Oxidative Medicine and Cellular Longevity

Oxidative Stress as a Link between Cerebrocardiovascular and Psychiatric Disorders

Lead Guest Editor: Maria Luca
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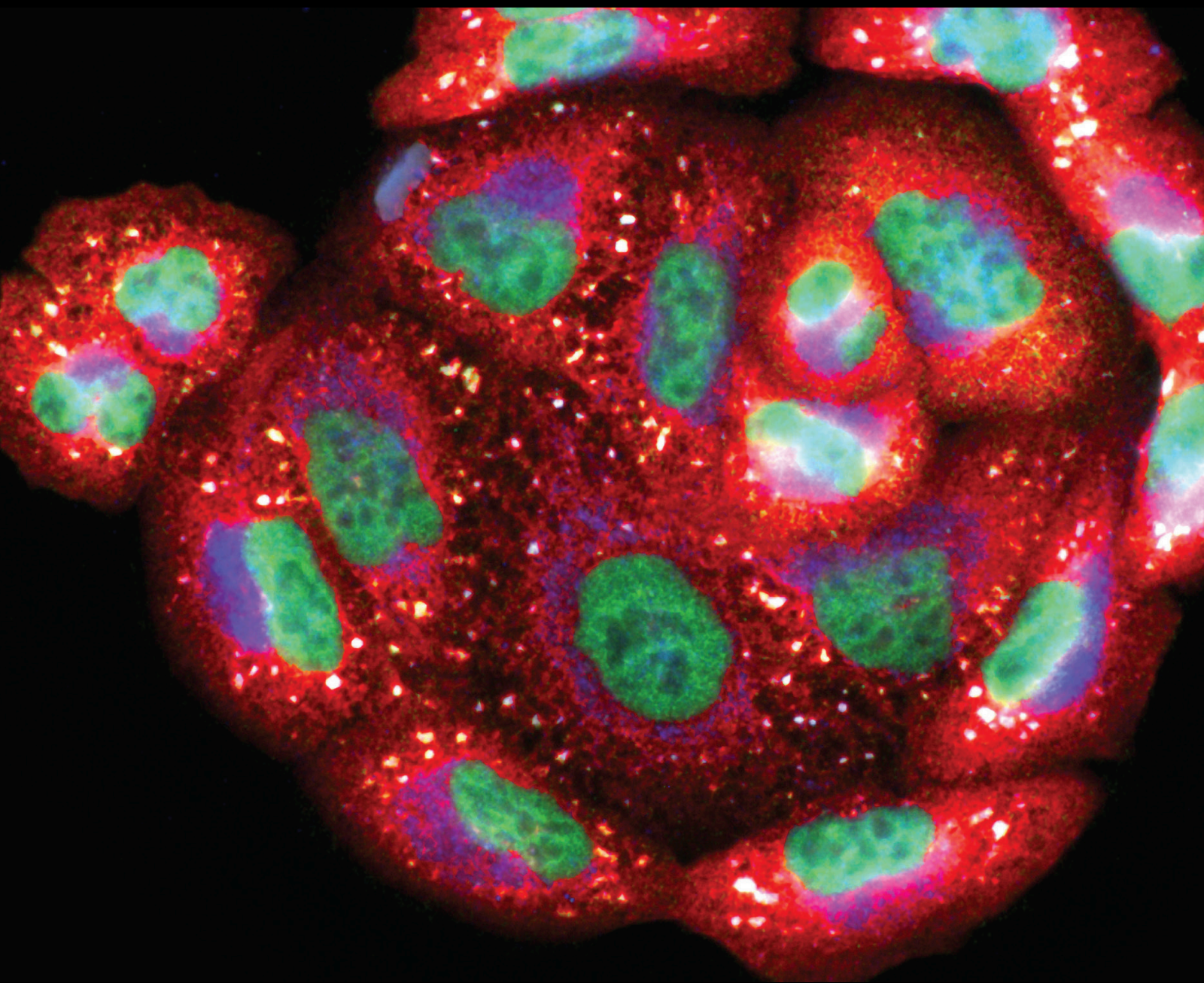

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Editorial

Oxidative Stress as a Link between Cerebrocardiovascular and Psychiatric Disorders

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Neuropsychiatric disturbances show high rates of comorbidity with cerebrocardiovascular disorders. In particular, depression and anxiety independently predict both cerebrovascular and cardiac events, which in turn represent a major cause of mortality in individuals suffering from psychiatric illnesses. On the other hand, depressive symptoms are extremely common in poststroke and vascular dementia.

From a molecular point of view, oxidative stress (OS) could not only represent the major contributor for the pathogenesis and progression of psychiatric and vascular disorders but also explain 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 neuropsychiatric and cerebrocardiovascular disorders.

Three papers focus on the role of OS in diabetes-related complications. In particular, P. Yang et al. reviewed current evidence on the role of the advanced glycosylation end products in the occurrence of OS-related cardiovascular complications in diabetes. N. Palachai et al. demonstrated the beneficial role of mulberry and ginger in reducing the metabolic alterations as well as the OS status and proinflammatory cytokines in male Wistar rats with metabolic syndrome. Finally, J. Wattanathorn et al. demonstrated the antioxidant and anti-inflammatory effect of *Oryza sativa* and *Anethum graveolens* in male Wistar rats with metabolic syndrome.

Two articles explore the detrimental effect of stress on health. More specifically, O. Hahad et al. reviewed the damaging effect of environmental noise on mental and cerebrocardiovascular health in relation to OS, with special focus on the autonomic nervous system, endocrine signaling, and vascular dysfunction. Instead, D. C. Wigger et al. studied the link between early life stress and the risk of cardiovascular disorders evaluating the expression of the myocardial oxytocin receptor and the enzyme cystathionine γ-lyase.

Two reviews summarize current knowledge on the relationship between cerebrocardiovascular disorders and neuropsychiatric disturbances. In particular, D. Lin et al. described the common OS pathways and risk factors shared by ischemic cardiocerebrovascular disorders and depression. M. Luca and A. Luca focused on the role of OS-related endothelial damage in the pathogenesis of both vascular depression and cognitive impairment, also commenting on the beneficial effect of aerobic physical exercise on these disorders.

Several authors studied the role of OS from a multidisciplinary point of view. In detail, L. Venturini et al. performed a pilot study supporting the anti-inflammatory and antioxidant effects of probiotic administration on chronic fatigue syndrome/myalgic encephalomyelitis. M. Castaldo et al. demonstrated that SH-SY5Y cells, whose proliferation, migration, and neurite outgrowth are improved by formyl peptide receptor-1, can stimulate NOX-dependent superoxide generation. N. Yu et al. reported the therapeutic effect of *Ganoderma lucidum triterpenoids*, an inhibitor of the ROCK signaling pathway, in improving cognitive performance and reducing hippocampal cell apoptosis in Alzheimer's disease model mice.

We believe that these contributions provide an updated and comprehensive view on the role of OS in neuropsychiatric and cerebrocardiovascular disorders.

Conflicts of Interest

The editors do not have conflicts of interest to declare regarding the publication of this issue.

Acknowledgments

We would like to express our gratitude to the authors and reviewers contributing to this special issue.

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Research Article

Ganoderma lucidum Triterpenoids (GLTs) Reduce Neuronal Apoptosis via Inhibition of ROCK Signal Pathway in APP/PS1 Transgenic Alzheimer's Disease Mice

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Guest Editor: Antonina Luca

Alzheimer's disease (AD) is the most common cause of dementia among senior citizen. Ganoderma lucidum triterpenoids (GLTs) have nutritional health benefits and has been shown to promote health and longevity, but a protective effect of GLTs on AD damage has not yet been reported. The objective of this research was to elucidate the phylactic effect of GLTs on AD model mice and cells and to explore its underlying mechanisms. Morris water maze (MWM) test was conducted to detect changes in the cognitive function of mice. Hematoxylin-eosin (HE) staining was applied to observe pathological changes in the hippocampus. Silver nitrate staining was applied to observe the hippocampal neuronal tangles (NFTs). Apoptosis of the hippocampal neurons in mouse brain tissue was determined by TUNEL staining. The expression levels of apoptosis-related protein Bcl2, Bax, and caspase 3/cleaved caspase 3; antioxidative protein Nrf2, NQO1, and HO1; and ROCK signaling pathway-associated proteins ROCK2 and ROCK1 were measured by western blot. *In vivo* experiments show that 5-month-old APP/PS1 mice appeared to have impaired acquisition of spatial learning and GLTs could reduce cognitive impairment in AD mice. Compared to normal mice, the hippocampus of APP/PS1 mouse's brains was severely damaged, while GLTs could alleviate this symptom by inhibiting apoptosis, relieving oxidative damage, and inactivating the ROCK signaling pathway. In *in vitro* cell experiments, $A\beta_{25\text{-}35}$ was applied to induce hippocampal neurons into AD model cells. GLTs promoted cell proliferation, facilitated superoxide dismutase (SOD) expression, and inhibited malondialdehyde (MDA) and lactic dehydrogenase (LDH) expression of neurons. Our study highlights that GLTs improve cognitive impairment, alleviate neuronal damage, and inhibit apoptosis in the hippocampus tissues and cells in AD through inhibiting the ROCK signaling pathway.

1. Introduction

Alzheimer's disease (AD), a widespread, progressive, nonreversible, and devastating set of neurodegenerative disorders, is characterized by progressive impairment of memory, motion disorders, and judging and reasoning abilities that eventually results in aphronesia [1, 2]. The pathological features mainly include senile plaques (SP) formed by extracellular amyloid β-protein ($A\beta$) deposition and endocellular neurofibrillary tangles (NFTs) formed by hyperphosphorylation of tau protein in neurons, along with the increasingly attenuate number and capacity of synapses and neurons

[3]. The NFTs are composed of amyloid fibrils, which are associated with synapse loss and neurodegeneration, and eventually lead to memory impairment and other cognitive problems [4]. The pathogenic causes linked with the incidence of AD include poor mental performance, traumatic brain injury, cerebral stroke, low social activity, age, social exclusion, and physical inactivity, and low education level [5]. There is an estimated 46.8 million people worldwide who suffered from AD or interrelated dementia disease in 2015, and the morbidity of AD throughout the world is anticipated to exceed 1.315 billion by year 2050 [6, 7]. The disease is clinically basically characterized by a severe dysfunction of cognition and ascensive degradation of memory, resulting in loss of self-care ability and eventually needing all-time medical solicitude [8]. Nevertheless, up to now, there appears no efficient cure or prevention for AD, and the treatments simply moderate symptoms without affecting the disease development, laying a tremendous millstone on public health and society.

Ganoderma lucidum (*G. lucidum*) is a Basidiomycetes fungus from the order Polyporales and acclaimed officinal agaric used as a folk remedy in Asia since ancient times as a result of its multitudinous health-promoting capabilities [9, 10]. It has been manifested that this eumycete is beneficial in preventing and treating high blood pressure, hyperglycemia, chronic bronchitis, hepatitis, asthma, cancer, heart diseases, and HIV [11–13]. *Ganoderma lucidum* triterpenoids (GLTs) is the major variety of bioactive and medicative components in *Ganoderma lucidum* (*G. lucidum*) [14]. The mother nucleus made of isoprene is an important chemical structure of GLTs [15]. But the chemical structures of GLTs are more sophisticated as a result of the highly enriched oxidized states of these compounds. Most GLTs exhibit a large scale of bioactivity, including anticancer [16], antihypertensive, anti-HIV-1, antiangiogenic, immunomodulatory [17], antiandrogenic, antioxidant, antihepatitis B, antimicrobial activities, and anticomplement [18]. GLTs are efficient as adjuvant therapies and enhance health when united with other pharmaceuticals to cure fatigue syndrome, hepatitis, and prostate cancer [19]. However, research investigating the mechanism and application of *G. lucidum* or GLTs in the treatment of diseases remains preliminary in terms of both the utilization efficacy and product type. Moreover, the therapeutic effect and molecular mechanism of GLTs on AD needs further research.

The present research was designed to evaluate the possible neuroprotective impact of GLTs on antiapoptosis in AD course. Firstly, we determined the effect of GLTs on cognitive disorder in APP/PS1 transgenic AD model mice compared to the control normal mice by place navigation test and spatial probe test. Furthermore, in consideration of the neurons, apoptosis is an important pathological process in AD; the roles of GLTs in the hippocampus' apoptosis of mice were investigated. What's more, we also probed into the function of GLTs on $A\beta_{25\text{-}35}$-induced hippocampal neuron cell AD model. In addition, it was found that ROCK signal pathway may participate in the regulation process of GLTs on AD. We hope that improved mechanistic understanding of these phenomena may lay the foundation for selecting new drugs and screening targets to treat this devastating disease.

2. Materials and Methods

2.1. Animal. Male APPswe/PS1dE9 (APP/PS1) transgenic mice with a C57BL/6J background and nontransgenic littermates (C57BL/6 mice) were purchased from the Jiangsu ALF Biotechnology Co., Ltd (Nanjing, China). All mice were housed under controlled room temperature (20–24°C) and humidity (60–80%) and received food and water ad libitum. Three-month-old APP/PS1 mice and C57BL/6 mice were used for this study, and the experimental protocol was approved by the Medicine Animal Welfare Committee of the First Affiliated Hospital of Hunan Normal University (Hunan Provincial People's Hospital).

2.2. Drug Treatment. GLTs were provided by the Supercritical Fluid Technology Research Center, Institute of Geochemistry, Chinese Academy of Sciences (Guiyang, China). Donepezil was purchased from Apharm Co., Ltd. (Daegu, South Korea). A total of 50 APP/PS1 mice were separated into five experimental groups: AD group ($10\,mL\,kg^{-1}$ normal saline), low-dose GLT group ($0.35\,g\,kg^{-1}$ GLTs), high-dose GLT group ($1.40\,g\,kg^{-1}$ GLTs), positive control group ($0.38\,g\,kg^{-1}$ donepezil), and solvent group ($10\,mL\,kg^{-1}$ edible oil). The above dosage was given by gavage once a day for 60 days. The other 10 C57BL/6 mice were in the normal control group ($10\,mL\,kg^{-1}$ normal saline).

2.3. Morris Water Maze (MWM) Test. An MWM test (recording system produced by Techman, Chengdu, China) was conducted after the intragastric gavage of GLTs and donepezil to determine changes in cognitive ability. The MWM experiments were divided into place navigation test and spatial probe test. The mice in place navigation test were trained once a day in the morning lasting for 9 days. The mice were put into the water from any of the three quadrants outside the safe platform to the wall of the pool, and the time of finding the safe platform within 120 s (escape latency) and the length of the swimming path (search distance) were recorded, and the mice were allowed to stay on the platform for 10 s. If the security platform cannot be found after 120 s, it is recorded as 120 s, and the mice were guided to the security platform. The place navigation test's results, including the escape latency and search distance, were represented as the average of the results obtained in 1-9 days. The safety platform was removed on the 10th day to conduct spatial probe test. The mice were put into the water from the same entry point, and the search time (exploration time) of the mice in the quadrant of the original safety platform within 120 s was recorded, as well as the percentage of the swimming distance of the rats in the quadrant of the original safety platform in the total distance (exploration distance percentage). The experiments were carried out at 8 am and 3 pm to preclude the influence of circadian rhythm, and the laboratory was kept quiet, and the temperature and light intensity were as consistent as possible.

2.4. Hematoxylin-Eosin (HE) Staining. The morphological changes of the CA1 area of the mouse hippocampus was observed by pathological examination. At the end of the MWM experiment, the mice were cut and their brain tissue was removed and quickly placed on the ice tray along the sagittal suture. The brain tissues were fixed in 4% formalin solution at 4°C for 8 h, taken out in a 70% ethanol solution for 5 min, then placed in 80%, 90%, 95%, and absolute ethanol for gradient dehydration for 4 h each time, respectively; finally, tissues were immersed in xylene for 30 min, and then embedded in paraffin. Continuous coronal sections at the optic chiasma area (including the hippocampus) were taken, and the slice thickness was 3 μm. Each specimen was taken for 10 consecutive slices for the HE tests.

2.5. Silver Staining. Silver nitrate staining was applied to observe neuronal tangles (NFTs) in the CA1 area of the mouse hippocampus. In brief, the paraffin section was dewaxed and placed in a 20% silver nitrate aqueous solution and immersed in a 37°C incubator for 30 min in the dark; after distilled water washing for 3 min, 10% of the formaldehyde solution was treated for several seconds until the section was yellow; after washing with distilled water for 5 min, the ammonia silver droplets were applied to dye for 40 s; 10% formaldehyde was again treated for 2 min and then 5% sodium thiosulfate solution was used to fix for 5 min. Finally, NFTs were observed under an optical microscope.

2.6. TUNEL Staining. Apoptosis of neurons in the CA1 area of the mouse hippocampus was measured by TUNEL staining (Roche, Nutley, NJ, USA). The paraffin-embedded tissue was cut into 4-5 μm thick sections. The sections were then incubated in 50 μL of the TUNEL mixture (47.5 μL of TUNEL label containing fluorescein isothiocyanate-conjugated dUTP and 2.5 μL of TUNEL enzyme) in a humidified chamber (60 min, 37°C). Control sections were incubated with 50 μL of TUNEL label solution containing no TUNEL enzyme. Sections were photographed and TUNEL-positive nuclei were detected with IP Lab Imaging Analysis Software (Fairfax, VA, USA). Apoptotic index was calculated using TUNEL-positive nuclei/total number of nuclei×100 automatically.

2.7. Western Blotting Analysis. RIPA lysate (Beyotime, Shanghai, China) was used to obtain total proteins, 100 μg of which were segregated using SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. TBST containing 5% skim milk was used for membrane incubation for 1 h. Then, the membranes experienced incubation with primary antibodies including Anti-Bax antibody (ab77566, 1 μg/mL, Abcam, Cambridge, MA, USA), anti-Bcl-2 antibody (ab196495, 1 : 500, Abcam), anti-Caspase-3 antibody (ab13847, 1 : 500, Abcam), anti-Cleaved Caspase-3 antibody (ab2302, 1 μg/mL, Abcam), anti-Nrf2 antibody (ab137550, 1 : 500, Abcam), anti-Heme Oxygenase 1 antibody (ab13243, 1 : 2000, Abcam), anti-NQO1 antibody (ab2346, 0.3 μg/mL, Abcam), anti-ROCK1 antibody (EP786Y) (ab45171, 1 : 2000, Abcam), anti-ROCK2 antibody (ab71598, 1 μg/mL, Abcam), and anti-GAPDH (ab181603, 1 : 10000, Abcam) at 4°C overnight. The membranes were washed in TBST three times and incubated with anti-rabbit IgG H&L (HRP) secondary antibody (ab6721, 1 : 2000, Abcam) at room temperature for 1.5 h. After washing using TBST thrice, the membranes were subjected to color reaction by ECL Plus from Life Technology, and GAPDH was detected as control groups.

2.8. Primary Culture of Hippocampal Neurons. C57BL/6 mice born for 0-48 h were taken out and the brain was taken aseptically after sacrifice. The hippocampus tissue was isolated, washed in a Petri dish containing D-Hank's solution, and then 1-2 mL 0.02% EDTA and 0.25% trypsin digest were added into the dish; the hippocampus tissues were cut into small pieces, then transferred into a 10 mL centrifuge tube for digesting for 10-15 min; then, 4-6 mL DMEM/F12 medium containing 20% fetal bovine serum to terminate digestion was added to the decomposition liquor; the digestive solution was filtered with nylon sieve, centrifuged at 1000 rpm for 10 min, and cells were collected. Hippocampus cells were seeded in a 96-well culture plate at 1×10^6 cells at 200 μL per well and incubated in a 37°C, 5% CO_2 incubator. After 7 days of culture, cells were used for follow-up experiments.

2.9. AD Cell Model and Drug Administration. Amyloid β-protein 25-35 ($A\beta_{25\text{-}35}$, Sigma-Aldrich, St. Louis, MO, USA) was applied to induce hippocampal neuron cells into AD cells. At first, $A\beta_{25\text{-}35}$ was diluted to a concentration of 5 μg/μL with sterile physiological saline and incubated at 37°C for one week to become aggregated $A\beta_{25\text{-}35}$. Hippocampal neuron cells were divided into six groups: blank control group (control), model group ($A\beta_{25\text{-}35}$), low-dose GLT group (3.0 μmol L^{-1} GLTs), middle-dose GLT group (30.0 μmol L^{-1} GLTs), high-dose GLT group (300.0 μmol L^{-1} GLTs), and vehicle group (drug control group which added an equivalent medium and 20 μmol L^{-1} $A\beta_{25\text{-}35}$). Hippocampal nerve cells in the treatment group were added with GLTs at the above dosage, and the model group was added with the same amount of culture medium. After 24 h of culture, $A\beta_{25\text{-}35}$ with a concentration of 20 μmol L^{-1} was added to both the treatment group and the model group, and the culture was continued for 24 h. The blank control group was given the same amount of medium.

2.10. MTT Assay. Hippocampus cells were inoculated into the 96-well plates at a density of 1×10^4 cells/well and stored in DMEM with FBS in it. At 37°C, cells were incubated for 4 h and 50 μL of MTT (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was added into each well and had their growth condition observed at the 12th h. To solubilize the crystals, 150 μL dimethylsulfoxide (DMSO) was added into each well after the supernatant was subsequently removed. The optical density (OD) was measured at 590 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.11. Determination of SOD, MDA, and LDH Levels. After drug treatment, the levels of superoxide dismutase (SOD), malondialdehyde (MDA), and lactic dehydrogenase (LDH) in the hippocampal neuron cell were then measured using a

SOD test kit, MDA test kit, and LDH test kit, which were purchased from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). The experiment was conducted in accordance with the instructions of the kit. The parallel experiment was repeated three times.

2.12. Flow Cytometry Analysis. To detect cell apoptosis, PE Annexin V (BD Biosciences, San Jose, CA, USA) was applied, keeping to the instructions of the manufacturers. An FACSCalibur FCM (BD Biosciences) was used to observe cell apoptosis. Experiments in triplicate helped to reduce errors. FACS Diva software was adopted at data analysis.

2.13. Statistical Analysis. Experimental data analyses are performed with GraphPad Prism v6.0 statistical software and presented as mean ± standard deviation (SD) of results from three or more independent repetition experiments. Student's *t*-test was used to compare the differences between two groups. One-way ANOVA was applied to analyze three groups or above. P values < 0.05 accepted as the scale for statistical significance.

3. Results

3.1. Ganoderma lucidum Triterpenoids (GLTs) Alleviate Cognitive Impairment of AD Mice. To explore the effect of GLTs on the 3 month APP/PS1 mouse's symptoms of dementia in behavior, it was detected with Morris water maze (MWM) test. The MWM test in our study included place navigation test and spatial probe test to analyze and infer the learning and memory, spatial orientation, and cognitive level of AD mice. As the results of place navigation test showed in Figures 1(a) and 1(b) , compared to the normal C57BL/6 mice, the average escape latency of occult platform (Figure 1(a)) and average search distance (Figure 1(b)) increased in AD mice. But the average escape latency period was shortened and the average search distance was reduced after gavage of GLTs. The results of the spatial probe test revealed that GLTs increased space exploration time (Figure 1(c)) and exploration distance percentage (Figure 1(d)), alleviating cognitive impairment of AD mice. These results suggest that the 5-month-old APP/PS1 mice presented impaired acquisition of spatial learning and GLTs could reduce cognitive impairment in AD mice.

3.2. Effects of GLTs on the Hippocampal Tissue Structure and Neuronal Tangles in the Hippocampus of AD Mice. In order to more intuitively reflect the effect of GLTs on the improvement of the hippocampal tissue of AD mice, HE staining was applied and presented in Figure 2(a). In the normal control group, the nerve cells were arranged neatly and round; the cell structure was intact and the cell membrane and nucleus were clear; and there was no obvious swelling and necrosis. The nerve cells in the AD group were extremely disordered and irregular in size and shape. The number of nerve cells was dramatically reduced, and the cell structure was blurred. After gavage of GLA in AD mice, high integrity of nerve cells was maintained and there was no obvious necrosis of nerve cells (Figure 2(a)). As neurofibrillary tangles (NFT) are an important pathological feature of AD patients, the number of NFT in the cytoplasm was detected. As showed in Figure 2(b), in the AD model mice, the number of neurons in the cortex and hippocampus decreased and the number of NFT greatly increased. Compared to the model group mice, the number of neurons in the cortex and hippocampus increased and the number of NFT in the cytoplasm significantly decreased in the GLT group.

3.3. Effects of GLTs on Apoptosis and Oxidative Damage in the Hippocampal Area of AD Mice through ROCK Signaling Pathway. To explore the mechanisms by which GLTs improve cognitive impairment in AD mice, apoptosis, oxidative damage, and ROCK signaling pathways were investigated. As TUNEL assay, compared to the normal control group, the number of apoptotic positive cells in the AD group observably increased, but the number of apoptotic positive cells in the GLT group and the positive drug group prominently decreased (Figure 3(a), $P < 0.05$). Subsequently, the expression levels of apoptosis-related protein Bax, Bcl2, and caspase 3/cleaved caspase 3 in different groups were detected. The Bax and caspase 3/cleaved caspase 3 protein expression were dramatically elevated while Bcl2 protein expression decreased in the AD group. However, GLTs could reduce the apoptosis of neurons. Compared to the AD group, GLTs markedly reduced Bax and caspase 3/cleaved caspase 3 protein expression and increased Bcl2 protein (Figure 3(b), $P < 0.05$). Then, the expressions of antioxidative protein Nrf2 and the downstream antioxidant enzymes NQO1 and HO1 were detected in the AD mouse hippocampus (Figure 3(c)). The Nrf2, NQO1, and HO1 protein levels were notably declined in the AD group, while GLTs recovered the antioxidative protein levels in the AD mice. As displayed in Figure 3(d), the protein expression of ROCK1 and ROCK2 in the hippocampal tissues were dramatically enhanced in the AD group, but GLTs attenuated the increase of ROCK signaling pathway-associated proteins in AD mice ($P < 0.05$). It leads to the conclusion that GLTs inhibit apoptosis, relieve oxidative damage, and inactivate the ROCK signaling pathway to play a protective role in AD mice.

3.4. The Protective Effect of GLTs on Hippocampal Neuron AD Model Cells. To figure out the protective effect of GLTs on AD *in vitro*, AD model cells were established by $A\beta_{25\text{-}35}$ treatment. Cell proliferation activity of neurons under $A\beta_{25\text{-}35}$ or GLT treatment was determined by MTT assay. Compared to normal neurons, $A\beta_{25\text{-}35}$ notably inhibited cell viability, while GLTs alleviated the inhibitory effect of $A\beta_{25\text{-}35}$ on neuron proliferation (Figure 4(a), $P < 0.05$). To explore the antioxidant effects of GLTs, the expression level of SOD, MDA, and LDH were measured. As presented in Figure 4(b), the SOD expression level was observably declined in $A\beta_{25\text{-}35}$-treated neurons ($P < 0.05$). Conversely, different concentrations of GLTs could facilitate SOD expression. However, the MDA (Figure 4(c)) and LDH (Figure 4(d)) expressions in the $A\beta_{25\text{-}35}$ group were markedly aggrandized compared to the control group ($P < 0.05$). GLTs decreased the MDA and LDH levels to protect the hippocampal neurons. Then, the effect of different concentrations of GLTs on neurons' apoptosis was detected (Figure 4(e)). The

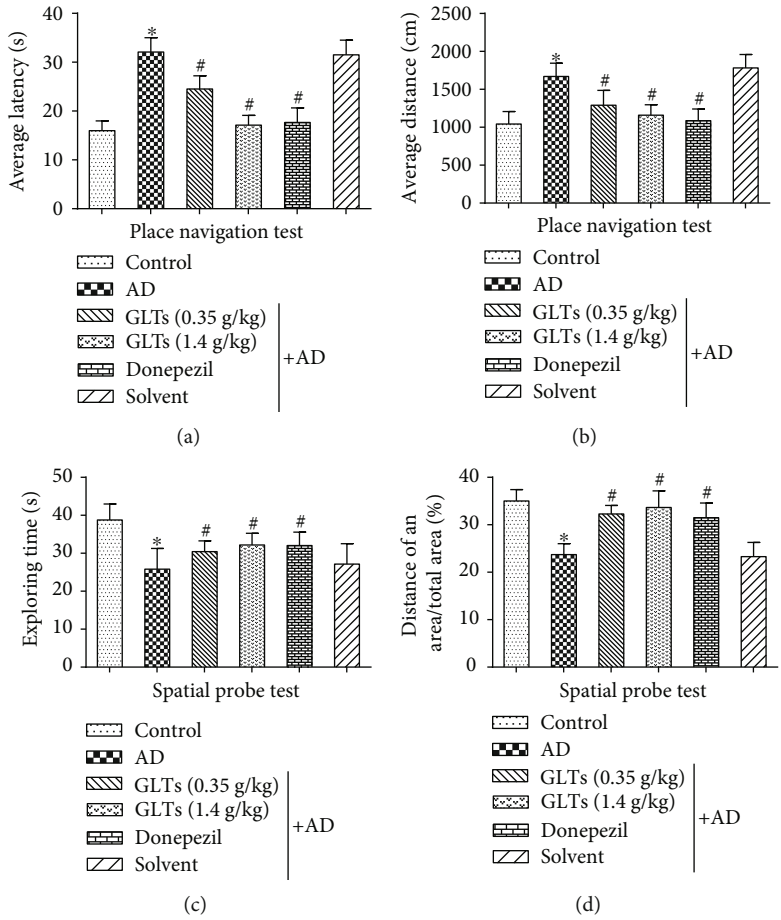


Figure 1: GLTs alleviate cognitive impairment of APP/PS1 transgenic AD mice. (a) The average escape latency of place navigation test in 1-9 days in different groups. (b) The average search distance of place navigation test in 1-9 days in different groups. (c) The space exploration time of spatial probe test in different groups. (d) The exploration distance percentage of spatial probe test in different groups. $N = 6$; $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AD group.

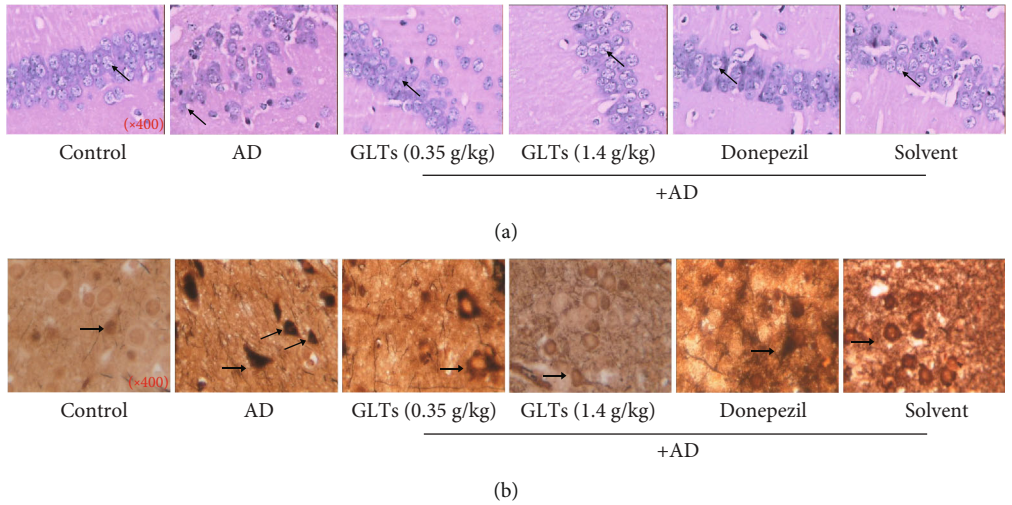


Figure 2: Effects of GLTs on the hippocampal tissue structure and neuronal tangles of the hippocampus of APP/PS1 transgenic mice. (a) HE staining was applied to present pathological changes in hippocampus tissues of mice in each group (×400). (b) The number of neuronal tangles (NFTs) in the CA1 area of the mouse hippocampus was detected by silver staining in each group (×400), $N = 6$.

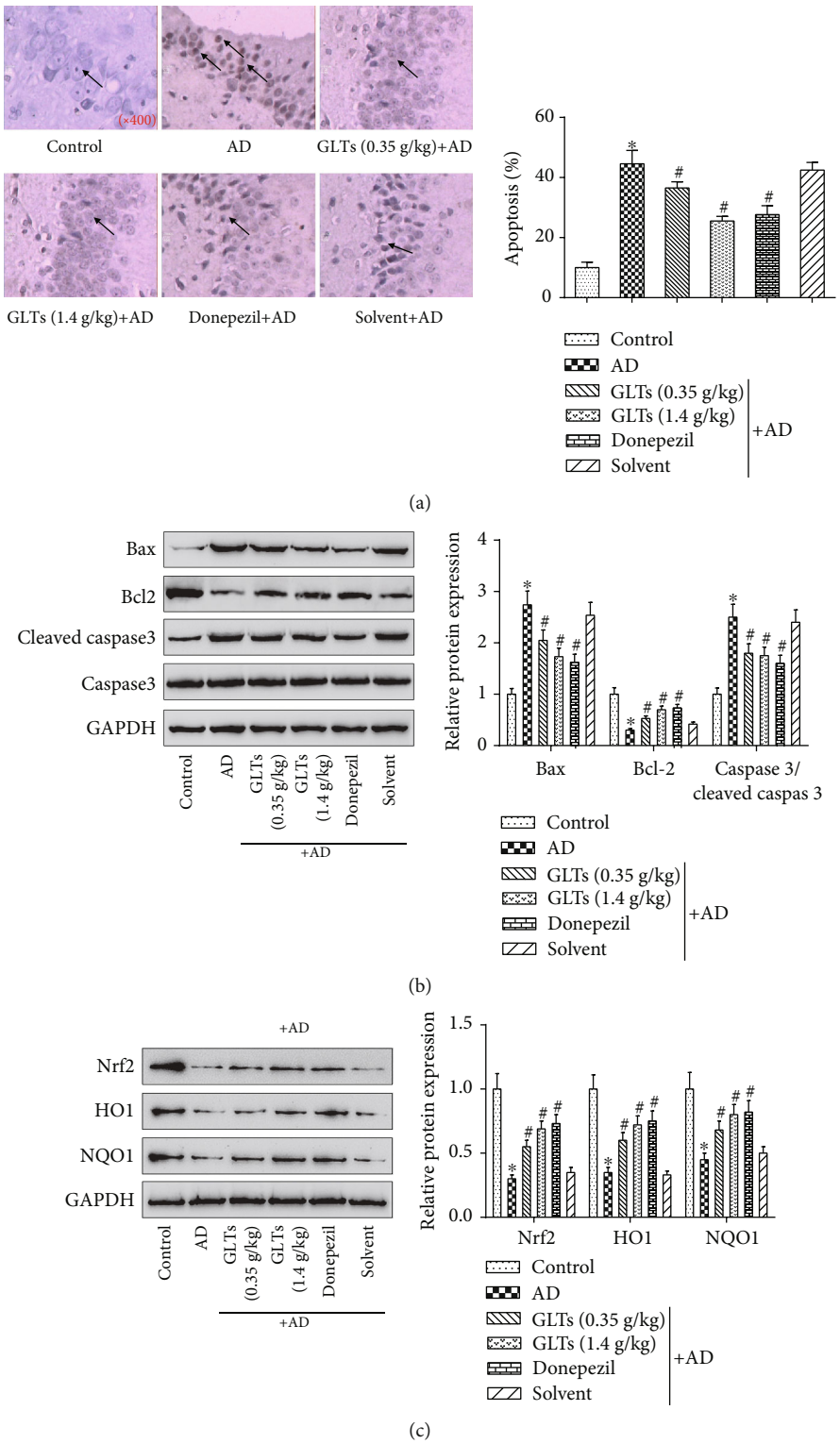
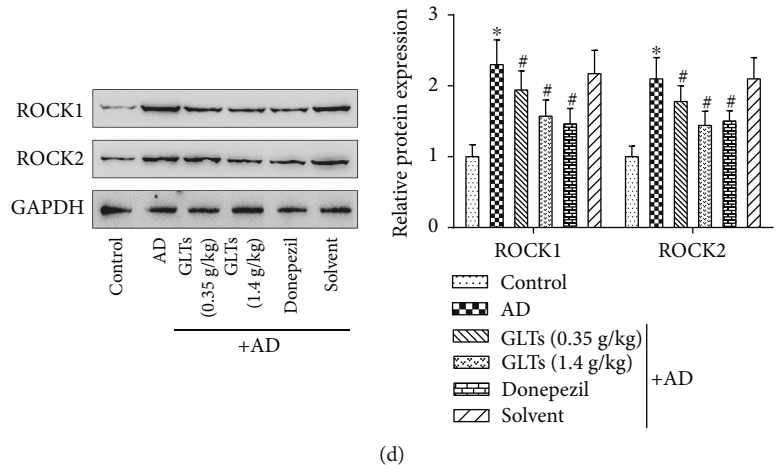


Figure 3: Continued.



(d)

Figure 3: Effects of GLTs on apoptosis and oxidative damage in hippocampal area of APP/PS1 transgenic mice through ROCK signaling pathway. (a) The number of apoptotic positive cells in the CA1 area of the mouse hippocampus was measured in each group by TUNEL assay (×400). (b) The expression of apoptosis-related protein Bax, Bcl2, and caspase 3/cleaved caspase 3 in each group was detected by western blot. (c) Western blot analysis of the antioxidative proteins Nrf2, HO1, and NQO1 expression levels in the mouse hippocampus. (d) The protein expression of ROCK signaling pathway-associated proteins ROCK1 and ROCK2 in hippocampal tissues was determined. $N = 6$; $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the AD group.

apoptosis rate was prominently fortified in the $A\beta_{25\text{-}35}$ group, while GLT treatment could restrain apoptosis of the hippocampal neurons ($P < 0.05$). The results suggested that GLTs play an antioxidant and inhibit apoptosis of the hippocampal neurons.

3.5. GLT Protects Hippocampal Neurons by Inhibiting the ROCK Signaling Pathway. In order to explore whether GLTs protect hippocampal neurons by inhibiting ROCK signaling pathway and apoptosis, the ROCK signaling pathway inhibitor Y-27632 was added in the process of the experiment. The results in Figure 5(a) revealed that $A\beta_{25\text{-}35}$ remarkably promoted Bax and caspase 3/cleaved caspase 3 protein expression and inhibited Bcl2 protein expression. While GLT or Y-27632 treatment memorably restrained Bax and caspase 3/cleaved caspase 3 protein expression and facilitated Bcl2 protein expression ($P < 0.05$). Next, the ROCK1 and ROCK2 proteins were detected by western blot (Figure 5(b)). $A\beta_{25\text{-}35}$ prominently facilitates ROCK1 and ROCK2 protein expressions compared to normal neurons ($P < 0.05$). The inhibitory effect of GLTs on ROCK1 and ROCK2 proteins was consistent with that of Y-27632. Collectively, these data indicated that GLTs inhibit apoptosis and deactivate the ROCK signaling pathway to protect the hippocampal neurons from AD.

4. Discussion

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by continuous cognitive decline and worsening of daily living performance, with no effective treatment as yet, and lays a tremendous burden on human society [20, 21]. As the pathogenic mechanism of AD is intricate and multifaceted, there is currently no effective therapeutic drugs to prevent or treat AD. Two types of drugs, acetyl choline esterase (AChE) inhibitor and N-methyl D-aspartate (NMDA) receptor antagonist, have been clinically prescribed for patients to improve their symptoms, yet without halting or reversing the pathological process [22, 23]. For centuries, many medicinal herbs and dietary supplements have been applied to ameliorate cognitive function and relieve symptoms associated with AD [24, 25]. *G. lucidum*, an edible medicinal mushroom, has diverse bioactivities including antidiabetes, antitumor, and immunomodulation and has been proved to ameliorate health and longevity for centuries in the Orient [26]. Huang et al. demonstrated that the polysaccharides from *G. lucidum* could ameliorate cognitive function and neural progenitor proliferation in a mouse model of AD [27]. However, as the major variety of bioactive and medicative components of *G. lucidum*, the therapeutic effect and molecular mechanism of GLTs on AD are not clear yet. In the present research, the APP/PS1 transgenic mouse model *in vivo* and $A\beta_{25\text{-}35}$-induced hippocampal neuron cell model *in vitro* of AD had been established to investigate the effect of GLTs on the therapeutic action of AD. Animal experiments showed that 5-month-old APP/PS1 mice revealed impaired acquisition of spatial learning and GLTs could reduce cognitive impairment in AD mice. Besides, the pathological features of AD include the presence of amyloid plaques, neuronal death, and neurofibrillary tangles (NFTs) [28]. We also found that the number of NFT in the cytoplasm in AD mice significantly decreased after GLT treatment. Subsequent cell experiments also showed that GLTs alleviated the inhibitory effect of $A\beta_{25\text{-}35}$ on neuron proliferation and had antioxidant effects. Here, we evidence that GLTs ameliorated cognitive dysfunction in transgenic AD model mice.

More recently, apoptosis has been attached great concern as an essential determinant in the pathological course of AD [29]. Apoptosis is a sequence of programmed events leading to the activation of caspases and cell disintegration [30]. Apoptosis plays crucial roles in tissue homeostasis, and squint towards being upregulated in neurodegenerative

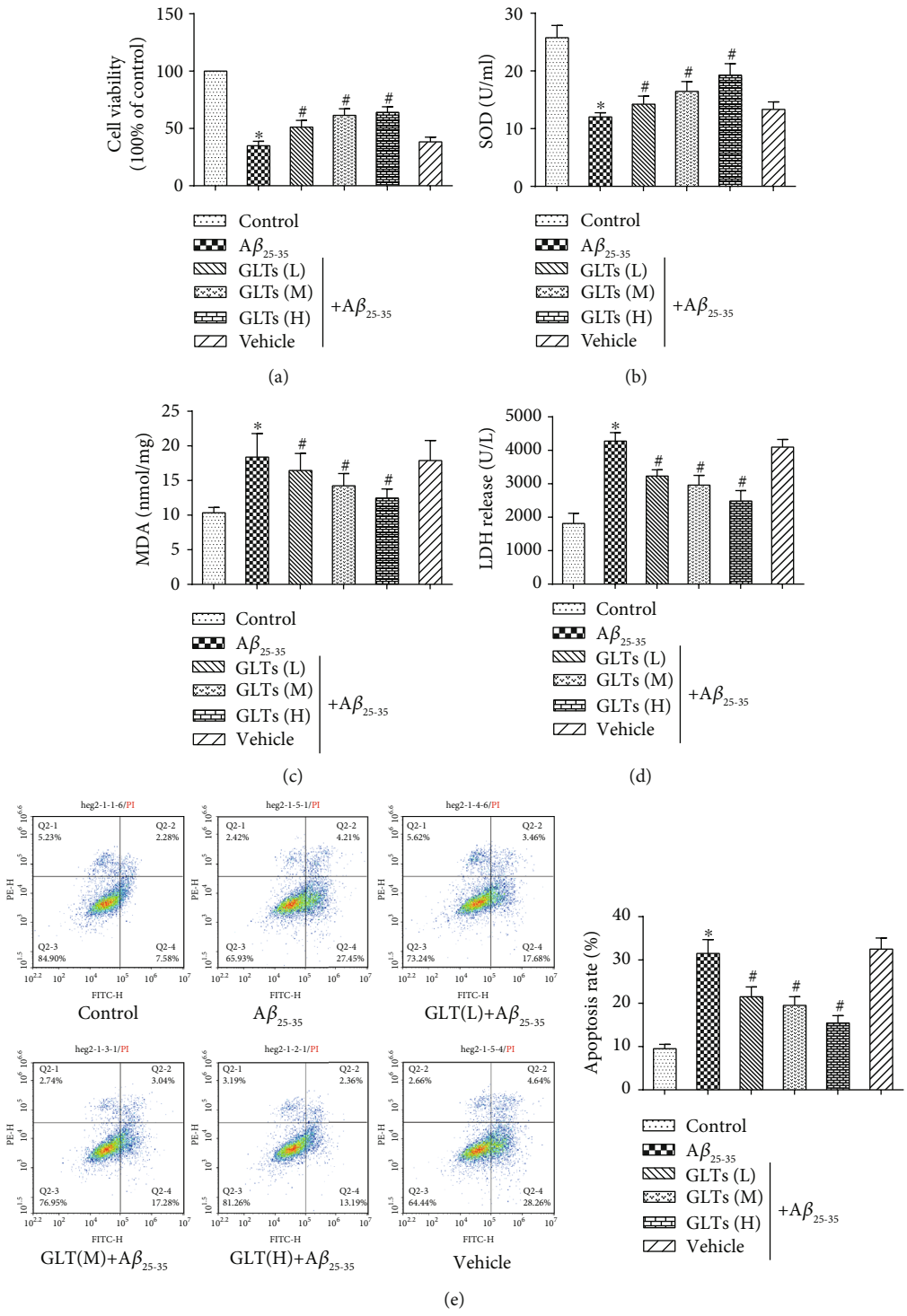


Figure 4: The protective effect of GLTs on hippocampal neuron AD model cells. Hippocampal neuron AD model cells were induced by $A\beta_{25\text{-}35}$ treatment. (a) Cell proliferation activity of neurons under $A\beta_{25\text{-}35}$ or GLT treatment was determined by MTT assay. (b) The SOD expression level in each group was measured by SOD test kit. (c) The MDA expression level in each group was measured by MDA test kit. (d) The LDH expression level in each group was measured by LDH test kit. (e) The apoptosis rate in each group was detected by flow cytometry analysis. $N = 3$; $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the $A\beta_{25\text{-}35}$ group.

disease [31]. In AD, the processes have been proverbially studied but the contribution to neuronal death remains unclear. In our study, the effect of GLTs on apoptosis of hippocampal tissues and neurons in AD mice was studied in detail. GLTs decreased apoptosis rate and the expression of apoptosis-related protein Bax, Bcl2, and caspase 3/cleaved caspase 3 in both hippocampal tissues and neuron cells to play a protective role in AD mice.

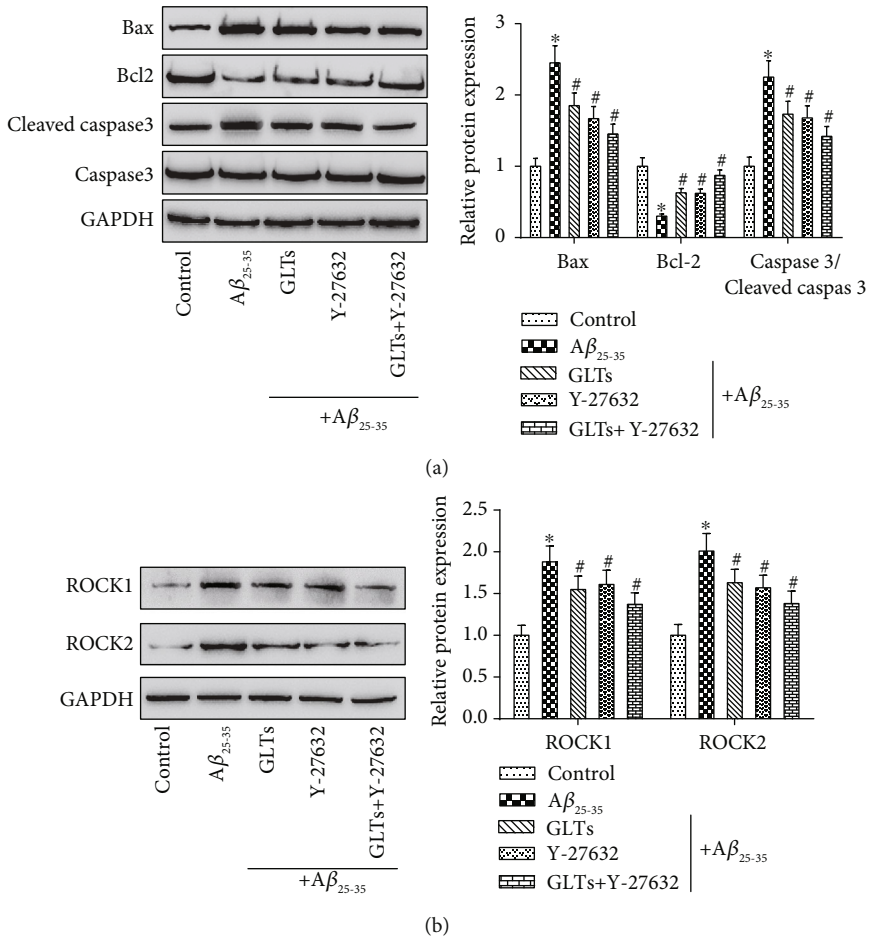


Figure 5: GLTs protect the hippocampal neurons from $A\beta_{25\text{-}35}$ damage by inhibiting the ROCK signaling pathway. The ROCK signaling pathway inhibitor Y-27632 was added in the process of experiment. (a) The expression of apoptosis-related protein Bax, Bcl2, and caspase 3/cleaved caspase 3 in each group was detected by western blot. (b) The protein expression of ROCK signaling pathway-associated proteins ROCK1 and ROCK2 in each group was determined. $N = 3$; $^{*}P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to $A\beta_{25\text{-}35}$ group.

What's more, the ROCK signaling pathway had also been found to be involved in AD progression. We found the protein expression of ROCK1 and ROCK2 in hippocampal tissues and neuron cells were dramatically enhanced in AD mice, but GLTs attenuated the increase of ROCK signaling pathway-associated proteins in AD mice. Consistent with our findings, Park et al. identified that RhoA-ROCK signaling pathway was activated in $A\beta_{42}$-induced blood–brain barrier (BBB) [32]. Moreover, as a pivotal adjuster of cytoskeletal proteins, ROCK1/ROCK2 activity is considerable for the structural maintenance of neuronal processes which underlie synaptic transmission and cognitive functions [33]. These findings, along with our research results, indicate that ROCK signaling is the most determinate risk factor of AD [34]. In addition, oxidative stress damage in the hippocampus of the brain is the crucial pathologic changes during the early phase of AD [35]. Patients with slight cognitive impairment had been identified to have oxidative damage before evolving into AD [36]. Our results disclosed that antioxidative protein Nrf2, HO1, and NQO1 in the hippocampus of transgenic AD model mice was observably reduced, and GLT treatment counteracted this attenuation. This result indicated that GLTs could potentially exert a protective effect on AD. Similar to our results, Özevren et al. provide evidence for *G. lucidum* protects rat brain tissue against trauma-induced oxidative stress [37]. So, we concluded that GLTs relieved oxidative damage and inactivated the ROCK signaling pathway to play a protective role in AD mice and the multitarget effects of GLTs may have desirable advantages for the treatment of multifactorial neurodegenerative diseases such as AD.

However, the limitations of this research have yet to be considered. Despite progress in revealing therapeutic potentials of *G. lucidum* or GLTs, the complete regulation molecular targets of GLTs require additional research disclosure. Besides, the application of GLTs to the treatment of human diseases, including AD, requires more rigorous and scientific verification and clinical trials.

In conclusion, we discovered that GLTs relieved cognitive impairment and decreased NFT numbers in APP/PS1 transgenic AD model mice by inhibiting apoptosis and inactivating the ROCK signaling pathway. Besides, we revealed

that GLTs facilitated hippocampal neuron proliferation and played antioxidant effect *in vitro* experiments. These findings may provide new clues for future therapeutic target research for this deadly disease.

Data Availability

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

Ethical Approval

The guidelines for the care and use of animals were approved by the Medicine Animal Welfare Committee of First Affiliated Hospital of Hunan Normal University (Hunan Provincial People's Hospital.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Nanhui Yu and Yongpan Huang contributed equally to this work.

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Research Article

Maternal Separation Induces Long-Term Alterations in the Cardiac Oxytocin Receptor and Cystathionine γ-Lyase Expression in Mice

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We recently showed that blunt chest trauma reduced the expression of the myocardial oxytocin receptor (Oxtr), which was further aggravated by genetic deletion of the H_2S-producing enzyme cystathionine γ-lyase (CSE). Exogenous H_2S supplementation restored myocardial Oxtr expression under these conditions. Early life stress (ELS) is a risk factor for cardiovascular disease by affecting vascular and heart structures. Therefore, we tested the hypotheses that (i) ELS affects cardiac Oxtr and CSE expressions and (ii) Oxtr and CSE expression patterns depend on the duration of stress exposure. Thus, two stress paradigms were compared: long- and short-term separation stress (LTSS and STSS, respectively). Cardiac Oxtr expression was differentially affected by the two stress paradigms with a significant reduction after LTSS and a significant increase after STSS. CSE expression, which was significantly reduced in $Oxtr^{-/-}$ knockout hearts, was downregulated and directly related to Oxtr expression in LTSS hearts ($r = 0.657$, $p = 0.012$). In contrast, CSE expression was not related to Oxtr upregulation in STSS. Plasma Oxt levels were not affected by either ELS paradigm. The coincidence of LTSS-induced reduction of cardiac Oxtr and reduced CSE expression may suggest a novel pathophysiological link between early life adversities and increased risk for the development of cardiovascular disorders in adulthood.

1. Introduction

Maternal separation (MS) is a robust and widely used animal model for inducing early life stress (ELS), a paradigm that is applied using different protocols, especially regarding the duration and time point of stress exposure [1, 2]. Depending on the type of ELS exposure, rodents showed adaptive responses resulting in resilience to stressors encountered later in life, ultimately resulting in psychological and physiological well-being [3, 4]. Our previous studies in mice revealed that "mild" short-term separation stress (STSS) induced by MS during postnatal days (PND) 14-16 resulted in reduced depressive-like behavior in adulthood [5] and epigenetically regulated activation of synaptic plasticity gene expression in the hippocampal formation, which was paralleled by an increase in dendritic complexity and number of excitatory spine synapses [6]. In contrast, we observed that "chronic" long-term separation stress (LTSS) induced by

MS from PND 1 to PND 21 and subsequent social isolation increased depressive-like behavior in adult males with elevated hippocampal Oxtr gene expression upon an adult stress challenge [5, 7]. Another study demonstrated that intracerebroventricular Oxt injections protected against the development of ELS-induced depressive-like behaviors through modulation of hippocampal mitochondrial function and neuroinflammation [8].

It is well established that Oxt acts not only as a neuromodulator, released from hypothalamic neurons, regulating social-emotional behavior [9], e.g., mother-child relationship [10], but also as a peripheral hormone. Beyond promotion of parturition and lactation [11], Oxt also critically influences peripheral organ functions [12]. In particular, Oxt exerts cardioprotective effects via negative chronotropic and inotropic properties [13], release of nitric oxide [14], anti-inflammatory and antioxidative properties [15], and modulating glucose utilization [16]. Since ELS, such as childhood maltreatment, may provide a critical programing factor for the development of coronary artery disease (CAD), diabetes [17], and hypertension [18] at later life periods, it is tempting to speculate that these effects might at least in part be mediated by ELS-induced changes of Oxt function. This hypothesis is supported by studies in animal models showing that MS results in cardiac changes including cardiomyocyte hypertrophy as well as cardiac fibrosis [19]. More data is available showing that MS results in changes on the vascular level by misprogramming of resistance artery smooth muscles [20], increased vasoconstriction [21], and blood pressure [22, 23]. These alterations are induced by superoxide production and endothelial dysfunction [24], inflammation [25], and sensitizing of the renal and sympathetic systems [26].

Hydrogen sulfide (H_2S) produced by vascular and cardiac activity of CSE [27] is another factor known to exert protective effects in the cardiovascular (CV) system by relaxation of vascular smooth muscles, thereby inducing vasodilation and reduction of blood pressure [28]. Interestingly, these effects are also reported for the Oxtr system [14]. Finally, both the H_2S [29] and oxytocin [30] systems exert their protective effects via antioxidant properties. However, this data might only suggest a possible interaction between the cardiac H_2S and Oxtr system in ELS. The H_2S-releasing salt NaHS attenuated the ELS-induced colonic epithelial damage, oxidative stress, and inflammation [31]. We have recently shown that the slow-releasing H_2S donor GYY4137 restored the myocardial Oxtr expression in mice lacking the H_2S-generating enzyme cystathionine γ-lyase (CSE) that had undergone cigarette smoke exposure to induce chronic obstructive pulmonary disease (COPD) prior to blunt chest trauma [32]. Hence, we hypothesized that ELS affects cardiac Oxtr expression and that these changes are dependent on the dose or duration of stress exposure. To test these hypotheses, we measured circulating Oxt plasma levels and cardiac tissue Oxtr protein expression in two different ELS paradigms: "chronic" LTSS and "mild" STSS. These measurements were complemented by analysis of the cardiac CSE expression, guided by the hypothesis that CSE is linked to stress-induced Oxtr changes in the heart.

2. Material and Methods

2.1. Animal Models

2.1.1. Housing Conditions. C57BL/6 mice were used for the present study. All experimental animals were bred in our animal facility and housed on a 12 h light-dark cycle with food and water provided ad libitum. During pregnancy, the home cages were cleaned once a week to minimize pregnancy stress. After delivery of the pups (day of birth = postnatal day, PND 0), the home cages were not cleaned for the first 16 PND to minimize stress for the mother and her pups. To prevent potential litter effects, a split litter design was used, and males from different litters were randomly assigned to the four different experimental groups, two different stress groups, and the respective control groups (see below). Animals for the respective experiments were derived from at least 7 litters per group, with the exception of the Oxt plasma concentration analysis in the LTSS animals. Litter size was between 6 and 8 animals with random distribution of male : female ratio; however, only litters with near-equal male : female ratio were used for experiments. All animals were handled in accordance with the German guidelines for the care and use of animals in laboratory research. The protocols were approved by the ethics committee of the government of the state of Saxony-Anhalt (§8 TSchG; AZ: 42502–2-1272).

2.2. "Chronic" Long-Term Separation Stress (LTSS)

2.2.1. LTSS Paradigm. Pups of this group were exposed to daily MS from PND 1 to PND 21 by removing them from the home cage and individually placing them in isolation boxes (13 × 13 cm, covered with paper bedding) for 3 h each day (9:00–12:00), which allowed olfactory and auditory but no visual or body contact with their separated siblings. During the first week, the isolation boxes were placed in a humidified incubator at 32°C. The dam remained undisturbed in the home cage. Prior to the return of the pups, fresh nesting material was provided in order to distract the mother from "overmothering" her pups during reunion. After weaning on PND 21, the animals were housed individually until the time of the respective experiment.

2.2.2. Control Animals (CON). Animals of this control group lived undisturbed with their mother and littermates. After weaning on PND 21, they were group housed with a maximum of 6 same-sex individuals until the time of the respective experiment on PND 64. Each experiment was conducted with a parallel individual control group. The control group used was identically treated as described here [7].

2.3. "Mild" Short-Term Separation Stress (STSS)

2.3.1. STSS Paradigm. Pups of this group were separated from their mother on PND 14-16 using the same separation conditions as described for the LTSS group (see above). After the last separation session on PND 16, the pups remained undisturbed until weaning on PND 21. On PND 21, the animals were reared in groups with a maximum of 6 individuals until the onset of the experiments on PND 64.

2.3.2. Control Animals (CON). Animals of the control group were treated and housed as described for the control animals of the LTSS experiment.

2.4. Homozygous and Heterozygous Oxtr Knockout. Male $Oxtr^{-/-}$ and $Oxtr^{+/-}$ knockout mice were maintained on a mixed 129 × C57BL/6J genetic background as described by Takayanagi et al. [33]. For immunohistochemical analyses, two animals were used, and for Western blot analyses, one animal was used.

2.5. Immunofluorescence. Control hearts were embedded in Tissue-Tek O.C.T.™. Cryosections (7 μm) were acetone-fixed and immunolabeled with the following antibodies: rabbit anti-Oxtr (1 : 1000, Sigma-Aldrich, St. Louis), mouse anti-smooth muscle actin (SMA, 1 : 100, Dako, Agilent, Santa Clara), Alexa Fluor goat anti-rabbit 488 (1 : 1000, Invitrogen, Carlsbad), and Alexa Fluor goat anti-mouse 555 (1 : 1000, Invitrogen, Carlsbad). Images were captured with a Leica DMI6000B microscope and edited with ImageJ 1.46.

