Neuropsychiatric Disturbances and Diabetes Mellitus: The Role of Oxidative Stress

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Editorial Board

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Editorial

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Oxidative stress (OS) plays a fundamental role in the pathogenesis of diabetes mellitus, neurodegenerative disorders (i.e., Alzheimer's disease (AD)), and psychiatric disturbances (i.e., depression). Interestingly, it could represent the common molecular basis explaining the high rates of comorbidity between these disorders.

This special issue is aimed at improving the current knowledge about the role of OS in the occurrence and progression of diabetes and neuropsychiatric disorders as well as its contribution to their comorbidity.

Three papers focus on OS and microbiota. In particular, L. Dumitrescu et al. underline that a peculiar microbiota type could enhance brain inflammation and OS thus favoring the occurrence of proteinopathies such as AD and Parkinson's disease. M. Luca et al. deepen the role of dysbiosis in AD, depression, and type 2 diabetes mellitus (T2D). Moreover, H.-X. Cui et al. relate the hypoglycemic effects of berberine fumarate in diabetic rats to its modulation of intestinal microflora (i.e., increase of *Bacteroidetes* and decrease of *Lachnospiraceae*).

Several contributions explore the role of OS from a multidisciplinary point of view. More specifically, G. Z. Réus et al. highlight that depression and T2D share OS, inflammation, and metabolic disturbances as common pathophysiological mechanisms, while B. Carpita et al. discuss maternal diabetes (and the related oxidative and inflammatory condition) as a risk factor for the development of autism spectrum disorders in the offspring. On the other hand, W. Ohnon et al. report the neuroprotective effects (reduction of TNF-α, IL-6, and other proinflammatory molecules) exerted by the combined extract of *Ozyza sativa* and *Anethum graveolens* administered to rats with experimentally induced metabolic syndrome and stroke. C. Chen et al. demonstrate that irbesartan pretreatment protects pancreatic β-cells from apoptosis while improving glucose levels and insulin secretion in streptozotocin-induced acute prediabetic mice. In addition, the findings by R. Li et al. indicate that Bailcalin, both in vitro and in vivo, improves cardiomyocyte hypertrophy in T2D-induced mice via antioxidative and lipid-lowering effects.

Three articles address the imbalance between reactive oxygen species production and antioxidant defense systems characterizing metabolic syndrome and diabetes. R. Vona et al. provide an overview of the biomarkers of OS in metabolic syndrome and associated diseases and comment on the possible therapeutic implications of natural antioxidants. M. Maciejczyk et al., evaluating redox homeostasis and oxidative damage in the cerebral cortex and hypothalamus of rats under insulin resistance conditions, describe an adaptive (although ineffective in terms of neuroprotection) increase in the antioxidant defense systems. On the other hand, A. Passaro et al. report, in patients with T2D, lower levels of paraoxonase-1 and lipoprotein phospholipase A2, two enzymes that, preventing the oxidative transformation of high- and low-density lipoproteins, may reduce the atherogenic risk.

All the papers collected in this special issue contribute to the current knowledge on the role of OS in neuropsychiatric disturbances and diabetes mellitus.

Conflicts of Interest

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

Acknowledgments

Special thanks go to the authors and the reviewers for their precious contribution.

Maria Luca
Maurizio Di Mauro
George Perry

Research Article

Bailcalin Protects against Diabetic Cardiomyopathy through Keap1/Nrf2/AMPK-Mediated Antioxidative and Lipid-Lowering Effects

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Previous studies demonstrated that Bailcalin (BAI) prevented cardiac injuries under different disease models. Whether BAI protected against type 2 diabetes mellitus- (T2DM-) associated cardiomyopathy was investigated in this study. T2DM was established by the combination of streptozotocin injection and high-fat diet in mice. BAI was administered daily for 6 months. After evaluating cardiac functions, mice hearts were removed and processed for morphological, biochemical, and molecular mechanism analyses. Neonatal rat cardiomyocytes (NRCM) were isolated and treated with high glucose and palmitate (HG/Pal) for *in vitro* investigation. BAI significantly ameliorated T2DM-induced cardiomyocyte hypertrophy, interstitial fibrosis, and lipid accumulation accompanied by markedly improved cardiac functions in diabetic mice. Mechanically, BAI restored decreased phosphorylation of AMPK and enhanced expression and nuclei translocation of Nrf2. In *in vitro* experiments, BAI also prevented NRCM from HG/Pal-induced apoptosis and oxidative stress injuries by increasing p-AMPK and Nrf2 accumulation. The means by which BAI restored p-AMPK seemed to be related to the antioxidative effects of Nrf2 after silencing AMPK or Nrf2 in NRCM. Furthermore, BAI regulated Nrf2 by inhibiting Nrf2 ubiquitination and consequent degradation mediated by Keap1. This study showed that BAI alleviated diabetes-associated cardiac dysfunction and cardiomyocyte injuries *in vivo* and *in vitro* via Keap1/Nrf2/AMPK-mediated antioxidation and lipid-lowering effects. BAI might be a potential adjuvant drug for diabetes cardiomyopathy treatment.

1. Introduction

A pooled analysis of 4.4 million participants showed that the global prevalence of diabetes has quadrupled since 1980 [1, 2]. About 8% of the people worldwide have been diagnosed with diabetes [1]. The cardiovascular system has been demonstrated to be one of the most suffering systems [1]. Diabetes could offset any decrease in cardiovascular morbidity and mortality obtained from the control of other cardiovascular risk factors [1, 2]. Diabetic cardiomyopathy (DCM) has been accepted as one of the most common and severe complications. DCM was characterized with the structure and function impairments of the myocardium, which were independent of hypertension- or coronary artery disease-induced cardiomyopathy. Currently, no effective treatments

have been used for preventing DCM, and even some traditional treatments have been demonstrated to have adverse effects for DCM therapy. However, increasing investigations have suggested that natural products, derived from green vegetables, fruits, teas, and daily foods, might be potential drugs for treatment or adjuvant treatment of DCM.

Many underlying mechanisms have involved in the development and progress of DCM [3]. Oxidative stress and lipid toxicity are two main pathogenesis of DCM induced by T2DM [4]. The redox dynamic equilibrium state is disturbed in the progress of diabetes because of decreased antioxidant and increased oxidant in cardiomyocytes, which causes the accumulation of reactive oxygen species (ROS) [4]. Excessively accumulated ROS may react quickly and nonspecifically with proteins, lipids, and DNA, resulting in DNA

damage, metabolism aberration, and cell apoptosis [4]. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a cytoprotective transcription factor that controls the expression of many antioxidant factors [5]. Under unstressed normal conditions, Nrf2 is arrested in cytoplasm for quick ubiquitin degradation by binding with an E3 ubiquitin ligase substrate adaptor (Kelch-like ECH-associated protein 1 (Keap1)) [5]. Keap1 possesses several highly reactive cysteines, upon modification by sensing oxidants and electrophiles, which could prevent Keap1-mediated Nrf2 proteasomal degradation, thereby resulting in nuclear accumulation of Nrf2. Nrf2 binds to antioxidant response elements (AREs) in the nucleus to activate the transcription of many antioxidant genes [5]. Activation of Nrf2 has been demonstrated to be beneficial for several cardiovascular disorders including hypertension, heart failure, and diabetic cardiomyopathy. The expression of Nrf2 was significantly downregulated at the late stage of DCM. Studies have demonstrated that restoring the expression and nucleus translocation of Nrf2 could prevent cardiomyocyte from diabetes-associated injuries *in vitro and in vivo* [6]. Lipid toxicity, another important pathological factor in DCM, is caused by dysregulation of enzymes and signaling pathways involved in triacylglycerol, phospholipid, and sphingolipid metabolisms [7]. Adenosine monophosphate-activated protein kinase (AMPK) is a key enzyme in regulating lipid metabolism and utilization [7]. Activated AMPK could promote glycolysis and fatty acid oxidation, autophagy initiation, and glycogen, lipid, and protein syntheses; however, the activity of AMPK was significantly downregulated in DCM. Genetic or pharmaceutic activation of AMPK could effectively decrease lipid accumulation and improve cardiac functions in DCM. Therefore, simultaneous pharmacological regulation of Nrf2 and AMPK might be a beneficial strategy in preventing DCM.

Baicalin (BAI) is a flavonoid component extracted from the traditional Chinese medicine *S. baicalensis*. BAI was shown to inhibit oxidative stress by reinforcing the endogenous antioxidant system through regulating the nuclear translocation of Nrf2 and HO-1 expressions in the alcoholic liver mouse model [8]. In a cerebral ischemia/reperfusion (I/R) mouse model, BAI ameliorated hyperglycemia-exacerbated I/R injuries through regulating mitochondrial functions in an AMPKα1-dependent manner [9]. More importantly, BAI has been demonstrated to decrease blood lipids and inflammation [10] in a randomized, double-blind, placebo-controlled trial. In cardiovascular diseases, BAI could prevent embryonic cardiovascular malformation by suppressing the excessive production of ROS and autophagy [11]. BAI has been shown to protect against pressure overload-induced cardiac remodeling through regulating AMPK [12] or PPARs [13]. So far, the role and underlying mechanism of BAI in T2DM-associated DCM have not been reported. Herein, we used an experimental T2DM mouse model to clarify the above formulated issues.

2. Materials and Methods

2.1. Materials.
BAI was purchased from Shanghai Winberb Medical S&T Development Co. Ltd. (Shanghai, China). BAI was provided by the manufacturer with a purity of >99%, which was determined by high-performance liquid chromatography. MG132 was purchased from Beyotime (S1748).

2.2. Animals.
Male C57BL/6J mice (age: 9-11 weeks, weight: 24.5-25.5 g) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Science (Beijing, China). All mice were housed for adapting to environment for one week before establishing the diabetic mouse model. All mice have free access to food and water with a 12 h light/dark cycle. All animal experiments abided by the laboratory animal guidelines published by the United States National Institutes of Health (NIH Publication, revised 2011) and were approved by the Animal Care and Use Committee of Zhengzhou University.

2.3. Diabetic Mouse Model and BAI Treatment.
The type 2 diabetes model was established according to the method used in the previously published literature [14, 15]. Briefly, mice were fed with high-fat diet (HFD) to induce insulin resistance or normal diet (ND) for 3 months. Type 2 diabetes was induced in these insulin-resistant mice with intraperitoneal injection of streptozotocin (STZ) dissolved in 0.1 M citrate buffer pH 4.5 at the dose of 100 mg/kg [14, 15]. Blood glucose was examined, and mice with blood sugar > 250 mg/dL were defined with diabetes and used for following experiments.

Four groups were included in this study: ND with vehicle treatment group (VEH, $n = 12$), ND with BAI treatment group (BAI, $n = 12$), HFD with vehicle treatment after STZ injection group (DM, $n = 16$), and HFD with BAI treatment after STZ injection group (DM/BAI, $n = 16$). BAI was administered to mice by a gastric tube for 4 months. BAI was administered at a dosage of 100 mg/kg/d for every day at 2 pm in the BAI and DM/BAI groups. The dose of BAI was chosen according to previous studies [14, 15]. The VEH and DM groups received the same volume of vehicle for 4 months.

2.4. Isolation and Treatment of Cardiomyocytes

2.4.1. Isolation of Cardiomyocytes.
Neonatal rat cardiomyocytes (NRCM) were isolated according to a published protocol [16, 17]. Briefly, neonatal rats (0-3-day-old Sprague-Dawley rats) were anesthetized with carbon dioxide and sacrificed by cervical dislocation. Rat hearts were removed and minced into pieces of about 1 mm³. Minced cardiac tissues were transferred to a glass bottle and resuspended in trypsin-collagenase 2 buffer. Then, the glass bottle was put into a preheated cell vibrator for 30 min with 100 rpm at 37°C. The cell suspensions were collected into a 50 mL centrifuge tube. Enzyme activity was terminated by addition of horse serum. Fresh trypsin-collagenase 2 buffer was added into the remaining tissue pieces for cellular digestion for another 30 min. Repeated cellular digestion was performed until completely dissociating tissue fragments. Collected cell suspensions were pooled, filtered, centrifuged for 5 min at 1000 rpm, and then resuspended in 12 mL cardiomyocyte culture medium. Cardiomyocytes were isolated from cardiac fibroblasts through delayed adherent culture for 1 hour in a precoated culture dish. After 1 hour of incubation in a

humidified incubator at 37°C and 5% CO_2, cardiac fibroblasts adhered to the culture dish while cardiomyocytes remained to suspend in culture medium. Cardiomyocytes were seeded at a density of 2×10^5/mL for following experiments.

2.4.2. siRNA Transfection. To knock down Nrf2 and AMPKα, respectively, Nrf2 siRNA (sc-156128), AMPKα2 siRNA (sc-155985), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. siRNAs were transfected into NRCM for 18-24 h by using lipofectamine 200 (Invitrogen). Transfection efficiency was evaluated by Western blot for Nrf2 and AMPK protein expression quantifications, respectively.

2.4.3. Treatment of NRCM. NRCM were treated with high glucose and palmitate (HG/Pal) to mimic T2DM-associated NRCM injuries according to published protocol [18]. Briefly, NRCM were cultured in medium containing D-glucose with a final concentration of 33 mM and palmitate with a final concentration of 200 μM, which could not cause cellular apoptosis in a period of 36 hours in experiment (data not shown). After testing the effects of BAI at different concentrations in NRCM, a final concentration of 20 μM was used for following experiments.

2.5. Cardiac Function Evaluation by Echocardiography and Pressure-Volume Loop. Transthoracic echocardiography (Echo) was performed to evaluate mouse cardiac functions by a high-resolution Echo system (Esaote S.P.A., Genoa, Italy) for small animals, equipped with a 10 MHz microprobe. The cardiac functions of mice were assessed under condition of anesthesia with 1.5% isoflurane. The left ventricle (LV) dimension was acquired at a parasternal short axis view. The heart rate (HR), LV end-diastolic diameter (LVEDd), LV end-systolic diameter (LVEDs), LV end-diastolic volume (LVVd), LV end-systolic volume (LVVs), LV ejection fraction (EF), and LV fractional shortening (FS) were calculated by system software. Pressure-volume analysis was performed according to published protocol [19]. After isolating the mouse carotid artery, a 1.4 French Millar catheter transducer was inserted into mouse LV under conditions of anesthesia with 1.5% isoflurane. The PVAN data analysis software was used for data analysis.

2.6. Examination of Pathological Morphology Changes in Mice Hearts. Mice hearts were harvested and fixed in 10% formalin for 12-24 h at room temperature. After dehydration, mice hearts were embedded in paraffin for cutting into 4-5 μm thick slices. The slices were stained with hematoxylin and eosin (HE) for examining general morphology and cardiomyocyte surface area (CSA). To observe cardiac fibrosis, the slices were subjected to Masson trichrome staining. The collagen deposition was shown in blue areas. Oil Red staining was performed to examine lipid accumulation in mouse heart tissue. Mice hearts were harvested freshly and embedded in optimal cutting temperature (OCT) medium for frozen sections at a thickness of 8-10 μm. The frozen sections were fixed in 10% formalin for 5 minutes at room temperature and then stained with the Oil Red O reagent for 1 h. After washing with 10% isopropanol, the frozen sections were counterstained with hematoxylin for 30 seconds. All of these stained slices were photographed by the Nikon microscope. The Image-Pro Plus 6.0 software was used to analyze the CSA in HE staining and collagen content in PSR staining.

2.7. Western Blot Assay. Snap frozen heart tissue was homogenized, or cardiomyocytes were sonicated in RIPA buffer. Total proteins were extracted and separated by 8%, 10%, or 12% SDS-PAGE gels (according to molecular weight) and then transferred to a nitrocellulose membrane, which was blocked with 5% FBS for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study are ANP (CST, 1 : 500), BNP (CST, 1 : 500), β-MHC (CST, 1 : 500), p-AMPKα (CST, 1 : 1000), T-AMPKα (CST, 1 : 1000), p-ACC (CST, 1 : 500), T-ACC (CST, 1 : 500), c-caspase 3 (CST, 1 : 500), BAX (CST, 1 : 1000), β-actin (CST, 1 : 500), Nrf2 (ABCAM, 1 : 500), HO-1 (ABCAM, 1 : 500), CPT-1 (ABCAM, 1 : 500), PGC1-α (ABCAM, 1 : 500), 3-NT (ABCAM, 1 : 500), 4-HNE (ABCAM, 1 : 500), Keap1 (ABCAM, 1 : 200), and ubiquitin (ABCAM, 1 : 500). On the next day, blots were washed with Tris-buffered saline containing 0.05% Tween 20 and then incubated with horseradish peroxidase- (HRP-) conjugated secondary antibody for 1 h at room temperature. The enhanced chemiluminescence kit (Thermo) was used to visualize corresponding blots. Image Lab 3.0 was used for blot analysis.

2.8. Isolation of RNA for Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was isolated using the TRIzol Reagent (Invitrogen, CA, USA) or NRCM according to manufacturer's instruction. The NanoDrop ND-1000 spectrophotometer was used to determine RNA concentrations and purities. The first strand complimentary DNA (cDNA) synthesis kit was purchased for reverse transcription of RNA according to manufacturer's instruction. 2 μg total RNA was reversely transcribed into cDNA. RT-qPCR was performed in a 20 μL reaction buffer, including 10 μL SYBR green dye, 1 μL primers, 1 μL templet, and 8 μL H_2O, in the Roche RT-qPCR system. Primers used in this study were listed in Table 1. Fluorescence intensity was measured to monitor the amplification of target genes. Comparative cycle time (CT) was used to assess fold changes among different groups.

2.9. Nuclei Extraction. To investigate nuclei translocation of Nrf2, the nuclei isolation kit (Nuc-201, Sigma) was purchased to extract nuclei from cardiomyocytes both ex vivo and *in vitro* according to the manufacturer protocol. Briefly, 50 mg cardiac tissue of each sample was homogenized, or 1 $\times 10^7$ cardiomyocytes were sonicated in 300 mL cold lysis buffer, and then, 600 mL cold 1.8 M Cushion Solution was added. Finally, the mixture was transferred into a new tube preloaded with Sucrose Cushion Solution. Tubes were centrifuged at 30000 g for 30 min. Nuclei were deposited at the bottom of tubes, and the cytoplasmic component existed in the supernatant layer. The supernatant and sediments were used for protein extraction and Western blot analysis.

TABLE 1

Genes	Genus	Forward primer	Reverse primer
TGF-β	Mouse	GCTGAACCAAGGAGACGGAA	GGGCTGATCCCGTTGATTTC
Collagen I	Mouse	GTAACGATGGTGCTGTTGGTG	CACCATTGGCACCTTTAGCG
Collagen III	Mouse	CCCTGGTCCACAAGGATTACA	CACCAGAATCACCCTTGCCT
CTGF	Mouse	AGAACTGTGTACGGAGCGTG	GTGCACCATCTTTGGCAGTG
MMP2	Mouse	AACGGTCGGGAATACAGCAG	TGGTAAACAAGGCTTCATGGG
MMP9	Mouse	CAGACGTGGGTCGATTCCAA	CGCGGCAAGTCTTCAGAGTA
ANP	Rat	TTCTCCATCACCAAGGGCTTC	CACCGCACTGTATACGGGATT
BNP	Rat	GAAGGACCAAGGCCTCACAAA	AACTTCAGTGCGTTACAGCC
NOQ1	Rat	CCACGCAGAGAGGACATCAT	TCAGATTCGACCACCTCCCA
BAX	Rat	TTCATCCAGGATCGAGCAGA	AATTCGCCTGAGACACTCGC
CPT-1	Rat	CCTACCACGGCTGGATGTTT	TACAACATGGGCTTCCGACC
PGC1-α	Rat	AACTCTCTGGAACTGCAGGC	GCTTTGGCGAAGCCTTGAAA

2.10. Coimmunoprecipitation and Ubiquitination Analysis.

Protein A+G Agarose was purchased from Beyotime Biotechnology (China, P2012) and was preserved at 4°C. The protein A+G Agarose was upside down for sufficient suspension before use. The culture medium was discarded, and cells were washed 3 times, and then, 1 mL RIPA lysate was added into each dish to lyse cells completely. To avoid nonspecific binding, normal rabbit IgG (1 μg) and protein A+G (20 μL) were added into each sample (containing 1 mg protein) for incubation 2 h at 4°C on a rotary platform, and then, the supernatant solution was collected for coimmunoprecipitation after 2500 rpm centrifugation for 5 minutes. 1 μg of primary antibody was added into each sample for overnight at 4°C on a rotary platform. Protein A+G (40 μL) was added into each sample for incubation for 3 hours on a rotary platform at 4°C. The supernatant solution was discarded after 2500 rpm centrifugation for 5 minutes; the precipitate was washed with PBS with 2500 rpm centrifugation for 5 min × 5 times, and then, 40 μL 1X SDS-PAGE electrophoretic sample buffer was added for resuspension of the precipitate. The collected protein lysate was boiled at 100°C for 10 min and then were loaded on 10% SDS-PAGE for Western blot and ubiquitin analyses. In this study, we isolated Nrf2 first by immunoprecipitation and then detected the ubiquitin by using anti-Ub antibody.

2.11. Measurement of MDA, GSH, GSSG, and SOD. 50 mg

heart tissue of each sample was homogenized in cold lysis buffer according to the manufacturer's protocol. Total proteins were collected by centrifugation at 12000 rpm for 15 min at 4°C. The CBA protein quantitative kit (Thermo) was used to determinate the protein concentration. The lipid peroxidation malondialdehyde (MDA) assay kit (S0131), GSH and GSSG Assay Kit (S0053), and total superoxide dismutase (SOD) assay kit (S0101) with WST-8 were purchased from Beyotime Co. (Shanghai, China). The examinations were performed according to the manufacturer's instructions.

2.12. Statistical Analysis. Data were presented as means ±

SEM. Comparisons among different groups were performed by one ANOVA, followed by the LSD test using SPSS 19.0. If heterogeneity of variance presented, ANOVA was performed after logarithmic transformation of the data. Statistical significance was registered as $p < 0.05$.

3. Results

3.1. BAI Prevented Diabetes-Induced Cardiac Remodeling.

After evaluating cardiac functions with Echo and pressure-volume loops, body weight and heart and lung weights (HW and LW), as well as tibia length (Tib), were recorded. As shown in Figure 1(a), the HW/Tib ratio was significantly increased in diabetic mice compared with nondiabetic mice. HE staining presented cardiomyocyte hypertrophy and inflammatory cell infiltration (Figures 1(b) and 1(c)). BAI treatment effectively decreased the HW/Tib ratio and prevented cardiomyocyte hypertrophy and inflammatory cell infiltration induced by diabetes (Figures 1(a)–1(c)). The HW, LVW, and LVW/Tib were also significantly increased in the DM group compared to the VEH or BAI group; BAI attenuated diabetes-induced increase of HW, LVW, and LVW/Tib. Cardiac remodeling-associated biomarkers, including ANP, BNP, and β-MHC, were significantly upregulated in diabetic mice hearts (Figure 1(d)), which were attenuated by BAI (Figure 1(d)). The blood glucose (BG) or triglyceride (TG) levels were significantly increased in the DM or DM/BAI group compared to VEH or BAI (Figure S1, A-D). No difference existed between the DM and DM/BAI groups at the beginning of the experiment (Figure S1, A and C); however, BAI treatment significantly decreased BG and TG in the DM/BAI group compared to the DM group at the end of this experiment (Figure S1, B and D). Although BAI treatment reduced BG and TG significantly after 6 months of treatment, the BG and TG in the DM/BAI group was far more than that in the VEH or BAI group (Figure S1, B). Therefore, the protective effects of BAI in DCM might rely on other regulatory mechanisms.

3.2. BAI Alleviated Diabetes-Induced Myocardial Fibrosis.

Masson trichrome staining was applied to assess cardiac fibrosis. As shown in Figure 2, diabetes caused distinctive
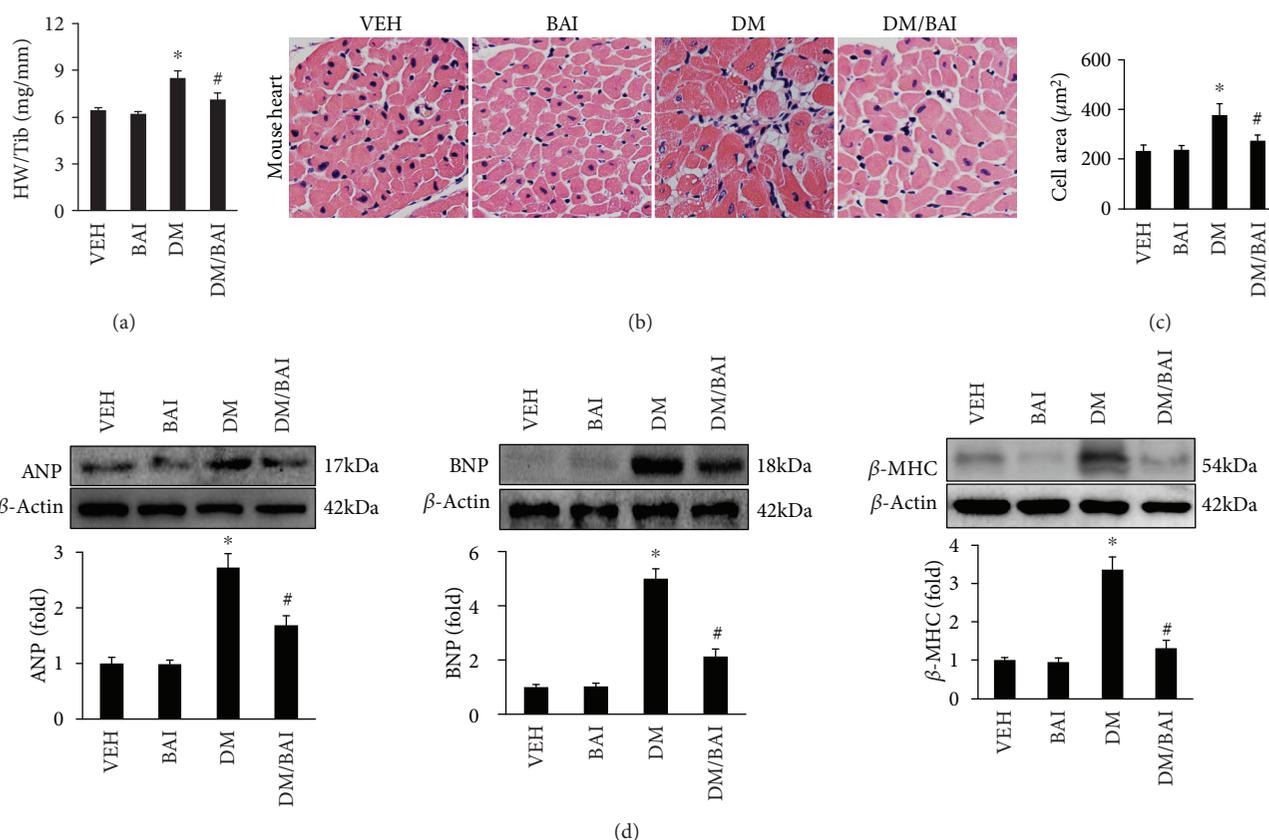
Figure 1: BAI attenuated T2DM-induced cardiac remodeling: (a) heart weight (HW)/tibia length (Tib), (b) HE staining of the left ventricle, (c) calculated cardiomyocyte surface area (CSA), and (d) the expression of cardiac remodeling-associated biomarkers, including ANP, BNP, and β-MHC. $N = 6$/group. T2DM: type 2 diabetes mellitus; VEH: vehicle; DM: diabetes mellitus; BAI: Bailcalin. $^*p < 0.05$ compared with the VEH or BAI group; $^\#p < 0.05$ compared with the DM group.

interstitial and perivascular fibrosis in the heart (Figure 2(a)), which was attenuated by BAI (Figures 2(a) and 2(b)). RT-PCR analysis showed increased expression of profibrotic genes, including TGF-β, collagen I, collagen III, CTGF, MMP2, and MMP9, increased in the DM group (Figure 2(c)), and BAI effectively blocked these changes (Figure 2(c)).

3.3. BAI Improved Diabetes-Induced Cardiac Dysfunction.

Echocardiography was performed to evaluate cardiac function before sacrificing mice at the end of this experiment. There was no difference of HRs among different groups (Figure 3(a)). The results evidenced that LVEDd, LVEDs, LVVd, and LVVs in the DM group were significantly increased (Figures 3(b)–3(e)), but LVEF and FS (Figures 3(f) and 3(g)) were decreased compared with the nontreated group. However, BAI markedly decreased LVEDd, LVEDs, LVVd, and LVVs and increased LVEF and FS compared with the DM group (Figures 3(b)–3(g)). In pressure-volume loop analysis, DM caused significantly decreased dp/dt and -dp/dt and increased Tau compared to the VEH or BAI group (Figures 3(h)–3(j)); however, BAI improved these changes (Figures 3(h)–3(j)). Thus, BAI could significantly attenuate T2DM-associated mouse cardiac dysfunction.

3.4. BAI Improved Lipid Metabolism in the Diabetes Mouse Heart.

Lipotoxicity is more severe in T2DM than in T1DM, which is also a main characteristic in T2DM patients. Therefore, it is meaningful to investigate whether BAI could effectively attenuate lipid accumulation in the T2DM mouse heart. Oil Red staining showed the massive lipid accumulation in the T2DM mouse heart (Figure 4(a)), which could be alleviated by BAI (Figure 4(a)). Furthermore, the level of cardiac triglyceride was significantly increased in the DM mouse heart and was decreased by BAI (Figure 4(b)). Moreover, we found that the phosphorylation of AMPK, a key regulator in cardiac lipid metabolism, was significantly downregulated (Figure 4(c)), and BAI effectively restored AMPK phosphorylation (Figure 4(c)). The phosphorylated AMPK could phosphorylate ACC to blunt its activity. Decreased AMPK was accompanied by decreased ACC phosphorylation but increased accumulation of malonyl-CoA, which caused CPT-1 inhibition. In this study, we also presented that phosphorylation of ACC and expression of CPT-1 were significantly downregulated in the DM mouse heart (Figures 4(d) and 4(e)), and BAI restored ACC phosphorylation and CPT-1 expression (Figures 4(d) and 4(e)). Thus, our investigation suggested that the beneficial effects of BAI on lipid metabolism might be associated with the

(a)

(b)

(c)
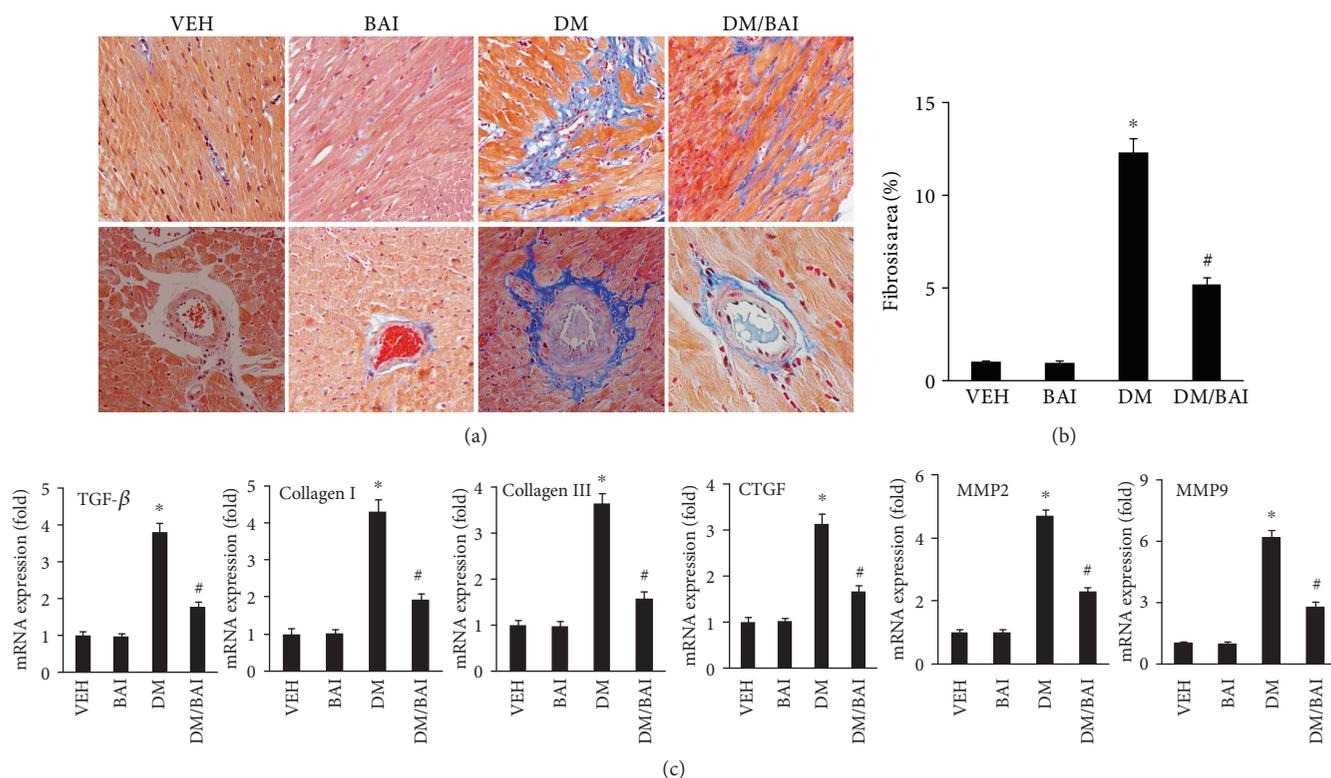
Figure 2: BAI alleviated T2DM-induced cardiac fibrosis: (a) Masson staining for cardiac fibrosis in the mouse left ventricle, (b) the calculated fibrosis area among different groups, and (c) the mRNA expression of fibrosis-associated biomarkers, including TGF-β, collagen I, collagen III, CTGF, MMP2, and MMP 9. $N = 6$/group. VEH: vehicle; DM: diabetes mellitus; BAI: Bailcalin. $^*p < 0.05$ compared with the VEH or BAI group; #$p < 0.05$ compared with the DM group.

AMPK-ACC-CPT-1 pathway. In addition, we also examined the expression of PGC1-α, a key regulator for fatty acid β-oxidation, and the result showed DM-induced down-regulation of PGC1-α, which could be upregulated by BAI (Figure 4(e)).

3.5. BAI Decreased Oxidative Stress in DCM.
Oxidative stress has been demonstrated to exert a key role in the development of DCM [4]. As shown in Figure 5, the T2DM mouse heart exhibited markedly increased oxidative stress evidenced by increased expression of the nitrosative stress indicator 3-NA and lipid peroxidation indicator 4-HNE (Figures 5(a) and 5(b)); both of which were downregulated by BAI (Figures 5(a) and 5(b)). Lipid peroxidation production and the activity of enzymes related to oxidative stress were investigated. Malondialdehyde (MDA), a key indicator of lipid peroxidation, and GSSG, a key indicator of oxidative stress, were significantly increased in the T2DM mouse heart (Figures 5(c) and 5(d)), but GSH and SOD were decreased (Figures 5(e) and 5(f)). These indicated the destroyed equilibrium between prooxidative stress and antioxidant stress substrates in T2DM. Obviously, BAI restored GSH and SOD but decreased MDA and GSSG (Figures 5(c)–5(g)).

3.6. BAI Enhanced Nrf2 Activity.
Natural compounds of flavonoids have been shown to have potent antioxidative stress function, and one of the underlying mechanisms relates to the regulation of the Nrf2/ARE pathway. Moreover, some

previous investigations also indicated that BAI could regulate the activity of Nrf2. In this study, our results showed that expression of Nrf2 was significantly downregulated (Figures 6(a) and 6(b)), and downstream genes of Nrf2, including HO-1 and NQO1 (Figure 6(c)), were also down-regulated in the T2DM mouse heart. However, BAI restored the Nrf2 expression, and nucleus translocation resulted in overexpression of HO-1 and NOQ1 (Figure 6(a)–6(c)). Accordingly, activation of the Nrf2 pathway underlies the protection provided by BAI against T2DM-associated oxidative stress injuries in the mouse heart.

3.7. BAI Exerted Antioxidative Effects via AMPK Dependent on Nrf2.
NRCM were prepared to investigate the potential BAI mechanism of action *in vitro*. The expression and nuclear translocation of Nrf2 were increased in a concentration-dependent (0, 5, 10, and 20 μM) manner after BAI treatment (Figure 7(a)), while p-AMPKα showed no significant change induced by BAI (Figure 7(a)). NRCM were treated with HG/Pal to establish the diabetes-associated cellular model. HG/Pal treatment caused a significant reduction of p-AMPK and Nrf2 (Figures 7(b) and 7(c)), and BAI treatment effectively increased the expression of p-AMPK and Nrf2 (Figures 7(b) and 7(c)). The lipid peroxidation indicator 4-HNE was also significantly accumulated in HG/Pal-treated NRCM, which could be prevented by BAI (Figure 7(d)). Meanwhile, the expression of proapoptosis proteins, including BAX and cleaved caspase 3 (c-caspase 3), was upregulated in NRCM
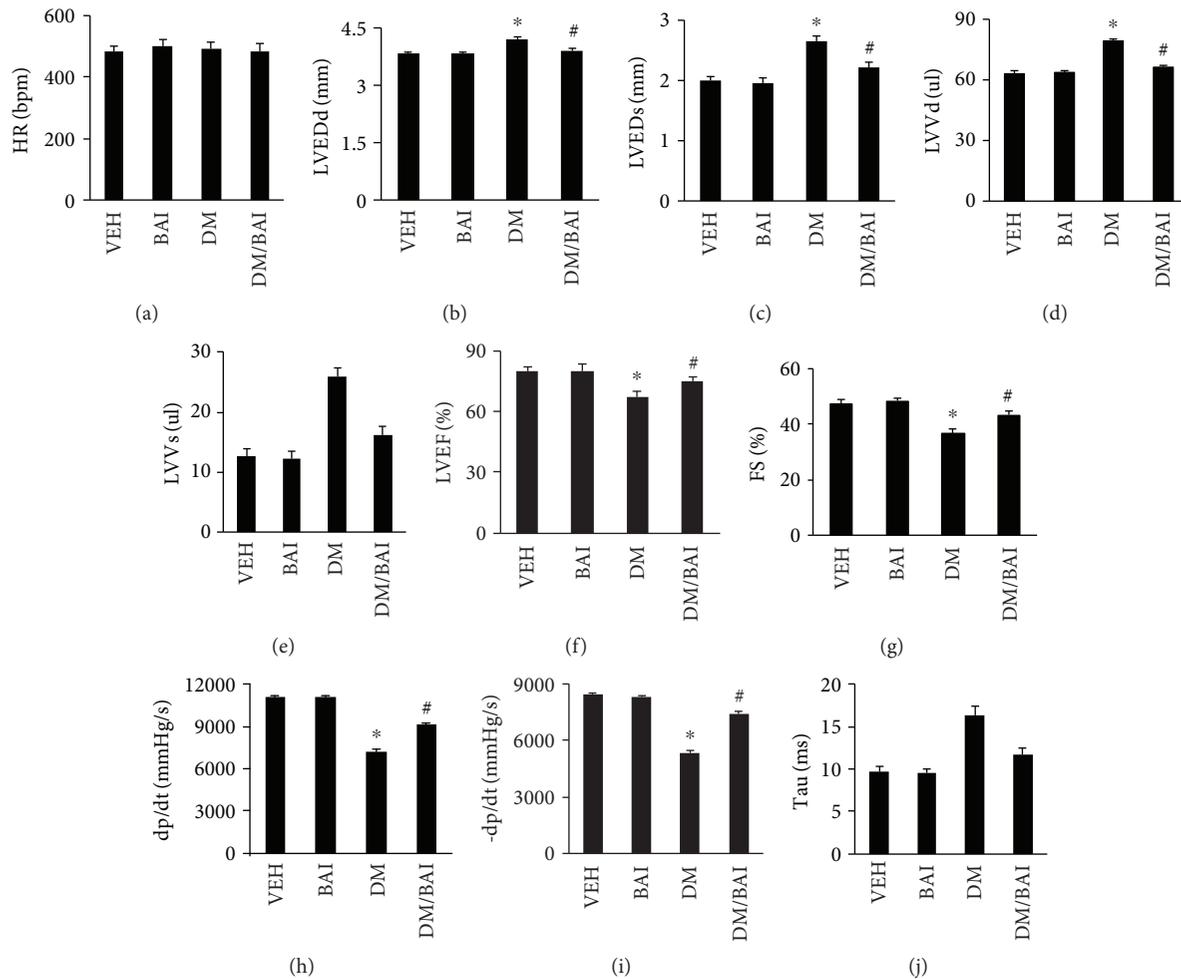
FIGURE 3: BAI improved T2DM-induced cardiac dysfunction. Parameters of cardiac functions examined by echocardiography: (a) heart rate (HR), (b) left ventricle (LV) end-diastolic diameter (LVEDd), (c) LV end-systolic diameter (LVEDs), (d) LV end-diastolic volume (LVVd), (e) LV end-systolic volume (LVVs), (f) LV ejection fraction (EF), and (g) LV fractional shortening (FS); parameters examined by pressure-volume loop: (h) dp/dt, (i) -dp/dt, and (j) Tau. $N = 12$ in the VEH and BAI groups; $N = 16$ in the DM and DM+BAI groups. VEH: vehicle; DM: diabetes mellitus; BAI: Bailcalin. $^*p < 0.05$ compared with the VEH or BAI group; $\#p < 0.05$ compared with the DM group.

(Figure 7(d)), and BAI could markedly inhibit their expression (Figure 7(d)). These results indicated that BAI could attenuate T2DM-associated cardiomyocyte injuries by enhancing the expression of Nrf2 and p-AMPK *in vitro*.

To investigate the interaction between AMPK and Nrf2, specific siRNAs were used to silence Nrf2 and AMPK, respectively. As shown in Figures 7(e) and 7(f), siRNA transfection successfully inhibited the expression of Nrf2 and AMPK. BAI could not increase the expression of Nrf2 or AMPK after transfection with siRNA for AMPK or Nrf2, respectively, in normal cardiomyocytes (Figures 7(e) and 7(f)). HG/Pal treatment increased expression of ANP and BNP (Figures 7(g) and 7(h)), which were inhibited by BAI (Figures 7(g) and 7(h)). BAI inhibited HG/Pal treatment used for mimicking T2DM effects that induced overexpression of ANP and BNP after AMPKα silencing; however, BAI failed to show similar effects after Nrf2 silencing (Figures 7(g) and 7(h)). Nrf2 silencing caused downregulation of HO-1 and NQO1 (Figures 7(e) and 7(i)). BAI treatment could still upregulate NQO1 expression after silence

of AMPKα (Figure 7(i)). The MDA and GSSG were significantly accumulated in NRCM after HG/Pal treatment (Figures 7(j) and 7(k)), which were blunted by BAI (Figures 7(j) and 7(k)). BAI also prevented HG/Pal-induced increase of MDA and GSSG after silence of AMPKα but showed no effect on MDA and GSSG after Nrf2 silencing (Figures 7(j) and 7(k)). BAI could restore CPT-1 and PGC1-α reduced by HG/Pal (Figures 7(l) and 7(m)), but this function was eliminated after silence of AMPK and Nrf2, respectively (Figures 7(l) and 7(m)). Finally, BAI treatment could inhibit BAX mRNA expression with or without AMPKα2 silencing but could not inhibit BAX mRNA expression after Nrf2 silencing (Figure 7(n)). Through the above descriptions, we could conclude the following.

3.8. BAI Prevented Nrf2 Ubiquitination and Modified Keap1. To further investigate the underlying mechanism for BAI-mediated accumulation of Nrf2 in nuclei, experiments were designed to detect the status of Nrf2 ubiquitination and Keap1 modification after BAI treatment. After treatment of
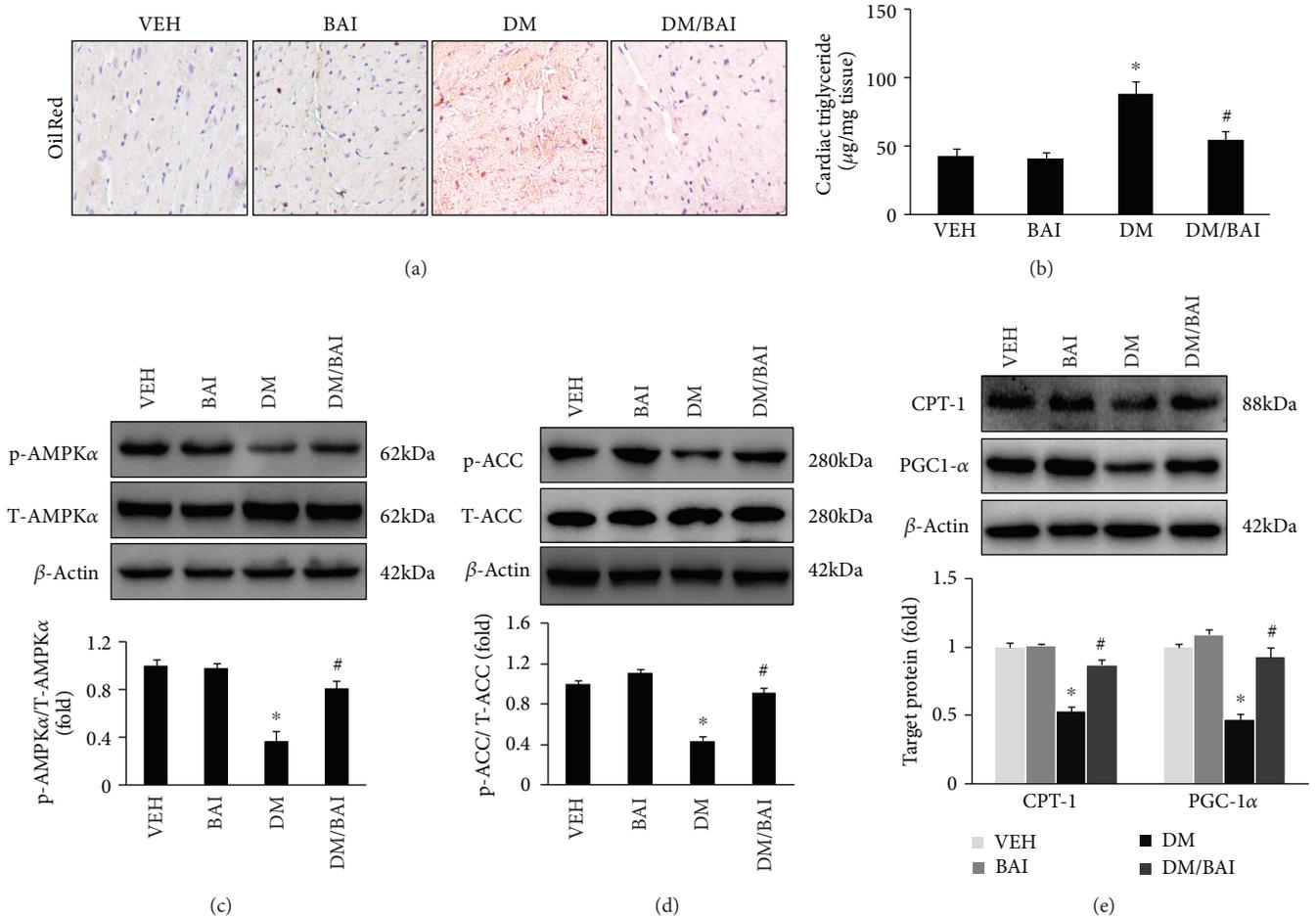
FIGURE 4: BAI decreased lipid accumulation in the heart of the T2DM mouse: (a) Oil Red staining for examining lipid accumulation, (b) cardiac triglyceride level in the mouse heart, (c) expression of phosphorylated AMPKα (p-AMPKα) and total AMPKα (T-AMPKα), (d) expression of p-ACC and T-ACC, and (e) expression of CPT-1 and PGC1-α; β-actin was selected as internal reference. $N = 6$/group. VEH: vehicle; DM: diabetes mellitus; BAI: Bailcalin. *$p < 0.05$ compared with the VEH or BAI group; #$p < 0.05$ compared with the DM group.

NRCM with MG132, a proteasome-specific inhibitor, or BAI, Nrf2 and Keap1 were immunoprecipitated for examining the ubiquitination of Nrf2 and Keap1, respectively. As presented in Figure 8, Nrf2 expression was upregulated after treatment with MG132 and BAI alone or in combination. But expression of Keap1 was downregulated in different treatment groups compared to the control group (Figure 8(a)). Meanwhile, the ubiquitination of Nrf2 (Figure 8(b)) instead of Keap1 (Figure 8(c)) was significantly reduced in the combination treatment (MG132 and BAI) group. These results definitely showed that enhanced expression of Nrf2 was, at least partly, regulated by inhibiting ubiquitination-mediated Nrf2 degradation; however, the decreased Keap1 expression seemed not to be due to ubiquitination degradation. Previous studies showed that some phytochemicals, such as sulforaphane [20] and quercetin [21], induced the production of modified Keap1, which was about 150 kDa. As shown in Figure 6(d), a blot of about 150 kDa was detected after treatment with BAI. Thus, the downregulation of Keap1 mediated by BAI might be associated with the formation of a modified Keap1 protein complex.

4. Discussion

T2DM, composing about 90-95% of the diabetes cases, is the most world spread metabolic disease [2, 3, 22]. Several key pathological abnormalities, including hyperglycemia, hyperlipidemia, insulin resistance, and abnormal insulin secretion because of impaired β-cell function, have been demonstrated to be involved in T2DM patients [2, 3, 22]. The T2DM animal models should cover all of the key pathogenic abnormalities listed above. One of the nongenetic models of T2DM used in this study (combined with HFD and STZ injection) has been suggested to mimic most of these metabolic abnormalities observed in human T2DM [14, 15]. In this study, T2DM-induced cardiomyopathy was characterized by cardiac remodeling, morphological abnormality, fibrosis, lipid accumulation, and cardiac dysfunction. But these pathological changes of the heart could be remarkably prevented by BAI treatment. Mechanistically, BAI could effectively increase phosphorylation of AMPKα resulting in improved lipid metabolism and decreased lipotoxicity and also promote the accumulation and nuclei translocation of Nrf2 to protect
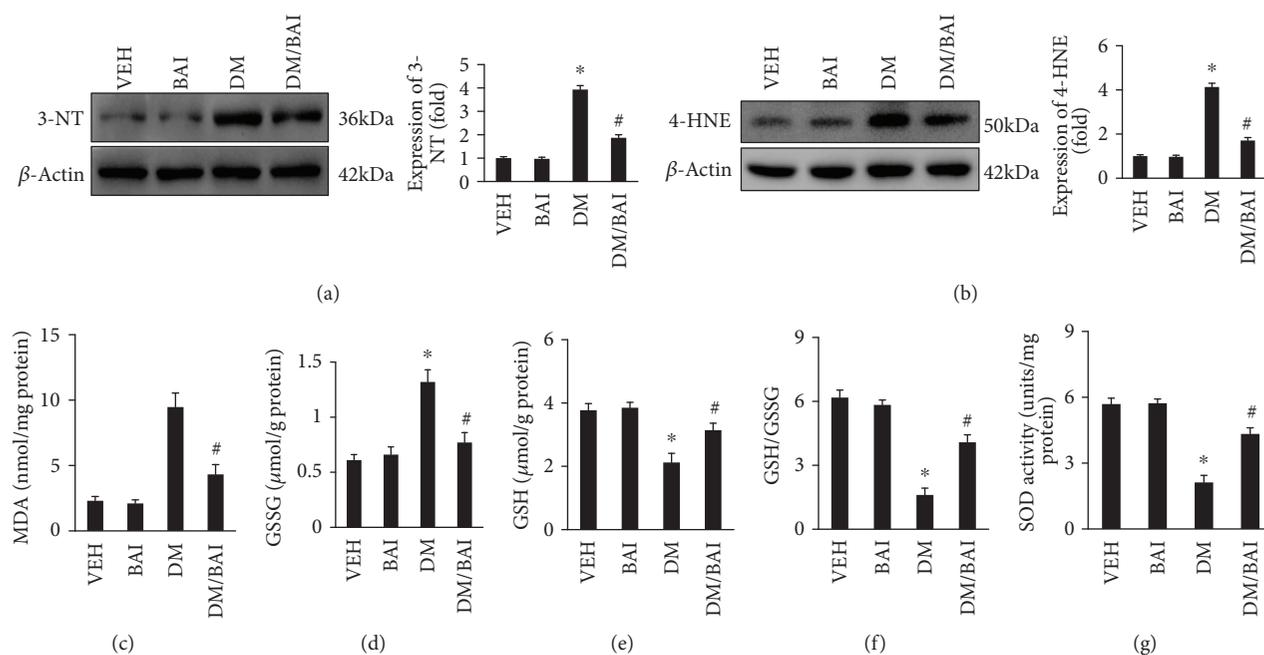
FIGURE 5: BAI decreased oxidative stress in DCM. Representative blots and calculated expression levels of (a) 3-NT and (b) 4-HNE, examining the (c) MDA, (d) GSSG, (e) GSH, (f) GSH/GSSG ratio, and (g) SOD; $N = 6$/group. VEH: vehicle; DM: diabetes mellitus; BAI: Bailcalin. $^*p < 0.05$ compared with the VEH or BAI group; $\#p < 0.05$ compared with the DM group.
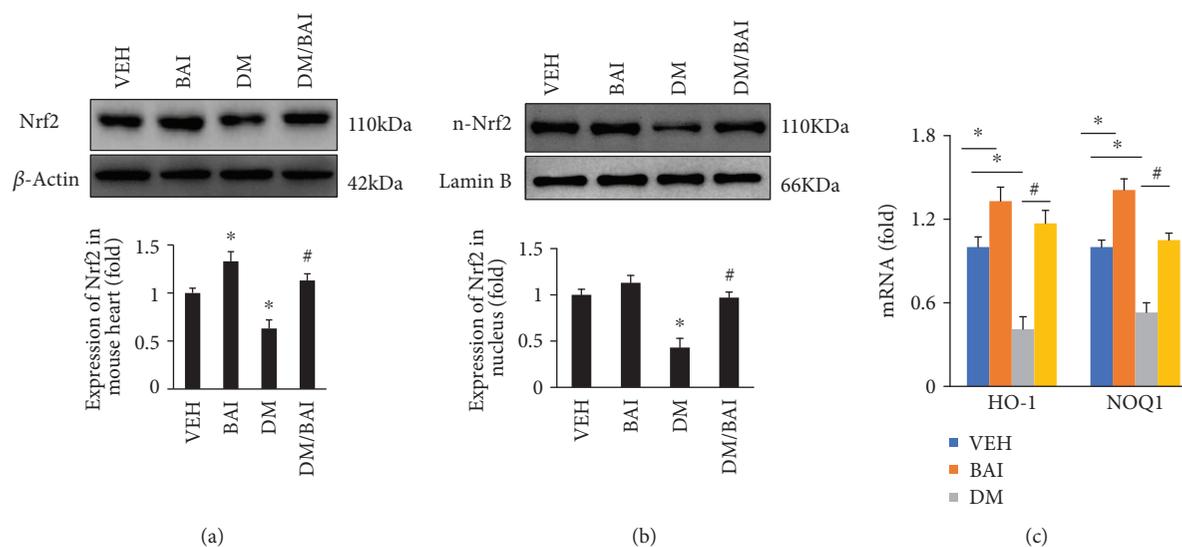
FIGURE 6: BAI enhanced Nrf2 activity. Representative blots and calculated expression levels of (a) Nrf2 in mouse heart tissue and (b) Nrf2 in the nucleus and the (c) mRNA expression level of HO-1 and NOQ1. β-Actin was selected as the internal reference. $N = 6$/group. VEH: vehicle; DM: diabetes mellitus; BAI: Bailcalin. $^*p < 0.05$ compared with the VEH or BAI group; $\#p < 0.05$ compared with the DM group.

against oxidative stress in T2DM mice hearts. Through silencing of Nrf2 in HG/Pal-treated NRCM, we showed that the regulation of AMPK by BAI might be associated with its function for counteracting oxidative stress in cardiomyocyte. Finally, we also demonstrated that BAI might prevent the combination of Keap1 and Nrf2, resulting in decreased ubiquitination and consequent degradation of Nrf2.

Activation of AMPK plays essential roles in regulating lipid metabolism in a mammalian heart [23]. Phosphorylated AMPK (p-AMPK) could promote fatty acid to enter into the mitochondria for fatty acid oxidation (FAO) via regulation of carnitine palmitoyl CoA transferase 1 (CPT-1), which is the rate-limiting enzyme of FAO in the heart [23]. CPT-1 could be inhibited by malonyl-CoA, which is a direct downregulation target of acetyl-CoA carboxylase (ACC) [23]. Activated AMPK could inhibit the activity of ACC to reduce production of malonyl-CoA resulting in CPT-1 activation [23]. In the progress of diabetic cardiomyopathy, the activity of AMPK and CPT-1 was significantly downregulated, but the activity of ACC was upregulated [18]. It has also been

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)
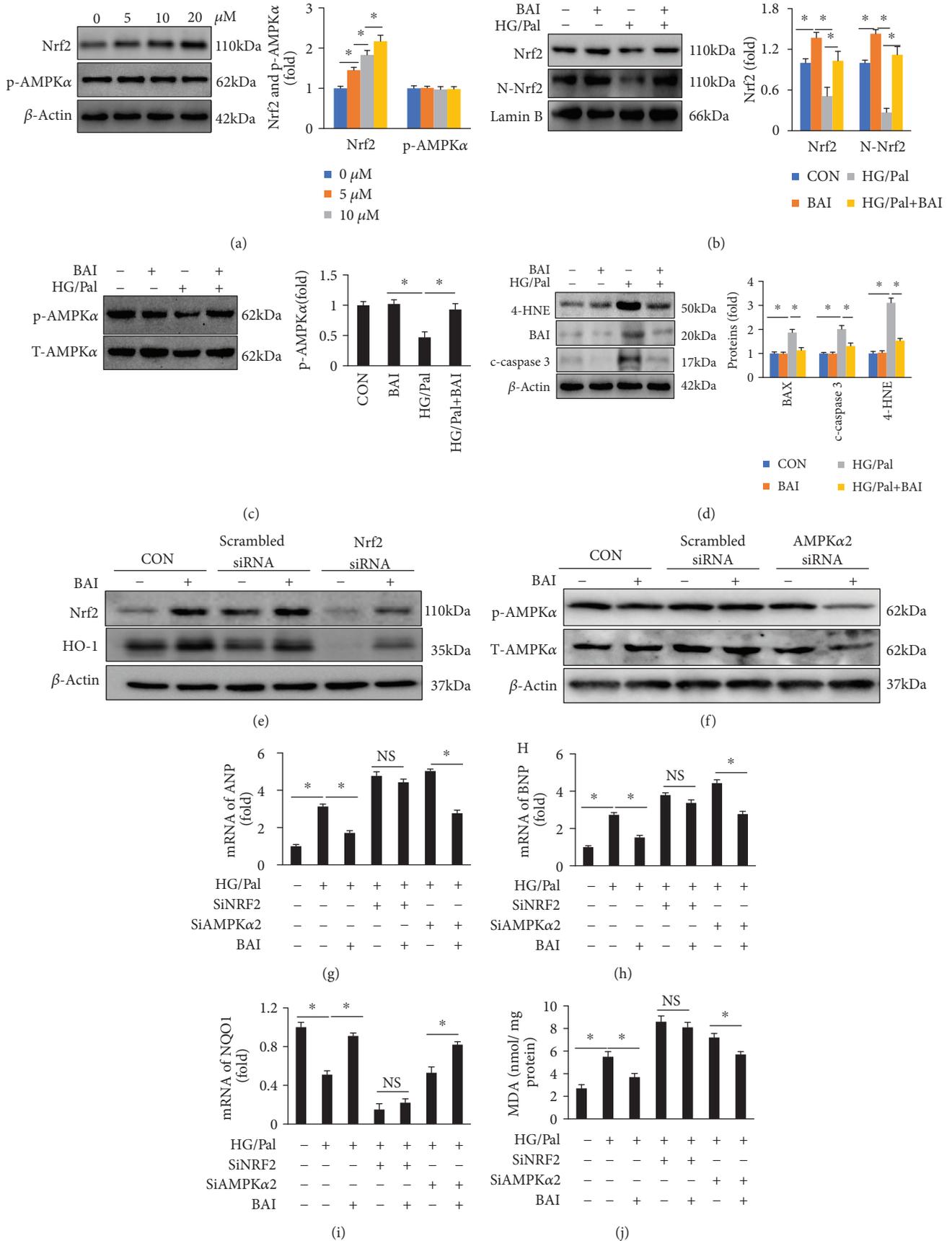
Figure 7: Continued.

