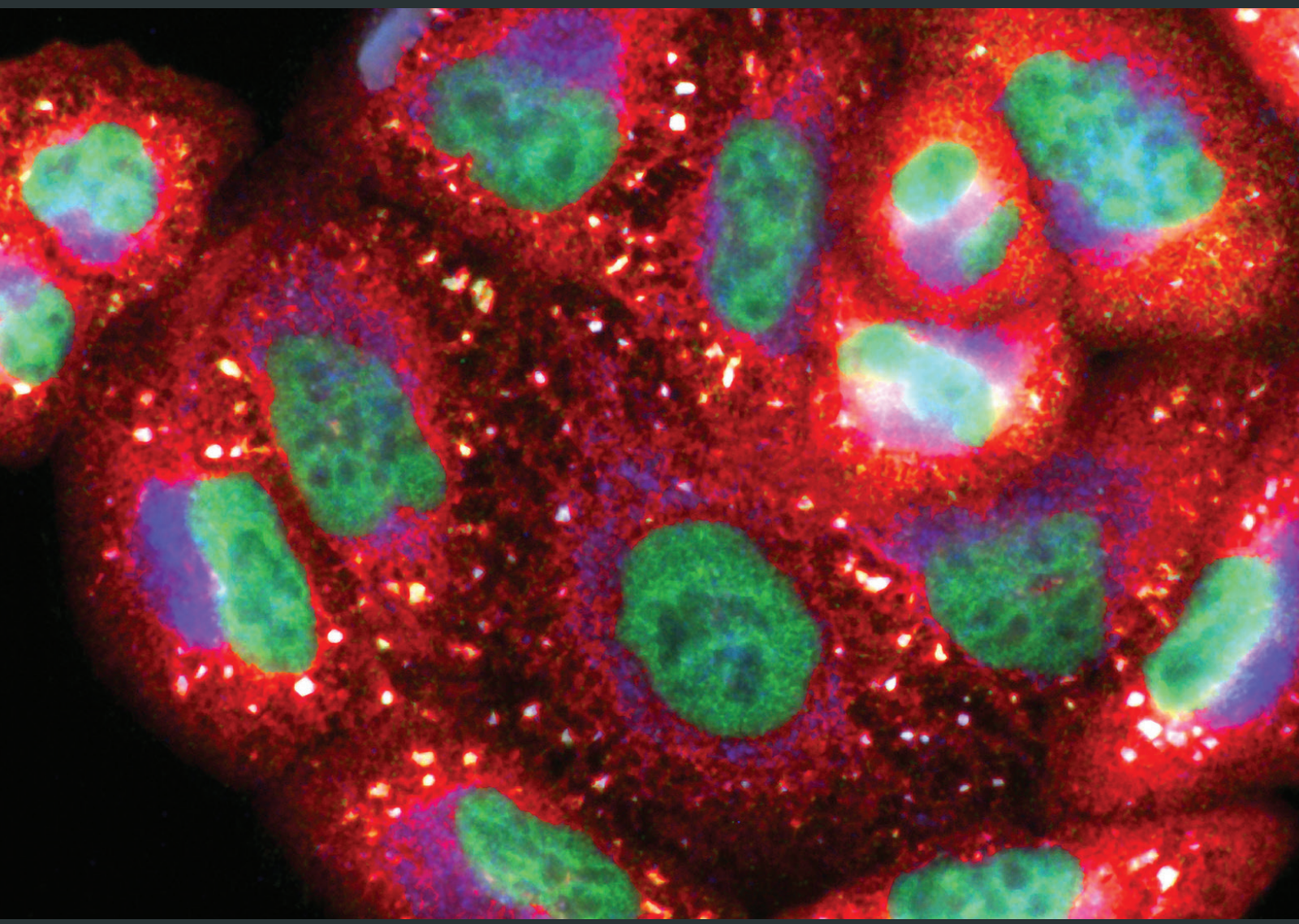
Oxidative Medicine and Cellular Longevity

Antioxidant, Anti-Inflammatory, and Microbial-Modulating Activities of Nutraceuticals and Functional Foods 2018

Special Issue Editor in Chief: Ilaria Peluso
Guest Editors: Débora Villaño Valencia, Chung-Yen Oliver Chen, and Maura Palmery



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Editorial

Antioxidant, Anti-Inflammatory, and Microbial-Modulating Activities of Nutraceuticals and Functional Foods 2018

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The antioxidant and anti-inflammatory activities of nutraceuticals are current research topics in the area of the management and prevention of oxidative stress-related diseases. This issue includes preclinical research aimed to evaluate the effect of pure compounds (P. Aguilar-Alonso et al., "Evaluation of Oxidative Stress in Cardiomyocytes during the Aging Process in Rats Treated with Resveratrol"; W.-R. Hao et al., "Cafestol Inhibits Cyclic-Strain-Induced Interleukin-8, Intercellular Adhesion Molecule-1, and Monocyte Chemoattractant Protein-1 Production in Vascular Endothelial Cells"; and H. Sun et al., "The In Vitro Antioxidant Activity and Inhibition of Intracellular Reactive Oxygen Species of Sweet Potato Leaf Polyphenols"), extracts from plant (W. Huang et al., "Antioxidant and Anti-Inflammatory Effects of Blueberry Anthocyanins on High Glucose-Induced Human Retinal Capillary Endothelial Cells"; Z. Liao et al., "Protective Role of Antioxidant Huskless Barley Extracts on TNF-α-Induced Endothelial Dysfunction in Human Vascular Endothelial Cells"; H. Sun et al. "The In Vitro Antioxidant Activity and Inhibition of Intracellular Reactive Oxygen Species of Sweet Potato Leaf Polyphenols"; and K. Wei Chan et al., "Defatted Kenaf (*Hibiscus cannabinus* L.) Seed Meal and Its Phenolic-Saponin-Rich Extract Protect Hypercholesterolemic Rats against Oxidative Stress and Systemic Inflammation via Transcriptional Modulation of Hepatic Antioxidant Genes"), fungal (X. Wang et al., "Antifatigue Potential Activity of *Sarcodon imbricatus* in Acute Excise-Treated and Chronic Fatigue Syndrome in Mice via Regulation of Nrf2-Mediated Oxidative Stress) or animal (Y. Zhang et al., "Acute Toxicity, Antioxidant, and Antifatigue Activities of Protein-Rich Extract from *Oviductus ranae*") sources, and a defatted kenaf seed meal (DKSM) (K. Wei Chan et al.).

In vitro data illustrate that blueberry anthocyanin-rich extract (W. Huang et al.), huskless barley extract (Z. Liao et al.), and sweet potato leaf polyphenols (H. Sun et al.) improve the redox status by reducing intracellular reactive oxygen species (H. Sun et al. and W. Huang et al.) and/or by increasing the capacity of antioxidant enzymes (W. Huang et al. and Z. Liao et al.). Two of these research papers (Z. Liao et al. and W. Huang et al.) also show that constituents in the huskless barley extract and sweet potato leaf polyphenols enable the reduction of monocyte chemotactic protein 1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and/or nuclear factor-kappa B (NF-κB) in human endothelial cells, which are in line with the concerted modulation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and NF-κB in inflammation and oxidative stress. However, H. Sun et al. note that the inhibitory effect of individual polyphenolic compounds present in sweet potato leaves on the intracellular ROS is not related to their antioxidant activity, suggesting that the polyphenolic compounds may attenuate the sources of ROS

generation. However, the precaution must be taken in the extrapolation of the *in vitro* findings to humans because most absorbed polyphenols are present in metabolized forms. Moreover, the protein-rich extract of *Oviductus ranae* (PEOR) displays a strong antioxidant effect in ethanol-induced oxidative stress mice model, despite its weak radical scavenging and ferric-reducing capacities *in vitro* (Y. Zhang et al.).

Both PEOR (Y. Zhang et al.) and *Sarcodon imbricatus* (X. Wang et al.) have an antifatigue effect in mice and upregulate antioxidant enzymes. In particular, *Sarcodon imbricatus* (X. Wang et al.) induces Nrf2, superoxide dismutase (SOD), haem oxygenase-1 (HO-1), and catalase (CAT). Upregulation of HO-1 has been reported in vascular endothelial cells treated with the diterpene cafestol (W.-R. Hao et al.). The latter, found in coffee, can inhibit ICAM-1, MCP-1, and mitogen-activated protein kinases (MAPK) pathway. Antioxidant and anti-inflammatory properties of DKSM and its phenolic-saponin-rich extract (PSRE) observed in hypercholesterolemic rats are likely modulated via the activation of the Nrf2-antioxidant responsive element (ARE) pathway (K. Wei Chan et al.). On the contrary, in rats treated with resveratrol, the decrease in nitric oxide and lipoperoxidation in the cardiac tissues is not accompanied by the induction of antioxidant enzymes (CAT and SOD).

In addition to the antioxidant and anti-inflammatory effects of huskless barley extracts, the polysaccharide extracts inhibit the synthesis of angiotensin-converting enzyme (ACE) *in vitro* (Z. Liao et al.), with the alkaline extracts being more pronounced probably due to the abundance of phenolic compounds as compared to the water extracts. The modulation of ACE expression is also found with blueberry anthocyanins and anthocyanidins (W. Huang et al.), but the effect is divergent, for example, malvidin downregulates the expression and some malvidin glycosides result in the upregulation.

Therefore, caution must be taken when interpreting preclinical data that evaluate the effects of natural functional compounds on antioxidation, anti-inflammation, and other pharmacological actions. In this context, E. Toti et al. (Non-Provitamin A and Provitamin A Carotenoids as Immunomodulators: Recommended Dietary Allowance, Therapeutic Index, or Personalized Nutrition?) underscore that preclinical evidence must be confirmed in human interventions before any recommendations can be made for carotenoids. This point can also be extended to polyphenols and other dietary agents. For example, both β-carotene and lycopene at pharmacological doses affect immune functions. However, large clinical trials do not support β-carotene supplementation. On the other hand, although lycopene supplementation for regulation of immunity seems more promising than β-carotene, more robust human studies with adequate power and duration are needed in order to confirm this effect.

In conclusion, we hope that this special issue adds knowledge of preclinical data of the potential health effects of nutraceuticals. However, these results only provide supports for future studies, particularly human trials, but not give indications for supplementation.

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Research Article

Antifatigue Potential Activity of *Sarcodon imbricatus* in Acute Excise-Treated and Chronic Fatigue Syndrome in Mice via Regulation of Nrf2-Mediated Oxidative Stress

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Sarcodon imbricatus (SI), a precious edible fungus, contains 35.22% of total sugar, 18.33% of total protein, 24 types of fatty acid, 16 types of amino acid, and 8 types of minerals. Encouragingly, it is rich in potential antioxidants such as total polyphenols (0.41%), total sterols (3.16%), and vitamins (0.44%). In the present study, the antifatigue properties of SI and its potential mechanisms of action were explored by the experiments on acute excise-treated mice and chronic fatigue syndrome (CFS) mice. SI (0.25, 0.5, and 1 g/kg) significantly enhanced exercise tolerance in the weight-loaded forced swimming test (FST) and rota-rod test (RRT) and reduced the immobility in the tail suspension test on CFS mice. SI markedly increased the levels of glycogen in the liver and adenosine triphosphate (ATP) in the liver and muscle and decreased the lactic acid (LD) and blood urea nitrogen (BUN) content in both acute swimming-treated mice and CFS mice. SI improved the endogenous cellular antioxidant enzyme contents in the two mouse models by improving the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and reducing reactive oxygen species (ROS) and malondialdehyde (MDA) levels in serum, liver, and muscle, respectively. In CFS mice, the enhanced expression levels of nuclear factor erythroid-2-related factor 2 (Nrf2), SOD1, SOD2, heme oxygenase-1 (HO-1), and catalase (CAT) in the liver were observed after a 32-day SI administration. Our data indicated that SI possessed antifatigue activity, which may be related to its ability to normalize energy metabolism and Nrf2-mediated oxidative stress. Consequently, SI can be expected to serve as a novel natural antifatigue supplement in health foods.

1. Introduction

Fatigue, caused by fierce stress from physical and mental work, is a decreased performance under subhealthy conditions [1]. Fast-paced lifestyles, intense competitive pressures, and irregular eating and drinking habits put people at risk of fatigue. If fatigue symptoms cannot be alleviated in time, patients will experience chronic fatigue syndrome (CFS), which is defined as the persistent or recurrent severe fatigue (more than 6 months) accompanied by apathetic, tender lymphadenopathy, body aches, headaches, unrefreshing sleep, inattention, and lower work efficiency [2]. CFS increases the risk of neuropsychiatric problems, such as depression and anxiety [3]. Depression mood is recognized as an important characteristic indicator within CFS patients [4].

Oxidative stress, a well-characterized factor, has received widespread attention as a bridge between fatigue and CFS. Oxidative stress is triggered by the overproduction of reactive oxygen species (ROS), and it attacks large molecules and cell organs [5] which leads to an injured body. Increased oxidative stress and decreased antioxidant defenses are positively correlated with the severity of symptoms in CFS [6]. Free radicals are regarded as an important indicator of impairment of skeletal muscle function, and intense exercise induces excessive ROS production. Lipid peroxidation caused by ROS leads to structural damage and cell or organelle dysfunction [7]. According to previous literature reports,

skeletal muscle and liver mitochondria were susceptible to lipid peroxidation-induced damage during exercise [8]. And prolonged oxidative stress will trigger CFS [9]. Nuclear factor-erythroid 2-related factor 2/antioxidant responsive element (Nrf2/ARE) is one of the most important defense mechanisms of the body's cells against oxidative damage [10].

Till now, the etiology of CFS is still not clear. Although some medicines including immunostimulants, immunosuppressants, antidepressants, hypnotics, analgesics, and antihistamines were used for the treatments to CFS-related diseases, no satisfactory effects were obtained in clinics due to no optimistic long-term efficacy and various side effects [11, 12]. Since nutrient supplementation positively enhances exercise capacity, researches attempt to seek a safe and effective anti-CFS agent from natural products, which people can take as the "tonics." Recently, China has carried out preclinical studies and clinical trials of CFS, with special emphasis on the use of traditional Chinese herbal medicine [13, 14]. Fungus, containing various nutritional ingredients, exhibits multiple activities such as antioxidation and antifatigue [15, 16]. Antioxidant active ingredients in natural medicines mainly include polysaccharides, polyphenols, tetraterpenes, sterols, and vitamins, which realize antioxidant activity by scavenging free radicals, terminating the progress of chain oxidation, and improving the antioxidant capacity of the body [17]. *Sarcodon imbricatus* (SI), belonging to the family of *Basidiomycotina* and *Aphyllophorales*, is an edible and medicinal fungus, widely distributed in Central Europe and in North America [18] and also produced in Tibet, Gansu, Anhui, northwestern Yunnan, and western Sichuan of China [19]. Although SI is described to show various pharmacological activities including anti-inflammation and anticancer in the folk, previous studies mainly focused on its chemical component analysis and polysaccharide isolation [20, 21]. Our group has already confirmed the immunomodulatory property of *S. imbricatus* water extracts in the cyclophosphamide- (CTX-) induced immunosupressive mouse model, which is related to its modulation on oxidative stress [22]. Encouragingly, based on these data, we speculated that SI has certain effects on improving exercise endurance and relieving fatigue due to its oxidation resistance.

For this purpose, in the present study, we analyzed the components of SI systematically first and then investigated its antifatigue effect properties and potential mechanisms in acute excise-treated and CFS mouse models. Valuable and useful information about the bioactivity of SI as a functional food supplement will be provided in our data.

2. Materials and Methods

2.1. Plant Material and Preparation. SI were collected from the broad-leaved forest area of Yunnan in September 2015, which are taxonomically identified by the Engineering Research Center of Chinese Ministry of Education for Edible and Medicinal Fungi, Jilin Agricultural University, Changchun, China. Dried SI were pulverized into powder by a flour mill and sieved through an 80-mesh sieve. It was dark brown and stored in a desiccator for subsequent experiments.

2.2. Measurement of the SI Components

2.2.1. Main Components. The main nutrition and quality components of the SI fruiting body were systematically determined according to the previous studies and national standards. The Folin-Ciocalteu method [23], UV spectrophotometric assay [24, 25], HPLC methods [26], phenol-sulfuric acid determination [27], 3,5-dinitrosalicylic acid colorimetric estimation [28], vanillin-glacial acetic acid and perchloric acid colorimetric spectrophotometry [29], the aluminium chloride colorimetric method [30], the periodate oxidation method [31], the petroleum benzine extraction method [32], the ashing method [33], and the Kjeldahl method [34] were used to analyze the levels of polyphenols, total content of carotenoids and sterols, vitamins, total sugar, reducing sugar, triterpenoids, flavonoids, mannitol, crude fat, total ash, and total protein, respectively.

2.2.2. Amino Acid Analysis. SI was hydrolyzed using 6 mol/L of HCl at 110°C for 24 h. After vacuum drying, the samples were dissolved in 1 mL of a buffer with pH 2.2. A quantitative analysis of the amino acids was carried out using an automatic amino acid analyzer (L-8900, Hitachi, Japan).

2.2.3. Minerals. Minerals of SI were carried out according to previous studies with some modifications [35]. Briefly, SI (0.5 g of each time) was placed in a porcelain citrus pot and completely ashed and then dissolved in nitric acid (5 mL). The digestion procedure was set as follows: raising the room temperature to 120°C, 0–5 min; holding at 120°C, 6–7 min; raising from 120 to 180°C, 8–17 min; and holding at 180°C, 18–32 min. After cooling at room temperature, the solution was transferred into a 50 mL volumetric flask and diluted to 50 mL with deionized water. Subsequently, the levels of potassium (K), sodium (Na), calcium (Ca), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), selenium (Se), mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb) were detected by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Thermo Elemental, Franklin, MA).

2.2.4. Fatty Acids. SI was extracted using a ratio of chloroform : methanol 2 : 1 (v : v), evaporated under the conditions of 80°C, and then mixed with potassium hydroxide-methanol solution (4 g potassium hydroxide : 100 mL methanol) at 50°C for 10 min. 1 mL of 20% BF_3 solution was added to the samples, and then the samples were incubated at 50°C for another 15 min. Finally, the samples were mixed with hexane. The hexane layer was washed with water until neutral, and the levels of fatty acids were analyzed using a gas chromatography-mass spectrometer (QP2010, Shimadzu, Japan).

2.3. Animal Care and Experimental Procedure. Experimental protocol was approved by the Institution Animal Ethics Committee of Jilin University (20160208). One hundred and ten Kunming male mice (4–6 weeks, 18–22 g, specific pathogen-free (SPF) grade) (SCXK (JI)-2017-0001) purchased from the lab animal center of Jilin University were housed under a controlled environment at a temperature of

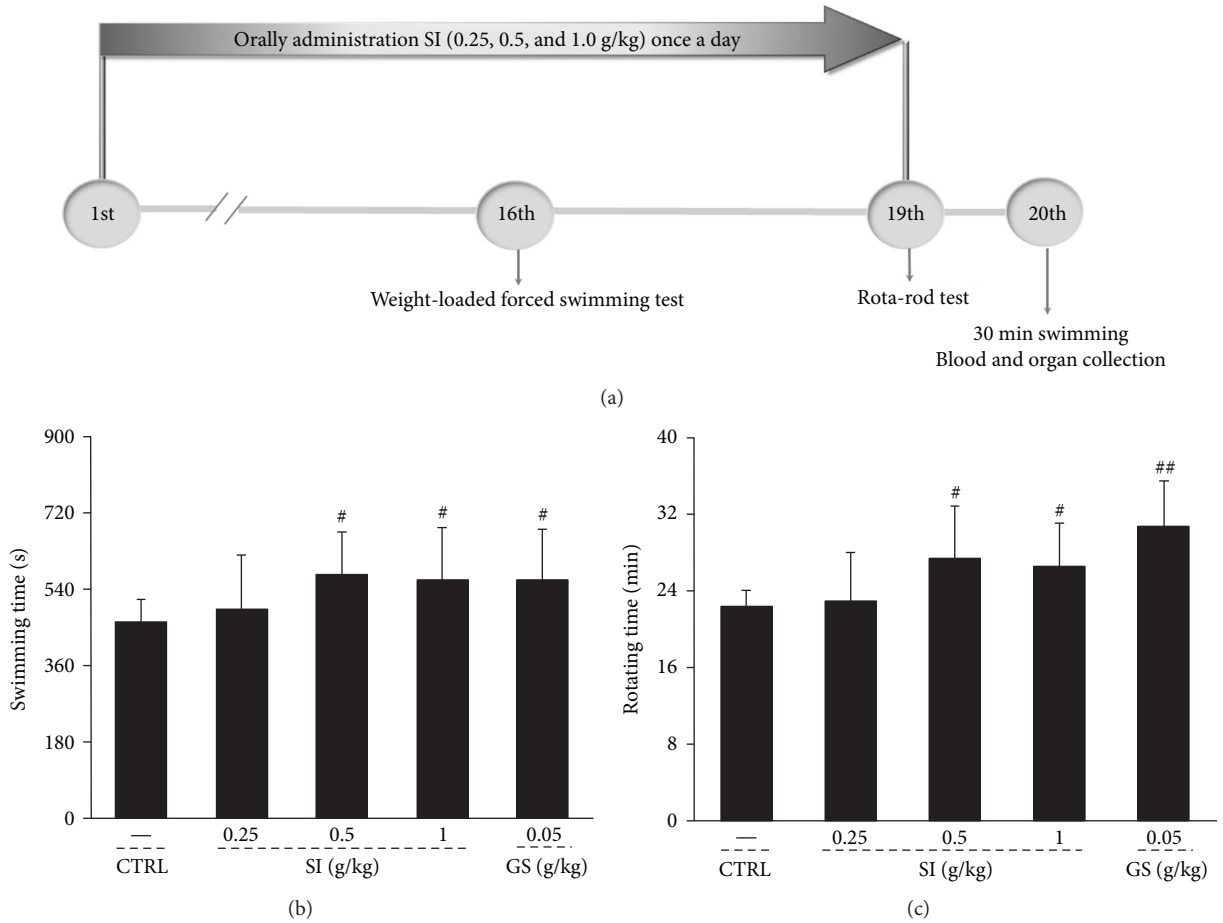

Figure 1: (a) The experimental protocol and drug administration procedure on acute exercise-treated mice. The effects of SI and GS on (b) weight-loaded forced swimming test and (c) rota-rod test in normal mice. Data were analyzed using a one-way ANOVA followed by Dunn's test and expressed as means ± SD ($n = 10$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group. SI: *S. imbricatus*; GS: *Ginsenoside*.

22 ± 2°C and moderate humidity of 50 ± 10% with a 12/12 h light/dark cycle and fed an autoclaved standard chow and water *ad libitum*. Mice were acclimatized for one week and then were used in the following experiments.

2.3.1. Acute Excise-Treated Mouse Model Establishment and Agent Treatment Procedure. Fifty mice were taken out randomly according to body weight and divided into five groups ($n = 10$/group) and orally treated with 0.5% of sodium carboxymethyl cellulose (CMC-Na, 0.2 mL/20 g) (control mice), 0.05 g/kg of ginsenoside (GS, 0.2 mL/20 g) dissolved in 0.5% of CMC-Na (positive control mice), and SI at doses of 0.25 g/kg (0.2 mL/20 g), 0.5 g/kg (0.2 mL/20 g), and 1.0 g/kg (0.2 mL/20 g) dissolved in 0.5% of CMC-Na once per day for 18 days. Oral gavage treatment was performed at 9 : 00 every day. At the 16th day and the 19th day, after SI administration for 30 min, the weight-loaded forced swimming test (FST) and rota-rod test (RRT) were performed to evaluate the endurance capacity of mice in each group, respectively. At the 20th day, all mice were forced to swim for 30 min without loads, and then blood was sampled from the caudal veins. After sacrificing, liver and muscle were collected from each mouse rapidly. The detailed experimental protocol and drug administration are shown in Figure 1(a).

2.3.2. CFS Mouse Model Establishment and Agent Treatment Procedure. Based on previous reports [36, 37], fifty mice were exposed to different stimuli including cold water swimming (15°C ± 1°C) for 10 min, exhaustive running 15 min, rota-rod for 15 min, and sleep deprivation once per day for 4 weeks. The same stressor was not applied continuously for two days. Another 10 mice receiving no stimuli for 4 weeks serve as the control group. FST was applied to test whether the CFS mice were established successfully. At the 29th day, CFS mice were divided into five groups randomly ($n = 10$/group) and orally administrated with 0.5% of CMC-Na (0.2 mL/20 g) (model mice), 0.05 g/kg of GS (0.2 mL/20 g) dissolved in 0.5% of CMC-Na (positive control mice), and SI at doses of 0.25 g/kg (0.2 mL/20 g), 0.5 g/kg (0.2 mL/20 g), and 1.0 g/kg (0.2 mL/20 g) dissolved in 0.5% of CMC-Na once per day for 32 days. Mice received different stimuli from the 29th day to the 57th day every other day. The control group mice were orally treated with 0.5% of CMC-Na (0.2 mL/20 g) for 32 days. At the 58th day, 60th day, and

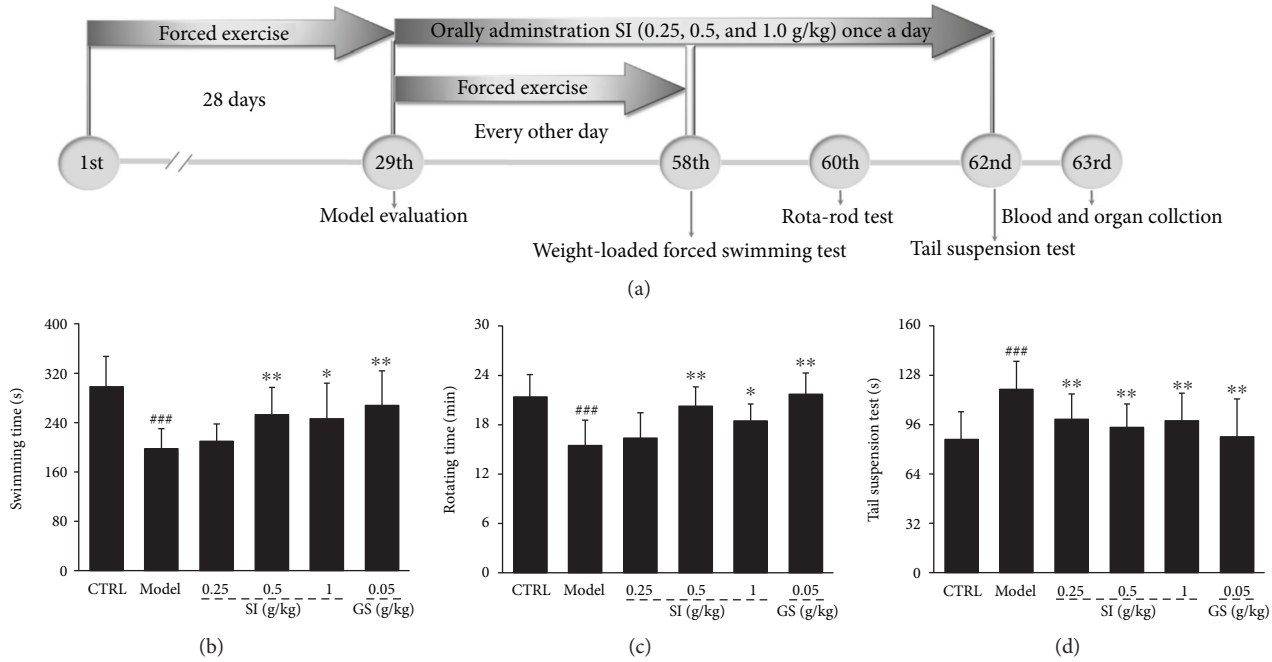

Figure 2: (a) The experimental protocol and drug administration procedure on CFS mice. The effects of SI and GS on (b) weight-loaded forced swimming test, (c) rota-rod test, and (d) tail suspension test in CFS mice. Data were analyzed using a one-way ANOVA followed by Dunn's test and expressed as means ± SD ($n = 10$). $^{###}P < 0.001$ versus the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the model group. SI: *S. imbricatus*; GS: *Ginsenoside*.

62nd day, FST, RRT, and tail suspension test (TST) were performed, respectively. At the 63rd day, blood was sampled from the caudal veins. After sacrificing, the liver and muscle were collected from each mouse rapidly. The detailed experimental protocol and drug administration are shown in Figure 2(a).

2.4. Animal Behavioral Tests

2.4.1. Weight-Loaded Forced Swimming Test. Mice were placed individually in a swimming pool (height: 30 cm, diameter: 25 cm) at 25°C ± 1°C, in which the mice could swim freely but were prohibited to touch the bottom. Lead blocks of 10% body weight were loaded on the tail root of each mouse. The mice were assessed to be exhausted when they failed to rise to the surface of water to breathe within a period of 8 s. Their exhaustive swimming time was recorded.

2.4.2. Rota-Rod Test. Mice were, respectively, placed on a rota-rod (ZB-200, Chengdu Taimeng Science Technology Co., Ltd., Chengdu, China) at 15 rpm for training three times. In the formal test, mice were placed on the rota-rod at 15 rpm, respectively, until they were exhausted and dropped from the rod. The total running time was recorded.

2.4.3. Tail Suspension Test. The tail suspension time not only reflects the state emotion of the animal's psychological endurance but also displays the animal's physical endurance. Mice were, respectively, suspended 1 m above the floor using an adhesive tape, positioned about 1 cm from the tip of the tail. The total duration of immobility, which can be defined as motionless hanging without any struggling movements, was recorded during the last 4 min of the whole 6 min test period.

2.5. Sample Preparations and Analysis of Biochemical Parameters. Serum was isolated by centrifugation at 4000 rpm for 15 min at room temperature. One part of the liver and muscle was homogenized to 10% solution with normal saline at 4°C. The levels of blood urea nitrogen (BUN) (C013-2), lactic acid (LD) (A019-2), adenosine triphosphate (ATP) (A003-1), glycogen (A043) (Nanjing Jiancheng Biological Company, Nanjing, China), reactive oxygen species (ROS) (CK-E91516), superoxide dismutase (SOD) (CK-E20348), malondialdehyde (MDA) (CK-E20347), and glutathione peroxidase (GSH-Px) (CK-E92669) (Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) in serum, liver, and muscle were detected by ELISA method according to the manufacturer's instructions.

2.6. Western Blot Analysis. One part of liver tissues obtained from CFS mice was extracted with lysis buffer (RIPA with protease and phosphatase inhibitor) for 30 min on ice and then centrifuged at 10000 rpm for 10 min at 4°C to remove the precipitate. The concentration of total protein was determined by a bicinchoninic acid (BCA) protein assay kit (Merck Millipore, USA). An equal amount of denatured protein samples (40 μg) was loaded per well for 12% SDS-polyacrylamide gel electrophoresis (Bio-Rad, USA) and transferred to PVDF membranes. The membranes were blocked using 5% bovine serum albumin (BSA) at room temperature for 2 h. The blots were incubated with the appropriate concentration of specific antibody overnight at

Table 1: Main components of SI.

Compounds	Contents (%)	Compounds	Contents (%)	Compounds	Contents (%)
Total sugar	35.22	Mannitol	9.40	Polyphenols	0.41
Reducing sugar	3.41	Crude fat	3.02	Carotenoids ($\times 10^{-3}$)	0.21
Triterpenoids ($\times 10^{-2}$)	4.12	Total ash	9.30	Sterols	3.16
Flavonoids ($\times 10^{-2}$)	2.05	Total protein	18.33		

SI: *Sarcodon imbricatus*

Table 2: The composition of vitamins in SI.

Compounds	Contents (mg/kg)	Compounds	Contents (mg/kg)	Compounds	Contents (mg/kg)
Vitamin A	0.12	Vitamin B_3 ($\times 10^3$)	3.16	Vitamin D_2 ($\times 10^2$)	1.10
Vitamin B_1	ND①	Vitamin B_6	ND②	Vitamin D_3	ND③
Vitamin B_2	28.68	Vitamin C ($\times 10^3$)	1.06	Vitamin E	ND④

SI: *Sarcodon imbricatus*; ND①: not detected (the detection limit was 0.54 mg/kg); ND②: not detected (the detection limit was 2.92 mg/kg); ND③: not detected (the detection limit was 0.08 mg/kg); ND④: not detected (the detection limit was 1.32 mg/kg).

4°C. Primary antibodies Nrf2 (ab137550), SOD1 (ab16831), SOD2 (ab131443), heme oxygenase-1 (HO-1) (ab25901), catalase (CAT) (ab7970) (Abcam, Cambridge, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ABS16) (Merck Millipore, Darmstadt, Germany) were diluted at 1 : 2000. The bonds were washed with TBS buffer plus 0.1% Tween-20 for five times and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (sc-3836) (Santa Cruz Biotechnology, Santa Cruz, USA) for 4 h at 4°C. The bands were established and fixed by an ECL Advance kit. The quantification of protein expression was determined using the ImageJ 1.46 software (Rasband, Bethesda, MD, USA).

2.7. Statistical Analysis. The data were analyzed using SPSS 16.0 software (IBM Corporation, USA). The results were presented as means ± standard deviation (SD), and the statistical significance of each difference was determined using a one-way analysis of variance (ANOVA) followed by Dunn's test. In the analysis results, $P < 0.05$ was considered to indicate significant differences.

3. Results

3.1. Composition of SI. For general nutrition, the SI fruiting body contains 35.22% of total sugar, 3.41% of reducing sugar, 0.04% of triterpenoids, 0.02% of flavonoids, 9.40% of mannitol, 3.02% of crude fat, 9.30% of total ash, 18.33% of total protein, 0.41% of total polyphenols, 3.16% of total sterols (Table 1), and 0.44% of vitamins (Table 2). Among 35 types of fatty acid detected, 24 types of them were found in the SI fruiting body (Table 3). Automatic amino acid analysis showed that the SI fruiting body consists 16 kinds of amino acids, including essential amino acids. Among them, 1.11% of aspartic acid (Asp), 3.04% of glutamic acid (Glu), and 1.10% of alanine (Ala) were noted, which shows higher levels than do other amino acids (Table 4). For mineral elements, the SI fruiting body contains K (3957.0 mg/100 g), Fe (78.4 mg/100 g), Ca (68.0 mg/100 g), Zn (10.9 mg/100 g), Na (14.5 mg/100 g), Mn (3.3 mg/100 g), and Cu (3.3 mg/100 g) (Table 5).

3.2. SI-Enhancing Exercise Capacities of Normal Mice and CFS Mice. The animal behavioral experiment intuitively reflects the antifatigue properties of test agents [38]. SI showed similar enhancing effects on exercise endurance of normal mice and CFS mice as that of GS. In normal mice, SI improved the exhaustion swimming time > 20.9% compared with control mice in FST ($P < 0.05$; Figure 1(b)). 0.5 g/kg and 1.0 g/kg of SI prolonged the excise time up to 18.7% and 19.3% compared with the control mice in RRT ($P < 0.05$; Figure 1(c)). In CFS mice, compared with the control group, the reduced exercise time was observed in FST ($P < 0.001$; Figure 2(b)) and RRT ($P < 0.001$; Figure 2(c)), and the increased immobility duration was found in TST ($P < 0.001$; Figure 2(d)). After 32-day oral administration of SI, compared with CFS mice, SI resulted in >24.3% and > 19.1% enhancements on exercise time in FST ($P < 0.05$; Figure 2(b)) and RRT ($P < 0.05$; Figure 2(c)), and a >21.2% reduction on immobility duration time in TST ($P < 0.01$; Figure 2(d)).

3.3. Effects of SI on the Levels of BUN, LD, ATP, and Glycogen in Serum and Organs of Acute Excise-Treated Mice and CFS Mice. In acute excise-treated mice, the 18-day SI treatment resulted in 21.2% and 18.6% reduction on the BUN and LD in serum compared to control mice ($P < 0.05$; Figure 3(a)). SI at a dose of 0.5 g/kg enhanced the ATP levels by 13.6% and 8.0% in the liver and muscle of acute excise-treated mice ($P < 0.05$; Figure 3(b)). Furthermore, compared with the control group, SI only significantly enhanced the glycogen levels in the liver ($P < 0.05$; Figure 3(c)), but not in the muscle ($P > 0.05$; Figure 3(c)) in acute excise-treated mice.

In CFS mice, compared with the control group, the enhanced levels of BUN ($P < 0.05$; Figure 3(d)) and LD ($P < 0.05$; Figure 3(d)) in serum and the decreased levels of hepatic glycogen ($P < 0.05$; Figure 3(f)) and ATP in the liver ($P < 0.05$; Figure 3(e)) and muscle ($P < 0.01$; Figure 3(e))

Table 3: The composition of fatty acids.

Compounds	Contents (‰)	Compounds	Contents (‰)	Compounds	Contents (‰)
Octoic acid (C8:0)	ND①	Heptadecenoic acid (C17:1) ($\times10^{-2}$)	3.02	Docosanoic acid (C22:0) ($\times10^{-2}$)	8.30
Capric acid (C10:0)	ND②	Stearic acid (C18:0) ($\times10^{-1}$)	4.70	Eicosatrienoic acid (C20:3n6)	ND⑦
Undecanoic acid (C11:0) ($\times10^{-2}$)	0.51	Trans-oleic acid (C18:1n9t) ($\times10^{-2}$)	3.61	Erucic acid (C22:1n9)	ND⑧
Lauric acid (C12:0) ($\times10^{-2}$)	1.72	Oleic acid (C18:1n9c)	4.89	Eicosatrienoic acid (C20:3n3) ($\times10^{-2}$)	1.92
Tridecanoic acid (C13:0) ($\times10^{-2}$)	0.40	*trans*-Linoleic acid (C18:2n6t) ($\times10^{-2}$)	0.62	Arachidonic acid (C20:4n6)	ND⑨
Myristic acid (C14:0)	0.07	Linoleic acid (C18:2n6c)	15.26	Tricosanoic acid (C23:0) ($\times10^{-2}$)	1.32
Myristoleic acid (C14:1)	ND③	Arachidic acid (C20:0) ($\times10^{-2}$)	4.43	Docosadienoic acid (C22:2n6) ($\times10^{-2}$)	2.20
Pentadecanoic acid (C15:0)	0.72	γ-linolenic acid (C18:3n6)	ND⑤	Tetracosanoic acid (C24:0) ($\times10^{-2}$)	5.12
Pentadecenoic acid (C15:1)	ND④	Eicosanoic acid (C20:1n9)	ND⑥	Eicosapentaenoic acid (C20:5n3)	ND⑩
Hexadecanoic acid (C16:0)	1.97	α-Linolenic acid (C18:3n3) ($\times10^{-2}$)	5.34	Nervonic acid (C24:1n9) ($\times10^{-1}$)	0.05
Palmitoleic acid (C16:1)	0.07	Heneicosanoic acid (C21:0) ($\times10^{-2}$)	0.41	Docosahexaenoic acid (C22:6n3)	ND⑪
Heptadecanoic acid (C17:0)	0.07	Eicosadienoic acid (C20:2)	0.04		

ND①: not detected (the detection limit was 4.20 mg/kg); ND②: not detected (the detection limit was 3.83 mg/kg); ND③: not detected (the detection limit was 2.82 mg/kg); ND④: not detected (the detection limit was 2.64 mg/kg); ND⑤: not detected (the detection limit was 2.51 mg/kg); ND⑥: not detected (the detection limit was 4.77 mg/kg); ND⑦: not detected (the detection limit was 2.68 mg/kg); ND⑧: not detected (the detection limit was 2.42 mg/kg); ND⑨: not detected (the detection limit was 4.66 mg/kg); ND⑩: not detected (the detection limit was 3.31 mg/kg); ND⑪: not detected (the detection limit was 4.33 mg/kg).

Table 4: The composition of amino acids in SI.

Compounds	Contents (%)	Compounds	Contents (%)	Compounds	Contents (%)
Aspartic acid (Asp)	1.11	Valine (Val)	0.62	Lysine (Lys)	0.61
L-Threonine (Thr)	0.64	DL-Methionine (Met)	0.40	Histidine (His)	0.10
Serine (Ser)	0.38	Isoleucine (Iso)	0.56	Arginine (Arg)	0.60
Glutamic acid (Glu)	3.04	Leucine (Leu)	0.88	Proline (Pro)	0.54
Glycine (Gly)	0.45	Tyrosine (Tyr)	0.34		
Alanine (Ala)	1.10	Phenylalanine (Phe)	0.54		

SI: *Sarcodon imbricatus*

Table 5: The composition of minerals (including heavy metals) in SI.

Compounds	Contents (mg/100 g)	Compounds	Contents (mg/kg)
Kalium (K) ($\times 10^2$)	39.57	Selenium (Se)	ND①
Natrium (Na)	14.52	Lead (Pb)	ND②
Calcium (Ca)	68.04	Mercury (Hg)	ND③
Cuprum (Cu)	3.31	Arsenic (As)	ND④
Ferrum (Fe)	78.42	Cadmium (Cd)	ND⑤
Zinc (Zn)	10.92	Chromium (Cr)	ND⑥
Manganese (Mn)	3.30		

SI: *Sarcodon imbricatus*; ND①: not detected (the detection limit was 2 mg/kg); ND②: not detected (the detection limit was 1 mg/kg); ND③: not detected (the detection limit was 0.1 mg/kg); ND④: not detected (the detection limit was 0.5 mg/kg); ND⑤: not detected (the detection limit was 0.5 mg/kg); ND⑥: not detected (the detection limit was 1 mg/kg).

were noted. SI displayed similar effects as that of GS except for those on muscle glycogen levels, which were only enhanced after GS administration ($P < 0.05$; Figure 3(f)). Compared with nontreated CFS mice, SI treatment resulted in 23.0% and 15.4% decrement on serum levels of BUN ($P < 0.01$; Figure 3(d)) and LD ($P < 0.05$; Figure 3(d)), respectively. Moreover, SI increased the ATP levels by 12.4% and 19.0% in the liver ($P < 0.05$; Figure 3(e)) and muscle ($P < 0.05$; Figure 3(e)) of CFS mice. SI only showed beneficial effects on hepatic glycogen levels, which were enhanced by 17.4% in SI-treated CFS mice ($P < 0.05$; Figure 3(f)).

3.4. Effects of SI on the Levels of Oxidative Stress Factors in Acute Excise-Treated Mice and CFS Mice. Excessive ROS destroys the balance between oxidation and antioxidation, resulting in the occurrence of oxidative stress [39]. MDA, a polyunsaturated fatty acid peroxide degradation product, indirectly reflects the degree of cellular attack and damage by free radicals. SOD is against the damage from oxygen free radicals; meanwhile, GSH-Px helps lipid peroxides be catalyzed by reduced glutathione (GSH) [40, 41]. SI showed similar regulatory effects on the levels of oxidative stress-related factors in acute excise-treated mice (Table 6) and CFS mice (Table 7) as that of GS. Compared with the control group, 18-day SI oral administration strongly reduced the levels of ROS ($P < 0.05$) and MDA ($P < 0.05$) and enhanced the levels of SOD ($P < 0.05$) and GSH-Px ($P < 0.05$) in serum and liver of 30 min swimming-treated mice (Table 6). In muscle, SI only reduced the ROS levels and enhanced the SOD concentration ($P < 0.05$), but failed to significantly influence the levels of MDA and GSH-Px ($P > 0.05$; Table 6).

CFS model establishment procedures resulted in levels of MDA and ROS increasing strongly and SOD and GSH-Px reducing in serum, liver, and muscle ($P < 0.05$; Table 7). After the 32-day gavage treatment, SI at doses of 0.5 and 1.0 g/kg decreased the serum, liver, and muscle levels of MDA and ROS back to the normal horizon ($P < 0.05$). Furthermore, SI resulted in 19.4–48.0% enhancement on SOD and 13.2–53.4% enhancement on GSH-Px levels in serum, liver, and muscle compared with the model group ($P < 0.05$).

3.5. The Regulatory Effects of SI on Nrf2 Signaling in the Liver of CFS Mice. In order to further reveal the potential mechanisms of antifatigue activities of SI in CFS mice, the expression levels of Nrf2, SOD1, SOD2, HO-1, and CAT in the liver were detected via Western blot. Nrf2 combined with ARE regions of antioxidant enzyme genes and activated these genes for transcription. The levels of Nrf2, SOD1, SOD2, HO-1, and CAT were remarkably downregulated in CFS mice compared with the control group ($P < 0.05$; Figure 4). Compared with the CFS model group, 32-day SI treatment strongly upregulated the expression of Nrf2 and the content of four antioxidant enzymes in the liver ($P < 0.01$; Figure 4). All results indicated that the Nrf2/HO-1 signal pathway can be activated by SI in the tested concentration range.

4. Discussion

Fatigue is a common physiological phenomenon and also accompanies with various diseases [42]. In the present study, a comprehensive and systematic experiment was performed to investigate the antifatigue activities of SI and the underlying mechanisms related to oxidative stress in acute excise-treated mice and CFS mice. SI is rich in polysaccharides, proteins, amino acids, and potential antioxidants such as polyphenols, sterols, and vitamins. Both polysaccharides and amino acids have been reported to improve the exercise capability, especially the amino acids, which can markedly retard the catabolism of protein in the muscle during exercise [43–45]. Gly, Pro, and Arg presented from the porcine placenta extract improve glycogen content and CAT and SOD activities and lower the blood levels of LD and alanine aminotransferase [46]. Polyphenolic compounds

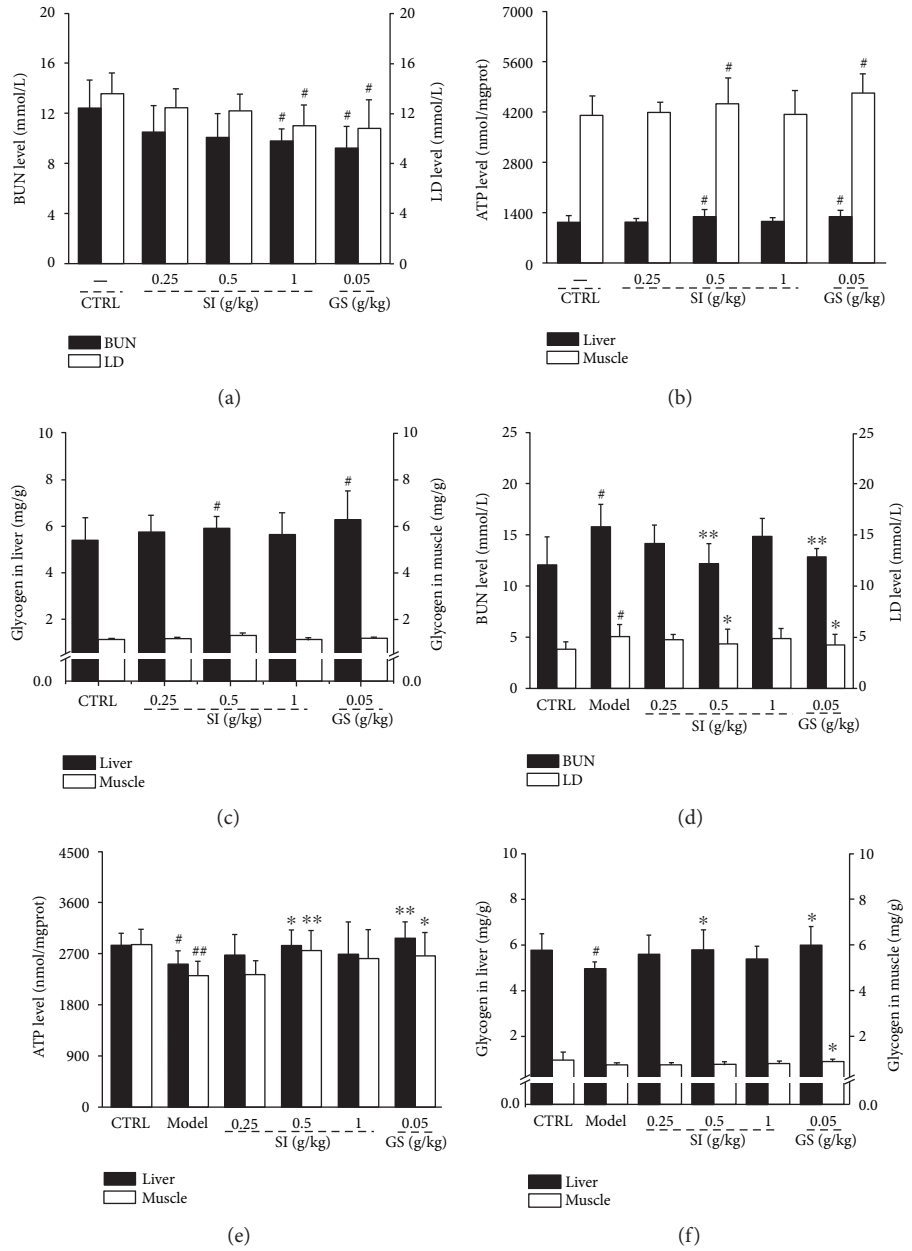

Figure 3: Mice were treated with SI (0.25, 0.5, and 1.0 g/kg) and GS (0.05 g/kg) for 18 days. After a 30 min swimming, the levels of (a) BUN and LD in serum, (b) ATP, and (c) glycogen in the liver and muscle were detected via ELISA kit. CFS mice were treated with SI (0.25, 0.5, and 1.0 g/kg) and GS (0.05 g/kg) for 32 days. The levels of (d) BUN and LD in serum, (e) ATP, and (f) glycogen in the liver and muscle were detected via ELISA kit. Data were analyzed using a one-way ANOVA followed by Dunn's test and expressed as means ± SD ($n = 10$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the model group. SI: *S. imbricatus*; GS: *Ginsenoside*.

are involved in neutralizing free radicals, modulating the enzymatic activity, and decomposing peroxide mechanisms to produce antioxidant activities [47]. Sterols are reported to be of great benefit to human health due to their antioxidant activities [48]. Vitamin C enhances the antioxidant capacity of the body mainly by scavenging hydroxyl radicals and cutting off the chain reaction. Meanwhile, vitamin C can work synergistically with vitamin E to exert an antioxidant effect by converting the oxidized form of α-tocopherol back to α-tocopherol [49]. The antifatigue activity of SI may be related to its rich potential antioxidative nutrient elements. Furthermore, it was confirmed that the heavy metals detected in SI were all within normal limits. In our preliminary experiments, the acute toxicity test showed that SI failed to influence the body weights, water and diet intakes, and organ functions of mice during a 7-day observation. All data reflect the safety of SI in animal experiments.

FST and RRT are used widely to assess the physical strength and the degree of fatigue in animals [50]. The immobility time of the tail suspension test, to a certain extent, can reflect the animal's muscle strength and emotions [37]. The relief from fatigue is the most important factor to improve

Table 6: The effects of SI on oxidative stress-related factors in serum, liver, and muscle of acute excise-treated mice.

		CTRL	SI (g/kg)			GS (g/kg)
			0.25	0.5	1	0.05
Serum	MDA (nmol/mL)	8.4 ± 2.0	8.0 ± 1.4	7.9 ± 2.2	6.9 ± 1.3$^{\#}$	6.7 ± 1.7$^{\#}$
	ROS (U/mL)	461.0 ± 20.0	442.6 ± 21.9	401.6 ± 17.8$^{\#\#}$	415.4 ± 28.6$^{\#}$	381.9 ± 11.9$^{\#\#}$
	SOD (U/mL)	74.5 ± 12.0	83.6 ± 5.7	85.8 ± 11.0$^{\#}$	86.3 ± 13.0$^{\#}$	85.1 ± 8.8$^{\#}$
	GSH-Px (U/mL)	418.4 ± 90.8	518.2 ± 170.1$^{\#}$	539.2 ± 156.8$^{\#}$	559.2 ± 38.7$^{\#\#}$	568.8 ± 97.4$^{\#\#}$
Liver	MDA (nmol/mgprot)	5.2 ± 0.7	4.4 ± 0.3$^{\#}$	4.8 ± 0.3	3.7 ± 0.4$^{\#\#}$	4.6 ± 0.5$^{\#}$
	ROS (FI/gprot)	22103.5 ± 7400.4	25042.9 ± 9027.8	17296.6 ± 1974.0$^{\#}$	14914.6 ± 4638.8$^{\#\#}$	18780.5 ± 5160.0$^{\#}$
	SOD (U/mgprot)	234.9 ± 48.8	273.3 ± 49.8	340.7 ± 91.8$^{\#\#}$	256.3 ± 49.3	343.1 ± 49.4$^{\#\#}$
	GSH-Px (μmol/gprot)	780.0 ± 195.0	818.3 ± 84.9	856.3 ± 19.4$^{\#}$	790.8 ± 85.1	886.2 ± 140.6$^{\#}$
Muscle	MDA (nmol/mgprot)	23.7 ± 4.6	24.2 ± 2.2	22.7 ± 2.2	21.6 ± 2.7	22.3 ± 2.1
	ROS (FI/gprot)	61278.3 ± 8914.2	59534.4 ± 4636.2	53426.1 ± 333.9$^{\#}$	51847.6 ± 5748.2$^{\#}$	47689.1 ± 6061.2$^{\#\#}$
	SOD (U/mgprot)	115.5 ± 23.4	122.4 ± 30.3	187.3 ± 24.6$^{\#\#}$	179.6 ± 19.4$^{\#}$	204.5 ± 29.7$^{\#\#}$
	GSH-Px (μmol/gprot)	653.9 ± 75.4	663.3 ± 43.1	702.4 ± 32.8	660.9 ± 46.8	756.2 ± 65.9$^{\#}$

Treatment with SI (0.25 g/kg, 0.5 g/kg, and 1.0 g/kg) and GS (0.05 g/kg) for 18 days; after a 30 min swimming, the levels of MDA and ROS and the activities of SOD and GSH-Px in serum, liver, and muscle were detected. The data were analyzed using a one-way ANOVA followed by Dunn's test and expressed as m eans ± SD (n = 10/group). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group. SI: *S. imbricatus*; GS: *Ginsenoside.*

Table 7: The effects of SI on oxidative stress-related factors in serum, liver, and muscle of CFS mice.

