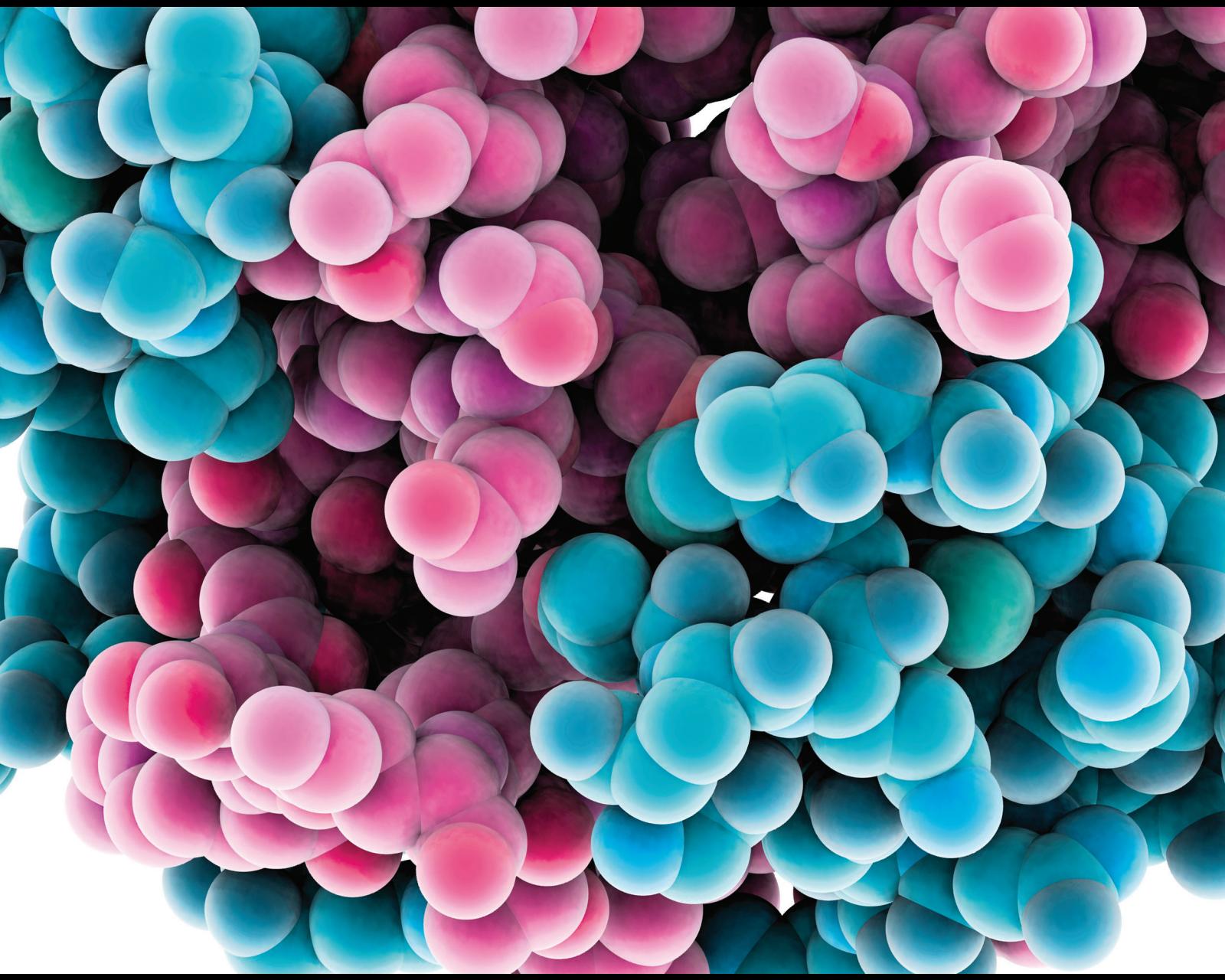
Journal of Diabetes Research

Diabetic Endothelium Dysfunction, Cardiovascular Complications, and Therapeutics

Guest Editors: Yunzhou Dong, Yong Wu, Hyoung Chul Choi, and Shuangxi Wang



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Editorial Board

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Editorial

Diabetic Endothelium Dysfunction, Cardiovascular Complications, and Therapeutics

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Diabetes affects the life quality of a number of people largely through the cardiovascular complications [1]. Vascular endothelial cells play a major role in maintaining vascular homeostasis [2]. Dysfunction of the vascular endothelium is a critical factor in the pathogenesis of diabetic micro- and macrovascular diseases [2, 3]. The fundamental mechanism contributing to vascular disease, nephropathy, retinopathy, and neuropathy has yet to translate into effective therapeutics [4]. Uncovering novel mechanism governing endothelium dysfunction, new concepts about biological pathways involved in diabetic tissue injury, and identification of new therapeutics are of significance [5]. Circulating endothelial progenitor cells (EPCs) in diabetes are reduced and dysfunctional [6–8], suggesting EPC as a biomarker for diabetes and a prospective target for regenerative medicine [9].

Although significant strides have been made, the molecular mechanism of diabetic complications in vascular diseases and the effective treatment remain largely unknown. In this special issue, investigators have identified novel mechanism and therapeutics from their original research or review articles on the role of endothelial dysfunction in the etiology and pathogenesis of the micro- and macrovascular complications of diabetes, as well as therapeutic practice. Among the top gear, diabetes badly affects the function of retina and kidney. X. Cai and J. F. McGinnis systemically reviewed the mechanism of neovascularization, endoplasmic reticulum stress, inflammation, and aberrant angiogenesis in the pathogenesis of diabetic retinopathy and proposed some novel ideas for the DR treatment such as nanoceria, stem cells, miRNAs, and CRISPR/Cas9 technology. P. Li et al. demonstrated that inhibition of Na^+/H^+ exchanger 1 attenuates renal dysfunction by AGEs in rats, likely by the reduction of oxidative stress. In (pre)clinic studies, several groups have obtained promising outcomes in animal models or patients. W. Yu et al. suggested that Curcumin can improve cardiomyocyte function by inhibiting oxidative stress and apoptosis via the activation of Akt pathway; Z. Liu et al. proved that vitamin B6 prevents endothelial dysfunction, insulin resistance, and hepatic lipid accumulation in *ApoE*-null mouse model fed with high fat diet; R.-M. Cazeau et al. revealed that vitamin C and E administration can improve endothelium function in type 1 diabetic adolescents, while P. Yun et al. wrote an article showing that long-term administration of acarbose can effectively reduce the risk of the incidence of major adverse cardiovascular events in acute coronary syndrome patients, and possibly by the improvement of endothelium function. Further, S. Ghosal and B. Siniha reevaluated the use of Gliptins in cardiovascular patients in clinics, and M. Jamiolkowska et al. suggested that real-time continuous glucose monitoring (RT-CGM) may help in the detection of glycaemic variability, a newly recognized cardiovascular risk factor in adolescent type 1 patients.

As reported in recent years, endothelial progenitor cells (EPCs) are reduced in circulation [8, 9]; therefore, EPC homeostasis is critical in endothelial regeneration after injury. H.-Y. Tsai et al. demonstrated that Coenzyme Q10 can

improve EPC function through AMP-kinase activation in diabetic condition, suggesting several benefits of Coenzyme Q10 for diabetic patients with cardiovascular complications such as atherosclerosis and hypertension.

Taken together, the articles in this special issue could further help researchers to understand the complexity of the diabetic complications in cardiovascular system and provide some new ideas to fight against diabetic cardiovascular complications.

Yunzhou Dong
Yong Wu
Hyoung Chul Choi
Shuangxi Wang

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Research Article

Curcumin Protects Neonatal Rat Cardiomyocytes against High Glucose-Induced Apoptosis via PI3K/Akt Signalling Pathway

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The function of curcumin on NADPH oxidase-related ROS production and cardiac apoptosis, together with the modulation of protein signalling pathways, was investigated in cardiomyocytes. Primary cultures of neonatal rat cardiomyocytes were exposed to 30 mmol/L high glucose with or without curcumin. Cell viability, apoptosis, superoxide formation, the expression of NADPH oxidase subunits, and potential regulatory molecules, Akt and GSK-3β, were assessed in cardiomyocytes. Cardiomyocytes exposure to high glucose led to an increase in both cell apoptosis and intracellular ROS levels, which were strongly prevented by curcumin treatment (10 μM). In addition, treatment with curcumin remarkably suppressed the increased activity of Rac1, as well as the enhanced expression of $gp91^{phox}$ and $p47^{phox}$ induced by high glucose. Lipid peroxidation and SOD were reversed in the presence of curcumin. Furthermore, curcumin treatment markedly inhibited the reduced Bcl-2/Bax ratio elicited by high glucose exposure. Moreover, curcumin significantly increased Akt and GSK-3β phosphorylation in cardiomyocytes treated with high glucose. In addition, LY294002 blocked the effects of curcumin on cardiomyocytes exposure to high glucose. In conclusion, these results demonstrated that curcumin attenuated high glucose-induced cardiomyocyte apoptosis by inhibiting NADPH-mediated oxidative stress and this protective effect is most likely mediated by PI3K/Akt-related signalling pathway.

1. Introduction

Diabetes mellitus (DM) is becoming a global health problem that is afflicting millions of people. According to the investigation conducted by the International Diabetes Federation (IDF), the incidence of DM is rapidly increasing and the total number of people with DM will reach 592 million in 2035 [1]. Studies have indicated that diabetic people have a 2- to 5-fold increased risk of developing heart failure [2] and that more than 50%–80% of diabetic patients die from diabetic cardiovascular complications [3]. Diabetic cardiomyopathy (DCM), as a major complication of DM, was initially proposed by Rubler in 1972 [4]. DCM is characterized by structural and functional cardiac disorder occurring independently of coronary artery disease and hypertension [5]. Although many research studies have attempted to elucidate its underlying mechanisms, the aetiology of DCM has never been directly determined. Numerous studies utilizing experimental animal models and clinical diabetes patients reported that diabetes enhances cardiomyocyte apoptosis not only simply in animals but also in patients [6, 7]. Thus, cell death by apoptosis likely plays an important role in triggering the pathogenic changes in DCM [8]. Cardiomyocyte apoptosis can cause a loss of cardiac contractile muscle tissue, which eventually leads to left ventricular remodeling [9].

Both type 1 and type 2 DM are associated with long-standing hyperglycemia. Chronic hyperglycemia has been shown to directly participate in the pathogenesis of DM-induced cardiac injury by promoting excessive oxidative stress in the heart [10], which increases cardiomyocyte apoptosis in both human and experimental DCM. Overproduction of reactive oxygen species (ROS) and a diminished antioxidant

defence system are linked to enhanced oxidative stress in the heart in DM. Consequently, if the balance between ROS generation and ROS scavenging systems is broken, superoxide accumulates and results in cellular damage or dysfunction. Given the injurious effects of ROS in DCM, increasing attention has been placed on the administration of antioxidant agents as a compensatory therapeutic approach in DCM [11].

Curcumin, a major constituent derived from the root of *Curcuma longa*, has been used as a spice and food additive in India since ancient times. Today, interest in curcumin has grown rapidly due to its diverse array of biological and pharmacological activities, and it was shown to have the potential to treat inflammatory and cardiovascular diseases and cancer [12]. Curcumin has antioxidant properties that are responsible for its cardioprotective effect by enhancing antioxidant defences and eradicating ROS [13]. Additional studies have indicated that C66, a curcumin analogue, has a protective role against high glucose-induced cardiac damage via inactivation of the JNK pathway [14]. Our early stage study demonstrated that curcumin reduces cardiomyocyte remodeling and improves cardiac dysfunction by inhibiting inappropriate apoptosis in diabetic rats [15], but the mechanism through which curcumin inhibits cardiomyocyte apoptosis and oxidative stress remains unknown.

Therefore, this study was performed to determine the action of curcumin against high glucose-induced cardiac injury and elucidate the molecular mechanism of cardiomyocyte protection by exposing primary neonatal rat cardiomyocytes to a high concentration of glucose.

2. Materials and Methods

2.1. Animals. One- to three-day-old Sprague-Dawley rats were obtained from the experimental animal centre at Hubei University of Science and Technology. The Committee of Experimental Animals of Hubei University of Science and Technology approved this study. All animals used in this study were cared for and experimented on in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.2. Primary Culture of Neonatal Rat Cardiomyocytes and Treatment. Neonatal rat cardiomyocytes were isolated as previously described with slight modifications [16]. The cardiomyocytes were cultured in DMEM containing 10% FBS (Gibco Life of Cells, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified air containing 5% CO_2 at 37°C. When the cardiomyocytes reached 70%–80% confluence, the cells were randomized into the experimental groups: 5.5 mmol/L D-glucose as the normal (NG) group, 30 mmol/L D-glucose (Sigma, USA) as the high glucose (HG) group, or identical concentrations of mannitol as an osmotic control group containing 5.5 mmol/L D-glucose plus 24.5 mmol/L mannitol for 24 h in the presence or absence of curcumin (10 μmol/L). A subset of cardiomyocytes were exposed to LY294002 for 1 h before administration of high glucose and curcumin.

2.3. Assessment of Cell Viability. Cell viability was assessed with a Cell Counting Kit-8 assay kit (CCK-8, Dojindo Molecular Technologies, Japan) in 96-well plates following the instructions from the manufacturer.

2.4. Analysis of Biochemical Parameters. Lactate dehydrogenase (LDH) and aspartate amino transferase (AST) released into the culture medium as well as the malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in cells were determined using the associated enzyme activity assay kits (Nanjing Jiancheng Bioengineering Research Institute, China).

2.5. Intracellular ROS Measurement. Intracellular superoxide anions were examined using the fluorescence probe dihydroethidium (DHE). Cardiomyocytes were cultured in a dark chamber at 37°C for 30 min after the application of 10 μmol/L DHE (Life Technology, USA) and were washed twice with PBS. Images of the cardiomyocytes were captured and analysed immediately under an inverted fluorescence microscopy (Olympus IX71, Japan).

Intracellular ROS accumulation was assessed by DCFH-DA staining (Beyotime, China). Cultured cells were incubated in DMEM with 10 μmol/L DCFH-DA at 37°C for 30 min. ROS production was detected by a Bio-Tek fluorometric imaging plate reader (excitation at 485 nm and emission at 528 nm).

Furthermore, intracellular ROS was also measured by high performance liquid chromatography (HPLC) (SHIMADZU, LC-20AD, Japan) using a DHE fluorescent probe as previously described [17]. Briefly, cardiomyocytes were treated with DHE (10 μmol/L) for 30 min and then incubated with 0.1% Triton X-100 dissolved in PBS to permeabilize the cell membrane. Protein determination was performed using a Bicinchoninic Acid (BCA) protein assay kit (Beyotime, China). Then, 100 μL of cell lysate was added to an equal volume of 0.2 mol/L $HClO_4$ in methanol, and the mixture was put on ice for 2 h to precipitate the proteins. Afterwards, the resulting mixture was centrifuged at 20,000 g at 4°C for 30 min. Then the supernatant was collected and neutralized with 1 mol/L potassium phosphate buffer (pH 2.6). The supernatant was spun again for 15 min and subjected to the HPLC analysis (excitation at 490 nm and emission at 596 nm).

2.6. TUNEL Assay. Apoptotic cardiomyocytes were detected using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay kit obtained from Roche Applied Science. Briefly, 4% paraformaldehyde and 0.1% Triton X-100 were used to fix and permeabilize the cardiomyocytes on glass slides. After rinsing the cardiomyocytes with PBS, they were added and incubated with the TUNEL reagents according to the instructions from the manufacturer. Images were captured under a fluorescence microscope (Olympus BX53, Japan) and the proportion of TUNEL-positive cells was estimated using the following formula: TUNEL-positive cardiomyocytes/total number of cardiomyocytes × 100%.

2.7. Flow Cytometry. Flow cytometry was performed using an Annexin V-FITC Apoptosis Detection Kit (Best Bio, China) following the manufacturer's protocol. Briefly, after being treated with the appropriate drugs, the cardiomyocytes were harvested with trypsin and washed with cold PBS. Afterwards, the collected cardiomyocytes were isolated by centrifugation, resuspended in 500 μL of 1x binding buffer, and treated with 5 μL Annexin V-FITC and 5 μL PI for 15 min at 4°C in the dark. Apoptotic cardiomyocytes were detected by flow cytometer (Becton Dickinson, USA).

2.8. Determination of Rac1 Activity. Rac1 activity was determined using a Rac1 Activation Assay Kit (Millipore, USA) following the instructions from the manufacturer. Cultured cardiomyocytes were homogenized with 1x MLB. Rac-GTP was immunoprecipitated using PAK1-PBD agarose beads coated with an anti-active Rac-GTP mouse monoclonal antibody. Finally, the extracts were analysed by western blot.

2.9. Western Blot Analysis. Cardiomyocytes were lysed with 1x RIPA lysis buffer (Cell Signalling Technology, USA). After centrifugation, the lysates were clarified, and the supernatants fractions were isolated. Protein concentrations in cells were defined by the BCA protein assay. Approximately 30–50 μg of protein was loaded and separated by SDS-PAGE gels and then transferred to a PVDF membrane. After blocking the membrane with 5% nonfat milk, the following primary antibodies were used for western blot: Bcl-2, Bax, Akt, GSK-3β, phospho-Akt (Ser473), phospho-GSK-3β (Ser9) (Cell Signalling Technology, USA), gp91^{phox}, p47^{phox}, and β-actin (Santa Cruz Biotechnology, USA). Then the membrane was probed with appropriate secondary antibodies. Finally, the blots were visualized using a chemiluminescence system (Pierce Biosciences, USA). Image analysis software (Gene-Tools from SynGene) was used to quantify the immunoblots.

2.10. Statistics. The values are expressed as mean ± SD from repeated experiments. Statistical analysis was performed using ANOVA, and a P value of <0.05 was considered to indicate a significant difference for all the values.

3. Results

3.1. Curcumin Inhibited High Glucose-Induced Cardiomyocytes Injury. To ascertain the role of curcumin in cell survival, we examined the viability of primary cultured neonatal rat cardiomyocytes incubated with different doses of curcumin for 24 h using a CCK-8 assay. As presented in Figure 1(a), compared with the NG group, cell viability was markedly decreased at the high glucose concentration of 30 mmol/L, and mannitol (30 mmol/L) employed as an osmotic control agent did not mimic the effects of 30 mmol/L D-glucose. In the cardiomyocytes exposed to high glucose, curcumin treatment increased cell viability in a dose-dependent manner.

LDH and AST are oxidoreductase enzymes that are present in the cytosol of animals and plants. They are highly stable enzymes that can be used to evaluate tissue and cell damage. As shown in Figures 1(b) and 1(c), the amounts of LDH and AST released by the cardiomyocytes were much higher in the HG group than those in the NG group. Interestingly, the cardiomyocytes exposed to high glucose and treated with curcumin released significantly lower amounts of LDH and AST into the medium than the cardiomyocytes undergoing high glucose alone.

3.2. Curcumin Abrogated High Glucose-Induced Cardiomyocytes Apoptosis. Apoptotic cardiomyocytes were detected by both TUNEL staining and flow cytometry. The TUNEL assay showed few apoptotic cardiomyocytes in the NG group and a greater number of apoptotic cardiomyocytes in the HG group. However, cotreatment with high glucose and curcumin (10 μM) abrogated the increase in TUNEL-positive cells triggered by high glucose (Figures 2(a) and 2(b)).

The flow cytometric analysis also suggested that the administration of curcumin resulted in an evident decrease in the number of apoptotic bodies compared to cardiomyocytes exposed to high glucose that were not treated with curcumin (Figures 2(c) and 2(d)).

It is well known that apoptotic-related proteins regulate the progression of apoptosis. Thus, we performed further experiments to investigate if these regulatory proteins contributed to the effect of the inhibition of high glucose-induced apoptosis by curcumin. As indicated by immunohistochemical staining (Figures 3(a) and 3(b)), in contrast to the NG group, the HG group had reduced Bcl-2 expression and enhanced Bax expression. As we predicted, curcumin significantly enhanced Bcl-2 expression and reduced Bax expression in cardiomyocytes exposed to high glucose. Western blot showed that the Bcl-2/Bax ratio was remarkably reduced in the HG group compared to the NG group (Figures 3(c) and 3(d)). After treatment with curcumin, Bcl-2 expression was elevated, Bax expression was greatly reduced, and the Bcl-2/Bax ratio was significantly upregulated.

3.3. Curcumin Decreased High Glucose-Induced ROS Generation, Reduced MDA Content, and Increased SOD Activity in Cardiomyocytes. MDA formation and SOD activity, indexes of lipid superoxide, and oxygen free radical levels were measured in the cardiomyocytes. The MDA level in cardiomyocytes was significantly increased in the HG group. In contrast, SOD activity was found to be decreased when comparing the HG group with the NG group. The cardiomyocytes exposed to high glucose with curcumin markedly decreased the MDA level and enhanced SOD activity compared to those not treated with curcumin (Figures 4(a) and 4(b)).

ROS (a key executor of oxidative stress) was measured by DHE staining, DCFH-DA staining, and HPLC assay, which indicated that the ROS level in the HG group was much higher than that in the NG group. However, the HG-induced increase in ROS was strongly blocked by treatment with curcumin (Figures 4(c)–4(f)).

3.4. Curcumin Attenuated High Glucose-Induced Expression of NADPH Oxidase Isoforms in Cardiomyocytes. Because NADPH oxidase activation is directly related to increased

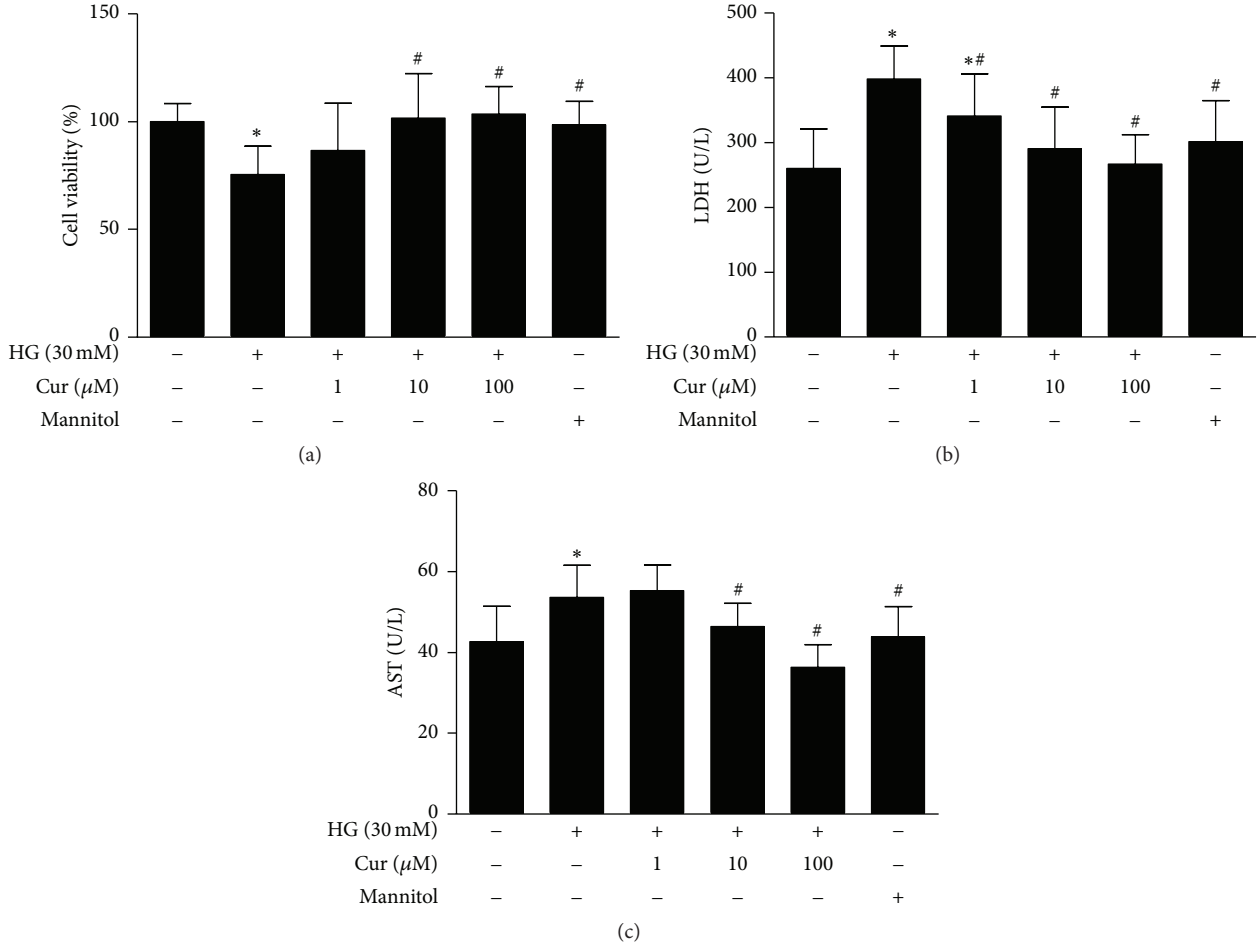

Figure 1: Curcumin increased cell viability and inhibited injury in cardiomyocytes exposure to high glucose. (a) Cell viability was examined with a CCK-8 assay. (b) Curcumin decreased the level of LDH in the supernatant. (c) Curcumin decreased the level of AST in the supernatant. Values are presented as mean ± SD. $^{*}P < 0.05$ versus NG group. $^{\#}P < 0.05$ versus HG group. $n = 10$.

oxidant production induced by hyperglycemia [18], we evaluated Rac1 activity and gp91^{phox} and p47^{phox} (NADPH subunits) expression. As presented in Figure 5, Rac1 activity in cardiomyocytes was much higher in the HG group than in the NG group. Moreover, increased expression of gp91^{phox} and p47^{phox} was detected in the HG group, while Rac1 activity and the expression of gp91^{phox} and p47^{phox} were markedly inhibited by curcumin in the cardiomyocytes exposed to high glucose. These data suggest that the protective role of curcumin against HG-induced cardiac injury is largely through inhibition of NADPH oxidase-mediated ROS production.

3.5. Curcumin Activated the PI3K/Akt/GSK-3β Signalling Pathway in Cardiomyocytes. The activation of the PI3K/Akt signalling pathways is well known to inhibit HG-induced apoptosis [19]. Therefore, we targeted the PI3K/Akt signalling pathway to determine the mechanism through which curcumin inhibits HG-induced apoptosis. Akt and GSK-3β phosphorylation were markedly decreased in the HG group as compared with the NG group. Cardiomyocytes treated with curcumin showed a remarkable increase in expression of Akt and GSK-3β phosphorylation. Pretreatment with the PI3K inhibitor LY294002 reversed the increased effect of curcumin on Akt and GSK-3β phosphorylation. In parallel with that, the expression of Bcl-2 and Bax regulated by curcumin was abolished by treatment with LY294002 and curcumin failed to reduce gp91^{phox} and p47^{phox} expression levels when LY294002 was applied. These results indicate that PI3K/Akt signalling may be involved in the inhibition of apoptosis by curcumin in cardiomyocytes exposed to high glucose (Figure 6).

4. Discussion

DCM is frequently seen in asymptomatic diabetic patients. It is now recognized as left ventricular dysfunction associated with increasing the danger of heart failure without hypertension and coronary artery disease or valvular heart diseases [6]. DM-induced left ventricular dysfunction includes impaired systolic and diastolic function, but diastolic dysfunction can occur prior to systolic dysfunction and can be characterized as the early phase of DCM [20].

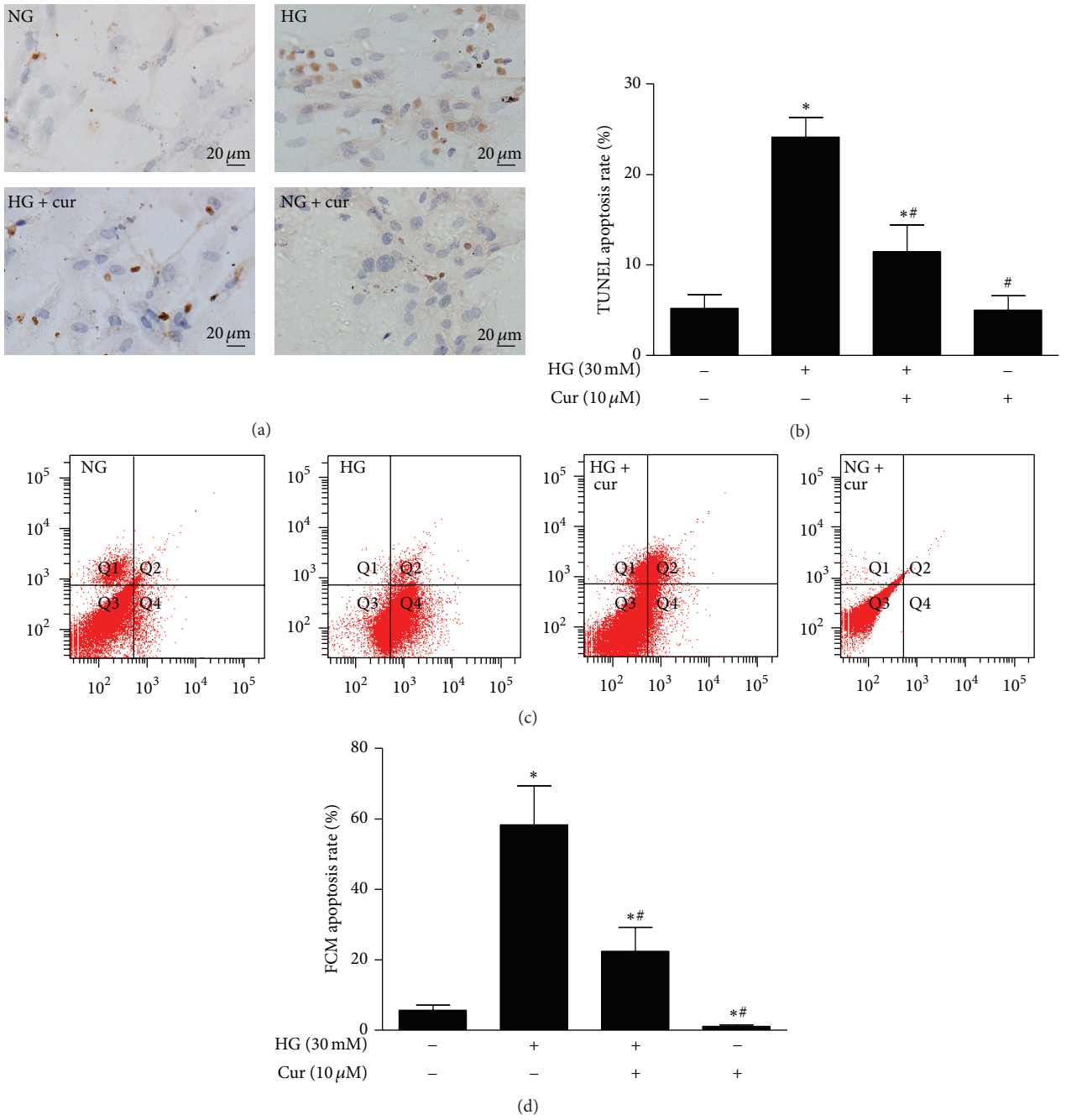

Figure 2: Curcumin inhibited high glucose-induced cardiomyocytes apoptosis. (a) Representative images of apoptotic cardiomyocytes stained by TUNEL (magnification = 400x, bar is 20 μm). (b) Quantitative analysis of TUNEL staining. (c) Representative images of apoptotic cardiomyocytes observed using FCM. (d) Quantitative analysis of the FCM results. Values are presented as mean ± SD. $^{*}P < 0.05$ versus NG group. $^{\#}P < 0.05$ versus HG group.

Several studies have shown a close correlation between left ventricular diastolic dysfunction and myocardial apoptosis and have shown that cardiac function can be improved by sufficient control of myocardial apoptosis [21]. Massive loss of cardiomyocytes due to various apoptotic stimuli occurs, resulting in fibrosis and, eventually, heart failure due to the lack of cardiomyocyte proliferation greatly limiting the generation of new cardiomyocyte. In this context, a clear reduction in cardiomyocyte apoptosis is regarded as a latent therapeutic strategy for the treatment of DCM. In the current work, we found that curcumin lessened cardiomyocyte apoptosis induced by high glucose and observed a curcumin-induced reduction in Bax, which plays a crucial role in mitochondrion-mediated apoptosis by being inserted into the mitochondrial outer membrane and resulting in the release of proapoptotic factors. In contrast, Bcl-2 is

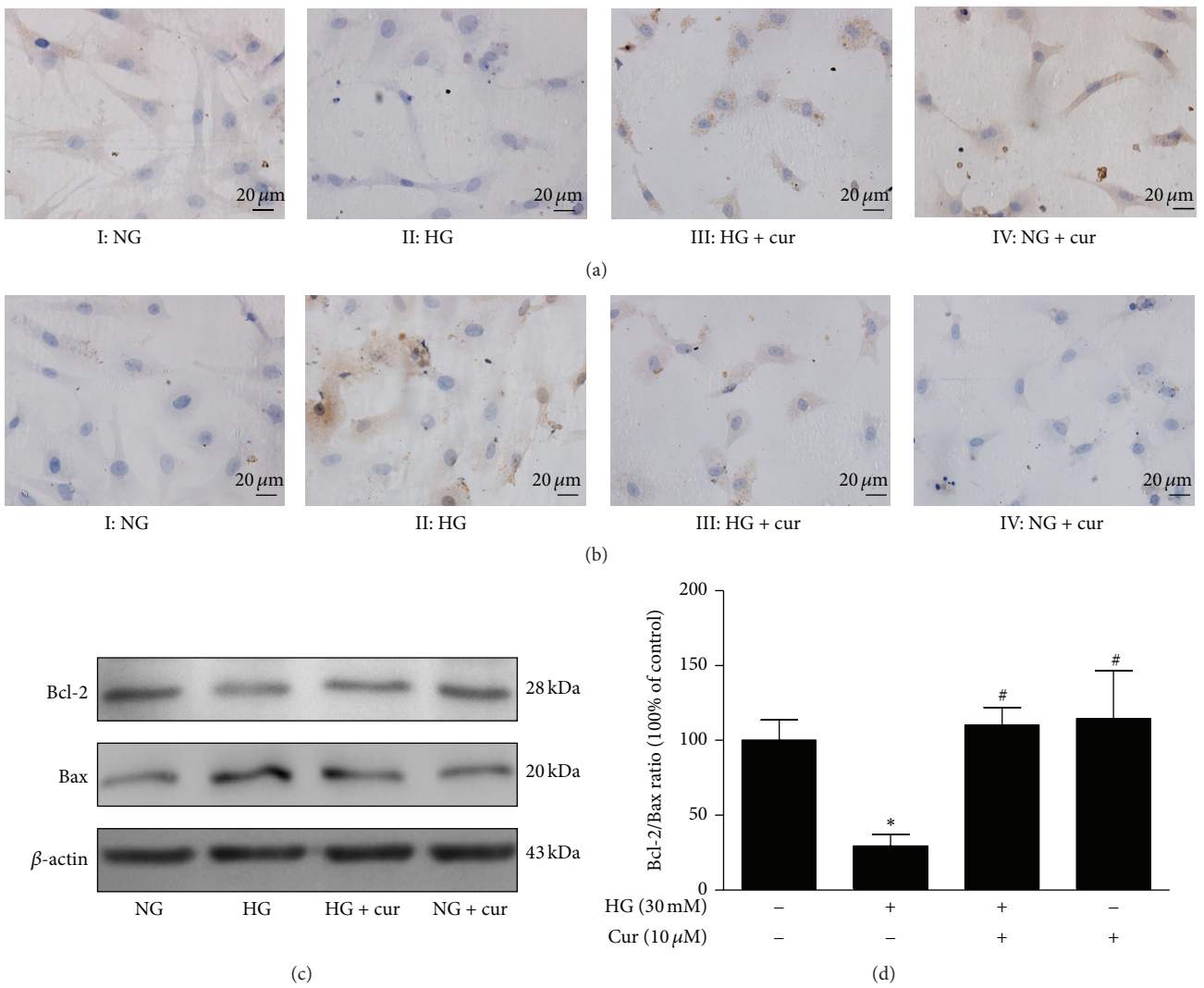

FIGURE 3: Curcumin regulated the expression of Bax and Bcl-2 in cardiomyocytes exposed to high glucose. (a) Representative images of Bcl-2 by immunohistochemical staining (magnification = 400x, bar is 20 μm). (b) Representative images of Bax by immunohistochemical staining (magnification = 400x, bar is 20 μm). (c) Representative images of Bax and Bcl-2 expression by western blot. (d) Quantitative analysis of the Bcl-2/Bax ratio. $n = 3$. Values are presented as mean ± SD. $^{*}P < 0.05$ versus NG group. $^{\#}P < 0.05$ versus HG group.

an antiapoptotic protein that prevents Bax oligomerization and was increased by curcumin treatment. Thus, curcumin's cardioprotective effects are possibly mediated by normalization of the Bcl-2/Bax ratio.