2.6. Immunoblotting. On PND 64, mice were sacrificed by decapitation. Hearts were dissected, perfused with PBS, and immediately frozen in liquid nitrogen. For the lysate fractioning, whole hearts were mechanically homogenized in RIPA buffer containing phenylmethanesulfonyl fluoride (PMSF). Total lysates were obtained by centrifugation for 15 min at 4°C with 1000 × g. The membrane fraction used for Oxtr/-Gapdh Western blot analyses was gained by centrifugation of total lysates for 30 min at 4°C with 12,000 × g. In preliminary tests, this fraction showed the highest protein expression level. Protein concentrations were measured using BCA assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham). 50 μg protein was separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and blotted to a polyvinylidene fluoride (PVDF) membrane (Membrane Hybond-P, GE Healthcare, Chalfont St. Giles). Visualization was performed using a ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules). The following antibodies were used: rabbit anti-Oxtr (1 : 1000, Sigma-Aldrich, St. Louis), mouse anti-Gapdh (1 : 500, Thermo Scientific, Waltham), horseradish peroxidase (HRP) goat anti-mouse (1 : 10,000, Invitrogen, Carlsbad), and HRP swine anti-rabbit (1 : 1000, Dako, Agilent, Santa Clara). Optical densities of protein bands were determined using ImageJ 1.46. Oxtr was normalized to the housekeeping gene Gapdh.

2.7. Immunohistochemistry. Additional groups of LTSS and STSS and their respective control animals as well as $Oxtr^{-/-}$ knockout hearts were formalin-fixed and paraffin-embedded. 4 μm sections were stained with the following antibodies: rabbit anti-Oxtr (1 : 50, Proteintech, Manchester), rabbit anti-CSE (1 : 1000, Proteintech, Manchester), and secondary alkaline phosphatase- (AP-) conjugated goat anti-rabbit IgG (1 : 25, Jackson ImmunoResearch Europe Ltd., Cambridgeshire). The chromogen used for the AP reaction was AP red (Dako REAL Detection System, Dako, Denmark). Images were captured with a 10x lens using a Zeiss Axio Scope A1 microscope, and four 800,000 μm^2 sections per animal were quantified with Zeiss AxioVision Rel. 4.9.1 image analysis software. Results are presented as densitometric sum red.

2.8. Plasma Collection and Oxt Plasma Determination. After decapitation, 500 μl blood was collected and 10 μl EDTA solution (0.8 mg/ml EDTA, Carl Roth, Karlsruhe) was added. Next, 7.5 μl of aprotinin solution (10 mg/ml aprotinin, Sigma-Aldrich, St. Louis) was added to 500 μl of EDTA blood and centrifuged at 4°C for 5 min at 1600 × g. The supernatant was stored at -80°C until measurement. Oxt plasma levels were determined via radioimmunoassay (RIA, RIAgnosis, Sinzing, Germany).

2.9. Statistics. The statistical analysis was processed using SPSS software packages (Version IBM SPSS Statistics 24). For quantitative analyses, data are presented as median and interquartile range, if not stated otherwise. Data were tested for normal distribution using a Kolmogorov-Smirnov test. Due to the small sample size, nonparametric tests were used. The Mann-Whitney U test was performed for group comparisons. Correlations between Oxtr and CSE expressions were calculated using the Spearman rho test. Results were defined as significant at $p \leq 0.05$.

3. Results

3.1. Localization of Oxtr in Cardiac Tissue of $Oxtr^{-/-}$, $Oxtr^{+/-}$ Knockout, and Wild-Type Mice. Oxtr immunohistochemical staining was performed in left ventricular (LV) heart tissues (Figures 1(a), 1(b), 1(e), and 1(f)). Oxtr was visible in cardiomyocytes. The lack of Oxtr immunoreactivity in $Oxtr^{-/-}$ knockout mice and Western blot analyses in cardiac tissue of $Oxtr^{-/-}$, $Oxtr^{+/-}$ knockout, and wild-type mice confirmed the specificity of our antibody. Oxtr protein was significantly downregulated in cardiac tissue of both $Oxtr^{-/-}$ and $Oxtr^{+/-}$ mice compared to wild-type mice (Figures 1(a), 1(b), 1(e), 1(f), and 1(i)). Immunofluorescence microscopy with costaining of Oxtr and smooth muscle actin (SMA) revealed that Oxtr is located in smooth muscle cells of the arterioles (Figure 2).

3.2. CSE Expression in LV Heart Tissue of $Oxtr^{-/-}$ Knockout and Wild-Type Mice. CSE staining in LV heart tissue in $Oxtr^{-/-}$ knockout mice showed a reduction of CSE protein compared to LV tissue of wild-type animals (Figures 1(c), 1(d), 1(g), and 1(h)).

3.3. Cardiac Oxtr and CSE Expression Is Downregulated after LTSS Exposure. Quantitative Western blot analyses revealed a significant reduction of Oxtr protein expression in cardiac tissue of LTSS-exposed mice normalized to their unstressed controls ($p < 0.001$; Figures 3(a) and 3(b)). These results were corroborated by immunohistochemical stainings which confirmed the significant downregulation of Oxtr in cardiac tissue of LTSS-exposed animals compared to their controls ($p = 0.038$; Figures 3(c) and 3(d)). Immunohistochemistry revealed a significant downregulation of CSE protein in animals exposed to LTSS compared to their respective controls ($p = 0.038$; Figures 3(e) and 3(f)).

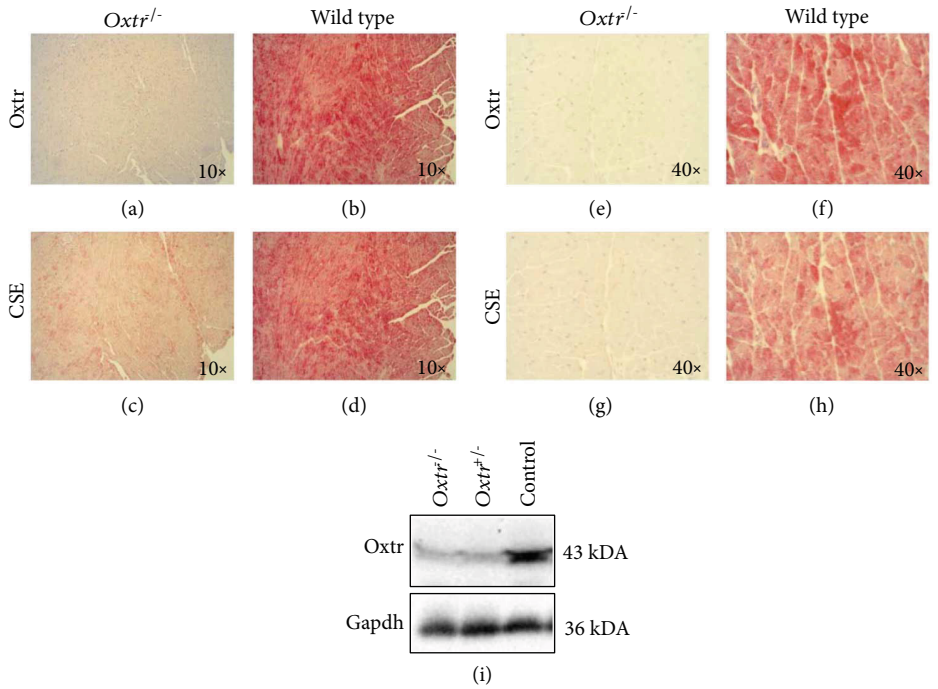

Figure 1: Immunohistochemical staining of Oxtr and CSE in the LV heart tissue in an $Oxtr^{-/-}$ knockout heart and a wild-type heart (×10 and ×40). Expression of Oxtr was absent in $Oxtr^{-/-}$ knockout heart (a, e) and clearly visible in wild-type myocardial tissue (b, f). CSE was significantly reduced in $Oxtr^{-/-}$ knockout hearts (c, g) compared to wild-type tissue (d, h). Western blot of $Oxtr^{-/-}$, $Oxtr^{+/-}$ knockout, and control hearts confirming the specificity of the Oxtr antibody. Oxtr protein expression was substantially reduced in $Oxtr^{-/-}$ and $Oxtr^{+/-}$ knockout heart tissue compared to controls (i). LV: left ventricular; Oxtr: oxytocin receptor; CSE: cystathionine γ-lyase.

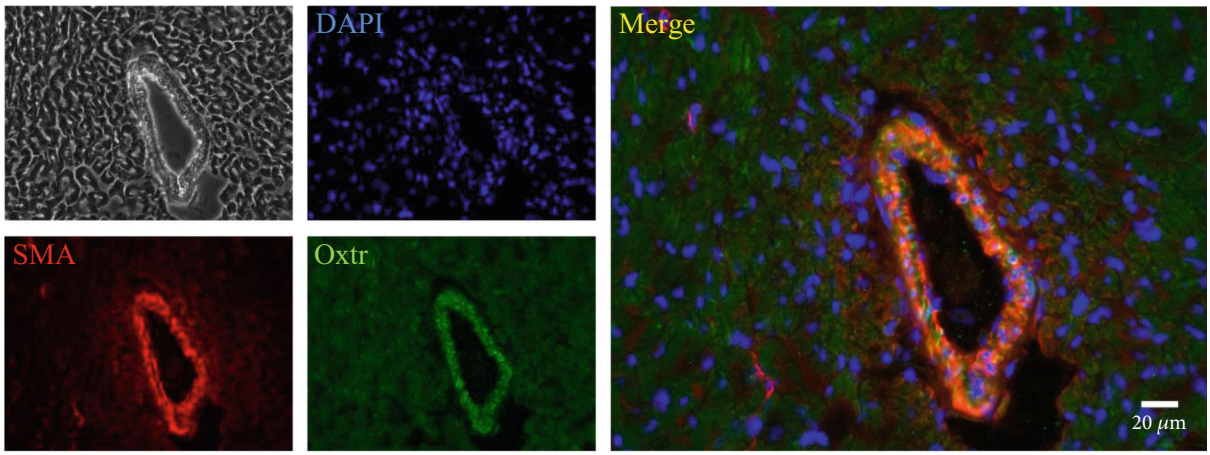

Figure 2: Immunofluorescence of the LV stained for Oxtr and SMA. Oxtr colocalized with SMA in a large arteriole indicating that Oxtr was highly expressed in arteriolar resistance vessels. LV: left ventricle; Oxtr: oxytocin receptor; SMA: smooth muscle actin.

3.4. Cardiac Oxtr Expression Is Upregulated after STSS. Quantitative Western blot analyses revealed an upregulation of cardiac Oxtr protein expression after exposure to STSS normalized to controls ($p < 0.001$; Figures 4(a) and 4(b)). These results were in line with immunohistochemical stainings that confirmed elevated Oxtr in the heart of STSS-exposed mice compared to unstressed controls ($p = 0.022$; Figures 4(c) and 4(d)). Immunohistochemistry revealed that CSE protein expression remained unchanged in animals exposed to STSS compared to their respective controls ($p = 0.710$; Figures 4(e) and 4(f)).

3.5. Oxtr and CSE Interaction Effects in LTSS and STSS. Correlation analyses between cardiac Oxtr and CSE expression yielded a linear correlation for LTSS and their respective controls ($r = 0.657$, $p = 0.012$; Figure 5(a)), whereas no statistically significant relation could be detected for STSS and their controls ($r = -0.033$, $p = 0.553$; Figure 5(b)).

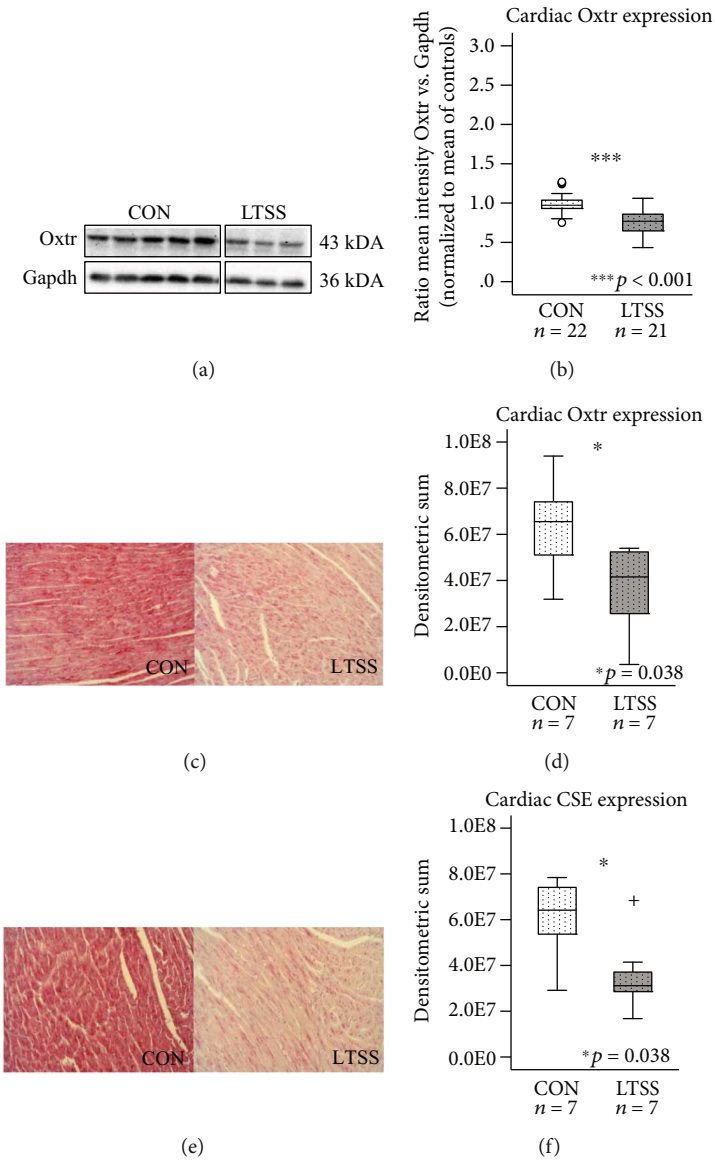

Figure 3: Western blot and immunohistochemical analyses of cardiac Oxtr and CSE expression in the LTSS paradigm. Representative Western blot of Oxtr protein expression (a) and quantitative results (b) revealed significantly reduced Oxtr expression in cardiac tissue of LTSS-exposed animals. Oxtr (d) and CSE (f) expression was significantly reduced in LTSS-exposed animals compared to their respective controls in immunohistological staining. Corresponding exemplary pictures of the LV myocardium are shown in (c, e). Data given as box plots (median, interquartile range, minimum, and maximum). $^{+}$Extreme value; Oxtr: oxytocin receptor; CSE: cystathionine γ-lyase; LTSS: long-term separation stress; CON: control; LV: left ventricular.

3.6. Adult Oxt Plasma Levels Remain Unchanged after LTSS or STSS. No significant differences in the Oxt plasma concentrations were found between mice exposed to LTSS (5 pg/ml (4 pg/ml; 11 pg/ml); $n = 4$) and their respective controls (7 pg/ml (4 pg/ml; 16 pg/ml); $n = 6$; $p = 1.0$). In addition, no difference was found between mice exposed to STSS (62 pg/ml (45 pg/ml; 77 pg/ml); $n = 18$) and their respective controls (54 pg/ml (48 pg/ml; 89 pg/ml); $n = 19$; $p = 0.964$).

4. Discussion

The present study tested the hypotheses (i) whether two different ELS paradigms, i.e., "chronic" LTSS and "mild" STSS, affect the Oxtr and CSE expression in adult cardiac tissue, (ii) if this effect is "dose"-dependent, and (iii) whether ELS-induced changes of Oxtr and CSE expression are correlated. In line with these hypotheses, we found that early postnatal-induced changes of adult cardiac Oxtr expression were critically dependent on the "dose" of ELS exposure, while LTSS resulted in reduced Oxtr expression in adult cardiac tissue compared to controls; the opposite was found after STSS, i.e., upregulation of Oxtr expression. Finally, our study provides further evidence for an interaction between the CSE/H_2S and oxytocinergic systems: in LTSS-exposed animals, reduced Oxtr expression was directly correlated with the CSE expression, which may indicate a functional interaction of these systems.

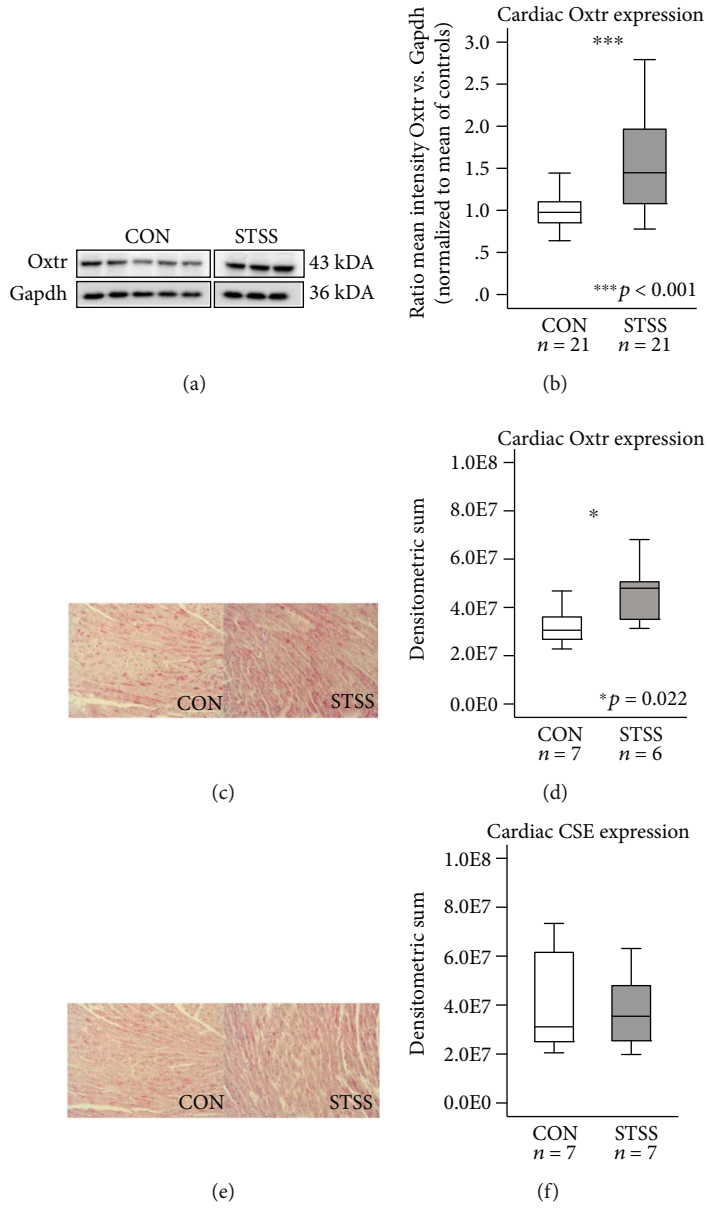

Figure 4: Western blot and immunohistochemical analyses of the cardiac Oxtr and CSE expression in the STSS paradigm. Representative Western blot of Oxtr protein expression in the STSS paradigm (a) and quantitative results (b) revealed significantly elevated Oxtr expression in STSS-exposed animals. Immunohistochemistry of Oxtr and CSE expression in the STSS-exposed animals compared to their respective controls revealed upregulated Oxtr expression after STSS (d). No significant changes in CSE expression were detectable after STSS (f). Corresponding exemplary pictures of LV myocardium are shown in (c, e). Data given as box plots (median, interquartile range, minimum, and maximum). Oxtr: oxytocin receptor; CSE: cystathionine γ-lyase; STSS: short-term separation stress; CON: control; LV: left ventricular.

Recently, we showed in adult mice that combining cigarette smoke exposure with acute blunt chest trauma (physical trauma) markedly reduced cardiac Oxtr expression [32]. The present study revealed that neonatal chronic psychological trauma due to ELS exposure (LTSS paradigm) also leads to a long-term reduction in cardiac Oxtr expression. Interestingly, we found that these effects were dependent on the "stress-dose": while "chronically" stressed LTSS animals displayed decreased Oxtr protein expression, "mild" STSS-exposed mice showed the opposite response, i.e., increased Oxtr expression. Applying the same stress paradigms, we previously demonstrated that LTSS increased depressive-like behavior in adulthood, an effect that was associated with alterations of the Oxtr expression in the hippocampus [7]. In contrast, STSS attenuated depressive-like behavior, paralleled by dendritic length, dendritic complexity, and spine number in the hippocampus, thus suggesting stress-induced adaptations of neuronal structures [5, 6]. Therefore, we conclude that "chronic" LTSS aggravates vulnerability, whereas "mild" STSS yields an opposite response by inducing adaptive processes that may promote resilience. In view of the CV protective functions of the Oxt/Oxtr system, it is tempting to

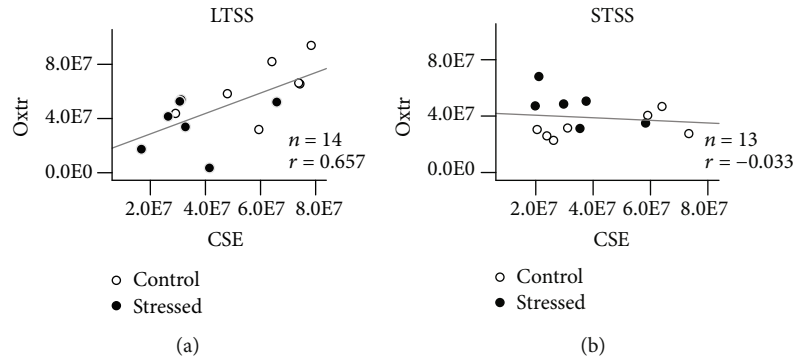

Figure 5: Regression analysis of Oxtr and CSE in the LV myocardium of LTSS- and STSS-exposed animals. Positive linear correlation between the Oxtr and CSE expression was detected in the LTSS paradigm (a). No significant correlation between Oxtr and CSE was found in the STSS paradigm (b). Oxtr: oxytocin receptor; CSE: cystathionine γ-lyase; LTSS: long-term separation stress; STSS: short-term separation stress; LV: left ventricular.

speculate that LTSS-induced long-term reduction of cardiac oxytocinergic functions may confer a risk for developing CV dysfunctions. In fact, it was shown that low Oxtr expression in infarcted LV tissue coincided with aggravated myocardial injury [15] underlining that Oxtr reduction leads to adverse CV health, whereas elevated Oxtr expression induced by exposure to "mild" STSS might reflect cardioprotective effects. This hypothesis is supported by studies showing that increased Oxtr expression dampens atherosclerosis and protects against myocardial infarction [15, 34].

The lack of changes in Oxt plasma levels which we found in mice exposed to LTSS or STSS is in contrast to other studies reporting increased Oxt plasma levels after stress exposure in rodents [35, 36]. However, in these studies, Oxt plasma levels were measured 5 to 30 min after stress exposure whereas our analyses presented here were performed weeks after stress exposure at resting conditions. Due to its short circulating half-life [37], basal Oxt plasma levels might have normalized in adulthood after ELS exposure. In fact, our finding is in good agreement with our previous study in women with CM experiences, whose basal Oxt plasma levels were unchanged in adulthood [38], and another study also showing that Oxt plasma levels are independent of the dose of maltreatment [39]. Nevertheless, this does not exclude that ELS may exert a "programming" effect on acute Oxt release during stress challenges at later life periods.

The Oxtr is expressed in cardiomyocytes [40], in endothelial cells [41], and in the vascular wall of large vessels [42] where it exerts both negative ino- and chronotropic [13], antihypertrophic [43], and vasodilatatory effects, the latter via NO activation [41]. Our experiments presented here show that the Oxtr is expressed not only in smooth muscles of large vessels and isolated primary cells but also in smooth muscle cells of arterioles. Arterioles as the main regulators of vascular resistance contribute to blood pressure regulation via smooth muscle-mediated changes in vessel diameter. Follow-up analyses will assess if ELS induces additional changes in Oxtr expression directly in the arteriolar system.

We also showed reduced cardiac CSE expression in LTSS animals. Moreover, this reduction was positively correlated with the degree of Oxtr downregulation, which might suggest a direct interaction between the oxytocinergic and CSE/H_2S systems in the heart, which is altered in response to LTSS. Similar to the Oxtr, CSE is also expressed in cardiomyocytes, endothelial and smooth muscle cells and, consequently, is involved in blood pressure regulation via H_2S action [44]. Evidence for an interaction between Oxtr and CSE was provided in our previous study, which showed that *CSE*$^{-/-}$ knockout mice displayed a reduced cardiac Oxtr expression, which, in turn, was restored to the level of the control myocardial tissue by application of exogenous H_2S [32]. In the present study, the reduced CSE expression observed in *Oxtr*$^{-/-}$ knockout mice further confirms a link between myocardial CSE and Oxtr expression. Moreover, the downregulation of Oxtr and CSE expression observed in the LTSS-exposed animals may contribute to CV pathology. In view of a possible reciprocal regulation between the Oxtr and CSE systems and the fact that mice lacking CSE show reduced endothelial-mediated vasorelaxation [45], this provides further evidence for a crucial role of Oxtr in blood pressure regulation. This view is further supported by the fact that Oxtr activation enhances baroreceptor sensitivity and, thus, enhances the capacity of blood pressure control [46, 47]. Finally, in ovariectomized spontaneously hypertensive rats, Oxtr blockade causes adverse cardiac remodeling [48] and monocrotaline-induced pulmonary hypertension leads to right ventricular Oxtr downregulation [49]. In contrast to the findings after LTSS, CSE expression was unchanged in the STSS animals. This suggest that any CSE-Oxtr interplay may be more relevant for vulnerable changes after LTSS but not for STSS, where, e.g., the dopaminergic system may be more involved [5].

5. Limitations of the Study

As methodological consideration, it should be pointed out that there was a substantial difference in the immunohistochemical CSE and Oxtr expression and in plasma Oxt concentrations in the two control groups for the two ELS paradigms. Since the two stress paradigms were run consecutively, two separate control cohorts were mandatory to

obtain separate baseline data for each ELS paradigm. As previously shown [5], control animals from different cohorts may be divergent in basal parameters, as it is apparent in our study as well. Similar 10-20-fold variations of baseline plasma Oxt levels have been reported in a study on murine Oxt plasma levels using the same method as in the present experiment [50]. Furthermore, our data do not directly prove the causality between CSE and Oxtr since our experimental design allows only a first description of this relationship. Further studies will elucidate if both factors are pathophysiologically linked to each other.

6. Conclusion

Taken together, we show here for the first time that "chronic" ELS results in long-term reductions of myocardial Oxtr and CSE expression, which last until adulthood and which might be indicative of a biological interaction between the oxytocinergic and H_2S systems. These alterations may reflect substantial biological pathways underlying microvascular and CV dysregulation in later life and may be viewed as "maltreatment" scar, i.e., a long-term negative outcome of ELS. The "chronic" LTSS stress paradigm provides a suitable model system, in which the cellular mechanisms underlying ELS-related CV disorders can be identified and characterized under experimentally controlled conditions.

Data Availability

The data that support the findings of this study are available from the corresponding author on request.

Conflicts of Interest

The authors declare no competing interests.

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Review Article

Oxidative Stress-Related Endothelial Damage in Vascular Depression and Vascular Cognitive Impairment: Beneficial Effects of Aerobic Physical Exercise

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Oxidative stress- (OS-) related endothelial damage is involved in the occurrence and progression of several disorders, such as vascular depression and dementia. It has been reported that moderate, aerobic, physical exercise could reduce OS and inflammation, thus limiting the cardiovascular risk factors while improving endothelial homeostasis, mood, and cognition. In this review, we will discuss about the role of OS and OS-related endothelial damage in vascular depression and vascular cognitive impairment. Then, we will comment on the effects of physical exercise on both disorders.

1. Oxidative Stress, Endothelium, and Physical Exercise

During physical exercise, the production of reactive oxygen species (ROS), including hydroxyl radical (OH-), hydrogen peroxide (H_2O_2), and superoxide (O_2-), increases. The fine balance between ROS and antioxidants, namely, redox homeostasis, is involved in various physiological processes including cellular signaling, gene expression, cellular growth, neurotransmitter release, and synaptic plasticity [1, 2]. Hence, a ROS overproduction can lead to detrimental effects for the cells. In fact, containing unpaired electrons, ROS are highly reactive molecules that can oxidize several cellular compounds, among which DNA, lipids, and proteins [3]. The main endogenous sources of ROS are probably the mitochondria, followed by peroxisomes and endoplasmic reticulum, while exogenous ROS-inducing agents are pollutants, xenobiotics, bacteria, radiations, diet, excessive physical exercise, etc. [4]. The imbalance between ROS generation and antioxidant substances determines oxidative stress (OS), condition favoring the occurrence and progression of several chronic illnesses such as dementia, movement disorders, depression, and cardiovascular disturbances [5–7]. In addition, ROS overproduction induces an inflammatory status, characterized by a release of cytokines, prostaglandins, and chemokines whose pivotal role in the occurrence of the previously mentioned disorders has been fully elucidated [8]. Among the conditions accompanied by sustained OS, overtraining has been demonstrated to determine an excessive production of ROS, thus favoring DNA damage, lipid peroxidation, and necrosis [9]. Conversely, moderate aerobic physical exercise leads to a transient elevated ROS production, an increased muscle contractility, an improvement of insulin sensibility, and a better regulation of vasodilatation [10]. Furthermore, moderate physical exercise improves the endogenous antioxidant defense system, stimulating the expression of superoxide dismutase 1, superoxide dismutase 2, glutathione peroxidase, and glutathione reductase, while reducing the concentration of several inflammatory markers, including interleukin- (IL-) 6, homocysteine, and tumor necrosis factor-alpha [11]. From a clinical point of view, the beneficial effects of moderate physical exercise result in a lower incidence of ischemic stroke, heart attacks [12], obesity, and diabetes [11] among trained individuals. Exercise seems to exert

its beneficial role both directly, improving the insulin sensitivity and the mobilization of fatty acids and glucose, and indirectly, by favoring weight loss [13]. Moreover, a recent animal study has demonstrated that exercise increases the endothelial nitric oxide synthase (eNOS) expression and activation both in the vascular wall and in the perivascular adipose tissue, thus restoring their anticontractile capacity with the consequent improvement of the vascular function [14]. The latter mechanism could explain, at least in part, the beneficial role of exercise training on several acute and chronic disorders related to vascular dysfunction, including vascular cognitive impairment (VCI) and vascular depression (VaD). In this review, we will discuss about the role of OS and OS-related endothelial damage in VCI and VaD. Then, we will comment on the effects of physical exercise on both disorders.

2. The Role of Vascular Endothelium in Depression

2.1. Vascular Depression. Depression is one of the most frequent mental disorders (lifetime prevalence of 20.6%) that, according to the World Health Organization projections, could become the leading cause of disability by 2030 [15, 16].

The most frequent depressive symptoms are sad mood, thoughts of guilt, lack of interest, sleep disorders, lack of appetite, psychomotor retardation or agitation, cognitive deficits, and suicidal ideation [17, 18]. Among the different neurobiological mechanisms contributing to the occurrence of depression, it is possible to include the serotoninergic, norepinephrinergic, and dopaminergic deficits, the dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, the hyperproduction of proinflammatory mediators, and an altered gut-brain axis [19]. Despite the median age at depression onset typically being the early adulthood [20], depressive symptoms are common in the elderly and are associated with reduced quality of life and elevated mortality [21]. Over the years, several factors have been variably associated with the occurrence of late-life depression, including social isolation, poor family support, low education, marital status [22], Parkinson's disease, diabetes, cancer, and cerebrovascular disorders [22–24]. The association between cerebrovascular disorders and depression in late-life has been so frequently reported that the concept of "*vascular depression*," subtype of late-life depression frequently predisposed or perpetuated by an ischemic damage of frontosubcortical circuits, emerged [25]. As a matter of fact, a recent meta-analysis supported the association between brain silent lesions due to vascular damage (white matter lesions (WMLs)) and late-life depression [26]. WMLs are commonly observed in the elderly; they are frequently related to structural and functional cerebrovascular pathology and are facilitated by the endothelial dysfunction [27–29].

2.2. Vascular Endothelium and Vascular Depression. Vascular endothelium is a single cellular layer that lines the walls of all blood vessels. Releasing both vasodilators (i.e., nitric oxide) and vasoconstrictors (i.e., endothelin), it regulates the vasomotor tone, thus exerting a fundamental role in the prevention of cerebrocardiovascular disorders [30]. Endothelial dysfunction could contribute to the occurrence of VaD, as discussed below.

Firstly, an association between risk factors for endothelial damage (i.e., hyperglycemia, hypertension, and dyslipidemia) and depression has been reported [31, 32]. Moreover, homocysteine, a toxic amino acid derived from dietary methionine and representing a strong risk factor for cerebrocardiovascular disorders (due to vasodilatation impairment), has been found to be elevated in depressed patients [33]. Interestingly, depression is frequently characterized by functional alterations affecting crucial components of the blood-brain barrier (BBB), such as a reduced E-cadherin expression (resulting in less efficient tight junctions) and an impaired function of claudin, a protein contributing to the formation and maintenance of the BBB. These functional changes are favored by the prooxidative and proinflammatory status [34]. In fact, OS seems to directly upregulate the endothelial expression of NMDA receptor in the neurovascular unit, thus mediating endothelial excitotoxicity [35]. Another OS-related condition characterizing depression is the activation of metalloproteinases 2 and 9, proteins demonstrated to increase the BBB permeability and stimulate the inflammatory status [34, 36, 37]. A dysregulated function of metalloproteinases, altering the neurovascular unit, favors neuronal damage and cognitive impairment. Consistently, high levels of metalloproteinase 9 have been detected in the vascular walls, senile plaques, and neurons of brains affected by Alzheimer's disease (AD) [38]. Interestingly, physical exercise has been repeatedly shown to reduce the levels of metalloproteinases [39, 40] and to improve the endothelial function, also through its stimulatory effect on eNOS [41, 42].

The since here discussed molecular alterations could account, at least in part, for the frequent association between depression and neurological conditions (i.e., traumatic brain injury, stroke, and multiple sclerosis) characterized by endothelial dysfunction and inflammation [34]. Concerning the latter, depression is characterized by the release of inflammatory molecules inducing vascular damage, such as IL-6- and cytokine-like leptin, an anorexogenic agent linked to the eating disturbances frequently associated with late-life depression [43–45]. In the latter type of depression, the endothelial damage is further stimulated by the age-related dysfunctional hyperactivation of glial cells, determining the release of several proinflammatory cytokines and ROS hyperproduction [46, 47]. Finally, the autonomic dysfunction characterizing mood disorders may as well sustain endothelial dysfunction [48, 49]. For example, an imbalance between sympathetic and parasympathetic tone, common in the elderly, has been related to myocardial infarction and ischemic stroke [30]. As a matter of fact, the relationship between vascular damage and depression seems to be "bidirectional": if, on the one hand, endothelial damage could sustain depression, on the other hand, depression itself represents a cerebrocardiovascular risk factor. As a result, elderly depressed patients frequently show an impairment in activities of daily living as well as cognitive decline and increased medical comorbidity [50].

3. Vascular Cognitive Impairment and Endothelium

The term *vascular dementia*, recently substituted by the more inclusive term "*vascular cognitive impairment*" (VCI), encompasses a range of cognitive disorders strictly related to vascular disease [51]. Despite the large variability in the esteems, VCI is still considered the second most common type of dementia, after AD, accounting for 15-20% of individuals affected by dementia [52]. Epidemiological studies reported that cerebrovascular disorders increase from 3.5 to 47 times the risk of dementia, depending on the severity of the vascular event (i.e., silent WMLs, transient ischemic attacks, minor stroke, and stroke) [53]. The differences in the epidemiological data can be reconducted in the variety in terms of frequency, features, and prognosis characterizing the disorders classified under the umbrella term "VCI." It should be noted, in fact, that the many faces of VCI reflect the heterogeneous nature of the vascular damage and its distinct contribution to the occurrence of cognitive decline. Hence, among VCI disorders, it is possible to include multi-infarct (cortical vascular) dementia, small vessel (subcortical vascular) dementia, strategic infarct dementia, hypoperfusion dementia, hemorrhagic dementia, hereditary vascular dementia (including the cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy CADASIL), and AD with cerebrovascular disease (cooccurrence of neurodegeneration and vascular damage) [54]. Cerebral small vessel disease is the most common form of brain vascular disorder [55] and is held responsible not only for vascular dementia but also for vascular parkinsonism, sometimes difficult to distinguish from idiopathic Parkinson's disease and atypical parkinsonism [56, 57].

Vascular brain pathology is anything but rare in the elderly and could cause cognitive deficit ranging from mild cognitive impairment to dementia [58]. From a clinical point of view, VCI is characterized by extremely variable cognitive deficits, highly dependent on vascular damage localization and severity, as previously stated. However, due to the frequency of small vessel disease, clinical manifestations related to the frontostriatal circuit impairment, including executive function and attention deficits, are usually observed [59]. The most studied risk factors variably associated with VCI are represented by old age, low educational level, smoking status, hypertension, diabetes, obesity, and hypercholesterolemia [60]. These factors, some of them modifiable, cooccur in a scenario of age-related modifications exerting a detrimental effect on the endothelium. In fact, from a molecular point of view, aging is characterized by an imbalance between oxidants and antioxidants. The consequent ROS overproduction in the vascular system, including brain circulation, could be partially related to the overexpression of NADPH oxidases, characterizing the elderly [61]. In addition, OS determines the hyperactivation of glycogen synthase kinase (GSK-3) and, consequently, an alteration of mitochondrial permeability, recognized to exert a pivotal role in the pathogenesis of atherosclerosis [62, 63]. Moreover, aging is characterized by microglial hyperactivation. This condition represents a defensive response to inflammation; however, particularly when sustained, it constitutes a dangerous source of free radicals, ultimately favoring OS and neurodegeneration [50]. The latter could be further facilitated by the aging-related impairment of the brain autoregulation myogenic tone and the BBB breakdown, leading to the passage of immune cells and toxins into the brain [64, 65]. On the light of what has been said, the interplay between inflammation and OS could represent the biological basis of endothelial damage and BBB break failure occurring in VaD [66, 67].

4. Physical Exercise in Vascular Depression and Vascular Cognitive Impairment

The pharmacological treatment of depression and dementia in the elderly is frequently complicated by poor compliance, drug side effects, and interactions due to multiple therapies. Therefore, nonpharmacological approaches, as monotherapy or in add-on to pharmacological agents, are receiving increasing interest.

Physical exercise exerts beneficial effects in terms of psychological well-being, quality of life, and depressive symptoms [68]. In fact, it has been demonstrated to be as effective as the antidepressant sertraline and to reduce depressive symptoms in nonresponders to antidepressants [69, 70]. Notably, a relatively recent meta-analysis confirmed the benefit of exercise on depression, even in comparison with active usual care (i.e., social contact) [71]. Since vascular dysfunctions are shared by both late-life depression and VCI [72], VaD could be more than a risk factor for VCI. In fact, a continuum from late-life depression to VCI could be hypothesized. In this context, physical exercise may represent a viable strategy to prevent cognitive impairment in the elderly. In fact, studies carried out on middle-aged individuals with normal cognition identified moderate cardiorespiratory fitness as a protective factor against cognitive impairment, being able to improve attention and executive function performances [73, 74]. These findings could find an explanation in some molecular mechanisms highlighted in humans and animal models of AD. For instance, physical exercise seems to improve the clearance of Aβ plaques and hyperphosphorylated tau protein [75]. On the other hand, physical exercise limits OS, favoring an increase of the peripheral insulin-like growth factor 1, stimulating the activity of superoxide dismutase and decreasing the NADH oxidase activity [76]. A further mechanism supporting the beneficial role of physical exercise in VCI is represented by the stimulation of the neurogenesis in the subventricular and subgranular zone and an increased angiogenesis of capillaries from existing vessels [76]. Interestingly, randomized controlled trials have confirmed that physical exercise may delay the cognitive decline characterizing AD [77] and VCI [78, 79]. Unfortunately, despite the recommendations of the World Health Organization and the encouraging evidence, physical inactivity is still a major concern when considering middle-aged individuals [80]. Future studies are needed in order to strengthen the current evidence regarding the role of physical exercise in both VaD and VCI and raise awareness on this issue.

5. Conclusion

The previously discussed evidence highlights the beneficial effects of moderate, aerobic, physical exercise in reducing the cerebrocardiovascular risk factors, as well as improving endothelial homeostasis, mood, and cognition. Hence, the elderly should be encouraged to maintain an active lifestyle to reduce the risk of disabilities in late-life.

Conflicts of Interest

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

Authors' Contributions

Maria Luca and Antonina Luca contributed equally to this work.

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Review Article

Advanced Glycation End Products: Potential Mechanism and Therapeutic Target in Cardiovascular Complications under Diabetes

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The occurrence and development of cardiovascular complications are predominantly responsible for the increased morbidity and mortality observed in patients with diabetes. Oxidative stress under hyperglycemia is currently considered the initial link to diabetic cardiovascular complications and a key node for the prevention and treatment of diabetes-related fatal cardiovascular events. Numerous studies have indicated that the common upstream pathway in the context of oxidative stress in the cardiovascular system under diabetic conditions is the interaction of advanced glycation end products (AGEs) with their receptors (RAGEs). Therefore, a further understanding of the relationship between oxidative stress and AGEs is of great significance for the prevention and treatment of cardiovascular complications in patients with diabetes. In this review, we will briefly summarize the recent research advances in diabetes with an emphasis on oxidative stress and its association with AGEs in diabetic cardiovascular complications.

1. Introduction

Diabetes and its associated complications present a global burden in terms of human health and economics [1], of which the prevalence is rising at an exponential rate worldwide. According to data from the International Diabetes Federation (IDF), the currently estimated total number of 18- to 99-year-old diabetic patients is approximately 425 million globally. Moreover, the number of adult diabetes patients is expected to continue to increase over the next several decades due to aging, urbanization, and changes in diet and physical activity. The number of adult diabetes patients was expected to increase to 693 million by 2045 [2, 3]. Hyperglycemia and insulin resistance can affect various tissues and organs throughout the body, causing chronic complications of multiple systems and organs, especially the cardiovascular system [4, 5]. Pathological remodeling of the heart is characterized by left ventricular concentric hypertrophy and perivascular and interstitial fibrosis, leading to diastolic dysfunctions [6]. Diabetic macroangiopathy includes atherosclerosis of the aorta, coronary arteries, cerebral arteries, renal arteries, and peripheral arteries, while diabetic microangiopathy includes diabetic retinopathy and diabetic nephropathy [7, 8].

Both macro- and microvascular complications adversely affect the quality of life of patients with diabetes [9, 10]. The death risk from major adverse cardiovascular events in diabetic patients is higher than that in nondiabetic patients. Cardiovascular disease is more severe and extensive in the former than in the latter, with a worse prognosis and earlier onset. Patients with type 2 diabetes are 2-4 times more likely to develop heart failure than nondiabetic patients [11]. Approximately 70-80% of diabetes patients eventually die from cardiovascular complications [12]. Moreover, approximately 3/4 patients with type 2 diabetes have a variety of cardiovascular risk factors, such as hypertension, dyslipidemia, and obesity. The clusters of these risk factors can directly promote the occurrence of cardiovascular complications in diabetes [13].

Although diabetes treatment has undergone a transformation from simple control of hyperglycemia to multi-risk-factor management, the prevention and control of cardiovascular

complications in this population still remain challenging. Sustained blood-glucose elevation is the initiating factor in the pathogenesis of diabetic cardiovascular complications. However, the mechanisms by which hyperglycemia can affect the cardiovascular system have not been adequately addressed.

Various hyperglycemia-elicited metabolic and hemodynamic derangements have been proposed to contribute to cardiovascular complications in diabetes [6]. The currently identified mechanisms include increased oxidative stress [14, 15], activation of protein kinase C (PKC) [16], chronic inflammation [17, 18], mitochondrial dysfunction [19], and activation of the renin-angiotensin system (RAS) [20]. Among these, increased oxidative stress is considered to be the initial core mechanism leading to diabetic cardiovascular diseases [21]. Therefore, understanding how oxidative stress is controlled in the context of cardiovascular complications is helpful for developing effective therapeutics against diabetes.

Advanced glycation end products (AGEs) are a general term for a class of heterogeneous compounds mainly derived from nonenzymatic saccharification (Maillard reactions) of reducing sugar on proteins, lipids, and nucleic acids. AGEs can increase the production of reactive oxygen species (ROS), thereby initiating intracellular oxidative stress [22]. Conversely, the increase in ROS production can in turn promote the production of AGEs, thereby forming a vicious circle between oxidative stress and AGEs. In this review, we will briefly summarize the recent research advances in *in vitro* and *in vivo* model systems of diabetes with an emphasis on oxidative stress and its association with AGEs in cardiovascular complications under diabetic conditions in an effort to provide some evidence for potential cardiometabolic-targeted therapies for diabetes.

2. Oxidative Stress

Oxidative stress is defined as an imbalance between oxidization and antioxidation, which subsequently leads to multiple negative effects on cellular metabolism. "ROS" is a general term for active substances composed of oxygen in the body or in the natural environment, including free radicals (hydroxyl, superoxide) and nonradicals (hydrogen peroxide, singlet oxygen molecules). They are continuously generated and eliminated during redox reactions in life activities. ROS generation mainly stems from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [23]. Other sources include xanthine oxidase (XO) and nitric oxide synthase (NOS) decoupling.

Normally, a proper amount of ROS as a signaling molecule is indispensable for regulation of transcription factors, expression of apoptosis genes, and antibacterial and anti-inflammatory effects. During pathological conditions, however, when the ROS level exceeds the buffering capacity of antioxidant enzymes and antioxidants, the balance between oxidation and antioxidation shifts the trend to oxidization, resulting in oxidative stress [24]. It has been reported that increased ROS levels can stimulate mitogen-activated protein kinase (MAPK), tyrosine kinase, Rho kinase, and transcription factor (NF-κB, AP-1, and HIF-1) activation [25–27]. Furthermore, ROS can inactivate protein tyrosine phosphatase (PTP), increase the intracellular free calcium ion concentration, and modulate the expression and activation of proinflammatory genes [28]. Changes in these intracellular signals can lead to endothelial dysfunction and myocardial remodeling.

3. Oxidative Stress in Diabetic Cardiovascular Complications

Increased oxidative stress is considered the initial core mechanism leading to diabetic cardiovascular diseases [21, 22]. In diabetic cardiovascular complications, NADPH oxidase is activated under conditions of hyperglycemia [29], catalyzing the formation of superoxide anions, and the superoxide anion undergoes a series of reactions to convert to hydroxyl groups, resulting in excessive ROS synthesis and subsequently leading to oxidative stress. In addition, the antioxidant capacity of the defense system, including enzymatic antioxidants (superoxide dismutase), nonenzymatic antioxidants (vitamin C, coenzyme Q10), and metal complexes (copper-binding proteins), is decreased by hyperglycemia [30, 31]. Oxidative stress triggers inflammation, endothelial dysfunction, cardiomyocyte hypertrophy and apoptosis, and myocardial fibrosis, which subsequently lead to decreasing left ventricular compliance, diastolic dysfunction, and finally heart failure, arrhythmia, and/or even sudden cardiac death.

3.1. Oxidative Stress and Inflammation. As previously reported, oxidative stress and inflammation interact with each other to promote diabetic cardiovascular complications [32]. ROS directly or indirectly activate NF-κB, transforming growth factor-β (TGF-β), MAPK, protein kinase C (PKC), stress-activated protein kinase, etc. They thereby trigger inflammation and myocardial fibrosis in the cardiovascular system in diabetes [33, 34]. The expression of inflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) was demonstrated to promote cardiac fibroblast proliferation, thereby increasing collagen synthesis and ultimately leading to myocardial fibrosis [35]. Inflammatory factors are in turn considered to increase ROS [36]. NF-κB can increase the expression of inducible nitric oxide synthase (iNOS), which promotes the generation of nitric oxide. Excessive nitric oxide and peroxyl radicals react to form peroxynitrate, which subsequently increases mitochondrial permeability and ROS production.

3.2. Oxidative Stress and Endothelial Cell Dysfunction. In addition, the sustained elevation of blood glucose can cause endothelial cell dysfunction [37, 38]. Accumulated superoxide and nitric oxide interact rapidly to form a highly active intermediate, peroxynitrite. Peroxynitrite is a strong cytotoxic oxidant that can cause nitrosylation, nitration, and oxidative damage of biomolecules such as proteins, lipids, and DNA in endothelial cells [39, 40]. Simultaneously, peroxynitrite can cause the unfolding of endothelial NOS to form superoxide, and superoxide can continue to react with nitric oxide to form peroxynitrite, forming a vicious circle [41].

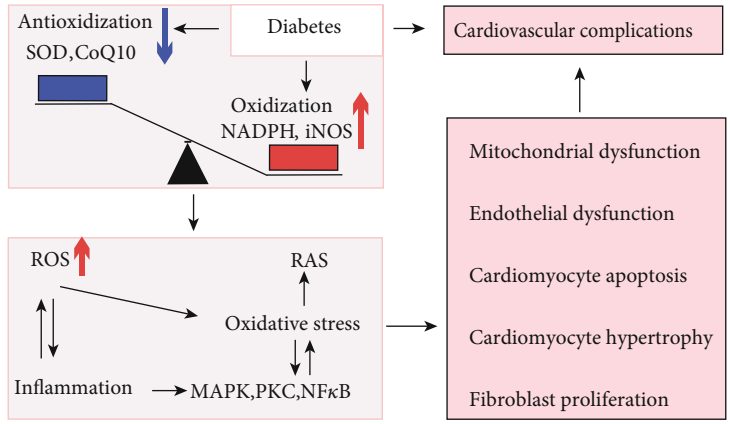

Figure 1: Oxidative stress plays an important role in diabetic cardiovascular complications. An impaired balance between oxidization and antioxidant activity in the diabetic cardiovascular system results in more pronounced ROS generation and oxidative stress. Oxidative stress interacts with inflammation and neurohumoral mechanisms, thereby promoting mitochondrial dysfunction, endothelial dysfunction, cardiac fibrosis, and hypertrophy.

3.3. Oxidative Stress and Cardiac Hypertrophy. Oxidative stress also plays a key role in promoting cardiac hypertrophy in diabetes [42, 43]. Under the condition of hyperglycemia, ROS activate neurohumoral mechanisms such as the renin-angiotensin-aldosterone system, endothelin-1, and the sympathetic nervous system [44, 45]. Excessive activation of the renin-angiotensin-aldosterone system can induce cardiac hypertrophy and abnormal cardiac functions [46]. Studies have shown that the angiotensin-converting enzyme inhibitor lisinopril can inhibit 8-hydroxydeoxyguanosine and hydroxyl radicals in cardiomyocytes in diabetic rats and thus alleviate cardiac inflammation, fibrosis, and hypertrophy [47].

3.4. Oxidative Stress and Mitochondrial Dysfunction. Moreover, ROS-mediated activation of mitochondrial uncoupling proteins, increased proton leakage, and oxidative phosphorylation uncoupling can result in reduced adenosine triphosphate (ATP) production, thereby inducing cardiomyocyte apoptosis [48, 49]. The oxidative phosphorylation respiratory chain enzyme complex is composed of mitochondrial DNA and nuclear DNA-encoding subunits involved in the oxidative phosphorylation of mitochondria [50, 51]. On the one hand, increased ROS can directly damage mitochondrial DNA and membranes [52]. In addition to changes in mitochondrial ATP production, abnormalities in mitochondrial-surface membrane ion channels and sarcomere-associated proteins can also cause cardiac dysfunction [53]. The calcium signaling pathway is a prerequisite for myocardial cell contraction and relaxation. The sarcoplasmic calcium pump is an important component of the calcium signaling pathway, which relaxes cardiomyocytes by isolating calcium ions. ROS can cause dysfunction of the sarcoplasmic reticulum, leading to the accumulation of calcium ions in the cells, followed by cardiac dysfunction, arrhythmia, and heart failure [54].

All the above studies have shown that oxidative stress in the heart can cause endothelial and myocardial metabolic abnormalities through inflammation, mitochondrial damage, glucose metabolism disorders, and other mechanisms, eventually leading to myocardial contractile and diastolic dysfunction or even heart failure. Moreover, the abnormal elevation of oxidative stress can further cause glucose metabolism disorders, thereby creating a vicious circle if not controlled (Figure 1).

4. AGE Metabolism in Diabetic Cardiovascular Complications

The nonenzymatic glycosylation reaction, also known as the Maillard reaction, was proposed by Maillard in the early 20th century [55]. AGEs are highly heterogeneous and exist in many different forms *in vivo*. Typical AGEs include pentosidine, carboxymethyl lysine (CML), carboxyethyl lysine (CEL), pyralline (Pyr), argpyrimidine (ArgP), and cross-linked AGEs [56, 57]. Once synthesis is finished, AGEs can accumulate with aging and can be used as a biomarker of aging [58]. AGEs function mainly through binding to specific receptors [59]. Currently identified receptors for AGEs include RAGE, macrophage scavenger receptors type I and type II, oligosaccharyl transferase-48, 80K-H phosphoprotein, and galectin-3, among which, RAGE is the major one [60]. The downstream targets of RAGE include NADPH oxidase, MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38-mediated signaling pathways, and ultimately NF-κB [61, 62].

Generally, the Maillard reaction occurs in some slower-renewing proteins, such as type IV collagen, laminin, and elastin, and the rate of AGE generation is relatively slow [63]. However, the formation and accumulation of AGEs have been known to progress at an accelerated rate in diabetes [64]. On the one hand, sustained hyperglycemia causes more rapid nonenzymatic glycosylation of the abovementioned proteins and results in the increase in AGEs. On the other hand, AGEs are scarcely degraded and remain for a long time in tissues even if glycemic control is improved [65]. The clearance of AGEs is mediated by specific receptors

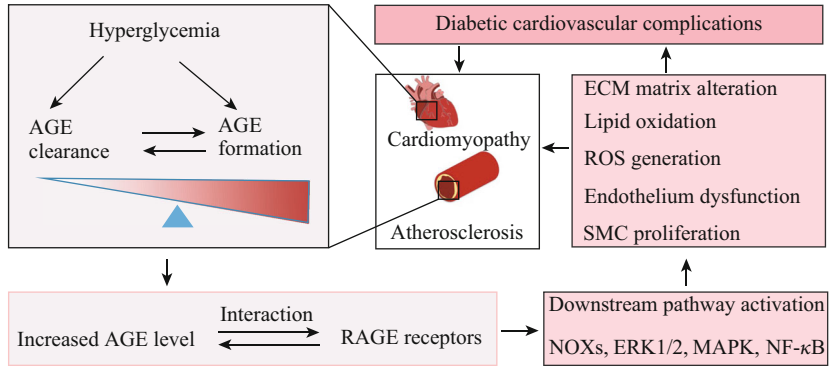

Figure 2: AGEs play an important role in diabetic cardiovascular complications. Under the condition of hyperglycemia, the balance between AGE formation and clearance is impaired in the diabetic cardiovascular system which thereby result in increased AGEs. AGEs interact with AGE receptors and then promote diabetic cardiovascular complications.

on macrophages via internalization or by extracellular proteolytic systems that decompose them into relatively lower molecular weight AGEs, which are finally cleared by the kidneys [66]. When nephritic insufficiency occurs in diabetes, AGEs increase in the circulation and further aggravate renal dysfunction [67]. Therefore, previous studies have paid more attention to the relationship between AGEs and diabetic nephropathy. Studies have confirmed that plasma AGE levels are closely related to the development of diabetic glomerular sclerosis, tubulointerstitial fibrosis, and mesangial cell proliferation [68, 69].

Increasing evidence indicates that AGEs are also involved in the occurrence and development of cardiovascular diseases. The increase in plasma AGEs is more pronounced in diabetic patients with coronary heart disease than in patients without coronary heart disease. Nin et al. [70] confirmed that plasma AGE levels are associated with all-cause mortality in fatal or nonfatal coronary artery disease. Steine et al. and Berg et al. [71, 72] found that left ventricular dysfunction in patients with type 1 diabetes is associated with plasma AGE levels. Jia et al. [73] also found that the level of tissue AGEs was independently associated with cardiac systolic dysfunction in diabetic patients with heart failure compared with diabetic patients without heart failure. Spadaccio and colleagues [74] showed that the risk of restenosis in diabetic patients with high plasma AGEs who undergo percutaneous coronary intervention (PCI) is relatively higher.

5. The Mechanism of AGEs in Diabetic Cardiovascular Complications

The effects of AGEs in the diabetic cardiovascular system are mediated by receptor-dependent and nonreceptor-dependent pathways (Figure 2). First, endothelial cell dysfunction is the starting event of atherosclerosis. AGEs can directly modify extracellular matrix proteins of endothelial cells, including type IV collagen and laminin [75, 76]. This process destroys the normal structure and function of blood vessels, and cardiac fibrosis is accelerated [77]. AGEs not only damage endothelial cells but also induce apoptosis and dysfunction of endothelial progenitor cells [78]. Ueda et al. [79] reported that serum AGE levels are independent risk factors for the number and function of circulating endothelial progenitor cells. In addition, AGEs can directly stimulate the production of vascular endothelial cell growth factor (VEGF), leading to increased vascular permeability or even vascular wall edema [80].

Additionally, circulating AGEs can increase lipid oxidation and deposition in atherosclerotic plaques and promote the infiltration of macrophages and T cell migration and proliferation, thereby promoting atherosclerosis [81]. AGE-induced LDL glycosylation results in the blocking of receptor-mediated LDL removal. Furthermore, increased glycosylation reduces the cholesterol reverse transport ability of HDL, thus promoting the deposition of lipids in blood vessel walls and resultant plaque formation [82, 83]. Recent studies have also shown that the binding of AGEs to the platelet membrane receptor CD36 induces thrombus formation, which may be an important mechanism by which AGEs promote cardiac ischemic events in diabetic patients [84].

In addition, a recent study confirmed that the AGE-RAGE axis interacts with the RAS, which contributes to the proliferation of cardiac fibroblasts and cardiomyocyte hypertrophy in diabetes as well [85]. Moreover, it was reported that AGEs can upregulate RAGE expression via the activation of NF-κB [86]. As mentioned before, activated NF-κB binds to specific DNA sequences, regulating corresponding gene transcription and accelerating the emergence of cardiovascular complications. It is conceivable that the positive feedback loops between AGEs and RAGE-downstream pathways could create a vicious cycle, thus promoting cardiovascular complications in diabetes.

6. Crosstalk between AGEs and Oxidative Stress in Diabetic Cardiovascular Complications

Oxidative stress and the AGE-RAGE axle pathway are not independent processes. Accumulating evidence has suggested that the crosstalk between AGE-RAGE and oxidative stress plays an important role in the context of cardiovascular complications of diabetes [64]. AGE-RAGE interaction

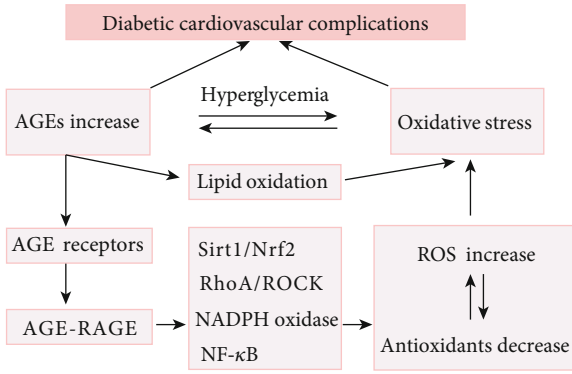

Figure 3: The interaction between AGE-RAGE axle and oxidative stress plays an important role in diabetic cardiovascular complications. On the one hand, AGE-RAGE axle activation can result in the activation of diverse signal transduction cascades, thereby including the generation of ROS and accelerating oxidative stress. On the other hand, excessive oxidative stress can in turn accelerate the generation of AGEs in the diabetic cardiovascular system.

results in the activation of diverse signal transduction cascades and downstream pathways such as MAPK, ERK1/2, p38, and NF-κB, thereby including the generation of ROS and accelerating oxidative stress and the emergence of cardiovascular complications in diabetes [87, 88]. Blockade of RAGEs attenuates vascular oxidative stress and the development of atherosclerosis.