		CTRL	Model	SI (g/kg)			GS (g/kg)
				0.25	0.5	1	0.05
Serum	MDA (nmol/mL)	22.4 ± 2.6	28.2 ± 1.5$^{\#}$	21.8 ± 3.0	20.2 ± 1.4**	22.1 ± 0.9	19.9 ± 1.2**
	ROS (U/mL)	203.3 ± 19.4	263.1 ± 9.0$^{\#}$	237.3 ± 15.1	232.5 ± 21.2*	221.7 ± 16.8*	224.4 ± 23.5*
	SOD (U/mL)	158.2 ± 11.2	129.7 ± 7.6$^{\#}$	137.6 ± 8.7	139.7 ± 11.9	154.8 ± 23.6*	142.2 ± 21.1*
	GSH-Px (U/mL)	230.4 ± 29.8	192.0 ± 15.5$^{\#}$	209.2 ± 27.0	203.5 ± 19.8	218.7 ± 21.2*	220.8 ± 10.6*
Liver	MDA (nmol/mgprot)	4.5 ± 0.5	5.6 ± 0.7$^{\#}$	5.3 ± 1.3	5.5 ± 0.7	4.5 ± 0.9*	4.5 ± 0.6*
	ROS (U/mgprot)	384.8 ± 41.6	449.2 ± 9.4$^{\#}$	420.3 ± 29.6	372.2 ± 39.8*	379.1 ± 29.7*	358.7 ± 20.2**
	SOD (U/mgprot)	136.1 ± 12.2	105.4 ± 8.2$^{\#\#}$	125.3 ± 25.1	128.5 ± 24.5*	120.4 ± 30.2	136.9 ± 23.9*
	GSH-Px (U/mgprot)	172.7 ± 13.6	110.5 ± 13.2$^{\#\#}$	163.9 ± 23.5*	169.5 ± 24.5*	157.6 ± 16.8	168.2 ± 27.4*
Muscle	MDA (nmol/mgprot)	6.1 ± 1.2	7.5 ± 1.1$^{\#}$	6.7 ± 1.2	6.3 ± 1.4	6.1 ± 0.7	6.0 ± 0.8*
	ROS (U/mgprot)	336.5 ± 39.8	402.2 ± 26.8$^{\#\#}$	423.2 ± 34.2	358.0 ± 37.9**	369.7 ± 38.0*	349.6 ± 36.2**
	SOD (U/mgprot)	289.6 ± 48.6	210.2 ± 19.8$^{\#}$	277.7 ± 35.4	297.2 ± 66.7*	311.1 ± 29.7**	287.9 ± 42.9*
	GSH-Px (U/mgprot)	167.7 ± 61.7	150.6 ± 30.7$^{\#}$	160.0 ± 47.1	170.5 ± 33.2*	165.2 ± 48.1	174.0 ± 47.3*

Treatment with SI (0.25 g/kg, 0.5 g/kg, and 1.0 g/kg) and GS (0.05 g/kg) for 32 days in CFS mice; the levels of MDA and ROS and the activities of SOD and GSH-Px in serum, liver, and muscle were detected. The data were analyzed using a one-way ANOVA followed by Dunn's test and expressed as means ± SD (n = 10/group). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the model group. SI: *S. imbricatus*; GS *Ginsenoside.*

the exercise endurance. SI significantly prolonged exhaustive swimming time and rata-roding time in normal mice and CFS mice and reduced the immobility time in TST. In order to further confirm the antifatigue activity of SI, biochemical indexes in serum, liver, and/or muscle were also analyzed. SI reduced serum levels of BUN and LD, increased the ATP content in the liver and muscle, and enhanced the concentration of hepatic glycogen. The content of BUN reflects the protein catabolism and the body tolerance to exercise, which serves as a biochemical index to evaluate the degree of fatigue [51]. In the course of vigorous movement, aerobic energy supply changes into anaerobic glycolysis in the muscle, and muscle glycogen is rapidly consumed, which produces a large amount of LD. The buildup of LD in the muscle and blood can cause a decrease in muscle capacity, further leading to exercise-induced fatigue [52]. These alterations were significantly attenuated by SI treatment. LD accumulation can alter the acidic environment in muscle and blood, resulting in overconsumption of phosphate, which hinders ATP synthesis [53]. ATP is the most direct and fastest source of energy. As the energy supplier during ATP production, mitochondria can be damaged by excessive deposition of oxygen free radicals, which will further delay the ATP synthesis [54]. Glycogen, reflecting the body's ability to resist exercise fatigue, can be consumed rapidly under strenuous exercise continuously to provide energy for muscle fiber contraction [55]. All present data confirmed the antifatigue properties of SI.

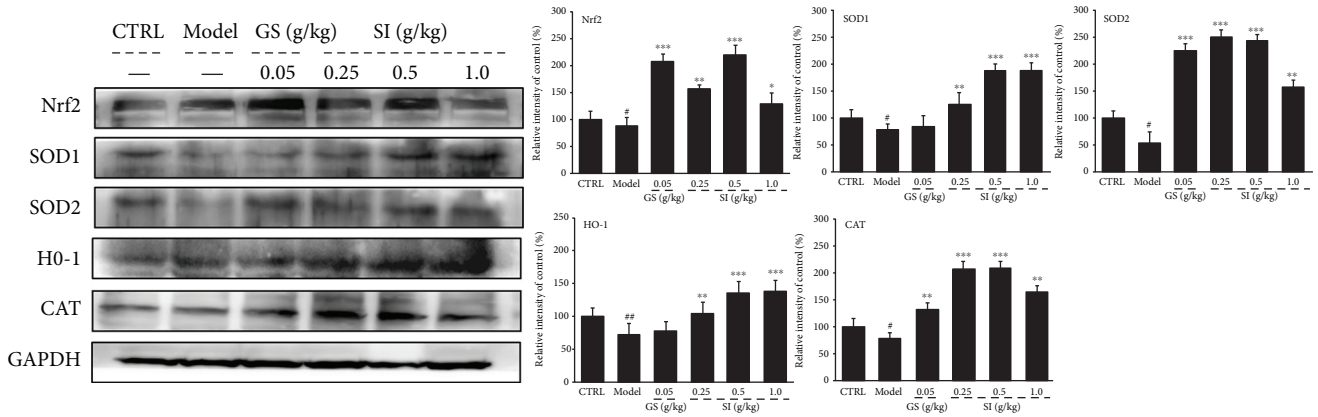

Figure 4: CFS mice were treated with SI (0.25, 0.5, and 1.0 g/kg) and GS (0.05 g/kg) for 32 days. The expression levels of Nrf2, SOD1, SOD2, HO-1, and CAT in the liver were detected via Western blot. Quantification data were normalized by related GAPDH and expressed as me ans ± SD ($n = 10$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the model group. SI: *S. imbricatus*; GS: *Ginsenoside*.

As reported, acute strenuous exercise and high consumption of energy could accelerate the occurrence of free radicals such as ROS and reactive nitrogen species (RNS) and induce severe oxidative stress bursting [56]. Patients with chronic fatigue syndromes have higher levels of free radicals [57]. Oxidative stress results from the imbalance between oxidant attack, which is due to free radical production, and antioxidant defense, which limits exercise capacity and mitochondrial functions [58]. Overproduced free radicals will attack the fatty acids on the cell membrane and eventually metabolize into MDA, which directly reflect the degree of lipid peroxidation [59]. The accumulation of ROS disturbs the balance of body metabolism and further leads to fatigue symptoms [60]. As important antioxidant enzymes, SOD and GSH-Px are natural scavengers of ROS in bodies [61]. Encouragingly, SI not only regulated the levels of these prooxidant and antioxidant factors in serum, liver, and muscle of acute excise-treated mice and CFS mice but also modulated the expression levels of Nrf2 signaling-related proteins in the liver of CFS mice. Nrf2 is the key regulator of cellular oxidation in the transcriptional level, which directly controls the concentration of SOD, HO-1, and CAT [62]. HO-1 helps to convert heme into biliverdin, which, in turn, is converted into bilirubin, a potent antioxidant [63]. When ROS accumulates excessively, Nrf2 is activated and accumulated in the cytoplasm [64]. Nrf2-deficient mice exhibit extreme vulnerability to oxidative stress in hepatic and gastric tissues [65]. Via improving the activity of Nrf2 in bodies, the oxidative stress damage can be effectively prevented [66]. It has demonstrated in numerous *in vivo* studies that activation of Nrf-2 can counteract oxidative stress and thus reduce fatigue [67]. Generally, the basic intracellular expressions of Nrf-2 are not sufficient to completely suppress oxidative stress. At this point, antioxidant compounds exhibit extraordinary potential to increase the inducible expression of Nrf-2, thereby contributing to a production of large quantities of antioxidants. Altogether, SI shows antifatigue activities in acute exercise-treated mice and CFS mice via regulating Nrf2 signaling-mediated oxidative stress.

In the present study, we only analyzed the antifatigue activities of the SI fruiting body, but not its mycelium obtained by submerged fermentation. The advantages of submerged fermentation have been reported widely such as the shorter growth cycle, stability chemical composition, and controllable biosynthesis processes. Encouragingly, the optimum submerged fermentation conditions for SI mycelium culture have been obtained by the previous study [21]. In our subsequent experiments, the differences in antifatigue activities between the SI fruiting body and SI mycelia will be investigated.

In conclusion, we first demonstrated the antifatigue effects of SI in acute excise-treated mice and CSF mice. SI increased exercise endurance in FST and RRT and reduced the immobility time in TST of CFS mice. SI reduced the levels of BUN and LD, enhanced ATP and glycogen storage, and promoted antioxidant ability by suppressing MDA and ROS levels and increasing SOD and GSH-Px levels. Further data reveal that SI displays the antifatigue ability via regulating Nrf2-mediated oxidative stress. Taken together, our results suggested that SI might be a good candidate for developing a new antifatigue functional food supplement.

Abbreviations

Ala: Alanine
ANOVA: Analysis of variance
ARE: Antioxidant responsive element
Asp: Aspartic acid
ATP: Adenosine triphosphate
BCA: Bicinchoninic acid
BSA: Bovine serum albumin
BUN: Blood urea nitrogen
CAT: Catalase
CFS: Chronic fatigue syndrome
CTX: Cyclophosphamide
CMC-Na: Sodium carboxymethyl cellulose
ELISA: Enzyme-linked immunosorbent assay
FST: Forced swimming test

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
Glu: Glutamic acid
GS: Ginsenoside
GSH: Glutathione
GSH-Px: Glutathione peroxidase
HO-1: Heme oxygenase-1
RRT: Rota-rod test
LD: Lactic acid
MDA: Malondialdehyde
Nrf2: Nuclear factor-erythroid 2-related factor 2
ROS: Reactive oxygen species
SD: Standard deviation
SOD: Superoxide dismutase
TST: Tail suspension test.

Conflicts of Interest

The authors have declared that there is no conflict of interest.

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Research Article

Protective Role of Antioxidant Huskless Barley Extracts on TNF-α-Induced Endothelial Dysfunction in Human Vascular Endothelial Cells

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Oxidative stress and inflammation are considered as two key factors that contribute to the development of atherosclerosis. This study was to investigate the antioxidant capacity of huskless barley and to explore its protective functions through the regulation of the antioxidant defense and inflammatory response in human umbilical vein endothelial cells (HUVEC). The oxygen radical absorbance capacity (ORAC), ferric-reducing antioxidant power (FRAP), and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) scavenging capacity of water and alkali extracts of the polysaccharides from nine huskless barley varieties were investigated *in vitro*. The antioxidant properties of the alkaline extracts were more pronounced than those of the water extracts. The results from the cell model showed that pretreatment of HUVEC with the water or alkaline extracts of the polysaccharides from the huskless barley cultivars QHH and NLGL decreased the levels of reactive oxygen species (ROS), monocyte chemotactic protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) but increased the level of superoxide dismutase (SOD) and maintained cell viability. Huskless barley polysaccharide extracts exhibited the vasodilatory effect of inhibiting angiotensin-converting enzyme (ACE) production. These discoveries revealed the potent protective functions of barley in oxidative damage and a potential role for barley in preventing chronic inflammation in cardiovascular diseases.

1. Introduction

The vascular endothelium is the epithelial tissue that lines the blood vessels. This tissue serves as a regulator of the vascular wall function. Changes in its structure and function form the common pathological basis of cardiovascular diseases. Endothelial dysfunction is considered as an important step in the development of atherosclerosis (AS), which is associated with an inflammatory response and the increased interaction of platelets and monocytes with the vessel wall [1, 2]. A number of inflammatory mediators are released by the endothelial cells in response to localized injury or trauma. The primary inflammatory mediators are categorized into four main groups: lipids, plasma enzymes, cytokines, and chemokines [3]. Among these mediators, tumor necrosis factor-alpha (TNF-α), a prototypical proinflammatory cytokine that is found in atherosclerotic lesions, can exert direct effects on vascular endothelial cells to induce the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) in leukocytes and other inflammatory cells. Accordingly, TNF-α facilitates the progression of atherosclerotic cardiovascular disease [4]. Considerable

evidence has shown that antioxidative, anti-inflammatory, and antihypertensive effects may be highly correlated with the inhibition of AS [5, 6].

Reactive oxygen species (ROS) may reduce the availability of endothelial nitric oxide (NO). Additionally, the reaction between ROS and NO forms a more reactive peroxide, nitroso peroxide, which further augments endothelial dysfunction [7]. Moreover, increased levels of vascular superoxide inhibit the vascular extracellular superoxide dismutase (ecSOD) activity, which leads to the production of more superoxide, inhibits the function of NO, and causes endothelial damage [8]. Therefore, antioxidants can reduce oxidative stress by directly scavenging ROS, increasing the SOD activity, and regulating NO to improve endothelial function. Together, these processes have the effects of preventing and treating hypertension.

Grains, cereals, vegetables, and fruits are good sources of antioxidants including polyphenols, flavonoids, and polysaccharides [9, 10]. As a major cereal crop that is used extensively as an industrial commodity and in fermentation products, barley is a functional cereal that is rich in dietary fiber. Dietary fiber (mainly non-starch polysaccharides) plays an important role in the health benefits associated with whole grain consumption, which has increasingly attracted the interest of scientists [11]. The physiological and functional properties of dietary fiber depend on the food source, extraction method, chemical composition, structure, and particle size [12, 13]. Knutsen and Holtekjolen reported that the water extracts of the polysaccharides consisted mostly of beta-glucans whereas the alkaline extracts consisted of arabinoxylans (AX) [14]. The carbohydrate composition in different barley varieties differs considerably; thus, the selection of the variety is also important.

Although it is widely recognized that dietary fiber possesses considerable antioxidant capacity, the antioxidative capacity of barley remains unknown. In the present study, we validated the antioxidant capacity of water and alkali extracts of the polysaccharides from nine huskless barley varieties *in vitro* by measuring the oxygen radical absorbance capacity (ORAC), ferric-reducing antioxidant power (FRAP), and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) scavenging capacity. Moreover, the protective role of barley in cells was studied. We hypothesized that huskless barley extracts may slow the process of atheroma formation by inhibiting TNF-α-induced cell adhesion through the suppression of superoxide anion production in human umbilical vein endothelial cells (HUVEC). The purpose of this study was to explore the inhibitory effects of the polysaccharide extracts of barley on the vascular inflammatory process in TNF-α-induced HUVEC and to elucidate the mechanism of the antioxidative action. We conducted research on the protection of vascular endothelium by investigating the expression of several molecules in TNF-α-induced HUVEC that had been pretreated with polysaccharide extracts from two representative huskless barley cultivars. Moreover, the results from two extraction methods (water and alkaline) were compared to help understand and analyze the active component in extracts.

2. Materials and Methods

2.1. Materials and Chemicals. Nine different cultivars of huskless barley were obtained as gifts from Diqing Shangri-La Huskless Barley Development Co. Ltd., Yunnan, China. Their codes and names are listed as follows: (a) Black and white huskless barley 80 days (BW80) from Xianggelila; (b) JiuGe (JG) from Xianggelila; (c) DiQing 3 (DQ3) in the winter season; (d) long and black huskless barley (LB) from Xianggelila; (e) YunQing 2 (YQ2); (f) GuiBaDingGeNa (GBDGN) from Deqin; (g) short and white huskless barley (SW) from Xianggelila; (h) QingHaiHuang (QHH) in the winter season; and (i) NanLongGeNa (NLGN) from Yanmen in Deqin.

A human umbilical vein endothelial cell (HUVEC) line was purchased from Zhongqiao Xinzhou Biological Technology Co. Ltd. (Shanghai, China). Dulbecco's phosphate-buffered saline (PBS), M199 medium, 2,2′-azobis(2-methylpropionamide) dihydrochloride (AAPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), and TNF-α were obtained from Sigma Chemical Co. Ltd. (Nanjing, China). Trolox (6-hydroxy-2,5,7,8-tetramethylchromate-2-carboxylic acid) was obtained from Acros Organics (Shanghai, China). A reactive oxygen species (ROS) assay kit was obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Streptomycin and penicillin were purchased from Life Technologies (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for superoxide dismutase (SOD), monocyte chemotactic protein 1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1), and human angiotensin-converting enzyme (ACE) were purchased from Boster Biotechnology Inc. (Wuhan, China). All chemicals were analytical grade.

2.2. Extractions of Huskless Barley Polysaccharide. The extraction method was based on the Association of Official Analytical Chemists (AOAC) 2009.01 protocol with minor modifications. Briefly, 10 g of a milled defatted sample, in duplicate, was subjected to sequential enzymatic digestion by heat-stable α-amylase (50 μL, 80°C water bath for 1 h), papain (15 mg, 60°C water bath for 1 h), and glucoamylase (400 μL, 60°C water bath for 1 h) to remove the starch and protein. The slurry was incubated twice with distilled water at 80°C for 3 h. After centrifugation at 3000 rpm for 30 min, the supernatant was combined with the water washings of the residue, and this mixture was precipitated in ethanol (100%, 3 volumes) with continuous stirring. The precipitate was washed with acetone, dried under nitrogen, and labeled as hot water-extracted polysaccharide (WE). To prepare the alkaline extracts of the polysaccharides, the residue was washed twice with NaOH (0.5 mol/L, 40 mL) in a 70°C water bath for 2 h. The alkaline washings were centrifuged at 3000 rpm for 30 min. The mixture was neutralized with 0.5 M HCl. The supernatant was dialyzed with three times its volume of distilled water and precipitated in ethanol (100%, 3 volumes) with continuous stirring. The precipitate was washed with acetone, dried under nitrogen, and labeled as alkaline-extracted polysaccharide (AE).

2.3. 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid) Diammonium Salt-Scavenging Capacity Assay. The ABTS free radical-scavenging activity of each extracted sample was determined according to the method described by Arts et al. [15]. Various levels (4, 2, 1, 0.5, 0.25, and 0.125 g/L) of the samples and a standard solution of Trolox in DMSO were prepared and assayed. The absorbance at 734 nm of the resulting oxidized solution was detected. The results were expressed in terms of the Trolox equivalent antioxidant capacity (TEAC), that is, micrograms of Trolox equivalent per gram of dry weight (mg Trolox/g dried weight (DW)). The half maximal inhibitory concentration (IC_{50}, g/L) was also determined.

2.4. Ferric-Reducing Antioxidant Power Assay. The FRAP assay was conducted using a modified version of the method described by Benzie and Strain [16]. Aliquots of various levels (4, 2, 1, 0.5, 0.25, and 0.125 g/L) of the samples were added to the FRAP reagent. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity was calculated from the linear calibration curve and expressed as micromoles of $FeSO_4$ equivalent per gram of dry weight (μmol $FeSO_4$/g DW).

2.5. Oxygen Radical Absorbance Capacity Assay. The radical-scavenging activity was assayed using the improved ORAC method as described previously [17]. The fluorescence decay curves of fluorescein in the presence of WE, AE, and Trolox at various concentrations were generated with MikroWin Microplate Data Reduction 2000 (Mikrotek Laborsysteme GmbH, Overath, Germany). The ORAC value was calculated from the slope of the sample equation dividing the slope of the Trolox curve obtained for the same assay. The final ORAC value is expressed as micromoles of Trolox equivalent per gram of dry weight (μmol Trolox equivalent (TE)/g DW).

2.6. Endothelial Cell Culture and Treatment. Human umbilical vein endothelial cells are considered a model system for studying the oxidative stress and anti-inflammatory and anti-hypertension activities in the vasculature. The cells were cultured according to a laboratory protocol. After stabilization in a reduced serum medium for 4 h prior to the beginning of the actual experiment, the cells were treated with water extracts of QHH (WE-QHH), alkaline extracts of QHH (AE-QHH), water extracts of NLGN (WE-NLGN), and alkaline extracts of NLGN (AE-NLGN) (10 mg/L) for 18 h in a separate set of experiments followed by the stimulation with TNF-α (10 μg/L). Dimethylsulfoxide (DMSO) was used in the control. At the end of the specified incubation period, the supernatants were collected and stored at −80°C until further analysis of MCP-1 and VCAM-1.

2.7. Cell Viability Assay. Human umbilical vein endothelial cells (20000 cells/well) were cultured according to the treatment above after which 20 mL of the stock solution of MTT (5 mg/mL) was added to each well for 4 h. Finally, the incubation medium was removed, and the formazan crystals were dissolved in 150 μL of DMSO. The MTT reduction was measured as the absorbance at 490 nm using a StatFax-2100 Microplate Reader (Awareness Technology Inc., Palm City, FL). The background absorbance of the control wells was subtracted. The analysis was performed in triplicate. Viability (%) = OD (experiment group)/OD (control) × 100%.

2.8. Reactive Oxygen Species Assay. A DCFH-DA detection kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to assess the ROS level in HUVEC. The cells were seeded in 6-well plates, treated with the various samples, and incubated for 24 h. After washing the cells with the reduced-serum medium, 10 μmol/L DCFH-DA was added to each well, and the cells were incubated at 37°C for 20 min. The cells were then washed thoroughly with reduced-serum medium to remove the DCFH-DA that did not enter the cells. The cells were collected in 1 mL of PBS after dissociation, and the fluorescence was immediately recorded using a LB 941 TriStar Microplate Reader (Berthold Technologies, Bad Wildbad, Germany) using 485 nm excitation and 535 nm emission filters. The total fluorescence intensity of the cells in each well was recorded, and ROS generation was measured as the fold increase over the untreated control.

2.9. Enzyme-Linked Immunosorbent Assay. The levels of SOD, MCP-1, VCAM-1, and ACE in the supernatants were quantified using the solid-phase sandwich ELISA kits. The assay procedures were performed according to the instructions in the kit protocol booklets. The samples were used to conduct a protein assay and were suitably diluted in the standard diluent buffer. The absorbance of the resulting yellow color was measured at 450 nm using a StatFax-2100 Microplate Reader (Awareness Technology Inc., Palm City, FL). The reader was controlled via Hyper Terminal Applet ELISA software.

2.10. Statistical Analysis. The data are presented as the mean values ± standard deviation (SD) and were analyzed using the analysis of variance (ANOVA). The data figures were prepared using GraphPad Prism version 5.02 (GraphPad Software Inc., CA, USA). Duncan's multiple range tests ($P < 0.05$) were conducted to analyze their differences. A two-way ANOVA was used to analyze the differences among the alkaline extracts and water extracts of the nine barley varieties. The statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Antioxidant Capacity of Huskless Barley Polysaccharide Extracts In Vitro. Table 1 shows the results for the antioxidant capacity of the water and alkali extractions from the nine huskless barley varieties *in vitro*, including the ABTS, FRAP, and ORAC data. The IC_{50} values for the AEs in the ABTS radical assay ranged from 1.74 to 2.84 g/L with a mean value of 2.12 g/L. The mean values for the TEAC, FRAP, and ORAC assays were 8.73 mg/g DW, 91.95 μmol/g DW, and 381.39 μmol TE/g DW, respectively. The IC_{50} values for the WEs in the ABTS radical assay ranged from 7.41 to 13.43 g/L with a mean value of 10.59 g/L. The mean values for the TEAC, FRAP, and ORAC assays were 1.79 mg/g

Table 1: Summary of the antioxidant capacities of huskless barley *in vitro*.

Sample		ABTS		FRAP (μmol $FeSO_4$/g DW)	ORAC (μmol TE/g DW)
		IC_{50} (g/L)	TEAC (mg/g DW)		
Black and white huskless barley (BW80)	AE	2.01 ± 0.06^{b}	9.18 ± 0.09^{d}	84.1 ± 1.98^{e}	652.45 ± 13.03^{a}
	WE	$9.91 \pm 0.09^{h,i}$	1.86 ± 0.04^{k}	36.7 ± 0.70^{i}	396.57 ± 7.16^{e}
JiuGe (JG)	AE	2.22 ± 0.14^{c}	8.32 ± 0.09^{f}	131.1 ± 3.63^{a}	539.96 ± 3.60^{b}
	WE	9.68 ± 0.16^{h}	$1.91 \pm 0.05^{j,k}$	26.5 ± 1.95^{k}	237.35 ± 9.51^{i}
DiQing 3 (DQ3)	AE	2.84 ± 0.07^{e}	6.50 ± 0.18^{h}	71.6 ± 1.35^{f}	$147.81 \pm 8.50^{l,m}$
	WE	11.41 ± 0.11^{k}	1.62 ± 0.08^{l}	15.8 ± 0.75^{m}	82.71 ± 2.50^{n}
Long and black huskless barley (LB)	AE	$2.10 \pm 0.22^{b,c}$	8.79 ± 0.08^{e}	51.1 ± 1.36^{g}	205.54 ± 9.57^{j}
	WE	11.74 ± 0.09^{l}	1.57 ± 0.04^{l}	41.6 ± 0.93^{h}	127.78 ± 8.00^{m}
YunQing 2 (YQ2)	AE	1.93 ± 0.04^{b}	9.56 ± 0.05^{c}	111.1 ± 4.15^{b}	515.44 ± 4.01^{c}
	WE	7.41 ± 0.08^{f}	2.49 ± 0.04^{i}	41.8 ± 1.41^{h}	348.24 ± 13.56^{f}
GuiBaDingGeNa (GBDGN)	AE	1.74 ± 0.14^{a}	10.61 ± 0.10^{a}	92.1 ± 2.52^{d}	467.55 ± 21.09^{d}
	WE	12.17 ± 0.03^{m}	1.52 ± 0.07^{l}	31.6 ± 2.10^{j}	268.00 ± 5.29^{h}
Short and white huskless barley (SW)	AE	1.74 ± 0.15^{a}	10.61 ± 0.10^{a}	100.0 ± 4.10^{c}	322.41 ± 11.00^{g}
	WE	10.26 ± 0.05^{j}	1.80 ± 0.06^{k}	$34.9 \pm 1.92^{i,j}$	71.49 ± 4.50^{o}
QingHaiHuang (QHH)	AE	2.58 ± 0.07^{d}	7.16 ± 0.03^{g}	81.4 ± 2.62^{e}	$248.89 \pm 11.05^{h,i}$
	WE	13.43 ± 0.14^{n}	1.37 ± 0.05^{m}	41.8 ± 1.70^{h}	153.67 ± 9.58^{l}
NanLongGeNa (NLGN)	AE	$1.90 \pm 0.03^{a,b}$	9.72 ± 0.075^{b}	$97.2 \pm 3.46^{c,d}$	$332.49 \pm 4.08^{f,g}$
	WE	9.30 ± 0.08^{g}	1.98 ± 0.055^{j}	18.6 ± 1.75^{l}	172.56 ± 7.17^{k}

AE: alkaline extract; WE: water extract. Different letters in the same column indicate significant differences ($P < 0.05$, as indicated by Fisher's least significant difference (LSD) test).

DW, 32.61 μmol/g DW, and 251.06 μmol TE/g DW, respectively. There were significant differences between the alkaline and water extracts in the ABTS, FRAP, and ORAC results ($P < 0.001$). Each alkaline extract possessed a much higher antioxidant capacity than the water extract for each huskless barley variety (Figure 1).

The various huskless barley varieties exhibited different antioxidant capacities using the different models. The extracts AE-GBGN and AE-SW had the strongest ABTS-scavenging capacity with the highest TEAC values (both 10.61 mg/g DW) and the lowest IC_{50} values (both 1.74 g/L). The extracts AE-NLGN, AE-YQ2, and AE-BW80 also had high ABTS-scavenging capacities with values over 9.00 mg/g DW TEAC. The extract WE-QHH showed the lowest ABTS-scavenging capacity, but AE-QHH was not the lowest of the nine AEs. The extract AE-JG had the highest FRAP value (131.1 μmol/g DW), followed by AE-YQ2 (111.1 μmol/g DW), AE-SW (100.0 μmol/g DW), AE-NLGN (97.2 μmol/g DW), and AE-GBGN (92.1 μmol/g DW). Both the AE and WE from BW80 had the best oxygen radical-absorbance capacity. The extract WE-BW80 was also superior to the AEs of most varieties. The ORAC values for AE-BW80 and WE-BW80 were 652.45 and 396.57 μmol TE/g DW, respectively. Moreover, AE-JG and AE-YQ2 had high oxygen radical-absorbance capacities, with ORAC values of over than 500 μmol TE/g DW. The extracts of QHH and NLGL showed moderate antioxidant activities *in vitro*, and their AEs and WEs were used to further study the mechanism of antioxidative action in the cell model.

3.2. Effect of Huskless Barley Polysaccharide Extracts on Cell Viability. To assess whether the inhibitory effect of barley on the stimulation by TNF-α could be attributed to its effect on cell viability, we examined the cytotoxic effect of water and alkaline extracts of the huskless barley polysaccharides using the MTT assay, which provides rapid and precise results for cellular growth (Figure 2). A concentration of 10 μg/L TNF-α substantially decreased the cell viability of HUVEC from 100% to 22.56% ($P < 0.001$) whereas the huskless barley extract pretreatments effectively reduced the effect of TNF-α, facilitating cell proliferation and vascular reproduction. The viabilities of the cells treated with WE-QHH, AE-QHH, WE-NLGN, and AE-NLGN were 80.91%, 69.66%, 79.76%, and 70.04%, respectively. The water extracts seemed to be more compatible with cellular growth than the alkaline extracts.

3.3. Effects of Huskless Barley Polysaccharide Extracts on SOD and ROS in Cells. The addition of TNF-α (10 μg/L) greatly decreased the level of SOD in the HUVEC (Figure 3(a)). However, when treated with the dietary fiber extracts from barley, the HUVEC presented higher levels of SOD production. Compared to the HUVEC that were stimulated with TNF-α alone, the SOD production of the barley-treated HUVEC was found to be 2.27, 2.62, 3.01, and 4.14 times higher ($P < 0.001$). The addition of the extracts from the huskless barley decreased the ROS values in the endothelial cells. The water and alkaline extracts from QHH resulted in 10.5% and 11.2% inhibition of ROS, respectively. The

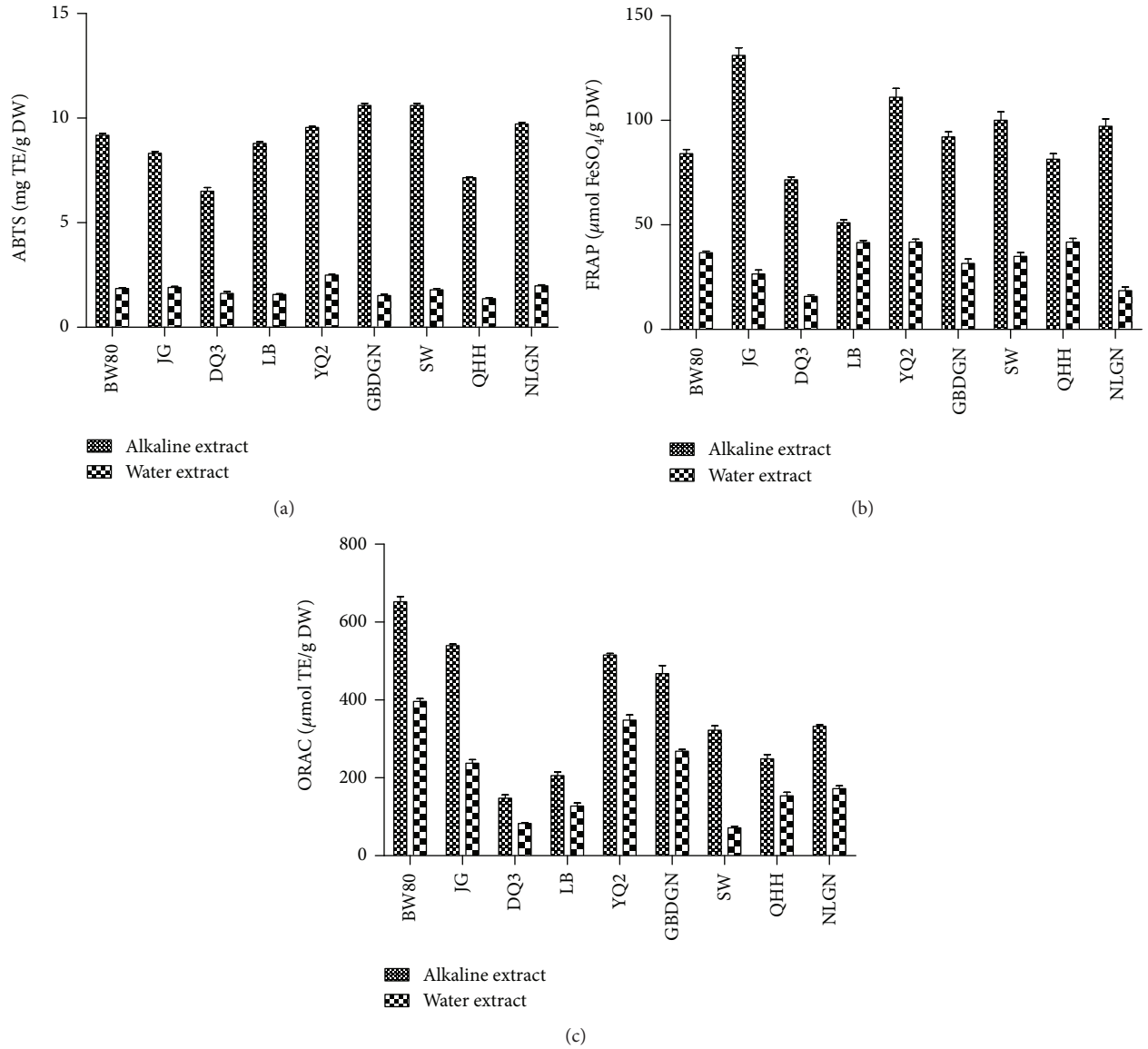

Figure 1: Antioxidant capacity of various huskless Barley extracts *in vitro*. (a) ABTS-scavenging capacity; (b) ferric-reducing antioxidant power (FRAP); and (c) oxygen radical-absorbance capacity (ORAC).

presence of NLGL decreased the ROS production by 24.7% (WE) and 35.2% (AE) (Figure 3(b)). Similar to the antioxidant capacity *in vitro*, the alkaline extracts seemed to have more pronounced antioxidant activity in cells than the water extracts, and NLGL possessed better antioxidant activity than QHH.

3.4. Effects of Huskless Barley Polysaccharide Extracts on the TNF-α-Induced MCP-1 and VCAM-1 Protein Expression in Cells. Figures 4(a) and 4(b) show that 10 μg/L of TNF-α significantly increased the production of MCP-1 and VCAM-1 in the endothelial cells ($P < 0.05$) compared to the control group. Both types of polysaccharide extracts from the two cultivars of huskless barley affected the protein levels of endothelial MCP-1. The WE-QHH, AE-QHH, WE-NLGN, and AE-NLGN extracts inhibited the TNF-α-induced MCP-1 levels by 60%, 62.9%, 88.6%, and 84.3%, respectively ($P < 0.001$). Similar to the effects on the MCP-1 protein, the levels of the VCAM-1 adhesion molecule in the cells that were pretreated with WE-QHH, AE-QHH, WE-NLGN, and AE-NLGN decreased by 39%, 48%, 62%, and 74%, respectively, compared with the TNF-α-stimulated group ($P < 0.001$). The extract of NLGL also possessed better anti-inflammatory activity than that of QHH. The difference between the effects of the water extracts and alkaline extracts on the MCP-1 protein expression was not significant. However, the alkaline extract inhibited the VCAM-1 protein expression by the cells more than the water extract for QHH and NLGL ($P < 0.05$), which was consistent with their antioxidant capacity.

3.5. Effects of Huskless Barley Polysaccharide Extracts on the TNF-α-Induced ACE Protein Expression in Cells. We investigated the effect on ACE production (Figure 5) in our study. When treated with TNF-α (10 μg/L), the level of ACE in

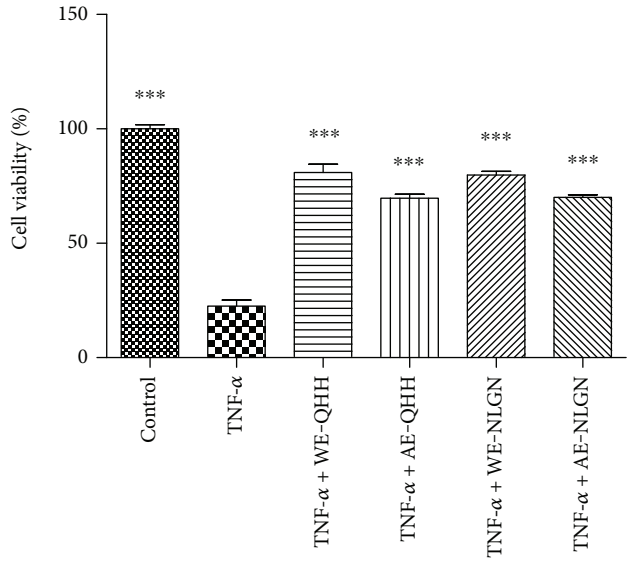

Figure 2: Effects of the water and alkaline extracts from QHH and NLGN on cell viability. *** indicates $P < 0.001$ versus the TNF-α group.

the endothelial cells increased significantly ($P < 0.001$). The polysaccharide extracts of huskless barley caused a significant reduction in the TNF-α-induced levels of ACE in the endothelial cells ($P < 0.001$). The extracts WE-QHH, AE-QHH, WE-NLGN, and AE-NLGN inhibited the ACE levels by 23%, 59%, 67%, and 76%, respectively, compared with the TNF-α-induced group. Similarly, the inhibitory effects of the alkaline ACE extracts were more pronounced than those of the water extracts, and the effects of NLGL were more pronounced than those of QHH.

4. Discussion

Huskless barley has attracted the attention of researchers and food processors in recent years for its potential health benefits. Huskless barley is recognized as a functional grain because it contains high levels of polysaccharides and phytochemicals [18]. In the present study, nine huskless barley varieties exhibited good antioxidant capacity *in vitro* using various methods (ABTS, FRAP, and ORAC). The huskless barley varieties exhibited different antioxidant capacities due to their different polysaccharide and phytochemical compositions. Djurle et al. reported differences in the carbohydrate composition in six different varieties of barley kernels [19]. For example, the KVL 301 variety had much lower extractability (76%) of mixed-linkage $(1 \rightarrow 3)$, $(1 \rightarrow 4)$-beta-D-glucan after extrusion than the other varieties (91–98%). The antioxidant properties of the alkaline extracts were more pronounced than those of water extracts *in vitro* and in cells. The different constituents of the water extracts (beta-glucans) and alkali extracts (arabinoxylans) might contribute to the differences in their bioactivities [14]. Sawicki et al. considered that in addition to the dietary fiber, other ingredients in the outer bran layer, including phenolic acids, alkylresorcinols, lignans, phytosterols, and tocols, may also contribute to health benefit outcomes [11]. Zhu et al. found that the extracts from four varieties of dehulled highland barley (*Hordeum vulgare* L.) showed excellent antioxidant activities as determined by ORAC and cellular antioxidant activity (CAA) assays; additionally, these varieties showed potent antiproliferative activity towards HepG2 human liver cancer cells. The bound phenolics make a significant contribution to antioxidant and anticancer activities in this model [20]. The alkaline extracts had higher total phenolic and flavonoid content than the water extracts in our tested barley varieties. For example, each gram of WE-QHH, AE-QHH, WE-NLGN, and AE-NLGN contained 1.28, 4.20, 2.89, and 5.14 mg of gallic acid equivalent, respectively. The total phenolic content of the alkaline extracts was nearly two to three times that of the water extracts, which may be another major reason for superior antioxidant activities of the alkaline extracts. The HPLC profiles of the extracts also showed that phenolic compounds of the alkaline extracts were more than those of the water extracts, in which phenolic acids (e.g., gallic acid, chlorogenic acid, 3,5-dicaffeoylqunic acid, and 4,5-dicaffeoylqunic acid) and flavonoids (e.g., rutin and astragalin) were detected (see the supporting document). The antioxidant properties of the dietary fiber from huskless barley bran also showed that the DPPH (1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl) radical-scavenging activity and ferric-reducing antioxidant power increased with an increase in the total phenolic content [21].

The nine huskless barley varieties evaluated here exhibited antioxidant capacities *in vitro*, and two representative barley cultivars, QHH and NLGN, showed antioxidant action as indicated by the increases in SOD and decreases in the ROS in the human umbilical vein endothelial cells. Superoxide dismutase is an enzyme that converts deleterious O_2 into less harmful H_2O_2 [22]. Superoxide dismutase is highly crucial for oxidative stress tolerance [23] and is recognized as an antioxidative defense against endothelial damage [24, 25]. Reactive oxygen species have destructive actions on both proteins and DNA and are therefore regarded as pathogenic, resulting in cellular death and arterial disease [26, 27]. Increases in ROS are associated with the accumulation of highly reactive free radicals, which exert deleterious effects. Moreover, overexpression of ROS may cause other side effects such as DNA mutations and genetic instability [28]. Therefore, the capacity of water and alkali extracts of the polysaccharides from huskless barley to increase SOD and to decrease ROS indicates that huskless barley can be used as an agent to alleviate harmful effects and protect the cells from oxidative impairment.

In this study, water and alkaline extracts of the polysaccharides from QHH and NLGN inhibited the vascular inflammatory process in TNF-α-induced endothelial cells by reducing the expression of the MCP-1 and VCAM-1 proteins. Cell adhesion molecule MCP-1 accumulates in large quantities during various inflammatory diseases [29, 30]. As a proinflammatory cytokine, TNF-α can induce the expression of chemokines, cytokines, and cell adhesion molecules in vascular endothelial cells. Stimulation of MCP-1 gene expression is associated with oxidation-reduction-sensitive mechanisms [31, 32]. Cell adhesion molecule VCAM-1 is

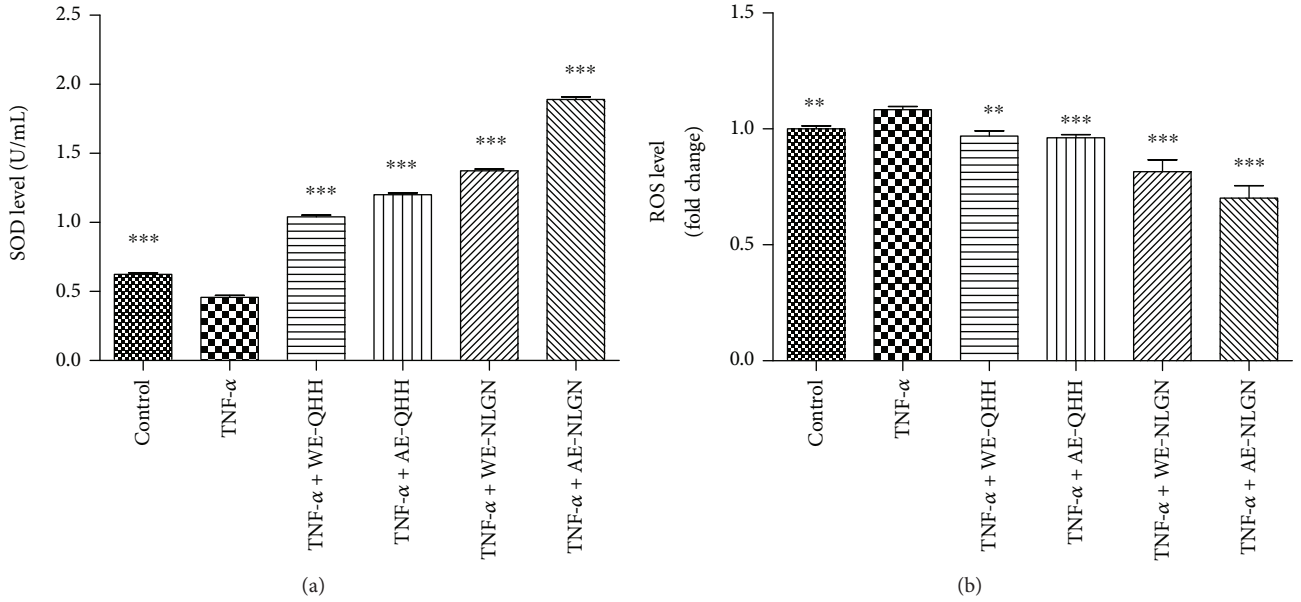

Figure 3: Effects of water and alkaline extracts from QHH and NLGN on (a) SOD expression and (b) ROS levels in the TNF-α-induced HUVEC. $**$ and $***$ indicate $P < 0.01$ and $P < 0.001$, respectively, versus the TNF-α group.

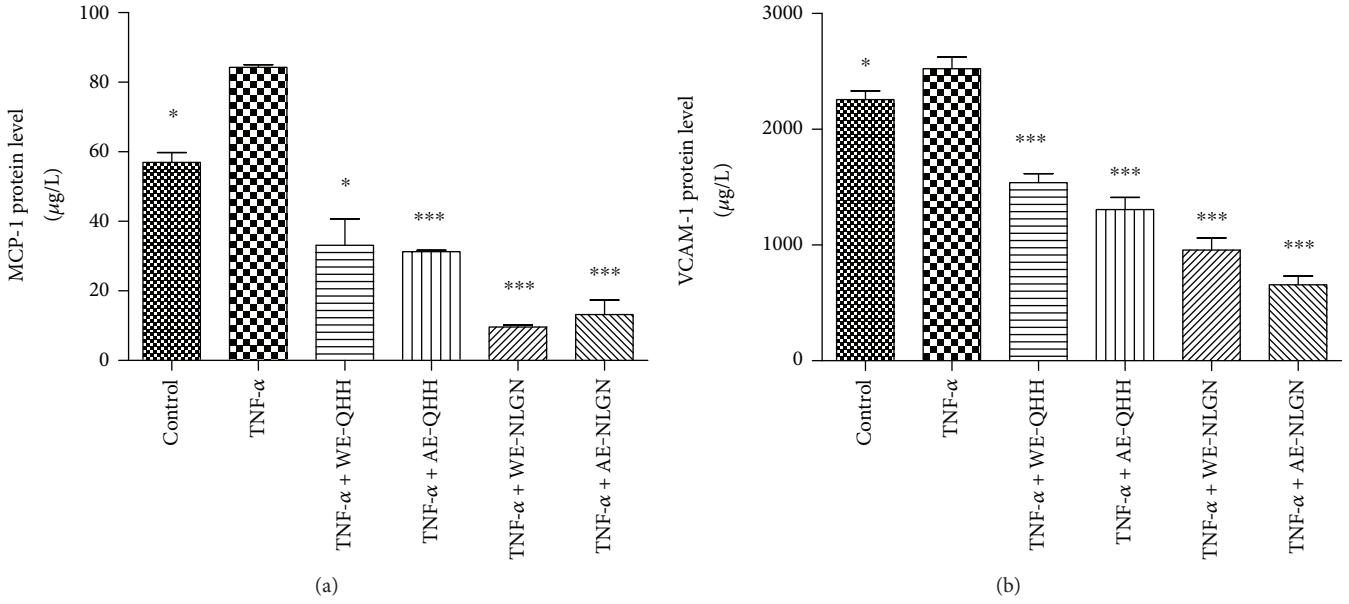

Figure 4: Effects of the water and alkaline extracts from QHH and NLGN on (a) MCP-1 and (b) VCAM-1 expression in the TNF-α-induced HUVEC. $*$ and $***$ indicate $P < 0.05$ and $P < 0.001$, respectively, versus the TNF-α group.

also important in the inflammatory responses and plays a significant role in cell adhesion and cell signal transduction [4]. Cybulsky et al. found that an early form of cell lesion was significantly diminished by a low level of VCAM-1, which suggested that VCAM-1 contributed to the initiation of AS [33]. In endothelial cells, this adhesion molecule has been revealed to actively participate in the vital functions of immune surveillance and inflammation and the migration of leukocytes from the blood into tissues [34]. The inhibition of the TNF-α-stimulated MCP-1 and VCAM-1 expression indicates that huskless barley might contribute to anti-inflammatory activity.

In addition, WE-QHH, AE-QHH, WE-NLGN, and AE-NLGN exhibited vasodilatory effects by inhibiting the production of ACE. Angiotensin-converting enzyme, a carboxyl-terminal dipeptidyl exopeptidase, indirectly leads to hypertension by causing blood vessels to constrict, which is highly correlated with cardiovascular disease [35]. Its mechanism is the conversion of the decapeptide angiotensin I to the potent vasoconstrictor-octapeptide angiotensin II

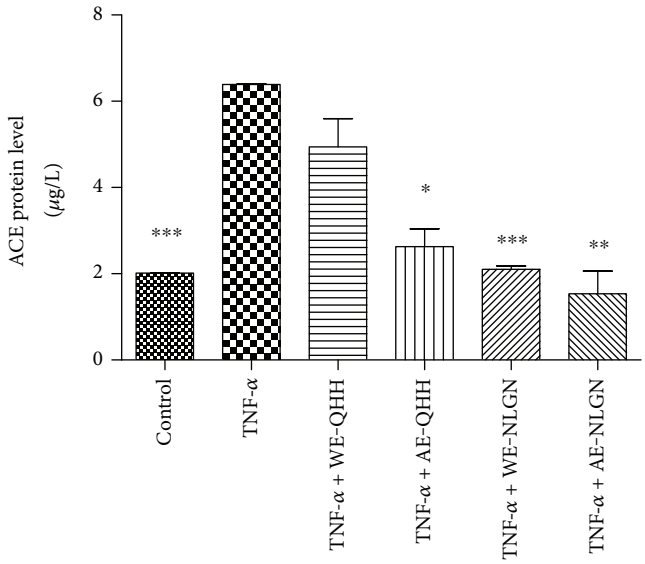

Figure 5: Effects of the water and alkaline extracts from QHH and NLGN on ACE expression in the TNF-α-induced HUVEC. $*$, $**$, and $***$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, versus the TNF-α group.

(Ang II). Several studies have shown that Ang II has crucial proinflammatory actions in the vascular wall, including the production of reactive oxygen species that in turn increase the expression of inflammatory cytokines. The inhibition of ACE reduces the Ang II receptor 1 (AT1) expression in HUVEC, which is thought to be the mechanism by which it decreases adhesion molecule production [36]. Therefore, it is reasonable to conclude that huskless barley possesses the potential to reduce the risk of cardiovascular disease through its antihypertensive effects on the vasculature.

In this study, huskless barley extracts exhibited antioxidant capacity. Alkaline-extracted polysaccharide possessed better antioxidant capacity and higher phenolic content than hot water-extracted polysaccharide. Since polysaccharides usually present as complex insoluble bound esters with phenolic compounds [37], phenolics might contribute part of antioxidant function. However, because of a small quantity of phenolics in polysaccharide extracts and the complexity of the natural crude extracts, it is rather difficult to characterize every compound and elucidate the structure. Further phytochemical analysis and the detailed composition of polysaccharide from huskless barley will require identification by LC-MS and NMR in the future. In addition, the huskless barley extracts were found to protect HUVEC against TNF-α-induced oxidative stress. However, the mechanism on antioxidant function of huskless barley extracts was unknown. Previous studies reported that dietary antioxidants could regulate oxidative stress in cells by activating Nrf2 (nuclear factor erythroid 2-related factor-2) or its related genes, such as wheat bran feruloyl oligosaccharides increasing SOD, catalase, and glutathione peroxidases via Nrf2 signalling [38]. Whether huskless barley extracts protect HUVEC against TNF-α-induced oxidative stress via Nrf2 signalling or its related genes will require further study in the future.

5. Conclusions

The results of the present study showed that the nine huskless barley varieties had good antioxidant capacities *in vitro*, including ABTS-scavenging capacity, ferric-reducing antioxidant power, and oxygen radical-absorbance capacity. The antioxidant properties of the alkaline extracts were more pronounced than those of the water extracts. Two representative huskless barley cultivars, QHH and NLGN, alleviated the negative effects of TNF-α by blocking the overexpression of the levels of several key proteins, MCP-1, VCAM-1, and ACE, in the HUVEC. Moreover, these cultivars increased the level of SOD and maintained the cell viability. Thus, these cultivars exerted antioxidant, anti-inflammatory, and antihypertensive effects on vascular endothelial cells and have a potential as potent agents to prevent cardiovascular diseases.

Abbreviations

AAPH:	2,2′-Azobis(2-methylpropionamide) dihydrochloride
ABTS:	2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ACE:	Angiotensin-converting enzyme
AE:	Alkaline extracted polysaccharide
Ang II:	Angiotensin II
ANOVA:	Analysis of variance
AOAC:	Official analytical chemists
AS:	Atherosclerosis
AT1:	Ang II receptor 1
AX:	Arabinoxylans
BW80:	Black and white huskless barley 80 days
CAA:	Cellular antioxidant activity
DCFH-DA:	2′,7′-Dichlorodihydrofluorescein diacetate
DMSO:	Dimethylsulfoxide
DPPH:	(1,1-Diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl)
DQ3:	DiQing 3
DW:	Dried weight
ecSOD:	Extracellular superoxide dismutase
ELISA:	Enzyme-linked immunosorbent assay
FRAP:	Ferric reducing antioxidant power
GBDGN:	GuiBaDingGeNa
HUVEC:	Human umbilical vein endothelial cells
IC_{50}:	Half maximal inhibitory concentration
JG:	JiuGe
LB:	Long and black huskless barley
MCP-1:	Monocyte chemotactic protein 1
MTT:	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
NLGN:	NanLongGeNa
NO:	Nitric oxide
OD:	Optical density
ORAC:	Oxygen radical absorbance capacity
PBS:	Phosphate-buffered saline
QHH:	QingHaiHuang
ROS:	Reactive oxygen species
SD:	Standard deviation
SOD:	Superoxide dismutase

SW: Short and white huskless barley
TE: Trolox equivalent
TEAC: Trolox equivalent antioxidant capacity
TNF-α: Tumor necrosis factor-alpha
VCAM-1: Vascular cell adhesion molecule 1
WE: Water extracted polysaccharide
YQ2: YunQing 2.

