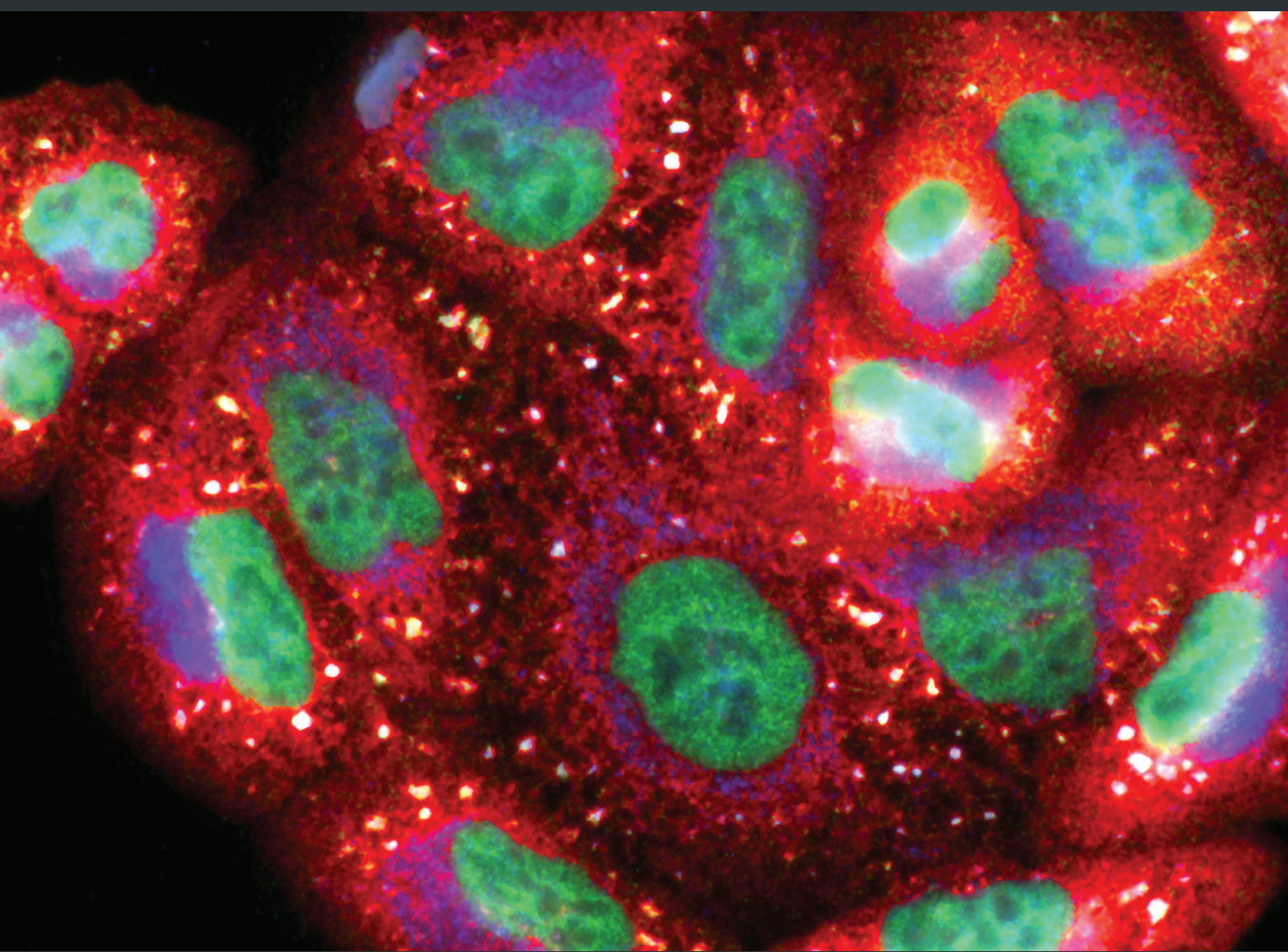
Oxidative Medicine and Cellular Longevity

Nutrients and Diet: A Relationship between Oxidative Stress, Aging, Obesity, and Related Noncommunicable Diseases

Lead Guest Editor: Rodrigo Valenzuela
Guest Editors: Undurti N. Das, Luis A. Videla, and Carolina G. Llorente



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Editorial

Nutrients and Diet: A Relationship between Oxidative Stress, Aging, Obesity, and Related Noncommunicable Diseases

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Nowadays, the significant increase in the prevalence of obesity worldwide is directly associated with an increased risk of developing noncommunicable diseases such as diabetes, cardiovascular disease, dyslipidemias, inflammatory disease, cancer, and even neurodegenerative diseases. In this regard, obesity and chronic diseases are not only limited to developing countries, nor to the adult population. On the contrary, the greatest increase in the incidence of obesity in the last 20 years has been observed in developing countries and young population.

Important risk factors involved in obesity development comprehend (i) high-energy intake; (ii) excessive fat consumption (especially saturated fat and transfatty acids); (iii) excessive intake of simple carbohydrates (sucrose, glucose, and fructose); (iv) insufficient intake of vegetables and fruits (sources of natural antioxidant), legumes (dietary fiber), and fish and seafood (sources of polyunsaturated fatty acid); and (v) unhealthy lifestyle (sedentarism among other factors like consumption of tobacco, alcohol, and drugs). These factors directly favor oxidative stress, which is a relevant metabolic disturbance related to the development of other pathologies.

In this sense, diet chemical composition and food preparation play a relevant and direct role in regulating several of the metabolic and molecular pathways involved in the prevention and treatment of obesity and its related comorbidities. For this reason, it is necessary to identify and understand molecular pathways involved in these events in order to develop nutritional strategies that contribute to the prevention and treatment of oxidative stress linked with the obesity.

In this special issue, the impact of specific diet and nutritional interventions destined to prevent or attenuate obesity-related oxidative stress injuries is presented. By reading the different articles, readers will be able to identify relevant aspects underlying molecular changes generated by oxidative stress as a consequence of obesity in different study models. The risk of developing metabolic syndrome and cardiovascular alterations in humans, as well as dietary interventions using foods or bioactive compounds (polyphenols or other micronutrients) to treat these alterations, is addressed in this issue. It should be noted that some of the studies published here suggest the protective effect of bioactive compounds and exercise on oxidative stress and other alterations generated by obesity. Thus, in this special issue, results about reproduction, oxidative stress, aging, obesity, and related noncommunicable diseases and their interaction with nutrients and diet are reported.

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Research Article

Attenuation of High-Fat Diet-Induced Rat Liver Oxidative Stress and Steatosis by Combined Hydroxytyrosol- (HT-) Eicosapentaenoic Acid Supplementation Mainly Relies on HT

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Pharmacological therapy for nonalcoholic fatty liver disease (NAFLD) is not approved at the present time. For this purpose, the effect of combined eicosapentaenoic acid (EPA; 50 mg/kg/day) modulating hepatic lipid metabolism and hydroxytyrosol (HT; 5 mg/kg/day) exerting antioxidant actions was evaluated on hepatic steatosis and oxidative stress induced by a high-fat diet (HFD; 60% fat, 20% protein, and 20% carbohydrates) compared to a control diet (CD; 10% fat, 20% protein, and 70% carbohydrates) in mice fed for 12 weeks. HFD-induced liver steatosis (i) was reduced by 32% by EPA, without changes in oxidative stress-related parameters and mild recovery of Nrf2 functioning affording antioxidation and (ii) was decreased by 42% by HT, concomitantly with total regain of the glutathione status diminished by HFD, 42% to 59% recovery of lipid peroxidation and protein oxidation enhanced by HFD, and regain of Nrf2 functioning, whereas (iii) combined EPA + HT supplementation elicited 74% reduction in liver steatosis, with total recovery of the antioxidant potential in a similar manner than HT. It is concluded that combined HT + EPA drastically decreases NAFLD development, an effect that shows additivity in HT and EPA effects that mainly relies on HT, strengthening the impact of oxidative stress as a central mechanism underlying liver steatosis in obesity.

1. Introduction

Oxidative stress is a disequilibrium condition in which the cellular redox balance is shifted towards a more oxidizing status that may trigger adaptation of cellular functions [1]. Depending on the antioxidant level of different cell types, the concentration of reactive oxygen species (ROS) achieved, and the duration of the exposure, oxidative stress may trigger beneficial responses under mild conditions and potentially harmful ones beneath severe situations, as a typically hormetic phenomenon [2]. In the latter case, oxidative stress development plays a role in the pathogenesis of several liver diseases, including alcoholic liver disease, haemochromatosis, Wilson's disease, chronic hepatitis C, and nonalcoholic fatty liver disease (NAFLD) [3]. NAFLD is characterized by excess triglyceride (TG) deposition in the hepatocyte followed by development of inflammatory (nonalcoholic steatohepatitis (NASH)) and fibrogenic responses [4] as shown in patients with obesity and insulin resistance [5–7]. Liver steatosis is also observed in rodents subjected to high-fat diets (HFDs) [8], which are considered adequate experimental models to understand the underlying mechanisms that may support dietary and/or nutritional interventions preventing or treating NAFLD [9, 10]. In addition to liver steatosis and oxidative stress, HFDs containing 45% to 75% of their calories as fat for 12 to 16 weeks induce overweight, insulin resistance,

a proinflammatory status, apoptosis, and n-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) depletion [8–11], diets that are characterized by being sufficient in macro- and micronutrients [12, 13].

Regardless of the high prevalence and increasing incidence of adult and paediatric NAFLD, no pharmacological therapy for NAFLD or NASH is approved at the present time, weight loss and exercise being the mainstay of treatment [14]. Considering that NAFLD is a multifactorial entity, it has been considered that combined therapies may achieve higher rates of responses and improved outcomes than monotherapies [14–16]. Supporting this contention, combined therapies using (i) thyroid hormone- (T_3-) docosahexaenoic acid (DHA) prevent ischemia-reperfusion-induced liver inflammatory injury [17] and (ii) DHA-extra virgin olive oil (EVOO) attenuates HFD-dependent hepatic steatosis [18], whereas (iii) combination drug treatments have been proposed in the case of uncontrolled hypertension [19] and (iv) high-potency statins combined with ezetimibe or pioglitazone were recommended for the resolution of NAFLD or NASH [20]. Based on the hepatoprotective effects of the EVOO component hydroxytyrosol (HT) [21, 22] and the n-3 LCPUFA eicosapentaenoic acid (EPA) [23], we hypothesized that the combined supplementation with EPA and HT could alter HFD-induced biochemical changes associated with steatosis. For this purpose, general metabolic parameters were measured concomitantly with the fatty acid (FA) composition and degree of steatosis, the oxidative stress status, and the activity of antioxidant enzymes controlled by the redox-sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in the liver of mice subjected to HFD (60% of the total calories as fat for 12 weeks).

2. Methods

2.1. Animals and Diet Supplementation. Weaning male C57BL/6J mice weighing 12–14 g (Bioterio Central, ICBM, Faculty of Medicine, University of Chile) were randomly assigned to each experimental group ($n = 8$ per experimental group) and were allowed free access to control diet (CD) or HFD. The CD composition (expressed as % total calories) was 10% fat, 20% protein, and 70% carbohydrate, with a caloric value of 3.85 kcal/g, and HFD composition was 60% fat, 20% protein, and 20% carbohydrate, with a caloric value of 5.24 kcal/g (Rodent Diet, product data D12450B and D12492, Research Diet Inc., USA). FA composition of CD and HFD was previously described [9]. Animals received water ad libitum and were housed on a 12 h light/dark cycle from days 1 to 84 (12 weeks). EPA, isolated from fish oil (Golden Omega S.A., Chile) as TAG (50% EPA, 5% DHA, and 5% of other n-3 FAs; 15% saturated fatty acid (SFA) (principally palmitic acid), and 25% MUFA (principally oleic acid)), was administered at 50 mg/kg/day dosage. HT (elaVida™, DSM Nutritional Products Company, Netherlands) was given at doses of 5 mg/kg/day, and control groups received isovolumetric amounts of saline orally, conforming eight experimental groups, namely, (a) CD (control), (b) CD + EPA, (c) CD + HT, (d) CD + EPA + HT, (e) HFD, (f) HFD + EPA, (g) HFD + HT, and (h) HFD + EPA + HT. The doses of EPA or HT used in this study were used according to previous research; namely, EPA (50 mg/kg) represents 50% of the dose of EPA + DHA used by Valenzuela et al. [8], whereas HT at 5 mg/kg exhibits protective effects against HFD [24]. Weekly controls of body weight and diet intake were performed through the whole period, and at the end of the 12th week the animals were fasted (6–8 h) and anesthetized with isoflurane (Lunan Baxter Pharmaceuticals Co. Ltd., Shandong, China), and blood samples were obtained by cardiac puncture for the determination of serum aspartate transaminase (AST) and alanine transaminase (ALT), together with the oxidative stress status of the liver. Liver samples were either frozen in liquid nitrogen for determination of FA composition or fixed in phosphate-buffered formalin, embedded in paraffin, stained with haematoxylin-eosin, and analysed by optical microscopy in a blind fashion describing the presence of steatosis and inflammation, both graded as absent, mild, moderated, and severe [25].

2.2. Ethics. All animal procedures in this study were in strict adherence to the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6–23, revised 1985) and were approved by the Bioethics Committee for Research in Animals, Faculty of Medicine, University of Chile (CBA protocol 0580 FMUCH).

2.3. Biochemical Analyses and Oxidative Stress Markers. Serum AST and ALT activities ($U\,L^{-1}$) were measured using specific diagnostic kits (bioMérieux SA, Marcy l'Etoile, France). Total fat content in liver (mg/g) was evaluated according to Bligh and Dyer [26], and triacylglycerol (TAG) content ($mg\,g^{-1}$) was measured using specific kits according to the manufacturer's instructions (Cayman Chemical Company, Michigan, USA). Livers from anesthetized animals were perfused in situ with a cold solution containing 150 mM KCl and 5 mM Tris (pH 7.4) to remove blood for protein carbonylation and glutathione assessments. Protein carbonyl concentration was determined by a fluorometric assay (Cayman Chemical Company, Michigan, USA) after adjusting the total protein concentration to $7.5\,mg\,mL^{-1}$ per sample. Reduced glutathione (GSH) and glutathione disulphide (GSSG) contents were assessed with an enzymatic recycling method [27]. The antioxidant capacity of serum, serum and liver thiobarbituric acid reactive substances (TBARS), and hepatic F-8 isoprostanes were determined by colorimetric assays (Cayman Chemical Company, Michigan, USA).

2.4. Determination of Liver Antioxidant Enzyme Activities. The liver activity of CAT was measured according to the method of Lück [28]. Assessment of SOD activity was carried out with a commercial assay kit (Cayman Chemical Company; Michigan, USA) according to the manufacturer's instructions. GPX activity was determined using the method described by Paglia and Valentine [29]. GR activity was determined according to Horn [30], NADPH-quinone oxidoreductase 1 (NQO1) activity was measured according to the method of Ernster et al. [31], glutathione-S-transferase (GST) activity was determined according to the method

described by Habig et al. [32], and γ-glutamyl transpeptidase (GGT) activity was determined following the method of Satomura et al. [33]. To develop the experimental specific conditions for evaluating the liver activity of these enzymes, we followed the methods previously published by Rincón-Cervera et al. [34] and Valenzuela et al. [24].

2.5. Gene Expression Assays. Total RNA was isolated from liver samples using TRIzol (Invitrogen, Paisley, UK), according to the supplier's protocols. Purified RNA (2 μg) was then treated with DNase (DNA-free kit; Ambion, Austin, TX, USA) and used to generate first-strand cDNA with M-MLV Reverse Transcriptase (Invitrogen, Paisley, UK), utilizing random hexamers (Invitrogen, Paisley, UK) and dNTP mix (Bioline, London, UK), according to the manufacturer's protocol. The resultant cDNA was amplified with specific primers for mice in a total volume of 10 μL. Table 1 depicts the gene-specific primer sequences used in the study. Primer optimization and real-time quantitative PCR were performed according to Rincón-Cervera et al. [34].

2.6. Assessment of Liver DNA-Binding Activity of Nrf2. Nuclear extracts from liver tissue (left lobe) were obtained using a commercial extraction kit (Cayman Chemical Company, Michigan, USA). Nrf2 DNA-binding activity was assessed with commercial ELISA kits (Cayman Chemical Company, Michigan, USA), according to the manufacturer's instructions. Values were expressed as percentage of Nrf2 DNA binding with respect to a positive control provided by the ELISA kit.

2.7. FA Profile. Quantitative extraction of total lipids from liver was carried out according to Bligh and Dyer [26]. Liver samples were homogenized in ice-cold chloroform/methanol (2 : 1 *v*/*v*) containing 0.01% butylated hydroxytoluene in an Ultra-Turrax homogenizer (Janke & Kunkel, Stufen, Germany). Total lipids from liver samples were extracted with chloroform/methanol (2 : 1 *v*/*v*). Fatty acid methyl esters (FAMEs) from total liver fat were prepared as previously described [34] and analysed according to Valenzuela et al. [9].

2.8. Statistical Analysis. Statistical analysis was performed with GraphPad Prism version 6.1 (GraphPad Software, San Diego, CA, USA). Values shown represent the mean ± SEM for the number of separate experiments indicated. Two-way ANOVA and Bonferroni's post hoc test assessed the statistical significance of differences between mean values, with $p < 0.05$ being considered significant. Pearson's coefficient was used to assess associations between variables.

3. Results

3.1. General Parameters, Food and Energy Intake, and Liver Function-Related Indexes. Mice in the different experimental groups exhibiting comparable initial body weights showed 75% to 95% increases in their final body weights when given CD, with an average body weight gain of 12.4 ± 0.7 g ($n = 64$) that was enhanced by 85% by HFD alone (group e; Table 2). EPA or EPA + HT supplementation in HFD mice generated a significant reduction in body weight gain, 20% and 33%, respectively, whereas HT supplementation was not significant in this parameter (Table 2). In all experimental groups, dietary intake was comparable, but energy consumption was higher in mice subjected to HFD without or with EPA, HT, and EPA + HT supplementation over values in the respective CD groups. Under these conditions, serum AST and ALT levels were comparable in all groups, whereas liver weight in the HFD + EPA + HT group was reduced by 18% ($p < 0.05$) compared to CD values. Furthermore, hepatic total fat was comparable in mice subjected to CD without or with supplementations, similarly to liver TG levels; however, hepatic fat increased by 193% due to HFD, and liver TGs were elevated by 210% over CD values ($p < 0.05$). The latter two parameters in HFD mice were not altered by EPA or HT, but decreased by 75% and 63% ($p < 0.05$) by EPA + HT supplementation, respectively (Table 2).

3.2. Liver Morphological Characteristics. Mice subjected to CD without or with EPA, HT, and EPA + HT supplementation exhibited normal liver histology (Figures 1(a)–1(d)) and showed comparable liver steatosis scores measured according to [35] (Figure 1(i)). HFD for 12 weeks elicited macrovesicular liver steatosis (Figure 1(e)) with 6.5-fold elevation in the steatosis score compared to the CD group ($p < 0.05$), a change that was decreased by 32%, 42%, and 74% by EPA, HT, and EPA + HT supplementation, respectively ($p < 0.05$) (Figure 1(i)).

3.3. Liver FA Composition. Total liver SFA, MUFA, and PUFA levels were comparable in all groups subjected to CD (Table 3). However, (i) total SFAs showed 35% increase by HFD over CD values ($p < 0.05$), which was decreased by 25%, 41%, and 22% by EPA, HT, and EPA + HT supplementation ($p < 0.05$); (ii) total MUFAs were not modified in all experimental groups; and (iii) total PUFAs were decreased by 35% by HFD over CD levels ($p < 0.05$), a change that was reduced by 35%, 38%, and 38% by EPA, HT and EPA + HT supplementation, respectively ($p < 0.05$) (Table 3). In relation to total LCPUFAs, mice subjected to CD and EPA or EPA + HT supplementation showed 26% or 34% increases over CD alone ($p < 0.05$), whereas HFD-fed animals exhibited 41% reduction over CD values, an alteration that was reversed by 58%, 38%, and 68% by EPA, HT, and EPA + HT supplementation, respectively ($p < 0.05$) (Table 3). Similarly, HFD induced (i) 35% decrement in n-6 LCPUFA levels ($p < 0.05$) compared to CD values, with EPA, HT, and EPA + HT eliciting 20%, 18%, and 30% recovery, respectively ($p < 0.05$); (ii) 53% reduction in n-3 LCPUFAs ($p < 0.05$) over CD values, whereas EPA, HT, and EPA + HT reached 161%, 74%, and 169% rescue versus HFD alone, with 115% and 130% enhancement by EPA and EPA + HT being found in mice given CD alone; and (iii) 37% increase in the n-6/n-3 LCPUFA ratio ($p < 0.05$), which was lowered by 54%, 32%, and 51% by EPA, HT, and EPA + HT ($p < 0.05$), respectively (Table 3).

3.4. Blood Plasma and Liver Oxidative Stress-Related Parameters. Mice subjected to CD showed similar values of

Table 1: Gene-specific primer sequences used in the study.

mRNA	Forward primer	Reverse primer
Nrf2	AAGCTTTCAACCCGAAGCAC	TTTCCGAGTCACTGAACCCA
Gst	TGCAGACCAAAGCCATTCTC	ACGGTTCCTGGTTTGTTCCT
Ggt	ATGTGGACACCCGATGCAGTATT	TGTCTTGCTTGTAGTCAGGATGGTTT
β-Actin	ACTGCCGCATCCTCTTCCTC	CTCCTGCTTGCTGATCCACATC

Sequences are listed in the $5' \rightarrow 3'$ direction. *Nrf2*: nuclear factor erythroid 2-related factor 2; *Gst*: glutathione-S-transferase; *Ggt*: γ-glutamyl transpeptidase.

the antioxidant capacity of plasma when given EPA, which was enhanced by 65% by HT and EPA + HT supplementation ($p < 0.05$); however, HFD elicited 58% reduction in animals without or with EPA treatment that was enhanced by 120% in HT and EPA + HT groups ($p < 0.05$) (Figure 2(a)). Animals given CD without and with supplementations exhibited no significant changes in liver total GSH equivalents (Figure 2(b)), in the levels of GSH (Figure 2(c)) and GSSG (Figure 2(d)), in GSH/GSSG ratios (Figure 2(e)), and in the content of TBARS (Figure 2(f)), F-8 isoprostanes (Figure 2(g)), or protein carbonyls (Figure 2(h)). HFD led to significant decreases in total GSH equivalents (30%), GSH content (34%), and GSH/GSSG ratios (44%), with no alteration in GSSG levels, whereas the contents of TBARS, F-8 isoprostanes, and protein carbonyls were increased by 154%, 157%, and 215%, respectively, over the CD group, changes that were comparable to those found in the HFD + EPA group (Figures 2(b)–2(h)). Compared to the group given HFD alone, HT and EPA + HT recovered to CD values hepatic total GSH equivalents and GSH/GSSG ratios (Figures 2(b) and 2(e)); furthermore, HT and EPA + HT recovered the contents of liver GSH by 77% and 84% (Figure 2(c)), TBARS by 59% and 68% (Figure 2(f)), F-8 isoprostane by 42% and 48% (Figure 2(g)), and protein carbonyls by 43% and 47% (Figure 2(h)), respectively. Under these conditions, antioxidant parameters in plasma (antioxidant capacity) and liver (GSH levels) were significantly correlated ($r = 0.84$; $p < 0.004$), whereas liver GSH contents were inversely associated with those of TBARS ($r = -0.98$; $p < 0.0001$), F-8 isoprostanes ($r = -0.94$; $p < 0.0002$), and protein carbonyls ($r = -0.93$; $p < 0.0004$).

3.5. Liver Nrf2 DNA Binding; mRNA Expression of Nrf2, GST, and GGT; and Activity of Enzymes Controlled by Nrf2. Mice subjected to CD without and with supplementations revealed comparable values of Nrf2 DNA-binding capacity and in the mRNA expression of Nrf2, GST, and GGT, which were significantly decreased by 68%, 77%, 76%, and 59% over CD values by HFD ($p < 0.05$) (Figures 3(a)–3(d)). HFD-induced decrease in Nrf2 DNA binding exhibited 33% and 64% recovery by EPA and HT, respectively, whereas EPA + HT achieved total recovery (Figure 3(a)); similarly, Nrf2 mRNA levels were recovered by 32%, 55%, and 93% by EPA, HT, and EPA + HT, respectively (Figure 3(b)). Compared to the group given HFD alone, GST mRNA levels were recuperated by 22%, 25%, and 67% by EPA, HT, and EPA + HT, respectively (Figure 3(c)); likewise, EPA, HT, and EPA + HT improved by 8%, 48%, and 70% HFD-induced reduction in GGT mRNA expression (Figure 3(d)).

EPA, HT, and EPA + HT did not elicit significant changes in the activities of the studied antioxidant enzymes in mice fed CD; however, HFD alone decreased those of CAT (66%), SOD (62%), GPX (45%), GR (60%), NQO1 (66%), GST (76%), and GGT (59%) ($p < 0.05$) (Figures 4(a)–4(g)). In HFD-treated animals, (i) EPA did not alter GPX, NQO1, GST, and GGT activities (Figures 4(c), 4(e), 4(f), and 4(g)), but it recovered by 54%, 30%, and 36% those of CAT, SOD, and GR, respectively (Figures 4(a), 4(b), and 4(d)), and (ii) HT improved SOD activity by 31% (Figure 4(b)), whereas (iii) HT and HT + EPA totally recuperated the activities of CAT, GPX, GR, NQO1, GSR, and GGT (Figures 4(a), 4(c)–4(g)).

3.6. Correlations. Liver steatosis score was significantly correlated with the content of hepatic TAGs ($r = 0.94$; $p < 0.0002$) and fat content ($r = 0.97$; $p < 0.0001$) and inversely associated with the antioxidant capacity of plasma ($r = -0.75$; $p < 0.02$). The hepatic levels of the antioxidant GSH exhibited an inverse association with the prooxidant parameters, TBARs ($r = -0.98$; $p < 0.0001$), protein carbonyls ($r = -0.92$; $p < 0.0004$), and F-8 isoprostanes ($r = -0.92$; $p < 0.0012$), but revealed a direct correlation with the DNA binding of the redox-sensitive transcription factor Nrf2 ($r = 0.94$; $p < 0.0004$). Moreover, the DNA-binding activity of Nrf2 was significantly associated with the activities of the antioxidant enzymes CAT ($r = 0.94$; $p < 0.0003$), SOD ($r = 0\,0.95$; $p < 0.0001$), GPX ($r = 0.89$; $p < 0.002$), GR ($r = 0.92$; $p < 0.0005$), NQO1 ($r = 0.89$; $p < 0.002$), GST ($r = 0.93$; $p < 0.0005$), and GGT (r = 0.89; $p < 0.002$).

4. Discussion

Mice subjected to a HFD comprising 60% of the total calories as fat for 12 weeks developed macrovesicular steatosis as evidenced histologically, with a 6.5-fold increase in the steatosis score over the CD values, in agreement with previous studies using either the same dietary protocol [8–10, 18, 34] or alternate procedures [11–13]. Under these conditions, total SFAs were increased by HFD, whereas total PUFAs including n-6 and n-3 LCPUFAs were reduced, changes that may contribute to fatty liver development. HFD-induced liver steatosis score was correlated with the significant enhancements in the contents of liver total fat and TGs, changes that were elicited under conditions of a comparable dietary intake but increased energy intake, and negatively associated with oxidative stress development. The latter phenomenon was characterized by significant alterations in the GSH status lowering the antioxidant potential of the liver with the consequent lipid peroxidation and protein oxidation responses, shown

Table 2: General and hepatic parameters in control mice (CD) and high-fat diet (HFD) animals subjected to EPA, HT, and EPA plus HT. Values are presented as means 10 ± 8 mice per experimental group. Significant differences between the groups are indicated by the letter identifying each group ($p < 0.05$), by two-way ANOVA and Bonferroni posttest

	Control diet (CD)				High-fat diet (HFD)			
	Saline	EPA	HT	EPA + HT	Saline	EPA	HT	EPA + HT
General parameters	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
Initial body weight (g)	14.71 ± 1.02	14.56 ± 1.11	14.38 ± 0.99	14.51 ± 0.91	14.26 ± 0.74	14.77 ± 1.02	14.31 ± 0.86	14.56 ± 0.97
Final body weight (g)	26.67 ± 3.51^{e,g}	25.47 ± 4.24^{e,g}	28.79 ± 1.76 ^{e,g}	26.96 ± 4.44	37.15 ± 5.01^{a,b,c,d}	33.13 ± 3.56^{c}	36.02 ± 3.15^{a,b,c,d}	30.00 ± 6.60^{e,g}
Total body weight increment (g)	11.95 ± 3.74^{e}	10.91 ± 4.44^{g}	14.42 ± 1.88^{h}	12.45 ± 4.02	22.89 ± 5.40^{a,b,c,d}	18.36 ± 2.99^{c}	21.71 ± 2.91^{a,b,c,d}	15.44 ± 6.11^{e,g}
Liver weight (g)	1.25 ± 0.23^{h}	1.04 ± 0.18	1.17 ± 0.14	1.07 ± 0.19	1.12 ± 0.11	1.19 ± 0.23^{h}	1.23 ± 0.16^{h}	1.03 ± 0.77^{a,fg}
Food and energy intake								
Dietary intake (g/day)	5.08 ± 0.44	5.24 ± 0.21	5.18 ± 0.32	4.99 ± 0.69	5.29 ± 0.37	5.18 ± 0.30	5.15 ± 0.41	5.24 ± 0.38
Energy intake (kcal/day)	19.59 ± 1.28^{e,f,g,h}	20.18 ± 1.42^{e,f,g,h}	19.95 ± 1.87^{e,f,g,h}	19.20 ± 1.36^{e,f,g,h}	27.74 ± 2.31^{a,b,c,d}	27.15 ± 2.24^{a,b,c,d}	26.99 ± 2.19^{a,b,c,d}	27.5 ± 2.04^{a,b,c,d}
Liver parameters								
AST (U/L)	152.67 ± 15.02	143.00 ± 15.11	142.67 ± 12.31	145.33 ± 11.44	151.83 ± 16.65	150.33 ± 9.38	152.50 ± 9.07	151.00 ± 14.87
ALT (U/L)	62.50 ± 6.63	64.00 ± 10.41	63.33 ± 7.63	59.33 ± 7.63	70.83 ± 6.69	69.83 ± 7.67	69.33 ± 8.67	64.67 ± 10.48
Hepatic fat g/100 g liver)	4.98 ± 0.88^{e,f,g,h}	3.81 ± 0.68^{e,f,g,h}	4.91 ± 1.31^{e,f,g,h}	3.13 ± 0.57^{e,f,g,h}	14.59 ± 1.69^{a,b,c,d,h}	10.12 ± 1.06^{a,b,c,d,h}	11.26 ± 1.11^{a,b,c,d,h}	8.49 ± 1.05^{a,b,c,d,e,f,g}
Hepatic TAG (mg/g liver)	33.05 ± 7.19^{e,f,g,h}	30.38 ± 5.52^{e,f,g,h}	29.48 ± 3.95^{e,f,g,h}	27.74 ± 5.09^{e,f,g,h}	102.47 ± 9.47^{a,b,c,d,h}	56.70 ± 5.74^{a,b,c,d,}	77.89 ± 7.21^{a,b,c,d,h}	49.94 ± 1.45^{a,b,c,d,e,f,g}

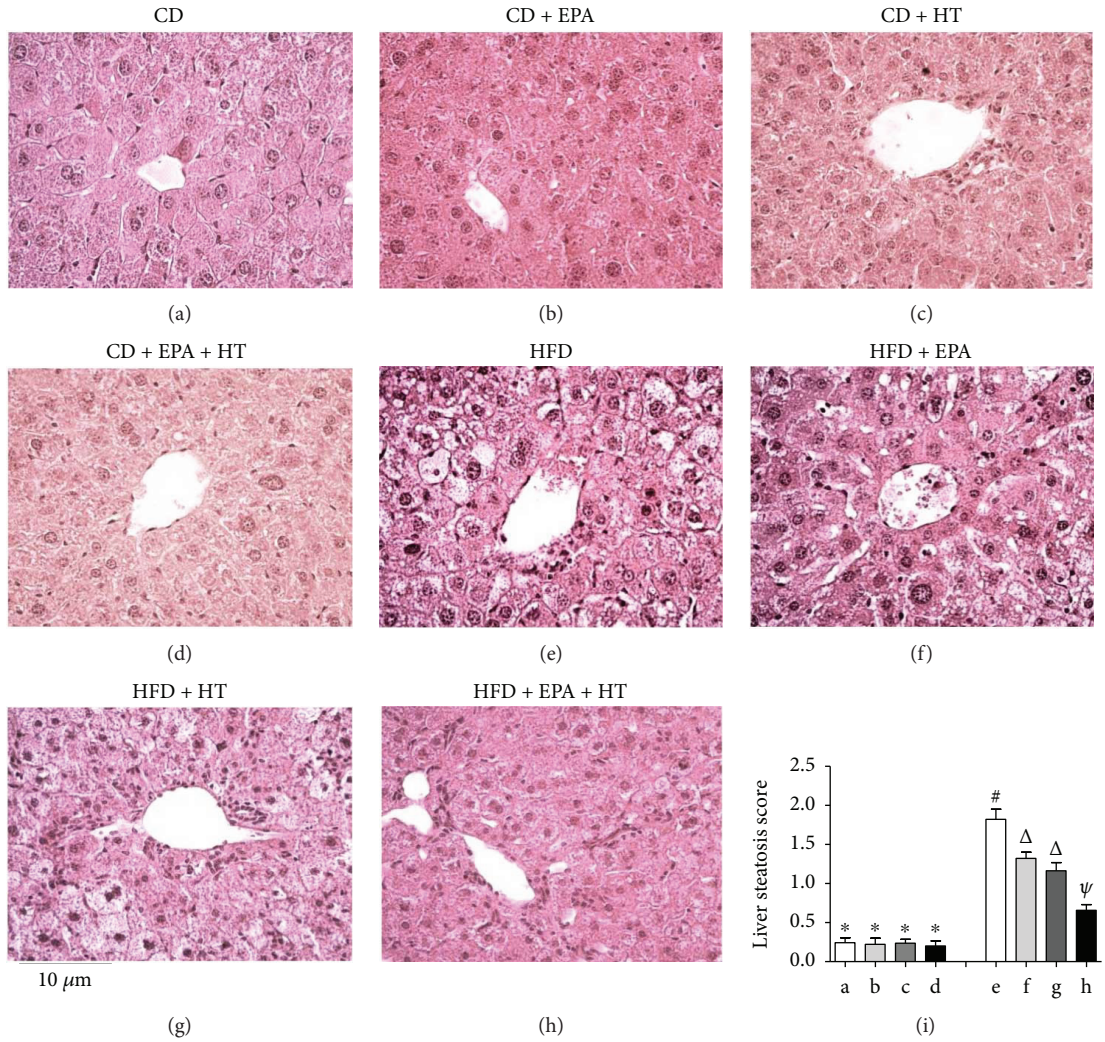

Figure 1: Liver histological assessment in mice subjected to control diet (CD) and high-fat diet (HFD) without and with eicosapentaenoic acid (EPA), hydroxytyrosol (HT), and EPA + HT supplementation. Representative liver sections from animals given (a) CD, (b) CD + EPA, (c) CD + HT, (d) CD + EPA + HT, (e) HFD, (f) HFD + EPA, (g) HFD + HT, and (h) HFD + EPA + HT (haematoxylin-eosin liver sections from 8 animals per experimental group; original magnification ×40). (i) Liver steatosis scores [24] expressed as means ± SEM for 8 animals per experimental group. Groups sharing the same symbol are not significantly different among them according to two-way ANOVA and the Bonferroni posttest ($p < 0.05$). *, $^{\#}$, $^{\Delta}$ and $^{\Psi}$ indicate the significant differences between the experimental groups.

by the elevations in the levels of hepatic TBARs, F-8 isoprostanes, and protein carbonyls, which were inversely correlated with those of GSH. Enhancement of the hepatic oxidative stress status by HFD may be contributed by the drastic decrease in the operation of Nrf2 shown by the lowered Nrf2 DNA-binding capacity and Nrf2 mRNA expression compared to CD, leading to reduced mRNA expression and/or activity of the antioxidant enzymes controlled by Nrf2. Loss of liver Nrf2 activity under sustained oxidative stress conditions triggered by HFD may be related to (i) the prevailing high free-radical level promoting protein oxidation (Figure 2(h)) and inactivation and/or (ii) the increase in the expression of the Nrf2 inhibitor Kelch-like ECH-associated protein 1 (Keap1) which supports continuous proteasomal Nrf2 degradation [36], a mechanism that remains to be evaluated after HFD feeding.

EPA is one of the most important n-3 LCPUFAs due to its roles as (i) DHA precursor, (ii) regulator of hepatic lipid metabolism, which is accomplished by activation of peroxisome proliferator-activated receptor α (PPAR-α) favouring FA oxidation and downregulation of sterol regulatory element-binding protein 1c (SREBP-1c) reducing de novo lipogenesis [23], and (iii) inhibitor of nuclear factor-κB (NF-κB) limiting inflammatory processes, actions that are shared by DHA [37]. At the dosage of 50 mg/kg/day, EPA supplementation significantly increased the hepatic content of EPA as well as that of DHA, both in mice subjected to CD or HFD. Under these conditions, however, HFD-induced liver steatosis was reduced by only 32% by EPA, without alterations in oxidative stress-related parameters and mild recovery of Nrf2 functioning. This is probably related to transformations that EPA could undergo in the

Table 3: Total hepatic fatty acid profile in control mice (CD) and high-fat diet (HFD) animals subjected to EPA, HT, and EPA plus HT. Values are presented as mean % of fatty acid methyl esters (FAME) 10 ± 8 mice per experimental group. Significant differences between the groups are indicated by the letter identifying each group ($p < 0.05$), by two-way ANOVA and Bonferroni posttest. Saturated fatty acids (SFA) are 10 : 0, 12 : 0, 14 : 0, 16 : 0, 18 : 0, 20 : 0, 22 : 0, and 24 : 0. Monounsaturated control mice (CD) and high-fat diet (HFD) animals subjected to EPA, HT, and EPA plus HT d fatty acids (MUFA) are 14 : 1, 16 : 1, 18 : 1, 20 : 1 n-9, and 22 : 1 n-9 to 24 : 1. Polyunsaturated fatty acids (PUFAs) are 18 : 2 n-6 (linoleic acid (LA)), 18 : 3 n-6, 18 : 3 n-3 (α-linolenic acid (ALA)), 20 : 2 n-6, 20 : 3 n-6, 20 : 3 n-3, 20 : 4 n-6 (arachidonic acid (ARA)), 20 : 5 n-3, 22 : 5 n-3 (docosapentaenoic acid (DPA)), and 22 : 6 n-3. Long-chain polyunsaturated fatty acids (LCPUFA) are 20 : 2 n-6: 20 : 3 n-6: 20 : 4 n-6, 20 : 5 n-3 (eicosapentaenoic acid (EPA)), 22: 5 n-3, and 22 : 6 n-3 (docosahexaenoic acid (DHA)).

	Fatty acid composition (g per 100 g FAME)							
	Control diet (CD)				High-fat diet (HFD)			
	Saline	EPA	HT	EPA + HT	Saline	EPA	HT	EPA + HT
Most relevant fatty acids	(a)	(b)	(c)	(d)	(e)	(g)	(f)	(h)
16 : 00	34.6 ± 3.6^{e}	33.8 ± 3.5^{e}	31.2 ± 3.8^{e}	34.7 ± 3.3^{e}	$47.9 \pm 4.1^{a,b,c,d,f,g,h}$	38.7 ± 2.9^{e}	36.9 ± 3.1^{e}	37.2 ± 3.2^{e}
18 : 01	25.3 ± 2.5	24.3 ± 2.7	26.5 ± 2.2	23.9 ± 2.6	26.8 ± 2.8	23.5 ± 2.3	24.4 ± 2.9	24.7 ± 2.6
18 : 2, n-6 (AL)	10.4 ± 1.3	9.98 ± 1.2	10.2 ± 1.1	9.75 ± 1.1	10.9 ± 1.3	9.71 ± 1.0	10.5 ± 1.4	10.1 ± 1.2
18 : 3, n-3 (ALA)	1.15 ± 0.1^{e}	1.14 ± 0.06^{e}	1.10 ± 0.1^{e}	1.12 ± 0.05^{e}	$0.81 \pm 0.03^{a,b,c,d,f,g,h}$	1.09 ± 0.1^{e}	1.11 ± 0.1^{e}	1.07 ± 0.2^{e}
20 : 4, n-6 (AA)	9.93 ± 1.5^{e}	8.64 ± 1.5^{e}	10.5 ± 1.7^{e}	8.98 ± 1.6^{e}	$6.61 \pm 0.6^{a,b,f,h}$	7.85 ± 1.1	7.84 ± 1.4	8.56 ± 1.2^{e}
20 : 5, n-3 (EPA)	$1.03 \pm 0.1^{b,d,e,g,h}$	$3.98 \pm 0.5^{a,c,e,f,g,h}$	$1.12 \pm 0.1^{b,d,e,g,h}$	$4.16 \pm 0.6^{a,c,e,f,g,h}$	$0.34 \pm 0.05^{a,b,c,d,f,g,h}$	$2.14 \pm 0.2^{a,b,c,d,e,f}$	$0.99 \pm 0.1^{c,d,e,g,h}$	$2.36 \pm 0.2^{a,b,c,d,e,f}$
22 : 6, n-3 (DHA)	$3.96 \pm 0.3^{b,d,e}$	$6.94 \pm 0.5^{c,d,e}$	$4.12 \pm 0.2^{b,d,e}$	$7.12 \pm 0.7^{b,d,e}$	$1.97 \pm 0.1^{a,b,c,d,f,g,h}$	$3.85 \pm 0.4^{b,d,e}$	$3.16 \pm 0.3^{c,d,e}$	$4.03 \pm 0.4^{b,d,e}$
Total SFA	37.8 ± 3.4^{e}	38.9 ± 3.9^{e}	35.4 ± 3.3^{e}	37.4 ± 3.4^{e}	$51.2 \pm 4.9^{a,b,c,d,f,g,h}$	38.6 ± 3.5^{e}	30.4 ± 3.2^{e}	39.9 ± 3.4^{e}
Total MUFA	29.5 ± 2.2	27.9 ± 2.8	31.3 ± 2.9	28.8 ± 2.6	27.7 ± 2.5	32.9 ± 3.0	30.5 ± 3.1	30.9 ± 3.1
Total PUFA	32.7 ± 2.9^{e}	33.2 ± 3.2^{e}	33.3 ± 3.5^{e}	33.8 ± 3.5^{e}	$21.1 \pm 1.9^{a,b,c,d,f,g,h}$	28.5 ± 2.5^{e}	29.1 ± 2.2^{e}	29.2 ± 2.5^{e}
Total LCPUFA	$15.6 \pm 1.2^{b,d,e,f}$	$19.8 \pm 1.6^{a,c,e,f,g,h}$	$16.2 \pm 1.4^{b,d,e,f}$	$20.9 \pm 2.1^{a,c,e,f,g,h}$	$9.13 \pm 0.5^{a,b,c,d,f,g,h}$	$14.4 \pm 1.1^{b,d,e,f}$	$12.1 \pm 0.7^{a,b,c,d,e,g,h}$	$15.3 \pm 1.3^{b,d,e,f}$
Total n-6 LCPUFA	$10.4 \pm 0.8^{b,e,f,g,h}$	$8.80 \pm 0.7^{a,c,e}$	$10.8 \pm 1.0^{b,e,f,g,h}$	$9.10 \pm 0.9^{e,f,}$	$6.71 \pm 0.5^{a,b,c,d,g,h}$	$8.08 \pm 0.6^{a,c,e}$	$7.90 \pm 0.6^{a,c,d,e}$	$8.78 \pm 0.7^{a,c,e}$
Total n-3 LCPUFA	$5.12 \pm 0.2^{b,d,e,f,g,h}$	$11.0 \pm 0.4^{a,c,e,f,g,h}$	$5.33 \pm 0.2^{b,d,e,f,g,h}$	$11.8 \pm 0.4^{a,c,e,f,g,h}$	$2.42 \pm 0.2^{a,b,c,d,f,g,h}$	$6.32 \pm 0.5^{a,b,c,d,e,f}$	$4.20 \pm 0.3^{a,b,c,d,e,g,h}$	$6.52 \pm 0.5^{a,b,c,d,e,f}$
n-6/n-3 LCPUFA ratio	$2.03 \pm 0.3^{b,d,g,h}$	$0.80 \pm 0.05^{a,c,e,f,g,h}$	$2.03 \pm 0.2^{b,d,g,h}$	$0.77 \pm 0.04^{a,c,e,f,g,h}$	$2.77 \pm 0.3^{b,d,g,h}$	$1.28 \pm 0.1^{a,b,c,d,e,f}$	$1.88 \pm 0.2^{c,d,f,g,h}$	$1.35 \pm 0.1^{a,b,c,d,e,f}$

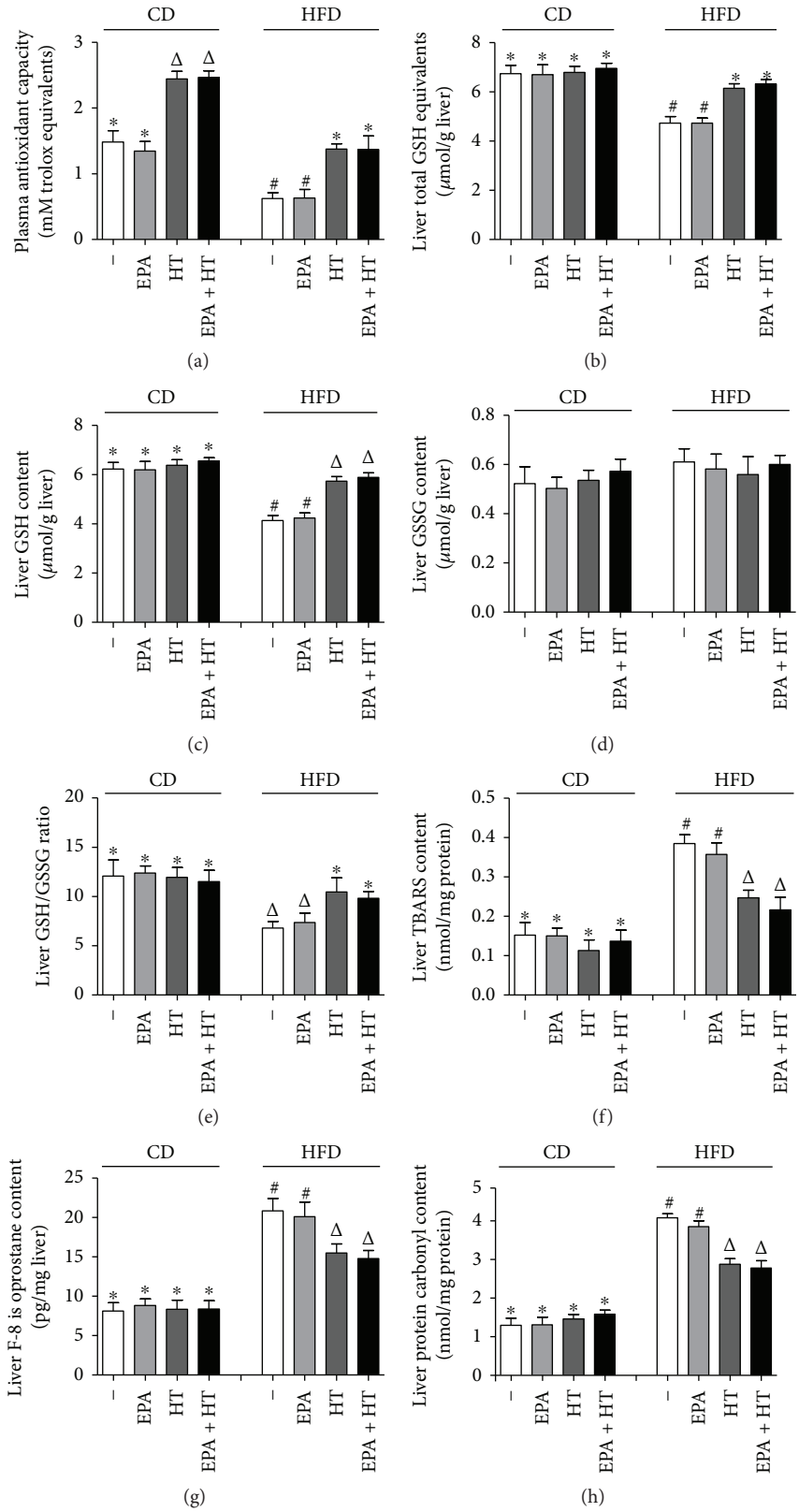

Figure 2: Liver oxidative stress-related parameters in mice subjected to control diet (CD) and high-fat diet (HFD) without (–) and with eicosapentaenoic acid (EPA), hydroxytyrosol (HT), and EPA + HT supplementation. Antioxidant capacity of plasma (a) and contents of total GSH equivalents (b), GSH (c), GSSG (d), GSH/GSSG ratios (e), TBARs (f), F-8 isoprostanes (g), and protein carbonyls (h). Values are means ± SEM for 8 animals per experimental group. Groups sharing the same symbol are not significantly different among them according to two-way ANOVA and the Bonferroni posttest ($p < 0.05$). GSH: reduced glutathione; GSSG: glutathione disulphide; TBARs: thiobarbituric acid reactants. *, $^{\#}$ and $^{\Delta}$ indicate the significant differences between the experimental groups.

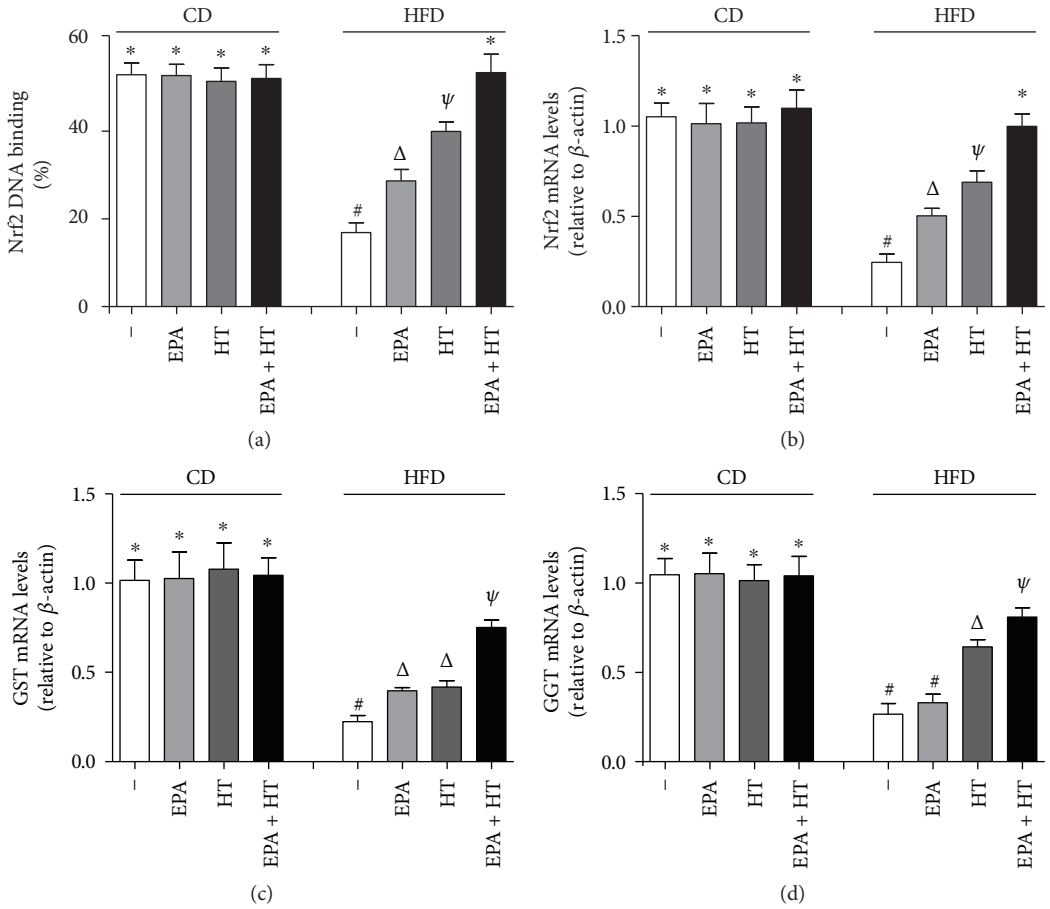

Figure 3: Liver Nrf2 DNA binding (a) and mRNA expression of Nrf2 (b), GST (c), and GGT (d) in mice subjected to control diet (CD) and high-fat diet (HFD) without (–) and with eicosapentaenoic acid (EPA), hydroxytyrosol (HT), and EPA + HT supplementation. Values are means ± SEM for 8 animals per experimental group. Groups sharing the same symbol are not significantly different among them according to two-way ANOVA and the Bonferroni posttest ($p < 0.05$). Nrf2: nuclear factor erythroid 2-related factor 2; GST: glutathione-S-transferase; GGT: γ-glutamyl transpeptidase. $^{*, \#, \Delta}$ and $^{\Psi}$ indicate the significant differences between the experimental groups.

liver, producing metabolic products such as DHA, E-resolvins [38], epoxy-derivatives [39], and/or J_3 isoprostanes [40], thus limiting the concentration needed for cell signaling.

HT is a polyphenol present in EVOO that has a powerful antioxidant action [41] regulating different signaling pathways associated with the intracellular redox state [42]. This is in agreement with the significant increases in the antioxidant capacity of plasma by HT observed in CD and HFD groups, an effect that is correlated with (i) the total regain towards CD values of the glutathione status (total GSH equivalents and GSH/GSSG ratio), with 77% recovery of GSH levels that were decreased by HFD, and (ii) the partial recovery (42% to 59%) of parameters related to free radical-induced lipid peroxidation (TBARs and F-8 isoprostanes) and protein carbonylation that were enhanced by HFD, indexes negatively correlated with GSH levels. Under these conditions, reduction in HFD-induced liver oxidative stress status by HT is associated with enhancement (55%) in the mRNA expression of Nrf2, with 64% regain in its DNA-binding capacity, responses that may contribute to an increase in the antioxidant potential of the liver [24]. This contention is supported by the total recovery of the activity of the enzymes controlled by Nrf2 that were decreased by HFD, namely, CAT, GPX, GR, NQO1, GST, and GGT, with the partial regain in that of SOD observed after HT supplementation. An additional effect elicited by HT favouring the antioxidant potential of the liver under HFD-induced oxidative stress conditions is the enhancement in the content of total LCPUFAs including that of total n-3 LCPUFAs, possibly by decreasing their oxidative deterioration [43]. This effect of HT is likely to promote PPAR-α activation increasing the FA oxidation capacity of the liver with concomitant SREBP-1c downregulation reducing de novo lipogenesis [43], thus in agreement with the 42% reduction found in HFD-induced steatosis score that correlated with the decreases in the fat and TG contents.

HFD-fed mice subjected to combined supplementation with EPA + HT showed a significant greater and additive antisteatotic effect (74% reduction) compared to that elicited by the separate HT (42% diminution) and EPA (32% decrease) treatments, with hepatic fat and TG contents being significantly lower than those induced by HFD alone. However, the effects of EPA + HT along with HFD feeding were either (i) similar to those achieved by HT alone, namely, total

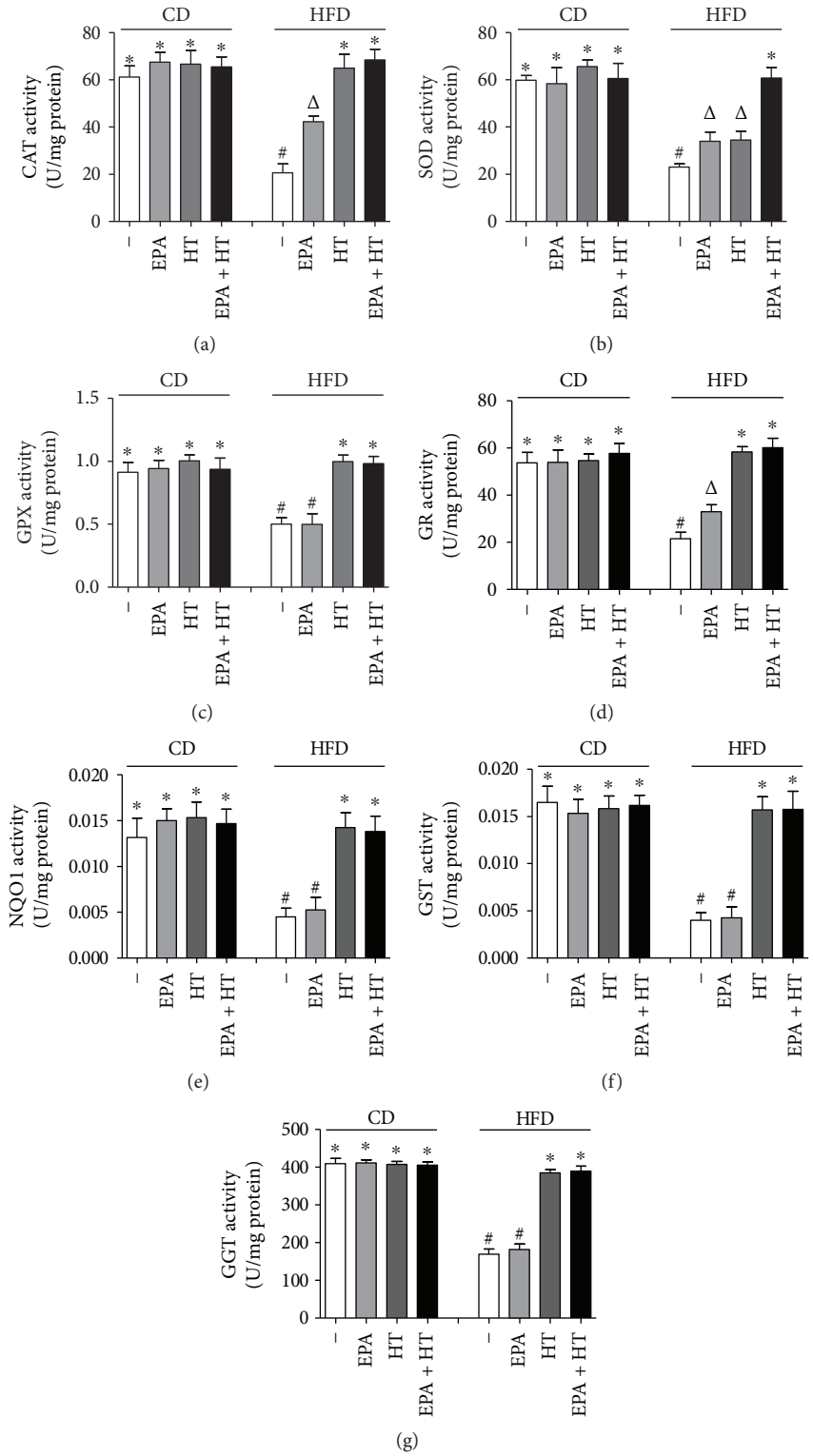

Figure 4: Liver activities of CAT (a), SOD (b), GPX (c), GR (d), NQO1 (e), GST (f), and GGT (g) in mice subjected to control diet (CD) and high-fat diet (HFD) without (–) and with eicosapentaenoic acid (EPA), hydroxytyrosol (HT), and EPA + HT supplementation. Values are means ± SEM for 8 animals per experimental group. Groups sharing the same symbol are not significantly different among them according to two-way ANOVA and the Bonferroni posttest ($p < 0.05$). CAT: catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase; GR: glutathione reductase; NQO1: NADPH-quinone oxidoreductase 1; GST: glutathione-S-transferase; GGT: γ-glutamyl transpeptidase. *, $^{\#}$ and $^{\Delta}$ indicate the significant differences between the experimental groups.

regain in n-3 LCPUFA levels, plasma antioxidant capacity, glutathione status, and CAT, GPX, GE, NQO1, GST, and GGT activities, or (ii) showed partial to total recovery transition of Nrf2 functioning and SOD activity, when values for HT alone and EPA + HT are compared. These data indicate that the antisteatotic and antioxidant effects produced by combined EPA + HT supplementation in HFD feeding are mainly due to the HT component, with EPA having a limited contribution at the dosages employed. This conclusion points to antioxidation as a major mechanism underlying attenuation of HFD-induced steatosis by natural products. In agreement with this proposal, mitigation of HFD-induced liver deleterious effects is also attained by both (i) an EVOO type having the highest antioxidant content (859 mg polyphenols/kg; antioxidant capacity of 7156 μmol eq. Trolox/L) compared to those having polyphenol levels of 116 or 407 mg/kg and antioxidant capacities of 3378 or 4841 μmol eq. Trolox/L, respectively [34], and (ii) a regular rosa mosqueta oil (RMO) containing α- and γ-tocopherols compared to a RMO type devoid of tocopherols [44], findings that establish a threshold for the content of antioxidant components of natural products to achieve beneficial effects. In addition to the antioxidant effect of natural products in fatty liver, reduction of HFD-induced liver steatosis by EPA, HT, and EPA + HT supplementation may result from a direct activation of hepatic lipases such as patatin-like phospholipase domain-containing protein 3 (PNPLA3) which hydrolyses acylglycerols including TGs [45], an aspect that remains to be elucidated in the present model.

5. Conclusion

Data presented show that combined EPA + HT supplementation in mice significantly attenuates HFD-induced hepatic steatosis, an effect that mainly relies on HT with a limited contribution of EPA. Under these conditions, the antisteatotic effect of HT is associated with the enhancement in the antioxidant potential of the liver, which partially recovers n-3 LCPUFA levels thus favouring FA oxidation through PPAR-α upregulation [35], while limiting de novo lipogenesis via SREBP-1c downregulation [43]. Additional mechanisms of HT action include (i) prevention of HFD-induced reduction in the desaturation capacity of the liver, with recovery in the activity of Δ5 and Δ6 desaturases promoting n-3 LCPUFA repletion [43]; (ii) reduction in the oxidative stress-dependent liver protein carbonylation triggered by HFD, thus decreasing the lipogenic response associated with the endoplasmic reticulum stress (ERS) developed [46, 47], an effect that may be contributed by normalization of SFA levels increased by HFD, FAs that also trigger ERS [48]; and (iii) amelioration of drug-induced cardiotoxicity involving oxidative stress and mitochondrial dysfunction [49], which suggest enhancement in electron transport chain capacity and FA oxidation potential [21]. These considerations and the previous suggestions concerning the adequacy of combination therapies [14–20] reinforce the impact of dietary interventions including safety components addressing oxidative stress as a central mechanism underlying liver steatosis in obesity in particular and other noncommunicable diseases in general.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Conflicts of Interest

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

Authors' Contributions

Rodrigo Valenzuela and Luis A. Videla conceived and designed the experiments. Rodrigo Valenzuela, Francisca Echeverría, Andrés Bustamante, Daniela Álvarez, Macarena Ortiz, Sandra A. Soto-Alarcon, Patricio Muñoz, and Alicia Corbari performed the experiments. Rodrigo Valenzuela, Francisca Echeverría, Andrés Bustamante, and Luis A. Videla analysed the data. Rodrigo Valenzuela, Francisca Echeverría, Andrés Bustamante, and Luis A. Videla contributed reagents/materials/analysis tools. Rodrigo Valenzuela and Luis A. Videla wrote the paper. All authors read and approved the final manuscript.

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Research Article

The Ability of Different Ketohexoses to Alter Apo-A-I Structure and Function In Vitro and to Induce Hepatosteatosis, Oxidative Stress, and Impaired Plasma Lipid Profile in Hyperlipidemic Zebrafish

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In the current study, we have tested the nonenzymatic glycation activities of ketohexoses, such as tagatose and psicose. Although tagatose-treated apoA-I (t-A-I) and psicose-treated apoA-I (p-A-I) exerted more inhibitory activity you cupric ion-mediated low-density lipoprotein (LDL) oxidation and oxidized LDL (oxLDL) phagocytosis into macrophage than fructose-treated apoA-I (f-A-I). In the lipid-free state, t-A-I and f-A-I showed more multimerized band without crosslinking. Since t-A-I lost its phospholipid binding ability, the rHDL formation was not as successful as f-A-I. However, injecting t-A-I showed more antioxidant activities in zebrafish embryo under the presence of oxLDL. Three weeks of consumption of fructose (50% of wt in Tetrabit/4% cholesterol) showed a 14% elevation of serum triacylglycerol (TG), while tagatose-administered group showed 30% reduction in serum TG compared to high cholesterol control. Fructose-fed group showed the biggest area of Oil Red O staining with the intensity as strong as the HCD control. However, tagatose-consumed group showed much lesser Oil Red O-stained area with the reduction of lipid accumulation. In conclusion, although tagatose treatment caused modification of apoA-I, the functional loss was not as much severe as the fructose treatment in macrophage cell model, zebrafish embryo, and hypercholesterolemic zebrafish model.

1. Introduction

It has been widely accepted that glycation is a major process that degenerates protein function and structure, which is a direct outcome of chronic metabolic diseases, such as diabetes [1], atherosclerosis [2], and aging [3]. Fructose treatment can cause glycation up to 10 times higher with the production of advanced glycated end (AGE) products than that of glucose, and higher fructose consumption promotes triglyceride synthesis [4, 5]. It has been known that major target of glycation via Maillard reaction is serum hemoglobin (Hgb), glycated Hgb level has been used as a diagnostic marker of diabetes [6]. Previous studies have concluded that glycation could occurs in high-density lipoproteins (HDL) and apolipoproteins in blood [7, 8].

High-density lipoprotein-cholesterol (HDL-C) is inversely associated with the incidence of cardiovascular disease [9] and is directly related to longevity [10]. HDL has antioxidant and anti-inflammatory potential [11]. Apolipoprotein A-I is the principal protein of HDL, exerting to suppress atherosclerosis

[12]. Our study group have reported that fructose-mediated apoA-I glycation results in the acute loss of the beneficial functions of apoA-I and HDL with respect to its antisenescence, antioxidant, and antiatherosclerosis activities [8, 13, 14] and that could be suggested due to the oligomerization (crosslinking of monomeric apoA-I to form dimers, trimers, tetramers, etc.). It is a process of multimerization which can contribute to amyloid production and impairment of lipoprotein functionality. The functionality and structural modifications coupled with increased protein degradation lead to severe health disorders [2, 3].

As fructose is a ketohexose family, there might be a possibility that other ketohexose can cause similar glycation process and physiological effects. Among ketohexoses, D-tagatose is the epimer of D-fructose differing only in the positioning of hydroxyl group on the 4th carbon. Tagatose has grabbed attention as a potent candidate of antidiabetic agents [15] and has been established as the safe sugar (GRAS) by World Health Organization (WHO) for use in food and beverages. It has been reported that dietary supplementation of tagatose in type 2 diabetes leads to weight loss and raises the HDL-C levels [16]. Unfortunately, this study was not placebo-controlled.

Not only D-tagatose, D-psicose, which is a C-3 epimer of D-fructose, has lesser sweetness than sucrose with no calories, rather exhibits hypoglycemic, hypolipidemic, and antioxidant activities [17–20]. The supplementation of D-psicose in the diet of male rats suppressed the hepatic fatty acid synthase and glucose 6-phosphate dehydrogenase enzymes and thereby reduces adipose tissue weight [19]. These properties make it a more promising agent for ameliorating diabetes and its related conditions [21]. However, the explanation for the beneficial effect of tagatose and psicose has not yet been deciphered, especially in serum proteins regarding glycation and its physiological mechanism. Since there has been no report about the potential effect of tagatose and psicose in lipoprotein metabolism and there is a possibility that both of them can affect serum protein via nonenzymatic glycation process, hence we tested the putative effect of tagatose treatment as well as fructose and psicose.

2. Materials and Methods

2.1. Materials. Cholesterol (# C-3045) was purchased from Sigma (St. Louis, MO, USA). D-tagatose (FW 180.16, cat #T1501), D-fructose (FW180.16, cat # F0060), and D-psicose (FW 180.16, cat # P1699) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Purification of apoA-I. Human plasma was used to purify ApoA-I using several techniques such as ultracentrifugation, column chromatography, and organic solvent extraction method described by Brewer et al. [22]. The purified apoA-I was lyophilized at −80°C until use.

2.3. Treatment of Ketohexose to apoA-I. Lipid-free apoA-I (10 mg/mL) in its processed state was kept in 200 mM potassium phosphate/0.02% sodium azide buffer (pH 7.4) supplemented with each of the ketohexose (final 250 mM) for up to 90 hrs in an incubator with 5% CO_2 at 37°C. The measure of advanced glycation was performed spectrophotometrically using fluorescence at 440 nm (emission) and 370 nm (excitation), the procedure was however slightly modified [23].

2.4. Synthesis of Reconstituted HDL and Analysis. Table 1 indicates the synthesis and characterization of rHDL containing each ketohexose. Discoidal rHDL was prepared by the sodium cholate dialysis method [24] using initial molar ratios of palmitoyl oleoyl phosphatidylcholine (POPC) : cholesterol : apoA-I : sodium cholate of 95 : 5 : 1 : 150. As the rHDL particles employed in the procedure were pure enough and exhibited extreme uniformity, further processing was deemed unnecessary. PAGGE or native polyacrylamide gradient gel electrophoresis was carried out in order to determine the size of the rHDL particles and to compare them with standard globular proteins (Cat# 17-0445-01 Amersham Pharmacia, Uppsala, Sweden). The Pharmacia Phast system was obtained from GE Healthcare, Uppsala, Sweden. Gel Doc® XR (Bio-Rad, Hercules, CA, USA) complemented with Quantity One software, version 4.5.2 was employed to compare the relative movement of particles, while its content of protein was measured by Lowry method but improvised by as modified Markwell et al. [25] with standard as BSA.

2.5. Circular Dichroism and Fluorospectroscopy. The assaying technique of circular dichroism (CD) spectroscopy (J-715 Spectropolarimeter Jasco, Tokyo, Japan) was used to unravel the quantity of alpha-helices prevalent in the free and bound protein-lipid interaction states. The circular light absorption pattern was obtained from 250–190 nm at 25°C, 0.1 cm was path length, 1.0 nm was bandwidth, speed was 50 nm/min, and a 4 sec response time. The purified protein specimens were made fructose free by dialysis against TBS, self-ligation was averted by dilution of the lipid-free apolipoproteins [26] to 0.07 mg/mL and to 0.1 mg/mL of the lipid-bound apolipoprotein. Four scans were obtained and averaged.

