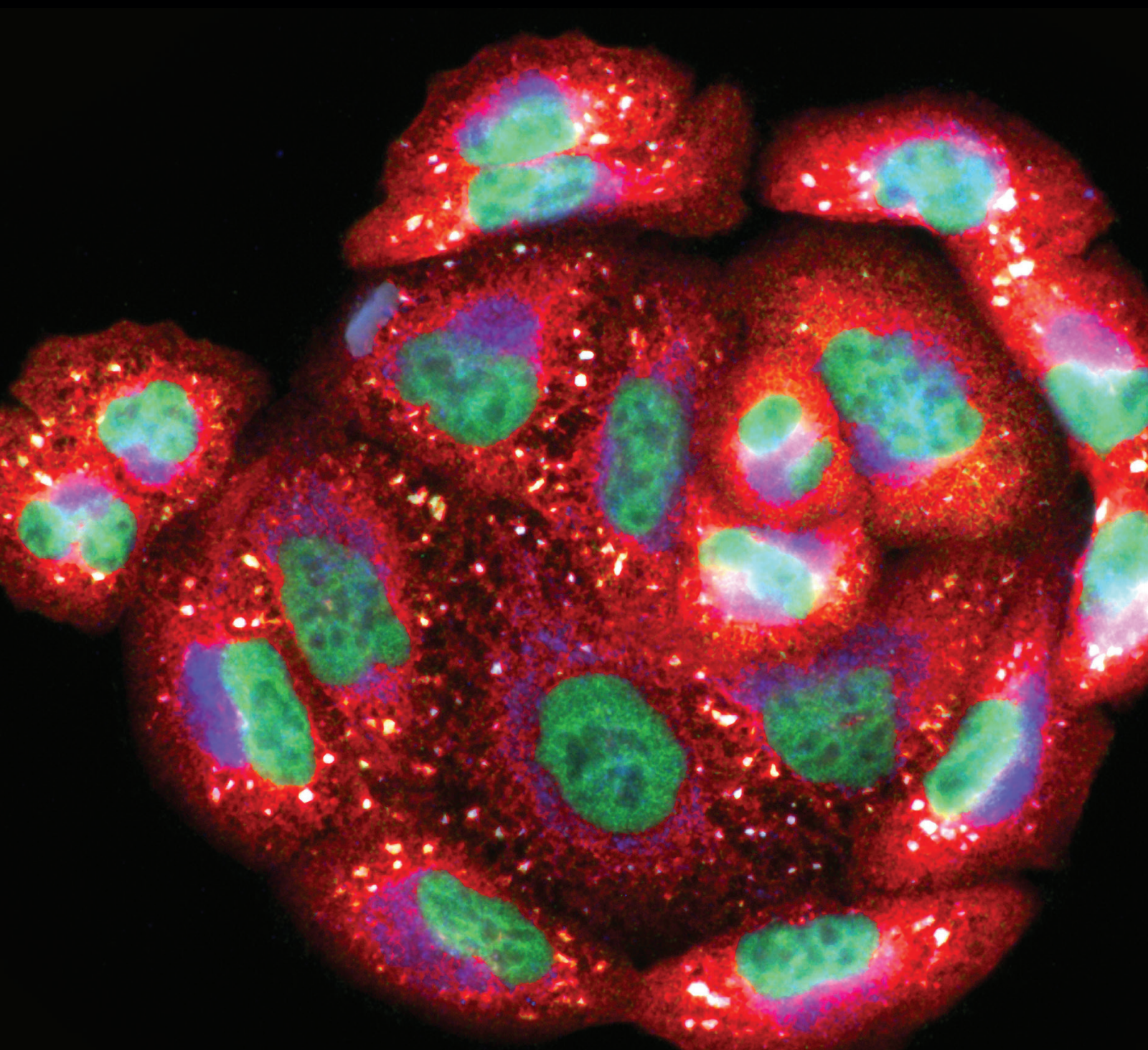
Oxidative Medicine and Cellular Longevity

Redox Control of Vascular Biology

Lead Guest Editor: Jaideep Banerjee
Guest Editors: Sabyasachi Sen, Mithun Sinha, and Jayeeta Ghose



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Editorial Board

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Editorial
Redox Control of Vascular Biology

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Oxidative stress and nitrosative stress are defined as a disruption of redox signaling and control. Homeostatic concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a crucial role as secondary messengers in many intracellular signaling pathways; however, excess ROS can lead to cell and tissue injuries [1, 2]. The major sources of vascular ROS are NAD(P)H oxidase, mitochondrial-derived superoxide, and uncoupled nitric oxide synthase [3]. Increasing studies have shown a close relationship between angiogenesis and oxidative stress in both physiological and pathological conditions, and oxidative damage plays a major role in vascular dysfunction, leading to endothelial dysfunction and inflammation.

As the field of redox biology employs new techniques to quantitatively measure the amount of ROS/RNS generated, innovative strategies are being developed to therapeutically target redox imbalance in vascular disease. This issue consists of 7 papers highlighting different therapeutic approaches targeting redox damage in cardiomyopathy, hypertension, skin disease, and diabetic retinopathy.

L. Chang et al. studied the *role of ZYZ-803 molecule in acute myocardial infarction* (AMI). Their work showed that ZYZ-803 protects heart tissues against acute myocardial ischemia via the targeting of ERS-related necroptosis by downregulating the RIP3-CaMKII pathway. The molecule further regulates H_2S and NO homeostasis via releasing both H_2S and NO. This opens up avenues to test the molecule as a candidate in AMI therapeutics.

In the review by X. Yang et al., the authors discuss about *therapeutic application of traditional Chinese medicines for treating coronary heart diseases* (CHD). Increased oxidative stress, disturbed lipid metabolism, and increased inflammation are critical factors in the occurrence and development of atherosclerosis and subsequent CHD. The authors emphasize the unique advantage of traditional herbal medicine as they do not pose therapeutic side effects. Some of the herbs with medicinal properties as discussed by the authors include Ginseng, Astragalus, Rehmannia, Ophiopogon root, Rhodiola rosea, Codonopsis pilosula, Atractylodes macrocephala, Astragalus, and Fructus crataegi.

In the review by D. Xian et al., the authors provide an overview of the current knowledge of the link between *oxidative stress* (*OS*) *and angiogenesis and their roles in certain skin diseases*. The authors view that there are two main mechanisms implicated in the area bridging angiogenesis and OS. One is a VEGF-dependent signaling pathway, HIF/VEGF signaling, while another is a VEGF-independent signaling pathway (CEP/TLR2/MyD88 axis and ROS/ATM/p38α pathway). They opined that both OS and angiogenesis participate in the development of certain skin diseases like psoriasis and atopic dermatitis. A large spectrum of proangiogenic factors mediate in psoriasis, including VEGF, HIF-1α, TNF, angiopoietins, IL-8, IL-17, and TGF-α. VEGF could enhance the migration of leukocytes into psoriatic skin and increase oxygen consumption, further activating HIF-1α and perpetuating the angiogenic/inflammatory cycle of psoriasis. Thus, ROS-VEGF signaling may be a potential target for the treatment of psoriasis.

Diabetic retinopathy (DR) is a leading cause of visual impairment and morbidity around the world. Endothelial dysfunction in the retinal blood barrier accompanying the hyperglycemic state is considered to be the major insult for

the onset and progression of DR [4]. In the review by N. Mahajan et al., the authors discuss *how impaired biochemical redox pathways contribute to diabetic retinopathy* highlighting the role of increased influx in polyol, accumulation of advanced end glycation products (AGE), enhanced activation of hexosamine, protein kinase C (PKC), and tissue renin-angiotensin system (RAS). The authors summarize that the overall effects of the metabolic abnormalities result in augmentation of ROS (reactive oxygen species) and RNS (reactive nitrogen species) production and associated oxidative and nitrosative damage, thereby leading to retinal vascular dysfunctions in DR. They also discuss the importance of metabolic memory caused by epigenetic changes such as a modified DNA methylation pattern, altered histone modifications of key regulatory proteins and altered microRNA (miRNA) expression leading to altered mitochondrial enzymes, damage to mitochondrial DNA, and altered mitochondrial ETC complexes. These result in superoxide formation, and depletion of antioxidants, eventually causing inflammation and apoptosis of retinal and endothelial cells during DR.

Oxidative stress associated with hyperlipidemia, hypertension, obesity, and IR, collectively referred to as Metabolic Syndrome (MS), is considered a risk factor for cardiovascular disease (CVD). Commonly used in traditional Asian and African medicine against hypertension, obesity, and hypercholesterolemia, in their work, using an MS rat model, I. Pérez-Torres et al. studied the antioxidant properties of *a medicinal herb*, *Hibiscus sabdariffa Linnaeus* (*HSL*), *in myocardial protection* against ischemia/reperfusion damage. The authors observed that cardiac mechanical performance, coronary vascular resistance, and activities of antioxidant enzymes were restored and oxidative damage was limited in HSL-treated rats compared to the untreated ones with MS. I. Pérez-Torres et al. concluded that HSL mediated myocardial protection during ischemia and reperfusion occurs through the antioxidant substances that it possesses such as PCA, anthocyanins, cyanidin-3-glucoside, quercetin, and polyphenols.

The study by M. Nitiéma et al. investigated whether oral administration of the ethyl acetate fraction of *Lannea microcarpa trunk barks* (*LMAE*) *corrects vascular dysfunction and angiotensin* (*Ang*) *II-induced hypertension in mice*. LMAE contains sterols, triterpenes, coumarins, and anthraquinone. Hemodynamic and echocardiographic parameters in vivo and vascular reactivity to acetylcholine (ACh) and $CaCl_2$ ex vivo were studied on isolated aortas. Results showed that LMAE prevents Ang II-induced hypertension and vascular dysfunction through a reduction of oxidative stress linked to COX-2 and NOX-2 pathway and inhibition of calcium entry.

Cells shed small vesicles called microparticles (MPs) including exosomes, which are trapped in tissues or released into bodily fluids. They harbor proteins and surface antigens specific to cells they originate from. MPs also mediate critical actions in intercellular communication and transmit biological messages by acting as paracrine vehicles.

Of interest and because of their easy detection using a variety of techniques, circulating MPs were recognized as biomarkers for cell activation and cross talk between different cell types. High plasma numbers of MPs were reported in many cardiovascular and metabolic disturbances, which are closely associated with insulin resistance and low-grade inflammation. They have been associated with adverse effect on the heart and vasculature. The review by T. Benameur highlights the *involvement of microparticles in cardiovascular complications associated with diabetes* and discusses the molecular mechanisms that underpin the pathophysiological role of MPs in the onset and progression of cellular injury.

Conflicts of Interest

The guest editors declare that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

All the guest editors wrote the editorial and contributed to and approved the final editorial.

J. Banerjee
J. Ghose
M. Sinha
S. Sen

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Research Article

ZYZ-803 Mitigates Endoplasmic Reticulum Stress-Related Necroptosis after Acute Myocardial Infarction through Downregulating the RIP3-CaMKII Signaling Pathway

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Acute myocardial infarction (AMI) is a leading cause of morbidity and mortality worldwide, and both cardiac necroptosis and endoplasmic reticulum stress (ERS) have been involved in the pathophysiology of AMI. ZYZ-803 is a hybrid molecule of a dual donor for gasotransmitters H_2S and NO. The aim of the present study is to investigate the antinecroptosis role and potential mechanisms of ZYZ-803 in the setting of ERS during AMI injury. *In vivo*, ZYZ-803 preserves cardiac function and reduces infarct size significantly after 24-hour left coronary artery ligation through revising H_2S and NO imbalance. In addition, ZYZ-803 relieves ERS and necroptosis in an AMI heart. *In vitro*, ZYZ-803 ameliorates ERS-related necroptosis induced by tunicamycin, and such effect has been depending on the receptor-interacting protein 3- (RIP3-) Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) signaling pathway. These findings have identified a novel antinecroptosis potential of ZYZ-803, providing a valuable candidate for cardioprotection in acute myocardial ischemia.

1. Introduction

Acute myocardial infarction (AMI) has been a leading cause of morbidity and mortality worldwide driven by an increased ageing population [1]. Damage to the coronary artery by vulnerable atherosclerotic plaques accounts for approximately 70% of all myocardial infarction events as well as by other pathogenic factors including a coronary spasm, emboli, and dissection [2–4]. The detrimental complications of AMI involving cardiac rupture, cardiogenic shock, dysrhythmic events, pericardial disease, and heart failure are responsible for the low rates of survival in patients [5]. Although pharmacological and catheter-based reperfusion (e.g., fibrinolysis and percutaneous coronary intervention) can provide mortality benefits, adverse events such as systematic bleeding and reperfusion failure of microvascular flow are still a driver of unsatisfactory outcomes. Thus, it is urgent to identify new strategies like the identification and characterization of novel cardioprotective drug candidates that can be developed for AMI intervention.

Pathologically, vast cardiac cell death following sharp ischemic insult is a hallmark process of AMI [5]. Programmed cell death and passive occurring necrosis are traditionally thought as the two main types of ischemic cardiomyocyte

death, and the former has become a key target for its gene-directed and regulated property in the field of cardioprotective study. Notably, necroptosis, or regulated necrosis, has recently been shown to be an important factor linked to ischemic heart injury [6–11]. As such, necroptosis holds particular appeal as a pharmacological target for cardioprotection. Important in necroptotic signaling is receptor-interaction protein 3 (RIP3). This protein serves as the core player in cardiac necroptosis along with RIP1-RIP3-MLKL (mixed lineage kinase domain-like protein) and RIP3-CaMKII- (Ca^{2+}-calmodulin-dependent protein kinase-) dependent pathway [9, 11, 12]. Targeting to these signaling pathways offers myocardial protection [8, 10, 13–17]. As such, targeting of these signaling systems could be important in the treatment of AMI.

Cardiomyocytes are rich in endoplasmic reticulum (ER) needed for contractive processes and metabolism. For this reason, cardiomyocytes are susceptible to endoplasmic reticulum stress (ERS) induced by exogenous injury such as ischemia and hypoxia. ERS is an evolutionarily conserved response to functional disturbances in cell ER function induced by genetic and environmental insults that can lead to cell death. The hallmark of ERS could be indicated by upregulation of some related genes such as activating transcription factor 6 (ATF6), CCAAT/enhancer-binding protein homologous protein (CHOP), glucose-related protein 78 (GRP78), and calreticulin [18]. A significant player in these processes is changes in cellular calcium pools; particularly, overload of intracellular Ca^{2+} is a hallmark of aberrant ERS [19]. Numerous researchers have revealed the pathophysiologic role of ERS in various diseases such as diabetes, neurodegeneration, cancer, and ischemic heart disease [20, 21]. In myocardial infarction (MI), metabolic disturbance resulted from acute hypoxia, and hypoglycemia frequently generates excessive misfolded protein accumulation, leading to aberrant ERS. This process can initiate secondary cell death, usually via the induction of apoptosis [21–25]. Although it has been reported that necroptosis is an alternative modality of ERS-induced downstream cell death in the L929 cell line [26], it remains unverified in myocardial cells. Furthermore, inhibition of ERS can improve insulted cardiac function, reduce adverse remodeling, and decrease cardiac apoptosis during MI [27, 28]. So, targeting to ERS and its possible downstream necroptosis should be a valuable way for MI prevention.

ZYZ-803 is a hybrid molecule synthesized by our laboratory that consists of S-propargyl-cysteine (a H_2S donor) and furoxan (a NO donor). This molecule is designed to slowly release the gasotransmitters of H_2S and NO that are important in cardiovascular function [29, 30]. It has been demonstrated that ZYZ-803 has a vasorelaxant effect on rat aortic rings [29] and angiogenesis role in the mouse ischemic hind limb model [31]. ZYZ-803 also showed cardioprotective potential in isoprenaline-induced heart failure [32]. The effects of ZYZ-803 are mediated via the interplay between H_2S and NO that act cooperatively to prevent cardiac damage. Since dysregulation of H_2S and NO homeostases exists during myocardial infarction, and aberrant ERS should possibly result in downstream cardiac necroptosis, targeting to ERS-related myocardial necroptosis during AMI as well as H_2S and NO imbalance is a promising cardioprotective scheme. In the current study, we intend to investigate the cardioprotective effects of ZYZ-803 on acute myocardial infarction both *in vivo* and *in vitro*. Additionally, the underlying mechanisms of action ascribed to ZYZ-803 have been addressed with a focus on ERS-related myocardial necroptosis and the subsequent signaling pathway involved.

2. Materials and Methods

2.1. Animals and Experimental Protocols. Adult male C57BL/6 mice (8 weeks) were acquired from the Sippr-BK Experimental Animal Center (Shanghai, China) and housed under pathogen-free conditions with free access to food and water. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Ethics Committee of Experimental Research, Shanghai Medical College, Fudan University. Mice were randomly assigned into 5 groups: sham group, model group, and (2, 4, and 8 mg/kg/day) ZYZ-803-pretreated group. ZYZ-803 was dissolved in 0.1% DMSO for intraperitoneal administration for 5 days before myocardial infarction operation. The model and ZYZ-803-pretreated groups had induced myocardial infarction by permanent ligation of the left anterior descending coronary artery following previous description [33]. Briefly, mice were intubated by a 22-gauge intravenous catheter and ventilated with a mouse ventilator after isoflurane anesthetization. The epigastric hair of the mouse was sheared to expose the surgical field, and then, the thoracic cavity was opened at the third intercostal space in order to permanently ligate the left anterior descending coronary artery using the 8-0 Prolene suture at 1-2 mm distal below the left auricular appendix. When the top of ventriculus sinister was observed blanching, it was confirmed infarction. Animals in the sham group were just performed with thoracotomy without ligation to the left anterior descending coronary artery.

2.2. Echocardiography. After 24-hour infarction, mice were anesthetized using 1.5% isoflurane in a 95% mixed oxygen and fastened on a heating pad. Parameters for heart function were acquired by transthoracic two-dimensionally directed M-mode echocardiography dynamically using a high-resolution ultrasound system (Vevo 770; VisualSonics Inc., Toronto, ON, Canada) with a mechanical scan probe, and the transducers for the ventricular structure were at frequency of 10 MHz to provide spatial resolutions. Left ventricular ejection fraction (LVEF, %), left ventricular fractional shortening (LVFS, %), left ventricular systolic diameter (LVSD, mm), and left ventricular systolic volume (LVSV, μl) were calculated automatically by the system.

2.3. Infarct Size. Infarct size was determined by TTC and Evans blue dye. In brief, after 24-hour infarction, mice were anesthetized by 1.5% isoflurane, intubated using a catheter, and ventilated with a mouse ventilator. Thoracotomy was done again, and 2% Evans blue in PBS (pH 7.4) was slowly

perfused into ventriculus sinister from the tip of hearts to visualize the area at risk (AAR) until the mouth and limbs of mice turned into blue. Then, mouse hearts were harvested rapidly and excised into 6 sections before they were incubated in 1% TTC solution (in PBS, pH 7.4) for 15 minutes at 37°C. And then, heart sections were fixed in 4% paraformaldehyde for 24 hours until they were digitally scanned by a scanistor. Scanned photographs were assessed by ImageJ software (NIH, Boston, USA). Infarct size (IS) was calculated as the ratio of IS/AAR.

2.4. Histopathology. After 72-hour AMI, the mouse hearts were isolated and fixed in 4% paraformaldehyde (pH 7.4) for 24 hours at room temperature and then embedded in paraffin in order to be serially divided into sections of 5 μm thickness. Standard hematoxylin and eosin (HE) staining was performed to observe histopathological changes, and Sirius red dye was applied to evaluate collagen deposition. The protocols for HE staining and Sirius red dye followed the manufacturers' direction of the corresponding kits (G1120 and G1470, Beijing Solarbio Science & Technology Co. Ltd., China), and then, sections were photographed by an optical microscope (Zeiss Inc., Oberkochen, Germany).

2.5. Measurements of H_2S and NO Concentrations in Serum and Heart Tissues. H_2S concentration in serum was detected according to the previous method [32]. In brief, 75 μl serum was mixed in 250 μl zinc acetate (1%, *w*/*v*), 425 μl deionized water, 133 μl N-dimethyl-p-phenylenediamine sulfate (20 mmol/l in 7.2 mmol/l HCl), and 133 μl $FeCl_3$ (30 mmol/l in 1.2 mmol/l HCl), and then, the mixture was incubated for 10 minutes at room temperature. Next, 250 μl trichloroacetic acid (10%) was added and then centrifuged at 14,000 rpm for 5 minutes. The absorbance at 670 nm was set to detect the changes in each sample. The H_2S level in mouse heart tissues was evaluated by a commercial ELISA (enzyme-linked immunosorbent assay) kit (N06478, Shanghai Jining Company, China) for mouse H_2S according to the manufacturer's direction. The concentration of NO in serum and heart tissues was assayed by the commercial Total Nitric Oxide Assay Kit (S0023, Beyotime Institute of Biotechnology, China) in accordance with the manufacturer's protocol.

2.6. Primary Neonatal Rat Ventricular Cardiomyocyte. Primary neonatal rat ventricular cardiomyocytes (NRVCs) were isolated from 1-day neonatal rats using the Pierce Primary Cardiomyocyte Isolation Kit (88281, Pierce Biotechnology, Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, the freshly dissected hearts from newborn rats were placed in cold Hank's Balanced Salt Solution (HBSS) and minced into 1-3 mm^3 pieces. Then, the minced heart tissues were washed in cold HBSS twice to remove blood before adding cardiomyocyte isolation enzyme mixture (including papain and thermolysin). The tissues with enzyme mixture solution were incubated in a 37°C incubator for 30 minutes. After gently removing the enzyme solution, the tissues were washed twice in cold HBSS. The complete Dulbecco's modified Eagle medium (DMEM) for primary cardiomyocyte isolation was added to break the tissue by pipetting up and down 25-30 times until the tissues were primarily a single-cell suspension. The appropriate cell suspension was plated with 0.1% cardiomyocyte growth supplement into culture vessels and incubated in a 5% CO_2 incubator at 37°C until next treatment procedures.

2.7. Cell Viability Assay. Cell viability after ZYZ-803 and/or tunicamycin administration was assayed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as previously described [31]. Primary cardiomyocytes were cultured in 96-well plates and incubated for 12 hours. After pretreatment with designed concentration of ZYZ-803 for 1 hour and following 48-hour tunicamycin (Tuni) administration, 0.5 mg/ml MTT was added into cell DMEM for another 4-hour incubation. Then, dimethylsulfoxide was added into wells to dissolve the formazan crystal. Absorbance at 570 nm in a microplate reader (M1000, TECAN, Austria GmbH, Austria) was set to measure changes in each group.

2.8. LDH and cTnI Measurement. Serum LDH and cTnI were assayed using related commercial assay kits (C0016, Beyotime Institute of Biotechnology, China, and E-EL-M1203c, Elabscience Biotechnology Co. Ltd., China, respectively) according to the manufacturers' instructions. For LDH detection, the cell supernatant or serum was incubated with prepared LDH working solution in the dark for 30 minutes at room temperature. Absorbance at 490 nm was measured using a microplate reader (M1000, TECAN, Austria GmbH, Austria). For the cTnI assay, serum was added into the well plate precoated with the mouse cTnI specific antibody; then, a biotinylated antibody specific for cTnI and avidin-horseradish peroxidase (HRP) conjugate were added successively to each well plate and incubated. After free components were washed away, the substrate solution was added into each well. Finally, stop solution was added to terminate the enzyme-substrate reaction, and then, the color turns yellow. The optical density was measured spectrophotometrically at 450 nm using a microplate reader (M1000, TECAN, Austria GmbH, Austria).

2.9. Hoechst 33342 and Propidium Iodide Staining. Cellular necrosis was evaluated using the Apoptosis and Necrosis Assay Kit (C1056, Beyotime Institute of Biotechnology, China) according to the manufacturer's direction. Briefly, cardiomyocytes were cultured on slides for 72 hours. After designed administration of ZYZ-803 and/or Tuni, cells were washed twice in PBS and added with Hoechst and propidium iodide (PI). Then, cell slides were incubated on ice for 20-30 minutes and photographed using a fluorescent microscope (Zeiss Inc., Oberkochen, Germany). Cells with strong red and blue fluorescence were defined as necrosis positive. ImageJ software (NIH, Bethesda, MD, USA) was used to count positive necrotic cells.

2.10. Intracellular $[Ca^{2+}]_i$ Evaluation. Overload of intracellular Ca^{2+} concentration is defined as a marker of ERS and cell injury [29]. Fluo-3/AM (Dojindo Lab, USA) is a fluorescent Ca^{2+}-indicator probe and was used to measure the level of intracellular $[Ca^{2+}]_i$. After designed treatments with ZYZ-803 and/or Tuni, cardiomyocytes were washed with cold

HBSS and loaded with 5 μM fluo-3/AM for 30 min at 37°C. Then, cells were washed twice to remove the dye before another 30 minutes was allowed to hydrolyze fluo-3/AM. A confocal microscope (Zeiss Inc., Oberkochen, Germany) at a wavelength of 480 nm excitation/525 nm emission was used to photograph the intracellular $[Ca^{2+}]_i$. Fluorescence intensity was analyzed by ImageJ software (NIH, Bethesda, MD, USA).

2.11. Small RNA Interference. Small interfering (si)RNA oligos for rat RIP3 gene were purchased from Thermo Fisher (Silencer® Select siRNA s80756). Target sequence for RIP3 is 5′CGUGAACUCGAAGAAGAUATT3′. For RNA interference, cardiomyocytes in six-well plates were transfected with final concentration of 10 nM siRNA using the X-tremeGENE siRNA Transfection Reagent (Cat. No. 04476093001, Roche) according to the manufacturers' instructions. The medium was replaced at 6-hour posttransfection, and silencing efficiency was determined by real-time PCR and Western blot 48 hours after transfection.

2.12. Real-Time Quantitative RT-PCR. Cardiomyocyte total RNA was abstracted using TRIzol (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's direction. Reverse transcription cDNA was prepared using the Primer Script RT Reagent Kit (TaKaRa Biotechnology Co. Ltd., Dalian, China). An amplified outcome of PCR was detected using the CFX96 Real-Time PCR Detection System (Bio-Rad). Primer sequences of RIP3 are forward primer: AAAC CACTGAGCGAGCATCC and reverse primer: TCCCTG AAATGTGGACAGGC.

2.13. Coimmunoprecipitation. Immunoprecipitation (IP) lysis buffer (20 mM Tris-HCl, 150 mM NaCl, and 1% Triton X-100, pH 7.5) was used to lyse mouse heart tissues, and the lysis was then centrifuged at 12,000 rpm for 10 minutes to remove cell debris. Supernatant of lysis was incubated with IgG antibody or target protein antibody and Protein A/G Plus-Agarose (Santa Cruz Biotechnology) at 4°C on a shaker overnight. The immunoprecipitated complex was collected by centrifugation and washed 4 times. The final pellet was resuspended in sample buffer for the following SDS-PAGE analysis.

2.14. Protein Immunoblotting. Total proteins of cells were extracted by the NuPAGE 1x LDS Sample Buffer (Invitrogen, USA), while tissue proteins were extracted in RIPA buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl, pH 7.0) containing phosphatase and protease inhibitor cocktail (Thermo Scientific, USA). The protein extracts were boiled in 96°C water bath before loading onto sodium dodecyl sulfate polyacrylamide gels and transferring to nitrocellulose membranes (PALL, USA). Then, the transferred membranes were blocked in 5% nonfat milk in TBS (Amresco, Solon, OH, USA) containing 0.1% Tween-20 for 2 hours at room temperature. Next, the membranes were incubated with the primary antibodies of target proteins overnight at 4°C, followed by binding with HRP-conjugated or fluorescent secondary antibodies. Final detection of protein blots was performed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) or Odyssey imaging system (LI-COR Biosciences, USA).

2.15. Cell Immunofluorescent. NRVCs were cultured in confocal cell dishes for 48 hours before ZYZ-803 pretreatment and Tuni-induced injury. Then, cell dishes were washed in PBS twice and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Next, after being washed in PBS, 0.2% Triton X-100 was used to permeate cells for 5 minutes. Cells were blocked in 5% BSA for 30 minutes at room temperature before successive incubation with primary antibodies and fluorescent secondary antibodies. Finally, cell dishes were covered with fluorescent quencher and observed by a confocal laser scanning microscope (Carl Zeiss, USA).

2.16. Drugs and Reagents. ZYZ-803 was synthesized and purified as described previously [29]. Tunicamycin (HYA0098) was purchased from MedChem Express (Shanghai, China). TTC and Evans blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to CSE and RIP3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to calreticulin, ATF6, CHOP, phosphorylated CaMKII, and total-CaMKII were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to MLKL was purchased from Signalway Antibody (College Park, MD, USA). Antibodies to GRP78, RIP1, and CBS were purchased from Proteintech (Rosemont, IL, USA). Antibody to GAPDH (MB001) was purchased from Bioworld (Nanjing, China). HRP-conjugated secondary antibodies were from Jackson Laboratories (West Grove, PA, USA). Goat anti-rabbit and donkey anti-mouse fluorescent secondary antibodies were purchased from LI-COR (Lincoln, NE, USA).

2.17. Statistical Analysis. The software GraphPad Prism v.5.01 (GraphPad Software, La Jolla, CA, USA) was used to analyze experimental data. Values are expressed as mean ± SEM. Comparison between two groups was performed using the Student *t*-test (two-tailed), while variance among multiple groups was assessed by one-way ANOVA with Bonferroni *post hoc* analysis. Statistical significance for each test was defined as $^*P < 0.05$ at least.

3. Results

3.1. ZYZ-803 Dose-Dependently Blocks Deterioration of the Cardiac Function after AMI. In order to investigate the protective potential of ZYZ-803 against AMI, based on our previous study [31] and preexperiments (Figure S1), we chose 2, 4, and 8 mg/kg/day ZYZ-803 to pretreat mice daily and intraperitoneally for 5 days before permanent ligation of the left anterior descending coronary artery. Cardiac function of experimental mice indicated by regional ventricular wall motion was assessed by transthoracic echocardiography. As displayed from the echocardiographic results, deterioration of the mouse left ventricle systolic function could be obviously observed in the model group compared to the sham one, which was rescued by ZYZ-803 in a dose-dependent manner (Figure 1(a)). Similarly, the statistical values of cardiac parameters LVEF, LVFS, LVSD,

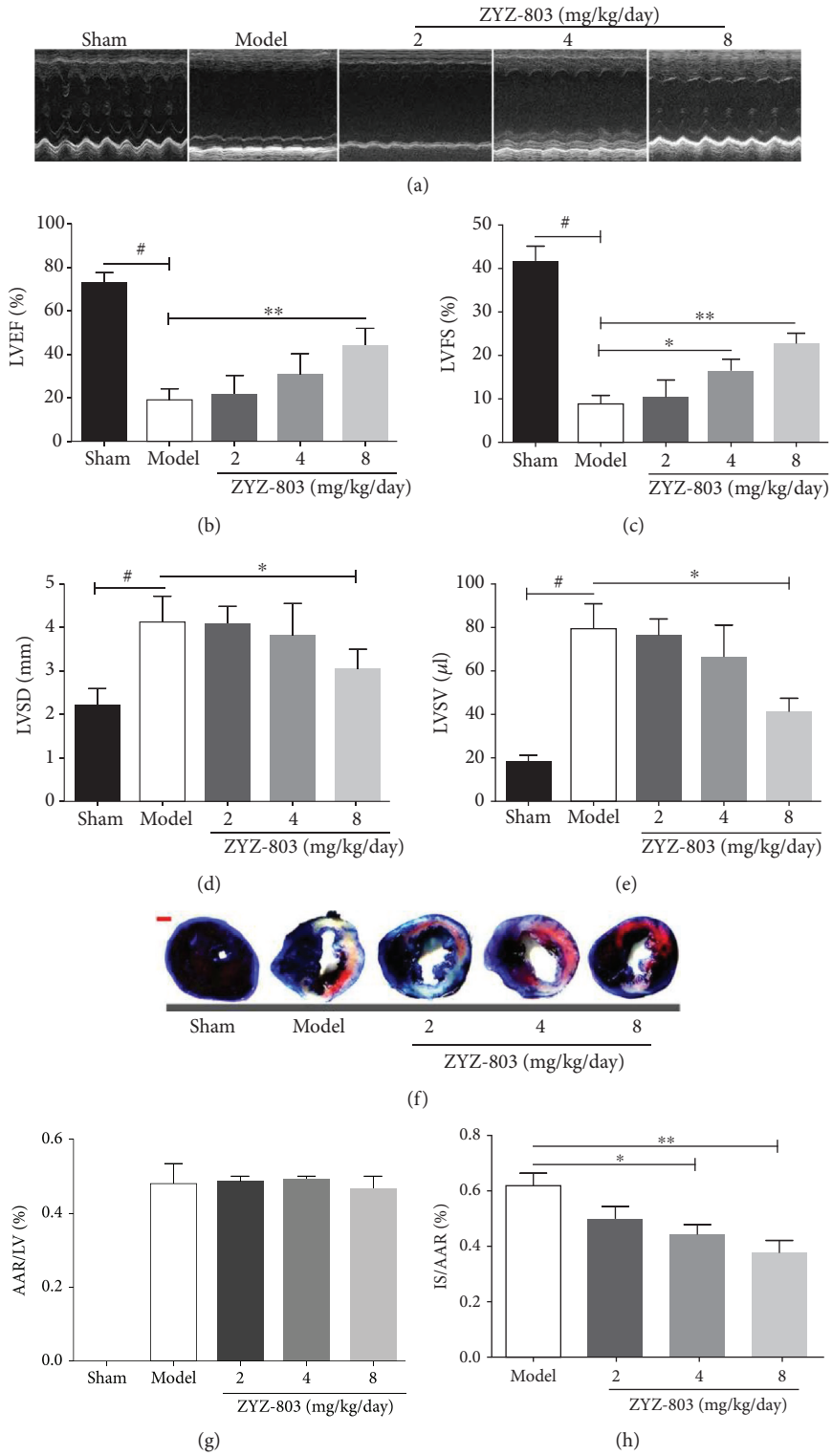


Figure 1: Pretreatment of ZYZ-803 preserves the left ventricular function and palliates the myocardial infarction scale. Mice were suffered from 24-hour ligation of the left anterior descending coronary artery after 5-day ZYZ-803 preconditioning. (a) Representative echocardiographs from transthoracic two-dimensionally directed M-mode echocardiography. (b–e) Statistical data of (b) left ventricular ejection fraction (LVEF (%)), (c) left ventricular fractional shortening (LVFS (%)), (d) left ventricular systolic diameter (LVSD (mm)), and (e) left ventricular systolic volume (LVSV (μl)), assessed by echocardiography ($n = 6$ mice in each group). Data are mean ± SEM, one-way ANOVA. $^{\#}P < 0.001$ versus sham group; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the model group. (f) Representative photographs for IS and AAR in the heart by TTC and Evans blue dye from each group. Scale bar (red): 1 mm. Quantitative data of (g) AAR/LV and (h) IS/AAR. AAR: area at risk; IS: infarct size; LV: left ventricle ($n = 6$ mice per group). Compared to the model group, $^{**}P < 0.01$ and $^{*}P < 0.05$. Data are mean ± SEM, one-way ANOVA.

and LVSV were reduced in groups of ZYZ-803 pretreatment when compared to the model group (Figures 1(b)–1(e) and Table S1); especially, these parameters in the high-dosage group (8 mg/kg/day) were much more significantly decreased ($^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the model). In addition, ZYZ-803 pretreatment showed no side effect to cardiac function in sham mice (Table S1), and the heart rate of mice in each group after AMI and ZYZ-803 pretreatment presented no apparent change (Figure S4). These data indicated that ZYZ-803 could play a preservation role in the injured cardiac function after AMI.

According to clinical research, myocardial infarct size is a detrimental factor for AMI to develop into heart failure [34]. So, it is quite necessary to evaluate the impact of ZYZ-803 on infarction scale change in order to further confirm its protective potential after AMI. TTC-Evans blue dye has usually been used to measure the infarction scale by presenting infarct size (IS) with white color and area at risk (RRA) without blue color. As the results show, there was no significant difference between the model and ZYZ-803 groups in RRA (Figures 1(f) and 1(g)). However, ZYZ-803 markedly decreased the IS/AAR ratio at the dosages of 4 and 8 mg/kg/day as compared to the model group (Figures 1(f) and 1(h); $^{*}P < 0.05$, $^{**}P < 0.01$), implicating ZYZ-803 improved cardiac function after AMI.

3.2. ZYZ-803 Decreases the Level of Serum Biomarkers for Myocardial Necrosis and Improves the Compromised Cardiac Compliance. In the clinical practice, evaluation of serum biomarkers is a cornerstone for AMI diagnosis [35, 36]. LDH has been a broad-spectrum enzymatic marker for myocardial injury, and cTnI, a component of the myocardial cell, is a specifically sensitive marker for myocardial necrosis. So, we chose these two biomarkers for further evaluation. From the data in Figures 2(a) and 2(b), apparently, the serum LDH and cTnI levels in ZYZ-803 groups were decreased compared to the model group ($^{**}P < 0.05$, $^{***}P < 0.01$), implying that ZYZ-803 protected against myocardial necrosis.

At the necropsy level, histopathologic change of heart tissue has been considered as a key indicator of AMI injury in animal experiments. Because histopathological change results from a subacute pathological process during AMI [5], mouse hearts were harvested after 72-hour AMI, and then, HE staining and Sirius red dye were utilized to testify the effect of ZYZ-803 on heart histopathology. As shown in Figures 2(c) and 2(d), when compared to the sham group, there were significant catastrophic changes of disorganized cell arrangement, neutrophil infiltration and cytolysis from HE staining, and plenty of collagen deposition indicating myocardial fibrosis from Sirius red dye in the heart tissues of the model group. However, ZYZ-803 pretreatment could positively improve these compromised histopathological changes of cardiac compliance in comparison with the model one (Figures 2(c) and 2(d)). These results further demonstrated the protective potential of ZYZ-803 in AMI.

3.3. Protection of ZYZ-803 against AMI Injury Is Mediated by Its Counterbalancing to CBS/CSE/eNOS Synthase Systems. The regulation of ZYZ-803 to H_2S and NO has been demonstrated in the mouse ischemic hind limb and heart failure [31, 32]. However, whether ZYZ-803 could protect against AMI injury through the regulation to H_2S and NO systems has not been confirmed. So here, the concentration of H_2S and NO in mouse serum and heart tissues was assessed, and the expression and activity of related synthases were detected. Figures 3(a) and 3(b) showed the results of serum H_2S and NO levels by defined chemical methods, and disastrously decreased levels of serum H_2S and NO in AMI mice were observed in the model group, which were both apparently reversed by ZYZ-803 pretreatment ($^{\#}P < 0.01$, $^{**}P < 0.01$). Similarly, decreased H_2S and NO concentrations were blocked by ZYZ-803 pretreatment in mouse heart tissues (Figures 3(c) and 3(d); $^{*}P < 0.05$, $^{**}P < 0.01$). As to the change of related synthases, abnormal expressions of H_2S synthase cystathionine-γ-lyase (CSE) and cystathionine β-synthase (CBS) were found in the model mice, and similar results were verified in the activity of endothelial NO synthase (eNOS), the main synthase of NO in the heart (Figures 3(e)–3(h); $^{\#}P < 0.01$). However, these enzymatic changes were expectedly salvaged by preadministration of ZYZ-803 as demonstrated in Figures 3(e)–3(h) ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). These results reflected that the dysregulation of H_2S and NO homeostases during AMI and ZYZ-803 pretreatment could correct this imbalanced H_2S-NO systems to protect against myocardial injury.

3.4. Cardiac ERS Injury and Necroptosis during AMI Are Mitigated by ZYZ-803. As stated above, both aberrant ERS and necroptosis are involved in the pathological process of AMI. To clarify whether ZYZ-803 could reduce ERS and necroptosis during AMI, we evaluated the change of related protein markers in mouse heart tissues using Western blotting. Results in Figures 4(a) and 4(b) illustrated a significant increase in the expression of GRP78, calreticulin, and CHOP in AMI mice compared to the sham group ($^{\#}P < 0.01$), which was blocked significantly by ZYZ-803 preconditioning ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). These results manifested amelioration of ZYZ-803 to aberrant ERS in AMI. Meanwhile, protein markers of necroptosis including RIP1, RIP3, MLKL, and phospho-CaMKII were measured in each group. The data showed that RIP1, RIP3, and phospho-CaMKII except MLKL were upregulated in the model mice (Figures 4(c)–4(f); $^{\#}P < 0.001$), while only RIP3 and phospho-CaMKII were apparently decreased by ZYZ-803 pretreatment (Figures 4(c)–4(f); $^{*}P < 0.05$, $^{**}P < 0.001$). These data revealed occurrence of aberrant ERS and necroptosis during AMI which were both blocked by ZYZ-803 preconditioning and also implied that cardiac necroptosis may be a downstream disastrous result of aberrant ERS during AMI.

3.5. ZYZ-803 Alleviates Necroptosis Induced by Abnormal ERS in Primary Cardiomyocytes. To further verify the speculation that abnormal ERS may be an upstream inducer for myocardial necroptosis and that ZYZ-803 may play a similar role to this speculated ERS-related necroptosis just like in animals, an in vitro injury model of abnormal ERS induced

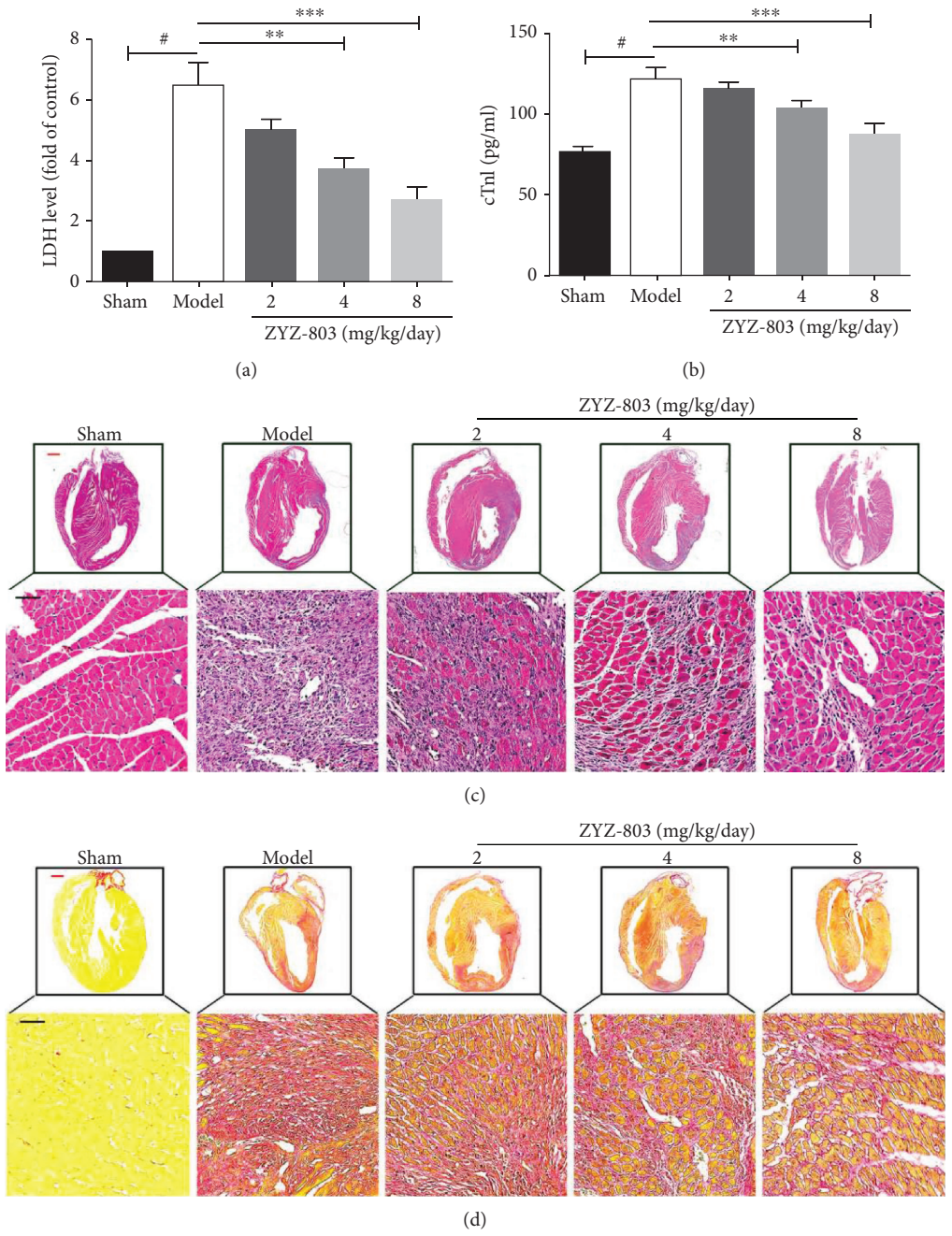


Figure 2: ZYZ-803 decreases the level of serum biomarkers for myocardial necrosis and improves the compromised cardiac compliance. (a, b) Both serum LDH and cTnI were decreased by ZYZ-803, $n = 6$ mice per group; compared to the model group, $^{**}P < 0.01$ and $^{***}P < 0.001$. Data are mean ± SEM, one-way ANOVA. (c) Hematoxylin and eosin staining of heart tissue sections from experimental animals to evaluate the severity of histopathologic changes after AMI. (d) Sirius red dye for evaluation of collagen deposition after mouse AMI. Red bars: 2.5 mm; black bars: 100 μm.

by a typical ERS agonist tunicamycin (Tuni) was employed. 2 μg/ml Tuni was chosen to treat primary neonatal ventricular cardiomyocytes (NRVCs) for 48 hours, and cell death was observably induced (Figure S2). The results, as shown in Figures 5(a) and 5(b), revealed that cellular necrosis indirectly indicated by cell viability and LDH release was obviously increased after Tuni treatment ($^{\#}P < 0.001$), which was ameliorated by pretreatment of ZYZ-803 ($^{*}P < 0.05$, $^{**}P < 0.01$). Likewise, after ZYZ-803 pretreatment, an obviously decreased level of necrosis (Figures 5(c) and 5(d); $^{*}P < 0.05$, $^{***}P < 0.001$) and intracellular Ca^{2+} (Figures 5(e) and 5(f); $^{*}P < 0.05$, $^{***}P < 0.001$) were observed by means of a fluorescent probe when compared to the Tuni group. In addition, upregulated expression of ERS-related proteins including ATF6, GRP78, calreticulin, and CHOP induced by Tuni was blocked by ZYZ-803 (Figure 5(g); $^{\#}P < 0.01$, $^{**}P < 0.01$, and $^{***}P < 0.001$). Furthermore, corresponding with the changes of ERS protein expression,

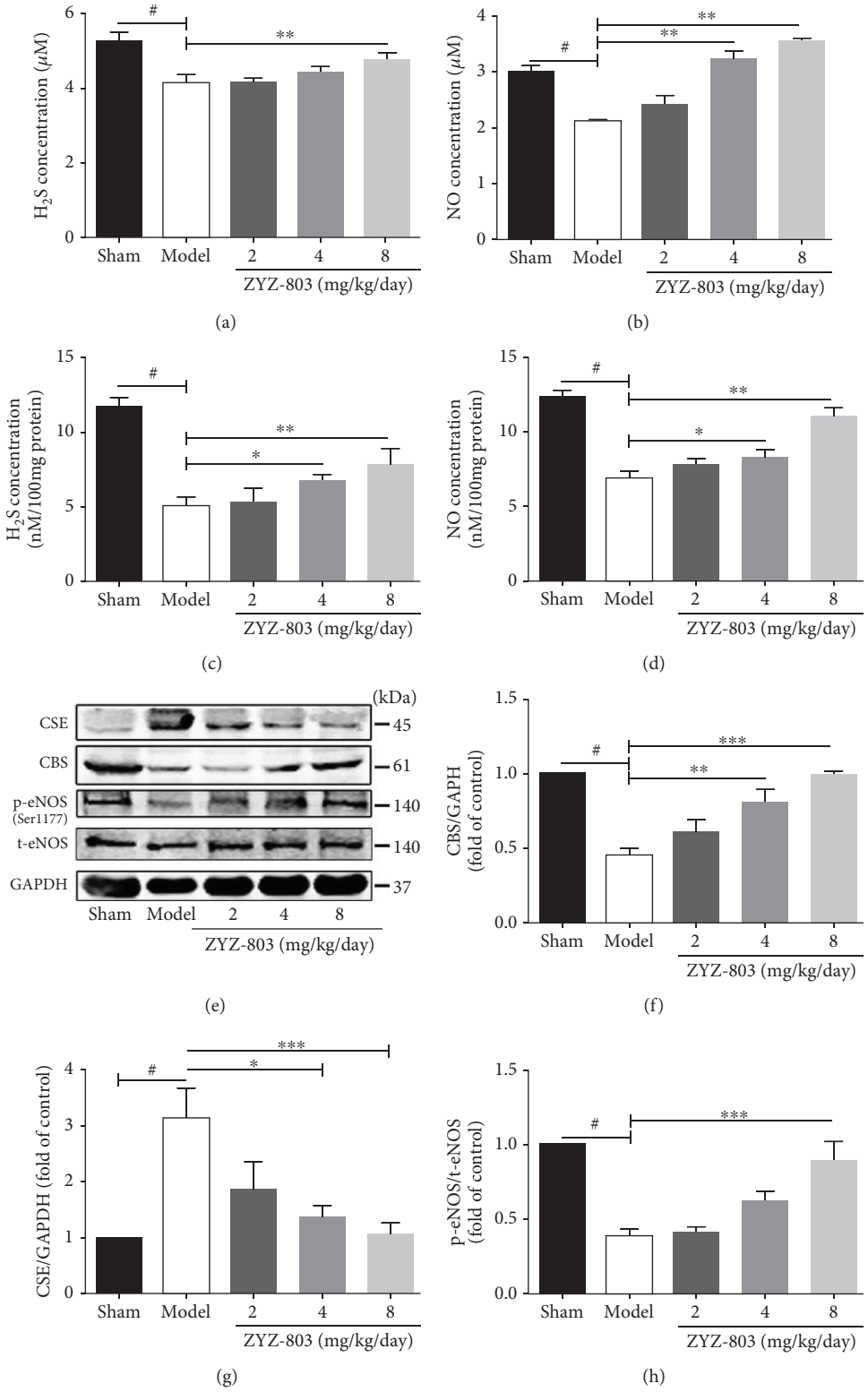


Figure 3: ZYZ-803 preserves H_2S and NO homeostases through regulating balance of the endogenous enzyme levels. (a, b) Averaged data of serum H_2S and NO concentration in experimental mice ($n = 6$ mice each group). (c, d) Averaged data of H_2S and NO contents in mouse heart tissues ($n = 6$ mice each group). (c) Representative graphs of protein expression detected by Western blotting. (d–f) Statistical analysis graphs of p-eNOS/t-eNOS, CBS, and CSE; each protein is normalized by GAPDH ($n = 4$ independent experiments). Data are presented as mean ± SEM, one-way ANOVA. $^{\#}P < 0.01$ versus sham; compared to the model group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

