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Lead Guest Editor: Érika B. Rangel
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Editorial Board

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Editorial

Micro- and Macrovascular Complications in Diabetes Mellitus: Preclinical and Clinical Studies

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Diabetes mellitus (DM) is a worldwide public health problem that affects millions of people from all age, gender, and racial and ethnic groups. DM is the leading cause of blindness and amputation and contributes substantially to kidney disease, cardiomyopathy, and cerebrovascular and peripheral artery diseases. Of importance, recent advances in biology and medicine have introduced new technologies to study the molecular pathology underlying DM-related complications, the development of novel strategies to treat these conditions, and the evaluation of outcomes.

The present special issue has been designed to stimulate the continuing efforts to develop novel drugs and therapeutic targets and new perspectives of combined therapies for decreasing micro- and macrovascular complications in DM. It includes one review article and ten original research papers, from leading and emerging scientists with diverse expertise and interests, and covers three thematic areas: (a) epidemiology and pathogenesis of DM-related complications; (b) microvascular complications (nephropathy, retinopathy, and neuropathy); and (c) macrovascular complications (cardiovascular disease, stroke, and peripheral artery disease). In all these thematic areas, pathophysiological and molecular mechanisms are discussed and novel drug-target therapies, as well as stem cell-based therapy, are documented in either preclinical or clinical studies. Of importance, future preclinical studies and smaller clinical trials are warranted before proceeding to pivotal trials.

In the paper of the present special issue entitled "Prevalence of Chronic Complications, Their Risk Factors, and the Cardiovascular Risk Factors among Patients with Type 2 Diabetes Attending the Diabetic Clinic at a Tertiary Care Hospital in Sri Lanka," M. H. Arambewela et al. reported the prevalence of micro- and macrovascular complications of 3,000 patients with type 2 DM (T2DM) and their risk factors in a single center. To note, the study comprised predominantly female patients (~75%), which are usually underscored in epidemiologic studies. Their main findings included that increased age, disease duration, and glycated hemoglobin (HbA_{1c}) were the main risk factors for microvascular disease and diabetic foot, while age was the only risk factor for macrovascular complications. In addition, occurrence of coronary artery disease (CAD), peripheral neuropathy, diabetic foot, and lower extremity amputation was significantly higher among male individuals when compared to female individuals. Collectively, gender differences may be taken into account when providing healthcare management and allocate resources for prevention and treatment of DM-related complications [1].

Besides traditional risk factors for macrovascular DM-related complications, emerging data of single nucleotide polymorphisms (SNPs) provide new insights for the risk of hyperglycemia and the development of these complications and their response to current treatment. In the study of X. Ma et al. entitled "Polymorphisms in the Glucagon-Like Peptide

1 Receptor (*GLP-1R*) Gene Are Associated with the Risk of Coronary Artery Disease in Chinese Han Patients with Type 2 Diabetes Mellitus: A Case-Control Study," eleven haplotype-tagging SNPs for GLP-1R were tested. They found that patients with the GG genotype at rs4714210 had a lower CAD risk when compared to patients with other genotypes, even when other known CAD risk factors were evaluated. GLP-1R agonists are associated with a decrease in well-known risk factors for heart disease, such as HbA_{1c}, fasting blood glucose, body weight, waist circumference, systolic and diastolic blood pressures, total cholesterol, and triglycerides [2]. Moreover, understanding GLP-1R polymorphism may help predict individual response to CAD treatment, although randomized controlled and multicenter trials are further required to verify the importance of GLP-1R polymorphism.

In the Evaluation of Lixisenatide in Acute Coronary Syndrome (ELIXA) multicenter randomized and placebo-controlled trial, 6,068 patients with T2DM who had a recent coronary event were enrolled into this trial and followed for a median of 25 months [3]. The addition of lixisenatide, a GLP-1 agonist, to standard care did not significantly alter the rate of major cardiovascular (CV) events or other serious adverse events, including renal and vascular disease occurrence. Further exploratory analysis of the ELIXA trial documented that lixisenatide reduced the progression of urinary albumin-to-creatinine ratio in macroalbuminuric patients and was associated with a lower risk of new-onset macroalbuminuria after adjustment for HbA_{1c} and other traditional renal risk factors [4]. In the study entitled "Retinopathy, Neuropathy, and Subsequent Cardiovascular Events in Patients with Type 2 Diabetes and Acute Coronary Syndrome in the ELIXA: The Importance of Disease Duration" of J. P. Seferovic et al., both retinopathy and neuropathy were associated with a primary composite outcome (CV death, nonfatal MI (myocardial infarction), stroke, or hospitalization for unstable angina); CV composite (CV death, nonfatal MI, stroke, and hospitalization for heart failure (HF)); myocardial infarction; HF hospitalization; and all-cause mortality. However, when retinopathy and neuropathy were adjusted to T2DM duration, no association was found, and only T2DM duration was related to those outcomes. These findings point out the relevance of adjusting DM duration to other variables when clinical trials are critically analyzed, as well as when novel therapeutic strategies are tested in DM setting.

Stem cell-based therapy is considered a promising strategy for the treatment of DM and DM-related complications [5]. In the review of this special issue entitled "Addressing Stem Cell Therapeutic Approaches in Pathobiology of Diabetes and Its Complications", B.-Y. Peng et al. summarized the main findings of stem cell-based therapy, with a focus on multipotent stem cells (hematopoietic stem cells and mesenchymal stem cells (MSCs) from different sources) and pluripotent stem cells (embryonic stem cells and inducible pluripotent stem cells) in micro- and macrovascular DM-related complications in both T1DM and T2DM settings. Pathophysiological and molecular mechanisms in DM, such as the increase in oxidative stress and advanced glycation end products (AGEs) accumulation and the decrease in endothelial progenitor cells function, were addressed. The broad spectrum of stem cell pleiotropic properties was also discussed, including paracrine effects (immunomodulation, secretion of growth, and anti-apoptotic and antifibrotic factors) and direct differentiation into damaged tissue. Of importance, efficacy of stem cell-based therapy requires further studies addressing the homing capacity, the most important source of stem cells and their route of injection, the number of infusions, and the severity of tissue damage and endpoints, which ultimately will reach definite conclusions about the therapeutic potential of stem cells and their impact on clinical outcomes [6].

As documented in several clinical trials, experimental findings on stem cell-based therapy for human disease are controversial [7]. MSC-based therapy reduced the severity of acute allograft kidney rejection and opportunistic infection after kidney transplant [8], although not affecting allograft survival and kidney function. Similarly, a single infusion of allogeneic MSCs stabilized or improved estimated glomerular filtration ratio in patients with moderate to severe DN [9]. However, no benefits of MSCs infusion were noted in patients with acute kidney injury after cardiac surgery [10]. In the study of K. W. Lee et al. entitled "Renal Ischemia-Reperfusion Injury in a Diabetic Monkey Model and Therapeutic Testing of Human Bone Marrow-Derived Mesenchymal Stem Cells," a cynomolgus monkey model of streptozotocin-induced DN combined to acute renal ischemia-reperfusion injury, MSC treatment promoted amelioration of functional parameters and attenuated morphological kidney damage. The results obtained from this study will contribute to set the basis for establishing further investigation on the therapeutic potential of MSCs for treatment of kidney disease in other preclinical and clinical studies.

Endothelial dysfunction (ED) plays also a critical role in DM-related complications and represents an imbalance in the production of vasodilator factors, which results in prothrombotic and atherogenic effects in the vasculature [11]. Furthermore, ED in DM setting is associated with vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, prooxidation, impaired coagulation, and decreased nitric oxide production. CKD is a potential contributor to the pathogenesis of ED [12]. M. N. Coutinho et al., in their study entitled "There Is No Impact of Diabetes on the Endothelial Function of Chronic Kidney Disease Patients" highlighted endothelial dysfunction in T2DM patients with CKD and compared their findings to nondiabetic patients with CKD. In DM-CKD patients, they observed more frequently obesity, previous MI, myocardial revascularization, and a higher number of endothelial progenitor cells and pulse wave velocity when compared to non-DM-CKD patients. Surprisingly, uremic toxins were the main determinants of ED in DM-CKD patients and not DM per se. These findings unravel important aspects of endothelial progenitor cell modulation in DM-CKD setting and have important biological implications for therapeutic application of stem cells therapy in that population.

However, future studies are urgently needed to fully gain mechanistic insights when uremic toxins and hyperglycemia are both present.