The analysis of molar ellipticity at 222 nm revealed the content of alpha-helices. Circular dichroism spectra with ketohexose-treated apoA-I in lipid-free and lipid-bound are represented in Supplementary Figure 2.

The perusal of the wavelengths of fluorescence (WMF) particularly of the Trp residues was done by a LS55 spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA) using WinLab software package 4.00 (Perkin-Elmer). The excitation wavelength was chosen to be 295 nm in order to steer clear of the interference from tyrosine fluorescence. Emissions were categorically checked from 305–400 nm.

2.6. Purification of Low-Density Lipoprotein (LDL) and Its Oxidation. LDL ($1.019 < d < 1.063$) was obtained from human plasma and purified by ultracentrifugation, the density was adjusted by adding NaCl, followed by centrifugation at 100,000g for 22 hours at 10°C temperature (Himac CP-90α Hitachi, Tokyo, Japan). The oxidized LDL (oxLDL) was procured postincubation with $CuSO_4$ (final concentration, 10 μM) for 4 hr at 37°C. Subsequently, it was filtered (0.2 μm) and investigated by using a thiobarbituric acid

Table 1: Synthesis and characterization of rHDL containing each ketohexose (fructose, tagatose, and psicose).

rHDL	Molar composition	WMF (nm)	Size[a] (Å)	Number of apoA-I/particle[b]	α-Helicity (%)
Native-A-I	95 : 5 : 1	337 ± 0.1 (344 ± 0.5)[c]	109–94		54.4 (32.6)[c]
H_2O-A-I	95 : 5 : 1	335 ± 0.5 (344 ± 0.5)	109–94	2, 3, 4	82.6 (51.4)
f-A-I	95 : 5 : 1	342 ± 1.6 (344 ± 0.5)	109–94	2, 3, 4, 5	38.4 (38)
t-A-I	95 : 5 : 1	335 ± 1.2 (344 ± 0.5)	93	2, 3, 4, 5	34.7 (15.4)
p-A-I	95 : 5 : 1	335 ± 0.4 (344 ± 0.5)	93	2, 3, 4, 5	35 (25.4)

[a]Determined from 8% to 25% native-gradient gel electrophoresis with densitometric scanning analysis. [b]Determined from BS_3-crosslinking and 8–25% SDS–PAGGE. [c]The numbers in the parentheses indicate the proteins in the lipid-free state. H_2O-A-I: H_2O-treated apoA-I; f-A-I: fructose-treated apoA-I; t-A-I: tagatose-treated apoA-I; p-A-I: psicose-treated apoA-I.

reacting substances (TBARS) assay to establish the degree of oxidation [27].

2.7. Cell Culture. The human monocyte cell line, THP-1, was acquired from the (ATCC, #TIB-202™; Manassas, VA, USA) and sustained in RPMI1640 (Hyclone, Logan, Utah) with 10% fetal bovine serum (FBS) supplemented. The cell line below 20 passages were used and incubated in phorbol 12-myristate 13-acetate (PMA; final 150 nM) supplemented medium in 24-well plates for 48 hours at 37°C in a humidified incubator (5% CO_2) to induce macrophage differentiation.

2.8. LDL-Phagocytosis Assay. The differentiated and the adhered macrophages were coincubated with 400 μL of fresh RPMI1640 medium supplemented with 1% FBS, 50 μL of oxLDL [1 mg of protein/mL in phosphate-buffered saline (PBS)], and 50 μL of each rHDL (1 mg/mL) for 48 hr at 37°C in a humidified incubator to test antiatherosclerotic activity [14]. Subsequently, the cells were washed thrice with PBS and then fixed in 4% paraformaldehyde for 10 minutes. Then the fixed specimens were further rinsed with 100% polypropylene glycol, then stained with Oil Red O staining solution (0.67%), and finally washed with distilled water. THP-1 macrophage-derived foam cells were then imaged and photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at 600x magnification.

2.9. Western Blot. After 48 hr incubation, the harvested cell was lysed by treatment of RIPA buffer (Radioimmunoprecipitation assay buffer, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)). Cell lysates were analyzed by western blot analysis using antihuman apoA-I antibody (Ab7613; Abcam, Cambridge, UK) and GAPDH (Ab8229, Abcam), antitumor necrosis factor (TNF)-α (SC52746, Santa Cruz, CA, USA). Protein content from each lysate was measured using Bradford assay (Bio-Rad, Hercules, CA, USA) before loading equal amounts of protein (25 μg/lane) into 13% SDS-PAGE gels.

2.10. Zebrafish. Wild-type zebrafish and their embryos were sustained as per the standard protocols [28] permitted by the Committee of Animal Care and Use of Yeungnam University (Gyeongsan, Korea). The zebrafish, larvae, and embryos were maintained in a system cage and 6-well plates at 28°C during exposure to the 14 : 10 hrs light : dark cycle.

2.11. Microinjection of Zebrafish Embryos. The Pneumatic PicoPump of the make PV820; World Precision Instruments, Sarasota, FL, USA was used for microinjecting the day one of fertilized embryo or 1 day postfertilization (dpf). This PV280 also had a magnetic manipulator (MM33; Kantec, Bensenville, IL, USA) fitted with a pulled microcapillary pipette-using device (PC-10; Narishige, Tokyo, Japan). The oxLDL (13 ng of protein) and ketohexose-treated apoA-I (50 ng of protein) were coinjected in total 100 nL volume as our previous report [14]. Following injection, live embryos were observed under a stereomicroscope (Motic SMZ 168; Hong Kong) and photographed using a Motic cam2300 CCD camera.

2.12. Imaging of Reactive Oxygen Species (ROS). After treatment with oxLDL in the presence of apoA-I, the increased ROS measures the extent of general oxidative stress were seen using dihydroethidium (DHE, cat # 37291; Sigma, St. Louis, MO) [29]. The image of the embryo stage was procured through fluorescence observation (Ex = 588 nm and Em = 605 nm) using a Nikon Eclipse TE2000 microscope (Tokyo, Japan). The measurement of fluorescent area in the embryo was done by a computer-guided morphometry using the Image Proplus software (version 4.5.1.22; Media Cybernetics, Bethesda, MD, USA).

2.13. In Vivo Test Using Hypercholesterolemic Zebrafish. The AB stain of Zebrafish was locally purchased. The preservation of zebrafish and procedures were permitted by the Committee of Animal Care and Use of Yeungnam University (Gyeongsan, Korea).

Tetrabit powder (47.5% crude protein, 6.5% crude fat, 2.0% crude fiber, 10.5% crude ash, vitamin A [29,770 IU/kg], vitamin D_3 [1860 IU/kg], vitamin E [200 mg/kg], and vitamin C [137 mg/kg]; Tetrabit Gmbh D49304, Melle, Germany) was mixed with fructose or tagatose (final concentrations, 50% in tetrabit [wt/wt]). The mixture was lyophilized post its complete dissolution in water, the remainder was further ground into a fine powder. The tetrabit powder in isolation and also mixed with the ketohexose was blended with diethyl ether solution of cholesterol to create a 4% cholesterol. We described the preparation of food diet of zebrafish in our previous report containing 4% cholesterol (final concentration) high cholesterol diet (HCD) [30]. The same procedure was used to prepare mixture of the normal

Table 2: Serum profile of zebrafish after consumption of high cholesterol diet and ketohexose for 3 weeks.

	ND[1] ($n = 70$)	HCD[2] ($n = 70$)	HCHF[3] ($n = 70$)	HCHT[4] ($n = 70$)
Weight (mg)/height (mm)	9.2 ± 1.6	$9.9 \pm 2.0^{*}$	8.9 ± 1.5	9.3 ± 1.9
Total cholesterol (mg/dL)	108 ± 5	$385 \pm 15^{**}$	$330 \pm 2^{**}$	$407 \pm 34^{**}$
Tricylglyceride (mg/dL)	232 ± 10	$271 \pm 51^{**}$	$311 \pm 31^{**}$	$194 \pm 13^{**}$
Glucose (mg/dL)	54 ± 16	$73 \pm 18^{**}$	$105 \pm 25^{**}$	$83 \pm 26^{**}$
GOT (Karmen/mL)	178 ± 4	$221 \pm 31^{**}$	$271 \pm 27^{**}$	$191 \pm 17^{**}$
CETP activity (% CE-transfer/4 h)	37 ± 1	$48 \pm 6^{**}$	$49 \pm 1^{**}$	$42 \pm 0^{**}$

[1]ND: normal diet, Tetrabit®: Tetrabit (47.5% crude protein, 6.5% crude fat, 2.0% crude fiber, 10.5% crude ash, containing vitamin A [29,770 IU/kg], vitamin D3 [1860 IU/kg], vitamin E [200 mg/kg], and vitamin C [137 mg/kg]). [2]HC: high cholesterol (4% cholesterol in ND, wt/wt). [3]HCHF: high cholesterol and high fructose (50% fructose in HCD, wt/wt). [4]HCHT: high cholesterol and high tagatose (50% tagatose in HCD, wt/wt). Data were analyzed by ANOVA Bonferroni t-test. Levels of significance were represented in the form of $^{**}P < 0.001$, $^{*}P < 0.05$ when compared with initial (ND) group. TC: total cholesterol; TG: triglycerides; GOT: glutamate oxaloacetate transaminase; CETP: cholesteryl ester transfer protein.

diet (ND) to remove the possibility of artifact caused by the solvent (diethyl ether).

The groups ($n = 70$) were fed with the assigned diet (10 mg/day/fish) without any exception; the details have been shown in Table 2. The zebrafish were kept at $28 \pm 1^{\circ}$C under a 14 : 10 hrs light : dark cycle.

2.14. Blood Analysis. After consumption of high cholesterol diet and ketohexose for 3 weeks, blood was aspirated from the hearts of the fish and mixed with 5 μL of 1 mM PBS-ethylenediaminetetraacetic acid (EDTA) and was subsequently transferred in EDTA-treated tubes. The plasma (30–40 μL) was separated by centrifugation from 10 zebrafish samples. Plasma total cholesterol (TC) and triglycerides (TG) were measured by using commercial assay kits (Wako Pure Chemical, Osaka, Japan). The concentration of glutamic oxaloacetic transaminase (GOT) was determined using a commercially available assay kit (Asan Pharmaceutical, Hwasung, Korea).

2.15. Plasma Cholesteryl Ester Transfer Protein (CETP) Activity. The cholesteryl ester- (CE-) donor consisting of apolipoprotein A-I (apoA-I) and cholesteryl oleate, reconstituted HDL (rHDL), was produced [31] with trace quantities of [^{3}H]-cholesteryl oleate (TRK886, 3.5 μCi/mg of apoA-I; GE Healthcare).

The CE-transfer reaction occurred in a 300 μL reaction mixture comprising of uniformly diluted zebrafish plasma (50 μL) as a cholesteryl ester transfer protein (CETP) source. The [^{3}H]-CE-rHDL (50 μL, 0.25 mg/mL) and human LDL (50 μL, 0.25 mg/mL) were used as cholesteryl (CE)-donor and CE-acceptor, in that order. Postincubation at 37°C, this reaction was stopped through centrifugation at 10,000g for 3 minutes at 4°C. The supernatant containing CE-acceptor (150 μL) was taken for to scintillation counting, and the percentage transfer of [^{3}H]-CE from rHDL to LDL was calculated.

2.16. Histologic Analysis. Briefly, after the zebrafish were sacrificed, the liver was fixed with 4% paraformaldehyde for 24 hr. The stained liver samples were subsequently entrenched in Tissue-Tek OCT compound (Thermo, Walldorf, Germany) and frozen. Further, 7 μm sections of these tissues were mounted on 3-APS (3-aminopropyl-triethoxysilane) coated slides and viewed under a Leica microcryotome (model CM1510s, Heidelberg, Germany). Seven successive sectioned slides of each zebrafish were first stained with Oil Red O and then counterstained with hematoxylin which highlighted the fatty streak lesions. To compare the extent of oxidative stress in these tissues, the totality of reactive oxygen species (ROS) was seen with dihydroethidium (DHE, cat # 37291; Sigma, St. Louis, MO) [14, 26] postmicrotome sectioning by using a Nikon Eclipse TE2000 microscope (Tokyo, Japan). Section fluorescence was measured through a computer-aided morphometry using Image Proplus software (version 4.5.1.22; Media Cybernetics, Bethesda, MD, USA).

2.17. Statistical Analysis. Resultant output were statistically analyzed as the mean±SD obtained from three independent experimental repetitions. Comparisons between results was made by Students t-test and one-way ANOVA (Bonferroni t-test) using SPSS program (version 12.0; SPSS Inc., Chicago, IL, USA). The values were tested for significance at $P < 0.001$, $P < 0.05$.

3. Results

3.1. Glycation by Ketohexose Treatment. Under high dosage of ketohexose (final 50 mM) treatment in the lipid-free state, without crosslinking reaction, all ketohexose-treated apoA-I showed more multimerized pattern than control (H_2O-treated apoA-I) as shown in Figure 1(a). After 72 hr incubation, tagatose-treated apoA-I (t-A-I) showed the strongest multimerization pattern from SDS-PAGE (Figure 1(a)) with the highest yellowish fluorescence (Figure 1(b)) from the Maillard reaction. Psicose treatment showed 2nd strongest multimerization and fluorescence.

Native polyacrylamide gel electrophoresis without sample boiling revealed that the ketohexose-treated apoA-I showed different band distribution and mobility in the lipid-free state as shown in Supplementary Figure 1A. They had an additional band around 88–89 Å and faster mobility in lower band position around 58–60 Å, while H_2O-treated

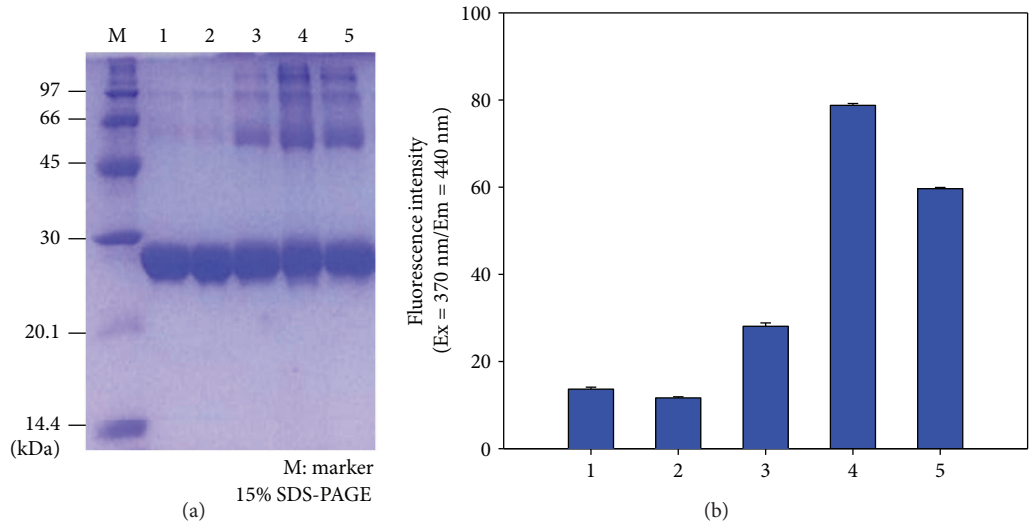

Figure 1: Electrophoretic characterization of modified apoA-I by each ketohexose. (a) Electrophoretic patterns of the glycated apoA-I in the lipid-free state on native gel (8–25% native gradient gel electrophoresis. Lane 1: native A-I, Lane 2: H2O-A-I, Lane 3: f-A-I, Lane 4: t-A-I, and Lane 5: p-A-I. (b) Glycation extent of apoA-I by each ketohexose treatment (Lane 1: native A-I, Lane 2: H2O-A-I, Lane 3: f-A-I, Lane 4: t-A-I, and Lane 5: p-A-I).

apoA-I or native apoA-I showed different electromobility with a major band around 71 Å. In the lipid-bound state, as shown in Supplementary Figure 1B, ketohexose-treated apoA-I showed decreased particle size around 93–95 Å, while H_2O-treated apoA-I showed distinct two bands around 95 and 109 Å. t-A-I-rHDL showed the weakest band intensity and the more band in the bottom, lipid-free apoA-I.

3.2. Phospholipid-Binding Ability. Up to 120 min incubation with DMPC (dimyristoyl phosphatidylcholine), t-A-I showed almost loss of phospholipid-binding ability, while f-A-I and p-A-I also showed impairment of binding ability (Figure 2). H_2O-treated apoA-I showed the fastest phospholipid-binding ability with half time for clearance ($T_{1/2} = 14$ min).

3.3. Inhibition of Cupric Ion-Mediated LDL Oxidation. During 120 min incubation, cupric ion-treated LDL showed the highest elevation of absorbance at 234 nm (A_{234}). There was no notable difference of A_{234} between ketohexose-treated apoA-I in the lipid-bound state.

Although t-A-I-treated LDL showed more oxidized product than that of n-A-I-treated LDL, t-A-I exhibited better antioxidant activity than f-A-I from monitoring of conjugated diene (A_{234}) as shown in Figure 3. Agarose electrophoresis also showed that f-A-I-treated LDL revealed the fastest electromobility, indicating the extent of oxidation. t-A-I- and p-A-I-treated LDL showed slower electromobility than f-A-I, suggesting their superior antioxidant ability than f-A-I.

3.4. Uptake of oxLDL into Macrophage. In the presence of ketohexose (final 5 mM) and oxLDL, fructose treatment caused the most severe extent of uptake of oxLDL (green fluorescence) and increased the level of ROS (red fluorescence) as shown in Figure 4. Although oxLDL was slightly less uptaken by fructose than oxLDL alone treatment, production of ROS

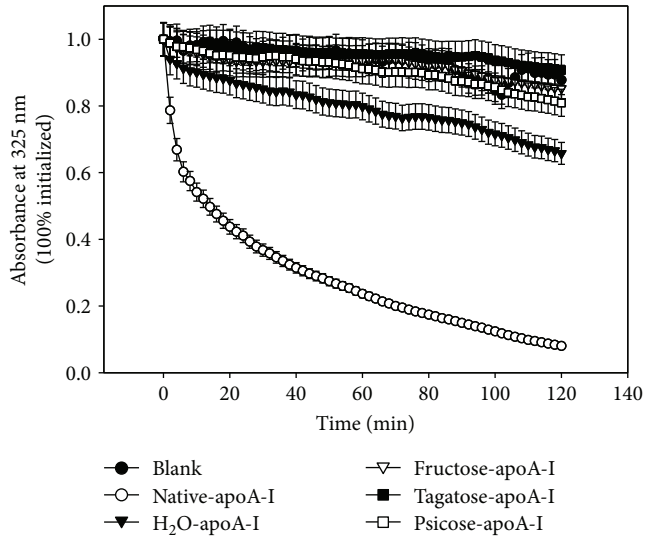

Figure 2: Phospholipid-binding ability of the ketohexose-treated apoA-I measured at an absorbance of 325 nm.

was more elevated in fructose treatment. These results suggest that basal level of fructose, even in final 5 mM, could enhance more atherogenic process via foam cell formation. However, treatment with tagatose and psicose showed much lesser uptake of oxLDL (green) and smaller ROS production (red). Similarly, the treatment of f-A-I caused the increased uptake of oxLDL and ROS production, while t-A-I- and p-A-I-treated THP-1 cell showed much less oxLDL uptake and ROS level (Figure 5). Taken together, the staining revealed that fructose or f-A-I treatment caused the highest production of ROS level, an indicator of oxidative stress in the cell.

3.5. Western Blotting. From immunodetection with apoA-I antibody, under the presence of 2 μM of protein, f-A-I-

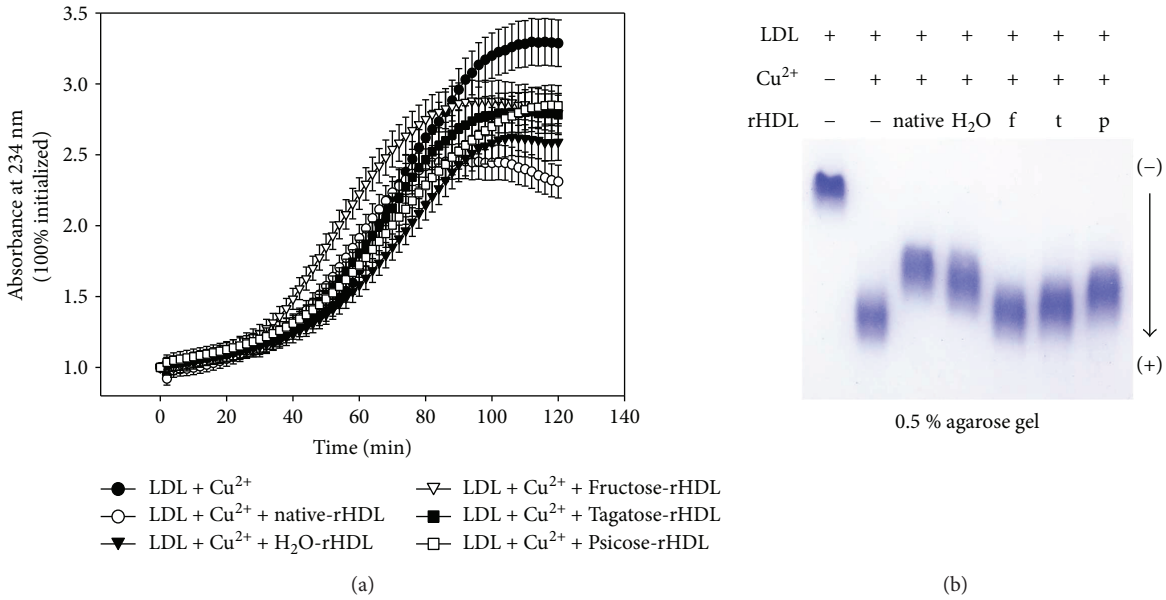

Figure 3: Antioxidant ability of apoA-I, which was treated by each ketohexose, against oxidation of LDL. (a) Detection of conjugated diene midst copper-mediated LDL oxidation. Continuous monitoring of conjugate diene level at absorbance 234 nm (A_{234}) during copper-mediated oxidation in the presence of either rHDL containing native apoA-I or ketohexose treated apoA-I. (b) Electromobility of LDL, which was oxidized by Cu^{2+} under presence of ketohexose treated apoA-I-rHDL, on 0.5% agarose gel.

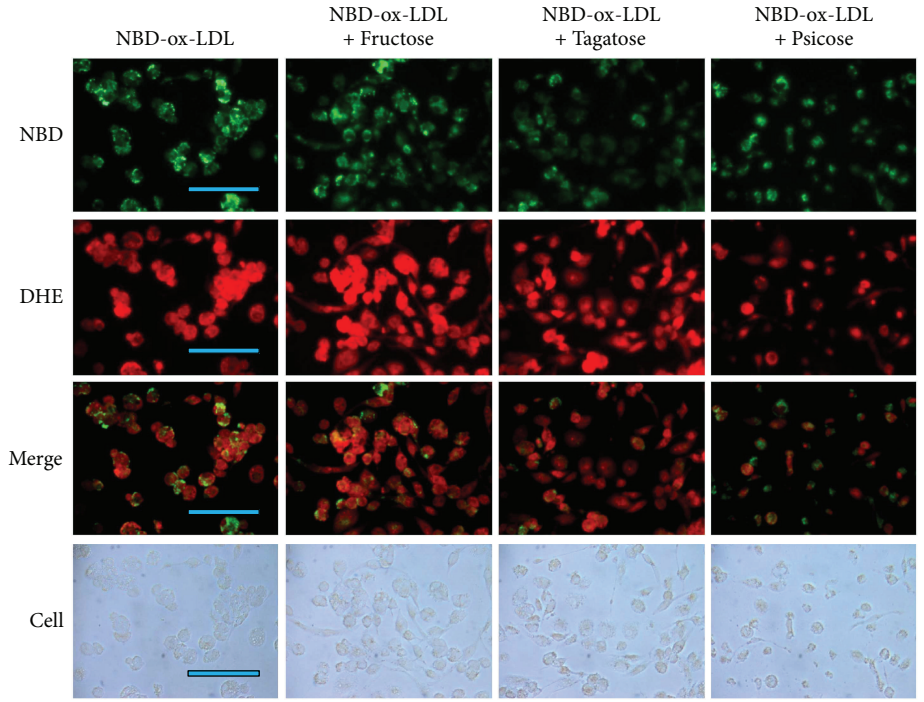

Figure 4: Cellular uptake of oxLDL containing NBD-cholesterol in the presence of each ketohexose (fructose, tagatose, and psicose). PMA-differentiated macrophages were incubated with 50 μL of oxLDL (1 mg/mL), 14 μL of ketohexose alone (175 mM, at final 5 mM), and 436 μL of RPMI1640 media. After incubation with oxLDL and each ketohexose for 48 hrs, cells were washed by PBS (phosphate-buffered saline) and green fluorescence (Ex = 488 nm, Em = 535 nm) intensity was detected. In order to compare production of reactive oxygen species (ROS), red fluorescence image (Ex = 588 nm, Em = 605 nm) was obtained after dihydroethidium (DHE) staining. Cell images were observed and photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan). The bar in the photo indicates 100 μm.

treated cell showed the strongest multimer band of apoA-I in the lysate of macrophage, while t-A-I-treated cell showed a less multimeric band of apoA-I as similar as native apoA-I (Figure 6). The ApoA-I band was not detected in ketohexose alone (final 5 mM) treated cell as control (lane 1–4, Figure 6), suggesting there was no detectable apoA-I in the

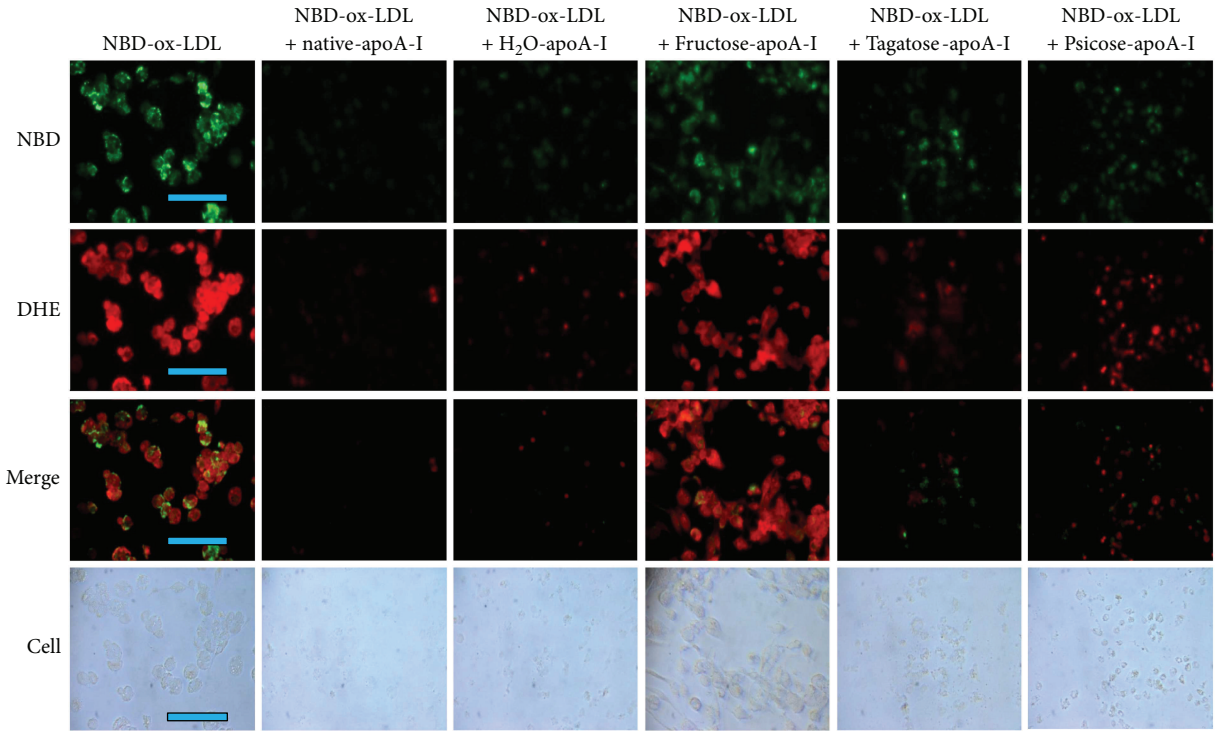

Figure 5: Cellular uptake of oxLDL in the presence of rHDL-containing ketohexose-treated apoA-I and NBD-cholesterol. PMA-differentiated macrophages were incubated with 50 μL of oxLDL (1 mg/mL), 50 μL of each rHDL (0.7 mg/mL, at final 2 μM), and 400 μL of RPMI1640 media. After incubation with oxLDL and each rHDL for 48 hrs, cells were washed by PBS (phosphate-buffered saline) and green fluorescence (Ex = 488 nm, Em = 535 nm) intensity was detected. In order to compare production of reactive oxygen species (ROS), red fluorescence image (Ex = 588 nm, Em = 605 nm) was obtained after dihydroethidium (DHE) staining. Cell images were observed and photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan). The bar in the photo indicates 100 μm.

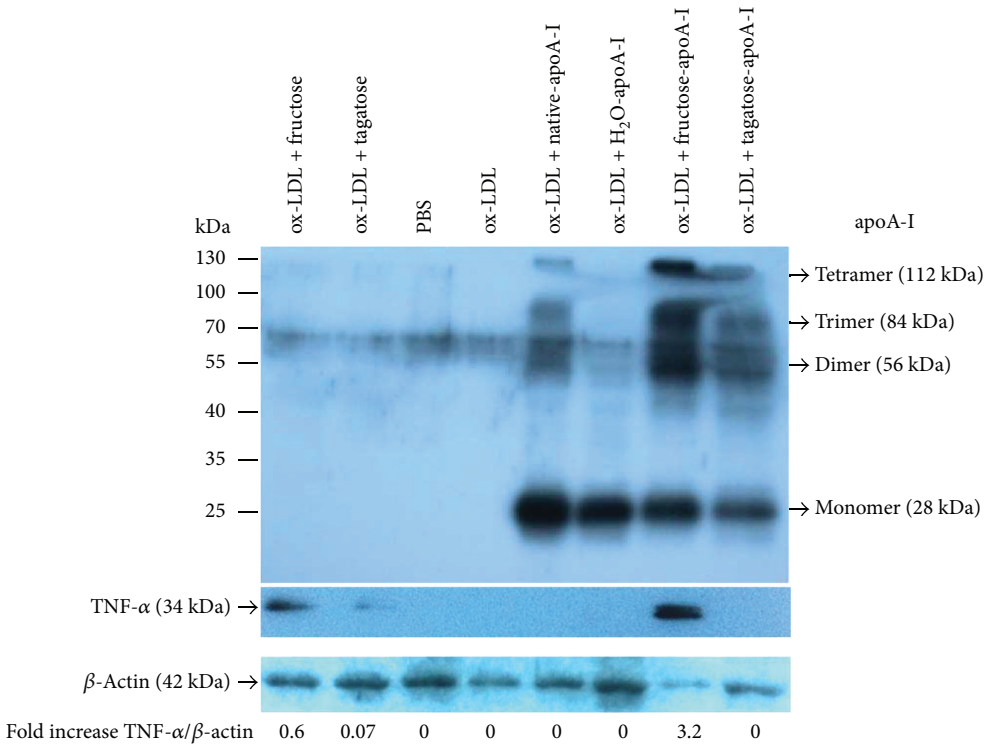

Figure 6: Western blot analysis. An equal amount of protein (13 μg per lane) was loaded on 15% SDS-PAGE. ApoA-I (ab7613; Abcam), tumor necrosis factor (TNF)-α (sc52746; Santa cruz biotechnology), and β-actin antibodies (ab8229; Abcam) were used.

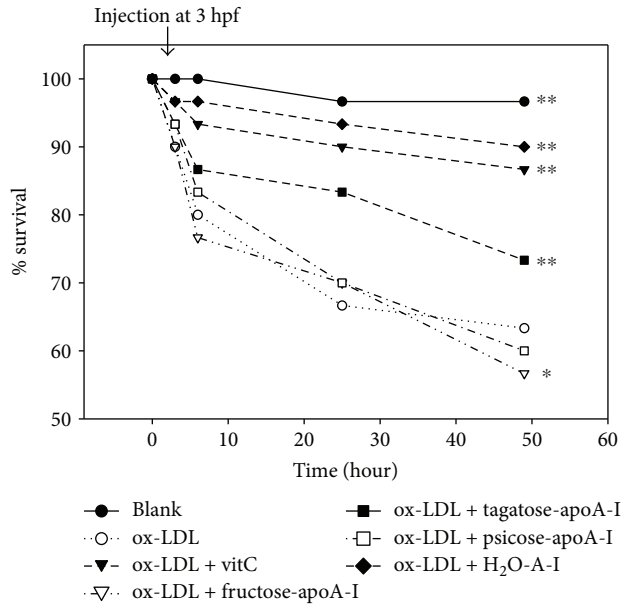

Figure 7: Survival curve of zebrafish embryos after co-injection of oxLDL (13 ng of protein) plus vitamin C (final concentration, 1 μM) or each ketohexoses apo-A-1 during 48 hr incubation post-injection. $^{*}P < 0.05$ vs oxLDL; $^{**}P < 0.01$ vs oxLDL.

macrophage. Interestingly, TNF-alpha band appeared only in fructose alone treated and f-A-I-treated models.

3.6. Microinjection of Ketohexose into Zebrafish. Microinjection of oxLDL (13 ng of protein) alone into zebrafish embryo resulted in the highest embryo death with around 63% survival as shown in Figure 7, suggesting that oxLDL could cause acute inflammatory death. Under the presence of oxLDL, f-A-I-injected embryo showed the lowest survivability, indicating that the existence of f-A-I made more exacerbation of the inflammatory death. Surprisingly, t-A-I-injected embryo showed higher survivability than oxLDL alone control, while coinjection of Vit-C resulted in the highest survival. At 24 hr postinjection, f-A-I-injected embryo showed the slowest embryo development speed as shown in Figure 8, as well as oxLDL alone, injected embryo. oxLDL alone was treated as a reference category to perform the statistical analysis.

3.7. Consumption of Tagatose Caused Hypotriglyceridemia in Zebrafish. After 3 weeks feeding of HCD with or without the ketohexose (50% wt/wt), fructose-administered group (HCHF) and tagatose-administered group (HCHT) showed higher than 90% survival similar with HCD control group, suggesting that excess feeding of ketohexose (50% wt/wt) was well tolerated. In the serum profile, HCD-alone-administered group showed an increment of TC and TG which was found to be significant when the HCD data was compared to the normal. Fructose-fed group showed 15% decrease of serum TC, while tagatose-administered group showed the similar level of serum TC compared with HCD group. However, the serum TG level was 14% more increased in fructose group, while tagatose-fed group showed 30% decrease of serum TG compared with HCD control. The fructose-fed group showed 1.6-fold higher serum TG level than the tagatose-fed group. Fructose-fed group also showed the highest serum glucose level, 43% more increased than HCD group. However, tagatose-fed group showed smaller glucose level than the fructose-fed group and these results were statistically significant. Serum GOT was also elevated in HCD group (Table 2), suggesting that hyperlipidemia is associated with acute hepatic inflammation. The fructose-fed group showed an increment of 22% in the serum GOT level; nonetheless, tagatose-fed group showed 15% more decreased than that of HCD group.

Serum CETP activity was more enhanced by HCD consumption than ND group, $48 \pm 4\%$ and $37 \pm 1\%$ of CE-transfer, respectively. However, HCHT group showed significantly decreased CETP activity up to 42% CE-transfer, while HCHF group showed similar CETP activity as HCD group. These results suggest that HC consumption can cause enhancement of CETP activity as our previous report and tagatose consumption could diminish the activity.

3.8. Histologic Analysis. From H & E staining, HC consumption caused more infiltration of inflammatory cells in hepatic microsections as shown in Figure 9. However, HCHT-consumed group showed much less infiltration of the cells, while HCHF group showed a similar level of the infiltration. Oil Red O staining revealed that HC group showed a remarkable increase of red intensity compared with normal group, suggesting cholesterol consumption caused fatty liver change. HCHF group showed stronger red intensity than HC group, indicating that fructose consumption accelerates the fatty liver change. However, surprisingly, HCHT group showed almost no Oil Red O-stained area as similar level as the normal group. These results suggest that tagatose consumption can ameliorate inflammatory aggravation and fatty liver change caused by cholesterol consumption.

4. Discussion

Generally, glycation is associated with structural modification and functional loss of serum proteins, especially hemoglobin and apolipoproteins. Our research group and several others have reported that glycation of apolipoprotein is involved in the critical process of diabetes, atherosclerosis, and senescence [11, 14]. Previous reports suggested that among ketohexoses, tagatose tastes like sucrose and is useful as a low-calorie sweetener. Tagatose has lower glycemic index compared to the other sweeteners and ketohexoses. It has been reported that a low glycemic index diet over many years lowers the risk of developing type 2 diabetes, CVD, and other metabolic risks [32]. Moreover, tagatose appears to have antihyperglycemic effect and obesity control drug by reducing the blood glucose among normal and prediabetic participants possibly through inhibiting intestinal disaccharidases and glucose transport. Tagatose decreases the level of postprandial glucose and modulates the insulin response through inhibition of glycogenolysis in the liver [33, 34].

Interestingly, t-A-I showed highest formation of multimer than f-A-I and p-A-I (Figure 1(b)), suggesting that tagatose could modify more BS^3-crosslinking site. However, there

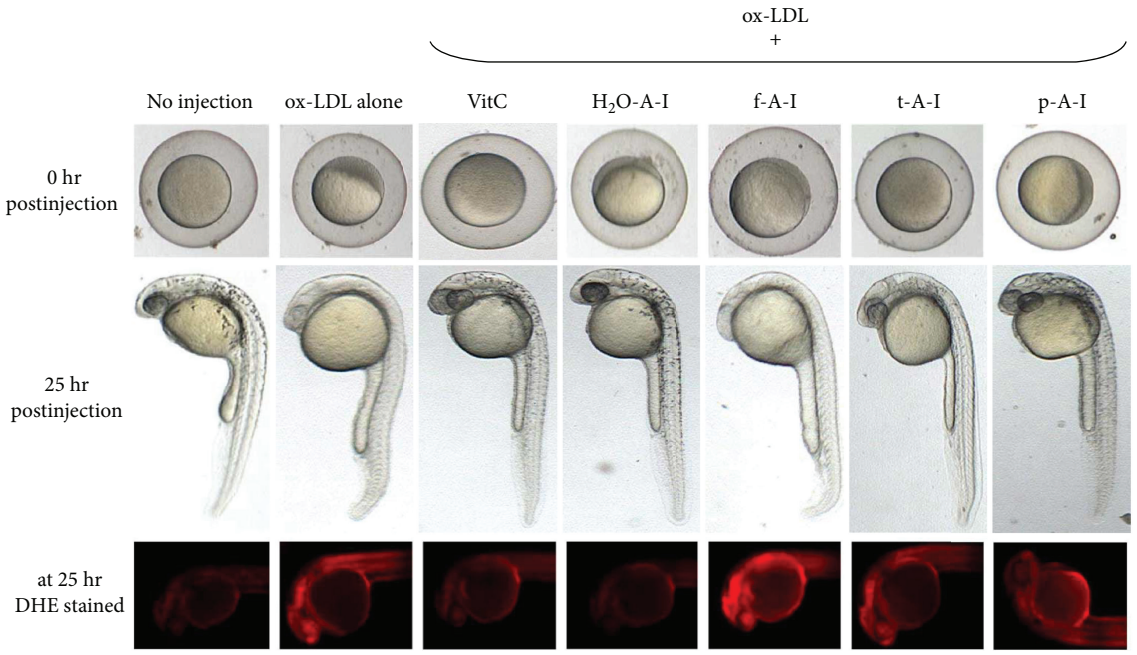

Figure 8: Change in embryo stage after coinjection of oxLDL and ketohexose-treated apoA-I at 0 and 25 hr postinjection. In order to compare production of reactive oxygen species, red fluorescence image (Ex = 588 nm, Em = 605 nm) of zebrafish embryo was obtained using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) after dihydroethidium (DHE) staining as described in the text. f-A-I: fructose-treated apoA-I; t-A-I: tagatose-treated apoA-I; p-A-I: psicose-treated apoA-I; H_2O-A-I: H_2O-treated apoA-I.

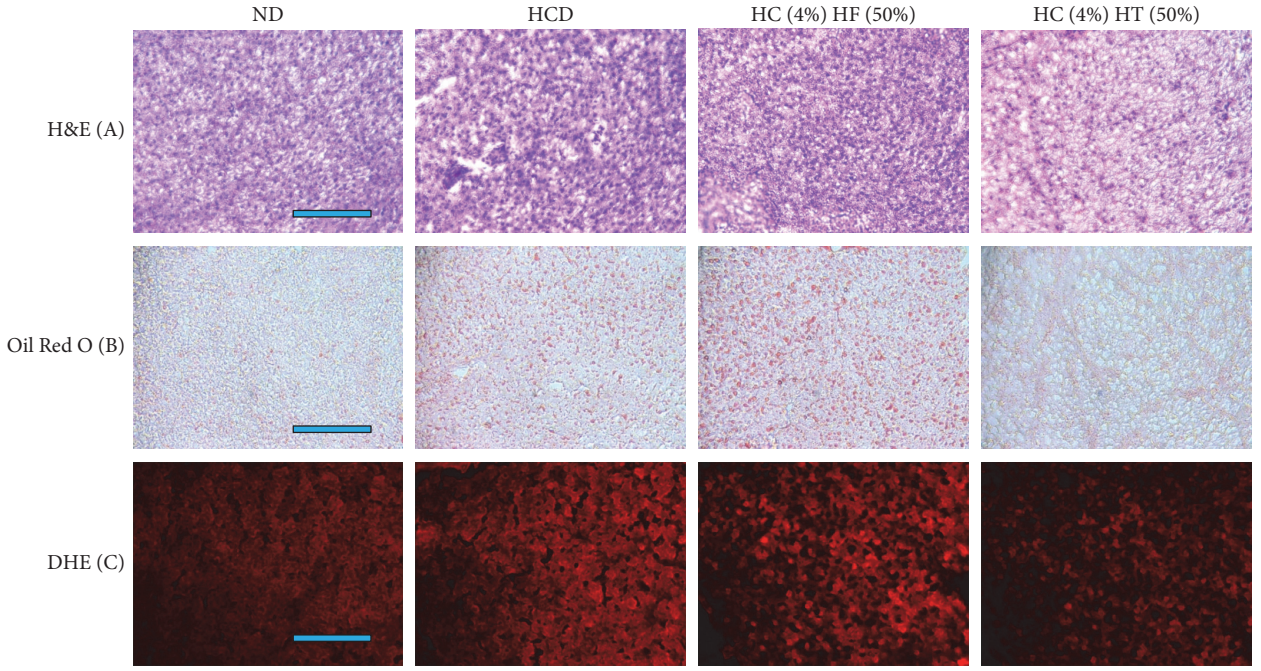

Figure 9: Representative micrographs for histological assessments of hepatic microsections. The bar in the photo indicates 100 μm. (a) Infiltrated inflammatory cells were visualized by hematoxylin and eosin staining. (b) Extent of fatty liver change was measured by Oil Red O staining. (c) Production of reactive oxygen species were determined by DHE staining.

was no notable difference in the crosslinking efficiency between ketohexose-treated apoA-I in rHDL state, although all ketohexose-treated apoA-I showed much more dimerization than control (H_2O treated).

The multimerization ability and phospholipid-binding ability of t-A-I might suggest that critical amino acid is modified by the tagatose treatment. However, the modification caused less damage of beneficial functions of apoA-I and rHDL.

More uptake of cholesterol from oxLDL into macrophage is associated with increased production of ROS (Figure 4). Especially in the presence of fructose, the extent of cholesterol uptake was similar compared with oxLDL alone, however, the production of ROS was stronger than oxLDL alone. Among ketohexose-treated apoA-I, f-A-I showed the highest uptake of NBD-oxLDL and production of ROS, suggesting that coincidence of oxLDL phagocytosis and oxidative stress (Figure 5). As shown in Figure 6, immunodetection with TNF-α antibody revealed that fructose-treated (lane 1) or f-A-I-treated (lane 7) cell lysate showed distinct band, while tagatose or t-A-I-treated cell did not.

The proinflammatory properties of fructose appeared again in the zebrafish embryo with the highest mortality and the slowest development speed as similar as an oxLDL alone injection. These results make a good agreement with the previous report that embryos injected with oxLDL alone had a more attenuated developmental speed than native LDL-injected embryos [35]. Furthermore, coinjection of fructosylated apoA-I and oxLDL exacerbated the embryo death with the slowest developmental speed, while native apoA-I showed a protective effect. While p-A-I showed more production of ROS in the macrophage and less protective effect against the oxLDL mediated embryo death, however, t-A-I showed more protective effect both in the macrophage and the embryo.

After 3 weeks feeding, HCD consumption caused infiltration of inflammatory cells and fatty liver change based on our previous finding in mouse model and recent reports draw attention on the deleterious effect of HCD consumption that caused severe steatohepatitis along with early liver fibrosis [36–38]. As shown in Table 2, tagatose-fed group showed mild weight loss effect compared with HCD control group. In human study, Donner et al. showed that 12 months of oral administration of tagatose resulted in weight loss effect, from 109 ± 14.7 kg to 105.3 ± 14.4 kg, along with a reduction in glycohemoglobin [16]. Although the study was based on a small number of subjects ($n = 8$), the weight loss effect made a good agreement with the current result. In a similar study on hypercholesterolemic mice, Police et al. revealed that in comparison to sucrose, an equal amount of tagatose did not elevate the risk of hyperglycemia, hyperlipidemia, and resulted in a lesser extent of hypercholesterolemia and atherosclerosis [39].

Surprisingly, fructose-treated group (HCHF) showed reduction in body weight and serum TC level compared with HCD group. More interestingly, the HCHF group revealed lower body weight than ND group, suggesting that excess dosage of fructose could result in severe weight loss. However, the group showed severe inflammation in hepatic tissue (Figure 9). This acute weight loss might be connected with fatty liver change and hepatosteatosis. The HCHF group showed the highest serum TG and inflammatory level. Similar reports of hepatic lipid accumulation, inflammation, and oxidative stress were shown by Sapp et al. and Jaiswal et al. after fructose treatment in larval zebrafish and L6 skeletal muscle cells [40, 41]. Our results make a good agreement that f-A-I-treated cell showed the highest elevation of TNF-α.

5. Conclusions

To conclude, tagatose may modify several functions of apoA-I such as phospholipid-binding ability and multimerization ability. However, the antioxidant and anti-inflammatory activity of apoA-I was not impaired in the macrophage and zebrafish embryo model. In hypercholesterolemic zebrafish model, tagatose-consumed group showed a reduction in serum TG, GOT, and CETP activity.

Disclosure

All authors have indicated they have no financial relationships relevant to this article to disclose.

Conflicts of Interest

The authors declare no competing financial interest.

Authors' Contributions

Dhananjay Yadav and Suk Jeong Kim performed the experiments; Suk Jeong Kim prepared the first draft of this manuscript; Myung Ae Bae and Jae-Ryong Kim analyzed the data; and Kyung-Hyun Cho wrote the paper and supervised the study project.

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Supplementary Materials

Supplementary Figure 1: electrophoretic patterns of the glycated apoA-I in the lipid-free (A) and lipid-bound states on native gel electrophoresis. Supplementary Figure 2: circular dichroism spectra with ketohexose-treated apoA-I in lipid-free (A) and lipid-bound states (B). f-A-I: fructose-treated apoA-I; t-A-I: tagatose-treated apoA-I; p-A-I: psicose-treated apoA-I. *(Supplementary Materials)*

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Research Article

Di-(2-Ethylhexyl) Phthalate Increases Obesity-Induced Damage to the Male Reproductive System in Mice

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Objective. This study evaluated the effects of di-(2-ethylhexyl) phthalate (DEHP) and obesity on male reproductive organ function in male mice and the potential mechanism of male secondary hypogonadism (SH) in such mice. *Methods.* 140 mice were assigned to six groups for 12 weeks: normal, DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high. The effects of DEHP and obesity upon the reproductive organs were determined by measuring sperm count and motility, relative testis and epididymis weight, hormone level, and pathological changes. Oxidative stress was evaluated by determining malondialdehyde, T-AOC, SOD, GSH, H_2O_2, CAT, and GSH-PX in testicular tissues. Nrf2 and Keap1 protein were measured by Western blotting. *Results.* DEHP and obesity reduced sperm count and motility, relative testis and epididymis weight, and testosterone level but increased the levels of MDA, H_2O_2, leptin, and estradiol. Pathological injury was observed in the testicular Leydig cells. Moreover, the activity of CAT, SOD, and GSH-Px enzymes was inhibited. Nrf2 protein expression was reduced but that of Keap1 was increased. *Conclusions.* DEHP and obesity jointly caused damage to male productive function. Oxidative stress in testicular tissue, and a high level of leptin, may provide some evidence to clarify the mechanisms of male SH with DEHP and obesity.

1. Introduction

Obesity is a multifactorial condition with syndromic and nonsyndromic variants. During 2011–2014, the prevalence of obesity in adults in the United States of America was over 36% [1]. In contrast, a 2014 study of chronic disease and nutrition in the Chinese population revealed that the prevalence of obesity and excessive weight gain was 11.9% and 30.1% among adults [2]. Moreover, the prevalence of obesity and excessive weight gain in adults had increased by 230% and 84% since 1992, respectively; further increases in obesity are expected in the future [2].

Previous studies have shown that obesity has an impact upon male reproduction [3]. For example, male obesity is associated with an increased incidence of low sperm concentration and a progressively low motile sperm count [3]. Even in the absence of organic disease in the hypothalamo-pituitary axis, the prevalence of secondary (hypogonadotropic) hypogonadism (SH) in obese men has also been demonstrated in several studies [4–6]. The pathogenesis and clinicopathological correlates of obesity-associated SH have not been fully elucidated yet. The mechanisms involved in the association of male SH and obesity are complex. However, male obesity has been associated with lower plasma testosterone levels [7, 8]. Since the development of the male reproductive organs and male secondary sexual characteristics is promoted by androgens, and since spermatogenesis is closely related to androgen secretion, it follows that reduced levels of testosterone may contribute to male SH in obesity [9].

Endocrine disrupting chemicals are exogenous substances that have the ability to change endocrine function and cause adverse effects at the level of the organism, its

progeny, and/or (sub) populations of organisms; these chemicals can cause the abnormal development of reproductive organs and reproductive dysfunction [10]. Di-(2-ethylhexyl) phthalate (DEHP), a form of endocrine disrupting chemicals, is widely used as a plastic plasticizer for synthetic polymers. Humans are widely exposed to DEHP, because of its use in many daily products, including vinyl flooring, wall covering, plastic bags and covers, food containers, cosmetics, and toys [11]. Therefore, obese people can easily come into contact with DEHP. Worryingly, DEHP has well-documented anti-androgenic effects [11]. In China, it is common for obese men to be exposed to DEHP, and we should therefore consider the effects of such exposure on androgens. We hypothesized that there is likely to be a joint action between obesity and DEHP upon male reproduction and that low levels of testosterone levels might be the key mechanism underlying this effect.

Leptin is considered to be the most important factor in regulating the reproductive axis, and high leptin levels have been found in obese males [12]. Our previous study found that high leptin level was one of the mechanisms responsible for reducing the level of testosterone in obese males [9]. However, it remains unclear as to what the exact changes are in obese males exposed to DEHP. We therefore wanted to investigate whether leptin levels are the key factor regulating testosterone levels in obese males exposed to DEHP.

Oxidative stress has also been found to be a highly influential factor upon male reproduction [13]. In our previous study, we proved that oxidative stress can damage testicular tissue in obese males. Therefore, in this present study, we attempted to ascertain the effect of obesity and DEHP on the function and development of reproductive organs in male mice. In addition, we evaluated the possible mechanisms (high leptin level and oxidative stress in testicular tissue) underlying the joint-damaging effect of obesity and DEHP upon the male reproductive system.

2. Materials and Methods

2.1. Animals, Diet, DEHP Exposure, and Grouping

2.1.1. Animals. A total of 140 4/5-week-old C57BL/6J male mice were obtained from the Experimental Animal Center, China Medical University, Shenyang, China. Mice were fed standard laboratory chow for the 1st week to allow them to adjust to their new environment. Animals were housed individually in a temperature and humidity-controlled room ($25 \pm 2^{\circ}$C and $55 \pm 10\%$, resp.) on a 12-hour light/dark cycle with free access to food and water. All experimental procedures were conducted in conformity with the institutional guidelines for the care and use of laboratory animals in China Medical University, Shenyang, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication number 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

2.1.2. Diet. Mice were randomly assigned to a standard laboratory diet (10% calories from fat, 20% calories from protein, and 70% calories from carbohydrates, 3.85 kcal/g) ($n = 20$) or a home-made high-fat diet, which contained 45% kcal from fat, as the high-fat diet group ($n = 120$) [9]. The high-fat diet was made up of 73% standard chow diet plus 20% lard, 7% casein (Aoboxing Biotech Company Ltd., Beijing, China) and trace amounts of multiple vitamins.

2.1.3. DIO (Diet-Induced Obesity) Definition. After 8 weeks on high-fat diets, the 40 mice in the upper tertile of body weight gain (which had been fed the high-fat diet) were defined as DIO mice, according to the method used by Levin et al. [14]. The remaining 80 mice in the intermediate and lower tertile of body weight gain were discarded from this study.

2.1.4. DEHP Exposure. After 8 weeks of feeding on a normal or high-fat diet, 10 mice fed upon the normal diet were given an oral gavage once a day for 4 weeks with 100 mg/kg of DEHP. 30 mice ($n = 10$ each group) fed the high-fat diet were also given an oral gavage once a day for 4 weeks with different doses of DEHP (30 mg/kg body, 100 mg/kg body, and 300 mg/kg body).

2.1.5. Grouping Methodology. Six different groups of mice were created and analyzed in this study.

(1) Ten mice fed a normal diet for 12 weeks were defined as the normal group (normal group).

(2) Ten mice fed a normal diet for 12 weeks and exposed to DEHP (100 mg/kg body weight) for 4 weeks (from the 8th week) were defined as the DEHP exposure group (DEHP group).

(3) Ten DIO mice exposed to a high-fat diet only for 12 weeks were defined as the DIO group (DIO group).

(4) Ten DIO mice exposed to a 12-week high-fat diet and 4 weeks of DEHP (30 mg/kg body weight, from the 8th week) were defined as the high-fat and DEHP low exposure group (DIO + DEHP low group).

(5) Ten DIO mice exposed to a 12-week high-fat diet and 4 weeks of DEHP (100 mg/kg body weight, from the 8th week) were defined as the high-fat and DEHP middle exposure group (DIO + DEHP middle group).

(6) Ten DIO mice exposed to a 12-week high-fat diet and 4 weeks of DEHP (300 mg/kg body weight, from the 8th week) were defined as the high-fat and DEHP high exposure group (DIO + DEHP high group).

2.2. Experimental Procedures. As shown in Figure 1, 140 mice were allowed to adjust to their new environment for 1 week. Ten mice were fed a normal diet for 12 weeks. Ten mice were fed a normal diet for 12 weeks and then exposed to DEHP for 4 weeks. The remaining 120 mice were fed a high-fat diet for 8weeks. Then, 40 mice were defined as DIO mice. Ten of the DIO mice were exposed to a high-fat diet for 4 weeks. The other 30 DIO mice were exposed to both a high-fat diet and DEHP at different doses (30, 100, and 300 mg/kg body weight). All mice were sacrificed after 12 weeks of feeding.

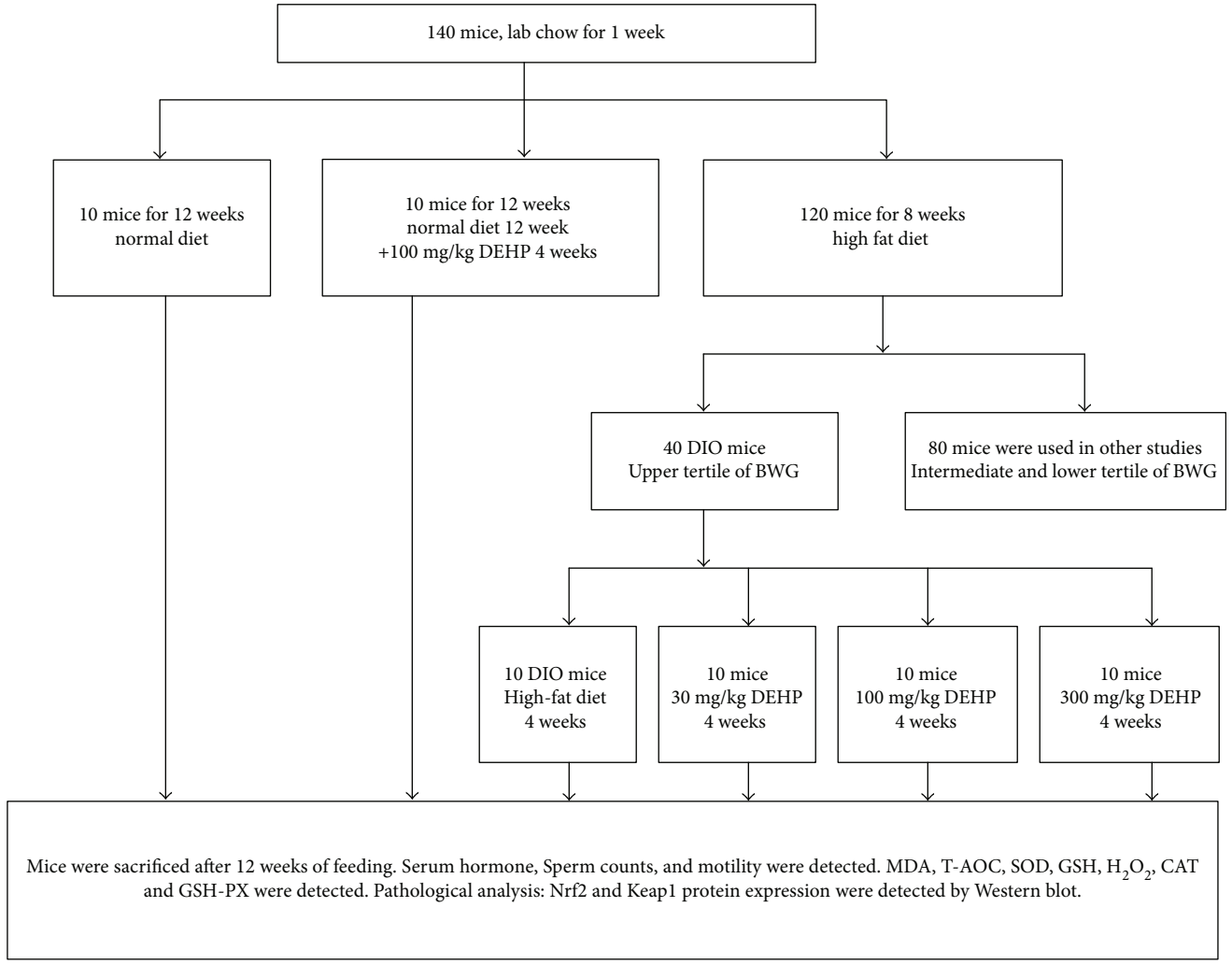

Figure 1: The flowchart of the animal experiment. BWG: body weight gain.

2.3. Tissue Processing and Assays. Twenty-four hours after receiving the last dose, animals were anesthetized with ether and blood samples were obtained from the vena cava. Serum was separated from whole blood for the measurement of hormones (testosterone, estradiol, and leptin). Immediately after blood samples were collected, the epididymis was rapidly excised, and sperm count and motility were analyzed. Retroperitoneal fat, epididymal fat, epididymis, testis, kidney, and liver were each dissected and weighed.

One testicle of mice ($n = 10$/group) in each group was prepared for 5% or 10% homogenate in order to determine the MDA, T-AOC, SOD, GSH, H_2O_2, CAT, and GSH-Px levels; five testicles from the 10 mice in each group were immediately frozen at −80°C for protein expression studies. Five testicles from the 10 mice in each group were prepared for light microscopy and transmission electron microscopy.

All substance contents and enzyme activities were normalized to the protein which was measured by the method described by Deng et al. [15] using bovine serum albumin as a standard. Each sample was tested in duplicate.

2.4. Cauda Epididymal Sperm Count and Motility Measurements. Male C57BL/6J mice (fed 12 weeks) were weighed and anesthetized. The left epididymis was immediately removed. The epididymis and the vas deferens were dissected away from the fat. In a six-well plate, the epididymis and vas deferens from each animal were placed in a well containing 1.0 ml of M2 buffer. The epididymis was then cut at the junction between the corpus and cauda epididymis, and the cauda was placed into a well with 1.0 ml of M2 buffer. Several cuts were made in the cauda epididymis with scissors, and the tissue was gently pressed to release sperm. Sperm was also expressed from the vas deferens in a separate well and then removed from the plate. The pressed sperm from the cauda epididymis was collected in an Eppendorf tube. Using a hemocytometer, sperm counts were determined as the number of sperm per microliter.

Sperm count and motility were assessed in accordance with World Health Organization (WHO) guidelines [16] (≥200 sperm counted for each sample). Sperm count was determined by counting on a hemocytometer. Sperm motility was assessed blinded under a light microscope, classifying 200 sperm per animal as either progressive motile, nonprogressive

motile, or immotile. Motility was then expressed as a percentage of the total motile population (progressive motility and nonprogressive motility). Detailed methods can be found in our prior study [9].

2.5. Pathological Analysis [9]

2.5.1. Light Microscopy. A portion of each testicle was cut into 4 μm thick pieces and fixed in 4% paraformaldehyde. Regular hematoxylin and eosin (HE) staining was performed for morphological observation with an AX-70 microscope (Olympus, Japan).

2.5.2. Transmission Electron Microscopy. A portion of each testicle was cut into fragments (1 mm × 1 mm × 1 mm), fixed in 2.5% glutaraldehyde made up in 0.1 M phosphate buffer (pH 7.2), postfixed in 1.0% OsO_4, dehydrated in a progressive ethanol and acetone solution, embedded in Epon812, sectioned with an LKB ultramicrotome, and stained with uranyl acetate followed by lead citrate, then observed by H-600 microscopy and photographed.

2.6. Hormone Detection. Leptin, testosterone, and estrogen were detected by ELISA methods. All methods were performed according to the instructions provided with the ELISA kit. The leptin kit was purchased from Merck Millipore (Parmstadt, Germany), the testosterone kit from Enzo Life Science Inc. (NY, USA), and the estradiol kit from Cayman Chemical Company (Ann Arbor, MI, USA).

2.7. MDA, T-AOC, SOD, GSH, H_2O_2, CAT and GSH-PX Assays. Analysis kits for MDA, T-AOC, SOD, GSH, H_2O_2, CAT, and GSH-PX assays were provided by Beyotime Biotechnology (Jiangsu, China). The GSH-PX, CAT, SOD, H_2O_2, and T-AOC contents were measured using assay kits in strict accordance with the manufacturer's instructions. MDA contents were expressed as nmol·mg^{-1} protein, T-AOC, SOD, CAT and GSH-PX contents were expressed as U·mg^{-1} protein, GSH contents were expressed as mgGSH·g^{-1} protein and H_2O_2 content was expressed as mmol·g^{-1} protein.

2.8. Western Blotting. The testes were washed twice in ice-cold phosphate-buffered saline (PBS). RIPA buffer (50 μL) was supplemented with 1 mmol/L PMSF, 1 μg/mL of leupeptin, 1 mmol/L β-glycerophosphate, 2.5 mmol/L sodium pyrophosphate, and 1 mmol/L Na_3VO_4 and placed on ice for 20 min, followed by centrifugation for 20 min at 12,000g and 4°C. Next, 50 μg of total protein from each sample was resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking in PBST containing 4% skimmed milk for 2 h at room temperature, the polyvinylidene fluoride membranes were incubated with rabbit polyclonal anti-Nrf2 antibody (ab31163, diluted 1 : 1000; Abcam) and anti-keap1 antibodies (ab119403, diluted 1 : 1000; Abcam) in PBST overnight at 4°C. The membranes were then washed three times in PBST and incubated in peroxidase-conjugated AffiniPure secondary antibodies (diluted 1 : 5000; ZSGB-BIO, Beijing, China) in PBST for 2 h at room temperature. Detection was carried out by chemiluminescence using ECL solution (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was in triplicate, at least.

3. Results

3.1. Body Weight. A higher body weight was observed in DIO (27.43 ± 1.60 g), DIO + DEHP low (26.89 ± 1.38 g), DIO + DEHP middle (27.85 ± 1.24 g), and DIO + DEHP high (26.62 ± 1.28 g) mice in comparison to age-matched controls (25.24 ± 1.80 g) and DEHP (25.25 ± 0.99 g) mice at 8 weeks ($P < 0.05$). At 12 weeks, the weight of the DEHP (27.64 ± 1.34 g), DIO (29.66 ± 2.39 g), and DIO + DEHP middle (27.92 ± 1.10 g) mice was higher than the control mice ($P < 0.05$). The weight of the DIO mice was significantly higher than the other 5 groups of mice ($P < 0.05$) (Figure 2).

3.2. Reproductive Organs, Sperm Count and Motility, and Sex Hormone Levels in the 6 Experimental Groups of Mice. DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, DIO + DEHP high mice and control mice did not exhibit significant differences in the absolute mean weight of testes, epididymis, or seminal vesicles at 12 weeks (not shown in the results). However, as shown in Table 1, there was a significant reduction in the relative testis and epididymis weight in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice compared to the control mice ($P < 0.01$). There was also a significant reduction in the relative epididymal weight of the DIO + DEHP middle and DIO + DEHP high mice compared with the DEHP mice ($P < 0.05$). Also, the relative epididymis weight in the DIO + DEHP high mice was lower than that in the DIO, DIO + DEHP low, and DIO + DEHP middle mice ($P < 0.01$).

As shown in Table 1, there was a significant reduction in the relative liver and kidney weight in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice compared to the control mice ($P < 0.01$). There was a significant reduction in the relative liver weight of the DIO + DEHP low mice compared with the DEHP mice ($P < 0.05$). Also, the relative liver and kidney weight in the DIO + DEHP middle and DIO + DEHP high mice was lower than that in the DEHP and DIO mice ($P < 0.05$).

As also shown in Table 1, there was a significant increase in the relative epididymal and retroperitoneal fat weight in the DEHP, DIO, DIO + DEHP low, and DIO + DEHP middle mice compared to the control mice ($P < 0.05$). There was a significant decrease in the relative epididymal fat weight in the DIO + DEHP middle and DIO + DEHP high mice compared with the DEHP mice ($P < 0.05$). There was a significant decrease in the relative epididymal and retroperitoneal fat weight in the DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice compared with the DIO mice ($P < 0.05$), and there was a significant decrease in the relative retroperitoneal fat weight in the DIO + DEHP high mice compared with the DEHP mice ($P < 0.05$).

Furthermore, as shown in Table 1, there was a significant decrease in sperm motility and sperm count in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO +

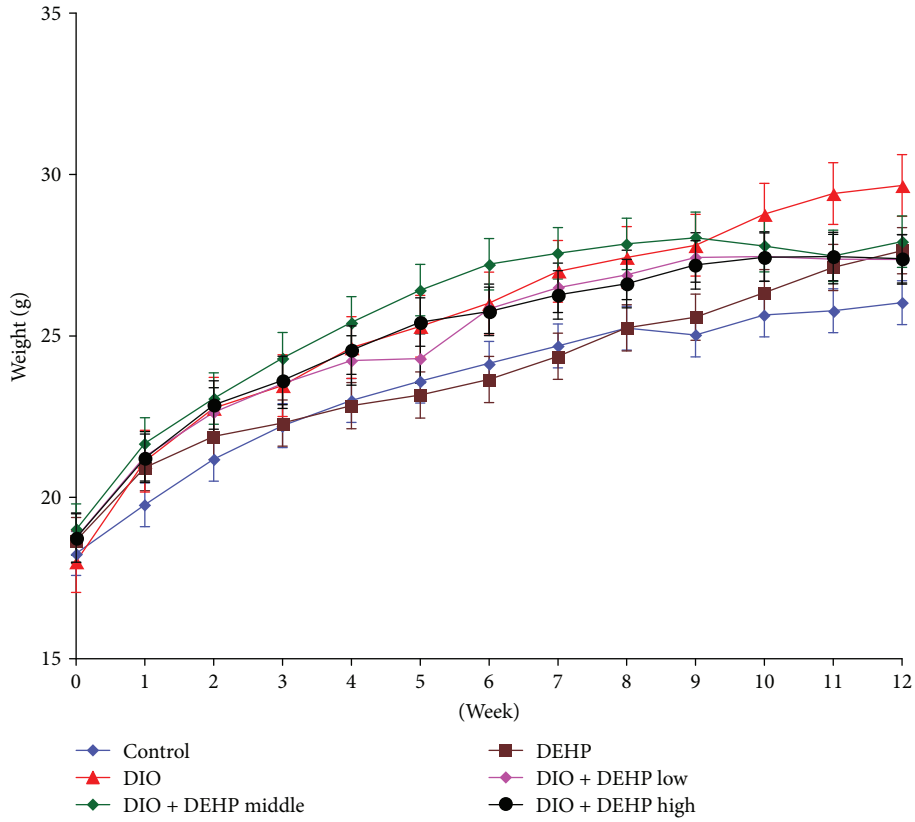

Figure 2: The body weight changes in the six experimental groups in week 12.

DEHP high mice compared to the control mice ($P < 0.01$). There was a significant decrease in sperm motility and sperm count in the DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice compared with the DEHP mice ($P < 0.05$). In addition, sperm motility in the DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice was lower than that in the DIO mice ($P < 0.05$) and the sperm count of the DIO + DEHP high mice was lower than that in the DIO mice ($P < 0.05$).

