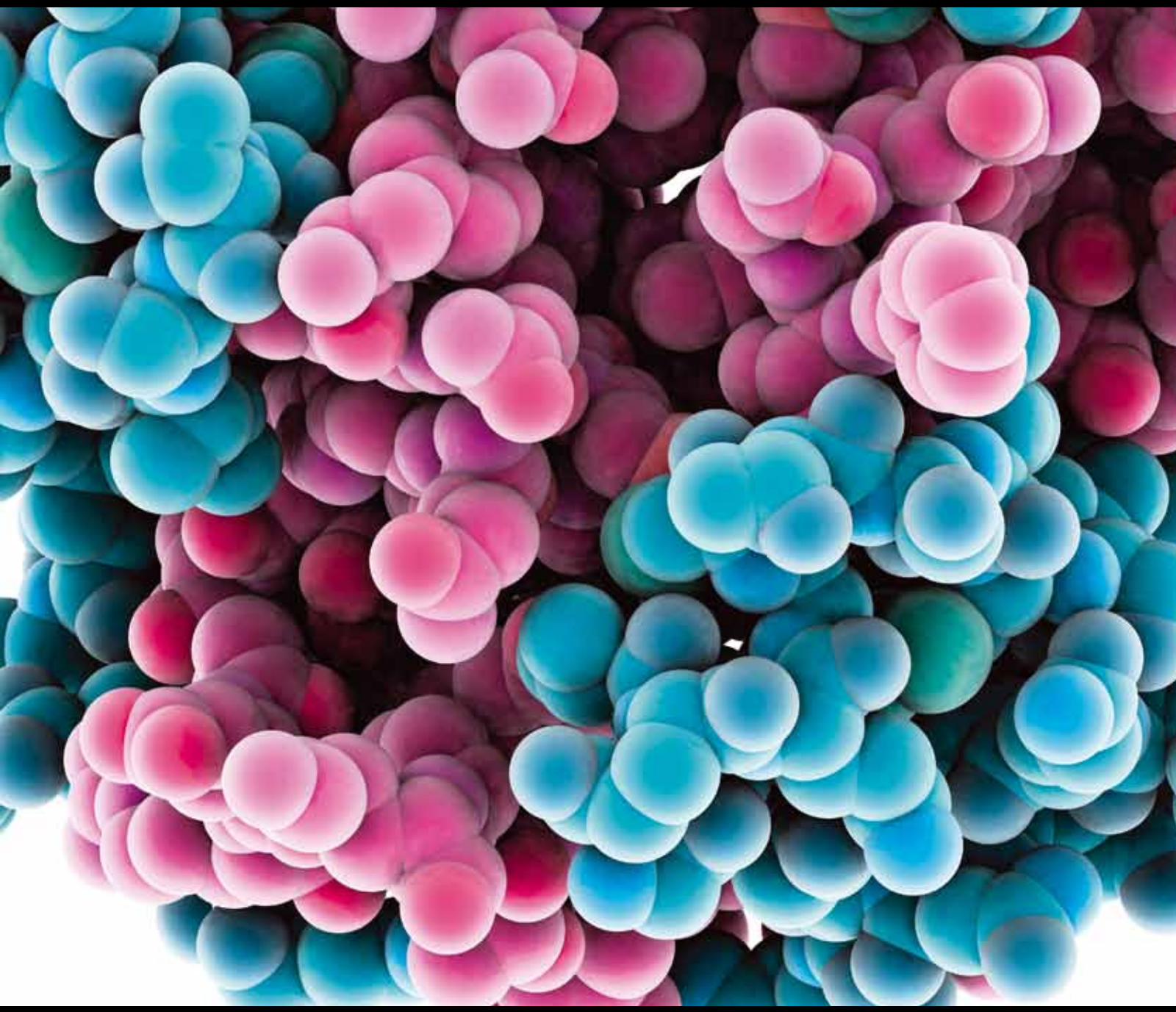
Journal of Diabetes Research

Chronic Vascular Complications in Diabetes

Guest Editors: Weiping Jia, Aimin Xu, Alex Chen, Jiarui Wu, and Jianping Ye



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Editorial Board

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Editorial

Chronic Vascular Complications in Diabetes

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With high prevalence of type 2 diabetes worldwide, diabetic complications have been a major health problem, which reduce life quality and induce premature death in adults. The prevalence of type 2 diabetes is closely related to obesity and aging, two major risk factors for metabolic disorders. The diabetic complications, including retinopathy, nephropathy, neuropathy, skin ulcer, and atherosclerosis, are risk factors for mortality of patients. Retinopathy, nephropathy, and skin ulcer are representatives of microvascular complications, which often happen in insulin-deficient diabetic conditions, such as type 1 diabetic patients and some late-stage type 2 diabetic patients when β-cells completely lose function. Atherosclerosis that is often found in type 2 diabetes is a complication of macrovascular disease that increases the risk for myocardial infarction and strokes. However, atherosclerosis is receiving more and more appreciation in type 1 diabetes. Endothelial cells that line the inner vessel have been extensively studied in the mechanism of the diabetic complications. Impaired endothelial cell function is a common cellular mechanism for most of the diabetic complications (retinopathy, nephropathy, skin ulcer, and atherosclerosis). The cells are exposed to the high level of glucose in diabetic condition and subjected to glucose-induced stresses. The stress may cause overproliferation and deficiency in proliferation of endothelial cells, which contribute to dysfunction of microcirculation. Prevention and treatment of the complications are dependent on protection of the endothelial cell functions in most cases. In this issue, the mechanism of endothelial cell dysfunction was investigated in several studies to understand the molecular events by which the endothelial cells are impaired by hyperglycemia. This topic is enhanced with another group of studies about intervention of the endothelial complications with antioxidant compounds and new drug candidates. These studies will help readers to understand the current status and gain new insights into the diabetic complications.

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Research Article

Impact of High Glucose and Proteasome Inhibitor MG132 on Histone H2A and H2B Ubiquitination in Rat Glomerular Mesangial Cells

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Background. Hyperglycemia plays a pivotal role in the development of diabetic nephropathy (DN) and may be related to epigenetic metabolic memory. One of the most crucial epigenetic mechanisms is histone modification, which is associated with the expression of a fibrosis factor in vascular injury. *Aim* .In this study, we investigated the ubiquitination of histones H2A and H2B to explore the epigenetic mechanisms of DN. *Materials and Methods*. The GMCs were cultured as follows: normal group, high glucose group, mannitol group, and intervention group. After 12 hr, 24 hr, and 48 hr, histones ubiquitination, transforming growth factor-β (TGF-β), and fibronectin (FN) were measured using WB, RT-PCR, and IF. *Result*. High glucose can induce the upregulation of FN. H2A ubiquitination in GMCs increased in high glucose group ($P < 0.01$), whereas it decreased significantly in intervention group ($P < 0.05$). In contrast, H2B ubiquitination decreased with an increasing concentration of glucose, but it was recovered in the intervention group ($P < 0.05$). Expression of TGF-β changed in response to abnormal histone ubiquitination. *Conclusions*. The high glucose may induce H2A ubiquitination and reduce H2B ubiquitination in GMCs. The changes of histone ubiquitination may be due in part to DN by activating TGF-β signaling pathway.

1. Introduction

Diabetic nephropathy (DN) is one of the most devastating microvascular complications of diabetes, which remains the most common cause for end stage renal disease (ESRD). The prevalence of diabetes and the patients suffering from diabetic microvascular complications is increasing worldwide [1]. Nearly one-third of patients with diabetes develop nephropathy, and early diagnosis is critical in preventing long-term kidney loss [2]. However, the mechanisms that cause DN have not been completely clarified, and the treatment options are limited.

Hyperglycemia plays a pivotal role in activating various inflammatory pathways in the development and progression of DN. It induces the fibrotic factor transforming growth factor-β (TGF-β) and fibronectin (FN), the renin-angiotensin-aldosterone system (RAAS), and advanced glycation end products both directly and via gene transcription, which leads to thickening of the glomerular and tubular basement membranes, progressive accumulation of extracellular matrix (ECM) proteins, interstitial fibrosis, and glomerulosclerosis [3–6]. FN is one of the main ingredients of ECM and an important symbol of cell injury. The upgrade expression of FN will eventually lead to the development of diabetic nephropathy. Clinical trials have reported that strict glycemic control reduces the progression of diabetic complications over time. Diabetic complications, including chronic kidney disorders such as DN, have been shown to persist and progress even after glucose control has been achieved, possibly as a result of prior episodes of hyperglycemia that are considered the epigenetic metabolic memory [7]. Preliminary work using endothelial cells has shown that transient episodes of hyperglycemia can induce changes in gene expression that are dependent on the modification of histone tails (i.e., methylation) [8]. The persistence of such modifications has not been fully explained. Additional

studies regarding the epigenetic mechanisms involved are necessary to provide valuable new insights into the pathology of DN.

Posttranslational modifications of the aminoterminal tails of nucleosomal histones, including acetylation, methylation, ubiquitination, phosphorylation, and sumoylation, play key roles in modulating the chromatin structure and gene transcription that have been implicated in regulating the metabolism of diabetic complications [9]. The modification of histones by ubiquitination is a prominent epigenetic mark that may influence changes in gene expression and involves a variety of chromatin-based events, such as gene silencing and repair of DNA damage [10]. The majority of histone ubiquitination occurs on chromatin by the addition of a single ubiquitin molecule via isopeptide linkage to a specific lysine residue on the C-terminal tail of histones H2A and H2B. To a lesser extent, histones H1, H3, and H4 can be ubiquitylated in vivo, and ubiquitination of different histones has distinct functions [11]. However, the effects of histone ubiquitination on DN are unclear.

Recent research has indicated that histone modification is directly or indirectly related to diabetic attacks [12–15]. Histone acetylation can activate the TGF-β signaling pathway, which plays an important role in DN renal fibrosis. Similarly, DN is associated with increased renal H3K9 and H3K23 acetylation, H3K4 dimethylation, and H3 phosphorylation at serine 10, which enhances chromatin unfolding and gene expression [16, 17]. To date, it is unknown whether histone ubiquitination is involved in interstitial fibrosis and glomerulosclerosis in DN or whether the effects of hyperglycemia on such epigenetic events can be mediated through TGF-β signaling pathways. MG132, a proteasome inhibitor, is suggested to attenuate hypertension-induced cardiac remodeling and dysfunction by downregulating the levels of TGF-β_1 [18]. Whether ubiquitin proteasome inhibitors can inhibit renal fibrosis which was followed by activation of the TGF-β signaling pathway in diabetic nephropathy remain unclear. So, additional research to develop new treatments for DN is necessary. In this study, we evaluated the influence of high glucose on the induction of histone H2A ubiquitination, reduced histone H2B ubiquitination in GCMs, and changes in the expression of TGF-β followed by abnormal histone ubiquitination. MG132, which acts as a ubiquitin proteasome inhibitor, may prevent the alterations in H2A and H2B ubiquitination induced by high glucose.

2. Materials and Methods

2.1. Cell Culture. Cell culture media and fetal bovine serum were purchased from Hyclone (USA). Rat glomerular mesangial cells (HBZY-1) were purchased from the Preservation Center at Wuhan University and maintained in low glucose DMEM with 10% fetal bovine serum at 37°C and 5% CO_2. GMCs were used between the 2nd and 5th passages for all experiments and randomly divided into the following six groups:

(i) the normal control group (NC group), with medium containing 5.6 mmol/L glucose,

(ii) the 10 mmol/L glucose group (HG1 group), with medium containing 10 mmol/L glucose,

(iii) the 20 mmol/L glucose group (HG2 group), with medium containing 20 mmol/L glucose,

(iv) the 30 mmol/L glucose group (HG3 group), with medium containing 30 mmol/L glucose,

(v) the osmotic pressure group as a control (OP group), with medium containing 5.6 mmol/L glucose + 22.4 mmol/L mannitol,

(vi) the MG132 intervention group (MI group), with medium containing 30 mmol/L glucose + 1 μmol/L MG132. MG132 was added to the culture medium to block the ubiquitination of histone.

Each group had been cultured for 12 hr, 24 hr, and 48 hr.

2.2. Protein Extraction and Western Blot. Total proteins were isolated from GMCs using a total protein extraction kit (Kaiji, Shanghai, China). The protein concentration was determined using BCA analysis (Beyotime, Shanghai, China). Western blotting was performed as previously described [19]. Immunoblot analysis was performed using anti-ubiquityl-histone H2A (mouse, 1 : 1000; Millipore, USA), anti-ubiquityl-histone H2B (rabbit, 1 : 1000; CST, USA), and anti-GAPDH and actin (rabbit, 1 : 1000; Beyotime, Shanghai, China). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were obtained from the Beyotime Institute of Biotechnology, Shanghai, China. Proteins were detected using the enhanced chemiluminescence system and ECL Hyperfilm (Millipore, USA).

2.3. RNA Extraction and Semiquantitative PCR. Total RNA was extracted from GMCs using an RNA extraction kit (Tiangen Biotech, Beijing, China). A total of 500 ng RNA was reverse transcribed at a final volume of 20 μL using a Takara RNA PCR kit (Baoshengwu, Dalian, China). Four microliters of cDNA were amplified in a gradient thermal cycler (Eppendorf, Germany) using the PCR Master Mix (Baoshengwu, Dalian, China). The results were determined using a UV transilluminator and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The primer sequences were as follows: histone H2A ubiquitination (uH2A), forward, 5′-GCA CCC TGA CCT TGC CTA T -3′, reverse, 5′-CCT TCC CAG ACT CCA CCA T-3′, histone H2B ubiquitination (uH2B), forward, 5′-CGC CTG GCT CAT TAC AAC-3′, reverse, 5′-CTT GGT TTC CGA CA-3′, transforming growth factor-β (TGF-β), forward, 5′-ATG GTG GAC CGC AAC AAC-3′, reverse, 5′-GAG CAC TGA AGC GAA AGC-3′, FN, forward, 5′- TGC CGA ATG TAG ATG AGGA -3′, reverse, 5′-AAA TGA CCA CTG CCA AAGC -3′, and GAPDH, forward, 5′-CCT CAA GAT TGT CAG CAA T -3′, reverse, 5′-CCA TCC ACA GTC TTC TGA GT-3′.

2.4. Immunofluorescence. Cells were grown on coverslips in 6-well plates. After overnight adherence, the cells were treated with media containing high glucose, mannitol, and the MI

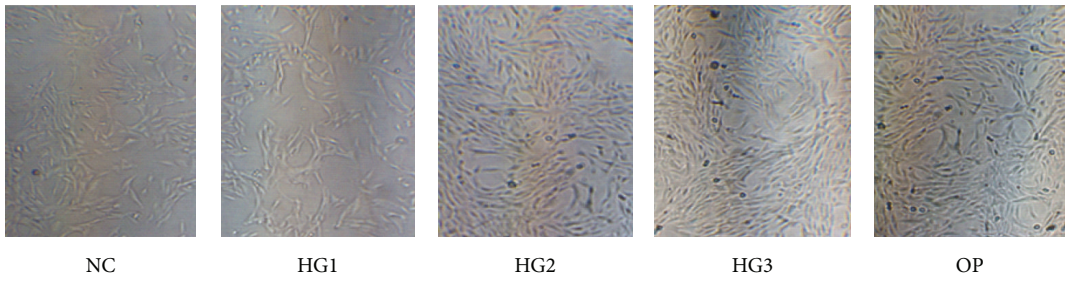

Figure 1: Cell picture in each group of GMCs by inverted phase contrast microscope (×100). No significant changes in cell morphology.

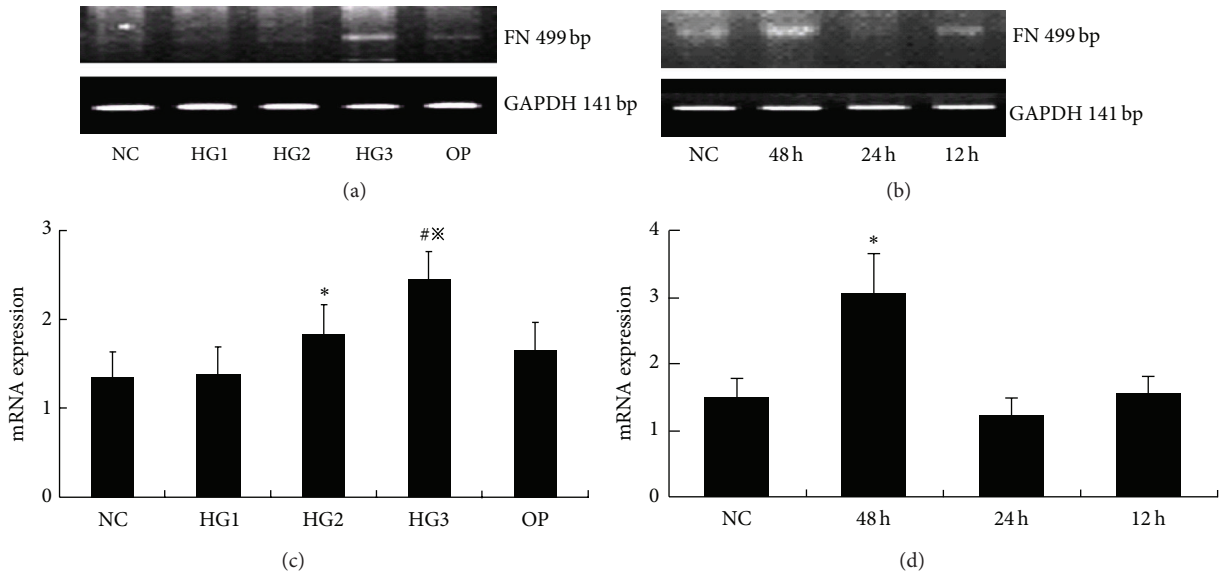

Figure 2: The mRNA levels of FN in each group of GMCs. (a) RT-PCR strip chart for different concentrations of glucose. FN mRNA increased in the high glucose group, especially in HG3 group. (c) The corresponding relative gray value statistics graph of the mRNA level. $^{*}P < 0.05$ versus NC group, $^{\#}P < 0.01$ versus NC group, and $^{※}P < 0.05$ versus HG2 group. (b) RT-PCR strip chart for different times. The expression of FN mRNA increased over time. (d) The corresponding relative gray value statistics graph of the mRNA level. $^{*}P < 0.01$ versus NC group.

media for 24 hr. The cells were fixed in 4% paraformaldehyde (Pierce) and permeabilized in 0.2% Tween 20 (Sigma) for 10 min after being washed briefly with PBS. The cells were blocked with 5% goat serum for 1 hr at room temperature and incubated overnight with primary antibodies followed by washes with PBS. The cells were incubated for 40 min with the appropriate secondary antibody conjugated to the FITC fluorescent dye. DAPI (4′,6′-diamino-2-phenylindole) was used to stain the nucleus in the cells. The coverslips were washed and mounted onto slides using fluorescent mounting medium (Beyotime, Shanghai, China). The control cells were incubated without a primary antibody. Images were taken with a $DMIRE_2$ laser scanning confocal microscope (Leica, Germany).

2.5. Statistics. All experimental data were obtained from three independent experiments performed in triplicate. Data were expressed as the mean ± SD ($\overline{x} \pm s$), which were analyzed using SPSS 11.5 statistical software. Statistical differences were calculated using one-way analysis of variance (one-way ANOVA) to compare more than two groups, followed by the LSD test for multiple comparisons. $P < 0.05$ was defined as statistically significant.

3. Results

The cellular morphology in different glucose culture medium is not significantly changing (Figure 1), but the expression of FN increased in the high glucose group over time, especially in HG3 group for 48 hr (Figure 2).

After 48 hr in culture, Western blot analysis showed low H2A ubiquitin expression in the NC group. Expression was higher in the high glucose group compared to the NC group ($P < 0.01$) in a concentration dependent manner. The strongest expression was in the 30 mmol/L high glucose group ($P < 0.01$). In contrast, H2B ubiquitination expression was strong in the NC group. There were no significant differences in H2B ubiquitination expression in the 10 mmol/L high glucose group compared to the NC group ($P = 0.327$). Expression was lower in the 20 and 30 mmol/L high glucose groups compared to the NC group ($P < 0.01$) and was the

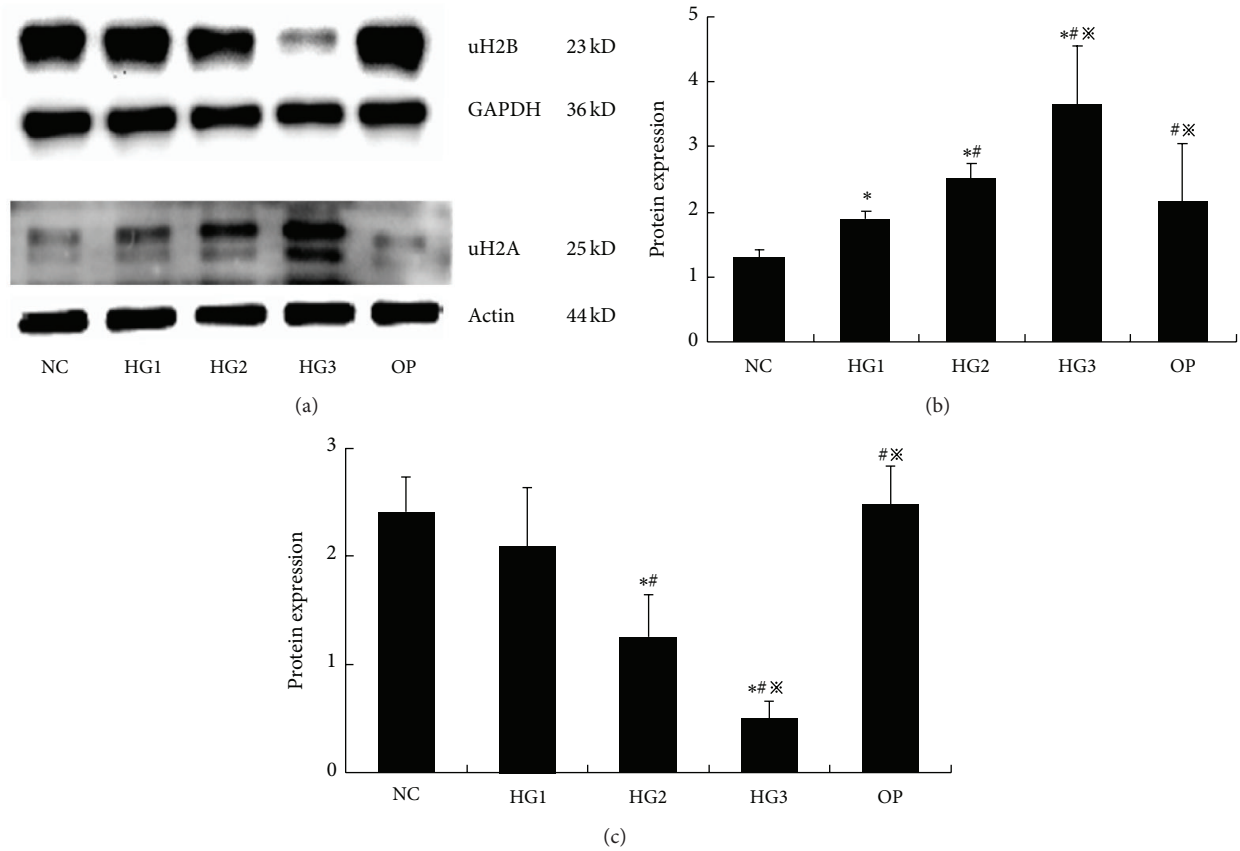

FIGURE 3: The expression of histone H2A and H2B ubiquitination in each group of GMCs by Western blot (48 hr). (a) uH2A and uH2B proteins at different glucose concentrations and high osmotic pressure at 48 hr: uH2A increased in the high glucose group and uH2B decreased as the glucose concentrations increased; they had the most significant changes in the HG3 group, but there were no apparent differences between the NC group and the OP group. (b) The gray graph shows the relative statistical values of uH2A for each group. The expression of the uH2A increased in the high glucose group, especially, in the HG3 group. $^{*}P < 0.01$ versus NC group, $^{\#}P < 0.03$ versus HG1 group, and $^{※}P < 0.05$ versus HG2 group. (c) The gray graph shows the relative statistical values of uH2B for each group. The expression of the uH2B proteins decreased by concentration dependency, obviously in HG3 group. $^{*}P < 0.01$ versus NC group, $^{\#}P < 0.03$ versus HG1 group, and $^{※}P < 0.05$ versus HG2 group.

weakest in the 30 mmol/L high glucose group. There were no differences between the OP and NC groups regarding the ubiquitination of H2A and H2B ($P > 0.05$) (Figure 3).

The expression of the uH2A protein increased at various time intervals in the HG3 group, particularly after 24 hr in culture. The increased expression of uH2A at various time points was statistically significant ($P < 0.01$). Expression of the uH2B protein was significantly reduced in a time dependent manner ($P < 0.01$) (Figure 4). After MG132 intervention, the expression of uH2A was significantly decreased compared to expression in the 30 mmol/L glucose group ($P < 0.05$). In contrast, the expression of uH2B was recovered in the MI group compared to the HG3 group ($P < 0.05$) (Figure 5). The same results were detected using cell immunofluorescent staining and laser scanning confocal microscopy applications (Figure 6).

The expression of TGF-β mRNA increased in the high glucose group compared to the NC group ($P < 0.01$) in a concentration and time dependent manner (Figure 7). And it decreased along with the normalization of uH2A and H2B protein expression ($P < 0.05$), although the mRNA levels of uH2A and H2B were not statistically different in each group (Figure 8).

4. Discussion

Hyperglycemia plays an important role in the development and progression of DN, which can induce the expression of FN and cause cell injury, but the DNA sequence changes cannot solely explain the heritable patterns of gene expression. However, hyperglycemic memory may explain why intensive glucose control has failed to improve cardiovascular outcomes in patients with diabetes, although the molecular mechanisms of this phenomenon remain to be elucidated. The current study found histone modifications can change the state of evacuation and aggregation in chromatin by affecting compatibility between the histones and double-stranded DNA and influencing the affinity of transcription factors for structural gene promoters, which can regulate the

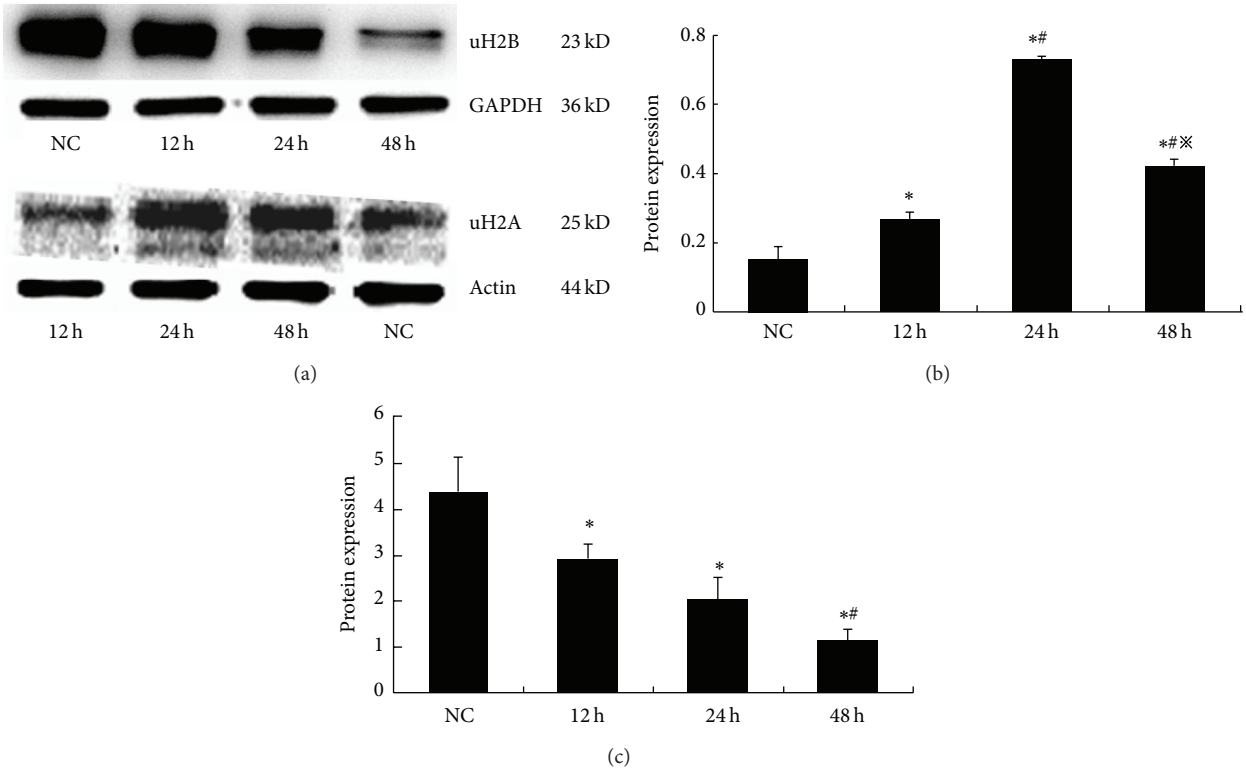

Figure 4: The expression of histone H2A and H2B ubiquitination in GMCs induced by 30 mmol/L glucose at various times determined using Western blot. (a) Compared to the NC group, uH2A expression increased after stimulation with 30 mmol/L glucose after 24 hr, but the uH2B expression decreased over time. (b) The gray graph shows the relative statistical values for uH2A protein expression at various time points for each group. The expression of uH2A was increased at 24 h sharp. $^{*}P < 0.01$ versus NC group, $^{\#}P < 0.05$ versus 12 h, and $^{※}P < 0.05$ versus 24 h. (c) The gray graph shows the relative statistical values for uH2B protein expression at various time points for each group. And the change of uH2B expression was a time dependent reduction. $^{*}P < 0.01$ versus NC group, $^{\#}P < 0.05$ versus 12 h, and $^{※}P < 0.05$ versus 24 h.

expression of genes [20]. Recent studies have shown that diabetes-induced epigenetic changes can affect gene expression in vascular endothelial cells and vascular smooth muscle cells and long-lasting changes in epigenetic modifications at key inflammatory gene promoters following exposure to diabetic conditions [7, 21]. Histone acetylation attenuates epidermal growth factor signaling, which has a key role in the development of DN, and genome-wide studies have shown cell-type specific changes in histone methylation patterns under conditions of DN [22, 23]. And in diabetic retinopathy, histone acetylation was significantly increased in retinas from diabetic rats and contributed to the hyperglycemia-induced upregulation of proinflammatory proteins and thereby to the development of diabetic retinopathy [24]. In DN, histone acetylation, specific histone acetyl transferases, and histone deacetylases significantly enhanced TGF-β1-induced gene expression in rat mesangial cells and in glomeruli from diabetic mice and augmented glomerular dysfunction linked to diabetic nephropathy [25]. Histone methylation has also gained much attention as potential molecular mechanisms underlying metabolic memory and DN. The specific Set7 methyltransferase is the best characterised lysine enzyme, which showed high expression in DN. Furthermore, the contribution of Set7 to the aetiology of diabetic complications may extend to other transcriptional events through methylation of nonhistone substrates [26, 27]. However, little is known about histone ubiquitination in diabetic nephropathy. In this study, we found high glucose may cause cell damage, induce the ubiquitination of histone H2A, and reduce the ubiquitination of histone H2B in GMCs.

The results indicate that histone H2A and H2B ubiquitination may be involved in the development and progression of DN as an epigenetic mechanism. Although the mechanisms of action vary for different histones, ubiquitination of histone H2A K119 may induce DN and ubiquitination of histone H2B K120 has been shown to delay the onset of DN.

TGF-β has been implicated in various human disorders, including vascular and renal diseases, and is a primary fibrotic factor. Diabetic nephropathy (DN) is a chronic renal complication characterized by thickening of the glomerular and tubular basement membranes and progressive accumulation of extracellular matrix (ECM) proteins, such as type I and type IV collagens, fibronectin, and laminin in the tubular interstitium and mesangium. TGF-β increases ECM accumulation and plays a major role in the development of chronic renal diseases through the induction of a downstream effector, which is a connective tissue growth factor, and by decreasing matrix degradation through the inhibition of

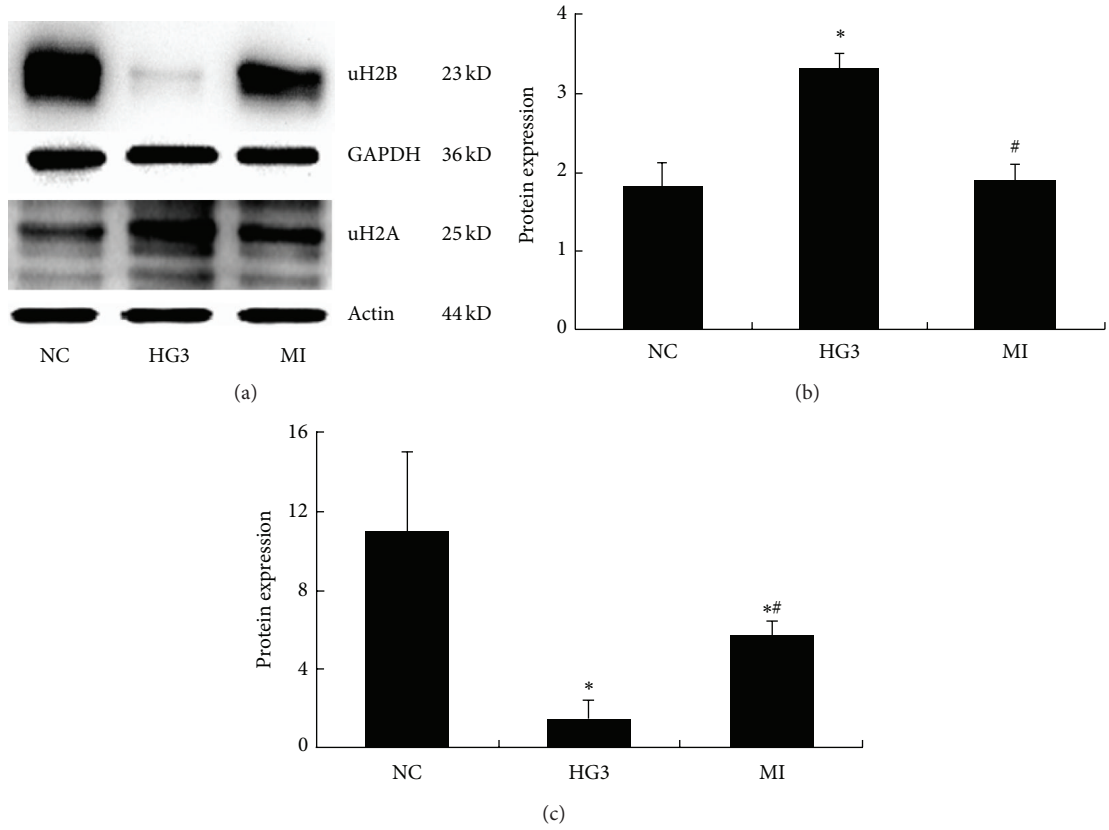

Figure 5: The expression of histone H2A and H2B ubiquitination after intervention with MG132 determined using Western blot (uH2A for 24 hr and uH2B for 48 hr). (a) Western blot strip chart. (b) The gray graph shows the relative statistical values of uH2A for each group. Compared with the NC group, the expression of the uH2A protein was significantly increased in the HG3 group. After MG132 intervention, the protein expression of uH2A decreased. $^{*}P < 0.01$ versus NC group and $^{\#}P < 0.05$ versus 1 HG3 group. (c) The gray graph shows the relative statistical values of uH2B for each group. Compared with the NC group, the expression of the uH2B protein was significantly decreased in the HG3 group. And after MG132 intervention, uH2B relative to the normal group changed. $^{*}P < 0.01$ versus NC group and $^{\#}P < 0.05$ versus 1 HG3 group.

proteases or activation of protease inhibitors [28]. The TGF-β signaling pathway is controlled by many factors, including histone modification and epigenetic chromatin marks, such as histone H3 lysine methylation in TGF-β1-induced gene expression in rat mesangial cells under normal and high glucose conditions. TGF-β1 has been shown to increase the expression of ECM-associated genes, the connective tissue growth factor collagen-α1, and plasminogen activator inhibitor-1. Increased levels of histone H3 K4 methylation associated with active genes and decreased levels of histone H3 K9 methylation at these gene promoters accompany changes in expression [29]. TGF-β1 also increased the expression of H3 K4 methyltransferase SET7/9 and recruitment to these promoters. SET7/9 gene silencing with siRNAs significantly attenuated TGF-β1-induced ECM gene expression [9]. In this study, we did not examine changes in the mRNA levels of uH2A and uH2B as a consequence of uH2A and uH2B proteins stimulation by high glucose. This implies that there is no difference in the gene order of histones H2A and H2B, except for posttranslational modifications, including histone ubiquitination. We observed that the mRNA level of TGF-β dramatically increased followed by changes in uH2A and uH2B proteins. In summary, changes in uH2A and uH2B protein expression induced by high glucose in GMCs may enhance the activation of TGF-β and influence the pathogenesis of DN.

A recent study reported that the ubiquitin proteasome inhibitor MG132 has an antifibrotic function. MG132 exerts an antifibrotic effect by simultaneously downregulating type I collagen and a tissue inhibitor of metalloproteinase-1 and upregulating metalloproteinase-1 production in human dermal fibroblasts [30]. Tubular injury in a rat model of type 2 diabetes was shown to be prevented by MG132 by reducing renal tubule interstitial fibrosis [31]. Several studies have shown that MG132 has an effect on mitigating renal fibrosis by inhibiting the expression of kidney fibronectin mRNA in rats with early diabetic nephropathy and could improve proteinuria and other symptoms [32].

Research on histone ubiquitination is scarce, and inhibitors that can effectively and specifically block the ubiquitination of histones have not been described. The process of histone ubiquitination is similar to the ubiquitination of other proteins. MG132 is a specific ubiquitin proteasome inhibitor that can inhibit activation of the TGF-β

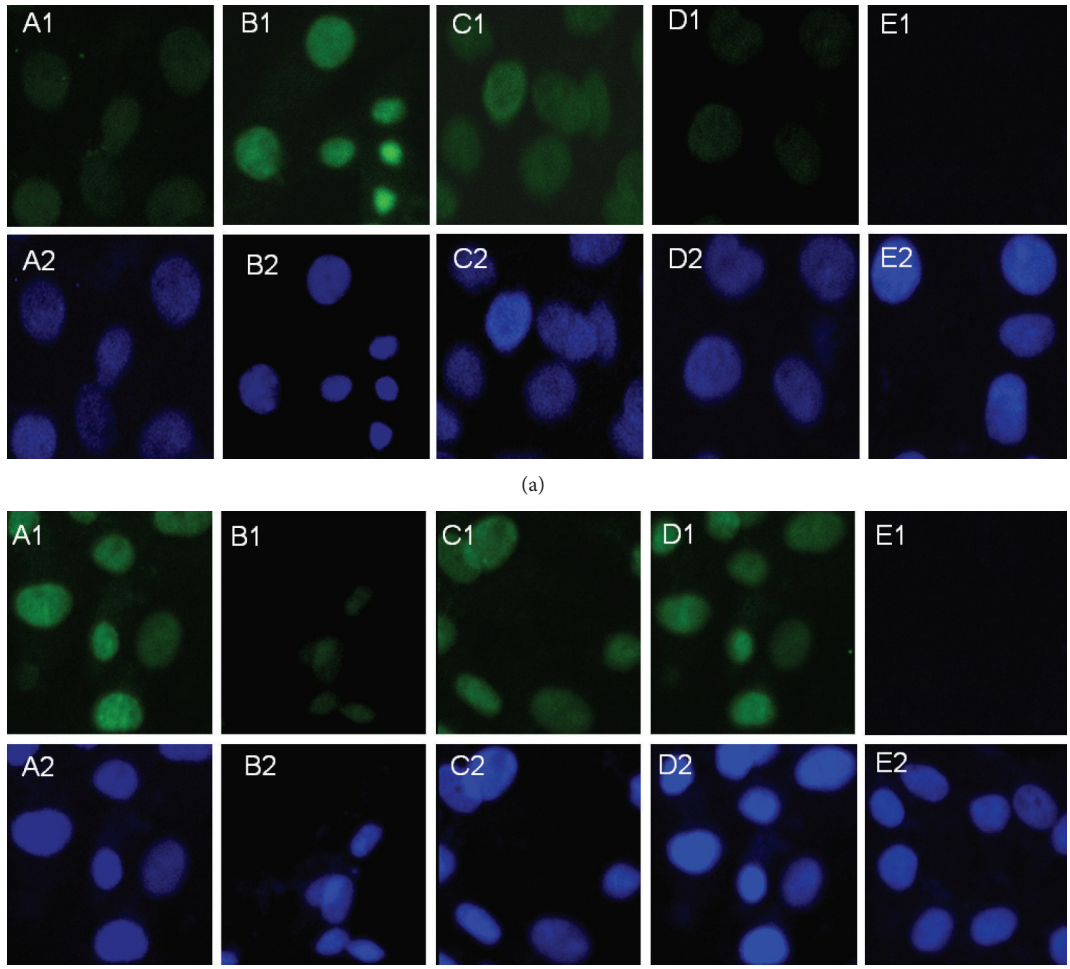

Figure 6: The expression of histone H2A and H2B ubiquitination in GMCs by immunofluorescent staining and laser scanning confocal microscopy applications. A1, NC group, B1, HG3 group, C1, MI group, D1, OP group, E1, negative control, and A2, B2, C2, D2, and E2 represent the corresponding groups of nuclear stains. (a) uH2A protein and (b) uH2B protein. uH2A and uH2B proteins were detected in the nucleus as green fluorescence overlapping with the blue fluorescence emitted by the nuclear stains DAPI. The uH2A protein was not detected in the NC group, but it was prominent in the HG3 group. The protein was strongly detected in the MG132 intervention group, which did not significantly change as a result of high osmotic pressure. The results for the uH2B protein were in contrast to the uH2A results.

signaling pathway, which is important in the development of fibrosis in DN [33]. However, there is not any evidence in the literature about whether MG132 can inhibit histone ubiquitination disorders or eliminate epigenetic metabolic memory to treat DN. Our experiments show that disorders involving histone H2A and H2B ubiquitination can exhibit an apparent reversal trend based on treating rat glomerular mesangial cells with MG132 and 30 mmol/L high glucose. After eliminating the dysfunction of histone ubiquitination, the expression of TGF-β mRNA was inhibited following MG132 intervention. This suggests that ubiquitin proteasome inhibitors may have a positive function in the treatment of diabetic nephropathy by inhibiting the disorders involving histone H2A and H2B ubiquitination that affects gene expression of TGF-β.

The proteasome inhibitor MG132 induces apoptosis. For example, MG132 inhibited the PI3K/Akt and NFκB pathways, promoted mitochondrial depolarization, and decreased the concentration of mitochondrial antiapoptotic protein. MG132 also mediated activation of p38-JNK1/2 signaling and enhanced selective apoptosis in glioblastoma cells [34, 35]. MG132 is promising for cancer treatment because it markedly inhibited the growth of malignant tumor cells and arrested cells in the G2/M phase of the cell cycle, and the cells become apoptotic [36]. Strom and Panlsen reported that MG132 inhibited the ubiquitin proteasome, which degrades the apoptosis inducer NGFI-B and is a nuclear receptor [37]. Thakur suggested that MG132 inhibits histone deacetylation by increasing the degradation of histone deacetylase induced by green tea polyphenols in a prostate cancer cell line, followed by cell arrest and apoptosis [38]. Therefore, the ubiquitin proteasome inhibitor MG132 could inhibit the proteasome and induce apoptosis. Possible answers regarding the causes of histone ubiquitination were identified in this study, but the detailed mechanisms involved will require additional research. Determining the specific inhibitors of

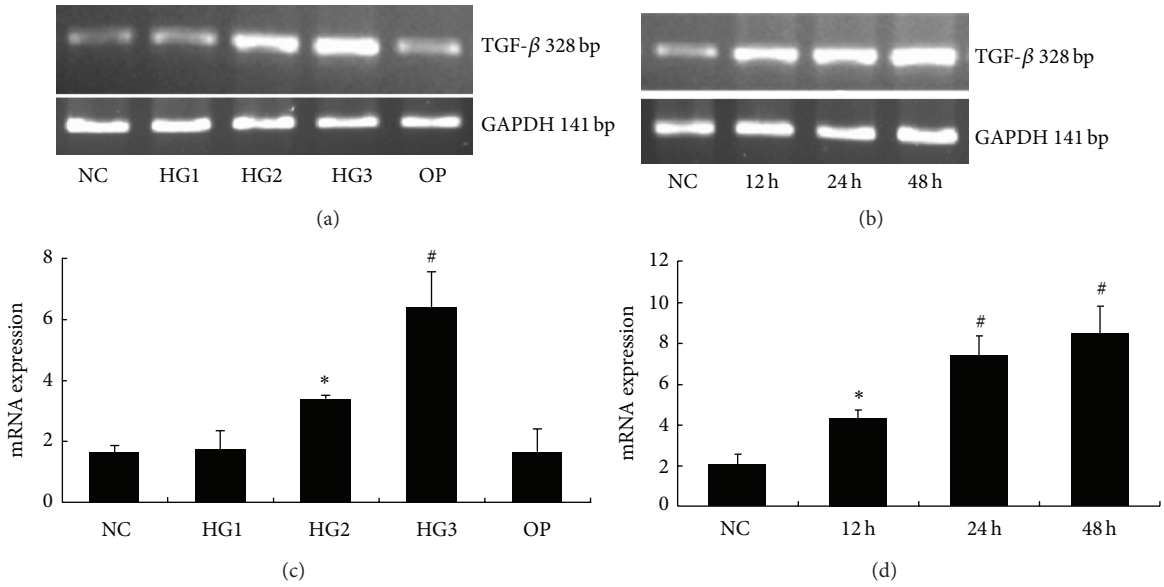

Figure 7: The mRNA levels of TGF-β in each group of GMCs. (a) RT-PCR strip chart for different concentrations of glucose. (c) The relative gray value statistics graph of the mRNA level for different concentrations of glucose. (b) and (d) The RT-PCR strip chart and the relative gray value statistics graph of the mRNA level for different times. $^{*}P < 0.05$ versus NC group and $^{\#}P < 0.01$ versus NC group.

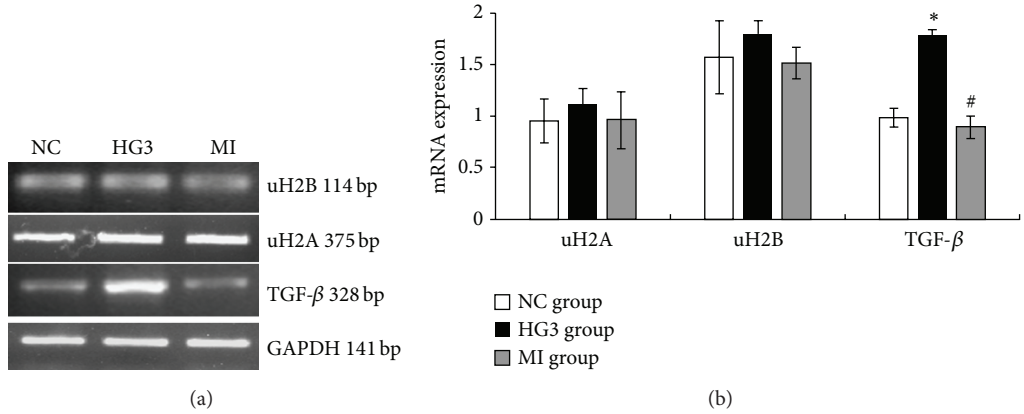

Figure 8: The mRNA levels of uH2A, uH2B, and TGF-β in GMCs induced by high glucose and inhibited by MG132. (a) RT-PCR strip chart. (b) The relative gray value statistics graph of the mRNA level. Compared with the NC group, the mRNA levels of uH2A and uH2B were not different between the groups. The expression of TGF-β mRNA was greatly increased in the HG3 group and decreased in the MI group compared to the HG3 group. $^{*}P < 0.01$ versus NC group and $^{\#}P < 0.01$ versus 1 HG3 group.

histone ubiquitination and discovering the role of histone ubiquitination in diabetic nephropathy renal fibrosis remain a challenge.

In conclusion, we demonstrated that the high glucose may induce the ubiquitination of histone H2A and reduce the ubiquitination of histone H2B in GMCs. The changes of histone ubiquitination in GMCs could activate TGF-β signaling pathway involved in the pathogenesis of diabetic nephropathy.

Acknowledgments

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Research Article

Notch Signaling Molecules Activate TGF-β in Rat Mesangial Cells under High Glucose Conditions

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The involvement of the Notch signaling pathway in the cellular differentiation of the mammalian kidney is established. Recently, the dysregulation of Notch signaling molecules has been identified in acute and chronic renal injuries, fibrosis models, and diabetic kidney biopsies. The canonical Notch ligand , Jagged1, is upregulated in a transforming growth factor-beta- (TGF-β-) dependent manner during chronic kidney disease. TGF-β, a central mediator of renal fibrosis, also is a major contributor to the development of diabetic nephropathy. To explore the roles and possible mechanisms of Notch signaling molecules in the pathogenesis of diabetic nephropathy, we exposed cultured rat mesangial cells to a γ-secretase inhibitor (DAPT) or high glucose and measured the expression of Notch signaling molecules and the fibrosis index. Notch pathway-related molecules, TGF-β, and fibronectin increased with exposure to high glucose and decreased with DAPT treatment. Our results suggest that the Notch signaling pathway may precipitate diabetic nephropathy via TGF-β activation.

1. Introduction

Diabetic nephropathy is a common microvascular complication of diabetes with a poorly understood pathogenesis. Hemodynamic changes and disorders of glucose metabolism resulting from genetic factors, hyperglycemia, and/or the actions of angiotensin II and other cytokines can precipitate in the development of diabetic nephropathy. Notably, activation of the Notch signaling pathway can induce the formation of glomerular and tubular lesions that are characteristic of this disease [1, 2]. The Notch ligand, Jagged1, and its target gene product, Hes1, are elevated in renal biopsies from diabetic nephropathy patients, further implicating Notch activation in this disease [3–5]. Despite evidence that Notch activation is present in diabetic nephropathy specimens *in vivo*, no reports have investigated Notch signaling during the development of diabetic nephropathy.

The early stages of diabetic nephropathy are associated with changes in certain cytokines, growth factors, and adhesion molecules, including TGF-β, a central mediator of the fibrotic response. Enhanced fibronectin (FN) deposition ultimately leads to glomerulosclerosis and tubulointerstitial fibrosis, which are characteristics of end-stage diabetic nephropathy [6]. The levels of Jagged1, Jagged2, and Notch1, 4 were upregulated significantly in a human kidney epithelial cell line (CC-2554) with TGF-β1 treatment in a dose-dependent manner [7]. Whether the Notch signaling pathway is involved directly in the pathogenesis of diabetic nephropathy remains unclear.

To elucidate the molecular events involved in Notch signaling that may precipitate diabetic nephropathy, we investigated whether abnormal levels of Notch could initiate signs of diabetic nephropathy in an *in vitro* model. To determine the effects of high glucose on Notch pathway molecules, glomerular mesangial cells (GMCs) were cultured with various concentrations of glucose (5.6, 15, 25, or 35 mmol/L) and different times (12 h, 24 h, 48 h). The expression levels of Notch pathway components in GMC then were measured using Western blotting, RT-PCR, and immunofluorescent

staining and laser scanning confocal microscopy. Our preliminary results support that Notch signaling molecules may contribute to this disease via TGF-β activation.

2. Materials and Methods

2.1. Cell Culture. Rat mesangial cells (HBZY-1) were cultured at 37°C with 5% CO_2 in low glucose DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells were analyzed at the first and third passages. Once cells reached 90% confluency, they were serum deprived in 0.2% FBS for 24 h. Subsequently, cells were stimulated with various media in the absence or presence of glucose at various concentrations. Prior to high-glucose exposure, some cells were exposed to 1 μmol/l N-(N-(3, 5-difluorophenacetyl)-l-alanyl-S-phenyl-glycine t-butylester (DAPT), which can inhibit Notch pathway that was added to the low glucose medium for 2 h. GMCs were randomly divided into the following six groups:

(i) the normal control group (NC group), with medium containing 5.6 mmol/L glucose;

(ii) the 15 mmol/L glucose group (HG1 group), with medium containing 15 mmol/L glucose;

(iii) the 25 mmol/L glucose group (HG2 group), with medium containing 25 mmol/L glucose;

(iv) the 35 mmol/L glucose group (HG3 group), with medium containing 35 mmol/L glucose;

(v) the osmotic pressure group as a control (OP group), with medium containing 5.6 mmol/L glucose +19.4 mmol/L mannitol;

(vi) the DAPT intervention group (HD group), with medium containing 25 mmol/L glucose +1 μmol/L DAPT.

2.2. RNA Isolation and Reverse Transcription-PCR. Cells were collected at 12 h, 24 h, or 48 h time points following glucose or DAPT exposure and immediately were washed in ice-cold phosphate-buffered saline (PBS). Cells then were lysed in 1 mL of TRIzol reagent, and total RNA was isolated as described previously. Samples were reverse transcribed (RT) to cDNAs using SuperScript II reverse transcriptase (Chengdu, Break) according to the manufacturer's instructions. The cDNAs were amplified in a 25 μL PCR mixture containing template cDNA (4 μL), 1 μL each forward and reverse primers, and 12.5 μL 2× FTC2000 PCR Master Mix (Funglyn Biotech, Canada). Primers were targeted to amplify Jagged1, Notch1, Hes1, FN, TGF-β, and GAPDH gene regions (Shengong, Shanghai, China). The following primer sequences were used: Notch1, sense, 5′-CAT CTC CGA CTT CAT CTA TC-3′, antisense, 5′-TCT CCT CCT TGT TGT TC TG-3′; Jagged1, sense, 5′-GCT GGG AAG GAA CAA CC-3′, antisense, 5′-CCT GGA GGG CAG ATA CAC-3′; Hes1, sense, 5′-CGG ACA AAC CAA AGA CC-3′, antisense, 5′-AAG CGGGTC ACC TCG TTC A-3′; FN, sense, 5′-TGC CGA ATG TAG ATG AGG A-3′, antisense, 5′-AAA TGA CC ACT GCC AAA GC-3′; TGF-β, sense, 5′-ATG GTG GAC CGC AAC AAC-3′, antisense, 5′-GAG CAC TGA AGC GAA AGC-3′; GAPDH, sense, 5′-CCT CAA GAT TGT CAG CAA T-3′, antisense, 5′-CCA TCC ACA GTC TTC TGA GT-3′.