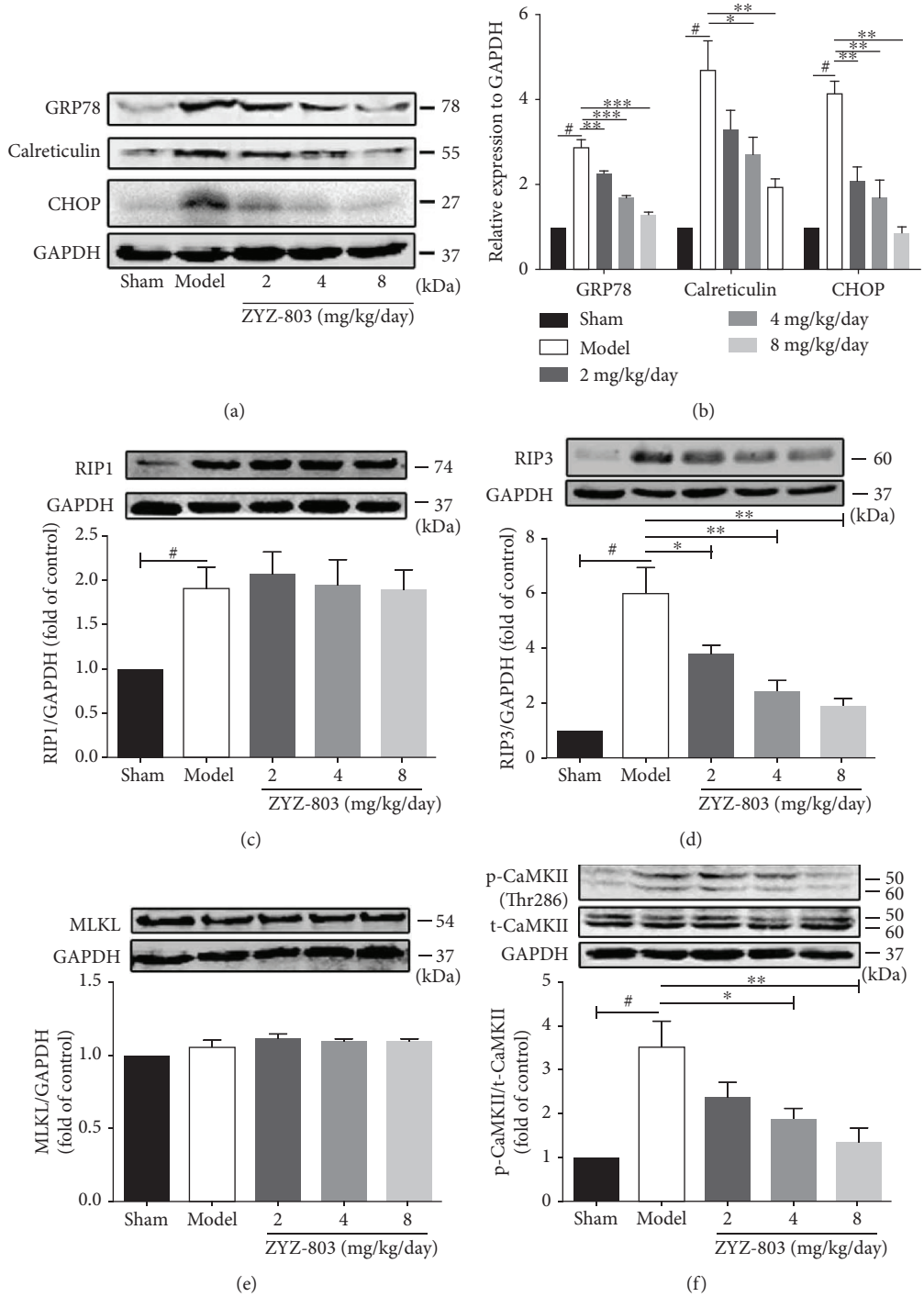
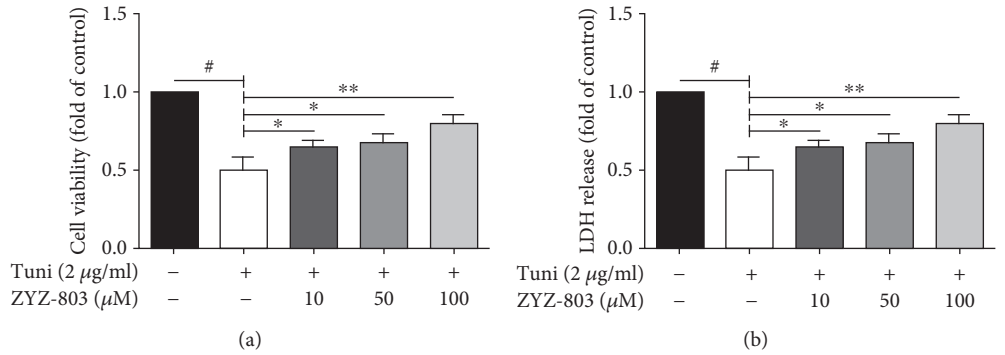


Figure 4: ZYZ-803 protects the heart from AMI injury by downregulating abnormal ERS and cardiac necroptosis. (a) Representative pictures of aberrant ERS-related protein expressions. (b) Analytical data of protein GRP78, calreticulin, and CHOP evaluated by Western blotting ($n = 4$ independent experiments). (c–f) Data of protein RIP1, RIP3, CaMKII, and MLKL expression ($n = 4$ independent experiments). Data are defined as mean ± SEM, one-way ANOVA. $^{\#}P < 0.01$ compared to the sham group; in comparison to the model group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

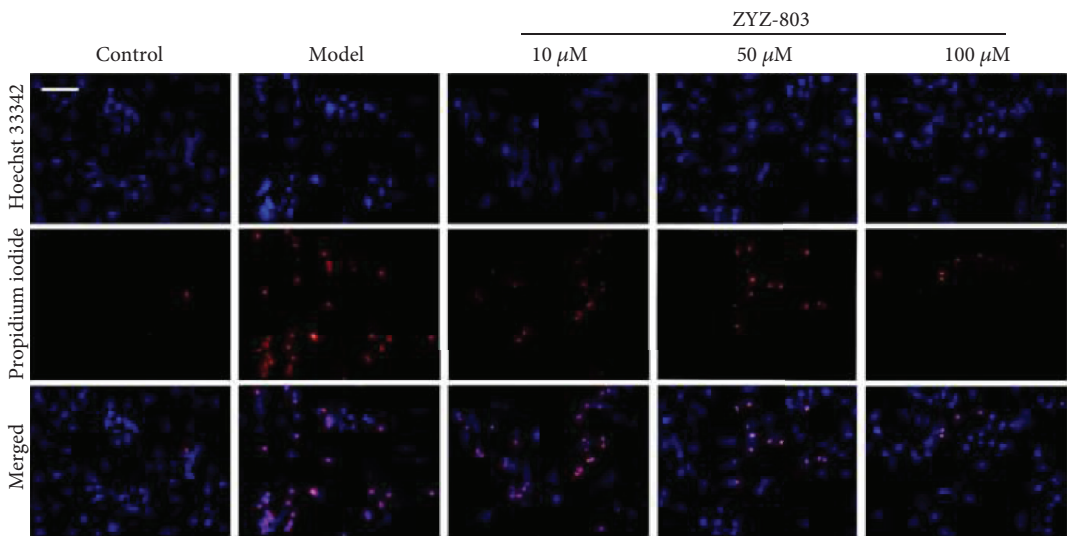
preadministration of ZYZ-803 significantly blocked the upregulated level of RIP3 and phospho-CaMKII except RIP1 and MLKL (Figure 5(h); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$), suggesting that downregulation of ZYZ-803 to ERS-related necroptosis in cardiomyocytes might be beyond depending on RIP1 and MLKL. These data demonstrated that ZYZ-803 could reduce cardiomyocyte necroptosis induced by abnormal ERS, and this is dependent on the RIP3-CaMKII signaling pathway.

3.6. ZYZ-803 Downregulates the RIP3-CaMKII Pathway to Reduce ERS-Related Necroptosis. To further explore



(a)

(b)



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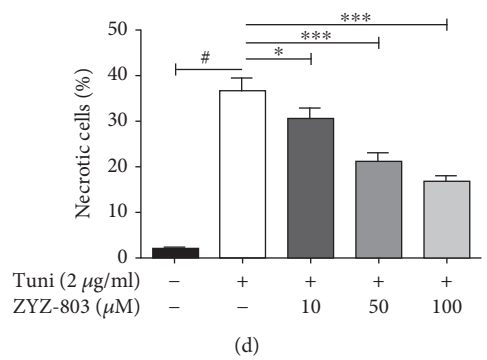

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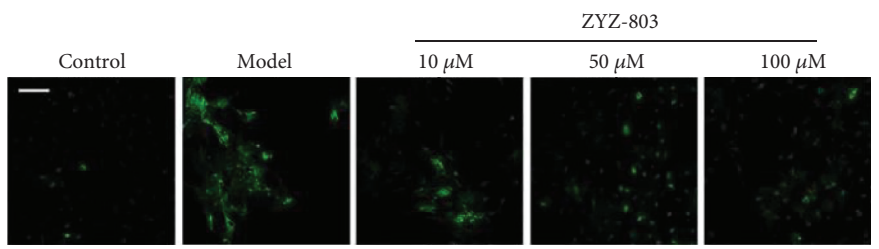


(e)

Figure 5: Continued.

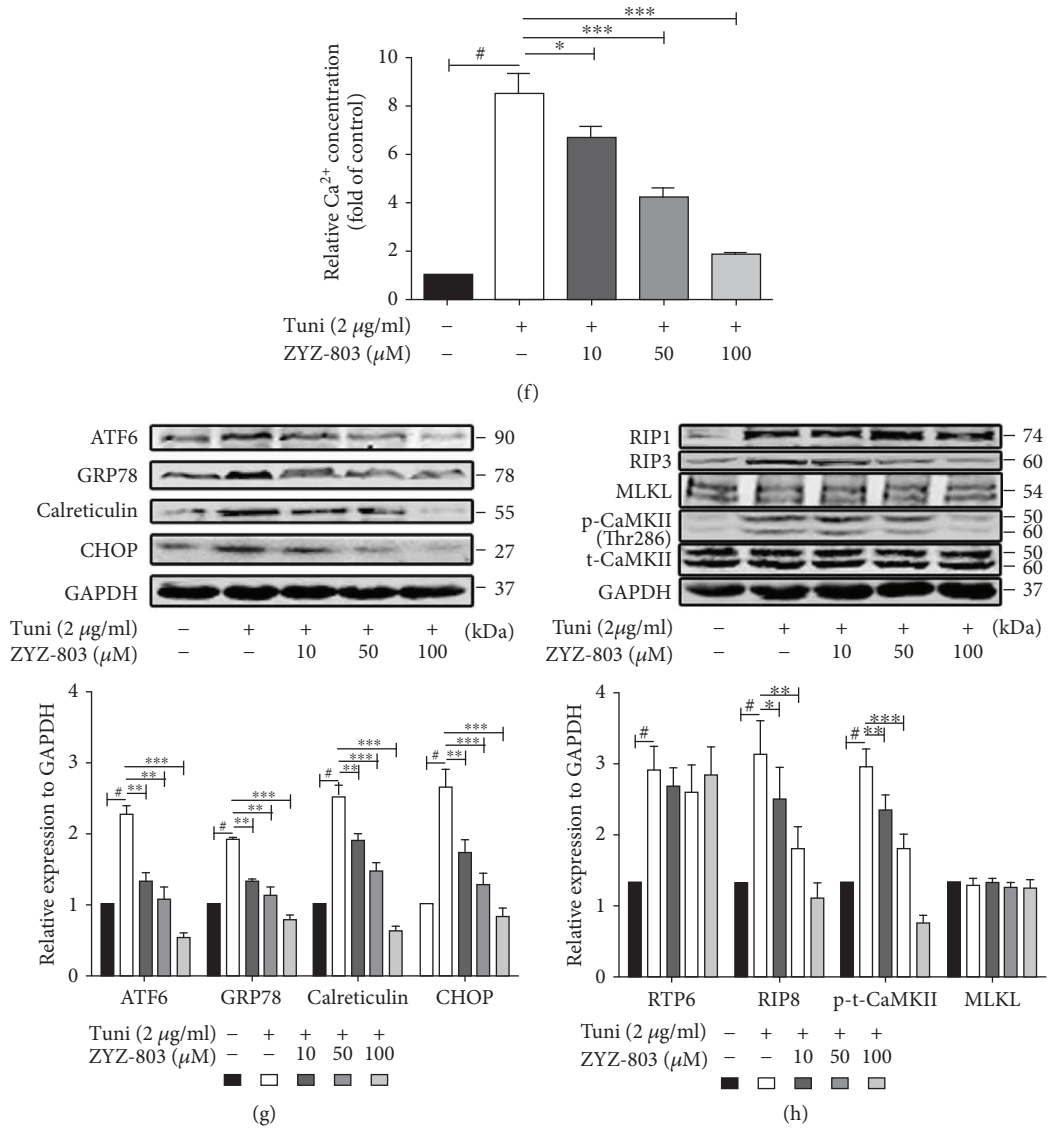


Figure 5: Cardiomyocyte necroptosis induced by abnormal ERS is alleviated by 1-hour pretreatment of ZYZ-803 *in vitro*. ERS was induced by tunicamycin 2 μg/ml for 48 hours. (a) Influence of ZYZ-803 on cell viability by the MTT assay. (b) Averaged data of LDH release from cardiomyocytes. (c) Representative photographs of cell necrosis assayed by Hoechst 33342 and propidium iodide staining; scale bar (white): 50 μm. (d) Data analysis of necrotic positive cells. (e) Intracellular $[Ca^{2+}]_i$ concentration visualized by confocal microscopy using the fluo-3/AM probe, a fluorescent Ca^{2+}-indicator dye; scale bars (white): 50 μm. (f) Statistic data of fluorescence intensity indicated intracellular calcium concentration. (g) Data of ERS-related protein expression. (h) Analysis of the expression of necroptosis protein markers. Data are defined as mean ± SEM, one-way ANOVA. $^{\#}P < 0.01$ compared to the control group; in comparison to the model group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. $n = 4$ independent experiments.

whether ZYZ-803 reduces cardiac necroptosis through downregulating RIP3-CaMKII signaling, we used small RNA interference and protein inhibitor to verify the effect of ZYZ-803 to ERS-induced necroptosis. As the results illustrated, transfection of RIP3-siRNA resulted in remarkable block of upregulated mRNA and protein expression induced by Tuni, which was similar to the effect of ZYZ-803 pretreatment without *RIP3* knockdown (Figures 6(a) and 6(b); $^{\$}P < 0.01$, $^{\$\$}P < 0.001$, $^{*}P < 0.05$, and $^{***}P < 0.001$). Furthermore, after RIP3-siRNA transfection, paralleled results were observed in the downstream of RIP3 including LDH release and CaMKII activity (Figures 6(c) and 6(d); $^{\$}P < 0.01$, $^{\$\$}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{***}P < 0.001$). Meanwhile, downregulation of phospho-CaMKII by ZYZ-803 was similar to the CaMKII inhibitor KN93, and cotreatment of ZYZ-803 and KN93 could further decrease phospho-CaMKII (Figure 6(e); $^{*}P < 0.05$, $^{**}P < 0.01$). These results suggested that ZYZ-803 could downregulate the RIP3-CaMKII signaling pathway to reduce cardiomyocyte necroptosis.

Based on the above point that ZYZ-803 could downregulate RIP3-CaMKII pathway, we next tried to detect the effect of ZYZ-803 to the interaction of RIP3 and CaMKII. In cardiomyocytes, as shown in the immunofluorescent graphs

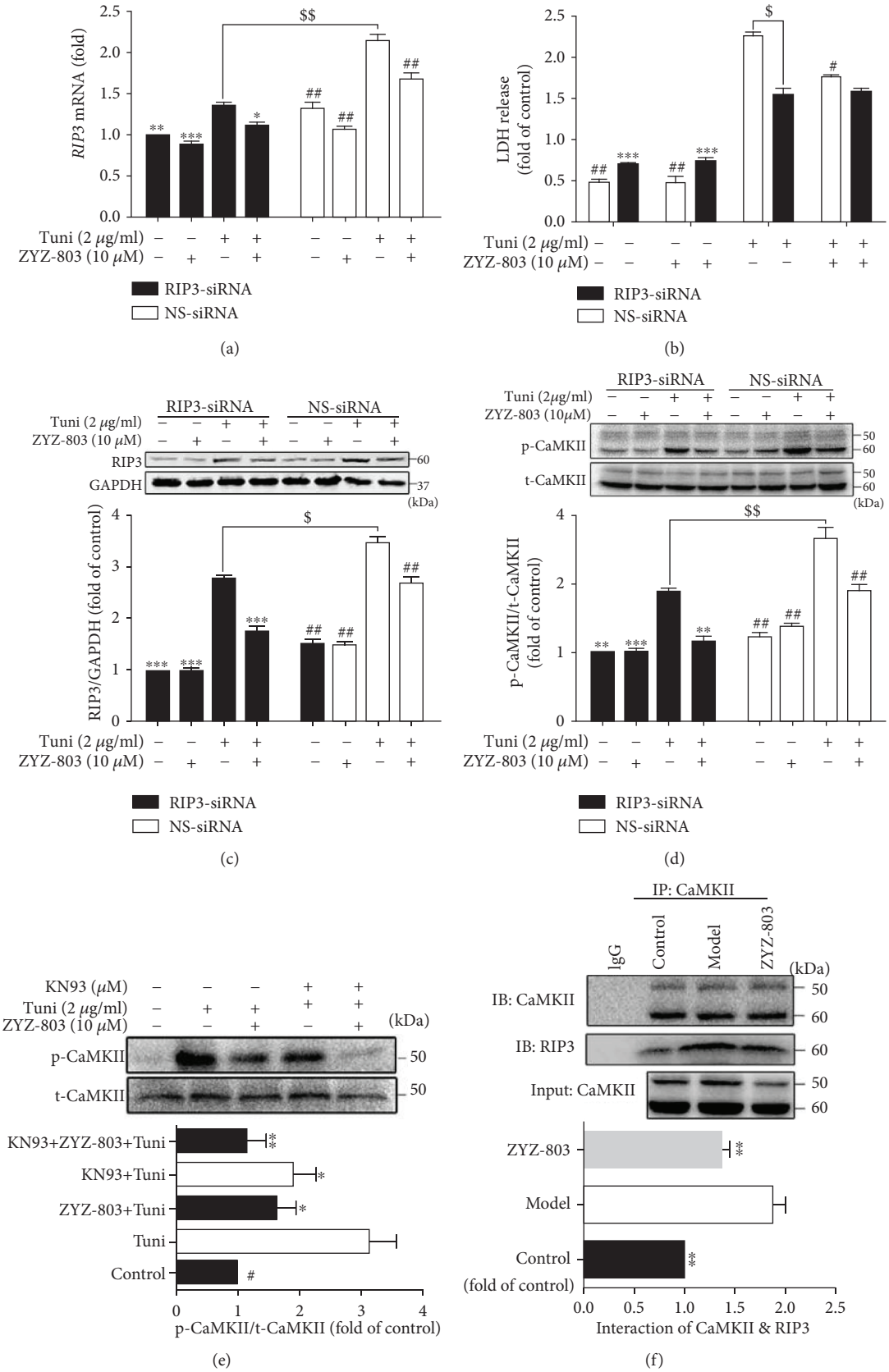
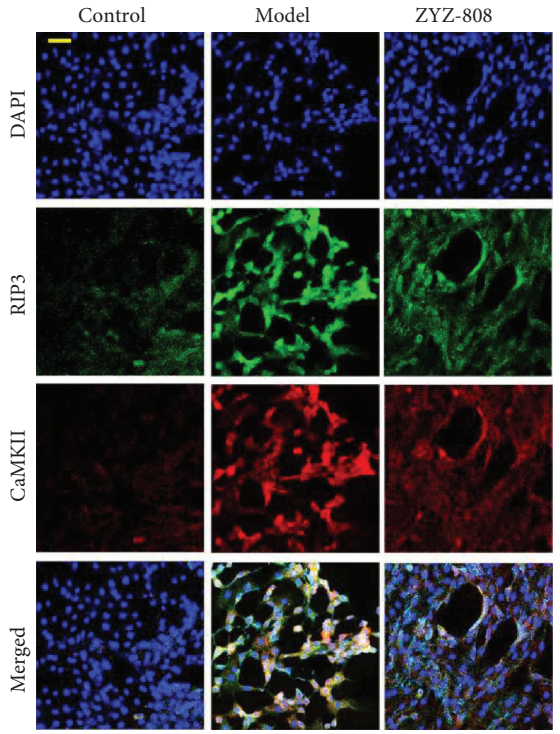


Figure 6: Continued.



(g)

Figure 6: ZYZ-803 downregulates RIP3-CaMKII signaling in ERS injury. (a) Data of *RIP3* expression in cardiomyocytes treated by RIP3-siRNA/NS-siRNA. (b) LDH release of RIP3 knockdown and WT cardiomyocytes pretreated by ZYZ-803 (10 μM) for 1 h before tunicamycin injury. (c) Protein expression of RIP3 in cardiomyocytes ($n = 3$ independent experiments). (d, e) Analytical data of p-/t-CaMKII expression in cardiomyocytes ($n = 3$ independent experiments). (f) Coimmunoprecipitation of RIP3 and CaMKII was blocked by ZYZ-803 preconditioning in heart tissues ($n = 3$ independent experiments). Data are defined as mean ± SEM, one-way ANOVA. Compared with the Tuni group (NS-siRNA), $^{\#}P < 0.001$, $^{\#\#}P < 0.001$; compared to the Tuni group (RIP3-siRNA), $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; Tuni (NS-siRNA) versus the Tuni group (RIP3-siRNA), $^{\$}P < 0.01$, $^{\$\$}P < 0.05$. (g) Immunofluorescent colocalization was decreased by ZYZ-803 in cardiomyocytes; scale bar (yellow): 50 μm.

in Figure 6(g), colocalization of RIP3 and CaMKII by double immunofluorescent staining in the ZYZ-803 group was apparently reduced compared to the model one. Additionally, formation of an immunoprecipitated complex between RIP3 and CaMKII in AMI heart tissues was significantly decreased in the ZYZ-803 preintervention group (Figure 6(f); $^{**}P < 0.01$, $^{***}P < 0.001$). These data further verified the regulation of ZYZ-803 to the RIP3-CaMKII signaling pathway as the paradigm shows in Figure 7.

4. Discussion

AMI is largely caused by epicardial coronary artery blockage from vulnerable plaques. This supports the rationale of using traditional approaches like fibrinolytic and antithrombotic therapies as well as percutaneous and surgical revascularization approaches to eliminate these obstructions. Some newly developed strategies, as described herein, focus on cardioprotection. This strategy exploits the use of drug entities that protect the heart from stress-induced injury, to prevent cell necrosis and to promote recovery. Cardiac necroptosis plays a pivotal pathological role in myocardial ischemic injury and has been seen as a new therapeutic target for ischemic heart injury [37, 38]. Meanwhile, H_2S and NO are recognized as gasotransmitters with widely pathophysiological roles in cardiovascular diseases, and both of them are dysregulated in the pathogenesis of myocardial ischemic injury, which can be rescued by H_2S and/or NO donors through different mechanisms including complex interplays of H_2S and NO [30, 39, 40]. However, there are no reports about the role of H_2S or NO in cardiac necroptosis during myocardial ischemia. From this perspective, in view of the proangiogenic role of ZYZ-803 by cooperative effect of H_2S and NO in heart failure [32], we inspiredly evaluated the cardioprotective potential of ZYZ-803 pretreatment after myocardial injury by targeting cardiac necroptosis *in vivo* and *in vitro*. The current results from animal experiments indeed revealed that ZYZ-803 pretreatment could block cardiac function damage and histopathologic deterioration in a dose-dependent manner (Figures 1 and 2 and Table S1), and cardiac necroptosis was also apparently decreased by ZYZ-803 (Figures 4(c)–4(f)). In parallel, cardiomyocyte necrosis induced by oxygen-glucose deprivation (OGD) and tunicamycin was significantly

alleviated by ZYZ-803 pretreatment (Figure S3(a), and Figures 5(a)–5(f)). Thus, these results firstly provide evidences for the cardioprotection of ZYZ-803 pretreatment through prevention of cardiac necroptosis during acute myocardial ischemia injury.

It has been widely recognized that the disturbance of endogenous production of gasotransmitters NO and H_2S is associated with the pathology of AMI [41, 42]. Thus, it is theoretically applicable to generate protective effect on AMI injury through rescuing the dysregulation of these two small gasotransmitters. Previous studies from our lab have revealed that exogenous H_2S donors such as sodium hydrosulfide and S-propargyl-cysteine can reverse dysregulation in H_2S homeostasis following AMI injury through different mechanisms [33, 42–45], and the deteriorative change after myocardial infarction is prevented by various NO donors [46–48]. In fact, it has been reported that synchronous administration of NO and H_2S donors could generate additive protective effect in ischemic heart injury [48]. In our research, we are the first to explore a novel combined donor of H_2S and NO, namely, ZYZ-803, in acute myocardial ischemia. Results showed that decreased levels of H_2S and NO in serum and heart tissues following AMI are reversed by ZYZ-803 pretreatment (Figures 3(a)–3(d)). Moreover, ZYZ-803 could reverse the aberrant expressions of H_2S synthase CBS and CSE in AMI in addition to increasing the activity of eNOS (Figures 3(c)–3(f)). Of note, in our results, we detected upregulated expression of CSE but a low H_2S level after AMI, which is contrary to the previous study [45]. The different time points of measurement of CSE and the H_2S level in the current study could be one of the reasons. Additionally, because CSE is sensitive to the change of the H_2S level, we speculate that it may be a transiently compensatory mechanism of CSE expression in risk cardiomyocytes to respond to the sharply decreased H_2S after acute myocardial ischemia. Considering that other H_2S synthetases, CBS, for example, can also contribute to H_2S generation, though not mainly as CSE in the cardiovascular system, we detected the change of CBS expression and found that CBS was decreased after AMI, which was blocked by ZYZ-803. These results just demonstrated an imbalanced change of H_2S synthetase systems that could be revised by ZYZ-803. On the other hand, it is very interesting that ZYZ-803 could also influence the synthases of H_2S and NO besides its direct release. We believe that there must be some unknown and complicated regulation mechanisms between H_2S and NO molecules and their synthases. However, more daringly, we speculate that partial H_2S and NO molecules released from ZYZ-803 might be a positive signal to stimulate their synthases for more H_2S and NO productions. By and large, from these results, we conclude that ZYZ-803 may play its protective role through cooperatively regulating the homeostases of H_2S and NO during AMI injury.

As mentioned, abnormal ERS is involved in the pathogenesis of AMI, and cardiac necroptosis has been revealed as a pivotal event in myocardial ischemia. In addition, in L929 cells, it has been shown that sustained ERS can result in necroptosis [26]. Thus, sustained ERS and necroptosis likely occur concurrently, and necroptosis might be a secondary outcome of sustained ERS during acute myocardial ischemia. In fact, we reported on the occurrence of sustained ERS and necroptosis in AMI mice and OGD cardiomyocytes, and the results clearly showed that ZYZ-803 preconditioning downregulated the expression of markers linked to these processes (Figure 4, Figure S3(b)). Furthermore, in order to verify the speculation that abnormal ERS may also induce necroptosis in cardiomyocytes, tunicamycin, a typical agonist for sustained ERS, was used to treat NRVCs, and indeed, necroptosis was remarkably induced besides sustained ERS, which was detected by decreased cell viability and increased LDH release, positive necrotic cells, intracellular Ca^{2+} overload, and representative protein markers. However, this necroptosis induced by sustained ERS was alleviated by pretreated ZYZ-803 (Figure 5). Therefore, these results implied that ZYZ-803 could ameliorate necroptosis secondary to ERS in the condition of acute myocardial ischemia.

It is recognized that the classical signaling pathway of necroptosis is dependent on the RIP1-RIP3-MLKL axis, namely, "RIP1-dependent" signaling. Typically, this pathway is usually induced by TNF-α and involves caspase-8 and the death receptor TNFR1 [37, 49]. At this context, uncleaved RIP1 and activated RIP3 form a complex called the "necrosome," which further recruits MLKL, a pseudokinase, to execute necroptosis [50, 51]. In L929 cells, sustained ERS promotes necroptosis via the RIP1-RIP3-MLKL signaling pathway [26]. However, in our work, even though obvious overexpression of RIP1 and RIP3 appeared, upregulated CaMKII activity rather than change of MLKL expression was observed both in AMI mice and in tunicamycin-induced NRVCs (Figures 4(c)–4(h) and Figure 5(h)). The possible explanation for this difference may be that MLKL does not participate in the regulation of cardiac necroptosis induced by aberrant ERS just as its negligible role in cardiac necroptosis was triggered by ischemic-reperfusion and oxidative stress [11]. Furthermore, it is interesting to note that only RIP3 and phospho-CaMKII were downregulated by ZYZ-803 preconditioning in the condition of our research (Figures 4 and 5). At this point, these results suggest that ZYZ-803 mediates its effects on ERS-related necroptosis in myocardium through the RIP3-CaMKII pathway rather than the classical "RIP1-RIP3-MLKL" axis.

Finally, in terms of the mechanisms of action for ZYZ-803, we believe that the alleviation of ERS-mediated necroptosis by ZYZ-803 is dependent on the downregulation of RIP3-CaMKII signaling in cardiomyocytes. Previous studies have shown that RIP3 knockout decreases myocardial necroptosis in myocardial infarction [9]. Similarly, in our study, RIP3 knockdown by siRNA blocked RIP3 overexpression and CaMKII phosphorylation following ERS-related necroptosis with decreased LDH release, similar to the effects seen in the ZYZ-803 pretreatment groups (Figures 6(a)–6(d)). However, ZYZ-803 pretreatment failed to further downregulate CaMKII phosphorylation after RIP3 knockdown, again suggesting an upstream effect of ZYZ-803 on RIP3. Furthermore, downregulation of ZYZ-803 to phospho-CaMKII was similar to that of

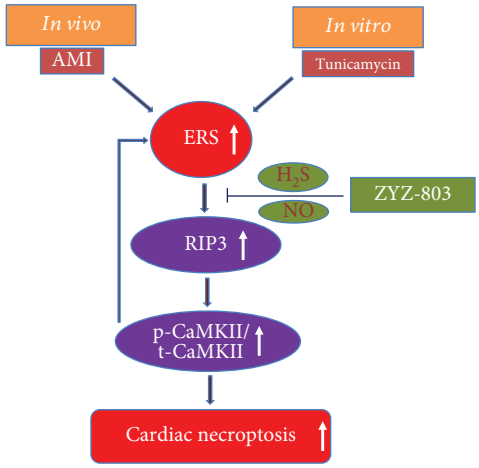


Figure 7: Schematic diagram for the new mechanism of ZYZ-803 in ER stress-related cardiac necroptosis. Aberrant ERS could be evoked during AMI and by its typical agonist tunicamycin in cardiomyocytes, which potentially induces myocardial necroptosis through the RIP3-CaMKII pathway. ZYZ-803, a dual donor for gasotransmitters H_2S and NO, could downregulate this ERS-related necroptosis at upstream of RIP3-CaMKII signaling through H_2S and NO balance and finally protects the heart from acute myocardial ischemia injury.

the CaMKII inhibitor KN93 (Figure 6(e)). Interestingly, cotreatment of ZYZ-803 and KN93 had an additive inhibition to CaMKII (Figure 6(e)). Because *S*-sulfhydration and *S*-nitrosylation of CaMKII, respectively, by H_2S and NO could inhibit activity of CaMKII [52, 53], we speculate that as a dual donor for H_2S and NO, ZYZ-803 might *S*-sulfhydrate and *S*-nitrosylate CaMKII to indirectly reduce phospho-CaMKII, which is beneficial to block cardiac necroptosis. From these results, we demonstrated that alleviation of ERS-related cardiac necroptosis by ZYZ-803 pretreatment is mediated by downregulating RIP3-CaMKII signaling. So, we finally were in turn to verify that ZYZ-803 could downregulate the interaction of RIP3 and CaMKII as determined using fluorescent colocalization in cells and immunoprecipitate blotting in animal tissues (Figures 6(e) and 6(f)). However, we have yet to determine the cellular target of ZYZ-803 that occurs upstream of RIP3. As such, further experiments will determine the mechanism of ERS-related necroptosis in myocardial injury in order to reveal the effect and targets of H_2S and NO in our model.

In conclusion, we herein have been the first to reveal that (1) the RIP3-CaMKII axis is mainly involved in ERS-related necroptosis in AMI; (2) the cardioprotective role of ZYZ-803 in AMI is realized by its regulation to aberrant H_2S and NO homeostases via releasing both H_2S and NO; (3) ZYZ-803 alleviates ERS-related necroptosis during AMI through downregulating necroptosis signaling RIP3-CaMKII. In closing, we report that ZYZ-803 protects heart tissues against acute myocardial ischemia via the targeting of ERS-related necroptosis. This work supports future studies in the development of ZYZ-803 as a clinically significant candidate for use in the treatment of AMI.

Abbreviations

AAR: Area at risk
AMI: Acute myocardial infarction
ATF6: Activating transcription factor 6
CaMKII: Ca^{2+}-calmodulin-dependent protein kinase
CBS: Cystathionine β-synthase
CHOP: CCAAT/enhancer-binding protein homologous protein
CSE: Cystathionine-γ-lyase
DMEM: Complete Dulbecco's modified Eagle medium
eNOS: Endothelial NO synthase
ER: Endoplasmic reticulum
ERS: Endoplasmic reticulum stress
GRP78: Glucose-related protein 78
HBSS: Hank's Balanced Salt Solution
HE: Hematoxylin and eosin
HRP: Horseradish peroxidase
IP: Immunoprecipitation
IS: Infarct size
LDH: Lactic dehydrogenase
LVEF: Left ventricular ejection fraction
LVFS: Left ventricular fractional shortening
LVSD: Left ventricular systolic diameter
LVSV: Left ventricular systolic volume
MI: Myocardial infarction
MLKL: Mixed lineage kinase domain-like protein
NRVCs: Neonatal rat ventricular cardiomyocytes
Phospho-CaMKII: Phosphorylated CaMKII
RIP1/3: Receptor-interacting protein 1/3
TTC: Triphenyltetrazolium chloride
Tuni: Tunicamycin.

Data Availability

The data used to support the findings of this study are included in the article and the supplementary information file. And the datasets are available from the corresponding authors on reasonable request.

Disclosure

The abstract of this manuscript has been presented as GW29-e1272 in the Journal of the American College of Cardiology after the 29th Great Wall International Congress of Cardiology (GW-ICC).

Conflicts of Interest

The authors declare no conflict of competing financial interests.

Authors' Contributions

YZZ, YCM, and LLC conceived and designed the study. FFM, BT, RZ, YX, TD, and XMX helped do some experiments. WG and YX helped analyze and interpret the data.

YZZ and YCM critically reviewed the manuscript. LLC and ZJW confirmed most of the experiments. LLC wrote the manuscript. Lingling Chang and Zhijun Wang contributed equally to this work.

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Supplementary Materials

Table S1: data of parameters indicating cardiac function in each group especially supplemented with the sham+ZYZ-803 group, which showed no side effect of ZYZ-803 preconditioning to the normal heart. Figure S1: cytotoxicity test of ZYZ-803 to cardiomyocytes showed that the ZYZ-803 dosage of more than 200 μM emerged cardiac injury. Primary cardiomyocytes were treated with ZYZ-803 for 1 hour. Data are defined as mean ± SEM, one-way ANOVA. Compared to group 0 μM, $^{*}P < 0.05$, $^{***}P < 0.001$. $n = 3$ independent experiments. Figure S2: induction of cardiomyocyte death by tunicamycin (Tuni) treatment showed that Tuni treatment at 2 μg/ml for 48 h could remarkably induce cardiomyocyte death. Data are defined as mean ± SEM, one-way ANOVA. Compared to group 0 μg/ml, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. $n = 3$ independent experiments. Figure S3: protection of ZYZ-803 against myocardial injury induced by oxygen-glucose deprivation (OGD). Primary cardiomyocytes with or without 1 h ZYZ-803 preadministration were treated with glucose-free medium and incubated in 95% N_2 and 5% CO_2 for 16 hours. (a) Assay of LDH release revealed reduction of ZYZ-803 to cardiomyocyte death induced by OGD. (b) Immunoblotting of protein expressions for ERS and necroptosis illustrated downregulation of ZYZ-803 to ERS and necroptosis induced by OGD. Data are defined as mean ± SEM, one-way ANOVA. Compared to the OGD group, $^{\#}P < 0.01$, $^{*}P < 0.05$, and $^{**}P < 0.01$. $n = 3$ independent experiments. Figure S4: no significant change of the heart rate in each experimental group. Figure S5: effect of ZYZ-803 to apoptosis. Primary cardiomyocytes with or without 1 h ZYZ-803 preadministration were treated with 2 μg/ml Tuni for 48 hours, and then, apoptosis protein markers were detected by Western blotting. Data are defined as mean ± SEM, one-way ANOVA. Compared to the model group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. $n = 3$ independent experiments. Figure S6: influence of ZYZ-803 to RIP3 and CaMKII detected by immunohistochemistry. Scale bar (black): 100 μm. *(Supplementary Materials)*

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Research Article

Ethyl Acetate Fraction of *Lannea microcarpa* Engl. and K. Krause (Anacardiaceae) Trunk Barks Corrects Angiotensin II-Induced Hypertension and Endothelial Dysfunction in Mice

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Traditional remedies prepared from *Lannea microcarpa* leaves, barks, roots, and fruits are used to treat many diseases including hypertension. This study investigated whether oral administration of the ethyl acetate fraction of *Lannea microcarpa* trunk barks (LMAE) corrects angiotensin (Ang) II-induced hypertension in mice. Its effects on vascular function were specifically investigated. Experiments explored hemodynamic and echocardiographic parameters *in vivo* and vascular reactivity to acetylcholine (ACh) and $CaCl_2$ *ex vivo* on isolated aortas. Mice received LMAE for 3 weeks (50 mg/kg/day) by oral gavage. In the last two weeks of treatment, mice were implanted with osmotic minipumps delivering NaCl (0.9%) or Ang II (0.5 mg/kg/day). LMAE completely prevented the increase in systolic and diastolic blood pressure induced by Ang II. Echocardiographic and kidney parameters were not affected by the different conditions. LMAE abrogated Ang II-induced impairment of ACh-induced relaxation without affecting that of sodium nitroprusside. LMAE also completely prevented $CaCl_2$-induced contraction in KCl-exposed aorta *ex vivo*. The extract alone did not modify superoxide ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$) production in femoral arteries from control mice but significantly limited Ang II-induced $O_2^{\cdot-}$ production. These effects were associated with reduced expression of inducible isoform of cyclooxygenase- (COX-) 2 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform NOX-2 in aortas. Finally, phytochemical analysis showed that LMAE contains sterols, triterpenes, coumarins, and anthraquinone. These results showed that LMAE prevents Ang II-induced hypertension and vascular dysfunction through a reduction of oxidative stress linked to COX-2 and NOX-2 pathway and inhibition of calcium entry. This study provides pharmacological basis of the empirical use of *Lannea microcarpa* trunk bark extract against hypertension.

1. Introduction

Hypertension is one of the most widespread and modifiable risk factors for cardiovascular disease worldwide [1, 2]. Despite improvements of antihypertensive treatments, 20-30% of patients with hypertension are resistant to at least three blood pressure-lowering drugs [3, 4]. The use of nonpharmacological treatments to lower blood pressure has been growing in the recent years and especially the administration of nutraceutical supplements based on botanicals and traditional medicine and pharmacopoeia [5].

Lannea microcarpa, commonly known as African grape, is widely distributed in the sub-Saharan region and is known for its medicinal properties. Leaf, bark, root, and fruit preparations from *Lannea microcarpa* are traditional remedies used to treat many conditions including mouth blisters, rheumatism, sore throats, dysentery, conjunctivitis, stomatitis, skin eruptions, ulcers, and high blood pressure [6, 7].

Pharmacologically, trunk bark extract of *Lannea microcarpa* has antioxidant activity [8–10] and can mitigate the damaging effects of oxidative stress on cells [11]. *Lannea microcarpa* extracts have also anti-inflammatory properties [12] and induced vascular relaxation *via* the inhibition of phosphodiesterases [13].

Physiologically, the renin-angiotensin system (RAS) is a master regulator of blood pressure and cardiovascular control of neural and endocrine functions. Moreover, the RAS participates in the onset and the evolution of cardiovascular-related diseases including hypertension, heart failure, chronic kidney disease, coronary artery disease, and stroke [14, 15]. The main mediator peptide of RAS is angiotensin II (Ang II) [16, 17] which activates AT1 receptors (AT1R) directly in the vascular smooth muscle leading to strong vasoconstriction. Sustained activation of ATIR leads to anion superoxide (O_2^-) overproduction from NADPH oxidase which in turn is responsible for the reduction of bioavailability of the endothelium-derived nitric oxide ($NO^\cdot$) and then for the endothelial dysfunction [18, 19]. Also, Ang II stimulates the synthesis and the secretion of aldosterone and consequently alters the absorption of renal sodium and water [20, 21]. One of the best experimental models of human hypertension is the chronic infusion of Ang II. This model displays an increased blood pressure, an impaired endothelium-dependent relaxation, and vascular inflammation [22]. The present study was designed to investigate whether oral administration of the ethyl acetate fraction of *Lannea microcarpa* trunk barks (LMAE) corrects Ang II-induced hypertension in mice, with a specific interest on its effects on vascular functions.

2. Materials and Methods

2.1. Plant Material. The *Lannea microcarpa* Engl. and K. Krause (Anacardiaceae) trunk barks were collected in January 2012 in the area of Loumbila (zone of savannah), located at 20 km in the northeast of Ouagadougou (Burkina Faso). The plant samples were authenticated at "Herbier National du Burkina (HNBU)" located at the "Département Environnement et Forêt/Centre National de la Recherche Scientifique et Technologique" (DEF-CNRST), Ouagadougou (Burkina Faso), where the voucher specimen had been deposited under number HNBU 361.

The collected sample was air-dried, deprived of solar light and dust, and was powdered using a mechanical grinder. The resulting powder was used for preparation of extracts for antioxidant and biological investigations.

2.2. Preparation of the Lyophilized Aqueous Decoction and Fractions with Dichloromethane and Ethyl Acetate. One hundred grams of powder of stem barks of *Lannea microcarpa* was extracted by decoction using 500 mL water distilled during 30 min. The aqueous solution was filtered and then centrifuged at 650×*g* for 5 min. The supernatant was lyophilized (14.78 g) for the various tests. Another preparation of the same concentration was used for fractionation.

The sequential extraction method with the aqueous decoction was performed with two organic solvents based on their polarity. A fractionation from the aqueous decoction (100 mL) was carried out starting with dichloromethane and then ethyl acetate. Exhaustion by the dichloromethane (3 × 50 mL) and ethyl acetate (3 × 50 mL) followed by dry evaporation (35°C) led to the fractions with dichloromethane (LMDCM, 25.2 mg) and with ethyl acetate (LMAE, 457.5 mg), respectively, used for pharmacological, antioxidant, and phytochemical investigations. LMAE was used in the present study because it represented the most effective extract in inducing vascular relaxation, in terms of pD_2 in the mice aorta and pig coronary artery compared to the aqueous decoction extracts (data not shown).

The dose of LMAE used in the present report was based on our preliminary studies (unpublished data). Invasive blood pressure measurements in rats showed that the 80% of effective dose of LMAE able to reduce hypertension was 0.5 mg/kg. Because it is assumed that only 1% of total compounds could be absorbed in the digestive tract, the dose used was then 50 mg/kg/day.

2.3. Ethics Statement. The procedure followed in the present study was approved by the local ethics committee ("Comité d'éthique en expérimentation animale Pays de la Loire"; CEEA.2011.40) in agreement with the guidelines and authorization with the French Ministry of Agriculture regulations based on the European Community.

2.4. Animals. Four groups of 8 male Swiss mice (6 to 8 weeks old and weighing 32 to 36 g) were used: (i) group receiving infusion of saline by an osmotic pump for 2 weeks (NaCl), (ii) group receiving Ang II (0.5 mg/kg/day, Sigma-Aldrich; St. Quentin Fallavier, France) by an osmotic pump for the 2 weeks (Ang II) [23], (iii) group receiving LMAE (50 mg/kg body weight/day, oral gavage, suspended in 2% of Tween 80 solution) during 3 weeks and infusion by an osmotic pump for the 2 last weeks (NaCl+LMAE), and (iv) group receiving LMAE (50 mg/kg body weight/day, oral gavage, suspended in 2% of Tween 80 solution) during 3 weeks and Ang II by an osmotic pump for the last 2 weeks (Ang II+LMAE).

Ang II and NaCl were delivered *via* unprimed osmotic minipumps (Model 1002, Alzet Osmotic Pumps, Cupertino, CA, USA) that were subcutaneously implanted into the back of mice (details of the surgical procedure were given in Supplemental Materials). All experiments were conducted in mice housed in a temperature-controlled animal facility with a 12-hour light/dark cycle and free access to rodent chow and tap water. At the end of the protocol, mice were euthanized. The blood, heart, kidneys, aorta, and femoral artery were collected for further examinations.

2.5. Blood Pressure and Heart Rate Measurements. Noninvasive blood pressure (systolic and diastolic) and heart rate were measured in conscious mice using the tail-cuff plethysmography system (Letica, Barcelona, Spain). All mice were trained with the device to accustom them to the procedure for 1 week prior to the start of the protocol. For blood pressure and heart rate determination, 6 consecutive measurements were obtained daily and averaged.

2.6. Echocardiography Examination. Cardiac function was measured as previously described [24]. Briefly, transthoracic echocardiography was performed on anesthetized (1.5% isoflurane) mice using the Vevo 770 ultrasound echograph from FUJIFILM VisualSonics (Toronto, ON, Canada) with a 30 MHz imaging transducer. Parasternal short-axis images were obtained in M-mode. Systolic and diastolic diameters, stroke volume, cardiac output, ejection and fractions, and left ventricle posterior and anterior wall (LVPW, LVAW) thickness in the systolic and diastolic phase were evaluated.

2.7. Biochemical Parameters. At the end of the protocol, blood was collected and centrifuged at 4°C for 10 min at 900×*g*. Plasma samples were frozen in liquid nitrogen and stored at -80°C until assayed. Biochemical analyses were performed with plasma using a Konelab™ 20 Clinical Chemistry Analyzer (Thermo Scientific™, Waltham, MA, USA) by assaying sodium (Na^+), chloride (Cl^-), urea and creatinine.

2.8. Myography. Ex vivo vasorelaxation experiments were conducted on thoracic aorta according to the method previously described [25]. Upon mouse euthanasia, the thoracic aorta was removed and pinned in a dissecting dish and cleaned of fat and connective tissue. Segments of the aorta (2 mm in length) were mounted on myographs (Danish Myo Technology, Aarhus, Denmark) filled with physiological salt solution (PSS). The composition of PSS (in mM) was 130 NaCl, 14.9 $NaHCO_3$, 3.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.6 $CaCl_2 \cdot H_2O$, 1.2 KH_2PO_4, and 11 glucose. The PSS was continuously kept at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2 at pH 7.4. Endothelium-dependent vasodilatation was evaluated by cumulative addition of Ach (1 nM-10 μM, Sigma-Aldrich) in order to construct a concentration-response curve on aortic rings precontracted with the thromboxane analogue A2 agonist, 9,11-dideoxy-9α,11α-methanoepoxy PGF2α (U46619, Merck Chemicals Ltd., Nottingham, UK) (80% of the maximal contractile response), as previously described [23].

Concentration-response relaxation to sodium nitroprusside (SNP, 1 nM-10 μM, Sigma-Aldrich) was also studied after precontraction of the aortas with U46619 (80% of the maximal contractile response).

In another set of experiment, the effects of LMAE on calcium-induced contractile responses of $CaCl_2$-exposed mice vessels were conducted on Swiss mice (healthy, untreated mice) thoracic aorta.