3.3. Light Microscopy in the 6 Experimental Groups of Mice. To confirm the effects of exposure to the high-fat diet and DEHP on morphological changes in testicular tissue, we performed HE staining. Light microscopic images showed that morphological changes had occurred in the testicular cells after 12 weeks (Figure 3). In the control group (Figure 3(a)), the structure of the seminiferous tubules was normal and complete with slight edema in the Leydig cells. The arrangement of Sertoli cells and germ cells appeared to be slightly irregular in the DIO (Figure 3(b)), DEHP (Figure 3(c)), DIO + DEHP low (Figure 3(d)), and DIO + DEHP middle (Figure 3(e)) groups. In the DIO + DEHP high group (Figure 3(f)), the Leydig cells showed edema. The number and lines of Sertoli cells and germ cells reduced obviously.

3.4. Electron Microscopy in the 6 Experimental Groups of Mice. Electron microscopy was performed on the mouse testes in week 12. In the control group (Figure 4(a)), abundant organelles were found in the Leydig cells. We also found smooth and rough endoplasmic reticulum, with only minimal lysosomes and lipid droplets. The chromatin had a light color, the Leydig cells had normal morphology. In the DIO group (Figure 4(b)), DEHP group (Figure 4(c)), DIO + DEHP low group (Figure 4(d)), and the DIO + DEHP middle group, the cytoplasm and organelles were reduced. The mitochondria were swollen and deformed with an increased number of lipid droplets. An irregular karyotype and heterochromatin side set was identified in the Leydig cells from the DIO group (Figure 4(b)), DEHP group (Figure 4(c)), DIO + DEHP low group (Figure 4(d)), and the DIO + DEHP middle group (Figure 4(e)). In the DIO + DEHP high group (Figure 4(f)), the Leydig cells showed vacuolization of the nucleus and cytoplasm; the mitochondria were swollen and deformed, and the number of organelles was reduced.

3.5. Serum Sex Hormone and Leptin Levels in the 6 Experimental Groups of Mice. As shown in Table 2, DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice exhibited decreased fasting levels of testosterone at 12 weeks ($P < 0.05$). Furthermore, the testosterone level of DIO + DEHP high mice was significantly lower than either DEHP or DIO mice ($P < 0.05$).

DEHP, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice exhibited increased fasting levels of estradiol at 12 weeks ($P < 0.05$; Table 2). The levels of estradiol in DEHP, DIO + DEHP low, DIO + DEHP middle, and

Table 1: The reproductive organs weight, sperm motility, count, and retroperitoneal, and epididymal fat weight in 12 weeks ($\bar{x} \pm SD$).

Group	n	Relative Tes. weight (g/100 g)	Relative epididymis weight (g/100 g)	Relative Sem. weight (g/100 g)	Relative liver weight (g/100 g)	Relative kidney weight (g/100 g)	Relative Epi fat (g/100 g)	Relative Ret fat (g/100 g)	Sperm motility (%)	Sperm count ($\times 10^6$/ml)
Control	10	0.85 ± 0.06	0.29 ± 0.02	0.98 ± 0.11	4.48 ± 0.30	1.36 ± 0.10	1.25 ± 0.37	0.26 ± 0.09	31.03 ± 3.08	4.75 ± 0.50
DEHP	10	0.71 ± 0.06^{b}	0.27 ± 0.01^{b}	0.96 ± 0.14	3.97 ± 0.24^{b}	1.24 ± 0.04^{b}	2.47 ± 0.61^{b}	0.69 ± 0.16^{b}	23.47 ± 2.24^{b}	4.05 ± 0.44^{b}
DIO	10	0.74 ± 0.07^{b}	0.26 ± 0.01^{b}	0.94 ± 0.11	3.83 ± 0.16^{b}	1.24 ± 0.07^{b}	3.04 ± 0.82^{b}	0.92 ± 0.37^{b}	23.33 ± 3.94^{b}	3.89 ± 0.41^{b}
DIO + DEHP low	10	0.69 ± 0.08^{b}	0.26 ± 0.01^{b}	0.95 ± 0.09	3.73 ± 0.20^{bc}	1.20 ± 0.07^{b}	2.35 ± 0.39^{bf}	0.68 ± 0.16^{bef}	20.49 ± 2.03^{bce}	3.60 ± 0.44^{bc}
DIO + DEHP middle	10	0.69 ± 0.14^{b}	0.25 ± 0.02^{bc}	0.95 ± 0.18	3.52 ± 0.20^{bdf}	1.17 ± 0.03^{bce}	1.84 ± 0.50^{adf}	0.45 ± 0.15^{af}	19.19 ± 1.17^{bdf}	3.51 ± 0.42^{bc}
DIO + DEHP high	10	0.69 ± 0.05^{b}	0.23 ± 0.02^{bdfhj}	0.89 ± 0.16	3.32 ± 0.23^{bdf}	1.14 ± 0.05^{bdf}	1.70 ± 0.25^{df}	0.42 ± 0.13^{df}	17.15 ± 1.78^{bdf}	3.42 ± 0.50^{bde}

Data are mean ± SD. $^{b}P < 0.01$ denotes statistical significance compared with the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ denote statistical significance compared with the DEHP group; $^{e}P < 0.05$ and $^{f}P < 0.01$ denote statistical significance compared with the DIO group; $^{h}P < 0.01$ denotes statistical significance compared with the DIO + DEHP low group; $^{j}P < 0.01$ denotes statistical significance compared with the DIO + DEHP middle group. Testis: Tes; seminal vesicles: Sem; retroperitoneal: Ret. epididymal: Epi. Relative Tes. weight = testis weight/body weight × 100. Relative epididymis weight = epididymis weight/body weight × 100. Relative Sem. weight = seminal vesicle weight/body weight × 100. Sperm motility = total motile sperm/all count sperm × 100. Relative liver weight = liver weight/body weight × 100. Relative kidney weight = kidney weight/body weight × 100. Relative Ret fat weigh = retroperitoneal fat weight/body weight × 100. Relative Epi fat weight = epididymal fat weight/body weight × 100.

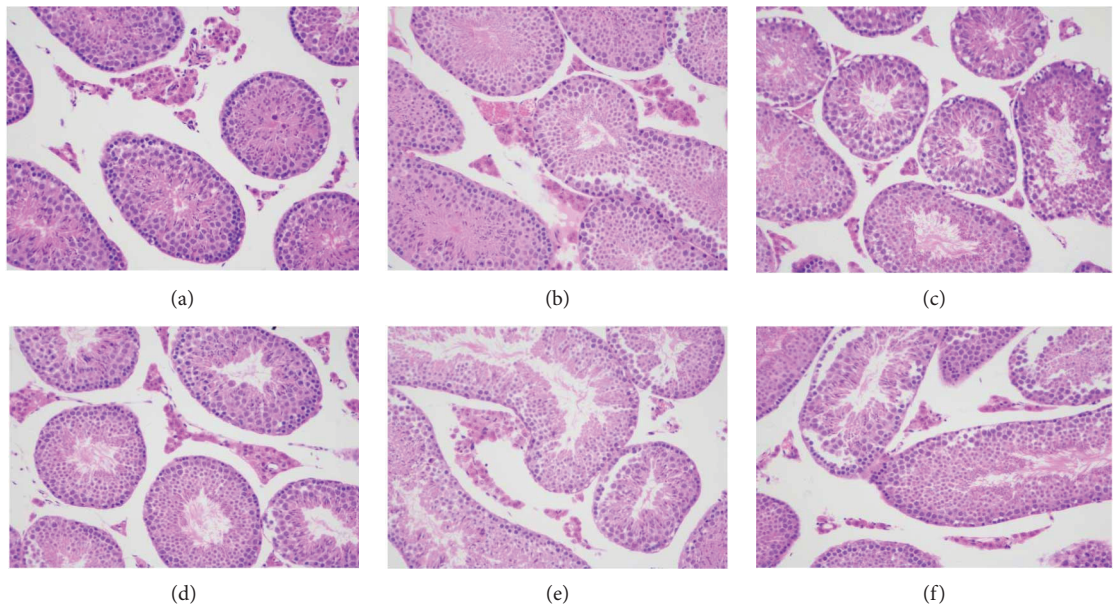

Figure 3: Light microscopic changes of the Leydig cells in six experimental groups in week 12. Light microscopic images showing morphological changes in testicular cells in week 12. Images show the control group (a), DIO group (b), DEHP group (c), DIO + DEHP low group (d), DIO + DEHP middle group, and (e) DIO + DEHP high group (f). Sections were stained with HE staining. Magnification ×40.

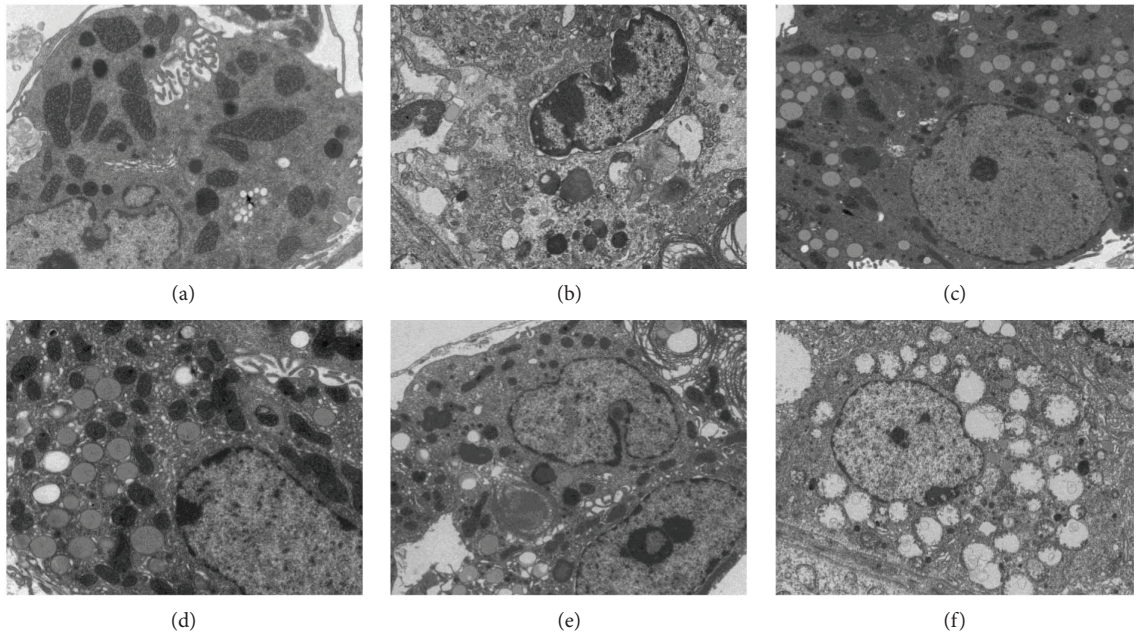

Figure 4: Electron microscopy changes in Leydig cells of the six groups of mice in week 12. Electron microscopy graphs showing lipid droplets, irregular karyotype, and heterochromatin side set of Leydig cells in the DIO group (b), DEHP group (c), DIO + DEHP low group (d), DIO + DEHP middle group, (e) and DIO + DEHP high group (f) compared to the control group (a).

DIO + DEHP high mice were significantly higher compared to the DIO mice ($P < 0.01$; Table 2).

DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice exhibited increased fasting levels of leptin at 12 weeks ($P < 0.05$; Table 2). The levels of leptin in DIO + DEHP middle and DIO + DEHP high mice were significantly higher compared to the DEHP, DIO, and DIO + DEHP low mice ($P < 0.01$; Table 2).

3.6. MDA, T-AOC, SOD, GSH, H_2O_2, CAT, and GSH-PX Levels of Testicular Tissue. The effect of obesity and DEHP on biomarkers of oxidative stress is shown in Table 3.

At 12 weeks, obesity and DEHP had caused an increase in the levels of MDA in testis tissue to 130%, 127%, 152%, 155%, and 164% of the control in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice,

Table 2: Testosterone, estradiol, and leptin level in the 6 experimental groups ($\bar{x} \pm SE$).

Group	n	Testosterone (ng/ml)	Estradiol (ng/ml)	Leptin (ng/ml)
Control	10	4.38 ± 0.15	29.69 ± 2.38	1.71 ± 0.11
DEHP	10	2.99 ± 0.23^{b}	80.36 ± 9.69^{b}	4.01 ± 0.72^{a}
DIO	10	3.02 ± 0.21^{b}	47.14 ± 7.01^{d}	4.07 ± 0.81^{a}
DIO + DEHP low	10	2.35 ± 0.42^{b}	90.42 ± 6.35^{bf}	6.01 ± 0.76^{b}
DIO + DEHP middle	10	2.36 ± 0.30^{b}	94.04 ± 6.91^{bf}	9.10 ± 0.95^{bdfh}
DIO + DEHP high	10	2.18 ± 0.23^{bce}	86.49 ± 10.00^{bf}	10.04 ± 0.68^{bdfh}

Data are mean ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ denote statistical significance compared with control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ denote statistical significance compared with the DEHP group; $^{e}P < 0.05$ and $^{f}P < 0.01$ denote statistical significance compared with the DIO group; $^{h}P < 0.01$ denotes statistical significance compared with the DIO + DEHP low group.

Table 3: MDA, T-AOC, SOD, GSH, H_2O_2, CAT, and GSH-PX levels of testis tissue of the 6 experimental groups in 12 weeks ($\bar{x}$±SD).

Group	n	MDA (nmol/mg prot)	T-AOC (U/mg prot)	SOD (U/mg prot)	GSH (mgGSH/g prot)	H_2O_2 (mmol/g prot)	CAT (U/mg prot)	GSH-PX (U/mg prot)
Control	10	3.3 ± 0.5	0.5 ± 0.2	196.7 ± 37.7	114.8 ± 15.6	3.1 ± 0.58	16.0 ± 0.9	55.7 ± 6.8
DEHP	10	4.3 ± 0.5^{b}	0.5 ± 0.2	90.5 ± 28.4^{b}	99.5 ± 13.5^{a}	2.8 ± 0.61	14.1 ± 1.0^{b}	49.8 ± 10.9
DIO	10	4.2 ± 0.8^{b}	0.5 ± 0.1	135.6 ± 19.4^{b}	91.8 ± 16.3^{b}	7.6 ± 1.7^{b}	14.4 ± 1.8^{b}	40.4 ± 7.1^{b}
DIO + DEHP low	10	5.0 ± 0.7^{bce}	0.4 ± 0.1^{c}	106.2 ± 25.6^{be}	73.7 ± 17.6^{bdf}	8.2 ± 1.5^{bd}	14.3 ± 1.1^{b}	39.3 ± 5.6^{bc}
DIO + DEHP middle	10	5.1 ± 0.7^{bcf}	0.4 ± 0.1^{ade}	89.6 ± 35.2^{bf}	47.6 ± 11.1^{bdfh}	9.7 ± 2.36^{bdfg}	13.2 ± 0.9^{beh}	29.5 ± 3.1^{bdeg}
DIO + DEHP high	10	5.4 ± 0.7^{bdf}	0.3 ± 0.1^{bdfg}	68.4 ± 10.9^{bfh}	38.2 ± 11.8^{bdfh}	11.4 ± 2.2^{bdfhi}	11.4 ± 1.0^{bdfh}	26.3 ± 6.2^{bdfh}

Data are mean ± SD. $^{a}P < 0.05$ and $^{b}P < 0.01$ denote statistical significance compared with the control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ denote statistical significance compared with the DEHP group; $^{e}P < 0.05$ and $^{f}P < 0.01$ denote statistical significance compared with the DIO group; $^{g}P < 0.05$ and $^{h}P < 0.01$ denote statistical significance compared with the DIO + DEHP low group; $^{i}P < 0.05$ denotes statistical significance compared with the DIO + DEHP middle group.

respectively ($P < 0.01$). Furthermore, the levels of MDA in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly higher compared to the DEHP and DIO mice ($P < 0.05$; Table 3).

At 12 weeks, the obesity and DEHP had reduced T-AOC levels to 80% and 60% of the controls in the DIO + DEHP middle and DIO + DEHP high mice, respectively ($P < 0.05$). The levels of T-AOC in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly lower compared to the DEHP mice ($P < 0.05$). Levels of T-AOC in DIO + DEHP middle and DIO + DEHP high mice were significantly lower compared to the DIO mice ($P < 0.05$). We also found that the levels of T-AOC in DIO + DEHP high mice were significantly lower compared to the DIO + DEHP low mice ($P < 0.05$; Table 3).

At 12 weeks, the obesity and DEHP had reduced SOD levels to 46%, 68.9%, 54%, 45.6%, and 34.7% of the control in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice, respectively ($P < 0.01$). Furthermore, the levels of SOD in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly lower compared to the DIO mice ($P < 0.05$). We also found that the levels of SOD in DIO + DEHP high mice were significantly lower compared to the DIO + DEHP low mice ($P < 0.05$; Table 3).

At 12 weeks, obesity and DEHP had reduced GSH levels to 87%, 80%, 64%, 41%, and 33% of the controls in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice, respectively ($P < 0.05$). Levels of GSH in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly lower compared to the DEHP and DIO mice ($P < 0.01$). We also found that the levels of GSH in DIO + DEHP middle and DIO + DEHP high mice were significantly lower compared to the DIO + DEHP low mice ($P < 0.01$; Table 3).

At 12 weeks, obesity and DEHP had increased H_2O_2 levels by 2.53-fold, 2.65-fold, 3.13-fold, and 3.68-fold relative to the controls in the DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice, respectively ($P < 0.01$). The levels of H_2O_2 in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly higher compared to the DEHP mice ($P < 0.05$). Furthermore, levels of H_2O_2 in DIO + DEHP middle and DIO + DEHP high mice were significantly higher compared to the DIO mice ($P < 0.05$). We also found that levels of H_2O_2 in DIO + DEHP middle and DIO + DEHP high mice were significantly higher compared to DIO + DEHP low mice ($P < 0.05$). Furthermore, the high-fat diet and DEHP had increased H_2O_2 levels by 1.18-fold in the DIO + DEHP middle and the DIO + DEHP high mice ($P < 0.05$; Table 3).

At 12 weeks, obesity and DEHP had reduced CAT levels to 88%, 90%, 89%, 83%, and 71% of the controls in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice, respectively ($P < 0.01$). CAT levels in DIO + DEHP high mice were significantly lower compared to DEHP mice ($P < 0.05$). Furthermore, the levels of CAT in the DIO + DEHP middle and DIO + DEHP high mice were significantly lower compared to DIO mice ($P < 0.05$). We

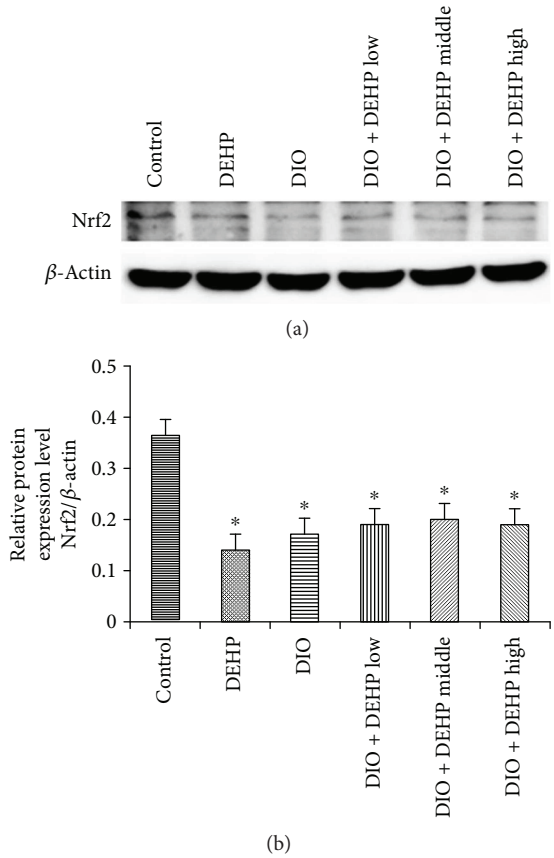

Figure 5: Expression of Nrf2 protein in the 6 experimental groups of mice. The upper bands (a) depict representative findings in the control, DIO, DEHP, DIO + DEHP, DIO + DEHP middle, and DIO + DEHP high groups. The lower bar graphs (b) show the results of the semiquantitative measurement of Nrf2. Each bar represents mean ± SE. $n = 4$. * indicates a significant difference from the control group, $P < 0.05$.

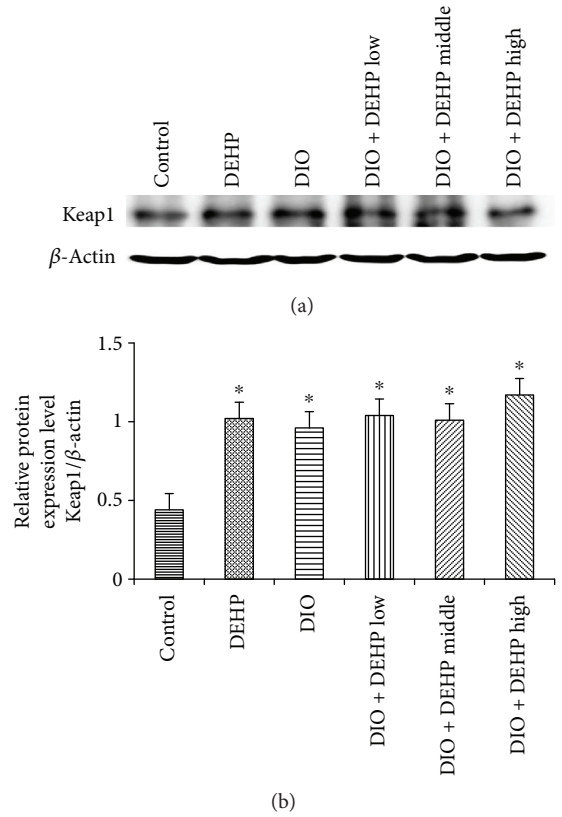

Figure 6: Expression of Keap1 Protein in the 6 experimental groups of mice. The upper bands (a) depict representative findings in the control, DIO, DEHP, DIO + DEHP, DIO + DEHP middle, and DIO + DEHP high groups. The lower bar graphs (b) show the results of the semiquantitative measurement of Keap1. Each bar represents mean ± SE. $n = 4$. * indicates a significant difference from the control group, $P < 0.05$.

also found that the levels of CAT in DIO + DEHP middle and DIO + DEHP high mice were significantly lower compared to DIO + DEHP low mice ($P < 0.05$; Table 3).

At 12 weeks, the high-fat diet and DEHP had reduced GSH-PX levels to 73%, 71%, 53%, and 47% of the controls in the DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice, respectively ($P < 0.01$). The levels of GSH-PX in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly lower compared to DEHP mice ($P < 0.05$. Furthermore, the levels of GSH-PX in DIO + DEHP middle and DIO + DEHP high mice were significantly lower compared to DIO mice ($P < 0.05$). We also found that the levels of GSH-PX in DIO + DEHP middle and DIO + DEHP high mice were significantly lower compared to the DIO + DEHP low mice ($P < 0.05$; Table 3).

3.7. Expression of Nrf2 Protein in the 6 Experimental Groups of Mice. Levels of Nrf2 protein were detected by Western blotting. Results indicated that Nrf2 expression was inhibited in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high groups compared to the control group (Figure 5). Obesity and DEHP significantly reduced levels of Nrf2 protein to 39%, 48%, 53%, 55%, and 52% of the controls in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high group ($P < 0.05$).

3.8. Expression of Keap1 Protein in the 6 Experimental Groups of Mice. Levels of Keap1 protein were detected by Western blotting. Results indicated that Keap1 expression increased in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high groups compared to the control group (Figure 6). Obesity and DEHP significantly increased the levels of Keap1 protein by 130%, 117%, 135%, 129%, and 167% in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high groups, respectively, compared to the controls ($P < 0.05$).

4. Discussion

In this study, the weight of DEHP mice was higher than the control mice; however, there was no significant difference in weight between the DEHP and control group prior to exposure to DEHP. We found that DEHP, as an estrogenic endocrine disruptor, may increase the weight of mice. Kim et al. found that DEHP exposure may affect body mass

change in early life through changes of obesity-related markers [17]. In another study, Lv et al. suggested that chronic DEHP exposure could induce obesity by interrupting energy homeostasis [18]. After joint exposure to a high-fat diet and DEHP, the weight of DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were higher than the control mice, although this difference was only statistically significant in the DIO + DEHP middle mice. However, the weight of DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high DIO mice was lower than the DIO mice. Consequently, we did not identify a joint effect of DEHP and obesity upon body weight in mice. There are three possible reasons for this: (1) there was a different mechanism of body weight gain between the high-fat diet and DEHP; (2) the exposure time was not long enough; and (3) DEHP exerted a toxicity effect. The joint effect of a high-fat diet and DEHP upon body weight should therefore be investigated more thoroughly.

Twelve weeks after joint exposure to a high-fat diet and DEHP, we found the following effects: (1) a reduction in the relative epididymis coefficient; (2) a decline in sperm motility; and (3) pathological damage to the Leydig cells (as shown by both light microscopy and transmission electron microscopy). Consequently, male obesity and DEHP may concomitantly cause hypogonadism.

Testosterone is the most important sex hormone in males and plays a critical role in testis development, spermatogenesis, and the maintenance of normal masculinization. Other studies have found that lower plasma testosterone levels played an important role in male hypogonadism caused by obesity [7, 8]. In this study, we identified significantly lower serum testosterone levels in DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice when compared to the control group. Furthermore, the testosterone level of DIO + DEHP high mice was significantly lower than DEHP and DIO mice. Consequently, there was a joint effect leading to reduced testosterone levels in DIO + DEHP high mice.

To investigate the mechanisms underlying the joint effect of DEHP and obesity on low testosterone levels, we determined the levels of leptin and oxidative stress in the testicular tissue. Leptin is expressed predominantly in adipose tissue and can reduce appetite and increase energy expenditure [19]. Leptin also plays an important role in male reproduction [20, 21]. Leptin receptors are also distributed in the testicular tissue [22] which suggests that leptin has a direct effect upon the testis. Previous work has found that leptin levels are inversely correlated with testosterone level in both boys and adult males [23, 24]. In the present study, DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice exhibited increased fasting levels of leptin at 12 weeks. The serum concentration of leptin was inversely correlated with testosterone at 12 weeks. Consequently, in line with our previous findings [9], our present data indicate that leptin levels in obese mice were higher than in normal mice. Isidori et al. also found a clear relationship between high leptin levels and low testosterone levels in obese males [25]. High levels of leptin can inhibit testosterone levels in obese males [25].

DEHP is known to exert weak estrogenic properties. Furthermore, DEHP acts as an endocrine disruptor owing to its ability to compete with endogenous steroid hormones binding to receptors [26]. There is sufficient evidence in rodents that phthalate exposure causes developmental and reproductive toxicities; DEHP may cause dysmorphic disorders of the genital tract in infant males [27]. Rats exposed to DBP/BBP/MBP (toxic metabolite products of DEHP) during the perinatal period are known to induce reproductive disorders, such as low sperm counts [28]. Lv et al. suggested that chronic DEHP exposure could induce obesity and increase leptin levels [18] while Sena et al. found that tributyltin chloride (a type of environmental estrogen) can increase leptin level in female rats [29]. In the present study, compared to the control mice, the leptin levels of DEHP mice were higher, and the testosterone levels were lower. DEHP may also increase the levels of leptin in mice, in a manner similar to the fact that high leptin levels can inhibit testosterone levels in obese males. High leptin levels can inhibit testosterone levels during exposure to DEHP. In order to identify if there was a joint effect on leptin level when mice were jointly exposed to obesity and DEHP, we designed an experiment featuring 3 groups for 3 levels of DEHP exposure. We found that the levels of leptin in DIO + DEHP middle and DIO + DEHP high mice were significantly higher compared to DEHP, DIO, and DIO + DEHP low mice. Thus, obesity and DEHP had a joint effect on leptin level. That is to say that high leptin levels may be one of the main mechanisms underlying the low testosterone level caused by the concomitant exposure of mice to obesity and DEHP.

With regards to the mechanism of high leptin levels and low testosterone observed in obese mice in this study, Smith et al. found that high leptin levels were related to low kisspeptin levels [30]. High leptin may reduce testosterone level by downregulating the expression of kisspeptin [31]. Yuan et al. considered that a reduced p-STAT3 protein level in testicular tissue was related to leptin resistance and sex hormone dysregulation [32]. Yi et al. further found that obesity can inhibit testosterone biosynthesis by disrupting the testicular leptin transduction pathway (LEP–JAK2–STAT3 signal pathway) in the testis [33]. Therefore, the role of high leptin inhibition not only occurs at the hypothalamic-pituitary level but also at the gonadal level. The mechanisms underlying the joint effect of obesity and DEHP on leptin level should be studied further.

Oxidative stress in testicular tissue is another important factor to consider. Oxidative stress results from the production of oxygen radicals in excess of the antioxidant capacity of the stressed tissue. Increasing testicular oxidative stress may lead to subsequent hypospermatogenesis [34].

Testicular oxidative stress may also be associated with reduced testosterone levels in obese males [9, 35]. To investigate the mechanisms of low testosterone levels induced by the joint exposure to obesity and DEHP, we determined some markers of oxidative stress: MDA, T-AOC, SOD, GSH, H_2O_2, CAT, and GSH-PX expression.

At 12 weeks, MDA and H_2O_2 levels were higher in the DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice than in the control group.

Furthermore, the levels of MDA and H_2O_2 in DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice were significantly higher compared to DEHP and DIO mice. There is evidence that H_2O_2, besides acting as independent signaling molecules, may also interrelate to form an oxidative death cycle [34, 36]. Obesity and DEHP jointly caused oxidative damage within the testicular tissue. These results suggest that obesity and DEHP induced excessive oxidative stress and may affect the histological structure and function of the testicular tissue.

The levels of several antioxidant enzymes (SOD, GSH, CAT, and GSH-PX) were found to be reduced in DEHP, DIO, DIO + DEHP low, DIO + DEHP middle, and DIO + DEHP high mice when compared to the control group at 12 weeks. Similar to our prior study, Erdemir et al. also found that SOD and GSH-PX were reduced in male rat offspring when the mother was obese [37]. Other researches have shown that exposure to endocrine disruptors which diminish the intratesticular concentration of testosterone may also inhibit the testicular expression of antioxidant enzymes such as GPx, SOD, and catalase [38, 39]. We also found that the levels of GSH, CAT, and GSH-PX in DIO + DEHP high mice were significantly lower compared to DEHP and DIO mice. Obesity and DEHP jointly reduced the level of antioxidant enzymes in the male testis tissue.

This study tries to identify the mechanism underlying the effect of oxidative stress caused by the joint effects of obesity and DEHP. NFE2-related factor 2 (Nrf2) is a central regulator of antioxidant and detoxification gene expression in response to electrophilic or oxidative stress [40]. Under homeostatic conditions, Nrf2 is repressed via cytoplasmic tethering and ubiquitination, mediated by the redox-sensitive Kelch-like ECH-associated protein 1 (Keap1) [41–43] and is constitutively degraded via the ubiquitin-proteasome pathway in the cytoplasm [43]. In this study, Keap1 expression increased while Nrf2 expression decreased along with the activities of other enzymatic antioxidants. Thus, oxidative stress caused by obesity and DEHP in the testes may be improved by inhibiting the Nrf2 antioxidant pathway. Thus, high MDA and H_2O_2 levels, and low GSH, CAT, and GSH-PX levels, may contribute to the low levels of testosterone induced by obesity and DEHP in testicular tissue. The low expression of Nrf2 and high expression of Keap1 may have contributed to the low expression of GSH, CAT, and GSH-PX.

In conclusion, the joint exposure of mice to obesity and DEHP caused pathological damage to the Leydig cells, increased serum leptin levels, and caused reductions in sperm count, motility, relative epididymis weight, and testosterone level. The activity of GSH, CAT, and GSH-PX enzymes was also reduced, as was the expression of Nrf2 . However Keap1 expression increased. We conclude that high levels of leptin and oxidative stress in testicular tissue may provide some evidence to clarify the mechanisms of male SH in obesity and DEHP.

Conflicts of Interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

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Review Article

Preventive and Therapeutic Role of Functional Ingredients of Barley Grass for Chronic Diseases in Human Beings

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Barley grass powder is the best functional food that provides nutrition and eliminates toxins from cells in human beings; however, its functional ingredients have played an important role as health benefit. In order to better cognize the preventive and therapeutic role of barley grass for chronic diseases, we carried out the systematic strategies for functional ingredients of barley grass, based on the comprehensive databases, especially the PubMed, Baidu, ISI Web of Science, and CNKI, between 2008 and 2017. Barley grass is rich in functional ingredients, such as gamma-aminobutyric acid (GABA), flavonoids, saponarin, lutonarin, superoxide dismutase (SOD), K, Ca, Se, tryptophan, chlorophyll, vitamins (A, B1, C, and E), dietary fiber, polysaccharide, alkaloid, metallothioneins, and polyphenols. Barley grass promotes sleep; has antidiabetic effect; regulates blood pressure; enhances immunity; protects liver; has anti-acne/detoxifying and antidepressant effects; improves gastrointestinal function; has anticancer, anti-inflammatory, antioxidant, hypolipidemic, and antigout effects; reduces hyperuricemia; prevents hypoxia, cardiovascular diseases, fatigue, and constipation; alleviates atopic dermatitis; is a calcium supplement; improves cognition; and so on. These results support that barley grass may be one of the best functional foods for preventive chronic diseases and the best raw material of modern diet structure in promoting the development of large health industry and further reveal that GABA, flavonoids, SOD, K-Ca, vitamins, and tryptophan mechanism of barley grass have preventive and therapeutic role for chronic diseases. This paper can be used as a scientific evidence for developing functional foods and novel drugs for barley grass for preventive chronic diseases.

1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world and has the highest dietary fiber content; its malt for functional food is not only the world's largest material for beer, but also often used as one of 300 species being used in Chinese herbal medicine. Regular consumption of whole grain barley and its hydroalcoholic extract reduces the risk of chronic diseases (diabetes, cancer, obesity, cardiovascular disease, etc.), based on phytochemicals including β-glucan, phenolic acids, flavonoids, lignans, tocols, phytosterols, and folate [1, 2]. Barley with preventive inflammatory and cardiovascular diseases has exhibited activities against all human platelet agonists inhibited both cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism, which elevated the SOD and GSH-Px activities [3].

Barley with cold and frost tolerance of growing at 4000 m is a key for ancient Tibetans climb to 3400 m [4]; Tibetan Plateau is an important origin and domestication base of cultivated barley [5]. Human *Flt3* ligand from barley is a glycoprotein including α(1,3)-fucose and α(1,2)-xylose, which showed expression of human growth factor in barley grains with active protein [6]. The amino acid concentration in

barley grass irradiated by artificial light (red 9 + blue 1) is greater than that by natural light, which can increase γ-tocopherol by 100% red light [7], but cyanogenic glucosides content is 4% less than that by sunlight [8]. The accumulation of lutonarin (isoorientin-7-*O*-glucoside) and 3-feruloylquinic acid ($C_{17}H_{20}O_9$) and xanthophyll-cycle pigments is greatly increased by high photosynthetically active radiation and ultraviolet exposure in barley leaves [9]. Chronic disease of human beings is associated with the five evolutionary stages of the major dietary guidelines (i.e., the healthiest major dietary guidelines for modern humans): fruits or vegetables, grass or Cyperaceou, cereals (rice, wheat, millet, beans, barley, and corn), polished rice or wheat flour, and white rice or wheat flour + grass powder [10].

Barley grass (BG) has young green leaves and stem of vegetative growth stage from seedling at 10 days after sprouting (barley sprout) to elongation stage (barley green) for nutritional peak before the start of reproductive cycle of barley [11–13]; however, *Vrs2* is associated with floral architecture by regulating hormonal homeostasis and gradients in barley [14]. BG is not only consumed as a popular green-colored drink [15], but also used in preventive chronic diseases, especially circulatory disorders, anticancer, reducing obesity, antidiabetes, anti-arthritis, reducing cholesterol, antioxidant, and anti-inflammation [12]. Light can promote cytokinin degradation and the formation of bioactive cytokinins in barley leaves, which has a positive correlation between cytokinin oxidase/dehydrogenase activity and senescence in most cases [16]. The amino acid and vitamin C content in hydroponic BG are higher than those in organic soil [17]. In spray-dried barley grass powder with good solubility and small size, its contents of the chlorophyll, flavonoids, and SOD enzyme activity are 56.7%, 68.1%, and 47.9% of vacuum freeze-dried powder with high nutrition and good color, respectively [18]. Although BG has played an important role in human health, coevolution and functional ingredients as well as major mechanism in therapeutic role between preventive chronic diseases and young barley grass for functional foods of human beings are unclear.

2. Functional Ingredients of Barley Grass

Barley grass is rich in nutritious and functional ingredients, in which major ingredients content according to dried barely grass include dietary fiber 29.5%, protein 27.3%, fat 4.57%, vitamin A 20.5 mg/100 g, vitamin C 251.6 mg/100 g, Ca 479.4 mg/100 g, S 305.5 mg/100 g, Cr 0.14 mg/100 g, Fe 23.3 mg/100 g, Mg 183.2 mg/100 g, K 3384 mg/100 g, chlorophyll 528.5 mg/100 g, SOD 440.0 U/g, catalase 839 U/g, lutonarin 342.9 mg/100 g, saponarin 726.2 mg/100 g, total flavonoid 0.53%, total polyphenol 1.06%, ABTS (RC50) 53.3 μg/mL, GABA 150.5 mg/100 g, and tryptophan 810.0 mg/100 g (see Table 1). Generally, the content of nutritious and functional ingredients is very different depending on the growth stage of barley grass or processing technology or various cultivars; for example, the sodium content in mountainous region is low but high in saline and alkaline land as well as vegetable land, and the content of dietary fiber at seedling stage is low but high at elongation stage. There are greater differences of saponarin and lutonarin contents in barley leaves at the growth stage; in particular, its lutonarin content at shooting period is 6.4 times higher than that at one leaf period, and its saponarin content in two leaves at one period is 6.5 times higher than that at heading period [11]. There are greater differences of tryptophan contents in barley leaves under three light sources [7]. Many studies have shown that BG contains significant quantities of Ca, Fe, Zn, K, Mg, folic acid, β-carotene, chlorophyll, pantothenic acid, vitamin C, and vitamin B12 [12]. Mean contents of chlorophyll (SPAD value), soluble solids, betaine, and flavonoid in BG of 100 cultivars are 44.53, 70.39 mg/g fresh weigh (FW), 2333.99 μg/g FW, and 4114.25 μg/g FW, respectively [25]. BG contains 30 times thiamine ($C_{12}H_{16}N_4OS$) and 11 times Ca than that of cow's milk, 6.5 times carotene and 5 times Fe content of spinach, 7 times vitamin C ($C_6H_8O_6$) in oranges, 4 times thiamine in whole wheat flour [12, 26], 2 times protein in barley grains [27], its total flavonoids and alkaloids are 2.1 times, 10.7 times, and GABA 37.8 times of brown rice [10].

2.1. GABA and Amino Acids. Gamma-aminobutyric acid is an inhibitory neurotransmitter that reduces neural excitability in the mammalian central nervous system with three subclasses of receptors, namely, relaxing, antianxiety, and anticonvulsive; alleviates pain; regulates sleep; and increases cognitive and reproductive effects [28]. GABA ($C_4H_9NO_2$), glutamic acid ($C_5H_9NO_4$), and $CaCl_2$ play significant roles in alleviating cold-induced effects by restoration of membrane integrity [29]. Barley bran is more efficient than wheat bran in the GABA production [30]. GABA contents of BG for Fudamai 1 and Fan 11 are 143~183 mg/100 g and 125~151 mg/100 g, respectively [24]. GABA can alleviate oxidative damage of H^+ and Al^{3+} toxicities in BG by activating antioxidant defense and reducing the carbonylated proteins [31]. BG contains 20 amino acids with energy production, cell building, and regeneration, especially 8 essential amino acids [12, 19].

2.2. Flavonoids. Higher dietary flavonoid intake associated with gastric cancer risk decreased in European population [32]. The microbiome contributes to diminished postdieting flavonoid levels and ameliorates excessive secondary weight gain [33]. Barley green contains total flavonoids of 1.12% and DPPH free radicals scavenging potential of 78.52%; however, betaine and total flavonoids can be kept at room temperature, but soluble protein and soluble total sugar and SOD could be better kept in cold storage [34]. The total flavonoid contents in BG increased from 273.1 to 515.3 CE mg/100 g between 13 and 56 days after sprouting; however, lutonarin (isoorientin-7-*O*-glucoside) has stronger radical scavenging activity than saponarin (isovitexin-7-*O*-glucoside); its antioxidant ability is improved with growth time, which exhibited high total polyphenol (44.37–55.07%) [13]. Total flavonoid extraction in BG is 94.66 mg/100 g [35]. There are 37 flavonoids, and hydroxycinnamates in BG include saponarin ($C_{27}H_{30}O_{15}$), lutonarin, isoorientin ($C_{21}H_{20}O_{11}$), isoscoparine ($C_{22}H_{21}O_{11}$), *C*-glycosyl flavones, *O*-glycosyl-*C*-glycosyl flavones, *O*-diglycosyl flavones, isoscoparin-7-

Table 1: Functional and nutrient compositions of dried barley grass.

Composition contents	Mean ± SD	Range	*N*	References
Calories (KJ/100 g)	1333 ± 315	1055~1607	4	[12, 19]
Sodium (mg/100 g)	328.2 ± 288.4	50~833	6	[12, 19]
Carbohydrates (%)	57.9 ± 5.1	55.2~64.0	4	[12, 19]
Dietary fiber (%)	29.5 ± 15.5	2.0~39.1	5	[12, 19]
Protein (%)	27.3 ± 4.3	19.9~34.1	7	[12, 19, 20]
Fat (%)	4.57 ± 1.31	2.30~6.03	6	[12, 19, 20]
Vitamin A (mg/100 g)	20.5 ± 4.7	14.4~25.0	4	[12, 19]
Vitamin B1 (mg/100 g)	0.61 ± 0.40	0.05~1.19	5	[12, 19, 20]
Vitamin B2 (mg/100 g)	1.56 ± 0.65	0.24~2.41	5	[12, 19, 20]
Vitamin B3 (mg/100 g)	7.18 ± 7.39	2.20~16.49	4	[12, 19]
Vitamin B6 (mg/100 g)	1.12 ± 0.97	0.18~2.58	5	[12, 19]
Vitamin B12 (mg/100 g)	1.16 ± 0.26	0.81~1.40	4	[12, 19, 20]
Vitamin C (mg/100 g)	251.6 ± 239.1	19.4~548.0	5	[19]
Vitamin E (mg/100 g)	15.0 ± 14.1	6.1~46.1	4	[19, 20]
Calcium (Ca, mg/100 g)	479.4 ± 172.5	330~819	6	[19–21]
Phosphorus (P, mg/100 g)	380.4 ± 60.7	283~430	5	[19, 21]
Chromium (Cr, mg/100 g)	0.14 ± 0.06	0.09~0.21	3	[19]
Copper (Cu, mg/100 g)	1.66 ± 1.25	0.72~3.50	6	[19–21]
Sulfur (S, mg/100 g)	305.5 ± 6.4	301~310	2	[19]
Iron (Fe, mg/100 g)	23.3 ± 10.1	10.0~41.2	6	[19–21]
Magnesium (Mg, mg/100 g)	183.2 ± 46.0	110~247.1	7	[19, 21]
Manganese (Mn, mg/100 g)	3.94 ± 1.56	2.3~5.4	5	[19, 21]
Molybdenum (Mo, mg/100 g)	0.048 ± 0.006	0.042~0.053	3	[19]
Potassium (K, mg/100 g)	3384 ± 649	2400~4300	7	[19, 21]
Zinc (Zn, mg/100 g)	3.43 ± 1.36	1.80~5.68	6	[19, 21]
Chlorophyll (mg/100 g)	542.9 ± 88.2	438~662	5	[17, 19, 22]
Superoxide dismutase (U/g)	440.0 ± 39.8	416~1382	4	[19]
Catalase (U/g)	839.0 ± 142.7	675~935	3	[19]
Lutonarin (mg/100 g)	342.9 ± 92.3	200.0~540.0	21	[11, 23]
Saponarin (mg/100 g)	726.2 ± 250.1	300.0~1260.0	21	[11, 23]
Total polyphenol (%)	1.06 ± 0.02	1.03~1.08	4	[13]
Total flavonoid (mg/100 g)	526.2 ± 52.7	487.5~593.4	5	[13, 17]
ABTS (RC50, μg/mL)	53.3 ± 9.1	41.8~63.8	4	[13]
GABA (mg/100 g)	150.5 ± 24.2	125~183	4	[24]
Tryptophan (mg/100 g)	736.7 ± 569.0	290~1400	6	[7]

O-glucoside derivatives, 7-*O*-[6-acyl]-glucoside, and -7-*O*-[6-acyl]-glc-4′-glucoside of isovitexin [36]. The major flavonoids from BG extract are isovitexin-7-*O*-glucoside (54.17%) and isoorientin-7-*O*-glucoside (33.36%) [37]. The major flavonoid antioxidants in BG are the flavone-*C*-glycosides, saponarin, and lutonarin [38]. Lutonarin and saponarin account for 71–75% of ten phenolics for BG, which contain 24.0 mg/100 g lutonarin and 14.0 mg/100 g saponarin [23, 39]. BG from Syrian contains the derivatives of flavonols, quercetin ($C_{15}H_{10}O_7$), and isorhamnetin ($C_{16}H_{12}O_7$), but flavonoids with glycosylation and acylation as well as hydroxycinnamate glycosides, esters, and amides in methanolic extracts from different regions of the world [40].

2.3. Enzymes. BG contains 300 enzymes of body utilization, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX, cellular imaging), aspartate aminotransferase (association with vitamin B6), cytochrome oxidase and hexokinase (association with mitochondria), deoxyribonuclease, fatty acid oxidase, malic dehydrogenase (allosteric regulation), nitrate reductase, RNase, P4D1, nitrogen oxyreductase, peroxidase, peroxidase catalase, phosphatase, phospholipase, polyphenol oxidase, transhydrogenase, and glycosyl isovitexin; but enzymes are not found in cooked foods [41, 42]. L-Phenylalanine ammonia lyase is the first enzyme in the biosynthesis of phenylpropanoid-derived plant compounds such

as flavonoids, coumarins, and the cell wall polymer lignin [43]. Antioxidant enzymes in BG include SOD, CAT, POD, APX, lipid peroxidation, protein oxidation, DNase activity, and DNA damage, which are oxidative biomarkers in response to Al^{3+} stress [44]. SOD has powerful anti-inflammatory activity; CAT is one antioxidant enzyme, which may provide resistance against many diseases, such as cancer [45]. The erythrocyte zinc and SOD activity are influenced by metabolic syndrome, plasmatic glucose, body mass index, and waist circumference [46]. Mean of SOD activity in barley leaves is 4.11 ± 1.31 U/mg [47]. Zn^{2+}, Cu^{2+}, and Mn^{2+} can inhibit significant CAT and SOD at higher contents, but Cd^{2+}, Hg^{2+}, and Pb^{2+} significantly restrain CAT and SOD at different contents in BG [48]. H_2S is a signaling molecule in plants and animals; H_2S treatment maintains higher POD activity in gibberellic acid-treated layers and higher SOD, POD, CAT, and APX activities in non-GA-treated barley aleurone layers [49].

2.4. Minerals. An increase in K^+ intake is a major nutritional approach in preventing hypertension, heart, and Alzheimer's disease as well as improving cognitive performance by decreasing inflammation and oxidative stress [10, 50]. Chronic kidney disease can cause cardiovascular disease and mortality, which is related with vascular calcification and abnormal electrolytes; however, hypocalcemia can cause mortality in patients with heart failure [51]. Due to their sulfide and quercetin mechanism in the treatment of chronic diseases, garlic and onion have anticancer properties; prevent cardiovascular and heart diseases; have anti-inflammatory properties; reduce obesity; have antidiabetic, antioxidant, antimicrobial, neuroprotective, and immunological effects; and so on [52]. BG contains the highest mineral content, especially potassium, calcium, iron, and sulfur (see Table 1); its K is 14.3 times, Ca 33.2 times, Fe 13.4 times, sulfur 3.3 times of brown rice [53]. The *qK1*/*qMg1*/*qCa1* region between markers Bmag0211 and GBMS0014 on chromosome 1H is shown to have large additive effects for Mg, Ca, and K concentrations in grains [54].

2.5. Chlorophyll. Chlorophyll and heme are the fundamental pigments of life. The biosynthetic pathways of methane production include chlorophyll, heme, and vitamin B12 [55]. The chlorophyll and soluble protein content in BG decreased with increasing seeding rate [22]. Photosystem II core dimers began to dissociate monomers at 40–50°C for heated (1°C/min) barley leaves, and chlorophyll-containing protein complexes appeared at 57–60°C [56]. The rate of CO_2 fixation and chlorophyll contents decreased, but flavonoids and carotenoids as well as enzyme activity increased, when etiolated barley seedlings at UV-B irradiation (312 nm) for 5 h [57]. Chlorophyll has anti-inflammatory and antioxidant properties and reduces fecal, urinary, and body odor [12]. Chlorophyll derivatives may play a significant role in anticancer activity, because it exhibits similar antimutagenic effect to 3-methylcholanthrene [58]. Chlorophyll content in barley grass is 542.9 mg/100 g (see Table 1); its extraction yield is 1364.6 mg/100 g [59]. The chlorophyll and total flavonoids content in barley grass under optimum combined drying conditions are 600.6 ± 19.2 and 569.5 ± 14.5 mg/100 g, respectively [17].

2.6. Vitamins. Fruit and vegetable (400 g/day) are associated with higher blood vitamin contents, especially antioxidant and B vitamins [60]. Diabetes is an oxidative inflammatory stress disease; however, it is necessary to monitor their vitamin B12 contents [61]. High vitamin C is used as homeostasis of brain-resident microglia [62]. Vitamin D deficiency is prevalent worldwide, which can prevent diabetes, cancer, depression, and so on [63]. Vitamins can treat nonalcoholic steatohepatitis (V_E) and chronic hepatitis C virus (V_{B12} and V_D), reduce gallstones (V_C), aphthous stomatitis (V_{B12}), and inflammatory bowel disease (V_D and V_{B1}), and so on [64]. The vitamin content (vitamin C, 0.52%, vitamin E, 73.06 mg/kg) of BG in Sebastian is higher than that in Malz (0.50%, 61.84 mg/kg) and AF Lucius (0.51%, 6.78 mg/kg) [65]. BG includes vitamin A 20.5 mg/100 g, V_{B1} 0.61 mg/100 g, V_{B2} 1.56 mg/100 g, V_{B6} 1.12 mg/100 g, V_E 15.0 mg/100 g, and V_C 251.6 mg/100 g (see Table 1).

2.7. Polyphenols. 500 polyphenols are distributed across a wide variety of foods; a protective role of dietary polyphenols against chronic diseases includes preventing cardiovascular diseases, diabetes, and cancer [66], due to their antioxidant and anti-inflammatory properties, and improving blood pressure and lipids as well as insulin resistance, which may reduce the risk of all-cause mortality [67]. The total polyphenol contents in BG increased from 776.6 to 1060.1 GAE mg/100 g between 13 and 40 days after sprouting, but decreased at day 56 to 982.6 GAE mg/100 g, in which it has higher antioxidant activity at 40 days after sprouting; $ABTS^+$ scavenging assay, the RC50 values of BG, decreased from 111.0 to 53.3 μg/mL between 13 and 40 days then increased to 55.3 μg/mL on day 56 [13]. The total phenolic content is 26.55 mg/100 g on 23 days after the sowing, but 13.91 mg/100 g on 56 days for BG, which are major hydroxycinnamic acid, orientin, isoorientin, and isovitexin derivatives [39].

3. Preventive Chronic Disease of Barley Grass

BG has the complete abundant nutrition including chlorophyll, superoxide dismutase, lutonarin, saponarin, vitamins, minerals, and eight essential amino acids [12, 68], but heat will destroy lots of nutritional values. Barley sprouts with saponarin showed anti-inflammatory and antioxidant activities; BG possess lots of health effects which include antioxidant, hypolipidemic, antidepressant, and antidiabetes [69]. Its manufacture is organically barley leaves squeezing, juice of low temperature, spray-dried in 3 seconds to stabilize grass powder [12]. BG has lots of health effect, such as hypolipidemic, hypoglycemic, preventive constipation, and anticancer, antioxidant, and anti-inflammatory activities [12]. Daily consumption of barley grass powder promotes sleep; regulates blood sugar and pressure; enhances immunity and liver function; detoxifies acne skin; improves gastrointestinal function; prevents constipation; has anticancer and anti-inflammatory effects; alleviates atopic dermatitis; loses weight and hypolipidemic; reduces gout and hyperuricemia; prevents heart

disease; has bone injury recovery, lustihood, and anti-fatigue effects; repairs memory; has antiaging effect; and so on [10].

3.1. Promote Sleep and Functional Ingredients of Barley Grass. Barley grass powder with higher GABA, Ca, K, and tryptophan contents is a very effective functional food in promoting sleep [70]. Sleep symptoms are associated with intake of specific dietary nutrients including Ca (OR = 0.83) and K (OR = 0.70) [71]. Barley grass powder for Yungong brand contains 62 times more GABA and 99 times more Ca as well as 31 times more K than that for polished rice [70]. The effective foods of improving sleep for modern people are polished rice or wheat flour plus BG powder and its products [70].

3.2. Antidiabetes and Functional Ingredients of Barley Grass. BG and its extract can scavenge oxygen free radicals, improve health, based on protective vascular diseases, and impair the pancreas endocrine of diabetic patients [72, 73]; its dietary fiber has a significant reduction in fasting blood sugar and blood glucose [74]. Saponarin in BG can control the postprandial blood glucose of diabetes [75]. Barley grass powder (1.2 g/day) within two months can significantly reduce fasting blood sugar, glycated hemoglobin, total cholesterol, and low-density lipoprotein (LDL) cholesterol, but significantly increase the high-density lipoprotein (HDL) cholesterol levels [76]. Hexacosanol in barley leaf can improve cholesterol metabolism by decreasing cholesterol synthesis [77]. Adenosine 5′-monophosphate-activated protein kinase in barley sprouts can regulate cell glucose that is a target for drugs against diabetes and obesity; however, policosanol content of 10 days after sprouting (3.437 g/kg) is 3 times higher than that of 5 days (1.097 g/kg) [78]. Polyamines in barley cells can increase under stress conditions, and it has a similar insulin function and antiglycating effect; however, the raising circulation of polyamines stop the glycation reaction under hyperglycemic concentrations [79].

3.3. Regulating Blood Pressure and Functional Ingredients of Barley Grass. The analogy porphyrin heads between chlorophyll (Mg) and hemoglobin (Fe) have an important therapeutic effect for chlorophyll in hemoglobin deficiency [12]. Chlorophyll and heme biosynthesis are regulated to adapt environment and plant development; 5-aminolevulinic acid provides for tetrapyrrole synthesis (Mg and Fe), however rapid dark inhibition of 5-aminolevulinic acid ($C_5H_9NO_3$) synthesis in BG [80]. Saponarin is a flavonoid found in BG that possesses potent regulating blood pressure [81]. Barley grass helps blood flow and digestion as well as general toxification of human's body, which related to superoxide dismutase and lutonarin as well as saponarin [12]. Barley grass powder with lowering hypertension has higher minerals (K and Ca) and GABA as well as lower Na [82]; its K, Ca, and GABA contents at autumn sowing under cold and high altitude (2010 m) are 3110, 845, and 377.46 mg/100 g, respectively [70]. Total free amino acid concentration varied very smaller, but the greater change from glutamate to GABA in BG and induced GABA gene expressions under cold acclimation and frost tolerance [83].

3.4. Enhance Immunity and Functional Ingredients of Barley Grass. Structural complexity of arabinoxylan of polysaccharide can be responsible for the immunomodulatory activity in young barley leaves [84]; high altitude cultivars (1200~3500 m) showed higher arabinoxylan (39.8–68.6%), anthocyanin (11.0–60.9%), β-glucan (7.5–30.8%), and metal chelating activity (16.6–43.2%) than plains (97–126 m) [85]. β-1,3-1,4-Glucan is a major accumulating component in cell wall of BG [86]. Glucuronoarabinoxylan and rhamnogalacturonan-I polysaccharide branched with arabinogalactan II side chain with immunostimulatory can be important for expression given the association with macrophage stimulatory activity in barley leaf [87].

3.5. Protective Liver and Functional Ingredients of Barley Grass. Barley sprouts with abundant saponarin possess the liver-protective effect by inhibiting the inflammatory response induced by alcohol [69]. Saponarin showed hepatoprotection and antioxidation against liver damage of CCl_4 in vitro and in vivo [88]. SOD enzymes are separated into three types of genes such as SOD1 (CuZn-SOD), SOD2 (Mn-SOD), SOD3 (extracellular SOD) in mammals. SOD1 (CuZn-SOD) deficiency can cause universal free radical damage and developing liver cancer in life [89].

3.6. Beauty Anti-Acne/Detox and Functional Ingredients of Barley Grass. Some exciting research has found that BG has the strongest ability to degrade six organophosphate pesticides; however, degradation of six pesticides (10 mg/L) in a 15% solution of young green barley leaves for 3 h at 37°C and pH 7.4 is malathion (100%, $C_{10}H_{19}O_6PS_2$) = chlorpyrifos (100%, $C_9H_{11}Cl_3NO_3PS$) > parathion (75%, $C_{10}H_{14}NO_5PS$) > diazinon (54%, $C_{12}H_{21}N_2O_3PS$) > guthion (41%, $C_{10}H_{12}N_3O_3PS$) > methidathion (23%, $C_6H_{11}N_2O_4PS_3$) [90]. Barley metallothioneins (MTs) have difference in intracellular homeostasis of metal ions specifically Cu detoxification, most MTs are downregulated by more Zn or Cd, and expression of MT1a, MT2b1, MT2b2, and MT3 in barley leaves increased more than 50 times during 10 d after germination [91].

3.7. Antidepressant and Functional Ingredients of Barley Grass. Depression not only is one of the most widely associated mental disorders in the world, but also has been associated with the risk to develop cancer, dementia, obesity, diabetes, blood pressure, atherosclerosis, epilepsy, and stroke. There is an important role for GABAergic, glutamatergic, and cholinergic receptors in the pathomechanism of depression [92, 93]. The reduce epilepsy and preventive dementia [10] as well as antidepressant of the young green barley leaf are regulated by inhibiting the hippocampus levels increased of nerve growth factor [94]. The vitamins and minerals in BG can prevent the stress-related psychiatric disorders of depression [95].

3.8. Improve Gastrointestinal Function and Functional Ingredients of Barley Grass. Young barley leaf powder with the water-insoluble dietary fiber can increase the fecal volume and laxative action by stimulating gut tract by the pH lowering [15]. BG is very effective in the treatment of

ulcerative colitis [96], pancreatitis, and disorders of the gastrointestinal tract [15]. A germinated barley foodstuff is helpful to reduce ulcerative colitis and improves symptoms by promoting the probiotics growth [97]. Selenium-enriched barley grass has significant ameliorative effect on ethanol-induced gastric ulcer in mice [98].

3.9. Anticancer and Functional Ingredients of Barley Grass. BG inhibits the cancer cell growth by the combined effects of high alkaline, strong antioxidative, phytochemicals, flavonoids, and chlorophyll [99, 100]. There is a very good antitumor effect for the phytochemical mixtures of BG in breast cancer [100]. BG can be served as health food for dialysis patients based on its absorbed exogenous functional ingredients applied from the outside [101]. Green barley extract has anticancer effect by its antiproliferative and proapoptotic functions on leukemia and lymphoma as well as breast cancer cells of human beings [102]. BG tricin can inhibits melanin production in melanoma cells, based on a hydroxyl group at the C-4′ position and methoxy groups at the C-3′,5′ positions of the tricin skeleton [103]. Yunnan province has the lowest cancer mortality and is the largest producer of anticancer barley, but Shanghai in China has the highest cancer mortality, which is associated with the sharp decline of barley [104].

3.10. Anti-Inflammation and Functional Ingredients of Barley Grass. BG has anti-inflammatory properties and heals the intestinal lining, which is used for gastrointestinal tract disorders, pancreatitis, recovering illness, and the treatment of ulcerative colitis [15, 96, 105]. Saponarin from barley sprouts is a very important functional ingredient of natural anti-inflammation [106]. Barley grass extract is very effective in the treatment of rheumatoid arthritis than that of SOD consumption [107, 108], which may be served as a supplement in the treatment of urologic and gynaecologic disorders as well as airway infections [12]. BG extract with antioxidant and anti-inflammation can be used as natural drug for the treatment of patients with rheumatoid arthritis by scavenge ROS and downregulate TNF-α production from peripheral blood and synovial fluid of patients [108]; however, green barley juice is part of arthritis therapy [109].

3.11. Antioxidants and Functional Ingredients of Barley Grass. Natural antioxidants in plant major include polyphenols, flavonoids, vitamins, and volatile chemicals [110]. Barley is among the most stress-tolerant crops, its flag leaf γ-tocopherol, glutathione and succinate content by same genes encoding enzymes of the pathways producing antioxidant metabolites [111]. The antioxidant phytonutrients of barley grass include the superoxide dismutase, 2″-*O*-glucosyl isovitexin (2″-*O*-GIV), and protoheme [112–114]. Flavonoids (lutonarin and saponarin) with antioxidative effects have been isolated from young barley [99, 115], in which lutonarin and saponarin contents in barley grass increase with UV exposure [36]. Saponarin in barley grass possesses strong antioxidant activities, which can prevent diseases caused by oxidative damage such as various cancers, inflammations, and cardiovascular diseases [81]. Isoorientin and orientin possessed potent antioxidant effects with IC_{50} values of 20.765 ± 651.1 and 27.565 ± 657.36508 M (DPPH) and 5.765 ± 650.3 and 8.265 ± 650.36508 M (ABTS), respectively [39]. Barley leaves extracted by methanol and ethanol may be alternatives to synthetic antioxidants in the food industry [114]. Barley leaf powder can be incorporated into raw minced pork as natural additives to retard oxidation [116]. Feeds supplemented with barley leaves containing antioxidants enhanced pork quality by increasing the levels of unsaturated oleic and gondoic acids [117].

3.12. Hypolipidemic and Functional Ingredients of Barley Grass. Barley green can modulate lipid metabolism, resist lipid peroxidation, improve vascular endothelium, and prevent atherosclerosis [118]. This 30% inhibition of hyperlipidemic atherosclerosis by barley leaf is associated with a decrease in plasma lipids and an increase in antioxidative abilities [73]. 2″-*O*-Glycosyl isovitexin from BG is more effective than α-tocopherol towards fatty acid esters at higher levels [119]. Barley sprout contains 4.97% fat, 52.6% polysaccharide, 34.1% protein, vitamins, minerals, and polyphenols, which show significant lipid-lowering [77].

3.13. Antigout/Hyperuricemia and Functional Ingredients of Barley Grass. Barley grass reduces blood uric acid, which has lots of benefits on feces metabolism, lipids metabolism, liver function, and antioxidant system for human [120]. A fermented barley extract can reduce uric acid effect on hyperuricemia [121]; however, SOD and alkaloid are focused on the treatment of arthritis, bursitis, and gout [122, 123]. A fermented barley extract P reduces serum uric acid by increasing its urinary excretion [124].

3.14. Preventive Cardiovascular Diseases and Functional Ingredients of Barley Grass. BG antioxidation may contribute to the prevention of cancer and metabolic disorders as well as cardiovascular diseases [115]. BG can prevent thrombosis and cardiovascular diseases by enhancing better blood viscosity and flow [125]. Normal tryptophan metabolism of barley grass is a developing appropriate therapies for the symptoms of cardiovascular disease patients [126].

3.15. Antihypoxia/Anti-Fatigue and Functional Ingredients of Barley Grass. BG is rich in flavones that have antihypoxia and anti-fatigue effects on humans, especially the total contents of lutonarin and saponarin amounting to 17.0% [127]. The barley seedling (1 g/mL) has significant effect on anti-fatigue in mice, especially the exhausting swimming and antianoxic time with significant longer, which reduced blood glucose significantly of diabetes inducted by alloxan ($C_4H_4N_2O_5$) and gastric ulcer induced by alcohol [128].

3.16. Preventive Constipation and Functional Ingredients of Barley Grass. Young barley leaf powder has lots of health effects, such as preventive constipation [129]. The dietary fiber of germinated barley alleviates constipation via the proliferation of the colonic crypts in loperamide ($C_{29}H_{33}ClN_2O_2$)-administered rats [130]; however, dietary fiber content of BG is 29.5% (see Table 1).

Table 2: Functional ingredients of barley grass for therapeutic chronic disease.

Number	Chronic disease	Functional ingredients	References
1	Promote sleep	GABA, Ca, K, tryptophan, vitamin C	[70, 71, 137]
2	Antidiabetes	Saponarin; dietary fiber, Ca; AMP-activated protein kinase, polyamines; GABA; SOD	[74, 75, 78, 79, 138, 139]
3	Regulating blood pressure	Saponarin; lutonarin, K, Ca; GABA	[12, 81, 82, 140, 141]
4	Enhance immunity	Arabinoxylan; polysaccharide; GABA	[84, 87, 142]
5	Protective liver	Saponarin; SOD; GABA	[69, 88, 89, 143]
6	Beauty anti-acne/detox	Metallothioneins	[91]
7	Antidepressant	GABA; saponarin; vitamins and minerals	[10, 69, 93, 95, 144]
8	Improve gastrointestinal	Dietary fiber; selenium; GABA	[15, 98, 145]
9	Anticancer	Alkaline, flavonoids, chlorophyll; tricin; SOD	[99, 100, 103, 139]
10	Anti-inflammation	Chlorophyll; saponarin; SOD; GABA; tryptophan	[12, 106–108, 145, 146]
11	Antioxidants	Chlorophyll; lutonarin, saponarin; isoorientin and orientin; γ-tocopherol, glutathione; SOD, flavonoid, protein P4D1; GABA	[12, 39, 81, 111–114, 147]
12	Hypolipidemic	Saponarin; α-tocopherol; 2″-*O*-glycosyl isovitexin, polysaccharide	[69, 77, 119]
13	Antigout/hyperuricemia	Alkaloid, SOD	[122, 123]
14	Preventive cardiovascular diseases	Saponarin; tryptophan; vitamins (A, B1, C, E), SOD; K, Ca; GABA	[72, 81, 87, 126, 136, 140, 148]
15	Antihypoxia/anti-fatigue	Flavones (lutonarin, saponarin)	[127]
16	Preventive constipation	Dietary fiber	[130]
17	Alleviated atopic dermatitis	GABA, SOD	[131, 132, 149]
18	Preventive heart disease	K, GABA	[10, 50, 150]
19	Calcium supplement	Ca	[10, 50, 70, 134]
20	Improve cognition	GABA, K, SOD	[10, 28, 50, 151]

3.17. Alleviated Atopic Dermatitis and Functional Ingredients of Barley Grass. $GABA_B$ receptor is a new therapeutic way to treat inflammatory skin diseases [131]. Combined administration of fermented barley extract P and GABA alleviated atopic dermatitis by regulating the Th1/Th2 balance to a Th1-immune response [132]. GABA (377.46 mg/100 g) of Yungong BG is 62.5 times and 37.8 times of polished and brown rice [10, 70].

3.18. Preventive Heart Disease and Functional Ingredients of Barley Grass. Western countries have more incident of coronary heart disease than that of stroke and diabetes for Asian countries, based on the loss of K and Mg as well as dietary fiber of major food from whole wheat to wheat flour [10]. An increase in K^+ intake can prevent heart disease which associate with decreasing inflammation and oxidative stress [10, 50]. K (3110 mg/100 g) of Yungong BG is 31 times of polished rice [10, 70].

3.19. Calcium Supplement and Functional Ingredients of Barley Grass. Calcium homeostasis is paramount physiological and pathophysiological importance in health and disease [133]. BG can be used as the prevention or treatment of osteoporosis [134]. Yungong BG has the health effect due to the highest Ca content (845 mg/100 g) that is 99.6 times of polished rice [10, 70].

3.20. Improve Cognition and Functional Ingredients of Barley Grass. GABA and K have increasing cognitive effects [10, 28, 50] due to higher concentration, such that GABA (377.46 mg/100 g) and K (3110 mg/100 g) of Yungong BG is 62.5 times and 31 times of polished rice [10, 70].

3.21. Preventive Other Diseases and Functional Ingredients of Barley Grass. BG of Yungong brand has also lustihood, bone injury recovery, antiaging, losing weight, reducing blood fat [10]. Carotene is a fat-soluble vitamin, which plays a very important role in the health of the retina, lungs, gastrointestinal tract, brain, and immune system [135]. Vitamin B1 (daily intake 2.0 mg) is a water-soluble vitamin, which has a favorable impact on the digestive, cardiovascular, and nervous systems [136].

4. Major Mechanisms of Functional Ingredients of Barley Grass for Preventive Chronic Disease

4.1. GABA Mechanism for Preventive Chronic Diseases. GABA ($C_4H_9NO_2$) in BG promotes sleep, is antidiabetic, regulates blood pressure, enhances immunity, protects liver, is antidepressant, improves gastrointestinal function, is anti-inflammatory and antioxidant, prevents cardiovascular and heart diseases, alleviates atopic dermatitis, increases cognition, and so on (Table 2). Sleep is regulated by

neurotransmitter systems of GABA and dopamine signaling, which improves the sleeping quality [152]. GABA has a very important intraislet transmitter in adjusting islet-cell secretion and anti-inflammatory and immunoregulatory activities, which can treat diabetes by promoting the regenerative functions and against apoptosis of β-cell [138]. GABA can inhibit an increase in blood pressure and accelerate growth hormone secretion, however GABA associated with CO_2 concentrations [141]. Baclofen ($C_{10}H_{12}ClNO_2$) of $GABA_B$ receptor agonist regulates Toll-like receptor 3 and Toll-like receptor 4 signaling in glia and immune cells, which has the therapeutic role in neuroinflammatory disease [142]. Hepatic encephalopathy is related with a regional reduce GABA levels in the visual cortex due to liver failure and cerebral osmolytic disorders [143]. Antidepressant effect of ascorbic acid and ketamine in tail suspension test may involve an activation of $GABA_A$ receptors and a possible inhibition of $GABA_B$ receptors [144]. GABA and GABA receptor function can modulate gastrointestinal motility and inflammation [145]. Antioxidants L-carnitine ($C_7H_{15}NO_3$) and D-methionine ($C_5H_{11}NO_2S$) regulate cortical electrical spike activity through $GABA_A$ receptor activation [147]; $GABA_B$ receptor positive allosteric modulators are very important in the treatment of alcoholism [153]. GABA can treat cardiovascular diseases that is associated with glycoprotein VI in platelet membrane, such as stroke and myocardial infarction, in which GABA inhibited platelet activation stimulated by convulsed and prolonged the closure time of whole blood and the occlusion time of platelet plug formation [148, 149]. The central mechanisms are an interaction between angiotensin II receptor 1 and interleukin-1 beta with decrease GABA expression in the paraventricular nucleus contributing to progression in heart failure [150]. Bone marrow mesenchymal stem cell transplantation can improve cognitive impairment via upregulating the hippocampal GABAergic system in a rat model of chronic cerebral hypoperfusion [154].