2.3. Western Blotting. Harvested cells were washed gently with PBS twice and then were pelleted at 1000 rpm for 5 min at 4°C. Cell pellets were lysed in 300 μL of sucrose buffer (0.125 mL 1.0 M Tris pH 7.5, 0.125 mL 3.0 M NaCl, 25 μL 10% SDS, 25 μL TritonX, 100 μL protease inhibitor) per 100 mm culture dish. Lysates were shaken on ice for 15 minutes, and whole-cell protein extracts were obtained. SDS-PAGE sample loading buffer (5×) was added, and cell fractions were boiled in 95°C asepsis water. Proteins were probed with antibodies raised against Jagged1 (rabbit, 1 : 400, YiXin Company, China), Notch1 (Zhongshan zs-6014, Beijing, China), Hes1 (rat, 1 : 400, Santa Cruz, USA), and β-actin (mouse, 1 : 1000, Beyotime Institute of Biotechnology, China). TGF-β antibody (rabbit, 1 : 1000, CST, USA), GAPDH (mouse, 1 : 1000, Beyotime, China), and Smad4 antibody (rabbit, 1 : 500, Santa Cruz, USA).

2.4. Immunofluorescence Microscopy. We treated GMC with 5.6 mmol/L or 25 mmol/L glucose in the presence or absence of DAPT for 24 h. Cells were grown on coverslips in 6-well plates. After overnight adherence, the cells were treated with media containing high glucose, mannitol, and the DAPT media for 24 h. The cells were fixed in 4% paraformaldehyde (Pierce) and permeabilized in 0.2% Tween 20 (Sigma) for 10 min after being washed briefly with PBS. The cells were blocked with 5% serum for 1 h at room temperature and incubated overnight with primary antibodies followed by washes with PBS. The cells were incubated for 40 min with the appropriate secondary antibody conjugated to the FITC fluorescent dye. The coverslips were washed and mounted onto slides using fluorescent mounting medium (Beyotime, Shanghai, China). The control cells were incubated without a primary antibody. Images were taken with a $DMIRE_2$ laser scanning confocal microscope (Leica, Germany).

The following antibodies were used for immunofluorescence: goat anti-Notch1 (1 : 50; zs-6014; Beijing, China), rat anti-Hes1 (1 : 50; sc-166378), and rabbit anti-Jagged1 (1 : 50 Yixin, Shanghai, China).

2.5. Statistical Analyses. All values are represented as means ± standard errors (S.E.) from at least three independent experiments. Statistical significance was assessed using ANOVA. Significance was set at $P < 0.05$. All data were analyzed using SPSS statistical software.

3. Results

After 24 h culture, compared with normal glucose controls, the protein expression levels of all Notch signaling molecules were significantly increased in GMC in HG2 group ($P < 0.05$). In the OP group, the expression of Notch pathway components was statistically similar to NC Group ($P > 0.05$) (Figure 1). RT-PCR confirmed these trends with respect to mRNA expression (Figure 2).

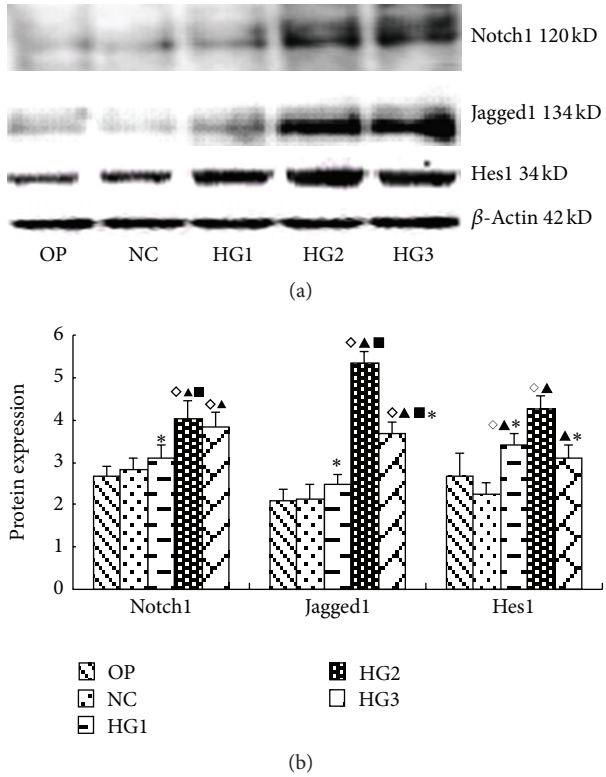

Figure 1: The expression of Notch pathway components as measured by Western blotting following treatment of GMC with various glucose concentrations for 24 h. (a) Notch-associated molecule proteins at different glucose concentrations and high osmotic pressure at 24 h. Notch-associated molecules increased in the high glucose group; they had the most significant changes in the HG2 group, but there were no apparent differences between the NC group and the OP group. (b) The gray graph shows the relative statistical values of Notch-associated molecules for each group. The expression of the Notch-associated molecules increased in the high glucose group, especially, in the HG2 group. $^{\lozenge}P < 0.05$ versus OP group; $^{\blacktriangle}P < 0.05$ versus NC group, $^{\blacksquare}P < 0.05$ versus HG group; $^{*}P < 0.05$ versus HG2 group.

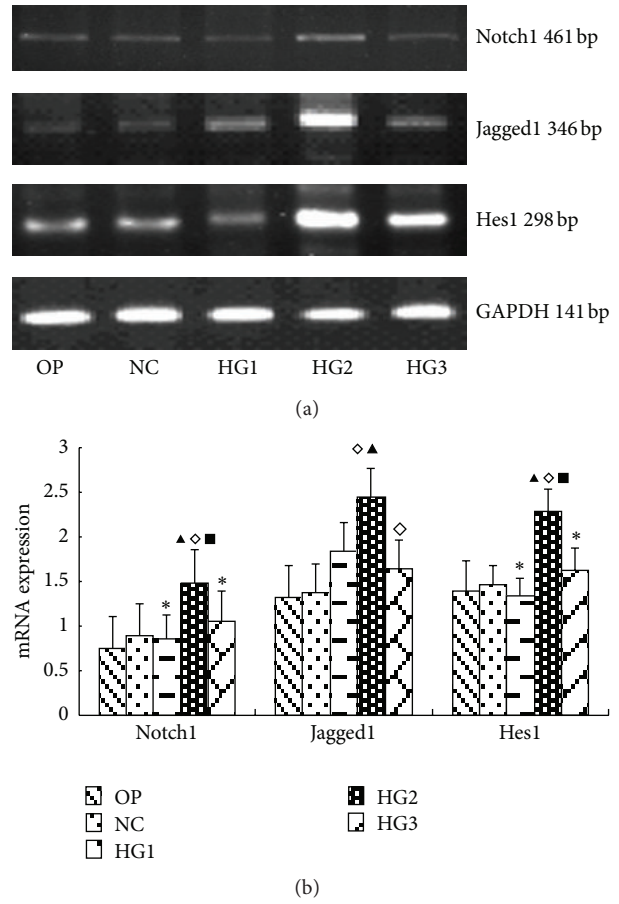

Figure 2: The mRNA levels of Notch pathway components in each group of GMCs. (a) RT-PCR strip chart for different concentrations of glucose. pathway components mRNA increased in the high glucose group, especially in HG2 group. (b) The corresponding relative gray value statistics graph of the mRNA level. $^{\lozenge}P < 0.05$ versus OP group; $^{\blacktriangle}P < 0.05$ versus NC group; $^{\blacksquare}P < 0.05$ versus HG1 group; $^{*}P < 0.05$ versus HG2 group.

Cells cultured in medium with or without high glucose (25 mmol/L) were harvested at various time points following exposure (12 h, 24 h, or 48 h). At the 24 h time point, all Notch-related proteins were significantly enhanced ($P < 0.05$). Notch1 protein was increased at both the 12 h and 24 h time points, with the 24 hr increase being more pronounced (Figure 3). The mRNA expression levels of all analyzed Notch signaling components were significantly upregulated at the 12 h time point ($P < 0.05$). The expression of Notch1 mRNA was enhanced significantly only at the 12 h time point. The expression levels of Hes1 mRNA were upregulated significantly at the 12 h and 24 h ($P < 0.05$) time points, with the 12 h increase being more obvious (Figure 4).

Following treatment with DAPT, the protein expression levels of all Notch pathway components (Notch1, Jagged1, and Hes1) were decreased significantly ($P < 0.05$) (Figure 5). The same results were detected using RT-PCR (Figure 6).

In the cell immunofluorescent staining and laser scanning confocal microscopy applications, the Notch1 protein was weakly expressed in the cytoplasm of cells cultured in 5.6 mmol/L glucose. Following treatment with 25 mmol/L high glucose for 24 h, Notch1 expression levels increased significantly in the cytoplasm but remained undetectable in GMC nuclei. In GMC treated with DAPT, the expression of Notch1 was decreased significantly in the cytoplasm compared with cells exposed to 25 mmol/L glucose group. The Jagged1 protein was expressed in the cytoplasm and nuclei of cells cultured under normal glucose conditions. Upon treatment with 25 mmol/L glucose for 24 h, Jagged1 expression increased significantly. Compared with the 25 mmol/L glucose group, the expression of Jagged1 in the DAPT-exposed group was decreased in cytoplasmic and nuclear compartments, with weaker expression in cell nuclei. The Hes1 protein was weakly expressed in the cytoplasm of GMC treated with 5.6 mmol/L glucose. Following treatment with 25 mmol/L glucose for 24 h, Hes1 expression increased significantly in the cytoplasm and nuclei of GMC. The levels of Hes1 protein in the DAPT-exposed group were decreased

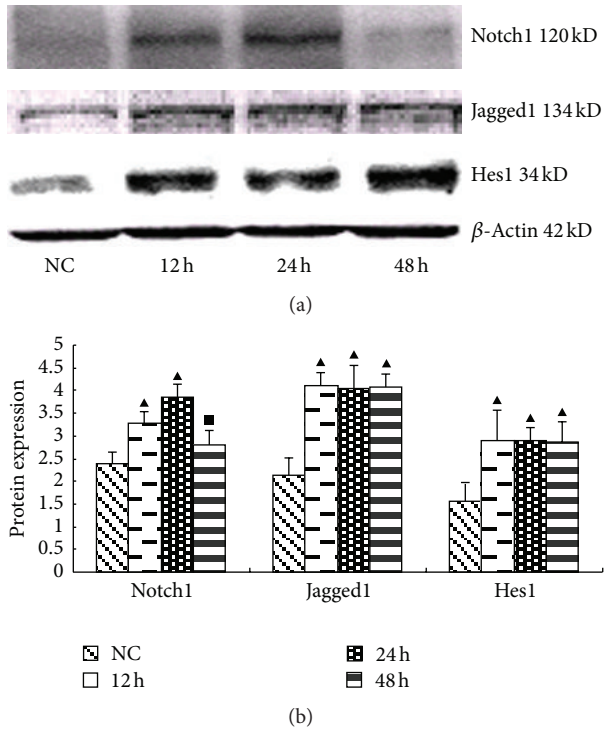

Figure 3: The expression of Notch pathway components in GMCs induced by 25 mmol/L glucose at various times determined using Western blot. (a) Western Blot strip chart for different times. At the 24 h time point, all Notch-related proteins were significantly enhanced. Notch1 protein was increased at both the 12 h and 24 h time points, with the 24 h increase being more pronounced. (b) The gray value statistics graph of the protein level. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus 12 h group; $^{\blacksquare}P < 0.05$ versus 24 h group.

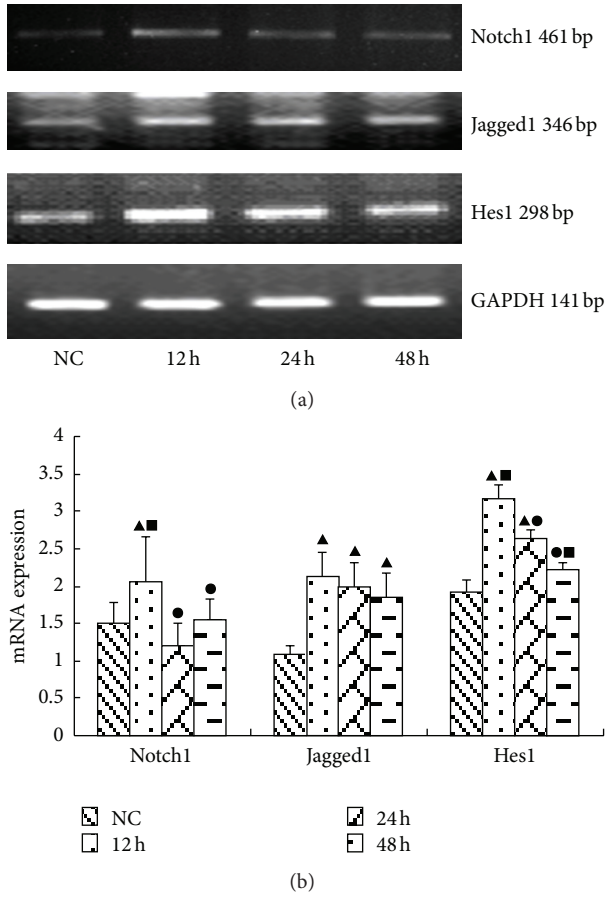

Figure 4: The mRNA levels of FN in each group of GMCs. (a) RT-PCR strip chart for different times. The mRNA expression of all Notch signaling components were significantly upregulated at the 12 h time point. The expression of *Notch1* mRNA was enhanced significantly at the 12 h time point. The expression levels of *Hes1* mRNA were upregulated significantly at the 12 h and 24 h ($P < 0.05$) time points, with the 12 h increase being more obvious. (b) The corresponding relative gray value statistics graph of the mRNA level. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus 12 h group; $^{\blacksquare}P < 0.05$ versus 24 h group.

in cytoplasmic and nuclear compartments, compared with the high glucose-treated group, with weaker expression in nuclei than in the cytoplasm (Figure 7).

Compared with the NC group, the expression of the TGF-β and Smad4 protein was significantly increased in the HG2 group. After DAPT intervention, they decreased (Figures 8 and 9). The mRNA expression of the FN and TFG-β changed in the same trend (Figure 10).

4. Discussion

Notch proteins are a family of single transmembrane proteins. In mammals, four Notch genes specify four types of Notch receptors (Notch1, 2, 3, and 4) [8, 9]. All Notch-associated proteins contribute to processes involved in kidney development. Hes1 is a Notch target gene that encodes a transcriptional regulator. The expression status of Hes1 is a marker for activation of the Notch signaling pathway [10]. Notch1 may participate in the development of fibrosis, which is correlated with glomerular sclerosis [11].

Aberrations in the expression patterns of Notch pathway components affect normal development of the kidney [12]. By assessing the expression of Notch-associated molecules, one can indirectly measure the strength of Notch signaling. Moreover, the expression, transport, and degradation of Notch-related molecules are subject to several regulatory factors [13]. For instance, the interaction of the Notch receptor with its ligand near the cell surface can trigger the Notch signaling pathway and subject the receptor to second and third digestion reactions [14]. After the third digestion reaction, Notch can be activated, and the Notch intracellular domain (NICD) can be released into the cytoplasm for subsequent translocation to the nucleus. In the nucleus, the NICD combines with the DNA binding protein CSL to activate the transcription of target genes (Hes, Hey, and Snail) with the Mam cofactor and p300 coactivator [15–17]. In addition to regulation at the receptor-ligand level, the Notch pathway is modulated by ubiquitination, degradation, protein transport levels, and so on.

A previous report suggested that the expression levels of Jagged1, Notch1, and Hes1 were increased in cultured

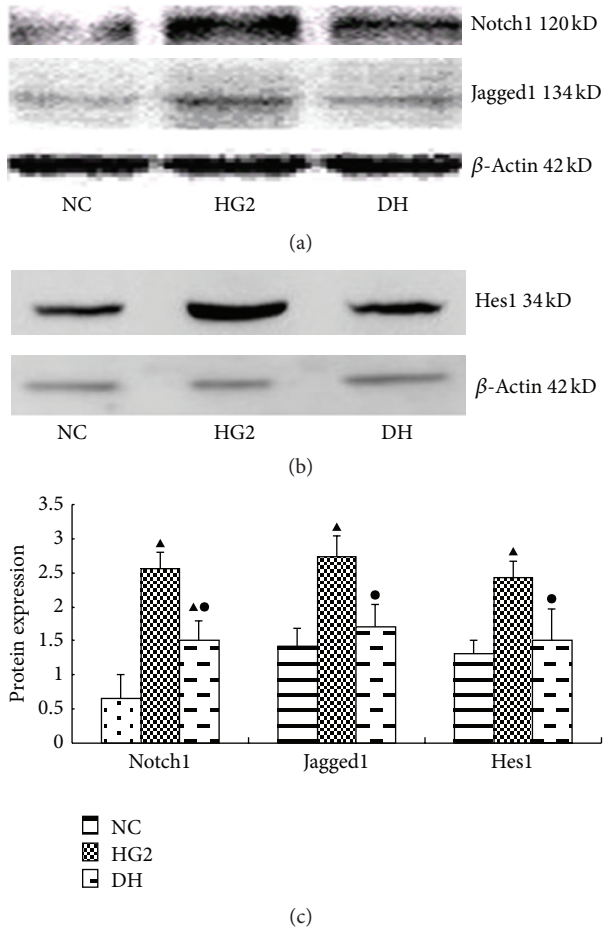

Figure 5: The expression of Notch pathway components after intervention with DAPT determined using western blot for 24 h. (a) Western blot strip chart for Notch1 and Jagged1. (b) Western blot strip chart for Hes1. (c) The gray graph shows the relative statistical values for Notch pathway components in each group. Compared with the NC group, the expression of the Notch pathway components protein was significantly increased in the HG2 group. After DAPT intervention, the protein expression of Notch pathway components decreased. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus HG2 group.

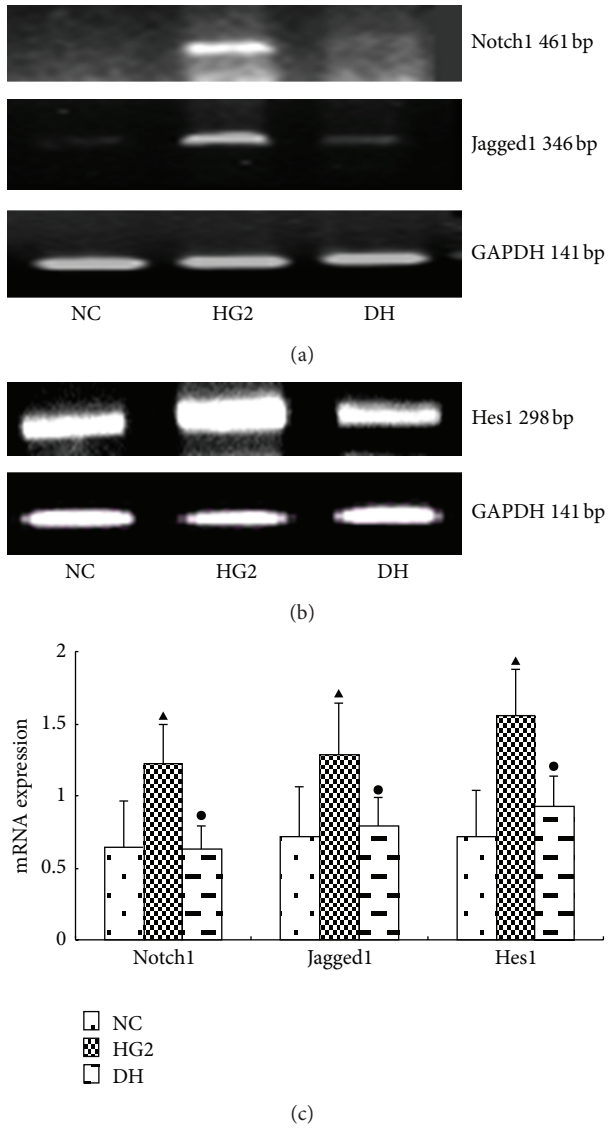

Figure 6: The expression of Notch pathway components after intervention with DAPT determined using PCR for 12 h. (a) RT-PCR strip chart for Notch1 and Jagged1. (b) RT-PCR strip chart for Hes1. (c) The gray graph shows the relative statistical values for Notch pathway components in each group. Compared with the NC group, the expression of the Notch pathway components mRNA was significantly increased in the HG2 group. After DAPT intervention, the mRNA expression of Notch pathway components decreased. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus HG2 group.

podocytes and human embryonic kidney cells following treatment with high glucose [18]. Similarly, we found that high glucose can activate Notch signaling (i.e., increase Notch1 expression) and upregulate related molecules in mesangial cells. Jagged1 and TGF-β expression correlate with glomerular lesions in a diabetic rat model and with diabetic focal segmental glomerulosclerosis in patients. Inactivation of genes related to the Notch signaling pathway can reduce fibrosis in tubular epithelial cells and increase the specificity of Notch1, causing excessive proteinuria [19] and accelerating the development of tubulointerstitial fibrosis [20]. Jagged1 is upregulated in a ureteral obstruction model in a TGF-β-dependent manner [7].

The TGF-β signaling pathway is composed of the TGF-β superfamily, the TGF-β receptor, the Smads protein family, and its regulatory gene. Each component represents subtypes [21]. The TGF-β superfamily includes the TGF-β subfamily (TGF-β1–6), activin, and bone morphogenetic proteins [22]. TGF-β can increase the expression of Jagged1 and Hes1 in human kidney (HK) epithelial cells [5]. Following treatment with TGF-β1 for 24 h, Jagged1 and Hes1 mRNAs are increased in HK-2 cells [23]. In our study, we find that the protein expression of Smad4 increased significantly in the HG2 group. After DAPT intervention, the protein expression of Smad4 decreased. This revealed that TGF-β pathway indeed is activated in our study.

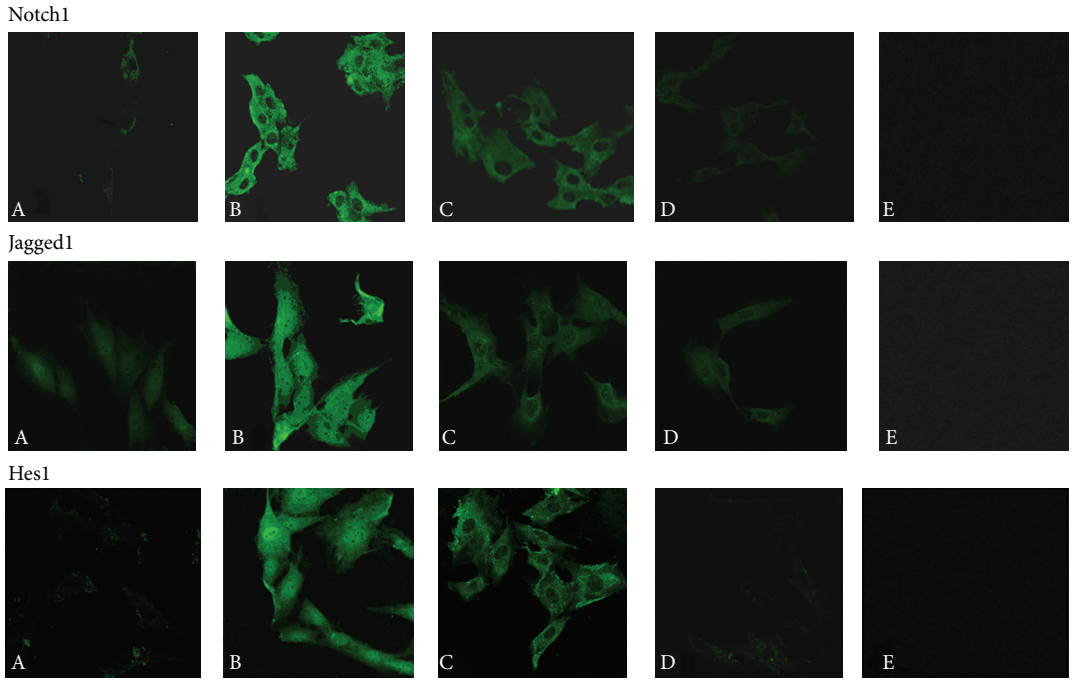

Figure 7: The expression of Notch pathway components in GMCs by immunofluorescent staining and laser scanning confocal microscopy applications. A, NC group; B, HG2 group; C, HD group; D, OP group; E, Negative control. Notch pathway component proteins were detected in the cell as green fluorescence. The Notch pathway components protein was little detected in the NC group, but it was prominent in the HG2 group, which did not significantly change as the result of high osmotic pressure. The protein was less detected in the DAPT intervention group. Original magnification ×630.

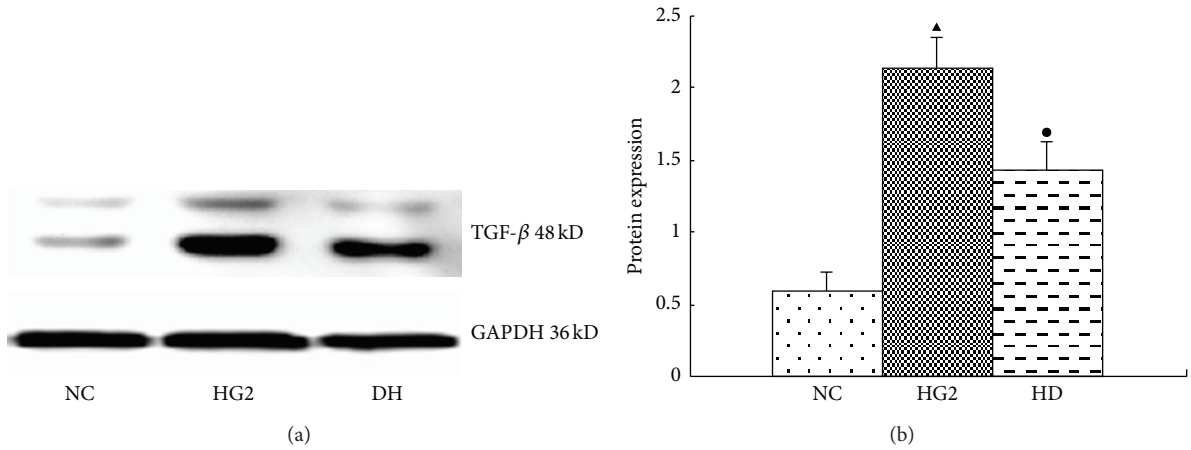

Figure 8: The expression of TGF-β after intervention with DAPT determined using western blot for 24 hr. (a) Western blot strip chart. (b) The gray graph shows the relative statistical values for TGF-β in each group. Compared with the NC group, the expression of the TGF-β protein was significantly increased in the HG2 group. After DAPT intervention, the protein expression of TGF-β decreased. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus HG2 group.

TGF-β1 can promote the synthesis and deposition of the mesangial matrix, and TGF-β is a key factor in the development of renal fibrosis [24, 25] and tubulointerstitial fibrosis [26]. Previous studies have identified a positive feedback loop between TGF-β and Notch signaling in keratinocytes, and the upregulation of TGF-β can increase the expression of the Notch ligand, Jagged1 [27, 28]. Our study shows that the expression levels of Notch signaling molecules were significantly decreased in all DAPT-exposed groups compared with cells treated with high glucose in the absence of DAPT. This finding indicates that DAPT can inactivate the Notch pathway despite a high glucose background.

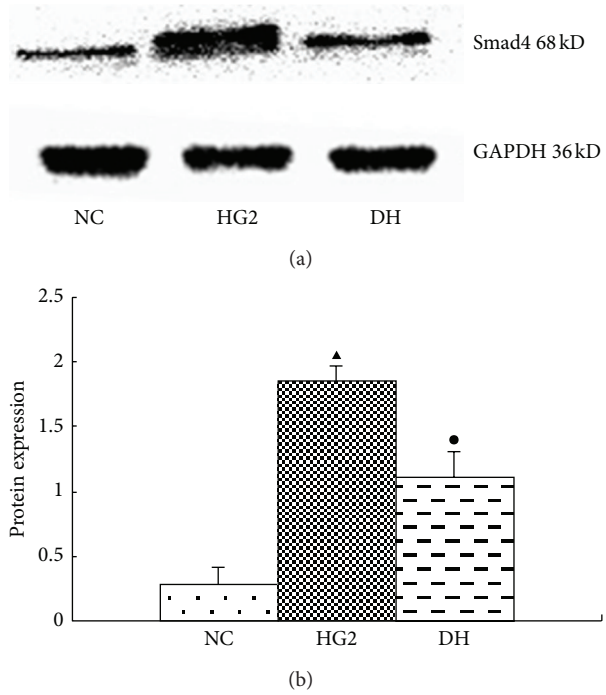

Figure 9: The expression of Smad4 after intervention with DAPT determined using western blot for 24 hr. (a) Western blot strip chart. (b) The gray graph shows the relative statistical values for Smad4 in each group. Compared with the NC group, the expression of the Smad4 protein was significantly increased in the HG2 group. After DAPT intervention, the protein expression of Smad4 decreased. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus HG2 group.

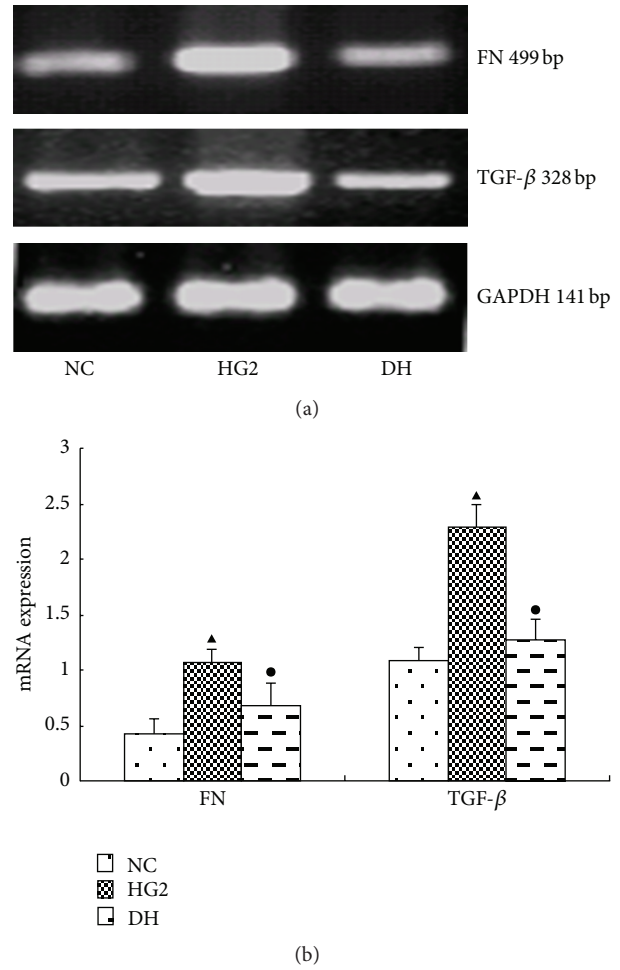

Figure 10: The expression of FN and TFG-β after intervention with DAPT determined using PCR for 12 hr. (a) RT-PCR strip chart for FN and TFG-β. (b) The gray graph shows the relative statistical values for FN and TFG-β in each group. Compared with the NC group, the expression of the FN and TFG-β mRNA was significantly increased in the HG2 group. After DAPT intervention, the mRNA expression of FN and TFG-β decreased. $^{\blacktriangle}P < 0.05$ versus NC group; $^{\bullet}P < 0.05$ versus HG2 group.

Although our experiments indicate that high glucose exposure can activate Notch signaling and that DAPT can reduce the expression of TGF-β and FN, there is no convincing evidence to prove that DAPT can prevent the occurrence of diabetic nephropathy. Thus, additional studies using animal models are warranted to confirm our *in vitro* results.

5. Summary

We report that high glucose can upregulate the expression of Notch signaling in GMC while also upregulate the expression of TGF-β, Smad, and FN. Activation of the Notch signaling pathway could induce TGF-β signaling pathway, which is involved in the pathogenesis of diabetic nephropathy.

Conflict of Interests

The authors declare that there is no conflict of interests, financial or otherwise.

Acknowledgments

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Research Article

The Effect of Dantonic Pill on β-Catenin Expression in a Rat Model of Streptozotocin-Induced Early Stage of Diabetic Nephropathy

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Diabetic nephropathy (DN) is one of the most common causes of end-stage renal failure. This study was performed to determine the effect of Dantonic Pill (DP) treatment on β-catenin expression in a rat model of streptozotocin- (STZ-) induced early-stage DN, with irbesartan treatment as a positive control. Including an analysis of the general metabolic index and renal function, immunohistochemical staining and reverse transcription real-time PCR for β-catenin were performed in the renal cortex of the rat models every 4 weeks. After the treatments of DP and irbesartan, the albuminuria level, kidney weight/body weight, and thickness of the glomerular basement membrane were decreased, but the expression of β-catenin was not downregulated in the renal cortex. The effective drug target of DP to ameliorate albuminuria and renal hypertrophy should not inhibit the upregulated expression of β-catenin in rats with STZ-induced early-stage diabetic damage.

1. Introduction

Diabetes mellitus presents a significant health concern because this disorder leads to long-term complications throughout the body involving the renal and other systems [1]. Diabetic nephropathy (DN) evolves into a progressive fibrosing kidney disease. Wnt pathway components have been reported to be associated with various kidney diseases including DN [2]. Regulating the β-catenin protein levels to control the activation of Wnt-responsive target genes is referred to as the canonical Wnt/β-catenin pathway. Wnt proteins interact with receptor proteins and stabilize the downstream transcription regulator β-catenin by inhibiting β-catenin phosphorylation, which reportedly affects tubule formation and epithelial differentiation [3]. High glucose levels (HG) increased the phosphorylation of β-catenin and reduced the nuclear β-catenin levels. The destabilization of β-catenin was correlated with the increased expression of other profibrotic factors in mesangial cells [4]. Impaired β-catenin signaling is one prominent pathologic reaction responsible for the ECM metabolism induced by HG in mesangial cells [5].

"Herbal medicines" and "herbal remedies" are interchangeable terms that are used to refer to treatments containing various mixtures of herbs. People choose to take herbal medicines as alternatives to orthodox medicines due to their supposed low levels of toxicity and their "natural" origins. Thousands of years ofclinic practice in traditional Chinese medicine (TCM) have accumulated a considerable number of formulae that exhibit reliable in vivo efficacy and safety. The "Dantonic Pill" (DP), also known as the "Cardiotonic Pill," has shown significant therapeutic benefits in patients with DN [6]. Although the DP has been widely used for many years, systematic scientific evidence and proof of efficacy are generally lacking compared with synthesized chemical medicines [7]. Chinese herbal formulae consist of multiple herbs and are therefore liable to produce a large number of metabolites that may act on multiple targets in the body.

The molecular mechanisms that underlie the progression of DN to end-stage renal failure are not well defined, thus limiting access to potential therapeutic targets. Therefore, a likely therapeutic strategy may be the modulation of the β-catenin levels and/or function. Numerous clinical studies have defined proteinuria as a major marker of the decline

of renal function and treatment with irbesartan, which reduces urinary albumin excretion, as a positive control. However, to date, whether DP has an effect on the β-catenin levels in early-stage DN remains unclear. This study was designed to establish diabetic animal models to observe the interventional effects of DP on β-catenin expression in early-stage diabetic kidney injury rats and to provide pathogenic and theoretical evidence of DN in these rats.

2. Materials and Methods

2.1. Materials. This study was performed at the Endocrinology Department, Laboratory of the First Hospital Affiliated to Xi'an Jiaotong University from October 2009 to January 2011. Male SD rats (weighing 180–200 g; clean grade) were selected from the Experimental Animal Center of the Medical College of Xi'an Jiaotong University and cared for using standardized methods. The rats were maintained under temperature-controlled conditions (22°C ± 2°C) and artificial 12 h light/dark cycles with food and water ad libitum. All rats were randomly divided into four groups: the nondiabetic control rats with vehicle treatment (control group, $n = 21$), the streptozotocin- (STZ-) induced diabetic nephropathy rats with vehicle treatment (STZ + vehicle group, $n = 21$), the STZ-induced diabetic rats with irbesartan treatment (STZ + irbesartan group, $n = 21$), and the STZ-induced diabetic rats with DP treatment (STZ + DP group, $n = 21$). In each group, 7 rats were evaluated every 4 weeks until the end of the study, which involved a 12-week observation period. The study was approved by the Institutional Animal Ethical Committee.

2.2. Methods

2.2.1. Model Establishment and Drug Administration. Streptozotocin (10 g/L) was single-peritoneally injected at a dose of 60 mg/kg body weight to establish the diabetic model. The blood glucose levels derived from the caudal vein were, respectively, measured at 72 h and on the 6th day after the injection. The model standard was confirmed by both of the blood glucose levels >16.6 mmo/L. The rats in the STZ + irbesartan and STZ + DP groups were administered with irbesartan 50 mg/(kg·day) and DP 500 mg/(kg·day), respectively, starting 4 weeks after model establishment. Every 4 weeks, 24-hour urine was reserved, blood was collected from the heart, and rats were sacrificed under anesthesia. Renal cortex tissue (100 mg) was used to extract total RNA, and the remaining tissues were fixed with 4% paraformaldehyde for pathological examination.

2.2.2. Renal Biochemical and Functional Detection. The serum creatinine (serum Cr) concentration was determined using a commercial assay kit (BeckmanCoulter, Miami, FL, USA). The urine creatinine concentration was measured using an assay based on Jaffe's reaction according to the manufacturer's protocol (Creatinine Assay Kit). The creatinine clearance rate (Ccr) was calculated using the following equation: Ccr[ml/(min·kg body weight)] = [urinary Cr (μmol/L) × urinary volume (ml)/serum Cr (μmol/L)]/[1/1440 (min)] × [1000/body weight (g)]) [8]. The urine protein level was detected by radioimmunoassay following the manufacturer's protocol. The kidney weight/body weight (KW/BW, %) was measured.

2.2.3. Renal Pathological Examination. The renal glomerulus and renal tubule mesenchymal lesions were observed following HE and PAS staining. Twenty renal glomeruli were randomly selected from the cortical area under 400x magnification by an experienced pathologist to measure the index of mesangial expansion. The index of mesangial expansion was scored by a quantitative estimate of the mesangial zone width in each glomerulus, expressed as a function of the total glomerular area [9]: 0, normal glomeruli; 1, matrix expansion occurring in up to 50% of the glomerulus; 2, matrix expansion occurring in 50–75% of the glomerulus; and 3, matrix expansion occurring in 75–100% of the glomerulus.

2.2.4. Renal Electron Microscopy Morphometric Evaluation. The electron microscopy morphometric evaluation was performed as described below. A portion of the renal tissues was cut into 1 mm cubes, fixed in 2.5% glutaraldehyde, and post-fixed in 1% osmium tetroxide. The samples were dehydrated in a graded alcohol series and embedded in Epon 812. Four ultrathin sections (60 nm) were cut with a diamond knife continuously and stained with uranyl acetate and lead citrate. The thickness of glomerular basement membrane (GBM) was examined with a Hitachi H-7650 (80 kV) transmission electron microscope (JEM100SX, Japan).

2.2.5. Determination of β-Catenin Protein Expression by Immunohistochemistry (IHC). Paraffin sections were routinely dewaxed, hydrated, and dipped in a 0.03 volume fraction of H_2O_2 and methyl alcohol. Then, a microwave was used to repair the antigen, and the tissues were blocked with normal caprine serum and dribbled with attenuated antibody I (antirabbit). The rat β-catenin antibody was diluted at the ratio of 1 : 50, stained with biotin-labeled caprine antirabbit antibody I and diaminobenzidine, restained with hematine, dehydrated, cleared, and sealed with glycerin gelatin. Stained renal glomeruli and tubules that were randomly selected from 20 sights were observed under 400x magnification by an experienced pathologist. The positive staining rate was evaluated as the following: ≤25% positive cells (+), 26–50% positive cells (++), 51–75% positive cells (+++), and >75% positive cells (++++); each scored as 1, 2, 3, and 4 points, respectively, for the statistical analysis [10].

2.2.6. Determination of β-Catenin mRNA Expression by Quantitative Real-Time PCR. Total RNA was extracted from the renal tissues with the Trizol method according to the manufacturer's protocol. cDNA was synthesized by reverse transcription using a kit (TaKaRa Company, Dalian, CA). The expression of β-catenin mRNA was detected by quantitative real-time polymerase chain reaction (RT-PCR). β-actin was used as the internal reference. The total volume of the PCR was 20 μL, with 30 ng of cDNA as the template. After an initial denaturation step at 95°C for 3 min, 40 cycles

of 95°C for 30 sec and 60°C for 30 sec for annealing and extension were run on a CFX384 Dice real-time PCR system (Bio-Rad Laboratories, Inc., CA). A melting curve analysis was performed after the amplification was completed. The mRNA levels were normalized to the β-actin levels of the respective control and presented as a ratio. The primers (designed and synthesized by Dalian Baosen Company) and their sequences, product lengths, and reactive conditions are shown in Table 1.

2.2.7. Statistical Analysis. The measurement data were expressed as the mean ± SD. Significant differences among the groups were analyzed using a factorial-designed ANOVA. The method of simple linear correlation analysis was employed to measure the correlation between two variables. P values < 0.05 were considered significant. SPSS 16.0 software was used in this study.

3. Results

3.1. General Group Comparisons. There were four groups of rats in the study, and the metabolic indices, such as body weight, food intake, water intake, urine output, and blood glucose levels, for each group are summarized in Table 2. The food intake, water intake, urine output, and blood glucose levels in the rats with STZ-induced diabetes were higher thanthose in the normal control rats ($P < 0.05$). The body weights were lower in the diabetic rats than in the normal rats. However, there were no significant differences in any of the metabolic indices among the diabetic rats treated with vehicle, DP, and irbesartan during the 12 weeks ($P > 0.05$).

3.2. Effect of DP and Irbesartan on Renal Functional and Pathological Examination. The renal pathological examination by HE and PAS staining indicated that the mesangial matrix was increased and the renal tubule was vacuolarly degenerated in the diabetic rats. The urine protein levels, Ccr, KW/BW, mesangial expansion index, and GBM thickness were significantly higher in the diabetic rats compared with the normal rats ($P < 0.05$). After 12 weeks of treatment with DP and irbesartan, compared with vehicle alone, the urine protein levels, KW/BW, and GBM thickness were decreased in the diabetic rats, but Ccr and the mesangial expansion index were not ($P < 0.05$ and $P > 0.05$, resp.). The effects of DP and irbesartan on the renal functional and pathological examination in the diabetic rats are shown in Table 3 and Figure 1.

3.3. Effect of DP and Irbesartan on the mRNA and Protein Expressions of β-Catenin. For 12 weeks, the β-catenin mRNA expression in the STZ-induced diabetic rats was significantly upregulated compared with the control group ($P < 0.05$). The β-catenin mRNA expression in the STZ + DP and STZ + irbesartan groups was reduced, but these changes were not significantly different compared with the levels in the STZ + vehicle group ($P > 0.05$). Immunohistochemical staining of β-catenin in the STZ + vehicle group was greater than that of the control group ($P < 0.05$), but the staining in the STZ + DP and STZ + irbesartan groups was not less compared with the STZ + vehicle group ($P > 0.05$). The effect of DP and irbesartan on the β-catenin mRNA and protein expression levels is shown in Figure 2.

Correlation Analysis. There was no significant correlation between the mRNA and protein expression levels of β-catenin and the urinary protein level or the KW/BW in the diabetic rats ($P > 0.05$).

4. Discussion

DN is characterized by definite renal morphological and functional alterations. The features of early diabetic renal changes are glomerular hyperfiltration and glomerular and renal hypertrophy. This disease is characterized by thickening of GBM and mesangial matrix expansion [11]. The data presented here demonstrate that the long-term model of STZ-induced diabetes is associated with the early stage of diabetic nephropathy. By 16 weeks, renal hypertrophy, hyperfiltration, and proteinuria were established in our model. At that time, the earliest changes of glomerular mesangial matrix accumulation, but not collapse of the glomerular capillaries, were detectable, as previously reported [12]. In our experiments, the DP herbal preparation and irbesartan attenuated the early-stage nephropathic symptoms in the diabetic rats, which were characterized by proteinuria, kidney hypertrophy, and thickening of the glomerular basement membrane. There were no effects of the DP and irbesartan treatments over the 12 weeks on distinctive metabolic indices, especially hyperglycemia, induced in the diabetic rats. Therefore, it should be noted that these treatments possess a protective effect against the diabetic renal damage that is independent of lowering the plasma glucose level, which is in agreement with other studies [13]. In this regard, we can conclude that the therapeutic dose of DP (500 mg/(kg·day)) is at least equally effective as that of irbesartan (50 mg/(kg·day)) in early-stage diabetic nephropathy rats.

The principle of formulation in TCM has been adopted to guide the selection of herbs (herb matching) in the multicomponent herbal formulae prescribed for the treatment of diseases. The most important and effective pharmacological ingredients identified in DP are danshensu and Panax notoginseng [14]. Dan Shen, as the main herb in DP, has been identified as containing lipophilic tanshinones such as tanshinone IIA and hydrophilic phenolic acids particularly including danshensu [15]. Danshensu downregulated the expression of fibronectin and collagen-1 induced by high glucose levels in human peritoneal mesothelial cells [16]. In the water extract of San Qi, Panax notoginseng downregulates the expression of β-tumor growth factor [17], vascular endothelial growth factor [18], and connective tissue growth factor (CTGF) [19] and inhibits the overproduction of advanced glycation end products [20] to protect the kidney in a diabetic model. Thus, we confirmed that DP therapy to treat diabetic renal complications should be effective.

Previous studies revealed that the Wnt pathway was identified to be associated with DN. The binding of specific Wnt proteins to receptor/coreceptor complexes transduces

Table 1: Nucleotide sequence of the primers used in real-time PCR.

Gene	Primers	Nucleotide sequence 5′-3′	Length (bp)	Temperature (°C)
β-catenin	Forward	AACGGCTTTCGGTTGAGCTG	118	60
	Reverse	TGGCGATATCCAAGGGCTTC		
β-actin	Forward	TGCCTTTGTGCACTGGTATG	152	60
	Reverse	CTGGAGCAGTTTGACGACAC		

Table 2: Metabolic effects of STZ-induced diabetes and treatments in rats.

Parameters	Body weight (g)	Food intake (g)	Water intake (mL)	Urine output (mL)	Blood glucose (mmol/L)
0 w					
Control ($n = 7$)	443.33 ± 39.28*	24.92 ± 8.59*	40.0 ± 28.5**	10.80 ± 5.45**	4.48 ± 0.15**
STZ ($n = 7$)	303.29 ± 46.61	56.21 ± 5.25	300.03 ± 56.91	197.43 ± 42.55	28.07 ± 8.91
4 w					
Control ($n = 7$)	455.20 ± 29.54*	25.30 ± 4.6*	40.00 ± 12.51**	10.80 ± 5.45**	4.28 ± 0.35**
STZ + vehicle (n=7)	303.29 ± 46.61	56.21 ± 5.25	300.00 ± 56.92	217.43 ± 42.55	28.07 ± 8.91
STZ + DP ($n = 7$)	309.37 ± 44.57	50.28 ± 8.44	227.86 ± 38.06	171.43 ± 23.87	28.28 ± 7.18
STZ + irbesartan ($n = 7$)	346.83 ± 39.28	52.25 ± 9.80	253.43 ± 44.40	184.14 ± 34.51	24.97 ± 1.03
8 w					
Control ($n = 7$)	521.71 ± 42.59*	23.14 ± 5.81*	30.00 ± 11.00**	14.93 ± 2.46**	4.13 ± 0.41**
STZ + vehicle ($n = 7$)	331.88 ± 71.39	50.81 ± 8.27	227.50 ± 41.75	156.50 ± 20.74	28.88 ± 8.32
STZ + DP ($n = 7$)	296.29 ± 25.12	59.01 ± 7.36	232.33 ± 33.69	171.00 ± 25.82	28.33 ± 6.53
STZ + irbesartan ($n = 7$)	311.00 ± 51.78	52.25 ± 8.08	241.25 ± 54.36	178.75 ± 44.52	25.70 ± 1.28
12 w					
Control ($n = 7$)	582.33 ± 85.35*	22.33 ± 5.05*	40.00 ± 10.95**	12.35 ± 4.73**	4.16 ± 0.54**
STZ + vehicle ($n = 7$)	328.22 ± 55.96	51.77 ± 8.60	214.44 ± 44.82	160.33 ± 38.63	28.06 ± 4.87
STZ + DP ($n = 7$)	326.38 ± 54.35	53.56 ± 7.36	246.25 ± 38.89	199.38 ± 34.12	28.28 ± 1.77
STZ + irbesartan ($n = 7$)	353.00 ± 43.17	46.35 ± 4.58	232.86 ± 25.63	189.29 ± 38.90	27.20 ± 1.91

* $P < 0.05$ versus STZ + vehicle rats ** $P < 0.01$ versus STZ + vehicle rats.

Table 3: Renal functional detection and pathological changes of STZ-induced diabetes and treatments in rats.

Parameters	Urinary protein (mg/24 h)	Ccr (mL/min·kg)	KW/BW (%)	Mesangial expansion index
0 w				
Control ($n = 7$)	0.12 ± 0.07*	2.90 ± 0.47*	0.65 ± 0.09*	0.22 ± 0.02*
STZ ($n = 7$)	0.32 ± 0.08	26.43 ± 7.50	1.30 ± 0.03	0.60 ± 0.02
4 w				
Control ($n = 7$)	0.10 ± 0.04*	3.64 ± 1.59*	0.74 ± 0.09*	0.35 ± 0.08*
STZ + vehicle ($n = 7$)	0.39 ± 0.02	13.98 ± 2.99	1.40 ± 0.03	0.64 ± 0.04
STZ + DP ($n = 7$)	0.34 ± 0.02*	14.15 ± 3.31	1.26 ± 0.02*	0.46 ± 0.05
STZ + irbesartan ($n = 7$)	0.20 ± 0.03*	13.51 ± 3.08	1.25 ± 0.04*	0.56 ± 0.02
8 w				
Control ($n = 7$)	0.18 ± 0.02*	1.88 ± 0.46*	0.65 ± 0.04*	0.38 ± 0.08*
STZ + vehicle ($n = 7$)	0.63 ± 0.04	19.40 ± 6.31	1.39 ± 0.08	0.69 ± 0.01
STZ + DP ($n = 7$)	0.30 ± 0.01*	18.83 ± 2.31	1.22 ± 0.09*	0.64 ± 0.05
STZ + irbesartan ($n = 7$)	0.23 ± 0.04*	17.08 ± 7.10	1.28 ± 0.06*	0.62 ± 0.02
12 w				
Control ($n = 7$)	0.13 ± 0.02*	1.30 ± 0.48*	0.63 ± 0.06*	0.40 ± 0.03*
STZ + vehicle ($n = 7$)	0.74 ± 0.04	15.81 ± 3.77	1.25 ± 0.06	0.79 ± 0.02
STZ + DP ($n = 7$)	0.27 ± 0.05*	15.52 ± 1.50	1.20 ± 0.04*	0.68 ± 0.05
STZ + irbesartan ($n = 7$)	0.26 ± 0.02*	14.19 ± 1.66	1.17 ± 0.09*	0.74 ± 0.05

* $P < 0.05$ versus STZ + vehicle rats.