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Figure 1: reversed-phase high performance liquid chromatographic (HPLC) profile ($\lambda = 280$ nm) of mixed phenolic standards. The following 9 peaks represent chemical standards: (1) gallic acid, (2) neochlorogenic acid (3-caffeoylquinic acid), (3) chlorogenic acid (5-caffeoylquinic acid), (4) cryptochlorogenic acid (4-caffeoylquinic acid), (5) rutin (quercetin-3-*O*-rutinoside), (6) isoquercetin (quercetin-3-*O*-glucoside), (7) isochologenic acid B (3,5-dicaffeoylqunic acid), (8) astragalin (kaempferol-3-O-glucoside), and (9) isochrologenic acid C (4,5-dicaffeoylqunic acid). Figure 2: reversed-phase high performance liquid chromatographic (HPLC) profiles ($\lambda = 280$ nm) of the phenolic compounds from different huskless barley extracts. (A) Water extract from NanLongGeNa. (B) Water extract from QingHaiHuang. (C) Alkaline extract from NanLongGeNa. (D) Alkaline extract from QingHaiHuang. The following peaks were identified: (1) gallic acid, (3) chlorogenic acid (5-caffeoylquinic acid), (4) rutin (quercetin-3-*O*-rutinoside), (5) isochologenic acid B (3,5-dicaffeoylqunic acid), (6) astragalin (kaempferol-3-O-glucoside), and (7) isochrologenic acid C (4,5-dicaffeoylqunic acid). *(Supplementary Materials)*

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Review Article

Non-Provitamin A and Provitamin A Carotenoids as Immunomodulators: Recommended Dietary Allowance, Therapeutic Index, or Personalized Nutrition?

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Vegetables and fruits contain non-provitamin A (lycopene, lutein, and zeaxanthin) and provitamin A (β-carotene, β-cryptoxanthin, and α-carotene) carotenoids. Within these compounds, β-carotene has been extensively studied for its health benefits, but its supplementation at doses higher than recommended intakes induces adverse effects. β-Carotene is converted to retinoic acid (RA), a well-known immunomodulatory molecule. Human interventions suggest that β-carotene and lycopene at pharmacological doses affect immune functions after a depletion period of low carotenoid diet. However, these effects appear unrelated to carotenoids and retinol levels in plasma. Local production of RA in the gut-associated lymphoid tissue, as well as the dependency of RA-induced effects on local inflammation, suggests that personalized nutrition/supplementation should be considered in the future. On the other hand, the differential effect of RA and lycopene on transforming growth factor-beta suggests that lycopene supplementation could improve immune functions without increasing risk for cancers. However, such preclinical evidence must be confirmed in human interventions before any recommendations can be made.

1. Introduction

Major dietary non-provitamin A (lycopene, lutein, and zeaxanthin) and provitamin A (β-carotene, β-cryptoxanthin, and α-carotene) carotenoids have different biological activities and efficacy, depending on their food content, dietary intake, bioavailability, and bioconversion [1]. The intestine and liver are crucial organs for vitamin A uptake and liver accounts for the majority of retinoid stores [2, 3]. The provitamin A carotenoid, β-carotene, is a significant source of vitamin A in the diet. β-Carotene $'$ oxygenase-1 (BCO1) and β-carotene 9$'$,10$'$ oxygenase-2 (BCO2) are the two known carotenoid cleavage enzymes in humans [4]. In rats, both BCO1 and BCO2 are highly expressed in the liver and intestine, localized in hepatocytes and mucosal epithelium, and BCO1 is also expressed in hepatic stellate cells [4]. Both enzymes have provitamin A and non-provitamin A as preferential substrates, respectively, and genetic variations of these enzymes have been suggested within the factors affecting carotenoid status in humans [5, 6].

β-Carotene is known as an antioxidant, but its prooxidant activity in some conditions accounts for its adverse effects [6]. In particular, β-carotene failed to prevent cancer in two large clinical trials: the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study; α-tocopherol 50 mg and β-carotene 20 mg/d) [7] and the Beta-Carotene and Retinol Efficacy Trial (CARET; β-carotene 30 mg/d and retinyl palmitate 25,000 IU) [8]. Moreover, β-carotene supplementation increased lung cancer risk in smokers [9, 10] and the overall mortality [11, 12]. On the other hand, a safer profile for non-provitamin A carotenoids (up to 20 mg/d for lutein and 75 mg/d for lycopene) has been

suggested [13]. Lycopene has been extensively studied [14], and encapsulation has been suggested to improve bioavailability for therapeutic use in many conditions, including immune-mediated diseases [15].

Retinol bound to the retinol-binding protein (RBP) is a source of retinoic acid (RA) [2, 16], and the latter is metabolized by cytochrome P450 26 (CYP26) [3]. After uptake, retinol can be oxidized by ubiquitously expressed alcohol dehydrogenases (ADH) to form retinaldehyde (retinal) which is then metabolized into RA by retinaldehyde/aldehyde dehydrogenases (ALDH) in the liver [3, 17, 18]. ALDH are also expressed in the gut-associated lymphoid tissue (GALT) [3]. Although RA is the major active metabolite affecting the immune system, non-provitamin A carotenoids are active in immune modulation [19]. Furthermore, it has been reported that BCO1 could yield acycloretinal from lycopene [20] and that lycopene-derived BCO2 metabolites could mediate in some circumstance signals similar to that induced by retinoic acid receptor (RAR) ligands [21].

In this review, we aim to discuss the potential role of carotenoids as immunomodulators, on the light of their intake and safety.

2. Carotenoid Sources

The major carotenoids present in food products are β-carotene, α-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin [22] (Table 1). With the exception of egg yolk rich in lutein, the main sources of these compounds in human diet are of plant origin; they are widely distributed in the plastids of flowers, leaves, seeds, and roots. Orange, yellow- and green-colored vegetables are the rich sources; lycopene is found abundantly in tomatoes and their related products and is also present in fruits, such as watermelon and pink grapefruit [23]. Citrus fruits, papaya, and peaches contain significant levels of β-cryptoxanthin. The xanthophylls lutein and zeaxanthin are mainly found in leafy green vegetables, such as spinach or broccoli [24]. Likewise, an emerging source of carotenoids is the by-products of industry processing of fruits and vegetables [25].

Contents of carotenoids vary widely because their syntheses are greatly influenced by a wide variety of factors, including climate, soil, cultivar, and cultivation [26]. Further, their profile in berries changes with ripening stage, with higher levels of α-carotene and lycopene in advanced ripening [27]. In addition to preharvest factors, their contents can be affected by all treatments during postharvest because their highly unsaturated structures with conjugated double bonds make them very susceptible to oxidative reactions and dimerization. For example, cutting of vegetables increases the exposure to oxygen and releases enzymes from the cell vacuoles of plant parenchyma, which further promote their degradation. Excessive exposure to sunlight also decreases the content of carotenoids in harvested products [28]. Degradation of carotenoids can be diminished by storage at low temperatures, protection from light (packaged in dark containers), or package under modified atmospheres. However, the impact of thermal treatments on carotenoids appeared mixed. For example, nonthermally treated tomatoes had higher amounts of carotenoids compared to thermally treated ones and similar results were observed with carrot [29]. However, home culinary techniques, such as boiling in hot water, cause partial degradation and isomerization of both β-carotene and lycopene. Current industrial processing techniques as high-pressure treatment tend to preserve or even increase the content of carotenoids [30].

3. Dietary Intake, RDA, and Retinol Equivalents

Dietary data on consumption of carotenoids were in the past usually expressed as β-carotene, β-carotene equivalents, or retinol equivalents, and only more recently, carotenoid food composition databases have been developed. There is a general consensus regarding that the contribution of dietary carotenoids from food sources depends not only on their contents in foods but also on the frequency of their consumptions. Estimated intakes of carotenoids vary widely on individual, regional, and national levels, and significant seasonal variations have also been reported in some countries [31]. Furthermore, assessment of carotenoid intake is a complex matter mainly because of the high variability within and between subjects, the degree of imprecision in data collection, and discrepancies in carotenoid food composition databases, which reflect in different intakes of carotenoids in the literature.

Studies on dietary carotenoids are few, and the main results of one of the few comparative studies are presented in Table 2 [32], where the assessment of carotenoid intakes was carried out by a Food Frequency Questionnaire (FFQ) at the individual level of five countries. It should be noticed that the population in this study was a group in a determined area of each of the five participant countries (ca. per country). Thus, subjects might not necessarily be representative of the overall population although it was assumed that they followed a typical dietary pattern of their countries. Moreover, it should not be ignored that FFQ overestimates carotenoid intake [33], especially of lutein and zeaxanthin when comparing with 3-day food records. Table 2 summarizes carotenoid intake in some countries from the representative literature with a larger sample size. The total carotenoid intakes range between 5.42 and 15.44 mg/d; however, comparisons should be considered with caution since, as shown, sample size and methodology differ between studies.

In a review from Maiani et al. [1], a calculation of the relative contribution of each carotenoid to total carotenoid intake, according to FAO Food Balance Sheet data from several European countries, was performed. Lutein + zeaxanthin and β-carotene were those most frequently found in European diet (48% and 33%, respectively, on a total carotenoid intake of 11.8 mg/d). No formal dietary recommendation for carotenoids has yet been established, and the European Food Safety Authority (2006) had decided that the existing evidence was insufficient to establish a recommended dietary allowance (RDA) or adequate intake (AI) for β-carotene and other carotenoids [34]. In most European countries, the recommended intake was established based on the assumption that 4.8 mg β-carotene is needed to meet the requirement of 800 micrograms of vitamin A (conversion factor 6). In

Table 1: Carotenoid food/spice content.

Range mg/100 g	α-Carotene	β-Carotene	β-Cryptoxanthin	Lutein + zeaxanthin	Lycopene
20–50		Carrot, paprika, peppers red			Tomatoes
10–20	Carrot	Carrots, peppers red		Chard, chicory greens, kale, paprika, pepper, spinach, turnip greens,	Catsup, tomatoes
5–10	Peppers red, pumpkin, carrot juice	Acai berry drink, carrot juice, carrots, chili powder, kale, parsley, pumpkin, spinach, turnip greens	Pepper, red or cayenne paprika	Basil, parsley, radicchio, watercress	Guavas, tomato juice, tomato soup
1–5	Carrot, chili powder, pepper	Apricots, broccoli, cabbage Chinese, cherries, chicory greens, endive, lettuce (green and red leaf), melons, oregano, parsley, peas green, peppers green, plums, pumpkin, sweet potato, thyme, watercress	Chili powder, squash	Broccoli, brussels sprouts, carrot, fava, lettuce (green and red leaf), oregano, parsley, peas green, pistachio, pumpkin, thyme, tomatoes, zucchini	Grapefruit (pink and red), papayas, watermelon

From: United States Department of Agriculture Agricultural Research Service (USDA) Food Composition Databases (https://ndb.nal.usda.gov/ndb/).

Table 2: Comparison of carotenoid intake (mg/d) in adults reported in several countries.

Population (subjects)	α-Carotene	β-Carotene	β-Cryptoxanthin	Lutein + zeaxanthin	Lycopene	Dietary methods	Ref.
Australia, $N = 3100$	1.25/1.13 (m/w)	5.14/5.27 (m/w)	0.32/0.35 (m/w)	1.62/1.70 (m/w)	7.11/6.26 (m/w)	FFQ	[39]
Costa Rica, $N = 459$	0.45/0.73 (m/w)	3.41/4.67 (m/w)	0.38/0.55 (m/w)	2.41/2.89 (m/w)	5.45/5.77 (m/w)	FFQ and 7-day diary	[40]
France, $N = 76$	0.74	5.84	0.45	2.50	4.75	FFQ	[32]
France, $N = 12{,}741$	—	3.14/3.79 (m/w)	—	—	—	6-day food diary	[41]
Ireland, $N = 828$	—	—	—	1.60	—	166-item FFQ	[42]
Italy (INRAN-SCAI study), $N = 2313$	0.15/0.18 (m/w)	3.07/3.01 (m/w)	—	3.79/3.73 (m/w)	7.10/5.64 (m/w)	3-day food diary	[43, 44]
Japan JACC Study Group, $N = 3095$	—	2.11 (m)	—	—	—	35-item FFQ	[45]
Korea National Health and Nutrition Examination Survey, $N = 24{,}377$	0.56	3.62	0.55	2.300	2.22	1-day 24 h recall	[46]
Spain, $N = 70$	0.29	2.96	1.36	3.25	1.64	FFQ	[32]
Spain (EPIC cohort), $N = 41{,}446$	0.27	1.31	0.22	0.84	3.0	Dietary history questionnaire	[47]
Rep Ireland, $N = 76$	1.23	5.16	0.78	1.56	4.43	FFQ	[32]
The Netherlands, $N = 75$	0.68	4.35	0.97	2.01	4.86	FFQ	[32]
USA, $N = 584$	0.69/0.79 (m/w*) 0.98/0.91 (m/w**)	3.28/0.63 (m/w*) 4.09/3.82 (m/w**)	0.15/0.17 (m/w*) 0.16/0.13 (m/w**)	1.47/1.56 (m/w*) 2.88/2.25 (m/w**)	6.07/5.35 (m/w*) 5.79/4.64 (m/w**)	118-items FFQ	[48]
USA, $N = 2787$	0.78 (w)	4.40 (w)	0.18 (w)	0.30 (w)	6.34 (w)	FFQ	[49]
UK, $N = 71$	1.04	5.55	0.99	1.59	5.01	FFQ	[32]
UK (EPIC Norfolk cohort), $N = 14{,}803$	0.41/0.40 (m/w)	2.07/2.04 (m/w)	0.41/0.46 (m/w)	1.10/1.14 (m/w)	1.43/1.29 (m/w)	7-day diary	[50]

FFQ: food frequency questionnaire; m/w: men/women; JACC: Japan Collaborative Cohort; EPIC: European Prospective Investigation into Cancer and Nutrition. *Hispanics and **Non-Hispanics.

other countries, for example in USA, a conversion factor of 12 for β-carotene and 24 for other carotenoids such as β-cryptoxanthin was applied [35]. For very complex matrices (i.e., spinach), human studies have revealed an even higher conversion factor for β-carotene such as 1 : 21 for a fruit/vegetable mix or 1 : 26 for vegetables [36]. Conclusions of many epidemiological studies revealed that a plasma level of 0.4 μmol/L β-carotene should be aimed at in order to benefit from the preventive health potential. This concentration can be achieved with consumption of 2–4 mg/d β-carotene [37], far below the supplemented dose used in the ATBC study [7] and the CARET study [8], in which an increased risk of lung cancer was noted in heavy smokers taking high doses (5 to 10 times the dose previously indicated of 2–4 mg/d) of β-carotene for long periods.

Consumption of foods rich in β-carotene is highly recommended since it is associated with a lower risk of chronic diseases and to ensure the intake of a sufficient amount of antioxidants. Healthy diet, which realistically contains 100–500 g/d of fruit and vegetables, shall contain a high proportion of carotenoid-rich food. On the other hand, proposed intake recommendations for some non-provitamin A carotenoids are 10–20 mg/d for lutein and 5.7–15 mg/d for lycopene [38].

4. Bioavailability and Accessibility

Bioavailability of dietary xanthophylls is varied widely between individuals and subject to the influence of many intrinsic and extrinsic factors [51]. Bioavailability is defined as the portion of the ingested nutrients that are absorbed in the small intestine, enter in the circulation, and become available for utilization or storage in organs [52–54]. Before nutrients in foods, beverages, or nutraceuticals are absorbed in the intestine, they must be made themselves ready for the transportation from the chyme in the lumen to enterocytes, a process defined as bioaccessibility. In the case of lipid-soluble carotenoids, ingested carotenoids must be first released from the food matrix, transferred into lipid emulsion, incorporated into the micelles containing pancreatic lipases and bile salts, and then available for transport into enterocytes [54–56]. The micelles act as a polar carrier from the hydrophilic chyme to the mucosal cell surface for the uptake through passive diffusion [57]. The factors influencing carotenoid bioaccessibility and bioavailability can be categorized to carotenoid-related and unrelated groups. The carotenoid-related includes dosage, chemical structure (isomeric forms), and interactions between carotenoids, and the unrelated includes cooking, nutrient composition of co-consumed foods, particle size of digested foods, biometrics of consumers, efficiency of micellarization, and transport from the enterocytes to the lymph system [36, 57–61]. Thus, carotenoid contents in foods may not be well correlated with their bioavailability and the ultimate bioefficacy because of the interference of negative effectors [62]. Among the unrelated factors, presence of dietary fat, heat treatment, and reduced particle size have a noticeable positive effect whereas dietary fibers and proteins have a negative effect [62]. Mechanical processing, including chopping and chewing, help reduce particle size and release carotenoids from chloroplasts and tissue for the bioaccessibility [63–65]. The amounts of naturally occurring lipids are rather low in most carotenoid-rich fruits and vegetables so that 3–5 g of fat intake per day is essential for the optimal absorption of carotenoids [66, 67]. Further, the presence of dietary fats, particularly long-chain fatty acids, for example, oleic acid, is more beneficial for the absorption of nonpolar carotenoids (carotenes) than that of polar ones (xanthophylls) [62, 68–70] because polar carotenoids can be more easily transferred from emulsified lipid to micelles [71]. Dietary fibers, the principle components of plant foods, compromise carotenoid release from food matrixes, and both fibers and proteins inhibit the incorporation of carotenoids into the micelles [60, 72]. While heating during cooking can degrade most nutrients in foods, such a treatment increases the bioavailability of certain nutrients, such as lycopene [73]. Therefore, understanding factors influencing bioaccessibility and bioavailability of carotenoids is crucial to achieving their ultimate bioefficacy.

5. Encapsulation

Nutrient bioavailability precedes its bioactivity at target tissues. In order to obtain the maximum bioefficacy of any given nutrients whose bioaccessibility and bioavailability are not satisfactory, a number of strategies are sought for their improvements. For example, encapsulation with food grade or related Generally Recognized As Safe (GRAS) materials has emerged as a novel strategy to improve the bioavailability and bioactivity of phytonutrients, including carotenoids. This encapsulation technology can include, but not limited to, microemulsions, matrix systems, solid dispersions, reassembled proteins, cross-linked polysaccharides, and liposomes [74–81]. The encapsulation, such as liposomes and emulsions, can stabilize carotenoids from possible degradation in the harsh gastrointestinal environment [82]. Nanoencapsulation is defined as a technology involving the formation of active loaded particles with diameters ranging from 1 to 1000 nm [83]. Particularly, polymeric nanoencapsulation has been adopted as one of preferred methods due to its higher loading capacity and better stability [84–86] and has been proven effective to augment bioavailability of carotenoids. For example, in a feeding study with male Swiss albino mice, Arunkumar et al. [87] reported that lutein nanoencapsulated by chitosan triphosphate was accumulated in a larger concentration in plasma, liver, and eyes as compared to the control. Furthermore, using an *in vitro* Caco-2 cell model, Yi et al. [88] found that solid lipid nanoentrapment significantly improved cellular uptake of β-carotene. Vishwanathan et al. [89] found in a small clinical trial that lutein supplemented in a stable hydrophilic nanoemulsion was 1.3-fold more bioavailable as evidenced in its serum status compared to lutein delivered in a pill. Thus, encapsulation can be a promising technology to enhance carotenoid bioaccessibility and bioavailability and to navigate precise delivery to target tissues such as eyes, brain, or/and skin for the maximum health benefits.

However, clinical data supporting their applications remain largely lacking.

6. Safety and Efficacy of Carotenoids

It is well known that an excess of retinoids induces teratogenic effects [90, 91] and affects xenobiotic metabolism [92]. Although β-carotene is not teratogenic [9], high doses of β-carotene and vitamin E can be prooxidant and toxic [93, 94] and increase cancer risk. In particular, despite that high intake of β-carotene reduces the risk of many cancers (Table 3), the effect on breast cancer risk depends on estrogen receptor (ER) and progesterone receptor (PR) statuses [95] (Table 3). In general, the relationships between carotenoids and cancer risk depend on type of carotenoids and site of cancer, but the supplementation never confirms the suggestions from intake data (Table 3). Moreover, the increased risk of lung cancer after β-carotene supplementation had been reported in smokers and people drinking ≥11 g ethanol/d (ATBC study) [7]. The ATBC (20 mg/d) and CARET (30 mg/d) studies also showed increased risk for intracerebral hemorrhage [96], cardiovascular diseases [97, 98], and hyperlipidemia (in asbestos-exposed subjects) [98]. On the contrary, lycopene supplementation decreased LDL cholesterol [99] and blood pressure [100], at doses of ≥25 and > 12 mg/d, respectively, and lycopene has been suggested for preventing the toxic effects of antineoplastic drugs [101].

The overall mortality increased after β-carotene supplementation [102–104] at a dose of >9.6 mg/d [104]. On the contrary, for non-provitamin A carotenoids, an Observed Safe Level (OSL) of 20 mg/d for lutein and 75 mg/d for lycopene [13] has been suggested and an acceptable daily intake (ADI) of 53 mg/d has been proposed for zeaxanthin [105]. The positive effect of lutein and zeaxanthin on age-related macular degeneration is well known [106].

In the ATBC study, an induction of cytochrome P450 enzymes (CYP450) in male smokers supplemented with β-carotene has been reported [10]. Since CYP450 is the primary metabolizer of xenobiotics in humans, interactions between medication use and dietary supplements can occur. In this context, β-carotene supplementation (25,000 IU twice daily, 28 days) did not affect pharmacokinetics of nelfinavir and its active metabolite M8 in HIV-1-infected individuals [107], whereas a mixed supplement (400 IU/d of vitamin E, 500 mg/d of vitamin C, and 6 mg/d of β-carotene twice daily, 6 months) decreased cyclosporine A in renal transplant recipients [108]. Therefore, potential nutraceutical-drug interactions must be evaluated on the basis of the pharmacokinetics. Furthermore, interactions between alcohol and RA precursors are well documented and the combination of β-carotene with ethanol results in hepatotoxicity [109].

In particular, competitive inhibition of ADH could account for this adversity [110] and for the less adverse effects of non-provitamin A carotenoids (Table 3 and Table 4).

In the CARET study, β-carotene increased from 17 to 210 μg/dL after 4 months of supplementation [111], whereas circulating lycopene concentrations between 2.17 and 85 μg/dL were inversely associated with prostate cancer risk [112]. It shall be noted that such an association did not exist at concentrations greater than 85 μg/dL [112]. It has been reported that circulating lycopene, rather than dietary lycopene, decreases stroke risk [113]. In this context, dietary guidance should consider upper limits for food-derived bioactive substances [114]. Also, efficacy should be determined in order to establish a therapeutic index of non-nutrient phytochemicals in foods and beverages [115].

7. Carotenoids and the Immune System

It is widely recognized that vitamin A deficiency decreases both humoral and cellular immune responses [16, 139] and that RA regulates innate immune response [140]. Vitamin A deficiency was associated with incidence of tuberculosis in human immunodeficiency virus- (HIV-) negative subjects [141] and in HIV-infected patients after antiretroviral therapy [142]. In addition, carotenoid concentrations were lower in tuberculosis cases before antiretroviral therapy [142]. However, in the ATBC study, β-carotene (20 mg/d) increased the risk of pneumonia in those who had initiated smoking at 21 years or later age [143] and the incidence of common cold in people undertaking strenuous exercise [144]. On the other hand, vitamins (vitamin C 120 mg, β-carotene 6 mg, and α-tocopherol 15 mg) with zinc (20 mg) and selenium (100 μg) decreased the infectious events in elderly subjects [145]. However, low levels of vitamin A and carotenoids are associated not only with immunodeficiency but also with inflammation and autoimmunity and both systemic and GALT immune dysfunctions [18]. Patients with rheumatoid arthritis [146, 147], systemic lupus erythematosus [146], celiac disease [148], and/or Crohn's disease [149] had lower serum concentrations of carotenoids [149], β-carotene [146, 147], and/or retinol [146, 148]. Concerning non-provitamin A carotenoids, in the Third National Health and Nutrition Examination Survey (NHANES III), high serum lycopene concentrations were associated with lower mortality in patients with systemic lupus erythematosus [150].

Despite the potential concerted modulation of redox and inflammatory status, in a review of studies that investigated the effect of supplementation with antioxidant-rich foods or nutraceuticals on combined markers of redox and inflammatory status in humans, overall improvement in both markers of redox and inflammatory status was observed only in 27 studies of the 88 studies analyzed and only 28.6% (2/7) of the interventions with carrot, tomato, or lycopene-derived tomato (Lyc-O-mato) improved at least one marker of redox or inflammatory status [151]. Some serum inflammatory cytokines, such as tumor necrosis factor- (TNF-) α and interleukin- (IL-) 6, are also called adipomyokines [152] and are not specific markers of immune function, whereas their ex vivo production from peripheral blood mononuclear cells can be an index of immune response.

Table 5 describes major findings of human intervention studies [153–173] that investigated the effect of β-carotene, lycopene, mixed supplements, or carotenoid-rich juices and diet (fruits/vegetables) on immune function assays, including the *in vivo* test of cell-mediated immune response delayed-

Table 3: Carotenoids and cancer risk.

	β-Carotene	α-Carotene	β-Cryptoxanthin	Lycopene	Lutein + zeaxanthin
High intake				Ovarian (postmenopausal) ↓ [116]	
High intake	Bladder ↓ [117]	Bladder ↓ [117]	Bladder ↓ [117]	Bladder ↔ [117]	Bladder ↔ [117]
Supplement	Bladder ↑ [118]				
High intake	Breast (ER+, ER+/PR+) ↑ [95] (ER−/PR+ or ER−/PR−) ↓ [95]	Breast ↓ [95]	Breast ↓ [95]	Breast (ER−/PR+ or ER−/PR−) ↓ [95]	Breast (ER−/PR+ or ER−/PR−) ↓ [95]
Supplement	Gut (colorectal) ≈↑ [119]				
High intake	Gut (esophageal) ↓ [120]	Gut (esophageal) ↓ [120]	Gut (esophageal) ↓ [120]	Gut (esophageal) ↓ [120]	Gut (esophageal) ↓ [120]
High intake	Gut (gastric) ↓ [121, 122]	Gut (gastric) ↓ [121, 122]		Gut (gastric) ↔ [121, 123]	Gut (gastric) (lutein) ↔ [121]
Supplement	Gut (liver) ↔ [124]				
High intake	Gut (pancreatic) ↓ [125]	Gut (pancreatic) ↔ [125]	Gut (pancreatic) ↓ [125]	Gut (pancreatic) ≈↓ [125]	Gut (pancreatic) ↔ [125]
Supplement	Gut gastric ≈↑ [126]				
Supplement	Gut intestinal ↑ [126]				
High intake	Hodgkin lymphoma ↓ [127]	Hodgkin lymphoma ↓ [127]	Hodgkin lymphoma ↔ [127]	Hodgkin lymphoma ↔ [127]	Hodgkin lymphoma ↓ [127]
High intake	Lung ↓ [128]				
Supplement	Lung ↑ [7, 129]				
High intake	melanoma ≈↓ [130]				
Supplement	Oral ↔ [131]				
High intake	Oral ↓ [132]	Oral ↓ [132]	Oral ↓ [132]	Oral ↓ [132]	
High intake	Prostate ↔ [133]	Prostate ↓ [133]		Prostate ↓ [112, 133]	
Supplement	Prostate ↔ [134]				

≈ ns. increase or decrease; ↓: decrease; ↑: increase; ↔: no change; ER: estrogen receptor; PR: progesterone receptor.

Table 4: Effects of lycopene and β-carotene supplementation on cardiometabolic outcomes.

	Lycopene	Lutein	β-Carotene
Blood lipids	↓ Cholesterol [99] ↔ Cholesterol [136]	↔ [135]	↑ Cholesterol and triglycerides (asbestos-exposed) [98]
Diabetes/insulin resistance		↔ Insulin resistance [135]	↔ Type 2 diabetes [137]
Diabetic macrovascular disease			↔ [138]
Metabolic syndrome		↓ [135]	
Blood pressure	↓ [100, 136]	↔ [135]	
CVD and nonfatal myocardial infarction			↑ [97, 98]
Stroke	↓ [113]		
Intracerebral hemorrhage			↑ [96]
CV death			↑ [103]

↓: decrease; ↑: increase; ↔: no change; CVD: cardiovascular disease; CV: cardiovascular.

type hypersensitivity (DTH) and/or ex vivo assays of innate (i.e., natural killer (NK) activity and oxidative burst) and adaptive immunity (i.e., lymphocyte proliferation and cytokine production).

Increased levels of β-carotene [155, 156, 158–160, 163–165, 167–169, 172], lycopene [159, 161, 162, 167–169, 172], and lutein [167, 168] as well as of antioxidant vitamins (vitamin E and/or C) in the case of mixed supplements (Table 5) were found in response to treatment. Furthermore, increases in plasma carotenoid from 2.03 to 3.05 μM were reported after 8 weeks of a consumption of 8 servings/d of vegetables and fruits, including carrots, green beans, peas, broccoli, zucchini, tomatoes, kohlrabi, Brussels sprouts, red cabbage, cauliflower, spinach, lettuce, radishes, cucumbers, fennel, apples, pears, kiwis, bananas, peaches, nectarines, cherries, strawberries, and red currants [173].

β-Carotene inhibited the ultraviolet light- (UV-) induced immunosuppression, evaluated with a DHT test in both healthy and elderly subjects, whereas contrasting results were reported on DHT when lycopene, β-carotene, or mixed supplements were used without UV irradiation (Table 5).

Data from ex vivo markers of adaptive immunity do not support an effect of lymphocytes' proliferation, whereas results concerning cytokine production are of difficult interpretation due to the differences in the dosage and duration of carotenoid supplementation and the use of carotenoid depletion periods (Table 5). In a longitudinal study of four periods, each lasting 2 weeks (weeks 1–2: low-carotenoid period; weeks 3–4: 330 mL tomato juice; weeks 5–6: 330 mL carrot juice; weeks 7–8 : 10 g dried spinach powder), tomato juice consumption increased IL-2 and IL-4 secretion compared with that at the end of the depletion period, whereas no effects were observed after carrot juice and spinach powder [170] (Table 5).

The same group [169] observed, in a crossover design, that ex vivo IL-2 production increased after carrot juice only in the arm depletion-carrot juice-depletion-tomato juice. TNF-α increased after the first supplementation (both juices) but only with carrot juice after the second supplementation [169]. Moreover, IL-2 further increased after supplementation and lymphocyte proliferation increased in both groups after the end of the first juice supplementation period despite that it did not change after carrot or tomato juice consumption compared with that at the end of the first low-carotenoid period [170]. Authors reported that this immunomodulation could not be explained by changes in the plasma carotenoid concentrations [170] and that provitamin A effect can be excluded because plasma retinol levels did not change after juice supplementation.

Concerning innate immunity, conflicting results were reported for oxidative burst-induced reactive oxygen species (ROS) production, whereas NK activity resulted to be increased in the majority of the studies (Table 5). However, the maximal increase in NK activity has been observed 1 week after juice supplementations had been stopped and the increase in NK cell activity is not associated to increase in NK percentage [157].

Accordingly, results on lymphocyte subsets are conflicting. Despite that in older subjects β-carotene (30 mg/d, 2 months) increased plasma β-carotene and the percentage of NK, without affecting plasma retinol [174], many studies did not observe any effect on lymphocyte subsets after β-carotene supplementation [153, 158, 159, 165–167, 172, 173, 175]. Moreover, in a randomized controlled trial (RCT), β-carotene (30 mg/d) supplementation for 3 months in subjects with colonic polyps or colon cancers increased CD4 count only in cancer patients who had a lower percentage of CD4 than in patients with polyps and in controls [176]. On the other hand, β-carotene (60 mg/d) increased $CD4^+$ cell counts only in patients with AIDS who have greater than 10 cells/microliters [177]. In HIV patients, β-carotene (60 mg/d, 3 months) increased NK, but not CD4 [178]. On the contrary, others reported that in HIV patients, β-carotene (60 mg/d orally three times daily and at 1 month and 3 months) did not change T cell subsets and NK, despite the increase in serum β-carotene [175]. Contrasting results came from supplementation with β-carotene in doses ranging from 60 mg/d to 180 mg/d on CD4 count in HIV patients

Table 5: Effects of carotenoid and carotenoid-rich food and beverages on test of immune function.

Subjects (study)	Treatment	Outcomes [ref.]
Healthy (RCT)	β-Carotene (15–120 mg), 4–7 wk	↔ lymphocyte proliferation [153], ROS production [154] ↑ DTH (30 mg) versus control (↓ after UV exposure response only in the placebo group) [155]
Elderly (RCT)	β-Carotene (8.2, 30, 50, and 90 mg), 3–6 wk to 10–12 y	↑ DTH (30 mg) versus control (↓ after UV exposure response only in the placebo group) [156] ↑ NK activity [157] ↔ production of IL-12 and IFN-γ (50, 90 mg) [157] ↔ DTH (50 and 90 mg), production of IL-2 [158], and lymphocyte proliferation [158, 159]
Smokers (RCT)	β-Carotene (40 mg), 4 and 6 wk	↓ ROS production [160]
Type 2 diabetes (RCT)	Lycopene (10 mg/d), 8 wk	↔ DHT [161, 162]
Elderly (RCT)	Lycopene (13.3 mg), 12 wk	↔ lymphocyte proliferation [159]
Elderly (RCT)	Mixed supplement β-Carotene (0.75 mg), vitamin C (90 mg), and vitamin E (20 mg), 1 y β-Carotene (6 mg), vitamin C (120 mg), and vitamin E (15 mg), 1–2 y	↑ DHT [163] ↔ DHT [164], lymphocyte proliferation [165]
Healthy (RCT)	Mixed supplement β-Carotene (12 mg), vitamin E (288 mg), and vitamin C (375 mg), 6 and 10 wk β-Carotene (30 mg), lycopene (15 mg), and lutein (9 mg), 5 wk	↑ DTH [166] ↔ lymphocyte proliferation, ROS production [166] ↔ DHT [167] ↓ IL-2 [167] and ROS [167, 168] production versus depletion (↑)
Healthy (RCT/longitudinal)	Carrot juice (330 mL, 21.6–27.1 mg β-carotene, and 13.1–15.7 mg α-carotene), 2 wk	↑ TNF-α versus depletion (arm carrot juice-tomato juice, arm tomato juice-carrot juice) [169] ↑ IL-2 versus depletion (arm carrot juice-tomato juice) [169] ↔ lymphocyte proliferation and IL-4 production [169, 170] ↑ NK activity [169]
Healthy (longitudinal)	Dried spinach powder 10 g (11.3 mg lutein and 3.1 mg β-carotene), 2 wk	↔ lymphocyte proliferation, IL-2 and IL-4 production [170]

Table 5: Continued.

Subjects (study)	Treatment	Outcomes [ref.]
Healthy (RCT/longitudinal)	Tomato-based drink (Lyc-o-Mato) (5.7 mg lycopene, 1 mg β-carotene, and 1.8 mg α-tocopherol), 26 days Tomato juice (330 mL, 37.0–40 mg lycopene and 1.5 mg β-carotene), 2 wk	↓ TNF-α production [171] ↔ IFN-γ production (versus baseline, ↑ in placebo versus baseline) [171] ↔ lymphocyte proliferation [169, 170], IL-2 and IL-4 production [169] ↑ TNF-α versus depletion (arm tomato juice-carrot juice) [169] ↑ IL-2 and IL-4 production versus depletion (↑) [170], ↑ NK activity [169]
Elderly (RCT)	Tomato juice (330 mL, 47.1 mg lycopene), 8 wk	↔ DTH, lymphocyte proliferation [172] ↓ IL-2 production (versus baseline, ns versus water) [172] ↑ activity of NK, IL-4, and TNF-α production (versus baseline, ns versus water) [172]
Healthy (RCT)	Vegetables and fruit: 2, 5, or 8 servings/d, 4 wk	↔ NK activity, IL-2, IL-12, IFN-γ, TNF-α production, lymphocyte proliferation [173]

↓: decrease; ↑: increase; ↔: no change; d: days; DTH: delayed-type hypersensitivity; IFN: interferon; IL: interleukin; mo: months; NK: natural killer cells; RCT: randomized controlled trials; TNF: tumor necrosis factor; UV: ultraviolet light; wk: weeks; y: years.

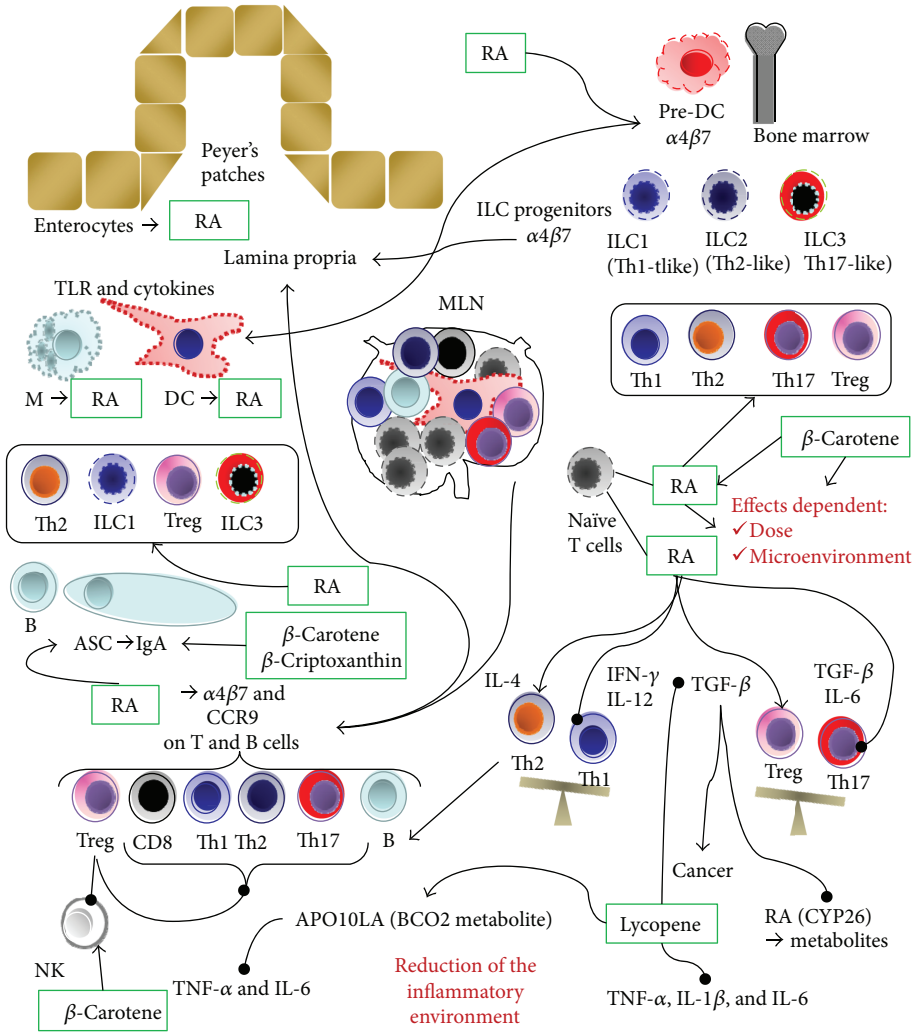

Figure 1: Immunomodulatory effects of carotenoids and retinoic acid. $\alpha4\beta7$: $\alpha4\beta7$ integrin; APO10LA: Apo-10′-lycopenoic acid; ASC: antibody-secreting cells; BCO2: β-carotene 9′,10′ oxygenase-2; CCR9: C-C chemokine receptor 9; CYP26: cytochrome P450 26; DC: dendritic cells; IFN: interferon; Ig: immunoglobulin; IL: interleukin; ILC: innate lymphoid cells; M: macrophages; MLN: mesenteric lymph nodes; NK: natural killer; RA: retinoic acid; TGF: transforming growth factor; Th: T helper; TLR: Toll-like receptor; TNF: tumor necrosis factor; Treg: regulatory T. →: homing and improvement; −•: inhibition.

[175, 177, 179–183], and data from a recent meta-analysis does not support β-carotene supplementation for increased CD4 cell count in patients with HIV [184]. However, GALT resulted to be depleted of CD4 also after restoration of blood CD4 by combined antiretroviral therapy (cART) [185]. In particular, it has been reported that HIV patients had defective gut homing of C-C chemokine receptor 9 (CCR9) and gut-homing $\beta7$ integrin on T helper cells producing IL-17 (Th17) [185]. In this context, it is well known that RA induces the gut-homing molecules $\alpha4\beta7$ integrin and CCR9 in B and T (CD4 and CD8) cells [2, 3, 139] (Figure 1). RA can also induce $\alpha4\beta7$ integrin and CCR9 on type 1 and 3 innate lymphoid cells (ILCs), but does not lead to CCR9 expression on type 2 ILCs [3, 18]. In terms of cytokine production, ILC1, ILC2, and ILC3 cells are Th1-like, Th2-like, and Th17-like cells, respectively [186] (Figure 1). Although plasticity has been suggested between ILC2/ILC1 and between ILC3/ILC1, ILC2 has been involved in asthma, lung fibrosis, esophagitis, and atopic dermatitis; ILC1 in chronic obstructive pulmonary disease and Crohn's disease; and ILC3 in psoriasis and obesity-associated inflammation [187]. Furthermore, ILC1 and ILC3 induce the polarization of inflammatory macrophages M1 [139]. Therefore, innate immunity can affect local inflammation.

In addition to the enterocytes' production, RA is also produced by stromal cells in the lamina propria (LP) and mesenteric lymph nodes (MLN), as well as by dendritic cells (DC) and macrophages [3] in the GALT. DC are major RA producers in LP, Peyer's patch, and MLN [188] (Figure 1). Preclinical studies suggest that the expression of gut-homing molecules by DC precursors in marrow is regulated by RA [18] (Figure 1). These cells migrate in the gut and induce oral tolerance by inducing regulatory T cells (Treg) [18]. RA induces also RA-producing $CCR7^+$ DC that migrate to the MLN and induce gut homing in T cells [18] (Figure 1). RA production by DC is regulated by many local signals.

Microbial-derived signals, by Toll-like receptor (TLR) 2 and TLR5, as well as butyrate produced by commensal bacteria, induce ALDH expression in DC [3, 18]. Besides IL-4 from ILC2 and Th2 cells, transforming growth factor beta (TGF-β) may also induce ALDH expression [3, 18]. The effects of RA on Th subsets depend on the local microenvironment [2, 3, 139].

In physiological conditions, RA produced by DC inhibits the differentiation of naïve T cells to Th17 cells by blocking IL-6, IL-21, and IL-23 signaling in naïve T cells [3]. RA-primed DC induce the production of the anti-inflammatory cytokine IL-10 in Tregs [3], and RA itself promotes TGF-β-mediated Treg conversion of naïve T cells [2, 3] (Figure 1). TGF-β is also involved in IgA class switching [189], and RA induces the expression of $\alpha 4\beta 7$-integrin and CCR9 on B cells and antibody-secreting cells (ASC) [2, 189] (Figure 1). Furthermore, DC-derived RA, plus IL-5, IL-6, or TLR signals, has a primary role in the polarization of B cells in favor of IgA-producing ASC, by inducing IgA class switching in B cells [3, 18, 139, 189], and it has been suggested that oral RA administration before vaccine can increase the secretion of IgA into gut secretions [91]. Concerning pro-vitamin A carotenoids, some preclinical studies suggest an effect on humoral immunity (Figure 1). In mice, 50 mg/kg β-carotene for 21 d increased the concentrations of IgA and the numbers of ASC in the jejunum [190]. Also, β-cryptoxanthin (5–10 mg/kg, 14 and 21 d) in rabbit increased the blood CD4, IL-4, and humoral immunity (IgG, IgM, and IgA) [191].

During inflammation, IL-1 enhances an IL-6-induced shift of the Treg/Th17 balance towards Th17 cells [3], and RA promotes, in the presence of IL-15, the secretion of IL-12 and IL-23 by DC, inducing the IFN-γ-producing Th1 and Th17 cells, and enhances the IL-4-mediated induction of Th2 [3, 18, 140]. On the other hand, in deficiency state, there are marked increases of ILC2 cell proliferation and cytokine (IL-4, IL-5, IL-6, IL-9, and IL-13) production, and, at the same time, the proliferation and function of ILC3 subset are suppressed [139].

It has been also suggested that RA has a dose-dependent effect: at pharmacological or high doses (10 nM and higher), RA inhibits Th17 and Th1 cells and induces Treg, whereas at physiological low doses (1 nM), RA favors Th17 cell differentiation [3, 16] (Figure 1). Th17 is involved in Crohn's disease [192], and the anti-$\alpha 4\beta 7$ integrin therapeutic antibody (vedolizumab) targets gut-homing Th17 [193]. Although a reduced Treg/Th17 balance is often associated with inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, the potential role of vitamin A or RA treatments is controversial [3].

IL-6 has a primary role in Th17 induction (Figure 1), and a recent meta-analysis reported that tomato supplementation was associated with significant reductions in IL-6 [136]. In a study using an animal model of ulcerative colitis (dextran sulfate sodium), β-carotene decreased colon IL-6 (5, 10, and 20 mg/kg), TNF-α (10 and 20 mg/kg), and IL-17 (20 mg/kg) and reduced plasma lipopolysaccharide [194]. On the other hand, intragastric lycopene administration (5 mg/kg [195]; 1, 2, and 4 mg/kg [196]) reduced TNF-α, IL-1β, IL-6, and/or TGF-β in a rat model of Alzheimer's disease and inhibited the β-amyloid-induced upregulation of TLR4 in the choroid plexus [195]. The effect on TGF-β has implication also in cancer (Figure 1). Lycopene inhibited TGF-β-induced migration, invasion, and adhesion activity of human liver adenocarcinoma SK-Hep-1 cells (2.5 μM) [197] and decreased TGF-β1 mRNA levels in fibroblasts [198]. On the contrary, the role of RA in cancer is controversial.

Despite that RA is required for the expansion of tumor-reactive CD8 T cells, the induction of the TGF-β-producing Treg may inhibit tumor immunosurveillance [188]. In this context, TGF-β reduced the expression of CYP26, inhibiting the breakdown of RA [3] (Figure 1). Therefore, non-provitamin A carotenoids could have anti-inflammatory properties without compromising cancer immunosurveillance and could not increase cancer risk as observed after β-carotene supplementation (Table 3). However, although the activity of β-carotene on immune function could be due to its conversion to vitamin A and RA [19], it has been suggested that apo-10′-lycopenoic acid (apo10LA), a BCO2 metabolite of lycopene, activates the RAR, reducing IL-6 and IL-1β [199]. In mice, APO10LA at 10 mg/kg diet for 24 weeks reduced diethylnitrosamine-initiated, high-fat diet-(HFD-) promoted hepatic tumorigenesis, lung tumor incidence, and hepatic TNF-α and IL-6 concentrations [200]. Data from BCO2-knockout (BCO2-KO) and wild-type mice suggest that IL-6 inhibition and chemoprevention could depend on BCO2 expression [201]. Therefore, the role of metabolites from non-provitamin A carotenoids deserves future investigation.

8. Conclusion

From the reviewed data, the total carotenoid intakes range from 5.42 to 15.44 mg/d (Table 2) and the suggested recommended intake range are 2–4.8 mg/d for β-carotene [34, 37], 10–20 mg/d for lutein, and 5.7–15 mg/d for lycopene [38]. Higher intakes from foods rather than supplementation with β-carotene have been associated with healthy effects (Table 3 and Table 4), whereas more promising results came from lycopene supplementations (Table 4). However, the majority of the available data came from epidemiological studies and meta-analysis that include few RCT (<15) [99, 100, 136], with a small sample size (<100), and no supplementation data on cancer risk is available. Therefore, large-scale intervention studies are warranted to substantiate the health effects of lycopene.

Despite the antioxidant activity of β-carotene, the major provitamin A carotenoid, its prooxidant activity in smokers and alcohol drinkers justifies its adverse effects in doses ranging from 20 mg/d to 30 mg/d [96–98, 143]. The overall mortality increased after β-carotene supplementation at doses >9.6 mg/d [104], and potential food/drug or supplements/alcohol interactions can be also taken into account due to competition for and/or induction of metabolism enzymes [10, 108–110].

On the contrary, non-provitamin A carotenoids could have a safer profile (20 mg/d for lutein, 75 mg/d for lycopene, and 53 mg/d for zeaxanthin) [13, 105] than β-carotene. The latter is converted to RA with immunomodulatory effects (Figure 1).

Human intervention studies that investigated the effects of carotenoids on immune function involve β-carotene, lycopene, or food sources and suggest that carotenoids affect immune function only after a depletion period and at doses (≥30 mg/d β-carotene and lycopene) (Table 5) higher than recommended intakes. Some effects, unrelated to carotenoids and retinol plasma levels, have been observed after the end of the supplementation period. Furthermore, results on lymphocyte subsets are conflicting. In this context, local production of RA can affect the GALT and lymphocyte gut homing. The effect of RA on T-helper subsets depends on local microenvironment and inflammatory status. In this context, although RA is the major active metabolite affecting the immune system, preclinical data suggest that lycopene metabolites derived from BCO2 can modulate immune function by reducing the inflammatory cytokine IL-6 (Figure 1). In this context, there is a growing interest in BCO2 metabolites [202] and it is well known that based on genetic polymorphisms of BCO1 it is possible to cluster subjects as strong responders or weak responders to carotenoids [203, 204]. BCO1 polymorphisms also affect non-provitamin A carotenoids, such as lutein [205, 206] and lycopene [206]. This body of evidence suggests that personalized nutrition/supplementation should be considered in the future.

On the other hand, preclinical studies suggest that the differential effect of RA and lycopene on TGF-β can account for the safer profile of lycopene in the context of cancer incidence (Figure 1).

However, on the light of the different effects of RA at physiological and pharmacological doses [3, 16] (Figure 1), more studies are needed in order to establish the therapeutic index for lycopene and caution must be taken to extrapolate preclinical data to clinical uses. Furthermore, the majority of human interventions report the effects of lycopene on immune function administering mixed supplements or tomato products with lycopene ranging from 15 to 47.1 mg (Table 5). These doses are near or over the higher value of the suggested recommended intake (5.7–15 mg/d) [38], raising a safety concern.

In conclusion, although lycopene supplementation for immune-regulation seems more promising than β-carotene, human studies with adequate power and duration are needed in order to confirm this hypothesis.

Conflicts of Interest

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

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Research Article

Evaluation of Oxidative Stress in Cardiomyocytes during the Aging Process in Rats Treated with Resveratrol

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The substantial increase in the number of elderly people in our societies represents a challenge for biology and medicine. The societies of the industrialized countries are subject to a progressive aging process that translates into an increase in the cardiovascular risk of the population. In the present work, the activity of catalase and superoxide dismutase was evaluated, as well as markers of oxidative stress (concentration of nitric oxide and total lipoperoxidation in its main components: malondialdehyde and 4-hydroxyalkene) in cardiomyocytes during the aging process in rats treated with resveratrol. Rats were divided into 4 groups according to the following categories: control (without treatment), negative control group (administered with physiological solution with 10% ethanol), positive control group (administered with vitamin E, 2 mg/kg/day), and group administered with resveratrol (10 mg/kg/day); these groups in turn were divided into 2, 4, 6, and 8 months of treatment. The analysis of nitric oxide showed a decreased level in the cardiac tissue in the groups treated with resveratrol; the same occurs when total lipoperoxidation is analyzed. The enzymatic activity studied (catalase and superoxide dismutase) did not present significant changes with respect to the controls. It is concluded that the cardioprotective effect of resveratrol is due to the antioxidant effect and other antiaging effects and not to the activation of the enzymes catalase and superoxide dismutase.

1. Introduction

The substantial increase in the number of elderly people in our societies represents a challenge for biology and medicine. The societies of the industrialized countries are subject to a progressive aging process that translates into an increase in the cardiovascular risk of the population. Aging is a multifactorial process in which numerous hypotheses have been postulated in order to explain the degenerative molecular processes that act in it [1, 2]. Harman in 1956 was the first to formulate the hypothesis that the biochemical processes generated largely by the cellular oxidative metabolism are responsible for numerous pathophysiological alterations and that could favor the molecular processes associated with aging [3].

The hypothesis of oxidative stress in aging refers to the completion of the genetic program that governs the sequence and duration of several ontogenetic phases and is linked to the expenditure of a defined sum of energy. The level of oxidative stress depends on the speed of generation of oxidants and antioxidant defense levels, which are genetically controlled but are also influenced by epigenetic factors. This oxidative stress exerts a regulatory influence on gene expression and is different at different stages of development [4].

Table 1: Main cardioprotective activities of resveratrol.

Activity	Concentration	Reference
Reduction of cardiovascular structural and functional deterioration in CHF.	5.0 mg/kg	Ahmet et al. [36]
Attenuation of postinfarct cardiac remodeling and contractile dysfunction.	2.5 mg/kg	Raj et al. [37]
Activation of a novel deacetylating pathway and attenuation of cardiac oxidative stress in diabetic heart.	10.0 mg/kg	Bagul et al. [38]
Induces autophagy and protects hearts from doxorubicin-induced toxicity.	5.0–50.0 mg/kg	Dutta et al. [39]
Prevents oxidative stress induced cardiomyocyte injury mainly by preserving the activities of critical antioxidant enzymes.	2.5 mg/kg	Movahed et al. [40]
Increase of expression of AK1 and IDPm on ventricular modeling.	1.0 mg/kg	Lin et al. [41]
Reverse of abnormalities in diastolic heart function associated with high-fat feeding in obese prone rats.	2.5 mg/kg	Louis et al. [42]
Suppression of sympathetic neural remodeling process after myocardial infarction.	1.0 mg/kg	Xin et al. [43]
Beneficial effects on myocardial function, coronary perfusion, EC function, and vascular tone.	10.0 mg/kg	Joshi et al. [44]
Protection against recurrent stroke.	25.0 mg/kg	Clark et al., [45]

Numerous studies show that, although the maximum lifetime could be altered by varying the metabolic rate, the total energy expended during life (metabolic potential) remains constant and is a characteristic of the species. A mechanism by which the metabolic rate influences the development and aging can be determined through modulations in the levels of oxidative stress [5].

