H_2O_2 addition. (d) Cells pretreated with rCramoll for 30 minutes prior to H_2O_2 addition. (∗) Significant differences in relation to H_2O_2.

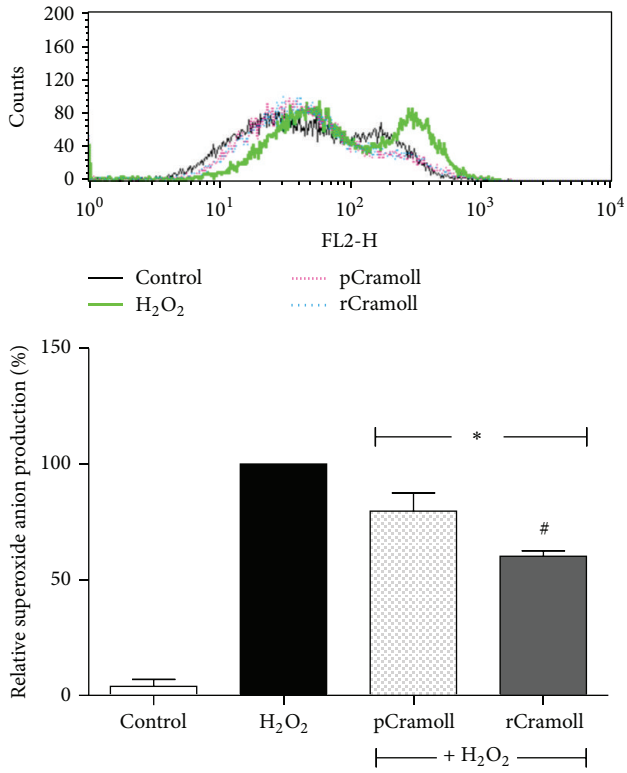

FIGURE 4: Effect of pCramoll and rCramoll on the H_2O_2-induced accumulation of mitochondrial superoxide anion in Vero cells, as observed by flow cytometry. (∗) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

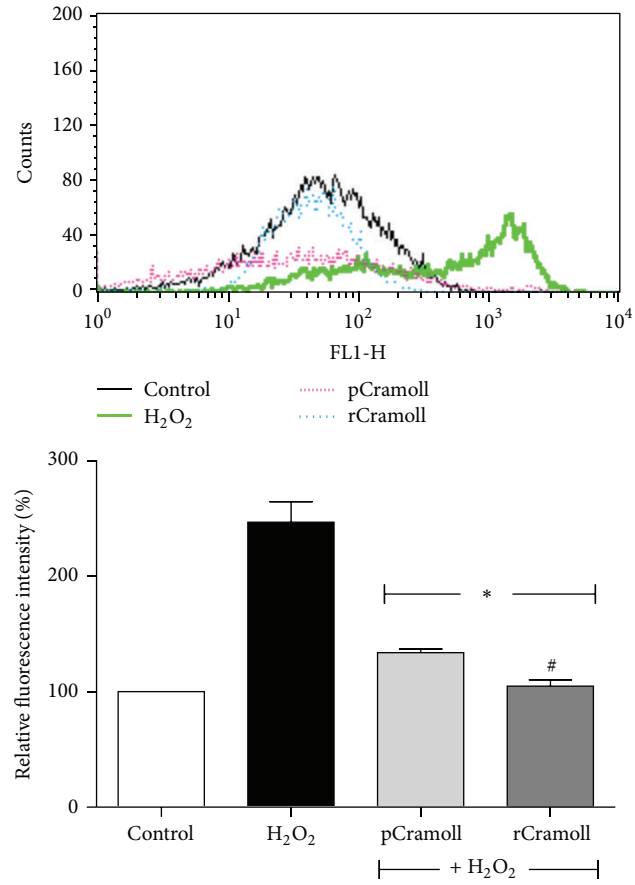

FIGURE 5: Effect of pCramoll and rCramoll on H_2O_2-induced loss of ΔΨm in Vero Cells. (∗) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

trigger (cancer, neurodegenerative, and cardiovascular disorders) [1, 15]. In the present work, H_2O_2 induced significant toxicity, reducing the cell viability to 34.96% (Live/Dead kit) and suppressing the cell proliferation. The pretreatment with different concentrations of both rCramoll and pCramoll lectins had a significant cytoprotective effect reestablishing the cell viability and proliferation to the rates near to those found in the control cells.

The secondary production of other mitochondrial ROS (such as superoxide) induced by H_2O_2 is one of potential mechanisms of cell damage [16]. In this study, we measured the superoxide generation using MitoSOX probe. The treatment of cells with H_2O_2 increased significantly the superoxide production by the mitochondria ($P < 0.05$). This effect was attenuated in both lectins-pretreated groups. These results indicate that the anticytotoxic effects of both lectins were related to the inhibition of mitochondrial ROS production.

Mitochondria play a critical role in maintaining the physiology of the cell and its dysfunction is an important pathway in the cell death [17]. It is well known that several proapoptotic mediators (cytochrome c, AIF, Smac/DIABLO, and endoG) are located in the mitochondria. These factors are released into the cytosol where they activate diverse enzymatic reactions that lead to the specific degradation of proteins and DNA during apoptosis [18]. The conservation of ΔΨm is essential for mitochondrial integrity and functions [19]. H_2O_2 induces mitochondrial dysfunction by the loss of ΔΨm [20]. Our results showed that native and recombinant Cramoll are able to inhibit the deleterious effects of H_2O_2 on the ΔΨm.

Although studies on oxidative-stress-induced cell dysfunction are focused on the alterations in the mitochondrial bioenergetic and proapoptotic compounds release, lysosomes are also susceptible to the oxidative stress and have been associated with necrotic, autophagic, and apoptotic cell death [21, 22]. Different kinds of hydrolytic enzymes (proteases, lipases, nucleases, glycosidases, phospholipases, phosphatases, and sulfatases) are present in this organelle and damage in this structure caused by toxic agents as H_2O_2 can lead to the leakage of these enzymes in the cytoplasm resulting in the vanishing of the AO red labeling, as observed in our study [23–25]. The lysosomal proteases such as cathepsins family (A, B, D, and L) are potent activators of apoptotic effectors [26]. The lectins tested in this study were able to prevent lysosomal damage induced by H_2O_2 and promoting the cell survival.

Furthermore, the activation of endonucleases leading to the genomic DNA fragmentation is one of the most representative events during apoptosis [27]. The protective actions of the tested lectins against DNA ladder fragmentation induced by H_2O_2 in Vero cells were confirmed by TUNEL analysis.