Cumulative evidence suggests that both cardiomyocyte apoptosis and oxidative stress contribute to the pathogenesis and development of diabetic cardiovascular complications [22]. Previous researches have provided direct evidence that the sustained generation of ROS during oxidative stress leads to cardiomyocyte apoptosis, which contributes to the development of DCM [23]. Emerging evidence has confirmed that activation of NADPH oxidase-generated ROS signalling is related to apoptosis in cardiomyocytes exposed to a hyperglycemic environment [24]. NADPH oxidase contains two membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$) and four cytosolic regulatory subunits, including $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac1. Rac1 plays a crucial role in the assembly of NADPH oxidase, which generates superoxide [25] and is a central factor in NADPH-mediated cardiomyocytes apoptosis in response to high glucose levels [26]. Therefore, the deleterious consequences of overactivation of NADPH oxidase in the form of diabetic cardiovascular complications have been well established. NADPH oxidase can impair the redox balance, thus inducing or exacerbating intracellular oxidative stress and resulting in abnormal ROS production. Therefore, inhibition of excessive ROS produced by NADPH oxidase appears to be another possible target for preventing the development of DCM [27]. Consistent with previous studies, we also found that lipid peroxidation levels were enhanced when the cardiomyocytes were exposed to high glucose, which was accompanied by an elevation in ROS generation as a result of the activation of NADPH

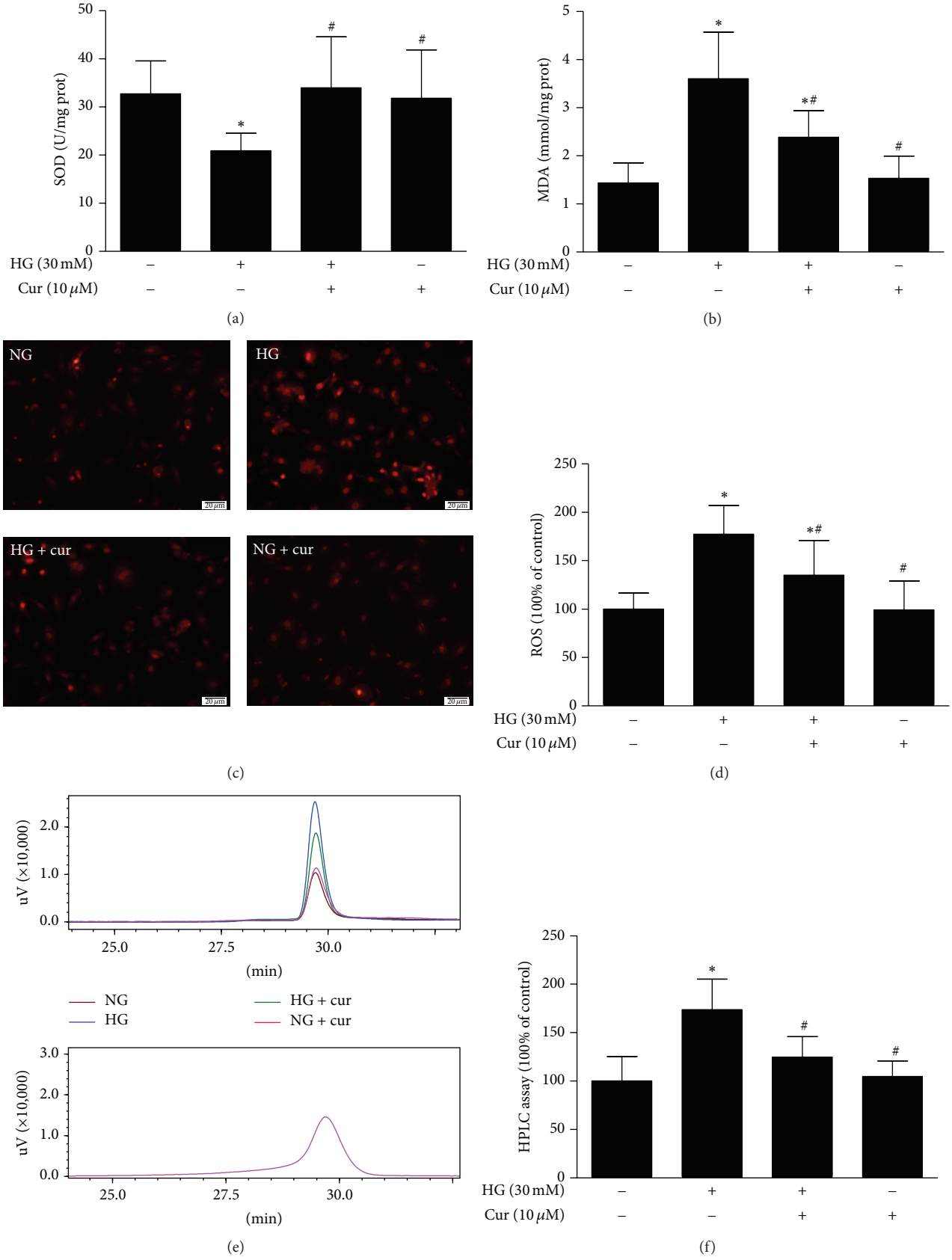

Figure 4: Curcumin suppressed high glucose-induced cardiomyocytes oxidative stress. (a) Curcumin enhanced SOD activity in cardiomyocytes ($n = 12$). (b) Curcumin reduced MDA level in cardiomyocytes ($n = 10$). (c) Representative images of DHE staining ($n = 3$). (d) Quantification of DCFH-DA staining (n = 11-12). (e) Representative images of HPLC assay (n = 4–6). (f) Quantification of HPLC assay. Values are presented as mean ± SD. $^{*}P < 0.05$ versus NG group. $^{\#}P < 0.05$ versus HG group.

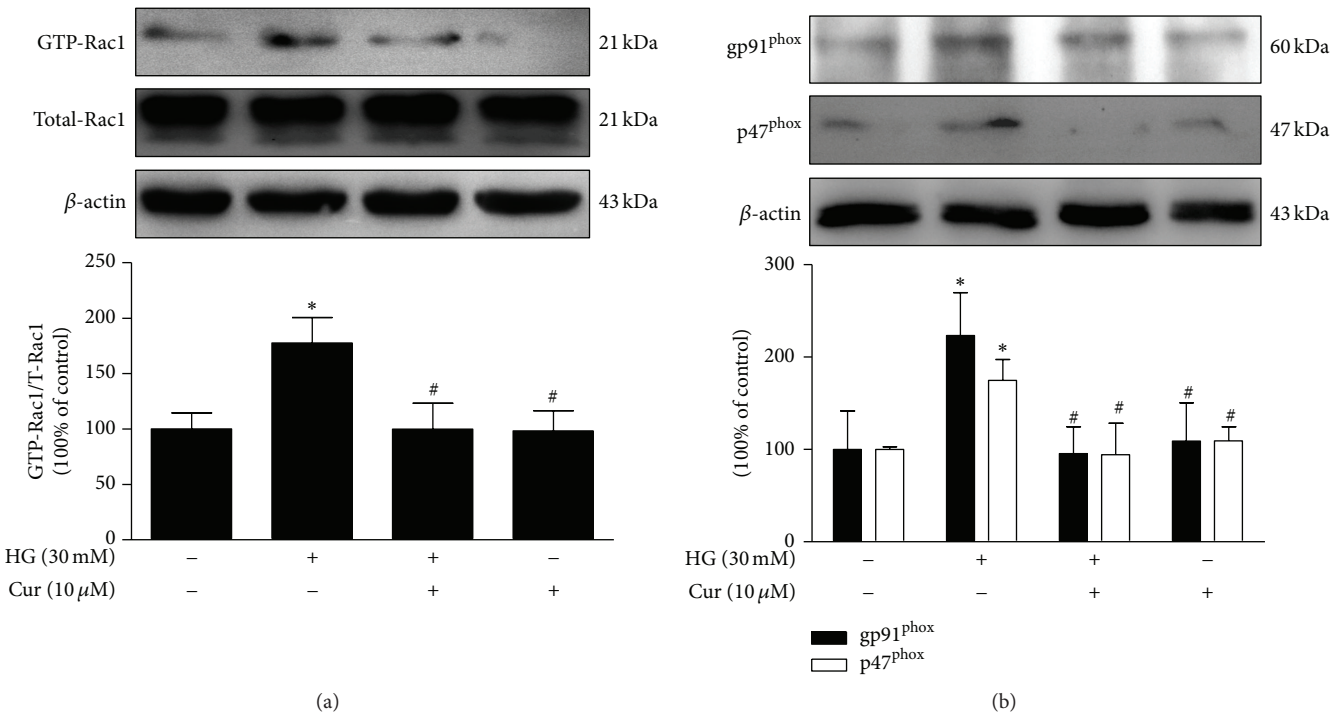

Figure 5: Curcumin decreased high glucose-induced Rac1 activity and the expression of gp91^{phox} and p47^{phox}. (a) Rac1 activity assay. (b) Western blots analysis of gp91^{phox} and p47^{phox} expression. $n = 3$. Values are presented as mean ± SD. $^{*}P < 0.05$ versus NG group. $^{\#}P < 0.05$ versus HG group.

oxidase. High glucose triggers NADPH oxidase activation by improving Rac1 activation and enhancing gp91^{phox} and gp47^{phox} expression. In agreement with earlier studies that showed that curcumin has a multitude of cardioprotective effects attributed to its efficient antioxidant capacity, we found that treatment with curcumin suppressed a hyperglycemia-induced rise in ROS generation through inactivation of NADPH oxidase.

Impaired Akt/GSK-3β signalling pathway has been shown to be involved in the development of metabolic disorders. Akt is responsible for the modulation of cardiovascular functions linked with cardiac growth and survival, contractile function, and coronary angiogenesis [28]. In particular, Akt1 has been demonstrated to play an antagonist role against pathological cardiac hypertrophy, which is an inevitable precursor of heart failure. Consistent with these observations, Akt1$^{-/-}$ mice displayed multiple heart defects, including enhanced cardiac growth and insufficient cardiomyocyte contractility [29]. Furthermore, Akt2 exerts a particularly important impact on the regulation of glucose metabolism and supports cell survival by restraining apoptosis via activation or inactivation of a number of target proteins involved in the process of apoptotic cascades [30, 31]. GSK-3β, a critical downstream element of the Akt pathway, participated in physiological and pathological processes such as regulation of glycogen synthesis and disposal, as well as cell death [32]. It is well accepted that glucose utilization is decreased and FFA oxidation is increased in the diabetic heart [33]. This substrate utilization shift has been known to contribute to the pathogenesis of DCM. Thus, activating Akt phosphorylation and inhibiting GSK-3β activity may be considered as cardioprotective actions, as they maintain the physiological growth and functions of the heart and promote cell survival. In the present study, our results indicated that cardiomyocytes exposed to high glucose have strikingly decreased expression levels of Akt and GSK-3β phosphorylation, which is in accordance with the previous report [34]. Interestingly, the beneficial roles exerted by curcumin in high glucose-induced cardiac injury such as enhancement of Akt and GSK-3β phosphorylation, reduction of gp91^{phox} and p47^{phox} expression, and regulatory apoptosis-related proteins were negated by the application of LY294002, which indicate that the PI3K/Akt/GSK-3β signalling pathway may be responsible for the inhibition of high glucose-induced cardiac injury by curcumin.

In summary, curcumin exerts cardioprotection against high glucose-induced cardiomyocyte apoptosis, and these effects were shown to possibly be due to efficient prevention of NADPH oxidase-derived oxidative stress and preservation of Akt and GSK-3β phosphorylation in vitro. Therefore, curcumin may be a feasible novel drug for the treatment of DCM.

Conflict of Interests

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

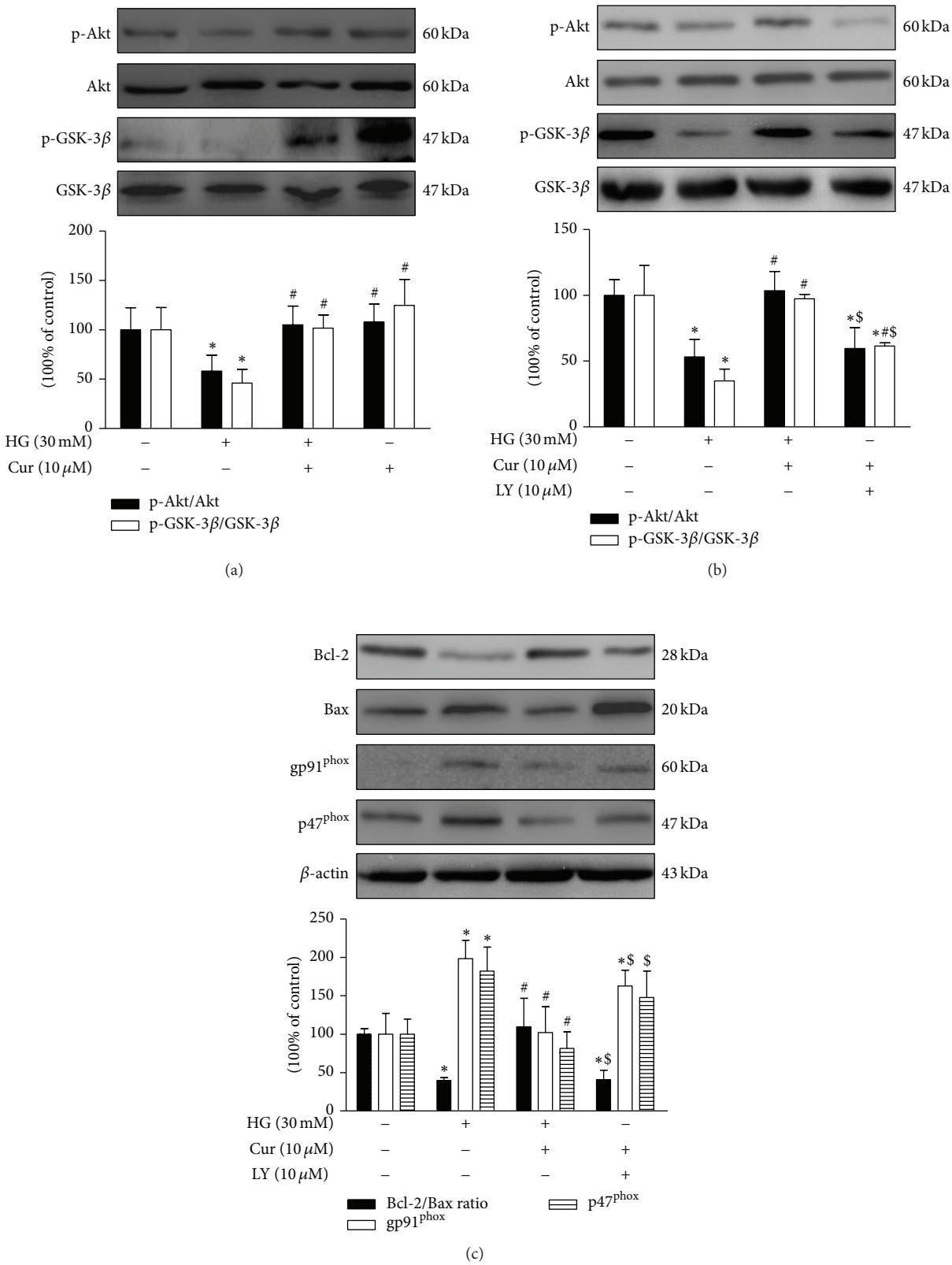

Figure 6: Curcumin inhibited high glucose-induced apoptosis and oxidative stress via activation of Akt in cardiomyocytes. (a) Curcumin upregulated Akt and GSK-3β phosphorylation levels. (b) Increased Akt and GSK-3β phosphorylation by curcumin were abolished by treatment with LY294002. (c) The effects on the Bcl-2/Bax ratio and the expression of gp91^{phox} and p47^{phox} by curcumin were blocked by treatment with LY294002. $n = 3$. Values are presented as mean ± SD. $^{*}P < 0.05$ versus NG group. $^{\#}P < 0.05$ versus HG group. $^{\$}P < 0.05$ versus curcumin treatment group.

Authors' Contribution

Wei Yu and Wenliang Zha contributed equally to this work.

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Review Article

Diabetic Retinopathy: Animal Models, Therapies, and Perspectives

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Diabetic retinopathy (DR) is one of the major complications of diabetes. Although great efforts have been made to uncover the mechanisms underlying the pathology of DR, the exact causes of DR remain largely unknown. Because of multifactor involvement in DR etiology, currently no effective therapeutic treatments for DR are available. In this paper, we review the pathology of DR, commonly used animal models, and novel therapeutic approaches. Perspectives and future directions for DR treatment are discussed.

1. Introduction

Diabetic retinopathy (DR) is one of the major complications of diabetes and is the leading cause of blindness among working people in developed countries. The symptoms are elevated blood sugar levels, blurred vision, dark spots or flashing lights, and sudden loss of vision. The development of DR can be divided into nonproliferative DR (NPDR; subdivided into mild, moderate, and severe stages) with microaneurysms, hard exudates, hemorrhages, and venous abnormalities [1, 2] and proliferative DR (PDR; advanced stage) with neovascularization, preretinal or vitreous hemorrhages, and fibrovascular proliferation [1, 2]. Development of glaucoma, retinal detachment, and vision loss may also happen at this stage. DR may cause macular edema when blood and fluid leak into the retina caused by swelling of the central retina [3]. DR is not easily diagnosed at early stages but is more readily noticed with the advanced stages or with edema. Multiple techniques have been used for detection, diagnosis, and evaluation of this disease including fundoscopic photography, fluorescence angiography, B-scan ultrasonography, and optical coherence tomography (OCT) [4].

2. Pathology and Molecular Mechanism of DR

Initially, DR was considered a microvascular complication of endothelial dysfunction, as it is characterized by capillary basement membrane (BM) thickening, pericyte and endothelial cell loss, blood-retinal barrier (BRB) breakdown and leakage, acellular capillaries, and neovascularization [5, 6]. However, it is currently acknowledged that before the typical features of DR occur and can be clinically diagnosed, cellular, molecular, and functional changes are evidenced in the retina [7, 8], where all types of retinal cells are affected including ganglion cells [5, 6, 9]. Also, thinning of the inner nuclear layer (INL), reduction in synapse numbers and synaptic proteins, changes in dendrite morphology, and retinal pigment epithelium (RPE) dysfunction occur in DR and result in the gradual loss of retinal function [9]. In addition, glia activation and innate immunity/sterile inflammation [5, 6] occur early in DR. Therefore, DR is not only a vascular disease but also a neurodegenerative disease.

DR shares numerous similarities in its etiology and pathology with other neovascular diseases which have been documented to be associated with chronic inflammation,

including increased vascular permeability, edema, inflammatory cell infiltration, tissue destruction, neovascularization, proinflammatory cytokines, and chemokines in the retina [3, 10]. Some of the potential risk factors leading to the pathology of other neovascular diseases also contribute to the pathology of DR.

Diabetes is the number one risk factor for the development of DR. Type 1 diabetes (juvenile diabetes, in which no insulin is made) is more likely to develop vision loss than type 2 diabetes (adult onset diabetes with insufficient insulin synthesis). In addition, race (Hispanic and African Americans), smoking, hyperglycemia (high blood sugar), hypertension (high blood pressure), and hyperlipidemia (high cholesterol) or dyslipidemia are also high risk factors [11, 12]. Vascular endothelial growth factor (VEGF) elevation induces a decrease in the tight-junction proteins and breakdown of the BRB [13], an increase of leukostasis within retinal vessels [14], inflammation [15, 16], upregulation of ICAM-1 (intercellular adhesion molecule-1) expression, an increase in all NOS (nitric oxide synthase) isoforms [17], and a metabolic imbalance in inorganic phosphate [18], all of which have been reported to contribute to DR pathology. Multiple interconnecting biochemical pathways, including an increased polyol pathway, elevated hexosamine biosynthesis pathway (HBP), activation of protein kinase C (PKC), hemodynamic changes, and advanced glycation end product (AGE) formation [5, 6, 14, 19], have also been found to play key roles in development of DR. RhoA is a small guanosine-5′-triphosphate-binding protein and acts as a GTPase. The RhoA/mDia-1 (mammalian diaphanous homolog-1)/profiling-1 [20] or RhoA/ROCK1 (Rho-associated coiled-coil-containing protein kinase 1) [21] pathways have been shown to be involved in the pathology of DR via triggering microvascular endothelial dysfunction. Activation of these pathways leads to the increase of growth factors such as VEGF and insulin-like growth factor-1 (IGF-1), activation of the renin-angiotensin-aldosterone system (RAAS), subclinical inflammation, and capillary occlusion [14]. Also increased endoplasmic reticulum (ER) stress and oxidative stress [22] resulting from deregulation of ER and mitochondrial quality control by autophagy/mitophagy, RPE dysfunction, genetic variants, and epigenetic changes in chromatin, such as DNA methylation, histone posttranslational modifications affecting gene transcription, and regulation by noncoding RNAs [23–26], have also been shown to be associated with DR. Interestingly, deletion of transforming growth factor-β (TGF-β) signaling results in undifferentiated pericytes that cause retinal changes in structure and function which mimic those of DR [27]. Loss of other gene functions such as BMP2 (bone morphogenetic protein 2) [28] and Toll-like receptor 4 [29] has been implicated in the pathogenesis of DR. Activation of the P2X7 receptor, a member of ligand-gated membrane ion channels, resulted in the formation of large plasma membrane pores that exacerbate the development of DR through induction of inflammation [30]. Recently, a prooxidant and proapoptotic thioredoxin interacting protein (TXNIP) was shown to be highly upregulated in DR and by high glucose (HG) in retinal cells in culture. TXNIP binds to thioredoxin (Trx) inhibiting its oxidant scavenging and thiol-reducing capacity. Hence, prolonged overexpression of TXNIP causes ROS/RNS stress, mitochondrial dysfunction, inflammation, and premature cell death in DR [31]. Collectively, hyperglycemia-induced vascular dysfunction and subsequent tissue damage have been proposed to act through the following four main pathways [32, 33]: (1) increased polyol pathway flux, in which cytosolic redox imbalance occurs with an increased NADH/NAD^+ ratio via the sorbitol pathway resulting in a decrease in cytosolic NADPH and cellular functions, (2) increased AGE formation, in which nonenzymatic glycosylation of proteins and production of AGEs alter gene expression and AGEs also induce the synthesis of numerous inflammatory cytokines, (3) activation of PKC via the formation of intracellular diacylglycerol (DAG) and AGEs, which contributes to the generation of ROS which induces VEGF and multiple other growth factors and transcription factors, and (4) increased hexosamine pathway flux, in which fructose-6-phosphate is converted to glucosamine-6-phosphate and finally to uridine diphosphate *N*-acetyl glucosamine. This modification results in changes in gene expression and protein function. However, each of the four major pathways is linked by overproduction of superoxide and increased generation of ROS [33], which provides a common target for potential treatment.

3. Animal Models

At present, most animal models of DR are rodents, mice, and rats. Based on the experimental approaches to induce DR, these models can be classified as chemically induced, spontaneous, and genetically created. However, knowledge of the molecular mechanisms underlying the initiation and development of DR is insufficient and largely unknown because there are no reliable and appropriate good animal models of spontaneous diabetes in which phenotypic characteristics exactly mimic the pathogenesis of clinical DR. Although various traditionally used animal models of DR present a number of pathological changes similar to those of human DR, several pathological characteristics of human DR, such as retinal neovascularization, cannot yet be fully mimicked in any existing animal model of DR [34].

3.1. Chemically Induced Model. The commonly used streptozotocin (STZ) or alloxan induced DR animal models (rats or mice) exhibit rapid onset of hyperglycemia (3 days after treatment) and some of the symptoms of early DR (type I diabetes), such as loss of retinal pericytes and capillaries, thickening of the vascular basement membrane, vascular occlusion, and increased vascular permeability [3, 34, 35]. However, variability of pathological characteristics, such as loss of retinal capillaries, ganglion cell death, and reduction of retinal function, has been reported among different species and even within the same species [3, 34, 35].

3.2. Akita Mice. The Akita ($Ins2^{Akita+/-}$) mouse, a spontaneous diabetes model for early stage of DR (type I diabetes), is caused by a missense mutation in the diabetogenic *Insulin 2* gene (*Ins2*) and is characterized by a rapid onset of hyperglycemia and hypoinsulinemia and marked reduction

of insulin secretion by 4 weeks of age [36]. Significant increases in vascular permeability were seen when measured at 12 weeks after hyperglycemia. The thickness of the inner plexiform layer (IPL) and INL in the peripheral region was decreased and the number of ganglion cells was significantly reduced when measured at 22 weeks after hyperglycemia [37]. Recently, Hombrebueno et al. reported that the Akita mice exhibit progressive thinning of the retina and cone loss from 3 months onwards, severe impairment of synaptic connectivity at the outer plexiform layer (OPL), and significant reduction in the number of amacrine and ganglion cells [38, 39]. ER stress associated proteins were upregulated in this mouse model [40]. The transportation of proinsulin from the endoplasmic reticulum (ER) to the Golgi apparatus is blocked, and instead the mutant proinsulin is accumulated in the ER forming complexes with BiP (binding immunoglobulin protein) which are eventually degraded [41].

3.3. Kimba Mice. The Kimba mice were generated by microinjection of human $VEGF_{165}$ isoform driven by a photoreceptor-specific promoter (rhodopsin). Pathological changes in the retinal vasculature, focal fluorescein leakage, relatively mild degree, and slow onset of neovascularization were shown at 3-4 weeks of age and stable retinopathy persisted for 3 months, which resembles NPDR and early stage of PDR [1]. A thinner outer nuclear layer (ONL) and INL, severe and extensive outer and inner retinal neovascularization, hemorrhage, retinal detachment [1], microaneurysm, leaky capillaries, capillary dropout [42], leaky blood vessels, and BRB loss [42, 43] were presented in this mouse model. However, the mice overexpressing photoreceptor-specific hVEGF are not on a hyperglycemic background and do not induce choroidal neovascularization [1, 42].

3.4. Akimba Mice. The Akimba ($Ins2^{Akita}VEGF^{+/-}$) mouse, generated from the Kimba ($VEGF^{+/-}$) (trVEGF029) and the Akita ($Ins2^{Akita}$) mice, is a model for advanced DR [42]. This model retains the parental retinal neovascularization with hyperglycemia and displays the majority of signs of advanced clinical DR including more diffuse vascular leakage (compared to the more focal leakage in Kimba mice) and the BRB disruption, which was linked to decreased expression of endothelial junction proteins, pericyte dropout, and vessel loss [42, 43]. With aging, Akimba mice exhibit enhanced photoreceptor loss, thinning of the retina, more severe and progressive retinal vascular pathology, capillary nonperfusion, much higher prevalence and persistence of edema, and retinal detachment [42]. Plasmalemma vesicle associated protein (PLVAP) is an endothelial cell specific protein which is absent in intact BRB but is significantly increased in Akimba mice (and also in Kimba mice). Therefore PLVAP plays an important role in the regulation of BRB permeability [43].

3.5. db/db Mice. The db/db ($lepr^{db}$) mouse, a spontaneous diabetic model of type 2 diabetes [44, 45], is caused by a mutation in the leptin receptor gene. It exhibits high glial activation, progressive loss of ganglion cells, and significant reduction of neuroretinal thickness. Significant abnormal retinal function is pronounced at 16 weeks of age. In addition, significantly higher levels of glial fibrillary acidic protein (GFAP, a marker for glial cells) expression, increases in accumulation of glutamate, and downregulation of abundant neurotransmission genes were found at 8 weeks of age [44]. Also, breakdown of the BRB is a hallmark of the db/db mice [46] and RPE dysfunction is concomitant with sustained hyperglycemia [45]. Proteomic analysis of 10-week-old retinas from db/db and wild type mice showed that 98 membrane proteins, out of a total of 844, were significantly differentially abundant in db/db versus wild type mice, in which 80 were downregulated and 18 were upregulated in the db/db retinas [47]. The major proteins decreased are synaptic transmission proteins, especially the vesicular glutamate transporter 1 (VGLUT1) [47], which is responsible for the loading of glutamate into synaptic vesicles and is expressed at the ribbon synapses in the photoreceptors and "ON" bipolar cells [48].

3.6. New Animal Models. In recent years, two new animal models were reported. One is a transgenic mouse overexpressing insulin-like growth factor-1 (IGF-1), which develops the most retinal alteration seen in human diabetic eyes on a nonhyperglycemic background [49] and exhibits progressive development of vascular alteration (from NPDR to PDR), increased VEGF level, BRB breakdown, vascular permeability, and glial alteration with age (3 months and older) [49, 50]. Retinal neurodegeneration was seen at 6 months of age with the number of bipolar and ganglion cells reduced and a 40% reduction of ONL and INL thickness was observed in 7.5-month-old mice. Microarray analysis on 4-month-old retinas, with evidence of NPDR and gliosis [50], revealed upregulation of genes associated with retinal stress, gliosis, and angiogenesis. Increased GFAP immunostaining was seen at 1.5 months of age and was maintained throughout the entire life. Activation of ERK signaling was detected at 3 months and was more pronounced at 7.5 months. In addition, expression of oxidative stress markers was increased; in particular a striking upregulation of all three subunits of NADPH oxidase, impaired glutamate recycling, and significantly higher levels of TNF-α and MCP-1 were seen at 7.5 months [51]. The other model is the hyperhexosemic marmosets (*Callithrix jacchus*) which, with a 30% galactose- (gal-) rich diet for two years, develops significantly high blood glucose levels, vascular permeability, macular edema, increased number of acellular capillaries, pericyte loss, vascular BM thickening, increased vessel tortuosity in the retinas, and microaneurysms. High-speed spectral domain OCT (SD-OCT) scan reveals significant thickening of the foveal and the juxtafoveal area resulting from intraretinal fluid accumulation. Also there are potential break in the RPE and discontinuous photoreceptor layers in the macular area starting at 15 months of galactose feeding. All these characteristics have striking similarities to the human DR [52].

4. Current Therapies

During the nonproliferative stages, treatment is usually not recommended because normal visual function is not disturbed at these stages. However, at the advanced stages, the

PDR, treatment has to be undertaken. Traditional approaches for treatment of DR and associated microvasculature and neovascularization include laser treatment, optimizing blood glucose level, and controlling blood pressure. Currently, laser treatment (photocoagulation) to stop the leakage and scattered laser burns to shrink abnormal blood vessels and prevent retinal detachment are effective and are widely employed and are the primary treatment strategy. Surgical treatment to remove the vitreous (vitrectomy) is usually taken for advanced PDR in type I diabetes if persistent vitreous hemorrhage or severe tractional retinal detachment occurs. Intravitreal injection of anti-VEGF (Avastin, Lucentis, and Eylea) and corticosteroids to prevent abnormal blood vessel growth are effective and are also beneficial treatments for PDR [2, 19, 53].

Clinical trial (ClinicalTrials.gov number: NCT01627249) phase III study (660 adults) with intravitreal injection of Aflibercept, Bevacizumab, or Ranibizumab for diabetic macular edema (DME) showed that visual acuity was improved, and Aflibercept is more effective when the initial visual acuity is worse [54]. A five-year clinical trial study reported that intravitreal injection of 0.5 mg Ranibizumab with prompt (124 patients) or deferred (111 patients) focal/grid laser treatment for diabetic macular edema resulted in the maintenance of vision gains obtained by the first year through 5 years in most of the eyes [55]. However, another clinical trial study (322 of 582 eyes) showed that repeated intravitreal Ranibizumab injections for DME may increase the risk of sustained elevation of intraocular pressure or the need for ocular hypotensive treatment [56] and a risk of stroke [2]. Another clinical trial, phase I/II study, evaluating the safety and bioactivity of intravitreal injection of a designed ankyrin repeat protein (MP0112) for specific and high-affinity binding to VEGF in patients with DME, showed reduction of edema and improvement of visual acuity, although several patients showed inflammation [57]. An ongoing clinical trial eliminates the source of inflammation from a new preparation [57].

DR associated pathological factors, molecular signaling pathways, and other mechanisms underlying the pathology of DR, as well as the direct pathological defects (retinal degeneration, synaptic connection impairment and cell loss, accumulation of glutamate, etc.), provide a broad spectrum of potential new therapeutic targets for the treatment of DR. Therapeutic treatment strategies targeting these molecules, components, or defects, including various factors, hyperglycemia- and glutamate-triggered pathways, and microvascular impairment and angiogenesis, have been shown to produce an effective outcome [11, 58, 59]. Chinese traditional medicine HF (He-Ying-Qing-Re formula), in which chlorogenic acid, ferulic acid, and arctin were identified as major components, was shown to have anti-DR effects, although hyperglycemia was not significantly inhibited. Its action on suppression of activation of AGEs and endothelial dysfunction occurs by inactivation of AGEs receptor and their downstream Akt signaling pathway [60]. Deletion of placental growth factor prevents DR by inactivation of Akt and inhibition of the HIF1α-VEGF pathway [11, 61]. Recently, angiopoietin-like 4 (ANGPTL 4) was identified as a potential angiogenic factor which was upregulated in the PDR patients and was shown to be independent of VEGF levels and localized in the area of retinal neovascularization. Neutralizing ANGPTL4 antibody can inhibit the angiogenic effect in PDR patients with low VEGF levels or produce an additive effect with anti-VEGF treatment for inhibition of VEGF expression [62].

Preclinical therapies targeting other factors have been reported. A single intravitreal injection of a vector expressing insulin-like growth factor binding protein-3 (IGFBP-3) into diabetic rat retina after 2 months of diabetes restores normal insulin signal transduction via regulation of the insulin receptor/TNF-α (tumor necrosis factor-alpha) pathway and leads to the reduction of proapoptotic markers or increases of antiapoptotic markers and the restoration of retinal function [63]. Blockage of TNF-α by intravitreal and intraperitoneal delivery of anti-TNF-α antibody in STZ-induced mice and Akita mice resulted in a dose-dependent prevention of increased retinal leukostasis, acellular capillary, BRB breakdown, and cell death [64]. Intraperitoneal injection of anti-VEGFR1 antibody (MF1) prevents vascular leakage and inhibits inflammation associated gene expression and abnormal distribution of tight-junction proteins in STZ-induced mice and Akita mice [65].

Fenofibrate is a peroxisome proliferator-activated receptor-α (PPAR-α) agonist and is known for clinical treatment for dyslipidemia. Recently, it was shown to significantly ameliorate retinal vascular leakage and leukostasis in DR of STZ-induced diabetic rats and Akita mice through downregulation of ICAM-1, MCP-1 (monocyte chemoattractant protein-1), and NF-κB (nuclear factor-kappa B) signaling [66]. Clinical studies demonstrated that Fenofibrate has protective effects on progression of proliferative DR in type 2 diabetic patients [67, 68]. Now, the use of this medication for DR is approved [69].

Omega-3 polyunsaturated fatty acid (ω-3PUFA) has been shown to be decreased in STZ-induced diabetic rat retina [70]. ω-3PUFA rich diets enhanced glucose homeostasis and preserved retinal function in db/db/mice, but the effect is independent of preservation of retinal vasculature integrity, inflammatory modulation, and retinal neuroprotection [71].

5. Novel Potential Therapeutic Targets

Because of the complicated etiology of DR, drugs such as inhibitors for signaling pathways and growth factors have been shown to be effective for the treatment of DR but have limitations. Currently, intravitreal injection of anti-VEGF and corticosteroids are popular therapeutics, but a high proportion of patients (~40%) do not respond to these therapies [58, 72]. This implies that other factors or pathways, independent of VEGF, are involved in the development of microvasculature and neovascularization. Therefore, there is an urgent need for finding potential target candidates and for the development of new treatment strategies for DR therapy.

Epigenetic chromatin modifications (DNA methylation, histone posttranslational modifications, and regulation by noncoding RNAs), acting on both *cis*- and *trans*-chromatin

structural elements, can be regulated by TXNIP [25]. Aberrant epigenetic modifications have been identified in DR and implicated in the progression of DR [25, 26]. MicroRNAs (miRNAs) are a group of noncoding RNA sequences which are short and highly conservative and can posttranscriptionally control gene expression by degradation or repression of target mRNAs. They are implicated in a variety of biological activities including modulation of glucose, angiogenesis, and inflammatory responses, as well as pathogenesis of diabetes and related complications such as DR [10]. However, conflicting data were seen with different miRNAs. It has been shown that retinal miRNA expression was altered in early DR rats induced by STZ, in which miRNAs were differentially regulated compared to the controls without DR [73]. Downregulation of miR-200b has been shown to increase VEGF expression, and polycomb repressive complex 2 (PRC2) (histone methyltransferase complex) represses miR-200b through its histone H3 lysine-27 trimethylation. Thus inhibition of PRC2 through histone methylation of miR-200b increases miR-200b and reduces VEGF in STZ-induced diabetic rats [74]. The $3'$-untranslated region ($3'$-UTR) of mRNA sequence contains regulatory regions including binding sites for miRNAs to repress translation and degrade mRNA transcripts. In DR rats, miRNA-195 was significantly upregulated after one month of diabetes, and the antioxidant enzyme MnSOD level was reduced. *In situ* hybridization indicated that miR-195 was overexpressed in the cells of INL and ONL and ganglion cell layers, but sirtuin 1 (SIRT1) was downregulated. SIRT1 is involved in many biological processes including cell survival and metabolism and miR-195 binds to the $3'$-UTR of SIRT1 to regulate its expression. Intravitreal injection of miR-195 antagomir leads to downregulation of SIRT1, thus preventing DR damage caused by SIRT1-mediated downregulation of MnSOD [75]. Collectively, increasing amounts of data demonstrate the active involvement and critical role of miRNAs in development of DR, although the exact mechanisms by which miRNA or miRNAs act are not known. Increased knowledge of how miRNAs function as therapeutic agents will lead to their effective use in the treatment of DR.