Taking a step forward in our understanding of DN pathogenesis, C. Wang et al. evaluated in their study entitled "Artificially Cultivated *Ophiocordyceps sinensis* Alleviates Diabetic Nephropathy and Its Podocyte Injury via Inhibiting P2X7R Expression and NLRP3 Inflammasome Activation" the therapeutic potential of *Ophiocordyceps sinensis* (ACOS), which corresponds to a fungus-caterpillar complex formed after the fungus infects the larva of the moth. ACOS has been used for centuries in China and Asian countries. Mechanistically, ACOS reduced the expression of the P2X7 receptor (P2X7R) and NLRP3 inflammasome (NLRP3, ASC, and caspase 1) and downstream effectors (IL-1β and IL-18), yet decreasing podocyte injury *in vitro* and *in vivo* in a rat model of DN (low dose of streptozotocin and high-fat diet). P2X7 receptors are highly expressed on macrophages and are essential components of proinflammatory signaling in multiple tissues. In diabetic patients, renal P2X7R expression is associated with severe mesangial expansion, impaired glomerular filtration ratio, and increased interstitial fibrosis. These findings were similarly found in P2X7R-deficient mice [13]. Correspondingly, P2X7R activation enhanced the release of MCP-1 in human mesangial cells cultured under high-glucose conditions. Therefore, inhibition of P2X7R may represent a novel target in DN setting. The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is a newly recognized and potent inflammatory mediator that induces inflammatory responses in several disorders, including DN. There is an interconnectivity between NLRP3 inflammasome, inflammation, and oxidative stress, which are activated by damage-associated molecular patterns (DAMPs, e.g., hyperlipidemia, hyperglycemia, receptor for AGES, islet amyloid polypeptide protein, and oxidized low-density protein) and ultimately drive micro- and macrovascular DM-related complications [14]. A key aspect of NLRP3 inflammasome activity in DN nephropathy is its modulation by antidiabetic drugs used in routine clinical practice, such as insulin, biguanides, sodium-glucose cotransporter-2 (SGLT2) inhibitors, sulfonylureas, thiazolidinediones, and dipeptidyl peptidase-4 (DPP-4) inhibitors [15].

One of these drugs, the SGLT2 inhibitors (empagliflozin, dapagliflozin, and canagliflozin), led to significantly lower rates of death from cardiovascular causes (38%), hospitalization for heart failure (35%), and death from any cause (32%) in T2DM patients, as documented in the randomized controlled trial EMPA-REG Outcomes ($n = 7{,}020$ individuals) with a 3.1-year follow-up [16]. Besides its effect on macrovascular DM-related complications, empagliflozin was associated significantly with lower rates of incident or worsening of DN (12.7% vs. 18.8%). Doubling of the serum creatinine and renal replacement therapy initiation also decreased by 44% and 55%, respectively [17]. In the study of E. H. Cho et al. entitled "Potent Oral Hypoglycemic Agents for Microvascular Complication: Sodium-Glucose Cotransporter 2 Inhibitors for Diabetic Retinopathy," they studied 49 individuals with T2DM under SGLT2 inhibitors (empagliflozin or dapagliflozin) treatment. They found that these drugs decreased the risk of diabetic retinopathy (DR) progression, even when adjusted for age, duration of DM, initial DR grade, and HbA_{1c} levels. These findings identified gaps that need to be addressed in further studies, yet full spectrum of SGLT expression and its role in the eye is poorly understood [18].

The pathogenesis of DR includes several hyperglycemia-mediated mechanisms, e.g., protein C kinase and polyol pathway activation, AGE accumulation, and increase in hexosamine pathway flux. These mechanisms promote changes in retinal blood flow, increase in vascular permeability, ED, altered growth factor signaling, thickening of capillary basement membrane, pericyte loss, microaneurysms, hemorrhages, apoptosis, and increase of reactive oxygen species [18]. One of these features, the thickening of capillary basement membrane is associated with deposition of extracellular matrix components, such as fibronectin, collagen IV, and laminin, which lead ultimately to microvascular occlusion and retinal hypoperfusion. In the study of G. Song et al. entitled "Effects of High Glucose on the Expression of LAMA1 and Biological Behavior of Choroid Retinal Endothelial Cells," laminin alpha-1 (LAMA1) expression was investigated in retinal choroidal vascular endothelial cells (RF/6A line). Surprisingly, authors found an inverse correlation of LAMA1 expression and glucose concentration, as well as a lower capacity of proliferation, migration, and adhesion of retinal choroidal vascular endothelial cells. These findings indicate that LAMA1 may exert a protective effect against DR, although future *in vitro* and *in vivo* studies are needed to verify these findings. Moreover, recreating the microenvironment of the eye will provide mechanistic insights of LAMA1 role in DR setting.

Peripheral artery disease (PAD) is particularly debilitating as it may result in acute and chronic inferior limb lesions and amputations in DM setting. In addition, PAD is extremely costly for the patient and financially impacts the economy as a range of prophylactic actions and drug-related and endovascular/surgical treatments may be essential. Diabetic foot affects 15% of diabetic patients, yet neuropathy, neuroischemia, and infections have an interconnectivity in determining the worsening of the lesions [19]. Moreover, almost 85% of all amputations are preceded by a foot ulceration that deteriorates to a severe gangrene or infection. In the paper entitled "Distribution of Microbes and Drug Susceptibility in Patients with Diabetic Foot Infections in Southwest China," M. Wu et al. evaluated the microbial distribution through collection of deep ulcer secretion and drug susceptibility among diabetic foot ulcers (DFUs), using Wagner classification, in 428 hospitalized patients. They documented a distinct distribution and type of bacteria in accordance to Wagner classification (grades 3-5 had mainly gram-negative bacilli) and duration of DFUs (chronic ulcer was also associated with gram-negative bacilli in 54.2%). Of importance, knowledge of the microbial etiology in DFUs and understanding antibiotic resistance is critical for an effective management and treatment of these infected lesions. Likewise, conventional diagnostic methods combined to molecular techniques for

bacterial identification and quantification may represent a powerful approach in DFU setting.

A promising therapy for diabetic foot is the bacteria-killing nanotechnology Bio-Kil socks, as addressed in the paper of D. Lu et al. and entitled "Insoles Treated with Bacteria-Killing Nanotechnology Bio-Kil Reduce Bacterial Burden in Diabetic Patients and Healthy Controls". Their findings showed that Bio-Kil socks efficiently reduced bacterial growth in both diabetic patients and healthy individuals, mainly in Gram-positive bacteria, which has important implications for the design of future studies. Recent advances in the field point out to the development of insoles with arch design and ulcer isolations for effective stress reduction in a diabetic foot, and these novel insoles are designed with a skin-like material [20]. To note, these findings may positively impact the outcomes of diabetic patients with DFUs.

Collectively, the present special issue provides new findings in micro- and macrovascular diabetic complications and future management of these complications. Of importance, further knowledge of the pathophysiological and molecular mechanisms involved in the onset and progression of DM-related complications is needed for not only to treat these complications but also to curtail their progression, which ultimately may provide a better care to patients.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Research Article

Retinopathy, Neuropathy, and Subsequent Cardiovascular Events in Patients with Type 2 Diabetes and Acute Coronary Syndrome in the ELIXA: The Importance of Disease Duration

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Introduction. We investigated the association of diabetic retinopathy and neuropathy with increased risk of recurrent cardiovascular (CV) events in 6068 patients with type 2 diabetes mellitus (T2DM) and recent acute coronary syndrome (ACS) enrolled in the Evaluation of Lixisenatide in Acute Coronary Syndrome (ELIXA). *Methods*. History of retinopathy and neuropathy as well as duration of T2DM were self-reported at screening. Proportional hazards regression models were used to assess relationships between retinopathy, neuropathy, and recurrent CV events. *Results*. At screening, retinopathy and neuropathy were reported in 10.7% and 17.5% of patients, respectively, while 5.7% reported both. When adjusted for randomized treatment only, both retinopathy and neuropathy were associated with a primary composite outcome (CV death, nonfatal MI, stroke, or hospitalization for unstable angina) (retinopathy: HR 1.44, 95% CI 1.19–1.75; neuropathy: HR 1.33, 95% CI 1.12–1.57), CV composite (CV death, nonfatal MI, stroke, hospitalization for heart failure (HF)) (retinopathy: HR 1.57, 95% CI 1.31–1.88; neuropathy: HR 1.38, 95% CI 1.19–1.62), myocardial infarction (retinopathy: HR 1.38, 95% CI 1.08–1.76; neuropathy: HR 1.26, 95% CI 1.02–1.54), HF hospitalization (retinopathy: HR 2.03, 95% CI 1.48–2.78; neuropathy: HR 1.71, 95% CI 1.30–2.27), and all-cause mortality (retinopathy: HR 1.65, 95% CI 1.28–2.12; neuropathy: HR 1.43, 95% CI 1.14–1.78). When included in the same model, and adjusted for T2DM duration, there were no independent associations of either with CV outcomes, while T2DM duration remained strongly associated with all outcomes. Addition of demographic characteristics and CV risk factors did not further alter these relationships. *Conclusions*. In patients with T2DM and recent ACS, a history of retinopathy and/or neuropathy and longer T2DM duration could be considered clinical markers for high risk of recurrent CV events. This trial is registered with the ELIXA (Evaluation of Lixisenatide in Acute Coronary Syndrome), ClinicalTrials.gov registration number NCT01147250.

1. Introduction

Cardiovascular (CV) risk in type 2 diabetes mellitus (T2DM) was associated with disease duration and severity of hyperglycemia in many studies [1–3]. In the Framingham Heart Study, duration of T2DM significantly and positively related to the risk of coronary heart disease mortality, but not morbidity, or CV disease (CVD) morbidity or mortality [2]. The Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) trial, on the contrary, showed that T2DM duration was independently associated with the risk of macrovascular complications and death [3]. In a meta-analysis of five trials, intensive glycemic control led to a reduction of nonfatal myocardial infarction (MI) incidence by one-sixth, with no significant effect on the incidence of nonfatal stroke, and both CV or all-cause mortality [4].