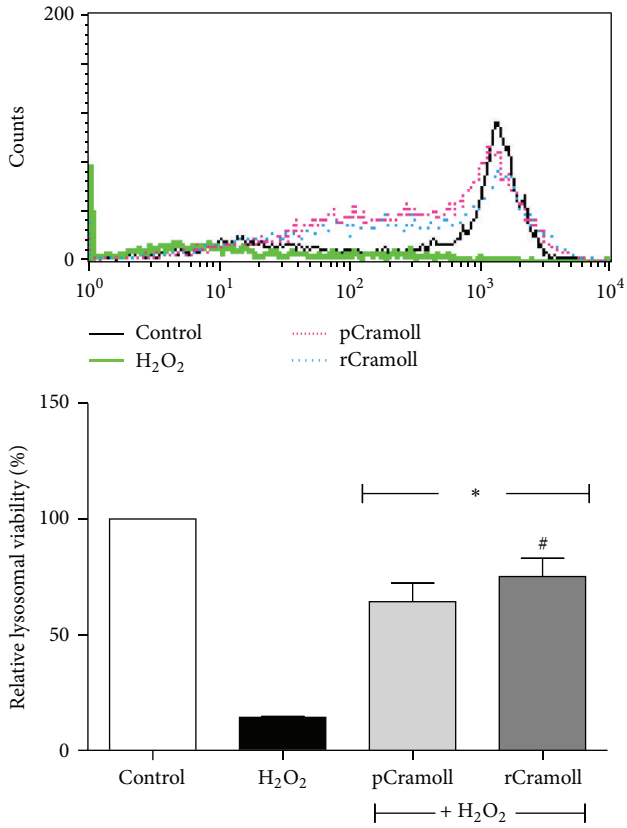

Figure 6: Effect of pCramoll and rCramoll on the lysosomal damage induced by H_2O_2 in Vero cells. (∗) Significant differences in relation to H_2O_2. (#) Significant differences between lectins.

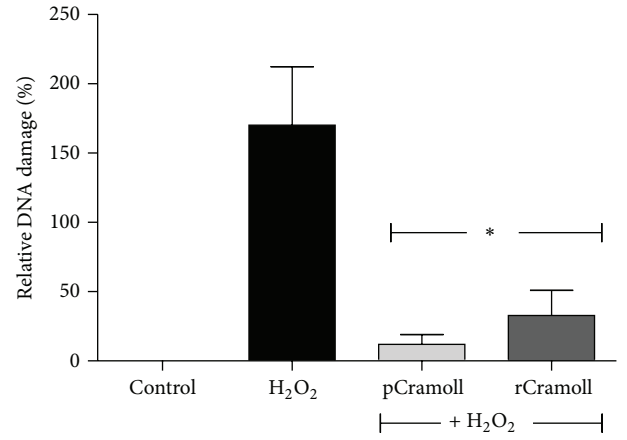

Figure 7: Effect of pCramoll and rCramoll on the DNA damage induced by H_2O_2 in Vero cells by TUNEL analysis using flow cytometry. (∗) Significant differences in relation to H_2O_2.

pCramoll and rCramoll share several biophysical properties, the ability to recognize Glc/Man moieties, and the same pH dependent dimer-tetramer equilibrium. However, the tetramers of rCramoll are composed of intact monomers due to the absence of natural fragmentation process, and this characteristic is related to the little enhancement in its stability when probed with acidification, high temperatures, or hydrostatic pressure [10, 11]. As different degrees of protective effects were observed in pCramoll and rCramoll (these differences could be also observed in other biological activities of these proteins [10, 28]), we decided to examine the effects of H_2O_2 on their structures using intrinsic fluorescence emission. Cramoll has four tryptophan residues and two of them (40 and 109) are located in the protein core [9]. Tryptophan is easily oxidized by hydrogen peroxide by photooxidation in the presence of oxygen [29, 30]. Oxidative damage distorts the hydrophobic surface and the hydrophobic core of proteins, and partial aromatic amino acid substitution, caused by hydrogen abstraction, results in a decrease in the fluorescence emission. This work showed a decrease in the intrinsic fluorescence emission for both proteins when treated with H_2O_2 at 1 mM, suggesting that the different effects of each lectin could be related to other factors, such as sugar affinity.

In summary, pCramoll and rCramoll ameliorate the H_2O_2-induced oxidative stress and cell death. The protective effects were related to the inhibition of mitochondrial superoxide generation, the reestablishment of $\Delta\Psi m$, and the blocking of the deleterious effects of oxidative stress on lysosomal integrity and DNA fragmentation, promoting cell survival and proliferation. Although the molecular mechanisms responsible for the antioxidant properties of pCramoll and rCramoll remain to be elucidated, some studies carried out by our and other research groups have demonstrated that some lectins act directly as scavengers of ROS [31–33]. However, we cannot rule out the possibility that pCramoll and rCramoll act indirectly on the antioxidant enzymes triggering detoxification mechanisms that protect the cells against oxidative stress induced by H_2O_2 treatment. Further studies are needed to investigate in more detail the molecular mechanisms involved in the protective role of lectins against the damage caused by the oxidative stress (Figure 8).

Abbreviations

AO:	Acridine orange
CFSE:	Carboxyfluorescein diacetate succinimidyl ester
Con A:	Concanavalin A, lectin from *Canavalia ensiformis* seeds
Cramoll 1,4:	Preparation containing isolectins 1 and 4 from *Cratylia mollis* seeds, pCramoll
Cramoll 1:	Isolectin 1 from *Cratylia mollis* seeds
DMSO:	Dimethyl sulfoxide
H_2O_2:	Hydrogen peroxide
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
O_2^{-}:	Superoxide anions
$OH^{\bullet}$:	Hydroxyl radicals
PBS:	Phosphate buffered saline
rCramoll:	Recombinant Cramoll 1
ROS:	Reactive oxygen species