Reactive oxygen species (ROS), the primary causative factor for a variety of diseases, have been shown to play an important role in promoting DR [12, 58, 76]. As a treatment target, evidence from preclinical and clinical studies indicates that antioxidant therapies which directly target ROS-producing enzymes are beneficial, although the outcome of large clinical trials has been less promising [76]. However, nuclear factor erythroid 2-related factor 2 (Nrf2), the regulator of phase II enzymes system and the network of cytoprotective genes [77, 78], is still attractive. Its activators have been proven effective in prevention of the development and progression of DR [79]. Here, we specifically point out that nanomedicine attracts more attention in the past several years because it has been beneficial in a variety of medical applications including its promising effects on disease therapy [80, 81]. We have been using cerium oxide nanoparticles (nanoceria) to treat several animal models for ocular diseases and demonstrated their nontoxic and long-lasting effectiveness in delaying retinal degeneration in *tubby* mice [82] and inhibiting retinal and choroidal neovascularization [83]. Due to their unique physicochemical features, nanoceria themselves exhibit superoxide dismutase and catalase activities under redox conditions and can upregulate phase II enzymes [84] and regulate the common antioxidant gene network downstream of Trx [85]. Nanoceria have an atom-comparable size which enables them to freely cross the cellular and nuclear membrane barriers. In addition, they do not need repeat dosing as is required by other antioxidants. Thus one single dose produces sustained protective effects [82–84] which suggests their great potential to be excellent agents for the treatment of DR.

Stem cells emerged as a regenerative therapeutic strategy for treatment of a variety of diseases because they are undifferentiated and retain their stem cell characteristics and possess the potential to differentiate into many different cell types under certain biological conditions [86, 87]. Stem cells have been obtained from multiple sources and have been shown to have a great potential for tissue repair and ocular disease treatment [87, 88]. Human embryonic stem cells (hESCs) can differentiate into more than 99% pure RPE cells and integrate into the host RPE layer and become matured. Phase I/II clinical trials for assessing the tolerability and safety of subretinal transplantation of hESC-derived RPE cells in patients with Stargardt's macular dystrophy (ClinicalTrials.gov number: NCT01345006) and advanced dry AMD (ClinicalTrials.gov number: NCT01344993) have shown that hESCs improve visual acuity [89]. Assessment of their medium- and long-term safety, graft, and survival in patients is ongoing [90]. Mesenchymal stromal cells (MSCs) have been shown to have multiple effects including tissue repair, secretion of neuroprotective growth factors, suppression of host immune response, and lowering glucose levels [91, 92]. Bone marrow derived MSCs have been reported to be differentiated into retinal cells and rescue retinal degeneration in several animal models [91]. Clinical trial phase I assessing their effects on visual acuity in patients with retinitis pigmentosa (RP) (ClinicalTrials.gov number: NCT01068561) has been completed and phase I/II in patients with AMD and Stargardt (ClinicalTrials.gov number: NCT01518127) will be completed in December 2015 (also see review [92]). However, no clinical study of therapeutic effects of MSCs in DR has been reported. Progress has also been made in using several classes of stem cells (EPCs, endothelial progenitor cells; ASCs, adipose stromal cells; PSCs, pluripotent stem cells) to stimulate both neuroregeneration and vascular regeneration in the diabetic retina [92]. EPCs are circulating cells and can be recruited to the sites of vessel damage and tissue ischemia and promote vascular healing and reperfusion [93]. Clinical studies have shown that altered numbers of EPCs were found in patients of type I and type II diabetes with NPDR and PDR, suggesting that EPCs are potential biomarkers for DME and PDR and may be used as therapeutic modalities to treat DR [72]. Preclinical study of STZ-induced diabetic rats receiving a single intravitreal injection of human derived ASCs at two months after diabetes onset showed significant decreases in vascular leakage and apoptotic cells and downregulation of inflammatory gene expression and improved rod b-wave amplitude within one week after injection [94]. Furthermore,

mouse ASCs (mASCs) were intravitreally injected into 5-week-old Akimba mice, and the mASCs integrated and associated with retinal microvasculature. Injection of TGF-β1-preconditioned mASCs into P9 Akimba pups resulted in a great decrease in capillary dropout areas and avascular areas [95]. These results suggest that regenerative medicine could be a permanent solution for fighting diabetes and associated complications.

Nevertheless, as we previously mentioned, DR has a complicated etiology and involves many factors. Among these causative factors, genetic background seems to contribute most heavily and current approaches for the treatment of DR can only delay the disease progression and do not provide a complete treatment or cure for DR. Correction of the defective gene(s) appears to be potentially the most effective way for DR treatment (see below). In the clinic, the challenge faced is the lack of detection methods for as yet unknown early clinical symptoms which would enable immediate and proper treatment for inhibition of the progression of NPDR to PDR.

6. Perspective and Future Direction for DR Treatment

With wide exploration of the etiology of the diseases using modern molecular techniques, one finds that almost all the diseases are linked with mutation(s) of a specific gene or multiple genes. Current effective gene therapy methods involve gene replacement therapy in which the defective copy of the gene is replaced by the wild type allele to compliment the defect; or knockdown of the defective gene by RNA interference (RNAi) silences the effects of the mutated gene; or introduces a gene to produce a product causing cell apoptosis (http://www.ghr.nlm.nih.gov/handbook). None of the above mentioned strategies can completely eliminate the products or effects of the defective genes indicating that the diseases cannot be completely cured. CRISPR/Cas9-mediated genome editing, which emerged as a new therapeutic strategy for defective gene repairing, has attracted significant attention in recent years. Indeed, at the 2015 annual ARVO (the association for research in vision and ophthalmology) meeting, several laboratories reported their progress in using this approach to correct (or repair) mutant gene sequences from patient-derived induced pluripotent stem cells (iPSCs) for treatment of inherited ocular diseases such as retinitis pigmentosa, AMD, and other retinal diseases [96–99]. Considering the similarity in the pathogenesis of AMD and DR, CRISPR/Cas9-mediated selective engineering of genes associated with DR or angiogenesis is expected to produce positive and effective treatment of DR.

Conflict of Interests

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

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Research Article

Vitamin B6 Prevents Endothelial Dysfunction, Insulin Resistance, and Hepatic Lipid Accumulation in *Apoe*$^{-/-}$ Mice Fed with High-Fat Diet

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Backgrounds. VitB6 deficiency has been associated with a number of adverse health effects. However, the effects of VitB6 in metabolic syndrome are poorly understood. *Methods.* VitB6 (50 mg/kg/day) was given to *Apoe*$^{-/-}$ mice with hkdigh-fat diet (HFD) for 8 weeks. Endothelial dysfunction, insulin resistance, and hepatic lipid contents were determined. *Results.* VitB6 administration remarkably increased acetylcholine-induced endothelium-dependent relaxation and decreased random blood glucose level in *Apoe*$^{-/-}$ mice fed with HFD. In addition, VitB6 improved the tolerance of glucose and insulin, normalized the histopathology of liver, and reduced hepatic lipid accumulation but did not affect the liver functions. Clinical and biochemical analysis indicated that the levels of VitB6 were decreased in patients with fatty liver. *Conclusions.* Vitamin B6 prevents endothelial dysfunction, insulin resistance, and hepatic lipid accumulation in *Apoe*$^{-/-}$ mice fed with HFD. Supplementation of VitB6 should be considered to prevent metabolic syndrome.

1. Introduction

Vitamin B6 (VitB6) includes pyridoxal, pyridoxine, and pyridoxamine, which function as essential cofactors for enzymes involved in various metabolic activities, which include amino acid, fat, and glucose metabolism [1]. The phosphate ester derivative pyridoxal 5′-phosphate (PLP) is the biologically active form of this vitamin and reflects long-term body storage [2]. Studies have shown that low plasma PLP concentrations are associated with increased risk of cardiovascular disease (CVD) [3, 4].

Nutrient overload is associated with high incidence of chronic metabolic diseases, including obesity, insulin resistance, and type 2 diabetes [5]. Prolonged exposure to high concentrations of saturated fatty acids leads to oxidative stress and endoplasmic reticulum stress, which may impair insulin signaling [6]. Moreover, supplementation of a high-fat diet (HFD) with branched-chain amino acids caused insulin resistance, as a part of metabolic syndrome [7]. Metabolic syndrome is associated with a risk of CVD and is a common early abnormality in the development of type 2 diabetes. In patients with nonalcoholic fatty liver disease (NAFLD), metabolic abnormalities have been reported in 33% to 100% of cases [8]. Patients presenting with NAFLD need to be examined for the presence of the components of the metabolic syndrome and their complications [9]. We also previously reported that apoptosis of liver cells contributes to liver dysfunction [10].

The identification of the link between VitB6 and metabolic syndrome including insulin and NAFLD might help to define novel nutritional and pharmacological approaches for the treatment of diabetes, obesity, and insulin resistance. Here, we reported that administration of VitB6 prevents endothelial dysfunction, insulin resistance, and hepatic lipid accumulation in $Apoe^{-/-}$ mice fed with high-fat diet. Clinically, deficiency of VitB6 should be considered as a high risk factor of NAFLD.

2. Materials and Methods

2.1. Materials. Human recombinant insulin was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against Akt (pAkt), GLUT4, glycogen synthase kinase-3β (GSK3), forkhead box protein O (FOXO), and GAPDH were purchased from Santa Cruz Biotechnology (Dallas, TX). The secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). VitB6, acetylcholine (ACh), sodium nitroprusside (SNP), and phenylephrine were from Sigma-Aldrich Company. All drug concentrations are expressed as final working concentrations in the buffer.

2.2. Animals and Experimental Protocols. Male $Apoe^{-/-}$ mice were purchased from Hua-Fu-Kang Animal Company (Beijing, China). All animals were housed in temperature-controlled cages with a 12-hour light-dark cycle and given free access to water and normal chow. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

Model of hyperlipidemia was induced by feeding mice with HFD containing 0.21% cholesterol and 21% fat (Research Diets Inc., D12079B). This diet was administered at 6 weeks of age and continued for 8 consecutive weeks. At 6 weeks of age, VitB6 (50 mg/kg/day) was also added to the drinking water for 8 weeks. The animal protocol was reviewed and approved by the Animal Care and Use Committee of Hunan Normal University.

2.3. Determinations of Serum Lipid Profiles and Liver Functions. Blood was sampled from mice for determination of total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and albumin (ALB). Serum levels of TB, AST, ALT, AP, and ALB were determined by commercial kits (Nanjing Jiancheng Biology Company, Nanjing, China).

2.4. Organ Chamber. In vivo or *ex vivo* organ chamber study was performed as described previously [11]. Mice were sacrificed under anesthesia by intravenous injection with pentobarbital sodium (30 mg/kg). The descending aorta isolated by removing the adhering perivascular tissue carefully was cut into rings (2-3 mm in length). Aortic rings were suspended and mounted to organ chamber by using two stainless hooks. The rings were placed in organ baths filled with Krebs buffer of the following compositions (in mM): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 0.6; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; EDTA, 0.026; pH 7.4 at 37°C; and they were gassed with 95% O_2 plus 5% CO_2, under tension of 1.0 g, for 90-minute equilibration period. During this period, the Krebs solution was changed every 15 min. After the equilibration, aortic rings were challenged with 60 mM KCl. After washing and another 30-minute equilibration period, contractile response was elicited by phenylephrine (1 μM). At the plateau of contraction, accumulative ACh (0.01, 0.03, 0.1, 0.3, 1, and 3 μM) or SNP (0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM) was added to induce the relaxation.

2.5. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT). As described previously [12], glucose (2.0 g/kg) was given to mice (i.p.) after an overnight fast. Blood glucose (BG) levels were then measured at indicated times with a portable glucose meter (LifeScan, Milpitas, CA) after tail snipping. For ITT, mice were injected with insulin (0.55 IU/kg, i.p.) after 6-hour fast. BG levels were measured at indicated times with a portable glucose meter (LifeScan, Milpitas, CA) after tail snipping.

2.6. HE or Oil Red O Staining. Histological specimens were taken at the end of the study period for all mouse groups as described previously [13]. For each mouse, liver segments were fixed in 4% buffered formaldehyde and embedded in paraffin for histological analysis. Sections (5 μm) were stained with either hematoxylin or eosin. Degree of severity of liver fibrosis was derived from blind analysis of each of the animals in each group. To determine hepatic lipid accumulation, frozen liver sections were stained with 0.5% Oil Red O for 10 min, washed, and counterstained with Mayer's hematoxylin for 45 sec. Data for Oil Red O staining were presented as the mean percentage of stained area to a total hepatic region in 10 fields from each liver section. Quantitative analysis was performed using analySIS-FIVE program (Olympus Soft Imaging System, Münster, Germany).

2.7. Western Blotting. The protocol for western blot was described as previously with some modifications [14]. Liver tissues were homogenized and the protein content in supernatant was assayed by BCA protein assay reagent (Pierce, USA). 20 μg proteins were loaded to SDS-PAGE and then transferred to membrane. Membrane was incubated with 1 : 1000 dilution of primary antibody, followed by 1 : 2000 dilution of horseradish peroxides-conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare, USA). The intensity (area × density) of the individual bands on western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area. The average of density for the bands in control group is considered as 100%.

2.8. Measurement of Cholesterol and Triglyceride Contents in Liver. Lipids in mouse liver were extracted as described by Folch et al. [15, 16]. Cholesterol and triglyceride levels in extracted lipids were measured enzymatically using the reagents from Cayman Chemical (Ann Arbor, MI) according to the manufacturer's instruction.

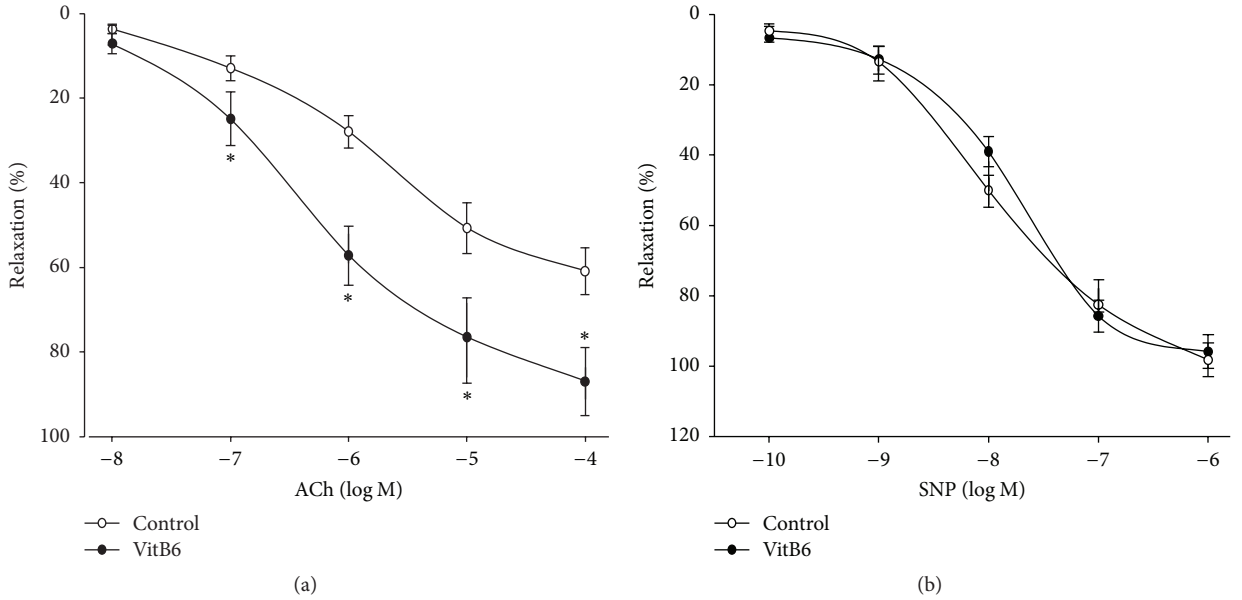

Figure 1: Administration of VitB6 prevents endothelial dysfunction in *Apoe*$^{-/-}$ mice fed with high-fat diet. Male *Apoe*$^{-/-}$ mice at the age of 6 weeks received high-fat diet and VitB6 (50 mg/kg/day) administration in drinking water for 8 weeks. At the end of experiments, mice were sacrificed under anaesthesia. The descending aortas were isolated and cut into rings. (a) ACh-induced endothelium-dependent relaxation and (b) SNP-induced endothelium-independent relaxation were determined by organ chamber as described in Section 2. All data were expressed as mean ± SEM. N is 10–15 in each group. $^{*}P < 0.05$ versus control.

2.9. Statistical Analysis. The results were expressed as mean ± SEM. One-way ANOVA followed by t-test was used for two groups' comparison. $P < 0.05$ was considered significant.

3. Results

3.1. VitB6 Prevents Endothelial Dysfunction in Apoe$^{-/-}$ *Mice Fed with HFD.* Endothelial dysfunction has been identified as an early hallmark of CVD, such as atherosclerosis and hypertension [17–20]. We firstly determined whether VitB6 prevents endothelial dysfunction in mice with metabolic syndromes. The hyperlipidemia model was induced by feeding *Apoe*$^{-/-}$ mice with HFD [21]. As indicated in Table 1, HFD in *Apoe*$^{-/-}$ mice dramatically increased serum levels of triglyceride, cholesterol, and LDL, indicating that the model is successfully established. Importantly, the random level of blood sugar was also increased in *Apoe*$^{-/-}$ mice fed with HFD. However, treatment of these mice with VitB6 did not alter the levels of triglyceride, cholesterol, and LDL, except for random level of blood glucose.

The endothelial function was determined by using ACh. As shown in Figure 1(a), ACh-induced vasorelaxation was significantly improved by VitB6. The SNP-induced vasorelaxation was not affected by VitB6 (Figure 1(b)), demonstrating that the protective effects of VitB6 on vascular function are limited to endothelium.

3.2. VitB6 Enhances Insulin Sensitivity in Apoe$^{-/-}$ *Mice Fed with HFD.* Insulin resistance is a high risk factor of endothelial dysfunction in CVD [22]. We next examined whether VitB6 improves insulin sensitivity in HFD-fed *Apoe*$^{-/-}$ mice. As shown in Figure 2(a), injection of D-glucose dramatically increased the levels of blood glucose (BG) in HFD-fed *Apoe*$^{-/-}$ mice. The peak level of BG was about 600 mg/dL after 30 minutes. The level of BG was back to the basal level after 90 minutes. However, administration of VitB6 delayed and lowered the peak levels of BG (470 mg/dL). After 90 minutes of glucose injection, the level of BG was also back to the basal level. These data indicate that VitB6 increases the tolerance of glucose.

The protective effect of VitB6 on glucose metabolism was further confirmed by measuring the sensitivity of insulin (Figure 2(b)). By injecting exogenous insulin into HFD-fed *Apoe*$^{-/-}$ mice, the levels of BG were reduced to 40% of basal level at the 60th minute and then went back to 80% of basal

Table 1: Serum sugar and lipid levels in *Apoe*$^{-/-}$ mice.

	WT	*Apoe*$^{-/-}$		
	ND	ND	HFD	HFD + VitB6
Glucose (mM)	6.5 ± 1.3	8.2 ± 1.3	13.5 ± 0.8	10.2 ± 0.9*
Cholesterol (mM)	3.7 ± 0.5	10.5 ± 2.1	28.4 ± 5.3	26.4 ± 4.9
Triglyceride (mM)	0.7 ± 0.2	0.8 ± 0.2	1.6 ± 0.3	1.5 ± 0.5
HDL-C (mg/L)	128 ± 15	252 ± 30	267 ± 38	257 ± 39
LDL-C (mg/L)	109 ± 14	249 ± 23	417 ± 53	435 ± 67

After 8-week administration of VitB6 in *Apoe*$^{-/-}$ mice fed with high-fat diet, serum sugar levels and lipid levels were determined. WT: wild-type; ND: normal diet; HFD: high-fat diet; HDL: high density lipoprotein; LDL: low density lipoprotein. All data were expressed as mean ± SEM. N is 10–15 in each group. $^{*}P < 0.05$ versus *Apoe*$^{-/-}$ mice fed with HFD.

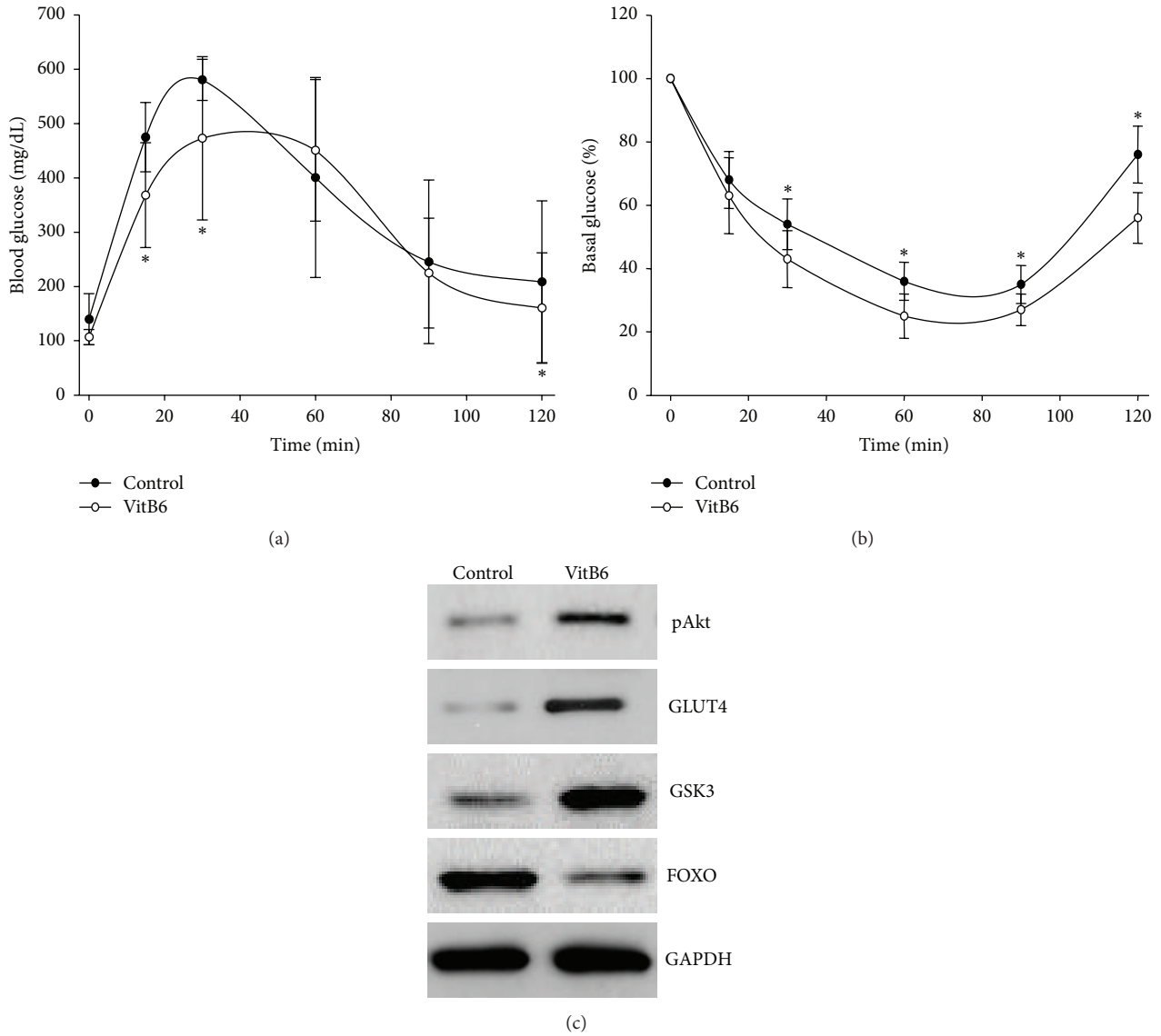

Figure 2: VitB6 improves insulin resistance in $Apoe^{-/-}$ mice fed with high-fat diet. Male $Apoe^{-/-}$ mice at the age of 6 weeks received high-fat diet and VitB6 (50 mg/kg/day) administration in drinking water. At the 8th weekend after VitB6 treatment, (a) GTT and (b) ITT were evaluated as described in Section 2. All data were expressed as mean ± SEM. N is 10–15 in each group. $^{*}P < 0.05$ versus control. (c) Homogenates of liver tissues were subjected to perform western blotting analysis to assay the levels of pAKt, GLUT4, GSK3, and FOXO. The picture is a representative blot from 10–15 mice.

level at the 120th minute. However, VitB6 further reduced the level of BG at the 90th minute to 25%. After the 120th minute, the level of BG was 55% of the basal level. Collectively, this suggests that VitB6 enhances insulin sensitivity in mice.

3.3. Increased Hepatic Levels of pAkt, GSK3, and GLUT4 Proteins and Decreased FOXO Protein Expression in VitB6-Treated $Apoe^{-/-}$ Mice. The beneficial effects of VitB6 on insulin resistance were further examined by assaying the hepatic levels of pAkt, GSK3, GLUT4, and FOXO, which are proteins related to glucose metabolism [23]. As depicted in Figure 2(c), compared to HFD-fed $Apoe^{-/-}$ mice, the levels of pAkt, GSK3, GLUT4, and BG were increased and the level of FOXO was reduced in HFD-fed $Apoe^{-/-}$ mice with VitB6, further supporting the notion that VitB6 improves insulin resistance in mice.

3.4. VitB6 Treatment Prevents Hepatic Lipid Accumulation in Mice. NAFLD is characterized by insulin resistance [24]. Thus, we detected the liver function in these mice. In Table 2, the markers of liver function, such as ALB, ALP, ALT, AST, and TB, were comparable in HFD-fed $Apoe^{-/-}$ mice with or without VitB6 treatment. Histological analysis of HE staining in liver sections from mice at the end of the experiment

Table 2: The indexes for liver function in mice.

	Control	VitB6
AST (IU/L)	132.2 ± 27.9	157.4 ± 31.8
ALT (IU/L)	186.7 ± 21.3	195.7 ± 28.6
TB (mg/dL)	0.13 ± 0.07	0.15 ± 0.09
ALP (IU/L)	255.5 ± 32.8	279.8 ± 35.8
ALB (g/L)	17.2 ± 1.4	20.6 ± 16.8

After 8-week administration of VitB6 in $Apoe^{-/-}$ mice fed with high-fat diet, serum levels of AST, ALT, TB, ALP, and ALB were determined. All data were expressed as mean ± SEM. N is 10–15 in each group.

Table 3: The levels of serum VitB6, homocysteine, folate, and VitB12 in patients with fatty liver.

	Control (57)	Patients (49)
VitB6 (PLP, nM)	55.8 ± 10.7	23.9 ± 8.1*
VitB12 (pg/mL)	686.7 ± 21.3	518.7 ± 28.6*
folic acid (ng/mL)	8.3 ± 0.7	7.7 ± 0.9
Homocysteine (nM)	15.8 ± 2.8	22.8 ± 5.4*

Serum levels of VitB6, VitB12, folic acid, and homocysteine were determined in patients with fatty liver and control subjects. All data were expressed as mean ± SEM. $^*P < 0.05$ versus control.

(Figure 3(a)) revealed that HFD caused marked neurosis and fibrosis, which was reversed by VitB6 treatment, suggesting that VitB6 is effective to protect the liver.

The typical feature of NAFLD is the elevated hepatic lipid accumulation [25, 26]. We next investigated whether VitB6 prevents hepatic lipid accumulation in hyperlipidemia mice by Oil Red O staining (Figure 3(a)). Compared to control HFD-fed $Apoe^{-/-}$ mice, the contents of liver triglycerides (Figure 3(b)) and cholesterol (Figure 3(c)) were decreased, demonstrating that VitB6 prevents hepatic lipid accumulation in mice and is potentially considered to serve as prevention of NAFLD.

3.5. Plasmatic Lower Levels of VitB6 in Patients with Fatty Liver. Finally, in order to establish the clinical association between VitB6 deficiency and NAFLD, we performed clinical and biochemical analysis. As described in Table 3, fifty-seven healthy humans and forty-nine patients had a clinical and biochemical analysis completed in the study. Compared to the healthy human subjects, the levels of folic acid were similar in patients with fatty liver. The levels of VitB12 were lightly increased. However, the levels of homocysteine in NAFLD patients were significantly increased, consistent with other reports [27, 28]. Most importantly, we found that the levels of VitB6 were lower in NAFLD than control healthy humans. These results indicate that deficiency of VitB6 might be a risk factor of NAFLD clinically.

4. Discussion

In the present study, we provide the first evidence that administration of VitB6 prevents endothelial dysfunction, insulin resistance, and hepatic lipid accumulation in $Apoe^{-/-}$ mice fed with HFD *in vivo*. Clinically, the serum level of VitB6 is low in patients with NAFLD. Our data not only indicate that VitB6 protects endothelial function and improves insulin resistance, but also imply that low VitB6 status might be a risk factor of NAFLD, as a component of metabolic syndrome.

The major discovery in the present study is that VitB6 produces several beneficial effects to prevent metabolic syndrome, such as insulin resistance and NAFLD. Traditionally, VitB6, in the form of PLP, is the coenzyme of 5 enzymes in these metabolic pathways: cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CGL), cytoplasmic and mitochondrial serine hydroxymethyltransferase (cSHMT and mSHMT), and glycine decarboxylase (GDC) in the mitochondria [29]. In this way, VitB6 regulates the transsulfuration pathway which contributes to homocysteine regulation and provides cysteine synthesis and consists of sequential reactions catalyzed by CBS and CGL. CBS catalyzes the condensation of homocysteine and serine to form cystathionine in a reaction that is subject to positive allosteric regulation by S-adenosylmethionine (SAM), whereas CGL catalyzes the cleavage of cystathionine to yield α-ketobutyrate, ammonia, and cysteine. Because both CBS and CGL require PLP as a coenzyme, inadequate VitB6 status might lead to impaired regulation of cellular homocysteine concentration. High levels of homocysteine impair endothelial function and cause metabolic syndrome including insulin resistance and lipid accumulation in liver. HHCY might play a role in the pathogenesis of vascular disorders and is considered as an independent risk factor for atherosclerosis [30]. From our observations, supplementation of VitB6 should be a helpful therapy to improve endothelial dysfunction and metabolic syndrome. Of course, the mechanism of VitB6 in prevention of metabolic syndrome needs further investigations.

We also identified VitB6 deficiency as a new risk factor of NAFLD. Obesity, metabolic syndrome, and type 2 diabetes mellitus are strictly related and are key pathogenetic factors of NAFLD, the most frequent liver disease worldwide. NAFLD is a clinicopathological syndrome including a wide spectrum of liver damage instances, ranging from hepatic steatosis to nonalcoholic steatohepatitis (NASH) to cirrhosis [31]. Epidemiologic studies showed that low VitB6 nutritional status is associated with increased risk of CVD, venous thrombosis, stroke, and possibly colon cancer [32]. Although a connection between VitB6 status and lipid metabolism has appeared periodically for more than 80 years, there is no evidence to support the role of PLP in NAFLD. To our knowledge, this is the first study to investigate whether marginal VitB6 deficiency affects hepatic lipid accumulation in human adults. We observed a significant decrease of plasma PLP concentration in patients with NAFLD. A potential mechanism responsible for the observations of lower plasma VitB6 level linking to NAFLD is impairment of PUFA interconversion because it has been reported that marginal VitB6 deficiency decreases plasma (n-3) and (n-6) PUFA concentrations in healthy men and women [33]. Further investigation should focus on the direct target of VitB6 on regulation of lipid metabolism in liver.

A limitation of this study is that $Apoe^{-/-}$ mouse is suitable for studying atherosclerosis resulting from hypercholesterolemia. Additionally, this mouse has several intriguing

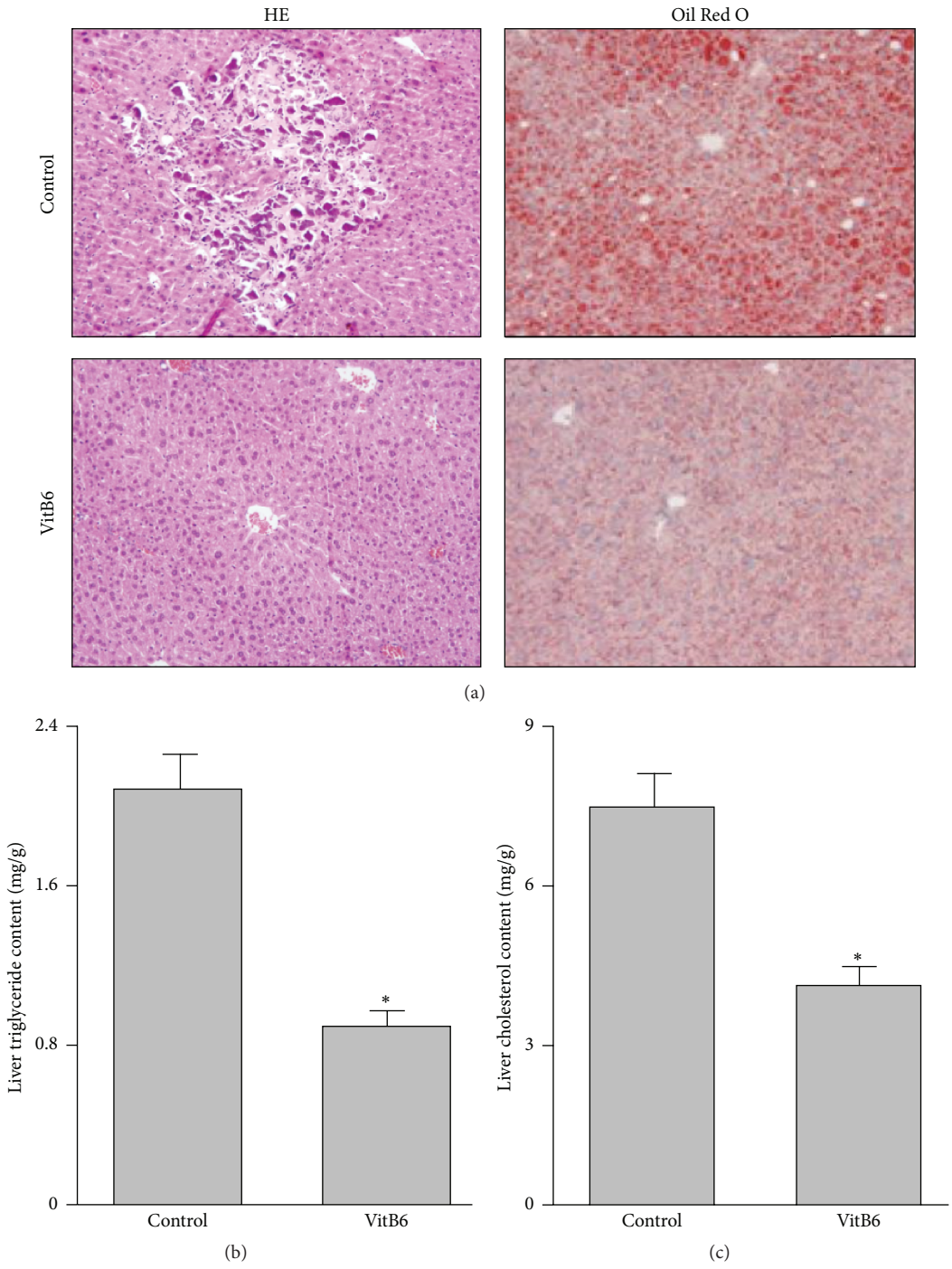

FIGURE 3: VitB6 reduces hepatic lipid accumulation in *Apoe*$^{-/-}$ mice fed with high-fat diet. Male *Apoe*$^{-/-}$ mice at the age of 6 weeks received high-fat diet and VitB6 (50 mg/kg/day) administration in drinking water. At the end of experiments, mice were sacrificed under anaesthesia. (a) Histological analysis of liver tissue by HE or Oil Red O staining. (b and c) Liver lipids were extracted and hepatic triglyceride and cholesterol levels were assayed using a commercial kit. The quantitative data were expressed as mean ± SEM. N is 10–15 in each group. $^{*}P < 0.05$ versus Control.

characteristics. First, *Apoe*$^{-/-}$ mice show obesity-resistant phenotype, resulting in remarkable insulin sensitivity. Second, this mouse has hepatic steatosis due to impairment of VLDL secretion from liver. Third, this mouse basically possesses endothelial dysfunction damaged from excess beta lipoprotein. It would be better to investigate the metabolic effects of vitamin B6 on wild-type mice with diet-induced metabolic disorders, such as C57B16 strain.

In summary, the results of this study have shown that low VitB6 status has substantial effects on metabolism including

glucose and fatty acid. The results of this study also demonstrate that the deficiency of VitB6 might be a risk factor of NAFLD.

Conflict of Interests

The authors confirm that there is no conflict of interests.

Authors' Contribution

Zhan Liu and Peng Li designed and performed all experiments, analyzed the data, and wrote the paper. Zhi-Hong Zhao, Yu Zhang, and Zhi-Min Ma collected the clinical samples. Zhan Liu and Shuang-Xi Wang conceived the project and wrote the paper. Zhan Liu and Peng Li contributed equally to this work.