(k)

(l)

(m)

(n)
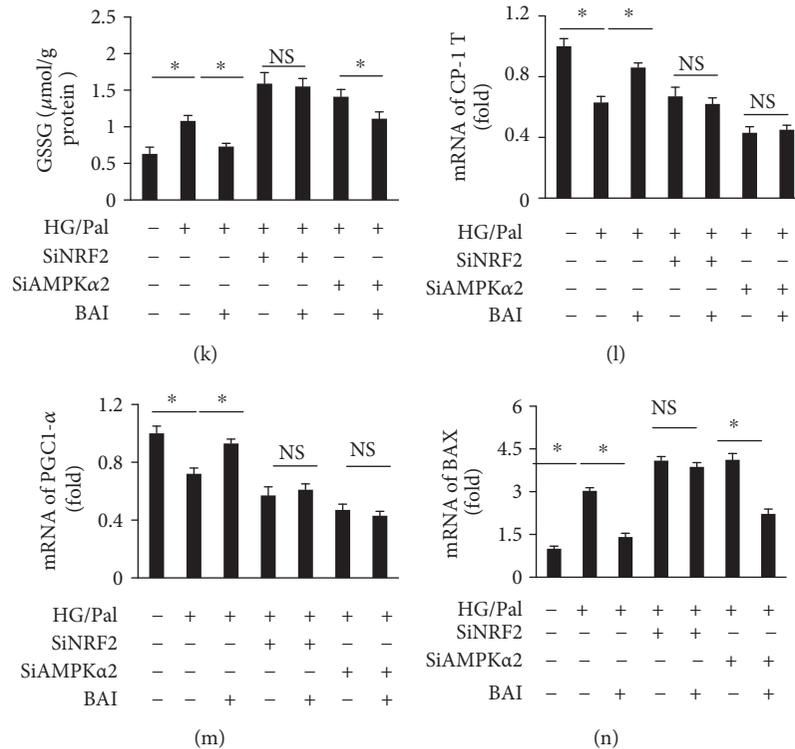
Figure 7: BAI exerted antioxidant effects via AMPK-dependent effects on Nrf2. (a) BAI increased Nrf2 but not p-AMPKα in a concentration-dependent manner. (b) BAI restored the expression and nucleus translocation of Nrf2 in HG/Pal-treated NRCM. (c) BAI restored p-AMPKα in HG/Pal-treated NRCM. (d) BAI inhibited the expression of 4-HNE, BAX, and cleaved caspase 3 (c-caspase 3) induced by HG/Pal treatment in NRCM. (e) Representative Western blots of Nrf2 and HO-1 expressions with or without Nrf2 silencing; (f) representative blots of p-AMPKα and total AMPKα (T-AMPKα) expressions with or without AMPKα silencing; mRNA expressions of (g) ANP, (h) BNP, and (i) NQO1; examination accumulation of (j) MDA and (k) GSSG; mRNA expressions of (l) CPT-1, (m) PGC1-α, and (n) BAX. HG: high glucose; Pal: palmitate; BAI: Bailcalin. β-Actin was selected as the internal reference. The final concentration of $20\,\mu$M BAI was used in experiments; each experiment was repeated three times independently. $^*p < 0.05$ compared with the indicated group.

reported that the activity and expression of PGC1-α significantly reduced in the late stage of DCM, resulting in aggravated mitochondrial damage [24]. The expression of PGC1-α could also be restored after AMPK activation. Consistent with previous studies, we found that phosphorylation of AMPK and expression of CPT-1 and PGC1-α were reduced in the T2DM mouse heart and HG/Pal-treated NRCM; however, BAI could effectively reduce these pathologic changes. These results indicated that the protective effects of BAI for attenuating DCM might be partially ascribed to the regulation of the AMPK pathway.

Oxidative stress is another important pathological process in the development and progress of DCM [4]. Although many mechanisms, including inflammation, autophagy, cardiac hypertrophy, and fibrosis, along with cardiomyocyte apoptosis, have been demonstrated to be involved in the pathological remodeling induced by diabetes, cardiac injuries caused by these pathological mechanisms could be significantly improved via strategies targeting oxidative stress [4]. Because few specific antioxidants are effective for DCM treatment, it is necessary to develop chemicals and drugs against oxidative stress during DCM. Nrf2 has been suggested to be a potential target for many chronic diseases, including neurodegenerative diseases, metabolic diseases, and cardiovascular diseases [5]. Nrf2 could induce the expression of a broad

panel of antioxidant genes, like heme oxygenase-1 (HO-1), glutathione-S-transferase, NAD(P)H:quinone oxidoreductase- (NQO-) 1, and superoxide dismutase, via binding to antioxidant response elements (AREs) [5, 6]. Nrf2 has also been verified to downregulate in the heart of diabetic animals and patients. In experimental diabetic models, Nrf2 deficiency enhanced oxidative and nitrosative stresses and led to early-stage cardiac injuries and dysfunction [3]. The diabetic model with Nrf2 deficiency displayed exaggerated cardiac fibrosis and increased ROS production. Treatment with Nrf2 inducers, such as sulforaphane and myricetin, could effectively protect mice hearts from diabetes-induced injuries [25, 26]. In this study, we exhibited that BAI could increase the expression of Nrf2, HO-1, SOD, and NQO1. Our results definitely indicate that BAI might be an effective inducer of Nrf2 for protecting against DCM.

Next, we would discuss the underlying relationship between AMPKα and Nrf2 with BAI treatment. Some other studies have suggested that hyperphosphorylation of AMPK promoted Nrf2 activation and nuclei translocation [27–29]. Our data demonstrated that BAI could not stimulate the AMPK phosphorylation at the baseline in vitro but could activate Nrf2 in vivo and in vitro at both baseline and pathological status. These results suggested that BAI might not directly regulate the AMPK activity. After AMPKα silencing
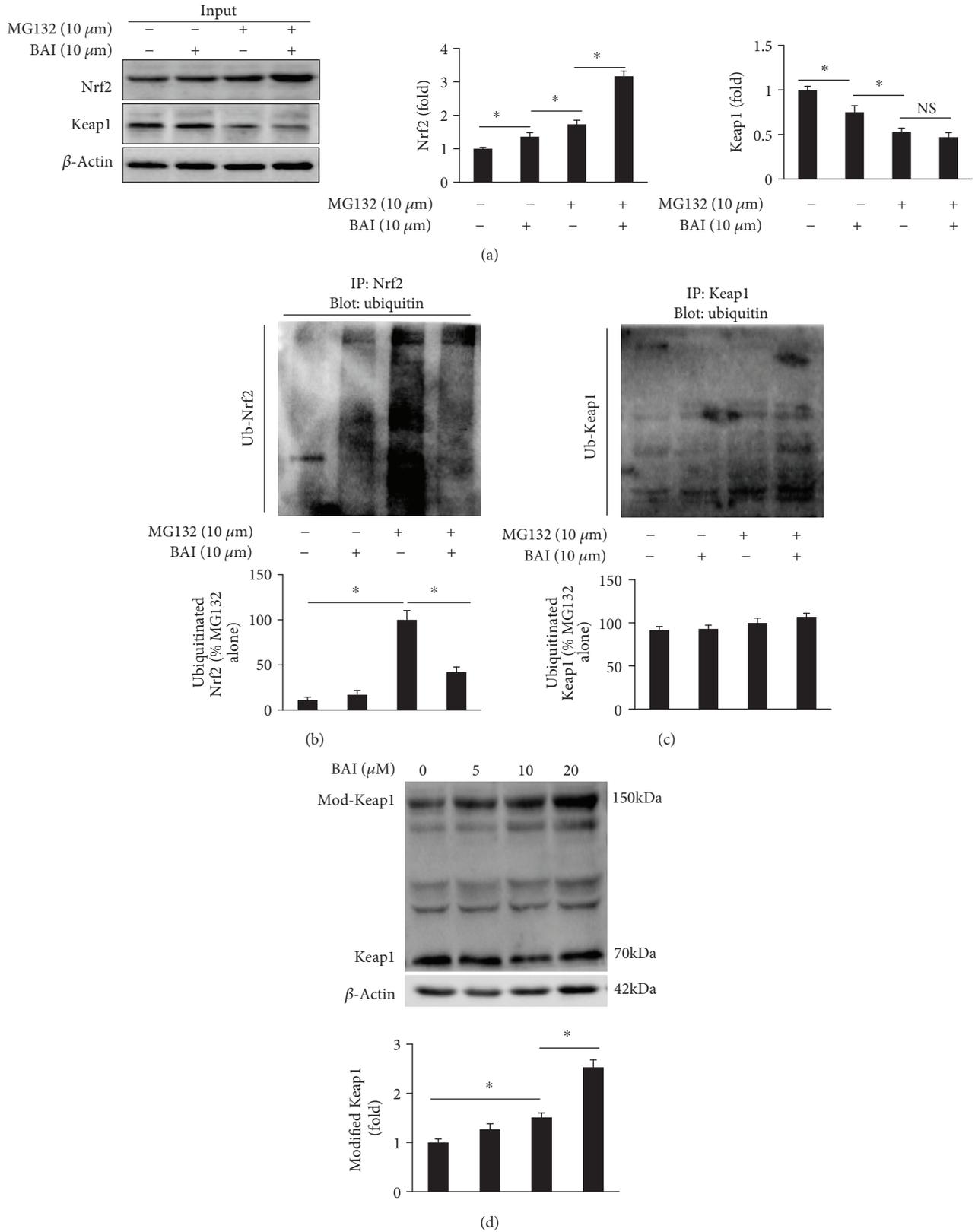
FIGURE 8: BAI modified Nrf2 ubiquitination: (a) representative blots of Nrf2 and Keap1. (b) Proteins were immunoprecipitated with anti-Nrf2 antibody for ubiquitin detection. (c) Proteins were immunoprecipitated with anti-Keap1 antibody for ubiquitin detection. (d) Detection of modified Keap1 after BAI treatment. NRCM were pretreated with MG132 ($10\,\mu M$) for 1 h and then incubated with or without BAI ($20\,\mu M$) for 12 h; each experiment was repeated three times independently. $^*p < 0.05$ compared with the indicated group.

by siRNA, we further showed that BAI-mediated AMPKα phosphorylation might be associated with Nrf2 regulation. The underlying mechanisms for reduced AMPK phosphorylation in DCM were partly ascribed to excessive oxidative stress [30], because excessive oxidative stress contributes to lipid peroxidation resulted in accumulation of 4-HNE [30]. Overproduction of 4-HNE inhibits the activity of LKB1 resulting in decreased AMPK phosphorylation [31]. The present study showed that BAI treatment inhibited 4-HNE accumulation via enhancing Nrf2 activity. A previous study also supported this view that Nrf2 could restore AMPK phosphorylation via reducing 4-HNE accumulation in DCM [15].

Nrf2 was regulated by an E3 ubiquitin ligase complex containing Keap1, Cullin3 (CUL3), and RING-box protein 1 (RBX1) [5]. Under normal conditions, ubiquitinated Nrf2 is degraded by a highly effective proteasomal degradation system, and this constitutive degradation maintains the activity and quantity of Nrf2 at a very low level in variety of cells [5]. The Keap1-GUL3-RBX1 complex will be destroyed when exposed to electrophiles and ROS, which modify the cysteine residues in Keap1, resulting in Nrf2 accumulation in the nucleus and activation of ARE-associated gene transcription [5]. In this study, a significant reduction of Nrf2 ubiquitination, but not Keap1 ubiquitination, was observed *in vitro*. Previous study demonstrated that cysteine residues (C151) in Keap1 were essential for repressing Nrf2 nucleus translocation [32]. Several chemical compounds, including TBHQ [32], sulforaphane [32], and quercetin [21], could induce C151-dependent Keap1 changes which resulted in protection of Nrf2 against Keap1-mediated ubiquitination degradation. According to previous studies, cysteine residue (C151) often participates in reversible disulfide bonds resulting in altered electrophoretic mobility [21, 32]. Previous studies suggested that some compounds, such as TBHQ, sulforaphane, and quercetin, could modify the cysteine residue in Keap1 resulting in an apparent molecular mass about 150 kDa. In this study, we also showed that BAI treatment increased the modified Keap1 in an apparent molecular mass about 150 kDa. Therefore, we concluded here that BAI-induced increase in Nrf2 expression was at least partially due to inhibition of Nrf2 ubiquitination degradation by modifying cysteine residues in Keap1.

Besides this canonical pathway of Keap1-Nrf2 pathway activation, the Akt/GSK-3β pathway and EKR signaling have also been demonstrated to be involved in regulating Nrf2 activity and nucleus accumulation [25, 33]. It also has been reported that BAI might be involved in regulation of Akt/GSK-3β [34] and ERK [35] signaling pathways; so, some other mechanisms might be also involved in promoting Nrf2 overexpression and nuclei translocation after BAI treatment; more experiments are needed to clarify these potential regulating mechanisms.

5. Conclusion

Overall, our study strongly indicates that BAI could effectively alleviate diabetes-associated cardiac injuries. The underlying mechanisms might be associated with restored AMPK activity and enhanced Nrf2 expression. Moreover, BAI was shown to regulate AMPK activity by controlling oxidative stress via activation of Nrf2. BAI might be a valuable adjuvant drug for the treatment of diabetes cardiomyopathy.

Data Availability

Readers are able to access data in this manuscript at any time if they request. If the reader wants to access the data, please email to the first author (Ran Li, email address: fcclir2@zzu.edu.cn) or the corresponding author (Fang Wang, fccwangf4@zzu.edu.cn) at any time.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ran Li and Yuan Liu contributed equally to this work.

Acknowledgments

Supplementary Materials

Supplementary Figure 1: BAI attenuated diabetes-induced cardiac hypertrophy: (A) tibia length (Tib), (B) heart weight (HW), (C) left ventricle weight (LVW), and (D) LVE/Tib. N = 12 in the VEH and BAI groups; N = 16 in the DM and DM+BAI groups; $p < 0.05$ compared with the VEH or BAI group; $^{\#}p < 0.05$ compared with the DM group. Supplementary Figure 2: BAI reduced blood glucose and plasma triglycerides: (A) the blood glucose (BG) at the beginning of the experiment, (B) the BG at the end of this experiment, (C) the plasma triglycerides (TG) at the beginning of the experiment, and (D) the plasma TG at the end of this experiment. N = 12 in the VEH and BAI groups; N = 16 in the DM and DM+BAI groups; $p < 0.05$ compared with the VEH or BAI group; $^{\#}p < 0.05$ compared with the DM group. *(Supplementary Materials)*

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Review Article

Biomarkers of Oxidative Stress in Metabolic Syndrome and Associated Diseases

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Metabolic syndrome (MS) represents worldwide public health issue characterized by a set of cardiovascular risk factors including obesity, diabetes, dyslipidemia, hypertension, and impaired glucose tolerance. The link between the MS and the associated diseases is represented by oxidative stress (OS) and by the intracellular redox imbalance, both caused by the persistence of chronic inflammatory conditions that characterize MS. The increase in oxidizing species formation in MS has been accepted as a major underlying mechanism for mitochondrial dysfunction, accumulation of protein and lipid oxidation products, and impairment of the antioxidant systems. These oxidative modifications are recognized as relevant OS biomarkers potentially able to (i) clarify the role of reactive oxygen and nitrogen species in the etiology of the MS, (ii) contribute to the diagnosis/evaluation of the disease's severity, and (iii) evaluate the utility of possible therapeutic strategies based on natural antioxidants. The antioxidant therapies indeed could be able to (i) counteract systemic as well as mitochondrial-derived OS, (ii) enhance the endogenous antioxidant defenses, (iii) alleviate MS symptoms, and (iv) prevent the complications linked to MS-derived cardiovascular diseases. The focus of this review is to summarize the current knowledge about the role of OS in the development of metabolic alterations characterizing MS, with particular regard to the occurrence of OS-correlated biomarkers, as well as to the use of therapeutic strategies based on natural antioxidants.

1. Introduction

Metabolic syndrome (MS) represents worldwide public health issue. It is characterized by a group of metabolic risk factors in the same person. The key factors are obesity, measured by waist circumference and body mass index (BMI), dyslipidemia, increased blood pressure, hyperglycemia, and insulin resistance [1].

A persistent increase of MS is a risk factor for type 2 diabetes (T2D), cardiovascular diseases (CVD), and premature mortality [1].

The pathogenesis of MS is very complex and not yet clear. Several studies support the concept that oxidant/antioxidant imbalance may play an important role in its manifestations [1, 2].

Increased biomarkers of oxidative stress (OS) and decreased antioxidant defenses have been measured in blood of patients with MS suggesting an *in vivo* overproduction of oxidizing species [1–6]. In particular, it has been reported that patients with MS have decreased antioxidant protection, in the form of depressed serum vitamin C and α-tocopherol concentrations, decreased superoxide dismutase (SOD) activity, and increased protein and lipid oxidation levels [1–6].

Many studies are focused on preventing OS in MS. Recent literature data suggest that diets that are rich in whole grain cereals, fruits, and vegetables, with low animal fat consumption, can ameliorate OS status [7, 8].

This review is aimed at presenting an overview on the role of OS in the pathogenesis of MS and the related diseases.

In particular, it is focused on (i) mitochondrial redox state and dysfunction, (ii) most reported and validated biomarkers of stress in metabolic disease manifestations, and (iii) benefit of various nutritional antioxidants. In addition, the role of "gut microbiota" in MS will be described.

2. Oxidative Stress: An Overview

OS results from unbalanced production of reactive oxygen and nitrogen species (ROS and RNS, respectively) associated with decreased amount/expression and impaired activity of antioxidant systems. When available in appropriate low amounts, ROS and RNS act as signal transduction molecules driving cell activities and providing cell protection [9]. On the other hand, when produced in excess, as in the case of inflamed tissues, they can generate further highly reactive species able to oxidize irreversibly proteins, lipids, and nucleic acids. Very important is the oxidative modification of critical enzymes or regulatory sites, whose redox modification triggers cell signaling alteration and programmed cell death [9].

2.1. ROS and RNS in Physiology and Pathophysiology. A suitable equilibrium between ROS and RNS generation and antioxidant levels allows, without any sort of damage, either the cross talk between cells or the control of fundamental intracellular functions, such as cell-cell interactions, proliferation, differentiation, migration, and contraction. These activities are carried out through the direct or indirect reversible-redox modification of critical targets located within catalytic enzymes or regulatory sites [9]. These targets include iron-sulfur clusters, metals, flavin and nicotinamide cofactors, and quinones. The most relevant physiological targets are protein thiol/disulfides, with particular regard to those present within catalytic enzymes or regulatory sites, as well as in receptors, channels and transporters, transcription factors, kinases, and phosphatases. The reversible thiol oxidation products include sulfenic acid (sulfenylation) and the adducts with $^{\bullet}$NO-derived species (S-nitrosylation), glutathione (S-glutathionylation), and H_2S (S-sulfhydration). These oxidative modifications represent the finest regulation of redox-based signaling affecting protein activity, protein–protein interactions, and protein location, inducing conformational and functional changes of fundamental target macromolecules involved in redox signaling. A slight imbalance by oxidant formation can be counteracted by a performance activity of the antioxidant system, which allows cells to return to their physiological status. In pathological condition, such as inflammation, atherosclerosis, ischemia/reperfusion injury, and diabetes mellitus, the oxidant-generating enzymes in tissues are activated to produce higher amounts of ROS and RNS at nonphysiological locations. These amounts, in association with the impaired activity of the antioxidant systems, lead to the irreversible oxidation of proteins, lipids, and nucleic acids [9, 10]. This unbalanced redox equilibrium, called OS, triggers cell signaling alteration leading to loss of essential cellular functions, senescence, and programmed cell death [9, 10].

2.2. Sources of ROS and RNS. ROS and RNS are chemical heterogeneous molecules that include radical species, such as superoxide anion ($O_2^{\bullet}$), hydroxyl radicals ($^{\bullet}$OH) and nitric oxide ($^{\bullet}$NO), and nonradical species, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and peroxynitrite (ONOO-). They are continuously produced within the cells particularly as (i) the result of the electron transfer processes in the mitochondria and (ii) the activity of several inflammation-linked enzymes, such as NADPH oxidase, nitric oxide synthase (NOS), xanthine oxidase, myeloperoxidase (MPO), lipoxygenase (LOX), and cyclooxygenase (COX) [9]. Interestingly, $O_2^{\bullet}$ and $^{\bullet}$NO are poor oxidizing molecules, with behavior linked to their chemical structure and their thermodynamic properties. These characteristics are expressed by the standard one-electron reduction potential, a parameter suitable to predicting the hierarchy of radical reactivity in terms of ability to transfer the unpaired electron to any oxidized species [11]. The values range from positive one-electron reduction potential (highly oxidizing species) to negative one-electron reduction potential (highly reducing species) [11]. The one-electron reduction potential of $O_2^{\bullet}$ is 0.94 V [11], while the one of $^{\bullet}$NO is -0.80 V [12]. These values are by far lower than the ones taken for the most reactive unspecific $^{\bullet}$OH (2.3 V) and confer to $O_2^{\bullet}$ a low reactivity and to $^{\bullet}$NO to be almost unreactive with most of the biological molecules. Notwithstanding their poor oxidizing potential, $O_2^{\bullet}$ and $^{\bullet}$NO represent the central nucleus of cellular oxidative chemistry, because they both directly and indirectly control the intracellular redox-based signaling and are the precursors of the most important oxidizing species produced under pathological conditions. Compared to many free radicals, $O_2^{\bullet}$ has a relatively long half-life that permits diffusion inside the cell where it can be converted to H_2O_2 by the action of SOD. At low concentrations, H_2O_2 diffuses in intracellular compartments and reacts with specific protein residues, such as cysteine, methionine, and selenocysteine, assuming a key role in the regulation of the intracellular redox signaling [9]. At higher concentrations and in the presence of transition metals, H_2O_2 generates however the strong oxidant $^{\bullet}$OH, which randomly reacts with all the most important macromolecules, inducing lipid peroxidation and oxidation of protein, DNA, and carbohydrates, leading to impairment of their activity and functions. $^{\bullet}$NO is generated via enzymatic reaction catalyzed by the different isoforms of NOS. Moreover, $^{\bullet}$NO can regulate intracellular signaling by directly inducing posttranscriptional reversible modification of cysteine residues in proteins, i.e., s-nitrosylation. On the other hand, the activation of intracellular pathways leading to ROS formation allows $^{\bullet}$NO to rapidly react with $O_2^{\bullet}$ and to produce the strong oxidant ONOO$^-$ [1, 13]. The oxidative chemistry mediated by ONOO$^-$ is led by its direct reactivity with several cellular targets (including CO_2, hemoproteins, DNA, lipids, and protein residues such as thiols, tyrosine, tryptophan, and methionine) or indirect- CO_2-dependent oxidations mediated by strong oxidizing radicals, such as $^{\bullet}NO_2$ (nitrogen dioxide radical) and carbonate radical ($CO_3^{\bullet}$). Importantly, with the further increase of NOS activity such as that characterizing inflammatory conditions, $^{\bullet}NO_2$ rapidly reacts with

•NO forming dinitrogen trioxide (N_2O_3), which in turn mediates nitrosylation of target compounds, such as thiols. Interestingly, under inflammatory conditions the increased OS in tissues can lead to the consumption of substrates or to the oxidation of cofactors, in the specific case of (6R)-5,6,7,8-tetrahydro-L-biopterin essential for the correct NOS activity promoting $O_2^•$ instead of •NO formation, referred to as endothelial NOS (eNOS) uncoupling. This condition increases further OS and decreases •NO bioavailability leading to hypertension and vascular dysfunction in animal models of obesity, increasing further the risk of CVD diseases [13–15].

The prooxidant heme-containing MPO is released by activated neutrophils and monocytes/macrophages in tissues where it catalyzes the conversion of H_2O_2 to hypohalous acids (HOX; X=Cl, Br, and SCN) in the presence of halide and pseudo-halide ions [16]. The major reactive species produced by MPO under physiological conditions are hypochlorous acid (HOCl), hypothiocyanous acid (HOSCN), and species with nitrating activity in the presence of NO_2^- and H_2O_2. These species are key contributors to the physiological microbicidal activity of phagocytes. However, excessive generation of MPO-derived oxidants has been linked to tissue damage in many diseases, especially in those characterized by acute or chronic inflammation [17].

2.2.1. Mitochondrial Functions and OS. Mitochondria are crucial, multifunctional organelles, which actively regulate cellular homeostasis. Their main function is to produce energy as adenosine triphosphate (ATP) via citric cycle (tricarboxylic acid cycle, Krebs cycle). Other cell functions include ionic homeostasis, production and regulation of ROS, lipid and carbohydrate utilization, pH regulation, steroid hormone synthesis, calcium homeostasis, thermogenesis, and cell death [18–20].

As mentioned before, mitochondria are the primary intracellular site of oxygen consumption and the major source of ROS, most of them originating from the mitochondrial respiratory chain. Dysfunction of the respiratory chain may lower the ATP production, increase ROS production, reduce antioxidative capacity, and induce apoptosis. ROS, highly reactive molecules radicals, and nonradicals have the ability to capture electrons from molecules (proteins and nucleic acids) with whom they get in contact, leading to cell damage. In healthy cells, an intricate homeostatic system regulates and maintains optimal mitochondrial function. A failure of this system is observed in obesity, asthma, and metabolic syndrome progression [21]. Mitochondrial dysfunction, characterized by proapoptotic Bax and Bcl-xS proteins, reduces the expression of the antiapoptotic protein Bcl-xL and cytochrome C release and partially activates caspase cascade, high protein carbonyl content, and 8-hydroxy-2¢-deoxyguanosine. Growing evidence suggests that these dysfunctions have a strong relationship with various MS components, resulting in clinical complications [22, 23]. Cheng and Almeida declare that mitochondrial dysfunction is an early pathophysiological event in insulin resistance and obesity development [24]. Mitochondrial dysfunction leads to the activation of stress pathways, which reduce cellular sensitivity to insulin, limiting nutrient influxes and preventing further damages. Chronically, in organs such as liver and skeletal muscle, it appears as reduced mitochondrial metabolism and insulin resistance, following consequent hyperinsulinemia and different nutrient storages in adipose tissue [25–27]. In addition to this, in epithelial and vascular endothelial cells, mitochondrial dysfunction interferes with •NO synthesis leading to oxide/nitrate stress.

Several studies demonstrate that in different tissues, mitochondria adapt physically to nutrient availability and that obesity causes mitochondrial OS and dysfunction [28, 29]. O'Keefe and Bell proved that, apparently, high caloric intake raises plasma free fatty acid (FFA) and glucose levels, which are closely associated with high ROS generation and obesity exacerbation [1, 30]. Conversely, calorie restriction alleviates sarcopenia [31].

In cardiomyocytes of young patients, an excess of body weight impairs mitochondrial function also in the absence of heart failure and diabetes [32]. Moreover, in humans and mouse models, obesity results in mitochondrial dysfunction, skeletal muscle, and adipose tissue [33].

Mitochondria are subject to dynamic processes in order to establish a control system related to survival or cell death and adaptation to changes in the metabolic environment of cells. The mitochondrial dynamic includes several processes such as fusion and fission, biogenesis, and mitophagy. Modifications of the mitochondrial dynamic in organs involved in energy metabolism such as the pancreas, liver, skeletal muscle, and white adipose tissue could be of relevance for the development of insulin resistance, obesity, and type 2 diabetes. Metabolic status can condition the number, form, and function of mitochondria, influencing organ function. Conversely, changes in mitochondrial dynamic influence organ metabolism. Mitochondrial biogenesis is critical for the normal function of cells, and it can be produced in response to an oxidative stimulus. Mitochondrial fusion is linked to increased ATP production, while the inhibition of this process is associated with ROS production [34–36].

2.3. Detection of ROS and RNS in the Biological System. To characterize ROS/RNS and to understand their role in the mechanisms leading to the different pathological conditions and diseases, the specific detection and quantification of the oxidants produced are fundamental. Luminescence-based techniques, such as fluorescence, chemiluminescence, and bioluminescence, have been applied to biological systems (cell-free systems, *in vitro* cell cultures, and *in vivo* animal models) to detect directly cellular oxidizing species, such as $O_2^•$, H_2O_2, •NO, and $ONOO^-$. These methods rely to the use of specific probes, such as dihydrorhodamine, dihydroethidium, dihydrodichlorofluorescein, Amplex Red, and boronates. All of these are able to interact with the formed oxidants and to provide information about the different species produced in the studied system. Initially, these methods have been used to study the changes of the redox environment in cell systems. Subsequently, with increasing knowledge on chemical reactivity and suitable/useless end product formation, several limitations on the use of these probes have been proved [37–39]. These limitations deal with

the chemical features of the probes as well as their reactivity, such as (i) secondary chemical interactions between probe end products, the probe-derived radicals, and the oxidants generated in the studied system; (ii) redox cycling and generation of ROS by probes and their oxidation products; (iii) probe interaction with metals and with the intracellular antioxidants; (iv) intracellular compartmentalization of probes and sites of oxidant formation; and (v) probe sensitivity to light and pH changes [37–39]. Another method suitable for ROS and RNS identification in biological systems is the electron paramagnetic resonance spectroscopy (EPR) that, in combination with the spin trapping technique, allows identifying oxidants such as $O_2^{\bullet}$, $^{\bullet}OH$, and $^{\bullet}NO$. The spin trapping technique permits the species-specific and time-dependent detection of products, showing typical EPR spectral characteristics. The signals obtained from the different formed radicals may be compared to complementary simple control experiments using inhibitors/scavenger/competitor compounds. The main restrictions to the application of the EPR-spin trapping technique are (i) low rate/concentration of oxidant formation in the biological system, (ii) low trapping rate constant of the used probes, and (iii) reduction of the EPR-active spin adduct to EPR silent product(s) by endogenous antioxidants/reducing systems [40].

Recent literature data on this topic illustrate that the simultaneous use of different techniques, i.e., EPR, HPLC, fluorescence, and LC-MS, is the best way to identify ROS and RNS in biological systems. Each of them provides useful information concurring to identify the formed species by detecting the specific adducts and end products [38–40].

2.4. Tissue Antioxidant Systems. Cells contain both enzymatic and nonenzymatic antioxidants that work together to regulate ROS and RNS concentrations. The primary antioxidant enzymes in tissues that are able to detoxify directly oxidizing species include SOD, glutathione peroxidase (Gpx), and catalase (CAT), and bilirubin. Other fundamental antioxidant enzymes such as glutathione reductase (GR), peroxiredoxins (Prx), thioredoxin (Trx), thioredoxin reductase (TrxR), and glutaredoxins (Grx) also contribute to cellular protection by reducing oxidized critical thiols in key enzymes/proteins and maintaining the suitable intracellular redox state [1, 9, 41]. The nonenzymatic system includes endogenous compounds, such as reduced glutathione (GSH), uric acid, bilirubin, and ceruloplasmin, as well as dietary low-molecular-weight compounds, such as vitamins (vitamins A, C, and E), β-carotene, flavonoids, polyphenols, and zinc and selenium. The latter are the key components of enzymes such as Gpx and TrxR [1, 9, 41]. SODs are a group of metalloenzymes that can be compartmentalized in different cellular districts, such as cytosol (CuZn-SOD), mitochondria (Mn-SOD), and the extracellular matrix (EC-SOD). The main activity of this enzyme is to catalyze the conversion of $O_2^{\bullet}$ to H_2O_2.

SODs then contribute to control ROS intracellular concentration and, in conjunction with CAT and GPx, to maintain the flux of H_2O_2 suitable for redox regulation of intracellular signaling. GPx is an enzyme dependent on the micronutrient selenium and plays a crucial role in the

reduction of lipid oxidation and peroxide detoxification. Moreover, together with GR, it plays a critical role in GSH metabolism, reducing glutathione disulfide (GSSG) to GSH, by the NADPH-dependent mechanism. Prxs are thiol-specific antioxidant enzymes that reduce various cellular peroxide substrates, including H_2O_2 and ONOO-, using cysteine-containing active sites. TrxR and Grx have catalytic-redox-active cysteines and catalyze the reduction of protein mixed disulfides. Trx in particular has function in DNA and protein repair by reducing ribonucleotide reductase as well as methionine sulfoxide reductases.

Some of these antioxidants, proteins, and redox enzymes belong to a network connected through substrates and products. Together with oxidized/reduced glutathione and oxidized/reduced Trx, the redox couple $NADP^+/NADPH$ is crucial for the intracellular redox homeostasis. Indeed, it provides reducing equivalents for the two main detoxifying enzyme systems (GR/GPx/GSH and TrxR/Trx/Prx) involved in H_2O_2 removal, in maintaining the suitable thiol/disulfide intracellular equilibrium linked to redox signaling (sulfenic acids, S-nitrosothiols, S-glutathionylation, etc.) [9]. $NADP^+/$-NADPH and the redox enzymes also have a key role in controlling smooth muscle vascular relaxation through the regulation of the intracellular calcium concentration from the potassium channels, the sarco(endo)plasmic reticulum calcium ATPase (SERCA) pump, ion channels controlling membrane potential, and contractile-enhancing systems [42]. The main source of intracellular NADPH is the oxidative branch of the pentose phosphate pathway, with the cofactor being produced by the rate-limiting glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Other sources of NADPH are the cytoplasmatic and mitochondrial isoforms of isocitrate dehydrogenases, formyltetrahydrofolate dehydrogenases, methylenetetrahydrofolate reductase, and the NADP-dependent malic enzymes [43].

Low-molecular-weight compounds are fundamental antioxidant molecules because they can directly detoxify ROS and RNS and repair oxidized biological targets [1, 9, 41]. GSH is the predominant non-protein low-molecular-weight compound (0.5–10 mM) in animal cells and is the major cellular redox buffer, so that its concentration and the one GSSG are commonly used to calculate the GSH/GSSG ratio, universally used as an indicator of the cellular redox environment [9, 11, 23]. GSH reacts with several oxidizing species, including $^{\bullet}OH$, HClO, and ONOO⁻, and is the substrate of GPx in its peroxide detoxification activity.

Vitamin C or ascorbic acid, the first antioxidant defense in tissues, is a biologically ubiquitous and water-soluble antioxidant able to donate an electron to potentially damaging oxidizing radicals such as $^{\bullet}OH$, alkoxyl radical (RO$^{\bullet}$), peroxyl radical (ROO$^{\bullet}$), thiyl radical (GS•), and tocopheroxyl radicals (TO$^{\bullet}$). It has been shown that physiological concentrations of ascorbic acid inhibit oxidation of LDL, lipid, and protein. An important feature of ascorbic acid is acting synergistically with vitamin E and protecting this vitamin from the oxidative modifications. Vitamin E is indeed lipid-soluble, it is the primary antioxidant in LDL and lipid membrane, and its one-electron oxidation product,

the α-tocoperoxyl radical, can be reduced by ascorbic acid. There are eight naturally occurring vitamin E isoforms, namely, α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. α-Tocopherol acts as a radical-scavenging antioxidant against lipid peroxidation by scavenging lipid-centered $ROO^{\bullet}$ before it attacks lipid in other molecules to yield lipid hydroperoxide (LOOH), and a lipid radical ($L^{\bullet}$), which propagates the chain oxidation. β-Carotene is a powerful singlet oxygen quencher and exhibits additional, strong antioxidant properties. It is very reactive to peroxyl radicals and DNA damage, but less so to $^{\bullet}OH$ and $O_2^{\bullet-}$.

Uric acid is synthesized mainly in the liver, intestines, and the vascular endothelium as the end product of an exogenous pool of purines, is a strong ROS and RNS scavenger (such as $^{\bullet}OH$, $ONOO^-$, and HClO), and has metal-chelating properties in blood plasma as in saliva.

Bilirubin has been proved to be a powerful antioxidant in human blood plasma both in its unconjugated form and complexed with serum albumin. In addition to its ability to inhibit membrane lipid peroxidation acting in synergy with the membrane-bound α-tocopherol, bilirubin can scavenge both ROS and RNS and protect proteins from ROS- and RNS-mediated oxidative damage [41]. Prooxidant and antioxidant systems are shown in Figure 1.

2.5. Biomarkers of OS Damage. In addition to the indirect measurement of ROS and RNS levels, oxidized molecules reflect the damage mediated by OS in cells and tissues, and their measurement can be indicative for the occurrence of OS in a specific disease, as well as the potential efficacy of clinical treatments.

As already mentioned, ROS and RNS generically can react with all the macromolecules of biological importance in cell and tissues, generating oxidative modification in lipids, DNA, and proteins that, in some cases, can be the footprint of the oxidant generated [44, 45].

Most of these oxidized molecules can be measured in plasma by using ELISA kits or by HPLC.

Polyunsaturated fatty acids (PUFAs), in particular linoleic and arachidonic acid, are important targets of lipid peroxidation. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are the most investigated end products of lipid oxidation. MDA is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products. It is a specific marker of omega-3 and omega-6 fatty acid peroxidation [46]. 4-HNE is an α, β-unsaturated aldehyde that derives from the oxidation of essentially arachidonic and linoleic acid, which are the two most represented fatty acids in biomembranes [47]. It is toxic at high concentrations (from $1\,\mu M$ to $10\,\mu M$), while it plays a role in signaling activities at lower concentrations (under $1\,\mu M$) [47].

ROS and RNS generate a large number of oxidative modifications in DNA including nucleotide oxidation, strand breakage, loss of bases, and adduct formation. The $^{\bullet}OH$ radical can react with all purine and pyrimidine bases, as well as deoxyribose backbone, generating various products, the most common one being 7,8-dihydroxy-8-oxo-24$'$-deoxyguanosine (8oxodG), which can be assessed noninvasively in the urine [48–49].

The main cellular targets of ROS and RNS in tissues are doubtless the highly concentrated proteins, which undergo posttranscriptional oxidative modifications (oxidation, carbonylation, nitrosylation, and nitration) of specific amino acid residues (cysteine, aromatic amino acids, histidine, and methionine). Some of these modifications are reversible (i.e., some oxidation products and nitrosylation of thiol groups), while others are irreversible (carbonylation and nitration) and can lead to the alteration of protein expression and activity. The reversible oxidative modifications are involved in the physiologic redox regulation of cellular signaling and are involved in particular cysteine in specific proteins or enzymes. Cysteine oxidation usually starts with the formation of sulfenic acid (-SOH), disulfide bridges (S-S-), S-glutathionylation (protein-SSG), and S-nitrosylation (-SNO) [10, 50]. The reversibility is linked to the activity of low-molecular-weight antioxidants, such as GSH and vitamin C, as well as the activity of antioxidant enzyme, such as GR, Grx, Trx/TrdR, and Prdx. Protein S-nitrosylation (addiction of -NO group) can be induced by RNS, such as $^{\bullet}NO$, nitroxyl, and $ONOO^-$. This thiol modification, and its reversibility, plays a pivotal role in cell physiology and signaling, so that S-nitrosylation has been regarded as functionally equivalent to protein phosphorylation and dephosphorylation [50].

The irreversible oxidative protein modification can allow to the definitive alteration of protein expression and activity, which inevitably reflects on cellular trafficking and redox signaling. This is particular true for cysteine residues in specific proteins or enzymes in which the oxidation processes, going further sulfenic acid formation, proceeds with the formation of sulfinic (-SOOH) and sulfonic (-SOOOH) acids, leading to the irreversible inactivation of their activity [9]. The carbonyl groups can be generated after the attack of the $^{\bullet}OH$ radical against the residues of proline, lysine, and arginine [51–53]. Carbamylated proteins are interesting to use as biomarkers, because they may quantitatively reflect the burden of pathological conditions (inflammation and uremia) and are present in plasma or whole blood. Finally, protein tyrosine residues can undergo nitration reaction (addiction of -NO_2 group) to form 3-nitrotyrosine and can be generated through several pathways including their selective reaction with ONOO- and the derived strong oxidants, such as $^{\bullet}OH$, $^{\bullet}CO_3$, and $NO_2^{\bullet}$, with nitrite anion at acidic pH, or with activated peroxidases in the presence of nitrite anion [52]. Protein nitration induces a loss and gain of protein function, compete with protein phosphorylation, stimulate the autoimmune response, and affect protein turnover. Biomarkers of OS are shown in Table 1.

3. Oxidative Stress in Diabetes and Metabolic Syndrome: State of the Art

Elevated OS in individual with T2D and MS has been shown to be one of the major risk factors for an increased risk of cardiovascular disease [1, 2, 5, 53]. T2D is a metabolic disease
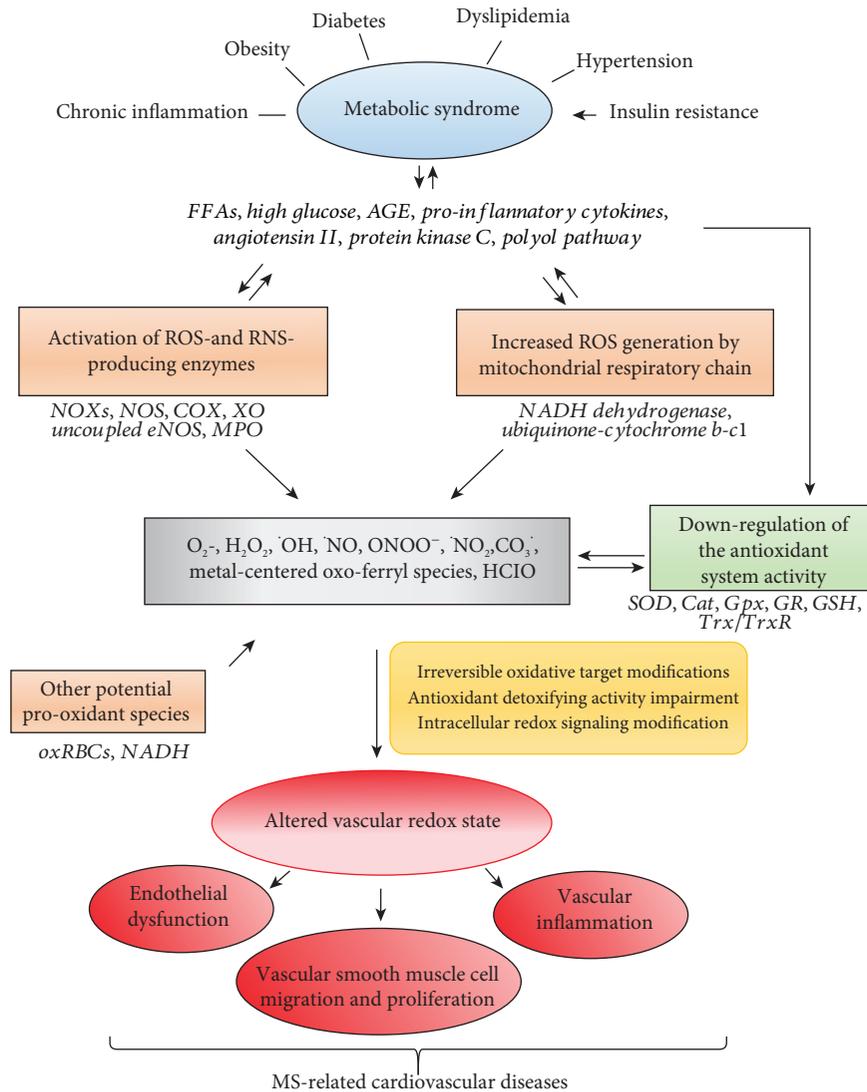
FIGURE 1: Oxidative stress and vascular implications in metabolic syndrome. Metabolic syndrome (MS) is characterized by risk factors having tissue oxidative stress (OS) as hallmark. Indeed, they are characterized by increased release and accumulation of proinflammatory mediators, such as free fatty acids (FFAs), high glucose levels, advanced glycation end-products (AGE), cytokines, and angiotensin II, as well as to the activation proinflammatory signals linked to the stimulation of protein kinase C (PKC) and polyol pathways. These conditions boost the increase in reactive oxygen (ROS) and nitrogen species (RNS) formation in tissues and in the vasculature through the activation of the related producing enzymes in the cytosol as well as in the mitochondria. The cytosolic enzymes include the different isoforms of NADPH oxidase (NOXs), nitric oxide synthase (NOS), cyclooxygenase (COX), xanthine oxidase, protein kinase C (PKC), uncoupled endothelial NOS (eNOS), and myeloperoxidase (MPO). Other potential sources of ROS and RNS are the oxidized RBCs (oxRBCs) and the increased NADH amounts. The former, forming in the vasculature under significant OS conditions, behave as prooxidant cells able also to release oxidant species. The latter, increased at expenses of NADPH under hyperglycemic conditions, can induce mitochondrial deregulation and ROS formation. Noteworthy for the MS-associated cardiovascular complications is the reduction in $^\bullet$NO bioavailability in the vasculature, notwithstanding the NOS upregulation. Indeed, the simultaneous increase in the concentration of $^\bullet$NO and $O_2^{\bullet-}$ allows these radicals to react fast generating the strong oxidant peroxynitrite (ONOO$^-$), which deeply affects intracellular redox chemistry. The MS-associated diseases are also characterized by the downregulation of the antioxidant systems, including the depletion of GSH concentration and the decrease of the activity of the detoxifying enzymes, such as superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (Gpx), glutathione reductase (GR), and the couple constituted by thioredoxin (Trx) and thioredoxin reductase (TrxR). In addition, as in a vicious cycle, the increased ROS and RNS formation can further worse MS-related diseases by affecting in turn the intracellular pathway generating the proinflammatory mediators, as well as decrease the activity of the antioxidant systems. All these conditions result in the irreversible accumulation of oxidation products in proteins, lipids, and sugars, which allow the impairment of intracellular redox signaling and detrimentally affect vascular biology by promoting vascular inflammation, endothelial dysfunction, and vascular remodeling. These alterations underlie the typical MS-associated cardiovascular complications, such as coronary atherosclerotic disease, arterial hypertension, left ventricular hypertrophy, diastolic dysfunction, coronary microvascular disease, and autonomic dysfunction.

TABLE 1: Biomarkers of oxidative stress.

Biomarkers	Features
MDA (malondialdehyde)	Marker specific of omega-3 and omega-6 fatty acid peroxidation
HNE (4-hydroxy-2 nonenal)	Marker of arachidonic and linoleic acid oxidation
Sulfenic acid (SOH)	Marker of cysteine residue oxidation in specific proteins or enzymes (reversible)
Sulfinic (-SOOH) and sulfonic (-SOOOH) acids	Marker of cysteine residue oxidation in specific proteins or enzymes (irreversible)
Protein nitrosylation	Marker of cysteine residue modification induced by RNS (reversible)
Nitrated proteins	Irreversible tyrosine residue modification induced by RNS
Carbamylated proteins	Generated following the attack of the $^\bullet$OH radical to the residues of proline, lysine, and arginine
Oxidized LDL	Generated by LDL oxidation with ROS, RNS, and carbon-centered radicals

associated with increased formation of ROS and RNS, as well as decreased antioxidant potential [54]. The precise mechanism by which OS may accelerate the development of complications in diabetes is partly known [1]. T2D is characterized by chronically elevated blood glucose levels, which may be caused by increased insulin resistance and glucose intolerance. Persistent hyperglycemia in T2D causes nonenzymatic protein glycation and oxidative degeneration. In the early stage of protein glycation, the aldehyde group of sugar reacts with amino acids to produce Schiff's base, which undergoes a series of modifications to form Amadori rearrangement products [55]. The accumulation of advanced glycation end products (AGE) contributes to diabetic complications through direct tissue damage and activation of specific AGE receptors (RAGE) [56]. AGEs and RAGE interaction elicit OS generation in various types of cells and subsequently evoke proliferative, inflammatory, and thrombogenic reactions, playing an important role in the development and progression of diabetes-associated disorders [57]. Biomarkers of protein glycation in T2D are glycated hemoglobin (HbA1c) and glycated low-density lipoprotein (gl-LDL). HbA1c is more negatively charged than hemoglobin and has a higher oxygen affinity therefore reducing gaseous exchange to tissues. Glycated LDL loses its affinity for the LDL receptor, but it is not very inflammatory. Biomarker of oxidative degeneration in T2D is an oxidized low-density lipoprotein (ox-LDL) that, in contrast to gl-LDL, is a proinflammatory and proatherogenic particle containing protein adducts and inflammatory lipids that promote atherosclerosis. Studies have shown that ox-LDL within the vascular endothelium leads to the expression of monocyte chemoattractant protein-1 (MCP-1), known to promote vascular endothelial dysfunction and to increase thrombogenicity [58]. Increased amounts of ox-LDL, carbonylation of cellular proteins, and NADPH oxidase activity can occur in MS leading to enhance ROS formation, which indicates increased risk of atherosclerosis and myocardial infarction as well as increased OS in MS patients [58, 59]. Vascular endothelial dysfunction results in increased ROS production and decreased bioavailability of $^\bullet$NO due to the formation of ONOO$^-$-derived species with oxidizing/nitrating activity. Moreover, ONOO$^-$ has been reported to inactivate prostacyclin synthase leading to the accumulation of inflammatory and prothrombotic eicosanoids [60, 61]. Finally, in endothelial cells, both gl-LDL and ox-LDL drive mitochondrial

dysfunction and high levels of ROS production, especially of $O_2^{\bullet-}$ [62, 63].

MS, characterized by impaired glucose metabolism, dyslipidemia hypertension, and abdominal obesity, is frequently associated with T2D. As for diabetes, OS plays a pivotal role in the pathogenesis of the MS and in the progression of its complications. In MS, ROS production could be increased by an accumulation of plasma FFAs [64] that increases ROS and RNS formation and release in endothelial and vascular smooth muscle cells [64, 65]. FFAs constitute a trigger for ROS increase in adipose tissue, by stimulating NOX and lowering the activity of the antioxidant enzymes [5]. The exposure of adipose tissues to OS results in the development of systemic inflammatory state, which contributes to obesity-associated vasculopathy and cardiovascular risk [66].

Currently, hypertension is considered a primary risk factor for MS linked to the decreased $^\bullet$NO bioavailability and/or to the FFA-mediated ROS increase in blood plasma. This link has been investigated in animal models of hypertension, in which ROS production, endothelial dysfunction, and lipid peroxidation markers have been found increased as a consequence of fructose administration [1]. The role of OS in hypertension has been proved also by the positive effects on ROS production and blood pressure measured after the antioxidant administration in animal models of hypertension.

A number of studies, in human's as well as in animal's models, have stated that most diseases associated with MS, such as obesity, insulin resistance, hypertension, dyslipidemia, and diabetes, cause mitochondrial OS, altered mitochondrial morphology, and oxidative phosphorylation functions, as well as the activation of mechanisms leading to the induction of mitophagy and apoptosis (Figure 2) [1]. In particular, the accumulation of free cholesterol, ox-LDL, and glycated HDL has been reported to mediate endothelial dysfunction through the ROS-mediated impairment of mitochondrial functions and adipocytokines release boosting the atherogenic processes [5, 67–70]. Increased protein carbonyls and lipid peroxidation markers together with decreased activities of mitochondrial antioxidant enzymes (SOD and Gpx) have been measured in adipose tissues from obese patients [71].

Furthermore, the MS-linked pathological conditions, as mentioned before, have been reported to be linked to chronic inflammation mediated by hyperglycemia, FFA and AGE accumulation, and systemic insulin resistance [1, 72]. Several
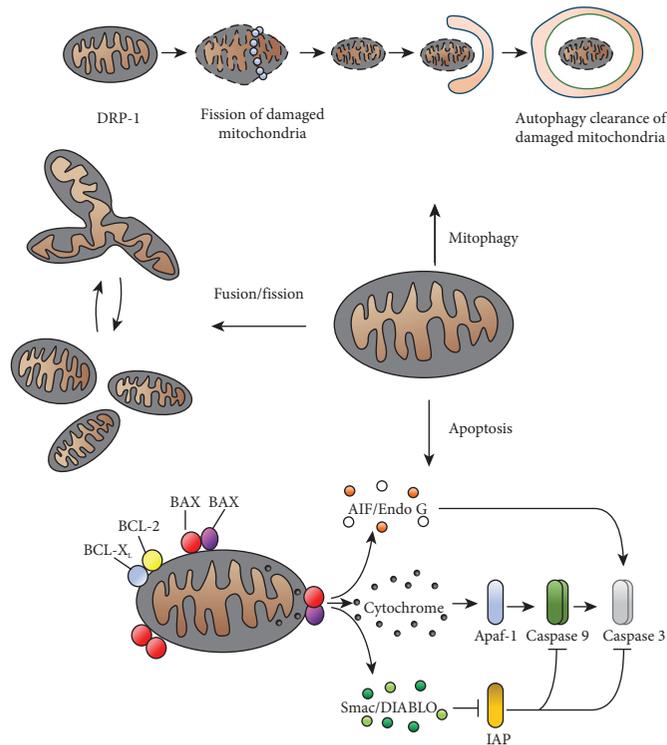
FIGURE 2: Mitochondria dynamics. Summary of major molecular events related to the mitochondria: apoptosis, mitophagy, and process of fusion/fission. In the apoptotic process, the function of BCL-2 family proteins in the control of integrity mitochondrial outer membrane is shown. In particular, the proapoptotic proteins BAX and BAK heterodimering open a channels, which allows the release of proteins such as the cytochrome-c, Smac/DIABLO, Endo G/AIF. These latter, in turn, led to caspase cascade activation, which induces the apoptotic modifications.

proinflammatory mediators, known to promote OS, are released in the vasculature where they boost endothelial activation and dysfunction, causing morbidity and mortality in a large number of MS patients. Among these mediators, inflammatory cytokines (leptin, tumor necrosis factor, and interleukin-6) [73] and high amounts of glucose [74] and FFAs [75] have been recognized to play a pivotal role in the deregulation of the activity of ROS- and RNS-producing systems, such as NADPH oxidases, NOS, and mitochondrial oxidases [1, 76].

The following paragraphs will analyze in detail the contribution of each oxidant source to OS occurrence in MS.

3.1. NADPH Oxidases in MS. NADPH oxidases are multisubunit enzymes present in seven isoforms, called NOX 1–5, and dual oxidase DUOX proteins 1–2 [76]. These enzymes are expressed in a wide variety of cell types, including endothelial cells and vascular smooth muscle cells, in which the isoforms NOX2/NOX4 and Nox1/NOX4, respectively, are mainly expressed [76].

The increase in NOXs mediated by $O_2^{\bullet}$ production has been detected in several MS-related diseases [1, 71, 76, 77]. In an animal model of mice overexpressing one of the NOX subunits (p22phox), an increase in vascular ROS concentration was found. Interestingly, in this study it was observed that a nutrition with a high-fat regimen induced the occurrence of inflammatory status and adipogenesis favoring

obesity and MS phenotype appearance [78, 79]. Moreover, it has been found that FFAs and hyperglycemia promote protein kinase C activation. The activation of this protein is involved in OS-mediated endothelial dysfunction by promoting NOX phosphorylation [77, 79], by activating mitochondrial oxidases [78, 80] as well as the signaling linked to the MAPK p38-mediated NOX induction through endothelin-1 signaling [81, 82]. FFAs and glucose accumulation have been shown to enhance in endothelial and vascular smooth muscle cells, kidney cells, and adipose tissue, lowering in addition the antioxidant enzyme activity [5, 83]. Interestingly, in the endothelium of diabetic mice, FFA-mediated increase in $O_2^{\bullet-}$ production has been reversed by the specific NOX inhibitor, diphenyleneiodonium [84].

Hyperglycemia has been found to promote the NOX-dependent increase in ROS formation in vascular smooth muscle cells, endothelial cells, and cardiomyocytes [78, 85, 86]. High glucose concentration promotes NOX activation through the stimulation of pathways linked to the protein kinase C and the Ca^{2+}/calmodulin-dependent protein kinase and/or in the presence of AGE as well as of angiotensin II [1, 72, 78, 87].

The angiotensin II-mediated protein kinase C-dependent NOX activation has been reported to induce mitochondrial dysfunction in cardiomyocytes and endothelial cells [87, 88]. In addition, high glucose can also stimulate ROS production by inducing mitochondrial dysregulation through

the alteration of internal membrane potential and the increase in the flux of electron transfer donors (NADH and FADH) into the mitochondrial respiratory chain [78]. Despite its involvement in hypertension onset, angiotensin II has been found to inhibit the insulin activity by its binding to the angiotensin type 1 receptor [89], which, in addition to NOX activation, contributes to the occurrence of vascular insulin resistance, endothelial dysfunction, apoptosis, and inflammation. Finally, hyperglycemia could modify cell signaling and antioxidant defense through the activation of the polyol sugar oxidation pathway, which reduces the intracellular amount of NADPH [90]. This cofactor is indeed essential for the activity of several enzymes (GR and TrxR) fundamental for the maintenance of the suitable intracellular GSH concentration and redox balance [9, 10]. In human endothelial cells, AGE, formed by the nonenzymatic reaction of reducing sugars with proteins, lipids, and nucleic acids, promoted NOX activation [91]. Interestingly, the administration of RAGE to diabetic mice partially counterbalances vascular dilation and inhibits NOX expression, confirming the key role of AGE/RAGE signaling in regulating OS and endothelial dysfunction in diabetes [92].

3.2. NOSs in MS. The major sources of $^{\bullet}$NO in the heart tissue are (i) endothelial NOS (eNOS), mainly expressed in the coronary and cardiac endothelium; (ii) neuronal NOS (nNOS), mainly located in the cardiac myocytes [93]; and (iii) inducible enzyme isoform (iNOS), expressed in cardiac myocytes or in neutrophils migrated in this tissue under inflammatory conditions [94]. Among these isoforms, eNOS and iNOS expression was found increased in the heart of diabetic animals [95]. In agreement, by EPR spectroscopy, other authors in blood measured increased amounts of $^{\bullet}$NO and augmented levels of some $^{\bullet}$NO-derived oxidation products (lipid peroxidation, 3-nitrotyrosine formation, and nitrite and nitrate concentration) [94]. Notwithstanding the evidences highlighting its activation, in MS-related disease $^{\bullet}$NO biological activities are in general downregulated due to a dysfunction of the radical metabolism and availability. As reported above, the activation of NOXs measured in the MS-related diseases could lead to increased ROS production. These may decrease $\bullet$NO availability by (i) forming, through the fast direct reaction between $^{\bullet}$NO and $O_2^{\bullet}$ and the vasoconstrictor and cytotoxic oxidant $ONOO^-$; (ii) eNOS uncoupling, which can produce $O_2^{\bullet}$, instead of $^{\bullet}$NO, under OS-mediated depletion of the enzyme cofactors, such as (6R)-5,6,7,8-tetrahydro-L-biopterin; and (iii) the NF-κB-mediated release of proinflammatory cytokines (IL-6 and TNF-α) able to inhibit NOS activity [72, 94]. This is the case of dyslipidemia, hyperglycemia, insulin resistance, and hypertension, which affect NOS activity through ROS formation increase mediated by several sources (NOXs and xanthine oxidase, dysregulated mitochondria, and uncoupled NOS) activated by FFAs, high glucose, AGE, angiotensin II, etc. Other intracellular pathways have been reported to alter $^{\bullet}$NO formation in MS by upregulating or downregulating the different NOS isoforms. In particular, in the myocardium, the activation of the leptin/STAT3 pathway upregulates the gene for iNOS, while that linked to leptin/JAK2/IRS-1

stimulates the Akt-mediated eNOS activity. A reduction in NOS activity has been found (i) as a consequence of the modification (OGlcNAcylation) of eNOS protein at the Akt site (condition favoring endothelial dysfunction in diabetic vascular complications); (ii) in hyperocholesterolemia, which impaired the signaling linked to soluble guanylate cyclase (the key enzyme of the $\bullet$NO signaling pathway); (iii) following PKC activation, which results in the reduction of eNOS activity linked to the phosphorylation of Thr495, which contrarily to the phosphorylation at Ser1177 did not increase the enzyme activity; and (iv) following interaction between AGE and their soluble receptor, which in endothelial cells decreases both eNOS expression and activity [94].

3.3. Mitochondrial Oxidases in MS. The mitochondrial respiratory chain, specifically complexes I (NADH dehydrogenase) and III (ubiquinone-cytochrome b-c1), is the main source of intracellular $O_2^{\bullet}$. Hyperglycemia and obesity allowed mitochondrial dysfunction, including ROS formation, impairing physiological respiration and decreased ATP production in the heart of animal models [96]. Hyperglycemic-linked mitochondrial dysfunction has been linked to the downregulated expression of the peroxisome proliferator-activated receptor gamma coactivator 1a (PGC-1a), a key factor for mitochondrial metabolism involved in oxidative phosphorylation [97]. FFAs, such as ox-LDL, can be the cause of mitochondrial dysfunction and/or apoptosis by impairing oxidative phosphorylation and ROS production in vascular endothelial cells and in cardiomyocytes [98, 99]. AGE and protein kinase C induce mitochondrial ROS formation in through NOX activation [76, 99, 100].

3.4. Red Blood Cells as Potential ROS and RNS Sources in MS. In addition to the biomarkers listed above, there are experimental studies that suggest red blood cells (RBCs) as possible gender-associated biomarkers implicated in the progression of MS [101].

RBCs, under physiological conditions, exert a scavenging activity towards reactive oxygen and nitrogen species often overproduced in morbidity states, for example, in inflamed tissues. Their deformability is an important prerequisite for vascular "antioxidant" functions. Conversely, under conditions of systemic OS, RBCs have an altered redox state and consequently a loss of their structural and functional characteristics, becoming in turn a source of reactive species and contributing to vascular damage.

A significant increase in RBCs displaying morphological alterations has been detected in patients with MS. In particular, differences in terms of cell aging, cell adhesion, and/or aggregation have been found. These data are in line with other literature data indicating erythrocytes as possible biomarkers of vascular disease [102].