The presence of functional endothelium was assessed by the ability of ACh (10 μM, Sigma-Aldrich) to induce more than 80% relaxation of vessels precontracted with U46619. In some rings, the endothelium was denuded by gently rubbing the intimal space with forceps. Endothelium-denuded aorta ring was considered effectively removed when 10 μM of ACh caused less than 10% relaxation. After testing the response of the vessels to PSS containing KCl 134 mM, $CaCl_2$, H_2O 1.6 mM, and without NaCl (indicated concentrations of KCl substituted for equimolar amounts of NaCl, 130 mM). The bathing solution was replaced by a "calcium-free" depolarizing medium (Ca_0-KCl PSS; this PSS contains 80 mM KCl). Each preparation was exposed to Ca_0-KCl PSS concentration. After a 45 min washout period, cumulative additions of $CaCl_2$ (10^{-5} to 10^{-2} M) were repeated two times, separated by 60 min washout periods, and consecutive concentration-response curves constructed. When LMAE (500 μg/mL) was used, it was added 5 min before the cumulative addition of $CaCl_2$ on vessels exposed to Ca_0-KCl PSS [26]. LMAE was prepared freshly in distilled water and DMSO (Sigma-Aldrich) with a final concentration of 0.02% of DMSO. After each experiment, the ring length was measured using a micrograduated magnification eyepiece.

2.9. Superoxide ($O_2^{\cdot-}$) and $NO^{\cdot}$ Spin Trapping by Electron Paramagnetic Resonance (EPR) Studies. The method for $O_2^{\cdot-}$ detection was previously described [24]. The femoral arteries isolated from all mice were dissected and allowed to equilibrate in deferoxamine-chelated Krebs-Hepes solution containing 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH; 500 mM, Noxygen, Mainz, Germany), deferoxamine (25 mM, Sigma-Aldrich), and diethyldithiocarbamate (DETC; 5 mM, Sigma-Aldrich) at 37°C) for 45 min. The arteries were then frozen using liquid nitrogen.

$NO^{\cdot}$ detection was performed using DETC as a spin trap as previously described [24]. The isolated femoral arteries were incubated for 45 min in a solution containing Krebs-Hepes buffer (BSA, 20.5 g/L, Sigma Aldrich), $CaCl_2$ (3 mM), and L-arginine (0.8 mM, Sigma-Aldrich). Diethyldithiocarbamate-iron(II) complex ($Fe[DETC]_2$) solution was added to the vessel and incubated for 45 min at 37°C. The arteries were then immediately frozen in plastic tubes using liquid nitrogen.

Both $O_2^{\cdot-}$ and $NO^{\cdot}$ measurements were performed on a table-top x-band spectrometer miniscope (MS200; Magnettech, Berlin, Germany). Recordings were made at 77°K, using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 180 s of sweep time, and 4 scans. Values are expressed as the amplitude of signal per mg weight of dried femoral artery.

2.10. Western Blotting. Aorta samples were frozen in liquid nitrogen and homogenized in buffer containing 500 μL sodium dodecyl sulfate (SDS, 20%), 100 μL sodium orthovanadate, 50 μL Na-pyrophosphate, 200 μL Tris-HCl 500 mM (pH 7.4), and 400 μL antiprotease. The suspensions were centrifuged at 15,000×*g* for 15 min at 4°C. Supernatants containing the proteins were collected and stored at -80°C until use. Proteins (40 μg) were separated using 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Invitrogen, Carlsbad, CA). After electrophoresis, proteins were

transferred to nitrocellulose membranes and the membrane were then saturated at room temperature for 1 h in TBS-T (20 mM Tris base, 61.5 mM NaCl pH 7.8, and 0.1% Tween 20) buffer containing 5% BSA. The membrane was incubated with primary antibody for 2 h at room temperature. The murine polyclonal antibody for cyclooxygenase 1 (COX-1, 1 : 1000, Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal antibody for cyclooxygenase 2 (COX-2, 1 : 500, BD Pharmingen, San Jose, CA), mouse monoclonal antibody for NADPH oxidase 2 (NOX-2: gp91-phox, 1 : 1000, Santa Cruz Biotechnology), and goat polyclonal antibody for NADPH oxidase 4 (NOX-4, 1 : 1000, Santa Cruz Biotechnology) were used. The same membrane was used to determine β-actin expression (loading) control using a polyclonal anti-mouse β-actin antibody (1 : 5000, Sigma Aldrich). The membrane was then incubated for 90 min at room temperature with the horseradish peroxidase- (HRP-) conjugated secondary antibody. Membranes were washed at least three times in Tris buffer solution containing 0.05%. The bands were visualized using the enhanced chemiluminescence system and quantified by densitometry. Images analysis were performed using ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA).

2.11. DPPH˙ Assay. The radical scavenging activity was performed using 2,2-diphényl-1-picrylhydrazyl (DPPH, Sigma-Aldrich) as previously described [27, 28]. Briefly, the absorbance of 10 μL of samples, standard, and Trolox (Sigma-Aldrich) added to 200 μL of DPPH was measured at 490 nm after 30 min incubation in the dark at room temperature using a Bio-Rad spectrophotometer (Model 680, Japan). The result was expressed as Trolox (Sigma-Aldrich) equivalent antioxidant capacity according to the following equation: TEAC = extract antiradical power (ARP)/Trolox ARP, where ARP was the amount of antioxidant necessary to decrease the initial DPPH˙ concentration by 50% (ARP = 1/IC50).

2.12. Ferric Reducing Antioxidant Power (FRAP) Assay. FRAP assay was performed in extract, fractions, and Trolox as previously described [29]. The mixture of 0.5 mL of samples with 1.25 mL of phosphate buffer and 1.25 mL of aqueous solution of potassium hexacyanoferrate (1%, Prolabo, Paris, France) was incubated for 30 min at 50°C. Then, 1.25 mL of trichloroacetic acid (10%, Sigma-Aldrich) was added and centrifugated at 3000×g for10 min. Distilled water (0.625 mL) and $FeCl_3$ solution (0.125 mL, 0.1%) were added to the upper layer solution (0.625 mL), and the absorbance was measured at 700 nm using a spectrophotometer (Agilent, Santa Clara, CA) equipped with UV-visible ChemStation software. Trolox was used to the plot calibration curve. FRAP activity of samples was expressed in mmol Trolox equivalent/gram of dry extract.

2.13. Phytochemical Screening by Liquid Medium. Phytochemical screening of the decoction and fractions of stem barks of *Lannea microcarpa* was conducted following the Ciulei method [30]. Phytochemical groups were determined in the different samples using the following characterization tests: iron chloride test, Shibata test, Liebermann-Büchard test, foam index, and fluorescence to the UV lamp 365 nm for identification of tannins, flavonoids, sterols and triterpenes, saponins, and coumarins, respectively. Borntranger's test was used for the detection of anthraquinones and emodols. Alkaloids were characterized by the reactions of Dragendorff and Mayer. The reducing compounds were characterized by the reaction of the Fehling reagent. For the anthocianosides tests, 1-2 sodium hydroxide pellets were added to 1 mL of extract with the appearance of a blue color the presence of anthocyanins in the extract.

2.14. Phytochemical Analysis of Extracts by Thin-Layer Chromatography (TLC). The LMAE fraction was loaded on Silica gel 60 F_{254} plates (Merck). The elution was carried out using two solvent systems: (i) n-hexane/ethyl acetate/toluene (3/1/1 $v/v/v$) was used to migrate anthocianosides, coumarins, sterols and triterpenes, and tannin compounds and (ii) ethyl acetate/formic acid/distilled water (6/1/1 $v/v/v$) was used to migrate saponin compounds.

Samples (10 mg/mL, 10 μL) were directly loaded as spot into the TLC plates. The coumarin compounds were observed under a UV lamp (254/366 nm) after spraying with a specific developer. The developer 5% KOH in 95% methanol was used to show the presence of anthracenosids and coumarins. Anisaldehyde-sulfuric acid was used to detect saponins, and 2% $FeCl_3$ in 95% methanol was used to detect sterols, triterpenes, and tannins.

2.15. Statistical Analysis. Data were analyzed using GrapPad Prism 5.02 software (GrapPad Software, San Diego, CA, USA). The results are expressed as mean ± SEM. For animal experiments, n represented the number of mice. Blood pressure, heart rate, and myography experiment values were compared using a two-way ANOVA with Bonferroni post hoc test. Data of western blot and electron paramagnetic resonance experiments as well as DPPH˙ and FRAP assays were compared using one-way ANOVA followed by a Bonferroni post hoc test. $^*p < 0.05$ was considered to be significant.

3. Results

3.1. Blood Pressure and Heart Rate. In mice receiving saline alone and saline (NaCl 0.9%) plus LMAE (Figures 1(a) and 1(b)), systolic blood pressure and diastolic blood pressure were stable throughout the experiment. As expected, Ang II increased the systolic and diastolic blood pressure, which became significant at day 8. In the other group of mice, LMAE completely prevented Ang II-induced hypertension (Figures 1(c) and 1(d)). Also, independently from treatment, heart rate values were not modified in any group of mice throughout the experiments (Figures 1(e) and 1(f)).

3.2. Heart and Kidney Function. Cardiac parameters measured by echocardiography (Table 1) were not affected by the different treatments throughout the study.

No significant changes in heart and kidney weights were observed in all groups of mice (Supplemental Figures 1A and

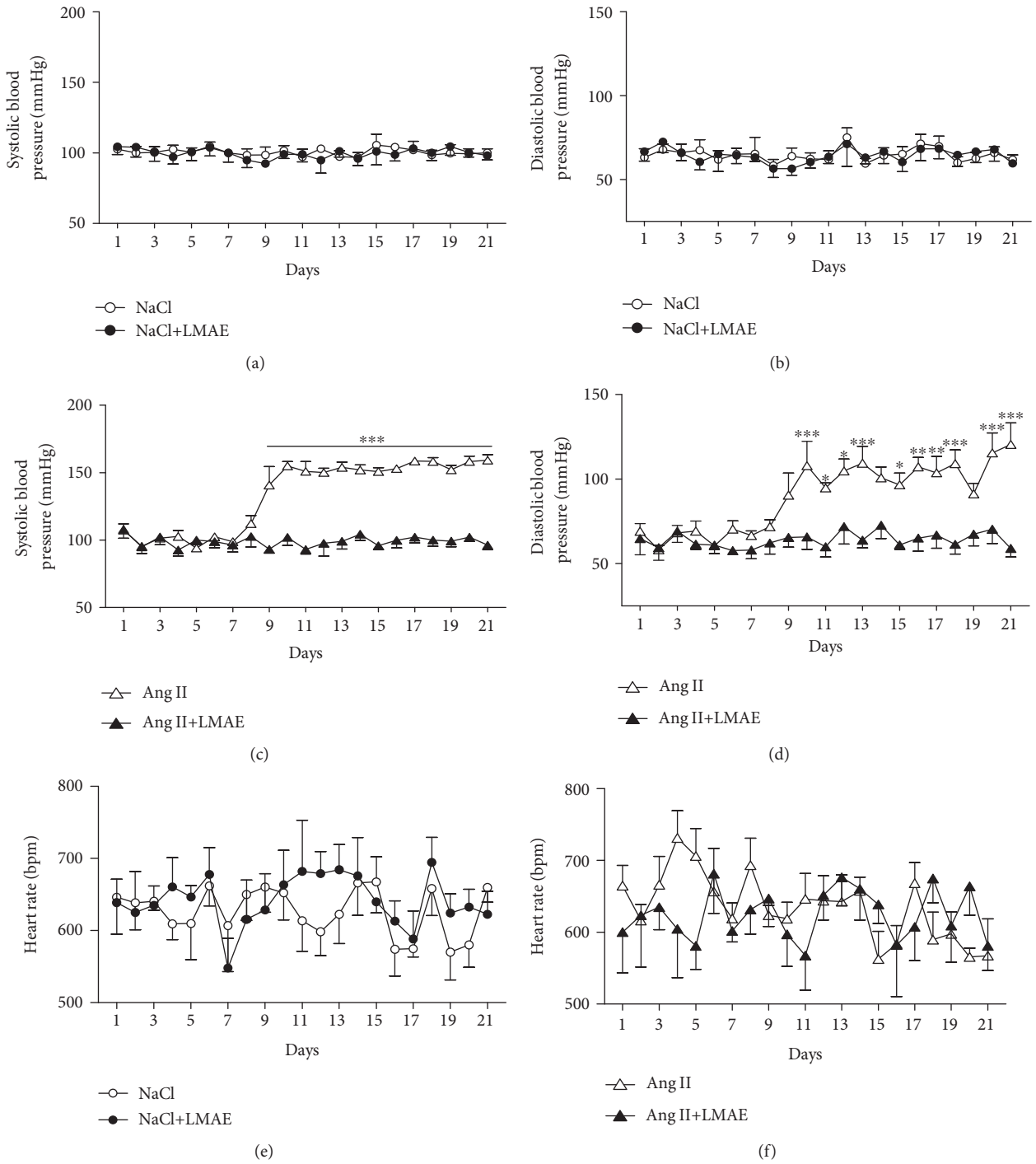

Figure 1: Effects of LMAE on systolic and diastolic blood pressure and heart rate in an Ang II-induced hypertension model. LMAE (50 mg/kg) was administered daily by oral gavage for 3 weeks, and osmotic minipumps delivering NaCl (0.9%) or angiotensin II (0.5 mg/kg/day) were implanted 1 week after the start of the LMAE pretreatment. The effect of LMAE was studied in control mice (a, b, and e) and Ang II-treated mice (c, d, and f). Systolic blood pressure (a, c) and diastolic blood pressure (b, d) were measured daily as well as the heart rate (e, f). Angiotensin II (Ang II) treatment induced hypertension that was prevented by LMAE. NaCl: mice treated with NaCl, control; NaCl+LMAE: mice treated with LMAE and NaCl; Ang II: mice treated with Ang II; Ang II+LMAE: mice treated with LMAE and Ang II. NaCl and Ang II groups were treated with an equal volume of vehicle (2% of Tween 80). Results are given as means ± SEM. $n = 7$-8/group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

1B). Also, neither LMAE nor Ang II affected kidney markers (creatinine, urea) and electrolytes (Na^+ and Cl^-) in all groups of mice (Supplemental Figure 1C–1F).

3.3. Vascular Function. ACh-induced endothelium-dependent relaxation was significantly reduced in the aortas of Ang II-treated mice compared to other groups of mice.

Table 1: Effect of LMAE on echocardiography parameters in an Ang II-induced hypertension model.

	NaCl	NaCl+LMAE	Ang II	Ang II+LMAE
Diastolic diameter (mm)	4.4 ± 0.4	4.5 ± 0.2	4.4 ± 0.3	4.4 ± 0.3
Systolic diameter (mm)	3.2 ± 0.3	3.3 ± 0.2	3.0 ± 0.4	3.2 ± 0.4
Stroke volume (mL)	46.2 ± 8.6	51.7 ± 8.0	51.8 ± 5.3	48.7 ± 6.5
Cardiac output (mL/min)	36.0 ± 17.6	22.1 ± 4.2	27.5 ± 16.6	22.6 ± 5.9
Ejection fraction (%)	53.6 ± 3.9	54.4 ± 5.6	59.8 ± 7.3	54.7 ± 6.8
Shortening fraction (%)	27.6 ± 2.4	28.2 ± 3.9	32.2 ± 5.0	28.4 ± 4.2
Diastolic LVAW (mm)	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.3
Diastolic LVPW (mm)	0.9 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
Systolic LVAW (mm)	1.3 ± 0.1	1.5 ± 0.3	1.4 ± 0.2	1.4 ± 0.4
Systolic LVPW (mm)	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.2

LMAE (50 mg/kg) was administered daily by oral gavage for 3 weeks, and osmotic minipumps delivering NaCl (0.9%) or angiotensin II (0.5 mg/kg/day) were implanted 1 week after the start of the LMAE pretreatment. Cardiac function was evaluated by echocardiography. This table shows the systolic and diastolic diameters, stroke volume, cardiac output, ejection and fractions, and left ventricle posterior and anterior wall (LVPW, LVAW) thickness in the systolic and diastolic phase. NaCl: mice treated with NaCl, control; NaCl+LMAE: mice treated with LMAE and NaCl; Ang II: mice treated with Ang II; Ang II+LMAE: mice treated with LMAE and Ang II. NaCl and Ang II groups were treated with an equal volume of vehicle (2% of Tween 80). The results are expressed as mean ± SEM. $n = 8$/group.

LMAE extract prevented Ang II-induced endothelial dysfunction (Figures 2(a) and 2(b)). LMAE prevented both the decreased sensitivity (pD_2) and reduced maximal relaxation (Emax) to ACh induced by Ang II (Figures 2(c) and 2(d)) with a relaxation profile similar to the control groups (NaCl), LMAE (NaCl+LMAE).

Relaxation to SNP and vasoconstriction induced by 80 mM KCl were similar in the four groups studied (Figures 2(e) and 2(f)).

3.4. O_2^- and $NO^\cdot$ Production in the Femoral Artery. LMAE did not affect the O_2^- level in the femoral artery of control animals. Ang II treatment significantly increased the O_2^- level. This was completely prevented by LMAE treatment (Figure 3(a)).

$NO^\cdot$ production in the femoral artery was not statistically different in the four groups studied, although LMAE seemed to slightly increase its production in control vessels (Figure 3(b)).

3.5. NADPH and COX Pathway Evaluation. Protein expression of enzymes involved in the modulation of reactive oxygen species production, including COX-1, COX-2, NOX-2, and NOX-4, was analyzed (Figure 4(a)). The aortas from Ang II-treated mice displayed a nonsignificant increased expressions of COX-1 and COX-2 compared to those isolated from either NaCl or NaCl+LMAE mice. Interestingly, LMAE treatment significantly decreased COX-2 but not COX-1 expressions in the aortas isolated from Ang II-treated mice compared to those from Ang II-treated mice alone (Figures 4(b) and 4(c)).

Ang II treatment significantly increased aortic expressions of NOX-2 compared to nonhypertensive groups, without affecting NOX-4 (Figures 4(a), 4(d), 4(e)). LMAE treatment prevented Ang II-induced increase of NOX-2 expression in the aorta (Figure 4(d)).

3.6. In Vitro Characterization of LMAE Antioxidant Activity. Antioxidant activity of aqueous decoction extract (LMaq) and its fractions dichloromethane (LMDCM) and ethyl acetate (LMAE) of *Lannea microcarpa* was investigated *in vitro* using Trolox equivalent antioxidant capacity (TEAC) assay and FRAP assay. LMAE was the most potent antioxidant extract with an antioxidant capacity similar to the standard, Trolox. LMaq had a lower antioxidant capacity compared to LMAE, and LMDCM antioxidant capacity was almost negligible (Figure 5(a)).

The FRAP assay measured the reductive activity of Fe^{3+}. LMAE displayed a higher reductive activity compared to LMaq and LMDCM that showed similar effects (Figure 5(b)).

3.7. Ex Vivo Characterization of LMAE on Vasoconstriction. The effect of LMAE was also investigated on calcium-induced contraction ex vivo. Cumulative concentrations of $CaCl_2$ (10^{-5}-10^{-2} M) were added to aortic rings with intact- or denuded endothelium in Ca^{2+}-free Krebs solution, containing 80 mM KCl to activate voltage-dependent calcium channels (VDCCs) (Figure 6). Compared to vehicle (0.02% of DMSO), preincubation with LMAE (500 μg/mL) dramatically inhibited $CaCl_2$-induced contraction of the aortas with (Figure 6(a)) and without functional endothelium (Figure 6(b)).

3.8. Phytochemical Characterization of Lannea microcarpa Trunk Bark Extracts. The phytochemical study of the powder of *Lannea microcarpa* trunk barks showed the presence of steroids and triterpenoids in the dichloromethane fraction. The ethyl acetate fraction showed the presence of anthraquinone, steroids, triterpenoids, and coumarins. The residual aqueous fraction showed the presence of tannins and saponins. The qualitative phytochemical analysis in liquid medium of *Lannea microcarpa* trunk bark extracts is summarized in Table 2. The representation of

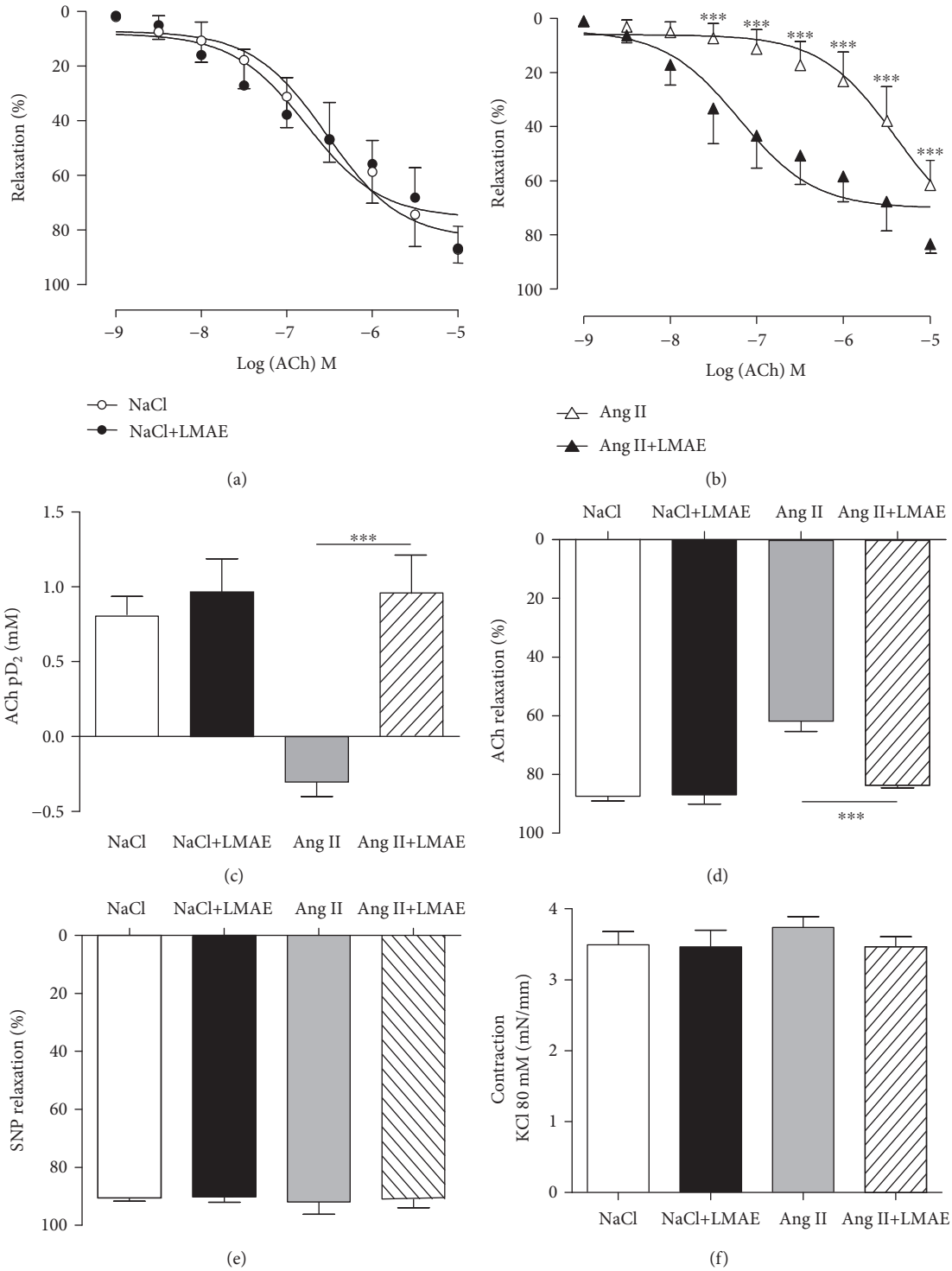

Figure 2: Effect of LMAE on vascular function *ex vivo*. LMAE (50 mg/kg) was administered daily by oral gavage for 3 weeks, and osmotic minipumps delivering NaCl (0.9%) or angiotensin II (0.5 mg/kg/day) were implanted 1 week after the start of the LMAE pretreatment. The effect of LMAE on Ach-induced vasorelaxation was studied in aortic rings precontracted with U46619 isolated from control mice (a) and Ang II-treated mice (b). Sensitivity to acetylcholine represented by pD2 (c) and maximal effect (Emax) (d) were measured. The same aortic rings were relaxed with SNP, Emax (e); after precontraction with KCl (80 mM), Emax (f). NaCl: mice treated with NaCl, control; NaCl+LMAE: mice treated with LMAE and NaCl; Ang II: mice treated with Ang II; Ang II+LMAE: mice treated with LMAE and Ang II. NaCl and Ang II groups were treated with an equal volume of vehicle (2% of Tween 80). The results are expressed as mean ± SEM. $n = 7$-8/group; $^{***}p < 0.001$.

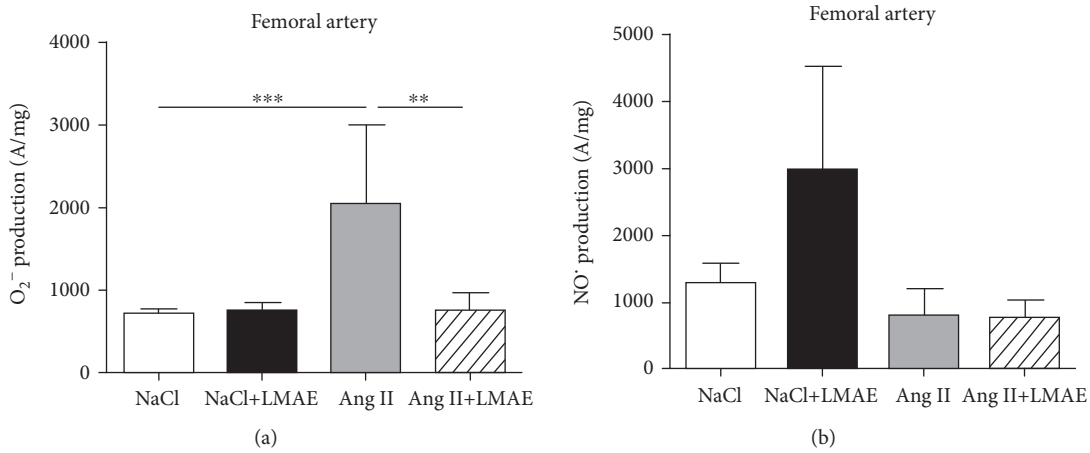

Figure 3: Effects of LMAE on superoxide and nitric oxide production *ex vivo*. LMAE (50 mg/kg) was administered daily by oral gavage for 3 weeks, and osmotic minipumps delivering NaCl (0.9%) or angiotensin II (0.5 mg/kg/day) were implanted 1 week after the start of the LMAE pretreatment. Nitric oxide ($NO^{\cdot}$) (a) and superoxide (O_2^{-}) (b) production was measured in femoral arteries. NaCl: mice treated with NaCl, control; NaCl+LMAE: mice treated with LMAE and NaCl; Ang II: mice treated with Ang II; Ang II+LMAE: mice treated with LMAE and Ang II. NaCl and Ang II groups were treated with an equal volume of vehicle (2% of Tween 80). The results are expressed as mean ± SEM. $n = 7$-8/group; $^{*}p < 0.05$.

revealed phytochemical groups by the TLC was illustrated in Supplemental Figure 2.

4. Discussion

We report that LMAE completely prevented Ang II-induced hypertension without modification of cardiac nor kidney functions. The beneficial effects of LMAE treatment was associated with improvement of endothelial dysfunction and decrease of arterial O_2^{-} production. This effect was probably due to the capacity of LMAE to reduce expression of prooxidant enzymes such as COX-2 and NOX-2 in the aorta. It can also be linked to the strong antioxidant properties of LMAE *in vitro*. Finally, LMAE prevented $CaCl_2$-induced contraction in the KCl-exposed aorta ex vivo. Altogether, this study established for the first time the mechanism underlying the antihypertensive action of LMAE which seemed to target mainly blood vessels.

Our aim was to provide pharmacological basis for the use of traditional remedies such as the extract of *Lannea microcarpa* trunk barks to treat hypertension in a well-established animal model. This was based on preliminary studies reporting potent antioxidant properties of *Lannea microcarpa* extracts and fractions obtained from the leaves, seed, and fruit. Moreover, LMAE had been shown to induce vasorelaxation of the rat thoracic aorta *via* inhibition of phosphodiesterases [13]. All of these effects may concur to the potential protective effect of LMAE. However, these studies have been performed in normotensive animals and the antihypertensive effect of LMAE was not characterized and the mechanism underlying its actions not completely understood.

In the present report, we demonstrate that LMAE is efficient against Ang II-induced hypertension in mice. Of particular interest was that LMAE did not possess hypotensive property in normotensive mice. LMAE did not affect the cardiac or kidney structure and function in an Ang II-induced hypertension model. These results indicate that the antihypertensive effect of LMAE is mainly due to its vascular action, leading to the reduction of vascular resistance. Correction of endothelial dysfunction has been reported previously in the same experimental model of hypertension using red wine polyphenols [31] or microparticles bearing sonic hedgehog [23]. With regard to red wine polyphenols, prevention of vascular NADPH oxidase induction and preservation of arterial $NO^{\cdot}$ availability during Ang II administration likely contributed to this effect. For microparticles bearing sonic hedgehog, increased $NO^{\cdot}$ production and reduction of oxidative stress concurred to endothelial protection.

We showed that Ang II treatment impaired endothelium-dependent relaxation to Ach and LMAE completely prevented endothelial dysfunction. It is well established that vascular oxidative stress induced endothelial dysfunction most likely by inactivating $NO^{\cdot}$. In the present work, LMAE alone did not modify O_2^{-} and $NO^{\cdot}$ productions in the femoral arteries but significantly reduced O_2^{-} in the vessels from angiotensin II-treated mice. Thus, LMAE might exert its protective effect by decreasing oxidative stress. Although we did not observe an increase in $NO^{\cdot}$ production in the vessels of Ang II-treated mice, the reduced level of O_2^{-} may lead to increase in $NO^{\cdot}$ bioavailability. This might be explained by the fact that the affinity of $NO^{\cdot}$ issued by eNOS is greater in acting with O_2^{-} within the endothelial cell compared to its affinity with the spin trap used, namely, DETC. Thus, $NO^{\cdot}$ may be blunted by the O_2^{-} release to form $ONOO^{-}$. This hypothesis is reinforced by the potent antioxidant properties of LMAE *in vitro*, comparable to those of the reference control, Trolox.

The mechanism by which LMAE decreased oxidative stress was further examined by assessing changes in the expression of endogenous prooxidant enzymes in the arterial wall. Among these enzymes, different reports had shown the importance of COX-2 and NADPH oxidase in the

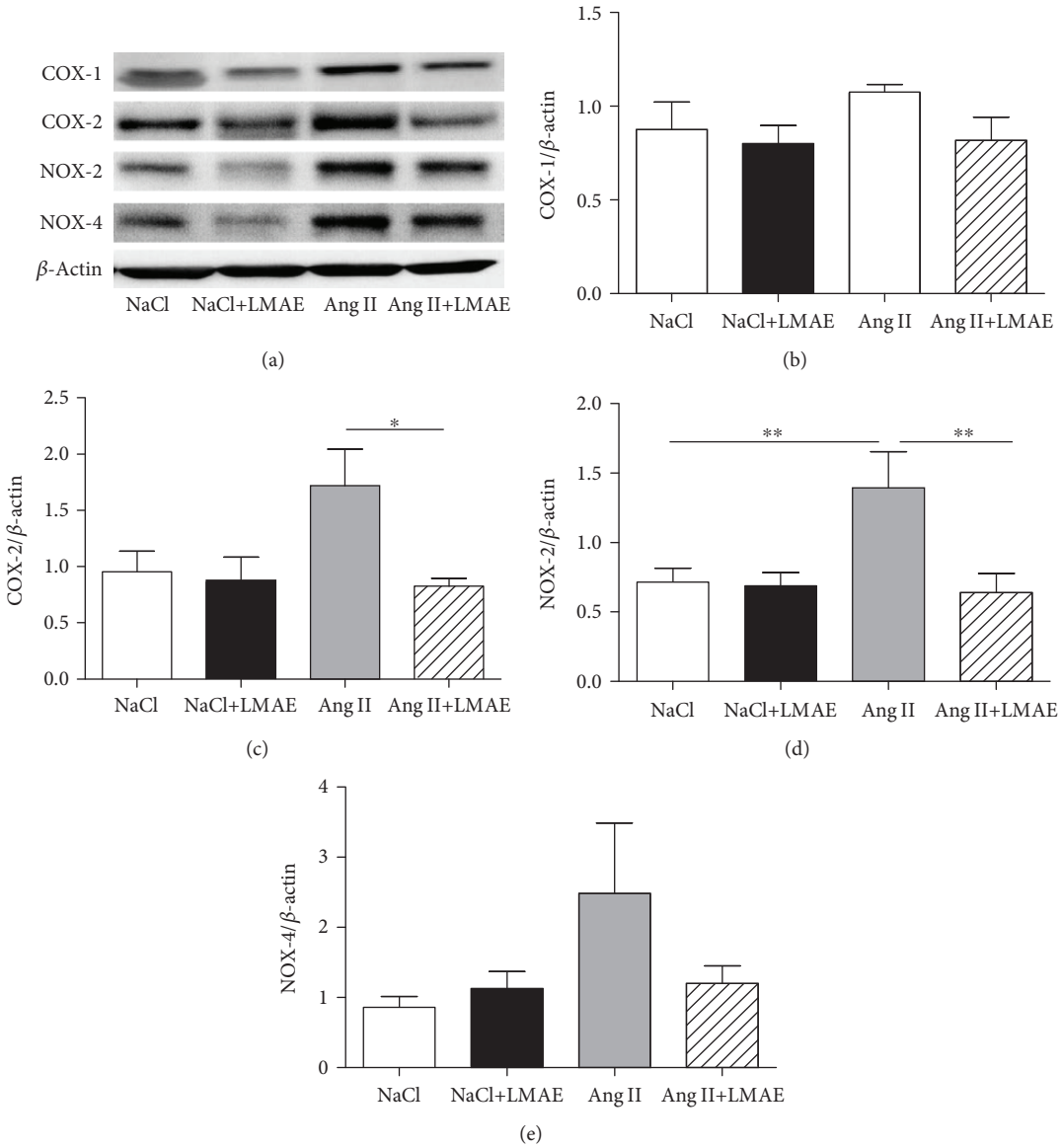

Figure 4: Effects of LMAE on the protein expression of prooxidant enzymes *ex vivo*. LMAE (50 mg/kg) was administered daily by oral gavage for 3 weeks, and osmotic minipumps delivering NaCl (0.9%) or angiotensin II (0.5 mg/kg/day) were implanted 1 week after the start of the LMAE pretreatment. The protein expression of prooxidant enzymes was measured in isolated aortas. A representative image of the blots is shown in (a). Quantitative evaluation of COX-1 (b), COX-2 (c), NOX-2 (d), Ang II under grey histogram and NOX-4 (e) protein expressions was performed. NaCl: mice treated with NaCl, control; NaCl+LMAE: mice treated with LMAE and NaCl; Ang II: mice treated with Ang II; Ang II+LMAE: mice treated with LMAE and Ang II. NaCl and Ang II groups were treated with an equal volume of vehicle (2% of Tween 80). The results are expressed as mean ± SEM. $n = 5$-6/group; $^*p < 0.05$.

endothelial dysfunction, oxidative stress, and progression of Ang II-induced hypertension [23]. Indeed, COX-2 activation induced the release of COX-derived vasoconstrictor metabolites [32]. Also, in the arterial wall of rats, both membrane-bound NADH/NADPH oxidase activity and the expression of different several NADPH oxidase subunits were increased [31]. Using the experimental model similar to the present study, the concomitant overproduction of reactive oxygen species from NADPH oxidase and/or mitochondria and the activation of COX-2/TP receptor pathway provoked vascular dysfunction including endothelial dysfunction, increased vascular reactivity, and hypertension [23]. In the present study, LMAE treatment reduced the expression of both the inducible isoform of cyclooxygenase, COX-2, and the NADPH oxidase isoform NOX-2 of the mouse aortas. The antioxidant property of LMAE could act in synergy with its ability to regulate the expression of prooxidant enzyme to mitigate the damaging effects of oxidative stress and exert vasculoprotection [33, 34]; such mechanism could be supporting the antihypertensive effect of LMAE.

Supporting this hypothesis, it is known that hypertension is associated with increased vascular reactivity to vasoconstrictor agents. Increased cytosolic calcium, *via* either voltage-dependent calcium channels, receptor-dependent

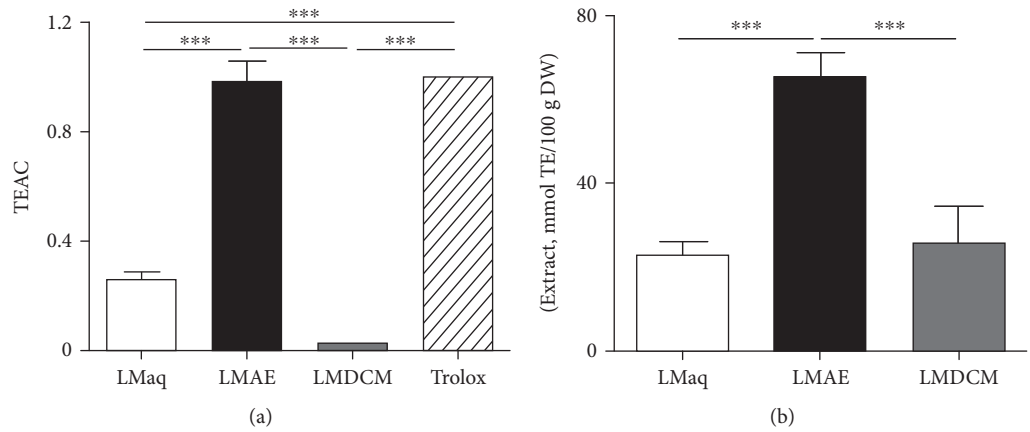

Figure 5: *In vitro* antioxidant activity of *Lannea microcarpa* trunk bark extracts. Antioxidant activity of aqueous decoction extract (LMaq) and its fractions dichloromethane (LMDCM) and ethyl acetate (LMAE) of *Lannea microcarpa* was investigated *in vitro* using $DPPH^{\cdot}$ (a) assay and FRAP assay (b). TEAC: Trolox equivalent antioxidant capacity; TE: Trolox equivalent; DW: dry weight. The results are expressed as mean ± SEM of triplicate; $^{***}p < 0.001$.

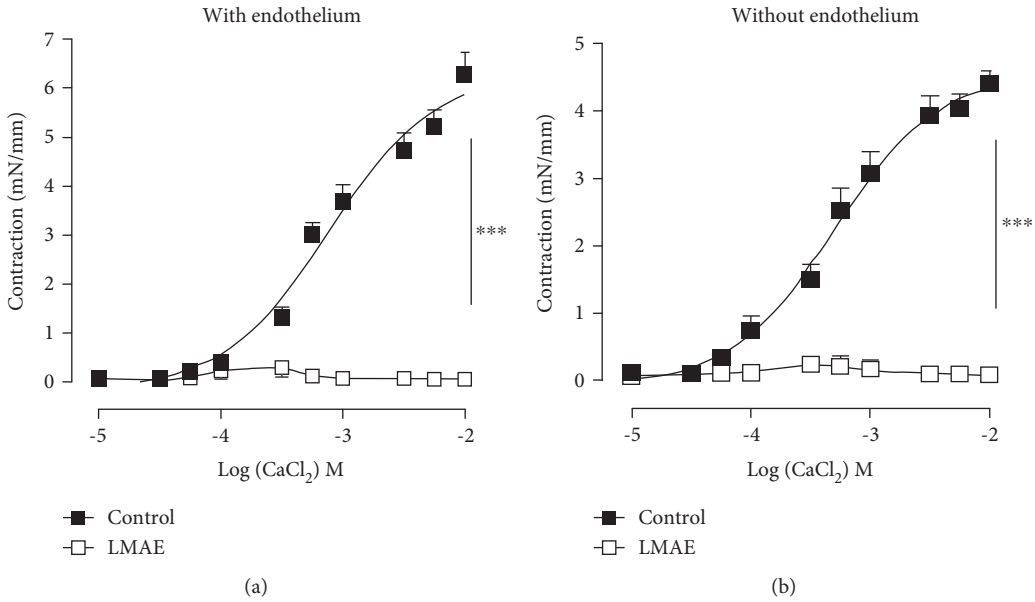

Figure 6: Effect of LMAE on CaCl2-induced contraction *ex vivo*. Cumulative concentrations of CaCl2 (10^{-5}-10^{-2} M) were added to aortic rings with intact (a) or denuded (b) endothelium in Ca^{2+}-free Krebs solution, containing 80 mM KCl, to activate voltage-dependent calcium channels (VDCCs). Aortic rings were either preincubated without vehicle (0.02% of DMSO) or LMAE (500 μg/mL). The results are expressed as mean ± SEM. ($n = 5$). $^{***}p < 0.001$.

calcium entry, or release of Ca^{2+} from intracellular stores, in addition to calcium sensitization of contractile proteins participates to vascular hyperactivity observed in hypertension. Notably, the contractile response to agonist relative to calcium entry is primarily due to voltage-dependent calcium channels beside the involvement of calcium entry *via* receptor-operated calcium channels [35, 36]. In the present study, LMAE completely prevented $CaCl_2$-induced contraction in the KCl-exposed mice aorta *ex vivo*. Thus, LMAE might exert its protective effects *via* inhibition of calcium entry in response to vasoconstrictor agonists at the level of smooth muscle cells in addition to its action on the endothelium.

Finally, although further studies are needed to characterize the compounds that support the antihypertensive properties of LMAE on the vascular wall, the phytochemical analysis of LMAE extracts showed that it contained sterols, triterpenes, coumarins, and anthraquinone. All of these compounds were previously described to have antioxidant and vasodilator properties [37–40].

5. Conclusion

This report is the first report demonstrating that LMAE corrects angiotensin II-induced hypertension and endothelial dysfunction in the aorta in an *in vivo* model. Furthermore,

Table 2: Qualitative phytochemical analysis of trunk bark extracts from *Lannea microcarpa*.

Plant solvent extraction	Phytochemical groups										
	Flavonoid aglycone	Emodols	Sterols and triterpenes	Alkaloids	Coumarins	Flavonoids	Anthraquinone	Tannins	Saponins	anthocianosides	Reducing compounds
DCM	-	-	+	-	-	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*
AE	*nd*	*nd*	+	*nd*	+	-	+	*nd*	*nd*	*nd*	*nd*
aq	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	+	+	+	+

LMDCM: dichloromethane; AE: ethyl acetate; aq: aqueous decoction. Keys: + = present; - = absent; nd = not determined.

we decipher the mechanisms involved in hypertension correction: reduction of COX-2- and NOX-2-induced oxidative stress and inhibition of calcium entry. Therefore, the present study represents a pharmacological basis of the empirical use of *Lannea microcarpa* trunk bark extract against hypertension.

Data Availability

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

Conflicts of Interest

The authors declared no conflict of interest.

Authors' Contributions

MN and RS performed the experiments, acquired and analyzed data, interpreted and discussed the results, and wrote and revised the manuscript. CK, PM, NO, and FBK performed the experiments and acquired data. LB, SO, and IPG interpreted and discussed the results. RA conceived and designed the experiments, interpreted and discussed the results, and wrote and revised the manuscript. All authors read and approved the final manuscript. Mathieu Nitiéma and Raffaella Soleti contributed equally to this work.

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Supplementary Materials

Supplemental Figure 1: heart and kidney weights and kidney parameters. Mean relative heart (A) and kidney (B) weights after 21 days of treatment with LMAE and Ang II. Plasma levels of sodium (Na^+) (C), chloride (Cl^-) (D), urea (E), and creatinine (F). The results are expressed as mean ± SEM. $n = 7$-8/group. Supplementary Figure 2: TLC plate images from ethyl acetate fraction of L. microcarpa trunk barks. Photographs represent anthracenosides, coumarins, saponins, triterpenoids and sterols, and tannins, respectively. Silica gel 60 F254 plates; LMAE: ethyl acetate fraction; LMAE': ethyl acetate fraction hydrolysed by acid chlorhydrique 10% solution. *(Supplementary Materials)*

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Review Article

Emerging Roles of Redox-Mediated Angiogenesis and Oxidative Stress in Dermatoses

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Angiogenesis is the process of new vessel formation, which sprouts from preexisting vessels. This process is highly complex and primarily involves several key steps, including stimulation of endothelial cells by growth factors, degradation of the extracellular matrix by proteolytic enzymes, migration and proliferation of endothelial cells, and capillary tube formation. Currently, it is considered that multiple cytokines play a vital role in this process, which consist of proangiogenic factors (e.g., vascular endothelial growth factor, fibroblast growth factors, and angiopoietins) and antiangiogenic factors (e.g., endostatin, thrombospondin, and angiostatin). Angiogenesis is essential for most physiological events, such as body growth and development, tissue repair, and wound healing. However, uncontrolled neovascularization may contribute to angiogenic disorders. In physiological conditions, the above promoters and inhibitors function in a coordinated way to induce and sustain angiogenesis within a limited period of time. Conversely, the imbalance between proangiogenic and antiangiogenic factors could cause pathological angiogenesis and trigger several diseases. With insights into the molecular mechanisms of angiogenesis, increasing reports have shown that a close relationship exists between angiogenesis and oxidative stress (OS) in both physiological and pathological conditions. OS, an imbalance between prooxidant and antioxidant systems, is a cause and consequence of many vascular complains and serves as one of the biomarkers for these diseases. Furthermore, emerging evidence supports that OS and angiogenesis play vital roles in many dermatoses, such as psoriasis, atopic dermatitis, and skin tumor. This review summarizes recent findings on the role of OS as a trigger of angiogenesis in skin disorders, highlights newly identified mechanisms, and introduces the antiangiogenic and antioxidant therapeutic strategies.

1. Introduction

The complex process, regulated by proangiogenic and antiangiogenic factors, scientifically understood as the beginning formation of new blood vessels from existing ones, is known as angiogenesis [1]. New blood vessel formation, based on the balance of proangiogenic and antiangiogenic factors, is overwhelmingly responsible for most physiological events, such as embryogenesis, organ regeneration, body growth and development, skin renewal, and wound healing [2–4]. In the skin, angiogenesis is reactivated during skin renewal, wound healing, and tissue repair; furthermore, in these conditions, many angiogenic factors are released by activated keratinocytes and some inflammatory cells and jointly function to promote skin recovery and rejuvenation [5]; however, this process may be impaired by excessive angiogenic factors. In certain pathological conditions, these factors become overmuch and the balance between angiogenic promoters and inhibitors shifts, resulting in an angiogenic switch. The most well-known conditions where this switch is seen are malignant and inflammatory skin disorders as well as other pathological events, e.g., age-related macular degeneration, rheumatoid arthritis, tumor growth, proliferative retinopathies, and skin diseases (psoriasis, atopic dermatitis (AD), systemic sclerosis (SSc), cutaneous carcinoma, etc.) [5–8]. Either physiological or pathological angiogenesis is in need

of initial mediation by various proangiogenic factors, consisting of endothelial growth factor (VEGF), fibroblast growth factors (FGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), placental growth factor (PGF), angiopoietin-1 (Ang-1), and transforming growth factor-β (TGF-β) [9]. These proangiogenic factors subsequently induce a continuous recruitment of inflammatory cells to participate in the pathological process, which in turn serve as a substantial source of reactive oxygen species (ROS) [10, 11]. More importantly, excessive ROS trigger oxidative stress (OS), further promoting angiogenesis, damaging cells/tissue, and resulting in a variety of pathological changes [12].

OS is frequently considered as an imbalance of redox originating from the overproduction of prooxidants (e.g., ROS, reactive nitrogen species (RNS), nitric oxide (NO), and lipid peroxides) or from the insufficiency of antioxidants/antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Figure 1) [13, 14]. Although the definition of OS is controversial, OS is currently regarded as a state in which stationary ROS/RNS transiently or gradually accumulate and ascend, further damaging cellular constituents and disturbing cellular metabolism [15]. Based on its intensity, OS is classified as basal OS (BOS), low-intensity OS (LOS), intermediate intensity OS (IOS), and high-intensity OS (HOS). OS, meanwhile, is categorized as mild OS (MOS), temperate OS (TOS), and severe OS (SOS) according to its degree [16]. In the process of OS, multiple redox signaling pathways are involved, primarily containing the mitogen-activated protein kinases/activator protein-1 (MAPK/AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Janus kinase-signal transducer and activator of transcription (JAK-STAT), nuclear factor erythroid 2-related factor (Nrf-2), phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt), and Toll-like receptor- (TLR-) mediated signal transduction pathway [17]. Through these signaling pathways, OS mediates in physiological or pathological events. For example, MOS contribute to cell survival, whereas SOS may damage macromolecules (DNA, proteins, and lipids) and organelles (mitochondria and membranes), even the whole tissues (Figure 1) [18]. ROS, the major contributors to OS, including oxygen-centered radical species (superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($R^{-}O_2^{\bullet}$)) and nonradical compounds (ozone (O_3), hypochlorous acid (HOCl), and hydrogen peroxide (H_2O_2)), are often generated by various categories of cells like endothelial cells (ECs), perivascular adipocytes, epithelial cells, smooth muscle cells, and adventitial fibroblasts [17]. In a physiological context, ROS have important roles in cell/tissue physiological processes including cell signaling, homeostasis, skin regeneration/renewal, and wound healing. In the skin in particular, ROS are mainly responsible for cell damage in the ageing process. ROS in low concentrations, nevertheless, participate in a substantial number of physiological cell redox signaling pathways to maintain redox equilibrium [19]; more importantly, ROS generated from immune cells are potently available for host defense [20]. As germicides or an important player in cellular signaling, they are also vital to wound healing and skin repair, while high-level ROS create a redox imbalance in the skin further causing severe "oxidative stress," eventually leading to DNA, cell, and tissue damage [21–27]. Several studies have demonstrated that in high concentration, H_2O_2 could induce endothelial injury; however, H_2O_2 in low concentration generally stimulates angiogenesis in wound healing and skin repair [28]. Accumulating evidence also supports that ROS as well ROS-mediated OS are involved in the process of physiological and pathological angiogenesis [29, 30] and closely implicated in the pathogenesis and exacerbation of angiogenesis-related diseases containing dermatoses, neurodegenerative disorders, cardiovascular diseases, and metabolic disorders [31–38]. In this review, we provide an overview of the current knowledge of the link between OS and angiogenesis and their roles in certain skin diseases as well as the emerging therapeutic strategies.