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Research Article

Effect of Vitamins C and E on Endothelial Function in Type 1 Diabetes Mellitus

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Background/Objectives. Endothelial dysfunction due to hyperglycemia-induced oxidative damage is an important predictor of future cardiovascular risk in patients with type 1 diabetes mellitus (T1DM) and is present in adolescent T1DM. We hypothesized that combined treatment with the antioxidant vitamins C and E might improve endothelial function (EF) and other biochemical risk factors in adolescents with T1DM. *Subjects/Methods*. Open-label antioxidant supplementation was given for six weeks with endpoint measurements collected at baseline and study completion. Endpoints measured included EF and plasma measurements of biochemical endothelial risk. *Results*. Two males and 7 females were studied. Mean age was 12.9 ± 0.9 yrs; mean T1DM duration was 5.5 ± 2.5 yrs; mean BMI was 22.1 ± 3.8 kg/m^2; and mean hemoglobin A1c was 9.3 ± 1.1%. No differences were found for EF, high sensitivity CRP, total antioxidant capacity, adiponectin, or endothelial progenitor cells (EPCs) between before and after combined vitamin C and E therapy. *Conclusions*. Our negative study results do not support previous findings of decreased oxidative damage, improved endothelial function, and increased vascular repair capacity with antioxidant therapy. Longer term studies may be needed to determine the effects, if any, of combined antioxidant therapy on EPCs, EF, and markers of micro- and macrovascular complications in T1DM.

1. Introduction

Type 1 diabetes mellitus (T1DM) is a chronic medical condition requiring intensive treatment and optimal glycemic control in order to prevent and/or delay onset and/or progression of long term complications. Cardiovascular disease (CVD) is the major cause of death for patients with T1DM in the United States. The mortality and morbidity of CVD are strikingly increased in individuals with T1DM compared to their nondiabetic counterparts [1, 2] with a 6–13-fold higher risk of cardiovascular death [3]. Atherosclerosis, the major pathophysiologic precursor of cardiovascular disease and ultimate cause cardiovascular morbidity and mortality, begins in childhood and adolescence [4]. Endothelial dysfunction is an early marker of atherosclerosis that begins early in T1DM and is directly involved in diabetes-induced microvascular and macrovascular complications [1, 3, 5–9]. Although clinically manifested diabetes-related microvascular (e.g., retinopathy, nephropathy, and neuropathy) and macrovascular complications (e.g., cardiac disease and peripheral vascular disease) are rare during childhood or adolescence, it is evident that early potentially modifiable vascular functional and structural changes begin soon after diagnosis.

Endothelial dysfunction, vascular inflammation, build-up of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall are characteristic features of atherosclerosis [10]. The development of atherosclerosis in T1DM begins in childhood and adolescence as a largely

silent process as shown by increased intima-media thickness (IMT) of the carotids and aorta [11, 12], impaired endothelial function, and altered endothelial repair capacity. Impaired endothelial function and altered endothelial repair have been observed in T1DM. Previous studies have not only found reduced flow mediated vasodilation [11, 13, 14], a measure of endothelial function, but also decreased CD34+ CD133+ CD31+ circulating progenitor cells (CPCs) cells with increased CD34+ CD45− endothelial colony forming cells (ECFCs) [15], an indicator of altered endothelial repair, in children and adolescents with T1DM. Additionally, hyperglycemia-induced oxidative stress plays a pivotal pathophysiological role in impairing endothelial function in T1DM [6, 7]. Numerous studies have demonstrated that hyperglycemia not only increases free radicals but also impairs endogenous antioxidant defense in T1DM. Children with T1DM have evidence of increased oxidative stress in blood and reductions in various aspects of antioxidant defense, including reduced endogenous levels of vitamins C and E, glutathione, decreased levels of superoxide dismutase, total antioxidant capacity (TAC), increased levels of malondialdehyde, and oxidized LDL [16, 17].

While optimal glycemic control has been associated with improved cardiac and peripheral vascular function and the prevention of later vascular complications, discovering a means of reducing or eliminating these early vascular changes of CVD seen in childhood that is either independent of or augmenting optimal glycemic control would be a major medical breakthrough. Enhanced antioxidant intake (either via diet or supplementation) may be one such means of reducing the risk of diabetes-related microvascular and macrovascular complications later in life, increasing the lifespan and wellness, and reducing anxiety and frustration in patients with T1DM.

The objective of this study was to determine effects of antioxidant therapy, specifically vitamins C and E, on antioxidant capacity, inflammatory markers (CRP and IL6), and endothelial function, and define their interrelationships in adolescents with T1DM. We also set out to determine the effects of one month of combined antioxidant therapy on numbers of endothelial colony forming cells (ECFCs: CD34+ CD133+ CD45−) in adolescents with T1DM. We hypothesized that antioxidant vitamin therapy would decrease oxidative damage thereby improving endothelial function and increase vascular repair capacity in adolescent T1DM.

2. Materials and Methods

2.1. Subjects. Nine children and adolescents aged 8–15 years (mean age ± SD) with T1DM were included in this study. These subjects were recruited from Pediatric Diabetes Clinics Nationwide Children's Hospital in Columbus, Ohio. Informed consent was obtained from a parent or legal guardian and informed assent was obtained from the subject. The study was approved by the Institutional Review Board of Nationwide Children's Hospital. The initial diagnosis of T1DM will have been made clinically. They must have been started on insulin therapy immediately after diagnosis and never have received an oral hypoglycemic agent.

For inclusion, subjects were between 8 and 15 years of age with BMI ≤ 95% tile for age, pubertal stage of Tanner stages 2–4, BP ≤ 95% tile for age, on insulin therapy since diagnosis, fasting c-peptide <0.4 ng/mL, normal thyroid function tests, random urine albumin to creatinine ratio <0.02 mg albumin/mg creatinine, and creatinine ≤1.0 mg/dL. Subjects with BMI ≥ 95% tile, BP > 95% tile, Tanner 1 or 5 pubertal status, pregnancy, smoking, history of oral hypoglycemic use, acanthosis nigricans, fasting c-peptide ≥0.4 ng/mL, abnormal thyroid function tests, random urine albumin to creatinine ratio ≥0.02 mg albumin/mg creatinine, creatinine >1.0 mg/dL, or use of any medications other than insulin, levothyroxine with stable dosage, or oral contraceptives were excluded from the study.

2.2. Protocol. This was an open-label antioxidant supplementation for six weeks with endpoint measurements collected at baseline and study completion. The subjects were seen twice at the Clinical Research Center of the Clinical and Translational Study Center of The Ohio State University. The first visit occurred the morning after a minimum of 10-hour fasting. Subjects were instructed to take their usual insulin the day before their study visit and to take any morning basal insulin. Subjects using continuous insulin pump therapy were continued on their usual basal insulin rates. Morning rapid acting insulin injection or insulin pump boluses were held. Baseline blood samples were drawn for total plasma antioxidant capacity (TAOC), high-sensitivity C-reactive protein (hsCRP), and measurement of endothelial progenitor cells (EPCs). Female participants were evaluated during the first two weeks of their menstrual cycle in an attempt to minimize the possible effects of hormonal changes on endothelial function [18, 19]. Endothelial function was then measured as described below. After completion of the measurements subjects took their morning rapid acting insulin injection or insulin pump bolus and were given breakfast. The second study visit was identical to the first with measurement of all endpoints. Subjects were instructed to return all medications at visit two.

2.3. Endothelial Function Measurement. Endothelium dependent vasodilatory response was quantified as the mean post-upper arm occlusion forearm vascular resistance (FVR) and the percent change in forearm vascular resistance from pre- to postocclusion. Forearm blood flow (FBF) was measured using strain gauge venous occlusion plethysmography using a Hokanson AI6 plethysmograph. Two minutes of baseline FBF was recorded after which the upper arm cuff was inflated to 200 mmHg pressure for 5 minutes to occlude flow. It was then released and FBF was measured for one minute. FVR was calculated by dividing mean arterial blood pressure by FBF. Arterial blood pressure was measured using an automated sphygmomanometer. This method of testing endothelial function assesses resistance vessel function. Results closely correlate with results from endothelial function assessed by intra-arterial acetylcholine infusion [20] and correlate well with the nitrite/nitrate ratio, an index of NO synthesis [21].

2.4. Vitamin Supplementation. Prior to discharge, subjects were started on combined vitamins C and E. We scaled our supplement dosing to account for subject size, as shown in Table 1; these doses are equivalent to a dose of 1 g of vitamin C and 400 IU of vitamin E per day in a 75 kg adult [16]. Subjects were instructed to take the medication once daily and were instructed to continue their routine diabetes care between visits.

2.5. Laboratory Assays. High-sensitivity C-reactive protein (hsCRP) and total plasma antioxidant capacity (TAOC), a measure of oxidative stress, were measured for each subject at their baseline fasting glucose level. TAOC is a nonspecific assay of antioxidant defense which measures the ability of constituents in plasma to absorb oxidation (BioVision Research Products, Mountain View, CA). Adiponectin was measured using a kit from R&D Systems Inc. Minneapolis, MN, Cat. Number 1065. ECFCs were measured using polychromatic flow cytometry methods. A 50 μL volume anticoagulated peripheral blood was incubated with 50 μL 3% BSA in PBS (without Ca++ and Mg++) at room temperature for 30 min. In dark, fluorescence labeled antibodies (2.5 μL of each), PE-AC133, FITC-CD34, and PECy5-CD45, were added and incubated for 30 min at room temperature. FACS lysis buffer (450 μL) was then added and incubated for 30 min at room temperature in dark. Samples were then analyzed on FACS Caliber flow cytometer, where total counts are >400,000 cells. Intra-assay variability from ~100 μL whole blood was <5%.

2.6. Statistical Analysis. Data were analyzed using a paired t-test. Results are presented as mean ± SD.

Table 1: Antioxidant dosage.

Dosing body weight	Daily vitamin C dose (mg)	Daily vitamin E dose (IU)
<30 kg	250 mg	100 IU
30–60 kg	500 mg	200 IU
>60 kg	750 mg	300 IU

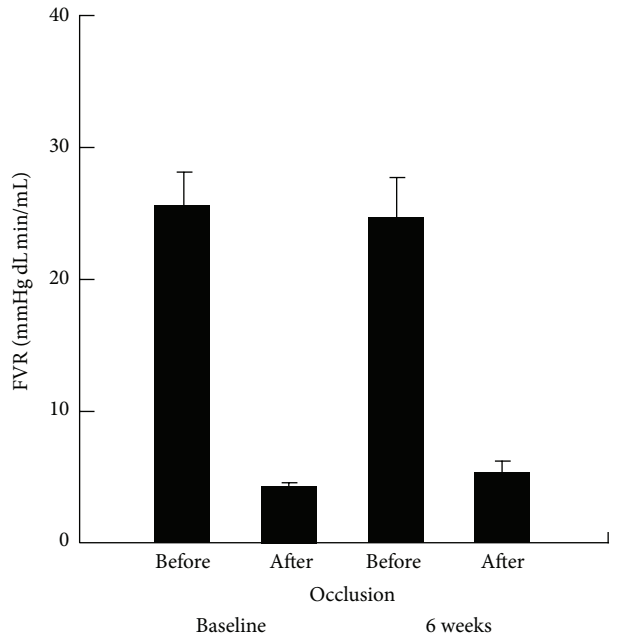

Figure 1: FVR response before and after occlusion before and after combined vitamin C and E therapy.

3. Results

Two males and 7 females were studied. Mean age was 12.9 ± 0.9 years (range, 11.2 to 13.9 years): mean duration of diabetes was 5.5 ± 2.5 years (range, 0.2 to 9.5 years); mean BMI was 22.1 ± 3.8 kg/m^2 (range 18.2 to 29.4 kg/m^2); and mean hemoglobin A1 was 9.3 ± 1.1% (range, 8.2% to 11.3%). No differences were seen in the FVR response to occlusion between before and after combined vitamin C and E therapy (Figure 1). No differences were seen in hsCRP, TAOC, adiponectin, or EPC percent before or after vitamin C and E therapy (Table 2).

4. Discussion

Oxidative stress plays a significant role in the chronic complications of insulin-dependent diabetes mellitus. Our study set out to determine the effects of antioxidant therapy on antioxidant capacity, inflammatory markers, endothelial function, and numbers of ECFCs, in adolescents with T1DM. The oxidative stress that impairs endothelial function in T1DM is induced by hyperglycemia [6, 7]. This hyperglycemia also impairs the endogenous antioxidant defense in T1DM. Children with T1DM have evidence of reduced levels of endogenous antioxidants vitamins C and E [16, 17]. Under normal conditions, vitamin E suppresses the propagation of lipid peroxidation and scavenges free radicals; and vitamin C with vitamin E inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation and inhibit Fenton- and Haber-Weiss-type reactions (i.e., generating hydroxyl radicals (OH) from hydrogen peroxide (H_2O_2) and superoxide (O_2^-)) [22–25]. We hypothesized that antioxidant vitamin therapy would decrease oxidative damage thereby improving endothelial function and vascular repair capacity in adolescent T1DM. This hypothesis proved to be incorrect as we found no changes in postocclusive FVR, hsCRP, TAC, adiponectin, or ECFCs.

Low vitamin C levels in type 1 diabetes are associated with increased transcapillary albumin escape [26] and increased atherosclerotic damage as evidenced by a negative relationship to IMT in children [27]. Previous studies with ascorbic acid (vitamin C) had suggested potential benefit. Antioxidant treatment with acute intravenous ascorbic acid or 10 days of oral ascorbic acid blocks the dilatory effect of hyperglycemia on endothelial function during hyperglycemic clamp [28] or oral glucose tolerance testing [29] in healthy adults. A 12-hour ascorbic acid infusion restores endothelial function to normal

Table 2: Biochemical markers and ECFCs at baseline and after 6 weeks of vitamin C and E therapy in adolescents with type 1 diabetes.

	hsCRP (ng/mL)	TAOC (Trolox equivalent/L)	Adiponectin (ng/mL)	ECFC (%)
Baseline	0.63 ± 0.85	95 ± 6	3970 ± 1150	0.027 ± 0.015
Vitamins C and E	0.74 ± 0.88	102 ± 12	4330 ± 1280	0.032 ± 0.037

levels in adults with recent onset or intermediate duration, well-controlled type 1 diabetes [30, 31]. In patients with poorly controlled type 1 diabetes ascorbic acid infusion, alone, only partially restores endothelial function. Acute ascorbic acid infusion blocks the acute effects of hyperglycemia in adolescents with type 1 diabetes [32]. Therapeutically, vitamin C decreases transcapillary albumin escape [26] and urinary albumin excretion [33] in adults with T1DM. A previous study by Varvaroksa et al. found in children with T1DM the combination of vitamins C and E decreased hemoglobin A1c and glycated protein levels and increased superoxide dismutase and reduced glutathione [16]. However, based on our results, combined vitamin C and E antioxidant intake via supplementation did not improve endothelial function, ECFCs, or other nontraditional risk factors.

Limitations of our study may include the short duration of vitamin C and E supplementation for 6 weeks which may have been inadequate to significantly decrease oxidative damage, improve endothelial function, and increase vascular repair capacity. The strategy (e.g., dosage and duration) was chosen based on studies in adults, in many varying trials, which showed that vitamin C (at doses 1 g/day or less) and vitamin E (at doses of 400 IU/day or less) were consistently shown to be safe and in many specific settings relevant to this study to have efficacy in reducing oxidative stress in vivo and/or affecting markers of disease. Our study utilized these two supplements in combination, primarily since we do not know if water soluble or fat soluble strategies are best in this setting and since it is clear that these and other small molecule antioxidants are compartmentalized in vivo and interact to affect cellular redox status [34]. Also other studies have suggested that lower doses of multiple antioxidants may indeed be superior (and safer than) to mega doses of one constituent [35–37]. Also our small sample size may not have been large enough to demonstrate significant differences. The lack of a normal control group is also another limitation to this study. The differences seen were small and even with a sample size large enough to find statistical significance are unlikely to be of any clinical significance.

5. Conclusions

Our negative study results do not support previous findings of decreased oxidative damage, improved endothelial function, and increased vascular repair capacity with antioxidant therapy. Further research into other means of reducing or eliminating vascular changes of CVD seen in childhood that is either independent of or augmenting optimal glycemic control is indeed warranted. Ideally these studies would begin at time of diagnosis of T1DM before the development of diabetic complications. Longer term studies are necessary to determine the effects, if any, of combined antioxidant therapy on ECFCs, endothelial function, and markers of microvascular and macrovascular complications in T1DM.

Abbreviations

BSA: Body surface area
ECFC: Endothelial colony forming cells
EPC: Endothelial progenitor cells
FBF: Forearm blood flow
FVR: Forearm vascular resistance
hsCRP: High-sensitivity C-reactive protein
TAOC: Total plasma antioxidant capacity
T1DM: Type 1 diabetes mellitus.

Conflict of Interests

The authors declare no conflict of interests.

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Clinical Study

Effect of Acarbose on Long-Term Prognosis in Acute Coronary Syndromes Patients with Newly Diagnosed Impaired Glucose Tolerance

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Objective. To investigate the effect of acarbose therapy on the long-term prognosis of patients with acute coronary syndromes (ACS) complicating newly diagnosed impaired glucose tolerance (IGT). *Methodology*. 135 patients hospitalized for ACS who had been newly diagnosed with IGT were randomly assigned to acarbose group (150 mg/day, $n = 67$) or control group (no acarbose, $n = 68$). All cases in each group were given the same elementary treatment. Mean follow-up was 2.3 years. The incidence of major adverse cardiovascular event (MACE) and carotid intima-middle thickness (CIMT) were statistically analyzed. *Results*. During the mean follow-up of 2.3 years, the risk of recurrent MACE in acarbose group was decreased significantly compared with that in control group (26.67% versus 46.88%, $P < 0.05$); at the same time, thickening of the CIMT was significantly slower than the control group ((1.28 ± 0.42) mm versus (1.51 ± 0.64) mm, $P < 0.05$). *Conclusions*. Acarbose can effectively reduce the risk of MACE in ACS patients with newly diagnosed IGT, simultaneously retarding the progression of carotid intima-media thickness.

1. Introduction

As gradual steps into aging society and changes of lifestyle, the prevalence of impaired glycometabolism and coronary artery disease (CAD) increases rapidly in China. Impaired glucose tolerance (IGT) has been regarded as a prediabetic state in which postprandial blood glucose is between normal glucose tolerance and overt diabetes mellitus (DM). It is well known that IGT is an independent risk factor of cardiovascular events (CV) and cardiovascular-associated mortality [1]. Acarbose can effectively reduce postprandial blood glucose and the progression from IGT to Type 2 DM (T2DM). STOP-NIDDM study had proved that acarbose therapy reduced the risk of any CV by 49%, of an acute myocardial infarction (AMI) by 91%, and of developing hypertension by 34% in IGT patients [2]. Emerging evidence suggested a high prevalence of unrecognized IGT and/or DM in patients admitted to hospital with acute coronary syndrome (ACS) [3]. Furthermore, such dysglycaemia has been shown to be associated with an increase in cardiovascular mortality. Therefore, we have reason to postulate that acarbose treatment may reduce major adverse cardiovascular event (MACE) in patients with ACS complicating impaired glycometabolism. No related research has been reported so far. Thus, the goal of present study is to determine whether acarbose can reduce the risk of recurrent MACE in ACS patients with newly diagnosed IGT.

2. Research Design and Methods

2.1. Study Patients. From March 2010 to August 2013, we randomly selected 426 cases of patients who were hospitalized in our two hospitals due to ACS. ACS was diagnosed by the presence of acute ischemic symptoms lasting ⩾20 min within 48 h before admission to hospital and electrocardiographic changes consistent with ACS [4]. Acute myocardial infarction (AMI) was diagnosed when creatine kinase-MB levels increased to at least twice the upper limit of normal or when troponin T levels were >0.1 ng/mL. Patients without AMI

Table 1: Baseline characteristics of study patients and frequencies of medication usage before admission.

	Control group ($n = 68$)	Acarbose group ($n = 67$)	P value
Basic characteristic			
Age, year	61.62 ± 4.58	62.24 ± 5.16	0.461
Male, n (%)	42 (61.76)	39 (58.21)	0.673
Smoking, n (%)	31 (45.59)	28 (41.79)	0.657
AMI, n (%)	43 (63.24)	46 (68.66)	0.506
Hypertension, n (%)	46 (67.65)	45 (67.16)	0.637
LVEF, %	52.13 ± 4.81	51.74 ± 5.25	0.653
Revascularization (PCI/CABG), n (%)	38 (55.88)	41 (61.19)	0.531
BMI, kg/m^2	25.82 ± 2.45	26.05 ± 3.24	0.427
Medications			
β-blocker, n (%)	16 (23.53)	13 (19.40)	0.559
ACE-I/ARB, n (%)	47 (69.12)	45 (67.16)	0.808
CCB, n (%)	18 (26.47)	20 (29.85)	0.662
Statin, n (%)	63 (92.65)	61 (91.04)	0.734
Aspirin, n (%)	64 (94.12)	62 (92.54)	0.713

AMI: acute myocardial infarction; PCI: percutaneous coronary intervention; CABG: coronary artery bypass grafting; BMI: body mass index; ACE-I: angiotensin-converting enzyme inhibitor; ARB: angiotensin II receptor blocker; CCB: calcium channel blocker.

were considered to have unstable angina pectoris (all cases confirmed by percutaneous or computed tomography coronary angiography). Exclusion criteria were as follows: (1) cardiogenic shock or pulmonary edema (Killip classification ⩾ II) at admission, (2) history of diabetes, (3) history of hepatic diseases or/and renal dysfunction (serum creatinine level >2 mg/dL), (4) severe gastrointestinal disease or malignant tumors, and (5) female patients given sex hormone replacement therapy. All cases underwent the standard 75 g oral glucose tolerance test (OGTT) two weeks after admission, and we used the WHO criteria to classify the OGTT results. IGT was defined as having a fasting plasma glucose (FPG) <6.1 mmol/L and a postprandial glucose level at 120 min after the glucose load (2 hPG) ⩾7.8 mmol/L but <11.1 mmol/L. 135 cases were newly diagnosed with IGT. The present study followed Helsinki principle which was reviewed and approved by the Ethics Committee; all patients and their families provided informed consent.

2.2. Methods. 135 IGT patients were randomly allocated, using random numbers generated by a computer, into the following two groups: the control group (68 cases) and acarbose group (67 cases). Each group was given standard medical therapy of CAD (including nitrate medications, ACE-I/ARB, β-blockers, statins, and antiplatelet drugs). Acarbose group was given additional acarbose (Bayer Pharmaceutical Co., Germany, three times/day, 25 mg/time start, and gradually increasing the amount to 50 mg/time in 2 weeks) on the base of fundamental treatment. Carotid intima-media thickness (CIMT) was measured using Siemens SEQUOIA512 ultrasonography, taking the point under 1 cm of carotid sinus as detection point and accounting the average of the left and right CIMT as the results. The carotid IMT was measured at baseline, and follow-up of all subjects, ΔCIMT, indicated the changes in CIMT. All patients were guided to take diet and exercise therapy, and having outpatient clinic or telephone follow-up for 1.0–4.5 years, the mean follow-up was 2.3 years. Incidence of MACE (including fatal cardiovascular events, nonfatal reinfarction, new-onset angina, cerebral stroke, and severe heart failure) was recorded.

2.3. Statistical Analysis. Statistical analysis was performed using SPSS 13.0. Continuous variables were expressed as mean and standard deviation, and categorical variables were expressed as using numbers and percentages. Comparisons among the two groups were performed using Student's t-test and paired t-test for continuous variables and χ^2 test for categorical variables. Values for P less than 0.05 were accepted as statistically significant.

3. Results

3.1. Clinical Background. The clinical profile of the subjects was shown in Table 1. There was no significant difference in age, gender, profiles for traditional risk factors, and past medication history between the two groups ($P > 0.05$). Of the total of 135 cases in the average follow-up of 2.3 years, 11 patients dropped out during the study which was 8.15% of all subjects (7 patients in the acarbose group: 5 male and 2 female; 4 patients in the control group: 2 male and 2 female). The cause was severe abdominal distension and diarrhea for 6 cases in the acarbose group, or lost for 1 case in acarbose group and 4 cases in control group, respectively; the incidence of serious gastrointestinal adverse reactions between the two groups was statistically significant ($\chi^2 = 6.373$, $P = 0.012$).

3.2. The Risk of Recurrent MACE. Recurrent MACE was observed in the 124 cases that completed the study. Table 2

Table 2: MACE among the study patients in the mean 2.3-year follow-up (n, %).

	Control group ($n = 64$)	Acarbose group ($n = 60$)
Cardiovascular death, n (%)	5 (7.81)	3 (5.00)
Nonfatal reinfarction, n (%)	7 (10.94)	2 (3.33)
New-onset angina, n (%)	9 (14.06)	5 (8.33)
Cerebral stroke, n (%)	4 (6.25)	2 (3.33)
Severe heart failure, n (%)	5 (7.81)	4 (6.67)
Total MACE, n (%)	30 (46.88)	16 (26.67)

MACE: major adverse cardiovascular events.

demonstrated the numbers of MACE among subjects in the mean 2.3-year follow-up. The incidence of total MACE in the acarbose group and control group was 26.67% and 46.88%, respectively; there was significant difference between the two groups ($\chi^2 = 5.420$, $P = 0.020$). In total MACE, the death of 3 patients in acarbose group and 5 patients in control group was due to cardiovascular events, and the cardiovascular caused mortality between the two groups was of no significant difference ($\chi^2 = 0.406$, $P = 0.524$). However, the incidence of the secondary end-point events (nonfatal reinfarction, new-onset angina, cerebral stroke, and severe heart failure) of the acarbose group was prominently lower than that of control group (21.67% versus 39.06%, $\chi^2 = 4.410$, $P = 0.036$).

3.3. Laboratory and Ultrasonography Examination. Table 3 shows the level of biochemical indicators, CIMT, blood pressure, and BMI of the two groups before and after treatment. All the indexes were not significantly different between the two groups before treatment ($P > 0.05$). After treatment, 2 hPG, HbA1c, CIMT, and ΔCIMT of the acarbose group were significantly lower than control group (t value was 8.731, 6.198, 2.440, and 18.622, resp., $P < 0.05$ or 0.01), while FPG, TC, TG, LDL-C, systolic pressure, diastolic pressure, and BMI had no statistical significance between the two groups after treatment (t value was 1.528, 0.242, 1.102, 0.214, 1.201, 0.883, and 1.866, resp., $P > 0.05$).

4. Discussion

Postprandial hyperglycemia is a hallmark characteristic in individuals with IGT and early T2DM and has been established as a key pathophysiological component of the mechanism underlying the development of diabetic complications [5]. Fluctuations in glucose levels following a meal are strongly associated with micro- and macrovascular complications not only in patients with T2DM, but also in individuals with IGT [6]. The risk for developing CAD or other MACE was increased almost threefold in subjects with IGT compared to people with normal glucose tolerance [7]. Kataoka et al. [8] found that multibranch coronary artery lesion which was common in T2DM patients had already emerged in the IGT stage. Recent study indicated that IGT was an independent risk factor for AMI and simultaneously increased the risk of recurrent CV after AMI [9]. Furthermore, Kitada et al. [10] showed that postprandial blood glucose above 8.9 mmol/L would increase the risk of recurrent MACE nearly one-fold in patients with AMI. There is a high prevalence of unrecognized dysglycaemia in patients with ACS; the present study found that 31.69% of the patients with ACS were newly diagnosed IGT; it was similar to previous researches [3, 11]. Thus, early intervention to IGT in ACS patients with the aim of reducing recurrent MACE should be anticipated.

IGT is a prediabetic state; several management strategies have been proposed for this early stage of dysglycaemia, including lifestyle modification and pharmacotherapies (e.g., acarbose, metformin, and thiazolidinediones) [12–14]. Although lifestyle modification is a vital part of dysglycaemia management, it is often insufficient to maintain long-term glycaemic control. Given that acarbose has a relatively modest efficacy of blood glucose lowering and can be administered to patients with heart failure and mild to moderate renal insufficiency, acarbose is safer than other drugs mentioned above for glucose management in ACS patients, because management of glucose levels within a given range and with minimal risk of hypoglycemia is recommended for the treatment of hyperglycemia in patients with ACS [15]. In the present study, 2 hPG and HbA1c of the acarbose group significantly reduced compared with those of control group ($P < 0.01$); this result was not unexpected. The starting dose of acarbose was semiquantity and gradually increased to normal dose (50 mg/time, three times/day), so only 6 patients (8.96%) withdrew from the trial due to severe gastrointestinal side effects. The other patients were well tolerated and with no hypoglycemia.

Previous studies [2, 16] had suggested that acarbose was useful in reducing the risk of MACE in patients with IGT; its cardiovascular protective effect might be due to the reduction of postprandial hyperglycemia and glucose variability, increased insulin sensitivity, induction of moderate weight loss, restoration of endothelial function, and so forth [17]. In the above mechanisms, the improvement of vascular endothelial function is reasonably important. Endothelial dysfunction plays an important role in the development of atherosclerosis and predicts CV outcomes independent of conventional CV risk factors [18]. Although the mechanism by which postprandial hyperglycemia induces endothelial dysfunction is not fully understood, oxidative stress-mediated disruptions in nitric oxide homeostasis are implicated as key role [19]. Endothelium-derived nitric oxide (NO) is one of the most potent known endogenous vasodilators and it plays an important role in the control of coronary blood flow by regulating vascular tone. Kato et al. [20] found acarbose improved postprandial endothelial function by improvement of postprandial hyperglycemia in patients with newly diagnosed T2DM. This notion had been recently reinforced by the finding that 24 weeks of acarbose monotherapy in newly diagnosed patients with T2DM was associated with increased levels of both fasting and postprandial glucagon-like peptide 1 (GLP-1), NO levels, and nitric oxide synthase (NOS) activity [21]. Thus, acarbose seemed to favorably affect endothelial function in the coronary arteries and contributed

Table 3: Biochemical indicator level, CIMT, BMI, and blood pressure of two groups between pre- and posttreatment (mean ± SD).

	Control group		Acarbose group	
	Pretreatment $n = 68$	Posttreatment $n = 64$	Pretreatment $n = 67$	Posttreatment $n = 60$
FPG, mmol/L	5.84 ± 0.33	5.95 ± 0.54	5.92 ± 0.42	5.78 ± 0.69
2 hPG, mmol/L	8.76 ± 0.49	9.46 ± 1.22	8.98 ± 0.54	$7.64 \pm 1.08^{\#,\blacktriangle}$
HbA1c, %	6.28 ± 0.23	6.36 ± 0.51	6.30 ± 0.28	$5.92 \pm 0.24^{\#,\blacktriangle}$
Systolic, mmHg	148.54 ± 8.63	$140.27 \pm 6.25^{*}$	150.16 ± 10.38	$138.82 \pm 7.14^{\blacktriangle}$
Diastolic, mmHg	93.26 ± 5.34	$88.51 \pm 4.62^{*}$	94.13 ± 7.18	$87.73 \pm 5.18^{\blacktriangle}$
TC, mmol/L	6.24 ± 1.03	$5.06 \pm 0.96^{*}$	6.38 ± 1.35	$5.03 \pm 0.87^{\blacktriangle}$
TG, mmol/L	2.36 ± 0.58	$1.74 \pm 0.52^{*}$	2.45 ± 0.62	$1.65 \pm 0.38^{\blacktriangle}$
LDL-C, mmol/L	3.07 ± 0.66	$2.56 \pm 0.52^{*}$	3.18 ± 0.75	$2.58 \pm 0.58^{\blacktriangle}$
BMI, kg/m^2	25.86 ± 2.45	25.64 ± 2.75	26.02 ± 3.47	$24.65 \pm 3.13^{\blacktriangle}$
CIMT, mm	1.23 ± 0.46	$1.49 \pm 0.54^{*}$	1.24 ± 0.52	$1.28 \pm 0.41^{\#}$
ΔCIMT, mm	0.22 ± 0.07		$0.05 \pm 0.02^{\#}$	

$^{*}P < 0.05$, versus the control group before treatment; $^{\#}P < 0.05$, versus the control group after treatment; $^{\blacktriangle}P < 0.05$, versus the acarbose group before treatment.

to an improved long-term prognosis. The present study showed that acarbose could effectively reduce total risk of MACE in ACS patients with newly diagnosed IGT ($P < 0.05$). This cardiovascular benefit mostly owed to the reduction of secondary endpoint events ($P < 0.05$), while the decline of cardiovascular disease mortality was not statistically significant ($P > 0.05$), which might be related to not long enough follow-up time and not enough number of patients enrolled.

The absolute value and change of CIMT are both indirect indicators of coronary atherosclerosis and independent predictors of long-term CV [22, 23]. Previous study had confirmed that acarbose could slow the progression of CIMT in patients with IGT or T2DM [24, 25], and Koyasu et al. [26] found that acarbose also could retard CIMT thickness and plaque formation in CAD patients with newly diagnosed IGT. The present study showed that acarbose slowed the progression of CIMT in ACS patients with IGT, which also indirectly suggested that acarbose delayed the development of coronary atherosclerosis in patients with IGT. In the present study, BMI in acarbose group had a distinctly downtrend compared with the control group ($P = 0.064$); it suggested that moderate loss of weight may be one of the possible reasons for the cardiovascular benefit.

In summary, the present study indicates that acarbose can effectively and safely retard the CIMT thickness and reduce the risk of recurrent MACE in ACS patients with newly diagnosed IGT. Therefore, acarbose can improve the prognosis of these patients.

Conflict of Interests

The authors declared that they have no conflict of interests in this work.

Authors' Contribution

Peng Yun and Ai-ming Du contributed equally to this work.

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Research Article

Inhibition of NA^+/H^+ Exchanger 1 Attenuates Renal Dysfunction Induced by Advanced Glycation End Products in Rats

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It has been recognized that sodium hydrogen exchanger 1 (NHE1) is involved in the development of diabetic nephropathy. The role of NHE1 in kidney dysfunction induced by advanced glycation end products (AGEs) remains unknown. Renal damage was induced by AGEs via tail vein injections in rats. Function and morphology of kidney were determined. Compared to vehicle- or BSA-treated rats, AGEs caused abnormalities of kidney structures and functions in rats, accompanied with higher MDA level and lower GSH content. Gene expressions of NHE1 gene and TGF-β1 in the renal cortex and urine were also increased in AGEs-injected rats. Importantly, all these detrimental effects induced by AGEs were reversed by inhibition of NHE1 or suppression of oxidative stress. These pieces of data demonstrated that AGEs may activate NHE1 to induce renal damage, which is related to TGF-β1.

1. Introduction

Diabetic nephropathy is one of the most important complications in diabetes and is also the leading cause of renal failure in adults. AGEs are a heterogeneous group of products in which protein and lipids are covalently bound to sugar residues under hyperglycemic and oxidative stress situations, which is proposed to play a major role in the pathogenesis of diabetic nephropathy [1]. However, the mechanisms involving the pathogenesis of renal damage induced by AGEs were poorly understood.

The Na^+/H^+ exchanger (NHE) is a protein that is expressed in many mammalian cell types [2], exchanging one intracellular H^+ for an extracellular Na^+. In this way, it regulates intracellular pH value and cell volume [3]. To date, nine isoforms (NHE1-9) have been identified. NHE1 is ubiquitously distributed in most tissues, which is localized in the membrane and sensitive to amiloride. It is involved in signaling transduction and regulation of cell functions [4]. Our previous studies have indicated that hyperactivity of NHE1 exchanger is related to the vascular injury associated with high glucose or hyperglycemia [5–7]. Cariporide, similar to amiloride, as a selective NHE1 inhibitor, prevents the process of vasculopathy in diabetic rats [8, 9]. Although its role in diabetic vascular complication has been extensively investigated, whether NHE1 mediates diabetic nephropathy and the pathogenic mechanism remain unclear.

It has been reported that AGEs-induced hyperglycemic memory phenomenon [10–12] is very similar to the persistent NHE1 activation in diabetic nephropathy [13]. These findings suggest that the mass accumulation of AGEs may involve activation of NHE1 in the pathogenesis of nephropathy. Our previous studies have also demonstrated that AGEs activate NHE1 to induce proliferation of vascular smooth muscle cell [14]. Therefore, we hypothesized that activation of NHE1 may be a critical step in the signal transduction of AGEs-induced

renal damage. Our results demonstrate that cariporide, via inhibition of NHE1, normalized the redox status to protect renal function in rats injected with AGEs.

2. Materials and Methods

2.1. Materials. Cariporide, N-acetylcysteine (NAC), Bovine serum albumin (BSA, cat. A1933, reagent ≥ 98%), and D-glucose were purchased from Sigma Company. Antibody to AGEs receptor (Ab-RAGE) was purchase from Santa Cruz Company. BCA protein assay kit was brought from PIECE Company.

2.2. Animals. Male Sprague-Dawley rats (8 ± 2 weeks old, 180 ± 20 g) were purchased from the Center of Experiment Animals, Central South University (Changsha, China). All rats were housed individually in cages at a room temperature of 21 ± 1°C with a 12 h light/dark cycle and were given free access to food and water. At the end of the experiments, rats were placed in individual metabolic cages and 24-hour urine samples for three consecutive days before sacrifice were collected. After fasting for 12 h, the rats in each group were anesthetized with sodium pentobarbitone (30 mg/kg, I.P.) and exsanguinated. The right kidneys were collected after perfusion with 40 mL of ice-cold PBS and stored at −80°C. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Central South.