4.2. Flavonoids Mechanism for Preventive Chronic Diseases. Flavonoids especially saponarin and lutonarin in BG have antidiabetic effect; regulate blood pressure; protect liver; have antidepressant, anticancer, anti-inflammatory, antioxidant, and hypolipidemic effects; prevent cardiovascular diseases; have antihypoxia and anti-fatigue effects; and so on (Table 2). Saponarin from young barley leaves can regulate gluconeogenesis and glucose uptake by activated AMPK in a calcium-dependent manner [155]. Saponarin and lutonarin with regulating blood pressure in BG can promote blood flow, digestion, and detoxification of whole body [12]. The liver-protective effect of saponarin is blocked nitric oxide synthase and cyclooxygenase expression; however, antidepressant of saponarin in BG is associated with its anti-inflammation and antioxidation [69]. Anti-inflammation of saponarin in barley is LPS-induced macrophages via inhibition of NF-κB, ERK, and p38 signaling [106]. Saponarin/lutonarin (4.5/1) from BG has very strong antioxidation using cod liver oil, ω-3 fatty acids, phospholipids, and blood plasma [156]. Twenty-five secondary metabolites (saponarin, lutonarin, etc.) are putatively identified in healthy and diseased barley leaves, which play a role in adaptation to unfavorable growth conditions [157].

4.3. SOD Mechanism for Preventive Chronic Diseases. SOD in BG has antidiabetic, anti-inflammatory, antioxidant, antigout and hyperuricemia, and anticancer effects, prevents cardiovascular disease, alleviates atopic dermatitis, improves cognition, and so on (Table 2). Oxygen radical absorbance capacity values are associated with the increases in CAT and SOD activities and the reduction in reactive oxygen species, in which the extract has a significant therapeutic and preventive potentials of cancer and diabetes [139]. Zinc is cofactor of SOD with antioxidant defense in type 2 diabetes by regulating the glutathione metabolism and metallothionein expression, competing with Fe and Cu in the cell membrane, inhibiting nicotinamide adenine dinucleotide phosphate-oxidase enzyme ($C_{21}H_{29}N_7O_{17}P_3$) [158]. Gout patients have oxidative stress and HDL protective effects against atherosclerosis dependent on paraoxonase-1 activity, which correlated positively with SOD, negatively with malondialdehyde, and oxidized LDL [159]. SOD is a characteristic of cardiovascular alterations in hypertension and diabetes, which is associated with alterations in vascular structure and function [160]. Atopic dermatitis patients can be damaged by oxidants, which is evident from an increase of malondialdehyde and a decrease of SOD and CAT enzymatic antioxidants [149]. Hippocampal-dependent cognitive functions have the aid of production of new neurons and dendritic structures; however, oxidative stress plays an important part in the SOD-deficient and radiation environment, which can be effective use of SOD deficiency in cognitive functions and identify therapeutic methods [151]. The natural ROS-scavenging compounds for vitamins and SOD of barley grass are crucial and promising therapeutic strategies for vascular repair [161].

4.4. K-Ca Mechanism for Preventive Chronic Diseases. K or Ca in BG promotes sleep, has antidiabetic effect, regulates blood pressure, prevents cardiovascular and heart diseases, is a calcium supplement, increases cognition, and so on (Table 2). Large-conductance Ca^{2+}-activated K^+ channels regulate rhythmicity for sleep-wake in suprachiasmatic nucleus neuronal actions [162]. The role of Ca^{2+}-activated K^+ channels for endothelial cell in uterine vascular dysfunction of diabetes, especially the impaired function of IKCa channels [163]. Small conductance Ca^{2+}-activated K^+ channels not only expressed in the paraventricular nucleus play a key role in the regulation of arterial blood pressure and cardiovascular function [140], but also inhibited lots of situations of atrial fibrillation in the heart under normal and pathophysiological conditions [164]. Barley seedling extracts inhibited RANKL-induced osteoclast differentiation with alteration of IκB degradation, c-Fos, and NFATc1 molecules in osteoclastogenesis [134].

4.5. Vitamins Mechanism for Preventive Chronic Diseases. Vitamins in BG promote sleep; have antidepressant, antioxidant, hypolipidemic, gastrointestinal, and anti-inflammatory effects; prevent cardiovascular diseases; and so on (Table 2).

Vitamin B1 has a favorable impact on the sleep and gastrointestinal and cardiovascular systems, which stimulates the brain and improves the psycho-emotional state [136]. V_C treatment can prevent the spatial memory impairment of chronic sleep deprivation by the antioxidant defense mechanisms of the hippocampus [137]. Vitamins in BG have very effective antistress capabilities by preventing reduction in the wheel-running activity and hippocampal mRNA of brain-derived neurotrophic factor in response to restraint stress [95]. The vitamin E has unique therapy chronic diseases, antioxidation and anti-inflammations, based on scavenge active nitrogen, inhibit cyclooxygenase- and 5-lipoxygenase-catalyzed eicosanoids, as well as suppress proinflammatory signaling [165]. Antioxidants vitamins C and E in BG may prevent cardiovascular diseases, which decrease total cholesterol, LDL cholesterol, and oxygen free radicals [72].

4.6. Tryptophan Mechanism for Preventive Chronic Diseases. Tryptophan in BG promotes sleep, has anti-inflammatory effect, prevents cardiovascular diseases, and so on (Table 2). Tryptophan may improve sleep quality in stress-vulnerable individuals carrying the 5-hydroxytryptamine transporter-linked promoter region S-allele [166]. Kynurenine and tryptophan as well as their ratio will contribute to the interplay between inflammation, metabolic syndrome, mood disturbance, anemia, and cardiovascular diseases [146]. The kynurenine pathway of tryptophan degradation in preventive cardiovascular diseases is very important by an inducible indoleamine 2,3-dioxygenase-mediated tryptophan metabolism [126]. Lineage-specific duplications of genes associated in the transport of nutrients to developing seeds and the mobilization of carbohydrates in barley grains [167].

5. Conclusion Remarks and Future Perspectives

The data summarized in current review point out that major mechanism and more than 30 functional ingredients of barley grass exert potent preventive exceed 20 chronic diseases. The result reveals coevolution between preventive chronic diseases and young barley grass for functional foods of human beings. We can suggest that chronic diseases of human beings are associated with six major dietary structures: (i) fruits/vegetables, (ii) young grass/barley grass, (iii) carnivorous, (iv) cereal crops (rice, wheat, millet, beans, barley, and corn), (v) polished rice or wheat flour, and (vi) white rice or wheat flour + grass powder. The modern diet of polished rice and wheat flour is the key for the outbreak of chronic diseases in human beings, but white rice (wheat flour) + barley grass powder is the healthiest major dietary guidelines for modern humans. Although functional ingredients of barley grass for preventing and treating chronic diseases seem a complicated task, the barley grass powder is the best functional foods of cell nutrition and detoxification in marketable nutritional and health products all over the world, which is associated with coevolution for the similar center of origin (Africa, especially Ethiopia) between human beings and barley, because a health product can prevent and cure more than 20 chronic diseases which has not been reported, which may still open new venues for therapeutic interventions. Regular consumption of barley has 8000 years history, which may become a successful and safe strategy to treat chronic disease conditions. These data support that barley grass powder is rich in GABA, flavonoids, SOD, K-Ca, vitamins, and tryptophan, which are known to play a pivotal role in many chronic diseases.

This review provides important information and effective strategies that will guide future research and production of functional foods, especially GABA, flavonoids, SOD, K-Ca, vitamins, and tryptophan for prevention and treatment of chronic diseases. BG of Yungong brand can prevent more than 20 chronic diseases, which is associated with its rich functional components, based on its growth stage from autumn via winter to summer, drying process under sunlight at 1900–2300 m at spring with dry and windy conditions; Yunnan province at 1900–2300 m can plant barley in the four seasons (spring, summer, autumn, and winter) and may be associated with the rich and complex enzyme system in BG. Further researches are indispensable to resolve lots of problems, such as a better understanding of the interconnection between other 20 functional ingredients and preventing chronic diseases in clinical trials as well as ecological contribution. Further studies are necessary to unravel major pathological mechanism of coevolution between preventive chronic diseases and young barley grass for functional foods of human beings. This review may be used as a starting point for novel nutraceuticals, functional foods, or complementary and alternative drugs to maintain or improve the chronic diseases in barley grass.

Conflicts of Interest

The authors declare that they have no conflicts of interest whatsoever to declare.

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Research Article

Dietary Total Antioxidant Capacity and Dietary Polyphenol Intake and Prevalence of Metabolic Syndrome in Polish Adults: A Nationwide Study

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Specific classes and subclasses of polyphenols have been studied for their potential effects on noncommunicable diseases, but studies on association between dietary polyphenol intake (DPI) and dietary total antioxidant capacity (DTAC) and MetS (metabolic syndrome) are scarce. Therefore, the aim of this study was to determine associations between DTAC and DPI and the prevalence of MetS and its components in the Polish adult population. Subjects (5690) were participants of the Polish National Multicentre Health Examination Survey (WOBASZ II study) performed in 2013-2014. MetS was defined according to the International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) criteria. Daily food consumption was assessed by 24-hour dietary recall. DTAC and DPI were evaluated using the data of food consumption and antioxidant potential of foods, measured by FRAP (ferric reducing antioxidant potential) method, and total polyphenol content in foods, measured by Folin-Ciocalteu assay. Logistic regression models were used to assess the relationship between DTAC and DPI and MetS and its components. Crude, age-adjusted, and multivariable-adjusted models were performed. This study demonstrated that in Polish women, high DPI and high DTAC were significantly associated with a reduced odds ratio for the prevalence of MetS components, such as elevated blood pressure and diabetes. In contrast, in men, high DPI and high DTAC did not have the potential to alleviate MetS components.

1. Introduction

Metabolic syndrome (MetS) is a clustering of risk factors, such as central obesity, elevated fasting glucose, elevated triglycerides, reduced high-density lipoprotein cholesterol, and elevated blood pressure, that together culminate in the increased risk of diabetes mellitus type 2 (DM2) and cardiovascular disease (CVD). The first formalized definition of MetS was proposed in 1998 by the World Health Organization (WHO) [1]. Over the past years, various diagnostic criteria have been presented by different organizations. Most recently, these have come from the International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) [2].

The prevalence of MetS varies widely across population and depends on several factors, such as age, gender, socioeconomic status, education level, and lifestyle [3–5]. MetS is

diagnosed in a third of the US population and a quarter of the European population [6, 7]. A consequence of metabolic syndrome is CVD and DM2, which are the leading public health problems with high socioeconomic cost. Currently, the global cost of diabetes is $825 billion dollars per year [8], while CVD costs US $555 billion per year [9] and €210 billion per year [10].

Several studies have reported that oxidative stress, caused by the imbalance between prooxidants and antioxidants in the living organism system, plays an important role in the prevalence of MetS [11, 12]. The overproduction of reactive oxygen species may impair insulin signalling pathways and lead to endothelial damage. This causes insulin resistance and promotes acceleration of the atherogenic process [13]. Moreover, oxidative stress and endothelial dysfunction are known to be associated with inflammation and can contribute to hypertension [14].

Modification of lifestyle, including healthy nutrition, is the primary approach for MetS prevention [15]. It was found that the dietary total antioxidant capacity (DTAC), which may increase after dietary polyphenol intake (DPI), has a significant influence on the serum total antioxidant status [16]. Some studies indicated that higher consumption of specific classes of polyphenols [17, 18] and DPI [18] and higher DTAC [19–21] have favourable effects on metabolic disorders in different population. Predominant sources of the dietary polyphenols in Polish population are tea, coffee, and apples [22, 23].

The objective of this study was to determine the associations between DTAC and DPI and the prevalence of MetS and its specific components in Polish adult population. To our knowledge, this is the first attempt to estimate the relationships between DTAC and MetS in a cross-sectional Polish study.

2. Material and Methods

2.1. Study Population. Subjects were participants of the Polish National Multicentre Health Examination Survey (WOBASZ II study) performed in 2013-2014, which was a cross-sectional study aimed at investigating the determinants of chronic noncommunicable diseases in a random sample of Polish residents aged 20 years or older. The project was conducted by the Institute of Cardiology in Warsaw in cooperation with 5 medical universities in Poland. The randomization had two stages. In each of 16 voivodeships, 6 communities were drawn, including 2 small (up to 8000 inhabitants), 2 medium (8000–40000 inhabitants), and 2 large (over 40000 inhabitants) ones, plus province capitals (unless it was selected as one of the communes). Thereafter, 70 men and 70 women were drawn from each community. Altogether, 15120 subjects (including 1557 respondents who were unavailable) of both genders were drawn from the Department of State Registry database, the Ministry of Internal Affairs (PESEL register). A total of 6170 respondents participated in the study, which was 45.5% of the total sample. Whereas, dietary recalls were obtained from 5690 people (2554 men and 3136 women). The aims and methods of the WOBASZ II study have been described in detail elsewhere [24, 25]. The participants provided written informed consent, and the study protocol was approved by the Bioethics Committee of the National Institute of Cardiology (number 1344).

2.2. Demographic and Lifestyle Information. Sociodemographic and lifestyle characteristics such as age, gender, educational level, occupational activity, physical activity, smoking habits, and alcohol intake were collected from self-reported standardized questionnaires. Educational level was classified as under middle (primary, vocational, and partial secondary education), middle (secondary and partial academic education), and high (licentiate or university education). Physical activity at leisure for at least 30 min a day was assessed as low (once a week or less), middle (2-3 times a week), and high level (≥4 times a week). Physical activity assessment was described in detail in a previous substudy [26]. Smoking status was categorised as smoking at least one cigarette a day. Alcohol intakes were calculated as gram pure ethanol a day.

2.3. Clinical Measurements. The measurements of body mass, height, waist circumference, and blood pressure were performed by trained nurses, using standardized procedures. Body weight was measured without footwear and without top garments, to the nearest 0.1 kg. Height was measured in the standing position without footwear, to the nearest 0.5 cm. Waist circumference was measured at the level of the umbilicus, using a measuring tape, to the nearest 0.5 cm. Body mass index (BMI) was calculated as body mass in kilograms divided by squared height in meters. A BMI of 18.5–24.9 kg/m^2 was defined as normal body mass, a BMI of 25.0–29.9 kg/m^2 was classified as overweight, and a BMI of over 30.0 kg/m^2 was determined as obesity. Blood pressure was measured three times on the right arm, in a sitting position, at one-minute intervals, using automatic devices AND UA-631, approved by AAMI (Association for the Advancement of Medical Instrumentation). The average value from the second and the third measurements was used for the analysis.

Determinations of fasting glucose and blood lipids were carried out at a single location, Diagnostyka Central Laboratory at the Institute of Cardiology in Warsaw, which holds a CDC certificate (the Centre for Disease Control, Lipid Standardization Program, Atlanta, USA) and the European certificate RIQAS (Random International Quality Assessment Scheme). Measurements of fasting glucose and blood lipids were performed using enzymatic-colorimetric methods on the analyzer Cobas 6000, manufactured by Roche.

2.4. MetS Criteria. MetS criteria were adopted according to the International Diabetes Federation (IDF) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) definition [2]. MetS was diagnosed when at least three of five risk factors have been identified: (1) elevated waist circumference (for European population: ≥94 cm for men and ≥ 80 cm for women); (2) elevated triglycerides (≥150 mg/dl–1.7 mmol/l); (3) reduced HDL cholesterol (<40 mg/dl–1.0 mmol/l for men and <50–

1.3 mmol/l for women); (4) elevated blood pressure (systolic ≥ 130 and/or diastolic ≥ 85 mmHg) or hypertension in interview; and (5) elevated fasting glucose (≥100 mg/dl–5.6 mmol/l) or diabetes in interview.

2.5. Dietary Assessment. Dietary assessment was performed by qualified interviewers, using a single 24-hour dietary recalls. Food portion sizes were estimated using an album with photographs of the most consumed food products established by the National Food and Nutrition Institute, Warsaw, Poland. It was found that 367 of the variety of foods were sources of polyphenol intakes among the whole population of study. These products were grouped in 5 food categories, namely, beverages (alcoholic and nonalcoholic), cereals, fruit, vegetables, and other food products (e.g., chocolates, nuts, and seeds). Individual components of complex dishes were extracted using dish recipes obtained from the Polish Food Composition Tables [27].

2.6. Estimation of DTAC and DPI. DTAC and DPI were determined by multiplying the daily consumption of individual food items by antioxidant capacity of foods and polyphenol contents in these food items. Antioxidant potential of foods, measured by FRAP method (ferric reducing antioxidant potential), was mostly taken from Polish databases [28, 29], and some missing values were complemented with another database [30]. Data on the total polyphenol content in foods, measured by Folin-Ciocalteu assay, were obtained from Polish databases [28, 29], as well as the online Phenol-Explorer database [31].

2.7. Statistical Analysis. Statistical analyses were performed using a Statistical Analysis System (SAS) software, version 9.2. 2 (SAS Institute Inc., Cary, NC). Continuous variables were presented as means and standard deviations (SD) and categorical variables as counts and percentages. Kruskal-Wallis test was used for comparison of continuous variables and chi-square test for categorical variables across categories of DPI and DTAC. Logistic regression models were used to assess the relationship between DPI and DTAC and MetS and its components. Crude, age-adjusted, and multivariable-adjusted models were performed. The results were presented as odds ratios (ORs) and 95% confidence intervals (CIs) of the associations between MetS and its specific components and tertiles (T1–T3) of DPI and DTAC. The level of significance for two-sided tests was considered to be at $p < 0.05$.

3. Results

Demographic and lifestyle characteristics of the 5690 participants (2554 men and 3136 women), stratified by occurrence of MetS, are given in Table 1. The mean age of the studied population was 50.08 ± 16.44 y, and the mean BMI was 27.17 ± 5.19 kg/m^2. It was found that MetS criteria have been met by 36% of participants (39% men and 33% women). Subjects of older age, higher BMI, low education level, low leisure time physical activity, lack of employment, and high alcohol intake were more likely to have MetS.

Anthropometric characteristics and biomarkers of metabolic syndrome by tertiles of DPI and DTAC are presented in Tables 2 and 3, respectively. Higher DPI and higher DTAC were significantly associated with lower fasting glucose and lower blood pressure, but only in women. There was no significant association in men.

The odds ratios for MetS and its specific components by tertiles of DPI and DTAC were evaluated individually for men and women, using multiple logistic regression analysis (Tables 4 and 5, resp.). Three models were tested: model 1—crude data, model 2—data adjusted for age, and model 3—data adjusted for age, BMI, educational level, leisure time physical activity, smoking, and alcohol intake. The first tertile (T1) in each model was adopted as a reference. For both genders, the associations between DPI and MetS as well as between DTAC and MetS were not significant after adjustment for multiple covariates (model 3). In men, higher DPI and higher DTAC were associated with reduced odds of diabetes between T3 and T1 (OR = 0.673, 95% CI = 0.495–0.917 and OR = 0.684, 95% CI = 0.506–0.925, resp.), but only when the crude data were taken into consideration (model 1). In a single model (model 2) and in a multiple adjustment model (model 3), associations were not statistically significant. Ambiguous results were found for DPI and HDL-cholesterol. In men, higher DPI was significantly associated with lower HDL cholesterol (T3 versus T1; model 3; OR = 1.410, 95% CI = 1.080–1.842); such association was not found for women.

In women, individuals with higher DPI had lower blood pressure (Table 4). When the analysis was stratified by multiple covariates (model 3), the odds of elevated blood pressure were 22.3% lower in T2 (OR = 0.777, 95% CI = 0.620–0.975) and 30.6% lower in T3 (OR = 0.694, 95% CI = 0.556–0.867) in comparison to T1. Higher DTAC was associated with 22.8% lower odds of elevated blood pressure between T3 and T1 in model 3 (OR = 0.772, 95% CI = 0.619–0.963) (Table 5). In addition, higher DPI and DTAC in women were significantly associated with reduced odds of elevated blood glucose (FG ≥ 100 mg/dl (5.6 mmol/dl) or diabetes in interview), but only in model 1. After adjustment for confounding factors (model 3), significant associations were found only for diabetes (FG ≥ 126 mg/dl (7 mmol/l) or diabetes in interview) between T2 and T1. In women, higher DPI was associated with 28.2% lower odds of diabetes (OR = 0.718, 95% CI = 0.520–0.992), whereas higher DTAC was associated with 27.9% lower odds of diabetes (OR = 0.721, 95% CI = 0.522–0.997).

4. Discussion

In the present study, we evaluated the relationship between DPI and DTAC and MetS and its specific components in Polish representative population of both genders. Inverse associations were evident in women between high DPI, high DTAC, and MetS components, such as elevated blood pressure and diabetes (in a middle tertile). In contrast, in men, high DPI and high DTAC did not have the potential to alleviate MetS components. Moreover, men who consumed more polyphenol had lower HDL-cholesterol. These

Table 1: General characteristics of studied population ($N = 5690$) according to prevalence of metabolic syndrome.

	Total	Metabolic syndrome		p
		No	Yes	
Gender, *N* (%)	5690 (100)	3662 (64.36)	2028 (35.64)	
Men	2554 (44.89)	1554 (60.85)	1000 (39.15)	<0.0001
Women	3136 (55.11)	2108 (67.22)	1028 (32.78)	
Age (years), mean ± SD	50.08 ± 16.44	46.11 ± 16.32	57.25 ± 14.04	<0.0001
Age, *N* (%)				
20–39	1724 (30.30)	1485 (86.14)	239 (13.86)	<0.0001
40–59	2202 (38.70)	1346 (61.13)	856 (38.87)	
>60	1764 (31.00)	831 (47.11)	933 (52.89)	
BMI (kg/m^2), mean ± SD	27.17 ± 5.19	25.44 ± 4.59	30.24 ± 4.76	<0.0001
BMI, *N* (%)				
<18.5 kg/m^2	91 (1.61)	91 (100.00)	0 (0.00)	
18.5–24.9 kg/m^2	1985 (34.91)	1758 (88.58)	227 (11.42)	<0.0001
25–29.9 kg/m^2	2120 (37.25)	1283 (60.53)	837 (39.47)	
>30 kg/m^2	1494 (26.23)	505 (33.78)	989 (66.22)	
Educational level, *N* (%)				
Under middle	2274 (39.96)	1235 (54.29)	1039 (45.71)	<0.0001
Middle	2140 (37.62)	1425 (66.60)	715 (33.40)	
High	1276 (22.42)	1002 (78.49)	274 (21.51)	
Leisure time physical activity, *N* (%)				
Low level	2614 (45.94)	1601 (61.23)	1013 (38.77)	<0.0001
Middle level	1478 (25.98)	1035 (70.06)	443 (29.94)	
High level	1598 (28.08)	1028 (64.32)	570 (35.68)	
Currently working, *N* (%)				
Yes	2980 (52.37)	2127 (71.39)	853 (28.61)	<0.0001
No	2710 (47.63)	1534 (56.59)	1176 (43.41)	
Currently smoking, *N* (%)				
Yes	1647 (28.95)	1049 (63.69)	598 (36.31)	0.0575
No	4043 (71.05)	2411 (59.64)	1632 (40.36)	
Alcohol intake (g ethanol/day), mean ± SD	5.74 ± 12.25	5.50 ± 11.80	6.18 ± 13.01	0.0368
Energy intake (kcal/day), mean ± SD	1965.0 ± 847.9	1975.0 ± 831.8	1946.9 ± 876.1	0.0839
DPI (mg/day), mean ± SD	2025.0 ± 892.0	2036.0 ± 886.5	2005.2 ± 901.9	0.1251
DTAC (mmol/day), mean ± SD	12.31 ± 7.37	12.34 ± 6.86	12.27 ± 8.22	0.2567

N: number; BMI: body mass index; DTAC: dietary total antioxidant capacity; DPI: dietary polyphenol intake.

differences between genders can be explained by different nutrition habits of both genders and different cut-off points set as criteria for HDL-cholesterol.

To date, only a limited number of epidemiological studies evaluated the association between DPI and MetS and its components. Our results regarding this association between higher DPI and lower risk of diabetes and hypertension in women are in accordance with those found in the HAPIEE (Health, Alcohol and Psychosocial factors In Eastern Europe cohort) study [32, 33]. Similarly, an observational cohort analysis of the nondiabetic participants in the PREDIMED (PREvencion con DIeta MEDiterranea) trial showed that higher DPI was associated with a reduced risk of diabetes in elderly persons at high risk of cardiovascular disease [34]. Moreover, another HAPIEE study indicated that high DPI was negatively associated with MetS components, such as waist circumference, blood pressure, HDL-cholesterol, and triglycerides in women and fasting plasma glucose in both genders [18].

Most of the current epidemiological studies have shown that higher consumption of polyphenols have been related to decreased risk of MetS components, such as diabetes [17, 18, 32, 34–37], hypertension [17, 18, 33, 38–41], central obesity [17, 18], dyslipidemia [17, 18, 42, 43], and incidence of cardiovascular events [44], although knowledge on this subject is still scarce and the results are inconsistent. Some studies indicate on the positive impact of dietary total polyphenol intake on lower prevalence of MetS and its components [18, 32–34, 42], while others show these associations only for selected subclasses of polyphenols [17, 35, 37–40]. Our study added to the current knowledge regarding positive results of dietary total polyphenol intake on lower

Table 2: Anthropometric characteristics and biomarkers of metabolic syndrome by tertiles of DPI and by gender, expressed as mean ± SD ($N = 5690$).

	Tertile of DPI, men ($N = 2554$)				Tertile of DPI, women ($N = 3136$)			
	T1 mean ± SD (range) 1153 ± 323 (174–1599)	T2 mean ± SD (range) 1969 ± 211 (1600–2346)	T3 mean ± SD (range) 3092 ± 785 (2347–9048)	p	T1 mean ± SD (range) 1152 ± 313 mg (140–1587)	T2 mean ± SD (range) 1904 ± 186 mg (1588–2239)	T3 mean ± SD (range) 2904 ± 691 mg (2240–8793)	p
BMI (kg/m^2)	27.30 ± 4.44	27.42 ± 4.67	27.53 ± 4.53	0.7198	27.18 ± 5.64	27.00 ± 5.65	26.71 ± 5.66	0.0946
WC (cm)	97.31 ± 12.16	97.39 ± 13.04	97.00 ± 12.04	0.8493	88.34 ± 13.79	88.48 ± 14.06	87.18 ± 13.46	0.0566
TC (mmol/l)	5.22 ± 1.33	5.17 ± 1.27	5.23 ± 1.40	0.7300	5.18 ± 1.24	5.17 ± 1.19	5.23 ± 1.23	0.3279
LDL-C (mmol/l)	3.19 ± 1.03	3.17 ± 1.04	3.21 ± 1.04	0.7121	3.10 ± 1.04	3.08 ± 0.98	3.16 ± 1.03	0.1525
HDL-C (mmol/l)	1.33 ± 0.40	1.32 ± 0.43	1.33 ± 0.46	0.6841	1.53 ± 0.42	1.53 ± 0.41	1.53 ± 0.43	0.8516
TG (mmol/l)	1.80 ± 1.92	1.74 ± 1.53	1.76 ± 2.21	0.4551	1.38 ± 0.81	1.37 ± 0.81	1.32 ± 0.75	0.3001
FG (mmol/l)	5.69 ± 1.59	5.62 ± 1.31	5.63 ± 1.85	0.9001	5.56 ± 1.69	5.34 ± 1.20	5.26 ± 0.96	0.0006
SBP (mmHg)	135.5 ± 19.1	134.3 ± 18.0	133.5 ± 17.4	0.1357	130.1 ± 20.6	127.0 ± 19.3	125.8 ± 19.0	<0.0001
DBP (mmHg)	81.8 ± 11.5	81.3 ± 11.1	81.5 ± 10.1	0.8439	79.9 ± 10.6	78.7 ± 10.7	79.0 ± 10.6	0.0242

BMI: body mass index; WC: waist circumference; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; FG: fasting glucose; SBP: systolic blood pressure; DBP: diastolic blood pressure.

Table 3: Anthropometric characteristics and biomarkers of metabolic syndrome by tertiles of DTAC and by gender, expressed as mean ± SD ($N = 5690$).

	Tertile of DTAC, men ($N = 2554$)				Tertile of DTAC, women ($N = 3136$)			
	T1 mean ± SD (range) 5.98 ± 2.02 (0.47–8.83)	T2 mean ± SD (range) 11.24 ± 1.42 (8.84–13.84)	T3 mean ± SD (range) 19.89 ± 8.00 (13.84–95.69)	p	T1 mean ± SD (range) 6.28 ± 2.03 (0.32–9.18)	T2 mean ± SD (range) 11.39 ± 1.28 (9.19–13.73)	T3 mean ± SD (range) 19.12 ± 8.31 (13.74–191.82)	p
BMI (kg/m^2)	27.29 ± 4.51	27.43 ± 4.59	27.53 ± 4.54	0.6124	27.15 ± 5.71	26.99 ± 5.77	26.75 ± 5.48	0.3117
WC (cm)	97.41 ± 12.37	96.92 ± 12.89	97.37 ± 11.99	0.4135	88.30 ± 14.09	88.37 ± 13.96	87.32 ± 13.26	0.1440
TC (mmol/l)	5.17 ± 1.34	5.19 ± 1.29	5.25 ± 1.38	0.2944	5.17 ± 1.24	5.20 ± 1.24	5.21 ± 1.18	0.4813
LDL-C (mmol/l)	3.14 ± 1.00	3.18 ± 1.06	3.25 ± 1.04	0.2028	3.09 ± 1.04	3.12 ± 1.01	3.13 ± 1.01	0.4652
HDL-C (mmol/l)	1.32 ± 0.39	1.32 ± 0.42	1.34 ± 0.47	0.6955	1.52 ± 0.41	1.54 ± 0.43	1.54 ± 0.42	0.5762
TG (mmol/l)	1.81 ± 1.99	1.80 ± 2.19	1.68 ± 1.48	0.5592	1.38 ± 0.87	1.35 ± 0.73	1.34 ± 0.76	0.5926
FG (mmol/l)	5.69 ± 1.50	5.63 ± 1.81	5.62 ± 1.47	0.4232	5.50 ± 1.60	5.39 ± 1.27	5.35 ± 1.27	0.0080
SBP (mmHg)	135.4 ± 19.0	134.3 ± 18.5	133.6 ± 17.0	0.1637	129.3 ± 20.5	127.8 ± 19.8	125.8 ± 18.7	0.0008
DBP (mmHg)	81.6 ± 11.5	81.4 ± 11.1	81.5 ± 10.2	0.8380	79.3 ± 10.8	79.3 ± 10.7	79.0 ± 10.4	0.8367

BMI: body mass index; WC: waist circumference; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; FG: fasting glucose; SBP: systolic blood pressure; DBP: diastolic blood pressure.

blood pressure and occurrence of diabetes in Polish women in a cross-sectional study. It should be pointed that polyphenols are characterized by low bioavailability and their maximal plasma concentrations are reached within the first two hours after ingestion and fall to baseline levels within 8–12 hours. Therefore, the protective effects of polyphenols on MetS may be reached through a long-term regular, daily consumption [45].

Polyphenol-rich foods, such as fruits and vegetables, may exert antioxidant activity, which are the sum of the effects of polyphenols and antioxidant vitamins [16]. In our study, after adjustment for confounding factors, DTAC was significantly associated with lower odds of elevated blood pressure and diabetes (in a middle tertile) in women. However, population studies that examine the relationship between the DTAC and MetS and its components are limited. Similarly to our findings, the results of the EPIC (European Prospective Investigation into Cancer and Nutrition) study showed that the DTAC may play an important role in reducing the risk of diabetes in women [46]. Whereas, the longitudinal TLGS (Tehran Lipid and Glucose Study) showed that higher DTAC was positively associated with lower occurrence of MetS, abdominal obesity, and hypertension [19]. Findings of the Hertfordshire Cohort Study suggested that DTAC may have important protective effects on glucose tolerance, especially in older obese women

Table 4: Odds ratios (OR) (95% CI (confidence interval)) for metabolic syndrome and its specific components by tertiles of DPI and by gender ($N = 5690$).

Characteristic	Tertile of DPI, men ($N = 2554$)			Tertile of DPI, women ($N = 3136$)		
	T1 (ref.) 174–1599	T2 1600–2346	T3 2347–9048	T1 (ref.) 140–1587	T2 1588–2239	T3 2240–8793
Metabolic syndrome						
Crude OR (95% CI)	1	0.990 (0.815–1.204)	1.074 (0.885–1.305)	1	0.813 (0.678–0.976)	0.820 (0.683–0.983)
Adjusted OR (95% CI)[1]	1	1.024 (0.836–1.254)	1.155 (0.944–1.412)	1	0.910 (0.745–1.111)	1.025 (0.840–1.250)
Adjusted OR (95% CI)[2]	1	0.908 (0.720–1.144)	1.044 (0.830–1.312)	1	0.861 (0.688–1.078)	0.952 (0.761–1.191)
WC ≥ 94 cm (men), ≥80 cm (women)						
Crude OR (95% CI)	1	0.992 (0.815–1.208)	1.065 (0.875–1.298)	1	0.908 (0.750–1.098)	0.883 (0.730–1.068)
Adjusted OR (95% CI)[1]	1	1.010 (0.822–1.241)	1.113 (0.906–1.367)	1	0.994 (0.806–1.226)	0.987 (0.802–1.215)
Adjusted OR (95% CI)[2]	1	0.981 (0.737–1.307)	1.079 (0.812–1.432)	1	1.033 (0.779–1.370)	1.080 (0.818–1.426)
TG ≥ 150 mg/dl (1.7 mmol/l)						
Crude OR (95% CI)	1	0.889 (0.725–1.088)	0.939 (0.768–1.148)	1	0.992 (0.805–1.221)	0.867 (0.702–1.071)
Adjusted OR (95% CI)[1]	1	0.890 (0.726–1.090)	0.941 (0.769–1.150)	1	1.055 (0.853–1.306)	0.964 (0.776–1.197)
Adjusted OR (95% CI)[2]	1	0.859 (0.691–1.066)	0.899 (0.726–1.115)	1	1.066 (0.849–1.338)	0.937 (0.743–1.182)
Low HDL-C < 40 mg/dl (1.0 mmol/l) (men), <50 mg/dl (1.3 mmol/l) (women)						
Crude OR (95% CI)	1	1.102 (0.851–1.429)	1.425 (1.111–1.828)	1	0.889 (0.734–1.077)	0.926 (0.765–1.120)
Adjusted OR (95% CI)[1]	1	1.106 (0.853–1.433)	1.433 (1.117–1.840)	1	0.921 (0.759–1.119)	0.985 (0.812–1.195)
Adjusted OR (95% CI)[2]	1	1.080 (0.818–1.426)	1.410 (1.080–1.842)	1	0.925 (0.751–1.140)	0.939 (0.762–1.157)
SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or hypertension in interview						
Crude OR (95% CI)	1	0.904 (0.737–1.109)	0.855 (0.698–1.047)	1	0.743 (0.625–0.884)	0.638 (0.536–0.759)
Adjusted OR (95% CI)[1]	1	0.922 (0.741–1.146)	0.869 (0.701–1.077)	1	0.782 (0.634–0.965)	0.703 (0.572–0.863)
Adjusted OR (95% CI)[2]	1	0.956 (0.759–1.203)	0.887 (0.706–1.113)	1	0.777 (0.620–0.975)	0.694 (0.556–0.867)
FG ≥ 100 mg/dl (5.6 mmol/dl) or diabetes in interview						
Crude OR (95% CI)	1	1.049 (0.859–1.281)	1.023 (0.838–1.249)	1	0.766 (0.629–0.934)	0.692 (0.566–0.845)
Adjusted OR (95% CI)[1]	1	1.106 (0.894–1.370)	1.125 (0.910–1.390)	1	0.845 (0.681–1.049)	0.854 (0.687–1.061)
Adjusted OR (95% CI)[2]	1	1.048 (0.839–1.310)	1.057 (0.848–1.319)	1	0.821 (0.652–1.034)	0.883 (0.700–1.130)
FG ≥ 126 mg/dl (7 mmol/l) or diabetes in interview						
Crude OR (95% CI)	1	0.922 (0.691–1.231)	0.673 (0.495–0.917)	1	0.649 (0.488–0.862)	0.534 (0.397–0.720)
Adjusted OR (95% CI)[1]	1	1.023 (0.752–1.392)	0.829 (0.598–1.150)	1	0.752 (0.556–1.017)	0.739 (0.538–1.014)
Adjusted OR (95% CI)[2]	1	0.964 (0.697–1.333)	0.797 (0.566–1.121)	1	0.718 (0.520–0.992)	0.780 (0.558–1.091)

WC: waist circumference; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; FG: fasting glucose. [1]Analysis adjusted for age; [2]analysis adjusted for age, BMI, educational level, leisure time physical activity, smoking, and alcohol intake.

[47]. A case-control study showed that higher dietary total antioxidant capacity is inversely related to prediabetes morbidity [48].

In our study, the prevalence of MetS was identified in 36% of Polish adults, with higher prevalence in men than in women. It was found that the prevalence of MetS in Polish population was increased from 25% to 36% between 2003–2005 and 2013-2014 [7]. Our findings correspond with the results of the NHANES (National Health and Nutrition Examination Survey) study, which showed the increase of MetS among US adults (from 29% to 34% between 1988–1994 and 1999–2006, with a greater increase of MetS in women) [49]. In our study, MetS was dependent on various factors, such as gender, age, BMI, education level, leisure time physical activity, employment, and alcohol intake, which is in accordance with other studies [3–7].

Our study has some limitations. The participation rate in the WOBASZ II study was rather low (45.5%), but typical for cross-sectional studies. This problem was discussed in a previous publication [24]. Next, food intakes were estimated with the single 24-hour dietary recall method that does not reflect habitual or long-term food consumption. However, the 24-hour recall is a common method, which is useful to estimate mean food intakes in large groups of participants. Moreover, this study might have underestimated intakes of some polyphenols and the dietary antioxidant potential of some foods, which have been omitted in the dietary interview or were not included in the polyphenol and antioxidant databases. It was previously found that polyphenol intake estimated according to various databases may substantially differ [50]. Finally, polyphenols in foods and dietary antioxidant capacity can interact with other constituents of foods, which may contribute to the association with prevalence of metabolic disorders. Despite such limitations, this study included a large randomly selected sample of Polish adult population, and clinical measurements and

Table 5: Odds ratios (OR) (95% CI (confidence interval)) for metabolic syndrome and its specific components by tertiles of DTAC and by gender ($N = 5690$).

	Tertile of DTAC, men ($N = 2554$)			Tertile of DTAC, women ($N = 3136$)		
	T1 (ref.) 0.47–8.83	T2 8.84–13.84	T3 13.84–95.69	T1 (ref.) 0.32–9.18	T2 9.19–13.73	T3 13.74–191.82
Metabolic syndrome						
Crude OR (95% CI)	1	1.010 (0.831–1.227)	1.038 (0.855–1.261)	1	0.946 (0.788–1.134)	0.900 (0.750–1.080)
Adjusted OR (95% CI)[1]	1	1.057 (0.863–1.294)	1.099 (0.899–1.345)	1	1.005 (0.823–1.227)	1.126 (0.922–1.376)
Adjusted OR (95% CI)[2]	1	0.985 (0.782–1.242)	1.010 (0.803–1.270)	1	1.019 (0.815–1.274)	1.043 (0.832–1.306)
WC ≥ 94 cm (men), ≥80 cm (women)						
Crude OR (95% CI)	1	0.911 (0.749–1.109)	1.088 (0.893–1.326)	1	1.013 (0.837–1.225)	0.946 (0.783–1.143)
Adjusted OR (95% CI)[1]	1	0.934 (0.760–1.147)	1.124 (0.915–1.381)	1	1.021 (0.828–1.259)	1.048 (0.852–1.288)
Adjusted OR (95% CI)[2]	1	0.798 (0.598–1.066)	1.020 (0.766–1.356)	1	1.163 (0.877–1.543)	1.065 (0.808–1.403)
TG ≥ 150 mg/dl (1.7 mmol/l)						
Crude OR (95% CI)	1	1.083 (0.885–1.326)	0.962 (0.786–1.179)	1	0.994 (0.806–1.226)	0.939 (0.761–1.159)
Adjusted OR (95% CI)[1]	1	1.085 (0.886–1.328)	0.964 (0.787–1.181)	1	1.020 (0.823–1.263)	1.035 (0.834–1.284)
Adjusted OR (95% CI)[2]	1	1.037 (0.836–1.288)	0.941 (0.759–1.169)	1	1.016 (0.809–1.276)	0.999 (0.793–1.258)
Low HDL-C < 40 mg/dl (1.0 mmol/l) (men), <50 mg/dl (1.3 mmol/l) (women)						
Crude OR (95% CI)	1	1.111 (0.860–1.435)	1.269 (0.988–1.629)	1	0.965 (0.797–1.169)	0.942 (0.778–1.140)
Adjusted OR (95% CI)[1]	1	1.115 (0.863–1.440)	1.274 (0.992–1.636)	1	0.977 (0.805–1.186)	0.994 (0.819–1.207)
Adjusted OR (95% CI)[2]	1	1.149 (0.873–1.513)	1.296 (0.991–1.696)	1	1.059 (0.861–1.304)	0.939 (0.760–1.159)
SBP ≥ 130 mm Hg or DBP ≥ 85 mm Hg or hypertension in interview						
Crude OR (95% CI)	1	0.815 (0.664–1.000)	0.817 (0.666–1.002)	1	0.876 (0.737–1.041)	0.709 (0.596–0.843)
Adjusted OR (95% CI)[1]	1	0.820 (0.660–1.020)	0.806 (0.649–1.001)	1	0.878 (0.712–1.083)	0.789 (0.643–0.969)
Adjusted OR (95% CI)[2]	1	0.816 (0.648–1.028)	0.812 (0.646–1.021)	1	0.891 (0.711–1.117)	0.772 (0.619–0.963)
FG ≥ 100 mg/dl (5.6 mmol/dl) or diabetes in interview						
Crude OR (95% CI)	1	0.965 (0.790–1.178)	1.016 (0.833–1.240)	1	0.854 (0.700–1.040)	0.737 (0.603–0.902)
Adjusted OR (95% CI)[1]	1	1.018 (0.822–1.261)	1.096 (0.887–1.354)	1	0.888 (0.716–1.102)	0.899 (0.723–1.118)
Adjusted OR (95% CI)[2]	1	0.991 (0.792–1.239)	1.060 (0.850–1.321)	1	0.859 (0.683–1.082)	0.907 (0.718–1.146)
FG ≥ 126 mg/dl (7 mmol/l) or diabetes in interview						
Crude OR (95% CI)	1	0.793 (0.592–1.062)	0.684 (0.506–0.925)	1	0.701 (0.527–0.934)	0.619 (0.461–0.830)
Adjusted OR (95% CI)[1]	1	0.889 (0.651–1.213)	0.821 (0.596–1.131)	1	0.754 (0.556–1.022)	0.851 (0.621–1.166)
Adjusted OR (95% CI)[2]	1	0.896 (0.645–1.245)	0.871 (0.623–1.216)	1	0.721 (0.522–0.997)	0.873 (0.625–1.220)

WC: waist circumference; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; FG: fasting glucose. [1]Analysis adjusted for age; [2]analysis adjusted for age, BMI, educational level, leisure time physical activity, smoking, and alcohol intake.

demographic and lifestyle information were based on the standardized procedures.

5. Conclusion

This study demonstrated that in Polish women, high DPI and high DTAC were significantly associated with a reduced odds ratio for the prevalence of MetS components, such as elevated blood pressure and diabetes. In contrast, in men, high DPI and high DTAC did not have the potential to alleviate MetS components.

Conflicts of Interest

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

Authors' Contributions

Małgorzata Elżbieta Zujko conceived the idea for the manuscript. Małgorzata Elżbieta Zujko and Anna Waśkiewicz contributed to the study design, analyzed the data, interpreted the results, and wrote the paper. Anna Maria Witkowska contributed to data analysis and interpretation and critically revised the manuscript. Tomasz Zdrojewski, Krystyna Kozakiewicz, and Wojciech Drygas critically revised the manuscript. Anna Waśkiewicz, Danuta Szcześniewska, Tomasz Zdrojewski, Krystyna Kozakiewicz, and Wojciech Drygas provided the data of the WOBASZ II study. Danuta Szcześniewska contributed on data analysis. All authors edited and approved the final version of the manuscript. Małgorzata Elżbieta Zujko and Anna Waśkiewicz contributed equally to this work.

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Research Article

Antiaging of Cucurbitane Glycosides from Fruits of *Momordica charantia* L.

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Methanol extracts of *Momordica charantia* L. fruits are extensively studied for their antiaging activities. A new cucurbitane-type triterpenoid (1) and nine other known compounds (2–10) were isolated, and their structures were determined according to their spectroscopic characteristics and chemical derivatization. Biological evaluation was performed on a K6001 yeast bioassay system. The results indicated that all the compounds extended the replicative lifespan of K6001 yeast significantly. Compound 9 was used to investigate the mechanism involved in the increasing of the lifespan. The results indicated that this compound significantly increases the survival rate of yeast under oxidative stress and decreases ROS level. Further study on gene expression analysis showed that compound 9 could reduce the levels of *UTH1* and *SKN7* and increase *SOD1* and *SOD2* gene expression. In addition, it could not extend the lifespan of the yeast mutants of *Uth1*, *Skn7*, *Sod1*, and *Sod2*. These results demonstrate that compound 9 exerts antiaging effects via antioxidative stress and regulation of *UTH1*, *SKN7*, *SOD1*, and *SOD2* yeast gene expression.

1. Introduction

Fruits of *Momordica charantia* L. are edible healthy vegetable in Asia and commonly known as bitter melon or bitter gourd because of their bitter taste. Given their nutritional potential, they are used as traditional Chinese herbal medicine to treat several ailments, such as diabetes, constipation, abdominal pain, kidney stones, piles, pneumonia, and improve appetite [1–5]. *M. charantia* contains biologically active phytochemicals, such as polysaccharides, proteins, flavonoids, glycosides, saponins, steroids, alkaloids, essential oils, and triterpenes [5–10]. Many of these phytochemicals exhibit antitumor, anti-inflammatory, immunomodulation, and antidiabetic activities and the ability to reduce oxidative stress [5].

Aging is a dominating risk factor for age-related diseases, including cancer, metabolic disease, cardiovascular disease, and neurodegenerative illnesses [11]. As the aging population is increasing dramatically throughout the world, aging has drawn great attention because of huge expenses for medical care and serious consequences of the related diseases. Interventions that delay aging were found to have a greater effect on the quality of life compared with disease-specific approaches [12]. In our previous studies [13–17], a yeast mutant K6001 was employed in the bioassay system, and ganodermasides A–D, phloridzin, nolinospiroside F, and parishin with significant antiaging potential from natural sources were obtained.

Basing on the K6001 bioassay system, we isolated one novel cucurbitane glycoside (1) and nine known cucurbitane-type triterpenoids (2–10) from the fruits of *M. charantia* L. (Figure 1). Essential studies on the action mechanism suggested that these cucurbitane glycosides could improve the antioxidative properties of yeasts. The yeast genes of youth 1 (*UTH1*), skinhead-7 (*SKN7*), and superoxide dismutase (*SOD*) may also be involved in the action.

2. Material and Methods

2.1. General. The chemical reagents used were of HPLC grade and purchased from TEDIA (Rhode Island, USA). The others were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

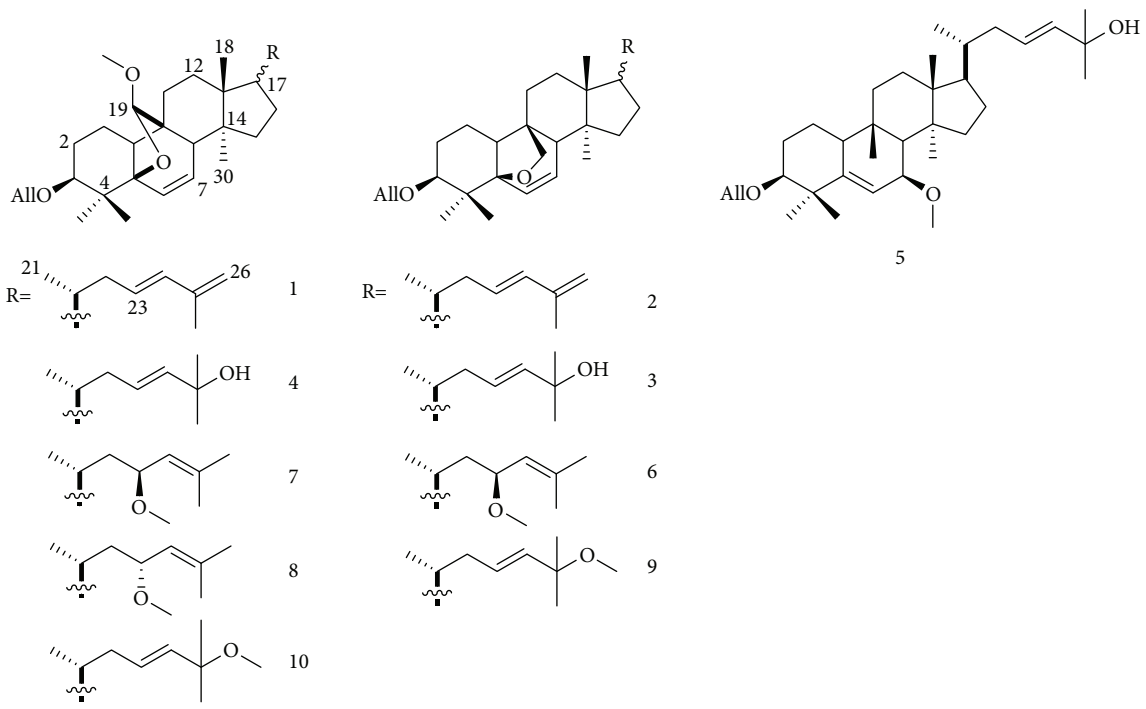

Figure 1: Chemical structures of compounds 1–10.

The preparative HPLC system was equipped with two ELITE P-230 pumps and an UV detector. Optical rotations were determined on a JASCO P-1030 digital polarimeter. High-resolution ESI-TOF-MS analyses were performed on an Agilent Technologies 6224A Accurate-Mass TOF LC/MS system (Santa Clara, CA, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV III-500 spectrometer (Bruker, Billerica, MA, USA). Column chromatography was performed over the silica gel (200–300 mesh, Yantai Chemical Industry Research Institute, Yantai, China) or reversed phase C18 (Octadecylsilyl, ODS) silica gel (Cosmosil 75C_{18}-OPN, Nacalai Tesque, Japan).

2.2. Plant Material and Yeast Strains. Fruits of *M. charantia* were purchased from Liangzhu market of Hangzhou, Zhejiang Province, China, 2011. The identity of this plant was confirmed by an associate professor Liurong Chen, and a voucher specimen (number 20110712) was preserved in Zhejiang University Institute of Materia Medica. The yeast strains BY4741 and mutants of *uth1*, *skn7*, *sod1*, and *sod2* with K6001 background are from Prof. Matsuura in Chiba University, and K6001 strain with W303 background is from Prof. Breitenbach in Salzburg University.

2.3. Extraction and Isolation. About 1.5 kg (dry weight) of the material was smashed and extracted with methanol (MeOH) for 3 days with shaking at room temperature. The extract was filtered and concentrated to obtain a crude extract (224 g), which was partitioned with ethyl acetate (EtOAc) and water. The active EtOAc layer (30 g) was subjected to a silica gel open column with *n*-hexane/acetone (99 : 1, 98 : 2, 95 : 5, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 20 : 80, and 0 : 100) and acetone/MeOH (50 : 50 and 0 : 100). The EtOAc layer yielded nine fractions after the combination based on TLC analysis. The eighth active fraction of 4.0 g (13.9 g in total) was subsequently separated by ODS open column with MeOH/H_2O (60 : 40, 70 : 30, 75 : 25, 80 : 20, 90 : 10, and 100 : 0), and 11 samples were obtained (fr.1–fr.11). The four active samples of fr.6–fr.9 were further separated as follows:

Fr.6 (600 mg) was introduced to an ODS open column with MeOH/H_2O (70 : 30, 73 : 27, 75 : 25, 77 : 23, 80 : 20, 83 : 17, 85 : 15, and 100 : 0) to yield nine samples (fr.6-1–fr.6-9). Fr.6-6 (72 mg) was further separated by a silica gel open column chromatography with dichloromethane (CH_2Cl_2)/MeOH (100 : 0, 99 : 1, 98 : 2, 97.5 : 2.5, 95 : 5, 90 : 10, and 0 : 100), and the sixth active fraction (11.6 mg) was purified by HPLC [C30-UG-5 (φ10 × 250 mm, Nomura Chemical), mobile phase: acetonitrile (MeCN)/H_2O (90 : 10), flow rate: 3 mL/min, and detector: 210 nm] and yielded compound 1 (4.6 mg, t_R = 33.5 min) and compound 2 (1.0 mg, t_R = 30.7 min).

Fr.7 (277 mg) was separated in a silica gel open column with chloroform ($CHCl_3$)/MeOH (100 : 0, 98 : 2, 95 : 5, 90 : 10, and 0 : 100) and yielded fr.7-1–fr.7-11. Subsequently, fr.7-7 (63 mg) was subjected to ODS open column chromatography with MeOH/H_2O (70 : 30, 75 : 25, 80 : 20, 90 : 10, and 100 : 0), and the second fraction (37.8 mg) was purified by HPLC [C30-UG-5 (φ10 × 250 mm, Nomura Chemical), mobile phase: MeCN/H_2O (62 : 38), flow rate: 3 mL/min, and detector: 210 nm] and yielded compound 3 (10.6 mg, t_R = 15.9 min), compound 4 (1.8 mg, t_R = 17.1 min), and compound 5 (1.9 mg, t_R = 18.7 min).

Fr.8 (340 mg) was introduced to a silica open column with *n*-hexane/$CHCl_3$ (50 : 50, 30 : 70, and 0 : 100) and $CHCl_3$/MeOH (97 : 3, 95 : 5, 90 : 10, and 0 : 100) to give fr.8-1–fr.8-9. Then, fr.8-4 (49 mg) was purified by HPLC

[CAPCELL PAKC$_{18}$, Shiseido (φ10 × 250 mm), mobile phase: MeCN/H$_2$O (63 : 37), flow rate: 3 mL/min, and detector: 210 nm] and yielded compound 6 (10.2 mg, t_R = 20.2 min), compound 7 (4.9 mg, t_R = 22.9 min), and compound 8 (1.3 mg, t_R = 32.0 min).

Fr.9 (600 mg) was separated by silica gel open column with CHCl$_3$/MeOH (100 : 0, 100 : 1, 100 : 2, 100 : 3, 100 : 5, 90 : 10, and 0 : 100), and fr.9-1–fr.9-8 were obtained. Then, fr.9-4 (332 mg) was further separated by ODS open column with MeOH/H$_2$O (90 : 10, 95 : 5, and 100 : 0), and the third fraction (42 mg) was purified by HPLC [CAPCELL PAKC$_{18}$, Shiseido (φ10 × 250 mm), mobile phase: MeCN/H$_2$O (60 : 40), flow rate: 3 mL/min, and detector: 210 nm] and yielded compound 9 (5.0 mg, t_R = 28.7 min) and compound 10 (8.0 mg, t_R = 33.8 min).

2.3.1. Compound 1. White solid; $[\alpha]_D^{20}$-78.6 (*c* 0.2, CH$_3$OH); high-resolution ESI-TOF-MS *m/z* 653.4029, calculated for $C_{37}H_{58}O_8Na$ $(M+Na)^+$ 653.4024; data of ^{1}H NMR and ^{13}C NMR are shown in Table 1.

2.3.2. Charantoside IV (2). Colorless solid; $[\alpha]_D^{28}$-143.9 (*c* 0.16, CH$_3$OH); MS *m/z* 623 $(M+Na)^+$, $C_{36}H_{56}O_7Na$; ^{13}C NMR (125 MHz, pyridine-d_5): δ = 15.4, 19.3, 19.3, 19.3, 20.6, 21.5, 24.2, 26.0, 28.0, 28.7, 31.4, 33.8, 37.2, 39.4, 40.5, 40.5, 45.7, 45.9, 49.3, 51.0, 52.7, 63.7, 69.7, 72.9, 73.5, 76.6, 80.5, 85.5, 86.3, 104.3, 115.1, 130.3, 130.4, 134.6, 135.1, and 142.9. The structure was identified based on comparison of MS, ^{1}H, and ^{13}C NMR data with literature [18].

2.3.3. Momordicoside F_2 (3). White solid; $[\alpha]_D^{20}$-101.0 (*c* 0.94, CHCl$_3$:CH$_3$OH = 1 : 1); MS *m/z* 641 $(M+Na)^+$, $C_{36}H_{58}O_8Na$; ^{13}C NMR (125 MHz, pyridine-d_5): δ = 15.4, 19.3, 19.3, 20.6, 21.5, 24.3, 26.0, 28.0, 28.6, 31.3, 31.3, 31.4, 33.8, 37.0, 39.4, 39.9, 40.5, 45.7, 45.8, 49.3, 50.6, 52.7, 63.7, 69.7, 70.1, 72.9, 73.5, 76.6, 80.5, 85.5, 86.3, 104.2, 124.6, 130.4, 134.6, and 142.1. The structure was identified based on comparison of MS, ^{1}H, and ^{13}C NMR data with literature [19].

2.3.4. Goyaglycoside-b (4). White solid; $[\alpha]_D^{20}$-100.7 (*c* 0.2, CH$_3$OH); MS *m/z* 671 $(M+Na)^+$, $C_{37}H_{60}O_9Na$; ^{13}C NMR (125 MHz, pyridine-d_5): δ = 15.3, 19.1, 19.3, 20.4, 21.7, 23.7, 25.3, 27.8, 28.6, 31.3, 31.3, 31.3, 34.3, 37.0, 39.5, 40.0, 42.0, 42.6, 45.7, 48.6, 48.7, 50.8, 58.1, 63.7, 69.7, 70.1, 72.2, 74.2, 77.0, 83.9, 86.0, 102.8, 112.8, 124.7, 132.0, 133.6, and 142.1. The structure was identified based on comparison of MS, ^{1}H, and ^{13}C NMR data with literature [4].

2.3.5. Karaviloside III (5). White solid; $[\alpha]_D^{28}$+70.9 (*c* 0.1, CH$_3$OH); MS *m/z* 657 $(M+Na)^+$, $C_{37}H_{62}O_8Na$; ^{13}C NMR (125 MHz, pyridine-d_5): δ = 16.0, 18.5, 19.4, 23.1, 26.3, 28.3, 29.3, 29.4, 29.6, 30.8, 31.3, 31.3, 33.2, 34.8, 35.4, 37.1, 39.8, 40.0, 42.4, 46.7, 48.7, 49.3, 50.6, 56.6, 63.8, 69.7, 70.1, 72.5, 73.9, 76.1, 78.0, 88.3, 105.4, 119.5, 124.2, 142.2, and 148.4. The structure was identified based on comparison of MS, ^{1}H, and ^{13}C NMR data with literature [20].

Table 1: ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) data of compound 1 in pyridine-d_5.

Position	Compound 1	
	δ_H (*J* in Hz)	δ_C
1α	1.46	19.2
1β	1.91	—
2α	1.75	27.8
2β	2.17	—
3α	3.73 (br s)	83.9
4	—	39.5
5	—	86.0
6	6.18 (dd, 2.0, 9.7)	133.6
7	5.63 (dd, 3.6, 9.7)	132.0
8β	3.15 (br s)	42.7
9	—	48.6
10α	2.48 (dd, 5.6, 12.7)	42.1
11α	1.75	23.7
11β	1.68	—
12α	1.60	31.3
12β	1.55	—
13	—	45.8
14	—	48.7
15α	1.31	34.3
15β	1.31	—
16α	1.94	28.7
16β	1.32	—
17α	1.50	51.2
18	0.91 (s)	15.3
19	4.91 (s)	112.8
20	1.50	37.3
21	0.95 (d, 5.6)	19.4
22α	1.83	40.5
22β	2.32	—
23	5.76 (m)	130.4
24	6.32 (d, 15.3)	135.1
25	—	143.0
26α	4.96 (s)	115.1
26β	5.10 (s)	—
27	1.92 (s)	19.3
28	0.83 (s)	25.3
29	1.47 (s)	21.7
30	0.90 (s)	20.4
–OCH$_3$	3.52 (s)	58.1
1′	5.52 (d, 7.8)	102.8
2′	3.94 (dt, 2.6, 7.6)	74.2
3′	4.76 (d, 2.8)	72.2
4′	4.23 (td, 2.8, 9.3)	69.7
5′	4.50 (m)	77.0
6′α	4.43 (m)	63.7
6′β	4.57 (m)	—

2.3.6. Charantoside VI (6). White solid; $[\alpha]_D^{28}$-67.2 (*c* 0.48, CH_3OH); MS *m/z* 655 $(M+Na)^+$, $C_{37}H_{60}O_8Na$; ^{13}C NMR (125 MHz, pyridine-d_5): $\delta = 15.2$, 18.8, 19.3, 20.3, 20.6, 21.5, 24.2, 26.0, 26.3, 28.0, 29.1, 31.5, 33.8, 34.2, 39.4, 40.5, 43.4, 45.7, 45.9, 49.3, 51.7, 52.7, 55.7, 63.7, 69.7, 72.9, 73.5, 76.6, 76.8, 80.5, 85.6, 86.3, 104.2, 127.8, 130.4, 134.5, and 135.5. The structure was identified based on comparison of MS, ^1H, and ^{13}C NMR data with literature [18].

2.3.7. Charantagenin E (7). Colorless solid; $[\alpha]_D^{28}$-104.3 (*c* 0.11, CH_3OH); MS *m/z* 685 $(M+Na)^+$, $C_{38}H_{62}O_9Na$; ^{13}C NMR (125 MHz, pyridine-d_5): $\delta = 15.1$, 18.8, 19.1, 20.4, 20.4, 21.7, 23.7, 25.3, 26.2, 27.8, 29.0, 31.4, 34.2, 34.2, 39.5, 42.0, 42.6, 43.4, 45.7, 48.5, 48.7, 51.8, 55.7, 58.0, 63.7, 69.7, 72.2, 74.2, 76.9, 77.0, 83.9, 86.0, 102.8, 112.8, 127.8, 132.0, 133.5, and 135.5. The structure was identified according to the comparison among MS, ^1H, and ^{13}C NMR data in literature [21].

2.3.8. Charantoside II (8). White solid; $[\alpha]_D^{20}$-67.1 (*c* 0.2, CH_3OH); MS *m/z* 685 $(M+Na)^+$, $C_{38}H_{62}O_9Na$; ^{13}C NMR (125 MHz, pyridine-d_5): $\delta = 15.2$, 18.5, 19.1, 19.3, 20.4, 21.6, 23.7, 25.2, 26.2, 27.7, 28.8, 31.4, 33.2, 34.2, 39.5, 42.0, 42.6, 43.7, 45.8, 48.5, 48.7, 51.7, 56.0, 58.0, 63.6, 69.6, 72.2, 74.1, 75.2, 76.9, 84.0, 85.9, 102.9, 112.8, 128.3, 132.0, 133.5, and 134.9. The structure was identified based on comparison of MS, ^1H, and ^{13}C NMR data with literature [18].

2.3.9. Momordicoside G (9). White solid; $[\alpha]_D^{28}$-90.2 (*c* 1.0, CH_3OH); MS *m/z* 655 $(M+Na)^+$, $C_{37}H_{60}O_8Na$; ^{13}C NMR (125 MHz, pyridine-d_5): $\delta = 15.5$, 19.3, 20.6, 21.5, 24.3, 26.0, 26.5, 26.9, 28.0, 28.6, 31.5, 33.8, 36.7, 39.4, 40.1, 40.5, 45.7, 45.9, 49.3, 50.6, 50.7, 52.7, 63.7, 65.3, 69.7, 72.9, 73.5, 75.3, 76.6, 80.6, 85.5, 86.3, 104.2, 128.8, 130.4, 134.6, and 138.1. The structure was identified based on comparison of MS, ^1H, and ^{13}C NMR data with literature [19].

2.3.10. Goyaglycoside-d (10). White solid; $[\alpha]_D^{28}$-124.9 (*c* 0.1, CH_3OH); MS *m/z* 685 $(M+Na)^+$, $C_{38}H_{62}O_9Na$; ^{13}C NMR (125 MHz, pyridine-d_5): $\delta = 15.3$, 19.1, 19.3, 20.4, 21.6, 23.7, 25.3, 26.4, 26.9, 27.8, 28.6, 31.3, 34.3, 36.8, 39.5, 40.1, 42.0, 42.6, 45.7, 48.5, 48.7, 50.5, 50.8, 58.1, 63.7, 69.6, 72.2, 74.1, 75.3, 77.0, 83.9, 86.0, 102.8, 112.8, 128.9, 132.0, 133.6, and 138.0. The structure was identified based on comparison of MS, ^1H, and ^{13}C NMR data with literature [4].

2.4. Acid Hydrolysis and Sugar Analysis of Compound 1. The absolute configuration of sugar moiety of compound 1 was determined according to a previously reported method [22, 23]. Briefly, compound 1 (0.5 mg) in anhydrous 2.0 M HCl in MeOH (1 mL) was heated at 80°C with reflux for 4 h. The reaction solution was evaporated and partitioned between chloroform and water. The residue of aqueous layer was heated with 0.5 mg L-cysteine methyl ester in pyridine (200 μL) at 60°C for 1 h; then, *o*-tolyl isothiocyanate dissolved in 100 μL pyridine (7 mg/mL) was added to the reaction mixture and further reacted at 60°C for 1 h. After that, the reaction mixture was dried and analyzed by LC/HRESIMS with the following conditions: Agilent Extend C18 column (3.5 μm, 3.0 × 100 mm); DAD detection, 210 nm; $t = 0$ min CH_3OH/H_2O/formic acid (30 : 70 : 0.1), $t = 15$ min CH_3OH/H_2O/formic acid (60 : 40 : 0.1); and flow rate: 0.45 mL/min.

The allose thiocarbamate standards were prepared in the same procedure. Given that L-allose is limitedly available, the retention time of L-allose thiocarbamate derivative was obtained by reacting D-allose with D-cysteine methyl ester. The basis of this approach is the fact that the t_R values of D- and L-enantiomers are reversed when D-cysteine methyl ester is used [22].

2.5. Lifespan Assay. The bioassay method was performed as described in a previous study [13]. Briefly, K6001 or mutants with K6001 background were grown on a YPGalactose medium consisting of 3% galactose, 2% hipolypeptone, and 1% yeast extract or on a YPGlucose medium containing 2% glucose instead of galactose. Agar plates were prepared by adding 2% agar to the medium. For screening, the K6001 yeast strain was first incubated in the galactose medium for 24 h with shaking and then centrifuged. The yeast pellet was washed with PBS three times. The cells were then diluted and counted using a hemocytometer, and approximately 4000 cells were plated on glucose agar plates containing different concentrations of samples. The plates were stored in an incubator at 28°C. After 48 h, the yeast cells in the plates were observed with a microscope. For each plate, 40 colonies were selected randomly, and the number of their daughter cells was counted and analyzed.

2.6. Antioxidative Stress Method. Antioxidative stress assay was performed as previously described with minor modification [16]. BY4741 yeast was inoculated in 5 mL of YPGlucose medium and cultured at 28°C with shaking for 24 h. The yeast cells at 0.1 OD_{600} were transferred in 20 mL of new YPGlucose medium and incubated with compound 9 at 1 and 3 μM or resveratrol (Res, positive control) at 10 μM for 12 h.

For the first method, 5 μL aliquot after double dilution from each group was dropped in the same YPGlucose agar plate mixed with 9 mM H_2O_2, and the plate was incubated at 28°C for 4 days. The growth rates of the yeast cells in different groups were compared and photographed.

Another antioxidative stress assay was used to validate the accuracy of the experiment. Approximately 200 cells mixed with the test samples were spreaded on YPGlucose agar plates with or without 5 mM H_2O_2 and cultured at 28°C for 48 h. The survival rates of the sample groups were counted and compared with those of the control group.

2.7. Determination of ROS Level in Yeast. The ROS assay procedure was the same with a previous study [17]. BY4741 yeast cells were cultured as described in the experiment above and incubated with compound 9 at 1 or 3 μM for 23 h. Changes in intracellular ROS levels of the yeast were determined using an ROS assay kit (Beyotime, Jiangsu, China) and a fluorescent plate reader (Spectra Max M2, Molecular Devices, San Francisco, CA, USA). A total of 1 mL of cultured broth was obtained, treated with 10 μM DCFH-DA at 28°C in dark, and then shaken by vortexing at 160 rpm at 15 min intervals for 1 h. The yeast cells were subsequently washed

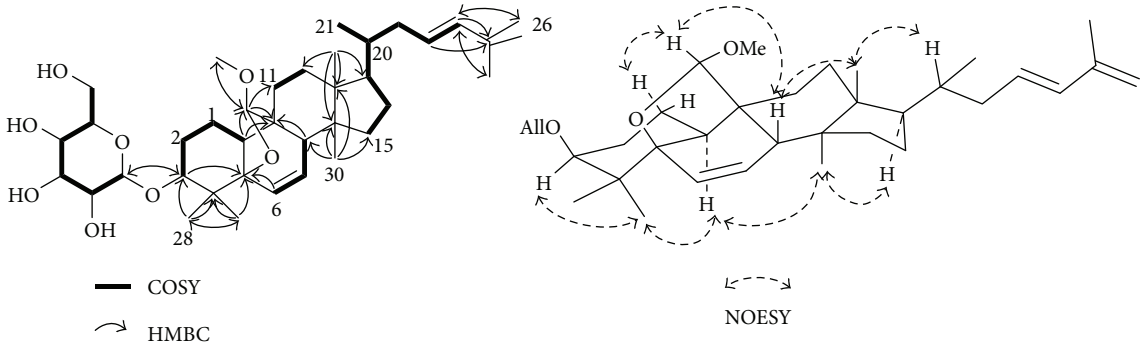

Figure 2: Gross structure of compound 1 with ^{1}H-^{1}H COSY, selected HMBC, and NOESY correlations.

with PBS, and their DCF fluorescence was measured by a fluorescent plate reader at excitation and emission wavelengths of 488 and 525 nm, respectively.