Tang et al. reported that AGEs can activate NF-κB to increase the expression of inducible NO synthase (iNOS) through RAGE/RhoA/ROCK-mediated and AMPK-mediated signaling pathways, which subsequently promote the generation of NO in endothelial cells [89]. Hegab et al. found that treatment of cardiomyocytes with AGEs for 24 h significantly increased ROS production [90]. Chen et al. suggested that AGEs can induce oxidative stress through the Sirt1/Nrf2 axis by interacting with RAGEs under diabetic conditions [91]. AGEs were also reported to increase the expression and activity of NADPH oxidase in endothelial cells, which is an important source of oxidative stress in diabetic cardiovascular complications [92, 93]. Increased NADPH oxidase activity results in the generation of ROS and the depletion of cellular antioxidants such as glutathione, glutathione peroxidase, superoxide dismutase, and catalase.

It was reported that AGEs are involved in a vicious cycle of oxidative stress [94]. AGEs modulate oxidative stress, and excessive oxidative stress can in turn accelerate the generation of AGEs [95] such as CML [96]. Taken together, these data suggest that the crosstalk between the AGE-RAGE axis and oxidative stress is highly involved in the context of diabetic cardiovascular complications (Figure 3). A deeper study of this relationship will facilitate the designing of new drugs and provide new prospects and methods for the prevention and treatment of diabetes and its cardiovascular complications. Drugs that deplete AGEs in the cardiovascular system or block their interaction with oxidative stress may be proper candidates.

7. Current and Future Therapies against Diabetic Cardiovascular Complications

Lifestyle changes, balanced energy intake, and glucose- and lipid-lowering drugs are the currently available treatment strategies for diabetes patients with cardiovascular complications [97]. A detailed description of therapeutic approaches for diabetes with cardiovascular complications is beyond the scope of this review. Since AGEs, oxidative stress, and their interactions are highly related to the progression of diabetic cardiovascular complications, therapies that involve AGEs and oxidative stress may help reduce the cardiovascular complications in diabetic patients (Table 1).

7.1. Traditional Antidiabetic Agents. Oscillating glucose is more deleterious to endothelial function and oxidative stress in type 2 diabetic patients [98, 99]. Therefore, the key point for preventing and delaying the occurrence and development of diabetic cardiovascular complications lies in blood-glucose control [100]. In theory, a drug that lowers blood sugar confers cardiovascular protection, but actually, selective antidiabetic agents are limited. In contrast, some antidiabetic drugs even increase the risk of death from cardiovascular disease [101].

The UKPDS posttrial study and DCCT/EDIC showed that intensive blood-glucose control not only reduces the risk of microvascular disease in diabetes patients but also significantly reduces the total mortality rate of cardiovascular disease [102]. Intensive blood-glucose control with metformin is currently accepted to reduce the risk of cardiovascular disease in diabetes [103]. Its cardiovascular protection effect was attributed to the antioxidant properties that lead to the reduction of XO activity and lipid peroxidation in patients with type 2 diabetes [104]. Treatment of diabetic rats with metformin was also found to decrease the plasma levels of AGEs [105], thereby reducing oxidative stress and cardiac remodeling.

However, the conclusion of three other intensive hypoglycemic trials, ADVANCE, ACCORD and VADT, suggested that it may be difficult to effectively reduce cardiovascular risk in patients with type 2 diabetes simply by intensive glucose control [106]. Intensive hypoglycemic therapy increases the risk of hypoglycemia, which has been demonstrated to be significantly correlated with severe cardiovascular events in diabetes patients [107]. Moreover, since rosiglitazone was reported to be associated with a significant increase in the risk of myocardial infarction and death from cardiovascular events [108], the concern regarding the cardiovascular safety of antidiabetics has markedly increased even though, subsequently, the RECORD trial emphasized that rosiglitazone does not increase the risk of cardiovascular events [109].

7.2. Antioxidants. According to the "metabolic memory" theory, hyperglycemia-induced metabolic changes will last for a long time even after the blood-glucose level returns to normal [106, 110]. Clearly, oxidative stress is considered the key cause of cardiovascular complications of diabetes. Therefore, antioxidant therapy and hypoglycemic therapy are equally important.

Table 1: Therapeutic candidates against diabetic cardiovascular complications.

Category	Examples	Mechanisms of act
Traditional hypoglycemic agents	Metformin	Antioxidant properties and possible effects on the reduction of AGE
Antioxidants	Vitamin C and vitamin E	Antioxidant properties
	ACEIs and ARBs	Reduce angiotensin II-induced oxidative stress
	Statins	Reduce lipid peroxidation
AGE-RAGE inhibitors	ALT-711	AGE cross-link breaker
	Aminoguanidine	Inhibit AGE formation
	Soluble RAGE	Competitively combine with AGEs
New hypoglycemic agents	GLP-1 receptor agonists	Not fully understood
	DDP-4 inhibitors	Not fully understood
	SGLT-2 inhibitors	Not fully understood

Antioxidants such as vitamins and vitamin analogs are widely used clinically. Vitamin C is a water-soluble vitamin that has significant antioxidant effects. Vitamin C infusion was reported to improve endothelial function and cardiac diastolic function. However, it did not alter the exercise capacity in type 2 diabetes [111]. Vitamin E can reduce lipid peroxidation in patients with noninsulin-dependent diabetes, which is important for the treatment of early diabetic cardiovascular complications [112]. Specifically, in patients with haptoglobin genotype-2 (Hp2-2), vitamin E has been shown to be associated with an approximately 35% reduction in cardiovascular diseases in both type 1 diabetes and type 2 diabetes [113, 114]. This reduction was mediated partly by an improvement in the function of HDL. However, some clinical trial data suggest that vitamin E does not affect the development of cardiovascular diseases in some patients [115]. For vitamin E to be clinically used in diabetes, an additional large prospective study will be needed.

NADPH oxidase is the major source of ROS in oxidative stress under the condition of diabetes. Since angiotensin II can activate NADPH oxidase to increase oxidative stress through the AT1 receptor [116, 117], the administration of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) is currently the regular treatment for diabetes with cardiovascular complications. Statins were first known to reduce cholesterol synthesis through competitive inhibition of hydroxymethyl glutarate mononyl coenzyme A (HGM-CoA) reductase. An increasing number of studies have revealed that statins work as antioxidants beyond their lipid-lowering effect [118, 119]. In patients receiving different treatments, the antioxidant state is independently affected but is significantly more pronounced in patients on statins [120]. This effect is mediated mainly by reducing the expression of NADPH oxidase subunit and increasing the expression of antioxidant enzyme [121].

7.3. AGE-RAGE Axis Inhibitors. Pharmacological inhibitors of AGEs include aminoguanidine (AG), pyridoxamine (a natural vitamin B6 derivative), benfotiamine, ACEIs, ARBs, statins, N-phenylthiazole bromide (ALT-711), and thiazolidinediones [122–124]. AG is a nucleophilic hydrazine complex that inhibits AGE formation by combining early glycosylation, glycogen oxidation products, acetaldehyde products, etc., subsequently attenuating AGE and ROS formation both *in vivo* and *in vitro* [125]. However, AG has been limited in further clinical usage because it interferes with several important regulatory systems and has yielded toxic side effects (such as flu-like symptoms, anemia, and gastrointestinal reactions) in clinical trials.

Reactive carbonyl compounds are precursors for the formation of AGEs, which significantly accelerate the formation of AGEs. Pyridoxamine acts as a nucleophilic compound that scavenges carbonyl compounds and is a possible mechanism by which pyridoxamine inhibits the formation of AGEs [126]. Nagai et al. [127] suggested that some metal ions, such as copper ions, can participate in the autooxidation of glucose and early glycation products, playing an important role in the formation of AGEs. Triethylenetetramine (TETA) was reported to induce antidiabetic changes by targeting these copper-mediated pathogenic mechanisms [31].

N-Phenylthiazole bromide (such as ALT-711 and TRC4186) can cleave the protein cross-linking structure in AGEs and reduce the accumulation of tissue AGEs. *In vitro*, collagen that cross-linked with AGEs incubated with ALT-711 was found to be more rapidly broken down by metalloproteinases [128]. Resveratrol treatment significantly reduces oxidative stress in the kidneys of rats with diabetes by downregulating RAGE [129]. Irisin alleviates AGE-induced inflammation and endothelial dysfunction by inhibiting ROS-NLRP3 inflammasome signaling [130]. However, the clinical application of the abovementioned agents in diabetes needs further study.

The molecular structure of RAGE includes its extracellular domain, transmembrane structure, and intracellular structure. Soluble RAGE (sRAGE) only contains the extracellular segment and can competitively combine with AGEs but cannot complete signal transduction, thus blocking the harmful effects of AGEs [131]. Studies have demonstrated that a high plasma level of sRAGE is independently associated with a low recurrence of atrial

fibrillation after catheter ablation in diabetic patients [132]. AGEs and RAGE are increased in the atherosclerosis plaques in apoE$^{-/-}$ mice, whereas sRAGE treatment significantly reduces these changes [133]. Another study found that sRAGE can stabilize plaque and inhibit inflammatory factors such as cyclooxygenase-2 (COX-2), VCAM-1, and monocyte chemoattractant protein-1 (MCP-1), thereby reducing endothelial cell dysfunction [134].

7.4. New Hypoglycemic Agents. Although a series of drug candidates was reported to reduce oxidative stress and/or the AGE-RAGE axis, therapeutics that adequately address diabetic cardiovascular injuries have yet to be established clinically. Many trials of hypoglycemic therapy have also failed to prove that lowering blood glucose can effectively improve cardiovascular complications in patients with diabetes. Notably, several newly developed hypoglycemic drugs have been shown to exert a beneficial effect on the cardiovascular system beyond their ability to lower blood-glucose levels.

Glucagon-like peptide-1 (GLP-1) can stimulate endogenous insulin release. However, the half-life of GLP-1 is very short; once produced, it is degraded by dipeptidyl peptidase-4 (DDP-4) in short time [135]. New hypoglycemic agents, including DDP-4 inhibitors and GLP-1 receptor agonists, have been shown to have a beneficial effect on the cardiovascular system. The DPP-4 inhibitor sitagliptin is the first hypoglycemic agent to exhibit a comprehensive cardiovascular safety profile proven by a large randomized clinical trial [136]. The result of that study (TECOS) showed that sitagliptin therapy does not increase the incidence of cardiovascular endpoint events, which fully demonstrates its cardiovascular safety. According to the LEADER trial, the addition of the GLP-1 analog liraglutide to conventional treatment significantly reduced the incidence of cardiovascular end point events in patients with type 2 diabetes compared with the placebo group [137, 138].

Sodium-glucose cotransporter 2 (SGLT-2) inhibitors reduce the glucose concentration by selectively inhibiting SGLT2 in proximal renal tubules, thereby reducing glucose reabsorption and promoting glucose excretion. According to the EMPA-REG OUTCOME trial, an SGLT-2 inhibitor (empagliflozin) significantly reduced the risk of cardiovascular death in diabetes patients [139]. This result from EMPA-REG OUTCOME showed that patients with type 2 diabetes who were at high risk of cardiovascular events had a significantly reduced risk of cardiovascular death (lowered by 38%) when empagliflozin was added to the standard regimen [140]. The drug also reduced all-cause death risk (by 32%) and hospitalizations due to heart failure (by 35%) [141]. Animal studies revealed that empagliflozin treatment significantly reduces oxidative stress in cardiac tissues with no blood pressure reduction or improvement of cardiac autonomic dysfunction [142].

Currently, evidence of the effect of these new hypoglycemic agents on diabetic cardiovascular complications may be mediated through their ability beyond antidiabetic effects [143]. However, the direct mechanisms of these new hypoglycemic agents are not fully understood.

8. Conclusions

In summary, it is widely accepted that diabetes aggravates cardiovascular diseases and that patients with diabetic cardiovascular complications experience worse clinical outcomes. The abovementioned data suggest that AGE-RAGE axis inhibition or blockade of its interaction with oxidative stress is a novel therapeutic strategy for preventing cardiovascular complications in diabetes. The master role of AGEs and oxidative stress in cardiovascular complications of diabetes has been widely recognized, but many of these mechanisms are not yet clear and need further clarification since AGEs not only modulate oxidative stress but also in turn are affected by oxidative stress.

Fortunately, a series of drug candidates were reported to reduce oxidative stress and/or the AGE-RAGE axis. In addition, several new glucose-lowering drugs have also been found to exert a protective effect on the cardiovascular system beyond blood-glucose control in this population. A deeper study of these mechanisms and drugs will facilitate the designing of new drugs and provide new ideas for the prevention and treatment of diabetes and associated cardiovascular complications.

Conflicts of Interest

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

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Research Article

NOX2-Dependent Reactive Oxygen Species Regulate Formyl-Peptide Receptor 1-Mediated TrkA Transactivation in SH-SY5Y Cells

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Several enzymes are capable of producing reactive oxygen species (ROS), but only NADPH oxidases (NOX) generate ROS as their primary and sole function. In the central nervous system, NOX2 is the major source of ROS, which play important roles in signalling and functions. NOX2 activation requires $p47^{phox}$ phosphorylation and membrane translocation of cytosolic subunits. We demonstrate that SH-SY5Y cells express $p47^{phox}$ and that the stimulation of Formyl-Peptide Receptor 1 (FPR1) by N-fMLP induces $p47^{phox}$ phosphorylation and NOX-dependent superoxide generation. FPR1 is a member of the G protein-coupled receptor (GPCR) family and is able to transphosphorylate several tyrosine kinase receptors (RTKs). This mechanism requires ROS as signalling intermediates and is necessary to share information within the cell. We show that N-fMLP stimulation induces the phosphorylation of cytosolic Y490, Y751, and Y785 residues of the neurotrophin receptor TrkA. These phosphotyrosines provide docking sites for signalling molecules which, in turn, activate Ras/MAPK, PI3K/Akt, and PLC-γ1/PKC intracellular cascades. N-fMLP-induced ROS generation plays a critical role in FPR1-mediated TrkA transactivation. In fact, the blockade of NOX2 functions prevents Y490, Y751, and Y785 phosphorylation, as well as the triggering of downstream signalling cascades. Moreover, we observed that FPR1 stimulation by N-fMLP also improves proliferation, cellular migration, and neurite outgrowth of SH-SY5Y cells.

1. Introduction

Neurotrophic factors, such as neurotrophin-nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), and neurotrophin-3 and 4 (NT-3 and NT-4), represent a family of biomolecules necessary for neuronal survival and plasticity. They support growth and differentiation of both developing and mature neurons by binding transmembrane receptors and, in turn, stimulating protein tyrosine phosphorylations in downstream signalling cascades. Neurotrophic factors also play a key role in neurodegenerative diseases and neuropsychiatric disorders such as Bipolar Disorders (BD), depression, and eating disorders. Neurotrophins bind the tyrosine kinase receptors (RTKs) Trk; CNTF binds the CNTF receptor complex and GDNF family members signal through the tyrosine kinase receptor c-Ret [1]. NGF, BDNF, NT-3, and NT-4 also bind the p75 neurotrophin receptor ($p75^{NTR}$), a member of the TNF receptor superfamily [2], which is a potential cell death receptor and whose activity is nullified by Trk tyrosine kinase signalling [3, 4]. Trk receptors belong to a family of three RTKs and each neurotrophin binds to a specific member of Trk family with NGF binding to TrkA, BDNF and NT-4 binding to TrkB and NT-3 binding to TrkC [5, 6].

TrkA receptor activation results in phosphorylation of Y670, Y674, and Y675 localized in the activation loop of the

kinase cytoplasmic domain, which enhances tyrosine kinase activity of the receptor [5]. Y490, Y751, and Y785 of TrkA are the main phosphorylated tyrosine residues in the juxtamembrane region, in the tyrosine kinase domain, and in the intracellular C-terminal tail, respectively [5, 7]. These phosphorylated tyrosines create docking sites for the recruitment of proteins containing PTB or SH2 domains. Phosphotyrosine 490 interacts with Shc, Frs2, and other adapter molecules, which trigger the activation of Ras/MAPK and PI3K/Akt pathways. Y751 phosphorylation is essential for PI3K docking and activation, whereas phosphorylated Y785 recruits PLCγ1 and activates PKC [5, 7].

Formyl-peptide receptors (FPRs) belong to the G protein-coupled receptor (GPCR) family and are associated with pertussis toxin- (PTX-) sensitive Gi proteins [8]. Their main function is to detect the presence of noxious molecules and to drive cells till the site of release of harmful molecules. The sensing function of FPRs includes the detection of pathogens and of formylated peptides derived from mitochondrial peptides. Other endogenous ligands include the antimicrobial peptide LL-37, the anti-inflammatory lipid lipoxin A4, the proinflammatory molecule SAA, urokinase and its receptor, resolvins, beta amyloid $(A\beta)_{1\text{-}42}$ peptide, prion protein $(Prp)_{106–126}$, humanin, neuropeptides, the dual pro- and anti-inflammatory protein annexin 1, chemokine variants, vasoactive intestinal peptide, and pituitary adenylate cyclase-activating polypeptide (PACAP) [9–16].

Human FPR1, FPR2, and FPR3 are functional members of the FPR family. FPR1 binds efficiently to the formylated peptide N-formyl-methionyl-leucyl-phenylalanine (N-fMLP), whereas FPR2 is activated by WKYMVm peptide [17]. FPR3 does not bind to N-fMLP, and shares some nonformylated peptide ligands with FPR2 [18]. However, a set of formylated peptides have been identified as natural agonists for FPR3 [19]. FPR1 and FPR2 are expressed in several cell types [20, 21], whereas FPR3, is expressed in monocytes, dendritic cells [8, 16], and HUVEC cells [22]. FPR2 is also expressed on nuclear membranes of cancer cells [23], whereas FPR3 is localized within the cytoplasm [24]. Overall, binding of specific agonists to FPR1 is associated with proinflammatory responses, while FPR2 is an unusual receptor because it can convey opposite signals, depending on the ligands and on different receptor domains used by distinct agonists [25, 26]. Endocrine cells and neurons in the central nervous system (CNS) also express FPR1 [8, 27], whereas neurons of the vomeronasal organ express the other members of the FPR family [28, 29]. FPRs signalling exerts a modulatory effect on anxiety-like behaviours. In fact, *Fpr1*$^{-/-}$ mice exhibit normal spatial memory and learning capacity, but reduced anxiety-like behavior, increased exploratory activity, and impaired fear memory [30]. *Fpr2/3*-deficient mice show increased explorative behaviour and reduced fear, as well as a distinct profile of behaviour characterized by reduced anxiety [31].

RTK transactivation mediated by GPCR agonists represents a mechanism to increase the information exchange across the entire cell and to coordinate the multitude of physiological or pathological stimuli to which a cell is exposed. Several molecular mechanisms are responsible for RTK transactivation by GPCRs. They include the activation of metalloproteases, the activation of cytosolic membrane-associated tyrosine kinases, and NADPH oxidase-dependent reactive oxygen species (ROS) generation [32]. Over the last years, six homologs of the NADPH oxidase have been identified: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 and are referred as the NOX family of NADPH oxidases [33] which produces ROS as their primary and sole function. NOX2 consists of the cytosolic proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac1, and of membrane-bound subunits $p22^{phox}$ and $gp91^{phox}$. In the nervous system, NOX2 is the most important source of ROS and its activation requires phosphorylation and membrane translocation of the $p47^{phox}$ subunit. In phagocytic and nonphagocytic cells, FPR stimulation by cognate agonists results in MEK- and PKC-dependent $p47^{phox}$ phosphorylation and, in turn, in ROS generation [9, 20, 34, 35], which act as intracellular second messengers by activating several redox signalling cascades.

In different cell types, FPR stimulation induces phosphorylation of Y951, Y996, and Y1175 residues of VEGFR2 [36] and of Y1313, Y1349, and Y1356 residues of the HGF receptor [35], as well as EGFR transactivation [37], suggesting that FPRs are able to integrate GPCR signalling with tyrosine kinase activity of RTKs even in the absence of direct stimulation by growth factors [32]. ROS play a crucial role in these cross-talk mechanisms. In fact, blockade of NADPH oxidase functions by siRNA or by specific inhibitors prevents EGFR, VEGFR2, and c-Met transphosphorylation [35–37].

Trk receptors can also be activated via GPCR stimulation without the involvement of neurotrophins [38]. In human monocytes, cross talks between FPR1, EGFR, and TrkA are essential for receptor-mediated activation and to modulate proinflammatory mediators [39]. Adenosine can activate TrkA phosphorylation through an adenosine 2A receptor, a member of the GPCR family [40] and the neuropeptide PACAP, which acts through a GPCR, transactivates TrkA [41]. Furthermore, the activation of angiotensin receptor type-2 transactivates TrkB [42].

Herein, we show that the SH-SY5Y neuroblastoma cell line expresses NOX2 components, FPR1, and TrkA, and that FPR1 stimulation by formylated peptides induces NADPH-dependent ROS generation, as well as the phosphorylation of Y490, Y751, and Y785 residues of TrkA. Cytosolic phosphotyrosines of TrkA act as docking sites for signalling proteins that, in turn, activate Ras/MAPK, PLC-γ1/PKC, and PI3K/Akt intracellular cascades. Furthermore, FPR1-mediated TrkA transactivation promotes cell proliferation, wound healing, and neurite outgrowth of SH-SY5Y cells.

2. Materials and Methods

2.1. Reagents. SH-SY5Y cell line (ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Monza, Italy) containing 15% foetal bovine serum (FBS) (Invitrogen). After reaching 80% confluence, cells were serum-starved for 24 hours and stimulated with N-fMLP (Sigma) at the final concentration of 0.1 μM for 2, 5, or 10 minutes. In other experiments,

serum-deprived cells were preincubated for 16 hours with pertussis toxin (PTX) (Sigma) at a final concentration of 100 ng/mL, or with 5 μM cyclosporin H (CSH) (Sigma) for 30 minutes, or with 5 μM rottlerin (Sigma) for 1 hour, or with 100 μM apocynin (Sigma) for 2 hours, or with 10 μM GW441756 (Sigma) for 1 hour, before the stimulation with N-fMLP for 5 minutes. SDS-PAGE reagents were obtained from Bio-Rad (Hercules, CA, USA). Anti-phosphoAkt(S473) (cat. no. 4060), anti-phosphoP38MAPK(T180, Y182) (cat. no. 4511), anti-CD133 (cat. no. 5860), anti-phosphoTrkA(Y490) (cat. no. 9141), and anti-phosphoTrkA(Y785) (cat. no. 4168) were from Cell Signalling Technology (Danvers, MA, USA). Anti-phosphoTrkA(Y751) (cat. no. 44-1342G) was from Life Technologies. Anti-phosphop47^{phox}(S359) (cat. no. GTX55429) was from GeneTex (Irvine, CA, USA). Anti-phosphoERK 1/2 (cat. no. SC-81492), anti-PKCα (cat. no. SC-8393), anti-PKCδ (cat. no. SC-937), anti-phosphoPKCδ (T507) (cat. no. SC-11770), anti-ERK 1/2 (cat. no. SC-514302), ant-Akt (cat. no. SC-8312), anti-TrkA (cat. no. SC-398728), anti-tubulin (cat. no. SC-8035), anti-rabbit (cat. no. SC-2357), and anti-mouse (cat. no. SC-2005) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein A-horseradish peroxidase was from Thermo Scientific (Little Chalfont, Buckinghamshire, UK).

2.2. Protein Extraction and Western Blot Analysis. Western blot assay was performed as previously described [43] on whole or membrane lysates. Proteins were purified from growth-arrested SH-SY5Y cells stimulated or not with 0.1 μM N-fMLP, in the presence or absence of the appropriate amounts of selective inhibitors. Whole lysates were obtained by incubation with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 10 μM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/mL aprotinin, 10 μg/mL pepstatin, and 10 μg/mL leupeptin) for 45 min at 4°C [44]. Membrane proteins were purified by incubating SH-SY5Y cells with a buffer containing 10 mM Tris-HCl, 1 mM $CaCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonylfuoride, 10 μg/mL aprotinin, 10 μg/mL pepstatin, and 10 μg/mL leupeptin (Buffer I). Samples were centrifuged at 400 × g for 10 minutes at 4°C, to obtain a cytosolic (supernatant) and membrane (pellet) fraction. Membrane fraction was washed three times in Buffer I and incubated overnight at 4°C in constant agitation with a buffer containing 125 mM Tris-HCl, 1 mM PMSF, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL pepstatin, and 10 μg/mL leupeptin (Buffer II) [45]. Bio-Rad protein assay was used to determine protein concentration (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins (40-60 μg, see legends to figures) were separated on 8%, 10%, or 12% SDS-PAGE (Bio-Rad), depending on molecular weight of analyzed protein. Proteins were electroblotted onto an immobilion-P PVDF membrane (Thermo Fisher Scientific), and aspecific binding sites were blocked by incubating membranes at room temperature with a solution of 5% nonfat dry milk in Tris-buffered saline 0.1% Tween for 1 hour. After overnight incubation at 4°C with primary antibodies, membranes were washed and incubated at room temperature for 1 hour with peroxidase-conjugated mouse or rabbit IgG. The expression of targeted proteins was detected by an ECL chemiluminescence reagent kit and visualized by autoradiography. A Discover Pharmacia scanner equipped with a sun spark classic densitometric workstation was used to evaluate band densitometry. The equal amount of loaded protein was determined by reprobing the same filters with an anti-α-tubulin or anti-CD133 antibody. All western blot experiments are representative of at least four independent experiments.

2.3. Proliferation Assay. SH-SY5Y cells (4×10^4) were seeded in a 24-well plate and cultured in DMEM supplemented with 15% FBS with or without 0.1 μM N-fMLP, in the presence or absence of the appropriate amounts of selective inhibitors. The number of trypan blue-positive and trypan blue-negative cells was counted at 24, 48, and 72 hours, by direct counting using Burker's chamber, as previously described [46]. Five independent experiments were performed in triplicate.

2.4. Wound Healing Assay. Wound healing assay was performed as previously described [47]. SH-SY5Y cells were cultured until 100% confluences with DMEM containing 15% FBS, at 37°C and 5% CO_2. The cell monolayer was scratched with an 80 μm diameter sterile tip, and the plates were washed with PBS to remove the detached cells. Once the wound injury was induced, cells were serum-deprived for 24 hours and incubated with 0.1 μM N-fMLP or with the vehicle, in the presence or absence of the appropriate amounts of selective inhibitors. An image was captured in the same area of the plates 0, 24, and 48 hours after the wound. Images were taken by using the Leica AF6000 Modular System and processed by using the Leica LAS AF lite software. ImageJ software was used to quantify the covered surface from four independent experiments.

2.5. Reactive Oxygen Species Assay. Generation of intracellular ROS was determined by measuring 2′,7′-dichlorodihydrofluorescein-diacetate (H2DCFDA; Sigma) oxidation into the fluorescent 2′,7′-dichlorofluorescein (DCF). Briefly, 4×10^4 SH-SY5Y cells were seeded in a 12-well plate and cultured at 37°C, 5% CO_2 with DMEM supplemented with 15% FBS. Cells were then serum-deprived for 24 hours and stimulated for different times with 0.1 μM N-fMLP in the presence or absence of the appropriate amounts of selective inhibitors. Cells were then incubated for 45 minutes at 37°C with 50 μM H2DCFDA, and the oxidization to the fluorescent DCF was analyzed on the FACS flow cytometer BD Biosciences Accuri C6 Flow Cytometer (BD Biosciences). Five independent experiments were performed in triplicate.

2.6. Neurite Outgrowth Assay. Neurite formation was determined by plating 10^4 cells into wells of 12-well plates in triplicate and cultured with DMEM supplemented with 15% FBS. Cells were then incubated with 0.1 μM N-fMLP or with 100 ng/mL NGF for increasing times. Five images/well were recorded and analyzed for neurite elongation after 24, 48, and 72 hours using ImageJ software plugin NeuronJ from five

independent experiments. The length of neurites was measured starting from the soma in each area. Untreated cells were used as controls. The morphometric analysis was performed on the images obtained under inverted-phase-contrast microscopy (Leica AF6000 Modular System) and processed by using the Leica LAS AF light software.

2.7. Statistical Analysis. All the data presented are expressed as mean ± standard error mean (SEM) and are representative of three or more independent experiments. For the statistical analyses, the comparisons were made by two-way analysis of variance (ANOVA). Differences were considered significant at a value of $p < 0.05$. All the analyses were performed with GraphPad Prism version 7 (GraphPad Softweare, San Diego, CA, USA).

3. Results and Discussion

3.1. FPR1 Stimulation by N-fMLP Induces NOX2 Activation in SH-SY5Y Cells. The human neuroblastoma SH-SY5Y cell line is characterized by a catecholaminergic phenotype, since it can synthesize both dopamine and noradrenaline [48] and represents an *in vitro* model widely used in neuropsychiatric research [48–50]. NOX2 is expressed in SH-SY5Y cells [51, 52], as well as in the brain, in the microglia, astrocytes, and neurons [33], which also express NOX1 and NOX4 [33]. Induction of neuronal apoptosis in response to the brain-derived neurotrophic factor is mediated by NOX2 [53], which is also involved in long-term potentiation and learning [54, 55] and in NMDA receptor signalling [56]. Learning and memory are impaired in NOX2 and $p47^{phox}$ knockout mice [57]. Furthermore, there is evidence for a role of microglial NOX2 in inflammatory neurodegeneration [58, 59] and in the injury of the nervous system, as demonstrated by the observation that NOX2 inhibition or knockdown improves the outcome of the spinal cord injury model in mice [60]. FPR-mediated NADPH oxidase-dependent ROS generation results also involved in the progression of Alzheimer's disease, mainly due to the activation of redox-sensitive pathways [61]. NOX2 activation requires $p47^{phox}$ phosphorylation and its membrane translocation [33, 62].

We observed that, in SH-SY5Y cells, N-fMLP induces time-dependent phosphorylation of $p47^{phox}$ within the first 5 min, which decreases after 10 min of stimulation (Figure 1(a)). SH-SY5Y cells were also treated with PTX, which ADP-ribosylates Gi alpha subunit conjugated to FPR1, or with cyclosporin H, a competitive antagonist of FPR1. The results show that $p47^{phox}$ phosphorylation is completely prevented by preincubation with PTX, or cyclosporin H (Figure 1(b)), suggesting that FPR1 is crucially involved in NADPH oxidase activation. Pretreatment with apocynin (Figure 1(c)), which prevents serine phosphorylation of $p47^{phox}$ and, in turn, NADPH oxidase activation, significantly reduces $p47^{phox}$ phosphorylation. Accordingly, stimulation for different times with N-fMLP induces NOX2-dependent ROS generation with a maximum of ROS production occurring at 5 min (Figure 1(d)) which is prevented by preincubation with PTX, or ciclosporin H, or apocynin (Figure 1(e)).

3.2. FPR1 Stimulation by a Formylated Peptide Induces NOX2-Dependent TrkA Transactivation. Survival of sympathetic and sensory neurons, axon growth and synapse formation, neurotransmitter and neuropeptide synthesis [63] are mediated by NGF which binds TrkA and induces its homodimerization followed by autophosphorylation of each monomer. The NPXY and the YLDIG motif, located in the juxtamembrane region and in the C-terminus of TrkA, respectively, are then phosphorylated creating docking sited for signalling molecules [64]. Y490, Y751, and Y785 represent the main phosphotyrosine residues of TrkA in the juxtamembrane, in the tyrosine kinase, and in the intracellular C-terminal domains, respectively [5, 7].

Cross-communication between GPCRs and RTKs provides the connection between the wide variety of GPCRs and the strong signalling ability of RTKs to modulate intracellular pathways involved in many biological functions. SH-SY5Y cells express both FPR1 [65] and TrkA [66] receptors. We analyzed FPR1-mediated TrkA transactivation in these cells, and in time-course experiments, we observed that the incubation with 0.1 μM N-fMLP elicits the phosphorylation of Y490, Y751, and Y785 residues of TrkA with the highest levels of phosphorylation occurring at 5 min (Figure 2(a)). Preincubation of SH-SY5Y cells with PTX or with cyclosporin H, before the incubation with N-fMLP, completely prevents tyrosine phosphorylation of TrkA (Figure 2(b)), strongly suggesting that TrkA transphosphorylation depends on FPR1 activation. ROS play an important role in RTK transactivation since they can inactivate, by oxidation, cysteines positioned in the catalytic site of protein tyrosine phosphatases (PTPs) [35, 36, 67]. Prevention of PTP action promotes the phosphorylated state of a RTK and, in turn, its transactivation. Several PTPs, such as NEAP/DUSP26, MEG2, SHP-1, are associated with TrkA [68–70]. We preincubated SH-SY5Y cells with apocynin before FPR1 stimulation, and we observed that N-fMLP-induced phosphorylation of Y490, Y751, and Y780 residues of TrkA is prevented (Figure 2(c)). Incubation of SH-SY5Y cells with PTX, or cyclosporin H, or apocynin, without N-fMLP stimulation, for the same times, does not affect the expression levels of TrkA (Supplementary Figure 1). These results demonstrate that NOX2-dependent ROS generation mediates the cross-talk between FPR1 and TrkA. RTK transphosphorylation can also occur via metalloprotease-mediated proteolytic cleavage of a pro-ligand, which generates a ligand able to bind and to transactivate an RTK. We cannot exclude that N-fMLP can promote the release of NGF via the activation of metalloproteases.

3.3. FPR1-Induced TrkA Transactivation Triggers the Ras/MAPK Pathway. Phosphorylated tyrosine 490 of TrkA provides a docking site for the Shc domain. A phosphotyrosine site on Shc recruits Grb2, which is bound to the exchange factor SOS that represents a scaffold for Ras. Activation of Ras is essential for neuronal differentiation, as well as for promoting survival of neuronal subpopulations [71], and is promoted by neurotrophin-dependent phosphorylation of RasGRF1 [72]. Active Ras triggers intracellular signalling through cRaf, PI3 kinase (PI3K),

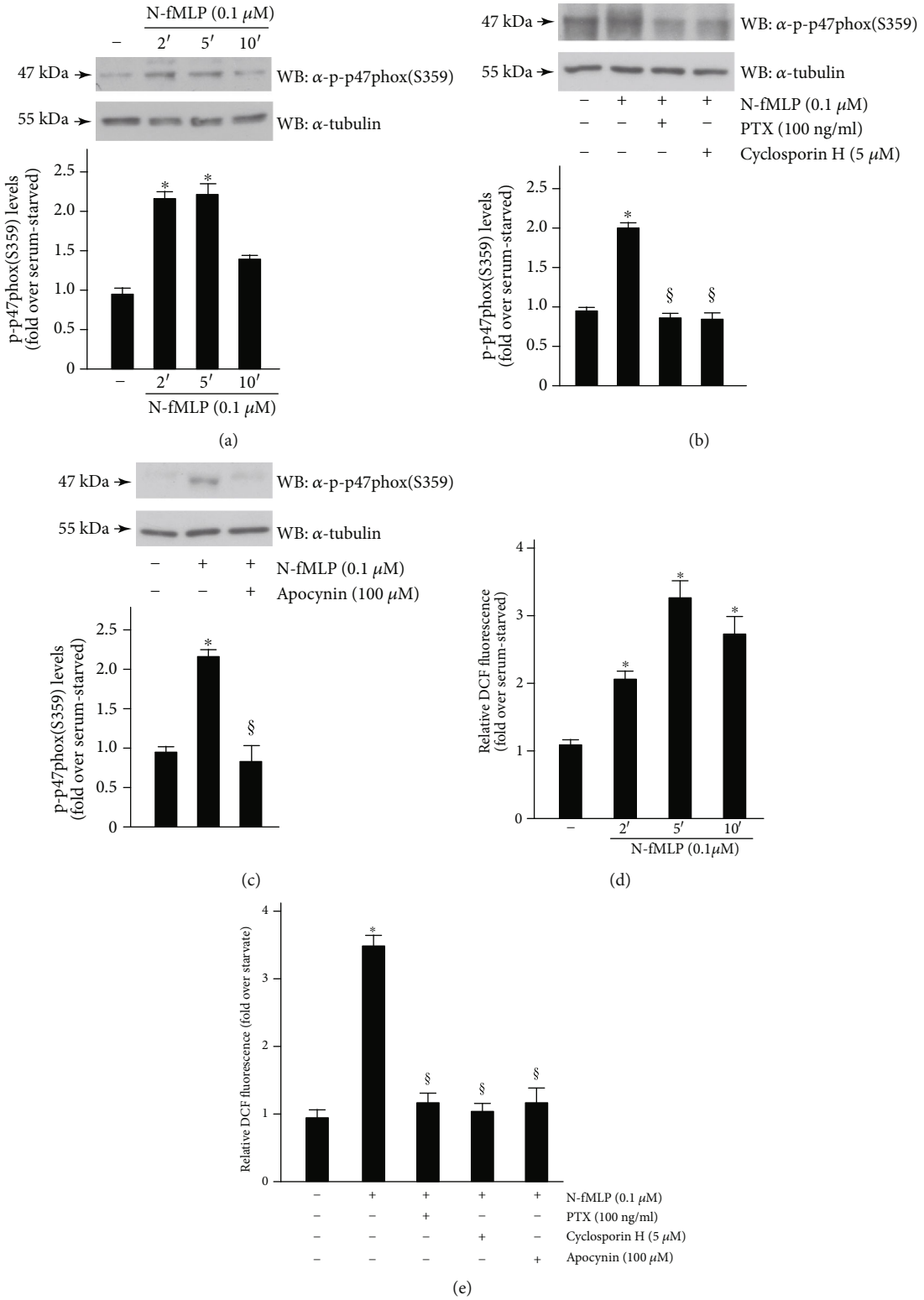

Figure 1: FPR1 stimulation induces NOX2 activation. SH-SY5Y cells were serum-starved for 24 hours and (a) stimulated for 2, 5, or 10 minutes with 0.1 μM N-fMLP or (b) preincubated with 100 ng/mL PTX, or with 5 μM cyclosporin H, or (c) with 100 μM apocynin before the stimulation for 5 minutes with 0.1 μM N-fMLP. Sixty micrograms of whole lysates (a, b, and c) was incubated with a phospho-p47^{phox}(S359)-specific antibody (α-p-p47^{phox}(S359)), and an anti-tubulin (α-tubulin) antibody was used as a control for protein loading. Band densitometry was evaluated through a scanner equipped with a densitometric workstation. Serum-starved SH-SY5Y cells were (d) stimulated with N-fMLP for increasing time or (e) preincubated with PTX, or cyclosporin H, or apocynin before the stimulation with 0.1 μM N-fMLP for 5 minutes. Detection of ROS was determined by measuring the level of DCF. $^{*}p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP stimulated cells.

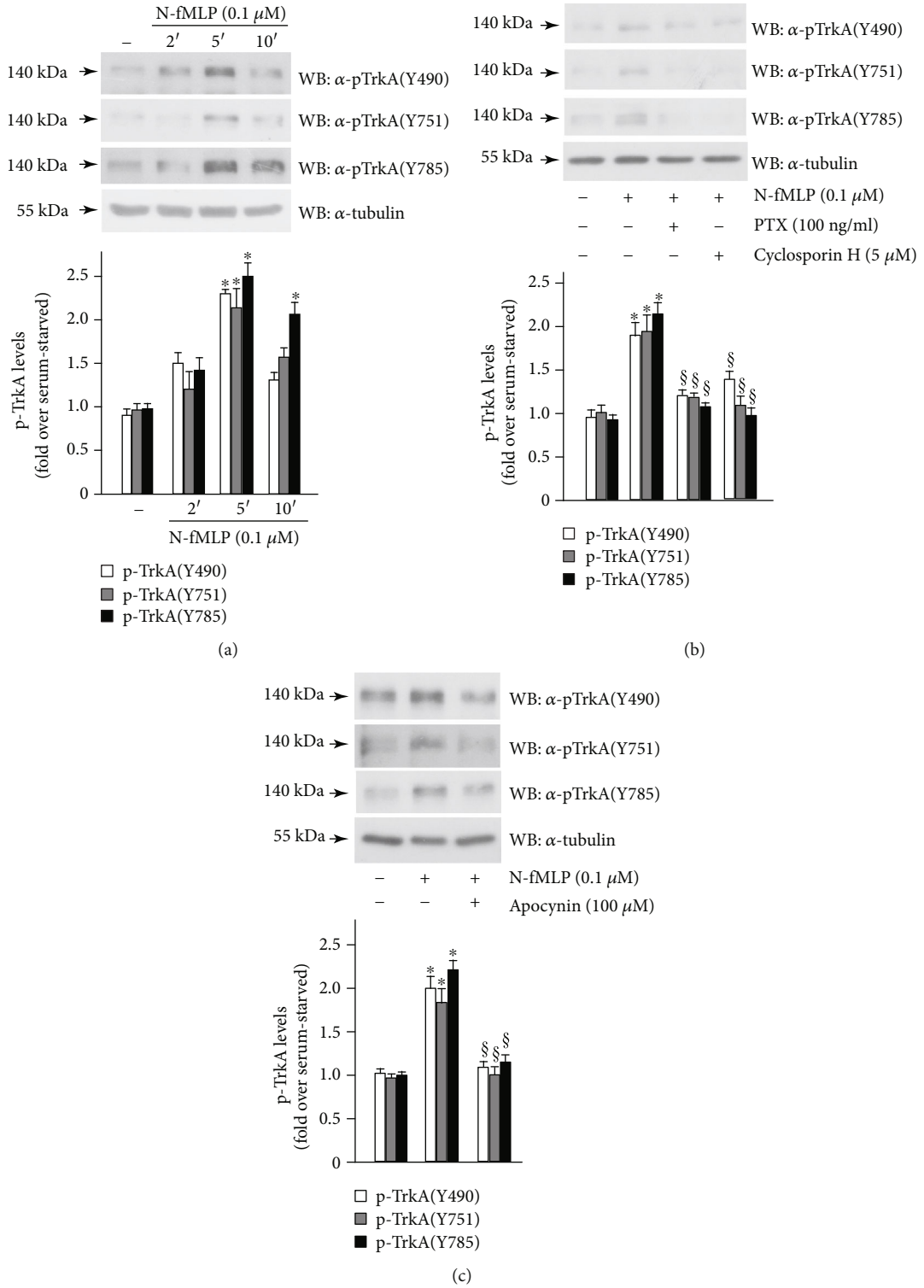

Figure 2: FPR1 stimulation triggers TrkA transactivation. SH-SY5Y cells were growth-arrested for 24 hours and (a) stimulated with N-fMLP for increasing time or (b) preincubated with PTX or cyclosporin H or (c) pretreated with apocynin, before the stimulation with 0.1 μM N-fMLP for 5 minutes. Fifty micrograms of total protein lysates was resolved on 10% SDS-PAGE and immunoblotted with anti-phospho TrkA (Y490) (α-pTrkA(Y490)), or anti-phospho TrkA (Y751) (α-pTrkA(Y751)), or anti-phospho TrkA (Y785) (α-pTrkA(Y785)) antibodies. An anti-tubulin (α-tubulin) antibody was used as a control for protein loading. Bar graphs show the densitometric analysis performed on phosphorylated bands. All the experiments are representative of four independent experiments. $^{*}p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP-stimulated cells.

and p38MAP kinase (p38MAPK) pathways. Raf phosphorylates Mek1/2 which, in turn, phosphorylates ERK1/2 on serine and threonine residues.

We analyzed ERK activation in FPR1-stimulated SH-SY5Y cells, and in western blot experiments, we observed that N-fMLP induces time-dependent phosphorylation of ERK1/2 with the maximum levels of phosphorylation occurring at 5 min (Figure 3(a)). Preincubation of SH-SY5Y cells with PTX or cyclosporin H, before N-fMLP stimulation, prevents ERK phosphorylation (Figure 3(b)). We also pretreated cells with apocynin (Figure 3(c)) or with GW441756 (Figure 3(d)), a potent and selective inhibitor of the ATP-binding site of the TrkA receptor, which in turn prevents its tyrosine phosphorylation and kinase activity. We observed that preincubation with apocynin or GW441756 before N-fMLP stimulation completely prevents ERK phosphorylation. On the other hand, incubation of unstimulated serum-deprived cells with PTX, or cyclosporin H, or apocynin, or GW441756, for the above indicated times, does not modulate the expression levels of ERKs (Supplementary Figure 1). These results suggest that N-fMLP-mediated ERK1/2 activation depends on a PTX-sensitive GPCR, NOX2-dependent ROS generation, and on FPR1-dependent TrkA transactivation.

In neurons, p38MAPK activation depends on the intracellular signalling cascade triggered by Ras-mediated binding of the exchange factor RalGDS, which results in Ral activation and Src recruitment [73]. In neuronal cells, p38MAPK can be also activated via neurotrophin-dependent activation of G proteins Rin and Rit, which belong to the Ras family [74, 75]. We observed that N-fMLP stimulation of SH-SY5Y cells for 5 min induces a significative increase of p38MAPK phosphorylation, which is prevented by preincubation with PTX or cyclosporin H (Figure 3(e)).

3.4. N-fMLP-Dependent Phosphorylation of Y490 and Y751 Residues of TrkA Triggers the PI3K/Akt Pathway. Phospho-Y490 of TrkA provides a recruitment site for Shc, which allows a link also for the PI3K pathway [72], and Y751 phosphorylation is essential for PI3K docking and activation [7]. PI3K generates 3-phosphate phosphoinositides, which show several effects on the development and survival of several populations of neurons. Class I of PI3Ks catalyzes *in vivo* the conversion of phosphatidylinositol (4,5)-bisphosphate into phosphatidylinositol (3,4,5)-trisphosphate. They also convert phosphatidylinositol into phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate into phosphatidylinositol (3,4)-bisphosphate *in vitro*. Class I of PI3Ks are activated via Ras-dependent or independent pathways [76, 77]. 3-Phosphate phosphoinositides recruit and activate phosphoinositide-dependent protein kinase 1 (PDK1) which phosphorylates and activates the serine/threonine kinase Akt.

The activity of glycogen synthase kinase 3 (GSK3) is negatively regulated by serine phosphorylation mediated by Akt [78], and, in neurons, phosphorylation-mediated GSK3 inhibition promotes the prosurvival effects induced by TrkA activation.

The PI3K/Akt/GSK3 signalling cascade may represent a diagnostic and pharmacological target for psychiatric illnesses. In human lymphocytes, PI3K levels are impaired in patients affects with schizophrenia [79] and Akt has been identified as a possible susceptibility gene for schizophrenia [80]. Furthermore, alterations of GSK3 activity represents a schizophrenia risk factor [81].

In SH-SY5Y cells, we observed time-dependent phosphorylation on Ser473 residue of Akt upon stimulation with N-fMLP (Figure 4(a)), which is prevented by preincubation with PTX, or with an FPR1 antagonist (Figure 4(b)). N-fMLP-induced Akt(Ser 473) phosphorylation is also hampered by a NOX2 inhibitor (Figure 4(c)) or by a TrkA inhibitor (Figure 4(d)). SH-SY5Y cells were also incubated, for the above indicated times, with PTX, or cyclosporin H, or apocynin, or GW441756 alone, and we observed that these treatments do not modulate the total expression levels of Akt (Supplementary Figure 1). Taken together, these results demonstrate that N-fMLP triggers ROS-dependent phosphorylation of Y490 and Y751 residues of TrkA which, in turn, provide docking sites for PI3K/Akt signalling.

3.5. FPR1-Mediated Phosphorylation of Y785 Residue of TrkA Provides a Docking Site for PLCγ1/PKC Pathway Activation. Following TrkA activation by NGF, PLCγ1 is recruited to a phosphorylated Y785 residue. TrkA mediates phosphorylation and activation of docked PLCγ1, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate in diacylglycerol (DAG) and inositol triphosphate (IP3). The presence of these two signalling molecules activates almost all PKC isoforms and many intracellular enzymes. In neuronal cells, PKCδ is required for NGF-promoted neurite outgrowth [82] and PKCα is one of the main targets for the regulation of genes involved in neurite [83]. In the CNS, PKCα, β, and γ are most extensively expressed [84] and influence neuronal signalling by short-, medium-, and long-term mechanisms [85]. In frontolimbic structures involved in mood regulation, such as hippocampus and amygdala, PKC isoenzymes are highly expressed [86] and are inhibited by lithium and valproic acid (VPA) [87]. Moreover, the regulation of processes impaired in BD, such as neuroinflammation, oxidative stress, neuroplasticity, glutamatergic neurotransmission, neurotransmitter release, and neuronal excitability involve PKC signalling [88–93].

In SH-SY5Y cells, we observed that in response to the FPR1 agonist, PKCα and PKCδ translocate to the membrane and a significant increase in their level is detectable after 5 min of exposure (Figure 5(a)). Preincubation with PTX or cyclosporin H prevents membrane translocation of PKCα and PKCδ, suggesting that it depends on FPR1 activation. Furthermore, an anti-phosphoPKCδ(Thr507) antibody detects PKCδ phosphorylation and activation in cells exposed for 5 min to N-fMLP but not in cells preincubated with apocynin or rottlerin, a specific PKCδ inhibitor, which prevents PKCδ tyrosine phosphorylation and activation (Figure 5(b)). Incubation of SH-SY5Y cells with PTX, or cyclosporin H, or apocynin, or rottlerin, without stimulation with the formylated peptide, does not affect the expression levels of PKCα and PKCδ (Supplementary Figure 1). These

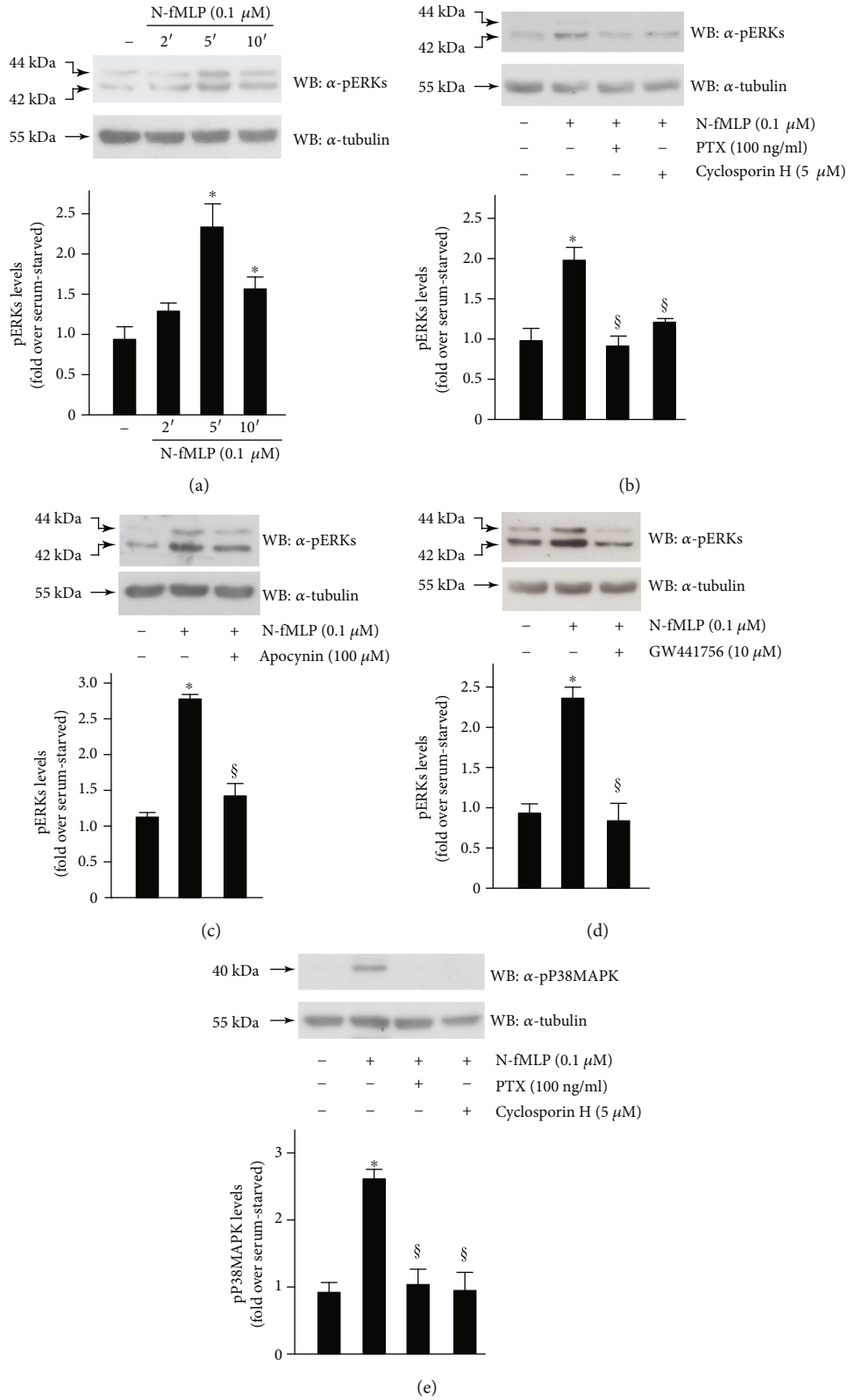

Figure 3: N-fMLP-induced Y490 phosphorylation is crucially involved in ERK activation. Serum-deprived SH-SY5Y cells were (a) stimulated for 2, 5, or 10 minutes with 0.1 μMN-fMLP or (b and e) pretreated with PTX or cyclosporin H, or (c) with apocynin, or (d) with GW441756, before the stimulation with N-fMLP for 5 minutes. Forty micrograms of whole lysates was incubated with (a, b, c, and d) an anti-phospho-ERK (α-pERK) antibody or (e) with an anti-phospho-P38MAPK (α-pP38MAPK) antibody. An anti-tubulin (α-tubulin) antibody was used as a control for protein loading. The data are representative of five independent experiments. Densitometric analysis was performed as described in Materials and Methods. $^{*}p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP-stimulated cells.

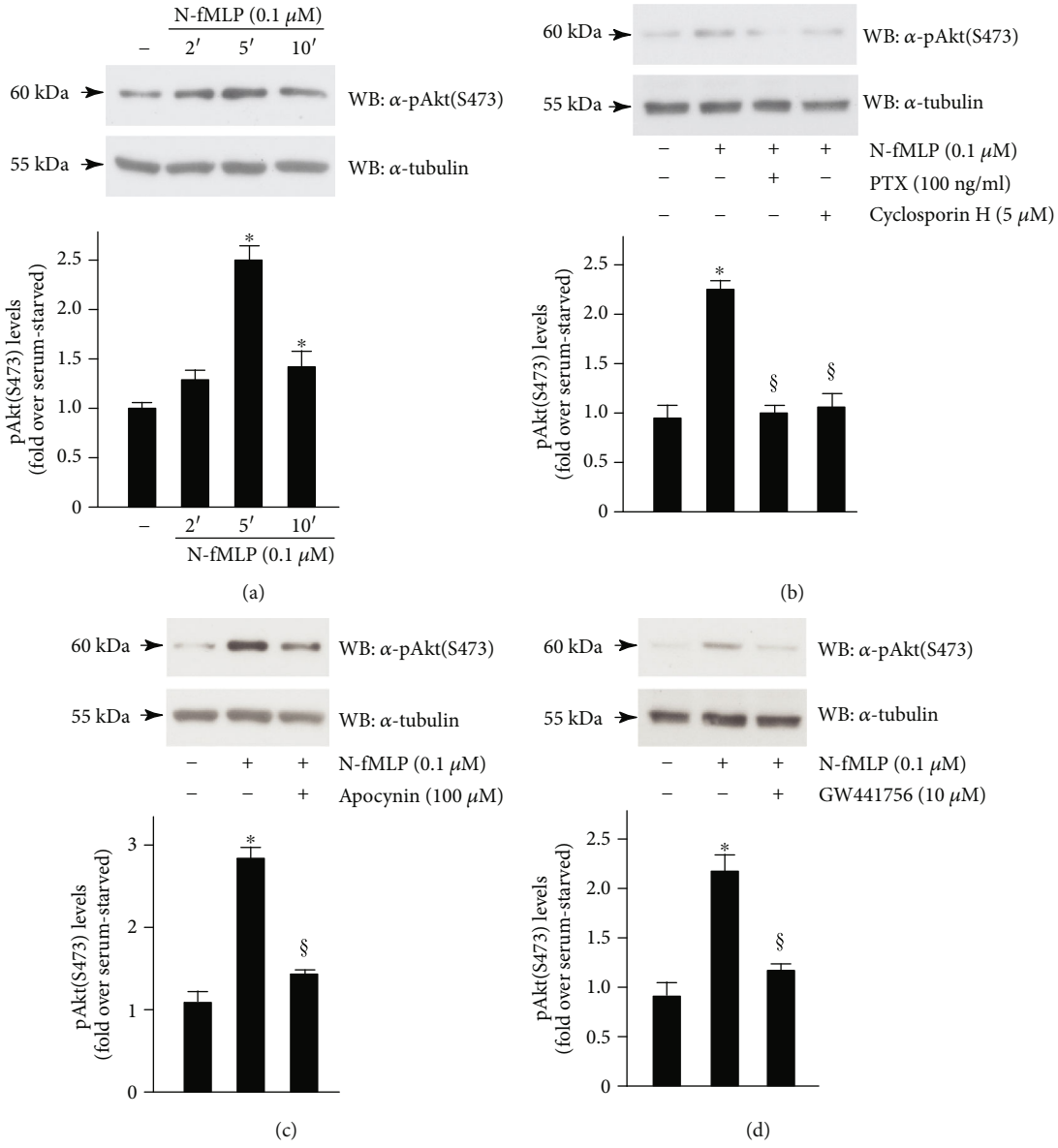

Figure 4: FPR1-mediated phosphorylation of Y751 residue of TrkA provides a docking site for PI3K/Akt pathway activation. SH-SY5Y cells were growth-arrested for 24 hours and (a) stimulated for 2, 5, or 10 minutes with 0.1 μM N-fMLP or (b) pretreated with PTX or cyclosporin H or (c) preincubated with apocynin or (d) with GW441756, before N-fMLP stimulation. Fifty micrograms of whole lysates was immunoblotted with an anti-phospho Akt(S473) (α-pAkt(S473)) antibody. An anti-tubulin (α-tubulin) antibody was used as a control for protein loading. All the experiments are representative of five independent experiments. $^{*}p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP-stimulated cells.

results indicate that PKC activation requires FPR1-mediated phosphorylation of Y785 residue of TrkA and NOX2-dependent ROS generation.

3.6. FPR1-Mediated TrkA Transactivation Promotes Cell Proliferation, Wound Healing, and Neurite Outgrowth. NGF stimulates growth, survival, differentiation, and maintenance of peripheral sensory and sympathetic neurons, both after injury and during development. TrkA plays a key role in neuron proliferation, differentiation, and survival in both peripheral and CNS [94, 95]. Neurotrophic factors hamper cell death, support neuronal proliferation and maturation, and improve the growth of affected neurons, as well as survival and regeneration of neurons [96, 97]. There is also increasing evidence indicating the involvement of neurotrophic factors in the survival, anti-inflammation, proliferation, and differentiation of nonneuronal tissues [98]. Overall, the systemic stimulation with NGF is related with enhanced biological activity of TrkA-expressing cells and is not connected with the induction of tumor cell proliferation [99–101].