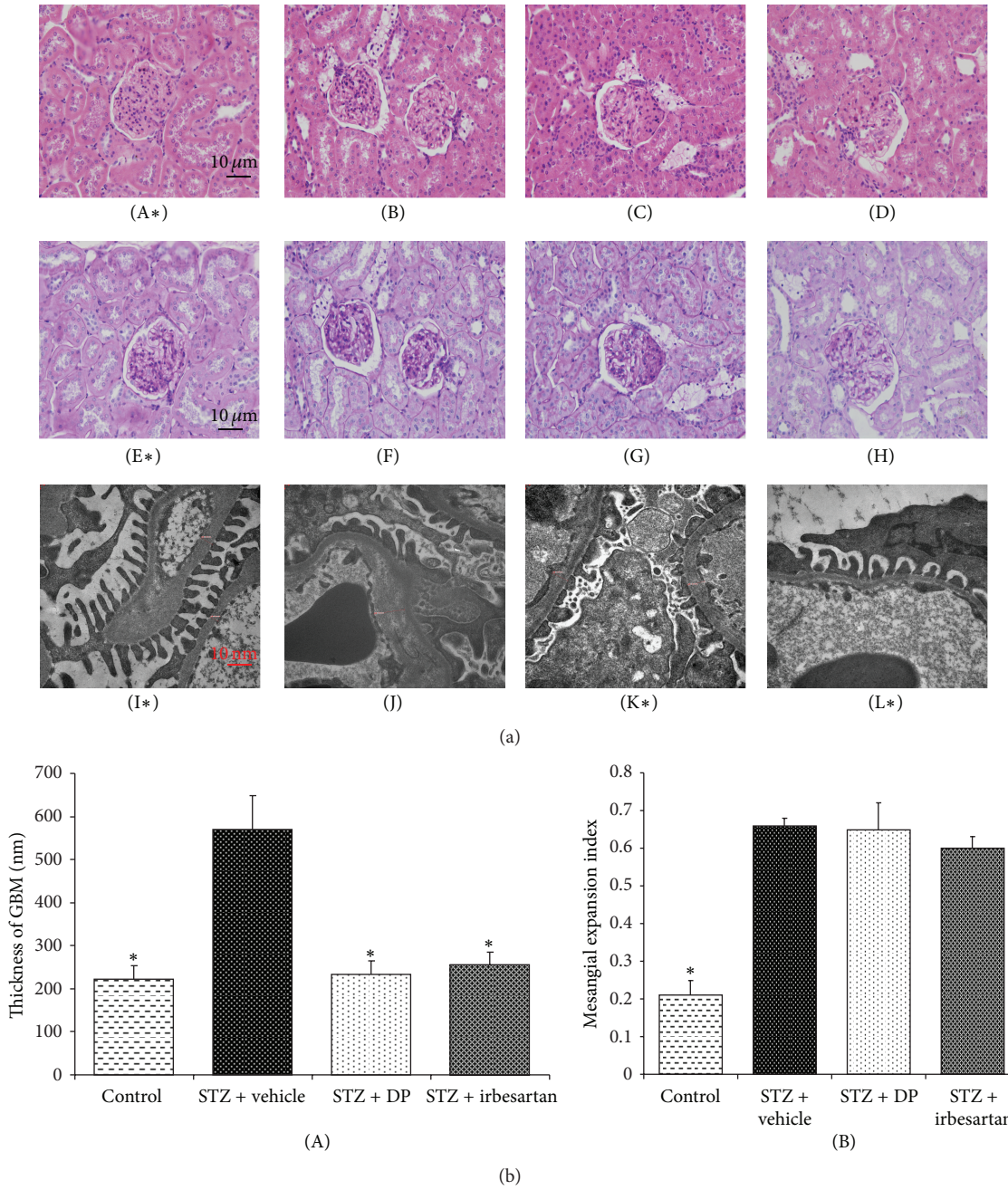

Figure 1: Effect of DP and irbesartan treatments on the renal pathological changes. (a) Renal pathological changes of mesangial expansion were examined by histological staining (HE and PAS), and thickness of the GBM was determined by electron microscopy, as described in the materials and methods section. (A)(E)(I) Normal renal cortex in the control group. (B)(F)(J) STZ + vehicle group. (C)(G)(K) STZ + DP (500 mg/(kg·day) group. (D)(H)(L) STZ + irbesartan (50 mg/(kg·day)) group. (b) Thickness of the GBM (A) and mesangial expansion index (B) expressed as a quantitative estimate score. All values are the means ± SD ($n = 7$). $P < 0.05$, compared with the STZ + vehicle group.

intracellular signals through either β-catenin-dependent or β-catenin-independent pathways. There are generally two pools of β-catenin in cells: one that is tightly associated with cadherins at cell-cell junctions, and the other that is "free" in the cytosol/nucleus. The latter pool is involved in gene transcription regulation. In the resting state, cytosolic/nuclear β-catenin must be maintained at a very low level through the rapid turnover of free β-catenin [21]. Including the Wnt proteins, β-catenin activity is also regulated by tumor necrosis factor [22], N-cadherin, and matrix metalloproteinase (MMP) [23]. Moreover, ROS liberate the cadherin-sequestered pool of β-catenin to promote signaling [24]. The target genes of β-catenin are known to mediate inflammation, angiogenesis, and fibrosis through the upregulation of

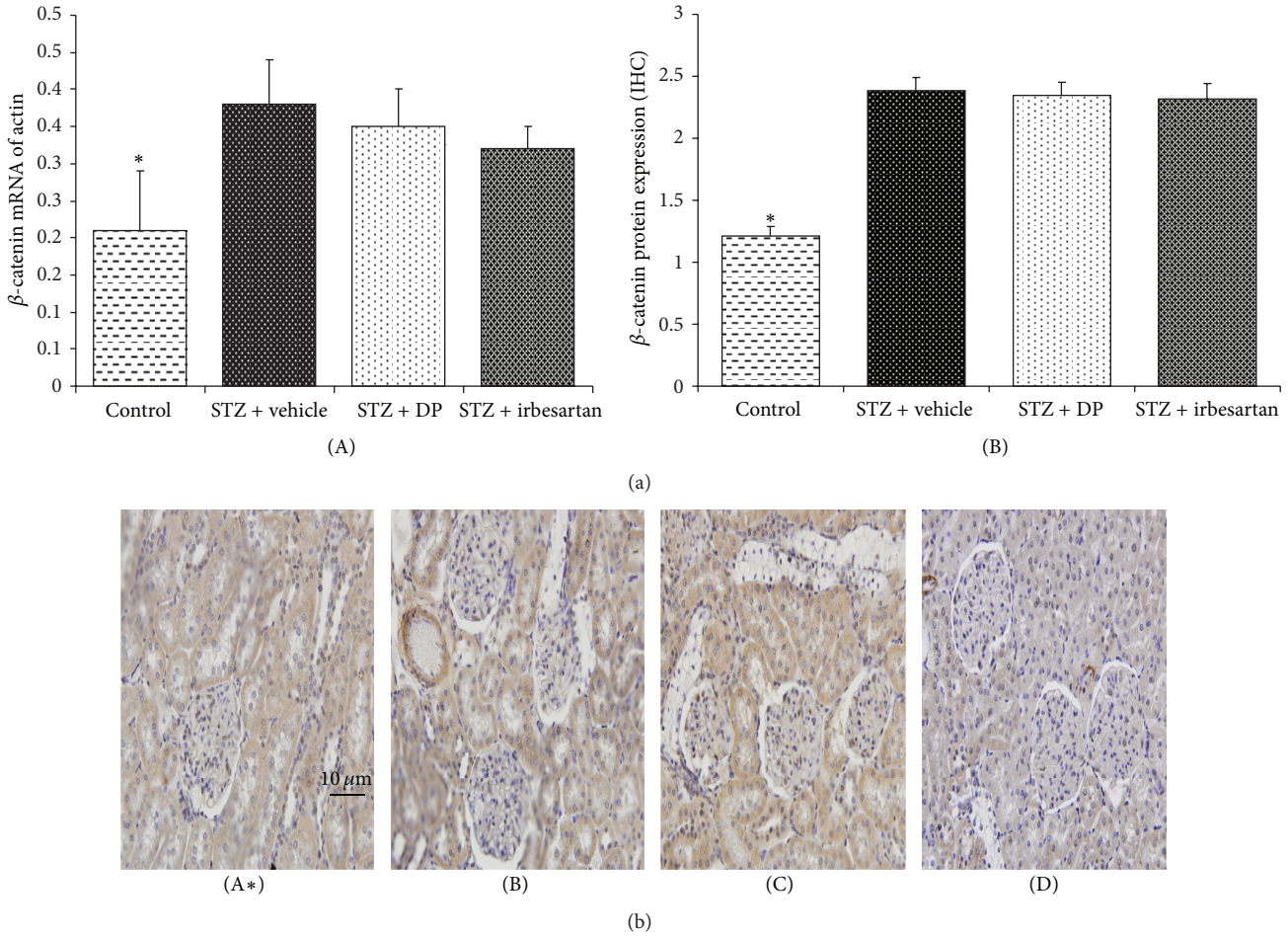

Figure 2: Effect of DP and irbesartan treatments on the renal expressions of β-catenin in the rats. (a) Effect of DP and irbesartan treatments on the renal mRNA and protein expressions of β-catenin as described in the materials and methods section. (A) The relative levels of the β-catenin mRNA were assessed by real-time PCR, and the results were normalized to β-actin. (B) The expression of the β-catenin protein expressed as a quantitative estimate score. (b) The protein expression specific to β-catenin ((A)–(D)) detected by immunohistochemistry (400x magnification): (A) normal glomerulus in the control; (B) STZ + vehicle group; (C) STZ + irbesartan (50 mg/(kg·day)) group; and (D) STZ + DP (500 mg/(kg·day)) group. All values are the means ± SD ($n = 7$). $^*P < 0.05$, compared with the STZ + vehicle group.

intercellular adhesion molecule [25], plasminogen activator inhibitor-1 [26], and CTGF [27], which are important factors participating in the pathogenesis and progression of DN [28]. Therefore, the development of STZ-induced diabetic renal disease appears to be a complex process involving β-catenin.

Immunohistochemistry and real-time PCR analyses showed that the expression of β-catenin in the renal cortex was upregulated in our untreated diabetic rats compared with the nondiabetic control rats during the 16 weeks of this study. A similar study also showed upregulated β-catenin levels in both the renal cytosol and nuclei of the streptozotocin-induced diabetic rats [29]. After 12 weeks of treatment with either DP or irbesartan, the decrease in the β-catenin expression levels was too slight to be significantly different compared with the diabetic control rats. These results suggested that DP and irbesartan decrease urinary albumin and renal hypertrophy to prevent renal injury in the early stage of DN through a mechanism that does not involve the inhibition of β-catenin. The explanation is that in experimental models the kinetics concerning the development of fibrosis is faster (weeks or a few months at best) compared with that of humans (years). Therefore, the treatment in animals might have to be started relatively early, before the irreversible destruction of the renal structure (i.e., before reaching a point of no return). The molecular mechanisms that underlie the progression of DN to end-stage renal failure are not well defined, thereby limiting access to potential therapeutic targets. Because albuminuria caused by diabetic nephropathy is an independent risk factor for cardiovascular events and death [30], a strategy to protect the kidney by reducing the effects of early diabetic kidney disease is the first step in treating this disease. To the best of our knowledge, there has been no study to investigate the therapeutic mechanism of DP on DN via the regulation of β-catenin.

In summary, DP may contribute to the further understanding of the mechanisms of DN and to the development of novel strategies for both its prevention and management.

However, our understanding of the specificities of β-catenin is very limited in DN due to the lack of reliable approaches to selectively assess its function. Such studies are important not only for the quality control of herbal medicine but also for the development of new pharmaceutical products in the prevention and management of diabetic nephropathy.

Conflict of Interests

The authors report no conflict of interests.

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Research Article

Protection Effect of Endomorphins on Advanced Glycation End Products Induced Injury in Endothelial Cells

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Endomorphins (EMs) have a very important bridge-function in cardiovascular, endocrinological, and neurological systems. This study is to investigate the effects of EMs on the synthesis and secretion of vasoactive substances induced by advanced glycation end products in primary cultured human umbilical vein endothelial cells (HUVECs). Firstly, HUVECs were stimulated with AGEs-bovine serum albumin (AGEs-BSA), bovine serum albumin (BSA), or both AGEs-BSA and EMs together, respectively. Then, HUVEC survival rate was calculated by MTT assay, the levels of NO, endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) were detected by colorimetric analysis, and the contents of endothelin-1 (ET-1) were detected by ELISA. The mRNA levels of eNOS and ET-1 were measured by RT-PCR. The expression of p38 mitogen-activated protein kinase (p38 MAPK) was detected by immunofluorescence assay. The results showed that the mRNA expression and secretion of eNOS were significantly enhanced after incubation with EMs compared to those with AGEs-BSA, while the secretion of NO and iNOS, mRNA expression, and secretion of ET-1 had opposite changes. The fluorescence intensity of p38MAPK in nuclear was decreased after pretreatment with EMs compared to incubation with AGEs-BSA. *Conclusion*. The present study suggests that EMs have certain protection effect on AGEs-BSA-induced injury in HUVEC.

1. Introduction

Overwhelming evidence proved that the formation and accumulation of advanced glycation end products (AGEs) progress in a normal aging process and at an accelerated rate under diabetes [1, 2]; an increase in the steady-state levels of highly reactive dicarbonylic compounds may lead to the formation of AGEs, while an increase in the generation of AGEs can be partly explained by the process of non-enzymatic glycosylation of proteins. These proteins appear to contribute to diverse cellular functions, such as the specific recognition and degradation of AGEs-modified proteins [3]. So far, several AGE-binding proteins have been identified, including AGE-R1, AGE-R2, AGE-R3, RAGE, and macrophage scavenger receptors type I and type II. In endothelial cells, AGEs exert adverse effects on mitochondrial function, with elevated production of reactive oxygen species (ROS), and consequently increased oxidative stress leading to cellular dysfunction and even cell death. AGEs also increase the formation of intracellular ROS, NO, and nitric oxide synthase (NOS) and stimulate ceramides as well as the MAPK cascade, which activates different targets including transcription factors through intermediate molecules such as NF-κB [4–6]. Therefore preventing the endothelial cell from AGE-triggered injury may improve diabetes-associated vascular complications.

The endogenous opioid peptides, endomorphin 1 (Tyr1-Pro2-Trp3-Phe4-NH2, EM1) and endomorphin 2 (Tyr1-Pro2-Phe3-Phe4-NH2, EM2), which were discovered in 1997 by Champion et al., have higher affinity and are more selective

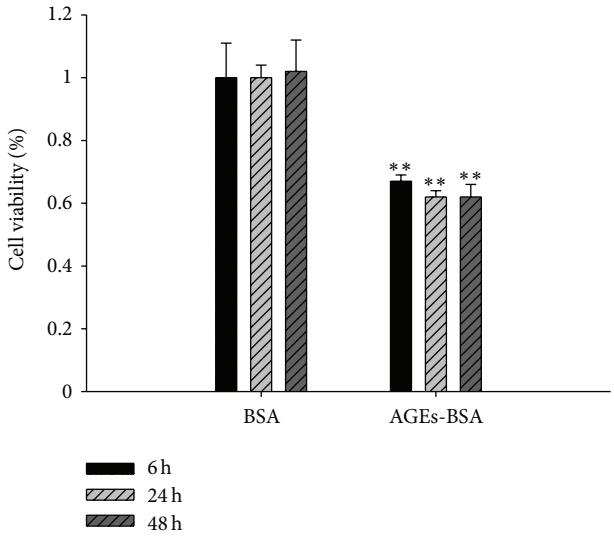

Figure 1: Effect of AGEs-BSA on cell viability determined by MTT test. HUVECs were treated with AGEs-BSA (100 mg/L) or BSA (100 mg/L) for 6 h, 24 h, 48 h. Viability was calculated as the percentage of living cells in treated cultures compared to those in control cultures. Each value represents the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus BSA group.

for the μ-opiate receptor than other opioid substances [7]. Many studies indicated that the endogenous opioid system played roles in the regulation of the cardiovascular system in a variety of species [8, 9], such as rabbits [10], rats [11, 12], and mice [13] Furthermore, Jaffe et al. [14] reported that vasodilator responses to endomorphin 1 were mediated by a nitric oxide-dependent mechanism and may act as an endothelium-dependent vasodilator agent in rat. However, the precise molecular mechanisms by which EMs inhibit AGE-induced injury in endothelial cells have not yet been thoroughly elucidated. The purpose of this study is to investigate the inhibitory effects and to involve mechanisms of EMs on AGEs induced-oxidative stress and apoptosis in human umbilical vein endothelial cells.

2. Materials and Methods

2.1. Reagents. Endomorphins was synthesized by Shanghai Hanhong Chemical Co., Ltd (Shanghai, China). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials (Hangzhou, China). NO and endothelial nitric oxide synthase (eNOS) assay kits were obtained from Jian-Cheng Biological Engineering Institute (Nanjing, China). Rabbit anti-human P38 (H174) antibody, FITC-conjugated goat anti-rabbit antibody were obtained from Bioworld Technology, Inc. (Minneapolis, USA). BSA was purchased from Sigma (St. Louis, MO, USA). The primers, Taq polymerase, dNTP, and Rnasin were provided by TaKaRa Bio Inc. (Otsu, Shiga, Japan).

2.2. Preparation of AGEs. AGEs-BSA was produced by incubation of 10 mg/mL BSA with 100 mM glucose in 150 mM phosphate-buffered saline (PBS), pH 7.4 at 37°C for 6 weeks [15]. Control BSA was incubated in the same conditions without glucose. Unbound sugar was removed by centrifugation filtration with Centricon filter cartridges. AGEs-BSA was identified by fluorescence spectrophotometer.

2.3. Cell Culture and Treatment. Before the study, we recruited mothers who assented and gave written consent to contributing 10 cm of umbilical cord postpartum, and were isolated according to a previous reported method [16] with minor modifications. Cultured cells were identified as endothelial by their morphology and the presence of von Willebrand factor. Briefly, the cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 mg/mL). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was refreshed every two days. For experiments, cells treated with endomorphins (10 μM, 1 μM, 0.1 μM, or 10 nM) were exposed to these substances for 2 h before treatment with AGEs-BSA.

2.4. Cell Viability Assay. Cells were incubated in 96-well plates at a density of 5×10^3 cells with 200 μL culture medium per well. After cells were incubated according to the aforementioned group, 30 μL medium containing 5 mg/mL MTT (Sigma, USA) was added to each well. Following a 4 h incubation period, 100 μL 10% SDS was added. And then, after overnight incubation in darkness, the dissolved MTT crystals were quantified. Optical densities were obtained using a test at a wavelength of 570 nm.

2.5. Chemiluminescence Analysis of NO. Levels of the nitric oxide (NO) derivative nitrite were determined in the conditioned medium of HUVEC with the Griess reaction [17]. After cells were incubated according to the aforementioned grouping, 100 μL culture solutions of each well was collected and put into the counterpart well of another plates, then NO production in cells was measured by Griess method and according to the indication on the NO assay kit. Optical density was read in a microplate reader at 540 nm. Each experiment was performed in triplicate.

2.6. Determination of eNOS and iNOS Activity. After cells were incubated according to the aforementioned grouping, 200 μL culture solutions of each well was collected and put into the counterpart well of another plates, then eNOS and iNOS expression in cells were measured according to the instructions given in the NOS assay kit. Optical density was read in a microplate reader at 530 nm. Each experiment was performed in triplicate.

2.7. ELISA Analysis of Endothelin-1. A specific sandwich enzyme-linked immunosorbent (ELISA) employing monoclonal antibody was used to determinate the level of ET-1; the ELISA was performed according to the instructions given in the ET-1 ELISA kit by Ad Litteram Diagnostic Laboratories (USA). Optical density was read in a microplate reader at 450 nm. Each experiment was performed in triplicate.

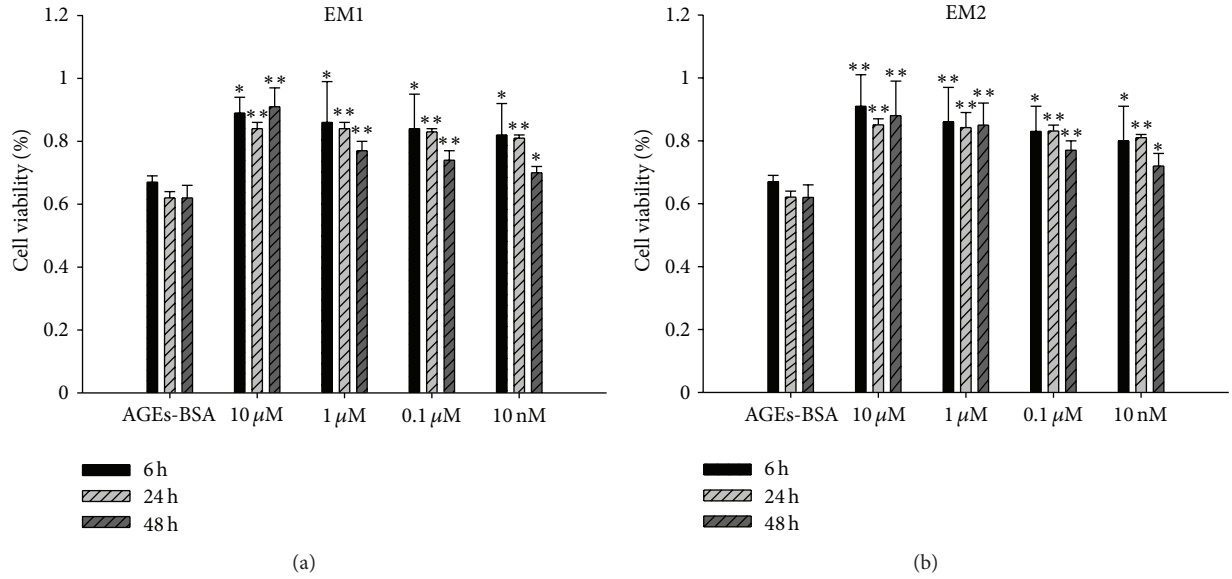

Figure 2: Effect of EM1, EM2 on cell viability determined by MTT test. HUVECs were treated with EMs (10 μM, 1 μM, 0.1 μM, 10 nM) for 2 h before treatment with AGEs-BSA (100 mg/L) for 6 h, 24 h, 48 h. Viability was calculated as the percentage of living cells in treated cultures compared to those in control cultures. Each value represents the mean ± SD ($n = 3$). Statistical analysis compared with AGEs-BSA group by ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$.

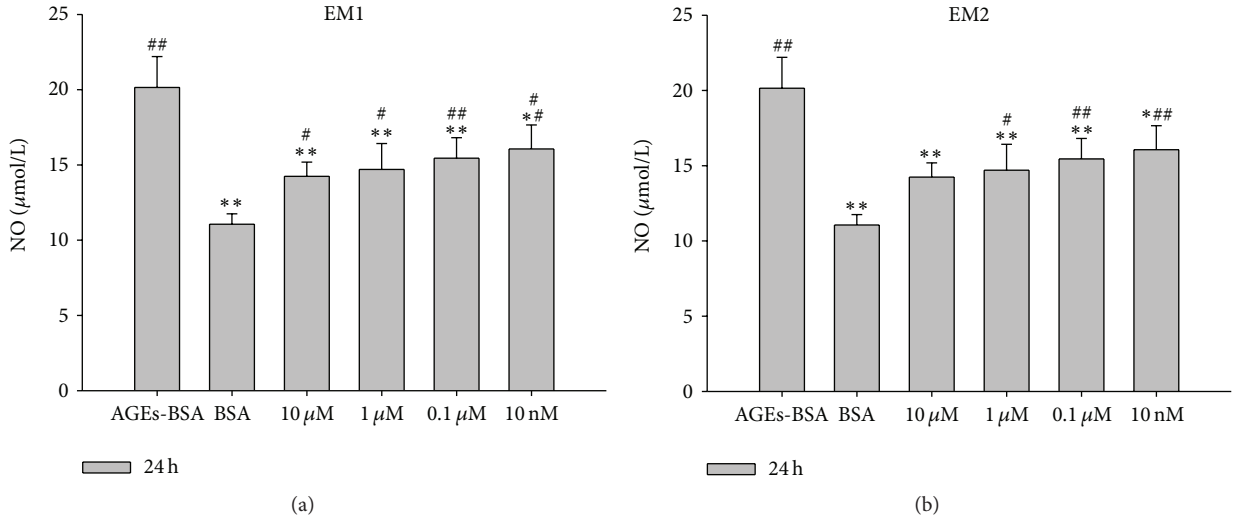

Figure 3: Effect of EM1, EM2 on NO concentration determined by Griess reaction test in HUVEC. Each data is expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$ versus AGEs-BSA group, $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ versus control (BSA) group.

2.8. Real-Time RT-PCR Analysis for eNOS and ET-1 mRNA Level. After incubation, the cells were washed twice with PBS and the total mRNA was extracted by Trizol. Thereafter, it was reverse-transcripted under following conditions: 37°C for 15 min, 85°C for 5 sec, and the cDNA product was stored at −80°C. For the PCR, 3 μL of the cDNA products of each sample was amplified with Taq DNA polymerase, using a primer pair specific to human eNOS, ET-1, and β-actin in a 25 μL reaction volume; the primer sequences and PCR condition were described in Table 1. PCR cycle conditions were 95°C for 30 sec, 95°C for 5 sec, 60°C for 30 sec for 50 cycles, with an initial denaturation at 94°C for 5 min and a final extension of 5 min at 72°C. The resulting data were analyzed by Rotor-Gene Real-Time analysis software 6.1. The relative mRNA expression level of each targeted gene was calculated by $2^{-\Delta\Delta Ct}$.

2.9. Immunofluorescence Staining. Immunofluorescence staining was performed as described previously [18]. Cells were fixed with 4% paraformaldehyde (pH 7.4) for 15 min at 4°C and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After being blocked with 5% normal bovine serum for 30 min, cells were incubated with p38

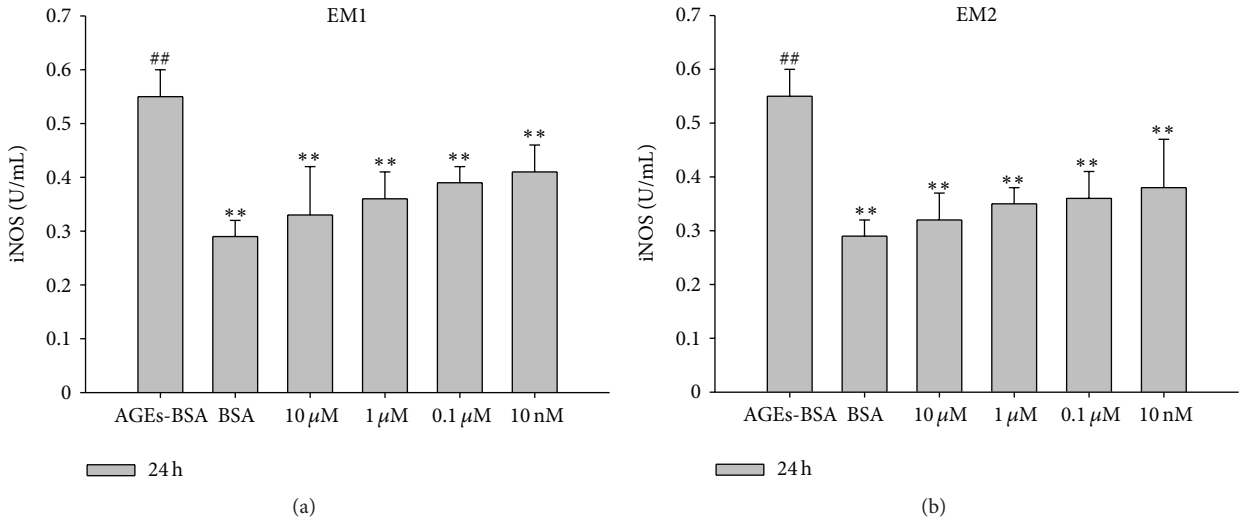

Figure 4: Effect of EM1, EM2 on iNOS level in HUVEC. Each data is expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$ versus AGEs-BSA group, $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ versus control (BSA) group.

Table 1: Primers used for RT-PCR.

Gene name	Primer sequences	T_m (°C)	Cycles	Length (bp)	Accession number
ET-1	F: 5′-TCAGAGGAACACCTAAGACAA-3′ R: 5′-TGCTCGGTTGTGGTCACATA-3′	63.3	35	123	NM-001955.3
NOS	F: 5′-GCTGTCTGCATGGACCTGGA-3′ R: 5′-TCCACGATGGTGACTTTGGCTA-3′	64.8	38	119	NM-000603.3
β-actin	F: 5′-GCAAGCAGTATGACGAGT-3′ R: 5′-CTGCGCAAGTTAGGTTTTGTC-3′	64.3	10	112	NM-001101.3

MAPK antibody (1 : 100 dilution) at 4°C overnight followed by FITC-conjugated secondary antibody (1 : 50 dilution, 1 h). Images were obtained using fluorescence microscope (IX81, Olympus, Japan).

2.10. Statistical Analysis. Statistical evaluations were performed using one-way ANOVA followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant. Data are expressed as mean ± SE of at least three independent experiments.

3. Results

3.1. Effect of EMs on Cell Viability. Exposure of HUVEC to AGEs-BSA (100 mg/L) for 6 h, 24 h, and 48 h significantly decreased the cells viability significantly compared to that of BSA (100 mg/L, as osmotic control) ($P < 0.01$, Figure 1). The cell viability was decreased at 6 h and reached minimal level at 48 h after AGEs-BSA treatment. Whereas pretreatment with EM1 and EM2 (10 μM, 1 μM, 0.1 μM, 10 nM) significantly increased the cells viability compared to AGEs-BSA group, the function was obvious at 24 h, 48 h compared to 6 h ($P < 0.005$ versus $P < 0.05$), and high concentration was more obvious than low concentration, which indicated that EMs can attenuate the reduction of cell viability by AGEs-BSA in a time- and concentration-dependent manner (Figure 2).

3.2. Effect of EMs on NO Production. As is shown in Figure 3, the NO production in HUVEC was 11.06 ± 0.69 μM after incubation for 24 h in the control group and was 20.15 ± 2.05 μM in the AGEs-BSA group, which was notably higher than that of control group ($P < 0.005$), while the NO production in HUVEC were 14.24 ± 0.95 μM, 14.70 ± 1.72 μM, 15.45±1.36 μM, 16.06±1.60 μM after incubation for 24 h in EM1 pretreated group as the concentrations of 10 μM, 1 μM, 0.1 μM, 10 nM, which were notably lower than that of AGEs-BSA group ($P < 0.005$, 0.05). These results indicated that EM1 inhibited the NO production in a concentration-dependent manner in HUVEC stimulated by AGEs-BSA. The same results were observed in EM2 group.

3.3. Effect of EMs on iNOS Secretion. In the control group (see Figure 4), the secretion of iNOS was 0.29 ± 0.03 U/mL after incubation for 24 h and was 0.55 ± 0.05 U/mL in AGEs-BSA treated group, which was significantly increased to the control group ($P < 0.005$). While the iNOS secretion in EM1, EM2 pretreated groups were 0.33 ± 0.09 U/mL, 0.36 ± 0.05 U/mL, 0.39 ± 0.03 U/mL, 0.41 ± 0.05 U/mL; 0.32 ± 0.05 U/mL, 0.35 ± 0.03 U/mL, 0.36 ± 0.05 U/mL, 0.38 ± 0.09 U/mL as the concentrations of 10 μM, 1 μM, 0.1 μM, 10 nM, which were significantly decreased to the AGEs-BSA treated group ($P < 0.005$, 0.05), these results indicated that EMs efficiently and

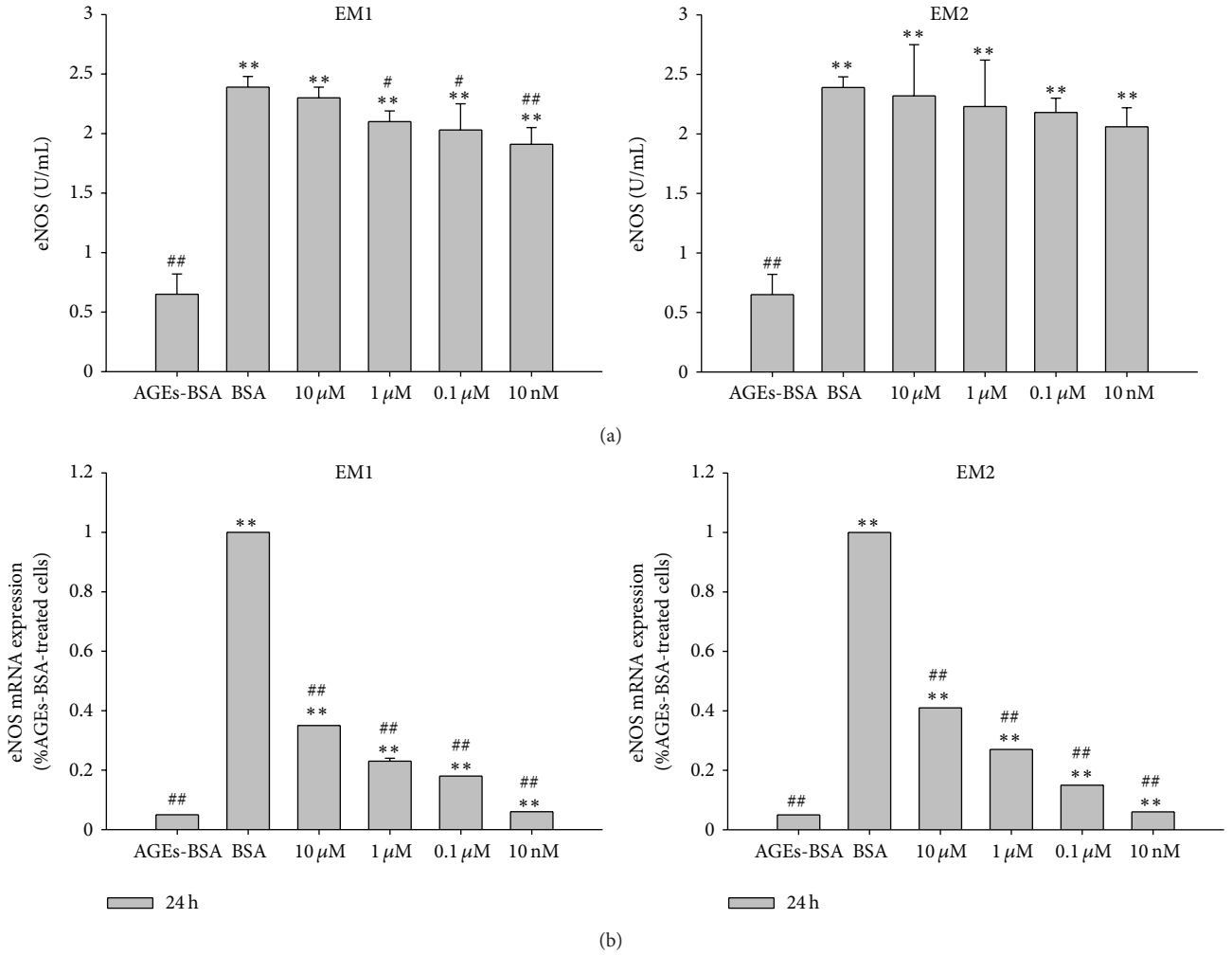

Figure 5: Effect of EM1, EM2 on eNOS secretion determined by ELISA test in HUVEC (a). HUVECs were incubated according to the aforementioned grouping. Each value represents the mean ± SD ($n = 3$); mRNA expression level of eNOS after treatment with AGEs-BSA and EM1 or EM2, using BSA treated cells as reference control (b). The parameter Ct was derived for each cDNA sample and primer pair; for a given sample, Ct values for β-actin were subtracted from the Ct of each candidate gene reaction to arrive at a ΔCt value. The mean ΔCt from all control reactions was then subtracted from the ΔCt of each treated sample to arrive at ΔΔCt. The relative fold change was calculated by the expression $2^{-\Delta\Delta Ct}$. Each data is expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$ versus AGEs-BSA group, $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ versus control (BSA) group.

concentration-dependently inhibited the iNOS secretion in HUVEC.

3.4. Effect of EMs on eNOS Secretion, mRNA Level of eNOS. In the control group (see Figure 5(a)), the secretion of eNOS was 2.39 ± 0.09 U/mL after incubated for 24 h, and that of AGEs-BSA treated group was 0.65 ± 0.17 U/mL in, which was significantly decreased compared to the control group ($P < 0.005$). While the secretions of eNOS in EM1, EM2 pretreated groups were 2.30 ± 0.09 U/mL, 2.10 ± 0.09 U/mL, 2.03 ± 0.22 U/mL, 1.91 ± 0.14 U/mL; 2.32 ± 0.43 U/mL, 2.23 ± 0.39 U/mL, 2.18 ± 0.12 U/mL, 2.06 ± 0.16 U/mL as the concentrations of 10 μM, 1 μM, 0.1 μM, 10 nM, which were significantly higher compared to the AGEs-BSA treated group ($P < 0.005$, 0.05), these results indicate that EMs pretreatment abrogated the decrease efficiently, and in a concentration-dependent manner. Similar results were observed for the mRNA level of eNOS (Figure 5(b)). These results indicate that EMs efficiently inhibited the decrease of eNOS expression and secretion stimulated by AGEs in HUVEC.

3.5. Effect of EMs on ET-1, mRNA Level of ET-1. In the control group (Figure 6(a)), the secretion of ET-1 was 0.76 ± 0.03 ng/mL after incubated for 24 h and was 0.99 ± 0.08 ng/mL in AGEs-BSA treated group, which was significantly higher than that in the control group ($P < 0.005$). While the ET-1 secretions in EM1, EM2 pretreated groups were 0.85 ± 0.03 ng/mL, 0.87 ± 0.06 ng/mL, 0.88 ± 0.01 ng/mL, 0.89 ± 0.04 ng/mL; 0.76 ± 0.03 ng/mL, 0.78 ± 0.13 ng/mL, 0.81 ± 0.06 ng/mL, 0.85 ± 0.01 ng/mL as the concentrations of 10 μM, 1 μM, 0.1 μM, 10 nM, which were significantly lower than the AGEs-BSA treated group ($P < 0.005$, 0.05), these results

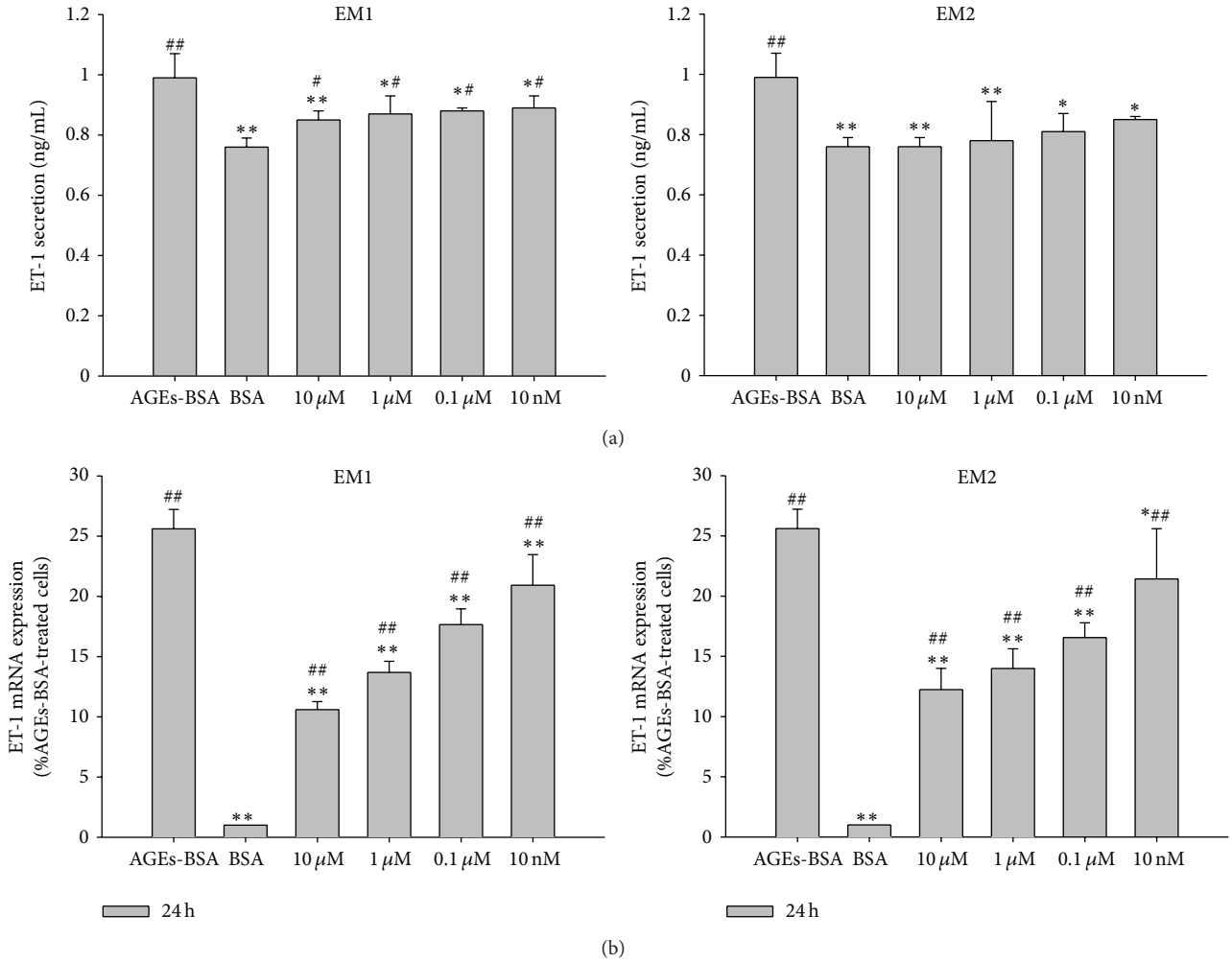

Figure 6: Effect of EM1, EM2 on ET-1 secretion determined by ELISA test in HUVEC (a). HUVECs were incubated according to the aforementioned grouping. Each value represents the mean ± SD ($n = 3$); mRNA expression level of ET-1 after treatment with AGEs-BSA and EM1 or EM2, using BSA treated cells as reference control (b). The parameter Ct was derived for each cDNA sample and primer pair; for a given sample, Ct values for β-actin were subtracted from the Ct of each candidate gene reaction to arrive at a ΔCt value. The mean ΔCt from all control reactions was then subtracted from the ΔCt of each treated sample to arrive at ΔΔCt. The relative fold change was calculated by the expression $2^{-\Delta\Delta Ct}$. Each data is expressed as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$ versus AGEs-BSA group, $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ versus control (BSA) group.

indicated that EMs pretreatment abrogated the increase efficiently in a concentration-dependent manner. Similar results were observed for the mRNA level of ET-1 (Figure 6(b)). These results indicated that EMs efficiently inhibited the ET-1 mRNA expression and ET-1 secretion in HUVEC.

3.6. Effect of EMs on p38 MAPK. In this study, our investigation tries to ascertain whether EMs inhibit the AGEs-induced dysfunction in endothelial cells through p38 MAPK activities. As noted in Figures 7(b) and 8(a), the fluorescence intensity of p38 MAPK in the nucleus was obviously elevated in AGEs-treated HUVECs relative to that in BSA-treated group (Figures 7(a) and 8(b)). However, in EMs pretreated groups (Figures 7(c)–7(f)) and Figures 8(c)–8(f)), the fluorescence intensity of p38 MAPK in the nucleus was similar to BSA group, obviously weaker compared to AGEs-BSA group. Therefore, these results implied that EMs inhibited the expression of p38 MAPK in the nucleus induced by AGEs.

4. Discussion

Vascular endothelial cells play an important role in modulating anti-thrombus and maintaining the natural function of vascular by secreting many active substances. AGEs, high blood glucose, oxide-LDL, and inflammatory factor are the main factors that induce endothelial cells injury [19, 20]. Once endothelial cell were damaged, it would result in dysfunction and abnormal secretion of active substances (e.g. NO, NOS, ET-1, and prostacyclin PGI_2).

NO is a strong oxidant and one of the most important mediators in the regulation of endothelial cell functions, which is synthesized by three isoforms of NO synthases

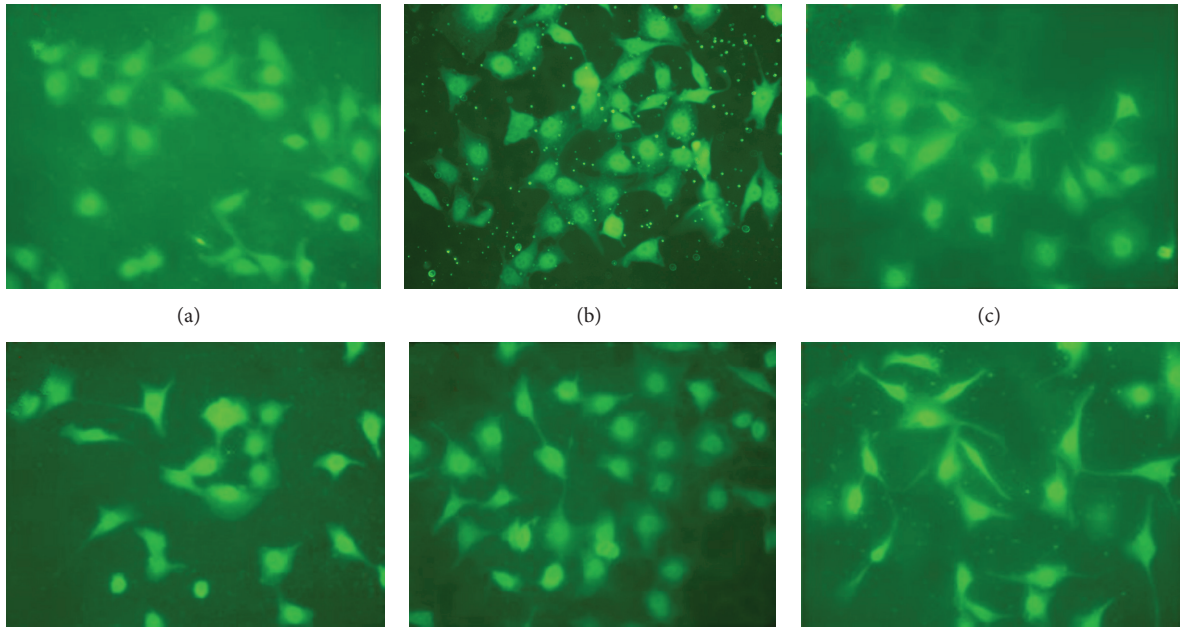

Figure 7: Immunofluorescence studies of EM1 on p38 MAPK in HUVECs. Cells were fixed, and incubated with p38 MAPK antibody and a FITC-conjugated second antibody. Pictures were taken at 400x magnification. ((a) BSA, (b) AGEs-BSA, (c) 10 μM, (d) 1 μM, (e) 0.1 μM, (f) 10 nM).

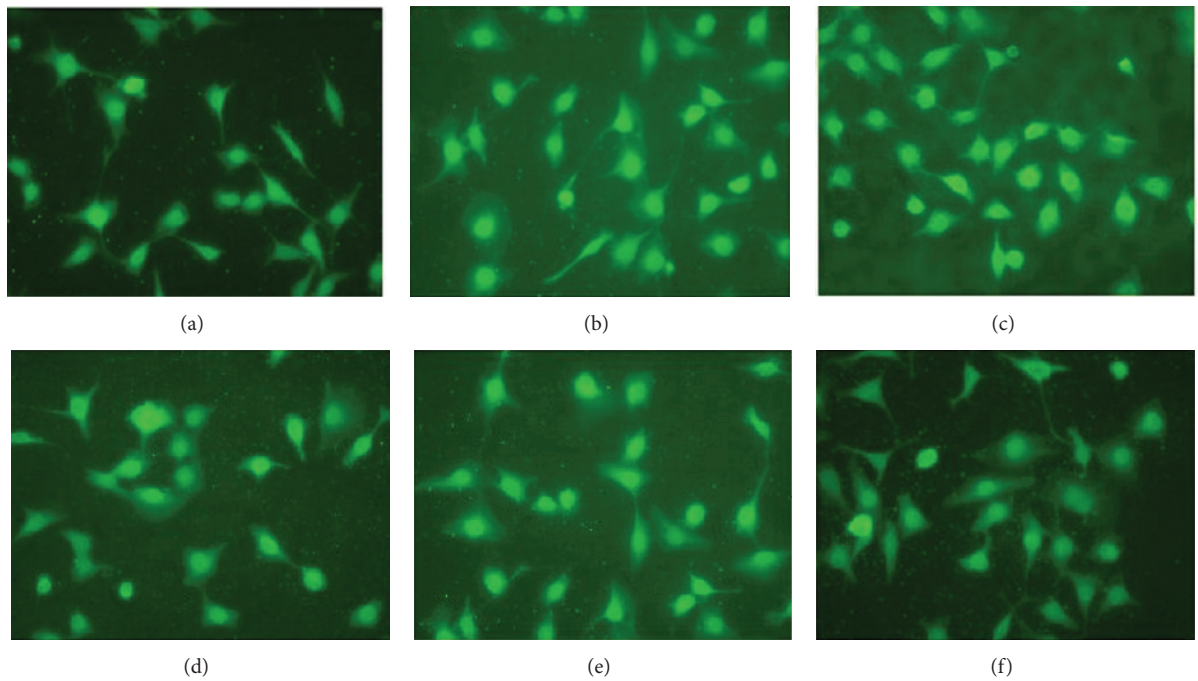

Figure 8: Immunofluorescence studies of EM2 on p38 MAPK in HUVECs. Cells were fixed and incubated with p38 MAPK antibody and a FITC-conjugated second antibody. Pictures were taken at 400x magnification. ((a) AGEs-BSA, (b) BSA, (c) 10 μM, (d) 1 μM, (e) 0.1 μM, (f) 10 nM).

(NOS), that is, eNOS, iNOS, and nNOS. eNOS is constitutively expressed and there is particularly continuous NO production during physiological conditions [21]. Our results suggested that incubation with AGEs (100 mg/L) for 24 h led to an significantly increase in the NO, iNOS production and a decrease in the secretion and the mRNA expression of eNOS compared to control group in HUVECs (Figures 3–5); these effects were strikingly reversed by EM1 and EM2 pretreatment, these findings were in line with some previous reports [22, 23]. It is well recognized that NO produced by eNOS is described as "low output" pathway whereas iNOS generates NO in a "high output" manner which causes cell or organ dysfunction and apoptosis [24]. Evidence indicated that NOS isoform expression (particularly iNOS) is altered and NO is oversupplied in some pathologic conditions [25, 26]. Based on our data, we inferred that AGEs can alter NOS isoform expression by decreasing eNOS expression and stimulating iNOS oversupplied in HUVECs, resulting in overproduction of NO, which was due to enhanced iNOS expression. EMs can enhance NOS activity by up-regulating eNOS expression and decreasing iNOS production, leading to a health production and bioavailability of NO. It is indicated that EMs can attenuate the dysfunction of NOS induced by AGEs in HUVECs. EM1 showed the same effects as EM2 in our observation. A large amount of studies reported that EMs can regulate NOS expression in mammalian cells, such as human bone marrow stromal cells [27], human macrophages [28], and mice peritoneal macrophages [29]; however, to our knowledge, there was no report in HUVECs.

ET-1, a member of potent vasoconstrictor polypeptide family, has been characterized as one of the most potent endogenous vasoconstrictors [30]; the balance between NO and ET-1 is critical for the regulation of vascular tone. Our study clearly indicated that EM1 and EM2 pretreatment down-regulated the mRNA expression and the plasma concentration of ET-1, which was up-regulated by AGEs-BSA (Figure 6). We think that EMs inhibited ET-1 expression through increased NO production which is synthesised by eNOS and decreased NO production which is synthesised by iNOS, leading to a balance between ET-1 and NO, potentially contributing to endothelial function.

It was shown earlier that p38 MAPK directly phosphorylates c-Jun [31, 32]; protein phosphatases and p38 MAPK interact in various cell systems and have been implicated in the regulation of diverse cellular responses together [33]. In HUVECs, AGEs induce differentiation accompanied by activation of ERK, JNK, and p38 MAPK pathways [34]. These datum are consistent with our observation that p38 MAPK is activated by AGEs (100 mg/L); furthermore, our study showed that p38 MAPK activation was weakened in EMs pretreated groups relative to AGEs groups. The results indicated that the rescue effect of EMs on the AGEs-induced injury may be mediated, at least in part, by the p38 MAPK pathway. It is acknowledged that the research means and methods we used in the study on signal pathway seemed too monospecific, which needs to be demonstrated by various experimental methods and different angles. That is our next working direction and emphasis in future.

In conclusion, endomorphins can attenuate the HUVEC dysfunction of synthesising and secreting NO, eNOS, iNOS, ET-1 induced by AGEs and may inhibited p38 MAPK signal pathway in nucleus stimulated by AGEs. These findings have partly revealed the molecular mechanism of endomorphins on protecting HUVECs from injuries induced by AGEs and thereby may provide the pharmacologic basis for the treatment of endothelial dysfunction in diabetes.

Acknowledgments

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Research Article

Serum Proteome Changes in Healthy Subjects with Different Genotypes of *NOS1AP* in the Chinese Population

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Type 2 diabetes and its chronic complications have become a worldwide epidemic nowadays. However, its molecular mechanism is still unknown. We have previously identified a novel variant rs12742393 of *NOS1AP* for type 2 diabetes susceptibility in the Chinese population. In this study, we analyzed the total serum profiling among three genotypes of rs12742393 to discover potential crosstalk under the variant and the disease through proteomic analyses for the first time. We used OFFGEL peptide fractionation, LC-MS/MS analysis, and label-free quantification to profile the fasting human serum samples of the genotypes in rs12742393 ($n = 4$, for CC, AC, and AA, resp.). Four proteins were identified, including apoA4, alpha1-ACT, HABP2, and keratin 10, with blood levels changed significantly between CC and AA homozygotes of rs12742393. Compared with AA group, the levels of apoA4 increased ($P = 0.000265$), whereas the concentration of alpha1-ACT, HABP2, and keratin 10 decreased in CC group ($P = 0.011116$, 0.021175, and 0.015661, resp.). Then we selected additional fasting serum samples for ELISA and western blot validation. However, no significant differences were identified by neither ELISA nor western blot ($P > 0.05$). The protein profiling changes between the genotypes of rs12742393 indicated that this SNP might play a role in the development of type 2 diabetes.

1. Introduction

Nitric oxide synthase 1 adaptor protein (NOS1AP), also named as CAPON, regulates the neuronal nitric oxide synthase (nNOS) activity and has an effect on nitric oxide (NO) release by binding N-methyl-d-aspartate receptors (NMDARs) [1]. Recent studies have shown that nNOS is also localized on insulin secreted granules in addition to neuronal tissues and can be activated by increasing intracellular calcium which is a known response to glucose stimulation on β cells [2, 3]. Several studies have suggested that both nNOS and NO are directly involved in insulin secretion as well as insulin resistance [4–7]. It was indicated that the interaction between nNOS and glucokinase (GCK) can affect GCK localization and activity and thus influenced glucose-stimulated insulin secretion (GSIS) in cultured β cells [4]. Furthermore, a novel mechanism for β-cell dysfunction has also been described in which nNOS, as a key protein linking cholesterol and glucose metabolism, can be dimerized to impair GCK activity and reduce GSIS on the insulin granules [8]. In addition, genetic studies have implied that the variations in *NOS1AP* are associated with reduced glucose lowering effect in sulfonylurea users as well as increased incidence of type 2 diabetes in patients taking calcium channel blockers [9, 10]. Though the studies on how the variants influenced the diseases were limited, one functional study showed that rs12742393 could affect *NOS1AP* gene expression through influencing transcription factor binding [11]. Our previous study showed evidence that rs12742393 in *NOS1AP* was involved in type 2 diabetes susceptibility in the Chinese population, with C

allele as the risk allele (OR 1.17, 95% CI 1.07–1.26; $P = 0.0005$) [12]. However, the association was not replicated in the European descent [13].

Recently, with the development of genomics and bioinformatics, proteome is widely used to describe all the proteins as well as their various modifications regarding the impact of environment and other stimuli within the whole body. Proteomics allows global screening of complex samples and provides qualitative and quantitative evidence for altered protein expression. Based on the information and initial data, we hypothesized that rs12742393 of *NOS1AP* might have an effect on the development of diabetes in the Chinese population. To test this hypothesis, we investigate the different protein profiling according to the different genotypes (AA, AC, and CC) of rs12742393 by proteomics technology.

2. Materials and Methods

2.1. Clinical Sample Collection and Preparation. Twelve healthy participants with normal glucose regulation were selected for proteomic investigation, including four CC homozygote, four AC heterozygote, and four AA homozygote individuals. However, we just selected CC and AA carriers for the final statistical analysis and validation, since they can be divided into two distinct groups based on the HCA and PCA. All the individuals for the proteomic analysis were matched strictly with age, sex, BMI, glucose, and lipid related parameters (HbA1c, fasting plasma glucose, OGTT-2 h glucose, triglyceride, total cholesterol, low density lipoprotein, and high density lipoprotein). Then forty-eight healthy participants with twenty-four CC homozygote and twenty-four AA homozygote carriers were included for western blot as well as ELISA validation. All these validation samples were matched with age, BMI, blood glucose, and lipid levels. Serum samples were collected in fasting state and then stored at −20°C before the analysis. The study was approved by the institutional review board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Written informed consent was obtained from each participant.

2.2. In-Solution Digestion and Peptide OFFGEL Fractionation. The crude plasma was diluted and treated by the albumin-removal manipulation with some modifications to collect plasma proteins [14, 15]. Then the protein mixtures were handled as described to obtain the total peptide mixtures for identification [16]. For p*I*-based peptide separation, we used the 3100 OFFGEL Fractionator (Agilent Technologies, Böblingen, Germany) with a 12-well setup. Electrofocusing of the peptides is performed at 20°C and 50 μA until the 100 kVh level was reached. All fractions were evaporated by centrifugation under vacuum and maintained at −20°C. Prior to MS analysis, samples were desalted by Empore C18 47 mm Disk (3 M).

2.3. Label-Free Shotgun Proteomic Identification. Each fractionated peptide was dissolved in 20 μL 0.1% formic acid and loaded into the RP column. RP-HPLC was performed using an Agilent 1100 Capillary system (Agilent Technologies) with C18 column (150 μm i.d., 100 mm length, Column Technology Inc., Fremont, CA). The mass spectral data were acquired on a LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA) equipped with an electrospray interface operated in positive ion mode. The mass spectrometer was set as one full MS scan was followed by ten MS/MS scans on the ten most intense ions from the MS spectrum with the following dynamic exclusion settings: repeat count, 2, repeat duration, 0.5 min, and exclusion duration, 1.5 min.

2.4. Database Searching and Protein Identification. All MS/MS spectra were searched using MASCOT algorithm against the human International Protein Index (IPI) database (version 3.73), in which each genuine protein sequence was followed by a reversed amino acid sequence. Carbamidomethylation (57.0214 Da) was searched as a fixed modification on cysteine, and oxidation (15.9949 Da) was set as a variable modification on methionine. Only one missing cleavage site was allowed. All output results were combined together using the in-house software named BuildSummary to delete the redundant data. Searches were conducted against the Human International Protein Index protein sequence database to control the false discovery rate at 1% and all spectral peptide count had a ΔCn score of at least 0.1. Finally, the NSFC (normalized spectral abundance factors) score was calculated for representing the abundance of each protein in the serum [17].