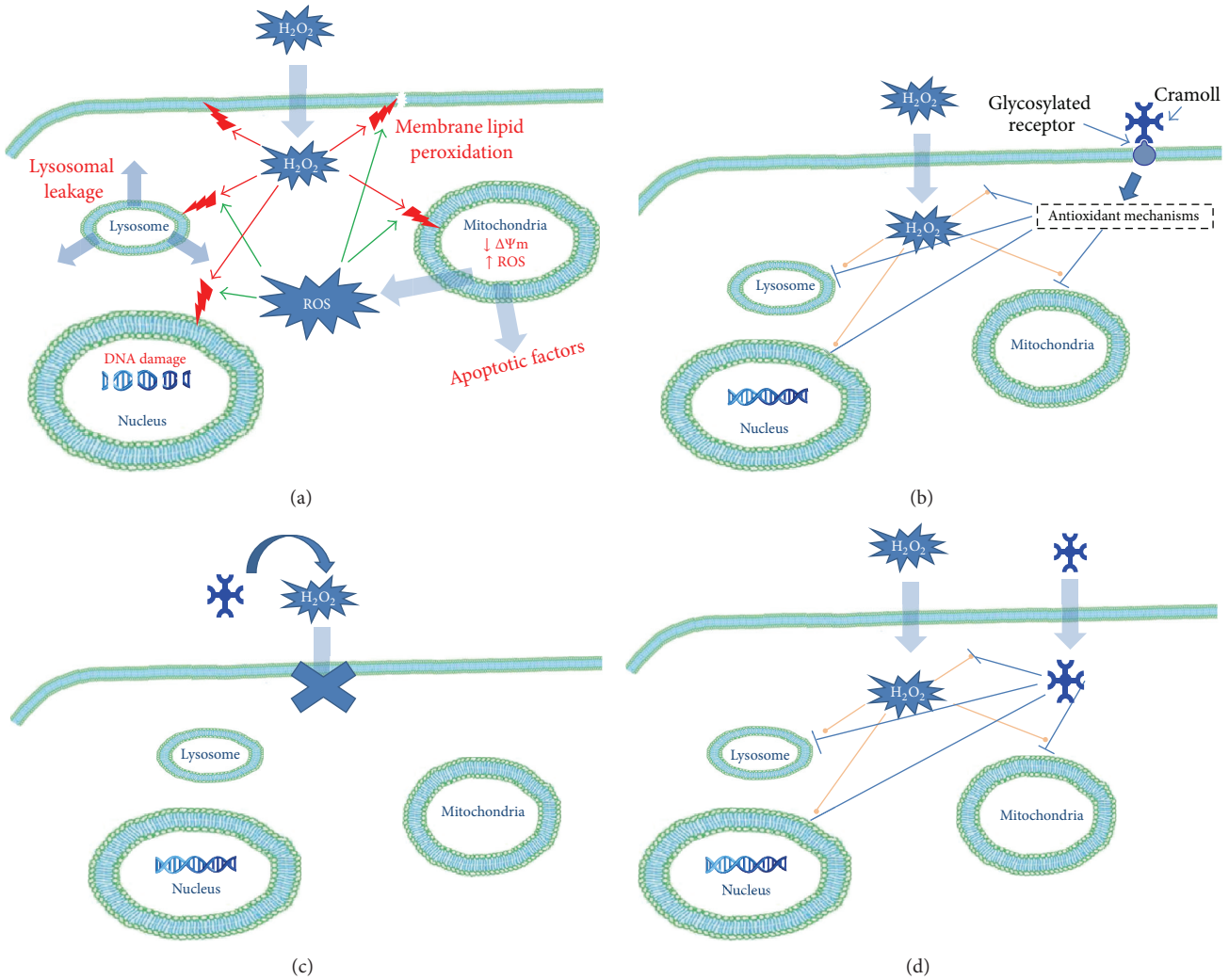

Figure 8: Schematic overview of the protective effects of pCramoll and rCramoll on the H_2O_2-induced cell death. (a) H_2O_2 can induce cell dysfunction due its capacity to interact directly or indirectly with organelles and cell membrane causing lipid peroxidation, leakage of lysosomal content, DNA damage, decrease in the mitochondrial membrane potential, and increase of mitochondrial ROS production, thereby disrupting cellular function and integrity. (b) The binding of both pCramoll and rCramoll to specific glycosylated targets on the surface of Vero cells or intracellularly can trigger the antioxidant mechanisms of cells that prevent the deleterious effects of H_2O_2 on organelles such as mitochondria, lysosomes, and nucleus, promoting cell survival and proliferation. (c and d) Alternatively, both lectins can act directly as H_2O_2 or/and secondary ROS scavengers, neutralizing the harmful effects of oxidative stress.

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
ΔΨm: Mitochondrial membrane potential.

Conflict of Interests

The authors confirm that this paper content has no conflict of interests.

Authors' Contribution

Maria Tereza dos Santos Correia and Regina Celia Bressan Queiroz de Figueiredo are equal contributors.

Acknowledgments

The authors are grateful to the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE).

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Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2015, Article ID 606981, 8 pages
http://dx.doi.org/10.1155/2015/606981

Research Article

Protective Effect of Edaravone in Primary Cerebellar Granule Neurons against Iodoacetic Acid-Induced Cell Injury

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Received 15 August 2014; Revised 19 October 2014; Accepted 22 October 2014

Academic Editor: Claudio Cabello-Verrugio

Edaravone (EDA) is clinically used for treatment of acute ischemic stroke in Japan and China due to its potent free radical-scavenging effect. However, it has yet to be determined whether EDA can attenuate iodoacetic acid- (IAA-) induced neuronal death *in vitro*. In the present study, we investigated the effect of EDA on damage of IAA-induced primary cerebellar granule neurons (CGNs) and its possible underlying mechanisms. We found that EDA attenuated IAA-induced cell injury in CGNs. Moreover, EDA significantly reduced intracellular reactive oxidative stress production, loss of mitochondrial membrane potential, and caspase 3 activity induced by IAA. Taken together, EDA protected CGNs against IAA-induced neuronal damage, which may be attributed to its antiapoptotic and antioxidative activities.

1. Introduction

Stroke is the second leading cause of mortality among most developed countries [1]. Cerebral ischemia is the most common type of stroke and accounts for 87% of all stroke cases [2]. Brain hypoxia and glucose deprivation are the primary pathophysiological features of cerebral ischemia, which lead to cerebral infraction [3]. After hypoxia, the balance between generation and clearance of reactive oxidative species (ROS) is compromised, and overproduction of ROS as byproducts by mitochondria may result in activation of mitochondria-dependent apoptotic pathway in acute ischemia stroke [4]. More recent lines of evidence have suggested that an excessive ROS generation may lead to mitochondrial membrane depolarization to induce the apoptosis cascade, which results in functional and structural damage to neuronal cells [5]. Mitochondrial dysfunction and excessive oxidative stress play a vital role in the pathogenesis of neurodegenerative diseases, including cerebral ischemia [3, 6]. Therefore, neuroprotective agents that scavenge free radicals and maintain mitochondrial function should be an effective therapeutic strategy for treating ROS-related disorders, especially ischemic stroke [7].