2.8. Real-Time Quantitative PCR Analysis. BY4741 yeast cells were cultured in glucose medium following the addition of 0, 1, 3 μM compound 9. RNA was extracted from yeast cells in the exponential phase through the hot-phenol method. Reverse transcription was performed using a cloned AMV first-strand cDNA synthesis kit (Invitrogen, California, USA) with oligo (dT) adaptor primers and 5 μg of yeast total RNA. Real-time PCR was performed using the CFX96-Touch (Bio-Rad, Hercules, USA) and SYBR Premix EX Taq™ (TaKaRa, Otsu, Japan). Thermal cycling parameters for *UTH1* and *SKN7*: 40 cycles, 94°C for 15 s, 55.4°C for 15 s, and 68°C for 20 s; for *SOD1* and *SOD2*: 40 cycles, 94°C for 15 s, 60°C for 25 s, and 72°C for 10 s. Primers used were as follows: for *UTH1*, sense 5′-CGC CTC TTC CTC TT-3′ and antisense 5′-ACC ATC GGA AGG TTG TTC AG-3′; for *SKN7*, sense 5′-AGT TGT CAG CGA CGG TCT TT-3′ and antisense 5'-GCT GTG GCA CCA TCT AGG TT-3′; for *SOD1* sense 5′-CAC CAT TTT CGT CCG TCT TT-3′ and antisense 5′-TGG TTG TGT CTC TGC TGG TC-3′; for *SOD2*, sense 5′-CTC CGG TCA AAT CAA CGA AT-3′ and antisense 5′-CCT TGG CCA GAA GAT CTG AG-3′; for *TUB1*, sense 5′-CCA AGG GCT ATT TAC GTG GA-3′ and antisense 5′-GGT GTA ATG GCC TCT TGC AT-3′. The amount of *UTH1*, *SKN7*, *SOD1*, and *SOD2* was normalized to that of *TUB1*.

2.9. Statistical Analysis. One-way analysis of variance was performed using GraphPad Prism biostatistics software (San Diego, CA, USA) to analyze the data. Significant differences were compared by two-tailed multiple *t*-tests with Student–Newman–Keuls test. Data were expressed as means ± SEM of triplicate experiments. A $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Structure Elucidation of Compound 1. Compound 1 has the molecular formula $C_{37}H_{58}O_8$ as determined by HR-ESIMS measurement. The ^{1}H NMR data showed six methyl groups at δ_H 0.83 (3H, s), 0.90 (3H, s), 0.91 (3H, s), 0.95 (3H, d, $J = 5.6$ Hz), 1.47 (3H, s), and 1.92 (3H, s), along with six olefinic protons at δ_H 4.96 (1H, s), 5.10 (1H, s), 5.63 (1H, dd, $J = 3.6, 9.7$ Hz), 5.76 (1H, m), 6.18 (1H, dd, $J = 2.10$, 9.7 Hz), and 6.32 (1H, d, $J = 15.3$ Hz). Several multiple peaks at δ_H 3.94–4.76 and the signal of an anomeric proton [δ_H 5.52 (1H, d, $J = 7.8$ Hz)] indicated the existence of a sugar moiety. The ^{13}C NMR data revealed the presence of 37 carbon signals. With the combined signals of ^{13}C NMR and DEPT, the 37 carbon signals were attributed to six olefinic carbons (δ_C 115.1, 130.4, 132.0, 133.6, 135.1, and 143.0), one anomeric carbon (δ_C 102.8), one oxygenated quaternary carbon (δ_C 86.0), six oxymethines (δ_C 69.7, 72.2, 74.2, 77.0, 83.9, and 112.8), one oxymethylene (δ_C 63.7), one methoxy group (δ_C 58.1), four quaternary sp^3 carbons (δ_C 39.5, 45.8, 48.6, and 48.7), four methines (δ_C 37.3, 42.1, 42.7, and 51.2), seven methylenes (δ_C 19.2, 23.7, 27.8, 28.7, 31.3, 34.3, and 40.5), and six methyl groups (δ_C 15.3, 19.3, 19.4, 20.4, 21.7, and 25.3). Detailed analysis of the ^{1}H-^{1}H COSY spectra led to the determination of the partial structures depicted by the bonds (Figure 2, in bold bonds). In the HMBC spectrum, these partial structures were connected to yield the following gross structures: H-3 to C-5; H-6 to C-5; H-8 to C-9; H-10 to C-9; CH_3-18 to C-12, C-13, C-14, and C-17; H-19 to C-9, C-10, C-11, and $-OCH_3$; CH_3-28 to C-3, C-4, and C-29; CH_3-29 to C-4, C-5, and C-28; CH_3-30 to C-8, C-13, C-14, and C-15; H-23 to C-25; H-24 to C-25, C-26, and C-27; H-26 to C-24; CH_3-27 to C-24; and $-OCH_3$ to C-19. The signals of H-3 to C-1′ of allose and anomeric proton H-1′ of allose to C-3 in the HMBC indicated the location of the sugar moiety (Figure 2). The β anomeric configuration of allose was determined from its coupling constant J (7.8 Hz) of anomeric protons (δ_H 5.52). The absolute configuration of the sugar moiety was further confirmed by the degradation of compound 1 and through the comparison of the retention time of its aldose thiocarbamate derivative ($t_R = 9.287$ min) with those of the following aldose thiocarbamate standards: L-cysteine-D-allose ($t_R = 9.127$ min) and D-cysteine-D-allose ($t_R = 7.460$ min).

The relative stereochemistry of compound 1 was deduced by nuclear overhauser enhancement spectroscopy (NOESY) analysis. As shown in Figure 2, the broad singlet signal of H-3 appeared at δ_H 3.73 thereby suggested the α configuration of this proton. The NOESY correlations of

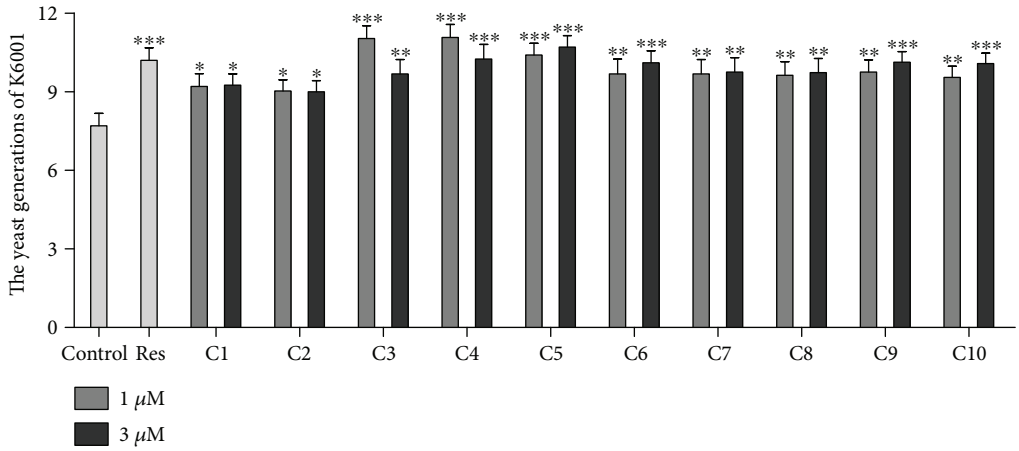

Figure 3: Effect of compounds 1–10 on the replicative lifespan of K6001 yeast strain. The average lifespan of K6001 was as follows: control (7.70 ± 0.48); Res at 10 μM ($10.20 \pm 0.42^{***}$); compound 1 at 1 μM ($9.20 \pm 0.49^{*}$) and at 3 μM ($9.25 \pm 0.42^{*}$); compound 2 at 1 μM ($9.03 \pm 0.43^{*}$) and at 3 μM ($9.00 \pm 0.42^{*}$); compound 3 at 1 μM ($11.03 \pm 0.53^{***}$) and at 3 μM ($9.68 \pm 0.55^{**}$); compound 4 at 1 μM ($11.08 \pm 0.50^{***}$) and at 3 μM ($10.25 \pm 0.56^{***}$); compound 5 at 1 μM ($10.40 \pm 0.45^{***}$) and at 3 μM ($10.70 \pm 0.45^{***}$); compound 6 at 1 μM ($9.68 \pm 0.57^{**}$) and at 3 μM ($10.10 \pm 0.46^{***}$); compound 7 at 1 μM ($9.68 \pm 0.55^{**}$) and at 3 μM ($9.75 \pm 0.55^{**}$); compound 8 at 1 μM ($9.63 \pm 0.52^{**}$) and at 3 μM ($9.73 \pm 0.55^{**}$); compound 9 at 1 μM ($9.75 \pm 0.47^{**}$) and at 3 μM ($10.13 \pm 0.41^{***}$); and compound 10 at 1 μM ($9.55 \pm 0.42^{**}$) and at 3 μM ($10.08 \pm 0.39^{***}$) ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control).

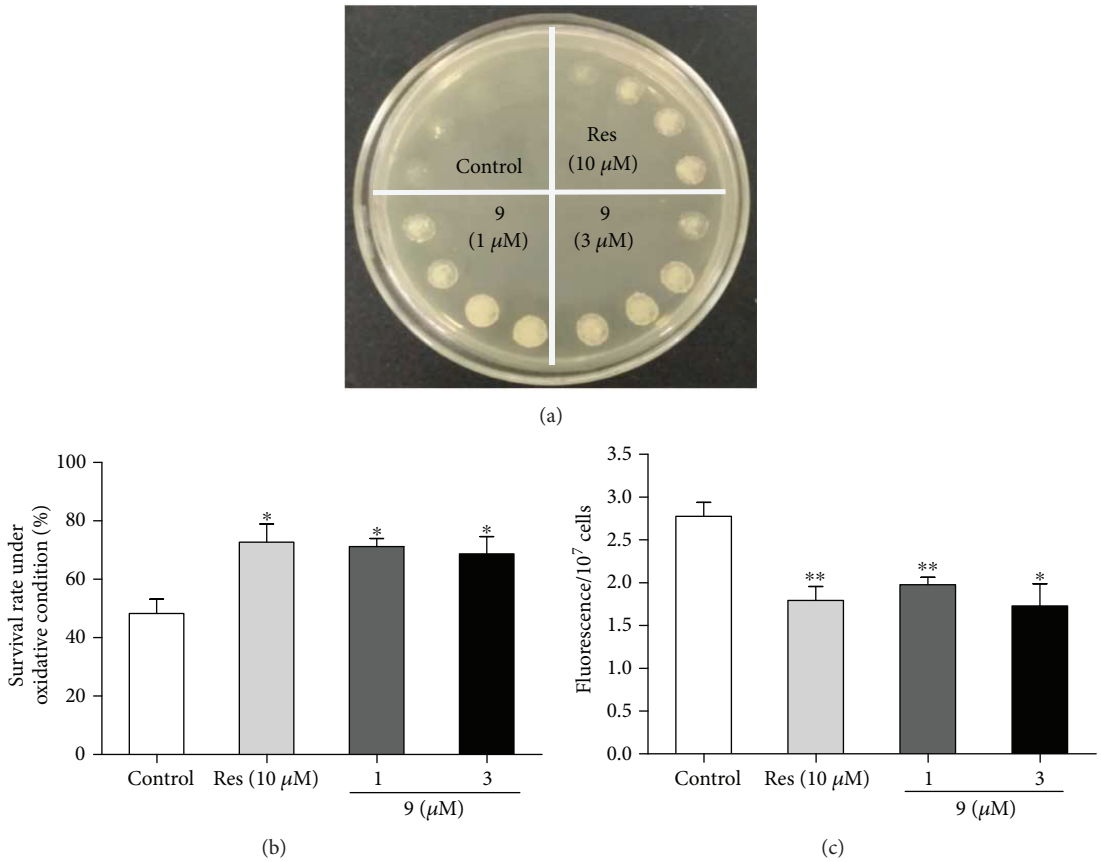

Figure 4: Effect of compound 9 on the antioxidative ability of yeast cells and ROS level of yeast. (a) BY4741 yeast cells in the control group and compound 9-treated groups were dropped in the same YPGlucose agar plate mixed with 9 mM H_2O_2. After four days, the growth of yeast cells in different groups was photographed. (b) Effect of compound 9 on the survival rates of yeast under oxidative stress condition. Control, 48.31 ± 4.94; Res, $72.74 \pm 6.19^{*}$; compound 9 at 1 μM, $71.21 \pm 2.75^{*}$and at 3 μM, $68.73 \pm 5.89^{*}$. The experiment was conducted at least thrice. Vertical bars represent the mean ± SEM of three assays ($^{*}P < 0.05$). (c) The change of ROS level of yeast after administration compound 9 at 1, 3 μM. Control, $2.77 \pm 0.15^{**}$; Res, $1.79 \pm 0.15^{**}$; compound 9 at 1 μM, $1.98 \pm 0.08^{**}$, and at 3 μM, $1.73 \pm 0.22^{*}$. Vertical bars represent the mean ± SEM of 6 repeats ($^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the control).

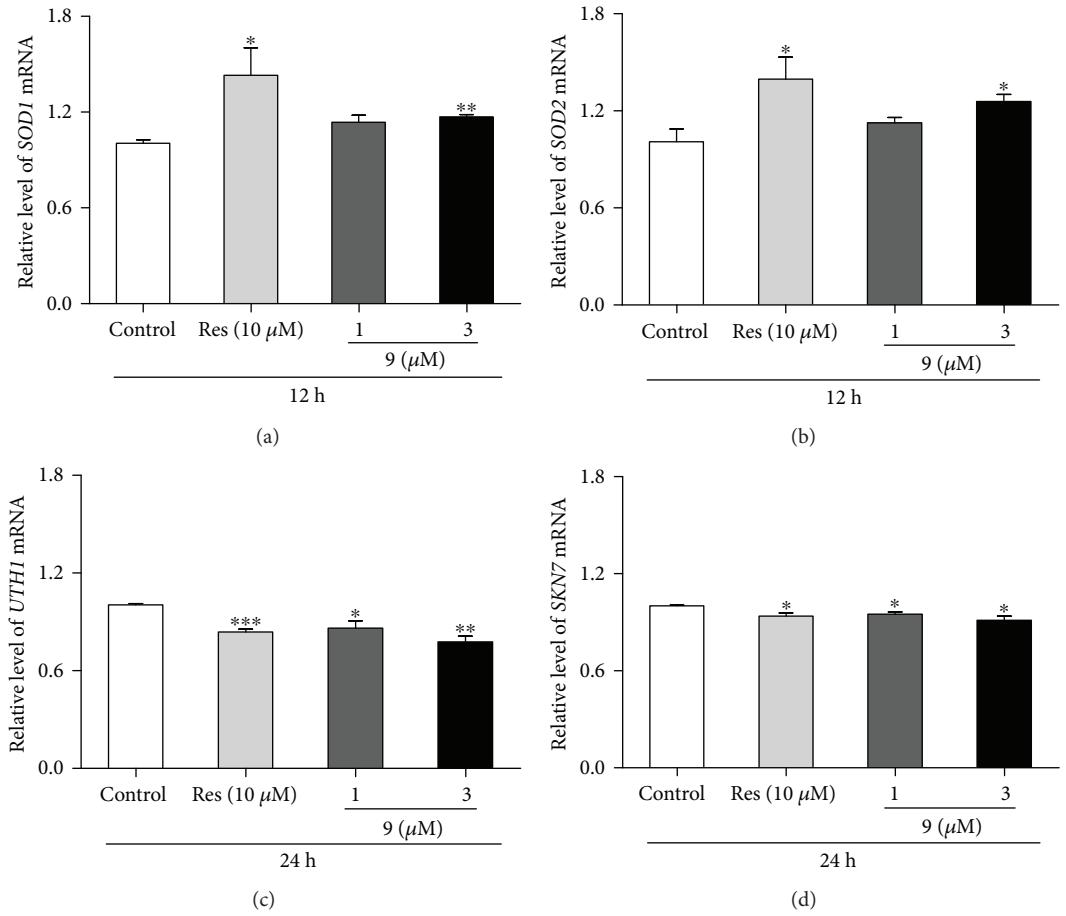

Figure 5: Effects of compound 9 on *SOD1* (a), *SOD2* (b), *UTH1* (c), and *SKN7* (d) yeast gene expression. The gene levels of BY4741 yeast cells were tested after treated with compound 9 at 1 and 3 μM. Compound 9 significantly increased *SOD1* and *SOD2* yeast gene level at 12 h and inhibited *UTH1* and *SKN7* yeast gene expression at 24 h. Amounts of the mRNA above were normalized to that of *TUB1*. The results were displayed as mean ± SEM for three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group).

major cross-peaks of H-3α/Me-28, Me-28/H-10, H-10/Me-30, and Me-30/H-17 indicated the α-orientation of these protons. The correlations between H-1β/H-19, H-19/H-8, H-8/Me-18, and Me-18/H-20 suggested the β-orientation of these groups. The double bond at C-23 and C-24 was elucidated by COSY correlations, whereas the transgeometry was determined from the coupling constant, $J_{23\text{-}24} = 15.3$ Hz.

The above evidence suggested that compound 1 was structurally similar to (19*R*, 23*E*)-5β, 19-epoxy-19-methoxycucurbita-6, 23-25-trien-3β-ol 3-O-β-D-allopyranoside (Figure 1).

3.2. Identification of the Known Compounds. Compounds 2–10 (Figure 1) were identified by comparing their spectroscopic data with those in literature.

3.3. Antiaging Activity in K6001 Yeast Strain. All isolated cucurbitane triterpenoids (1–10) were tested for antiaging activity through the K6001 bioassay method at different optimum concentrations. All the compounds at 1 and 3 μM extended the replicative lifespan of K6001 significantly (Figure 3), demonstrating that the cucurbitane triterpenoids isolated from *M. charantia* L. fruits have antiaging effect in yeast.

3.4. Compound 9 Improves the Oxidative Resistance and Decreases ROS Production of Yeast. Studies on mechanism of action were conducted with compound 9 because of its abundance and good activity. Oxidative stress is one of the primary causes of aging, as indicated in various model organisms [24]. Therefore, the effect of compound 9 on the oxidative resistance of yeast was first tested. The growth of yeast cells was inhibited at 9 mM H_2O_2, whereas incubation with compound 9 at 1 or 3 μM remitted the inhibition (Figure 4(a)). The effect was further confirmed in another assay. As shown in Figure 4(b), the survival rate of the control group was 48.31% ± 4.94%, whereas that in the experimental groups increased to 71.21% ± 2.75% (compound 9 at 1 μM, $P < 0.5$) and 68.73% ± 5.89% (compound 9 at 3 μM, $P < 0.5$). The experiments indicated that compound 9 enhances the oxidative resistance of yeast cells. Furthermore, we detected the ROS level of yeast after administration compound 9 at 1 and 3 μM. As we expected, the ROS level of yeast in the resveratrol and compound 9 groups were significantly decreased compared with the control

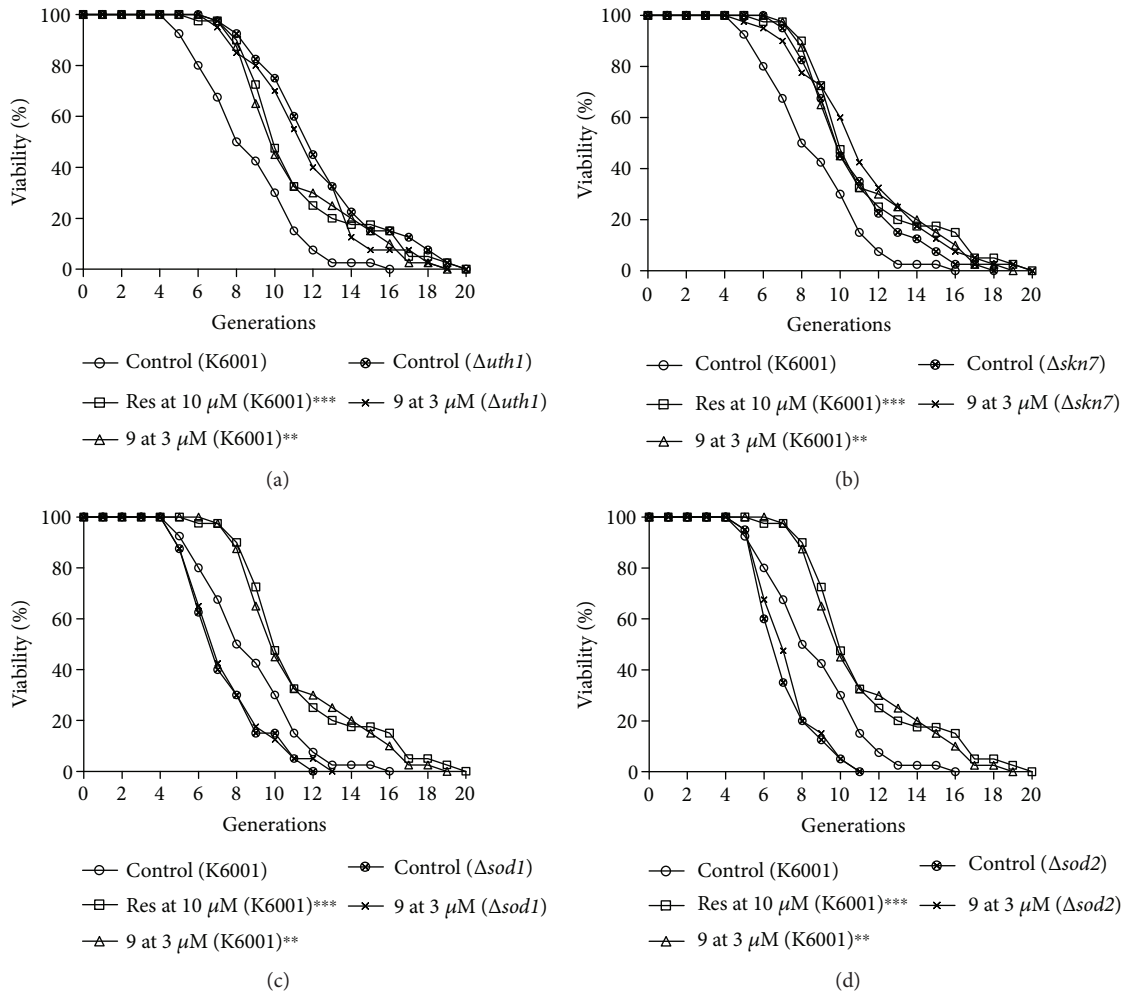

Figure 6: Effect of compound 9 on the replicative lifespan of *uth1* (a), *skn7* (b), *sod1* (c), and *sod2* (d) mutants. The average lifespan of K6001 in the control group was 7.93 ± 0.41; Res at 10 μM, $10.45 \pm 0.52^{***}$; and compound 9 at 3 μM, $10.33 \pm 0.49^{**}$. (a) The average lifespan of Δ*uth1* in the control group was 11.60 ± 0.51 and compound 9 at 3 μM, 10.95 ± 0.46. (b) The average lifespan of Δ*skn7* in the control group was 9.88 ± 0.41 and compound 9 at 3 μM, 10.40 ± 0.53. (c) The average lifespan of Δ*sod1* in the control group was 6.55 ± 0.32 and compound 9 at 3 μM, 6.65 ± 0.34. (d) The average lifespan of Δ*sod2* in the control group was 6.28 ± 0.25 and compound 9 at 3 μM, 6.50 ± 0.25 ($^{**}P < 0.01$ and $^{***}P < 0.001$).

group (Figure 4(c), $P < 0.01$, $P < 0.01$, and $P < 0.05$), respectively. These results suggested that compound 9 extended the replicative lifespan via inhibition of oxidative stress.

3.5. Compound 9 Extends Yeast Lifespan via Modification of UTH1, SKN7, SOD1, and SOD2 Gene Expression. It is well known that antioxidative stress is one of mechanisms of action for antiaging. *UTH1* gene essentially takes part in oxidative stress regulation, and deletion of *UTH1* gene will lead to extend the replicative lifespan of yeast [25]. *SKN7* is upstream gene and is a stress response transcription factor in *Saccharomyces cerevisiae* [26]. Superoxide dismutases (SOD) are major ROS scavenging enzymes and can convert superoxide anion to hydrogen peroxide [24]. Real-time PCR analysis was performed to examine the molecular mechanism of compound 9-mediated lifespan extension. The significant gene expression reduction or increase of *UTH1*, *SKN7*, *SOD1*, and *SOD2* was observed in the compound 9 treatment groups (Figure 5). These results suggested that compound 9 produced antiaging effect via regulation *UTH1*, *SKN7*, *SOD1*, and *SOD2* yeast gene expression.

3.6. Antiaging Effects of Compound 9 Diminished in Uth1, Skn7, Sod1, and Sod2 Mutations with K6001 Background. To investigate the role of these genes in the antiaging activity of compound 9, we used the mutants of *uth1*, *skn7*, *sod1*, and *sod2*. As shown in Figure 6, compound 9 at 3 μM did not affect the replicative lifespan of *uth1* (Figure 6(a)) or *skn7* mutants (Figure 6(b)), neither of *sod1* (Figure 6(c)) or *sod2* mutants (Figure 6(d)). These results were further indicated that these four genes were involved in the mechanism of action of compound 9.

4. Conclusions

A novel cucurbitane-type triterpenoid and nine known compounds were isolated and identified from the fruits of *M. charantia*. All the compounds showed antiaging effect in

yeast. The antiaging activities of these cucurbitane-type triterpenoids depended on their antioxidative ability and the regulation of the *UTH1*, *SKN7*, *SOD1*, and *SOD2* yeast genes. Apart from being one of the most well-known vegetables and frequently used as a traditional medicine because of its health benefits, *M. charantia* has potential as an antiaging functional food.

Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Authors' Contributions

Xueli Cao and Yujuan Sun contributed equally to the article.

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Research Article

Characterization of Portuguese Centenarian Eating Habits, Nutritional Biomarkers, and Cardiovascular Risk: A Case Control Study

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Background and Aims. Eating habits may contribute to longevity. We characterized the eating habits and cardiovascular risk (CVR) biomarkers in Portuguese centenarians (CENT) compared to controls. *Methods and Results*. Centenarians ($n = 253$), 100.26 ± 1.98 years, were compared with 268 controls (67.51 ± 3.25), low (LCR) and high (HCR) CVR (QRISK®2-2016). Anthropometric and body composition were evaluated by bioimpedance. Abdominal obesity, BMI, and fat mass (FM) cut-offs were according to the WHO. Sarcopenia was defined by muscle mass index cut-off $\leq 16.7\,kg/m^2$. Daily red meat intake, adjusted for age and gender, was sarcopenia protective (OR = 0.25, 95% CI = 0.096–0.670, $P = 0.006$); however, it contributes for FM excess (OR = 4.946, 95% CI = 1.471–16.626, $P = 0.01$), overweight, and obesity (OR = 4.804, 95% CI = 1.666–13.851, $P = 0.004$). This centenarian eating habit (2%) contrasts to HCR (64.3%). The history of red meat ($P < 0.0001$) and canned/industrialized food intakes ($P < 0.0001$) was associated with HCR. Basal metabolism was lower in centenarians versus LCR/HCR (CENT = 1176.78 ± 201.98; LCR = 1356.54 ± 170.65; HCR = 1561.33 ± 267.85; $P < 0.0001$), BMI (CENT = 21.06 ± 3.68; LCR = 28.49 ± 4.69; HCR = 29.56 ± 5.26; $P < 0.0001$), waist circumference (CENT = 85.29 ± 10.83; LCR = 96.02 ± 11.71; HCR = 104.50 ± 11.84; $P < 0.0001$), and waist-hip ratio (CENT = 0.88 ± 0.07; LCR = 0.92 ± 0.08; HCR = 1.01 ± 0.08; $P < 0.0001$). CENT had lower total cholesterol, LDL cholesterol, non-HDL cholesterol, and cholesterol/HDL ratio than controls. *Conclusions*. Frequent consumption of red meat, cholesterol, and heme iron rich may contribute to obesity and increased CVR. The low frequency of this consumption, observed in centenarians, although associated with sarcopenia, may be one of the keys to longevity.

1. Introduction

According to the WHO, very old individuals are a rapidly growing age group around the globe, thanks to the improvements in medicines, as well as the modification lifestyle. This nutrition characteristic is a key component for achieving good health [1]. Adults approaching 70 years will more likely face problems of caloric excess, leading to overweight or obesity [2].

There are several methods to evaluate the eating habits [3]. Retrospective methods are a good tool for assessing past eating habits [4]; however, they have some limitations, particularly in populations such as the elderly and children groups [3]. Photographic models may play an important role

when used in conjunction with retrospective methods of food intake assessment [5]. In epidemiological studies, the choice of method to use depends on many factors. The food frequency questionnaire (FFQ) is a method regularly used in epidemiological studies. Its use makes possible to evaluate the habitual frequency of food consumption over longer periods of time. It is considered the most practical and informative method to evaluate the relation of causality between food consumption and disease [6]. The structure of the FFQ is usually composed of a predefined food list and a section with the frequency of consumption. Some FFQs are semiquantitative, defining a mean reference portion consumed, so that individual reports define whether their consumption was higher, equal, or lower than the average portions presented in home measures [7].

Findings from a meta-analysis indicate that high consumption of red meat, in particular, processed meat, is associated with higher all-cause mortality [8]. Epidemiologic studies have linked the consumption of red or processed meat with obesity, type 2 diabetes, cardiovascular disease (CVD), and cancer [9, 10]. A meta-analysis of 12 cohort studies showed a 20% increase risk of diabetes per 120 g/day increase in red meat intake and, for processed red meat, a 57% increase risk per 50 g/day increase [11].

Adipose tissue is an active endocrine organ that effects insulin sensitivity and production of insulin-like growth factors and increases the oxidative stress and chronic low-grade inflammation affecting immune response [12]. In obesity, increased release from the adipose tissue of free fatty acids, TNF-α, and resistin and reduced release of adiponectin lead to the development of insulin resistance. Cancer death rates increase, mostly as a consequence of the aging of the population. A healthy diet and control of obesity based on abundant and variable plant foods, high consumption of cereals, olive oil as the main fat, low intake of red meat, and moderate consumption of wine reduced the risk of CVD and cancer [13].

The pathophysiology of sarcopenia is complex, having not modifiable contributory factors, including the aging process, leading to reduced sex hormones and mitochondrial dysfunction [14]. In addition, some subjects will experience neurodegenerative disease with aging that will have detrimental effects in terms of muscle signaling and function [15]. Increases in fat mass may contribute to the loss of muscle mass that ultimately leads to sarcopenic obesity through increased inflammation and upregulation of protein degradation via the ubiquitin-proteasome pathway [16].

In obesity, the presence of inflammatory factors may have detrimental effects on amino acid utilization and/or insulin signaling pathways involved in the stimulation of muscle synthesis following food intake [17].

The physiological and morphological changes in skeletal muscle with advancing age are characterized by overall declines in size and number of skeletal muscle fibers, mainly the type 2 or fast-twitch muscle fibers, and a marked infiltration of fibrous and adipose tissue into the skeletal muscle [18].

There is a physiological decline in food intake with aging. The reasons are multifactorial (interindividual variations) and may include alterations in the hedonic qualities of food (decreased odor and taste sensations), increased gastrointestinal satiation signals, and a decline in the central feeding drive [19]. The type of diet and eating habits may determine, throughout a nutrigenetic interaction, the levels of reactive species, oxidative stress, and chronic disease development, namely, cardiovascular ones [20]. Nutrients affecting gene expression and genomic integrity modulate disease processes such as cancer, cardiovascular disease, and neurodegenerative disorders [21]. The high consumption of red meat, saturated fatty acids, and cholesterol may be associated with increased risk of diabetes, CVD, and mortality risk [22]. Free radicals and neuroinflammation processes underlie many neurodegenerative conditions [23]. The diets identified as Alzheimer's disease protectors were associated with higher intake of vegetables, fruit, whole grains, fish, and legumes and with lower intake of high-fat dairies, processed meat, and sweets [24]. Currently, besides nutrition longevity influence via complex epigenetic mechanisms [25], emerging research techniques such as nutrigenomics, metabolomics, and proteomics indicate that the type of food and dietary restriction can lead to cell health status capable of modulating apoptosis, reactive oxygen species and reactive nitrogen species detoxification, and gene response, towards disease prevention and longevity [23].

For all these reasons and because there are still no studies in all Portuguese population on this field, we went to characterize the eating habits and nutritional and cardiovascular biomarkers from Portuguese centenarians, to compare them with both high and low cardiovascular risk (CVR) controls.

2. Methods

2.1. Study Patients. We studied from 2012 to 2015 a total of 521 subjects, both genders, being 253 centenarians (CENT) (100.26 ± 1.98 years old) 197 women (77.9%) and 56 men (22.1%). The control group included 268 subjects (67.51 ± 3.25 years old), being 164 women (61.2%) and 104 men (38.8%). This group had both low (LCR) and high cardiovascular risks (HCR); calculations were based on QRISK2-2016 [26]. Centenarians, from all the regions of Portugal, were identified, enrolled, and evaluated at their usual place of residence, as previously described [27]. Centenarian individuals, although uniformly distributed throughout the country, predominated in the Castelo Branco District, followed by Lisbon. The area of Castelo Branco District, surrounded by mountains in the orographic aspect, is mainly rural. On the other hand, the area of Lisbon is mainly an urbanized area. At the time of the interview, most of the centenarians (69.2%) reported having lived most of the life in the interior of the country and only 30.8% in coastal regions. Most of them (51%) lived in small villages for most of their life, but it is noteworthy that one part (30.4%) reported having lived in a city environment. Although all the centenarian individuals presented a capacity for understanding and communication (being an exclusion criterion otherwise), the centenarian men of the present study presented cognitive scores superior to those of centenarian women. The control group included patients recruited from the Heart and Vessels Department of Santa Maria Hospital

and from a primary health care center in Lisbon, Portugal. Hospital de Santa Maria is a reference hospital at the national level, and as such, the controls are not all of the Lisbon region but of several regions of the country.

2.2. Nutrition Data. Anthropometric and body composition analyses were evaluated by bioimpedance, using a portable tetrapolar bioelectrical equipment, the Tanita® BC-420MA (Tanita Corporation of America Inc., Illinois, USA) device to estimate weight, body mass index (BMI), fat mass (FM), muscle mass (MM), and resting metabolic rate (RMR). The MM and FM indexes were calculated [kg/height (m^2)]. Exclusion criteria for bioimpedance measurements were previously described [27].

Data were collected by applying a semiquantitative food frequency questionnaire, based on a validated FFQ for a Portuguese population [28]. The questionnaire used was composed of a list of food groups with 10 items (red meat, fish, eggs, sweets, dairy products, vegetables, legumes, fruits, oilseeds, and canned food) and one closed section with five categories of frequencies of consumption. A photographic manual was used, published by the Institute of Public Health Dr. Ricardo Jorge, I.P., [29] as a visual support for the identification of multiples and submultiples of the middle portion. Data were statistically analyzed in order to know the differences of consumption of food groups between the centenarians and the control group of both high and low CVR.

2.3. Biomarkers and Cardiovascular Risk. Participants or their direct supervisors were asked to provide access to the latest routine blood analyses. The following biochemical data, obtained by laboratory routine analysis measured in certified labs, were collected when available: glucose, total cholesterol (TC), LDL-C, HDL-C, non-HDL-C, triglycerides (TG), uric acid, urea, and creatinine, or calculated: non-HDL-C.

Dyslipidemia was defined when one of the following conditions was present: TC ≥ 200 mg/dL, TG ≥ 150 mg/dL, LDL-C ≥ 100 mg/dL, and HDL-C ≤ 40 mg/dL in men or ≤50 mg/dL in women [30].

The abdominal obesity (cm), BMI (kg/m^2), and the cut-off for FM by gender were established in agreement with WHO guidelines [31]. Sarcopenia was defined by muscle mass index cut-off ≤ 16.7 kg/m^2 [32].

CVR was calculated using a QRISK 2-2016 risk calculator program (https://qrisk.org), based on age, gender, ethnicity, smoking habits, diabetes status, angina or heart attack in a 1st degree relative age below 60 years, chronic kidney disease (stage 4 or 5), atrial fibrillation, hypertension, and rheumatoid arthritis and also based on cholesterol/HDL ratio, systolic blood pressure, and body mass index [26, 33].

2.4. Ethical Considerations. This study was approved by the Scientific and Ethics Committees of the Lisbon Academic Medical Centre (Faculty of Medicine of the University of Lisbon and Santa Maria Hospital) and by the National Commission for Data Protection and was conducted in agreement with the Helsinki Declaration. All the participants gave their written informed consent in order to be included in the study.

2.5. Statistical Analysis. Statistical analysis was performed using the computer software for Windows SPSS, version 20.0 (SPSS Inc., Chicago). The results of quantitative variables were expressed as the mean ± standard deviation and for qualitative categorical variables as the number and percentage. To test the normality of all variables, the Kolmogorov-Smirnov test was applied. Categorical variables were compared with the chi-square with the Z-proportion test or Mann–Whitney *U* test. The comparison of means between groups of numeric variables and normally distributed means was performed by one-way analysis of variance (ANOVA) or Kruskal-Wallis test, followed by the Tukey test. The values of nonnormal parameters are presented in median and interquartile range. Numeric variables were related by the application of Pearson or Spearman correlation coefficients. Binary and multivariate logistic regression analysis was performed. As the measure of association, the odds ratio (OR) was used with the respective 95% confidence interval. All the tests were considered statistically significant if $P < 0.05$.

3. Results

There were differences in the frequency of food groups' consumption between centenarians and controls, except for the oilseed group (see Table 1). As shown in Figure 1, the daily intake of red meat, adjusted for age and gender, was a protective factor for sarcopenia (OR = 0.25, 95% CI: 0.096–0.670, $P = 0.006$); however, it contributes for FM excess (OR = 4.946, 95% CI: 1.471–16.626, $P = 0.01$), overweight, and obesity (OR = 4.804, 95% CI:1.666–13.851, $P = 0.004$). Only 2% of the centenarians reported this eating habit in the opposite 64.3% of the HCR group. In Figure 2, we can see that the frequency history of red meat intake was associated with higher CVR ($\chi^2 = 239.807$; df = 8, $P < 0.0001$), in the same way of canned food intake ($\chi^2 = 225.321$; df = 8, $P < 0.0001$).

Basal metabolism (Kcal) was lower in centenarians and higher in the HCR group (Figure 3) (CENT = 1176.78 ± 201.98 versus LCR = 1356.54 ± 170.65 versus HCR = 1561.33 ± 267.85; $P < 0.0001$). Compared with controls, centenarians also had a lower BMI (CENT = 21.06 ± 3.68 versus LCR = 28.49 ± 4.69 versus HCR = 29.56 ± 5.26; $P < 0.0001$) (Figure 4), waist circumference (cm) (CENT = 85.29 ± 10.83 versus LCR = 96.02 ± 11.71 versus HCR = 104.50 ± 11.84; $P < 0.0001$) (Figure 5(a)), and waist-hip ratio (CENT = 0.88 ± 0.07 versus LCR = 0.92 ± 0.08 versus HCR = 1.01 ± 0.08; $P < 0.0001$) (Figure 5(b)).

Considering the biochemical parameter values of CVR, particularly, lipidogram and lipid profile, there were significant differences between the results obtained between the group of centenarian individuals and those of the low-risk and high-risk control groups (Table 2).

Total cholesterol ($P < 0.0001$), LDL-C ($P < 0.0001$), and non-HDL cholesterol ($P < 0.0001$) levels were lower in the centenarian group and differed significantly from

Table 1: Frequency of food consumption and comparison between centenarians (CENT) and low (LCR) and high (HCR) cardiovascular risk control groups. The amount/day and repetition refers to the main meal.

		LCR, *n* (%)	HCR, *n* (%)	CENT, *n* (%)	*P* value
Number of meals/day	1–3	52 (15.5)	99 (29.5)	185 (55.1)	<0.0001
	4-5	46 (34.3)	43 (32.1)	45 (33.6)	
	6 or more	1 (16.7)	5 (83.3)	0 (0.0)	
Amount/day	Mini	6 (4.1)	5 (3.4)	136 (92.5)	<0.0001
	Medium	70 (30)	89 (38.2)	74 (31.8)	
	Full	19 (26.4)	38 (52.8)	15 (20.8)	
	Very full	3 (21.4)	10 (71.4)	1 (7.1)	
Repetition	No	73 (20.3)	94 (26.2)	192 (53.5)	<0.0001
	Yes	24 (25.3)	46 (48.4)	25 (26.3)	
Red meat	Never/4x a year	3 (4.2)	1 (1.4)	68 (94.4)	<0.0001
	>4x a year, <1x a month	7 (9.7)	4 (5.6)	61 (84.7)	
	1–3x a month	14 (14.6)	14 (14.6)	68 (70.8)	
	1–6x a week	43 (27.4)	74 (47.1)	40 (25.5)	
	1–3x a day	33 (33.7)	63 (64.3)	2 (2)	
Fish	Never/4x a year	1 (2.6)	1 (2.6)	36 (94.7)	<0.0001
	>4x a year, <1x a month	2 (2.7)	1 (1.3)	72 (96)	
	1–3x a month	12 (12.4)	25 (25.8)	60 (61.9)	
	1–6x a week	68 (28.5)	108 (45.2)	63 (26.4)	
	1–3x a day	17 (37.8)	21 (46.7)	7 (15.6)	
Eggs	Never/4x a year	4 (16.7)	5 (20.8)	15 (62.5)	0.009
	>4x a year, <1x a month	17 (18.3)	23 (24.7)	53 (57)	
	1–3x a month	42 (26.6)	60 (38)	56 (35.4)	
	1–6x a week	35 (17.7)	62 (31.3)	101 (51)	
	1–3x a day	2 (20)	6 (60)	2 (20)	
Sweets	Never/4x a year	11 (8.1)	21 (15.6)	103 (76.3)	<0.0001
	>4x a year, <1x a month	11 (13.6)	24 (29.6)	46 (56.8)	
	1–3x a month	33 (33.7)	29 (29.6)	36 (36.7)	
	1–6x a week	27 (23.5)	51 (44.3)	37 (32.2)	
	1–3x a day	18 (28.1)	30 (46.9)	16 (25)	
Dairy	Never/4x a year	4 (26.7)	3 (20)	8 (53.3)	0.001
	>4x a year, <1x a month	2 (4.7)	10 (23.3)	31 (72.1)	
	1–3x a month	8 (18.2)	10 (22.7)	26 (59.1)	
	1–6x a week	19 (22.6)	18 (21.4)	47 (56)	
	1–3x a day	67 (21.9)	114 (37.3)	125 (40.8)	
Vegetables	Never/4x a year	1 (50)	1 (50)	0 (0)	<0.0001
	>4x a year, <1x a month	1 (50)	1 (50)	0 (0)	
	1–3x a month	4 (23.5)	11 (64.7)	2 (11.8)	
	1–6x a week	35 (36.1)	58 (59.8)	4 (4.1)	
	1–3x a day	59 (15.6)	84 (22.3)	234 (62.1)	
Legumes	Never/4x a year	1 (20)	3 (60)	1 (20)	<0.0001
	>4x a year, <1x a month	13 (50)	12 (46.2)	1 (3.8)	
	1–3x a month	40 (38.1)	60 (57.1)	5 (4.8)	
	1–6x a week	40 (26.3)	70 (46.1)	42 (27.6)	
	1–3x a day	6 (3.1)	10 (5.1)	180 (91.8)	

Table 1: Continued.

		LCR, *n* (%)	HCR, *n* (%)	CENT, *n* (%)	*P* value
Fruits	Never/4x a year	2 (100)	0 (0)	0 (0)	0.040
	>4x a year, <1x a month	0 (0)	4 (66.7)	2 (33.3)	
	1–3x a month	3 (13.6)	10 (45.5)	9 (40.9)	
	1–6x a week	16 (15.4)	34 (32.7)	54 (51.9)	
	1–3x a day	79 (22.1)	107 (29.9)	172 (48)	
Oilseeds	Never/4x a year	30 (17.3)	53 (30.6)	90 (52)	0.401
	>4x a year, <1x a month	28 (21.9)	38 (29.7)	62 (48.4)	
	1–3x a month	23 (22.5)	37 (36.3)	42 (41.2)	
	1–6x a week	15 (25.9)	23 (39.7)	20 (34.5)	
	1–3x a day	4 (22.2)	4 (22.2)	10 (55.6)	
Canned	Never/4x a year	25 (9)	41 (14.7)	212 (76.3)	<0.0001
	>4x a year, <1x a month	29 (37.2)	32 (41)	17 (21.8)	
	1–3x a month	31 (40.8)	40 (52.6)	5 (6.6)	
	1–6x a week	12 (25.5)	34 (72.3)	1 (2.1)	
	1–3x a day	3 (25)	9 (75)	0 (0)	

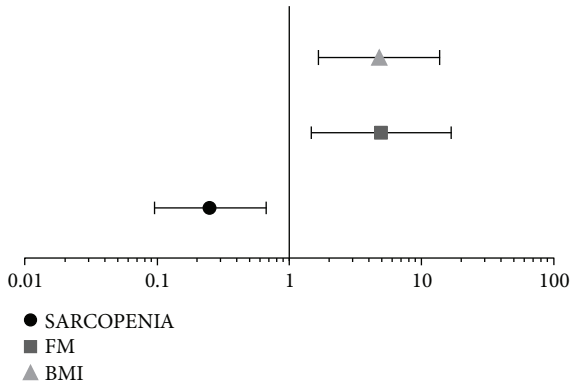

Figure 1: Odds ratio of daily intake of red meat, adjusted for age and gender concerning sarcopenia, fat mass excess, and overweight/obesity. The *x*-axis is in logarithmic scale (log 10).

either the low or the high cardiovascular risk control subgroups (Table 2).

In relation to LDL values, there were no significant differences between LCR and HCR subjects ($P = 0.161$, Tukey test). In cholesterol/HDL cholesterol ratio, there was no significant difference between centenarians and LCR subjects ($P = 0.960$, Tukey test) (Table 2).

4. Discussion

As far as we know, this is an original work in human longevity which investigates some aspects of eating habits, anthropometry, basal metabolism, and blood parameters. We sought to know the history of the eating habits of Portuguese centenarians and verify if these habits were or not coincident with the history of the dietary profile of younger individuals, some of them with HCR and others with LCR, whose probable life expectancy, according to the projection of the 2011 census, does not exceed 84 years [34].

We applied a semiquantitative food frequency questionnaire using photographic models because it was considered to be the most appropriate for the population studies [35, 36]. The 24-hour questionnaire is a retrospective method considered the one with the best accuracy to estimate food intake [37]. However, as mentioned in epidemiological studies in the elderly, the required repetition of the previous 24-hour questionnaire may be more inaccurate in comparison with a food frequency questionnaire in which participants report their past eating habits in a single interview. A large part (71.9%) of the centenarians studied was institutionalized so the present eating habits were very different from the past ones. In the centenarians, it was possible to observe the difference in the ease to recall past eating habits in relation to the most recent ones.

The food history showed that the frequency of consumption of legumes, fruits, and vegetables is higher and red meat consumption is lower in the centenarians compared to the control group. The latter was frequently ingested with larger and repeated food portions (Table 1). Both aspects are indicative that the daily caloric intake of the centenarians may be lower than that of the controls and that by consuming foods with health benefits (vitamins, bioactive compounds, and dietary fiber) more often supports the idea that can promote longevity (Figure 2). These data may lead to a reflection on the importance of eating habits such as caloric overload and in particular that associated with red meat ingestion in longevity.

The centenarians had distinguished themselves from controls in all food groups that have been evaluated with the exception of oilseed ingestion. There are studies that indicate the excess consumption of red meat as a negative impact related to good health since this consumption was associated with obesity, type 2 diabetes, CVD, cancer [10], and higher all-cause mortality [8], and accumulating scientific evidence has indicated that high consumption of red meat, especially processed meat, may be associated with an increased risk of major chronic diseases [22].

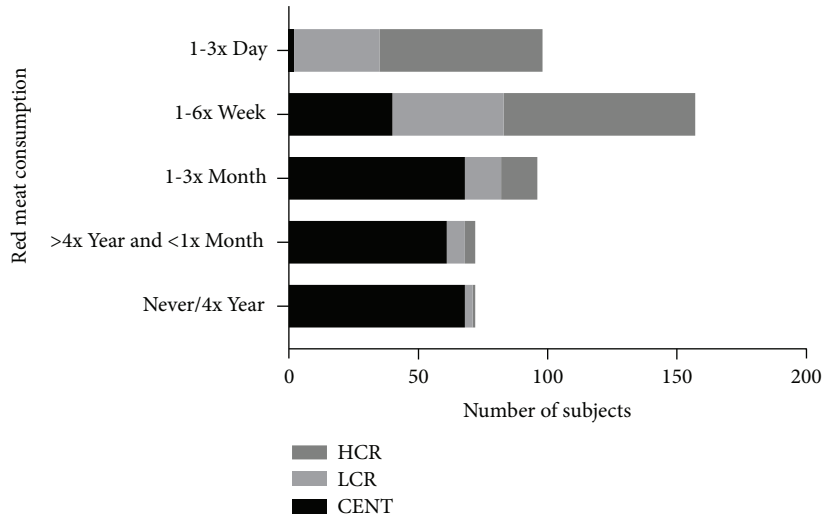

Figure 2: Distribution of the frequencies of red meat intake during most of the life among the groups: centenarians (CENT), low cardiovascular risk (LCR) control group, and high cardiovascular risk (HCR) control group.

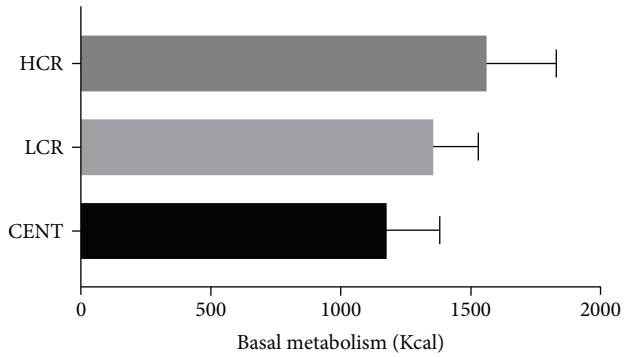

Figure 3: Basal metabolism of all groups: centenarians (CENT), low cardiovascular risk (LCR) control group, and high cardiovascular risk (HCR) control group.

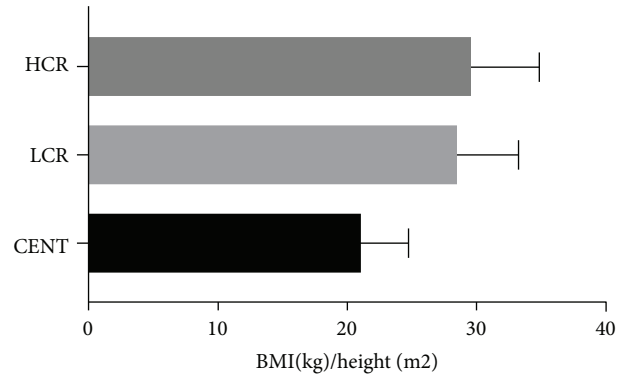

Figure 4: BMI (kg)/height (m^2) of all groups: centenarians (CENT), low cardiovascular risk (LCR) control group, and high cardiovascular risk (HCR) control group.

We found that the individuals with the highest CVR were those who had the highest frequencies of red meat consumptions (Figures 2, 5(a), and 5(b)). In fact, this consumption, in particular, processed meat, is associated with a higher incidence of CVD such as coronary heart disease, heart failure, and stroke in addition to other pathologies [22].

Red meat, on the other hand, is a source of heme iron [38]. Free heme may catalyze oxidant processes involving several components of biological systems, resulting in tissue damage and ultimately leading to disease. Actually, heme-catalyzed oxidations can damage lipids, proteins, DNA, and other nucleic acids and various components of biological systems. A major pathway involves reactions of lipids with heme: LOOH (lipid hydroperoxide) + Fe-ligands (heme) ➔ LOOFe ligands ➔ LO* (lipid alkoxy radical) + *OFe ligands (heme oxyradical). The alkoxy radicals and the heme oxy radicals can initiate further oxidations some of which would result in oxidative chain reactions. Heme catalysis of oxidation is the strongest oxidizing system for developing tissue damage. These heme-catalyzed oxidations can lead to the initiation of biochemical and cellular damage and subsequently disease processes [39]. Also, the formation of *N-nitroso* compounds in the intestine conditioned by the ingestion of red meat may lead to oxidative stress and DNA damage [40]. High red meat consumption was associated with modestly higher concentrations of plasma GGT and hs-CRP, whereas high whole grain bread consumption was related to modestly lower concentrations of GGT, ALT, and hs-CRP [41]. The association of red meat consumption with increased levels of hs-CRP could be modified by high whole grain bread consumption [41].

These facts highlighted the hypothesis that dietary factors may modulate these biomarkers, which may be potential mediators related to the risk of diabetes and CVD [41]. Even more, the discovery of a link between L-carnitine ingestion, gut microbiota metabolism, and CVD risk revealed a new pathway linking dietary red meat ingestion with atherosclerosis pathogenesis pointing out the role of gut microbiota in this pathway suggesting a new potential therapeutic target for preventing CVD [42].

Red meat is known to have higher contents of saturated fat and cholesterol [38]; this fact agrees with our observations

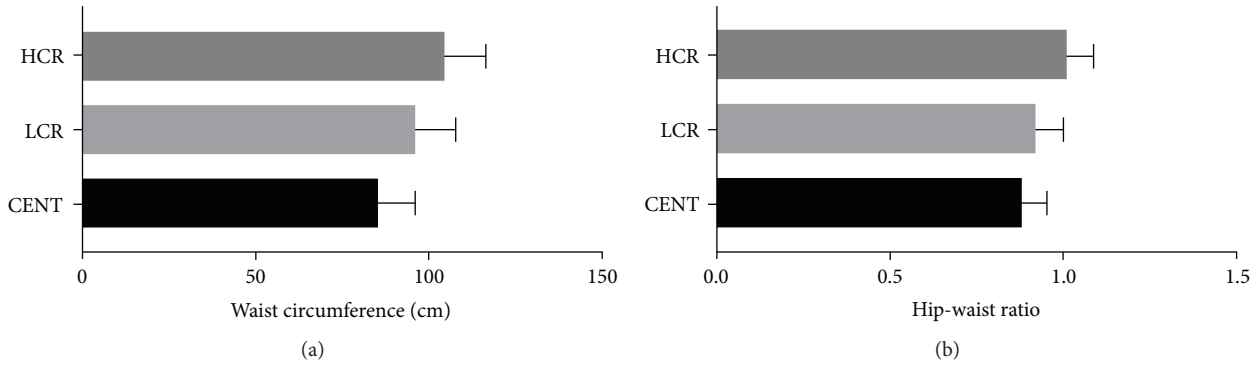

Figure 5: (a) Waist circumference (cm) of all groups: centenarians (CENT), low cardiovascular risk (LCR) control group, and high cardiovascular risk (HCR) control group. (b) Waist-hip ratio of all groups: centenarians (CENT), low cardiovascular risk (LCR) control group, and high cardiovascular risk (HCR) control group.

Table 2: Lipid profile comparison between centenarians (CENT) and low (LCR) and high (HCR) cardiovascular risk control groups.

	CENT	LCR	HCR	*P*
Total cholesterol (mg/dL)	178.81 ± 42.36[a,b]	213.51 ± 46.98	194.84 ± 42.91[a]	<0.0001
HDL cholesterol (mg/dL)	47.00 [38.00–56.00][a]	56.00 [49.00–67.00][b]	47.00 [38.00–55.00]	<0.0001*
Triglycerides (mg/dL)	106.00 [86.00–134.30][a]	94.00 [71.00–133.00][b]	117.00 [91.00–156.50]	0.001*
LDL cholesterol (mg/dL)	96.4 [78.5–129.0][a,b]	123.8 [104.7–151.17]	122.3 [93.6–145.85]	<0.0001*
Non-HDL cholesterol (mg/dL)	127.33 ± 38.75[a,b]	153.08 ± 43.29	145.99 ± 39.95	<0.0001
Ratio total cholesterol/HDL cholesterol	3.71 [3.02–4.41][b]	3.66 [3.06–4.16][b]	4.03 [3.36–4.89]	0.001*

[a]Different from LCR. [b]Different from HCR. *Results expressed in median [IQR 25–75]. Kruskal Wallis Test was used.

revealing that centenarians (24.1%) have low hypercholesterolemia frequency than controls of low (75.8%) and high (78.9%) CVR. Additionally, the cholesterol/HDL ratio was statistically higher ($P = 0.017$) in the high-risk subgroup (4.24 ± 1.18) compared to centenarians (3.81 ± 1.09) (Table 2). We assumed that the centenarians have low CVR since they reached extreme longevity. We observed that they differ from the other groups, namely, the HCR group having lower values of total cholesterol, LDL cholesterol, non-HDL-C, and cholesterol/HDL ratio. LDL-C and non-HDL-C are atherogenic factors, the latter including TG-rich lipoproteins, cholesteryl ester-enriched remnants of TG-rich lipoproteins, and lipoprotein(a) with great predictive CVR value [43].

Excess meat consumption was associated with an increase in fat mass, obesity, and waist circumference and increased waist-hip ratio associated with the HCR group (Figures 4, 5(a), and 5(b)). As observed for red meat intake, our results support this observation, since the frequency (at least 1x a week) of consumption of red meat ($\chi^2 = 239.807$; $df = 8$, $P < 0.0001$) as well as canned/industrialized foods ($\chi^2 = 225.321$; $df = 8$, $P < 0.0001$) was associated with HCR individuals compared to the other groups.

Similarly to that observed with red meat, a higher frequency of canned/industrialized food consumption in HCR individuals compared to LCR and centenarians (72.3% versus 25.5% versus 2.1%, resp., consumed at least 1x per week) was observed. It is known that polyphosphates are commonly used as an additive in industrially processed food and may increase serum phosphate levels leading to vascular damage and cardiovascular morbidity inducing aging processes [44].

Concerning meat consumption, however, we found a benefit in relation to a possible contributor to prevent sarcopenia, as verified by Rondanelli et al. [45]. The underlying cause of sarcopenia is unclear but may include a lower basal rate of protein synthesis in aged muscle. Meats are nutrient-rich sources of protein which are potently stimulatory for muscle protein synthesis and may aid in mediating gains in muscle mass and strength when combined with exercise program [46].

Although beneficial for the prevention of sarcopenia, however, red meat consumption may increase the risk of stroke. In fact, red meat is a source of saturated fatty acids and cholesterol. Some studies have indicated that a high intake of saturated fatty acids increases total cholesterol levels, LDL, and triglycerides, which could increase the risk of stroke [47]. No sarcopenic obesity was observed either in the controls or in the centenarians, which were mostly eutrophic.

It was verified that the group of centenarians consumed more vegetables/legumes/fruits than the control groups (HCR and LCR, Table 1) that may contribute to longevity. Epidemiological studies suggest a role of fruits and vegetables, in protection against disease of aging [23], and the WHO considers that these should be the main foods to be ingested [48]. Actually, the exogenous antioxidants, greatly relevant for longevity, such as vitamin C (ascorbic acid/ascorbate), vitamin E (tocopherols, tocotrienols), carotenoids

(α-carotene, β-carotene, zeaxanthin, lutein, lycopene, β-cryptoxanthin, etc.), polyphenols (flavonols, flavanols, anthocyanins, isoflavones, and phenolic acid), and trace elements (selenium, zinc), predominate in dietary sources derived mainly from the vegetable kingdom [20].

It must be considered protein supplementation in patients with sarcopenia with no medical contraindications [49], which can contribute to improve not only the muscular mass but also the cognitive aspects [50].

The basal metabolism decreases with age [51], which was also observed in our study. It was found that individual HCR controls had an increased basal metabolism compared to the other groups (LCR and centenarians). We assume that the centenarians had a low CVR profile; otherwise, they would not have reached that age.

5. Study Strengths and Limitations

In this case-control study design, the sample size is adequate according to what has been previously explained [27]. The group of centenarians is compared with a group of younger individuals assuming that the probability of reaching 100 years is remote for the control group. On the other hand, it is also assumed that the CVR of centenarians is small compared to the control group since otherwise they would not have reached 100 years. Estimation of energy and nutrient intake may be considered a study limitation although the frequency of consumption and food portion size were evaluated.

6. Conclusions

Centenarians have a different food history than the control population. Frequent consumption of red meat contributes to obesity and increased CVR, since LDL-cholesterol and heme iron of red meat that catalyze oxidations may lead to atherosclerosis disease processes. Menus mainly with vegetables and legumes and less red meat, observed in centenarians, although associated with sarcopenia, may promote a longer life span.

Disclosure

An earlier version of this paper was presented as an abstract at the 19th European Congress of Endocrinology, held in Lisbon: Endocrine Abstracts (2017) 49: OC5.4.

Conflicts of Interest

The authors declare no conflict of interest.

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Research Article

Role of Exercise-Induced Cardiac Remodeling in Ovariectomized Female Rats

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Myocardial extracellular matrix (ECM) is essential for proper cardiac function and structural integrity; thus, the disruption of ECM homeostasis is associated with several pathological processes. Female Wistar rats underwent surgical ovariectomy (OVX) or sham operation (SO) and were then divided into eight subgroups based on the type of diet (standard chow or high-triglyceride diet/HT) and exercise (with or without running). After 12 weeks, cardiac MMP-2 activity, tissue inhibitor of metalloproteinase-2, content of collagen type I, the level of nitrotyrosine (3-NT) and glutathione (GSH), and the ratio of infarct size were determined. Our results show that OVX and HT diet caused an excessive accumulation of collagen; however, this increase was not observed in the trained animals. Twelve weeks of exercise promoted elevation in the levels of 3-NT and GSH and similarly an increase in MMP-2 activity of both SO and OVX animals. The high infarct-size ratio caused by OVX and HT diet was mitigated by physical exercise. Our findings demonstrate that ovarian estrogen loss and HT diet caused collagen accumulation and increased ratio of the infarct size. However, exercise-induced cardiac remodeling serves as a compensatory mechanism by enhancing MMP-2 activity and reducing fibrosis, thus minimizing the ischemia/reperfusion injury.

1. Introduction

Premenopausal women have a lower risk of developing cardiovascular disease (CVD) compared to age-matched men; however, this sex advantage for women gradually disappears after the onset of menopause, suggesting that sexual hormones have a strong influence on cardiometabolic parameters [1]. Several physiological changes which develop during menopause may also influence the incidence and manifestation of CVD, such as weight gain, obesity, and its comorbidities [2]. In the pathogenesis of CVD, studies have shown that both estrogen deficiency and obesity contribute to structural and tissue remodeling, as well as to the changes in cardiac function [3–5].

Myocardial extracellular matrix (ECM) serves as an important mediating factor in cardiac development, homeostasis, and remodeling [6]. The most abundant structural components of the ECM are collagens, particularly collagen type I and collagen type III, which are produced primarily by fibroblasts [7], and its synthesis and degradation is essential for normal cardiac structure and function [8]. During pathological conditions, cardiac failure and remodeling are characterized by collagen accumulation, myocyte loss, and impaired rearrangement of cardiac structure [9–11], proving

that disruption of ECM/collagen homeostasis is a key factor for the progression of cardiac dysfunction. Degradation of fibrillar collagens and other ECM proteins is catalyzed by matrix metalloproteinases (MMPs), which are a family of zinc-dependent proteases with more than 25 members. MMP-2 is one of the most commonly known among the aforementioned proteins and an enzyme that is constitutively abundant in almost all cell types and characterized by its degrading effect of the denatured collagen (gelatin) and other extracellular matrix proteins [12]. Similar to other enzymes, MMPs are regulated by naturally occurring inhibitors called tissue inhibitors of metalloproteinases (TIMPs), preventing excessive ECM degradation by MMPs [13]. The functional balance between MMPs and TIMPs determines cardiac remodeling [14].

Physical exercise is widely unanimous as a nonpharmacological therapeutic tool for the prevention and treatment of CVD. The latter can initiate cardiovascular adaptations, including reduction in blood pressure, and promotes cardiac remodeling by the development of physiological hypertrophy and reduction of cardiac fibrosis [15, 16]. Furthermore, exercise-mediated cardioprotection has been linked to the activation of antioxidant defense mechanisms and reduction of metabolic risk factors [17]. In our earlier study, we verified that a 12-week voluntary exercise combined with calorie restriction (CR) could attenuate the metabolic parameters, which are frequently linked to major cardiovascular risks in an estrogen-deficient state [18].

We hypothesized that a 12-week voluntary exercise could be an effective strategy in modifying the heart remodeling effect caused in an estrogen-deficient state. Thus, the aim of the current study was to investigate the potential protective effects against the deregulatory and detrimental effects of MMP-2 and collagen content, linked with the detection of necrotic ratio after ischemia/reperfusion injury.

2. Materials and Methods

2.1. Animals. Female Wistar rats weighing 180–200 g were obtained from Toxi-Coop Zrt., Hungary, and acclimated for at least 1 week prior to experimental use and were maintained under controlled conditions of illumination (12/12 h light/dark cycle) and room temperature (20–23°C). All experimental procedures were performed in accordance with the standards of the European Community guidelines on the care and use of laboratory animals and had been approved by the Institutional Ethics Committee.

2.2. Surgery. Following the one-week acclimation period, female Wistar rats of 10 weeks of age were subjected to either ovariectomy surgery (OVX) or sham operation (SO) under anesthesia with thiopental (5 mg/100 g i.p.). During OVX, a bilateral dorsolateral incision was made and the ovaries were removed. On the contrary, the ovaries of SO animals were exteriorized to create similar stress but were not removed. After a 4-week resting period, and to verify the OVX-induced menopause, the serum estrogen levels (Quantikine rat Estrogen ELISA kit, R&D Systems Inc.) were checked using estrogen quantitative enzyme-linked immunosorbent assay (ELISA) [19].

2.3. Experimental Design of Dietary Period and Exercise Training. OVX and SO female rats were randomly divided into 8 new subgroups based on type of diet (standard chow (CTRL) and high-triglyceride diet (HT)) and exercise (with or without running) for 12 weeks. The rats in the CTRL subgroup were fed with a laboratory chow, while the animals in the HT subgroup were subjected to a diet composed of 40% fat content mixed with 60% standard chow. The latter dietary animal groups were further randomly divided into running and sedentary subgroups. The running animals were placed individually into cages fitted with a running wheel, with a free access to the wheel for 24 h per day for 12 weeks. The exercising protocol defined as a voluntary wheel-running model was selected in an effort to isolate the effects of exercising from the additional stress associated with forced exercise protocols [20]. Animals from sedentary subgroups were placed for the same period in standard holding cages. At the end of the experimental period, the estrus phase of SO rats was checked by Giemsa staining to ensure that all SO animals were killed at the same stage of the phase (proestrus phase). All rats were sacrificed, and heart tissues were collected and either mounted into a Langendorff perfusion system to detect ischemia/reperfusion injury ex vivo (10 rats of each group) or were clamped, frozen in liquid nitrogen right after excision, and then stored at −80°C for later use in biochemical analysis (10 rats of each group). The experimental design of the study is shown in Figure 1.