We observed that N-fMLP stimulation for 24, 48, and 72 hours induces a time-dependent proliferation of SH-SY5Y serum-deprived cells (Figure 6(a)). It is prevented by

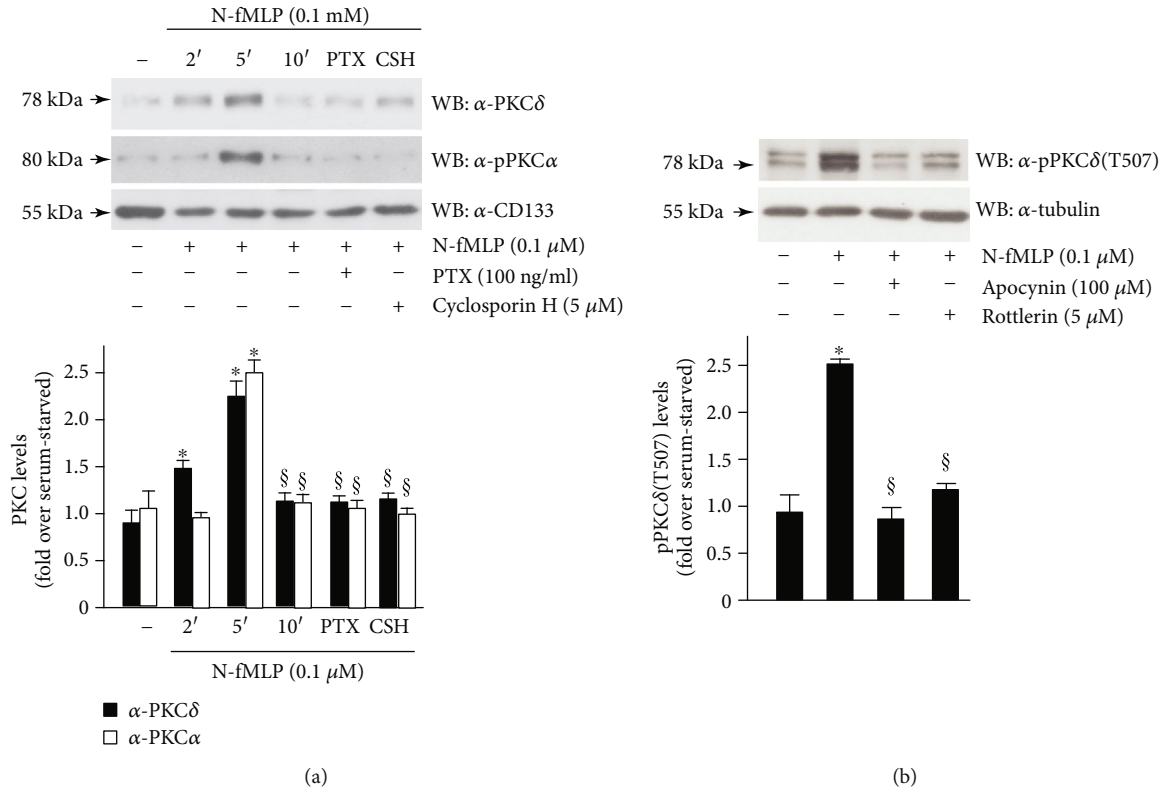

Figure 5: FPR1-mediated TrkA transactivation triggers PLCγ1/PKC pathway activation. SH-SY5Y cells were serum-deprived for 24 hours and (a) stimulated for 2, 5, or 10 minutes with N-fMLP or pretreated with PTX or cyclosporin H before the stimulation for 5 minutes with N-fMLP. (b) Cells were also preincubated with apocynin or rottlerin before N-fMLP stimulation. Fifty micrograms of membrane lysates was immunoblotted with (a) an anti-PKCδ (α-PKCδ) or anti-PKCα (α-PKCα) antibody or (b) with an anti-phosphoPKCδ (T507) (α-pPKCδ(T507)) antibody. An anti-CD133 (α-CD133) or an anti-tubulin (α-tubulin) antibody was used as a control for protein loading. $^{*}p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP-stimulated cells.

preincubation with PTX, or cyclosporin H, or GW441756 suggesting that it depends on FPR1 activation and FPR1-mediated TrkA transactivation (Figure 6(a)).

NGF plays a role also in the repair process, and the pharmacological effect of NGF in accelerating wound healing was demonstrated in both normal and healing-impaired mice [102]. NGF and TrkA are differentially expressed during tissue repair, and NGF represents a bridging factor between all the cells implicated in the healing process. During tissue reorganization and wound healing, NGF might affect epithelization or contraction by priming structural or immune resident/infiltrating cells, or via the stimulation of other profibrogenic factors.

PI3K/Akt and Ras/MAPK pathways play a key role in NGF-promoted wound healing [103, 104]. These two signalling cascades are triggered by NGF-dependent phosphorylation of Y490 and Y751 residues of TrkA, which are also transphosphorylated upon FPR1 activation by N-fMLP (Figure 2(a)). FPR1 has chemotactic properties, and its function is to detect the appearance of harmful molecules, driving cells till the site of their release. Therefore, to evaluate whether FPR1 stimulation promotes wound closure, we analyzed SH-SY5Y cells in an *in vitro* wound healing assay. We observed that N-fMLP induces a more prompt cell migration after both 24 and 36 hours, compared to untreated cells (Figure 6(b)). Preincubation with PTX, or an FPR1 antagonist, or a TrkA inhibitor prevents N-fMLP-dependent wound closure (Figure 6(b)), suggesting that it depends on FPR1 activation and TrkA transactivation.

In primary cultured dorsal root ganglion cells from normal mice, resolvin D1, which efficiently binds FPR2, stimulates neurite outgrowth [105]. FPR2 also promotes neuronal differentiation, with a longer and higher number of primary neurites per cell [106], and inhibition of FPR2 signalling reduces the length of axons and dendrites, suggesting that FPR2 is involved in axonal and dendritic outgrowth [107]. NGF induces TrkA phosphorylation on a Y785 residue, which is also transphosphorylated by FPR1 activation (Figure 2(a)) and which represents a docking site for the activation of the PLCγ1/PKC pathway. PKCδ and PKCα are required for NGF-promoted neurite outgrowth and for the regulation of genes involved in neurite [82, 83]. Therefore, we analyzed the ability of FPR1 to promote neurite outgrowth, and we observed neurite formation after 24 and 48 hours of treatment with N-fMLP (Figure 6(c)).

4. Conclusions

Herein, we demonstrate that in neuroblastoma SH-SY5Y cell line, FPR1 stimulation by its agonist results in NOX2-dependent ROS generation and, in turn, in TrkA transactivation. The observation that apocynin prevents TrkA

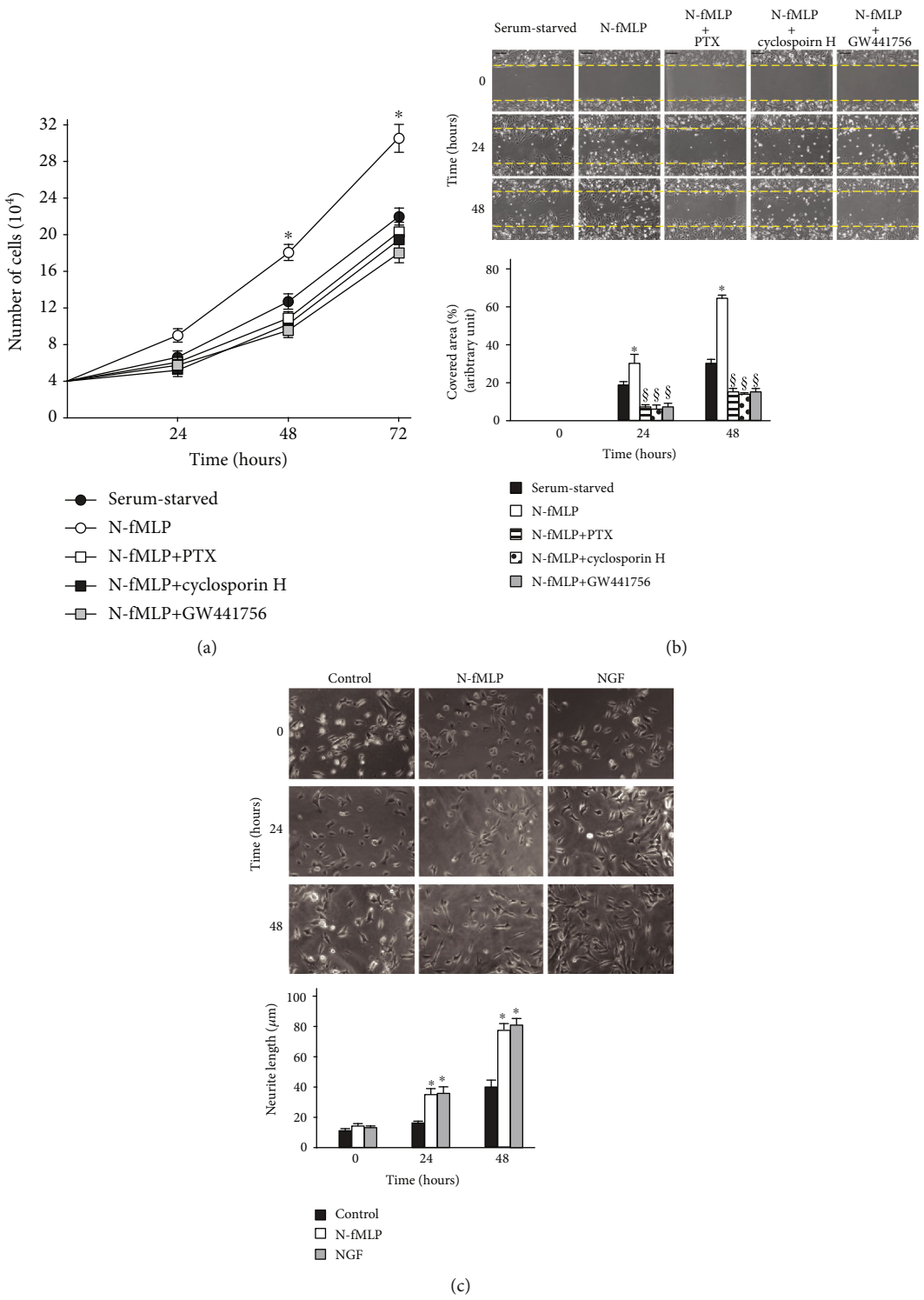

Figure 6: FPR1-mediated TrkA transactivation promotes SH-SY5Y cell proliferation, migration, and neurite outgrowth. (a) SH-SY5Y cells were grown in the presence or absence of 0.1 μM N-fMLP and preincubated or not with PTX, or cyclosporin H, or GW441756. The cellular proliferation graph is representative of five independent experiments. Cell count was determined at 24, 48, and 72 h after plating (for all groups, 10^4 cells/well). (b) Representative images (top) and bar graph quantization (bottom) of SH-SY5Y cell migration from 4 independent experiments. Cells were incubated with 0.1 μM N-fMLP or vehicle in the presence or absence of PTX, or cyclosporin H, or GW441756. Images were acquired at 0, 24, and 48 hours after wound injury (scale bar: 20 μm). $^*p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP-stimulated cells. (c) Representative images (top) and bar graph quantization (bottom) of SH-SY5Y neurite outgrowth from five independent experiments. Neurite length was measured in untreated SH-SY5Y cells (control) or treated with 0.1 μM N-fMLP (N-fMLP) or 100 ng/mL NFG (NGF) up to 48 hours. Neurite length was measured at different times (0, 24, and 48 hours). Arrows show neurite formation. $^*p < 0.05$ compared to unstimulated cells. $^{\S}p < 0.05$ compared to N-fMLP-stimulated cells.

transphosphorylation and the downstream signalling cascades triggered by this receptor highlights the role of ROS in cross-talking between FPR1 and TrkA. We also demonstrate that, as a result of FPR1-mediated TrkA transactivation, phosphotyrosine Y490, Y751, and Y785 of TrkA provide docking sites for the activation of Ras/MAPK, PI3K/Akt, and PLCγ1/PKC signalling cascades. These promote some of the downstream responses triggered by NGF stimulation, such as cell proliferation, migration, and neurite outgrowth.

The phospho-antibodies that we used to assess TrkA phosphorylation are not specific for TrkA. In fact, the anti-phospho TrkA(Y785) can recognize also Y816 of TrkB, whereas anti-phospho TrkA(Y490) detects TrkA, TrkB, and TrkC phosphorylated isoforms. On the other hand, the anti-phospho TrkA(Y751) recognizes only TrkA. However, in many experiments, we blocked TrkA tyrosine kinase activity by using GW441756, which selectively inhibits TrkA and, in turn, TrkA signalling, (Figures 3, 4, 6(a), and 6(b)). Therefore, our results strongly suggest that TrkA is the isoform transactivated by FPR1. Nevertheless, we cannot exclude that also TrkB and TrkC, as well as other tyrosine kinase receptors, can be transactivated by FPR1 in SH-SY5Y cells. Further studies are necessary to investigate the potential contribution of other tyrosine kinase receptors in FPR1-mediated transactivation.

MAPKs play key physiological roles in the mature CNS and represent important targets for the actions of CNS-active drugs [108–110]. MAPK/ERK signalling is responsive to several drugs in the mesocorticolimbic system and is altered upon acute and chronic exposure to drugs [111]. MAPK/ERK pathway plays also an important role in fear memory reconsolidation processes, both in terms of molecular events and brain structures implicated [112]. MAPK/ERK pathway mediates many of the effects of neurotrophic factors and promotes neurite outgrowth. Accordingly, our results strongly suggest that FPR1-dependent MAPK/ERK activation promotes neurite outgrowth in SH-SY5Y cells. Mood stabilizers, such as lithium, VPA, and carbamazepine, represent a class of drugs effective in BD treatment. VPA activates the MAPK/ERK cascade [108] and induces morphological changes of human neuroblastoma cells, such as the presence of prominent growth cones and long neurites. Our results show that N-fMLP triggers the activation of FPR1/TrkA(Y490)/MAPK/ERKs cascade, suggesting that FPR1-mediated ERK activation might represent a promising therapeutical approach for BD treatment.

The regulation of PI3K/Akt/GSK3 signalling is involved in the etiology of depression and mood disorders [113, 114]. In animal models, Akt deletion elicits behaviour modifications that reflect the psychiatric appearance evocative of anxiety, schizophrenia, and depression [115]. The therapeutic effects of several psychiatric drugs are mediated by inhibition of the PI3K/Akt/GSK3 signalling. For instance, lithium, a mood stabilizer widely used for the treatment of depression, schizophrenia, and other mental illnesses, inhibits the GSK3 signalling [116]. In human lymphocytes, PI3K(p110) levels are altered in patients affected by schizophrenia [79]. On the other hand, Akt1 and Akt3 have been identified as possible susceptibility genes for schizophrenia [80] and Akt2 has been associated with anxiety- and depression-like behaviors [117]. Furthermore, Akt activity is decreased in some brain regions of major depression patients [118], and phosphorylated Akt levels are decreased in a depression animal model [119]. GPCRs and RTKs are involved in the activation of the PI3K/Akt/GSK3 pathway, and the selective activation of these receptors or GPCR-mediated RTK transactivation may be effective in treating some neuropsychiatric disorders. In SH-SY5Y cells, we observe that FPR1 activation by N-fMLP triggers TrkA(Y751) transactivation, which provides a docking site for the activation of the PI3K/Akt pathway. Phosphorylated Akt might be involved in supporting the survival of immature neurons and in contributing to cell proliferation and the initial phase of neurite outgrowth [120]. Modulation of TrkA transactivation may provide a new therapeutic strategy for the treatment of depression, schizophrenia, and other mental illnesses. Lithium, which inhibits PI3K/Akt/GSK3 signalling [116], is a mood stabilizer widely used for the treatment of these disorders. However, the molecular mechanisms of the regulation of signalling activity by lithium are poorly understood, and the identification of key signalling cascades is critical to identify novel therapeutic targets. A better comprehension of the complex PI3K/Akt/GSK3 pathway and of its activation may allow an improvement both for the diagnosis and treatments.

PKC isoenzymes are highly expressed in the brain where they play a key role in regulating pre- and postsynaptic neurotransmission. The observation that in cortical homogenates of postmortem patients affected by BD, PKC activity is increased in comparison with healthy controls [121] and that commonly used mood stabilizers inhibit PKC activity, highlight the importance of active PKC signalling in BD [122, 123]. Acute lithium treatment transiently activates PKC, whereas chronic lithium exposure results in a downregulation of PKC isoenzymes in several hippocampal structures but not in cortical and subcortical districts [110]. Neurotrophic factors regulate gene expression and synaptic plasticity via PKC signalling [124]. NGF modulates PKC activity [125], PKC influences NGF expression [126], and the interdependence of these signalling cascades modulates the fine-tuning of the synaptic strength [84]. Moreover, the activation of PKC by TrkA is required for the induction of neurite outgrowth in PC12 cells [127]. In SH-SY5Y cells, we observe that FPR1 stimulation provides a docking site in Y785 of TrkA for the binding of PLCγ and, in turn, for PKC activation. Membrane translocation of PKCα and PKCδ, observed in N-fMLP-stimulated SH-SY5Y cells, strongly suggests that FPR1-mediated TrkA transactivation can further support neurite outgrowth.

Oxidative stress is also implicated in BD pathophysiology [128], and scavengers of ROS show pleiotropic nonspecific PKC inhibition [129]. Quercetin administration can prevent lipid peroxidation in the prefrontal cortex, hippocampus, and striatum in a mouse model of mania induced by paradoxical sleep deprivation, as well as inhibits hyperlocomotion and oxidative stress in these districts [130]. This study provides a new aspect of the role of ROS in neuronal cells. We demonstrate that FPR1-induced ROS generation plays a key role in TrkA transactivation and, in turn, in signalling

cascades triggered by this receptor, as well as in modulating cell proliferation, migration, and neurite outgrowth.

FPR1-mediated transactivation of TrkA provides further opportunities for drug discovery approaches for neuropsychiatric disorders driven by an increase of TrkA activity. The comprehension of signalling pathways responsible for TrkA transphosphorylation and of the intracellular cascades triggered by TrkA transactivation can contribute to identify new drugs efficient to interfere with targets within the FPR1 pathway. Our results also suggest that drugs able to target simultaneously FPR1 and TrkA might have enhanced therapeutic effects in neuropsychiatric disorders, compared with targeting the receptors separately.

Data Availability

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

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: representative western blot (top) and densitometric bar graph (bottom) analysis obtained from four independent experiments performed on whole lysates. SH-SY5Y cells were serum starved for 24 hours and incubated or not with PTX, cyclosporin H, apocynin, or GW441756. Lysates were immunoblotted with anti-TrkA (α-TrkA), or anti-Akt (α-Akt), or anti-PKCα (α-PKCα), or anti-PKCδ (α-PKCδ), or anti-ERKs (α-ERKs) antibodies. An anti-tubulin (α-tubulin) antibody was used as a control for protein loading. *(Supplementary Materials)*

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Research Article

Modification of Immunological Parameters, Oxidative Stress Markers, Mood Symptoms, and Well-Being Status in CFS Patients after Probiotic Intake: Observations from a Pilot Study

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The present study discusses about the effects of a combination of probiotics able to stimulate the immune system of patients affected by Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). To this purpose, patients diagnosed according to Fukuda's criteria and treated with probiotics were analyzed by means of clinical and laboratory evaluations, before and after probiotic administrations. Probiotics were selected considering the possible pathogenic mechanisms of ME/CFS syndrome, which has been associated with an impaired immune response, dysregulation of Th1/Th2 ratio, and high oxidative stress with exhaustion of antioxidant reserve due to severe mitochondrial dysfunction. Immune and oxidative dysfunction could be related with the gastrointestinal (GI) chronic low-grade inflammation in the lamina propria and intestinal mucosal surface associated with dysbiosis, leaky gut, bacterial translocation, and immune and oxidative dysfunction. Literature data demonstrate that bacterial species are able to modulate the functions of the immune and oxidative systems and that the administration of some probiotics can improve mucosal barrier function, modulating the release of proinflammatory cytokines, in CFS/ME patients. This study represents a preliminary investigation to verifying the safety and efficacy of a certain combination of probiotics in CFS/ME patients. The results suggest that probiotics can modify the well-being status as well as inflammatory and oxidative indexes in CFS/ME patients. No adverse effects were observed except for one patient, which displayed a flare-up of symptoms, although all inflammatory parameters (i.e., cytokines, fecal calprotectin, ESR, and immunoglobulins) were reduced after probiotic intake. The reactivation of fatigue symptoms in this patient, whose clinical history reported the onset of CFS/ME following mononucleosis, could be related to an abnormal stimulation of the immune system as suggested by a recent study describing an exaggerated immune activation associated with chronic fatigue.

1. Introduction

Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) is a pathologic condition characterized by persistent and unexplained relapsing fatigue, physical and cognitive, that is worsened by physical and mental exertion [1]. It is a multisystemic condition in which immune functions, mitochondrial function, and cardiovascular, neurological, and endocrine systems are compromised [2–4]. The estimated prevalence of CFS/ME in the worldwide population is between 1 and 0.4% with an age onset of 30-40 years and a prevalence in female subjects [5, 6], and increasingly frequently, also children are diagnosed with CFS/ME, probably as a result of an increased dissemination of knowledge about the disease [7]. Since specific markers for this disease are yet unknown, consequently, the diagnosis is carried out

according to the exclusion criteria. It is possible that in the past, many CFS/ME cases were unrecognized or that incorrect diagnoses have been made. For example, a diagnosis of mental and behavioral disorder for patients with CFS/ME is not uncommon, supported by the observation that these patients often complain difficulty of concentration or look like they have depression [8].

CFS/ME is a condition that is very debilitating both in terms of severity and duration of illness [9, 10]. Since the first description, many researchers have carried out studies in various directions to search for the possible etiologic factors, for example, the role of infectious or chemical and/or physical agents. The infectious hypothesis is based on the observation that CFS/ME often arises following virus infection or reactivation (i.e., Epstein Barr Virus (EBV)). In favor of the toxic hypothesis, there are a lot of data in the literature describing CFS/ME onset after exposure to toxic agents [11, 12]. All researchers agree with the occurrence of an impaired immune response in CFS/ME, particularly as concerns Natural Killer (NK) cell activity [13, 14]. Moreover, a modification in the T-helper cell 1 (Th1)/T-helper cell 2 (Th2) balance leading to an increase in Th2 activation profile, a decreased cytotoxic activity of neutrophils, an altered functionality of B cell subpopulations, and a different ability in metabolizing xenobiotic agents has been described [15–18]. But the main hypothesis about the aetiology of CFS/ME is the infectious one, based on observation that the onset is frequently associated with EBV infection or reactivation.

Patients with CFS/ME exhibit an abnormal immune response to exercise, oxidative stress system dysfunction, and modification in the gene expression profile of immune cells [19]. The increased expression of toll-like receptor 4 correlated with postexertional malaise, which is the main feature of ME/CFS, suggests the existence of an impaired interaction between these immune receptors and pathogenic or xenobiotic agents [20].

Moreover, in CFS patients, structural and functional mitochondrial dysfunctions are documented which lead to increased oxidative stress indexes in response to exercise or inflammation and exhaustion of antioxidant reserve; amelioration of symptoms might occur in relation to antioxidant administration [21–28]. This immune and oxidative dysfunction is associated with gastrointestinal (GI) chronic low-grade inflammation in the lamina propria and intestinal mucosal surface, leaky gut, bacterial translocation, and a particular microbiota composition, overall promoting further immune and oxidative dysfunction. As reported in the literature, bacterial species can modulate the functions of the immune and oxidative systems and the administration of some probiotics can improve mucosal barrier function, modulating the release of proinflammatory cytokines, in CFS/ME patients [21–31]. Chronic inflammation within the gut exaggerates enteric autonomic activation and impairs the anti-inflammatory cholinergic system leading to systemic low-grade neuroendocrine-immune activation that in turn induces a vicious circle, an additional intestinal inflammation and dysfunction as well as the trigger of all inflammatory systemic cascade responsible of CFS/ME symptomatology. It has been also suggested that CFS/ME could be an autoimmune disease for the frequently described relapsing-remitting course and for a higher prevalence in females than in males, which suggests a different gene regulation under the influence of sex hormones [32–34]. The hypothesis of an immune deregulation and of a disrupted tolerance might be related with gastrointestinal (GI) disturbances, frequently observed in CFS/ME patients [35]. The key for the interpretation of this disease may reside in the tolerance mechanisms, in which mesenteric lymph nodes (MLNs) exert a central role in addition with the signals from commensal microbiota [36]. Most lymphocytes and antibodies are produced in the gut, and MLNs are localized at a pivotal area for the control of immune anatomy and migration, forming the crossing border between mucosal immunity and the remainder immune system. The imbalance between Th1 and Th2 immune response observed in CFS/ME could be ascribed to changes in the intestinal barrier functions, which in turn, could trigger autoimmune processes [37]. In support of this hypothesis, patients with this syndrome often complain gastrointestinal disorders with the persistence of a low-grade inflammation in the lamina propria of the intestinal mucosa [38]. It is widely reported that CFS/ME arises from, and is perpetuated by, a mucosal deregulated immune response triggered by unknown etiological factors in a genetically susceptible individual. Emerging research studies on the microbial flora of CFS/ME patients have reported a different fecal microbial composition with a reduction of *E. coli* and Bifidobacterium spp. and a significant rise in the *Enterococcus* sp. prevalence [39]. Similarly, in the small and large intestines of patients with chronic enteritis, a decrease of Bifidobacterium spp. was observed [40]. The gut microbiota plays a major role in the immune system functioning: in general, wealth and diversity of bacterial species in the ecosystem bowel are considered indicators of good health [41, 42]. By contrast, a reduced bacterial biodiversity characterizes different pathological situations, such as inflammatory bowel disease, allergic diseases, type 2 diabetes, and autism [42–45]. Some studies claim that microbial diversity within the gut is also positively associated with mental well-being [46]. Several clinical studies suggest that probiotic administration could represent a preventive and therapeutic strategy for allergic and chronic inflammatory diseases for their capacity to modify the gut microbial composition, improve mucosal barrier function, and downregulate proinflammatory cytokines [47]. In recent years, the knowledge about gut microbiota has been largely improved and the ability of some cultivated bacterial species to modulate the functions of the immune system has been demonstrated [48, 49]. Among these, lactobacilli act as immunoregulators through interaction of lipoproteins with toll-like receptor 2 (TLR2) and of peptidoglycans with nucleotide-binding oligomerization domain-containing protein 2 (NOD2) [50]. In the hypothesis that an altered gut microbiota with a mucosal barrier dysfunction and an aberrant intestinal immunity are involved in the pathogenesis of CFS/ME, a modification of gut microbiota could be one strategy to control the development and/or progression of this disorder. Therapeutic target of probiotic administration in CFS is multisystemic. Probiotics improve mucosal barrier functions reducing low-grade chronic inflammation

Table 1: Probiotic protocol.

1st week	Enterelle 2 cps bid
2nd week	Bifiselle 2 cps bid
3rd week	Ramnoselle 2 cps bid
4-8th week	Enterelle 2 cps Citogenex 2cp Rotanelle 2 cps

as well as bacterial translocation. They modulate the gut-brain neuroendocrine axis which benefits the autonomic nervous system (ANS) inflammatory vicious circle leading to improvement in mood symptoms, pain sensitivity, and cognitive functions. Moreover, they seem to reduce oxidative stress indexes, improve antioxidant defense and mitochondrial function, and modulate immune system response [50]. With the aim to evaluate the effectiveness of some cultivated probiotics to modulate immune functions and counteract the symptoms of CFS/ME, we conducted a randomized clinical trial on patients diagnosed according to Fukuda's criteria treated with probiotics known to have a therapeutic effect in experimental animal models of autoimmune encephalomyelitis [51].

2. Materials and Methods

2.1. Study Population. Patients with CFS/ME diagnosis according to Fukuda's criteria referred to the clinic of Internal Medicine and Geriatrics ASP-IDR Santa Margherita (Pavia, Italy) who gave their consent to participate in the trial were enrolled for the study. The exclusion criteria were as follows: lack of consent, overlap with other diseases, early diagnosis of psychiatric illness, somatoform disorders, abuse of alcohol and drugs, antibiotic therapy, and presence of risk factors for probiotic sepsis according to Boyle's criteria [52].

The study was approved by the local Ethics Committee, and before starting treatment, all the patients signed a written consent to participate in the study in accordance with the Declaration of Helsinki (1964).

2.2. Probiotics. Four different mixtures of probiotics (Bromatech s.r.l, Milano, Italy) were employed for treatments: *Enterococcus faecium* and *Saccharomyces boulardii* (Enterelle); *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis* (Bifiselle); *Bifidobacterium longum AR81* (Rotanelle); *Lactobacillus casei* and *Bifidobacterium lactis* (Citogenex); and *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* (Ramnoselle) according to the schedule of Table 1.

All patients were treated for eight weeks. Probiotics were administered to patients without modifying their usual diet. Nonpathogenic *E. coli* and some fungi (i.e., *Saccharomyces boulardii*) have been demonstrated to be beneficial in CFS/ME patients [29].

Probiotic mixture was selected in relation to the literature data in order to positively interfere in clinical history of CFS/ME.

The probiotics were selected due to their previous documented pharmacological actions in order to affect the intestinal dysbiosis, to reinforce the intestinal mucosal barrier, and to modulate the immune system. The probiotics selected specifically improve mucosal barrier function (mucus and tight junction formation) [53, 54] and compete with gut preexistent pathogens to enhance gut permeability and low-grade gut inflammation and to reduce bacterial translocation. Modulation of the gut immune (GALT) and systemic immune systems depends on probiotic interaction with TLRs activating dendritic cells to trigger modulation of polarizing program (which is different in relation to probiotic combination) to stimulate T1 or regulatory T cell profile [55, 56].

The consequent reduced local and systemic inflammation improves the immune and oxidative system functions [57, 58] and affects the modulation of gut-brain interactions with mood leading to better cognitive performances [51, 59, 60]. The literature suggests that administration of *L. casei*, *L. acidophilus*, and *B. lactis* leads to cognitive improvement in CFS/ME patients [51]. High dose of *L. casei* strain Shirota (LcS) significantly increases both fecal lactobacillus and Bifidobacterium spp. and is associated with anxiety reduction in CFS/ME, suggesting their effect on gut-brain axis modulation with psychological benefit [61].

2.3. Laboratory Analysis. The *erythrocyte sedimentation rate (ESR)* was assessed by a modified Westergren Method: venous blood samples (5–10 mL) were taken in vacutainer tubes under sterile conditions from patients and controls between 08:30 and 10:30 am. Serum was obtained from freshly drawn and rapidly centrifugated. Serum was quickly frozen at −70°C and stored until processed.

Reactive oxygen metabolites were determined photometrically by performing the d-ROM test (Diacron International, s.a.s., Grosseto, Italy) on heparinized plasma.

For *immunophenotyping of leukocytes*, fresh blood samples were collected by venipuncture in EDTA separator tubes and promptly applied to peripheral blood mononuclear cell (PBMC) isolation by Ficoll Density Gradient, using LSM 1077 Lymphocyte Separation Medium (PAA, Pasching, Austria) and centrifugation at 2200 rpm for 20 minutes at 20°C. The intermediate layer consisting of PBMC was recovered, washed in Hanks's medium (PAA) containing 0.1% BSA and 0.5 mM EDTA, and stained with monoclonal antibody against CD3, CD4, CD8, and CD19 (MBL International, Woburn, MA). Flow cytometric analysis was performed through an Epics XL cytometer (Beckman Coulter).

For *quantitative serum immunoglobulin test*, serum fraction of peripheral blood samples was obtained to detect the levels of the three major classes of immunoglobulin (IgG, IgA, and IgM). Ig concentrations were measured by nephelometric technique using the BN Prospec Nephelometer Analyzer and commercially available kits from Dade Behring, Marburg, Germany.

Urinary free cortisol (UC) was measured in the urine collected over 24 hours, making a night dexamethasone suppression test, by liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.

Determination of dehydroepiandrosterone sulfate (DHEA-S) concentration was measured by chemiluminescence technique on the automatic device "Immulite 2000" Siemens® Los Angeles, CA, USA.

For *determination of fecal calprotectin (CAL)*, the stool samples were prepared and analyzed for calprotectin levels according to the manufacturer's instructions (PhiCal Calprotectin ELISA Kit; Immunodiagnostic, Bensheim, Germany). Calprotectin levels, expressed as micrograms per gram of feces, were determined in a stool homogenate obtained with the addition of an extraction buffer containing citrate in a weight/volume ratio of 1 : 50 with the quantitative ELISA method at OD 450 nm.

For *determination of C-reactive protein (CRP)*, quantitative concentration of CRP was determined in serum fraction by immunoturbidometric method by means of the Abbott Architect c-800 system and using 6K26 MULTIGENT CRP Vario (Abbott Laboratories, Illinois, USA).

2.4. Statistical Analysis. Statistical analysis was performed with the commercial software SPSS (for Windows, version 20.0; SPSS Inc., IBM, Armonk, NY, USA) according to the appropriate tests for each considered variable. One-way analysis of variance was applied for comparison among groups. Tukey's test and Bonferroni's corrections were used as post hoc tests. The t-test was applied for means comparisons. p values less than 0.05 will be considered significant.

2.5. Clinical Evaluations. Health status, quality of life, and mood were assessed both at the beginning and at the end of the study administering a short battery of 4 questionnaires. The *SF-36 Health Survey* is a polyvalent short form health survey composed of 36 questions that yields two summary indexes, physical and mental, respectively, PCS and MCS [62]. *Chadler's scale* is used to measure the severity of fatigue [63]. The *Beck Depression Inventory I (BDI-I)* and *Beck Depression Inventory II (BDI-II)* are used for measuring the severity of depression [64, 65].

3. Results

The present study was carried out from January 2010 to January 2014 at the Internal Medicine and Geriatrics Department of Pavia University at the ASP-IDR Hospital Santa Margherita (Pavia, Italy). 13 patients diagnosed with ME/CFS who met the criteria for CFS/ME defined by Fukuda's criteria were enrolled [1]. Four of 13 recruited patients discontinued treatment early, before the 8-week protocol of going out of the study. The statistical analysis was performed on 9 of 13 patients enrolled. All eligible patients gave consent to diet supplementation with probiotics, according to the protocol described in Materials and Methods.

The probiotics used to treat patients were chosen to consider the possible pathogenic mechanisms of CFS/ME syndrome which has been associated with an impaired immune response against a hypothetical infectious agent and with a dysregulation of Th1/Th2 ratio. Each patient received a probiotic combination of different types of bacteria able to counteract pathogens, reinforce the mucosal barrier, and modulate the immune system. More specifically, the probiotic mixture (Enterelle) is composed of *Enterococcus faecium UBEF-41*, *Saccharomyces cerevisiae* sub. *boulardii*, and *Lactobacillus acidophilus LA 14*. *E. faecium* and *S. boulardii* are bacterial strains with a competitive action against antibiotic-resistant microorganisms such as *E. coli*, *C. difficile*, and *C. albicans*. Particularly, *S. boulardii* stimulates IgA production [66]; *L. acidophilus* regulates dendritic cell activation and maturation [67]. The other probiotic mixtures contain different Bifidobacterium spp.: *B. longum*, *B. breve*, *B. bifidum*, *B. infantis* (Bifiselle), and *B. longum AR81* strain (Rotanelle).

Bifidobacteria are anaerobic microorganisms that colonize the intestine and counteract proliferation and metabolic activities of other bacteria helping with the removal of nitrogen compounds derived from putrefaction processes triggered by the proteolytic bacteria *Klebsiella*, *Proteus*, *Clostridia*, and *Bacteroides* [68]. Moreover, a lot of the metabolites produced by Bifidobacteria are short-chain fatty acid able to stimulate the immune system and induce the differentiation of dendritic cells. In particular, *B. longum* stimulates Th1 subset and antiviral action and *B. bifidum* stimulates T-helper cell 17 (Th17) subset [69–71]. *Lactobacillus casei* was demonstrated to be able to rebalance Th1/Th2 subsets and stimulate the innate immune response mediated by NK cells and macrophages through the stimulation of IL-10 and IL-12 secretion [72].

In order to obtain a multifunctional stimulation and a functional rebalancing of the immune system, two different types of probiotic preparations were administered to CFS/ME patients: *Lactobacillus rhamnosus* combined with *Lactobacillus casei* (Ramnoselle) and *Lactobacillus casei* combined with *Bifidobacterium lactis* (Cytogenex). Clinical evaluations to assess well-being status and laboratory tests to state inflammatory indexes were performed before and after probiotic administrations.

The evaluation of well-being status was performed by applying Chadler's scale and the Short Form Health Survey (SF-36), before and after probiotic intake to assess, respectively, physical (PCS) and mental (MCS) components. In Figure 1, the mean values of PCS and MCS obtained by applying the SF-36 survey are shown, before, during, and after taking the probiotic protocol. Comparing the indexes obtained during and after probiotic intake with the basal values, we observed a progressive reduction of Chadler's scale score indicating a reduction of fatigue and a progressive increase of both PCS and MCS indexes indicating an improvement of both physical and mental conditions after probiotic administration. All these results agree with an overall increase in the quality of life of patients.

In Figure 2, the values obtained by applying the two different Beck Depression Inventory (BDI-I and BDI-II) tests are reported. The results of both tests showing a reduction of indexes during and after probiotic protocol in comparison with the basal values indicate an improvement of mood according to the reduced perception of fatigue observed by means of PCS tests.

In order to evaluate the probiotic capacity to modify inflammatory chronic condition, a series of analyses

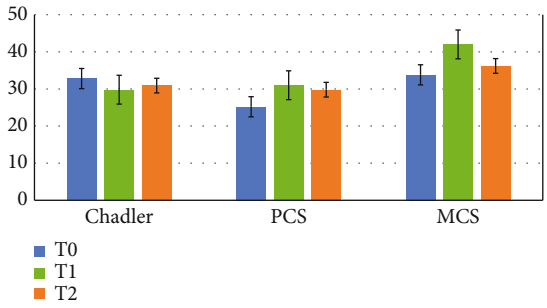

Figure 1: Health status indexes. Chadler's scale score and PCS and MCS indexes, respectively, for physical and mental condition before and after probiotic protocol. T0: mean basal values; T1: mean values after 4 weeks of probiotic protocol; T2: mean values after 8 weeks of probiotic protocol ($n = 9$).

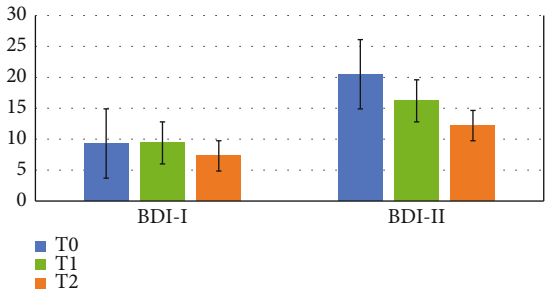

Figure 2: Mood indexes. Mean values of Beck Depression Inventory tests (BDI-I and BDI-II) before and after probiotic protocol. T0: mean basal values; T1: mean values after 4 weeks of probiotic protocol; T2: mean values after 8 weeks of probiotic protocol ($n = 9$).

evaluating different indexes were carried out; specifically, urinary cortisol (UC), fecal calprotectin (CAL), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) were measured. The relative increases/decreases of the fold change values after taking probiotics with respect to the baseline were calculated for each parameter, and the mean values obtained were compared. The results are reported in Figure 3, showing the increase of urinary free cortisol (2.3x), ESR (1.7x), and DHEA-S (1.4x) and a reduction of about 30% of CRP values after probiotic intake. The results obtained indicated that although CRP reduces after probiotic intake, other inflammatory indexes increase at the end of the study versus baseline. The differences were not statistically significant.

It is well known that the ESR index is vulnerable to misinterpretation in clinical practice unlike CRP, which is sensitive to subtle changes in the acute phase response and falls quickly once inflammation subsides, because of its short half-life. For this reason, in chronic inflammatory conditions, the accuracy and sensitivity of ESR and CRP is a topic of debate because of age, gender, and adiposity. For example, high ESR/low CRP discordance is frequently observed in women, likely associated with their propensity to develop connective tissue disorders as reported by some studies [73].

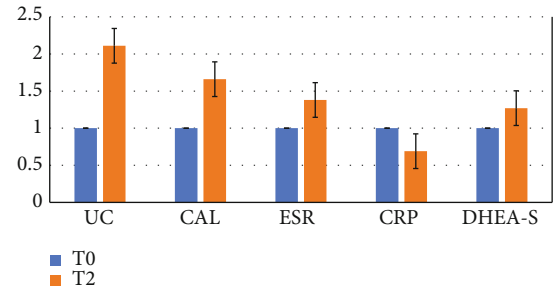

Figure 3: Inflammatory parameters. Inflammatory indexes (fold change values) following probiotic administration. UC: urinary free cortisol; CAL: fecal calprotectin; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; DHEA-S: dehydroepiandrosterone sulfate ($n = 9$).

Our data shows that the more sensitive test for systemic inflammation (CRP) ameliorates after probiotic administration.

As concerns calprotectin, which is a specific marker of intestinal inflammation, patients showed basal values higher than the normal range (2.5-10x), with an increase of these values after probiotic treatment. The fecal calprotectin (CAL) increase does not agree with systemic inflammatory index (CRP) reduction as well as with the improvement in clinical symptoms (such as mood improvement and reduction in fatigue symptoms) observed; it is possible that probiotic administration modulating the inflammatory, immune, and nervous enteric systems might initially induce a local and transient increase of local inflammation; further data and/or successive controls are necessary to better clarify the significance of the data obtained.

Urinary free cortisol (UC) as well as DHEA-S increases after probiotic intake; it must be noted that the levels of urinary cortisol as well of DHEA-S are very low in the CFS/ME patients studied (in accordance with the literature data) and that the level of urinary cortisol is within the normal range after probiotic administration. Cortisol and DHEA-S levels are closely linked with stress as well as with the body's ability to cope with stressful conditions; moreover, it has to be noted that patients with CFS/ME usually exhibit low cortisol levels. The increase (to normal) of these hormones we observed seems to indicate the ability of probiotic administration to interfere with the neuroendocrinology of stress, increasing stress hormone production; moreover, amelioration of stress response might be associated with the obtained clinical data in terms of reduction of fatigue and amelioration of mood symptoms as well as quality of life.

To evaluate the ability of probiotics to stimulate the immune system, serum levels of immunoglobulins (IgM, IgG, and IgA) and the prevalence of CD4 and CD8 lymphoid cell subsets were determined. In Figure 4, the average values of these parameters (fold change values) after probiotic intake are shown. Considering the mean values, after treatment with probiotics, a significant increase of IgM (of about 3x over the basal values) was observed, but no changes as concerns IgG and IgA serum levels were observed; further data and/or successive controls are necessary to better clarify the significance of the data obtained. A reduction of

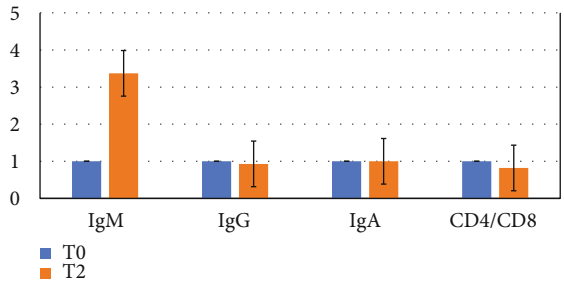

Figure 4: Immunological parameters. Immunoglobulin levels and CD4+/CD8+ lymphocytes ratio (fold change values) following probiotic intake ($n = 9$).

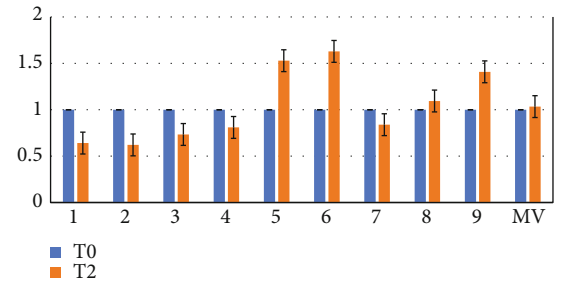

Figure 5: Oxidative stress. Oxidative stress index (d-ROMs) in each patient following probiotic intake. T2 levels compared to T0 levels (baseline) (fold change values, T0/T2 ratio). MV: mean values ($n = 9$).

CD4/CD8 ratio with a mean index value of 1.78 vs. 2.06 was obtained. Three patients showed a higher reduction (more than 50%), 1 patient a slight reduction (about 5%), and in 5 patients, this index was unchanged.

In Figure 5, the d-ROM index before and after probiotic protocol is reported. Similarly to CD4/CD8 ratio, a slight reduction of mean values was obtained but a great variability among patients.

Particularly, patients with very low d-ROM values in T0 (Group A) increase oxidative production in T2; conversely, patients with normal d-ROM values at T0 (Group B) decrease oxidative production after probiotic intake as reported in Figure 6.

Comparison between Group A and Group B at T0 (Figure 7) indicates that patients with lower d-ROM values in T0 have greater degree of depression (higher levels in BDI tests) and greater symptoms of fatigue (higher Chadler's scale score). Moreover, Group A shows higher levels of UC and lower physical and mental indexes of quality of life (higher levels in SF-36 indexes both in PCS and MCS) than patients in Group B.

Following probiotic intake, amelioration of fatigue (Chadler's score), improvement of mood and quality of life indexes (MCS and PCS), reaching significant values for mental health, and urinary cortisol level increase were observed in Group A as suggested by fold change values by comparing T0 and T2 results (Figure 8, fold change values, T0/T2 ratio).

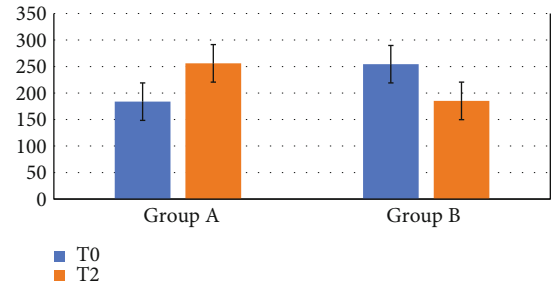

Figure 6: Oxidative stress index (d-ROMs) in the two groups of patients at T0 and at T2. Group A: very low d-ROM values at T0; Group B: normal d-ROM values at T0 ($n = 9$). Patients with very low d-ROM values in T0 (Group A) increase oxidative production in T2; patients with normal d-ROM values at T0 (Group B) decrease oxidative production after probiotic intake.

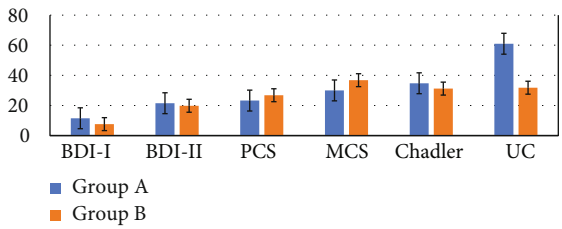

Figure 7: Inflammatory parameters, health status indexes, and mood indexes. Group A at T0 and Group B at T0 compared ($n = 9$). Lower d-ROM in T0 is associated with greater degree of depression (BDI) and fatigue (Chadler's scale score), higher levels of UC, and lower physical and psychological quality of life (PCS and MCS lower level). UC level is higher in Group A than in Group B in T0.

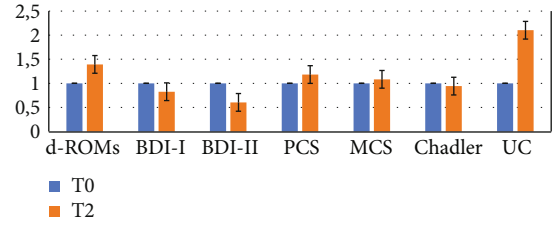

Figure 8: Inflammatory parameters, health status indexes, and mood indexes. Group A at T0 and at T2 compared (fold change values, T0/T2 ratio) ($n = 9$).

Except for d-ROM levels, after taking the probiotic protocol, similar results were obtained in Group B, as shown in Figure 9 (fold change values, T0/T2 ratio) where we reported the fold change values obtained by comparing T0 and T2 results of Group B.

To explain the individual difference in the effects of probiotic intake, we analyzed the possible correlation between d-ROM changes and psychophysiological states of CFS/ME patients. Our results suggest a nonsignificant correlation between d-ROM and Chadler's scale ($p = 0.346$,

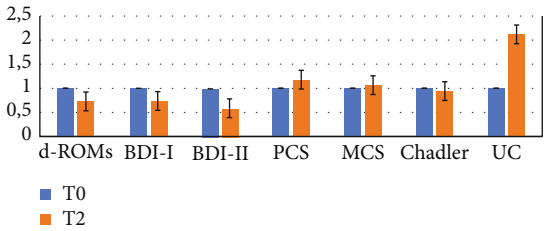

Figure 9: Inflammatory parameters, health status indexes, and mood indexes. Group B at T0 and at T2 compared (fold change values, T0/T2 ratio) ($n = 9$).

$t = 1.0097$), between d-ROM and BDI-I and BDI-II inventory ($p = 0.389$, $t = 0.91882$; $p = 0.184$, $t = 1.4734$, respectively), and between d-ROM and PCS indexes ($p = 0.708$, $t = 0.39086$). Interestingly, the analysis between d-ROM and MCS indexes shows a significant correlation with $p = 0.043$ and $t = 2.4659$ underlying the potential probiotic beneficial effect on mood and psychological state. The data obtained show that patients with very low levels of stress oxidative response (lower d-ROM basal levels) increase oxidative stress after therapy but exhibit a similar trend of clinical response to probiotic administration. Further data and/or successive controls are necessary to better clarify the significance of the data obtained.

4. Discussion

The changes in clinical and laboratory features show a modulation of intestinal and systemic inflammation as well as an improvement in fatigue and mood symptoms after the probiotic protocol supplementation in patients enrolled. The results suggest that the probiotic ability to counteract the main features of CFS/ME, i.e., chronic fatigue, immune imbalance, and psychophysical discomfort, affects the well-being status of patients. Our data agreed with a recent study which demonstrated that some bacterial strains belonging to the Bifidobacterium genus improve mood and quality of life in patients with inflammatory bowel disease (IBD); the improvements were associated with changes in brain activation patterns indicating a reduction of limbic reactivity due to probiotic activity [74].

Probiotics modulate the host's defenses including the innate and acquired immune system [75] playing an important role in the prevention and in the therapeutic approach of infectious diseases and of chronic inflammation, particularly of the digestive tract. A lot of studies, particularly those based on the most advanced technologies, have demonstrated that probiotics can interfere with commensal and/or pathogenic microorganisms because of synergic and antagonist mechanisms between different bacterial groups [76]. This interaction is crucial during prevention and treatment of infections to gut microbial equilibrium retrieval. Probiotics also act on microbial products like toxins on host products (e.g., bile salts) and food components resulting in toxin inactivation and host detoxification. The use of probiotics aims at stabilizing or reconstituting the physiological balance between the intestinal microbiota and its host. However, it must be stressed that no specific probiotic is able to prevent or treat all kinds of diseases, because the ability of probiotics depends on the kind of molecules expressed in the gut of patients and on the metabolic properties of components secreted by the bacterial strains. The main target cells are gut epithelial and gut-associated immune cells. The interaction of probiotics with host cells by adhesion itself might already trigger a signaling cascade leading to immune modulation. Alternatively, release of soluble factors can trigger signaling cascades in immune or epithelial cells.

The manipulation of the gut flora cannot however be considered a precision intervention because of the lack of knowledge about microbial communities that harbor the human gut and, particularly, on the complex relationships among the different species. Finally, although probiotics are considered safe, there are concerns about their use in people with highly compromised immune systems, and in premature infants, as revealed by some infection events as a consequence of probiotic intake in immunosuppressed children and in severely debilitated patients [52, 77, 78]. Although these events occurred rarely, it is necessary to take into account the possibility of bacterial translocation in the presence of an increased intestinal permeability frequently occurring in gut inflammation and of an incomplete, not yet well-established, microbial colonization, which represents a common condition for newborns and preterm infants [79]. On the other hand, the evidence for the efficacy of probiotic supplementation demonstrated well-established benefits [80].

The observations here reported and obtained from a pilot study aimed at exploring the feasibility of an adjuvant treatment in CFS/ME patients. Even with a low sample size, our results showed that in CFS/ME patients, the administration of some combination of probiotics could be practicable and safe. The probiotics used were able to improve psychophysical well-being of patients. No adverse effects were observed in all patients. One patient showed an exacerbation of symptoms at the beginning of the therapy, and for this reason, the treatment was discontinued. It was not possible to ascertain whether this event was related to the assumption of probiotics. We were not able to ascertain whether this effect was a consequence of the treatment or due to an intrinsic factor of that subject such as the preexistence of an overactive immune system. A recent published study highlighted that a chronic fatigue condition with diagnostic features overlapping those observed in CFS/ME condition arises in a small proportion of patients who were treated with interferon alpha to stimulate the immune system [81]. Considering that CFS/ME is a condition in which the immune system seems to be constantly subjected to activation stimuli, it is worthwhile, for further studies, to consider the possibility that an exaggerated stimulation of the immune system could cause a worsening of symptoms. To avoid this event, a larger monitoring of the immune activation status of patients to be treated and the choice of those probiotics having a prevalent immune regulation activity, avoiding those with high stimulatory effects, would be desirable.

In the context of a low sample size, the data reported here show that treatment with probiotics can be viable and safe.

The clinical laboratory data obtained are representative of a population of patients with CFS/ME treated with a combination of probiotic microorganisms administered at an appropriate dosage and chosen according to their specific therapeutic targets.

Data Availability

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

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Review Article

The Role of Oxidative Stress in Common Risk Factors and Mechanisms of Cardio-Cerebrovascular Ischemia and Depression

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The public health sector faces a huge challenge as a result of the high prevalence and burden of disability caused by ischemic cardio-cerebrovascular disease (CVD) and depression. Although studies have explored the underlying mechanisms and potential therapies to address conditions, there is no treatment breakthrough, especially for depression which is highly influenced by social stressors. However, accumulating evidence reveals that CVD and depression are correlated and share common risk factors, particularly obesity, diabetes, and hypertension. They also share common mechanisms, including oxidative stress (OS), inflammation and immune response, cell death signaling pathway, and microbiome-gut-brain axis. This review summarizes the relationship between ischemic CVD and depression and describes the interactions among common risk factors and mechanisms for these two diseases. In addition, we propose that OS mediates the crosstalk between these diseases. We also reveal the potential of antioxidants to ameliorate OS-related injuries.

1. Introduction

Epidemiological data indicate that cardio-cerebrovascular diseases (CVD) and depression pose a huge global disease burden. The Global Burden of Disease 2016 (GBD 2016) study showed that CVD were the number one reason of years of life lost (YLLs) globally [1], whereas GBD 2017 demonstrated that stroke and ischemic heart disease (IHD) had the highest mortality and disability-adjusted life-years (DALYs) in China in 2017 [2]. Also, GBD 2017 reported that major depressive disorder was the third cause of years lived with disability (YLDs) after low back pain and headache disorders [3]. Therefore, there is a need to understand the underlying mechanisms and find effective therapies to control CVD and depression. A previous study demonstrated the relationship between cardio-cerebrovascular ischemia and depression [4] and clarified the vital role of oxidative stress (OS) in the pathogenesis of these two prevalent diseases. However, how OS works and its relationship with other mechanisms still remain unclear and there is little compelling evidence showing depression can cause ischemic CVD via some clear mechanisms, although many studies proved depression acting as a dependent or independent risk factor for ischemic CVD [5]. Herein, this review summarizes studies in the last five years and focuses on the risk factors and mechanisms of ischemic CVD and depression. It explores the central role of OS in the connection between the two diseases in detail, aimed at finding emerging targets in future therapies.

2. Overview of Cardio-Cerebrovascular Ischemia

Ischemic CVD are diseases caused by a lack of blood supply because of changes in blood vessels or blood flow, leading

to multiple organ dysfunction and even death. The main types of ischemic CVD are cerebral ischemic stroke and IHD. But it is widely known that the main pathophysiology of IHD is coronary blood flow reduction caused by coronary artery atherosclerosis [6]; therefore, the term "coronary heart disease (CHD)" is often used to describe this syndrome. Thus, this review focuses on cerebral ischemia and CHD. In this section, their prevalence, common risk factors and mechanisms are discussed. Moreover, either ischemic stroke or CHD gives rise to depression.

2.1. Overview of Ischemic Stroke. Of all strokes, ischemic stroke accounts for 87%, and the remaining 13% are hemorrhagic strokes. According to the GBD 2016, there were about 80.1 million cerebrovascular patients worldwide, among whom 67.6 million had an ischemic stroke and 2.7 million died [4]. Generally, ischemic stroke symptoms manifest as sudden confusion or difficulty in speaking and sudden facial numbness and weakness of limbs [7, 8]. The high prevalence and disability associated with this condition are tightly associated with the risk factors and pathophysiological mechanisms, which are discussed in the next sections. As for risk factors, approximately 90% of them are health-related (such as high systolic blood pressure (BP), obesity, hyperglycemia, and hyperlipidemias), whereas 74% are behavioral risk factors and 29% are air pollution risk factors [4]. When it comes to mechanisms, they are associated with abnormal cell metabolism, cellular dysfunction, and various pathological events (such as immune responses, inflammatory reactions, apoptosis, and OS) [9, 10]. These often lead to the incomplete blood-brain barrier (BBB), loss of cell integrity, acute neuronal death, and early/secondary brain injuries [11].

Diseases of postischemic stroke including motor and sensory deficits, aphasia, and psychological distress affect patients' recovery to a large extent [12]. According to previous studies, one-third of stroke survivors experience depression, anxiety, or apathy. A systematic review and meta-analysis covering 50 studies revealed the prevalence of depression among stroke survivors to be 29% (95% confidence interval (CI) 25-32) [13]. In another meta-analysis covering 61 observational studies, the pooled frequency estimate of poststroke depression (PSD) was 31% (95% CI 28-35), though the proportional frequency varies across studies [14]. Moreover, in women, a prior history of depression and major physical disability was a significant predictor of PSD occurring within the first six months [15]. Taken together, cerebral ischemic stroke is a prevalent intractable disease with complicated mechanisms and it has a close relationship with depression, requiring effective prevention and treatment for both.

2.2. Overview of Cardiac Ischemia. Similar to ischemic stroke, CHD has a high incidence and some common risk factors and mechanisms at the cellular and tissue level. Data showed that CHD accounted for 43.2% of CVD deaths in 2016, including more than 3.6 million people in the United States [4]. In terms of risk factors, despite the variations in populations (e.g., age, sex, country of origin, and ethnic groups), traditional risk factors include unhealthy dietary, high systolic BP, high total cholesterol (TC) level, and high fasting plasma glucose level [16, 17]. Nevertheless, near 80% of CVD could be prevented after maintaining the levels of three risk factors mentioned above [18]. And a seventeen-year follow-up study showed that 64% female and 45% male CHD deaths could have been prevented by avoiding hypertension, smoking, and high TC (≥240 mg/dL) [19]. When it comes to mechanisms, the pathogenesis of ischemic stroke occurs in CHD patients who have extra biological defects, including cardiac autonomic dysfunction, endothelial and platelet dysfunction, and elevated catecholamine levels. These activities result in cardiac malfunctions and depression [5]. Different from the incidence of PSD, approximately 20% of CHD patients have major depression, and another 20% have minor depression at any given point in the course of their illness [5]. To sum up, CHD is a multifactor disease with complex mechanisms and its relationship with depression requires further studies.

3. Overview of Depression

Depression is a mental state characterized by a pessimistic sense of inadequacy and a despondent lack of activity, affecting a fairly large population worldwide. Nearly a fifth of the world's population experience one episode of depression at some point in their life, and the World Health Organization (WHO) predicted that this disease would rank the first as the cause of disease burden by 2030 [20]. Major symptoms of depression include low mood, anhedonia, poor appetite and sleep, feelings of worthless, and having suicidal thoughts. Unlike ischemic CVD, risk factors related to depression include psychosocial stressors and biological factors. The former involves factors like poor financial situation, marital status, and life events; the latter have genetic and gender predisposition and other health conditions like obesity [21] and diabetes [22]. Mechanism underlying depression includes the monoamine hypothesis, hypothalamic-pituitary-adrenal axis, inflammation, neuroplasticity, neurogenesis [20], and OS [23–26]. Recently, inflammation and neurogenesis hypotheses have gained more acceptances [27, 28].