Additionally, duration of T2DM and severity of hyperglycemia are strong and consistent risk factors for the development and progression of microvascular diabetic complications—retinopathy (DR), neuropathy (DN), and nephropathy [5–8]. For example, in the very large and diverse T2DM population in ADVANCE, T2DM duration was independently associated with the risk of DR and nephropathy [3]. In the Maastricht Study, a T2DM-enriched population-based cohort study prediabetes, T2DM, and measures of hyperglycemia were independently associated with DR and DN [9]. Improved metabolic control was associated with 25% risk reduction of microvascular composite endpoint in the United Kingdom Prospective Diabetes Study (UKPDS) [10]. Similar effect has been shown for individual complications, DR [5, 11], DN [6, 11], and nephropathy [7, 11, 12].

The association of diabetic nephropathy and increased risk of CVD has been revealed in multiple patient cohorts. Data from United States community-based study demonstrated that individuals with diabetic nephropathy were at four- and threefold higher risk for CV death and all-cause death, respectively, compared to those without [13]. In the ADVANCE study, both albuminuria and reduced estimated glomerular filtration rate (eGFR) were independently and continuously associated with the risk for CV and kidney outcomes in T2DM patients [14]. Similarly, among patients with type 1 diabetes followed for over 30 years, the development of diabetic nephropathy was associated with higher risks of CVD and renal events. However, this effect was almost entirely eliminated by adjustment for updated mean HbA1c which led to the conclusion that glycemic exposure correlates very strongly with CVD and mortality, and that this is partly mediated by hyperglycemia-induced renal disease [15]. Steno-2 study, including T2DM patients with microalbuminuria, showed that long-term intensified therapy reduced the risk of CV events, as well as diabetic nephropathy, DR, and DN [16]. Also, the risk of microalbuminuria has been shown to increase with T2DM duration [17].

Potential associations, of DR and DN with CV outcomes, whether causal or only predictive, have received less attention. We therefore investigated the association of DR and DN with increased risk of recurrent CV events, in patients with a recent acute coronary syndrome (ACS).

2. Materials and Methods

2.1. Study Design and Patients. The ELIXA (Evaluation of Lixisenatide in Acute Coronary Syndrome) was a randomized, double-blind, placebo-controlled trial designed to assess the effects of lixisenatide added to current T2DM therapy on CV morbidity and mortality in 6068 patients with a recent ACS.

We examined the primary composite (CV death, nonfatal MI, stroke, or hospitalization for unstable angina), CV composite (CV death, nonfatal MI, stroke, and hospitalization for heart failure (HF)), each of its components, and all-cause mortality. Details of the trial design, entry criteria, and the main results have been reported previously [18, 19].

For this post hoc analysis, all 6068 ELIXA participants were included. Self-reported historical data on DR and DN were collected at screening. Patients were asked to answer "yes," "no," or "unknown" on the presence of DR and/or DN. If DR was present, date of diagnosis was recorded, as well as information on photocoagulation and vitrectomy. However, these interventions were not analyzed further due to a small number of events. Presence of DN was defined as a report of either sensory/motor or autonomic neuropathy. Only "yes" responses were used to define the exposure variables for all subsequent analyses. Blood samples included in this analysis were done at screening by a central laboratory. Duration of T2DM was evaluated based on medical record review or self-report at the screening visit.

2.2. Statistical Analysis. Baseline characteristics of patients were stratified by the presence of DR and/or DN. Descriptive data are presented as the mean ± standard deviation for normally distributed variables and as median (25–75th percentile) for nonnormally distributed variables. Categorical variables are expressed as proportions and were compared by the chi-square test, while continuous variables were compared using t-tests or Wilcoxon rank-sum tests, as appropriate. Two proportional hazards regression models were used to assess the association between DR, DN, and recurrent CV events. Multivariable proportional hazards models were used to assess the association between DR, DN, and primary composite endpoint, CV composite endpoint, components, and all-cause mortality. Both DR and DN were included in the first model and adjusted for the duration of T2DM. In the second model, demographic characteristics and CV risk factors (age, sex, race, body mass index (BMI), baseline HbA1c, smoking, history of hypertension (HT), heart rate, total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides) were added to the previous model. Both models were adjusted for randomized study treatment. Predictors of DR and DN were determined from multivariable logistic regression model using forward stepwise selection including all variables in Table 1. Two-sided p values < 0.05 were considered significant. No adjustment was made for multiple comparisons. Analyses were performed using the Stata version 13.1 (StataCorp., College Station, TX, USA).

Table 1: Characteristics of all patients at baseline according to the presence of retinopathy and/or neuropathy.

Characteristic	All patients $n = 6068$	No retinopathy/ neuropathy $n = 4705$	Retinopathy and/ or neuropathy $n = 1363$	p value
Age (years)	60.3 ± 9.7	59.7 ± 9.7	62.3 ± 9.2	<0.001
Male sex (n (%))	4207 (69.3)	3379 (71.8)	828 (60.7)	<0.001
Body weight (kg)	84.9 ± 19.4	84.1 ± 18.9	87.6 ± 20.9	<0.001
Body mass index (kg/m^2)	30.2 ± 5.7	29.9 ± 5.6	31.2 ± 6.0	<0.001
Duration of T2DM (years)	7.4 (2.8, 13.6)	6.0 (2.0, 11.7)	12.4 (7.0, 20.2)	<0.001
Categories of T2DM duration (n (%))				<0.001
≤1 year	964 (15.9)	914 (19.4)	50 (3.7)	
>1–≤5 years	1294 (21.3)	1130 (24.0)	164 (12.0)	
>5–≤10 years	1359 (22.4)	1076 (22.9)	283 (20.8)	
>10 years	2451 (40.4)	1585 (33.7)	866 (63.5)	
Race (n (%))				<0.001
White	4576 (75.4)	3471 (73.8)	1105 (81.1)	
Black	221 (3.6)	171 (3.6)	50 (3.7)	
Asian	771 (12.7)	669 (14.2)	102 (7.5)	
Other	500 (8.2)	394 (8.4)	106 (7.8)	
Region (n (%))				<0.001
Africa/Near East	296 (4.9)	228 (4.8)	68 (5.0)	
Asia Pacific	703 (11.6)	615 (13.1)	88 (6.5)	
Eastern Europe	1587 (26.2)	1115 (23.7)	472 (34.6)	
North America	807 (13.3)	564 (12.0)	243 (17.8)	
South and Central America	1944 (32.0)	1600 (34.0)	344 (25.2)	
Western Europe	731 (12.0)	583 (12.4)	148 (10.9)	
Smoking status (n (%))				<0.001
Current	709 (11.7)	579 (12.3)	130 (9.5)	
Former	2746 (45.3)	2184 (46.4)	562 (41.2)	
Never	2612 (43.1)	1941 (41.3)	671 (49.2)	
Diastolic blood pressure (mmHg)	77 ± 10	77 ± 10	76.3 ± 10	<0.001
Systolic blood pressure (mmHg)	130 ± 17	129 ± 17	131 ± 17	<0.001
Heart rate (beats/min)	70 ± 10	70 ± 10	71 ± 10	0.027
Fasting plasma glucose (mg/dl)	148.3 ± 51.6	145.2 ± 49.1	159.3 ± 58.1	<0.001
Glycated hemoglobin (%)	7.7 ± 1.3	7.6 ± 1.3	8.0 ± 1.2	<0.001
Glycated hemoglobin (mmol/mol)	61 ± 14	60 ± 14	64 ± 13	<0.001
Total cholesterol (mg/dl)	153.5 ± 44.6	151.3 ± 43.5	161.1 ± 47.3	<0.001
HDL cholesterol (mg/dl)	42.9 ± 10.9	42.6 ± 10.6	44.1 ± 11.7	<0.001
LDL cholesterol (mg/dl)	78.5 ± 35.3	77.1 ± 34.7	83.4 ± 36.9	<0.001
Triglycerides (mg/dl)	137.2 (99.1, 195.6)	136.3 (100.0, 192.9)	141.6 (99.1, 208.0)	0.021
eGFR (ml/min/1.73m^2)	76 ± 21	77 ± 21	71 ± 22	<0.001
Albuminuria (n (%))				<0.001
<30 mg/g	4441 (74.3)	3579 (77.2)	862 (64.2)	
≥30–<300 mg/g	1148 (19.2)	819 (17.7)	329 (24.5)	
≥300 mg/g	389 (6.5)	237 (5.1)	152 (11.3)	
Medical history at randomization (n (%))				
Hypertension	4635 (76.4)	3463 (73.6)	1172 (86)	<0.001
Heart failure	1358 (22.4)	923 (19.6)	435 (31.9)	<0.001
Stroke	331 (5.5)	204 (4.3)	127 (9.3)	<0.001
Peripheral arterial disease	393 (6.5)	227 (4.8)	166 (12.2)	<0.001
Atrial fibrillation	366 (6.0)	247 (5.2)	119 (8.7)	<0.001
Percutaneous coronary intervention	4079 (67.2)	3263 (69.4)	816 (59.9)	<0.001
Coronary artery bypass grafting	507 (8.4)	363 (7.7)	144 (10.6)	<0.001

Table 1: Continued.