Oxidative stress is a situation in which the cells are exposed to a prooxidant environment that can affect the homeostasis of the redox state. In parallel, the defensive antioxidant mechanisms were developed to counteract the action of reactive oxygen species (ROS) overgeneration, resulting in a tissue vulnerability against the action of these reactive molecules, which seem to participate in some degenerative processes of biological systems [6].

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a nonflavonoid natural polyphenol belonging to the family of stilbenes that is produced in 72 plant species in response to an exogenous factor such as UV radiation or pathogens such as bacteria or fungi. It consists of two aromatic rings joined by a methylene bridge [7].

The interest in the cardioprotective nature of resveratrol arose from the study by Douste-Blazy et al. [8], in which cardiovascular risk factors (1985–1987) were studied in two population samples from the French regions of Strasbourg and Toulouse. Resveratrol seems to be, among others, responsible for a nutritional fact called "French paradox", since the French are Europeans who eat more saturated fats and yet have a lower risk of cardiovascular disease than other Europeans, such as the English, who, like the French, follow a diet rich in saturated fats. Table 1 summarizes the activities and concentrations at which resveratrol presents cardioprotective activity [9].

From the end of the 80s, a group of biomarkers that directly or indirectly provide information on the concentration of different types of reactive oxygen species (ROS) and nitrogen (RNS) began to be introduced in the measurement of oxidative stress in the human organism [10]. It has even been suggested that there are specific biomarkers for certain diseases [11]. Some of the markers of oxidative stress are listed above.

Nitric oxide ($NO^{\bullet}$): increasing ROS concentrations decrease the amount of bioactive $NO^{\bullet}$ by chemical inactivation to form toxic peroxynitrite ($ONOO^{-}$). Peroxynitrite in turn can "uncouple" the NOS (nitric oxide synthase) endothelial enzyme to become a dysfunctional superoxide-generating enzyme, which contributes to vascular oxidative stress [12].

Malondialdehyde (MDA): it is a final product of oxidation that is generated after the oxidation of biological membranes. This compound is the most common of those known as lipoperoxides and is used as a marker of oxidative stress in plasma and tissues [13–16]. Some works on aging have analyzed the relationship of MDA with age, using MDA and other products of lipoperoxidation as direct markers of oxidative stress [17].

Catalase (CAT): CAT as a part of the antioxidant system is involved in the destruction of H_2O_2 generated during cellular metabolism. This enzyme is characterized by its high reaction capacity but relatively little affinity for the substrate [18].

Superoxide dismutase (SOD): these enzymes catalyze the conversion of the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), in one of the fastest catalyzed reactions known [19].

2. Materials and Methods

2.1. Experimental Animals. Male Wistar rats, 3 months old, were obtained from the Bioterio Claude Bernard of the Benemérita Universidad Autónoma de Puebla. The animals were kept under standard conditions of a bioterium with dark-light cycles of 12 hours and temperature of 21°C, with access to water and food ad libitum. For the experiment, they were divided into 4 groups according to their administration and divided into the following categories: control (without treatment), negative control group (administered with physiological solution with 10% ethanol), positive control group (administered with vitamin E, 2 mg/kg/day), and group administered with resveratrol (10 mg/kg/day), which were distributed to be treated during different periods of time (2, 4, 6, and 8 months). Vitamin E was chosen as a

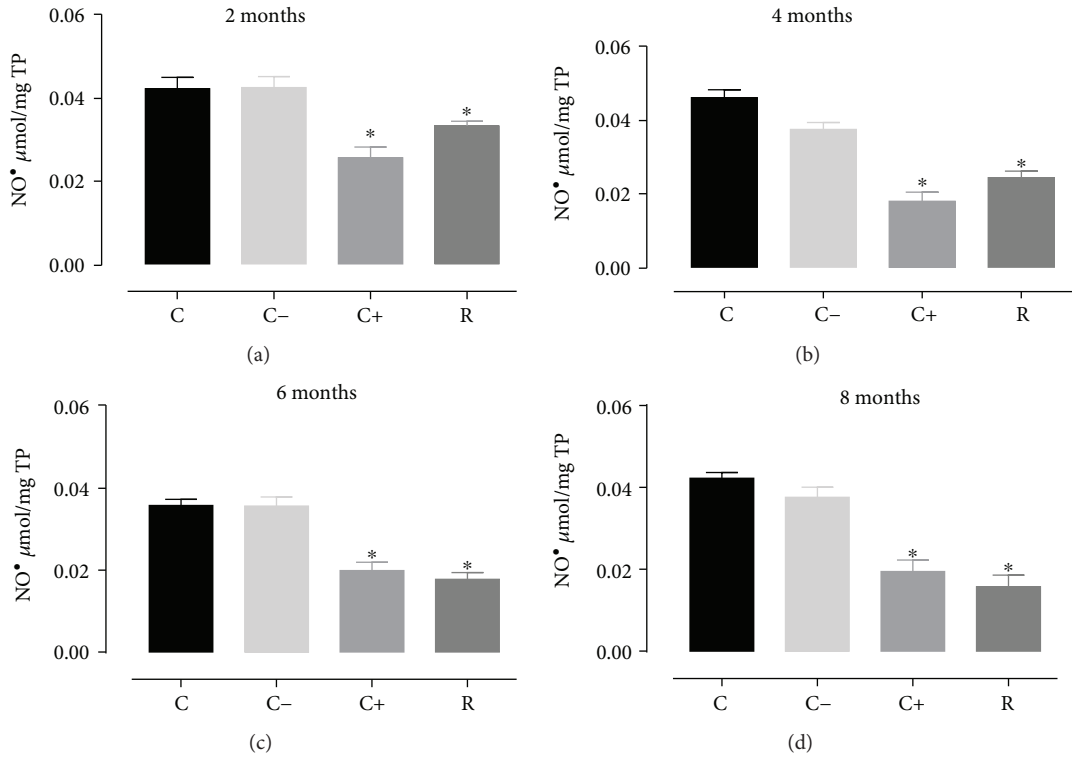

Figure 1: Concentration of nitric oxide in cardiomyocytes during the aging process (2, 4, 6, and 8 months) in rats treated with resveratrol. Results obtained with $n = 7$ animals per group analyzed in triplicate. A one-way ANOVA was realigned, followed by a Dunnett's multiple comparison test, $p < 0.05$. C: control, C−: negative control, C+: positive control, R: resveratrol. $^{*}p < 0.05$, statistically significant difference with respect to the control group.

positive control because it is considered the antioxidant par excellence for the human body; the doses used of vitamin E and resveratrol were the ones that reported the best results in the previous study [20].

The administration was carried out in all cases orally (cannula). The resveratrol corresponded to the trademark Lemi & Jo Resveratrol® *(Polygonum cuspidatum)*. All the procedures followed the rules according to the "Guide for the Care and Use of Laboratory Animals" of Mexico and approved by the Institutional Committee for the Care and Use of Animals. All efforts were focused to minimize the suffering of the animals.

2.2. Heart Homogenate Obtention. The hearth tissue was weighted and homogenized in PBS solution (pH 7.2–7.4) in a ratio 1 : 4, at 5340 g during 5 minutes in a Tissue-Tearor BioSpec mod. 985370, taking care of cold line in ice; one part of homogenate was separated for SOD activity, then the homogenate was centrifuged at 14850 *g* for 30 minutes in a cooled centrifuge, and the supernatant was separated for posterior analysis. The cold chain was maintained (−70°C) as much as possible, and dry ice was used for the thawing on the day the samples were worked. For the homogenate and the supernatant, ice was used to maintain at 4°C on the day of analysis.

2.3. Quantification Techniques

2.3.1. Quantification of Nitrites. $NO^{\bullet}$ was determined by nitrite concentration evaluation. The nitrites were measured by using the Griess reaction. Griess reagent was composed of equal volumes of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1.32% sulfanilamide in 60% acetic acid (Sigma). The colorimetric reaction was made in 100 μL of supernatant and 100 μL Griess reagent. The volume was adjusted to 1 mL by adding distilled water. The absorbance of the samples was determined at 540 nm in a spectrophotometer and compared to a standard curve of $NANO_2$ in each assay. Results were expressed as micromoles of nitrite per milligram of protein (μM of $NO_2^{-\bullet}$/mg of protein) [21].

2.3.2. Quantification of Total Proteins (TP). It was performed by the Sedmak and Grossberg method [22] using a standard curve of bovine serum albumin as standard. In the case of cardiomyocytes, the proteins were quantified in 1 μL of the supernatant of the homogenate plus 500 μL of the color reagent (Coomassie blue 0.06%), taking it to one mL with distilled water.

2.3.3. Quantification of Malondialdehyde (MDA) plus 4-Hydroxyalkene (4-HDA). 650 μL of N-methyl-2-phenylindole solution was dissolved in a mixture of acetonitrile : methanol (3 : 1), 200 μL of sample and was placed in a vortex for 3-4 seconds. 150 μL of 99% methanesulfonic acid was added and vigorously mixed; the tubes were covered, then incubated at 45°C for 40 minutes. They were cooled to room temperature and centrifuged for 15 minutes at 850 *g*. Finally, the absorbance at 586 nm was read against a reagent blank in a spectrophotometer (SpectrumVis SP1105) at 586 nm. The

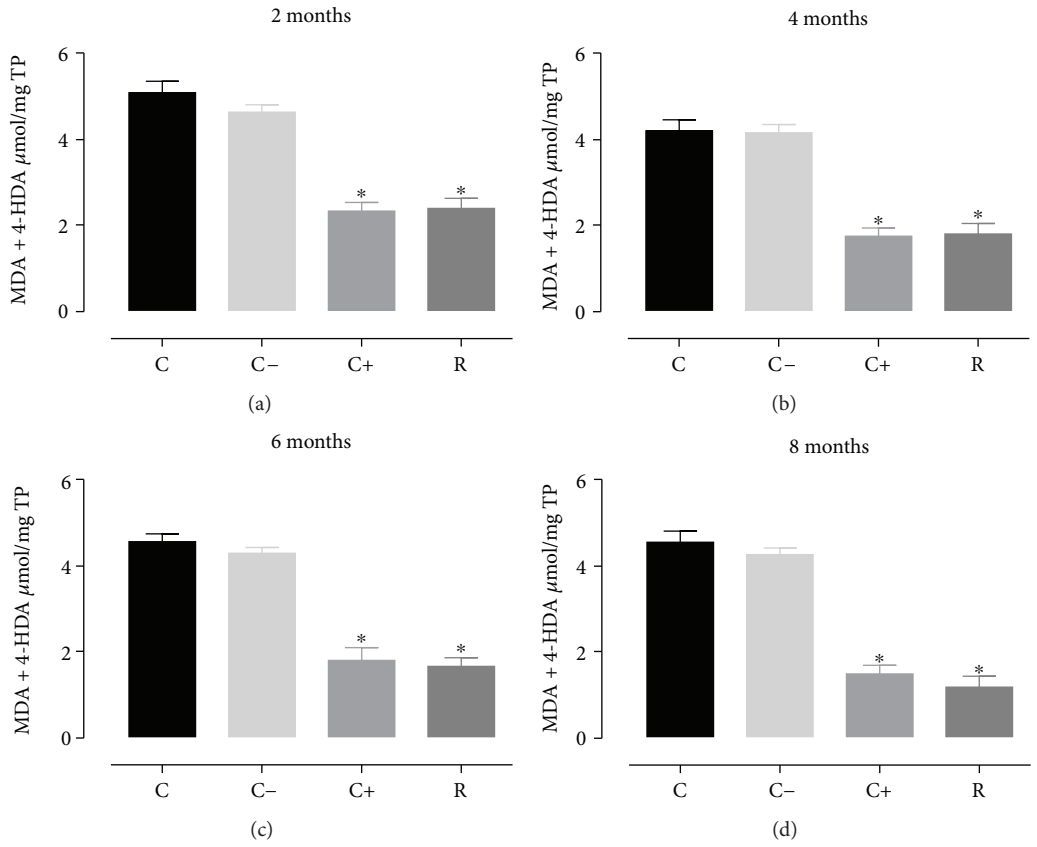

Figure 2: Concentration of MDA plus 4-HDA in cardiomyocytes during the aging process (2, 4, 6, and 8 months) in rats treated with resveratrol. Results obtained with $n = 7$ animals per group analyzed in triplicate. A one-way ANOVA was realigned, followed by a Dunnett's multiple comparison test, $p < 0.05$. C: control, C−: negative control, C+: positive control, R: resveratrol. $^{*}p < 0.05$, statistically significant difference with respect to the control group.

concentration of MDA plus 4-HDA was determined by interpolating the optical density of the samples on a standard curve of 1,1,3,3-tetramethoxypropane (0.5 to 10 μL), which was determined in parallel in each trial.

2.3.4. Quantification of Malondialdehyde (MDA). 650 μL of N-methyl-2-phenylindole solution was dissolved in a mixture of acetonitrile : methanol (3 : 1), 200 μL of sample and was placed in a vibro agitator for 3-4 seconds. 150 μL of 35% of hydrochloric acid was added and vigorously mixed; the tubes were covered, then incubated at 45°C for 60 minutes. They were cooled to room temperature and centrifuged for 15 minutes at 850 g. Finally, the absorbance at 586 nm was read against a reagent blank in a spectrophotometer (SpectrumVis SP1105) at 586 nm. The concentration of MDA plus 4-HDA was determined by interpolating the optical density of the samples on a standard curve of 1,1,3,3-tetramethoxypropane (0.5 to 10 μL), which was determined in parallel in each trial.

The concentration of 4-hydroxyalkenals was calculated as the difference between the concentrations obtained using methanesulfonic acid minus the concentration obtained using hydrochloric acid [23].

2.3.5. Catalase Activity in Cardiomyocytes. 34 μL of supernatant of cardiomyocytes was mixed in a quartz cuvette with 333 μL of hydrogen peroxide; 30 mM plus 50 mM phosphate buffer, pH 7.4, were added. During 2 minutes of reaction, the difference in optical density per minute at 240 nm was determined and compared to a blank without reaction [24].

2.3.6. Superoxide Dismutase (SOD). 100 μL of cardiomyocyte homogenate was mixed in a quartz cuvette with 2.8 mL of tris-HCL buffer solution; 8.20 and 50 μL of EDTA solution were added. 50 μL of the pyrogallol solution was then added, and after 10 seconds of reaction, the optical density difference per minute at 420 nm was determined, against a blank without reaction.

2.4. Statistical Analysis. The results are the mean of 7 animals analyzed in triplicate ± the standard deviation (SD). One-way ANOVA and Dunnett's multiple comparison test were used as a posttest to evaluate statistically significant differences with respect to the control group for MDA, MDA plus 4-HDA, 4-HDA, and nitric oxide. An unpaired parametric Student t-test analysis of two stems was used for enzymatic activities. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

There are still numerous challenges ahead in relation to understanding aging [25, 26]; nevertheless, although the aging process is complex and multifactorial, López-Otín

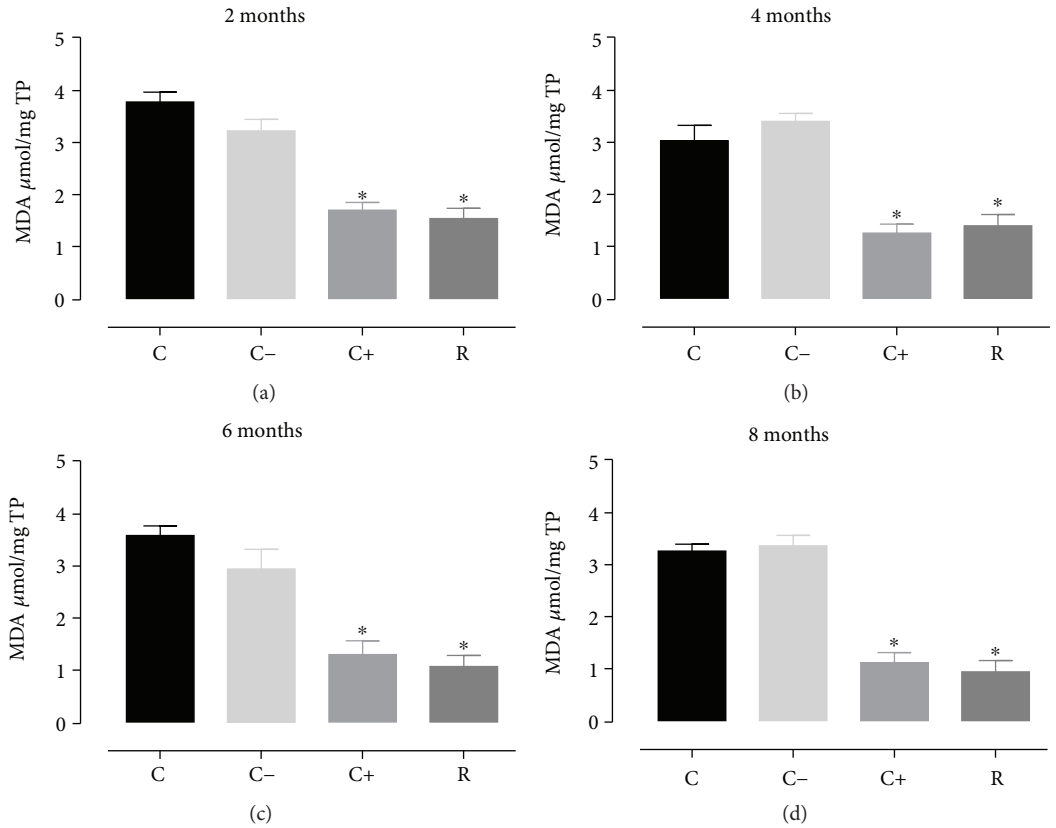

Figure 3: Concentration of MDA in cardiomyocytes during the aging process (2, 4, 6, and 8 months) in rats treated with resveratrol. Results obtained with $n = 7$ animals per group analyzed in triplicate. A one-way ANOVA was realigned, followed by a Dunnett's multiple comparison test, $p < 0.05$. C: control, C−: negative control, C+: positive control, R: resveratrol. $^{*}p < 0.05$, statistically significant difference with respect to the control group.

et al. [27] described nine hallmarks that could explain this process: genomic instability, telomere wear, epigenetic alterations, proteostasis loss, dysregulated nutrient detection, dysfunction mitochondrial, cellular senescence, depletion of stem cells, and alteration of intercellular communication. The interesting thing would be that every one of these theories converges at some point on oxidative stress. For example, the mitochondrial theory of free radicals of aging proposes that progressive mitochondrial dysfunction occurs with aging resulting in increased production of ROS, which in turn causes greater mitochondrial deterioration and overall cellular damage [28].

Oxidative stress produces changes in the generation of free radicals that accumulate in the aging process; one of the most affected organs is the heart, producing damage in the cardiomyocytes [29], so it was decided to analyze the $NO^{\bullet}$ which normally is a protective vasodilator agent and a second messenger, when is overgenerated in some situations such as aging process, heart and brain ischaemia, hypertension among others is a NRS (nitrogen reactive species) generator of oxidative stress [30]. The results obtained showed a significant decrease ($p < 0.05$) of nitric oxide in all treated groups. The vitamin E group (positive control) tended to decrease nitric oxide levels more, but after 8 months of treatment, both groups (positive control and resveratrol) had the same effect (Figure 1). As observed, the prolonged treatment with resveratrol consecutively decreased nitric oxide levels until 8 months of treatment, when compared with vitamin E (positive control); vitamin E has better antioxidant effect until reaching 8 months of treatment. These results showed that in the longer treatment time (during the aging process), the resveratrol decreases the concentration of $NO^{\bullet}$ in cardiac cells, from 18.49% decrease in the group of 2 months of treatment to 62.67% decrease in the group of 8 months of treatment with resveratrol.

The decreased concentrations of nitric oxide were also accompanied by the total lipoperoxidation products, which are here represented by its main components malondialdehyde plus 4-hydroxyalquenals during the aging process in rats treated with resveratrol. When reactive species are not scavenged by antioxidant systems, they induce harmful processes to cells such as lipoperoxidation, which involves the conversion of polyunsaturated fatty acids into highly reactive aldehydes [29] which in turn increase oxidative stress. Figure 2 shows that during the aging process the rats treated with resveratrol presented a significantly decreased production ($p < 0.05$) of the total lipoperoxidation products with respect to the control group and negative control; there were no differences in the resveratrol group with respect to the positive control (vitamin E).

With respect to malondialdehyde, it was observed that the aging process in rats treated with resveratrol and the

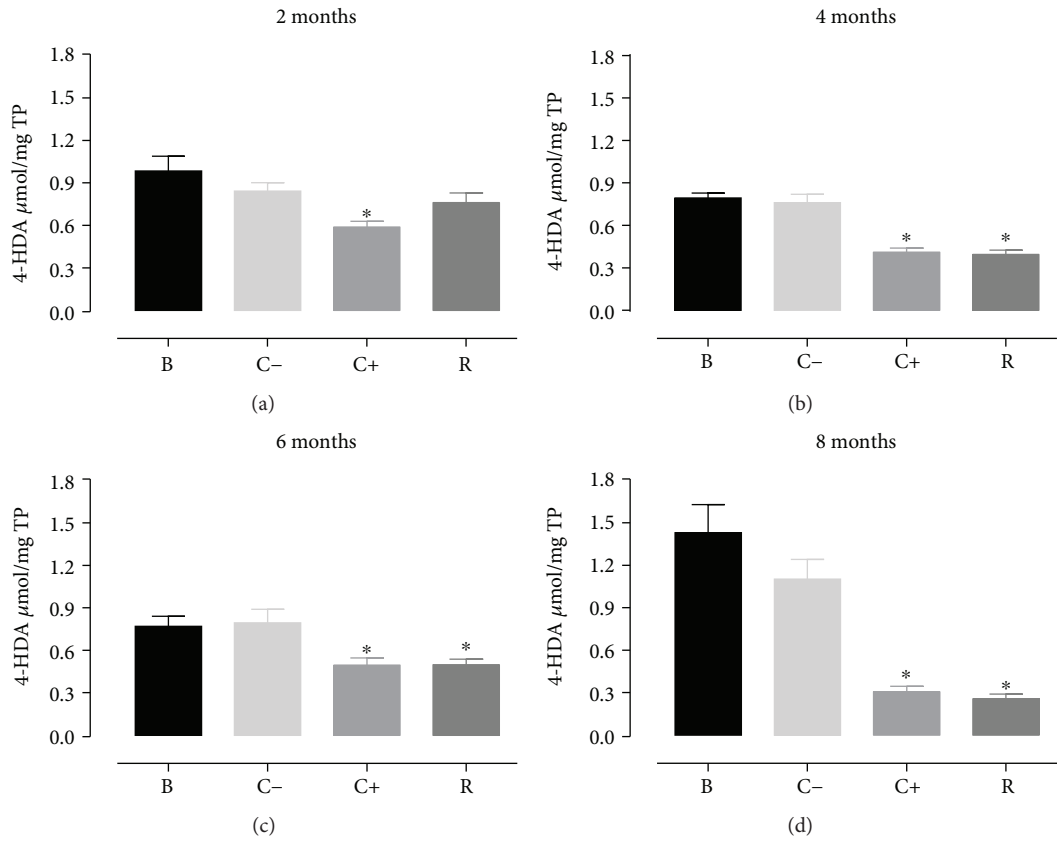

Figure 4: Concentration of 4-HDA in cardiomyocytes during the aging process (2, 4, 6, and 8 months) in rats treated with resveratrol. Results obtained with $n = 7$ animals per group analyzed in triplicate. A one-way ANOVA was realigned, followed by a Dunnett's multiple comparison test, $p < 0.05$. B: white, C−: negative control, C+: positive control, R: resveratrol. $^{*}p < 0.05$, statistically significant difference with respect to the control group.

rats treated with vitamin E showed significant decreases in the generation of malondialdehyde (Figure 3) but without differences between these two groups, presenting a similar situation in the levels of 4-hydroxyalkenals (Figure 4).

Regarding the CAT and the SOD, contrasting to results found by Hosoda et al. [31] who reported the decrease of intracellular ROS through the induction of SOD, our findings showed that there were no differences in the levels of SOD or catalase during the study period; however, this must be carefully analyzed, since it is known that when an antioxidant supplementation is used to compensate systemic oxidative stress promoted by an imbalance derived from various oxidants, most of the main antioxidant enzymes are negatively regulated and/or are not activated [32].

No differences were observed neither with respect to the negative control nor with the target (Tables 2 and 3), which shows that the activity of these two enzymes does not change significantly during the aging process and the administration of an exogenous antioxidant (such as resveratrol or vitamin E), but it should also be considered that many studies have been carried out administering mega doses of vitamin E [33] or resveratrol [34]. We worked at resveratrol levels of 10 mg/kg of weight and the amount of vitamin E needed (in this case 2 mg/kg) to cover the recommended daily intakes of vitamin E in rats; the objective was to observe the effect of a chronic dose and not an acute one on oxidative stress.

The comparison of the negative control group (10% ethanol) with the target group did not show significant differences in any of the months of treatment, so it is concluded that the vehicle does not interfere in the normal aging process.

4. Conclusion

The administration of resveratrol during the aging process in rat cardiomyocytes showed a significant decrease in the markers of nitric oxide oxidative stress and total lipoperoxidation. The major lipoperoxidation product in heart was malondialdehyde compared to 4-hydroxyalkenals probably due to the presence of the ω group fatty acids. The administration of resveratrol during the aging process could help to decrease levels of oxidative stress during the aging process.

Since there are no significant differences in the activity of the enzymes catalase and superoxide dismutase with respect to their targets in cardiomyocytes during the aging process in rats treated with resveratrol, it could be concluded that the antioxidant activity exerted by resveratrol in

Table 2: Catalase activity during aging in rats treated with resveratrol.

	Data				*P* value				
	B	C−	C+	R	C− versus B	C+ versus B	R versus B	C− versus R	R versus C+
2 months									
Mean	83.60	84.13	87.17	92.02	0.882	0.391	0.176	0.148	0.392
Std. deviation	8.47	3.70	6.40	12.98					
4 months									
Mean	51.03	51.6	47.18	44.63	0.908	0.413	0.202	0.596	0.202
Std. deviation	8.59	9.61	8.38	9.14					
6 months									
Mean	62.48	59.98	59.42	53.26	0.465	0.581	0.060	0.209	0.050
Std. deviation	8.27	2.93	11.63	7.48					
8 months									
Mean	33.25	35.76	32.17	34.75	0.396	0.690	0.548	0.600	0.174
Std. deviation	5.37	3.58	3.47	2.56					

B: blank group, C−: negative control group (ethanol 10%), C+: positive control (vitamin E 2 mg/kg), R: resveratrol group (10 mg/kg).

Table 3: Superoxide dismutase activity during aging in rats treated with resveratrol.

	Data				*P* value				
	B	C−	C+	R	C− versus B	C+ versus B	R versus B	C− versus R	R versus C+
2 months									
Mean	15.11	14.34	14.24	15.63	0.228	0.128	0.492	0.124	0.094
Std. deviation	1.08	1.12	0.51	1.60					
4 months									
Mean	15.83	15.57	17.21	16.56	0.821	0.184	0.484	0.260	0.313
Std. deviation	2.36	1.84	1.06	1.24					
6 months									
Mean	14.71	15.91	16.04	15.81	0.088	0.142	0.195	0.880	0.780
Std. deviation	1.52	0.78	1.66	1.48					
8 months									
Mean	14.99	15.70	15.75	15.80	0.160	0.120	0.186	0.929	0.931
Std. deviation	0.66	0.90	0.92	1.28					

B: blank group, C−: negative control group (ethanol 10%), C+: positive control (vitamin E 2 mg/kg), R: resveratrol group (10 mg/kg).

cardiomyocytes during the process of aging in rats treated with resveratrol is not due to the activation of the enzymes catalase and superoxide dismutase but apparently to the direct antioxidant effect of resveratrol (and vitamin E). However, this result should be analyzed from several perspectives, since the molecular basis of the pharmacological effects of resveratrol is its multiple effects, ranging from a direct physical interaction to indirect modulations such as in the expression levels [35].

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Research Article

Cafestol Inhibits Cyclic-Strain-Induced Interleukin-8, Intercellular Adhesion Molecule-1, and Monocyte Chemoattractant Protein-1 Production in Vascular Endothelial Cells

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Moderate coffee consumption is inversely associated with cardiovascular disease mortality; however, mechanisms underlying this causal effect remain unclear. Cafestol, a diterpene found in coffee, has various properties, including an anti-inflammatory property. This study investigated the effect of cafestol on cyclic-strain-induced inflammatory molecule secretion in vascular endothelial cells. Cells were cultured under static or cyclic strain conditions, and the secretion of inflammatory molecules was determined using enzyme-linked immunosorbent assay. The effects of cafestol on mitogen-activated protein kinases (MAPK), heme oxygenase-1 (HO-1), and sirtuin 1 (Sirt1) signaling pathways were examined using Western blotting and specific inhibitors. Cafestol attenuated cyclic-strain-stimulated intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein- (MCP-) 1, and interleukin- (IL-) 8 secretion. Cafestol inhibited the cyclic-strain-induced phosphorylation of extracellular signal-regulated kinase and p38 MAPK. By contrast, cafestol upregulated cyclic-strain-induced HO-1 and Sirt1 expression. The addition of zinc protoporphyrin IX, sirtinol, or Sirt1 silencing (transfected with Sirt1 siRNA) significantly attenuated cafestol-mediated modulatory effects on cyclic-strain-stimulated ICAM-1, MCP-1, and IL-8 secretion. This is the first study to report that cafestol inhibited cyclic-strain-induced inflammatory molecule secretion, possibly through the activation of HO-1 and Sirt1 in endothelial cells. The results provide valuable insights into molecular pathways that may contribute to the effects of cafestol.

1. Introduction

Cardiovascular disease (CVD) has a high mortality rate worldwide and has become a critical health concern, particularly in consideration of population aging. An epidemiological study suggested that moderate coffee consumption is inversely related to death due to CVD [1]. However, research evaluating mechanisms underlying the favorable relationship between coffee consumption and reduction in risk factors for CVD is extremely limited. Endothelial inflammation is

associated with a high risk of adverse cardiovascular events [2, 3] and plays a key role in the development of CVD [4]. Several natural compounds present in coffee, such as phenolic compounds, flavonoids, and caffeic acid derivatives, have been reported to possess an anti-inflammatory property [5]. This anti-inflammatory property is likely responsible for the favorable relationship between coffee consumption and a low CVD mortality rate [6, 7]. Among the natural compounds, cafestol, a diterpene molecule found in the cherries of *Coffea arabica*, possesses various properties, including anti-inflammatory [8, 9] and antiangiogenic properties [10, 11]. However, the effect of cafestol on vascular endothelial cells remains to be clarified.

Chronic inflammation in endothelial cells produces various inflammatory mediators that exacerbate endothelial dysfunction [12]. Endothelial dysfunction caused by inflammation plays a dominant role in the pathogenesis of CVD, including atherosclerosis, hypertension, and diabetes-induced vasculopathy and vascular remodeling [13, 14]. Vascular endothelial cells are permanently exposed to mechanical stretching. Mechanical stretching, particularly cyclic strain, modulates the function of vascular endothelial cells by regulating the expression of many genes. In vascular endothelial cells, cyclic strain has been shown to increase reactive oxygen species (ROS) production, leading to the upregulation of cell adhesion molecules and cytokines [15, 16]. In addition, the cyclic straining of endothelial cells activates several proteins involved in the regulation of gene expression, including mitogen-activated protein kinases (MAPK) [17]. The MAPK family includes extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38, which are believed to be among the major regulators of proatherogenic inflammatory gene expression in endothelial cells. Adhesion molecules and inflammatory cytokines regulated by cyclic strain have been identified in endothelial cells, including intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein- (MCP-) 1, and interleukin- (IL-) 8 [15, 16, 18]. Adhesion molecules and inflammatory cytokines may play a pivotal role in the pathogenesis of CVD [19]. However, the effects of cafestol on cyclic-strain-stimulated inflammatory molecule production remain unclear.

Many natural dietary compounds are believed to provide protection against oxidative stress, and a few compounds have been reported to induce genes involved in antioxidant defense through the activation of nuclear E2-related factor 2 (Nrf2) or sirtuin 1 (Sirt1) [20, 21]. The MAPK family plays an essential role in the transduction of extracellular signals to cellular responses through a cascade of phosphorylation events [22]. Cyclic strain stimulated Nrf2 expression, resulting in the subsequent expression of antioxidant enzymes, such as heme oxygenase-1 (HO-1), in stretched endothelial cells [23]. The transcription factor Nrf2 alleviates cyclic-strain-induced IL-8 expression by upregulating the expression of HO-1 [24]. We recently reported that cafestol inhibits urotensin II-induced IL-8 expression and cell proliferation via Nrf2/HO-1-dependent mechanism in endothelial cells [25]. In addition, Sirt1 is the human ortholog of the yeast silent information regulator 2 (Sir2) protein that extends the lifespan of lower organisms [26]. By interacting with several target proteins, Sirt1 performs various cellular functions including endothelial protection from vascular diseases [27]. Sirt1 exerts anti-inflammatory effects through the modulation of cytokine levels in human umbilical vein endothelial cells (HUVECs) [28]. Several natural and synthetic compounds activate Sirt1 and promote endothelial homeostasis [27, 29]. However, the effect of cafestol on MAPK, HO-1, and Sirt1 expression in cyclic-strain-activated vascular endothelial cells remains to be determined. In this study, we investigated the effects of cafestol on the modulation of cyclic-strain-stimulated inflammatory cytokine production and identified the intracellular mechanism that may be responsible for the putative effects of cafestol.

2. Material and Methods

2.1. Antibodies and Reagents. Pure cafestol (dissolved in dimethyl sulfoxide) and all other chemicals of the reagent grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). All enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcam (Cambridge, UK). Antibody-directed phosphorylated ERK, phosphorylated p38, and phosphorylated JNK antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA, USA). Anti-MCP-1 was purchased from Sigma-Aldrich. Anti-ERK, anti-p38, anti-JNK, anti-HO-1, anti-Sirt1, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Endothelial Cell Culture. HUVECs were obtained from PromoCell (Heidelberg, Germany), as previously described [30]. All endothelial cells used in this study were from the third to fourth passages.

2.3. In Vitro Cyclic Strain on Cultured Endothelial Cells. Endothelial cells cultured on a flexible membrane base were subjected to cyclic strain produced by a computer-controlled application of sinusoidal negative pressure, as described previously [31].

2.4. ELISA of Proinflammatory Molecules. For the detection of tumor necrosis factor-α (TNF-α), ICAM-1, MCP-1, IL-6, and IL-8 in the supernatant, cells were treated with and without cafestol for 12 h and then treated with cyclic strain for 24 h. After centrifugation at 1000 rpm for 10 min, the supernatant was collected to measure TNF-α, ICAM-1, MCP-1, IL-6, and IL-8 levels in the cell medium through ELISA. Commercially available ELISA kits (Abcam, Cambridge, UK) were used according to the manufacturer's protocol [30].

2.5. Intracellular ROS Analysis. Cellular ROS were analyzed using the fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (Thermo Fisher Scientific, Waltham, MA, USA), which passively diffuses into the cell and is cleaved and oxidized to 2′,7′-dichlorofluorescein (DCF), as described previously [32].

2.6. RNA Extraction and Quantitative Polymerase Chain Reaction Analysis. Total RNA was extracted from vascular endothelial cells by using the TRIzol method according to

the protocol recommended by the manufacturer (Thermo Fisher Scientific). The extracted RNA was used to synthesize single-stranded complementary (c)DNA by using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), as described previously [30]. HO-1 messenger (m)RNA was quantified using TaqMan Gene Expression Master Mix (Applied Biosystems) with specific primers in an ABI 7300 Real-Time PCR System (Applied Biosystems). TaqMan gene expression assay kits containing specific primers for HO-1 and GAPDH were obtained from Applied Biosystems. Specific primers for GAPDH were used to normalize the amount of the sample added. Samples were quantified in triplicate during three separate experiments.

2.7. Western Blot Analysis. After each experiment, cells were washed twice with cold PBS and harvested in 150 μL of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/L leupeptin, 1 mg/L aprotinin, and 2 mM dithiothreitol). Cell homogenates were centrifuged at 10,000 ×g for 20 min at 4°C. The resulting supernatant was used as a cellular protein. Samples containing 40 μg cellular proteins were resolved by electrophoresis and then transferred to nitrocellulose membranes. Western blot analysis was performed as described previously [30]. The data of protein bands on Western blots were quantified using ImageJ densitometry analysis software (National Institutes of Health, Bethesda, MD, USA).

2.8. Sirt1 Short Interfering (si) RNA Transfection. Sirt1 siRNA and control siRNA obtained from Santa Cruz were transfected using the Lipofectamine reagent, and the experiments were performed as previously described [24].

2.9. Statistical Analysis. All experiments were repeated at least three times. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using Student's *t*-test or analysis of variance, where appropriate, followed by Dunnett's multiple comparison test, by using Prism Version 3.0 for Windows (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of Cafestol on ICAM-1, MCP-1, and IL-8 Secretion in Cyclic-Strain-Treated HUVECs. Endothelial cells cultured on flexible membrane bases were subjected to deformation to produce an average level of strain (−20 kPa, 1 Hz). The levels of cytokines released into culture media were measured. Figure 1(a) shows the mean levels of cytokines measured using ELISA in three separate experiments. The levels of TNF-α and IL-6 were not affected by cyclic strain treatment for 24 h. By contrast, the levels of IL-8, ICAM-1, and MCP-1 increased significantly after the application of cyclic strain for 24 h compared with static control cells. To evaluate the effects of cafestol on inflammatory protein expression in cyclic-strain-treated HUVECs, MCP-1 protein expression was detected using Western blot analysis. As shown in Figure 1(b), cyclic strain treatment increased MCP-1 protein expression, and cafestol (3 and 10 μM) attenuated this increase in MCP-1 protein expression. Next, we evaluated the effect of cafestol on the secretion of the inflammatory proteins MCP-1, ICAM-1, and IL-8 by using ELISA. As shown in Figures 1(c)–1(e), pretreatment with cafestol (3 and 10 μM) for 12 h significantly inhibited cyclic-strain-induced ICAM-1, IL-8, and MCP-1 protein secretion.

3.2. Antioxidative Effects of Cafestol on Cyclic-Strain-Induced ROS, ICAM-1, MCP-1, and IL-8 Production. Increased ROS production in response to cyclic strain in HUVECs has been described [15, 16, 31]. Therefore, we examined ROS production in HUVECs in response to cyclic strain. Exposure to cyclic strain for 2 h led to the intracellular accumulation of ROS. Following the validation of cyclic-strain-dependent DCF fluorescence, we evaluated whether cyclic-strain-induced ROS production and inflammatory molecule protein secretion could be reduced through ROS inhibition by cafestol. As shown in Figure 2(a), the induction of ROS production by cyclic strain was prevented by pretreatment with the antioxidant *N*-acetylcysteine (NAC) and cafestol. Moreover, pretreatment with NAC and cafestol blocked the production of inflammatory molecules, including IL-8, ICAM-1, and MCP-1, in response to cyclic strain (Figure 2(b)). These results suggest that cafestol inhibits cyclic-strain-induced IL-8, ICAM-1, and MCP-1 production through ROS inhibition.

3.3. Cafestol Inhibits Cyclic-Strain-Activated MAPK Signaling Pathways. The cyclic straining of endothelial cells activates several proteins, including MAPK, which are believed to be among the major regulators of inflammatory gene expression [33]. HUVECs were treated with cyclic strain for different time periods, and cell lysates were immunoblotted with specific antibodies. As shown in Figures 3(a)–3(c), cyclic strain induced the phosphorylation of ERK, JNK, and p38 with a peak at 30 min in HUVECs. To investigate how cafestol affects cyclic-strain-induced MAPK phosphorylation, HUVECs were treated with 10 μM cafestol for 12 h prior to cyclic strain treatment for 30 min. Figure 3(d) shows that cafestol treatment (10 μM) significantly prevented the cyclic-strain-induced phosphorylation of ERK and p38. These results indicate that the inhibition of MAPK signaling pathways may be associated with the modulatory effect of cafestol on cyclic-strain-treated HUVECs.

3.4. Cafestol Enhances HO-1 Expression in Cyclic-Strain-Treated Endothelial Cells. Natural products have been demonstrated to activate HO-1 to inhibit cyclic-strain-induced IL-8 expression in vascular endothelial cells [24]. We investigated how cafestol affected HO-1 expression in the presence of cyclic strain. Figure 4(a) shows that cyclic strain treatment only slightly stimulated HO-1 mRNA expression, and pretreatment with cafestol (10 μM) enhanced HO-1 upregulation. Figure 4(b) shows that parallel to results observed in mRNA expression, the treatment of HUVECs with cyclic strain for 12 h slightly upregulated HO-1 protein expression and cafestol enhanced HO-1 protein expression. To further investigate whether decreased inflammatory molecule protein expression observed in cafestol-pretreated cells was

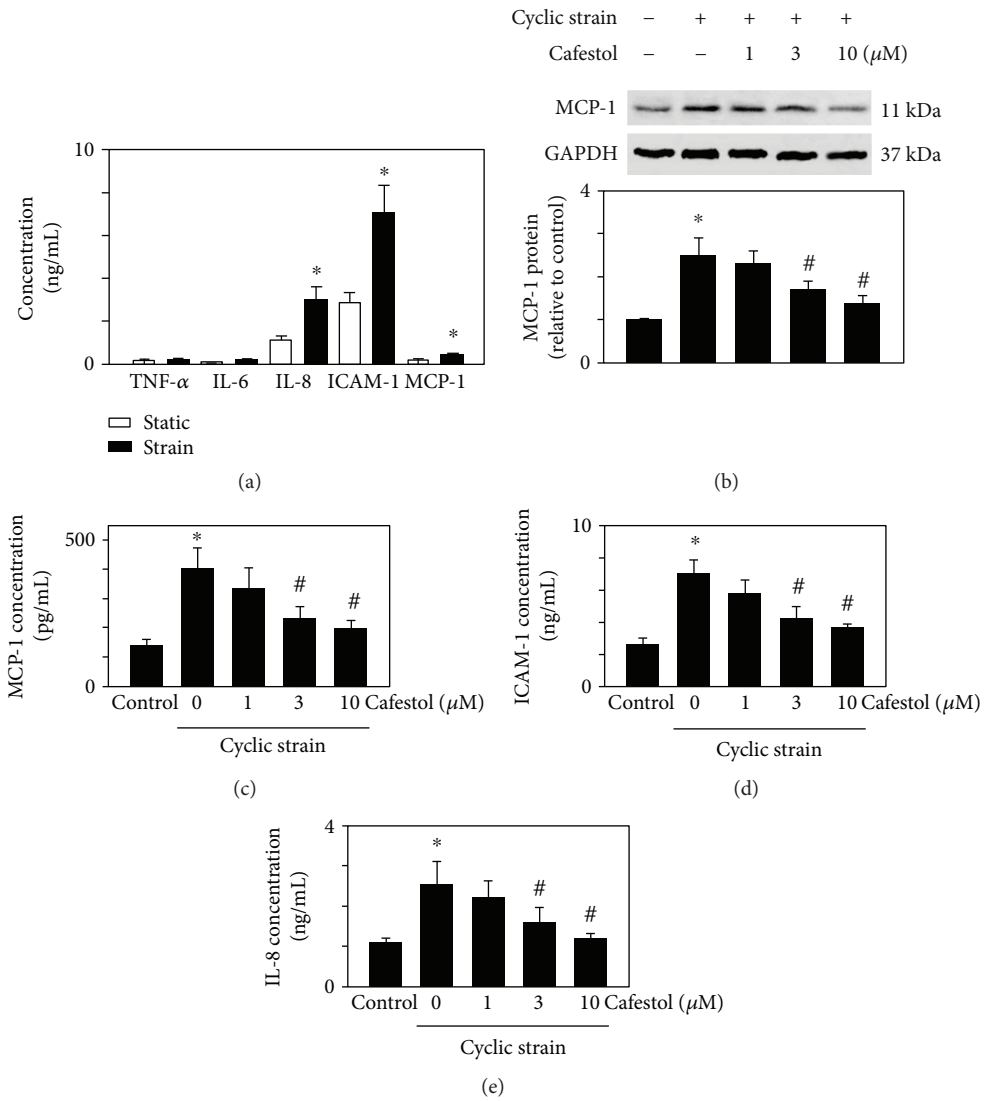

Figure 1: Cafestol inhibits cyclic-strain-induced inflammatory molecule secretion in HUVECs. HUVECs grown on Flexcell plates were subjected to cyclic strain for 24 h. Control cells were left under static conditions. (a) Effect of cyclic strain (−20 kPa) applied for 24 h on the production of cytokines. Values are the mean ± SEM ($n = 3$). $^*P < 0.05$ versus static controls. (b) MCP-1 expression was detected using Western blot analysis with the corresponding antibody. GAPDH staining was used as a normalization control. The upper panels are representative of three independent experiments. Lower panel: the bar graph shows the fold increase in protein expression compared with static control cells. Soluble MCP-1 (c), ICAM-1 (d), and IL-8 (e) production in culture media was evaluated using ELISA; $n = 4$, $^*P < 0.05$ versus the static control group. $^\#P < 0.05$ versus the cyclic strain group.

dependent on HO-1 activity, HUVECs were treated with zinc protoporphyrin IX (ZnPP), a potent competitive inhibitor of HO enzyme activity, for 30 min, followed by cafestol for 12 h before exposure to cyclic strain for 24 h. Figure 4(c) shows that the addition of ZnPP (1 μM) attenuated the cafestol-mediated modulatory effect. These results indicate that HO-1 activity may participate in the inhibitory effect of cafestol on cyclic-strain-induced IL-8, ICAM-1, and MCP-1 production in HUVECs.

3.5. Effects of Sirt1 Activation by Cafestol and Sirt1 Inhibition on Cyclic-Strain-Induced Inflammatory Molecule Protein Secretion. Accumulating evidence indicates that Sirt1 plays a crucial role in cardiovascular cell function in aging and disease [27]. A recent study showed that Sirt1 expression decreased in aged and atherosclerotic vessels *in vivo* [34]. On the basis of these results, we examined whether the modulation of inflammatory molecule production by cafestol in cyclic-strain-stimulated cells is mediated by Sirt1. Cafestol upregulated the Sirt1 protein level in cyclic-strain-treated HUVECs (Figure 5(a)). By contrast, the Sirt1 inhibitor sirtinol attenuated the induction of Sirt1 by cyclic strain but enhanced the expression of ICAM-1, IL-8, and MCP-1 proteins (Figure 5(b)). The role of Sirt1 in the inhibition of cyclic strain-induced expression of ICAM-1, IL-8, and MCP-1 by cafestol was also examined by the silencing of Sirt1. Cells transfected with Sirt1 siRNA, followed by treatment with cafestol (10 μM, 12 h), abrogated the inhibitory effect of

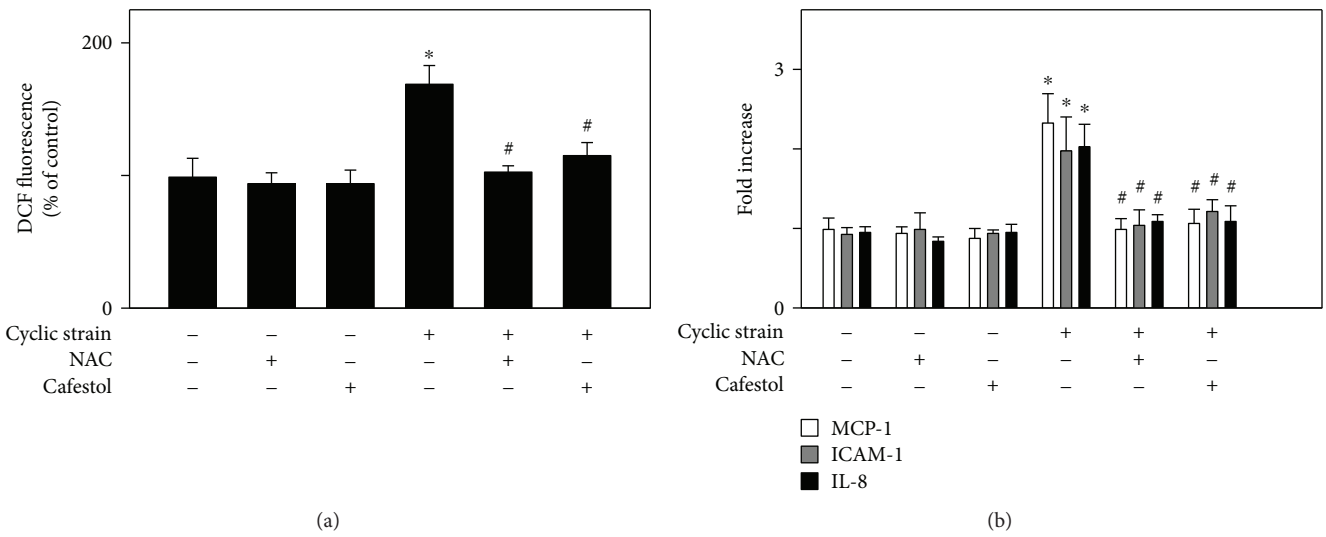

Figure 2: Antioxidative effect of cafestol on cyclic-strain-induced ROS production and inflammatory molecule secretion in HUVECs. Cells were pretreated with cafestol (10 μM) for 12 h or NAC (10 mM) for 2 h and then exposed to cyclic strain for 24 h. (a) ROS production was assayed using DCF. (b) Protein expression levels were examined using ELISA. The bar graph shows the fold increase in protein secretion compared with control cells. Results are shown as the mean ± SEM ($n = 5$). $^{*}P < 0.05$ versus untreated controls; $^{\#}P < 0.05$ versus cells exposed to cyclic strain.

cafestol on the cyclic strain-induced expression of ICAM-1, IL-8, and MCP-1 secretion. In contrast, the control siRNA (100 nM) failed to block the inhibitory effect of cafestol. These results suggest that the effect of cafestol is linked to upregulated Sirt1 expression, and the inhibitory effect of cafestol on cyclic-strain-induced ICAM-1, IL-8, and MCP-1 protein secretion in endothelial cells at least partially depends on Sirt1.

4. Discussion

According to the literature review, moderate coffee consumption appears to be safe and is associated with neutral to beneficial effects on most of the studied health outcomes [35]. The major natural products in coffee that partly explain its beneficial effects are diterpenes, such as cafestol [36]. Natural products with advantages such as a potent anti-inflammatory effect and high availability have received considerable attention in recent years [37]. Inflammatory disorders usually involve many complicated mechanisms and pathways; therefore, only one drug is not adequate for treating inflammation. The use of a combination of drugs is a practical and beneficial approach. Natural products usually have multiple target interactions and have a strong therapeutic effect; therefore, combinations of natural compounds are expected to be useful in the treatment of acute and chronic inflammatory diseases in practice [38]. The role of hemodynamic forces in the pathogenesis of CVD is receiving increasing attention. Because the chemokines ICAM-1, MCP-1, and IL-8 regulate immune cell adhesion and integration with endothelial cell processes [39], they can become potential therapeutic targets. Our experiments demonstrated that among the several cytokines examined, mechanical stretching enhances the production of IL-8, MCP-1, and ICAM-1 in human endothelial cells. MCP-1, which exhibits potent monocyte chemotactic activity, is believed to be one of the crucial molecules involved in CVD [40]. The increased surface expression of ICAM-1 by cyclic-strain-activated vascular endothelial cells and local production of IL-8 may provoke leukocyte chemotaxis to the overinflated regions of the vessel and cause additional damage to the vasculature, leading to the exacerbation of vascular injury [41]. These observations may provide an explanation for an early link between the mechanical stretching of vascular walls and the prediction of CAD risk. In this study, we observed that cafestol acted as a potent inhibitor of cyclic-strain-stimulated ICAM-1, MCP-1, and IL-8 production in endothelial cells. These findings further support the anti-inflammatory effect of cafestol.