Possible implications of OS in the integrity and function of RBCs have been also found in patients with non-insulin-dependent diabetes [103]. In particular, it has been demonstrated that in these patients, RBCs show ultrastructural alterations that could be countered by treatment with the antioxidizing drug *N*-acetylcysteine [103].

Importantly, altered RBC could represent a biomarker playing a critical role in the cardiovascular complications associated with metabolic diseases.

3.5. Gut Microbiota in MS. The human intestinal tract constitutes a nutrient-rich environment colonized by a highly diversified microbial species, collectively called "gut microbiota." Endotoxin derived by microbial species triggers inflammation, leading to MS and contributing to OS. Growing evidence indicates that nutrients and environmental factors are tightly associated with ROS/RNS generation and gut microbiota and that nutritional factors such as certain natural compounds and nutraceuticals may ameliorate oxidative through the change of the microbiota [104]. Diets with elevated intake of high-energy foods have long been considered as a factor in the onset of MS and related diseases. Several studies on animals and humans have showed that consumption of diets rich in sugars and fat induces obesity, dyslipidemia, and insulin resistance, three of major MS components [105–109]. Nevertheless, the mechanisms by which these diets contribute to the pathogenesis of MS are not completely understood.

Studies on healthy adults have revealed that the gut microbial composition is highly host-specific. In fact, there is an elevated interindividual variation in terms of species, strain composition, and abundance and that this may contribute to variations in normal physiology and metabolism [109–113]. Moreover, it has been suggested that gut microbiota specificity is also determined by host genotype, age, sex, and health state [113, 114].

Gut microbiota participates in food digestion through two main catabolic pathways categorized as saccharolytic, with the production of the majority of short-chain fatty acid (SCFA), or proteolytic, which also induces SCFA formation, but leads to other co-metabolites potentially toxic predominantly renally cleared [115–117]. Furthermore, it has been reported that microbiota constitutes and regulates the intestinal mucosal barriers, controls nutrient uptake and metabolism, assists with maturation of immunological tissues, and prevents propagation of pathogenic microorganisms [117–120].

Studies on animal models and humans have also shown that changes in diet are associated with alterations in the composition and diversity of the community gut microbes and metabolic functions [109, 113, 121–128].

In particular, in animal models it has been shown that consumption of fructose, a major component of the Western-style diet, alters gut microbiota composition and induces several markers of the metabolic syndrome, inflammation, and OS. The fructose-rich diet also results in increased plasma levels of nonesterified fatty acid, plasmatic levels of bacterial lipopolysaccharide (LPS), and tumor necrosis factor (TNF-α) [125].

In humans, it has been suggested that both quantity and type of diet (fat or carbohydrate) can modify gut microbiota composition and also this can determine the development and rising incidence of obesity and MS [109, 122, 129–132].

Recent dietary interventions in subjects with high risk of developing CVD have shown that diets rich in fruit and vegetables improve cardiovascular health and impact on the gut microbiota [133–135]. In particular, the FLAVURS study showed that increasing the consumption of fruit and vegetables up to six 80 g portions daily caused a significant increase in total urinary flavonoids, vitamin C, and other phytochemicals [135–137].

Another strategy of modulating intestinal microbiota is by using of prebiotics. They are defined as live microorganisms used to reestablish an appropriate intestinal balance. They may potentially act through different mechanisms including pH modulation, antibacterial compound production, and competition with pathogens [113, 117, 138].

Typical prebiotics are mainly, although not all, dietary sources of fibers. In some studies, prebiotic administration is associated with both improved glycemic control and plasma lipid profiles [117, 128, 139, 140].

It is possible that there may be other factors or mediators yet to be discovered that link gut microbiota to MS. More research is needed to elucidate the several mechanisms underlying diet–gut microbiota–host relationships that determine the development of MS. Main functions of bacteria in the gut are shown in Figure 3.

3.6. Antioxidant Systems and Antioxidant Therapy in MS. A significant decrease in the activity/expression of antioxidant systems has been found in MS patients. Cu, ZnSOD, Gpx, and CAT activity was lower in RBCs of obese women compared with cells of a normal weight group [141]. Interestingly, under inflammatory conditions, which characterize also MS, the expression of Gpx isoform 1 and MnSOD has been found downregulated as a consequence of the suppression of the transcription factor p53, leading to the increase in ROS formation and oxidative damage [142]. In addition to this, the decrease in p53 expression leads to adipose tissue inflammation, local insulin resistance, and cardiometabolic disorders [1, 143]. Furthermore, in addition to several biomarkers of OS (ox-LDL, 3-nitrotyrosine, monocyte $O_2^{\bullet}$ formation, and NOX activity), low amounts of the antioxidant defense nuclear factor E2-related factor 2 (Nrf2) have been measured in blood of patients with MS. Nrf2 is an important antioxidant factor involved in controlling the nuclear transcription of antioxidant enzymes, such as GR, aldo-keto-reductase, heme-oxygenase-1, and γ-glutamyl-cysteine synthetase [144, 145].

Decreased levels of GSH have been measured in blood plasma as well as in RBCs [141] of MS patients, as well as reduced GSH/GSSG ratio animal models of obesity [50]. Interestingly, in addition to GSH depletion, a decreased activity of glucose-6-phosphate dehydrogenase, GR, and Gpx has been found in the serum of T2D patients [146]. As the main intracellular source of NADPH, a suitable activity of glucose-6-phosphate dehydrogenase is essential to maintain intracellular redox equilibrium. Therefore, the hyperglycemia-dependent inhibition of its activity in diabetic patients may have important repercussions on the entire NADPH-dependent antioxidant detoxifying system controlling the intracellular redox status. Moreover, under hyperglycemic conditions, the polyol pathway is the preferential way of glucose consumption [146]. The increased
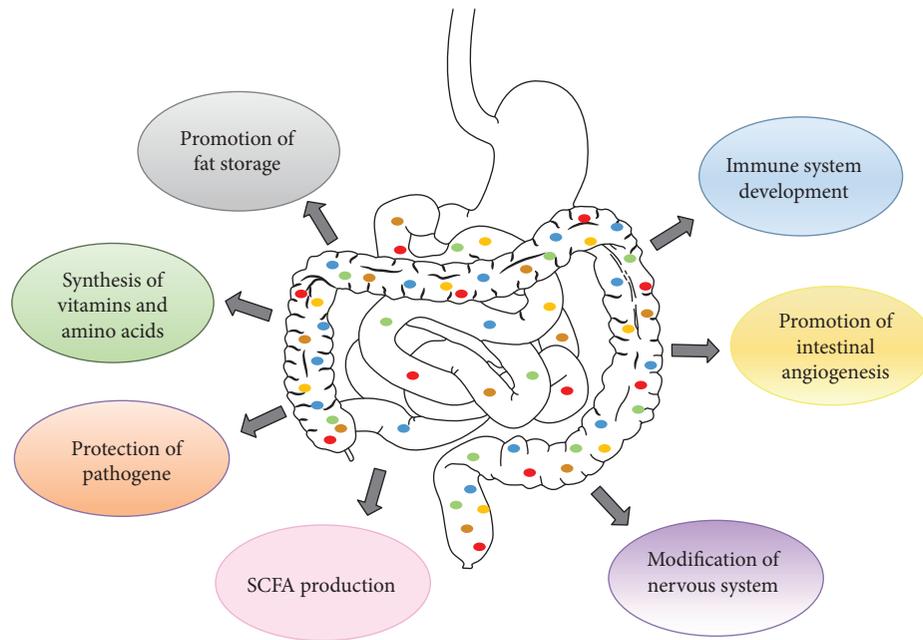
FIGURE 3: Main functions of bacteria in the gut. SCFAs: short-chain fatty acids.

glucose flux in this pathway leads to (i) increased NADPH consumption in the conversion of glucose to sorbitol, (ii) increased NADH concentration, and (iii) decreased GSSG concentration linked to the downregulation of GR activity [146]. The increased NADH concentration could contribute to the increased OS linked to hyperglycemia, by favoring an overflux and recycling of NADH through the mitochondrial electron transport chain leading to ROS hyperproduction [146]. Moreover, under NADPH depletion conditions, the enzymes that use this cofactor as an electron donor cofactor, including NOXs and NOS, could also undergo to malfunction with important collateral effects such as vasoconstriction and platelet aggregation.

Finally, significantly elevated TrxR activity and Trx content have been measured in adipocytes present in subcutaneous tissue of obese people with metabolic disturbances, compared to metabolically healthy obese and control subjects [147]. The Trx/TrxR system could have a protective role in MS adipocytes and could represent an adaptive response to the altered redox environment [147, 148].

Therapeutic strategies aimed at reducing OS and enhancing antioxidant defense have been employed to restore redox balance and consequently to reduce cardiac dysfunction in the MS and related CDV diseases.

Some foods and some specific nutritional components have received special attention for the treatment of MS and related diseases. A diet with a higher total antioxidant capacity (TAC) in plasma has been associated with low OS low risk of abdominal obesity and diabetes mellitus. TAC is an indicator of diet quality defined as the sum of antioxidant activities of the pool of antioxidants present in a food [149]. Its concept was introduced because the evaluation of individual antioxidants does not reflect the antioxidant capacity of a whole diet or the interactions and synergistic effects of various antioxidants.

Mediterranean diet takes an adequate intake of fruits, vegetables, cereals, legume, fish, nut, red wine, and olive oil, which contain several nutritional components with anti-inflammatory and antioxidant properties [149, 150].

The PREDIMED study, a multicenter, randomized, controlled clinical trial, has shown that a Mediterranean diet increases TAC levels in subjects at high risk of cardiovascular disease [151].

The majority of clinical trials and studies in animal models of MS have been performed by using bioactive nutraceuticals from functional food, in particular vitamins (such as vitamins C and E), carotenoids, flavonoids, polyphenols, and selenium, which prevent the CVD in MS patients, although the underlying mechanisms were not fully elucidated for most of these compounds [152].

Vitamin C is an essential nutrient mainly found in fruits, especially citrus (lemon and orange), and vegetables (pepper and kale), whose benefits are associated with its antioxidant and anti-inflammatory properties. It produces its antioxidant effect primarily by quenching damaging free radicals and other reactive oxygen and nitrogen species and therefore preventing molecules such as ox-LDL [149].

Vitamin E, a fat-soluble phenolic compound contained in vegetable oils, is able to reduce OS and total cholesterol in patients with MS [153]. In diabetic patients, the supplementation of vitamins C and E, alone or together, significantly lowered hypertension, decreased hyperglycemia, and increased antioxidant activity, in particular the one of SOD and Gpx [153, 154]. Contrarily, in another study the combination of vitamins C and E did not affect body weights, glycated hemoglobin, LDL, or triglyceride amounts of patients with the MS or T2D [154]. On the other hand, vitamin C administration in an animal model contributed to lower hypertension by preventing the OS-mediated oxidation of the cofactor BH4 preventing NOS uncoupling [155].

With respect to the above vitamins, polyphenols and flavonoids showed a greater efficacy in reducing metabolic and cardiac dysfunctions characterizing MS.

It has been reported that resveratrol (3,5,40-trihydroxystilbene), a phenolic compound mainly found in red grapes and derived products (red wine, grape juice), exerts antioxidant and anti-inflammatory activities, by inhibiting NFκB signaling [156]. In an animal model, it protects the heart, coronary artery, and liver from dysfunctions generated by a high-fat and sugar-fed diet [157, 158]. In obese patients, it reduces insulin, glucose, and lipid concentrations in blood, as well as improving mitochondrial functions in skeletal muscle [159]. In a randomized, double-blind, placebo-controlled trial conducted on 50 MS patients treated for 8 weeks with red yeast rice-olive extract supplement, containing monacolin K and hydroxytyrosol, a reduction in total cholesterol, triacylglycerol, and ox-LDL has been demonstrated. No effects on plasmatic levels of MDA and 8-OHdG were detected [160].

Quercetin, a flavanol naturally present in vegetables, fruits, green tea, or red wine, reduces blood pressure and ox-LDL in blood plasma in overweight subjects with high cardiovascular disease risk [161]. Improved cardiac functions have been obtained by quercetin in animal models submitted to high-carbohydrate and high-fat diet [162].

Moreover, it has been reported that curcuminoid, sulforaphane, and epigallocatechin were effective in controlling hyperglycemia and improving the antioxidant response mediated by Nrf2 [163–165].

Curcuminoids are bioactive principles of the famous dietary spice, having a polyphenolic structure. A meta-analysis of randomized controlled trials shows that supplementation with curcuminoid-piperine combination significantly improves oxidative and inflammatory status in patients with MS [166].

3.7. Mitochondrial Dysfunction as Therapeutic Target. As reported above, mitochondria could contribute to the pathogenesis of MS with increased ROS production, alteration of energy metabolism, and dysfunction of the mitophagy process. Therefore, it is possible that it develops a strategy aimed at preventing mitochondrial dysfunction [167, 168]. The focus of these studies was to choose compounds with antioxidant capacity showing high mitochondrial affinity, such as SOD mimetics (pharmacological mimetics of antioxidant enzyme), coenzyme Q10 (a vitamin-like, lipid-soluble component of the mitochondrial electron-transport chain), and MitoQ (a triphenylphosphonium-conjugated derivative of coenzyme Q). Studies *in vitro* showed that SOD mimetics were effective in reducing ROS and restoring mitochondrial function [169], while in animal models of obesity, SOD mimetic and ONOO$^-$ scavenger improved glucose tolerance [169]. Contrasting results were obtained in patients with MS, in which the addition of coenzyme Q10 to regular clinical therapy reduced diastolic dysfunction in children with cardiomyopathy [170], while the supplementation with the antioxidant was not sufficient to reduce hypertension in patients with the MS [170, 171]. In animal models of obesity, coenzyme Q10 supplementation reduced ROS formation and lipid peroxidation and ameliorated pressure parameters [172].

The use of MitoQ in animal models ameliorated cardiac dysfunction in rats subjected to ischemia/reperfusion [173] and decreased adiposity, hypercholesterolemia, and hypertriglyceridemia in high fat-fed mouse in models of MS [174]. No studies have yet been undertaken regarding the use MitoQ in clinical studies for human MS. The combined supplementation of MitoQ and MitoTEMPOL, another mitochondria-targeted antioxidant, improved mitochondrial activity and protected the coronary artery in rats subjected to ischemia/reperfusion [175].

4. Conclusions

Several lines of experimental and clinical evidences indicate that OS and redox imbalance play a pivotal role in the development of CDV linked to MS. The risk factors associated with MS, including obesity, diabetes, dyslipidemia, hypertension, and impaired glucose tolerance, are characterized by the persistence of OS-mediated chronic inflammatory conditions, as indicated by the occurrence of specific biomarkers in tissues from animals as well as from MS patients. OS may occur by multiple mechanisms, with prominent roles attributable to mitochondrial dysfunction, ROS/RNS-producing enzyme activation, and impairment of antioxidant system activity. Beyond its occurrence, it remains unclear whether OS may be the cause or consequence of MS. This is particularly important for mitochondrial-derived OS and dysfunction, which could be the main sources of oxidative damage and metabolic alterations in MS. Controversial findings have been obtained by using antioxidant therapies aimed at counteracting systemic as well as mitochondrial-derived OS. Other studies and clinical trials, by clarifying the mechanisms linking OS to MS, will allow developing specific and more suitable therapeutic approaches aimed not only at the control of oxidizing species formation but also at developing preventive strategies focused on limiting the occurrence and the progression of the cardiovascular risk factors.

Several factors link gut microbiota to MS. In fact, many data suggest that the gut microbiota has important physiological functions having a direct impact not only on host metabolism but also on gut mucosal barrier development and both local and systemic immune functions. Targeting gut microbiota composition or metabolic functions with natural and safe compounds, such as pro- or prebiotics, to promote a healthier profile might represent, indeed, a promising tool for prevention and treatment of obesity and correlated diseases.

Since OS has emerged as a central player in chronic metabolic diseases, it is imperative to (i) explore further the mechanisms that disrupt the normal equilibrium between oxidative and antioxidative processes; (ii) integrate the current therapies with various nutritional antioxidants such as flavonoids, arginine, vitamin C, vitamin E, carotenoids, resveratrol, and selenium capable of neutralizing OS; and (iii) prevent mitochondrial dysfunction to enhance defenses against OS.

Abbreviations

8oxodG:	8-Oxo-2$'$-deoxyguanosine
AGE:	Advanced glycation end products
ATP:	Adenosine triphosphate
BH4:	(6R)-5,6,7,8-Tetrahydro-L-biopterin
CAT:	Catalase
COX:	Cyclooxygenase
CuZnSOD:	Copper-zinc superoxide dismutase
CVD:	Cardiovascular diseases
DUOX:	Dual oxidases
EC-SOD:	Extracellular superoxide dismutase
eNOS:	Endothelial nitric oxide synthase
EPR:	Electron paramagnetic resonance
FFAs:	Free fatty acids
Gl-LDL:	Glycated low-density lipoprotein
Gpx:	Glutathione peroxidase
GR:	Glutathione reductase
Grx:	Glutaredoxin
GSH:	Reduced glutathione
GSSG:	Oxidized glutathione
HDL:	High-density lipoprotein
HNE:	4-Hydroxy-2-nonenal
IL-6:	Interleukin-6
iNOS:	Inducible nitric oxide synthase
LDL:	Low-density lipoprotein
LOOH:	Lipid hydroperoxide
LOX:	Lipoxygenase
LPS:	Lipopolysaccharide
MCP-1:	Monocyte chemoattractant protein-1
MDA:	Malondialdehyde
MnSOD:	Manganese superoxide dismutase
MPO:	Myeloperoxidase
MS:	Metabolic syndrome
NFκB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS:	Neuronal nitric oxide synthase
NOS:	Nitric oxide synthase
NOXs:	NADPH oxidase isoforms
Nrf2:	Nuclear factor E2-related factor 2
OS:	Oxidative stress
ox-LDL:	Oxidized low-density lipoprotein
PGC-1a:	Proliferator-activated receptor gamma coactivator 1a
PKC:	Protein kinase C
Prx:	Peroxiredoxin
PUFAs:	Polyunsaturated fatty acids
RAGE:	Soluble receptor of AGE
RBCs:	Red blood cells
RNS:	Reactive nitrogen species
RO$^\bullet$:	Alkoxyl radical
ROO$^\bullet$:	Peroxyl radicals
ROS:	Reactive oxygen species
RS$^\bullet$:	Thiyl radicals
SCFA:	Short-chain fatty acids
SOD:	Superoxide dismutase
T2D:	Type 2 diabetes
TAC:	Total antioxidant capacity
TNF-α:	Tumor necrosis factor
TNF-α:	Tumor necrosis factor alpha
TO$^\bullet$:	Tocopheroxyl radicals
Trx:	Thioredoxin
TrxR:	Thioredoxin reductase.

Conflicts of Interest

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

Authors' Contributions

Elisabetta Straface and Donatella Pietraforte contributed equally to this work.

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Review Article

Gut Microbiota in Alzheimer's Disease, Depression, and Type 2 Diabetes Mellitus: The Role of Oxidative Stress

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Gut microbiota consists of over 100 trillion microorganisms including at least 1000 different species of bacteria and is crucially involved in physiological and pathophysiological processes occurring in the host. An imbalanced gastrointestinal ecosystem (dysbiosis) seems to be a contributor to the development and maintenance of several diseases, such as Alzheimer's disease, depression, and type 2 diabetes mellitus. Interestingly, the three disorders are frequently associated as demonstrated by the high comorbidity rates. In this review, we introduce gut microbiota and its role in both normal and pathological processes; then, we discuss the importance of the gut-brain axis as well as the role of oxidative stress and inflammation as mediators of the pathological processes in which dysbiosis is involved. Specific sections pertain the role of the altered gut microbiota in the pathogenesis of Alzheimer's disease, depression, and type 2 diabetes mellitus. The therapeutic implications of microbiota manipulation are briefly discussed. Finally, a conclusion comments on the possible role of dysbiosis as a common pathogenetic contributor (via oxidative stress and inflammation) shared by the three disorders.

1. Introduction

1.1. Gut Microbiota. Recent technological advances have increased the interest on the relationship between the microorganisms inhabiting the gut (gut microbiota) and human health. The gastrointestinal tract hosts over 100 trillion microorganisms including at least 1000 different species of bacteria [1]. In humans, about 1/3 of gut microbiota is "common," while the other 2/3 is different from one individual to another, providing our "personal identity" [2]. Despite the difficulties in defining a "good" microbiota, data suggest that, in adulthood, a healthy microbiota is characterized by the community stability and the species diversity. More specifically, despite lifestyle and food changes, the *Firmicutes* (such as Lactobacillus) and the *Bacteroides* represent the main bacterial phyla in the gut [3] followed by *Proteobacteria*, *Actinobacteria* (such as Bifidobacterium), and *Cyanobacteria* [2], which constitute an ecological community entertaining a beneficial relationship with the host [4]. As a result, an imbalance of the intestinal bacteria representation

(dysbiosis) could lead to different diseases, ranging from inflammatory bowel disease to obesity, diabetes, and asthma, as well as Parkinson's disease, Alzheimer's disease (AD), and depression [5].

1.2. The Gut-Brain Axis. The term "gut-brain axis" refers to a crosstalk between the brain and the gut involving multiple overlapping pathways, including the autonomic, neuroendocrine, and immune systems as well as bacterial metabolites and neuromodulatory molecules [6].

In particular, millions of nerves end in the gastrointestinal tract mucosa, constituting the enteric nervous system which regulates the intestinal functions and communicates with the brain through the vagus nerve. The latter is responsible for the transmission of signals from the brain to the gastrointestinal tract (through the autonomic nervous system) and vice versa [7]. The presence of dysbiosis, causing the breakdown of the intestinal permeability, can lead to an inflammatory condition not limited to the gut, since the proinflammatory cytokines can get into the bloodstream

and reach the brain [8]. The importance of inflammation should not be underestimated, since several evidences support its crucial role in several chronic disorders, such as type 2 diabetes [9], AD [10], and depression [11]. Apart from the cytokines, other mediators can send signals from the gut to the brain through the vagus nerve. In fact, especially after a meal rich of fats and carbohydrates, a subgroup of specialized intestinal cells, named enteroendocrine cells, releases hormones and peptides, such as 5-hydroxitryptamine (5-HT), cholecystokinin (CKK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) [12]. These mediators exert many important functions. For example, PYY and GLP-1 inhibit the intestinal peristalsis and improve glucose metabolism attenuating pancreatic islet hypertrophy and the insulin resistance [13]. Moreover, these peptides, binding their cognate receptors located in the nucleus of the solitary tract and in the hypothalamus, induce the sense of satiety and modulate the energy expenditure [14]. In addition, GLP-1 seems to be able to upgrade hippocampal neural plasticity improving cognition [15] and to stimulate receptors located in the amygdala and in the hippocampus, thus exerting anxiolytic and antidepressant effects [16].

Interestingly, mice assuming a high-fat diet are more likely to present lower levels of short-chain fatty acids (SCFAs) than rodents assuming a low-fat diet [17]. Moreover, low levels of butyrate have been related with a higher risk to develop type 2 diabetes in humans [18]. SCFAs are involved in neurotransmission, since they modulate the synthesis of several neurotransmitters regulating behaviour and cognition. In particular, both butyric and propionic acid enhance the expression of tyrosine and tryptophan hydroxylase, enzymes involved in the synthesis of dopamine, noradrenaline, and serotonin [19]. Interestingly, evidence suggests that several bacteria could directly produce neurotransmitters, including gamma-aminobutyric acid (GABA) and serotonin. Animal studies demonstrate that Lactobacillus rhamnosus is able to modulate GABA receptor expression in the brain, thus exerting a beneficial role in the treatment of mood disorders [20]. Concerning serotonin, it is mainly produced by the gut enterochromaffin cells and modulates several physiological processes (i.e., mood regulation, sleep, and sexual behaviour). Literature data suggest that Escherichia and Enterococcus are able to produce serotonin both directly and through the production of SCFAs [21].

In summary, gut microbiota and brain are strictly intertwined and communicate through different ways, including the production of bacteria metabolites, cytokines, and neurotransmitters [4, 19, 20]. On this ground, it is not surprising that it has been hypothesized that the gut microbiota could play a pivotal role in the pathogenesis of chronic disorders such as depression, AD, and diabetes.

1.3. Microbiota, Inflammation, and Oxidative Stress. Among the various functions of gut microbiota, the regulation of oxidative stress (OS) is probably the most fascinating one [22]. The gastrointestinal tract is rich of sources of nitric oxide (NO), such as intestinal tissues (i.e., mast cells, smooth muscle, and neural plexus), leukocytes, and commensal anaerobes. It has been demonstrated that a high nitrate intake

increases both nitrite concentration and NO. In fact, gut lactobacilli and bifidobacteria are able to convert nitrate and nitrite in NO while enhancing the release of NO by host epithelial cells [23]. In addition to NO production from nitrate, gut streptomycetes and bacilli produce NO through their NO synthetase (NOS) from L-arginine [23]. NO is the principal neurotransmitter of the nonadrenergic, noncholinergic enteric nervous system and is released by the activation of NMDA receptors by glutamate. While nanomolar concentrations of NO exert a neuroprotective function being involved in signaling and apoptosis, excessive NO production is noxious, being associated with neuroinflammation, cellular damage, axonal degeneration, and neurodegenerative disorders [10]. An aberrant production of NO leads to detrimental effects due to the generation of reactive oxygen species (ROS), such as superoxide anions (which form the highly reactive peroxynitrite ion, responsible for protein nitrotyrosilation and inhibition of mitochondrial functions) and hydrogen peroxide, which forms the highly reactive hydroxyl radical that is responsible for lipid peroxidation and DNA damage [24]. The main site of the production of ROS and reactive oxygen nitrogen species (RONS) is represented by the mitochondria, primary energy centre involved in the oxidative reactions leading to adenosine triphosphate (ATP) generation. Recent studies have underlined the existence of an intertalk between host and microbiota mediated by mitochondria [25, 26]. Gut microbiota metabolites, SCFAs in particular, influence mitochondrial function reducing ROS production [27]. Other studies have reported that SCFAs are also associated with a reduction of telomere shortening and DNA damage by altering chromatin structures and inducing the production of the antioxidant glutathione [28] and increasing COX-2 activity [29].

If on the one hand, commensal bacteria exert beneficial effects against OS, on the other hand, pathogens (i.e., Salmonella and E. coli) are able to degrade sulphur amino acids leading to hydrogen sulfide (H_2S) production in the gut. High levels of H_2S are responsible of several negative effects in the host, such as the inhibition of COX activity and the shifting of the metabolism towards glycolysis, thus leading to an increased lactate and decreased ATP production [30]. Moreover, it has been demonstrated that the exposure to high levels of H_2S leads to a decreased mitochondrial oxygen consumption and an overexpression of proinflammatory mediator genes, IL-6 in particular [31].

Considering the important role of the functionally microbiota-derived active substance on host immunological and inflammatory functions, it is apparent that the maintenance of a "healthy microbiota" becomes fundamental for the individual's wellbeing. See Figure 1 for a visual presentation of how dysbiosis favors OS.

2. Alzheimer's Disease

Epidemiological studies reported that 50 million people are affected by dementia worldwide [32] with an age-specific incidence ranging from 5 per 1000 at the age 65-70 to 80 per 1000 for individuals older than 85 years [33].
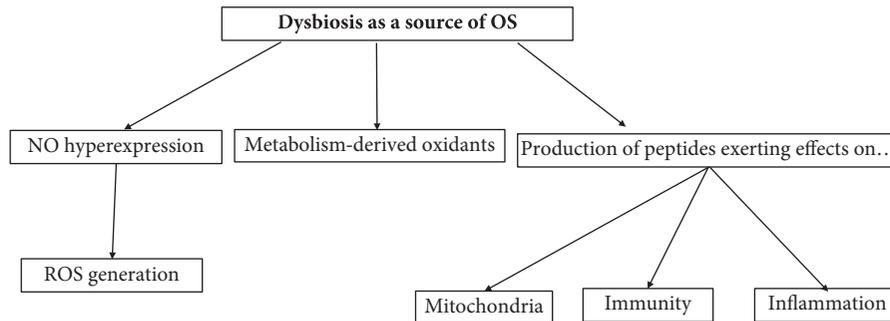
FIGURE 1: Dysbiosis favors oxidative stress and affects the immunological and inflammatory status of the host. NO: nitric oxide; OS: oxidative stress; ROS: reactive oxygen species.

AD is the most frequent neurodegenerative disorders and form of dementia in the elderly [34].

During the early stages of the "typical" form of AD, the main symptom is represented by episodic memory impairment then accompanied by other cognitive domain deficits (visuospatial, attention, language, and executive functions) leading to a loss of abilities of daily living and dementia [35].

To date, thanks to biomarker research, AD is considered a slowly chronic progressive brain disease that can be diagnosed several years before the clinic onset, during a "preclinical stage" [36]. In fact, the pathological hallmarks of AD, including hippocampus atrophy, extracellular amyloid-β (Aβ) plaques, and intracellular neurofibrillary tangles of hyperphosphorylated tau protein can be identified up to decades before the occurrence of cognitive decline and behavioural disturbances [37].

Despite some conflicting results, the role of several genetic [38, 39] and modifiable environmental risk factors in the pathogenesis of AD has been documented [40]. Among the modifiable risk factors, special attention has been given to the role of gut microbiome alteration in the maintenance of the chronic age-related low inflammation [41].

2.1. Oxidative Stress and Alzheimer's Disease: The Role of Gut Microbiome. From a pathophysiological point of view, as mentioned before, the AD brain is characterized by Aβ1-42 aggregation and neurofibrillary tangles with a consequent immune response activation driven by activated microglia. This immune response activation initially allows Aβ clearance, but, during aging, it undergoes alterations thus leading to a progressive deposition of Aβ plaques which inexorably determines synaptic dysfunction, neuron death, neuroinflammation, and OS [42].

In particular, in the AD brain, several sources of ROS production have been demonstrated: (1) mitochondrial dysfunction determined by a cytochrome c oxidase deficiency [43] as well as by an alteration of their permeability due to the OS-related hyperactivation of glycogen synthase kinase (GSK-3) [44], (2) endoplasmic reticulum dysfunction due to its engagement in the elimination of abundant misfolded proteins, such as hyperphosphorylated tau protein [45], (3) metal ions (i.e., copper, iron, and zinc) accumulated in the neuritic plaques [46], and (4) age-related microglial cell hyperactivation with a subsequent high expression and

activation of NADPH oxidase resulting in overproduction of hydrogen peroxide [47].

It should be also emphasized that the CNS is particularly vulnerable to OS for several reasons, including its high oxygen consumption, its use of different reactive species in the signalling process, and its scarce antioxidant metabolism [48].

Although it is still debated whether OS represents the determinant or the immediate consequence of the neurodegenerative processes, OS is indubitably involved in the key events driving the progressive neuronal loss [10]. Markers of lipid peroxidation have been, in fact, detected in biological samples of both AD animal models [49] and patients [50] as well as high levels of protein oxidation markers, such as carbonyls [51]. In this scenario, while a "well-balanced" gut microbiota seems to exert a positive role in the reduction of ROS production via SCFA such as N-butyrate [27], a dysbiosis may lead to systemic inflammation determining, over the years, microglia activation, BBB damage, and consequent crossing of pathogens and immune cells [52].

The most recent theories, in fact, consider AD not only as a result of a confined brain inflammation but also as the consequence of peripheral inflammatory reaction [53]. To support these theories, an association between antimicrobial response and Aβ production has been reported. Indeed, Aβ exerts an antimicrobial peptide role against several pathogens such as bacteria [54] and viruses [55]. It could therefore be hypothesized a periodic, growing with aging, Aβ production following new infections or reactivation of latent brain infections. Moreover, it has been demonstrated that some microbes are able to contribute to Aβ accumulation producing, themselves, a microbial amyloid. The latter, being similar to the cerebral one, easily reaches the brain due to the age-related increased permeability of gastrointestinal mucosa and BBB; being recognized by the immune cells, microbial amyloid determines a massive release of inflammatory cytokines thus sustaining chronic inflammation in AD [41].

Unfortunately, it has been demonstrated that the *age-related reduction of microbiota biodiversity*, with the relative abundance of Proteobacteria and decrease of Bifidobacteria, contributes to the occurrence of dementia not only through the significant reduction of beneficial SCFAs but also through interfering with lipid metabolism. In

particular, Bifidobacteria could exert a fundamental hypocholesterolemic role both *directly*, reducing the absorption and production of cholesterol and facilitating its faecal elimination [56], and *indirectly*, increasing the serum levels of leptin, an antiobesity hormone recently associated with hippocampal long-term potentiation and memory impairment prevention [57, 58]. Considering that lipids exert a fundamental role in APP trafficking and processing thus influencing the Aβ oligomer production, the maintenance of microbiota biodiversity appears to be fundamental. Moreover, it should be noted that a dysfunctional lipid metabolism has been found to be associated with anxiety [59], which has been in turn linked to subcortical amyloidosis in nondemented patients [60].

A recent double-blind controlled trial demonstrated that treating AD patients with a probiotic formulation containing Lactobacilli and Bifidobacteria normalized the serum triglyceride levels and improved cognitive performances, thus confirming the role of microbiota in the maintenance of a balanced lipid metabolism [61].

Another mechanism through which microbiota protects against cognitive impairment is represented by the documented ability of Lactobacilli to reduce ammonia concentration in rats [62].

Ammonia is one of the end-products of protein catabolism, historically implicated in the pathogenesis of AD [63]. Due to its neurotoxic nature even at low concentrations, an efficient astrocyte-modulated brain ammonia detoxification through the formation of glutamine by glutamine synthetase (GS) is crucial [64]. With aging, a lower brain GS activity has been demonstrated with the consequent elevation of ammonia concentration. In turn, hyperammonemia causes a progressive mitochondrial dysfunction, thus determining an increase in ROS production and superoxidase levels, while favoring the decrease of cytochrome c oxidase, superoxide dismutase, and glutathione peroxidase [65].

In conclusion, in an era marked by therapeutic failures despite scientific efforts, deepening the knowledge of the diversified mechanisms through which microbiota could exert a role in counteracting cognitive impairment appears to be of fundamental importance. Further research on the effectiveness of microbiota manipulation as a therapeutic tool is needed.

3. Depression

Depression is probably the most common mental disorder, affecting more than 17% of the American general population [66]. It is characterized by depressed mood, apathy, anhedonia, sleep disturbances, appetite or weight changes, psychomotor retardation [67] or agitation, cognitive impairment, thoughts of guilt, and recurrent thoughts about death or suicide [68]. This mental disorder, strongly associated with fatigue, loss of productivity, and increased mortality, represents an economic burden for public health [69].

Even if the aetiology of depression is still unclear, several neurobiological mechanisms seem to play a role in its occurrence such as (1) the reduction of serotonin, norepinephrine, and dopamine; (2) the alteration of the hypothalamic-pituitary-adrenal (HPA) axis with the consequently elevated plasmatic cortisol level; and (3) the imbalance between proinflammatory and anti-inflammatory mediators [70]. As discussed below, both animal and human studies have demonstrated a certain role of an "altered" microbiota in this biological scenario.

3.1. Gut Microbiota in Depression: The Role of Oxidative Stress. Several studies have reported an imbalance between elevated levels of proinflammatory cytokines, such as IL-1, IL-6, IL-8, IL-12, tumor necrosis factor-alpha (TNF-α), and decreased level of anti-inflammatory cytokines, such as transforming growth factor-beta and IL-10 in patients suffering from depression [70, 71]. Moreover, nonspecific inflammatory markers (i.e., acute phase protein, α1-antitrypsine, haptoglobin, fibrinogen, and C-reactive protein) have been found to be high in depressed patients [72].

From a biochemical point of view, as mentioned before, the activation of the inflammatory pathway is characterized by a hyperproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with a consequent damage of DNA, proteins, mitochondria, and cell membranes [73]. The presence of an oxidative and nitrosative stress is supported by the detection in depressed patients of high levels of by-products of lipid peroxidation such as malondialdehyde and 4-hydroxynonenal [74]. Moreover, if on the one hand, depression is characterized by an increased oxidative and nitrosative stress pathway, on the other hand, endogenous antioxidants such as zinc, glutathione, coenzyme Q10, melatonin, and vitamin E have been found to be decreased. These aforementioned substances, involved in the mitochondrial functioning and in the regulation of cAMP/circadian gene, may lead, if deficient, to neurodegeneration and decreased neurogenesis and neuroplasticity [75]. Probably, the major expression of an "altered" gut microbiota lays just in these oxidative mechanisms. In fact, while germ-free mice present a reduced antioxidant enzyme activity (i.e., catalase, glutathione peroxidase, and superoxide) [76, 77], an altered microbiota can stimulate the NADPH oxidase [78] and the NO synthesis [79], thus inducing OS.

The "depression-associated bacteria" are able to induce depressed mood both directly, producing valeric acid (adenosine A1 receptor inverse agonist) [80], and indirectly, promoting the production of kynurenine from tryptophan [81].

Moreover, while in physiological conditions, gut microbiota is separated from the systemic immune system by the epithelial barrier, in pathological conditions, it can reach the mesenteric lymph nodes determining the activation of monocytes and macrophages and the consequent production of inflammatory mediators with antibacterial properties, such as lysozyme [82].

In normal conditions, the gut epithelial barrier is protected from OS and inflammation by a class of ubiquitously expressed intracellular proteins, the heat shock proteins (HSP). These proteins are chaperones and play a role in the synthesis and folding of other proteins [83].

Due to their role in the repair and stabilization of proteins, evidence suggests that the synthesis of the HSPs is strongly enhanced in physical and psychological stressful

conditions [84]. Nevertheless, if on the one hand, the upregulation of the HSPs can be considered as a cellular "defence" mechanism, on the other hand, their release in the extracellular matrix, occurring during cellular necrosis or apoptosis, can be remarkably harmful. Extracellular HSPs, in fact, are able to stimulate an inflammatory response leading to an increased proinflammatory cytokine secretion [84]. The demonstration of high plasmatic concentration of extracellular HSP70 in depressed patients has led to hypothesize that it could play a role in the occurrence of mood disorders [85]. Interestingly, the physiological epithelial HSP tone is influenced by gut microbiota activity and diversity. Several Bifidobacteria and Lactobacilli are, in fact, strong inducers of gut epithelial HSPs, thus contributing to gut protection [86].

The relationship between depression and microbiota has been known for several decades, when animal studies reported that stressed mice presented marked reductions of the number of gut lactobacilli [87]. Recently, human studies have confirmed this early observation, reporting a less represented microbial diversity, with a relative abundance of *Bacteroidetes* and a reduction of *Lachnospiraceae*, in depressed patients [88]. In addition, chronic depressed subjects present high plasmatic levels of immunoglobulin (Ig) A and IgM antibodies against the LPS of *Enterobacteriaceae*, thus supporting the hypothesis that microbiota could play a role in the occurrence of depression probably sustaining a chronic inflammatory status [82]. Moreover, stressed germ-free mice present high circulating levels of ACHT and corticosterone, depression-sustaining hormone [89].

On these grounds, even if literature data still report conflicting results, the interest in treating depression through the administration of probiotics is growing so that the term "psychobiotics" is increasingly used. However, despite probiotics are able to decrease the plasmatic levels of cortisol with a consequent psychological wellbeing and a reduction of depressive symptoms [90], literature data are still inconsistent. In fact, while some studies demonstrated the efficacy of probiotic augmentation in the reduction of depressive symptoms in drug-resistant patients [91, 92], the administration of probiotics alone seems to be ineffective [93].

Nonetheless, it is undeniable that the potential usefulness of probiotics in major depression is a fascinating and worthy of investigation topic, also considering the elevated tolerability of these compounds.

4. Type 2 Diabetes Mellitus

Diabetes and obesity, two world epidemics, represent a global challenge for health care [94].

The 2016 *Global Report on Diabetes* by the World Health Organization (WHO) states that in 2014 the prevalence of diabetes reached up to 8.5% in the adult population, while more than 1 in 10 adults were obese [95]. Diabetes mellitus was the 7th cause of death in 2016 and is projected to move up in the rankings by 2030 [96, 97].

Hence, research pertaining obesity, metabolic syndrome, and type 2 diabetes mellitus (T2DM) has been focusing on the identification of potentially modifiable dysfunctions, among which dysbiosis of the gut microbiota has been attracting much interest [98–101].

As previously stated in this review, gut microbiota plays a fundamental role in physiological and pathophysiological processes occurring in the host and it is apparent how diet could directly influence the fine balance of the intestinal ecosystem [102–105].

4.1. Gut Microbiota in Obesity, Insulin Resistance, and T2DM: The Role of Oxidative Stress. Obese individuals are frequently characterized by insulin resistance, a condition associated with low-grade subclinical inflammation leading to hyperglycemia and favoring the onset of T2DM [106].

An animal-model study demonstrated how gut microbiome transplant from twin mice discordant for obesity to germ-free mice influenced the metabolic arrangement of the host. More specifically, mice receiving faecal transplant from obese donors had a higher tendency to develop obesity compared to mouse receiving the transplant from a lean donor. Interestingly, diet modulated the colonizing of the microbiome and acted as a protector from developing the obesity phenotype [107].

Differences between obese and lean people both in terms of concentration and type of resident bacterial population have been reported. Overall, individuals with a low bacterial richness are characterized by marked adiposity, insulin resistance, and dyslipidaemia [108]. *Firmicutes* and *Bacteroides* phyla account for the 90% of the adult gut flora. An imbalance between the two species has been linked to obesity with conflicting results, but it seems that a reduced production of the microbiota-induced fermentation product butyrate (exerting anti-inflammatory and antioxidative properties and increasing insulin sensitivity in mice) and an increased release of LPS (already described as endotoxin) could favor obesity; the latter is characterized by a microbiota prone to harvest energy from diet [109, 110].

Concerning T2DM, gut alterations lead to an enrichment in membrane transport of sugars, branched-chain amino acid transport and sulfate reduction, decreased butyrate biosynthesis, and OS response that could account for the proinflammatory state characterizing diabetes. Interestingly, patients suffering from T2DM show a dysbiotic condition characterized by a decrease of butyrate-producing bacteria (such as Firmicutes, Roseburia intestinalis, and Faecalibacterium prausnitzii) and an increase in opportunistic pathogens. Despite this unfavorable condition, the gut environment of T2DM attempts to limit OS, with an increase in functions related to OS resistance, such as catalase, peroxiredoxin, and glutathione reductase. In general, despite the role of some metabolites (such as butyrate) needs to be further confirmed, it has been hypothesized that dysbiosis in T2DM could be related to qualitative, more than quantitative, changes in the gastrointestinal ecosystem and a condition of OS that could increase the risk of diabetes complications [101, 111].

While scientific evidence supports the link between gut microbiota and metabolic syndrome [100], the relationship between inflammation, gut microbiome, and metabolic alterations has not yet been clearly elucidated.

A potential interconnection between these three factors could be represented by diet; indeed, it has been reported that a high-fat diet leads to an increase in the proportion of lipo-polysaccharide- (LPS-) containing microbiota and in the LPS plasma levels, thus leading to the so-called endotoxemia. The latter contributes to the occurrence of insulin resistance and accounts for chronic low-grade inflammation and OS characterizing metabolic syndrome. The circulation of endotoxins is made possible through the permeability alterations and the changes in composition affecting the gut [112, 113]. LPS directly sustain insulin resistance and inflammation via toll-like receptor (TLR) signaling. TLR4 in particular serves as a coreceptor for the monocyte differentiation antigen CD14 and mediates the LPS-induced inflammatory cascade and innate immune response [113, 114].

The discussed findings have practical implications. In fact, it can be inferred that the manipulation of intestinal ecosystem could interfere with the dysbiosis-related prooxidative and proinflammatory mechanisms described above. Preliminary reports support this hypothesis. In fact, the use of antibiotics in mice has been related to reduced endotoxemia, OS, and inflammation [115]. In addition, *Lactobacillus*, *Bifidobacterium*, and *Enterobacter halii* are bacterial candidates for the treatment of obesity, even though more studies are needed to confirm their efficacy [116]. In terms of future perspectives, the characterization of faecal metagenome could help identifying people at high risk of developing metabolic and inflammatory complications [18].

5. Gut-Brain Axis: Therapeutic Perspectives

As stated above in the sections dedicated to AD, depression, and diabetes, several studies have reported the usefulness of microbiota manipulation in the treatment of these disorders [61, 91, 115]. In recent years, special attention has been given by researchers to probiotic supplementation with promising results [117–119]. Despite further insights on the efficacy of dysbiosis restoration in these disorders are needed, the gut-brain axis cannot be simply dismissed as a "fashionable topic" [120].

6. Conclusion

Dysbiosis has been demonstrated to exert regulatory functions on inflammation and OS and represents a pathogenetic contributor shared by AD, depression, and T2DM [8, 22, 41, 81, 101], three disorders characterized by a prooxidative and proinflammatory condition [42, 70, 111]. The gut-brain axis can account for the molecular similarities linking these disorders, also confirmed by the high rates of comorbidity between depression and T2DM, which in turn increase the risk of dementia. Metabolism, cognition, and mood are strictly intertwined; if glucose toxicity can directly interfere with the cognitive functions, the insulin pathway is involved in amyloid formation, while depression can precipitate neuronal damage through inflammatory mechanisms [121]. Deepening the knowledge on the pathogenetic mechanisms of these burdening disorders could open new scenarios. In fact, the manipulation of the gut environment could be

further investigated as a preventive and/or therapeutic tool with (potentially) a good safety profile.

Conflicts of Interest

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

Authors' Contributions

Maria Luca and Maurizio Di Mauro contributed equally to this work.

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Review Article

Relationship of Oxidative Stress as a Link between Diabetes Mellitus and Major Depressive Disorder

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Both conditions, major depressive disorder (MDD) and diabetes mellitus (DM) are chronic and disabling diseases that affect a very significant percentage of the world's population. Studies have been shown that patients with DM are more susceptible to develop depression, when compared to the general population. The opposite also happens; MDD could be a risk factor for DM development. Some mechanisms have been proposed to explain the pathophysiological mechanisms involved with these conditions, such as excess of glucocorticoids, hyperglycemia, insulin resistance, and inflammation. These processes can lead to an increase in damage to biomolecules and a decrease in antioxidant defense capacity, leading to oxidative stress.

1. Introduction

1.1. Diabetes Mellitus. Diabetes mellitus (DM) is characterized by hyperglycemia due to changes in the production or action of insulin; the chronicity of this condition is associated with damage, dysfunction, and insufficiency of target systems such as cardiovascular and central nervous systems [1]. The physiopathology of DM is related to changes in β-pancreatic cells that compromise the synthesis and secretion of insulin, together with resistance to the action of insulin in peripheral tissues. Insulin secretion is controlled by several factors, including nutrients, hormones, and neural factors [2]. One of the roles of insulin is to influence inflammatory reactions by it acting on oxidative stress and in the release of cytokines [3]. The inflammatory component in the physiopathology of DM is evidenced by the involvement of the factor nuclear

kappa B (NF-κB), which is one of the transcription factors that control the production of proinflammatory cytokines. The NF-κB pathway binds the inflammatory and metabolic responses and represents a point of connection for a better understanding of metabolic diseases [4]. In addition, chronic conditions of low-grade inflammation appear to play an important role in the pathogenesis of renal failure, one of the consequences of DM [5]. Hyperglycemia, a frequent condition in DM, is related to cellular and tissue damage, due to changes in cell signaling, gene transcription, and protein and lipid changes [6].

1.2. Major Depressive Disorder. Major depressive disorder (MDD) has high morbidity, and nearly 350 million people are affected worldwide. The physiopathological mechanism is not widely understood, but is believed to have a

multifactorial origin, involving dysfunction in multiple brain areas such as the hippocampus, prefrontal cortex, nucleus accumbens, and amygdala [7]. Moreover, MDD pathophysiology is associated to an inflammatory process due to microglial activation, elevated cytokine release, and increased oxidative stress, with astrocyte atrophy and alteration in glutamatergic system regulation, which may lead to local damage [8]. These processes may also activate the enzyme indoleamine 2,3-dioxygenase, diverting tryptophan to the kynurenine pathway, causing the production of active neurotoxic metabolites [9]. In fact, it is known that microglial cells regulate the activation and progression of several neuroimmune pathways that are mediated by macrophages, growth factors, cytokines, and others. In addition, they also initiate the formation of intracellular multiprotein complexes, the inflammasomes, which in turn cleave precursor forms of interleukin-1β (IL-1β) in its active form [10]. The inflammatory process, when exacerbated, can cause a significant increase in the production and expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β, as well as reactive species of oxygen (ROS) and nitric oxide, contributing to the neuroinflammatory and neurodegenerative processes associated with psychiatric disorders, including MDD [11]. In another recent study, it was identified that MDD, associated or not to posttraumatic stress disorder, presents changes in the cytokines and increased oxidative stress [12], thus demonstrating that the association of several factors contributes to the pathophysiology of MDD.

1.3. Oxidative Stress. The term oxidative stress is used to characterize the imbalance between the production of ROS and antioxidant defenses. Elevated levels of ROS cause damage to lipids, proteins, and DNA [13], and it is associated with several diseases including cancer, DM, cardiovascular, neurodegenerative diseases, and MDD [14].

Under normal physiological conditions, there is formation of ROS and reactive nitrogen species (RNS) that act as a messengers and also regulate intracellular signal transduction pathways involved with survival and cell death, being removed by several mechanisms of antioxidant defense, such as catalase and superoxide dismutase enzymes [15]. However, when in excess, they are harmful to the metabolism, mainly because of being able to inactivate important cellular molecules which are necessary for the regulation and homeostasis [16]. Antioxidants are a defense system for the body against these and all free radicals. They act by eliminating or by preventing their transformation into products that are less toxic to cells [17].

There is an important relationship between some diseases and oxidative stress, because they participate in vital processes, such as inflammation, glucose homeostasis, and cell survival [18]. In DM, hyperglycemia induces increased oxidative stress through several biochemical processes [19], including the glucose self-activation, increase of glycation and diacylglycerol, and the activation of protein kinase C and polyol pathways. Also, this process causes the progression and complications of DM due to increased free radicals and decreased antioxidant enzymes leading to an increase in

lipid peroxidation [20]. A significant increase in oxidative stress in diabetic patients compared to controls was observed, but this change appears to be more evident during disease progression and complications [21, 22].

Oxidative stress also may be related to some psychiatric disorders. There is evidence that in the MDD patients, excess of ROS may be a relevant mechanism related to immune activation [23], increased oxidation of monoaminergic neurotransmitters [24], and lipid peroxidation [25]. Furthermore, in MDD there is also a decrease in important antioxidant substances as well as a lower activity of the antioxidant enzymes [26].

1.4. Diabetes Mellitus, Major Depressive Disorder, and Oxidative Stress. Studies have shown that patients with DM have a higher risk to develop MDD, when compared to the general population [27–30]. Patients with MDD, as well as the use of antidepressant drugs, could be risk factors for the development of DM [31–34]. In addition, depression in patients with DM is a major cause of poor self-care, which are very important for these patients to avoid future complications, for example, renal, ocular, and neurological damage [35].

It is believed that the glucose accumulation in the extracellular space due to DM can cross the blood brain barrier (BBB) and affect specific brain areas involved with memory and mood regulation [36, 37]. On the other hand, MDD may be correlated with insulin resistance due to higher levels of glucocorticoid and a decrease in insulin sensitivity [38, 39]. It was proposed by Watson [40] that DM and other diseases such as cancer and dementias are accelerated or caused by failure of the endoplasmic reticulum to generate sufficient oxidative redox potential for disulphide bonds to be formed. Indeed, genomics, epigenomics, and exposomics methods are suggested to characterize redox components and their functional organization in health and disease [41].

The pathophysiological mechanism involved when both DM and MDD are together is still not clear. One of these mechanisms could be related to the oxidative stress (Table 1). In fact, oxidative stress plays an important role in the development and progression of DM due to higher free radical production, damage to cell constituents, and impairment in the antioxidant defense enzymes, such as superoxide dismutase and catalase [42, 43]. MDD also is characterized by activated oxygen and nitrogen species pathways, leading to lipid, protein, and DNA damage [44–47].

Experimental studies have been shown that alloxan-diabetic rats displayed a depressive-like behavior in the forced swimming test [48, 49], while the treatment with the antidepressant imipramine [48] and with the antioxidant N-acetylcysteine (NAC) [49] was able to reverse the depressive-like behavior, thus showing that both antidepressant and antioxidant could improve depressive behavior induced by the animal model of diabetes. The treatment with clonazepam, a positive GABA$_A$ receptor modulator, alone or in combination with insulin also reversed the depressive-like behavior in diabetic rats [50–52]. Interestingly, the treatment with insulin and clonazepam was able to restore the antioxidant status in the brain of diabetic rats [52]. A study carried

TABLE 1: Summary of changes associated to oxidative stress in diabetes mellitus and major depressive disorder.

Species/model	Damage	Antioxidant effect	Reference
Alloxan-diabetic rats	Depressive behavior	N-Acetylcysteine and imipramine displayed antidepressant effects	[48, 49]
Diabetic rats	Depressive behavior and oxidative stress	Clonazepam and insulin reversed the depressive behavior and restored the antioxidant status	[50, 52]
STZ-diabetic rats	Depressive behavior	Hydrogen sulfide induced antidepressant effects	[53]
STZ-diabetic rats	Depressive behavior, oxidative stress, and inflammation	Metformin plus ascorbic acid reduced the depressive behavior and had antioxidant and anti-inflammatory effects	[54]
STZ-nicotinamide-diabetic rats	Depressive behavior	Ascorbic acid had antidepressant effects, reduced oxidative stress, and inflammation	[55, 56]
STZ-diabetic rats	Depressive behavior	Aloe vera displayed antidepressant, antioxidant, and antidiabetic effects	[56]
MDD and bipolar disorder patients	Severity of symptoms was associated to glucose levels and the number of episodes to glucose toxicity	—	[63]

out by Tang et al. [53] demonstrated that hydrogen sulfide (H2S), a signaling molecule in the brain, with antioxidant activity was able to reverse the depressive-like behavior in streptozotocin- (STZ-) induced diabetic rats. The authors suggested that this behavioral change was associated to a reduction in oxidative stress in the hippocampus. Recently, Shivavedi et al. [54] showed that a combination treatment with metformin and ascorbic acid reduced the depressive-like behavior, oxidative stress and inflammation, and elevated monoamine levels in STZ-induced diabetic rats. It was suggested that the antidepressant effects exercised by metformin and ascorbic acid in diabetic rats were associated with a reduction in blood glucose and oxidative stress and increased plasma insulin levels [54]. Ascorbic acid, a natural antioxidant, was proposed as a potential strategy against comorbid depression-like behavior in diabetic rats. It was revealed that ascorbic acid treatment reduced the depressive behavior in STZ-nicotinamide-induced diabetic rats [55]. Also, it was demonstrated that ascorbic acid reduced oxidative stress, hyperglycemia, and inflammation [55, 56]; on the other hand, positive results with Aloe vera treatment were found, which has antioxidative, neuroprotective, and antidiabetic effects. The study revealed that Aloe vera displayed antidepressant effects in STZ-induced diabetic rats, and these effects were suggested to be related to hypoglycemic and antioxidant properties of Aloe vera in the hippocampus [56].

Some studies have reported a potential therapeutic for ebselen, a glutathione peroxidase mimetic and which can contribute to regulation of cell function [57, 58]. Experimental studies revealed that treatment with ebselen reduced diabetes-associated atherosclerosis in apolipoprotein E/GPx1 double-knockout mouse [59], prevented islet apoptosis, and preserved the β-cell mass and function in Zucker diabetic fatty (ZDF) rats [60]. Also, ebselen treatment in human erythrocytes from patients with uncontrolled diabetes exerted glycation-inhibiting properties [61]. Contrarily, a randomized, crossover trial with DM patients did not show improvement in the oxidative stress

profile and it did not affect the endothelium-dependent vasodilation [62]. There are no studies evaluating the effects of ebselen in depression; however, a study demonstrated that ebselen due to its capacity to inhibit the inositol monophosphatase could be an alternative treatment for bipolar disorder, comparable to lithium [58]. Future studies evaluating the efficacy of ebselen in depression and DM comorbidity could be interesting.

A human study with MDD and bipolar disorder patients revealed no association with mood disorder symptoms and insulin resistance or increased glucose toxicity [63]. However, the same study demonstrated effects for severity of mood disorders on glucose levels and in the number of mood episodes on glucose toxicity. In addition, β-cell function and insulin resistance were associated with immune-inflammatory, ROS, and RNS pathways, which in turn induced glucose toxicity [63]. Contrarily, a recent cohort study revealed that higher levels of systemic oxidative stress, marked by DNA/RNA damage from oxidation (8-oxodG/8-oxoGuo) in patients with DM, were not associated with higher risk for psychiatric diseases, such as unipolar depression, anxiety, bipolar disorder, and schizophrenia [64]. Discrepancies in these studies may be related to the type of marker studied, study time, and psychiatric disorder conditions analyzed.

2. Conclusion

The imbalance between ROS formation and the antioxidant system can result in several pathological alterations that are related to both psychiatric and metabolic diseases, and these changes are evident mainly in progressive and chronic pathologies such as DM and MDD.

Although few studies have evaluated the relationship of oxidative stress when MDD and DM are present in the same patient, oxidative stress oxidative redox potential may be the key factor in triggering comorbidities such as MDD associated with DM and vice versa; nevertheless, many other factors such as inflammation, hyperglycemia, and insulin

resistance are also involved, although all these conditions increase the levels of oxidative stress.

Further studies evaluating medications with antidepressant and antioxidant effects that can reduce oxidative stress may be clinically important to prevent comorbid MDD in DM condition.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

The Combined Extract of Black Sticky Rice and Dill Improves Poststroke Cognitive Impairment in Metabolic Syndrome Condition

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Despite the increase in cognitive deficit following stroke in metabolic syndrome (MetS) condition, the therapeutic strategy is still limited. Since oxidative stress and neuroinflammation play the crucial roles on the pathophysiology of aforementioned conditions, the cognitive enhancing effect of the combined extract of *Oryza sativa* and *Anethum graveolens* was considered based on their antioxidant, anti-inflammation, and neuroprotective effects together with the synergistic effect concept. Male Wistar rats weighing 180-220 g were induced metabolic syndrome-like condition by using a high-carbohydrate high-fat diet (HCHF diet). Then, reperfusion injury following cerebral ischemia was induced by the occlusion of right middle cerebral artery and treated with the combined extract of *O. sativa* and *A. graveolens* (OA extract) at doses of 0.5, 5, and 50 mg/kg BW once daily for 21 days. Spatial memory was assessed every 7 days throughout the experimental period. At the end of the study, neuron and glial fibrillary acidic protein- (GFAP-) positive cell densities, the oxidative stress status, AChE, and the expression of proinflammatory cytokines (TNF-α, IL-6) in the hippocampus were determined. The results showed that OA extract at all doses used in this study significantly improved memory together with the reductions of MDA, TNF-α, IL-6, AChE, and density of GFAP-positive cell but increased neuron density in the hippocampus. Taken together, OA is the potential cognitive enhancer in memory impairment following stroke in MetS condition. The possible underlying mechanism may occur partly via the reductions of oxidative stress status, GFAP-positive cell density, and neuroinflammatory cytokines such as TNF-α and IL-6 together with the suppression of AChE activity in the hippocampus. This study suggests that OA is the potential functional ingredient to improve the cognitive enhancer. However, further clinical research is required.

1. Introduction

Currently, the prevalence of metabolic syndrome (MetS), a cluster of metabolic disorders, is continually rising to 39-46% in every ethnic and age group [1]. It has been reported that this prevalence is increasing with the advanced age [2]. Accumulative lines of evidence during the last decade reveal that MetS is closely associated with stroke risk [3–5]. It

has been demonstrated that the adjusted risk ratios for incident ischemic stroke associated with MetS are in the range of 2.1-2.47 [6–9]. Stroke is regarded as the important cause of disability. A high proportion of stroke survivors develop cognitive impairment within 3 months after stroke [10]. This defect produces the great impact on the quality of life of the patients. Accumulative lines of evidence demonstrate that oxidative stress imbalance and neuroinflammation play the

crucial role on the pathophysiology of MetS, stroke, and cognitive impairment [11–16]. Based on the important role of both oxidative stress and inflammation mentioned earlier, they were considered as the target for neuroprotection.

According to the traditional folklore, food is served not only as a source of nutrients and energy but also as the tool for promoting health. Recently, food polyphenol intervention has been considered as the potential neuroprotection food. The recent study reveals that a polyphenol especially anthocyanin-rich diet can improve brain damage in an animal model of MetS induced by a high-carbohydrate high-fat diet via the attenuation of brain oxidative stress status [17] and inflammation [18]. Both *Oryza sativa*, *L. indica* (black sticky rice), and *Anethum graveolens* Linn. (dill) are commonly consumed in Thailand. They exhibit antioxidant [19, 20] and anti-inflammation effects [21, 22]. In addition, they also possess neuroprotective effect [23–25]. Based on the pharmacological effects of both herbs mentioned earlier and a synergistic effect according to the traditional folklore, we hypothesized that the combined extract of black sticky rice and dill should provide neuroprotection against stroke in an animal model of MetS. Due to the lack of available data, we aimed to determine the neuroprotective of the combined extract of black sticky rice and dill against cerebral ischemia in an animal model of MetS induced by a high-carbohydrate high-fat diet (HCHF diet). The possible underlying mechanisms were also explored.