Characteristic	All patients $n = 6068$	No retinopathy/ neuropathy $n = 4705$	Retinopathy and/ or neuropathy $n = 1363$	p value
Qualifying ACS event (n (%))				<0.001
STEMI	2666 (44.0)	2187 (46.5)	479 (35.2)	
NSTEMI	2348 (38.7)	1817 (38.6)	531 (39.0)	
Unstable angina	1042 (17.2)	693 (14.7)	349 (25.6)	
Missing	9 (0.1)	6 (0.1)	3 (0.2)	
Antihyperglycemic therapy (n (%))				
Metformin	4243 (69.9)	3367 (71.6)	876 (64.3)	<0.001
Sulfonylureas	2266 (37.3)	1779 (37.8)	487 (35.7)	0.16
Insulin	2891 (47.6)	1948 (41.4)	943 (69.2)	<0.001
Thiazolidinediones	128 (2.1)	92 (2.0)	36 (2.6)	0.12
Alpha-glucose inhibitor	181 (3.0)	150 (3.2)	31 (2.3)	0.08
Dipeptidyl peptidase 4 inhibitor	226 (3.7)	176 (3.7)	50 (3.7)	0.90
Other	485 (8.0)	384 (8.2)	101 (7.4)	0.37

Data is presented as means ± SD, median (25–75th percentile), or percentages. HDL: high-density lipoprotein; LDL: low-density lipoprotein; MI: myocardial infarction; ACS: acute coronary syndrome; eGFR: estimated glomerular filtration rate.

3. Results

3.1. Baseline Characteristics. Demographic and clinical characteristics of the 6068 patients are shown in Table 1. In the whole population, the mean age was 60.3 years and known T2DM duration was 7.4 years. However, the reported duration of T2DM varied widely, with 15.9% of participants having known T2DM for less than 1 year, 21.3% ≤5 years, 22.4% >1–≤5 years, and 40.4% longer than10 years (Supplementary Figure 1). Of the whole population, 1363 (22.5%) reported DR and/or DN. DR was reported in 651 (10.7%) patients, DN in 1060 (17.5%) patients, and both in 348 (5.7%) patients (Figure 1). Of 651 who reported DR, 159 patients (24.4%) had prior photocoagulation, and 32 patients (4.9%) had vitrectomy.

Patients who had DR and/or DN were significantly older (mean 62.3 vs. 59.7 years) and had longer known duration of T2DM (mean 12.4 vs. 6.0 years) than those with neither complication. Smaller proportions of patients with DR and/or DN reported shorter T2DM duration of <1 year (3.7% vs 19.4%) and ≤5 years (12.0% vs. 24.0%). The distribution of T2DM duration in all patients, as well as in those with and without retinopathy and/or neuropathy, is shown in Figure 2. Participants with DR and/or DN also had higher BMI, were also more likely to be nonsmokers, and had significantly higher glycated hemoglobin, fasting plasma glucose, and total and LDL cholesterol. The subgroup with DR and/or DN also had more evidence of renal disease reflected as increased albumin-to-creatinine ratio in comparison with those without DR and/or DN. Also, they were more likely to have history of CV disease (HT, HF, stroke, peripheral arterial disease, and atrial fibrillation). Finally, patients with DR and/or DN used more frequently metformin and insulin, while other glucose-lowering agents were similarly distributed among groups (Table 1).

3.2. Retinopathy and/or Neuropathy and CV Outcomes. In univariate analysis, DR was significantly associated with primary and CV composite endpoint, all-cause and CV death, and all CV events except stroke ($p = 0.068$), while DN was associated with primary and CV composite endpoint, all-cause death, and all nonfatal CV events, but not CV death ($p = 0.08$, Table 2). When both DR and DN were included in the same model, along with T2DM duration, there were no independent associations of either with any of the outcomes, while duration of T2DM remained highly significantly associated with all outcomes (Table 2). Furthermore, the addition of demographic characteristics and CV risk factors to the previous model resulted in neither DR nor DN being associated to any of the outcomes, but identified duration of T2DM as an independent predictor of CV events, beyond DR, DN, and CV risk factors (Table 2). There were no significant interactions between DR and DN in adjusted and unadjusted models. It has previously been reported that no significant interactions were detected with respect to prespecified patient subgroups for the ELIXA

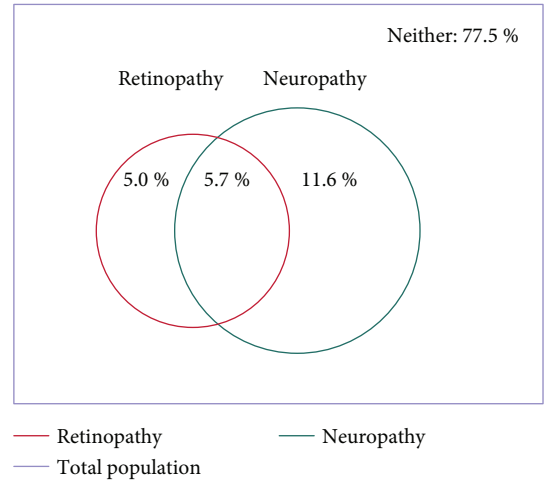

Figure 1: Prevalence of retinopathy and/or neuropathy.

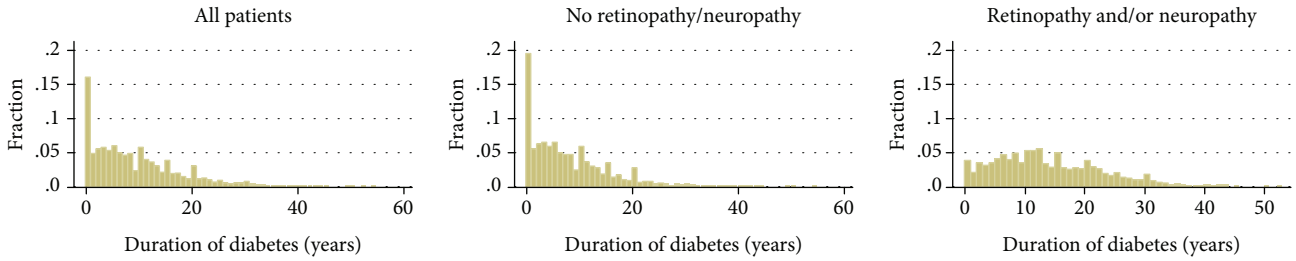

Figure 2: Distribution of T2DM duration in all patients, patients with no retinopathy/neuropathy and patients with retinopathy and/or neuropathy.

primary composite outcome, which included T2DM duration < 10 vs. >10 years [19]. In addition, no statistically significant interactions were found with respect to DR, DN, or duration of T2DM as a continuous variable.

3.3. Predictors of Retinopathy and/or Neuropathy. In both univariate and multivariate analysis, the most significant predictors of both DR and DN were duration of T2DM and insulin use (Table 3). The relationships between presence of DR and DN with duration of T2DM are shown in Supplementary Figures 2a–b. In multivariate analysis, other highly statistically significant ($p < 0.001$) predictors of DR were history of HF and stroke (Table 3) and of DN higher weight were previous percutaneous coronary intervention (PCI), total cholesterol, history of peripheral arterial disease, and previous stroke (Table 3). These models were effectively able to discriminate between patients with and without DR (area under the curve (AUC) 0.81) and DN (AUC 0.76).

4. Discussion

In this post hoc analysis of a large population selected for having a recent ACS event, together with T2DM, less than one-fourth of patients reported a history of DR, DN, or both. This was a smaller proportion than found in other studies of CV outcomes in T2DM, in which many patients had CV risk factors but not necessarily a completed event. For example, the prevalence of DR in the Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) study—which also relied on patient self-reports—was almost three times higher in a more severely compromised patient cohort with T2DM, chronic kidney disease, and anemia, but also twice as long T2DM duration [20]. The widely varying duration of T2DM in the ELIXA, and the inclusion of a large fraction with very short duration of T2DM, allowed our analysis to examine the relationships between duration of T2DM, presence of DR or DN, and risk of subsequent CV events.

The variability of previously reported associations between DR and/or DN and subsequent CV events could stem from variable T2DM duration and comorbidities of analyzed patient cohorts. The positive association of DR and CV events was observed in various patient cohorts [21–23]. In a recently published study with a large cohort of T2DM patients without the history of CV diseases, the risk of developing CV death, nonfatal MI, or stroke was about 40% higher in patients with DR and DN [24]. In the meta-analysis including 11,505 patients, DR was associated with 1.7-fold increased risk for CV events [25]. However, in the TREAT study, enrolling 4038 patients with T2DM, chronic kidney disease, and anemia, DR was not independently associated with a higher risk of renal or CV morbidity or death, possibly due to longer duration of T2DM and more comorbidities [20].

Our findings on DN are partially in accordance with previously published studies that revealed an association between autonomic DN and CV events [26]. Other studies have shown that DN was associated with a twofold risk increase for peripheral vascular disease [27]. In our study, we investigated the association of both DN and outcomes, but not peripheral vascular disease. Therefore, our findings on the large cohort of T2DM patients with recent ACS provide novel insight into the association between DN and recurrent CV events. These findings indicate the importance of the duration of T2DM as a traditional risk factor [28].