2.3. Preparation of AGEs. AGEs were prepared *in vitro* as described previously [14]. Briefly, BSA (50 mg/mL) was incubated with D-glucose (0.5 M) in PBS supplemented with penicillin (100 U/L) and streptomycin (100 mg/L) for 12 weeks at 37°C under sterile environments and darkness. After incubation, the solutions were dialyzed against PBS (pH 7.4) at 4°C for 48 h to remove free glucose, following separation of AGEs into aliquots, and stored at −20°C. The protein concentration was measured with the method of BCA. AGE-specific fluorescence was determined using 370 nm excitation and 440 nm emission wavelengths by using a spectrofluorometer (Shimadzu, Beijing Beyond Technology Development Co). BSA was incubated in the same conditions without D-glucose and served as control of AGEs.

2.4. Preparation of Renal Slices. As described in details previously [15], the isolated kidneys were immediately placed in 5 mL ice-cold Krebs buffer and kept on ice. The slices were rinsed two times in 5 mL oxygenated Krebs buffer each for 3 min at 25°C in an oxygen environment with constant shaking and then transferred to 3 mL oxygenated Krebs in designated Erlenmeyer flasks and equilibrated for 10 min at 37°C prior to different treatments.

2.5. Examinations of Renal Function. The creatinine levels in both serum and urine were detected using alkaline picric acid method under the guidance of commercial kits (Nan Jing Jian Cheng Bioengineering Institute, China). The creatinine clearance was calculated on the basis of urinary creatinine, serum creatinine, urine volume, and body weight as described previously [16]. Blood urea nitrogen level was measured using urea enzymatic colorimetric kit (Nan Jing Jian-Cheng Bioengineering Institute, China). 24-hour urinary protein was determined by the BCA method (Beyotime Institute of Biotechnology, China).

2.6. Determination of Kidney Histopathology. As described in details previously [17], HE staining was performed to determine kidney histopathology. The severity of renal damage was estimated by the following parameters: (1) total glomerular surface area and (2) mesangial matrix injury score expressed by mesangial surface area/glomerular total surface area.

2.7. Determinations of MDA, GSH, LDH, and TGF-β1 in the Urine. Kidney was homogenized using an ElectroMotion glass homogenizer (Ningbo Scientz Biotechnology Co., China) as describe previously [18]. After centrifugation, the supernatant was kept under −80°C before determinations of MDA, LDH, and GSH. The protocols of GSH and MDA measurements were remanded by commercial kits (Nan Jing Jian Cheng Bioengineering Institute, China). Urinary TGF-β1 was quantified by ELISA using commercial kits (BioSource, Camarillo, CA, USA) according to the manufacturer's instructions.

2.8. RT Polymerase Chain Reaction (PCR). The protocol of RT-PCR was described previously by us [19]. In short, total RNA was extracted from each renal cortex tissue using 1 mL Trizol reagent (GIBCO, USA) according to the manufacturer's protocol. RNA concentrations were determined by the A260/280 ratio using a spectrophotometer and the quality was assessed on a 1.5% ethidium bromide-agarose gel. Absorbance ratios between 1.90 and 2.15 indicated pure RNA samples. Three micrograms of total RNA were reverse transcribed with oligo-dT primer and M-MLV reverse transcriptase (TIANGEN, China). One microgram of the reaction mixture was used in each PCR containing a pair of specific primers for rat NHE1, TGF-β1, and GAPDH. The sequences of the NHE1 primers specific for rats were sense 5′-CACGCTGTGGAATGCT-3′ and antisense 5′-GAAGATGTCCGAGATGC-3′. PCR product was 289 bp. Sequences of the TGF-β1 specific for rats were sense 5′-GCCAAGACCCTAACA-3′ and antisense 5′-CACTGAAGTCCACCAA-3′. PCR product was 381 bp. GAPDH mRNA was codetected with sense 5′-CAATGTATCCGTTGTGG-3′ and antisense 5′-GTCCAGGGTTTCTTACTC-3′. PCR product was 307 bp. Target sequences were amplified at 1°C below Tm using the same amount of cDNA for all primer sets, and the cycle number was adjusted between 30 and 35 to yield visible products within the linear amplification range. PCR products then were run on 1.5% agarose gels and photographed under ultraviolet light. Densities of bands were measured by scanning densitometry with Image J analysis system software and normalized to GAPDH in the same sample.

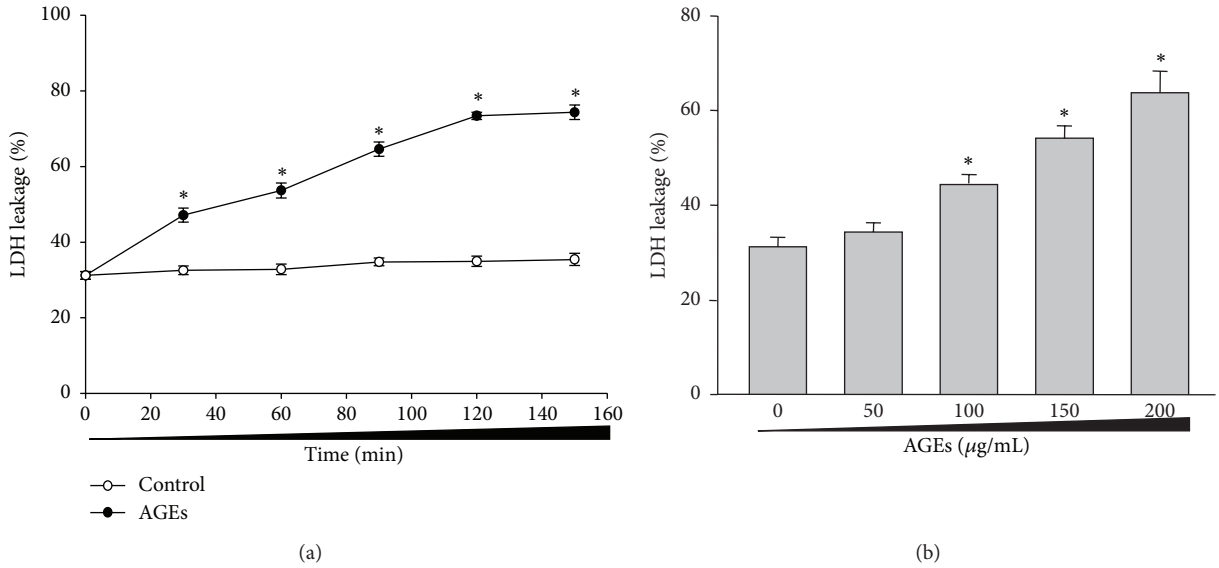

Figure 1: AGEs time-/dose-dependently increase LDH leakage in isolated renal cortex from rats. Cortex from isolated rat kidney was sliced into small pieces with the thickness of 0.3–0.5 mm. The slice was incubated with AGEs as indicated times and concentrations. LDH leakage was assayed in slice of renal cortex. (a) Time course of AGEs on LDH leakage. (b) Dose course of AGEs on LDH leakage. All data were expressed as mean ± SD. N is 5 in each group. $^{*}P < 0.05$ versus control (0).

2.9. Western Blotting. As described previously [19], tissues were homogenized on ice in cell-lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin) and 1 mM PMSF. Cell was lysated with cell-lysis buffer. The protein content was assayed by BCA protein assay reagent (Pierce, USA). 20 μg proteins were loaded to SDS-PAGE and then transferred to membrane. Membrane was incubated with a 1 : 1000 dilution of primary antibody, followed by a 1 : 2000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare). The intensity (area X density) of the individual bands on Western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area. We used control as 100%.

2.10. Ex Vivo Experimental Designs. Renal slices were divided into 7 groups. Group 1 included the following: slices incubated with culture medium of DMEM/F12; group 2 included the following: slices incubated with culture medium containing BSA (200 μg/mL); group 3 included the following: slices incubated with culture medium containing cariporide (1 μM, H-car); group 4 included the following: slices incubated with culture medium containing AGEs (200 μg/mL); group 5 included the following: slices incubated with culture medium containing AGEs plus cariporide (0.1 μM, L-car); group 6 included the following: slices incubated with culture medium containing AGEs plus cariporide (1 μM); group 7 included the following: slices incubated with culture medium containing AGEs plus antibody of AGEs receptor (5 μg/mL, Ab-RAGE). Slices were incubated with these treatments for 2 hours.

2.11. In Vivo Experimental Design. SD rats were divided into 5 groups: group 1: control group; group 2: BSA-injected group; group 3: AGEs-injected group; group 4: AGEs-injected plus NAC treatment group; group 5: AGEs-injected plus cariporide treatment group. Rats in group 1 were fed with regular diet and tap water. Rats in group 2 received tail vein injection of BSA (100 mg/kg/day). Rats in group 3 received tail vein injection of AGEs (100 mg/kg/day). Rats in group 4 received tail vein injection of AGEs plus gavage with NAC (200 mg/kg/day). Rats in group 5 received tail vein injection of AGEs plus gavage with cariporide (1 mg/kg/day). The injection was performed on rats under anesthesia with diethyl ether. All treatments were performed for 12 consecutive weeks in rats fed with regular diet and tap water. No rat died during the whole experiment.

2.12. Statistical Analyses. All values are expressed as mean ± S.E.M. The results were carried out by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test for multiple comparisons with SPSS 11.5. A p value of 0.05 or less was considered significant.

3. Results

3.1. AGEs Time-/Dose-Dependently Induce LDH Leakage in Isolated Renal Cortex from Rats. In order to test the hypothesis, we firstly investigated whether AGEs caused renal dysfunction in rats by measuring LDH leakage in isolated renal cortex from rats, which is an indicator of loss of membrane integrity [20]. As shown in Figure 1(a), incubation of rat renal cortex slice with AGEs (100 μg/mL) from 20 to 160 minutes increased LDH leakage in a time-dependent

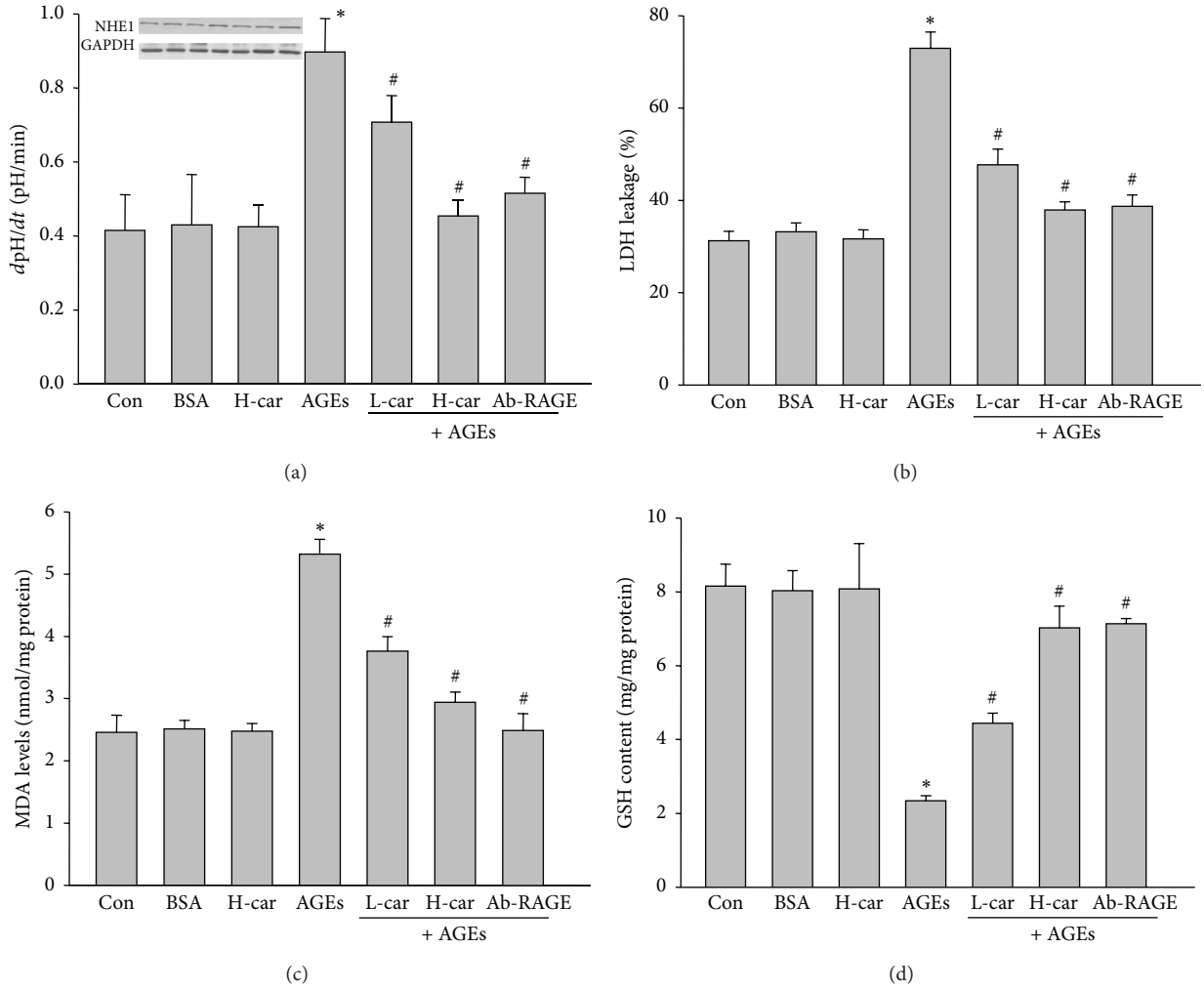

Figure 2: *Ex vivo* inhibition of NHE1 by cariporide reverses AGEs-increased LDH leakage in rat renal cortex. Sliced renal cortex was incubated with BSA, H-car (1 μM cariporide), L-car (0.1 μM cariporide), and Ab-RAGE (antibody of AGEs receptor). After treatment, (a) LDH leakage and NHE1 protein expression, (b) GSH content, (c) MAD level, and (d) NHE1 activity were measured, respectively. All data were expressed as mean ± SD. N is 5 in each group. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs alone.

manner. BSA control had no effects on LDH leakage. Following treatment of 50–200 μg/mL for 2 hours, LDH leakage significantly began to increase when the concentration of AGEs is 100 μg/mL or above (Figure 1(b)). These pieces of data indicate that AGEs induce LDH leakage in isolated renal cortex, which is time/dose dependent.

3.2. AGEs Activates Cariporide-Sensitive NHE1 in Cultured Renal Cortex Cells. We next determined whether activation of NHE1 mediates AGEs-increased LDH leakage. Cariporide, a selective NHE1 inhibitor, which has been identified by us and others [7, 21], was used to inhibit NHE1 activity in this section of the present study. As indicated in Figure 2(a), AGEs, but not BSA, dramatically increased NHE1 activity in cultured renal cortex cells. Though cariporide did not inhibit NHE1 activity in cells without AGEs treatment, it significantly reduced NHE1 activity in AGEs-treated cells at low or high dose, indicating that AGEs activate NHE1, which is cariporide sensitive, consistent with our previous report [14]. The effects of cariporide on AGEs-induced NHE1 activation were mirrored by blocking receptor of AGEs by using specific antibody (Ab-RAGE), further supporting that AGEs via its receptor activate NHE1.

3.3. Inhibition of NHE1 by Cariporide Abolishes AGEs-Increased LDH Leakage in Renal Cortex. We then detected LDH leakage in renal cortex slice treated by AGEs. As depicted in Figure 2(b), the increased LDH leakage by AGEs was abolished by cariporide at low or high dose and Ab-RAGE, demonstrating that AGEs-induced LDH leakage enhancement is possibly related to its receptor and subsequent NHE1 activation.

3.4. Cariporide Normalizes the Redox State in AGEs-Treated Rat Renal Cortex. Inhibition of NHE1 has been reported to suppress oxidative stress in vascular system [22, 23].

Table 1: General parameters in rats.

Groups	BG (mM)	BW (g)	KI (g/kg)
Vehicle	5.71 ± 0.59	385.81 ± 11.67	3.18 ± 0.272
BSA	5.94 ± 0.67	383.35 ± 9.59	3.41 ± 0.194
AGEs	6.07 ± 0.71	356.40 ± 5.94	5.07 ± 0.537*
AGEs + NAC	5.29 ± 0.32	368.35 ± 4.38	3.75 ± 0.237#
AGEs + car	5.80 ± 0.54	373.80 ± 3.10	3.62 ± 0.142#

The rats received a tail vein injection of AGEs (100 mg/kg) followed by treatment with N-acetylcysteine (200 mg/kg/day) or cariporide (1 mg/kg/day). 12 weeks later, all rats were sacrificed under anesthesia and blood glucose (BG), body weight (BW), and kidney index (KI) were determined. All data were expressed as mean ± SD. N is 10–15 in each group. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs alone.

Thus, we hypothesized that cariporide may inhibit AGEs-induced oxidative stress to maintain membrane integrity in isolated renal cortex. We evaluated the levels of oxidative stress by determinations of MDA and GSH, two markers of oxidative stress in cells [24]. In Figures 2(c) and 2(d), either cariporide or Ab-RAGE reduced the AGEs-increased MDA and AGEs-decreased GSH levels in renal cortex, suggesting that inhibition of NHE1 by cariporide normalizes the redox state in AGEs-treated rat renal cortex.

3.5. In Vivo Administration of Cariporide Inhibits AGEs-Induced Kidney Hypertrophy and Glomerular Sclerosis in AGEs-Injected Rats. AGEs are the major factors in diabetic nephropathy [25]. *Ex vivo* experiments indicated the beneficial effects of cariporide on AHGEs-induced membrane disruption of renal cortex. We next investigated the *in vivo* effects of cariporide on AGEs-induced renal dysfunction. As shown in Table 1, 12-week injection of AGEs via tail vein did not affect the body weight and blood sugar levels in rats. However, AGEs dramatically increased the weight of kidney, as indicated by kidney index. Treatment of cariporide failed to alter body weight and blood glucose levels but inhibited the hypertrophy of kidney.

The hypertrophy of kidney induced by AGEs was further supported by morphological analysis by HE staining. As indicated in Figure 3(a), compared to BSA-treated rats, widespread glomerular sclerosis was observed in AGEs alone challenged rats, as well as increased mesangial matrix injury score (Figure 3(b)), cell numbers (Figure 3(c)), and glomerular volume (Figure 3(d)) by quantitative analysis. In contrast, all these effects induced by AGEs were corrected by cariporide intervention. Inhibition of oxidative stress by NAC also mimicked these effects induced by cariporide in AGEs-injected rats. Taking these data together, it suggests that inhibition of NHE1 protects kidney structure in AGEs-injected rats, which is related to suppression of oxidative stress.

3.6. Cariporide Inhibits Ages-Induced Renal Dysfunction in Rats. The protective effects of cariporide in kidney of AGEs-injected rat were further confirmed by analysis of renal function. Compared to BSA-injected rats, AGEs remarkably caused renal dysfunction as increased serum creatinine (Figure 4(a)), blood urea nitrogen (Figure 4(b)), and urinary albumin excretion (Figure 4(c)) and decreased clearance of creatinine (Figure 4(d)). As expected, cariporide reversed AGEs-induced enhancements of serum creatinine, blood urea nitrogen, and urinary albumin excretion and reduction of clearance of creatinine. The effects of cariporide on renal function were also copied by NAC. These data suggest that activation of NHE1 and oxidative stress are key steps contributing to AGEs-induced renal dysfunction.

3.7. AGEs via NHE1 Activation Induces Oxidative Stress in Rat Kidney. In order to establish the relationship between NHE1 activation and oxidative stress in AGEs-induced renal damage, we examined the levels of oxidative stress in cariporide-treated AGEs-injected rats. As indicated in Figures 5(a) and 5(b), both increased MDA and decreased GSH were induced by AGEs in rats, which were reduced by cariporide or NAC, indicating cariporide via inhibition of NHE1 suppress oxidative stress in AGEs-injected rats.

3.8. Cariporide Inhibits Gene Expressions of NHE1 and TGF-β1 in Kidneys from AGEs-Injected Rats. We finally determined the effects of cariporide on TGF-β1, which is a key mediator for diabetic nephropathy [26]. Compared with BSA-treated rats, AGEs increased NHE1 and TGF-β1 gene expressions, as determined by RT-PCR (Figure 6(a)). Cariporide obviously downregulated NHE1 and TGF-β1 gene expressions to the level of BSA-treated group. The reductions of NHE1 and TGF-β1 gene expressions induced by NAC were weaker than cariporide. The effects of cariporide and NAC on TGF-β1 gene expression were further confirmed by assaying urinary TGF-β1 excretion (Figure 6(b)). Collectively, it indicates that NHE1 is a potential target of cariporide to prevent renal function in AGEs-injected rats.

4. Discussion

The present study demonstrates that AGEs *ex vivo* or *in vivo* cause glomerular sclerosis and renal dysfunction, which is abrogated by NHE1 inhibition, blockage of AGEs receptor, and suppression of oxidative stress. Mechanistically, the detrimental effects of AGEs on kidney function might be related to activation of its receptor and sequent activation of NHE1, resulting in upregulation of oxidative stress. In this way, cariporide, a selective NHE1 inhibitor, normalizes the redox state in renal cortex and functions.

AGEs can accumulate in diverse biological settings, such as diabetes, inflammation, renal failure, and aging [27]. Many studies support that interactions between AGEs and its

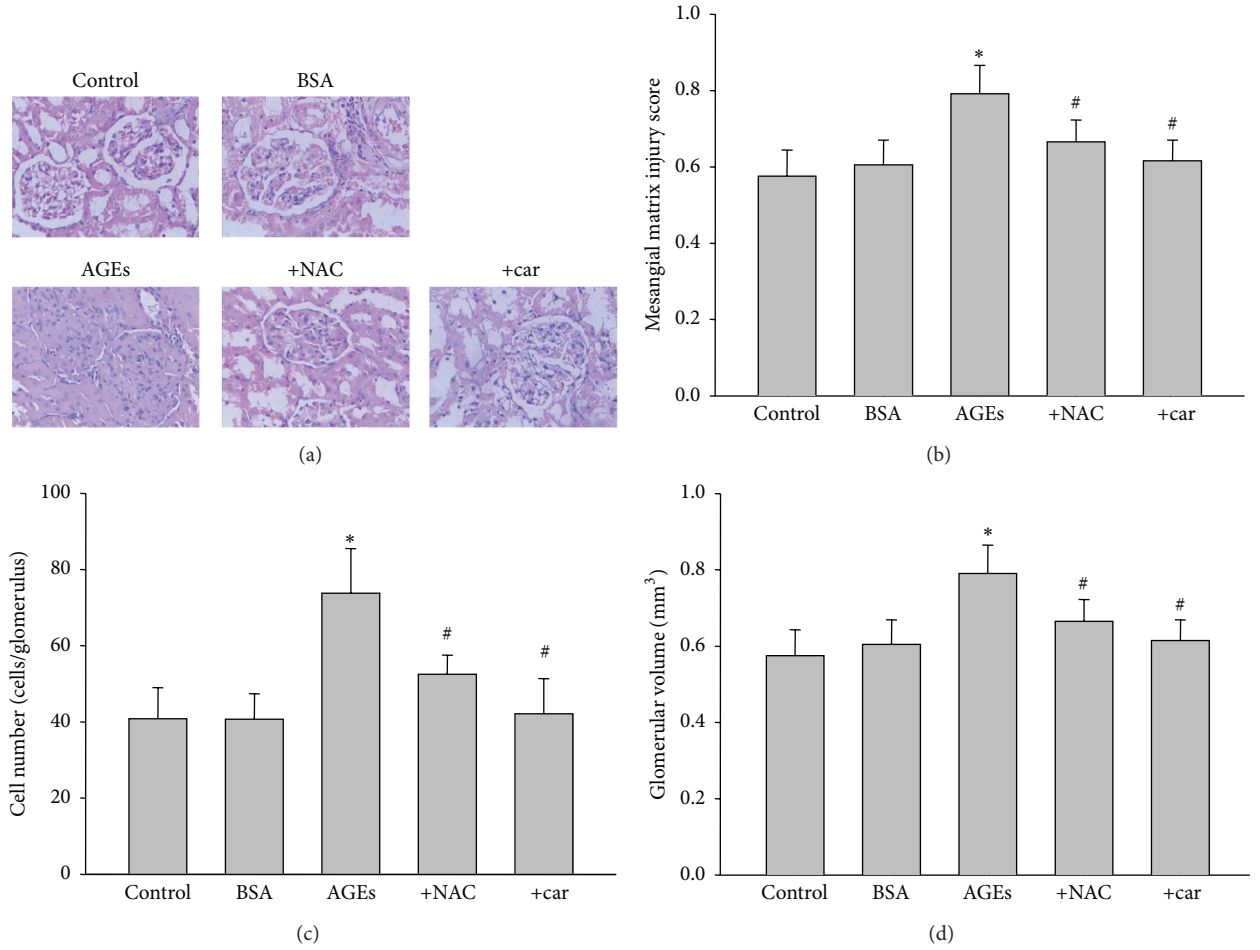

Figure 3: Inhibition of NHE1 improves AGEs-induced abnormality of glomerular structure in rats. The rats received a tail vein injection of AGEs (100 mg/kg) followed by treatment with or without N-acetylcysteine (200 mg/kg/day) or cariporide (1 mg/kg/day) for 12 weeks. At the end of experiments, rats were sacrificed under anesthesia. (a) Morphology of glomerular in kidney by HE staining. (b) Mesangial matrix injury score, (c) cell numbers in glomerular, and (d) glomerular volume were determined. Data are expressed as mean ± S.E.M. N is 10–15 in each group. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs alone.

receptor are involved in the pathogenesis of diabetic complications, in particular, nephropathy [1, 25, 28]. The formation and accumulation of AGEs adducts in various tissues are associated with altered protein structure and function. In addition, AGEs are able to activate intracellular signaling by binding to specific receptors [29]. A number of AGEs inhibitors and crosslink breakers, such as aminoguanidine and ALT-711, have been shown to prevent the formation and break the crosslink of AGEs [30, 31]. However, they could not affect the cellular interactions of the existing AGEs with its receptor. In this present study, we injected exogenous AGEs into rats to mimic diabetic nephropathy and examined the effects of NHE1 selective inhibitor cariporide on kidney function. We observed that AGEs alone resulted in the increase of glomerular sclerosis and renal dysfunction, which are reversed by cariporide. This is the major discovery of this study.

Another important discovery is that NHE1 inhibition reduces oxidative stress induced by AGEs in diabetic nephropathy. AGEs may be linked to the increased reactive oxygen species (ROS) by decreasing antioxidative enzyme including superoxide dismutase and catalase, diminishing glutathione stores [32]. Our study indicated that AGEs significantly increased production of MDA and lower GSH content, while treatment with cariporide and NAC dramatically decreased MDA concentration and restored GSH level. Our previous study has showed that cariporide could prevent oxidative stress reaction mediated by high glucose [6, 33]. These findings confirmed that there was a close relationship between the oxidative stress and the changed activity of NHE1. During the pathologic process of AGEs in renal damage, NHE1 may be also activated by ROS due to AGE-RAGE reaction because ROS is also a well-known activator of NHE1 [34]. It should be noted that this study has been examined only in animal experiment; the precise mechanism remains to be determined in cell studies.

TGF-β1 is a multifunctional cytokine produced by tubular, interstitial, and glomerular cells. It stimulates the synthesis of ECM such as collagen and laminin and blocks ECM degradation through inhibition of matrix metalloproteinase

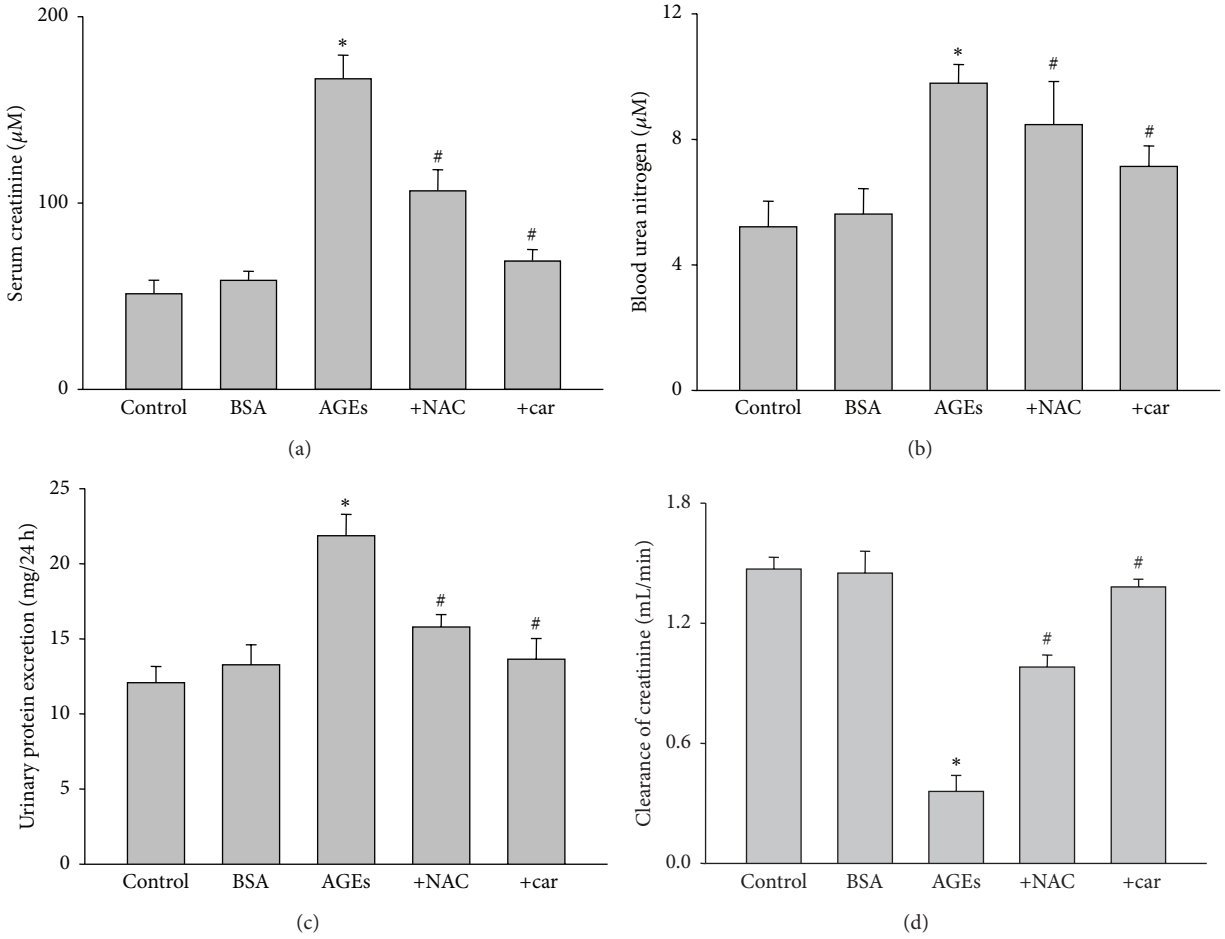

Figure 4: *In vivo* administration of cariporide prevents renal dysfunction in AGEs-injected rats. The rats received a tail vein injection of AGEs (100 mg/kg) followed by treatment with or without N-acetylcysteine (200 mg/kg/day) or cariporide (1 mg/kg/day) for 12 weeks. At the end of experiments, (a) serum creatinine level, (b) blood urea nitrogen, (c) urinary protein excretion, and (d) clearance of creatinine were measured, respectively. All data were expressed as mean ± SD. N is 5 in each group. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs alone.

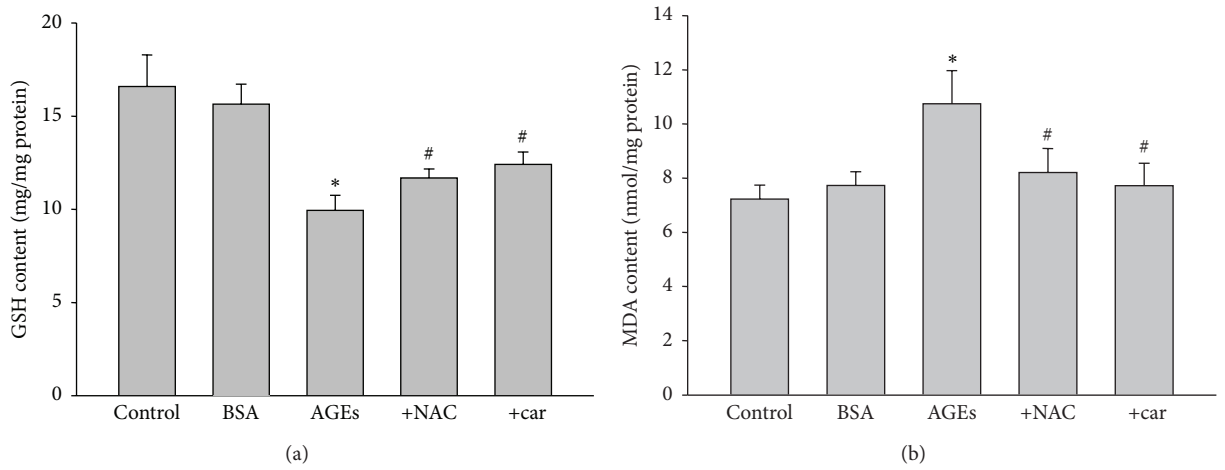

Figure 5: Cariporide recues AGEs-induced oxidative stress in kidney of rats. The rats received a tail vein injection of AGEs (100 mg/kg) followed by treatment with or without N-acetylcysteine (200 mg/kg/day) or cariporide (1 mg/kg/day) for 12 weeks. At the end of experiments, rats were sacrificed under anesthesia. (a) GSH content and (b) MAD level were determined. All data were expressed as mean ± SD. N is 10–15 in each group. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs alone.

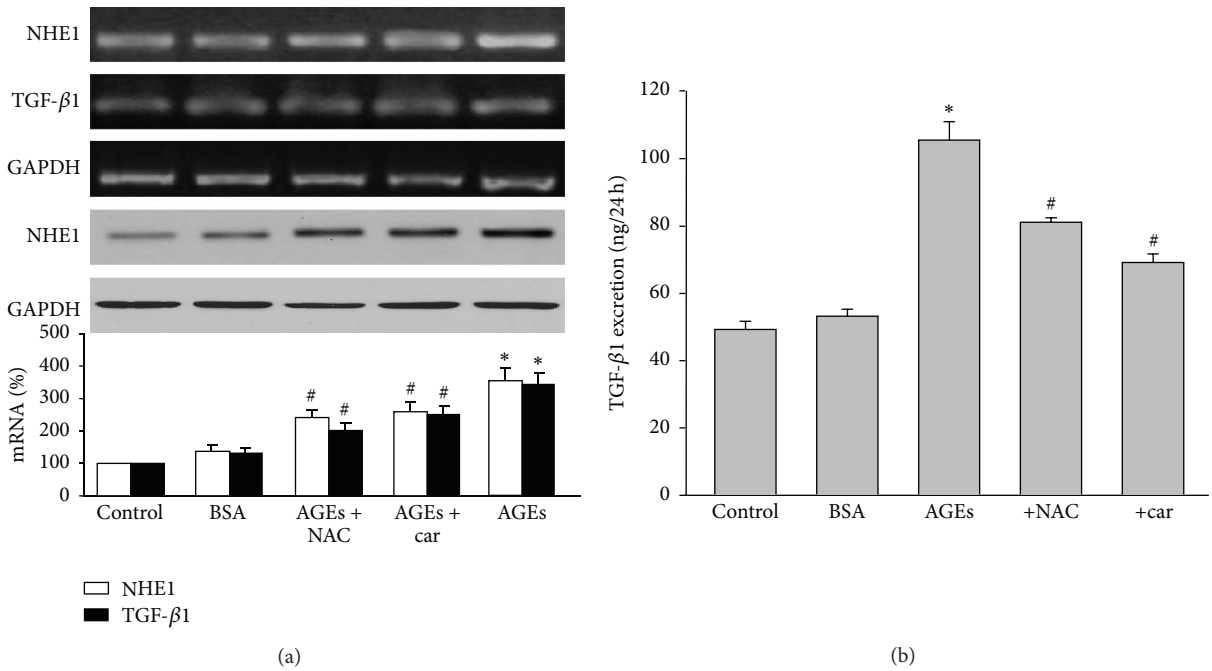

Figure 6: Cariporide reduces the gene expressions of NHE1 and TGF-β1 in renal cortex from AGEs-injected rats. The rats received a tail vein injection of AGEs (100 mg/kg) followed by treatment with or without N-acetylcysteine (200 mg/kg/day) or cariporide (1 mg/kg/day) for 12 weeks. At the end of experiments, rats were sacrificed under anesthesia. (a) mRNA levels of NHE1 and TGF-β1 and protein level of NHE-1 were assayed by RT-PCR or Western blot. (b) TGF-β1 excretion of rat urine. All data were expressed as mean ± SD. N is 10–15 in each group. $^{*}P < 0.05$ versus BSA. $^{\#}P < 0.05$ versus AGEs alone.

[35]. It is considered to be the strongest cytokine to glomerulosclerosis in diabetic nephropathy and a last major mediator induced by a variety of damaging factors in the pathological process [36]. Therefore, we tentatively put forward that NHE1 function as a platform to link AGEs with TGF-β1 pathway. In this study, AGEs treated alone rats showed widespread fibrosis in renal glomerulus, parallel increase of TGF-β1 expression in the renal cortex, and urinary TGF-β1 excretion. Cariporide treatment congruously decreased the above index and prevented renal glomerulus fibrosis, suggesting that NHE1 plays a decisive role in activation of TGF-β1 pathway of renal injury induced by AGEs. It is worth more detailed studies in the future to further confirm the precise pathway.