2. Role of OS in Angiogenesis

With further knowledge of angiogenesis, the pathogenesis of angiogenesis to some extent gradually becomes clear. It arrives at a consensus that ROS-mediated OS plays a crucial role in the development of angiogenesis. Moreover, two signal pathways of angiogenesis mediated by OS have been identified. One is the VEGF-dependent signaling pathway, while another is the VEGF-independent pathway [10, 39, 40].

2.1. Generation of ROS in Angiogenesis. At present, it is demonstrated that ROS-mediated redox signaling has a central role in angiogenesis. ROS act as a double-edged sword in the vasculature. In a physiological process, ROS work as an important component of signaling events and play an important role in cellular differentiation and maintenance of homeostasis [41]. However, overproduction of ROS (O^{-2} and H_2O_2) in turn contributes to neovascularization [21]. In this process, two endogenous ROS sources are mainly involved in the angiogenesis; one is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of the NOX family, and another is mitochondrial electron transport chain reactions [21, 31, 42, 43]. NADPH oxidase, a major source of ROS in ECs, generates $O_2^{\bullet-}$ by transferring electrons from NADPH to oxygen. There are seven isoforms of NADPH oxidases expressed in mammals, namely, Nox1, Nox2 (previously gp91phox), Nox3, Nox4, Nox5, Duox1, and Duox2. This NADPH oxidase homologue consists of the following subunits: gp91phox (newly termed Nox2), p22phox, p40phox, p47phox, p67phox, and GTPase Rac1 [35, 44–46]. NADPH oxidase may be activated by diverse growth factors including VEGF, angiopoietin-1, ischemia, and hypoxia, and then, ROS derived from NADPH oxidase mediate in VEGFR-2 autophosphorylation [45, 47]. Apart from NADPH oxidase, ROS, the intracellular ROS in particular, were as well originated from the mitochondria. In the mitochondria, over 95% of oxygen consumed by cells affords water molecule production via redox reactions. But at complexes I and III in the transport chain, less than 4% of oxygen, which is reduced to superoxide anion instead of water, can generate OS [31, 45].

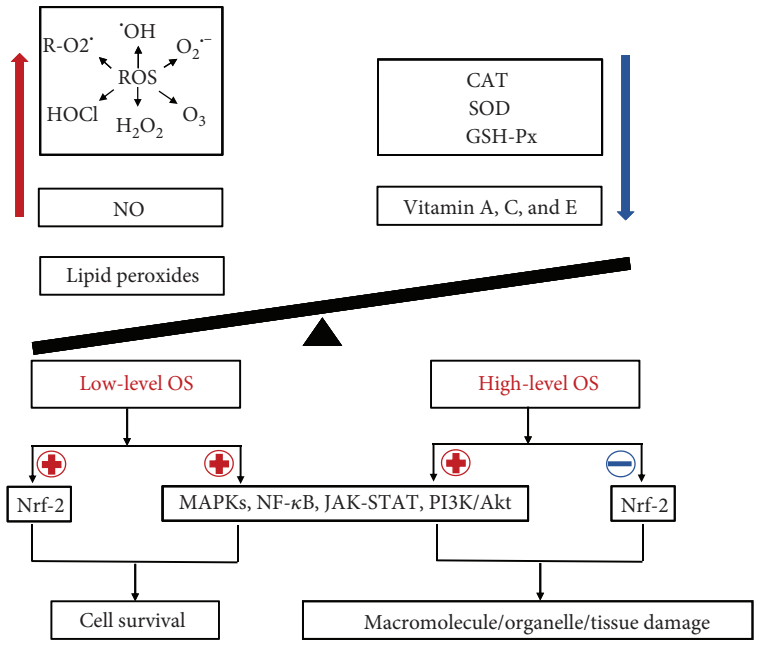

Figure 1: The process of oxidative stress (OS) generation. OS occurs when prooxidants (e.g., ROS, NO, and lipid peroxides) outbalance antioxidant defenses (e.g., SOD, CAT, and GSH-px). OS mediates in physiological or pathological events by activating/suppressing multiple redox signaling pathways (e.g., Nrf-2, MAPK, NF-κB, PI3K/Akt, and JAK-STA). For example, high-level OS may induce the damage of macromolecules (DNA, proteins, and lipids), organelles (mitochondria and membranes), and even the whole tissues, whereas low-level OS may contribute to cell survival. In this process, ROS, including radical and nonradical ROS such as $O_2^{\bullet-}$, $^{\bullet}OH$, $R^{-}O_2^{\bullet}$, O3, HOCl, and H_2O_2, play a pivotal role in the generation of OS. $\oplus$ means "to promote or enhance"; $\ominus$ means "to inhibit or suppress."

2.2. OS and Physiological Angiogenesis. Angiogenesis is physiologically essential for skin renewal, wound healing, tissue repair, skeletal remodeling, individual reproduction, etc. Among these physiological events, wound healing is a typical process involving angiogenesis and OS [20]. In this process, angiogenesis is induced by tissue hypoxia and ROS in either a VEGF-dependent way or a VEGF-independent way [48]. Low-concentration ROS facilitate angiogenesis in mouse wound healing and skin repair, which are involved in VEGF and its receptor signaling [49]. As the potent inducer of VEGF, hypoxia inducible factor 1 (HIF1) activated by ROS promotes angiogenesis via triggering VEGF expression during wound repair [50]. Thus, angiogenesis could be induced by ROS-mediated OS in a VEGF-dependent manner in wound healing. Increasing evidence has verified that VEGF can promote angiogenesis via binding to VEGF receptor-2 (VEGFR-2) in endothelial cells. The binding of VEGF to VEGFR-2 allows to activate a series of signal transduction molecules, including phospholipase C gamma (PLCγ) and phosphatidylinositol 3-kinase (PI3K), further stimulates the Raf-MAPK-ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway, and finally facilitates angiogenesis [51, 52]. It is therefore considered that VEGF/-VEGFR-2 signaling is a crucial signal transducer in both physiologic and pathologic angiogenesis. Apart from VEGF, other soluble factors like PDGF play an important role in angiogenesis during wound healing, which is dependent on H_2O_2 for its biological function [53]. Upon activation of the PDGF pathway, signaling occurs via the PI3K/Akt complex pathway and MAPK molecules [54]. Besides, endogenous 2-ω-carboxyethyl pyrrole (CEP), one major member of the carboxyalkyl pyrrole (CAP) family, is recognized by Toll-like receptor2 (TLR2) on endothelial cells and then activates MyD88-dependent signaling to promote angiogenesis at the wound site, which, in turn, accelerates wound healing. Consequently, OS also acts as the chief mediator of the VEGF-independent pathway in angiogenesis during the wound repair process [10].

2.3. OS and Pathological Angiogenesis. Pathological angiogenesis, fundamentally similar to physiological angiogenesis, is also affected by OS in VEGF-dependent and VEGF-independent ways, which proceeds in an unbalanced and uncontrolled fashion, finally resulting in an excessive and abnormal vascular pattern [55].

2.3.1. VEGF-Dependent Signaling Pathway. As one of the major angiogenesis factors, VEGF stimulates EC proliferation and migration via binding to VEGFR-2 regardless of physiological status or pathological condition, further activates several downstream signaling cascades, such as mitogen-activated protein kinases (MAPKs), PI3k/AKT, or eNOS, and eventually leads to physiological or pathological angiogenesis. Physiological angiogenesis like wound angiogenesis has been discussed before, and the mechanism of pathological angiogenesis is as follow.

The VEGF signal is essential for homeostasis and vascular development, which is always influenced by ROS [35]. Increasing evidence has indicated that most OS-related angiogenesis depends on VEGF involvement. Xia et al.

showed that NADPH oxidase-dependent ROS stimulated VEGF secretion and facilitated excessive angiogenesis in a tumor microenvironment through the HIF-1α-mediated VEGF pathway, further promoting tumor growth [56]. ROS from follicle-stimulating hormone (FSH) triggered HIF-1α signal and activated the VEGF signaling pathway by binding HIF-1α to the VEGF promoter and further accelerated excessive angiogenesis and finally contributed to ovarian epithelial cancer progression [57]. Likewise, products of oxidation exemplified by oxidized phospholipids (OxPLs) stimulate VEGF expression both *in vivo* and *in vitro*, thereby interacting with VEGFR-2 and triggering angiogenesis [58]. Especially, oxidized low-density lipoproteins (OxLDL) originated from OS could strongly induce HIF-1a and VEGF expression in monocyte macrophages and significantly enhance tube formation in cocultured endothelial cells [59–61]. As Toll-like receptor (TLR) ligands, poly (I:C) and lipopolysaccharide (LPS) both generated from OS, are also able to encourage angiogenesis via stimulating VEGF secretion or production and activate HIF-1α and the TLR pathway in a TLR-dependent manner [55, 62]. In addition, nitric oxide (NO) is considered to be one of the major contributors to angiogenesis and it has a capability of increasing the expression of HIF-1α and VEGF, thereby leading to angiogenesis [63]. Thus, ROS-promoting angiogenesis is dependent on VEGF and the HIF-1α/VEGF/VEGFR-2 pathway is a key molecular mechanism of OS-mediated angiogenesis [60].

2.3.2. VEGF-Independent Signaling Pathway. Apart from the VEGF-dependent pathway, another novel mechanism of OS-mediated angiogenesis in a VEGF-independent manner recently has been demonstrated. Nowadays, because of some malignant tumors being resistant to anti-VEGF therapy, it is widely considered that the existence of VEGF-independent signaling is mainly responsible for this treatment-resistant event. In most cases, this resistance to anti-VEGF is linked with inflammation and infiltration of myeloid cells, which could create substantial oxygen tension and result in the accumulation of CEPs and finally accelerate neovascularization in a VEGF-independent manner [10]. There are two main VEGF-independent signaling pathways involved in angiogenesis, the CEP/TLR2/MyD88 axis and ROS/ataxia-telangiectasia mutated (ATM)/p38α pathways [10, 64]. The former mediates proangiogenesis and involves the accumulation of new lipid oxidation products, e.g., CAP protein adducts [55, 65]. CEP acts as a potential biomarker for OS-induced vascular disorders and has the same proangiogenic effect as VEGF *in vitro* [66]. It has been demonstrated that TLRs not only serve as guardians of innate immunity but also function as prominent contributors to angiogenesis [67]. At present, it has been discovered that several proangiogenic ligands of TLRs produced by OS promote angiogenesis in a VEGF-independent way, such as CEP (a TLR2 ligand), macrophage-activating lipopeptide-2 (MALP-2) (a TLR2/6 ligand), and LPS (a TLR4 ligand). The molecular pattern of CEP, for example, is recognized by TLR2 on endothelial cells and triggers the MyD88-dependent signal to accelerate neovascularization [68]. LPS could stimulate endothelial sprouting directly *in vitro* through a TRAF6-mediated activation of NF-κB and JNK [69]. Angiogenesis is also induced via GM-CSF by TLR2/6 ligand binding to its receptor [70–72].

On the other hand, the latter, namely, ATM kinase, known for its function in the regulation of cell cycle and DNA damage repair, has been identified as an alternative mediator of OS-induced angiogenesis [73–75]. Remarkably, compared to CEP-TLR2 in angiogenesis, ATM in angiogenesis is uniquely limited to promoting the pathological process and ATM activation enhances no other cells but endothelial cell proliferation, which provides a probability for anti-ATM therapy [73]. As the downstream of ATM in endothelial cells, p38 is also involved in response to ROS; diminishing of ATM also suppressed angiogenesis even in the absence of VEGF inhibitors, suggesting a VEGF-independent proangiogenic role of ATM [73]. Figure 2 sketches two pathways of OS-mediated angiogenesis.

3. OS and Angiogenesis in Dermatoses

Growing evidence supports that OS and angiogenesis are both closely implicated in the occurrence and development of some skin diseases, such as psoriasis, AD, malignant melanoma (MM), Behcet's disease (BD), and scleroderma. However, the specific mechanism still remains unclear; thus, we concentrate on recent findings to present the possible mechanism of OS and angiogenesis in these cutaneous diseases.

3.1. OS and Angiogenesis Associated with Psoriasis. Psoriasis is a common chronic inflammatory skin disease approximately affecting 2% of the population. It characteristically manifests as erythema and papules/plaques accompanied by thick silvery-white scales. Nowadays, there is a wide range of options available for the treatment of psoriasis, such as topical therapies, phototherapy, older small-molecule systemic agents (e.g., methotrexate, cyclosporine, acitretin, and fumaric acid), the newer oral phosphodiesterase-4 inhibitor apremilast, and the biologics (e.g., etanercept, adalimumab, infliximab, and ustekinumab) [76]. Despite that these therapies offer a certain efficacy, patients scarcely get satisfaction with substantial psoriatic lesion clearance, symptom relief, and improvements in quality of life [77]. Thus, there is a pressing need to develop some novel effective remedies. Although the etiology of psoriasis still remains unclear, it is thought that oxidative and angiogenic mechanisms both get involved in the pathological process of psoriasis. As one of major pathological features of psoriasis, angiogenesis has been persistently studied and various proangiogenic mediators have been identified in the psoriatic skin. Heidenreich et al. revealed a large spectrum of proangiogenic factors to mediate in psoriasis, including VEGF, HIF-1α, TNF, angiopoietins, IL-8, IL-17, and TGF-α [78].VEGF expression, in particular, remarkably elevated in the psoriatic serum and lesions. Moreover, ROS induced VEGF releasing from various cell types, whereas VEGF in turn promoted endothelial cell migration and proliferation through an increase of intracellular ROS. Thus, the VEGF pathway may be a crucial link between OS and angiogenesis in psoriasis, especially for the HIF-1α/VEGF signaling pathway playing a synergistic role in the neovascularization of psoriasis [79, 80]. By

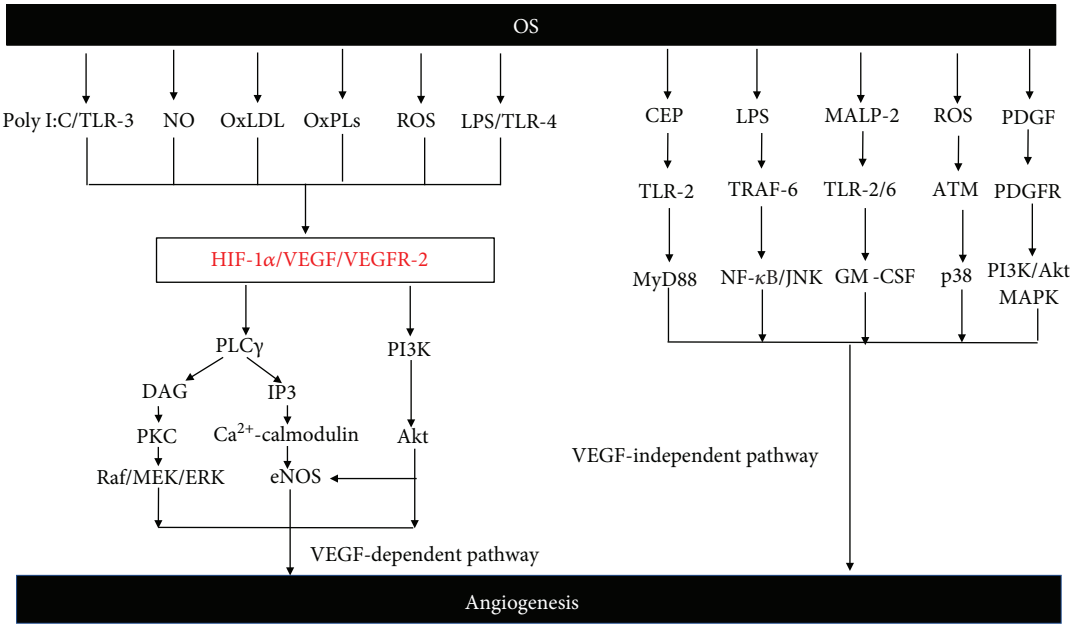

Figure 2: Schematic illustration of angiogenesis signaling pathways induced by OS. According to different responses to OS, two signal pathways of angiogenesis are covered, namely, the VEGF-dependent signaling pathway and VEGF-independent signaling pathway. In the VEGF-dependent pathway, ROS, NO, OxLDL, and OxPLs strongly stimulate the expression of HIF-1a and VEGF. Meanwhile, Poly I:C and LPS promote the expression of HIF-1α and VFGF by coupling with their specific receptors (TLR3 and TLR4). These further combine to the downstream receptor VEGFR-2 and facilitate angiogenesis by activating the HIF-1α/VFGF/VEGFR-2 signaling pathway in a VEGF-dependent manner. On the other hand, many mediators are involved in the VEGF-independent pathway, including CEP, LPS, MALP-2, PDGF, and ROS. CEP/yyMALP-2 initially couples to their receptors (TLR2/6), then sensitizes specific downstream targets (e.g., MyD88 and GM-CSF), and finally promotes angiogenesis. Meanwhile, ROS activate the P38 pathway via inducing the activation of ATM and ultimately result in angiogenesis. Besides, LPS is considered to induce angiogenesis through a TRAF6-mediated activation of NF-κB and JNK. As another soluble mediator, PDGF triggers PI3K/Akt and MAPK signaling by binding to its receptor and promotes neovascularization.

upregulating the expression of cell adhesion molecules, VEGF could enhance the migration of leukocytes into the psoriatic skin and increase oxygen consumption, further activating HIF-1α and perpetuating the angiogenic/inflammatory cycle of psoriasis [30, 81]. Furthermore, OxPLs afford the pathogenesis of psoriasis through enhancing VEGF generation from keratinocytes [30]. Besides, Elias et al. discovered that epidermal VEGF knockout mice scarcely appeared acanthosis after barrier disruption, suggesting an important contributor for VEGF to the development of keratinocyte hyperplasia. Hence, ROS-VEGF signaling may be a potential target for the treatment of psoriasis. However, the specific relationship between OS and angiogenesis in psoriasis requires to be further studied, which is conducive to fully clarify the pathogenesis of psoriasis and expand the optimal treatments for this disease.

3.2. OS and Angiogenesis Associated with AD. AD, a chronic inflammatory skin disease, adversely affects many people especially young children [82]. The current management of AD covers avoidance of triggering factors, skin care, and anti-inflammatory therapy (mostly topical corticosteroids and topical calcineurin inhibitors). Once these first-line approaches are unsuccessful, systemic therapy or phototherapy tends to be carried out as second-line treatment [83]. After being treated with these vehicles, most symptoms may be relieved. However, long-term use of the above therapeutic probably causes many side effects such as skin atrophy and dryness, photoaging, and potential occurrence of cutaneous malignancies [84]. Therefore, some novel therapies are needed for the management of AD. The pathogenesis of AD is complex and still poorly understood. Recently, emerging evidence suggests that OS is a potential key factor in the pathogenesis of AD [85]. OS is implicated in AD for several decades and remains present throughout the disease, including the onset of AD, the development of AD, and the exacerbation of AD. Moreover, excessive ROS overwhelm and destroy the skin antioxidant defense, which ultimately lead to AD progression and exacerbation [86]. Apart from OS, angiogenesis, as a hallmark of chronic inflammatory disorders, also gets involved in AD [87]. Several angiogenic factors contribute to the presence of angiogenic switch in the AD skin, such as VEGF, Angs, and IL-17. It has been demonstrated that angiogenesis is dysregulated in AD patients or models and high-level VEGF is detected in AD patient lesions. Meanwhile, Chen et al. discovered that a progressive increase of VEGF-A mRNA appeared in the skin of an AD mouse model [88]. Taken together, both OS and angiogenesis are mainly responsible for the pathogenesis of AD and the VEGF pathway may be a potential link between OS and angiogenesis in AD. Therefore, specific inhibitors targeting various mediators (e.g., VEGFs), receptors (e.g., VEGFRs and Tie-2), and oxides offer a promising foreground for the treatment of AD [7].

3.3. OS and Angiogenesis Associated with MM. MM, a malignant tumor of melanocytes, is accounted for about 10% of skin cancers, but it is responsible for over 90% of skin cancer deaths. For years, the cornerstones of cancer treatment have been surgery, chemotherapy, and radiation therapy. During the last decade, new strategies emerge from antitumor therapy for MM, including immunotherapy (e.g., checkpoint blockades) and targeted therapy (e.g., protein kinase inhibitors) or their combination [89]. Despite of extensive novel approaches serving for MM, the response rate is rarely higher than 20% and drug resistance is very common [90]. Up to date, rarely effective treatment has been approved for MM due to these reasons. As a result, it is urgent to invent other alternatives and targeted therapies [91, 92]. Compared to other tumors, MM is known for abundant ROS that exist in the primary tumor environment [93, 94]. ROS from OS at one time had been recognized as a powerful weapon for the immune system to kill tumor cells [95]. However, once MM cells successfully escape ROS-induced apoptosis, persistent ROS tend to favor melanoma survival, proliferation, and metastasis through activating several related pathways [96]. Thus, ROS and ROS-mediated OS are closely implicated in different stages of MM. Elevated ROS could trigger the occurrence of OS, which further disrupt the homeostasis of melanocytes, affect the epigenetic regulation, and induce gene mutation, ultimately leading to cancer generation [97]. Accordingly, much efforts have been made to battle melanoma by using antioxidants so far [98]. Moreover, ROS and ROS-mediated OS would promote MM angiogenesis in a VEGF-dependent or VEGF-independent manner; in their publication, Bald et al. as well have emphasized the importance of the vascular network for MM [99]. Several angiogenic factors (e.g., VEGF, bFGF, PIGF, PDGF, IL-8, and Ang-1) have been found to highly express in primary skin MM, and these mediators further promote MM angiogenesis and metastasis [100]. In addition, intratumoral hypoxia encourages the consequent expression of HIF-α transcription factors, in turn modulating VEGF and transcriptional product expression and mediating in cell growth, metabolism, and death [101, 102]. Meanwhile, preclinical studies indicate that the inhibitors targeting VEGF or VEGFR are effective in slowing the growth and metastasis of MM in murine models [103, 104]. Apart from VEGF, PDGF and its receptor PDGFR-β are responsible for MM angiogenesis. PDGF signaling is also implicated in angiogenesis in a VEGF-independent fashion. Therefore, OS and angiogenesis play vital roles in the development of MM; VEGF and PDGF signaling, moreover, may be the key link to OS and angiogenesis, which probably become the potential targets for the treatment of MM [105].

3.4. OS and Angiogenesis Associated with BD. BD, a chronic and recurrent vasculitis disease, is characterized by various clinical manifestations including skin lesions, oral/genital ulcer, ocular symptoms/lesions, joint signs, and organ involvements [106–108]. Glucocorticoids, azathioprine, cyclophosphamide, and cyclosporine A are currently the mainstay of treatments in vasculo-Behcet's disease, but long-term use of these drugs may induce some systemic adverse reactions [109]. Once immunosuppressive and corticosteroid therapies fail, biologic agents (e.g., infliximab, alemtuzumab, and adalimumab) can help for vascular lesions. However, high cost may be an obstacle to their widespread application [110]. Although BD etiology keeps being unknown, recently, growing evidence supports that elevated OS and insufficient antioxidant capacity are primarily involved in the pathogenesis of BD [111–113]. In the process of BD attack, ROS overproduction from OS may in turn accelerate OS aggression, then lead to tissue damage, and ultimately result in the pathological and clinical manifestations of BD. More importantly, we have demonstrated in our previous studies that there is an abnormal OS indeed existing in BD and a skewed redox balance remains present throughout this disease [114]. Apart from the OS-mediated mechanism, vascular endothelial activation is also considered to be a major one in BD [115–117]. Nowadays, it has been confirmed that several angiogenesis-promoting molecules (namely, angiogenic promoters) get involved in the pathogenesis of BD, including IL-8, matrix metalloproteinases, E-selectin, vascular endothelial-cadherin, and VEGF [118]. Among them, VEGF, the dominant factor controlling angiogenesis, was found to highly express in BD serum and elevated-level VEGF was proportional to BD activity [119–121]. VEGF, at the same time, plays an active role in the maintenance and growth of vascular endothelial cells. Kamoun et al. thought that high-level VEGF was closely associated with high concentration of NO from OS in BD [122]. Thus, OS and angiogenesis are crucial in BD pathogenesis and OS zealously mediates in the process of angiogenesis. However, further studies are needed to investigate the underlying mechanisms of OS-mediated angiogenesis in BD, in order to develop new therapeutic strategies for BD patients to suppress OS and angiogenesis.

3.5. OS and Angiogenesis Associated with Scleroderma. Scleroderma, also known as systemic sclerosis (SSc), is a chronic immune-mediated connective tissue disease involving the skin, blood vessels, systemic organs, lungs, kidney, and gastrointestinal tract in particular [91]. SSc consists of two clinical subsets: one is limited cutaneous SSc (lc-SSc) and another is diffuse cutaneous SSc (dc-SSc). Because skin sclerosis can cause joint contracture, disability, and poor quality of life, various systemic treatments (e.g., penicillamine, cyclophosphamide, methotrexate, azathioprine, mycophenolate mofetil, intravenous immunoglobulin, and tyrosine kinase inhibitors) have been applied to alleviate the symptoms. These treatments, however, may cause severe side effects and offer inconsistent efficacy [123]. Phototherapy, another approach used to relieve skin sclerosis, provides a local effect on the skin without systemic involvement, but it alone cannot completely reverse skin sclerosis and it is just used as an adjunctive therapy together with other antifibrotic treatments (i.e., corticosteroids and pentoxifylline) [124]. Recently, it is thought that OS plays an important part in promoting scleroderma development, though SSc pathogenesis remains obscure [125]. Murrell proposed that an abnormal generation of ROS should be responsible for

most of the pathologic features of SSc [126]. For example, ROS could stimulate the production of profibrotic cytokines (including PDGF and TGF-β) and proinflammatory factors, accelerate the activation and proliferation of fibroblasts, promote the synthesis of type I collagen, and induce vascular dysfunction [127]. By targeting ROS-generating NADPH oxidase, fibroblast activation and experimental skin fibrosis are inhibited *in vitro* and *in* vivo [128]. On the other hand, several abnormalities in regulating angiogenic responses in scleroderma indicate that aberrant angiogenesis may be another important pathogenic factor of scleroderma [129]. Hummers et al. have found that high levels of angiogenic factors were measured in patients with scleroderma, e.g., VEGF, PDGF-BB, FGF2, and PlGF [130]. Meanwhile, increased-level VEGF and VEGFR have been discovered in the serum and skin samples from scleroderma patients [131–135]. Besides, HIF-1a is more prevalent in SSc patients than normal subjects [136]. Accordingly, the pathogenesis of scleroderma is closely associated with OS and abnormal angiogenesis but further studies focused on the link between OS and angiogenesis in SSc are still needed, which may lead to the development of a new way for scleroderma treatment.

3.6. OS and Angiogenesis Associated with Rosacea. Rosacea is a common chronic inflammatory dermatosis, clinically characterized by erythema of the central face, episodic flushing, papules, and pustules [137, 138]. Skin care and pharmacologic treatments are the pillars of effective management of rosacea. Apart from existing topical agents (sodium sulfacetamide, azelaic acid, metronidazole, and the alpha-adrenergic agonist brimonidine) and systemic medications (tetracyclines, beta-blockers and isotretinoin), new therapies including serine protease inhibitors and mast cell stabilizers may ameliorate rosacea symptoms [139]. However, some of these approaches have not been approved by the Food and Drug Administration. Though the exact pathogenesis of rosacea needs to be clarified, OS and oxidation of lipids are considered as crucial factors to trigger and aggravate the inflammatory processes of rosacea. Increased OS and decreased antioxidants are determined in systemic circulation of rosacea [140, 141]. OS, in addition, is complicated in vascular changes, inflammation, and oxidative tissue damage in rosacea [142]. Therefore, antioxidants may be a potential strategy for treating rosacea. As an essential process in chronic inflammatory dermatoses, angiogenesis also contributes to the development of rosacea [143–145]. Amal et al. reported that VEGF expression elevated in cutaneous lesions of rosacea and was consistent with vascular histological changes which clinically presented as erythema and telangiectasia [146]. VEGF, indeed, has an important impact on the angiogenesis process, responsible for telangiectasia and increased vascular permeability, leading to cutaneous inflammation and the presence of papules, pustules, and nodules in rosacea [147, 148]. Thus, attenuation of OS and VEGF may be relevant approaches for the therapy of rosacea. However, more research should be carried out to clarify the relationship of OS and angiogenesis and provide a novel therapeutic way for rosacea.

4. Therapeutic Implications

Given that OS and OS-mediated angiogenesis have important roles in promoting various dermatoses, it should be fully suitable to develop novel therapies for skin disorders aimed at both aspects (Figure 3). As a major regulator of angiogenesis, VEGF and its pathway are considered as key targets for antiangiogenic therapy [149, 150]. Some effective drugs targeting VEGF have emerged from the pharmaceutical industry to inhibit new vessel formation.

4.1. Antiangiogenic Agents in the Management of Skin Diseases. Based on successful phase III trials, antiangiogenic therapeutics, anti-VEGF agents in particular (e.g., sorafenib, bevacizumab, and sunitinib), have entered the clinical practice in the USA and elsewhere. Strategies have been developed to inhibit the VEGF signaling pathway including anti-VEGF antibody therapy (e.g., bevacizumab), anti-VEGFR antibody therapy (e.g., ramucirumab), inhibitors of VEGFR-2 tyrosine kinases (e.g., apatinib), and inhibitors of angiogenic receptor tyrosine kinases (e.g., sunitinib, pazopanib, sorafenib, and regorafenib) [151]. Due to their antiangiogenic, antioxidative, and antiproliferative effects, phytochemicals are beneficial in the battle against cutaneous carcinoma [152]. Intraperitoneal injection of recombinant thrombospondin type 1 repeat domain (rTSR1) or a disintegrin-like and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) could potently inhibit subcutaneous melanoma growth by diminishing angiogenesis, promoting apoptosis, and decreasing cell proliferation in the tumor tissue [153]. Antiangiogenic agent AE-941 from extracts of cartilage potentially provides a beneficial effect to treat cutaneous and systemic diseases especially for psoriasis [154]. It is speculated that cannabinoids have a potential role in treatment of psoriasis by controlling angiogenesis and inflammation through decreasing HIF-1α and VEGF levels [155]. Meanwhile, Kuang et al. also demonstrated that topical sunitinib ointment contributed to attenuate imiquimod-induced psoriasis-like inflammation through regulating the proliferation and apoptosis of keratinocytes via suppressing p-Stat3 and VEGF expression [156]. Besides, thalidomide effectively works in skin disorders such as BD through inhibition of VEGF- and FGF-2-mediated angiogenesis [157].

4.2. Agents against OS in the Management of Dermatoses. On the other side, it is quite beneficial to skin disorder recovery by employing OS-targeted drugs like antioxidants. Because OS-dependent angiogenesis is an important contributor to the progression of cancers, antioxidants may overcome the limitations of anti-VEGF therapy, especially in relation to tumor resistance. Related documents revealed that glabridin ameliorated imiquimod-induced psoriasis-like inflammation on BALB/c mice skin through improvement of antioxidant status and downregulation of proinflammatory cytokines [158]. By decreasing lipid peroxidation and modulating Ca^{2+} release, colchicine significantly induced protective effects on OS in the neutrophils of BD patients [159]. More importantly, high-dose vitamin C could effectively work in the skin diseases of MM and AD owing to its antioxidant

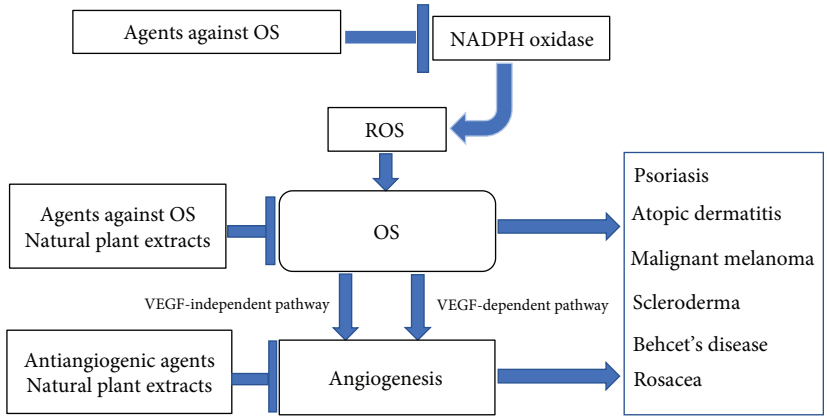

Figure 3: Strategies for dermatoses through mediating OS-induced angiogenesis and OS. Several novel therapies used for OS or angiogenesis-related skin disorders have been developed, including antiangiogenic agents, agents against OS, and natural plant extracts. These vehicles are potentially effective in management of dermatoses (e.g., psoriasis, AD, MM, scleroderma, BD, and rosacea) through mediating OS or angiogenesis-associated signal pathways.

protection [160]. Apart from the traditional antioxidants, NADPH oxidase, a key enzyme generation of ROS in neovascularization, potentially becomes the important target of pharmacological inhibitors. And NOX inhibitors are the most promising therapeutic option for diseases associated with OS. Among them, traditional NADPH oxidase inhibitors, such as apocynin and diphenylene iodonium, have no specificity and little isoform selectivity. Instead, several novel NOX inhibitors (GKT137831, ML171, and VAS2870) exhibit improved specificity for NADPH oxidases and NOX isoform selectivity [161].

4.3. Natural Plant Extracts in the Management of Dermatoses. Nowadays, natural extracts from plants increasingly arrest the attention from medical fields and pharmaceutical industry. Numerous natural extracts, like tea polyphenol, proanthocyanidins, and allicin, are potently beneficial to various skin disorders. As the main active ingredient of tea polyphenol, epigallocatechin-3-gallate (EGCG) could prevent OS-induced damage and suppress angiogenesis to avail against skin cancer and psoriasis basing on its antioxidant, antitumor, and antiangiogenic properties [162, 163]. Due to their powerful antioxidation, antiangiogenesis, antiproliferation, and antioncogenesis, proanthocyanidins have a wide utilization in the management of various OS-related and angiogenic complaints [164, 165]. Phenolic metabolites [166]. Moreover, we have proposed in our previous publications that proanthocyanidins are good for the treatment of psoriasis, AD, allergic purpura, SSc, rosacea, skin cancer, and other dermatoses [167]. Besides, our recent finding reveals that allicin, an active substance from garlic, has a favorable efficacy on BD by attenuation of OS and balance of oxidant/antioxidant status [168].

5. Conclusion

In summary, there are two main mechanisms implicated in the area bridging angiogenesis and OS; one is the VEGF-dependent signaling pathway (HIF/VEGF signaling), while another is the VEGF-independent signaling pathway (CEP/TLR2/MyD88 axis and ROS/ATM/p38α pathway). It is clear that OS and OS-derived angiogenesis are important contributors to the progression of chronic diseases and tumors. There is no doubt that both OS and angiogenesis participate in the development of certain skin diseases; however, a deeper understanding of the mechanisms behind OS and OS-dependent angiogenesis is necessary. There is a need for an investigation of multifaceted pathways involved in OS-induced angiogenesis in dermatoses and a specific target discriminating pathological vasculature from the physiological one. Therefore, in addition to the anti-VEGF drugs and OS inhibitors or antioxidants, it is necessary to develop some newly specific target strategies.

Conflicts of Interest

No financial or other conflicting interest exists.

Authors' Contributions

Dehai Xian and Jing Song contributed equally to this work.

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Research Article

Myocardial Protection from Ischemia-Reperfusion Damage by the Antioxidant Effect of *Hibiscus sabdariffa Linnaeus* on Metabolic Syndrome Rats

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Cardiovascular diseases (CVD) constitute one of the most prevalent health problems worldwide, being strongly associated with metabolic syndrome (MS). Oxidative stress (OS) is present in both CVD and MS. Infusions of *Hibiscus sabdariffa Linnaeus* (HSL) have antioxidant properties and could therefore decrease the presence of OS in these diseases. The aim of this study was to evaluate myocardial protection during ischemia/reperfusion due to the antioxidant effect of HSL infusion (3%) on a MS rat model induced by the administration of 30% sucrose in drinking water. We determined in control, MS, and MS + HSL rat hearts ($n = 6$ per group) cardiac mechanical performance (CMP), coronary vascular resistance (CVR), and activities of manganese and copper/zinc superoxide dismutases (Mn and Cu/Zn-SOD), peroxidases, glutathione peroxidase (GPx), catalase (CAT), glutathione s-transferase (GST), glutathione reductase (GR), and glutathione (GSH). We also determined lipoperoxidation (LPO), total antioxidant capacity (TAC), and the nitrate/nitrite ratio (NO_3^-/NO_2^-). The treatment with the HSL infusion restored the CMP ($p = 0.01$) and CVR ($p = 0.04$) and increased the Mn- ($p = 0.02$), Cu/Zn-SOD ($p = 0.05$), peroxidases ($p = 0.04$), GST ($p = 0.02$) activity, GHS ($p = 0.02$), TAC ($p = 0.04$), and NO_3^-/NO_2^- ($p = 0.01$) and decreased the LPO ($p = 0.02$) in the heart of MS rats undergoing ischemia/reperfusion. The results suggest that the treatment with an infusion from HSL calices protects the cardiac function from damage by ischemia and reperfusion through the antioxidant activities of the substances it possesses. It favors antioxidant enzymatic activities and nonenzymatic antioxidant capacity.

1. Introduction

Cardiovascular diseases (CVD) constitute one of the most prevalent health problems worldwide. These diseases have been strongly linked with metabolic syndrome (MS) which constitutes a complex condition associated with pathologies such as high blood pressure (BP), hypertriglyceridemia (TG), obesity, hyperinsulinemia, insulin resistance (IR), and oxidative stress (OS). OS is caused by a loss of the balance between the generation of reactive oxygen species (ROS) [1] and the capacity of the systems having antioxidant properties to neutralize ROS or to repair the resulting

damage. OS is the result of an imbalance of the cellular redox state [2]. Furthermore, ROS excess has been involved in CVD, including aortic dilatation, aortic dissection, cardiac arrhythmias, coronary heart disease, left ventricular hypertrophy, and congestive heart failure [3].

Several experimental models in animals have been used to understand the participation of ROS in MS-associated CVD [4]. In our laboratory, we have studied a MS rat model that consumes chronically 30% sucrose in drinking water. In this model, glucose metabolism and the energy transfer in the heart are altered and CVD are favored [5]. Experiments in the isolated rat heart from male MS rats subjected to ischemia/reperfusion showed the development of lethal arrhythmias. Furthermore, the addition of insulin to the perfusion liquid administered to these hearts affected their mechanical work [6]. Other authors have reported that a high-sucrose diet for 2.5 weeks favored the development of early abnormalities of diastolic function followed by alterations in myocardial structure and systolic dysfunction 10 weeks after the end of the treatment [7]. Rats treated with 8% sucrose showed hypertension and tachycardia after 2 weeks of treatment [8]. In this last model, the cardiomyocytes were more susceptible to damage by oxidation since their antioxidant capacity was lower than their oxidative capacity, when compared to other cells. The OS present in the heart of the MS rats is characterized by the decrease in the activities of the enzymes SOD, CAT, and GPx [9]. The mitochondria of the heart of MS rats are decoupled, increasing the production of ROS, and this condition alters cardiac function [10].

Moreover, many studies have reported that the intake of the antioxidants contained in some foods contributes in diminishing the oxidation processes at the endogenous level, thus reducing the negative consequences derived from OS in diverse organs. *Hibiscus sabdariffa Linnaeus* (HSL) is known as aleluya in Cuba, meśta in India, and jamaica flower in México. HSL has been used to treat liver diseases, hypercholesterolemia, hypertriglyceridemia, gastrointestinal disorders, and hypertension [11]. The HSL calyces have compounds such as protocatechuic acid (PCA), anthocyanins, quercetin, catechins, and polyphenols that protect cellular components from damage by oxidation [12], decreasing lipid peroxidation (LPO), increasing the activities of CAT, GPx, and SOD [13], and participating in the regeneration of other antioxidants (vitamins C and E). It increases the GSH concentration and inhibits the xanthine oxidase (XO) activity and the angiotensin-converting enzyme (ACE). It also has an anti-inflammatory effect by modulating cyclooxygenase 2 and inducible nitric oxide (iNOS) synthase [14] and avoiding the prostaglandin E_2 and nitric oxide (NO) synthesis [15]. It prevents liver cell apoptosis by inhibiting the activation of p-JNK and p38 MAPK transcription factors. It promotes low-serum TG and low-lipoprotein level density during inflammation, and it increases high-density lipoproteins, inhibiting the development and/or progression of atherosclerosis [16]. The perfusion using 12 polyphenols obtained from of HSL at doses ranging from 125 to 500 μg/mL diminished systolic function in a recent work determining cardiac function in Langendorff preparations of rat hearts. The authors found negative inotropic, negative chronotropic, and positive lusitropic effects. The effect was attributed to calcium entry, its release, and reuptake [17], but the authors did not study the effect of these polyphenols on oxidative stress reduction and its implications for improving heart function. In addition, the HSL extract reverts fibrosis, inflammation, and hypertrophy and prevents heart failure in a rat model of myocardial infarction by isoprenaline [18]. Likewise, the chronic administration of an aqueous extract of HSL significantly attenuated and reversed cardiac hypertrophy in 2K-1C hypertensive rats through negative inotropic and chronotropic effects. This model is characterized by vascular ROS production. The protective mechanism was associated with the high concentrations of anthocyanins and vitamin C contained in the extract [19].

Here, we hypothesized that HSL infusion might have a positive effect reducing OS and thus prevent abnormalities in the MS rat heart. Therefore, the objective of this study was to evaluate myocardial protection from damage by ischemia/reperfusion due to the antioxidant effect of 3% HSL infusion in a MS rat model.

2. Material and Methods

2.1. Infusion. The HSL calyces were acquired in Chilapa de Alvarez (high zone in Guerrero, México). For the infusion preparation, 30 g of HSL calyces was incubated in a liter of boiling drinking water (95–100°C) for 10 min. The solution was then left to cool. It was filtered and 300 g sucrose was added. It was stored at 4°C until consumption. To determine total anthocyanin content of the infusion, 100 μL was added to 50 mL of buffers ($NaC_2H_3O_2$, 4 M) at pH 1 and 4.5, and the absorbance was measured at 520 and 700 nm and compared against a blank cell, filled with distilled H_2O. The difference in the absorbance was used for calculating the cyanidin-3-glucoside (total monomeric anthocyanin) as described by the method of Lee et al. [20]. Total flavonoid content was determined by the method of Zhishen et al. [21]. In brief, 100 μL of HSL infusion was added to 2175 μL of distilled H_2O plus 75 μL of 5% $NaNO_2$ and incubated for 3 min. Then, 150 μL of 10% $AlCl_3$ was added and the solution was incubated for 5 min. 0.5 mL of 1 M NaOH was added to the mixture, and it was shaken vigorously in a vortex. The absorbance was measured at 510 nm. The calibration curve was done using quercetin as standard. Total estimation of vitamin C was determined by the method of Jagota and Dani [22]. In brief, 100 μL of the HSL infusion was added to 200 μL of 0.20 mM Folin-Ciocalteu reagent. The mixture was shaken vigorously in a vortex for 5 seconds and incubated for 10 min. The absorbance was measured at 760 nm. The calibration curve was obtained using an ascorbic acid standard solution. The 3% HSL infusion contained 136.50 ± 58.50 mg/L of cyanidin-3-glucoside, 18.37 ± 0.48 mg/L of quercetin, and 1.45 ± 0.03 mM of vitamin C.

2.2. Animals. The Laboratory Animal Care Committee of the National Institute of Cardiology "Ignacio Chávez" in Mexico

approved the experimental design, and experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals of NIH. Weanling male rats weighing 200-250 g were used, $n = 6$ per group. The groups were as follows: control (Ctr), 30% sucrose fed (MS)—sucrose was added in their drinking water, and 30% sucrose fed plus HSL extract (MS + HSL) at the concentration of 30 g/L. The animals were placed in plastic boxes and were kept under 12 h light/obscurity cycles and environmental temperature ranging from 18 to 26°C. They were fed commercial rodent pellets (PMI Nutrition International Inc., LabDiet 5008, Richmond, IN) ad libitum. Weight of the rats and determinations of blood pressure (BP) were taken at the end of the experimental period of 12 weeks. BP was determined using a tail cuff attached to a pneumatic pulse transducer method (Narco Bio-Systems Inc., Houston, TX, USA), in compliance with the method described by Pérez-Torres et al. [23].

2.3. Isolated Heart Perfused by the Langendorff Method. The animals were anesthetized and given anticoagulants. After a thoracotomy, the heart was exposed, and with the help of a silk thread, the ascending aorta was referred. The heart was removed, placed in isotonic saline at 4°C, and connected to the perfusion system through the ascending aorta as previously described [24]. After an adaptation period of 30 min, the experimental perfusion conditions were established as previously described [25]. The determinations of left intraventricular pressure (LIVP), perfusion pressure (PP), and the values of HR (heart rate) and left intraventricular pressure (LIVP), cardiac mechanical performance (CMP), and coronary vascular resistance (CVR) were done as previously reported [25]. After, at the end of the experiments in the isolated perfused heart, the organ was stored at -30°C. It was homogenized in 0.25 mM sucrose solution, and total proteins were determined by the Bradford method as previously published [26]. The retroperitoneal fat tissue was also dissected and weighed. Blood samples were centrifuged for 20 min at 936 g and 4°C, in order to collect the serum in aliquots of 400 μL and stored at -30°C for quantification of biochemical variables.

2.4. Mn and Cu/Zn Superoxide Dismutase and Peroxidase Activities. The activities of SOD enzyme isoforms were determined in the homogenate of the heart by nondenaturing gel electrophoresis and nitro blue tetrazolium (NBT) staining as described by Pérez-Torres et al. [23]. The NBT stain for O_2 was viewed by UV light exposure for another 10 min. Riboflavin and TEMED in the presence of UV light and oxygen produce ROS; NBT and SOD compete with them. Where SOD is present, the gel remains transparent, whereas reduced NBT turns it purple-blue.

For the peroxidase activity, 35 μL of horseradish peroxidase was loaded to a final concentration of 178.5 μg as standard and 100 μg of protein in the same conditions of the native gel as previously described. To observe the activity of the peroxidases, the gel was washed with distilled water three times, during 5 minutes, and it was then incubated with a mixture of 0.003 mg/mL 3,3′,5,5′-tetramethylbenzidine dissolved in a solution of ethanol : acetic acid : water (1 : 1 : 1 *v*/*v*) with H_2O_2 for 10 minutes in the dark. In these conditions, where peroxidases are present, the gel remains transparent and 3,3′,5,5′-tetramethylbenzidine is oxidized showing a green coloration. The gels for SOD isoform and peroxidase activities were analyzed by densitometry with the Kodak Image® 3.5, and activities were calculated following the technique described by Pérez-Torres et al. [23].

2.5. Glutathione Peroxidase. GPx activity was determined by a previously reported technique [27]. Activity is expressed in μmol of NADPH oxidized/min/mg protein.

2.6. Glutathione S-Transferase. The activity of GST was determined spectrophotometrically in 100 μg of protein, according the technique described by Beutler [28]. The sample was incubated and monitored for 10 min at 37°C at 340 nm. Values of GST activity were expressed in U/min/mg of protein. A unit of activity of GST is expressed in μmol of GS-DNB conjugate formed/min/mg of protein at 37°C.

2.7. Glutathione Reductase. The GR activity was determined according to the method described by Agarkov et al. [29]. The absorbance was read at 340 nm. GR activity is expressed in U/min/mL of protein.

2.8. GSH Concentration. The GSH concentration in 100 μg of protein of heart homogenate was made according to a previous method by Ellman [30], and absorbance was read at 412 nm. The calibration curve was done with GSH at concentrations from 5 to 25 μmol.

2.9. Lipid Peroxidation (LPO). LPO was determined by a previously reported technique [23], after extracting the products to an n-butanol phase and measuring the absorbance at 532 nm. The calibration curve was obtained using tetraethoxypropane as standard.

2.10. Evaluation of Total Antioxidant Capacity (TAC). The total antioxidant capacity was measured by a previously reported technique [31]. The calibration curve was obtained using Trolox.

2.11. Nitrate and Nitrite Ratio Quantification. NO_3^- was reduced to NO_2^- by the Cu-Cd reaction as previously reported [23]. The calibration curve was obtained with KNO_2 solution of 5–0.156 nM. The absorbance was measured at 540 nm.

2.12. Biochemical Variables. Commercially obtained ELISA kits were used for the determination of some serum biochemical variables from the rats, such as glucose, cholesterol, TG, and insulin. The HOMA index of resistance to insulin was calculated. HOMA − IR = (insulin μU/mL)* (glucose mM/L)/22.5.

2.13. Histological Preparation. The histological sections of the left ventricle were done after the ventricle had been washed in 0.9% NaCl for 30 sec. and fixed by immersion in phosphate buffer with 10% formalin (pH 7.4) for 24 h. The sections were processed according to conventional histological

procedures by hematoxylin-eosin stain. A Carl Zeiss light microscope (Carl Zeiss Axio Imager Z2, West Germany) with objective EC Plan-Neofluar 10x, with an HP Z800 computer and HP ZR30W screen, was used to analyze the histological sections. The photomicrographs were studied using densitometry with the SigmaScan Pro 5 Image Analysis software. The density values are expressed as pixel units.