2.5. Western Blot Analysis of Four Proteins. We additionally collected forty-eight serum samples with twenty-four CC homozygote and twenty-four AA homozygote carriers for western blot validation. 2 uL of each individual serum sample diluted to 1/40 with 2*SDS was subjected to PAGE-gel electrophoresis, and then proteins in the gel were transferred to a nitrocellulose membrane (Whatman International Ltd., England). The membranes were incubated first with the appropriate primary antibodies overnight at 4°C (apolipoprotein A4 mouse mAb, #5700 from cell signaling technology; keratin 10 rabbit polyclonal Ab, ab97764 from Abcam Ltd., Cambridge, MA; antialpha1-antichymotrypsin mouse mAb, LF-MA0166, AbFrontier; anti-HABP2 rabbit polyclonal Ab, ab81490 from Abcam Ltd., Cambridge, MA, resp.) and then incubated with HRP-conjugated secondary antibodies for 1 h. Signals were detected by enhanced chemiluminescence system (ECL-plus, Amersham PharmaciaBiotech). Gray scale of the protein bands was calculated using Image J software. To decrease the system discrepancy, we used GAPDH with 1 : 1000 dilution (GAPDH rabbit mAb, cell signaling 14C10) as the loading control. Relative levels of target protein in the serum were calculated by the proportion of density ratio of sample bands to that of the loading control band.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis. Human apolipoprotein A4 (apoA4) ELISA kit (Cat. no. KT-50113, Kamiya Biomedical Company) and human alpha1-antichymotrypsin (alpha1-ACT) ELISA kit (Cat. no. KT-498, Kamiya Biomedical Company) were available to quantify

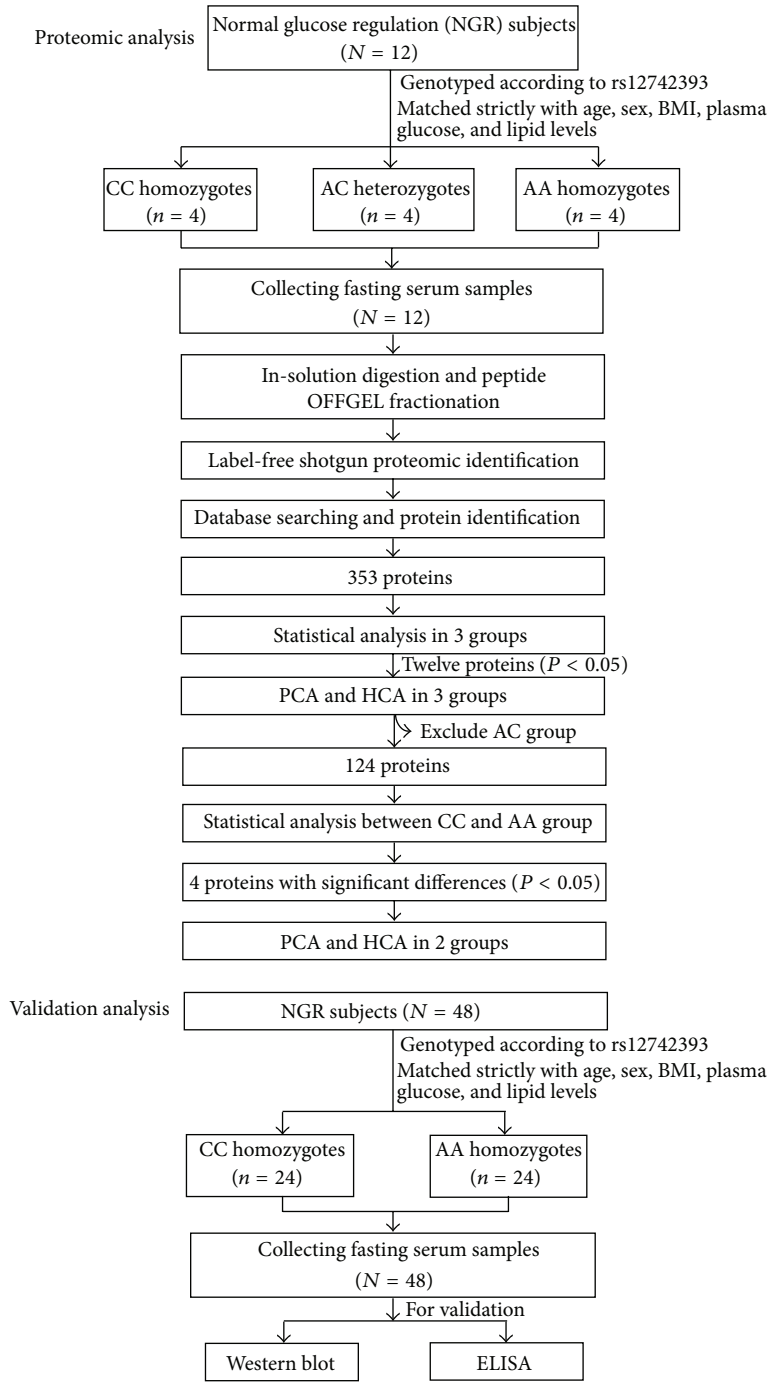

Figure 1: Flow chart of the study.

the protein levels. Original and 1 : 2500 diluted serum samples were prepared for apoA4 and alpha1-ACT detection, respectively, and then standard procedures were followed by the instructions of each ELISA kit. The absorbance of each sample at 450 nm was recorded using a Bio-Rad microplate reader model.

2.7. Statistical Analysis. Data were shown as mean ± standard error (str) for normally distributed values. Differences between groups for normally distributed variables were compared using two-tailed t-test. All calculations were performed with SAS (version 8.0; SAS Institute, Cary, NC). A P value below 0.05 was considered statistically significant.

3. Results

3.1. Semiquantitative Proteomic Identification in the Serum. We analyzed differential protein profile in three groups

Table 1: Clinical characteristics of the four CC carriers and four AA carriers.

	CC genotype (risk allele)	AA genotype (nonrisk allele)	P value
Male/female	4/0	4/0	>0.05
Age	54.0 ± 4.06	54.25 ± 0.48	0.9550
BMI (kg/m^2)	26.28 ± 0.83	26.38 ± 0.56	0.9244
FPG (mmol/L)	5.01 ± 0.15	5.01 ± 0.17	0.9831
2 h glucose (mmol/L)	5.32 ± 0.33	5.68 ± 0.44	0.5413
HbA1c (%)	5.7 ± 0.12	5.8 ± 0.29	0.7620
TC (mmol/L)	4.98 ± 0.40	5.0 ± 0.38	0.9652
TG (mmol/L)	1.74 ± 0.39	2.63 ± 0.28	0.1136
HDL (mmol/L)	1.01 ± 0.11	1.10 ± 0.07	0.5329
LDL (mmol/L)	3.23 ± 0.31	3.24 ± 0.35	0.9919

Data were shown as mean ± str.
FPG: fasting plasma glucose; HbA1c: glycated hemoglobin A1c; TC: total cholesterol; TG: triglyceride; HDL: high density lipoprotein; LDL: low density lipoprotein.

using shotgun proteomics and label-free quantitative strategy (Figure 1). The proteins were identified with criteria corresponding to an estimated false discovery rate of 1%. After combining the MS/MS data generated from all experiments, 62,523 peptide counts leading to identification of 1,725 unique peptides were assigned to 353 protein groups in twelve serum samples. For semiquantitative analysis, protein identified at least in seven samples was selected in our data.

3.2. HCA and PCA Presentation. To visualize the global pattern related to type 2 diabetes, we used HCA and PCA in this study. As shown in Figure 2, HCA and PCA can completely divide the CC and AA carriers into two distinct groups. Therefore, we excluded AC group and only compared the other two groups (CC and AA homozygotes) to investigate the different protein profiling. Finally, 124 proteins were selected for statistical analysis and further validation between CC and AA groups (see Supplementary material (Table 1) available online at http://dx.doi.org/10.1155/2013/357630).

3.3. Clinical Data. Twelve subjects were selected for the proteomic analysis, but only eight subjects with four CC carriers and four AA carriers were selected for further validation based on the PCA and HCA results (Table 1). Additional forty-eight samples were selected for western blot and ELISA validation, with twenty-four CC carriers and twenty-four AA carriers (Supplementary Table 2).

3.4. Statistical Analysis for Significantly Changed Proteins. To obtain significantly changed proteins related to diabetes, the 124 proteins were ranked based on the quantitative data and showed that four proteins including apolipoprotein A4 (apoA4), alpha1-antichymotrypsin (alpha-1-ACT), keratin 10, and hyaluronan-binding protein 2 (HABP2) had a significant difference ($P < 0.05$) between AA and CC groups. Compared with AA group, the levels of apoA4 increased ($P = 0.000265$),

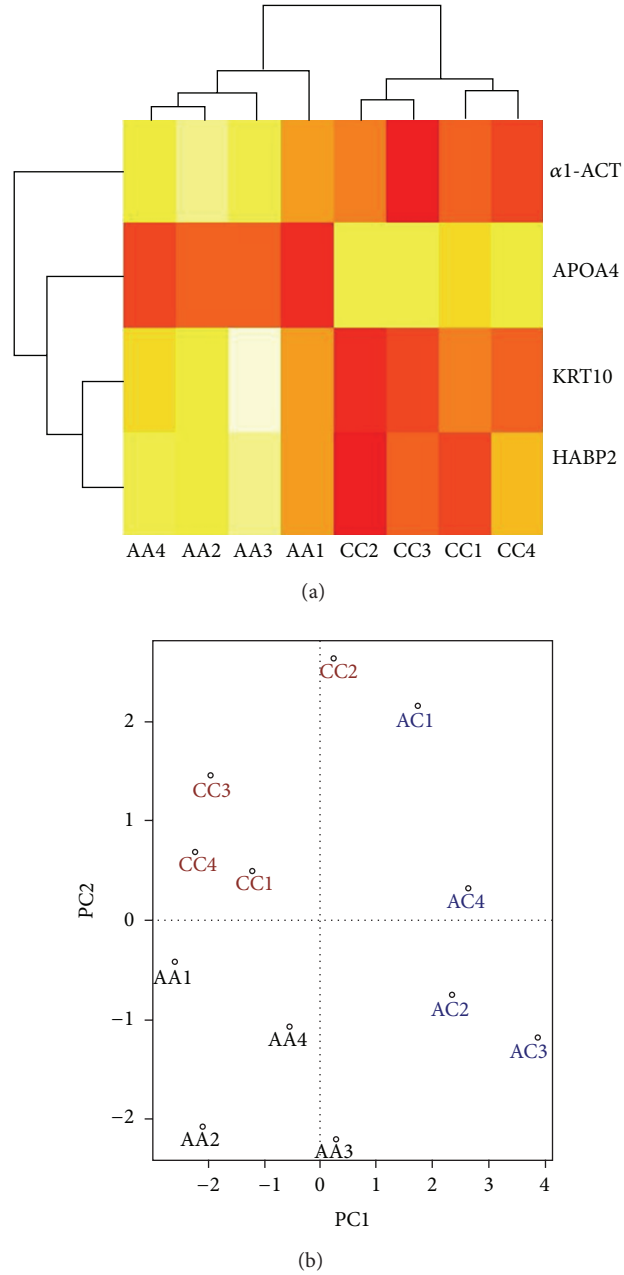

Figure 2: Global relationships were visualized by performing "hierarchical cluster analysis" (HCA (a)) and "principal component analysis" (PCA, (b)) according to the significantly changed proteins. AA1-AA4 and CC1-CC4 represent the eight samples for proteomics analysis.

whereas the concentration of alpha1-ACT, keratin 10, and HABP2 decreased in CC group ($P = 0.011116$, 0.015661, and 0.021175, resp.). These four proteins are involved in the lipoprotein metabolism, acute inflammatory response, and epidermis development as well as cell adhesion (Table 2).

3.5. Semiquantification Analysis by Western Blot. ApoA4, alpha1-ACT, keratin 10, and HABP2 were validated by western blot in additional serum samples from twenty-four CC

Table 2: Identification of proteins with significant differences between CC carriers and AA carriers based on NSAF value.

IPI ID	Protein name	CC carriers mean NSAF	AA carriers mean NSAF	P value
00009865.4	Keratin10	−8.038	−6.938	0.015661
00847635.1	Alpha-1-antichymotrypsin	−6.037	−5.501	0.011116
00304273.2	Apolipoprotein A4	−8.339	−11.107	0.000265
00746623.2	Hyaluronan-binding protein 2	−9.435	−8.623	0.021175

NSAF: normalized spectral abundance factors.

homozygotes and twenty-four AA homozygotes. The integrated densities of all the target proteins were normalized by GAPDH. No significant differences were replicated in any of the four proteins by western blot. However, the differences of keratin 10 showed some trend between CC and AA groups ($P = 0.067$).

3.6. Quantification Analysis by ELISA. In order to determine the levels of four proteins as identified by LTQ, ELISA was used to analyze human apoA4 and alpha1-ACT levels (ELISA kits available) in expanded serum samples ($n = 48$, the same as used in western blot). All the absorbance was adjusted by the standard blank calibrate sample and the target protein concentrations were calculated according to the formula derived from the standard calibrators. However, no significant differences were observed in either of the two proteins between the two groups.

4. Discussion

In this study, the RP-HPLC-MS/MS coupled with quantitative analysis strategy was applied to investigate the different protein profiling of rs12742393 genotypes in *NOS1AP*. The present study investigated the possible function of *NOS1AP* using proteomic analysis for the first time.

Our data showed an increasing level of the apoA4 in the CC homozygote carriers, while it showed decreasing levels of alpha1-ACT HABP2 as well as keratin 10 compared with AA homozygote carriers. These proteins were involved in lipoprotein metabolism, acute phase of inflammation or infection, and cell proliferation or migration, as well as epidermis development, which might contribute to the development of diabetes.

Apolipoprotein A4 (apoA4), secreted by small intestine in response to fat absorption, contributed a lot to lipid absorption, reverse cholesterol transport [18], anti-inflammatory response, [19] and, particularly, glucose homeostasis. For example, apoA4 had a direct effect on enhancing glucose-stimulated insulin secretion in pancreatic islets. ApoA4 knockout mice also showed impaired glucose tolerance and insulin secretion while exogenous apoA4 administration to apoA4$^{-/-}$ mice could improve glucose tolerance through increasing insulin secretion [20]. Moreover, genetic variants in apoA4 also indicated an association with fasting glucose levels [21]. Therefore, apoA4, the only upregulated protein in the risk allele carriers in our research, might have an association with type 2 diabetes through the interaction with NOS1AP (or nNOS). The mechanism should be elucidated by other functional studies.

Alpha1-antichymotrypsin (alpha1-ACT) is an acute phase protein produced in the liver, which is induced during inflammation response. Elevating levels of alpha1-ACT were observed in the hepatocellular carcinoma patients using 2D-LC MALDI-MS/MS [22]. Moreover, recent studies have confirmed that IL-1β could modify IL-6-induced acute phase protein production, such as alpha1-ACT, through a complex intracellular crosstalk between STAT3 and NF-κB-mediated signal transductions [23]. This evidence might provide us some new perspectives to find the internal links between alpha1-ACT and diabetes.

Hyaluronan-binding protein 2 (HABP2) is an HA-binding extracellular serine protease, which is involved in the extrinsic pathway of blood coagulation and fibrinolysis. Although HABP2 is mainly produced in the liver, many studies have demonstrated its expression in pulmonary endothelium cells, which contributed a lot to the lung injury [24–27]. Up till now, no direct evidence has linked HABP2 with diabetes; however, its ligand, hyaluronan, has been suggested to interact with CD44 and PKC and reduce inflammation in diabetic nephropathy mice [28]. Our data showed a decreased HABP2 level in CC carriers, which might give us some hints to find the crosstalk between this protein and diabetes.

Keratin 10 is a member of the type I (acidic) cytokeratin family, which belongs to the superfamily of intermediate filament (IF) proteins. Due to its classic role in the epidermis development, mutations in *KRT10* were associated with epidermolytic hyperkeratosis [29, 30]. However, keratin 10 also had an interaction with both protein kinase B (PKB) and atypical PKCδ, which could impair the activation of the kinase and inhibit their intracellular translocation [31]. As we know, PKB and PKC pathways could regulate glucose homeostasis; thus, the impairment of PKB and PKCδ by keratin 10 might induce potential metabolic disorders [32, 33]. Further research should be performed to elucidate the mechanism.

Overall, it was the first time to investigate the potential function of susceptible variant to type 2 diabetes using proteomic technology. We found four proteins that might associate with the mechanism of diabetes. However, the intrinsic interaction between these proteins and NOS1AP and the mechanism by which rs12742393 in *NOS1AP* mediated still need further research to elucidate. In addition, the results of proteomic analysis were not replicated in western blot or ELISA in the expanded samples; thus, we could not totally exclude the "false positive" of proteomic analysis. The reasons

that we did not validate the results in neither western blot nor ELISA were probably due to the minor differences in clinical characteristics between the two sets of subjects (subjects for proteomic analysis and for validation analysis). For example, BMI of the subjects in the proteomic analysis (mean BMI > 26 kg/m^2) is higher than that in the validation analysis (mean BMI < 24 kg/m^2); thus, it might have an effect on the glucose and lipid metabolism potentially. In addition, sex should be also taken into consideration in the analysis. Therefore, we should match the glucose and lipid related traits more strictly to exclude the interpretation besides increasing the sample size. However, as what we described in the method, the quality controls of the proteomic analysis were strict; thus, we believed that the proteins that showed significant differences between the two groups were not just an incidental result. Therefore, it might be due to the different characteristics between the two sets of subjects and limited samples in the validation analysis. More samples should be involved in the study to perform the validation test.

We detected four proteins showing significant differences between CC and AA carriers of rs12742393 in *NOS1AP*. Although we failed to validate these differences in the large sample cohort, we suggested that these four proteins might associate with the development of type 2 diabetes in subgroup of patients through the crosstalk with NOS1AP protein, which might provide us a new perspective to the mechanism of type 2 diabetes.

Conflict of Interests

There is no conflict of interests.

Authors' Contribution

Feng Jiang and Congrong Wang contributed equally to this paper.

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Clinical Study

Effects of Glucose Load and Nateglinide Intervention on Endothelial Function and Oxidative Stress

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We analysed endothelial function and oxidative stress in patients with abnormal glucose metabolism, the effect of glucose load, and the impact of nateglinide. 109 participants were grouped into newly diagnosed diabetes, prediabetes, and control. Fasting plasma glucose (FPG), postprandial plasma glucose (PPG), glycosylated haemoglobin (HbA_{1c}), and glycated albumin (GA) varied significantly among the study groups ($P < 0.01$). Nitric oxide (NO) and insulin resistance index (HOMA-IRI) levels were markedly different between the newly diagnosed diabetes and the control ($P < 0.01$). Glucose loading lowered flow-mediated endothelium-dependent dilation (FMEDD), NO, and superoxide dismutase (SOD) ($P < 0.01$). Fasting and glucose loading FMEDD, FPG, PPG, HbA_{1c}, and GA were negatively correlated ($r = -0.4573, -0.4602, -0.3895, -0.3897$, and $r = -0.4594, -0.4803, -0.4494, -0.3885$; $P < 0.01$), whereas NO, SOD, and HOMA-β were positively correlated ($r = 0.2983, 0.3211, 0.311$, and $r = 0.1954, 0.361, 0.2569$; $P < 0.05$). After the treatment with nateglinide, significant decreases in FPG, PPG, GA, HbA1C, endothelin-1(ET-1), malondialdehyde (MDA), and HOMA-IRI were observed, whereas FMEDD, NO, and SOD increased ($P < 0.01$). Thus, the study demonstrated the adverse effect of glucose load on endothelial function and oxidative stress. Nateglinide lowers blood glucose, reduces insulin resistance and oxidative stress, and improves endothelial function in newly diagnosed diabetes.

1. Introduction

The incidence of diabetes is increasing at an alarming rate worldwide. The prevalence of diabetes, reported as about 346 million by WHO, is predicted to advance further in the future if not treated [1]. Diabetes, a chronic metabolic disorder characterised by hyperglycaemia and glucose intolerance, is associated with both macrovascular and microvascular complications [2]. Diabetes-induced vascular complications such as cardiovascular, cerebrovascular, and peripheral vascular diseases adversely affect the life expectancy and quality of life of the patients.

At the cellular level, endothelial and vascular smooth cell dysfunction along with abnormal coagulation system is associated with the pathogenesis of diabetes-linked vascular complications [3]. Among these factors, vascular endothelial dysfunction is a key pathological factor in promoting diabetic vascular complications. Oxidative stress, one of the major mechanisms responsible for vascular endothelial dysfunction, is mediated through increased reactive oxygen species (ROS). Elevated ROS is generated through diverse biochemical pathways impacted by hyperglycaemic conditions [4]. In normal vascular endothelium, regulatory mediators, such as NO, prostanoids, endothelin-1(ET-1), and angiotensin II, ensure vascular homeostasis [5]. However, ROS scavenges NO, thus limiting its availability for vascular homeostasis [6]. Compromised endothelial regeneration and angiogenesis processes ultimately lead to diabetes-associated micro- and macrovascular complications [7].

While studying the effect of glucose fluctuations on oxidative stress in patients with diabetes, it was observed that there was an increased triggering of oxidative stress

under conditions of glucose fluctuations or glucose swings when compared with chronic sustained hyperglycaemia [8]. Glycaemic variability is being related to the occurrence as well as the increase in severity of coronary artery disease (CAD) in type 2 diabetic patients [9]. Fluctuations in FPG within the normal limits in nondiabetic healthy individuals are associated with aggravated arterial stiffness [10]. However, Major-Pedersen et al. (2008) reported that oral glucose load does not cause endothelial dysfunction in healthy individuals when the mean glucose level is at 5.6 mmol/L and insulin is at 27.2 mmol/L, at 2 hours after glucose [11]. Nevertheless, a recent study has shown that oral glucose load attenuated flow-mediated dilation in brachial artery of healthy individuals [12].

Progression of type 2 diabetes is characterised by loss of early insulin secretion, leading to postprandial hyperglycaemia. This ultimately leads to insulin resistance [5]. Usage of an insulin secretagogue can selectively enhance early meal-induced insulin secretion, resulting in improved postprandial hyperglycaemia, thus preventing type 2 diabetes. Nateglinide, an insulin secretagogue, has been shown to selectively enhance early meal-induced insulin secretion, leading to meal time glucose control. An earlier study demonstrated that nateglinide can effectively normalise the glycaemic response curve after an oral glucose load in patients with impaired glucose tolerance (IGT) [13].

In the light of these earlier observations, the objective of the present study was to detect vascular endothelial function and oxidative stress levels in patients with abnormal glucose metabolism and to understand the changes after the glucose load. The study also aimed at analysing the impact of nateglinide on vascular endothelial function and oxidative stress in newly diagnosed patients with diabetes.

2. Patients and Methods

2.1. Study Population and Study Design. The study protocol was approved by the Ministry of Health Beijing Hospital Ethics Committee. Informed consent was obtained from all the participants.

The study participants were recruited from Beijing Hospital Internal Medicine, Endocrinology Clinic from June to December of 2010. Inclusion criteria were no previous history of (1) diabetes or impaired glucose regulation (2) hypertension, and (3) dyslipidemia. Subjects were aged between 35 and 65 years (average age of 52.62 ± 8.51 years), of which 49 were male and 60 were female. All subjects were unrelated and belonged to Han Dynasty. Exclusion criteria were (1) diabetes with acute complications such as diabetic ketoacidosis, diabetic lactic acidosis, hyperosmolar nonketotic hyperglycaemic coma and hypoglycaemia syndrome, (2) secondary diabetes, (3) hypertension, coronary heart disease, dyslipidaemia, vascular inflammation, vascular stenosis and atherosclerotic peripheral vascular lesions, (4) patients with abnormal renal function (serum creatinine > 178 μmol/L, blood urea nitrogen > 9 mmol/L) and abnormal liver function (transaminases greater than twice the normal), (5) serious autoimmune and blood diseases, (6) presence of fever, infectious diseases as well as dementia and other mental illnesses, (7) current use of antihypertensive drugs, lipid lowering drugs and other drug, which affect endothelial function, (8) patients who refused treatment and/or comprehensive review after 4 weeks. 109 participants in the recruitment met the above the criteria. All participants underwent oral glucose tolerance tests (OGTT), conducted flow-mediated endothelium-dependent dilation (FMEDD), and detected other indicators.

2.1.1. Data Collection. Patients' demographics, vital statistics (height, weight, body mass index (BMI), blood pressure) and medical history were collected from all participants. Fasting plasma glucose (FPG), postprandial plasma glucose (PPG), glycosylated haemoglobin (HbA_{1c}), glycated albumin (GA), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), creatinine (CRE), alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid (UA), serum insulin (INS), serum C-peptide nitric oxide (NO), endothelin-1(ET-1), malondialdehyde (MDA), and superoxide dismutase (SOD) were detected in all participants.

2.1.2. Diagnostic Criteria for Diabetes. Diagnosis of diabetes was based on the WHO specified diagnostic criteria (2006). On the basis of the results of the oral glucose tolerance test (OGTT), patients were diagnosed to have either diabetes (FPG ≥ 7.0 mmol/L or 2 h plasma glucose (PG) ≥ 11.1 mmol/L), IGT (2 h PG 7.8–11.0 mmol/L), or impaired fasting glucose (IFG) regulation (OGTT FPG 6.1–6.9 mmol/L) [14]. Normal glucose metabolism was defined by OGTT values 6.0 mmol/L or less (FPG) and 7.7 mmol/or less (2 h PG). On the basis of OGTT results, 109 participants were separated into 3 groups, namely, newly diagnosed type 2 diabetes group (n = 43; 25 males and 18 females with mean age of 53.40 ± 8.99 years), prediabetes group (n = 33; 12 males and 21 females with mean age of 52.88 ± 9.20 years), and control group (n = 33; 12 males and 21 females with mean age of 51.36 ± 7.15 years).

Among the newly diagnosed diabetic patients, 32 (19 males, 13 females, mean age of 54.94 ± 7.41 years) patients who signed informed consent were given nateglinide (120 mg tid), 3 patients were provided diet management and exercise, 3 were administered intensive insulin therapy, 3 other patients were on other antidiabetic drug therapy and 2 patients were lost to follow-up. In the newly diagnosed type 2 diabetes group, medication compliance was 98.10%.

Only the 32 diabetic patients treated with nateglinide had a repeat of all measurements, and the weight of the 32 diabetic patients was measured after 4 weeks of treatment.

2.1.3. Treatment Protocol. 32 diabetic patients were treated with nateglinide at a dosage of 120 mg tid. Analyses regarding the changes of endothelial function and oxidative stress in these patients were compared pre- and posttherapy after 4 weeks of treatment interval.

2.2. Laboratory Methods. The various biochemical tests performed included FPG, PPG, total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), creatinine

(CRE), alanine aminotransferase (ALT), aspartate aminotranferase (AST), uric acid (UA), serum insulin (INS), serum C-peptide, and glycosylated haemoglobin (HbA1c). These tests were carried out in biochemical laboratory. Determination of glycated albumin (GA) was performed using a test kit provided by the Japanese Asahi Kasei company.

Other biochemical tests included NO estimation by nitrate reductase method, MDA by thiobarbituric acid (TBA) method, total superoxide dismutase (SOD) by xanthine oxidase method, and ET-1 by enzyme linked immunosorbent assay (test kit from Nanjing Jiancheng Bioengineering Institute and the American R and B Company; kit sensitivity: 0.5 μg/L).

2.3. Flow-Mediated Endothelium-Dependent Dilation (FMEDD). FMEDD was performed using Philips (Italy) Philips IU22 high-resolution multifunction colour Doppler ultrasonic diagnostic apparatus with a probe frequency of 4–8 MHz. Subjects were asked to rest comfortably in supine position at least 10 min before the test, with the right arm above the elbow for imaging the brachial artery 2–15 cm above the antecubital crease [15]. The longitudinal section image was taken by adjusting the probing depth and gaining recognition to measure the brachial artery diameter as *D0* (simultaneous to electrocardiographic *R* wave).

2.4. Reactive Hyperaemia Test. Blood pressure cuff was tied on the forearm and was inflated to 280 mmHg for 5 min. Reactive hyperaemia results by deflation during which brachial artery image is recorded after 50 s to 60 s and at 90 s, as brachial artery diameter (*D1*). Reactive hyperaemia (in percentage) is calculated as brachial artery diameter after expansion as follows:

$$\text{FMEDD} = \frac{(D1 - D0)}{D0} \times 100\% \quad (\text{normal reference values } (11 \pm 2)\%), \tag{1}$$

see [16].

During the entire testing process, the ultrasonic probe was used at a fixed location by the same ultrasound professional who used the same testing equipment to operate. The persons measuring the above measurements did not know which group the patients belonged to.

2.5. Insulin Sensitivity and β-Cell Function Evaluation. HOMA of insulin sensitivity and basal β-cell function are calculated as

$$\text{HOMA-IRI} = \frac{\text{FPG} \times \text{fasting insulin (Fins)}}{22.5}, \tag{2}$$

see [17]. Assume

$$\text{HOMA } \beta\text{-cell function}\,(\text{HOMA-}\beta) = \frac{20 \times \text{Fins}}{(\text{FPG} - 3.5)}. \tag{3}$$

2.5.1. Glucose Load. Various tests were measured both while fasting and 2 hours after the consumption of 75 g oral glucose.

2.6. Statistical Analysis. Data entry was performed using epidata3.0 and double parallel entry. All data were analysed by SAS9.1.2 software. Results of continuous variables were presented as mean ± standard deviation. For comparisons between the 3 groups, analysis of variance (ANOVA) was used in case of normal distribution and homogeneity of variance between groups. Nonparametric test methods were used for incomplete variance and in case of not meeting the normal distribution. Multiple comparison methods were used to check if the differences between the two groups were statistically significant. Repetitive measure ANOVA was used for comparison between fasting and 2 hours after glucose load. To compare pre- and post-treatments of newly diagnosed type 2 diabetes, the single sample *t*-test was used if the difference met the normal distribution; signed rank test was used if the difference did not meet the normal distribution. Pearson's and Spearman's correlation analyses were used to examine correlations between the various indicators. Multiple comparison methods were used to check statistical significance in differences between the two groups. For all analyses, *P* value of less than .05 was considered to be statistically significant.

3. Results

3.1. Analysis of Clinical Metabolic Parameters in Different Glucose Metabolism Status and after Glucose Load. BMI, liver, and kidney functions were comparable among prediabetes, newly diagnosed type 2 diabetes, and control groups ($P > .05$). Although ALT and AST values were non-significantly elevated in newly diagnosed type 2 diabetes groups when compared with the control group, uric acid level was significantly increased in the former group. ($P < .05$) (Table 1).

Statistically significant differences were observed in FBG, PPG, DPG, HbA_{1c}, and GA among the control group, prediabetes group, and newly diagnosed type 2 diabetes group ($P < .001$). However, increase in total cholesterol, TG, and LDL-C was not significantly different in these 3 groups ($P > .05$). HDL-C and HOMA-IRI were significantly different between newly diagnosed type 2 diabetes group and control group ($P < .05$) (Table 2).

Both at fasting and at 2 hours after glucose load, FMEDD and SOD showed a significant difference among the prediabetes, newly diagnosed type 2 diabetes, and control groups ($P < .01$). NO was significantly lowered in both prediabetic and newly diagnosed type 2 diabetes group at fasting and 2 hours after glucose load, when compared with control group ($P < .01$). In addition, fasting plasma NO was significantly decreased in newly diagnosed type 2 diabetes when compared with prediabetes group ($P < .01$). Among the three groups, FMEDD, NO, and SOD levels were significantly decreased after the glucose load when compared with fasting level ($P < .01$). However, the levels of MDA and ET-1 at fasting and 2 hours after glucose load did not differ significantly between the 3 groups. ($P > .05$) In the control group and the newly diagnosed type 2 diabetes group, ET-1 and MDA increased significantly ($P < .01$) (Table 3). Additional data on correlation analysis is provided in Online Resource 1.

Table 1: Analysis of the general clinical data.

Group	Control group	Prediabetes groups	Newly diagnosed type 2 diabetes group	F/χ^2 value	P value
Number of patients (M/F)	33 (12/21)	33 (12/21)	43 (25/18)		
Age (years)	51.36 ± 7.15	52.88 ± 9.20	53.40 ± 8.99	1.78[a]	.411
BMI	24.76 ± 3.60	25.75 ± 3.13	25.69 ± 2.93	2.55[a]	.280
ALT	25.58 ± 6.84	26.91 ± 12.00	31.49 ± 14.21	3.30[a]	.192
AST	26.03 ± 5.64	26.12 ± 7.55	28.74 ± 10.35	1.07[a]	.586
CRE	65.30 ± 10.44	66.19 ± 13.96	65.60 ± 13.32	0.04	.959
UA	302.21 ± 73.79	324.12 ± 74.42	348.60 ± 84.44	3.31	.040*

M/F: male/female; BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CRE: creatinine; UA: uric acid.
[a]Nonparametric analysis method was used.
*Significant between newly diagnosed type 2 diabetes and control groups.

Table 2: Analysis of relative biochemical indicators.

Group	Control group	Prediabetes groups	Newly diagnosed type 2 diabetes group	F/χ^2 value	P value
Number of patients (M/F)	33 (12/21)	33 (12/21)	43 (25/18)		
FPG	5.43 ± 0.45	6.08 ± 0.53	7.54 ± 1.17	72.87[a]	<.001*
PPG	6.49 ± 0.89	8.80 ± 1.09	14.55 ± 3.19	90.78[a]	<.001*
DPG	1.19 ± 0.59	2.72 ± 1.31	7.01 ± 2.45	78.85[a]	<.001*
HbA_1C (%)	5.96 ± 0.31	6.50 ± 0.01	7.40 ± 0.97	67.02[a]	<.001*
GA	12.73 ± 1.00	13.71 ± 0.93	16.59 ± 1.81	75.93[a]	<.001*
TC	5.48 ± 0.55	5.52 ± 0.97	5.84 ± 1.74	2.44[a]	.296
TG	1.51 ± 0.49	1.80 ± 0.90	2.16 ± 1.17	5.78[a]	.056
LDL-C	3.07 ± 0.61	3.14 ± 0.75	3.23 ± 0.71	0.48	.618
HDL-C	1.36 ± 0.39	1.24 ± 0.26	1.17 ± 0.26	6.27[a]	.043**
HOMA-IRI	1.85 ± 1.43	2.69 ± 2.15	3.48 ± 2.74	7.68[a]	.022**
HOMA-β	80.49 ± 59.30	81.45 ± 66.45	52.99 ± 41.58	5.35[a]	.069

FPG: fasting plasma glucose; PPG: postprandial plasma glucose; DPG: diphosphoglycerate plasma glucose; HbA_1C: glycosylated haemoglobin; GA: glycated albumin; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; HOMA-IRI: homeostasis model method of assessment-insulin resistance index; HOMA-β: homeostasis model method of assessment β-cell function.
[a]Nonparametric analysis method used.
*Significant among the 3 groups.
**Significant between newly diagnosed type 2 diabetes and control group.

The effect of nateglinide treatment on vascular endothelial function and oxidative stress in patients with newly diagnosed type 2 diabetes was also analysed. FPG, PPG, DPG, HbA1C, GA, and HOMA-IRI were significantly decreased after nateglinide therapy ($P < .001$); LDL-C also decreased significantly after nateglinide treatment ($P < .05$). Significant decrease in the levels of ALT and AST were also recorded after treatment ($P < .05$). The decrease in TC, TG, and CRE and the increase in HDL-C and HOMA-β after treatment were not significant ($P > .05$) (Table 4).

Treatment with nateglinide significantly increased FMED, NO, and SOD levels both at fasting and 2 hours after glucose load in patients newly diagnosed with type 2 diabetes, ($P < .001$) However, ET-1 and MDA decreased significantly after treatment ($P < .001$). After the treatment, the average weight had no significant difference comparing with the previous ($P > .05$) (Table 5).

4. Discussion

4.1. Clinical Metabolic Parameters under Different Glucose Metabolism. In obese and overweight population, impairment of endothelial function appears even before the signs of metabolic disorders and cardiovascular diseases are evident [18]. A dose-response relationship between BMI and FMEDD has been demonstrated [19]. However, in the present study, BMI was observed to be not significantly different among the study groups. Although FMEDD decreased with increase in BMI, the difference was not statistically significant ($P > .05$). Thus, the present study did not demonstrate the influence of BMI on endothelial function among the prediabetic, type 2 diabetic, and normal individuals. Oxidative stress and endothelial dysfunctions are observed to increase with high uric acid concentration [20]. Our study too demonstrated significant increase in the levels of uric acid in patients with

Table 3: Differences between FMEDD, NO, ET-1, MDA, after SOD after glucose load.

Group		Control group	Prediabetes groups	Newly diagnosed type 2 diabetes group	F/χ^2 value	P value
Number of patients (M/F)		33 (12/21)	33 (12/21)	43 (25/18)		
FMEDD	Fasting	17.79 ± 3.91	15.91 ± 4.40	12.16 ± 2.93	28.81[a]	<.001*
	After glucose load	14.07 ± 3.15	11.99 ± 4.37	9.42 ± 3.18	16.09	<.001*
	P value	<.001	<.001	<.001		
NO	Fasting	174.76 ± 67.25	153.69 ± 61.67	128.02 ± 52.91	12.28[a]	.002***
	After glucose load	153.63 ± 60.06	138.13 ± 60.79	117.90 ± 48.25	9.43[a]	.009**
	P value	<.001	.0006	<.001		
ET-1	Fasting	1.44 ± 1.51	1.62 ± 1.29	1.93 ± 1.79	1.87[a]	.392
	After glucose load	1.66 ± 1.54	1.54 ± 1.47	2.13 ± 1.86	2.17[a]	.338
	P value	<.001	.3878	.0046		
MDA	Fasting	5.41 ± 1.32	5.72 ± 1.18	5.76 ± 1.29	0.8	.454
	After glucose load	6.03 ± 1.62	6.14 ± 1.64	6.31 ± 1.65	0.49[a]	.783
	P value	<.001	.1938	.0003		
SOD	Fasting	169.24 ± 22.35	143.94 ± 17.37	118.51 ± 17.26	63.73[a]	<.001*
	After glucose load	159.85 ± 22.57	127.88 ± 14.24	100.93 ± 17.37	77.17[a]	<.001*
	P value	.0052	.0011	.0002		

FMEDD: flow-mediated endothelium-dependent dilation; NO: nitric oxide; ET-1: endothelin-1; MDA: malondialdehyde; SOD: superoxide dismutase.
[a]Using nonparametric analysis method.
*Significant among the 3 groups.
**Significantly different in newly diagnosed type 2 diabetes and control groups.
***Significantly different in newly diagnosed type 2 diabetes and control groups, prediabetes groups, and newly diagnosed type 2 diabetes group.

diabetes compared with the control population. FMEDD was also significantly decreased in patients with diabetes. In addition, uric acid showed a significant negative correlation with fasting SOD and SOD after glucose load. These results suggest a role for uric acid in oxidative stress in patients with diabetes. People with dyslipidaemia, hypertension, and so forth were excluded in this study, it will lead to a somewhat selected population of patients with T2DM and does not represent the majority of the population of diabetic patients who often have high blood pressure as well as hyperlipidaemia. Because hypertension, coronary heart disease, dyslipidaemia, vascular inflammation, vascular stenosis, and atherosclerotic peripheral vascular lesions could affect the vascular endothelial function, the patients with dyslipidaemia, hypertension, and so forth were excluded in order to avoid confounding factors.

Significant differences in the levels of plasma glucose (FPG, PPG, DPG), HbA1C, and GA among the study groups demonstrate that these parameters reflect the state of glucose metabolism in an individual. Dyslipidaemia is one of the main factors responsible for endothelial dysfunction [21]. Increase in TG is associated with increased free fatty acid (FFA) production resulting in rise in mitochondrial ROS production. Moreover, reduction of intracellular GSH antioxidant defence system with increase in FFA further increases oxidative stress [22]. However, since in this study, patients with abnormal blood lipid profile were excluded, increase in the levels of TC, TG, and LDL-C, although not significant, suggests that endothelial function and oxidative stress are influenced by dyslipidaemia.

4.2. Glucose Load on Oxidative Stress and Endothelial Function. Many clinical ultrasound data have shown that, for early diabetes, FMEDD was impaired both while fasting as well as after glucose load but nitroglycerin-mediated endothelium-independent dilation (NMEID) had no significant differences when compared with normal subjects [23, 24]. The fact that nonendothelium-dependent vascular dilation function and vascular smooth muscle cell functions are normal in early diabetic people suggests that endothelial dysfunction precedes vascular structural changes during the progress of diabetic vascular disease in chronic diabetes. In this study, FMEDD (fasting and 2 hours after the glucose load) was negatively correlated with FPG, PPG, HbA1C, GA, and positively correlated with NO, SOD, and HOMA-β. This suggests that blood glucose levels can impact endothelial dysfunction. At the same time, a significant correlation between FMEDD, NO, and SOD indicates the strong effect of NO and antioxidative stress on endothelium-dependent vasodilation. The negative correlation between FMEDD (2 hours after glucose load) and LDL-C may be due to increased oxidative stress associated with the acute blood glucose fluctuations. This is supported by earlier findings that increased oxidative stress is triggered in diabetic patients under conditions of glucose fluctuations or glucose swings when compared with

Table 4: Comparison of biochemical parameters in newly diagnosed type 2 diabetes before and after treatment with nateglinide.

Group	Before taking nateglinide	After taking nateglinide	Difference in levels before and after nateglinide treatment	*P* values
Number of patients (M/F)	32 (19/13)	32 (19/13)		
Weight	71.35 ± 10.47	70.81 ± 9.52	0.54 ± 4.13	.805
FPG	7.44 ± 0.69	6.74 ± 0.52	0.69 ± 0.49	<.001*
PPG	14.16 ± 2.53	10.11 ± 2.09	4.05 ± 2.43	<.001
DPG	6.72 ± 2.28	3.36 ± 2.07	3.36 ± 2.34	<.001
HbA_1C (%)	7.26 ± 0.58	6.73 ± 0.35	0.53 ± 0.50	<.001*
GA (%)	16.43 ± 1.03	14.39 ± 0.64	2.04 ± 0.81	<.001
TC	5.54 ± 0.81	5.22 ± 0.84	0.32 ± 0.76	.062*
TG	2.10 ± 1.07	1.81 ± 0.58	0.30 ± 0.87	.063*
LDL-C	3.17 ± 0.71	2.84 ± 0.60	0.33 ± 0.55	.002
HDL-C	1.18 ± 0.26	1.22 ± 0.20	0.04 ± 0.25	.252*
HOMA-IRI	3.62 ± 2.91	2.86 ± 2.18	0.76 ± 1.75	.008
HOMA-β	56.69 ± 45.04	59.86 ± 44.71	3.17 ± 25.03	.072
ALT	31.63 ± 14.67	28.31 ± 11.19	3.31 ± 7.73	.044*
AST	28.97 ± 10.80	26.66 ± 8.10	2.31 ± 4.61	.003*
CRE	66.75 ± 13.31	68.22 ± 12.79	1.47 ± 6.27	.451*
UA	353.56 ± 92.01	358.28 ± 76.40	4.72 ± 62.86	.869*

FPG: fasting plasma glucose; PPG: postprandial plasma glucose; DPG: diphosphoglycerate plasma glucose; HbA_1C: glycosylated haemoglobin; GA: glycated albumin; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; HOMA-IRI: Homeostasis model method of assessment (HOMA) insulin resistance index; HOMA-β: HOMA β-cell function; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CRE: creatinine; UA: uric acid.
*Using nonparametric analysis method.

Table 5: Changes in FMEDD, NO, ET-1, MDA, and SOD in newly diagnosed type 2 diabetes after treatment with nateglinide.

Group		Before taking nateglinide	After taking nateglinide	Difference in levels before and after nateglinide treatment	*P* values
Number of patients (M/F)		32 (19/13)	32 (19/13)		
FMEDD	Fasting	12.20 ± 3.21	16.06 ± 4.23	3.86 ± 4.11	<.001
	After glucose load	9.4 ± 3.43	12.81 ± 4.01	3.41 ± 4.50	.0002
NO	Fasting	134.15 ± 58.55	173.64 ± 72.44	39.49 ± 35.22	<.001*
	After glucose load	123.76 ± 53.62	145.18 ± 62.97	20.52 ± 50.96	<0.001*
ET-1	Fasting	2.16 ± 1.81	1.40 ± 1.23	0.75 ± 0.92	<.001*
	After glucose load	2.31 ± 1.88	1.85 ± 1.56	0.46 ± 0.73	<.001*
MDA	Fasting	5.64 ± 1.22	4.66 ± 1.01	0.98 ± 0.77	<.001*
	After glucose load	6.19 ± 1.59	5.39 ± 1.03	0.80 ± 0.95	<.001*
SOD	Fasting	118.53 ± 17.30	146.81 ± 21.02	28.81 ± 15.48	<.001
	After glucose load	99.84 ± 17.09	125.28 ± 16.58	25.44 ± 13.00	.0002

FMEDD: flow-mediated endothelium-dependent dilation; NO: nitric oxide; ET-1: endothelin-1; MDA: malondialdehyde; SOD: superoxide dismutase.
*Using nonparametric analysis method.

chronic sustained hyperglycaemia [8]. Moreover, oxidation of LDL-C to form Ox-LDL-C, can also increase superoxide anions (O_2^-), leading to endothelial cell dysfunction [25].

Many studies have indicated that endothelial dysfunction is present in prediabetes groups, including IGT or IFG [26–28]. In this study, FMEDD, NO, and SOD during fasting and 2 hours after glucose load were significantly different among the study groups. Several studies have shown that HOMA-IRI is significantly increased and HOMA-β significantly reduced in patients with diabetes [29, 30]. Results of our study are consistent with these observations. In this study HOMA-IRI and BMI, FPG, PPG, HbA1C showed significant positive correlations whereas HOMA-β and FPG, HbA1C, and GA were negatively correlated. This suggests that with high blood glucose levels, β-cell function was reduced and insulin resistance was more apparent as oxidative stress can aggravate pancreatic β cells dysfunction by promoting early apoptosis of β cells. In addition, HOMA-β and FMEDD (fasting and

2 hours after glucose load) were positively correlated. With reduced β-cell function, the NO synthesis may be inhibited, which damages the endothelial NO synthase (eNOS), leading to endothelial dysfunction [31].

Acute blood glucose fluctuations after glucose load can trigger a series of adverse reactions, including increased insulin resistance, oxidative stress, and endothelial dysfunction. Endothelial cells in a stable high glucose environment have a certain degree of adaptive capacity, whereas the blood glucose fluctuations impaired endothelial ability to adapt [32]. In a prospective follow up study in type 2 diabetic patients, postprandial hyperglycaemia was suggested as an independent risk factor for cardiovascular disease [33]. Number of animal experiments confirmed that acute glucose fluctuations increased adhesion of monocytes to vascular endothelium, which had more impact leading to endothelial dysfunction compared with changes due to persistent hyperglycaemia [34, 35]. Glucose fluctuations after glucose load can quickly inhibit brachial artery endothelium-dependent vasodilation in patients with impaired glucose tolerance and diabetes [36–38]. Our study results are also in agreement with these observations, as FMEDD, NO, and SOD after glucose load were significantly decreased compared with that while fasting. In contrast, ET-1 and MDA were significantly higher among newly diagnosed diabetes group. At the same time, a positive correlation was seen for DPG and FPG, PPG, HbA1C, GA, HOMA-IRI, and TG. Both fasting and 2 hours glucose load DPG and FMEDD, NO, SOD were negatively correlated. Thus, the vulnerable endothelial function in diabetes is damaged further after glucose fluctuations.

4.3. Effect of Nateglinide on Endothelial Function and Oxidative Stress. Many antioxidant drugs are being used to reduce the toxicity of high glucose by acting against ROS and related pathways to reduce oxidative stress. In this study, we evaluated the effect of nateglinide therapy on endothelial function and oxidative stress in patients with newly diagnosed type 2 diabetes. Nateglinide is believed to reduce glucose autoxidation and hence reduce the generation of oxygen free radicals in the polyol pathway and in the glycolytic pathway by lowering blood sugar [39]. In the study by Major-Pedersen et al. (2008), nateglinide could improve endothelial function by reducing postprandial hyperglycaemia and stimulating early phase insulin secretion in patients with insulin resistance [11]. Nateglinide reduces oxidative stress and restores carotid artery intima-media thickness by strict control of blood glucose in patients with type 2 diabetes [40]. In this study, the average weight had no significant difference comparing with the previous after the treatment. Therefore, an impact on endothelial function and oxidative stress of the change in weight was firstly excluded. In this part, FPG, PPG, DPG, HbA1C, GA, HOMA-IRI, ET-1, MDA were significantly decreased in posttreatment condition with nateglinide when compared with pretreatment. FMEDD, NO, and SOD during fasting and 2 hours after glucose load were significantly higher. LDL-C, ALT, and AST levels were also significantly reduced after treatment with nateglinide. Although TC and TG decreased and HDL-C and HOMA-β increased after nateglinide treatment, the changes were statistically nonsignificant ($P > .05$).

On the basis of these findings, we conclude that nateglinide can significantly lower blood glucose, reduce insulin resistance, improve endothelial function, and reduce oxidative stress. The mechanism involved in nateglinide-mediated improvement of endothelial function and oxidative stress may be as follows: (1) by acting on pancreatic β cells, nateglinide may rapidly and briefly inhibit adenosine triphosphate (ATP) sensitive potassium channel (K_{ATP} channel) to remodel early phase insulin secretion, which has a similar physiological pattern of insulin secretion. Early phase secretion of insulin can act directly on the liver and inhibit hepatic glucose output to reduce postprandial blood glucose levels, which can reduce the effect of blood glucose fluctuation on endothelial function and oxidative stress; (2) nateglinide can inhibit lipolysis and reduce free fatty acid levels and oxidative stress; (3) early phase insulin secretion can maintain normal endothelial function, thus inhibiting the secretion of inflammatory factors.

Hence, reducing the blood glucose fluctuations to mitigate endothelial dysfunction and oxidative stress is beneficial as it delays the development of diabetic vascular complications.

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Research Article

Sex Disparities in Diabetes Process of Care Measures and Self-Care in High-Risk Patients

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Patients with chronic diabetic complications experience high morbidity and mortality. Sex disparities in modifiable factors such as processes of care or self-care activities have not been explored in detail, particularly in these high-risk patients. Sex differences in processes of care and self-care activities were assessed in a cross-sectional analysis of the Pathways Study, an observational cohort of primary care diabetic patients from a managed care organization (N = 4,839). Compared to men, women had decreased odds of dyslipidemia screening (adjusted odds ratio (AOR) 0.73, 95% CI 0.62–0.85), reaching low-density lipoprotein goal (AOR 0.70, 95% CI 0.58–0.86), and statin use (AOR 0.69, 95% CI 0.58–0.81); women had 19% greater odds of reaching hemoglobin A1c <7% (95% CI 1.02–1.41). There were no sex differences in hemoglobin A1c testing, microalbuminuria screening, or angiotensin-converting enzyme inhibitor use. Women were less likely to report regular exercise but had better adherence to healthy diet, glucose monitoring, and self-foot examination compared to men. Patterns of sex differences were consistent in subjects with diabetic complications. Significant sex disparities exist in diabetes process of care measures and self-care, even amongst patients known to have chronic diabetic complications.

1. Introduction

In the United States, diabetes mellitus affects 26 million people [1] and its chronic vascular complications are associated with significant morbidity [2, 3], disability [4], and mortality [5]. Chronic complications from diabetes account for approximately $58 billion in excess medical expenditures per year [3]. Common risk factors for microvascular and macrovascular diabetic complications include age, duration of diabetes [6], hyperglycemia [7–9], and high blood pressure [10]; therefore multiple complications commonly develop in the same patient [5, 6]. In addition to the management of cardiovascular risk factors, prevention of diabetes complications also involves diabetes self-care such as diet, exercise, self-monitoring of blood glucose, and self-foot examination [11]. As a result, the American Diabetes Association (ADA) has established clinical practice guidelines regarding standard diabetes care, which include recommendations for diabetes process of care measures (frequency of laboratory testing, clinical goals, and recommended medications) and self-care [11].

A few studies have suggested that adherence with these diabetes clinical practice guidelines varies by sex. Women with diabetes have been reported to have worse blood pressure, lipid, and glycemic control compared to men [12], even amongst those known to have cardiovascular disease [13, 14]. Furthermore, diabetic women tend to be less physically active than men [15]. However, sex differences in laboratory testing and other self-care behaviors have not been explored in detail, nor is it known whether these sex disparities persist

in high-risk patients, such as those who already have a history of a diabetic complication. Identification of modifiable factors related to diabetes outcomes is imperative if the rate of adverse outcomes is to be decreased, and evaluation of sex-specific differences provides an opportunity to develop strategies to reduce sex-related health disparities in diabetes care.

This study examined the associations between sex and selected diabetes process of care measures and self-care activities in a cohort of primary care patients with diabetes. This study also examined whether sex differences in diabetes process of care measures and self-care activities were detectable in the subgroup of subjects with a history of diabetic complications, a particularly high-risk group for adverse outcomes.

2. Materials and Methods

2.1. Participants. We conducted a cross-sectional analysis of baseline data from the Pathways Study, which has been described previously [16, 17]. In brief, the Pathways Study is a prospective, observational cohort of the prevalence and impact of depression on patients with diabetes at Group Health (GH), a large nonprofit health maintenance organization (HMO) in Washington and Idaho, USA. GH maintains a registry of diabetes patients and their guideline-recommended test results. Nine primary care clinics were chosen for patient recruitment based on the number of diabetic patients, ethnic diversity, and proximity to Seattle, WA, USA. For the study, 9,063 potential candidates were identified from the GH diabetes registry (Figure 1). In 2001-2002, surveys were sent to these patients regarding demographic information, diabetes characteristics, diabetic complications, and self-care. Diabetic complications included retinopathy, nephropathy, neuropathy, cerebrovascular, cardiovascular, peripheral vascular disease, or metabolic (hypoglycemia, diabetic ketoacidosis, or hyperosmolar nonketotic coma). Of those identified, 1,222 patients were excluded from the study due to no diabetes, gestational diabetes, cognitive impairment, severe illness, deceased, disenrollment from GH, language or hearing problems, or other reasons. Of the remaining 7,842 eligible patients for the study, 4,839 (61.7%) returned the survey of which 4,467 (92.3%) gave permission to link survey data with automated data from GH regarding laboratory tests, pharmacy records, hospitalizations, and outpatient visits. The study protocol was approved by GH and University of Washington institutional review boards.

2.2. Measures. Baseline hemoglobin A1c and low-density lipoprotein (LDL) were ascertained closest to the date of the baseline epidemiologic survey, up to 12 months prior to study enrollment. Microalbuminuria was defined as a urine albumin to creatinine ratio (UACR) >17 mg/g for women and >25 mg/g for men, based on sex-specific cutoffs [18]; given the large proportion of missing data, microalbuminuria was ascertained up to 24 months prior to study enrollment. Estimated glomerular filtration rate (eGFR) was calculated using Chronic Kidney Disease-Epidemiology (CKD-EPI) equations [19]. Chronic kidney disease (CKD) stage was determined by eGFR and microalbuminuria, using the National Kidney Foundation Kidney Disease Outcomes Quality Initiative classification system [20]. History of hypertension was based on ICD-9 code 401.x [21]. Computerized pharmacy records were used to identify patients who were prescribed any insulin, oral diabetic medication, HMG-CoA reductase inhibitor (statin), angiotensin-converting enzyme (ACE) inhibitor, or angiotensin receptor blocker (ARB) in the 12 months prior to study enrollment. For simplicity we will use the generic term ACE inhibitor to refer to either an ACE inhibitor or ARB.