In conclusion, we have identified that inhibiting NHE1 with cariporide exhibited marked protection from AGEs-mediated renal damage. NHE1 may function as a structural scaffold to link AGEs with TGF-β1 signaling in renal damage. These findings suggest that NHE1 is a promising target for the treatment of diabetic nephropathy and makes cariporide a promising drug for the future treatment of diabetic nephropathy.

Conflict of Interests

The authors confirm that there is no conflict of interests.

Authors' Contribution

Peng Li, Fu Wang, and Geng-Rong Chen designed and conducted the experiments and analyzed data. Ping Xu and Li-Ying Liu gave a lot of useful suggestions to this project. Ya-Ling Yin and Shuang-Xi Wang analyzed data, conceived the project, and wrote the paper. Peng Li and Geng-Rong Chen contributed equally to this work.

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Research Article

Coenzyme Q10 Attenuates High Glucose-Induced Endothelial Progenitor Cell Dysfunction through AMP-Activated Protein Kinase Pathways

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Coenzyme Q10 (CoQ10), an antiapoptosis enzyme, is stored in the mitochondria of cells. We investigated whether CoQ10 can attenuate high glucose-induced endothelial progenitor cell (EPC) apoptosis and clarified its mechanism. EPCs were incubated with normal glucose (5 mM) or high glucose (25 mM) enviroment for 3 days, followed by treatment with CoQ10 (10 μM) for 24 hr. Cell proliferation, nitric oxide (NO) production, and JC-1 assay were examined. The specific signal pathways of AMP-activated protein kinase (AMPK), eNOS/Akt, and heme oxygenase-1 (HO-1) were also assessed. High glucose reduced EPC functional activities, including proliferation and migration. Additionally, Akt/eNOS activity and NO production were downregulated in high glucose-stimulated EPCs. Administration of CoQ10 ameliorated high glucose-induced EPC apoptosis, including downregulation of caspase 3, upregulation of Bcl-2, and increase in mitochondrial membrane potential. Furthermore, treatment with CoQ10 reduced reactive oxygen species, enhanced eNOS/Akt activity, and increased HO-1 expression in high glucose-treated EPCs. These effects were negated by administration of AMPK inhibitor. Transplantation of CoQ10-treated EPCs under high glucose conditions into ischemic hindlimbs improved blood flow recovery. CoQ10 reduced high glucose-induced EPC apoptosis and dysfunction through upregulation of eNOS, HO-1 through the AMPK pathway. Our findings provide a potential treatment strategy targeting dysfunctional EPC in diabetic patients.

1. Introduction

Diabetes is a metabolic disease clinically expressed by chronic hyperglycemia that has been extensively reported to be linked to several micro- and macrovascular diseases that significantly impair the quality of life. Among diabetic vascular complications, foot ulcers represent the first cause of hospitalization in diabetic patients and a significant cause of health care costs [1]. Despite promising therapeutic strategies, morbidity and mortality in diabetic patients with peripheral artery disease have not been markedly improved over the last decade. Clinical studies have indicated that diabetic patients have impaired endothelial function and are prone to suffer from severe organ damage due to poor collateral vessel formation in response to tissue ischemia [2]. Accumulating evidence suggests that circulating endothelial progenitor cells

(EPCs) are mainly derived from the monocyte/macrophage lineage, and are capable of forming new blood vessels in ischemic tissues through a process of vasculogenesis [3, 4]. It has been reported that in patients with diabetes the numbers and function of circulating EPCs are decreased, although the mechanisms underlying this decrease are poorly understood [5, 6]. Clinical and experimental studies have indicated that micro- or macrovascular complications associated with diabetes may be due to the reduced count and impaired functionality of circulating EPCs [5, 6].

Coenzyme Q (CoQ10), also known as ubiquinone, acts as an electron transport carrier from complex I or complex II to complex III within the inner mitochondrial membrane and exerts an important role in maintaining bioenergy homeostasis in mitochondria [7, 8]. Additionally, CoQ10 is also an antioxidant which neutralizes free radicals and inhibits cellular apoptosis [7]. There is a considerable amount of data suggesting that supplementation of CoQ10 has beneficial effects on cardiovascular disease, metabolic syndrome, and diabetes by improvement of mitochondrial membrane potential and counteracts oxidative stress in myocytes [9, 10]. Recent reports have also shown that CoQ10 augments endothelial function of type 2 diabetic patients and ischemic heart disease [11, 12]. However, whether treatment with CoQ10 improves dysfunctional EPC and prevents apoptosis in high glucose conditions remains unclear. In this study, we aim to investigate whether administration of CoQ10 could attenuate high glucose-induced apoptosis of EPCs and whether nitric oxide (NO) could be involved in the corresponding signaling pathway in this process. Our findings provide novel evidence that CoQ10 could be a potential therapeutic strategy to diminish ischemia-induced tissue damage by enhancement of EPC function in diabetic patients.

2. Materials and Methods

2.1. Cell Culture and Reagents. Total mononuclear cells (MNCs) were isolated by density gradient centrifugation with Histopaque-1077 (1.077 g/mL, Sigma) from peripheral blood of healthy young volunteers. Briefly, 5×10^6 MNCs were seeded in EGM-2MV medium (Cambrex, East Rutherford, NJ, USA), with supplements (hydrocortisone, human epidermal growth factor, R^3-insulin-like growth factor 1, human fibroblast growth factor, vascular endothelial growth factor (VEGF), gentamicin, amphotericin B, vitamin C, and 20% fetal bovine serum) on 0.1% fibronectin-coated plate. The medium was changed after every four days of culture, and EPCs appeared within 7–15 days after the start of the MNC culture. EPCs were used passage numbers 3 through 7. Additionally, we identified the EPCs by the antibodies CD34, CD133, KDR, CD31 (Santa Cruz), and vWF (Neomarkers) using immunofluorescence. The fluorescent images were obtained by a laser scanning confocal microscope. Coenzyme Q10 was purchased from Sigma, dissolved in 0.04% Lutrol F127, and then diluted in culture medium.

2.2. EPC Viability. After cells were cultured with different glucose conditions for 4 days or CoQ10 for 1 day, EPCs were treated with MTT (0.5 mg/mL, Sigma). The cells were lysed with dimethyl sulfoxide and measured at 550/650 nm.

2.3. EPC Migration. The migratory function of the EPCs was evaluated by a modified Boyden chamber (Transwell, Costar). Briefly, 4×10^4 EPCs were placed in the upper chambers of transwell plates with serum-free endothelial growth medium. In the lower chambers, stromal cell-derived factor 1 (SDF-1) (50 ng/mL) was supplemented to the medium placed at 37°C incubation. After incubation for 24 hours, the membrane of chamber was washed by PBS twice and stained using lectin-FITC (UEA-1 lectin, Sigma). Then the upper membrane side was scraped with a cotton ball and fixed with 2% paraformaldehyde. The migrated cells in lower membrane side were counted by 6 random high-power (×100) microscopic fields by fluorescence microscopy.

2.4. EPC Senescence. EPCs aging was evaluated with a Senescence Cell Staining kit (Sigma, USA). The EPCs were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 6 min. Then the cells were incubated at 37°C without CO_2 in fresh X-gal staining solution (1 mg/mL X-gal, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$; pH6). After 6–8 hours, β-galactosidase-positive cells appeared (green). The level of senescence of EPCs was evaluated by calculating relative percentages of green-stained and total cells.

2.5. Mitochondrial Apoptosis. Mitochondrial apoptosis was determined by JC-1 assay (BD Pharmingen). The EPCs were harvested by trypsinization and incubated with JC-1. After 15 min, the cells were washed with PBS twice. Apoptosis was detected by a change in JC-1-labeled fluorescence from red to green with flow cytometer and analyzed with Cell Quest Alias software, as described in the literature [13].

2.6. Mitochondrial Function. Mitochondrial function was determined by measurement of mitochondrial membrane potential via rhodamine 123 (Rh123), as described in [14]. After treatment with high glucose or CoQ10, the cells were stained with Rh123 (5 mM, Sigma) and incubated at 37°C for 30 min. The intensity of fluorescence (relative fluorescence units) was measured at excitation wavelength 485-nm and emission wavelength 530-nm by a fluorescence microplate reader.

2.7. Nitric Oxide (NO) and Reactive Oxygen Species (ROS) Production. NO production was determined by staining with 3-amino,4-aminomethyl-2′,7′-difluorofluorescein (DAF-FM) diacetate (10 μM, Molecular Probes) for 30 min. The intensity of fluorescence (relative fluorescence units) was evaluated at excitation wavelength 495-nm and emission wavelength 515-nm by a fluorescence microplate reader.

ROS was determined by H_2O_2 detection using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA 20 μM, Molecular Probes) as a probe. The intensity of fluorescence was evaluated at excitation wavelength 485-nm and emission wavelength 530-nm by a fluorescence microplate reader.

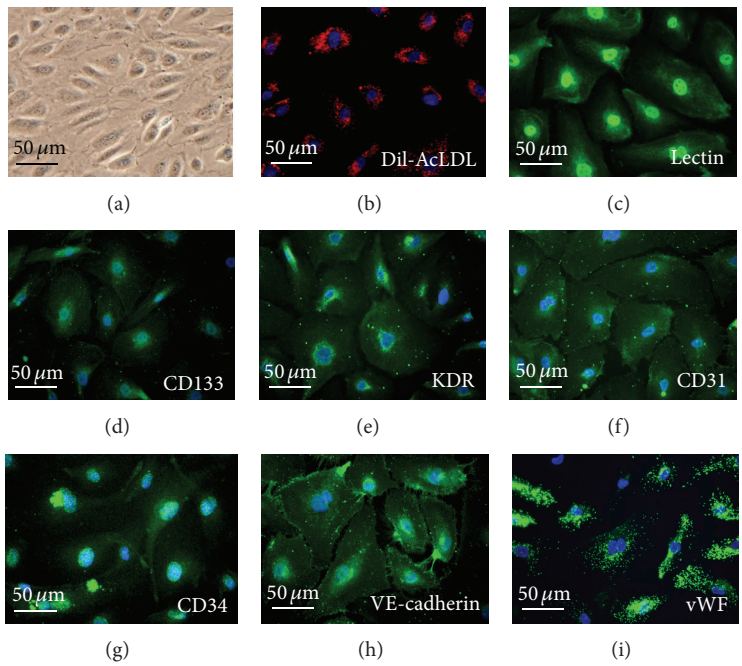


Figure 1: Morphology and characterization of EPCs from peripheral blood. (a) Peripheral blood mononuclear cells (MNCs) were plated on a fibronectin-coated culture dish on the fourteenth day. EPCs were also characterized by immunofluorescence staining for the expression of (b) DiI-AcLDL, (c) lectin, (d) CD133, (e) kinase insert domain receptor (KDR), (f) platelet/endothelial cell adhesion molecule-1 (CD31), (g) CD34, (h) VE-cadherin, and (i) Von Willebrand factor (vWF). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for the nuclei (blue).

2.8. Western Blotting. EPCs were lysed in protein lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 1 mM PMSF, and 1 μg/mL aprotinin, pepstatin, and leupeptin). The proteins were separated by SDS-PAGE and transferred to PVDF membrane. Membranes were probed with antibodies against β-actin, eNOS, phosphorylated eNOS (Millipore, Billerica, MA, USA), Akt, phosphorylated Akt (Cell Signaling, Denvers, MA), AMPK, phosphorylated AMPK (Sigma, St. Louis, MO, USA), activated-caspase 3, Bcl-2, and HO-1 (Cell Signaling, Danvers, MA). The protein blots were detected by chemiluminescence detection using ImageQuant LAS 400.

2.9. Animals. Nude mice were purchased from the National Laboratory Animal Center, Taiwan. This animal study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 1996) and all experimental procedures involving the animals received the approval of the Institutional Animal Care Committee of National Yang-Ming University (Taipei, Taiwan). Experimental mice received unilateral hindlimb surgery to induce ischemia, which involved excision of the right femoral artery as previously described [15]. The femoral artery was ligated at the proximal and distal portions. The blood perfusion of hindlimb was measured by a laser Doppler perfusion imaging system (Moor Instruments Limited, Devon, UK). At 24 hours after surgery, EPCs, high glucose-cultured EPCs, and high glucose combined with CoQ10-cultured EPCs were labeled with PKH26 (2 nM, Sigma) for 5 min. The PKH26-labeled cells (1×10^5) were injected into the ischemia limbs of operated nude mice. The control animals received the same volume of normal medium without EPCs.

2.10. Statistical Analysis. Statistical analyses were executed using SPSS software (version 14; SPSS, Chicago, IL, USA). Unpaired Student's t-test or analysis of variance was used to evaluate comparisons between groups. Significance was attained when a P value was less than 0.05.

3. Results

3.1. Characterization of Human EPC. As shown in Figure 1, MNCs were cultured on a fibronectin-coated dish on the fourteenth day (a). Most cells expressed DiI-acLDL uptake simultaneously (b) and fluorescein isothiocyanate UEA-1 (lectin, green (c)) binding. Then EPCs were characterized by immunofluorescence detection of CD133 (d), KDR (e), CD31 (f), CD34 (g), VE-cadherin (h), and vWF (i).

3.2. Effects of CoQ10 on EPC Viability, Migration, and Senescence in High Glucose Conditions In Vitro. To clarify the effects of CoQ10 on high glucose-induced viability and migratory function, MTT and modified Boyden chamber assays were used to evaluate cell viability and migration. EPCs were cultured for 4 days in high glucose medium (25 mM) with and without the indicated concentrations of CoQ10 (5–20 μM). High glucose environment decreased cell viability and attenuated EPC migration by 25% and 30%, respectively.

However, treatment with CoQ10 significantly improved EPC viability and migration in high glucose conditions (Figures 2(a) and 2(b)).

We further examined whether CoQ10 improves EPC senescence under high glucose condition. Compared with the control group, EPCs incubated with high glucose showed a significant increase in senescence (β-galactosidase-positive cells) by 28%. As shown in Figure 2(c), administration of CoQ10 for 24 hours attenuated high glucose-induced senescence by 30%.

3.3. CoQ10 Recovered High Glucose-Suppressed Mitochondrial Function of EPCs. Mitochondrial function was analyzed by measurement of mitochondrial membrane potential. Rhodamine 123 (Rh123) emits green fluorescence and localizes in mitochondria and was used to determine mitochondrial membrane potential by accumulation of green fluorescence. As shown in Figure 2(d), high glucose reduced mitochondrial membrane potential of EPCs by 20%; however, treatment with CoQ10 significantly reversed this mitochondrial effect.

3.4. CoQ10 Improved High Glucose-Induced EPCs Apoptosis. During cellular apoptosis, disruption of the mitochondrial membrane potential ($\Delta\Psi_m$) is one of the earliest intracellular events. To elucidate mitochondrial apoptosis of EPCs, JC-1 assay was used to analyze cellular apoptosis by the change in JC-1-derived fluorescence from red to green. Therefore, decline of mitochondrial membrane potential during apoptosis was represented by a decrease in red fluorescence intensity. The data shows that mitochondrial membrane potential ($\Delta\Psi_m$) of high glucose-cultured EPCs fell by 22% and administration of CoQ10 reversed the mitochondrial membrane potential by 25%. Therefore, CoQ10 recovered mitochondrial apoptosis in high glucose-stimulated EPCs (Figure 3(a)).

To further assess the effect of CoQ10 on antiapoptosis capacity in EPCs, we determined the expression of associated apoptosis proteins. The results showed that high glucose increased activated-caspase 3 and decreased Bcl-2 expressions; however, treatment with CoQ10 (10 μM and 20 μM) significantly inhibited activated-caspase 3 and increased Bcl-2 expressions in high glucose-cultured EPCs (Figure 3(b)).

3.5. CoQ10 Activates AMPK Pathway of EPCs in High Glucose Conditions In Vitro. To identify the possible mechanistic pathway of CoQ10 to recover EPC functions suppressed by high glucose, phosphorylated-AMPK expression was investigated in cultured EPCs exposed to high glucose. As shown in Figure 4(a), phosphorylated-AMPK expression was not enhanced in response to high glucose stimulation, but treatment with CoQ10 (10 μM) significantly upregulated phosphorylated-AMPK expression.

We further examined whether CoQ10 reduces high glucose-stimulated NO and ROS production. The data shows that high glucose reduced NO production of EPCs by 28% and treatment with CoQ10 reversed NO production by 30% (Figure 4(b)). On the other hand, ROS production was promoted by high glucose medium (increased 28%), and high glucose promoted-ROS production was significantly attenuated by treatment with CoQ10 (Figure 4(c)).

Moreover, administration of CoQ10 upregulated phosphorylation of Akt, eNOS and increased HO-1 expressions of EPCs in high glucose conditions (Figures 4(d)–4(f)). Of note, these effects were significantly nullified by administration of AMPK inhibitor. Furthermore, the administration of a NO inhibitor (L-NAME) reduced the activation of CoQ10-promoted HO-1 in the high glucose environment. These results suggested CoQ10 could activate eNOS, AKT, and HO-1 expressions through the AMPK pathway in high glucose-induced EPCs.

3.6. CoQ10 Improves High Glucose-Suppressed EPC Function through AMPK, NO, and HO-1 Pathways. To confirm that HO-1, NO, and AMPK pathways are involved in the effects of CoQ10 on cell functions suppressed by high glucose, EPCs were pretreated with compound C (AMPK inhibitor, 10 μM), L-NAME (NO inhibitor, 100 μM), and SnPP IX (HO-1 inhibitor, 5 μM) for 1 hour before CoQ10 treatment. As shown in Figure 5(a), treatment of EPCs with compound C, L-NAME, and SnPP IX reduced CoQ10-improved migratory capacity by 25%, 33%, and 23%, respectively. In addition, administration of compound C, L-NAME, and SnPP IX significantly reversed CoQ10-improved mitochondrial apoptosis, mitochondrial membrane potential, and ROS production (Figures 5(b)–5(d)).

Western blot analysis revealed that high glucose increased activated-caspase 3 expression, and treatment with CoQ10 suppressed this effect. Additionally, CoQ10-induced activated-caspase 3 protein was significantly downregulated by AMPK and NO inhibitors (34% and 32%, resp.) after CoQ10 treatment in high glucose conditions (Figure 5(e)). Moreover, the antiapoptotic protein Bcl-2 was downregulated by high glucose stimulation but recovered by CoQ10 treatment. However, this upregulation was reduced by AMPK and NO inhibitors by 40% and 45%, respectively. In addition, administration of HO-1 siRNA (10 nM) enhanced CoQ10 suppressed activated-caspase 3 protein in high glucose medium (Figure 5(f)). These data indicate that CoQ10 improved EPC function and attenuated cellular apoptosis through AMPK, NO, and HO-1 pathways.

3.7. CoQ10-Treated EPCs Transplantation Improves Hindlimb Perfusion. To further elucidate whether CoQ10 improves EPCs' angiogenic function, EPCs, high glucose-treated EPCs, and high glucose-treated EPCs incubated with CoQ10 were separately transplanted into ischemic hindlimbs in nude mice. EPCs were labeled with PKH26 fluorescence after being cultured with high glucose or high glucose treated with CoQ1K0 (10 μM). Blood flow of normal saline mice kept constant throughout the study, by about 50% of that measured in the nonischemic limb (3 weeks after operation). By contrast, mice transplanted with EPC were cultured with high glucose and CoQ10 medium and had a better blood flow recovery than only high glucose cultured EPC (Figure 6). These data suggest that blood flow in ischemic hindlimbs

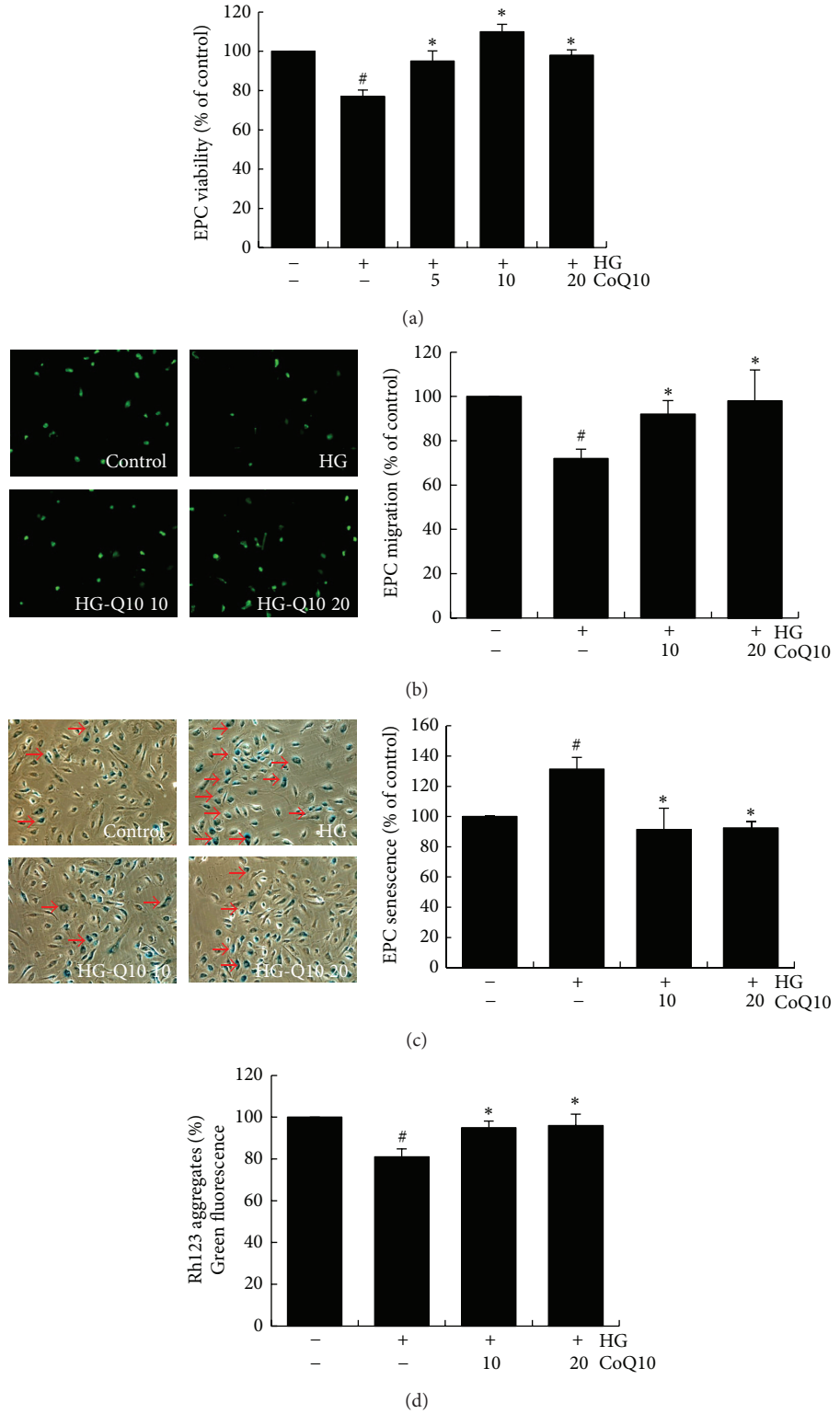


Figure 2: Effects of CoQ10 on EPC viability, migration, senescence, and mitochondrial function under high glucose conditions. Cells were cultured with glucose (25 mM) for 3 days, followed by treatment with CoQ10 (5 μM, 10 μM, and 20 μM) for 24 hr. (a) EPC viability was analyzed by MTT assay. (b) A Boyden chamber assay was used with SDF-1 as chemoattractive factor for EPC migration. The migrated cells were stained with fluorescein isothiocyanate UEA-1 (lectin) (green) and counted under the fluorescence microscope. (c) EPC senescence was analyzed by senescence-associated acidic-β-galactosidase activity assay. (d) Cell mitochondrial function was measured by staining with rhodamine 123- (Rh123-) derived green fluorescence 5 mM for 20 min. Data are mean ± SE; $n = 6$; $^{\#}P < 0.05$ versus control (5 mM glucose); $^{*}P < 0.05$ versus high glucose (HG).

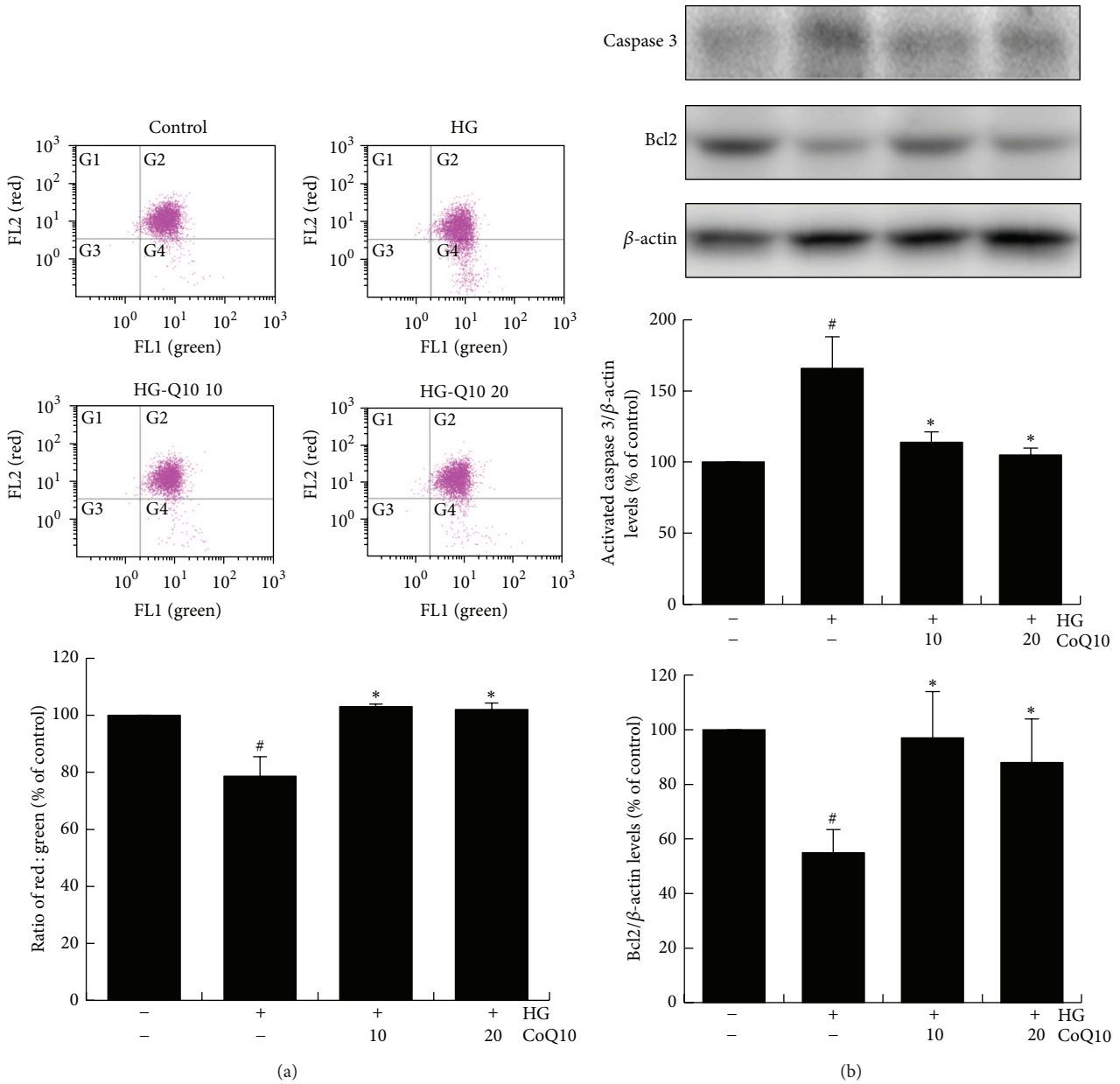


Figure 3: Effect of CoQ10 on EPC apoptosis under high glucose conditions. EPCs were incubated with CoQ10 (10 μM) for 24 hrs in high glucose medium. (a) Mitochondrial apoptosis was detected by JC-1 assay by flow cytometry. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was performed by the change in JC-1-derived fluorescence from red to green. The ratio of red/green fluorescence represented $\Delta\Psi_m$ in EPCs. (b) Expression of activated-caspase 3 and Bcl-2 protein levels were assessed by western blot analysis. Data are mean ± SE; $n = 4$; $^{\#}P < 0.05$ versus control (5 mM glucose); $^{*}P < 0.05$ versus high glucose (HG).

could be improved by transplantation with healthy EPCs and CoQ10-treated EPCs cultured under high glucose conditions.

4. Discussion

The defects in EPC functions and behavior may underlie some of the vascular complications associated with diabetes, such as endothelial dysfunction, that predispose a diabetic patient to diffuse atherosclerosis and impaired neovascularization after ischemic events [6, 16, 17]. Consequently, the idea of using EPCs as a therapeutic agent has grown in popularity. To the best of our knowledge, this is the first study to show the benefit of CoQ10 on high glucose-suppressed EPC functions. Administration of CoQ10 improved EPC functions, decreased ROS, increased NO production, and attenuated cellular apoptosis, with the mechanism of these effects being shown to involve AMPK, eNOS, and HO-1 pathways. Transplantation of CoQ10-treated EPCs under high glucose conditions into ischemic hindlimbs improved blood flow recovery more than in those that received only high glucose-incubated EPCs. Our findings provide novel evidence that CoQ10 could

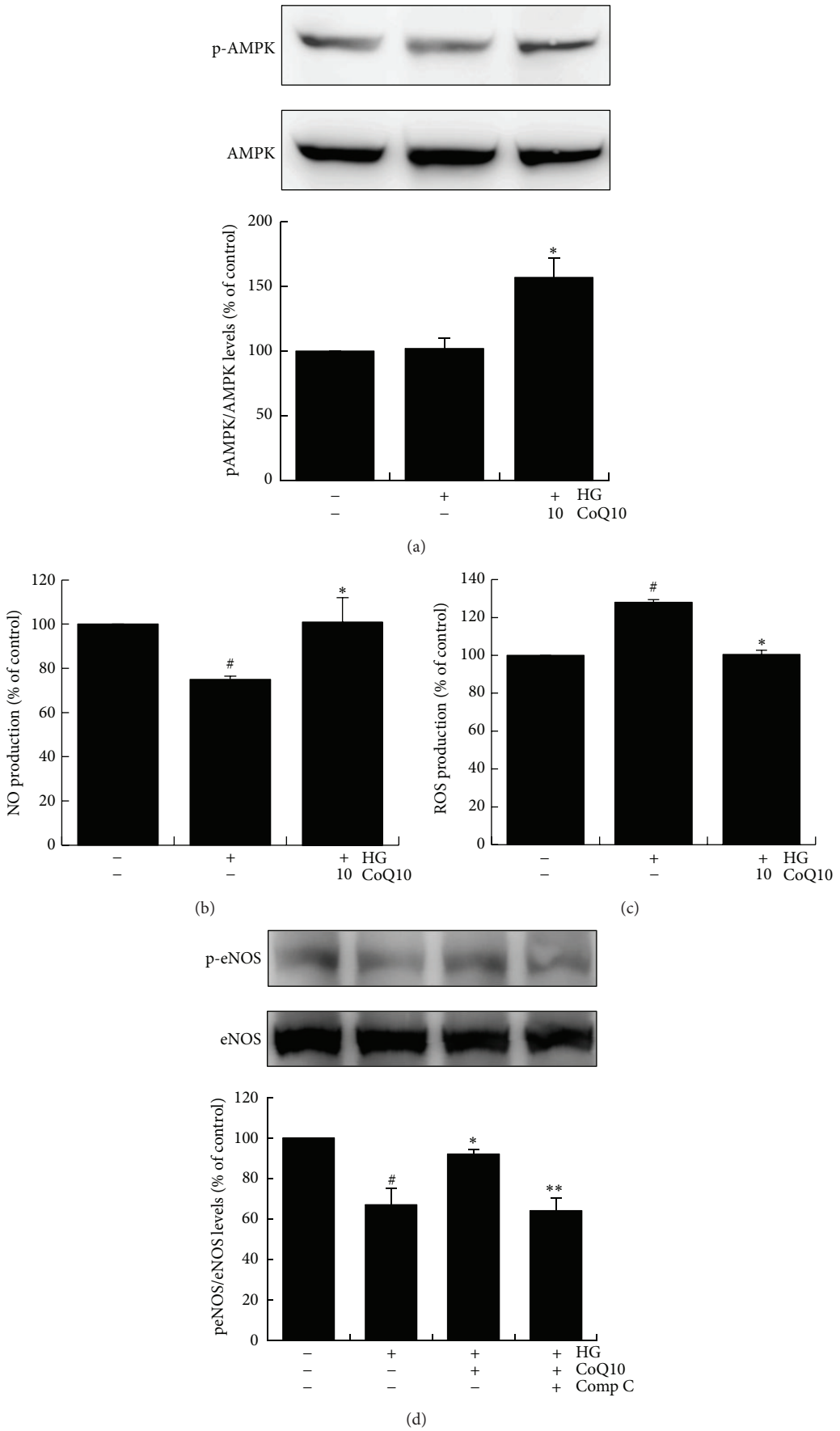


Figure 4: Continued.

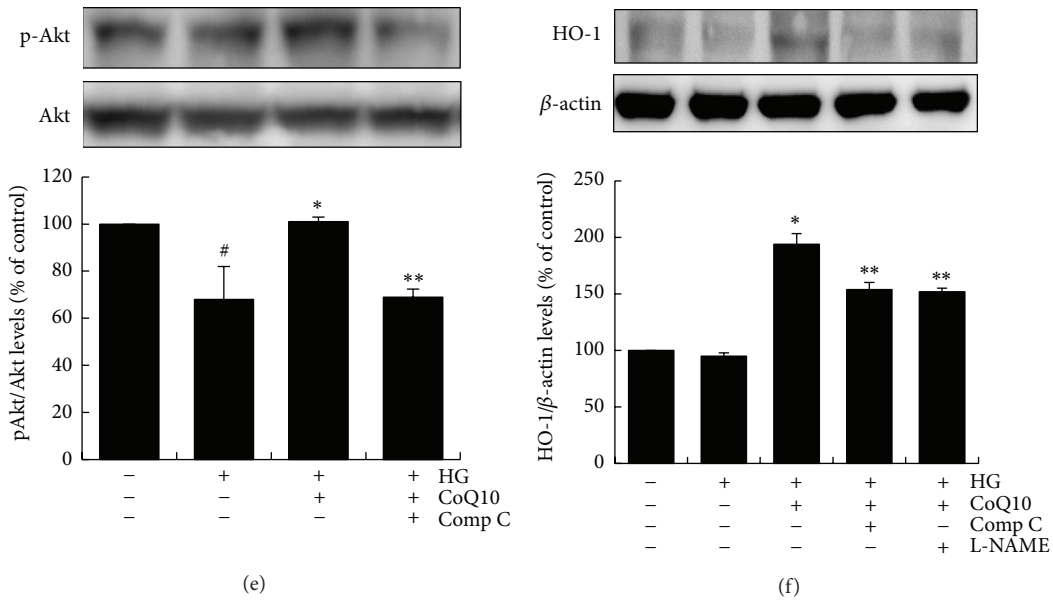


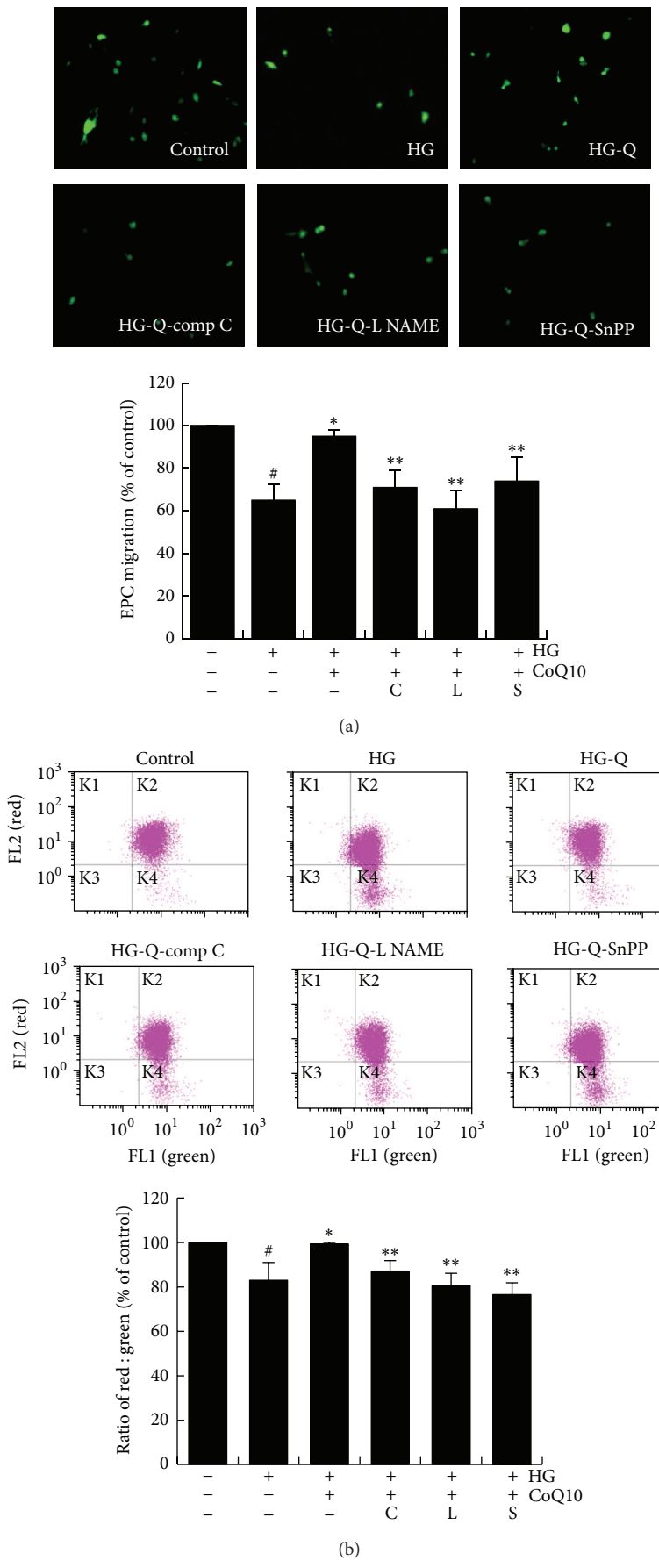
Figure 4: CoQ10 attenuates ROS and activates NO and AMPK pathway of EPCs under high glucose conditions. EPCs were incubated with CoQ10 (10 μM) under high glucose conditions. (a) AMPK protein phosphorylation levels of EPCs were analyzed by western blot. Data are mean ± SE; $n = 6$. (b) NO production of EPCs was assessed by staining with NO fluorescent indicator 3-amino,4-aminomethyl-2′,7′-difluorofluorescein (DAF-FM) diacetate (10 μM) for 30 min. (c) ROS production of EPCs was assessed by staining with DCFH-DA (10 μM) for 20 min. The fluorescence intensity was measured using a fluorescent microplate reader. ((d), (e), and (f)) Expressions of Akt protein phosphorylation, eNOS protein phosphorylation, and HO-1 protein were assessed by western blot analysis. Comp C: component C, AMPK inhibitor; L-NAME, NO inhibitor. Data are mean ± SE; $n = 4$; $^{\#}P < 0.05$ versus control (5 mM glucose); $^{*}P < 0.05$ versus high glucose (HG); $^{**}P < 0.05$ versus HG-CoQ10.

be a potential therapeutic agent to diminish high glucose-attenuated EPC angiogenic functions in diabetic patients.