2.14. Statistical Analysis. Statistical analysis and graphs were performed with a SigmaPlot 12.3 program (version 2016, Systat Software Inc., San Jose, CA, USA). The data are presented as the mean ± SE. Statistical significance was determined by one-way ANOVA test, followed by the post-hoc Tukey test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. General Variables. Table 1 shows general characteristics of the experimental animals. The insulin, HOMA-index, TG, intra-abdominal fat, and BP were significantly elevated in MS in comparison with Ctr ($p = 0.001$). The administration of HSL reduced these variables in comparison to MS ($p < 0.04$). Cholesterol and glucose remained at normal levels in all groups.

3.2. Perfusion of the Isolated Heart. Figure 1(a) describes the coronary vascular resistance (CVR) in the experimental groups. In the Ctr group, a CVR of 4 mmHg/mL/min in the initial period was found and it increased 60% at the end of the reperfusion period. These variations are normal under this condition. The MS group had a 50% increase in CVR in comparison with the Ctr group in the initial period, and it increased 2-fold in the reperfusion period showing a statistically significant difference ($p = 0.01$). After the treatment with the HSL infusion, CVR was restored in MS rats ($p = 0.04$) and it was in a value similar to the one found in Ctr rats. Figure 1(b) shows that the CMP in Ctr hearts is decreased by 20% at the end of the reperfusion period. This diminution reflects the damage generated by the global ischemia, in addition to the damage caused by reperfusion. The same behavior was observed in MS + HSL hearts. In comparison, MS hearts without treatment had a CMP that was decreased by 50% when compared to the Ctr and SM + HSL groups ($p = 0.03$ and $p = 0.01$, respectively).

3.3. SOD Isoform Activities. In the heart homogenate of the MS rats, the Mn-SOD activity was not significantly modified when compared to that found in Ctr rats. However, the HSL treatment significantly increased the activity of these isoforms in MS rats ($p = 0.02$, Figure 2(a)). The Cu/Zn-SOD activity in heart homogenate from the MS rats showed a significant decrease in comparison to the activity found in Ctr and MS + HSL rat hearts ($p = 0.02$, $p = 0.05$, respectively, Figure 2(b)).

3.4. Glutathione-Dependent Enzymes in the Heart. Figure 3(a) shows that the activity of the peroxidases in the heart homogenates was significantly decreased in MS rats in comparison with Ctr and MS + HSL rats ($p < 0.01$ and $p = 0.04$, respectively). Figure 3(b) shows that the activity of GPx was significantly decreased in the MS group when compared to the Ctr group ($p = 0.03$). However, the treatment with HSL in MS rats only showed a tendency to increase its activity without reaching a significant change ($p = 0.06$). Figure 4(a) shows that the activity of GST was significantly decreased in the MS group in comparison to the Ctr group ($p = 0.01$). The treatment with HSL in MS rats significantly increased the GST activity ($p = 0.02$). The enzymatic activity of the GR was not significantly increased in the MS vs. the Ctr group. When the activity of the enzyme was compared with that from the MS + HSL group, it only showed a tendency to increase without reaching a statistically significant change ($p = 0.06$, Figure 4(b)).

3.5. GSH Levels. Figure 5(a) shows that the GSH in the heart homogenate was significantly diminished in the MS group when compared to the Ctr group ($p = 0.01$). The treatment with the HSL infusion in MS rats significantly increased the GSH levels ($p = 0.02$).

3.6. Catalase Activity. The MS rats showed a significant decrease in CAT activity in comparison to Ctr rats ($p < 0.001$). The HSL infusion did not significantly modify the CAT activity in the MS rats (Figure 5(b)).

3.7. Oxidative Markers in Heart Homogenate. The NO_3^-/NO_2^- ratio was significantly decreased in MS rats in comparison with Ctr and MS + HSL rats ($p = 0.05$ and $p = 0.01$, respectively, Figure 6(a)). Regarding LPO, which is a LPO marker, the MS group showed a significant increase in its levels in comparison to the Ctr and MS + HSL groups ($p < 0.001$ and $p = 0.02$, respectively, Figure 6(b)). In addition, Figure 6(c) shows that the TAC in the heart homogenate was decreased in MS when compared to its level in Ctr rats ($p = 0.007$). The HSL treatment significantly increased its level ($p = 0.04$).

3.8. Heart Histology. Figure 7(a) shows the section of a myocardium from the Ctr group in which contraction bands are arranged in compact bundles of myocytes, separated by fibrous bands. It is possible to distinguish focally ovoid nuclei and intercalated disks, as part of the normal histologic aspect of the myocardium. In the hearts from the MS group, there are slight changes in relation to the Ctr group. A variable undulation in the bundles of myofibrils is observed, and it is possible to point out that they are narrower than those in the controls in the longitudinal section (Figure 7(b)). There is also edema between them, loss of striations, and focally incipient necrosis. We did not find hemorrhage, hypereosinophilia, or inflammatory cells or polymorph nuclear leukocytes. In the HSL-treated hearts, there were clearly less changes in the morphology than those found in the MS group. The hearts from this group showed similar characteristics to those found in the Ctr group (Figure 7(c)). All representative microphotographs of the Ctr, MS, and MS + HSL groups were taken from areas irrigated by the left anterior descending coronary artery, from the tip of the heart and from the anterior wall of the left ventricle and approximately two-thirds anterior to the ventricular septum.

Table 1: General characteristic of experimental groups.

Variables	Ctr	MS	MS + HSL
Glucose (mmol/L^{-1})	114.6 ± 4.3	115.3 ± 3.5	105.1 ± 6.9
Insulin (μU/mL^{-1})	4.8 ± 0.6	**18.1±4.0****	**7.4 ± 1.0***
HOMA index	1.3 ± 0.1	**4.9±1.2****	**2.1 ± 0.3***
Triglycerides (mg/dL^{-1})	56.1 ± 4.0	**132.0±15.5****	**90.0 ± 11.4***
Cholesterol (mg/dL^{-1})	43.4 ± 1.3	41.6 ± 1.7	43.6 ± 4.1
Intra-abdominal fat (g)	3.3 ± 0.3	**10.8±1.3****	**6.4 ± 0.5***
Systolic blood pressure (mmHg)	115.1 ± 0.2	**138.1±0.8****	**128.1 ± 2.7***
Body mass (g)	812.2 ± 18.7	821.1 ± 22.4	816.1 ± 16.3

Data are mean ± SE, $n = 6$ each group. Statistically significant at $^{*}p < 0.04$ MS vs. MS + HSL; $^{**}p = 0.001$ Ctr vs. MS. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*.

Figure 8 describes the densito-photometric analysis of myocyte bundle areas which showed a significant decrease in the myocardium from the MS group in comparison to the Ctr and MS + HSL groups ($p < 0.001$ and $p = 0.01$, respectively).

4. Discussion

MS is a cluster of pathologies that includes hyperlipidemia, hypertension, obesity, and IR and is considered as risk factors for CVD [32]. Cardiomyopathy in the MS is characterized by complex changes in the mechanical, biochemical, structural, and electrical properties of the heart. In both MS and CVD, the redox equilibrium is altered towards OS [33]. Traditional medicine has allowed people to identify several nutrients, food supplements, herbs, and spices which exhibit anti-MS and antiobesity effects [34]. HSL is one of the medicinal herbs commonly used in traditional Asian and African medicine against hypertension, obesity, and hypercholesterolemia. It has been described that a crude extract of HSL exhibits antihypertensive and cardioprotective effects on hypertensive rats [35]. The aim of this study was to evaluate the myocardial protection induced by a 3% infusion of HSL against ischemia-reperfusion damage due to its antioxidant effect on a MS rat model.

4.1. Isolated Perfused Heart and Hypertension. Our results show that there are altered CVR, CMP, and anatomical changes in the hearts from MS rats after an ischemic insult and that reperfusion further alters this condition. These changes have been previously reported [6]. In the MS model induced by a high-sucrose diet, there is decreased mitochondrial function that contributes to a diminution of the ATP levels reaching the myofibrils, leading to a decrease in CMP [5]. In this paper, we found that the treatment with an HSL infusion improves CMP and CVR, by decreasing the ROS exacerbation. It has been reported that HSL infusion might also act on the heart by promoting a Ca^{2+} flow. Both of these actions, reduction of OS and Ca^{2+} flow, contribute to negative inotropic and chronotropic effects.

Anthocyanins such as delphinidin and cyanidin-3-0-sambubiosides are present in HSL and may block Ca^{2+} channels in vascular smooth muscle, reducing vasoconstriction [36]. It has also been reported that HSL polyphenols ameliorate cardiac dysfunction and vasodilatation, via modulation of intracellular Ca^{2+} entry and reuptake in the heart [17]. Another report showed that oral consumption of HSL enhanced cardiac Na^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase activities in a rat model of hypertension [37]. The finding of vasorelaxation in isolated rat coronary arteries indicated that there is a Ca^{2+} inhibitory action by flavonoids such as quercetin via the inhibition of extracellular Ca^{2+} influx-induced contraction, reduced intracellular-free Ca^{2+} concentration, and an inhibition of the inward Ca^{2+} currents through voltage-dependent Ca^{2+} channels [38].

Other metabolic pathways have been proposed to explain the beneficial effects of HSL on the heart. A recent study reported both negative inotropic and chronotropic actions of HSL aqueous extract in isolated atria and attributed this action to hibiscus acid, the main phenolic acid present in HSL [39], and to the polyphenol and resveratrol present in the HSL infusion. These compounds reversed remodeling and improved inotropic function in a rodent model of heart failure [40]. Another study reported a beneficial effect of the HSL extract in isolated hearts indicating that the extract modulates negative inotropic effects through the antagonistic action on the β-adrenergic receptor [41]. In this model, a significant increase in the vasoconstriction in aortic rings by norepinephrine was associated to damage in the endothelium. The HSL treatment decreased this damage, and this was associated with low BP [13].

In addition, another study showed that HSL effectively ameliorated the systolic dysfunction of the heart. This was evidenced by a significant rise in the development of left ventricular pressure and in cardiomyocyte hypertrophy. The HSL extract reduced gene expression of the myocyte hypertrophic machinery and contributed to a reduction of cardiac fibrosis improving ventricular compliance and relaxation. This was associated with a decrease of OS [42]. The above finding suggests that the inotropic and chronotropic actions by HSL infusion in the heart of the MS rats can contribute to restored CMP and CVR. This would be

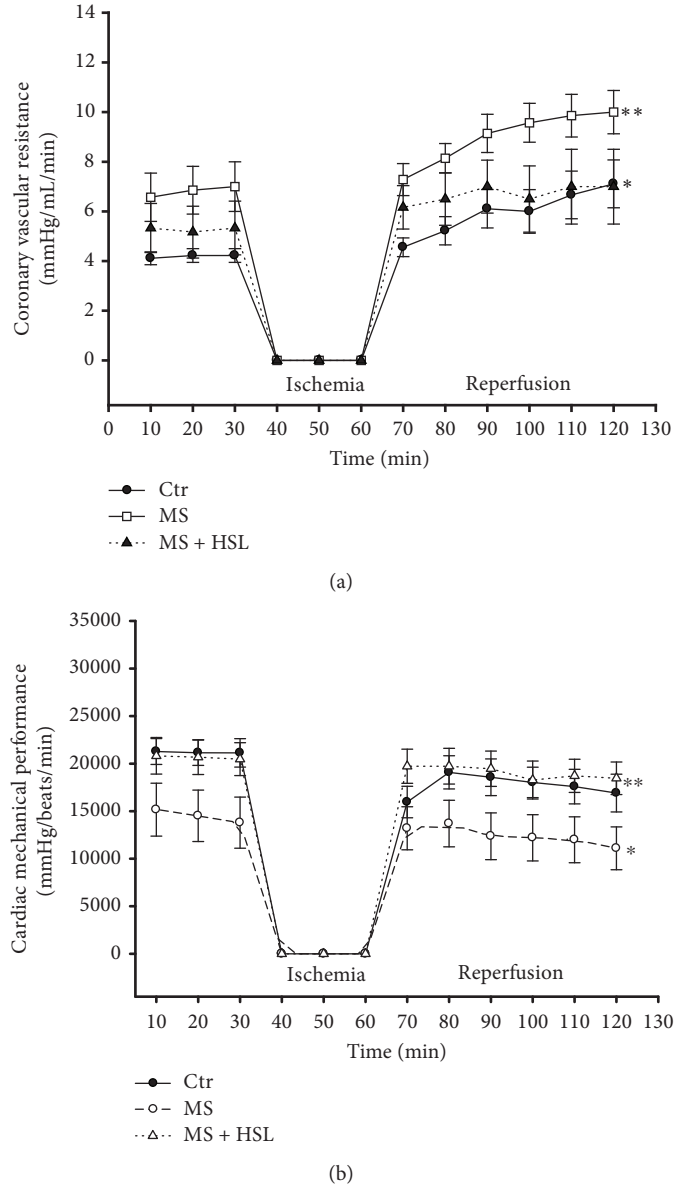

Figure 1: Isolated perfused heart in experimental rats. (a) Coronary vascular resistance of hearts with global ischemia (30 min) and reperfusion (60 min) periods ($n = 6$); $^{*}p = 0.01$ Ctr vs. MS and $^{**}p = 0.04$ MS vs. MS + HSL. (b) Cardiac mechanical performance of isolated hearts with global ischemia (30 min) and reperfusion (60 min) ($n = 6$); $^{*}p = 0.03$ Ctr vs. MS and $^{**}p = 0.01$ MS vs. MS + HSL. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus.*

partly reflected in the BP decrease in MS rats. In turn, this may also favor cardiac mechanics.

Polyphenols present in HSL activate the PI3 K pathway in the endothelium. This pathway is capable of upregulating nitric oxide (NO) via phosphorylation of eNOS. It also suppresses spontaneous Ca^{2+} events in isolated cardiac myocytes and in the isolated rat heart thus decreasing cardiac arrhythmias [17]. Furthermore, the NO synthesized by this pathway can increase endothelial vasorelaxation and lead to a decrease and/or increase in BP and in the NO^{3-}/NO^{2-} ratio, which is an index of NO release. This was shown by our results where there was a normalization of the cardiac function and BP.

Herrera-Arellano et al. have demonstrated in a clinical trial that in patients with stage I and II hypertension, the HSL extract has antihypertensive action. Promotion of diuresis or ACE inhibition could underlie this antihypertensive action [43]. When HSL is ingested for a long term, there is heart hypertrophy in the spontaneously hypertensive rats and a decrease in BP and left ventricular mass. There is also an increase in the surface area and in the length and density of myocardial capillaries [44]. In addition, the anthocyanins delphinidin and cyanidin-3-0-sambubiosides present in HSL have an antihypertensive effect thanks to two synergistic and complementary mechanisms of action: the first is by acting as diuretics, probably due to pharmacokinetics similar to those of aldosterone antagonists (increases the elimination of water and natriuresis without modification in the excretion of potassium), and the second is by competition with the ACE [43].

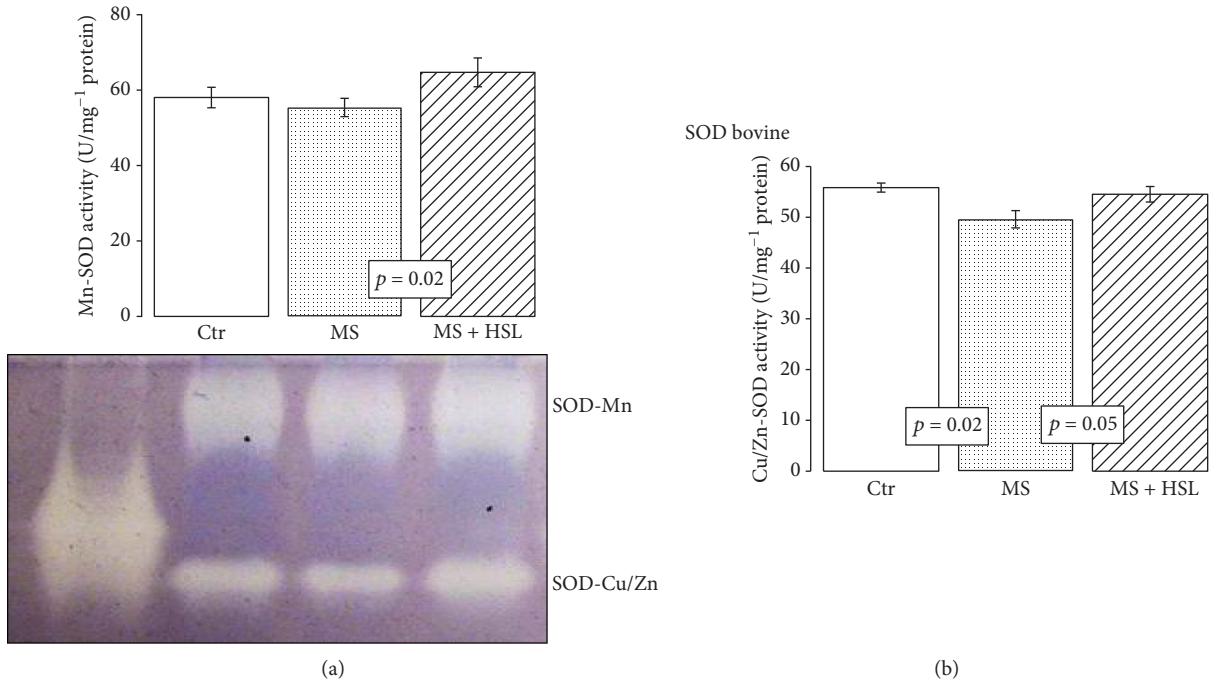

Figure 2: Densitophotometric analysis of activity of super oxide dismutase isoforms in the heart homogenates of the experimental rats; a native gel representative of the Mn-SOD and Cu/Zn-SOD activities is presented. (a) Manganese isoform and (b) copper/zinc isoform. Native gel electrophoresis with 10% polyacrylamide. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*. Data are expressed in mean ± SE ($n = 6$ rats in each group).

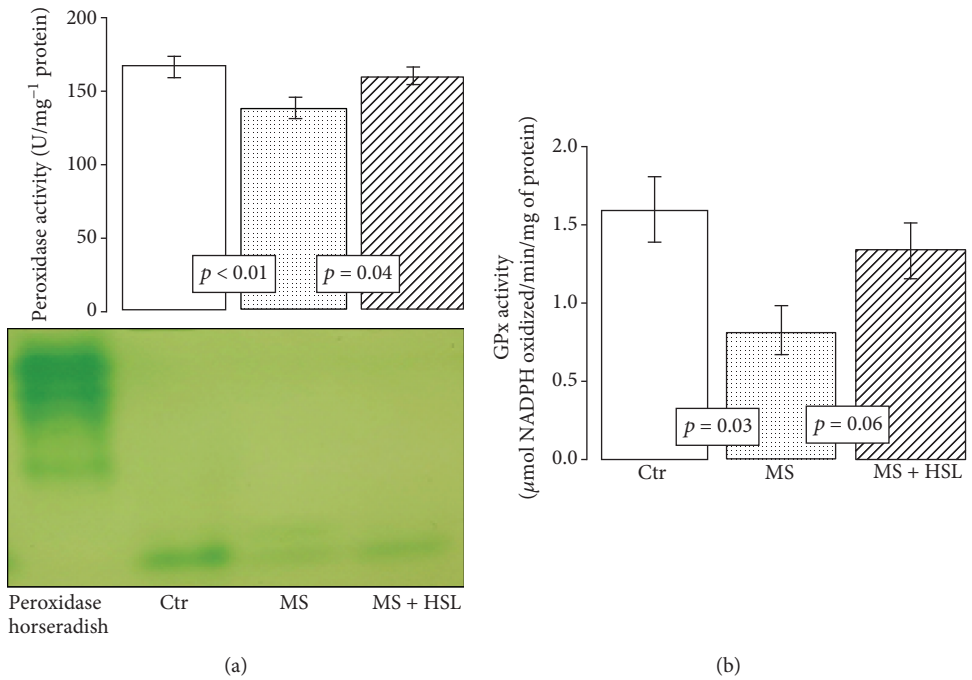

Figure 3: (a) Densitophotometric analysis of peroxidase activities. A native gel electrophoresis with 10% polyacrylamide is shown between (a) and (b). Under these conditions, where peroxidases are present, the gel remains transparent and the 3, 3′,5,5′-tetramethylbenzidine is oxidized showing a green coloration. (b) GPx activity has a tendency to increase its activity when comparing MS + HSL vs. MS. However, the change was not statistically significant ($p = 0.06$ NS). Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*. Data are expressed in mean ± SE ($n = 6$ rats in each group).

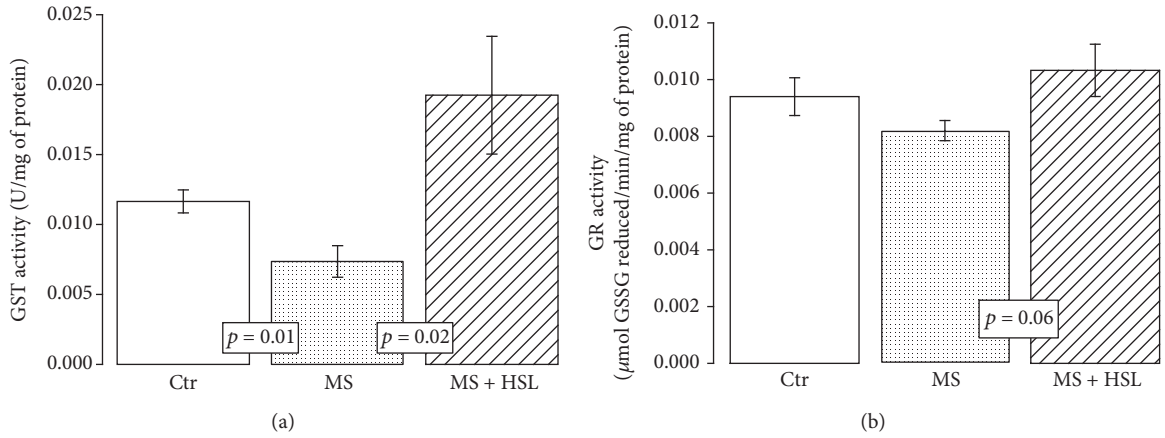

Figure 4: (a) Glutathione S-transferase activity in the experimental groups and (b) effect of the treatment with HSL infusion on glutathione reductase activity in the heart homogenate. There was a tendency to an increase in the activity of GR between MS + HSL and MS; however, it did not reach a statistically significant level ($p = 0.06$ NS). Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*. Data are expressed in mean ± SE ($n = 6$ rats in each group).

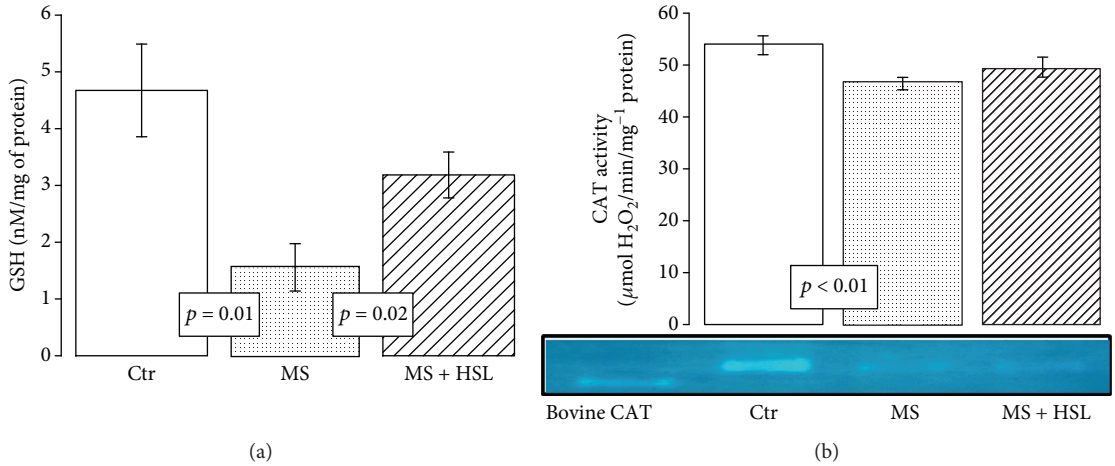

Figure 5: Effect of treatment with HSL infusion. (a) Reduced GSH concentrations. (b) Densitophotometric analyses of CAT activity in the heart homogenate. In (b), a native representative gel of the CAT activity is shown. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*. Data are expressed in mean ± SE ($n = 6$ rats in each group).

4.2. Antioxidant Enzymes: SOD, CAT, GPx, and Peroxidases. During ischemia and reperfusion, the myocardium produces ROS which play a major role in the reperfusion injury. ROS production causes Ca^{2+} reentry, infiltration by inflammatory cells, platelet activation, NO production, metabolic alterations, and endothelial dysfunction. These changes contribute to the background of OS that characterizes the MS model [23]. The activity of antioxidant enzymes is regulated according to cellular requirements, since their production can be induced or inhibited by endogenous effectors. Among the main antioxidant enzymes are SOD isoforms. There are three isoforms of this enzyme, Cu/Zn-SOD located in the cytoplasm, Mn-SOD that is present in the mitochondrial matrix, and EC-SOD located in plasma [45]. An elevation of SOD isoforms by HSL treatment may contribute to reduce superoxide (O_2^-) in the heart of the MS rats, and this can in part help improve cardiac function. However, this leads to an increase in H_2O_2. To detoxify this peroxide, cardiomyocytes have two systems. The first is CAT which is a hemoprotein with four heme groups, located in peroxisomes and mitochondria. This enzyme is responsible for detoxifying H_2O_2, following a concentration gradient and converting it into H_2O and molecular O_2 [46]. The HSL treatment did not modify this enzyme's activity; its levels were low and contributed to OS and to the deterioration of cardiac function. However, this may be due to the overproduction of H_2O_2. When this substrate is used by CAT, it inhibits its activity. However, the PGx enzyme selenoprotein found in the mitochondrial matrix and in the cytoplasm can also detoxify the H_2O_2 and other organic hydroperoxides converting them into H_2O and molecular O_2. This enzyme does not depend on the concentration gradient being dependent on the presence of $NADPH^+$ and GSH [47]. The increased tendency of the GPx and elevated peroxidase activities by HSL treatment suggest that HSL treatment can modulate the activity of these enzymes, favoring an increase in their activity, thus contributing to the decrease of chronic OS in the heart. Previous studies showed that the HSL extract significantly increased

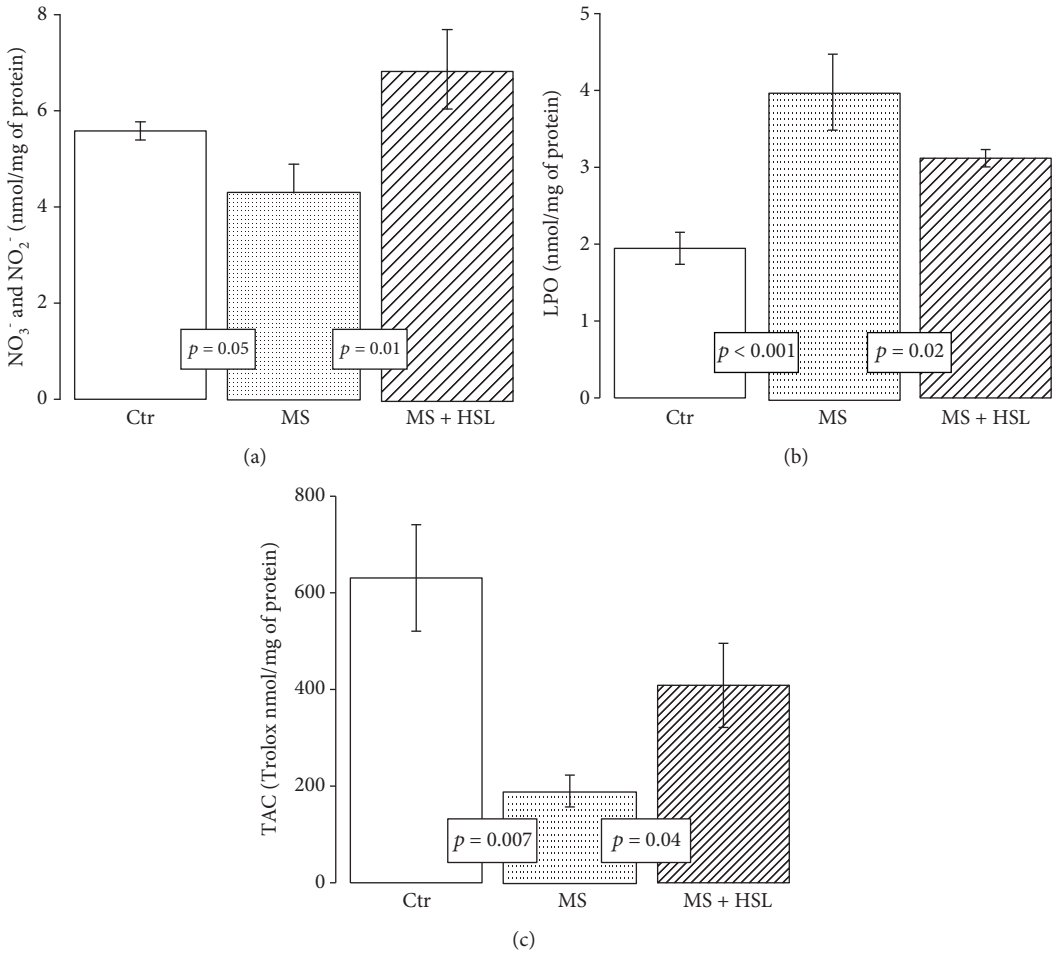

Figure 6: (a) Nitrate and nitrite ratio in the heart homogenates from the experimental groups. (b) Lipid peroxidation levels and (c) total antioxidant capacity. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*. Data are expressed in mean ± SE ($n = 6$ rats in each group).

GPx and SOD in *Cyprinus carpio* hepatocytes by carbon tetrachloride toxicity [48]. Also, HSL treatment overexpressed the GPx in renal ischemia-reperfusion injury in mice by acetaminophen toxicity [49]. In addition, the extract of *Hibiscus rosa-sinensis*, another flower of the *Malvaceae* family, significantly raised GSH levels and SOD and CAT activities and decreased LPO in myocardial ischemia/reperfusion in rats [50]. In addition, GPx catalyzes the reduction of H_2O_2. To carry out its antioxidant activity, GPx requires GSH, which is a critical molecule in the defense against OS that maintains the reducing environment of the cardiomyocytes producing its oxidized form GSSG. The GR reverts this substrate to its reduced form [51].

4.3. Glutathione and GR Activity. GSH is the most versatile antioxidant because of its variety of functions that include the detoxification of xenobiotics or their metabolites. It is the largest source of endogenous antioxidants, and it inhibits the radicals -OH and O_2. It also regenerates vitamins E and C, reconverting them to their active form, and it acts as a cofactor for the GPx enzymes. Approximately 90% of the GSH present in the cardiomyocytes is found in the cytoplasm, while 10% is located in the mitochondria. Eighty-five percent of the total cellular GSH is free, and the rest is bound to proteins. It is transported by amino acids through the plasma membrane acting as a storage source of cysteine [52]. GSH raises and, together with the tendency to increase the GR activity, suggests that HSL infusion may contribute to GSH increase through its regeneration by the GR activity in the heart of the MS rats. The HSL treatment may also contribute the GSH increase through the PCA present in the HSL calyces, what attenuates the OS [53]. In addition, another study showed that the polyphenol extract of HSL increases GSH in the damaged liver by acetaminophen [54]. However, the GSH concentration not only comes from this metabolic pathway but also depends on its precursor amino acids cysteine, glutamate, and glycine or on a decrease/increase in the activity of the enzymes that synthesize it such as γ-glutamyl-cysteine synthetase and GSH synthetase [47].

4.4. Glutathione S-Transferase Activity, TAC, and LPO. On the other hand, GST is the enzyme involved in xenobiotic metabolism and in the protection of damage caused by peroxidated lipids. It catalyzes the GSH ionization to the form of a nucleophilic thiolate anion which reacts spontaneously with nucleophilic components that are closely located. This

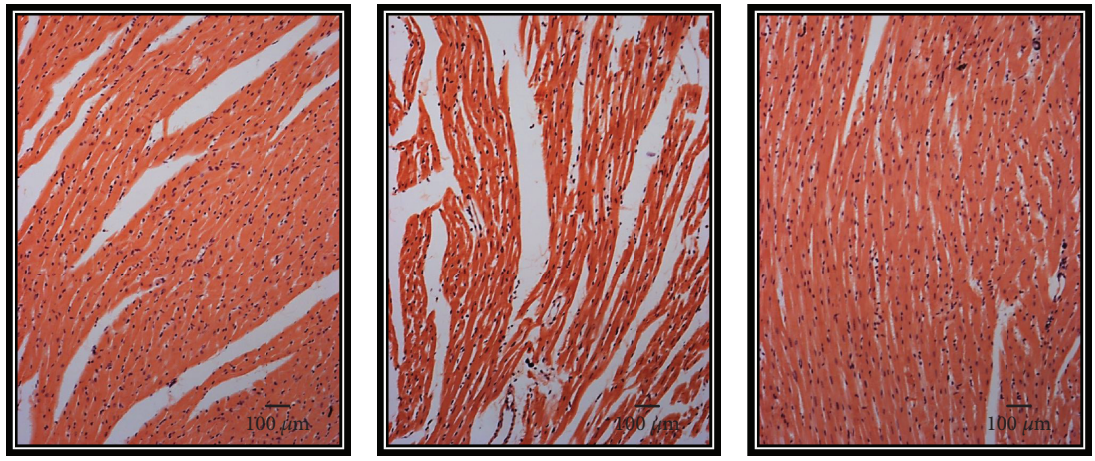

Figure 7: (a–c) Representative photomicrographs of heart tissue after of reperfusion from the three experimental groups. 5 fields per sample were analyzed. (a) Ctr, (b) MS, and (c) MS + HSL. Values are the mean ± SE ($n = 6$). The tissue was processed according to conventional histological procedures, and histological sections were made and stained by hematoxylin-eosin stain at 10x. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*.

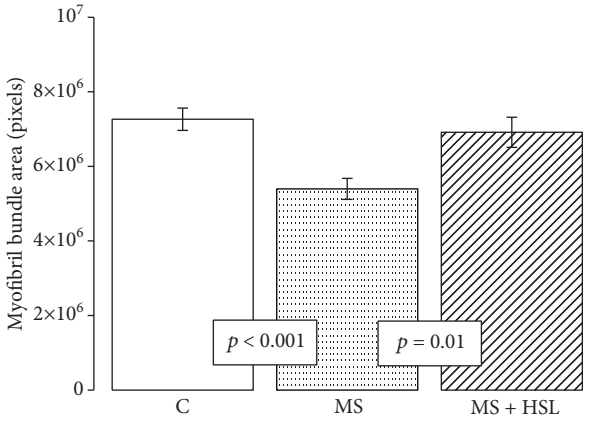

Figure 8: Densitophotometric analysis of myocyte bundle areas in experimental groups. Ctr: control; MS: metabolic syndrome; MS + HSL: metabolic syndrome plus *Hibiscus sabdariffa Linnaeus*. Values are the mean ± SE ($n = 6$).

reaction is followed by the conjugation of the substrate, the formation of the product, and its release. The conjugation increases the solubility of the toxic products, facilitating their excretion from the cell [55], and decreases the LPO rate, which may cause toxicity. HSL treatment increases GST activity and TAC while decreasing LPO. This suggests that the decreased GST activity in the heart of the MS rats favors OS, which is evidenced by the LPO increase. The antioxidant properties of the HSL infusion can contribute in raising GST activity associated with the decrease in LPO. This, in turn, increases the TAC. A recent study in the aortic aneurysm of Marfan syndrome patients showed that an infusion of 2% HSL increased GST activity and TAC and this was associated with LPO decrease [56]. In addition, another study showed a decrease in GST activity related to an increase in ROS in hypertension [57]. In the same way, it has been described that GSTA4 expression is downregulated in the adipose tissue from obese insulin-resistant C57BL/6 J mice and in humans with obesity-linked IR [58]. This model is characterized by IR and hyperinsulinemia which can contribute in inhibiting the GST activity, increasing the LPO, and decreasing the TAC in the heart of MS rats. Furthermore, it has been described that IR and hyperinsulinemia lead to structural abnormalities in the heart, such as increased left atrial size and left ventricular mass [59]. Also, the treatment of HSL infusion decreases the HOMA index; this effect may be due to the presence of cyanidin-3-glucoside which can over express the GLUT4 transporter and can increase the signaling of insulin by the cells [60]. Moreover, *in vitro* studies have shown that the polyphenolic fraction from the aqueous HSL extracts increases the TAC [55]. This may be because the HSL polyphenols participate as captors of ROS in a second line of defense when they have not been neutralized by the enzymatic antioxidant system [32]. They also increase the antioxidant capacity of the nonenzymatic system, favoring an increase of TAC in the heart of MS rats. In the H9c2 cardiomyoblast cells, the protective effect of HSL on doxorubicin-induced cytotoxicity was through the attenuation of ROS production and by inhibition of xanthine oxidase activity, by the reduction of LPO and by the elevation of the antioxidant enzyme activity. Therefore, TAC was observed [61]. In addition, the hydrophilic antioxidants present in HSL have scavenging properties through which they are able to inhibit the free radical mechanism of LPO [62].

On the other hand, the decrease in serum TG by the HSL treatment could be explained by the amount of soluble fiber present in the calices of the HSL plant. It has been estimated that each 250 mL of HSL infusion contains 166 mg of fiber [11].

5. Conclusion

Based on these results, it can be concluded that treatment with HSL infusion protects the cardiac function during

ischemia and reperfusion through the action of the antioxidant substances that it possesses such as PCA, anthocyanins, cyanidin-3-glucoside, quercetin, and polyphenols, thus favoring antioxidant enzymatic activities and nonenzymatic antioxidant capacity.

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 have no personal or financial conflict of interest in relation to the present study. This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Authors' Contributions

Israel Pérez-Torres and Juan Carlos Torres-Narváez share the first authorship of this paper.

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Review Article

Perturbed Biochemical Pathways and Associated Oxidative Stress Lead to Vascular Dysfunctions in Diabetic Retinopathy

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Diabetic retinopathy (DR) is a vascular insult that accompanies the hyperglycemic state. Retinal vasculature holds a pivotal role in maintaining the integrity of the retina, and any alteration to retinal vasculature affects retinal functions. The blood retinal barrier, a prerequisite to vision acuity, is most susceptible to damage during the progression of DR. This is a consequence of impaired biochemical pathways such as the polyol, advanced end glycation products (AGE), hexosamine, protein kinase C (PKC), and tissue renin-angiotensin system (RAS) pathways. Moreover, the role of histone modification and altered miRNA expression is also emerging as a major contributor. Epigenetic changes create a link between altered protein function and redox status of retinal cells, creating a state of metabolic memory. Although various biochemical pathways underlie the etiology of DR, the major insult to the retina is due to oxidative stress, a unifying factor of altered biochemical pathways. This review primarily focuses on the critical biochemical pathways altered in DR leading to vascular dysfunctions and discusses antioxidants as plausible treatment strategies.

1. Introduction

The retina, a transparent tissue of the eye, has an intricate arrangement of neurons and requires highly dedicated circulation to meet its metabolic requirements and functioning of neurotransmission, phototransduction, and complex interaction of metabolites, growth factors, and vasoactive agents. Retinal circulation is a regular geometrically arranged network of vessels with a complex three-dimensional architecture. It mainly is supplied by two vasculatures: the choroid and retinal vessels, where the endothelial cells lining the vessels integrate the normal physiology of the retina [1]. The central retinal artery enters via the optic nerve ensuring blood flow and exchange of gases and nutrients, while the central retinal vein is involved in the removal of waste products that move away from the retina. An important normal physiological function of retinal vasculature is maintenance of the inner blood-retinal barrier (iBRB), which prevents nonspecific permeation of retinal neuropile by macromolecules yet facilitates exchange of respiratory gases, amino acids, salts, sugars, and some peptides [2].

The most sensitive part of the retina is the outer region which constitutes one-third of the retina and is devoid of blood vessels. The absence of blood vessels serves as a special adaptation for visual functioning, but poses a great challenge in the maintenance of continuous energy requirements [1]. The outer blood-retinal barrier that is formed between the tight junctions of retinal pigment cells maintains ionic concentrations in the avascular region of the retina and the interstitial space for neurotransmission. Alternatively, the metabolic need of photoreceptor cells is maintained by choroid vessels. Thus, these efficient blood retinal barriers serve as major anatomical adaptation, with attainment of demanding metabolic requirements of the retina and without compromising its conductive extracellular microenvironment [1, 2]. This intricate retinal vasculature is sensitive to various systemic disorders with diabetes being the most common and perhaps well-studied metabolic insult that has a profound influence on retinal vessels. Retinal vascular dysfunction commences soon after the onset of diabetes and is characterized by impaired microvasculature and transport through the blood retinal barrier which may be an important factor in the initiation and progression of the vascular lesions

in diabetic retinopathy [3, 4]. Various studies on diabetes conclude that increased blood flow and impaired autoregulation are key features of diabetic retinopathy [5].

2. Diabetic Retinopathy

Diabetic retinopathy is one of the leading causes of visual impairment and morbidity across the globe [6]. Type 1 and type 2 diabetes damages the blood vessels in the retina, which may lead to microvasculature complications; however, the incidence of DR is higher in type 1 patients than in those with type 2 diabetes [7]. Amongst 468 million people estimated with diabetes mellitus worldwide [8], approximately 90 million suffer with some form of diabetic retinopathy, [7].

DR is classified into nonproliferative DR (NPDR) and proliferative DR (PDR) stages on the basis of the presence of visible ophthalmologic changes and manifestation of retinal neovascularization [9]. In the NPDR stage, sex, onset, and duration of type 1 diabetes and HbA1c levels are suggested to be the key pointers implicated in NPDR development [10]. Diabetic maculopathy accompanies the NPDR stage and has been considered as the main reason for the loss of vision. The NPDR stage is primarily consequent to hyperglycemic conditions which weaken the capillary walls resulting in microaneurysms. This is followed by the rupture of vessels which leads to accumulation of fatty deposits and lipid by-products [11]. Ensuing this, an obstruction in the nerve fibre layer is observed that results in white fluffy spots known as cotton wool spots. The NPDR stage ranges from mid, moderate, and severe, where the microaneurysms are followed by venous beading and cotton wool spots along with severe microvascular complications [12].

NPDR is then followed by a proliferative state of the retinal tissue. The PDR stage is a consequence of ischemic conditions that arise due to obstruction in the NPDR stage. The higher metabolic requirement of retinal tissue poses the need for neovascularization which is due to the release of angiogenic signals. Retinal detachment and this neovascularization with the proliferation of the fibrovascular tissue is a characteristic of the PDR stage [13]. These newly formed vessels are leaky, fragile, and misdirected, and with age the shrinkage of the vitreous humour causes them to tear and result in sudden vision loss. If a greater force is created, it may lead to tractional retinal detachment. Despite severe complications in the PDR stage, macular oedema is the main reason behind the loss of visual acuity. Regardless of these varied stages characterising DR, the major progressive change leading to retinopathy in diabetic patients remains speculative in terms of microvascular and biochemical complications leading to oxidative/nitrative stress.

3. Microvascular Complication

Microcirculation involves the transfer of nutrients and removal of waste products and regulates the fluctuating hydrostatic pressure of the eye. Normally, fundamental metabolic and myogenic autoregulatory mechanisms guarantee satisfactory advancement of these microcirculatory functions [14]. Microvascular endothelial cells are believed to be targets of hyperglycemic damage since they are not capable of lowering the glucose transport rate when glucose concentrations are high, stimulating intracellular hyperglycemia. This has been thought to be the key event in microvascular endothelial damage, diminished accessibility of nitric oxide, increased permeability, increased leukocyte attachment, and procoagulant action [14].

In one of the recent studies on high sucrose- (HSu-) treated rats, a decrease in the thickness of the inner retinal layers was observed. Nevertheless, neither apoptotic cells nor retinal neural markers were identified in the retinas of HSu-treated animals. Also, no progression was recognized in the permeability of the blood-retinal barrier as well as tight junction proteins. Likewise, these parameters stayed unaltered in the retina regardless of the increase in the number of retinal microglial cells. Thus, a prediabetic rodent demonstrates that the retinal structure is influenced by the diminishing internal layers, without vascular and inflammatory changes [15]. Therefore, inconspicuous auxiliary changes may be seen as an early unsettling influence in the development of DR which could be reversed by preventive strategies at this stage, before it results in irreversible damage to the retina. Progression from diabetes to diabetic retinopathy underlies changes in the haemodynamics or vascular geometry [16]. Haemodynamic factors like perfusion pressure, vascular resistance, blood viscosity, and vascular geometry influence the circulation of blood flow to the retina. Regardless of this fact, the components involved in haemodynamic modifications have not been fully elucidated [14, 16].

In an earlier study, an analysis was performed to assess the relationship between the assessed haemodynamic highlights and the progression of DR. Vessel bifurcations on fundus images and factors like nodal pressure, volumetric blood flow, wall shear stress, blood flow velocity, and Reynolds number were investigated over a period of three years [17]. The analysis revealed critical changes in haemodynamic parameters related with perceptible changes to vessel geometry, especially in the venular network, and these changes were articulated to be three years prior to DR onset [17]. Therefore, these findings altogether suggest a role of the microvasculature and its geometry in the progression of DR, but further studies in this area are yet to be undertaken to fully elucidate the role of altered vascular biology in the progression of DR.

However, vascular modifications are considered to be the major insult for the onset and progression of DR, including altered blood flow, dyslipidemia, basement membrane thickening, loss of pericytes, and platelet aggregation along with neuroglial damage [18]. These changes have been suggested to be the result of alteration/disruption of biochemical mechanisms required for the normal functioning of a cell. A unifying mechanism of hyperglycemia and induced oxidative stress in retinal cells has been regarded as one of the crucial players in causing alteration(s) in various biochemical pathways that have been shown to be interconnected with vascular insult [19, 20]. Furthermore, an increase in the stressful conditions eventually leading to apoptosis of the cells associated with retinal vasculature is the major effect of these pathways. Cardinal biochemical pathways proposed

to be involved in the DR includes increased flux of the polyol pathway [21, 22], advanced glycation end product/receptors of advanced glycation end product (AGE/RAGE) pathway [10, 23], hexosamine pathway [24], PKC activation [25], tissue (renin-angiotensin system) RAS [26], and histone modifications which are emerging as key events in the development of DR. The overall effects of these metabolic abnormalities are hypothesized to result in augmentation of (reactive oxygen species) ROS and (reactive nitrative species) RNS production and associated oxidative and nitrosative damage [20, 27] which are key mediators in inducing vascular dysfunctions and related insult to retinal circulation (Figure 1).

4. Increased Flux in the Polyol Pathway

Although the polyol pathway is a minor glucose metabolism pathway, it is considered to play a pivotal role in retinopathy [21, 28]. The first and rate-limiting step of this pathway is the conversion of excess glucose to sorbitol, using NADPH as a cofactor, a reaction catalyzed by an enzyme aldose reductase. The sorbitol formed is then converted to fructose by a slow reaction involving sorbitol dehydrogenase [22]. Also, the existence of polymorphism in the aldose reductase gene (C106T) has been shown to be associated with the increased susceptibility to retinopathy in individuals with type 1 (diabetes mellitus) DM [29]. Numerous studies have been carried out to establish the role of increased polyol production in DR. In one of the studies, both rat and human retinal endothelial cells showed increased aldose reductase immunoreactivity. In addition, rat and human retinas exposed to high glucose in organ culture increased the production of sorbitol corroborating excess aldose reductase activity to be one of the mechanisms in the development of DR [30]. Dagher et al. [30] have suggested that the polyol pathway mediates the increase in apoptosis of neurons and attenuation of GFAP- (glial fibrillary acidic protein-) immunostained astrocytes along with the increase in the levels of sorbitol and fructose in the retina. Indeed, this is due to the nonpermeability of sorbitol which results in osmotic damage [21, 31]. Moreover, fructose produced by the polyol pathway gets phosphorylated to fructose-3-phosphate [32], which in turn is broken down to 3-deoxyglucosone; both these molecules are strong glycosylating agents that result in the formation of AGEs. Additionally, the increased flux of the polyol pathway results in depletion of cellular NADPH, affecting the production of reduced glutathione and nitric oxide thereby resulting in antioxidant imbalance [33]. Moreover, it has further been suggested that the polyol pathway is the only mechanism of glucose toxicity responsible for the spectrum of neural and vascular abnormalities [34]. Aldose reductase has been extensively studied as a molecular target for DR, and inhibitors targeting aldose reductase are expected to be beneficial against DR. Inhibitors such as sorbinil and beta-glucogallin (BGG) have been shown to deplete sorbitol accumulation and reduce oxidative stress [33]. Various in silico studies have identified 2-benzoxazolinone derivatives effective against ALR2 (aldose reductase 2) and can reduce AGE and oxidative stress [35]. Additionally, studies on human aldolase reductase have revealed huperzine A, rosmarinic acid, and luteolin 78 to possess aldose reductase inhibition potential [36] and hence could be potential molecules in targeted therapeutics.