As mentioned above, ischemic CVD leads to depression with an incidence rate of one-third in patients after stroke and one-fifth in CHD patients. In fact, depression can increase the risk for ischemic CVD; that is, depressed people are more likely to have stroke or CHD than nondepressed individuals. A 12-year follow-up study reported that depression causes a 2-fold increase in odds of stroke [29]. The results were supported by another study that showed an enhanced risk of cerebral ischemia in depressed subjects in a meta-analysis [30], and Booth et al. found that patients experiencing stressful life events had a 33% increased risk of total stroke in another meta-analysis [31]. Five meta-analyses reported a 60-80% increased risk of CHD in participants with depression [5], although there were different diagnostic questionnaires and criteria included in the studies. In summary, depression, a frequently occurring disease has a bidirectional relationship with ischemic CVD and partially shares common risk factors (such as obesity and diabetes) and mechanisms (such as inflammation and OS) with CVD, providing a new direction for future research.

4. Common Risk Factors among Ischemic CVD and Depression

Studies have documented that obesity, diabetes, and hypertension are common risk factors for stroke, CHD, and depression. Progression of the diseases in relation to those factors involves overactivation of OS; this is discussed in the next section.

4.1. Obesity. Obesity is defined by WHO as Body Mass Index (BMI) greater than 30 kg/m^2. Obesity is a serious public health problem in modern society, with an increasing proportion of the obese population in developed and developing countries by years. Studies have confirmed that obesity is a complicated disease that increases the risk of chronic diseases such as CVD, diabetes, and depression. In particular, experiments have demonstrated that obesity as a state of chronic inflammation with amplified OS plays a role in the occurrence of ischemic stroke [32, 33], and a nationwide population-based study concluded that obesity, especially the metabolically unhealthy type, raised the risk of stroke [34]. Besides ischemic stroke, obesity is considered as an independent risk factor for cardiovascular disease. However, recent epidemiological data have revealed a growing interest in "obesity paradox" theory; that is, overweight and mildly obese individuals may have a decreased or similar outcome of mortality compared with their normal-weight counterparts after CVD has been established, despite the increased risk of developing CVD in the obese. This theory stresses the importance of classifying obesity when talking about obesity-related diseases [35, 36]. Similar to the effect of obesity on CVD, there was a significant association between obesity and depression and more severe depressive symptoms were observed in the obese group compared with a normal-weight group, based on a meta-analysis of 18 studies that enrolled 51,272 participants [37]. Therefore, obesity is associated with ischemic CVD and depression. After investigating the mechanisms of obesity in these disorders, a vast array of data from human studies have indicated that there are complex pathological processes influenced by environmental, genetic, and epigenetic interactions [38]. Mechanisms like inflammation and gut-brain axis which are involved in the pathogenesis and OS (Figure 1 A) also play a crucial role [39].

4.2. Diabetes. Diabetes is a variable carbohydrate metabolic disorder caused by a combination of genetic and environmental factors, usually characterized by insufficient secretion or utilization of insulin. The International Diabetes Federation (IDF) recently reported that about 425 million adults worldwide suffered from diabetes in 2017. China has the largest number of diabetic patients (114.4 million) in the world. As a serious chronic disease, diabetes causes many complications and increases the risks of acquiring other diseases, including ischemic CVD and depression, if not managed. Diabetes contributes to ischemic CVD. After correction for other risk factors, diabetics have more than double the risk of ischemic stroke compared to nondiabetics, and stroke accounts for approximately 20% of deaths in diabetics [40–42]. Also, prediabetics and the duration of diabetes increase stroke risk [7]. Dysregulation of metabolism in diabetes adversely affects vascular wall, and this causes vascular dysfunction, making patients more prone to atherosclerosis; this significantly increases their risk of myocardial infarction (MI) and CHD [43]. The findings are supported by an observation of a cohort CVD study with 71,745.4 person-years, showing that diabetes had a significant impact on the residual lifetime risk of CHD in middle-aged men and women [44]. Diabetes promotes the onset and progression of depression. People with diabetes (either type 1 or type 2) are twice likely to have depression compared to nondiabetics [45]. This is explained by two possible mechanisms. The first one is the psychosocial burden of chronic disease [46], whereas the second one is connected to biochemical changes in diabetes, such as inflammatory and psychoneuroendocrine mechanisms [47, 48]. Presently, diabetes affects a large population worldwide and has connections with both CVD and depression.

The underlying mechanisms explaining this relationship are similar to those of obesity. The brain is susceptible to glucose, and the risk of stroke increases by OS through complex mechanisms [49, 50], and diabetes worsens results of acute coronary syndrome due to overactivation of OS [43]. Recent studies have proved that reducing OS response via antioxidant treatment is able to ease the depressive-like behavior caused by diabetes [51] and implicated gut microbiota as a common mechanism mediated by OS in diabetes and depression [23, 52]. In conclusion, diabetes is an important risk factor for ischemic CVD and depression, and its pathogenesis is closely related to OS (Figure 1 B).

4.3. Hypertension. Two recent studies published in The Lancet on hypertension control in high-, middle-, and low-income countries revealed an increasing trend of hypertension prevalence over time [53]. It is widely acknowledged that high BP is one of the most important risk factors for stroke and heart disease [54]. Several studies have demonstrated a significant increase in the risk of depression in hypertension patients. In cases of stroke, there is a strong, direct, linear, and continuous relationship between high BP and stroke risk [7]. The mechanism of ischemic stroke caused by hypertension has been relatively clear, among which OS (Figure 1 C) occupies an important position [55]. When considering heart disease, detailed studies over the past few decades have identified hypertension as a leading cause of CHD, especially myocardial ischemia. Importantly, the formation of atherosclerotic plaques, associated with OS, was involved in many processes, from the classic chain reaction of hypertension to the development of CHD and myocardial ischemia [56]. Parallel to CVD, depression has links with high BP. A Dutch study showed that depressed elderly patients with higher BP showed more symptoms of apathy than the controls with normal BP [57]. A Latin American study illustrated that even after adjusting for many potential confounders, hypertension was an independent predictor of depressive symptoms among Mexican community-dwelling elders [58]. In short, hypertension is another important common risk factor for the mentioned illnesses and OS also takes part in the pathological processes.

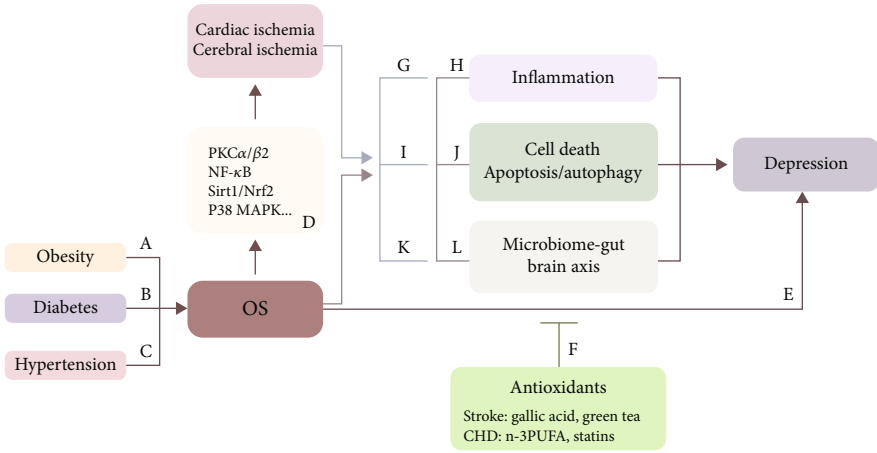

Figure 1: The role of OS associated with common risk factors and mechanism for ischemic CVD (stroke and CHD) and depression. A–C Obesity, diabetes, and hypertension promote the development of ischemic CVD and depression by increased OS and decreased anti-OS reactions. Moreover, this phenomenon can be reversed by using antioxidants. D OS independently affects stroke via mtDNA, oxLDL, and epithelium and affects CHD via Sirt1/Nrf2 pathway, p38 MAPK pathway, NF-κB/p65 pathway, and PKCα/β2 pathway. E OS facilitates depression by NOX1-derived ROS and prooxidative and proinflammatory events. F Overactive OS contributes to depression, while administrating antioxidants ameliorates depressive symptoms by using gallic acid and green tea in stroke, as well as n-3 PUFA and statins in CHD. G Ischemic patients develop depression through inflammatory reactions. H OS can interact with inflammation through common molecules, such as GSK-3 and NLRP3 inflammasome. I A cascade of reactions in postischemic depression, when OS influences apoptosis through the Bcl-2/Bax pathway and mitochondrial dysfunction in J. K Microbiome-gut-brain axis contributes to the progression of depression in objects with ischemic CVD by impacting the immune system and brain activity as well as by spreading diseases through the enteric nervous system. L Studies have found the role of OS in promoting abnormal protein aggregation, brain lesions, and gut dysbiosis in this axis.

5. The Inherent Connection between Risk Factors and Mechanisms of Ischemic CVD and Depression

This section provides a brief overview of the common mechanisms associated with ischemic CVD and depression, describes the inherent connection among risk factors, pathological changes, and the two disorders, and identifies the central role of OS in the network (Figure 1). In recent years, these mechanisms mentioned earlier including OS, inflammation and immune response, cell death signaling pathway, and microbiome-gut-brain axis can be mediated by OS.

5.1. Oxidative Stress and Antioxidative System. Oxidation-reduction is beneficial for physiological functions under normal conditions, and imbalanced redox resulting from excessive productions of free radicals (FR) and weakened antioxidative defenses can cause multiple pathological pathways, inducing mitochondrial dysfunction and destruction of homeostasis. Excess production of reactive oxygen species (ROS), especially mitochondrial ROS (mitoROS) produced by mitochondrial respiration, is detrimental to body health. Redundant mitoROS exert an adverse effect on metabolic pathways, such as molecular oxidation and impairment of ATP synthesis [59]. Subsequently, there is intracellular ionic imbalance and activation of intracellular proteases, lipases, and ribonucleases due to energy deficits, which is fatal to cells and organs. Moreover, overactive Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase (NOX) can bring an excess of superoxide [60] and hydroxyl radicals and ions to aggravate injuries [61]. In contrast, reduced antioxidant levels are negatively correlated with prognosis in some diseases like CVD and depression. Since nonenzymatic substances (e.g., melatonin and ursolic acid) can detoxify ROS [62], and antioxidant enzymes (e.g., superoxide dismutase) and catalase (CAT) have effects on scavenging superoxide radicals; declined levels of those antioxidants negatively affect health in varying degrees [63, 64]. Inhibition or lack of nuclear factor erythroid 2-related factor (Nrf2) which recognizes the antioxidant response element (ARE) and protects cells from ROS accumulation may increase injury to patients; however, this can be reversed by administration of antioxidants [65], such as dimethyl fumarate and monomethyl fumarate [66]. It is therefore not surprising that alterations in OS and anti-OS levels are found in ischemic CVD and depression as discussed earlier.

Oxidative stress can independently and directly affect stroke, CHD (Figure 1 D) and depression (Figure 1 E). After cerebral ischemia induction, energy expenditure in the brain can promote OS activity resulting in DNA damage and molecular peroxidation. For example, a case-control study about cerebral ischemia presented a significant association between elevated ROS and low mitochondrial DNA (mtDNA) in peripheral blood leukocytes [67]. Another biomarker is oxidized low-density lipoprotein (oxLDL) whose increase revealed a worse prognosis [68] and a higher prevalence of cognitive impairment in stroke survivors than the controlled group [69]. Moreover, ROS impede blood circulation and destroy the epithelium by affecting the blood vessels, hence triggering their overreaction to contractile agents, with observations of increased platelet aggregation, endothelial cell permeability, and focal endothelial cell lesions [70, 71].

In CHD, OS may contribute to vascular epithelial injury via the Sirt1/Nrf2 and p38 MAPK pathways [72], as well as the NF-κB/p65 pathway [73]. Moreover, NOX activation facilitates the initiation and progression of coronary artery disease (CAD) through the PKCα/β2 signaling pathway [74]. Similar mechanisms involving active OS action also happens in depression. Studies indicate that NOX1-derived ROS induces the oxidation of NMDA receptor 1 (NR1) in the prefrontal cortex to facilitate depressive-like behaviors [75], whereas a longitudinal study suggests a cascade of prooxidative and proinflammatory events in the development of depression [76].

Furthermore, OS acts as a link between ischemic CVD and depression (Figure 1 F). There is a positive correlation between an elevated serum malondialdehyde (MDA) level at admission and an increased risk of depression after acute stroke, especially minor stroke [77]. Nevertheless, antioxidants can attenuate this correlation. Gallic acid [78], *Hypericum androsaemum* L. [79], and green tea induce a reduction in depressive symptoms and OS, restoring normal behavior and antioxidant endogenous defenses [80]. Similarly, greater depressive symptoms related to higher OS can be treated using omega-3 polyunsaturated fatty acid (n-3 PUFA); this mitigates OS and thus improves depression in CAD patients [81]. Also, the use of statins reduces the risk of depression in individuals after a heart attack, supporting the role of oxidative and inflammatory processes in depression [82].

To sum up, OS directly influences the pathogenesis of ischemic CVD or depression through various signaling pathways, and it also acts as a link between ischemic CVD and depression. Of note, anti-OS may open the door to rational and novel therapies for the two diseases.

5.2. Oxidative Stress and Risk Factors. As mentioned above, OS has relations with the three risk factors of CVD and depression and this section provides an in-depth discussion.

5.2.1. Obesity. Animal and human studies have identified the relationship between obesity and OS (Figure 1 A). Cerebral ischemia in gerbil study revealed that there were elevated levels of OS indicators (dihydroethidium and 8-hydroxyguanine (8-OHdG)) and reduced levels of antioxidant enzymes (superoxide dismutase (SOD1) and SOD2) in the obese gerbils compared to nonobese, both in pre- and postischemic phases. But this obesity-induced oxidative damage could be attenuated by pretreated fucoidan which had antioxidant properties [83]. In CHD, research suggests that obese patients with myocardium are more susceptible to ischemia compared to nonobese people, with enhanced levels of ROS and ROS-producing enzymes (i.e., p47phox, xanthine oxidase) and reduced antioxidant activity (mitochondrial aldehyde dehydrogenase and heme oxygenase-1) [84]. In depression, obese mice fed with high-fat diet had severe depressive behaviors, which could be reversed by ondansetron treatment via restoration of brain prooxidant/antioxidant balance [85] and by allicin via activation of the Nrf2 pathway [86]. Apart from preclinical investigations, human studies have indicated that postpartum depression affects one in seven women, and obese women have an increased risk of depression through neurooxidation and neuronitrosation [87, 88].

5.2.2. Diabetes. Diabetes can affect ischemic CVD and depression via OS, and antioxidant therapy possibly improves these conditions (Figure 1 B). Whereas a study emphasized the protection of glucagon-like peptide 1 (GLP-1) agonists against oxidative and apoptotic damage in a diabetic mouse model [89], another study supported the role of metformin in improving neurological functions and OS status via the AMPK/mTOR signaling pathway in acute stroke patients with type 2 diabetes [90]. In heart disease, a clinical trial enrolling 3766 adults with prevalent diabetes mellitus illustrated that increased levels of 8-oxo-2′-deoxyguanosine, a biomarker of oxidative DNA damage, were independently associated with elevated cardiovascular mortality [91]. In depression, recent studies have proposed that one possible mechanism linking diabetes and depression was the increase in lipid peroxidation and decrease in antioxidant activity in the hippocampal and prefrontal cortices, which are the areas of the brain associated with mood [92, 93].

5.2.3. Hypertension. Altered levels of antioxidants and oxidative biomarkers have demonstrated the role of OS in ischemic or depressed patients with hypertension (Figure 1 C). In salt-loaded stroke-prone spontaneously hypertensive rat (SHRSP), OS from multiple sources affected its stroke susceptibility [94]. However, curcumin could delay the occurrence of stroke and improve the survival of SHRSP through decreasing ROS levels as well as improving endothelium-dependent relaxation of the carotid artery via uncoupling the protein 2 signaling pathway [95, 96]. In heart disease, studies found that pomegranate peel extract may alleviate CHD caused by hypertension by reducing coronary angiotensin-converting enzyme (ACE), OS, and vascular remodeling [97], which also occurs in linagliptin [98]. In patients with depression and hypertension, there are decreased glutathione peroxidase-1 (GPx-1) and SOD-1 activities but increased concentrations of MDA and H_2O_2, in comparison with the controls [99]. These results have revealed the role of OS in risk factors for CVD and depression to a full extent.

5.3. Oxidative Stress and Other Mechanisms. Many studies have shown the involvement of several mechanisms in CVD and depression, including inflammatory and immune response, apoptosis and autophagy, and microbiome-gut-brain axis. Although all can affect disease independently, they may act as a link between the two diseases and may interact with OS or other signaling pathways to aggravate the diseases.

5.3.1. Inflammation and Immune Response. Inflammatory processes and immunoreaction participate in the pathogenesis of ischemic stroke [100], CHD [101], and depression, and they have common molecules with OS. Inflammation and immune reactions exist in ischemic CVD and depression. Initiated by stagnant blood flow after ischemic stroke, activation of intravascular leukocytes and the release of proinflammatory mediators trigger inflammation which

decreases the integrity of the BBB leading to the release of danger-/damage-associated molecular patterns (DAMP) from injured neurons. This in turn induces the production of cytokines like interleukin- (IL-) 1β and tumor necrosis factor (TNF). Such a process then feeds back into the inflammatory cascade via cytokine and chemokine, thereby causing great damages [102]. In ischemic stroke survivors, Ferrarese et al. observed peak levels of TNF-α at day 1 and IL-6 and TNF-α at day 4, as well as a long-lasting activation of these two cytokines in peripheral blood cells [103]. Innate and adaptive immune responses also occur in ischemia, accompanied by the activation of microglia [104], neutrophils, T cells, and B cells [105]. Elsewhere, multiple human translational and preclinical studies have revealed that inflammation facilitates the development of atherosclerosis and evokes immunoreactions via the release of cytokines, such as TNF-α, IFN-γ, and IL-6 [106]. Similar to ischemic CVD, numerous studies have suggested that inflammatory and immune processes occur in depression. For example, higher IL-6, IL-18, and C-reactive protein (CRP) levels have been found in depressed individuals [107, 108]. Moreover, a study indicated that assembly of the inflammasome can act as a key point between the inflammation and immune activities [109], and depression was shown to be in connection with proinflammatory activation of the peripheral innate immune system with relative inactivation of the adaptive immune system [110].

Inflammation and immune response may be a link between ischemic CVD and depression (Figure 1 G). These mechanisms facilitate the progression of depression in patients after ischemic CVD. For instance, a 2-year prospective study showed that depression was prevalent in patients after ischemic stroke, and IL-6 was positively correlated with the risk of PSD [111]. As for CHD subjects, results conveyed elevated levels of inflammation, manifested as higher levels of CRP, IL-6, and plasma vascular endothelial cell growth factor (VEGF), in CHD patients with depression under the condition of hypothalamic-pituitary-adrenocortical (HPA) axis hypoactivity and activation of the kynurenine pathway [112]. On the contrary, animals receiving Resolvin D1 which is a metabolite of n-3 PUFA that can diminish neutrophil accumulation in the ischemic myocardium showed decreased depression-like symptoms via better performance in the forced swim tests [113]. This mechanism increases the risk of ischemic CVD among depressed individuals [5].

Oxidative stress can mediate inflammatory and immune activities in the mentioned diseases (Figure 1 H). In cerebral ischemia, ROS leads to the phosphorylation of glycogen synthase kinase 3 (GSK-3) which inhibits nuclear translocation of element-binding protein (CREB) and Nrf2-ARE pathway, leading to elevated levels of proinflammatory and inflammatory cytokines [114, 115]. Besides, oxidative damage to platelets and endothelial cells is also involved in the inflammatory response, with overactive leucocyte activities [116]. Similarly, in depressed individuals, OS took part in inflammatory reactions by activating NOD-like receptor protein 3 (NLRP3) inflammasome to activate IL-1β and IL-18 [117].

5.3.2. Cell Death Signaling Pathway. What is generally known is that the cell death signaling pathway is closely associated with the development of ischemic CVD and depression, and it relates to OS. Firstly, cell death, especially apoptosis and autophagy, is frequently observed in the two disorders. The receptor-interacting protein 1 kinase- (RIP1K-) mediated necroptosis contributes to neuronal and astrocytic cell death in ischemic stroke via the autophagic-lysosomal pathway [118], and the p53-dependent pathway and the calpain-caspase-3 pathway play a part in apoptosis of neural cells [119, 120]. In comparison, endothelial progenitor cells facilitate coronary atherosclerotic heart disease via autophagy and activation of the mTOR signaling pathway [121]. However, myocardial cathepsin D can protect against cardiac remodeling and malfunction through promoting myocardial autophagic flux [122], and Phellinus Linteus Mycelium (PLM) alleviates myocardial ischemia-reperfusion by suppressing proapoptotic signaling and regulation of autophagic signaling [123]. Similar to ischemic CVD, cell death signaling has been observed in depression. The TNF-like weak inducer of apoptosis (TWEAK) induces apoptosis in resident brain cells in the cortex and hippocampus to cause lupus-associated neurobehavioral deficits including depression and cognitive dysfunction [124], whereas chronic stress induces depressive-like behavior and hippocampal neuropathology by regulating autophagy via the PI3K/Akt/mTOR signaling [125].

Secondly, cell death may link ischemic CVD and depression (Figure 1 I), although evidence for this comes from few studies. In a bilateral internal carotid artery occlusion mouse model, cerebral ischemia causes depressive-like behaviors through the caspase-8/9-dependent neural cell apoptosis [126]. In patients with acute myocardial infarction comorbid with depression, escitalopram may directly confer cardioprotection by inhibiting proapoptotic pathways [127].

Thirdly, OS mediates the cell death signaling pathway, especially apoptosis, to affect the mentioned diseases (Figure 1 J). Several studies have shown that ROS could stimulate apoptosis, necrosis, and their combined pathway in ischemia [128], possibly by mediating antiapoptotic protein, B cell lymphoma-2 (Bcl-2), and the proapoptotic apoptosis regulator, Bcl-2-associated X protein (Bax) protein [129]. Furthermore, OS mainly correlates with intrinsic apoptosis. After OS-induced mitochondrial dysfunction, the released cytochrome c (Cytc) binds to the apoptotic protease to form apoptosomes, contributing to DNA damage and apoptotic cell death [129]. Similar to mechanisms in ischemia, caspase-9 is activated and apoptosome is formed to induce apoptosis in depressed animals [130]. In terms of autophagy, it has been reported that excessive ROS adjusts the transcriptional regulatory mechanisms in the nucleus to promote autophagy, but the underlying mechanisms are not well known [131].

5.3.3. Microbiome-Gut-Brain Axis. Recent studies have focused on the microbiome-gut-brain axis. The microbiome-gut-brain axis plays a role in either ischemic CVD or depression. It has been reported that after stroke, intestinal dysbiosis not only alters the immune balance of the small intestine with an increase in regulatory T cells and a reduction in interleukin- (IL-) 17-positive $\gamma\delta$ T cells but also suppresses

trafficking of effector T cells from the gut to the leptomeninges [132, 133]. In the myocardial infarction model after three-week arterial occlusion, rats began to exhibit depression-like symptoms with increased gastric retention rates [134]. In depression, after surveying a large microbiome population cohort and analyzing fecal metagenomes, researchers indicated a potential role of microbial γ-aminobutyric acid production and provided population-scale pieces of evidence for microbiome relating to mental health [135].

The latest studies have documented that the microbiome-gut-brain axis can be a link between ischemic CVD and depression (Figure 1 K). The 2017 named series and the psychoneuroimmunology research reported that commensal microbes may impact the immune system and brain activity via behavioral and immunological responses to social stresses, which can be attenuated by healthy dietary [136]. Moreover, the enteric nervous system (ENS) structure and neurochemistry are similar to the CNS; therefore, pathogenic mechanisms inducing CNS disorders might also give rise to ENS dysfunction and nerves, interconnecting the ENS and CNS, can be conduits for spreading of disease [137]. This collection of work describes a connection between the microbiota, brain, behavior, immunity, stroke, and depression, to provide an important rationale for extending the work in the future. Contrarily, a study showed that depressive disorder and gastrointestinal dysfunction after myocardial infarction were associated with abnormal metabolism of tryptophan-5-hydroxytryptamine, an important substance in the gut-brain axis [134].

Oxidative stress also plays a role in regulating the microbiome-gut-brain axis related to ischemia and depression (Figure 1 L). Intestinal microbiome might increase ROS and promote abnormal protein aggregation and brain lesions, which can result in alteration of gut properties and microbiota [138]. For instance, ischemic stroke intensifies gut barrier breakdown by overproduced ROS and aggravates microbiota alterations, followed by the translocation of a selective bacterial strain to the surrounding tissues. As a consequence, it promotes poststroke infections [139]. In depression, several studies suggest that ROS has connections with gut dysbiosis [140], one of which illustrates that increased chronic apical periodontitis and lipopolysaccharide levels probably lead to depression via OS-induced hypernitrosylation and neuroprogressive processes [141].

6. Conclusion

Based on the mechanisms described in the previous sections, we make the following conclusions (Figure 2). Notably, ischemic CVD and depression are highly prevalent and are major causes of disability. These two disorders are interrelated; that is, stroke survivors are likely to develop depression and depressed individuals have a higher risk for stroke and CHD than nondepressed people [29–31]. Additionally, these two diseases share some common risk factors, such as obesity, diabetes, and hypertension. The underlying mechanisms involved in many pathological processes, such as inflammation, cell death (apoptosis and autophagy), microbiome-gut-brain axis, and OS, also participate in both diseases. Notably, OS is in the center of this web. These three risk factors are associated with OS and can trigger the development and aggravate the progression of these diseases via overactivation of OS and attenuation of antioxidant activities. In addition, OS directly increases the risk of depression in patients with cardiovascular diseases, whereas it increases the risk of cardiovascular diseases in depressed people. Thirdly, high levels of ROS promote the pathogenesis of ischemic CVD and depression via OS-induced inflammation, cell death signaling, and microbiome-gut-brain axis. In summary, the common risk factors increase the production of OS and reduce antioxidant defenses, thereby promoting the occurrence and development of interacted ischemic CVD and depression. Application of antioxidants can mitigate OS-related injuries and diseases. Due to the central role of OS in these two illnesses, inhibition of OS may provide novel and promising therapeutic strategies for the two conditions.

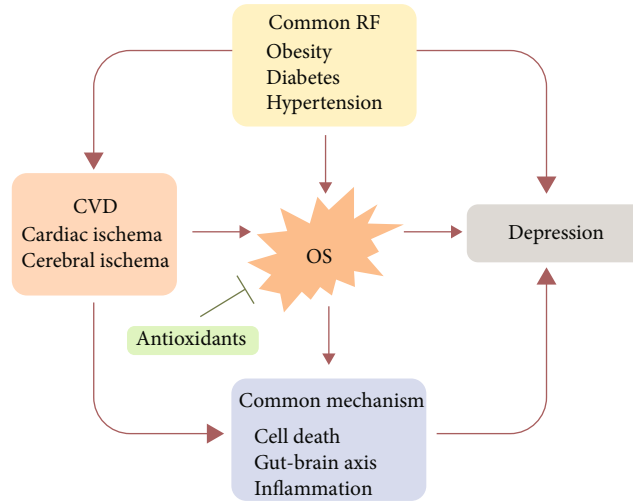

Figure 2: The relationship among OS, common risk factors, and common mechanism of ischemic CVD (stroke and CHD) and depression. The common risk factors, obesity, diabetes, and hypertension, can affect either ischemic stroke or depression. Moreover, they can exert influence via overactive OS activity and downregulated antioxidant defenses. On the other hand, OS acts as one of the common mechanisms promoting postischemic depression and it also interacts with inflammation, cell death signaling pathway (apoptosis and autophagy), and gut-brain axis to exacerbate the process of postischemic depression.

7. Limitations and Perspectives

The previous studies investigating the relationship between hypertension and depression have been limited to the elderly population, and some meta-analyses have contradictory results due to high heterogeneity and other reasons such as the following: (1) The clinical manifestation of some types of stroke or depression varies among patients, and there may be some biases when studying such patients. (2) The small sample size or short follow-up time of some studies may introduce some variations in the results. This requires

further longitudinal, large-sample size, cohort studies to provide more conclusive outcomes.

The inconsistency among clinical diagnostic criteria and the lack of uniform and timely evaluation tools may increase errors in the data, which makes head-to-head comparisons among studies unsuitable. In particular, there are many types of depression which are diagnosed with different diagnostic guidelines. This will reduce the comparability among studies.

Many animal experiments cannot adequately eliminate the interference of other factors, and hence, the impact of certain factors on disease phenotypes may affect the results [5]. This calls for the development of precise and stable animal models of stroke or depression, which will provide accurate assessment of disease progression and pathogenesis.

Despite the mechanisms discussed in this review, there are no clear cellular or molecular level pathways that explain the pathogenesis of stroke and depression. Therefore, appropriate in vitro models of apoplexy or depression are urgently required.

Some antioxidants exert anti-inflammatory, antiapoptotic, and antioxidative effects simultaneously. So when they have certain curative effects on diseases, they cannot be fully explained only by antioxidation effect [79–82]. This also suggests further studies are required to reveal other mechanisms of oxidative stress in diseases.

According to the data reviewed in this article, we propose the following research directions: (1) Should antioxidation therapy be given to depression patients to prevent ischemic cardiovascular disease? Are patients with ischemic stroke and myocardial infarction suitable for antioxidant therapy to prevent later depression? (2) What is the timing of antioxidant intervention? (3) Will a combination of antioxidant therapy produce better outcomes in patients with depression or cardiovascular and cerebrovascular diseases? All of these questions are yet to be answered, and therefore, future multicenter randomized controlled clinical studies with large samples and more scientific and reasonable experimental verification are needed.

Conflicts of Interest

The authors declare that they have no competing interests.

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Research Article

Cerebroprotective Effect against Cerebral Ischemia of the Combined Extract of *Oryza sativa* and *Anethum graveolens* in Metabolic Syndrome Rats

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The novel strategy against ischemic stroke in metabolic syndrome (MetS) targeting at oxidative stress and inflammation has gained attention due to the limitation of the current therapy. Due to the antioxidant and anti-inflammation of the combined extract of *Oryza sativa* and *Anethum graveolens*, the cerebroprotective effect against cerebral ischemia in MetS condition has been focused. Since no data were available, this study was set up to determine the effects of the combined extract of *Oryza sativa* L. and *Anethum graveolens* Linn. against ischemic stroke in the animal model of metabolic syndrome. The possible underlying mechanism was also further investigated. Male Wistar rats (180-220 g) were fed with high-carbohydrate high-fat diet (HCHF diet) to induce metabolic syndrome-like condition. Then, MetS rats were subjected to reperfusion injury at the right middle cerebral artery. The combined extract of *O. sativa* and *A. graveolens* (OA extract) at doses of 0.5, 5, and 50 mg/kg BW was fed once daily for 21 days. Neurological assessment was performed every 7 days throughout the experimental period. At the end of study, brain infarction volume, neuron and glial fibrillary acidic protein- (GFAP-) positive cell density, the oxidative stress status, the expressions of proinflammatory cytokines (NF-κB, IL-6), and eNOS in the cortical area together with the expression of VCAM-1 and the histological changes of common carotid artery were determined. It was found that OA extract decreased brain infarction, neurological score, oxidative stress status, and inflammatory mediators but increased eNOS expression in the cortical area; the increased VCAM-1 and intima-media thickness together with the reduction of lumen diameter of common carotid artery of MetS eats with MCAO were also mitigated by OA extract. These data suggest the cerebroprotective effect of OA, and the underlying mechanism may occur partly via the improvement of oxidative stress status, inflammation, and brain blood supply.

1. Introduction

Nowadays, the prevalence of metabolic syndrome, an important noncommunicating disease, is continually rising and it is regarded as the critical health problem in both the developed and developing countries. The prevalence of metabolic syndrome (MetS), a cluster of conditions associated with glucose intolerance, insulin resistance (IR), central obesity, dyslipidemia, and hypertension [1–3], in Asia has increased rapidly and continually. MetS also increases the risk of many deleterious disorders, especially stroke [4].

Stroke has been recognized as a leading cause of morbidity and mortality worldwide. It has been predicted that the number of global stroke deaths should be 7.8 million within 2030 if the effective vascular disease prevention program is not implemented. The most common type of stroke worldwide is ischemic stroke [5]. It has been reported that the pathophysiology of ischemic stroke especially in the aged population is attributed to MetS and atherosclerosis. Since MetS is an important cause of atherosclerosis, a key pathophysiological factor of ischemic stroke [5], the prevalence of ischemic stroke in MetS also increases. Stroke in MetS often induces poor outcomes [6] and produces both financial and psychological burdens for the country. Despite increasing their importance, the effective pharmacological agents which can effectively prevent and treat cerebral ischemia with MetS are still in an unsatisfaction level. Therefore, the novel neuroprotectant is required.

In recent years, many reviews have been published about the effects and potential benefits of herbal medicine in MetS and stroke [7–8]. It has been revealed that some herbal medicines may improve the brain microcirculation, protect against ischemic reperfusion injury, possess neuroprotective properties, and inhibit apoptosis in MetS and ischemic stroke patients [7, 9]. In Thailand, numerous plants are reputed for disease treatment and health promotion. Both *Oryza sativa* L. and *Anethum graveolens* Linn. are also reputed for both actions. In addition, they also possess a potent antioxidant activity [10, 11]. Based on this reputation and the crucial role of oxidative stress on the pathophysiology of MetS and stroke, the possibility of these medicinal plants to attenuate the diseases such as ischemic stroke in MetS condition has gained attention. Since no data concerning this issue are available, this study was set up to determine the effects of the combined extract of *Oryza sativa* L. and *Anethum graveolens* Linn. against ischemic stroke in the animal model of metabolic syndrome. In addition, the possible underlying mechanism was also investigated.

2. Materials and Methods

2.1. Plant Material Preparation and Extraction. Grains of black sticky rice (*Oryza sativa* L. indica) and aerial part of dill (*Anethum graveolens* Linn.) used in this study were collected from Khon Kaen province in September to October. The voucher specimens (ICAM 12001 and ICAM 12002) were authenticated by Associate Professor Panee Sirisa-ard, the pharmacognosy expert from the Faculty of Pharmacy, Chiangmai University, and kept at the Integrative Complementary Alternative Medicine Research and Development Center, Khon Kaen University. All samples were cleaned and dried in an oven (Memmert GmbH, USA) at 60°C for 72 hours. Following this process, the aqueous extract of *O. sativa* and 95% hydroalcoholic extract of *A. graveolens* were prepared by using the maceration technique for 24 hours at room temperature. The extracts were subjected to a 3000 rpm centrifugation for 10 minutes. Then, they were filtered with a filter paper (Whatman No. 1), dried by using a freeze dryer, and kept at -20°C until used. The yields of *O. sativa* and *A. graveolens* were 10 and 26 percent, respectively. To prepare the combined extract (OA extract), the extracts of *O. sativa* and *A. graveolens* were mixed at a ratio which produced the highest benefit for ischemic stroke in metabolic syndrome according to our pilot study [12].

2.2. Determination of Total Phenolic Compound Contents. The Folin-Ciocalteu colorimetric method was used to determine the total phenolic compound content of OA extract [13]. Briefly, 158 μL of distilled water, 20 μL of the tested substances, and 1000 μL of 50% Folin-Ciocalteu phenol reagent (Sigma-Aldrich, USA) were mixed and subjected to an 8-minute incubation period at 37°C. Following this step, an aliquot 20% Na_2CO_3 (Sigma-Aldrich, USA) at the volume of 30 μL was added and incubated in the dark room for 2 hours at room temperature. At the end of incubation period, an absorbance at 765 nm was measured. The content of total phenolic compounds was expressed as mg gallic acid equivalent (GAE)/mg sample. Various concentrations of gallic acid (Sigma-Aldrich, USA) ranging from 1 to 500 μg/mL were prepared for a standard reference.

2.3. Determination of Total Flavonoid Content. The total flavonoid content of OA extract was determined via the modified aluminium chloride colorimetric method [14]. Briefly, 100 μL of 2% $AlCl_3{\cdot}6H_2O$ (2 g dissolved in 100 mL of methanol) and 100 μL of the tested substances were mixed and incubated for 30 minutes at room temperature. After the incubation period, the absorbance at 415 nm was measured. Results were expressed as μg quercetin equivalent (QE)/mg sample.

2.4. Determination of Antioxidant Activities

2.4.1. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay. This assay was performed based on an ability to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical of the tested substances [15]. Briefly, 50 μL of various concentrations ranging from 1, 5, 10, 50, 100, 250, 500, and 1000 μg/mL of OA extract was mixed with 0.25 mL of 0.15 mM DPPH solution. Following this step, the incubation of the mixture for 30 minutes in a dark room was carried out. Then, an absorbance at 517 nm was measured using a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo Electron Corporation, IL, USA). L-Ascorbic acid was used as a standard reference. $DPPH^{\cdot}$ blank solution was prepared by mixing 300 μL $DPPH^{\cdot}$ solution with 2.7 mL of methanol. The percent inhibition of DPPH radical was calculated according to the following formula: $\%\text{DPPH inhibition} = ((A_{\text{DPPH}}\text{-}A_{\text{extract}})/A_{\text{DPPH}}) \times 100$. A_{DPPH} was the absorbance value of the DPPH· blank solution. A_{extract} was the absorbance value of the sample solution. 50% inhibition (IC50) value was calculated using the graph showing the relation between the percent inhibition and the extract concentration [15, 16].

2.4.2. Ferric-Reducing Antioxidant Power (FRAP) Assay. The assessment of FRAP assay was performed based on the ability to change ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) [17]. In brief, FRAP reagent was freshly prepared by mixing 5 mL of 10 mM TPTZ (Sigma-Aldrich, USA) solution, 5 mL of 20 mM ferric

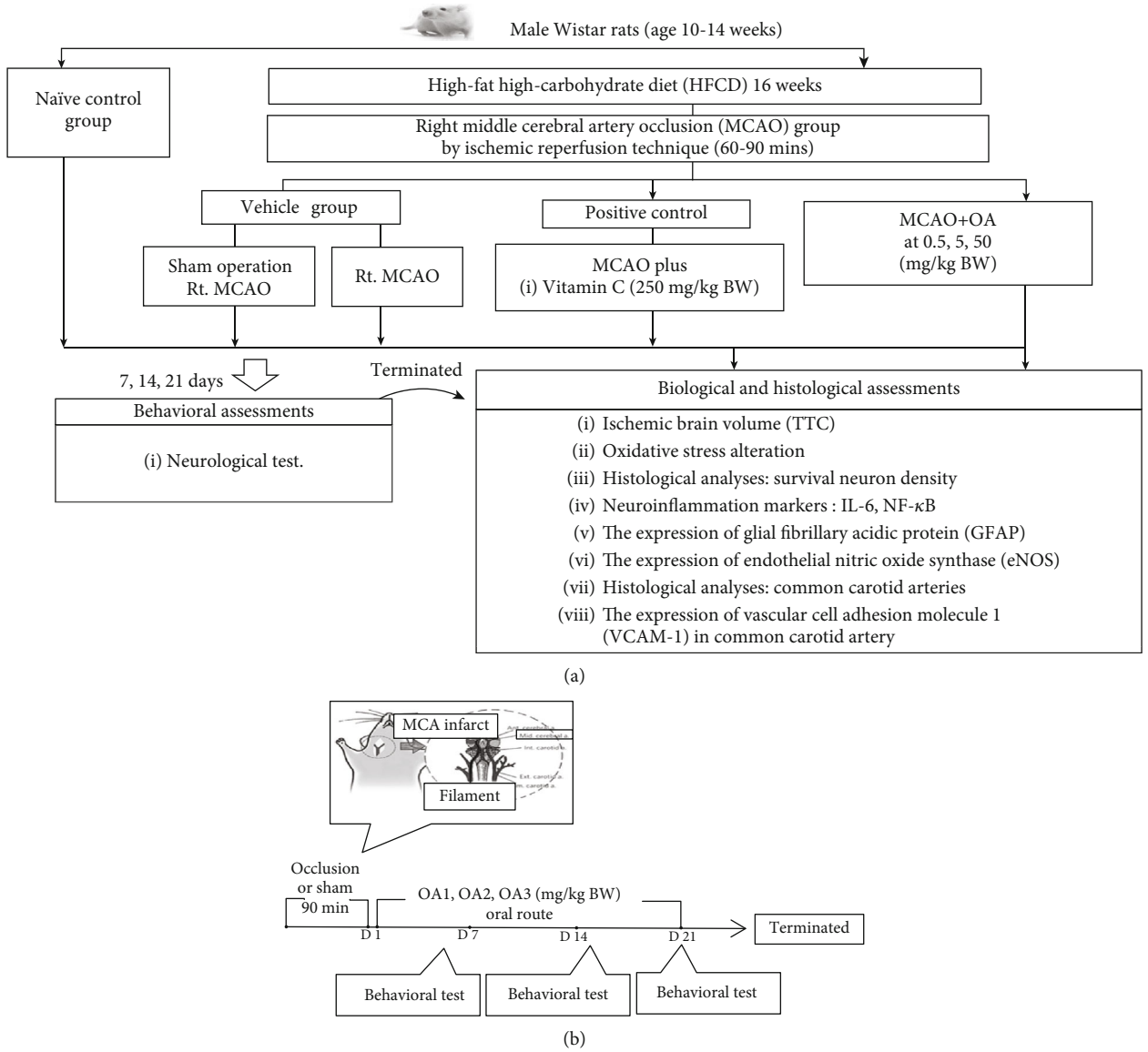

Figure 1: Schematic diagram showing all experimental procedures. (a) Experimental protocol of OA extract treatment and the determination of various parameters. (b) Right MCAO induction and schedule for OA extract treatment. IL-6: interleukin-6; NF-κB: nuclear factor-kappaB; HCHF: high-carbohydrate high-fat 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.

chloride solution ($FeCl_3$) (Sigma-Aldrich, USA), and 50 mL of 300 mM acetate buffer, pH 3.6 (Sigma-Aldrich, USA) together. Then, the mixture was mixed with 190 μL of FRAP reagent and 10 μL of samples and incubated for 10 minutes at 37°C. The absorbance was measured at 593 nm, and L-ascorbic acid was used as a standard reference. Results were expressed as the EC50 value.

2.5. Determination of Cyclooxygenase 2 (COX-2) Inhibition Activity. The effect of OA extract on the activity of cyclooxygenase 2 (COX-2), an enzyme playing a key role in inflammation, was also determined. Briefly, the mixture containing 150 μL of 100 mM Tris-HCl buffer pH 8.0, 10 μL of 50 nM of COX-2, 10 μL of 0.5 μM of heme, and 10 μL of various concentrations of OA extract was added to a 96-well plate. Then, 20 μL of 10 μM of N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) and 20 μL of 100 μM arachidonic acid (Cayman Chemical, USA) were added and incubated for 5 minutes at room temperature. At the end of incubation period, an absorbance at 590 nm was measured using a microplate reader. Indomethacin was used as a standard reference. The percent inhibition of COX-2 was calculated, and the results were expressed as EC50 [18].

2.6. Experimental Protocol. Adult male Wistar rats, age 10-14 weeks, weighing 180-220 grams were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. Rats were housed in standard metal cages and maintained in 12 : 12-hour light : dark cycle at 22 ± 2°C. Animals were given food and water ad libitum. This study

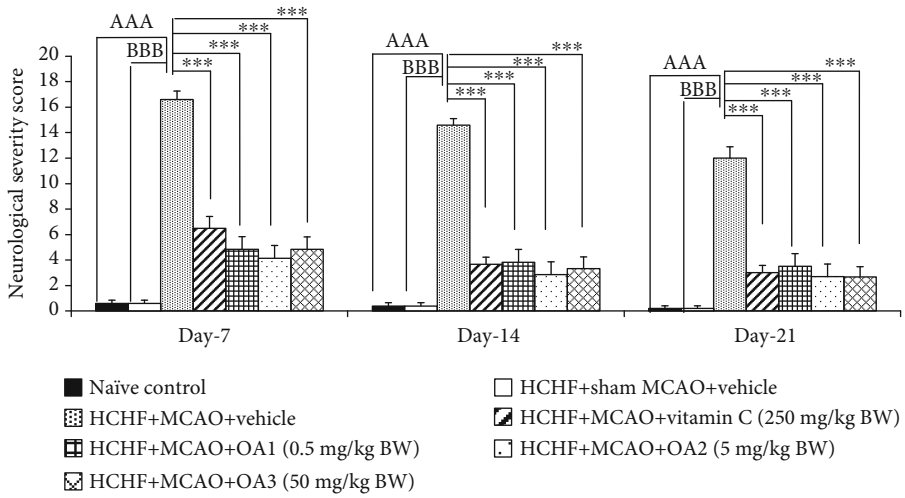

Figure 2: Effect of OA extract on the neurological scores. Data are presented as mean ± SEM. ^{AAA}p value < 0.001; compared to naïve intact rats, ^{BBB}p value < 0.001; compared to sham operation which received HCHF diet and vehicle and $^{***}p$ value < 0.001; compared to MCAO rats which received HCHF 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.

was approved by the Institutional Animal Care and Use Committee, Khon Kaen University, Khon Kaen, Thailand (AEKKU 30/2558).

The experimental animals were divided into 7 groups ($n = 6$/group) as the following. Group I naïve intact: rats were fed with normal diet comprised of 42% carbohydrate, 24% protein, and 4.5% fat and received no treatment. Group II HCHF+sham operation+vehicle: all rats were fed with high-carbohydrate high-fat (HCHF) diet and subjected to sham operation and vehicle treatment. Group III HCHF+MCAO+vehicle: all rats were fed with HCHF diet and subjected to the temporary occlusion of right middle cerebral artery (Rt. MCAO) followed by reperfusion and vehicle treatment. Group IV HCHF+MCAO+vitamin C: rats were subjected to Rt. MCAO followed by reperfusion and treated with vitamin C at a dose of 250 mg/kg BW. Groups V-VII HCHF+MCAO+OA extracts (OA1, OA2, and OA3): all animals in these groups were subjected to Rt. MCAO followed by reperfusion and treated with OA extracts at doses of 0.5, 5, and 50 mg/kg BW, respectively.

All animals in groups II-VII were fed with high-carbohydrate high-fat diet comprised of 35.83% of fat, 35.54% of carbohydrate, and 28.6 3% of protein for 16 consecutive weeks in order to induce metabolic syndrome condition. The metabolic syndrome rats (MetS rats) which showed the body weight change more than 40%, fasting plasma glucose (FPG) more than 100 mg/dL, the systolic or diastolic blood pressure higher than 130 and 85 mmHg, respectively, and the atherogenic index higher than the control group were selected for inducing ischemic and reperfusion injury [12]. After the operation, rats were administered the assigned substances once daily for 21 consecutive days via oral route. The neurological score was evaluated every 7 days throughout the study period. At the end of the study, brain infarction volume, the density of survival neuro, and the density of glial fibrillary acidic protein- (GFAP-) positive cells were evaluated. In addition, the oxidative stress status, the nuclear factor-kappa B (NF-κB), the expression of proinflammatory cytokine (IL-6), the endothelial nitric oxide synthase (eNOS) in the cerebral cortex, the expression of vascular cell adhesion molecule 1 (VCAM-1) in the common carotid artery, and the structural changes in the common carotid artery were also determined. The schematic diagram showing the study protocol is shown in Figure 1.

2.7. Focal Cerebral Ischemia/Reperfusion Induction. Rats were subjected to an anesthetization by administering pentobarbital sodium at doses of 50 mg/kg BW (Tianjin Kemiou Chemical Reagent Co., Ltd., Tianjin, China) via intraperitoneal route. Following this step, a silicone-coated 4-0 monofilament nylon (USS DG; Tyco Healthcare Group Lp, CT, USA) was gently inserted from the lumen of the right common carotid artery (CCA) and then passed through the internal carotid artery (ICA) approximately 17-18 mm from the bifurcation in order to occlude the right middle cerebral artery. After 90 minutes of intraluminal occlusion, the nylon monofilament was withdrawn to allow the reperfusion process. The identical operation except the intraluminal occlusion was performed in the sham operation group. After operation, rats were cared until they recovered from anesthesia and they were returned to the cage [12].

2.8. Neurological Assessment. Neurological score assessment was graded at 24 hours after the induction of reperfusion injury by using the modified neurological severity scores (mNSS), one of the most common methods used in the animal study of stroke. The 18 scale mNSS was performed based on the assessment of neurological functions including motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), reflex, and balance tests. According to this assessment, the higher score indicated the more severity of neurological deficit [19].

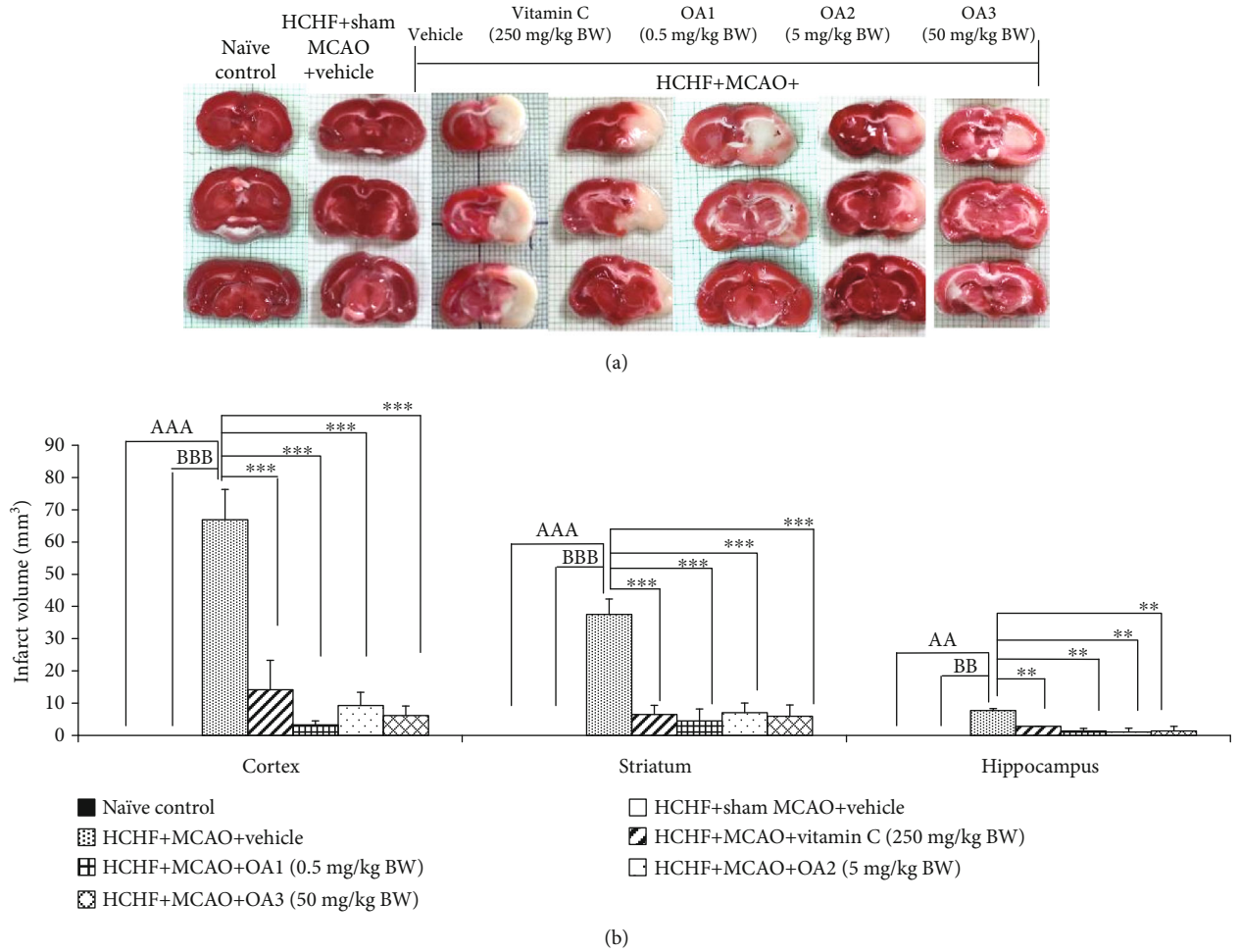

Figure 3: Effect of OA extract on brain infarct volume in the cortex, striatum, and hippocampus. Data are presented as mean ± SEM ($n = 6$/group). $^{AA,AAA}p$ value < 0.01 and 0.001; compared to naïve intact rats, $^{BB,BBB}p$ value < 0.01 and 0.001; compared to sham operation which received HCHF diet and vehicle and $^{**,***}p$ value < 0.01 and 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.

2.9. Evaluation of Brain Infarct Volume. After the neurological score assessment, rat brains were removed and prepared as coronal sections at 2 mm thick. The brains were immediately incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma-Aldrich, St. Louis, MO, USA) in the dark at room temperature for 15 minutes and turned over every 5 min. Then, all brain slices were captured using a digital camera (Sony HDR-SR11 Handycam Camcorder; Sony Co. Ltd., Japan), and the infarct size was calculated with the computerized image analysis system [20].

2.10. Histological Study

2.10.1. Nissl Staining. The brains of the experimental animals were transcardially perfused with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3 and postfixed in the same fixative overnight at 4°C. Then, they were immersed in a cryoprotectant containing 30% sucrose (Merck, Germany) solution at 4°C for 72 h. Serial sections of the frozen tissues were cut at 20 μm thick using cryostat (Thermo Scientific™ HM525 Cryostat). All sections were placed on slides coated with 0.3% gelatin containing 0.05% aluminium potassium sulfate (Sigma-Aldrich, USA). Following this step, the sections were stained with 0.2% cresyl violet solution (Sigma-Aldrich, USA) for 8 minutes, rinsed with distilled water, and dehydrated with 70%, 95%, and 100% alcohols, respectively (RCI LabScan, Thailand). Then, the sections were immersed in xylene 2 times for 5 minutes each and mounted with DPX mountant (Merck, Germany). The determination of neuron density was performed based on the stereotaxic coordinates from the rat brain atlas as the following: anteroposterior 2.5-4.5 mm and mediolateral 0.2-1.0 mm [21] under the Olympus light microscope model BH-2 (Japan) at 40x magnification. Results were expressed as density of neurons per 255 μm^2.

2.10.2. Immunohistochemistry. Brain tissue sections for immunohistochemistry evaluation were prepared as mentioned earlier in Section 2.10.1. The prepared sections were immersed in 0.01 M sodium citrate buffer (pH 6.0) and

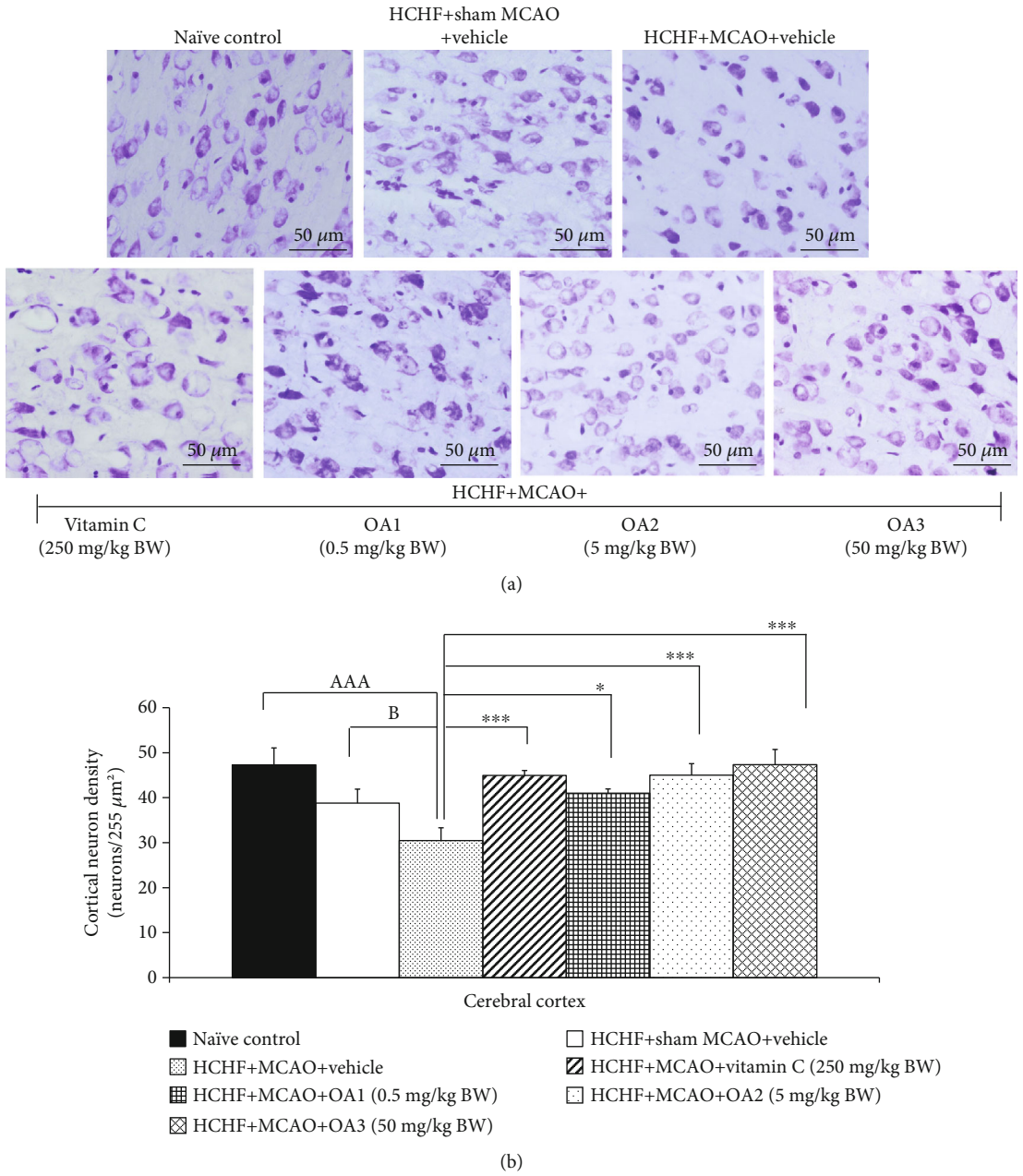

Figure 4: Effect of OA extract on neuron density in the cerebral cortex. (a) Light microscope of coronal sections in the cerebral cortex was stained with cresyl violet at 40x magnification. (b) Density of survival neurons in the cerebral cortex. Data are presented as mean ± SEM ($n = 6$/group). ^{AAA}p value < 0.001; compared to naïve intact rats and $^{**,***}p$ value < 0.01, 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.

heated using the microwave oven for 10 minutes. After letting cool at room temperature, all sections were washed for 5 minutes with phosphate buffer saline (PBS) (3 times) and incubated in 0.3% hydrogen peroxide for 20 minutes at room temperature. Then, sections were washed for 5 minutes with PBS 3 times and incubated in the solution consisting of 0.3% Triton X-100 (Fluka Chemika, Buchs, Switzerland), 1% (w/v) bovine serum album (BSA), and 10% normal goat serum for 20 minutes at room temperature. After the incubation process, the sections were washed with PBS (3 times for 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 process, sections were washed and incubated with a REAL™ EnVision™ Detection System, peroxidase/DAB+ rabbit/mouse, (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Then, sections were washed with PBS and incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, USA) for 5 minutes. Negative

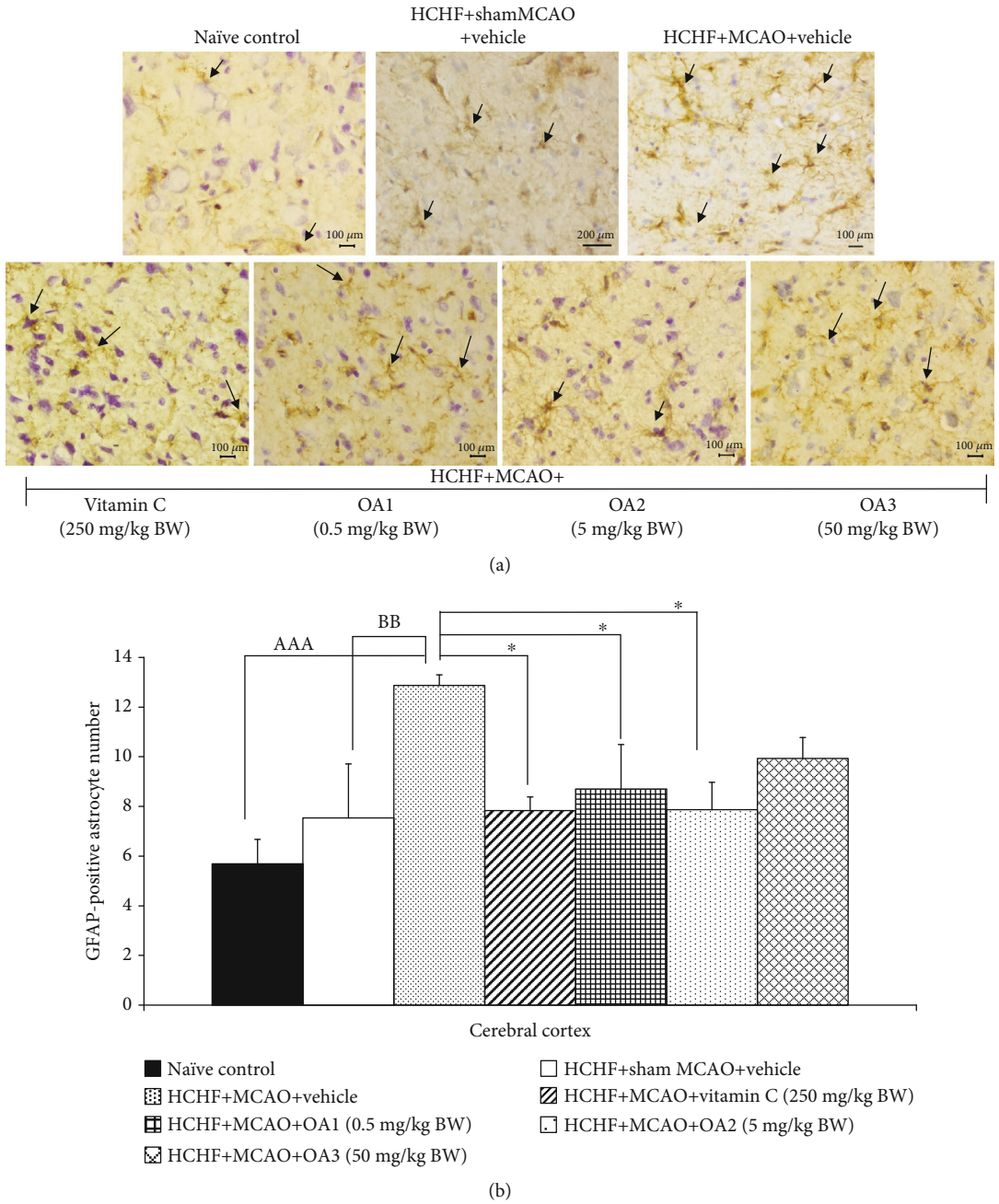

Figure 5: Effect of various doses of OA extract on the density of GFAP-positive cell in the cerebral cortex. (a) Immunostaining for GFAP-positive cell in the cerebral cortex. GFAP-positive cell or astrocytes were stained brown (arrow). Magnification, 40x; scale bar = 100 μm. (b) GFAP-positive cells in the cerebral cortex. 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 all; 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.

control sections were prepared using the identical process without an exposure to primary antibody. Positive staining was shown as a brown color. All sections were mounted on slides coated with gelatin and counterstained with cresyl violet. Then, they were dehydrated with graded alcohols, cleared with xylene, and mounted with DPX mountant. Numbers of positive cells in cerebral cortex were counted by a trained technician who was blind to this experimental design. Results were shown as mean ± SEM.