Cyclic strain induced ROS production *in vitro* [15, 16, 31]. In the current study, we found that both cafestol and NAC, a synthetic precursor of glutathione, blocked cyclic-strain-mediated ROS production, as measured by decreased DCF fluorescence. We also found that cafestol attenuated the cyclic-strain-induced phosphorylation of ERK, and p38. Cyclic strain induces ROS production and subsequently leads to the activation of MAPK signaling [42]. Therefore, it is reasonable to speculate that cafestol attenuates cyclic-strain-induced MAPK phosphorylation through the inhibition of ROS production. Apart from direct antioxidative reactivity, natural products, including cafestol, may also activate some intracellular signaling pathways, such as the Nrf2/HO-1 pathway, to prolong the cellular defense response [24, 25]. HO-1 catalyzes the rate-limiting step in heme degradation, leading to the generation of biliverdin and CO. Biliverdin and bilirubin, formed due to the action of biliverdin reductase, are potent antioxidants. In addition, CO, a major product of HO-1 activity, plays a protective role in both physiology and pathological conditions [43]. In the present study, cyclic strain alone only weakly or insignificantly induced HO-1 expression. However, cafestol pretreatment

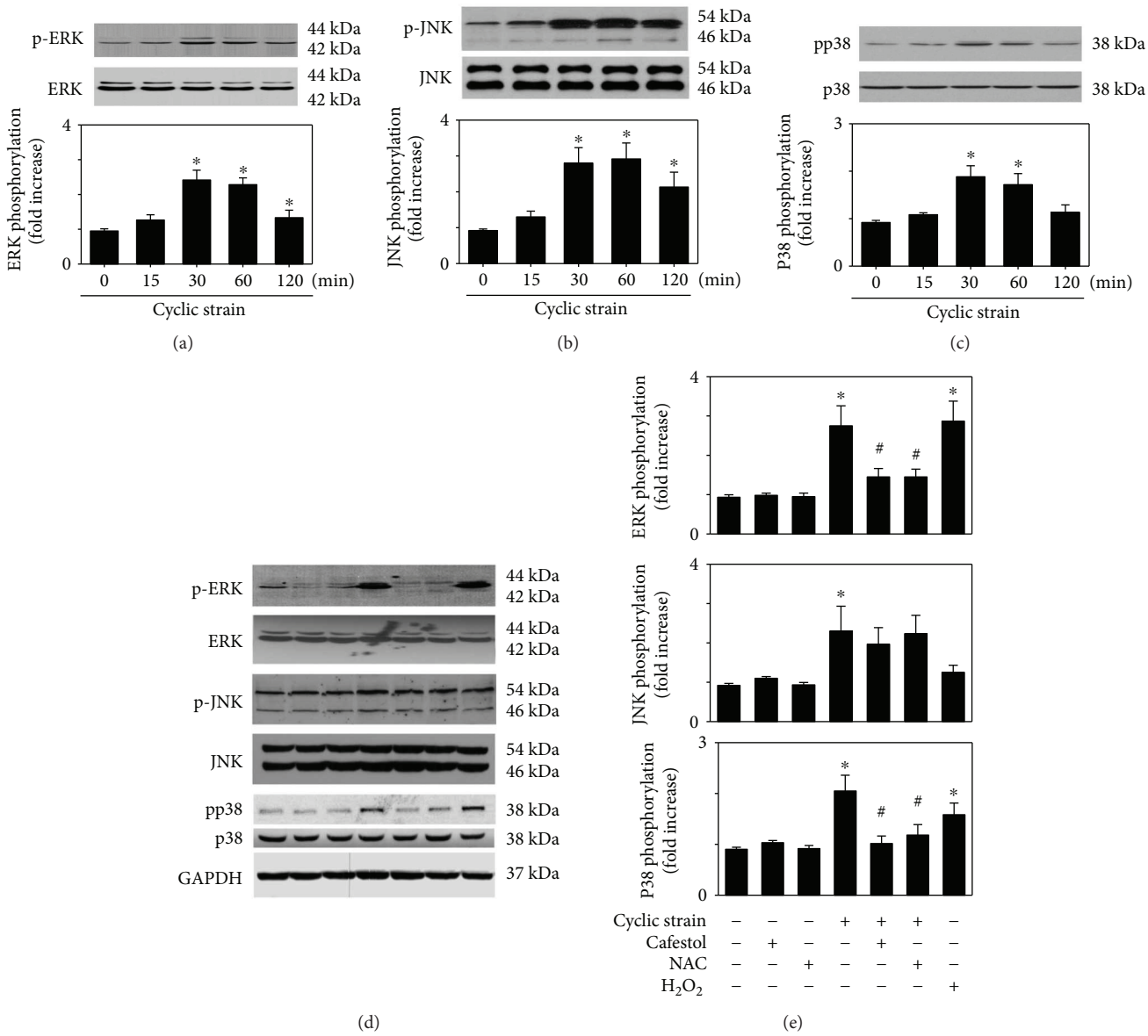

Figure 3: (a–c) Effects of cyclic strain on MAPK phosphorylation. Representative Western blots of phosphospecific and total ERK (a), JNK (b), and p38 (c) from cell lysates collected at the indicated times after cyclic strain treatment. Optical density measurements were obtained to determine the relative amounts of phosphorylated MAPK normalized by the respective total MAPK. The values (mean ± SEM, $n = 4$) indicate the fold change in phosphorylation relative to static controls for each individual experiment. $*$ indicates a significant difference from the static control ($P < 0.05$). (d) Effects of cafestol on cyclic-strain-induced phosphorylation of MAPK. Upper panels: Western blots of phospho-ERK, phospho-JNK, and phospho-p38 in HUVECs pretreated with cafestol (10 μM) for 12 h and then treated with cyclic strain for 30 min. Lower panel: quantitative analysis of stretch-induced phosphorylation of MAKP. Results are representative of four individual experiments and expressed as the mean ± SEM ($n = 4$). $^{*}P < 0.05$ versus untreated controls; $^{\#}P < 0.05$ versus cells exposed to cyclic strain.

enhanced the increase in the HO-1 level, and the modulatory role of HO-1 was confirmed by the addition of the competitive inhibitor Znpp. These results provide further evidence suggesting that cafestol enhances HO-1 expression and thus scavenges excess free radicals produced by cyclic strain. Furthermore, the present data suggest that the anti-inflammatory action of cafestol may be, at least partly, due to its induction of HO-1 expression.

To date, several natural and synthetic substances, say ergothioneine, have been reported to activate Sirt1 and promote endothelial homeostasis [29, 44]. In addition, numerous studies support a cardioprotective role for sirtuin activators (e.g., resveratrol), as well as other emerging modulators of protein acetylation, including curcumin, honokiol, oroxilyn A, quercetin, epigallocatechin-3-gallate, bakuchiol, tyrosol, and berberine, and the development of sirtuin-activating compounds, such as nutraceuticals, for the management of chronic diseases has attracted considerable research interest in recent years [45]. Here we found that cafestol also enhanced Sirt1 expression in cyclic-strain-

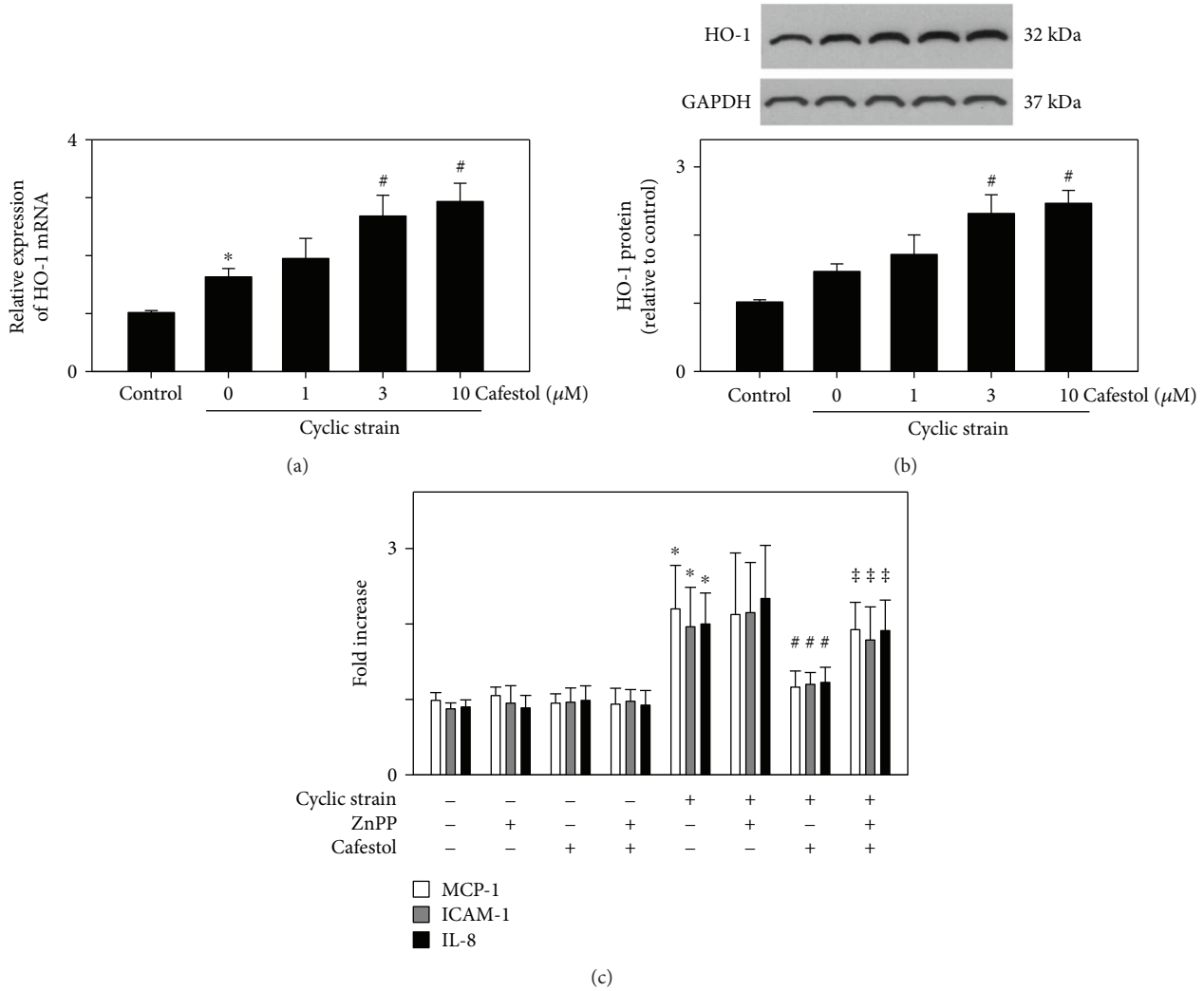

Figure 4: Effects of cafestol on HO-1 expression in the presence of cyclic strain treatment. HUVECs were treated with cafestol 12 h prior to cyclic strain treatment for 12 h. (a) The mRNA level of HO-1 was analyzed through qPCR and normalized to GAPDH. (b) Immunoblotting of HO-1 and GAPDH was performed, and the bands were quantitated using ImageJ. The data represent the mean ± SEM of three independent experiments. $^{*}P < 0.05$ versus untreated control; $^{\#}P < 0.05$ versus cells exposed to cyclic strain. (c) Effects of the inhibition of HO-1 on protein expression levels as examined using ELISA. HUVECs were pretreated for 30 min with Znpp, and cafestol (10 μM) was then added 12 h prior to cyclic strain treatment for 24 h. Data represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ versus untreated controls; $^{\#}P < 0.05$ versus cells exposed to cyclic strain. $^{\ddagger}P < 0.05$ versus cells exposed to cyclic strain plus cafestol treatment.

treated HUVECs. In the presence of the Sirt1 inhibitor sirtinol or Sirt1 silencing, no cafestol-mediated inhibitory effect was observed on cyclic-strain-induced ICAM-1, IL-8, and MCP-1 protein secretion. These results indicate that cafestol suppresses cyclic-strain-induced ICAM-1, IL-8, and MCP-1 protein secretion, possibly through the modulation of Sirt1 expression. Nevertheless, additional investigations are needed to fully characterize the interaction between Sirt1 and cyclic-strain-induced inflammatory protein expression.

5. Conclusion

Cafestol suppressed the secretion of ICAM-1, MCP-1, and IL-8 and inhibited the phosphorylation of ERK, and p38 MAPK in cyclic-strain-treated HUVECs. The mechanism of action of cafestol appears to be associated with the upregulation of HO-1 and Sirt1. On the basis of our study results, coffee consumption might be considered a preventive strategy for CVD. The results of this study might just be "the tip of the iceberg," and additional studies are required to understand the diverse and interrelated roles of coffee consumption in disease prevention. Cafestol appears to reduce the total expression of inflammatory molecules in endothelial cells through multiple mechanisms. This study provides new insights into the anti-inflammatory properties of cafestol. The results of this study support the potential application of cafestol against inflammation-dependent disorders. The upregulation of HO-1 and Sirt1 expression and the suppression of cyclic-strain-induced ICAM-1, MCP-1, and

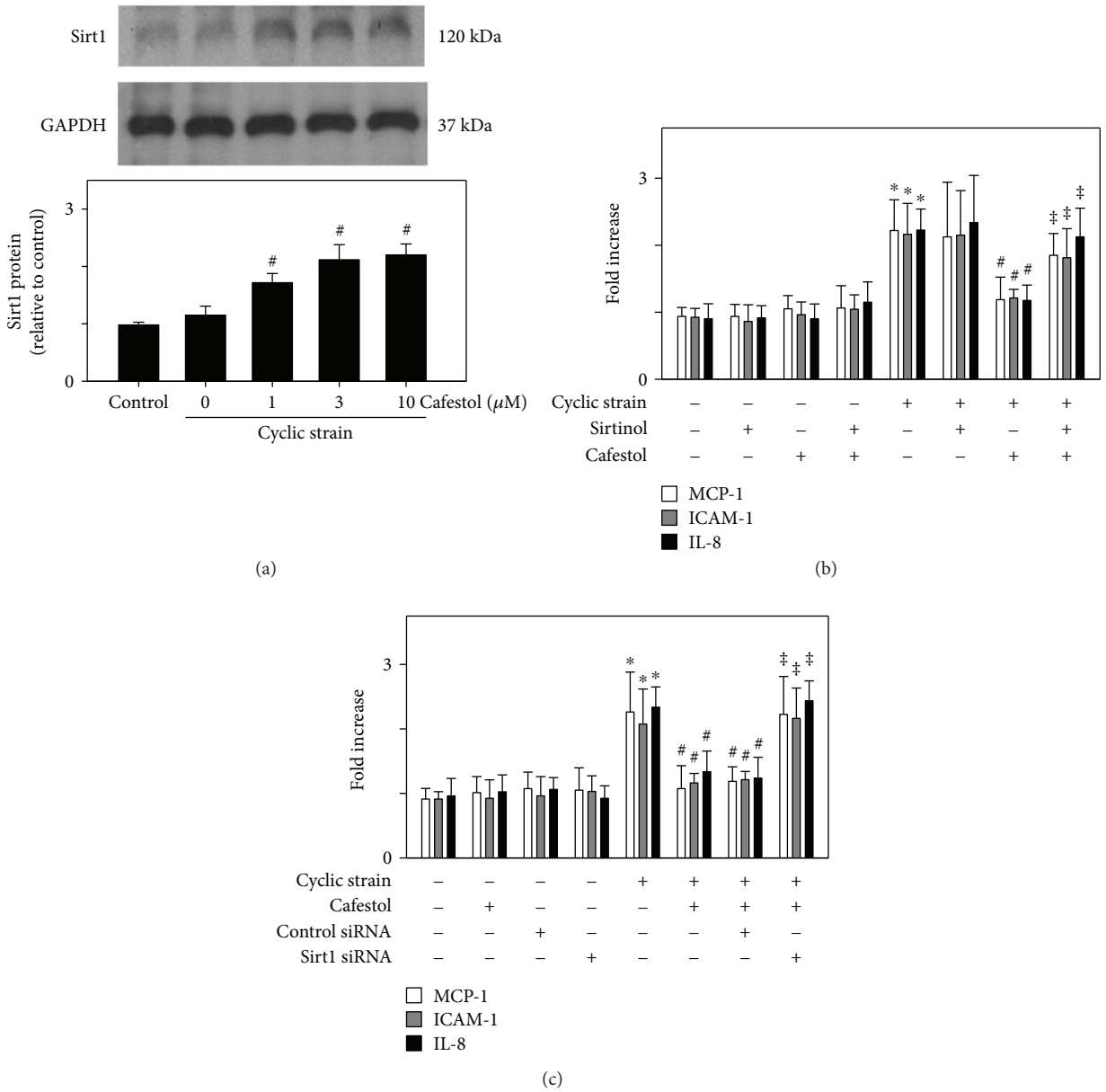

Figure 5: Effects of cafestol and the Sirt1 inhibitor sirtinol on cyclic-strain-induced inflammatory molecule protein expression in HUVECs. (a) Effects of cafestol on Sirt1 expression. Cells were pretreated with cafestol for 12 h or sirtinol for 2 h and then exposed to cyclic strain for 24 h. Protein expression was examined using Western blotting. The data are representative of three independent experiments. The bar graph shows the fold increase in protein expression compared with control cells. (b) Effects of the inhibition of Sirt1 by sirtinol on protein expression levels as examined using ELISA. HUVECs were pretreated with cafestol for 12 h or sirtinol for 2 h prior to cyclic strain treatment for 24 h. (c) Effects of Sirt1 siRNA on protein expression levels as examined using ELISA. Transfected cells were pretreated with 10 μM cafestol for 12 h, then subjected to cyclic strain treatment for 24 h. Data represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ versus untreated controls; $^{\#}P < 0.05$ versus cells exposed to cyclic strain. $^{\ddagger}P < 0.05$ versus cells exposed to cyclic strain plus cafestol treatment.

IL-8 secretion by cafestol may be some of the possible mechanisms responsible for the protective effect of cafestol on the cardiovascular system.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Po-Yuan Chen, Tzu-Hurng Cheng, Hung-Hsing Chao, and Ju-Chi Liu conceived and designed the experiments; Wen-Rui Hao performed the experiments; Wen-Rui Hao, Po-Yuan Chen, and Tzu-Hurng Cheng analyzed the data; Li-Chin Sung, Chun-Chao Chen, and Jin-Jer Chen contributed the reagents and materials; and Po-Yuan Chen,

Tzu-Hurng Cheng, Hung-Hsing Chao, and Ju-Chi Liu wrote the paper.

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Research Article

Defatted Kenaf (*Hibiscus cannabinus* L.) Seed Meal and Its Phenolic-Saponin-Rich Extract Protect Hypercholesterolemic Rats against Oxidative Stress and Systemic Inflammation via Transcriptional Modulation of Hepatic Antioxidant Genes

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The present study aimed to investigate the antioxidant and anti-inflammatory properties of defatted kenaf seed meal (DKSM) and its phenolic-saponin-rich extract (PSRE) in hypercholesterolemic rats. Hypercholesterolemia was induced using atherogenic diet feeding, and dietary interventions were conducted by incorporating DKSM (15% and 30%) or PSRE (at 2.3% and 4.6%, resp., equivalent to the total content of DKSM-phenolics and saponins in the DKSM groups) into the atherogenic diets. After ten weeks of intervention, serum total antioxidant capacities of hypercholesterolemic rats were significantly enhanced by DKSM and PSRE supplementation ($p < 0.05$). Similarly, DKSM and PSRE supplementation upregulated the hepatic mRNA expression of antioxidant genes (Nrf2, Sod1, Sod2, Gsr, and Gpx1) of hypercholesterolemic rats ($p < 0.05$), except for Gpx1 in the DKSM groups. The levels of circulating oxidized LDL and proinflammatory biomarkers were also markedly suppressed by DKSM and PSRE supplementation ($p < 0.05$). In aggregate, DKSM and PSRE attenuated the hypercholesterolemia-associated oxidative stress and systemic inflammation in rats, potentially by enhancement of hepatic endogenous antioxidant defense via activation of the Nrf2-ARE pathway, which may be contributed by the rich content of phenolics and saponins in DKSM and PSRE. Hence, DKSM and PSRE are prospective functional food ingredients for the potential mitigation of atherogenic risks in hypercholesterolemic individuals.

1. Introduction

Cardiovascular diseases (CVDs) remain as the leading cause of global mortality for the past 15 years. In 2015, CVDs had claimed 17.7 million lives, accounting for approximately 45% of all noncommunicable diseases deaths and 31% of all deaths globally [1]. Atherosclerosis is the core pathological element that underlies CVDs, contributing to over 80% of CVD-related fatalities worldwide [2]; while hypercholesterolemia is one of the most prominent risk factors for developing atherosclerosis [3]. Although hypercholesterolemia is related to excessively elevated levels of circulating total and

non-high-density lipoprotein cholesterols in the blood, it is not solely a metabolic disorder of cholesterol homeostasis. Instead, hypercholesterolemia is indispensably associated with exacerbation of oxidative stress and inflammation, which culminates in the impairment of vascular reactivity and progression of atherogenesis [4]. The hypercholesterolemic environment activates major oxidant-producing enzymes including xanthine oxidase, NADPH oxidases (NOX), and myeloperoxidase, resulting in the excessive generation of reactive oxygen species (ROS) and oxidative stress consequently. Oxidative stress abrogates endothelial nitric oxide (NO) availability, uncouples endothelial nitric oxide synthase (eNOS), and enhances the oxidation of entrapped low-density lipoprotein (LDL) within the subendothelial space, thereby eliciting the vascular inflammation response by recruiting monocytes into the tunica intima. The unregulated uptake of oxidized LDL (oxLDL) by differentiated monocytes (macrophages) leads to the formation of foam cells, producing numerous proinflammatory and oxidative stress markers, cytokines, and growth factors, which further aggravate the atherogenic process [4–7].

Improvements in awareness of CVDs, their risk factors, and preventive behaviors have been evident around the world. The appeal in the relationship between diet and health coupled with consumer acceptance for the concept of functional foods, and better understanding of its determinants, has stimulated exponential growth of the global functional food market recently. According to the latest market report, the global cardiovascular health market was valued at USD 8.2 billion in 2016 [8]. Due to the strong correlation between hypercholesterolemia, oxidative stress, and inflammation in the pathogenesis of atherosclerosis, the search for cardioprotective functional food ingredients that possess strong antioxidant and anti-inflammatory properties in addition to cholesterol-lowering effects is receiving increasing attention from related authorities, researchers, manufacturers, and consumers [9–13].

Kenaf (*Hibiscus cannabinus* L.) is a commercial fibre crop, cultivated mainly for its stem and stalk for the production of biocomposites, paper, fibre boards and bioplastics, and biofuel. Kenaf seed is one of the major by-products from the kenaf plantation, and its seed oil has been extensively studied for its potential as functional edible oil [14–17]. Defatted kenaf seed meal (DKSM) is the secondary waste product yielded from the kenaf seed oil extraction process, which accounts for over 75% of its seed mass. Recently, DKSM has been increasingly proven and attested to be a novel functional edible flour with highly nutritive, antioxidative, antihypercholesterolemic, and anticancer properties. Furthermore, our findings also showed that phenolics and saponins are the two major bioactives in DKSM that correspond to the aforementioned nutraceutical properties [18–22]. Aside from the previous reports on antioxidant properties of DKSM and its bioactive-rich extract based on chemical assays, their antioxidant effects under physiological or pathological conditions have not been delved into. Furthermore, studies of anti-inflammatory properties of DKSM and PSRE in a hypercholesterolemic *in vivo* model have not been reported hitherto. Hence, the objectives of the present study were to investigate the antioxidant and anti-inflammatory properties of DKSM and PSRE supplementation via a hypercholesterolemic rat model. In addition, their modulatory effects on the hepatic mRNA level of antioxidant genes were also studied. PSRE was prepared and tested along with DKSM at the equivalent levels of total DKSM-phenolics and saponins in order to determine the possible contributing roles of both bioactives in the *in vivo* antioxidant and anti-inflammatory properties of DKSM. To date, this is the first study to report on the antioxidant and anti-inflammatory properties of DKSM and PSRE supplementation in a hypercholesterolemic rat model.

2. Materials and Methods

2.1. Materials. Ingredients of rat diets, that is, standard rat chow, cholesterol, cholic acid, palm oil, corn starch, full cream milk powder, and eggs, were purchased from Specialty Feeds (Glen Forrest, Australia), Amresco (Solon, OH, USA), Santa Cruz Biotechnology Inc. (Dallas, TX, USA), Yee Lee Edible Oil Sdn. Bhd. (Perak, Malaysia), Thye Huat Chan Sdn. Bhd. (Penang, Malaysia), Eaga Exports Pty Ltd. (South Perth, Australia), and Lay Hong Berhad (Klang, Selangor, Malaysia), respectively. Simvastatin was purchased from Pfizer (New York, NY, USA), while potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All solvents of analytical grade were purchased from Merck (Darmstadt, Germany). Fixative solution (RCL2®) was purchased from Alphelys (Plaisir, France). Rat oxidized low-density lipoprotein (oxLDL) and interleukin 6 (IL-6) ELISA kits were purchased from Cusabio (Wuhan, Hubei, China), while rat tumour necrosis factor-alpha (TNF-α) and C-reactive protein (CRP) ELISA kits were purchased from EMD Millipore, Merck (Darmstadt, Germany). GenomeLab™ GeXP Start Kit and RNA isolation kit (GF-TR-100 RNA Isolation Kit) were purchased from Beckman Coulter Inc. (Brea, CA, USA) and Vivantis (Selangor, Malaysia), respectively. Magnesium chloride ($MgCl_2$) and DNA Taq polymerase were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of DKSM and PSRE. Kenaf seeds (variety V 36) were obtained from the Malaysian Kenaf and Tobacco Board in Pasir Putih, Kelantan, Malaysia, and DKSM was produced following the defatting procedures of our previous study [19]. Briefly, ground kenaf seeds were homogenized at 9500 rpm (Ultra-turrax T25 basic, IKA®-WERKE GmbH & Co. KG, Staufen, Germany) with n-hexane at the ratio of 1 : 2 (w : v) for 15 min. Then, the mixture was filtered through Whatman number 2 filter paper. The residue (DKSM) was reextracted twice accordingly and dried in an oven at 50°C for 3 h to remove residual solvent. Finally, DKSM was passed through a 30-mesh sieve and kept in −20°C for further use. Proximate analysis showed that DKSM contained 57.09% carbohydrate, 26.19% protein, 9.34% moisture, 6.65% ash, and 0.73% fat [19]. Besides, DKSM also contained 16.95% crude fibre.

Table 1: Composition (g/100 g diet) and energy distribution of diets.

	Rat Groups					
	NC	AD/Statin	DKSM-Low	DKSM-High	PSRE-Low	PSRE-High
Ingredient						
Ground standard rat chow	100.0	60.0	45.0	30.0	57.7	55.4
DKSM			15.0	30.0		
PSRE					2.3*	4.6#
Palm oil		20.0	20.0	20.0	20.0	20.0
Full cream milk powder		15.0	15.0	15.0	15.0	15.0
Egg yolk		1.5	1.5	1.5	1.5	1.5
Cholesterol		2.0	2.0	2.0	2.0	2.0
Cholic acid		0.4	0.4	0.4	0.4	0.4
Starch		1.1	1.1	1.1	1.1	1.1
Total	100.0	100.0	100.0	100.0	100.0	100.0
Energy distribution						
Protein (% kcal)	22.2	13.2	14.1	15.0	12.9	12.5
Carbohydrate (% kcal)	65.8	35.7	35.6	35.5	36.0	36.4
Fat (% kcal)	12.0	51.1	50.3	49.5	51.1	51.1
Total caloric value (kcal/100 g diet)	360.8	479.3	476.1	473.0	478.2	477.0

*Based on 15.36% (*w*/*w*) extraction yield, which corresponds to the equivalent level of total DKSM-phenolics and saponins in the "DKSM-Low" group; #based on 15.36% (*w*/*w*) extraction yield, which corresponds to the equivalent level of total DKSM-phenolics and saponins in the "DKSM-High" group.

Phenolic-saponin-rich extract (PSRE) containing the total phenolics and saponins of DKSM was prepared according to the extraction procedures in our previous work [18]. In brief, DKSM was refluxed in 50% aqueous ethanol for 3 h in the ratio of 1 : 15 (*w* : *v*). Then, the mixture was filtered through Whatman filter paper number 2. Finally, solvents in the filtrate were evaporated under reduced pressure (Rotavapor R210, Buchi, Flawil, Switzerland) followed by lyophilization (VirTis BenchTop K Freeze Dryer, SP Industries, Warminster, PA, USA) to obtain PSRE. In order to estimate the recoveries of DKSM-phenolics and saponins in PSRE, DKSM residue obtained from the aforementioned procedure was extracted with methanol under sonication for 1 h. Then, the mixture was centrifuged at 7500 rpm for 10 min at 25°C. Subsequently, the supernatant was subjected to determination of total phenolic and saponin contents, respectively, by Folin–Ciocalteu reagent and vanillin-sulphuric acid assays [18, 23, 24]. The recoveries of phenolics and saponins in PSRE from DKSM were estimated at 97.2 ± 0.1% and 92.5 ± 1.8%, respectively.

Characterization of targeted bioactives in PSRE, that is, phenolics and saponins, was reported in our previous work [18], and the same batch of DKSM and PSRE was used in the present study. From our study [18], total phenolic content of PSRE was estimated at 34.44 mg/g sample, with *p*-coumaric acid (27.72 mg/g sample), caffeic acid (5.75 mg/g sample), (+)-catechin (0.86 mg/g sample), and gallic acid (0.11 mg/g sample) detected as the major phenolics present. Besides, PSRE was found to contain total and steroidal saponins of 128.66 and 0.83 mg diosgenin equivalents/g sample, respectively. Correspondingly, DKSM contains about 5.29 mg/g sample of total phenolics, which was composed of 4.26 mg of *p*-coumaric acid, 0.88 mg of caffeic acid, 0.13 mg of (+)-catechin, and 0.02 mg of gallic acid. Total saponin and steroidal saponin contents of DKSM were estimated at 19.76 and 0.13 mg diosgenin equivalents/g sample, respectively.

2.3. Animal Study. Approval for the animal study was granted by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (Animal Ethics Approval Number: UPM/IACUC/AUP-R065/2013). The study was conducted in accordance with the guidelines for the use of animals. Forty-two male Sprague-Dawley rats (6 weeks old, 130–150 g) were housed in individual plastic cages under the controlled condition of 12/12 h light/dark cycle, at 25 to 30°C. During the acclimatization period (1 week), all rats were fed with standard rat chow (*ad libitum*) and given free access to water. After that, the rats were randomly assigned into 7 different groups, each consisting of 6 rats, that is, NC: rats were fed with standard rat chow; AD: rats were fed with an atherogenic diet containing 20% palm oil, 2% cholesterol, and 0.4% cholic acid; DKSM-Low and DKSM-High: rats were fed with a similar diet to the AD group except for the replacement of rat chow with DKSM (15% and 30% of total diet, resp.); PSRE-Low and PSRE-High: rats were fed with a similar diet to the AD group except for the replacement of rat chow with PSRE respectively at the level of 2.3% and 4.6%, of the total diet (based on 15.36% extraction yield from DKSM, which are corresponded to the equivalent levels of total DKSM-phenolics and saponins in the DKSM groups); and Statin: rats were fed with an atherogenic diet and administrated with simvastatin (10 mg/kg body weight/day) through oral gavage. All diet compositions and caloric values are depicted in Table 1. As shown in Table 1, replacement of DKSM and PSRE with rat chow did not significantly alter the energy distribution of atherogenic diets. Food was given based on daily isocaloric value of 30 kcal/100 g body

Table 2: Gene name, accession number, and primer sequences used in GeXP multiplex analysis.

Gene name	Accession number	Primer sequence (with universal tag)	
		Forward	Reverse
Nrf2/Nfe2l2	NM_031789.2	AGGTGACACTATAGAATATCAGT TACAACTGGATGAAG	GTACGACTCACTATAGGGAGACT CATGGTCATCTACAAAT
Sod1	NM_017050	AGGTGACACTATAGAATAATATG GGGACAATACACAA	GTACGACTCACTATAGGGATCCA ACATGCCTCTCT
Sod2	NM_017051	AGGTGACACTATAGAATACAGGT TGCTCTTCAGC	GTACGACTCACTATAGGGAAACT CTCCTTTGGGTTCT
Gsr	NM_053906.2	AGGTGACACTATAGAATAAATAA ACTGGGGATTCAGAC	GTACGACTCACTATAGGGAAGTA GATTTTCACATTGTCTTTG
Gpx1	NM_030826	AGGTGACACTATAGAATATTGAG AAGTTCCTGGTAGGT	GTACGACTCACTATAGGGATTTT CTGGAAATCAGGTGT
B2m[a]	NM_012512	AGGTGACACTATAGAATAATGCT TGCAGAGTTAAACA	GTACGACTCACTATAGGGATGCA TAAAATATTTAAGGTAAGA
Kan(r)[c]		GGTGACACTATAGAATAATCATC AGCATTGCATTCGATTCCTGTTTG	GTACGACTCACTATAGGGAATTC CGACTCGTCCAACATC
Hprt1[a,b]	NM_012583	AGGTGACACTATAGAATATCCTC ATGGACTGATTATG	GTACGACTCACTATAGGGACTGG TCATTACAGTAGCTCTT
Rpl13a[a]	NM_173340	AGGTGACACTATAGAATAATGGG ATCCCTCCAC	GTACGACTCACTATAGGGAATTT TCTTCTCCACATTCTT

[a]Housekeeping genes; [b]normalization gene; [c]internal control supplied by Beckman Coulter Inc. (Brea, CA, USA).

weight for 10 weeks, and prefiltered tap water was supplied in water-dispensing bottles *ad libitum*. After 10 weeks of dietary intervention, all rats were euthanized (exsanguination by cardiac puncture under anesthesia by ketamine (100 mg/kg) and xylazine (10 mg/kg)) after an overnight fast. Fasting sera were obtained via centrifugation of collected bloods. Rats' livers were carefully excised, cleaned, and preserved in RCL2® solution at −80°C.

The effects of DKSM and PSRE supplementation on the cholesterol metabolism of experimental rats from the present study have been reported [22]. Biochemical analysis on rats' sera showed that atherogenic diet feeding had successfully induced hypercholesterolemia and liver steatosis in rats, as evidenced by significant elevations in hepatosomatic index and hepatic lipid content as well as levels of circulating total and LDL cholesterol, as compared to the NC group. Dietary supplementation of DKSM (DKSM-Low and DKSM-High groups), PSRE (PSRE-Low and PSRE-High groups), and simvastatin (Statin group) exerted superior antihypercholesterolemic properties in the rats, with significant suppressions of elevated total and LDL cholesterol levels. Besides, supplementation of DKSM and PSRE significantly enhanced the high-density lipoprotein (HDL) cholesterol level of hypercholesterolemic rats. Furthermore, supplementations of DKSM, PSRE, and simvastatin had successfully improved the hepatosteatosis of hypercholesterolemic rats by the significant lowering of hepatosomatic indexes and hepatic lipid contents.

2.4. Serum Total Antioxidant Capacity. Serum total antioxidant capacity of experimental rats was assessed using a modified Trolox equivalent antioxidant capacity (TEAC) assay described by Katalinic et al. [25] and Chan et al. [18]. $ABTS^{\bullet+}$ stock solution was prepared by reacting 7 mM of ABTS with 2.45 mM of potassium persulfate. After 18 h of incubation in the dark at room temperature, the stock solution was diluted with phosphate buffer saline to the absorbance of 0.70 ± 0.02 at 734 nm (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan). Subsequently, 50 μL of diluted serum was reacted with 950 μL of adjusted $ABTS^{\bullet+}$ solution for 10 min, and the absorbance was measured at 734 nm (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan). Trolox was used as standard, and the serum total antioxidant capacity of experimental rats was expressed as mg Trolox equivalent antioxidant capacity (TEAC)/mL serum.

2.5. Hepatic mRNA Levels of Antioxidant Genes. The primers for the gene expression study were designed by referring to the *Rattus norvegicus* gene sequences from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nucleotide/) and tagged with an 18-nucleotide universal forward and 19-nucleotide universal reverse sequence, respectively. Primers were supplied by Integrated DNA Technologies (Singapore) and reconstituted in RNAse-free water. The primer sequences of 5 antioxidant genes, 3 housekeeping genes, and an internal control (Kanr) for the rat hepatic multiplex panel are shown in Table 2.

Rat hepatic RNA was extracted using an RNA isolation kit according to the manufacturer's instructions, while the processes of reverse transcription (RT) and polymerase chain reaction (PCR) were conducted according to the GenomeLab™ GeXP Start Kit protocol. Multiplex universal reverse primers and 50 ng extracted RNA were used for RT in an XP Thermal Cycler (BIOER Technology, Hangzhou, Zhejiang, China) under the following conditions: 48°C for 1 min, 37°C for 5 min, 42°C for 60 min, 95°C for 5 min, and then held at 4°C. Subsequently, the cDNA product (9.3 μL) was mixed with 2 μL of 200 nM forward universal primers,

4 μL 25 mM $MgCl_2$, 0.7 μL of Thermo Start Taq DNA polymerase, and 4 μL of 5x PCR Master Mix buffer and subjected to PCR in an XP Thermal Cycler (BIOER Technology, Hangzhou, Zhejiang, China) under the following conditions: initial denaturation at 95°C for 10 min, followed by two-step cycles of 94°C for 30 s and 55°C for 30 s, ending in a single-extension cycle of 68°C for 1 min.

The PCR products obtained from previous steps were analyzed using GeXP GenomeLab Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA). In brief, 1 μL of PCR products was mixed with 38.5 μL sample loading solution and 0.5 μL DNA size standard 400 (provided in the GenomeLab GeXP Start Kit) on a 96-well sample plate before loading on the machine. Results were analysed with the Fragment Analysis Module of the GeXP system software and normalized on the Express Profiler software.

2.6. Circulating Oxidized Low-Density Lipoprotein and Proinflammatory Biomarkers. Fasting sera of rats were subjected to immunoassays (ELISA kits) for determination of circulating oxidized LDL (oxLDL) and proinflammatory biomarkers according to the manufacturer's instructions. The levels of circulating oxLDL and C-reactive protein (CRP) were expressed in ng/mL and μg/mL serum, respectively, while levels of circulating tumour necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) were determined as pg/mL serum.

2.7. Statistical Analysis. All results are reported as mean ± standard deviation ($n = 6$). One-way analysis of variance (ANOVA), accompanied with Tukey's multiple comparison test (GraphPad Prism 6.01, GraphPad Software Inc., La Jolla, CA, USA), was conducted to identify significant differences between samples ($p < 0.05$).

3. Results and Discussion

3.1. Serum Total Antioxidant Capacity. Oxidative stress is closely associated with the pathogenesis of atherosclerosis [6]. Thus, serum/plasma total antioxidant capacity (TAC) may represent a useful tool in assessing the global oxidative stress and antioxidant defense levels in experimental animals and human subjects [26–30]. Trolox equivalent antioxidant capacity (TEAC) assay is one of the most common assays employed in the assessment of serum TAC based on the spectrophotometric measurement of $ABTS^{\bullet+}$ cation reduction (decay of green-blue chromophore absorbance) by serum antioxidative components, in comparison to the control antioxidant, Trolox (hydrophilic analogue of vitamin E) [31].

Figure 1 depicts serum TAC of experimental rats after 10 weeks of dietary intervention. Atherogenic diet feeding significantly lowered serum TAC of hypercholesterolemic rats in the AD group ($p < 0.05$). This finding is in agreement with several *in vivo* studies involving diet-induced hypercholesterolemic/hyperlipidemic rats [10, 32, 33]. The depletion of serum TAC was probably due to the override of *in vivo* antioxidant defense by excessive generation of oxidants/ROS under hypercholesterolemic condition. In contrast, simvastatin treatment significantly improved serum TAC of

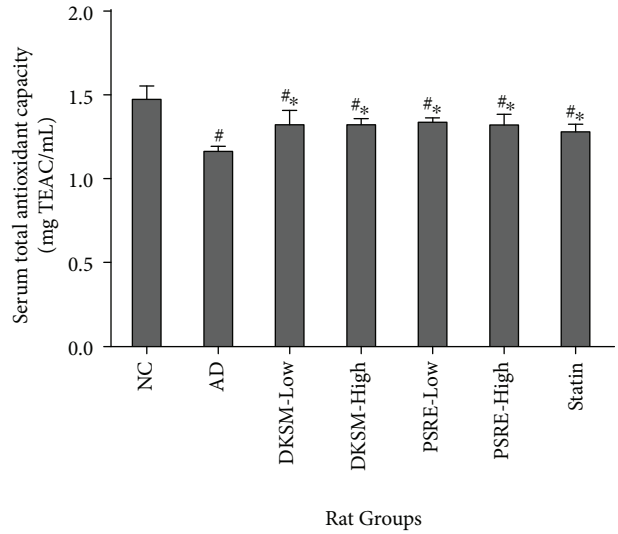

Figure 1: Serum total antioxidant capacity after 10 weeks of dietary intervention. Data represent the mean of each group, accompanied with standard deviation. Symbols "*" and "#" respectively represent significant difference between samples in comparison to the AD and NC groups ($p < 0.05$).

hypercholesterolemic rats ($p < 0.05$), affirming the pleiotropic antioxidant properties of simvastatin [34].

As compared to the AD group, supplementation of DKSM and PSRE effectively counteracted the decrease in serum TAC induced by hypercholesterolemia ($p < 0.05$). The improvement in serum TAC in hypercholesterolemic rats was probably due to high antioxidant properties of DKSM and PSRE, which is supported by our previous studies using *in vitro* assays based on different mechanisms [18, 19]. Consumption of an antioxidant-rich diet (e.g., fruits and vegetable which are rich in polyphenols) is strongly correlated with the improvement of antioxidant status and the attenuation of atherogenic risks in human subjects [28, 35–37].

In the present study, phenolics and saponins may have substantially contributed to the *in vivo* antioxidant properties of DKSM and PSRE since serum TAC between the DKSM groups and their corresponding PSRE groups (which contained the equivalent levels of DKSM-phenolics and saponins with the DKSM groups) were insignificantly different ($p > 0.05$). In agreement to our previous study [18], phenolics and saponins had significantly contributed to the antioxidant properties of PSRE and DKSM. This postulation is further supported by a number of studies reporting on the *in vivo* antioxidant properties of major phenolic compounds detected in DKSM and PSRE, that is, *p*-coumaric acid, caffeic acid, (+)-catechin, and gallic acid. For instance, oral administration of *p*-coumaric acid and gallic acid at the dosage of 100 mg/kg body weight for 2 weeks was found to greatly improve the cardiac and hepatic total antioxidant capacities of healthy rats [38, 39], while dietary supplementation with caffeic and coumaric acids (0.2% of total diet) for 6 weeks effectively enhanced the *in vivo* antioxidant capacity of hypercholesterolemic rats [40]. On the other hand, plasma and urine TAC of Wistar rats was significantly increased following 10 days of intraperitoneal administration of catechin

Table 3: Hepatic antioxidant gene expressions after 10 weeks of dietary intervention.

Rat groups	Hepatic antioxidant genes (relative expression)				
	Nrf2	Sod1	Sod2	Gsr	Gpx1
NC	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.20	1.00 ± 0.20	1.00 ± 0.11
AD	$0.68 \pm 0.19^{\#}$	$0.56 \pm 0.10^{\#}$	$0.67 \pm 0.12^{\#}$	$0.57 \pm 0.11^{\#}$	$0.61 \pm 0.12^{\#}$
DKSM-Low	$1.21 \pm 0.08^{*}$	$0.76 \pm 0.12^{*\#}$	0.88 ± 0.12	$0.82 \pm 0.08^{*}$	$0.50 \pm 0.04^{\#}$
DKSM-High	$1.23 \pm 0.18^{*\#}$	$0.76 \pm 0.09^{*\#}$	$1.02 \pm 0.17^{*}$	$0.83 \pm 0.12^{*}$	$0.66 \pm 0.09^{\#}$
PSRE-Low	$1.02 \pm 0.16^{*}$	$1.57 \pm 0.09^{*\#}$	$2.09 \pm 0.17^{*\#}$	$1.28 \pm 0.04^{*\#}$	$1.71 \pm 0.32^{*\#}$
PSRE-High	$1.16 \pm 0.17^{*}$	$1.53 \pm 0.11^{*\#}$	$2.18 \pm 0.40^{*\#}$	$1.37 \pm 0.17^{*\#}$	$1.64 \pm 0.29^{*\#}$
Statin	$1.69 \pm 0.04^{*\#}$	$1.75 \pm 0.03^{*\#}$	$1.74 \pm 0.12^{*\#}$	$1.42 \pm 0.23^{*\#}$	$2.04 \pm 0.28^{*\#}$

Symbol "$*$" within the same column indicates significant difference in comparison to the AD group ($p < 0.05$); symbol "#" within the same column indicates significant difference in comparison to the NC group ($p < 0.05$); abbreviations: Nrf2: nuclear factor erythroid 2-related factor 2; Sod1: cytosolic superoxide dismutase; Sod2: mitochondrial superoxide dismutase; Gsr: glutathione-disulfide reductase; Gpx1: glutathione peroxidase 1.

mixture (23 mg/kg body weight) [41]. Besides phenolic compounds, saponins have also been proposed as a group of dietary phytochemicals with distinctive *in vivo* antioxidant properties [42, 43]. For example, supplementation with total saponins extracted from three medicinal species of *Dioscorea* and dry root tuber of *Trichosanthis kirilowii* were found to effectively improve the *in vivo* antioxidant capacity of myocardial ischemic rats and carbon tetrachloride-intoxicated mice, respectively [44, 45].

3.2. Expression of Hepatic Antioxidant Genes. Endogenous antioxidant defense plays a critical role in restoring the cellular redox imbalance caused by oxidative insults, whilst consumption of high antioxidative phytochemicals (particularly phenolic compounds) has been implicated in the enhancement of endogenous antioxidant defense via modulation of multiple redox mechanisms [46, 47]. Table 3 shows the mRNA levels of hepatic antioxidant genes (nuclear factor erythroid 2-related factor 2 (Nrf2 or Nfe2l2), cytosolic superoxide dismutase (Sod1), mitochondrial superoxide dismutase (Sod2), glutathione-disulfide reductase (Gsr), and glutathione peroxidase 1 (Gpx1)) in the experimental rats, as influenced by different dietary interventions.

After 10 weeks of atherogenic diet feeding, hepatic antioxidant gene expression (Nrf2, Sod1, Sod2, Gsr, and Gpx1) in the AD group was adversely altered ($p < 0.05$) in comparison to the NC group, suggesting the manifestation of hypercholesterolemia-induced oxidative stress in these rats. On the contrary, hepatic antioxidant gene expressions in the Statin group were significantly enhanced ($p < 0.05$). In consonance with previous studies, diet-induced hypercholesterolemia has been associated with the exacerbation of oxidative stress and compromised endogenous antioxidant defense in the experimental animals, while statin treatment is the effective pharmaceutical approach in reversing these deleterious impacts [10, 13, 32, 48, 49].

In the present study, supplementation with DKSM and PSRE substantially improved the endogenous antioxidant defense of hypercholesterolemic rats via transcriptional modulation of hepatic antioxidant genes. In comparison to the AD group, hepatic Nrf2 gene expression of all DKSM- and PSRE-supplemented rats was significantly elevated by 1.5- to 1.8-folds ($p < 0.05$). DKSM supplementation resulted in the upregulation of hepatic Sod1, Sod2, and Gsr expressions, especially in the DKSM-High group ($p < 0.05$). However, supplementation with DKSM did not improve the hepatic Gpx1 gene expression of hypercholesterolemic rats ($p > 0.05$). Similarly, PSRE supplementation upregulated the hepatic gene expressions of Sod1, Sod2, Gsr, and Gpx1 of hypercholesterolemic rats by 2- to 3-folds ($p < 0.05$). Except for Nrf2, both PSRE groups exhibited superior upregulatory effects in the expressions of all studied hepatic antioxidant genes than their corresponding DKSM groups, which contained an equivalent level of total DKSM-phenolics and saponins ($p < 0.05$). This is probably due to the enhancement in the release of bioactives (phenolics and saponins) from the DKSM matrix during the heated reflux extraction process, which may then result in the better bioavailability and bioefficiency of PSRE. Appropriate increase in the extraction temperature will disrupt the integrity of the cell wall, thus facilitating the release of bound bioactives from the matrix and enhancing the solubility and diffusion coefficient of bioactives into the extraction solvent, leading to the optimal recovery of bioactives in the extract [50, 51]. For instance, heated reflux extraction (80°C) of *Pterodon emarginatus* vogel seeds with 70% aqueous ethanol provided the highest phenolic recovery as compared to the nonthermal extraction processes [52], while a higher recovery of chickpeasaponin B1 was observed when the aqueous-ethanolic extraction processes were carried out under heated reflux condition (90°C) in comparison to the nonthermal ultrasonic extraction [53].

In the present study, results from hepatic antioxidant gene expression analysis aggregately suggest that supplementation with DKSM and PSRE could have activated the hepatic Nrf2-ARE pathway in the experimental rats and consequently improved their endogenous antioxidant defense against hypercholesterolemia-induced oxidative stress. Besides, supplementation with PSRE at an equivalent level of DKSM-phenolics and saponins produced similar or superior modulatory effects on the hepatic antioxidant gene expressions than on their corresponding DKSM counterparts. This finding signifies the contributory roles of phenolics and saponins as the dietary Nrf2-ARE-activating factors in DKSM and PSRE. In recent years, activation of Nrf2-ARE has been proposed as the targeted therapeutic pathway

Table 4: Circulating oxidized low-density lipoprotein and proinflammatory biomarkers after 10 weeks of dietary intervention.

Rat groups	oxLDL (ng/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)	CRP (μg/mL)
NC	26.90 ± 2.29	20.75 ± 3.86	3.08 ± 0.18	798.42 ± 55.95
AD	$35.90 \pm 3.71^{\#}$	$83.00 \pm 19.08^{\#}$	$4.22 \pm 0.20^{\#}$	$1018.56 \pm 155.09^{\#}$
DKSM-Low	$21.49 \pm 0.83^{*\#}$	$46.75 \pm 7.72^{*\#}$	3.66 ± 0.25	$749.31 \pm 42.60^{*}$
DKSM-High	$15.35 \pm 2.85^{*\#}$	$45.25 \pm 9.78^{*\#}$	$2.81 \pm 0.40^{*}$	$624.41 \pm 42.07^{*\#}$
PSRE-Low	$23.79 \pm 1.72^{*}$	$50.50 \pm 8.39^{*\#}$	$3.23 \pm 0.30^{*}$	$727.67 \pm 67.66^{*}$
PSRE-High	$16.81 \pm 1.34^{*\#}$	$33.00 \pm 2.00^{*}$	$3.44 \pm 0.88^{*}$	$725.22 \pm 54.81^{*}$
Statin	$20.85 \pm 5.64^{*\#}$	$52.50 \pm 6.56^{*\#}$	$3.92 \pm 0.68^{\#}$	$683.39 \pm 77.96^{*}$

Symbol "$*$" within the same column indicates significant difference in comparison to the AD group ($p < 0.05$); symbol "#" within the same column indicates significant difference in comparison to the NC group ($p < 0.05$); abbreviations: oxLDL: oxidized low-density lipoprotein; TNF-α: tumour necrosis factor-alpha; IL-6: interleukin 6; CRP: C-reactive protein.

for a wide array of degenerative and immunological diseases, particularly CVDs, whilst a number of dietary phytochemicals especially polyphenols, isothiocyanates, organosulfur compounds, saponins, and curcumin are prominent natural activators of this pathway [46, 54–56]. Nrf2 is a critical transcription factor that regulates the antioxidant responses against oxidative insults. Once it is activated, Nrf2 binds to the antioxidant response element (ARE) in the nucleus to upregulate a vast array of antioxidative and electrophile detoxification genes, such as Sod, Gsr, and Gpx [57]. Antioxidant enzymes are the core pillars of endogenous antioxidant defense that cohesively shield our body from oxidative damage and its related pathogenesis [58]. For instance, Sod is one of the most effective primary antioxidant enzymes that catalyses the conversion of superoxide anions to hydrogen peroxide, while Gpx renders hydrogen peroxide and other organic hydroperoxides (e.g., lipid peroxide) into inert end products. On the other hand, Gsr serves as an important secondary antioxidant enzyme that maintains the proper function of primary antioxidant enzymes (e.g., Gpx) by catalysing the reduction process of glutathione disulfide (GSSG) to glutathione (GSH) with NADPH as the reducing cofactor.

Interestingly, simvastatin treatment and PSRE supplementation did not only neutralize the deleterious effects of hypercholesterolemia on the transcriptions of hepatic antioxidant genes but their hepatic expressions of antioxidant genes were upregulated to a higher degree than those of the NC group ($p < 0.05$). In the present study, PSRE supplementation and simvastatin treatment significantly improved the severity of hypercholesterolemia in the experimental rats and thus produced a milder oxidative stress condition as compared to the AD group. Mild/moderate oxidative stress, simvastatin, and polyphenols have been previously reported as the activators of the Nrf2-ARE pathway by inducing the dissociation of Nrf2 from the Kelch-like ECH-associated protein-1 (Keap1) and consequently upregulating the expressions of its downstream antioxidant genes [55, 59, 60]. Although the hepatic Nrf2 expression between the NC and PSRE groups were indifferent ($p > 0.05$), the higher expressions of other hepatic antioxidant genes in the PSRE and Statin groups could be possibly explained by the enhancement of Nrf2-ARE activation through the combinatorial effects of improved oxidative stress condition as well as the inductions by simvastatin or PSRE bioactives. In agreement, the similar findings have been previously observed in the diet-induced hyperlipidemic rats, supplemented with phenolic-rich extract from *Clinacanthus nutans* and simvastatin [10].

3.3. Circulating Oxidized Low-Density Lipoprotein and Proinflammatory Biomarkers. Chronic hypercholesterolemia triggers excessive ROS generation, compromises endogenous antioxidant defense, and consequently results in the formation of oxidatively modified LDL/oxidized LDL (oxLDL) [4]. Circulating oxLDL level is one of the most important oxidative stress-related biomarkers, which is strongly correlated to the prevalence of atherosclerotic CVD [61–63]. Circulating oxLDL and proinflammatory biomarker levels of experimental rats after 10 weeks of dietary intervention are depicted in Table 4. In comparison to the NC group, atherogenic diet feeding significantly elevated the circulating oxLDL level of the AD group ($p < 0.05$), indicating the successful induction and advanced manifestation of hypercholesterolemia-induced oxidative stress in these rats. In contrast, dietary supplementation with DKSM and PSRE effectively lowered the circulating oxLDL level of hypercholesterolemic rats by 34% to 57%, in a dose-dependent manner of DKSM-High $\geq$ PSRE-High $\geq$ Statin $\geq$ DKSM-Low $\geq$ PSRE-Low $>$ AD ($p < 0.05$). Remarkably, supplementation with higher concentration of DKSM (DKSM-High group) exhibited superior LDL oxidation inhibitory activity than simvastatin treatment ($p < 0.05$). Since there was no significant difference in the circulating oxLDL levels between the DKSM groups and their corresponding PSRE groups ($p > 0.05$), it is suggested that phenolics and saponins could have contributed to the antioxidant and LDL oxidation inhibitory properties of DKSM and PSRE. This deduction is further supported by our previous studies, of which the phenolic-saponin-rich fraction obtained via partial purification of DKSM ethanolic extract exhibited superior antioxidant properties than its bioactive-deficient counterpart [18]. Furthermore, some of the major phenolics in DKSM and PSRE, that is, *p*-coumaric acid, caffeic acid, and (+)-catechin, have been previously reported as promising natural inhibitors against LDL oxidation via *in vitro* and *in vivo* models [64–67].