Diabetes mellitus has reached epidemic proportions worldwide and is associated with a large economic burden and markedly increased risk of cardiovascular diseases. A large body of evidence suggests a causal link between diabetic hyperglycemia and the development of vascular complications [18]. However, the mechanisms that underlie diabetic hyperglycemia-induced vascular complications remain to be determined. Circulating EPCs are derived mainly from the monocyte/macrophage lineage, and are capable of forming new blood vessels through a process of vasculogenesis [3, 4]. Clinical studies have reported that EPCs are markedly decreased in diabetic patients, and EPCs from diabetic patients show reduced capacity to induce angiogenesis in vitro [19]. Importantly, impaired postischemic EPC mobilization in diabetic animals has been demonstrated previously [20]. The impairment of EPC functions and behavior may promote some of the vascular complications associated with diabetes that predispose diabetic patients to diffuse atherosclerosis and attenuated neovascularization after ischemic events.

It is well known that a causal link between diabetic hyperglycemia and the development of macrovascular and microvascular complications. Macrovascular complications include coronary artery disease, atherosclerosis and peripheral vascular disease, and microvascular complications include retinopathy, nephropathy and neuropathy [21]. Recent evidence suggests that NO has a crucial role in maintaining EPC and endothelial cells function [22]. Long-term exposure to high glucose conditions might enhance EPCs senescence and decrease cell numbers and functional competencies of EPCs via NO-related mechanisms [6]. Moreover, high glucose induces cytochrome-C release and promotes apoptosis of endothelial cells due to downregulation of NO bioavailability [23]. In line with previous reports, our results indicated that high glucose impaired Akt/eNOS activity of EPCs, as well as NO production, and administration of CoQ10 recovered eNOS activation through the AMPK pathway.

CoQ10, also known as ubiquinone-10 or ubiquinol-10, is well understood as an important component in the oxidative phosphorylation of mitochondria and the production of adenosine triphosphate [24]. CoQ10 is stored in the mitochondria of cells as a mobile lipophilic electron carrier and regulates respiratory chain activity. In addition, CoQ10 involves NAD(P)H-oxidoreductase-dependent reactions such as in NO synthesis in Golgi and plasma membranes [25]. Previous studies have indicated that CoQ10 attenuates cellular apoptosis in corneal fibroblasts by inhibition of mitochondrial depolarization [13, 26] and prevents HUVEC apoptosis through suppression of mitochondria dependent caspase 3 protein. However, there has been no report investigating the direct effect of CoQ10 on EPC in vitro. In our study, we described that administration of CoQ10 improved high glucose-suppressed EPC function and reversed apoptosis of



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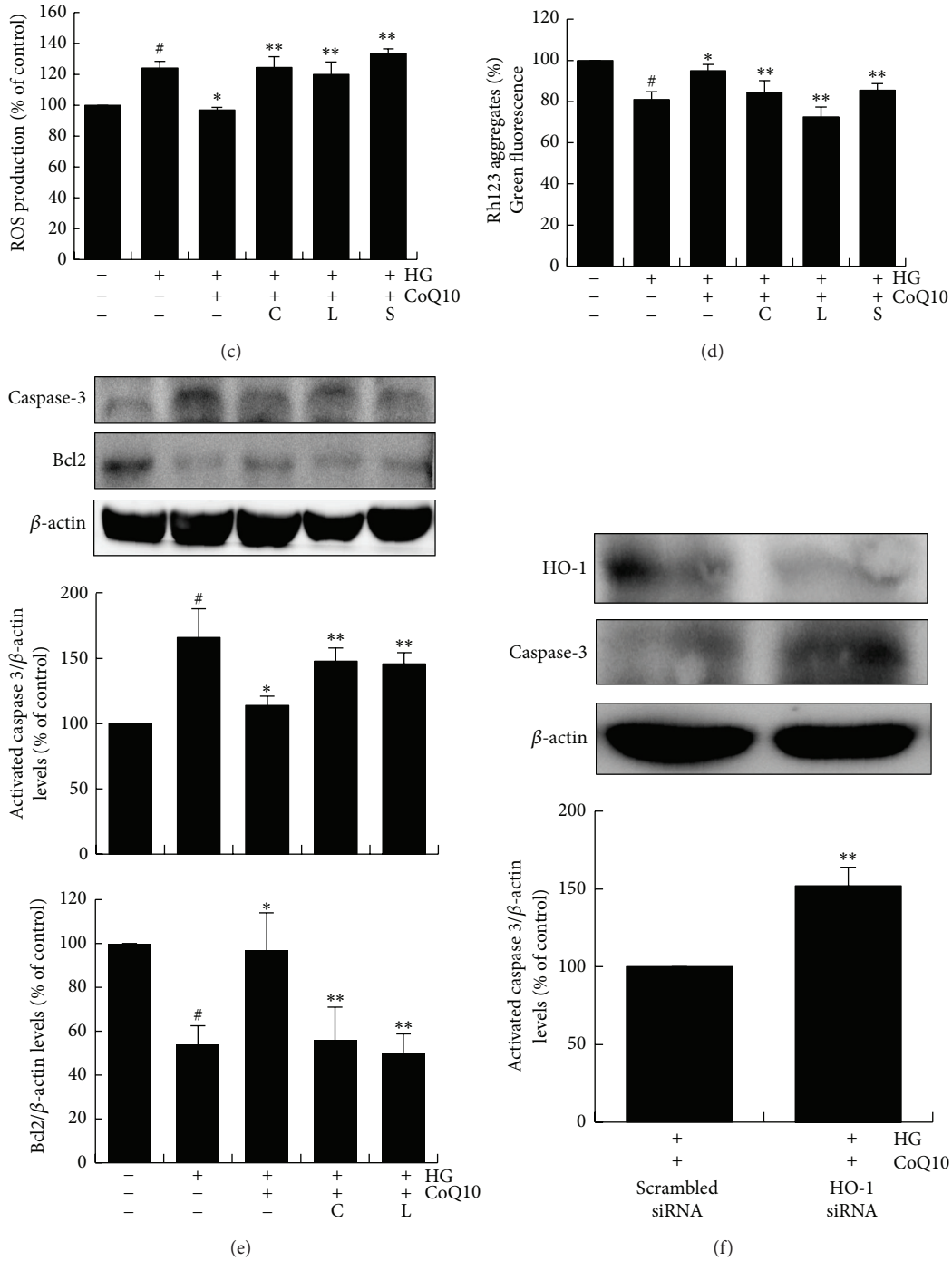

Figure 5: CoQ10 improves high glucose-induced EPCs dysfunction by upregulation of eNOS and HO-1. Cells were cultured with comp C (20 μM), L-NAMEA (100 μM), and SnPP IX (10 μM) for 60 min before CoQ10 incubation under high glucose conditions. (a) EPC migration was measured by Boyden chamber assay. The migrated cells were stained with fluorescein isothiocyanate UEA-1 (lectin) (green) and counted under the fluorescence microscope. (b) Mitochondrial apoptosis was detected by JC-1. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by the change in JC-1-derived fluorescence from red to green. The ratio of red/green fluorescence represented $\Delta\Psi_m$ in EPCs. (c) ROS production of EPCs was assessed by staining with DCFH-DA. (d) The mitochondrial function was measured using Rh123 dye (5 mM), and the fluorescence intensity was measured at 485-nm excitation and 530-nm emission using a fluorescent microplate reader. (e) Expressions of activated-caspase 3 and Bcl2 protein were assessed by western blot. (f) Cells were transfected with scramble and HO-1 siRNA (10 nM), respectively, in CoQ10-treated EPCs under high glucose conditions, and the expressions of activated-caspase 3 and HO-1 protein were detected by western blot. Comp C: component C, AMPK inhibitor; L-NAME, NO inhibitor; Snpp IX, HO-1 inhibitor. Data are mean ± SE; $n = 4$; $^{\#}P < 0.05$ versus control (5 mM glucose); $^{*}P < 0.05$ versus high glucose (HG); $^{**}P < 0.05$ versus HG-CoQ10.

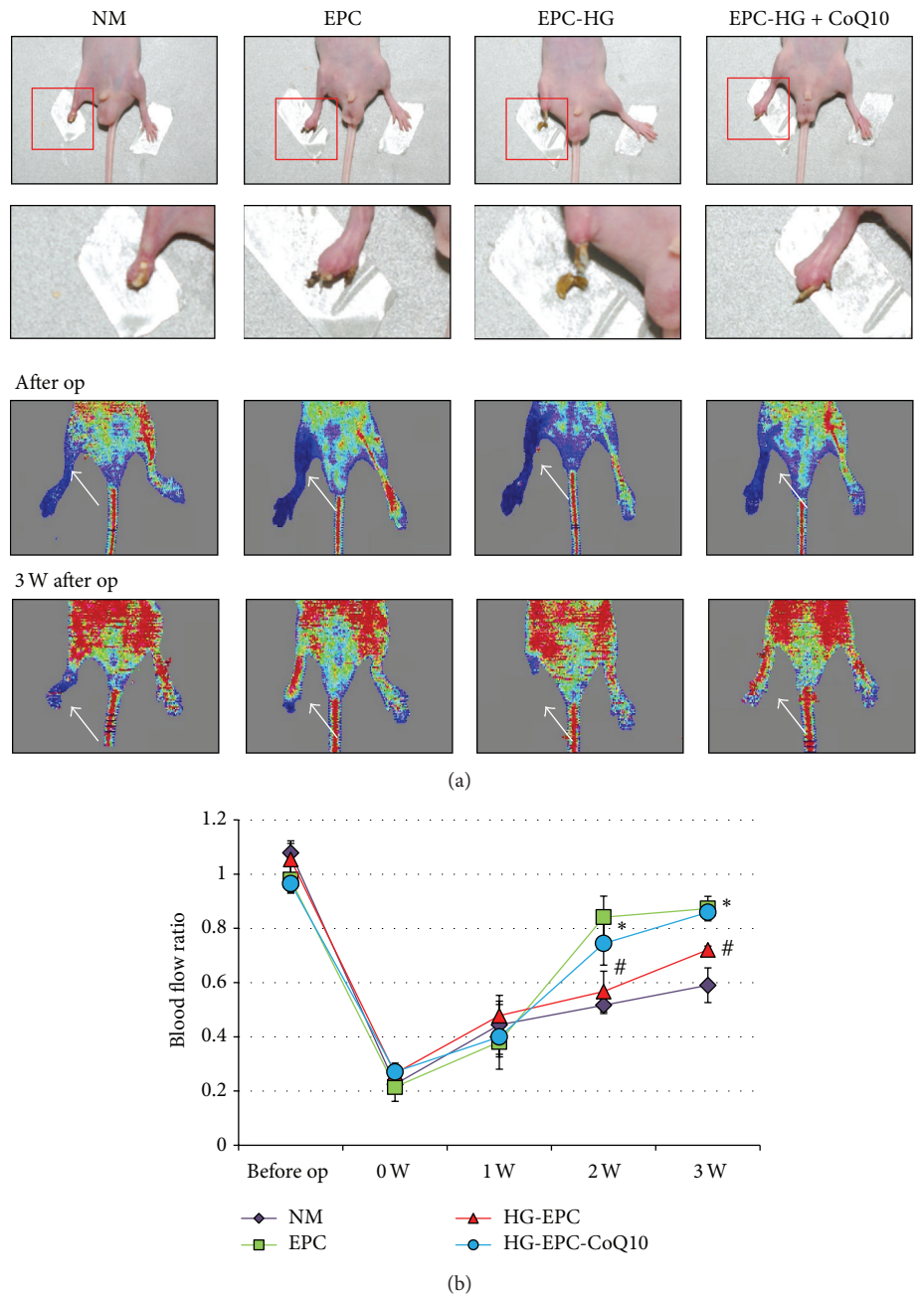

Figure 6: Effect of CoQ10-treated EPCs in high glucose medium transplantation on hindlimb perfusion. (a) Serial laser Doppler analyses of hindlimb perfusion revealed before and 3 weeks after hindlimb ischemia surgery in nude mice, which received a transplant with normal saline, EPCs, high glucose-treated EPCs, and high glucose-treated EPC incubated with CoQ10. Low or no perfusion is displayed as blue, whereas the highest perfusion is displayed as red. Arrows indicate ischemic (right) limb after hindlimb ischemia surgery. (b) Quantification analysis of perfusion recovery by laser Doppler perfusion imaging rations (ischemic/normal hindlimb) over time in the different groups. Results are mean ± SE; $n = 4$; $^{\#}P < 0.05$ versus control (5 mM glucose); $^{*}P < 0.05$ versus high glucose (HG).

EPCs by enhancement of mitochondrial function, enhanced Bcl-2 expression, and downregulation of caspase 3, which suggest that CoQ10 might improve EPC functional impairment and survival in diabetic patients. Additionally, CoQ10 was shown to reduce ROS production under high glucose conditions and improve EPC function by upregulation of eNOS and HO-1 through the AMPK pathway. These findings are consistent with previous reports and suggest that activation of CoQ10 might reduce hyperglycemia-induced mitochondrial dysfunction and promote mitochondrial biogenesis in EPCs exposed to high glucose [27, 28].

CoQ10, an antioxidant, scavenges free radicals and inhibits apoptosis in the mitochondria of cells. A recent study indicated that CoQ10 inhibited caspase 3 dependent apoptosis which was regulated by mitochondrial signaling in high glucose-treated HUVECs [29]. Moreover, CoQ10 displayed

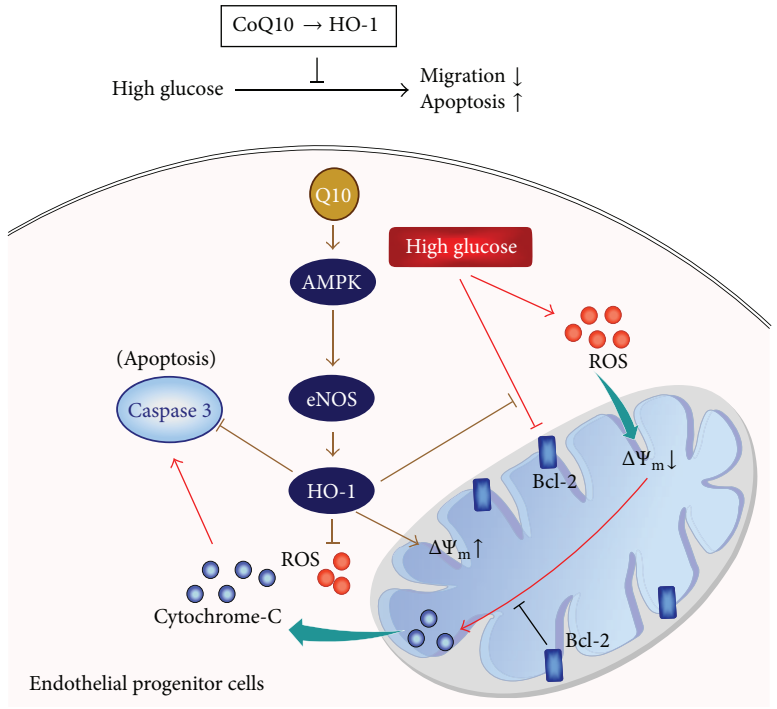

FIGURE 7: The schematic diagram summarizes possible mechanisms by which CoQ10 reduces hyperglycemia-induced endothelial progenitor cell damage. In high glucose condition, CoQ10 improved EPCs migration and apoptosis by ROS and caspase 3 downregulation and mitochondrial membrane potential ($\Delta\Psi_m$) and Bcl-2 upregulation through HO-1 pathway.

an antiapoptotic effect by suppressing mitochondrial membrane depolarization, cytochrome-C release, and caspase activation [14, 30]. CoQ10 has the ability to mediate oxidative stress and prevent diabetic endotheliopathy by eNOS activation [30]. In the current report, we showed that high glucose impaired Akt/eNOS phosphorylation and NO production, reduced EPC functional activity, and increased cell apoptosis. Treatment with CoQ10 improved Akt/eNOS phosphorylation and NO production and reversed high glucose-induced EPC damage.

Besides, Li et al. indicated that AMPK transgenic mice were resistant to hyperglycemia-induced impairment in endothelium dependent relaxation and reendothelialization of injured carotid arteries through HO-1 [31]. In our study, we found that HO-1 expression was upregulated by CoQ10, and administration of SnPP IX (HO-1 inhibitor) blocked CoQ10-improved EPCs' migration and apoptosis. These results extended previous studies suggesting that HO-1 overexpression in EPCs promoted reendothelialization and inhibited neointimal hyperplasia in injured vessels [32]. The beneficial effects may provide a new viewpoint for the use of CoQ10 therapy for its vascular protective properties in diabetic patients with critical limb ischemia or provide a clinical incentive to improve dysfunctional EPCs before cell therapy.

5. Conclusion

This study provides a notion that CoQ10 has beneficial effects in high glucose-induced EPC apoptosis and dysfunction in vitro. These data may clarify the underlying mechanisms responsible for the benefit of CoQ10 on the treatment of diabetic vasculopathy and cardiovascular diseases (Figure 7).

Disclaimer

Funding agencies had no role in study design, data collection, analysis, decision to publish, or preparation of the paper.

Conflict of Interests

The authors report no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hsiao-Ya Tsai, Po-Hsun Huang, Jaw-Wen Chen, and Shing-Jong Lin conceived and designed the experiments. Hsiao-Ya Tsai and Po-Hsun Huang performed the experiments. Hsiao-Ya Tsai, Po-Hsun Huang, Feng-Yen Lin, and Shing-Jong Lin analyzed the data. Jia-Shiong Chen and Chih-Pei Lin contributed reagents/materials/analysis tools. Hsiao-Ya Tsai, Po-Hsun Huang, and Shing-Jong Lin wrote the paper.

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Research Article

Impact of Real-Time Continuous Glucose Monitoring Use on Glucose Variability and Endothelial Function in Adolescents with Type 1 Diabetes: New Technology—New Possibility to Decrease Cardiovascular Risk?

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Children with type 1 diabetes (T1DM) are the high-risk group of accelerated atherosclerosis. Real-time continuous glucose monitoring (RT-CGM) provides possibilities for the detection of glycaemic variability, newly recognized cardiovascular risk factor. The aim of the study was to assess the usefulness of RT-CGM as an educational tool to find and reduce glycaemic variability in order to improve endothelial function in T1DM adolescents. Forty patients aged 14.6 years were recruited. The study was based on one-month CGM sensors use. Parameters of glycaemic variability were analyzed during first and last sensor use, together with brachial artery flow-mediated dilatation (FMD) to assess endothelial function. In the whole group, FMD improvement was found (10.9% to 16.6%, $p < 0.005$), together with decrease in all studied glycaemic variability parameters. In patients with HbA_1c improvement compared to the group without HbA_1c improvement, we found greater increase of FMD (12% to 19%, $p < 0.005$ versus 8.2% to 11.3%, $p = 0.080$) and greater improvement of glucose variability. RT-CGM can be considered as an additional tool that offers T1DM adolescents the quick reaction to decrease glycaemic variability in short time observation. Whether such approach might influence improvement in endothelial function and reduction of the risk of future cardiovascular disease remains to be elucidated.

1. Introduction

Type 1 diabetes mellitus (T1DM) is a well-established risk factor of accelerated cardiovascular disease, and, on the other hand, cardiovascular disease is the major cause of mortality in this group of patients. The relative risk for coronary heart mortality in T1DM patients is seven times higher than in matched counterparts without the disease and two times higher compared to risk of T2DM patients [1, 2]. Lately, it has been postulated that the main reason is not only chronic hyperglycaemia or other traditional risk factors, but frequent hypo- and hyperglycaemia episodes that accompany the disease daily course, and a new cardiovascular risk factor—excessive glycaemic variability—has been postulated. Blood glucose instability may contribute, perhaps even more than elevated HbA_1c, to the development of diabetes complications [3, 4]. These extreme glucose fluctuations lead to endothelial dysfunction and accelerated atherosclerosis, independently of average glucose concentrations, probably in mechanism of oxidative stress occurrence [5, 6]. Alterations in endothelial function precede the development of morphological changes and contribute to atherosclerotic lesion development and progression [7]. It is now clear that vascular disease begins in childhood and progresses silently until complications, such as stroke or myocardial infarction, later appear. T1DM in childhood is recognized as a high-risk factor for premature atherosclerosis [8–10].

Appreciation of the role of the vascular endothelium throughout the atherosclerotic disease process has led to the development of a range of invasive and noninvasive

Table 1: Basic characteristic of the study group.

	Study group	HbA_1c before the study		Improved HbA_1c after 3-month follow-up	
		<7.5%	≥7.5%	Yes	No
Number of patients	40	8	32	27	13
Age (years)	14.6 ± 2.1	15 ± 1	14.5 ± 2	14.4 ± 2	15.1 ± 1
Gender (boys/girls)	19/21	4/6	15/15	15/12	4/9
Diabetes duration (yrs)	7.4 ± 3.6	6.5 ± 2.7	7.6 ± 3.8	7 ± 4	8 ± 2.2
Height (m)	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.0
Body mass (kg)	59 ± 9.9	64 ± 3.8	58 ± 10	58 ± 10	62 ± 8
Body mass index (kg/m^2)	21.6 ± 3.1	21 ± 1.8	21.8 ± 3	22 ± 3	21 ± 2.4
SDS-BMI	0.6 ± 1.0	0.3 ± 0.8	0.7 ± 1	0.8 ± 1	0.2 ± 0.8
Cholesterol (mg/dL)	167.2 ± 29.1	138.3 ± 9.8	174.4 ± 27.9	169.9 ± 34.4	161.5 ± 11.6
LDL (mg/dL)	88.0 ± 25.7	73.5 ± 10.1	91.7 ± 27.2	88.4 ± 28.6	87.3 ± 19.1
HDL (mg/dL)	64.7 ± 16.7	48.0 ± 3.6	68.8 ± 16.0	67.2 ± 16.7	59.4 ± 15.9
TG (mg/dL)	70.7 ± 21.1	80.8 ± 26.9	68.1 ± 19.1	69.8 ± 16.7	72.4 ± 16.7
SBP (mmHg)	116.1 ± 7.1	125.0 ± 6.5	113.8 ± 5.2	116.6 ± 8.0	114.9 ± 4.7
DBP (mmHg)	71.1 ± 4.9	74.0 ± 5.5	70.3 ± 4.6	71.8 ± 4.8	69.5 ± 5.1
HbA_1c (%) before study	9.3 ± 1.5	7.2 ± 0.2	9.8 ± 1.2	9.0 ± 1.3	10 ± 1.7

Data are presented as mean ± SD.

techniques which permit evaluation of different aspects of its function [11]. At present, several noninvasive imaging techniques used in children offer an opportunity to study the relationship of surrogate markers to the atherosclerosis development. The use of these techniques may help to identify high-risk individuals in preclinical phase who may benefit from active therapy to prevent clinical disease. Endothelial function, that is, the vasodilator response to increased blood flow (flow-mediated vasodilatation, FMD), can now be accomplished using high-resolution ultrasound. FMD is an early endothelial dysfunction marker, used to detect minimal endothelial changes in children and adolescents [12].

T1DM poses a challenge to the goal of maintaining adequate glucose variability for years. From the 1990s, a new device has been introduced to the market to support patients in achieving this goal. Real-time continuous glucose monitoring system (RT-CGM) continuously measures the glucose level in interstitial fluid of subcutaneous tissue [13]. It provides the ability to track blood glucose trends and, thanks to warning alarms, prevents dramatic glucose variability before these changes become problematic. The potential clinical benefit of this technology as a tool to assist with optimization of glucose control has been demonstrated in several recent clinical studies, reviewed in [14].

The studies demonstrate that strict T1DM control helps not only in HbA_1c and glycaemic variability reduction, but also in diminishing oxidative stress level and dyslipidemia. Both of these are predominant in children and adolescents with T1DM, playing a key role in endothelial dysfunction and leading to early atherosclerosis development [15–17]. Some recent data indicate that even up to thirty percent of pediatric T1DM patients have impaired endothelial function, independently associated with A_1c and related to endothelial nitric oxide synthase T(-786)C polymorphism to some extent [18].

Noteworthily, there are no studies so far assessing possible influence of the RT-CGM device use on the cardiovascular system neither in T1DM adults nor in children. We hypothesized that use of RT-CGM via reduction of glycaemic variability might influence improvement of endothelial function. Therefore, we aimed firstly to assess brachial artery flow-mediated dilatation before and after short-term use of real-time continuous glucose monitoring in adolescents with T1DM. Secondly, we aimed to define a possible group of young patients who would benefit most from the use of this method.

2. Subjects and Methods

2.1. Patients. The entire study involved a total of forty adolescents (21 girls) at the age of 11–18 years—mean 14.6 ± 2.1, with the diagnosis of T1DM—who had been treated by continuous subcutaneous insulin infusion (CSII). Mean disease duration was 7.4 ± 3.7 years and mean HbA_1c level at the baseline was 9.35 ± 1.53%. Primary eligibility requirements were diabetes duration above 1 year, exogenous insulin requirement at least 0.5 j/kg/day, and insulin pump therapy for at least 0.5 years. Patients were excluded from the study if they presented other diseases that could affect the outcome (additional autoimmune disease, hypertension, hyperlipidemia, obesity, and early microangiopathy) as well as having education difficulties and presenting a lack of cooperation. We enrolled patients who have never used real-time continuous glucose monitoring system before but expressed their willingness to participate in the study. All of the patients used either a sensor augmented insulin pump (Paradigm 722, Medtronic MiniMed, Northridge, CA) or Guardian RT (Medtronic, Northridge, CA) device when the original pump (Paradigm 715, Medtronic or AccuCheck Spirit, Roche) did not have a sensor option. We used "enlite" sensors (Medtronic) in all patients. Table 1 presents the basic characteristic of the study group.

2.2. Study Design. The study was based on one-month continuous glucose sensors use combined with technical training and proper education of the patients and their caregivers concerning insulin therapy, diet, and physical activity. There was also the written instruction of operating system given to all of the patients. Children and their parents were able to contact the diabetology team if any problem occurred. Every patient was supplied with 4-5 sensors, changed every 6-7 days, to provide the 4-week trial. First sensor was inserted by medical staff. After the first and last sensor usage, the obtained data were uploaded to the computer using the CareLink Pro (Medtronic). Clinical issues were analyzed and discussed together with diabetologist. During the study, patients were told to live normal life adjusting the insulin therapy based on sensor records, confirmed with SMBG measurements, by themselves or prior telephone consultations. They also calibrated sensors 4 times a day using the personal glucometer. Next, we performed the analysis of computer uploaded glucose variability parameters that included mean blood glucose, standard deviation (SD) for the mean glucose, and area under the curve (AUC) for glucose level >140 mg/dL and <70 mg/dL, minimum and maximum glucose levels.

2.3. Laboratory Analyses. The level of glycated hemoglobin (HbA_1c) was determined by high-pressure liquid chromatography (HPLC) at the baseline and 3 months after the study. Depending on the initial value of HbA_1c, the group was divided into well-controlled group (with HbA_1c <7.5%, mean 7.25 ± 0.19%) and poorly controlled group (with HbA_1c >7.5%, mean 9.88 ± 1.22%). Decrease of HbA_1c by at least 0.5% after 3-month follow-up was the criterion to divide the study group into improved group versus not improved group.

2.4. Ultrasound Measurements. The ultrasound measurement procedure was conducted between 8.00 and 10.00 AM after a fasting period of 8–12 hours. Examinations of the brachial and carotid arteries were performed with Hewlett Packard Sonos 4500 apparatus, using a 7.5 MHz linear transducer. Ultrasound examination of the right brachial arteries was performed in longitudinal sections 2–10 cm above the elbow, according to guidelines [19].

The principle is to induce vasodilatation in the proximal (brachial) artery by postischemic (forearm) enhanced flow. All lumen diameter measurements were scanned at end diastole by use of the R-wave of the electrocardiogram. First scans were taken at rest and second scans during reactive hyperemia. Increased flow was induced by deflating a pneumatic tourniquet placed on the right forearm, inflated to the pressure about 50 mmHg above the patient's resting systolic blood pressure for 4.5 min. The postischemic scan was performed 45–120 seconds after cuff deflation. Flow-mediated dilatation (FMD) was derived from the percentage change of the brachial artery diameter after ischemia of the forearm from baseline. Nitric oxide-dependent FMD of the brachial artery was assessed at baseline (before RT-CGM sensor placement) and after one month of sensors usage.

The study was approved by the Bioethics Committee, Medical University of Białystok, Poland. Caregivers and children were informed about the purpose and nature of the study. The caregivers gave a written consent, whereas the children expressed a spoken consent before examination.

2.5. Statistical Analysis. The statistical analysis was performed using the Statistica 9.0 software (Krakow, StatSoft). To determine the differences between the study groups for variables with normal distributions, the Student t-test was applied. The Student t-test for paired variables was used to compare the variables within the respective groups at baseline, after one month (glycaemic variability parameters), and after 3 months (HbA_1c values). Since in this study the variables satisfied the conditions of normal distribution, no other tests were applied. The correlations between studied variables were assessed using Pearson correlation. All the results are presented as the mean ± standard deviation (SD). Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Metabolic Control and Glucose Variability. Our analysis of metabolic control in the study group was based on HbA_1c level, which has significantly decreased in entire study group after three months from 9.35 ± 1.5% to 8.81 ± 1.8% ($p < 0.001$). Significant HbA_1c improvement, which we considered as at least 0.5%, 3 months after the study was observed in 68% of the patients (27 children). In the group with improvement, HbA_1c decreased from 9.03 ± 1.4% to 8.04 ± 1.2% ($p < 0.001$). The group with no improvement in HbA_1c showed significantly worse metabolic control already at the beginning of the study: 10.03 ± 1.7% versus 9.03 ± 1.4% ($p = 0.054$ compared to group with improvement), and that difference has grown much more after three-month follow-up: 10.42 ± 1.6 versus 8.04 ± 1.2% ($p < 0.001$) (Table 2). In the group of children with optimal metabolic control (HbA_1c <7.5%), mean HbA_1c level decreased from 7.25 ± 0.2% to 6.73 ± 0.2% ($p = 0.006$), and in the group with poor metabolic control ($HbA_1c \geq 7.5\%$), mean HbA_1c level after 3-month follow-up decreased from 9.88 ± 1.2% to 9.33 ± 1.16% ($p = 0.003$) (Table 3).

Glucose variability parameters during the last week sensor have improved compared to results of first week sensor use in the entire study group. We noticed decrease of mean glycaemia, SD of mean glycaemia, and maximum glucose values and AUC for hyperglycaemia, while minimum glucose values increased and AUC for hypoglycaemia did not change (Table 2). The significance of that improvement was related to the HbA_1c level changes. Mean glucose decreased significantly in all the patients after a monthly usage of RT-CGM, but some other parameters have changed significantly only in group with metabolic improvement. SD for mean glucose in these patients decreased from 60.74 mg/dL during the first sensor usage to 51.67 mg/dL during the last sensor usage ($p = 0.010$). Area under the curve (AUC) >140 mg/dL decreased from 41.23 using the first sensor to 21.22 using the last sensor ($p < 0.001$). Maximal glucose level changed from 344.37 mg/dL to 317.41 mg/dL ($p = 0.004$). The positive effect of RT-CGM use lost some statistical significances in group without HbA_1c improvement (Table 2).

Table 2: Glucose variability during the study in the whole group and depending on HbA_1c improvement.

		Study group	HbA_1c improvement	No HbA_1c improvement
HbA_1c (%)	Before the study	9.35 ± 1.5	9.03 ± 1.35	10.03 ± 1.71
	After the study	8.81 ± 1.8	8.04 ± 1.33*	10.42 ± 1.60*
	p	<0.001	<0.001	0.102
Mean glucose (mg/dL)	1st week	168.18 ± 33.9	164.44 ± 37	175.92 ± 22.77
	Last week	144.8 ± 22.9	138.22 ± 31*	158.46 ± 21.19*
	p	<0.001	<0.001	<0.001
Mean glucose SD (mg/dL)	1st week	61.25 ± 14.5	60.74 ± 15	62.31 ± 12.43
	Last week	53.55 ± 14.1	51.67 ± 14	57.46 ± 21.19
	p	0.002	0.010	0.050
AUC > 140 mg/dL	1st week	43.29 ± 26.1	41.23 ± 29	47.56 ± 18.47
	Last week	25.23 ± 16.9	21.22 ± 15*	33.58 ± 17.41*
	p	<0.001	<0.001	<0.001
AUC < 70 mg/dL	1st week	0.58 ± 0.61	0.73 ± 0.6*	0.29 ± 0.36*
	Last week	0.53 ± 0.53	0.62 ± 0.5	0.35 ± 0.36
	p	0.591	0.439	0.576
Max glucose (mg/dL)	1st week	365.15 ± 74.5	344.37 ± 57	380.6 ± 100.08
	Last week	328.6 ± 67.2	317.41 ± 55	354.92 ± 82.98
	p	0.002	0.004	<0.001
Min glucose (mg/dL)	1st week	51.15 ± 12.5	49.85 ± 13	53.85 ± 10.05
	Last week	47.5 ± 6.4	46.07 ± 5*	50.46 ± 7.31*
	p	0.045	0.136	0.107
FMD (%)	Before the study	10.9 ± 6.6	12.22 ± 5.41	8.18 ± 8.15
	After the study	16.67 ± 8.5	19.27 ± 7.40*	11.29 ± 8.35*
	p	<0.001	<0.001	0.089

Results of RT-CGM are presented as mean ± SD.
* $p < 0.05$: significant difference between two subgroups, considering the same time sensor usage (first or last week).

Table 3: Glucose variability depending on the initial HbA1c level.

		HbA_1c before the study	
		<7.5%	≥7.5%
HbA_1c (%)	Before the study	7.25 ± 0.19*	9.88 ± 1.22*
	After the study	6.73 ± 0.47*	9.33 ± 1.62*
	p	0.006	0.003
Mean glucose (mg/dL)	1st week	127.75 ± 9.84*	178.28 ± 29.90*
	Last week	126.75 ± 23.26*	149.31 ± 8.12*
	p	0.8	<0.001
Mean glucose SD (mg/dL)	1st week	46.75 ± 6.94*	64.88 ± 13.57*
	Last week	44.75 ± 3.49*	55.75 ± 14.95*
	p	0.286	0.003
AUC > 140 mg/dL	1st week	14.75 ± 6.94*	50.46 ± 24.33*
	Last week	13.00 ± 3.52*	28.29 ± 17.65*
	p	0.157	<0.001
AUC < 70 mg/dL	1st week	0.98 ± 0.46*	0.49 ± 0.61*
	Last week	0.68 ± 0.28	0.58 ± 0.62
	p	0.203	0.957
Max glucose (mg/dL)	1st week	279.75 ± 25.93*	375.26 ± 70.36*
	Last week	291.50 ± 14.92	339.13 ± 71.90
	p	0.14	<0.001
Min glucose (mg/dL)	1st week	42.75 ± 1.75*	52.25 ± 13.17*
	Last week	48 ± 5.45	47.37 ± 6.65
	p	0.045	0.005
FMD (%)	Before the study	16.25 ± 4.32*	9.57 ± 6.44*
	After the study	27.81 ± 6.49*	13.89 ± 6.43*
	p	0.002	<0.001

Results of RT-CGM are presented as mean ± SD.
* $p < 0.05$: significant difference between two subgroups, considering the same time sensor usage (first or last week).