2. Materials and Methods

2.1. Plant Material Preparation and Extraction.
Oryza sativa, *L. indica*, and *Anethum graveolens* Linn. were collected from Khon Kaen Province. They were harvested during September-October. They were authenticated by Associate Panee Sirisa-ard, pharmacognosy expert from Faculty of Pharmacy, Chiang Mai University, who served as the consultant of High Human Performance and Health Promotion Research Institute, Khon Kaen University, Thailand. Voucher specimens (ICAM 12001 and ICAM12002) were deposited at Integrative Complementary Alternative Medicine Research and Development Center, Khon Kaen University. For the preparation, the grains of black sticky rice and aerial part of dill were cleaned and dried in the oven (Memmert GmbH, USA) at 60°C for 72 hours. Then, the water extract of black sticky rice and 95% hydroalcoholic extract were prepared by using maceration technique. In brief, the plant materials mentioned earlier were subjected to the 24-hour maceration at room temperature. The extracts were harvested, centrifuged at 3000 rpm for 10 min, and filtered with Whatman no. 1 filter paper. The filtrates were collected and subjected to a freeze drying process. The percentage yields of *O. sativa* and *A. graveolens* were 10 and 26, respectively. The yielded extracts were stored at -20°C until use. The combination extract (OA extract) was prepared by mixing *O. sativa* and *A. graveolens* at a ratio of 1 : 6 based on our pilot data which showed that this ratio showed the highest potential for treating cerebral ischemia in metabolic syndrome.

2.2. Measurement of Total Phenolic Compound Contents.
The total phenolic content of OA extract was determined by using the Folin-Ciocalteu colorimetric method. In brief, $1000 \mu l$ of a 50% Folin-Ciocalteu phenol reagent (Sigma-Aldrich, USA) and $158 \mu l$ of distilled water were mixed with $20 \mu l$ of the tested substances (OA extract and gallic acid) and incubated at 37°C for 8 minutes. At the end of the incubation period, $30 \mu l$ of 20% Na_2CO_3 (Sigma-Aldrich, USA) was added, mixed, and incubated at room temperature in the dark room for 2 hours. Then, the absorbance at 765 nm was recorded. Results were expressed as mg gallic acid equivalent (GAE)/mg per 0.1 g of OA extract. Various concentrations of gallic acid (Sigma-Aldrich, USA) ranging from 0 to $500 \mu g/ml$ were used to prepare the standard calibration curve [26].

2.3. Measurement of Flavonoid Content.
The modified aluminium chloride colorimetric method was used to measure the flavonoid content of OA extract [27]. Aliquots of 1.5 ml of the tested substances were added to equal volumes of a solution of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 ml methanol). Then, the mixture was subjected to a vigorous shake and incubated at room temperature for 30 minutes. At the end of the incubation period, an absorbance at 415 nm was measured. The contents of flavonoids were expressed as μg quercetin equivalent/mg extract.

2.4. Antioxidant Property Assessment

2.4.1. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Radical Assay.
This method measured an antioxidant activity of the tested substance on the basis of the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical [28]. Briefly, 0.25 ml of 0.15 mM DPPH solution was mixed with $50 \mu l$ of OA extract solution of varying concentrations (1, 5, 10, 50, 100, 250, 500, and $1000 \mu g/ml$). After a 30-minute incubation period in a dark condition, an absorbance at 517 nm was recorded using a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo Electron Corporation, IL, USA). Corresponding blank was prepared, and L-ascorbic acid was used as standard reference. Extract concentration which provided 50% inhibition (IC50) was calculated using the graph by plotting inhibition percentage against extract concentration [28, 29].

2.4.2. Ferric Reducing Antioxidant Power (FRAP) Assay.
The antioxidant potential to change ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) of OA extract was measured by using FRAP assay. FRAP reagent was freshly prepared by mixing 25 ml of 300 mM acetate buffer (Sigma-Aldrich, USA), pH 3.6, and 2.5 ml of 10 mM TPTZ (Sigma-Aldrich, USA) solution in 2.5 ml of 20 mM ferric chloride solution ($FeCl_3$) (Sigma-Aldrich, USA) together. Then, $190 \mu l$ of the FRAP reagent and $100 \mu l$ of distilled water were mixed with $10 \mu l$ of OA extract at various concentrations ranging from 1 to $1000 \mu g/ml$. The mixture was incubated at 37°C for 10 minutes. After the incubation, an absorbance was recorded at 593 nm [30]. Ascorbic acid was used as the positive control, and results were expressed as the EC50 value.

2.5. Measurement of Acetylcholinesterase Inhibitory (AChEI) Activity.

AChE suppression activity of OA extract was determined by a colorimetric method according to the method of Ellman et al. [31]. This method is based on the determination of a yellow color of 5,5′-dithiobis (2-nitrobenzoic acid) produced by the hydrolysis of acetylcholine by acetylcholinesterase (AChE). In brief, various concentrations of OA extract at the volume of 25 μl each were added to the reaction mixture containing 50 μl of Tris-HCl (50 mM, pH 8.0) (Sigma-Aldrich, USA), 75 μl of 3 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, USA), 25 μl of 15 mM thiocholine iodide (ATCI) (Sigma-Aldrich, USA), and 25 μl of AChE (0.22 U/ml) (Sigma-Aldrich, USA). After mixing, the reaction mixture was incubated at room temperature for 5 minutes, the absorbance at 412 nm was recorded with a microplate reader (iMark™ Microplate Absorbance Reader). Percentage of inhibition was calculated by comparing the rate of enzymatic hydrolysis of ATCI for the samples to that of the blank (50% aqueous methanol in buffer). Donepezil (1-32 mM) (ARICEPT®, USA) was used as a reference standard. The AChE inhibition activity of each sample was expressed in terms of EC50. Every sample was assessed in triplicate.

2.6. Determination of Cyclooxygenase-2 (COX-2) Activity.

The activity of cyclooxygenase-2 (COX-2), an enzyme playing an important role in inflammatory event, was assessed. Briefly, the reaction mixture containing 150 μl of 100 mM Tris-HCl buffer, 10 μl 0.5 μM of heme, 10 μl of 50 nM COX-2, and 10 μl of OA extract was mixed in 96-well microliter plates. The reaction was initiated with 20 μl of 100 μM arachidonic acid and 20 μl of 10 μM of TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine) (Cayman Chemical, USA). Then, the plate was incubated at room temperature for 5 minutes. The absorbance at 590 nm was measured at the end of the incubation period by using a microplate reader. Indomethacin was used as a standard compound. The percentage of COX-2 inhibition was calculated, and results were expressed as the EC50 value [32].

2.7. Finger Print Chromatogram Assessment.

The phenolic profiles of OA extract consisting of cyanidin-3-glucoside (Sigma-Aldrich, USA), gallic acid (Sigma-Aldrich, USA), and quercetin-3-O-rutinoside (Sigma-Aldrich, USA) were determined by high-performance liquid chromatography (HPLC). Chromatography was performed by using a Waters® system equipped with a Waters® 2998 photodiode array detector. Chromatographic separation was performed using Purospher® STAR, C-18 encapped (5 μm), LiChroCART® 250-4.6, and HPLC-Cartridge, Sorbet Lot no. HX255346 (Merck, Germany). The mobile phase (HPLC grade) consisted of 100% methanol (solvent A) (Fisher Scientific, USA) and 2.5% acetic acid (solvent B) (Fisher Scientific, USA) in deionized (DI) water was used to induce gradient elution. The gradient elution was carried out at a flow rate of 1.0 ml/min with the following gradient: 0-17 min, 70% A; 18-20 min, 100% A; and 20.5-25 min, 10% A. The sample was filtered (0.45 μm, Millipore), and a direct injection of the tested sample at the volume of 20 μl on the column was performed. The chromatograms were recorded at 280 nm using the UV detector, and data analysis was performed using Empower™ 3.

2.8. Experimental Protocol.

Adult male Wistar rats weighing 180-220 grams at the ages between 10 and 14 weeks were obtained from National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The animals were housed in a group of 6 per cage in the standard metal cages at $22 \pm 2°C$ on a 12 : 12 h light : dark cycle and ad libitum access to food and water. All experimental protocols used in this study had been approved by the Institutional Animal Care and Use Committee, Khon Kaen University, Khon Kaen, Thailand (AEKKU 30/2558). After 1 week of acclimatization, rats were divided into seven groups ($n = 6$) as follows:

(1) Group I (naïve intact): rats in this group received a normal diet (4.5% fat, 42% carbohydrate, and 24% protein) and received no treatment

(2) Group II (HCHF+sham operation+vehicle): all rats in this group received a HCHF diet and subjected to sham operation and vehicle treatment

(3) Group III (HCHF+MCAO+vehicle): animals in this group were HCHF diet-treated rats which were subjected to reperfusion after the occlusion of right middle cerebral artery (Rt. MCAO) and treated with vehicle

(4) Group IV (HCHF+MCAO+vitamin C): all animals were HCHF diet-treated rats which were subjected to reperfusion after the occlusion of right middle cerebral artery (Rt. MCAO) and treated with vitamin C at dose of 250 mg/kg BW

(5) Groups V-VII (HCHF+MCAO+OA) (OA1, OA2, and OA3): all rats in these groups were HCHF diet-treated rats which were exposed to reperfusion injury after the occlusion of right middle cerebral artery (Rt. MCAO) and treated with OA extract at various doses ranging from 0.5, 5, to 50 mg/kg BW

Rats in group II-group VII were fed a high-carbohydrate high-fat diet (HCHF; 35.83% fat, 35.54% carbohydrate, and 28.63% protein) in order to induce metabolic syndrome. After 16 weeks of the feeding period, rats which showed the percentage change of body weight more than 40 percent, fasting plasma glucose more than 100 mg/dl, systolic blood pressure more than 130 or diastolic blood pressure more than 85 mmHg, and the atherosclerosis index (total serum cholesterol/total serum HDL-C) higher than the control group were selected for inducing reperfusion injury after the occlusion of Rt. MCAO. Then, the animals were orally given the assigned substances once daily for 21 days. Spatial memory was assessed by using the Morris water maze test every 7 days throughout a 21-day study period. At the end of the study period, neuron density, the oxidative stress status, AChE, density of glial fibrillary acidic protein- (GFAP-) positive cell, and the expressions of proinflammatory cytokines

(a)

(b)
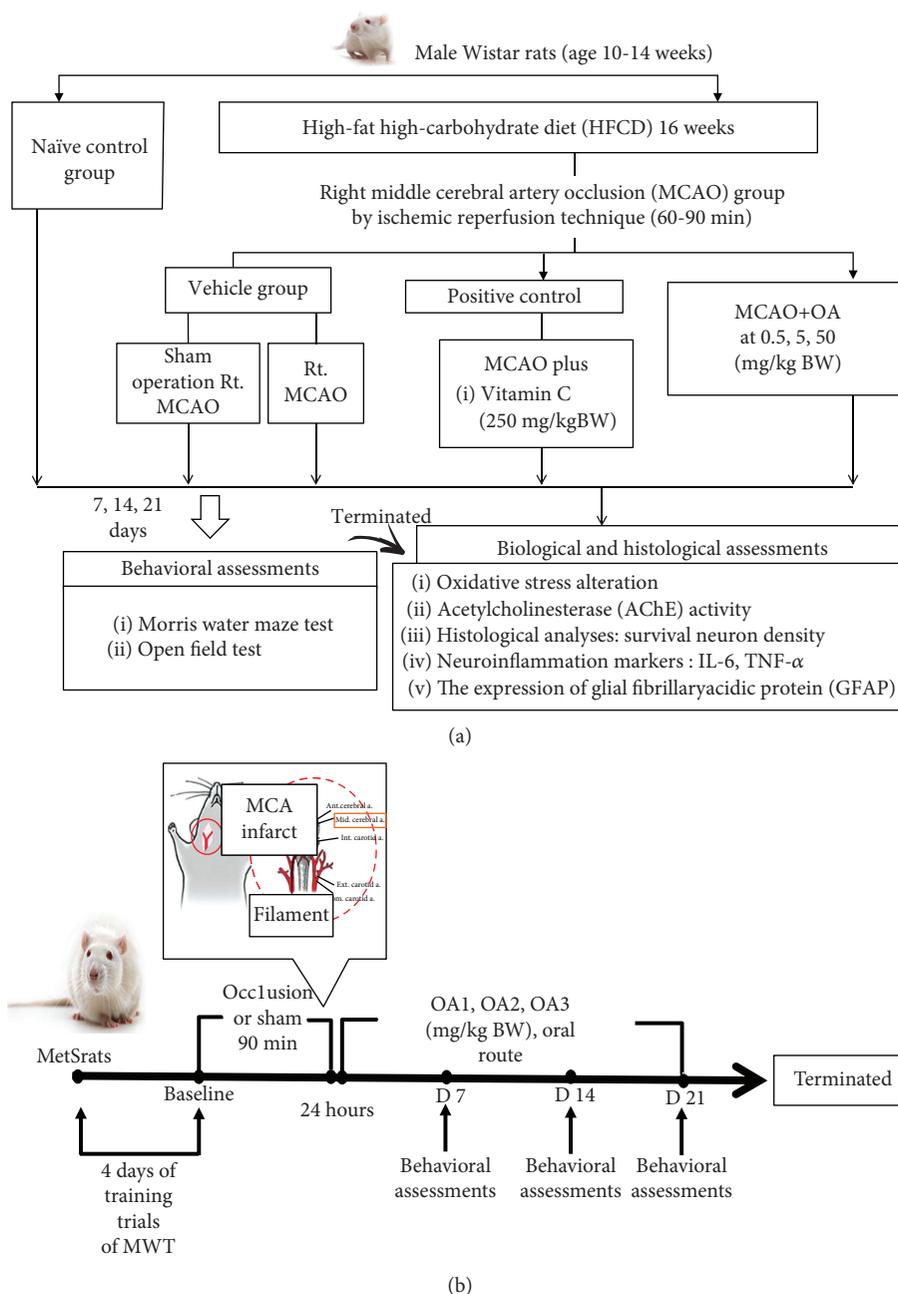
FIGURE 1: Schematic diagram showing all experimental procedures. (a) Experimental protocol of OA extract treatment and the determination of various parameters. (b) Right MCAO induction and schedule for OA extract treatment. IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; HCHF: high- high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.

(TNF-α, IL-6) in the hippocampus were determined. The schematic diagram showing the experimental protocol was shown in Figure 1.

2.9. Focal Cerebral Ischemia/Reperfusion Induction. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg BW; Tianjin Kemiou Chemical Reagent Co. Ltd., Tianjin, China). Then, the monofilament was inserted via the right common carotid artery, and the right external carotid artery (ECA) was exposed through a

ventral midline neck incision and was ligated proximally. A silicone-coated nylon monofilament (4-0) suture (USS DG; Tyco Healthcare Group LP, CT, USA), with its tip rounded by heating near a flame, was inserted 17-18 mm into the right internal carotid artery (ICA) from distal to the carotid bifurcation, to occlude the origin of the MCA. The suture was fixed, and the incision was closed. Following 90 min of ischemia, the nylon suture was withdrawn to allow reperfusion. The sham-operated rats underwent identical surgery, but the nylon suture was not inserted. After 1 week of operation,

rats in groups V-VII were subjected to the assigned treatments once daily for a period of 21 days.

2.10. Spatial Memory Assessment.

Spatial memory was assessed by using the Morris water maze test. The water maze apparatus is a circular pool at the diameter of 147 cm and filled with tap water to the depth of 40 cm. The surface was covered with nontoxic powder. The pool was divided into 4 equal quadrants, and the removable escape platform was placed in the center on one quadrant below the water level. The location of the platform was invisible, and it remained there throughout the training. Each animal was trained to memorize the location of the platform by forming the association information between its location and the location of platform by using external cues. After 4 training sessions, animal was placed in the water in the starting quadrant and allowed to swim until the animal found and climbed onto the platform. The time which the animal spent to reach the hidden platform was recorded as escape latency. The retention time was determined 24 h later by exposing the animal to subject to the same situation as mentioned earlier except that the immersed platform was removed and the time which the animal spent for swimming in the quadrant previously located platform was regarded as retention time [25].

2.11. Locomotor Assessment.

The effect of the developed product on locomotor activity was assessed by using an open field test, the most frequently used method. A 90 cm square plexiglass chamber with a 70 cm height was used for behavioral evaluation. The tests were performed in a room lit by a 60 W light bulb 1.75 m above the center of the open field. Each animal was placed into the center of the open field chamber and allowed to explore the apparatus for 5 minutes. Every time both hind paws entered one of these squares, a crossing was recorded. In addition to the number of crossing, the number of center square entries was also recorded. The number of licking, rearing, and grooming was also monitored by using a video tracking system [33].

2.12. Histological Study

2.12.1. Nissl Staining.

For histopathological analysis, the brains were perfused transcardially with fixative solution containing 4% paraformaldehyde (Sigma-Aldrich, USA) in 0.1 M phosphate buffer pH 7.4 overnight at 4°C. Then, they were infiltrated with 30% sucrose (Merck, Germany) solution for 72 h at 4°C. Serial sections of tissues were cut frozen on a cryostat (Thermo Scientific™ HM 525 Cryostat) at 20 μm thick. All sections were picked up on slides coated with 0.3% aqueous solution of gelatin containing 0.05% aluminium potassium sulfate (Sigma-Aldrich, USA). The triplicate coronal sections of brains were immersed in 0.2% cresyl violet (Sigma-Aldrich, USA) for 8 minutes, rinsed with double distilled water, and dehydrated through graded alcohols (70, 95, and 100% 2x) (RCI Labscan, Thailand). The sections were cleared with xylene for 5 minutes (2 times) and mounted using DPX mountant (Merck, Germany). The evaluation of neuron density in the hippocampus was performed under an Olympus light microscope model BH-2 (Japan) at 40x magnification. Counts were performed in three adjacent

fields, and the mean number was calculated and expressed as density of neurons per 255 μm^2.

2.12.2. Immunohistochemistry.

Brain sections containing the hippocampus were prepared as mentioned in Section 2.12.1. All sections were picked up on slides coated with 0.3% aqueous solution of gelatin containing 0.05% aluminium potassium sulfate (Sigma-Aldrich, USA). The sections were heated using a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 10 minutes and cooled at room temperature. The sections were subjected to a 5-minute washing step with phosphate-buffered saline (PBS) for 3 times and incubated in 0.3% hydrogen peroxide at room temperature for 20 minutes. At the end of the incubation period, the sections were subjected to a 5-minute washing with PBS for 3 times. Then, they were incubated in the mixture containing 0.3% Triton X-100 (Fluka Chemika, Buchs, Switzerland), 1% (w/v) bovine serum albumin (BSA), and 10% normal goat serum at room temperature for 20 minutes. Following this process, the sections were washed with PBS for three times (5 minutes each) and incubated with primary anti-GFAP (Abcam, Cambridge, MA, USA) at a dilution of 1 : 500 (diluted in the solution containing 0.01 M PBS with 1% Triton X-100 and 10% normal serum) at 4°C overnight. Following this step, the sections were washed and incubated with REAL™ EnVision™ Detection System, Peroxidase/DAB+ rabbit/mouse (Dako, Glostrup, Denmark) at room temperature for 30 minutes. At the end of the incubation period, the sections were rinsed with PBS and incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, USA) for 5 minutes. Positive staining was recognized as a brown color. Negative control sections were subjected to the same procedures except the exposure to primary antibody. The sections were mounted on gelatin-coated slides, counterstained with cresyl violet, dehydrated with graded alcohols, cleared with xylene, and mounted with DPX mountant. The numbers of positive cells in an area of 255 μm^2 were counted. The data were shown as mean and standard error. Cell counts were carried out by a technician who is blind to the experimental design. All measurements were repeated for three times, and the mean value was used.

2.13. Brain Homogenate Preparation.

Hippocampi were isolated, weighed, and homogenized with a buffer consisting of 50 volume of 0.1 M phosphate-buffered saline. Then, the brain homogenates were centrifuged at 3000g for 15 min at 4°C. Supernatant of tissue homogenates was collected and used for the determinations of acetylcholinesterase (AChE) and oxidative stress markers including malondialdehyde (MDA) and the activities of main scavenger enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). The protein concentration in brain homogenate was also determined by using a Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

2.14. Biochemical Assessments

2.14.1. Oxidative Stress Marker Assessment.

The level of malondialdehyde (MDA), a lipid peroxidation product, in

the brain was determined by determining the accumulation of thiobarbituric acid reactive substances (TBARSs) according to the method of Ohkawa et al. and Harishekar and Kiran [34, 35]. In brief, $50\,\mu$l of sample was mixed with the solution containing $50\,\mu$l of 8.1% sodium dodecyl sulphate (SDS) (Sigma-Aldrich, USA), $375\,\mu$l of 0.8% of thiobarbituric acid (TBA) (Sigma-Aldrich, USA), $375\,\mu$l of 20% acetic acid (Sigma-Aldrich, USA), and $150\,\mu$l of distilled water (DW). Then, the mixture was boiled in a water bath at 95°C for 1 hour and cooled immediately under tap water. Then, $250\,\mu$l of DW and $1250\,\mu$l of the solution containing n-butanol and pyridine (15 : 1 v/v) (Merck, Germany) were added and mixed together and centrifuged at 4000 rpm for 10 minutes. The upper layer was separated, and the absorbance at 532 nm was measured. 1,3,3-Tetramethoxy propane (0-15 μM) (Sigma-Aldrich, USA) was used as the standard, and the level of MDA was expressed as ng/mg protein.

SOD activity was monitored based on the inhibition of nitroblue tetrazolium (NBT) reduction [36]. In brief, $20\,\mu$l of sample was mixed with the reaction mixture containing 57 mM phosphate buffer solution (KH_2PO_4) (Sigma-Aldrich, USA), 0.1 mM EDTA (Sigma-Aldrich, USA), 10 mM cytochrome C solution (Sigma-Aldrich, USA), $50\,\mu$M of xanthine solution, and $20\,\mu$l of xanthine oxidase solution (0.90 mU/ml) at 25°C. The absorbance at 415 nm was measured. SOD enzyme (Sigma-Aldrich, USA) activities at the concentrations of 0-25 units/ml were used as the standard, and the results were expressed as units/mg protein.

Catalase activity measurement was carried out spectrophotometrically by measuring the decrease in absorbance of H_2O_2 [37]. In brief, $50\,\mu$l of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0) (BDH Chemicals Ltd., UK), $25\,\mu$l of H_2SO_4 (Sigma-Aldrich, USA), $150\,\mu$l of 5 mM KM_nO_4 (Sigma-Aldrich, USA), and $10\,\mu$l of tissue homogenate were mixed thoroughly, and changes in an absorbance of the reaction solution at 490 nm were determined. CAT enzyme (Sigma-Aldrich, USA) at the concentration range of 0-100 units/ml was used as the standard, and the result was expressed as units/mg protein.

In order to determine the activity of GSH-Px, the mixture containing $10\,\mu$l of 1 mM dithiothreitol (DTT) (Sigma-Aldrich, USA) in 6.67 mM potassium phosphate buffer (pH 7), $100\,\mu$l of 1 mM sodium azide (Sigma-Aldrich, USA) in 6.67 mM potassium phosphate buffer (pH 7), $10\,\mu$l of 50 mM glutathione (Sigma-Aldrich, USA) solution, $100\,\mu$l of 30% hydrogen peroxide (BDH Chemicals Ltd., UK), and $20\,\mu$l of sample solution was mixed and incubated at room temperature for 5 minutes. Then, $10\,\mu$l of 10 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) (Sigma-Aldrich, USA) was added, and the optical density at 412 nm was recorded at 25°C over a period of 5 min [38]. The standard calibration curve was prepared by using GSH-Px enzyme (Sigma-Aldrich, USA) at the concentration range of 0-5 units/ml. GSH-Px activity was expressed as units/mg protein.

2.14.2. In Vivo Assessment of AChE.

The activity of rat brain AChE was determined by the method of Ellman with a slight modification [31]. In brief, a mixture containing a $20\,\mu$l of sample solution, $200\,\mu$l of 0.1 mM sodium phosphate buffer (pH 8.0) (Sigma-Aldrich, USA), and $10\,\mu$l of 0.2 M DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid)) (Sigma-Aldrich, USA) was mixed and incubated at room temperature for 5 minutes. Then, $10\,\mu$l of 15 mM acetylcholine thiochloride (ACTI) (Sigma-Aldrich, USA) was added and incubated for 3 minutes. The change in absorbance of light was measured at 412 nm for 3 minutes at regular intervals of 30 seconds using a microplate reader (iMark™ Microplate Absorbance Reader). The activity of AChE was calculated according to the equation below and expressed as nmol/min·mg protein.

$$\text{AChE activity} = \left(\frac{\Delta A}{(1.36 \times 10^4)} \right) \times \left(\frac{1}{(20/230)} \right) C, \quad (1)$$

where ΔA = the difference of absorbance/minute and C = protein concentration of brain homogenate.

2.15. Western Blotting Analysis.

The hippocampus was homogenized in the mammalian protein extraction reagent (M-PER; Pierce Protein Biology Product, Rockford, IL, USA), with protease inhibitor cocktail (1 : 10) (Sigma-Aldrich, USA). Then, the samples were centrifuged at 12,000g for 10 minutes at 4°C, and the supernatant was collected and stored on ice for protein determination by using a Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). For each animal, 80 μg of hippocampal lysate was adjusted to appropriate concentration by using Tris-Glycine SDS-PAGE loading buffer (Bio-Rad, USA) and heated at 95°C for 10 minutes. In addition, $20\,\mu$l of tissue sample protein was loaded onto SDS-polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Biotinylated broad-range molecular weight markers (Bio-Rad) were loaded onto the gels as well. After electrophoretic separation, proteins were transferred to a nitrocellulose membrane by electroblotting, washed with 0.05% TBS-T, and incubated overnight at 4°C in blocking buffer (PBS containing 1% Tween-20 (T-PBS) and 6.5% non-fat dry milk). Membranes were then incubated overnight at 4°C with polyclonal rabbit TNF-α (#3707) and IL-6 (#12912) primary antibodies (Cell Signaling Technology, USA; dilution 1 : 1000), rinsed with T-PBS for 30 minutes, and incubated with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, USA; dilution 1 : 2000) at room temperature for 1 hour. The bands were visualized and quantitated by using the ECL detection systems (GE Healthcare) and LAS-4000 luminescent image analyzer (GE Healthcare). Band intensities were measured for statistical analysis using the ImageQuant TL v.7.0 image analysis software (GE Healthcare). The expression was normalized using β-actin (Cell Signaling Technology, USA; dilution 1 : 2000). Data were presented as a relative density to the control normal group.

2.16. Statistical Analysis.

All data are expressed as mean ± standard error of mean (SEM). Statistical significance was evaluated by using one-way analysis of variance (ANOVA), followed by the post hoc (Tukey) test. The statistical significance was regarded at P values < 0.05.

TABLE 1: Characterization of OA extract including phenolic compositions and biological activities.

Parameters	Units	*O. sativa*	*A. graveolens*	OA extract	Standard reference
Total phenolic	μg GAE/mg extract	824.62 ± 90.98	941.54 ± 41.63	1724.10 ± 159.73[aaa,bb]	—
Total flavonoids	μg quercetin/mg extract	47.40 ± 2.98	23.81 ± 0.32	62.18 ± 1.03[aaa,bbb]	—
Antioxidant activities					
DPPH	EC50 (mg/ml)	0.123 ± 0.01	0.065 ± 0.02	0.014 ± 0.003[aa]	0.008 ± 0.002, ascorbic acid
FRAP	EC50 (mg/ml)	2.56 ± 0.37	2.99 ± 0.055	1.19 ± 0.24[a,b]	2.56 ± 0.14, ascorbic acid
Neuronal marker					
AChE inhibition	EC50 (mg/ml)	2.03 ± 1.08	2.94 ± 0.62	1.98 ± 0.15	0.67 ± 0.001, donepezil
Inflammatory marker					
COX-2 inhibition	EC50 (mg/ml)	9.37 ± 1.18	142.44 ± 15.82	1.73 ± 0.44[bbb]	0.02 ± 0.001, indomethacin

Data are presented as mean ± SEM. [a,aa,aaa]P value < 0.05, 0.01, and 0.001, respectively, compared with *O. sativa* and [b,bb,bbb]P value < 0.05, 0.01, and 0.001, respectively, compared with *A. graveolens*. OA extract: the combined extract of *O. sativa* and *A. graveolens*.

3. Results

3.1. Characterization of the Combined Extract of O. sativa and A. graveolens (OA Extract).

The characterization of the combined extract of *O. sativa* and *A. graveolens* or OA extract was shown in Table 1. *O. sativa* extract and *A. graveolens* contained the phenolic compounds at the concentrations of 824.62 ± 90.98 and 941.54 ± 41.63 μg GAE/mg extract while the concentration of this substance in the combined extract of *O. sativa* and *A. graveolens* (OA extract) was 1724.10 ± 159.73 μg GAE/mg extract. In addition, the flavonoid contents in *O. sativa* and *A. graveolens* were 47.40 ± 2.98 and 23.81 ± 0.32 μg quercetin/mg extract, respectively. It was found that the concentration of flavonoid content in OA extract was 62.18 ± 1.03 μg quercetin/mg extract. The contents of phenolic compounds and flavonoids in OA extract were significantly higher than those in *O. sativa* (*P* value <0.001 all) and *A. graveolens* (*P* value <0.01 and 0.001). The antioxidant activities were assessed by using DPPH and FRAP assays. The current results showed that EC50 of *O. sativa* and *A. graveolens* via DPPH assay were 0.123 ± 0.01 and 0.065 ± 0.02 mg/ml while EC50 of both substances mentioned earlier via FRAP assay were 2.56 ± 0.37 and 2.99 ± 0.055 mg/ml, respectively. It was found that EC50 of OA extract via DPPH and FRAP assays were 0.014 ± 0.003 and 1.19 ± 0.24 mg/ml, respectively. The antioxidant activity of OA extract assessed via DPPH assay showed the significant higher potent activity than the antioxidant activity of *O. sativa* (*P* value < 0.01) while the antioxidant activity of OA extract assessed via FRAP assay showed the significant higher potent activity than the activity of both *O. sativa* and *A. graveolens* (*P* value < 0.05 all). *O. sativa* also showed that EC50 of acetylcholinesterase suppression and cyclooxygenase-2 (COX-2) suppression activities were 2.03 ± 1.08 and 9.37 ± 1.18 mg/ml, respectively. EC50 of both mentioned activities of *A. graveolens* were 2.94 ± 0.62 and 142.44 ± 15.82 mg/ml, respectively. The present data showed that EC50 of acetylcholinesterase suppression and cyclooxygenase-2 (COX-2) suppression activities of OA extract were 1.98 ± 0.15 and 1.73 ± 0.44 mg/ml, respectively. It is also clearly shown that COX-2 suppression activity of
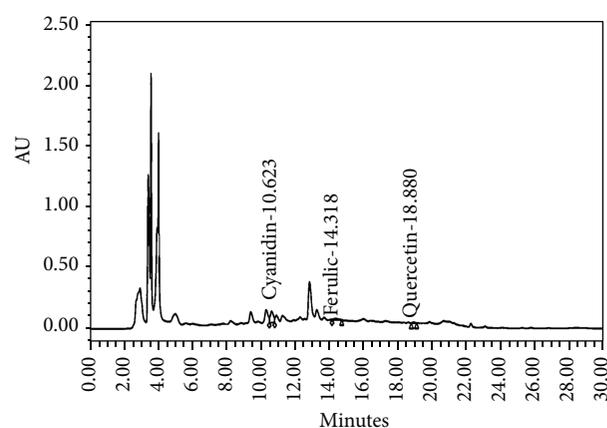
FIGURE 2: The chromatogram finger print of OA extract (100 mg/ml) using Purospher® STAR, C-18 encapped (5 μm), LiChroCART® 250-4.6, and HPLC-Cartridge, Sorbet Lot no. HX255346 (Merck, Germany) with guard column (Merck, Germany).

OA extract was significantly potent than that of *A. graveolens* (*P* value < 0.001).

The fingerprint chromatogram of OA extract was also determined, and data were shown in Figure 2. The contents of cyanidin-3-glucoside, quercetin, and ferulic acid in OA extract were 0.52 ± 0.003 μg Cyn-3-glu/50 mg of extract, 3.00 ± 0.58 μg QE/50 mg of extract, and 1.16 ± 0.12 μg ferulic acid/50 mg of extract, respectively.

3.2. Cognitive Enhancing Effect of OA Extract.

Figure 3 showed that no significant changes in escape latency among various groups were observed at 7 and 14 days after MCAO. However, metabolic syndrome rats induced by a HCHF diet which subjected to MCAO and received vehicle significantly increased escape latency (*P* value < 0.01, compared to the HCHF+sham operation+vehicle group). Interestingly, this change was attenuated by vitamin C and OA extract at doses of 0.5, 5, and 50 mg/kg (*P* value < 0.05, 0.01, 0.001, and 0.01, respectively, compared to the HCHF+MCAO+vehicle group). In addition, MCAO also decreased retention time
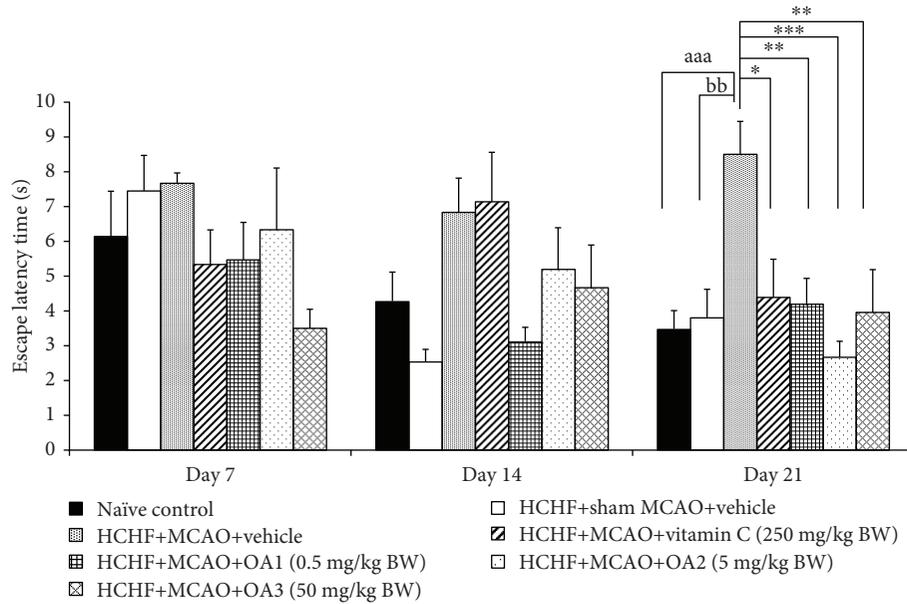
FIGURE 3: Effect of OA extract on escape latency time. Data are presented as mean ± SEM ($n = 6$/group). [aaa]P value < 0.001, compared to naïve intact rats; [bb]P value < 0.01, compared to sham operation which received HCHF diet and vehicle; and [*,**,***]P value < 0.05, 0.01, and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.
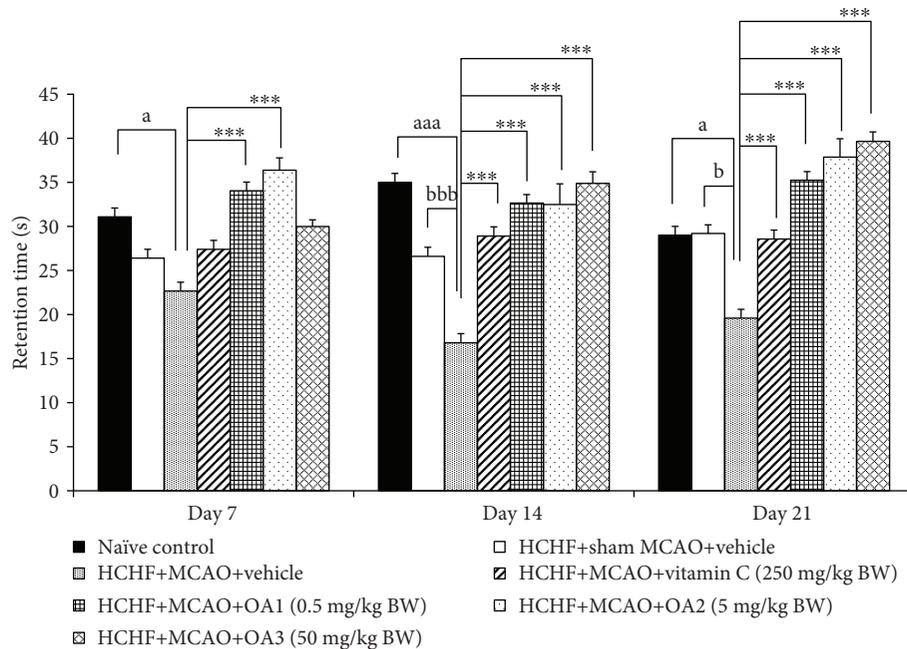
FIGURE 4: Effect of OA extract on retention time. Data are presented as mean ± SEM ($n = 6$/group). [a,aaa]P value < 0.05 and 0.001, respectively, compared to naïve intact rats; [b,bbb]P value < 0.01 and 0.001, respectively, compared to sham operation which received HCHF diet and vehicle; and [***]P value < 0.001, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.

in metabolic rats which received vehicle at 14 and 21 days after MCAO (P value < 0.001 and 0.05, respectively, compared to the HCHF+sham operation+vehicle group).

These changes were also mitigated both at 14 and 21 days after MCAO (P value < 0.001 all, compared to the HCHF+MCAO+vehicle group) as shown in Figure 4. To
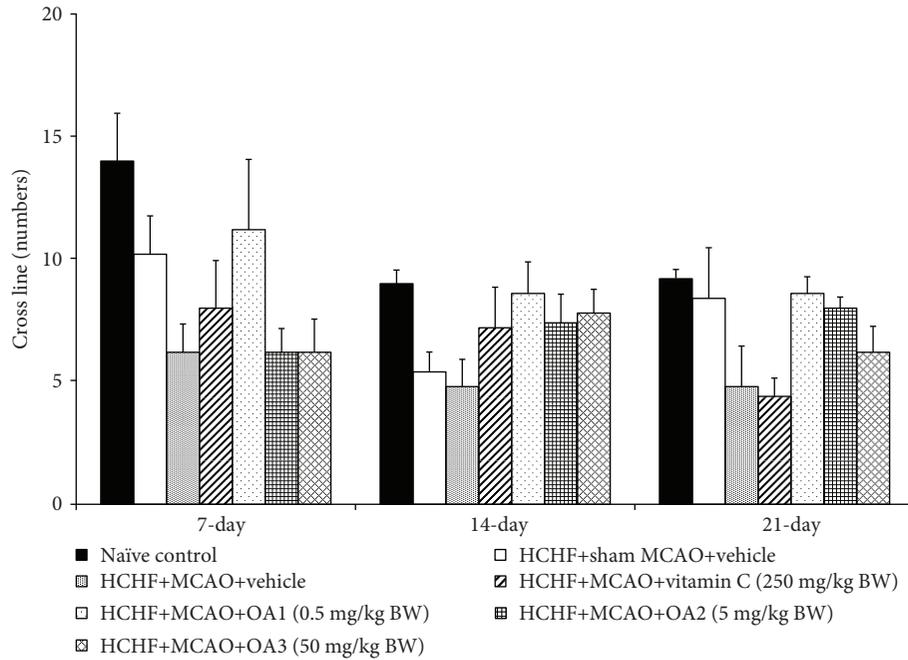
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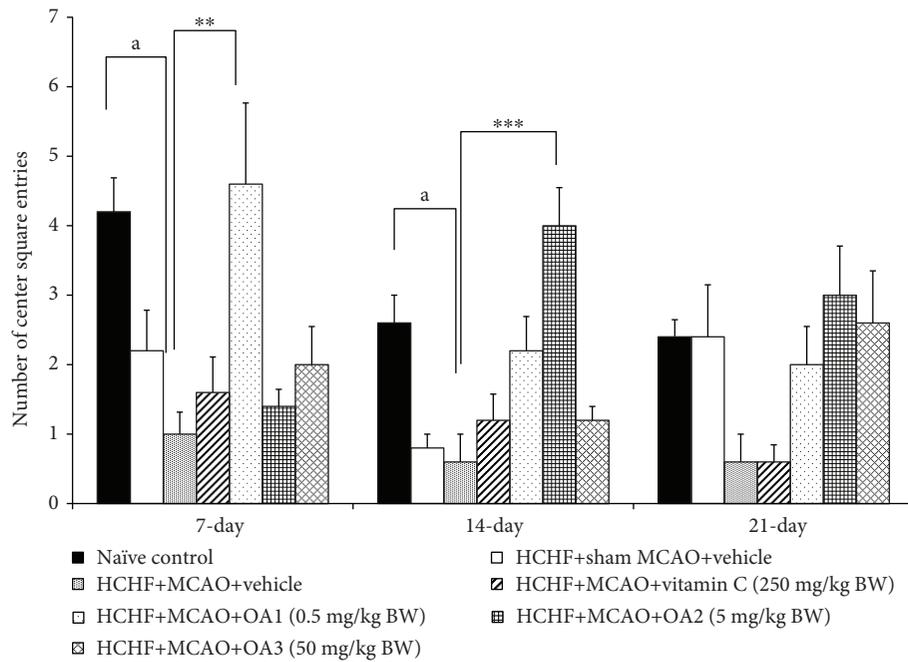
(b)

FIGURE 5: Effect of OA extract on locomotor activities: (a) the number of cross line and (b) the number of center square entries. Data are presented as mean ± SEM ($n = 6$/group). [a]P value < 0.05, compared to naïve intact rats and [**,***]P value < 0.01 and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.

assure that the cognitive enhancing effect observed in this study was not the false positive, the effect of OA extract on locomotor activity was also evaluated and results were shown in Figures 5 and 6. Our data showed that obese rats which subjected to MCAO and received vehicle (HCHF+MCAO+vehicle) significantly decreased the number of center square entry at days 7 and 14 after MCAO

(P value < 0.05 all, compared to the naïve control). However, low and medium doses of OA extract could counteract the reduction of this parameter at 7 and 14 days after MCAO, respectively (P value < 0.01 and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle). No other significant changes were observed.
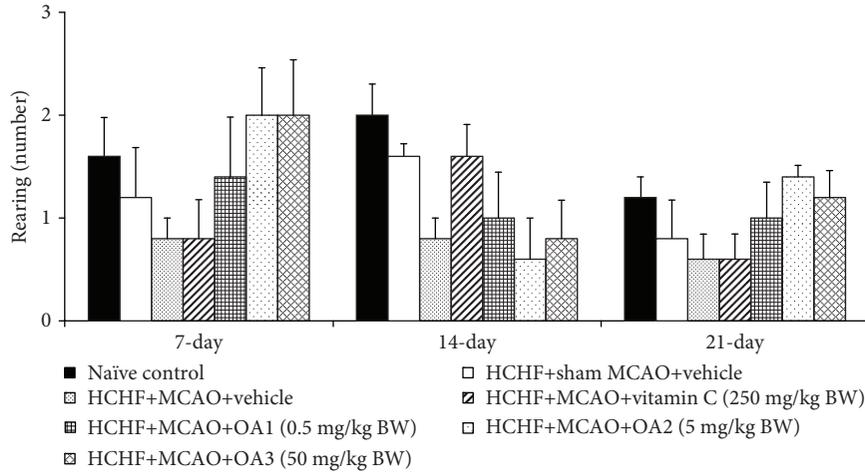
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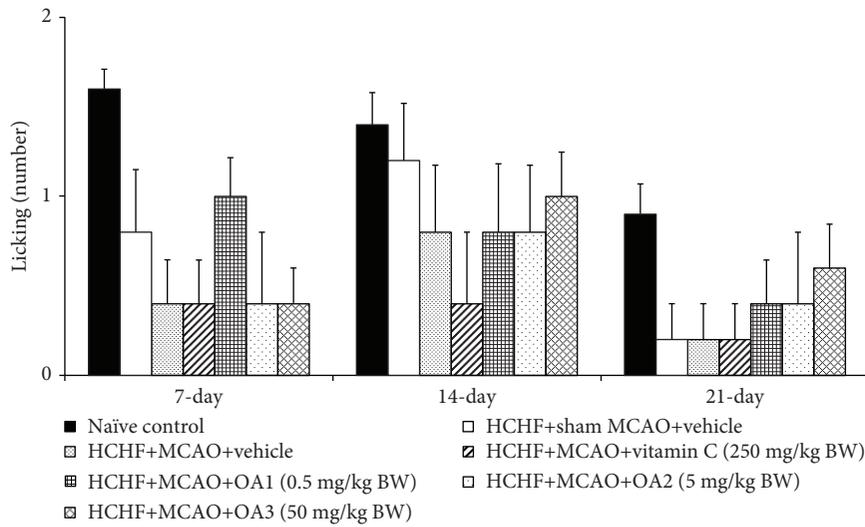
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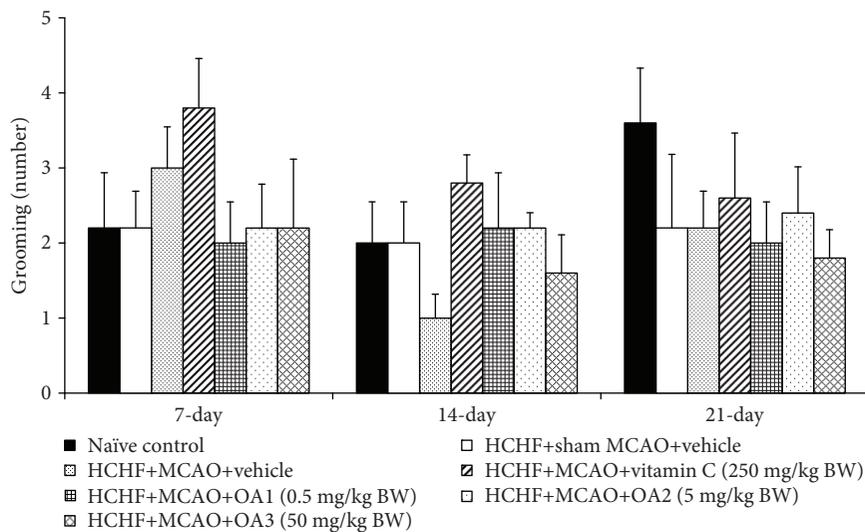
(c)

Figure 6: Effect of OA extract on exploratory activities: (a) licking, (b) rearing, and (c) grooming behaviors. Data are presented as mean ± SEM ($n = 6$/group). HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.
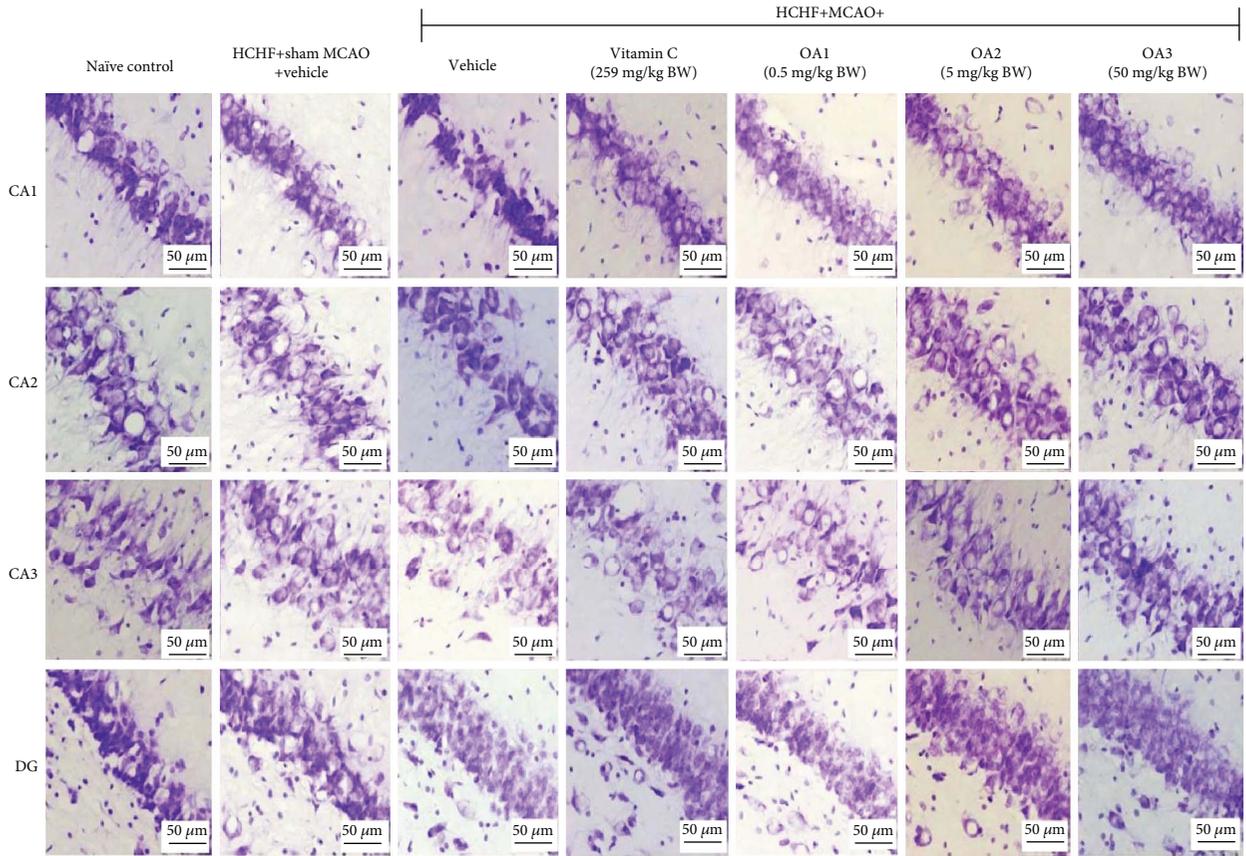
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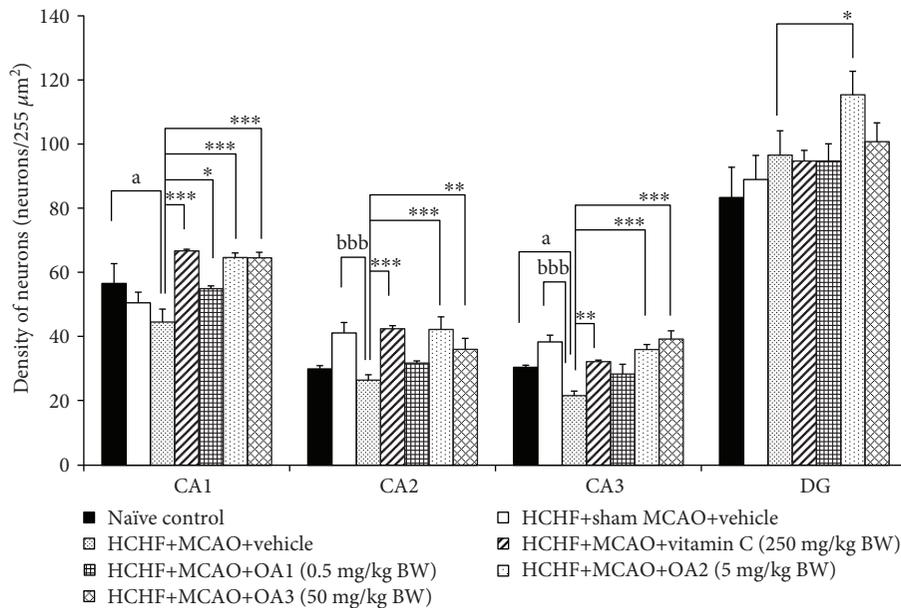
(b)

FIGURE 7: Effect of OA extract on neuron density in various subregions of the hippocampus. (a) Light microscopy of coronal sections in CA1, CA2, CA3, and dentate gyrus of the hippocampus which were stained with cresyl violet at 40x magnification. (b) Density of survival neurons in CA1, CA2, CA3, and dentate gyrus of the hippocampus. Data are presented as mean ± SEM ($n = 6$/group). [a]P value < 0.05, compared to naïve intact rats; [bbb]P value < 0.001, compared to sham operation which received HCHF diet and vehicle; and [*,**,***]P value < 0.05, 0.01, and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.
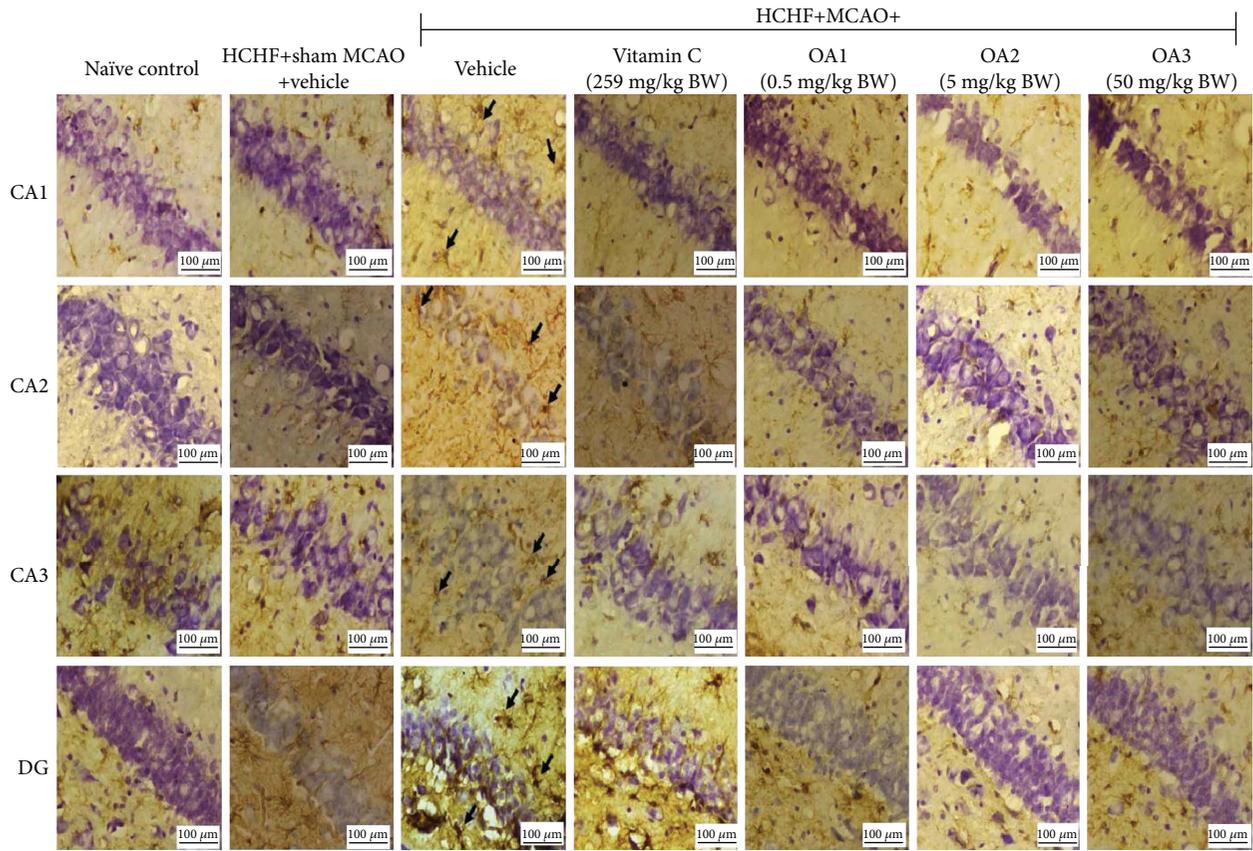
(a)
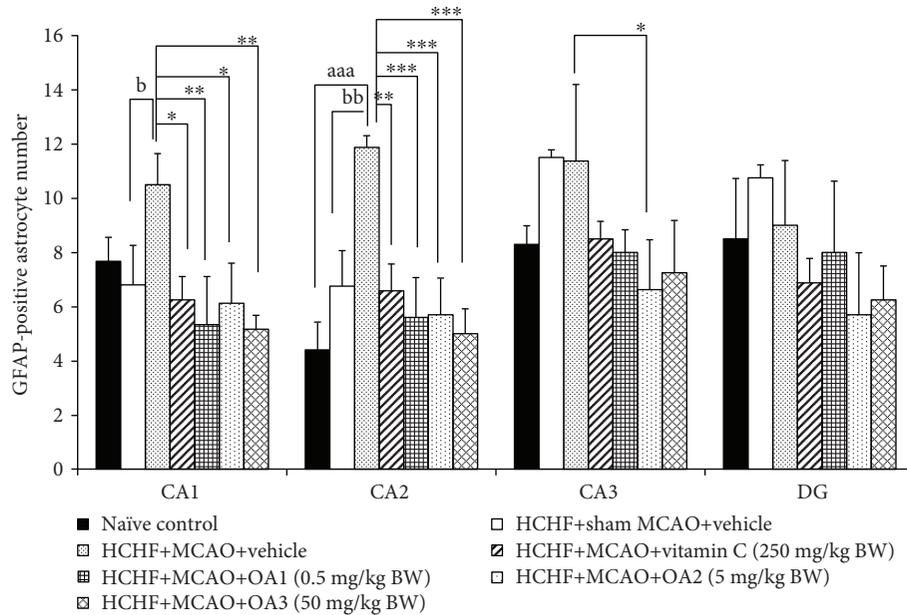
(b)

FIGURE 8: Effect of various doses of OA extract on the density of GFAP-positive cell. (a) Immunostaining for GFAP-positive cell in the hippocampus. GFAP-positive cell or astrocytes were stained brown (arrow). Magnification, 40x; scale bar = 100 μm. (b) GFAP-positive cells in CA1, CA2, CA3, and dentate gyrus of the hippocampus. Data are presented as mean ± SEM ($n = 6$/group). [aaa]P value < 0.001, compared to naïve intact rats; [b,bb]P values < 0.05 and 0.01, respectively, compared to HCHF+MCAO+vehicle; and [*,**,***]P values < 0.05, 0.01, and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively; GFAP: glial fibrillary acidic protein.
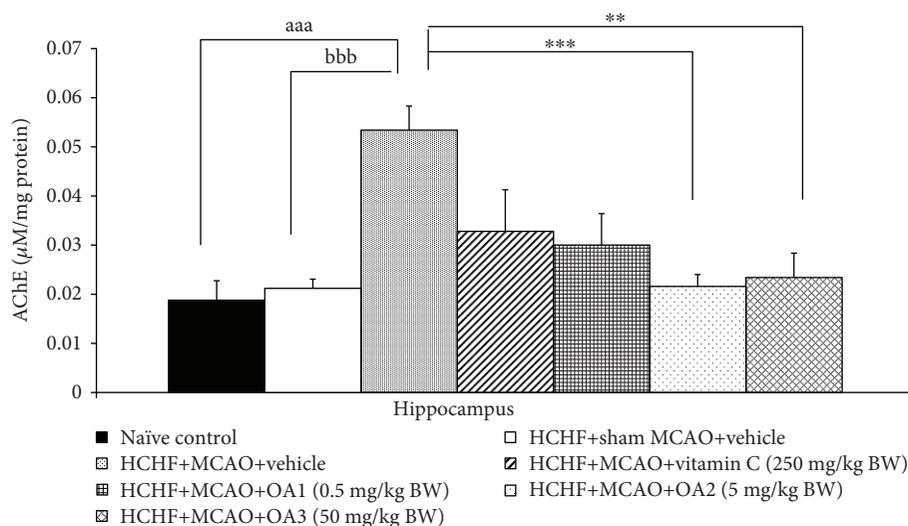
FIGURE 9: The effect of various doses of OA extract on acetylcholinesterase activity in the hippocampus. Data are presented as mean ± SEM ($n = 6$/group). [aaa]P value < 0.001, compared to naïve intact rats; [bbb]P value < 0.001, compared to sham operation which received HCHF diet and vehicle; and [**,***]P value < 0.01 and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.

3.3. Histological Changes in the Hippocampus.

Since our data demonstrated that OA extract significantly improved spatial memory, the effect of OA extract in the hippocampus, the area contributing an important role on spatial memory [26], was also explored. Figure 7 showed that MCAO significantly decreased neuron density in CA2 and CA3 (P value < 0.001 all, compared to the HCHF+sham operation+vehicle group). Vitamin C significantly increased neuron densities in CA1, CA2, and CA3 (P value < 0.001, 0.001, and 0.01, respectively, compared to the HCHF+MCAO+vehicle group). It was found that the medium dose of OA extract produced the significant increase in neuron densities in CA1, CA2, CA3, and dentate gyrus (P value < 0.001, 0.001, 0.001, and 0.05, respectively, compared to the HCHF+MCAO+vehicle group). The low dose of extract significantly increased neuron density in CA1 while the high dose of extract showed the significant increase in neuron density in both CA2 and CA3 (P value < 0.01 and 0.001, respectively, compared to the HCHF+MCAO+vehicle group).