Due to the critical roles of circulating TNF-α, IL-6, and CRP in the pathogenesis of atherosclerosis, these proinflammatory biomarkers are frequently used as promising panel for the assessment of cardiovascular risks [68, 69]. In the present study, hypercholesterolemia had evidently induced systemic inflammation in the experimental rats fed on an atherogenic diet. In comparison to the NC group, significant elevations of circulating TNF-α, IL-6, and CRP levels by 4-, 1.4-, and 1.3-folds, respectively, were observed in the hypercholesterolemic rats from the AD group ($p < 0.05$). After 10 weeks of DKSM and PSRE supplementation, circulating TNF-α levels of hypercholesterolemic rats were markedly reduced by 39 to 60% in a dose-dependent order, that is, PSRE-High ≥ DKSM-High ≥ DKSM-Low ≥ PSRE-Low ≥ Statin > AD ($p < 0.05$). A significantly lower circulating IL-6 level in the experimental rats was observed in the DKSM-High (−33.4%), PSRE-Low (−23.5%), and PSRE-High (−18.5%) groups in comparison to the AD group ($p < 0.05$), while simvastatin treatment (Statin group) and DKSM supplementation at a lower level (DKSM-Low group) produced insignificant lowering effects on the circulating IL-6 level ($p > 0.05$). Similar to the trend of the TNF-α level, supplementation with DKSM and PSRE effectively repressed the circulating CRP level of hypercholesterolemic rats in a dose-dependent manner, that is, DKSM-High ≥ Statin ≥ PSRE-High ≥ PSRE-Low ≥ DKSM-Low > AD ($p < 0.05$). Furthermore, there were no significant differences in the levels of proinflammatory biomarkers between DKSM groups and their corresponding PSRE groups ($p > 0.05$), suggesting that phenolics and saponins might be the key bioactives that have contributed to the anti-inflammatory properties of DKSM and PSRE.

Elevated level of oxLDL is correlated with the upregulation of proinflammatory mediators (e.g., TNF-α, IL-6, and CRP) in human subjects [62]. In the present study, the level of circulating oxLDL was strongly correlated with the level of CRP ($r = 0.9390$) and moderately correlated with the TNF-α ($r = 0.5682$) and IL-6 ($r = 0.5892$) levels, thus affirming the etiological role of hypercholesterolemia-induced oxidative stress in eliciting systemic inflammation and higher atherogenic risk in the rats. Moreover, these correlations also suggest that the lower systemic inflammation observed in DKSM- and PSRE-supplemented rats may in part be due to the inhibition of LDL oxidation by these dietary interventions. Supplementation with DKSM and PSRE modulated the upregulation of hepatic antioxidant gene expressions as well as the enhancement of circulating nonenzymatic low molecular weight antioxidant levels (as evidenced by improvement in serum TAC) in the hypercholesterolemic rats. Thus, it is postulated that these antioxidative effects might have advantageously controlled hypercholesterolemia-induced ROS overproduction in the rats and therefore reduced the severity of LDL oxidative damage and systemic inflammation. Although PSRE supplementation showed superior upregulatory effects than its DKSM counterpart in the hepatic expressions of antioxidant genes, similar effects were not observed in TAC, oxLDL, and proinflammatory biomarker assays. This is possibly due to the relatively high levels of DKSM and PSRE used in the present study, hence resulting in the optimal *in vivo* antioxidant and anti-inflammatory effects (i.e., plateau portion of the dose-response curve) observed under the tested physiological condition. On the other side, DKSM and PSRE supplementation might have exhibited an all-or-none effect notwithstanding the transcriptional changes in the *in vivo* model. This is probably due to the posttranscriptional modifications that produced therapeutic effects to the same degree irrespective of the degree of transcriptional changes induced. Investigations on dietary effects of DKSM and PSRE on endogenous antioxidant defense in hypercholesterolemic animal models at posttranscriptional and translational levels are suggested for further studies.

4. Conclusion

DKSM and its derived PSRE supplementation improved *in vivo* antioxidant defense of hypercholesterolemic rats possibly via transcriptional activation of hepatic Nrf2-ARE pathway and improvement of serum TAC. The enhancement in endogenous antioxidant defense therefore meritoriously inhibited the oxidation of LDL and systematic inflammatory response in the hypercholesterolemic rats. Phenolics and saponins are suggested as the key antioxidant and anti-inflammatory bioactives in DKSM and PSRE. Finally, DKSM and PSRE could be potentially used as cardioprotective functional food ingredients in counteracting hypercholesterolemia-associated oxidative stress and systemic inflammation.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Acute Toxicity, Antioxidant, and Antifatigue Activities of Protein-Rich Extract from *Oviductus ranae*

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The paper investigated the preparation, amino acid composition, acute toxicity, and *in vitro* and *in vivo* antioxidant, coupled with *in vivo* antifatigue activities of protein-rich extract of *Oviductus ranae* (PEOR). The results indicated that PEOR possesses high-safety property with maximum tolerated dose (MTD) higher than 20 g/kg in mice, shows weak scavenging capacities against hydroxyl, superoxide anion, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, as well as ferric-reducing antioxidant power *in vitro*, but exerts strong antioxidant effect in ethanol-induced oxidative stress mice model; it can decrease malonaldehyde (MDA) and protein carbonyl (PCO) formation and increase total superoxide dismutase (T-SOD) activity and glutathione (GSH) synthesis. Besides the strong *in vivo* antioxidant activity, PEOR in a dose of 400 mg/kg also has antifatigue effect in mice, and it can prolong the exhaustive swimming time, reduce the elevated blood urea nitrogen (BUN) and blood lactic acid (BLA) caused by intense exercise. The *in vivo* activity of PEOR may be contributed by its absorbed amino acids, due to the fact that eight antioxidant amino acids and twelve glucogenic ones were found in it. This study will provide an evidence for the clinical use of PEOR as a dietary supplement for antioxidant and antifatigue in the same oral dose (400 mg/kg).

1. Introduction

Some harmful factors including overconsumption of drinking and smoking, X-ray irradiation, organic pollutants, and heavy metals can cause the overproduction of reactive oxygen species (ROS), which subsequently destroy the dynamic equilibrium between ROS generation and elimination to induce oxidative stress [1]. Moreover, under the physiological condition of oxidative stress, excess ROS can directly react with protein and DNA, as well as lipid to damage their structures and functions [2], leading to cell death and aging [3], coupled with some diseases, such as inflammation [4], immune deficiency [5], Parkinson's disease [6], Alzheimer's disease [7], and cancer [8]. Due to the fact that levels or activities of endogenous antioxidants, such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalases (CAT), are always lower than the level demanded for free radical-scavenging, it is usually necessary to supplement exogenous antioxidant when facing oxidative stress [9]. Another critical role of antioxidant lies in its positive effects on chronic fatigue syndrome (CFS) [10]. An increasing number of papers have demonstrated that antioxidant also exerts antifatigue activity *in vivo*, especially for some natural products [11–14].

Proteins are a kind of macromolecular substances consisting of different amino acids, which were found

throughout most organs and tissues in the body. In addition to innate physiological functions comprising biological catalysis, DNA replication, muscle contraction, and molecules transportation [15], they also exhibit diverse biological activities, and antioxidant activity is one of the beneficial functions for human beings [16–18].

The Chinese brown frog, *Rana chensinensis*, is one of the famous economic animals farmed in China. It belongs to small amphibious frog with the mature male body size of 52~64 mm and female of 58~64 mm [19]. The natural populations of *R. chensinensis* mainly distribute in wet woodlands and mountains at low altitude ranging from 600 m to 1300 m in Northeastern China. In 1987, the wild *R. chensinensis* was listed as one of the national key-protected wild medicinal materials by the Chinese government, and its artificial breeding achieved large-scale reproduction in the 1990s [20, 21]. The economic value of *R. chensinensis* mainly depends on its dried oviduct, *Oviductus ranae* (OR), a traditional Chinese medicine (TCM) used in China for hundreds of years. OR was originally described in Compendium of Materia Medica in the Ming dynasty and listed in Chinese Pharmacopeia since 1985 [22]. Traditionally, OR was consumed as a tonic for the remedies of debilitation, insomnia, neurasthenia, respiratory symptoms, and climacteric syndrome [23]. Modern pharmacological studies have revealed that OR displays a wide range of activities including immune-enhancement [24], antiaging [25], antifatigue [26], antioxidation [27], anti-osteoporosis [28], and estrogen-like effects [29]. OR is composed of proteins, lipids, steroids, vitamins, nucleic acids, and trace elements [30]; among them, proteins are the main constituents present in it; in most cases, their contents are more than 50% [24].

A great number of basic and experimental studies regarding oxidative stress have been performed to reveal the probable mechanisms involving the regulation of the imbalance between prooxidant and antioxidant system. These mechanisms provided major insights into oxidative stress and have advanced the clinical trials and approaches, resulting in successful prevention and diagnosis, as well as therapies [31]. Many natural antioxidants based on plants and other living organisms have been scientifically confirmed as effective therapeutic agents. Moreover, with the increase of healthy lifestyle pursuit, more and more people have consumed the natural antioxidants routinely. Furthermore, some individuals, especially athletes and sport professionals, are also eager for the antioxidant-possessing antifatigue functions. To date, numerous papers have reported the antioxidant activities of extracts or isolated compounds from TCM, which has become one of the most abundant sources of novel antioxidant discovery [32]. It is therefore important to obtain efficient natural antioxidants that can also exert antifatigue activities without any damages on the healthy consumers from TCM. OR is a precious TCM with high protein content, but, to our knowledge, there is little information on the antioxidant-related activities of proteins in it. Thus, in present study, with the aim at obtaining the potential of proteins from OR for becoming an antioxidant supplement with high-safety property. The protein-rich extract from OR (PEOR) was prepared and analyzed, and the acute toxicity and in *vitro* and in *vivo* antioxidant activities of PEOR were assessed. Then the in *vivo* antifatigue effect of PEOR was further evaluated.

2. Materials and Methods

2.1. Materials. OR samples were obtained from Jilin Huangzhihua Pharmaceutical Co. Ltd (Jilin, Changchun, China) and identified by Prof. Guangshu Wang, School of Pharmaceutical Sciences, Jilin University (Jilin, Changchun, China). Specimen of OR (voucher number HG-2035) was preserved in Jilin Engineering Research Center for Agricultural Resources and Comprehensive Utilization, Jilin Institute of Chemical Technology (Jilin, Jilin, China).

Ginsenoside (2.5 mg of Rg3 per 100 mg) was from Dalian Fusheng Pharmaceutical Co. Ltd (Liaoning, Dalian, China). The bovine serum albumin (BSA) was purchased from Nanjing Jiancheng Biotechnology Co. Ltd (Jiangsu, Nanjing, China). The majority of chemicals used for the *in vitro* antioxidant evaluation were obtained from Civi Chemical Technology Co. Ltd (Shanghai, China) including 1, 10-phenanthroline, vitamin C (VC), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), and phenazine methosulphate (PMS). Other solvents and reagents were analytical grade and provided by Sigma Aldrich Chemical Co. Ltd (St. Louis, MO, USA).

Amino acid mixture standard solution including glycine, L-alanine, L-cystine, L-methionine, L-leucine, L-isoleucine, L-valine, L-glutamic acid, L-aspartic acid, L-phenylalanine, L-arginine, L-lysine, L-threonine, L-histidine, L-tyrosine, L-serine, L-proline, and ammonium chloride was obtained from Wako Pure Chemical Industries Ltd (Tokyo, Japan).

Biochemical indicators including aspartate transaminase (AST), alanine transaminase (ALT), glucose (GLU), triglycerides (TG), creatinine (CRE), and blood urea nitrogen (BUN) were determined by an AU2700 Beckman coulter chemistry analyzer (Beckman Coulter, Brea, CA, USA).

Reagent kits for the determination of malonaldehyde (MDA), total superoxide dismutase (T-SOD), glutathione (GSH), protein carbonyls (PCO), BUN and hepatic glycogen (HG) were obtained from Jiancheng Biotechnology Co. Ltd (Nanjing, Jiangsu, China).

Blood lactic acid (BLA) was determined by a Lactate Scout+ analyzer (EKF Diagnostics, Cardiff, WAL, England).

2.2. Preparation of PEOR. Prior to experiment, OR samples were grinded into power and sieved to 100 mesh. PEOR was prepared according to the reported method [24] with some modifications. About 10 g of OR powers were soaked in 1000 ml of phosphate buffered saline (PBS) solution (pH 6.5) at room temperature for 12 h, treated with ultrasonic wave at a power of 300 W for 2 h (Voshin, VS-200UE, Jiangsu, China). Then the mixture was centrifuged at 4500 rpm for 20 min at 4°C, the supernatant was filtered using a hollow-fiber membrane (0.45 μm, GE Healthcare Life Sciences, Pittsburgh, PA, USA), and the precipitant was extracted twice as the above-mentioned method. The filtrate was mixed with ammonium sulfate (80% saturation) to

produce precipitant, which was dissolved in distilled water and dialyzed for 24 h (MD10, Viskase, Darien, IL, USA). The dialysate was centrifuged at 4500 rpm for 20 min at 4°C, then the supernatant was freeze-dried, giving 1.02 g of PEOR, and the extraction yield was 10.2%.

2.3. General Chemical Analysis. Protein content of PEOR was determined by Bradford method using BSA as standard [33]. Moisture was determined via drying PEOR in an oven at 105°C for 6 h, and ash was determined by heating PEOR overnight at 550°C. Lipid content of PEOR was determined by Soxhlet method using petroleum ether as solvent [21, 34, 35].

2.4. Amino Acid Composition of PEOR. 50 mg of PEOR was mixed with 5 ml of 6 N hydrochloric acid at 110 ± 1°C for 24 h under the protection of nitrogen atmosphere [36]. Before analysis, the pH value of hydrolysate solution was adjusted to 2.2 with 4 mol/l LiOH. Then the amino acid composition was analyzed by a fully automated amino acid analyzer (L-8900 Hitachi, Tokyo, Japan). The analytical conditions were as follows: chromatographic column, cation exchange resin 4.6 mm × 60 mm; temperature of column oven, 57°C; mobile phase, citric acid-sodium citrate at a flow rate of 0.25 ml/min; chromogenic agent, ninhydrin solution at a flow rate of 0.125 ml/min; temperature of derivatization, 135°C; sample size, 20 μl; detection wavelength, 570 nm/440 nm.

2.5. Animals and Acute Toxicity of PEOR

2.5.1. Experimental Animals. SPF-graded ICR mice (aged 4 weeks, weighing 20 ± 2 g, half male and half female) were purchased from the Experimental Animal Center of Jilin University (approval number SCXK (Ji) 2008-0005, Jilin, Changchun, China). Animals were feed in polypropylene cages and allowed free access to food and water. The rearing conditions were as follows: temperature of 20 ± 2°C, relative humidity of 60 ± 10%, and a 12 h-light/dark regime. Animal experiments were conducted based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 8023, revised 1978) and approved by the Animal Care and Welfare Committee of Jilin Institute of Chemical Technology.

2.5.2. Acute Toxicity of PEOR. Acute toxicity evaluation was conducted based on the guideline of Organization for Economic Cooperation and Development (OECD) for acute oral toxicity and previous work [21, 37] with some modifications. Eighty mice (half male and half female) were randomly divided into four groups (20 in each group, in each group 10 per sex); prior to administration, animals were fasted for 12 h and had free access to water. PEOR was dissolved in distilled water (3 ml/100 g BW) and orally treated to mice in doses of 5 g/kg BW (administration once), 10 g/kg BW (administration twice in 12 h), and 20 g/kg BW (administration three times in 24 h); mice in normal control (NC) group were orally treated with equal amount of distilled water. After a single dose administration, mortality and clinical signs associated with toxicity were observed and recorded daily for consecutive two weeks; body weight changes were measured before and after administration on the 14th day.

On day 14, after being weighed, animals were fasted for 12 h (free access to water) and anesthetized with pentobarbital sodium in a dose of 50 mg/kg BW intraperitoneally. Blood samples were collected from orbit into nonheparinized Eppendorf tubes for the determination of serum biochemical parameters including AST (substrate method), ALT (substrate method), GLU (hexokinase method), TG (GPO-PAP method), CRE (sarcosine oxidase method), and BUN (urease/glutamate dehydrogenase method) using an AU2700 Beckman coulter chemistry analyzer (Beckman Coulter, Brea, CA, USA). Then animals were euthanized with carbon dioxide, and a complete necropsy was performed. Some vital organs comprising liver, spleen, kidney, and testes/ovaries were harvested and weighed. Relative organ weight was calculated according to the following formula:

$$\text{Relative organ weight}(\%) = \frac{\text{organ weight}}{\text{body weight}} \times 100. \quad (1)$$

Organs collected from animals were preserved in formalin solution (10%, pH 7.4) for the further histopathologic examination.

2.6. In Vitro Antioxidant Activity of PEOR

2.6.1. Hydroxyl Radical-Scavenging Assay. Hydroxyl radical-scavenging assay of PEOR was conducted based on the method reported by You et al. [38] with some modifications. PEOR samples were dissolved in distilled water to prepare solutions at different concentrations (2, 4, 6, 8, and 10 mg/ml). 2 ml of PEOR solution and 1 ml of PBS solution containing 0.75 mmol/l 1, 10-phenanthroline (pH 7.4) were mixed together. Subsequently, 1 ml of 0.75 mmol/l $FeSO_4$ and 1 ml of H_2O_2 solution (0.12%, *v*/*v*) were added. After being incubated at 37°C for 60 min, the absorbance of mixture (A_s) was determined at 536 nm using an UV-visible spectrophotometer (722 N, Jingke Scientific Instrument Co. Ltd., Shanghai, China). The other two reaction systems in the absence of H_2O_2 and PEOR samples were used as normal control (A_c) and blank (A_0) solutions, respectively. VC at concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml were used as positive control. The hydroxyl radical-scavenging rate was calculated as the following formula:

$$\text{Hydroxyl radical} - \text{scavenging rate}(\%) = (A_s - A_0) \times \frac{100}{(A_c - A_0)}. \quad (2)$$

2.6.2. DPPH Radical-Scavenging Assay. DPPH radical-scavenging activity of PEOR was determined using the previously reported method [39] with some modifications. PEOR samples were dissolved in distilled water to prepare solutions at concentrations of 2, 4, 6, 8, and 10 mg/ml. 2 ml of PEOR solution and 2 ml of 0.1 mmol/l DPPH ethanol solution were mixed and reacted in the dark for 30 min at room temperature. Then the absorbance of the mixture was measured at 517 nm (A_s). The reaction system in the absence of DPPH was used as normal control (A_c), system in the absence of

PEOR used as blank solution (A_0). VC at concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml was used as positive control. The DPPH radical-scavenging rate was calculated by the following equation:

$$\text{DPPH radical} - \text{scavenging rate}(\%) = (A_s - A_c) \times \frac{100}{A_0}. \quad (3)$$

2.6.3. Superoxide Anion Radical-Scavenging Assay. Superoxide anion radical-scavenging activity of PEOR was assessed by the method reported by Li et al. [40] with some modifications. PEOR samples were dissolved in distilled water to prepare solutions at concentrations of 1, 2, 3, 4, and 5 mg/ml. 1 ml of PEOR solution and 3 ml of Tris-HCl buffer (16 mmol/l, pH 8.0) containing 0.5 ml of NADH solution (470 μmol/l) and 0.5 ml of NBT solution (300 μmol/l) were mixed, and then 0.5 ml of PMS solution (60 μmol/l) was added to start the reaction. After being incubated at room temperature for 5 min, the absorbance of the mixture was read at 560 nm (A_s), mixture without PEOR samples was used as blank control (A_0). VC at concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml was used as positive control. The superoxide anion radical-scavenging rate was estimated by the following equation:

$$\text{Superoxide anion} - \text{scavenging rate}(\%) = (A_0 - A_s) \times \frac{100}{A_0}. \quad (4)$$

2.6.4. Reducing Power Assay. Reducing power was assayed according to the method reported by Wang et al. [34] with some modifications. Different concentrations (8, 10, 12, 14, and 16 mg/ml) of PEOR solutions were prepared. 1 ml of PEOR solution, 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6), and 2.5 ml of potassium ferricyanide solution (1%, *w*/*v*) were mixed and incubated at 50°C for 20 min; 2.5 ml of trichloroacetic acid (10%, *v*/*v*) was added. The mixture was centrifuged at 3000 rpm for 10 min, 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride solution (0.1%, *w*/*v*), and then the absorbance at 700 nm was measured. VC at concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml was used as positive control.

2.7. In Vivo Antioxidant Activity of PEOR. Sixty male ICR mice (aged 4 weeks, weighing 20 ± 2 g) were randomly divided into six groups (10 mice per group) as follows: normal control (NC), positive control (PC), oxidative stress model control (MC), and three PEOR-treated groups. Prior to experiment, animals were fasted for 12 h. Mice in PEOR-treated groups were administered with PEOR solution (2 ml/100 g BW) by oral gavage once a day in doses of 100, 200, and 400 mg/kg BW for 30 consecutive days, mice in PC group were treated with VC in a dose of 200 mg/kg BW, and mice in NC and MC groups were dosed with equal amount of distilled water. Dose selection of PEOR was based on the results of preliminary test (data not shown).

On the last day, after being fasted for 12 h (free access to water), except the mice in NC group, others were orally administered with a solution of 50% (*v*/*v*) ethanol to induce oxidative stress in a dose of 12 ml/kg BW. After 6 h, animals were anesthetized with pentobarbital sodium, and blood samples were collected from orbit to prepare serum for the determination of T-SOD and MDA by being centrifuged at 4°C, 4000 rpm for 10 min. Then animals were euthanized with carbon dioxide, livers were immediately dissected, washed, homogenized in physiological saline, and centrifuged at 4°C, 4000 rpm for 10 min to obtain supernatant for the quantification of GSH and PCO. The MDA (thiobarbituric acid method), T-SOD (hydroxylamine method), GSH (spectrophotometric method), and PCO (spectrophotometric method) levels were determined according to the methods described in the instructions of kits (Jiancheng Biotechnology Co. Ltd, Nanjing, Jiangsu, China) [41, 42].

2.8. In Vivo Antifatigue Activity of PEOR. The *in vivo* antifatigue evaluation of PEOR was designed and performed according to our previous work and reported method [13, 43, 44] with some modifications.

2.8.1. Experimental Design. Before experiment, male ICR mice (aged 4 weeks, weighing 20 ± 2 g) swam twice a day (10 min each time) within one week to accustom themselves to swimming; mice that failed to learn swimming were eliminated. Then 90 animals were randomly divided into three groups (30 animals each) as normal control (NC), positive control (PC), and PEOR-treated group. After being fasted for 12 h, mice in NC group were orally administered with distilled water, mice in PC group were treated with ginsenoside (2.5 mg Rg3 per 100 mg) in a dose of 50 mg/kg BW, and mice in PEOR-treated group were administered with PEOR in a dose of 400 mg/kg BW once a day for 30 consecutive days. Then the animals in each group were further divided into three subgroups of 10 mice each according to following three test/determination section (Figure 1).

2.8.2. Exhaustive Swimming Test. On the last day, animals were allowed to rest for 30 min after oral gavage, and ten mice in each group were attached to the tail of a tin wire (about 5% of body weight). Then mice were placed in a plastic swimming pool (50 cm × 50 cm × 40 cm) with temperature of 25 ± 1°C and depth of 30 cm. Exhaustive swimming time was recorded as the time when animals failed to rise to the surface to breathe within 10 s.

2.8.3. Determination of BUN and HG. After the last administration, mice in the second subgroup were allowed to rest for 30 min then forced to swim without load. After swimming for 30 min, animals were anesthetized with pentobarbital sodium, and blood samples were collected from orbit to prepare serum for the quantification of BUN using kit (urease-Berthelot method, Jiancheng Biotechnology Co. Ltd, Nanjing, Jiangsu, China). Then mice were euthanized with carbon dioxide, and livers were harvested and homogenized for the determination of HG using kit (spectrophotometric method, Jiancheng Biotechnology Co. Ltd, Nanjing, Jiangsu, China).

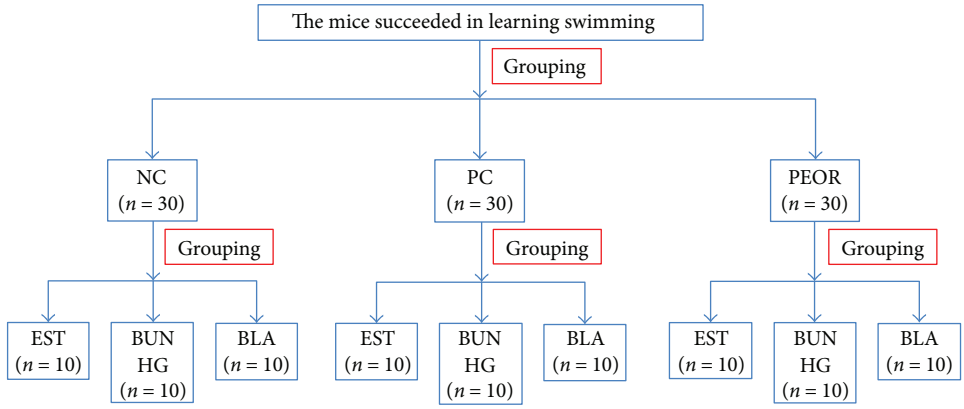

FIGURE 1: The flow chart for experimental design of antifatigue evaluation of PEOR. NC: normal control (distilled water); PC: positive control (ginsenoside, 50 mg/kg BW); PEOR (400 mg/kg BW); EST: exhaustive swimming test; BUN: blood urea nitrogen; HG: hepatic glycogen; BLA: blood lactic acid.

2.8.4. Determination of BLA. Mice in the third subgroup were also subjected to a forced swimming without load, before swimming, blood samples were taken from the eyeball for the quantification of BLA (C_1) using a Lactate Scout+ analyzer (EKF Diagnostics, Cardiff, WAL, England). After swimming for 30 min, another blood samples were immediately collected for the determination of BLA (C_2), and after resting for 20 min, blood samples were taken again for the determination of BLA (C_3). The area under the curve of BLA (AUC_{BLA}) was calculated as the following formula:

$$AUC_{BLA}\left(\frac{\text{mmol}}{\text{l}}\right) = 5 \times (C_1 + 3 \times C_2 + 2 \times C_3). \quad (5)$$

2.9. Statistical Analysis. Experimental data was expressed as mean ± SD (standard deviation), and statistical analysis was performed using a SPSS19.0 software (SPSS Inc., Chicago, USA). For results of the *in vitro* evaluation, *t*-test was used to evaluate the significance of distances between two means, and for results of the *in vivo* evaluation, Levene's test was used to detect the homogeneity of variances, if homogeneous, one-way analysis of variance (ANOVA) was operated.

3. Results

3.1. General Chemical Analysis. The total protein content of PEOR was found to be 80.35 ± 2.71%. In addition, PEOR contains 2.64 ± 0.15% lipids, 7.41 ± 0.28% ash, and 1.12 ± 0.06% moisture.

3.2. Amino Acid Analysis of PEOR. The chromatograms of amino acid standard mixture and PEOR sample were shown in Figure 2, and amino acid composition of PEOR was summarized in Table 1. Seventeen amino acids were noted in PEOR, seven of them were essential amino acids, which accounted for 41.9%. The top three amino acids present in PEOR were threonine, aspartic acid, and serine with threonine as the highest content of 120 mg/g.

3.3. Acute Toxicity of PEOR. During the period of 14 days, no death and noticeable clinical signs associated with toxicity were found in NC and all PEOR-treated groups.

3.3.1. Body Weight. As shown in Table 2, the body weights of mice increased gradually during the study period, when compared with NC group, no significant differences in body weight changes were observed.

3.3.2. Relative Organ Weight. The effects of PEOR on relative weight of vital organs including liver, kidney, spleen, and testis/ovary were demonstrated in Table 3. No significant differences in relative organ weight were noted between NC and PEOR-treated groups.

3.3.3. Biochemical Parameter. Some biochemical parameters (AST, ALT, GLU, TG, CRE, and BUN) reflecting the pathological changes of vital organs were determined, and results were shown in Table 4. Statistical analysis of these parameters indicated that there were no significant differences between NC and PEOR-treated groups.

3.3.4. Histopathological Examination. The microphotographs of histopathological observation of liver, spleen, and kidney in NC and PEOR-treated groups were exhibited in Figure 3. When compared with NC group, any obvious tissue changes were not found.

3.4. In Vitro Antioxidant Activity of PEOR. The *in vitro* scavenging capacities of PEOR against hydroxyl, DPPH, and superoxide anion radicals, as well as ferric ion-reducing power, were shown in Figure 4, and the corresponding half inhibitory concentration (IC_{50}) value was expressed in Table 5. In the range of 2~10 mg/ml, hydroxyl radical-scavenging activity of PEOR increased with the increase of sample concentration, the highest scavenging rate against hydroxyl radical of PEOR was 86.35 ± 1.82%, and the IC_{50} value was 4.85 ± 0.06 mg/ml, which was much lower than that of positive control (VC) with the IC_{50} value of 0.0476 ± 0.0005 mg/ml (Figure 4(a)). As for DPPH radical, in the range of 2~10 mg/ml, PEOR also displayed radical-

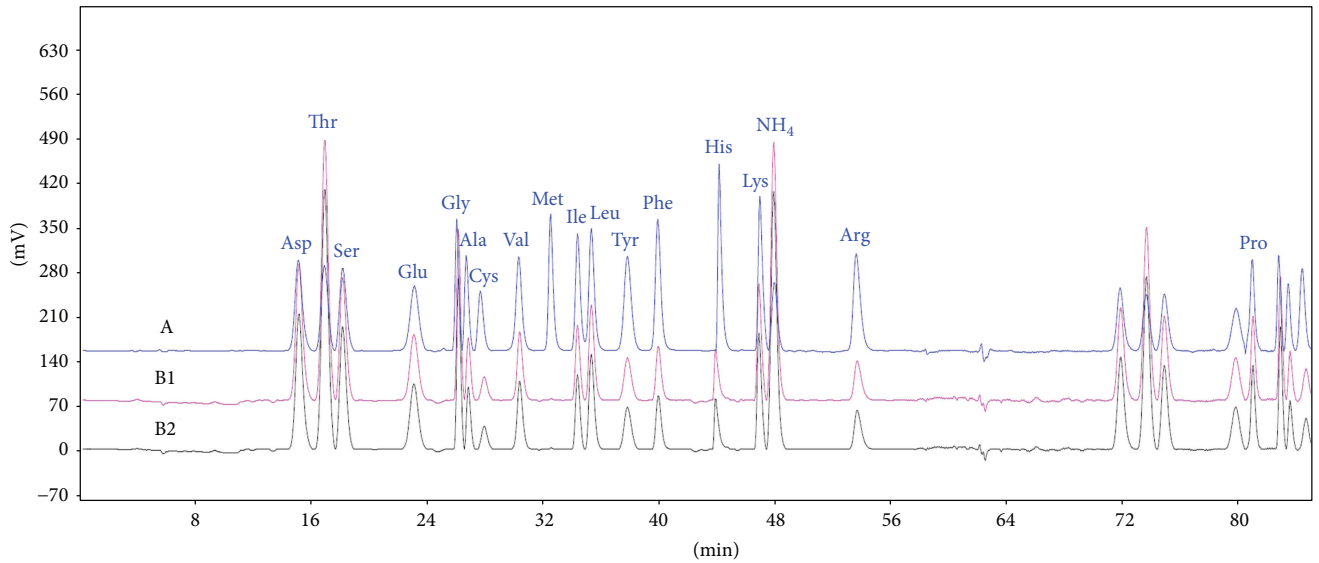

Figure 2: Chromatograms of amino acid standard mixture (A) and PEOR sample (B1 and B2). Asp: aspartic acid; Thr: threonine; Ser: serine; Glu: glutamic acid; Gly: glycine; Ala: alanine; Cys: cystine; Val: valine; Met: methionine; Ile: isoleucine; Leu: leucine; Tyr: tyrosine; Phe: phenylalanine; His: histidine; Lys: lysine; Arg: arginine; Pro: proline. Two parallel injections of sample.

Table 1: Amino acid composition of PEOR.

Amino acid	Content (mg/g)	Percent composition (%)
Aspartic acid	69.4	10.4
[a]Threonine	120	18.0
Serine	53.3	7.98
Glutamic acid	51.8	7.75
Glycine	44.3	6.63
Alanine	30.8	4.61
Cystine	37.5	5.61
[a]Valine	28.5	4.27
[a]Methionine	1.13	0.17
[a]Isoleucine	28.1	4.21
[a]Leucine	36.4	5.45
Tyrosine	25.1	3.76
[a]Phenylalanine	23.6	3.53
Histidine	13.9	2.08
[a]Lysine	42.0	6.29
Arginine	25.5	3.82
Proline	37.1	5.55
Total	668	100

[a]Essential amino acid.

Table 2: Effects of PEOR on body weight in mice.

Sex	Group	Initial weight (g)	Final weight (g)	Weight gain (g)
Male	NC	19.0 ± 1.02	31.3 ± 2.27	12.3 ± 2.13
	5 g/kg	19.4 ± 1.29	30.8 ± 2.07	11.4 ± 1.78
	10 g/kg	19.1 ± 0.91	30.9 ± 1.93	11.8 ± 1.51
	20 g/kg	19.6 ± 1.42	31.1 ± 2.38	11.5 ± 1.87
Female	NC	18.2 ± 0.72	28.7 ± 1.87	10.5 ± 1.33
	5 g/kg	18.7 ± 0.94	28.4 ± 1.39	9.71 ± 0.78
	10 g/kg	19.1 ± 0.62	28.8 ± 2.12	9.65 ± 1.49
	20 g/kg	18.5 ± 0.79	29.2 ± 1.83	10.7 ± 1.24

No statistically significant differences were noted; NC: normal control; values are the means ± SD ($n = 10$).

scavenging activity, which increased with the elevation of PEOR concentration, and the highest scavenging rate was 65.23 ± 1.22%, but inferior to VC, the IC_{50} values of PEOR and VC against DPPH radical were 4.98 ± 0.37 mg/ml and 0.036 ± 0.0011 mg/ml, respectively (Figure 4(b)). In the range of 1~5 mg/ml, PEOR exhibited scavenging activity against superoxide anion radical in a good linear relationship to sample concentration ($R^2 = 0.9971$), and the highest scavenging rate was 89.6 ± 2.43%, but its activity was still much lower than VC; the corresponding IC_{50} values were 2.58 ± 0.02 mg/ml and 0.0332 ± 0.0006 mg/ml, respectively (Figure 4(c)). In the range of 8~16 mg/ml, the absorbance (A) of PEOR at 700 nm increased with the increase of sample concentration. When $A_{700\,nm}$ was 0.2, the concentration of PEOR was 11.8 ± 0.02 mg/ml, and the corresponding concentration of VC was 0.0395 ± 0.0002 mg/ml (Figure 4(d)).

3.5. In Vivo Antioxidant Activity of PEOR

3.5.1. Effects of PEOR on MDA. As shown in Figure 5(a), MDA contents decreased with the increase of PEOR dose; the lowest content of MDA was 5.28 ± 1.27 mmol/l, 2.6-fold lower than that in MC, and 1.8-fold lower than that in PC group. Statistical analysis of MDA contents indicated that there were significant differences ($P < 0.01$) between NC and MC groups. When compared with MC group, significant differences ($P < 0.01$) were found in PC and all PEOR-treated

Table 3: Effects of PEOR on relative organ weight in mice.

Sex	Group	Liver (%)	Kidney (%)	Spleen (%)	Testis/ovary (%)
Male	NC	4.26 ± 0.49	1.33 ± 0.23	0.27 ± 0.05	0.51 ± 0.09
	5 g/kg	4.35 ± 0.53	1.29 ± 0.17	0.26 ± 0.07	0.52 ± 0.08
	10 g/kg	4.22 ± 0.57	1.31 ± 0.28	0.26 ± 0.05	0.53 ± 0.07
	20 g/kg	4.43 ± 0.54	1.30 ± 0.19	0.28 ± 0.04	0.49 ± 0.08
Female	NC	4.13 ± 0.38	1.17 ± 0.18	0.21 ± 0.04	0.038 ± 0.006
	5 g/kg	4.18 ± 0.42	1.15 ± 0.16	0.19 ± 0.05	0.042 ± 0.008
	10 g/kg	4.16 ± 0.37	1.16 ± 0.19	0.20 ± 0.06	0.039 ± 0.005
	20 g/kg	4.19 ± 0.43	1.12 ± 0.15	0.21 ± 0.06	0.041 ± 0.007

No statistically significant differences were noted; NC: normal control; values are the means ± SD ($n = 10$).

Table 4: Effects of PEOR on biochemical parameter in mice.

Sex	Group	AST (U/l)	ALT (U/l)	GLU (mmol/l)	TG (mmol/l)	CRE (μmol/l)	BUN (mmol/l)
Male	NC	143 ± 12.1	31.9 ± 4.08	4.53 ± 0.58	2.13 ± 0.08	45.2 ± 6.13	7.18 ± 0.55
	5 g/kg	142 ± 11.6	33.0 ± 3.34	4.41 ± 0.51	2.04 ± 0.17	47.6 ± 7.47	6.79 ± 0.46
	10 g/kg	141 ± 12.3	32.7 ± 3.93	4.49 ± 0.53	2.05 ± 0.09	43.9 ± 6.08	7.06 ± 0.68
	20 g/kg	142 ± 11.2	32.3 ± 4.22	4.51 ± 0.64	2.03 ± 0.07	45.8 ± 5.66	7.36 ± 0.71
Female	NC	118 ± 10.7	26.9 ± 3.52	4.38 ± 0.69	1.84 ± 0.12	39.6 ± 5.73	7.73 ± 0.91
	5 g/kg	117 ± 9.14	28.1 ± 3.83	4.75 ± 0.53	1.87 ± 0.28	37.2 ± 5.48	8.06 ± 0.54
	10 g/kg	121 ± 8.76	27.1 ± 2.89	4.63 ± 0.62	2.06 ± 0.15	36.8 ± 7.61	7.94 ± 0.62
	20 g/kg	118 ± 10.2	27.7 ± 3.23	5.06 ± 0.68	1.83 ± 0.26	38.4 ± 6.33	8.15 ± 1.23

No statistically significant differences were noted; NC: normal control; values are the means ± SD ($n = 10$).

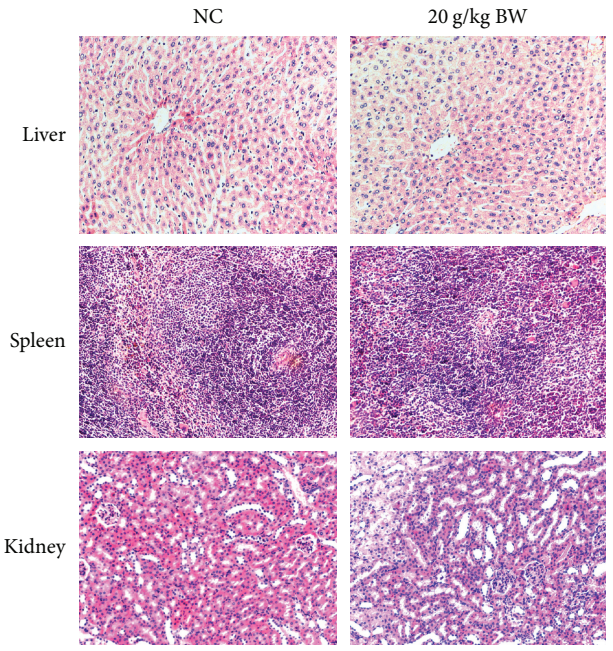

Figure 3: Representative microphotographs of the liver, spleen, and kidney of mice at 200× from NC and 20 g/kg BW of PEOR-treated groups.

groups. Significant differences ($P < 0.01$) were also noted compared with PC group.

3.5.2. Effects of PEOR on GSH. As shown in Figure 5(b), when compared with NC, significant differences ($P < 0.01$) in GSH contents were observed in MC group, and significant differences ($P < 0.01$) were also found between MC and other groups. GSH contents increased with the increase of dose; when PEOR dose was 400 mg/kg, the content of GSH reached 17.85 ± 3.82 mg/gprot, which was a little higher than that in PC (16.36 ± 3.82 mg/gprot), but no statistically significant differences were noted.

3.5.3. Effects of PEOR on T-SOD. As shown in Figure 5(c), T-SOD activities in MC decreased significantly ($P < 0.01$) compared with NC. When compared with MC, significant differences ($P < 0.01$) in T-SOD were found in PC and all PEOR-treated groups. Oral administration of PEOR can increase T-SOD activities in a dose-dependent manner ($P < 0.01$), when PEOR dose reached 400 mg/kg, the T-SOD activity was 234.6 ± 14.5 U/ml, which was significantly ($P < 0.01$) higher than that in PC (179.6 ± 21.0 U/ml).

3.5.4. Effects of PEOR on PCO. As shown in Figure 5(d), PCO contents in MC group were significantly higher ($P < 0.01$) than those in NC. When compared with MC, significant differences ($P < 0.01$) in PCO were found in PC and all

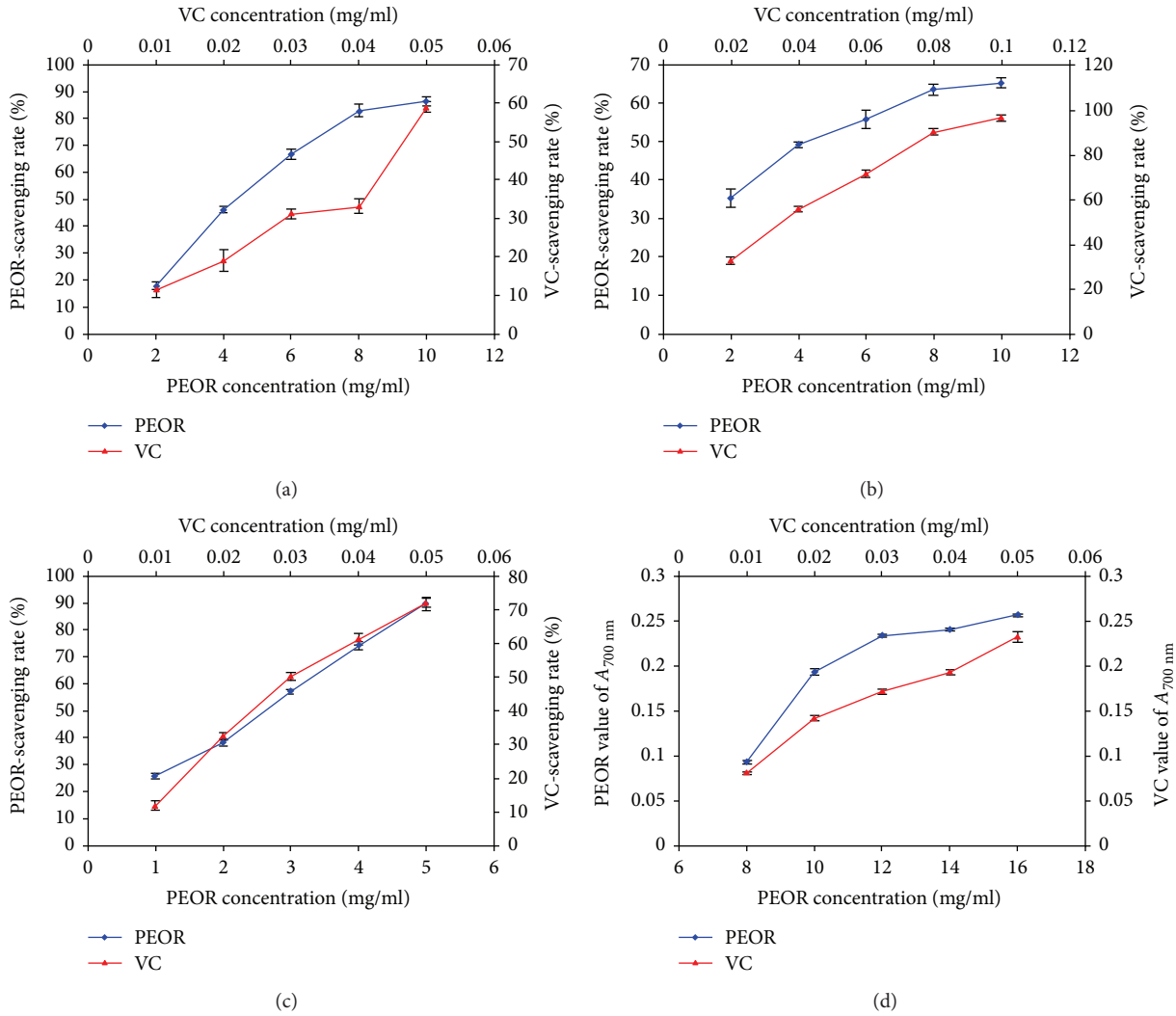

Figure 4: The *in vitro* antioxidant activities of PEOR using VC as a positive control. (a) Hydroxyl radical-scavenging activity; (b) DPPH radical-scavenging activity; (c) superoxide anion radical-scavenging activity; (d) reducing power. Data was expressed as the mean ± SD ($n = 3$).

Table 5: The IC_{50} values and reducing power of PEOR and VC.

Sample	IC_{50} (mg/ml)			Reducing power (mg/ml)[a]
	Hydroxyl radical	DPPH radical	Superoxide anion	
PEOR	4.85 ± 0.06**	4.98 ± 0.37**	2.58 ± 0.02**	11.8 ± 0.02**
VC	0.0476 ± 0.0005	0.036 ± 0.0011	0.0332 ± 0.0006	0.0395 ± 0.0002

[a]The corresponding concentrations of PEOR and VC, when $A_{700\ nm} = 0.2$; data was expressed as the mean ± SD ($n = 3$); symbol indicates statistically significant differences, **$P < 0.01$ versus VC group.

PEOR-treated groups. PCO contents decreased in a dose-dependent manner ($P < 0.01$), and significant differences ($P < 0.01$) were noted in 400 mg/kg of PEOR-treated group compared with PC (1.16 ± 0.37 nmol/mgprot versus 2.62 ± 0.99 nmol/mgprot).

3.6. In Vivo Antifatigue Activity of PEOR

3.6.1. Exhaustive Swimming Test. As shown in Figure 6, there were significant differences ($P < 0.01$) between NC and PEOR-treated groups, and the swimming time was 6.38 ± 2.26 min, 1.9-fold longer than that in NC. When compared with PC, significant differences ($P < 0.05$) were found in PEOR-treated group, approximately 1.5-fold longer than that in PC.

3.6.2. Effects of PEOR on BUN. As shown in Figure 7, when compared with NC, BUN contents in PC and PEOR-treated groups were significantly ($P < 0.05$) lower by 18% and 17%, respectively.

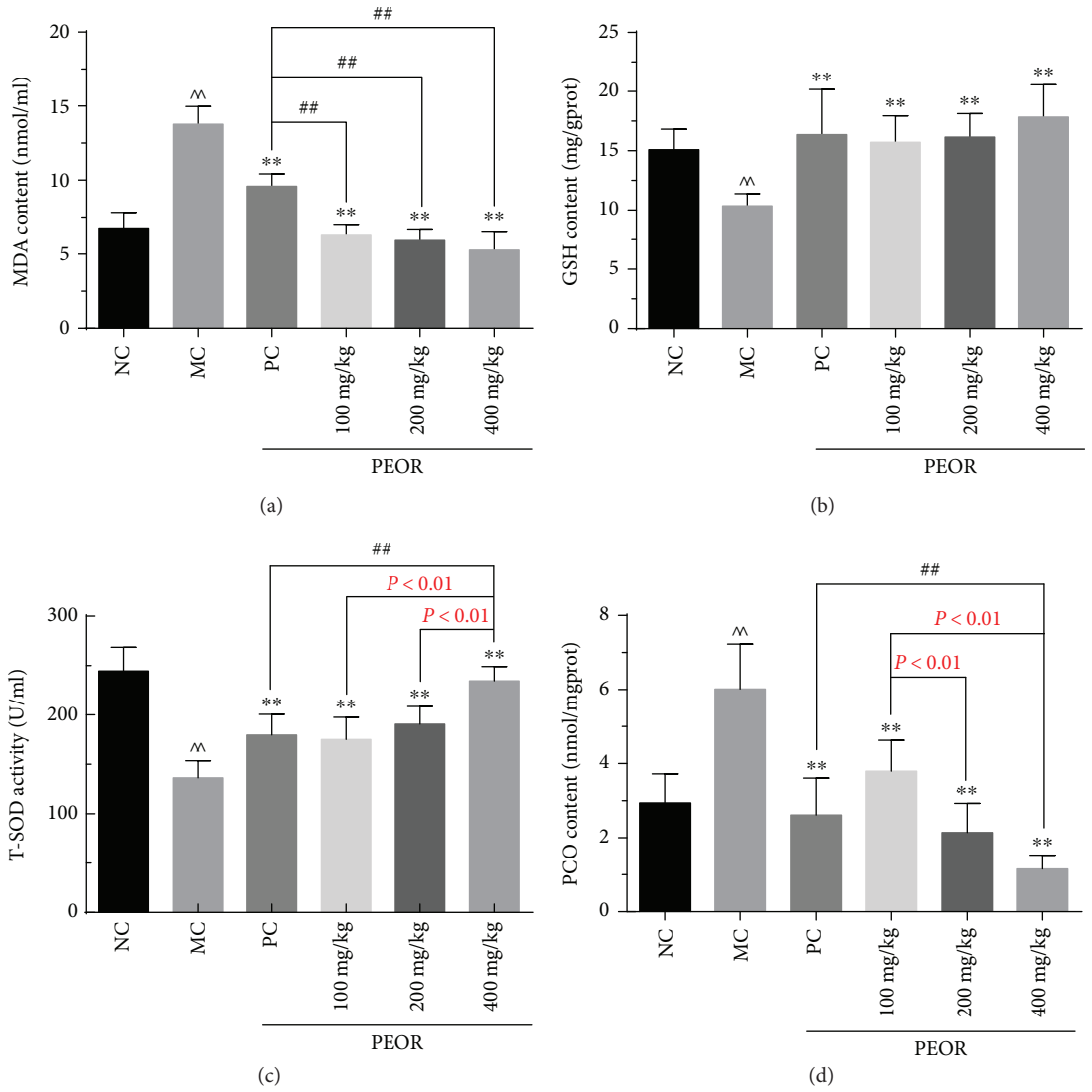

Figure 5: Effects of PEOR on (a) MDA, (b) GSH, (c) T-SOD, and (d) PCO. Data denoted were means + SD ($n = 10$). Different symbols indicate statistically significant differences, $^{\wedge\wedge}P < 0.01$ as compared with NC group; $^{**}P < 0.01$ as compared with MC group; $^{\#\#}P < 0.01$ as compared with PC group. MDA: malonaldehyde; GSH: glutathione; T-SOD: total superoxide dismutase; PCO: protein carbonyls; NC: normal control; MC: model control; PC: positive control (VC in a dose of 200 mg/kg BW).

3.6.3. Effects of PEOR on HG. As shown in Figure 8, HG contents in PEOR-treated group was 37.66 ± 11.49 mg/g and that in NC was 35.83 ± 11.49 mg/g; no significant differences were found. There were statistically significant differences ($P < 0.01$) in HG between PC and NC groups, the HG content in PC was 56.88 ± 13.3 mg/g, 1.59-fold higher than that in NC and 1.51-fold higher than that in PEOR-treated group.

3.6.4. Effects of PEOR on BLA. BLA content at different time points ($t_{\text{before swimming}}$, $t_{0\,\text{min after swimming}}$, and $t_{20\,\text{min after swimming}}$) was determined. As shown in Table 6, before swimming, no significant differences in BLA were noted among groups. When compared with NC group, at 0 min after swimming, the BLA contents in PC ($P < 0.01$) and PEOR-treated groups ($P < 0.01$) significantly decreased. After resting for 20 min, the elevated BLA levels of all groups were reduced, and no statistically significant differences were observed. The AUC_{BLA} value was calculated and found that there were significant differences ($P < 0.05$) in PC and PEOR-treated groups compared with NC group. The AUC_{BLA} value in PEOR-treated group was similar to that in PC group (115.4 ± 24.7 mmol/l versus 114.4 ± 19.4 mmol/l).

4. Discussion

Since *Oviductus ranae* (OR) is a precious TCM with abundant protein contents, thus, in this paper, the protein-rich extract of OR (PEOR) was prepared and analyzed. The results indicated that PEOR contains 80.35 ± 2.71% protein, which comprises seventeen amino acids, seven of them are essential amino acids with total contents of 41.9% (Figure 2 and Table 1).

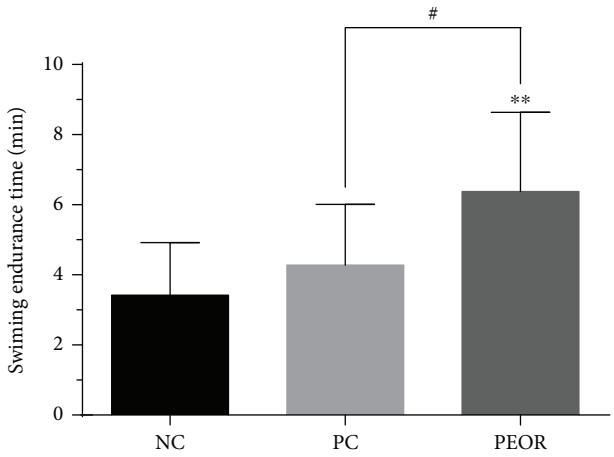

Figure 6: Effects of PEOR on exhaustive swimming endurance time. Data denoted were means + SD ($n = 10$). Different symbols indicate statistically significant differences, $^{**}P < 0.01$ as compared with NC; $^{\#}P < 0.05$ as compared with PC. NC: normal control; PC: positive control (ginsenoside in a dose of 50 mg/kg BW); PEOR (400 mg/kg BW).