2.4. Measurement of MMP-2 Activity. MMP-2 activity was measured from heart samples using gelatin zymography. Fifty micrograms of protein samples were electrophoresed on 8% polyacrylamide gel copolymerized with gelatin (20 mg/ml; type A from porcine skin; Sigma). After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated for 20 hours at 37°C in an incubation buffer. Staining was performed using 0.05% Coomassie Brilliant Blue followed by destaining with aqueous 4% methanol and 8% acetic acid. A protein ladder (Spectra Multicolor Broad Range Protein Ladder, Thermo Scientific) was used to identify the 2 enzyme isoforms (MMP-2, 72 kDa and 64 kDa). Zymograms were digitally scanned and the intensity of the bands quantified by using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.5. Measurement of Total Glutathione (GSH + GSSG). Heart samples were homogenized in a solution composed of 0.25 M sucrose, 20 mM Tris, and 1 mM dithiothreitol (DTT) and centrifuged at 15,000 ×g for 30 min at 4°C. The supernatant fractions were collected, and then 0.1 M $CaCl_2$, 0.25 M sucrose, 20 mM Tris, and 1 mM DTT were added. After incubation at 0°C for 30 min and further centrifugation at 21,450 ×g for 60 min at 4°C, a clear cytosolic fraction was used for enzyme assays. A solution of 125 mM Na phosphate and 6.0 mM EDTA was used as a diluent buffer for the stock solution of glutathione (GSH), glutathione reductase, 5,5′ dithio-bis-2-nitrobenzoic acid (DTNB), and β-nicotinamide

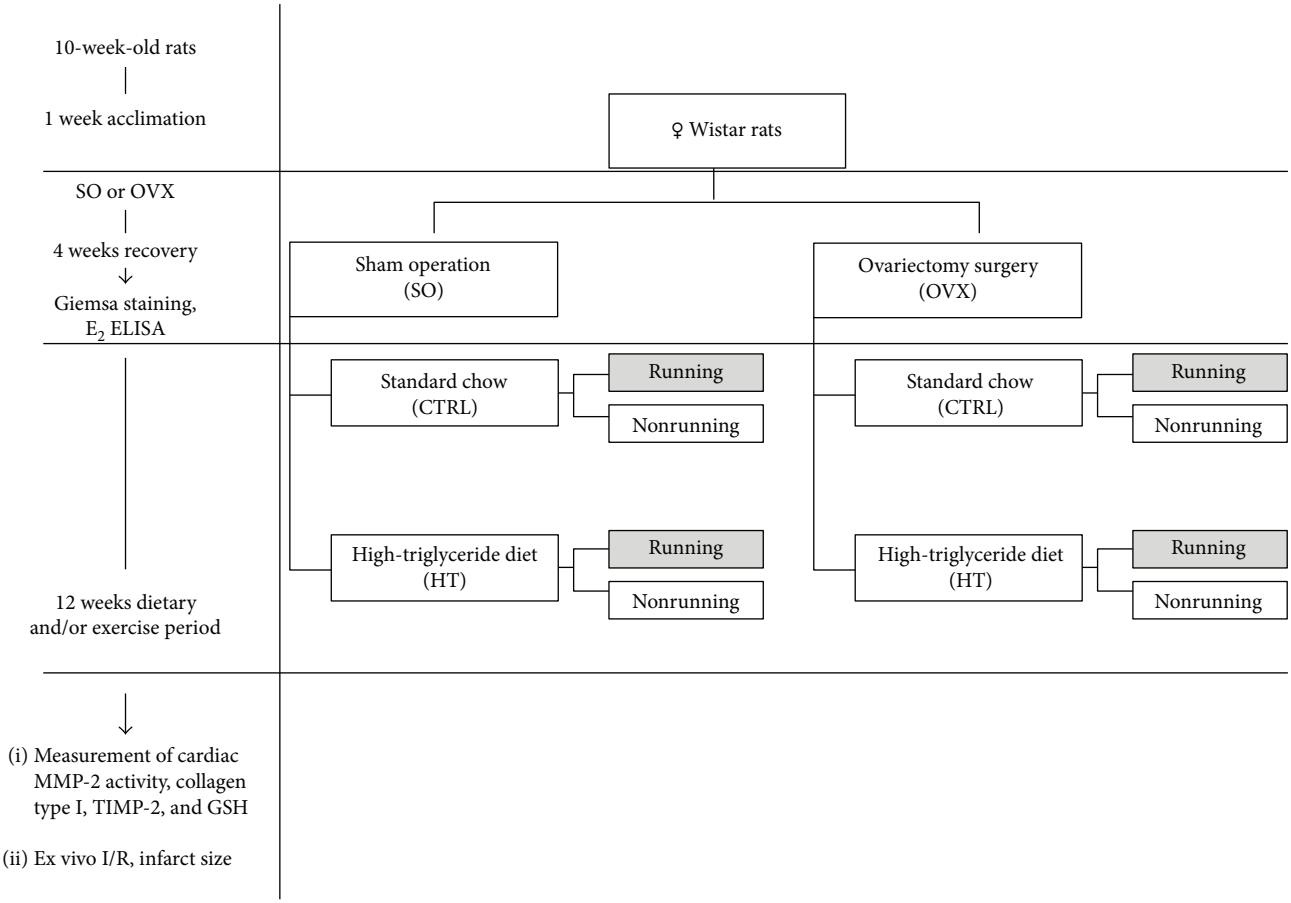

Figure 1: The experimental protocol of the study. SO = sham-operated; OVX = ovariectomized; CTRL = standard chow; HT = high-triglyceride diet.

adenine dinucleotide phosphate (β-NADPH). A total volume of 40 μl of each blank, standard, or heart sample and equal volumes of DTNB stock solution (20 μl) and β-NADPH (140 μl) were added to each well and then incubated at 25°C for 5 min. A 10 μl volume of glutathione reductase was used to start the reaction, and the absorbance was measured at 405 nm in a microplate reader after 10 min from the initiation of the reaction.

In the spectrophotometric assay for total GSH, GSH was sequentially oxidized by DTNB and reduced by NADPH in the presence of glutathione reductase. Total glutathione values were expressed as nmol/mg protein.

2.6. Determination of Cardiac 3-NT, Collagen Type I, and TIMP-2. At the end of the 12-week treatment period, the cardiac samples were clamped and frozen after excision. The samples were homogenized (Ultra-Turrax T8; 2×30 s) in phosphate buffer (pH 7.4) and then centrifuged at 2000 r.p.m. for 20 min at 4°C. Cardiac 3-NT, collagen type I content, and TIMP-2 were assayed with commercial kits purchased from GenAsia, Shanghai. Optical density was measured at 450 nm (Benchmark Microplate reader; Bio-Rad). Protein content was determined using a commercial protein assay kit (Bio-Rad Labs), and aliquots (20 μl) of the diluted samples (15× or 25× with distilled water) were mixed with 980 μl of distilled water and 200 μl Bradford reagent. After mixing and following 10 min incubation, the samples were assayed spectrophotometrically at 595 nm. Referring to protein values, the cardiac 3-NT levels were defined as pmol/mg protein, collagen type I was defined as pg/mg protein, and TIMP-2 level was expressed as ng/mg protein.

2.7. Ischemia/Reperfusion Protocol. After anesthetization, heart tissues were rapidly excised and placed in ice-cold Krebs-Henseleit buffer solution consisting of 11.2 mM glucose, 1.24 mM KH_2PO_4, 20.1 mM $NaHCO_3$, 119 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, and 1.24 mM $MgSO_4$ and then mounted onto a Langendorff perfusion system. A retrograde perfusion was applied for the hearts via the aorta at constant pressure of 75 mmHg with the Krebs-Henseleit buffer bubbled with 5% CO_2 and 95% O_2 at 37°C. After perfusion, local ischemia was induced by occlusion of the left anterior descending coronary artery (LAD) for 30 min, after which it was followed by reperfusion for 120 min. At the end of each experiment, the LAD was reoccluded and perfusion stopped, and the hearts were stained with 1% Evans blue solution injected into the aorta to reveal the area at risk. Heart samples were frozen at −20°C overnight.

2.8. Measurement of Infarct Size. Frozen heart-tissue samples were cut into 2 mm thick cross-sectional slices and immersed in 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution

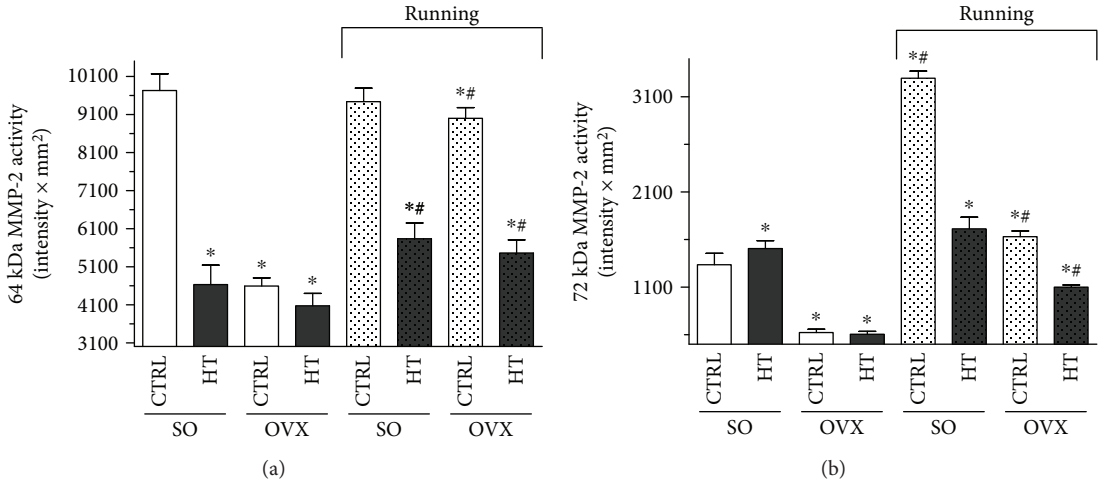

Figure 2: (a) Effects of 12-week wheel-running exercise and nutrition on the cardiac 64 kDa MMP-2 activity (expressed as intensity × mm^2). Results are shown as means ± S.E.M. $n = 12$. (b) Effects of 12-week wheel-running exercise and nutrition on the cardiac 72 kDa MMP-2 activity (expressed as intensity × mm^2). Results are shown as means ± S.E.M. $n = 12$. Statistical significance: $^*p < 0.05$ relative to the SO CTRL group, and $^\#p < 0.05$ shows a significant difference between the running and nonrunning groups. SO = sham-operated; OVX = ovariectomized; CTRL = standard chow; HT = high-triglyceride diet.

prepared in phosphate buffer saline (pH 7.4) for 10 min at 37°C. After TTC staining, the tissue slices were transferred into formalin (10%) solution for 10 min and then placed in phosphate buffer (pH 7.4). Following this incubation, both sides of each slice were photographed with a digital camera. Infarct size was calculated as the percentage of the area at risk.

2.9. Statistical Analysis. The results are expressed as means ± S.E.M. Differences between groups were calculated using ANOVA test, and $p \leq 0.05$ was considered as significant.

3. Results

3.1. Evaluation of Cardiac MMP-2 Activity in Response to Estrogen Depletion, Exercise, and Nutrition. To better understand the mechanism of how estrogen deficiency, type of diet, and exercise training can influence cardiac fibrosis, we evaluated the activity of 64 and 72 kDa MMP-2 isoforms. At the end of the 12-week experimental period, we found a significantly lower ($^*p < 0.05$) activity of 64 kDa MMP-2 in sham-operated (SO/HT) and ovariectomized (OVX/CTRL and OVX/HT) sedentary rats compared with the SO/CTRL group. Exercise training resulted in a significant increase ($^\#p < 0.05$) in the SO/HT and OVX groups compared with nonrunning counterparts. Comparing the MMP-2 activity of running animals, we observed that the high-triglyceride diet in both the SO and OVX animals caused significantly reduced ($^*p < 0.05$) values compared with SO/CTRL.

The activity of 72 kDa MMP-2 isoform was significantly decreased ($^*p < 0.05$) with ovariectomy and high-triglyceride diet (OVX/HT) in sedentary rat hearts. However, voluntary wheel-running exercise caused a significant elevation ($^*p < 0.05$) in each SO and OVX subgroup compared with SO/CTRL animals. Hearts from the running SO/CTRL animals exhibited the highest activity of 72 kDa MMP-2 isoform. In addition, significant differences ($^\#p < 0.05$) were observed between running and nonrunning counterparts in the case of the SO/CTRL, OVX/CTRL, and OVX/HT groups. Data are shown in Figures 2(a) and 2(b).

3.2. Determination of Cardiac 3-NT Level. Figure 3(a) shows that cardiac levels of 3-NT were significantly decreased ($^*p < 0.05$) in the OVX/CTRL and OVX/HT groups compared with the 3-NT values of the SO/CTRL group. The 3-NT level in the hearts of SO rats decreased with the high-triglyceride diet, but this trend did not reach a statistical significance. Exercise training resulted in a significant increase ($^\#p < 0.05$) in both SO and OVX rats fed with high-triglyceride diet compared with nonrunning counterparts, except in OVX/CTRL rats, in which the 3-NT values were substantially lower ($^*p < 0.05$) than in the SO/CTRL group.

3.3. Measurement of Cardiac GSH. Cardiac GSH levels were measured at the end of 12 weeks of the experimental period by spectrophotometric assay. A significant decrease ($^*p < 0.05$) of GSH was found in heart samples of the sedentary sham-operated (SO/HT) and ovariectomized (OVX/CTRL and OVX/HT) rats compared with the SO/CTRL group. As a result of voluntary exercise training, GSH levels displayed a significant elevation ($^\#p < 0.05$) compared with nonrunning counterparts. Data are presented in Figure 3(b).

3.4. Evaluation of Cardiac TIMP-2 Concentration. To determine the role of the MMP/TIMP system on ECM turnover, cardiac TIMP-2 was also examined. Our results reveal that exercise training significantly enhanced the TIMP-2 values, which was diminished by estrogen depletion and high-triglyceride diet. Significant elevation ($^\#p < 0.05$) was noted between the running and nonrunning counterparts. Data are shown in Figure 4(a).

3.5. Concentration of Cardiac Collagen Type I. To test the hypothesis that exercise training might modulate the

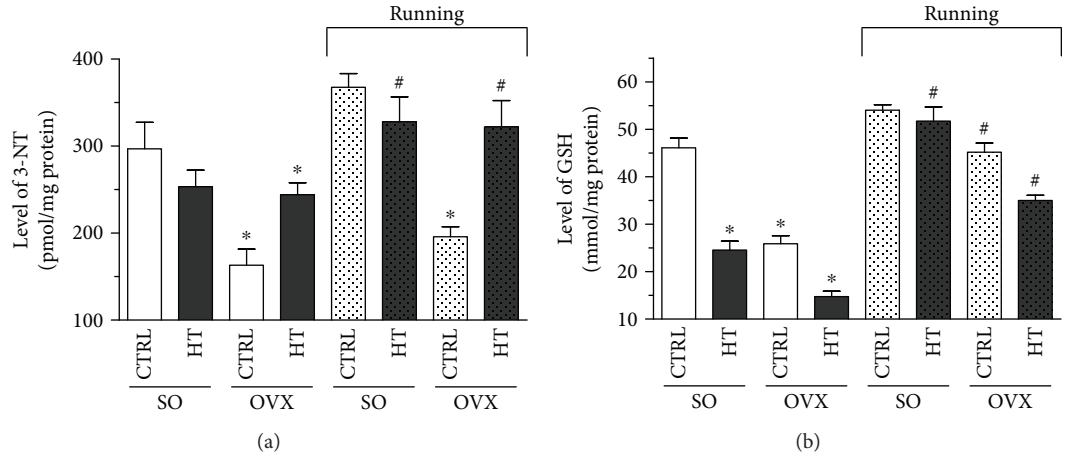

Figure 3: (a) Effects of 12-week wheel-running exercise and nutrition on the cardiac nitrotyrosine (3-NT; expressed as pmol/mg protein). Results are shown as means ± S.E.M. $n = 6$–8. (b) Effects of 12-week wheel-running exercise and nutrition on the glutathione level (GSH; expressed as nmol/mg protein). Results are shown as means ± S.E.M. $n = 6$–8. Statistical significance: $^{*}p < 0.05$ relative to the SO CTRL group, and $^{\#}p < 0.05$ shows a significant difference between the running and nonrunning groups. SO = sham-operated; OVX = ovariectomized; CTRL = standard chow; HT = high-triglyceride diet.

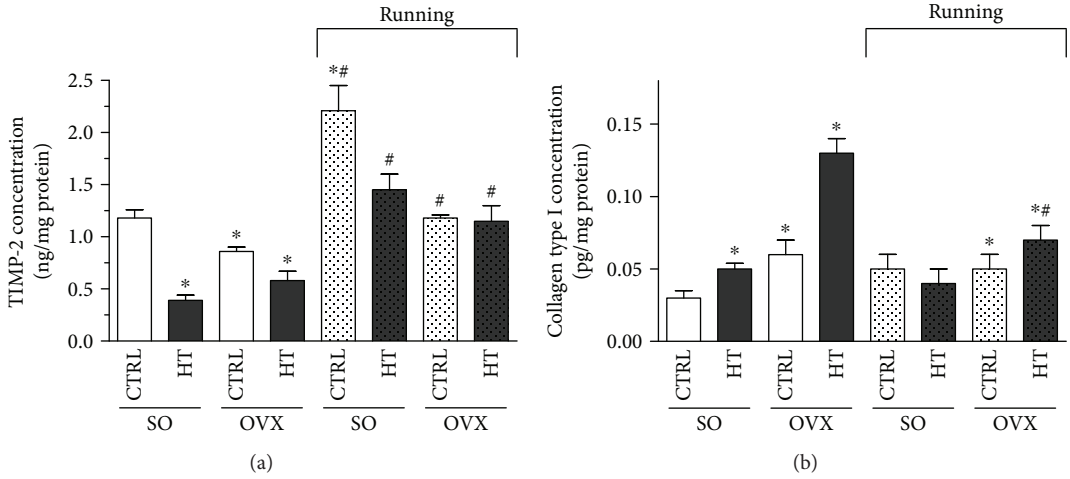

Figure 4: (a) Effects of 12-week wheel-running exercise and nutrition on the cardiac TIMP-2 level (expressed as ng/mg protein). Results are shown as means ± S.E.M. $n = 5$–8. (b) Effects of 12-week wheel-running exercise and nutrition on the cardiac collagen type I accumulation (expressed as pg/mg protein). Results are shown as means ± S.E.M. $n = 6$–9. Statistical significance: $^{*}p < 0.05$ relative to the SO CTRL group, and $^{\#}p < 0.05$ shows a significant difference between the running and nonrunning groups. SO = sham-operated; OVX = ovariectomized; CTRL = standard chow; HT = high-triglyceride diet.

accumulation of fibrotic tissue, cardiac collagen type I was measured by ELISA. While estrogen withdrawal and high-triglyceride diet resulted in an excessive accumulation of collagen type I, physical exercise training significantly ($^{\#}p < 0.05$) reduced the collagen accumulation in the heart of OVX rats fed with high-triglyceride diet. Data are presented in Figure 4(b).

3.6. Myocardial Infarction Extension. Figure 5 clearly shows that estrogen depletion and high-fat diet significantly increased ($^{*}p < 0.05$) the necrotic extension of the heart myocardial infarction compared with the SO/CTRL group. However, 12 weeks of exercise abolished the detrimental effects of ovariectomy and high-triglyceride diet with a significant reduction of infarcted area in each running group.

4. Discussion

Cardiovascular disease (CVD) is one of the major causes of death, and it can lead to heart failure including cardiac remodeling, cardiac apoptosis, and fibrosis. Inflammation, disruption of antioxidant states, and estrogen depletion are the risk factors that can lead to cardiac hypertrophy. Exercise training which is a safe nonpharmacological therapeutic tool for the prevention and treatment of CVD promotes beneficial effects, such as decreased aging-induced cardiomyocyte apoptosis, decreased risk of heart failure, and improved cardiac function. A growing number of studies have addressed that alterations in the tightly regulated ECM homeostasis can have a profound influence on the structure and function of the heart [8, 21]. Cardiac fibrosis is

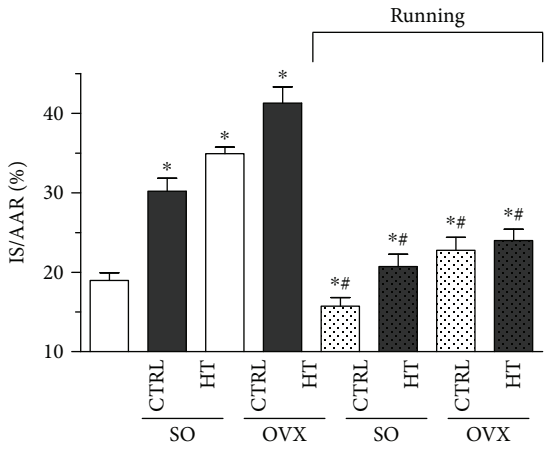

Figure 5: Effects of 12-week wheel-running exercise and nutrition on the extension of myocardial infarction. Infarct size is demonstrated as a percentage of the area at risk. Results are shown as means ± S.E.M. $n = 8$–10. Statistical significance: $^{*}p < 0.05$ relative to the SO CTRL group, and $^{\#}p < 0.05$ shows a significant difference between the running and nonrunning groups. SO = sham-operated; OVX = ovariectomized; CTRL = standard chow; HT = high-triglyceride diet.

characterized by an excessive deposition of ECM proteins, especially collagens, leading to a pathological remodeling with increased myocardial stiffness, hypertrophy, and acute cardiac injury such as myocardial infarction [10]. Increased deposition of interstitial collagen is resulted from aging [22], myocardial ischemia [23], inflammatory processes [24], diabetes [25, 26], and hormones [27]. It has emerged that sexual hormones and their receptors play a key role in the regulation of ECM proteins. The interplay of MMP-2, peroxynitrite ($ONOO^{-}$), and glutathione (GSH) in heart tissue shows new insight into the pathophysiology of the heart in estrogen-deficient conditions.

We examined the mechanism of estrogen depletion-induced collagen accumulation and fibrosis, which may occur through MMP-2 downregulation and cardiac hypertrophy. Our results clearly show that estrogen withdrawal and high-triglyceride diet caused a significant increase of collagen content and reduced cardiac levels of 3-NT and GSH. However, 12 weeks of moderate physical exercise could attenuate the OVX-induced heart fibrosis via GSH/3-NT and MMP-2 regulation. Our previous findings proved that elevation of blood pressure caused by OVX may participate in these changes [1, 28]. The antioxidant effects of endogenous and exogenous estrogen may play a critical role in eliciting vasoprotective effects, the oxidative stress increased in postmenopausal women and in animals [29, 30]. Pedram et al. showed that estrogen prevents against myofibroblast development and production of collagen and fibronectin proteins that cause cardiac remodeling [4], while E_2 reduces the turnover of ECM and exerts protective effects against cardiomyocyte apoptosis, estrogen withdrawal leading to left ventricular hypertrophy, collagen deposition, and increased sensitivity to constrictive agents, such as angiotensin II [31]. In accordance with the literature, our findings show that ovariectomy caused damage to cardiac morphology with collagen type I content enhancement. Fibrosis is multifactorial, and the molecular mechanisms related to the regulation of ECM metabolism involve multiple signaling pathways. It is widely accepted that inactivity or sedentary lifestyle, reduction in circulating estrogen level, aging process, and oxidative stress can cause excessive accumulation of collagen matrix and increased progression of cardiac dysfunction [32]; however, the mechanism responsible for the reduction of cardiac fibrosis induced by physical training is not fully identified. In this current study, we focused on the role of voluntary physical exercise and the type of diet in the modifying effects of MMP-2 regulation. MMPs and their tissue inhibitors regulate the profile of ECM both in normal and pathological conditions, so the balance between MMPs and TIMPs determines cardiac remodeling [33]. In a previous study, Felix et al. proved that ovarian hormone deprivation caused significant damage to cardiac morphology, whereas the low intensity of aerobic exercise prevented the increase in cardiac fibrosis. However, they did not examine the regulatory effects of MMP-2 [34]. Kwak et al. investigated the alterations of collagen profile in response to exercise training and proved that physical training protected against age-related downregulation of active MMPs [15]. Our research group recently demonstrated that a 12-week voluntary exercise significantly increased the MMP-2 activity, indicating the protective effects of exercise against collagen accumulation and fibrosis. MMP-2 degrades the ECM proteins which are responsible for cardiac remodeling. In addition, we also found that exercise training caused a reduction in collagen type I content and improved the MMP/TIMP profile resulting in protective effects against cardiac injuries. In the present study, 12 weeks of exercise training significantly decreased the infarct size, mitigating the estrogen withdrawal, high-triglyceride diet, and obesity-related enhanced ratio of necrotic area after ischemia/reperfusion. Many studies support the notion that women in menopausal and postmenopausal periods have greater risk for CVDs, including myocardial infarction (MI), which is related to an increase in oxidative stress and a reduction in nitric oxide (NO) bioavailability [35, 36]. Physical exercise has become a nonpharmacological therapeutic option in the prevention and treatment of CVD in both women and men. Exercise-induced improvement in myocardial capillarization, intracellular redox balance, and endothelial dysfunction by increasing of NO production can minimize the ratio of infarct size [37, 38]. Almeida et al. reported that exercise training decreased the protein expression of one of the main pathways generating ROS and also increased the antioxidant enzyme catalase, which contributed to improvement in cardiac function and remodeling process in ovariectomized rats after MI [31]. Clinical findings also demonstrated that cardiac fibrosis is strongly associated with obesity and contributes to cardiac dysfunction in obese women. Kosmala et al. concluded that abnormalities of the left ventricular function are related to the changes in the MMP/TIMP system that might promote the attenuation of ECM degradation, mainly due to the downregulation of MMP-2 in obese women [3]. Our experimental protocol with exercise training in both sham-operated and ovariectomized rats resulted in a significant increase in the

MMP-2 activity and diminished the collagen accumulation in the heart, representing an important protective strategy to treat cardiac pathologies. The mechanism related to exercise-induced collagen turnover and cardioprotection is due to the MMP/TIMP profile and the result of the activation pathways of MMP. MMP-2 can be activated by proteolytic and nonproteolytic ways. Proteolytic activation of the 72 kDa zymogen occurs by removal of its autoinhibitory propeptide to render an active 64 kDa MMP-2 or by post-translational modification caused by peroxynitrite ($ONOO^-$) in the presence of cellular glutathione (GSH) [39]. The ability of $ONOO^-$, which is a product of superoxide anion and nitric oxide (NO), to suppress [40, 41] or activate [39] MMP-2 is controversial. Rajagopalan et al. found that $ONOO^-$ enhanced the gelatinolytic activity of unpurified MMP-2 in smooth muscle cells [42]. Oxidative stress-induced post-translational modifications can result in the activation of MMP-2. The presence of the intracellular level of glutathione and $ONOO^-$ causes S-glutathiolation and conformational changes and results in an active MMP form [43].

GSH is a nonenzymatic antioxidant in the cells, and its depletion is considered as an important biomarker of oxidative stress [44]. A significant reduction of the GSH level was noted in our present study in response to estrogen withdrawal and high-triglyceride diet. The duration of 12 weeks of exercise training could restore the GSH level of the heart in agreement with others [44, 45]. GSH plays a critical role in cardiac function, maintaining redox homeostasis [17]. Frasier et al. demonstrated that physical exercise preserves cardiac glutathione pools and decreases myocardial damage after ischemic insult [46].

The complexity of functional properties of MMPs poses some limitations, and the activation of MMP-2 is not uniformly concordant, especially in cardiovascular pathologies. Moreover, there are no similar studies which investigated the role of lifestyle changes (estrogen withdrawal, exercise, and type of diet) in the activation and regulation of MMP-2.

In conclusion, the 12 weeks of exercise caused enhancement in the levels of cardiac 3-NT and GSH. Elevation in 3-NT and GSH levels with activation of 72 kDa MMP-2 may play a compensatory role against cardiac fibrosis. These data are in line with our further findings that exercise-induced activation of MMP-2 and the improved balance between MMP and TIMP contribute to cardioprotection and serves as a therapeutic agent in cardiac remodeling.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Renáta Szabó and Zoltán Karácsonyi contributed equally to this paper as first authors.

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Review Article

Honey and Diabetes: The Importance of Natural Simple Sugars in Diet for Preventing and Treating Different Type of Diabetes

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Diabetes is a metabolic disorder with multifactorial and heterogeneous etiologies. Two types of diabetes are common among humans: type 1 diabetes that occurs when the immune system attacks and destroys insulin and type 2 diabetes, the most common form, that may be caused by several factors, the most important being lifestyle, but also may be determined by different genes. Honey was used in folk medicine for a long time, but the health benefits were explained in the last decades, when the scientific world was concerned in testing and thus explaining the benefits of honey. Different studies demonstrate the hypoglycemic effect of honey, but the mechanism of this effect remains unclear. This review presents the experimental studies completed in the recent years, which support honey as a novel antidiabetic agent that might be of potential significance for the management of diabetes and its complications and also highlights the potential impacts and future perspectives on the use of honey as an antidiabetic agent.

1. Introduction

Diabetes mellitus is one of the top diseases in modern times, with more than 285 million people estimated in 2010 and about 438 million people predicted for 2030 in all over the world [1]. Diabetes prevalence may be genetically determined or can be developed during lifetime at any age. This disease takes no account of age for example, but scientific studies reveal that it is more common in developing countries than in the rest of the world (developed countries and third world countries) [1]. The increasing incidence may be due to demographic changes and undesirable result of risk factors such as obesity and sedentary life.

What is in fact diabetes mellitus? Diabetes is a metabolic disorder with multifactorial and heterogeneous etiologies. The high blood sugar level is the "symptom" known for diabetes, but other symptoms should not be ignored: increased thirst and hunger, unexplained fatigue, increased urination, blurred vision, and unexpected weight loss. Two types of diabetes are common among humans: type 1 diabetes that occurs when the immune system attacks and destroys insulin. This type of diabetes is believed to be genetically determined but also environmental factors are important in the determination of the disease. The symptoms of this type of diabetes generally start quickly, in a matter of weeks. Type 2 diabetes, the most common form, may be caused by several factors, the most important being lifestyle, but also it may be determined by different genes. This type of disease is developed during several years, and the symptoms are also not noticeable; for this reason, many people find themselves with diabetes without specific or unusual symptoms. Type 2 diabetes is most of the time related to overweight or obese state.

Although diabetes mellitus is a chronic disease of endocrine diagnosis and remains the major cause of mortality worldwide [2–5], it is not a death sentence.

Nowadays, the medical world is turning more and more on the health benefits of natural products, medicinal herbs, and also honey, in the management of this illness. Together with classic medical treatment, using recipes of traditional

medicine, including the use of apicultural products (i.e., honey), the diabetic patients can maintain the normal level of insulin in the blood and also their overall health condition.

Honey composition comprises more than 200 components, with fructose, glucose, and water as main substances. Honey was used in folk medicine back in time at the beginning of our era, but their health benefits were based only on eye observations, without having any basis for scientific support. Only in the last decades, the scientific world was concerned in testing and explaining the benefits of honey. These research studies explain to a large extent many medicinal effects of honey such as antioxidant [6–11], hepatoprotective [12–14], cardioprotective [15–17], antibacterial [18–23], anti-inflammatory [24–26], or antitumor [27–30].

For a long time, there has been a myth that honey could not be used in diabetic patient's diet, due to the high content of carbohydrates from its chemical composition. Considering the background of the research team that has been working on characterization of different types of honey from Romania and worldwide and the determination of its biological properties for a long period, we considered being appropriate to gather in a review, literature studies that may answer the question: is honey a good substitute for sugar in diabetic diet? Are natural simple sugars important in preventing and treating diabetes mellitus?

Therefore, the present study acknowledged different scientific studies, demonstrating the use of honey in diabetes mellitus: preclinical and clinical studies, animal model studies, and human studies that demonstrate the potential impact of honey on this complex disease.

2. Fructose and the Hypoglycemic Effect of Honey

Fructose content of honey varies from 21 to 43% and the fructose/glucose ratio from 0.4 to 1.6 or even higher [31–34]. Although fructose is the sweetest naturally occurring sweetener, it has a glycemic index of 19, compared to glucose which has 100 or sucrose (refined sugar) with 60 [35]. Different studies reveal the hypoglycemic effect of honey, but the mechanism of this effect remains unclear. It was suggested that fructose, selective mineral ions (selenium, zinc, copper, and vanadium), phenolic acids, and flavonoids might have a role in the process [10, 11, 31, 33, 36, 37].

There is evidence that fructose tends to lower blood glucose in animal models of diabetes [38, 39]. Mechanisms involved in this process may include reduced rate of intestinal absorption [40], prolongation of gastric emptying time [41, 42], and reduced food intake [43, 44]. Fructose stimulates glucokinase in hepatocytes, which plays an important role in the uptake and storage of glucose as glycogen by the liver. Glucose on the other hand, which is present beside fructose in honey, enhances the absorption of fructose and promotes its hepatic actions through its enhanced delivery to the liver [45, 46].

The pancreas is an important organ in diabetes, because it secrets two glucose-regulating hormones—insulin and glucagon—and honey might protect this organ against oxidative stress and damage with its antioxidant molecules, this being another potential mechanism of hypoglycemic effect of honey [32, 47].

Different studies were made on the effect of fructose on glycemic control, glucose-regulating hormones, appetite-regulating hormones, body weight, food intake, and oxidation of carbohydrates or energy expenditure [38, 44, 48–61].

Fructose administrated alone or as part of sucrose molecule in normal rats improved glucose homeostasis and insulin response compared to rats which received glucose [62]. Other studies show that fructose supplementation in normal or type 2 model of diabetic rats produced lower levels of plasma insulin and glucose, more than other administrated sugars [38].

3. Animal Model Experiments

Different animal models were used to study the possible hypoglycemic effect of honey. The most used experimental tool for inducing type 1 and type 2 diabetes is streptozotocin and alloxan of appropriate doses [63–66].

A study of six weeks [67] on healthy nondiabetic rats fed with a honey-containing diet exhibits good results: weight was reduced statistically significant, but no significant decreasing for glycosylated hemoglobin or food intake was observed.

Long-term honey feeding in Sprague-Dawley rats (52 weeks) produces a significant decrease of HbA1c levels but increases HDL cholesterol [68]. In sucrose-fed and sugar-free diet-fed rats, in the same experiment, HDL cholesterol levels were decreased and no other differences were observed for other lipids. Weight gain was similar for honey and sugar-free diet-fed rats but less compared to sucrose-fed rats.

Busseroles et al. [69] fed healthy rats with 65 g/100 g combined fructose and glucose or a honey-based diet for two weeks and the level of blood fructose, serum vitamin E, and serum vitamin E/triglycerides increased, while glucose content remains unchanged and triglyceride content decreased.

Feeding healthy rats with a diet containing 20% honey for 33 days, Nemoseck et al. [70] obtained significant decrease of triglycerides, leptin content, body weight, food/energy intake, and epididymal fat weight but not significantly glucose decrease, total cholesterol decrease, adiponectin, and C-reactive proteins. This experiment shows that longer period of feeding must be used, to obtain significant results.

Erejuwa et al. [11, 47] found no significant differences in fasting blood glucose or body weight in honey-fed rats.

If honey was demonstrated to have hypoglycemic effect in healthy animals, the same beneficial effect was observed in induced diabetic animals. A very important observation regarding honey and diabetes is that honey augments the antihyperglycemic effect of standard antidiabetic drugs in induced diabetes [10, 33].

Rabbits with diabetes induced by alloxan were used in one experiment, and three types of sweeteners were used for feeding the animals [65]. Pure honey of *Apis florea* and *Apis dorsata* and adulterated honey were given in different doses in a rabbit's diet, and a dose-dependent rise in blood glucose was registered.

Another study [66] of alloxan-induced diabetic rats fed with honey and healthy rats fed with fructose shows different results: glucose decreased significantly in alloxan-induced diabetic rats and not significantly in fructose-fed rats. Body weight increased in healthy fructose-fed rats, and hypoglycemic effect and also the same effect were found for streptozotocin-induced diabetic rats [71]. Table 1 summarizes the preclinical studies on healthy and induced diabetic animals, using honey solution or other sweeteners in their diet.

4. Honey versus Sugars in Human Clinical Trials

Human diet must have all types of nutrients required in the metabolic transformations and life support. Water, proteins, lipids, carbohydrates, vitamins, minerals, amino acids, and bioactive compounds are needed by the human body, and all of these compounds are taken from the diet. Maintaining a healthy life, equilibrate diet, and intake of each and every one of these nutrients is the key factor of health in general. Different diseases have as a starting point unbalances in metabolism, because of lack or excess of one or more nutrients.

Diabetes, as stated before, represents the high level of blood sugars due to low or no insulin production in the body. Experimental studies on animals suggest the beneficial effects of honey as a diet supplement and encouraging results on control of diabetes mellitus and additional complications are presented in medical studies; the experiments and reports on humans (healthy or diabetic) are rather sparse.

The published studies present favourable effects of honey in both healthy and diabetic subjects [16, 31, 72–76]. Since oxidative stress is implicated and mainly responsible for diabetes development, the antioxidant effects of honey are very important in this disease management [77].

The study of Al-Waili [78] on healthy, diabetic, or patients with hypertriglyceridemia shows promising results, when honey was used in their diet, compared with dextrose and sucrose. Thus, lipid profile was improved, normal and elevated C-reactive protein was lowered, and also homocysteine value and triacylglycerol were decreased in patients with hypertriglyceridemia. In diabetic patients, honey compared with dextrose caused a significantly lower rise of plasma glucose level (PGL). Honey caused greater elevation of insulin compared to sucrose; after different time of consumption, it reduces blood lipids, homocysteine, and CRP in normal subjects. The conclusion was that honey compared with dextrose and sucrose caused lower elevation of PGL in diabetics. This experimental study on healthy, diabetic, and hyperlipidemic human subjects demonstrates the different intake rate of refined sugar and honey, the raising of blood sugar and also raising their insulin levels.

Sugar is a refined product, obtained from different natural sources, but follows a technological process, leading to an almost pure substance—sucrose—highly used in modern life in the food industry.

Honey, on the other hand, being also a natural sweet product, has a complex composition, but compared to sugar, it has a lower glycemic index and energetic value. When we talk about refined sugar, it is easy to state the exact chemical composition, very simple actually, but talking about honey, many aspects should be considered regarding its composition. Botanical and geographical origins determine the specific composition and properties of all types of honeys.

Table 2 presents comparatively the chemical composition of refined sugar and honey.

The fact that refined sugar is almost 100% sucrose, and very small amounts of other components compared to honey, makes the last one, an important sweetener, with almost 80% simple sugars from the total chemical composition (35–40% fructose and 30–35% glucose).

Even though the exact mechanism by which honey may have beneficial effects upon blood glucose is not very clear; from comparative experiments, some conclusions about the importance of fructose in honey are available. Fructose is known to stimulate glucokinase in hepatocytes, which plays an important role in the uptake and storage of glucose as glycogen by the liver [79], the amount of fructose in honey being very important for its hypoglycemic effects.

A study on humans [80] evaluated for a large period of time wherein a group of twenty adult patients with type 2 diabetes volunteered to stop their medication and use honey as treatment for their disease. This nonrandomized, open clinical trial aiming to study the safety and efficiency of honey as unique treatment revealed interesting results (Table 3).

Besides glycemic index (GI), peak incremental index (PII) is used to assess the glycemic effect (the effect on blood glucose level after ingestion of various foods) [81].

C-peptide is considered a good marker of insulin secretion, being cosecreted with insulin by the pancreatic cells as a by-product, with no biological activity of its own [82], of the enzymatic cleavage of proinsulin to insulin. Scientific studies regarding the effects of honey on insulin and C-peptide levels are controversial in healthy and diabetic patients [54, 83, 84].

A study made in the National Institute of Diabetes in Cairo, Egypt, on twenty diabetic young patients and ten healthy nondiabetic ones try to elucidate this controversy [73]. Glucose, sucrose, and honey were administrated diluted with 200 ml water, according to the patient's weight (amount of sugar/honey = weight of the subject in kg $\times$ 1.75, with a maximum of 75 g). The diluted sugars and honey were ingested in the morning by every participant, one week apart for each sugar type, the whole test lasting for three weeks. Blood tests were made before ingestion and after every 30 min postprandial of sugars, until 120 min (2 hours). Serum C-peptide level and glucose assay were measured for all blood samples.

The glycemic index and peak incremental index were lower both in patients and control group, when honey was used compared to glucose and fructose, but the level of C-peptide was different in patients and control group.

Honey causes a postprandial rise of plasma C-peptide levels compared to sucrose and glucose in nondiabetic patients, suggesting that honey might have a direct stimulatory effect on the healthy beta cells of the pancreas [73].

Table 1: Preclinical studies on animal models regarding the effect of honey on induced diabetes mellitus.

Ref.	Animal models	Applied treatment	Obtained results
[10]	60 diabetic rats divided into 6 groups: (1) distilled water, (2) honey, (3) glibenclamide, (4) glibenclamide and honey, (5) metformin, and (6) metformin and honey	Distilled water, honey, glibenclamide, glibenclamide and honey, and metformin or metformin and honey treatment orally once a day for 4 weeks	Honey significantly increased insulin (0.41 ± 0.06 ng/ml), decreased hyperglycemia (12.3 ± 3.1 mmol/L), and fructosamine (304.5 ± 10.1 μmol/L). Glibenclamide and metformin alone significantly reduced hyperglycemia, but combined with honey, produced significantly much lower blood glucose (8.8 ± 2.9 or 9.9 ± 3.3 mmol/L, resp.) compared to glibenclamide or metformin alone (13.9 ± 3.4 or 13.2 ± 2.9 mmol/L).
[11]	Diabetic rats (6 rats/group) induced by streptozotocin (STZ) 60 mg/kg	Distilled water (0.5 ml/day) Honey (0.2 g/kg/day, 1.2 g/kg/day, and 2.4 g/kg/day) oral gavage for 4 weeks	Total antioxidant status (TAS), activities of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) were significantly reduced, while superoxide dismutase (SOD) activity was upregulated in kidneys of diabetic rats. Lipid peroxidation (TBARS) and fasting plasma glucose (FPG) were significantly elevated while body weight was reduced in diabetic rats. Honey significantly increased body weight, TAS, and activities of CAT, GPx, GR, and GST in diabetic rats.
[12]	Adult male Sprague-Dawley rats; diabetes induced by STZ (60 mg/kg body weight)	Tualang honey (1.0 g/kg body weight)	Tualang honey supplementation in diabetic rats reduces elevated levels of AST and ALT and also produces a hepatoprotective effect in STZ-induced diabetic rats.
[14]	6 groups of 6 rats/group	(1) Control rats feed with standard pellet diet and water; (2) diabetic rats as untreated diabetic control; (3) diabetic rats treated with honey 1.0 g/kg BW for 21 days; (4) hyper cholesterol rats: cholesterol (1.5%) and cholic acid (0.5%) mix with diet; (5) hyper cholesterol rats treated with honey (1.0 g/kg BW for 21 days); and (6) diabetic rats treated with glibenclamide (0.5 mg/kg)	Honeybee treatment significantly decreases blood glucose level in diabetic rats. TC, TG, LDL, and VLDL are significantly decreased whereas HDL significantly increases. The SGPT, SGOT, and CRP were significantly decreased.
[33]	8 groups of diabetic rats (5–7 animals/group)	Treatments/groups: (1) distilled water (0.5 ml); (2) honey (1.0 g/kg); (3) metformin (100 mg/kg); (4) metformin and honey; (5) glibenclamide (0.6 mg/kg); (6) glibenclamide and honey; (7) metformin and glibenclamide; and (8) metformin, glibenclamide, and honey orally, once a day for 4 weeks	Malondialdehyde (MDA) levels, glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities were significantly elevated while catalase (CAT) activity, total antioxidant status (TAS), reduced glutathione (GSH), and GSH : oxidized glutathione (GSSG) ratio were significantly reduced in the diabetic kidneys. CAT, glutathione reductase (GR), TAS, and GSH remained significantly reduced in the diabetic rats treated with metformin and/or glibenclamide. In contrast, metformin or glibenclamide combined with honey significantly increased CAT, GR, TAS, and GSH.
[47]	Diabetic (2 groups) and nondiabetic rats (2 groups)	Diabetic rats were administered distilled water (0.5 ml/d) and Tualang honey (1.0 g/kg/d). Nondiabetic rats received also distilled water (0.5 ml/d) and Tualang honey (1.0 g/kg/d)	The honey-treated diabetic rats had significantly reduced blood glucose levels [8.8 (5.8) mmol/L; median (interquartile range)] compared with the diabetic control rats [17.9 (2.6) mmol/L].

Table 1: Continued.

Ref.	Animal models	Applied treatment	Obtained results
[65]	8 groups of rabbits (6 animals/group); groups I to IV were normal and healthy (nondiabetic) and groups V to VIII were diabetic induced by alloxan monohydrate	Group I: untreated control received 20 ml of water orally. Groups II–IV treated orally with 5, 10, and 15 mg/kg BW honey diluted up to 20 ml/kg with distilled water. Groups V–VI treated with tolbutamide (250 mg and 500 mg). Group V: diabetic control, treated with 20 ml of water. Groups VI–VIII treated orally with 5, 10, and 15 ml/kg BW of honey diluted to 20 ml with distilled water	Oral administration of pure honeys in 5 ml/kg/doses could not produce a significant ($P > 0.05$) increase in glucose levels in normal and alloxan-diabetic rabbits whereas the adulterated honey significantly raised the blood glucose levels in normal and hyperglycemic rabbits even at this low dosage.
[66]	48 matured male Wistar rats separated into 6 groups	Group 1a: control had standard rat chow for 3 weeks. Group 1b: fed with honey along with standard rat chow for 3 weeks. Group 2a: alloxan-induced diabetes and standard rat chow for 3 weeks. Group 2b: alloxan-induced diabetes, fed with honey and standard rat chow. Group 3a: standard rat chow and fructose for 3 weeks. Group 3b: standard rat chow fructose for three weeks than honey along with standard rat chow and fructose for 3 weeks	At the end of three weeks, it was found that daily ingestion of honey for 3 weeks progressively and effectively reduced blood glucose level in rats with alloxan-induced diabetes. Honey also caused a reduction in hyperglycemia induced by long-term ingestion of fructose, albeit to a lesser degree than its effect on alloxan-induced hyperglycemia. Honey could not reduce blood glucose in controlled rats that received neither alloxan treatment nor fructose ingestion, even though it caused an increase in body weight, irrespective of other substances concomitantly administered to the rats.
[67]	40 six-week-old Sprague-Dawley rats	A powdered diet that was either sugar-free or which contained 8% sucrose, 8% mixed sugars as in honey, or 10% honey freely for 6 weeks	HbA1c and triglyceride levels were significantly higher in all sugar treatments compared with rats fed with a sugar-free diet.
[68]	55 Sprague-Dawley rats aged approximately 8 weeks	3 experimental diets were prepared to contain no sugar, 7.9% sucrose, or 10% honeydew honey	Weight gain was substantially reduced in honey-fed rats compared with those given a sucrose-based diet; the finding that consuming honey increases HDL cholesterol levels is still a significant result though. There have been strong associations seen between low HDL cholesterol levels and the increased risk of cardiovascular disease.
[71]	36 rats divided into 6 groups of 6 animals. Diabetes was induced by STZ (60 mg/kg; ip)	Diabetic rats received distilled water (0.5 ml/day), honey (1.0 g/kg/day), and metformin (100 mg/kg/day) or a combination of metformin (100 mg/kg/day) and honey (1.0 g/kg/day) orally for four weeks. Similarly, two groups of nondiabetic rats received distilled water (0.5 ml/day) and honey (1.0 g/kg/day)	Honey significantly increased GSH, TAS, and activities of CAT and GR in diabetic rats while FPG, MDA levels, and SOD activity were decreased. The final results indicate that honey exerts hypoglycemic effect and ameliorates renal oxidative stress.

Although honey has lower GI than sugar (Table 2), an average value for honey is presented [85], according to fructose/glucose ratio, and GI value of different honeys is also different [86].

Twenty healthy subjects from Erciyes University, Kayseri, Turkey, were subjected voluntarily to a test of ingesting 50 g of pure glucose in 250 ml water and an amount of honey that corresponds to 50 g glucose (accordingly to the physico-chemical analysis of honey used in the test). Capillary blood samples were taken from the finger in the next morning after sugar consumption and again every 15 minutes after second ingestion of sugars in the next day, until 120 minutes. Serum glucose and serum insulin level decreased after 2 hours of honey intake, and C-peptide level increased slightly 2 hours after honey intake. This study demonstrates how different types of honey, having different GI values, influence the parameters usually measured for diabetes control in a different manner [85].

Sixty healthy subjects aged 18 to 30 years, enrolled in one experiment in Isfahan University of Medical Science, Iran [87], receive 80 g of honey and 80 g sucrose dissolved in 250 ml water once a day for six weeks. Systolic blood pressure

Table 2: Average chemical composition of honey compared to sugar.

Component/100 g	Honey*	Sugar
Glycemic index	58	60
Calories	300 Kcal	387 Kcal
Sugars	80.0 g	99.9 g
Lipids	0.02 g	—
Protein	0.3 g	—
Calcium	6.0 mg	1.0 mg
Iron	0.42 mg	0.01 mg
Magnesium	2.0 mg	—
Phosphorus	4.0 mg	—
Zinc	0.22 mg	—
Potassium	52.0 mg	2.0 mg
Vitamin C	0.5 mg	—
Vitamin B2	0.038 mg	0.019 mg
Vitamin B3	0.121 mg	—
Vitamin B5	0.068 mg	—
Vitamin B6	0.024 mg	—
Vitamin B9	2.0 μg	—
Water	17.0 g	0.03 g

*Values specified for honey represent an average of floral and honeydew honey.

(SBP), diastolic blood pressure (DBP), and fasting blood sugar (FBS) were determined from each participant at the beginning and in the end of the study. No significant change was registered in SBP and DBP in both groups at the beginning and in the end of the study, but FBS registered a significant reduction in the honey group at the end of the study, compared to the sucrose group [87].

Different studies mentioned before show that honey consumption reduces body weight but also blood glucose in healthy and diabetic patients compared to sugar intake. A study on type 2 diabetic patients consuming natural honey shows that body weight may be reduced and blood lipids and glucose as well [31]. The study consists of 58 patients with type 2 diabetes, with fasting blood sugar of 110–220 mg/dl, with same oral hypoglycemic drugs, but no insulin treatment. The experimental group ($n = 25$) receives natural honey for eight weeks following an experimental scheme, and the control group ($n = 23$) did not receive honey or other sweeteners. The participants continued their usual diet over the study period. The body weight and fast blood sugar were measured every 2 weeks, and constant decreasing was registered [31]. Scientific studies reviewed by Erejuwa et al. [12, 33] demonstrate that fructose and oligosaccharides from honey contribute to its hypoglycemic effect. In addition to lowering oxidative stress and hyperglycemia, honey consumption ameliorates other metabolic disorders associated with diabetes, such as reduced levels of hepatic transaminases, triglycerides, and glycosylated hemoglobin (HbA1c) and increased HDL cholesterol [12, 31].

Several honey types from different parts of the world ameliorate metabolic abnormalities in type 1 and type 2 diabetic patients [36, 73, 88]. These studies investigate the acute effects of honey on hyperglycemia and metabolic disorders, because the diabetic parameters were measured postprandial in studies which last from two to eight weeks. Table 3 summarizes the clinical studies on humans, applied treatment, and the main obtained results.

5. Honey in Diabetic Wound Healing

Besides the health benefits of ingesting honey in diabetes, another important use of honey could be in managing diabetic wounds [89]. These wounds are not like typical wounds, they are slower in healing or they do not heal at all, leading to complications that conventional medications do not work.

Honey was used in alternative medicine for healing different wounds since ancient times, the use of honey in diabetic wound management being more recent. Diabetic patients sometimes suffer from different complications such as arterial disease, vascular problems, ulcerations, and foot complications [90, 91].

Even if diabetic wounds are similar to wounds from normal patients, the healing process in the former is very slow and problematic and the medical costs are extremely high. Honey is a potential candidate to be used in these treatments because it is available, natural, and not expensive. But how can honey work at the wound site? The honey diluted with water or different body fluids forms hydroxyl radicals and hypochlorite anions at the wound site. The antioxidants present in the honey act through two different mechanisms in a wound: first, antioxidants fight against microorganisms and lower the infection in the wound [75, 92, 93]; second, the same antioxidants reduce the reactive oxygen species and inflammation caused by the wound, helping in the healing process [94–96].

The antimicrobial activity of honey is due to acidic pH, osmotic effect, hydrogen peroxide, and nitric oxide. The presence of nitric oxide metabolites in honey as well as the production of NO products by honey in different body fluids improves the healing process [74, 80, 97].

Debridement, wound odor, scar formation, and inflammation control are very important in diabetic wound management [89]. The slow healing process in diabetic wounds is due to the peripheral arterial diseases and peripheral neuropathy that occur with diabetes; the blood vessels tend to shrink, reducing blood circulation in the respective areas. The nerves do not receive enough blood (nutrients) and may become damaged and more vulnerable to injury. The stimulating tissue growth when honey is used is due to the chemical composition, the presence of assimilable sugars, vitamins, amino acids, and phenolics that increases oxygen and nutrients in the wound area [98, 99].

Numerous studies show evidence of successful honey treatments against diabetic wounds all over the world [100–105]. Honey applications reduce wound ulcer pain and size and deodorization of the wound, and reduction of healing time and are safe and there are no side effects.

A recent study [106] brings new evidence in demonstrating the effects of Manuka honey in wound healing. The

Table 3: Clinical studies regarding the effect of honey in human diabetic subjects.

Ref	Research groups	Honey/sugars treatment schemes	Obtained results
[16]	17 subjects (control group) 38 subjects (experimental group)	70 g sucrose daily for 30 days in the control group and 70 g of honey in the experimental group	Honey caused a mild reduction in body weight (1.3%) and body fat (1.1%), reduced total cholesterol (3%), LDL-C (5.8), triacylglycerol (11%), FBG (4.2%), and CRP (3.2%), and increase HDL-C (3.3%) in normal subject and in patients honey cause reduction in total cholesterol by 3.3%, LDL-C by 4.3%, triacylglycerol by 19%, and CRP by 3.3%.
[31]	48 type II diabetic patients: Honey group Control group	1 g/kg BW/day for 2 weeks; 1.5 g/kg BW/day for next 2 weeks; 2 g/kg BW/day for next 2 weeks; and 2.5 g/kg BW/day for the last 2 weeks	Body weight, total cholesterol, low-density lipoprotein cholesterol, and triglyceride decreased, and high-density lipoprotein cholesterol increased significantly in the honey group. The levels of hemoglobin A (1C) increased significantly in the honey group.
[36]	24 healthy subjects, 16 type II diabetic subjects 6 patients with hypertension	12 healthy subjects receive inhalation with distilled water for 10 min; after one week, they received inhalation of honey solution (60% wt/*v*) for 10 min. 12 healthy subjects received inhalation of 10% dextrose for 10 min	Honey inhalation significantly reduced random blood glucose level from 199+/−40.9 mg/dl to 156+/−52.3 mg/dl after 30 min. Fasting blood glucose level was reduced after honey inhalation during 3 hr postinhalation, which was significant at hour 3. Intensity of hyperglycemia was significantly lowered in glucose tolerance test when patients received honey inhalation.
[54]	32 type II (noninsulin-dependent) diabetic patients	Diet of 25 g glucose, fructose, or lactose or 30 g honey, 50 g white bread, 125 g white rice or potatoes, and 150 g apples or 260 g carrots	Blood glucose and plasma insulin were measured at zero time and then at 15, 30, 60, 90, and 120 min after the meal. Counting the blood glucose increase after glucose as 100%, the corresponding increases in glycemia for other carbohydrates were fructose, 81.3%; lactose, 68.6%; apples, 46.9%; potatoes, 41.4%; bread, 36.3%; rice, 33.8%; honey, 32.4%; and carrots, 16.1%.
[73]	20 young type I diabetic patients in the experimental group; 10 healthy nondiabetics in the control group	Calculated amount of glucose, sucrose, and honey (amount = weight of the subject in kg × 1.75 with a maximum of 75 g/patient)	Honey, compared to sucrose, had lower GI and PII in both patients and control groups. In the patient group, the increase in the level of C-peptide after using honey was not significant when compared with glucose or sucrose.
[76]	30 individuals with a proven parental (mother or father) history of type II diabetes mellitus	Glucose diet supplementation Honey diet supplementation	The plasma glucose levels in response to honey peaked at 30–60 minutes and showed a rapid decline as compared to that of glucose. Significantly, the high degree of tolerance to honey was recorded in subjects with diabetes as well, indicating a lower glycemic index of honey.
[78]	48 subjects: healthy and diabetic and with hyperlipidemia	(i) Dextrose solution (250 ml of water containing 75 g of dextrose) or honey solution (250 ml of water containing 75 g of natural honey) (ii) Dextrose, honey, or artificial honey (250 ml of water containing 35 g of dextrose and 40 g of fructose) (iii) Honey solution, administered for 15 days (iv) Honey or artificial honey (v) 70 g of dextrose or 90 g of honey in patients with type 2 diabetes mellitus (vi) 30 g of sucrose or 30 g of honey in diabetic patients	Healthy subjects: dextrose elevated PGL at 1 and 2 hours and decreased PGL after 3 hours. Honey elevated PGL after 1 hour and decreased it after 3 hours. Elevation of insulin and C-peptide was significantly higher after dextrose than after honey. Dextrose slightly reduced cholesterol and low-density lipoprotein cholesterol (LDL-C) after 1 hour and significantly after 2 hours and increased TG after 1, 2, and 3 hours. Artificial honey slightly decreased cholesterol and LDL-C and elevated TG. Honey reduced cholesterol, LDL-C, and TG and slightly elevated high-density lipoprotein cholesterol (HDL-C). Honey consumed for 15 days decreased cholesterol, LDL-C, TG, CRP, homocysteine, and PGL but increased HDL-C. Hypertriglyceridemic patients: artificial honey increased TG, but honey decreased TG. In patients with hyperlipidemia, artificial honey increased LDL-C, while honey decreased LDL-C. Honey decreased cholesterol, LDL-C, and CRP after 15 days.

Table 3: Continued.

Ref	Research groups	Honey/sugars treatment schemes	Obtained results
			In diabetic patients, honey compared with dextrose caused a significantly lower rise of PGL. Elevation of PGL was greater after honey than after sucrose at 30 minutes and was lower after honey than it was after sucrose at different intervals. Honey caused elevation of insulin compared to sucrose after different intervals and lower elevation of PGL in diabetics.
[80]	20 adult patient volunteers suffering from type 2 DM and its associated metabolic disorders from 30 to 65 years and both sexes	Honey dose of 2 g/kg BW/day, (i) 50 ml (60 g) honey was dissolved in water (ratio of 1 : 3) and given before meals twice daily; (ii) the remaining 25 ml (30 g) was used for sweetening purposes	Honey consumption resulted in more hyperglycemia in these patients but without diabetic ketoacidosis (DKA) or hyperglycemic hyperosmolar state (HHS). Longer-term honey consumption resulted also in weight reduction in all the patients, and control of the blood pressure in the patients, who had hypertension before the honey intervention. The cardiovascular status improved in the patients, who had coronary heart disease (CHD) before the intervention.
[88]	50 patients with type I diabetes mellitus 30 controls without diabetes	The honey dose: 1.75 g/kg BW Sucrose dose: 1.75 g sugar/kg BW	The GI and PII of either sucrose or honey did not differ significantly between patients and controls. Both the GI and PII of honey were significantly lower when compared with sucrose in patients and controls. In both patients with diabetes and controls, the increase in the level of C-peptide after the honey was significant when compared with either glucose or sucrose.

results reported by the authors, based on the capacity of this type of honey to improve the responsiveness to oxidative damage, as well as stimulation of cell proliferation, could help to understand how Manuka honey develops its healing effect on wounds.

Although, some guidelines for honey applications must be used such as natural unheated honey should be used in treatments and stored in dark glass bottles in cool places. Different medical grade honey with standardized antibacterial activity for use in wound treatments are known, such as Apiban (Apimed: Cambridge, New Zealand), Woundcare 18+ (Comvita: Te Puke, New Zealand), and Medihoney (Capilano: Richmonds, Queensland, Australia) [99]. If these honeys are not available, any dark honey with high antibacterial activity may be used.

6. Conclusions

Considerable evidence from experimental studies shows that the honey may provide benefits in the management of diabetes mellitus. The benefits could be a better control of the hyperglycemic state, limiting other metabolic disorders and diminishing the deleterious effects on different organs that may produce diabetic complications. Anyway, there are some data and literature with contrary discussions regarding the use of honey in diabetic diseases.

Animal models of diabetes were employed chemically (streptozotocin or alloxan), and this may not entirely reflect the development of type 2 diabetes in humans. More studies on animals are necessary but following other animal models, closer than human type 2 diabetes.

Optimal doses for human consumption must be established, and longer period experiments must be developed, due to the fact that diabetes mellitus is a chronic disease.

Answering the main question of the study, it is true that honey may be used as a potential antidiabetic agent that has the potential to reduce the complications of diabetes, long-term studies using honey as an alternative or a complementary therapy in human subjects suffering from type 2 diabetes mellitus are needed, with a larger number of patients, randomized clinical trials set up with different levels of diabetes, treated with different doses of honey, following both short-term and long-term treatment.

As stated recently [107], "The use of honey in diabetic patients still has obstacles and challenges and needs more large sample sized, multicenter clinical controlled studies to reach better conclusions."

Abbreviations

ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
BW: Body weight
CAT: Catalase
CHD: Coronary heart disease
CRP: C-reactive protein
DBP: Diastolic blood pressure
DKA: Diabetic ketoacidosis
DM: Diabetes mellitus
FBG: Fasting blood glucose
FBS: Fasting blood sugar
FPG: Fasting plasma glucose
GI: Glycemic index

GPx: Glutathione peroxidase
GR: Glutathione reductase
GSH: Reduced glutathione
GSSP: Oxidized glutathione
GST: Glutathione-S-transferase
HbA1C: Glycated hemoglobin
HDL: High-density lipoproteins
HHS: Hyperglycemic hyperosmolar state
MDA: Malondialdehyde
NO: Nitric oxide
PII: Peak incremental index
PGL: Plasma glucose level
SBP: Systolic blood pressure
SGOT: Serum glutamic oxaloacetic transaminase
SGPT: Serum glutamate pyruvate transaminase
SOD: Superoxide dismutase
STZ: Streptozotocin
TAS: Total antioxidant status
TBARS: Thiobarbituric acid reactive substances
TC: Total cholesterol
TG: Triglyceride
VLDL: Very low-density lipoprotein.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All authors contributed equally to this paper.

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Review Article

Nutrients and Oxidative Stress: Friend or Foe?

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There are different types of nutritionally mediated oxidative stress sources that trigger inflammation. Much information indicates that high intakes of macronutrients can promote oxidative stress and subsequently contribute to inflammation via nuclear factor-kappa B- (NF-κB-) mediated cell signaling pathways. Dietary carbohydrates, animal-based proteins, and fats are important to highlight here because they may contribute to the long-term consequences of nutritionally mediated inflammation. Oxidative stress is a central player of metabolic ailments associated with high-carbohydrate and animal-based protein diets and excessive fat consumption. Obesity has become an epidemic and represents the major risk factor for several chronic diseases, including diabetes, cardiovascular disease (CVD), and cancer. However, the molecular mechanisms of nutritionally mediated oxidative stress are complex and poorly understood. Therefore, this review aimed to explore how dietary choices exacerbate or dampen the oxidative stress and inflammation. We also discussed the implications of oxidative stress in the adipocyte and glucose metabolism and obesity-associated noncommunicable diseases (NCDs). Taken together, a better understanding of the role of oxidative stress in obesity and the development of obesity-related NCDs would provide a useful approach. This is because oxidative stress can be mediated by both extrinsic and intrinsic factors, hence providing a plausible means for the prevention of metabolic disorders.

1. Introduction

There are different types of nutritionally mediated oxidative stress sources that trigger inflammation. Oxidative stress plays a crucial role in the development of numerous human diseases [1]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced continuously in the body via oxidative metabolism, mitochondrial bioenergetics, and immune function [2]. The most frequent forms of ROS include superoxide anion, hyphochlorous acid, hydrogen peroxide, singlet oxygen, hypochlorite, hydroxyl radical, and lipid peroxides, which are involved in the progression, growth, death, and differentiation of cells. They can bind with nucleic acids, enzymes, membrane lipids, proteins, and other small molecules [1]. Short-term postprandial mitochondrial oxidative stress causes inflammation, which is mainly mediated by nuclear factor-kappa B (NF-κB) [3]. Conversely, long-term chronic overconsumption contributes to obesity, which induces permanent states of inflammation via the generation of white adipose tissue which secretes proinflammatory factors [4]. Extensive research has shown that high-glucose and a high-fat diet mediate inflammation, which suggests that oxidative stress may alter cellular physiological processes [5, 6].

Substantial evidence highlights the detrimental impact of diets high in refined carbohydrates and saturated fat [7]. Cardiovascular disease (CVD), obesity, type 2 diabetes, and nonalcoholic fatty liver disease are attributed to the overconsumption of foods high in carbohydrates and saturated fats, the saturation of nutrient storage, and sedentary lifestyles [8, 9]. Studies exploring the influence of a Westernized dietary pattern on inflammatory diseases, such as colorectal cancer [10], have consistently shown a similar trend. Such findings highlight the fundamental idea that diet quality

can impact immune function and systematic inflammation. In a study by Song et al. [11] focusing on carbohydrate and refined-grain intake and metabolic syndrome outcome in Korean men and women, women were shown to have a greater likelihood of metabolic syndrome with refined-grain consumption compared to the men, suggesting that refined-grain intakes are linked to a high level of inflammation.

The prevalence of obesity has doubled from 1980 to 2008 worldwide. In 2008, more than 50% of men and women in the WHO European Region were overweight, and nearly 20% of men and 23% of women were obese [12]. Nearly 1.5 billion people worldwide are obese or overweight which increases their risk of developing inflammatory disturbances, CVD, nonalcoholic fatty liver disease, coronary heart disease, and type 2 diabetes [13, 14].

The effects of oxidative stress are related to the type of macronutrients consumed and their absolute quantity [15]; both of these aspects contribute to oxidative stress and may favor the development of obesity and obesity-related noncommunicable diseases (NCDs) [16]. However, the molecular mechanisms of nutritionally mediated oxidative stress are complex and poorly understood. Therefore, this review aimed to explore how dietary choices exacerbate or dampen oxidative stress and inflammation. We also discussed the implications of oxidative stress in the adipocyte and glucose metabolism and obesity-associated NCDs. A better understanding of the role of oxidative stress in obesity and the development of obesity-related NCDs would provide a useful approach. This is because oxidative stress can be mediated by both extrinsic and intrinsic factors, hence providing a plausible means for the prevention of metabolic disorders.

2. Oxidative Stress

The harmful effects of free RNS and ROS radicals cause a potential biological damage, namely, nitrosative stress and oxidative stress, respectively [17]. ROS are generated in normal aerobic metabolism as a by-product; however, when the level is increased under stress, it may cause basic health hazard [18]. The mitochondrion is the predominant cell organelle in ROS production [19]. It generates adenosine triphosphate (ATP) via a series of oxidative phosphorylation processes [19]. During this process, one or two electron reductions instead of four electron reductions of oxygen have occurred, which subsequently leads to the formation of H_2O_2 or $O_2^{\bullet}$, and convert to other ROS [19]. The major form of RNS includes nitric oxide (NO) and peroxynitrite (ONOO−) [17]. When excess NO is present, this reaction leads to the formation of nitrogen dioxide radical [17]. Higher NO concentration leads to the formation of N_2O_3 and this usually results in nitrosation [17].

Oxygen free radicals, including alkyl peroxyl radical ($^{\bullet}$OOCR), hydroxyl radical ($OH^{\bullet-}$), and superoxide anion radical ($O_2^{\bullet-}$), are potent initiators in lipid peroxidation, the role of which is well-established in the pathogenesis of diseases [18]. Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced [18]. Thus, end products of lipid peroxidation, for example, F2-isoprostanes, 4-hydroxy-2-nonenol (4-HNE), and malondialdehyde (MDA), are accumulated in biological systems [18]. DNA bases are very susceptible to ROS oxidation, and the major detectable oxidation product of DNA bases is 8-hydroxy-2-deoxyguanosine [18]. Oxidation of DNA bases can cause mutations and deletions in both nuclear and mitochondrial DNA. Mitochondrial DNA is relatively prone to oxidative damage due to its proximity to ROS and its deficient repair capacity compared to that of the nuclear DNA [18]. These oxidative modifications cause functional changes in structural and enzymatic proteins, which may lead to substantial physiological impact [18]. In addition, redox modulations of transcription factors also increase or decrease their specific DNA binding activities and thereby altering gene expression [18].

3. Nutritionally Mediated Oxidative Stress

3.1. High Carbohydrates. Much information indicates that high intakes of macronutrients can promote oxidative stress and subsequently contribute to inflammation via NF-κB-mediated cell signaling pathways [20]. Dietary carbohydrates are important to highlight here because they may contribute to the long-term consequences of nutritionally mediated inflammation [21]. Dietary carbohydrate intake has gained attention among researchers because of the associations between a high glycemic index (GI) or glycemic load (GL) diet with diabetes, obesity, cancer, and coronary heart disease [22, 23]. High GL diets have been characterized as a common feature of Western culture; they are heavy in added sugars and refined carbohydrates [24]. By contrast, low GI foods were found to decrease postprandial glycemia in overweight/obese [23] and type 2 diabetes patients [25]. Consistent relationships between high GI and diabetes have been demonstrated in observational and cohort studies [26–28].

The high GI of white rice may lead to high oxidative stress [29]. Most Asian populations consume large amounts of rice as a staple food; thus, dietary carbohydrate intake plays a substantial role in the development of metabolic diseases in Asian populations. In support of this, a positive relationship between rice intake or total carbohydrates and diabetes has been demonstrated in Japanese women [30, 31]. In addition to diabetes outcome, a high intake of refined-grain was also positively linked to fasting blood glucose and triglyceride levels and negatively associated with high-density lipoprotein (HDL) cholesterol in Asian Indian and Korean populations [11, 32], indicating that a high GI diet may negatively impact health.

The elevation of oxidative stress is linked to chronic inflammation [33]; other sources may also further increase the accumulation of proinflammatory cytokines in a "vicious cycle" [34]. In cultured adipocytes, ROS promotes the production of cytokine interleukin-6 (IL-6) and proinflammatory monocyte chemotactic protein-1 (MCP-1) expression [35, 36]. In the adipose tissue, this can activate macrophage infiltration and subsequently result in a proinflammatory environment [37, 38]. ROS can also stimulate signal transduction pathways (mainly via NF-κB), which activates the production of tumor necrosis factor-α (TNF-α) and IL-6 [35, 39, 40]. Further, oxidative stress can also promote cells

into cellular senescence, particularly adipocyte senescence, partly via cellular oxidation damage [41, 42]. Adipocyte senescence may recruit macrophages and elevates the production of proinflammatory cytokines [42, 43].

Excessive high caloric intake from either a high-carbohydrate or high-fat diet will cause more substrates to enter into mitochondrial respiration [44]. Subsequently, the number of electrons donated to the electron transport chain may increase [45]. Upon reaching a threshold voltage, extra electrons might back up at complex III with further donations to molecular oxygen, which produces high levels of superoxide [45].

Intriguingly, extremely high amounts of carbohydrates may lead to the reduction of insulin binding and the downregulated transcription of insulin receptor expression in the skeletal muscle [46]. High insulin and glucose levels may decrease insulin binding to the insulin receptor in adipocytes [47], negatively affecting Akt activity. The accumulation of ROS/RNS or a reduction of antioxidant capacity due to increased carbohydrate metabolism in insulin target tissues may change the phosphorylation status of these signaling pathways, subsequently resulting in deactivation. Indeed, exposure to hydrogen peroxide (H_2O_2) promotes a significant loss in distal and proximal insulin signaling and decreased glucose transport in muscles and adipocytes *in vitro* [48].

Evidence from an epidemiological study demonstrated that the consumption of refined carbohydrates, such as fructose-rich syrups, potentially leads to the epidemics of type 2 diabetes and obesity [49, 50]. Indeed, fructose-rich syrups may potentially pose a risk of diabetes and CVD [49]. Animal model studies further demonstrate that feeding normal rats fructose-rich diets may induce several endocrine and metabolic derangements, interfering with many organs and tissues [51, 52]. Because the liver is predominantly responsible for fructose metabolism and uptake, several studies are focusing on hepatic glucose metabolism [53]. Although the molecular link underlying fructose detrimental effects and carbohydrate metabolism requires further elucidation, most of the experimental studies indicate that oxidative stress could play a central role [54, 55]. In this regard, a key mode of action to explain this relationship is via fructose-induced oxidative stress which subsequently leads to impaired carbohydrate metabolism. Data from animal experiments have shown a greater likelihood of inflammation after the administration of fructose [51]. Such findings highlight the association of insulin resistance and fructose and its role in hepatic metabolism and carbohydrate metabolism against the anabolic pathway and impaired glucose tolerance [52, 55, 56]. Castro et al. [53] further demonstrated that fructose may modulate the liver glucokinase activity via the production of ROS. These data imply that numerous metabolic changes induced by fructose in the liver are more likely initiated by an increase of fructose phosphorylation by fructokinase, followed by adaptive changes that attempt to switch the substrate flow from mitochondrial metabolism to energy storage [53].