Consistent with prior reports, our analysis found that DR and/or DN were associated at baseline with longer T2DM duration, worse glycemic control, more insulin use, and history of prior manifest CV disease. Furthermore, both DR and DN were associated with increased risk of recurrent CV events, especially HF hospitalization. However, these associations were no longer statistically significant after adjustment for duration of T2DM, which remained a strong independent predictor of CV events [20, 29, 30]. Thus, DR and DN as well as duration of T2DM appear to be predictors of increased CV risk but, unlike diabetic nephropathy, not themselves are contributors to this risk.

While other relevant studies also adjusted clinical outcomes for the duration of T2DM—among other demographic characteristics and CV risk factors—none of them adjusted for only the known duration of the disease, which makes our findings unique. In a high-risk T2DM patient cohort used in the Veterans Affairs Diabetes Trial (VADT) (1791 subjects) [31], the duration of T2DM was significantly related to CV events. However, to the best of our knowledge, no studies included similar patient cohort, T2DM patients with recent ACS.

There are several possible explanations for the duration of T2DM being a strong independent predictor of recurrent CV events in our analysis. Longer duration of the disease may have a direct effect on progression of atherosclerotic lesions, increasing the risk of a recurrent CV event. In addition, it might be associated with autonomic DN and reduced heart rate variability, increasing the risk of CV death, which was not the case in our cohort. A long-term increase in

Table 2: Multivariable modeling.

Hazard ratio (95% CI)		Primary composite endpoint $N = 805$ events	CV composite endpoint $N = 913$ events	Cardiovascular death $N = 315$ events	Myocardial infarction $N = 531$ events	Stroke $N = 127$ events	Heart failure hospitalization $N = 249$ events	Death $N = 434$ events
Univariate**	Retinopathy	1.44 (1.19–1.75)*	1.57 (1.31–1.88)*	1.58 (1.17–2.13)*	1.38 (1.08–1.76)*	1.56 (0.97–2.51)	2.03 (1.48–2.78)*	1.65 (1.28–2.12)*
	Neuropathy	1.33 (1.12–1.57)*	1.38 (1.19–1.62)*	1.29 (0.99–1.68)	1.26 (1.02–1.54)*	1.59 (1.07–2.37)*	1.71 (1.30–2.27)*	1.43 (1.15–1.78)*
	T2DM duration (per 5 years)	1.17 (1.13–1.22) *	1.19 (1.15–1.23)*	1.22 (1.16–1.29)*	1.19 (1.14–1.25)*	1.08 (0.98–1.19)	1.30 (1.23–1.38)*	1.22 (1.17–1.28)*
Model 1**	Retinopathy	1.07 (0.86–1.32)	1.13 (0.94–1.31)	1.12 (0.80–1.56)	1.00 (0.77–1.31)	1.27 (0.74–2.16)	1.21 (0.85–1.73)	1.13 (0.85–1.50)
	Neuropathy	1.10 (0.92–1.31)	1.11 (0.94–1.31)	1.01 (0.76–1.34)	1.02 (0.82–1.28)	1.43 (0.93–2.20)	1.23 (0.90–1.66)	1.12 (0.88–1.42)
	T2DM duration (per 5 years)	1.16 (1.12–1.21)*	1.18 (1.14–1.22)*	1.21 (1.14–1.29)*	1.19 (1.14–1.25)*	1.04 (0.94–1.16)	1.28 (1.20–1.36)*	1.21 (1.15–1.27)*
Model 2**	Retinopathy	1.07 (0.86–1.33)	1.13 (0.92–1.38)	1.12 (0.80–1.57)	1.00 (0.76–1.32)	1.28 (0.75–2.19)	1.15 (0.81–1.65)	1.15 (0.87–1.53)
	Neuropathy	1.02 (0.85–1.23)	0.99 (0.84–1.18)	0.91 (0.68–1.22)	1.00 (0.78–1.24)	1.27 (0.82–1.99)	0.96 (0.70–1.31)	1.03 (0.81–1.31)
	T2DM duration (per 5 years)	1.10 (1.05–1.15)*	1.12 (1.07–1.16)*	1.14 (1.07–1.22)*	1.13 (1.08–1.20)*	0.95 (0.85–1.07)	1.21 (1.13–1.30)*	1.13 (1.07–1.19)*

Primary composite endpoint: CV death, nonfatal MI, stroke, or hospitalization for unstable angina; CV composite endpoint: CV death, nonfatal MI, stroke, hospitalization for heart failure (HF); $^{*}p \leq 0.05$. Univariate: adjusted for randomized study treatment only; model 1: adjusted for retinopathy, neuropathy, T2DM duration, and randomized study treatment; model 2: model 1+ age, sex, race, BMI, baseline HbA1c, smoking, history of hypertension, heart rate, total cholesterol, LDL cholesterol, and triglycerides; $^{**}p \leq 0.05$.

Table 3: Predictors of retinopathy and neuropathy multivariate models.

Parameter	Retinopathy		Neuropathy	
	OR (95% CI)	$\|Z\|$ score	OR (95% CI)	$\|Z\|$ score
Duration of diabetes (per 5 years)	1.48 (1.41–1.56)	14.64	1.31 (1.25–1.37)	11.52
Insulin use	2.77 (2.23–3.46)	9.13	2.19 (1.86–2.58)	9.36
Weight (per 5 kg)	—	—	1.09 (1.07–1.11)	8.83
Previous PCI	0.75 (0.61–0.91)	2.94	0.60 (0.51–0.70)	6.32
Total cholesterol (per 10 mg/dl)	—	—	1.05 (1.04–1.07)	6.23
History of PAD	—	—	1.88 (1.45–2.43)	4.82
History of HF	1.65 (1.35–2.02)	4.82	1.32 (1.12–1.56)	3.23
Previous stroke	2.06 (1.51–2.82)	4.54	1.86 (1.42–2.45)	4.45
Diastolic blood pressure (per 10 mmHg)	—	—	0.88 (0.82–0.95)	3.25
Sulphonylurea use	—	—	1.29 (1.11–1.51)	3.24
Previous CABG	0.72 (0.52–0.98)	2.11	0.66 (0.51–0.86)	3.06
Presence of hypertension	1.46 (1.11–1.91)	2.69	1.39 (1.12–1.72)	3.00
Body mass index (per 5 kg/m^2)	1.12 (1.04–1.22)	2.99	—	—
Glucose (per 10 mg/dl)	1.02 (1.0-1.04)	2.29	1.02 (1.01–1.03)	2.87
eGFR (per 10 ml/min/1.73m^2)	0.94 (0.90–0.98)	2.66	—	—
Male sex	—	—	1.25 (1.06–1.47)	2.60
HDL cholesterol (per 10 mg/dl)	1.11 (1.02–1.20)	2.53	—	—
Age (per 5 years)	—	—	1.05 (1.01–1.10)	2.20
HbA1c (per 1%)	1.09 (1.00–1.19)	1.98	—	—

Model AUC = 0.81 (retinopathy) and model AUC = 0.76 (neuropathy). HF: heart failure; PCI: percutaneous coronary intervention; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; CABG: coronary artery bypass grafting; HbA1c: glycated hemoglobin; PAD: peripheral artery disease.

oxidative stress in T2DM patients may cause increased risk of CV death. Also, longer exposure to hyperglycemia may simply reflect greater exposure to other, perhaps unmeasured, CV risk factors.

The major limitation of our study was the assessment of DR and DN which was based on patients' answers to self-report questionnaires. Therefore, the prevalence of both might have been underestimated. Such potential misclassification bias might have potentially weakened our results, and, therefore, the real association of DR and DN with recurrent CV events could be stronger than presented in this analysis. Another important limitation is the fact that T2DM duration was evaluated based on medical record or self-report and could therefore be underestimated, as patients often remain undiagnosed for many years. Also, having in mind that the study cohort included high-risk patients with T2DM and recent ACS, the results may not be applicable to other populations such as those without CV disease or with a less advanced stage of it. In addition, the design of this study was cross-sectional and therefore does not provide information on whether or not DR and DN developed before or after the onset of any CV disease antecedent to the qualifying ACS event. Finally, our findings should be considered post hoc and hypothesis-generating.

5. Conclusions

In a population with recent ACS together with T2DM, recurrent CV events, DR, and DN were all strongly associated with the T2DM duration. A history of either DR or DN was associated with increased risk of recurrent CV events. As these associations were eliminated by adjustment for the duration of T2DM, which remained a strong independent predictor of recurrent CV events, the link between DR and/or DN and these events is not likely to be a causal one. However, history of DR, DN, or both and longer duration of T2DM could be considered clinical markers for high risk of recurrent CV events. It is important to point out that the presence of both DN and DR is expected to positively correlate with patients' level of interaction with healthcare providers. Furthermore, it is expected that their level of healthcare provider interaction would correlate negatively with the frequency and magnitude of adverse outcomes.

Data Availability

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

Disclosure

The company, Sanofi, played no role in the design and execution of this analysis.