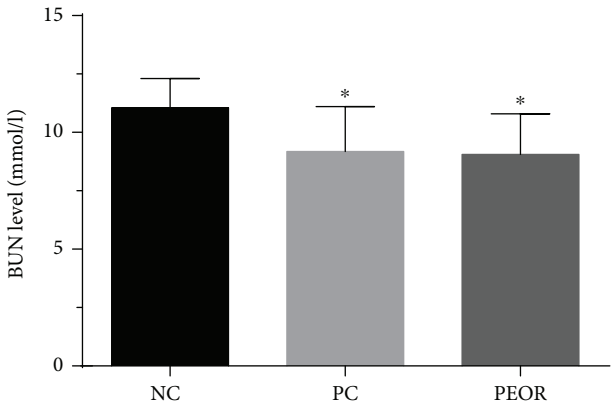

Figure 7: Effects of PEOR on BUN. Data denoted were means + SD ($n = 10$). Symbol indicates statistically significant differences, $^{*}P < 0.05$ as compared with NC group. BUN: blood urea nitrogen; NC: normal control; PC: positive control (ginsenoside in a dose of 50 mg/kg BW); PEOR (400 mg/kg BW).

In view of increasing number of therapeutic risks caused by the use of natural products [45–47] and our previous work [21], where we found that OR possesses high-safety property, in present study, only a single-dose oral toxicity with an observation of 14-day interval was conducted to evaluate the safety of PEOR, and 20 g/kg was taken as an upper limit dose. During the observation period, no death and noticeable clinical signs associated with toxicity were found in NC and PEOR-treated groups; there were also no significant changes in body weights (Table 2), suggesting that the maximum tolerated dose (MTD) of PEOR may be higher than 20 g/kg in mice.

Then a complete necropsy and serum biochemical and histopathological examinations were performed to assess the harmful effects of PEOR on inner organs. During necropsy, any noticeable abnormalities were not noticed, and

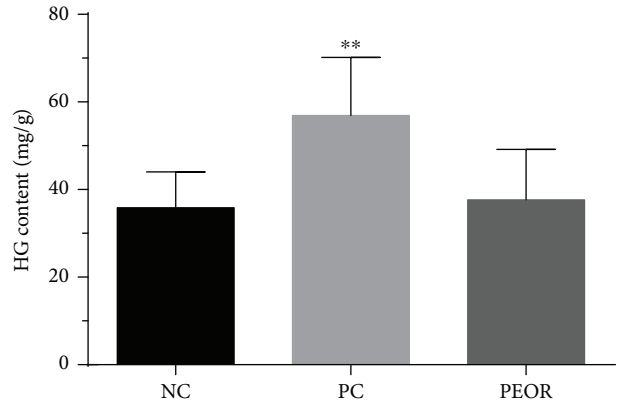

Figure 8: Effects of PEOR on HG. Data denoted were means + SD ($n = 10$). Symbol indicates statistically significant differences, $^{**}P < 0.01$ as compared with NC group. HG: hepatic glycogen; NC: normal control; PC: positive control (ginsenoside in a dose of 50 mg/kg BW); PEOR (400 mg/kg BW).

Table 6: Effects of PEOR on BLA.

Group	Before swimming (C_1) (mmol/l)	0 min after swimming (C_2) (mmol/l)	20 min after swimming (C_3) (mmol/l)	Area under the curve (AUC_{BLA}) (mmol/l)
NC	2.88 ± 0.79	6.38 ± 1.27	3.28 ± 0.67	142.8 ± 22.7
PC	2.72 ± 0.66	4.63 ± 0.94**	3.13 ± 0.61	114.4 ± 19.4*
PEOR	2.84 ± 0.84	4.59 ± 1.04**	3.24 ± 1.17	115.4 ± 24.7*

Data denoted were means ± SD ($n = 10$). Different symbols indicate statistically significant differences, $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with NC group. BLA: blood lactic acid; NC: normal control; PC: positive control (ginsenoside in a dose of 50 mg/kg BW); PEOR (400 mg/kg BW). $AUC_{BLA} = 5 \times (C_1 + 3 \times C_2 + 2 \times C_3)$.

there were no significant differences in relative weights of vital organs including liver, kidney, spleen, and testis/ovary between NC and PEOR-treated groups (Table 3). Serum biochemical parameter is another important profile to detect the *in vivo* injury degree of organs, for example, ALT and AST are closely related to the function of the liver, while CRE and BUN are important biomarkers of renal toxicity [48, 49]. When compared with NC, no significant differences in AST, ALT, GLU, TG, BUN, and CRE were found in PEOR-treated groups (Table 4), indicating that oral administration of PEOR has no harm on inner organs, which was further confirmed by the results of histopathological examination, where any obvious changes in liver, spleen, and kidney were not found compared with NC even in the maximum dose of 20 g/kg (Figure 3).

In antioxidant assay, the *in vitro* evaluation was firstly conducted to obtain the antioxidant potential of PEOR. The results showed that PEOR exhibits certain scavenging capacities against hydroxyl, DPPH, and superoxide anion radicals, as well as certain reducing power to ferric ion in different concentrations, and activities increased with the increase of concentration (Figure 4 and Table 5). The free radical-scavenging activity of PEOR may be contributed by some of its amino acid residues, which may provide active hydrogen

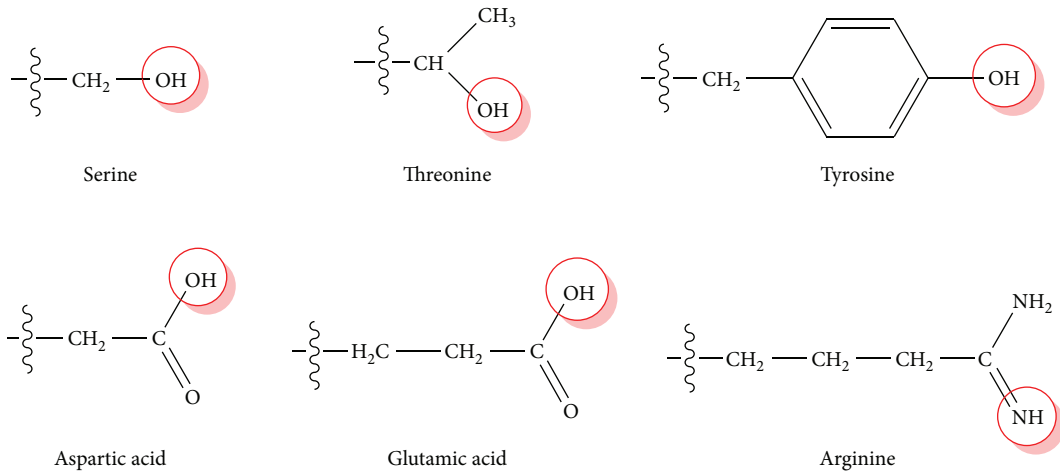

Figure 9: Hydrogen-donor side chains of amino acids present in PEOR.

to destroy free radicals in liquid medium [50]; as shown in Table 1 and Figure 9 six amino acids with hydrogen-donor side chains were present in PEOR. However, when compared with VC, a well-known water-soluble antioxidant, the free radical-scavenging capacities and ferric ion-reducing power of PEOR were significantly ($P < 0.01$) lower; the IC_{50} values were about 100-fold higher than those of VC (Table 5). These disappointing results were consistent with the general finding that proteins or large polypeptides show lower free radical-scavenging capacities than their short peptides and amino acids, owing to the fact that smaller molecules are inclined to interact with free radicals more effectively [51]. In order to verify this hypothesis, the *in vivo* antioxidant evaluation of PEOR was further performed.

Based on the results of previous study [41], an ethanol-induced oxidative stress mice model was taken to evaluate the *in vivo* antioxidant activity of PEOR using VC in a dose of 200 mg/kg as positive control. Four antioxidant biomarkers including MDA (product of lipid peroxidation), GSH (endogenous antioxidant), T-SOD (antioxidase), and PCO (product of protein oxidation) were selected and determined. As shown in Figure 5, when compared with NC, significant differences ($P < 0.01$) in MDA, GSH, T-SOD, and PCO were found in MC, suggesting that an ethanol-induced oxidative stress mice model was well established. When compared with MC, significant differences ($P < 0.01$) in MDA, GSH, T-SOD, and PCO were noted in PEOR-treated groups, and the positive effects enhanced with the increase of dose; in the case of T-SOD and PCO, a dose-dependent ($P < 0.01$) manner was observed. When compared with PC, significant differences ($P < 0.01$) in MDA were found in all PEOR-treated groups, and similar tendencies ($P < 0.01$) in T-SOD and PCO were noted in 400 mg/kg of PEOR-treated group. These results revealed that oral administration of PEOR can reduce the oxidative stress caused by ethanol in mice and has more effects on MDA, T-SOD, and PCO than on GSH, especially for MDA. The 400 mg/kg in mice is a promising effective dose for antioxidant activity of PEOR. Its mechanism may involve the decrease of MDA and PCO formation and increase of T-SOD activity and GSH synthesis. The strong *in vivo* antioxidant activity of PEOR contradicted with the weak activity of *in vitro* evaluation, which further confirmed our speculation, that is, the absorbed amino acids and small peptides may be the real forms of PEOR to exert antioxidant effects. Previous studies have manifested that the types and compositions of absorbed protein digestive products are closely related to their activities [34], and some amino acids including threonine, cysteine, methionine, tryptophan, tyrosine, histidine, phenylalanine, glutamic acid, aspartic acid, and lysine show good antioxidant activities both *in vitro* and *in vivo* [52–54]. As shown in Table 1, there were eight antioxidant amino acids present in PEOR, that is, aspartic acid (69.4 mg/g), threonine (120 mg/g), glutamic acid (51.8 mg/g), methionine (1.13 mg/g), tyrosine (25.1 mg/g), phenylalanine (23.6 mg/g), histidine (13.9 mg/g), and lysine (42.0 mg/g), which accounted for approximately 52% of the total amount of amino acids present in PEOR; their existence may play an important role in the exertion of antioxidant effect *in vivo*.

Several findings have manifested that intense exercise-induced oxidative stress can cause the accumulation of free radicals and induce muscle fatigue [55, 56]. Exogenous dietary antioxidants can potentiate the scavenging effects of endogenous antioxidants to fight against fatigue [11]. In consideration of the strong *in vivo* antioxidant effects of PEOR, as well as its high safety, we then evaluated the *in vivo* antifatigue activity of PEOR in the promising dose found in antioxidant evaluation (400 mg/kg). In our previous work [43], we noticed that ginsenoside in a dose of 35 mg/kg in mice is a good positive control for the evaluation of antifatigue effect of natural product; in order to ensure the control full effectiveness, we slightly raised the dose and selected 50 mg/kg as the dose of positive control. The exhaustive swimming time, coupled with some biochemical indicators (BUN, HG, and BLA) reflecting fatigue degree, was determined to estimate the *in vivo* antifatigue effect of PEOR. As shown in Figures 6–8, PEOR in a dose of 400 mg/kg can prolong the exhaustive swimming time and reduce the elevated BUN content caused by intense exercise in mice, but little

effect on HG storage. As for BLA profile (Table 6), PEOR has little effect on BLA under resting-state conditions but significantly ($P < 0.01$) reduces the elevated BLA level induced by intense exercise. When compared with PC (ginsenoside, 50 mg/kg), PEOR in a dose of 400 mg/kg can significantly ($P < 0.05$) improve the exercise tolerance, indicating that PEOR (400 mg/kg) is also a promising candidate for the development of nature-based antifatigue supplement, owing to the fact that exhaustive swimming time is the most direct and potent index to measure the antifatigue activity of tested sample [57]. The antifatigue capacity of PEOR could be also attributed to the extraenergy supplied by its glucogenic amino acids including aspartic acid (69.4 mg/g), threonine (120 mg/g), serine (53.3 mg/g), glutamic acid (51.8 mg/g), glycine (44.3 mg/g), alanine (30.8 mg/g), valine (28.5 mg/g), methionine (1.13 mg/g), isoleucine (28.1 mg/g), histidine (13.9 mg/g), arginine (25.5 mg/g), and proline (37.1 mg/g), which accounted for about 75% of the total amino acids present in PEOR (Table 1). The exact mechanism regarding antifatigue effect of PEOR deserved to be studied in the near future. In addition, due to the fact that most of the proteins enter the bloodstream as single amino acids [58, 59], in this paper, we mainly discussed the active contributions of amino acids in PEOR. Meanwhile, contributions of small peptides absorbed in intestinal tract to the antioxidant and antifatigue effects of PEOR, especially the chemical structures and activity-favourable conformations, need to be further explored.

5. Conclusion

In summary, PEOR is mainly composed of seventeen amino acids with seven essential ones. It possesses high safety with MTD value upper than 20 g/kg in mice and exerts weak scavenging capacities against hydroxyl, DPPH, and superoxide anion radicals, as well as ferric ion-reducing power *in vitro*, but exhibits strong antioxidant activity in ethanol-induced oxidative stress mice model; its mechanism may involve the decrease of MDA and PCO formation, associated with the increase of T-SOD activity and GSH synthesis. The *in vivo* antioxidant effect of PEOR increased with the increase of dose; 400 mg/kg is a promising dose deserved to be further studied; in this dose, PEOR also shows antifatigue effect. There are six amino acids with hydrogen-donor side chains, eight antioxidant amino acids, and twelve glucogenic amino acids present in PEOR; they may play an important role in exertion of the *in vitro* and *in vivo* antioxidant activity, as well as the *in vivo* antifatigue effect of PEOR.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Hongli Zhou and Yang Zhang conceived and designed the experiments; Yang Liu performed the *in vivo* antioxidant evaluation; Kun Zhu determined the biochemical indicators; Yao Dong conducted the *in vitro* antioxidant evaluation; Hao Cui prepared and analyzed PEOR; Liping Mao and Xiaoxiao Xu did the *in vivo* antifatigue evaluation; Yang Zhang analyzed the data and wrote the manuscript, and Hongli Zhou revised it.

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Research Article

Antioxidant and Anti-Inflammatory Effects of Blueberry Anthocyanins on High Glucose-Induced Human Retinal Capillary Endothelial Cells

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Blueberries possess abundant anthocyanins, which benefit eye health. The purpose of this study was to explore the protective functional role of blueberry anthocyanin extract (BAE) and its predominant constituents, malvidin (Mv), malvidin-3-glucoside (Mv-3-glc), and malvidin-3-galactoside (Mv-3-gal), on high glucose- (HG-) induced injury in human retinal capillary endothelial cells (HRCECs). The results showed that BAE, Mv, Mv-3-glc, and Mv-3-gal enhanced cell viability ($P < 0.05$ versus the HG group at 24 h); decreased the reactive oxygen species (ROS, $P < 0.01$ versus the HG group both at 24 and 48 h); and increased the enzyme activity of catalase (CAT) and superoxide dismutase (SOD) ($P < 0.05$ versus the HG group both at 24 and 48 h). Mv could greatly inhibit HG-induced Nox4 expression both at 24 and 48 h ($P < 0.05$), while BAE and Mv-3-gal downregulated Nox4 only at 48 h ($P < 0.05$). Mv, Mv-3-glc, and Mv-3-gal also changed nitric oxide (NO) levels ($P < 0.05$). BAE and Mv-3-glc also influenced angiogenesis by decreasing the vascular endothelial cell growth factor (VEGF) level and inhibiting Akt pathway ($P < 0.05$). Moreover, Mv and Mv-3-glc inhibited HG-induced intercellular adhesion molecule-1 (ICAM-1, $P < 0.001$) and nuclear factor-kappa B (NF-κB) ($P < 0.05$). It indicated that blueberry anthocyanins protected HRCECs via antioxidant and anti-inflammatory mechanisms, which could be promising molecules for the development of nutraceuticals to prevent diabetic retinopathy.

1. Introduction

Diabetic retinopathy (DR) is a common complication of diabetes mellitus and a leading cause of vision loss and blindness in diabetic patients [1]. Hyperglycemia is known to be the main contributing factor in the progression of the disease, which triggers pathological metabolic and biochemical changes that damage the retinal cells [2]. In the development of DR, retinal microvascular dysfunction includes the loss of endothelial cells and pericytes, capillary occlusion and blood-retinal barrier breakdown, and endothelial cell hypertrophy and degeneration that lead to capillary nonperfusion and hypoxia [3, 4].

Dietary intake of phytochemicals, particularly anthocyanins, is being increasingly recognized as beneficial for modern human eye health [5]. Anthocyanins are known to have antioxidant, antimicrobial, antiviral, antiallergic, anticarcinogenic, anti-inflammatory, antimutagenic, and antiproliferative effects and thus may play an essential role in preventing various degenerative diseases including DR [6]. Anthocyanins from different vegetables and fruits, such as blueberry, bilberry, mulberry, maqui berry, blackcurrant, and black

soybean, could reduce retinal degeneration and improve visual function [7–10]. Their possible functional mechanisms include the attenuation of oxidative damage, alteration of retinal enzyme activity, inhibition of inflammation, stimulation of the immune system, reduction of platelet aggregation, and modulation of cholesterol synthesis and hormone metabolism [11, 12]. More and more scientists and consumers realize the vision-related benefits of anthocyanin-rich berries, and the anthocyanosides from berries are currently used in ophthalmology for their capacity to improve vision and prevent diabetic retinopathy [13].

Blueberries are rich in anthocyanins, whose content is believed to be the highest among all commonly consumed vegetables and fruits [14]. Blueberries are famous for their wide range of health benefits, including ameliorating diabetes, attenuating vascular problems, maintaining endothelial function, and preventing retinal diseases [15]. Many scientists have reported that blueberry components such as anthocyanins, polyphenols, and pterostilbene can protect corneal epithelial cells against light-induced retinal injury via antioxidative, antiangiogenic, and antiaging effects in vitro, and the bioactivity of blueberries in improving vision has been confirmed in vivo [13, 16, 17]. However, the function of blueberry anthocyanins on human retinal capillary endothelial cells (HRCECs) is still unknown. Liu et al. identified anthocyanin components of wild Chinese blueberries and found that malvidin glycosylated with hexose or pentose accounted for >46% of the total anthocyanin content in blueberries [18]. Our previous survey also confirmed that malvidin-3-glucoside (Mv-3-glc) and malvidin-3-galactoside (Mv-3-gal) were major anthocyanins in Brightwell rabbiteye blueberry (*Vaccinium ashei*) of Nanjing [19]. In the present study, the protective effects of blueberry anthocyanin extract, as well as malvidin and its glycosides (Mv-3-glc and Mv-3-gal) on high glucose-induced injury in HRCECs, were investigated to propose a functional mechanism for the role of antioxidants in eye nourishment.

2. Materials and Methods

2.1. Materials and Chemicals. Brightwell rabbiteye blueberry (*Vaccinium ashei*) was harvested in July 2016 from the orchards of Lishui in Nanjing. The plant species were authenticated by a blueberry expert at the Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences. The collected fruits were kept at −18°C in the dark.

Human retinal capillary endothelial cells (HRCECs, primary culture cells) were purchased from Jennio Biotechnology Co. Ltd. (Guangzhou, China). Malvidin (Mv), malvidin-3-glucoside (Mv-3-glc), malvidin-3-galactoside (Mv-3-gal), and trypsin were purchased from Sigma Aldrich (Shanghai, China). Fetal bovine serum (FBS) and DMEM medium were obtained from Gibco/Invitrogen (Shanghai, China). Penicillin and streptomycin were purchased from Life Technologies (Shanghai, China). Reactive oxygen species (ROS) Assay Kit, BCA (Bicinchoninic acid) Protein Assay Kit, and MTT Cell Proliferation Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Andygene human catalase (CAT), superoxide dismutase (SOD), and endothelial nitric oxide synthase (eNOS) Enzyme Activity Assay Kits, and nitric oxide (NO), angiotensin-converting enzyme (ACE), vascular endothelial cell growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1), and nuclear factor-kappa B (NF-κB) ELISA Kits were purchased from Xinzetianyou Biotechnology Co., Ltd. (Beijing, China). All chemicals and reagents were of analytical grade.

2.2. Antibodies. Rabbit monoclonal primary antibody against Nox4 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal primary antibody against Akt was purchased from MengZhouShi Ruiying Biological Technology Co. Ltd. (Jiaozuo, Henan, China). Mouse monoclonal primary antibody against VEGF was purchased from Abcam (Shanghai, China). Rabbit monoclonal primary antibody against β-actin was purchased from Sigma Aldrich (Shanghai, China). Goat anti-mouse/rabbit IgG HRP-linked secondary antibodies were purchased from Cell Signaling Technology Inc. (Shanghai, China). Primary antibodies were used at 1 : 1000 dilutions. Secondary antibodies were used at 1 : 4000 dilutions.

2.3. Extraction of Anthocyanins from Blueberries. Extraction of anthocyanins from blueberries was performed according to the method used previously by Liu et al. [18]. The stored blueberry fruits were thawed at room temperature and beaten to a pulp. The blueberry pulp (250 g) was soaked in 1000 mL of methanol containing 1% HCl solution for 24 h by mixing every two hours to extract anthocyanins. The extract was collected and centrifuged at 5000 ×g for 15 min. After in vacuo evaporation of the solvent at 40°C, the residue was dissolved in 300 mL double distilled water and extracted with 1 : 1 (*v*/*v*) ethyl acetate three times to remove impurities, such as phenolic acids. The water phase containing anthocyanins was collected and concentrated in vacuo to obtain the crude anthocyanin extract. The extract was further purified with AB-8 macroporous resin (Sigma Aldrich, China). The extract was subjected to column chromatography on 1000 g AB-8 macroporous resin for 24 h absorption and then eluted with double distilled water to remove fructose and protein. Anthocyanin fraction was eluted with 80% ethanol containing 1% HCl solution, concentrated in vacuo, and then dried using an Eyela FDU-1200 freeze dryer (Tokyo Rikakikai, Japan) to get blueberry anthocyanin extract (BAE) powder (0.3 g). Blueberry anthocyanin extract was characterized by HPLC-DAD to determine the exact amount of each compound in the extract. Malvidin glycosides were the dominant anthocyanins in the blueberries, accounting for 47.9% of total anthocyanin content, in which Mv-3-glc and Mv-3-gal were 17.2% and 22.6%, respectively (unpublished data).

2.4. Endothelial Cell Culture and Treatment. HRCECs were cultured in normal glucose (5.5 mmol/L) DMEM containing 10% FBS and 1% streptomycin and penicillin at 37°C in a 5% CO_2 humidified incubator. The third to fifth passage cells were used for all experiments at 80–90% confluence. HRCECs were quiesced in a reduced serum medium (not containing FBS) for 4 h before the experiment. Based on the preliminary experiments, a dose-response assay regarding

viability showed that the extract and compounds were not toxic to the cells at a concentration of less than 10 μg/mL. The cells were seeded in 6-well plates and pretreated with 10 μg/mL of BAE, Mv, Mv-3-glc, or Mv-3-gal for 24 h, respectively. Then, the sample cells were stimulated with high glucose (final concentration 30 mmol/L) for another 24 and 48 h. Normal glucose (NG: 5.5 mmol/L) group without pretreating blueberry anthocyanins was used as the control. High glucose (HG: 30 mmol/L) group without pretreating blueberry anthocyanins was used as the oxidative model. The supernatants were collected for ELISA analysis. The cells were prepared for Western blotting.

2.5. Cell Viability Detection. The cell viability was determined by MTT method. The cells were cultured with high glucose (30 mmol/L) for 24 and 48 h with or without BAE, Mv, Mv-glc, or Mv-gal pretreatment for 24 h. Ten microliters of MTT (5 mg/mL) was added to each well and cultured for 4 h. After the removal of MTT solution, cell crystal was dissolved by adding 100 μL DMSO (dimethylsulfoxide) and shaking 10 min slowly. The absorbance was measured at 550 nm on a Synergy H4 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The reader was controlled via Hyper Terminal Applet ELISA software. The cells were cultured only when normal glucose (5.5 mmol/L) was used as the control group. The blank group used the wells without cells. The cell viability was determined with the following formula: $\text{Cell viability}(\%) = (\text{sample group OD value} - \text{blank group OD value})/(\text{control group OD value} - \text{blank group OD value}) \times 100\%$.

2.6. Reactive Oxygen Species (ROS) Assay. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) detection kit was used to assess the ROS level in HRCECs. Briefly, the cells were seeded in 96-well plates, treated with different samples to incubate for 24 h and cultured with high glucose (30 mmol/L) for 24 and 48 h. After washing cells with phosphate-buffered saline (PBS), 10 μmol/L DCFH-DA was added to each well and reacted for 20 min at 37°C. The cells were collected after dissociation, and fluorescence was recorded by a Synergy H4 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) with 488-P excitation and 525-P emission filters. The total fluorescence intensity of cells in each well was noted, and ROS generation was measured as fold of the control (each NG group, 24 and 48 h).

2.7. ELISA Analysis and Western Blotting. The levels of CAT, SOD, NO, eNOS, ACE, VEGF, ICAM-1, and NF-κB were quantified from the supernatants using ELISA Kits or enzyme activity assay kits. The assay procedure was employed according to the kit protocol booklet instructions. The absorbance was measured at 450 nm on a Synergy H4 Multi-Mode Microplate Reader (BioTek Instruments, Inc. Winooski, VT, USA). The reader was controlled via Hyper Terminal Applet ELISA software. The total cell protein of the supernatant in each well was detected using BCA Protein Assay Kit.

Protein expression of Nox4, Akt, and VEGF was also analyzed by Western blotting performed on the HRCEC lysates. Data were normalized by reprobing the membrane with an antibody against β-actin, which was used as a loading control. Cell lysates from untreated cells were loaded on to every gel, and all data were expressed as fold of the corresponding control.

2.8. Statistical Analysis. All data presented are mean value ± standard deviation (SD) of three independent experiments. Figures were obtained using GraphPad Prism Version 5 (GraphPad Software, Inc., CA, USA). The data of each group used t-tests were to determine significant differences among different treatments (NG, HG, BAE, Mv, Mv-3-glc, and Mv-3-gal). Two-way analysis of variance (ANOVA) was used to analyze differences in culture time (24 and 48 h) and their interactions with treatment. The differences were considered significant at P value < 0.05.

3. Results

3.1. Effects of Blueberry Anthocyanins on Cell Viability. In this study, we observed that stimulation with high glucose concentration (30 mmol/L, HG: 64.03 ± 2.97%) for 24 h significantly decreased cell viability in comparison with normal glucose- (5.5 mmol/L, NG: 100 ± 7.04%) incubated cells ($P < 0.01$). MTT assay showed that 10 μg/mL of BAE, Mv, Mv-3-glc, and Mv-3-gal all significantly increased cell viability after 24 h of high glucose incubation ($P < 0.05$) (Figure 1). The effect of blueberry anthocyanin extract (BAE: 78.69 ± 4.75%) was lower than those of malvidin and its glycosides, while the effects of malvidin glycosides were stronger than that of malvidin (Mv: 82.78 ± 10.27%). Malvidin-3-glucoside (Mv-3-glc: 91.16 ± 7.77%) was better than malvidin-3-galactoside (Mv-3-gal: 86.49 ± 3.32%). These indicated that high glucose inhibited cell growth, while BAE, malvidin, and its glycosides protected the cell by inhibiting a decrease in cell viability caused by HG. Interestingly, the same effects were not found in cells incubated with HG for a long time (significant difference between 24 and 48 h: $P < 0.01$). The cells continued to grow over time with more cell numbers at 48 h than at 24 h in each well. However, no significant difference was observed between NG (100 ± 5.28%) and HG (106.01 ± 10.41%), while anthocyanin pretreatments seemed to decrease a little cell viability (BAE: 82.55 ± 8.90%, Mv: 94.42 ± 10.49%, Mv-3-glc: 89.76 ± 10.01%, and Mv-3-gal: 77.84 ± 5.57%).

3.2. Effects of Blueberry Anthocyanins on ROS, CAT, SOD, and Nox4 Levels. As shown in Figure 2, high glucose concentration significantly enhanced the ROS levels of HRCECs at both short-term and a long-term ($P < 0.001$). ROS levels of the HG groups at 24 and 48 h were 4.23 ± 0.41- and 4.53 ± 0.23-folds of the control (NG groups), respectively. BAE, Mv, Mv-3-glc, and Mv-3-gal all significantly inhibited HG-induced increase of ROS in HRCECs ($P < 0.01$ for 24 h; $P < 0.001$ for 48 h). HRCECs exhibited higher ROS levels at 48 h than those at 24 h ($P < 0.001$) except pretreatment with Mv-3-glc. Similarly, malvidin-3-glucoside possessed the strongest antioxidant effect, with nearly the same ROS levels as the control. Moreover, the ROS level of cells treated with Mv-3-glc was only 0.93 ± 0.23-fold of the control

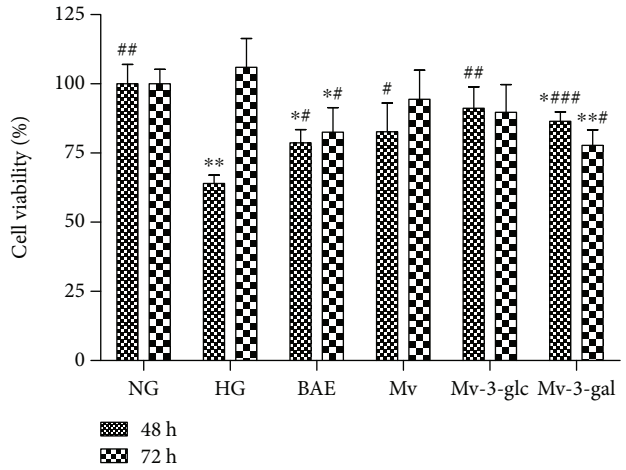

Figure 1: Effects of different treatment on cell viability in HRCECs exposed to high glucose for 24 and 48 h. $*$ and $**$ indicate $P < 0.05$ and $P < 0.01$, respectively, compared to each NG group; $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each HG group.

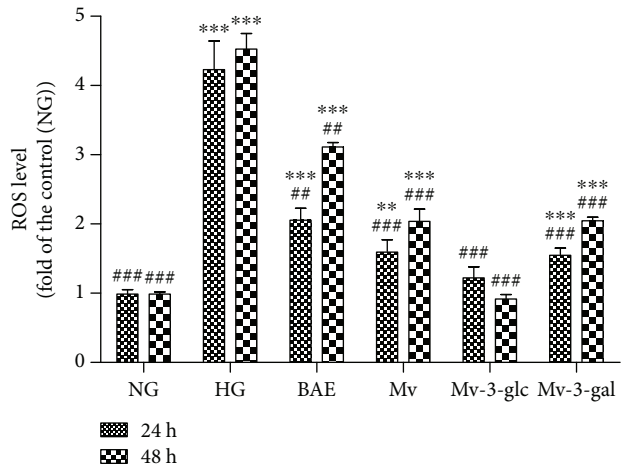

Figure 2: Effects of different treatment on ROS levels in HRCECs exposed to high glucose for 24 and 48 h. $**$ and $***$ indicate $P < 0.01$ and $P < 0.001$, respectively, compared to each NG group; $^{\#\#}$ and $^{\#\#\#}$ indicate $P < 0.01$ and $P < 0.001$, respectively, compared to each HG group.

when exposed to high glucose for 48 h. These data suggest that BAE, malvidin, and their glycosides significantly reduced the levels of ROS, showing an effective attenuation of high glucose-induced oxidative damage in HRCECs.

In this study, high glucose reduced the activity of the antioxidant enzymes CAT ($P < 0.01$ at 24 h) and SOD ($P < 0.05$ at 48 h) in HRCEC supernatants (Figure 3). BAE, Mv, Mv-3-glc, and Mv-3-gal all significantly enhanced CAT and SOD activity in HRCEC supernatants ($P < 0.05$). The CAT and SOD enzyme activity of cells pretreated with BAE, Mv, Mv-3-glc, and Mv-3-gal was even higher than that of the control (NG group). BAE and Mv-3-gal seemed to have more pronounced effects on SOD activity than Mv and Mv-3-glc. However, Mv and Mv-3-glc seemed to have more pronounced effects on CAT activity than BAE and Mv-3-gal. Both CAT and SOD enzyme activity decreased in a time-dependent manner (24 h versus 48 h: $P < 0.01$). Thus, blueberry anthocyanin malvidin and its glycosides could attenuate oxidative stress by upregulating the antioxidant enzymes activities in HRCECs.

Figure 4(a) showed that high glucose significantly enhanced Nox4 protein expression in cells to 2.17-fold of the control after 24 h stimulation ($P < 0.01$), but it had no significant change for 48 h stimulation. Only Mv could greatly inhibit Nox4 expression induced by high glucose stimulation for 24 h with inhibition rate at 45.96% ($P < 0.05$). BAE, Mv, and Mv-3-gal all could lower Nox4 levels in cells with 48 h HG stimulation ($P < 0.05$ for BAE and Mv-3-gal; $P < 0.01$ for Mv). However, Mv-3-glc did not affect the Nox4 protein expression in cells at both 24 and 48 hours. Nox4 protein expression also decreased in a time-dependent manner (24 h versus 48 h: $P < 0.01$). The results indicated that Mv could reduce superoxide production in endothelial cells by downregulating Nox4. Mv-3-glc inhibited the most ROS formation but did not inhibit Nox4 expression, which should exist some other mechanism for Mv-3-glc on antioxidant effects in HRCECs.

3.3. Effects of Blueberry Anthocyanins on NO, eNOS, and ACE Levels. In this study, high glucose could significantly increase the NO level ($P < 0.001$ at 24 h; $P < 0.01$ at 48 h) and eNOS activity ($P < 0.01$ at 24 h; $P < 0.05$ at 48 h) in HRCEC supernatants (Figures 5(a) and 5(b)). BAE slightly inhibited the increase of NO at 24 h ($P > 0.05$) but not as much as Mv-3-glc and Mv-3-gal ($P < 0.001$). However, Mv-3-glc and Mv-3-gal seemed to induce more NO than the HG group for long time incubation ($P < 0.01$ for Mv-3-glc and $P < 0.001$ for Mv-3-gal at 48 h). HRCECs produced higher NO at 24 h than those at 48 h. Enzyme activity of eNOS in HRCECs pretreated with BAE, Mv, Mv-3-glc, and Mv-3-gal was higher than that of NG and lower than that of HG. However, the differences among them were not always significant. Like CAT and SOD, eNOS enzyme activity decreased in a time-dependent manner (24 h versus 48 h: $P < 0.001$). The change of ACE content in the supernatants showed that high glucose-induced ACE expression ($P < 0.01$ at 24 h). Malvidin downregulated the HG-induced ACE expression, while BAE, Mv-3-glc, and Mv-3-gal further upregulated ACE expression (Figure 5(c)). ACE contents also decreased in a time-dependent manner (24 h versus 48 h: $P < 0.001$).

3.4. Effects of Blueberry Anthocyanins on High Glucose-Induced VEGF and Akt Levels. Figure 6 shows that high glucose concentration could significantly upregulate the VEGF production in HRCEC supernatants ($P < 0.01$ at 24 h; $P < 0.001$ at 48 h). BAE, Mv, Mv-3-glc, and Mv-3-gal significantly inhibited HG-induced VEGF production at 24 h ($P < 0.01$ for BAE, Mv, and Mv-3-glc; $P < 0.05$ for Mv-3-gal). Mv and Mv-3-glc also exhibited inhibitory effects at 48 h ($P < 0.01$). However, BAE and Mv-3-gal did not show the VEGF inhibitory effect at 48 h. Like the contents of NO and ACE and the enzyme activity of CAT, SOD, and eNOS, VEGF contents were decreased with a prolonged time (24 h

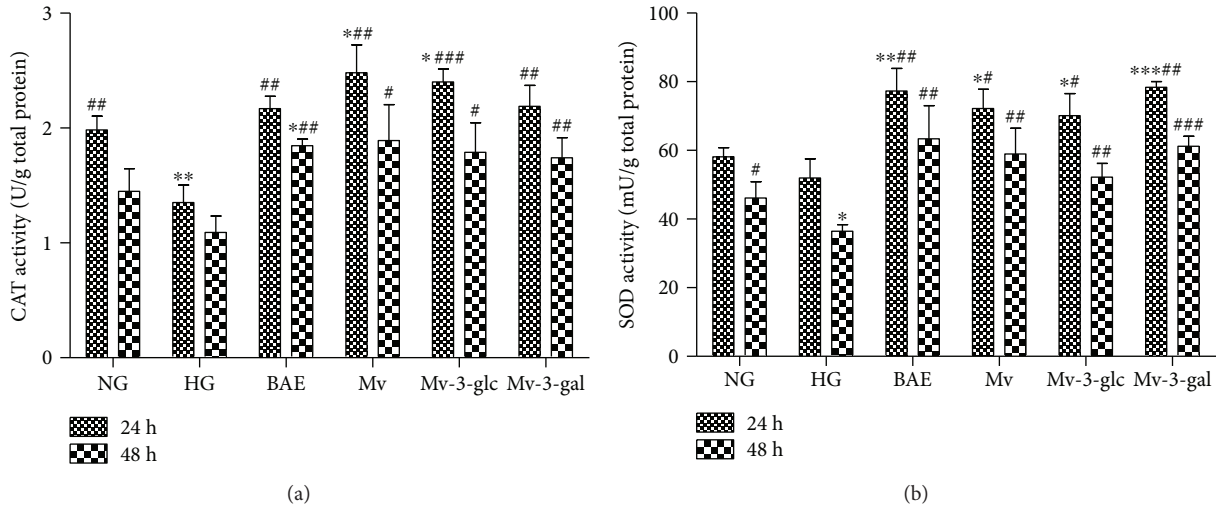

Figure 3: Effects of different treatment on CAT (a) and SOD activity (b) in HRCECs exposed to high glucose for 24 and 48 h. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each NG group; $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each HG group.

versus 48 h: $P < 0.001$). Mv-3-glc showed the strongest inhibitory effects against VEGF.

VEGF protein expression in cells was also determined by Western blotting (Figure 4(b)). Strangely, high glucose did not enhance VEGF protein expression in cells and even 24 h HG stimulation decreased VEGF level to 0.64-fold of the control ($P < 0.05$). BAE could greatly inhibit VEGF expression both at 24 h (0.59-fold of the control) and 48 h (0.73-fold) ($P < 0.001$ and $P < 0.01$ versus the control). Moreover, Mv-3-glc exhibited lower VEGF protein level (0.69-fold) than the control ($P < 0.05$). VEGF protein expression in cells at 24 and 48 h was different ($P < 0.05$). The VEGF levels at 48 h pretreated with BAE and Mv were higher than those at 24 h, which was consistent with the HG-stimulated group. However, the VEGF levels at 48 h pretreated with Mv-3-glc and Mv-3-gal were lower than those at 24 h, which indicated their capacity to downregulate the VEGF expression.

Moreover, high glucose could enhance the expression of Akt protein in cells to 1.18- ($P < 0.01$) and 1.11- ($P < 0.05$) folds of the control after 24 and 48 h stimulation, respectively. BAE, Mv-3-glc, and Mv-3-gal inhibited the increased Akt expression with HG stimulation for 24 h or 48 h. The Akt protein levels of BAE, Mv-3-glc, and Mv-3-gal at 24 h were 0.76-, 0.91-, and 1.09-folds of the control, respectively, and at 48 h, they were 1.06-, 1.01-, 0.89-folds of the control, respectively (Figure 4(c)). However, Mv did not affect the Akt protein expression in cells.

3.5. Effects of Blueberry Anthocyanins on High Glucose-Induced ICAM-1 and NF-κB. In this study, high glucose concentrations could significantly upregulate the ICAM-1 production in HRCEC supernatants ($P < 0.001$ both at 24 and 48 h). As shown in Figure 7(a), malvidin and Mv-3-glc greatly inhibited HG-induced ICAM-1 levels ($P < 0.001$ both at 24 and 48 h), particularly Mv exhibited a lower ICAM-1 content than NG at 24 h ($P < 0.01$). Mv-3-gal was also observed to have an ICAM-1 inhibitory effect compared to the HG group ($P < 0.01$ at 24 h and $P < 0.05$ at 48 h). However, BAE had no effects on ICAM-1 levels in HRCECs at 24 h. ELISA assay of NF-κB (p65) contents showed similar results. However, the effects were not always significant (Figure 7(b)). Moreover, Mv and Mv-3-glc exhibited strongest inhibitory effects at both 24 and 48 h ($P < 0.05$). The ICAM-1 contents and NF-κB (p65) levels of 48 h were lower than those of 24 h ($P < 0.001$) except Mv, which had a lower ICAM-1 content at 24 h than that at 48 h ($P < 0.01$).

4. Discussion

The chronic hyperglycemia-induced cell death in the endothelial cells of retinal vessels is well established [2]. Leal et al. found that high glucose, but not mannitol, decreased cell viability of rat retinal endothelial cells exposed to 30 mM glucose (high glucose) for 7 days (long-term exposure) [20]. Fan et al. also observed that stimulation with 30 mM glucose for 48 h and 72 h significantly decreased cell viability in comparison with 5.5 mM glucose-treated rat retinal capillary endothelial cells [1]. However, Wang et al. reported that high glucose-induced human retinal microvascular endothelial cell proliferation and enhanced the cell viability [2]. In the present study, cell viability of human retinal capillary endothelial cells was decreased by high glucose only when stimulated for 24 h but not for 48 h. Maybe the effect of cell proliferation counteracted the effect of cell death at 48 h, so there was no significant change in cell viability. Moreover, blueberry anthocyanin extract and its major constituents (malvidin glycosides) could have a particular protective effect on HRCECs, all enhancing cell viability. Their possible functional mechanisms include attenuation of oxidative damage, alteration of retinal enzyme activity, and inhibition of inflammation [6]. As a whole extract, BAE exhibited a reductionist activity, since crude extract also had some other noneffective components. This confirmed that anthocyanins, particularly

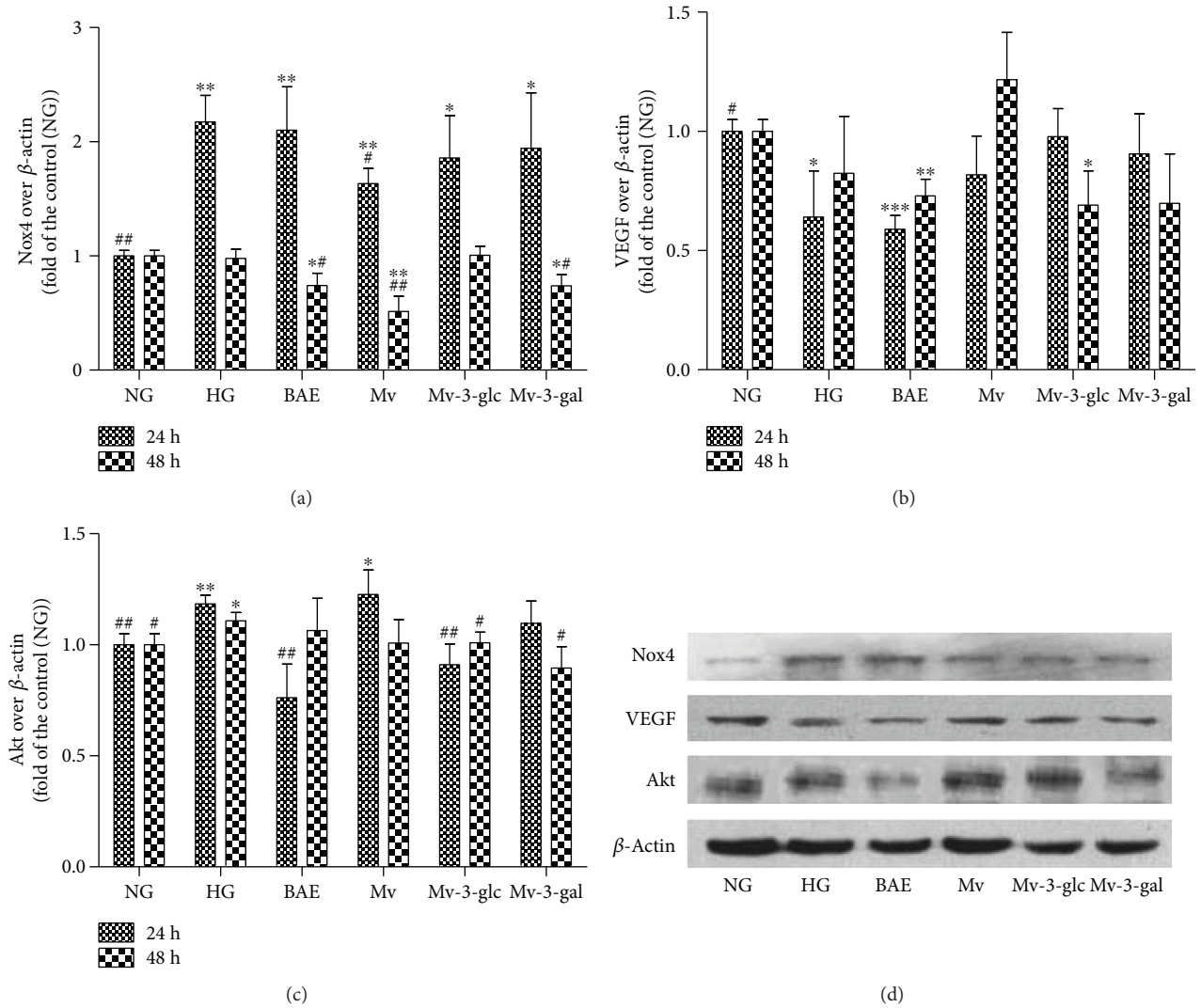

Figure 4: Effects of different treatment on Nox4 (a), VEGF (b), and Akt (c) protein expression in HRCECs exposed to high glucose for 24 and 48 h. (d) Representative Western blots of HRCECs exposed to high glucose for 24 h are shown. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each NG group; $^{\#}$ and $^{\#\#}$ indicate $P < 0.05$ and $P < 0.01$, respectively, compared to each HG group.

Mv-3-gal and Mv-3-glc, were effective components in blueberry extracts.

The upregulated reactive oxygen species (ROS) generation inferred the potent stimuli for the oxidative stress [21]. In this study, BAE, Mv, Mv-3-glc, and Mv-3-gal attenuated high glucose-induced oxidative stress in HRECE by reducing ROS levels. The activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases has been identified as an important source of ROS in vascular endothelial cells [22]. Nox4 is the major catalytic component of an endothelial NADPH oxidase. The self-perpetuating cycle between oxidative stress and inflammation contributes to the upregulation of NADPH oxidase, which increases ROS production [23]. The ROS can react with nitric oxide (NO) to form peroxynitrate leading to a reduction in NO bioavailability and subsequently impaired NO-dependent vasodilation [15]. However, Schröder et al. speculated that endogenous Nox4 protects the vasculature against inflammatory stress because loss of Nox4 results in the reduction of endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) expression, as well as NO production, which comprise an important antioxidant defense against endothelial oxidative damage [23]. This might explain why Nox4 did not change greatly with pretreatment with blueberry anthocyanin malvidin glycosides in this study.

The local production of NO mediates the function of endothelium-dependent vasodilation, which is synthesized from *L*-arginine by the enzyme eNOS [24]. Conversely, angiotensin-converting enzyme (ACE) is responsible for vasoconstriction by converting angiotensin I (AngI) into AngII (a potent vasoconstrictor) and inactivating the vasodilator bradykinin [25]. Vasodilation and vasoconstriction of blood vessels together affect blood pressure. In several pathological conditions including diabetes, endothelium-dependent vasodilation is reduced because of a decreased release of NO [15, 26]. Extracts from various plants full of anthocyanins can induce endothelium-dependent vasodilation probably by releasing NO or enhancing NO bioactivity

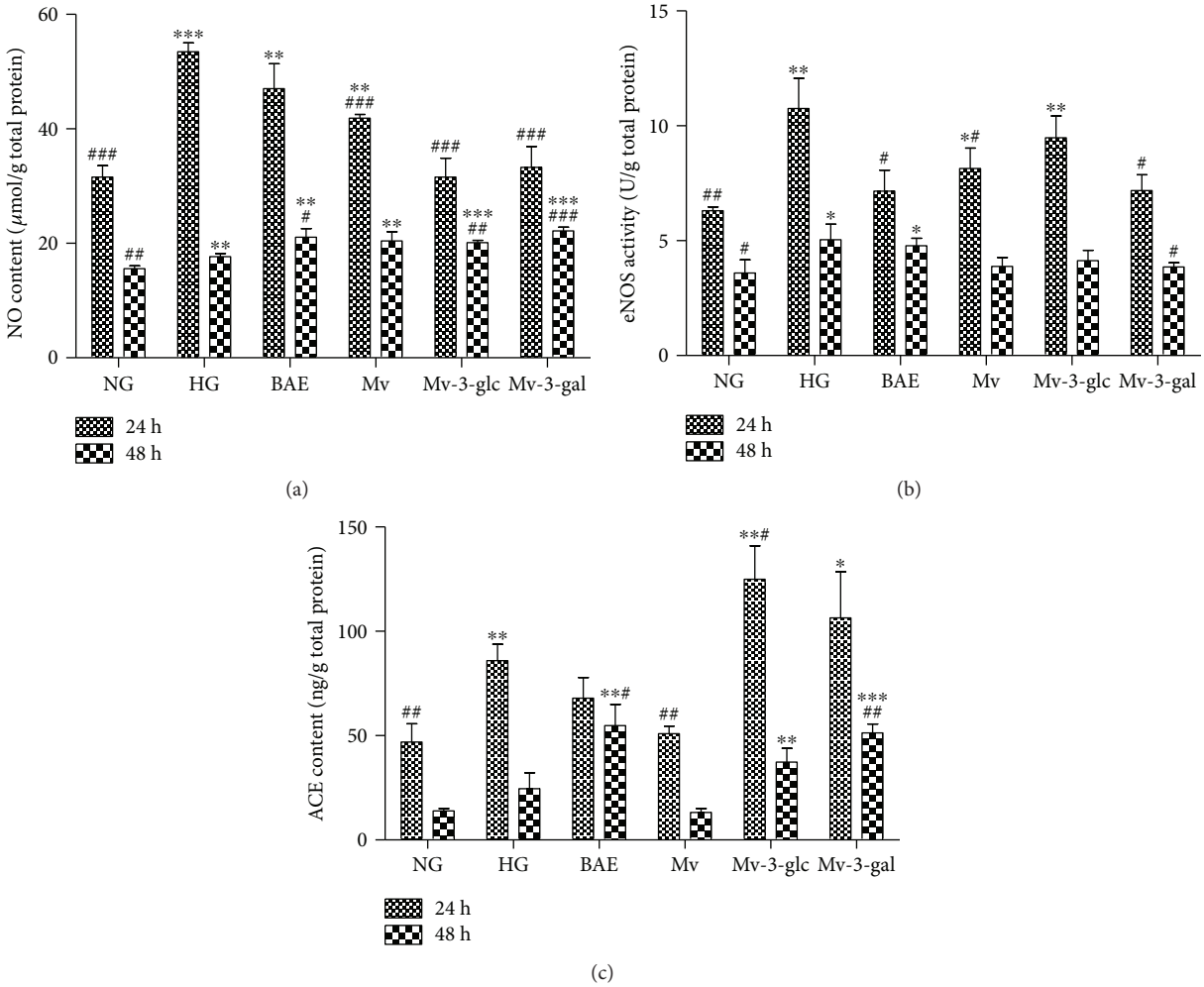

Figure 5: Effects of different treatment on NO contents (a), eNOS activity (b), and ACE contents (c) in HRCECs exposed to high glucose for 24 and 48 h. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each NG group; $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each HG group.

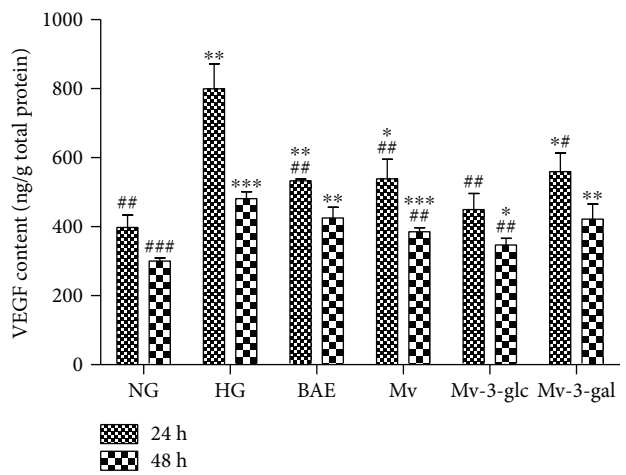

Figure 6: Effects of different treatment on VEGF contents in HRCECs exposed to high glucose for 24 and 48 h. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each NG group; $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each HG group.