2.10.3. Hematoxylin and Eosin (H&E) Staining Process. To determine the structural changes of common carotid artery, hematoxylin and eosin (H&E) staining assay was performed. Tissues were fixed in 10% formalin solution and embedded in

Table 1: The effect of various doses of OA extract on oxidative stress markers in the cerebral cortex.

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	0.14 ± 0.01	5.30 ± 0.24	5.39 ± 0.61	2.77 ± 0.70
HCHF+sham MCAO+vehicle	0.10 ± 0.02	5.01 ± 0.52	3.88 ± 0.12	1.15 ± 0.19
HCHF+MCAO+vehicle	$0.72 \pm 0.03^{aaa,bbb}$	$3.11 \pm 0.21^{aa,b}$	2.63 ± 0.19^{aaa}	0.51 ± 0.06^{aaa}
HCHF+MCAO+vitamin C (250 mg/kg BW)	$0.16\pm0.04^{***}$	$6.12\pm0.83^{***}$	$8.36\pm1.26^{***}$	$1.49 \pm 0.52^{*}$
HCHF+MCAO+OA1 (0.5 mg/kg BW)	$0.13\pm0.02^{***}$	3.55 ± 0.50	$7.58\pm0.74^{***}$	0.93 ± 0.28
HCHF+MCAO+OA2 (5 mg/kg BW)	$0.20\pm0.03^{***}$	$4.67\pm0.94^{**}$	$10.80\pm0.65^{***}$	$1.66 \pm 0.18^{*}$
HCHF+MCAO+OA3 (50 mg/kg BW)	$0.12\pm0.02^{***}$	3.66 ± 0.69	$8.67\pm2.28^{***}$	$1.68 \pm 0.09^{*}$

Data are presented as mean ± SEM ($n = 6$/group). $^{aa,aaa}p$ value < 0.01, 0.001; compared to naïve intact rats, $^{b,bbb}p$ value < 0.05, 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.

paraffin. Then, six serial sections (5 μm thick) were prepared and stained with hematoxylin and eosin (H&E). To investigate the detailed histomorphometric changes, the mean diameters of the vessels, tunica intima, and tunica media (μm/vessel) were evaluated [22, 23] by the ImageJ (version 1.52p) program.

2.11. Brain Homogenate Preparation. The cerebral cortex used in this study was homogenized in 50 volumes of 0.1 M phosphate buffer saline. The homogenate brains were centrifuged at 3000 g at 4°C for 15 min. The supernatant was kept, and the protein concentration was also measured by using a Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

2.12. Biochemical Assessments

2.12.1. Oxidative Stress Marker Assessment. The assessment of thiobarbituric acid reactive substances (TBARSs) was performed in order to measure the level of malondialdehyde (MDA) level, a lipid peroxidation product, in the cerebral cortex [24, 25]. Briefly, the mixture containing 50 μL of 8.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA), 375 μL of 20% acetic acid (Sigma-Aldrich, USA), 375 μL of 0.8% of thiobarbituric acid (TBA) (Sigma-Aldrich, USA), and 150 μL of distilled water (DW) was mixed with 50 μL of brain homogenate and heated in 95°C boiling water for an hour. After cooling, 250 μL of DW and 1,250 μL of the mixture of n-butanol:pyridine (15,1 *v*/*v*) (Merck, Germany) were added and mixed together. After the 4000 rpm centrifugation for 10 minutes, the separated butanol layer was collected and determined an absorbance at 532 nm. The standard reference was prepared by using the 1,3,3-tetramethoxy propane at the concentration range of 0-15 μM (Sigma-Aldrich, USA). The level of MDA was expressed as ng/mg protein.

The main scavenger enzyme activities including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were also determined. Catalase activity was measured via the decrease in the absorbance of H_2O_2 [26]. Briefly, 10 μL of enzyme tissue homogenate was mixed with 50 μL of 30 mM of H_2O_2 (in 50 mM phosphate buffer, pH 7.0) (BDH Chemicals Ltd., UK), 150 μL of 5 mM $KMnO_4$ (Sigma-Aldrich, USA), and 25 μL of H_2SO_4 (Sigma-Aldrich, USA). The solution was measured with microplate reading at 490 nm. CAT enzyme (Sigma-Aldrich, USA) at various concentrations ranging from 1 to 100 units/mL was used as a standard reference. Results were expressed in units of catalase per mg protein.

Superoxide dismutase (SOD) activity was determined based on the inhibition rate of cytochrome C reduction by the superoxide radical [27]. Briefly, the cocktail solution contains 57 mM phosphate buffer solution (KH_2PO_4) (Sigma-Aldrich, USA), 0.1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, USA), 10 mM cytochrome C solution (Sigma-Aldrich, USA), and 50 μM of xanthine solution. 200 μL of cocktail solution and 20 μL of 0.5-unit xanthine oxidase were mixed with 20 μL of samples or superoxide dismutase enzyme (Sigma-Aldrich, USA) standard at various concentrations ranging from 1 to 25 units/mL. The absorbance was measured using a microplate reader at 415 nm. Results were presented in units of SOD activity per mg protein.

Glutathione peroxidase (GSH-Px) activity was also assessed [28]. Briefly, the solution containing 10 μL of 1 mM dithiothreitol (DTT) (Sigma-Aldrich, USA) in 6.67 mM potassium phosphate buffer (pH 7), 100 μL of 1 mM sodium azide (Sigma-Aldrich, USA) in 6.67 mM potassium phosphate buffer (pH 7), 10 μL of 50 mM glutathione (Sigma-Aldrich, USA) solution, and 100 μL of 30% hydrogen peroxide (BDH Chemicals Ltd., UK) was mixed with 20 μL of samples. After the exposure to a 5-minute incubation at room temperature, 10 μL of 10 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) (Sigma-Aldrich, USA) was added. An absorbance was measured using a microplate reader at 412 nm. GSH-Px enzyme (Sigma-Aldrich, USA) at various concentrations ranging from 1 to 50 units/mL was used as a standard reference. GSH-Px activity was expressed as units per mg protein.

2.13. Western Blotting Analysis. Samples was homogenized in the solution containing mammalian protein extraction reagent (M-PER) (Pierce Protein Biology Product, Rockford, IL USA) and protease inhibitor cocktail (Sigma-Aldrich,

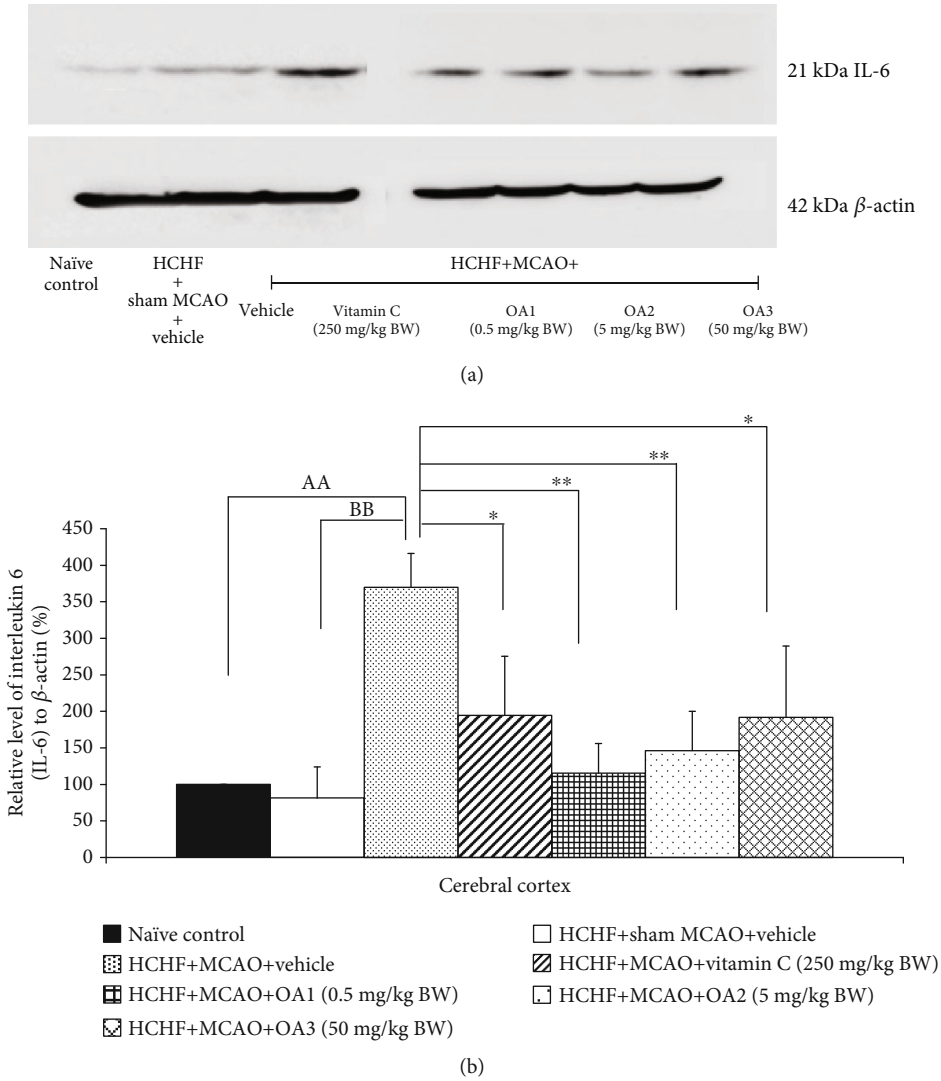

Figure 6: Effect of various doses of OA extract on the expression of IL-6 in the cerebral cortex. (a) Representative western blot showing the levels of IL-6. (b) Relative density of IL-6. Data are presented as mean ± SEM ($n = 6$/group). ^{AA}p value < 0.01; compared to naïve intact rats, ^{BB}p value < 0.01; compared to sham operation which received HCHF diet and vehicle and $^{*,**}p$ value < 0.05, 0.01, 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.

USA) at a ratio of 1 : 10, respectively. After the centrifugation at 12,000 g for 10 minutes at 4°C, the supernatant was harvested. The protein concentration was also determined by using a Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). An aliquot of 80 μg of sample lysate was added to Tris-Glycine SDS-PAGE loading buffer (Bio-Rad, USA) with an appropriate concentration and heated at 95°C for 10 minutes. Then, 20 μL of sample protein was loaded onto SDS-polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, biotinylated broad-range molecular weight markers (Bio-Rad) were also loaded onto the gels. After electrophoresis, samples were transferred to a nitrocellulose membrane, washed with 0.05% TBS-T, and incubated in blocking buffer containing 1% Tween-20 (T-PBS) and 6.5% nonfat dry milk at 4°C overnight. Then, membranes were incubated overnight at 4°C with polyclonal rabbit IL-6 (Cell Signaling Technology, USA; dilution 1 : 1000), anti-NF-κB p65 (Cell Signaling Technology, USA; dilution 1 : 500), anti-eNOS primary antibodies (Cell Signaling Technology, USA; dilution 1 : 1000), and anti-VCAM-1 antibody (Abcam, USA; dilution 1 : 1000). After washing with T-PBS for 30 minutes, they were incubated with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, USA; dilution 1 : 2000) for 1 hour at room temperature. The bands were visualized and quantitated by using the ECL detection systems (GE Healthcare) and the LAS-4000 luminescent image analyzer (GE Healthcare). Band intensities were determined using ImageQuant TL version 7.0 image analysis software (GE Healthcare). The expression was normalized using β-actin

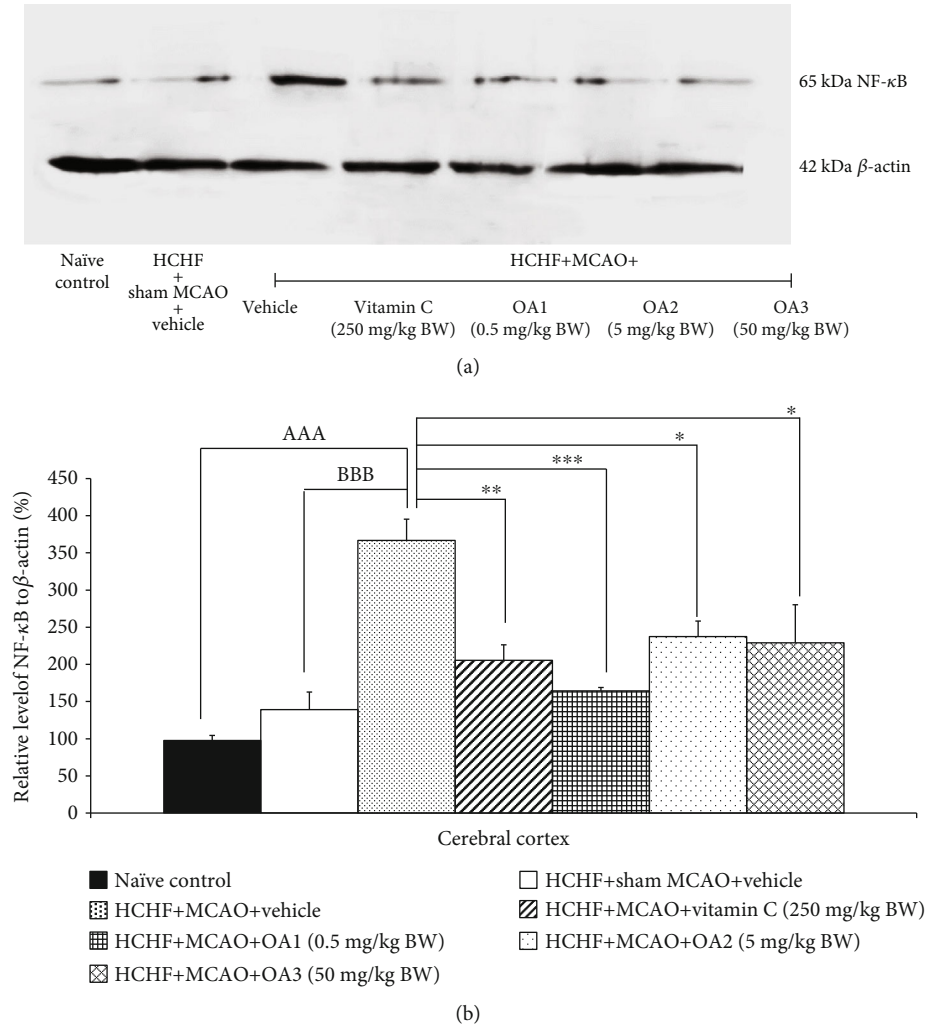

Figure 7: Effect of various doses of OA extract on the expression of NF-κB in the cerebral cortex. (a) Representative western blot showing the levels of NF-κB. (b) Relative density of NF-κB. Data are presented as mean ± SEM ($n = 6$/group). ^{AAA}p value < 0.01; compared to naïve intact rats, ^{BBB}p 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.

(Cell Signaling Technology, USA; dilution 1 : 2000). Data were expressed as a relative density to the control normal group.

2.14. Statistical Analysis. All data are presented as mean ± SEM. The significant differences among various groups were compared using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. p values of <0.05 were considered to be statistical significant. All statistical data were analyzed using SPSS software version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows).

3. Results

3.1. Changes of Neurological Score and Brain Infarction. The effect of the combined extract of *O. sativa* and *A. graveolens* on the neurological score is shown in Figure 2. HCHF diet which subjected to sham MCAO failed to produce the significant change on the neurological score throughout a 21-day study period. This suggested that MetS alone did not produce any change on the neurological score. MetS rats which subjected to MCAO and received vehicle showed an increase in the neurological score throughout the study period (p value < 0.001 all; compared to the HCHF+sham operation group). Vitamin C significantly decreased the neurological score of MetS rats with MCAO at 7, 14, and 21 days after treatment (p value < 0.05, 0.05, and 0.001, respectively; compared to HCHF+MCAO+vehicle). In addition, the neurological scores throughout the study period of MetS rats with MCAO were also improved by the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5, 5, and 50 mg/kg BW (p value < 0.001 all; compared to HCHF+MCAO+vehicle).

Based on the correlation between the neurological score and the brain infarcted volume after transient and

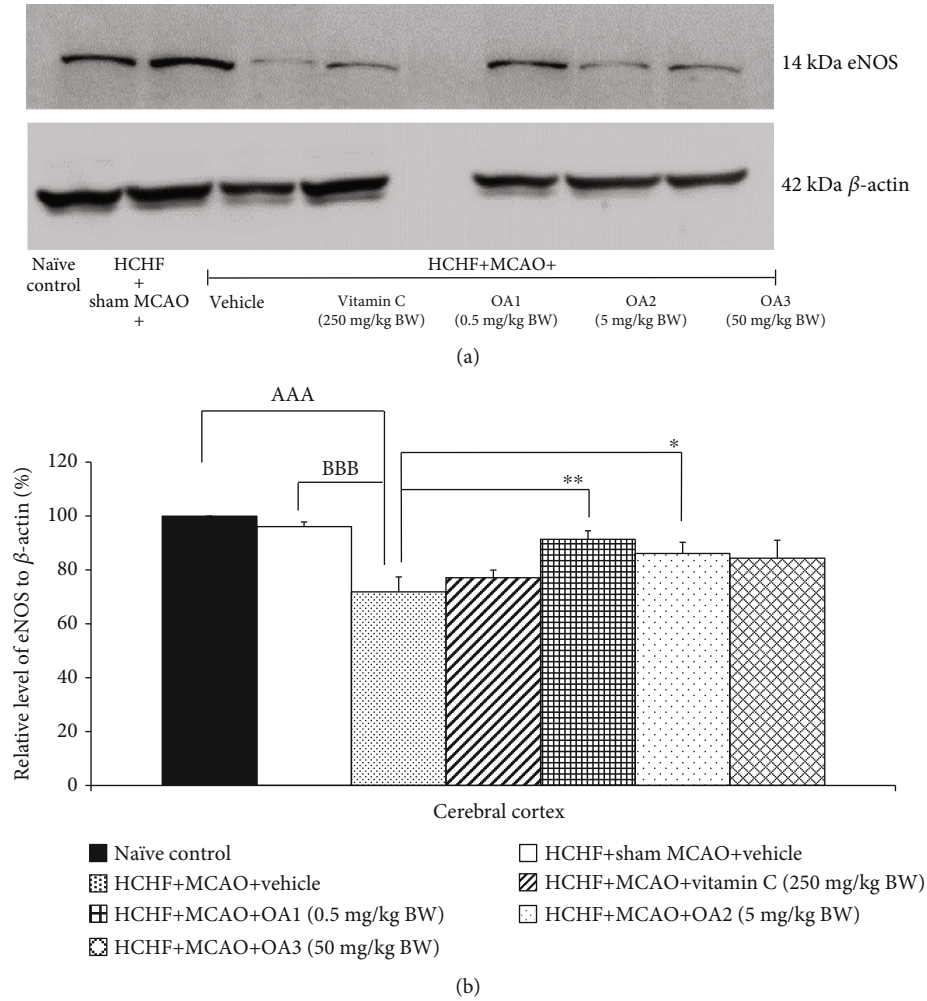

Figure 8: Effect of various doses of OA extract on the expression of endothelial nitric oxide synthase (eNOS) in the cerebral cortex. (a) Representative western blot showing the levels of eNOS. (b) Relative density of eNOS. Data are presented as mean ± SEM ($n = 6$/group). ^{AAA}p value < 0.001; compared to naïve intact rats, ^{BBB}p value < 0.001; compared to sham operation which received HCHF diet and vehicle and $^{*,**}p$ value < 0.05, 0.01, 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.

permanent focal cerebral ischemia in the rat [29], we also determined the changes of brain infarction volume and data are shown in Figure 3. It was found that no infarction volume were observed in the brain of the naïve intact and HCHF diet+sham operation groups as shown in Figure 3(a). However, MCAO induced the infarction area in the brain of MetS rats induced by HCHF diet. Vitamin C and all doses of the combined extract of *O. sativa* and *A. graveolens* significantly decreased the brain infarction area in the cortex, striatum, and hippocampus (p value < 0.001 all; compared to HCHF+MCAO+vehicle).

3.2. Brain Histological Changes. Previous study had demonstrated that cortical neuronal loss due to brain ischemia induced by the occlusion of MCAO was associated with the behavioral or mood impairments poststroke and it was set up as a target of stroke treatment [30]. Therefore, the neuron density in the cortical area has been investigated, and results are shown in Figure 4. MetS rats without MCAO showed no significant reduction in the cortical neuron density whereas the MetS rats with MCAO and received vehicle showed the significant reduction in the cortical neuron density (p value < 0.001; compared to naïve control). This change was mitigated by vitamin C and all doses of the combined extract of *O. sativa* and *A. graveolens* (p value < 0.001, 0.05, 0.001, and 0.001, respectively; compared to HCHF+MCAO+vehicle).

It has been demonstrated that astrocyte plays an essential role on the recovery of cerebral ischemia due to the physical and chemical inhibitory effect of astrocyte-rich glial scar on the recovery process [31, 32]. Therefore, the effect of the combined extract of *O. sativa* and *A. graveolens* on the changes of astrocyte glial cell was also explored, and results are shown in Figure 5. In this study, GFAP-positive-stained cells were used as an indirect indicator of astrocyte because GFAP is the most important marker of astrocyte [33]. There

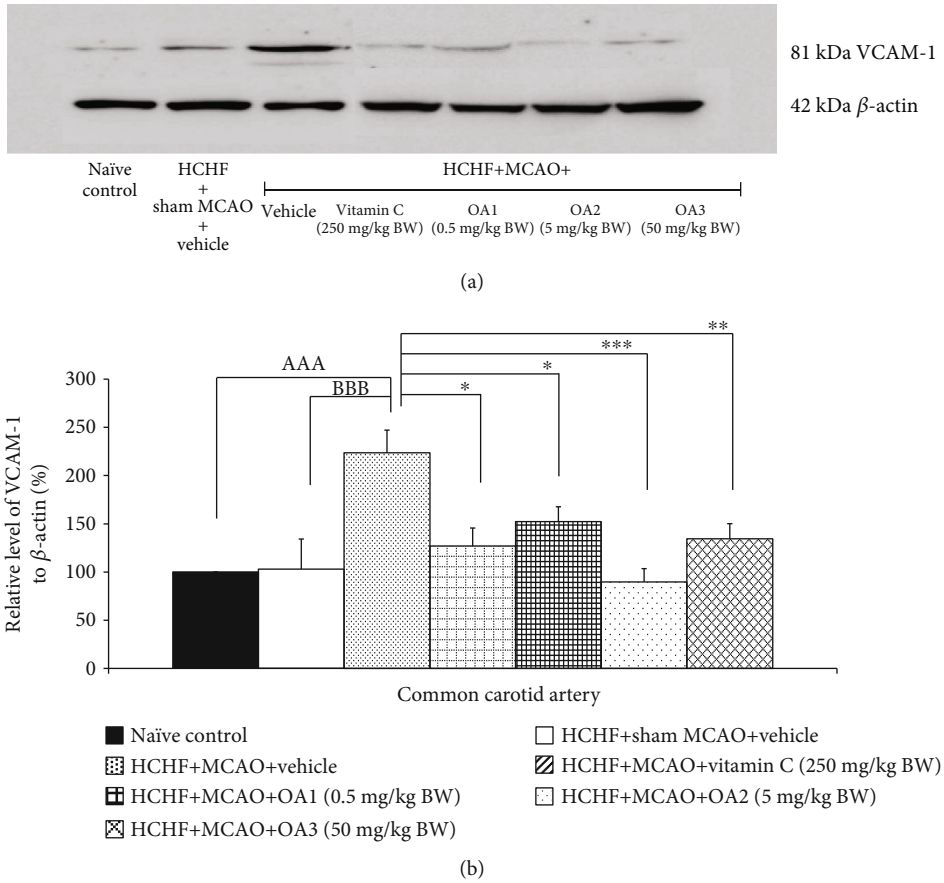

Figure 9: Effect of various doses of OA extract on the expression of vascular cell adhesion molecule 1 (VCAM-1) in common carotid artery. (a) Representative western blot showing the levels of VCAM-1. (b) Relative density of VCAM-1. Data are presented as mean ± SEM ($n = 6$/group). ^{AAA}p value < 0.001; compared to naïve intact rats, ^{BBB}p value < 0.001; compared to sham operation which received HCHF diet and vehicle and $^{*,**,***}p$ value < 0.05, 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.

are no significant change in the density of GFAP-positive-stained cell in the cortical area of MetS rats with sham operation. However, MetS rats with MCAO showed an increase in the density of GFAP-positive-stained cell in the area just mentioned (p value < 0.01; compared to HCHF+sham operation). This change was mitigated by vitamin C and the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5 and 5 mg/kg BW (p value < 0.05 all; compared to HCHF+MCAO+vehicle). The high dose of the combined extract of *O. sativa* and *A. graveolens* failed to produce the significant change of this parameter.

3.3. Brain Biochemical Changes. The effect of the combined extract of *O. sativa* and *A. graveolens* on brain oxidative stress status was also determined due to its important role in brain damage after stroke [34]. Table 1 showed that MetS rats with sham operation failed to produce the significant changes of all oxidative stress markers including MDA, SOD, CAT, and GSH-Px. MCAO significantly decreased the activities of CAT and GSH-Px but increased the MDA level (p value < 0.001 all; compared to HCHF+sham operation). Both vitamin C and the medium dose of the combined extract of *O. sativa* and *A. graveolens* increased the activities of SOD, CAT, and GSH-Px enzymes but decreased the MDA level in the cortical area (p value < 0.001 all, p value < 0.05 all, p value < 0.001 all, and p value < 0.001 all; compared to HCHF+MCAO). The low dose of the combined extract of *O. sativa* and *A. graveolens* increased the SOD activity but decreased the MDA level (p value < 0.001 all; compared to HCHF+MCAO) in the cerebral cortex. However, the high dose of the combined extract of *O. sativa* and *A. graveolens* increased the SOD and CAT activities but decreased the MDA level (p value < 0.001, 0.05, and 0.001, respectively; compared to HCHF+MCAO) in the area just mentioned.

The effect of the combined extract of *O. sativa* and *A. graveolens* on the inflammatory cytokines such as IL-6 and NF-κB was also investigated, and data are shown in Figures 6 and 7. MetS rats failed to show the significant modulation effects on the expressions of both IL-6 and NF-κB in the cortical area. However, the increase in the expression of both parameters in MetS rats was observed in MetS rats with MCAO (p value < 0.001 all; compared to HCHF+MCAO). Vitamin C and all doses of the combined extract used in this study significantly decreased the expressions of both IL-6 (p value < 0.05, 0.01, 0.01, and 0.05; compared to HCHF+MCAO) and NF-κB

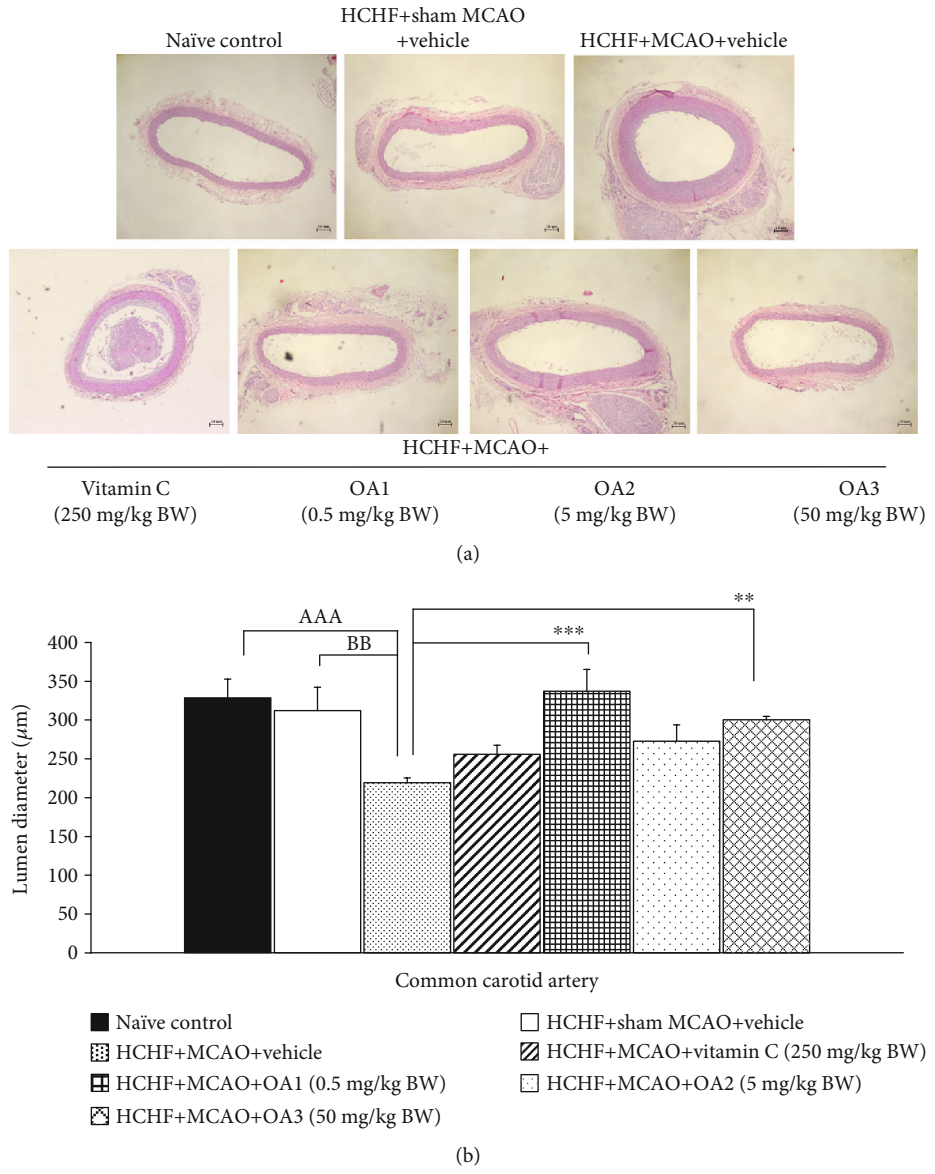

Figure 10: Effect of various doses of OA extract on histopathological appearance of common carotid artery. (a) Light microscope of common carotid artery stained with H&E at 40x magnification. (b) The length of lumen diameter in the common carotid artery. Data are presented as mean ± SEM ($n = 6$/group). ^{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 < 0.01, 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.

(p value < 0.01, 0.001, 0.05, and 0.04, respectively; compared to HCHF+MCAO) in a cortical area.

Figure 8 shows the effect of the combined extract of *O. sativa* and *A. graveolens* on the expression of eNOS on the cortical area. The changes of eNOS in MetS rats with sham operation failed to show the significant effect but the significant reduction of eNOS expression in the cerebral cortex was observed in MetS with MCAO (p value < 0.001; compared to HCHF+sham operation). This change was mitigated only in MetS rats with MCAO which received either low or medium dose of the combined extract of *O. sativa* and *A. graveolens* (p value < 0.05 and 0.01, respectively; compared to HCHF+MCAO).

3.4. Changes of Common Carotid Artery. Although the occlusion of a common carotid artery is a rare case, the common carotid artery is generally associated with occlusion of the distal vessels such as internal carotid artery and middle cerebral artery [35]. Therefore, we also explored the effect of the combined extract of *O. sativa* and *A. graveolens* on the changes of this artery, and results are shown in Figures 9–12. No significant changes in the lumen diameter, the expression of VCAM-1, and the thickness of both tunica intima and tunica media in the common carotid artery of MetS rats were found. MCAO significantly decreased the lumen diameter but increased the expression of VCAM-1 and the thickness of tunica intima and media

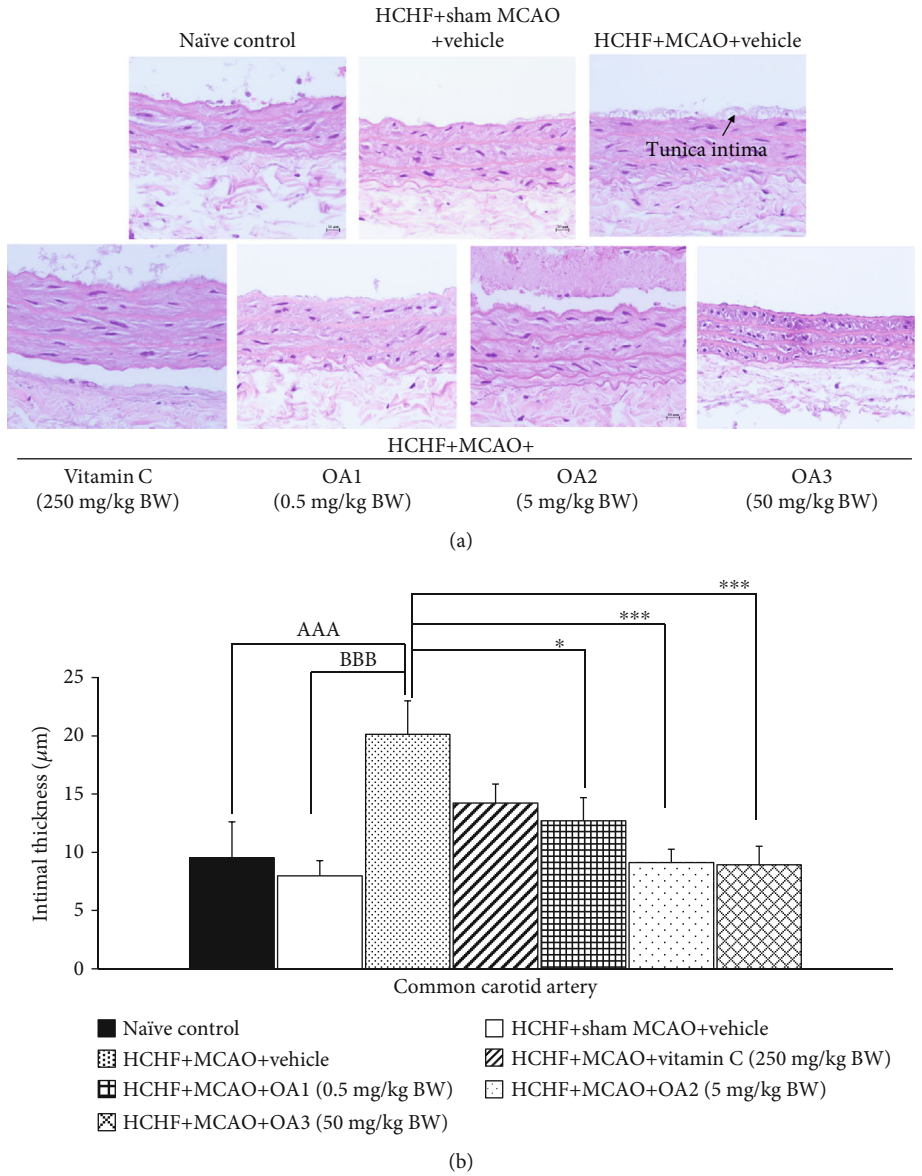

Figure 11: Effect of various doses of OA extract on histopathological appearance of common carotid artery. (a) Light microscope of common carotid artery stained with H&E at 40x magnification. (b) Tunica intima thickness in the common carotid artery. Data are presented as mean ± SEM ($n = 6$/group). ^{AAA}p 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 < 0.05, 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.

(p value < 0.01, 0.01, 0.001, and 0.01, respectively). Vitamin C treatment could decrease the VCAM-1 proliferation (p value < 0.05; compared to HCHF+MCAO) but failed to produce the significant changes in the lumen diameter and the thickness of both tunica intima and media of the common carotid artery. Interestingly, all doses of the combined extract of *O. sativa* and *A. graveolens* produced the significant decrease in VCAM-1 expression (p value < 0.05, 0.001, and 0.01, respectively; compared to HCHF+MCAO) and the thickness of tunica intima in the artery (p value < 0.05, 0.001, and 0.01, respectively; compared to HCHF+MCAO). The significant reduction in the thickness of the tunica media was observed only in MetS rats which received either the middle or the high dose of the combined extract of *O. sativa* and *A. graveolens* (p value < 0.05 and 0.001, respectively; compared to HCHF+MCAO) whereas the significant increase in the lumen diameter was observed only in MetS rats which received the combined extract of *O. sativa* and *A. graveolens* at doses of 0.5 and 50 mg/kg BW (p value < 0.001 and 0.01, respectively; compared to HCHF+MCAO).

4. Discussion

The current data have demonstrated that the combined extract of *O. sativa* and *A. graveolens* significantly improves

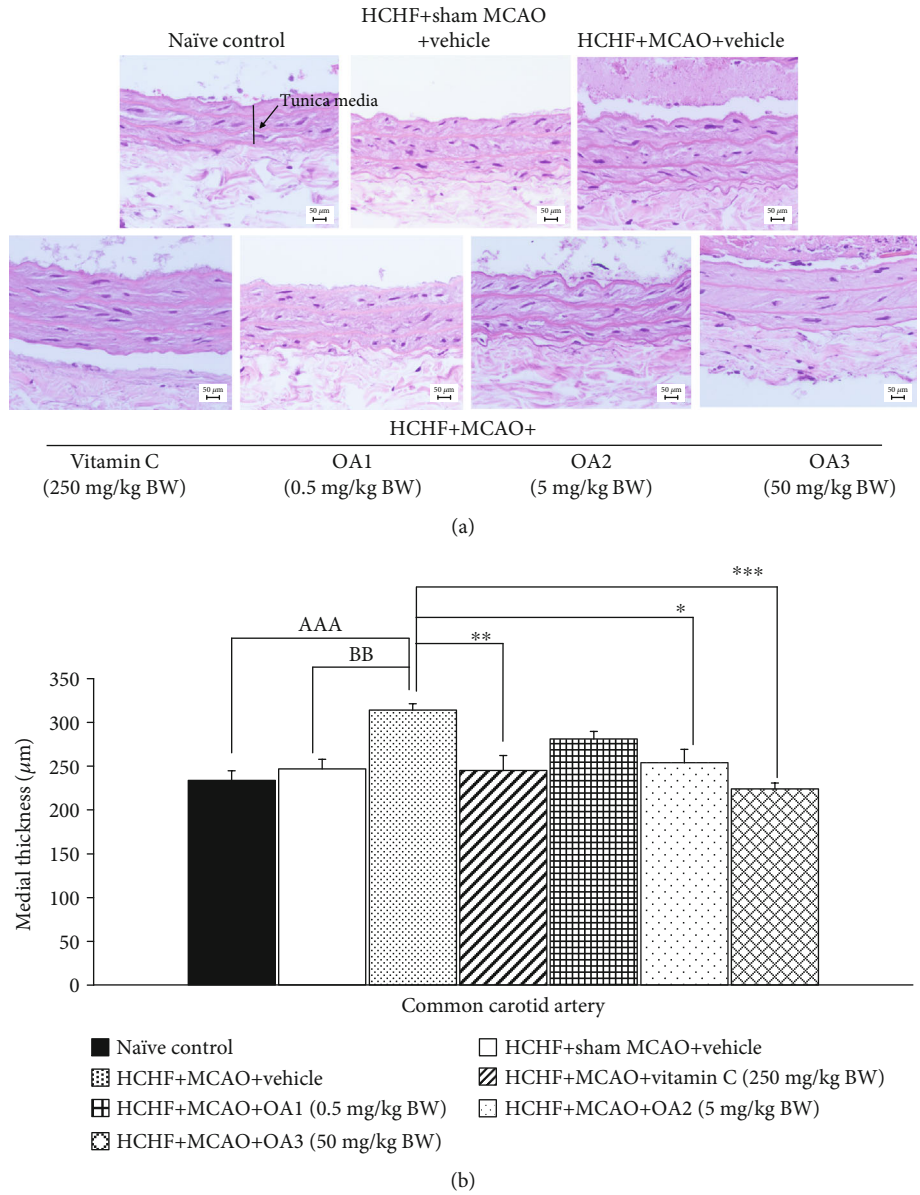

Figure 12: Effect of various doses of OA extract on histopathological appearance of common carotid artery. (a) Light microscope of common carotid artery stained with H&E at 40x magnification. (b) Tunica media thickness in the common carotid artery. Data are presented as mean ± SEM ($n = 6$/group). ^{AAA}p 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, 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.

neurological deficit in the animal model of cerebral ischemia in MetS condition. The combined extract also decreases the brain infarction, MDA, IL-6, and NF-κB levels but increases the activities of antioxidant enzymes, the expression of eNOS in the frontal cortex. It also increases the lumen diameter but decreases the thickness of both tunica intima and tunica media of the common carotid artery. In addition, the expression of VCAM-1 in the artery just mentioned also decreased.

Our data showed that MetS rats with sham operation failed to show the elevation of MDA whereas the previous study of Obadia and coworkers revealed the increase in brain MDA level. The possible explanation for the discrepancy might be associated with the different type of diet used for MetS induction. Our study used HCHF diet but the previous study used high-fat diet [36]. In addition, the selected cortical area for the investigation might be different because the current study focused on the changes of the frontal cortical area which represented the major area which is affected by the occlusion of MCA, but the previous study did not mention the cortical area that was investigated [37–39]. Aforementioned events contributed the crucial roles on brain infarction and cortical neuronal loss which in turn induced neurological deficit [31, 32, 34, 40]. It was found that cytokines also upregulated adhesion molecule expressions such

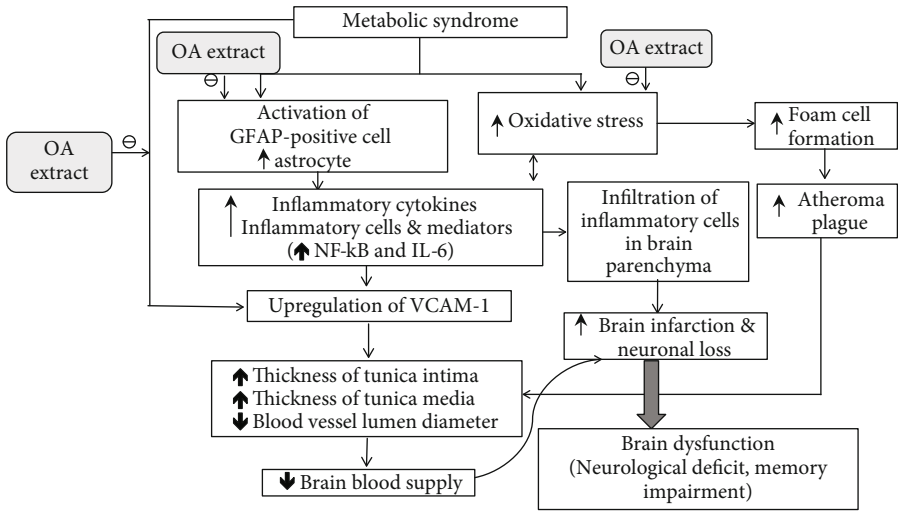

Figure 13: The possible underlying mechanism for the neuroprotective effects of the combined extract of *O. sativa* and *A. graveolens* in an animal model of metabolic syndrome with cerebral ischemic stroke.

as ICAM1 and VCAM-1 on the surfaces of cells of the cerebral vasculature which in turn allowed the infiltrate of inflammatory cells in brain parenchyma giving rise to the secondary damage of brain after ischemic reperfusion injury [40]. In addition to the factors mentioned earlier, the changes in blood vessels such as atherosclerosis which in turn played the role on the brain circulation also played the crucial role on the pathophysiology of ischemic stroke [41]. Since the severity of carotid atherosclerosis was a useful indicator of the risk of ischemic stroke [42], the change of adhesion molecule such as VCAM-1 in the common carotid artery was also investigated. VCAM-1 expression was observed in atherosclerotic conditions and early injury [43, 44]. Our data showed that ischemic reperfusion injury also increased the expression of VCAM-1 and the thickness of tunica media and tunica intima of the common carotid artery of MetS rats. Taken all together, MetS rats subjected to ischemic reperfusion injury increased VCAM-1 expression in the artery leading to the infiltrate of inflammatory cells in the blood vessel and the brain parenchyma. Then, the inflammatory cell increased both inflammation and oxidative stress. An elevation of oxidative stress also induced the formation of foam cell at the blood vessel resulting in atherosclerosis and decreased brain blood supply. The formation of foam cell and atheroma gave rise to the increase in intima-media thickness resulting in the reduction of lumen diameter and brain blood supply and brain infarction. In addition, the infiltration of inflammatory cell also produced both oxidative stress and inflammation resulting in infarction.

In addition, the expansion of ischemic lesions through microcirculatory disturbances after cerebral ischemia also occurred as a result of endothelial dysfunction [45]. Under the normal circumstance, endothelium-derived nitric oxide (eNO), a substance synthesized by endothelial nitric oxide synthase (eNOS), was regarded as an important signal transduction molecule which regulates and maintains brain microcirculation. It was demonstrated that the loss of eNOS gave rise to the reduction in cerebral blood flow resulting in the increase brain infarction area [46, 47]. Therefore, the reduction of eNOS expression in the cortical area observed in this study was also associated with the increased infarction area of the cortex in MetS rats subjected to ischemic reperfusion. Since the downregulation of eNOS was induced by IL-6 [48], we suggested that the suppression of eNOS in the cortical area of MetS rats subjected to MCAO occurred as the result of the elevation of IL-6 in this area. Based on aforementioned information, it has been suggested that substances possessing antioxidant activity such as vitamin C and the combined extract of *O. sativa* and *A. graveolens* can significantly decrease oxidative stress giving rise to the reduction of inflammatory cytokines such as NF-κB and IL-6 which in turn downregulates the expression of VCAM-1 leading to the reduction of intima-media thickness resulting in the increase in lumen diameter of the blood vessel especially the artery and gives rise to the improved brain blood supply and reduced brain infarction and neuronal loss. The reduction in inflammatory cytokines induced by either the antioxidant or the anti-inflammation activities of the combined extract of *O. sativa* and *A. graveolens* [12] also decreases the infiltration of inflammatory cell in the brain parenchyma leading to the improved brain infarction and neuronal loss. In addition, the reduction of IL-6 induced by the combined extract of *O. sativa* and *A. graveolens* also leads to the increase in eNOS expression and the improvement of brain blood supply which in turn decreases brain infarction and neuronal loss. The decreased oxidative stress status induced by the combined extract of *O. sativa* and *A. graveolens* can also decrease the formation of foam cell and atherosclerosis resulting in the improvement of brain infarction and neuronal loss. Moreover, the combined extract of *O. sativa* and *A. graveolens* can also decrease the expression of VCAM-1 directly via the suppression of oxidative stress [49] as shown in Figure 13.

It has been shown that anthocyanin-rich substance can protect against brain damage induced by oxidative stress [12, 50–54]. Therefore, the anthocyanin content in the combined extract of *O. sativa* and *A. graveolens* may contribute the role on the improvement of brain infarction and

dysfunction observed in this study. Since it contains numerous ingredients, the lack of a dose-dependent manner can be observed due to masking effect of other ingredients.

5. Conclusion

This study has clearly demonstrated that the combined extract of *O. sativa* and *A. graveolens* which is rich in anthocyanins can improve brain infarction and neuronal loss in MetS rats with cerebral ischemia induced by ischemic/reperfusion injury at MCAO. The possible underlying mechanism may occur partly via the reduction in oxidative stress status and inflammation together with the improvement of brain blood supply via the reduction of VCAM-1 and thickness of tunica intima and tunica media. In addition, the increases in eNOS expression in the cortical area and lumen diameter in the artery may also contribute the role. Therefore, the combined extract of *O. sativa* and *A. graveolens* is the potential neuroprotectant against MetS with cerebral ischemia. However, the clinical trial study is essential to confirm the health benefit of this substance.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Antimetabolic Syndrome Effect of Phytosome Containing the Combined Extracts of Mulberry and Ginger in an Animal Model of Metabolic Syndrome

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Due to the antimetabolic syndrome effect of mulberry and ginger together with the advantages of the synergistic effect and phytosome encapsulation technique, we hypothesized that phytosome containing the combined extracts of mulberry and ginger (PMG) should be able to manage MetS. PMG was developed and assessed the phenolic content and biological activities associated with the pathophysiology of MetS. The antimetabolic syndrome effect and the possible underlying mechanisms in the animal model of MetS were also assessed. Male Wistar rats induced MetS by subjecting to a 16-week high-carbohydrate high-fat diet. MetS rats were orally given PMG at doses of 50, 100, and 200 mg/kg for 21 days. They were determined metabolic parameter changes in serum, histomorphology changes of adipose tissue, the inflammatory cytokines such as IL-6 and TNF-α, oxidative stress status, PPAR-γ, and HDAC3 in adipose tissue. Our *in vitro* data showed that PMG increased phenolic contents and biological activities. PMG significantly improved MetS parameters including body weight gain, lipid profiles, plasma glucose, HOMA-IR, and ACE. In addition, the density and size of adipocyte, adiposity index, and weights of adipose tissues were also improved. Moreover, the decrease in TNF-α and IL-6, oxidative stress status, and HDAC3 expression together with the increase in PPAR-γ expression in adipose tissue was also observed. These data suggest that PMG exhibit antimetabolic syndrome and the possible underlying mechanism may be associated partly with the modulation effect on HDAC3, PPAR-γ, and adipose tissue. In addition, PMG also improves oxidative stress and inflammation in MetS. Therefore, PMG can be served as the potential supplement to manage MetS. However, a clinical trial study is essential to confirm this health benefit.

1. Introduction

Currently, the prevalence of metabolic syndrome (MetS), a complex disorder consisting of central obesity, hyperglycemia, hypertension, and hyperlipidemia [1], has been recognized as the global health problem [2]. MetS produces a great impact on the socioeconomic burden. In addition, it also produces numerous MetS-associated disorders including cardiovascular disorders and stroke. Despite its significance, the current therapy can successfully deal with some of the individual components such as hypertension, insulin resistance, and dyslipidemia. Unfortunately, such therapeutic success has not been shared by obesity, the other major component of the metabolic syndrome [3]. Therefore, the novel strategy that covers this component in MetS is still required.

Recent study has demonstrated that the alteration of adipose tissue plays a significant impact on whole-body metabolism and serves as a key driver for the development of these metabolic derangements [4]. The excess visceral tissue can induce numerous deleterious effects including

insulin resistance, dyslipidemia, and inflammation [5]. Based on the key role of adipose tissue mentioned earlier, it has been considered as one of the treatment targets. Recently, it has been demonstrated that natural phytochemical substance possessing anti-inflammatory property such as capsaicin can improve MetS [6]. Other spice-derived components possessing an anti-inflammatory effect such as *Zingiber officinale* can also improve the aforementioned condition [7]. In addition to the inflammation, oxidative stress also plays an important role on the pathophysiology of MetS [8]. Substances possessing antioxidant activity such as anthocyanin-rich substances also exhibit an antimetabolic syndrome effect [8].

Most of the active ingredients of the medicinal plants, fruits, and vegetables are unstable and highly labile. Moreover, most of these phytochemical substances are poorly absorbed and instable during food processing, distribution, or storage in the gastrointestinal tract [9]. Therefore, a strategy to overcome all of these limitations is required. Interestingly, phytosome technology, a technology to conjugate phytochemicals to phospholipids in order to produce lipid compatible molecular complexes, is reported to improve the stability and bioavailability of the phytochemical substances [10–13]. It can improve stability by decreasing the decay induced by environment [14, 15]. Based on the advantages of a phytochemical substance in ginger and mulberry fruit together with the benefit of phytosome technology on stability and bioavailability mentioned earlier, we hypothesized that the phytosome containing the extract of mulberry and ginger could improve metabolic syndrome in metabolic syndrome rats. The changes of adipocyte, oxidative stress status, inflammation, PPAR-γ, and epigenetic mechanism were also explored to investigate the possible underlying mechanism.

2. Materials and Methods

2.1. PMG Preparation. Rhizomes of ginger (*Zingiber officinale* Roscoe) were collected from Khon Kaen province, Thailand, and authenticated by the expert in pharmacognosy of the National Museum of THAI Traditional Medicine, Thailand (voucher specimen No. 0002402 and deposited at the National Museum of THAI Traditional Medicine), and mulberry fruit (*Morus alba* Linn. var. Chiangmai) was identified and kindly provided by Mr. Sombat Kongpa, the chief of Queen Sirikit Department of Sericulture Center (Udon Thani Province), Ministry of Agriculture and Cooperatives, Thailand (voucher specimen 61001 and deposited at the Research Institute of Human High Performance and Health Promotion). The samples of both plants were cleaned and dried with the oven (Memmert GmbH, USA) at 60°C for 72 hours. Then, they were grounded to fine powder. Powder of ginger was prepared as 50% hydroalcoholic extract whereas mulberry powder was prepared as 95% hydroalcoholic extract by using maceration techniques. Then, the extracts were centrifuged at 3,000 rounds per minute (rpm) for 10 minutes and filtered with Whatman No. 1 filter paper. The filtrate was dried by using a rotator evaporator and freeze dryer.

According to the phytosome preparation, phosphatidylcholine was selected as encapsulation matrix. Mulberry extract and ginger extract were mixed at the ratio of 1 : 1 (w/w). The combined extract of mulberry and ginger was dissolved in 100 ml of 50% ethanol whereas phosphatidylcholine was dissolved in 50 ml of dichloromethane. Following this process, the solution was mixed together with magnetic stirring at 25°C for 8 hours. Then, ethanol and dichloromethane were removed by using a rotary evaporator at 45°C for 3 hours. The solution was frozen at -80°C overnight and dried by using lyophilization (Labconco freeze dryer, Labconco Corporation, Kansas City, MO, USA) for 48 hours (-86°C, 0.008 mbar). The dry sample was packed and stored in a desiccator containing silica gel at 4°C.

2.2. Determination of the Fingerprint Chromatogram. The fingerprint chromatogram of PMG was determined by using the high-performance liquid chromatography (HPLC) analysis. Chromatography was performed by using a Waters® system equipped with a Waters® 2998 photodiode array detector. The separation of chromatogram was performed using Purospher® STAR, C-18 encapped (5 μm), LiChroCART® 250-4.6, and HPLC-Cartridge, Sorbet Lot No. HX255346 (Merck, Germany). According to this study, 100% methanol (solvent A) (Fisher Scientific, USA) and 2.5% acetic acid (solvent B) (Fisher Scientific, USA) in deionized (DI) water were used to produce the gradient of mobile phase. The gradient elution of mobile phase 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; 20.5-25 min, 10% A. The sample was filtered (0.45 μm, Millipore) and an aliquot of sample at the volume of 20 μl was directly administered. The chromatogram assessment was performed at 280 nm using a UV detector, and data analysis was performed using EmpowerTM3.

2.3. Measurement of Total Phenolic Compound Contents and Flavonoid Content. The amount of total phenolic compounds in a sample was determined by using the Folin-Ciocalteu colorimetric method in a microplate reader (iMark™ Microplate Absorbance Reader) [16].

The reagent consisting of 158 μl of distilled water and 20 μl of 50% v/v Folin-Ciocalteu reagent (Sigma-Aldrich, USA) was freshly prepared, mixed with 20 μl of the extract, and incubated for 8 minutes. Following this process, 30 μl of 20% Na_2CO_3 (Sigma-Aldrich, USA) was added and incubated at room temperature in a dark room for 2 hours. Then, an absorbance was measured at 765 nm. Result was expressed mg gallic acid equivalent (GAE)/mg extract. Various concentrations of gallic acid (Sigma-Aldrich, USA) were used as a standard calibration curve.

Flavonoid content was assessed by using the aluminum chloride method [17]. In brief, 100 μl of PMG at various concentrations was mixed with 100 μl of 2% methanolic aluminum chloride (Sigma-Aldrich, USA) and incubated at room temperature in a dark room for 30 minutes. At the end of incubation time, absorbance at 415 nm was measured against the suitable blank. Various concentrations of quercetin (Sigma-Aldrich, USA) were used for the standard

calibration curve preparation. Results were expressed as μg quercetin equivalent/mg extract.