3.2. High Animal-Based Proteins. In developed countries, meat composes a significant proportion of the normal diet and consists of 15% of the daily energy intake, 40% of daily protein, and 20% of daily fat [57]. Meat is high in dietary protein and saturated fatty acids (SFAs). Fermentation of the excessive proteins in the gut produces metabolites such as ammonia (NH3) and hydrogen sulfide (H2S), which are compounds known to trigger the toxicity of the mucosa [58]. Meat can be marketed fresh or processed, the latter of which includes curing, salting, stuffing, smoking, drying, and fermentation [59]. Although meat contains high amounts of dietary protein, it can also be a source of mutagens due to the presence of N-nitroso compounds (NOC) in processed meats and heterocyclic amines (HCA) and polycyclic aromatic hydrocarbons (PAH) during high-temperature cooking and grilling [60].

Research has shown an association between the intake of well-done red meat and colorectal cancer, which could be partially explained by the formation of carcinogenic HCA and PAH. Although meat is high in SFAs, a study evaluating the mechanisms behind this finding suggests that these associations are more likely caused by something other than SFA content. However, the formation of cyto and genotoxic lipid oxidation products, such as malondialdehyde (MDA), 4-hydroxy-2- nonenal (4-HNE), and N-nitroso compounds (NOC) catalyzed by heme-Fe during digestion, is regarded as the most plausible determinant that contributes to the increased risk of colorectal cancer [61, 62]. A high intake of red meat has been demonstrated to increase NOC formation in humans, which is related to the colonic development of the NOC-specific DNA adduct O6-carboxymethylguanine (O6-C-MeG) [63].

Free Fe^{2+} markedly increases during the cooking of uncured meats [63]. Conversely, nitrite curing prevents the degradation of heme-Fe through the stabilization of the porphyrin ring [63]. Heat treatment also causes a reduction of antioxidant enzymes, such as glutathione peroxidase [64, 65], and generates oxygen from oxymyoglobin, which contributes to the production of H_2O_2 [66]. Further, free Fe^{2+} catalyzes the Fenton reaction when oxidative processes are initiated [67]. Through this reactive nature, ROS results in oxidative damage to meat proteins, which further explains the high formation of 4-HNE and MDA when uncured pork is heated [68]. Compared to cooked meat, a slightly lower concentration of simple aldehydes was observed in overcooked uncured pork. This could be explained by the evaporation of aldehydes caused by the reduction of the prooxidant effect of oxymyoglobin when heated to above 75 °C or intense heating [69]. Rather, when meats are nitrite-cured, less degradation of the heat-stable NO-heme may contribute to a reduced release of Fe^{2+} to initiate oxidation processes, which subsequently results in a reduction of lipid oxidation. Because the Fenton reaction is a chain reaction, a higher dosage of oxidation products after digestion was expected [70]. A further study reported by Van Hecke et al. [63] showed that the antioxidant effect of nitrite-curing during digestion was significantly reduced in overcooked nitrite-cured pork. Consistent with the study reported by Van Hecke et al. [63], Okayama et al. [71] also found that a prolonged cooking time or a temperature reaching 80 °C increased the decomposition of nitrite. A 1 : 1 ratio of nitric oxide ($^{\bullet}NO$) to ROS activates lipid oxidation

whereas $^{\bullet}NO > ROS$ suppresses this process [72]. Accordingly, low residual nitrite caused by intense heating is more likely to alter the $^{\bullet}NO : ROS$ ratio; thus, nitrite could change from an antioxidant to prooxidant behavior, which might explain the increased formation of oxidation products in overcooked nitrite-cured meats. In an earlier study by Ayala et al. [73], MDA was shown to be absorbed in the bloodstream and produce lipid oxidation products that could reach tissues and cause DNA damage. Low lipid oxidation product levels in colonic digests are attributed to Schiff base formation with proteins, which thus binds with bacterial DNA [74] or is oxidized by bacterial aldehyde dehydrogenase activity. Collectively, the effect of nitrite curing of meat in the colonic step was predominant since it was linked to a low level of MDA but proportionally increased 4-HNE levels and doubled heptanal amounts in the overcooked and cooked meats [63].

In addition, the nitrite-curing of beef and pork also caused a twofold difference in heptanal levels in stimulated colonic digests compared to their counterparts [70]. Lipid aldehydes, such as 4-HNE and MDA, react with protein chains leading to protein aggregation, causing the protein to be less susceptible to pepsin activity [70]. Overcooked nitrite-cured pork has low concentrations of protein carbonyl compounds and lipid oxidation products before digestion; this likely occurs because the meat proteins are initially well-digested in the stomach, after which the low levels of residual protein bind with 4-HNE and MDA, which subsequently form in a later phase of digestion [70]. The rate of protein digestibility is vitally important in association to colorectal cancer because higher levels of residual protein reaching the colon could result in the formation of potentially harmful protein fermentation products, such as p-cresol, ammonia, indole, and phenol [75]. NOC can be stimulated either enzymatically or nonenzymatically via oxidation [76, 77]. This nonenzymatic stimulation of NOC can be generated by a hydroxyl radical-generating system containing H_2O_2, Cu^{2+}, Fe^{2+}, and ascorbic acid. All these compounds are present in meat. When H_2O_2, Cu^{2+}, or Fe^{2+} was eliminated from a reaction mixture with N-nitroso-N-methylpentylamine, the mutagenicity of these mixtures was reduced [76].

3.3. Excessive Consumption of Fats. An extensive body of systematic reviews of randomized trials [78, 79] and prospective cohort studies [78, 80] has urged for a reevaluation of dietary guidelines for consumption and a reappraisal of the impact of SFAs on health. Although research has demonstrated an association between SFAs and CVD [81], not all data demonstrated such a link. De Souza et al. [82] did not identify an association of SFA intake and CVD, coronary heart disease, ischemic stroke, or type 2 diabetes. Interestingly, a study has reported that the total fat and types of fat were inversely associated with total mortality [83]. Additionally, no association was reported between the total fat and types of fat with CVD mortality and myocardial infarction [83].

Substantial evidence has suggested that SFAs can boost proinflammatory signaling. The lengths of SFA chains can produce different physiological responses [84, 85], but many mechanisms are still debated. Long-chain SFAs including palmitate and myristate acids are typically known for their harmful effects against endothelial cells, which can induce apoptosis through the induction of NF-κB in human coronary artery endothelial cells (HCAECs) [86, 87]. Harvey et al. [86] showed that long-chain SFAs can promote proinflammatory endothelial cell phenotypes through the incorporation into endothelial cell lipids. Conversely, short- and medium-chain SFAs do not incorporate or contribute to lipotoxicity. Particularly, stearic acid stimulates the upregulation of *ICAM-1* human aortic endothelial cells (HAECs) via an NF-κB dependent manner [86].

Murumalla and Gunasekaran [88] reported that SFAs (lauric acid and palmitic acid) did not stimulate Toll-like receptors 4 (TLR4) or 2 (TLR2) in HEK-Blue cells transfected with TLR2 and TLR4. Despite the inverse association between SFAs and TLR4 or TLR2, not all studies agreed. Huang et al. [84] found that palmitic acid and lauric acid activated TLR2 and TLR4 in RAW264.7 macrophages and transiently transfected human monocytic (THP-1) monocytes. Data from human studies exploring the impact of SFAs on gene expression are limited, but evidence from epidemiological studies indicates the association between SFA consumption and CVD. Nonetheless, the meta-analyses of prospective studies exploring the relationship between CVD and SFA showed a consistent poor association. From the study reviewed, metaregressions conducted in randomized trials demonstrated that polyunsaturated fatty acids (PUFAs) replacing SFAs did not lead to any changes in CVD risk [89]. Inconclusive findings suggest that SFAs are generally grouped together although medium-chain SFAs may provide beneficial health effects such as preventing obesity and the inhibition of body fat accumulation [90]. The impact of high-SFA diet on gene expression in adipose tissue was also presented by Youseef-Elabd et al. [91]. In particular, an SFA diet led to an upregulation of genes such as integrin beta 2 (*ITGB2*), cathepsin S (*CTSS*), and interleukin-8 (*IL-8*) in moderately overweight individuals, suggesting that these changes were linked to diet-induced changes rather than obesity.

A high-fat diet (HFD) was demonstrated to be a significant risk factor for health. Animals feeding a long-term HFD show increased oxidative stress and dysfunctional mitochondria in several organs [92–94]. Several research studies have also indicated that high-fat consumption causes a significant reduction in auditory function [95, 96]. This study demonstrated that long-term HFD reduced auditory function and promoted age-related hearing loss [97]. From the study reviewed, feeding rats with a HFD for a period of 12 months may increase plasma triglycerides, total cholesterol, and nonesterified fatty acid levels, causing an increase in blood oxidative stress parameters. A HFD was shown to not only aggravate the lipid profile it also further enhanced ROS accumulation and triggered mitochondrial damage in the inner ear [97], suggesting enormous detrimental impacts of a HFD on health.

Several studies have corroborated this finding and found that increased caloric intake or obesity is associated with increased mitochondrial superoxide production. Data

reported by Anderson et al. [98] have shown that feeding a HFD to both mice and humans causes a significant elevation of H_2O_2 from the mitochondria isolated from the skeletal muscle. From the study reviewed, H_2O_2 emission was used as a surrogate of superoxide emission as mitochondrial superoxide and is converted to H_2O_2 by superoxide dismutase 2 (SOD 2). Further, ROS accumulation has also been found in mitochondria isolated from adipose [99], liver [100], and kidney [101] tissue in high-fat or obese-treated animals. In another study, Valenzuela et al. [102] found that liver enzyme activity such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase was significantly reduced by a HFD diet-fed mice.

Additionally, an adipogenic diet and the accumulation of adipose tissue can trigger oxidative stress in mammalian tissues. Some studies supported the hypothesis that HFD promotes inflammation in the intestine, particularly in the small intestine. This observation may represent an early event that precedes and predisposes the individual to insulin resistance and obesity [103]. de La Serre et al. [104] reported that HFD activates myeloperoxidase activity, an inflammation marker, in the ileum of obesity-prone Sprague-Dawley rats. A study by de Wit et al. [105] further supported that HFD activates macrophage migration inhibitory factor expression in the ileum of obesity-prone C57BL/6J mice. Consistent with studies reported by de La Serre et al. [104] and de Wit et al. [105], Ding et al. [106] and Cortez et al. [107] also found that TNF-α expression was activated after 2 to 6 weeks of HFD administration and led to weight gain and an increased body fat mass. High-fat consumption also stimulates Kupffer cells (the resident macrophages of the liver) in mice and causes an elevation of the M1-polarized population, which is linked to the pathogenesis of obesity-induced fatty liver disease and insulin resistance [108]. Consequently, obesity is associated with a marked increase in oxidative damage to all cellular macromolecules [14, 109, 110].

The mechanisms underlying the elevation of oxidative stress in metabolic disorders are not fully understood, but it is hypothesized that mitochondrial dysfunction [16], augmented by NADPH oxidase activity [111], and increased fatty acid oxidation [112] contribute to these phenomena. Most of the studies so far addressed abnormal gene expression in the adipose tissues and liver, accompanied by upregulated NADPH oxidase expression and downregulated antioxidative enzyme expression [113, 114]. HFD promotes dyslipidemia, which is associated with oxidative stress, an accumulation of some transition metals and elevated free radicals [115]. Fat accumulation has also been linked to systemic oxidative stress in mice and humans via the increased accumulation of ROS, accompanied by the improved expression of NADPH oxidase and the decreased expression of antioxidative enzymes [114]. Moreover, HFD provokes lipid peroxidation and oxidative stress, whereas NADPH oxidase activation deregulates the production of redox-sensitive transcription mRNA such as NF-κB and adipocytokines (fat-derived hormones) including plasminogen activator inhibitor-1, monocyte chemotactic protein-1 (MCP-1), IL-6, adiponectin, and other inflammatory cytokines form different metabolic tissues [116].

Additionally, HFD raises the level of chylomicrons in the intestine. These chylomicrons enter circulation and cause the generation of free fatty acids (FFAs), which are taken up by the liver. These hepatic FFAs may either enter the mitochondria for β-oxidation or be esterified into triglycerides [117, 118]. Triglycerides are either accumulated in hepatocytes as small droplets or generate very low-density lipoprotein (VLDL) which is thereby converted into low-density lipoprotein (LDL) [118]. An excessive LDL burden in the blood due to its excessive accumulation or lack of LDL-receptors in hepatocytes may form oxidized-LDL (Ox-LDL), which in turn is engulfed by macrophages to become foam cells. Subsequently, foam cells accumulate in the arterial endothelium to form plaque. Ultimately, these lead to cardiovascular and circulatory disorders such as thromboembolism, hypertension, atherosclerosis, and heart block [119–121]. Subsequently, the mitochondrial β-oxidation of FFAs is linked to the conversion of oxidized cofactors (NAD^+ and FAD) into reduced cofactors NADH and $FADH_2$ and is thereby reoxidized and restored back into NAD^+ and FAD by the mitochondrial respiratory chain. During reoxidation, NADH and $FADH_2$ transfer electrons to the first complexes of the respiratory chain. Most of these electrons then migrate up to cytochrome-c oxidase and thereby combine with protons and oxygen to form water. These intermediates may interact with oxygen and produce more and more superoxide anion radicals and other ROS [122–125]. Therefore, the high consumption of fat-rich diets promotes mitochondrial β-oxidation of FFAs and subsequently leads to an excess electron flow using cytochrome-c oxidase, which elevates the accumulation of ROS. Mitochondria are a vitally important cellular source of ROS; they oxidize the unsaturated lipids of fat deposits to cause lipid peroxidation. ROS and lipid peroxidation can consume vitamins and antioxidant enzymes [125, 126]. The depletion of these protective substances may hamper ROS inactivation and promote ROS-mediated damage and lipid peroxidation [114]. This HFD-induced ROS may stimulate the proinflammatory state and thereby activate the NF-κB transcription factor. Further, HFD also may trigger ROS or NF-κB, which induces NF-κB-dependent proinflammatory agents such as TNF-α, inducible nitric oxide synthase (iNOS), and interferon-γ (IFN-γ) [101, 127, 128]. These data converge to provide evidence supporting the role of oxidative stress induced by HFD in metabolic disorders. Surprisingly, an *in vitro* study showed that free fatty acids increased ROS accumulation, indicating that increased fatty acids in obesity may provide an extra source of additional electron transport chain substrates via the oxidation of fatty acids [111, 129]. In addition to the generation of ROS, the overproduction of nitric oxide (NO) through the activation of iNOS also causes an accumulation of RNS [130, 131]. Taken together, chronic consumption of high GI foods may cause oxidative stress via the formation of free radicals that are capable in destroying biological molecules and initiate abnormal cell growth through gene mutation [132]. Further, HCA formed during high-temperature cooking and grilling of meat may cause oxidation of

proteins and lipids, thereby resulting in oxidative stress and may subsequently increase the risk of chronic diseases [133], while the HFD may serve as a stimulus to elevate the systemic inflammatory response in the development of obesity, CVD, diabetes, and cancers [134–137]. Overall, these data imply that high-carbohydrate/high-calorie/high-fat diets stimulate oxidative stress by augmenting the inflammatory response and elevating inflammatory markers.

4. Molecular Connectivity of Oxidative Stress-Induced Diseases

4.1. Obesity and Adipocyte Dysfunction. Obesity has been recognized as a heritable disorder in recent decades [138]. It has become increasingly clear that sedentary lifestyles and an increased availability of inexpensive calorie-dense foods have played a pivotal role in creating an obesogenic environment, which has contributed to the obesity epidemic [139–141]. The individual heritability of obesity susceptibility genes and interaction of the nutrients in the obesogenic environment, particularly dietary macronutrients, including refined carbohydrates and saturated fats, are linked to weight gain and may subsequently contribute to obesity [142, 143]. Thus, the functions of obesity susceptibility genes may be associated with this major health concern.

Obesity is considered a chronic low-grade inflammatory stress condition modulated by immune cells via the infiltration of adipose tissue, along with metabolic stress when oversupplied with glucose and lipids in adipocytes [144, 145]. Inflammatory cytokines have been observed in many fat cells; they are involved in fat metabolism and are associated with all indices of obesity, particularly abdominal obesity [146]. The alterations of leptin and hypothalamic pituitary adrenal (HPA) axis dysfunction, adipocyte function, and fatty acid levels and oxidative stress have been suggested to play a vitally important role in obesity-associated inflammation [146]. In general, the association between excessive nutrient uptake (sugars, lipids, and fatty acids) and metabolic disturbances is modulated by several types of cells, such as adipocytes and resident or infiltrating immune cells including monocytes, T cells, mast cells, and macrophages, which indirectly modify adipocyte function and dysfunction [147, 148]. A study by Lim et al. [149] found that dietary fatty acids activate protease-activated receptor 2 (PAR2) expression, which is a new biomarker for obesity and a substantial contributor in metabolic dysfunction and inflammation.

Studies have shown that ROS is generated from hypertrophic adipocytes induced by a HFD. The expansion of fat mass occurs via two concomitant processes in white adipose tissue expansion: hyperplasia (increased numbers of fat cells associated with the differentiation of adipocyte precursors) and hypertrophy (increased size of fat cells) [150–152]. Several studies have shown a close relationship between ROS and fat mass expansion [153, 154]. Fat accumulation parallels with ROS, as demonstrated by an increase of ROS accumulation during adipocyte 3T3-L1 alteration [155, 156]. Leptin, a white adipose tissue-derived hormone, has been reported to promote the elevation of ROS accumulation in endothelial cells [157, 158]. NF-κB can be stimulated by leptin in an oxidant-dependent manner. This finding is linked with an increased expression of monocyte chemoattractant protein-1 (MCP-1), which enhances atherosclerosis by supporting the relocation of inflammatory cells [152, 159]. Further, leptin also activates ROS in vascular smooth muscle cells via the protein kinase C-dependent activation of NAD(P)H oxidase [160]. Leptin promotes the release of active macrophage lipoprotein lipase via an oxidative stress-dependent pathway, signifying a proatherogenic effect of leptin on macrophages in diabetes [157]. By contrast, the exposition of adipocytes to high ROS levels suppresses the secretion and expression of adiponectin [161], an adipokine that shows anti-inflammatory, antiatherogenic, and insulin-sensitizing properties [162]. Collectively, systemic oxidative stress-associated HFD and obesity may lead to insulin sensitivity of metabolic organs, which thus promotes the inflammatory response [163].

Obesity has been demonstrated to drive the development of insulin resistance. However, not all obese individuals develop type 2 diabetes mellitus or insulin resistance, indicating that the biological mechanism underlying the association between obesity and insulin resistance must be well-controlled under certain circumstances [164]. Obesity has become an epidemic and represents the major risk factor for several chronic diseases, including diabetes, CVD, and cancer [165]. Therefore, the present study focused on the detrimental impact of oxidative stress on diabetes, CVD, and cancer outcomes.

4.2. Diabetes. Type 2 diabetes is the most common metabolic disorder, affecting 422 million people worldwide in 2014 [166], with nearly half of all deaths attributable to high blood glucose [166]. Type 2 diabetes is currently the most common form of the disease, representing nearly 90–95% of diabetes mellitus cases. Diabetes mellitus is a complex and progressive disease that is accompanied by several complications such as nephropathy, retinopathy, neuropathy, and micro- and macrovascular damage [167].

Oxidative stress has been identified as a major risk factor in the development of diabetes [168]. Numerous risk factors including increased age, unhealthy dietary intake, and obesity all lead to an oxidative environment that may modify insulin sensitivity either via the elevation of insulin resistance or the impairment of glucose tolerance [169]. The mechanisms that implicate these diseases are complex and involve several cell signaling pathways [170]. Hyperglycemia is linked to diabetes and subsequently contributes to its progression and an overall oxidative environment [171]. Macro- and microvascular complications contribute to the morbidity and mortality of diabetic patients, and all these factors are associated with oxidative stress [172].

The derangement of molecular and cellular processes is common in type 2 diabetes, particularly in β cells. Pathophysiologically, ROS and RNS, such as H_2O_2, superoxide anion ($O_2^{\bullet-}$), NO, peroxynitrite (ONOO–), and hydroxyl radical ($OH^{\bullet}$), all contribute to primary physiologic and metabolic processes. Mitochondrial function impairment leads to a reduction in ATP generation capacity, which in turn leads to β cell glucose-stimulated insulin secretion (GSIS), the

NADPH complex, and Ca^{2+} signaling related to neurotransmission [173, 174].

Insulin resistance plays a predominant role in the development and progression of metabolic dysfunction associated with obesity. Insulin resistance refers to the impairment of the cellular response in insulin-sensitive tissues such as skeletal muscle, adipose, liver, and brain tissues [175–177]. Subsequently, this may lead to a reduction of glucose uptake, accompanied by the elevation of hepatic glucose output, and thereby contribute to plasma glucose concentrations [178]. The subsequent changes of glucose homeostasis may place a burden on pancreatic β cells to secrete and produce more insulin to restore normal blood carbohydrate levels [179]. Nonetheless, this compensatory mechanism may ameliorate glucose levels in an early or prediabetes condition, characterized by continuous insulin resistance and high exposure of β cells to blood glucose and lipids [180]. This may boost β cell failure and dysfunction and culminate in overt diabetes [176].

Pancreatic islets are highly vascularized and specialized structures that control the nutrient contents in the bloodstream and are mainly comprised of five cell types: α cells, β cells, δ cells, ghrelin cells (γ cells), and pancreatic peptide- (PP-) secreting cells [181]. Islets generate blood from the splenic branches and pancreaticoduodenal arteries and interact to increase dietary nutrients to secrete insulin from α and β cells into glucagon and the bloodstream, respectively (during nutrient-deprived conditions such as starvation and fasting) [175]. The pancreatic β cell response to glucose depends on the acute regulation of intracellular or extracellular ROS and RNS [173, 174]. The elevation of glycolytic flux promotes ATP production and oxidative phosphorylation, which subsequently results in the formation of $O_2^{\bullet-}$ released from the electron transport chain [182]. Additionally, an initial adaptive response is modulated through the pentose phosphate pathway in which surfeit glucose is converted to pentose and glucose carbon is deviated away from excessive oxidative and glycolysis phosphorylation. However, shuttling glucose in this direction may also increase NADPH oxidase (NOX) activity and subsequently lead to increased $O_2^{\bullet-}$ synthesis. Indeed, high glucose levels may increase ROS through other possible mechanisms, such as the generation of advanced glycation end products (AGEs) and glucose autoxidation [183].

Once insulin is released into the blood circulation by β cells in response to increased blood glucose levels, insulin exhibits its anabolic effects through the transmembrane insulin receptor (IR) in target tissues. Interaction with insulin fosters the autophosphorylation of the receptor with the phosphorylation and recruitment of insulin receptor substrate (IRS) proteins and the stimulation of other related downstream signaling cascades, such as protein kinase B (Akt) and phosphatidylinositol-3-kinase (PI3K) [184]. Akt has been identified as a primary regulator in vesicle translocation of glucose transporter type 4 (GLUT-4) to the plasma membrane, which is crucial in the intracellular uptake of free glucose in insulin-sensitive tissues [48].

Numerous studies have indicated that there is an association between increased nitrosylation and carbonylation of proteins in obese- or insulin-resistant phenotypes and insulin-sensitive tissues [110, 185–187]. This suggests that an insulin-resistant phenotype may promote the reduction of insulin receptor expression. Thus, prolonged hyperinsulinaemia and chronic hyperglycemia, along with increased ROS and RNS levels, are hypothesized to influence insulin receptor gene expression through the derangement of key transcription factors such as high mobility group AT-hook 1 (HMGA-1) [188]; they may also increase insulin receptor-desensitization, which under normal circumstances is a process under the negative-feedback control [189, 190]. Taken together, the development and progression of diabetes mellitus is associated with β cell dysfunction and insulin resistance, and this phenomenon is normally related to obesity [175].

4.3. Cardiovascular Disease. Oxidative stress is implicated in the progression and development of cardiovascular disease (CVD) [191]. Its burden is attributable to lifestyle factors, particularly smoking, alcohol consumption, sedentary lifestyles, and dietary intake [192]. In Malaysia, western dietary habits that are high in fat and low in dietary fiber lead to the increase in CVD incidence [193]. Chronic and low-grade inflammation has been suggested as a major pathophysiology in obesity and its associated diseases such as CVD [194]. C-reactive protein (CRP) has been shown to be an independent risk factor for the development of CVD [195, 196]. The elevation of CRP in obesity could be attributed to macrophage infiltration into the expanded adipose tissue and subsequently leads to the production and release of macrophage-derived proinflammatory cytokines such as IL-6 and TNF-α [197, 198].

One common feature of CVD is increased oxidative stress in the heart [199]. Specifically, systemic oxidative damage in patients with CVD was due to ROS accumulation and reduced antioxidant defense [200]. A HFD increased ROS accumulation and reduced antioxidant capacity, thus causing a variety of disorders including endothelial dysfunction, which is characterized by a decreased bioavailability of vasodilators, namely, NO, and promotes endothelium-derived contractile factors causing atherosclerotic disease [201]. One potential biological mechanism linking cardiac oxidative stress has been described by Ilkun and Boudina [201] and includes mitochondrial dysfunction, increased fatty acid oxidation, and increased NADPH oxidase activity. Ilkun and Boudina [201] demonstrated that the modes of action underlying cardiac pathology are complex and might include altered calcium homeostasis, lipid accumulation, abnormal autophagy, increased fibrosis and stiffness, increased oxidative stress, and mitochondrial dysfunction. Collectively, mitochondrial and extramitochondrial sources of ROS and a reduction of antioxidant defense mechanisms have occurred in the myocardium of human and animals [201].

4.4. Cancer. Research has demonstrated that high oxidative stress leads to cancer, including colorectal cancer [202]. Oxidative stress is hypothesized to be associated with obesity and cancer. A study in an animal obese model of nonalcoholic steatohepatitis supports these hypotheses, suggesting that the absence of adiponectin promotes hepatic tumor

formation and elevates oxidative stress [203]. Indeed, ROS plays a crucial role in cancer development [204, 205]. The elevation of ROS leads to increased mutation rates or susceptibility to mutagenic agents and thus contributes to DNA damage during the early stages of carcinogenesis [205]. The elevation of ROS has also been demonstrated in tumor proliferation via the ligand-independent transactivation of receptor tyrosinekinase [204], which can promote metastasis and the invasion of cancer cells [206]. Semenza [207] observed that ROS can promote the stabilization of hypoxia-inducible factor 1, a transcription factor of vascular endothelial growth factor, which facilitates tumor angiogenesis.

Intriguingly, data from a previous study have shown that insulin is a proliferation factor for prostate cancer; thus, the reduction of carbohydrates may subsequently decrease serum insulin and slow down prostate cancer proliferation [208]. Epidemiological studies have shown that patients with type 2 diabetes and obesity have a greater likelihood of having liver, colorectal, breast, and pancreatic cancers [209, 210]. These findings suggest that leptin [203, 211], insulin/insulin-like growth factor-1 [212, 213], adiponectin [203, 211], and inflammation [214, 215] are additive between type 2 diabetes or obesity and cancers. Fat accumulation is often linked with systemic oxidative stress via elevation of ROS [114]. A previous study stated that increased oxidative stress can lead to chronic inflammation, which in turn could modulate chronic diseases such as cancer [216]. Oxidative stress can trigger a wide range of transcription factors such as Wnt/β-catenin, NF-κB, and nuclear factor E2-related factor 2 (*Nrf2*) and thereby activates inflammatory pathways [216]. Taken together, these findings suggest that increased circulating or local ROS levels derived from the expansion of the adipose tissue in a tumor environment provoke oxidative stress within tumor cells and thereby lead to an increased risk for cancer progression in patients with type 2 diabetes or obesity.

5. Diet Ameliorates Oxidative Stress-Induced Diseases

Oxidative stress is increased in diabetic patients and cancer cells [217, 218]. Higher intracellular glucose concentrations can generate ROS via several pathways [219], and the progression and development of these diseases could be prevented by changing dietary habits [220]. It was evident that high-glucose and an animal-based protein diet and excessive fat consumption can promote oxidative stress [221], for example, excessive omega-6 stimulates inflammation [222]; however, there are other dietary choices (the Mediterranean and Okinawan diets of the Greek and Japanese populations) that can reduce inflammation [223]. Figure 1 summarizes the dietary intake pattern in relation to human health.

5.1. Whole Grains. Numerous components of the diet may promote inflammation. Whole grains comprised of germ, endosperm, and bran are rich in vitamins, fibers, minerals, and phytochemicals such as carotenoids, lignans, vitamin E, inulin, β-glucan, sterols, and resistant starch [224]. As an example, the fiber found in whole grain foods appears to play a role in immune-modulating functions [225, 226]. Fiber affects microbiota in the gut [227], which affects immune function [228]. In support of this, the intake of whole grains such as sorghum benefits the gut microbiota and indices associated with oxidative stress, obesity, inflammation, hypertension, and cancer [229]. Whole grain foods are rich in phytochemicals and provide protection against oxidative stress, which can result in inflammation. Polyphenol compounds present in wheat sprouts may benefit a certain group of the population because they appear to combat oxidative stress associated with obesity [230] and enhance glucose metabolism [231].

Data from a meta-analysis have demonstrated that a high intake of whole grain products is associated with a reduction of total cancer risk [232]. In a Scandinavian HELGA cohort study, intakes of whole grains were found to be inversely associated with colorectal cancer incidence [233]. A study by Tan et al. [202] and Tan et al. [234] further supported the role of a unique complex of bioactive constituents in brewers' rice, which is a rice by-product in the rice industry that exerts significant nutritional value to combat colon carcinogenesis. Anti-inflammatory effects of brewers' rice protect against oxidative stress and free radical damage by improved antioxidant enzymes such as MDA, SOD, and NO. They also inhibit DNA damage caused by ROS via the upregulation of the *Nrf2* signaling pathway. Several studies have also reached a similar finding, in which rice by-products have an antiproliferative activity against cancer [235, 236]. Strikingly, feeding with brewers' rice not only reduced the number of aberrant crypt foci (ACF) [237]; in fact, the relative proportions of natural antioxidant compounds in brewers' rice have also been reported to attenuate liver and kidney damage in azoxymethane-induced oxidative stress in rats, as reported by Tan et al. [238], suggesting that bioactive constituents present in whole grains may ameliorate oxidative stress.

In addition to the effects observed on cancer, germinated brown rice has been extensively studied in the past few decades. Germinated brown rice has a significant nutritional value. In addition to containing high amounts of minerals, vitamins, and fiber, germinated brown rice is also rich in a variety of bioactive compounds and has drawn a great deal of interest in the prevention of CVD risk. These bioactive compounds were demonstrated to have antioxidant activities that are suggested to alleviate CVD risk via the modulation of hepatic cholesterol metabolism and oxidative stress [239, 240]. Accordingly, germinated brown rice modulates lipid metabolism via the transcriptional regulation of peroxisome proliferator-activated receptor gamma (PPARγ), hepatic lipoprotein lipase (LPL), ATP-binding cassette, subfamily A (ABCA), v-akt murine thymoma viral oncogene homologue 1 and homologue 3 (AKT1 and AKT3), and adiponectin [239]. In this regard, natural components present in whole grain such as polyphenolic compounds have the potential to suppress proinflammatory immune signaling and subsequently improve lipid metabolism and inhibit cancer development [241].

Notably, the nutritional values of fiber components such as arabinoxylans and β-glucans are also found in whole

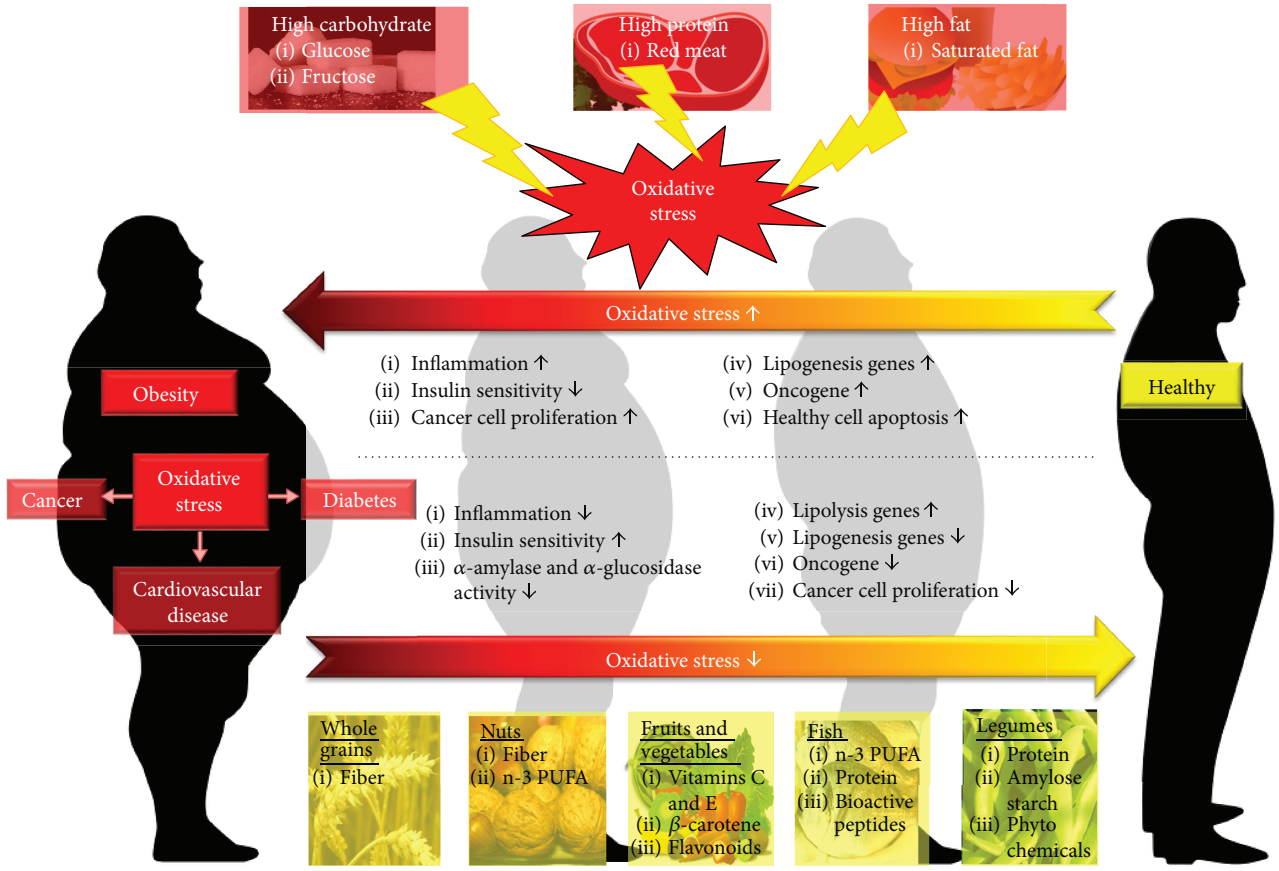

Figure 1: Dietary intake patterns affect human health state. High-carbohydrate and an animal-based protein diet and excessive fat consumption will eventually lead to obesity as well as other obesity-related diseases such as cardiovascular diseases (CVD), diabetes, and cancer. The key pathway involved in the pathogenesis is via the elevation of oxidative stress. Subsequently, inflammation occurs resulting in the reduction of insulin sensitivity, increased cancer cell proliferation, involvement of gene in lipogenesis, and cancer development of which is activated and accompanied by apoptosis of healthy cells. To revert these unhealthy conditions, consumption of healthy diet is essential. Healthy diet includes whole grains, nuts, fruits and vegetables, fish, and legumes. In general, a healthy diet contains dietary fiber, unsaturated fatty acids like monounsaturated fatty acid (MUFA) and n-3 polyunsaturated fatty acid (n-3 PUFA), protein, vitamins, minerals, and others health-promoting components. All these components exhibit antioxidant ability thereby reduce oxidative stress. The healthy diet could reduce inflammation, cancer development, and lipogenesis transcriptional expression. It also increases insulin sensitivity accompanied by the reduction of α-amylase and α-glucosidase activity. A healthy dietary pattern is crucial for maintaining good health.

grains. Studies have revealed a positive association between wheat and rye arabinoxylans and water-soluble maize on caecal fermentation, the reduction of serum cholesterol, and the production of short-chain fatty acids [242, 243]. Dietary fibers present in whole grain also play a central role to enhance immune function through the production of short-chain fatty acids, suggesting that increasing the intake of fermentable dietary fiber may be vitally important in reducing inflammation [244, 245]. Short-chain fatty acids may promote T helper cells, neutrophils, macrophages, and cytotoxic activity in natural killer cells [246]. Further, the fermentation of dietary fiber in the colon and changes in gut microbiota are associated with impaired gastrointestinal tolerance [247]. Together with the gut immune system, mucosal and colonic microflora prevent pathogenic bacteria from invading the gastrointestinal tract [248]. The intestinal flora salvages energy via the fermentation of undigested carbohydrates in the upper gut [246]. The predominant substrates are dietary carbohydrates and mucus, which escape digestion in the upper gastrointestinal tract [246]. These include nonstarch polysaccharides (such as hemicelluloses, celluloses, gums, and pectins), resistant starch, sugar alcohols, and nondigestible oligosaccharides [246]. The primary fermentation pathway produces pyruvate from hexoses in undigested carbohydrates [246]. Colonic bacteria use a wide range of carbohydrates to hydrolyze enzymes and produce methane, hydrogen, short-chain fatty acids (primarily butyrate, propionate, and acetate), carbon dioxide, and lactate [249]. In this regard, these components activate fermentation, increase bacterial and fecal mass, and ultimately lead to a stool bulking effect [246]. Overall, this suggests that the protective effect of whole grains on oxidative stress may be

mediated partly via the synergistic/additive effects of these bioactive components.

5.2. Nuts. When a landmark epidemiological study found that a high frequency of nut consumption was related to a reduction of CVD [250], nuts were brought from obscurity to prominence as a crucial health food. In the last 15 years since this first epidemiological study, scientific research on the health effects of nuts has not only focused on the area of coronary heart disease and its risk factors but has also extended to other areas of health. In addition, clinical trials have found that diets enriched with nuts reduce oxidative stress and inflammation [251] and alleviate endothelial dysfunction or insulin resistance [252]. Another clinical study consistently reported a hypocholesterolemic potential of regular nut consumption, which partly explains how walnuts reduce the risk of CVD [253].

Nuts are not only a high-fat and energy-dense food but they are also rich in bioactive constituents [254] that are believed to have anti-inflammatory and anticarcinogenic properties including folic acids and several phytochemicals [255, 256]. Notably, collective findings suggest that a protective role of nuts on colorectal and endometrial cancer prevention is possible [257–259].

A crucial underlying mechanism of action that has been proposed to explain an inverse relationship between the frequency of nut-enriched consumption and risk of obesity is unsaturated fatty acids. Healthy fats (unsaturated fatty acids) in nuts contribute to the prevention of diabetes and CVD risk. By contrast, nuts are complex food matrices that are also a source of other bioactive constituents, namely, tocopherols and phenolic compounds [260]. Compelling evidence suggests that monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) are more readily oxidized [261] and have a greater thermogenic effect [262] than do saturated fatty acids, which might contribute to less fat accumulation. Due to their unique fat and nonfat composition, nuts are more likely to mediate inflammation and oxidative stress.

Because nuts contain the abundance of unsaturated fatty acids, protein, and fiber, they are a highly satiating food [263]. Thereby, after consuming nuts, hunger is reduced and subsequent food intake is curtailed [264]. The physical structure of nuts may also lead to their satiety effect because they must be masticated, small enough for swallowing. Mastication stimulates nutrient, mechanical, and sensory signaling systems that may alter appetitive sensations [265]. Additionally, a small degree of fat absorption may occur after nut consumption because fat is found within the wall cellular structures that are not fully digested in the gut [266], which could be compounded by incomplete mastication [267]. Data from population-based studies indicate an inverse relationship between nut intake, such as almonds and CRP [268, 269]. Plasma IL-6 levels were reduced after a Mediterranean diet with nuts compared to a control diet [270, 271]. Similarly, previous studies reported by Zhao et al. [272] and Zhao et al. [273] also evaluated walnuts rich in PUFAs and, in particular, alpha-linolenic acid (ALA), in relation to proinflammatory cytokine production [273] and inflammatory markers [272] by blood mononuclear cells. The data showed that compared to the average American diet, the CRP levels were reduced by 75% in subjects consuming an ALA diet; conversely, levels in subjects consuming the linoleic acid (LA) diet decreased by 45% [272]. Indeed, reductions in multiple inflammatory markers such as IL-6, IL-1β, and TNF-α produced by cultured mononuclear cells were observed from subjects who consumed an ALA-enriched diet [273].

Based on the findings for marine-derived omega-3 PUFA, ALA would be expected to have anti-inflammatory properties. This was evaluated in a clinical study with a relatively small observed effect [274]. However, an *in vitro* study in which THP-1 cells were supplemented with LA, ALA, docosahexaenoic acid (DHA), and palmitic acid in the presence of lipopolysaccharide [275] showed a significant reduction in TNF-α, IL-1β, and IL-6 after treatment with DHA, ALA, and LA compared to palmitic acid, indicating that ALA present in walnuts elicits an anti-inflammatory response. Notably, cellular adhesion molecules are biochemical markers of endothelial dysfunction concomitantly with inflammation. In a further study focused on CVD outcomes, Zhao et al. [273] compared hypercholesterolemic subjects who consumed a diet high in ALA, a diet high in LA, and an American diet, respectively. The data showed that participants who consumed 15 g of walnut oil along with 37 g of walnuts/daily for 6 weeks demonstrated a reduction in CRP, cellular adhesion molecule soluble intercellular adhesion molecule (sICAM) 1, and E-selectin. Importantly, some research has emerged to suggest that CVD risk factors negatively affect endothelial function and are involved in the modulation of LDL cholesterol [276, 277]. In support of this, the acute consumption of walnuts oils is favorably affected and shows a better endothelial function [278]. Further, walnuts and walnut oil may influence inflammation, at least in part, via the elevation of cholesterol efflux, which is a reverse cholesterol transport that is crucial for the removal of cholesterol from peripheral tissues and indicates cardioprotective effects [253]. Taken together, nuts seem a good dietary choice for providing nutrients and preventing obesity and other chronic diseases. However, the bioactive components responsible for the effects that we stated above require further elucidation.

5.3. Fruits and Vegetables. Fruits and vegetables are rich in minerals, vitamins, and dietary fiber. High intakes of fruits and vegetables are inversely associated with mortality and the incidence of obesity-related diseases such as CVD, type 2 diabetes, and cancer [279]. Such protection has been accredited to antioxidant vitamins such as β-carotene, vitamin E, and vitamin C [280]. In general, more than 85% of the total antioxidants in fruits and vegetables are hydrophilic antioxidants [281]. Beta-carotene and vitamins E and C are vitally important for the proper regulation of physiological function [282]. The essential role of vitamin E in maintaining the oxidative-antioxidant balance is well-recognized, yet vitamin C can enhance the antioxidant protection [282]. Beta-carotene is usually found in bright-colored fruits and vegetables [283]. It has been demonstrated to maintain the immune system and exert an ability to decrease LDL-cholesterol oxidation through the modulation of antioxidant enzymes

[283]. In addition to the vitamin antioxidants stated above, other dietary components such as flavonoids may protect against oxidative stress. Flavonoids are plant polyphenolic compounds ubiquitous in fruits and vegetables. Flavonoids exert multiple biological activities such as antitumor effects, anti-inflammatory activity, antioxidant activity, and antimicrobial action, and they suppress platelet aggregation [284].

An animal study has demonstrated that a diet supplemented with β-carotene from fruit significantly downregulated the expression of fatty acid synthase, acetyl-CoA carboxylase, and fat synthesis-related genes [285]. Findings from a population-based study mirror some of those from preclinical data obtained from an *in vivo* study. Data from a population-based study reported that high intakes of fruits and vegetables significantly decreased energy consumption, waist circumference, body weight, and sagittal abdominal diameter in overweight and obese men and women [286].

Compelling epidemiological studies have revealed that intakes of fruits and vegetables induce protective cardiometabolic effects. A study showed that encapsulated fruit and vegetable-concentrated juice decreased total cholesterol, LDL-cholesterol, plasma TNF-α, and systolic blood pressure, in addition to increasing total lean mass [287]. The improvements in these indices could be attributed to the alteration of gene expression via several signaling pathways such as AMP-activated protein kinase (AMPK) and NF-κB associated genes [287]. Body composition, blood lipids, and systemic inflammation were improved in obese subjects after consuming fruits and vegetables and thus provide a useful approach for reducing the obesity-induced chronic diseases risk [288]. Further, fruits and vegetables can also prevent CVDs or assist with the restoration of function and morphology of vessels and the heart after injury. Fruits and vegetables are thought to protect against CVD by regulating lipid metabolism, protecting vascular endothelial function, suppressing platelet function, modulating blood pressure, inhibiting thrombosis, attenuating inflammation, alleviating ischemia/reperfusion injury, and reducing oxidative stress [289, 290].

In addition to the effects observed in obesity and CVD, a beneficial effect of fruit and vegetable consumption in human has also been reported on the incidence of type 2 diabetes. Data from a meta-analysis included a study from 1966 to 2014 that demonstrated that a high intake of fruit, particularly berries, and yellow, cruciferous, green leafy vegetables or their fibers, is negatively linked to type 2 diabetes [291]. As an example, lactucaxanthin (Lxn), a carotenoid in lettuce (*Lactuca sativa*), suppresses α-amylase and α-glucosidase activity both *in vitro* and in diabetic rats [292]. Such findings highlight the role of unique complexes of bioactive components in fruits and vegetables.

Fruits and vegetables not only reduce obesity, CVD, and diabetes but they also inhibit several cancers, demonstrating the numerous functional potentials of fruits and vegetables. Epidemiological studies have shown an inverse relationship between fruit and vegetable intakes and cancer risks such as colon, breast, and prostate cancers. This suppressive effect was mainly observed in cruciferous and green-yellow vegetables [293] via the modulation of genes involved in proliferation and glucose metabolism and the induction of several antioxidant genes [294]. Notably, dietary fiber in fruits and vegetables will undergo fermentation by gut microbiota, which may lead to the production of short-chain fatty acids. Short-chain fatty acids such as acetate [295], butyrate [296], and propionic acids [297] may have protective effects against cancers. It is possible that only certain types of fruits and vegetables confer protection against oxidative stress [283]. Since some bioactive compounds regulate the same gene expression and pathways targeted by drugs, diets high in fruits and vegetables in combination with medical therapies are being considered as a novel treatment strategy [298]. Overall, bioactive constituents in fruits and vegetables might be promising tools for the alleviation of a wide range of diseases [299].

5.4. Fish. Fish is an essential source of dietary protein, omega-3 fatty acids, and minerals. Nakamura et al. [300] demonstrated that individuals who consume fish daily were inversely associated with obesity compared to those with normal weight or underweight. The intake of fish has been linked to a reduced risk of obesity [301], yet the composition of fish often includes representative PUFA amounts, such as n-3 fatty acids, whose chemical structure makes them prone to peroxidation and are found abundantly in fatty fish. Therefore, our body becomes more susceptible to oxidative stress and subsequently activates the lipid peroxidation process [302]. Undoubtedly, PUFA intake is essential as they have well-established health benefits especially in preventing heart disease [303]. However, it is recommended to have an adequate vitamin E to match the increased of PUFA intake [304]. This is because lipophilic antioxidant vitamin E plays a vital role in protecting PUFA [305]. In addition, an animal study has shown that the vitamin E requirement is increased almost proportionally with the degree of unsaturation of the PUFA [304].

The consumption of lean fish has a beneficial impact on insulin sensitivity, glucose homeostasis, and lipid metabolism [306, 307]. Aadland et al. [306] further demonstrated that intakes of lean fish for 4 weeks reduced the ratio of total to HDL cholesterol in serum, decreased the VLDL concentration, and reduced fasting and postprandial triacylglycerol (TAG) compared to those with a nonseafood diet, suggesting the cardioprotective potential of lean-seafood intake. A similar dietary intake was also found to reduce the urinary excretion of metabolites involved in mitochondrial lipid and energy metabolism, possibly facilitating a higher lipid catabolism [308]. Intriguingly, lean fish contains relatively low amounts of marine n-3 fatty acids, and thereby the beneficial effects of fish are not solely ascribed to the lipid composition.

Dietary protein has been suggested as the most effective food macronutrient to provide a satiating effect. Therefore, protein-rich foods can facilitate in the modulation of food intake, promoting body weight loss and maintaining body weight thereafter. Glucagon-like peptide-1 (GLP-1) release stimulated by a high-protein meal is evoked by carbohydrate content. Indeed, cholecystokinin (CCK) and peptide YY (PYY) release is activated by a high-protein meal [309].

Fish not only contains macronutrients but also has a substantial antioxidant source due to its composition and offers a

relatively low level of saturated fat compared to other food items. Taurine, an amino acid that is abundantly found in fish, is a vital antioxidant source. Studies have shown that taurine can effectively combat metabolic syndrome by regulating glucose metabolism, reducing triglycerides to prevent obesity, regulating the renin-angiotensin-aldosterone and kallikrein-kinin systems to decrease blood pressure, and lowering cholesterol (particularly reducing VLDL + LDL cholesterol and promoting HDL cholesterol) to prevent diet-induced hypercholesterolemia [310].

Notably, the production of fish protein peptides (hydrolysates) maximizes the usage of fish protein because peptides have a health-promoting potential [311]. Techniques such as autolysis, thermal hydrolysis, and enzymatic hydrolysis have been developed to produce fish hydrolysates. The antiviral-, cardioprotective- (antihypertensive, antiatherosclerotic, and anticoagulant), analgesic-, antimicrobial-, antioxidative-, antitumor-, immunomodulatory-, neuroprotective, and appetite-suppressing activities have drawn attention from the pharmaceutical industry, which attempts to design the treatment and prevention of certain diseases [312]. Lassoued et al. [313] and Razali et al. [314] reported that peptides derived from fish proteins exhibit significant antioxidative activity in oxidative systems. The dietary intake of antioxidant compounds can strengthen the body's oxidant status and facilitate a balanced condition in terms of oxidants/antioxidants in the body.

In addition to fish and its protein peptides, neovastat (AE-941), a liquid extract derived from the cartilage of sharks, exerts antiangiogenic, anti-inflammatory, and antitumor properties both *in vitro* and *in vivo* [315]. These favorable effects are mediated via the suppression of matrix metalloproteinases (MMP)-2, MMP-9, and MMP-12 and the activation of tissue plasminogen activator enzymatic activities [315].

Another metabolic disorder is hypertension, which occurs when renin produces *angiotensin I* from *angiotensinogen*. The angiotensin I-converting enzyme (ACE) cleaves *angiotensin I* to *angiotensin II*, which is a potent vasoconstrictor [316]. Accordingly, Balti et al. [317] have sourced bioactive constituents from different types of fish in ACE-inhibitor activity studies with molecular weights of <10 kDa. From a review study, Balti et al. [317] found that bioactive peptides are suitable competitive inhibitors that can bind to the active site of ACE and thereby block its activity. Collectively, it remains unknown whether PUFA content or its antioxidant is responsible for its beneficial effects; thus, further study is necessary to conclusively resolve the question behind the anti-inflammatory effects of fish.

5.5. Legumes. Legumes are a primary component of the Mediterranean diet. They are rich in fiber and protein, which can facilitate in lowering energy density and reducing the glycemic response [318]. Legumes also contain B vitamins and minerals, such as potassium, calcium, and iron. Most of the nutritional value in legumes is contributed by their relative proportions of protein, fibers [319], and phytochemicals such as isoflavones, phytoestrogens, saponins, oligosaccharides, lectins, and phenolic compounds [320]. Due to their high nutritional values, legume intake has been demonstrated to have beneficial effects in the prevention of obesity and other related disorders [321].

Compared to those who rarely or never consume legumes, adults who consume legumes have a significantly lower body mass index and waist circumference. Children who consume legumes had smaller waist circumferences compared to those who never consume legumes [322]. Shinohara et al. [323] further demonstrated that ethanol extracts of chickpeas improved total lipid indices and gene expression associated with fatty acid metabolism in adipocytes. Studies have shown that enzymes involved in lipogenesis such as AMPK, acetyl-CoA carboxylase (ACC), and liver kinase B1 (LKB1) were inactivated by phosphorylation. Further, lipolysis was increased by the extract through the stimulation of palmitoyltransferase 1 (CPT1) and uncoupling protein 2 (UCP2), which has been reported as a crucial protein in fatty acid oxidation [323].

Starch digestibility and composition influence glycemic response. Legumes are high in amylose starch. Nonetheless, the digestion of high amylose starch is significantly lower compared to that of high amylopectin starch [324]. Yang et al. [325] reported a more sustainable plasma glucose level after a high-amylose meal compared to a high-amylopectin meal [325]. Furthermore, legumes have a high protein content; thus, the interaction of protein-starch may further hamper digestibility [326]. Moreover, high amounts of dietary fiber markedly reduced the extent and rate of legume starch digestibility. A high intake of fiber may promote satiety, enhance insulin resistance, and decrease the glycemic response [327]. Evidence from epidemiological studies shows that legume intake is negatively associated with fasting glucose levels [328].

Notably, data from large-scale epidemiological studies found that legume consumption is negatively associated with CVD mortality. Compared to the highest and lowest legume consumption, high legume consumption showed a 6% decreased risk of CVD [329]. Isoflavones are believed to have hypolipidemic activity by binding with estrogen receptors when circulating estrogen is low and thereby translocating to the nucleus, which interacts with a DNA sequence near the promoter region of target genes and results in DNA transcription [330]. Through this mechanism, isoflavone may act as a ligand for lipid-regulating proteins including PPAR, farnesoid X receptor, and liver X receptor, which facilitates cholesterol reabsorption, bile acid synthesis, and hepatic lipid synthesis [331].

In addition to the effects mentioned above, legumes have the potential to protect against cancers. For example, soy food protects against estrogen receptor-negative breast cancer [332]. A study reported by Guo et al. [332] demonstrated that in women with high soy intakes, tumor suppressor genes were upregulated (miR-29a-3p and IGF1R), and oncogenes were downregulated (KRAS and FGFR4). Consistent with the study reported by Guo et al. [332], green pea- (*Pisum sativum*) extracted lectin has also been reported to have antiproliferative activity against liver cancer cell lines [333]. Despite the limited available evidence to draw a firm conclusion, some studies suggest that legumes may be

potentially beneficial to some population segments. Collectively, future studies may elucidate the role of legumes in human health, yet their use within a balanced diet should be considered in the absence of clear contraindications.

6. Summary and Future Prospects

This review has provided clear evidence of the identification of known sources of nutritionally mediated oxidative stress as a mediating pathway for both risks of obesity and other obesity-associated diseases. Oxidative stress is a central player of metabolic ailments associated with high-carbohydrate and animal-based protein diets and excessive fat consumption. There is inconsistent research supporting the clinical use of antioxidant agents in preventing or delaying the onset and progression of metabolic disorders such as diabetic complications and cancer [334–337], and most clinical studies are limited in their sample size and duration of the study. Despite this, preclinical studies *in vitro* and animal experiments have provided in-depth insight into the modulation of these diseases. Several anti-inflammatory dietary sources such as whole grains, nuts, fruits and vegetables, and others can delay the onset of insulin resistance, prevent adipocyte and endothelial dysfunctions, and prevent tumor proliferation by reacting with oxidizing free radicals and inhibiting the inflammatory response. Therefore, more randomized clinical trials are warranted to evaluate the overall long-term effects of dietary intervention.

7. Conclusions

The available research strongly supports that a diet high in carbohydrates and animal proteins and excessive fat consumption produces ROS and subsequently leads to oxidative stress. The best dietary advice for the prevention and management of obesity and other metabolic disorders includes replacing refined carbohydrates with whole grains, increasing fruits and vegetables, substituting total and saturated fat with MUFAs, and consuming a moderate amount of calories with an ultimate goal of maintaining an ideal body weight. Overall, further studies are warranted to gain a better understanding of the types and the degree of ROS generation in relation to diet-induced metabolic disorders.

Conflicts of Interest

The authors declare that there are no conflicts of interests regarding the publication of this article.

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Research Article

Effect of N-Acetylcysteine on Antioxidant Defense, Oxidative Modification, and Salivary Gland Function in a Rat Model of Insulin Resistance

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Oxidative stress plays a crucial role in the salivary gland dysfunction in insulin resistance (IR). It is not surprising that new substances are constantly being sought that will protect against the harmful effects of IR in the oral cavity environment. The purpose of this study was to evaluate the effect of N-acetylcysteine (NAC) on oxidative stress and secretory function of salivary glands in a rat model of insulin resistance. Rats were divided into 4 groups: C—normal diet, C + NAC—normal diet + NAC, HFD—high-fat diet, and HFD + NAC. We have demonstrated that NAC elevated enzymatic (superoxide dismutase, catalase, and peroxidase) and nonenzymatic antioxidants (reduced glutathione (GSH) and total antioxidant capacity (TAS)) in the parotid glands of HFD + NAC rats, while in the submandibular glands increased only GSH and TAS levels. NAC protects against oxidative damage only in the parotid glands and increased stimulated salivary secretion; however, it does not increase the protein secretion in the both salivary glands. Summarizing, NAC supplementation prevents the decrease of stimulated saliva secretion, seen in the HFD rats affected. NAC improves the antioxidative capacity of the both glands and protects against oxidative damage to the parotid glands of IR rats.

1. Introduction

Type 2 diabetes is a metabolic disease involving a genetic predisposition that leads to progressive relative insulin deficiency and resistance of peripheral tissues to its action [1]. It was estimated that the number of people with type 2 diabetes will increase from year to year, and by 2030, it will reach 366 million worldwide [2]. This dramatic rise is largely due to excessive weight, obesity, and lack of physical activity and less due to population growth, aging, and urbanization [2]. Moreover, according to the WHO, more than 80% of diagnosed cases are type 2 diabetes and this is primarily due to insulin resistance (IR) [3]. IR exists in prediabetic state, often over many years before the onset of symptoms of type 2 diabetes. The pathogenesis of IR is not fully understood; however, it appears that oxidative stress (OS) is one of the key mechanisms [4–6]. Oxidative stress is a situation in which increased production of reactive oxygen species (ROS) leads

to cellular metabolism disorders and degradation of cellular structures, which results in oxidative organ damage and their dysfunction [7].

It has been proven that oxidative stress during high-fat diet-induced insulin resistance disrupts antioxidative systems of the parotid and submandibular salivary glands, leading to DNA, lipid, and protein oxidation and, in consequence, causing morphological changes of the glands, which is reflected in changes to the quality and quantity of saliva produced [8–10]. The data available confirms that salivary gland dysfunction manifests itself already at the IR stage and starting from this stage, diabetes can adversely affect oral health and the quality of life. It is well known that the changes in the salivary gland redox balance are responsible for salivary redox alterations [11, 12]. Considering the importance of the saliva in maintaining oral health, it is not surprising that the redox balance disturbances in the saliva dramatically increase the risk for development of oxidative stress-related oral maladies (dental caries, oral inflammatory infections like gingivitis, periodontitis, and oral mucosa ulceration, candidiasis, and burning mouth syndrome) [13–16]. These oxidative stress-related oral complications are diagnosed in almost all the patients affected by diabetes, even in well-controlled diabetes [17]. On the other hand, the presence of oral infections increases the risk and severity of diabetes. This relationship is due to the spread of the inflammatory mediators via bloodstream; moreover, the biological pathways that intensify diabetes are the same that intensify oral diseases [18, 19].

Synthetic antioxidants and nutrients with high antioxidant capacity showed the ability to prevent the oxidative damage and have been proposed as a unique therapeutic option for the treatment of the diabetes, IR, and their related complications [20–22]. N-Acetylcysteine (NAC) is an exogenous antioxidant, which works as a free radical scavenger. It is also a glutathione precursor regarded as one of the most important intracellular antioxidants. It has been proven that supplementation with N-acetylcysteine (NAC) activates antioxidant enzymes, prevents oxidative stress development, improves sensitivity towards insulin, and lowers insulin concentration in the serum of high-sugar diet-induced insulin resistant rats [20–23]. It was also shown that NAC supplementation prevents fructose-induced insulin resistance and hypertension [21] and inhibits development of diabetic peripheral neuropathies [24].

Scientific reports regarding the results of antioxidant supplementation on antioxidant defense, oxidative stress, oxidative damage, and function of the salivary glands in a rat model of insulin resistance are virtually nonexistent. Thus, the present study was undertaken to determine whether a chronic treatment of high-fat fed rats with N-acetylcysteine has a protective action against oxidative damage and prevents dysfunction of the salivary glands.

2. Materials and Methods

Male Wistar rats (cmdb outbred, 50–70 g, 4 weeks of age) after one week of adaptation were randomly assigned into four groups as follows: C—normal diet ($n = 10$), C + NAC—normal diet plus N-acetylcysteine ($n = 10$), HFD—high-fat fed ($n = 10$), and HFD + NAC—high-fat fed rats plus N-acetylcysteine ($n = 10$). For eight consecutive weeks of the experiment, the rats were fed high-fat diet (Research Diets Inc. cat. number D12492, composed of 59.8% fat, 20.1% protein, and 20.1% carbohydrates (kcal)) or normal diet (Agropol, Motycz, Poland, consisted of 10.3% fat, 24.2% protein, and 65.5% carbohydrates (kcal)). Every day, the rats from groups C + NAC and HFD + NAC were fed through an oral gavage N-acetylcysteine solution (500 mg/kg m.c; N-acetyl-L-cysteine A9165 Sigma). The dose of NAC was selected based on available literature. 500 mg/kg body weight of NAC is one of the more commonly used NAC doses that do not cause toxic symptoms in Wistar rats and have good antioxidants effects [25]. We decided on intragastric (i.g.) administration of NAC, because it is a dedicated route of administration to ensure that the animal receives a full dose of the drug in the experiment. Every morning, between the hours of 8 and 9, NAC was dissolved in saline immediately before administration and administered by gastric gavage in the amount of 2 mL/kg body weight by the one experienced person. Rats of the other groups received only intragastric saline solution in an amount of 2 mL/kg body weight.

The gastric administration of the liquid was always provided by the same 2 trained people (MM and JM). The rats were kept singly in standard cages and maintained at controlled temperatures (20-21°C), under standard condition of light from 6.00 a.m. to 6.00 p.m. and with free access to tap water.

Food consumption was measured once a week. Body weights were monitored every 2 days, and the amount of NAC was adjusted.

Rats were cared for in accordance with the principles and guidelines of the Institutional Committee for Ethics Use of Animals in the Medical University of Bialystok, Poland (protocol number 21/2017).

Eight weeks after being fed with the various diets, the rats were fasted for 12 hours and then anesthetized with phenobarbital (80 mg/kg of body weight). Rats were placed in a supine position on a heated (37°C) couch. Nonstimulated salivary secretion was measured for 15 minutes, using preweighted cotton balls inserted into the oral cavity underneath the tongue [26]. To evaluate the stimulated salivary secretory ability, rats were injected with pilocarpine hydrochloride (5 mg/kg BW, intraperitoneal, Sigma Chemical Co., St. Louis, MO, USA). Whole stimulated saliva was collected in a way analogous to the unstimulated secretion, 5 minutes after the pilocarpine administration, for 5 minutes [8]. The salivary flow was determined from the difference in the initial and final weight of the cotton balls. 1 mg of whole saliva was equal to 1 μL [27].

Following that, tail blood glucose analysis was done (Accu Chek, Roche) followed by blood sampled from the abdominal aorta. The salivary glands were rapidly dissected out, weighed (laboratory weight KERN PLI 510-3M), immediately freeze-clamped with aluminium tongs, frozen in liquid nitrogen, and stored at −86°C for subsequent analysis. Blood was placed into glass tubes with heparin and spinned (5 min, 4°C, 3000 ×g, MPW 351, MPW Med Instruments, Warsaw, Poland) to obtain the plasma. For plasma samples,

solution of BHT (10 μL 0.5 M BHT in acetonitrile per 1 mL of the plasma, BHT; Sigma-Aldrich, Germany) and protease inhibitor (Complete Mini, Roche, France) were added. Plasma samples were precooled in liquid nitrogen and stored at −86°C.

The salivary gland fragments were immediately used to estimate the reduced glutathione, and the remaining salivary gland fragments were frozen similarly to plasma. On the day of performing analysis, the remaining salivary glands were divided into pieces and diluted (1 : 10) in ice cold PBS. The salivary gland remnants were homogenized with addition of the protease inhibitor (1 tablet/10 mL of the buffer) (Complete Mini, Roche, France) and the addition of antioxidant butyl-hydroxytoluene (10 μL 0.5 M BHT in acetonitrile per 1 mL of the buffer) (BHT; Sigma-Aldrich, Germany), on ice (glass homogenizer; Omni TH, Omni International, Kennesaw, GA, USA), and after sonificated (1800 J/sample, 20 sec three times, on ice; ultrasonic cell disrupter, UP 400S, Hielscher, Teltow, Germany). The achieved salivary homogenates were centrifuged for 20 min, 4°C, 5000 ×g (MPW Med Instruments, Warsaw, Poland), and supernatants were analyzed the same day.

3. Biochemical Determinations

3.1. Enzymatic and Nonenzymatic Antioxidants. Catalase (CAT, EC 1.11.1.6) activity was analyzed in triplicate samples measuring the decrease in hydrogen peroxide (H_2O_2) consumption at 240 nm using Infinite M200 PRO Multimode Microplate Reader, Tecan [28].

Salivary peroxidase (Px, EC 1.11.1.7) activity was estimated colorimetrically measuring the absorbance changes at 412 nm in the reaction mixture containing DTNB (5,5′-dithiobis-2-nitrobenzoic acid), KI (potassium iodide), and H_2O_2 (hydrogen peroxide) [29].

Plasma glutathione peroxidase (GPx, EC 1.11.1.9) activity was measured colorimetrically at 340 nm estimating the conversion of NADPH to NADP+. One unit of GPx activity was defined as the amount of enzyme, which catalyzes the oxidation of 1 mmol NADPH per one minute [30].

Superoxide dismutase-1 (SOD-1, E.C. 1.15.1.1) activity was measured colorimetrically based on the epinephrine autoxidation at pH 10.2 in 37°C. One unit of SOD-1 activity was defined as the amount of enzyme, which inhibits oxidation of epinephrine by 50% [31].

The concentration of reduced glutathione (GSH) was analyzed colorimetrically by reaction with DTNB to give a complex that absorbs at wavelength 412 nm [32].

Total antioxidant status (TAS) was estimated in triplicate samples using a commercial kit according to the manufacturer's instructions (total antioxidant status (TAS) Randox (Crumlin, UK)). The ability of antioxidants contained in a sample to inhibit the formation of $ABTS^{\bullet+}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) radical cation was measured colorimetrically at wavelength 600 nm [33].

All assays were performed in a duplicate samples, except for the CAT and TAS determination (see above), and standardized to mg of the total protein.

3.2. Oxidative Damage Products. The concentration of advanced oxidation protein products (AOPP) was analyzed colorimetrically at 340 nm measuring the total iodide ion-oxidizing capacity of the sample [34].

The content of advanced glycation end products (AGEs) were analyzed spectrofluorimetrically measuring the specific AGE fluorescence (350/440 nm). The final results were expressed as fluorescence/mg of the total protein [34].

The concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) protein adducts was estimated using ELISA method (8-hydroxy-2′-deoxyguanosine ELISA Kit, USCN, Life Science, Wuhan, China; OxiSelect™ HNE Adduct Competitive ELISA Kit, Cell Biolabs Inc. San Diego, CA, USA, resp.). 8-OHdG and 4-HNE determination was carried out according to the manufacturer's instructions, and color changes were measured at 450 nm.

Total oxidant status (TOS) was measured in triplicate samples using commercial kit PerOx (TOS/TOC) (Immundiagnostik AG, Bensheim, Germany) based on the oxidation of Fe^{2+} to Fe^{3+} in the presence of oxidants comprised in a sample [34]. Changes in absorbance were measured colorimetrically at wavelength 450 nm.

Oxidative stress index (OSI) was calculated according to the formula OSI = TOS/TAS × 100% [35].

The total protein was determined colorimetrically using BCA (bicinchoninic acid) method with BSA (bovine serum albumin) as a standard (Thermo Scientific Pierce BCA Protein Assay Kit, Rockford, IL, USA).

All assays were performed in duplicate samples, except for the TOS determination (see above), and converted to mg of the total protein.

The insulin concentration was determined by ELISA using commercially available kits (Shibayagi Co., Gunma, Japan; Cell Biolabs) following the attached instructions. The insulin sensitivity was calculated using the HOMA index of insulin resistance (HOMA-IR, homeostasis model assessment of insulin resistance) = fasting insulin (U/mL) × fasting glucose (mM)/22.5 [36].

4. Data Analysis

Statistical analysis was done using Statistica 10.0 (Statsoft, Cracow, Poland). The Kolmogorov-Smirnov test showed no normal distribution of the obtained results, which was the reason for using nonparametric methods. The Kruskal-Wallis test was performed to analyze quantitative values between the four groups followed by nonparametric multiple comparison test to assess differences among the specific groups. The data were explained as median (minimum–maximum). The Spearman correlation coefficient was used to study correlation between nonparametric variables. The statistical significance was defined as $p \leq 0.05$.

5. Results

5.1. General Characteristics. At 8 weeks after treatment, body weights of the high-fat fed rats (HFD) were significantly higher compared to the control rats fed normal chow ($p = 0.03$). Chronic treatment with NAC prevented body

Table 1: Effect of NAC supplementation on body weight, fasting plasma glucose, insulin concentration HOMA-IR, food intake, unstimulated and stimulated saliva secretion, protein concentration, and salivary gland weight.