Self-care activities were assessed using the Summary of Diabetes Self-Care Activities (SDSCA), which is a brief questionnaire that asks how many days per week an activity was performed [22]. The SDSCA has been shown to be a reliable and valid measure of adherence to diabetes self-care in observational and interventional studies [23]. For this study, investigators selected five SDSCA questions regarding diet, exercise, blood glucose testing, and foot care that were considered the most clinically relevant for analysis (Table 1).

2.3. Outcomes. The primary outcomes of interest were sex-specific differences in the following diabetes process of care measures and self-care activities: (1) history of recommended laboratory testing (hemoglobin A1c, LDL, and microalbuminuria), (2) attainment of clinical targets (hemoglobin A1c < 7% and LDL < 130 mg/dL), (3) medication use (statins in all subjects and in those with LDL >130 mg/dL, ACE inhibitors in all subjects and in those with microalbuminuria), and (4) compliance with self-care (diet, exercise, and foot examination at least 3 times per week; blood glucose testing at least 3 times a week if on oral hypoglycemic agents only or 5 times a week if on insulin).

2.4. Data Analysis. Statistical analyses were performed using STATA version 12 (College Station, TX, USA) [24]. Significant sex differences in diabetes clinical process of care measures and self-care activities were determined using t-tests for continuous data and χ^2 tests for categorical data. Logistic regression models were used to calculate adjusted odds ratios (AORs) to determine if there were adjusted sex differences in compliance with diabetes process of care measures and self-care activities. Models regarding process of care measures were adjusted for age, race/ethnicity, marital status, education, smoking, body mass index, hemoglobin A1c (except for models where hemoglobin A1c testing and achievement of hemoglobin A1c <7% were outcomes of interest), history of hypertension, and CKD stage (except the model for microalbuminuria testing). Models regarding self-care activities were additionally adjusted for a history of major depression, which was strongly associated with both sex and self-care outcomes. Analyses were also performed on the subgroup of patients with a known history of at least one diabetic complication (N = 3,045), since these patients are at high risk for adverse outcomes. Sensitivity analyses demonstrated similar results in this subgroup as with subgroups of patients with specific diabetic complications (cardiovascular, cerebrovascular, or nephropathy).

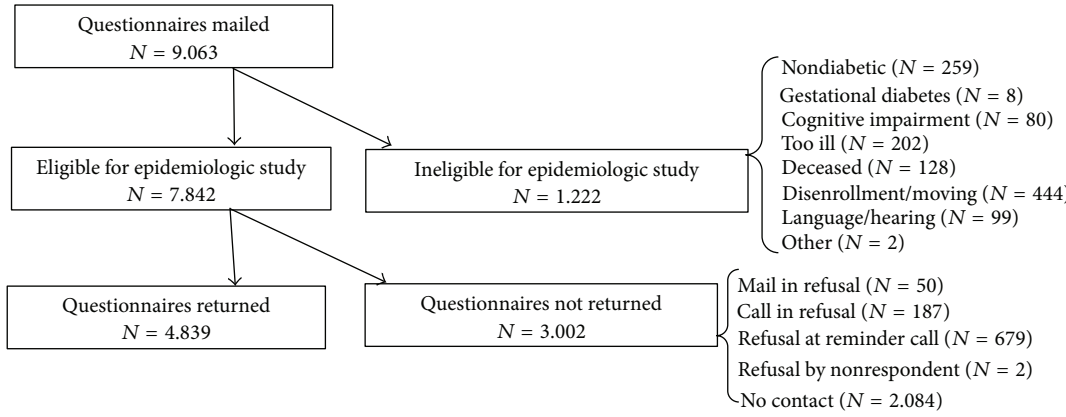

Figure 1: The Pathways Study subject recruitment.

Table 1: Selected questions from the Summary of Diabetes Self-Care Activities (SDSCA).

Self-care domain	Description	SDSCA question
Diet	≥5 servings of fruits/vegetables	On how many of the last SEVEN DAYS did you eat five or more servings of fruits and vegetables?
Diet	High fat foods	On how many of the last SEVEN DAYS did you eat high fat foods such as red meat or whole-fat dairy products?
Exercise	≥30 minutes exercise	On how many of the last SEVEN DAYS did you participate in at least 30 minutes of physical activity? (This means 30 minutes of continuous activity, including walking)
Blood glucose	Blood glucose testing	On how many of the last SEVEN DAYS did you test your blood sugar?
Foot care	Foot examination	On how many of the last SEVEN DAYS did you check your feet?

3. Results

3.1. Pathways Cohort Characteristics. Of the 4,839 subjects in the total cohort, 48.8% were women (Table 2). Men tended to be older, more frequently married, and had higher levels of education and income compared to women. Women had higher mean BMI and greater prevalence of hypertension (45.0% versus 41.0%) and major depression (14.1% versus 9.6%) than men. Men had a greater prevalence of microalbuminuria (46.7% versus 34.4%) and higher mean number of diabetic complications (1.4 ± 1.4 versus 1.3 ± 1.3) compared to women. There were similar patterns of sex differences in cohort characteristics amongst the subset of subjects known to have diabetic complications.

3.2. Diabetes Process of Care Measures. Approximately 86.7% of subjects had previous hemoglobin A1c testing and 61.4% had urine microalbuminuria screening; these test frequencies did not vary significantly by sex. In contrast, LDL testing was less frequent in women compared to men; only 52.6% of women had their LDL checked in the previous year compared to 59.0% of men ($P < 0.001$).

Both men and women had a mean hemoglobin A1c of 7.8%, and a similar proportion of subjects achieved a hemoglobin A1c <7%. Mean LDL was higher in women (115.0 ± 36.0 mg/dL) than in men (107.8 ± 33.6 mg/dL, $P < 0.001$), and a lower proportion of women achieved a target LDL of <130 mg/dL (67.3% versus 75.3% in men, $P < 0.001$). Statins were prescribed less frequently to women than men overall (26.5% versus 35.4%, $P < 0.001$) and in those with LDL levels above 130 mg/dL (24.2% versus 31.3%, $P = 0.02$). There were no sex differences in ACE inhibitor use overall or in the subset with microalbuminuria.

In adjusted multivariable logistic regression models (Table 3), a greater proportion of women were more likely to be guideline discordant than men for LDL testing (AOR 0.73, 95% CI 0.62–0.85), achievement of LDL target <130 mg/dL (AOR 0.70, 95% CI 0.58–0.86), any statin prescription (AOR 0.69, 95% CI 0.58–0.81), or statin prescription if LDL was greater than 130 mg/dL (AOR 0.61, 95% CI 0.41–0.91) compared to men. Women were more likely to achieve hemoglobin A1c target (AOR 1.19, 95% CI 1.02–1.41). There were no differences in the odds of hemoglobin A1c testing, microalbuminuria screening, or ACE inhibitor prescription by sex.

3.3. Diabetes Self-Care Activities. Women reported more frequent consumption of ≥5 servings of fruits or vegetables per day and less frequent consumption of high fat foods compared with men. In the week prior to study assessment, a lower proportion of women (55.3%) exercised at least 3 times a week compared to men (63.9%, $P < 0.001$). Frequency of blood glucose testing was similar by sex. Women tended to examine their feet more frequently than men.

In adjusted logistic regression models of self-care activities (Figure 2(a)), women were more likely to report high

Table 2: Pathways cohort characteristics by sex.

	All subjects			Subjects with diabetic complications		
	Women (N = 2,360)	Men (N = 2,479)	P	Women (N = 1,445)	Men (N = 1,600)	P
Age (years)	62.5 ± 14.0	63.8 ± 12.7	0.002	64.4 ± 13.6	66.0 ± 12.1	0.001
Race/ethnicity						
Non-Hispanic white	1,753 (74.3)	1,872 (75.5)	0.62	1,104 (76.4)	1,245 (77.8)	0.59
Non-Hispanic black	207 (8.8)	195 (7.9)		132 (9.1)	124 (7.8)	
Asian/Pacific Islander	236 (10.0)	236 (9.5)		119 (8.2)	131 (8.2)	
Other	164 (7.0)	176 (7.1)		90 (6.2)	100 (6.3)	
Married	1,238 (53.3)	1,924 (78.4)	<0.001	742 (52.1)	1,252 (78.7)	<0.001
≥High school education	1,640 (71.0)	1,918 (78.6)	<0.001	987 (69.7)	1,234 (77.9)	<0.001
Income ≥ $20,000/year	931 (51.4)	1,264 (61.9)	<0.001	521 (47.7)	755 (57.3)	<0.001
Smoker	181 (7.9)	231 (9.4)	0.05	102 (7.2)	125 (7.9)	0.47
Body mass index (kg/m^2)	32.3 ± 8.2	29.7 ± 5.7	<0.001	32.3 ± 8.2	29.8 ± 5.8	<0.001
Hypertension	980 (45.0)	939 (41.0)	0.008	753 (52.1)	758 (47.4)	<0.001
Major depression	331 (14.1)	237 (9.6)	<0.001	211 (14.7)	186 (11.6)	0.01
Diabetic complications						
Number of diabetic complications	1.3 ± 1.3	1.4 ± 1.4	<0.001	1.9 ± 1.1	2.0 ± 1.1	0.006
≥1 diabetic complication	1,445 (66.3)	1,600 (69.9)	0.01	—	—	—
Microalbuminuria	507 (34.4)	698 (46.7)	<0.001	486 (50.5)	633 (61.3)	<0.001
Laboratory testing						
Hemoglobin A1c (12 months)	2,043 (86.6)	2,153 (86.9)	0.77	1,366 (94.5)	1,521 (95.1)	0.51
LDL (12 months)	1,242 (52.6)	1,462 (59.0)	<0.001	843 (58.3)	1,082 (67.6)	<0.001
Microalbuminuria (24 months)	1,475 (62.5)	1,494 (60.3)	0.11	963 (66.6)	1,032 (64.5)	0.21
Laboratory results						
Mean hemoglobin A1c (%)	7.8 ± 1.5	7.8 ± 1.6	0.41	7.9 ± 1.6	7.9 ± 1.6	0.64
Hemoglobin A1c < 7%	692 (33.9)	683 (31.7)	0.14	417 (30.5)	429 (28.2)	0.17
Mean LDL (mg/dL)	115.9 ± 36.0	107.8 ± 33.6	<0.001	112.3 ± 35.3	104.9 ± 33.3	<0.001
LDL < 130 mg/dL	955 (67.3)	1,259 (75.3)	<0.001	671 (70.3)	969 (78.5)	<0.001
Medications						
Statin	577 (26.5)	809 (35.4)	<0.001	451 (31.2)	665 (41.6)	<0.001
Statin if LDL >130 mg/dL	110 (24.2)	122 (31.3)	0.02	81 (29.5)	93 (36.9)	0.07
ACE inhibitor	1,238 (56.8)	1,354 (59.2)	0.11	901 (62.4)	1,040 (65.5)	0.07
ACE inhibitor if microalbuminuria	342 (67.5)	455 (65.2)	0.41	334 (68.7)	424 (67.0)	0.54
Diet						
≥5 servings of fruits/vegetables (days/week)	4.1 ± 2.4	3.8 ± 2.5	<0.001	4.1 ± 2.4	3.8 ± 2.5	0.004
High fat foods (days/week)	2.8 ± 1.9	3.2 ± 2.0	<0.001	2.8 ± 1.9	3.1 ± 2.0	<0.001
Exercise						
≥30-minute exercise (days/week)	3.0 ± 2.4	3.5 ± 2.4	<0.001	2.8 ± 2.4	3.4 ± 2.4	<0.001
≥30-minute exercise, 3 times a week	1,276 (55.3)	1,568 (63.9)	<0.001	730 (51.8)	989 (62.4)	<0.001
Blood glucose testing (days/week)	4.5 ± 2.8	4.4 ± 2.9	0.23	4.7 ± 2.8	4.6 ± 2.8	0.34
Foot examination (days/week)	4.7 ± 2.6	4.4 ± 2.8	<0.001	4.8 ± 2.6	4.7 ± 2.7	0.09

Data are n (%) or mean ± SD.
LDL: low-density lipoprotein; ACE: angiotensin converting enzyme.

fruit and vegetable consumption (AOR 1.36, 95% CI 1.15–1.61), blood glucose testing (1.27, 95% CI 1.04–1.55), and self-foot examination (AOR 1.32, 95% CI 1.11–1.57) but less likely to report fatty food consumption (AOR 0.69, 95% CI 0.59–0.80) and regular exercise (AOR 0.72, 95% CI 0.62–0.85) compared to men.

Table 3: Logistic regression models of diabetes process measures for women compared to men in the pathways study.

	Unadjusted OR (95% CI)	*P*	Adjusted OR (95% CI)*	*P*
Hemoglobin A1c testing (12 months)	0.98 (0.83–1.15)	0.77	1.04 (0.68–1.61)	0.85
LDL testing (12 months)	0.77 (0.69–0.87)	<0.001	0.73 (0.62–0.85)	<0.001
Microalbuminuria testing (24 months)	1.10 (0.98–1.23)	0.11	1.15 (0.99–1.34)	0.07
Hemoglobin A1c < 7%	1.10 (0.97–1.25)	0.14	1.19 (1.02–1.41)	0.03
LDL < 130 mg/dL	0.68 (0.58–0.79)	<0.001	0.70 (0.58–0.86)	0.001
Statin	0.66 (0.58–0.75)	<0.001	0.69 (0.58–0.81)	<0.001
Statin if LDL >130 mg/dL	0.70 (0.52–0.95)	0.02	0.61 (0.41–0.91)	0.02
ACE inhibitor	0.91 (0.81–1.02)	0.11	0.97 (0.83–1.14)	0.71
ACE inhibitor if microalbuminuria	1.11 (0.87–1.41)	0.41	1.14 (0.86–1.50)	0.36

* Adjusted for age, race, marital status, education, smoking, body mass index, hemoglobin A1c (except models for hemoglobin A1c testing and hemoglobin A1c <7%), history of hypertension, and chronic kidney disease stage (except model for microalbuminuria testing).
LDL: low-density lipoprotein; ACE: angiotensin converting enzyme.

Table 4: Logistic regression models of diabetes process measures for women compared to men amongst subjects with diabetic complications.

	Unadjusted OR (95% CI)	*P*	Adjusted OR (95% CI)*	*P*
Hemoglobin A1c testing (12 months)	0.90 (0.65–1.23)	0.51	0.94 (0.66–1.34)	0.75
LDL testing (12 months)	0.67 (0.58–0.78)	<0.001	0.65 (0.54–0.77)	<0.001
Microalbuminuria testing (24 months)	1.10 (0.95–1.28)	0.21	1.06 (0.88–1.28)	0.51
Hemoglobin A1c < 7%	1.12 (0.95–1.31)	0.17	1.21 (1.01–1.44)	0.04
LDL < 130 mg/dL	0.65 (0.54–0.79)	<0.001	0.63 (0.51–0.78)	<0.001
Statin	0.64 (0.55–0.74)	<0.001	0.67 (0.56–0.79)	<0.001
Statin if LDL >130 mg/dL	0.71 (0.50–1.03)	0.07	0.64 (0.42–0.97)	0.04
ACE inhibitor	0.87 (0.75–1.01)	0.07	0.87 (0.73–1.03)	0.10
ACE inhibitor if microalbuminuria	1.08 (0.84–1.40)	0.54	1.10 (0.83–1.47)	0.51

* Adjusted for age, race, marital status, education, smoking, body mass index, hemoglobin A1c (except models for hemoglobin A1c testing and hemoglobin A1c <7%), and history of hypertension.
LDL: low-density lipoprotein; ACE: angiotensin converting enzyme.

3.4. Patients with Diabetic Complications. Based on adjusted logistic regression models of 3,045 patients with at least one known diabetic complication, men and women had similar odds of hemoglobin A1c and microalbuminuria testing (Table 4). Women in this subgroup had decreased odds of LDL testing (AOR 0.65, 95% CI 0.54–0.77), achievement of LDL goal <130 mg/dL (AOR 0.63, 95% CI 0.51–0.78), statin use overall (AOR 0.67, 95% CI 0.56–0.79), and statin use if serum LDL was >130 mg/dL (AOR 0.64, 95% CI 0.42–0.97). Women with at least one diabetic complication had greater odds of achieving target hemoglobin A1c <7% compared to their male counterparts. There were no sex-specific differences in ACE inhibitor use.

Amongst those with at least one diabetic complication, women had greater odds of high fruit and vegetable consumption (AOR 1.61, 95% CI 1.34–1.93), blood glucose monitoring (AOR 1.26, 95% CI 1.01–1.55), and self-foot examination (AOR 1.31, 95% CI 1.08–1.58) compared to men (Figure 2(b)). Women in this subgroup were less likely to consume fatty foods (AOR 0.69, 95% CI 0.58–0.81) or to exercise (AOR 0.69, 95% CI 0.59–0.82) compared to their male counterparts.

4. Discussion

This analysis found significant sex differences in diabetes process of care measures and self-care activities, even amongst the high-risk subgroup of subjects who were known to have chronic complications of diabetes. In general, women tended to have better glycemic control and adherence to recommended self-care compared to men. However, despite having higher LDL levels, women were less likely to be screened for dyslipidemia or to be prescribed statins compared to men. Women were also less likely to engage in physical activity than men.

This is the first study to report sex disparities in diabetes processes of care and self-care behaviors in the subgroup of patients with a history of at least one diabetic complication, which is surprising since these patients are at high-risk for additional diabetic complications and warrant aggressive diabetes care. Our results are congruent with previous findings of sex differences in diabetes process of care measures, particularly with respect to management of dyslipidemia. In a cross-sectional study of 3,849 patients with diabetes from five

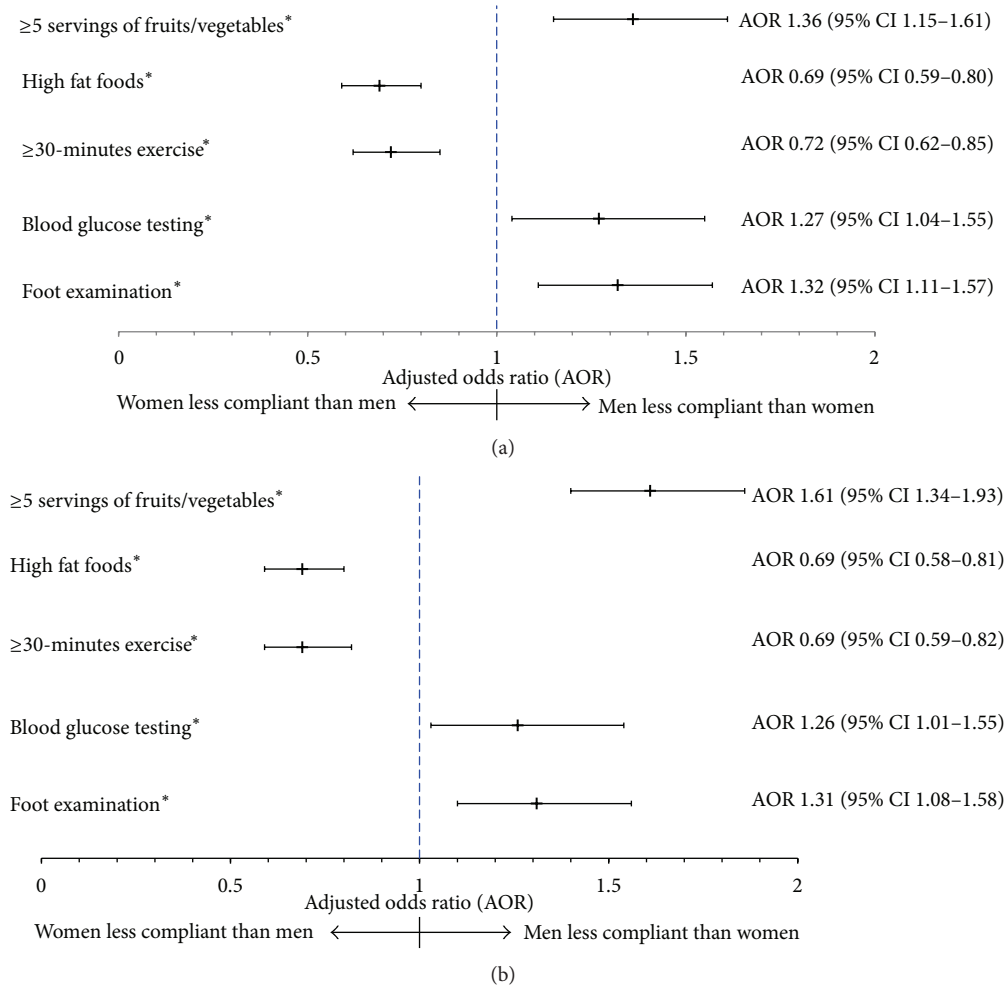

Figure 2: Logistic regression models of compliance with self-care activities for women compared to men in the overall cohort (a) and amongst subjects with at least one diabetic complication (b). Adjusted for age, race/ethnicity, marital status, education, smoking, body mass index, hemoglobin A1c (except model for hemoglobin A1c <7%), history of hypertension, and major depression. Models in (a) were also adjusted for chronic kidney disease stage.

academic medical centers, Wexler et al. found that women had higher cholesterol levels, were less likely to receive lipid lowering therapy, and when receiving lipid lowering therapy, were less likely to reach LDL targets compared to men [14]. Ferrara et al. found that, compared to men, diabetic women without cardiovascular disease received less frequent lipid testing and diabetic women with cardiovascular disease were treated less frequently with lipid lowering agents [25]. Although other studies have reported worse glycemic control in diabetic women compared to men [12, 14], we found that women had better glycemic control than men.

We also found significant sex differences in patterns of diabetes self-care. Diabetic women tended to be more physically inactive than men, which is consistent with findings from the Third National Health and Nutrition Examination Survey (NHANES III) [15]. This study builds on previous reports by demonstrating sex differences in other diabetes self-care activities. Diabetic men were consistently less adherent to recommendations regarding diet, blood glucose monitoring, and foot care than women. Likewise, these sex differences in diabetes self-care persisted in patients with known diabetic complications.

The observed sex differences in diabetes process of care measures and self-care activities are likely multifactorial and may be related to both provider and patient factors. Providers may perceive diabetic women to have a lower risk of cardiovascular disease and other diabetic complications compared to men, which may result in less aggressive monitoring and treatment in women. Whether sex influences management of diabetic patients has not been investigated; however, research in cardiovascular disease revealed that sex influenced how physicians managed chest pain [26], coronary heart disease [27], and cardiovascular disease prevention [28]. Since public awareness of cardiovascular disease in women remains suboptimal [29, 30], female patients may themselves underestimate their risk for diabetic complications and either fail to inquire about or decline routine diabetes care. Moreover, men and women may have differing beliefs in the benefit of self-care. In a survey of new patients to a diabetes education center, women were more likely than men to have a history of

previous diabetes education and had higher expectations that self-management would improve health outcomes; this may explain our finding that women had better glycemic control and overall better adherence to self-care than men [31]. Finally, biologic differences between men and women, due to estrogen or lack thereof, may result in differential outcomes in care. Sex hormones are associated with glucose tolerance [32], lipid metabolism [33], albuminuria [34], and coronary heart disease [35]. Diabetes has a greater adverse effect on serum triglyceride and LDL levels in women compared to men [36], and a recent meta-analysis of statin therapy for secondary cardiovascular prevention found that statins reduced all-cause mortality and stroke risk men but not in women [37].

It is important to recognize that the results of this study present an opportunity to improve the quality of diabetes care not only for women, but for men as well. Although women had less adequate screening and management of dyslipidemia and poorer adherence to exercise, they tended to have better glycemic control and adherence to other recommended self-care activities compared to men. Health care providers should pay particular attention to ordering recommended laboratory tests and medications in women and targeting patient education interventions regarding self-care to men. All patients should be encouraged to follow a regular exercise program and this should be heavily emphasized among women.

The strengths of this study include the large sample size of study subjects with comparable access to care. The study surveyed patients directly for variables related to diabetes self-care and we were able to adjust for several known confounding variables, including major depression. However, this study does have several limitations that are important to consider. The cross-sectional nature of this study is subject to unmeasured confounding and cannot establish causal relationships. We did not have access to actual blood pressure measurements and therefore could not adjust for the degree of control of hypertension. There was a large proportion of missing data for laboratory test results, particularly for LDL and microalbuminuria, which affects the validity and generalizability of these results. Pharmacy records could only capture medications prescribed within the GH system and do not reflect actual patient usage. Self-care activities were ascertained by self-reported measures rather than actual measurements. Although the SDSCA has been shown to be a reliable and valid measure of diabetes self-management [22], differential misclassification could occur if there were systematic differences in how men and women recall or report self-care. Finally, this study could not account for several factors that may contribute to the observed sex differences, such as differences in patient visit frequency, provider styles of care, or patient preferences by sex.

5. Conclusion

In conclusion, sex is associated with significant differences in diabetes process of care measures and self-care activities, even amongst subjects known to have chronic complications from diabetes. Women may benefit from more attention to dyslipidemia screening, lipid lowering treatment, and regular exercise, whereas men may require more encouragement in diabetes self-care including healthy diet, self-blood glucose monitoring, and self-foot examination. The findings from this study indicate an opportunity for intervention to reduce sex-related disparities in diabetes care. Although further studies are needed to elucidate the causes for these sex disparities, it is important for primary health care providers to be aware of the existence of sex differences in diabetes care such that these disparities may be eliminated.

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Research Article

Protective Effects of Resveratrol on TNF-α-Induced Endothelial Cytotoxicity in Baboon Femoral Arterial Endothelial Cells

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Endothelial injury induced by inflammatory factors plays a critical role in the pathogenesis of cardiovascular disease. Endothelial cell (EC) apoptosis, proliferation, migration, and cellular adhesion molecule (CAM) expression contribute to the development of atherosclerosis. We investigated the effects of resveratrol (0.1–100 μM) on the proliferation, migration, and CAM expression of primary cultures of baboon arterial endothelial cells (BAECs). In addition, we tested its effects under normal conditions as well as under inflammatory conditions induced by tumour necrosis factor-α (TNF-α) administered either by cotreatment, pretreatment, or posttreatment. Immunocytochemistry, MTT, wound-healing, and flow cytometry assays were performed. The resveratrol treatment significantly enhanced BAEC proliferation and attenuated TNF-α-induced impairment of proliferation at the optimal doses of 1–50 μM. Resveratrol at a high dose (100 μM) and TNF-α impaired BAEC migration, while low doses of resveratrol (1–50 μM) attenuated TNF-α-induced impairment of BAEC migration. Moreover, resveratrol inhibited TNF-α-induced ICAM-1 and VCAM-1 expression. Taken together, our results suggest that the resveratrol protects BAECs after inflammatory stimulation as well as ameliorates inflammatory effects at low concentrations. Consequently, resveratrol should be considered as a candidate drug for the prevention and treatment of inflammatory vascular diseases.

1. Introduction

Endothelial cell (EC) cytotoxicity induced by inflammatory factors plays a key role in the pathogenesis of cardiovascular disease. Tumour necrosis factor (TNF)-α, a pleiotropic proinflammatory cytokine involved in the pathogenesis of inflammatory, and vascular disease, can promote endothelial cell apoptosis and inflammation [1] by directly activating a number of cellular stress-sensitive pathways including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) [2]. These subsequently contribute to endothelial cell injury and cellular dysfunction [3]. Therefore, the inhibition of TNF-α-induced endothelial cell cytotoxicity can be important in preventing the cardiovascular disease and inhibiting its progression.

The resveratrol possesses many pharmacological properties including anticancer [4], anti-inflammation [5], and cardioprotective effects [6]. The resveratrol exerts direct cardiovascular protective effects by improving myocardial perfusion, reducing oxidant stress, and inhibiting platelet aggregation [7–9]. Recent studies have shown that resveratrol acts partially through the inhibition of cellular apoptosis and inflammation by inhibiting the NF-κB pathway [10, 11]. The beneficial effects of resveratrol on suggest that it could be an important pharmacological target for the treatment of cardiovascular disease. However, resveratrol has cell-specific [12, 13] and dose-dependent [14] effects on cellular proliferation or apoptosis. Studies of a wide range of concentrations of resveratrol administered to a uniform population of ECs are lacking. Such studies are required to comprehend the diverse and sometimes contradictory cellular effects of resveratrol [15]. Previous studies demonstrated that resveratrol has biphasic properties in relation to its concentration

on EC proliferation, with no effects at low concentrations (0.1–25 μM) and induced apoptosis at high doses (such as 100 μM) in human umbilical vein endothelial cells (HUVECs) [16].

The baboon is a well-characterized model for human biomedical studies including cardiovascular diseases [17, 18]. In the present study, we investigated the effects of a wide range of concentrations of resveratrol on cultured primary baboon arterial endothelial cells (BAECs) under normal conditions as well as under TNF-α-induced inflammatory condition in which cells underwent cotreatment, pretreatment, or posttreatment with resveratrol.

2. Methods

2.1. Materials. Unless otherwise indicated, all the reagents used in this study and their sources were as follows: resveratrol and 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, St. Louis, MO); anti-CD62E (E-selectin) (R&D Systems, Minneapolis, MN, Clone BB1G-E5); anti-CD54 (intercellular adhesion molecule-1) (BD Biosciences, San Jose, CA, Clone HA58); anti-CD106 (vascular cell adhesion molecule-1) (US Biological, Swampscott, MA, Clone 5K26T); cell culture media and supplies (Invitrogen, Carlsbad, CA).

2.2. BAEC Isolation and Culture. BAECs were isolated from baboon femoral arteries as previously described [17]. Briefly, a 2-3 cm segment of femoral artery was obtained under sterile surgical procedure by experienced veterinarians; procedures were approved by the Institutional Animal Care and Use Committee of Texas Biomedical Research Institute. The artery was incubated with 0.1% collagenase at 37°C for 15 min to digest extracellular tissue to allow the release of cells. The released cells were washed and seeded immediately on 1.0% gelatin-coated culture plates. Primary BAECs were cultured in F-12K growth medium supplemented with 20% foetal calf serum (FCS). Passages three and four of BAECs were used in this study. BAECs were treated with resveratrol at various concentrations in the presence or absence of TNF-α (10 ng/mL) for different time periods.

2.3. Immunocytochemistry. Primary BAECs were seeded into Lab-Tek multiwell chamber slides (BD Falcon 8-well Culture Slides) [18], fixed with 200 μL 4% formaldehyde at room temperature (RT) for 10 min and then blocked with 10% goat serum for 30 min at RT. Cells were then incubated with 300 μL of 1 : 400 or 1 : 800 diluted primary antibody (vWF: Sigma, St. Louis, MO; VE-Cadherin: Cell Signaling Technology, Danvers, MA) in 1% BSA in phosphate buffered saline (PBS) overnight at 4°C and then with FITC-labelled secondary antibody in 1% BSA in PBS for 1 h at RT in the dark. DAPI (0.5 μg/mL) was added for 5 min, and slides were mounted with 10 μL/well antifade solution (Invitrogen, no. S36936) in 1× PBS. Slides were observed by fluorescence microscopy.

2.4. MTT Assay. Cells were seeded in 96-well plates at a density of 1000 cells/well in 2% FCS F-12K growth medium for 24 h and treated with resveratrol at the designated concentrations (0.1–100 μmol/L) in the presence or absence of TNF-α (10 ng/mL) for different time periods. Treated cells were incubated with 20 μL (1 mg/mL) MTT for 4 h at 37°C to form formazan crystals by reacting with metabolically active cells. Subsequently, the formazan crystals were solubilized with 150 μL DMSO. The absorbance of each well was measured at A570 nm using a microplate reader. Cell viability was measured by the absorbance, normalized to cell numbers, incubated in control medium (considered 100%), and then determined relative to the control.

2.5. Wound-Healing Assay. Nearly confluent cell monolayers were "wounded" in a cross-shaped pattern with a sterile 200 μL pipette tip. The medium and dislodged cells were aspirated, and plates were replenished by either endothelial cell growth medium (ECGM) without FCS, which served as the control or by medium containing resveratrol (0.1–100 μM) with or without TNF-α (10 ng/mL). Images of the wound healing at 15 h were captured in the same scratched area localized to the right of the cross-shaped scratch with a 40x objective (Olympus IX70 microscope) and quantified by measuring the wound area with Image-Pro Plus software 6.0. Three fields per well were evaluated, and all experiments were performed in quadruplicate. Results were reported as the percentage of wound healing using the equation: % wound healing = [1 − (wound area at T15 h /wound area at T0 h)] × 100, where T0 h is the time immediately following wounding.

2.6. Flow Cytometry. The expression of proportions of cell specific cellular adhesion molecules was quantified by standard immunofluorescence cell sorting techniques [19, 20]. The following antibodies were used in this study: anti-human CD62E (clone BBIG-E5), anti-human CD54 (clone HA58), and anti-human CD106 (clone 5K267). Unstained and isotype controls were used to determine background staining. Flow cytometry analysis was performed using Cyan ADP (Becton Dickinson, San Jose, CA). Mean fluorescence intensity (MFI) values in respective gates were used to represent antigen expression. All experiments were performed three times and each time in triplicate.

2.7. Statistical Analysis. All quantitative variables were expressed as means ± SEM from at least three separate experiments. Comparisons between groups were made using one-way ANOVA followed by Dunnett's post hoc test. To evaluate the difference of two groups, we used two-tailed Student's t-test. $P < 0.05$ was considered statistically significant. SPSS version 17.0 (SPSS Inc., Chicago, IL) was used for statistical analyses.

3. Results

3.1. Primary BAEC Culture and Identification. Primary baboon arterial had a typical cobblestone shape (Figures 1(a) and 1(b)) and took 3–7 days to reach confluence depending

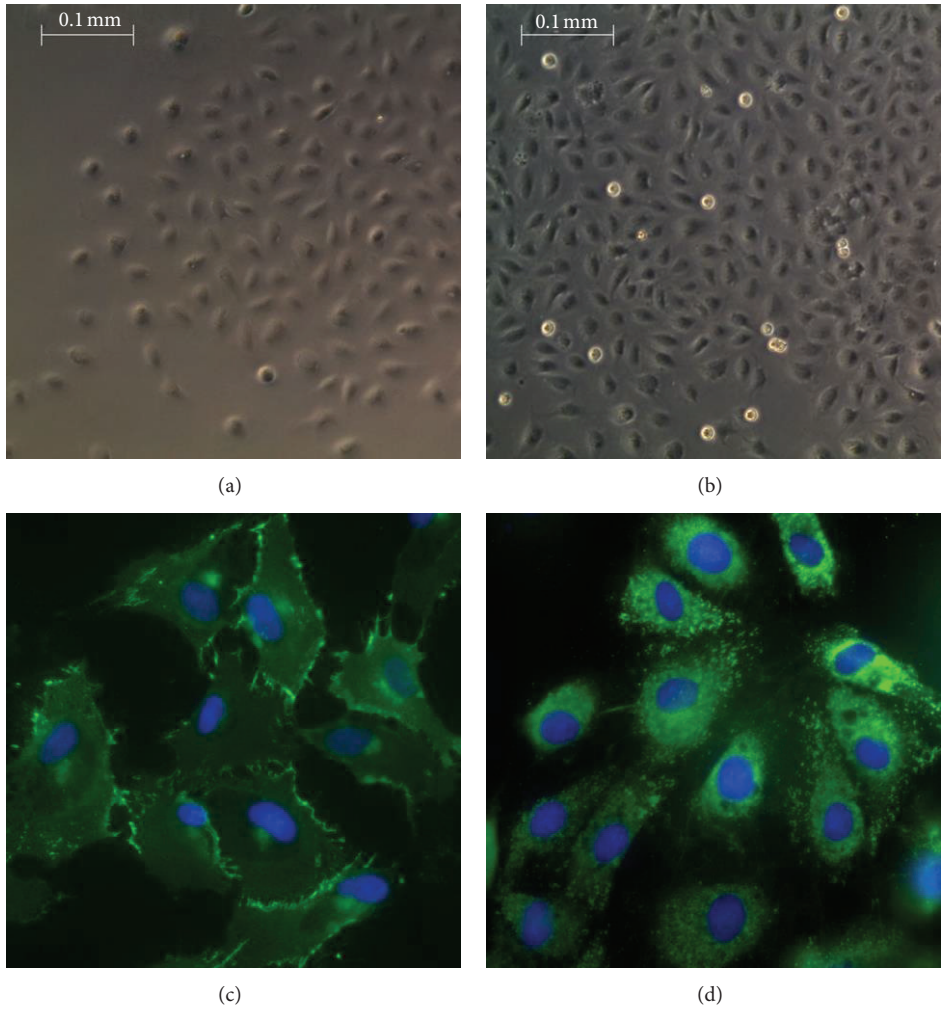

Figure 1: Morphology of primary baboon ECs cultured at day 3 (a) and day 7 (b). Isolated baboon femoral artery ECs showed a typical cobble stone shape (magnification ×40). Cells were stained with specific endothelial cell surface markers for VE-cadherin (c) and vWF (d) (magnification ×100). Immunofluorescence staining of baboon ECs with antibody to VE-cadherin and von Willebrand factor was conducted using an FITC-labelled secondary antibody. DAPI was used to stain the nuclei. Images were taken with an Eclipse E800 microscope.

on cell seeding density and number of passages. BAECs maintained proliferation ability for 30 populations or 10 passages, with stable expression of cellular adhesion molecules. Prior to their use, we stained BAECs with endothelial specific marker VE-cadherin (Figure 1(c)) and vWF (Figure 1(d)) to ensure the purity and healthy condition of the cells.

3.2. Resveratrol Enhances BAEC Proliferation and Attenuates TNF-α-Induced Impairment. We first tested the effect of various concentrations of resveratrol (0.1–100 μM) on the growth of primary BAECs (Figure 2(a)) and the extent to which resveratrol affected BAEC proliferation impaired by TNF-α when administered as cotreatment (Figure 2(b)), pretreatment (Figure 2(c)), or posttreatment (Figure 2(d)). Resveratrol (0.1–100 μM) highly significantly enhanced EC proliferation after 24 h incubation in the optimal range of 10–50 μM ($P < 0.05$, $P < 0.01$) (Figure 2(a)). TNF-α treatment alone (10 ng/mL) for 24 h significantly decreased ($P < 0.01$) BAEC proliferation by comparison with controls (Figure 2(b)). However, cotreatment with resveratrol at 1–50 μM alleviated cytotoxicity induced by TNF-α ($P < 0.01$). No effects were detected at 1 or 100 μM, suggesting a dose-dependent effect of resveratrol on baboon ECs subjected to inflammatory conditions. Pretreatment with resveratrol (0.1–100 μM) (Figure 2(c)) for 24 h also attenuated impairment of BAEC proliferation caused by incubation with TNF-α for 4 h ($P < 0.05$), especially at doses of 1–50 μM ($P < 0.01$). Additionally, posttreatment with resveratrol (0.1–100 μM) (Figure 2(d)) for 24 h attenuated TNF-α-induced impairment of BAEC proliferation, with the optimal dose being 10 μM ($P < 0.05$, $P < 0.01$). Together, the data indicated that the resveratrol could enhance primary baboon EC proliferation and could prevent cytotoxicity induced by TNF-α.

3.3. Resveratrol Attenuated BAEC Migration Impaired by TNF-α. We investigated whether resveratrol could affect BAEC migration with or without TNF-α treatment. As shown in Figure 3(a), resveratrol at a low concentration (10 μM;

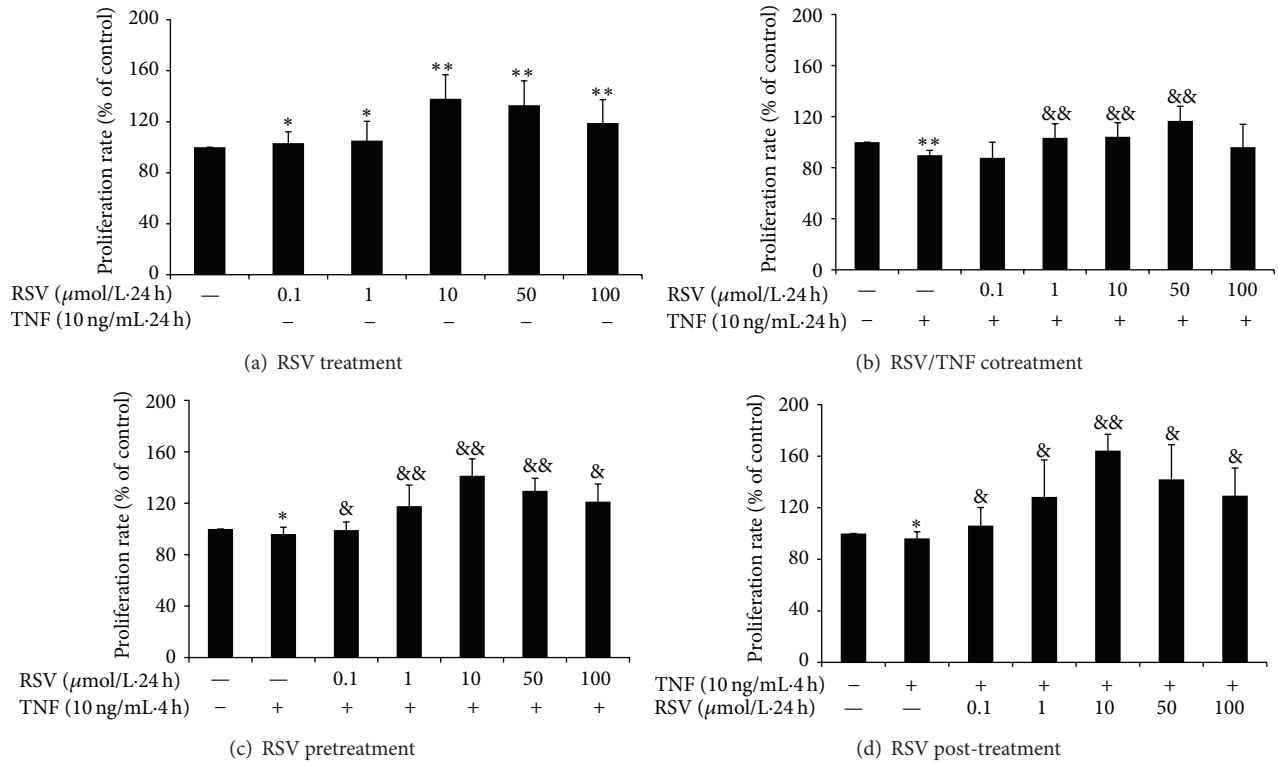

Figure 2: Effect of resveratrol on BAEC proliferation with or without TNF-α for 24 h. Resveratrol (0.1–100 μM) highly significantly enhanced EC proliferation after 24 h incubation in the optimal range of 10–50 μM (a). TNF-α (10 ng/mL) inhibited endothelial cell proliferation, while cotreatment with resveratrol (1–50 μM) reversed inhibition by TNF-α (b). BAECs were pretreated with resveratrol for 24 h then treated with TNF-α for 4 h; pretreatment with resveratrol ameliorated TNF-α-induced inhibition (c). BAECs were treated with TNF-α for 4 h then incubated with resveratrol for 24 h; posttreatment with resveratrol ameliorated TNF-α-induced inhibition (d). Data are expressed as a percentage of basal value (100%) and are the mean ± SEM from seven independent experiments, each conducted in quintuplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control and $^{\&}P < 0.05$; and $^{\&\&}P < 0.01$ versus TNF-α alone.

$P < 0.05$) increased wounded BAEC migration by comparison with controls, but, at a high concentration (100 μM; $P < 0.01$), it decreased the BAEC migration during 15 h. No effect was observed at concentrations of 0.1, 1, and 50 μM. As shown in Figure 3(b), TNF-α ($P < 0.01$) dramatically impaired BAEC migration at 15 h compared with controls, while resveratrol (1–50 μM) in the presence of TNF-α significantly increased the BAEC migration rate by comparison with TNF-α treatment alone ($P < 0.01$). No protective effect of 100 μM resveratrol was observed.

3.4. Resveratrol Inhibits TNF-α-Induced Expression of VCAM-1 (CD106) and ICAM-1 (CD54) in BAECs. To investigate whether resveratrol affected the level of inducible cell adhesion molecule expression of E-selectin (CD62E), ICAM-1 (CD54), and VCAM-1 (CD106), BAECs were analysed by flow cytometry. BAECs were cotreated (Figures 4(a) and 4(b)), pretreated (Figure 4(c)), or posttreated (Figure 4(d)) with resveratrol (10, 50 μM) and TNF-α. E-selectin, ICAM-1, and VCAM-1 expression on BAECs were significantly increased by TNF-α (10 ng/mL) stimulation at 4 h by comparison with controls ($P < 0.01$, Figure 4(a)). ICAM-1 and VCAM-1, but not E-selectin, remained elevated after 24 h ($P < 0.01$, Figure 4(b)). Resveratrol cotreatment did not inhibit the elevation in CAM expression elicited by TNF-α at 4 h (Figure 4(a)), but the expression of ICAM-1 and VCAM-1 significantly decreased in BAECs treated for 24 h with resveratrol (10, 50 μM) and TNF-α (10 ng/mL) by comparison with TNF-α treatment alone (10 ng/mL, $P < 0.01$, Figure 4(b)). Pretreatment with resveratrol (10, 50 μM) for 24 h (Figure 4(c)) inhibited the elevation of ICAM-1 and VCAM-1 expression induced by TNF-α stimulation ($P < 0.01$). BAECs that were treated with TNF-α for 4 h and then incubated with resveratrol (10, 50 μM, posttreatment) for 24 h (Figure 4(d)) exhibited significantly attenuated ICAM-1 (50 μM) and VCAM-1 (10, 50 μM) expression compared with controls ($P < 0.05$, $P < 0.01$).

4. Discussion

Recently, resveratrol has gained considerable attention because of its anticancer [4], anti-inflammatory [5], antidiabetes [21], and cardiovascular protective effects [6]. These properties have been reported in studies using experimental mouse, rat, and swine models [8, 22], but not primate models. Moreover, the effects of resveratrol on human are influenced by their vascular origin [23].

In this study, we investigated the effect of resveratrol on primary baboon femoral artery as an *in vitro* primate model. The advantages of this model are as follows. First, it

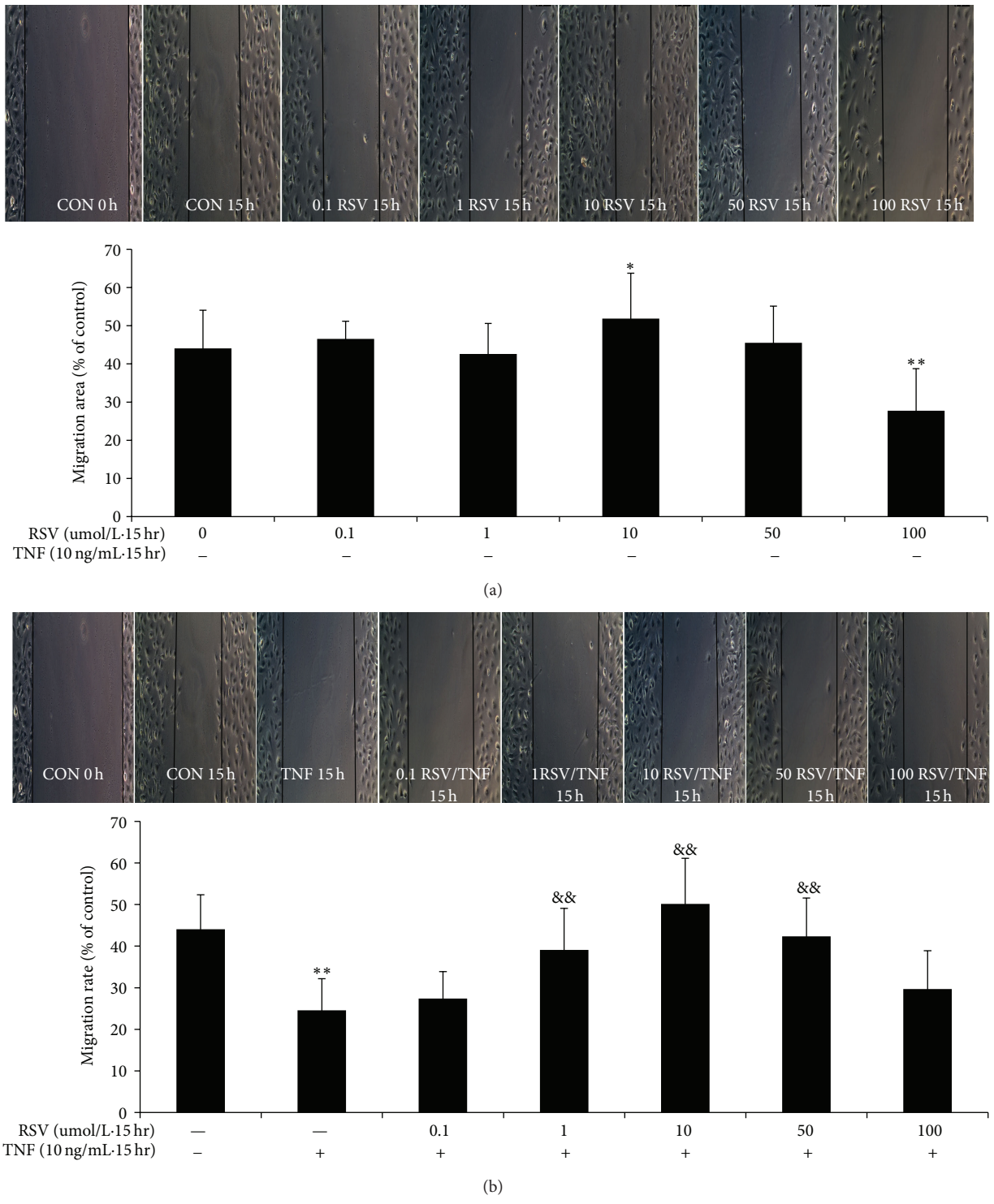

Figure 3: Effect of resveratrol on baboon endothelial cell migration with or without TNF-α (10 ng/mL) for 15 h. 10 μM resveratrol significantly increased BAEC migration, and 100 μM resveratrol significantly decreased BAEC migration (a). TNF-α administration (b) decreased BAEC migration by comparison with controls. Resveratrol (1–50 μM) attenuated impairment of BAEC migration by TNF-α when during incubation for 15 h (b). Data are the mean ± SEM from five independent experiments; each conducted in triplicate. $^{*}P < 0.05$; and $^{**}P < 0.01$ versus control, $^{\&}P < 0.05$; and $^{\&\&}P < 0.01$ versus TNF-α alone.

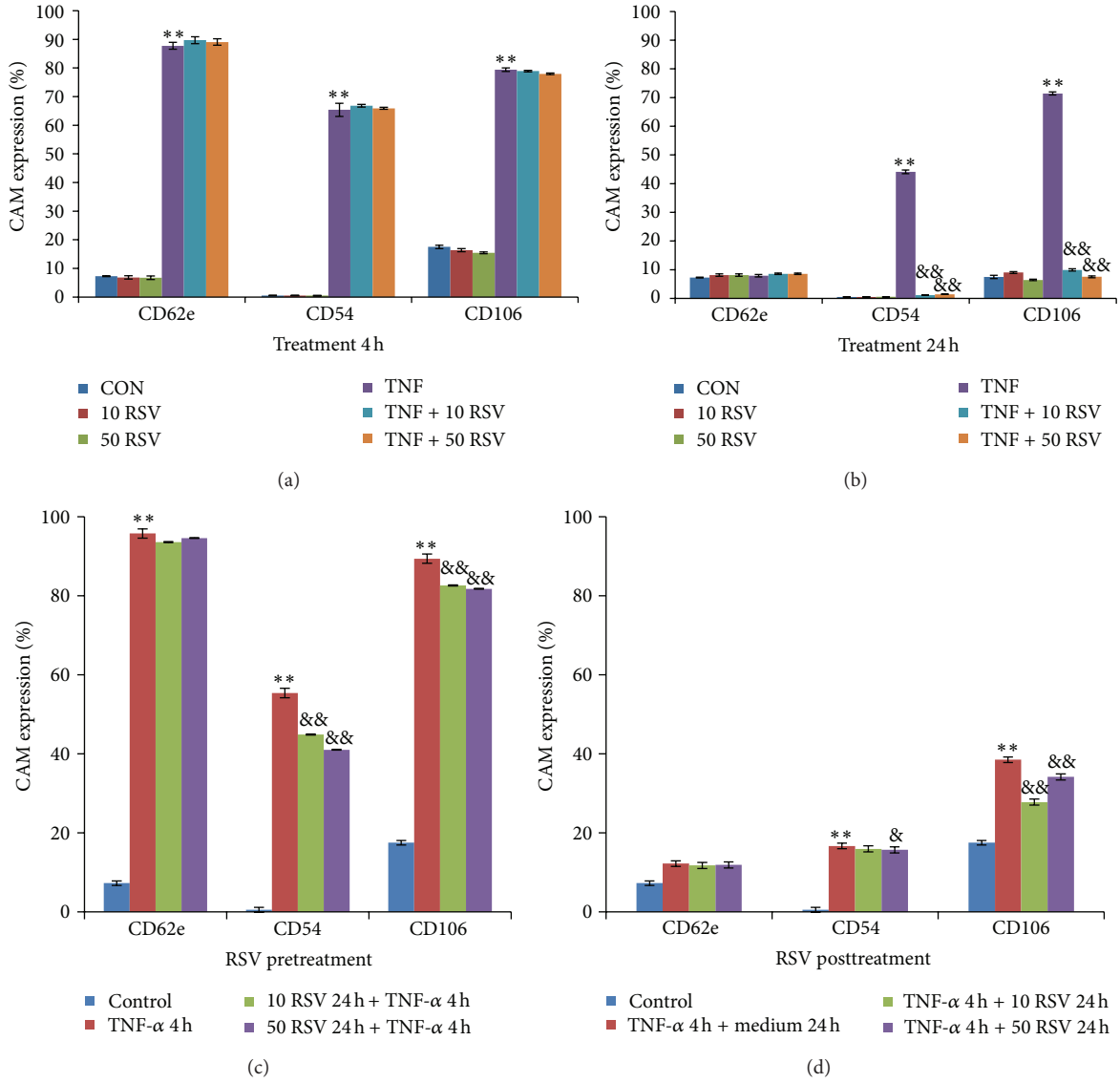

Figure 4: Resveratrol inhibited CAM expression induced by TNF-α (10 ng/mL). TNF-α significantly stimulated ICAM (CD54) and VCAM (CD106) expression after 4 H (a) and 24 h (b) treatment and significantly increased E-selectin (CD62e) expression after 4 h. Resveratrol (RSV) (10, 50 μM) inhibited expression of ICAM and VCAM in activated BAECs stimulated with TNF-α (10 ng/mL) during 24 h of cotreatment (b). Resveratrol (10, 50 μM) administered pretreatment (c) also significantly inhibited the expression of ICAM and VCAM activated by TNF-α, and significantly attenuated sustained ICAM and VCAM expression after TNF stimulation by posttreatment (d). Data are mean ± SEM from three independent experiments each performed in triplicate. $^{*}P < 0.05$; and $^{**}P < 0.01$ versus control and $^{\&}P < 0.05$; and $^{\&\&}P < 0.01$ versus TNF-α alone.

is easier to obtain primary macrovascular ECs from baboons in comparison with humans. Second, baboons exhibit a high degree of genetic, biochemical, physiological, and anatomical similarity to humans, and more closely resemble humans in comparison with other species in the pathology of vascular diseases [18]. Third, we preferred BAECs because they are hyperresponsive to TNF-α compared with venous ECs, and they may play a key role in macrovascular disease under adverse conditions [24]. Fourth, BAECs have similar responses to human aortic endothelial cells (HAECs) when stimulated with TNF-α and thus provide an effective model for translating therapies to human clinical trials [25]. BAECs can be used at an early stage of disease that may closely mimic the inflammation responses of ECs *in vivo*. We have established a protocol for primary BAEC culture and previously performed fundamental investigations on their morphology and functions [20, 26]. Additionally, Mikula-Pietrasik et al. recently demonstrated that the resveratrol and its derivatives have cell-specific effects, which were confirmed by having different effects on the proliferation of human from different sources [15]. Thus, we selected BAECs as our model for *in vitro* studies as a prelude to *in vivo* studies.