Edaravone (EDA) has been used to treat acute cerebral infraction since 2001, which is the first novel free radical scavenger approved in Japan [8, 9]. EDA has been shown to quench hydroxyl, peroxyl, and superoxide radicals and inhibit free radical-mediated lipid peroxidative damage in a rat middle cerebral artery occlusion (MCAO) model [10]. Furthermore, recent mechanistic research found that EDA suppressed delayed neuronal death induced by reperfusion, reduced long-term inflammatory reaction, and inhibited the expression of vascular endothelial growth factor mediated by the ischemic cascade [8, 11]. Thus, the therapeutic benefit of EDA is attributed not only to its antioxidative activity but also to its ability to regulate various signaling pathways.

Several studies have shown that iodoacetic acid (IAA) treatment could closely mimic the hypoxic/ischemia condition in nerve cells *in vitro* [12–14]. IAA, a chemical ischemia

stimulus, inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase irreversibly and induces cell death accompanied with an increase in ROS production, mitochondrial dysfunction, and loss of ATP [15–17]. These responses are very similar to changes observed in animal models of ischemic stroke [18]. Cerebellar granular neurons (CGNs) are primary rat neurons, obtained from cerebellum, which contain the largest homogeneous neuronal population in the brain, which have been widely used as an *in vitro* model for investigating the cellular and molecular mechanisms underlying neuronal apoptosis in neurodegenerative disorders [19–21]. Previously, researchers have demonstrated that IAA reduced cell viability via increasing ROS production in CGNs [22, 23]. It has yet to be determined whether EDA can attenuate IAA-induced neuronal death *in vitro*. Therefore, we herein used the *in vitro* CGNs model exposed to IAA to study the neuronal protective effects of EDA and to explore the possibility of its underlying mechanisms of action.

2. Materials and Methods

2.1. Chemicals and Reagents. EDA was purchased from Aladdin Reagent Co. (Shanghai, China). MTT, fluorescein diacetate (FDA), propidium iodide (PI), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), DNase, poly-L-lysine, and cytosine-D-arabinofuranoside were obtained from Sigma-Aldrich (St. Louis, MO, USA). Basal medium Eagle (BME), fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Mitochondrial membrane potential assay kit and caspase 3 activity assay kit were obtained from Beyotime (Shanghai, China). The cytotoxicity detection kit (LDH) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. Primary Cultures of Cerebellar Granule Neurons and Drug Treatment. CGNs were isolated from postnatal 8-day-old Sprague-Dawley rat pups (15–20 g) from the Animal Care Facility of Sun Yat-sen University according to the protocol described by Bilimoria and Bonni [24]. The density of viable cells in suspension was determined by cell count using trypan blue and adjusted to 2×10^6 cells/mL. Neurons were seeded into 96-well pates or 12-well plates which were precoated with 50 μg/mL poly-L-lysine and maintained in a humidified incubator with 5% CO_2 in air at 37°C. Twenty-four hours after plating, cytosine-D-arabinofuranoside (final concentration: 10 μM) was added to the culture medium to arrest the proliferating of nonneuronal cells. All animal studies were conducted according to guidelines of the Experimental Animal Care and Use Committee of Jinan University. The experimental protocols were approved by the Ethics Committee for Animal Experiments of Jinan University.

Unless otherwise stated, on day 7 of *in vitro* culture, CGNs were pretreated with EDA (3, 10, and 30 μM) for 2 h. IAA (50 μM) was then added for incubation for another 4 h to induce cell injury.

2.3. Immunofluorescence. Immunofluorescence staining with βIII-tubulin antibody was used to observe the morphology changes of the neurite. Briefly, CGNs were washed with HBSS and were fixed with 4% paraformaldehyde for 15 min at 4°C. The cells were then permeabilized with 0.1% Triton X-100 for 5 min. The fixed neurons were incubated in 10% horse serum for 1 h to block nonspecific protein interactions. The primary antibody (rabbit anti-mouse βIII-tubulin antibody, 1 : 400, Abcam) was added and the cells were incubated at room temperature for 3 h. After washing, the neurons were incubated with the secondary antibody (goat anti-rabbit IgG-FITC, 1 : 100, Invitrogen) for 30 min at room temperature. After washing twice with HBSS, the coverslips were mounted with antiquenching mounting medium and the neurites morphology changes of neurons were visualized using a fluorescence microscope at ×400 magnification.

2.4. MTT Assay. The cell viability was assessed using the MTT assay according to conditions described previously [25]. The absorbance at 570 nm was measured using a Wallac Victor3 V microplate reader (PerkinElmer, Netherlands). Cell viability was expressed as a percentage of the MTT reduction of control.

2.5. LDH Release Assay. The cytotoxicity inflicted to cells by IAA was also assessed by the LDH release assay. Determination of total and released LDH activity was performed according to the instructions accompanying the cytotoxicity detection kit (Roche). LDH released was normalized to a total LDH release and the results were shown as a percentage of total LDH activity.

2.6. FDA/PI Double Staining and Hoechst Staining. Viable neurons were stained with fluorescein formed from FDA, which is deesterified only by living cells. PI can penetrate cell membranes of dead cells to intercalate into double-stranded nucleic acids. Briefly, neurons were washed twice with ice-cold PBS. After incubation with 10 μg/mL of FDA and 5 μg/mL of PI for 15 min, the neurons were examined and photographed using a fluorescence microscope (Nikon Instruments Inc., Melville, NY).

Chromatin condensation was detected by staining the cell nucleus with Hoechst 33342. The cultures were fixed with 4% paraformaldehyde and washed with PBS before staining with Hoechst 33342 for 30 min. Thereafter, cell morphology was observed under a fluorescent microscope (Zeiss, Oberkochen, Germany). The percentage of apoptotic nuclei from five random fields in each well of different treatment groups was quantified and averaged.

2.7. Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$). The dye JC-1 was used as a molecular probe to measure $\Delta\psi_m$. The staining procedure was performed according to the manufacturer's instructions with minor modification. Briefly, CGNs were washed with HBSS and stained with 2 μM JC-1 for 10 min. Fluorescence intensity was measured on a microplate reader using 488 nm excitation and 529 nm/590 nm dual emissions. The mitochondrial accumulation of JC-1 is dependent on $\Delta\psi_m$ and is reflected by a shift in 529 nm and 590 nm emissions. Mitochondrial membrane

depolarization is indicated by a decrease in the ratio of 590 nm to 529 nm emissions.