Conflicts of Interest

JPS has no competing interests. RBL is a consultant and research support of Sanofi and a research support and spouse/partner of Sanofi, Amgen Inc., and Novartis

Pharmaceuticals Corporation. BC has no competing interests. RD is a research support of Sanofi. HCG is an advisory panel of Sanofi, Novo Nordisk Inc., Bristol-Myers Squibb Company, Roche Pharmaceuticals, AstraZeneca Pharmaceuticals LP, GlaxoSmithKline, Bayer HealthCare, LLC, Merck, Boehringer Ingelheim Pharmaceuticals Inc., and Eli Lilly and Company; a consultant of Sanofi; and a research support of Sanofi and Eli Lilly and Company. LVK has a relationship with Sanofi and Novartis Pharmaceuticals Corporation. FL is an employee of Sanofi. EFL is a consultant and spouse/partner of Sanofi; a research support of Amgen Inc., Novartis Pharmaceuticals Corporation, and Sanofi; and a research support and spouse/partner of Sanofi. APM has a relationship with Sanofi, Oxford University, Eli Lilly and Company, and BMS. JMcM has a relationship with GlaxoSmithKline, PPD Development LP, Merck, Oxford University, Amylin Pharmaceuticals Inc., Eli Lilly and Company, Parexel, Bayer HealthCare, LLC, Abbvie, and Sanofi. JLP is an advisory panel of Sanofi, a consultant of Sanofi, and a research support of Sanofi. MCR is a consultant of Eli Lilly and Company, AstraZeneca Pharmaceuticals LP, Sanofi, Valeritas, LLC, and Elcelyx Therapeutics Inc.; a research support of AstraZeneca Pharmaceuticals LP, Eli Lilly and Company, Sanofi, and Novo Nordisk Inc.; and has a relationship with Sanofi. These dualities of interest have been reviewed and managed by Oregon Health & Science University. SDS is a consultant of Novartis Pharma AG and Amgen Inc. and a research support of Abbott Laboratories Inc., Amgen Inc., Daiichi-Sankyo Inc., Novartis Pharma AG, Theracos, Boston Scientific, NHLBI, Lone Star Heart, and Sanofi. JCT has no competing interests. MAP is a consultant of Aastrom Biosciences Inc., Abbott Laboratories Inc., Amgen Inc., FibroGen Inc., Cerenis Pharmaceuticals, Concert Pharmaceuticals, GlaxoSmithKline, Hamilton Health Sciences, Medtronic, Merck, Roche Pharmaceuticals, Servier, Teva Pharmaceuticals, and University of Oxford; a research support of Amgen Inc., Celladon, Novartis Pharma AG, and Sanofi; and has a relationship with Novartis Pharma AG.

Authors' Contributions

The presented analysis was prepared jointly by all authors. The corresponding author and coauthors had full access to the data in the study, contributed to the writing of the manuscript, and had final responsibility for the decision to submit for publication.

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Supplementary Materials

Supplementary Figure 1. Patient distribution by T2DM duration categories widely varies: 15.9 % of participants have known T2DM for less than 1 year, 21.3% ≤5 years, 22.4% >1–≤5 years, and 40.4% longer than 10 years. Supplementary Figures 2a–b. Relationship of retinopathy and neuropathy with T2DM duration. *(Supplementary Materials)*

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Research Article

Potent Oral Hypoglycemic Agents for Microvascular Complication: Sodium-Glucose Cotransporter 2 Inhibitors for Diabetic Retinopathy

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The purpose of this study was to investigate the effects of sodium-glucose cotransporter 2 inhibitors (SGLT2i) on the progression of diabetic retinopathy (DR) in patients with type 2 diabetes. The medical records of 21 type 2 diabetic patients who used a SGLT2i and 71 patients with sulfonylurea (control) were reviewed retrospectively. The severity of DR was assessed using the Early Treatment Diabetic Retinopathy Study (ETDRS) scale. Fewer patients who used a SGLT2i than control patients with sulfonylurea showed progression of DR based on ETDRS scale (44% versus 14%, $P = 0.014$). Moreover, treatment with a SGLT2i was associated with a significantly lower risk of DR progression ($P = 0.021$), and this effect remained significant after adjusting for the age, duration of diabetes, initial DR grade, and HbA1c level by propensity score matching ($P = 0.013$). Treatment of type 2 diabetic patients with a SGLT2i slowed the progression of DR compared to sulfonylurea, which is independent of its effect on glycemic control. This study provides a foundation for further evaluation of the effect of SGLT2i on the progression of DR.

1. Introduction

The prevalence of type 2 diabetes is dramatically increasing worldwide, and an estimated 592 million people will have this disease by 2035 [1, 2]. Diabetic retinopathy (DR) is one of the major microvascular complications of diabetes and is also the leading cause of blindness among working-age people in developed countries [3, 4]. Reduction of hyperglycemia is the primary goal of most therapies for type 2 diabetes, and these therapies may also prevent or arrest the development of DR [5]. In addition to strict glycemic control, use of systemic agents in other therapeutic classes, such as candesartan and fenofibrate, can delay the progression of DR in patients with type 2 diabetes [6, 7].

The sodium-glucose cotransporter 2 inhibitors (SGLT2i) are a novel class of oral hypoglycemic agents that decrease the reabsorption of glucose in the renal proximal tubules [8, 9]. These agents can reduce the level of serum glycosylated hemoglobin (HbA1c), induce weight loss, and decrease blood pressure [8–10]. Among several SGLT2i, empagliflozin and dapagliflozin are now available in Korea, and clinicians usually recommended its use in combination with other hypoglycemic agents as a second- or third-line therapy for type 2 diabetes [11].

There are recent reports that SGLT2i also reduce macrovascular and microvascular complications by affecting vascular remodeling [12, 13]. This suggests that these drugs have renoprotective effects. Thus, the SGLT2i not only improve glycemic control but also have important hemodynamic and nonhemodynamic effects [14]. Because the pathogenesis of diabetic nephropathy and DR are similar [15], we hypothesized that SGLT2i may also protect against the progression of DR, which is a topic that has not yet been examined. We retrospectively examined the records of patients with type 2

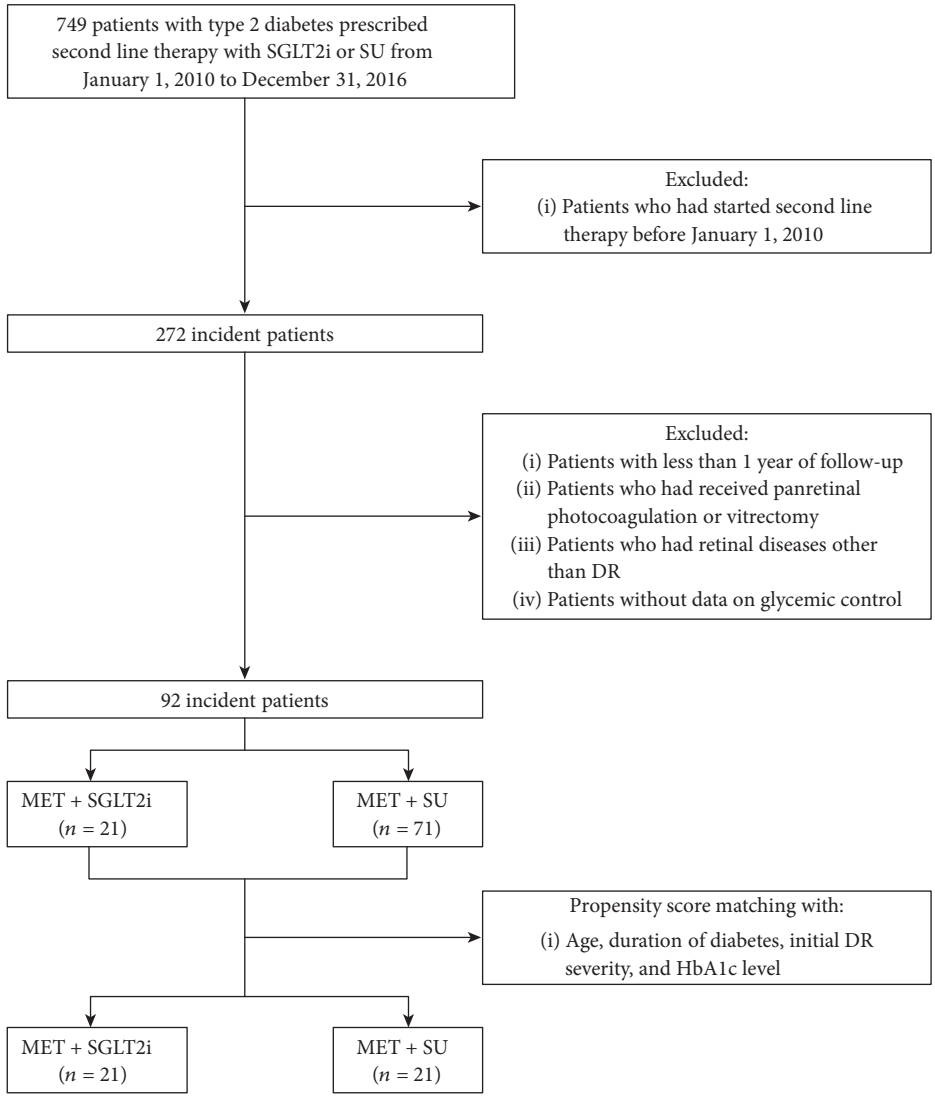

Figure 1: Flow chart of patients included in this study. DR = diabetic retinopathy; MET = metformin; SGLT2i = sodium-glucose cotransporter 2 inhibitor; SU = sulfonylurea.

diabetes to determine the effect of SGLT2i on the progression of DR.