5. AGE/RAGE Pathway

Elevated glucose and coupled perturbations in the pathways regulating glucose levels result in the formation of advanced glycation end products (AGEs) which are made from nonenzymatic glycoxidation and glycation of various biomolecules and sugar metabolites [37]. AGEs bind to their receptors termed as RAGE (receptor for advanced glycation end product) and trigger the cascade of inflammatory signals [38]. The AGE-modified plasma proteins have been also found to bind to AGE receptors on cells like macrophages, vascular endothelial cells, and vascular smooth cells affecting their functionality [37, 38]. AGEs accumulate in the circulation due to their inefficient renal clearance. Moreover, exogenous AGEs or dietary AGEs have also shown to be the reason for their accumulation in diabetic patients [39] and are considered to be the pivotal participants in inducing the ROS formation by altering the proteins, enzymes, and the genetic material of the mitochondria by glycation [40]. Their accumulation increases the vessel thickening and platelet aggregation leading to ischemic situation, a condition also responsible for the induction of growth factors and neovascularization [41]. Both intracellular and extracellular formations of AGEs in the retina are involved in destructive roles, as the alteration in protein chemistry distorts their structure [39]. Additionally, oxidative stress has also shown to accelerate the formation of the AGEs. Although the human body is self-sufficient in degrading the AGEs by ubiquitination and autophagy, the excess formation or intake results in their accumulation [37]. Additionally, AGEs are also accountable for permanent dysfunction of the mitochondrial enzymes due to glycation of the mitochondrial genetic material leading to "metabolic memory," a severe condition that is observed in DR [42]. It is an unresponsive state where even glucose control cannot prevent complications of DR. Besides these, the components of the extracellular matrix are subject to modification by AGE precursors.

AGEs have been corroborated to upregulate the expression of RAGE in retinal pericytes via ROS production [43] which is the earliest known alteration in the diabetic retinal vasculature [44]. AGE-RAGE interactions are responsible for NADPH-mediated ROS generation via activation of mitogen-activated protein kinase [38]. These interactions are also responsible for the translocation of NF-κB, decrease in the ratio of Bcl-2/Bax, and increased expression of vascular endothelial growth factor (VEGF), inflammatory cytokines, and adhesion molecules [45], which correlate with the development of DR. During diabetes, AGEs have been shown to accumulate in retinal pericytes, decreasing their survival, breakdown of the blood retinal barrier, and progression towards diabetic retinopathy [9]. Even the major AGE precursor methylglyoxal has been shown to mediate oxidative stress and impair nitric oxide- (NO-) mediated vasorelaxation and upregulate inflammatory markers in an animal

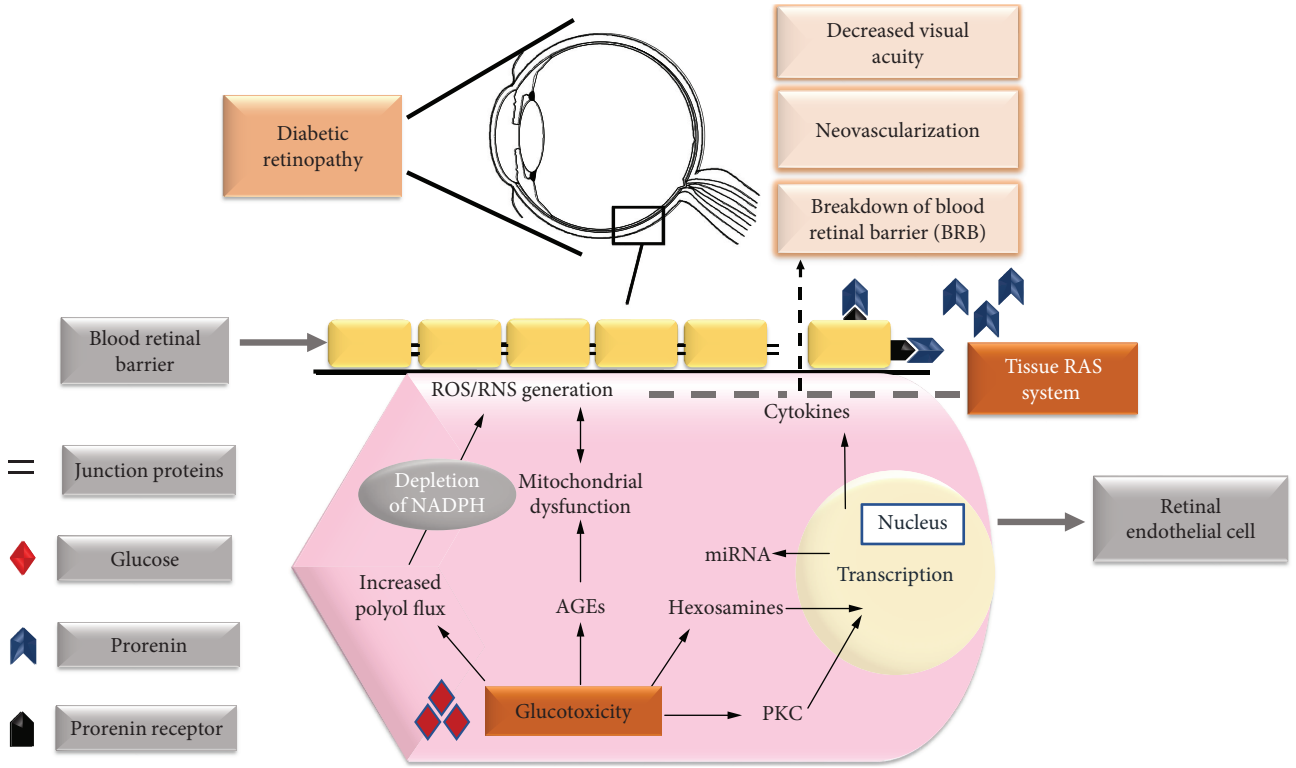

Figure 1: Effect of glucotoxicity on plausible biochemical pathways involved in pathogenesis of DR.

model of type 2 diabetes where the rats were fed with methylglyoxal [46]. Also, methylglyoxal in a similar study was shown to reduce retinal pigment epithelial cell viability via ER stress-dependent ROS production, mitochondrial membrane potential loss, and intracellular calcium increase [47]. In a recent study, retinal pigment epithelial cells were treated with chrysin, a naturally occurring flavonoid, to exploit its retinoprotective effects against diabetes-associated visual cycle impairment by targeting the AGE-RAGE pathway. It was demonstrated that chrysin treatment restored the retinoid visual cycle through blocking ER stress via AGE-RAGE activation in glucose-stimulated retinal pigment epithelial cells and diabetic eyes [48], highlighting the importance of the AGE-RAGE pathway in impaired visual cycle associated with diabetic retinopathy.

6. Hexosamine Pathway

Another crucial pathway involved in the pathogenesis of DR is the hexosamine pathway which itself is relatively a minor branch of glycolysis where fructose-6-phosphate is converted to glucosamine-6-phosphate, a reaction catalyzed by the first and rate-limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT) [49]. Chronic hyperglycemia leads to an enhanced influx through the hexosamine pathway which results in perturbation of retinal cells [50, 51]. In this pathway, glucose is metabolized into UDP-N-acetylglucosamine (UDPGlcNAc) [19]. Specific O-GlcNAc transferases (OGT) then modify various cytoplasmic and nuclear proteins by addition of the amino sugar N-acetylglucosamine (O-GlcNAc) from UDP-GlcNAc similar to phosphorylation modification at the ser/thr residues. Pancreatic β-cells express large amounts of both O-linked β-N-acetylglucosamine transferase (OGT) and O-GlcNAcase (OGA), suggesting the importance of O-GlcNAc in pancreatic β-cell functioning and survival under normal glucose conditions [52]. In addition, hyperglycemia-mediated enhanced O-GlcNAc modifications contribute towards increased β-cell death [53]. This imbalanced O-GlcNAc modification has been implicated in the etiology of microvascular complications related to DR as it regulates the fate of retinal vascular cells [54, 55]. Moreover, it has been observed that in retinal neuronal cells, this modification alters the neuroprotective effect of the insulin/Akt pathway [56].

Nucleoside diphosphate kinases (NDPK) are the enzymes which provide nucleoside triphosphates to the cells and hence play a pivotal role in mediating fundamental cellular processes [57–59]. NDPKs are histidine protein kinases, which transfer the phosphoryl group from the phosphohistidine active site to the histidine residue of the target protein. These histidine kinases maintain the metabolic status of the cell by regulating the levels of NTP in the cell [57]. The B isoform of this enzyme, NDPK-B, has been shown to phosphorylate the β subunit of the G protein, potassium channels ($K_{Ca}3.1$), and calcium channels (TRPV5), thus modulating their functions [60]. Moreover, NDPK-B has been documented in modulating vascular integrity [61]. NDPK deficiency has been shown to mimic vascular regression similar to DR. O-GlcNAcylation of FoxO1, a transcription factor, upregulates Ang 2 (angiopoietin 2) which is an initiator of vascular regression suggesting that the hexosamine pathway is responsible for O-GlcNAcylation of various proteins to

be the prime culprit underlying the changes in the molecular signals associated with microvasculature [62]. Corroborating the similar fact, another study has stated the role of NDPK-B deficiency in causing a diabetes-like vascular pathology by upregulating endothelial angiopoietin 2 in the retina of mice [49]. Hyperglycemia increases O-GlcNAcylation of retinal proteins in DR. O-GlcNAcylation of the p65 subunit of NF-κB in streptozotocin-induced DR mice has been shown to be responsible for hyperglycemia-induced activation of NF-κB and retinal ganglion cell death [51], further linking the involvement of imbalanced O-GlcNAc modification in the etiology of the microvascular complications in DR.

7. PKC Pathway

Diacylglycerol and PKC are also amongst the key players altered by hyperglycemia in DR [63, 64]. Mainly, three isoforms of PKC are reported in biological systems; viz., the conventional PKC isoforms (PKC-α, β1, β2, and γ) are activated by phosphatidylserine, calcium, and DAG or phorbol esters. The novel PKCs (PKC-δ, -θ, -η, and -ε) are activated by phosphatidylserine, DAG, or PMA (phorbol 12-myristate 13-acetate), and the atypical PKCs (PKC-ζ and -ι/λ) are not activated by either calcium, DAG, or PMA [65]. In the retina, hyperglycemia persistently elevates diacylglycerol (DAG) and activates downstream protein kinase C, evidently interconnecting PKC with associated microvascular changes [21, 66, 67]. The beta and delta isoforms of PKC are mainly activated, but increases in other isoforms have also been found in the retina [68]. Hyperglycemia activates PKC isoforms indirectly through the AGE-RAGE pathway [69] and polyol pathway [70] by increasing ROS. The DAG-PKC signaling pathway plays roles in vascular cells by regulation of permeability, contractility, extracellular matrix (ECM), cell growth, angiogenesis, cytokine actions, and leukocyte adhesions, processes seen to be altered in diabetes [71, 72]. Numerous studies have demonstrated the contribution of PKC activation in decreasing retinal blood flow. Studies focussed on PKC agonists and antagonists have revealed decreased or increased retinal blood flow, respectively. Introduction of phorbol esters, an agonist of PKC into the retina, declines retinal blood flow while this decrease in blood flow has been shown to be resolved by PKC inhibitors [67].

Amid many targets, a plausible mechanism employed by PKC to cause vasoconstriction and decreased retinal blood flow is the increase in the expression of a potent vasoconstrictor, endothelin A (ET-A) [73]. Its expression has been shown to increase in the retina of diabetic rats while intravitreous injection of the endothelin-A (ET-A) receptor antagonist prevented the decrease in retinal blood flow [73]. This declined retinal blood flow causes hypoxic conditions, which in turn is a robust inducer of VEGF, causing increases in permeability and microaneurysms [74, 75]. δ isoform of PKC and p38α mitogen-activated protein kinase (MAPK) activation increases the expression of Src homology 2 domain–containing phosphatase-1 (SHP-1), a protein tyrosine phosphatase, which dephosphorylates the PDGFβ receptor and induces pericyte apoptosis [38, 76]. Previously, it was shown that antiamyotrophic lateral sclerosis (ALS) drug riluzole attenuates pathological changes in oxygen-induced retinopathy, a surrogate model of DR [77]. More recently, a similar study on cultured retinal pericytes as well as in diabetic rats targeting PKCβ showed that anti-ALS drug riluzole attenuates monocyte chemotactic protein (MCP1), a cytokine elevated in vitreous humour and serum during DR [78] most probably by preventing the abnormal activation of PKC.

8. Renin-Angiotensin System

One of the hallmarks and early events of DR is the breakdown of the blood–retinal barrier (BRB), and one of the plausible reasons of this breakdown is renin-angiotensin system-(RAS-) mediated altered vascular permeability [79]. Tissue RAS, a paracrine system, is a property of numerous organs such as eye, brain, vessels, adrenal gland, testis, and kidney, which locally produces angiotensin (Ang) [80]. This system involves prorenin which binds to its receptor, termed as prorenin receptor (P)RR, which has been implicated in the pathogenesis of DR [81] and induces the production of VEGF through ERK1/2, and this signal cascade is known as the receptor-associated prorenin system (RAPS) [82, 83] which is considered to be responsible for the dysfunctional blood retinal barrier. A study on PDR patients has shown the levels of (P)RR to be high in their vitreous fluid samples than in nondiabetic control eyes, strengthening the implication of (P)RR in DR [84, 85]. Also, (P)RR and other RAS system components have also been detected in human PDR fibrovascular tissues, normal ocular tissues, and various human retinal cell lines, including retinal pigment epithelial cells [84, 86], while vitreous prorenin and Ang 2 levels have been reported to increase in PDR eyes [84, 87].

Ang 2, a vasoactive and angiogenic agent, along with VEGF which is a proangiogenic stimulus has been observed to be elevated in the vitreous fluid of PDR patients [88]. Additionally, an increase in VEGF and VEGFR-2 gene expression and an elevation of ocular active renin are indicative of the interaction of tissue RAS and VEGF wherein endothelial cell proliferation is observed as an outcome of the activated tissue RAS system [89], thus correlating the tissue RAS system and VEGF in the progression of DR. In addition, ATP6AP2 or prorenin receptor (P)RR is shown to interact and colocalize with the PDHB subunit of the PDH (pyruvate dehydrogenase) complex. It was observed that PDH activity is downregulated due to ATP6AP2 knockdown, and it leads to suppression of glucose-induced ROS generation in retinal pigment epithelial cells. Thus, ATP6AP2 is considered pathogenic due to its role in RAPS activation and mitochondrial ROS generation [86]. Therefore, blockade of (P)RR and other players of the RAS system might inhibit a series of events vital for vascular abnormalities represented in DR.

9. Metabolic Memory and Epigenetic Modifications

Numerous studies have suggested epigenetic modifications to be a significant contributor in DR development [90–92]. The duration of hyperglycemia decides whether improved

glycaemic control would be effective in DR [85], implicating that hyperglycemia exposure results in a phenomenon of metabolic memory and could be attributed to epigenetics [91]. Earlier, an anatomic observation of gradual reduction of capillary cells in DR suggested that "retinopathy neither appears promptly after the onset of hyperglycaemia nor arrests promptly on correction of the hyperglycaemia" [90].

The most primitive epigenetic modification is DNA methylation which has a correlation with DR progression as indicated by various studies. DNA methylation is a phenomenon where the methyl group is transferred from S-adenosylmethionine (SAM) to DNA molecules, a reaction catalyzed by DNA methyltransferases. DR patients have shown a significantly higher level of DNA methylation as compared to those without DR [92], indicating that higher DNA methylation is a key component in DR development. Moreover, the study also showed that the levels of DNA methylation remain constant in these DR patients, suggesting that this epigenetic modification occurred only during the early stage of the disease. Another study on DR patients found altered methylated CpG sites, further highlighting the role of epigenetics in DR [93]. Studies using animal models have also strengthened this data where modified methylation patterns have been seen under hyperglycemic conditions [94]. Another study revealed methylation and activation of the matrix metalloproteinase 9 (MMP-9) gene which is known to be associated with DR [95] that plays a role in accelerating the apoptosis of retinal vascular endothelia. In addition, transcription of MMP-9 is regulated by nuclear factor kappa B (NF-κB) whose activation is modulated by the acetylation of its p65 subunit. Histone deacetylase plays an important role in the acetylation-deacetylation of p65. In diabetic mice, histone deacetylase activity was found to be decreased and p65 acetylation was elevated leading to an increase in MMP-9 expression [96].

Another epigenetic alteration, histone modification, has also been a key contributor in DR pathophysiology [97]. The transcription activity of HDAC1/2/8 (histone deacetylase) was elevated in retinal endovascular cells, while the activity of HAT (histone acetyltransferase) and the expression of acetylated histone H3 were both decreased in streptozotocin- (STZ-) induced diabetic models. Moreover, these changes were found to be irreversible after the blood glucose of the rats was restored to normal level, indicating that DR development is to be associated with histone modifications and might be participants in the formation of the "metabolic memory" phenomenon.

Several studies implicated a major role of mitochondrial alteration due to epigenetic modifications as the key process in the induction of metabolic memory in DR. During DR, the mitochondrial homeostasis and dynamics are altered, creating a vicious cycle where the alteration of mitochondrial enzymes induces superoxide formation which in turn alters the organelle physiology. The sensitivity of the mitochondria is exclaimed due to the close proximity of the mitochondrial DNA (mtDNA) to the electron transport chain (ETC) and lack of histones. An increase in 8-OHdG in the diabetic retina confirms the mitochondrial susceptibility [94]. Additionally, the dysfunction of the repair pathways further complicates the mitochondrial damage [98]. The mtDNA replication also plays an important role in mtDNA damage experienced by the retina in diabetes, and these are under the control of superoxide, which is well known to be altered under hyperglycemic conditions. Thus, the regulation of mtDNA replication/repair machinery has the potential to prevent mitochondrial dysfunction and the development of diabetic retinopathy [99].

Histone modifications of the molecules regulating the redox status of the cell has been extensively studied, wherein the mitochondrial superoxide dismutase SOD2 depletion and inhibition of Nrf2 (nuclear factor- (erythroid-derived 2-) like 2), a transcription factor affecting antioxidants, has been observed. During the state of oxidative stress, Nrf2 translocates to the nucleus where it binds to the antioxidant response element (ARE). Keap1, an inhibitor of Nrf2, tethers it in the cytosol and leads to proteasomal degradation through cullin-3-dependent degradation [100]. Mishra et al. [101] have observed that hyperglycemia increased the binding of Sp1, a transcription factor at the Keap1 promoter, and enriched H3K4me1 and activated SetD7 (methyl transferase). This leads to inhibition of Nrf2 binding on antioxidant response element (ARE) leading to oxidative stress in the cell. In earlier studies, deletion in MnSOD (manganese superoxide dismutase) and Sod2 activity *in vivo* has been shown to increase oxidative damage in mitochondria and alters the mitochondrial function [102]. In another study, streptozotocin- (STZ-) induced diabetic rats showed an increase in H4K20me3, acetyl H3K9, and NF-κB p65 at the promoter and enhancer of retinal Sod2. Even the reversal of hyperglycemia failed to prevent increases in H4K20me3, acetyl H3K9, and NF-κB p65 at Sod2. Thus, increased H4K20me3 at Sod2 contributes to its downregulation and is responsible for the development of DR and metabolic memory phenomenon [103].

Dysregulated mitochondrial biogenesis also contributes to the phenomenon of metabolic memory. The nuclear-mitochondrial transcriptional factors and translocation of transcription factor A (TFAM) to the mitochondria are essential for transcription and replication of mitochondria and thus tightly control the biogenesis of the organelle [104]. An earlier study which investigated the effects of diabetes on nuclear-mitochondrial communication in the retina has uncovered that retinal mitochondrial biogenesis is under the control of superoxide radicals and is debilitated in diabetes, perhaps by diminished transport of TFAM to the mitochondria. Hence, regulation of biogenesis by pharmaceutical or molecular means might provide potential means to impede the development/progression of diabetic retinopathy [105]. Additionally, a good glucose control along with lipoic acid supplementation has shown to retard the progression of DR indicative of a major role of mitochondrial function in the progression of disease [105, 106].

10. Role of miRNA

MicroRNAs (miRNAs) are a class of 19 to 25 nucleotide bases, noncoding RNAs that regulate gene expression at the posttranscriptional level by annealing to their partially

complementary sequences in the target mRNAs resulting in translational repression or degradation of mRNAs, thereby depleting the protein expression [107]. miRNAs have been implicated in the regulation of genes involved in DR development, thus playing a role in epigenetic alterations of DR [108]. A total of 11 miRNAs (miR-182, miR-96, miR-183, miR-211, miR-204, miR-124, miR-135b, miR-592, miR-190b, miR-363, and miR-29c) displayed increased expression in the retinas of DM rats, whereas the expression of 6 miRNAs (miR-10b, miR-10a, miR-219-2-3p, miR-144, miR-338, and miR-199a-3p) was found to be decreased [109]. Moreover, it was observed in cultured human endothelium cells that miR-23b-3p regulates high-glucose-induced cellular metabolic memory through an SIRT1-dependent signaling pathway [110], while in a diabetic rat model, miR-126 was found to play a potential role in the pathogenesis of DR [111, 112]. Additionally, miRNA has also been implicated in regulating retinal neovascularization [112].

11. Interplay of Nitrosative Stress/Oxidative Stress and Inflammation

The biochemical mechanisms which are altered in DR probably via hyperglycemic conditions eventually culminate into cellular stress affecting retinal homeostasis. This stressful condition is induced either by the increased flux of these biochemical pathways along with alterations in the proteins involved in maintaining the metabolic energy homeostasis [96]. Also, the mitochondrial complexes are the prime victims due to an increase in reactive oxidative species [96] and nitrative species [113].

11.1. Nitrosative Stress. Nitrosative stress is prompted by a response of superoxide with nitric oxide (NO), which creates peroxynitrite and is ensnared in diabetic conditions. Increased nitrative stress could be prompted by protein nitration and damage membrane proteins and fatty acids, prompting changes in cell signaling transduction and upregulation of inflammatory reaction and initiating the apoptotic pathway. Hyperglycemic episodes are connected with increased nitrative stress, which can trigger the advancement in diabetic complications [113].

Mitochondria are additionally equipped for creating both RNS and ROS. NOS exist in three isoforms (endothelial (eNOS), neuronal (nNOS), and inducible (iNOS)) which catalyze the change of L-arginine into citrulline and NO. This response additionally requires flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin (BH4), heme, and calmodulin. These cofactors are essential (e.g., when BH4 levels are restricted) in light of the fact that NOS may move toward becoming uncoupled and produce superoxide rather than NO. Another plausibility is that, in states of high oxidative stress, NO and superoxide collaborate to produce ONOO−, an exceptionally receptive species equipped for nitrating tyrosine residues, thereby enhancing oxidative damage [113]. The accumulation of RNS shifts the homeostasis of the cell, resulting in nitrosative stress, which is a major phenomenal consequence of the stress conditions in the diabetic retina and is now emerging as a new insight for DR research [113]. NO, a multifunctional molecule that can change proteins by means of nitrosylation, is majorly found in reactive form during DR progression. In addition, nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), a specific marker for NO-producing neurons, was revealed to be positive in immunoreactive experiments on diabetic retina suggesting a role of NO in development of DR [114].

Augmentation in nitrosative stress has been revealed as the key player in worsening the pathogenesis of PDR [115]. Additionally, LPO (lipid peroxide), NO, and GSH (glutathione) levels were also shown to be significantly associated with the severity of diabetic retinopathy [116]. An increase of oxidative/nitrative pressure markers, for example, 4-hydroxynonenal and nitrotyrosine, was more elevated in retinal vasculature of diabetic rodents when contrasted with normoglycemic mature and adult rodent retinas [117].

On similar lines, a study showed that the onset of diabetes results in an increase in the nitrated proteins in the retina [114]. Furthermore, nitrotyrosine immunolabelling in the photoreceptor layer, ganglion cell layer, inner nuclear layer, and some Muller cell processes in the retina of diabetic rats points towards the impinging role of nitrosative stress in DR [114]. Conjointly, recent preclinical and clinical studies have indicated a plethora of factors contributing towards the role of NO in the pathogenesis of DR and have pointed towards a further investigation in the plight of therapeutics [118]. Another finding suggests that aminoguanidine treatment hinders capillary cell death and development of diabetic retinopathy [119, 120]. Moreover, it has also been found to decrease the hyperglycemia-induced increase in NO and its sequelae raise a plausibility that (RNS) assumes a major role in the pathogenesis of retinopathy. In any case, it is not conceivable at present to infer that aminoguanidine represses retinopathy exclusively through hindrance of NO generation. Therefore, approaches that specifically repress NO-intervened procedures will be important to absolutely assess the role of NO in the development of DR [121].

Calcium dobesilate (calcium,2,5-dihydroxybenzenesulfonic corrosive (CaD)), which is considered an angioprotective agent, is recommended as an elective treatment for diabetic retinopathy and other vascular diseases, in spite of the debate with respect to its clinical viability. Some clinical preliminaries did not report any helpful impact of CaD treatment, particularly in patients at the later phases of diabetic retinopathy. Notwithstanding, a few different clinical trials demonstrated enhancement in visual acuity after oral treatment. Diabetes increases tyrosine nitration in the retina, fundamentally in the ganglion cell layer, and treatment with CaD attenuates this increase in tyrosine nitration initiated by diabetes [122].

11.2. Oxidative Stress. Besides nitrative stress, a shift in the normal physiology due to oxidative stress is also a chief suspect in DR pathophysiology [123]. The balance of oxidants and antioxidants is a mediator of the fundamental cellular process including maintenance of the vascular system [124], which if disrupted can lead to threatening situations depending upon the severity of stress imposed and availability of their clearance system [125]. Unfortunately, imbalance

Table 1: Oxidants elevated in diabetic retinopathy.

Oxidants	Involvement in DR
Superoxide radicals	Accumulation in retinal cell mitochondria leads to mutations in the mtDNA and induces a phenomenon of "metabolic memory" [132, 133].
Hydroxy radicals	Generated by Fenton reaction and responsible for damage to retinal cells membrane and mtDNA as well as reduction of thinning of outer and inner nuclear layers of the retina [133–135].
Peroxyl radical/lipid peroxides	Lipid peroxidation chain reaction damages retinal cell membrane and creates a redox milieu, as evident by an increase in the vitreous of PDR patients [136, 137].
Hydrogen peroxide	Toxic radical species are increased in retinal cells, and treatment with obestatin prevents the H_2O_2-induced (retinal ganglion cells) RGC damage [133, 138].
Singlet oxygen	Excitation of oxygen through sunlight and radiation and higher oxygen consumption by retinal cells adds to the impaired redox status of the cell in DR [131].
Peroxynitrite	The reactive nitrogen species shifts the redox status of the cell towards destruction causing apoptosis of retinal endothelial cells [139].

Table 2: Antioxidants depleted in diabetic retinopathy.

Antioxidants	Mechanism of action	Effect on DR
Thioredoxin (Trx)	The negative regulator of thioredoxin, thioredoxin-interacting protein (TXNIP), and the dissociation of apoptosis signal regulating kinase-1 (ASK-1) from the oxidised thioredoxin are key players inducing apoptosis during DR [140, 141].	ASK-1 induces apoptosis of Neuro2a cells during DR. TXNIP is upregulated in Muller cells and leads to apoptosis of pericytes [140, 142].
Superoxide dismutase (SOD)	First line of defence against hyperglycemic induced superoxide anion radicals in the mitochondria, and the highest SOD activity is present in the retina to help scavenge the superoxide radicals generated via metabolism [143–145].	SOD downregulation in retinal endothelial cells induces apoptosis [146] and upon overexpression ameliorates and protects the mtDNA from oxidative damage in DR [144].
NADPH oxidase (Nox)	Nox4, an isoform of Nox enzyme, promotes retinal neovascularization through ROS-dependent regulation of the VEGF/VEGFR2 signalling pathway [147] and via various inflammatory signalling pathways [148].	Nox4 isoform gene (*NOX4*) is involved in DR [149] and is the predominant isoform expressed in the human retinal endothelial cells [150].
Vitamin E (α-tocopherol)	A nonenzymatic antioxidant which donates hydrogen atom to peroxy radicals and other radicals to maintain the redox status of the cell [151].	Its supplementation reduces oxidative stress in NPDR and PDR patients [152] interlinking an antioxidant role in the prevention of DR.
Vitamin C (ascorbic acid)	An antioxidant or a reducing agent that donates electrons to various radical species [153].	Prevents high-glucose and RAGE-induced apoptosis in pericytes and endothelial cells. Also preserves NO generated by endothelial cells and tightens the leaky endothelial permeability barrier [154].

in ROS formation and the scavenging system plays a role in the pathogenesis of diseases including DR as increased oxidative stress has been seen in the retinas of animal models of diabetes [126] as well as in DR patients. In addition, ROS buildup in the eye is considered to be a trigger for degeneration of retinal cells, neural cells, and vascular lesions in the progression of DR. Accumulation of ROS in the eye gradually activates the NF-κB and MAPK cascades that result in inflammation in the retinal tissue. Hence, the interplay of ROS and inflammatory cytokines has been a major area of target and a platform to prevent prognosis of the condition [9]. Along with inflammation, neurodegeneration is another potential target of oxidative stress. Degeneration of retinal ganglion cells (RGC) has been reported to be in close association with oxidative stress and inflammation, wherein the activation of microglial cells results in the neurotoxicity and apoptosis of the RGC. It has been observed that high-glucose free fatty acid cotreatment results in the upregulation of CD11b and ionized calcium-binding adapter 1 (Iba-1), markers of microglial cells, corroborating oxidative species as key players in neurodegeneration, mediated via inflammatory response [127]. Moreover, crocin, a bioactive component of saffron, has been proved to be a good therapeutic agent, due to observed reduction in ROS and NO levels and IL-1β and TNF-α and additionally with its neuroprotective effect by activating the PI-3/Akt pathway [127]. Another insight into the cause of vascular lesions is neural photoreceptors inducing oxidative stress and inflammatory changes. These photoreceptors are a major source of superoxide radicals (correct this other place also) in the diabetic retina which

Table 3: Antioxidants as treatment strategies in diabetic retinopathy.

Antioxidant	Effect on DR
Resveratrol (RSV)	Reduces ROS levels and cleavage of caspase 3 in BREC (bovine retinal endothelial cells). Additionally, RSV shows antiapoptotic effects *in vitro* and *in vivo* on Muller cells with addition of miR-29b [155, 156].
Citrus flavones (hesperidin)	Modulation of mitochondrial function and inhibition of caspase activation via a ROS-dependent p38 and JNK signalling pathway. Also protects retinal pigment cells from hyperglycemic effects [157, 158].
Citrus flavones (hesperetin)	Prevents early- or late-stage microvasculopathy by its antiangiogenic properties. Protects Muller cell processes and photoreceptors with an increase in basement membrane thickness in diabetic retina [159, 160].
Lipoic acid	Reduces VEGF levels and preserves retinal layer thickness and protects ganglion cells. Also, safeguards injured the retinas of diabetic rats by decreasing oxidative stress, partially via AMPK activation [161, 162].
Telmisartan	Increases neurotrophic factors such as BDNF, CNTF, and TH by decreasing caspase-3 activity and increasing GSH levels in the serum and diabetic retina [163].
Astaxanthin	Reduces hyperglycemia-induced abnormal proliferation and oxidative stress in retinal pigmented epithelial cells. Also downregulates retinal ganglion cell apoptosis by inhibiting oxidative stress [164, 165].
Hydrogen sulphide	Suppresses oxidative stress and exhibits neuroprotective effects on the retina and ablates oxidative stress and inflammation in STZ-induced diabetic rats. However, during PDR stage, increased H_2S levels are detected in the vitreous cavity and require further studies to understand H_2S's role in therapeutics [166, 167].
Tauroursodeoxycholic acid (TUDCA)	Suppresses inflammatory cytokines and molecules such as NF-kappa B, ICAM-1, and NOS (nitric oxide synthase). Also decreases the levels of VEGF and exerts neuroprotective effects in an experimental retinal detachment model [168, 169].
Curcumin	Exhibits hypoglycemic, antioxidant, and anti-inflammatory properties in diabetic rats. Additionally downregulates VEGF and has neuroprotective properties [170, 171].

is due to the contributory effect of both mitochondria and NADPH oxidase. This is further supplemented by the fact that diabetes-induced induction of the proinflammatory molecules iNOS and ICAM-1 was not observed by the removal of photoreceptor cells [128].

Epigenetic changes in the mitochondrial enzymes induced by ROS form a metabolic memory such that even controlled glycemic levels do not respite the symptoms of DR [98]. In retinal endothelial cells, hyperglycemia leads to ROS production, decreasing the levels of a class III histone deacetylase and increasing inflammatory responses from NF-κB [129]. Moreover, this high glucose-mediated ROS production and SIRT1 have been considered important in mediating memory phenomena of retinal endothelial cells [129].

The various species that are involved in oxidative stress includes free radical molecules like superoxide radicals, hydroxyl radicals, nonradicals like hydrogen peroxides and ozone, and reactive lipids like ketosamine and ketoaldehyde groups. These reactive species can be generated by endogenous factors including the electron transport chain of the mitochondria or from the polymorphonuclear cells [130]. Exogenous factors like UV and infrared radiations also contribute to the radical formation. These superoxide species can also lead to formation of peroxynitrate and other reactive nitrogen species, leading to nitrative stress. The eye which allows the light to penetrate every layer makes it susceptible to damage by oxidative or nitrative stress via exogenous factors [131]. Some of the culprit oxidants of DR are mentioned in Table 1. Moreover, oxidative stress also leads to a decline in certain antioxidants thus making it easier for the DR to manifest and progress. These include thioredoxin, superoxide dismutase, NADPH oxidase, Nrf2, vitamin C, and vitamin E and are listed in Table 2.

Although prevention of the progression of retinopathy in diabetic patients lies in the sole strategy of a better diet regimen and maintenance of normal glycemic conditions, new avenues for treatment are increasing with the evolution of better pharmacological targets to treat the severity of retinopathy. Since standard therapies pose a drawback in clinic such as resistance to anti-VEGF intravitreal injection and inflammatory conditions like macular oedema, an hour of need is to find better treatment strategies with lower side effects. Moreover, the balance between prooxidants and antioxidants is disrupted in DR; thus, clinical research involving the use of antioxidant therapies serves as a new direction in relieving the severity of this condition. Some of the antioxidant molecules studied as treatment strategies against DR are mentioned in Table 3. These molecules have shown to be involved in reducing the severity of the disease by exhibiting their effects on the pathways that lead to cellular damage. However, none of the treatments is efficient enough to revert the symptoms completely; further studies are needed to evaluate more potential antioxidants with specific bioactive properties in combating retinal pathophysiology associated with DR.

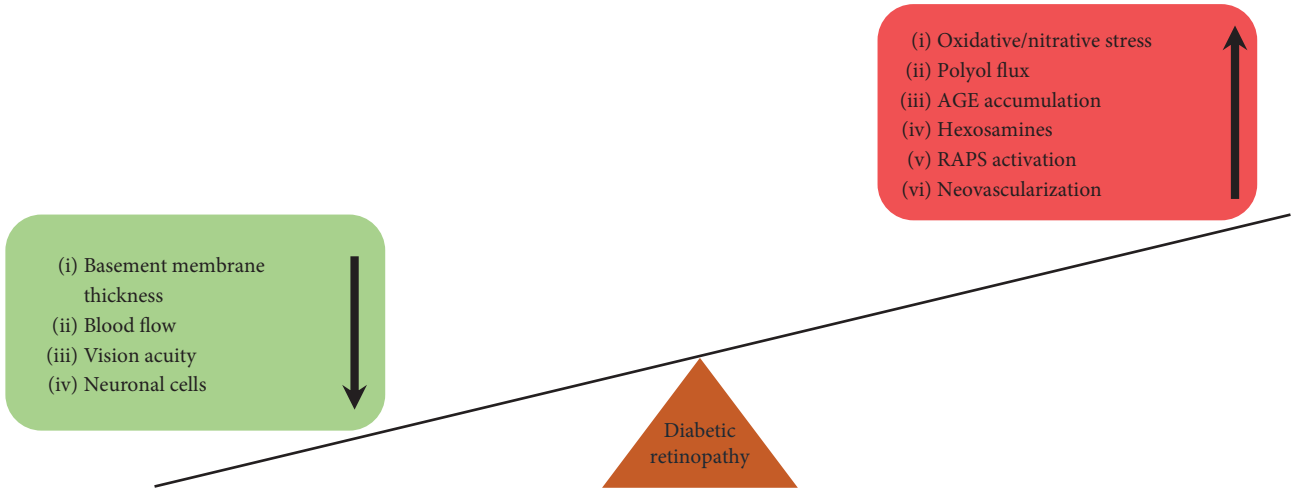

Figure 2: Perturbations in diabetic retinopathy.

12. Conclusions

Diabetic retinopathy, an acquired blindness, is amongst one of the leading conditions around the globe. The major insult in the progression of DR is the vascular dysfunction in the retinal blood barrier. This is a consequence of the impaired biochemical pathways such as the polyol, AGE/RAGE, hexosamine, PKC, tissue RAS, histone modifications, and altered miRNAs which are considered as major contributors in DR. These perturbations are summarized in Figure 2. The state of metabolic memory has also been implicated along with oxidative stress-induced damage to the mtDNA. This alteration in the mitochondrial complexes is the major inducer of radical species eventually resulting in depletion of antioxidants, leading to inflammation and apoptosis of retinal and endothelial cells during DR. Several treatment strategies aforementioned as the emerging area cardinally target the prooxidant/antioxidant status of the cells to protect from damage in DR. However, more studies are warranted to understand DR development and progression for the benefit of those individuals with retinal damage from diabetes.

Conflicts of Interest

The authors declare no conflicts of interest.

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Review Article

The Role of Traditional Chinese Medicine in the Regulation of Oxidative Stress in Treating Coronary Heart Disease

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Oxidative stress has been closely related with coronary artery disease. In coronary heart disease (CHD), an excess of reactive oxygen species (ROS) production generates endothelial cell and smooth muscle functional disorders, leading to a disequilibrium between the antioxidant capacity and prooxidants. ROS also leads to inflammatory signal activation and mitochondria-mediated apoptosis, which can promote and increase the occurrence and development of CHD. There are several kinds of antioxidative and small molecular systems of antioxidants, such as β-carotene, ascorbic acid, α-tocopherol, and reduced glutathione (GSH). Studies have shown that antioxidant treatment was effective and decreased the risk of CHD, but the effect of the treatment varies greatly. Traditional Chinese medicine (TCM) has been utilized for thousands of years in China and is becoming increasingly popular all over the world, especially for the treatments of cardiovascular diseases. This review will concentrate on the evidence of the action mechanism of TCM in preventing CHD by modulating oxidative stress-related signaling pathways.

1. Introduction

Coronary heart disease (CHD) is one of the primary reasons of death in the world, with 7.4 million deaths in 2013, being responsible for one-third of all deaths [1–3]. By 2020, it is forecasted that CHD will continue to be the prime and most prevalent threat to human life [4]. CHD is multifactorial and concerns intricate interactions between physiological, genetic, and lifestyle factors [5]. In past studies, traditional risk factors of CHD like diabetes, hypertension, smoking, and hyperlipidemia are linked with oxidative stress [6–8]. However, a number of studies have also associated oxidative stress with the mechanism of coronary atherosclerosis and have assessed the markers of oxidative stress, indicating that they can predict the occurrence of CHD [9]. Therefore, oxidative stress is one of the risk factors of CHD, which can affect the prognosis and reduce the survival time and quality of life of patients with CHD [10, 11].

Oxidative stress has been closely related with the mechanism of atherosclerosis and coronary artery disease. Oxidative stress may take place when the antioxidant ability is insufficient to decrease reactive oxygen species (ROS) and other free radicals. When oxidative stress occurs, ROS may generate oxidative modification or lipid peroxidation damage at the deoxyribonucleic acid (DNA) level and protein level with harmful consequences for the structure and function of the vascular system [12, 13]. In CHD, microvascular pathology revealed a higher level of ROS. The production of excess ROS generates endothelial cells and smooth muscle functional disorder, leading to a disequilibrium between the antioxidant capacity and prooxidants, thus leading to inflammatory signal activation and mitochondria-mediated apoptosis, which can promote and increase the occurrence and development of CHD [14, 15].

There are several types of vital antioxidative systems, including superoxide dismutase (SOD), catalase (CAT), and

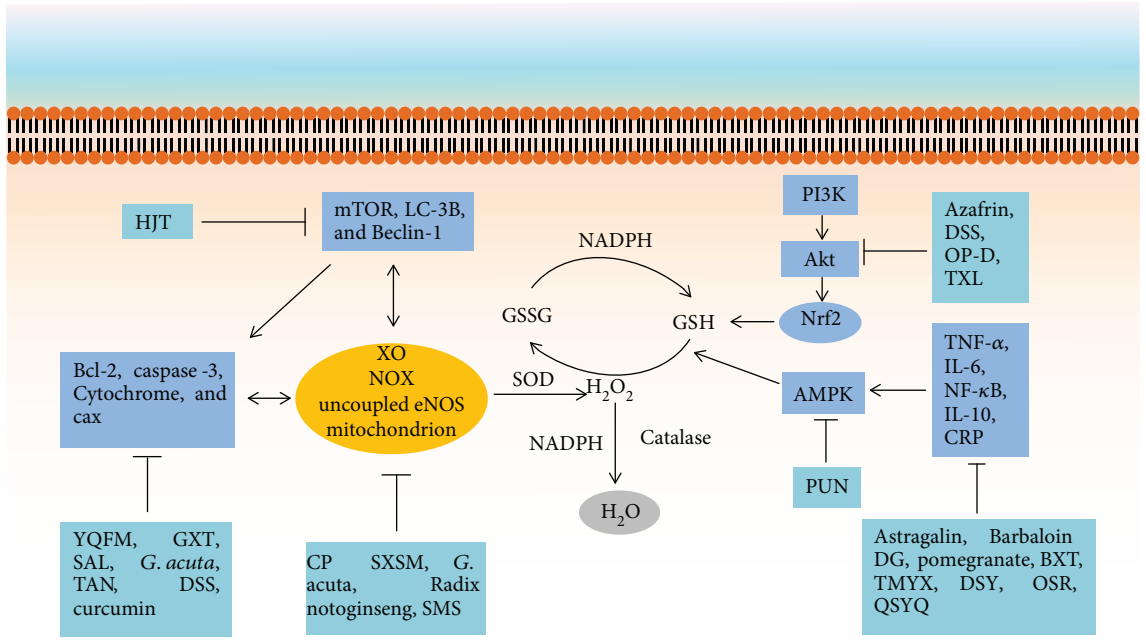

Figure 1: The mechanism of TCM in preventing CHD by oxidative stress-related signaling pathways. HJT: Hongjingtian injection; mTOR: mammalian target of rapamycin; LC-3B: light chain 3B; NADPH: nicotinamide adenine dinucleotide phosphate; GSSG: glutathione disulfide; GSH: glutathione; SOD: superoxide dismutase; PI3K: phosphoinositide 3-kinase; Akt: serine/threonine kinase; Nrf2: nuclear factor erythroid-2-related factor 2; AMPK: adenosine monophosphate-activated protein kinase; PUN: punicalagin; DSS: Danshensu; OP-D: Ophiopogonin D; TXL: Tongxinluo; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; NF-κB: nuclear factor-κB; IL-10: interleukin-10; CRP: C-reaction protein; DG: Dunye Guanxinning; TMYX: Tongmai Yangxin pill; DSY: Dan-Shen-Yin; OSR: oxysophoridine; QSYQ: Qi-shen-yi-qi; XO: xanthine oxidase; NOX: NADPH oxidase; eNOS: endothelial nitric oxide synthase; CP: cardiotonic pill; SXSM: Shenxian-shengmai; SMS: Shengmai San; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated protein X; YQFM: YiQiFuMai powder injection; SAL: salvianolic acid; TAN: tanshinone; GXT: Guanxintai; BXT: Bao-Xin-Tang; *G. acuta*: *Gentianella acuta*.

glutathione peroxidase (GSH-PX). There are also numerous crucial small molecular antioxidants such as β-carotene, ascorbic acid, α-tocopherol, and reduced glutathione (GSH) [16]. Studies have shown that antioxidant treatment was effective and decreased the risk of CHD [17]. Oxidative status records for a particular patient are generally inadequate, and specific antioxidants suitable for that patient are rarely prescribed [18] which influences treatment effectiveness. However, traditional Chinese medicine (TCM) has been utilized for thousands of years in China and is becoming increasingly popular all over the world, especially for the treatment of cardiovascular diseases [19]. Modern pharmacological research has indicated that many Chinese herbal extracts protect the development of cardiovascular diseases through their antioxidating effects [20–22]. A schematic diagram of the mechanisms of ROS is demonstrated in Figure 1. This review will concentrate on the current evidence of the action mechanism of TCM in preventing CHD by modulating oxidative stress-related signaling pathways.

2. Protective Effects of Traditional Chinese Medicine (TCM) and Its Constituent Compounds on Coronary Heart Disease

2.1. Myocardial Infarction (MI). MI is considered as one of the most common forms of ischemic heart disease and is one of the main reasons of death worldwide. A growing body of evidence has indicated that ROS can lead to cell loss following MI and is closely related to the generation of MI [23]. ROS reduction may represent a vital therapeutic target for relieving the damage caused by a MI. Therefore, targeting the production of ROS with all kinds of antioxidants has been shown to decrease oxidative stress-related injury and therefore improve MI status.

2.1.1. The Bioactive Ingredients of Traditional Chinese Medicine. Salvia miltiorrhiza: Salvia miltiorrhiza, a famous Chinese herb medicine, has been widely used in treating cardiovascular diseases [24]. Studies showed that it could relieve small artery circulation, decrease ROS production [25–27], restrain cell apoptosis [27–29], and protect the heart against ischemia-reperfusion injury [30–32]. *Salvianolic acid* (SAL, $C_{36}H_{30}O_{16}$; $C_{26}H_{22}O_{10}$) and *tanshinone* (TAN, $C_{18}H_{12}O_3$; $C_{19}H_{18}O_3$), hydrophilic and lipophilic compounds, are extracted from *Salvia miltiorrhiza* [33]. Wang et al. [34] studied the use of the MI models to evaluate the cardioprotective functions of SAL and TAN in rats. Both echocardiographic and infarct sizes were assessed after surgery, while gene activity was detected by microarray analysis and validated by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). These results depicted that SAL is possibly mediated by the downregulation of factors participating in oxidative stress and apoptosis, while TAN is

probably mediated by the suppression of intracellular calcium and cell adhesion pathways in the MI.

Danshensu (DSS, $C_9H_{10}O_5$), the main water-soluble ingredient of *Salvia miltiorrhiza*, has also been studied as a significant compound. In a study [35], DSS was detected in an ischemia-reperfusion (I/R) model to research its cardioprotective function. The results showed that DSS significantly reduced the level of creatine kinase and lactate dehydrogenase and that DSS had ROS scavenging activity and enhanced endogenous antioxidants such as SOD, CAT, malondialdehyde (MDA), GSH-PX, and heme oxygenase-1 (HO-1) activities through stimulation of the nuclear factor erythroid-2-related factor 2 (Nrf2) signaling pathway which was regulated by serine/threonine kinase (Akt) and extracellular signaling-regulated kinase 1/2 (ERK1/2) signaling pathway in a western blot analysis. The mechanism might be associated with the improvement of the antioxidant defense system by stimulating Akt/ERK1/2/Nrf2 signaling pathways [36, 37].

Astragalin: astragalin ($C_{21}H_{20}O_{11}$) is a flavonoid that is extracted from the leaves of *Rosa agrestis*, *persimmon*, or green tea seeds. A large number of studies have indicated that *astragalin* has wide pharmacological activities, covering anti-inflammatory, antioxidative, and other beneficial activities [38–40]. A study [41] that assessed the cardioprotective functions of *astragalin* against I/R injury in the rat heart by Langendorff apparatus has been conducted. The results revealed that *astragalin* pretreatment ameliorated myocardial function. SOD activity and the glutathione/glutathione disulfide (GSH/GSSG) ratio were dramatically enhanced, and the levels of MDA, tumor necrosis factor-α (TNF-α), intracellular ROS, and interleukin-6 (IL-6) were reduced in the *astragalin*-treated groups. Thus, *astragalin* displayed cardioprotective functions through its antiapoptotic, antioxidative, and anti-inflammatory activities [41–43].

Ophiopogonin D (OP-D): OP-D ($C_{44}H_{70}O_{16}$) is a significantly valid monomeric ingredient used in the Shenmai injection (SM-I). It is reported that it has a wide range of biological activities, including antiapoptotic effects, antioxidant, and anti-inflammatory actions [44–47]. The rat model of myocardial ischemia-reperfusion (MI/R) damage was produced by ligation of the left anterior descending coronary artery to study the protective actions and underlying mechanisms of *OP-D* and SM-I [48]. The study found that *OP-D* and SM-I act by inducing cardioprotection on MI/R injury by regulating cardiac function, reducing acetate dehydrogenase and creatine kinase (CK) generation, decreasing infarct size, and improving the injured cardiac structures. Cardioprotection by *OP-D* and SM-I was mediated by activating the phosphoinositide 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway and suppressing the nuclear factor-κB (NF-κB) signaling pathway [49, 50].

Curcumin: curcumin ($C_{21}H_{20}O_6$), used both as a seasoning and a traditional medicine, is a natural compound derived from the roots of *Curcuma longa L*. It has various pharmacological activities such as anti-inflammatory, antioxidant, and anticarcinogenic activities in different models [51–53]. In a study [54], the possible protective function of *curcumin* on cardiac function in MI/R rats was researched. The rats suffered from myocardial injuries through ligation of the left anterior descending coronary artery. Afterwards, lipid peroxidation products and antioxidant enzymes were evaluated in the myocardial tissue. The result showed that *curcumin* might decrease the venture of coronary heart disease via activating the JAK2/STAT3 signal pathway, reducing oxidative damage and suppressing myocardium apoptosis [55–57].

Punicalagin (PUN): PUN ($C_{48}H_{28}O_{30}$), a main bioactive constituent in pomegranate juice, has been tested for neuroprotective functions against cerebral ischemia-reperfusion (I/R) injury by antioxidative mechanisms [58, 59]. Another study [60] investigated if PUN offers cardioprotective effects against MI/R damage and the potential mechanisms. MI/R was achieved by ligating the left anterior descending coronary artery. PUN acts by ameliorating cardiac function and infarct size, decreasing serum creatine kinase-MB (CK-MB) and lactate dehydrogenase, and inhibiting myocardial apoptosis against MI/R damage. These results showed that PUN protects against I/R-induced ROS and myocardial damage by activating adenosine monophosphate-activated protein kinase (AMPK) [60, 61].