2.8. ROS Measurement. Intracellular ROS were measured using the redox-sensitive fluorescent probe DCFH-DA, which is hydrolyzed to nonfluorescent DCFH by intracellular esterase. DCFH is rapidly oxidized to the fluorescent DCF when reacting with intracellular ROS. CGNs were washed with PBS and were incubated with 20 μM DCF-DA for 1 h. Fluorescence was measured on a microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 515 nm.

2.9. Caspase 3 Activity Assay. CGNs were scraped off in HBSS, collected by centrifugation, and lysed at 4°C in cell lysis buffer containing 20 mM EDTA, 20 mM Tris (pH 7.5), and 1% Triton X-100. Lysates were centrifuged at 12000 g at 4°C for 5 min. Caspase 3 assay was performed on 96-well microplates using substrate peptides Ac-DEVD-pNA according to the manufacturer's instruction. The release of p-NA was qualified by determining the absorbance at 405 nm.

2.10. Statistical Analysis. All measurements were repeated 3 times. Data were expressed as mean ± SEM and were analyzed using GraphPad Prism V5.0 (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) and Dunnett's test were used to evaluate statistical differences. The value of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of EDA on IAA-Induced CGNs Death. We evaluated the protective effect of EDA on IAA-induced primary neuron death by immunofluorescence-based morphological analysis, MTT reduction and LDH leakage-based cell viability assay, and FDA/PI double staining. As shown in Figure 1(a), IAA clearly destroyed the gross morphology of the CGNs cell body and neuritic network. Pretreatment with EDA notably protected neurons from IAA-induced neurotoxicity (Figure 1(a)). For 4 h treatment, IAA concentration-dependently decreased the cell viability of CGNs. The survival rate reduced approximately by 50% when CGNs were exposed to 50 μM IAA (Figure 1(b)). The cell viability of CGNs pretreated with 3–30 μM EDA significantly increased in a concentration-dependent manner ($P < 0.05$ versus IAA treatment alone, Figure 1(c)) and was up to 85% of control at 30 μM EDA treatment. Pretreatment of EDA also reduced the level of LDH release compared with IAA-treated alone group ($P < 0.05$, Figure 1(d)). Moreover, cotreatment of EDA with IAA also significantly inhibited the decrease of cell viability induced by IAA in a concentration-dependent manner ($P < 0.05$ versus IAA treatment alone, Figure 1(e)).

The FDA/PI double staining was performed to further determine whether EDA attenuated cell injury after IAA treatment in CGNs. Simultaneous use of two fluorescent dyes allows a two-color discrimination of the population of live cells from the necrotic-cell population. As shown in Figure 2(a), IAA significantly increased cell necrosis as stained by red fluorescence, accompanying decrease in green fluorescent cells. Consistent with the result of MTT-based cell viability assay, pretreatment of EDA remarkably attenuated CGNs necrosis induced by IAA (Figures 2(a) and 2(b)).

3.2. EDA Prevents IAA-Induced CGNs Apoptosis. Apoptosis is morphologically characterized by cell shrinkage, chromatin condensation. To identify whether EDA reverses IAA-induced CGNs apoptosis, we used Hoechst 33342 staining to evaluate nuclear condensation. As shown in Figure 3(a), normal untreated cells appeared circular or elliptical where no condensation of the nucleus was observable. In contrast, bright condensed dots known as apoptotic bodies (as indicated by arrows) were clearly identified when treated with IAA. Pretreatment of EDA could mitigate IAA-induced apoptosis (Figure 3(a)). In addition, the count of apoptotic nuclei revealed that EDA significantly reduced IAA-induced apoptosis concentration-dependently (Figure 3(b)).

3.3. Effects of EDA on IAA-Induced Intracellular ROS Generation. IAA induces cell death accompanied with an increase in ROS production through irreversible inhibition of the glyceraldehyde 3-phosphate dehydrogenase [15–17]. To investigate whether EDA reduces ROS level after IAA treatment in CGNs, intracellular ROS production was detected by DCFH-DA probe. As shown in Figure 4, after exposure to IAA for 4 h, the level of ROS was increased by 3.3-fold in CGNs compared with untreated control. Pretreatment with EDA significantly suppressed this increase in intracellular ROS level. At 30 μM, EDA almost completely inhibited ROS production to a level equal to that of the normal control.

3.4. The Effects of EDA on $\Delta\psi_m$ in CGNs Induced by IAA. It has been reported that IAA induces mitochondrial dysfunction [17]. To investigate the protective effect of EDA against IAA-induced mitochondrial damage, $\Delta\psi_m$ in CGNs was assessed using JC-1 probe. As shown in Figure 5, IAA treatment resulted in a profound loss of $\Delta\psi_m$ in CGNs. When the cells were pretreated with EDA, the $\Delta\psi_m$ increased significantly and concentration-dependently compared with that of the IAA alone-treated group.

3.5. EDA Attenuates IAA-Induced Caspase 3 Activation in CGNs. Activation of caspase 3 plays a key role in cellular apoptosis. Figure 6 showed that IAA treatment caused a dramatic increase in caspase 3 activity. When CGNs were preincubated with EDA, the elevated caspase 3 activity induced by IAA was significantly reduced in a concentration-dependent manner.

4. Discussion

In the present study, we demonstrated that EDA protected against IAA-induced neurotoxicity in CGNs. Subsequent experiments to explore the mechanisms underlying the neuroprotective effect revealed that EDA significantly decreased neuronal apoptosis and intracellular ROS overproduction, maintained $\Delta\psi_m$, and attenuated caspase 3 activity in CGNs.