2.4. Determination of Biological Activities

2.4.1. Antioxidant Activity. Antioxidant activity of PMG was determined by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assays. According to DPPH assay, the stable DPPH assay was used for the determination of free radical-scavenging activity of PMG. In brief, 0.1 mM alcoholic solution of DPPH in methanol was prepared and 2 ml of this solution was mixed with 0.3 ml of PMG at various concentrations (1-100 mg/ml) and was allowed to react at 25°C. At the end of a 30-minute incubation time period, the absorbance value was measured at 517 nm against the blank via a microplate reader (iMark™ Microplate Absorbance Reader). L-Ascorbic acid was served as control. The radical-scavenging activity was expressed as percent inhibition of DPPH radical [18].

FRAP assay was carried out based on the ability of PMG to convert ferric tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous tripyridyltriazine (Fe^{2+}-TPTZ). FRAP working solution was prepared by mixing 300 mM acetate buffer (Sigma-Aldrich, USA), 10 mM TPTZ (Sigma-Aldrich, USA), and 20 mM ferric chloride ($FeCl_3$) (Sigma-Aldrich, USA) solutions at a ratio of 10 : 1 : 1, respectively. In brief, 190 μl of FRAP reagent was mixed with 10 μl of PMG and incubated at 37°C for 10 minutes. After the incubation time period, an absorbance at 593 nm was measured against blank [19]. Vitamin C was used as a positive control, and results were as expressed as EC_{50} value.

ABTS assay was also used to determine the free radical-scavenging activity of the PMG [20]. $ABTS^+$ solution was prepared by mixing the 7 mM ABTS (Sigma-Aldrich, USA) and 2.45 mM potassium persulfate ($K_2S_2O_8$) (Sigma-Aldrich, USA) at a ratio of 2 : 3. Then, 3 ml of the solution was reacted with 120 μl of distilled water, 30 μl of ethanol, and 30 μl of various concentrations of PMG. The absorbance was measured at 734 nm with a spectrophotometer (Pharmacia LKB-Biochrom 4060). Trolox was used as a positive control. Results were also expressed as EC_{50} value.

2.4.2. Assessment of Pancreatic Lipase. The working solution of lipase at a concentration of 10 ng/ml was prepared by dissolving lipase from porcine pancreas type II (Sigma, USA) in deionized (DI) water and centrifuged to a 16,000 rpm centrifugation for 5 minutes. Then, the supernatant was harvested for further used. In brief, 100 mM Tris buffer pH 8.2 and p-nitrophenyl laurate (pNP laurate) were used as the substrate. The pNP laurate was dissolved in 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100 to produce 0.08% w/v substrate solution and served as stock solution. This solution was heated in boiling water for 1 min to aid dissolution, mixed well, and then cooled to 25°C. The reaction mixture containing 70 μl of assay buffer, 90 μl of substrate solution, 30 μl of lipase, and 10 μl of PMG was mixed and incubated at 37°C for 2 hours. At the end of the incubation time period, the solution was centrifuged at 16,000 rpm for 1 minute and measured absorbance with a microplate reader (iMark™ Microplate Absorbance Reader) at 400 nm. [21]. The orlistat was used as a positive control.

2.4.3. Assessment of Cyclooxygenase-2 (COX-2). COX-2 inhibition was measured by using a colorimetric COX-2 inhibitor screening assay kit (COX Activity Assay Kit, item No.760151) (Cayman Chemical, USA). The effect of PMG on COX-2 inhibition activity was performed according to the manufacturer's protocol. Cox-2 working solution was prepared by dissolving COX-2 agent in 100 mM Tris-HCl buffer, pH 8.0 at a ratio of 1 : 100. In brief, the reaction mixture containing 150 μl of assay buffer, 10 μl of PMG, 10 μl of heme (Cayman Chemical, USA), 10 μl of COX-II working solution, 20 μl of 10 μM TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride) (Sigma, USA), and 20 μl of 100 μM arachidonic acid (Cayman Chemical, USA) was added to a 96-well microplates and incubated at 25°C for 30 minutes. At the end of the incubation time period, an absorbance at 590 nm was recorded and results were expressed as EC_{50} [22]. Indomethacin was used as a positive control.

2.5. Experimental Protocol. Male Wistar rats (weighting 180-220 g, 8 weeks old) were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The rats were kept under standard laboratory conditions with food and water ad libitum and housed in standard metal cages (6 per cage). Temperature was controlled at 23 ± 2°C on a 12 : 12 hour light-dark cycle. All procedures and experimental protocols were approved by the Institutional Animal Ethics Committee of Khon Kaen University (record no. IACUC-KKU 95/60). After 1 week of acclimatization, rats were divided into 7 groups ($n = 6$) as follows:

(1) Group I (ND+vehicle): all rats in this group were administered normal diet and treated with vehicle

(2) Group II (HCHF+vehicle): all rats in this group received high-carbohydrate high-fat (HCHF) diet and treated with vehicle

(3) Group III (HCHF diet+vitamin C): all rats in this group received HCHF diet and treated with vitamin C at a dose of 250 mg/kg BW

(4) Group IV (HCHF diet+simvastatin): animals in this group received HCHF diet and treated with simvastatin at a dose of 1.3 mg/kg BW

(5) Group V-VII (HCHF diet+PMG) (PMG50, PMG100, and PMG 200): all rats in these groups received HCHF diet and treated with PMG at various doses ranging from 50 and 100 to 200 mg/kg BW

Rats in group I were fed with normal diet (4.5% fat, 42% carbohydrate, and 24% protein) whereas rats in group II-VII were fed with HCHF diet (HCHF; 35% fat, 45% carbohydrate, and 20% protein) in order to induce metabolic syndrome. After 16 weeks of the feeding period, rats which

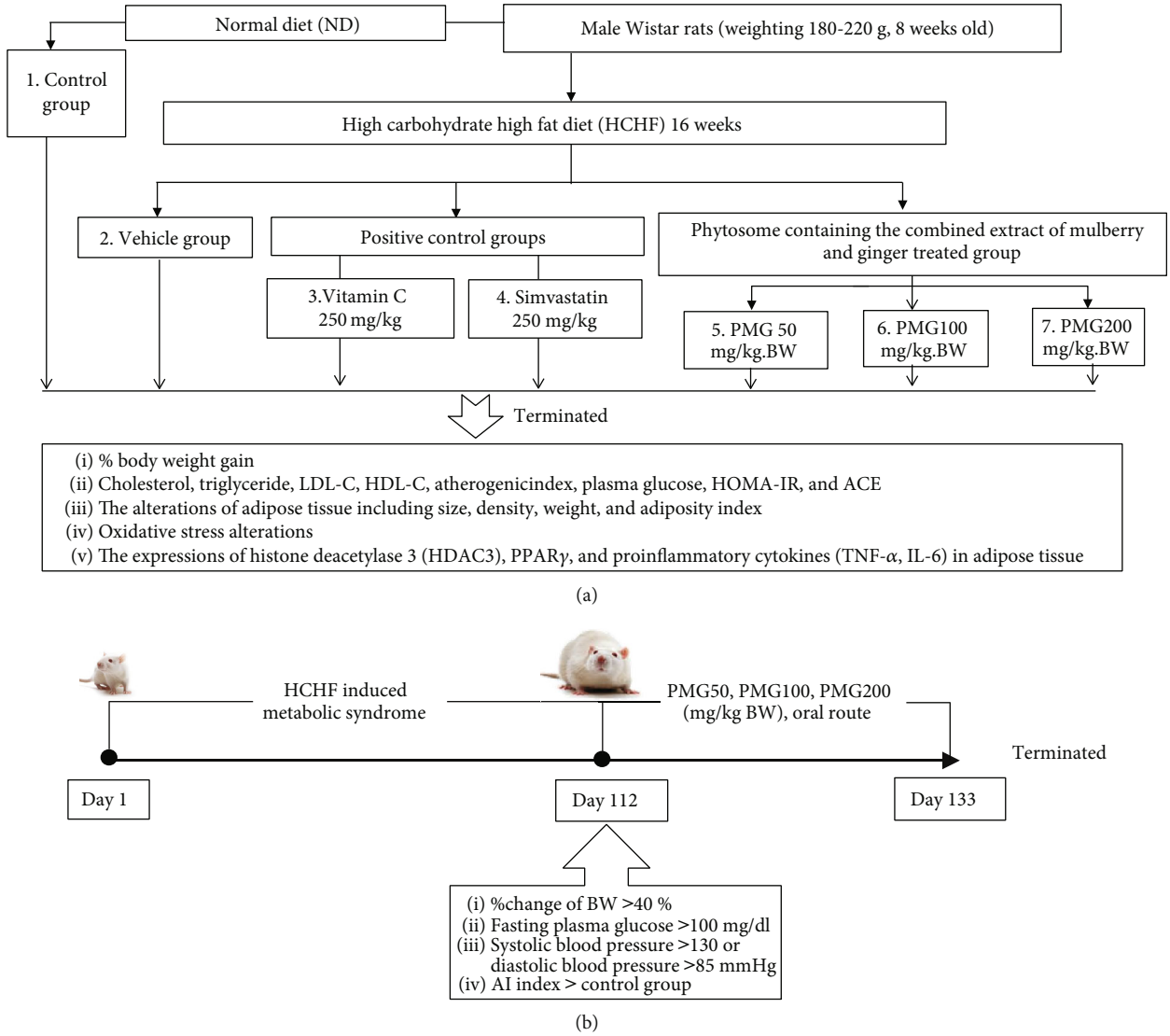

Figure 1: Schematic diagram showing all experimental procedures. (a) Experimental protocol of PMG treatment and the determination of various parameters. (b) Metabolic syndrome induction and schedule for PMG treatment. LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; ACE: angiotensin-converting enzyme; HDAC3: histone deacetylase 3; PPAR-γ: peroxisome proliferator-activated receptor gamma; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

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 further study. Then, the animals were orally given the assigned substances once daily for 21 days. Food and water consumption together with body weight was assessed every day throughout the study period. At the end of the study period, %body weight gain, cholesterol, triglyceride, LDL-C, HDL-C, atherogenic index, plasma glucose, HOMA-IR, and ACE were determined. In addition, the alterations of adipose tissue including size, density, weight, and adiposity index together with the oxidative stress status, the expressions of histone deacetylase 3 (HDAC3), PPAR-γ, and proinflammatory cytokines (TNF-α, IL-6) in adipose tissue were also determined. The schematic diagram showing the experimental protocol is shown in Figure 1.

2.6. Biochemical Assay

2.6.1. Plasma Lipid Profiles

(1) Determination of Total Plasma Cholesterol. The total plasma cholesterol was determined by "CHOD-PAP": enzymatic photometric test [23]. 10 μl of plasma or calibrator was mixed with 1,000 μl of cholesterol FS Reagent (DiaSys

Diagnostic Systems GmbH, Germany) and incubated at 25°C in a dark room for 20 minutes. Then, an absorbance at 500 nm was measured within 60 minutes by using a UV-spectrophotometer (Pharmacia LKB-Biochrom 4060). Total cholesterol was expressed as mg/dl and calculated as follows:

$$\text{Cholesterol (mg/dl)} = \left(\frac{A \text{ sample}}{A \text{ calibrator}}\right) \times \text{Conc.Cal.(mg/dl)}. \quad (1)$$

(2) Determination of Plasma Triglycerides. The enzymatic colorimetric test was performed by using glycerol-3-phosphate-oxidase (GPO) reagent (DiaSys Diagnostic Systems GmbH, Germany). 10 μl of plasma or calibrator was mixed with 1,000 μl of the prepared reagent and incubated at 25°C in a dark room for 10 minutes. The absorbance was measured at 500 nm within 60 minutes by using a UV-spectrophotometer (Pharmacia LKB-Biochrom 4060). Triglycerides was expressed as mg/dl and calculated as follows:

$$\text{Triglycerides (mg/dl)} = \left(\frac{A \text{ sample}}{A \text{ calibrator}}\right) \times \text{Conc.Cal.(mg/dl)}. \quad (2)$$

(3) Determination of Plasma Low-Density Lipoprotein Cholesterol (LDL-C). Plasma LDL-C was assessed based on the Friedewald equation [24] by using LDL-C select FS Reagent 1 and 2 (DiaSys Diagnostic Systems GmbH, Germany). In brief, 5 μl of plasma or Trulab L calibrator and 280 μl of reagent 1 were mixed and incubated at 37°C for 5 minutes. After the incubation time period, an absorbance at 595 nm (A1) was measured by using a microplate reader (iMark™ Microplate Absorbance Reader). Then, 70 μl of reagent 2 was added to the mixture and incubated at 37°C for 5 minutes. After that, the absorbance at 595 nm (A2) was measured. LDL-C was expressed as mg/dl and calculated as follows:

$$\text{LDL-C (mg/dl)} = \left(\frac{A \text{ sample}}{A \text{ calibrator}}\right) \times \text{Conc.Cal.(mg/dl)},$$
$$\text{A} = [(\text{A2} - \text{A1}) \text{ sample or calibrator}] - [(\text{A2} - \text{A1}) \text{ blank}] \quad (3)$$

(4) Determination of Plasma High-Density Lipoprotein Cholesterol (HDL-C). Plasma HDL was determined by using HDL-C select FS Reagent 1 and 2 (DiaSys Diagnostic Systems GmbH, Germany) [25]. In brief, 5 μl of plasma or Trulab L calibrator and 240 μl of reagent 1 were mixed and incubated at 37°C for 5 minutes. After the incubation time period, an absorbance at 595 nm (A1) was measured by using a microplate reader (iMark™ Microplate Absorbance Reader). Then, 60 μl of reagent 2 was added to the mixture and incubated at 37°C for 5 minutes. After the incubation, the absorbance at 595 nm (A2) was measured. HDL-C was expressed as mg/dl and calculated as follows:

$$\text{HDL-C (mg/dl)} = \left(\frac{A \text{ sample}}{A \text{ calibrator}}\right) \times \text{Conc.Cal.(mg/dl)},$$
$$\text{A} = (\text{A2} - \text{A1}) \text{ sample or calibrator} \quad (4)$$

2.6.2. Estimation of Atherogenic Index (AI Index). The atherogenic index (AI index), the most reliable indicator for the prediction of cardiovascular disease risk, was determined and calculated according to the following equation [26]:

$$\text{Atherogenic index (AI index)} = \frac{\text{total cholesterol (TC)}}{\text{high-density lipoprotein cholesterol (HDL-C) ratio}}. \quad (5)$$

2.6.3. Estimation of Plasma Glucose. All animals were fasted for 12 hours. After the food deprivation period, the basal blood glucose concentrations were measured. In brief, the blood sample was collected from tail vein and determined the blood glucose level by using ACCU-CHEK® Performa Blood Glucose Meter.

2.6.4. Estimation of HOMA-IR. At the end of a 12-hour fasting period, serum insulin and plasma glucose determinations were performed in order to calculate the homeostasis model assessment of insulin resistance (HOMA-IR) [27]. Blood samples were collected and immediately kept cool in ice bath. Then, they were centrifuged at 1,000 x g for 10 minutes at room temperature, according to the manufacturer's instructions. Serum was stored at -80°C until the assay. Insulin assay was performed using the Luminex™ kit (Millipore™, Billerica, MA) following the provided luminescence method. HOMA-IR was calculated according to the following equation:

$$\text{HOMA IR} = \frac{\text{serum insulin (mmol/l)} \times \text{blood glucose (mmol/l)}}{22.5 \text{ insulin assay}}. \quad (6)$$

2.6.5. Determination of Plasma Angiotensin-Converting Enzyme. Plasma angiotensin-converting enzyme was performed base on Serra et al.'s concept [28]. The enzymatic reaction was started by adding the 20 μl of plasma into the 50 μl of substrate solution Hip–Gly–Gly (100 mmol/l) (Sigma, USA) and incubated at 37°C for 35 minutes. The reaction was stopped by adding 120 μl of 3 M sodium tungstate (Sigma, USA) and 0.5 M sulfuric acid (Sigma, USA) and centrifuged at 2500 rpm for 10 minutes. After the centrifugation, the supernatant was placed into a 96-well microplate and mixed with 20 μl of 60 mM TNBS (Sigma, USA) and incubated at dark condition for 20 minutes. At the end of the incubation time period, an absorbance at 415 nm was recorded with a microplate reader

(iMark™ Microplate Absorbance Reader). The standard calibration curve was prepared by using ACE (Sigma-Aldrich, USA) at the concentration range of 0.001-1 units/ml. Results were expressed as units/mg protein.

2.7. Histological Procedure and Adiposity Assessment. After the scarification, fat pads from visceral and subcutaneous areas were removed and immersed into fixative solution containing 10% formalin (Sigma-Aldrich, USA) for 72 hours. Serial sections of tissues were cut frozen on cryostat (Thermo Scientific™ HM 525 Cryostat) at 10 μm thick. All sections were picked up on slides coated with 0.3% aqueous solution of gelatin containing 0.05% aluminum potassium sulfate (Sigma-Aldrich, USA). The observation was carried out after the hematoxylin-eosin (H&E) (Sigma-Aldrich, USA) staining process [29]. The determination of adipocyte cell diameter and density were performed from 3 randomly selected different fields of each area by using Olympus light microscope model BH-2 (Japan) under 40x magnification with PixeLINK PL-A6xx Capture and IT tool program. In addition, the adiposity index was calculated by the sum of intra-abdominal fat weights/body weight ratio × 100 and expressed as percentage of adiposity.

2.8. Assessment of Oxidative Stress Status in Adipose Tissues and Plasma. Adipose tissues were isolated and homogenized with 0.1 M potassium phosphate buffer solution, pH 7.4 (sample dilution 10 mg: PBS 50 μl). The derived homogenate was used for the determination of oxidative status, including the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) level. The protein concentrations in adipose tissue homogenate were assessed by using a Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) and measured the optical density at the wavelength of 280 nm.

According to assess SOD activity, the reaction solution was prepared by mixing the 0.2 M phosphate buffer solution (KH_2PO_4), pH 7.8 (Sigma-Aldrich, USA), 0.01 M EDTA (Sigma-Aldrich, USA), 15 M cytochrome C (Sigma-Aldrich, USA), and 0.5 mM of xanthine, pH 7.4 (Sigma-Aldrich, USA) together at the ratio of 25 : 1 : 1 : 50 (*v*/*v*). 20 μl of tissue homogenate was mixed with 200 μl of the reaction mixture and 20 μl of xanthine oxidase (0.90 mU/ml) (Sigma-Aldrich, USA). The optical density at 415 nm was measured. SOD enzyme (Sigma-Aldrich, USA) activities at the concentrations of 0-25 units/ml were used as standard, and the results were expressed as units/mg protein [30].

Base on the capability of the enzyme to break down, H_2O_2 was used for determining catalase activity. In brief, the reaction mixture containing 50 μl of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0) (BDH Chemicals Ltd., UK), 25 μl of 5 M H_2SO_4 (Sigma-Aldrich, USA), and 150 μl of 5 mM $KMnO_4$ (Sigma-Aldrich, USA) was mixed with 10 μl of sample. After mixing, an absorbance at 490 nm was recorded [31]. CAT enzyme (Sigma-Aldrich, USA) at the concentration range between 10 and 100 units/ml was used as standard, and the result was expressed as units/mg protein.

Glutathione peroxidase activity was assessed by mixing 20 μl of sample solution with the reaction mixture consisting of 10 μl of 1 mM dithiothreitol (DTT) (Sigma-Aldrich, USA) and 10 mM monosodium phosphate (NaH_2PO_4) in DW, 100 μl of 1 mM sodium azide (Sigma-Aldrich, USA) in 40 mM potassium phosphate buffer (pH 7.0), 10 μl of 50 mM glutathione (Sigma-Aldrich, USA) solution, and 100 μl of 30% hydrogen peroxide (BDH Chemicals Ltd., UK) and incubated at 25°C for 10 minutes. At the end of the incubation time period, 10 μl of 10 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) (Sigma-Aldrich, USA) was added and an absorbance at 412 nm was recorded [32]. GSH-Px enzyme (Sigma-Aldrich, USA) at the concentration range between 1 and 5 units/ml was used as standard. GSH-Px activity was expressed as units/mg protein.

The MDA level was also assessed according to the thiobarbituric acid reaction method [33]. The reaction mixture containing 50 μl of sample solution, 50 μl of 8.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA), 375 μl of 0.8% of thiobarbituric acid (TBA) (Sigma-Aldrich, USA), 375 μl of 20% acetic acid (Sigma-Aldrich, USA), and 150 μl of distilled water (DW) was boiled at 95°C in the water bath for 60 minutes. After boiling, it was cooled with tap water. After that, 250 μl of DW and 1,250 μl of the solution containing n-butanol and pyridine (Merck, Germany) at the ratio of 15 : 1 were added, mixed together, and centrifuged at 4,000 rpm for 10 minutes. The upper layer was separated and measured the absorbance at 532 nm. TMP (1,1,3,3-tetramethoxypropane) (0-15 μM) (Sigma-Aldrich, USA) was served as standard, and the level of MDA was expressed as ng/mg protein.

2.9. Western Blotting Analysis. Adipose tissue was homogenized and lysed in 1/5 (*w*/*v*) RIPA (radioimmunoprecipitation assay) buffer (Cell Signaling Technology, USA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Cell Signaling Technology, USA). The tissue homogenate supernatant of the middle layer of adipose tissue samples was isolated after the 12,000 g centrifugation at 4°C for 10 minutes. Protein concentration was determined by using a Thermo Scientific NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). In brief, 80 micrograms of tissue lysates were adjusted to appropriate concentration by using Tris-Glycine SDS-PAGE loading buffer and heated at 95°C for 10 minutes. Protein in tissue sample was isolated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by loading 20 μl of sample on SDS-polyacrylamide gel. Then, the separated bands were transferred to nitrocellulose membrane, washed with 0.05% TBS-T, and incubated in blocking buffer (5% skim milk in 0.1% TBS-T) at 25°C for 1 hour. After the blocking process, the nitrocellulose membrane was incubated with anti-HDAC3 (Cell Signaling Technology, USA; dilution 1 : 500), anti-PPAR gamma (Abcam, UK; dilution 1 : 1000), anti-IL-6 (Cell Signaling Technology, USA; dilution 1 : 500),

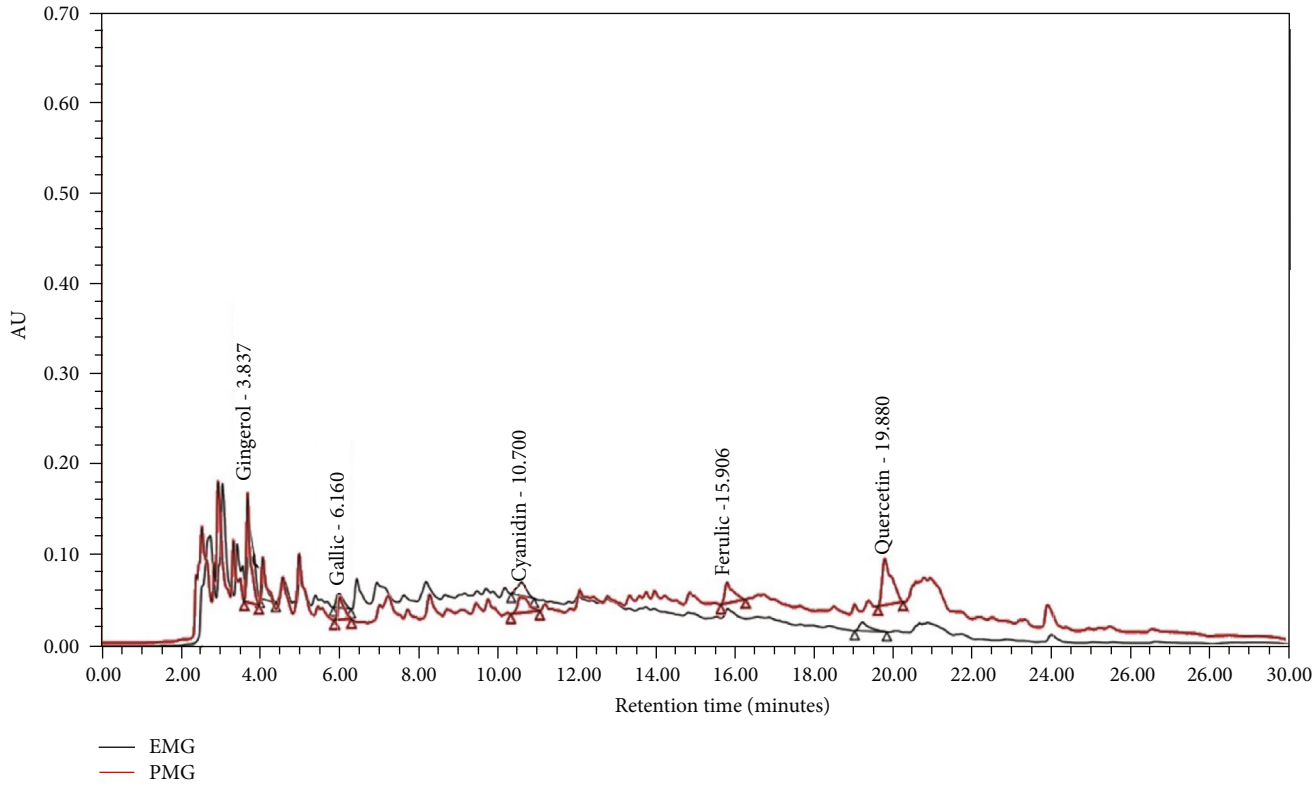

Figure 2: The HPLC profiles of gingerol, gallic acid, cyanidin-3-glucoside, ferulic acid, and quercetin-3-O-rutinoside of EMG and PMG. EMG: the combined extracts of mulberry and ginger; PMG: the phytosome containing the combined extracts of mulberry and ginger.

anti-TNF-α (Cell Signaling Technology, USA; dilution 1 : 500), and anti-β-actin (Cell Signaling Technology, USA; dilution 1 : 1000) antibodies at 4°C overnight. After an incubation time period, the nitrocellulose membrane was rinsed with TBS-T (0.05%) again and incubated with anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, USA; dilution 1 : 2000) at 25°C 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 ImageQuant TL v.7.0 image analysis software (GE Healthcare). The expression was normalized using anti-β-actin. Data were presented as a relative density to the naïve control group [34].

2.10. 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. Student's t test was used for comparison of the means for two groups. Statistical significance was regarded at p values < 0.05. All statistical data analyses were performed using SPSS version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows).

3. Results

3.1. Fingerprint Chromatogram and the Determination of Phenolic Compounds, Flavonoids, and Biological Activities. Figure 2 shows the fingerprint chromatogram of EMG and PMG whereas Table 1 shows the contents of phenolic compounds, flavonoids, and the important active ingredients in the combined extracts of mulberry and ginger (EMG) and phytosome containing the combined extracts of mulberry and ginger (PMG). Both EMG and PMG showed the similar chromatograms but differed in the concentration of the ingredients. It was found that EMG contained total phenolic and flavonoid contents at the concentrations of 243.00 ± 30.55 mg GAE/mg extract and 90.33 ± 15.40 μg quercetin/mg extract, respectively. It was found that each 50 mg of EMG contained gingerol, cyanidin-3-O-glucoside, quercetin-3-rutinoside, ferulic acid, and gallic acid at the concentrations of 9.45 ± 0.03 μg gingerol, 11.63 ± 0.06 μg Cyn-3-glu, 25.93 ± 0.17 μg Rutin, 12.89 ± 0.42 μg ferulic acid, and 11.26 ± 0.01 μg GAE. However, the contents of total phenolic, flavonoids, gingerol, cyanidin-3-O-glucoside, quercetin-3-rutinoside, ferulic acid, and gallic acid in PMG were 273.00 ± 5.77 mg GAE/mg extract, 137.00 ± 3.85 μg quercetin/mg extract, 19.23 ± 0.03 μg gingerol/50 mg extract, 16.01 ± 0.06 μg Cyn-3-glu/50 mg extract, 39.82 ± 0.40 μg Rutin/50 mg extract, 20.00 ± 0.18 μg ferulic acid/50 mg extract, and 47.89 ± 0.16 μg GAE/50 mg extract, respectively. These data clearly revealed that PMG contained all substance mentioned earlier higher than EMG (p value < 0.05, 0.001, 0.001, 0.01, 0.05, and 0.001) except total polyphenolic compound.

The biological activities associated with the pathophysiology of MetS such as antioxidant, antiobesity, and anti-inflammation were also assessed. The EC_{50} of an antioxidant

Table 1: Characterization of PMG including phenolic compositions and biological activities.

Parameters	Units	EMG	PMG	Standard reference
Total phenolic	mg GAE/mg extract	243.00 ± 30.55	273.00 ± 5.77	—
Total flavonoids	μg quercetin/mg extract	90.33 ± 15.40	137.00 ± 3.85*	—
Gingerol	μg gingerol/50 mg extract	9.45 ± 0.03	19.23±0.03***	—
Cyanidin-3-O-glucoside	μg Cyn-3-glu/50 mg extract	11.63 ± 0.06	16.01±0.06***	—
Quercetin-3-rutinoside	μg Rutin/50 mg extract	25.93 ± 0.17	39.82±0.40**	—
Ferulic acid	μg ferulic acid/50 mg extract	12.89 ± 0.42	20.00 ± 0.18*	—
Gallic acid	μg GAE/50 mg extract	11.26 ± 0.01	47.89±0.16***	—
Antioxidant activities				
DPPH	EC 50 (mg/ml)	39.97 ± 1.68	39.67 ± 0.93	0.03 ± 0.01, ascorbic acid
FRAP	EC 50 (mg/ml)	97.15 ± 3.17	41.28±0.37***	122.19 ± 12.82, ascorbic acid
ABTS	EC 50 (mg/ml)	58.46 ± 0.66	47.28 ± 1.89*	0.20 ± 0.002, trolox
Obesity marker				
Pancreatic lipase	EC 50 (mg/ml)	181.56 ± 17.87	138.15 ± 0.92*	0.002 ± 0.001, orlistat
Inflammatory marker				
COX-II	EC 50 (mg/ml)	81.25 ± 2.97	80.42 ± 1.85	0.02 ± 0.001, indomethacin

Data are presented as mean ± SEM. $^{*,**,***}p$ value < 0.05, 0.01, and 0.001, respectively, compared between EMG and PMG. EMG: the combined extracts of mulberry and ginger, PMG: phytosome containing the combined extracts of mulberry and ginger.

effect determined by DPPH assay of EMG and PMG were 39.97 ± 1.68 and 39.67 ± 0.93 mg/ml whereas EC_{50} which derived from FRAP were 97.15 ± 3.17 and 41.28 ± 0.37 and EC_{50} of both substances from ABTS assay were 58.46 ± 0.66 and 47.28 ± 1.89 mg/ml. The suppression activities of pancreatic lipase and COX-II were also determined. EC_{50} values of the suppression activity of pancreatic lipase of EMG and PMG were 181.56 ± 17.87 and 138.15 ± 0.92 mg/ml. It was found that the EC_{50} value of COX-II suppression effect of EMG and PMG was 81.25 ± 2.97 and 80.42 ± 1.85 mg/ml. The current data showed that the PMG exhibited more potent antioxidant activity via FRAP and ABTS and more potent activity of pancreatic lipase suppression activity than EMG (p value < 0.001, 0.01, and 0.01, respectively).

3.2. Antimetabolic Syndrome Effect Assessment. Table 2 showed the effect of PMG at various doses on metabolic parameters. HCHF diet significantly increased the percent of body weight gain, triglyceride, cholesterol, LDL-C, blood glucose, HOMA-IR, AI-index, and ACE activity but decreased HDL-C (p value < 0.05, 0.01, 0.001, 0.001, 0.01, 0.001, 0.01, 0.001, and 0.001, respectively; compared to naïve control). Vitamin C failed to show the positive modulation effects on the aforementioned parameters. Simvastatin and PMG at doses of 50 and 100 mg/kg BW significantly decreased the percent of body weight gain, triglyceride, cholesterol, LDL-C, AI-index, and ACE but increased HDL-C (p value < 0.01, 0.001, and 0.001; p value < 0.001 all; p value < 0.05, 0.01, and 0.01; p value < 0.001 all; p value < 0.01, 0.001, and 0.001; p value < 0.01, 0.001, and 0.001; p value < 0.05 all; compared to the HCHF diet-treated group). All doses of PMG could significantly improve HOMA-IR (p value < 0.05, 0.01, and 0.05, respectively; compared to HCHF diet-treated group). Interestingly, PMG at a dose of 200 mg/kg BW significantly decreased body weight gain, cholesterol, triglyceride, LDL-C, AI-index, plasma glucose, HOMA-IR, and ACE but increased HDL-C (p value < 0.001, 0.01, 0.001, 0.001, 0.001, 0.05, 0.05, 0.001, and 0.01, respectively; compared to HCHF diet-treated group).

3.3. Changes of Adipose Tissue. The effect of PMG on the weight of adipose tissue, size, and density of adipocyte together with adiposity index in both visceral and subcutaneous areas was determined, and data are shown in Figure 3 and Table 3. MetS rats showed the increase in weight of adipose tissue and size of adipocyte in both areas mentioned earlier (p value < 0.001 and 0.01; p value < 0.001 all; compared to the naïve control group). An adiposity index in the visceral area also increased (p value < 0.001; compared to the naïve control group). In addition, the density of adipocyte of MetS rats also decreased (p value < 0.001 all; compared to the naïve control group). Vitamin C failed to produce the significant changes of all parameters just mentioned in MetS rats. Simvastatin decreased the size but increased the density of adipocyte in the visceral area (p value < 0.05 and 0.01, respectively; compared to the HCHF-treated group). All doses of PMG significantly decreased the size of adipocyte in visceral and subcutaneous areas (p value $0 < 0.001$ all; p value $0 < 0.01$, 0.01, and 0.001, respectively; compared to the HCHF-treated group) but increased the density of adipocyte in the areas just mentioned (p value < 0.001 all; p value < 0.05, 0.001, and 0.001, respectively; compared to the HCHF-treated group). In addition, the decrease in

Table 2: The effect of various doses of PMG on metabolic parameters.

Parameters	ND+vehicle	HCHF+vehicle	HCHF+VitaminC	HCHF+Simvastatin	HCHF+PMG50	HCHF+PMG100	HCHF+PMG200
Body weight gain (%)	3.94 ± 0.44	6.71 ± 0.03^{a}	3.49 ± 1.01	2.33±0.75**	−5.47±0.55***	−7.89±0.45***	−6.42±1.18***
Cholesterol (mg/dl)	68.20 ± 3.32	100.20 ± 2.80^{aa}	85.20 ± 6.40	74.60 ± 4.95*	73.60±1.57**	69.20±2.03**	69.00±3.48**
Triglyceride (mg/dl)	72.80 ± 3.25	112.00 ± 1.41^{aaa}	101.00 ± 5.43	75.00±2.35***	75.80±3.80***	75.80±4.31***	73.60±2.54***
LDL-C (mg/dl)	28.20 ± 1.16	65.00 ± 3.08^{aaa}	58.80 ± 1.69	31.00±3.08***	33.00±1.58***	33.00±2.00***	32.40±1.63***
HDL-C (mg/dl)	59.40 ± 1.89	38.80 ± 1.53^{aa}	44.60 ± 1.72	54.60 ± 2.42*	53.80 ± 3.29*	54.60 ± 2.48*	54.80 ± 4.91*
Atherogenic index	1.16 ± 0.08	2.61 ± 0.16^{aaa}	1.94 ± 0.23	1.51±0.04**	1.05±0.06***	1.00±0.12***	1.04±0.08***
Plasma glucose AUC (mg h/dl)	107.17 ± 3.88	207.33 ± 25.98^{aa}	184.50 ± 5.72	201.17 ± 16.83	144.67 ± 5.20	137.17 ± 7.13	127.50 ± 5.59*
HOMA-IR	2.13 ± 0.09	3.36 ± 0.18^{aaa}	3.23 ± 0.19	3.01 ± 0.08	2.57 ± 0.08*	2.47±0.09**	2.57 ± 0.08*
ACE (units/mg protein)	0.03 ± 0.00	0.14 ± 0.00^{aaa}	0.10 ± 0.00	0.08 ± 0.00^{aa,**}	0.05±0.00***	0.05±0.01***	0.05±0.00***

Data are presented as mean ± SEM ($n = 6$/group). $^{a,\ aa,\ aaa}p$ value < 0.05, 0.01, and 0.001, respectively, compared to naïve control which received ND and vehicle and $^{*,**,***}p$ value < 0.05, 0.01, and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

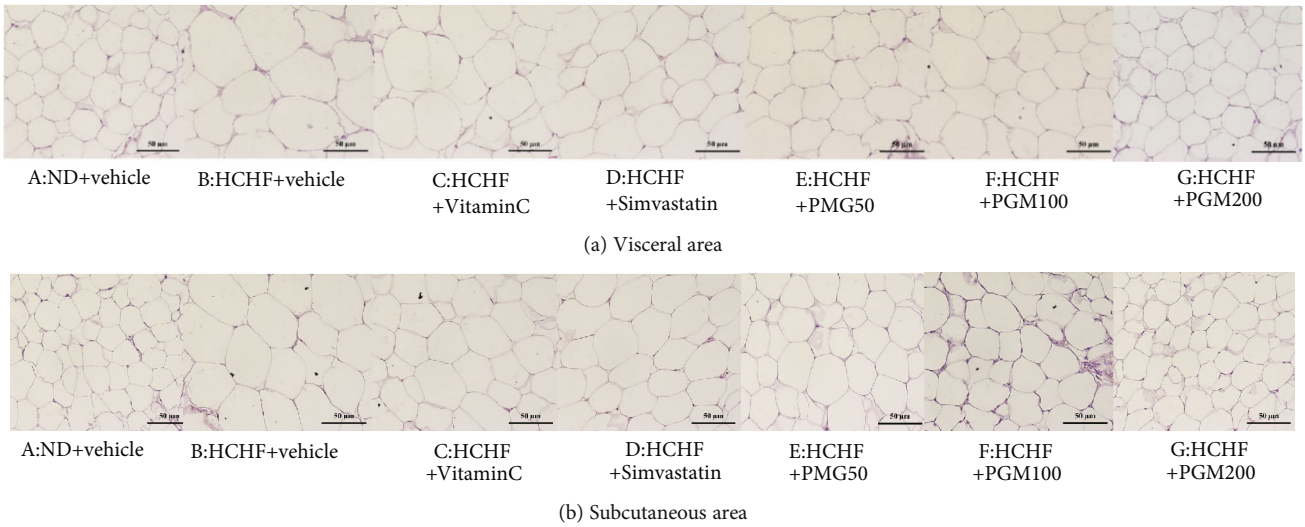

Figure 3: Light microscope of white adipose tissue was stained with hematoxylin and eosin (H&E) at 40x magnification. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

weight of adipose tissue and adiposity index in the visceral area was also observed in MetS rats which received PMG at doses of 100 and 200 mg/kg BW (p value < 0.001 all; compared to the HCHF-treated group).

3.4. Oxidative Stress Changes. The effect of PMG on oxidative stress markers in both visceral and subcutaneous areas together with oxidative stress markers in plasma is shown in Tables 4–6. HCHF diet significantly increased the MDA level in both visceral and subcutaneous areas of adipose tissue, and plasma of normal rats (p value < 0.001 all, compared to normal rats, which received normal diet and vehicle). Vitamin C and all doses of PMG significantly mitigated the elevation of the MDA level in both visceral and subcutaneous areas of adipose tissue, and plasma of metabolic syndrome rats, which fed with HCHF diet (p value < 0.001 all, compared to the HCHF-treated group). MetS rats induced by HCHF diet significantly decreased SOD activities in adipose tissue of both visceral and subcutaneous areas and in plasma (p value < 0.001 all; compared to naïve control). These changes were mitigated by vitamin C and PMG at doses of 50, 100, and 200 mg/kg in the visceral area (p value < 0.001, 0.001, 0.001, and 0.01, respectively, compared to the HCHF diet-treated group), subcutaneous area (p value < 0.05, 0.001, 0.001, and 0.001, respectively, compared to the HCHF diet-treated group), and plasma (p value < 0.001 all, compared to the HCHF diet-treated group).

In addition, metabolic syndrome rats induced by HCHF diet showed the significant decrease in CAT activity in adipose tissue of both visceral and subcutaneous areas and in plasma (p value < 0.001 all, compared to naïve control). However, vitamin C and PMG at doses of 100 and 200 mg/kg significantly increased CAT activity in the visceral area (p value < 0.01, 0.05, and 0.05, respectively, compared to HCHF diet-treated group). Moreover, vitamin C and PMG at all doses significantly increased CAT activity in the subcutaneous area (p value < 0.01 all, compared to the HCHF diet-treated group). Only PMG treatment can produce the significant increased CAT activity in serum (p value < 0.001 all, compared to the HCHF diet-treated group).

It was also found that HCHF diet produced the significant decrease in GSH-Px activity in adipose tissue of both visceral and subcutaneous areas and in plasma of normal rats (p value < 0.001 all, compared to naïve control). However, vitamin C and all doses of PMG could attenuate the change of this parameter in both visceral and subcutaneous areas (p value < 0.001 all, compared to the HCHF diet-treated group) and in plasma (p value < 0.05, 0.001, 0.001, and 0.001, respectively, compared to the HCHF diet-treated group).

3.5. Histone Deacetylase 3 (HDAC3) Expression Change. Effect of PMG on the expression of HDAC3 in adipose tissue was also determined, and results are shown in Figure 4. MetS rats which received HCHF significantly increased HDAC3 expression in adipose tissue (p value < 0.001, compared to naïve control). Simvastatin and PMG at a high dose significantly decreased HDAC3 expression in adipose tissue (p value < 0.001 all, compared to the HCHF diet-treated group).

3.6. Effect of PMG on PPAR-γ Expression. Figure 5 shows the effect of PMG on the expression of PPAR-γ in adipose tissue. MetS rats induced by HCHF diet significantly decreased PPAR-γ expression in adipose tissue (p value < 0.001, compared to naïve control). However, all interventions used in this study including vitamin C, simvastatin, and PMG at all doses significantly increased PPAR-γ expression in adipose

Table 3: The effect of various doses of PMG on adipose tissue alterations.

Parameters		ND+vehicle	HCHF+vehicle	HCHF+VitaminC	HCHF+Simvastatin	HCHF+PMG50	HCHF+PMG100	HCHF+PMG200
Size of adipocyte (μm)	Visceral area	46.10 ± 1.00	84.79 ± 1.31^{aaa}	81.46 ± 5.25	78.82 ± 1.56*	64.68±0.99***	63.30±1.53***	53.04±1.38***
	Subcutaneous area	33.37 ± 0.97	55.25 ± 1.50^{aaa}	51.29 ± 0.87	51.00 ± 1.24	48.50±1.43**	48.38±2.12**	43.20±1.90***
Density of adipocyte (adipocytes/225 μm^2)	Visceral area	62.33 ± 0.99	25.50 ± 0.43^{aaa}	26.33 ± 0.76	30.33±0.99**	37.67±0.42***	38.50±0.56***	47.32±0.61***
	Subcutaneous area	56.50 ± 1.45	30.33 ± 2.07^{aaa}	33.50 ± 1.80	32.33 ± 2.16	36.33 ± 1.74*	40.33±0.99***	43.67±4.72***
Adiposity index (AI) (%)	Visceral area	3.35 ± 0.21	5.85 ± 0.18^{aaa}	5.60 ± 0.18	5.06 ± 0.25	5.92 ± 0.09	3.49±0.43***	3.14±0.08***
	Subcutaneous area	3.42 ± 0.18	3.74 ± 0.15	3.54 ± 0.11	3.68 ± 0.09	3.60 ± 0.15	3.38 ± 0.12	3.16 ± 0.13
Weights of white adipose tissues (g/kg BW)	Visceral area	14.59 ± 0.84	32.29 ± 0.84^{aaa}	30.86 ± 1.09	30.87 ± 0.83	30.09 ± 0.70	16.92±0.86***	15.52±0.33***
	Subcutaneous area	14.91 ± 0.86	19.22 ± 1.00^{aa}	19.51 ± 0.56	18.79 ± 0.93	18.26 ± 0.56	16.36 ± 0.52*	15.62±0.50**

Data are presented as mean ± SEM (n = 6/group). $^{aa,\ aaa}p$ value < 0.01 and 0.001, respectively, compared to naïve control which received ND and vehicle and $^{*,**,***}p$ value < 0.05, 0.01 and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND; normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

Table 4: The effect of various doses of PMG on oxidative stress markers in the visceral area.

Treatment group	MDA (ng/mg protein)	SOD (units/mg protein)	CAT (units/mg protein)	GSH-Px (units/mg protein)
ND+vehicle	0.54 ± 0.05	16.52 ± 0.81	69.77 ± 3.98	17.67 ± 0.67
HCHF+vehicle	2.97 ± 0.15^{aaa}	4.11 ± 0.24^{aaa}	26.13 ± 1.66^{aaa}	4.37 ± 0.36^{aaa}
HCHF+VitaminC	1.14±0.14***	9.74±0.79***	48.52±4.37**	10.87±0.83***
HCHF+Simvastatin	2.46 ± 0.07*	5.93±0.49***	36.53 ± 2.45	5.13 ± 0.38
HCHF+PMG50	0.64±0.06***	9.94±0.49***	41.92 ± 2.34	10.57±0.83***
HCHF+PMG100	0.69±0.08***	9.21±0.57***	45.34 ± 2.50*	10.65±0.64***
HCHF+PMG200	0.51±0.03***	8.01±0.84**	45.40 ± 3.22*	10.77±1.27***

Data are presented as mean ± SEM (n = 6/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{*,**,***}p$ value < 0.05, 0.01, and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

Table 5: The effect of various doses of PMG on oxidative stress markers in the subcutaneous area.

Treatment group	MDA (ng/mg protein)	SOD (units/mg protein)	CAT (units/mg protein)	GSH-Px (units/mg protein)
ND+vehicle	0.34 ± 0.05	7.14 ± 0.23	77.91 ± 5.09	11.38 ± 0.37
HCHF+vehicle	1.70 ± 0.26^{aaa}	3.04 ± 0.22^{aaa}	40.11 ± 1.45^{aaa}	1.64 ± 0.19^{aaa}
HCHF+VitaminC	0.60±0.04***	5.94 ± 0.54*	63.59±3.51**	7.53±0.55***
HCHF+Simvastatin	1.31 ± 0.13	3.66 ± 0.43	46.07 ± 4.13	4.26 ± 0.34
HCHF+PMG50	0.40±0.02***	6.25±0.24***	62.35±5.41**	6.87±0.75***
HCHF+PMG100	0.36±0.04***	6.65±0.74***	64.95±3.72**	7.18±0.54***
HCHF+PMG200	0.39±0.02***	6.43±0.56***	63.38±4.54**	7.52±0.66***

Data are presented as mean ± SEM (n = 6/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{*,**,***}p$ value < 0.05, 0.01, and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

Table 6: The effect of various doses of PMG on oxidative stress markers in serum.

Treatment group	MDA (ng/mg protein)	SOD (units/mg protein)	CAT (units/mg protein)	GSH-Px (units/mg protein)
ND+vehicle	0.28 ± 0.04	7.14 ± 0.23	75.34 ± 2.51	10.14 ± 0.54
HCHF+vehicle	1.58 ± 0.23^{aaa}	1.25 ± 0.43^{aaa}	23.50 ± 6.85^{aaa}	1.48 ± 0.53^{aaa}
HCHF+VitaminC	0.49±0.02***	4.58±0.30***	55.72 ± 6.74	5.03 ± 0.42*
HCHF+Simvastatin	1.16 ± 0.12*	3.66 ± 0.43*	39.97 ± 4.30	3.79 ± 0.30
HCHF+PMG50	0.35±0.02***	6.26±0.24***	62.85±7.71***	7.79±0.46***
HCHF+PMG100	0.31±0.04***	6.66±0.74***	71.57±8.25***	7.93±1.14***
HCHF+PMG200	0.31±0.02***	6.43±0.56***	72.73±5.12***	7.59±0.31***

Data are presented as mean ± SEM (n = 6/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{*,***}p$ value < 0.05 and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

tissue (p value < 0.05, 0.01, 0.001, 0.001, and 0.001, respectively, compared to the HCHF diet-treated group).

3.7. Effect of PMG on Inflammatory Mediators. Figures 6 and 7 show that HCHF diet increased IL-6 and TNF-α in adipose tissue (p value < 0.001 all, compared to naïve control). However, the changes of IL-6 and TNF-α were mitigated by vitamin C, simvastatin, and all doses of PMG treatments (p value < 0.001 and 0.05; p value < 0.01 and 0.05; p value < 0.001 and 0.01; p value < 0.001 all; p value < 0.001 all; compared to the HCHF diet-treated group).

4. Discussion

The current study has clearly revealed that PMG significantly improves most biological activities which related to MetS.

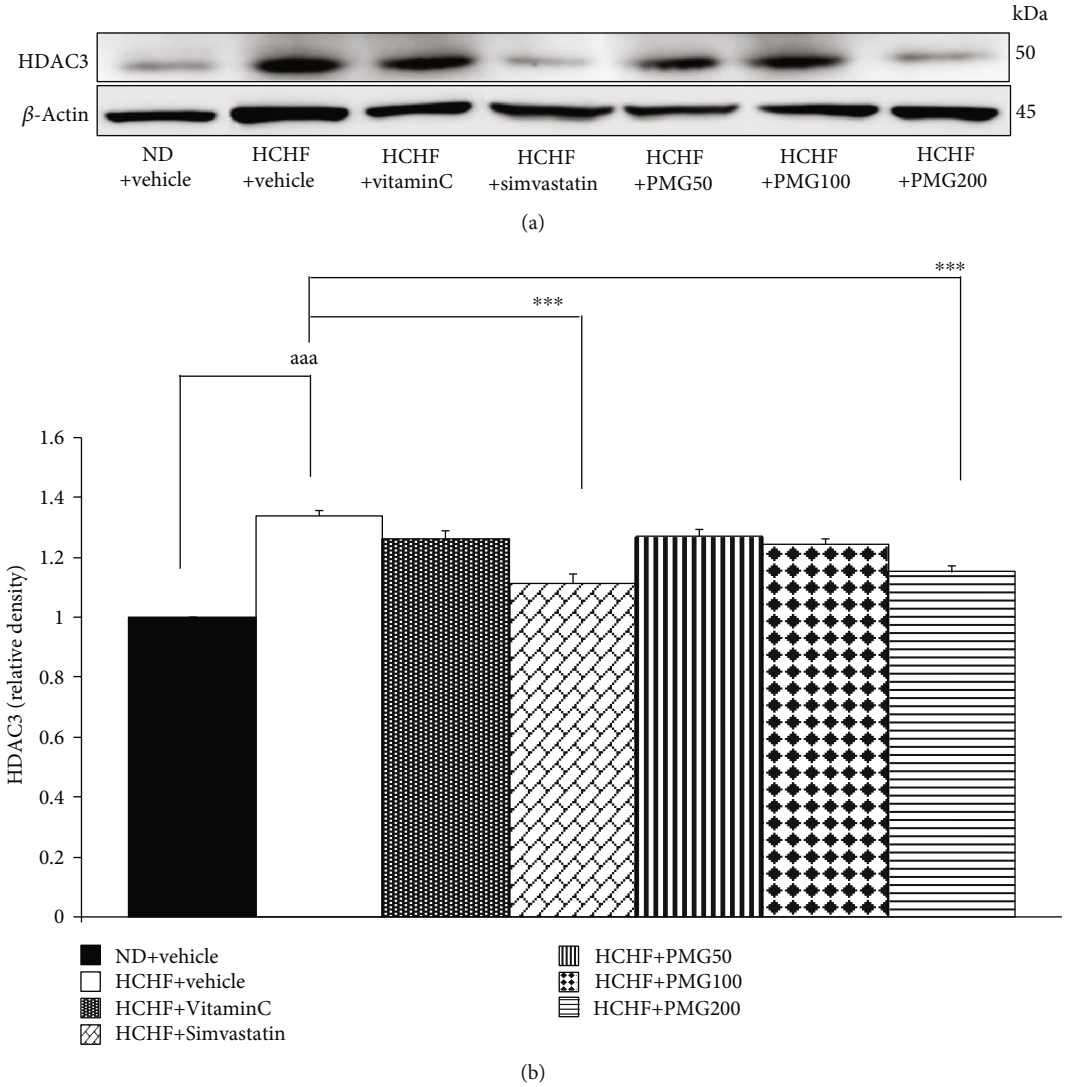

Figure 4: Effect of various doses of PMG on the expression of HDAC3 in adipose tissue. (a) Representative western blot showing the levels of HDAC3. (b) Relative density of HDAC3. Data are presented as mean ± SEM ($n = 6$/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{***}p$ value < 0.001, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

The possible explanation may be associated with the reduction of active ingredients lost due to the encapsulation technique. PMG also improves numerous changes in MetS including the symptoms of MetS, AI index, HDAC3, PPAR-γ, adipose tissue, oxidative stress, and inflammation.

Since PPAR-γ serves as the major regulator of adipogenesis, the changes of both PPAR-γ and adipose tissue are also investigated. The current results show that MetS rats induced by HCHF diet significantly decrease PPAR-γ expression in adipose tissue and density of adipocyte but increase the size of adipocyte. The reduction of adipocyte density observed in this study may be associated with the reduction of PPAR-γ in adipose tissue. This finding are in agreement with the study of He and coworkers which demonstrates that targeted deletion of PPAR-γ in fat tissue induces the marked reduction in the number of adipocytes [35]. The possible explanation for the reduction of adipocyte density may occur partly via the adipogenesis reduction in preadipocyte [36] and apoptosis of adipocyte cell [37]. In addition to the reduction of adipocyte density, the adipocyte hypertrophy is also observed. It has been shown that targeted deletion of PPAR-γ in fat tissue induces the marked reduction in the number of adipocytes, alongside a compensatory hypertrophy of the remaining cells [35]. Therefore, the adipocyte hypertrophy observed in this study may occur partly via the compensatory hypertrophy of the remaining cells mentioned earlier. In addition, it may also occur as the result of the lipid sequestration of adipose tissue and the accumulation of

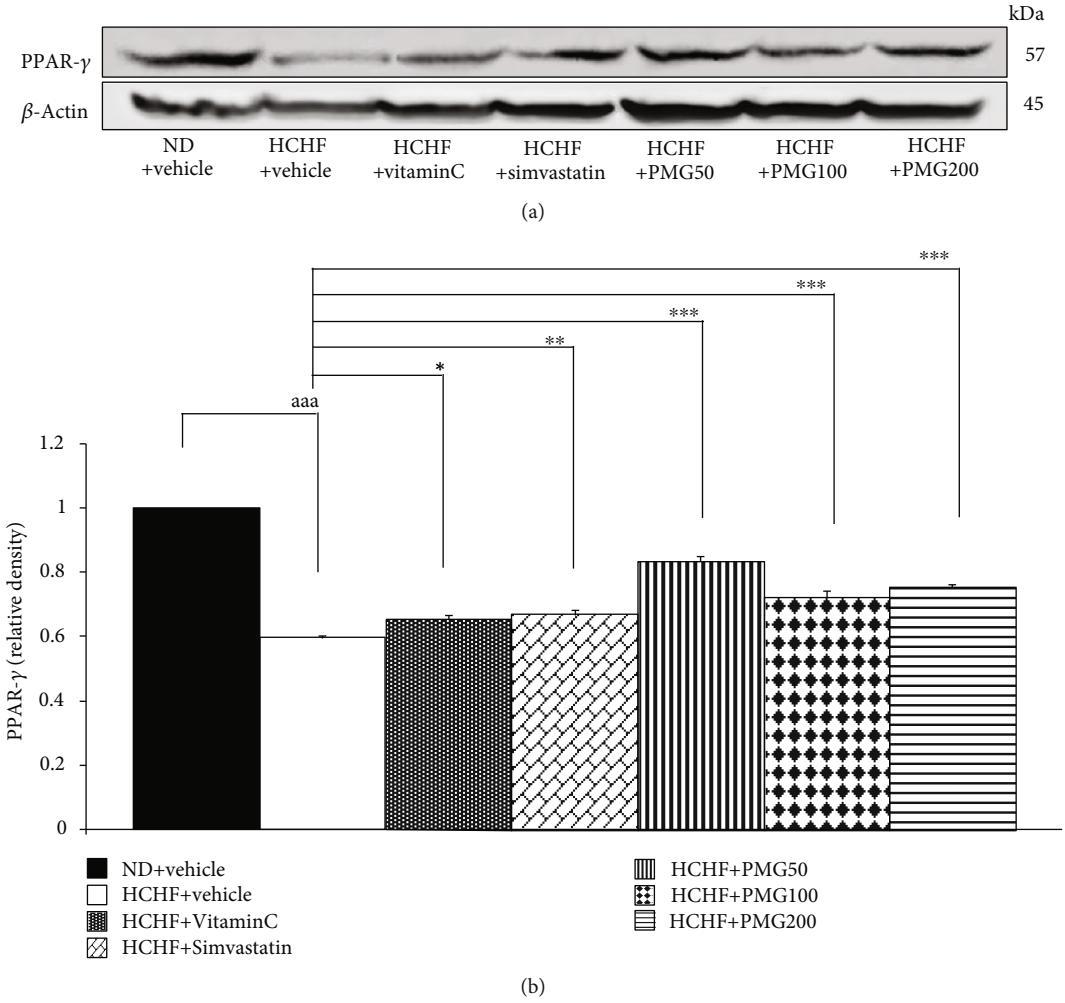

Figure 5: Effect of various doses of PMG on the expression of PPAR-γ in adipose tissue. (a) Representative western blot showing the levels of PPAR-γ. (b) Relative density of PPAR-γ. Data are presented as mean ± SEM ($n = 6$/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{*,**,***}p$ value < 0.05, 0.01, and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

triacylglyceride (TAG) in cytoplasmic lipid droplets (LDs) within adipocytes (fat cells) due to the caloric excess induced by MetS.

It has been revealed that adipocyte hypertrophy shows the positive correlation with insulin resistance (measured by HOMA-IR) and fasting plasma insulin in humans [38]. Therefore, insulin resistance and the elevation of plasma glucose in MetS rats may occur partly via adipocyte hypertrophy. However, PPAR-γ is also associated with gene regulating insulin sensitivity [39] and the regulation of glucose homeostasis via the decrease in gluconeogenesis and the increase in glycogen synthesis [40], so the reduction of PPAR-γ in adipose tissue may also play a role on both insulin resistance and the elevation of plasma glucose mentioned earlier. In addition, aforementioned effects, PPAR-γ also plays a role on lipid metabolism by decreasing triglyceride (TG) but increasing a high density of lipoprotein (HDL) [41]. Moreover, dyslipidemia observed in MetS also occurs as the results of adipocyte hypertrophy. Hypertrophic adipocyte induces the impairment of the process which incorporates FFAs into TGs leading to the decrease in FFA trapping and retention by adipose tissue which in turn gives rise to the elevation of FFA in plasma. This process that stimulates the triglyceride synthesis in the liver gives rise to the increase in VLDL and LDL but decreases HDL [42]. The dyslipidemia in MetS rats may also increase atherogenic index.