[27]. For example, blackcurrant prevented eye probably by increasing blood supply based on the endothelium vasodilation [28]. Strangely, the effects on NO production were not always consistent with the effects on eNOS activity in this study. The level of NO could not be completely determined by eNOS because endothelial NOS is only one of the three isoforms of nitric oxide synthase [29]. Other isoforms, such as neuronal NOS (nNOS) and inducible NOS (iNOS), should also be effective. Retinal capillary endothelial cells can express iNOS when stimulated by inflammatory mediators, such as IL-1β [21]. Moreover, nitrosative/oxidative stress might be related to NO levels. NOS can also catalyze superoxide anion production, depending on substrate and cofactor availability [29]. Anthocyanins exhibited high NO levels, which contributed to the effective antioxidant capacity and the vasodilatory effect. Our previous study found that malvidin and its glycosides could inhibit TNF-α-induced ACE expression and activity in the human umbilical vein endothelial cells [30]. However, blueberry anthocyanins did not downregulate ACE expression and were not detected in HRCECs in the present study. It seemed to be contradictory

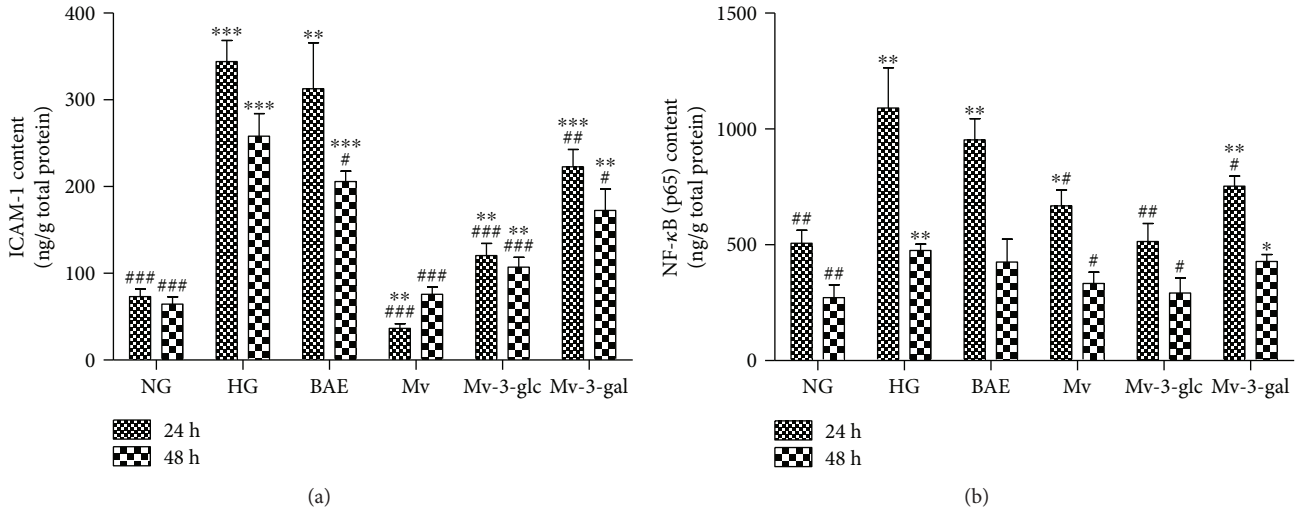

Figure 7: Effects of different treatment on ICAM (a) and NF-κB (p65) contents (b) in HRCECs exposed to high glucose for 24 and 48 h. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each NG group; $^{\#}$, $^{\#\#}$, and $^{\#\#\#}$ indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to each HG group.

that BAE and malvidin glycosides possessed simultaneous vasodilatory and vasoconstrictive effects at the same time. Clozel et al. also reported that the endothelin ETB receptor could mediate both vasodilation and vasoconstriction [31]. Further study on the mechanism of malvidin glycosides in the endothelium should be conducted in the future.

Under retinopathy state, the activity of NOS is spontaneously regulated to improve the formation of NO fighting for inflammation [29]. Moreover, the stimulus-secretion coupling of high glucose-induced the synthesis and the release of NO could interact with the vascular endothelial growth factor (VEGF) [32]. VEGF stimulates vasculogenesis and angiogenesis, which gives rise to the proliferative DR [33]. In the diabetic microangiopathic condition, microvascular permeability and the number of leucocytes sticking to the vascular endothelium are increased [6]. In the present study, high glucose stimulated VEGF secretion, and blueberry anthocyanin malvidin glycosides downregulated the VEGF expression.

Akt, the serine/threonine kinase, is a central node in cell signaling downstream responses, including cell survival, growth, proliferation, angiogenesis, glucose uptake, metabolism, and migration [34]. In endothelial cells, the PI3K-Akt pathway is robustly activated by VEGF, and Akt activates eNOS [35]. The release of NO produced by activated eNOS can stimulate vasodilation, vascular remodeling, and angiogenesis [36]. During diabetic retinopathy, Akt activation is aberrant. In the present study, blueberry anthocyanin malvidin glycosides inhibited Akt expression, therefore inhibiting eNOS activity and changing the NO level.

Nuclear factor-kappa B (NF-κB), an important protein complex that controls the transcription of DNA and cytokine production, is present in many cell types and participates in cell apoptosis and neovascularization [37]. On activation of the NF-κB pathway, the p65 protein interacts with the promoters to induce and maintain the state of inflammation [38]. It has been shown that the expression of NF-κB p65 in the retina can regulate the expression of intercellular adhesion molecule-1 (ICAM-1), which is the primary adhesion molecule responsible for inflammation in the pathogenesis of diabetic retinopathy [39]. Previous studies confirmed that Mv-3-glc could increase NO bioavailability as well as inhibit peroxynitrite-induced NF-κB activation [40]. Anthocyanin and phenolic acid metabolites were found to attenuate visible light-induced retinal degeneration in vivo via NF-κB suppression [41]. In the present study, blueberry anthocyanin malvidin glycosides also exhibited anti-inflammatory effects by inhibiting NF-κB in HRCECs.

5. Conclusions

In the present study, blueberry anthocyanin extract, as well as its major constituent malvidin and its glycosides, could protect human retinal capillary endothelial cells against high glucose-induced injury. BAE, Mv, Mv-3-glc, and Mv-3-gal promoted cell growth of HRCECs with higher cell viability than the high glucose-stimulated group. They had great antioxidant effects by decreasing ROS levels and increasing enzyme activity of CAT and SOD in HRCECs. Downregulation of Nox4 expression might be an antioxidant mechanism. Moreover, the upregulation of NO levels might be another antioxidant mechanism for blueberry anthocyanins, which contributed to vasodilatory effects. However, malvidin glycosides still possessed vasoconstrictory effects by increasing ACE contents in some case. Blueberry anthocyanins and malvidin glycosides changed VEGF levels in HRCECs and influenced the Akt pathway to some extent. Moreover, Mv and Mv-3-glc significantly inhibited HG-induced extracellular ICAM-1 and NF-κB (p65). BAE, Mv, Mv-3-glc, and Mv-3-gal protected cells in a time-dependent manner with the difference between 24 and 48 h HG stimulation. Incubation for long times may weaken the protective effects, which can be attributed to the irreparable oxidative damage caused by the prolonged stimulation of high glucose. The results

indicated that blueberries, as an excellent resource of anthocyanins, could improve human retinal capillary endothelial function and, thereby, might have the potential to prevent the progression of diabetic retinopathy.

Conflicts of Interest

There are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Wuyang Huang and Zheng Yan contributed equally to this work.

Acknowledgments

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Supplementary Materials

Figure 1. Chromatographic separation and UV detection ($\lambda = 520$ nm) of anthocyanins in blueberry anthocyanin extract. The following 14 peaks were identified: 1, delphindin-3-galactoside; 2, delphindin-3-glucoside; 3, cyaniding-3-galactoside; 4, petunidin-3-galactoside; 5, cyaniding-3-glucoside; 6, cyaniding-3-arabinoside; 7, petunidin-3-glucoside; 8, peonidin-3-galactoside; 9, petunidin-3-arabinoside; 10, peonidin-3-glucosidea; 11, malvidin-3-galactoside; 12, malvidin-3-glucoside; 13, malvidin-3-arabinose; and 14, acylated anthocyanin. *(Supplementary Materials)*

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Research Article

The *In Vitro* Antioxidant Activity and Inhibition of Intracellular Reactive Oxygen Species of Sweet Potato Leaf Polyphenols

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The *in vitro* antioxidant activity and inhibition of intracellular reactive oxygen species (ROS) of the total and individual phenolic compounds from Yuzi No. 7 sweet potato leaves were investigated in this study. Sweet potato leaf polyphenols possessed significantly higher antioxidant activity than ascorbic acid, tea polyphenols, and grape seed polyphenols. Among the individual phenolic compounds, caffeic acid showed the highest antioxidant activity, followed by monocaffeoylquinic acids and dicaffeoylquinic acids, while 3,4,5-tri-O-caffeoylquinic acid showed the lowest value. Sweet potato leaf polyphenols could significantly decrease the level of intracellular ROS in a dose-dependent manner. The order of the inhibiting effect of individual phenolic compounds on the intracellular ROS level was not in accordance with that of antioxidant activity, suggesting that there was no direct relationship between antioxidant activity and intracellular ROS-inhibiting effect. Sweet potato leaves could be a good source of biologically active polyphenols with multiple applications in the development of foods, health products, pharmaceuticals, and cosmetics.

1. Introduction

Reactive oxygen species (ROS) are a series of metabolic byproducts involved in degenerative and pathological processes in the human body [1]. Overproduction of ROS could disturb cellular redox balance, resulting in cell injury or apoptosis [2], further triggering oxidative damage of tissues and organs, which accelerates the development of various diseases, such as cancer, atherosclerosis, diabetes, chronic inflammatory disease, cardiovascular disease, and Alzheimer [3–6]. Although humans and other organisms have endogenous antioxidant defenses against ROS, these systems may sometimes not be sufficient to prevent the occurrence of cell damage [7].

Synthetic antioxidants are widely used in the food industry, to prevent the production of toxicities or mutagenicities that may present health hazards [8]. However, studies have shown that synthetic antioxidants have chemical toxicity, which can increase the risk of cancer and damage the liver [9–12]. Consequently, it is particularly important to look for natural antioxidants that can replace synthetic antioxidants. One of the natural antioxidants, plant polyphenols, which are widely found in fruits and vegetables, is known as potent antioxidant and free radial scavenger, and thus

possesses many biological activities, such as antioxidation, antiaging, and prevention of cardiovascular disease, cancer inhibition, anti-inflammatory effects, antiviral effects, antibacterial effects, and so forth [13–17]. That is to say, plant polyphenols have the potential to be widely used as natural antioxidants in food, cosmetic, pharmaceutical, and medicinal products [18].

Sweet potato leaves are the aboveground parts of sweet potato (*Ipomoea batatas* L.), which can be harvested several times a year, but most of the sweet potato leaves in China have been discarded or used as feed, causing serious environmental pollution and waste of resources [19]. In our previous study, it has been found that sweet potato leaves are rich in polyphenols, with the content ranging from 2.73 to 12.46 g/100 g dry weight (DW) [20, 21]. Meanwhile, the *in vitro* antioxidant activity of polyphenols extracted and purified from sweet potato leaves (cultivars: Yuzi No. 7 and Ximeng No. 1) was also investigated in our previous study, and the results showed that sweet potato leaf polyphenols possessed strong *in vitro* antioxidant activity, which is 2 times higher than ascorbic acid, tea polyphenols, and grape seed polyphenols [19]. The above-mentioned results indicate that sweet potato leaf polyphenols have great potential to be widely used in food, healthcare, pharmaceutical, and cosmetic industries. However, the study on sweet potato leaf polyphenols is just in its early stages. Most studies have focused on the extraction and purification of polyphenols from sweet potato leaves and the antioxidant activity *in vitro* [19, 22]. So far, no studies were undertaken to explore the inhibition of intracellular ROS.

Our previous study showed that the correlation coefficient between antioxidant activities of sweet potato leaves and polyphenols were the highest, followed by carbohydrate. There were negative correlation coefficients between antioxidant activity and protein, fat, and crude fiber [20, 21]. In addition, the antioxidant activity of polyphenols is largely up to their individual phenolic compound composition [19]. Iwai et al. [23] found that the antioxidant activity of dicaffeoylquinic acid was 2 times higher than that of monocaffeoylquinic acids. Our previous study showed that sweet potato leaf polyphenols were mainly composed of seven caffeoylquinic acids and a small amount of caffeic acid [19], which was similar to other researchers' reports [24–26]. However, there is little information about the contribution rate of different individual phenolic compounds from sweet potato leaves on the *in vitro* antioxidant activity and the inhibition of intracellular ROS.

Therefore, in the present study, photochemiluminescence (PCL) assay and oxygen radical absorbance capacity (ORAC) method were used to analyze the *in vitro* antioxidant activity of sweet potato leaf polyphenols and their individual phenolic compounds. Furthermore, H_2O_2 was used to induce and establish human hepatocyte LO2 oxidative stress model, and the inhibition of intracellular reactive oxygen species of sweet potato leaf polyphenols and their individual phenolic compounds were investigated. The purpose is to make the *in vitro* antioxidant activity and inhibition of intracellular reactive oxygen species of sweet potato leaf polyphenols clear and to further lay a theoretical foundation for the development and utilization of sweet potato leaf polyphenols.

2. Materials and Methods

2.1. Materials. Sweet potato leaf variety, Yuzi No. 7 (which was bred by hybridization, not a genetically modified organism), was collected from the Research Institute of Sweet Potato of the Chinese Academy of Agricultural Sciences (Xuzhou, China), which was planted with standard production practices in early March, 2015, and collected in the middle of August. Then, sweet potato leaves were washed, freeze dried, ground, and stored at 4°C in sealed aluminum foil bags for further use. The proximate composition and total polyphenol content (TPC, measured by Folin-Ciocalteu method [27, 28]) of Yuzi No. 7 sweet potato leaves were as follows: moisture 88.16 ± 0.51 g/100 g fresh weight, protein 22.09 ± 0.34 g/100 g dry weight (DW), fat 2.36 ± 0.07 g/100 g DW, dietary fiber 36.52 ± 0.75 g/100 g DW, carbohydrate 50.22 ± 0.85 g/100 g DW, gross energy 421.39 ± 1.05 kcal/100 g DW, ash 8.91 ± 0.76 g/100 g DW, and TPC 12.97 ± 0.82 g chlorogenic acid equivalent (CAE)/100 g DW.

Then, the extraction of polyphenols from sweet potato leaves was carried out according to the method described by Sun et al. [20, 21]), and the purification of crude polyphenol extract was carried out according to the method established by Xi et al. [19] and Sun et al. [27]. TPC of sweet potato leaf polyphenols after being purified by AB-8 resin was 87.56 ± 1.38%.

2.2. Reagents. Folin-Ciocalteu reagent, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), ascorbic acid, 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), chlorogenic acid, caffeic acid, 2′,7′-dichlorofluorescein diacetate (DCFDA),McCoy's 5A medium, methyl thiazolyl tetrazolium (MTT), dimethyl sulfoxide (DMSO), crystal violet, and chromatography grade acetonitrile and methanol were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The chromatography grade caffeoylquinic acid standards (3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, and 3,4,5-tri-O-caffeoylquinic acid) were purchased from AMRESCO Biotechnology Co. Ltd. (Solon, OH, USA). Tea polyphenols (TP) and grape seed polyphenols (GSP) were purchased from Yihe Biotechnology Co. Ltd., Xi'an, China. Fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences HyClone Laboratories (Logan, Utah, USA). Penicillin/streptomycin was purchased from Mediatech Inc. (Manassas, Virginia, USA). Trypsin (1 : 250, activity: 250 NFU/mg) was purchased from BioD BioTech Co. Ltd. (Beijing, China). Sodium fluorescein, sodium hydroxide, phosphate, and other analytical grade reagents were purchased from Beijing Chemical Reagents Co. (Beijing, China).

2.3. Extraction of Polyphenols from Sweet Potato Leaves. Extraction of polyphenols from sweet potato leaves was carried out according to the method described by Sun et al.

[20, 21]). Briefly, 10 g of leaf powder was extracted with 200 mL of 70% (*v*/*v*) ethanol for 30 min at 50°C and subjected to ultrasonic wave treatment (59 kHz). Following centrifugation at 5000 *g* for 10 min at 4°C, the residue was reextracted twice with 70% ethanol as described above. The supernatants were pooled, concentrated in a rotary evaporator at 45°C, and freeze dried to obtain a crude polyphenol extract.

2.4. Total Polyphenol Content. Total polyphenol content (TPC) was measured by the Folin-Ciocalteu method [27, 28]. The crude extract was dissolved in 100 mL distilled water; an aliquot (0.5 mL) was mixed with 1.0 mL of Folin-Ciocalteu reagent, previously diluted 10 times, and allowed to react at 30°C for 30 min. Subsequently, 2.0 mL of saturated Na_2CO_3 (10%, *w*/*v*) was added to the mixture. The following 30 min, absorbance was measured at 736 nm in a UV1101 spectrophotometer (Hitachi, Japan). A calibration curve consisting of chlorogenic acid standards, ranging from 0.02 to 0.10 mg/mL, was prepared. TPC was expressed as chlorogenic acid equivalent (CAE) on a dry weight (DW) basis. TPC of Yuzi No. 7 sweet potato leaves was 12.97 ± 0.82 g CAE/100 g DW.

2.5. Purification of Polyphenols from Sweet Potato Leaves by AB-8 Macroporous Resins. The purification of crude polyphenol extract was carried out according to the method established by Xi et al. [19] and Sun et al. [27]. Briefly, the crude polyphenol extract was dissolved in distilled water to get a crude polyphenol solution of 2.0 mg CAE/mL and adjusted to pH 3.0 using 2.0 mol/L HCl. The purification process was carried out in a glass column (1 cm × 10 cm) wet packed with pretreated AB-8 resin. The bed volume (BV) of the resins was 10 mL (equal to 5 g resin). The crude polyphenol solution was allowed to flow through the glass column at a flow rate of 1.0 mL/min (the volume ratio between crude polyphenol solution and BV was 5 : 1). After the adsorption equilibrium had been reached, the column was first washed with distilled water at a flow rate of 1.0 mL/min until the effluent was clear and then eluted by 70% (*v*/*v*) ethanol solution at a flow rate of 1.0 mL/min (the volume ratio between ethanol solution and BV was 3 : 1). The eluted solution was collected and concentrated in a rotary evaporator at 45°C to remove the ethanol and then freeze dried. The total polyphenol content of purified polyphenols from sweet potato leaves was 87.56 ± 1.38%.

2.6. Quantification of Individual Phenolic Compounds by Reversed-Phase HPLC (RP-HPLC). Individual phenolic compounds in polyphenols from sweet potato leaves were evaluated by RP-HPLC (Agilent Technologies, Palo Alto, CA, USA) according to the method described by Sun et al. [27]. Spectral data from 200 to 800 nm were recorded, and the polyphenol chromatogram was monitored at 326 nm. Caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, and 3,4,5-tri-O-caffeoylquinic acid were used as standard. Identification and quantitative analysis were done by comparison with standards. The amount of individual phenolic compound was expressed as g/100 g of purified sweet potato leaf polyphenols on a dry weight basis (g/100 g DW).

2.7. Antioxidant Activity Analysis

2.7.1. Photochemiluminescence Assay. Photochemiluminescence assay was carried out using an automated photo chemiluminescent (PCL) system (Photochem, Analytik Jena AG, Germany), according to the method reported by Cofrades et al. [29]. Briefly, 20 μL of sample solution at different concentrations (5, 10, and 20 μg/mL) was used in a commercial kit for antioxidant capacity determination. Ascorbic acid was used as the standard. The results were expressed as ascorbic acid equivalent (ACE) relative to sample solution volume (μg·ACE/mL).

2.7.2. Oxygen Radical Absorbance Capacity Assay. Oxygen radical absorbance capacity (ORAC) assay was carried out according to the method described by Sun et al. [27]. Briefly, all samples and reagents in the experiment were dissolved and diluted with phosphate buffer (0.075 M, pH 7.4). 20 μL sample solutions at different concentrations (5, 10, and 20 μg/mL) were added to 20 μL phosphate buffer and then mixed with 20 μL·63 nmol/L sodium fluorescein solution in a clear 96-well microplate and incubated at 37°C for 15 min. Then, 140 μL·18.28 mmol/L AAPH solution was rapidly added to the well. Fluorescence was read at 485 nm excitation and 535 nm emission until complete extinction. ORAC values were expressed as μg·Trolox equivalent (TE)/mL (μg·TE/mL).

2.8. Inhibition of Intracellular Reactive Oxygen Species of Sweet Potato Leaf Polyphenols

2.8.1. Cell Culture. Human hepatocyte LO2 cells were obtained from Bioye Biological Technology Co. Ltd. (Shanghai, China). Cells were cultured in McCoy's 5A culture medium, containing 10% of FBS and 1% of penicillin/streptomycin, at 37°C in an atmosphere of 5% CO_2 in air with controlled humidity.

2.8.2. Determination of H_2O_2 Concentration in the Establishment of LO2 Oxidative Stress Model. The H_2O_2 stock solution was diluted with McCoy's 5A culture medium without FBS into H_2O_2 working solution of different concentrations: 1, 5, 25, 50, 100, 200, 400, 500, and 1000 μM. LO2 cells were seeded into 96-well cell culture plates at a density of 5×10^3 cells/well and then incubated for 24 h. After that, the culture medium in 96-well cell culture plates was discarded, and the cells were washed once with 200 μL of PBS in each well. Subsequently, the H_2O_2 working solution of different concentrations was added to 96-well cell culture plates. Following incubation for another 6 h, the H_2O_2 working solution was discarded, and 100 μL of FBS-free culture medium was added to each well; then, 20 μL of 5.0 mg/mL MTT solution was added to each well. After incubation for another 4 h, the supernatant in each well was discarded. Then, the generated formazan precipitate was dissolved in 150 μL of DMSO, and absorbance was measured at 570 nm using a RT-6000 microplate reader (Rayto, Shenzhen,

China). The cell viability was calculated according to (1). The H_2O_2 concentration when the cell viability was reduced to 50% was chosen to be the optimal concentration to establish human hepatocyte LO2 oxidative stress model.

$$\text{Cell viability}(\%) = \frac{A_n}{A_0} \times 100, \quad (1)$$

wherein A_n was the absorbance value of H_2O_2 treatment group and A_0 was the absorbance value of blank control group.

2.8.3. Determination of the Concentration Range of Polyphenols from Sweet Potato Leaves. The sweet potato leaf polyphenol stock solution was diluted with FBS-free McCoy's 5A culture medium into sweet potato leaf polyphenol working solution of different concentrations: 25, 50, 100, 200, 400, 800, 1000, 1500, 2000, and 3000 μg/mL. LO2 cells were seeded into 96-well cell culture plates at a density of 5×10^3 cells/well and then incubated for 24 h. After that, the culture medium in 96-well cell culture plates was discarded and the cells were washed once with 200 μL of PBS in each well. Subsequently, the sweet potato leaf polyphenol working solution of different concentrations was added to 96-well cell culture plates. Following incubation for another 24 h, the sweet potato leaf polyphenol working solution was discarded and 100 μL of FBS-free culture medium was added to each well; then, 20 μL of 5.0 mg/mL MTT solution was added to each well. The cell viability test was carried out according to the protocol described in the last section.

2.8.4. Effect of Sweet Potato Leaf Polyphenols on the Cell Viability of Oxidative Stress LO2 Cells. The experiment was divided into 7 groups: blank control group, oxidative stress model group, Trolox-pretreated oxidative stress model group, ascorbic acid-pretreated oxidative stress model group, tea polyphenol-pretreated oxidative stress model group, grape seed polyphenol-pretreated oxidative stress model group, and sweet potato leaf polyphenol-pretreated oxidative stress model group.

LO2 cells were seeded into 96-well cell culture plates at a density of 5×10^3 cells/well and then incubated for 24 h. After that, the culture medium in 96-well cell culture plates was discarded and the cells were washed once with 200 μL of PBS in each well. Subsequently, the sweet potato leaf polyphenol working solution of different concentrations/Trolox solution of a certain concentration/ascorbic acid solution of a certain concentration/tea polyphenols solution of a certain concentration/grape seed polyphenols solution of a certain concentration was added to 96-well cell culture plates. Following incubation for another 24 h, the culture medium was discarded and the cells were washed once with 200 μL of PBS in each well. Then, H_2O_2 working solution of a certain concentration (which was used to establish LO2 oxidative stress model) was added to 96-well cell culture plates. Following incubation for another 6 h, the culture medium was discarded and 100 μL of FBS-free culture medium was added to each well; then, 20 μL of 5.0 mg/mL MTT solution was added to each well. The cell viability test was carried out according to the protocol described in the last section.

2.8.5. Effect of Individual Phenolic Compounds from Sweet Potato Leaves on the Cell Viability of Oxidative Stress LO2 Cells. The experiment was divided into 10 groups: blank control group, oxidative stress model group, sweet potato leaf polyphenol-pretreated oxidative stress model group, 3-O-caffeoylquinic acid-pretreated oxidative stress model group, 4-O-caffeoylquinic acid-pretreated oxidative stress model group, 5-O-caffeoylquinic acid-pretreated oxidative stress model group, 3,4-di-O-caffeoylquinic acid-pretreated oxidative stress model group, 3,5-di-O-caffeoylquinic acid-pretreated oxidative stress model group, 4,5-di-O-caffeoylquinic acid-pretreated oxidative stress model group, and 3,4,5-tri-O-caffeoylquinic acid-pretreated oxidative stress model group. The experimental procedure was the same as described in the above paragraph.

2.8.6. Effects of Sweet Potato Leaf Polyphenols on the Level of Intracellular Reactive Oxygen Species. The experiment was divided into 7 groups: blank control group, oxidative stress model group, Trolox-pretreated oxidative stress model group, ascorbic acid-pretreated oxidative stress model group, tea polyphenol-pretreated oxidative stress model group, grape seed polyphenol-pretreated oxidative stress model group, and sweet potato leaf polyphenol-pretreated oxidative stress model group.

LO2 cells were seeded into 96-well cell culture plates at a density of 5×10^3 cells/well and then incubated for 24 h. After that, the culture medium in 96-well cell culture plates was discarded and the cells were washed once with 200 μL of PBS in each well. Subsequently, the sweet potato leaf polyphenol working solution of different concentrations/Trolox solution of a certain concentration/ascorbic acid solution of a certain concentration/tea polyphenols solution of a certain concentration/grape seed polyphenols solution of a certain concentration was added to 96-well cell culture plates. Following incubation for another 24 h, the culture medium was discarded and the cells were washed once with 200 μL of PBS in each well. 10 μM of H_2DCF-DA was added to each well. After incubation away from light for 30 min, the cells were washed once with 200 μL of PBS in each well. Then, H_2O_2 working solution of a certain concentration (which was used to establish LO2 oxidative stress model) was added to 96-well cell culture plates. Following incubation away from light for another 30 min, the fluorescence intensity was read at 485 nm excitation and 530 nm emission using a microplate reader (Chameleon, Hidex, Turku, Finland). The level of intracellular reactive oxygen species was calculated as the following:

$$\text{The level of int racellular reactive oxygen species}(\%) = \frac{FI_n}{FI_0} \times 100, \quad (2)$$

wherein FI_n is the fluorescence intensity of the pretreated groups and FI_0 is the fluorescence intensity of the oxidative stress model group.

2.8.7. Effects of Individual Phenolic Compounds from Sweet Potato Leaves on the Level of Intracellular Reactive Oxygen Species. The experiment was divided into 10 groups: blank

Table 1: Individual phenolic compound composition of Yuzi No. 7 sweet potato leaf polyphenols.

Peak number	Retention time (min)	Identification*	Standard curve	R^2	Peak area[a]	Content (%, DW)[a]
1	1.47	5-CQA	$y = 11.372x - 0.428$	0.9962	54.72 ± 0.85	2.42 ± 0.07
2	1.91	3-CQA	$y = 9.909x + 0.286$	1.0000	19.63 ± 0.23	0.98 ± 0.02
3	2.10	4-CQA	$y = 25.894x - 17.128$	0.9988	32.27 ± 0.19	0.95 ± 0.01
4	2.92	CA	$y = 28.183x - 1.211$	1.0000	4.12 ± 0.07	0.09 ± 0.01
5	4.16	4,5-CQA	$y = 9.208x - 7.244$	0.9987	386.51 ± 3.68	21.38 ± 0.21
6	4.54	3,5-CQA	$y = 18.056x - 18.405$	0.9981	1292.36 ± 22.32	36.30 ± 0.19
7	4.88	3,4-CQA	$y = 15.353x - 12.021$	0.9987	371.93 ± 5.16	25.01 ± 0.42
8	6.87	3,4,5-CQA	$y = 6.218x - 5.158$	0.9949	26.84 ± 0.99	2.57 ± 0.08

*5-CQA: 5-O-caffeoylquinic acid; 3-CQA: 3-O-caffeoylquinic acid; 4-CQA: 4-O-caffeoylquinic acid; CA: caffeic acid; 4,5-CQA: 4,5-di-O-caffeoylquinic acid; 3,5-CQA: 3,5-di-O-caffeoylquinic acid; 3,4-CQA: 3,4-di-O-caffeoylquinic acid; and 3,4,5-CQA: 3,4,5-tri-O-caffeoylquinic acid. [a]Values were means ± SD of three determinations.

Table 2: Antioxidant activity of Yuzi No. 7 sweet potato leaf polyphenols, tea polyphenols, and grape seed polyphenols.

Samples*	Sample concentration (μg/mL)					
	5	10	20	5	10	20
	$\cdot O_2^-$ scavenging activity (μg·ACE/mL)			Oxygen radical absorbance capacity (μg·TE/mL)		
SPLP	14.57 ± 0.31[a]	30.56 ± 2.59[a]	62.71 ± 2.99[a]	22.35 ± 1.59[a]	33.72 ± 2.61[a]	55.68 ± 1.45[a]
TPP	3.60 ± 0.28[b]	7.29 ± 0.31[b]	10.62 ± 0.45[b]	16.67 ± 2.98[b]	32.23 ± 1.22[a]	43.53 ± 0.59[b]
GPP	3.02 ± 0.11[c]	3.18 ± 0.42[c]	6.73 ± 0.12[c]	13.75 ± 0.62[b]	29.21 ± 1.68[b]	43.54 ± 0.77[b]

*SPLP: total polyphenols from sweet potato leaves; TPP: total polyphenols from tea; GPP: total polyphenols from grape seeds. [a–c]Data in the same column that were significantly different were represented by different letters ($p < 0.05$).

control group, oxidative stress model group, sweet potato leaf polyphenol-pretreated oxidative stress model group, 3-O-caffeoylquinic acid-pretreated oxidative stress model group, 4-O-caffeoylquinic acid-pretreated oxidative stress model group, 5-O-caffeoylquinic acid-pretreated oxidative stress model group, 3,4-di-O-caffeoylquinic acid-pretreated oxidative stress model group, 3,5-di-O-caffeoylquinic acid-pretreated oxidative stress model group, 4,5-di-O-caffeoylquinic acid-pretreated oxidative stress model group, and 3,4,5-tri-O-caffeoylquinic acid-pretreated oxidative stress model group. The experimental procedure was the same as described in the above paragraph.

2.9. Statistical Analysis. All the above-mentioned experiments were performed for at least three replicates. The results were expressed as mean ± SD. Statistical analysis was carried out by means of one-way ANOVA followed by a Duncan multiple comparison test with the SAS version 8.1 software (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1. Quantification of Individual Phenolic Compounds by RP-HPLC. Table 1 showed that there were 7 caffeoylquinic acids and a small quantity of caffeic acid detected from Yuzi No. 7 sweet potato leaf polyphenols, which was in accordance with the previous reports [19–21, 24–26]. The three dicaffeoylquinic acids showed the highest contents, especially 3,5-di-O-caffeoylquinic acid (36.30 ± 0.19%, DW), followed by 3,4-di-O-caffeoylquinic acid (25.01 ± 0.42%, DW), 4,5-di-O-caffeoylquinic acid (21.38 ± 0.21%, DW), 3,4,5-tri-O-caffeoylquinic acid, monocaffeoylquinic acids, and caffeic acid. The composition of individual phenolic compounds in sweet potato leaves could be mainly attributed to the different genotypes and agroecological environment [30]. But in general, from the result obtained in this study regarding the composition of the individual phenolic compounds, sweet potato leaves could be a good source of biologically active compounds with multiple applications in the development of health products, functional foods, pharmaceuticals, and cosmetics. However, in pursuit of application in practical production, it is necessary to clarify the biological activities of each individual phenolic compound.

3.2. Antioxidant Activity

3.2.1. Antioxidant Activity of Total Polyphenols from Sweet Potato Leaves. The antioxidant activity of total polyphenols from Yuzi No. 7 sweet potato leaves was shown in Table 2. The $\cdot O_2^-$ scavenging activity showed significant dose dependence ($p < 0.05$). Under all tested concentrations, the $\cdot O_2^-$ scavenging activity of total polyphenols from sweet potato leaves was higher than that of total polyphenols from tea and grape seeds. When the concentration reached 20 μg/mL, total polyphenols from sweet potato leaves showed the highest $\cdot O_2^-$ scavenging activity (62.71 μg·ACE/mL), which was 4.90 and 8.32 times higher than that of total polyphenols from tea and grape seeds, respectively. The oxygen radical absorbance capacity of total polyphenols from sweet potato leaves also showed a significant dose-dependent relationship ($p < 0.05$). At the concentration of 20 μg/mL, the oxygen

Table 3: Antioxidant activity of individual phenolic compounds from sweet potato leaves.

Samples*	$\cdot O_2^-$ scavenging activity (μg·ACE/mL)	Oxygen radical absorbance capacity (μg·TE/mL)
SPLP	30.56 ± 2.59^{b}	33.72 ± 2.61^{c}
CA	51.12 ± 5.35^{a}	56.78 ± 4.12^{a}
3-CQA	22.97 ± 2.81^{c}	41.23 ± 1.06^{b}
4-CQA	19.36 ± 1.45^{c}	39.15 ± 1.58^{bc}
5-CQA	20.12 ± 2.79^{c}	42.58 ± 3.66^{b}
3,4-CQA	20.68 ± 1.55^{c}	39.91 ± 8.37^{bc}
3,5-CQA	21.69 ± 1.42^{c}	35.21 ± 2.11^{bc}
4,5-CQA	22.14 ± 2.15^{c}	42.16 ± 3.89^{b}
3,4,5-CQA	15.03 ± 1.12^{d}	32.21 ± 1.62^{c}

*The concentration of all tested samples was 10 μg/mL; SPLP: total polyphenols from sweet potato leaves; CA: caffeic acid; 3-CQA: 3-O-caffeoylquinic acid; 4-CQA: 4-O-caffeoylquinic acid;5-CQA: 5-O-caffeoylquinic acid; 3,4-CQA: 3,4-di-O-caffeoylquinic acid; 3,5-CQA: 3,5-di-O-caffeoylquinic acid; 4,5-CQA: 4,5-di-O-caffeoylquinic acid; 3,4,5-CQA: 3,4,5-tri-O-caffeoylquinic acid. $^{a-d}$Data in the same column that were significantly different were represented by different letters ($p < 0.05$).

radical absorbance capacity of total polyphenols from sweet potato leaves was the highest (55.68 μg·TE/mL), which was 1.28 times higher than that of total polyphenols from tea and grape seeds.

3.2.2. Antioxidant Activity of Individual Phenolic Compounds from Sweet Potato Leaves. The antioxidant activity of individual phenolic compounds from Yuzi No. 7 sweet potato leaves was shown in Table 3. For the $\cdot O_2^-$ scavenging activity, caffeic acid showed the highest value (51.12 μg·ACE/mL), which was much higher than total polyphenols from sweet potato leaves (30.56 μg·ACE/mL). However, the $\cdot O_2^-$ scavenging activities of all individual caffeoylquinic acids was lower than those of caffeic acid and total polyphenols from sweet potato leaves. Among which, the monocaffeoylquinic acids and dicaffeoylquinic acids showed no significantly different values and 3,4,5-tri-O-caffeoylquinic acid showed the lowest value (15.03 μg·ACE/mL).

For the oxygen radical absorbance capacity, all the individual phenolic compounds except 3,4,5-CQA showed higher values than total polyphenols from sweet potato leaves (33.72 μg·TE/mL). Caffeic acid showed the highest value (56.78 μg·TE/mL), which was significantly higher than other individual phenolic compounds ($p < 0.05$). There was no significant difference among the oxygen radical absorbance capacity of monocaffeoylquinic acids and dicaffeoylquinic acids.

Iwai et al. [23] reported that the radical scavenging activity of caffeoylquinic acid derivatives was positively correlated with the number of caffeoyl groups in their molecules. However, in our present study, there was no significant difference among the antioxidant activities of monocaffeoylquinic acids and dicaffeoylquinic acids, which was not in accordance with the report of Iwai et al. One of the possible reasons was that the methods used in determining antioxidant activity were different. In other words, different free radical scavenging activities were detected in the study of Iwai et al. and ours, for example, DPPH radical scavenging activity was detected in the study of Iwai et al., while the $\cdot O_2^-$ scavenging activity and oxygen radical absorbance capacity were detected in our present study. Moreover, Bendary et al. [31] reported that the antioxidant activity of polyphenols was mainly related to the number of phenolic hydroxyl groups in the molecules—the higher the phenolic hydroxyl number, the stronger the antioxidant activity. The molecular weight of mono- and dicaffeoylquinic acids was 354.31 and 516.45, respectively, and the phenolic hydroxyl group number in mono- and dicaffeoylquinic acids was 5 and 6, respectively. That is to say, the number ratio of phenolic hydroxyl groups in the same concentration of mono- and dicaffeoylquinic acids in the present study was 1.21, which possessed little difference. Therefore, at the same mass concentration, the antioxidant activity was not significantly different between mono- and dicaffeoylquinic acids. In addition, the antioxidant activity of polyphenols was related not only to the number of phenolic hydroxyl groups but also to the position of phenolic hydroxyl groups [32]. The position of phenolic hydroxyl groups and spatial conformation of different caffeoylquinic acids were different, making it very difficult to judge which one possesses higher antioxidant activity.

Although caffeic acid possessed the highest antioxidant activity, its content in the total polyphenols from sweet potato leaves was only 0.09%. By contrast, the antioxidant activity of mono- and dicaffeoylquinic acids was lower than that of caffeic acid, but the content of dicaffeoylquinic acids was predominant, accounting for 82.69% of the total polyphenols in sweet potato leaves. It can therefore be said that dicaffeoylquinic acids contribute most to the antioxidant activity of sweet potato leaf polyphenols.

3.3. Inhibition of Intracellular Reactive Oxygen Species of Sweet Potato Leaf Polyphenols

3.3.1. Determination of H_2O_2 Concentration in the Establishment of LO2 Oxidative Stress Model. MTT test result showed that, with the increase of H_2O_2 concentration, the cell viability of human hepatocyte LO2 cells decreased gradually (Figure 1(a)). When the concentration of H_2O_2 was 25 μM, the cell viability of LO2 was significantly lower than that of blank control (without H_2O_2 treatment). When the concentration of H_2O_2 reached 100 μM, the cell viability decreased to 50.17%. When the concentration of H_2O_2 reached 1000 μM, the cell viability was only 29.35%. Therefore, the following experiments were carried out with H_2O_2 working solution of 100 μM as the modeling agent.

3.3.2. Determination of the Concentration Range of Polyphenols from Sweet Potato Leaves. Compared to blank control (without sweet potato leaf polyphenol treatment), the sweet potato leaf polyphenol treatment under the concentration range of 25–800 μg/mL did not cause significant change of cell viability (Figure 1(b)). When the concentration

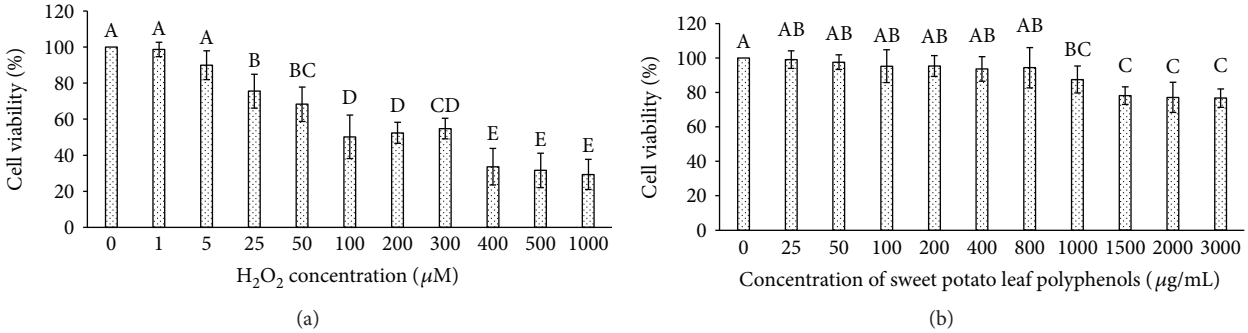

Figure 1: The effect of (a) H_2O_2 concentration and (b) sweet potato leaf polyphenol concentration on the cell viability of human hepatocyte LO2 cells. Values were means ± SD of five determinations. The different letters above the different bars mean that the cell viability was significantly different ($p < 0.05$).

of sweet potato leaf polyphenols reached 1000 μg/mL, the cell viability of human hepatocyte LO2 cells decreased to 87.48%. With the further increase of sweet potato leaf polyphenol concentration, the cell viability was further reduced. Therefore, the following experiments were carried out with sweet potato leaf polyphenol working solution of 0–800 μg/mL.

3.3.3. Inhibition of Intracellular Reactive Oxygen Species of Total Polyphenols from Sweet Potato Leaves. The effect of total polyphenols from sweet potato leaves on the cell viability of oxidative stress LO2 cells was shown in Figure 2(a). The total polyphenols from sweet potato leaves could significantly reduce the decline of LO2 cell viability induced by H_2O_2, and this effect possessed a dose-dependent manner. 25, 50, 100, 200, 400, and 800 μg/mL sweet potato leaf polyphenol working solutions were able to restore the cell viability to 53.32%, 59.40%, 66.78%, 71.67%, 92.23%, and 94.01%, respectively. When the concentration of sweet potato leaf polyphenols reached 50 μg/mL, the protective effect on LO2 cells was comparable to that of 100 μg/mL Trolox and ascorbic acid. However, at the same concentration (100 μg/mL), the protective effect of sweet potato leaf polyphenols, Trolox, ascorbic acid, and tea polyphenols showed no significant difference, which were all lower than that of grape seed polyphenols (the cell viability is 115.22%).

The effect of total polyphenols from sweet potato leaves on the level of intracellular reactive oxygen species was shown in Figure 2(b). Sweet potato leaf polyphenols decreased the level of intracellular reactive oxygen species significantly ($p < 0.05$) in a dose-dependent manner. When the concentration of sweet potato leaf polyphenols was 25 μg/mL, the level of intracellular reactive oxygen species was decreased to 87.14% compared to the oxidative stress model group (100%), which was equivalent to 100 μg/mL Trolox (82.51%) and ascorbic acid (89.30%). When the concentration of sweet potato leaf polyphenols was 50 and 100 μg/mL, the level of intracellular reactive oxygen species was decreased to 79.19% and 78.32%, respectively, showing no significant difference compared to 100 μg/mL Trolox. When the concentration of sweet potato leaf polyphenols was 200 and 400 μg/mL, the level of intracellular reactive oxygen species was decreased to 67.34% and 61.17%, respectively, reaching the level of blank control and 100 μg/mL tea polyphenols. When the concentration of sweet potato leaf polyphenols was 800 μg/mL, the level of intracellular reactive oxygen species was decreased to 51.95%, which was equivalent to 100 μg/mL grape seed polyphenols, and significantly lower than that of blank control.

3.3.4. Inhibition of Intracellular Reactive Oxygen Species of Individual Phenolic Compounds from Sweet Potato Leaves. The protective effect of individual phenolic compounds from sweet potato leaves on human hepatocyte LO2 oxidative stress was shown in Figure 3. Under the same sample concentration (100 μg/mL), the cell viability of LO2 pretreated by caffeic acid and 3-O-caffeoylquinic acid was highest (95.12% and 93.71%, resp., but no significant difference), followed by 3,5-di-O-caffeoylquinic acid (89.96%), 4,5-di-O-caffeoylquinic acid (88.79%), 3,4,5-tri-O-caffeoylquinic acid (84.41%), 5-O-caffeoylquinic acid (80.97%), 3,4-di-O-caffeoylquinic acid (78.22%), 4-O-caffeoylquinic acid (77.83%), and the total polyphenols from sweet potato leaves (66.78%) (Figure 3(a)). Moreover, the cell viability of LO2 pretreated by all individual phenolic compounds and total polyphenols from sweet potato leaves was significantly higher than that of the LO2 oxidative stress model (50.17%) ($p < 0.05$), and the cell viability of LO2 pretreated by 3-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid did not show a significant difference with that of blank control (100%).

The effect of individual phenolic compounds from sweet potato leaves on the level of intracellular reactive oxygen species was shown in Figure 3(b). All the tested samples decreased the level of intracellular reactive oxygen species significantly ($p < 0.05$) at the same concentration of 100 μg/mL; especially caffeic acid and 3-O-caffeoylquinic acid, the intracellular reactive oxygen species level of which was decreased by 50.34% and 48.88% of H_2O_2 control, respectively, even lower than that of blank control. The intracellular reactive oxygen species level did not show a significant difference among 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid,

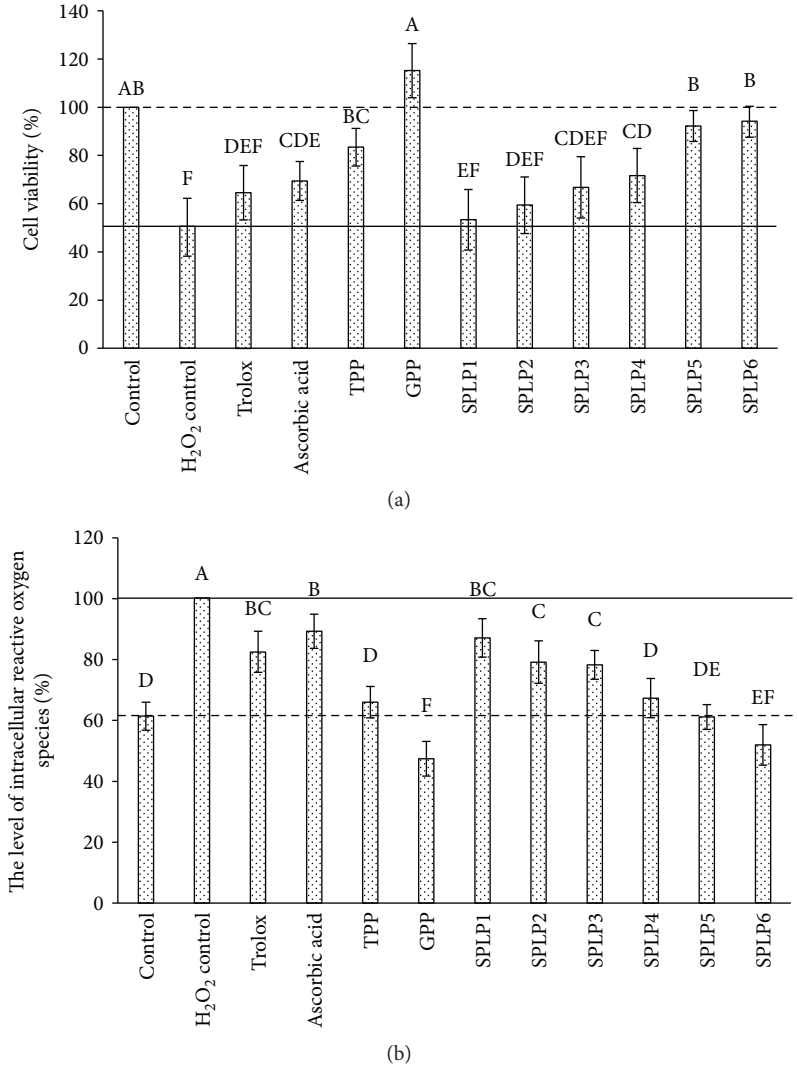

Figure 2: Protective effect of total polyphenols from sweet potato leaves on human hepatocyte LO2 oxidative stress. (a) The effect of total polyphenols from sweet potato leaves on the cell viability of oxidative stress LO2 cells. (b) The effect of total polyphenols from sweet potato leaves on the level of intracellular reactive oxygen species. Control was LO2 cells without H_2O_2 and antioxidant treatment; H_2O_2 control was the LO2 oxidative stress model group which was treated by H_2O_2 of 100 μM; Trolox, ascorbic acid, TPP, and GPP were LO2 cells pretreated by 100 μg/mL Trolox, ascorbic acid, tea polyphenols, and grape seed polyphenols, respectively, and then treated by 100 μM H_2O_2; SPLP1, SPLP2, SPLP3, SPLP4, SPLP5, and SPLP6 were LO2 cells pretreated by sweet potato leaf polyphenols of 25, 50, 100, 200, 400, and 800 μg/mL, respectively, and then treated by 100 μM H_2O_2; the dashed lines represented the values of the blank control group, while the solid lines represented the values of the LO2 oxidative stress model group. Values were means ± SD of five determinations. The different letters above the different bars mean that the cell viability or the level of intracellular reactive oxygen species was significantly different ($p < 0.05$).

3,4,5-tri-O-caffeoylquinic acid, and total polyphenols from sweet potato leaves. However, considering the obvious difference in the content of individual phenolic compounds in sweet potato leaf polyphenols, it can be said that dicaffeoylquinic acids contribute most to the inhibition of intracellular reactive oxygen species of sweet potato leaf polyphenols.

The sequence of the effect of individual phenolic compounds from sweet potato leaves on the level of intracellular reactive oxygen species was not in accordance with that of the antioxidant activity, suggesting that there is no direct relationship between the antioxidant activity and protective effect on human hepatocyte LO2 oxidative stress. It has been reported that improving the endogenic ROS-regulating ability is the key to improve the ability of antioxidative stress [33]. By regulating the expression of oxidative stress-related genes, improving the endogenic ROS-regulating ability, intervening the signal transduction pathway of oxidative damage-inducing apoptosis, and protecting or repairing mitochondrial function, the cell damage induced by oxidative stress could be prevented effectively [33]. That is to say, the preventive effect of sweet potato leaf polyphenols on the

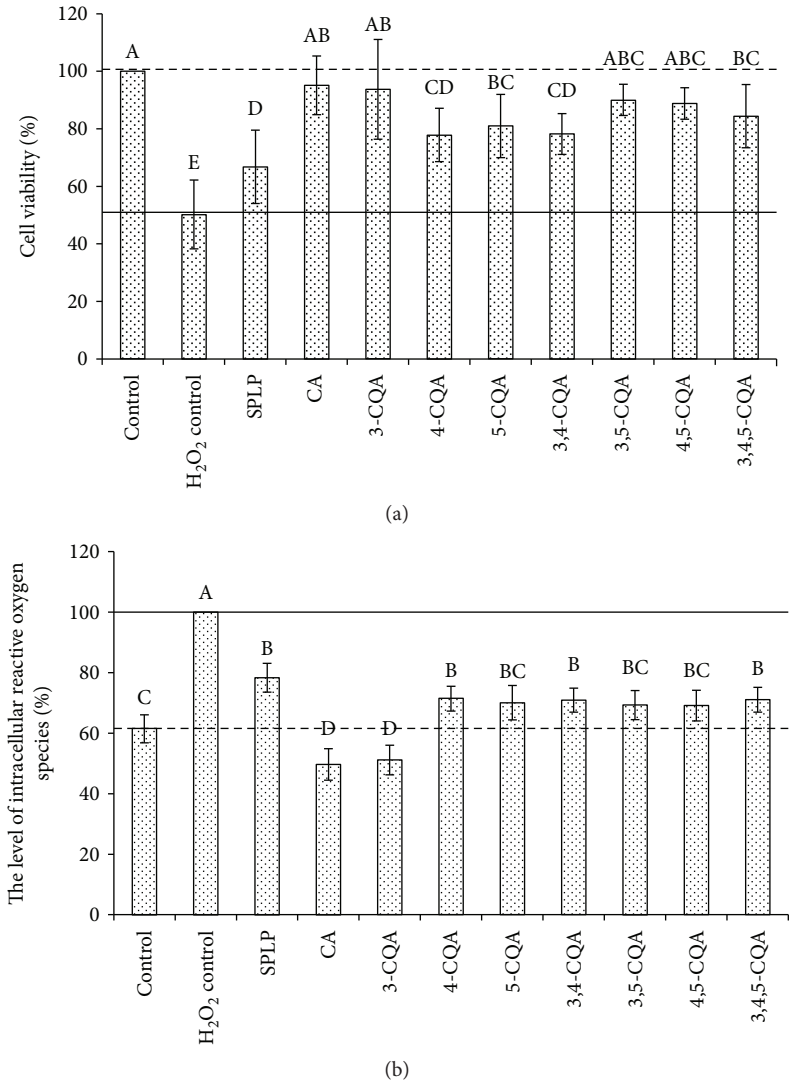

Figure 3: Protective effect of individual phenolic compounds from sweet potato leaves on human hepatocyte LO2 oxidative stress. (a) The effect of individual phenolic compounds from sweet potato leaves on the cell viability of oxidative stress LO2 cells. (b) The effect of individual phenolic compounds from sweet potato leaves on the level of intracellular reactive oxygen species. Control was LO2 cells without H_2O_2 and antioxidant treatment; H_2O_2 control was LO2 oxidative stress model group which was treated by H_2O_2 of 100 μM; SPLP, CA, 3-CQA, 4-CQA, 5-CQA, 3,4-CQA, 3,5-CQA, 4,5-CQA, and 3,4,5-CQA were LO2 cells pretreated by 100 μg/mL sweet potato leaf polyphenols, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,4,5-tri-O-caffeoylquinic acid, respectively, and then treated by 100 μM H_2O_2; the dashed lines represented the values of the blank control group, while the solid lines represented the values of the LO2 oxidative stress model group. Values were means ± SD of five determinations. The different letters above the different bars mean that the cell viability or the level of intracellular reactive oxygen species was significantly different ($p < 0.05$).

oxidative stress of LO2 cells might be performed by regulating the expression of oxidative stress-related genes, rather than directly through the antioxidant effect, which needs to be verified by further study.

4. Conclusion

The antioxidant activity of sweet potato leaf polyphenols was significantly higher than those of ascorbic acid, tea polyphenols, and grape seed polyphenols. Among the individual phenolic compounds present in sweet potato leaves, caffeic acid showed the highest antioxidant activity, followed by mono- and dicaffeoylquinic acids and 3,4,5-tri-O-caffeoylquinic acid showed the lowest antioxidant activity. The intracellular ROS-inhibiting activity of sweet potato leaf polyphenols was similar to that of Trolox. The sequence of the inhibiting effect of individual phenolic compounds from sweet potato leaves on the level of intracellular ROS was not in accordance with that of the antioxidant activity, suggesting that there is no direct relationship between the antioxidant activity and protective effect on human hepatocyte LO2 oxidative stress. Sweet potato leaves could be a good source of biologically active polyphenols with multiple applications in the development of functional foods, health products, pharmaceuticals,

and cosmetics. In pursuit of application in practical production, the biological activities of other compounds present in sweet potato leaves should also be investigated.

Conflicts of Interest

The authors declare that there is no competing financial interest.

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