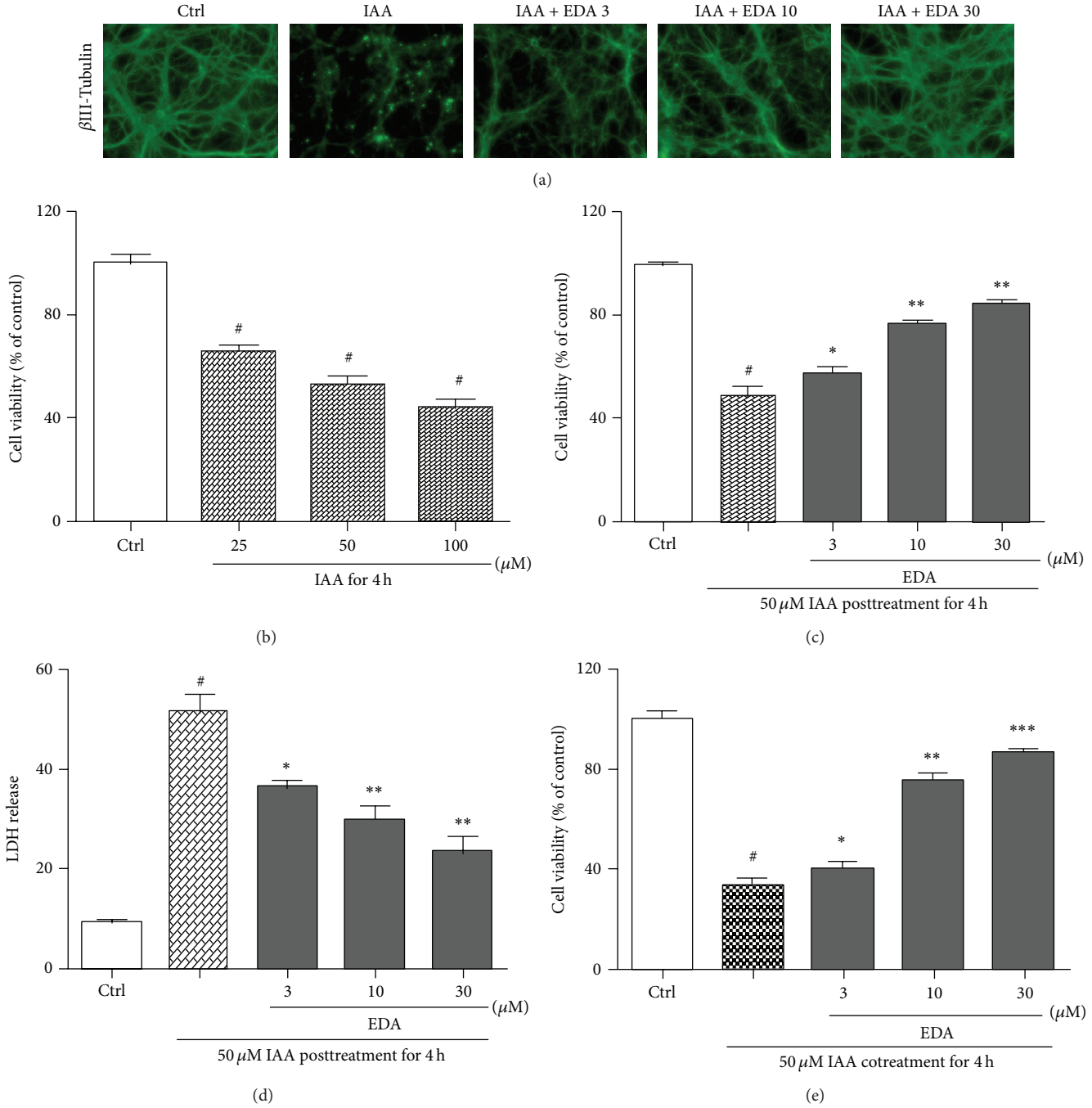

Figure 1: Protective effect of EDA against IAA-induced cells damage in CGNs. (a) Gross morphological change of CGNs cell body and neuritic network was determined by fluorescent immunostaining with antibody against βIII-tubulin (400x magnification). CGNs were pretreated with or without EDA for 2 h and then incubated with 50 μM IAA for another 4 h. (b) IAA-induced decrease in cell viability by MTT assay. CGNs were exposed to 25, 50, and 100 μM of IAA for 4 h. Cell viability was measured by MTT reduction assay. (c) EDA pretreatment attenuates IAA-induced neuronal loss in a concentration-dependent manner. CGNs were pretreated with or without EDA for 2 h and then incubated with 50 μM IAA for another 4 h. Cell viability was measured by MTT reduction assay. (d) Effect of EDA pretreatment on IAA-induced LDH release. Cells were treated as in (c) and LDH release level was detected by cytotoxicity detection kit. (e) Cotreatment of EDA with IAA inhibits cell viability decrease induced by IAA. CGNs were cotreated with EDA and IAA for 4 h. Cell viability was measured by MTT reduction assay. $^{\#}P < 0.001$ versus control (Ctrl); $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ versus IAA alone group.