Proliferation and migration of ECs may play a crucial role in vascular self-repair in normal physiological as well as pathological conditions. EC monolayer integrity is maintained via proliferation and migration of neighboring cells.

We first investigated different experimental settings to verify the effects of resveratrol on BAEC proliferation under normal and inflammatory conditions. Resveratrol highly significantly enhanced BAEC proliferation at the optimal concentrations of 10–100 μM, and it attenuated cellular impairment caused by TNF-α treatment when administered to the cells as cotreatment, pretreatment, or post-treatment. Data concerning the influence of resveratrol on EC growth have not been completely consistent. Previous studies demonstrated that resveratrol induces increasing levels of apoptosis in with increasing concentration (25–100 μM) and induces apoptosis at 100 μM by cleavage of caspase 3 [16, 27], while low doses (0.1–25 μM) had no effect. Csiszar et al. demonstrated that 10 μM resveratrol prevented EC apoptosis induced by cigarette smoke extract [28]. Our results were partially consistent with those studies. Moreover, Ungvari et al. and Brito et al. showed that resveratrol at a concentration range of 1–100 μM attenuated apoptosis mediated by oxidative stress (TNF-α, oxidized LDL, and peroxynitrite) [29, 30]. With respect to EC apoptosis, the impact of resveratrol depends on concentration as well as cell source. Our study suggested that resveratrol is beneficial in protecting BAECs subjected to inflammatory stimulation as well as preventing cell injury at low concentrations.

The translation of *in vitro* and *in vivo* findings from experiments of animals and humans is thought to depend largely on parent resveratrol plasma concentration and bioavailability. The greatest plasma concentrations of resveratrol have been reported to be 2.36 μM by Boocock et al. [31] and 4.2 μM by Brown et al. [32] when 5 g of transresveratrol was administered to humans. However, the physiological plasma levels are either not detectable or below the micromolar concentrations that are typically employed *in vitro* (~32 nM–100 μM) in research with human cells. However, tissue resveratrol levels may be higher than what is suggested based on plasma levels because resveratrol is lipophilic. Moreover, resveratrol has shown efficacy at very low concentrations [33] in studies with animal models of human diseases and dramatically opposite effects depending on dose [34]. Our present studies investigated the protective effects of low concentrations of resveratrol on baboon ECs. Additional experiments with baboons are needed to determine if the *in vitro* effects of resveratrol on inflammation reflect *in vivo* effects that can be achieved by oral ingestion of resveratrol and if oral ingestion is cardioprotective.

EC migration is an essential process for a variety of vascular functions such as tumour growth, vascular remodelling, and vascular wound healing. Our results indicated that resveratrol at a low concentration ameliorates impairment of BAEC migration induced by TNF-α (0.1–50 μM). We found that a low concentration of resveratrol had no inhibitory effect on BAEC migration. However, a high concentration (100 μM) of resveratrol decreased BAEC migration. In et al. [27] showed that resveratrol inhibited endothelial cell migration at a dose range of 10–100 μM in human and bovine brain EC because of its antiangiogenesis properties. Our results also demonstrated that low doses of resveratrol attenuated the impairment of BAEC migration caused by inflammatory conditions, suggesting a mechanism for its anti-inflammatory properties. Cicha et al. [35] observed that resveratrol dose-dependently inhibited EC migration at 1–20 μmol/L in a Rho-associated-kinase- (ROCK-) dependent manner. Resveratrol promoted proliferation and migration of cerebral by activation of phosphoinositide 3 kinase (PI3-K)/Akt and mitogen-activated protein kinase (MAPK)/ERK signaling pathways [36]. However, a recent study also revealed that polyphenols from olive oil and red wine reduce inflammatory EC migration in cultured through MMP-9 and COX-2 inhibition [37]. We showed that resveratrol protected BAEC migration from inflammatory conditions. However, the cell specific responses and molecular mechanisms require further investigation to fully resolve the contradictory observations of effects of resveratrol on EC migration.

Inflammation is defined in part by the upregulation of cell adhesion molecules on the surface of in response to cytokines. Adhesion molecule expression is the molecular basis of leukocyte-endothelium interactions and an important characteristic of inflammatory reactions, which are critical in atherogenesis [30]. We investigated the regulation of adhesion molecules by resveratrol with and without TNF-α in BAECs. We demonstrated for the first time that resveratrol inhibits TNF-α-induced expression of ICAM-1 and VCAM-1 during long-term incubation after TNF-α stimulation, as well as by pretreatment and cotreatment. There was no inhibitory effect of resveratrol on CAM expression during short-term incubation, and this characteristic may contribute to its time-dependent inhibition of NF-κB [10]. E-selectin, the earliest adhesion molecule upregulated during leukocyte recruitment, was markedly increased after 4-h stimulation. We observed no significant inhibitory effects of resveratrol on the expression of *E*-selectin, which may be restricted by its long-term incubation.

Resveratrol decreased TNF-α-induced ICAM-1 and VCAM-1 expression, which may protect cells from TNF-α-induced cytotoxicity. However, the underlying molecular mechanisms by which resveratrol exerts its physiological effects require further investigation. Together, our findings highlight the power of resveratrol to protect primary ECs in culture and suggest that resveratrol may offer an alternative therapy for the prevention and treatment of cardiovascular disease. Preclinical trials with baboons are planned to evaluate the protective effects of resveratrol.

5. Conclusions

In summary, our data suggest that resveratrol may protect baboon ECs from cytotoxicity induced by TNF-α. Resveratrol may provide a pharmacological approach for suppressing injury under inflammatory conditions and for reducing risk of cardiovascular disease and diabetes.

Acknowledgments

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Clinical Study

Relationship between Stage of Diabetic Retinopathy and Pulse Wave Velocity in Japanese Patients with Type 2 Diabetes

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Objectives. We investigated the relationship between the stage of diabetic retinopathy and pulse wave velocity (PWV). *Methods*. This was a cross-sectional study of 689 patients (406 men and 283 women) with type 2 diabetes who were admitted to our hospital from 2004 to 2007. Brachial-ankle pulse wave velocity (baPWV) was measured by an arterial pressure measurement device as PWV/ABI. Diagnosis of diabetic retinopathy was made by ophthalmologists based on the Davis classification: no diabetic retinopathy (NDR), simple retinopathy (SDR), pre-proliferative retinopathy (pre-PDR), and proliferative retinopathy (PDR). *Results*. There was a significant difference in PWV between patients without diabetic retinopathy (1657.0 ± 417.9 m/s (mean ± SD)) and with diabetic retinopathy (1847.1 ± 423.9 m/s) ($P < 0.001$). In addition, the stage of diabetic retinopathy was associated with aortic PWV (1657.0 ± 417.9 m/s in NDR ($n = 420$), 1819.4 ± 430.3 m/s in SDR ($n = 152$), 1862.1 ± 394.0 m/s in pre-PDR ($n = 54$), and 1901.1 ± 433.5 m/s in PDR ($n = 63$) ($P < 0.001$)). *Conclusions*. In patients with diabetic retinopathy, even in those with SDR, PWV was higher than that in patients without diabetic retinopathy. Physicians should therefore pay attention to the value of PWV and macroangiopathy regardless of the stage of diabetic retinopathy.

1. Introduction

Pulse wave velocity (PWV) has been used as a noninvasive clinical index of aortic stiffness. It is reported that PWV of patients with diabetes is higher than that of healthy subjects [1]. In a Japanese report of more than 10,000 healthy subjects (age 30 to 74 years), it is reported that the mean ± standard deviation values of PWV are 1331.0 ± 242.0 m/s in male and 1207.0 ± 245.0 m/s in female [2]. It is considered that chronic hyperglycemia causes the progression of arterial stiffness. Chronic hyperglycemia also causes progression of diabetic microangiopathy including diabetic retinopathy. Previous studies have shown that two-hour plasma glucose, glycated hemoglobin, and fasting plasma glucose concentrations are predictors of the development of retinopathy and nephropathy [3, 4]. It was reported that the association of hyperglycemia with retinopathy is stronger than that with nephropathy [3]. In addition, microangiopathy is a strong predictor of the development of the more serious long-term complications of diabetes such as blindness, end-stage renal disease, amputation [5], and cardiovascular disease [6]. Previous studies have shown that PWV, a marker of arterial stiffness, is associated with the presence of diabetic retinopathy [7–11].

Retinal capillary microaneurysms are the hallmark of diabetic retinopathy and its earliest reliable sign, and individual acellular capillaries are usually visible histologically in the earliest stages of diabetic retinopathy. As retinopathy becomes more severe, larger patches of acellular capillaries are seen. When lesions like cotton-wool spots, intraretinal microvascular abnormalities, venous beading, and retinal hemorrhages are prominent, diabetic retinopathy is considered pre-proliferative, and new vessels are likely to appear soon on the surface of the retina or optic disc. When new

vessels appear on the surface of the retina or optic disc, diabetic retinopathy is said to have entered the proliferative stage [12].

To our knowledge, no study has compared PWV with the stage of diabetic retinopathy. Therefore, we investigated the relationship between increased PWV and the stage of diabetic retinopathy.

2. Methods

2.1. Subjects. From January 2004 to December 2007, 732 Japanese patients with type 2 diabetes who were admitted to Keio University Hospital (Tokyo, Japan) were consecutively observed. Among them, 43 patients with acute illness (e.g., cardiovascular event, stroke, infection, etc.) were excluded from the evaluation. Consequently, a total of 689 patients with type 2 diabetes who were admitted due to having poor glycemic control were enrolled in this study. All of their purposes of admission were to control glucose metabolism and education for diabetes. The study protocol was approved by the ethical committee of the hospital. Informed consent was obtained from all patients.

2.2. Measurements. The diagnosis of diabetic retinopathy was made by ophthalmologists based on the Davis classification: no diabetic retinopathy (NDR); simple retinopathy (SDR); pre-proliferative retinopathy (pre-PDR); and proliferative retinopathy (PDR).

During hospitalization, fasting plasma glucose (FPG), 2-hour plasma glucose (PG), C-peptide (CPR), hemoglobin A1c (HbA1c), glycated albumin (GA), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea nitrogen (UN), creatinine (Cr), uric acid (UA) in blood, and 24-hour urine microalbumin were measured. HbA1c was determined by high-performance liquid chromatography (Toso, Tokyo, Japan) and presented as the equivalent National Glycohemoglobin Standardization Program (NGSP) value [13]. Furthermore, we measured systolic/diastolic blood pressure, height, weight, BMI, waist, and hip circumference.

Measurements of brachial-ankle PWV (baPWV) were carried out using an automatic waveform analyzer (Colin Medical Technology Corporation, Japan). Patients lay in the supine position during the test, and occlusion and monitoring cuffs were placed around both the upper and lower extremities. PWV was calculated using the formula: baPWV = $(D1 - D2)/T1$, where $D1$ is the distance from the heart to the left ankle, and $D2$ is the distance from the heart to the right upper arm. These distances were calculated automatically on the basis of the patient's height. The pressure waveforms obtained at two different sites were simultaneously recorded, and the time interval between the initial rise in the brachial and tibial pressure waveforms was determined as $T1$. ABI was calculated using the formula ABI = ankle systolic BP/brachial systolic BP.

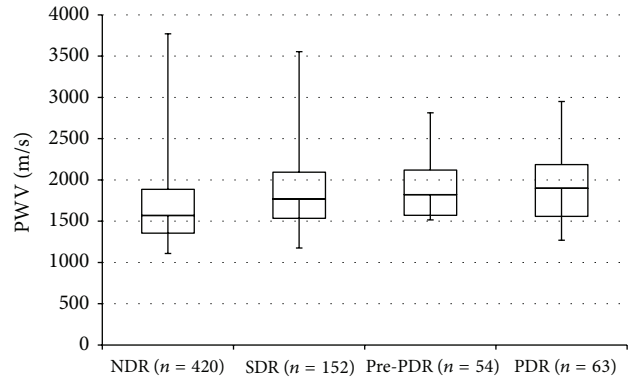

Figure 1: Pulse wave velocity (PWV) in NDR ($n = 420$), SDR ($n = 152$), pre-PDR ($n = 54$), and PDR ($n = 63$). Significant difference among groups ($P < 0.001$) was detected by the Kruskal-Wallis test. NDR: no diabetic retinopathy, SDR: simple diabetic retinopathy, pre-PDR: pre-proliferative diabetic retinopathy, and PDR: proliferative diabetic retinopathy.

2.3. Statistical Analysis. Demographic factors and baseline characteristics were summarized by diabetic retinopathy (DR) and NDR groups. They were compared between the DR and NDR groups using Mann-Whitney U test. Next, the patients were divided into four groups according to the stage of diabetic retinopathy (NDR, SDR, pre-PDR, or PDR) to investigate the relationship between each stage and the value of PWV by the Kruskal-Wallis test. The relationship between the PWV and each factor was evaluated with Spearman's correlation coefficient. The selected variables, which were statistically significant and clinically important, were included in nonparametric multiple regression models to evaluate the association between PWV and each stage of diabetic retinopathy adjusted for some covariates. The purpose of these multivariate analyses was to show the robustness of the results from the univariate analysis.

Data are presented as mean ± standard deviation (SD) in the text and tables. The significance level for all tests was two-sided, at 5%. All analyses were performed using SPSS 17.0 (SPSS; Chicago, IL, USA) and SAS 9.2 (SAS; Cary, NC, USA).

3. Results

Demographic factors and clinical baseline characteristics of patients are shown in Table 1. Among patients, durations of diabetes, age, SBP, and PWV were significantly higher in patients with retinopathy than in those without.

PWV in patients with diabetic retinopathy (1847.1 ± 423.9 m/s) was significantly higher than that in patients without diabetic retinopathy (1657.0 ± 417.9 m/s) ($P < 0.001$). Furthermore, there was a significant positive association between the stage of diabetic retinopathy and PWV. PWV was 1657.0 ± 417.9 m/s in NDR ($n = 420$), 1819.4 ± 430.3 m/s in SDR ($n = 152$), 1862.1 ± 394.0 m/s in pre-PDR ($n = 54$), and 1901.1 ± 433.5 m/s in PDR ($n = 63$) ($P < 0.001$) (Figure 1).

Some sensitivity analyses were performed to evaluate robustness of the results from the univariate analysis. Factors significantly correlated with the PWV by means of

Table 1: Clinical characteristics of patients with type 2 diabetes mellitus.

	Total	NDR	DR	*P* value
N (male/female)	689 (406/283)	420	269	
Duration (years)	12.0 ± 10.0	10.0 ± 9.5	16.0 ± 10.0	<0.001
Age (years)	62.2 ± 13.4	61.0 ± 14.3	65.0 ± 11.6	0.002
BMI (kg/m^2)	25.0 ± 5.5	25.6 ± 5.8	24.3 ± 4.7	0.001
SBP (mmHg)	132.0 ± 20.9	130.0 ± 20.0	135.0 ± 22.0	0.009
DBP (mmHg)	76.9 ± 14.0	77.0 ± 13.6	76.0 ± 14.5	0.907
HbA1c (%)	9.6 ± 2.0	9.7 ± 2.2	9.5 ± 1.7	0.233
LDL-C (mg/dL)	127.2 ± 37.1	128.0 ± 37.2	125.9 ± 37.2	0.295
PWV (m/s)	1731.2 ± 430.0	1657.0 ± 417.9	1847.1 ± 423.9	<0.001

Data are shown as mean ± SD. Comparison between patients without diabetic retinopathy (NDR) and with DR by Mann-Whitney's *U* test.
BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, LDL-C: low-density lipoprotein cholesterol, and PWV: pulse wave velocity.
HbA1c is presented as the National Glycohemoglobin Standardization Program (NGSP) value.

Table 2: Relationship between PWV and clinical factors by Spearman's correlation.

	Correlation coefficient	*P* value
BMI (kg/m^2)	−0.19	<0.001
Age (years)	0.61	<0.001
SBP (mmHg)	0.35	<0.001
DBP (mmHg)	−0.01	0.73
FPG (mg/dL)	−0.14	<0.001
HbA1c (%)	−0.20	<0.001
LDL-C (mg/dL)	−0.05	0.20
HDL-C (mg/dL)	0.00	0.98
TG (mg/dL)	−0.01	0.73
TC (mg/dL)	−0.04	0.33
ABI	0.07	0.06

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, FPG: fasting plasma glucose, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TG: triglyceride, TC: total cholesterol, and ABI: ankle brachial index.

Spearman's correlation coefficient were BMI, age, SBP, FPG, and HbA1c (Table 2). However, we decided that FPG and HbA1c should not be included in the multivariate analysis because they fluctuate by control of diabetes. We evaluated the association between the stage of diabetic retinopathy and PWV adjusted for the above-mentioned covariates by using nonparametric multiple regression analyses. As a result, when taking account of the covariates that have an effect on PWV, PWV tended to increase as the stage of diabetic retinopathy progression ($P < 0.001$).

4. Discussion

Measurement of aortic PWV is considered the gold-standard evaluation of arterial stiffness [14]. Values of PWV in patients with diabetes are higher than those in healthy people in the same generation [15]. The prevalence of arterial stiffness is increased in patients with type 2 diabetes, and these patients are at particularly higher risk for cardiovascular morbidity and mortality. Several studies have shown that diabetic retinopathy is associated with cardiovascular complications [16–18].

In the present study, PWV was significantly higher in patients with diabetic retinopathy than in those without. This finding supports the report that diabetic retinopathy is the microvascular complication with the strongest association with increased aortic stiffness [7]. In addition, there was a relationship between PWV and stage of diabetic retinopathy in Japanese patients with type 2 diabetes, in our study. We showed that the values of PWV in patients with diabetic retinopathy, even in those with SDR, were higher than those in patients without diabetic retinopathy. Henricsson et al. reported that the severity of diabetic retinopathy might be associated with survival, primarily owing to cardiovascular death in patients with diabetes [19]. While the severity of diabetic retinopathy might be important for prediction of macroangiopathy, physicians should pay more attention to macroangiopathy in patients with diabetic retinopathy, regardless of the stage.

Increased arterial stiffness is thought to be related to not only hyperglycemia but also to carbonyl and oxidative stress, chronic inflammation, endothelial dysfunction, and formation of advanced glycation end products (AGEs) [7]. It is reported that PWV is associated with the duration of diabetes and with the accumulation of fluorescent AGEs [20]. Besides, several reports indicate that the stage of diabetic retinopathy correlates with the accumulation of AGEs [21, 22]. Therefore, there is a possibility that the stage of diabetic retinopathy is associated with PWV through the accumulation of AGEs.

Several limitations should be taken into account when considering the results of this study. First, the cross-sectional study design and small sample size for each stage of diabetic retinopathy in our study make it difficult to infer the association between PWV and retinopathy. Second, we could not consider the effects of prescribed medication, for instance, anti-platelet agents, which could influence the state of both retinopathy and arterial stiffness. Third, the raw data might have deviated slightly because there was more than one PWV

technician and ophthalmologist. However, it was thought that the influence of bias was small because the technicians and ophthalmologists were experts and were not aware of this study when they carried out the examinations. Lastly, the patients with type 1 diabetes were not included in this study. In a recent meta-analysis of observational studies, diabetic retinopathy predicted all-cause mortality and cardiovascular events in patients with type 2 diabetes and also type 1 diabetes [18]. Based on these findings, physicians should pay attention to latent macroangiopathy in patients with not only type 2 diabetes but also type 1 diabetes who have diabetic retinopathy, even SDR.

In conclusion, this study suggested that PWV is significantly higher in patients with diabetic retinopathy than in those without, and that there is a relationship between the stage of diabetic retinopathy and PWV in Japanese patients with type 2 diabetes. Physicians should pay attention to latent macroangiopathy in patients with type 2 diabetes who have diabetic retinopathy, even SDR.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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Review Article

Zinc and Diabetic Retinopathy

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Zinc (Zn) is an important nutrient that is involved in various physiological metabolisms. Zn dyshomeostasis is often associated with various pathogeneses of chronic diseases, such as metabolic syndrome, diabetes, and related complications. Zn is present in ocular tissue in high concentrations, particularly in the retina and choroid. Zn deficiencies have been shown to affect ocular development, cataracts, age-related macular degeneration, and even diabetic retinopathy. However, the mechanism by which Zn deficiency increases the prevalence of diabetic retinopathy remains unclear. In addition, due to the negative effect of Zn deficiency on the eye, Zn supplementation should prevent diabetic retinopathy; however, limited available data do not always support this notion. Therefore, the goal of this paper was to summarize these pieces of available information regarding Zn prevention of diabetic retinopathy. Current theories and possible mechanisms underlying the role of Zn in the eye-related diseases are discussed. The possible factors that affect the preventive effect of Zn supplementation on diabetic retinopathy were also discussed.

1. Introduction

Zinc (Zn) is the second most abundant trace element in the human body and is an important nutrient and cofactor of numerous enzymes and transcription factors [1–3]. There are more than 300 catalytically active Zn metalloproteinase and more than 2000 Zn-dependent transcription factors. Zn is involved in homeostasis, immune responses, oxidative stress, apoptosis, and aging. Zn homeostasis results from the coordinated regulation by metallothioneins (MTs) and proteins in the Zrt/Irt-like protein (ZIP) and Zn transporter (ZnT) families [4–7]. These proteins are involved in the uptake, excretion, and intracellular storage/trafficking of Zn. Abnormalities in Zn homeostasis, such as its deficiency, may be associated with various pathogeneses of chronic diseases.

Metallothionein (MT) play a key role in scavenging of free radicals and is the main regulator of the intracellular transport and mobilization, storage, and transferring of Zn [8]. It is a cysteine-rich protein that binds metals such as Zn and copper and acts as an antioxidant that is very efficient in scavenging various free radicals or reactive oxygen species (ROS) [9, 10].

Diabetes mellitus affects 200 million people worldwide [11], including 20 million people in the United States alone [12]. Diabetic retinopathy (DR), a specific microvascular complication of diabetes, is the leading cause of blindness in working-aged persons in the United States [12]. The prevalence of DR increases with duration of diabetes [13], and nearly all persons with type 1 diabetes and more than 60% of those with type 2 have some retinopathy after 20 years.

Clinically, DR can be classified as nonproliferative DR (NPDR) and proliferative DR (PDR) [14]. NPDR is characterized ophthalmoscopically by the presence of microaneurysms and dot and blot hemorrhages. Severe NPDR (also called preproliferative DR) shows increased retinal microvascular damage as evidenced by cotton wool spots, venous beading, venous loops, and intraretinal microvascular abnormalities. If left untreated, PDR (characterized by abnormal retinal neovascularization) can develop. Clinically important outcomes of PDR are retinal and vitreous hemorrhage and tractional

retinal detachment [14], which ultimately result in blindness. Previous studies have shown that Zn supplementation attenuates oxidative changes at the early stage of diabetic rats, potentially preventing the early stages of DR, and delay its progression.

Several complications of diabetes may be related to increased intracellular oxidants and free radicals associated to decreases in intracellular Zn and Zn-dependent antioxidant enzymes [15]. Zinc effectively ameliorates diabetes-related complications in various animal models [16]. It is also an effective inducer of gene and protein expressions of MT, a potent antioxidant [17]. Therefore, the goal of the present paper was to summarize the information from the literature regarding (1) the role of Zn in diabetes, (2) the effect of Zn on the eye, and (3) the evidence that Zn prevents DR, as well as the possible mechanisms underlying this role. Finally, the future of Zn therapy for DR is briefly discussed.

2. Zinc and Oxidative Stress

It is well accepted that oxidative stress is elevated in various tissues that are associated with microvascular and macrovascular complications of diabetes [18, 19]. Increased oxidative stress contributes to the development of DR [20, 21], as an increase in reactive oxygen species (ROS) is considered a causal link between elevated glucose and other metabolic abnormalities that are important in its development [22]. Various antioxidants and nutrients have provided encouraging results in experimental models of DR [20, 21], although the results from clinical trials have been less conclusive [23–25]. In diabetic mice, overexpression of manganese superoxide dismutase (MnSOD), the enzyme responsible for scavenging mitochondrial superoxide, prevents early retinal lesions of retinopathy [26].

Zn has antioxidant properties and protects tissue from oxidative stress by two main mechanisms: (i) protection of protein sulfhydryl groups from free radical attack and (ii) reduction of free radical formation through the antagonism of redox-active transition metals, such as iron and copper (Cu) [27]. Each of these mechanisms results in decreased reactivity of sulfhydryl groups. The protection of protein sulfhydryls is thought to involve reduction of sulfhydryl reactivity through one of three mechanisms: (i) direct binding of Zn to sulfhydryl groups, (ii) steric hindrance as a result of Zn binding to another protein site in close proximity to the sulphydryl group, and (iii) a conformational change that results from Zn binding to another site on the protein. Some examples of proteins that Zn protects are dihydroorotase, DNA Zn-binding proteins (Zn fingers), and protein farnesyltransferase (Figure 1) [28].

As an antioxidant, Zn reduces the formation of free radicals by acting as an inhibitor of NADPH oxidase, which is an inducer of MTs (free radical scavengers) and an integral metal of Cu/Zn-SOD. ROS are known to activate NF-κB, which in turn, activates growth factors, antiapoptotic molecules resulting in cell proliferation (cancer), inflammatory cytokines, and adhesion molecules [29]. Zn also reduces inflammatory cytokine production by upregulating a Zn-finger protein, A20, which inhibits NF-κB activation via the TRAF pathway

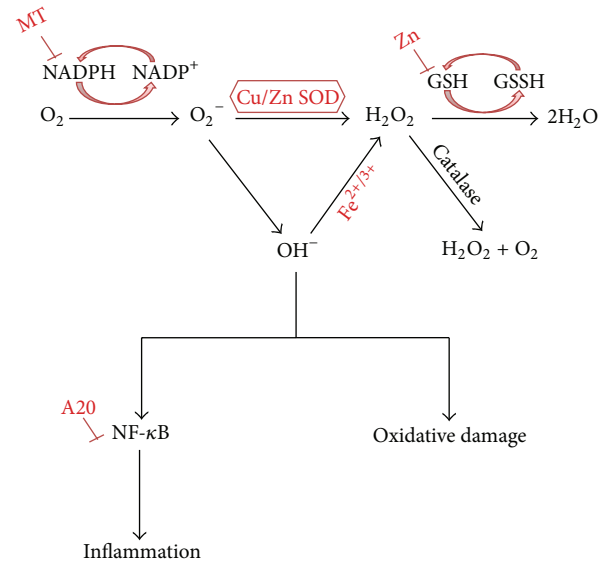

Figure 1: Proposed mechanism of zinc effect on oxidative stress and inflammation. Zinc attenuates oxidative damage and inflammation via MT, Cu/Zn SOD, Zn-finger protein, and itself.

[30]. Thus, Zn not only functions as an antioxidant, but also as an anti-inflammatory agent (Figure 1). In contrast to plasma Cu, plasma Zn concentrations and the Zn/Cu ratio were lower in diabetic subjects [31]. Meanwhile, it has been suggested that Zn supplementation protects against oxidative changes in the early stages of diabetes [32]. Zn supplementation also appears to have beneficial antioxidant effects in people with type 2 diabetes [33] (Figure 1).

3. Zinc and the Eye

Zn is indispensable to the growth and development of microorganisms, plants, and animals. It is found in all body tissues and secretions in relatively high concentrations, with 85% of whole body Zn found within the muscle and bones, 11% found in the skin and the liver, and the remaining found in all other tissues, with the highest concentrations in the prostate and parts of the eye [34], such as retina [35]. Zn appears to play an integral role in maintaining normal ocular function and is present in high concentrations in the ocular tissue, particularly in the retina and the choroid. Zn deficiency has been shown in a number of species to result in a variety of gross, ultrastructural, and electrophysiologic ocular manifestations.

Zn deficiency in rats dramatically affects ocular development. Severe Zn deficiency, when imposed upon rats during gestation, results in optic cup invagination failure, colobomata, retinal dysplasia, and occasionally anophthalmia in pups [36]. Acrodermatitis enteropathica is a rare early childhood disease, with multiple systemic manifestations caused by abnormalities in Zn metabolism. The ocular abnormalities include blepharitis, photophobia, conjunctivitis, corneal opacities, and cataracts [37]. Superficial punctate opacities, nebulous subepithelial opacities, and linear corneal

erosions have also been reported in cases of acrodermatitis enteropathica [37]. Recently, differential display was used to investigate gene expression in acrodermatitis enteropathica, and the results showed an insertional mutation that affects the mRNA of a Zn transport protein, resulting in decreased Zn absorption [38]. Recalcitrant corneal ulcers have been reported in conjunction with low serum Zn [39]. In addition, Zn has been indirectly linked with corneal ulcers because it may be required for the functional activity of collagenases [40].

Zn deficiency in humans results in poor dark adaptation and night blindness [41, 42], which in most cases can be reversed by Zn supplementation [43]. These alterations appear to be the consequence of defects in retinol processing in retinal pigment epithelial (RPE) cells during the visual cycle. The Zn metalloenzyme, retinol dehydrogenase, catalyzes the oxidation of retinol to retinal, and animal experiments and *in vitro* studies have demonstrated that the activity of retinol dehydrogenase can be impaired by Zn deficiency [44]. The above-mentioned changes in the localization of histochemically reactive Zn within the dark- and light-adapted states of the photoreceptor suggest that Zn possesses a unique role in the phototransduction process and/or the photoreceptor-RPE interaction, as occurs for vitamin A [45]. The presence of reactive Zn (Zn^{2+}) within photoreceptor terminals, and the evidence that exogenous Zn affects the electrophysiological activity of the distal retina, has led to the presumption that its corelease with glutamate may play an essential role in the modulation of information at the first synapse in the visual pathway. Although Zn release can be visualized in the outer synaptic layer of a retinal slice preparation, it cannot be ascertained with certainty that the release sites are at the presynaptic terminal and not from the mitochondria-rich inner segment or within the distal processes of photoreceptors and Müller cells. Synaptically released Zn may significantly influence neural processing in the vertebrate retina by modulating the activity of excitatory and/or inhibitory receptors as well as intracellular signaling proteins [46]. Therefore, the possibility exists that abnormal dark adaptation in Zn-deficient states is the result of an impaired Zn-dependent reaction in the visual cycle within the photoreceptors.

Cataracts are a common disease in older adults worldwide. Although some epidemiological studies have shown Zn involvement in the development of cataracts, the lowest concentration of Zn in crystalline lenses has been detected in patients with mature senile cataracts, while the highest concentrations have been detected in patients with traumatic cataracts [47]. However, the results from the largest randomized trial done in the United States showed no beneficial effect of supplement Zn with cupric oxide on the development or progression of cataracts [48].

Zn has a strong effect on the acceleration of MT synthesis [49]. Some studies have reported that MT-III plays a pivotal role as an endogenous neuroprotectant against light-induced retinal damage [50]. The mRNAs of MT isoforms (I–III) were upregulated in the murine retina by light exposure, and light-induced retinal damage is exacerbated in MT-III-deficient mice [50]. Furthermore, it has also been demonstrated that MT isoforms I and II do not have pivotal roles in protecting against light-induced retinal photoreceptor cell loss, whereas MT-III has neuroprotective effects, possibly due to its strong interaction with ROS.

Age-related macular degeneration is a leading cause of visual loss in older adults and is characterized by accumulation of membranous debris on both sides of the RPE basement membrane. This condition is thought to result from oxidative stress, and Zn deficiency is involved in its pathogenesis. It has been demonstrated that Zn [51, 52] and MT [53] levels are reduced in the RPE of the human eye in aged-related macular degeneration. In accordance with this, a study using a monkey model of early-onset aged-related macular degeneration [54] showed a four-fold decrease in retinal Zn content, decreased synthesis of MT, and increased oxidative stress in affected retinas compared to unaffected controls. In addition, it has been suggested that Zn supplementation prevents the appearance of age-related macular degeneration [55] and decreases the progression of the dry form of the disease [56]. These findings support the view that Zn deficiency is involved in the pathogenesis of age-related macular degeneration. Moreover, a clinical study showed a significant reduction in the Zn/Cu ratio in serum involved in the development of DR, thus demonstrating the importance of Zn in disease progression [57].

4. Zn Plays an Important Role in DR

Zn is a key element for maintenance of the structural and functional integrity of eukaryotic cells and tissues [58]. Many studies have addressed the importance of Zn as an antioxidant and therapeutic agent in several free radical initiation systems [59–61]. Some studies have reported the beneficial effects of antioxidants and Zn supplementation in preventing progression to advanced age-related macular degeneration, and people supplemented with antioxidants and Zn are less likely to lose visual acuity [62]. Moreover, Zn has been shown to protect the retina from diabetes-induced increased lipid peroxidation and decreased glutathione levels in rats either by stabilizing the membrane structure or by inducing MT synthesis [32]. Zn is essential for Cu-Zn SOD and inhibits diabetes-induced increases in plasma malondialdehyde and decreases in erythrocyte antioxidant defense enzymes [63]. Cu oxide functions as the active center of many cuproenzymes, including Cu-Zn SOD [64], and Cu deficiencies result in oxidative damage to lipids, DNA, and proteins [65]. The precise mechanism by which Zn and Cu exert their protective effects against retinal damage remains unclear, but there is a strong possibility that these nutrients help decrease oxidative damage.

It is apparent that DR can best be managed by tight glycemic control [21]. Moreover, antioxidant therapy may be a suitable approach for inhibiting intrinsic changes within the retinal capillary bed that leads to the development of DR. A previous study showed that alloxan causes a reduction in GSH levels and an increase in the levels of lipid peroxidation products (TBA reactants) in the retinas of diabetic rats, supporting the role of oxidative stress in the development of DR [32]. Many studies have addressed the importance of

antioxidants in the control of abnormalities in diabetic retinas [66–68]; however, many of these studies have indicated the inability of these antioxidants to lower blood hexose levels [20, 67, 69, 70]. Other studies have indicated the inability of some antioxidants to inhibit lipid peroxidation in diabetic eyes [71]. Above all, these studies reveal the ability of Zn to both minimize perturbations in plasma glucose levels in alloxan-diabetic rats and ameliorate deteriorative changes in the levels of TBA reactants and GSH in the retina [32].

The previous study indicates increased lipid peroxidation in the retinas of alloxan-treated rats that was associated with increased plasma glucose levels. On the other hand, the study reported elevated rates of liver lipid peroxidation accompanied by deterioration in glucose tolerance in GSH-depleted rats [72]. It has been suggested that in free radicals initiation systems, deterioration in glucose tolerance is attributed to impaired insulin action [73]. One study showed that impaired insulin-stimulated glucose transport across the cell membrane is a major mechanism underlying age-associated glucose intolerance in aged rats [74]. Free radicals are hypothesized to be one of the underlying causes of aging [75]. Initiating lipid peroxidation by free radicals in the lipid moiety of the cell membrane is presumed to result in distortion of the structural and functional integrity of the cell membrane or internal cellular components. This would interfere with the ability of insulin to initiate and propagate its normal sequence of actions [74], which may account, at least in part, for alloxan-induced hyperglycemia. Previous studies have shown that the treatment of alloxan-diabetic rats with Zn chloride resulted in the reduction of both plasma glucose levels and lipid peroxidation in the retinas of these rats [32]. The protective effects of Zn against increases in lipid peroxidation may be due to its ability to bind and stabilize cellular membranes against lipid peroxidation and disintegration [76]. An alternative protective mechanism of Zn may be its ability to induce MT synthesis. The high sulfhydryl content enables MT to efficiently scavenge oxyradicals [77, 78]. Another possible protective mechanism of MT is its ability to release Zn to bind to sites on membrane surfaces, thereby displacing adventitious iron and inhibiting lipid peroxidation [76]. Moreover, the suggested effect of Zn in inducing SH-rich MT synthesis may preserve the SH residue in many functional proteins. Therefore, Zn may preserve the structural and functional integrity of SH-dependent enzymes, including those that regulate glucose metabolism. More recently, it was hypothesized that MT, which is cysteine-rich, plays a role in nitric oxide signaling events via sequestration or release of Zn^{2+} by the unique thiolate clusters of the protein [79].

The protective effect of Zn against lipid peroxidation that is observed in the retinas of alloxan-diabetic rats could be of considerable importance for halting the progression of diabetes-related retinal degeneration, since a strong positive correlation has been found between lipid peroxidation products and vascular endothelial growth factor (VEGF) concentrations in the vitreous of patients with proliferative DR [68]. Moreover, one type of SOD, a major antioxidant enzyme, is Zn-dependent (Cu/Zn-SOD). Cu/Zn-SOD is a potent antioxidant enzyme that has recently been proposed to have a tumor-suppression effect [80]; therefore, its role in the protection against the development of DR should not be excluded. Thus, the importance of Zn as a protective antioxidant against DR may lie in its ability to initially exert good glycemic control, thus inhibiting the development of the deleterious consequences of hyperglycemia. In addition, Zn may be an important factor in inhibiting the progression of the intrinsic changes in diabetic retinas that eventually lead to the development of DR.

5. Possible Mechanisms of Zn Prevention of DR

As discussed above, Zn acts as a potent antioxidant. Therefore, the antioxidant action of Zn could be considered as the first possible mechanism. It is well known that hyperglycemia accelerates the formation of advanced glycation end products (AGEs), which have been implicated in the pathogenesis of DR [81]. They can stimulate ROS production in retinal pericytes, largely via activation of NADPH oxidase, which results in retinal pericyte apoptosis [82]. Other studies have suggested that the critical role of oxidative stress in pericyte apoptosis, as treatment of diabetic rats with a mixture of different antioxidants [20] or with trolox [66], was able to prevent pericyte loss in retina. As discussed above, Zn can prevent cells from oxidative damage, and NADPH oxidase is inhibited by zinc and SOD, which is both a zinc and copper-containing enzyme [29]. It is suggested that Zn might prevent retinal pericyte apoptosis via inhibition of NADPH oxidase in DR.

Ocular neovascularization, which is most potently caused by hypoxia and ischemia, is also a key component in DR [83, 84]. It has been convincingly demonstrated that hypoxia inducible factor-1 (HIF-1) and VEGF are involved in the initiation and progression of neovascularization in DR [85]. This has led to the finding of many new agents targeting VEGF [86]. Ischemia and hypoxia are similar conditions in cancer. Zn reduces inflammatory cytokine production by upregulating the Zn-finger protein, A20, which inhibits NF-κB activation via the TRAF pathway [30]. Some studies have shown that Zn supplementation can reduce VEGF expression by inhibiting NF-κB in prostate cancer [87]. It has been suggested that Zn might prevent neovascularization by inhibiting VEGF expression in DR (Figure 2). In addition, recent finding suggests that ZnT8 expression was reduced by ischemic insults and to restore the ZnT8 to its basal homeostatic levels can prevent retinas from ischemia induced injury [88]. Ischemia is also a key component in DR. Therefore, Zn supplement might rescue retina from DR (Figure 2).

Vascular leakage is also an important part of DR. p38 MAPK and NF-κB signaling pathways contribute to the regulation of claudin-5 expression, and these factors induce endothelial permeability [89]. It has been reported that Zn associates with p38 MAPK activation in the diabetic testis [90]. Moreover, recent studies have shown VEGF-induced vascular leakage in DR [91]. As discussed above, Zn might reduce VEGF by inhibiting NF-κB under conditions of hypoxia and ischemia; thus Zn might prevent vascular leakage in DR (Figure 2).

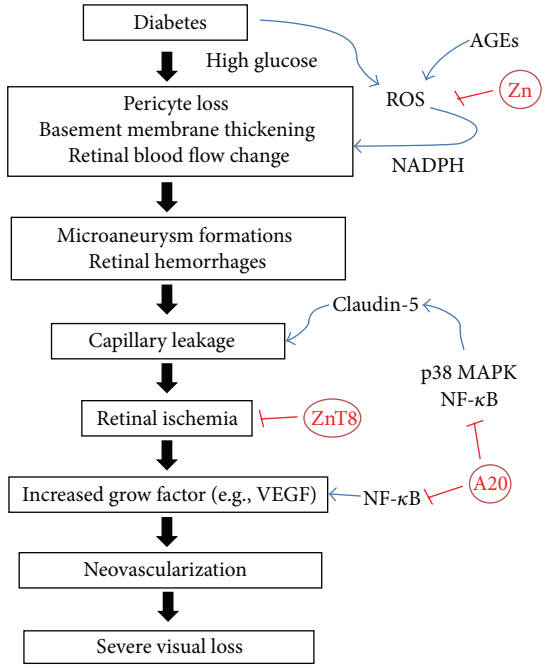

Figure 2: Proposed mechanism by which zinc protects from DR. Zinc protects DR by suppressing the pericyte apoptosis, capillary leakage, and neovascularization.

6. Conclusions

Increased oxidative stress plays an important role in many human diseases, such as diabetes and its complication. Zn supplementation seems beneficial for the patients with diabetes to control glucose levels. Zn as an antioxidant or via induction of MT attenuates ROS effect. Zn might protect retina from ROS induced pericytes apoptosis, capillary leakage, and neovascularization (Figure 2), thereby might have protective on DR. However whether this means that Zn supplementation can immediately be used to treat or prevent DR remains to be determined. A promising future for Zn supplementation will warrant further studies.

Abbreviations

AGEs: Advancedglycationendproducts
Cu: Copper
DR: Diabeticretinopathy
Znfingers: DNAZn-bindingproteins
HIF-1: Hypoxiainduciblefactor-1
MT: Metallothionein
MnSOD: Manganesesuperoxidedismutase
NF-κB: NuclearfactorkB
NPDR: NonproliferativeDR
PDR: ProliferativeDR
RPE: Retinalpigmentepithelialcells
ROS: Reactiveoxygenspecies
VEGF: Vascularendothelialgrowthfactor
Zn: Zinc
ZIP: Zrt/Irt-likeprotein
ZnT: Zntransporter.

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Research Article

Glucagon-Like Peptide-1 Analog Liraglutide Protects against Diabetic Cardiomyopathy by the Inhibition of the Endoplasmic Reticulum Stress Pathway

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Aim. This study aimed to investigate whether the glucagon-like peptide-1 analog liraglutide (LIRA) can protect against diabetic cardiomyopathy and explore the related mechanism. *Methods*. Rats were divided into 6 groups: a nondiabetic group, diabetic cardiomyopathy rats without LIRA treatment, diabetic cardiomyopathy rats with LIRA treatment (with high-, medium-, and low-dose, resp.), and diabetic cardiomyopathy rats treated with insulin. Cardiac function was examined by echocardiography before and after treatment. The histopathology of the heart was examined with H&E staining. The mRNA levels of XBP1, ATF4, and TRAF2 were analyzed by RT-PCR, and the expression of glucose-regulated protein 78 (Grp78), enhancer-binding protein homologous protein (CHOP), caspase-3, and caspase-12 was detected by western blot. *Results*. LIRA strongly improved cardiac function from both echocardiographic and histopathologic analyses, but insulin only partly increased cardiac function by improving FS and LVPW values. LIRA treatment can significantly decrease the expression of XBP1, ATF4, and TRAF2 ($P < 0.01$). LIRA also significantly downregulates the expression of Grp78, caspase-3 ($P < 0.01$), CHOP, and caspase-12 ($P < 0.05$). *Conclusions*. LIRA can protect against diabetic cardiomyopathy by inactivating the ER stress pathway. The improvement in cardiac function by LIRA is independent of glucose control.

1. Introduction

Diabetes mellitus is a serious, complex metabolic disease that affects approximately 4% of the population worldwide [1]. Cardiovascular complications are the leading cause of diabetes-related morbidity and mortality [2]. Although coronary atherosclerosis is the major cause of cardiovascular diseases in diabetic patients, diabetic cardiomyopathy increases the risk of heart failure independently of coexisting coronary artery disease, obesity, and hypertension [3, 4]. Diabetic cardiomyopathy refers to a disease process which affects the myocardium in diabetic patients causing a wide range of structural abnormalities, such as ventricular dilation prominent interstitial fibrosis and cardiomyocyte hypertrophy [5, 6], and eventually leads to left ventricular hypertrophy and diastolic and systolic dysfunction or a combination of these [7, 8]. The prevalence of diabetic cardiomyopathy may reach to ~60% in type 2 diabetic patients [9].

The pathogenesis of diabetic cardiomyopathy was intensively investigated during the past decade. Impaired calcium homeostasis, myocardial insulin resistance, increased lipid uptake, glucotoxicity, activation of the renin-angiotensin system, and increased oxidative stress are the major mechanisms [10]. Furthermore, the pathological role of endoplasmic reticulum (ER) stress is increasingly recognized.

The ER is a central organelle entrusted with lipid synthesis, protein folding, and protein maturation, and the ER is involved in the intrinsic pathway of apoptosis [11]. Various conditions, including hypoxia, ischemia, elevated protein synthesis, exposure to free radicals, hyperhomocysteinemia,

and gene mutations, can induce the pathological accumulation of unfolded proteins in the ER, a condition referred to as ER stress [12, 13]. ER stress plays a role in many pathological conditions, such as tumors, viral diseases, prion disease [14], and diabetic kidney disease [15]. Some complex homeostatic signaling pathways, such as the unfolded protein response (UPR), have evolved to deal with ER stress [16]. One action of UPR is to activate the expression of glucose-regulated protein 78 (Grp78), which is an ER resident protein that plays an important role in dealing with accumulated proteins. Moderate ER stress could alleviate injury triggered by stress, but severe and chronic stress could lead to apoptosis and induce many diseases. The activation of JNK and the transcriptional induction of CHOP and caspase-12-dependent pathways could initiate apoptotic processes [17, 18]. Recently more studies have strongly demonstrated the critical role of ER stress in the development of diabetic cardiomyopathy. The experimental evidence has been provided that two ER stress hallmarks, GRP78, and caspase-12 were upregulated in the diabetic rat hearts compared to normal rat hearts [19]. Whereas some drugs such as valsartan could relieve the ER stress-associated apoptosis, resulting in a significant prevention of cardiac remodeling [20].

Glucagon-like peptide 1 (GLP-1), a major incretin hormone, is released from L cells in gut in response to nutrients and potently stimulates glucose-induced insulin secretion [21]. In patients with type 2 diabetes, its secretion is diminished [22, 23], and incretin-based therapies have emerged as an important therapeutic option. Activation of the GLP-1 receptors enhanced insulin synthesis/secretion, suppressed glucagon secretion, slowed gastric emptying, and enhanced satiety [24].

The GLP-1R is fairly widely expressed in heart and vasculature [25, 26]. Therefore, in addition to its incretin effect, studies in both animals and humans have repeatedly shown a beneficial action of GLP-1 on cardiovascular system. Recent evidence has confirmed that GLP-1 increases myocardial glucose uptake independently of its ability to enhance insulin secretion [27–29] and increase survival of cardiac cells and cardiac function in rat models [30]. Treatment with GLP-1 analog liraglutide (LIRA) for 2 weeks in db/db mice downregulated genes involved in proapoptosis and endoplasmic reticulum (ER) stress [31].

In the current study, we investigated whether the GLP-1 analog, LIRA, improves diabetic cardiomyopathy in the STZ-induced diabetic rats. Our results indicate that GLP-1 analog, LIRA, improves cardiac function via the inhibition of ER stress in the rats with diabetic cardiomyopathy.

2. Materials and Methods

2.1. Animals Preparations and Experimental Protocol. Adult male SD rats (Animal Center of Jilin University, Changchun, Jilin Province), weighting 200–250 g, were studied. Rats were housed at 20–22°C on a 12-h light-dark cycle. Rats were separated into high-fat diet rats ($n = 110$) and control rats ($n = 10$). The former were fed with high-fat diet for 8 weeks and then given intraperitoneal injection of streptozotocin twice (STZ at 30 mg/kg, Sigma-Aldrich, USA, dissolved in citrate buffer, pH 4.5), and the latter were fed with regular chow and injected with the same dose of citrate buffer. Five weeks after the STZ injections, blood samples were harvested from the rat tail vein after 12 h of fasting. The levels of fasting blood glucose (FBG) were measured in spectrophotometry-based assays using commercially available kits (Invitrogen, USA). Those rats with FBG > 7.8 mmol/L were considered to be diabetic rats. The FBG of control rats is normal. STZ-induced diabetic rats were randomly studied in the following 5 different treated groups, non-LIRA group (DCM, $n = 8$), high dose of LIRA group (LH, 500 μg/kg, $n = 10$), medium dose of LIRA group (LM, 100 μg/kg, $n = 12$), low dose of LIRA group (LL, 50 μg/kg, $n = 10$), and insulin group (glargine 3.2 IU/kg, $n = 8$). At 8 weeks after LIRA or insulin treatment, rats were euthanized with ketamine HCl (50 mg/kg) and xylazine (10 mg/kg) for study. The levels of FBG, body weight, and cardiac function were measured by echocardiography at baseline and 8 weeks of treatment, and heart tissues were collected after 8 weeks of treatment. The investigation conforms to the Guide for the Care and Management of Laboratory Animals published by the Universities Federation for Animal Welfare (UFAW). The study protocols were approved by the Animal Care and Use Committee of the University of Jilin.

2.2. Echocardiographic Evaluation. Rats were measured with echocardiography to compare the development of diabetic cardiomyopathy. Two-dimensional and M-mode echocardiography images of rats were obtained using a commercially available 12 MHz linear array transducer system and an echocardiogram machine (HP, USA). M-mode recordings were of the left ventricle (LV) at the level of the mitral valve in the parasternal view using two-dimensional echocardiography guidance in both the short- and long-axis views. Pulsed-wave Doppler was used to examine mitral diastolic inflow in the apical four-chamber view. For each measurement, the data were averaged from three consecutive cardiac cycles. All measurements were made from digital images captured at the time of the study by the use of inherent analysis software (Sonos 5500 software packages).

2.3. Tissue Preparation and Hematoxylin-Eosin Staining. The LV was removed and sectioned into four slices along a plane parallel to the atrioventricular ring. The middle section was fixed in 4% buffered formalin, and 4 μm paraffin-embedded sections were prepared for hematoxylin-eosin (HE) staining. The remaining portion of the heart sample was stored at −80°C for western blot or semiquantitative reverse transcription PCR (RT-PCR) assays.

2.4. Western Blot Analysis. After extraction of myocardial proteins, equal amounts of the protein preparations were separated by 15% SDS-PAGE, as described in [32]. The separated proteins were transferred to nitrocellulose membranes (Invitrogen, USA) for 50 min at 120 V. The membrane was blocked with 5% nonfat milk in PBST (phosphate buffered saline, pH 7.6, containing 0.05% tween-20) for 2 h

at room temperature and then incubated with a primary antibody against Grp78 (1 : 1000, Santa Cruz, USA), caspase-3 (1 : 1000, Sigma, USA), CHOP (1 : 500 and 1 : 1000, resp., Stressgen, USA), and β-actin (1 : 600, Santa Cruz, USA) at 4°C overnight. After incubating with 1 : 4000 horseradish-peroxidase-(HRP-) conjugated anti-mouse/rabbit/goat IgG (Santa Cruz, USA), the blots were developed using enhanced chemiluminescence (PE Applied Biosystems, USA). The membranes were scanned densitometrically by Typhoon (Pharmacia, USA) and quantitated using Image Total Tech (Pharmacia, USA).

2.5. RNA Extraction and Semiquantitative RT-PCR. To evaluate the transcription of the myocardial ER stress-related factors, such as XBP1, ATF4 and TRAF2, semi-quantitative RT-PCR assays were performed. Total RNA was extracted from frozen myocardial tissues using TRIzol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Primers specific for XBP1, ATF4, and TRAF2 were synthesized and listed below: XBP1: sense: TGGCCGGGTCTGCTGAGTCCG, antisense: ATCCATGGGAAGATGTTCTGG; ATF4: sense: GTTGGTCAGTGCCTCAGACA, antisense: CATTCGAAACAGAGCATCGA; TRAF2: sense: ACCTGTGATGGCTGTGGC, antisense: TCTGTGAGGCTTGGGACT. β-actin was used as the internal control. Total cellular RNA (including HepG2 and HL-7702) was prepared using an RNA simple Total RNA kit (TIANGEN, China). Reverse transcription was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, USA), according to the manufacturer's protocol. Two microliters of RT reaction products was amplified by PCR in a volume of 50 μL under the following conditions: 94°C for 40 s, 60°C for 30 s, and 72°C for 30 s. After electrophoresis on a 1.5% agarose gel, the gel images of each PCR product were digitally captured with a CCD camera and analyzed with the NIH Imager beta version 2. Relative transcriptional values for each factor in the semi-quantitative RT-PCR are presented as the ratio of the signal value of the specific PCR product to that of β-actin.

2.6. Statistical Analysis. All data are presented as means ± SD. Statistical analyses were performed with SPSS13.0 software, using either student t-test or analysis of variance (ANOVA) with post hoc analysis as appropriate. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Development of DCM. The LV systolic parameters, including LV end-diastolic diameter (LVEDD) and LV posterior wall (LVPW), fractional shortening (FS), and ejection fraction (EF), significantly decreased in DCM group compared with CON group (Table 1). LV diastolic function variables expressed by the E-wave (early diastolic filling and early peak velocity) differed significantly in DCM rats compared with CON rats. A significant reduction in the E-wave velocity, a significant increase in the A-wave velocity, and a significant decrease in the E/A ratio was found, with an obvious decrease in FS and EF (Table 1). The altered cardiac diastolic performance is thought to result from reduced cardiac compliance. The results show that the diabetic cardiac muscle fibers were disordered, and many of the fibers were collapsed, according to the HE staining (Figures 1(a) and 1(b)). The levels of FBG in DCM rats were measured every week and showed a persistence of hyperglycemia of approximately 10 mmol/L from the 1st week after 5 weeks of two STZ treatments (hyperglycemic establishment) to the 8th week after hyperglycemia. Combined with the FBG data, histomorphological examination by HE staining confirmed the development of DCM.