2. Materials and Methods

2.1. Study Population. The medical records of 49 patients with type 2 diabetes who used SGLT2i (SGLT2i group) as add-on medication to metformin and were followed up by the Ophthalmology and Endocrinology Departments of Ajou University Hospital (Suwon, Korea) from January 2010 to December 2016 were retrospectively reviewed (Figure 1). The records of 700 patients with type 2 diabetes who received metformin and sulfonylurea for their diabetes during the same period were also reviewed as control group. Those with dipeptidyl peptidase-4 (DPP4) inhibitors, which may affect DR, were initially excluded from the study [16, 17]. Patients were also excluded if they had (i) no fundus photographs or fluorescein angiography results to grade DR severity, (ii) a history of laser photocoagulation or vitrectomy at initial presentation, (iii) the presence of a retinal pathology other than DR, and (iv) received follow-up for less than 1 year. This study complied with the Declaration of Helsinki and was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-MED-MDB-17-312).

2.2. Clinical Parameters. The demographic and clinical characteristics of the patients were obtained from their medical records. In particular, age, sex, duration of type 2 diabetes, prior history of hypertension and cardiovascular diseases (i.e., coronary artery disease or ischemic stroke (or transient ischemic attack)), serum lipid profile, estimated glomerular filtration rate (eGFR), and ophthalmic history (including DR severity and number of intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents) were recorded. Patients with eGFR less than 60 mL/min/1.73m^2 were excluded to avoid the effect of renal function.

Table 1: Clinical characteristics of patients in the SGLT2i and control groups before propensity score matching.

	SGLT2i ($n = 21$)	Control ($n = 71$)	P value
Age (years)	51.3 ± 9.7	57.8 ± 12.4	0.014*
Sex (male : female)	16 : 5	38 : 33	0.064
Follow-up period (months)	20.1 ± 7.8	25.1 ± 9.2	0.140
Medical history			
Duration of diabetes (years)	11.3 ± 8.9	11.5 ± 9.2	0.963
Presence of hypertension	10/21	37/71	0.717
Presence of CVD	2/21	8/71	0.822
Initial laboratory data			
HbA1c (%)	9.6 ± 2.2	8.2 ± 1.8	0.007*
Total cholesterol (mg/dL)	170.8 ± 45.5	167.4 ± 48.9	0.832
Triglycerides (mg/dL)	181.4 ± 129.7	162.9 ± 159.4	0.148
HDL cholesterol (mg/dL)	48.5 ± 11.6	44.5 ± 11.3	0.168
LDL cholesterol (mg/dL)	91.2 ± 35.3	97.9 ± 52.0	0.669
Last laboratory data			
HbA1c (%)	8.1 ± 1.3	7.6 ± 1.6	0.243
Total cholesterol (mg/dL)	156.3 ± 35.6	156.5 ± 40.9	0.981
Triglycerides (mg/dL)	162.0 ± 90.3	148.8 ± 113.8	0.694
HDL cholesterol (mg/dL)	48.9 ± 9.6	45.9 ± 10.3	0.335
LDL cholesterol (mg/dL)	75.3 ± 25.5	88.7 ± 35.6	0.221
Initial ETDRS score			0.314
20, 35 (mild NPDR)	3	22	
43, 47 (moderate NPDR)	13	29	
53 (severe NPDR)	2	13	
61, 65, 71, 75, 81 (PDR)	3	7	
DR severity (worsened : stable)	3 : 18	31 : 40	0.014*
No. of IVT	0.7 ± 1.2	1.2 ± 1.9	0.368

Data are presented as means ± standard deviations. CVD = cardiovascular disease; DR = diabetic retinopathy; ETDRS = Early Treatment Diabetic Retinopathy Study; HDL = high-density lipoprotein; IVT = intravitreal anti-VEGF injection; LDL = low-density lipoprotein; NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy; SGLT2i = sodium-glucose cotransporter 2 inhibitor. *$P < 0.05$.

The severity of DR was assessed using the Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale [18]. The ETDRS severity scale was determined from fundus photographs and simultaneously performed fluorescein angiography at initial presentation and after at least one year of follow-up by the same experienced retinal specialist (Y. R. Chung). DR progression was defined as an increase of 2 or more steps on the ETDRS severity scale during follow-up [19, 20].

2.3. Statistics. Categorical variables were compared using the chi-square test, and continuous variables were compared using the independent t-test or Mann-Whitney test, depending on the distribution. Statistical analysis were performed using PASW software (version 18.0, SPSS, Chicago, IL), and statistical significance was defined as a P value below 0.05.

To adjust for confounding factors in the analysis, 1 : 1 propensity score matching of the SGLT2i and the control groups was performed using logistic regression analysis, with matching for age, duration of diabetes, HbA1c level, and initial ETDRS score. Logistic regression was also used to identify the factors associated with the progression of DR.

3. Results

We ultimately enrolled 21 patients in the SGLT2i group and 71 patients in the control group (Table 1). Overall, the mean age was 56.3 ± 12.1 years, 54 (59%) were male, the mean duration of diabetes was 11.4 ± 9.1 years, and the mean follow-up period was 23.9 ± 12.4 months. Three patients in the SGLT2i group took empagliflozin and 18 took dapagliflozin. Patients using SGLT2i was younger than patients in the control group and had higher level of HbA1c. Significantly, fewer patients in the SGLT2i group had DR progression relative to the control group (44% vs. 14%, $P = 0.014$). The change of ETDRS scales in patients with DR progression is shown in Figure 2.

The glycemic control in diabetic patients could possibly affect the rate of DR progression, so differences between the 2 groups at baseline could have affected the results presented in Table 1. Thus, we performed propensity score matching to adjust for factors that could potentially influence DR progression (age, duration of diabetes, glycemic status (HbA1c), and initial DR severity). After propensity score matching (Table 2), the SGLT2i group still showed

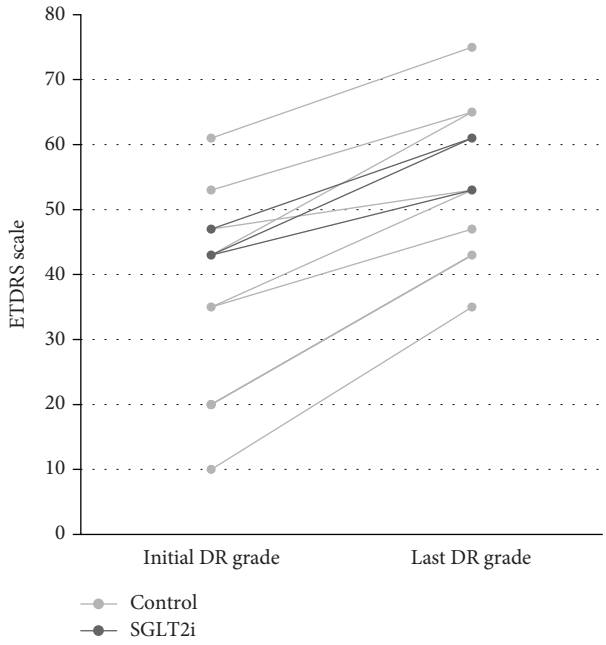

Figure 2: Change of ETDRS scales in patients with DR progression. DR = diabetic retinopathy; ETDRS = Early Treatment Diabetic Retinopathy Study; SGLT2i = sodium-glucose cotransporter 2 inhibitor.

less progression of DR ($P = 0.009$). The mean number of intravitreal anti-VEGF agent injections and HbA1c levels were not significantly different between the 2 groups in the unmatched analysis (Table 1) and the matched analysis (Table 2).

We performed logistic regression analysis to identify the factors associated with DR progression both in unmatched patients (Table 3) and in matched patients (Table 4). The results show that treatment with a SGLT2i was associated with a significantly lower risk of DR progression (odds ratio (OR) = 0.215, 95% confidence interval (CI) = 0.058–0.796, $P = 0.021$). This significant difference remained after propensity score matching for age, the duration of diabetes, initial DR grade, and HbA1c level (OR = 0.152, 95% CI = 0.034–0.674, $P = 0.013$).

4. Discussion

The SGLT2i are a newly introduced class of antihyperglycemic agents that were approved for patients with type 2 diabetes in 2013 and 2014 [8]. These drugs lower blood glucose by reducing glucose reabsorption in the renal proximal tubule, and they also induce weight loss and lower blood pressure [21, 22]. Several randomized controlled trials examined their effects on different cardiovascular outcomes [22, 23]. In particular, the EMPA-REG OUTCOME study showed that empagliflozin decreased the rate of hospitalization for heart failure and lowered the rates of cardiovascular and all-cause mortality in patients with established cardiovascular diseases but had no effect on the rates of myocardial infarction or stroke [22]. The CANVAS trial reported that canagliflozin reduced the risk of a composite outcome (cardiovascular death, myocardial infarction, and stroke) by 24% reduced renal complications in those with high risk for cardiovascular diseases but had no effect on myocardial infarction and stroke [23]. The CVD-REAL study, a large multinational study that compared canagliflozin, dapagliflozin, and empagliflozin with other glucose-lowering agents, reported that the use of a SGLT2i was associated with a lower risk of hospitalization for heart failure and all-cause death [24]. Taken together, these previous studies indicate that SGLT2i reduce cardiovascular mortality in patients with type 2 diabetes but have no apparent effect on myocardial infarction and stroke, which is the most common macrovascular complications of diabetes. Furthermore, no previous studies have examined the effect of SGLT2i on DR, which is the major microvascular diabetic complication.