When we compared children with initial optimal and poor glycaemic control, we noticed that the largest favourable changes were found in group with HbA_1c >7.5%. All glycaemic variability parameters, apart from AUC <70 mg/dL that remained stable, achieved improvement at level $p < 0.001$. In group with initial optimal HbA_1c, only minimum glucose level achieved significant improvement: 42.75 mg/dL versus 48 mg/dL, $p = 0.045$. Noteworthily, however, the glucose variability parameters were better at the beginning of the study and at the end as well in the group with initial optimal glycaemic control when compared appropriately with first and last sensor use in poor control group (Table 3).

3.2. Endothelial Function. In the whole study group, an increase of FMD of the brachial artery was observed (from 10.90 ± 6.6% to 16.67 ± 8.5%, $p < 0.005$) (Figure 1). This result depended on metabolic control improvement. A much greater increase of FMD was found in the patients with HbA_1c improvement (from 12.22 ± 5.4% to 19.27 ± 7.4%, $p < 0.005$) compared to the group without HbA_1c improvement (from 8.18 ± 8.2% to 11.29 ± 8.4%, $p = 0.080$) (Figure 2). As we have subdivided our patients into two groups depending on the initial HbA_1c level (>7.5% and <7.5%), we have found a greater increase of mean brachial artery FMD in a group with initial HbA_1c below 7.5% (from 16.25 ± 4.3% to 27.81 ± 6.5%, $p < 0.005$) than in a group with initial HbA_1c above 7.5% (from 9.57 ± 6.4% to 13.89 ± 6.4%, $p < 0.005$).

3.3. Correlation Analysis. We found that FMD before the study significantly correlated inversely with HbA_1c before

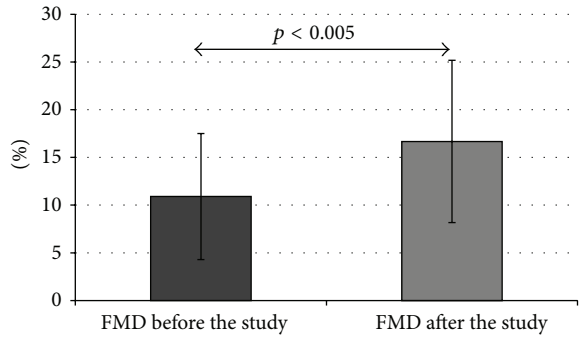

Figure 1: FMD in the whole study group before and after the study (data presented as mean ± SD).

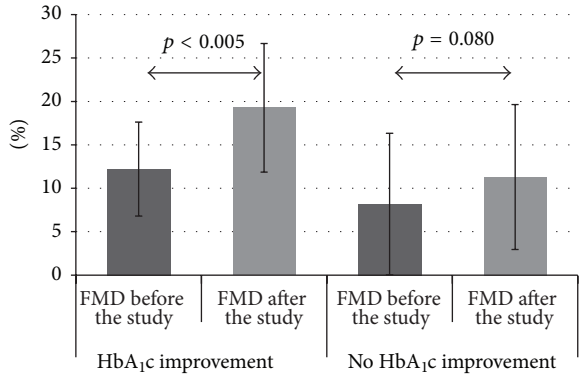

Figure 2: FMD changes depending on HbA_1c improvement in follow-up (data presented as mean ± SD).

the study ($r = -0.6$, $p < 0.001$), mean glucose before the study ($r = -0.40$, $p = 0.009$), and AUC >140 mg/dL before the study ($r = -0.35$, $p = 0.026$). FMD after the study significantly correlated inversely with HbA_1c after the study ($r = -0.78$, $p < 0.001$), mean glucose after the study ($r = -0.37$, $p = 0.016$), mean glucose from the whole study ($r = -0.44$, $p = 0.004$), and AUC >140 mg/dL for the whole study ($r = -0.34$, $p = 0.27$). No associations were observed between subclinical marker of atherosclerosis and total cholesterol, LDL-cholesterol, and triglycerides or between disease duration and onset either.

4. Discussion

The present study is the first to our knowledge to show that use of real-time CGM can be helpful in reduction of glycaemic variability in order to improve endothelial function in young T1DM patients without any symptoms of clinically present cardiovascular disease. Among the whole study group, we have observed significant endothelial function improvement, mainly in those patients who have also improved their HbA_1c level. In those patients, the initiation of RT-CGM caused both a rapid reduction of glucose variability and improvement in vascular function.

Glucose variability affects quality of life and can contribute to pathogenesis of diabetic complications [4, 20]. Continuous subcutaneous insulin infusion (CSII) and multiple daily insulin injections (MDI), established therapies for type 1 diabetes, are thought to prevent hyperglycaemia and deleterious glucose fluctuations. Patients using insulin pumps present with lower glycaemic variability, better glycaemic control, and treatment satisfaction compared with those using MDI [21, 22]. RT-CGM can be considered as one step further in achieving a safe way to target near normoglycaemia. Although there is still a debate as to whether RT-CGM can improve glycaemic control and improve quality of life, increasing amount of data of observational and RCT studies in the pediatric age groups found improvement in metabolic control with use of this device. Most of the RCT studies, including pediatric T1DM, have demonstrated that the frequency of the CGM use was significantly associated with the effect of lowering HbA_1c levels. Real-time CGM has been shown to lead to sustained reduction in MAGE and hypoglycaemia; nevertheless, benefits from sensor augmented pumps relate to sensor use frequency [13, 14]. Our results are in agreement with these studies, as we observed significant improvement in HbA_1c in almost 70% of patients and significant improvement in glucose variability parameters.

Recently, the close relationship between increased glucose variability and endothelial dysfunction has been demonstrated in several studies in both type 1 and type 2 diabetic patients [5, 23, 24]. Noteworthily, in the study of obese adults with or without metabolic syndrome and type 2 diabetes, results of CGM revealed the close connection between glycaemic variability and endothelial function. Interestingly, it appeared that glycaemic variability may be elevated even in nondiabetic, despite being obese, subjects and is also independently correlated with endothelial function [24]. Furthermore, one of Australian studies demonstrated that children with T1DM following continuous subcutaneous insulin infusion (CSII) initiation have early improvements in vascular function, blood pressure, and metabolic control associated with reduced glucose variability [23]. In our study, all of our patients have already used CSII. Moreover, we have gone a step further and used real-time glucose monitoring to give the patients the possibility of positive feedback and immediate reaction. In the discussed studies, patients were using CGM blindly, and the results were analyzed retrospectively, whereas in our study patients were given a possibility for dynamic modulation of the displayed glycaemic variability. They could react immediately by changing the insulin dosage, diet, physical activity, and lifestyle. In the abovementioned study, the effects were unfortunately not sustained after twelve months, and FMD and glucose variability returned to baseline levels, with deterioration of metabolic control. Poor adherence to CSII-related tasks, such as insulin bolusing for meals, is frequently seen in adolescents [25]. In our study, we have discovered that using real-time CGM may help to improve not only glucose variability parameters, but endothelial function as well, in patients already treated with CSII.

In some contrast to our results remains the study of Peña et al., where only hypoglycaemia, but not glucose variability, during continuous glucose monitoring related to impaired vascular endothelial function in children with T1DM [26].

However, this was not interventional study, like our study was. In our group, we found that AUC for hypoglycaemia and minimal glycaemia value in the whole study group did not change significantly after one-month glucose sensor use. Interestingly, however, in the group with initial optimal glycemic control, where the highest improvement in endothelial function was observed, we noticed higher minimal glucose value during last week of sensor use compared to the first sensor use and insignificantly decreased AUC for hypoglycaemia.

It has been speculated that oxidative stress is the link between glucose variability and vascular dysfunction. Both in vitro studies and animal models have demonstrated evidence of vascular inflammation and endothelial cell apoptosis following fluctuations in blood glucose levels, via the production of reactive oxygen species [27, 28]. Oscillating blood glucose may have much worse effect on endothelial function and oxidative stress than stable hyperglycaemia in diabetic as well as nondiabetic subjects.

CGM allows constant glucose variability control. Still, the gold-standard method to measure glucose variability in research and clinical practice was not established. Using just glycated hemoglobin (HbA_1c) as a glycaemic variability parameter has a number of limitations: it presents average glucose levels from the last three months, while the research shows that avoiding hypo- and hyperglycaemia episodes may be even more important. In some patients, it is even possible to reduce glycaemic instability without HbA_1c improvement. The superiority of glycaemic variability was presented in recent studies, which recommend that clinicians should focus first on limiting glucose variability, before attempting to reduce median blood glucose, that is, HbA_1c [29]. Standard deviation (SD), an index of the dispersion of data around mean blood glucose, was by design viewed as the simplest approach for the evaluation of glucose variability, beyond the simple determination of mean blood glucose. A clear consensus on the gold-standard method to measure glucose variability in clinical practice and research is still lacking, although a number of indicators have been proposed [30]. In our study, we have chosen generally accepted methods of the quality of blood glucose control and variability. These include the area under the curve (AUC) and the percentage of time inside, above, and below the blood glucose target. We have intentionally avoided the use of newly introduced parameters, such as continuous overlapping net glycaemic action (CONGA) or mean amplitude of glycaemic excursions (MAGE) due to reports showing the limitations of those methods and to the fact that those have not gained widespread use in clinical practice [24, 31].

The impact on endothelial function seems to be more effective in the patients who present better adherence. Our study has confirmed the correlation of FMD increase with metabolic control improvement. In our study, we named responders to CGM these patients who were able to improve the HbA_1c level at least by 0.5% after three months of follow-up. Almost 70% of our adolescents achieved this result, and what is more, responders achieved greater improvement in endothelial function as FMD increased in this group significantly more than in nonresponders. Patients who improved their metabolic control presented greater decrease of parameters of glucose variability—the average glucose, SD for the mean glucose, AUC >140 mg/dL, and maximal glucose level. FMD showed similar trend; greater increase was observed in patients with more significant HbA_1c improvement. Therefore, our study demonstrated that responders to RT-CGM benefit most in endothelial function improvement. Surprisingly, we observed the greatest increase in FMD in those with optimal glycaemic control from the onset. While HbA_1c in this subgroup decreased even more from 7.25% to 6.7%, changes in glycaemic variability parameters did not achieve statistical significance, except for the already abovementioned decrease in AUC for hypoglycaemia. We can speculate that it is due to their already initial high adaptation to diabetes-related tasks and educational potential and abilities to view and use real-time data to make insulin, nutrition, and lifestyle modifications. However, the mechanism is still unknown and requires further research. Our data provide important information on the role glycaemic variability may play in influencing cardiovascular risk even in children with T1DM and the possibility to undertake deliberate action to improve endothelial function in this group of high-risk patients.

4.1. Limitations of the Study. We are aware that there are certain limitations of our study implicating a careful interpretation of the conclusions. Metabolic control and endothelial function improvement might be caused by extra training and more intensive education schedule that was given to the patients and their caregivers rather than the effect of CGM itself. No control group went through the same training and monitoring without RT-CGM. An additional limitation is that the improvement of A_1c after 3 months was directly attributed to the 4 weeks of CGM use; there is no proof from the data that these two are directly related. The study was not controlled for physical activity. Our group numbers were quite small, with only 8 patients in the <7.5% group. Notwithstanding, the aim of the study was rather to demonstrate the additional benefits of RT-CGM device use in children and adolescents than to prove the superiority of CGM over SMBG in improving metabolic control and endothelial function.

5. Conclusions

RT-CGM can be considered as an additional, educational tool which offers type 1 diabetic adolescent the quick reaction to decrease glycaemic variability parameters in short-time observation. Whether such approach may allow for the improvement in endothelial function and further influence to reduce the risk of future cardiovascular disease remains to be elucidated. The best outcome was demonstrated in initially well-controlled patients (HbA_1c <7.5%), but also in RT-CGM responders, that significantly improved HbA_1c and glycaemic fluctuations in one-month system use. Significant FMD improvement was not evident in poorly controlled individuals.

Conflict of Interests

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

Authors' Contribution

Milena Jamiołkowska and Izabela Jamiołkowska contributed equally to the work.

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Review Article

Gliptins and Cardiovascular Outcomes: A Comparative and Critical Analysis after TECOS

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The issue related to macrovascular outcomes and intensive glycemic control was hotly debated after the publication of landmark trials like ACCORD, ADVANCE, and VADT. The only benefits seem to come from intervening early on in the disease process as indicated by the 10-year UKPDS follow-up. To complicate matters USFDA made it mandatory for modern drugs to conduct cardiovascular safety trials in high-risk populations after the 2008 rosiglitazone scare. This led to all the modern group of drugs designing cardiovascular safety trials (gliptins, GLP-1 agonists, and SGLT-2 inhibitors) to meet USFDA regulatory requirements. We saw publication of the first 2 randomized trials with gliptins published a year and a half back. On the face value SAVOR TIMI and EXAMINE satisfied the primary composite CV end-points. However, issues related to significant increase in heart failure and all-cause 7-day on-treatment mortality created a lot of confusion. FDA reanalysis of these data (especially SAVOR) raises a lot of doubts as far as CV safety of these groups of drugs was concerned. Hence, all eyes were on TECOS, which was published this year. We take a microscopic look at these trials trying to understand where we stand as from now on this issue.

1. Introduction

The adverse impact of oral antihyperglycemic agent on the cardiovascular system came into focus as early as 1971 with UGDP program [1]. The drug in question was a sulfonylurea. However, with the advent of second-generation sulfonylureas and nonusage of first-generation ones this controversy was put to rest (without proper investigation).

In early 2000 it was glitazones which came into the headlines with issues related to fluid accumulation (pedal edema and macular edema) [2]. Glitazone group of drugs were contraindicated in patients with NYHA Classes III and IV heart failure [3].

There was another development in 2005 which brought the issue of secondary end-points into focus. It was the PROactive study [4]. The results of this study were eagerly awaited, as this was the first time type 2 diabetic individuals with high CV risk were exposed to a CV outcomes trial. All the surrogate CV markers positively influenced by pioglitazone were put to test [5]. The primary end-points put to test over an average of 34.5-month follow-up were time to first death, nonfatal MI, stroke, acute coronary syndrome, major leg amputation, coronary revascularization, and leg revascularization.

The key findings are summarized in Table 1.

The primary end-points in this study failed to achieve statistical significance in spite of a significant glycemic difference between the two arms (−0.8% pioglitazone versus −0.3% placebo; $P < 0.0001$) [4]. It was a huge disappointment. However, it was at this point that we saw the secondary end-points gaining a lot of significance. Instead of being a negative trial PROactive was suddenly a positive trial. However, one of the secondary end-points, that is, heart failure, was grossly underhighlighted. There were significantly higher event rates related to heart failure in the pioglitazone arm (Table 2).

The main blow to modern drugs seeking regulatory approval came from a meta-analysis involving rosiglitazone. Nissen and Wolski presented data analyzing 42 randomized trials with control group and of 24-week duration [6]. The baseline characteristics are summarized in Table 3.

Table 1: PROactive primary end-point results [4].

	HR (95% CI)
Death	0.96 (0.78–1.18)
Nonfatal MI	0.83 (0.65–1.06)
Stroke	0.81 (0.61–1.07)
Major leg amputation	1.01 (0.58–1.73)
Acute coronary syndrome	0.78 (0.55–1.11)
Coronary revascularization	0.88 (0.72–1.08)
Leg revascularization	1.25 (0.90–1.73)

Table 2: PROactive heart failure data [4].

	Pio/placebo (%)	*P* value
Any heart failure report	11/8	<0.0001
Heart failure not requiring hospitalization	5/3	0.003
Heart failure requiring hospitalization	6/4	0.007
Fatal heart failure	1/1	0.634

Table 3: Baseline characteristics [6].

Age	57 years or less
Sex	Predominantly males
Average HBA1C	8.2%
Active comparators	(i) Placebo (ii) Metformin (iii) Sulfonylureas (iv) Insulin

There was a statistically significant 43% increase in myocardial infarction with a trend towards increased death from cardiovascular causes in the rosiglitazone arm compared to active comparators and placebo [6] (Table 4).

The very next year (2008) USFDA came up with "Guidance for Industry" [7]. The new guidance stressed on the sponsors recruiting an independent committee looking into the cardiovascular end-points in the phases II and III study programs. The primary and secondary end-points along with the methods employed for statistical analysis should be clearly mentioned. A two-sided 95% confidence interval for each assessment of risk ratio was formulated. The cut-off values set as mandatory requirements are presented in Table 5.

It was pointed out, however, that individuals recruited in phases II and III programs would mostly be younger with a short duration of diabetes (not the ideal population to assess CV outcomes). Hence, it was mandatory to conduct a CV safety outcomes trial even when there were no adverse signals in the phases II and III programs especially if the CV event rates were low [7].

The modern antidiabetic medications as a result of the abovementioned developments were exposed to a new set of laws.

Keeping in mind the USFDA CV safety requirements and the importance of secondary end-points especially heart failure, let us take an in-depth look at the recently published CV outcomes data with gliptins in focus.

Table 4: Adverse outcomes [6].

	Odds ratio (95% CI)	*P* value
Myocardial infarction (combined comparator drugs)	1.43 (1.03–1.98)	0.03
Death from CV causes (combined comparator drugs)	1.64 (0.98–2.74)	0.06

Table 5: Upper bound 2-sided 95% confidence interval cut-off for approval [7].

≥1.8	Not approvable
1.3–<1.8	Approvable (large CV safety trial required)
<1.3	Approvable (postmarketing CV trial may not be required)

2. The Gliptin Era

In mid-2000 we got a new group of antihyperglycemic agents (DPP 4 inhibitors or gliptins) for the management of type 2 diabetes. The advantages associated with this group were lack of hypoglycemia and weight neutrality [8]. Added to metformin, gliptins have an equivalent HBA1C reducing ability compared to sulfonylurea [9]. Subsequent data pointed at additional benefits associated with gliptins as far as CV surrogates were concerned [10]. Sitagliptin was associated with reduction in postmeal triglyceride rich apo-B levels [11]. Similarly, saxagliptin was associated with reduction in blood pressure in animal models [12].

All these positive effects pointed at the possibility of CV outcomes benefit with gliptins compared to placebo or other active oral comparators.

3. The Polled Phase 2/3 Saxagliptin Data

USFDA analyzed the pooled phases 2b and 3 saxagliptin data, which included a total of 8 studies and came out with their report in 2009 [13]. The primary MACE was the focus of attention. The major obstacle in the way of analyzing the data was deriving an accurate definition and terminology for the individual MACE events. Apart from the sponsor's definition of MACE, two more definitions were included in this analysis. The first was "Broad SMQ MACE" and the second "Custom MACE" [13]. SMQ stands for standardized MedDRA (Medical Dictionary for Regulatory Activities) queries. The terminologies used to define a disease entity were in accordance with a standardized definition. Custom MACE was a subset of "Broad SMQ MACE" which is more specific as far as defining the end-points was concerned supervised by 3 FDA reviewers [13]. Saxagliptin arm fared very well as far as the primary end-points were concerned (Table 6).

The MACE data indicates that both the custom and sponsor MACE achieved the FDA recommended cut-off value of less than 1.3 [13]. The ST custom MACE as a matter of fact reached statistical significance. The SMQ MACE was

Table 6: Saxagliptin polled phase 2b/3 MACE [13].

	Events (%): saxagliptin/placebo	OR (95% CI)
Sponsor MACE	0.5/1	0.5 (0.2–1.2)
Custom MACE (ST)	0.1/0.6	0.21 (0.04–0.8)
Custom MACE (ST + LT)	0.7/1.3	0.52 (0.3–1.0)
SMQ MACE (ST)	1.8/2.0	0.90 (0.6–1.5)
SMQ MACE (ST + LT)	3.1/3.2	0.96 (0.7–1.4)

diluted with the use of CPK as one of the preferred terms thereby increasing the number of events and the upper limit of the two-sided confidence interval crossing 1.3 [13].

In a similar analysis of data with linagliptin the sponsor MACE (0.36 [95% CI 0.17–0.78]) as well as FDA Custom MACE (0.34 [95% CI 0.15–0.75]) met the FDA cut-off criteria [14].

However, the number of CV events in the saxagliptin pooled data custom MACE was 40 and that in the linagliptin pooled data was 11 [13, 14]. This was an extremely small number to come to a definitive conclusion as far as CV safety was concerned. Hence, it was mandatory to undergo a dedicated CV safety trial. All the gliptins took to dedicated CV safety trials except vildagliptin.

4. Gliptins: CV Safety Data (SAVOR TIMI-53, EXAMINE, and TECOS)

SAVOR TIMI-53 (saxagliptin) and EXAMINE (alogliptin) were published in 2008 whereas TECOS (sitagliptin) was published in 2015. Let us take a look at the salient CV safety issues highlighted in these three landmark trials.

4.1. SAVOR TIMI-53 (Saxagliptin). The aim of this study was to analyze primary efficacy (superiority), primary safety (noninferiority), and secondary safety issues in an intention to treat (ITT) population. It was estimated from the annual primary CV event rate with placebo (2.8%) that the study should recruit 12,000 patients and run the trial for 2 years with 3-year follow-up (total of 5 years duration) to get hold of 1,040 CV events [15]. This would enable the investigators to generate adequate power to test the noninferiority hypothesis. However, after 10 months after recruitment the investigators realized that the numbers recruited would not yield the anticipated event rates and additional subjects (with established CVD) were recruited to increase the study population number to 16,500 [16].

This study differed significantly from the other CV trials on the following points:

(i) Baseline HBA1C was over a broader range (>6.5% to ≤12.0%) with a mean value of 8.0% (±1.4%) versus TECOS (HBA1C range: 6.5–8.0%) [15, 17].

(ii) There were patients without established CVD recruited in this trial (primary CV prevention cohort). Recruitment of this population was restricted to 25% of the whole study population [15].

The results were published in October 2014. The prominent primary end-point results were as follows:

(i) Primary efficacy end-point (superiority): HR 1.0 (0.89–1.12); $P = 0.99$. It failed the superiority test [16].

(ii) Primary safety end-point (noninferiority): $P < 0.001$ (passed the noninferiority test) [16].

The areas of concern from CV perspective are as follows:

(i) Although designed as a glycemic equipoise study, the end results revealed a statistically significant difference between the two arms as far as fasting plasma glucose and HBA1C were concerned. There were significantly more episodes of both minor ($P = 0.002$) and major ($P = 0.047$) hypoglycemic episodes in the saxagliptin arm compared to placebo [16].

(ii) There was a statistically significant 27% increased rate for hospitalization due to heart failure (HR 1.27 [1.07–1.51]; $P = 0.007$) [16].

Although a distinctive mechanism leading to heart failure was not obvious with gliptins, several groups speculated whether it was the impact of DPP-4 inhibitors on substrates like Neuropeptide-Y (NP-Y) and Substance P (SP) that could lead to this effect [18]. Others speculated whether the additional patients with established CVD recruited later in the trial could lead to this effect. Till date, we do not have any answer to this phenomenon. The next logical step would be to wait for another CV safety trial.

4.2. EXAMINE (Alogliptin). EXAMINE study group investigators recruited patients exclusively with established ACS. Since patients with established ASC were included in this trial, the placebo annual primary MACE was estimated at 3.5% [19]. As a result, recruiting a smaller population (5,400) followed up for approximately 40 months would generate adequate enough power to fulfill the HR cut-off [19].

At the end of the trial there was a statistically significant 0.36% greater HBA1C reduction ($P < 0.001$) with alogliptin compared to placebo (usual care) [20]. Once again the glycemic equipoise hypothesis was not satisfied.

The alogliptin arm achieved the primary end-point (death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke) noninferiority (HR 0.96; upper boundary of CI 1.16) [20].

With the adverse impact of saxagliptin in SAVOR TIMI-53 on hospitalization for heart failure as well as increased hypoglycemic episodes, all eyes were on EXAMINE as far as these parameters were concerned:

(i) At baseline 24.2% of the recruited patient population had congestive heart failure [19]. However, NYHA Class IV was an exclusion criterion. EXAMINE data revealed that there was no significant trend as far as hospitalization due to heart failure as a first event was concerned (HR: 1.07; 95% CI: 0.79–1.46) [20].

(ii) There were no significant differences between the two groups as far as the rates of hypoglycemic events were concerned.

On the one hand, the results of EXAMINE were a bit reassuring while on the other hand the absence of a clear cut answer as far as hospitalization from heart failure was concerned was discomforting. In an editorial on the post hoc analysis of heart failure, it was pointed out that there was a statistically nonsignificant increased risk for hospitalization due to heart failure (HR 1.19 CI: 0.89–1.57) [21]. As a matter of fact those without a background history of heart failure had a significant risk of developing it while being on alogliptin (HR 1.76; 95% CI: 1.07–2.90) [21].

Hence, all eyes were on the upcoming CV safety trial with gliptins and the one closest to publication after SAVOR TIMI-53 and EXAMINE was TECOS.

However, at this point FDA took note of the adverse signals emerging from these trials and conducted an analysis of their own.

5. FDA Analysis of SAVOR TIMI-53 and EXAMINE

5.1. Reanalysis of SAVOR TIMI-53. Four prominent issues were up for discussion [22]:

(i) The issue related to heart failure.

(ii) Comments that were sought on all-cause mortality.

(iii) Issues related to renal safety.

(iv) Any additional safety issues.

We will focus on the CV safety related issues only in this review.

While reanalyzing the sponsors existing data, FDA introduced the term mITT, which differed from ITT (intention to treat) in terms of the fact that the subject must have received the drug in question even if it is for a day. This "on-treatment" analysis was further subdivided into +7 days and +30 days' analysis (i.e., events occurring 7 days and 30 days after receiving the last dose of saxagliptin or placebo): the time-to-event analysis [22].

Another important point to remember at this point is that all-cause mortality was a stand-alone secondary end-point whereas hospitalization for heart failure was one of multiple secondary end-points (MACE+).

5.2. The Reanalysis Results

5.2.1. Primary MACE. Both the ITT (HR: 1.00; 95% CI: 0.89–1.12) and the mITT (HR: 1.00; 95% CI: 0.89–1.12) for primary MACE satisfied the FDA requirements (upper limit of CI <1.3) [22].

The individual components of primary MACE did not differ either.

5.2.2. Secondary End-Points (MACE+). The prespecified ITT secondary end-point composite (MACE+) had a hazard ration of 1.02 with the upper boundary of CI at 1.11 [22].

Amongst the individual secondary end-points, it was hospitalization due to heart failure, which was pointing the wrong way. The problem FDA identified was that this was not a prespecified stand-alone secondary end-point. Since this end-point analysis ended up with a statistically significant difference, FDA went ahead and reanalyzed the data. Although those in the secondary end-point arm could continue on in the trail, those experiencing a primary event were not followed up any more. This results in crunching of the high-risk population base as we go on. Hence, FDA designed a new analytical process whereby subjects with heart failure or other high-risk composites were included in the analysis (e.g., hHF or primary MACE; hHF or CV death) [22]. On-study ITT population was analyzed.

Of the 3 end-points analyzed (hHF or primary MACE, hHF or CV death, and hHF or all-cause death) hHF or CV death (HR: 1.14; 95% CI: 1.00–1.30) and hHF or all-cause death (HR: 1.16; CI: 1.03–1.30) had the lower boundary of CI at or above 1 [22]. Hence, the issue of hHF remained significant even in the reanalysis performed.

It was thought that the detrimental by-products of DPP-4 inhibition Neuropeptide-Y (NP-Y) and Substance P (SP) clubbed with ACE inhibition could lead to increased sympathetic activity and heart failure. However, the data indicated a higher and significant hazards ratio (HR 1.42; 95% CI: 1.09–1.88) for time to first hospitalization due to heart failure in those not on ACE inhibitors compared to those who were on it (HR 1.18; 95% CI: 0.94–1.48) [22]. Hence, the DPP-4 inhibition induced production of adverse by-products (NP-Y and SP) could not explain this end-point.

Although a lot of effort was spent analyzing why there was an increase in hospitalization due to heart failure, none of the hypothesis generated could explain this phenomenon.

All-Cause Mortality. This was a stand-alone secondary end-point.

This was another area of concern. Although the prespecified IIT analysis did not show any trend towards an increase in all-cause mortality (HR: 1.11; 95% CI: 0.96–1.27), the mITT analysis pointed at a statistically significant increase in 7-day death (HR: 1.23; 95% CI: 1.02–1.48) (Table 7) [22]. Once again we come across a number of speculations attempting to explain the increased rates of all-cause mortality. A sizable 25% of patients in SAVOR TIMI had a baseline HBA1C below 7%. This clubbed with statistically increased rates of hypoglycemia in the saxagliptin arm was touted as one of the reasons. However, the FDA analysis on the same and other points (heart failure, increased rates of arrhythmias, etc.) could not arrive at a definitive conclusion [22]. Nevertheless, the FDA document did not consider this increased risk as a pattern happening by chance.

5.3. Reanalysis of EXAMINE Trial. All eyes were on the reanalyzed heart failure and all-cause mortality data as the primary end-point was met.

5.4. Heart Failure Reanalysis Data. The same analytical method was employed as in the case of SAVOR TIMI. A composite of MACE or heart failure was looked into and the data was encouraging (HR: 0.982; 95% CI: 0.848–1.138) [23].

Table 7

	SAVOR TIMI 53	EXAMINE	TECOS
Primary composite CV end-points	HR 1.00 [CI: 0.89–1.12] [16]	HR 0.96 [CI: Upper Limit 1.16] [20]	HR 0.98 [CI: 0.88–1.09] [17]
Heart failure	HR 1.27 [CI: 1.07–1.51] [16] FDA reanalysis: HR 1.16 [CI: 1.03–1.30] [22]	HR 1.07 [CI: 0.79–1.46] [20] FDA reanalysis: HR 0.98 [CI: 0.84–1.13] [23]	HR 1.00 [CI: 0.83–1.20] [17] Composite analysis: HR 1.02 [CI: 0.90–1.15] [17]
All-cause mortality (7-day death) MACE: risk	HR 1.23 [CI: 1.02–1.48] [22]	Higher risk of MACE in the following: (i) Smokers, DM duration >10 yrs., metformin nonusers, insulin users, moderate-to-severe renal insufficiency [23]. (ii) Patients from US and Canada [23].	HR 1.01 [CI: 0.90–1.14] [17]
Hypoglycemia	HR 1.16 [CI: 1.08–1.25] [16]	6.7% (A) versus 6.5% (P) [19]	HR 1.12 [CI: 0.89–1.40] SH [17]

There were no signals of increased first hospitalization from heart failure. However, it should be mentioned at this point once again that EXAMINE trial excluded patients with NYHA Class IV and had the majority of individuals in the NYHA Class II category [23].

5.5. Mortality Signals. Patients recruited from US and Canada had a higher hazard ratio of MACE (HR: 1.28; 95% CI: 0.89–1.84) (Table 7) [23]. Although there were differences as far as baseline characteristics were concerned (longer duration of diabetes, more smokers, etc.), these factors could not explain why there was an increased mortality trend.

Increased hazard ratio for MACE was also observed in those with longer duration of diabetes (>10 years duration), moderate-to-severe renal disease, biguanide nonusers, and insulin users (Table 7) [23].

It was reassuring to note that all-cause mortality was not increased in this trial (HR: 0.876; 95% CI: 0.705–1.089) [23].

5.6. Post-FDA Reanalysis of Data in 2015. There were mixed reactions. Some schools of thought felt that the issue of heart failure was not replicated and hence there was some reassurance on this issue. On the other hand, the critics pointed out that there were inherent differences between the trials and hence it was not possible to come to a definite conclusion.

Hence, all eyes were on TECOS.

5.7. The TECOS Data

Baseline Characteristics in Brief. TECOS recruited patients with a compact HBA1C range (6.5–8.0%) and established cardiovascular disease. Patients with eGFR <30 mL/min were excluded from the trial [24].

The placebo annual rate of CV events was estimated at 2.5–3.0% for primary composite events. Hence, it was estimated that recruiting 14,1000 patients would result in approximately 611 CV events over 6 years [24].

5.8. The Results. Once again we are looking at a glycemic equipoise study. However, the end-of-study HBA1C difference of 0.29% reached statistical significance (95% CI: −0.32 to −0.27; $P < 0.0001$) [17].

Primary CV End-Points. Both PP (HR: 0.98; 95% CI: 0.88–1.09) and ITT (HR: 0.98; 95% CI: 0.88–1.09) analysis echoed the findings from SAVOR TIMI and EXAMINE [16, 17, 20]. Sitagliptin satisfied the FDA upper bound CI of <1.30 as far as primary CV outcomes were concerned. However, sitagliptin could not meet the superiority criteria (HR: 0.99; 95% CI: 0.89–1.10) [17].

5.9. The Areas under Focus. All eyes were focused on the heart failure and mortality issues.

The ITT analysis of hospitalization due to heart failure did not reveal any concerns (HR: 1.00; 95% CI: 0.83–1.20; $P = 0.98$). What was interesting to note was that unlike the previous studies TECOS utilized the FDA pattern and analyzed hHF or cardiovascular death composite for the ITT population. Once again the results were reassuring (HR: 1.02; 95% CI: 0.90–1.15; $P = 0.74$) [17].

All-cause mortality was also not adversely affected (HR: 1.01; 95% CI: 0.90–1.14) [17]. What was reassuring to note was that there were no adverse MACE signals as far as differing patient population, duration of diabetes, baseline HBA1C, baseline nonuse of biguanides, insulin usage, or use of ACE inhibitors were concerned [17]. This was in direct contrast to the findings from EXAMINE trial.

6. Conclusion

The issue of adverse impact of oral hypoglycemic agents and adverse CV signals can be traced back to UGDP in 1971 [1]. Similar trends were also observed in UKPDS 34 when adding metformin to sulfonylurea resulted in an increase in diabetes-related deaths and all-cause mortality [25].

However, these data were not given a lot of attention as there were issues related to trial design, analysis, and interpretation.

The modern trials on the contrary are well designed and analyzed. There is no running away from the fact that dedicated CV safety trials will be required for all the modern drugs to find a definitive place in treatment algorithm. With this background all the three randomized trials (SAVOR TIMI, EXAMINE, and TECOS) reassure us on the CV safety of DPP-4 inhibitors from the primary end-point perspective. What keeps the debate going are the issues related to increased hypoglycemia risk, hHF, and increased all-cause mortality in SAVOR TIMI and increased MACE risk in certain population in EXAMINE. TECOS was the picture-perfect trial pulling along the other drugs in this group towards the positive side and ruling out the fear of class effect as far as the adverse events were concerned.

However, it would be premature to say that the issue has been settled once and for all.

There are a couple of interesting studies lined up either to simplify the contentious issues or to complicate matters even further. CARMELINA with linagliptin would add on to the increasing experience with usage of DPP-4 inhibitors in high CVD risk diabetic population [26]. Another data would probably help put a lot of unanswered questions in perspective.

However, things might get a bit complicated with CAROLINA [27]. This study recruited patients with newly diagnosed treatment naïve diabetes or early on in the disease process with high CV risks profile. Patients were randomized to either glimepiride or linagliptin and would be followed up for 5 years. This would be the first in kind head-to-head comparison between a sulfonylurea and a gliptin on high CV risk patients. The duration of this trial is too short to answer this question, as we saw CV benefits appearing after 20 years in those with newly diagnosed type 2 diabetes [28]. However, the patient population in this trial is unique as they are newly diagnosed but already have established CVD (similar to the ORIGIN trial population [29]).

Overall, sitagliptin in TECOS trial came up with the most impressive results (pending FDA reanalysis). We need to keep at the back of our minds the issues related to saxagliptin and alogliptin in some special situations.

Abbreviations

ACCORD: Action to Control Cardiovascular Risk in Diabetes
ADVANCE: Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation
VADT: Veterans Affairs Diabetes Trial
UKPDS: United Kingdom Prospective Diabetes Study
SGLT-2: Sodium Glucose Cotransporter Protein 2
USFDA: United States Food and Drug Administration
SAVOR: Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus
EXAMINE: Examination of Cardiovascular Outcomes: Alogliptin versus Standard of Care in Patients with Type 2 Diabetes Mellitus and Acute Coronary Syndrome
UGDP: University Group Diabetes Study Program
NYHA: New York Heart Association
CV: Cardiovascular
MI: Myocardial infarction
DPP-4: Dipeptidyl peptidase-4
MACE: Major adverse cardiac events
ST: Short term
LT: Long term
TECOS: The Trial to Evaluate Cardiovascular Outcomes after Treatment with Sitagliptin
HR: Hazard ratio
CI: Confidence interval
NP-Y: Neuropeptide-Y
SP: Substance P
eGFR: Estimated glomerular filtration rate
hHF: Hospitalization due to heart failure
DM: Diabetes mellitus
SH: Severe hypoglycemia
CARMELINA: Cardiovascular Safety and Renal Microvascular Outcome with Linagliptin in Patients with Type 2 Diabetes Mellitus at High Vascular Risk
CAROLINA: Cardiovascular Outcome Study of Linagliptin versus Glimepiride in Early Type 2 Diabetes
ORIGIN: Outcome Reduction with Initial Glargine Intervention.

Conflict of Interests

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

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