Figure 8 showed that a HCHF diet failed to produce the significant changes in GFAP-positive cell in the hippocampus. MCAO significantly enhanced GFAP-positive cell in CA1 and CA2 metabolic syndrome rats (P value < 0.05 and 0.01, respectively, compared to the HCHF+sham operation+vehicle group). Vitamin C treatment significantly mitigated the changes mentioned earlier (P value < 0.05 and 0.01, respectively, compared to the HCHF+MCAO+vehicle group). Interestingly, the low and high doses of OA produced the significant reduction of GFAP-positive cell densities in CA1 (P value < 0.01 all, compared to the HCHF+MCAO+vehicle group) and CA2 (P value < 0.001 all, compared to the HCHF+MCAO+vehicle group). The medium dose of OA could suppress GFAP-positive cell densities in CA1, CA2, and CA3 (P value < 0.05, 0.001, and 0.05,

respectively, compared to the HCHF+MCAO+vehicle group).

3.4. Effect of OA Extract on Biochemical Parameters.

Based on the crucial role of acetylcholinesterase suppression on the memory-enhancing effect [17, 39–43], we also determined the effect of OA extract on AChE activity in the hippocampus and data were shown in Figure 9. MCAO significantly enhanced AChE activity in the hippocampus of metabolic syndrome rats induced by a HCHF diet which received vehicle (P value < 0.001, compared to the HCHF+sham operation+vehicle group). However, this change was mitigated by low and medium doses of OA extract (P value < 0.001 and 0.01, respectively, compared to the HCHF+ MCAO+vehicle group). Vitamin C and high dose of OA extract failed to modulate AChE activity in the hippocampus.

The effect of OA extract on oxidative stress markers including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) was also determined, and data were shown in Table 2. Sham operation and vehicle failed to produce the significant changes of the aforementioned parameters. MCAO significantly decreased SOD and GSH-Px activities but increased the MDA level (P value < 0.01, 0.001, and 0.001, respectively, compared to the HCHF+sham operation+vehicle group). Vitamin C mitigated the reduction of GSH-Px and the increase in the MDA level in metabolic syndrome rats subjected to MCAO (P value < 0.001 all, compared to the HCHF+MCAO+vehicle group). All doses of OA extract produced the significant reduction in the MDA level in metabolic syndrome rats with cerebral ischemia (P value < 0.001 all, compared to the HCHF+MCAO+ vehicle group). However, the significant increase in SOD and GSH-Px activities was observed only in metabolic syndrome rats with cerebral ischemia which received OA

TABLE 2: The effect of various doses of OA extract on oxidative stress markers in the hippocampus.

Treatment group	MDA level (ng/mg protein)	SOD activity (units/mg protein)	CAT activity (units/mg protein)	GSH-Px activity (units/mg protein)
Naïve control	0.16 ± 0.02	8.86 ± 1.52	4.81 ± 0.69	8.54 ± 0.59
HCHF+sham MCAO+vehicle	0.15 ± 0.04	8.73 ± 1.79	2.52 ± 0.88	10.37 ± 2.41
HCHF+MCAO+vehicle	$0.89 \pm 0.10^{aaa,bbb}$	$4.73 \pm 0.48^{aa,bb}$	0.62 ± 0.03^{aaa}	$3.77 \pm 0.26^{aa,bbb}$
HCHF+MCAO+vitamin C (250 mg/kg BW)	$0.37 \pm 0.08^{***}$	7.47 ± 0.59	1.94 ± 0.50	$6.25 \pm 0.66^{***}$
HCHF+MCAO+OA1 (0.5 mg/kg BW)	$0.37 \pm 0.06^{***}$	6.83 ± 0.19	1.45 ± 0.21	5.60 ± 0.87
HCHF+MCAO+OA2 (5 mg/kg BW)	$0.15 \pm 0.02^{***}$	5.01 ± 0.40	1.67 ± 0.25	8.87 ± 0.46
HCHF+MCAO+OA3 (50 mg/kg BW)	$0.25 \pm 0.07^{***}$	$8.01 \pm 1.48^{*}$	2.27 ± 0.27	$8.54 \pm 0.59^{**}$

Data are presented as mean ± SEM ($n = 6$/group). $^{aa,aaa}P$ value < 0.01 and 0.001, respectively, compared to naïve intact rats; $^{bb,bbb}P$ value < 0.01 and 0.001, respectively, compared to sham operation which received HCHF diet and vehicle; and $^{*,**,***}P$ value < 0.05, 0.01, and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle.

(a)

(b)
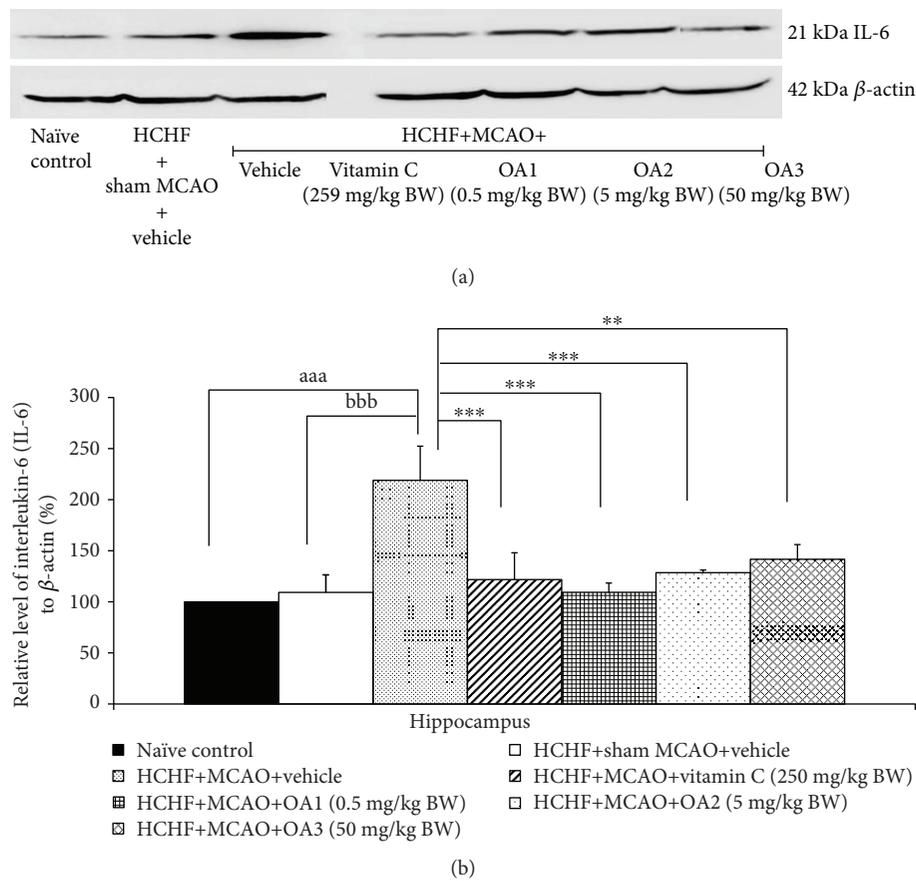
FIGURE 10: Effect of various doses of OA extract on the expression of IL-6 in the hippocampus. (a) Representative western blot showing the levels of IL-6. (b) Relative density of IL-6. Data are presented as mean ± SEM ($n = 6$/group). ^{aaa}P value <0.001, compared to naïve intact rats; ^{bbb}P value < 0.001, compared to sham operation which received HCHF diet and vehicle; and $^{**,***}P$ value < 0.01 and 0.001, respectively, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.

extract at dose of 50 mg/kg BW (*P* value < 0.05 and 0.01, respectively, compared to the HCHF+MCAO+vehicle group).

In addition, sham operation failed to produce the significant change of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels in metabolic syndrome rats with cerebral ischemia but MCAO significantly increased the levels of both substances mentioned earlier in the hippocampus (*P* value < 0.001 all, compared to the HCHF+sham operation+vehicle group). These changes were mitigated by

| | | 25 kDa TNF-α |

Naïve control | HCHF + sham MCAO + vehicle | HCHF+MCAO+

Vehicle | Vitamin C (259 mg/kg BW) | OA1 (0.5 mg/kg BW) | OA2 (5 mg/kg BW) | OA3 (50 mg/kg BW)

(a)

- ■ Naïve control
- ▨ HCHF+MCAO+vehicle
- ⊞ HCHF+MCAO+OA1 (0.5 mg/kg BW)
- ▨ HCHF+MCAO+OA3 (50 mg/kg BW)

- □ HCHF+sham MCAO+vehicle
- ◪ HCHF+MCAO+vitamin C (250 mg/kg BW)
- □ HCHF+MCAO+OA2 (5 mg/kg BW)

(b)
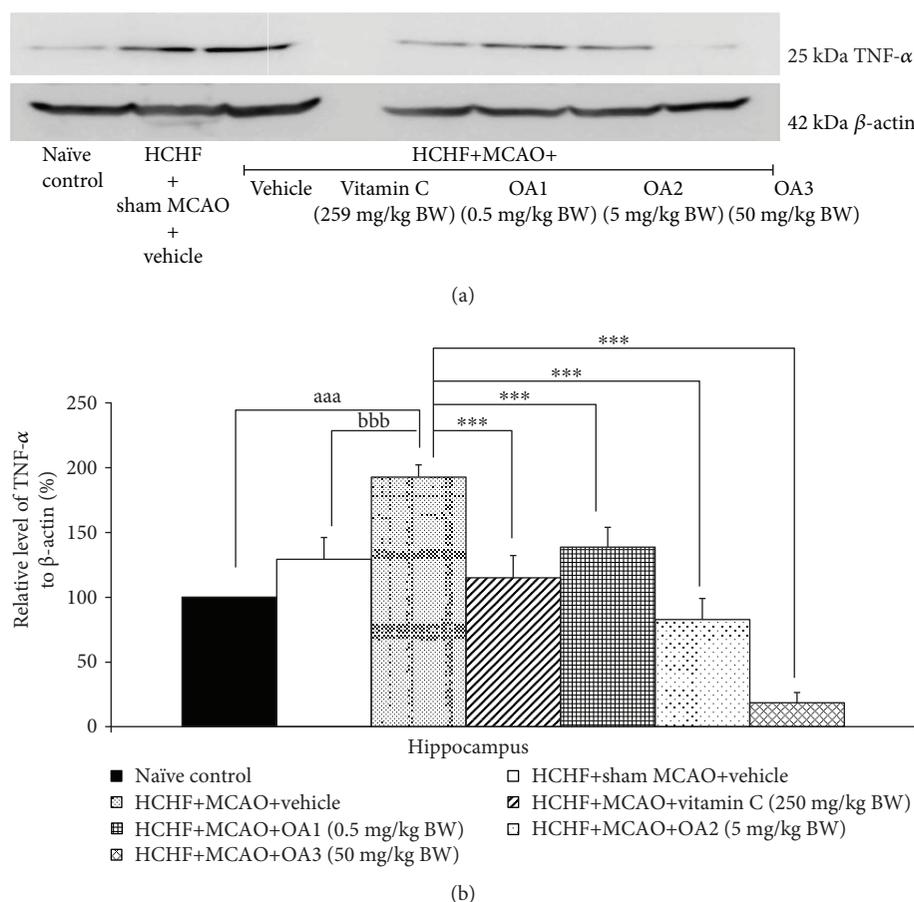
Figure 11: Effect of various doses of OA extract on the expressions of TNF-α in the hippocampus. (a) Representative western blot showing the levels of TNF-α. (b) Relative density of TNF-α. Data are presented as mean ± SEM (n = 6/group). [aaa]P value <0.001, compared to naïve intact rats; [bbb]P value <0.001, compared to sham operation which received HCHF diet and vehicle; and ***P value < 0.001, compared to MCAO rats which received HCHF and vehicle. HCHF: high-carbohydrate high-fat diet; MCAO: right middle cerebral artery occlusion; OA1, OA2, and OA3: the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW, respectively.

vitamin C and all doses of OA extract as shown in Figures 10 and 11.

4. Discussion

The current data clearly demonstrated that MetS rats induced by a HCHF diet failed to produce a significant increase in the MDA level and no significant reduction in antioxidant enzyme activities was observed in the hippocampus. In addition, no significant changes in TNF-α, IL-6, and hippocampal damage in various regions were observed. Reperfusion after cerebral ischemia (R/I) in MetS rats increased MDA, TNF-α, and IL-6 levels, but it decreased the activities of the main scavenger enzymes including SOD, CAT, and GSH-Px and neuron densities in CA2 and CA3 subregions of the hippocampus. These findings were in agreement with the previous studies which showed that R/I enhanced oxidative stress [44] which in turn destroyed the hippocampus, an area playing an important role in learning and memory [45, 46] leading to memory impairment [47]. It has been found that vitamin C and all doses of OA significantly decreased the MDA level, an oxidative stress

damage biomarker. However, no tight association between the concentrations of OA used in this study, and the alterations of antioxidant enzyme activities were observed. Although the reduction of the MDA level was observed at all doses used in this study, the elevation of antioxidant enzyme activity was observed only at high dose of OA. These data suggested that other factors might play a role on the reduction of oxidative stress. The recent study has demonstrated that the anthocyanins can act as antioxidant and remove the oxidative stress reactive species produced during reperfusion injury directly [48]. It also enhances glutathione but decreases the MDA level together with the increase in spatial memory [49]. In addition, it can also improve mitochondrial function leading to the reduction of oxidative stress production [50]. Based on the aforementioned effect of OA on oxidative stress changes and the positive modulation effect of anthocyanins mentioned earlier, we do suggest that the reduction of oxidative stress observed in this study may be explained partly via the direct antioxidant effect of anthocyanins in OA and the elevation of GSH-Px activity which in turn induces the reduction of the MDA level induced by anthocyanins. In addition, the effect to improve
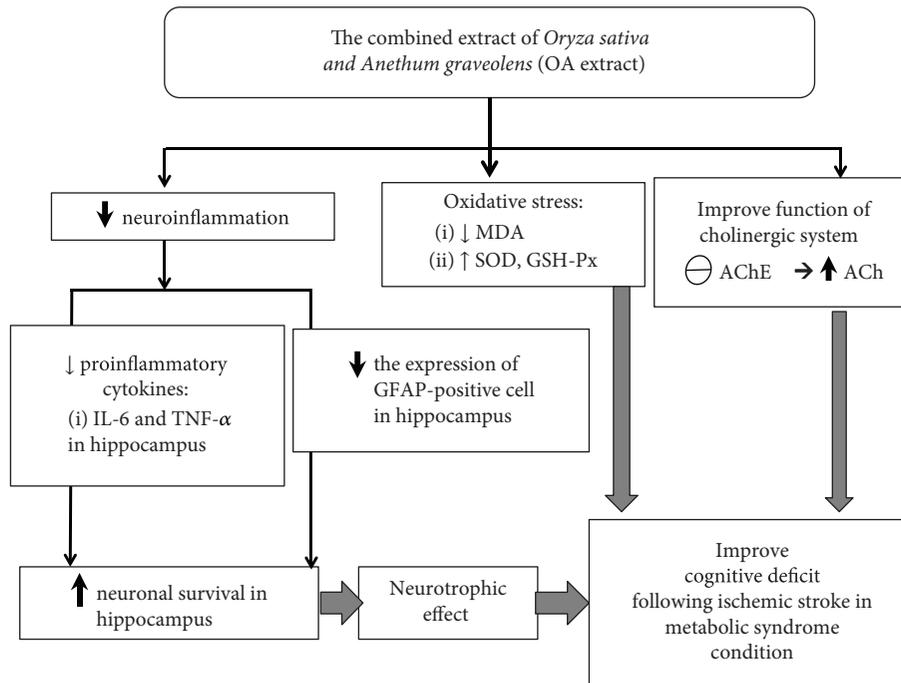
FIGURE 12: The possible underlying mechanism for the neuroprotective and cognitive enhancing effects of the combined extract of *Oryza sativa*, *L. indica*, and *Anethum graveolens* Linn. (OA extract) in an animal model of metabolic syndrome with cerebral ischemic stroke.

mitochondria functions induced by anthocyanins may also contribute a role on the reduction of oxidative stress observed in this study.

In addition to the oxidative stress, neuroinflammation also plays the role on the brain damage and memory deficit following ischemic injury [51]. Our data also showed that reperfusion injury following cerebral ischemia in metabolic syndrome rats increased TNF-α and IL-6 together with the cognitive impairment which were in agreement with the previous study [51–53]. After injury, injured neurons in the core and penumbra of the lesion and glial cells in the core produce proinflammatory mediators, cytokines, and reactive oxygen species, which activate both astrocytes and microglia [54]. Then, activated astrocytes can produce the proinflammatory cytokines including IL-6, TNFα, and others [55, 56]. The elevations of cytokines produce the detrimental effect to brain by inducing apoptosis and suppressing the hippocampal neurogenesis [57, 58]. However, the brain damage and the neuroinflammation can be attenuated by anthocyanins [59, 60]. In addition, anthocyanins can also decrease neuroinflammation and the density of GFAP-positive cell in the hippocampus and improve memory dysfunction [61]. In addition, our data also showed that OA suppressed AChE in the hippocampus of metabolic syndrome rats.

Based on all data mentioned earlier, we suggest that anthocyanins present in OA may exert the beneficial effect at multitarget sites. Anthocyanins may improve oxidative stress in the hippocampus via the increase in GSH-Px activity and the direct antioxidant effect of anthocyanins. The reduction in the oxidative stress production by mitochondria may also contribute the role, but this part still required a further investigation. In addition, anthocyanins in OA can also

decrease glial cell expression in the hippocampus resulting in the decrease in neuroinflammation leading to the increase in neurogenesis in various subregions of the hippocampus. Therefore, the density of functional neurons in the hippocampus increases giving rise to the improvement of memory performance. Since both glial cell expression and neurogenesis are under the influence of the growth factor, it is possible that anthocyanins can exert the effect to modulate the expression of the growth factor which in turn decreases GFAP-positive cell or glial cell especially astrocyte but enhances neurogenesis in the hippocampus. However, this requires further exploration. Moreover, OA also suppresses AChE activity in the hippocampus which in turn enhances an available ACh giving rise to the memory enhancement.

5. Conclusion

The current study clearly demonstrates that the combined extract of *O. sativa* and *A. graveolens* or OA is the functional ingredient to improve cognitive deficit following ischemic stroke in metabolic syndrome condition. The possible underlying mechanisms involve multitargets including the reduction of oxidative stress and neuroinflammation levels together with the suppression of AChE activity. Interestingly, OA also possesses neurotrophic effect and suppresses the expression of GFAP-positive cell in the hippocampus as shown in Figure 12. Based on the modulation effects of OA on various proteins mentioned earlier, the epigenetic modulation effect induced by OA is worth for further exploration.

Data Availability

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Hypoglycemic Mechanism of the Berberine Organic Acid Salt under the Synergistic Effect of Intestinal Flora and Oxidative Stress

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Both alterations to the intestinal microflora and chronic systemic inflammation predispose towards type 2 diabetes (T2D). Changes in the composition of the intestinal microflora are associated with glucose metabolism changes in rats with T2D. Here, we demonstrate that a berberine fumarate (BF) has a hypoglycemic effect by regulating the intestinal microflora and metabolism of diabetic rats. The T2D rats had disorders of glucose and lipid metabolism, an abnormal intestinal microflora, fewer butyrate-producing and probiotic-type bacteria, larger numbers of potentially pathogenic and sulfate-reducing bacteria, and tissue inflammation. Administration of berberine fumarate significantly ameliorated the metabolic disorder; increased the populations of Bacteroidetes, Clostridia, Lactobacillales, Prevotellaceae, and Alloprevotella; and reduced those of Bacteroidales, Lachnospiraceae, Rikenellaceae, and Desulfovibrio. In addition, it reduced inflammation, inhibiting the overexpression of TLR4 and p-JNK and increasing the expression of PI3K, GLUT2, and other proteins, which are closely related to oxidative stress, thereby promoting the metabolism of glucose.

1. Introduction

Type 2 diabetes (T2D) comprises a series of metabolic disorders caused by hypofunction of pancreas and insulin resistance. It is characterized by chronic hyperglycemia, which can result in many complications, such as heart disease, stroke, and chronic renal failure [1–3]. Not only is T2D associated with insulin resistance, but also it has been confirmed that changes in the intestinal microflora are involved in the low-grade chronic systemic inflammatory response that characterizes insulin resistance [4–6]. The microflora present in the human intestinal tract directly participate in digestion and absorption and influence energy metabolism in the body. Subtle changes in the species present and their proportions may lead to the development of various chronic metabolic diseases. In particular, accumulating evidence suggests that the intestinal microflora plays an important role in the pathogenesis of diabetes [7].

Therapeutic targeting of the microflora may have the potential to ameliorate insulin resistance and reduce the incidence of diabetic complications [8]. Conversely, disorders of the intestinal flora lead to inflammation and insulin resistance, exacerbating diabetes. Indeed, many studies have shown a correlation between changes in intestinal flora and markers of diabetes [9]. The intestinal microflora may influence the body mass, insulin sensitivity, bile acid metabolism, inflammation, and gastrointestinal hormone secretion by the host [10, 11]. Therefore, regulation of the intestinal microflora may be beneficial for glucose metabolism and ameliorate insulin resistance in the host. Release of lipopolysaccharide (LPS) by intestinal Gram-negative bacteria can initiate systemic inflammation, because LPS binds to and

promotes the expression of CD14 and toll-like receptor 4 (TLR4), which activates an intracellular signaling pathway that leads to the secretion of proinflammatory cytokines. Oxidative stress is an imbalance in cellular redox reactions which plays a key role in the pathogenesis of metabolic disorders and drug-induced injury. Oxidative stress is the result of reactive oxygen species (ROS) overproduction or a decline in antioxidant defense mechanisms. Several diseases, including obesity, metabolic syndrome, diabetes mellitus, and others, are well-known to be associated with excessive ROS production. Thus, agents or signaling pathways that counteract excessive ROS and/or modulate oxidative stress represent an attractive strategy for treating T2D. The same study also showed that antibiotics have beneficial effects on the metabolic abnormalities in obese mice, ameliorating the impaired glucose tolerance, body mass gain, metabolic endotoxemia, inflammation, and oxidative stress [12].

Berberine is an isoquinoline alkaloid extracted from the rhizomes of *Ranunculaceae coptis* or *Cortex phellodendri*. A number of clinical studies have shown that berberine has a significant hypoglycemic effect in patients with T2D [13]. However, the oral bioavailability of berberine is low (0.68%), and about half of the dose is not absorbed by the intestines [14, 15]. Studies have shown that berberine organic acids can reduce blood glucose and have the good effects of berberine hydrochloride [13]. Fumaric acid, an intermediate product of the tricarboxylic acid (TCA) or Krebs cycle, has been widely used in food, sustainable chemistry, and biomedical applications, and fumarate is often used in the treatment of chronic inflammatory skin diseases and autoimmune diseases [16–18]. It has been shown that fumarate releases free radicals in the process of scavenging inflammation, thus protecting nerve and glial cells [19], while numerous clinical trials have demonstrated the safety and immunomodulatory effects of oral fumarate [20–22]. Therefore, the study's aim was to test if BF may have a beneficial effect on the composition of the intestinal flora in type 2 diabetic rats.

2. Materials and Methods

2.1. Materials and Animals.
Streptozotocin (STZ) (purity > 98%) was purchased from Aladdin Bio-Reagent (Shanghai, China). ELISA kits were supplied by Jiancheng Biotech Sci. Inc., Nanjing, China.

Male Sprague-Dawley rats weighing 180–200 g were acclimatized to the experimental conditions of $20 \pm 2°C$, humidity $60 \pm 5\%$, 12 h light/dark cycle, and *ad libitum* access to food and water. Rats and food (high-fat, high-sucrose diet containing 20% fat, 20% sucrose, and 2.5% cholesterol) were purchased from the Laboratory Animal Center of Zhejiang Academy of Medical Sciences (Zhejiang, China; certificate number SCXK 2014-0001). The study was approved by the animal ethics committee of Zhejiang Academy of Medical Sciences. All animal procedures were conducted in accordance with the Chinese animal care guidelines, which conform with internationally accepted guidelines for the use of experimental animals.

2.2. Experimental Design.
Berberine fumarate (BF) was prepared by our laboratory to a purity of 98.8% [13]. T2D was induced by feeding rats with the high-fat, high-sucrose diet for 12 weeks, after which a single intraperitoneal injection of 50 mg/kg STZ dissolved in pH 4.2–4.8 citrate buffer solution (0.1 mol/L) was administered [23]. All rats were randomly divided into four groups ($n = 8$) as follows: NC (normal control) nondiabetic mice intragastric treated with distilled water, T2D rats intragastric treated with distilled water, 140 mg/kg·d metformin, or 500 mg/kg·d BF for 4 weeks.

After treatment was completed, blood was collected from a retro-orbital vein after 12–18 h of overnight fasting and centrifuged at $5120 \times g$ for 10 min. Fasting blood glucose (FBG) and plasma levels of fasting insulin (FINS), triglycerides (TG), total cholesterol (TC), total superoxide dismutase (T-SOD), glycosylated serum protein (GSP), glutathione peroxidase (GSH-PX), glucagon-like peptide 1 (GLP-1), and LPS-binding protein (LBP) were measured using ELISA kits. The homeostasis model assessment for insulin resistance (HOMA-IR) was also calculated. At the end of the experiment, pancreas, liver, and ileum samples were promptly excised from animals anesthetized with 10% chloral hydrate solution. The samples were rinsed with normal saline and fixed in 10% neutral-buffered formalin for histopathological examination, following hematoxylin-eosin (H&E) staining. Fresh small intestinal contents were collected from the ileum in a sterile environment and stored at −80°C until use.

2.3. Analysis of the Composition of the Bacterial Microflora.
Five samples from each group were used for analysis of the intestinal microbiota. Microbial genomic DNA was extracted from each fecal sample (0.1 g) using a Genomic DNA Isolation Kit (Sangon Biotech Co. Ltd., Shanghai, China). The construction of a high-throughput sequencing library and its sequencing using the Illumina MiSeq platform were completed by GENEWIZ (Suzhou, China). The concentration of DNA samples was measured using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). The sequencing libraries were constructed using MetaVx™ Library construction kits (GENEWIZ Inc., South Plainfield, NJ, USA). Multiple variable regions of 16S rDNA (V3, V4) were amplified using DNA as a template. Sequence analysis was performed using VSEARCH (1.9.6) software. Sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). Taxonomic annotation was conducted using a ribosomal database project (RDP) classifier. Based on the results of the OTU analysis, alpha and beta diversity analyses were performed for all samples, to provide information about species richness, evenness, and differences in community structures.

2.4. Semiquantitative RT-PCR Analysis.
Total RNA was isolated from ileum with TRIzol reagent (Sangon Biotech Co. Ltd., Shanghai, China). The expression levels of specific mRNAs were determined using semiquantitative RT-PCR analysis, normalizing to β-actin expression. One microgram of total RNA was reverse-transcribed using oligo dT and reverse transcriptase (Boya Co. Ltd., Shanghai, China). Then,

TABLE 1: Sequence of primers used for the RT-PCR assays.

Genes	Primer sequences	
	Forward primer ($5'\rightarrow3'$)	Reverse primer ($5'\rightarrow3'$)
TLR4	ATCATCCAGGAAGGCTTCCA	GCTGCCTCAGCAAGGACTTCT
JNK	TGACGCCTTATGTGGTGACT	TGATGTATGGGTGCTGGAGA
GLUT2	CCTGCTTGGTCTATCTGCTGTG	CCTTGCTTTGGCTTCCCC
PI3K	CATCACTTCCTCCTGCTCTAT	CAGTTGTTGGCAATCTTCTTC
β-Actin	GCCATGTACGTAGCCATCCA	GAACCGCTCATTGCCGATAG

cDNAs were amplified using oligonucleotide primers (Table 1) using a One-Step RT-PCR kit (Takara Co., Japan). The following PCR conditions were applied: denaturation at 95°C for 1 min, followed by 33 cycles (c-Jun n-terminal kinase (JNK) and phosphoinositol 3-kinase (PI3K): 95°C for 30 s, then 56°C for 1 min, and 72°C for 1 min) or 40 cycles (β-actin, TLR4, and facilitative glucose transporter 2 (GLUT2): 95°C for 30 s, then 60°C for 30 s), with a final extension at 72°C for 5 min. The PCR products were subjected to horizontal electrophoresis on 1.0% agarose gels, and images were captured using a Bio-Rad ChemiDoc imaging system (Hercules, CA, USA).

2.5. Western Blot Analysis. Ileal samples were homogenized in RIPA lysis buffer and centrifuged at $10001 \times g$ (30 min, 4°C) to obtain cleared lysates. The supernatant protein concentrations were then measured using a BCA Protein Assay Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China). For Western blot analysis, equal amounts of protein (50 μg/lane) were electrophoresed on 12% polyacrylamide gels, after which they were electrotransferred onto polyvinylidene fluoride membranes (Millipore, Marlborough, MA, USA). Membranes were then incubated for 3 h in blocking buffer (1x tris-buffered saline containing 0.1% Tween-20, and 4% nonfat milk) at room temperature and then overnight in the same buffer containing primary antibodies against TLR4 (1: 1000), p-JNK (1: 1000), GLUT2 (1:1000), PI3K (1:500), or β-actin (1: 1500) (Boster Biological Technology Ltd., Wuhan, China). For antibodies targeting phosphorylated proteins, bovine serum albumin (BSA) was used instead of nonfat milk. Membranes were then washed three times for 5 min and incubated for 2 h at 4°C with HRP-conjugated secondary antibodies (anti-rabbit or anti-rat) (Boster Biological Technology Ltd., Wuhan, China). Proteins were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway, NJ, USA) [24].

2.6. Statistical Analysis. All data are expressed as mean ± standard deviation (SD) and were analyzed using SPSS statistical software (SPSS 19.0, SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) with Duncan's test was used for intergroup comparison. $P < 0.05$ was considered to represent statistical significance.

3. Results and Discussion

3.1. The Effect of BF on Biochemical Indices in the Plasma of T2D Rats. Many studies have shown that berberine is an effective antihyperglycemic agent and has positive effects on diabetic complications, such as hypertension, hyperlipidemia, cardiovascular and cerebrovascular diseases, and peripheral nerve lesions [13, 25]. However, we hypothesized that the combination of berberine and fumaric acid could be safer and more effective [13].

As shown in Figure 1, BF treatment significantly increased plasma FINS, T-SOD, GSH-PX, and GLP-1 and reduced FBG, HOMA-IR, and plasma TG, TC, and GSP (all $P < 0.05$) in diabetic rats. Berberine can inhibit gluconeogenesis by regulating the function of β cells and can also promote glycolysis, thereby lowering blood glucose and lipid levels and ameliorating insulin resistance [26]. The effect of berberine to reduce blood lipid is the result of an improvement in antioxidant capacity, the promotion of lipid metabolism, and the inhibition of preadipocyte differentiation [13, 25]. Excessive oxygen-free radicals can trigger insulin resistance by modulating signal transduction [27], and insulin resistance may aggravate oxidative stress [28].

It has also been shown that berberine can ameliorate abnormalities in plasma gastrointestinal hormone levels, such as those of glucagon-like peptide- (GLP-) 1 and 2, insulin stimulating polypeptide [29], and pancreatic polypeptide. GLP-1 is mainly secreted by L cells distributed throughout the ileum. Berberine can increase the proliferation of L cells, increase glucagon and prohormone invertase synthesis, and enhance GLP-1 secretion in diabetic rats [30]. Therefore, BF may regulate glucose and lipid metabolism by promoting the synthesis and secretion of GLP-1 in the intestinal tract.

Many studies have shown that the human intestinal microflora can convert insoluble nutrients, such as proteins and carbohydrates, into soluble substances, to facilitate their absorption. It can also transform difficult-to-absorb polysaccharides into monosaccharides, metabolize glucose to form lactic acid, and participate in the metabolism of cholesterol, thereby having an important effect on the nutrients present in and absorbed from the intestine [31]. Microbiological studies have shown that there are significant differences in the type and number of intestinal bacteria in the intestines of diabetic patients and in those of healthy people [32, 33]. One study has also shown that the secretion of GLP-1 and other hormones is lower in high-fat diet-fed mice, while feeding additional dietary fiber or probiotics can significantly increase the secretion of the same hormones [34, 35], suggesting that the secretion of gastrointestinal hormones is influenced by changes in the intestinal microflora. Also, there was no significant difference between the BF group and the Me group.
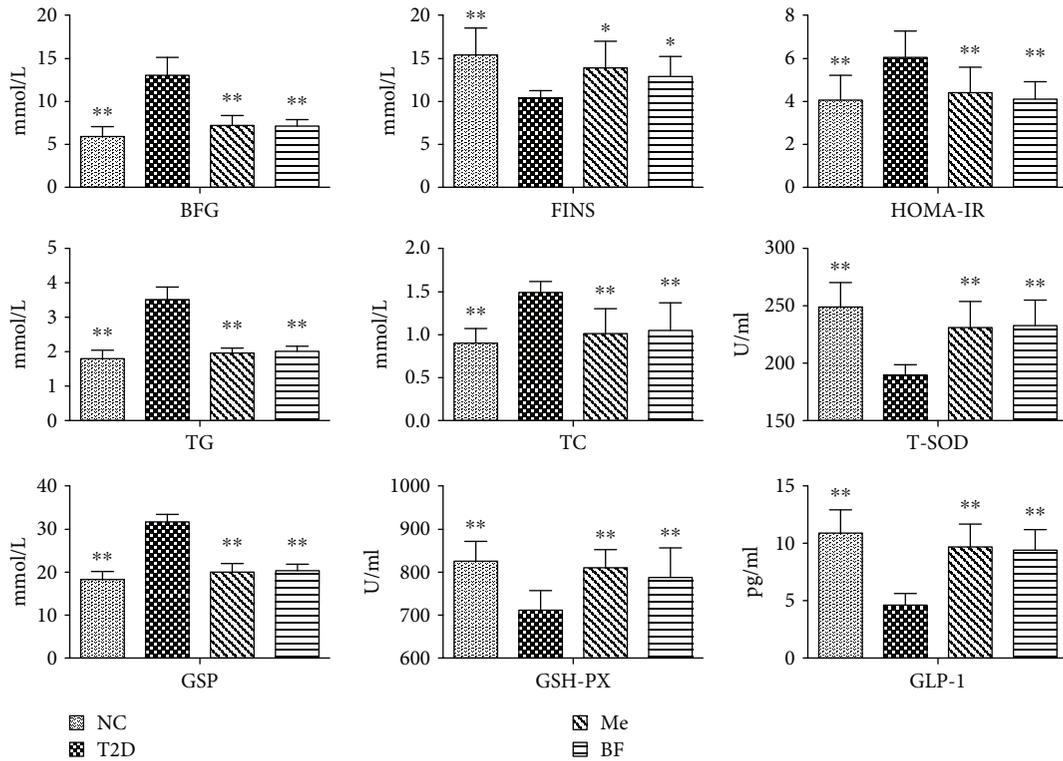
FIGURE 1: The effect of BF on the plasma index of T2D rats. The data were expressed as mean ± SD ($n = 8$), $^*P < 0.05$ and $^{**}P < 0.01$ vs T2D group.

3.2. Effect of BF on the Intestinal Microflora of Rats with T2D. The normal intestinal flora forms a natural barrier on the surface of the intestinal mucosa and participates in normal digestion and absorption in humans, but it also regulates immune function and prevents the invasion of pathogenic bacteria and opportunistic pathogens [36]. Thus, the intestinal microflora directly participates in nutrient digestion and absorption, energy supply, fat metabolism, immune regulation, and disease resistance [37, 38]. In addition, disorders in the composition of the microbiota may also be involved in the development of chronic metabolic diseases, such as obesity [39, 40] and diabetes [41, 42]. Previous findings have suggested that the beneficial effects of metformin (Me) on glucose metabolism may be in part microbially mediated [43, 44]. Berberine has been used clinically for many years to treat bacterial infections in the intestines, but in addition, after 4 weeks of berberine administration to high-fat diet-fed mice, their body mass, visceral fat content, blood glucose, and plasma lipid content had been reduced significantly. However, these effects were also associated with significant reductions in the proportions of Firmicutes and Bacteroidetes in the feces of the mice [45].

Here, alpha diversity analysis showed that the bacterial species richness and diversity in the intestines of the T2D group were significantly lower than those of the control rats ($P < 0.05$), but that these were largely normalized by treatment with metformin and BF (Figure 2). Nonmetric multidimensional scaling (NMDS) plots of these data are shown in

Figure 2(c). The stress for these was <0.114, indicating that NMDS accurately reflects the degree of difference between samples, which is indicated by the distance between each point. This technique demonstrates a clear difference between the T2D group and the other groups. Previous studies have shown that berberine administration can reduce the diversity of the intestinal microflora in rats and selectively increase the abundance of bacteria producing short-chain fatty acids, such as Blautia and Allobaculum [46].

As shown in Figure 2, representatives of eight main phyla were detected in the small intestinal contents of each group: Firmicutes, Bacteroidetes, Saccharibacteria, Proteobacteria, Actinobacteria, Deferribacteres, Tenericutes, and Cyanobacteria. Members of the Firmicutes and Bacteroidetes were dominant in all the groups. However, the relative abundance of Bacteroidetes in the T2D group (44.07%) was lower than in the NC group (61.46%), but this difference was largely abolished after treatment (Me: 54.54%; BF: 54.77%). An imbalance in the proportions of Firmicutes and Bacteroidetes in the intestinal microflora is associated with many diseases. The ratios of Firmicutes to Bacteroidetes (F/B) in the NC, T2D, Me, and BF groups were 0.54, 1.09, 0.74, and 0.73, respectively (Figure 2(d)). Previous studies have shown that Bacteroidetes are more abundant in patients with diabetes, such that the F/B ratio is lower [47]. Here, we show that the F/B ratio in the rat intestines was positively correlated with their host's blood glucose concentrations ($P < 0.01$) (Table 2), which is

(a)

(b)

(c)

(d)

(e)

Figure 2: Continued.

Anaeroplasmatales
Bacillales
Bacteroidales
Campylobacterales
Clostridiales
Coriobacteriales
Deferribacterales
Desulfovibrionales

Enterobacteriales
Erysipelotrichales
Gastranaerophilales
Lactobacillales
Mollicutes_RF9
Pasteurellales
Pseudomonadales
Unknown_order

(f)

Anaeroplasmataceae
Bacteroidaceae
Bacteroidales_S24-7_group
Christensenellaceae
Clostridiaceae_1
Clostridiales_vadinBB60_group
Coriobacteriaceae
Deferribacteraceae
Desulfovibrionaceae
Enterobacteriaceae
Erysipelotrichaceae
Family_XIII
Helicobacteraceae

Lachnospiraceae
Lactobacillaceae
Moraxellaceae
Pasteurellaceae
Peptococcaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae
Staphylococcaceae
Streptococcaceae
Unclassified
Unknown_family

(g)

Figure 2: Continued.

(h)

FIGURE 2: The effect of BF on the structure of intestinal flora of T2D rats. (a, b) Analysis of alpha diversity; (c) analysis of beta diversity; (d–h) the relative abundances of main species under different levels (phylum, class, order, family, and genus). $n = 5$, $^{*}P < 0.05$ and $^{**}P < 0.01$.

consistent with the findings of previous studies [48]. However, no significant correlation was found between F/B ratio and blood lipids, oxidative stress, and GLP-1.

The Clostridia, Bacteroidia, and Bacilli were the dominant bacterial classes identified. Compared with the NC group (55.56%, 29.23%), the relative abundance of Clostridia (44.24%) was lower and that of the Bacteroidia (42.77%) was higher, in the T2D group, but these differences were largely abolished by treatment (Me: 53.34%, 31.89%; BF: 51.50%, 30.01%) (Figure 2(e)). In addition, the relative abundance of Clostridia in the rat intestines was negatively correlated with their host's blood glucose [49, 50], as shown in Table 2

TABLE 2: Analysis of the correlation between abundance of intestinal flora and fasting blood glucose (FBG).

		F/B	Clostridia	Epsilon-proteobacteria	Bacillales	Lactobacilli
FBG	P	0**	0.012*	0.001*	0.006*	0.0541
	r	0.763	−0.552	0.683	−0.596	0.501

($P < 0.05$). Previous studies have shown that Clostridiales and Lactobacillales can ferment saccharides to form butyric acid or conjugate oleic acid, which can contribute to cell differentiation [51]. The present study shows that the Clostridiales and Bacteroidales were the dominant orders in the intestines of each group of rats. In addition, compared with the NC group (56.34%, 7.33%, and 20.53%), the relative abundances of Clostridiales (35.91%) and Lactobacillales (2.46%) were lower and that of Bacteroidales (47.31%) was higher, in the T2D group, and these differences were largely abolished by treatment (Me: 54.06%, 3.75%, and 28.14%; BF: 53.89%, 5.05%, and 29.26%) (Figure 2(f)). Moreover, there was no significant difference between the BF group and the Me group.

Previous studies have shown that lactic acid produced by bacteria can be used by other bacteria to produce butyric acid in the intestine, thereby promoting intestinal synthesis of mucin, which protects the intestinal mucosa [52]. Berberine has been shown to reduce the abundance of butyric acid-producing bacteria in the Clostridium coccoides and Clostridium leptum subgroups, and this is significant because butyric acid bacteria can degrade plant polysaccharides to provide additional energy for the host. Roseburia are butyric acid-producing bacteria, while Prevotella can cause the degradation of mucin. Previous animal experiments have shown that the presence of large numbers of Lactobacilli and Bifidobacteria are associated with diabetes resistance, while large numbers of Bacteroides and IV Clostridium can promote the development of diabetes [53]. Bacteroides and IV Clostridium can use glucose and lactic acid to synthesize short-chain fatty acids which cannot be used for mucin synthesis [54], but instead increase the permeability of the intestinal mucosa and promote inflammation [52]. These findings regarding the effects of specific bacterial groups have laid the foundation for the targeting of the intestinal microflora in T2D therapy.

In Figure 2(g), the Lachnospiraceae and Bacteroidales S24-7 are shown to be the dominant bacterial types in each rat group. Compared with the NC group (30.25%, 3.34%, 29.84%, and 6.34%), the relative abundances of Lachnospiraceae (44.58%) and Rikenellaceae (3.55%) were higher and those of Bacteroidales S24-7 (15.21%) and Prevotellaceae (1.47%) were lower, in the T2D group. In addition, there were also different distributions of families and genera within a phylum. For example, with regard to Bacteroidetes, compared with the NC group (0.58%), the relative abundance of Bacteroidaceae (1.77%) was higher, but those of S24-7 and Rikenellaceae were lower in the T2D group. Prevotella copri and Bacteroides vulgatus have previously been identified as the main species responsible for the association between the

biosynthesis of branched-chain amino acids (BCAAs) and insulin resistance, and in mice we have demonstrated that P. copri can induce insulin resistance, aggravate glucose intolerance, and augment circulating levels of BCAAs [8].

Figure 2(h) shows that the abundance of the Desulfovibrio genus (2.69%) of Proteobacteria was higher and that of Alloprevotella (0.92%) of Bacteria was lower in the T2D group than in controls (0.85%, 3.34%). Desulfovibrio species are sulfate-reducing bacteria that reduce sulfate to sulfide in the intestines. Sulfides have toxic effects on intestinal epithelial cells [55], inducing abnormal proliferation and metabolism of epithelial cells, which impairs intestinal barrier function [56, 57]. In addition, the abundance of Epsilon-proteobacteria is positively correlated with FBG ($P < 0.05$) (Table 2).

Previous studies have shown that hyperglycemia can result from a decrease in the proportion of anaerobes, especially Bacteroides [9]. Therefore, regulation of the intestinal microflora may be beneficial to the glucose metabolism and insulin sensitivity of the host. Furthermore, the study has shown that abundance of Lactobacilli positively correlates with FBG and glycosylated hemoglobin levels and that of Clostridia negatively correlates with FBG, glycosylated hemoglobin, and insulin levels [58]. As shown in Table 2, the abundance of Clostridia and Bacillales are negatively correlated with FBG ($P < 0.05$), while there is no clear correlation between the abundance of Lactobacilli and FBG ($P > 0.05$), perhaps because of the large number of changes in the composition of intestinal microflora. A considerable number of bacterial types used in probiotics, such as Bifidobacteria and Lactobacilli, exist in the intestinal tract of healthy people. However, the number of such bacteria in diabetic patients is significantly lower than that in healthy individuals, and therefore their numbers may be negatively correlated with FBG [59–61]. Studies have shown that high-fat diet leads to intestinal flora structure disorder by inducing oxidative stress, and intestinal flora can significantly regulate lipid metabolism [10]. However, no significant correlation was found between oxidative stress and lipid metabolism and bacteria.

3.3. The Effect of BF Administration on the TLR4/JNK/PI3K Signaling Pathway.

The effect of BF on insulin resistance in rats with T2D was at least partially mediated through effects on the intestinal microflora. In particular, changes in the intestinal flora can reduce the level of plasma lipopolysaccharide-binding protein (LBP) being produced and thereby help to reduce systemic inflammation (Figure 3).

The c-Jun n-terminal kinase (JNK) plays a vital role in the metabolic changes and inflammation induced by a

(a)

(b)

(c)

(d)

(e)
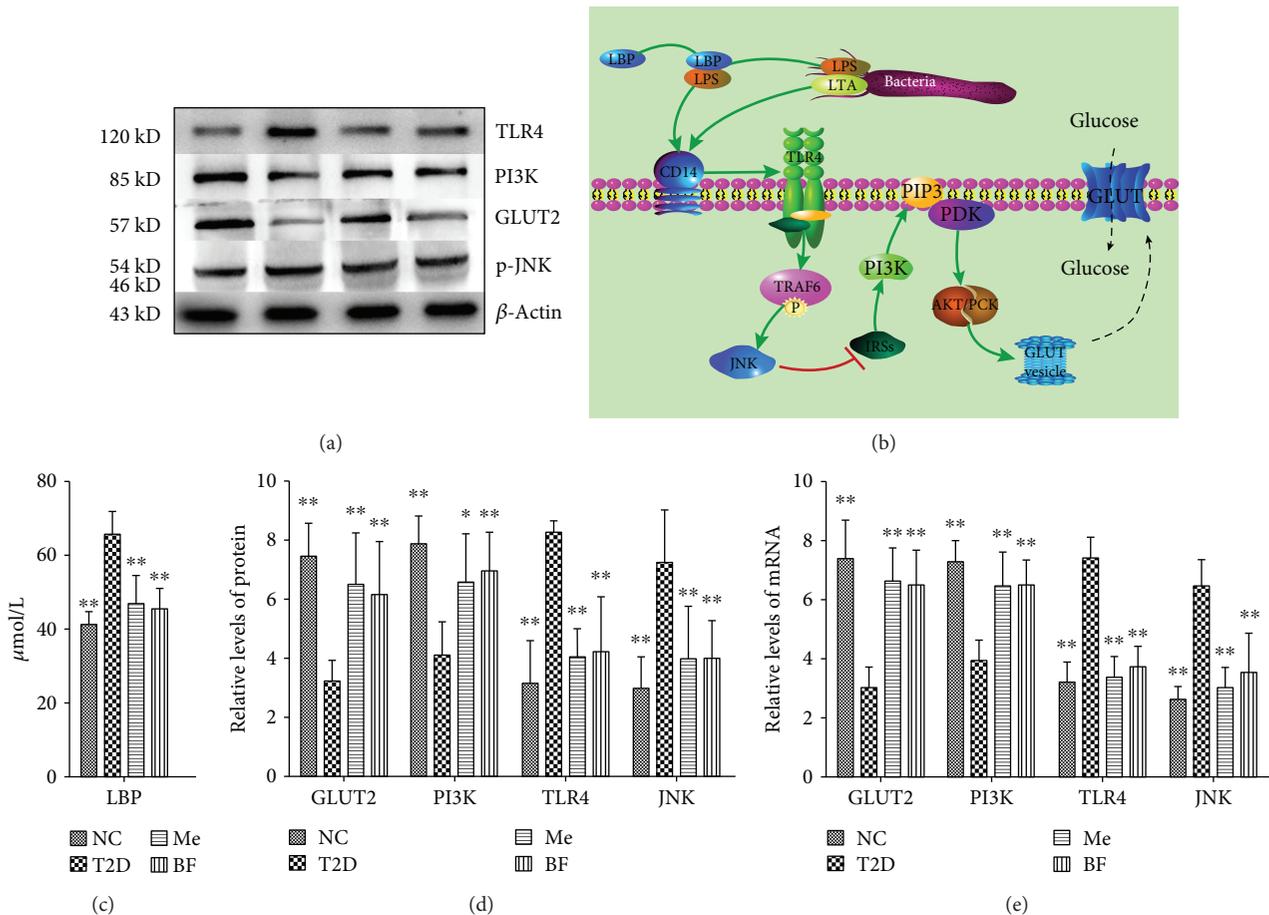
FIGURE 3: The results of Western blot and RT-PCR. (b) Signal pathways, "↑," activation and "T," inhibition. The data were expressed as mean ± SD ($n = 8$), $^*P < 0.05$ and $^{**}P < 0.01$ vs T2D group.

high-fat diet, which are involved in the pathogenesis of T2D [62–64]. Under diabetic conditions, the JNK pathway is activated in various tissues and has deleterious effects on both insulin sensitivity and pancreatic β-cell function. Activation of the JNK pathway interferes with insulin action and reduces insulin biosynthesis, and its inhibition in diabetic rats ameliorates insulin resistance and β-cell function, leading to an improvement in glucose tolerance. Thus, the JNK pathway is likely to play a central role in the progression of insulin resistance and β-cell dysfunction and could represent a potential therapeutic target for T2D [64].

The PI3K pathway is the main signal transduction pathway mediating insulin action in the liver, and inhibition of PI3K signaling leads to insulin resistance and potentially therefore obesity, fatty liver, T2D, and metabolic syndrome [65, 66]. In particular, downregulation of insulin receptor substrate- (IRS-) 2 can impair the effective transmission of the downstream PI3K signal and lead to insulin resistance [67–69]. GLUT2, the main glucose transporter in rodents and human hepatocytes, mediates the bidirectional transport of glucose, and therefore, its abnormal expression can lead to disorders of glucose metabolism.

As shown in Figure 3, LBP, TLR4, and JNK expressions were significantly higher, and GLUT2 and PI3K expressions were significantly lower, in T2D rats than in control rats, and these differences were largely abolished by BF treatment ($P < 0.05$). This suggests that BF may alleviate the inflammatory response and ameliorate insulin resistance through effects on the JNK and PI3K signaling pathways that are likely to improve glucose metabolism (Figure 3(b)).

In addition, oxidative stress is one of the key factors for the development of insulin resistance. A high-fat diet can induce more reactive oxygen species in the body, which can activate multiple intracellular signaling pathways, cause disorder of lipid metabolism, and produce insulin resistance [70]. In a high-glucose environment, cell inflammation is enhanced, and inflammatory cells can produce a large number of oxides, making oxidative stress possible.

3.4. The Protective Effect of BF on the Tissues of Diabetic Rats. Berberine can ameliorate ileal and systemic inflammation by inhibiting the activation of the TLR4 pathway and reducing the intestinal damage caused by LPS [71]. A microscopic examination of the liver of NC rats demonstrated a normal central vein and a narrow surrounding sinusoidal radiation, without any significant congestion of the liver sinuses or cell swelling. In contrast, the liver of STZ-induced diabetic rats
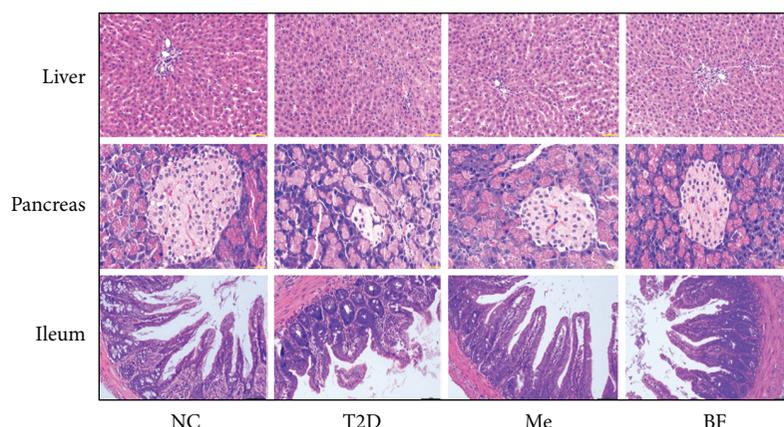
FIGURE 4: The protective effect of BF on liver, pancreas, and ileum of T2D rats (hematoxylin-eosin stain, ×200).

showed obvious pathology, in the form of nonradiating sinusoids, scattered necrotic cells showing pyknosis, and the formation of microvesicles in the cytoplasm of hepatocytes. However, the histopathology in the liver of treated rats was variable, but less marked.

The pancreatic islets of rats in the NC group appeared normal, whereas those of diabetic rats showed severe pathology, including necrosis, smaller size, and fewer cells. The pancreatic islets of rats in the BF and Me groups also showed injuries, but there was a significant improvement compared with the T2D group (Figure 4).

The ileal villi of the NC group were arranged neatly and compactly, with no endothelial discontinuities. In contrast, the ileal villi of diabetic rats were fewer in number, blunted, and lacked structural integrity, while mucosal inflammation was also apparent. However, in rats that had been treated for 6 weeks with BF and Me, the ileal villi were neatly arranged and more intact and numerous than those of the T2D group (Figure 4). When the organism is infected by gram-negative bacteria, lipopolysaccharide can activate signaling pathways, resulting in a large number of proinflammatory cytokines released. Berberine can reduce the release of lipopolysaccharide and ameliorate inflammation by reducing the level of LBP, thus alleviating intestinal injury and improving insulin resistance [72].

Abbreviations

T2D:	Type 2 diabetes
STZ:	Streptozotocin
BOAS:	Berberine organic acid salt
BF:	Berberine fumarate
NC:	Normal control
Me:	Metformin
FBG:	Fasting blood glucose
FINS:	Fasting insulin
TG:	Triglycerides
TC:	Total cholesterol
T-SOD:	Total superoxide dismutase
GSP:	Glycosylated serum protein
GSH-PX:	Glutathione peroxidase
HOMA-IR:	The homeostasis model assessment for insulin resistance
GLP-1:	Glucagon-like peptide 1
LPS:	Lipopolysaccharide
LBP:	LPS-binding protein
JNK:	c-Jun n-terminal kinase
PI3K:	Phosphoinositol 3-kinase
GLUT2:	Glucose transporter 2
TLR4:	Toll-like receptor 4.

Data Availability

All the figures and tables used to support the findings of this study are included within the article.

Additional Points

Chemical Compounds. Chemical compounds mentioned in this study are berberine hydrochloride (PubChem CID: 12456), fumaric acid (PubChem CID: 444972), streptozotocin (PubChem CID: 29327), sodium chloride (PubChem CID: 5234), water (PubChem CID: 962), citric acid (PubChem CID: 311), butyric acid (PubChem CID: 264), metformin (PubChem CID: 4091), alpha-D-glucose (PubChem CID: 79025), and triglyceride (PubChem CID: 10851).

Conflicts of Interest

The author claims that there is no conflict of interest. All authors have approved the final article.

Acknowledgments

Supplementary Materials

The corresponding experimental data associated with this article can be seen from the supplementary material files. *(Supplementary Materials)*

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Review Article

Oxidative Stress and the Microbiota-Gut-Brain Axis

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The gut-brain axis is increasingly recognized as an important pathway of communication and of physiological regulation, and gut microbiota seems to play a significant role in this mutual relationship. Oxidative stress is one of the most important pathogenic mechanisms for both neurodegenerative diseases, such as Alzheimer's or Parkinson's, and acute conditions, such as stroke or traumatic brain injury. A peculiar microbiota type might increase brain inflammation and reactive oxygen species levels and might favor abnormal aggregation of proteins. Reversely, brain lesions of various etiologies result in alteration of gut properties and microbiota. These recent hypotheses could open a door for new therapeutic approaches in various neurological diseases.

1. Introduction

The microbiota-gut-brain axis is a complex multidirectional cross-talk system between the gut microbiota, the enteric nervous system (ENS), and the brain. It acts as an adaptive interface with the environment and consists of a humoral pathway, based on the intestinal barrier, portal and systemic circulations, blood-brain barrier (BBB), and a neural pathway (via the vagus nerve) [1]. A strong interplay also exists with the neuroendocrine-immune network; therefore, the functional integrity of the axis is required for the homeostasis of several systems [1, 2].

Increasing evidence suggests that the gut microbiota is involved in several neurodegenerative disorders, such as Parkinson's disease (PD) and Alzheimer's disease (AD), as well as in acute central nervous system (CNS) injury, such as ischemic stroke [1, 2]. Interestingly, oxidative stress (OS) is also a key player in the pathogenesis of these disorders.

In this review, we summarize the available data concerning potential interactions between the microbiota-gut-brain axis and CNS's oxidative stress.

2. The CNS Oxidative Stress: Gut Microbiota Connection—A Plausible Hypothesis

Recent experimental evidence found that, in the presence of the microbiota, the epithelial lining of the gut generates physiological levels of OS. In return, these interfere both with the composition and functionality of the microbiota (e.g., anaerobes thrive in the presence of electron acceptors) and directly with the permeability of the intestine, thus increasing the chances of xenobiotic molecules reaching the systemic circulation and the CNS [3]. The oxidative reduction potential of the gut microbiota (i.e., the tendency and capacity of the microbiota to gain electrons) influences the homeostasis of the intestinal barrier as well [3], while the brain/CNS

modulates the level of OS within the intestine via the vagal cholinergic anti-inflammatory pathway [1, 4, 5]. All these may have direct or indirect (and possibly cumulating) consequences on the oxidative balance in the CNS, either by increasing the oxidant component or by interfering with the antioxidant system [2]. Therefore, one may speculate that gut dysbiosis may be both a cause and a consequence of increased levels of CNS OS [4], thus adding a new dimension to the interplay between the gut microbiota and the brain, also known as the microbiota-gut-brain axis.

3. Oxidative Stress and Its Role in CNS Health and Disease

3.1. General Considerations. OS is a type of reactive stress. As a biochemical concept, it is defined as the state of imbalance between oxidants and antioxidants, with relative excess of the former, resulting in the "disruption of redox signaling and control and/or molecular damage" [6]. Though the terminology may suggest that OS is only a disadvantageous by-product, it actually plays critical physiological roles, providing that it is maintained within a safe steady-state range (e.g., mitigating infections). At higher levels, however, OS is potentially neurotoxic, resulting in biomolecular damage, i.e., protein, lipid, and deoxyribonucleic acid (DNA) oxidation, which may result in a broad spectrum of cellular dysfunctions, culminating with cell death (Massaad and Klann, 2013, [6]). Its presence is intrinsic to the aerobic metabolism, virtually all chemical reactions involving molecular oxygen resulting in the generation of short-lived, highly unstable/reactive intermediate products, known as reactive oxygen species (ROS) [6–8].

3.2. Redox Reactions and the Biology of Oxidative Stress. Oxidants are chemical species able to remove and accept electrons from other atoms or electronegative atoms from other molecules [9]. Conversely, antioxidants are able to delay or prevent the effects of the oxidants, balancing the oxidative state of a system without becoming destabilized themselves [8].

The free radicals are oxidants able to remove and accept electrons from other atoms, meaning that they contain at least one unpaired electron but are stable enough to exist independently [6, 9].

The biological activity of free radicals, which includes toxic and beneficial effects, is related to their propensity for triggering reduction-oxidation or redox reactions that perpetuate in a domino-like fashion (i.e., reactions involving the transfer of an electron between two chemical species: the species gaining the electron is the "oxidant"/"oxidizing agent" which is "reduced," and the species losing the electron is the "reductant"/"reducing agent" which is "oxidized") [6, 8, 9].

ROS are mostly free radicals, but nonradical species are also produced. Other free radicals (either oxidant or reductant) are also generated endogenously, in physiologic conditions, as well as during interactions with exogenous factors (e.g., drugs, radiations, and xenobiotic toxins) [6, 8].

Molecular oxygen (O_2) itself is a free radical, having two unpaired electrons which cannot be reduced simultaneously

during chemical reactions, resulting in the production of ROS (e.g., superoxide anion, hydrogen peroxide, nitric oxide, perioxynitrite anion, and the hydroxyl and peroxyl radicals) [6, 8, 10].

The generation of ROS results either from physiological processes (via ROS-generating enzymes) or by interactions with potentially harmful exogenous factors [8]. Most ROS are generated as by-products of physiological processes that occur in various parts of the cell, with the main source being the mitochondria. Other sources of ROS include xanthine and flavin oxidases and cytochrome P450 [11].