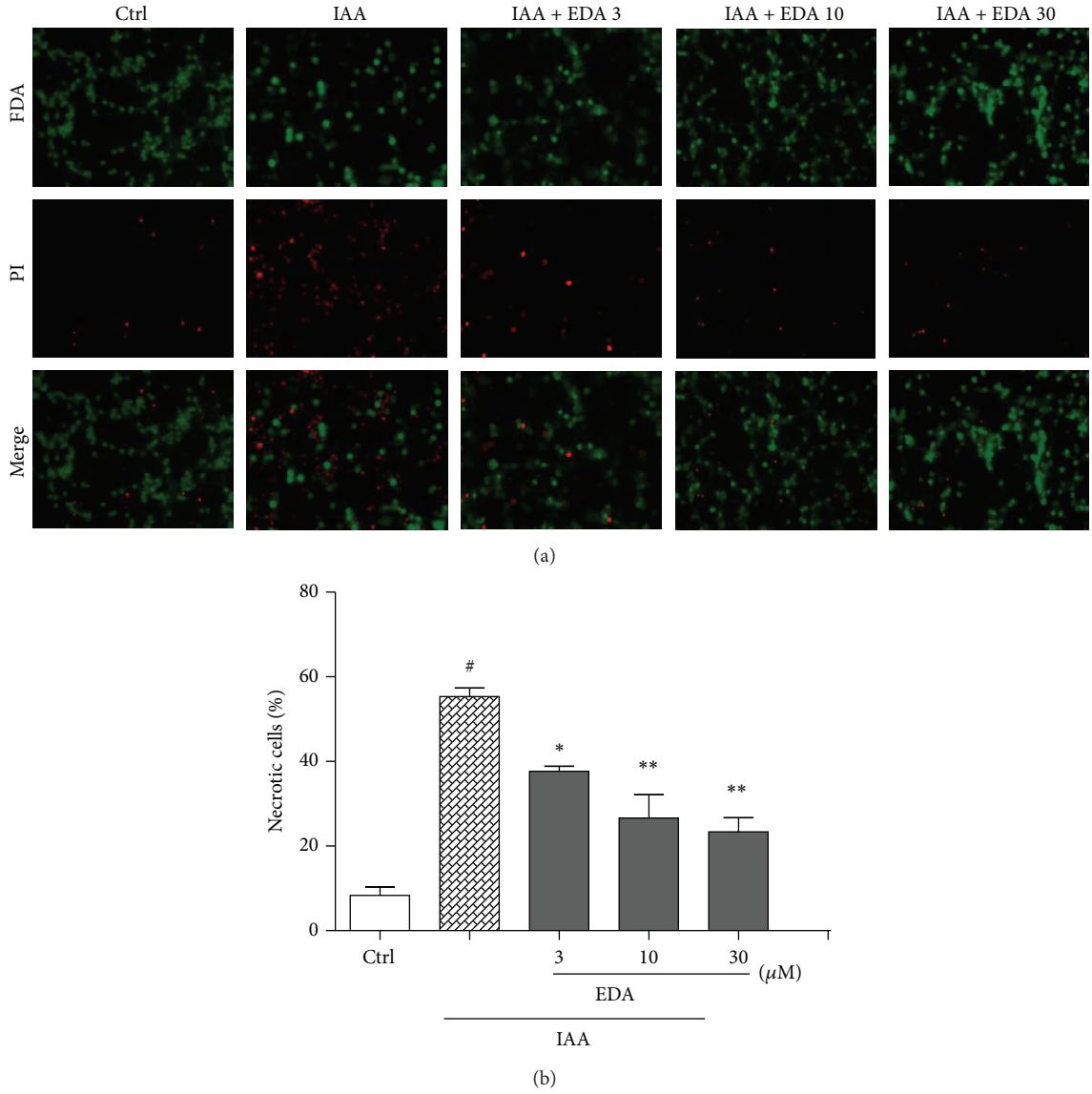

Figure 2: EDA attenuates CGNs neurosis induced by IAA. CGNs were preincubated with or without EDA for 2 h followed by exposure to 50 μM IAA for another 4 h. (a) CGNs were stained with FDA and PI (200x magnification). (b) Quantitative analysis of necrotic cells from three representative photomicrographs was represented as a percentage of the total number of cells counted. $^{\#}P < 0.001$ versus Ctrl; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus IAA alone group.

Cerebral ischemia impairs the normal neurological functions which are triggered by a complex series of biochemical and molecular mechanisms, such as excitotoxicity, oxidative stress, and apoptosis along with histological changes [26]. Cerebral ischemia triggers two general pathways of apoptosis: the intrinsic pathway, originating from mitochondrial release of cytochrome c and associated stimulation of caspase 3, and the extrinsic pathway, originating from the activation of cell surface death receptors, resulting in the stimulation of caspase 8. In addition, cerebral ischemia and reperfusion generate ROS, which causes DNA damage. Many studies have shown that EDA attenuated apoptosis in neuronal cells. Song et al. demonstrated that EDA suppressed the Bad protein overexpression and increased Bcl-2 protein expression, repaired the mitochondrial dysfunction, and maintained ATP level in PC12 cells [27]. Xiong et al. showed that EDA inhibited protein Bax expression and attenuated downregulation of Bcl-XL induced by rotenone [28]. Chen et al. also found that EDA inhibited cobalt chloride-induced apoptosis in PC12 cells via the regulation of Bcl-2 family and reduced caspase 3 activity [4]. Consistent with previous findings, our present study showed that EDA also significantly reduced IAA-induced CGNs apoptosis via suppression of caspase 3 activation and maintaining $\Delta\psi_m$. However, different from other studies, which applied oxygen-glucose deprivation, rotenone, and cobalt chloride as causes of cell injury, our present study used IAA to induce cell damage. It has been reported that oxygen-glucose deprivation induced

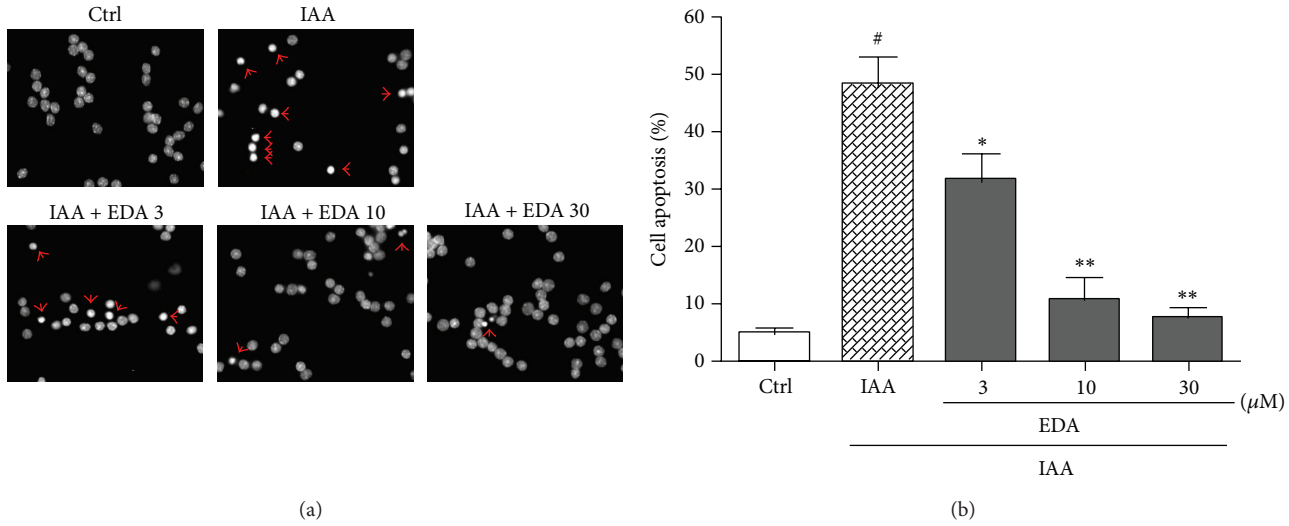

FIGURE 3: EDA attenuates IAA-induced CGNs apoptosis. CGNs were preincubated with or without EDA for 2 h followed by exposure to 50 μM IAA for another 4 h. (a) Cell apoptosis was assessed by Hoechst 33342 staining and observed by fluorescent microscopy (400x magnification). Apoptotic nuclei with condensed chromatin were indicated by red arrows. (b) The amount of apoptosis nuclei was counted from three representative photomicrographs and was represented as a percentage of the total number of nuclei counted. $^{\#}P < 0.001$ versus Ctrl; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus IAA alone group.