Barbaloin (BAR): BAR ($C_{21}H_{22}O_9$) is the major medicinal ingredient of *Aloe vera* belonging to the liliaceous plant group that has good antioxidant properties [62]. Zhang et al. [63] investigated if BAR offers cardioprotection in the MI myocardial damage. BAR was intragastrically administrated to rats before MI operation. The result showed that BAR pretreatment efficiently suppressed I/R-induced ROS and inflammatory effects by activating AMPK signaling in MI/R rat hearts [62–65].

Oxysophoridine (OSR): OSR ($C_{15}H_{24}N_2O_2$), a natural alkaloid from the Chinese herbal medicine *Sophora alopecuroides L.*, can play multiple pharmacological roles such as the suppression of oxidative stress and apoptosis [66, 67]. A study [68] assessed the cardioprotective effect of OSR against MI in rats. OSR decreased infarction size and the levels of myocardial enzymes, including the CK-MB, cardiac troponin T, and lactate dehydrogenase. A decreased level of MDA was noticed while increased levels of catalase, SOD, glutathione peroxidase activity, and nonenzymatic scavenger glutathione were also verified in OSR-treated rats. In addition, OSR suppressed the activities of various inflammatory cytokines [69, 70]. The results showed that OSR relieves myocardial damage in the rat model of acute myocardial infarction (AMI) and that the cardioprotective effects may be associated with antiapoptotic, anti-inflammatory, and antioxidative mechanisms.

Gentianella acuta (G. acuta): G. acuta ($C_{13}H_8O_2$) is extensively used for the therapy of coronary heart disease in Mongolian medicine. It is commonly known as "Wenxincao" in traditional Chinese medicine [71]. The potential protective effect of *G. acuta* on myocardial I/R injury by using the Langendorff apparatus in isolated rats was studied [72]. Some hemodynamic parameters were logged during the perfusion. These results showed that the *xanthones* from *G. acuta* dramatically ameliorated myocardial function and enhanced

the levels of SOD, succinate dehydrogenase (SDH), CAT, malate dehydrogenase (MDH), adenosine triphosphate (ATP), and the proportion of GSH/GSSG while inhibiting the levels of CK, MDA, and LDH. Moreover, *xanthones* could upregulate the Bcl-2 protein and downregulate the Bax protein. In short, *xanthones* from *G. acuta* displayed a cardioprotective effect on myocardial I/R damage via antioxidative and antiapoptosis activities [73–76].

Azafrin: Centranthera grandiflora Benth. is an ethnic drug known as Ye-Can-Dou-Gen (YCDG) and has been extensively used to cure cardiovascular system diseases in China. *Azafrin* ($C_{27}H_{38}O_4$), a carotene antioxidant, is one of the richest active compounds in YCDG [77]. Yang et al. [78] investigated the cardioprotective capacity of *azafrin* on the MI and MI/R damage to understand its potential myocardium preservation mechanisms. By experimental procedures, the results indicated that *azafrin* treatment significantly ameliorated heart function and infarct size in rats; reduced the levels of myocardial enzymes, cardiac troponin l (cTnI), and MDA; and increased SOD activity in vivo. In a word, *azafrin* displayed cardioprotective effects against myocardial damage through activation of the Nrf2-antioxidant response element (ARE) pathway [79].

2.1.2. Traditional Chinese Medicine Decoction. Bao-Xin-Tang (BXT): BXT is a Chinese herbal compound used to treat coronary heart disease and is made of *Codonopsis pilosula*, *Atractylodes macrocephala*, *Astragalus*, *Fructus crataegi*, etc. Previous studies have verified that it can ameliorate blood circulation to protect the myocardium of patients with MI [80]. A study [81] designed to explore if BXT offers cardioprotection against MI has been conducted. The rat model of MI was made by the ligation of the left anterior descending coronary artery. The data suggested that BXT could decrease the infarction size, myeloperoxidase, interleukin-6 (IL-6), and levels of C-reactive protein (CRP) and enhance SOD activities and anti-inflammatory media such as interleukin-10 (IL-10). Thus, the functions of BXT may be associated with antioxidant and anti-inflammation properties [82, 83].

Dan-Shen-Yin (DSY): DSY, including sandalwood *Fructus amomi* and *Salvia miltiorrhiza*, is a famous Chinese herbal formula which is extensively used for the therapy of CHD [84, 85]. A study [86] explored whether DSY could protect from MI. The left anterior descending branch of the coronary artery was ligated to induce myocardial ischemia in rats, measuring the infarction size, inflammation factor, and antioxidative enzyme activities. DSY decreased the infarction size, IL-6, CRP, TNF-α, and MAD, as well as enhanced SOD activities and glutathione [87, 88]. These results suggested that DSY plays a remarkable role against ischemic myocardial damage in rats, probably through an anti-inflammatory reaction and antioxidative properties.

2.1.3. Patented Drugs from Traditional Chinese Medicine. Dunye Guanxinning (DG): DG, a traditional Chinese herbal medicine formula, is extracted from the rhizomes of *Dioscorea zingiberensis* and is widely used for the treatment of angina, hyperlipidemia, and coronary heart disease [89, 90]. A study [91] explored that DG ameliorates myocardial I/R damage by suppressing caspase-1 activity and neutrophil infiltration. The result suggested that DG restrained neutrophil infiltration and decreased the interleukin-1 beta (IL-1β). In addition, DG suppressed caspase-1 activity and activatory AMPK phosphorylation in rat hearts. Thus, DG may be able to suppress the inflammatory response by the AMPK pathway [90, 91].

Hongjingtian injection (HJT): HJT is extracted from *Rhodiola rosea* and could prevent all kinds of vascular diseases like coronary heart disease and angina [92]. A study [93] assessed the cardioprotective effects of HJT. The experiments showed that HJT suppressed H/R-induced apoptosis and adjusted the expression of apoptosis-related proteins caspase 3 and Bcl-2. In addition, HJT obviously regulated the activity of the Akt, ERK/mTOR, and Akt/Beclin-1 pathways in cardiac cell autophagy. HJT prominently reduced the infarct size and ameliorated cardiac function and enhanced the light chain 3B (LC-3B) protein expression in the coronary ligation rat model. As a result, HJT reduced myocardial injury by adjusting the balance of apoptosis and autophagy and by decreasing ROS levels [94, 95].

Guanxintai (GXT): GXT, a Chinese compound formula, is often used in the treatment of cardiovascular diseases and is mainly composed of *Ginseng*, *Astragalus*, *Rehmannia*, *Ophiopogon root*, etc. Previous studies have verified the cardioprotective effects of GXT on the angina [96–99] and arrhythmia [100], as well as its inhibitory actions on blood lipid levels [101]. Yang et al. [102] studied the protective actions of GXT on ischemic cardiomyocytes and the related antioxidative effects. The research findings showed that GXT decreased the degree of myocardial cell injury and apoptosis and partly ameliorated cardiac function after MI. Furthermore, GXT restrained the ROS level and reduced NADPH oxidase (NOX) and mitogen-activated protein kinase (MAPK) protein expression. Therefore, the cardioprotective effects of GXT are exerted by the activity of the antioxidative NOX suppression [103, 104].

Cardiotonic pill (CP): CP includes *Salvia miltiorrhiza*, *Borneol*, and *Panax notoginseng* and is extensively used for the treatment of ischemic angina pectoris. A study [105] explored the underlying mechanisms of CP antioxidative activity. Male rats had left anterior descending artery ligation, and then, reperfusion was performed. The result suggested that CP decreased myocardial damage, ROS, and microcirculation disturbance. CP prominently suppressed I/R-induced NOX subunit p67phox, gp91phox, and p47phox protein expression. These data indicated that the CP alleviated I/R-induced rat myocardial damage and the disorder of microcirculation by inhibiting NOX activity [105–107].

Shenxian-shengmai (SXSM): SXSM oral liquid, a Chinese compound formula, has been widely used for bradyarrhythmias in clinical practice [108, 109]. MI, especially in right coronary-associated cardiac diseases, can give rise to bradyarrhythmias. A study [110] evaluated the functions of SXSM on bradyarrhythmias and cardiac insufficiency caused by myocardial I/R damage. Results showed that SXSM enhanced heart rate and protected from myocardial I/R damage. The study also discovered that SXSM ameliorated

Table 1: The role of traditional Chinese medicine (TCM) in the regulation of reactive oxygen species (ROS) in myocardial infarction (MI).

Type of TCM	TCM (molecular formula)	Type of study	Mechanism of action	References
The bioactive ingredients of TCM	SAL ($C_{36}H_{30}O_{16}$; $C_{26}H_{22}O_{10}$), TAN ($C_{18}H_{12}O_3$; $C_{19}H_{18}O_3$)	In vivo	Downregulation of factors participated in oxidative stress and apoptosis; inhibition of intracellular calcium and cell adhesion pathways	Wang et al. [129]
	Danshensu ($C_9H_{10}O_5$)	In vivo	Activation of the Akt/ERK1/2/Nrf2 signaling pathway	Yu et al. [35]
	Astragalin ($C_{21}H_{20}O_{11}$)	In vivo	Antiapoptotic, antioxidative, and anti-inflammatory activities	Qu et al. [41]
	OP-D ($C_{44}H_{70}O_{16}$)			
	Curcumin ($C_{21}H_{20}O_6$)	In vivo	Activating JAK2/STAT3 signal pathway, reducing oxidative damage and suppressing myocardium apoptosis	Liu et al. [111]
	Punicalagin ($C_{48}H_{28}O_{30}$)	In vivo	Activation of AMPK	Ding et al. [60]
	Barbaloin ($C_{21}H_{22}O_9$)	In vivo	Antioxidative, anti-inflammatory	Zhang et al. [93]
	OSR ($C_{15}H_{24}N_2O_2$)	In vivo	Antiapoptotic, anti-inflammatory, and antioxidative	Meng et al. [68]
	G. acuta ($C_{13}H_8O_2$)	In vivo	Activities of antioxidative and antiapoptosis	Wang et al. [81]
	Azafrin ($C_{27}H_{38}O_4$)	In vivo, in vitro	Activation of the Nrf2-ARE pathway	Yang et al. [158]
Traditional Chinese medicine decoction	Bao-Xin-Tang	In vivo	Antioxidant and anti-inflammation properties	Wang et al. [81]
	Dan-Shen-Yin	In vivo	Anti-inflammatory and antioxidant properties	Yan et al. [86]
Patented drugs from traditional Chinese medicine	Dunye Guanxinning	In vivo	Inhibits inflammasome activity through the AMPK pathway	Zhang et al. [91]
	Hongjingtian injection	In vivo, in vitro	Decreasing myocardial oxidative damage	Zhang et al. [93]
	Guanxintai	In vivo, in vitro	Reduced NOX and MAPK proteins	Yang et al. [158]
	Cardiotonic pill	In vivo	Inhibiting NOX activity	Yang et al. [105]
	Shenxian-shengmai	In vivo	Enhanced the activity of SOD and aggrandized the content of GSH	Zhao et al. [110]

TCM: traditional Chinese medicine; SAL: salvianolic acid; TAN: tanshinone; OP-D: Ophiopogonin D; OSR: oxysophoridine; *G. acuta*: *Gentianella acuta*; SOD: superoxide dismutase; GSH: glutathione; NOX: NADPH oxidase; MAPK: mitogen-activated protein kinase; AMPK: adenosine monophosphate-activated protein kinase; Nrf2: nuclear factor erythroid-2-related factor 2; Akt: serine/threonine kinase.

myocardial interstitial dilatation and the structural changes of myocardial cells. At the same time, SXSM protected myocardial cells against ROS induced by H_2O_2 and I/R damage by decreasing the intracellular levels of ROS. Furthermore, SXSM enhanced the activity of SOD and aggrandized the content of GSH by accelerating the glutamate-cysteine ligase catalytic subunit (GCLC) expression and GSH-Px activity, suggesting the antiarrhythmia and cardioprotective effects [111] (Table 1).

2.2. Ischemic Heart Failure

2.2.1. Patented Drugs from Traditional Chinese Medicine. Qi-shen-yi-qi (QSYQ): QSYQ, a formula used for the routine treatment of HF in China, includes *Radix*, *Astragali mongolici*, *Salvia miltiorrhiza Bunge*, *Flos Lonicerae*, *Scrophularia*, *Radix Aconiti Lateralis preparata*, and *Radix glycyrrhizae* and has been proven to ameliorate cardiac function by downregulating the Renin-Angiotensin-Aldosterone System (RAAS) activity [112, 113]. A study [114] surveyed the treatment with QSYQ ischemic heart failure prevention by alleviating oxidative stress and suppressing inflammation. Rats were processed by coronary artery ligation, and then, the indicators of fibrosis such as Masson dyeing, matrix metalloproteinases (MMPs) and collagens, and inflammation factors were detected. The study demonstrated that QSYQ ameliorated cardiac function via reducing the degree of myocardial fibrosis, TNF-a, NF-κB, and IL-6-STAT3 pathways and modulating angiotensin II-NADPH oxidase-ROS-MMP pathways [114, 115].

Tongxinluo (TXL): TXL is a prescription compound of Chinese medicine and has been verified as having anti-inflammatory, lipid-lowering, and antioxidant effects in ameliorating ischemic heart diseases [116]. A study [117] explored if TXL protected against the pressure overload-inducedischemic heart failure in mice. The transverse aortic constriction (TAC) operation was carried in mice to induce ischemic heart failure. TXL ameliorated cardiac function and relieved cardiac hypertrophy and myocardial fibrosis after treatment. Furthermore, TXL also enhanced

Table 2: The role of traditional Chinese medicine (TCM) in the regulation of reactive oxygen species (ROS) in ischemic heart failure and angina.

Type of disease	Type of TCM	TCM (molecular formula)	Type of study	Mechanism of action	References
Ischemic heart failure	Patented drugs from traditional Chinese medicine	Qi-shen-yi-qi	In vivo	Recovering angiotensin II-NADPH oxidase-ROS-MMP pathways	Li et al. [114]
		Tongxinluo	In vivo	Activation of the VEGF/Akt/eNOS signaling pathway	Wang et al. [117]
		YiQiFuMai powder injection	In vivo	Ameliorating cardiac function and structure damage, oxidative stress, and cell apoptosis and inhibiting the MAPK signaling pathways	Pang et al. [121]
Stable angina		Tongmai Yangxin pill	In clinic	Attenuating oxidative stress and inflammation	Cai et al. [127]

ROS: reactive oxygen species; MMPs: matrix metalloproteinases; VEGF: vascular endothelial growth factor; Akt: serine/threonine kinase; eNOS: endothelial nitric oxide synthase; MAPK: mitogen-activated protein kinase.

myocardial capillary density and reduced oxidative stress damage by activating the vascular endothelial growth factor (VEGF)/Akt/eNOS signaling pathway [118].

YiQiFuMai powder injection (YQFM): YQFM, a Chinese medicinal formula rediscovered on the basis of Shengmai San, is extracted from Panax ginseng, Ophiopogon japonicus, and Schisandra chinensis and is widely used to treat angina and ischemic heart failure [119, 120]. Another study [121] noticed the therapeutic effect of YQFM on coronary artery occlusion-induced ischemic heart failure. Ischemic heart failure was induced by coronary artery occlusion in mice. After treatment with YQFM, the result displayed that YQFM can reduce LDH and CK activities and levels of MDA, N-terminal pro-B-type natriuretic peptide (NT-proBNP), and hydroxyproline (HYP). Moreover, YQFM relieves coronary artery occlusion-induced ischemic heart failure by ameliorating the cardiac function and structure damage, oxidative stress, and cell apoptosis and suppressing the MAPK pathways [122–125].

2.3. Angina

2.3.1. Tongmai Yangxin (TMYX) pill. TMYX, a frequently used drug, is a Chinese compound formula used in the treatment of angina [126]. It mainly includes *rehmannia*, *Caulis Spatholobi*, *Ophiopogon*, *licorice*, *Polygonum multiflorum*, *donkey-hide gelatin*, *fructus schisandrae*, *Codonopsis pilosula*, *tortoise*, *dates*, and *cassia*. Metabolomics is a vital part of systems biology, which aims to monitor the changes of endogenous metabolites under physiological or pathological conditions. Cai et al. [127] analyzed the serum samples in clinical patients after oral administration of TMYX gathered from seven different clinical units in China. Using performance liquid chromatography, they tested metabolite profile changes in serum samples. Biomarkers, including metabolism, oxidative stress, and inflammation, were measured. The result indicated that after TMYX treatment, 10 biomarkers were reversed to normal conditions. These biomarkers participate mainly in energy metabolism, oxidative stress, and inflammation. As a result, TMYX has a therapeutic action via relieving myocardial energy disturbance, ROS, and inflammatory response [127–129]. The study, which is the first multicenter clinical study to reveal the basis and therapeutic mechanism of molecular biology of TMYX on the stable angina, can provide an objective index for the evaluation of the efficacy of TMYX in the stable angina pectoris, setting the stage for the clinical use of TMYX (Table 2).

2.4. Coronary Atherosclerotic Heart Disease

2.4.1. Single Chinese Herbal Medicines. Radix notoginseng: *Radix notoginseng*, a traditional Chinese medicine extracted from the roots of *Panax notoginseng*, is widely planted and used as an herbal medicine in Southern China. It indicates multiple biological activities, and it is also used as a therapeutic agent for coronary heart disease and peroxidation [130, 131]. A study [132] explored the cardioprotection effect of *Radix notoginseng* in cardiovascular system diseases related to hyperlipidemia and excess cholesterol. The rat model was established by using a dietary supplement to keep a high fat diet. *Radix notoginseng* led to a significant reduction in cholesterol and triglycerides, with a rise in the high-density lipoprotein-cholesterol. In addition, *Radix notoginseng* ameliorated antioxidant status through the SOD and glutathione peroxidase (GPx) activity and decreased the lipid peroxidation [133, 134]. The result showed that *Radix notoginseng* could ameliorate lipid distributions, suppress peroxidation, and enhance antioxidant enzymes activity, thereby decreasing the occurrence of CHD.

Pomegranate: pomegranate fruit is abundant in polyphenols, has an antioxidant activity, and has been suggested to have advantageous effects in cardiovascular disease [135]. The impacts of pomegranate on ROS and inflammation in the model of coronary heart disease in mice have been studied [136, 137]. Transgenic mice were treated with pomegranate extract [138]. Pomegranate could improve cardiac enlargement and electrocardiogram (ECG) abnormalities by reducing macrophage infiltration, lipid accumulation, ROS, and monocyte chemotactic protein-1 in transgenic mice with

Table 3: The role of traditional Chinese medicine (TCM) in the regulation of reactive oxygen species (ROS) in coronary atherosclerotic heart disease.

Type of TCM	TCM (molecular formula)	Type of study	Mechanism of action	References
Single Chinese herbal medicines	Radix notoginseng	In vivo	Inhibit peroxidation and increase the activity of antioxidant enzymes	Xia et al. [132]
	Pomegranate	In vivo	Reduced oxidative stress and inflammation	Al-Jarallah et al. [138]
Patented drugs from traditional Chinese medicine	Shengmai San	In vivo	Inhibit peroxidation and increase the activity of antioxidant enzymes	Yao et al. [140]

coronary atherosclerotic plaque. These results indicated that the protective effect of pomegranate against atherosclerosis may relate to reduce inflammation and ROS.

2.4.2. Patented Drugs from Traditional Chinese Medicine. Shengmai San (SMS): SMS includes *Panax ginseng*, *Schisandra chinensis*, and *Ophiopogon* and is a Chinese patent medicine used to treat CHD with antioxidative effects [139]. There was a study [140] which explored the influence of SMS on lipid peroxides and antioxidant reactions in the heart of cholesterol-raised rats. Antioxidant activities and ROS markers in the heart of rats were assessed. Results suggested that GSH-Px, glutathione-S-transferase (GST), and SOD activities were slightly improved after the SMS treatment [141] (Table 3).

3. Discussion

Awareness of the importance of ROS in CHD pathogenesis and the development of novel treatments has increased [142]. As a crucial resource of treatment, TCM has multiple bioactivities with antioxidative ability [143, 144]. As a result, we summarized the research progress of TCM on the treatment of CHD by regulating ROS.

During MI, mechanisms of pathogenesis are associated with a number of factors, like the large amount of free radical generation, enhanced inflammation, and apoptosis [145]. When MI/R injury happens, cardiac intracellular calcium overload can increase XO synthesis and NOX, resulting in a rapid increase of ROS generation [146]. OSR cardioprotection, CP, and SXSM against MI in rats were correlated with antioxidant properties, particularly regarding NOX. Meanwhile, inflammatory signaling pathways are related to the occurrence and development of CHD, and I/R injury is closely connected with increased inflammation [147]. Astragalin, Barbaloin, BXT, and DSY can act on various targets such as suppression of NOX and the rise of GSH, which efficiently decreases ROS injury after I/R damage. Moreover, the anti-inflammatory action of TCM is, at least partly, attributed to its antioxidant effects. A number of studies have also indicated that the upregulation of several antiapoptotic factors and proapoptotic genes, such as the Bcl-2 and Bax, plays a vital function in the ischemic tissue [148, 149]. Curcumin, *G. acuta*, HJT, and GXT offer cardioprotection by ameliorating heart functions, inhibiting the ROS of cardiac cells, enhancing the release of antioxidant enzymes, and restraining mitochondria disorder and cardiomyocyte apoptosis during I/R damage. There is growing evidence that the PI3K/Akt and Nrf2 pathways help in ROS resistance and play a key function in improving myocardial cell survival [150, 151]. Activating antiapoptotic signaling pathways such as Nrf2 and PI3K/Akt could adjust Bcl-2 and suppress caspase c activation. Therefore, a large number of studies have shown that TCM treatment, such as SAL, TAN, DSS, OP-D, and azafrin, could decrease cardiomyocyte ROS and apoptosis through activation of the Nrf2 and PI3K/Akt signaling pathways during I/R damage. AMPK can also upregulate the cell antioxidant enzymes such as SOD and catalase, thereby decreasing oxidative damage [152]. The activation of the AMPK signaling pathway during I/R injury has been thought to be a mechanism of treatment against ROS and myocardial damage [153]. Above all, PUN, Barbaloin, DG, and GXT have been noticed to improve mitochondrial damage and ROS by the AMPK signaling pathway.

In ischemic heart failure, the oxidative stress system is activated, thereby significantly promoting coronary arterial disease and damaging cardiac myocytes [154]. In this pathway, NOX plays an important role in the occurrence and progression of IHF [155, 156]. At the same time, increased oxidative stress combined with the activation of a variety of inflammatory and apoptosis pathways significantly influence the effect on the occurrence and development of ischemic heart failure [157]. TCM has been used to cure ischemic heart failure for thousands of years. A lot of TCMs, such as QSYQ, TXL, and YQFM, showed cardioprotection against HF by alleviation of apoptosis, inflammation, and ROS.

Increased oxidative stress, disturbed lipid metabolism, and increased inflammation are critical factors in the occurrence and development of atherosclerosis and subsequent CHD [158, 159]. Radix notoginseng, pomegranate, and SMS offer tissue damage protection, attributed to ROS, by decreasing lipid peroxidation and enhancing the activity of antioxidant enzymes. In angina, pathogenesis mainly involves energy metabolism, ROS, and inflammation [160]. TMYX may have therapeutic actions by ameliorating myocardial energy supply dysfunction and amino acid disorders and by reducing ROS and inflammation.

4. Conclusions

In conclusion, there is overwhelming evidence that oxidative stress is associated with the pathogenesis of CHD. TCM therapy has unique advantages in CHD. In recent years, Chinese medicine has made great progress in the treatment of CHD,

which can effectively ameliorate the symptoms of patients and improve the quality of life of patients. Compared with Western medicine, it has significant therapeutic effects, few side effects, and no obvious drug dependence. The treatment of this disease by TCM has a broad prospect, and it is worthy of further promotion and development.

Conflicts of Interest

All authors claim that there is no conflict of interests about the publication of this review.

Authors' Contributions

Xinyu Yang, Yanwei Xing, and Hongcai Shang designed the idea for drafting this review. Xinyu Yang and Tianmai He collected the documents and wrote the paper. Songjie Han contributed to Discussion. Xiaoyu Zhang and Yang Sun revised and edited the review. All authors commented on the manuscript.

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Review Article

Molecular Mechanisms Underpinning Microparticle-Mediated Cellular Injury in Cardiovascular Complications Associated with Diabetes

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Microparticles (MPs) are small vesicles shed from the cytoplasmic membrane of healthy, activated, or apoptotic cells. MPs are very heterogeneous in size (100–1,000 nm), and they harbor proteins and surface antigens specific to cells they originate from. Virtually, all cells can shed MPs, and therefore, they can be found in all body fluids, but also entrapped in tissues. Of interest and because of their easy detection using a variety of techniques, circulating MPs were recognized as biomarkers for cell activation. MPs were also found to mediate critical actions in intercellular communication and transmitting biological messages by acting as paracrine vehicles. High plasma numbers of MPs were reported in many cardiovascular and metabolic disturbances that are closely associated with insulin resistance and low-grade inflammation and have been linked to adverse actions on cardiovascular function. This review highlights the involvement of MPs in cardiovascular complications associated with diabetes and discusses the molecular mechanisms that underpin the pathophysiological role of MPs in the onset and progression of cellular injury in diabetes.

1. Introduction

Microparticles (MPs) are membrane-shed vesicles, ranging in size between 100 nm and 1,000 nm, that are released from the cytoplasmic membrane of activated and/or apoptotic cells. For many decades, MPs were considered inert cell debris or platelet dust derived from platelets which are rich in phospholipids and endowed of procoagulant capacity [1]. Later, the development of methods of genetic and protein profiling showed that MPs could transport cargo content including secretable and nonsecretable biological molecules such as active lipids and nucleic acids, such as coding (mRNA) and noncoding (e.g., microRNA and long noncoding RNA) RNA and DNA, in addition to membrane and cytosolic proteins to target cells [2] and are therefore recognized today as true vectors of intercellular communication and mediators of a variety of biological messages.

MPs are involved in the regulation of molecular processes within the emitting cell itself or other distant cells. Cells may export into the extracellular environment certain subcellular organelles and macromolecules or genetic material (e.g., mRNA or microRNA) because of their role in controlling certain cell functions inside the cell or because they are directly involved in the control of the process of MP shedding and release. The elimination of these molecules entrapped within MPs may alter the properties of the parent cells such

as the modulation of intracellular levels of some specific microRNAs or regulatory signaling molecules and second messengers [3].

The release of MPs to the extracellular environment will bring them in contact with neighboring cells, or if they reach systemic circulation, MPs can interact with cells of different types at remote sites within the body. MPs can interact with target cells in multiple ways ranging from a ligand-receptor type of interaction to their surface antigens, through membrane fusion with target cell or internalization which allows for dumping of MP cargo content inside the target cell [3]. Target cells, if they did not degrade MP content or eliminate it outside of the cell inside new vesicles, may respond to signaling molecules brought by MPs which eventually can alter cellular functions and responses within the recipient cell [4].

The shedding of MPs from cells is a natural mechanism, and virtually, any cell in the body is capable of releasing MPs into the extracellular environment. However, the cellular mechanisms governing the shedding of MPs are not fully elucidated. Many studies have reported that MPs are present in various body fluids and solid tissues from both healthy humans and animal models; however, the number of these MPs was found to be increased in pathological states and may constitute therefore good biomarkers for the prognosis and diagnosis of multiple pathologies [5]. In relation to cardiovascular diseases and complications, MPs from different cellular origins were reported to be increased in the blood of patients including those derived from circulating cells such as platelets, leukocytes, red cells, endothelial cells, and smooth muscle cells; however, much of circulating MPs detected were from platelet origin [5].

The current review article focuses on the involvement of MPs in diabetes-induced complications. Furthermore, it discusses the molecular mechanisms that underpin the pathophysiological role of MPs in the induction and progression of cellular injury associated with diabetes.

2. Differences between MPs and Other Extracellular Vesicles and Mechanisms of Formation

MPs are not the only vesicles of cell origin that can be found in body fluids; other types of extracellular vesicles are also documented such as exosomes and apoptotic bodies. However, the mechanisms controlling the liberation of these different vesicles are not the same (Table 1).

2.1. Formation and Release of MPs. MPs are directly released from the cytoplasmic membrane of cells. They are very heterogeneous in size (100–1,000 nm) and content but contain cytosolic cargo. The mechanisms of membrane remodeling implicated in the formation of MPs are not completely understood; however, much evidence indicates that the release of MPs happens after the activation of cells either chemically or physically or when cells undergo apoptosis. The number of MPs shed, and their qualitative content was reported to vary according to the types of stimuli at the origin of MP shedding [6, 7].

The release of MPs from cell membrane formation requires membrane remodeling following cell stimulation and the activation of cascades of intracellular signals. Bleb formation from plasma membranes, an indicator of MPs shedding, was shown to take place when an influx of calcium inside the cell occurs following cell activation. Following this intracellular calcium surge, cell blebbing is allowed via the activity of cysteine protease, μ calpain, which leads to the breakdown of cytoskeleton constituents, talin, and α-actinin [8]. In platelets, it has been reported that μ calpain inhibition prevented MP shedding and that μ calpain activation was mediated through an elevation of cyclic AMP (cAMP) levels and the subsequent activation of protein kinase A (PKA) [9].

It is in general well agreed that MPs are shed when the asymmetry of membrane phospholipid distribution between the inner and outer layers is disrupted or lost. In a basal condition, the negatively charged aminophospholipid, phosphatidylserine (PS), is essentially located in the inner leaflet of the cytoplasmic membrane. However, during cell activation or apoptosis, a prominent change in membrane asymmetry is the externalization of PS to the outer leaflet. The externalization of PS is an early indicator of the process of MP shedding. Because of its negative charge, PS externalization creates an excess of negative charges at the cell surface and imbalances the molecular masses of the two leaflets, which in turn could contribute to membrane destabilization and the formation of blebs. Maintaining membrane phospholipid asymmetry is pivotal for the proper function of cell membrane.

Loss or disruption of membrane asymmetry is tightly linked to cell activation and is associated with various disease states. Phospholipid asymmetry is maintained by the selective synthesis of specific phospholipids on each side of a cell membrane. Several proteins are involved in maintaining lipid asymmetry either by persevering or by disrupting the gradient of lipid distribution between the inner and out leaflets. There are three main classes of these protein: (i) flippase, directed toward the cytosol and ATP-dependent transporters; (ii) floppases, directed toward the extracellular environment and ATP-dependent transporters; and (iii) scramblases, bidirectional and ATP-independent transporters. Scramblases allow for a random distribution of lipids between the membrane bilayers. The flippases are very selective for PS, and their action is responsible for maintaining this phospholipid mainly sequestered at the inner leaflet of the cell membrane [10]. A sustained increase in intracellular calcium following cell activation or apoptosis inhibits flippases and therefore contributes to surface exposure of PS because of the overwhelming action of floppases [11, 12]. The disruption of cell membrane asymmetry and changes in masses of the two leaflets in addition to the calcium-induced proteolytic degradation of cytoskeleton caused by the activation of μ calpains lead to the formation of membrane blebs and MP release [12].

MPs exposing PS at their surface provide sites for the assembly of coagulation factors and participate thus to the process of hemostasis. Platelets have a very high rate of scrambling of membrane lipids and thus can rapidly generate MPs exposing PS which contributes in preventing bleeding;

Table 1: Major differences between extracellular vesicles found in body fluids.

	Apoptotic bodies	Microparticles (MPs)	Exosomes
Size of vesicles	>1,000 nm	100 nm–1,000 nm	<100 nm
Isolation method	Low to moderate speed centrifugation (<10,000 g)	High-speed centrifugation (<21,000 g)	Ultracentrifugations (>100,000 g)
Origin	Cytoplasmic membrane	Cytoplasmic membrane	Endocytic lysosomal system
Mechanism of shedding and release	Cytoplasmic membrane cytoskeletal rearrangement and loss of membrane asymmetry and release of bodies	Cytoplasmic membrane cytoskeletal rearrangement and loss of membrane asymmetry and release of bodies facilitated by calcium-dependent degradation of cytoskeleton	Fusion of multivesicular bodies (MVBs) with cytosolic membrane and exocytosis to extracellular environment
Most used techniques for detection and quantification	Flow cytometry, immunofluorescence, electron and atomic force microscopies, light scattering technique, western blotting, ELISA-based tests	Flow cytometry, immunofluorescence, electron and atomic force microscopy, light scattering technique, western blotting, ELISA-based tests	Immunofluorescence, electron microscopy, light scattering technique, western blotting, ELISA-based tests
Commonly used markers for detection	High levels of phosphatidylserine	Selectins, integrins, cell-specific surface antigens (cluster of differentiation or CD), phosphatidylserine	Tetraspanins (e.g., TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8, lactadherin, LAMP1

Abbreviations: ESCRT, endosomal sorting complexes required for transport; LAMP1, lysosomal-associated membrane protein 1; MFGE8, milk fat globule-EGF factor 8 protein; PDCD6IP, programmed cell death 6-interacting protein; TSG101, tumor susceptibility gene 101; TSPAN, tetraspanins.

however, an excessive shedding of MPs may cause thrombotic complications [12]. Sinauridze et al. [13] reported that one single platelet-derived MP had nearly the same procoagulant activity as one activated platelet despite the surface area was 2 orders of magnitude smaller suggesting that platelet-derived MPs have a procoagulant surface that is 50- to 100-fold more potent than activated platelets [13].

However, not all MPs appear to express PS at their surface. Connor et al. [14] demonstrated that in nonstimulated plasma that is poor in platelets, about 80% of platelet-shed MPs were unable to interact with annexin V. They also reported that the fraction of MPs that successfully interacted with annexin V was dependent upon the nature of the stimulus used. Naturally stimulating molecules such as collagen produced less MPs positively labeled with annexin V as compared to nonphysiological agonists such as calcium ionophore [14]. Furthermore, in our own hands, we observed that MPs exposing PS (positive for annexin V) represented, at the most, 50% of total MPs detected in the plasma from patients with metabolic syndrome, septic shock, and sleep apnea for instance [15–18]. Therefore, it is reasonable to suggest that we still lack some understating about the significance of shedding of these MPs that do not expose PS at their surface and that other cellular mechanisms are involved in their biogenesis, which warrants further research.

Other molecular events were identified to bridge the link between the sustained increase in intracellular calcium following activation and externalization of PS and MP shedding and release from cell membranes. It has been proposed that externalization of PS is related to the entry of calcium through membrane calcium channels, which follows depletion of intracellular calcium stores; this phenomenon is known as capacitative or store-operated calcium entry (SOCE), and it has been shown that this event together with the externalization of PS is partly regulated by actin cytoskeleton [19]. The small GTPase Rho A, which interacts with the extracellular signal-regulated kinase (ERK) pathway, was found to regulate SOCE and the surface externalization of PS through the rearrangement of actin cytoskeleton [20]. The phosphorylation of myosin light chain (MLC) causes actin/myosin-mediated contractile tension, leading thus to an increase in the pressure on the cytoplasm structure, which eventually causes membrane bleb formation. The phosphorylation of MLC was shown to be mediated, during apoptosis, by the serine/threonine kinase Rho-associated protein kinase- (ROCK-) I, one of the effectors downstream of the small GTPase Rho. ROCK-I was found be activated in apoptosis by proteolytic cleavage by caspase 3, and the active kinase can then simultaneously phosphorylate and hence activate MLC, then phosphorylate and thus inactivate MLC phosphatase [12, 21, 22].

2.2. Formation and Release of Exosomes and Apoptotic Bodies. Another example of extracellular vesicles found in body fluids including systemic circulation is exosomes that have a size ranging between 40 and 150 nm [23–25]. Exosomes are released upon the fusion of multivesicular bodies with cell membrane. Their content is very distinct from MPs owing to their specific mode of formation. The formation of exosomes takes place within endosomes by introversion of the delineating membranes causing the generation of multivesicular bodies. Subsequently, these multivesicular bodies merge with the cytoplasmic membrane releasing thus exosomes outside of the cell [26].

The detection of exosomes is supported by both structural and molecular features. Because of their small size, exosomes can be collected following high-speed centrifugations above 100,000 g. Exhaustive proteomic profiling of MPs and exosomes derived from many cell types found that exosomes are exclusively enriched in some stereotypic protein

markers such as TSG101, cell death 6-interacting protein (PDCD6IP), and tetraspanin 30 (CD63) while MPs do not express these markers [27, 28].

Cells undergoing apoptosis can release larger vesicles referred to as apoptotic bodies with sizes larger than 1,000 nm which are generated by the uncoordinated blebbing of cytoplasmic membrane and can carry nuclear parts and proteins. Under certain conditions, the number of apoptotic bodies exceeds those of exosomes and MPs and their content can differ between various body fluids [2].

These various types of membrane vesicles found in the extracellular environments can be discriminated according to multiple structural and physical properties. Sequential rounds of high-speed centrifugations are the most effective method to isolate cells, debris, apoptotic bodies, MPs, and exosomes. MPs are collected from the supernatant resulting from the elimination of cell debris by additional cycles of centrifugation with a speed ranging between 10,000 and 21,000 g whereas the isolation of exosomes requires ultracentrifugation speeds above 100,000 g (Table 1). It is possible to discriminate between apoptotic bodies, MPs, and exosomes by electron microscopy or flow cytometry, although owing to their detection limits, flow cytometers are believed not be optimal for the detection of exosomes because of their small size.

3. Composition of MPs

3.1. Protein Content of MPs. MP content mirrors the remodeling and rearrangement of the cytoplasmic membrane of the parent cell that is shedding them. Several studies including those involving molecular profiling methods such as proteomic techniques revealed that protein cargo content of MPs does not only depend on the nature of parent cells but also depend on the stimulating conditions at the origin of MP generation [29, 30]. It has been reported previously for example that MPs engineered *in vitro* from T lymphocytes using different stimuli carry key differences in their cargo protein content. It was found that MPs, generated from T lymphocytes activated with phytohemagglutinin (PHA) for 72 hours and then induced in apoptosis with additional stimulation for further 24 hours with phorbol-12-myristate-13 (PMA) and actinomycin D, expressed the morphogen sonic hedgehog (Shh) [7]; however, apoptotic T lymphocytes (stimulated with actinomycin D only for 24 hours) generated MPs which were deficient in Shh [6]. It has been observed that only the treatments of T lymphocytes with PHA, PMA, and actinomycin D or with PHA/actinomycin D were able to generate MPs carrying Shh [31]. However, the mechanisms behind this differential expression in protein content are not yet clear.

The differential expression of Shh translated into differences in the biological messages carried out by MPs generated from T lymphocytes. T lymphocyte MPs harboring Shh induced nitric oxide (NO) release from endothelial cells and reversed endothelial dysfunction causing ischemia/reperfusion of the left descending anterior coronary artery in mice [7] and improved endothelial dysfunction in a mouse model of angiotensin II-induced hypertension [32]. However, T lymphocyte MPs deficient for Shh reduced NO bioavailability in endothelial cells and caused endothelial dysfunction in mouse aortas [6]. MPs carrying Shh were found to activate the Shh signaling pathway in target cells [33].

Proteomic analysis of MPs derived from control and β-cells of islets of Langerhans stimulated with cytokines for 24 hours showed that several proteins were differentially expressed by the two populations of MPs. Multiple members of the tumor necrosis factor (TNF) transduction pathway were upregulated in MPs obtained from cells stimulated with cytokines, such as the TNF receptor superfamily member 1A, TNFα-induced protein 3, and TNF-interacting kinase receptor-interacting serine-threonine kinase 1 [34].

Xu et al. [35] performed a proteomic analysis of circulating MPs from newly diagnosed type-2 diabetics and healthy volunteers. The authors found 46 proteins that were differentially expressed by MPs between diabetic patients and healthy volunteers. Of these proteins, 20 proteins were increased in diabetic MPs and 17 others were only found in MPs from healthy subjects. Of interest, it was found that diabetic MPs expressed significantly higher levels of Ras-related protein Rap-1b (RAP1B), CD9, and integrin alpha-IIb (ITGA2B, CD41) [35]. All these molecules play a role in hemostasis and platelet activation and aggregation; however, the increase in CD41 expression is not surprising because this is an essential platelet glycoprotein and that platelet-derived MPs represent over 80% of all circulating MPs [5]. Xu and colleagues [35] also reported that the expression of S100A8 and S100A9 was significantly elevated in MPs from diabetic patients [35]. A previous proteomic study also found that S100A8 and S100A9 were highly increased in chronic diabetic foot exudates compared to exudates from split-skin donor sites of burn victims who are otherwise healthy [36]. Both S100A8 and S100A9 are inflammatory mediators, which can bind to advanced glycation end-products (AGEs) receptors and toll-like receptors (TLRs) to let go inflammatory cytokines [37]. The genetic deletion of S1009A in atherosclerosis-prone Apo E-deficient mice reduced atherogenesis [38]. These findings support reports indicating that MPs from diabetic patients, especially those from platelets, have a stronger procoagulant potential which contributes to the hypercoagulability in these patients and strengthens the evidence for a potential role of MPs in vascular pathogenesis in diabetes.

Because they originate from the cell membrane, MPs are enriched in membrane proteins from the parent cell such as integrins, glycoprotein Ib (GPIb), and P-selectin [2]. These surface markers expressed by MPs are crucial for their detection in flow cytometry according to their cellular origin and support the use of patterns of expression of specific subpopulations of MPs as markers for the onset and progression of disease.

MPs can also carry membrane receptors and ligands, in addition to cytosolic proteins such as transcription factors and enzymes that are functionally bioactive, including for instance phosphatidylinositol-3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) [2]. The identification of specific and differentially expressed surface markers

between MPs derived from healthy and abnormal cells, such as dysfunctional vascular cells, would provide tools for better disease diagnosis and progression monitoring.

3.2. Lipid Content of MPs. MPs consist of a lipidic bilayer membrane identical to the parent cell plasma membrane. Membranes of MPs compared to cell plasma membranes have higher flip-flop motions which facilitate the distribution and exchange of phospholipids between the membrane leaflets [2, 39]. MP membranes are shown to exhibit a high content of sphingomyelin and cholesterol which eventually contributes to their rigidity and resistance to degradation making them thus ideal carriers for various macromolecules including proteins and nucleic acids such as miRNAs [40, 41]. Macrovesicles can harbor also enzymes implicated in the metabolism of lipids such as phospholipases A2, C, and D and carry a variety of bioactive lipids such as free fatty acids which can modulate cellular responses in recipient cells [42]. The differential lipid composition of MPs may reflect the conditions of stimulation of the parent cell and vary according to the content in lipids in the extracellular environment surrounding the emitting cells.

It is anticipated that MPs from subjects suffering from metabolic disturbances and dyslipidemias may have a specific lipid and lipid metabolic enzyme composition compared to MPs from healthy subjects, which may eventually contribute to a differential effect of these MPs on recipient cells; however, this hypothesis warrants further investigation. The numbers of MPs from platelets [43] and those from T cells or neutrophils and those harboring TF [44] were found to have a negative correlation with plasma levels of plasma HDL cholesterol.

3.3. Nucleic Acids and MicroRNA Content of MPs. In addition to carrying bioactive lipids, membrane, and cytoplasmic proteins and other organelles and nuclear components from the parent cell, MPs can also carry nucleic acids, specifically mRNA and microRNA (miRNA), suggesting MPs can deliver genetic material to recipient cells [5]. MicroRNAs, which are small noncoding RNAs, play a key role in the epigenetic regulation of gene expression and control many metabolic and physiological processes associated with health and disease. Circulating MPs appear to be an efficient carrier for miRNA and a tool for their transfer to target cells where they can modify the physiological response of the cell.

It has been reported that platelets carry a rich and diverse content of more than 400 miRNAs [45, 46]. Importantly, the miRNA cargo content of platelet-derived MPs differs under disease conditions and upon cellular activation [47, 48]. The treatment of cells with thrombin, a potent platelet agonist, altered the miRNA signature of platelets and platelet-derived MPs with differential expression of miR-15a, miR-339-3p, miR-365, miR-495, miR-98, and miR-361-3p being reported [46, 47]. It is now becoming apparent that diabetes induces its own miRNA signature which may contribute to the onset of diabetes-induced disturbances. The pattern of expression of 5 plasma miRNAs, miR-15a, miR28-3p, miR-126, miR-223, and miR-320, was found to form a unique molecular signature enough to differentiate between individuals with and without diabetes [49]. Laffont et al. [47] reported that when platelets were activated with thrombin release, they selectively and preferentially released MPs rich in miR-223 [47]. Recently, Jansen et al. [50] reported in a cohort composed of 80 control subjects and 55 patients suffering from type-2 diabetes that endothelial-derived MPs from diabetics had a lower expression of miR-126 and miR-26a compared to controls. Intracellular miR-126 expression level was reported to define the regenerative capacity and proangiogenic capacity of $CD34^+$ peripheral blood mononuclear cells [51] and circulating angiogenic early outgrowth cells [52].

4. Methods of Detection of MPs

Using a variety of methods, it is quite easy to detect MPs in body fluids. These techniques include assessing the concentration of proteins carried by MPs, flow cytometry analysis using cell-specific antigen markers, or ELISA assays to detect the prothrombotic activity of MPs based on the assumption that all MPs expose PS at their surface and can thus interact with annexin V. More recently, new single-particle detection instruments have been used for the analysis of MPs, including the resistive pulse sensing (RPS) and the nanoparticle tracking analysis (NTA), which can measure the size distribution and the concentration of in-solution MPs [53, 54]. Because of the tendency of MPs, especially those of a platelet origin, to aggregate during the process of isolation by centrifugation, single-particle detection instruments (e.g., RPS and NTA) can detect a lower concentration of MPs. However, flow cytometers which are particle size-insensitive instruments can detect these large aggregates, and thus, a higher concentration is detected [53, 54].

Flow cytometry analysis is the most preferred technology nowadays to trace MPs because of its high speed of detection and the availability of more sensitive machines. With flow cytometry, there is a possibility to determine the total number of MPs as well as the cellular origins of MPs by targeting specific surface antigens expressed by MP particles from a specific cell origin. However, owing to the differences between flow cytometers in terms of their optical configuration and sensitivity of detection and because of the absence of standard units for the measurement of MPs, the comparison of data between laboratories is difficult which hinders the potential use of MPs as a diagnostic and/or prognostic tool for diseases due to the variability and nonreproducibility of results depending on the machine used [53].

The need for standardization of techniques of detection of MPs by flow cytometry was recognized by the Scientific Standardization Committee on Vascular Biology (SSCVB) of the International Society on Thrombosis and Hemostasis (ISTH), which launched an initiative over 6 years ago to standardize MP measurements using flow cytometry following a survey that highlighted that nearly 75% of laboratories use flow cytometry to count and phenotype MPs according to cellular origins in clinical samples (e.g., plasma). An inaugural collaborative workshop was established to determine both the resolution and the level of background noise of major flow cytometers used across several research laboratories.

The other objective of this workshop was to define the reproducibility of platelet-derived MP enumeration in human plasma between different instruments. The strategy followed was to use Megamix® fluorescent calibrated beads to allow for a reproducible setup of MP window analysis [55].

Lacroix et al. [56] tested 49 flow cytometers in 40 laboratories and found that the instruments tested had very heterogeneous forward scatter (FS)/FS channeling (FSC) resolution and machine background noise. The authors also reported that the use of Megamix® helped in identifying and fixing some of the parameters affecting FS/FSC resolution and that eventually 33 instruments tested were validated [56]. Nonetheless, because of the huge variety of optical designs in flow cytometers available on the market, it was possible to achieve an efficient universal standardization methodology to count platelet-derived MPs. The consortium observed, however, that the resolution was better and more homogenous in a subset of instruments using side scatter (SSC) instead of FSC. Thus, another set of beads was chosen to better respect the design of SSC-focused flow cytometers [57].

More recently, another workshop was organized by the SSCVB to test a new strategy for the standardization of MP detection by flow cytometers and to evaluate interinstrument reproducibility in detecting platelet-derived MPs. This new strategy used two different types of beads to suit instruments of different optical designs (Megamix-Plus® FSC or SSC beads). The study by Cointe et al. [58] included 44 laboratories across 17 countries and had 52 instruments registered for testing. Flow cytometers qualified for inclusion in the strategy of scandalization according to their resolution and low levels of background noise. All these instruments could correctly rank levels of platelet-derived MPs in a plasma. The interlaboratory variance in the enumeration of MPs was between 28 and 37%. The authors showed that the use of size-calibrated beads can successfully be utilized to standardize MP counting across different instruments and laboratories if special care is taken to consider the internal instrument behavior for size-related measurements and this independently of using FSC or SSC as the relative sizing parameter [58]. Despite this second study which only investigated parameters for the detection of platelet-derived MPs and only focused on the optimal scatter-based gating of the MP population, it is a very important initiative and an important step forward standardizing conditions of detection of MPs by flow cytometry.

One major challenge in using flow cytometers in detecting MPs is still the limit of the detection, and further technological developments are thus required to improve the detection of smaller MPs. Furthermore, additional efforts from the extracellular vesicle community are still warranted to improve standardization methods for MP detection for an optimal use of circulating MPs as routine laboratory diagnosis and/or prognosis tools.

5. MPs as Biomarkers of Cardiovascular Diseases

Increased numbers of plasma MPs were reported in several cardiovascular diseases associated with insulin resistance, inflammatory, and/or procoagulant states [5]. In these pathologies, platelet-derived MPs were found to represent the dominant subpopulation. However, MPs from other origins including endothelial cells, erythrocytes, or leukocytes were also found to be increased in certain pathological states such as metabolic syndrome [16], acute coronary syndrome [59, 60], severe hypertension [61], and type-2 diabetes [62]. Two key features of these disease states are endothelial dysfunction and impaired microcirculation with which the levels of circulating MPs frequently correlate indicating that MPs carry cellular messages that may contribute to the onset and progression of these pathologies.