The main cellular sites of ROS production are the mitochondrial complexes I (NADH-coenzyme Q oxidoreductase) and III (cytochrome c oxidoreductase), but also complex II (succinate-Q oxidoreductase) [12]. One of the major roles of the mitochondria is the production of energy in the form of adenosine triphosphate (ATP) through the process of oxidative phosphorylation (OXPHOS) [13]. This phenomenon takes place in the inner mitochondrial membrane, where four redox complexes form the electron transport chain (ETC). The production of ATP by cytochrome c oxidase (complex IV) in the final step of the ETC requires electrons that are transported from reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$) with the help of complexes I and II [12]. A high quantity of O_2 is needed in this process as it provides an efficient electron acceptor [7]. The addition of an electron to O_2 results in the formation of superoxide ($O_2^{-\bullet}$), which is the main precursor to many ROS [14]. Under normal conditions, the levels of superoxide resulting from the ETC are decreased by antioxidant enzymes such as manganese superoxide dismutase (SOD) and copper-zinc SOD that convert superoxide to hydrogen peroxide (H_2O_2) and O_2, the former being afterwards transformed to water through the action of catalase and glutathione peroxidase [15, 16]. However, this process is not perfect and the passage of electrons through the ETC also results in electron leakage and the subsequent formation of low levels of superoxide [17]. The addition of an electron to superoxide leads to the generation of ROS such as H_2O_2 and hydroxyl anion (^-OH)—the higher the amount of oxygen is, the higher is that of superoxide resulting in greater ROS production [7]. Hydroxyl radical ($HO^\bullet$) is very reactive, and its formation depends not only on the amount of oxygen but also on that of iron or copper ions that serve as catalytic factors in what is known as the Fenton reaction ($O_2^{-\bullet} + H_2O_2 \rightarrow HO^\bullet + OH^- + O_2$) [12].

Though the ETC is the major source of ROS production in the cell, it is not the only one. The action of monoamine oxidases inside the outer mitochondrial membrane leads to H_2O_2 formation, while the transfer of electrons from NADPH in the endoplasmic reticulum results in more ROS production [12]. Lipoxygenases, a class of enzymes that oxidize esterified and free polyunsaturated fatty acids (PUFA), also catalyze the transformation of arachidonic acid leading to hydroxyl radical and superoxide formation [18].

Peroxisomes contain various enzymes involved in the metabolism of lipids that generate ROS as part of their normal catalytic cycle. Beta-oxidation of fatty acids and the actions of glycolate oxidase and xanthine oxidase produce

superoxide and H_2O_2 [12]. Xanthine oxidases are also located in the cytoplasm, where they are involved in the metabolism of purines, a process that leads to superoxide formation [19].

ROS are also generated by leucocytes and microglia in the brain. When these cells are activated, they increase oxygen consumption in the process known as "respiratory burst" and use various enzymes in reactions that result in the production of ROS such as H_2O_2 or hypochlorous acid (HOCl) [12, 20].

3.3. The Physiological Roles of Oxidative Stress. The free radicals generated by the mitochondrial ECT are efficiently used by the innate immunity for mitigating infections [9]. OS also has beneficial/homeostatic roles in the CNS, with ROS/RNS playing important roles in several processes such as the growth of hippocampal progenitor cells, synaptic plasticity, and axonal path finding [9, 21–23]. Moreover, free radicals are also involved in cellular redox signaling and other prosurvival pathways, mediated by "redox sensors" that modulate the expression of certain enzymes, and are kept under control by soluble and insoluble "redox sinks" (e.g., glutathione and thioredoxin—see below) [10]. These redox-active proteins (sensors and sinks) may undergo rapid, reversible, and gradual oxidation of their numerous cysteine residues, buffering the free radicals and concomitantly allowing for an accurate perception of the intracellular levels of ROS (and thus for the fine tuning of the responses) [4, 8].

Thus, in physiological conditions, ROS are involved in interconnected processes such as inflammation, signaling transduction pathways, the immune response, and apoptosis. There is a dual relationship between OS and inflammation: OS can be induced by inflammatory responses, and inflammation can be triggered or enhanced by ROS through activation of nuclear factor-kappa B (NF-κB), which controls the expression of many genes, including some involved in inflammatory responses leading to the production of various cytokines [24]. Leucocytes induce OS in the process of phagocytosis by generating ROS in reactions catalyzed by NADPH oxidase, superoxide dismutase, and myeloperoxidase (MPO) [12]. MPO is a lysosomal enzyme that generates HOCl used as a powerful oxidative agent against pathogens [20]. ROS also seem to be involved in various signal transduction pathways playing a role in intracellular signaling and regulation in regard to cytokine and growth factor signaling, nonreceptor tyrosine kinases, protein tyrosine phosphatases, serine/threonine kinases, and nuclear transcription factors [14].

3.4. The Roles of Oxidative Stress in CNS Disorders. OS exerts most of its deleterious effects by inducing lipid peroxidation and by damaging nucleic acids and proteins. The main targets of lipid peroxidation are PUFA, such as arachidonic acid and linoleic acid, lipids that are found in abundance in the cell membrane [25]. The addition of hydrogen from ROS to PUFA leads to the formation of lipid peroxyl radical which interacts with another PUFA that then reacts with oxygen and forms another lipid peroxyl radical, generating a chain reaction [25]. Since the hydroxyl radical has a very high

chemical reactivity, it is the most effective ROS in inducing lipid peroxidation thus producing significant damage to the neuronal membrane. The cellular levels of hydroxyl radical depend on the amount of available oxygen, but also on those of iron and copper that catalyze the previously described Fenton reaction in which hydroxyl radical results from superoxide and H_2O_2 [12].

Nucleic acids like ribonucleic acid (RNA), nuclear DNA, or mitochondrial DNA (mtDNA) are targets of OS. The hydroxyl radical can permanently damage the DNA by inflicting injuries to purines, pyrimidines, and deoxyribose, but most notably, it is mtDNA that is prone to oxidative damage since mitochondria are the main site of ROS production and mtDNA is in direct contact with ROS [13, 26].

Protein oxidation may lead to changes in their function such as activation, inactivation, or gain of a new function, depending on the specific oxidative modification taking place with consequences on various signal transduction pathways [26].

There are multiple factors that make the brain particularly susceptible to OS. Most notably, the brain has a high rate of oxygen use, amounting to about 20% of total oxygen consumption, even though it represents only 2% of the body's total weight [7]. As we have previously discussed, the generation of ATP through the ETC leads to electron leakage and superoxide formation with subsequent ROS production. Since the brain utilizes large quantities of oxygen, it also generates a significant amount of ROS. Moreover, the brain has regions with high levels of iron which is used to generate even more hydroxyl radical. This increase in ROS production is met by a greater concentration of PUFA making the brain more susceptible to OS [27].

Besides OS occurring as a part of physiological processes that may be more or less related to normal aging, it is important to note that disease states lead to greater production of ROS and subsequent oxidative damage. As such, OS has been involved in the pathology of various chronic disorders of the brain such as AD [28, 29], PD [30], Huntington's disease [25], amyotrophic lateral sclerosis [31], multiple sclerosis [25], and depression [32], as well as in acute damage that occurs in stroke [33] or traumatic brain injury [34].

3.5. The CNS Antioxidant Metabolism and Gut Microbiota Interference. The CNS is highly susceptible to OS, and chronic OS is a putative mechanism in many of its diseases. This is explained by its narrow redox homeostatic window, the proper functioning of the CNS generating and requiring high levels of ROS (i.e., strong oxygen demand, with high oxidative metabolism and extensive use of ROS and other reactive species for intra- and intercellular signaling) [9, 11]. Other particularities contributing to the high susceptibility of the CNS to OS include its high content of redox-active transition metals (e.g., iron and copper) and PUFA (which are prone to peroxidation) and the presence of autoxidating neurotransmitters [9, 11, 35].

Complex gut microbiota microbe-microbe and microbiota-host interactions may also influence the oxidative state of the CNS, directly and indirectly, by interfering both with the level of ROS (endogenous and exogenous) and with the

antioxidant system [1, 2, 4]. These mechanisms are mostly speculative but are pertinent to the hypothesize that the oxidative state of the CNS could be regulated by the microbiota via the production of various metabolites (i.e., absorbable vitamins, short-chain fatty acids (SCFA), polyphenols, and highly diffusible antioxidant and oxidant gases), optimization of dietary energy harvest, regulation of the permeability of the intestinal barrier and BBB, immune system modulation, and prevention of extensive colonization by pathogenic microbes ([1, 2], Ravcheev and Thiele, 2012, [4, 36, 37]). The microbiota also produces considerable amounts of CNS neurotransmitters (e.g., dopamine, serotonin, and gamma-amino butyric acid) which modulate the local activity of the ENS and may correlate with their respective levels within the CNS, depending on the intestinal and BBB permeability [9]. Moreover, the microbiota may also produce neurotoxic and potentially neurotoxic substances (such as lipopolysaccharides and amyloid proteins) which may reach the CNS via the systemic circulation or the vagus nerve, promoting microglia activation and neuroinflammation, increasing the CNS production of ROS and/or making neurons more susceptible to OS [9].

The antioxidant metabolism of the CNS is relatively modest, but tightly regulated [8, 9]. The enzymatic antioxidants include SOD, which reduces the superoxide anion to O_2 and H_2O_2 and is essential for cell survival; glutathione and glutathione peroxidases, which are selenium-dependent and selenium-independent isoenzymes that use glutathione to catalyze the reduction of H_2O_2 and lipid peroxides; peroxiredoxins which are thiol-specific peroxidases found in the cytoplasm, nuclei, mitochondria, peroxisomes, and lysosomes that catalyze the reduction of hydroxyperoxides (including H_2O_2 and peroxynitrite); and catalase which converts H_2O_2 to water and oxygen, using iron or manganese as a cofactor, but has low CNS expression (i.e., 50 times lower than in hepatocytes) and minor roles at steady-state levels [2, 9, 11].

The presence of moderate levels of ROS activates transcription factors that increase the antioxidant potential (/defense), thus priming the CNS for exogenous OS and increasing the chances of cell survival [9]. Glutathione has a low expression in the CNS (about half of that found in other tissues) [11]. In its reduced form, it reacts nonenzymatically with free radicals and functions as the electron donor for the reduction of peroxide by glutathione peroxidases, resulting in glutathione disulfide. The latter can be regenerated to glutathione by a reductase which transfers electrons from NADPH (Tse, 2015, [11]). The low CNS levels of glutathione may limit glutathione peroxidase 4 activity, thus possibly explaining the high neuronal susceptibility to iron-related programmed cell death (i.e., ferroptosis) [9, 11]. However, glutathione peroxidase 1 is one of the most important antioxidant systems in the CNS. It is expressed in the microglia (but not in neurons) and is upregulated in response to injury, having a cytoprotective effect. Peroxiredoxin-thioredoxins are a NADPH-dependent enzymatic system which is highly expressed in neurons. It is involved in redox-transducing signaling and may be required for the efficient metabolism of H_2O_2 [9]. Another antioxidant system expressed by CNS cells consists of peroxiredoxins, which are responsible for the reduction of up to 90% of mitochondrial H_2O_2 and almost all cytoplasmic H_2O_2 (Tse, 2015, [11]). Also, an important cytoprotective pathway (arguably the most important) is the Kelch-like ECH-associating protein 1-nuclear factor erythroid 2-related factor 2-antioxidant response element (Keap1-Nrf2-ARE), which is highly expressed in neurons. It responds to both physiological and pathological/xenobiotic OS by modulating the expression of SOD, thioredoxin, peroxiredoxins, and glutathione peroxidases [4, 9]. The "redox sensor" protein NF-κB is also expressed in the CNS, activating the transcription of antiapoptotic proteins and inhibiting caspase-dependent cell death [4, 9, 11]. Higher levels of ROS, however, are proapoptotic, inhibiting the binding of NF-κB to the DNA [8, 11].

3.5.1. Nitrosative Stress and Antinitrosative "Defense."

The reactive chemical species generated by the activity of NADPH oxidase (Nox) are categorized as reactive nitrogen species (RNS) and may result in the so-called nitrosative stress (NS). NS typically accompanies OS [2, 6, 8].

Nitric oxide (NO) (i.e., the endothelium-derived relaxing factor) is another free radical and also the main neurotransmitter of the nonadrenergic noncholinergic ENS. It is a highly diffusible short-lived gas, synthesized endogenously from L-arginine and oxygen by various nitric oxide synthases (NOS), using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor [2]. It functions as a signaling molecule and neuroprotector at low levels, resulting in neurotoxic RNS and OS/NS with harmful neuroinflammatory repercussions at higher levels [2]. Its neuroprotective effects are mediated by the nitrosylation of the N-methyl D-aspartate (NMDA) receptors and caspases, limiting excitotoxicity, and apoptosis [2, 6]. The mechanism of NO toxicity is related to its interaction with other ROS resulting in the generation of highly reactive peroxynitrite, hydrogen peroxide, hypochlorite ions, and hydroxyl radical. Elevated NO levels also downregulate the secretion of brain-derived neurotrophic factor, reducing the neuronal survival and synaptogenetic processes [2, 6, 11]. At gastrointestinal levels, NO is released by ENS inhibitory motor neurons via the activation of NMDA receptors, as well as by infiltrating neutrophils and monocytes. In the CNS, it is generated in nanomolar amounts from L-arginine by the endothelial and neuronal NOS (eNOS and nNOS, respectively), while inducible NOS (iNOS) secretes higher, neurotoxic levels, in response to proinflammatory stimuli [2, 6]. The main producer of NO is the gut microbiota via the reduction of gastric nitrate and nitrite and denitrification. A higher nitrate intake may increase the production of nitrite, NO, and ammonium (NH_3) by certain salivary and also intestinal bacteria using L-arginine-dependent and L-arginine-independent pathways. This results in higher levels of NO within the intestinal tract and the CNS (e.g., NO is absorbed from the intestinal tract and may be scavenged by erythrocyte hemoglobin, reaching the CNS via the systemic circulation), with potentially deleterious consequences [2].

(a)	(b)	(c)	(d)
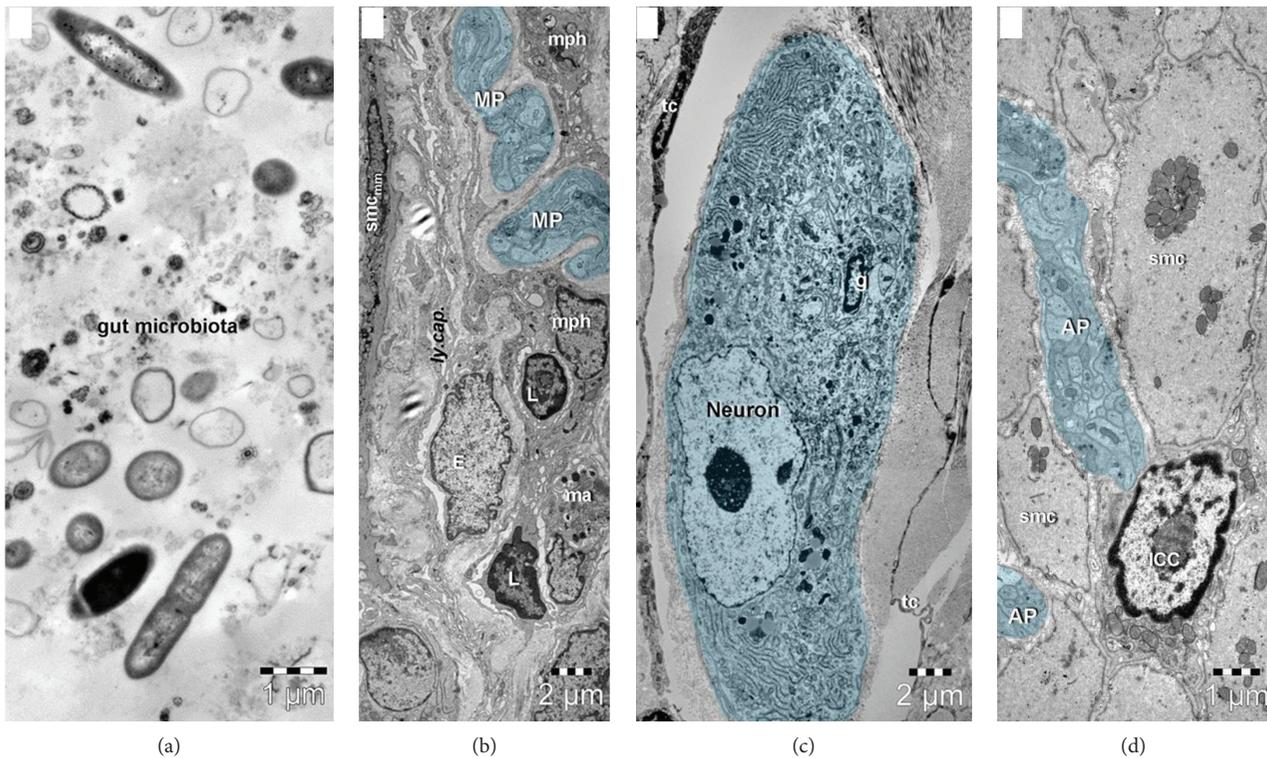
FIGURE 1: Transmission electron microscopy images of the (a) gut microbiota, (b, c) submucosal Meissner plexus, and (d) myenteric Auerbach plexus from the small intestine of 6-month-old mouse. (a) Various types of bacteria, nanoparticles, and vesicles with diverse dimensions from intestinal lumen. (b) Nerve endings of the submucosal plexus (MP—Meissner plexus) run along a lymphatic capillary (ly.cap.) between macrophages (mph), mast cells (ma), lymphocytes (L), and smooth muscle cells of muscularis mucosae (smc_{mm}). (c) A neuron and an enteric glial cell (g) from the submucosal Meissner plexus are surrounded by telocytes (tc). (d) Nerve fibers from the myenteric Auerbach plexus (AP) in contact with an interstitial Cajal cell (ICC) between smooth muscle cells (smc) of the muscularis.

3.5.2. Other CNS Antioxidants. Molecular hydrogen (H_2, dihydrogen) is another highly diffusible bioactive gas. It has antioxidant properties, reducing hydroxyl radicals and possibly peroxynitrite ($ONOO^-$), but not other reactive ROS/RNS, and raised interest over the past years due to its efficiency in ameliorating several disease processes associated to OS [36, 37]. Humans do not directly produce it, but the average microbiota generates about 1 liter per day during the process of fermentation [36–38]. The hydrogen-producing bacteria include anaerobic cocci, members of the Enterobacteriaceae family and certain strains of the Clostridium genus. These are usually accompanied by symbiotic counterparts that consume H_2 (e.g., methanogens, sulfate-reducing bacteria, and acetogens bacteria); thus, the production of H_2 varies between individuals and within the same individual, in relation to the composition of the microbiota and the diet. Since H_2 is produced by gut bacteria, but not humans, it is plausible to consider that gut dysbiosis may result in low H_2 production, limiting the availability of the gas to the CNS neurons and increasing their susceptibility to OS-related disorders [38].

4. The Gut-Brain Axis in Neurodegeneration

There is a two-way connection between our gut and our brain, which is important not only for the physiology of the digestive system but for good brain health as well. The ENS is the largest component of the autonomous nervous system, with a number of nervous cells similar to the spinal cord, and has integrative activity [39]. The vast majority (90%) of vagus nerve fibers are afferent, making the gut a possible large access door to brainstem and CNS [40].

Most neurodegenerative disorders are proteinopathies, meaning that they are associated with intraneuronal protein misfolding and aggregation. OS is another shared pathogenetic factor; however, the etiopathogeny of these diseases is incompletely understood and disease-modifying treatments are not available. Heiko Braak has made an important contribution when he unravelled the presence of aggregated α-synuclein in submucosal Meissner's and myenteric Auerbach's plexuses in the stomach and gut in PD patients, hypothesizing that the misfolded protein pathology might start in the intestine and ascend transynaptically to CNS neuron populations, resulting in neurodegeneration [Braak et al., 2006]. Considering the proximity of ENS neurons to the intestinal lumen, the gut microbes were considered as plausible triggering factors—for the morphologic relationship of nerve endings with other cells and structural elements (lymphatics, smooth muscle cells, and immune-competent cells) and gut microbiota (please see Figure 1). Later on, accumulating data suggested that gut microbiota might influence the aggregation and propagation of α-synuclein and Friedland and Chapman proposed the term *mapranosis* (i.e., microbiota-associated proteopathy and neuroinflammation)

in order to describe the influence of the microbiota on the brain [41].

5. Oxidative Stress and the Microbiota-Gut-Brain Axis in Parkinson's Disease

Sporadic PD is the second most common neurodegenerative disorder, after AD. It is a multifactorial disease, involving selective loss of central and peripheral aminergic neurons associated with intracytoplasmic aggregation of misfolded α-synuclein, i.e., the so-called Lewy bodies and Lewy neurites [42, 43]. Accumulating evidence supports the initial hypothesis of Braak, suggesting that the pathogenic process begins in the gut, progressing towards the CNS via the vagus nerve. Thus, the gut microbiota and gut dysbiosis are highly plausible contributing environmental factors to the development and progression of PD, as suggested by recent animal findings and indirect human data [11, 42–44].

The motor symptoms, which are still considered the hallmark of PD, become prominent in the later stages of evolution and are related to the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [11, 42]. The presence of OS is supported by the finding of lower SNpc glutathione levels and higher iron, H_2O_2, and lipid peroxidation, which may result in increased production of the highly toxic hydroxyl radicals and subsequent neuronal death [11, 45]. The particular susceptibility of SNpc neurons may also be related to the metabolism of dopamine itself. This involves monoamine oxidase enzymes which catalyze a deamination reaction generating hydrogen peroxide and ammonia. Neuronal activity (i.e., oxygen saturation) induces OS and dopamine oxidation results in several H_2O_2 molecules and electrophilic aldehyde metabolites. Dopamine reacts with molecular oxygen, forming dopamine semiquinone radical, which reacts with another dioxygen molecule to form dopamine quinine. Redox transition metals increase this reaction, and dopamine quinones may interact to form semiquinones. The oxidation products of dopamine metabolism may also enter redox cycling, forming superoxide anion and hydroxide peroxide, which contribute to the pathogenesis of PD [9] The mechanism of neuronal death in PD is incompletely understood, but the available evidence suggests that mitochondrial dysfunction and OS are key pathogenic pathways [11, 46]. Mitochondrial respiratory chain dysfunction (especially complex I deficiency) is present in PD and results in the production of excessive ROS, leading to apoptosis [11, 44, 46]. This is also supported by the cytotoxic effect induced on dopamine neurons by complex I inhibitors, such as 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) [11, 42, 43, 46]. The monogenic PD cases related to α-synuclein, parkin, phosphatase, and tensin homolog-induced putative kinase (PINK) also show mitochondrial dysfunction and high OS levels, supporting these as plausible mechanisms [11, 46]. The role of α-synuclein is still being debated, but a recent study found a conformationally distinct α-synuclein aggregate that induces mitochondrial damage and mitophagy [44]. Moreover, increased levels of OS and decreased free radical scavenger capacity exacerbate α-synuclein aggregation in animal

models [47]. Mitochondria are bacterial endosymbionts which maintain some pathogen-associated molecular patterns and release damage-associated molecular patterns, triggering innate immunity responses, which may result in even higher levels of OS [44, 46]. Considering the above, it has been hypothesized that some members of the gut microbiota may produce toxins (/antibiotics) targeting the mitochondria of the ENS and CNS that could result in subsequent neurodegeneration [46].

As already discussed, decreased production of H_2 by the microbiota has been proposed as an environmental factor which may interfere with the development and subsequent evolution of several diseases, including sporadic PD [38]. In a rat model of PD, 50%-saturated H_2 drinking water was successful in preventing nigrostriatal degeneration [36, 37]. H_2-water also prevented neuronal loss and reduced OS markers in the substantia nigra of a MPTP mouse model [37]. In humans, a pilot placebo-controlled, double blind, randomized trial found H_2-water to be beneficial, improving the motor ratings of PD patients [36, 37]. Though it is tempting to speculate that gut dysbiosis may result in low H_2 production, with a negative impact on PD evolution, further investigation is needed [38].

6. Oxidative Stress and the Microbiota-Gut-Brain Axis in Alzheimer's Disease

AD is the most common neurodegenerative disorder [48]. It is characterized by a progressive impairment in episodic memory and other cognitive domains, progressing towards dementia [49]. The neuropathology hallmarks of AD are cerebral extracellular amyloid plaques embodying amyloid-β (Aβ) that aggregates and adopts a β-sheet structure and intracellular neurofibrillary tangles comprising hyperphosphorylated tau protein [48, 50]. Brain atrophy emerges as a consequence of synaptic degeneration and neuronal death, notably involving the hippocampus [48]. Decades of research failed to fully elucidate its etiopathogenesis, whereas preventive or disease-modifying therapies are still missing. New insights into the mechanisms of AD are required in order to conceive effective treatments.

OS is currently regarded as a key process in the pathogenesis of AD. The high energetic demands of the nervous system enhance exergonic oxidative processes, subsequently exposing the neurons to ROS [49, 51]. Considering the substantial PUFA able to interact with ROS and low levels of glutathione responsible for impaired clearance of free radicals, neurons are particularly vulnerable to OS [11, 49, 52].

The role of oxidative damage in the pathogenesis of AD is reflected by altered activity of antioxidant enzymes (SOD, catalase) and increased levels of OS biomarkers (malondialdehyde, 4-hydroxynonenal, and F2-isoprostane protein are markers of lipid oxidative damage; protein carbonyls and 3-nitrotyrosine are products of protein oxidation, whereas 8-hydroxydeoxyguanosine reflects nucleic acid oxidation) in the blood and cerebrospinal fluid of patients with AD [11, 52, 53]. Moreover, the amount of oxidative markers is directly proportional to the degree of cognitive impairment [54] and brain weight [52]. It is noteworthy

that 8-hydroxydeoxyguanosine excess in the parietal cortex precedes the pathognomonic histopathological abnormalities of AD by decades, whereas patients with mild cognitive impairment have elevated levels of malondialdehyde, 4-hydroxynonenal, F2-isoprostane protein, and protein nitration products in the brain [52]. These findings suggest that OS is not merely a collateral event in the pathogenesis of AD, but rather an early prominent process. Inflammation, which is one of the key elements of AD pathogenesis [55], is an important OS trigger, and there is still no intervention to alleviate its effects, since it activates both favorable and unfavorable signaling pathways [56].

There is a mutual relationship between OS and $A\beta$ production and aggregation in AD—OS enhances $A\beta$ deposition, whereas $A\beta$ triggers oxidative reactions [11]. Interestingly, $A\beta$ aggregates facilitating OS are confined not only to extracellular regions but also to cellular organelles such as Golgi apparatus, endoplasmic reticulum, and mitochondria with emergent mitochondrial dysfunction [11]. Tau pathology is also linked to OS. Cells with overexpressed tau protein exhibit decreased NADH-ubiquinone oxidoreductase activity and mitochondrial dysfunction that generate ROS [11].

Multiple mechanisms underlie OS, particularly mitochondrial dysfunction, metal accumulation, hyperphosphorylated tau protein, and inflammation [52]. Mitochondrial dysfunction is promoted by defective ETC enzymes (i.e., cytochrome oxidase), mitochondrial DNA mutations, and inactivation of antioxidant mitochondrial enzymes (i.e., SOD) and leads to significant ROS production and scarce energy stores in hippocampal neurons of AD patients [11, 52]. Metal accumulation has been found in the hippocampus and amygdala of AD patients; copper and iron generate ROS by binding to $A\beta$, whereas zinc is a component of amyloid plaques [52]. Hyperphosphorylated tau protein burden is proportional to ROS levels [52]. Since neurofibrillary tangles exhibit decreased levels of 8-hydroxydeoxyguanosine despite marked OS, tau phosphorylation is supposedly involved in cytoprotection against oxidative damage [52]. Inflammation also triggers oxidative reactions [52]. $A\beta$ activates microglia and astrocytes that release cytokines, chemokines, and ROS [52]. Provided that OS contributes to $A\beta$ and tau pathology in AD, antioxidants are potential effective treatments by lowering ROS and protecting nervous cells from oxidative insult [52].

The intestinal microbiome seems to play a significant role in AD pathogenesis, as suggested by gut microbiota shifts towards proinflammatory bacteria in transgenic APP/PS1 mice [57] and declining amyloid plaque deposition and neuroinflammation (as indicated by reduced plaque-localized gliosis and modified microglial phenotype) in the same murine model treated with long-term broad spectrum combinatorial antibiotics [58].

Altered gut microbiota enhances the cerebral aggregation and deposition of $A\beta$ by immune, metabolite-mediated, endocrine, and neural pathways [50]. Amyloid proteins produced by bacterial populations (i.e., microbial amyloid) activate the innate immune system, subsequently inducing a response that entails TLRs and CD14 and elicits underrecognition of misfolded $A\beta$ with impaired $A\beta$ clearance [41]. A concomitant humoral reaction involves proinflammatory

cytokine activation with ensuing disruption of intestinal and blood-brain barriers [50]. Microbial metabolites such as hydrogen sulfide, trimethylamine, and SCFA are likely to be involved in AD pathogenesis [41]. Reduced plasmatic levels of enteric hormones with impaired signaling pathways have been reported in AD [59]. Ghrelin prevents synaptic degeneration and memory loss, leptin acts as a neuroprotective factor against $A\beta$ toxicity by directly regulating γ-secretase-mediated amyloidogenic pathway, and glucagon-like peptide 1 diminishes $A\beta$ load, whereas glucose-dependent insulinotropic polypeptide exerts neuroprotective effects [59]. Microbiota modulation by either probiotic mixtures (lactic acid bacteria and bifidobacteria) or anti-inflammatory bacterial metabolites such as SCFA increases the synthesis of enteric hormones and counteracts the progression of AD in animal models [59].

Regulatory interventions on microbiota also improve proteolytic pathways usually impaired in AD [59]. Declining hippocampal apoptosis and ubiquitin conjugate levels such as p27 and p53 in AD mice following administration of probiotics indicate enhanced proteasome functionality [59]. Facilitation of autophagy as proved by diminished cathepsin B activity (lysosomal enzyme associated with amyloid plaques in AD) as opposed to cathepsin L activity (lysosomal enzyme that amplifies α-secretase-mediated nonamyloidogenic pathway) also occurs [59].

Another suggested mechanism underlying microbiota-mediated cerebral amyloid accumulation is *cross-seeding of microbial amyloid* (i.e., promotion of amyloid-misfolded aggregates from one protein to a different one) via the autonomic nervous system in a manner similar to prion propagation [41, 60]. Additionally, distinct amyloid conformers interacting with cellular targets could induce different toxicities that might explain the existence of various AD phenotypes [61].

Provided that gut microbiota dysbiosis interferes with the bidirectional signaling of the gut-brain axis, modulating it through dietary or microbiotic interventions is presumably a potential therapeutic strategy [50].

Intestinal microbiota regulates several homeostatic functions. Recent data suggest that certain bacterial strains such as Lactobacilli are able to promote generation of physiological levels of ROS within epithelial cells [4]. Apart from their antimicrobial role, some ROS species such as H_2O_2 produced in this manner are involved in critical signaling pathways [4]. The Keap1/Nrf2/ARE pathway deals with OS by expressing genes that encode antioxidant and detoxification enzymes, hence assuring the intracellular redox hemostasis and cytoprotection [4]. Keap1/Nrf2/ARE also enhances antioxidant reactions and cellular repair mechanisms in inflammatory states [4]. NF-κB is another signaling pathway that generates proinflammatory cytokines and antibacterial factors [62]. Nevertheless, it is the concentration of ROS in the cell that provides the shifts towards beneficial or unfavorable processes (i.e., cell proliferation differentiation, cytokine release, cell death by apoptosis, or activation of the innate immune system) [62]. Both under- and overproduction of ROS elicit damaging cellular reactions, so maintaining a redox balance is essential [62]. Since altered

microbial population dysregulates ROS generation causing alteration of the resident microbiota profile, providing an anti-inflammatory milieu through probiotics is seemingly helpful [62].

7. Oxidative Stress and the Microbiota-Gut-Brain Axis in Ischemic Stroke

Ischemic stroke is an acute life-threatening condition and a leading cause of death and long-term neurological disability worldwide, with up to 40% of stroke cases not expected to recover autonomy. It results as a consequence of the interruption or severe reduction of the blood flow in the cerebral arteries, leading to oxygen and glucose deprivation, and the accumulation of waste metabolites in the affected area, with harmful effects on energy-dependent neuronal processes. The affected region of the brain is defined by two major areas of injury: the core and the surrounding region, known as the penumbra. Within the core, the brain tissue undergoes irreversible cellular damage resulting in almost instant neuronal death; the penumbra is a dysfunctional but still viable cerebral tissue characterized by moderate hypoperfusion and preserved structural integrity; it may evolve either towards necrosis or towards recovery [33].

In the setting of an acute stroke, the ensuing cerebral ischemia leads to anaerobic glycolysis and lactic acidosis subsequently promoting a prooxidant effect by increasing H^+ concentrations and excessive production of ROS. This is a common consequence of several types of brain insults, representing a fundamental mechanism of cerebral damage in stroke [63]. Along with the accumulation of excessive levels of ROS, the ROS scavenging capacity is decreased, presumably due to an impairment of the antioxidant defense systems. Experimental studies have shown that the expression of SODs, CATs, GPx, and glutathione is significantly reduced in animal models of stroke [64]. Furthermore, although rapid restoration of blood flow in the ischemic brain is essential to prevent neuronal death in the hypoperfused area surrounding the ischemic core, the required recanalization interventions may also result in tissue damage known as "reperfusion injury." During ischemia and reperfusion conditions, the accelerated ROS generation exceeding the endogenous antioxidant capacity is one of the main hallmarks in the pathogenesis of brain tissue destruction [65].

The ischemic cascade begins with depletion of cellular energy by failure of ATP synthesis. This adversely affects Na^+/K^+-ATP-ase pump and Ca^{2+} pump, resulting in plasma membrane depolarization and critical rise in intracellular calcium concentration, respectively. Furthermore, the initial calcium influx triggers a secondary intracellular toxic calcium overload. An important role in ischemic injury is also played by glutamate which is crucial for neuron degeneration when it acts as a toxic excitatory neurotransmitter. Moreover, activation of glutamate receptors (mostly NMDA) facilitates influx of calcium into neurons leading to excitotoxicity. During excitotoxicity, increasing the mitochondrial calcium concentration leads to the excessive production of ROS [63].

ROS have significant vascular effects ultimately influencing cerebral blood flow. Considering particular concentrations along with direct or indirect pathogenic pathways, the same free radical exerts divergent reactions. For instance, the superoxide is known for its biphasic induced effect on vascular tone which is remarkably complex, causing arterial relaxation if superoxide is produced from xanthine while excessive levels of superoxide in the presence of NADPH or high NADH concentrations cause cerebral arterial contraction. The effect of H_2O_2 on vascular tone has been largely investigated. In mice, in vivo application of H_2O_2 induces basilar artery dilation [33]. Nonetheless, high levels of H_2O_2 can produce vasoconstriction followed by vasodilatation. Low concentration of $ONOO^-$ induces vasodilatation of cerebral arterioles in vivo via activation of potassium channels. An animal model study reported that low concentrations of $ONOO^-$ produced contraction of the posterior cerebral artery following middle cerebral artery occlusion but higher concentrations induced vasodilatation and loss of myogenic activity [66].

ROS indirectly influence the platelet activity by reducing the antiplatelet properties of the endothelium as well as scavenging NO. Moreover, O_2^- in particular may induce spontaneous aggregation presumably as a consequence of reduced bioavailability of NO as a potent inhibitor of platelet activation. Experimental data have shown that platelets themselves generate ROS via enzymatic systems (NADPH, NOS, XO, and phospholipase A2) [67].

ROS have substantial cellular effects in stroke resulting in neural tissue demise and neuronal death. Two of the major consequences of ROS-induced brain injury are lipid peroxidation and protein denaturation. ROS also promote DNA modification derived by two separate mechanisms including oxidative alterations and endonuclease-mediated DNA fragmentation. Furthermore, following ROS-induced release from the mitochondria, cytochrome c forms the apoptosome and activates caspases that can cleave nuclear DNA repair enzymes increasing the oxidative DNA lesions. More than that, ROS can be involved in apoptotic pathways also by activating caspase-activated DNase which can cleave DNA resulting in apoptosis [68].

In addition to the aforementioned mechanisms, ROS mediate BBB dysfunction directly by oxidative damage, tight-junction alteration, cytoskeletal reorganization, and matrix metalloproteinases activation. It is well known that BBB is composed of the endothelial cells of the capillary wall, tight junctions among endothelia, basal membrane, pericytes, and astrocyte endfeet encircling the capillary. *In vitro* exposure of human umbilical vein endothelial cells to H_2O_2 induces redistribution of occludin and dissociation from zonula occludens-1 (ZO-1) [69]. Moreover, exposure of bovine pulmonary artery endothelial cells to H_2O_2 activates focal adhesion kinase leading to actin cytoskeleton reorganization and subsequently to increased permeability [70].

Over the last few years, considerable progress has been made regarding the role of the gut microbiota in stroke. However, plenty of concerns have remained unanswered. Following cerebrovascular events (either ischemic or hemorrhagic stroke), up to 50% of patients develop gastrointestinal complications consisting of dysphagia, gastrointestinal haemorrhage, constipation, or bowel incontinence. These

result in poor patient outcomes, including delayed patient rehabilitation, increased mortality rates, and degrading neurologic function [71].

A recent experimental study using a middle cerebral artery occlusion mouse model analyzed both the intestinal bacterial biomass and composition demonstrating that stroke promotes intestinal barrier breakdown and substantial microbiota alteration. The results also strongly indicated systemic dissemination of gut bacteria [72].

It was recently reported that the brain infarct volume after transient middle cerebral artery occlusion was decreased by 60% when intestinal microbial diversity was reduced in amoxicillin/clavulanate-treated mice [73]. This study also emphasized the trafficking of intestinal T cells to the meninges. The remodeling of intestinal microbiota after stroke defines gut dysbiosis and influences immunological changes [73]. As we mentioned before, neuroinflammation and OS are closely involved in cerebral ischemia-reperfusion injury. OS induces inflammation, while inflammation causes damage through OS. Previous researching evidence established that inflammation is a decisive step in the pathophysiology of ischemic stroke. Moreover, numerous studies have indicated that neuroinflammation ensuing stroke is a determinant factor of acute outcome and long-term prognosis for ischemic stroke [74]. Therefore, various experimental approaches have explored the therapeutic potential of immunomodulation. Commensal intestinal bacteria influence the host immune system and subsequently the disease mechanisms in several organs, including the brain. Intestinal commensal microbes appear to be a potent regulator of lymphocyte populations, including regulatory T (Treg) and $\gamma\delta$ T cells, both of which are involved in cerebral ischemic injury. $\gamma\delta$ T cells represent a considerable lymphocyte population with innate immune properties which can exacerbate ischemic brain injury by secreting IL-17 and generating chemotactic signals for neutrophils and monocytes. Proinflammatory cytokines inhibit brain repair due to an increased production of ROS-generating OS. On the other hand, Treg cells contribute to neuroprotection by secreting the anti-inflammatory cytokine IL-10 and down-regulating postischemic inflammation [75].

Summarizing, extensive stroke lesions lead to gut dysbiosis which consecutively affects stroke outcome via changes in T cell homeostasis, inducing a proinflammatory response and OS. Therapeutic transplantation of fecal microbiota in models normalizes brain lesion-induced dysbiosis and improves stroke outcome. Therefore, a target of stroke-induced systemic alterations is the gut microbiome, which is an important determinant with substantial impact on stroke outcome [63].

8. Conclusions

The CNS is highly susceptible to OS and chronic OS is involved in many CNS diseases. This may be explained by certain particularities: the CNS has a strong oxygen demand with a high oxidative metabolism but a modest endogenous antioxidant defense, it extensively uses ROS/RNS and other reactive species for intra- and intercellular signaling, it uses interneuronal signaling pathways that generate ROS (e.g.,

glutamate and calcium transients), it is abundant in redox-active transition metals (e.g., iron and copper) and PUFA (which are prone to peroxidation), it has a high glucose metabolism and high mitochondria activity, and it has auto-oxidating neurotransmitters.

The complex microbiota-host cross-talk occurring via the microbiota-gut-brain axis may influence the OS of the CNS, directly and indirectly, by interfering both with the local level of ROS/RNS and with the CNS antioxidant system. Among these, the production of potentially neurotoxic molecules, such as lipopolysaccharides, amyloid proteins, or antibiotics, which may reach the CNS via the systemic circulation or the vagus nerve, promoting microglia activation and the production of ROS and OS, should be further explored. Identification of microbiota biomarkers related to deleterious CNS OS also deserves further attention. The microbiota-gut-brain axis opens a gate for new therapeutic approaches of various neurological conditions.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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Research Article

Effects of Irbesartan Pretreatment on Pancreatic β-Cell Apoptosis in STZ-Induced Acute Prediabetic Mice

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The current study was performed to investigate the effects and potential effects of irbesartan pretreatment on pancreatic β-cell apoptosis in a streptozotocin- (STZ-) induced acute mouse model of prediabetes. Twenty-four male BALB/C mice (18–22 g) were randomly divided into three groups: normal control group (NC, $n = 6$), STZ group (STZ, $n = 8$), and irbesartan + STZ group (IRB + STZ, $n = 10$). In the IRB + STZ group, mice were administered irbesartan (300 mg/kg per day) by gavage for one week. The STZ group and IRB + STZ group received STZ (80 mg/kg by intraperitoneal (IP) injection once). The NC group received normal saline (80 mg/kg by IP injection once). Fasting blood glucose prior to STZ injection and presacrifice was analysed using samples withdrawn from the caudal vein to confirm the induction of prediabetes. Haematoxylin and eosin staining, immunohistochemical detection of insulin, and apoptosis analysis were performed. Reverse transcription-quantitative polymerase chain reaction was used to detect angiotensin II type 1 receptor (AT1R), caspase-3, and p38 mitogen-activated protein kinase (MAPK) mRNA expression. Blood glucose was significantly higher in the STZ group (9.01 ± 1.1089 vs 4.78 ± 0.7026) and IRB + STZ group (7.86 ± 1.1811 vs 4.78 ± 0.7026) compared with the NC group ($P < 0.05$). In comparison to the STZ group, the islet cell damage was marginally improved in the IRB + STZ group, and the IRB + STZ group had a significantly lower apoptotic rate than the STZ group (22.42 ± 8.3675 vs 50.86 ± 5.3395, $P < 0.001$). AT1R expression in the IRB + STZ group was lower than that in the STZ group (1.56 ± 1.2207 vs 3.92 ± 2.4392, $P < 0.05$). The mRNA expression of caspase-3 in pancreatic tissue was significantly lower in the IRB + STZ group than in the STZ group (0.90 ± 0.7272 vs 1.88 ± 1.0572, $P < 0.05$). Similarly, the IRB + STZ group also had lower p38MAPK levels than the STZ group (1.16 ± 1.0642 vs 2.55 ± 1.7925, $P > 0.05$). In conclusion, irbesartan pretreatment improved glucose levels and insulin secretion and decreased islet β-cell apoptosis to protect islet β cells in an STZ-induced acute prediabetic mouse model.

1. Introduction

Previous clinical studies have indicated that blockade of the renin-angiotensin system (RAS) using inhibitors of angiotensin-converting enzymes (ACEIs) or angiotensin-receptor blockers (ARBs) reduces the occurrence of diabetes and the incidence of complications associated with diabetes in hypertensive patients [1–4]. Similarly, patients with hypertension treated with irbesartan not only reduced blood pressure, but also improved metabolic parameters,

for example, blood glucose and lipid levels as well as liver function [5]. These studies suggest that ACEIs or ARBs may be beneficial for the prevention of diabetes. However, the underlying mechanisms involved remain unclear.

Irbesartan, a commonly used ARB, has been reported to have various beneficial effects beyond reduction in blood pressure. Clinical and experimental data indicate that irbesartan can ameliorate diabetic nephropathy by suppressing signalling from the receptor activator of nuclear factor kappa B (NF-κB) [6]. Additionally, irbesartan

may have a role in protecting against diabetes-related bone damage by blocking the detrimental effects of advanced glycation end product- (AGE-)/receptor for advanced glycation end product- (RAGE-) mediated oxidative stress [7]. Furthermore, irbesartan treatment can improve diabetes-related arteriosclerosis [8]. In the diabetic Zuker rat model with insulin-resistant obesity induced by fructose, irbesartan treatment led to improved glucose tolerance and insulin sensitivity [9]. Thus, irbesartan is a widely used ARB in clinical practice, especially in hypertensive patients with diabetes, and it is important to explore the effect of irbesartan in the prevention of diabetes further.

Prediabetes is the stage before DM in which not all of the symptoms or signs required to diagnose diabetes are present, but blood glucose is abnormally high. Epidemiological data shows that the estimated prevalence of prediabetes in China was much higher in 2013 than the estimate in 2008 (35.7% vs 15.5%) [10]. Prediabetes is often associated with obesity, hypertension, and dyslipidaemia. There are many studies that have investigated the effects of ACEI or ARB in diabetic models; however, as the treatment for diabetes is limited to the later stages when a diagnosis has been received, and even patients with well-controlled blood glucose gradually develop diabetes-related complications, it is vital to identify preventative methods. Hypertensive patients often have impaired glucose tolerance [11], and ACEIs and ARBs have both been reported to help prevent diabetes in patients with hypertension. Thus, the preventative function of ACEI or ARB in prediabetes should also be explored, as few studies have investigated their effects in this context.

The RAS is a vital determinant of blood pressure and intravascular volume. The major components involved in the system are renin, angiotensinogen, angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2, angiotensin II (Ang II), and its receptors Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R) [12]. In addition to the classical (circulating) RAS, there is a growing body of experimental evidence indicating that local RAS (complete or partial) exists in several tissues and has pivotal roles in a variety of developmental and disease processes [13]. Evidence has suggested that the pancreas has a local RAS in humans, rats, mice, and dogs, with expression of the essential RAS components, including Ang II and its receptors (AT1R and AT2R), detected in the pancreas. In the human pancreas, expression of AT1 receptors is confined to the β cells and endothelial cells of the islets. Emerging evidence suggests that the local RAS in pancreatic islets has functions in glucose homeostasis. In rats, islet blood flow appears to be suppressed by locally produced Ang II under normal conditions. In vitro, Ang II delays the first phase of insulin release in response to glucose. These findings suggest that locally generated Ang II may indirectly affect glucose homeostasis via somatostatin-mediated inhibition of insulin or glucagon secretion under certain conditions [12, 14, 15]. Ang II is a key bioactive peptide of the RAS. Initially, renin cleaves its substrate, angiotensinogen, to form the decapeptide angiotensin I (Ang I). Then, the two terminal amino acids are split by the biologically active dipeptide carboxypeptidase ACE to form the octapeptide Ang II. Ang II exerts its biological actions via two G-protein-coupled receptors, AT1R and AT2R, with the deleterious effects predominantly mediated via AT1R [16].

Emerging data support that chronic hyperglycaemia, AGEs, high fat, obesity, inflammation, and hypertension can activate the pancreatic RAS by increasing AT1R expression, inflammation, oxidative stress, and apoptosis in pancreatic β cells [17, 18]. Previous studies have illustrated the novel roles of the pancreatic RAS in pancreatic β-cell function. In diabetes, activation of the pancreatic RAS by hyperglycaemia and hyperlipidaemia can result in reduced insulin biosynthesis, insulin secretion, and islet blood flow, and also increased β-cell apoptosis, oxidative stress, and islet fibrosis [14, 19]. In our previous study, we demonstrated that high glucose promotes islet cells apoptosis in vitro [20]. However, the underlying mechanisms are poorly elucidated. High glucose activates the RAS and upregulates Ang II and AT1R expression. Ang II induces the production and activation of reactive oxygen species (ROS) via AT1R-mediated nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase and increases the expression of the apoptosis-related factors Bax and caspase-3 [20].

Understanding the relationship between the RAS and pancreatic β-cell function during prediabetes and identifying individuals that are at risk of developing specific pathologies will guide more effective personalized prevention and treatment. Therefore, the present study was aimed at investigating the effect of irbesartan pretreatment on pancreatic β-cell apoptosis in a streptozotocin- (STZ-) induced acute prediabetic mice.

2. Materials and Methods

2.1. Animals. Male BABL/C mice weighing approximately 18–22 g were purchased from the Animal Center of Guangxi Medical University (Nanning, China). Mice were humanely housed at $22 \pm 2°C$ with 12-h light/dark cycles. All animals had free access to food and water. All animal studies were approved by the ethical review committee of Guangxi Medical University and followed the regulations of the National Institutes of Health guidelines on the care and welfare of laboratory animals.

2.2. Drugs and Chemicals. Irbesartan was obtained from Sanofi (S.A., Paris, France), and STZ was purchased from Sigma-Aldrich Inc., (St. Louis, MO, USA). The appropriate pretreated dose of irbesartan was determined in preliminary experiments (supplemental materials, part 1).

2.3. Induction of Experimental Prediabetes. An acute prediabetic mouse model was successfully established using STZ in a previous study [21]. Prediabetes was induced in mice by a single intraperitoneal injection of STZ (80 mg/kg). Mice with mild hyperglycaemia (fasting glucose before sacrifice > 6 mmol/L and <16.7 mmol/L), mild pancreatic islet damage, and <60% apoptosis of pancreatic β cells were considered to indicate prediabetes, and these mice were included in the study.

2.4. Study Design. The animals were randomly divided into three groups: normal control group (NC group, $n = 6$), STZ group (STZ, $n = 8$), and irbesartan + STZ group (IRB + STZ, $n = 10$). In the IRB + STZ group, mice were administered irbesartan (300 mg/kg per day) orally by gavage for one week. The STZ group and IRB + STZ group received STZ (80 mg/kg by intraperitoneal (IP) injection once); the NC group received normal saline (80 mg/kg by IP injection once). Fasting blood glucose was taken before STZ injection and before sacrifice using blood from the caudal vein, and was used to confirm the induction of prediabetes. Mice were sacrificed by cervical dislocation after 12 h.

2.5. Haematoxylin and Eosin (H&E) Staining. Isolated pancreas samples were fixed in 4% formaldehyde. The tissues were sectioned and stained with H&E for morphological analysis.

2.6. Immunohistochemical Detection. Before dewaxing, the tissue sections were placed in a 60°C incubator. Following dewaxing and hydration, the tissue sections were immersed into xylene and then transferred to ethanol. The sections were incubated in 0.01 mol/L citrate buffer (pH 6.0) for antigen retrieval. Then, freshly prepared 3% hydrogen peroxide solution was added to eliminate endogenous peroxidase activity. Next, the sections were incubated with anti-insulin antibody (cat. no. BM0080; Wuhan Boster Biological Technology Ltd., Wuhan, China; 1 : 200), then incubated with a secondary antibody, and finally counterstained with haematoxylin.

2.7. Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) Assay. Tissues were sectioned as described above. The assay was performed according to the instructions of the TUNEL kit (cat. no. 11684817910; Roche Diagnostics, Basel, Switzerland). The sections were washed in PBS three times before incubation in diaminobenzidine for 3 min and analysed in mounting medium under a fluorescence microscope. The number of positive cells in five noncontinuous high power fields of vision was observed and counted under the microscope.

2.8. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was isolated from pancreas samples using TRIzol, and RT was then performed using the PrimeScript®RT reagent kit (cat. no. HRR037A; Takara Biotechnology Co. Ltd., Dalian, China). qPCR analysis was performed using a Mastercycler PCR machine (Mastercycler® X50s; Eppendorf, Hamburg, Germany). The relative quantification analysis for a given gene was performed using 7500 Fast Real-Time PCR (Applied Biosystems; Thermo Fisher Scientific Inc., Waltham, MA, USA). Oligonucleotide sequences are provided in the supplemental materials, part 2.

2.9. Statistical Analyses. SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous data with a normal distribution is expressed as the mean ± standard deviation ($x \pm s$). Analysis of variance was used to compare the means of multiple samples. When the data met the assumption of homogeneity of variances, the
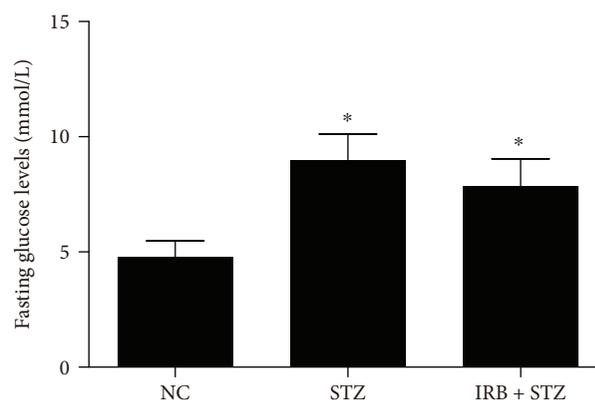
Figure 1: Fasting glucose levels after 12-hour intervention in each group. NC = normal control group ($n = 6$, received normal saline 80 mg/kg by intraperitoneal (IP) injection once), STZ = streptozotocin group ($n = 8$, received STZ 80 mg/kg by IP once), and IRB + STZ = irbesartan + streptozotocin group ($n = 10$, administered irbesartan 300 mg/kg per day by gavage for one week before receiving STZ 80 mg/kg by IP once). Data are presented as mean ± SD. ∗ compared with the NC group, $P < 0.05$.

Table 1: Fasting glucose levels after 12-hour intervention in each group.

Group	n	Glucose level (mmol/L)
NC	6	4.78 ± 0.7026
STZ	8	$9.01 \pm 1.1089^*$
IRB + STZ	10	$7.86 \pm 1.1811^*$

Notes: data are presented as mean ± SD. ∗ compared with the NC group, $P < 0.05$.

least significant difference test was used for the comparison of multiple samples. When the data did not meet the homogeneity of variance assumption, Games-Howell was used to compare multiple samples. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Changes in Blood Glucose. Compared with the control group, blood glucose was significantly increased in the STZ group (9.01 ± 1.1089 vs 4.78 ± 0.7026) and IRB + STZ group (7.86 ± 1.1811 vs 4.78 ± 0.7026) after injection of STZ ($P < 0.001$); however, all blood glucose levels were <10 mmol/L. The level of blood glucose elevation in the IRB + STZ group was smaller than that in the STZ group, but there was no significant difference between the two groups (Figure 1 and Table 1).

3.2. Effect of Irbesartan on Islet Morphology. In the NC group, the islet cells were clumped together with abundant capillaries between the groups of cells. The morphology of islet cells was normal, and the nuclei were round and clearly visible. After STZ injection, islet cells appeared swollen with

(a)

(b)

(c)
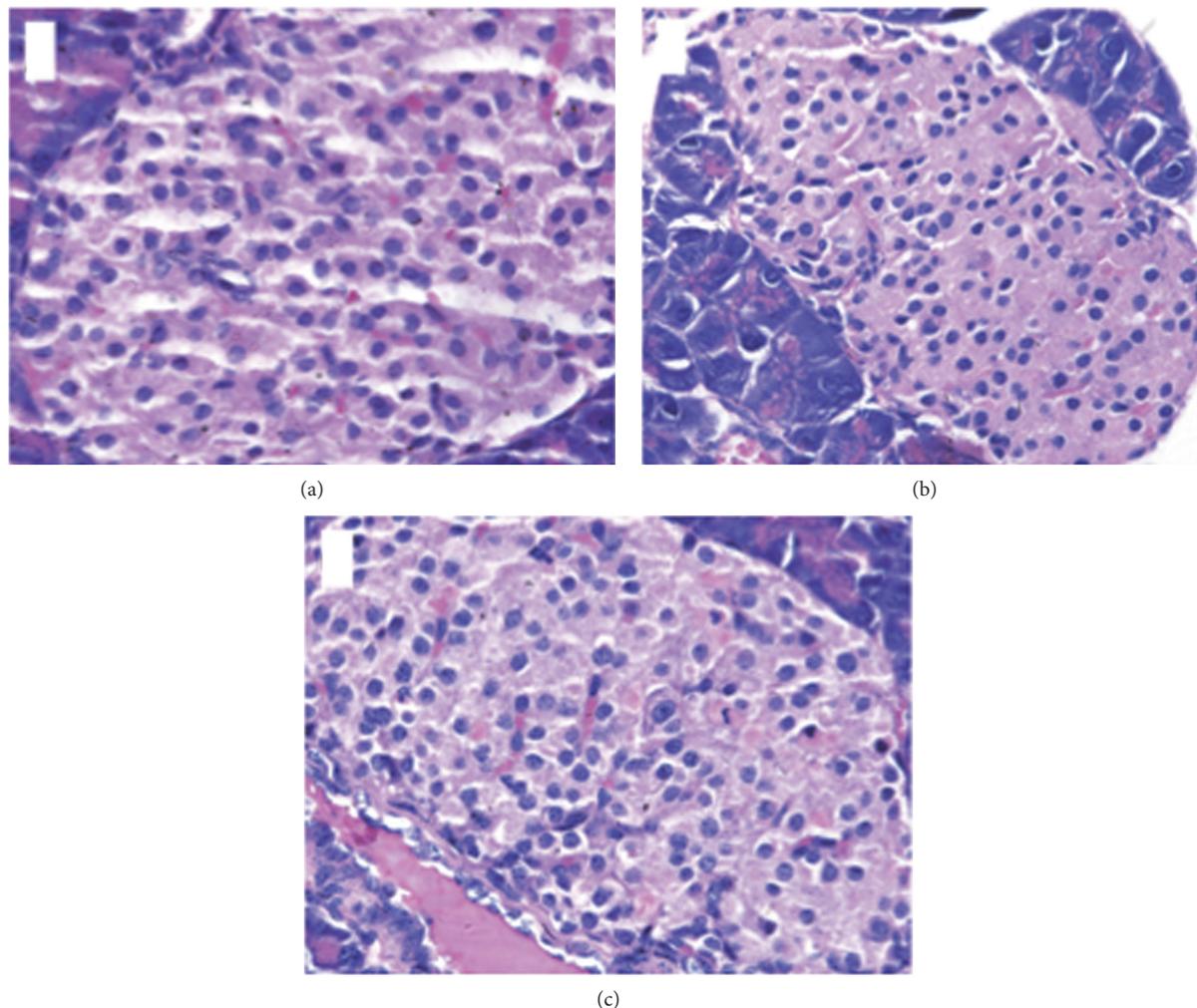
FIGURE 2: The HE staining of islet cells in mice in each group (400x). (a) NC group; (b) STZ group; and (c) IRB + STZ group.

hyaline degeneration, nuclear dissolution, and nuclear condensation. In comparison to the STZ group, the islet cells damage were marginally improved in the IRB + STZ group (Figure 2).

3.3. Apoptosis Is Reduced by Irbesartan in the Pancreas of STZ-Induced Mice.
Compared with the NC group, the apoptotic rates were significantly higher ($P < 0.001$) in the STZ group (50.86 ± 5.3395 vs 4.53 ± 1.0020) and IRB + STZ group (22.42 ± 8.3675 vs 4.53 ± 1.0020) were significantly higher ($P < 0.001$). However, the IRB + STZ group had a significantly lower rate of apoptosis compared with the STZ group (22.42 ± 8.3675 vs 50.86 ± 5.3395, $P < 0.001$), indicating that irbesartan preconditioning can partially protect islet cells from STZ by reducing the apoptotic rate of islet cells (Table 2, Figures 3 and 4).

3.4. Irbesartan Increases Insulin Levels in STZ-Induced Mice.
The level of insulin in the islet β-cells was significantly decreased in the STZ group compared with the NC group (0.44 ± 0.0423 vs 0.34 ± 0.0410, $P < 0.001$), indicating that STZ induced damage of pancreatic β cells, resulting in reduced insulin reserves. In the IRB + STZ group, insulin

TABLE 2: Apoptosis rate of pancreatic islet cells in each group.

Group	n	Apoptosis (%)
NC	6	4.53 ± 1.0020
STZ	8	$50.86 \pm 5.3395^{*}$
IRB + STZ	10	$22.42 \pm 8.3675^{*\#}$

Notes: data are presented as mean $\pm$ SD. $*$ compared with the NC group, $P < 0.05$. # compared with the STZ group, $P < 0.05$.

expression was higher than in the STZ group (0.38 ± 0.0315 vs 0.34 ± 0.0410, $P < 0.001$), but still lower than in the NC group (0.38 ± 0.0315 vs 0.44 ± 0.0423, $P < 0.001$) (Table 3 and Figure 5).