3.2. LIRA Improves Cardiac Function of DCM Rats. As shown in Table 1, LVPW and LVEDD in DCM rats were significantly increased compared with CON rats ($P < 0.05$). However, upon long-term treatment with LIRA, LVPW and LVEDD in LIRA treated rats were significantly decreased (LL, $P < 0.05$ for both; LM, $P < 0.05$ for LVPW and $P < 0.01$ for LVEDD; LH, $P < 0.01$ for LVPW and $P < 0.05$ for LVEDD), compared with DCM rats. On the contrary, insulin (INS) decreased the LVPW level (Table 1, $P < 0.01$) but not the LVEDD level. EF and FS were improved significantly in the group of the long-term treatment with middle dose of LIRA ($P < 0.05$ for EF, $P < 0.01$ for FS, resp.). INS also improved the FS reduction in DM rats ($P < 0.01$) without the effect on EF. In the four treated groups (INS, LL, LM, and LH), only LM group had a marked improvement in the E/A ratio ($P < 0.01$). There was no change in the FBG level between DCM with and without the 8-week LIRA treatment. According to the HE staining results from the cardiac myocytes, we found that the disordered diabetic cardiac muscle fibers were repaired by the addition of LIRA, especially in the LM group and LH group (Figures 1(e) and 1(f)). The treatment with either INS or a low dose of LIRA could not strongly improve the cardiac structure (Figures 1(c) and 1(d)).

3.3. LIRA Inhibits Cardiac Myocyte Apoptosis in DCM Rats. The expression of Caspase-3 is measured to evaluate the apoptosis of cardiac myocyte since ER stress-associated apoptosis is significantly associated with cardiac remodeling. Our Western blotting showed that caspase-3 was activated significantly in DCM rats (Figure 2, $P < 0.05$). However, caspase-3 was significantly inhibited in LIRA-treated group, especially in the LM group, which shows Lira can decrease the apoptosis of cardiomyocyte.

3.4. LIRA Decreases ER Stress-Induced Myocardial Apoptosis by Downregulating the Expression of CHOP and Grp78 and Inactivating Caspase-12. Grp78 is an important molecular chaperone localized in the ER, which is usually regarded as an indicator reflecting the activation of ER stress. In the DCM rats, Grp78 expression was abundant in myocardium, suggesting that myocardial ER stress is present in DCM rats. However, the Grp78 expression in DCM rats treated with LIRA was significantly attenuated compared with the DCM group (Figure 3(a), $P < 0.05$). Since CHOP is also essential for ER stress-induced cardiomyocyte apoptosis in

Table 1: Echocardiographic and fasting blood glucose data.

	CON	DCM	INS	LL	LM	LH
n	4	4	4	7	8	8
FBG (mmol/L)	2.83 ± 0.39	10.39 ± 1.78[b]	8.49 ± 1.86[a]	8.6 ± 3.7[a]	7.8 ± 1.86[a]	7.47 ± 2.95[a]
E/A	2.45 ± 0.99	0.32 ± 0.04[a]	0.39 ± 0.06[a]	0.31 ± 0.03[a]	1.38 ± 0.09[ad]	0.35 ± 0.1[a]
EF	88.25 ± 0.96	75.75 ± 4.57[b]	88.5 ± 0.58	74.56 ± 6.96[a]	87.63 ± 5.97[c]	75.13 ± 2.95[b]
FS	52.25 ± 0.96	44.5 ± 2.52[b]	53.25 ± 0.5[d]	39.33 ± 8.46[b]	52.13 ± 3.76[d]	38.88 ± 2.3[b]
LVPW (mm)	3.47 ± 0.4	4.53 ± 0.5[a]	3.23 ± 0.17[d]	3.31 ± 0.38[c]	3.3 ± 0.42[c]	2.72 ± 0.31[ad]
LVEDD (mm)	5.54 ± 0.99	6.54 ± 0.35[a]	6.27 ± 0.68[a]	5.47 ± 0.59[c]	4.88 ± 0.76[ad]	5.13 ± 0.26[c]

LVEDD: left ventricular end-diastolic diameter; LVPW: left ventricular posterior wall; E/A: the ratio of E and A (E: peak early transmitral filling velocity during early diastole; A: peak transmitral atrial filling velocity during late diastole); FS: fractional shortening; EF: ejection fraction; FBG: fasting blood glucose. [a]$P < 0.05$ versus CON rats; [b]$P < 0.01$ versus CON rats; [c]$P < 0.05$ versus DCM rats; [d]$P < 0.01$ versus DCM rats.

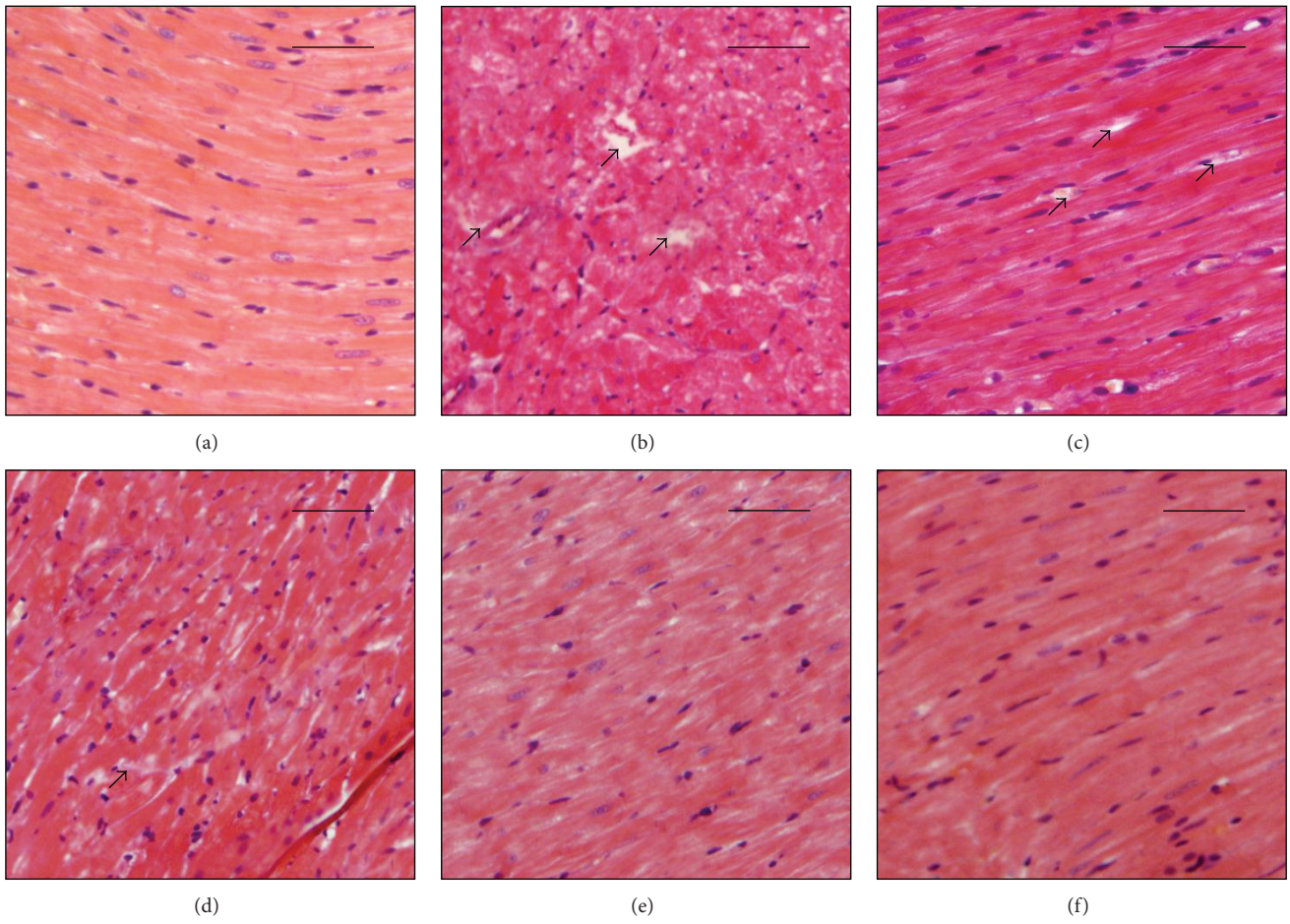

Figure 1: Histopathological improvement of LIRA-treated diabetic myocardium. The normal myocardium, diabetic myocardium and LIRA or ISN treated DCM myocardium were stained with HE staining (200x magnification). Representative samples of normal rats (a), diabetic cardiomyopathy rats (b), INS treated DCM rats (c), and LIRA treated DCM rats ((d) to (f)) were shown in the figure. Arrows indicate regions with ischemic myocyte degeneration in the subendocardial, subepicardial region and papillary muscles of the myocardium. Bar = 50 μm.

diabetes, we next examined the expression of CHOP and shows that the expression of CHOP was in parallel with the incidence of cardiomyocyte apoptosis in DCM rats. Interestingly, LIRA treatment at the middle and high doses decreased the expression of CHOP (Figure 3(b), LM and LH groups, both $P < 0.05$). Low-dose LIRA and insulin had no effect on CHOP expression (Figure 3(b)).

To further certify the ER stress, we observed caspase-12 activation in DCM rats. The results showed that caspase-12 activity was significantly increased in DCM rats compared with the CON (Figure 4, $P < 0.01$). When DCM rats were treated, only the LH group, but not the other treatment groups (including the LL, LM, and INS groups), showed a reduced level of caspase-12 activation (Figure 4, $P < 0.05$).

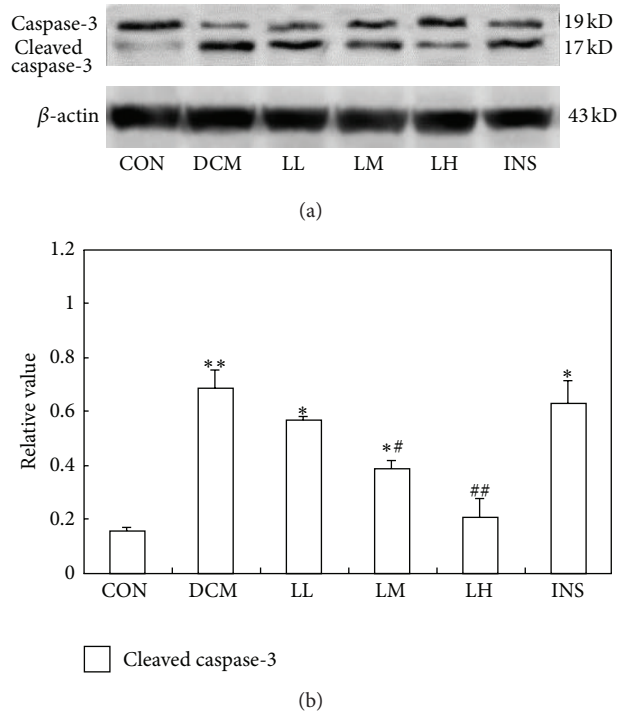

Figure 2: Effects of LIRA on the activation of caspase-3 in the DCM and CON. (a) Western blot was performed using each relevant antibody. β-actin was shown as a loading control. (b) Statistical analysis. Data were shown as mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CON rats, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ versus DCM rats.

3.5. LIRA Blocks the mRNA Transcription of Some ER Stress-Associated Factors. Because those factors of ATF4, TRAF2, and XBP1 are related to ER stress, we further analyzed their transcriptional levels using semi-quantitative RT-PCR. As shown in Figure 5, mRNA levels of ATF4, TRAF2, and XBP1 were significantly higher in the heart of DCM rats than that in CON rats ($P < 0.05$). On the contrary, LIRA treatment decreased them. INS did not change the levels.

4. Discussion

The current study demonstrates that GLP-1 exerts cardioprotective actions in experimental models of diabetic cardiomyopathy. While diabetes leads to myocardial structural and function abnormalities in vivo, administration of GLP-1 analog, LIRA, improved the disorder of cardiac muscle fibers and LV diastolic and systolic parameters. LIRA decreased some mRNA expression of ER stress-related factors and ER stress, which are associated with myocardial apoptosis. Thus, our data strongly suggest that activation of GLP-1 receptor protects against diabetic cardiomyopathy by the inhibition of the endoplasmic reticulum stress pathway.

Prior studies have demonstrated the presence of the GLP-1 receptor in the myocardium and even an increase in GLP-1 receptor in DCM [33]. This supports the idea that GLP-1 contributed to the beneficial extrapancreatic effects on heart independently of its role in pancreatic insulin

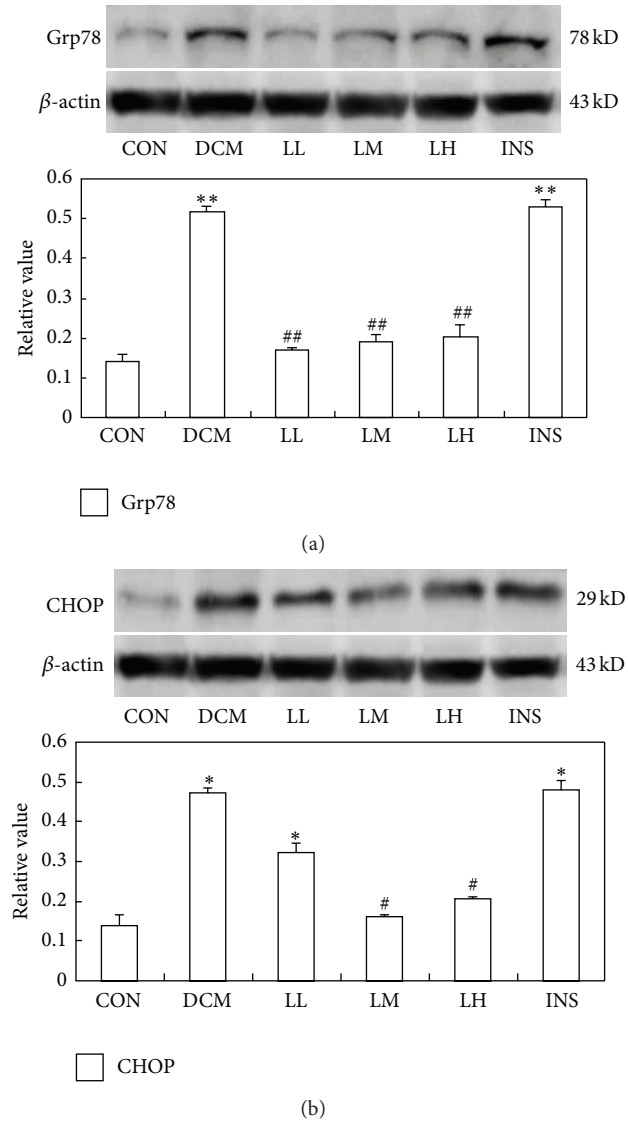

Figure 3: Immunoblot analysis for (a) Grp78 and (b) CHOP in the myocardium of DCM and CON rats. The upper trace of each panel shows representative blots of proteins in DCM and CON rats. The lower panels show the bar graphs summarizing the immunoblot data. Western blot was performed using each relevant antibody. β-actin was shown as a loading control. Data were shown as mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CON rats, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ versus DCM rats.

release. The current study further extends previous findings by demonstrating that activation of the GLP-1 receptor is beneficial to diabetic cardiomyopathy through decreasing ER stress.

Recent studies indicated that hyperglycemia-caused ER stress played an important role in diabetic cardiomyopathy [34], which is consistent with our study. The ER plays an essential role in the modification process after protein synthesis and is also where the disposal of abnormally folded proteins begins. Normally, the unfolded protein response (UPR) could result in upregulation of ER stress-associated

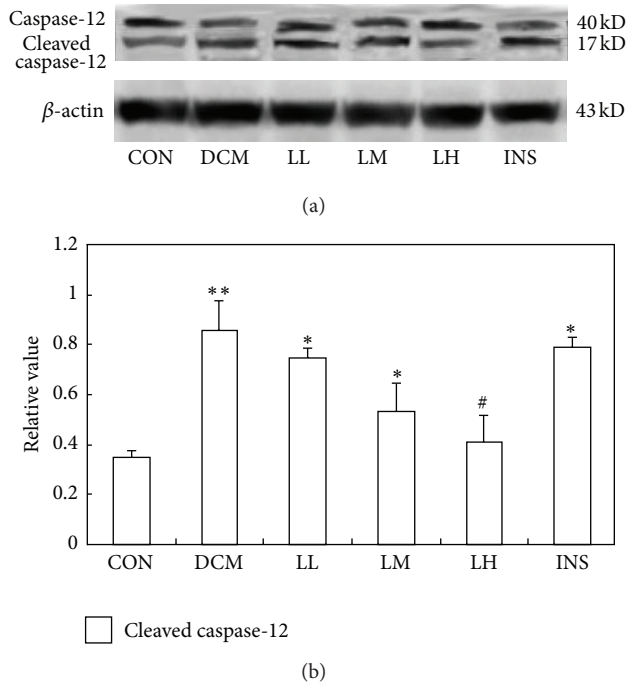

Figure 4: Effects of LIRA on the activation of caspase-12 in the DCM and CON. (a) Western blot was performed using each relevant antibody. β-actin was shown as a loading control. (b) Statistical analysis. Data were shown as mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CON rats, $^{\#}P < 0.05$ versus DCM rats.

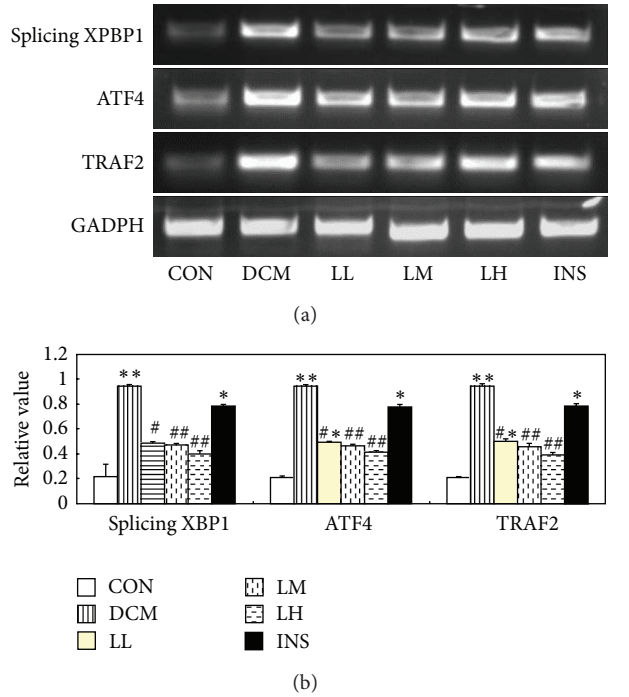

Figure 5: Semiquantification of mRNA levels of the ER stress-associated genes in the DCM and CON. (a) The ATF4, TRAF2, and splicing XBP1 were separated in 1.4% agarose gels. The relative value of each preparation is calculated by the gray numerical value of each specific product versus that of β-actin. (b) The average data of each preparation are evaluated based on three independent reactions and represented as mean ± S.D. Statistical differences of the data of each preparation compared with that of CON are illustrated as $^{*}P < 0.05$ and $^{**}P < 0.01$, and of DCM are illustrated as $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, respectively.

chaperone synthesis. In the diabetic cardiomyopathy rats, according to the metabolism drawback, the proteins in cardiac myocytes may suffer from UPR and trigger an increase in Grp78 protein. Actually, in our study, Grp78 is involved in ER stress in DCM rats and LIRA is responsible for the inhibition of Grp78 expression. Grp78 protein is an important molecular chaperone localized in the ER, which refers to the immunoglobulin heavy chain binding protein (Bip) and plays a vital role in the recognition of unfolded proteins. Grp78 also serves as a master modulator for the UPR network by binding to ER sensors, such as protein-kinase-R-(PKR-) like ER kinase, inositol-requiring 1 (IRE1). Activation of IRE1 induces X-box-binding protein mRNA splicing. The spliced XBP1 protein functions as a transcription factor, which induces the ER stress gene Grp78. We detected XBP1 mRNA, which is also overexpressed in the DCM rats and inactivated in the LIRA-treated DCM rats. This result is consistent with the upregulation of Grp78.

CHOP is the downstream protein of the apoptotic pathway and plays an important role in ER stress-induced apoptosis. CHOP can be activated by the overtranscription of ATF4, TRAF2, and XBP1 [35, 36]. Accumulation of CHOP can promote the transcription of ATF4, TRAF2, and XBP1, and overexpression of this factor can sensitize the ER stress of cells via increasing the expression of the CHOP protein. To explore the antidiabetic cardiomyopathy function of LIRA in DCM, CHOP is a key target. Our results showed that the expression of CHOP was significantly upregulated in DCM rats compared with CON rats. The overexpression of CHOP in DCM rats can be significantly inhibited by a medium and high dose of liraglutide (LM and LH groups), but there are no obvious changes in the LL group, which shows a dose effect of LIRA on the inhibition of the CHOP expression

Caspase-12 is exclusively located at the ER, and, following its activation, it can directly process downstream caspases in the cytosol, mainly caspase-9 and caspase-3 [37]. Caspase-12 mediated apoptosis is a specific apoptosis pathway of the ER, and apoptosis that occurred because of membrane or mitochondrial targeted signals would not activate caspase-12 [38]. In this study, caspase-12 also participates in ER stress in the DCM rats through enhancing its activity. This activated caspase-12 could only be blocked significantly by the addition of high-dose LIRA (LH group), which showed the same dose-dependent effect as CHOP protein. The release of TRAF2 is believed to be a biomarker for ER stress, which activates caspase-12. Furthermore, TRAF2-JNK is the third pathway in ER-associated apoptosis, which has been demonstrated to be vital in insulin resistance. Perhaps the upregulated mRNA of TRAF2 induced the activation of caspase-12 and the JNK apoptotic pathway. LIRA could inhibit ER stress via downregulating TRAF2 directly or indirectly. In our study, the increased induction of Grp78 and CHOP, the cleavage

of caspase-12, and JNK phosphorylation in diabetic cardiomyopathic rats paralleled with the destruction of cardiac function. The mechanism of the protection in LIRA group was triggered by the inactivation of the ER stress pathway, including CHOP, caspase-12, and JNK pathway in diabetic cardiomyopathic rats. However, the improvements in cardiac function by LIRA are independent of glucose control.

We employed echocardiography to evaluate the influence of LIRA on cardiac function for the first time and creatively found that the treatment of LIRA could protect against diabetic cardiomyopathy. It is interesting to note that, although high-dose LIRA showed the strongest effect on the inhibition of ER stress and histopathologic analysis also supported the results that high-dose LIRA could improve cardiac structure significantly, the echocardiographic evaluation showed that high-dose LIRA only decreased the parameters of LVPW and LVEDD. In contrast, insulin did not have significant effect on cardiac structure by histopathologic analysis, which was consistent with the results of ER stress. However, from echocardiography detection, insulin could improve the values of FS and LVPW, indicating a minor improvement on cardiac functions. All of this implied that the echocardiography results are not always paralleled with the molecular and histopathologic analyses. The phenomenon is worthy to be studied in detail in the future.

5. Conclusions

In conclusion, GLP-1 improves diabetic cardiomyopathy and heart function by the decrease of cardiac myocyte ER stress and subsequent myocardial apoptosis. This is an important finding because ER stress is one of the underlying mechanisms of diabetic complications. Thus, chronic treatment of GLP-1 analogs may significantly contribute to complication prevention in diabetes.

Abbreviations

CHOP: Enhancer-binding protein homologous protein
CON: Nondiabetic cardiomyopathy
DCM: Diabetic cardiomyopathy
E/A: The ratio of E and A
EF: Ejection fraction
ER: Endoplasmic reticulum
FS: Factional shortening
GLP-1: Glucagon like peptide-1
Grp78: Glucose-regulated protein 78
INS: Diabetic cardiomyopathy rats with insulin treatment
IRE1: Inositol-requiring 1
LH: Diabetic cardiomyopathy rats with LIRA high-dose treatment
LIRA: Liraglutide
LL: Diabetic cardiomyopathy rats with LIRA low-dose treatment
LM: Diabetic cardiomyopathy rats with LIRA medium-dose treatment
LVEDD: Left ventricular end-diastolic diameter
LVPW: Left ventricular posterior wall
PKR: Protein-kinase-R
UPR: Unfolded protein response.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Antibacterial Effect of Autologous Platelet-Rich Gel Derived from Subjects with Diabetic Dermal Ulcers In Vitro

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Background. Autologous platelet-rich gel (APG) is an effective method to improve ulcer healing. However, the mechanisms are not clear. This study aimed to investigate the antibacterial effect of APG in vitro. *Methods*. Platelet-rich plasma (PRP), platelet-poor plasma (PPP) and APG were prepared from whole blood of sixteen diabetic patients with dermal ulcers. Antibacterial effects against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were evaluated by bacteriostasis assay of APG, PRP, and APG-APO (APG combined with apocynin), with phosphate-buffered saline (PBS) and PPP as the control group. *Results*. (1) Compared to the PBS and PPP, the APG and APG-APO groups showed strong antibacterial activity against *Staphylococcus aureus*. There was no significant difference ($P > 0.05$) between APG and APG-APO. (2) Compared to PBS, APG, APG-APO, and PRP showed obvious antibacterial effects against *Escherichia coli* and *Pseudomonas aeruginosa*. No significant difference ($P > 0.05$) was revealed among the three groups. Compared to the PPP group, they did not show antibacterial effect against *Escherichia coli* and *Pseudomonas aeruginosa* ($P > 0.05$). *Conclusions*. APG has antibacterial effect against *Staphylococcus aureus* mediated by platelet activation in the diabetic patients with dermal ulcer, and does not present obvious antibacterial effect against *Escherichia coli* or *Pseudomonas aeruginosa*. Combination of APG and antibiotics may have synergistic antibacterial effect.

1. Introduction

Autologous platelet-rich gel (APG), prepared from whole blood, is a mixture of platelet-rich plasma (PRP), calcium, and thrombin. APG has been used to treat refractory diabetic dermal ulcers for several years. And it turns out to be effective in improving the healing of ulcers [1, 2]. Risk of infection decreases after the use of APG on surgical wounds, in addition to its effect on facilitating healing [3]. One study has revealed the antibacterial activity of APG against *Staphylococcus aureus* [4], and similar results have been shown in our previous research [5]. But in previously published studies outcomes about antibacterial activities of APG against other bacteria (such as *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*) are different. And no research has revealed the effects of APG from diabetic ulcer patients. The purpose of the study is to investigate the antibacterial activity of APG (from diabetic patients with dermal ulcers) against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* in vitro.

2. Materials and Methods

2.1. Patients. Sixteen patients with diabetic dermal ulcers, 11 men and 5 women, with an average (mean ± SD) age of 61 ± 10 years, were enrolled. The mean duration of diabetes was 8 ± 4 years. The mean HbA1c was 8.8 ± 2.2%. Antibiotics were used intravenously because of clinical importance, and peripheral venous blood was drawn about 8 to 12 hours after antibiotics

were used the last time. This study was approved by the ethics committee of West China-hospital. Informed consent was signed and obtained from all subjects.

2.2. Preparation of APG. EDTA disodium salt anticoagulated whole blood was obtained from the 16 subjects. Following centrifugation at 313 ×g for 4 minutes, erythrocyte concentrate was removed. PRP and PPP were prepared by centrifugation (1252 ×g) for 6 minutes from the remaining plasma. Thrombin (Heilongjiang Dilong Pharmaceutical Co., China) and calcium gluconate (Sichuan Beauty Sport HuaKang Pharma Co., China) were added to PRP; the gel-like mixture is called APG [6].

2.3. Determination of Platelet and Leukocyte Counts. Platelet and leukocyte counts were measured in samples of whole blood and PRP. Platelet enrichment degree [7] was calculated to evaluate the efficiency of the PRP production.

2.4. Evaluation of Antibacterial Activity. *Staphylococcus aureus* (ATCC6538), *Escherichia coli* (ATCC8099), and *Pseudomonas aeruginosa* (ATCC15422) (provided by West China School of Public Health, Sichuan University, China) were used to evaluate the antibacterial activity of APG. Bacteria incubated for 24 hours were diluted with sterile PBS; final bacterial count was 10^7 colony-forming units (CFU)/mL.

Experimental samples were divided into six groups: Group 1 (APG), Group 2 (APG-APO), Group 3 (PRP), Group 4 (PPP), Group 5, and Group 6 (PBS) (Table 1). The APG-APO group was added with apocynin (Sigma-Aldrich Co., USA) to block the possible antibacterial activity of leukocyte [7]. In the PRP group, no thrombin was added to activate the platelets. The PPP group was designed to exclude the antibacterial activity of antibiotics used before when compared to the APG and APG-APO groups. The 5th and 6th groups (PBS1 and PBS2) were designed as the double-blank control. The final bacterial count in each tube was 10^6 CFU/mL.

After 0, 1, 4, 6, 8, 12, and 24 h, a 0.05 mL sample was taken from each tube. Serial 10-fold dilutions of each sample were made, and 20 uL samples were plated on Mueller-Hinton plates (Beijing Land Bridge Tech Co., China). After 24 h incubation at 37°C, the number of viable bacteria was determined.

The antibacterial rate was calculated using the following formula [5]:

$$\text{antibacterial rate} = \frac{\text{bacteria counts} - \text{bacteria counts in control group}}{\text{bacteria counts in control group}}. \quad (1)$$

2.5. Statistical Analysis. The data are reported as mean ± standard deviation. Analysis was performed in SPSS 13.0, using repeated measures analysis for antibacterial activity and one-way ANOVA followed by Tukey's test for platelet count. A value of $P < 0.05$ indicates statistical significance.

Table 1: Components of each group.

Content (mL)	1 APG	2 APG-APO	3 PRP	4 PPP	5 PBS 1	6 PBS 2
Bacteria	0.1	0.1	0.1	0.1	0.1	0.1
PRP	0.5	0.5	0.5	—	—	—
PPP	0.4	—	0.45	0.95	—	—
Thrombin-calcium	0.05	0.05	—	—	—	—
Apocynin	—	0.4	—	—	—	—
PBS	—	—	—	—	0.95	0.95

3. Results

3.1. Platelet and Leukocyte Counts. The average volume of blood obtained from subjects was 45 mL, and an average of 4.5 mL PRP was harvested. The platelet count was (242.56 ± 72.33) $\times 10^9$/L in whole blood, while (1968.8 ± 874.58) $\times 10^9$/L in PRP with an average 8.1-fold enrichment of platelet concentration after the processing ($P < 0.05$), about (78.56 ± 20.79)% platelet enrichment degree. The leukocyte count of PRP was (5.75 ± 1.46) $\times 10^9$/L, similar to (5.58 ± 5.89) $\times 10^9$/L in whole blood.

3.2. Antibacterial Activity

3.2.1. Bacterial Counts of Staphylococcus aureus over 24 Hours. Bacterial counts in the APG and APG-APO groups showed a rapid and pronounced decrease compared to PBS group in the first 4 hours ($P < 0.05$) and were still lower than the PBS group in the following 6 hours, although no significant difference was noted ($P > 0.05$). Compared to PRP and PPP groups, bacteria counts in the APG and APG-APO groups reduced significantly ($P < 0.05$) during 24 hours. There were no statistical differences between the APG and APG-APO groups ($P > 0.05$) and between the PRP and PPP groups ($P > 0.05$) (Figure 1(a)).

3.2.2. Bacterial Counts of Escherichia coli over 24 Hours. Compared to PBS group, bacterial counts in the APG, APG-APO, PRP, and PPP groups significantly decreased ($P < 0.05$), while no statistical difference was shown among the four groups ($P > 0.05$) (Figure 1(b)).

3.2.3. Bacterial Counts of Pseudomonas aeruginosa over 24 Hours. Similar to results of *Escherichia coli*, bacterial counts of *Pseudomonas aeruginosa* in the APG, APG-APO, PRP, and PPP groups reduced significantly ($P < 0.05$) compared to the PBS group, while no statistical difference was shown among the four groups ($P > 0.05$) (Figure 1(c)).

3.2.4. Antibacterial Rate. Compared to PBS group, the antibacterial rate of APG against *Staphylococcus aureus* reached 77% in the first 4 hours and slowly declined to 61% at the 24th hour. The antibacterial rate against *Escherichia coli* was between 61% and 91%. APG showed lower antibacterial activity against *Pseudomonas aeruginosa* with antibacterial rate of 41% to 70% (Figure 2(a)).

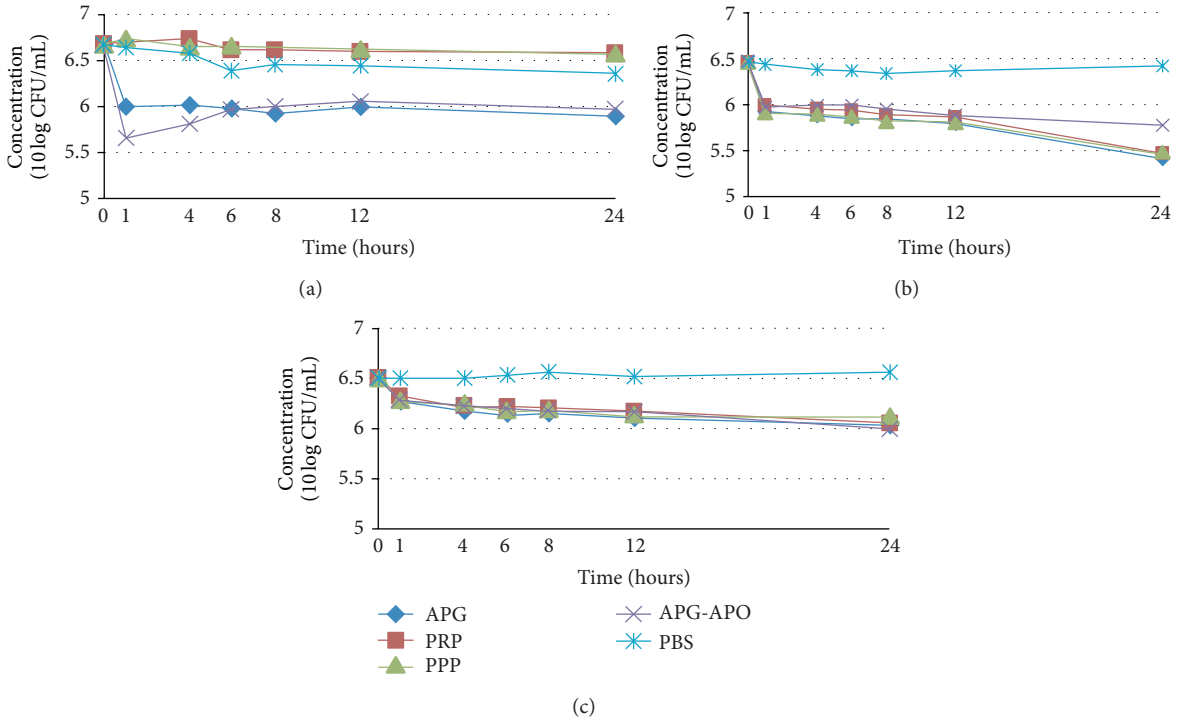

Figure 1: Effect of APG on the counts of various bacteria ((a) *Staphylococcus aureus*; (b) *Escherichia coli*; (c) *Pseudomonas aeruginosa*). APG: autologous platelet-rich gel; APG-APO: APG combined with apocynin; PRP: platelet-rich plasma; PPP: platelet-poor plasma; PBS: phosphate-buffered saline.

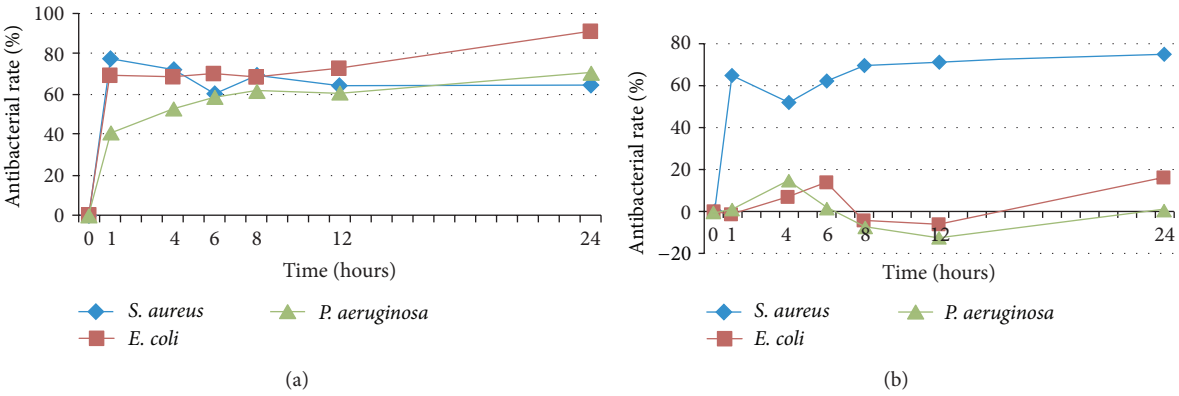

Figure 2: Antibacterial rate of APG against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* ((a) compared to PBS group; (b) compared to PPP group). *S. aureus*: *Staphylococcus aureus*; *E. coli*: *Escherichia coli*; *P. aeruginosa*: *Pseudomonas aeruginosa*.

Compared to PPP group, the antibacterial rate of APG against *Staphylococcus aureus* fluctuated between 52% and 77% over 24 hours, while the antibacterial rate of APG against *Escherichia coli* and *Pseudomonas aeruginosa* never reached 20% over 24 hours (Figure 2(b)).

4. Discussion

Antibacterial activity of APG against *Staphylococcus aureus* was revealed in this study. And similar effect of APG-APO is also found. This result is consistent with our previous study [5]. In addition, studies from Moojen et al. [4], Bielecki et al. [8], and Isaly and Beckley [9] indicate the similar antibacterial activity against *Staphylococcus aureus*. Compared to PRP, PPP almost does not have erythrocytes, leukocytes, and platelets, while other plasma components (such as proteins and antibiotics used) are similar to the PRP. In order to exclude the antibacterial activity of antibiotics used, the PPP group was designed as a positive control. Furthermore, apocynin was used to block the possible act of leukocytes on bacteria in this study. In this study, we found that there

was obvious antibacterial activity of APG when compared with the PPP group. Similar antibacterial activity of APG and APG-APO makes us conclude that the antibacterial effect is not derived from leukocytes. While compared to PRP, the antibacterial effect of APG is still profound. Because the components in APG and PRP are largely the same, except the extra added thrombin and calcium in APG, there is a reason for the contribution of the antibacterial activity to the activation of platelets.

While considering the effect against *Escherichia coli* and *Pseudomonas aeruginosa*, we revealed that similar antibacterial activity is found in the PRP, PPP, APG, and APG-APO groups when compared with the PBS group. Because of the negligible amount of platelets in PPP, the antibacterial activity of PRP, APG, and APG-APO cannot be attributed to the activated platelets and may be contributed to the antibiotics used before.

In fact, platelets play an important part in host-defense system. The abnormality of quantity and quality of platelets can exacerbate infection and increase its related mortality [10–14]. Thrombin, a strong agonist of platelets, was added in the processing of APG. Although the antibacterial mechanisms of APG are not clear, platelets may be play a role. Activated platelets could not only release various growth factors [7] that play an important role in improving the healing of ulcers, but also secret platelet microbicidal proteins (PMPs) [15]. PMPs contain a series of materials which have antibacterial activity, including platelet factor 4, regulated upon activation of normal T-cell expressed and secreted protein, connective tissue-activating peptide 3, platelet basic protein, thymosin beta-4, fibrinopeptide A, and fibrinopeptide B. PMPs could possibly play a role through the following mechanisms: contacting the bacterial membrane, changing the membrane permeability, entering the cell, and inhibiting the synthesis of big molecules [15].

In conclusion, antibacterial activity of APG against *Staphylococcus aureus* was further confirmed, and the effect may be attributed to the activation of platelets in APG. The effect of APG against *Escherichia coli* and *Pseudomonas aeruginosa* comes probably from previously used antibiotics. Therefore, APG itself may have no antibacterial activity against the two bacteria.

However, it is worth noting that the study in vitro is not as the same that as we meet in clinics, such as hyperglycemia, wound repair damage, and immunology change in diabetic patients with severe foot ulcers. Although decreased infection rate was observed by Kachel [3], more research of the antibacterial activity of APG in vivo should be done.

So far, the antibacterial mechanisms of APG are not clear; further investigation is needed to elucidate them.

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Research Article

Insulin Sensitivity and Beta-Cell Function Are Associated with Arterial Stiffness in Individuals without Hypertension

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Aim. We investigated the relationship between brachial-ankle pulse wave velocity (baPWV) and glucose levels, insulin sensitivity, and beta-cell function in Chinese individuals with or without hypertension. *Methods*. We recruited 3137 nondiabetic individuals whose age, body mass index (BMI), glucose levels, blood pressure (BP), lipids, hemoglobin A1C (HbA1c), baPWV, and insulin levels were measured. *Results*. In normotensive group, 2 h glucose levels ($\beta = 0.046$, $P < 0.001$) associated with baPWV, showed a significant increase in patients with NG as compared to those with DM ($P = 0.032$). The hypertensive group showed no such differences. The Matsuda index ($\beta = 0.114$, $P < 0.001$) and HOMA-β ($\beta = 0.045$, $P < 0.001$) were negatively correlated with baPWV while lnHOMA-IR ($\beta = 0.196$, $P = 0.076$) and the Quantitative Insulin Sensitivity Check Index (QUICKI) ($\beta = 0.226$, $P = 0.046$) showed a borderline negative correlation. BaPWV significantly decreased ($P = 0.032$) with an increase in insulin sensitivity in individuals with both normal BP and glucose tolerance. *Conclusions*. BaPWV was significantly associated with 2 h glucose levels, insulin sensitivity and beta-cell function in normotensive population, whereas in hypertensive individuals, BP was the dominant factor influencing arterial stiffness. Individuals with abnormal insulin sensitivity in the absence of diabetes and hypertension are also at an increased risk of arterial stiffness.

1. Introduction

It is well known that the prevalence of cardiovascular disease (CVD) is high among individuals with abnormal glucose tolerance, resulting in significant mortality and morbidity rates from macrovascular diseases in such individuals. A large number of population studies have focused on the relationship between arterial stiffness and glucose abnormality; however, the conclusions remain controversial. Among these studies, most have revealed a positive association between arterial stiffness and type 2 diabetes [1–4], while some have found fasting and postchallenge glucose levels and HbA1c levels, which reflect 2-3 months of glycemic control, to be independently related to accelerated stiffening of arteries [4–6]. However, the best predictor of arterial stiffness among these remains unclear. Some studies show that 2 h blood glucose levels are better predictors than fasting blood glucose levels and HbA1c levels [7, 8], while another study reported different results [6]. In addition, recent studies have suggested that 1 h or 30 min blood glucose levels during the oral glucose tolerance test (OGTT) may also demonstrate a stronger correlation with arterial stiffness [9–11]. Variability in populations and evaluated factors between different studies may have led to these inconsistencies.

Indeed, individuals with CVDs and hypertension, who also commonly display abnormal glucose metabolism [12], individuals were rarely included in these studies. Therefore, we wanted to determine whether plasma glucose levels had the same effects on arterial stiffness in individuals with and without hypertension as well as elucidate the glycemic status that exerted maximum influence on arterial stiffness. Measuring pulse wave velocity (PWV) is widely used to assess arterial stiffness in the clinical setting because of its convenience and noninvasiveness [13]. Recent studies showed that PWV, especially brachial-ankle PWV (baPWV), can be used as a tool for screening cardiovascular risk and as

a marker for assessing the severity of atherosclerotic vascular damage in the general population [14]. baPWV has been shown to be related with several factors such as age [15], blood pressure (BP) [16], dyslipidemia [17], and obesity [18]. Increased BP and glucose tolerance are also associated with increased insulin resistance (IR); however, few reports have described the relationship between IR and PWV. Therefore, we investigated the relationship between baPWV and glucose metabolism in a large sample of Chinese individuals with or without hypertension and delineated the impact of different glucose metabolism conditions on baPWV as well as the impact of the interaction between glucose levels and BP on arterial stiffness.

2. Subjects and Methods

2.1. Study Population. We recruited community-dwelling individuals aged 40–75 years living in the Gulou district, Nanjing, Jiangsu Province, China, from June 2011 to December 2011. A total of 10027 participants were invited by telephone or door-to-door visit to take part in this study. The protocol was approved by the ethics committee of The First Affiliated Hospital with Nanjing Medical University. After excluding individuals with a history of type 2 diabetes and cerebral, cardiovascular, peripheral artery, liver, and chronic kidney diseases, 3137 individuals were included in our study. The use of antihypertensive agents was also an exclusion criteria for our study.

2.2. Clinical and Biochemical Measurements. All measurements and procedures were performed from 7:30 to 11:30 AM following an overnight fast. The heights and weights of the subjects were measured with individuals dressed in only a single layer of clothes without shoes. Body mass index (BMI) was expressed as the weight in kilograms divided by the height in meters (kg/m^2). The waist circumference was measured in the standing position at the level of the umbilicus by traditional examiners, and the waist-to-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference. Fasting parameters, including levels of fasting plasma glucose (FPG), HbA1c, triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and total cholesterol, were measured in each subject using standardized procedures. A 75 g OGTT was performed with plasma glucose sampling done at 0, 30, and 120 min. HbA1c levels in peripheral blood were measured using high-performance liquid chromatography (HPLC, BIO-RAD Company, USA). Plasma glucose levels were measured using the hexokinase method, HDL levels using the accelerator selective detergent method, LDL levels using the liquid selective detergent method, triglyceride (TG) levels using the glycerol phosphate oxidase method, and total cholesterol levels using the enzymatic method. All measurements were analyzed by the autoanalyzer (Modular E170; Roche). Serum insulin concentrations were determined with the Phadebas Insulin Test (Pharmacia, Uppsala, Sweden) using a radioimmunosorbent technique. Glucose metabolic status was categorized as follows: normal glucose tolerance (NGT; fasting glucose, <5.6 mmol/L; 2 h glucose, <7.8 mmol/L), prediabetes mellitus (pre-DM; including impaired fasting glucose: 5.6 mmol/L ≤ fasting glucose < 7 mmol/L or impaired glucose tolerance: 7.8 mmol/L ≤2 h glucose < 11.1 mmol/L, among them 143 participants had both IFG and IFG in this group), and diabetes mellitus (DM; fasting glucose ≥ 7.0 mmol/L or 2 h glucose ≥ 11.1 mmol/L).

Blood pressures (BP) was measured using a standardized sphygmomanometer and quantified from the mean of 3 consecutive measurements taken with at least 5 min intervals of rest. Hypertension was diagnosed if the average systolic BP (SBP) was ⩾140 mmHg or diastolic BP (DBP) was ⩾90 mmHg. BaPWV was measured in the supine position using a waveform analyzer (VP-1000; Colin Co., Komaki, Japan) after at least 5 min of bed rest. Briefly, the PWV value was calculated as the distance/transit time ratio and expressed in centimeter per second (cm/s). The mean of the left and right baPWV (average baPWV) was obtained for all participants and used for analysis.

2.3. Statistical Analysis. IR was calculated using the mathematical formula of HOMA-IR by dividing the product of fasting blood insulin (FBI; lU/mL) and fasting blood glucose (FBG; mmol/L) by 22.5. The Quantitative Insulin Sensitivity Check Index (QUICKI) was also used for the measurement of IR by dividing 1 by the sum of the logarithms of FBI and FBG [18]. The Matsuda Insulin Sensitivity Index (Matsuda ISI), which measures hepatic and peripheral IR, was calculated by the following formula: 10000/(FBG × FBI × mean glucose during OGTT × mean insulin during OGTT) [19]. Beta-cell function was estimated by the following formula: (20 × insulin (mU/L))/(glucose (mmol/L) − 3.5)). Collated data were analyzed using SPSS 17.0 for Windows (Chicago, IL, USA). Data from individual subjects was expressed as mean ± standard deviation, and the differences between groups were compared by Student's t-test. χ^2-statistic was used to compare proportions. Stepwise multiple linear regression analysis was performed to determine the effect of study variables on the PWV value. HOMA-IR was not normally distributed, so we transformed the variables logarithmically for all multivariate analyses. Comparisons of means were analyzed by one-way analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant.

3. Results

3.1. Baseline Characteristics of Subjects. Table 1 shows the biochemical and clinical variables of the groups classified by glucose tolerance. All parameters differed significantly among the three groups. Among them, age and HDL, LDL and total cholesterol levels did not differ between the pre-DM and DM groups. The pre-DM group had a higher baPWV compared with the normal group, while the DM group had the highest baPWV.

3.2. Relationship between baPWV and Other Variables. Multiple regression analyses were performed to determine the relationship between each variable and baPWV value for

Table 1: Baseline characteristics of the participants.

	NGT (n = 1990)	Pre-DM (n = 843)	DM (n = 304)	P value	Age and gender Adjusted P
Age (year)	54.71 ± 8.21[a,c]	57.81 ± 8.50	58.18 ± 8.41	<0.001	
Gender (male/female)	708/1082	358/485	147/157	<0.001	
BMI (Kg/m^2)	23.40 ± 2.89[a,c]	24.78 ± 3.23[b]	25.79 ± 3.03	<0.001	<0.001
WHR	0.86 ± 0.67	0.89 ± 0.63	0.90 ± 630.79	<0.001	<0.001
FPG (mmol/L)	5.35 ± 0.35[a,c]	5.83 ± 0.52[b]	7.45 ± 1.85	<0.001	<0.001
30 min PG	8.52 ± 1.52[a,c]	10.17 ± 1.48[b]	12.82 ± 2.68	<0.001	<0.001
120 min PG	6.04 ± 1.02[a,c]	8.49 ± 1.35[b]	13.89 ± 3.78	<0.001	<0.001
HbA1c (%)	5.60 ± 0.37[a,c]	5.82 ± 0.400[b]	6.85 ± 1.32	<0.001	<0.001
HDL (mmol/L)	1.39 ± 0.35[a,c]	1.32 ± 0.32	1.29 ± 0.29	<0.001	<0.001
LDL (mmol/L)	1.83 ± 0.75[a]	2.93 ± 0.82	3.04 ± 0.82	<0.001	<0.001
TG (mmol/L)	1.38 ± 0.92[a,c]	1.74 ± 1.12[b]	1.97 ± 1.31	<0.001	<0.001
CHOL (mmol/L)	4.85 ± 0.96[a,c]	4.99 ± 1.03	5.11 ± 1.03	<0.001	<0.001
SBP (mmHg)	123.43 ± 15.40[a,c]	130.58 ± 16.13[b]	136.16 ± 18.89	<0.001	<0.001
DBP (mmHg)	76.72 ± 10.13[a,c]	76.69 ± 10.50[b]	82.02 ± 11.70	<0.001	<0.001
baPWV (cm/s)	1424.36 ± 272.94[a,c]	1561.87 ± 330.39[b]	1657.71 ± 327.62	<0.001	<0.001
INS0	11.60 ± 7.26	12.40 ± 8.52	13.73 ± 9.61	<0.001	<0.001
INS30	71.56 ± 54.28[c]	71.59 ± 56.57[b]	44.70 ± 40.01	<0.001	<0.001
INS120	48.74 ± 39.30[a,c]	90.37 ± 69.90	89.60 ± 65.86	<0.001	<0.001

BMI: body mass index, WHR: waist-hip ratio, FPG: fasting plasma glucose, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TG: triglyceride, CHOL: cholesterol, SBP: systolic blood pressure, and DBP: diastolic blood pressure.
INS0: fasting serum insulin, INS30: 30-minute serum insulin, INS120: 120-minute serum insulin, HbA1c: hemoglobin A1c, and baPWV: brachial-ankle pulse wave velocity.
[a]NGT versus Pre-DM, $P < 0.05$.
[b]Pre-DM versus DM, $P < 0.05$.
[c]NGT versus DM, $P < 0.05$.

the overall population and for the groups subdivided according to BP. Table 2 shows that age ($\beta = 0.355$, $P < 0.001$), working heart rate (WHR) ($\beta = 0.078$, $P < 0.001$), heart rate (HR) ($\beta = 0.171$, $P < 0.001$), SBP ($\beta = 0.489$, $P < 0.001$), TG ($\beta = 0.045$, $P = 0.001$) levels, and 2 h plasma glucose levels ($\beta = 0.052$, $P = 0.008$) were positively associated with baPWV, while BMI ($\beta = -0.086$, $P < 0.001$) was negatively associated with baPWV. After dividing the subjects into two groups, the result displays that besides the aforementioned variables, HDL ($\beta = -0.038$, $P = 0.037$) levels were also associated with baPWV when BP was normal. In contrast, only age ($\beta = 0.523$, $P < 0.001$), BMI ($\beta = -0.149$, $P = 0.007$), HR ($\beta = 0.243$, $P < 0.001$), SBP ($\beta = 0.474$, $P < 0.001$), and TG ($\beta = 0.097$, $P = 0.004$) levels were still significantly related to baPWV in the individuals with hypertension.

3.3. Association between baPWV and Glucose Status in Individuals with or without Hypertension. We divided the subjects with different BPs into subgroups on the basis of diagnostic criteria or HbA1c levels, which represent glucose control in the last 3 months. With regard to the normotensive individuals, after adjustment for age, gender, BMI, lipids levels, HR, SBP, DBP and WHR, Figure 1(a) shows that the pre-DM group had a higher baPWV compared with the NGT group ($P = 0.005$), while the DM group had the highest baPWV among the three groups (NGT versus DM, $P < 0.001$; pre-DM versus DM, $P = 0.019$). When the diagnostic criteria were replaced by HbA1c levels, similar differences were found among the three groups. However, the difference between the NGT and pre-DM groups was not significant (NGT versus pre-DM, $P = 0.079$). On the other hand, no differences were observed for the hypertensive individuals, irrespective of the glucose metabolism status.

3.4. Independent Association of baPWV with the Indices of IR, Insulin Sensitivity, and Beta-Cell Function. As shown in Table 3, the Matsuda index ($\beta = -0.114$, $P < 0.001$) and HOMA-β ($r = -0.045$, $P < 0.001$) were negatively correlated with baPWV after adjustment for age, gender, BMI, WHR, lipids, HR, and MAP, while lnHOMA-IR ($\beta = 0.196$, $P = 0.076$) and QUICKI ($\beta = 0.226$, $P = 0.046$) showed a borderline negative correlation with baPWV. Only Matsudas index was weakly but significantly associated with baPWV in the hypertensive group ($\beta = -0.126$, $P = 0.046$).

We further selected individuals with NGT from the normotensive population and grouped them according to the quartile of the Matsuda index. BaPWV significantly decreased with an increase in insulin sensitivity ($P = 0.032$) (Figure 2).

4. Discussion

In the present study that involved individuals with no history of diabetes or hypertensive medication, 2 h glucose

Table 2: Independent relationships between pulse wave velocity and clinical variables in the entire study population and in the individuals with or without hypertension.