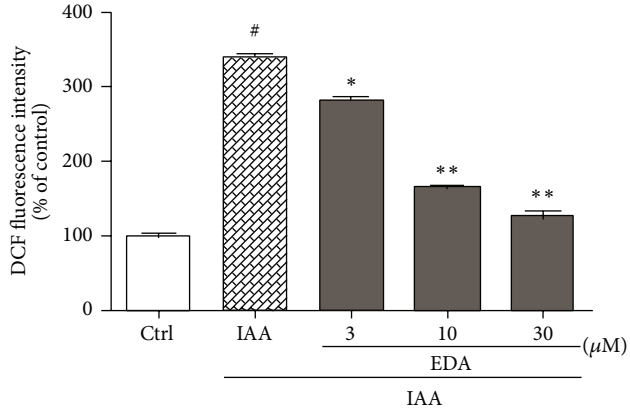

FIGURE 4: Effect of EDA on IAA-induced ROS production in CGNs. CGNs were preincubated with or without EDA for 2 h followed by exposure to 50 μM IAA for another 4 h. Intracellular ROS generation was evaluated by DCFH-DA probe. $^{\#}P < 0.001$ versus Ctrl; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus IAA alone group.

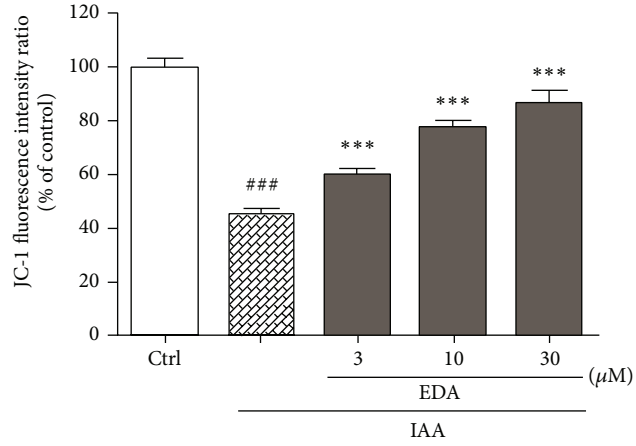

FIGURE 5: Effect of EDA on IAA-induced $\Delta\psi_m$ loss in CGNs. CGNs were preincubated with or without EDA for 2 h followed by exposure to 50 μM IAA for another 4 h. The $\Delta\psi_m$ was determined by JC-1. $^{\#\#\#}P < 0.0001$ versus control; $^{***}P < 0.01$ versus IAA alone group.

mitochondrial dysfunction and oxidative stress in neurons [29] and stimulated Ca^{2+}-dependent glutamate release in cortical slice cultures [30]. Both rotenone and cobalt chloride, as well as IAA, are mitochondrial inhibitors; however, they inhibit mitochondrial function at different sites of the mitochondrial respiratory chain and are inhibitors of mitochondrial respiratory complex I, prolyl hydroxylase, and glyceraldehyde 3-phosphate dehydrogenase, respectively [15, 17, 31, 32].

Free radical production is enhanced in both the ischemic core and penumbra following stroke injury, and this is believed to cause much of the damage seen in these regions [1]. Normally, oxidative stress is being caused by the imbalance between free radical production and degradation. Brain is most susceptible to oxidative stress due to high consumption of oxygen [33]. Natural formation of oxidants during mitochondrial electron transport and autooxidation of some neurotransmitters and in ischemic attacks of events during ischemia can result in oxidant formation and subsequent tissue damage [34]. Normally, free radicals are removed by antioxidant enzymes in living cells. The antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione (GSH). SOD catalyses the dismutation of the highly reactive superoxide anion ($O_2^{\bullet -}$) to O_2 and H_2O_2. CAT reacts with peroxide to form

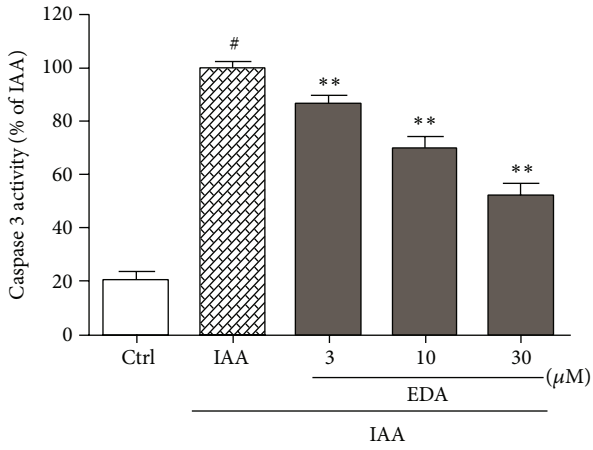

Figure 6: EDA inhibits caspase 3 activation in CGNs. CGNs were preincubated with or without EDA for 2 h followed by exposure to 50 μM IAA for another 4 h. Cells were lysed and caspase 3 activity was measured as described in Section 2.9. $^{\#}P < 0.001$ versus control and $^{**}P < 0.01$ versus IAA alone group.

water and molecular oxygen. GPX catalyses the reduction of organic peroxides using GSH and thereby protects cells from oxidative damage. Many studies have shown that protective effect of EDA was associated with the increased SOD activity and CAT, GSH levels in PC12 cells [35, 36]. Our present study indicated that EDA significantly decreased ROS production induced by IAA in CGNs. However, that EDA defenses ROS production induced by IAA in CGNs through direct scavenging ROS or through indirect elevating antioxidative enzymes needs to be further clarified.

5. Conclusion

In conclusion, the neuroprotective effect of EDA on IAA-induced neurotoxicity in CGNs was due to its antiapoptotic and antioxidative effects. However, the precise molecular events involved in its antioxidative and antiapoptotic actions need to be further elucidated.

Conflict of Interests

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

Acknowledgments

This work is partially supported by grants from the National Natural Science Foundation of China (NSFC 81303251 to Zaijun Zhang), China's "12.5" Innovative Drug Project (2012ZX09103101-055 to Yuqiang Wang), the Municipal Science and Technology Project of Guangdong (2012A080201009 to Yuqiang Wang), and the International Science and Technology Collaborative Project of Guangdong Department of Education (gjhz1102 to Yuqiang Wang).

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