3.5. Effect of Irbesartan on Gene Expression.
The AT1R mRNA expression was significantly increased in pancreatic tissue of the STZ group compared with the NC group ($P < 0.05$), suggesting that the pancreatic RAS was activated during apoptosis of pancreatic islet cells. Furthermore, AT1R expression was lower in the IRB + STZ group compared with the STZ group (1.56 ± 1.2207 vs 3.92 ± 2.4392, $P < 0.05$).
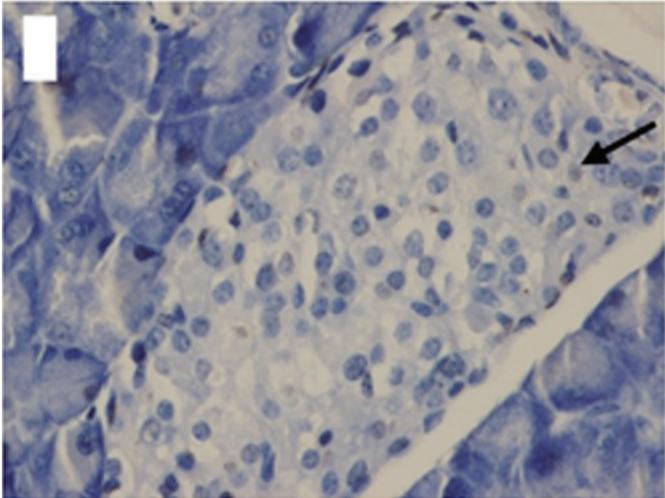
(a)
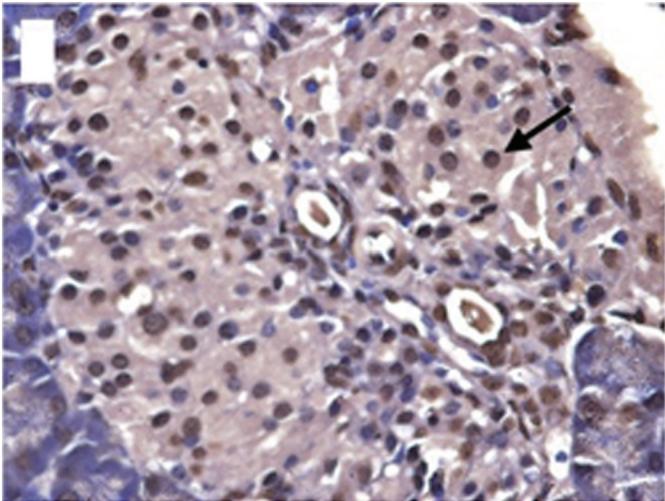
(b)
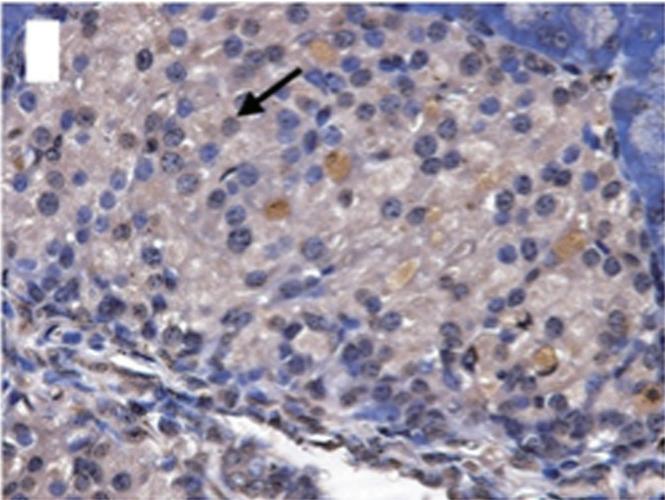
(c)

FIGURE 3: Apoptosis of islet cells in mice in each group (400x). The arrow pointed to the apoptosis islet cells. (a) NC group; (b) STZ group; and (c) IRB + STZ group.
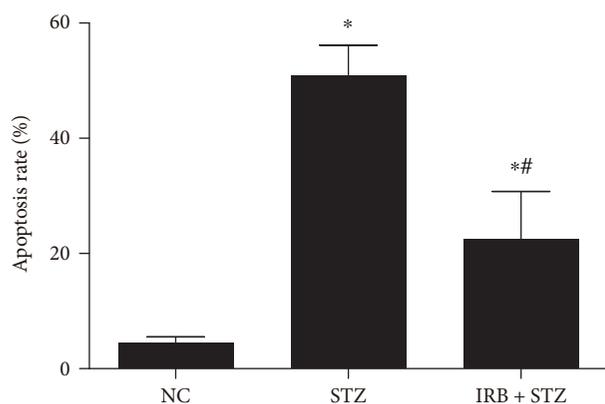
Figure 4: Apoptosis rate of pancreatic islet cells in each group. NC = normal control group ($n = 6$, received normal saline 80 mg/kg by intraperitoneal (IP) injection once), STZ = streptozotocin group ($n = 8$, received STZ 80 mg/kg by IP once), and IRB + STZ = irbesartan + streptozotocin group ($n = 10$, administered irbesartan 300 mg/kg per day by gavage for one week before receiving STZ 80 mg/kg by IP once). Data are presented as mean ± SD. ∗ compared with the NC group, $P < 0.05$. # compared with the STZ group, $P < 0.05$.

Table 3: Insulin expression in the pancreas of mice in each group.

Group	n	Insulin
NC	6	0.44 ± 0.0423
STZ	8	$0.34 \pm 0.0410^{*}$
IRB + STZ	10	$0.38 \pm 0.0315^{*\#}$

Notes: data are presented as mean ± SD. ∗ compared with the NC group, $P < 0.05$. # compared with the STZ group, $P < 0.05$.

There was approximately double the caspase-3 mRNA expression in the STZ group compared with the NC group ($P > 0.05$), suggesting that apoptosis of islet cells was induced by STZ. Furthermore, the mRNA expression of caspase-3 in pancreatic tissue was significantly lower in the IRB + STZ group than in the STZ group (0.90 ± 0.7272 vs 1.88 ± 1.0572, $P < 0.05$). Similarly, the IRB + STZ group also exhibited reduced p38 mitogen-activated protein kinase (MAPK) levels compared with the STZ group (1.16 ± 1.0642 vs 2.55 ± 1.7925, $P > 0.05$), although there was not up to statistical difference, further indicating that irbesartan attenuated apoptosis of pancreatic β cells (Table 4 and Figure 6).

4. Discussion

The most intriguing findings of the present study include that irbesartan pretreatment exhibited the following benefits in the prediabetic model: (1) improved blood glucose levels; (2) minimized the damage to pancreatic cells; (3) reduced pancreatic β-cell apoptosis; (4) increased insulin levels in the pancreatic β cells; and (5) reduced the expression of AT1R, p38MAPK, and caspase-3.

A previous clinical study suggested that when patients are diagnosed with diabetes the number of pancreatic β cells has been reduced by 50%, with β-cell apoptosis as the main cause

of the loss of β cells [22], which also indicates that β-cell apoptosis is the main pathological change in the prediabetic stage. If appropriate interventions are used at this stage, the prevalence of diabetes may significantly decrease and high-risk populations will benefit from preventative treatment. However, few studies have investigated the physiological and pathological processes of prediabetes. Thus, we first established a mouse model of prediabetes in this study. Our previous study demonstrated that a small dose of STZ can acutely induce β-cell apoptosis as a nondiabetic mouse model, which we consider to be a prediabetic model for the following reasons: (1) mild hyperglycaemia; (2) mild pancreatic islet damage; and (3) apoptosis of pancreatic β cells at <60% [21]. Thus, we were able to investigate the role of irbesartan in the prevention of diabetes.

A large body of data indicates that a local RAS exists in the pancreatic islet cells in both humans and several other animals. Activation of the pancreatic RAS, mainly through AT1R, acutely diminishes insulin secretion, which is due to reduced proinsulin biosynthesis and islet blood flow. The RAS also causes islet apoptosis and fibrosis, and may consequently contribute to islet cell dysfunction in type 2 diabetes [23]. It is well documented that RAS blockade using ACEIs or ARBs has a positive effect on the development processes of diabetes. For instance, treatment with an ACEI in diabetic mice reduced circulating glucose [18]. However, no studies have investigated the effects of RAS blockade in prediabetes. To the best of our knowledge, this is the first study to explore the influence of ARB irbesartan pretreatment on pancreatic β-cell function. Consistent with previous studies, the findings of the present study suggested that treatment of irbesartan prior to STZ intervention reduced the STZ-induced increase in blood glucose in the prediabetic mouse model, which provided experimental evidence indicating that irbesartan can improve glucose levels, not only in diabetes, but also in prediabetes.

The mechanisms by which ACEIs or ARBs improve glucose status and pancreatic β-cell function have not been fully elucidated. RAS blockade is associated with increased β-cell mass, improvement in first-phase insulin secretion, and normalization of islet morphology [24]. Furthermore, in a previous study, islet fibrosis was diminished and β-cell dysfunction was improved following treatment of rats with the ACE inhibitor ramipril for 24 weeks [17]. Similarly, other evidence from animal models has demonstrated that ACEIs and ARBs prevent the development of fibrosis [25]. Furthermore, ARBs and ACEIs increase glucose transporter (GLUT) 4 translocation to the membrane, resulting in increased skeletal muscle glucose uptake and insulin sensitivity in animal models [18, 26]. The present study provided further evidence that pretreatment with irbesartan attenuates pancreatic damage, reduces islet β-cell apoptosis, and increases insulin secretion, which affirms that irbesartan can prevent STZ-induced pancreatic islet damage in an acute prediabetic mouse model.

Emerging data support the notion that elevated glucose level and other factors can stimulate the activity of RAS components, such as Ang II and AT1R. Isolated human islets exposed to high glucose concentrations exhibited
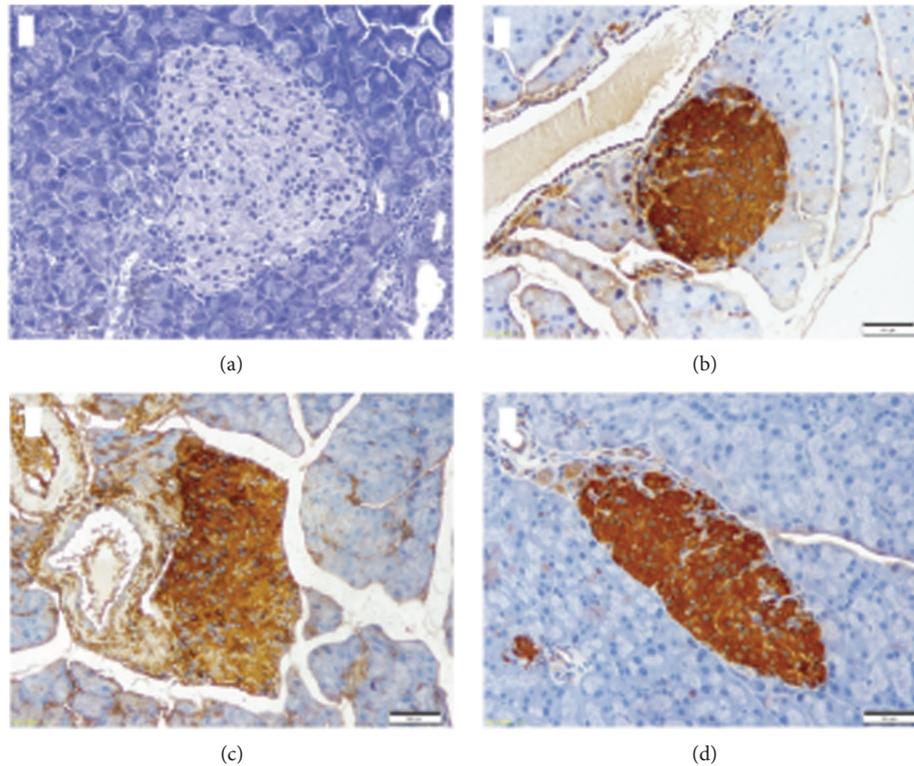
FIGURE 5: Immunohistochemical staining of insulin in pancreas of mice in each group (200×). (a) Negative control group; (b) normal control group; (c) STZ group; and (d) IRB + STZ group.

TABLE 4: AT1R, caspase-3, and p38MAPK expression levels in pancreas in each group.

Group	n	AT1R	Caspase-3	p38MAPK
NC	6	1	1	1
STZ	8	3.92 ± 2.4392*	1.88 ± 1.0572	2.55 ± 1.7925*
IRB + STZ	10	1.56 ± 1.2207#	0.90 ± 0.7272#	1.16 ± 1.0642

Notes: data are presented as mean ± SD. ∗ compared with the NC group, $P < 0.05$. # compared with the STZ group, $P < 0.05$.

enhanced expression of AT1R and ACE [27]. Notably, Ang II is involved in lipid- and glucose-induced inflammation, oxidative stress, and apoptosis via AT1R [18]. Pancreatic β cells have relatively low levels of antioxidants; thus, increased oxidative stress is more likely to induce apoptosis and decrease β-cell numbers [1]. There is a vicious circle, in which activation of the RAS, amyloid deposition, and oxidative stress-related changes result in elevated apoptosis, reduction of β-cell mass, and impairment of insulin secretion and insulin production, which in turn impairs lipid and carbohydrate homeostasis. Finally, lipotoxicity and glucotoxicity further deteriorate β-cell dysfunction and insulin resistance [27]. In an STZ-induced pancreatic β-cell apoptosis model, STZ increases the synthesis of ROS, causes mitochondrial damage, and reduces the formation of adenosine triphosphate in the pancreas, thus, resulting in islet β-cell damage [28]. The transfer of the methyl group from STZ to DNA causes damage, which induces a chain of

events that results in the fragmentation of the DNA [28]. Our present study was aimed at exploring the effects of irbesartan in preventing diabetes, and to determine whether the changes discussed were also present in prediabetes. Therefore, irbesartan pretreatment was performed prior to induction of an STZ-induced prediabetes model in mice to imitate the preventative use of ACEI or ARB in a clinical setting. Following injection of STZ, the expression of AT1R in the STZ group was markedly increased compared with that in the NC group, but this effect was attenuated by irbesartan pretreatment, indicating that the local pancreatic RAS was active in the prediabetic model, and irbesartan can diminish the activation of local pancreatic RAS.

Research has focused on the influence of ACEIs or ARBs on the pancreas and pancreatic β-cell function; however, the mechanistic pathway that mediates the effects is still poorly understood. Currently, the evidence indicates that two pathways may be involved: MAPK and caspase pathways. The signalling events in response to Ang II activate redox-sensitive signal molecules, such as MAPKs [29]. MAPKs are a family of serine/threonine kinases associated with the pathogenesis of vascular fibrosis and hypertension. In addition, MAPKs are also crucial regulatory proteins that control the cellular response to apoptosis, growth, and stress signals [30]. Key MAPKs include p38MAPK, extracellular signal-regulated kinases 1/2, and stress-activated protein kinase/c-Jun N-terminal kinases [31]. Particularly, p38MAPK is a vital component of the oxidative stress-sensitive pathways activated by Ang II in vascular smooth muscle cells, and it is also a key protein crucial for various cellular processes, including
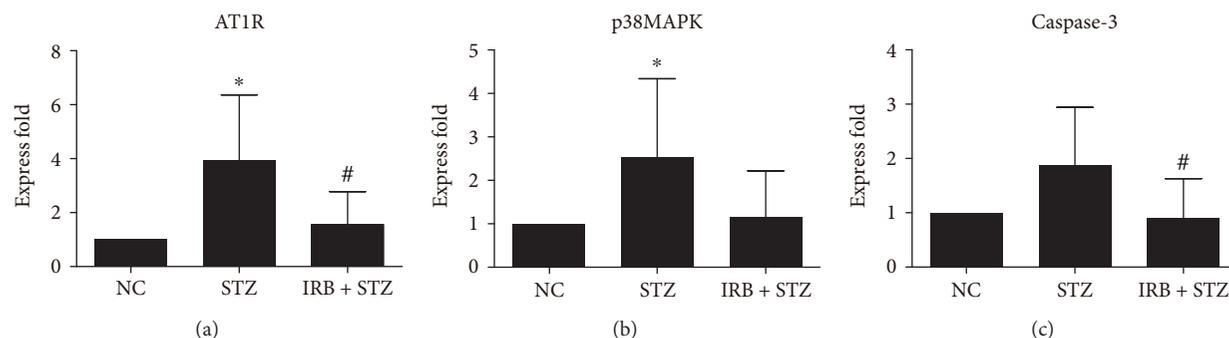
FIGURE 6: AT1R (a), p38MAPK (b), and caspase-3 (c) mRNA expression levels in pancreas in each group. NC = normal control group ($n = 6$, received normal saline 80 mg/kg by intraperitoneal (IP) injection once), STZ = streptozotocin group ($n = 8$, received STZ 80 mg/kg by IP once), and IRB + STZ = irbesartan + streptozotocin group ($n = 10$, administered irbesartan 300 mg/kg per day by gavage for one week before receiving STZ 80 mg/kg by IP once). Data are presented as mean ± SD. * compared with the NC group, $P < 0.05$. # compared with STZ group, $P < 0.05$.

cell differentiation, inflammation, cell growth, and cell death [32, 33]. Furthermore, Ang II can induce ROS generation via oxidative stress reactions, which in turn, activates the p38MAPK signalling pathway. Both p38MAPK and ROS molecules are involved in myocardial fibrosis formation [34]. Caspases are a unique class of aspartate-specific proteases that are the key components of the apoptotic response. Caspase-3 has a pivotal role in the intrinsic and extrinsic apoptotic pathways [35], and Ang II has been reported to stimulate caspase-3 activity [36]. The present study found that the early intervention with irbesartan reduced the STZ-induced activation of apoptosis signal pathways (p38MAPK and caspase-3) in prediabetes. We speculate that irbesartan blocked the effects of STZ-induced prediabetes by inhibiting Ang II and AT1R, and thus reducing the activation of the p38MAPK and caspase-3 pathways, ultimately improving pancreatic β-cell function in the acute prediabetic stage.

5. Conclusions

In summary, irbesartan pretreatment improved glucose levels and insulin secretion in the pancreatic islet and decreased islet β-cell apoptosis to protect islet β cells in an STZ-induced acute prediabetic mouse model. The underlying mechanism may be that irbesartan blocks activation of the pancreatic RAS by inhibiting AT1R expression, leading to decreased activity of p38MAPK and caspase-3-mediated apoptosis pathways. These actions protect the pancreatic islet β cells and may have a diabetes prevention effect. However, this study only tentatively explores the molecular changes that are associated with the effects of irbesartan during prediabetes. The specific molecular mechanisms and signal pathways still require further investigation.

Data Availability

The data used to support the findings of this study are included within the article and the supplementary information file.

Conflicts of Interest

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

Authors' Contributions

Cuihong Chen and Li Li are co-first authors.

Supplementary Materials

Preliminary experiments of the appropriate pretreated dose of irbesartan are provided in supplemental materials, part 1. Oligonucleotide sequences are provided in supplemental materials, part 2. (Supplementary Materials)

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Research Article

Distribution of Paraoxonase-1 (PON-1) and Lipoprotein Phospholipase A2 (Lp-PLA2) across Lipoprotein Subclasses in Subjects with Type 2 Diabetes

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Paraoxonase-1 (PON1) and lipoprotein phospholipase A2 (Lp-PLA2) may exert an important protective role by preventing the oxidative transformation of high- and low-density lipoproteins (HDL and LDL, respectively). The activity of both enzymes is influenced by lipidome and proteome of the lipoprotein carriers. T2DM typically presents significant changes in the molecular composition of the lipoprotein subclasses. Thus, it becomes relevant to understand the interaction of PON1 and Lp-PLA2 with the subspecies of HDL, LDL, and other lipoproteins in T2DM. Serum levels of PON1-arylesterase and PON1-lactonase and Lp-PLA2 activities and lipoprotein subclasses were measured in 202 nondiabetic subjects (controls) and 92 T2DM outpatients. Arylesterase, but not lactonase or Lp-PLA2 activities, was inversely associated with TD2M after adjusting for potential confounding factors such as age, sex, smoking, body mass index, hypertension, and lipoprotein subclasses (odds ratio = 3.389, 95% confidence interval 1.069–14.756). Marked difference between controls and T2DM subjects emerged from the analyses of the associations of the three enzyme activities and lipoprotein subclasses. Arylesterase was independently related with large HDL-C and small intermediate-density lipoprotein cholesterol (IDL-C) in controls while, along with lactonase, it was related with small low-density lipoprotein cholesterol LDL-C, all IDL-C subspecies, and very low-density lipoprotein cholesterol (VLDL-C) in T2DM ($p < 0.05$ for all). Concerning Lp-PLA2, there were significant relationships with small LDL-C, large IDL-C, and VLDL-C only among T2DM subjects. Our study showed that T2DM subjects have lower levels of PON1-arylesterase compared to controls and that T2DM occurrence may coincide with a shift of PON1 and Lp-PLA2 towards the more proatherogenic lipoprotein subclasses. The possibility of a link between the two observed phenomena requires further investigations.

1. Introduction

Several lines of evidence clearly suggest that oxidative stress (OxS) is implicated in the pathogenesis of type 2 diabetes mellitus (T2DM) and plays a critical role in the development of its frequent microvascular and macrovascular complications [1]. OxS appears to mediate hyperglycemia-induced tissue damage by influencing polyol and the hexosamine pathway, increasing intracellular formation of advanced glycation end-products (AGEs) and their receptors (RAGEs) etc. [2]. OxS is both a downstream and upstream event of these altered processes. As paradigmatic example in this context, AGEs-RAGE interaction is accelerated by reactive oxygen species (ROS) and, in the same time, promotes the formation of these oxidants by inducing mitochondria dysfunction and nicotinamide adenine dinucleotide phosphate oxidase (NOX) activation [1, 2].

Increase in ROS results in the accumulation of oxidative-damaged biomolecules, including the highly proatherogenic oxidized low-density lipoproteins (ox-LDLs) [3, 4]. These modified lipoproteins entail endothelial cell activation, dysfunction, and death and contribute to the onset and progression of the atherosclerotic process [4]. This detrimental action of ox-LDL is antagonized by high-density lipoproteins (HDLs) which are able not only to promote reverse transport of cholesterol but also to act as effective anti-inflammatory and antioxidant agents [5, 6]. A wealth of *in vitro* and *in vivo* evidence suggests that paraoxonase 1 (PON1) and lipoprotein-associated phospholipase A2 (Lp-PLA2) contribute to vasculoprotective function of HDL [7–10]. Both enzymes are able to hydrolyze, by different and still poorly known mechanisms, lipo-lactones, such as those resulting from oxidation of fatty acid or cholesterol-enriching lipid environment of HDL and LDL [7, 9, 11]. It has been suggested that the antioxidant-like function of PON1 and Lp-PLA2 may account for the several findings linking altered levels of enzyme activities and the risk of developing T2DM as well as its related clinical complications [12–17].

HDL and LDL are heterogeneous collection of particles which vary in size, density, lipid composition, proteome, and physiological role [18]. The different biochemical dynamics of lipoprotein subclasses inevitably result in a different affinity between them and accessory proteins, such as Lp-PLA2 and PON1 [19–22]. A limited number of studies addressed the distribution of PON1 and Lp-PLA2 in HDL and LDL subclasses, respectively, and generated inconsistent results [6, 20, 21, 23].

In T2DM, the primary quantitative lipoprotein abnormalities are elevated triglyceride levels and diminished HDL-C levels; concomitantly, the lipoproteins also change in structure, chemical composition, and size [24]. In particular, the proportions of circulating small dense LDL and HDL are increased, while there are fewer particles of the respective large subclasses, leading to a more proatherogenic setting [24]. This qualitative change in lipoprotein might have significant repercussion in stability and activity of Lp-PLA2 and PON1; indeed, it is well known that both are sensitive to their milieu, intended as lipid and proteome that surround the two enzymes [25, 26]. Besides, OxS, high glucose levels, and inflammation have been repeatedly shown to induce modifications in the PON1 structure that, in turn, compromise its biological function [9, 11, 27, 28]. Overall, this convergent evidence makes it relevant to discern the interplay between PON1 and Lp-PLA2 with lipoprotein subclasses in the diabetic state.

To address this still open question, the present population-based study sought to determine the link of T2DM with PON1 and Lp-PLA2 activities and, most importantly, to evaluate whether the disease might affect the distribution of these two enzymes across lipoprotein subclasses.

2. Materials and Methods

2.1. Subjects. The subjects examined in this study were enrolled among men/women attending the metabolic outpatient clinic of Sant'Anna University Hospital (Ferrara, Italy) and outpatients undergoing bone densitometry testing at the Menopause and Osteoporosis Centre of the University of Ferrara [29]. Exclusion criteria for subjects with and without T2DM were infection, acute or chronic disease (affecting liver, kidney, lungs, etc.), dementia, cancer, pregnancy, and alcohol consumption > 10 g daily. The diagnosis of T2DM was made in agreement with American Diabetes Association (ADA) criteria. The whole number of participants was 719 and included 574 nondiabetic subjects (controls) and 145 T2DM subjects.

At the point of study entry, all participants underwent a clinical (questionnaire plus blood pressure), physical (weight, height, and waist circumference), and routine biochemical investigation (plasma lipid profile and glucose) by trained personnel. Standard questionnaire was administered to collect main demographic and clinical data (history of CVD and other complications of DM, smoking, and current medications). Participants were deemed hypertensive when having a mean systolic blood pressure ≥ 140 mmHg and/or mean diastolic blood pressure ≥ 90 mmHg and/or when they were on active antihypertensive treatment. Patients were defined as dyslipidemic, according to the National Cholesterol Education Program Adult Treatment Panel III 2004 [30] guidelines, when total cholesterol ≥ 200 mg/dL and/or LDL-C ≥ 130 mg/dL and/or HDL-C < 40 mg/dL and <50 mg/dL for males and females, respectively, and/or mean triglycerides ≥ 200 mg/dL and/or when on active lipid-lowering treatment (10 and 15% of controls and T2DM patients, respectively).

The whole study conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was conducted accordingly to Good Clinical Practice guidelines. It was approved by the Local Ethics Committee; written informed consent was obtained from each patient and no personal information was available to the authors.

Of note, lipoprotein subclass analysis was carried out in a subsample of 292 subjects (202 controls and 90 T2DM patients) that will be referred with the term Lipoprint throughout the report.

2.2. Biochemical Assays.

Venous blood samples from all study participants were drawn after overnight fasting, and serum was stored at −80°C until analysis.

Arylesterase and lactonase activities of PON1 and Lp-PLA₂ activity in serum were measured by UV-VIS spectrophotometric assays in a 96-well plate format by using a Tecan Infinite M200 microplate reader (Tecan Group Ltd., Switzerland).

Arylesterase activity was measured by using phenylacetate as substrate [31]. A molar extinction coefficient (wavelength = 270 nm) of 1.3×10^3 L^{-1}·mol^{-1}·cm^{-1} was used for the calculation of enzymatic activity, which was expressed in kilo unit per liter. One unit of arylesterase activity accounts for 1 μmol of phenol produced in a minute under the conditions of the assay. Intra-assay CV was 3.8% whereas interassay CV was 9.7% [31].

Lactonase activity was assessed using gamma-thiobutyrolactone (TBL) as substrate, and Ellman's procedure was used to spectrophotometrically monitor the accumulation of free sulfhydryl groups via coupling with 5,5-dithiobis (2-nitrobenzoic acid) [31]. A molar extinction coefficient (wavelength = 410 nm) of 13.6×10^3 L^{-1}·mol^{-1}·cm^{-1} was used for the calculation of enzyme activity that was expressed in unit per liter. The intra-assay CV was 6.1% whereas the interassay CV was 9.8% [32].

Lp-PLA2 was assessed by using 2-thio PAF as substrate, which is hydrolyzed by the enzyme in sn-2 position, and the consequent formation of free thiols was detected by Ellman's procedure. A molar extinction coefficient (wavelength = 410 nm) of 13.6×10^3 L^{-1}·mol^{-1}·cm^{-1} was used for the calculation of enzyme activity, expressed in unit per liter. The intra-assay CV was 4.8% whereas the interassay CV was 10.1% [32].

Serum levels of total cholesterol, triglycerides, HDL-C, LDL-C, and glucose were evaluated by routine laboratory methods.

2.3. Analysis of Lipoprotein Subclasses.

Nine distinct lipoprotein subclasses were assessed in 25 μL of serum by nondenaturated polyacrylamide gel electrophoresis and the Lipoprint system (Lipoprint LDL system and Lipoprint HDL system; Quantimetrix Corporation, Redondo Beach, CA) according to the manufacturer's specifications. The lipoprotein subclasses determined were as follows: very low-density lipoprotein (VLDL), three IDL subclasses (large, medium, and small IDL), two LDL subclasses (small and large), and three HDL subclasses (small, medium, and large). The gels were scanned, and the relative area for each band was measured and adjusted for total cholesterol level. In-depth description of this method is available here [33].

2.4. Statistical Analysis.

Since the distribution of arylesterase, lactonase, and Lp-PLA2 analyzed by Kolmogorov-Smirnov test was skewed, the values were log transformed in order to approximate a normal distribution before being analyzed by parametric tests. Means of the variables examined were compared by using t-test while prevalence of categorical variables was compared by the χ^2 test. Pearson's correlation coefficient was used to evaluate the possible association

Table 1: Main characteristics of controls and T2DM subjects (total sample, $n = 719$).

	T2DM ($n = 145$)	Controls ($n = 574$)	p value
Age (years)	69 ± 11	64 ± 13	<0.001
Gender (women/men)	85/60	419/155	<0.001
BMI (kg/m^2)	33 ± 6	25 ± 5	<0.001
Smoking (% never/ex/current)	39/44/17	66/13/21	<0.001
Glucose (mg/dL)	147 ± 36	96 ± 10	<0.001
Hypertension (%)	85	40	<0.001

Data are expressed as means ± standard deviations for continuous variables and number or percentage within the group for categorical variables. BMI: body mass index.

between variables of interest. This test was followed by partial correlation or multiple regression analysis in order to check the independence of the observed univariate correlations. Multivariable logistic regression analysis was performed to evaluate whether arylesterase was independently related to T2DM. The covariates were included in the models if they were correlated with the dependent variable and/or not collinear with each other. In this test, arylesterase was classified as low if it was within the lower quartile. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. PON1-Arylesterase, PON1-Lactonase, and Lp-PLA2 Activities in Controls and T2DM Patients (Whole Sample, $n = 719$).

The main demographic and clinical characteristics of the subjects enrolled in the present study are summarized in Table 1. Diabetics were significantly older and had higher BMI compared to controls; besides, this group presented higher prevalence of men, smokers, and subjects with hypertension.

Among the three serum enzyme activities assessed in this study, only PON1-arylesterase exhibited a significant change between groups. More specifically, as compared to controls, arylesterase was significantly ($p < 0.001$) decreased by 20% in participants with T2DM (78 ± 25 vs. 99 ± 31 kU/L) (Figure 1(a)). In contrast, either lactonase ($p = 0.220$) or Lp-PLA2 ($p = 0.280$) was not significantly different between the two groups (Figures 1(b) and 1(c)).

In order to check whether the observed decrease in arylesterase activity was influenced by confounding factors of PON1 such as age and gender, we compared this activity between subgroups of 85 controls and 85 diabetics with similar age and prevalence of women/men (Supplementary Table 1). From this analysis, it emerged that the difference in arylesterase was less marked than that found in the whole sample (11%), but still significant ($p = 0.003$).

3.2. Evaluation of the Possible Effect of Lipoprotein Subclasses on the Relationship between Arylesterase and T2DM (Lipoprint Subsample, $n = 292$).

The quantification of lipoprotein subfraction distribution was carried out on a subsample, Lipoprint, including 202 controls and 90 T2DM subjects, in order to (1) identify additional potential
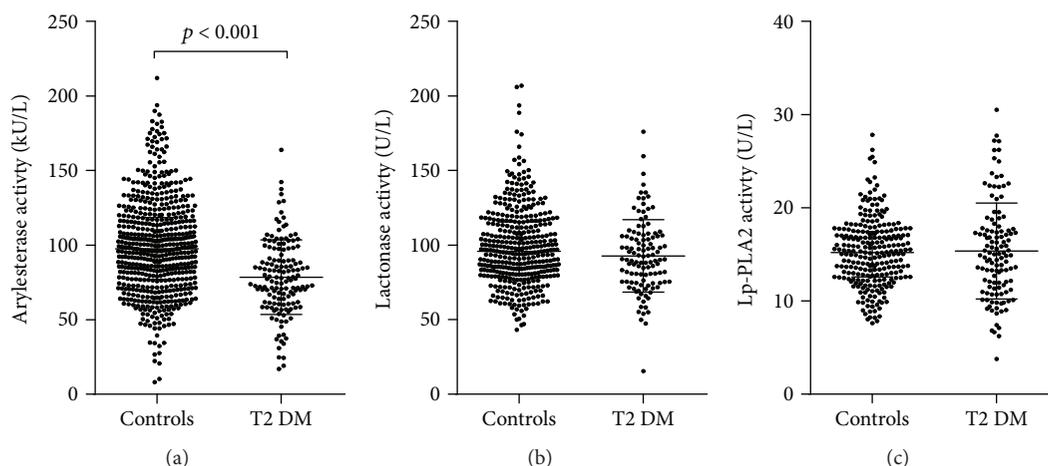
FIGURE 1: Scatter plots displaying PON1-arylesterase (a), PON1-lactonase (b), and Lp-PLA2 (c) activities in controls and T2DM patients.

confounders of the association between arylesterase activity and T2DM and (2) explore the effect of T2DM on the distribution of PON1 activities and Lp-PLA$_2$ on lipoprotein subclasses. Cases and controls included in this subset had similar demographic and clinical characteristics and equal difference pattern of the whole sample (Supplemental Table 2). Arylesterase, lactonase, and Lp-PLA$_2$ activities also followed a similar trend, with only arylesterase showing a significant ($p < 0.001$) decrease in diabetics compared to controls (75 ± 20 vs. 96 ± 31 kU/L) (Supplementary Table 2).

With regard to lipid and lipoprotein subfraction profile, T2DM patients exhibited the typical atherogenic profile (Table 2). Indeed, they had higher levels of triglycerides and lower levels of HDL-C compared to the controls ($p < 0.001$), with the latter group presenting however an increase in total cholesterol and LDL ($p < 0.001$ vs. T2DM). Moreover, diabetics showed higher levels of proatherogenic small LDL-C ($p < 0.001$), lower levels of large LDL ($p < 0.001$), and lower LDL size ($p < 0.01$). Both large HDL-C and small HDL-C were decreased in diabetics ($p < 0.001$); regarding the relative percentages, large HDL-C decreased and small HDL-C increased in T2DM compared to controls ($p < 0.001$ for all). The other changes are as follows: (1) large IDL-C levels were higher in TD2M patients compared to controls while small IDL-C followed an opposite trend and (2) VLDL-C levels were higher in T2DM subjects ($p < 0.001$).

The above results along with the reports showing that PON1 reside in various lipoprotein subclasses [20, 23] prompted us to consider their relative serum concentration as possible confounders of the observed relationship between arylesterase and T2DM. To address this hypothesis, we first examined the association between this PON1 activity and lipoprotein subclasses in the whole population sample (Table 3). We found that serum levels of the enzyme activity were positively related with large HDL-C as expressed in concentration and percentage ($p < 0.001$ for both), medium and small HDL-C ($p < 0.001$ for both), large LDL-C ($p < 0.05$), mean LDL size ($p < 0.001$), and small IDL-C ($p < 0.001$); it was negatively related with the percentage

TABLE 2: Lipid profile and distribution of lipoprotein subfractions in controls and T2DM subjects included in lipoprotein subsample.

	T2DM ($n = 90$)	Controls ($n = 202$)	p value
Total cholesterol (mg/dL)	188 ± 47	208 ± 32	<0.001
LDL-C (mg/dL)	114 ± 43	138 ± 30	<0.001
HDL-C (mg/dL)	34 ± 10	52 ± 9	<0.001
Triglycerides (mg/dL)	206 ± 91	88 ± 41	<0.001
HDL subfractions			
Large HDL-C (%)	25 ± 7	33 ± 7	<0.001
Medium HDL-C (%)	47 ± 3	45 ± 4	0.001
Small HDL-C (%)	28 ± 6	21 ± 5	<0.001
Large HDL-C (mg/dL)	9 ± 4	18 ± 6	<0.001
Medium HDL-C (mg/dL)	16 ± 5	23 ± 4	<0.001
Small HDL-C (mg/dL)	9 ± 3	11 ± 3	<0.001
LDL subfractions			
Large LDL-C (mg/dL)	51 ± 19	66 ± 20	<0.001
Small LDL-C (mg/dL)	17 ± 12	10 ± 10	<0.001
Mean LDL-C particle size (Å)	262 ± 5	268 ± 4	<0.01
IDL subfractions			
Large IDL-C (mg/dL)	14 ± 5	12 ± 4	<0.001
Medium IDL-C (mg/dL)	15 ± 7	15 ± 6	0.200
Small IDL-C (mg/dL)	12 ± 6	17 ± 5	<0.001
VLDL-C (mg/dL)	45 ± 13	34 ± 8	<0.001

Data are expressed as means ± standard deviations. HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low-density lipoprotein cholesterol; IDL-C: intermediate-density lipoprotein cholesterol.

of small HDL-C ($p < 0.001$) and that of small LDL-C ($p < 0.05$). Regarding the conventional lipid profile, arylesterase was associated positively with HDL-C ($p < 0.001$) and LDL-C ($p < 0.05$) and negatively with triglycerides ($p < 0.001$). Among the aforementioned correlations, only those involving large and medium HDL-C, LDL size, and small IDL-C remained significant after adjustment for

Table 3: Simple (r) and partial ($r_{partial}$) Pearson's correlation coefficients of the relationship between PON1-arylesterase and serum lipids and lipoprotein subfractions.

	Pearson's correlation coefficient (r)	Partial Pearson's correlation coefficient# ($r_{partial}$)
Total cholesterol	0.187	0.074
HDL-C	0.394**	*0.239**￼*
LDL-C	0.194*	0.063
Triglycerides	−0.275**	−0.100
Large HDL-C (%)	0.268**	0.077
Medium HDL-C (%)	−0.141	0.014
Small HDL-C (%)	−0.254**	−0.111
Large HDL-C (mg/dL)	0.381**	*0.193*￼*
Medium HDL-C (mg/dL)	0.376**	*0.234**￼*
Small HDL-C (mg/dL)	0.129*	0.065
Large LDL-C	0.209*	0.050
Small LDL-C	−0.175*	0.092
Mean LDL particle size	0.295**	*0.182*￼*
Large IDL-C	0.071	0.053
Medium IDL-C	0.114	0.110
Small IDL-C	0.375**	*0.268**￼*
VLDL-C	−0.061	0.050

*$p < 0.05$; **$p < 0.001$; significant partial correlation coefficients are highlighted in italics; #covariates: age, sex, smoking, hypertension, and BMI. HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL; very low-density lipoprotein cholesterol; IDL-C, intermediate-density lipoprotein cholesterol.

age, gender, BMI, hypertension, and smoking (scatter plots of these associations were displayed in Supplementary Figure 1).

We next performed multivariable logistic regression to evaluate whether the independent correlates of arylesterase that emerged from the previous analysis could influence the association between this activity and T2DM (Figure 2). This analysis showed that low arylesterase (i.e., activity level in the lower quartile) confirmed the inverse association between this activity and T2DM that emerged from between-group comparison (Figure 1(a)). It also showed that while covariates such as age, gender, BMI, hypertension, smoking, and HDL-C did not markedly affect the association, the further inclusion of large HDL-C, medium HDL-C, LDL size, and small IDL-C led to a drastic decrease in the odds ratio (nonadjusted model, OR = 8.561, 95% CI 3.322–22.112; fully adjusted model, OR = 3.389, 95% CI 1.069–14.756).

3.3. Associations of Arylesterase, Lactonase, or Lp-PLA$_2$ with Lipoprotein Subclasses in Controls and T2DM Patients (Lipoprint Subsample, n = 292). To investigate the possible effect of T2DM on the distribution of PON1-arylesterase, PON1-lactonase and Lp-PLA2 across lipoprotein subclasses, we measured the correlation between these variables separately in both controls and T2DM subjects Table 4. For the sake of simplicity, this paragraph will only deal with those

associations that remained significant after controlling for potential confounders (highlighted in bold in the table). Arylesterase was independently associated with HDL-C, large and medium HDL-C, mean LDL size, and small IDL-C ($p < 0.05$ after adjustment for all). The pattern changed among DM patients, where the correlations persisted for total cholesterol and HDL-C ($p < 0.05$ for both), small LDL-C ($p < 0.05$), all three IDL-C subclasses ($p < 0.01$ for all), and VDL-C ($p < 0.01$). Lactonase activity of PON1 was not correlated with any lipid/lipoprotein variables in controls but did correlate with mean LDL size ($p < 0.05$), large and medium IDL-C ($p < 0.001$ and $p < 0.05$, respectively), and VLDL-C ($p < 0.01$). Finally, Lp-PLA$_2$ was weakly associated with total cholesterol and LDL-C ($p < 0.05$) in controls, with these two correlations becoming stronger ($p < 0.01$) in T2DM patients. Besides, within this group, the enzyme activity was also associated with small LDL-C ($p < 0.01$), mean LDL size ($p < 0.05$), large IDL-C ($p < 0.01$), and VLDL-C ($p < 0.05$).

4. Discussion

In overall agreement with previous reports [13, 14, 23], our study showed that PON1-arylesterase, but not PON1-lactonase or Lp-PLA2, activity was decreased in T2DM patients compared to controls. Of note, the observed difference between groups remained significant after adjustment for potential confounding factors, including lipoprotein subclasses. The subsequent analysis of the association between the enzyme activities and lipoprotein subclasses revealed that (1) Lp-pLA2 was associated with small LDL-C, large IDL-C, and VLDL-C in the T2DM group but not in controls and (2) arylesterase was associated only with large/medium HDL-C in controls and only with some of the more proatherogenic ApoB lipoproteins in T2DM subjects.

There is general consensus that the cholesterol component does not completely capture the vascular protective effect of HDL, which is indeed beyond its role in blood lipid transport [6, 34]. Other aspects of HDL functionality include the ability to contrast OxS and exacerbated inflammatory responses of immune cells involved in atherosclerotic processes [34–36]. PON1 has been widely shown to be one of the major contributors of HDL ability to contrast the oxidative challenges against their carrier, LDLs, macrophages, and endothelial cells [37, 38]. Lp-PLA2 is also reported to be associated with lipoproteins (in particular LDL) and to play a role in redox processes occurring in blood vessels [21, 39]. The hypothesized catalytic mechanism strictly recalls that of PON1; it can hydrolyze biomolecules resembling platelet-activating factors (PAF) (its natural substrate) such as phospholipids (PLPs) or containing oxidized fatty acyl groups [9]. Despite the abundant lines of experimental and clinical evidence on these two enzymes, there is still a high degree of vagueness regarding the various aspects of their biochemistry, physiological role, and impact on individual health [9, 40, 41].

One of the few certainties in this confusing landscape is the intimate interaction between the lipoprotein environment surrounding PON1 or Lp-PLA$_2$ and their activities
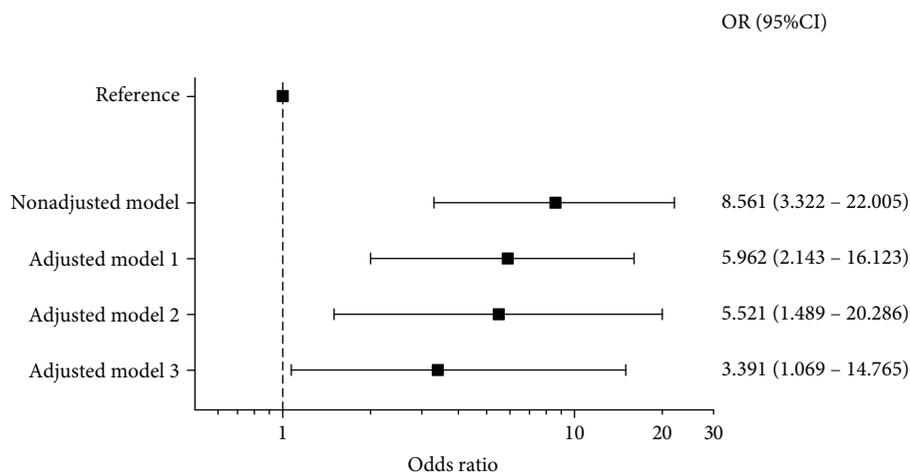
FIGURE 2: Box plots displaying unadjusted and multiadjusted odds ratio (95% confidence interval) for the association of a low PON1-arylesterase (activity level in the lower quartile) activity and T2DM model covariates: adjusted model 1: age, sex, smoking, body mass index, and hypertension; adjusted model 2: age, sex, smoking, body mass index, hypertension, and HDL-C; adjusted model 3: age, sex, smoking, body mass index, hypertension, large HDL-C, small HDL-C, LDL-particle size, and small IDL-C.

TABLE 4: Pearson's correlation coefficients of the relationship of arylesterase, lactonase, or Lp-PLA2 with serum lipids or lipoprotein subfractions in controls and in type 2 DM subjects.

| Lipoproteins | Controls | | | Type 2 diabetes | | |
	Arylesterase r	Lactonase r	Lp-PLA2 r	Arylesterase r	Lactonase r	Lp-PLA2 r
Total cholesterol	0.172*	0.201*	*0.265**	*0.315*	0.302**	*0.411*
HDL-C	*0.404**	0.159*	−0.100	*0.464**	0.168	−0.060
LDL-C	0.174*	0.154*	*0.232**	0.063	0.233*	*0.388**
Triglycerides	−0.169*	−0.009	0.183*	−0.246*	0.212*	0.189
Large HDL-C (%)	0.192*	0.011	0.108	−0.046	−0.064	−0.141
Medium HDL-C (%)	−0.094	−0.019	−0.096	0.013	−0.024	−0.021
Small HDL-C (%)	−0.195*	0.002	−0.045	0.073	0.100	0.151
Large HDL-C (mg/dL)	*0.257*	−0.068	0.077	0.124	0.067	−0.134
Medium HDL-C (mg/dL)	*0.223*	0.128	−0.011	0.245*	0.187	−0.124
Small HDL-C (mg/dL)	−0.163	0.108	0.007	0.226*	0.215*	−0.046
Large LDL-C	−0.041	0.146	0.312**	0.143	0.113	0.104
Small LDL-C	−0.196*	−0.044	0.117	*0.223*	0.263*	*0.314**
Mean LDL particle size	*0.244*	0.074	−0.059	0.079	−0.191	*−0.280**
Large IDL-C	0.098	0.097	0.051	*0.285*	*0.400**	*0.314*
Medium IDL-C	0.037	0.097	−0.004	*0.367**	*0.314*	0.205
Small IDL-C	*0.274**	0.188*	−0.018	*0.309*	0.077	0.056
VLDL-C	−0.008	0.005	−0.086	*0.288**	*0.338**	*0.283**

*$p < 0.05$; **$p < 0.001$; significant partial correlation coefficients (covariates: age, sex, smoking, hypertension, and BMI) are highlighted in italics. HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL: very low-density lipoprotein cholesterol; IDL-C: intermediate-density lipoprotein cholesterol. Lactonase was measured in 152/202 controls; Lp-PLA2 was measured in 111/202 controls.

[9, 27, 42]; these two accessory proteins are anchored to lipids and some apolipoproteins which also coordinate and modulate their catalytic activity [43]. Recent improvements in separation techniques have highlighted that PON1 and Lp-PLA$_2$ have a preferential, but not exclusive, distribution within HDL or LDL subclasses [20, 21, 23, 26, 44]. This may depend on the "broad" affinity of the enzymes with various apolipoproteins and lipid subspecies that may reach the most suitable combination in the small HDL$_3$ in the case of PON1 and small LDL for Lp-PLA$_2$. It was also demonstrated that when the composition and spatial location of phospholipid moiety or apolipoproteins such as APO A1 and A2 and Apo E change (as during HDL maturation), also the distribution of PON1 across HDL subclasses

varies as well [21, 22, 39]. This "flux" of PON1 was observed to occur during HDL maturation, but it might be a phenomenon also associated with diseases typically featuring quantitative and qualitative abnormalities of lipid/apolipoprotein profiles, such as T2DM [12, 45]. In the present study, we focused on T2DM, not only because of the association with the aforementioned qualitative change in lipoproteins but also for the cumulating reports showing a disease-related alteration in PON1 and Lp-PLA$_2$ activities [13, 21, 23].

To the best of our knowledge, this is the first study that evaluated whether the inverse association between arylesterase and T2DM is independent from such large spectrum of lipoprotein subclasses. Potential statistical interference of HDL subclasses on this association has been recently evaluated by Dullaart et al. [23] where the inverse relationship between PON1 and T2DM was modestly attenuated when the level of large HDL particles was included in the multivariable analysis. Our finding is overall consistent with the work of Dullaart et al., with a meaningful difference: the strength of the association largely decreased upon controlling for large and medium HDL-C and small IDL-C (from OR = 5.522, 95% CI 1.489–20.426 to OR = 3.389, 95% CI 1.069–14.756), suggesting that the distribution of PON1 in lipoprotein subclasses can, in part, explain the observed relationship. In addition, the data regarding arylesterase and lactonase were highly discrepant but this outcome was not either surprising or unprecedented [36]. Arylesterase, although referred as one of the two promiscuous activities of PON1 (the other is paraoxonase), is more frequently measured in epidemiological studies compared to the putative physiological activity, lactonase [28, 46, 47]. Arylesterase is minimally influenced by some prevalent PON1 genetic polymorphisms, discloses low interindividual variability, and is regarded as a better surrogate of PON1 concentration than the other two activities [47]. Furthermore, regarding the lack of significant association between Lp-PLA2 and T2DM, data on the association with T2DM and related CV complications are highly variable and divergent [16, 17, 21], and besides, it is still not clear whether high/low levels of Lp-PLA2 are beneficial or detrimental for human health [32, 48]. Some authors suggest that the possible explanation of this recurrent paradox may be related to the distribution of Lp-PLA2 among lipoprotein classes and subclasses [49]. In particular, Lp-PLA2 might be anti-inflammatory when is complexed with HDL, whereas it is proinflammatory (the hydrolysis of oxidized lipids generates the cytotoxic lysophosphatidylcholine [9]) when it resides in ApoB-containing lipoproteins [9, 32, 50].

In order to address the possibility of influence of the diabetic state over the distribution of Lp-PLA2 and PON1 activities across lipoprotein subspecies, we assessed the simple and adjusted correlation coefficients separately in controls and T2DM subjects. The finding that Lp-PLA2 was associated to the small LDL-C, one of the most important risk factors of CVD, is consistent with some studies on isolated lipoproteins showing that in healthy people [51, 52] and, mostly, in diabetics [21], the enzyme is preferentially located in this LDL subclass. Failure in detecting a significant correlation of Lp-PLA2 with small LDL-C in controls may be the result of the low levels of this subclass in this group (Table 2). Alternatively, it can also be hypothesized that ApoB lipoproteins may be enriched in active Lp-PLA2 in T2DM patients and this could reflect in a further selective increase in proatherosclerotic potentials of these particles. In turn, this change might contribute to the excess risk for CVD in people with diabetes.

Regarding PON1, the disappearance and appearance of association with large/medium HDL-C and the more proatherogenic ApoB lipoproteins, respectively, in T2DM csubjects may have two, not necessarily antithetic, explanations.

First, the phenomenon could merely depend on the change in lipoprotein profile. Within this assumption, the lack of correlation between arylesterase and large/medium HDL-C in the T2DM group may be the result of the marked decrease in the concentration of these subclasses (Table 2) and, thus, of the fraction of PON1 complexed with these particles. On the other hand, the exclusive association of arylesterase and lactonase with large/medium IDL, small LDL, and VLDL among T2DM patients might simply reflect the concomitant increase in the levels of these lipoproteins. As a proof of concept, PON1 has been detected within VLDL, VLDL remnants (i.e., large IDL), and small LDL in healthy individuals [19], but not in a sufficient amount to significantly contribute in arylesterase/lactonase total serum activities.

It can be also speculated that the redistribution of PON1 among HDL subclasses is caused by the disease itself. In T2DM patients, PON1 is still bound in HDL, as suggested by the strong correlation between arylesterase and total HDL-C, but most likely, it is more evenly distributed among the subspecies of this lipoprotein compared to controls. Besides, it has been reported that glycation and oxidation of HDL or directly of PON1 occurring in T2DM may cause the detachment of the enzyme from its host and, as consequence, affect its activity [53].

Some important limitations of the study need to be underpinned. Firstly, the procedure for assessing the distribution of lipoprotein measures the cholesterol amount associated to each subfraction subclasses, but not the particle number. Notwithstanding this limitation, Quantimetrix Lipoprint has been consistently described to afford a reliable quantitative determination of LDL/HDL/IDL subfractions [33, 54]; as proof of concept, the observed distribution of lipoprotein subclasses in our population sample is comparable with that reported by a number of studies including those dealing with particle quantification. Secondly, we were not able to measure the real fraction of PON1 or Lp-PLA2 activity in each lipoprotein subclass. However, with some exceptions [6, 20], our results on nondiabetic subjects are in overall concordance with other studies dealing with the detection of enzyme activity/mass in isolated subfractions [12, 19, 23, 55]. Thirdly, we cannot exclude that other confounders besides those considered in the multivariable analyses could bias our results. This is the case of putative modulators of PON1 activity such as hormonal therapies (e.g., oral contraceptives), nutrient components (in particular, vitamins E and C), drugs

(e.g., statins) or still not precisely identified environmental pollutants. Thirdly, the cross-sectional design did not allow to define a cause-effect relationship between enzyme activities, lipoprotein subclasses, and TD2M.

5. Conclusion

In conclusion, the present community-based population study showed that PON1-arylesterase activity is inversely associated with T2DM. Notably, we found that this relationship was independent of several confounding factors, including the lipoprotein subclasses that may carry PON1 itself. Our study also showed that the occurrence of T2DM could coincide with a shift of PON1 and Lp-PLA2 towards the more proatherogenic lipoprotein subclasses. The existence of a possible cause-effect link between decreased PON1 activities and its redistribution across lipoprotein subclasses required further investigations.

Data Availability

The data (included in an excel database) used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Acknowledgments

Supplementary Materials

Supplementary Figure 1: (figure legend) scatter plots displaying the correlation between Log10 PON1-arylesterase activity and HDL-C (A), large HDL-C (B), medium HDL-C (C), mean LDL particle size (D), and small IDL-C (E). Supplementary Table 1: main characteristics and serum arylesterase and lactonase activities of PON1, in controls and type 2 DM subjects with similar age and gender distribution. Supplementary Table 2: main characteristics and serum levels of PON1-arylesterase, PON1-lactonase, and Lp-PLA2 activities of controls and T2DM subjects (Lipoprint subsample, $n = 292$). (Supplementary Materials)

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Review Article

Oxidative Stress, Maternal Diabetes, and Autism Spectrum Disorders

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Autism spectrum disorders (ASD) are a group of early-onset neurodevelopmental conditions characterized by alterations in brain connectivity with cascading effects on neuropsychological functions. To date, in the framework of an increasing interest about environmental conditions which could interact with genetic factors in ASD pathogenesis, many authors have stressed that changes in the intrauterine environment at different stages of pregnancy, such as those linked to maternal metabolic pathologies, may lead to long-term conditions in the newborn. In particular, a growing number of epidemiological studies have highlighted the role of obesity and maternal diabetes as a risk factor for developing both somatic and psychiatric disorders in humans, including ASD. While literature still fails in identifying specific etiopathological mechanisms, a growing body of evidence is available about the presence of a relationship between maternal immune dysregulation, inflammation, oxidative stress, and the development of ASD in the offspring. In this framework, results from high-fat diet animal models about the role played by oxidative stress in shaping offspring neurodevelopment may help in clarifying the pathways through which maternal metabolic conditions are linked with ASD. The aim of this review is to provide an overview of literature about the effects of early life insults linked to oxidative stress which may be involved in ASD etiopathogenesis and how this relationship can be explained in biological terms.

1. Introduction

Brain development in a fetus and in the first years of life is pivotal in the shaping of the individual overall neuropsychological performance level [1–3]. Any alteration of the intrauterine environment at different stages of pregnancy, such as maternal metabolic pathologies, may lead to long-term condition in the newborn.

Given the above consideration, it should be noted how the rates of obesity and diabetes have experienced a steep increase in several countries, with a correspondent increasing number of studies in literature devoted to this topic [4]. There is a rich literature regarding the effect of excessive weight before pregnancy, particularly in combination with rapid weight gain during pregnancy: a condition generally described as a risk factor for gestational diabetes (GDM), which is glucose intolerance with the onset or first recognition during pregnancy. Up to 15% of pregnant women worldwide are esteemed to be affected from diabetes, and

approximately 87.5% of maternal diabetes are GDM. Only 7.5% are preexisting type 1 diabetes [5]. As such, maternal diabetes (and GDM in particular) is an important risk factor for conditions like, but not limited to, miscarriage, macrosomia [6], and neurodevelopmental impairments in the offspring [7–9]. In particular, several studies suggest that the offspring of GDM mothers presents more often language delay, poor motor development, and impaired recognition memory [10–12]. A more specific group of studies focused on the possible link between autism spectrum disorder (ASD) risk in the offspring of GDM mothers [13–18].

ASD is a neurodevelopmental condition characterized by a deficit in social interaction, communication issues, and repetitive, stereotyped behaviors [19–21]. ASD etiopathogenesis is still unclear [22]. However, good evidence for genetic correlates is available: specific genetic mutations can be, in fact, identified in about 20% of ASD cases, and twin studies estimate a heritability between 64 and 91% [23]. This strong genetic influence but the concomitant lack

of full concordance in monozygotic twins points to the relevance of environmental factors in the etiopathogenesis of the disorder [24, 25]. Although studies about ASD are focused mainly on children, the presence of this condition is particularly relevant also in adulthood, due to its quite extensive comorbidity with other psychiatric disorders. The presence of undetected ASD among seeking-treatment inpatients is clearly a subject of clinical relevance, highlighting a need for careful investigation of autistic symptoms both in clinical samples and in the general population. A growing body of studies in fact has shown that also the presence of autistic traits (that is, the presence of autism spectrum symptomatology not necessarily in its full-blown clinical presentation) may not only have an impact on increasing the severity of other mental conditions but also be considered as a risk factor for developing other disorders or toward suicidality [26, 27]. Despite that, autistic symptoms often remain undetected in clinical settings, especially in subjects with little cognitive impairments and moderate symptoms, hided by the manifestations of other comorbid disorders [28–31].

The aim of this paper is to review evidence from literature, regarding whether the effects of early life insults and that linked to oxidative stress in particular might be involved in short- and long-term risks for ASD and how this risk can be explained in biological terms.

2. Obesity, GDM, and Neurodevelopment: Toward a Comprehensive Model

A good number of studies on rodent or nonhuman primate models focused on the effects of maternal high-fat diet in the offspring. A regime of high-fat diet might vary depending on the studied specimen (mice, rats, and primates) and also on the specific experimental model. As a result, there is a certain degree of variation among these studies. In order to provide a general picture, a common high-fat chow for murine models ("HF"; Research Diets, D12492) provides 60% kcal from fat [32], while primate high-fat food (Test Diet; 5A1F; Purina Mills) provides 32% of calories from fat, and this lab food might be supplemented with calorically dense treats [33]. As reviewed by Sullivan et al. [34], researches on animal models proved that maternal high-fat diet impacts offspring behavior, socialization, and cognition; moreover, it affects reward pathways. Given this data, several studies focused on the biological pathways on such effects, although with controversial results. Most studies on behavioral effects on the offspring of maternal diet have been conducted in rodents. High-fat diet has been shown influencing offspring behavior by modifying maternal care: in particular, by leading to an increased nursing behavior [32, 35]. It has been suggested [34] that this feature might lead to hyperphagia, hypothalamic reprogramming of energy balance-regulating pathways, and—as a result—increased body weight in the offspring. Also, findings from another rodent model show that consumption of high-energy diet in mothers disrupted hippocampal function and thus impaired learning and

memory performance [36]. Moreover, maternal high-fat diet has been associated with heightened anxiety in both nonhuman primates and rodents [33, 37]. According to another study, maternal diet rich in polyunsaturated fatty acid produced a highly aggressive offspring, which displayed hyperlocomotion and decreased immobility in a swim test, due probably to an observed protein kinase C downregulation (in the whole brain except the hypothalamus) [38]. High-fat diet is also correlated with alteration in development of cognitive functions. Male offspring from rats exposed to a diet high in saturated or trans fats, once adults, display a deficit in cognitive spatial functioning [37]. Moreover, offspring from obese mothers show a decrease in hippocampal brain-derived neurotrophic factor production (BDNF) which causes an alteration in neurodevelopment for this area [39]. It is also interesting to note how maternal high-fat diet leads to an alteration in reward-based behavior such as food preference in the offspring, as many studies remark [40–43].

One of the most widely reported data about this topic [34] is the fact that high-fat diet consumption seems to expose the offspring to an increase in inflammatory cytokines, which interact with neural development; despite that, only few studies addressed how effects of inflammatory dysregulation can be modulated by the timing and the duration of exposition to such a diet [37].

Sullivan et al. [44] outline two main pathways by which high-fat diet consumption and obesity influence offspring neurodevelopment and subsequently behavior in animal models. Both immune and (neuro-)endocrine systems should be considered affected by this condition. Inflammation plays a clear role, as obesity is associated with elevated inflammatory cytokine production, due to the increase in adipose tissue, to the point that it has been considered as a state of chronic inflammation [45]. Inflammation exposure during gestation is not only associated with perinatal conditions such as premature birth and low birth weight but also associated with neurodevelopmental disorders like ADHD, ASD, and schizophrenia [46–49]. Inflammatory mediators are able to cross the blood-placenta barrier and interact with fetal neurodevelopment. While evidence from rodent models shows that the proinflammatory cytokine interleukin 6 impacts genes for cortical expression [50], it is known that maternal high-fat diet causes a raise of inflammatory markers, with microglial activation and protoinflammatory cytokines in the offspring hippocampus [44].