Under normal circumstance, PPAR-γ can also reverse macrophage infiltration in adipocyte and subsequently reduces inflammatory gene expression [43]. Therefore, the increase in the expressions of inflammatory cytokine such as IL-6 and TNF-α in adipose tissue in MetS rats may partly due to the decrease in PPAR-γ. PPAR-γ involves not only inflammation but also oxidative stress. PPARs also possesses anti-inflammatory and antioxidant properties by decreasing

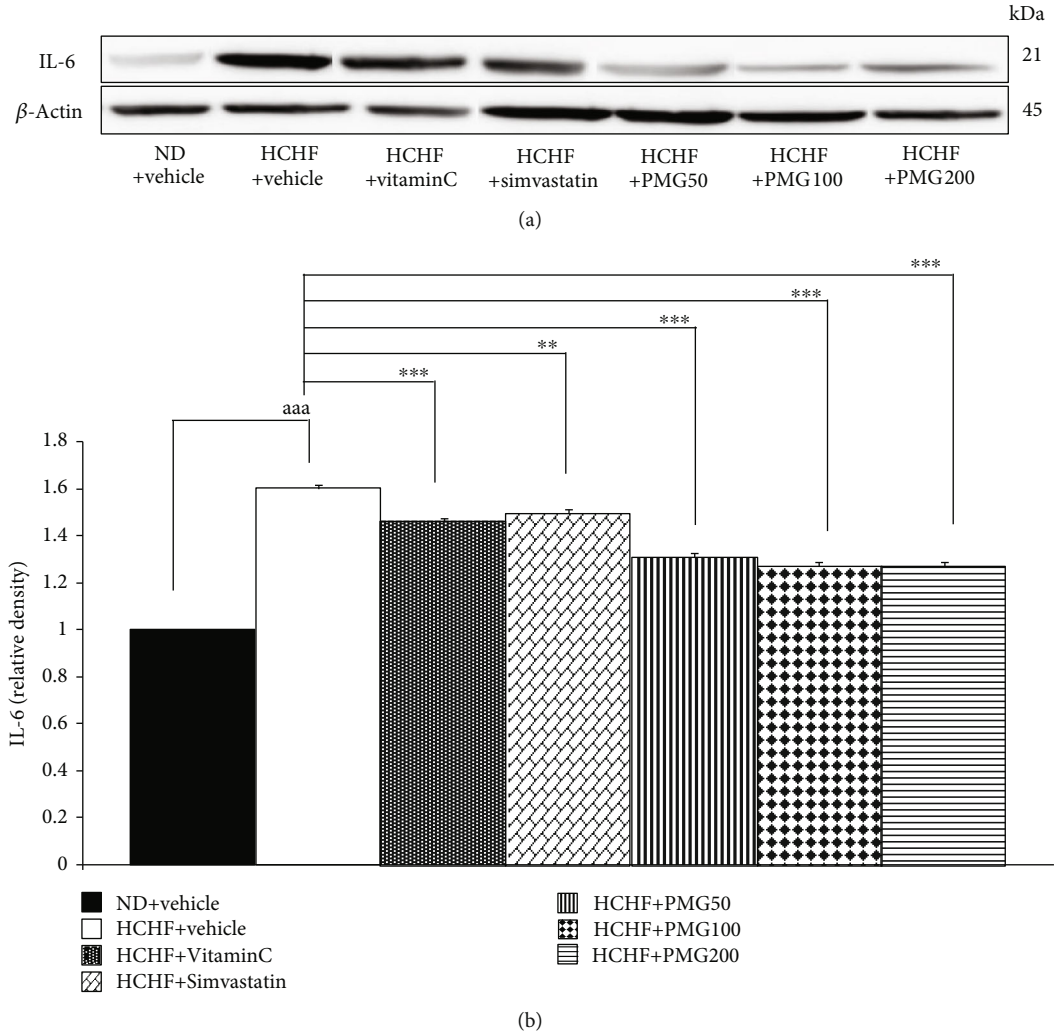

Figure 6: Effect of various doses of PMG on the expression of IL-6 in adipose tissue. (a) Representative western blot showing the levels of IL-6. (b) Relative density of IL-6. Data are presented as mean ± SEM ($n = 6$/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{**,***}p$ value < 0.01 and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

ROS production and upregulating the expression of antioxidant enzymes [44]. The current data also show the corresponding changes of oxidative stress markers. MetS rats showed the reduction of antioxidant enzymes such as SOD, CAT, and GSH-Px but increased the MDA level in adipose tissue from visceral and subcutaneous areas. The changes of oxidative stress markers just mentioned in serum also showed the same pattern.

MetS rats also demonstrate the high activity of angiotensin-converting enzyme (ACE) which indicates the stimulation of renin angiotensin system (RAS), a system playing an important role on the regulation of blood pressure [45]. The possible explanation for the stimulation of RAS resulting in the elevation of ACE may be associated with the secretion of aldosterone from adipocyte [46]. Therefore, the increased adipose tissue mass observed in MetS may possibly increase aldosterone which in turn stimulate the function of RAS resulting the increase ACE activity and finally increase angiotensin II resulting in hypertension.

Recent study has demonstrated that PPAR-γ function is regulated epigenetic modifications such as histone modification. The suppression of histone deacetylase 3 (HDAC3) can activate PPAR-γ expression [47]. Therefore, this information points out that the increase in HDAC3 in MetS rats may contribute the role on the reduction in PPAR-γ expression in adipose tissue especially at a high dose of the phytosome containing the combined extract of mulberry and ginger. This change in turn induces the increases in oxidative stress status, inflammation, dyslipidemia, insulin resistance, and hyperglycemia. In addition, it is also responsible for the increase adiposity, hypertrophic adipocyte. However, the current results show that HDAC3 failed to show the closed

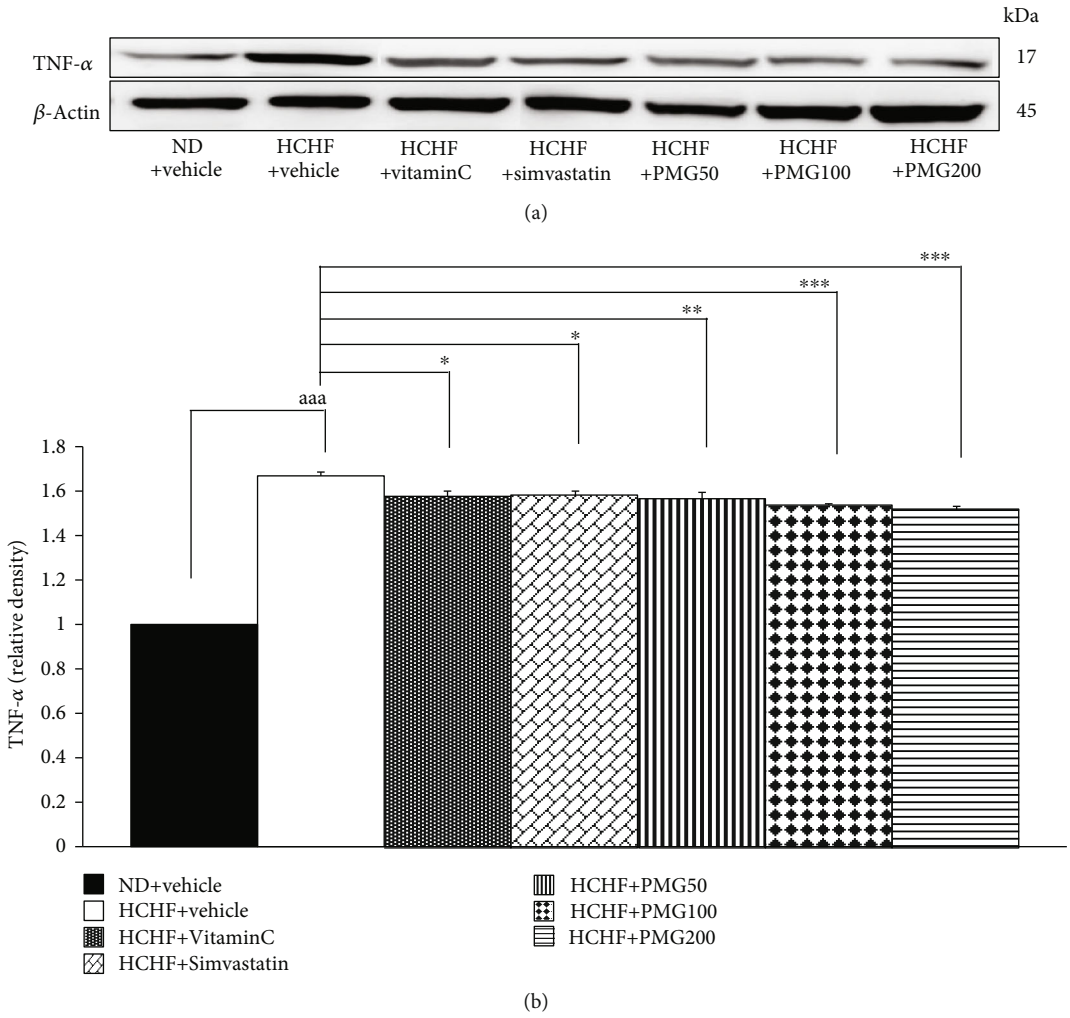

Figure 7: Effect of various doses of PMG on the expression of TNF-α in adipose tissue. (a) Representative western blot showing the levels of TNF-α. (b) Relative density of TNF-α. Data are presented as mean ± SEM ($n = 6$/group). ^{aaa}p value < 0.001, compared to naïve control which received ND and vehicle and $^{*,**,***}p$ value < 0.05, 0.01, and 0.001, respectively, compared to metabolic syndrome rats which received HCHF and vehicle. ND: normal diet; HCHF: high-carbohydrate high-fat diet; VitaminC: vitamin C at a dose of 250 mg·kg^{-1} BW; Simvastatin: simvastatin at a dose of 1.3 mg·kg^{-1} BW; PMG50, PMG100, and PMG200: the phytosome containing the combined extracts of mulberry and ginger at a dose of 50, 100, and 200 mg·kg^{-1} BW, respectively.

relationship with PPAR-γ. Therefore, other factors such as other modification processes of histone, DNA, and other factors influencing the transcription and translation processes may also play the role on the regulation of PPAR-γ.

However, the aforementioned changes can be improved by vitamin C, simvastatin, and all doses of phytosome containing the combined extract of mulberry and ginger. The possible underlying mechanism may occur partly via the increase in PPAR-γ expression in adipose tissue which in turn improves oxidative stress status, inflammation, dyslipidemia, insulin resistance, and hyperglycemia together with the reduction of adipose tissue in MetS rats. Vitamin C also exerts the positive modulation partly via the increase in PPAR-γ expression in adipose tissue. However, other mechanisms such as the alterations in adipokines may also contribute the role and required further investigation. Based on the activation effect of anthocyanins on PPAR-γ [48, 49], we suggest that the positive modulation effect of phytosome containing the combined extract of mulberry and ginger may be associated with anthocyanins [8] and gingerol [50].

5. Conclusion

Our study has clearly demonstrated that phytosome containing the combined extract of mulberry and ginger can improve MetS. The possible underlying mechanism occurs via the multipathway including the increase in PPAR-γ which in turn decreases adipose tissue, dyslipidemia, insulin resistance, inflammation, and oxidative stress. The epigenetic modification also plays the role especially at a high dose of the phytosome containing the combined extract of mulberry and ginger as shown in Figure 8. Therefore, this product can be served as the potential supplement to manage MetS.

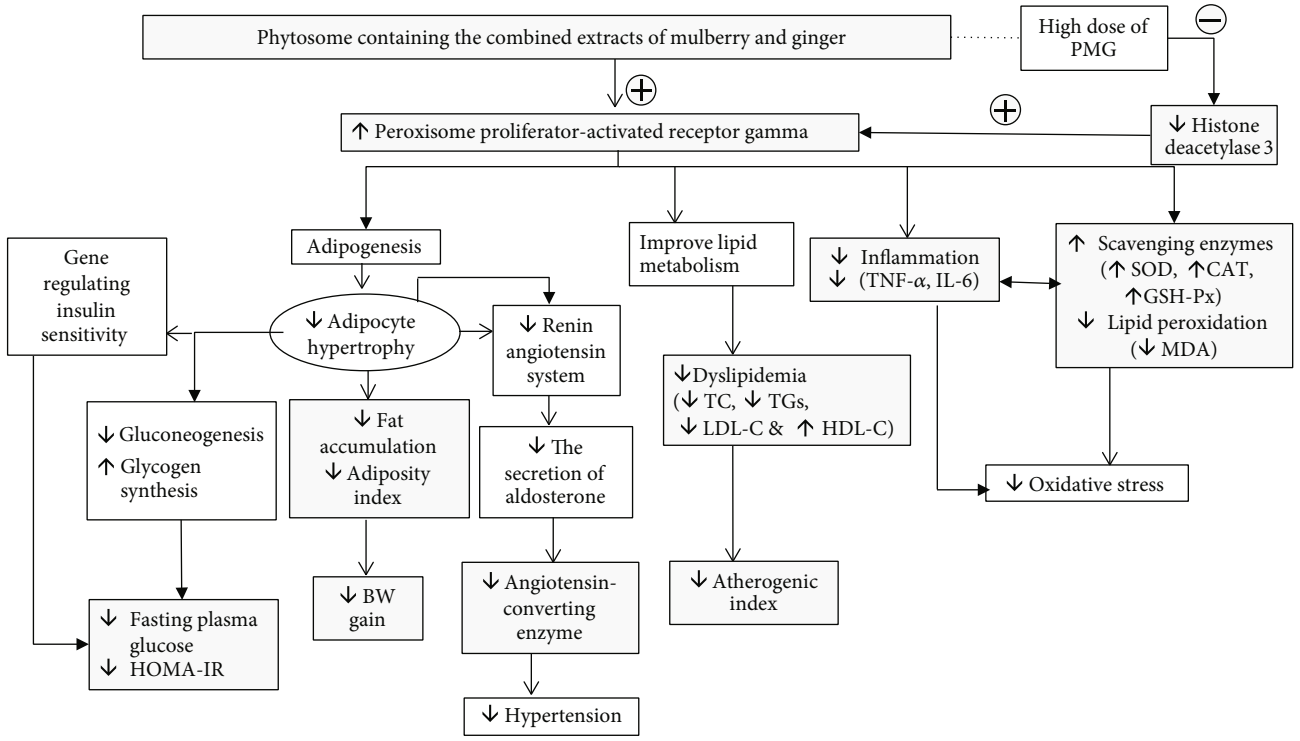

Figure 8: The schematic diagram demonstrated the positive modulation effect of PMG in the animal model of metabolic syndrome. HOMA-IR: homeostasis model assessment of insulin resistance; TC: total cholesterol; TGs: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; BW: body weight; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase; MDA: malondialdehyde.

However, a clinical trial study is essential to confirm this health benefit.

Data Availability

I confirm that data are available and will be provided on request because during this period, all data are in the process of petty patent registration.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Review Article

Environmental Noise-Induced Effects on Stress Hormones, Oxidative Stress, and Vascular Dysfunction: Key Factors in the Relationship between Cerebrocardiovascular and Psychological Disorders

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The role of noise as an environmental pollutant and its adverse effects on health are being increasingly recognized. Beyond its direct effects on the auditory system (e.g., hearing loss and tinnitus induced by exposure to high levels of noise), chronic low-level noise exposure causes mental stress associated with known cardiovascular complications. According to recent estimates of the World Health Organization, exposure to traffic noise is responsible for a loss of more than 1.5 million healthy life years per year in Western Europe alone, a major part being related to annoyance, cognitive impairment, and sleep disturbance. Underlying mechanisms of noise-induced mental stress are centered on increased stress hormone levels, blood pressure, and heart rate, which in turn favor the development of cerebrocardiovascular disease such as stroke, arterial hypertension, ischemic heart disease, and myocardial infarction. Furthermore, traffic noise exposure is also associated with mental health symptoms and psychological disorders such as depression and anxiety, which further increase maladaptive coping mechanisms (e.g., alcohol and tobacco use). From a molecular point of view, experimental studies suggest that traffic noise exposure can increase stress hormone levels, thereby triggering inflammatory and oxidative stress pathways by activation of the nicotinamide adenine dinucleotide phosphate oxidase, uncoupling of endothelial/neuronal nitric oxide synthase inducing endothelial and neuronal dysfunction. This review elucidates the mechanisms underlying the relationship between noise exposure and cerebrocardiovascular and psychological disorders, focusing on mental stress signaling pathways including activation of the autonomous nervous system and endocrine signaling and its association with inflammation, oxidative stress, and vascular dysfunction.

1. Introduction

Already in the beginning of the 20th century, the Nobel prize-winning bacteriologist Robert Koch predicted that "One day man will have to fight noise as fiercely as cholera and the plague". Indeed, of the most recent WHO Environmental Noise Guidelines for the European Region provides substantial evidence that links environmental noise exposure to adverse health outcomes [1]. According to estimations of the World Health Organization (WHO), exposure to traffic-related noise accounts for a yearly loss of more than 1.5 million years of healthy life in Western Europe with 61,000 years for ischemic heart disease, 45,000 years for cognitive impairment of children, 903,000 years for sleep disturbance, 22,000 years for tinnitus, and 654,000 years for annoyance [2]. According to conservative estimates for the European Region, exposure to noise from road traffic, railway, and aircraft leads to annoyance among 53 million and to sleep disturbance among 34 million adults, resulting each year in nearly 1.7 million additional prevalent cases of hypertension, 80,000 additional

cases of hospital admissions, and to 18,000 cases of premature mortality due to ischemic heart disease and stroke [3]. A large body of epidemiological and experimental studies demonstrated that exposure to traffic noise is associated with increased risk of cerebrocardiovascular disease such as stroke, arterial hypertension, ischemic heart disease, and myocardial infarction [4, 5]. A recent meta-analysis conducted on behalf of the WHO suggested a relative risk (RR) for the incidence of ischemic heart disease of 1.08 (95% confidence interval (CI): 1.01–1.15) per 10 decibel (dB) increase in road traffic noise exposure above 50 dB based on high-quality longitudinal studies [1]. Furthermore, environmental noise exposure has been established as a phenomenon causing annoyance and mental stress reactions, resulting in sympathetic and endocrine stress reactions (i.e., increased stress hormone levels) and psychological disorders such as depression and anxiety, all of which further impair cerebrocardiovascular function [6]. Importantly, chronic noise stress may generate cerebrocardiovascular risk factors on its own by influencing hemodynamics, hemostasis, oxidative stress, inflammation, vascular function, and autonomic tone, subsequently leading to manifest cerebrocardiovascular disease [7]. The present review discusses the role of environmental noise exposure in developing cerebrocardiovascular and psychological disorders as well as their bidirectional relationship focusing on sympathetic and endocrine stress responses with subsequent onset of inflammation, oxidative stress, and vascular endothelial dysfunction.

2. Noise Reaction Model

According to Babisch's noise reaction model, the "indirect/-nonauditory pathway" (compared to the "direct/auditory pathway," which describes effects on the auditory system by exposure to high levels of noise such as hearing loss and tinnitus) is crucial in determining adverse systemic health effects (Figure 1) [7, 8]. In this setting, low-level noise exposure interferes with communication, disturbs daily activities, and disrupts sleep, leading to sympathetic and endocrine activation and a number of cognitive and emotional reactions, including annoyance, depression, and mental stress. If the exposure persists over a period of time, the cognitive and emotional state of stress could then cause a pathophysiological cascade, resulting in increased stress hormone levels, blood pressure, and heart rate, which in turn favors the development of cerebrocardiovascular risk factors such as hypertension, arrhythmia, dyslipidemia, increased blood viscosity and blood glucose, and activation of blood clotting factors and the subsequent manifestation of cerebrocardiovascular disease such as stroke, ischemic heart disease, acute myocardial infarction, heart failure, and arterial hypertension [5, 6]. Of note, even short-term nocturnal aircraft noise exposure has been shown to be associated with takotsubo cardiomyopathy, a condition triggered by emotional stress and excessive stress hormone release (also known as broken-heart syndrome) [9]. Noise-induced annoyance has been proposed to play an intermediary role in disease development, i.e., the degree to which noise causes interference, annoyance, and mental stress may mediate the pathophysiological consequences and disease risk [10, 11]. Accordingly, traffic noise annoyance was shown to be an effect modifier or to be directly related to ischemic heart disease, hypertension, atrial fibrillation, myocardial infarction, stroke, and symptoms of cardiovascular disease [10–18].

Additionally, considering that noise annoyance represents mental stress, it has been shown to be associated with psychological symptoms and disorders such as depression and anxiety, with the important notion that different noise sources may induce different levels of annoyance (Figure 2) [19, 20]. Importantly, chronic mental stress per se is a well-established independent risk factor for both cerebrocardiovascular and psychological disorders, while these conditions are known to negatively affect each other in a bidirectional way [21, 22]. Given this framework, chronic noise annoyance/stress may impair adaptation and increase stress vulnerability, leading to decreased stress resistance in order to cope with the stressor [15]. Instead, as a consequence of stress and onset of psychological disorders, noise exposure may promote maladaptive coping mechanisms in the manner of life style risk factors, as shown by recent studies indicating that traffic noise was related to physical inactivity, smoking, and alcohol consumption [23–25]. Thus, besides the direct adverse cerebrocardiovascular effects of noise, an indirect pathway of adverse noise effects can be assumed causing cerebrocardiovascular disease via causing psychological disorder such as depression and anxiety.

3. Epidemiological Evidence for Health Effects of Noise

There is extensive epidemiological evidence for the significant relationship between environmental noise exposures, in particular for exposure to noise from road traffic, railway, and aircraft, and cerebrocardiovascular as well as psychological endpoints. In the following, an overview of studies for these endpoints shall be given. This review is based on a selective search of publications in PubMed from 2005 to 2019 with focus on systematic reviews, meta-analyses, and primary studies. We used the search terms: "noise exposure" in combination with "cardiovascular disease," "cardiovascular," "psychological," "psychiatric," "depression," and "anxiety," following an initial rapid review and selection of the articles based on the authors' expertise. Clear descriptions of study population characteristics, adjustment for common confounders, description of inclusion and exclusion criteria, and robust methodology were at least required.

3.1. Ischemic Heart Disease. As mentioned, the most recent meta-analysis from 2018 by Kempen et al. found a RR of 1.08 (95% CI: 1.01–1.15) for the incidence of ischemic heart disease per 10 dB increase in road traffic noise exposure for the relationship of road traffic noise, starting as low as 50 dB [1]. Similar findings were obtained by two other meta-analyses by Vienneau et al. and by Babisch, reporting a RR of 1.06 (95% CI: 1.03–1.09) and 1.08 (95% CI: 1.04–1.13), respectively, per 10 dB/dB(A) (A-weighted decibel) increase in aircraft and/or road traffic noise exposure [26, 27]. A large population-based study from Canada showed that an increase in traffic-related noise levels per

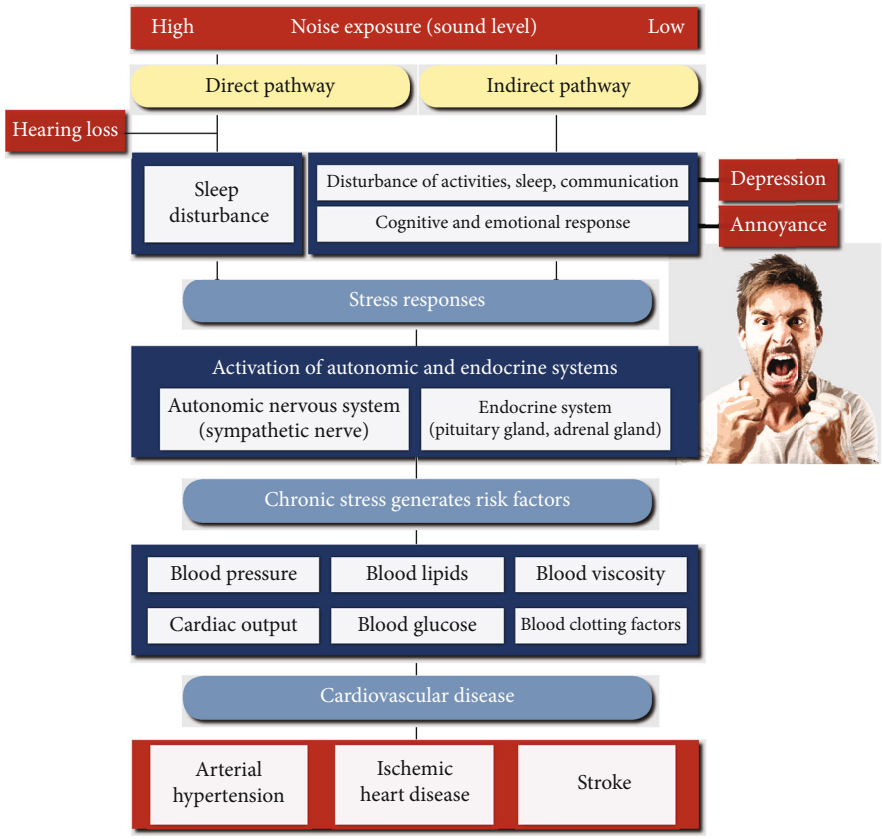

Figure 1: Proposed expanded noise reaction model characterizing the adverse health effects of environmental noise exposure. The direct pathway refers to effects on the auditory system by exposure to high levels of noise (e.g., hearing loss and tinnitus). The indirect pathway is associated with cognitive and emotional stress responses, leading to sympathetic and endocrine activation triggering alterations in cerebrocardiovascular risk factors. Additionally, chronic noise stress is associated with increased risk for psychological symptoms and disorders, which in turn impairs cerebrocardiovascular function. As a consequence, noise exposure may promote maladaptive coping mechanisms and decrease stress resistance, further negatively affecting cerebrocardiovascular function. Adapted from Babisch [7, 8] and Münzel et al. [5] with permission.

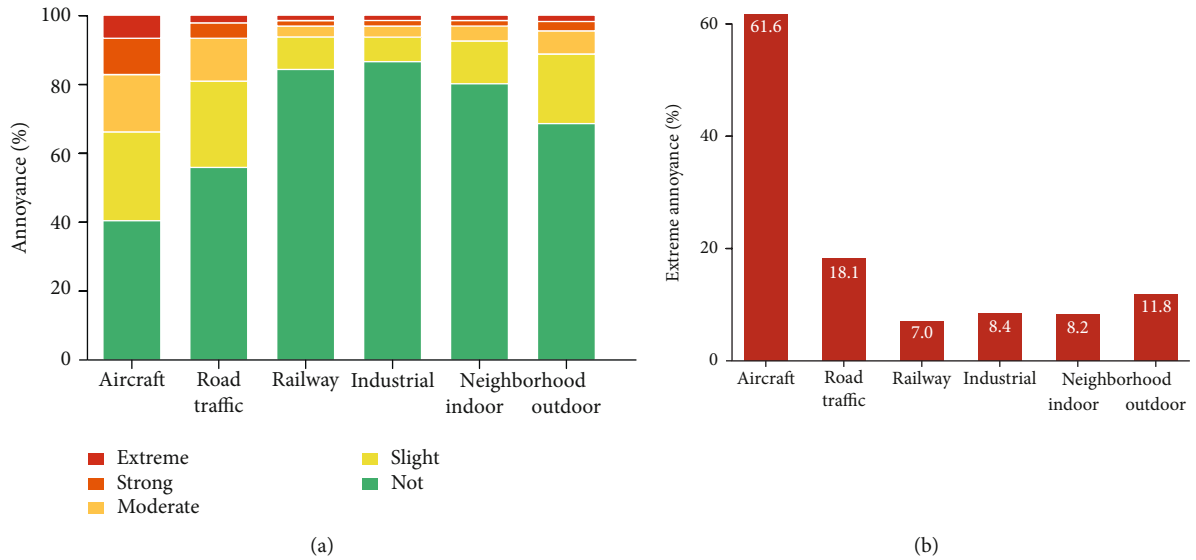

Figure 2: (a) Degrees of overall annoyance according to different sources of noise. (b) Sources of extreme annoyance. Adapted from Beutel et al. [19] with permission.

10 dB(A) was associated with a 9% (RR: 1.09, 95% CI: 1.01–1.18) higher risk of death from ischemic heart disease [28].

3.2. Hypertension. A meta-analysis of 24 cross-sectional studies by van Kempen and Babisch found a 3.4% (odds ratio (OR): 1.034, 95% CI: 1.011–1.056) higher probability of prevalent hypertension per increase of 5 dB(A) in road traffic noise [29]. However, prospective studies have also indicated an association between aircraft noise exposure and incident hypertension, thereby supporting the cross-sectional findings [30, 31]. The more recent study from 2017, based on data from the large Hypertension and Exposure to Noise near Airports (HYENA) study from 6 European countries, found that an increase in nocturnal aircraft noise exposure per 10 dB was associated with an OR of incident hypertension of 2.63 (95% CI: 1.21–5.71) [31]. Findings of a Taiwanese study suggested an OR of 2.15 (95% CI: 1.08–4.26) for prevalent hypertension in subjects exposed to high levels of road traffic noise (82.2 vs. 77.2 dB(A)) [32].

3.3. Myocardial Infarction. Three Scandinavian studies have reported on the association between road traffic noise exposure and myocardial infarction [33–35]. In a Swedish case-control study, an OR of 1.38 (95% CI: 1.11–1.71) was found for noise levels of <50 vs. ≥50 dB(A) after exclusion of subjects with hearing loss or exposure to noise from other sources [33]. Similar results were obtained in two large Danish cohorts, indicating a hazard ratio (HR) of 1.12 (95% CI: 1.03–1.21) per interquartile range increase in noise levels and an incidence rate ratio (IRR) of 1.12 (95% CI: 1.02–1.22) per increase of 10 dB, respectively [34, 35]. Concerning risk of death from cardiac causes, a nationwide study from Switzerland including more than 4 million individuals analyzed the association between traffic noise exposure and mortality due to myocardial infarction [36]. The results indicated a 3.8% (HR: 1.038, 95% CI: 1.019–1.058) higher risk for road traffic noise, a 2.6% (HR: 1.026, 95% CI: 1.004–1.048) higher risk for aircraft noise, and a 1.8% (HR: 1.018, 95% CI: 1.004–1.031) higher risk for railway noise per 10 dB increase in noise levels. In a Canadian study of nearly 28,000 lumber mill workers, an increased risk of fatal myocardial infarction in relation to occupational noise exposure was observed (RR: 1.05, 95% CI: 1.1–2.2 for ≥115 vs. <100 dB(A)) [37]. A German case-control study, which was based on secondary data of the Noise-Related Annoyance, Cognition, and Health (NORAH) project, using data of more than 1 million individuals living in the Rhine-Main region, showed that road traffic and railway noise exposure increased risk of myocardial infarction by 2.8% (OR: 1.028, 95% CI: 1.012–1.045) and by 2.3% (OR: 1.023, 95% CI: 1.005–1.042) per 10 dB increase, respectively, whereas the association was weaker for aircraft noise (OR: 0.993, 95% CI: 0.966–1.020) [38].

3.4. Stroke. Sorensen et al. found road traffic noise to increase the risk of hospitalization due to incident stroke by 14% (IRR: 1.14, 95% CI: 1.03–1.25) [39]. A large population-based study including 3.6 million individuals living around Heathrow airport in London showed that aircraft noise exposure during the day as well as at night was associated with increased risk of hospitalization due to incident stroke [40]. A RR of 1.29 (95% CI: 1.14–1.46 for >55 vs. ≤50 dB) was determined for nocturnal aircraft noise exposure, whereas a RR of 1.24 (95% CI: 1.08–1.43 for >63 vs. ≤51 dB) was estimated for daytime exposure. Comparable results were reported for stroke-related mortality in this study. A further study, likewise conducted in London including 8.6 million individuals living around, revealed road traffic noise exposure to be associated with an elevated risk of stroke-related hospitalization during the day (RR: 1.05, 95% CI: 1.02–1.09 for >60 vs. <55 dB in individuals aged ≥25 to 74 years) and at night (RR: 1.05, 95% CI: 1.01–1.09 for 55-60 vs. <55 dB in individuals aged ≥75 years) [41]. Results from a NORAH case-control study revealed that stroke risk was increased by 1.7% (OR: 1.017, 95% CI: 1.003–1.032) for road traffic noise and by 1.8% (OR: 1.018, 95% CI: 1.001–1.034) for railway noise per 10 dB increase [42]. This association was weaker in case of aircraft noise (OR: 0.976, 95% CI: 0.953–1.000).

3.5. Other Endpoints. Another NORAH-based case-control study examined the association between exposure to traffic noise and risk of heart failure or hypertensive heart disease [43]. The study showed that increased levels of road traffic (OR: 1.024, 95% CI: 1.016–1.032), railway (OR: 1.031, 95% CI: 1.022–1.041), and aircraft noise (OR: 1.016, 95% CI: 1.003–1.030) were found to be associated with increased risk (per 10 dB increase).

Based on cross-sectional data from the large, population-based Gutenberg Health Study (GHS) from Germany, Hahad et al. demonstrated that annoyance to different noise sources during the day and at night, including aircraft (OR: 1.09, 95% CI: 1.05–1.13), road traffic (OR: 1.15, 95% CI: 1.08–1.22), and railway annoyance (OR: 1.13, 95% CI: 1.04–1.22, at night and per point increase in annoyance), was dose-dependently related to a higher probability of prevalent atrial fibrillation (Figure 3(a)) [15].

A Taiwanese study analyzed the association between road traffic noise exposure and physician-diagnosed cardiovascular disease [44]. An elevated OR of 2.23 (95% CI: 1.26–3.93) for prevalent cardiovascular disease per 5 dB(A) increase in noise levels was found.

A nationwide study including 6 million older people (aged ≥65 years) residing near 89 airports in the United States showed that aircraft noise exposure per 10 dB increase was associated with a 3.5% (95% CI: 0.2–7.0) higher cardiovascular hospital admission rate comprising stroke, ischemic heart disease, heart failure, arrhythmia, and peripheral vascular disease [45].

Results of the National Health and Nutrition Examination Survey (NHANES) from the United States revealed that occupational noise exposure was associated with increased risk of angina pectoris (OR: 2.91, 95% CI: 1.35–6.26 for never exposed vs. current exposed) [46].

The relationship between road traffic noise and incident atrial fibrillation was also evaluated in a Danish prospective study, showing that an increase in noise levels per 10 dB was associated with a 6% (IRR: 1.06, 95% CI: 1.00–1.12) higher risk; however, this relationship was not independent of exposure to air pollution [47]. Since the growing demand

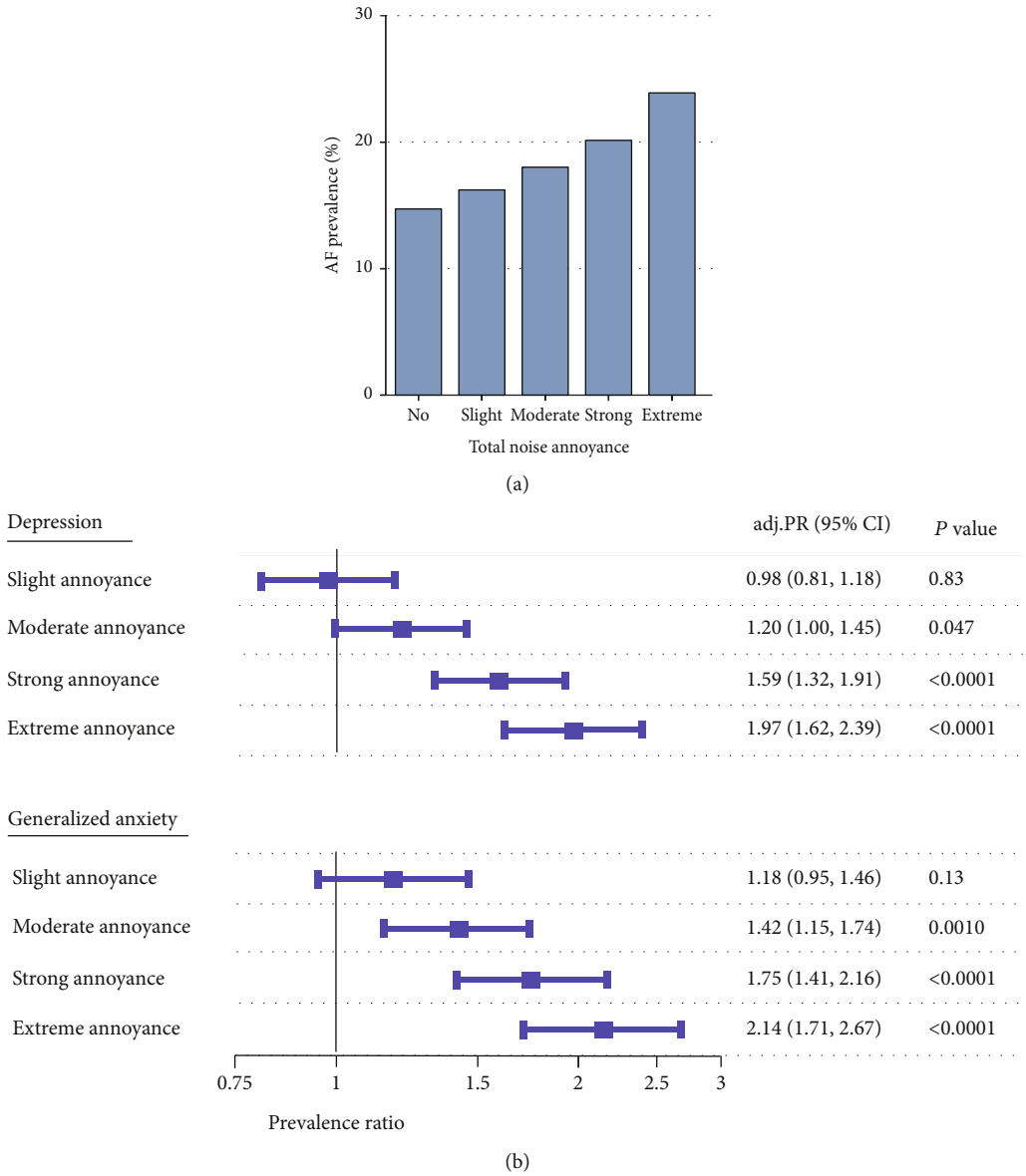

Figure 3: Associations between total noise annoyance, (a) atrial fibrillation, (b) depression, and generalized anxiety in the Gutenberg Health Study. AF: atrial fibrillation; adj. PR: adjusted prevalence ratio for sex, age, and socioeconomic status; CI: confidence interval. Adapted from (a) Hahad et al. [15] and (b) Beutel et al. [19] with permission.

for mobility is not only related to noise but also air pollution, it is important to differentiate between these two exposures in terms of evaluating health effects. However, a range of epidemiologic evidence supports the concept that both noise and air pollution independently contribute to disease development, while they also may interact with each other having additive negative effects [48, 49]. Importantly, exposure to air pollution was also shown to be independently associated with cerebrocardiovascular (e.g., stroke, ischemic heart disease, and hypertension) and psychological/psychotic disorders (e.g., depression, anxiety, and schizophrenia) and was found to share common pathophysiological pathways with noise exposure [50–53].

Furthermore, several studies have indicated substantial associations between traffic noise exposure and metabolic abnormalities, showing that higher exposure is associated with increased risk of obesity and diabetes mellitus [54–57], both well-established risk factors for cerebrocardiovascular [58, 59] and psychological disorders [60, 61].

3.6. Psychological Symptoms and Disorders. Another cross-sectional analysis based on data from the GHS including 15,010 subjects could demonstrate that depression and generalized anxiety disorder increased dose-dependently with the degree of total noise annoyance (highest annoyance rating of all analyzed categories of noise including aircraft, road traffic, and railway noise), even after adjustment for sex, age, and socioeconomic status [19]. Compared to no annoyance, the prevalence ratio (PR) for depression and generalized anxiety disorder increased steadily from slight, over moderate

and strong to extreme annoyance. In case of extreme annoyance, the PR for depression was 1.97 (95% CI: 1.62–2.39) and for generalized anxiety disorder 2.14 (95% CI: 1.71–2.67) (Figure 3(b)). In addition, there are studies showing a positive association between aircraft noise exposure and the intake of tranquilizing and sleep-inducing drugs and antidepressants [62, 63].

These results have been confirmed in the prospective German Heinz Nixdorf Recall (HNR) study, showing that road traffic noise was associated with an increased RR of high depressive symptoms at follow-up of 1.29 (95% CI: 1.03–1.62 for >55 vs. ≤55 dB(A)) [64]. A further NORAH-based case-control study examined the association between exposure to traffic noise and risk of incident depression [20]. Road traffic noise was associated with an OR of 1.17 (95% CI: 1.10–1.25) for noise levels of >70 dB, an OR of 1.23 (95% CI: 1.19–1.28) was found for aircraft noise levels of ≥50 to <55 dB, and in case of railway noise, the OR was 1.15 (95% CI: 1.08–1.22) for noise levels of ≥60 to <65 dB. The highest OR of 1.42 (95% CI: 1.33–1.52) was revealed for a combined exposure to noise from all three sources at noise levels above 50 dB.

A small case-control study from Italy showed an increased OR for prevalent generalized anxiety disorder (OR: 2.0, 95% CI: 1.0–4.2) and anxiety disorder not otherwise specified (OR: 2.9, 95% CI: 1.0–4.1) among subjects exposed to aircraft noise [65]. Recently, two Dutch studies analyzed the influence of traffic noise on depression and anxiety [66, 67]. Leijssen et al. demonstrated that exposure to road traffic noise was associated with increased prevalent depressed mood (OR: 1.65, 95% CI: 1.10–2.48 for ≥70 vs. 45-54 dB(A)), independent of ethnic and socioeconomic inequalities between groups of exposure [66]. The case-control study by Generaal et al. indicated an OR of 1.26 (95% CI: 1.08–1.47) for depressive disorder and 1.29 (95% CI: 1.11–1.50) for anxiety disorder per 3.21 dB(A) increase in traffic noise levels [67].

Moreover, occupational noise has been linked to psychological symptoms. A Korean study found occupational noise annoyance to increase depressive symptoms and suicidal ideation in men and women [68]. Compared to no annoyance, the OR for depressive symptoms were 1.58 (95% CI: 1.12–2.23) in men and 1.49 (95% CI: 1.05–2.11) in women; for suicidal ideation, the corresponding OR were 1.76 (95% CI: 1.29–2.40) in men and 1.41 (95% CI: 1.01–1.97) in women. A study of Egyptian airport workers found an increased prevalence of symptoms of anxiety along with other somatic symptoms in noise exposed workers (34% vs. 18% in controls) [69].

4. Noise and Experimental Studies in Animals and Humans

Experimental data provide mechanistic pathways by which noise exposure may trigger pathophysiological alterations and contribute to disease development. However, evidence is limited and the molecular mechanisms underlying the relationship between noise exposure, mental stress, and risk for cerebrocardiovascular and psychological disorders are not completely understood, yet. Taken together, it has been proposed that autonomic perturbation and sympathoadrenal activation induced by chronic noise stress may lead to increased levels of circulating stress hormones and subsequent oxidative stress-induced endothelial dysfunction, an early predictor for atherosclerosis, accompanied by the release of proinflammatory mediators and activation of prothrombotic pathways [70, 71]. This concept is well supported by a recent human study linking emotional stress with increased risk of cardiovascular disease by demonstrating that increased amygdala activity, a brain region involved in stress, is associated with arterial inflammation (increased plaque burden) by ^{18}F-fluorodexoyglucose PET/CT scanning technique [72]. Importantly, increased amygdala activity not only correlated with higher emotional stress burden but was also predictive for risk of future cardiovascular events (standardized HR: 1.59, 95% CI: 1.27–1.98), a finding that remained significant after multivariate adjustments.

4.1. Evidence from Human Studies. To date, only few studies of experimental, mechanistic nature have examined the underlying molecular mechanisms of noise effects in humans. The most comprehensive results in this context were achieved by Münzel and coworkers. In a field study of 75 healthy adults, Schmidt et al. evaluated the effects of simulated nighttime aircraft noise exposure on endothelial function as determined by flow-mediated vasodilation, sleep quality, and stress hormone levels [73]. Noise exposure was found to cause a dose-dependent worsening of endothelial function and sleep quality and to increase adrenaline levels proportionally, clearly in line with the implications of the "indirect pathway" of Babisch's noise reaction model. Of note, mental stress and depression per se were shown to contribute to endothelial dysfunction and atherosclerosis, while on the other hand, endothelial dysfunction and atherosclerosis may contribute to the incidence of depression via induction of hypothalamic-pituitary-adrenal axis overactivity, increased platelet activation, hypercoagulability, and activation of the inflammatory response [74, 75]. In addition, the pulse transit time was decreased, a parameter reflecting sympathetic arousal associated with elevated blood pressure, vascular tone, and stiffness. Interestingly, the worsening of endothelial function could be corrected by the single administration of the antioxidant vitamin C in a subgroup of subjects, which further suggests that an increased formation of reactive oxygen species and oxidative stress may be involved in the mediation of noise-induced endothelial dysfunction and cardiovascular complications.

A subsequent study by Schmidt et al. of 60 subjects with prevalent or with increased risk of ischemic heart disease found the above-described effects even more pronounced in this sample [76]. In addition to the previous findings regarding vascular function, sleep quality, and stress hormone levels, it has been shown that aircraft noise exposure is associated with increased systolic blood pressure (control group: 129.5 mmHg vs. noise group: 133.6 mmHg). Alongside these observations, further studies indicated that noise exposure causes autonomic imbalance by, e.g., increased heart rate, increased blood pressure, and sympathetic activation or parasympathetic withdrawal as well as increased arterial stiffness [77–81]. Overall, these results may provide key

pathophysiological mechanisms by which noise induces adverse health effects as shown by the results of epidemiological studies on ischemic heart disease, arterial hypertension, stroke, myocardial infarction, arrhythmia, heart failure, depression, anxiety, and metabolic abnormalities.

In addition, Chang et al. showed that environmental noise exposure has differential impact on arterial compliance and resistance vessels. Whereas a 1-dB(A) increase in noise exposure was associated with an augmentation of 1.25 (95% CI: 1.10–1.42) %mL/mmHg in arterial compliance, a decrease of 2.12 (95% CI: -2.51 to -1.80) kdynes$*$s/cm^5 in arterial resistance was observed [82]. The same authors also demonstrated that road traffic noise exposure ($\geq$ the median of noise levels) applied at specific frequencies may exert different impact on the risk for prevalent hypertension in 820 residents of central Taiwan with the 125 Hz frequency component displaying the most pronounced hypertensive effects (OR: 4.08, 95% CI: 1.57–10.63) [83]. Likewise, a retrospective cohort study identified the 4 kHz component of occupational noise as the most potent trigger of hypertension in 1,002 volunteers from 4 machinery and equipment manufacturing companies in Taiwan [84]. A 20 dB(A) increase in noise exposure at 4 kHz was associated with a 34% higher risk of hypertension (OR: 1.34, 95% CI: 1.01–1.77).

4.2. Evidence from Animal Studies. High noise levels (octave band noise: 80-100 dB, 8-16 kHz, 8 h/d for 20 d; 8 rats/group) increased plasma levels of stress hormones (corticosterone, adrenaline, noradrenaline, endothelin-1) and caused oxidative stress (increased malondialdehyde levels and decreased superoxide dismutase activity) in rats leading to an adverse cardiovascular phenotype as evidenced by severe endothelial dysfunction [85]. Other pathophysiological effects included higher mean arterial blood pressure and heart rate as well as higher levels of circulating nitrogen oxides (marker of inducible nitric oxide synthase activity) [85].

Recently, two comprehensive animal models in mice were established to study the molecular, nonauditory consequences of noise exposure. In our first study, mice were exposed to simulated aircraft noise for four days; in the control scenario, the mice were exposed to "white noise," both conditions exhibiting the same noise intensity (i.e., 72 dB(A)) [86]. Aircraft noise exposure caused an increased stress response as indicated by increased levels of cortisol, noradrenaline, dopamine, angiotensin II, and endothelin-1. In addition to increased systolic blood pressure, increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform 2 (Nox2), uncoupling of endothelial nitric oxide synthase (eNOS), inflammation in the vasculature, and subsequent endothelial dysfunction were observed (Figure 4) [86]. The induction of oxidative stress and inflammatory pathways in the vasculature was indicated by increased levels of 3-nitrotyrosine-positive proteins, lipid peroxidation products, and interleukin-6 (IL-6) and a more pronounced infiltration of proinflammatory macrophages into the aortic wall, respectively [86]. The presence of the two radical-forming enzymes, Nox2 and uncoupled eNOS, led to subsequent decreased vascular bioavailability of NO and thus to endothelial dysfunction and high blood pressure. Moreover, next-generation sequencing (Illumina RNAseq) revealed downregulation of genes encoding for antioxidant systems (e.g., intracellular SOD1, glutathione peroxidase-1, forkhead-box-protein O3), while an upregulation of proapoptotic factors for enhanced cell death (e.g., caspases, Fas, p38) was observed [86]. Interestingly, these effects were not seen in the control scenario with white noise, implicating that the stress-inducing character of aircraft noise exposure rather than noise exposure per se is crucial in determining adverse effects.

In our second study, aircraft noise exposure was furthermore found to induce cerebral oxidative stress and neuroinflammation, which was associated with a dysregulation of genes encoding the circadian clock, all of which caused systemic inflammation (e.g., increased expression of inducible NO synthase), oxidative stress, and endothelial dysfunction [87]. Of note, uncoupling and downregulation of neuronal NO synthase was observed, which will ultimately lead to impaired neuronal NO signaling and dysregulation of this important neuronal signaling molecule may explain, at least in part, the cognitive impairment in school children in response to noise [88]. Interestingly, noise-induced cerebral and vascular effects were present when mice were exposed during sleep but not awake phase, suggesting nighttime interference and impaired sleep quality leading to more stress as a crucial step. Genetic deletion of Nox2 not only prevented cardiovascular but also cerebral complications inflicted by noise clearly identifying oxidative stress as a major component in noise-triggered cerebrocardiovascular risk.

In summary, these findings indicate oxidative stress-induced endothelial dysfunction in response to noise exposure-related mental stress as key factors in the relationship between cerebrocardiovascular and psychological disorders. Importantly, environmental noise exposure appears to share common pathophysiological pathways with traditional cerebrocardiovascular risk factors such as obesity, diabetes mellitus, hypertension, and smoking with the main consequence of oxidative stress and endothelial dysfunction. However, as discussed above in Section 4.1 (last paragraph), specific frequency components of traffic noise exert differentially pronounced health effects in human subjects [83, 84] and, accordingly, translation of animal experimental data may be hampered by species differences in noise perception. Moreover, we have shown that white noise exposure, despite application of similar sound pressure levels and despite presence of a continuous band of frequencies in white (or pink) noise, displayed no adverse cardiovascular effects in mice [86], suggesting that the noise pattern (e.g., crescendo and diminuendo character, tantamount to increasing and decreasing intensity/loudness, of aircraft noise as well as particular breaks) may be of importance as well.

4.3. Oxidative Stress and Inflammation as Common Features of Psychological Disorders. The above-described mechanisms of noise-induced cerebral oxidative stress may also contribute to the onset of psychological disorders in response to chronic noise. Obviously, the origin of the mental stress plays a minor role for the activation of these stress response pathways, which will all converge at the level of oxidative stress and inflammation. Mice that were subjected to daily restraint

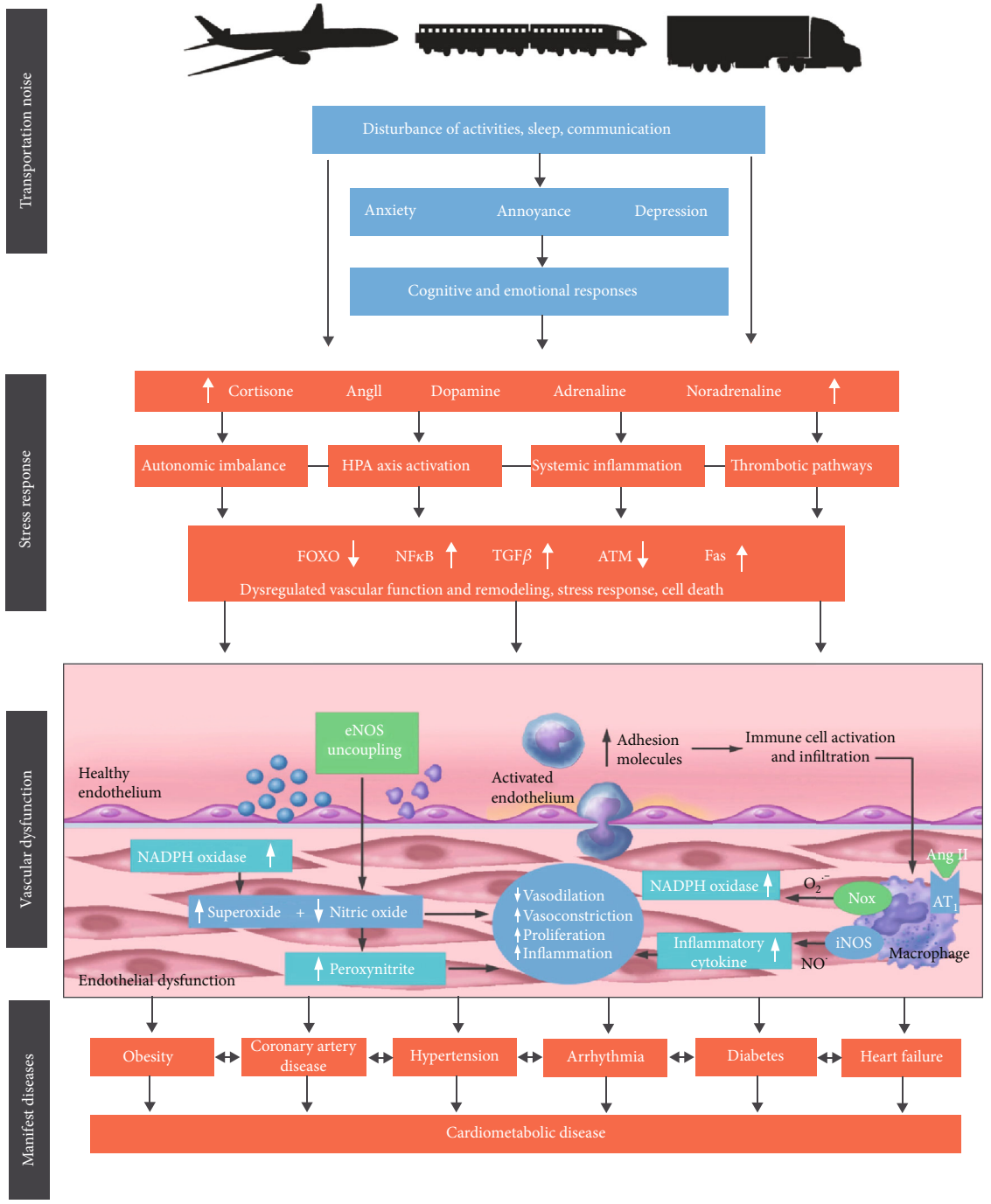

Figure 4: Noise causes annoyance and stress responses characterized by activation of the hypothalamic-pituitary-adrenal axis, oxidative stress-induced endothelial dysfunction, inflammation, thrombosis, and altered gene expression. Adapted from Münzel et al. [6]. Ang II: angiotensin II; AT_1: angiotensin receptor type 1; ATM: ataxia telangiectasia mutated; eNOS: endothelial nitric oxide synthase; Fas: cell death signaling molecule (CD95); FOXO: forkhead-box-protein O3; HPA: hypothalamic-pituitary-adrenal; iNOS: inducible nitric oxide synthase; NADPH: nicotinamide adenine dinucleotide phosphate; Nox: NADPH oxidase; NO: nitric oxide; O_2: oxygen; TGF: transforming growth factor. Adapted from Münzel et al. [6] with permission.

and cage-switch stress for one week developed severe inflammation and hypertension [89, 90].

Likewise, human data also support this concept as a meta-analysis (23 studies, 4,980 subjects) revealed a 0.55 of 1 standard deviation increase in oxidative stress markers among individuals with depression compared with those without depression, which was further supported by a negative correlation between depression and antioxidant status [91]. Another meta-analysis (10 studies, 1,308 subjects) found that oxidative stress markers 8-hydroxy-2′-deoxyguanosine and F2-isoprostanes are increased in depression [92]. Qualitatively, another meta-analysis came to the same

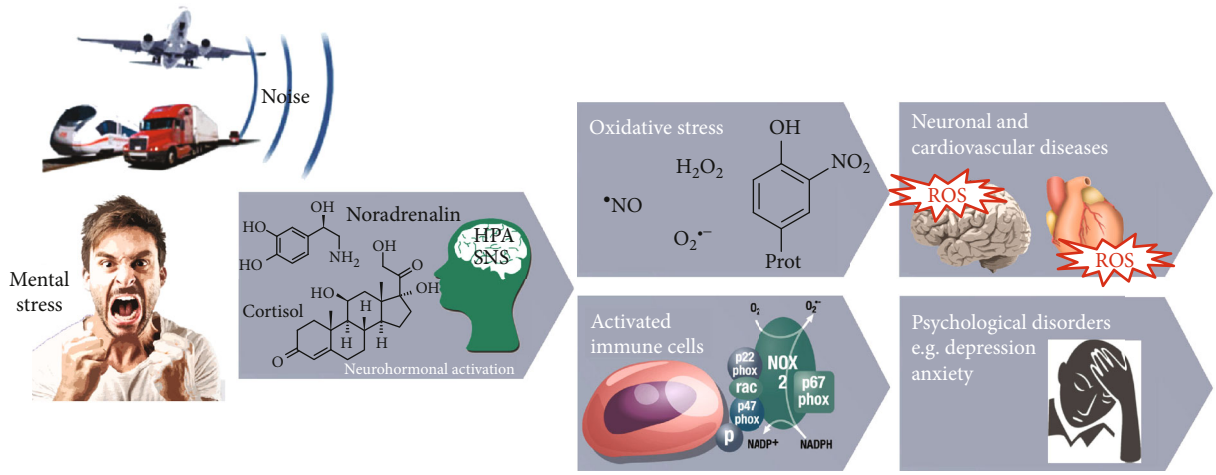

FIGURE 5: Environmental noise exposure and subsequent mental stress cause a stress reaction through activation of either the hypothalamus-pituitary-adrenal (HPA) axis with subsequent cortisol release or by the activation of the sympathetic nervous system (SNS) with subsequent catecholamine formation. As a consequence, cerebral and vascular inflammatory and oxidative stress pathways as well as altered gene expression become active, leading to endothelial dysfunction. Taken together, these consequences contribute and interact with traditional risk factors, leading to neuronal, cerebrocardiovascular, and psychological disorders. Modified from Münzel et al. [100] and adapted from Daiber et al. [71] with permission.

conclusion (29 studies, 3,961 subjects) and antidepressant therapy restored levels of oxidative stress markers and antioxidants [93]. A report on 96,989 individuals from two independent cohort studies revealed that higher plasma levels of the physiological antioxidant uric acid are associated with lower risk of depression hospitalization and lower antidepressant medication use [94]. Likewise, combining several inflammatory biomarkers (e.g., C-reactive protein, IL-6, tumor necrosis factor alpha) within a meta-analysis (53 studies, 2,467 cases and 2,360 controls) could differentiate individuals with bipolar disorder from healthy controls and indicate a specific mood-phase signature [95].

The impact of mental stress on oxidative stress pathways and inflammation was reviewed in full detail by Siegrist and Sies [96] as well as in two recent articles within the forum issue "Oxidative stress and redox signaling induced by the environmental risk factors mental stress, noise and air pollution" [97, 98]. Another review article put forward the concept that severe life stress is associated with cerebral oxidative stress with Nox2 as a major source [99].

5. Conclusions

In summary, the present review elucidates important mechanisms by which environmental noise exposure induces cerebrocardiovascular and psychological disorders (Figure 5). Noise interferes with communication, disturbs daily activities, and disrupts sleep, leading to mental stress. Upon chronic exposure, stress responses as evident by increased stress hormone levels lead to autonomic imbalance, oxidative stress, inflammation, and endothelial dysfunction, which then accelerates the development of cerebrocardiovascular risk factors and disease. Importantly, since noise exposure reflects mental stress, it favors the onset of psychological symptoms and disorders, which in turn is associated with cerebrocardiovascular dysfunction, highlighting the interrelationship between mental stress/psychological disorders and cerebrocardiovascular disease. Further studies, in particular with assessment of noise-induced cerebrocardiovascular and psychological consequences in context of one another, are warranted to gain more insight in the mechanisms underlying this relationship. In conclusion, environmental noise has to be acknowledged as an important risk factor for cerebrocardiovascular and psychological health, which has to be mentioned in corresponding current guidelines.

Conflicts of Interest

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

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