	C ($n = 10$) M (min–max)	C + NAC ($n = 10$) M (min–max)	HFD ($n = 10$) M (min–max)	HFD + NAC ($n = 10$) M (min–max)
Final body weight (g)	282 (260–298)	286 (261–319)	345 (326–364)**	305 (250–304)*
Glucose (mg/dL)	89 (86–93)	95 (92–97)	145 (130–161)**	95 (87–99)*
Insulin (mIU/mL)	79.70 (29.3–92.36)	80.48 (42.22–96.96)	166.42 (158.2–176.88)**	61.25 (39.55–81.18)*
HOMA-IR	3.64 (1.87–6.15)	5.67 (2.45–6.25)	19.60 (15.45–21.05)**	4.33 (2.54–5.10)*
Food intake (mg/day/rat)	17.68 (16.43–18.45)	16.92 (15.98–19.23)	11.82 (10.01–13.63)**	12.25 (10.21–13.89)**
Energy from chow (MJ/day/rat)	0.16 (0.15–0.18)	0.15 (0.14–0.19)	0.26 (0.22–0.29)**	0.27 (0.22–0.30)**
NS (μL/min)	0.43 (0.41–0.58)	0.43 (0.40–0.51)	0.40 (0.25–0.55)	0.46 (0.30–0.50)
SS (μL/min)	109.67 (104.58–121.37)	91.84 (82.20–102.8)	68.60 (56.38–70.40)**	115.25 (96.67–152.63)**
Parotid weight (mg)	76.22 (74.10–79.90)	79.52 (72.90–87.60)	99.46 (91.00–112.10)**	92.55 (81.10–112.20)**
Submandibular weight (mg)	211.64 (184.10–240.20)	208.52 (192.60–222.90)	250.04 (216.40–273.00)	264.38 (221.90–304.20)

C: control rats; C + NAC: control rats + N-acetylcysteine; HFD: high-fat fed rats; HFD + NAC: high-fat fed rats + N-acetylcysteine; HOMA-IR: homeostasis model assessment of insulin resistance; NS: nonstimulated saliva secretion; SS: stimulated saliva secretion: M (min–max): median (minimum–maximum). *$p < 0.05$ HFD and HFD + NAC; **$p < 0.05$ HFD and C; HFD + NAC and C.

weight gain, as body weights of HFD + NAC rats were significantly lower than rats fed only a high-fat diet ($p = 0.03$). HFD rats had higher glucose ($p = 0.0002$ and $p = 0.03$, resp.), insulin ($p = 0.003$ and $p = 0.0009$, resp.), plasma concentration, and HOMA-IR ($p = 0.001$ and $p = 0.001$, resp.) as compared to the control and HFD + NAC groups. HFD ($p = 0.01$) and HFD + NAC ($p = 0.01$) rats consumed significantly lesser chow than the control rats, while the energy intake was higher in HFD and HFD + NAC groups in relation to the control ($p = 0.01$ and $p = 0.01$, resp.) (Table 1).

The submandibular gland weights and the nonstimulated saliva secretions were similar among all the groups. The parotid gland weights of HFD rats were significantly higher as compared to the control rats ($p = 0.005$). NAC supplementation did not prevent the increase in weight of the parotid glands. These glands of HFD + NAC rats were significantly heavier than in the control group ($p = 0.03$). The HFD rats secreted less stimulated saliva than the rats given only normal chow ($p = 0.003$). Chronic treatment with NAC significantly increased stimulated saliva secretion in the HFD + NAC group in relation to the HFD group ($p = 0.002$) to a similar level as in the control rats given normal chow (Table 1).

5.2. The Plasma Oxidative Stress. The plasma total protein, 8-OHdG concentration, and GPx activity were comparable in all four groups (Table 2).

The HFD rats showed lower plasma SOD ($p = 0.005$) and CAT ($p = 0.03$) activities as well as TAS and GSH concentration ($p = 0.006$ and $p = 0.03$, resp.) as compared to the control rats fed normal chow. Chronic treatment with NAC significantly increased plasma SOD activity ($p = 0.01$) and GSH concentration ($p = 0.008$) as compared to HFD rats, to a similar level observed in the control rats, while significantly decreased plasma CAT activity ($p = 0.01$) in relation to the control rats. Chronic treatment with NAC significantly decreased plasma SOD activity and TAS concentration in the C + NAC group as compared to the control group (C), with $p = 0.01$ and $p = 0.03$, respectively (Table 2).

TOS ($p = 0.02$), AOPP ($p = 0.008$), 4-HNE protein adduct ($p = 0.03$) concentration, and OSI ($p = 0.002$) were increased in the plasma of the HFD group in relation to the control rats given normal chow (Table 2). NAC supplementation significantly decreased 4-HNE protein adduct ($p = 0.01$) and OSI ($p = 0.03$) in plasma of the HFD + NAC group as compared to the HFD rats, to a level observed in the control group (Table 2). Moreover, NAC prevented increase of the AOPP and TOS concentrations in the plasma of HFD + NAC rats, reaching values similar to the untreated control rats.

5.3. Parotid Glands. Effect of NAC supplementation on parotid glands SOD, CAT, and Px activities and GSH, TAS, TOS, OSI, 8-OHdG, AOPP, 4-HNE protein adduct, AGE, and total protein concentrations is given in Table 3.

There were no differences in AGE concentration in the parotid glands among the groups. HFD rats had lower parotid glands Px and SOD activities ($p = 0.04$ and $p = 0.03$, resp.) as well as GSH ($p = 0.03$) and TAS ($p = 0.003$) concentrations in relation to the control group fed normal chow. NAC supplementation significantly increased SOD, CAT, and Px activities ($p = 0.04$, $p = 0.02$, and $p = 0.01$, resp.) and GSH ($p = 0.08$) and TAS ($p = 0.005$) concentrations in the parotid glands of the HFD + NAC group as compared to the HFD group, to a similar level as in the control rats given normal chow. Moreover, NAC increased CAT activity in the parotid glands of the HFD + NAC groups in relation to the control ($p = 0.02$).

The HFD group showed higher TOS ($p = 0.01$), 8-OHdG ($p = 0.08$), AOPP ($p = 0.02$), 4-HNE protein adduct ($p = 0.002$) concentrations, and OSI ($p = 0.003$) in relation to the control group fed normal chow. NAC supplementation significantly decreased TOS ($p = 0.01$), 8-OHdG ($p = 0.02$), AOPP ($p = 0.04$), 4-HNE protein adduct ($p = 0.0009$), and OSI ($p = 0.03$) in the parotid glands of the HFD + NAC group as compared to the HFD group, to a similar level as in the untreated control rats.

Table 2: Effect of NAC supplementation on plasma SOD, CAT, and GPx activities and GSH, TAS, TOS, OSI, 8-OHdG, AOPP, 4-HNE protein adduct, AGE, and total protein concentrations.

Plasma	C ($n = 10$) M (min–max)	C + NAC ($n = 10$) M (min–max)	HFD ($n = 10$) M (min–max)	HFD + NAC ($n = 10$) M (min–max)
SOD (mU/mg of protein)	0.76 (0.65–0.88)	0.32 (0.20–0.55)**	0.27 (0.22–0.33)**	0.59 (0.48–0.73)*
CAT (nmol H_2O_2/min/mg of protein)	1.53 (1.00–1.78)	0.78 (0.47–1.28)	0.73 (0.61–0.96)**	0.66 (0.43–0.77)**
GPx (μU/mg of protein)	32.85 (17.65–39.99)	27.39 (18.25–36.32)	34.45 (24.97–50.43)	46.88 (33.14–53.10)
GSH (ng/mg of protein)	0.72 (0.55–0.92)	0.67 (0.46–0.94)	0.28 (0.18–0.47)**	0.91 (0.84–1.05)*
TAS (μmol/mg of protein)	0.62 (0.56–0.65)	0.42 (0.35–0.53)**	0.38 (0.34–0.41)**	0.48 (0.38–0.58)
TOS (μmol/mg of protein)	1.45 (0.77–1.86)	2.64 (1.04–4.16)	3.52 (2.95–4.24)**	2.08 (0.92–3.32)
OSI	227.85 (122.68–296.46)	604.77 (286.40–901.52)	927.7 (781.51–1211.9)**	446.87 (177.89–726.32)*
8-OHdG (pg/mg of protein)	0.02 (0.01–0.04)	0.19 (0.01–0.44)	0.12 (0.02–0.21)	0.18 (0.02–0.34)
AOPP (nmol/mg of protein)	1.55 (1.13–1.81)	2.67 (1.59–5.36)	3.81 (2.85–4.61)**	2.24 (1.54–2.57)
4-HNE protein adduct (μg/mg of protein)	30.42 (14.33–50.44)	36.29 (24.47–53.98)	70.47 (66.52–78.64)**	19.24 (2.46–42.48)*
AGE (fluorescence/mg of protein)	0.89 (0.76–0.98)	1.37 (0.86–2.61)	1.41 (1.04–1.94)	1.16 (0.73–1.15)
Protein (μg/mL)	4551 (4103–4964)	4423.9 (3588.6–5027.4)	4271.0 (3471.3–4848.8)	3886.2 (3432.0–5027.4)

C: control rats; C + NAC: control rats + N-acetylcysteine; HFD: high-fat fed rats; HFD + NAC: high-fat fed rats + N-acetylcysteine; M: median: min: minimum; max: maximum; SOD: superoxide dismutase; CAT: catalase, GPx: glutathione peroxidase; GSH: reduced glutathione; TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative status index; 8-OHdG: 8-hydroxy-d-guanosine; AOPP: advanced oxidation protein products; 4-HNE protein adduct: 4 hydroxynonenal protein adduct; AGE: advanced glycation end products. *$p < 0.05$ HFD and HFD + NAC; **$p < 0.05$ C + NAC and C; HFD and C; HFD + NAC and C.

Table 3: Effect of NAC supplementation on parotid glands SOD, CAT, and Px activities and GSH, TAS, TOS, OSI, 8-OHdG, AOPP, 4-HNE protein adduct, AGE, and total protein concentrations.

Parotid glands	C ($n = 10$) M (min–max)	C + NAC ($n = 10$) M (min–max)	HFD ($n = 10$) M (min–max)	HFD + NAC ($n = 10$) M (min–max)
SOD (mU/mg of protein)	0.62 (0.44–1.17)	0.37 (0.33–0.42)	0.29 (0.18–0.31)**	0.57 (0.36–0.68)*
CAT (nmol H_2O_2/min/mg of protein)	0.81 (0.59–0.97)	0.84 (0.53–1.13)	0.76 (0.09–1.09)	1.26 (1.2–1.74)*,**
Px (μU/mg of protein)	55.7 (51.4–59.5)	34.5 (25.5–60.6)	37.4 (33.5–39.6)**	52.9 (46.7–55.9)*
GSH (ng/mg of protein)	0.94 (0.95–0.99)	1.12 (0.70–1.55)	0.73 (0.52–0.88)**	1.18 (0.86–1.73)*
TAS (μmol/mg of protein)	0.67 (0.65–0.99)	0.65 (0.53–0.82)	0.32 (0.27–0.49)**	0.92 (0.66–1.13)*
TOS (μmol/mg of protein)	1.30 (0.76–1.78)	0.69 (0.36–0.98)	2.24 (1.98–2.54)**	1.25 (0.47–1.67)*
OSI	192.9 (116.0–258.0)	114.3 (44.0–172.0)	536.9 (403.6–620.6)**	165.8 (68.0–366.3)*
8-OHdG (pg/mg of protein)	1.47 (1.31–1.58)	1.19 (0.89–1.39)	1.91 (1.80–1.99)**	1.53 (1.16–1.57)*
AOPP (nmol/mg of protein)	10.0 (6.2–12.4)	9.6 (1.7–12.8)	23.4 (18.0–34.1)**	11.64 (7.90–14.6)*
4-HNE protein adduct (μg/mg of protein)	10.70 (2.16–18.9)	5.70 (1.59–10.54)	50.7 (30.4–57.3)**	1.78 (0.34–2.91)*
AGE (fluorescence/mg of protein)	0.41 (0.36–0.46)	0.38 (0.28–0.53)	0.41 (0.35–0.52)	0.48 (0.44–0.52)
Protein (μg/mL)	4419 (4054–4889)	3654 (3348–4031)	3324 (3000–3828)**	3292.52 (2930.30–3457.30)**

C: control rats; C + NAC: control rats + N-acetylcysteine; HFD: high-fat fed rats; HFD + NAC: high-fat fed rats + N-acetylcysteine; M: median; min: minimum; max: maximum; SOD: superoxide dismutase; CAT: catalase; Px: peroxidase; GSH: reduced glutathione; TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative status index; 8-OHdG: 8-hydroxy-d-guanosine; AOPP: advanced oxidation protein products; 4-HNE protein adduct: 4 hydroxynonenal protein adduct; AGE: advanced glycation end products. *$p < 0.05$ HFD and HFD + NAC; **$p < 0.05$ C + NAC and C; HFD and C; HFD + NAC and C.

Total protein in the parotid gland of HFD rats was decreased in relation to the control rats ($p = 0.007$). NAC supplementation did not prevent decrease of the protein content in the HFD group. Rats from the HFD + NAC had reduced protein concentration in the parotid gland as compared to the control rats (C) ($p = 0.009$).

Table 4: Effect of NAC supplementation on submandibular glands SOD, CAT, and Px activities and GSH, TAS, TOS, OSI, 8-OHdG, AOPP, 4-HNE protein adduct, AGE, and total protein concentrations.

Submandibular glands	C ($n = 10$) M (min–max)	C + NAC ($n = 10$) M (min–max)	HFD ($n = 10$) M (min–max)	HFD + NAC ($n = 10$) M (min–max)
SOD (mU/mg of protein)	0.33 (0.12–0.48)	0.36 (0.33–0.41)	0.33 (0.23–0.39)	0.37 (0.24–0.49)
CAT (nmol H_2O_2/min/mg of protein)	1.18 (0.98–1.36)	1.02 (0.76–1.27)	1.1 (0.8–1.4)	1.34 (1.11–1.65)
Px (μU/mg of protein)	48.36 (44.53–53.91)	38.8 (28.1–56.1)	32.76 (22.11–40.04)**	31.78 (29.24–35.30)**
GSH (ng/mg of protein)	1.09 (0.95–1.18)	1.31 (1.05–1.64)	0.56 (0.16–0.76)**	0.95 (0.83–1.21)*
TAS (μmol/mg of protein)	0.56 (0.43–0.73)	0.63 (0.44–0.81)	0.35 (0.22–0.46)**	0.61 (0.55–0.78)*
TOS (μmol/mg of protein)	0.61 (0.37–0.79)	0.58 (0.34–0.87)	1.6 (1.4–1.9)**	1.24 (1.05–1.35)*,**
OSI	118.47 (50.47–169.84)	89.92 (58.09–114.92)	471.10 (326.46–621.83)**	187.2 (179.4–280.7)*,**
8-OHdG (pg/mg of protein)	1.23 (1.02–1.41)	1.29 (1.11–1.47)	1.31 (1.01–1.87)	1.42 (1.36–1.49)
AOPP (nmol/mg of protein)	9.35 (7.90–11.01)	9.81 (9.32–12.57)	18.85 (17.69–23.31)**	15.47 (12.74–16.97)*,**
4-HNE protein adduct (μg/mg of protein)	7.29 (3.74–10.61)	32.82 (9.99–46.88)**	37.88 (27.41–45.84)**	21.27 (13.24–26.02)*,**
AGE (fluorescence/mg of protein)	0.26 (0.21–0.37)	0.5 (0.45–0.52)**	0.41 (0.39–0.45)**	0.32 (0.20–0.55)
Protein (μg/mL)	3804 (3567–3933)	3548 (3413–3673)	3210 (3080–3387)**	3282 (3160–3423)**

C: control rats; C + NAC: control rats + N-acetylcysteine; HFD: high-fat fed rats; HFD + NAC: high-fat fed rats + N-acetylcysteine; M: median; min: minimum; max: maximum; SOD: superoxide dismutase; CAT: catalase; Px: peroxidase; GSH: reduced glutathione; TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative status index; 8-OHdG: 8-hydroxy-d-guanosine; AOPP: advanced oxidation protein products; 4-HNE protein adduct: 4 hydroxynonenal protein adduct; AGE: advanced glycation end products. *$p < 0.05$ HFD and HFD + NAC; **$p < 0.05$ C + NAC and C; HFD and C; HFD + NAC and C.

5.4. Submandibular Gland. The effect of NAC supplementation on submandibular glands SOD, CAT, and Px activities and GSH, TAS, TOS, OSI, 8-OHdG, AOPP, 4-HNE protein adduct, AGE, and total protein concentrations is presented in Table 4.

The CAT and SOD activities and 8-OHdG concentration were similar between all groups. The HFD groups showed lower submandibular gland Px activity ($p = 0.03$), GSH ($p = 0.04$), and TAS ($p = 0.03$) concentrations as compared to the control. NAC supplementation increased the concentrations of GSH ($p = 0.03$) and TAS ($p = 0.04$) in the submandibular glands of the HFD + NAC group in comparison to the HFD rats; however, it did not prevent the decrease of Px activity. The specific activity of Px in the submandibular glands of HFD + NAC rats was significantly lower as compared to the control rats fed normal chow ($p = 0.03$).

The HFD group showed significantly higher submandibular gland: TOS ($p = 0.005$), AOPP ($p = 0.004$), 4-HNE protein adduct ($p = 0.01$), and AGE ($p = 0.04$) concentrations as well as OSI ($p = 0.03$) in relation to the rats fed normal chow (C). TOS ($p = 0.09$), 4-HNE protein adduct ($p = 0.03$), and AOPP ($p = 0.02$) concentration as well as OSI ($p = 0.01$) in the submandibular glands of HFD + NAC rats were significantly higher in relation to the control group given normal chow, and at the same time, significantly lower compared to the HFD rats ($p = 0.04$, $p = 0.03$, $p = 0.03$, and $p = 0.04$, resp.). NAC supplementation significantly increased 4-HNE protein adduct and AGE concentration in the submandibular glands of the C + NAC group ($p = 0.003$ and $p = 0.02$, resp.) in relations to the control.

Total protein in the submandibular gland of HFD rats was decreased in relation to the control rats ($p = 0.02$). NAC supplementation did not prevent decrease of the protein content in the HFD group. Rats from the HFD + NAC had reduced protein concentration in the submandibular gland as compared to the control rats (C) ($p = 0.009$).

5.5. Correlations. Positive correlation was shown between TAS and GSH concentrations in the submandibular gland ($p = 0.03$ and $r = 0.52$) as well as between 4-HNE protein adduct concentration in submandibular glands and plasma insulin ($p = 0.02$, $r = 0.63$) of HFD + NAC rats.

A positive correlation between SOD activity in the parotid glands and stimulated salivary flow ($p = 0.01$, $r = 0.56$) as well as negative relationship between SOD activity and plasma glucose concentration ($p = 0.02$, $r = -0.47$) of HFD + NAC rats were also noted.

6. Discussion

Saliva is secreted by the salivary glands, and it forms the liquid environment of the ecosystem of the mouth. Saliva determines homeostasis of the oral cavity due to the presence of organic and inorganic content, lubricating and buffering qualities, and specific and nonspecific defense mechanisms. In addition to the abovementioned host-protective properties, saliva is the first line of defense of the gastrointestinal tract against free radicals and their reactions with cellular components. The role of OS in the pathogenesis and development of salivary gland pathology in insulin resistance has been well established [8–11].

In the present study, we evaluated the antioxidant system and the parameters of oxidative stress in the salivary glands of high-fat fed rats supplemented with N-acetylcysteine

(NAC). Basically, in the presence of NAC, the antioxidant barrier was restored and the oxidative stress was decreased in both parotid and submandibular glands.

A chronic high-fat diet was found to effectively induce obesity, hyperglycaemia, hyperinsulinemia, general oxidative stress, and insulin resistance [36–38]. It is not surprising, therefore, that the applied model of an 8-week high-fat diet resulted in a shift of the oxidative/antioxidant plasma balance towards the oxidative status as well as a significant increase in the body weight of rats and decreased whole body insulin sensitivity assessed by a significantly higher medians of insulin and glucose in the blood and the HOMA-IR index as compared to rats fed a standard diet [36–38].

Our results are also consistent with the facts that NAC affects glucose metabolism and has a potential effect as a therapeutic agent against the onset of insulin resistance, oxidative stress, and its complications [20–22]. During the eight weeks of the experiment, NAC supplementation prevents the onset of plasma OS (Table 2), reduced hyperglycaemia and hyperinsulinemia as well as HOMA-IR compared to the rats given a high-fat diet only, to the level observed in the control group given normal chow (Table 1). Despite similar caloric intake, HFD + NAC rats showed lower final body weight compared to HFD rats, and this was in agreement with Diniz et al. [39]. The reason for reduced body weight of HFD + NAC rats in relation to the HFD group was not assessed in our study, but previous study suggested that reduced body weight was caused by a NAC-induced decrease in intestinal absorption of the ingested chow [39].

The major body site for producing superoxide anion is the mitochondria, which is an organelle-processing chemical energy contained in the energy substrates (glucose and fatty acids) into the energy of anhydride pyrophosphate bonds of the ATP (adenosine triphosphate). Under a balanced diet, electrons are passed through a mitochondrial electron transport chain in order to reduce molecular oxygen to water. However, 1–3% of all electrons "escape" from this chain resulting in the formation of small quantities of superoxide anions, which are easily converted into hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD). SOD works with other enzymes (peroxidase (Px), glutathione peroxidase (GPx), and catalase (CAT)) and nonenzymatic antioxidants (reduced glutathione (GSH)) that remove H_2O_2 and other reactive oxygen and nitrogen species (RNS), which allows the redox balance or the balance between ROS/RNS rates of production and their rates of elimination to be maintained [40]. In the case of a high-fat diet, the excess fatty acid supply in the respiratory chain increases dramatically, which results in the formation of excessive quantities of superoxide anions and subsequently other ROS and RNS [41]. The unbalanced reactive oxygen and nitrogen species during deficiency of antioxidant mechanisms can lead to development of oxidative stress and oxidative damage of cellular structures and dysfunction of cellular metabolism and its regulation [42]. Increased ROS can damage all cellular components. Therefore, to assess the presence, degree, and prognosis of OS, there are several markers of oxidative damage. The most common assessments to evaluate oxidative damage are 4-HNE protein adduct (for the lipids), advanced oxidation protein product (AOPP) (for the proteins), and 8-hydroxy-d-guanine-8-OHdG (for the DNA) [7].

Our results confirmed previous data showing that high-fat diet-induced insulin resistance increased the parotid and submandibular gland oxidative damage and decreased the antioxidant barrier of both types of glands as compared to the control. The parotid glands were more susceptible than the submandibular glands to oxidant attack generated in the course of high-fat diet-induced IR which we observed as a greater intensity and variety of oxidative damage, as already documented [8–11]. High-fat feeding-induced insulin resistance impaired stimulated salivary flow and affected the function of both glands in the course of protein synthesis/secretion, which was assessed as a significant decrease in the total protein concentration in both glands of the HFD rats in relation to the control and was in agreement with Kołodziej et al. [8].

The effect of NAC supplementation on the salivary glands of rats fed a high-fat diet is novel. Furthermore, this is the first report of an administration of any antioxidant agent aiming at protecting the salivary glands against OS in the course of high-fat feeding.

Administration of NAC has been reported to be beneficial in IR, diabetes, and their complications [20–22]. NAC is regarded as an antioxidant that acts either directly or by increasing intracellular GSH level [43]. Our results showed that NAC supplementation normalizes the antioxidant barrier in both the salivary glands of the high-fat fed rats (HFD + NAC rats), in comparison to the HFD group of rats. This is indicated by a significant TAS increase. TAS is one of the main parameters describing the antioxidant barrier; TAS is the sum of all antioxidants both in the parotid and in the submandibular glands. However, it is worth noticing that we observed a higher percentage TAS increase in the parotid (↑ 37%) than in the submandibular glands (↑ 8%) in the HFD + NAC versus HFD groups. In the submandibular glands of HFD + NAC rats, we observed only a significant increase of glutathione. Moreover, a positive correlation between TAS and GSH suggests that NAC normalizes the submandibular antioxidant barrier mainly by an increase of glutathione concentration. It should be, however, underlined that in the salivary antioxidant system, Px is by far the most important antioxidant, the only one synthesized in the salivary glands. Other salivary enzymes like SOD, CAT, and GPx or nonenzymatic antioxidants come mainly from the blood vessels and have only marginal antioxidant significance [44]. Persistent drop in Px activity in the submandibular salivary gland, despite NAC supplementation, is a proof that the antioxidant barrier is deficient in combating ROS. On the contrary, in the parotid glands of the HFD + NAC group, the increase of total antioxidant barrier was achieved not only by essential increase of GSH but also by essential increases of SOD, Px, and CAT activities, which indicates an activation of the enzymatic antioxidant barrier. The increase of the abovementioned antioxidant enzymes may be related to previously cited NAC-suppressed NF-κB (nuclear factor kappa B) activation and upregulation of the gene expression of these enzymes [45, 46]. High-fat

feeding has been shown to be associated with reduced mRNA expression of SOD1, CAT, and GPx in adipose tissue [47]. On the other hand, the negative correlation between SOD activity and blood glucose concentration in the parotid gland of HFD + NAC rats suggests that hyperglycaemia could be a significant factor in the downregulation of enzymatic antioxidant capacity, which was observed earlier [48].

The difference in parotid and submandibular antioxidant defense mechanisms is not understood. It may come from the fact that the parotid glands are the main source of antioxidants while the mandibular glands' share of the total salivary antioxidant capacity is insignificant [10, 44]. The parotid glands are also capable of more antioxidant response to counterbalance ROS, and presumably, the addition of exogenous antioxidant increases their antioxidant potential and allows the parotid glands to counteract the excess of ROS effectively. Essential reduction of the concentration of TOS (↓ 45%), 8-OHdG (↓ 20%), AOPP (↓ 49.7%), 4-HNE-protein adduct (↓ 97%), and OSI (↓ 70%) was observed in the parotid gland of the HFD + NAC group versus HFD rats. Furthermore, concentrations of the abovementioned parameters of oxidative stress in the parotid glands of the HFD + NAC group did not differ from concentrations in the parotid glands of the rats fed a standard diet, which proves that the 8-week NAC treatment prevents oxidative damage and allows maintaining the redox balance in these glands. As the salivary antioxidant capacity is determined mainly by the antioxidant efficiency of parotid glands, the results obtained are of great importance. It points to the fact that the oral cavity will be sufficiently protected against high-fat fed-induced enhanced ROS generation.

In the case of the submandibular gland, we observed a significantly higher OSI index (the oxidative stress index which evaluates relationship between the total oxidant status (TOS) and total antioxidant status (TAS)) and significantly higher TOS versus control group. NAC supplementation could not prevent oxidative damage in these glands, indicated by increased concentration of AOPP and 4-HNE protein adduct versus control glands. It should be stressed that our study showed some degree of a protective effect of NAC in decreasing oxidative stress in the submandibular glands of rats given high-fat chow. The NAC protective action was seen in significantly lower concentrations of TOS (↓ 23%), OSI (↓ 61%), AOPP (↓ 18%), and 4-HNE protein adduct (↓ 44%) in the submandibular glands of the HFD + NAC versus HFD rats. Moreover, NAC prevented an increase of AGE concentration in the submandibular glands of the HFD + NAC rats. AGE concentration in the submandibular glands of the HFD + NAC was similar to the control rats.

Since NAC can act as a prooxidants, it has been suggested to avoid its prophylactic administration in situations that are not accompanied by oxidative stress [49, 50], which is in accordance with our results. A significant increase in 4-HNE protein adduct and AGE concentration in the submandibular gland and a significant decrease in SOD activity and TAS concentration in plasma of C + NAC rats versus control confirm that in the healthy control, NAC raises the prooxidant milieu rather than prevents oxidative stress.

No correlation between antioxidant/oxidant products in the salivary glands and plasma was observed in any of the groups. This indicates that the redox balance in the salivary glands is independent from blood plasma and that the antioxidant mechanisms of the salivary glands and the intensity of oxidative stress are a result of actions within the salivary glands, not diffusion from the blood vessels. However, we noticed a positive correlation between insulin concentration and concentration of 4-HNE protein adduct in submandibular glands of the HFD + NAC group. This result is in agreement with the data that the hyperinsulinemia leads to insufficient scavenging of hydrogen peroxide. Hydrogen peroxide undergoes Fenton reaction to generate ·OH which is implicated in the onset and propagation of lipid peroxidation [51].

It should be emphasized that in humans, the parotid gland produces mainly stimulated saliva, while submandibular/sublingual salivary glands secrete without stimulation. Any quantitative or qualitative changes in stimulated saliva reflect solely parotid gland dysfunction. We noted that chronic treatment with NAC prevented dysfunction of parotid acinar machinery involved in stimulated saliva secretion, seen in the HFD rats affected. It should be underlined that high-fat feeding did not induce impairment in the non-stimulated saliva secretion, so it is not surprising that any protective effect of NAC was not observed. It is considered that disrupted secretion of stimulated saliva in patients with IR or diabetes is a result of neurotransmission disruption and has a multifactorial etiology that involves remodeling of the extracellular matrix, inflammation, degradation of acinar cells, and oxidative stress. NAC has an antioxidant, but also the anti-inflammatory and antiapoptotic properties. Moreover, NAC positively affects neurotransmission and inhibits the onset of diabetic peripheral neuropathies [24]. All these NAC properties presumably could facilitate function and communication of residual of the neural and acinar cells, which prevent reduced ability of the parotid glands to respond to external stimuli.

On the other hand, there is a positive correlation between SOD activity in the parotid glands of the HFD + NAC rats and stimulated saliva secretion rate, which suggests another mechanism preventing reduction of salivary secretion. It was shown that SOD activates muscarinic receptor M1 inducing extracellular signaling-regulated protein kinase and modulating synaptic transmission in neuroblastoma SK-N-BE cells [52]. Receptor M1 activation in the salivary glands stimulates water flow into glandular cells and increases secretion of stimulated saliva.

In contrast to saliva secretion, NAC did not prevent disruption to the protein synthesis mechanism in parotid and submandibular glands, which is observed as a significant decrease in the total protein content in both salivary glands of the HFD + NAC rats versus control. It therefore appears that factors other than OS, inflammation, neuropathies, or other NAC-related mechanisms influenced the reduction in the total protein content in both glands. It was documented that a reduced protein concentration in the salivary glands may be due to reduced food intake. Evidence showed that decreased food intake causes alterations in protein synthesis

or their exocytosis, which was indicated as a higher storage of granules/vehicles with secretory proteins and decreased protein concentrations in salivary glands [53]. On the other hand, the NAC dose used by us, however useful for the redox balance and physiological saliva secretion, may not be sufficient to prevent protein synthesis disruption in the salivary glands of HFD-fed rats.

Considering the protective effect of NAC against the salivary dysfunction induced by high-fat feeding, one should keep in mind that this effect could be secondary to the systemic effect of NAC (preventing of insulin resistance). Only kinetic studies could address this problem. However, Murley et al. [54] showed that thiol-containing drugs, which have some indications of NAC molecular mechanisms, by NF-κB pathway influenced the expression of antioxidant enzymes in SA-NH tumor cells.

7. Conclusions

(1) In summary, our results showed that chronic treatment of high-fat fed rats with NAC prevents the decrease of stimulated saliva secretion, seen in the HFD rats affected. Reduced protein concentration can be observed in parotid and submandibular glands of high-fat fed rats despite the chronic NAC treatment.

(2) Supplementation of NAC was effective in increasing the antioxidant barrier of both glands.

(3) NAC supplementation offered a protection to parotid glands against oxidative stress, while the severity and extent of oxidative damage to the submandibular glands of HFD + NAC rats were only reduced by the NAC treatment.

Conflicts of Interest

Authors declare no conflict of interest.

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Review Article

Supplementation of Micronutrient Selenium in Metabolic Diseases: Its Role as an Antioxidant

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Selenium is an essential mineral naturally found in soil, water, and some of the food. As an antioxidant, it is one of the necessary trace elements in human body and has been suggested as a dietary supplement for health benefit. Although the human body only needs a trace amount of selenium every day, plenty of recent studies have revealed that selenium is indispensable for maintaining normal functions of metabolism. In this study, we reviewed the antioxidant role of nutritional supplementation of selenium in the management of major chronic metabolic disorders, including hyperlipidaemia, hyperglycaemia, and hyperphenylalaninemia. Clinical significance of selenium deficiency in chronic metabolic diseases was elaborated, while clinical and experimental observations of dietary supplementation of selenium in treating chronic metabolic diseases, such as diabetes, arteriosclerosis, and phenylketonuria, were summarized. Toxicity and recommended dose of selenium were discussed. The mechanism of action was also proposed via inspecting the interaction of molecular networks and predicting target protein such as xanthine dehydrogenase in various diseases. Future direction in studying the role of selenium in metabolic disorders was also highlighted. In conclusion, highlighting the beneficial role of selenium in this review would advance our knowledge of the dietary management of chronic metabolic diseases.

1. Introduction

With its name derived from the Greek word "Selene," selenium has caught attention as a micronutrient since 1817, when it was first described as a by-product from sulphuric acid production. Although selenium is an essential element which is naturally occurring in the body, its endogenous level fluctuates across populations in different geographical areas, as well as different age groups in the same area, indicating that both environmental and internal factors may affect the selenium level [1, 2]. Both organic and inorganic forms of selenium can be absorbed by the small intestine and in turn can be widely distributed in various body tissues and render important biological functions, primarily through regulating the synthesis of selenoproteins [3]. Human selenoproteins are a series of 25 selenium-containing proteins whose synthesis requires insertion of a selenium-containing homolog of cysteine. The major role of multiple selenoproteins, such as glutathione peroxidase (GPX), thioredoxin reductase (TrxR), and iodothyronine deiodinases (IDD), is to act as important intracellular antioxidants in preventing oxidative injury [4]. Therefore, the importance of selenium supplementation in boosting up the internal antioxidative defence has been highlighted in recent years.

It was not until 1957 that the therapeutic role of selenium as a micronutrient was identified by Wrobel et al., who observed that selenium supplementation at a low dose can prevent a rat liver from necrosis [3]. Since then, mounting studies have suggested the beneficial effects of selenium supplementation in maintaining immune-endocrine function, metabolic cycling, and cellular homeostasis. In addition to its essential physiological function, the potential of selenium supplementation in remitting human pathological conditions, especially chronic metabolic disorders, has been frequently proposed. Wei et al. found that daily selenium intake has a negative correlation with metabolic syndromes [5]; however, the role of selenium supplementation as antioxidants in major metabolic syndromes, such as hyperlipidaemia and hyperglycaemia, has not yet been critically reviewed. Here, we retrieved studies from PubMed database

and systematically reviewed the biological activity and underlying mechanism of selenium in various metabolic diseases. Toxicity and recommended dose of selenium were reviewed and discussed. In addition, as the molecular action of selenium was less identified, we predicted and discussed the potential interaction on gene networks and signalling proteins upon selenium supplementation.

2. The Role of Selenium in Treatment of Hyperlipidaemia

Hyperlipidaemia refers to a phenomenon of abnormal high concentrations of lipid products and lipoproteins in the blood. It could be primarily caused by the genetic and familial factors, but in most of the cases, it is triggered by other metabolic disorders. Secondary hyperlipidaemia is a kind of metabolic abnormality involved in several chronic human diseases, such as diabetes and obesity. Healthy young subjects with higher dietary selenium intake (higher than 82.4 μg/day) showed lower level of sialic acid and triacylglycerol, and they exhibited reduced inflammatory response and prevalence of metabolic syndromes such as lipid profile impairment and insulin resistance [6]. It was found that increased hair selenium concentration in hyperlipidemic patients had adverse association with their lipid profiles [7]. Karita et al. showed that the selenium level in erythrocytes may be an indicative factor of decreased total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) after menopause in Japanese premenopausal and postmenopausal women [8]. This indicated the beneficial effect of selenium intake in regulating lipid metabolism. In contrast, another study found that plasma selenium level was raised in preaging cases (aged 59–71) of lipemia [9].

In rats with hyperlipidaemia caused by diazinon, one of the most organophosphate insecticides used in agriculture and industry, selenium supplementation in the form of sodium selenite (200 μg/kg/d) could normalize the serum thiobarbituric acid reactive substances (TBARS), total lipids, cholesterol, urea, and creatinine, which may be due to the induced antioxidant enzymes and glutathione content [10]. Additionally, nicotine reduced the intestinal intake of selenium and caused hyperlipidaemia in rats. Selenium supplementation (1 μg/kg/d) improved the hyperlipidaemic condition, as evidenced by the reduced expression of hydroxymethylglutaryl-CoA reductase (HMGCoA) and lipogenic enzymes [11]. In Triton WR-1339-induced hyperlipidaemia, supplementation of selenium in the form of diphenyl diselenide (10 mg/kg) increased the high-density lipoprotein cholesterol (HDL-C) while reduced the non-HDL and triglyceride in the serum of mice, indicating its hypolipidemic effect [12]. But another study suggested that this effect was independent to its antioxidant property [13]. Furthermore, it was found that hyperlipidaemia had a significant adverse effect on male fertility, while supplementation of inorganic selenium or selenium-enriched probiotics (equivalent to 0.05 μg/g Se) was suggested to improve fertility in humans and animals [14].

2.1. Arteriosclerosis. Kalkan et al. found that dyslipidemic patients with glycogen storage disease type I and type III, which did not lead to premature atherosclerosis, exhibited lower plasma concentration of selenium compared with healthy control [15]. Chan et al. found that selenium deficiency may be associated with reduced arterial function in patients, with higher potential of vascular incidents [16]. Supplementation of selenium in the form of selenium yeast (0.1 mg/kg) subsidized the cardiac enzymes, lipid peroxidation, and inflammation, indicating that it can improve myocardial performance by preventing oxidative damage [17]. Treatment of a formula containing selenium (10 ppm for 30 days) might modulate the lipid profile of hyperlipidaemic rats, mainly reducing the level of TC, non-HDL-C, and atherogenic index [13]. In contrast, another study in British adults showed that higher level of selenium in serum indicated an adverse cardiometabolic risk, with increased total and non-HCL cholesterol [18].

Supplementation of selenium was also suggested when antiatherogenic mode of nutrition was applied to patients, according to a study of 800 persons, in which the results indicated that sodium selenite treatment could give out a favourable outcome on the immune system [19]. Delattre and colleagues showed that treatment of LDL apheresis might be the direct cause of low plasma selenium in normocholesterolemic subjects [20]. This was further evidenced by the observation that LDL apheresis treatment, which eliminated cholesterol-containing LDL from bloodstream, could lower plasma level of selenium but not the other antioxidants including vitamin E and β-carotene [21]. However, an argument was raised on a long-term benefit of LDL apheresis treatment in reducing atherogenic cholesterol oxidation products (COP) in the plasma, and therefore acute drop of selenium by the treatment seemed not meaningful [22].

2.2. Hypercholesterolemia. The significance of serum selenium concentration was highlighted by the study from Galicka-Latala and colleagues. The lipid peroxidation marker malondialdehyde (MDA) had a negative correlation with serum selenium level in both normo- (plasma total cholesterol less than 5.2 mmol/L) and hypercholesterolemic (plasma total cholesterol greater than 5.2 mmol/L) patients, while the MDA, to be specific in low-density lipoprotein, was negatively associated with selenium level in patients diagnosed with hypercholesterolemia [23]. In an experimental model, deficiency of selenium in hypercholesterolemic animal led to lower expression of hepatic LDL receptor and HMG-CoA reductase but elevated apolipoprotein B (ApoB) level, which can be subsidized by selenium resupplementation (1 ppm) [24–26]. In high-fat diet-fed rats, treatment of selenite (0.173 mg/kg/d via gavage for 10 weeks) could suppress LDL-C in serum, triglyceride, and TC in the liver, which was probably due to the reduced expression of fatty acid synthase [27]. Kaur et al. found that supplementation of selenium (1 ppm) could diminish the high-fat diet-induced ROS levels by 29% and suppress the serum paraoxonase 1 but not platelet-activating factor acetylhydrolase, indicating its potential in limiting the complications of hypercholesterolemia [28]. Furthermore,

selenium supplementation (1 ppm) could restore the reduced T3 and T4 hormones in the serum of high-fat diet-fed rabbits, with improvement of type I iodothyronine 5′-deiodinase (5′-DI) in the liver, indicating that selenium is capable of regulating thyroid behaviours in hyperlipidaemic state [29, 30]. Selenium supplementation (2.5 mg/kg, i.p.) was also found to improve dysregulated renal morphology caused by hypercholesterolemia [31].

3. The Role of Selenium in Treatment of Hyperglycaemia

A lot of studies have revealed that hyperglycaemic patients exhibited selenium deficiency in the blood, though a study in diabetic Germans showed that blood selenium level was higher in patients with hyperglycaemia [32]. Compared with some contradicting outcomes in the therapeutic effect of selenium supplementation on type 2 diabetes mellitus, it is quite a consensus that selenium is beneficial for patients with type 1 diabetes mellitus as well as for treatment of hyperglycaemia-related complications.

3.1. Type 1 Diabetes Mellitus. It was found that selenium was distinctly decreased in the red blood cells of type 1 diabetic patients and was negatively correlated with the elastic and viscous component of whole blood viscosity, indicating the selenium deficiency in red blood cells may be associated with impaired haemorpheology of type 1 diabetic patients [33]. Another study showed that selenium level in erythrocyte was lower in type I diabetic groups [34]. Sheng et al. treated alloxan-induced diabetic mice with sodium selenite (via gavage, 2 mg/kg/d for 4 weeks) and found that selenite reduced blood glucose and improved glutathione (GSH) levels in the liver and brain of diabetic mice; nonetheless, selenite treatment in normal mice surprisingly reduced hepatic GSH level [35]. Using STZ-induced diabetic model, Guney and colleagues found that combination of vitamin E (60 mg/kg/d) and sodium selenite (1 mg/kg/d) treatment decreased blood glucose level by inducing expression and activities of several antioxidant enzymes, such as catalase, superoxide dismutase, and GPX [36]. Similar antioxidant treatment could also reverse the skin lipid peroxidation and subsequent damage [37]. Furthermore, Satyanarayana et al. found that half or single therapeutic dose of selenium (0.9 and 1.8 μg/200 g, resp.) had hypoglycaemic effect in alloxan-induced diabetic animal, while double dose of selenium (3.6 μg/200 g rat) increased blood glucose. Combination treatment of selenium improved the hypoglycaemic effect of gliclazide in both normal and diabetic animals [38]. Atalay et al. compared the effect of oral administration of sodium selenate (0.3 mg/kg/d) and doxycycline on STZ-induced hyperglycaemic rats and concluded that selenate can reduce blood glucose level without triggering significant loss of body weight. Selenate preserved thioredoxin-1 (TRX-1) level in skeletal muscle but not in the liver, while the protein carbonyl capacity and oxygen radical absorbance capacity in the liver were suppressed. In addition, free and total protein thiol levels were restored by selenate treatment (0.3 mg/kg, p.o.) in both the skeletal muscle and liver of diabetic rats [39]. Bajpai et al. had similar conclusion about the hypoglycaemic effect of sodium selenite, another inorganic form of selenium in STZ-induced diabetic rats. Treatment of selenite (10–30 μg/ml for 14 days) can reduce serum glucose and improve the wound closure of diabetic mice by normalizing the low levels of vascular endothelial growth factor (VEGF) and extracellular superoxide dismutase. It also improved angiogenesis in the wound site of diabetic rats [40]. Mechanistically, Chen et al. suggested that selenium (1 ppm) might play an insulin-like role to normalize the glucose metabolism and improve glucose uptake and metabolism in the liver of alloxan-induced diabetic animals [41]. Selenium supplementation (5 ppm/d for 4 weeks) could restore glucagon-like peptide 1 receptor (GLP-1R) expression and suppress insulin receptor substrate-1 (IRS-1) and Raf-1 in the liver, which may render hypoglycaemic effect on STZ-induced diabetic rats [42]. In addition, Kahya et al. showed that 1.5 mg/kg/d of sodium selenite treatment can improve brain and erythrocyte lipid peroxidation and plasma IL-1β and IL-4 levels due to the restoration of antioxidant status in STZ-induced diabetic rats [43]. Erbayraktar et al. compared the hypoglycaemic effect of different forms of selenium in STZ-induced diabetic rats and found that both sodium selenate and selenomethionine (2 μmol/kg/day via orogastric route for 12 weeks) can suppress elevation of blood glucose in diabetic mice. However, sodium selenate seemed to have a stronger effect in inducing GPX activity than selenomethionine [44]. Xu et al. examined the combination effect of low-dose insulin and selenium (180 μg/kg/d) in treatment of STZ-induced hyperglycaemia and found that this combination could facilitate reduction of blood glucose and lipid levels, with remarkable restoration of PI3K and GLUT4 in cardiac muscle, which eventually improved myocardial function [45]. Selenium supplementation (0.3 mg/kg Se) in the form of selenium-enriched *Catathelasma ventricosum* mycelia can normalize serum glucose, insulin, and antioxidant enzyme activity in STZ-induced diabetic mice and suppress α-amylase and α-glucosidase activities in *in vitro* gastric and intestinal models [46]. Supplementation of sodium selenite (intraperitoneal injection of 0.3 mg/d for 25 days) can increase vitamin E level in the liver and plasma of STZ-induced diabetic animals. Treatment of selenium can increase GPX activity and GSH concentration in the red blood cells and liver, which reduces TBARS concentration [47].

3.2. Type II Diabetes Mellitus. Anderson et al. found that in patients with type 2 diabetes the selenium level and antioxidant status in plasma remained normal, though 30% of the subjects may have Zn deficiency [48]. A clinical study conducted by Stranges et al. showed that selenium uptake (200 *u*g/d) had no significant beneficial effect to the incidence of type 2 diabetes. Nonetheless, in the highest tertile of baseline plasma selenium level, selenium statistically increased the risk for type 2 diabetes occurrence (hazard ratio, 2.70 (CI, 1.30 to 5.61)) [49]. Another study revealed that inactivation of selenium-dependent enzymes by glycation might eventually lead to oxidative stress in patients with type II diabetes [50]. Study on growing rats with developing obesity

and diabetes, from Mueller and colleagues, revealed that a recommended dietary level or superanutritional level of selenium uptake (1-2 mg/kg in diet), in the forms of either selenite or selenate in diets, increased the body weight of rats. The expression of GPX1 in the liver was upregulated by selenium supplementation, which then triggered overexpression of PTP1B and reduction of glutathionylation [51]. Wang et al. reported that overexpression of GPX1 may deliver a beneficial effect by changing pancreatic expressions of PDX1 and UCP2 via elimination of ROS and hyperacetylation of H3 and H4 histone in islet. However, in long term, it may lead to chronic hyperinsulinaemia by dysregulating beta cell mass and pancreatic content [52]. Surprisingly, Zhou et al. found that, instead of being an antioxidant, selenium might foster lipid peroxidation and decrease GSH/GSSG in the liver and promote ASK1/MKK4/JNK oxidative stress pathway [53]. These observations revealed a plausible mechanism underlying the action of selenium supplementation on the development of obesity and diabetes [51]. Furthermore, Faghihi et al. observed, in a clinical study of type 2 diabetes patients, that selenium intake (200 μg/d for 3 months) accelerated disease progression by increasing fasting plasma glucose, glycosylated haemoglobin A1c, and serum HDL-C level, indicating an unflavoured outcome of selenium uptake in type 2 diabetes despite the restoration of serum selenium level towards optimal concentration of antioxidant activity [54]. In contrast, an experimental observation in high-fat diet/STZ-induced type 2 diabetic rats showed that supplementation of selenium (180–500 μg/kg/d) can reduce blood glucose, cholesterol, and triglyceride level and improve antioxidant status and nitric oxide (NO) release [55]. Additionally, treatment of selenium-containing tea polysaccharides (Se-GTP, 200–800 mg/kg/d for 8 weeks) in high fructose-induced resistant animals could significantly improve hyperglycaemia and hyperinsulinemia and restore antioxidant and hepatic lipid levels. However, this does not prove the direct effect of selenium supplementation in improving type 2 diabetic condition as no comparative study has been made to understand the independent efficacy of tea polysaccharides without selenium [56]. Similar concern was raised by the research from Tanko et al., which showed selenium-enriched yeast (0.1–0.2 mg/kg/d via oral administration for 6 weeks) can improve cholesterol diet-induced type 2 diabetes mellitus in rats by reducing blood glucose and increasing antioxidant activities, yet it could not rule out the possibility of independent therapeutic effect of nonselenium components in the yeast [57].

3.3. Gestational Diabetes. Al-Saleh et al. measured the serum concentration of selenium in gestational diabetic patients, and the results showed that plasma selenium was significantly lower (102.3 versus 75.2 μg/L) [58]. Hawkes observed that pregnant women at between 12 and 34 weeks of gestation had a lower level of serum selenium, which was inversely correlated with increased fasting glucose, but not the insulin level, suggesting that selenium may affect glucose metabolism independent to insulin [59]. Bo and colleagues found that dietary intakes of selenium but not vitamins were significantly lower in hyperglycaemic subjects; in particular, the intake of selenium was negatively correlated with gestational hyperglycaemia. Selenium level was particularly lower in patients with impaired glucose tolerance [60]. However, maternal intake of selenium (6.3/95 μg/d, mean/maximum) had neither positive nor negative correlation with the incidence of advance beta cell autoimmunity in early childhood [61]. Guney et al. applied a combination treatment of vitamin E (60 mg/kg/d) and sodium selenite (1 mg/kg/d) onto diabetic pregnant rats and found that after 21 days of treatment, the abnormal lipid peroxidation (LPO) level in rats was significantly normalized, which may be related to the potent increase of antioxidant enzymes [62]. Asemi et al. conducted a RCT clinical study of selenium supplementation in patients with gestational diabetes. The results indicated that selenium (200 μg/d for 6 wk from weeks 24 to 28 of gestation) could significantly reduce fasting plasma glucose, serum insulin level, and insulin resistance. In addition, selenium could reduce serum high-sensitivity C-reactive protein and increase GSH, resulting in reduction of plasma MDA. However, there was no significant changes on β-cell function, lipid profiles, plasma NO, or total antioxidant capacity concentrations observed [63].

3.4. Hyperglycaemic Complications. The direct evidence of antioxidant effects of selenium in STZ-induced diabetes was obtained by Naziroglu and colleagues. Treatment of sodium selenite (0.3 mg/d for 21 days) improved vitamin E concentration, reduced MDA level in the plasma, and suppressed testicular lipid peroxidation, indicating that selenium supplementation may reduce reactive oxygen substances and improve testicular complications in diabetes [64]. Aliciguzel et al. found that in diabetic rats fed with 10% sucrose following alloxan injection, GPX activity was lower in the liver, brain, kidney, and heart in both early and late stages of diabetes [65]. Furthermore, Liu and colleagues found that supplementation of selenium in the form of Se-polysaccharide from *Catathelasma ventricosum* (100 mg/kg/d) could also reduce MDA and LDL-C in diabetic mice, which was associated with the increased antioxidant enzymes in the liver and kidney. These together with restoration of LDL-C rendered protective effect on the pancreas, liver, and kidney against peroxidative damage [66]. In addition, nanoparticles of selenium exhibited a beneficial effect (0.1 mg/kg via oral administration for 28 days) in improving the testicular tissue condition in STZ-induced diabetic rats. This was related to reduce lipid peroxidation and NO with increased glutathione content and antioxidant enzyme activities. Molecular studies showed that mRNA level of Bcl-2 was upregulated in testicular tissue of selenium nanoparticle-treated rats while Bax was suppressed. Treatment of selenium nanoparticles (0.1 mg of SeNPs/kg) increased PCNA expression as well as testicular function [67].

Faure et al. found that selenoprotein GPX activity in diabetic patients was lower than that in healthy subjects, which was associated with thrombosis and cardiovascular complications [68]. In STZ-induced diabetic animals, Ayaz et al. observed that sodium selenite treatment (i.p. 5 μmol/kg/d for 4 weeks) could prevent myofibril loss and reduce myocyte size. Selenium supplementation (5 μmol/kg/d) rendered

remission on discus intercalaris and nucleus in the heart and preserved myofilament and Z-lines [69]. Treatment of sodium selenite (10 μmol/kg/d for 3 weeks) corrected adenosine-induced negative chronotropic effect in STZ-induced diabetic animals, but selenium supplementation had a minimal effect on carbachol-induced inotropic and chronotropic responses in the left and right atria [70]. In the aorta of STZ-induced diabetic rats, sodium selenate treatment (0.3 mg/kg/d for 4 weeks) can improve isoproterenol-induced relaxation and contraction responses and preserve the morphology of smooth muscle cells. This may be related to the regulation on MMP-2 activity and protein loss in aorta, as well as the inhibition of tissue nitrite and protein thiol oxidation. Pathway study revealed that selenium supplementation might improve endothelin-1, PKC, and cAMP production in the aorta [71]. Aydemir-Koksoy et al. found that treatment of sodium selenite (0.3 mg/kg/d) could prevent depression in the left ventricular development pressure and the rates of changes in developed pressure in STZ-induced diabetic rats, and this effect was much greater than antioxidant treatment using vitamin E combined with omega-3 fish oil. The increase of myocardial oxidized protein sulfhydryl and nitrite concentration in the heart of diabetic rats was normalized by selenium supplementation [72]. Mechanism study revealed that myocardial MMP-2 and TIMP-4 were normalized, and selenite treatment increased expression of Tnl and α-actin in the heart of diabetic mice [73]. Liu et al. also revealed that high glucose-induced cardiomyocyte apoptosis could be attenuated by selenium supplementation through regulating TLR-4/MyD-99 signalling pathway and ROS formation [74]. Inhibition of NF-κB-mediated proinflammatory cytokine transcription and suppression of leukotriene pathway by sodium selenite treatment also contributed to the protective effect of selenium against diabetic cardiac hypertrophy [75]. Ng et al. observed that a water-soluble selenium-containing sugar rendered antioxidant activity in the aortae and prevented hyperglycaemia-induced endothelial dysfunction through reducing superoxide levels, as well as improving basal NO availability and vasoconstrictor prostanoids [76]. Combination of selenium with low-dose insulin can restore PI3K-mediated GLUT4 in cardiac muscle, which reduced damage and dysfunction of myocardial cells in STZ-induced diabetic rats [45].

Kornhauser et al. observed that, in type 2 diabetic patients, plasma selenium level was reduced. Serum concentration of GPX was significantly lower in diabetic patients with microalbuminuria than in those without nephropathy. Notably, microalbuminuria was negatively correlated with plasma level of selenium and GPX in patients with type 2 diabetes [77]. The role of selenium in diabetic nephropathy was evident by the observation that animal fed with selenium-deficient diet developed albuminuria and glomerular sclerosis as well as increased expression of TGF-β1 mRNA. Supplementation of selenium (0.27 mg/kg Se in diet) in the form of sodium selenite in diabetic rats improved glomerular sclerosis and tubulointerstitium [78]. Roy et al. observed that sodium selenate treatment (16 μmol/kg) could improve serum creatinine, urea, and albumin levels, as well as the renal antioxidant enzyme activities, such as superoxide dismutase (SOD), catalase, and GSH in STZ-induced diabetic rats. Selenate treatment could reduce lipid peroxidation and TGF-β1 in the diabetic rat kidney and improve cellular architecture of the kidney. This may lead to reduce apoptotic renal cells in diabetic mice [79]. In contrast, study from Bas et al. found that sodium selenite treatment (1 mg/kg for 28 days) had a minimal effect on diabetes-mediated toxicity in kidneys through improving lead nitrate-induced nephrotoxicity in nondiabetic animals [80].

Intraperitoneal injection of sodium selenite (5 μmol/kg/day) for 4 weeks did not significantly improve high blood glucose and body weight loss in diabetic animals, but seemed to improve diabetes-induced structural alterations in the mandible [81]. Ozdemir et al. observed in STZ-induced diabetes that intraperitoneal injection of 5 μmol/kg/d for 4 weeks could prevent deterioration of structural and ultrastructural changes in the long bones of diabetic rats [82].

In type 1 diabetic rats induced by STZ injection, treatment of sodium selenite (5 μg/kg/d, intraperitoneal injection for 4 weeks) could significantly improve liver antioxidant enzymes in diabetic rats. The ultrastructure of the liver tissue, including variation in staining quality of hepatocyte nuclei, density, and eosinophilia of the cytoplasm, focal sinusoidal dilatation and congestion, and number of abnormal mitochondria, was normalized by sodium selenite treatment [83]. Intraperitoneal injection of sodium selenite (1.5 mg/kg/d for 4 weeks) could improve the liver function of STZ-induced diabetic animals and increase the hepatic expression of superoxide dismutase, reduce glutathione, lactate dehydrogenase, pyruvate kinase, and hexokinase, which rendered inhibition to NO, MDA, and phosphoenolpyruvate carboxykinase (PEPCK) in the liver [84]. Supplementation of selenium in the form of sodium selenite (1 ppm in drinking water) reduced aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in diabetic rats, with a significant improvement in serum antioxidant enzymes and reduction of GSH level. Improvement of hepatic lipid accumulation and centrilobular hepatocyte degeneration was also observed [85]. In addition, treatment of sodium selenite (0.5 mg/kg/d for 4 weeks) could significantly reduce aldehyde oxidase and xanthine oxidase activities in the liver, but not in the kidney or heart, which might be associated with improvement of total antioxidant status after selenium supplementation [86].

4. The Role of Selenium in Treatment of Hyperphenylalaninemia

Phenylketonuria (PKU) is a born error in amino acid metabolism which leads to mildly or strongly elevated concentrations of the amino acid phenylalanine in the blood. PKU is the major cause of hyperphenylalaninemia. Studies have supported that in patients with PKU, the antioxidant defence in plasma and erythrocytes was decreased, which can be due to the secondary deprivation of micronutrients [87]. An observation from 156 patients with hyperphenylalaninemia showed that selenium was diminished in 25% of the subjects, 95% of which exhibited phenylketonuric phenotype [88]. The reason of low plasma selenium could be diet

related, as PKU patients are often required to take natural protein and phenylalanine-restricted diet, which brings risk of low selenium intake [89]. Plasma level of selenium was significantly lower in patients with phenylketonuria or milder hyperphenylalaninemia, consistent with low total antioxidant status. The plasma selenium was correlated with erythrocyte GPX activity, which was lower in phenylketonuria, but inversely associated with free triiodothyronine and thyroxine [90, 91]. In contrast, Artuch and colleagues showed that plasma selenium concentration in patients with phenylketonuria had no different change compared with the healthy population [92]. In maternal Czech women with hyperphenylalaninemia, reduction of serum and urinary selenium level was observed [93]. Selenium deficiency led to defective GPX activities and consequently an increased level of MDA and organic hydroperoxides in the serum [94]. Further study showed that selenium deficiency in phenylketonuria might be the aetiology of dysrhythmia and cardiac dysfunction [95, 96]. Selenium deficiency in phenylketonuria might cause reduced response to OKT3 mitogenesis via T-cell antigen receptor complex (TCR/CD3) [97]. Gassio et al. reported a consistent observation of low-serum selenium level in patients with phenylketonuria and found that selenium concentration was associated with worsen Conners' Continuous Performance Test measures (more omission errors, fluctuating attention and inconsistency of response times, and slowing reaction time as the test progressed) [98]. However, another study showed that the neuropsychological disturbance in phenylketonuria patients might be independent to selenium level, as plasma selenium seems to be normal in patients, while patients with lower selenium GPX had more severe neuropsychological disturbances [99].

The decreased level of serum selenium in phenylketonuric patients did not improve by dietotherapy [100, 101]. A study in Czech patients with phenylketonuria and hyperphenylalaninemia showed that controlled diet with low protein may cause serum selenium deficiency in adults, while prealbumin, zinc, and iron remained unchanged [102]. Consistent observation was found in another 12-year study on selenium status in 78 phenylketonuric children (aged 1–16) [103]. In patients with phenylalanine-restricted diet, intake of sodium selenite (115 μg/d) for 3 months could increase selenium level in plasma and blood cells and improve plasma GPX activity and left ventricular cardiac index, which led to decrease of thyroxin, free thyroxin, reverse triiodthyronin, TC, mean erythrocyte and thrombocyte volume, and lymphocytic CD2 expressions [104]. In patients with phenylalanine-restricted diet, selenium supplementation (1 μg/kg/d for 3 weeks) could reduce both concentrations of prohormone thyroxine (T4) and metabolic inactive reverse triiodothyronine (rT3), which could be probably due to the increase in activity of type I 5′-deiodinase [105, 106].

A pilot observation on 5 patients was conducted by Lombeck and colleagues, who showed that supplementation of selenium (45 μg/d) could render a normal selenium level in blood of phenylketonuric patients after a 4-week treatment, though GPX activity was only partially normalized [107]. However, Zachara et al. found that GPX activity in red blood cells of patients with phenylketonuria well indicated the functional restoration of selenium supplementation [108]. A possible mechanism underlying this discrepancy may be understood from the observation that selenium supplementation (0.13 mmol/kg/day) could only result in a short-term (within 10 days) but not long-term increase of plasma selenium level [109]. Using a special formula containing 31.5 μg/d selenium and 98 mg/d L-carnitine reduced lipid peroxidation and protein oxidative damage and improved GPX activity in phenylketonuric patients, indicating that selenium supplementation was important for the amelioration of neurological symptoms of phenylketonuria via regulating oxidative stress pathways [110]. Alves observed in a clinical study of phenylketonuric children that selenium supplementation could significantly increase serum selenium and GPX in erythrocytes, which in turn reduced serum concentration of free thyroxin and improved patient conditions [111].

5. Discussion and Conclusion

5.1. Toxicity of Selenium. Although the reviewed studies and some other ongoing investigations have been providing mounting evidence on the beneficial role of selenium in both healthy people and patients, it is necessary to pay attention to its toxicity which is probably due to overdose of daily intake from food and water. As selenium can be accumulated through the food chain, selenium contamination, especially in the aquatic environment, can lead to enrichment of selenium speciation, such as Se(IV), Se(VI), and selenomethionine in plants and fishes [112]. These selenium species may cause direct toxicity [113], which may be related to the induction of ROS-associated oxidative stress [114]. What is more, aquatic organisms exposed under high-dose selenium are taking a risk of organ damage and genome mutation [115, 116], making them susceptible in safety as human food. In this case, environmental selenium accumulation may bring primary risk (for selenium enrichment) and secondary risk (from unknown mutation-borne food toxicity) to the human body, and this yielded attempt to set up criteria for allowable selenium level in aquatic system by different organizations (Table 1, adopted from review by Sharma et al. [117]).

Chronic exposure of environment selenium has been demonstrated to be a high risk factor of health in human population. Selenium overdose in humans may develop selenosis [118, 119], though quite rare, and is possible to cause amyotrophic lateral sclerosis [120] regardless of races and ethnicities [121–123]. Mechanistically, cellular exposure of high-dose selenium can cause elevation of intracellular ROS, which is considered as the main mediator of selenium-induced cell toxicity [124]. Though selenium is overall regarded as an essential factor of antioxidant enzyme production, chemically, it is capable to react and form intramolecular disulfide bond (S-Se) with essential thiol groups, or cysteine resides in the substrates [125] and indirectly generates ROS. The increasing oxidizing cellular environment may then cause DNA damage and genome instability, leading to initiation of cell apoptosis [126, 127]. The oxidative stress-involving selenium toxicity might therefore lead to impaired immune function, cytotoxicity, genotoxicity, and carcinogenesis [128–130]. Overdose of selenium can be

Table 1: Allowable Se level in aquatic systems*

Organization	Criteria
United States Environmental Protection Agency	Se(IV) ≤ 257 μg/L; Se(VI) ≤ 417 μg/L
The French Institute of Industrial Environment and Risks	0.88 μg/L as probable no effect concentration (PNEC) for direct chronic effect; 0.97 μg/L as no observable effect concentration
British Columbia	PNEC = 2 μg/L for freshwaters and marine environment
Environment Canada	PNEC = 1 μg/L for freshwaters

*Adopted from a review by Sharma et al. [117].

lethal; as shown in rats, the LD_{50} values were 7, 138, and 6700 mg Se/kg.bw for selenite, selenium sulphide, and elemental Se, respectively [131]. In humans, daily intake of selenium at 4.9 mg/person/day was considered toxic in Chinese and Indian populations [4, 132, 133]. The issue of dose of intake shall be therefore taken into serious account when using selenium as a nutritional or therapeutic agent.

5.2. Dose Recommendation for Selenium Intake. Given the double-sword nature of selenium intake, the daily dose of intake is quite a critical issue for selenium as either nutritional supplements or therapeutics. In spite of the official guideline for the use of selenium as beneficial supplements in patients is not yet developed, a lot of efforts have been made to specify the selenium intake in a healthy population. The World Health Organization (WHO) has made recommendation on the dose of selenium for adults to be 30 to 40 μg/day and stated that daily intake up to 400 μg selenium shall be considered safe [134]. Recommended dose of selenium varies in different countries in consideration of differences in geographical and racial natures as well as in living styles of particular populations. Table 2 summarized recommendations on daily dose of selenium from official and/or nonofficial organizations in various regions, which was adopted from a recent review by Kieliszek and Blazejak [134]. Optimal dose of selenium intake in patients with metabolic diseases is difficult to estimate, but from several studies we retrieved in this review, it seems that daily supplementation of 31.5–200 μg Se is beneficial. There are not much literature for reference, since the limited amount of studies with inconsistent data quality, though most of these studies indicated patients with metabolic disorders, might need to take higher dose of selenium than the healthy population (82.4–200 μg).

Table 2: Recommendations on daily dose of selenium*

Counties/regions	Recommendations
Czech Republic	10–25 μg/day
China	7–4990 μg/day
Venezuela	200–350 μg/day
Poland	30–40 μg/day
Austria	48 μg/day
Great Britain	34 μg/day
USA	40–70 μg/day for men; 45–55 μg/day for women

*Adopted from a recent review by Kieliszek and Blazejak [134].

5.3. Proposed Mechanism of Action. Metabolic disorders are a series of diseases resulting from breakdown of internal homeostasis of the human body, which involves an infinite cycle of energy synthesis and waste production. Major metabolic dysregulation including hyperglycaemia, hyperlipidaemia, and hyperphenylalaninemia causes illness in multiple organs including the livers, kidney, and heart, leading to a series of diseases such as obesity, diabetes, phenylketonuria, and atherosclerosis. To further understand the molecular function of selenium, we retrieved genes related to multiple disorders (Table S1). Hyperphenylalaninemia seems to be caused by independent mechanism, while hyperglycaemia and hyperlipidaemia share a series of related genes (Figure 1, Table S2). By searching stitch 4.0 and STRING database, we found that selenium interacts with a series of selenoproteins, which secondarily interact with a series of proteins (Table S3). Particularly, it was noticed that xanthine dehydrogenase (XDH) is the interacting protein that connects the pathogenesis with molecular action of selenium. As superoxide-producing enzyme XDH and its converted form xanthine oxidase (XO) have been found increased in metabolic diseases [135, 136]. Although there has not been direct evidence showing that selenium treatment suppresses XDH and XO, a previous study showing the inverse correlation between selenium-associated GPX enzyme level with XDH level in diabetic rats [65] suggested that selenium may be related to the activity of xanthine metabolism. Future original study may focus on the role of XDH/XO system in the therapeutic role of selenium in metabolic disorders via GPX system.

5.4. Future Direction. Selenium has been proposed to be beneficial supplements for human health. Although high dose of selenium can definitely cause toxicity, the rational intake of selenium shall be safe and useful to not only healthy population but also patients with metabolic diseases. Efforts have been made to understand the action of selenium in metabolic diseases, and more clinical-relevant studies in the future are highly expected. The dose and form of selenium given to metabolic patients shall be standardised by official guidelines. Efficacy and safety of selenium supplementation in improving metabolic disorders shall be proven by long-term follow-up in patients. In addition, the mechanism underlying the action of selenium, which may be dependent

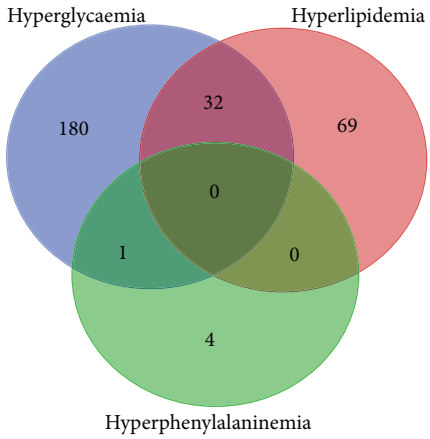

Figure 1: Venn diagram of interacting genes among various metabolic disorders. Disease-related genes were retrieved from the NCBI database, and Venn diagram was created to find any common gene involved in different metabolic diseases. 213 genes for hyperglycaemia (shown in blue circle), 101 genes for hyperlipidemia (shown in red circle), and 5 genes for hyperphenylalaninemia (shown in green circle) were collected. Hyperlipidemia and hyperglycemia share 32 common disease-related genes, while there is only one gene overlap between the groups of hyperglycaemia and hyperphenylalaninemia. No common gene is observed for the three diseases. Detail lists of genes were shown in Table S1.

or independent to internal antioxidant defence system, shall be studied by more experimental investigations.

In conclusion, we systemically reviewed the role of selenium as an antioxidant in various metabolic disorders. Selenium deficiency is observed in multiple metabolic diseases, including hyperglycaemia, hyperlipidaemia, and hyperphenylalaninemia. Supplementation of selenium may improve atherosclerosis, hypercholesterolemia, type 1 diabetes mellitus, and phenylketonuria, but its action remains controversial for type 2 diabetes mellitus. While regulation of hyperphenylalaninemia may go through an independent mechanism, hyperglycaemia and hyperlipidaemia may have shared mechanisms with a series of common genes involved. Toxicity of selenium was highlighted, and the window of selenium between beneficial and toxic doses shall be paid attention to recommend a proper dose of administration. The antioxidant role of selenium in metabolic diseases may be highlighted with the prediction that selenium-related proteins may interact with xanthine metabolism and superoxide-producing enzymes in metabolic diseases. Our study indicates the therapeutic potential of selenium supplementation as an antioxidant in the treatment of metabolic disorders.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Yibin Feng conceived the review. Ning Wang retrieved the literatures and drafted the paper. Hor-Yue Tan, Sha Li, Yu Xu, and Wei Guo helped to analyse the literatures. All authors revised the paper and approved the final manuscript.

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Supplementary Materials

Table S1: genes involved in major metabolic disorders. Table S2: genes of interests. Table S3: predicted partner genes of selenium proteins. *(Supplementary Materials)*

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