However, maternal obesity does expose the fetus not only to proinflammatory cytokines but also to an environment where nutrients and neuroendocrine agents like fatty acids, glucose, triglycerides, and leptin are higher. The most immediate effect of this is fetal hyperglycemia. Since glucose can cross the blood-placenta barrier but maternal insulin cannot, the fetus secretes its own insulin, which is also a growth factor involved in brain development. It has been thus hypothesized that hyperinsulinemia in the prenatal period might lead to an alteration in brain development and regulation [51].

Not only insulin but also leptin shows an increased level in obese and diabetic mothers [52]. Leptin receptors are

distributed—in humans—among several brain regions which play a central role in behavioral regulation such as the cortex, hippocampus, amygdala, thalamus, and hypothalamus. While linked to inflammatory response [53], leptin is also active in the hypothalamic pituitary adrenal (HPA) axis, a pivotal structure for stress response [44, 54]. As a result, it is interesting to note that higher levels of leptin were detected in ASD children compared to healthy, age-matched controls (Ashwood et al., 2008; [55]).

Leptin leads to consider the role of the HPA axis and its effect on behavior. The HPA axis is responsible for corticotropin-releasing hormone (CRH), and CRH and arginine vasopressin are synthetized in the paraventricular nucleus of the hypothalamus in response to stress. While maternal diet and extension obesity impact both the HPA axis and extrahypothalamic CRH neurons, the interaction of this pathway with neurodevelopment should be addressed [44]. In rat models, high-fat diet in midpregnancy mothers leads to an offspring with increased postnatal basal corticosterone levels in association with reduction of hippocampal and hypothalamic phospholipid-derived arachidonic acid [56]. The Sasaki et al. [57] rat model showed how high-fat diet consumption during pregnancy and lactation led to an offspring with decreased basal corticosterone levels but heightened response to stress with a slower restoration of baseline corticosterone. This offspring also showed an increase in glucocorticoid receptors in the amygdala, with a concurrent alteration in inflammatory gene expression for the hippocampus and amygdala.

In both human and rats, serotonin plays a crucial role for emotional regulation [44]. Inflammation leads to alteration in serotonin regulation, as the animal model described by Ishikawa et al. [58] suggests by showing how rats treated with cytokine-interferon alpha have decreased serotonergic axon density in the amygdala and the ventral medial prefrontal cortex. Offspring from mothers fed a high-fat diet have been reported displaying alteration in the hippocampus, including not only increased 5-HT1A receptors in the ventral hippocampus but also increased brain-derived neurotrophic factor in the dorsal hippocampus (Peleg-Raibstein et al., 2012). A nonhuman primate model showed that maternal high-fat diet consumption impaired the development of the serotonergic system, leading to a reduction of serotonin synthesis and increased anxiety behaviors in the female offspring [33]. This model also shows increased inflammation levels in the hypothalamus among mothers and offspring [33, 44].

Fewer studies investigated also dopamine pathways. A decrease in mesocorticolimbic dopamine sensitivity, with a concurrent decrease in locomotor activation in response to psychostimulant administration, has been found in rat offspring from mothers who have been fed with a high-fat diet during late gestation and lactation [59]. Another study stressed the involvement of genetic expression in this process, showing in a rat that maternal high-fat diet-induced obesity led to dopamine dysregulation in the offspring by the means of genome-wide methylation and gene regulation for dopamine reuptake trasporter, the μ-opioid receptor and preproenkephalin [60].

3. Maternal Metabolic Conditions and ASD

3.1. Oxidative Stress from GDM. As Rossignol and Frye [61] have demonstrated in a large systematic review, strong evidence is available in literature on the links between immune dysregulation, inflammation, oxidative stress, and the etiopathogenesis of autism. The above-considered role of oxidative stress in high-fat diet animal models sheds light, through different pathways, on the widely observed correlation in human population between GDM and poor performance of the offspring on standardized IQ tests and motor development assessments (Rizzo et al., 1997; Ratzon et al., 2000), as well as on abnormalities in the limbic system detected in ASD children samples [18].

To date, many epidemiological studies have proven obesity being a risk factor for development of neuropsychiatric disorders in humans; however, no clear causative link has been yet identified [62].

From an epidemiological point of view, the concomitant rise in prevalence of both obesity and neuropsychiatric disorders has been stressed in literature [63, 64], but it is important to remember how the increase of the latter disorder might be explained with the advancement of diagnostic tools and an improved awareness toward this kind of conditions [65].

In a study on 1004 mothers, diabetes and hypertension were more common among mothers of children affected by ASD and among mothers of children affected by developmental delays (DD) without ASD. Diabetes, in particular, was strongly associated with greater deficits in expressive language in children with ASD and to a less extent to impairment in visual reception, adaptive behavior, motor skills, and receptive/expressive language, as measured by the Social Communication Questionnaire, the Mullen Scales of Early Learning, and the Vineland Adaptive Behavior Scales [18].

Another study with longitudinal design, conducted in a population of 308 mothers with singleton pregnancies, reported different results. Among this population, normal weight women (128) have been divided from overweight (58), obese (52), and GDM ones (76). Among the infants, assessed at 6 and 18 months with the Bayley Scales of Infant Development-III (BSID-III), those born from mothers with pregestational obesity had significantly higher scores in both cognitive and language developments at 6 months of age; however, the authors observe that their absolute numbers are quite low thus suggesting a careful generalization [66].

A study on 2734 mother-child pairs, with an average of 6-year follow-up, found that mothers of children with ASD were significantly more often older and affected by GDM than the mothers of children typically developing (TD). Also, mothers of children with ADHD were more likely to be of lower education, to be obese, and to have used alcohol during pregnancy than the mothers of TD children. It should be noted that since intellectual disabilities (ID) but not other DD showed a pattern of risk increasing with obesity and GDM, the author suggests that ASD with ID may be etiologically distinct from ASD without ID [67].

A large study ($N = 165311$) of mother-child pairs, comprising 17,988 diabetic mothers, shows that there is a

significant prevalence, even though the odds ratio is rather low, for ID in children of mothers with diabetes. It is noteworthy that this risk factor is independent of other maternal features like tobacco smoke, ethnicity and race, educational level, birth weight, offspring gender, and hypertension. The authors point out that the link between diabetes in mothers and ID should be identified in maternal and fetal inflammation processes, which poses a risk for abnormal fetal brain development [68]. Furthermore, Wang et al. (2015) found that hypertension related more than pregnancy diabetes with ID in a large ($N = 123{,}922$) sample of mother-child couples. Huang et al. [69] meta-analysis on this topic shows that maternal diabetes (OR 1.15, $p < 0.0001$) and maternal hypertension, preeclampsia, or eclampsia (OR 1.33, $p < 0.0001$) act as a risk factor toward ID, thus strengthening the hypothesis that oxidative stress- and obesity-caused inflammation can disrupt neurodevelopment.

A similar meta-analysis, this time focusing on ASD, shows that maternal diabetes acts as a risk factor (OR 1.48) without significant heterogeneity ($I^2 = 9.1$, $p = 0.35$) (Xu et al., 2015). *In utero* exposure to inflammatory factors and hyperglycemia, exempli gratia, could be linked to an increase of free radical production and an impairment of antioxidant countermeasures, through raising the oxidative stress in the cord blood and the placental tissue [70, 71]. These data are even more interesting in the light of studies that take into account the association between maternal autoimmune diseases and subsequent diagnosis of autism in children. A large body of data has been collected on how a family history of autoimmune disorders has been reported more commonly among ASD children than in healthy controls [72, 73]. The Croen et al. [14] case study on 407 couples of mother-children with ASD and 2095 controls has found that maternal autoimmune conditions were significantly associated with ASD in children and asthma in particular. This is coherent with the reported data about midgestation infections by the influenza virus as a risk factor for autism [74, 75], and since the influenza virus cannot cross the placenta, the focus shifts from viral components to the maternal immune response [76–78].

3.2. Broadening the Perspective. As above stated, several contributions point out a relationship among GDM, preeclampsia, autoimmune activation, and, in a broader perspective, oxidative stress and inflammation in mothers of ASD children. However, it is clear from these data that disruption to the normal homeostasis of placental environment may increase the risk but not determine psychiatric disorders; thus, the outcome depends also on the other contextual factors which might be involved in the causative algorithm [79]. Maternal inflammatory states and GDM have the peak of their influence during the central part of the gestation [80], when maternal immune activation is also at full efficiency. According to [79], GDM interaction with maternal immune activation might then disrupt the *in utero* environment thus affecting fetal neurodevelopment, as suggested by the role played by interleukin 6 in several animal models [81–83].

Given the role for inflammatory response, oxidative stress and neurodevelopment disruption, it is also useful to point out that eclampsia and also preeclampsia have been considered a risk factor for ASD in many studies. Gardener et al. [84] extensive meta-analysis shows how preeclampsia has been reported being a significant risk factor for ASD, even if the effect size from the various studies is not always consistent. According to Walker et al. [85], preeclampsia is able to affect fetal neurodevelopment by causing an abnormal trophoblast differentiation during embryogenesis and by limiting fetus intake of nutrient and oxygen. This condition primes the syncytiotrophoblast to release proteins into the maternal bloodstream in an attempt to improve circulation. As a side effect, this release might rise baseline systemic inflammation, insulin resistance, and vascular endothelial changes in the mother. The oxidative stress progressive rise is of particular interest, as it could be integrated with the two-stage model of preeclampsia [85, 86]. In this framework, the first state of preeclampsia, characterized by a poorly perfused placenta, is not sufficient to produce the clinical manifestation of preeclampsia. However, this preliminary condition of poor perfusion and oxidative stress interacts with the maternal immune and vascular systems paving the way for the second stage, where the clinical symptoms of preeclampsia occur [86].

In this framework, a potential integration for etiopathogenetic mechanism may come from contribution that takes into account gene interactions and nutritional factors, which might occur at the time of the inflammation, a field which received rising attention from researchers. It has already been pointed out how deficiency of omega-3 fatty acids (n3FAs) might play a decisive role in the etiology of several neurodevelopmental disorders, namely, ASD and ADHD [87–90]. While fish oil supplementation during pregnancy and in the first 3 months after birth leads, at 4-year follow-up, to higher mental age in probands than in controls [91], lower plasma or rbc levels of n3FAs have been found in ASD patients compared to controls [92–94].

According to Field [95], genetic studies outline several connections between n3FA metabolism and neurodevelopmental disorders. Chromosome site 11q22-23, a location linked to ASD and ADHD, contains the genes for desaturases involved in FA conversion, as well as a dopamine receptor gene linked to bipolar disorder. Sites in the 6p21-23 region containing genes involved with fatty acid metabolism are also associated with ASD, ADHD, schizophrenia, and bipolar disorder. The genes for phospholipase A2s, enzymes responsible for transforming Sn-2 long chain fatty acid into free FA molecules, are near genes involved in ADHD, ASD, and bipolar disorder genes [96]. This led several scholars to question how dietary change, as an environmental genetic interaction, might affect etiopathogenesis of neurodevelopmental disorders ([97, 98]; Stevens et al., 1995).

Field [95] proposed to consider how parental age, a risk factor for ASD (less so for ADHD), might be linked to the aging effect on the maternal metabolism of fatty acids, as somatic cells might have defects in delta 6 desaturase activity causing a poorer conversion of fatty acids in older women. Other interesting evidence arises from studies that take into account pregnancy iron deficiency, a more recent trend than the one on fatty acids. Fe is known for its pivotal role in

myelination, synaptogenesis, and nerve cell metabolism as a whole; as such, iron deficiency has already been reported as a risk factor for abnormal development While iron deficiency might be associated with various conditions and given the fact that the placenta has an effective regulatory ability for iron transfer, it should be noted that obesity is a risk factor for iron deficiency [1, 3, 99–102]. Berglund et al. [103] noted how this interaction might act as a confounding factor for the link between oxidative stress, inflammation, and neurodevelopment impairments.

Many genetic pathways have been described for autism ([104]; Bae, Hong, 2018), several of which might be connected to the relationship between maternal metabolism, oxidative stress, and neurodevelopment. Of particular interest is the mTOR pathway. A serine-threonine kinase, mTOR (namely, its two protein complexes mTORC1 and mTORC2) is involved in a complex interaction, whose unifying feature could be identified in the integration of multiple intracellular and extracellular signals to coordinate several responses, including protein synthesis, growth, proliferation, and, in the central nervous system, also synaptic plasticity [21, 105]. In particular, mTORC1 and mTORC2 promote the transcription of genes involved in autophage inhibition, protein translation, carbohydrate metabolism, and lipogenesis. As such, they play a central role in the mechanisms of obesity and autoimmune disorders and also of cancer development and aging [106]. The role of mTor has been studied in a large variety of neuropsychiatric disorders, such as mood disorders, schizophrenia, and drug addiction, and it is involved with an alteration in dopaminergic transmission (Ryskalin et al., 2018). Dysregulation of mTOR is a common feature of several neurodevelopmental disorders, such as tuberous sclerosis, which has been associated with ASD in retrospective, prospective, and also meta-analytic studies [107]. Also, Angelman syndrome [108], Rett syndrome [109], CDKL-5 syndrome [110], and Phelan-McDermid syndrome (associated with SHANK 3 deletion and with a high comorbidity with ASD) [21] have been associated with mTOR pathways. This implication of mTOR in autism led to investigating its role as a potential target to understand and treat ASD [111]. Kalkman and Feuerbach [112] argued that ASD syndromes might be caused by mutations in genes that inhibit mTOR and that mTOR might lead to autophagy inhibition. This evidence may shed more light also on the association between allergies, as well as the wide spectrum of autoimmune disorders, and ASD. An interesting study [113] employed BTBR mice, a strain considered a representative model for ASD behavioral deficits [114], that features upregulation of the mTOR-S6K pathway and synaptic inhibition of the mTOR-ULK1 pathway. This animal model has been treated with acute systemic injection of insulin-like growth factor-II, showing an improvement of many major ASD-like behaviors and specifically of cognitive and social deficits, as well as of repetitive behaviors [113].

4. Concluding Remarks

In the framework of a growing interest about possible environmental factors involved in the etiopathogenesis of ASD, in particular during *in utero* life, increasing epidemiological reports highlighted the link between ASD and maternal metabolic conditions. Many studies stressed the crucial role for neurodevelopment of the exposition to oxidative stress, which is linked to inflammation, immune dysregulation, and thus, also to a wide variety of metabolic conditions. Despite that, the mechanisms through which oxidative stress might lead to developing ASD remain unclear and not specific, and maternal metabolic conditions have been associated also with different kinds of both somatic and neuropsychiatric disorders in the offspring. Moreover, most of the authors have focused only on singular mechanisms and/or specific metabolic conditions; thus, there is a lack of studies featuring a concomitant evaluation of a broader range of biochemical pathways.

It might be useful to highlight some of the limitations of the present inquiry. This is not a systematic review on the subject, and as such, it bears possible bias under the methodological and interpretative points of view. Moreover, we consider here only some of the many possible ethiopathogenetic paths of autism. As such, the large number of environmental and genetic variables implied in this process has been restricted. In addition, animal models on high-fat diet provide a good picture of some of the mechanisms that might be acting in ASD development, but due to some practical limitations (high-fat or high-energy diet is defined depending on the species), results from these data should be carefully weighted when comparing them to results from humans. Globally, while human clinical conditions such as diabetes mellitus, preeclampsia, or other metabolic conditions may elicit several neurodevelopmental disruptive pathways, it should be noted how these mechanisms, mostly related with oxidative stress, should be better considered in a wider framework of complex interaction with genetic underpinnings as well as with other environmental and neurobiological factors, not limited to the cases here discussed.

Further studies might allow to clarify this perspective, considering in particular the possible multifactorial etiopathogenesis of ASD, which feature the interaction between genetic and environmental conditions. Increasing the knowledge about this topic is of crucial interest for both clinical and research settings, as it may lead not only to improve therapeutic and prevention strategies but also to shed more light on the relationships between central and peripheral systems and between somatic and neuropsychiatric disorders.

Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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Research Article

Redox Balance, Antioxidant Defense, and Oxidative Damage in the Hypothalamus and Cerebral Cortex of Rats with High Fat Diet-Induced Insulin Resistance

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Oxidative stress is a key pathogenic factor in both neurogenerative and metabolic diseases. However, its contribution in the brain complications of insulin resistance is still not well understood. Therefore, the aim of this study was the evaluation of redox homeostasis and oxidative damage in the hypothalamus and cerebral cortex of insulin-resistant and control rats. 16 male Wistar rats were divided into two equal groups ($n = 8$): the control and high fat diet group (HFD). Prooxidant enzymes (xanthine oxidase and NADPH oxidase); enzymatic and nonenzymatic antioxidants [glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase-1 (SOD-1), and uric acid (UA)]; and oxidative damage products [advanced glycation end products (AGE), 4-hydroxynonenal (4-HNE), malondialdehyde (MDA), and 8-hydroxy-2′-deoxyguanosine (8-OHdG)] as well as the total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI), and total ferric reducing ability of sample (FRAP) were evaluated in the hypothalamus and cerebral cortex as well as serum/plasma of HFD-fed and control rats. The activity of prooxidant enzymes was significantly increased in the cerebral cortex and hypothalamus of HFD-fed rats vs. control rats. Additionally, we have showed enhanced antioxidant efficiency in the hypothalamus (↑CAT, ↑UA, ↑TAC, and ↑FRAP) and cerebral cortex (↑GPx, ↑CAT, ↑SOD-1, ↑UA, ↑TAC, and ↑FRAP) of HFD-fed rats. All of the oxidative damage markers (AGE, 4-HNE, MDA, 8-OHdG, and OSI) were significantly increased in the cerebral cortex of insulin-resistant rats, while only 4-HNE and MDA were markedly higher in the hypothalamus of the HFD group. Summarizing, the results of our study indicate an adaptive brain response to the increased production of free radicals under insulin resistance conditions. Despite the increase in antioxidative defense systems, this mechanism does not protect both brain structures from oxidative damages. However, the cerebral cortex is more susceptible to oxidative stress caused by HFD.

1. Introduction

Type 2 diabetes (T2DM, OMIM #125853) is one of the most common metabolic disorders referred to as "the epidemic of the 21st century" [1, 2]. The pathogenesis of T2DM involves two basic pathological defects: impaired insulin secretion and insulin resistance (IR), which may be defined as reduction or lack of insulin sensitivity to the target tissues, such as adipose tissue, muscles, and the liver [3]. An important role in the

pathogenesis of IR is attributed to free fatty acids (FFA) derived from a high fat diet (HFD). Excessive accumulation of FFA in cells inhibits glucose uptake, which is responsible for the malfunctioning of signal transduction pathways regulated by diacylglycerol (DAG) [3]. In these conditions, the increased activity of stress-induced serine-threonine kinases (e.g., JNK kinase) leads to phosphorylation of serine residues in the insulin receptor substrate (IRS) which blocks the effect of the insulin signaling pathway [4, 5]. It has been

proven that diet-induced IR impairs body functioning and significantly increases a risk of cardiovascular disease, hypertension, cancer, and osteoarthritis [4, 5]. However, more and more attention is paid to the possible complications of IR to the central nervous system (CNS) [6–8]. Although for many years the brain was thought to be a typical non-insulin-dependent organ, recent studies have indicated that insulin can cross the blood-brain barrier (BBB) and it is produced within the brain structures including the hypothalamus, cerebral cortex, cerebellum, amygdala, and hippocampus [8, 9]. In addition, the brain insulin activity is associated with regulation of neurotransmitter/neuromodulator secretion as well as acting a major role in synaptic plasticity, memory, learning processes, and neuronal apoptosis [9, 10]. The latest epidemiological studies have also shown the relationship between systemic IR and dementia, neuroinflammation, depression, or increased incidence of the Alzheimer's disease [6–8]. Although these disorders are characterized by diverse clinical picture, it is suggested that they can be explained by similar mechanisms of cerebral neurodegeneration [7].

A significant role in etiology of IR is also attributed to oxidative stress [11, 12]. Generally, oxidative stress is lack of balance between the formation of reactive oxygen species (ROS) and efficiency of enzymatic and nonenzymatic antioxidative systems [13]. This results in oxidative damage to cell components and thus leads to the impairment of cell structures and biological functions [14]. It is assumed that (in both target organs and the brain) the formation of oxidative stress is related to the increase in oxidation of FFA and glucose, as well as increased generation of oxygen free radicals in the mitochondrial respiratory chain [12, 15]. Additionally, the overproduction of ROS may be caused by the increased glycolysis under hyperglycemic conditions as well as intensification of nonenzymatic glycation (glycosylation) of cellular proteins [16]. The resulted oxidative products, especially advanced glycation end products (AGE), play a key role in the cerebral neurodegeneration [8, 16].

Despite a few reports about the role of oxidative stress in the brain complications of IR, the exact kind of brain oxidative damage is still unknown. There is also a lack of data comparing HFD-related oxidative stress in various brain structures, and thus, the aim of this study was the evaluation of redox homeostasis, enzymatic and nonenzymatic brain antioxidants, and oxidative damage in the different brain structures involved in energy homeostasis (hypothalamus) and cognition processes (cerebral cortex) of HFD-fed rats. This is also the first study that has compared oxidative stress on both systemic (serum/plasma) and local (brain) levels in HFD-induced IR.

2. Materials and Methods

2.1. Animals.
The protocol of the study was approved by the Local Ethical Committee for Animal Experiments of the Medical University of Bialystok, Poland (permission number 89/2015, 2015/109).

The research was conducted on male Wistar rats (cmdb outbred, 5 weeks of initial age, 50–70 g of initial weight). Throughout the whole experiment, a stable temperature

(20–21°C ± 2°C), humidity (40–60%), twenty-four-hour rhythm (12 h light/12 h dark cycle), and free access to food and drinking water were preserved. The rats were housed individually in standard cages and remained in constant eye contact with each other. After one week of adaptation, the rats were randomly divided into two equal groups:

(1) Control (C) ($n = 8$)

(2) High fat diet group (HFD) ($n = 8$)

The animals assigned to the control group received a standard laboratory rat chow containing 13.5 kcal% fat, 24 kcal% protein, and 62.5 kcal% carbohydrates (Agropol, Motycz, Poland) for a period of 8 weeks. The animals from the experimental group were fed HFD containing 59.8 kcal% fat, 20.1 kcal% protein, and 20.1 kcal% carbohydrates (Research Diets Inc., cat no. D12492) also for 8 weeks. Food consumption and body weights were monitored every 3 days. Body mass index (BMI) was also analyzed using the weight and the height (the length from the tip of the nose to the anus). BMI was calculated using the formula: BMI = body weight (g)/length^2 (cm^2) [17]. BMI between 0.45 g/cm^2 and 0.68 g/cm^2 were assumed to be normal values, whereas obesity was defined as BMI greater than 0.68 g/cm^2 [17].

After the 8 weeks of experiment and after an overnight fasting, animals were anesthetized by intraperitoneal injection with sodium phenobarbital in a dose of 80 mg/kg BW. Fasting tail-blood glucose analysis was done (Accu-Chek Active Blood Glucose Meter, Roche, Bayer, Germany), and blood samples were collected from the abdominal aorta. The samples were placed into glass tubes (to obtain serum) and glass tubes containing sodium heparin (to obtain plasma). The blood was then centrifuged (3000 × g, 4°C, 10 min), and to protect against sample oxidation and proteolysis, the antioxidant butylated hydroxytoluene (0.5 M solution in acetonitrile, 10 μL/1 mL; Sigma-Aldrich, Germany) and protease inhibitor (Complete Mini, Roche, France) were added [18]. Serum and plasma samples were precooled in liquid nitrogen and stored at −80°C until use.

The hypothalamus and cerebral cortex were collected by one and the same lab technician. The brain structures were freeze-clamped with aluminum tongs, precooled in liquid nitrogen, and stored at −80°C until assays. On the day of biochemical analysis, brain tissues were slowly thawed at 4°C, divided into small pieces, and diluted (1 : 15, $w : v$) in ice-cold phosphate buffered saline (PBS; 0.02 M, pH 7.0). To all samples, antioxidant butylated hydroxytoluene and protease inhibitor were added [2]. The brain samples were homogenized on ice (glass homogenizer; Omni TH, Omni International, Kennesaw, GA, USA) and sonicated (1800 J/sample, 20 sec × 3; ultrasonic cell disrupter, UP 400S, Hielscher, Teltow, Germany). Next, the homogenates were centrifuged (5000 × g, 4°C, 20 min), and the resulting supernatants were analyzed on the same day.

Plasma FFA were determined by gas chromatography (GC) [19]. Fasting insulin concentration was assessed by the ELISA method using commercial kit Insulin Rat ELISA

BioVendor (Brno, Czech Republic). To confirm IR, the insulin sensitivity was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) = fasting insulin (U/mL) × fasting glucose (mM)/22.5.

2.2. Biochemical Assays. The performed analysis included

(i) determination of prooxidant enzymes—NADPH oxidase (NOX; EC 1.6.3.1) and xanthine oxidase (XO; EC 1.17.3.2)

(ii) determination of enzymatic and nonenzymatic antioxidants—glutathione peroxidase (GPx; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.7), catalase (CAT; EC 1.11.1.6), Cu-Zn-superoxide dismutase-1 (SOD-1; EC 1.15.1.1), and uric acid (UA)

(iii) determination of total antioxidant/oxidant status—total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI), and the total ferric reducing ability of sample (FRAP)

(iv) determination of oxidative damage products—advanced glycation end products (AGE), 4-hydroxynonenal (4-HNE) protein adducts, malondialdehyde (MDA), and 8-hydroxy-2′-deoxyguanosine (8-OHdG)

All assays were performed in homogenates of the brain samples (hypothalamus and cerebral cortex). Additionally, enzymatic antioxidants (GPx, GR, CAT, and SOD-1) were evaluated in the serum samples, while another redox/oxidative stress biomarkers in rat plasma samples. Absorbance/fluorescence was measured using microplate reader Infinite M200 PRO Multimode Tecan (Tecan Group Ltd., Männedorf, Switzerland). All determinations were performed in duplicate samples (except for CAT, SOD-1, TAC, and TOS; see below), and all biochemical reagents were obtained from Sigma-Aldrich Germany/Sigma-Aldrich USA. The final results were standardized to one milligram of the total protein. The total protein content was estimated in duplicate samples using the colorimetric bicinchoninic acid (BCA) assay with bovine serum albumin as a standard (Pierce BCA Protein Assay Kit, Rockford, USA) [20].

2.3. Prooxidant Enzymes. Activity of prooxidant enzymes (NOX and XO) was assayed immediately after sample collection. NOX activity was analyzed by the luminescence assay using lucigenin as a luminophore [21]. One unit of NOX activity was defined as the amount of enzyme required to release 1 nmol of superoxide anion per one minute. XO activity was evaluated colorimetrically according to Prajda and Weber [22] by measuring the increase in absorbance of uric acid (UA) at 290 nm. One unit of XO activity was defined as the amount of enzyme required to release 1 μmol of UA per one minute.

2.4. Enzymatic and Nonenzymatic Antioxidants. GPx activity was assayed spectrophotometrically based on the conversion of NADPH to $NADP^+$ [23]. The absorbance was analyzed at

340 nm. It was assumed that one unit of GPx activity catalyzes oxidation of one millimole NADPH for one minute.

CAT activity was estimated spectrophotometrically by measuring the decomposition rate of hydrogen peroxide (H_2O_2) at 340 nm [24]. It was assumed that one unit of CAT activity degrades 1 micromole of H_2O_2 for one minute. CAT activity was analyzed in triplicate samples.

SOD-1 activity was assayed spectrophotometrically by measuring the cytosolic activity of superoxide dismutase by inhibiting oxidation of epinephrine to adrenochrome [25]. The absorbance was measured at 340 nm. One unit of SOD-1 activity was defined as the amount of enzyme which inhibits oxidation of epinephrine by 50%. SOD-1 activity was estimated in triplicate samples.

UA concentrations were estimated using the commercial colorimetric kit QuantiChrom™Uric Acid Assay Kit DIUA-250 (BioAssay Systems, Hayward, CA, USA). The absorbance was measured at 490 nm.

2.5. Total Antioxidant/Oxidant Status. TAC content was determined in triplicate samples using a colorimetric method with 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{*+}$) [26]. The absorbance was measured at 660 nm, and TAC was calculated from the calibration curve for Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). TOS content was analyzed bichromatically at 560/800 nm based on oxidation of Fe^{2+} to Fe^{3+} ions in the presence of the oxidants contained in the samples [27]. TOS was estimated in triplicate samples and expressed as μmol H_2O_2 equiv./L. Oxidative stress index (OSI) was calculated by dividing TOS by TAC content and expressed in % [28].

FRAP content was determined spectrophotometrically by measuring the ferric reducing ability of samples and using 2,4,6-tripyridyl-s-triazine (TPTZ) [29]. The absorbance was measured at 593 nm, and FRAP was calculated from the calibration curve for $FeSO_4$.

2.6. Proteins, Lipids, and DNA Oxidation Products. AGE content was determined spectrofluorimetrically by measuring characteristic fluorescence of AGE-derived compounds at 440/350 nm [30]. Immediately before the assay, the plasma samples were diluted 1 : 5 ($v : v$) in PBS (0.02 M, pH 7.0).

MDA concentration was assayed colorimetrically using the thiobarbituric acid reactive substances (TBARS) method with 1,3,3,3-tetraethoxypropane as a standard [31]. The absorbance was measured at 535 nm.

4-HNE and 8-OHdG concentrations were measured using commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA, USA, and USCN Life Science). The absorbance was measured at 405 nm.

2.7. Statistical Analysis. The data were processed using Statistica 12.0 (StatSoft, Cracow, Poland) and GraphPad Prism 7 (GraphPad Software, La Jolla, USA). The statistical analysis was performed using unpaired Student's t-test and Pearson's correlation method (p set at 0.05). In the lack of normal distribution of the results, the nonparametric Mann–Whitney U

TABLE 1: Effect of 8-week HFD on body weight, BMI, glucose, insulin, HOMA-IR, food and energy intake, and total protein concentration.

	C ($n = 8$)	HFD ($n = 8$)
Final body weight (g)	312.2 ± 15.2	$378.1 \pm 17.6^*$
BMI (g/cm^2)	0.52 ± 0.1	$0.66 \pm 0.3^*$
Fasting glucose (mg/dL)	99.8 ± 4.5	$169.6 \pm 15.1^*$
Fasting insulin (μU/mL)	4.7 ± 0.3	$54.6 \pm 4.1^*$
HOMA-IR index	1.4 ± 1.2	$21.3 \pm 1.6^*$
Plasma FFA (μmol/L)	79.5 ± 10.7	$174.3 \pm 10.2^*$
Food consumption (g/day)	23.2 ± 0.8	$17.1 \pm 0.6^*$
Energy intake (kJ/day)	266.2 ± 3.2	$338.9 \pm 5.1^*$
Cerebral cortex total protein content (μg/mL)	2515.0 ± 94.9	2159.0 ± 44.2
Hypothalamus total protein content (μg/mL)	1892.0 ± 121.7	1616.0 ± 66.4

FFA, free fatty acids; HOMA-IR, homeostatic model assessment of insulin resistance; HFD, high fat diet. Differences statistically important at $^*p < 0.05$.

test was used. The results were expressed as mean $\pm$ SD. The sample size was set based on a previously conducted pilot study (the power of the test was set at 0.9).

3. Results

3.1. General Characteristic of Rats. The energy intake in HFD rats was significantly higher (+27%) when compared to control animals. Therefore, despite the lowered food consumption (−26%), after 8 weeks of the experiment, the body weight as well as BMI were significantly increased (+21% and +27%, respectively) (Table 1). The increase of free fatty acids in plasma (+119%) of HFD-fed rats led to insulin resistance as fasting glucose and insulin levels as well as the HOMA-IR index were significantly higher (+70%, 12 times and 15 times, respectively) (Table 1). Increased amounts of ingested fats had no influence on the total protein concentration both in the cerebral cortex and hypothalamus.

3.2. Enzymatic and Nonenzymatic Antioxidants, Total Antioxidant/Oxidant Status, and Oxidative Damage Products in Plasma and Serum. Enzymatic antioxidant activity (GPx, CAT, and SOD-1) was significantly higher in the serum of HFD rats (+78%, +59%, and +46%); only GR activity remained unchanged when compared to the control (Table 2). Similarly, higher content of uric acid in the plasma of high fat diet-fed rats was observed (+44%). The total antioxidant/oxidant status and oxidative damage products (TAC, TOS, OSI, and FRAP) were also increased in animals which ingested high amounts of fats (+35%, +241%, +125%, and +66%, respectively). All of the estimated oxidative damage products (AGE, 4-HNE, MDA, and 8-OHdG) were significantly higher (+181%, +232%, +281%, and +30%, respectively) in the plasma of HFD animals (Table 2).

TABLE 2: Effect of 8-week HFD on enzymatic and nonenzymatic antioxidants, total antioxidant/oxidant status, and oxidative damage products in the rat's plasma and serum.

	C ($n = 8$)	HFD ($n = 8$)
GPx (mU/mg protein)	0.9 ± 0.09	$1.6 \pm 0.1^*$
GR (nU/mg protein)	55.3 ± 0.7	56.55 ± 0.2
CAT (nmol H_2O_2/min/mg protein)	3.9 ± 0.2	$6.2 \pm 0.2^*$
SOD-1 (mU/mg protein)	29.4 ± 1.3	$42.9 \pm 1.6^*$
UA (μg/mg protein)	3.4 ± 0.3	$4.90 \pm 0.3^*$
TAC (Trolox nmol/mg protein)	9.6 ± 0.2	$13.0 \pm 0.9^*$
TOS (nmol H_2O_2 equiv./mg protein)	36.9 ± 1.9	$126.0 \pm 5.0^*$
OSI	433.5 ± 32.7	$976.0 \pm 38.2^*$
FRAP (nmol/mg protein)	1.15 ± 0.3	$1.91 \pm 0.2^*$
AGE (AFU/mg protein)	29.7 ± 0.6	$83.6 \pm 0.2^*$
4-HNE (fg/mg protein)	51.5 ± 5.5	$170.8 \pm 10.6^*$
MDA (ng/mg protein)	70.2 ± 8.2	$267.4 \pm 15.5^*$
8-OHdG (pg/mg protein)	6.6 ± 0.3	$8.6 \pm 0.3^*$

4-HNE, 4-hydroxynonenal protein adducts; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AGE, advanced glycation end products; CAT, catalase; FRAP, ferric reducing ability of sample; GPx, glutathione peroxidase; GR, glutathione reductase; HFD, high fat diet; MDA, malondialdehyde; OSI, oxidative stress index; SOD-1, Cu-Zn-superoxide dismutase-1; TAC, total antioxidant capacity; TOS, total oxidant status; UA, uric acid. Enzymatic antioxidants (GPx, GR, CAT, and SOD-1) were determined in serum whereas other markers were assayed in plasma. Differences statistically important at $^*p < 0.05$.

3.3. Prooxidant Brain Enzymes. The activity of NOX and XO was significantly increased in the animals fed HFD both in the cerebral cortex (+31% and +26%) and hypothalamus (+22% and +27%) (Figure 1).

3.4. Enzymatic and Nonenzymatic Brain Antioxidants. The activity of enzymatic antioxidants (GPx, CAT, and SOD-1) in the cerebral cortex of the HFD-fed animals was markedly increased when compared to the control (+46%, +65%, and +40%, respectively) (Figure 2). On the other hand, in the hypothalamus of the HFD rats, only the activity of catalase was increased (+73%), whereas the activity of GPx and GR was significantly lower (−33% and −30% as compared to the control). Interestingly, uric acid content was increased in the cerebral cortex as well as in the hypothalamus (+32% and +117%) of the animals fed HFD (Figure 2).

3.5. Total Antioxidant/Oxidant Brain Status. HFD leads to an increase in the antioxidant/oxidant status in both studied brain structures (except OSI in the hypothalamus). TAC, TOS, OSI, and FRAP were significantly higher in the cerebral cortex of HFD animals (+28%, +44%, +33%, and 34%, respectively). Similarly, in the hypothalamus, an increase in TAC, TOS, and FRAP was observed (+22%, 95%, and 21%) whereas OSI remained unchanged when compared to the control (Figure 3).

3.6. Oxidative Brain Damage Markers. All of the oxidative damage markers (AGE, 4-HNE, MDA, and 8-OHdG)
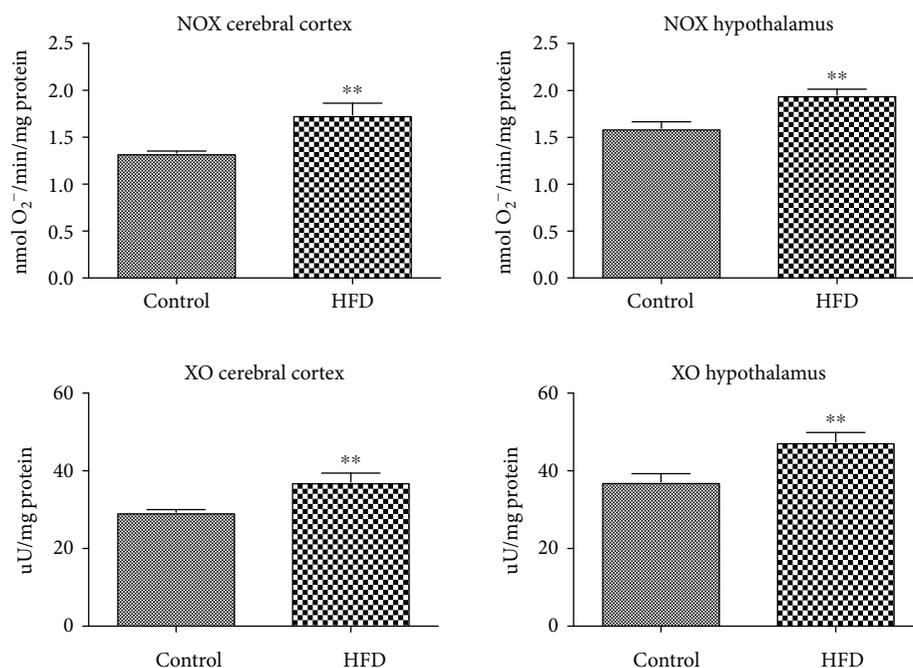
FIGURE 1: Prooxidant brain enzymes in the control and HFD-fed rats. NOX, NADPH oxidase; HFD, high fat diet; XO, xanthine oxidase. Differences statistically important at $^{**}p < 0.005$.

assayed in the cerebral cortex of HFD rats were significantly increased (+28%, +239%, +148%, and +55%) (Figure 4). In contrast, only 4-HNE protein adduct and MDA concentrations were markedly higher in the hypothalamus of high fat diet-fed animals (+248% and +82%).

3.7. Correlations. In the cerebral cortex of high fat-fed rats, we have shown a highly positive correlation between GPx activity and the TAC level ($r = 0.86$, $p = 0.001$), NOX and AGE ($r = 0.9$, $p < 0.0001$), and NOX and 4-HNE ($r = 0.62$, $p = 0.01$). In the HFD group, we have also demonstrated a positive correlation between the HOMA-IR index and AGE ($r = 0.7$, $p = 0.05$), as well as HOMA-IR and 4-HNE levels ($r = 0.82$, $p = 0.001$). There were no statistically significant correlations between the plasma/serum and brain OS biomarkers.

4. Discussion

This is the first study that compares both redox status, antioxidant defense, and oxidative damage between the hypothalamus and cerebral cortex of HFD-fed rats. We have demonstrated that chronic administration of HFD induces IR, which may be responsible for the redox imbalance, alternations in enzymatic and nonenzymatic brain antioxidants, and enhanced oxidative damage to the rats' cerebral cortex and hypothalamus. Moreover, we have observed a positive correlation between the brain oxidative damage and the HOMA insulin resistance index, which points at the involvement of systemic IR in the development of brain oxidative stress. Finally, although redox balance of both brain structures shifted towards the oxidative status, the intensity of oxidative damage was much greater in the rat's cerebral cortex.

Recently, a global epidemic of metabolic disorders caused by an improper diet and lack of physical activity is observed [32]. Excessive consumption of fat and cholesterol may lead to dyslipidemia, obesity, hyperglycemia, hyperinsulinemia, and whole-body IR [5]. Therefore, it is not surprising that the applied model of chronic (8 weeks) HFD resulted in higher plasma free fatty acids, elevated fasting glucose and insulin, and reduced insulin sensitivity indicated by the higher HOMA-IR index. Based on the available literature and diagnostic criteria of IR [32, 33], we have confirmed the occurrence of IR in all the HFD-fed animals. In this group, we have also observed a significant increase in body weight and body mass index (BMI), as well as general (plasma/serum) oxidative stress, which is consistent with the previous reports on the animal models of IR [2, 32].

It is beyond question that the main free radical source in the brain is the increased oxidation of glucose and free fatty acids in mitochondria [32, 34]. Of all the by-products generated during mitochondrial processes, superoxide anions ($O_2^{-\bullet}$) are formed in the largest quantities [34]. Up to 90% of ROS is produced during oxidative phosphorylation, while enhanced oxidation of energy substrates can further increase the formation of free radicals in the cell [2, 32]. Indeed, when caloric intake is enhanced, the number of electrons supplied to the respiratory chain is increased and generates more $O_2^{-\bullet}$. The production of $O_2^{-\bullet}$ is also catalyzed by the enzymes from the oxidoreductase class including NADPH oxidase (NOX) and xanthine oxidase (XO) [35]. The results of the presented study indicate a higher activity of ROS-generating enzymes (NOX and XO) in the hypothalamus and cerebral cortex of HFD-fed animals. Referring to the previous research, exposure to HFD stimulates inflammatory signaling in macrophages and adipocytes,
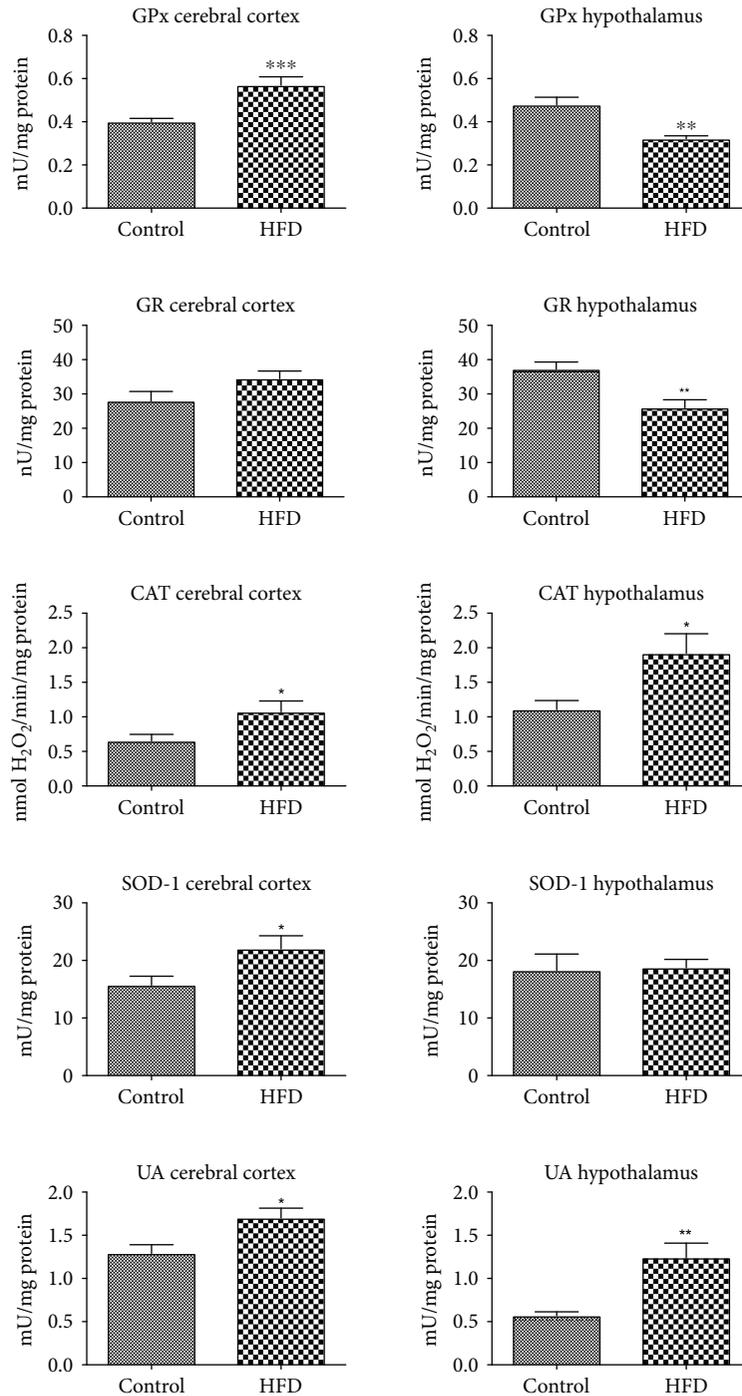
FIGURE 2: Enzymatic and nonenzymatic brain antioxidants in the control and HFD-fed rats. CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; HFD, high fat diet; SOD-1, Cu-Zn-superoxide dismutase-1; UA, uric acid. Differences statistically important at $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$.

which results in the NOX activation and increased production of proinflammatory cytokines (e.g., IL-1β, IL-6, and TNFα) [36]. It is well known that NOX and XO can dramatically increase a rapid consumption of oxygen during an oxidative respiratory burst in mitochondria [34]. They can also stimulate adipocytes to release MCP-1 (monocyte chemoattractant protein-1), which promotes the transformation of monocytes to macrophages and intensifies

inflammation [36]. Bearing in mind that the increased expression of cytokines (e.g., IL-1β, IL-6, and TNFα), chemokines, and proinflammatory enzymes (e.g., COX-2 and iNOS) have been reported in the IR brain [37, 38], the enhanced activity of NOX and XO may not only be an important source of ROS but can also induce an inflammatory response in the hypothalamus and cerebral cortex of HFD-fed rats.
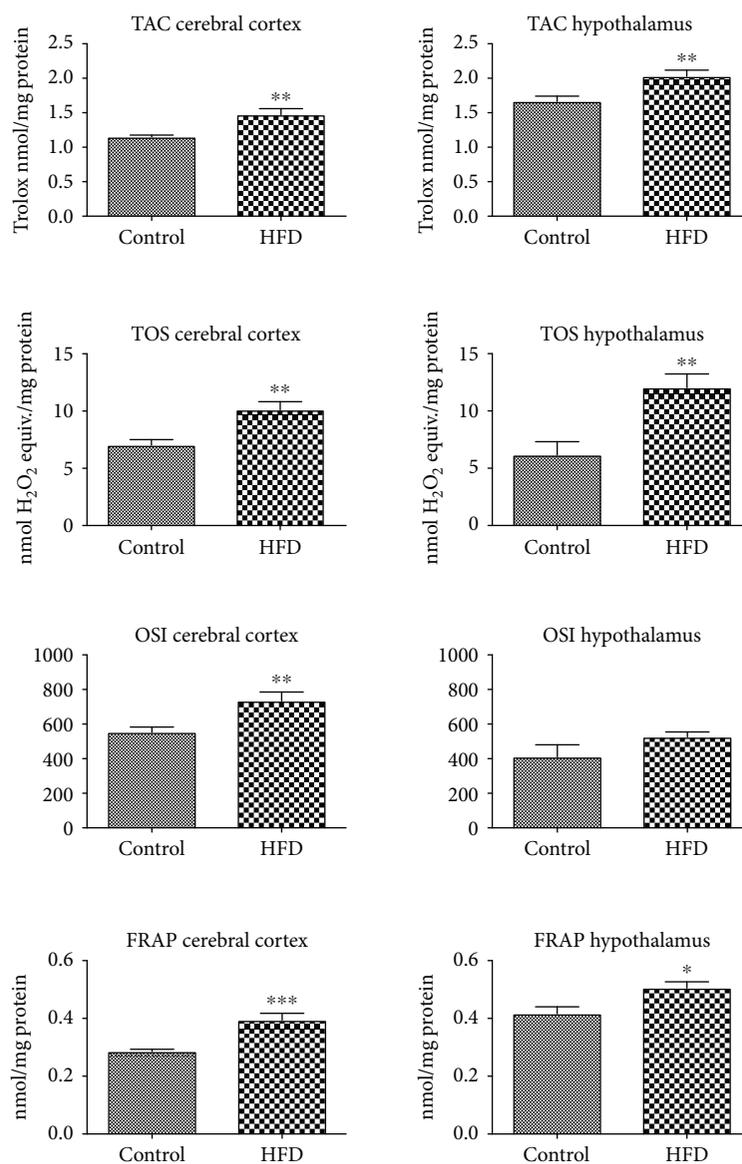
FIGURE 3: Total antioxidant/oxidant status in cerebral cortex and hypothalamus of the control and HFD-fed rats. FRAP, ferric reducing ability of sample; HFD, high fat diet; OSI, oxidative stress index; TAC, total antioxidant capacity; TOS, total oxidant status. Differences statistically important at $^*p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$.

To prevent the harmful effects of ROS, the brain has developed specialized antioxidative systems including enzymatic (SOD, CAT, and GPx) and nonenzymatic (UA and reduced glutathione) brain antioxidants. However, the total antioxidant effect is not a simple sum of individual antioxidants separately. The ability to scavenge free radicals and/or reduce oxidative damage in the cell depends on the mutual interactions of all brain antioxidants [35]. It is well known that a very important parameter to assess the redox balance in biological systems is the total antioxidant capacity (TAC) and the total oxidant status (TOS) [26]. TAC determines the overall ROS scavenging ability, whereas TOS can be defined as the total level of oxidants (ROS) in the sample [26, 27, 35]. Therefore, despite the increase in some antioxidative mechanisms (↑CAT and ↑UA) and the decrease of other antioxidant enzymes (↓GPx and ↓GR), the ROS

removal efficiency of the hypothalamus is more severe in HFD-fed rats (↑TAC and ↑FRAP) in comparison to the control. In our study, we have also showed enhanced antioxidant efficiency in the cerebral cortex of HFD-fed rats (↑GPx, ↑CAT, ↑SOD-1, ↑UA, ↑TAC, and ↑FRAP). Therefore, the elevated activity/level of antioxidants indicates an adaptive brain response to the increased production of ROS under IR pathology. It is undeniable that the strengthening of the antioxidant's barrier is the most important mechanism for limiting the formation of ROS and regulating their activity. However, do the antioxidant systems prevail over the free radical reactions? For the quantitative assessment of redox homeostasis disorders, we have used the oxidative stress index (OSI; TOS to TAC ratio), which is referred to as the "gold indicator of oxidative stress" [28]. We have demonstrated that in the HFD-fed rats, the level of oxidants
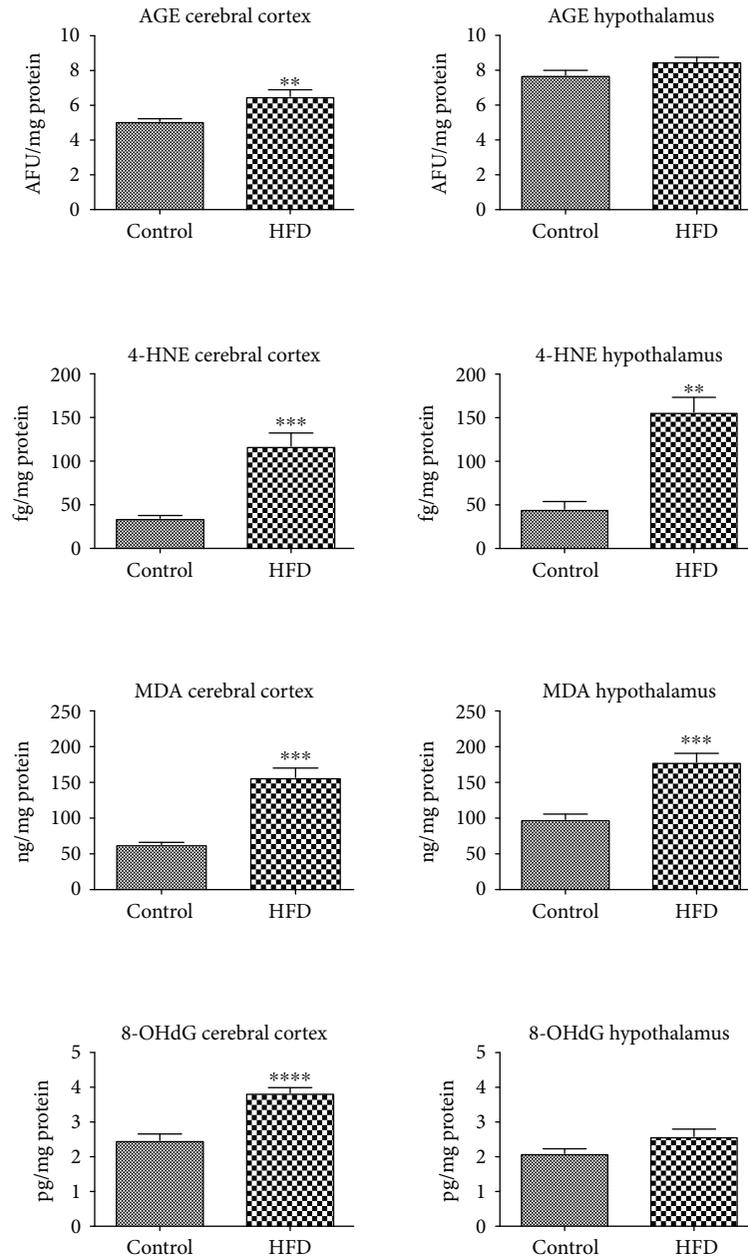
Figure 4: Oxidative damage to the cerebral cortex and hypothalamus of the control and HFD-fed rats. 4-HNE, 4-hydroxynonenal protein adducts; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AGE, advanced glycation end products; HFD, high fat diet; MDA, malondialdehyde. Differences statistically important at $^{**}p < 0.005$, $^{***}p < 0.0005$, and $^{****}p < 0.0001$.

(TOS) outweighs the antioxidant defense mechanisms (TAC), which leads to a shift in the redox balance in favor of the oxidation reactions. Significantly higher OSI in the cerebral cortex of HFD-fed rats suggests a greater extent of oxidative processes in comparison to the hypothalamus. Additionally, the disturbances in oxidant/antioxidant status have also been reported in the blood of HFD-fed animals (↑TAC, ↑TOS, ↑OSI, and ↑FRAP).

Redox abnormalities observed in the study may have consequences in the cellular manifestations of diet-induced IR. Despite the enhanced activity of the brain and blood antioxidative systems, we have shown an increase in the level

of oxidized biomolecules (↑AGE, ↑4-HNE, ↑MDA, and ↑8-OHdG) both in the brain and plasma of HFD-fed animals. It should be noted that the brain is a significant target for oxidative stress due to its increased oxygen consumption, limited detoxification mechanisms, and high content of prooxidative metal ions (mainly iron and copper) [39, 40]. Under these conditions, ROS may alter the cellular components resulting in the enhanced peroxidation of the membrane lipids. Indeed, we have demonstrated significantly higher levels of lipid oxidation products (↑4-HNE and ↑MDA) in both the hypothalamus and cerebral cortex of HFD-fed rats. It is well known that these

compounds, mainly 4-hydroxynonenal (4-HNE), can alter the fluidity and integrity of cells and can also react with proteins and nucleic acids, resulting in further oxidative damage [14]. 4-HNE is also implicated with ATP depletion, impaired glucose transport, and oxidative damage to the active centres of enzymes in the hippocampal and cortical neurons [41, 42]. In our study, in addition to the increased concentration of 4-HNE in both brain structures of HFD-fed rats, we also observed an increase in MDA levels. Malondialdehyde shows mutagenic and carcinogenic effects that can affect the brain cell's proliferation and lead to the apoptotic cell death [34, 40]. In addition, it has been shown that MDA and 4-HNE may increase permeability of the blood-brain barrier (BBB) [40, 43], which may be one of the causes of cerebral neurodegeneration. Deleterious consequences of lipid peroxidation on the brain may also be the induction of proinflammatory enzymes in macrophages [40] and thus stimulation of inflammation. In our study, this may be confirmed by the positive correlation between the 4-HNE level and the NOX activity in the cerebral cortex of HFD-fed rats.

The effect of free radical interactions is not only damage to the lipids but also oxidation of proteins and nucleic acids (DNA or RNA). Oxidative DNA damage is particularly dangerous for the brain because it can cause genetic instability and lead to neuronal cell death via apoptosis or necrosis pathways [7]. In our study, we showed significantly higher concentrations of DNA injury marker 8-OHdG in the cerebral cortex and plasma of HFD-fed rats vs. the control group. Both nuclear and mitochondrial DNA can undergo oxidative damage, which can result in a decrease in the ATP concentration (required for the sodium-potassium ATPase) and other transport proteins in the cell [42]. In our study we have also observed the elevated content of AGE (products of glycation and oxidation by reducing sugar) in the cerebral cortex of rats within the study group. It is believed that the increase in nonenzymatic glycation of proteins and lipids is the main cause of neurodegeneration and cerebral changes caused by aging [44]. Higher AGE levels have also been observed in many metabolic diseases, particularly in the hyperglycemic conditions [16, 45]. Binding of AGE to a specific receptor (RAGE) activates many transcription factors and inflammatory signaling pathways including NFKB, MAP-kinase, NJK, and p21RAS [16, 46]. The presence of RAGE receptors has been demonstrated on the surface of the endothelial cells, cardiomyocytes, dendritic cells, monocytes/macrophages and also the CNS neurons [16]. What is important, AGE may also increase the production of ROS by inducing the activity of prooxidant enzymes, especially NADPH oxidase and XO [14]. Additionally, oxidized proteins tend to form aggregates resistant to degradation, which favors the accumulation of altered proteins in cells and intensifies inflammation [32]. This seems to explain the positive correlation between the AGE content and NOX activity in the cerebral cortex of HFD-fed rats. Moreover, it suggests that glycooxidative damage to cells is directly responsible for the progression of chronic complications of IR and T2DM. It has been shown that serum AGE concentration correlates with the IR level assessed by the HOMA-IR

index [44, 45]. In our study, we have reported a highly positive correlation between the HOMA-IR index and the brain oxidative damage to the proteins (AGE) and lipids (4-HNE). This suggests that central IR may affect the development of cerebral complications of the disease. However, we did not observe any relationship between the blood and brain oxidative stress biomarkers, which indicates the different nature of oxidative damage at the central and local level. This may also indicate that oxidative modification products are formed directly in the brain and do not pass from the circulation through the BBB. Similarly, we did not notice any correlation between the antioxidant defense and redox homeostasis indicators. Thus, the processes taking place in the brain occur independently from the general (plasma/serum) oxidative stress.

An important conclusion from our study is the fact that the cerebral cortex is more strongly affected by oxidative stress than the hypothalamus. Increased concentrations of protein (↑AGE) and DNA (↑8-OHdG) oxidative modifications have been observed only in the cerebral cortex of HFD-fed animals. Although our experiment does not explain the differences between the different brain sensitivity to cellular oxidative stress, it can be assumed that the greater vulnerability to oxidation of the cerebral cortex may be caused by its greater ability to accumulate prooxidative metal ions [47]. Under these conditions, significant amounts of the hydroxyl radicals can be formed. It has been shown that even a slight increase in the transition metals content (e.g., Fe^{2+}, Cu^{2+}, Co^{2+}, V^{2+}, and Cr^{2+}) may significantly enhance $OH^\bullet$ production in the brain [39, 40]. It is well known that $OH^\bullet$ is able to react with all adjacent biomolecules. However, membrane lipids are the most exposed to $OH^\bullet$ and other ROS [34]. Cellular lipids are also a rich source of polyunsaturated fatty acids (PUFAs) that are particularly susceptible to oxidation via oxidative stress [39, 48]. Therefore, this is not surprising that the hypothalamic oxidative damage has only been reported to the lipid molecules.

In our study, the elevated rate of oxidative processes in the IR cerebral cortex is also demonstrated by an increase in OSI levels, suggesting higher production of ROS in comparison to the hypothalamus. The reason for the observed differences may also be the changes in brain bioenergetics and mitochondrial functioning. Indeed, it has been shown that the cerebral cortex and hypothalamus are characterized by different energy metabolisms which additionally changes with age [49]; however, the presented issue requires further research and observations.

When analyzing the results of our work, attention should also be paid to its limitation. Firstly, we evaluated only selected, though the most commonly used, biomarkers of redox homeostasis and oxidative stress. Secondly, the observed changes in the oxidant-antioxidant balance may be the result of not only IR but also other disturbances induced by HFD (e.g., obesity, hyperinsulinemia, hyperglycemia, or metabolic syndrome). In addition, we must not ignore the fact that oxidative stress may be the cause of diet-induced IR. We believe that only kinetic studies can explain this issue. However, it should be borne in mind that this is the first study in which the hypothalamic and cortical

oxidative stresses were compared in HFD-fed animals. We also characterized the redox balance at the systemic and local levels, which is an undeniable advantage of our work.

Data Availability

All of the data used to support the findings of this study are included within the article.

Conflicts of Interest

Authors declare no conflict of interest.

Acknowledgments

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