	The whole population (n = 3037)		Normotension (n = 2475)		Hypertension (n = 652)	
	Beta (95% CI)	P	Beta (95% CI)	P	Beta (95% CI)	P
Age	0.355 (0.323–0.378)	<0.001	0.312 (0.285–0.340)	<0.001	0.523 (0.440–0.606)	<0.001
BMI	−0.086 (−1.116–0.057)	<0.001	−0.060 (−0.090–−0.030)	<0.001	−0.149 (−0.233–−0.065)	0.007
WHR	0.078 (0.047–0.109)	<0.001	0.075 (0.044–0.106)	<0.001		NS
HR	0.171 (0.145–0.197)	<0.001	0.147 (0.121–0.174)	<0.001	0.243 (0.172–0.315)	<0.001
SBP	0.489 (0.449–0.529)	<0.001	0.403 (0.348–0.457)	<0.001	0.474 (0.356–0.539)	<0.001
TG	0.046 (0.011–0.081)	0.001	0.028 (−0.008–0.064)	0.057	0.097 (0.006–0.188)	0.004
HDL		NS	−0.038 (−0.058–−0.018)	0.037		NS
2 h PG	0.052 (0.013–0.090)	0.008	0.046 (0.006–0.087)	<0.001		NS
Adjusted r^2	0.524		0.402		0.377	

BMI: body mass index, WHR: waist-hip ratio, FPG: fasting plasma glucose, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TG: triglyceride, CHOL: cholesterol, SBP: systolic blood pressure, and DBP: diastolic blood pressure.
INS0: fasting serum insulin, INS30: 30-minute serum insulin, INS120: 120-minute serum insulin.
NS: nonsignificant.
Only significant correlations are listed.

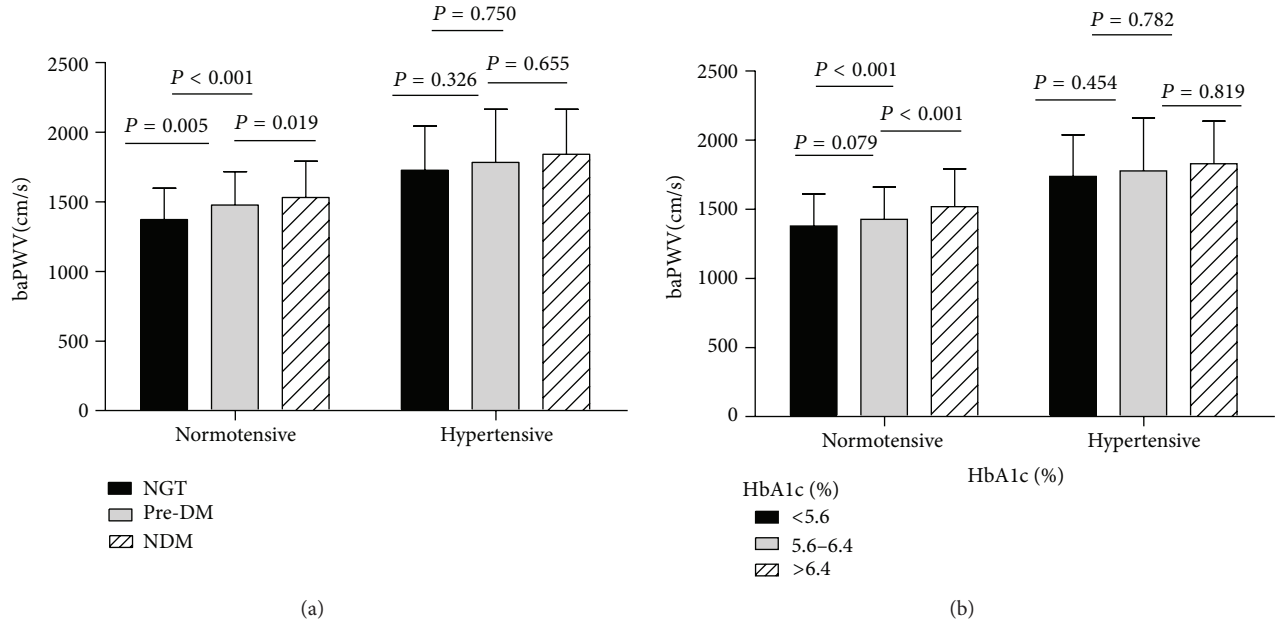

Figure 1: (a) Difference in PWV values across the glucose tolerance groups, after adjustment for age, gender, BMI, WHR, TG, lipids, HR, and MAP. P for trend = 0.000, 0.429 in normotensive and hypertensive groups, respectively. (b) Difference in PWV values across the HbA1c groups, after adjustment for age, gender, BMI, WHR, TG, lipids, HR, and MAP. P for trend = 0.001, 0.761 in normotensive and hypertensive groups, respectively.

was positively and independently associated with baPWV, even after adjusting for confounding factors such as age, BP, and lipid profile. These data are in agreement with a number of studies. In a study based on five cohorts of Asian origin, postchallenge glucose was found to be an independent predictor of CVD, and 2 h plasma glucose levels were a superior predictor of immature death compared with FPG levels [18]. Similarly, a study involving a nondiabetic population demonstrated that 2 h plasma glucose levels were more strongly associated with cardiometabolic risk factors and subclinical atherosclerosis compared with FPG or HbA1c levels [7].

Hypertension is one of the major risk factors for arterial stiffness, and previous studies [20–22] have shown that PWV was higher in hypertensive individuals than in individuals with normal BP. However, previous studies have rarely focused on the interaction of hypertension and glucose metabolism with baPWV. To investigate whether an association exists between 2 h glucose levels and baPWV among individuals with different BP conditions, we divided

Table 3: Independent association of brachial-ankle pulse wave velocity with indices of insulin resistance, insulin sensitivity, and beta-cell function.

	Normotensive			Hypertensive		
	Beta	P	Adjusted r^2	Beta	P	Adjusted r^2
Model 1, age, gender adjusted						
InHOMA-IR	0.508	<0.001	0.261	0.403	0.120	0.198
QUICKI	0.532	<0.001		0.383	0.153	
Matsuda	−0.226	<0.001		−0.086	0.195	
HOMA-β	−0.131	<0.001		−0.069	0.141	
Model 2, model 1 + BMI, WHR, HR, and MAP						
InHOMA-IR	0.257	0.043	0.378	0.369	0.125	0.337
QUICKI	0.223	0.023		0.456	0.067	
Matsuda	−0.131	<0.001		−0.143	0.023	
HOMA-β	−0.088	<0.001		−0.009	0.839	
Model 3, model 2 + LIPIDS						
InHOMA-IR	0.196	0.076	0.380	0.339	0.160	0.341
QUICKI	0.226	0.046		0.430	0.084	
Matsuda	−0.114	<0.001		−0.126	0.046	
HOMA-β	−0.082	<0.001		−0.005	0.903	

HOMA-IR: homeostasis model assessment of insulin resistance, QUICKI: Quantitative Insulin Sensitivity Check Index.
HOMA-IR was log-transformed for comparison.

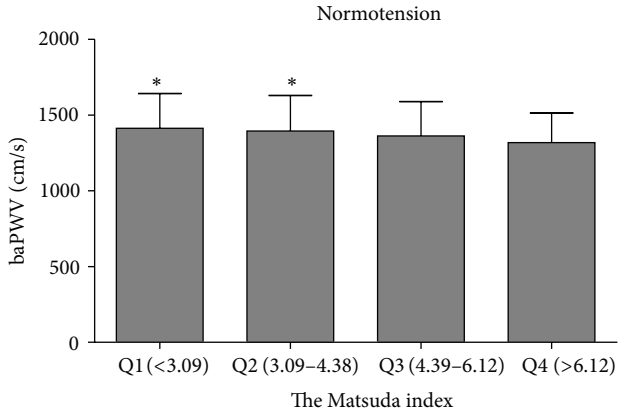

Figure 2: Difference in PWV values across the quartile of Matsuda's index among nondiabetic people in normotensive group, after adjustment for age, BMI, WHR, lipids, HR, and MAP. P for trend = 0.032; $^*P < 0.05$ versus Q4.

the subjects into two groups on the basis of the diagnosis of hypertension. Separate analysis of the population via multiple linear stepwise analysis, including compounding variables, revealed that 2 h glucose levels were related to baPWV in the normal group but not in the hypertensive group. To further investigate the influence of glucose metabolism and glycemic control on PWV in the normal and hypertensive population, we classified them on the basis of diabetes diagnostic criteria and HbA1c levels. After adjustment for age, gender, BMI, lipid levels, SBP, DBP, WHR, and HR, baPWV in the normotensive group was closely related to different glucose metabolism conditions and glycemic control, whereas glucose status, whether normal or pathological, rarely affected arterial stiffness in hypertensive individuals.

In hypertensive individuals, BP was the main determinant of baPWV despite abnormal glucose status; therefore, the condition of blood vessels may benefit more from intensive antihypertensive treatment than from blood glucose control. This hypothesis is supported by several previous studies. Tomita et al. [23] demonstrated that arterial stiffness was more directly related to BP than to blood glucose levels. The UK Prospective Diabetes Study (UKPDS) followed 1148 hypertensive patients with type 2 diabetes for an average of 8.4 years and observed that tight BP control rather than tight blood sugar control achieved a clinically important reduction in the risk of macrovascular and microvascular complications [24]. Another study by Czernichow et al. [25] demonstrated that BP was the most important metabolic syndrome component in relation to the structure and function of large arteries, thus reflecting our results and emphasizing the importance of BP control in arterial stiffness. Although there are many pharmacological strategies to reduce arterial stiffness, antihypertensive treatment seems to be the most powerful therapy at present [26].

Although our data corroborates those from a majority of previous studies, some controversial results do exist. Bruno et al. [1] recently demonstrated that PWV was significantly higher in hypertensive individuals with diabetes than in hypertensive individuals with normal glucose status. In a study involving 1375 never-treated hypertensive subjects, an association between arterial stiffness and glucose tolerance existed [27]. However, it is noteworthy that these studies did not exclude patients with a history of diabetes or antidiabetic treatment, and this may have affected the results. Moreover, Chen et al. [28] showed that PWV increased with the

duration of diabetes in individuals with or without hypertension, suggesting that arterial stiffness gradually worsened with a longer duration of diabetes, which was likely to be another reason for the differences detected in our study that involved patients with no history of diabetes.

The euglycemic hyperinsulinemia clamp studies have proved that 2 h glucose levels are associated with peripheral IR [29], which had an unclear contribution to arterial stiffness. Although many studies concluded that IR was associated with arterial stiffness [30–32], Okada et al. [33] claimed that low insulin levels, which indicate high insulin sensitivity, were also linked to the occurrence of atherosclerosis. Nevertheless, previous studies have not clearly demonstrated whether IR is associated with increased arterial stiffness independent of the clusters of other risk factors. Therefore, we explored the possibility that insulin-sensitivity may influence arterial stiffness in patients with different BP levels. Because HOMA-IR was only based on fasting glucose and insulin levels, we used the Matsuda index as well, which represents whole-body insulin sensitivity in the basal state and after the ingestion of a glucose load. Indeed, our analysis of the normotensive population found a strong association between decreased insulin sensitivity and baPWV, independent of other metabolic factors. However, only the Matsuda index was weakly but significantly associated with baPWV, making it difficult to conclude that IR was an independent risk factor in hypertensive patients.

Moreover, we estimated the association between baPWV and beta-cell function. To our knowledge, the relationship between beta-cell function and arterial stiffness has not been previously explored. Curtis et al. [34] demonstrated that beta-cell function, evaluated by HOMA-β, was a significant predictor of incident cardiovascular events, which could be the outcome of arterial stiffness. In our study, HOMA-β was significantly correlated with baPWV after adjustment for metabolic factors. Another novel aspect of our study is that not only individuals with diabetes but also those with IR in the absence of diabetes and hypertension are at risk of arterial stiffness. These results suggest that the influence of IR on arterial stiffness is independent of glucose tolerance status. Several clinical studies also support our conclusion to some extent. Insulin sensitizing agents significantly decreased both PWV [35, 36] and IMT [37] of the carotid artery of type 2 diabetes patients, irrespective of their glucose-lowering effects.

The mechanism linking IR and arterial stiffness is not completely understood. Chronic hyperinsulinemia accentuates the activity of the renin-angiotensin-aldosterone axis as well as the expression of angiotensin type 2 receptors in vascular tissue, leading to wall hypertrophy and fibrosis [38, 39]. In addition, IR causes decreased release of nitric oxide and dilation of vasculature, thereby increasing the possibility of damage to the vessel wall [40].

The influence of 30 min or 1 h glucose levels on arterial stiffness has not been clearly defined. In a study from Korea [9], 30 min postchallenge glucose levels, in contrast to FPG and 2 h glucose levels, were a weak but significant determinant of mean baPWV. However, this outcome was not reported in our study. This discrepancy may be due to the different population between studies; the Korean study selected individuals with impaired FPG and acute glucose excursion worse than those seen in the general population.

The role of BMI in hardening of arteries remains controversial, as Tomiyama et al. [41] showed that BMI was positively associated with PWV while Tomiyama et al. [15] and Li et al. [42] reported opposite results. Our findings were consistent with the latter study, where the colinearity between BMI and other cardiovascular risk factors may lead to the negative association. With regard to lipid metabolism, we found that TG and HDL levels were related to baPWV in individuals without hypertension. Only TG levels remained associated with baPWV in the hypertensive group, a finding consistent with that of previous reports [43, 44].

There were limitations to our study. First, because it is a cross-sectional research study, we cannot draw causal conclusions on blood glucose and PWV. Second, OGTT and the insulin index used were not the gold standard to diagnose IR. Third, we evaluated an exclusively Chinese population; therefore, the conclusions may not be applicable to other ethnic groups.

Nonetheless, our study was the first, as per our knowledge, to gauge the impact of glucose metabolism on baPWV in patients with different BP levels. Overall, our data indicated that baPWV was significantly associated with 2 h glucose levels in normotensive individuals, which may be connected with insulin sensitivity. In hypertensive individuals, BP was the dominant factor influencing arterial stiffness; neither glucose level nor islet function had a strong impact on baPWV, suggesting that intensive antihypertensive treatment may be more effective than glycemic control in reducing the incidence of cardiovascular events in these patients. Individuals with abnormal insulin sensitivity are also at increased risk of arterial stiffness, even in the absence of diabetes and hypertension.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

C. Meng and M. Sun have contributed equally to this work.

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Clinical Study

Association of the HLA-DQA1 and HLA-DQB1 Alleles in Type 2 Diabetes Mellitus and Diabetic Nephropathy in the Han Ethnicity of China

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HLA gene system is one of the most polymorphic regions of the human genome. The association of HLA class II genes in T1DM pathogenesis has been reported for several ethnicities. Associations of HLA class II genes with T2DM have revealed inconsistent results. Moreover, correlations between DN and HLA alleles remain unclear. We carried out DNA typing chip by specific medium resolution typing probes in 310 T2DM subjects (including 210 patients with DN and 100 patients without DN) in addition to 100 healthy controls. Differences were found between patients with T2DM and the control group in the frequencies of the HLA-DQA1*0301 (15.5% versus 8.0%, $P < 0.01$) and the HLA-DQA1*0501 alleles (16.6% versus 8.5%, $P < 0.01$). Differences were found between patients with DN and without DN in the frequencies of the HLA-DQA1*0302 (6.9% versus 13.5%, $P < 0.01$) and HLA-DQB1*0501 alleles (5.8% versus 14.5%, $P < 0.01$). Diabetes duration and systolic blood pressure were independent risk factors associated with DN (OR = 2.277 and 1.366, resp., $P < 0.05$), whereas the HLA-DQB1*0501 llele had a protective effect on DN (OR = 0.53, $P < 0.05$). These data suggest the HLA-DQA1*0301 and HLA-DQA1*0501 alleles are markers of susceptibility for T2DM, and the HLA-DQB1*0501 allele is associated with a protective effect on DN in Han ethnicity of China.

1. Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) worldwide [1]. It affects approximately 30% of patients with long-standing Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM) [2] and confers added risks of cardiovascular disease and mortality. Asians, including Chinese T2DM patients, have a higher prevalence of nephropathy, with 20% having clinical proteinuria and 40% microalbuminuria [3]. Multiple causes have been implicated in DN including hyperglycaemia, hypertension, inflammation, smoking, and dyslipidaemia [4, 5]. In addition, familial clustering of DN suggests genetic predisposition plays a role in the pathogenesis of this complication.

The HLA gene system is one of the most polymorphic regions of the human genome and one of the most extensively studied regions because of the association of variants at this locus with autoimmune, infectious, and inflammatory diseases [6]. It was proposed that both susceptible and protective alleles at the HLA DRB1, DQA1, and DQB1 loci were associated with the pathogenesis of some diseases [7]. T1DM is an autoimmune disease characterized by the selective destruction of pancreatic islets. Both genetic and environmental factors participate in the pathogenesis of the autoimmune process leading to the onset of this disease. T2DM is a complex heterogeneous group of metabolic disorders including hyperglycemia and impaired insulin action and/or insulin secretion, in which genetic factors play a complex and yet not clearly defined role. The association of HLA class II genes in T1DM pathogenesis has been reported for several ethnicities [8–10]. Studies on the association of HLA class II with T2DM have revealed inconsistent results, since an association [11], no association [12], and a weak link between HLA class II and T2DM have all been reported. Moreover, correlations between DN and HLA alleles remain unclear; the purpose of this study was to examine whether

the HLA-DQA1 and HLA-DQB1 alleles are associated with the etiology of T2DM and DN in the Han ethnicity of China using the gene chip technique.

2. Materials and Methods

2.1. Study Population. We selected T2DM subjects ($n = 310$) in Han ethnic group in the city of Tianjin, China, who were in-patients at the Department of Diabetic Nephropathy at Tianjin Medical University Affiliated Metabolic Disease Hospital. T2DM was diagnosed according to the 2007 American Diabetes Association diagnostic criteria. Exclusion criteria for this study were (1) diabetes secondary to known causes such as chronic pancreatitis and Cushing's syndrome and (2) T1DM defined by presentation with ketoacidosis or requirement of insulin therapy from the disease onset or positive GAD, IA-2A, or ICA antibody. Subjects were divided into groups with DN (DN1) and without DN (DN0) according to their 24 h albumin excretion rates (AERs). The group without DN ($n = 100$) consisted of patients who had been diagnosed with T2DM for at least 10 years and did not show albuminuria (AER < 30 mg/24 h). After ruling out urinary tract infection, hematuria, nephritis, and other conditions [11], the DN group was further subdivided into microalbuminuria group ($n = 104$, 300 mg/24 h > AER ≥ 30 mg/24 h) and overt albuminuria group ($n = 106$, AER ≥ 300 mg/24 h), with AERs determined in at least two consecutive overnight samples collected over a 3-to-6 month period.

Controls consisted of healthy individuals including 50 males and 50 females (mean age 57.5 ± 4.7 yr). For inclusion in the study, control subjects had normal fasting/random glucose levels and no family history of DM or other autoimmune diseases. All were of Han ethnicity of China.

2.2. Methods. Blood samples from all subjects were collected after obtaining written informed consent. The protocol was approved by the Ethics Committee of the Tianjin Medical University.

Peripheral blood samples were collected using EDTA-coated vacutainers. DNA was isolated according to the protocol of Sambrook et al. [13]. Unsymmetrical PCR was used to amplify the HLA-DQA1 and HLA-DQB1 exon 2 and exon 3. The amplification reactions contained 40 ng of DNA, 10 μM dNTP mix, 1X PCR buffer (Applied Biosystems), 2 U of Taq polymerase (AmpliTaq Gold, Applied Biosystems), and 1 μM of each primer in a total volume of 10 μL. Each PCR was optimized with respect to the concentration of Mg^{2+} ions. Reactions were carried out in an ABI Gene Amp PCR system 9700. We carried out DNA-typing chip by specific medium resolution typing probes designed according to the gene frequency of HLA-DQA1 and HLA-DQB1 [14]. The PCR products were labeled and hybridized with the probes on the chip. The gene typing of HLA-DQA1 and HLA-DQB1 was certified by scanning the hybridized products and analyzing the read-out with Perkin Elmer Scanarray 4000 software.

2.3. Statistical Analysis. The clinical and laboratory characteristics were expressed as means ± SD. Observed distributions of genotypes were analyzed for deviation from the Hardy-Weinberg equilibrium by chi-square (χ^2) tests. Because there were no differences in the HLA genotype frequencies between the microalbuminuria and overt albuminuria groups, we combined these two groups into a DN group for further data analyses. Comparisons between the diabetes with and without DN groups and those between the genotypic groups were performed with unpaired Student's t-tests and χ^2 analysis. To evaluate the independent contributions of the HLA genotype to the risk of DN, we performed multivariate logistic regression analysis; the analyses included possible confounders (age at diagnosis of diabetes, diabetes duration, hypertension, triglyceride level, total cholesterol level, and A1C). Odds ratios (ORs) and 95% CIs were calculated. All calculations were performed using SPSS, Version 13.0 for Windows (SPSS, Chicago, IL, USA). We considered $P < 0.05$ as statistically significant in all analyses.

Table 1: Clinical and biochemical parameters of the controls and diabetic subjects.

	T2DM patients		Controls
	Without DN group	DN group	
N	100	210	100
Age	57.8 ± 9.5	58.1 ± 8.7	57.5 ± 4.7
Gender (male/female)	47/53	105/105	50/50
Age at diagnosis of DM (years)	53.3 ± 10.5	51.7 ± 8.3$^{\#}$	—
Diabetes duration (years)	13.2 ± 2.7	16.1 ± 4.5†	—
BMI (kg/m^2)	25.5 ± 3.6*	25.8 ± 4.0*	23.1 ± 3.8
Hypertension (%)	47 (47%)*	152 (72.4%)$^{\dagger *}$	23 (23%)
SBP (mmHg)	136.2 ± 16.5*	144.1 ± 17.6$^{\dagger *}$	130.4 ± 15.7
DBP (mmHg)	80.7 ± 9.5*	83.2 ± 9.6$^{\dagger *}$	76.1 ± 8.9
FPG (mmol/L)	9.3 ± 5.8*	9.5 ± 6.4*	5.1 ± 3.7
HbA1c (%)	9.2 ± 4.3*	9.3 ± 5.7*	5.2 ± 2.8
Triglyceride level (mmol/L)	1.4 ± 0.7*	1.8 ± 0.9$^{\dagger *}$	1.1 ± 0.6
Total cholesterol level (mmol/L)	4.8 ± 1.2	4.9 ± 1.4	4.7 ± 1.5

Data are expressed as means ± SD and n (%); $^{*}P < 0.01$ versus controls; $^{\#}P < 0.05$ versus without DN group; $^{\dagger}P < 0.01$ versus without DN group.

3. Results

Clinical and biochemical parameters of the controls and diabetic subjects are summarized in Table 1. The BMI, DBP, SBP, FBG, HbA1c, and triglyceride levels were found to be significantly high ($P < 0.001$) in diabetic patients than controls. The patients with DN differed significantly with respect to age at diagnosis of diabetes, diabetes duration, blood pressure, and triglyceride level compared to the group without DN ($P < 0.05$, $P < 0.01$, $P < 0.01$, and $P < 0.01$, resp.).

Table 2: Frequency of HLA-DQA1 alleles in T2DM group versus control group.

HLA-DQA1 alleles	T2DM (n = 310)		Control (n = 100)		χ^2	P
	PN	AF	PN	AF		
DQA1*0101	50	0.081	18	0.090	0.174	0.677
DQA1*0102	65	0.105	20	0.100	0.038	0.845
DQA1*0103	62	0.100	19	0.095	0.042	0.837
DQA1*0104	50	0.081	20	0.100	0.726	0.394
DQA1*0201	43	0.069	21	0.105	2.670	0.102
DQA1*0301	96	0.155	16	0.080	7.182	0.007(*)
DQA1*0302	56	0.090	24	0.120	1.513	0.219
DQA1*0401	46	0.074	22	0.110	2.549	0.110
DQA1*0501	103	0.166	17	0.085	7.967	0.005(*)
DQA1*0601	49	0.079	23	0.115	2.442	0.118

PN: positive number; AF: antigen frequency; (*)P value < 0.05.

Table 3: Frequency of HLA-DQB1 alleles in T2DM group versus control group.

HLA-DQB1 alleles	T2DM (n = 310)		Control (n = 100)		χ^2	P
	PN	AF	PN	AF		
DQB1*0201	58	0.094	15	0.075	0.642	0.423
DQB1*0301	64	0.103	16	0.08	0.927	0.336
DQB1*0302	114	0.184	36	0.18	0.015	0.902
DQB1*0303	132	0.213	50	0.25	1.205	0.272
DQB1*0401	53	0.086	16	0.08	0.059	0.808
DQB1*0501	54	0.087	19	0.095	0.116	0.733
DQB1*0601	89	0.144	32	0.160	0.325	0.568
DQB1*0602	56	0.09	16	0.08	0.201	0.654

PN: positive number; AF: antigen frequency.

Table 4: Logistic regression analysis of association between T2DM and HLA alleles.

Independent variables	OR	95% CI	P
HLA-DQA1*0301	1.965	1.235–3.135	0.009
HLA-DQA1*0501	2.137	1.461–2.816	0.007

OR: odds ratio; significant if P value < 0.05.

Tables 2 and 3 show the frequencies of the HLA-DQA1 and HLA-DQB1 alleles between T2DM and control groups. Significant differences were detected between patients with T2DM and controls in the frequencies of the HLA-DQA1*0301 (15.5% versus 8.0%, $\chi^2 = 7.182$, $P < 0.01$) and HLA-DQA1*0501 alleles (16.6% versus 8.5%, $\chi^2 = 7.967$, $P < 0.01$). The frequency of other HLA-DQB1 alleles in T2DM group did not differ from control group ($P > 0.05$). After controlling for confounding variables (including age, gender, BMI, FBG, HbA1c, and age at diagnosis of DM), logistic regression analysis demonstrated that HLA-DQA1*0301 and HLA-DQA1*0501 alleles remained significantly associated with T2DM (OR = 1.965, $P < 0.01$; OR = 2.137, $P < 0.01$, resp.) (Table 4).

Table 5: Frequency of HLA-DQA1 alleles in T2DM patients with and without DN.

HLA-DQA1 alleles	DN0 (n = 100)		DN1 (n = 210)		χ^2	P
	PN	AF	PN	AF		
DQA1*0101	16	0.080	34	0.081	0.002	0.968
DQA1*0102	21	0.105	44	0.105	2.862	0.091
DQA1*0103	19	0.095	43	0.102	0.082	0.775
DQA1*0104	11	0.055	39	0.093	2.619	0.106
DQA1*0201	12	0.060	31	0.074	0.400	0.527
DQA1*0301	27	0.135	69	0.164	0.888	0.346
DQA1*0302	27	0.135	29	0.069	7.172	0.007(*)
DQA1*0401	10	0.050	36	0.086	2.516	0.113
DQA1*0501	38	0.190	65	0.155	1.214	0.270
DQA1*0601	19	0.095	30	0.071	1.034	0.309

PN: positive number; AF: antigen frequency; DN0: without DN group; DN1: DN group; (*)P value < 0.05.

Table 6: Frequency of HLA-DQB1 alleles in T2DM patients with and without DN.

HLA-DQB1 allele	DN0 (n = 100)		DN1 (n = 210)		χ^2	P
	PN	AF	PN	AF		
DQB1*0201	18	0.09	40	0.095	0.044	0.834
DQB1*0301	20	0.10	44	0.105	0.033	0.855
DQB1*0302	32	0.16	82	0.195	1.121	0.290
DQB1*0303	41	0.205	91	0.217	0.110	0.740
DQB1*0401	16	0.08	37	0.088	0.114	0.736
DQB1*0501	29	0.145	25	0.058	12.45	0.004(*)
DQB1*0601	24	0.12	65	0.155	1.332	0.249
DQB1*0602	20	0.1	36	0.086	0.337	0.562

PN: positive number; AF: antigen frequency; DN0: without DN group; DN1: DN group; (*)P value < 0.05.

Table 7: Independent risk factors of DN in diabetic patients.

Independent variables	OR	95% CI	P
Diabetes duration	2.277	1.180–4.39	0.014
SBP	1.366	1.149–1.779	0.021
HLA-DQB1*0501	0.53	0.296–0.85	0.036

OR: odds ratio; significant if P value < 0.05.

Tables 5 and 6 show the frequencies of the HLA-DQA1 and HLA-DQB1 alleles between the DN and non-DN groups. There were decreased frequencies of the HLA-DQA1*0302 and HLA-DQB1*0501 alleles in DN group compared to the non-DN group (6.9% versus 13.5%, $\chi^2 = 7.172$, $P = 0.007$; 5.8% versus 14.5%, $\chi^2 = 12.45$, $P = 0.004$, resp.).

To evaluate the independent contributions of the polymorphism to the risk of DN, multivariate logistic regression analyses of T2DM patients with and without DN were performed by integrating the possible confounders in Table 7. After adjusting for factors such as aging, triglyceride levels, hypertension, and diabetes duration by using a logistic regression model, diabetes duration and systolic

blood pressure were identified as independent and significant determinants of DN in these Chinese T2DM patients (OR = 2.277, $P < 0.05$; OR = 1.366, $P < 0.05$, resp.). The significance was retained for the HLA-DQB1*0501 allele ($P < 0.05$), but lost for the HLA-DQA1*0302 allele ($P > 0.05$).

4. Discussion

According to the China National Diabetes and Metabolic Disorders Study, the prevalence of diabetes in China in 2009 was 9.7% [15]. Over 90% of the Chinese diabetes patients are T2DM. Western lifestyle contributes a lot to the T2DM epidemic, and genetic determinants also influence T2DM susceptibility. Several genes involved in the affected metabolic pathways of T2DM have been regarded as candidates [16]. Additionally, recent data has indicated a role for certain HLA alleles in the pathogenesis of T2DM [11, 17]. To investigate the possible relationships between T2DM and the HLA alleles in the Han ethnic group, we analyzed these parameters in individuals of the same ethnicity in the city of Tianjin, China. The T2DM patients in this study showed a significant association with certain HLA-DQA1 alleles. The frequencies of the HLA-DQA1*0301 and HLA-DQA1*0501 alleles in T2DM group were 15.5% and 16.6%, respectively, which were significantly higher than those in normal group (approximately 8%); logistic regression analysis revealed that HLA-DQA1*0301 and HLA-DQA1*0501 alleles were nominally associated with susceptibility to T2DM.

The association of specific HLA genotypes with T2DM susceptibility/protection depends on the ethnicity and racial background of each population. Studies investigating the HLA-T2DM relationship are very limited, and the link between HLA and T2DM is still not conclusive. In two studies, using different genotyping methods, no association was found between T2DM and the HLA class II antigens (HLA-DR, HLA-DQ) in Punjabi Sikhs [19], while a positive association with HLA-DQA genes was reported for Belgians [20]. In Bahrainis, a population with a high prevalence of T2DM, T2DM was found significantly associated with both HLA-DRB1 and HLA-DQB1 genotypes, with some alleles appearing to confer susceptibility and others playing a protective role [11]. The inconsistencies reported in these studies may be accounted for by many potential factors, such as study design and sample size. Our finding that the HLA-DQA1*0301 and HLA-DQA1*0501 alleles are associated with the T2DM further complicates interpretation of these heterogeneous findings. Further studies in Chinese people with prospective data and more information on environmental factors are needed to better elucidate the effects of these genetic variants on diabetes risk and their interaction with environment.

DN is a serious microvascular complication of diabetes, and it is a leading cause of end-stage renal disease in Western countries and in China. Traditionally, metabolic and hemodynamic factors are the main causes of renal lesions in patients with T2DM and DN, and although several genetic and environmental factors are likely to contribute to its development and progression, the precise mechanism for this contribution is still unknown. Specific ethnic populations are at unusual high risk for developing kidney disease. Native Americans (the Pima Indians of the southwestern United States) [21] and African Americans have a high prevalence of diabetes and kidney disease [22]. The reasons for the increased risk have not been clearly identified, but it is likely that there is a very strong genetic basis for the observed susceptibility, although factors such as hyperglycemia and hypertension also may play an important role. In any event, a concerted effort to understand the specific susceptibility of these groups is of great importance to the prevention of the increased morbidity and mortality associated with the onset of DN.

When we examined the T2DM group who did not develop nephropathy, despite many years of diabetes, we found decreased frequencies of the HLA-DQA1*0302 and HLA-DQB1*0501 alleles compared to the non-DN group. Multivariate logistic regression analyses revealed that diabetes duration and systolic blood pressure were independent risk factors for the occurrence of chronic kidney disease in these patients and that the HLA-DQB1*0501 allele had a protective effect for DN. The existence of carriers of the HLA-DQB1*0501 allele with normoalbuminuria, despite long duration of diabetes, suggests that this allele is associated with reduced risk of DN. It is well known that chronic hyperglycaemia and the duration of diabetes are the most important risk factors for DN. DN progresses in some patients, despite good glycaemic control. Also, poor glycaemic control does not always lead to DN in younger onset patients, while still others develop severe DN. These facts suggest that the risk factors for DN are not necessarily the same among different patients and that the occurrence of DN may be influenced by genetic factors. Overall, our study indicates that the HLA-DQB1*0501 allele is associated with a protective effect against DN in the Han ethnicity of China. The mechanism underlying this protective association is still unknown, and further investigation of its functional role is needed.

5. Conclusion

The HLA-DQA1*0301 and HLA-DQA1*0501 alleles are markers of susceptibility for T2DM in the Han ethnicity of China, and the HLA-DQB1*0501 allele is associated with a protective effect on DN.

Conflict of Interests

There is no conflict of interests.

Authors' Contribution

All authors fulfill the criteria for authorship. Z.-J. Ma and L.-M. Chen contributed to designing and coordinating the study, also interpreting the findings, and drafting the paper. P. Sun, R. Zhang, and G. Guo contributed to drafting the protocol and the statistical analysis. All authors read and approved the final manuscript.

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Clinical Study

Relationship between Oxidant/Antioxidant Markers and Severity of Microalbuminuria in the Early Stage of Nephropathy in Type 2 Diabetic Patients

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A wide range of microalbuminuria cutoff values are currently used for diagnosing the early stage of nephropathy in type 2 diabetes (T2D). This study analyzed the relationships between oxidant and antioxidant markers of nephropathy and the severity of microalbuminuria. The study included 50 healthy controls (Group 1), 50 diabetic patients with no nephropathy (Group 2), 50 diabetic patients with nephropathy and a urinary albumin excretion (UAE) of 30–200 mg/24 h (Group 3), and 50 diabetic patients with UAE 200–300 mg/24 h (Group 4). Serum nitrotyrosine, conjugated dienes, 8-hydroxy-2′-deoxyguanosine (8-OHdG), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) levels were determined. Oxidative stress is increased in the early stage of nephropathy in patients with T2D. There was a significant correlation between the extent of microalbuminuria and markers of oxidative stress. Multiple linear regression analysis identified lipid oxidative stress as a possible independent marker for evaluating the degree of renal damage in diabetic nephropathy. Stratifying microalbuminuria values during the early stage of nephropathy might be an important factor in facilitating earlier and more specific interventions.

1. Introduction

Diabetic nephropathy (DN) is an important microvascular complication of diabetes and is widely recognized as the most common cause of the end-stage renal disease seen in clinical practice. The condition not only causes disability but is associated with a high mortality rate in diabetic patients as well [1]. Early treatment depends on a clear understanding of the mechanisms that underlie DN. Oxidative stress is becoming increasingly recognized as an important causative factor. Routine urine testing fails to detect DN in its early stages even though urinary protein results may be positive. This means that treatment is delayed and that therapeutic responses are far from optimal.

An imbalance between oxidation and antioxidation is thought to precede the development of renal lesions, and thereafter the degree of oxidation gradually increases in parallel with the progression of the disease [2]. Previous studies have shown that the extent of microalbuminuria affects long-term prognosis [3]. The emergence of microalbuminuria not only indicates the early stage of DN, but may also be the consequence of extensive damage of systemic endothelial cells [4]. These studies confirmed that although microalbuminuria was currently the best nontraumatic predictor of early stage of DN, advanced changes in renal structure may have already developed when microalbuminuria is first seen. It has also been shown that reducing urinary protein excretion is of therapeutic benefit to patients with clinically significant microalbuminuria [5]. However, the normal values for microalbuminuria currently used to diagnose DN may be too high, and the range of microalbuminuria values is probably too wide [6].

These findings highlight the need to clarify the relationship between markers of oxidative stress and severity of microalbuminuria based on urinary albumin excretion (UAE). This information would help to define a specific target for the prevention and treatment of DN.

In the present study, we determined the association between levels of oxidant and antioxidant markers and different degrees of microalbuminuria in patients with type 2 diabetes (T2D) and an early stage of nephropathy. We used multiple linear regression analysis to identify the predominant risk factors that determined the severity of microalbuminuria.

2. Materials and Methods

2.1. Subjects and Groups. Between December 2007 and December 2010, 200 subjects 18 years of age or older were recruited into the study from the Health Examination Center and the Department of Endocrinology at The First Clinical Hospital of Harbin Medical University. The study subjects were divided into four groups. Group 1 comprised 50 healthy control subjects without diabetes, Group 2 included 50 patients with T2D who did not have nephropathy, Group 3 comprised 50 patients with T2D and nephropathy with an UAE between 30 and 200 mg/24 h, and Group 4 included 50 patients with T2D and an UAE between 200 and 300 mg/24 h. Patients and control subjects were well generally matched in terms of demographic and physiological parameters (Table 1).

The diagnosis of type 2 diabetes mellitus was based on the 1999 World Health Organization (WHO) diagnostic criteria [7], and the presence of early-stage DN was based on the staging criteria proposed by Mogensen et al. [8]. The diagnostic criterion for microalbuminuria was an UAE between 30 and 300 mg/24 h, measured at least twice from overnight urine samples taken three times in the 6 months before entering the study. All control subjects adhered to a standardized diet based on 2007 China guideline for type 2 diabetes [9].

Pregnant and lactating women and diabetic patients with ketoacidosis or inadequately controlled blood glucose were excluded from entering the study. None of the patients had changes in urinary protein levels >50% in the 2 weeks before entering the study and none had proteinuria caused by heart, liver, kidney, urinary tract infection, or other diseases. In addition, none of the patients had received antioxidant drugs (i.e., vitamin E), antihypertensive medication, or lipid-lowering medication for at least 1 month prior to entering the study.

The study was conducted in accordance with the ethical principles of Declaration of Helsinki and was approved by the Ethical Committee of The First Clinical Hospital of Harbin Medical University. All participants provided informed consent prior to entering the study.

2.2. Assessments. Blood samples were obtained after an overnight fast. Samples (3 mL) of venous blood were incubated in a water bath for 30 minutes at 37°C and centrifuged at 4500 rpm for 10 minutes. The supernatants were stored at −70°C. Serum nitrotyrosine, conjugated dienes, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were measured by enzyme-linked immunosorbent assay (ELISA), in accordance with the procedures described in the assay kit (Netherlands HyCult Biotechnology b.v (Hbt). Serum superoxide dismutase (SOD) activity and total antioxidant capacity (T-AOC) levels were determined by spectrophotometry (TU-1900 UV-visible spectrophotometer). The assays were conducted according to the manufacturer's (Nanjing Blanching Institute of Biology) instructions.

Triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose, and glycosylated hemoglobin (HbA1c) levels were measured using routine clinical chemical assays.

2.3. Statistical Analysis. The statistical analysis was undertaken using SPSS version 17 software. The data were presented as means ± standard deviations (SDs). The variables were tested for normality using the Kolmogorov-Smirnov test. Parametric variables were compared using one-way analysis of variance (ANOVA). Associations between degree of microalbuminuria and measured parameters were analyzed using the Pearson correlation test. Multiple linear regression analysis was undertaken to identify independent risk factors. Values of $P < .05$ were considered statistically significant.

3. Results

Fasting plasma glucose levels and HbA1c levels were well matched between the three groups of diabetic patients and microalbuminuria levels (mg/24 h) reflected the levels intended in the study design (Table 1). In addition, patients in Group 2 had comparable levels of microalbuminuria as healthy control subjects.

As shown in Table 2, serum nitrotyrosine, conjugated dienes, and 8-OHdG levels were significantly higher in Groups 2, 3, and 4 than in Group 1 ($P < .001$), whereas SOD and T-AOC levels were significantly lower in Groups 2, 3, and 4 than in Group 1 ($P < .001$). Serum nitrotyrosine, conjugated dienes, and 8-OHdG levels were significantly higher and SOD and T-AOC were significantly lower in Group 3 than in Group 2 ($P < .001$). Levels of serum nitrotyrosine, conjugated dienes, and 8-OHdG were significantly higher in Group 4 than in Group 3 and SOD and T-AOC levels were significantly lower in Group 4 than in Group 3 ($P < .001$).

Serum nitrotyrosine ($r = 0.859$; Figure 1), conjugated dienes ($r = 0.867$; Figure 2), and 8-OHdG levels ($r = 0.826$; Figure 3) were positively correlated with microalbuminuria (all $P < .001$). As shown in Figures 4 and 5, serum SOD ($r = -0.659$) and T-AOC levels ($r = -0.609$) were negatively correlated with microalbuminuria ($P < .001$).

Multiple linear regression analysis of microalbuminuria and oxidant and antioxidant markers identified conjugated dienes as the predominant indicator of the severity of microalbuminuria (Table 3).

Table 1: Comparison of demographic and clinical characteristics ($n = 50$ per group).

Parameters	Group 1	Group 2	Group 3	Group 4
Age (years)	54.68 ± 6.47	55.66 ± 5.12	56.36 ± 3.97	56.98 ± 2.69
Female/male	27/23	28/22	25/25	26/24
Duration of diabetes (years)	—	7.3 ± 1.6	7.5 ± 1.8	7.7 ± 1.4
BMI (kg/m^2)	24.24 ± 1.22	24.78 ± 1.50	24.54 ± 1.72	24.92 ± 1.66
Triglycerides (mmol/L)	1.46 ± 0.39	1.41 ± 0.11	1.38 ± 0.15	1.43 ± 0.10
Cholesterol (mmol/L)	4.48 ± 0.13	4.49 ± 0.48	4.58 ± 0.48	4.53 ± 0.55
HDL-C (mmol/L)	1.31 ± 0.14	1.29 ± 0.19	1.25 ± 0.20	1.26 ± 0.21
LDL-C (mmol/L)	1.39 ± 0.63	1.47 ± 0.87	1.55 ± 0.93	1.67 ± 1.01
Systolic blood pressure (mmHg)	122.92 ± 9.16	124.08 ± 6.87	123.36 ± 9.81	124.52 ± 5.61
Diastolic blood pressure (mmHg)	76.90 ± 4.13	77.10 ± 5.13	77.72 ± 5.64	78.04 ± 4.62
Microalbuminuria (mg/24 h)	8.82 ± 1.49	14.83 ± 3.27	96.73 ± 41.56*,†	247.67 ± 24.01$^{*,\dagger,\#}$
Fasting plasma glucose (mmol/L)	4.96 ± 0.63	6.34 ± 0.74*	6.37 ± 0.82*	6.39 ± 0.76*
HbA1c (%)	5.93 ± 0.74	7.11 ± 0.50*	7.12 ± 0.63*	7.12 ± 0.61*

Mean ± standard deviation (SD), $^{*}P < .001$ versus Group 1, $^{\dagger}P < .001$ versus Group 2, and $^{\#}P < .001$ versus Group 3.

Table 2: Comparison of markers of oxidant and antioxidant ($n = 50$ per group).

Parameters	Group 1	Group 2	Group 3	Group 4
Nitrotyrosine (OD)	79.01 ± 2.04	87.12 ± 1.89*	93.18 ± 1.16*†	99.31 ± 1.15$^{*\dagger\#}$
Conjugated dienes (OD)	0.11 ± 0.02	0.97 ± 0.05*	1.62 ± 0.06*†	2.27 ± 0.09$^{*\dagger\#}$
8-OHdG (OD)	45.37 ± 2.52	51.45 ± 1.10*	55.94 ± 1.35*†	60.40 ± 1.74$^{*\dagger\#}$
SOD (NU/mL)	101.27 ± 5.02	86.71 ± 4.89*	82.59 ± 3.37*†	78.65 ± 1.52$^{*\dagger\#}$
T-AOC (U/mL)	9.50 ± 0.39	6.21 ± 0.23*	5.84 ± 0.41*†	5.44 ± 0.39$^{*\dagger\#}$

Mean ± standard deviation (SD), $^{*}P < .001$ versus Group 1, $^{\dagger}P < .001$ versus Group 2, and $^{\#}P < .001$ versus Group 3.

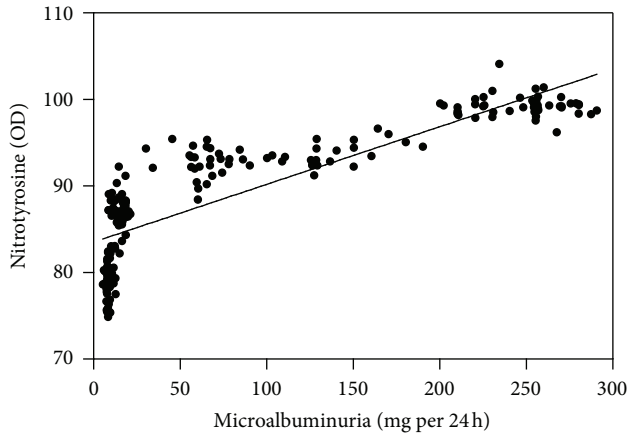

Figure 1: Correlation between microalbuminuria levels and nitrotyrosine.

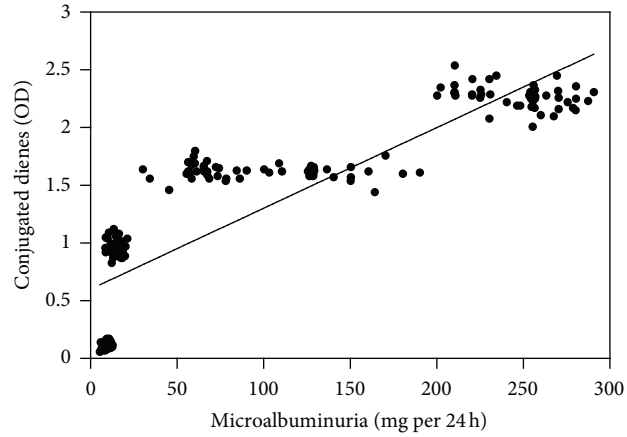

Figure 2: Correlation between microalbuminuria levels and conjugated dienes.

4. Discussion

We investigated the relationship between oxidative stress and severity of microalbuminuria in the early stages of nephropathy in patients with T2D. We were interested in finding out if different levels of microalbuminuria had clinical significance in patients in the same early stage of nephropathy. We also wanted to identify the main risk factor associated with the development of severe microalbuminuria. We, therefore, evaluated levels of different markers of oxidative stress in each of the four groups of subjects with different levels of microalbuminuria. We have selected different UAE cut-off values to analyze the relationship between oxidant/antioxidant markers and severity of microalbuminuria in an early stage of nephropathy in type 2 diabetic patients in our previous study, while UAE 30 mg/24 h and 200 mg/24 h were significantly for stratifying. There was a

Table 3: Multiple linear regression analysis between microalbuminuria and oxidant and antioxidant markers.

Variable	*B*	SEB	Beta	*T*	*P* value
Nitrotyrosine	5.999	1.488	0.465	4.032	.000*
Conjugated dienes	148.771	18.664	1.201	7.971	.000*
8-OHdG	2.486	1.548	0.146	1.605	.010*
SOD	−1.688	0.677	−0.160	−2.491	.014*
T-AOC	−34.886	3.815	−0.580	−9.145	.000*
Constant	−881.492	142.025	—	−6.207	.000*

*Significant values.

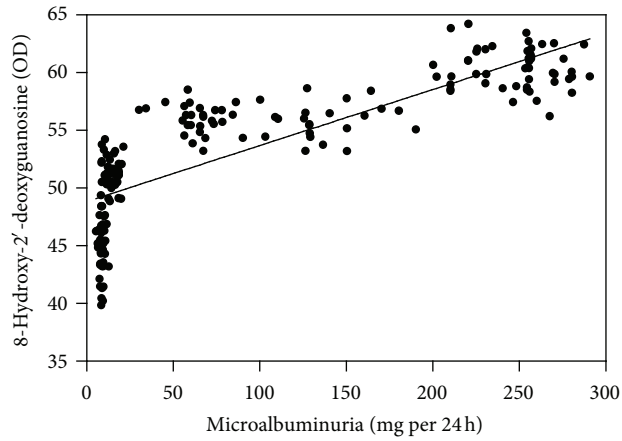

Figure 3: Correlation between microalbuminuria levels and 8-hydroxy-2′-deoxyguanosine.

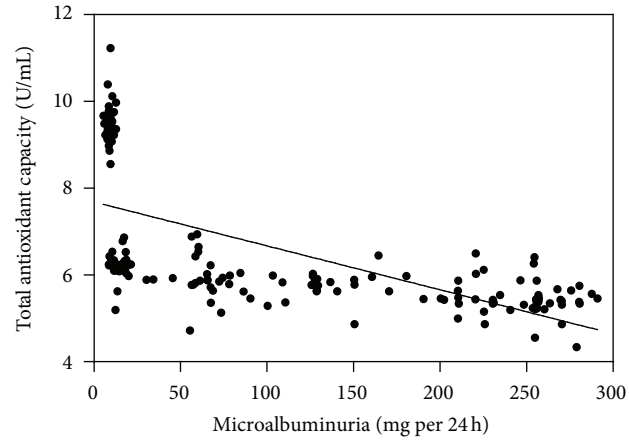

Figure 5: Correlation between microalbuminuria levels and total antioxidant capacity.

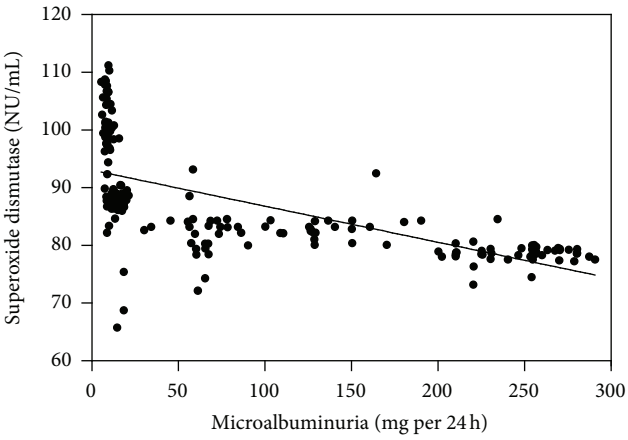

Figure 4: Correlation between microalbuminuria levels and superoxide dismutase.

significant correlation between the extent of microalbuminuria and markers of oxidative stress when selecting UAE 30 mg/24 h; the results are consistent with UAE 200 mg/24 h. However, UAE 200 mg/24 h was significantly higher than UAE 30 mg/24 h; post hoc results the two values were selected as thresholds in the current study.

The generation of reactive oxygen species (ROS) is known to increase in parallel with the degree of oxidative stress and is responsible for oxidative damage to biological macromolecules (i.e., proteins, lipids, and nucleic acids) [10]. Oxidative damage to proteins is believed to play an essential role in the pathogenesis of many diseases [11, 12]. Nitrotyrosine is formed by the interaction of tyrosine residues with reactive nitrogen during the posttranslational modification of proteins. In pathological conditions, nitrotyrosine levels increase in response to the generation of reactive oxygen and reactive nitrogen species. Indeed, the formation of nitrotyrosine is considered as a biomarker of reactive nitrogen species in vivo. A study examining renal biopsy specimens demonstrated increasing levels of nitrotyrosine patients with DN, indicating that nitrotyrosine may be involved in the development of renal lesions in these patients [13].

Conjugated dienes are lipid oxidation products, which can be further oxidized by polyunsaturated fatty acids found in low-density lipoprotein (LDL). Conjugated dienes' levels, therefore, indicate the degree of lipid oxidation. A number of studies have shown that serum conjugated diene levels are increased in patients with diabetes [14–16].

Hydroxyl radicals and superoxide anions have been shown to interact with DNA molecules, resulting in breakage of the DNA chain, modification of DNA bases, and cross-linking of DNA proteins. These changes ultimately result in oxidative damage and produce 8-OHdG. Thus, 8-OHdG has been widely used as a marker for oxidative DNA damage.

Oxidative DNA damage plays an important role in the pathogenesis of many diseases (i.e., tumor, coronary artery disease, and diabetes) and has been shown to damage renal

cells and mitochondrial DNA [17]. These changes may play a role in the development of DN. It has also been demonstrated that 8-OHdG levels are higher in diabetic patients than in healthy controls [16].

Our study indicated that the serum levels of nitrotyrosine, conjugated dienes, and 8-OHdG were elevated in patients with early DN and diabetes without nephropathy and that the levels of these markers increased in parallel with the severity of microalbuminuria. These findings indicate that protein, lipid, and DNA damage already exist in the early stages of DN in patients with T2D and that the levels of oxidative stress increases as microalbuminuria becomes more pronounced.

The multiple linear regression analysis identified conjugated dienes as an oxidant marker of the risk of severe microalbuminuria. This finding suggests that the oxidative damage of lipids has the greatest impact and plays a fundamental role in the pathogenesis and progression of microalbuminuria in an early stage of DN. Although there were no significant differences in the lipid profile among the groups, we can speculate that visceral fat accumulation plays an important role in renal damage. Interestingly, a new study in China has shown that the expansion of visceral adiposity is a risk factor for an elevated risk of 24-hour UAE, and with the expansion of visceral adiposity, the prevalence of heavy albuminuria increases [18]. The results are consistent with a previous study demonstrating that the visceral fat area is independently associated with microalbuminuria in Japanese adult patients with T2D [19]. Abdominal obesity is an important feature of T2D patients in Asian populations, which is often accompanied by large amounts of visceral fat accumulation. It is those amounts of visceral fat accumulation that would cause lipid oxidative stress and promote adipose tissue to secrete inflammatory adipokines, such as interleukin- (IL)-6, tumor necrosis factor-α (TNF-α), and macrophage chemoattractant protein-1 (MCP-1) in an early stage of DN [20, 21].

A decrease in antioxidant capacity breaks down the dynamic balance between oxidation and antioxidation in vivo. A growing amount of evidence indicates that the antioxidant capacity is decreased in diabetic patients [22, 23]. There are many enzymatic and nonenzymatic antioxidants in vivo, such as SOD, catalase (CAT), glutathione peroxidase, and vitamin E. Among these, SOD is the only substrate used as the superoxide anion scavenger enzyme, and as such it constitutes the first line of defense against ROS. T-AOC is an important marker of oxidation, which mainly reflects nonenzymatic but includes the activity of a minority of small molecular enzymatic systems. Animal and clinical studies have confirmed that antioxidant treatment plays an effective role in diabetes and DN [24–27]. Our results demonstrated that serum levels of SOD and T-AOC were lower than control subjects and decreased with severity of microalbuminuria in early DN and diabetes without nephropathy. These findings show that a decreased antioxidant activity already exists in diabetes at an early stage of DN and that antioxidant capacity weakens in parallel with the severity of microalbuminuria in T2D patients.

In conclusion, the oxidative stress increases in the early stage of nephropathy in patients with T2D relative to that in patients without nephropathy. However, in the same early stage of DN, there was a significant correlation between different levels of microalbuminuria and markers of oxidative stress. Serum conjugated dienes emerged as the main marker for evaluating kidney damage in DN. Stratifying microalbuminuria values during the early stage of nephropathy might be an important factor in facilitating earlier and more specific interventions.

The oxidative products assayed in our study are highly reactive, extremely unstable, and have a short half-life, all of which compromise accuracy. Our findings, therefore, need to be substantiated by other studies in larger numbers of patients from different centers.

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