Circulating MPs found in the plasma originate from vessel wall cells such as endothelial and smooth muscle cells and other circulating cells such as platelets, erythrocytes, and leukocytes. However, most of these MPs are derived from platelets which represent nearly 80% of the total population of circulating MPs. An important physiological role of platelets is the control of hemostasis by providing a membrane surface to enhance blood coagulation and promote the generation of the fibrin network in the hemostatic plug [63]. Platelets have a very high rate of scrambling of membrane lipids and when stimulated generate MPs most rapidly especially those exposing PS which contributes in preventing bleeding; however, an excessive shedding of MPs may cause thrombotic complications [12]. Platelets produce MPs which display a heightened prothrombin-converting activity and which provide an extended membrane surface to promote coagulation. It has been reported that MPs originating from platelets are 50- to 100-fold more procoagulant than activated platelets [13]. However, although high circulating levels of platelet-derived MPs were reported in many disease conditions, changes in nonplatelet-derived MPs, particularly those originating from endothelial cells, were found to contribute a key role in the pathogenesis of these disorders [15–18].

MPs are recognized today as promising biomarkers for the diagnosis and monitoring of disease progression because total levels of MPs or those from specific cellular origins were found to correlate with the severity or the progression of several disorders. An increase in the numbers of MPs from a specific cell population may represent a signature of injury and a marker of changes to which these cells are subjected to. For instance, endothelial MP levels were found to be good predictors for the health of the endothelium and to correlate with endothelial dysfunction. For example, high levels of endothelial MPs were found to correlate positively with arterial erectile dysfunction in patients with insulin resistance [64] and with endothelial dysfunction in patients suffering from chronic ischemic left ventricular dysfunction [65]. The circulating levels of platelet-released MPs were associated with carotid and intima/media thickness and corporal in mass in obese subjects [66]. Activated leukocyte-derived MPs ($CD62L^+$) were found to positively correlate with oxyhemoglobin desaturation index (ODI) in patients with obstructive sleep apnea which do not suffer from any cardiovascular comorbidities [18].

6. Circulating MPs in Insulin-Resistant States and Diabetes

6.1. Impact of Diabetes on MP Expression Profile. Much evidence shows that circulating numbers of MPs are more elevated in patients with diabetes and in experimental animal models of diabetes and they contribute to the development of cardiovascular complications associated with diabetes. A meta-analysis study by Li et al. [67] investigated the relationship of MPs with type-2 diabetes. The authors included 48 studies with a total number of 2,460 patients suffering from type-2 diabetes and 1,880 nondiseased volunteers, of which 34 studies were quantitatively assessed. The general analysis revealed that type-2 diabetes patients, regardless whether they had comorbidities or not, exhibited higher circulating levels of total MPs, platelet-, monocyte-, and endothelium-derived MPs in comparison to healthy volunteers. However, the count of leukocyte-derived MPs was similar between controls and patients [67].

Previously, Sabatier et al. [68] investigated circulating levels of MPs and determined their procoagulant properties in both type-1 and 2 diabetics and reported in general all diabetics had higher numbers of MPs compared to controls. However, the authors observed that diabetic patients exhibited a difference in the pattern of expression of MPs according to their cell origins and in their prothrombotic action. Patients with type-1 diabetes had higher levels of MPs derived from platelets and endothelial cells in addition to prothrombotic MPs compared to controls and type-2 diabetes patients [68]. Later, Feng et al. [69] reported that type-2 diabetes patients had elevated levels of procoagulant, platelet, leukocyte, and endothelial MPs compared to controls and endothelial MPs were the only population to positively correlate with endothelial dysfunction as assessed by flow-mediated dilation (FMD) in patients [69].

Koga et al. [70] stressed the importance of endothelial MPs as markers of vascular dysfunction in diabetes and showed that endothelial MPs were twofold higher in diabetics compared to control volunteers and that these MPs have even higher levels of diabetic patients suffering from coronary heart disease than those free from such a comorbidity [70]. In another study, Bernard et al. [71] evaluated the association between plasma levels of endothelial- and platelet-derived MPs and the presence of coronary noncalcified plaques in patients with diabetes. Researchers reported that MPs of endothelial origin ($CD144^+$) were highly elevated in patients exhibiting coronary noncalcified plaque [71]. Tsimerman et al. [72] reported that diabetic patients had high circulating levels of MPs originating from platelets and that patients also suffering from diabetic foot ulcers had the highest numbers of prothrombotic, endothelial, and platelet MPs compared to patients suffering from diabetes and coronary heart disease or retinopathy [72].

A study by Zhang and coworkers [73] investigated the impact of the copresence or not of obesity and type-2 diabetes on the profile of expression of circulating MPs. The authors reported that regardless of the presence or not of obesity, type-2 diabetics had high levels of total platelet-derived MPs or those from activated platelets which express fibrinogen, tissue factor (TF), or P-selectin. However, investigators did not find any specific effect of obesity on MP counts in the absence of type-2 diabetes [73]. The absence of differences in the presence of obesity alone is contradicting with previous reports indicating an effect of obesity on the numbers of circulating MPs [5]. This is potentially due to the small cohort of patients included in this study (5 to 11 patients per group) [73].

6.2. MP Expression in Type-2 Diabetes and Asymptomatic Atherosclerosis. Recently, Berezin et al. [74] investigated the phenotype of expression of circulating MPs between healthy volunteers and patients with type-2 diabetes either with angiographic evidence of asymptomatic atherosclerosis or without any known coronary atherosclerosis. The authors found that in comparison to controls and diabetics with no angiographic evidence of atherosclerosis, patients with asymptomatic atherosclerosis had elevated numbers of circulating platelet-derived ($CD41a^+$) and endothelial-derived ($CD144^+/CD31^+$ or $CD144^+$) MPs in addition to MPs from procoagulant endothelial cells ($CD31^+$/annexin V^+). Using a multivariate regression model, the authors found that MPs from procoagulant endothelial cells ($CD31^+$/annexin V^+) were an independent predictor for nonsymptomatic atherosclerosis [74].

6.3. Differential MP Origins and Endothelial Dysfunction in Obesity. High circulating levels of MPs from different origins were also observed in various studies of patients with obesity, which is closely associated with insulin resistance and considered number one contributor to the development of type-2 diabetes. Several studies in obese children suggested a predictive potential for MPs for future adverse cardiometabolic events. A cross-sectional study by Bruyndonckx et al. [75] investigated the relationship between microvascular endothelial dysfunction and numbers of MPs derived from endothelial cells and endothelial progenitor cells (EPCs) in a cohort composed of 57 obese and 30 age- and sex-matched and normal weight children. The noninvasive measurement of microvascular endothelial function by determining the peripheral arterial tonometry at the distal phalanx, that reflects endothelial function at the level of small resistance arteries, revealed that obese children had significantly impaired peripheral endothelial function in addition to a lower number of circulating EPCs and a high level of circulating endothelial-derived MPs ($CD31^+/CD42b^-$) compared to control children. Single regression and multivariate analysis showed that the peak response (or the ratio between postocclusion pulse-wave amplitude over the baseline amplitude) correlated negatively with endothelial-derived MPs but positively with EPCs count that were both independent determinants of the peak response together with systolic blood pressure [75].

6.4. MP Expression Profile in Metabolic Syndrome. Obesity is often associated with other comorbidities including insulin resistance, type-2 diabetes, dyslipidemias, and hypertension. This association of cardiometabolic disturbances is commonly referred to as the metabolic syndrome (MetS) [76,

77]. The pathophysiology of MetS is tightly related to insulin resistance and excess of fatty acids [78, 79] in addition to low-grade inflammation [77, 79, 80]. The combination of inflammatory factors with excessive production of reactive oxygen species (ROS) contributes to the development of cardiovascular disturbances in MetS [5]. Several studies investigated the count and phenotype of circulating MPs present in the plasma from patients with MetS. Agouni et al. [16] determined the count and phenotyped plasma MPs according to their cellular origins in 43 MetS patients and 37 volunteers. It was observed that MetS patients had higher numbers of total MPs in comparison to controls, in addition to prothrombotic MPs (annexin V^+) and those originating from platelets, endothelial cells, and erythrocytes [16]. These observations were also corroborated by the work of Helal et al. [81] in a bicentric cohort study of MetS patients. The authors also found that BMI correlated positively with the number of prothrombotic and endothelial-derived MPs [81].

These studies strongly indicate that MPs are potential biomarkers for the diagnosis, stratification, and follow-up of disease progression and development of cardiovascular comorbidities for metabolic disorders where insulin resistance is a central pathophysiological driver such as obesity, diabetes, and MetS. These findings also highlight the crucial role that MPs may play in the development of metabolic diseases and in the onset and/or maintenance of cardiovascular complications associated with these pathological states.

7. Pathophysiological Roles of MPs in Altering Cell Signaling and Causing Cell Injury in Insulin-Resistant States and Diabetes

In the last decade, many studies were conducted to investigate the role of MPs as active effectors in the onset and progression of diabetic cardiovascular complications. Cells subjected to pathological stimuli have modified intracellular responses, and thus, MPs generated from these cells can transfer these deleterious biological messages to recipient cells to modify in turn their cellular responses.

MPs can transfer biological information to recipient cells through multiple potential mechanisms although the exact process is not fully understood yet. These mechanisms of interaction with target cells may involve the following: (i) direct interaction between surface proteins in a ligand/receptor type of interaction; (ii) transfer to target cell of surface receptors, ligands, channels, proteins, genetic material, and lipids; (iii) merging between membranes of MPs and target cells; and (iv) internalization of MPs inside the target cell. The mechanism of interaction with recipient cell involving ligand and receptor interaction may rapidly either switch on or switch off intracellular signaling responses; however, the other means of interaction will involve more complex changes of cellular responses inside target cells. MPs can also interact with recipient cells through more than one of these mechanisms. For instance, it has been previously reported that MPs carrying the morphogen Shh were able to activate the morphogen's signaling pathway to enhance endothelial angiogenic process and NO release [7, 82]. However, two hours following this surface interaction, MPs carrying Shh were internalized by endothelial cells and enhanced the expression of antioxidant enzymes [83]. Figure 1 summarizes the mechanisms of interaction of MPs with recipient cells.

In the next section, we will discuss the role of MPs in the onset and maintenance of cellular dysfunction and injury associated with insulin resistant states and diabetes with special emphasis on the molecular mechanisms involved. The major alterations in circulating levels of MPs and their molecular contribution to diabetes-induced complications in humans and *in vivo* and *ex vivo* models are summerized in Table 2.

7.1. MPs and Vascular Dysfunction. Due to its direct exposure to blood stream, one major target of circulating MPs are endothelial cells, which play an important role in the maintenance of vascular homeostasis by achieving a balance between vasoactive agents (vasodilators and vasoconstrictors) and prothrombotic and antithrombotic factors in addition to a tight control of vascular permeability among other key actions. Physiological levels of shear stress regulate the homeostasis of endothelial cells; however, extreme disturbances in shear stress due to changes in vascular pressure can imbalance the release of endothelium-derived factors leading to endothelial dysfunction.

Endothelial dysfunction is referred to as the reduced capacity of vessels to dilate in response to the activation of endothelial cells by humoral or mechanical mediators such as shear stress or bradykinin. It is now widely recognized that endothelial dysfunction is an independent predictor of future adverse cardiovascular events which are widely associated with obesity and diabetes. Most notably, a decrease of endothelial NO production and bioavailability, a heightened release of vasoconstrictor mediators, and an enhanced oxidative stress, which altogether lead to alterations of vascular reactivity, cause vascular inflammation and vascular remodeling by affecting the levels of proteins and enzymes involved in these processes. It is expected that MPs may affect all these vascular events that contribute to endothelial dysfunction.

MPs produced from activated vascular cells, such as endothelial cells, are biomarkers of cell injury; however, they are also capable of interacting with neighboring cells in an autocrine or paracrine mechanism and modify therefore the cellular responses of target cells including the emitting cells themselves. Jenkins et al. [84] reported that disturbed blood flow in the forearm of healthy men by distal cuff occlusion was shown to increase endothelial MPs in the experimental arm by nearly 9-fold after 20 minutes only, while activated endothelial MPs expressing E-selectin ($CD62E^+$) increased by 4-fold [84]. Even though arteries in the forearm are less prone to atherosclerosis, it was possible to observe acute changes in activated endothelial MPs, suggesting that in atherosusceptible arteries from the elderly or individuals suffering from comorbidities that are associated with impaired endogenous endothelial repair mechanisms (e.g., obesity or type-2 diabetes), the effects might be more severe [84]. The acute reduction of physical activity for 5 consecutive days was also reported to increase the numbers of total and

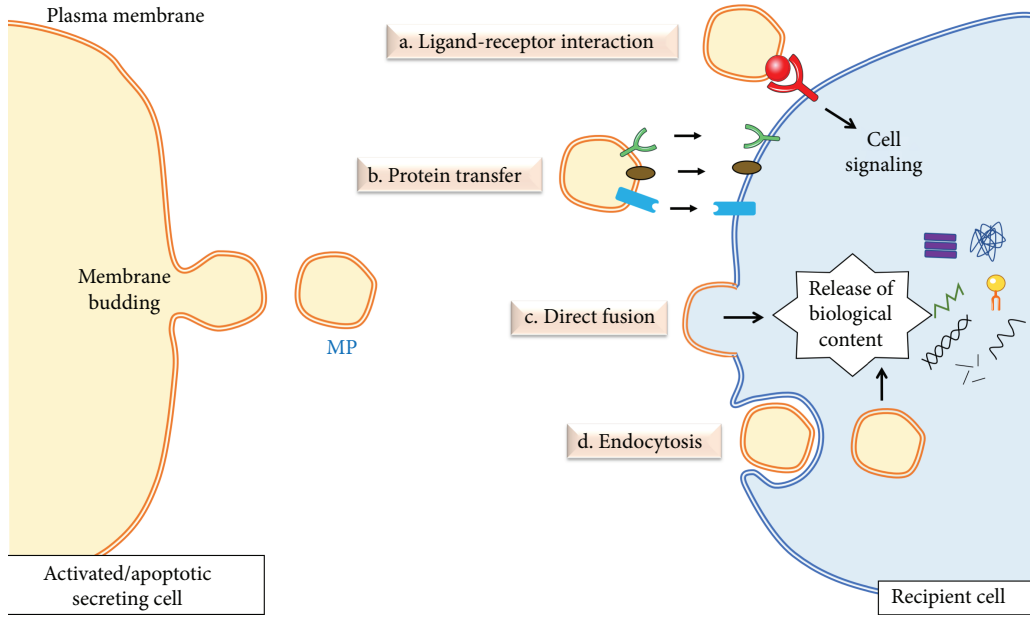

Figure 1: Mechanisms of interaction of MPs with recipient cells. MPs are first released from activated or apoptotic cells by direct budding of the plasma membrane. MPs can then directly interact with the target cell via ligand-receptor interaction which results in cell signaling (a), transfer proteins (i.e., adhesion molecules, MHC, and membrane receptors) from the MP vesicle to the surface of the target cell (b), or deliver the biological content of the MP to the target cell through either direct fusion of the MP with the plasma membrane of the target cell (c) or endocytosis (d). PS: phosphatidylserine, MHC: major histocompatibility complex, MP: microparticle.

activated endothelial-derived MPs in healthy volunteers, which was associated with an impairment in popliteal artery flow-mediated dilation (FMD) [85]. These studies indicate that acute changes in MP release not only are early biomarkers of cell injury but can also be considered active players and conveyers of biological message which contribute to changes in the vascular function.

Several studies have reported a relationship between numbers of total MPs or specific cell populations and endothelial dysfunction; however, few studies looked at the molecular mechanisms underpinning the actions of MPs on endothelial cells. Ishida et al. [86] found that streptozotocin-injected rats (a model of type-1 diabetes) had high numbers of plasma MPs originating from platelets ($CD61^+$) and activated platelets ($CD62P^+$) in addition to procoagulant MPs (annexin V^+) compared to control animals. They also found that the *ex vivo* exposure of carotids from control rats to diabetic MPs reduced endothelium-dependent relaxation by blunting eNOS protein expression [86]. Using a diet-induced obesity rat model, Heinrich et al. [87] reported that obese rats had higher circulating MP levels compared to chow diet-fed animals, including those of procoagulant, endothelial, platelet, leukocyte, monocyte, and T lymphocyte origins. The incubation of endothelial cells *in vitro* with MPs from obese rats enhanced the expression of vascular cell adhesion molecule 1 (VCAM-1) and increased oxidative stress indicating that MPs from insulin resistant animals induced endothelial dysfunction [87].

Previously, Martin et al. [88] observed that MPs engineered *in vitro* from apoptotic T lymphocytes reduced endothelium-dependent relaxation and impaired shear-mediated dilation in mouse aortas and small mesenteric arteries, respectively. These effects were related to a deceased expression of eNOS and overexpression of caveolin-1, a negative regulator of eNOS. Furthermore, the authors found that MPs derived from diabetic T lymphocytes or circulating total MPs from diabetic patients blunted eNOS protein expression in HUVECs [88]. Tesse and coworkers [89] have shown later that *ex vivo* exposure of mouse aortic rings to MPs from either apoptotic T lymphocytes or patients with diabetes caused impaired vascular response to vasoconstrictor agents. This effect was linked to an enhanced release of NO and prostacyclin mediated by NF-κB-induced upregulation of inducible forms of NOS (iNOS) and cyclooxygenase (COX-2). The authors also observed that *in vivo* injection of mice with MPs derived from apoptotic T lymphocytes impaired vascular response to vasoconstrictors, which was prevented by the concomitant administration of both NO and COX-2 inhibitors [89]. Interestingly, Tesse et al. [89] also observed that the vascular effects mediated by diabetic and T lymphocyte-derived MPs involved the interaction of Fas-ligand (FasL or CD95L) carried out by MPs its receptor (Fas or CD95) expressed by smooth muscle cells [89].

Agouni et al. [16] observed that MPs collected from patients suffering from MetS reduced endothelium-mediated vasodilation in aortas of mice that received an intravenous injection of MetS MPs in comparison to those that received MPs from MetS-free subjects. Of interest, the authors found that in spite the majority of MPs from MetS patients originated from platelets, the effects mediated by MetS MPs on endothelial function were mainly caused by MPs of nonplatelet origin [16]. The same group has subsequently found that MetS MPs could also interact with smooth muscle cells to cause vascular dysfunction [15]. The team

Table 2: Summary of the main studies reporting diabetes-induced complications associated with altered levels of circulating MPs in humans and *in vitro* and animal models.

Complications	Origins of MPs and markers used for detection	Cell and animal models	Humans	Key observations	References
Diabetic nephropathy	(i) Urinary podocyte MPs (annexin V^+/podoplanin$^+$)		Patients with uncomplicated type-1 diabetes	(i) Podocyte-derived MPs are higher in type-1 diabetes patients compared to healthy volunteers and increase under hyperglycemic clamp	[141]
	(i) Platelet MPs (AV^+/CD41$^+$) (ii) Endothelial MPs (AV^+/CD62E$^+$)		Chronic kidney disease (CKD) patients	(i) Positive correlation between platelet- and endothelial-derived MPs with p-selectin (marker of platelet activation) and vWF (marker of endothelial injury)	[142]
	(i) Endothelial MPs (CD144$^+$)		End-stage renal failure (ESRD) patients	(i) Endothelial MPs correlated with vascular dysfucntion *in vivo* (loss of flow-mediated dilation, elevated aortic pulse wave velocity, and common carotid augmentation index) (ii) *Ex vivo*, endothelial MPs from ESRD patients impaired endothelium-dependent relaxation and cGMP generation in aortic rings from Wistar rats	[143]
	Endothelial MPs (CD31$^+$/CD41$^-$)		End-stage renal failure (ESRD) patients	(i) Endothelial MP levels are independent predictors of all-cause and cardiovascular mortality in ESRD patients	[154]
	Urinary podocyte MPs (podocalyxin$^+$)	Mouse models of diabetic nephropathy		(i) Higher podocyte-derived MPs were observed in mouse models of diabetic nephropathy [streptozotocin- (STZ-) treated, OVE26, and Akita mice]	[140]
	(i) Annexin V^+ MPs (ii) Platelet MPs (annexin V^+/CD41$^+$, CD41$^+$/CD62P$^+$, CD41$^+$/CD142$^+$, CD41$^+$/CD154$^+$) (iii) Endothelial (annexin V^+/CD144$^+$, annexin V^+/CD62E$^+$, CD62E$^+$/CD142$^+$) (iv) Monocyte MPs (annexin V^+/CD14$^+$, CD14$^+$/CD142$^+$)		Patients with diabetic nephropathy	(i) Apart from TF (CD142$^+$)-positive MPs, all other subpopulations were higher in diabetics with CKD compared to diabetics without (ii) All MP subtypes correlated negatively with GFR	[147]
	(i) Endothelial MPs (CD144$^+$ and CD146$^+$)		Children with CKD	(i) Endothelial MPs were positively associated with blood pressure, age, disease duration, CRP, and parathyroid hormone and negatively associated with hemoglobin, GFR, and albumin	[145]

Table 2: Continued.

Complications	Origins of MPs and markers used for detection	Cell and animal models	Humans	Key observations	References
				(ii) Pulse wave velocity was independently related to endothelial MPs	
	(i) Endothelial MPs ($CD144^+$or $CD146^+$) (ii) Platelet MPs ($CD41^+$) (iii) Leukocyte MPs ($CD45^+$) (iv) Annexin V^+ MPs		Patients with chronic renal failure who were undialyzed (CRF) or hemodialyzed (HD)	(i) Endothelial MPs ($CD144^+$ and $CD146^+$) were higher in CRF and HD patients compared to nondiseased controls	[144]
Diabetic retinopathy	(i) Mesenchymal stem cell-derived extracellular vesicles (levels not measured) obtained in diabetes-mimicking conditions	Human retinal pericytes		(i) Mesenchymal stem cell-derived MPs stimulated retinal pericyte detachment and endothelial cell proliferation *in vitro*	[139]
	Vitreous MPs: (i) Annexin V^+ MPs (ii) Platelet MPs ($CD41^+$) (iii) Endothelial MPs ($CD144^+$)		Patients with diabetic retinopathy	(i) MPs promoted angiogenesis *in vivo* (endothelial cell migration and new vessel formation)	[137]
	(i) Platelet MPs ($CD62P^+$ and $CD63^+$)		Patients with diabetic retinopathy	(i) Platelet MP count increased with the progression of retinopathy	[155]
	(i) Monocyte MPs (annexin V^+/$CD14^+$)		Patients with diabetic retinopathy	(i) Monocyte MP levels significantly correlated with levels of platelet activation markers (platelet-drived MPs, $CD62P^+$, and $CD63^+$) and adhesion molecules (p-selectin and ICAM-1)	[156]
	(i) Monocyte MPs (annexin V^+/$CD14^+$)		Patients with diabetes and diabetic complications (nephropathy, retinopathy, or neuropathy)	(i) Elevated levels of monocyte-derived MPs correlated positively with platelet activation markers (platelet MPs, $CD62P^+$, and $CD63^+$)	[62]
Ischemic diseases and diabetic cardiomyopathy	(i) Platelet MPs ($CD62P^+$)	Rat model of streptozotocin-(STZ-) induced diabetes and cardiomyopathy		(i) MPs reduced endothelium-dependent relaxation and eNOS expression in carotid arteries	[86]
	(i) Endothelial MPs from apoptotic origin (annexin V^+/$CD31^+$) (ii) Activated endothelial MPs ($CD62E^+$)		Metabolic syndrome patients with chronic heart failure	(i) Patients with chronic heart failure had higher numbers of annexin V^+/$CD31^+$ MPs and lower levels of $CD62E^+$ MPs compared to patients without (ii) Biomarkers of biomechanical stress (NT-proBNP, OPG, and hs-CRP) were independent predictors	[74]

Table 2: Continued.

Complications	Origins of MPs and markers used for detection	Cell and animal models	Humans	Key observations	References
				of the decreased ratio of $CD62E^+$ MPs to annexin $V^+/CD31^+$ MPs	
	(i) Endothelial MPs (annexin $V^+/CD31^+$)		Stable coronary artery disease patients	(i) High levels of endothelial MPs were associated with a high risk of cardiovascular mortality and need for neurovascularization (ii) Endothelial MPs independently predicted future major adverse cardiovascular and cerebral events	[157]
	(i) Platelet MPs ($CD61^+$, $CD61^+/CD62P^+$, $CD61^+$/fibrinogen$^+$, $CD61^+/TF^+$)		Diabetic patients with atherosclerotic disease (e.g., stroke, ischaemic heart disease, or peripheral arterial disease)	(i) Soluble plasma p-selectin was higher in these patients, but no significant correlation was found between the levels of platelet-derived MPs and soluble plasma p-selectin	[158]
	(i) Endothelial MPs ($CD144^+$) (ii) Platelet MPs ($CD41^+$)		Type-2 diabetes patients with coronary noncalcified plaques	(i) Endothelial- and platelet-derived MPs correlated with high levels of hs-CRP (ii) Endothelial MP numbers were higher in the presence of noncalcified cornonary diseased segments	[71]
	(i) Endothelial MPs ($CD144^+$)		Patients with type-2 diabetes and coronary artery disease	(i) Significant association between elevated levels of endothelial MPs and endothelial injury *in vitro* and impaired endothelium-dependent vasodilation *in vivo* (ii) Elevated levels of endothelial MPs were a significant risk factor for coronary artery disease in type-2 diabetes patients	[70]
	(i) Apoptotic endothelial MPs (annexin $V^+/CD31^+$)		Coronary artery disease patients	(i) Elevated MP counts positively correlated with impaired cornonary endothelial-dependent vasodilation in coronary artery disease patients (ii) High levels of endothelial MPs were an independent predictor of the severity of endothelial dysfunction	[60]

Abbreviations: GFR, glomerular filtration rate; hs-CRP: high-sensitive C-reactive protein; ICAM-1, intracellular adhesion molecule-1; NT-proBNP, N-terminal pro b-type natriuretic peptide; OPG, osteoprotegerin; TF, tissue factor; vWF, von Willebrand Factor.

found that MetS MPs injected into healthy mice impaired vasocontractile response in the aorta by stimulating an inflammatory response in the vessel through the overexpression of iNOS increasing thus vascular NO production [15].

More recently, Safiedeen et al. [90] have reported that MPs engineered *in vitro* from apoptotic T lymphocytes or obtained from MetS patients activated the proinflammatory signaling pathway of endoplasmic reticulum (ER) stress, in cultured human aortic endothelial cells (HAECs) and *in vivo* in mouse aortas, which contributed to MP-induced endothelial dysfunction [90]. Protein synthesis and key post-translational modifications occur inside the ER. In situations associated with an increased demand on protein synthesis such as obesity or diabetes, there is an overload on the ER leading to the accumulation of misfolded and/or unfolded proteins within the ER lumen that results in the activation of the unfolded protein response (UPR) with the primary goal to improve ER homeostasis and clear the protein load that has accumulated to improve cell viability and survival [91, 92]. However, when the activation of the UPR pathway prolongs and becomes chronically switched on, this can lead to a situation of "ER stress," which is associated with the activation of adverse cellular responses that may lead to cell death [91, 92]. Several reports linked ER stress response activation to the onset of insulin resistance in obesity and diabetes [93, 94]. ER stress is also tightly associated with the activation of major inflammatory intracellular signaling response such as nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK). The extreme activation of ER stress may cause apoptosis [91, 92, 95, 96].

Endothelial cells undergoing apoptosis are dysfunctional, more procoagulant, and prone to adhere to platelets [97]. ER stress-mediated apoptosis involves, at least, three apoptotic subpathways: the first is through the activation of proapoptotic transcription factor C/EBP homologous protein (CHOP), the second is controlled by the activation of JNK signaling response, and the third is due to the activation of ER-associated caspases 3 and 12 (only found in rodents) [92, 95, 96]. ER stress response was shown to be activated in endothelial cells lining vascular areas with the highest susceptibility to develop atherosclerotic plaques and hence indicate an involvement of this pathway in the development of endothelial dysfunction [98].

Recently, it has been shown that high glucose disturbed NO signaling and impaired angiogenic capacity of cultured HUVECs through the activation of ER stress response [99]. Interestingly, Safiedeen et al. [90] reported that the exposure of HAECs to MetS or apoptotic T lymphocyte-derived MPs caused the activation of serval key ER stress markers, including the nuclear translocation of activating transcription factor (ATF)-6, and reduced endothelial NO production [90]. All these effects were interestingly prevented by the pretreatment of cells with a chemical chaperone, tauroursodeoxycholic acid (TUDCA), to improve the native functions of the ER and improve protein folding [90]. The intravenous injection of MetS or apoptotic T lymphocyte-derived MPs into mice impaired endothelium-mediated vasodilation in the aorta; however, the preinjection of animals with TUDCA prevented this deleterious effect on vascular function. The authors also found that the effects of MetS and apoptotic T lymphocyte-derived MPs on the activation of ER stress in HAECs involved two membrane receptor interactions, Fas/-FasL and low-density lipoprotein receptor (LDL-R), that were both been previously found to be implicated in the vascular effects of MetS or apoptotic T lymphocytes-derived MPs [15, 89, 100]. Furthermore, MetS and apoptotic T lymphocyte-derived MPs increased the protein expression of the neutral sphingomyelinase (SMase) that was found to contribute to the activation of ER stress response [101]. The neutralization of FasL expressed at the surface of MPs or the blockade of the LDL-R expressed at the surface of endothelial cells normalized the levels of protein of expression of SMase. Furthermore, the blockade of SMase action reversed the actions of MPs on NO signaling and ER stress response activation, suggesting that the effects of MPs are mediated through neutral SMase [90].

Altogether, these studies highlight the complex interactions of MPs from insulin resistant or diabetic patients with cells of the vessel wall to induce vascular dysfunction which eventually contributes to the development of comorbidities associated with these metabolic disorders. The deeper understanding of the molecular mechanisms involved will pave the way for the identification of novel therapeutic targets to reduce the burden of these cardiometabolic disturbances on the quality of patients' lives.

7.2. MPs and Ischemic Diseases. MPs are potential vectors of biological messages between cells and were found to play key roles in regulating angiogenesis in ischemic conditions. Numerous studies have demonstrated that human T lymphocyte-derived MPs carrying the morphogen Shh were able to regulate the proangiogenic activity of endothelial cells *in vitro* through the regulation of the expression of proangiogenic genes [82, 83]. Importantly, the proangiogenic effects of these MPs were further confirmed *in vivo* using mouse models of hindlimb ischemia. The authors found that the treatment of the ischemic mouse with MPs bearing Shh enhanced the postischemic reparative neovascularization by regulating NO pathway and modulating angiogenesis-related gene expression in these models [102]. In another study using an *in vivo* mouse model of angiogenesis, Benameur et al. [103] observed that MPs carrying peroxisome proliferator-activated receptor (PPAR)-α play a stimulatory role on neovascularization mediated by EPCs originating from the bone marrow. The MP-induced angiogenic effects were essentially mediated by the improvement of EPC differentiation and the enhancement of the proangiogenic activity of endothelial cells [103].

Diabetes is associated with a high risk of ischemic diseases and other associated complications as discussed in this review. Given the increasing clinical significance of MPs in various pathologies and their important biological functions as well as the importance of other cell-derived molecular partners, a growing number of studies have started investigating the contribution of MP-derived miRNA transfer in modulating the pathogenesis of many diseases. MPs harboring miRNA have been reported in multiple diseases including cancer and cardiovascular disorders [104]. It was observed

that the expression of endothelial-specific miRNA molecules in MPs shed from endothelial cells was significantly altered in diabetes mellitus with stable coronary artery diseases. In addition, endothelial-derived MPs bearing miR-126 were shown to promote vascular endothelial repair through the transfer of miRNA to the target cells. This effect was abolished under hyperglycemic conditions [105]. In another translational approach, it was further confirmed that diabetes mellitus correlated with impaired expression of not only endothelial miR-126 but also miR-26 in circulating MPs when compared to patients without diabetes mellitus. These results underscore the potential implication of endothelial-derived MPs in modulating vascular biology and consequently leading to diabetes mellitus vasculopathy [50]. Altogether, these findings support that diabetics with lower levels of miR-126 and miR-26 can be at a higher risk of concomitant coronary artery disease.

Consistent with previous findings published by Caporali et al. [106], a more recent research has provided a new level of understanding of the contribution of MPs to the development of diabetes-mediated vascular complications, with particular emphasis on the interaction between endothelial cells and pericytes in a rodent model of hindlimb ischemia. Results demonstrated that MPs secreted from diabetic endothelial cells and carrying miR-503 were subsequently delivered to neighboring pericytes to modify the permeability of vessels and alter the postischemic angiogenic process in limb muscles via the regulation of genes under the control of miR-503, such as VEGFA and EFNB2 (Ephrin B2) [107].

It has also been observed that *db*/*db* mice, a genetic rodent model of type-2 diabetes, exhibited elevated plasma MPs originating from endothelial cells compared to wild-type mice and that these MPs caused a reduction in cerebral microvascular density [108]. The authors found that MPs collected from *db*/*db* animals also impaired the properties of EPCs as evidenced by the failure of EPCs (obtained from control mice) prestimulated with MPs collected from *db*/*db* to improve ischemic damage following their administration into diabetic mice [108].

Because of the importance of tissue factor (TF) in regulating angiogenesis, the crucial role of MPs derived from microvascular endothelium that bear TF was demonstrated in enhancing collateral flow and capillary formation in a murine hindlimb ischemia model [109, 110]. TF-bearing microvascular endothelial-derived MPs accelerated the angiogenic process through a paracrine control of adjacent endothelial cells, via a mechanism implicating the β1-integrin pathway, Rac1-ERK1/2-ETS1 to form new and functional mature vessels. Furthermore, Edrissi et al. [111] reported that MPs shed during cerebral ischemia enhanced the permeability of endothelial cell layer. This increase in endothelial permeability was, at least, mediated by an accelerated apoptosis induced by the transfer of the activated TNFα pathway molecules, caspase 3, and Rho kinase delivered by MPs to the target cells [111]. Tsimerman et al. [72] also observed that MPs obtained from patients suffering from diabetes enhanced the procoagulant activity of endothelial cells. The prothrombotic activity was the highest in endothelial cells stimulated by MPs collected from diabetic patients with severe diabetic foot ulcers, suggesting a role for these MPs in thrombotic complications associated with diabetes [72].

These findings open up intriguing new therapeutic strategies based on the use of MPs as potential novel therapeutic tools in the treatment of critical limb ischemia and other ischemic diseases. Taking into account the different properties of MPs, much evidence supports the concept of expanding the MPs' use as efficient therapeutic tools in altered angiogenesis associated with ischemic diseases [112].

7.3. MPs and Myocardial Infarction. Diabetic patients are at a high risk of developing acute coronary syndromes (ACS). Due to their procoagulant and proinflammatory potential, MPs were found to contribute to the formation of thrombus and the progression of atherosclerotic disease. High circulating levels of MPs have been observed in ACS [113, 114]. ACS include unstable angina, non-ST segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) in addition to sudden death. ACS are frequently characterized by the buildup of vulnerable atherosclerotic plaques in coronary arteries and manifest following the plaque rupture and the occlusion of arteries by the formed thrombi [115].

Several studies reported high circulating levels of MPs from various cell origins in STEMI patients and found that their levels reflected systemic inflammation and cell injury and activation in addition to correlating with the size of ischemic cardiac tissue [116–120]. For instance, Porto et al. [116] observed that STEMI patients had higher levels of platelet- and endothelial-derived MPs in intracoronary blood compared to aortic blood and that these MPs correlated positively with thrombus scores [116]. Furthermore, it has been reported that the numbers of circulating MPs continued to increase in the short term following appropriate intervention in STEMI patients. Zhou et al. [117] followed up circulating levels of MPs over time in STEMI patients undergoing percutaneous transluminal coronary intervention (PCI) (before and up to 48 h after PCI). The authors found that platelet MPs increased directly after the PCI and continued to rise over time until the end of follow-up period, whereas the levels of leukocyte- and endothelial-derived MPs decreased after the PCI, but rose thereafter until the end of the follow-up period [117].

Recently, Chiva-Blanch et al. [118] compared the profile of MP expression between STEMI and NSTEMI patients. The authors found that STEMI patients had higher numbers of platelet-derived MPs compared to NSTEMI in spite of receiving a dual antiplatelet treatment, indicating that the process of platelet activation may be more implicated in the pathogenesis of STEMI. STEMI patients exhibited a higher plasma TF procoagulant activity compared to NSTEMI patients, which positively correlated with the number of MPs originating from platelets, monocytes, and those harboring TF [118].

However, MPs are not always playing a deleterious role in cardiac ischemic disease. The process of remote ischemic conditioning (RIC) is a good example for this. RIC consists of subjecting a remote area of heart muscle to brief reversible cycles of ischemia and reperfusion, conferring a general

protection to distant tissues and organs, which become resistant to injury caused by prolonged episodes of ischemia and reperfusion [121]. Despite the fact that effector pathways of RIC were described in the literature, the propagation patterns of cardioprotective signals between organs remain unclear [122]. Interestingly, it was shown that the release of MPs from the heart increased following a preconditioning procedure and these MPs were found to contribute to the forwarding of protective remote conditioning signals. This suggests that MPs exert a protective effect in ischemic heart diseases [123]. Very recently, Bueno-Betí et al. [33] reported that MPs engineered *in vitro* and harboring the morphogen Shh restored the vasculogenic properties of EPCs obtained from patients suffering from myocardial infarction to the levels of healthy volunteers [33].

7.4. MPs and Ischemic Stroke. Diabetes mellitus is recognized as an important risk factor for stroke and is associated with increased incidence of ischemic stroke at all ages [124, 125]; however, the chronic influence of stroke on cell activation and dysfunction processes remains poorly understood. Circulating levels of neural precursor cell (NCP)-derived MPs ($CD56^+/CD34^+$/annexin V^+) were found to be higher in stroke patients in comparison to high cardiovascular risk controls. This increase was chronically maintained when compared to the levels measured at the onset of attacks in patients with lower lesion volumes. However, a decreased level of NCP-derived MPs and increased levels of smooth muscle cell-derived MPs were observed in patients with higher volume of cerebral lesions. These data suggest that NCP-derived MPs can reflect an ongoing repair of the damaged brain tissue in patients and can be considered as a potential biomarker of stroke.

The specific underlying pathophysiological mechanisms of the involvement of MPs in stroke need further investigations [126]. MPs harboring TF (TF^+-MPs) are produced in various pathological conditions including atherosclerosis, cancer, acute coronary syndrome, and diabetes where they can trigger thrombosis cascade [127]. It is well documented that the presence of TF on MPs markedly increased their procoagulant activity [128]. In a study of acute ischemic stroke, 26% of stroke patients had diabetes; the authors reported significantly higher plasma numbers of TF^+-MPs and an elevated circulating concentration of TF pathway inhibitor (TFPI) in stroke patients compared to healthy controls. Moreover, one week following diagnosis, the activity of MPs was more elevated in stroke patients not treated with a tissue plasminogen activator (Alteplase) compared to their activity at the stroke attacks onset [129].

7.5. MPs and Diabetic Cardiomyopathy. Understanding of the underlying pathogenic mechanisms of diabetic cardiomyopathy is necessary for the early detection and control of this major cardiovascular complication of diabetes [130]. At early stages, diabetic cardiomyopathy is often unrecognized due to the lack of pathognomonic features. Indeed, these early stages are most often nonsymptomatic and only molecular and cellular alterations are taking place. Moreover, the insignificant alteration in the myocardial structure and function make the subclinical detection of diabetic cardiomyopathy extremely challenging in clinical practice. In addition, the subclinical state of the disease becomes more critical after an episode of ischemia. This will reduce the possibility to restore the heart function to normal level.

Interestingly, previous investigations provided evidence that miRNAs were found in human biofluids and considered as a novel potential category of biomarkers for type-2 diabetes [131] and suggested utilization of miRNA as promising biomarkers for the early diagnosis of subclinical diabetic cardiomyopathy [132, 133]. Promising preclinical studies suggested that the concept of miRNA and their specific antagonists could contribute to a better therapeutic control of heart failure to dyslipidemia, for example, in spite of the specificity and targeting challenges [134]. The potential of MP-contained miRNAs as a prominent biomarker for type-2 diabetes can be implicitly understood from the key roles that miRNAs play in the control of metabolic and cardiovascular pathophysiological processes [135].

The early detection of subclinical cardiomyopathy in diabetic patients using extracellular vesicles as biomarkers could be a future alternative of antidiabetic treatment. This can also serve as an early predictor of the diabetic myocardiopathy in patients with a high risk of diabetes-associated complications. Altogether, these new research streams highlight the crucial and emerging role of extracellular vesicles and their cargo in understanding cardiovascular physiology and the pathogenesis of cardiovascular disease associated with diabetes.

7.6. MPs and Diabetic Retinopathy. Diabetic retinopathy is another major, but common, complication of diabetes, which affects the microvasculature. It is an important contributor to vision loss and blindness in diabetic patients. Small vessels in the eye have pericytes that control the capillary tone, generate new capillaries, and protect the vessels against noxious molecules. Uncontrolled diabetes leads to the loss of pericytes and degradation of the blood retinal barrier which results in increased permeability and proliferation of endothelial cells [136].

Because of the association of diabetic retinopathy with progressive retinal capillary activation and proliferation, elevated vitreous levels of MPs from different cellular lineages were detected in patients with proliferative diabetic retinopathy. These MPs were especially of platelet, endothelial, and retinal origins. These MPs were able to stimulate cell migration and initiation of vessel formation, the key features of angiogeneis, in an *in vivo* Matrigel plug assay [137]. The levels of endothelial- and platelet-derived MPs were dramatically decreased in the vitreous body following a panretinal laser photocoagulation and after intravitreal treatment directed against vascular endothelial growth factor (VEGF), respectively [137]. These findings demonstrate to what extent MPs participate in the progression of proliferative diabetic retinopathy and highlight the role of MPs as indicators of therapy effectiveness and as efficient specific biomarkers for early diagnosis of retinal disturbances.

Different MP populations can exert different effects on the pathological neovascularization seen in diabetic

retinopathy. For example, Tahiri et al. [138] have shown that lymphocyte-derived MPs (LMPs) suppressed laser-induced choroidal neovascularization (CNV). Given the fact that macrophages carry proangiogenic properties in CNV, LMPs were able to modulate the angiogenic properties of macrophages, suggesting that LMPs can be considered potent therapeutic antiangiogenic factors in pathological choroidal neovascularization [138]. Furthermore, extracellular vesicles released from mesenchymal stem cells that were treated with high glucose increased angiogenesis and retinal neovascularization *in vitro* by promoting the dissociation of pericytes from adjacent endothelial cells which results in uncontrolled proliferation of endothelial cells, contributing thus to proliferative diabetic retinopathy [139].

7.7. MPs and Diabetic Nephropathy. Diabetic nephropathy is characterized by the loss of renal podocytes. Podocytes are crucial for maintaining the proper permeability of the glomerular filtration barrier. Upon podocyte injury and loss, the glomerular filtration barrier's permeability is increased which results in the permeation of proteins such as albumin to the urine, a clinical manifestation of diabetic nephropathy. Since the loss of podocytes is irreversible, there is a need for a marker that can detect early cellular injury to podocytes which will allow for early intervention before clinical manifestation of symptoms. Burger et al. [140] indicated that podocytes can shed MPs into urine and that these MPs are significantly elevated in diabetic nephropathy in *in vitro* and *in vivo* models, even before the presence of albuminuria [140]. Similarly, Lytvyn et al. [141] found that patients with uncomplicated type-1 diabetes had significantly elevated numbers of podocyte-derived MPs in comparison to healthy volunteers [141]. It is clear that shedding of MPs from podocytes happens before the onset of diabetic nephropathy which supports their use as a diagnostic marker of renal injury.

Diabetic nephropathy is also an important risk factor for cardiovascular complications in diabetic patients. Endothelial dysfunction is believed to develop at the early stages of diabetic nephropathy and cardiovascular disorders. Accumulating evidence is demonstrating that certain MP populations that are significantly correlated with impaired vascular function are elevated in chromic kidney disease (CKD) and end-stage renal failure patients such as MPs from platelets and endothelial cells [142–144]. Endothelial MPs ($CD144^+$ and $CD31^+/CD41^-$) strongly correlated with impaired vascular function *in vivo* and impaired relaxation in aortic rings in response to acetylcholine *in vitro* [143]. Lu et al. [142] also found that platelet and endothelial MPs positively correlated with markers of endothelial injury and platelet activation (von Willebrand factor and p-selectin, respectively) in patients with CKD [142]. Previously, Dursun et al. [145] studied the association between endothelial MPs ($CD144^+$), arterial stiffness, and atherosclerosis in children suffering from CKD. The authors reported that the numbers of plasma endothelial MPs were higher in dialysis patients in comparison to predialysis patients and healthy volunteers. Furthermore, it was found that endothelial MPs positively correlated with blood pressure, C-reactive protein (CRP), parathyroid hormone, and disease duration, while they correlated negatively with glomerular filtration rate (GFR) and albumin levels [145]. Therefore, we can understand that MPs are active players in endothelial dysfunction that can later progress to cardiovascular complications and are not only biomarkers of endothelial cell activation and injury.

MPs themselves can be used to monitor response to treatments. The antidiabetic agent teneligliptin and the lipid-lowering agent simvastatin were tested for their effect in protecting against endothelial dysfunction in patients with diabetic nephropathy to prevent the progression to cardiovascular disease seen in this population of patients. Since MPs are markers of and contributors to endothelial dysfunction, as discussed earlier, they were measured to monitor the protective effects of these medications. Teneligliptin strongly reduced the levels of plasma platelet-derived MPs and the plasminogen activator inhibitor, a hallmark of platelet activation, in diabetic patients receiving hemodialysis suggesting its protective effects in this population [146]. Likewise, the treatment of diabetic patients suffering from CKD with simvastatin resulted in a significant reduction of procoagulant MPs (PS positive), platelet MPs, and monocyte MPs, suggesting its beneficial effect in ameliorating endothelial dysfunction [147].

Altogether, MPs can serve as a diagnostic marker because they are noninvasive, predict early pathological changes in the endothelium, and can predict the risk of other diabetic complications. MPs can also be used for monitoring treatment effects. Finally, therapeutic agents can be developed to target MPs as they appear to have a role in the pathophysiology of diabetic nephropathy and other complications.

8. Future Directions

Multiple clinical and preclinical investigations assessed the impact of treatments of diabetes and its complications on plasma levels of MPs. Esposito et al. [148] evaluated the actions of two oral antidiabetics, pioglitazone and metformin, on the numbers of plasma MPs originating from endothelial cells and EPCs in newly diagnosed type-2 diabetes patients. This study found that following 24 weeks of treatment with pioglitazone, patients' plasma exhibited a decrease in endothelial MPs while the numbers of EPC-shed MPs increased [148]. Furthermore, type-2 diabetes patients who received miglitol, an oral antidiabetic drug, for a period of 4 months, had lower levels of MPs of platelet origin compared to controls [149]. Shimazu et al. [150] reported earlier that another orally available antidiabetic treatment, acarbose, also reduced circulating numbers of platelet-shed MPs in type-2 diabetes patients [150]. Similarly, teneligliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, significantly reduced plasma levels of platelet-shed MPs and plasminogen activator inhibitor in type-2 diabetes patients receiving hemodialysis [146]. The use of statins was also reported to affect the circulating levels of MPs patients with diabetes. Almquist et al. reported that simvastatin significantly reduced total PS-positive MPs, platelet-derived MPs, and monocyte-derived MPs in patients with diabetes and CKD [147].

Approaches to manage body weight were also observed to modify circulating levels of MPs from select cellular origins. Morel et al. [151] reported that a very low-calorie diet for 90 days caused a reduction in body weight in obese women and lowered plasma levels of MPs from different origins including procoagulant, leukocyte, and lymphocyte MPs. Furthermore, the diet improved several cardiac and metabolic vitals such as leptin levels and blood pressure [151]. Moreover, endothelial-derived MP levels were found to be reduced following a high-intensity exercise in overweight inactive participants [152].

Treatments based on the use of natural nanoparticles were also found to be effective in reducing the number of plasma MPs. For instance, Garavelo et al. [153] reported recently that the treatment of a rabbit model of atherosclerosis, fed a high-cholesterol diet, with natural nanoparticles obtained from medicinal plants (PTC) combined with trans-Sialidase (TS) for 6 weeks, reduced the number of total MPs in addition to those positive for *Mycoplasma pneumoniae* and oxidized LDL. Furthermore, the treatment of animals with PTC and TS reduced atherosclerotic plaque area and caused a positive remodeling of ascending aortic segment [153]. These findings highlight the potential of nanoparticle-based therapies in modulating the circulating levels of MPs.

Since MPs are implicated in the onset and progression of cardiovascular pathologies and diabetes-induced complications, they can be targeted therapeutically by many strategies. One strategy is controlling their release to reduce their levels; however, currently, there is a need to understand the mechanisms involved in the production and shedding of MPs to better and specifically target them. Another strategy could be the inhibition of their uptake by modulating specific surface lipids of MPs to prevent deleterious messages from reaching the recipient cells or inhibiting MP's surface ligands that interact with cell surface receptors, in order to prevent cell signaling. Finally, the modulation of the cargo content of the MPs to control the biological messages that they carry can be another strategy.

Disclosure

The statements made herein are solely the responsibility of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

AA and TB wrote the manuscript. AO, AP, AAA, and SM contributed to writing selected sections and critically revised the manuscript. AO developed the figure. AA coordinated the writing-up and the submission process. All authors approved the final version for submission.

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