Recent estimates suggest that the number of people with DR will increase dramatically from 127 million in 2010 to 191 million in 2030 [25]. Thus, the burden of DR and blindness must be considered when estimating the socioeconomic burden of type 2 diabetes. Treatment of classic risk factors, such as hyperglycemia and hypertension, can prevent or slow the progression of DR [26]. Laser photocoagulation and intravitreal injections of steroids or anti-VEGF agents can effectively treat complications in patients with preexisting DR, such as diabetic macular edema, vitreous hemorrhage, and proliferative changes [27]. However, these treatments are mainly for patients with late-stage DR and typically cannot provide full restoration of vision [27], so prevention of DR progression is needed to reduce the rate of irreversible complications.

The present investigation of the effect of SGLT2i showed that these agents slowed the progression of DR in patients with type 2 diabetes. The level of HbA1c was higher in patients with SGLT2i compared to control group, but the ratio of patients with DR progression was lower in patients with SGLT2i. We also found that SGLT2i still had a protective effect on DR after matching of patients by glycemic control state (based on HbA1c data) and initial DR grade. The final HbA1c levels also showed no differences between groups. The number of intravitreal anti-VEGF agent injections, which affect DR progression, was not different between groups. This suggests that SGLT2i protected against the progression of DR independently of their effect on lowering of blood glucose.

We did not investigate the mechanism underlying the protective effect of SGLT2i on DR, but other studies suggest possible clues. For example, early-stage DR is characterized by vascular hyperperfusion, with higher blood flow and larger vessel diameters [28–30]. This elevated blood flow can increase shear stress and cause vascular damage, which leads to endothelial dysfunction, disruption of the basement membrane, and remodeling of the extracellular matrix [31]. Recent studies of dapagliflozin reported that an effect independent of glucose lowering was responsible for prevention of arteriole wall thickening, reduction of arterial stiffness [12], reducing oxidative stress, and improving endothelial function [32]. Empagliflozin can also reduce glucotoxicity and oxidative stress and has anti-inflammatory and antifibrotic effects [33, 34].

Table 2: Clinical characteristics of patients in the SGLT2i and control groups after propensity score matching.

	SGLT2i ($n = 21$)	Control ($n = 21$)	P value
Age (years)	51.3 ± 9.7	49.4 ± 11.2	0.772
Sex (male : female)	16 : 5	12 : 9	0.190
Follow-up period (months)	20.1 ± 7.8	23.8 ± 13.6	0.512
Medical history			
Duration of diabetes (years)	11.3 ± 8.9	11.0 ± 10.4	0.565
Presence of hypertension	10/21	8/21	0.533
Presence of CVD	2/21	3/21	0.634
Initial laboratory data			
HbA1c (%)	9.6 ± 2.2	9.4 ± 1.9	0.930
Total cholesterol (mg/dL)	170.8 ± 45.5	167.2 ± 45.4	0.798
Triglycerides (mg/dL)	181.4 ± 129.7	136.1 ± 72.6	0.177
HDL cholesterol (mg/dL)	48.5 ± 11.6	44.6 ± 7.2	0.391
LDL cholesterol (mg/dL)	91.2 ± 35.3	100.4 ± 41.4	0.425
Last laboratory data			
HbA1c (%)	8.1 ± 1.3	7.9 ± 1.9	0.804
Total cholesterol (mg/dL)	156.3 ± 35.6	150.1 ± 34.8	0.622
Triglycerides (mg/dL)	162.0 ± 90.3	123.1 ± 46.9	0.153
HDL cholesterol (mg/dL)	48.9 ± 9.6	43.9 ± 7.7	0.131
LDL cholesterol (mg/dL)	75.3 ± 25.5	82.1 ± 26.8	0.516
Initial ETDRS score			0.872
20, 35 (mild NPDR)	3	5	
43, 47 (moderate NPDR)	13	13	
53 (severe NPDR)	2	2	
61, 65, 71, 75, 81 (PDR)	3	1	
DR severity (worsened : stable)	3 : 18	11 : 10	0.009*
No. of IVT	0.7 ± 1.2	1.5 ± 2.2	0.255

Data are presented as means ± standard deviations. CVD = cardiovascular disease; DR = diabetic retinopathy; ETDRS = Early Treatment Diabetic Retinopathy Study; HDL = high-density lipoprotein; IVT = intravitreal anti-VEGF injection; LDL = low-density lipoprotein; NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy; SGLT2i = sodium-glucose cotransporter 2 inhibitor. *$P < 0.05$.

Table 3: Logistic regression analysis of the effect of different variables on the progression of DR before propensity score matching in the SGLT2i and control groups.

Variable	OR (95% CI)	P value
Age	0.985 (0.951-1.021)	0.411
Sex (female)	1.455 (0.617-3.428)	0.392
Duration of diabetes	0.965 (0.919-1.015)	0.165
Hypertension	0.774 (0.331-1.808)	0.554
CVD	0.705 (0.170-2.929)	0.631
SGLT2i	0.215 (0.058-0.796)	0.021*
HbA1c	1.041 (0.841-1.288)	0.714
Total cholesterol	1.000 (0.991-1.009)	0.944
Triglycerides	0.996 (0.991-1.002)	0.178
HDL cholesterol	0.954 (0.907-1.003)	0.067
LDL cholesterol	1.004 (0.991-1.017)	0.589

Data are presented as odd ratios (95% confidence interval). CVD = cardiovascular disease; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SGLT2i = sodium-glucose cotransporter 2 inhibitor. *$P < 0.05$.

Metformin is the preferred initial agent for the treatment of type 2 diabetes, and an additional second-line agent is often considered if there is insufficient lowering of HbA1c after 3 months of monotherapy [35]. When prescribing a secondary oral hypoglycemic agent, its effects on vascular complications are an important consideration. We recently reported the association of DR with diastolic dysfunction in type 2 diabetic patients with cardiomyopathy [36], so efforts to prevent the progression of DR might also protect cardiac function. DPP4 inhibitors can protect against DR [16, 37], but their effect on DR remains controversial because they may aggravate vascular leakage [17]. SGLT2i may be a more suitable choice for secondary therapy, because they protect against the progression of DR and also reduce cardiovascular mortality [22, 24].

The major limitations of this study are its retrospective design and the small number of patients. Although we adjusted for confounding factors by propensity score matching, a prospective study with a larger number of patients is needed to confirm the protective effect of SGLT2i on the progression of DR. This study was also limited in that we only examined the progression of preexisting DR; further

Table 4: Logistic regression analysis of the effect of different variables on the progression of DR after propensity score matching in the SGLT2i and control groups.

Variable	OR (95% CI)	*P* value
Age	0.978 (0.917-1.043)	0.500
Sex (female)	1.173 (0.304-4.527)	0.817
Duration of diabetes	1.007 (0.944-1.073)	0.835
Hypertension	0.236 (0.054-1.035)	0.056
CVD	1.389 (0.204-9.445)	0.737
SGLT2i	0.152 (0.034-0.674)	0.013*
HbA1c	1.287 (0.916-1.809)	0.146
Total cholesterol	0.997 (0.982-1.012)	0.665
Triglycerides	0.980 (0.960-1.000)	0.050
HDL cholesterol	0.936 (0.854-1.026)	0.159
LDL cholesterol	1.003 (0.981-1.025)	0.808

Data are presented as odd ratios (95% confidence interval). CVD = cardiovascular disease; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SGLT2i = sodium-glucose cotransporter 2 inhibitor. *$P < 0.05$.

studies are needed to investigate the effect of SGLT2i on the onset of DR. Nevertheless, this pilot study provides important new information, because it is the first to document the effect of SGLT2i on the progression of DR in a clinical setting.

5. Conclusions

Taken together, the present study showed that treatment of type 2 diabetic patients with SGLT2i slowed the progression of DR, and that this protective effect was independent from their glucose-lowering effects. To our knowledge, this is the first study to show that SGLT2i slows the progression of DR. Further prospective randomized double-blind studies are needed to confirm these findings.

Data Availability

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

Conflicts of Interest

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

Authors' Contributions

Eun Hyung Cho and Se-Jun Park contributed equally to this work.

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Research Article

There Is No Impact of Diabetes on the Endothelial Function of Chronic Kidney Disease Patients

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Background. Patients with chronic kidney disease (CKD) and type 2 diabetes mellitus (DM) have increased risk of endothelial dysfunction, cardiovascular disease, and mortality. Several studies have separately analyzed endothelial function in these populations. However, data of patients with both CKD and DM are scarce. The aim of this study was to evaluate whether the presence of DM has any additional effect on the endothelial dysfunction of CKD patients. *Methods*. We measured endothelial progenitor cells (EPCs), stromal-derived factor 1 alpha (SDF-1α), serum and urinary nitric oxide (NO), flow-mediated dilation (FMD), and pulse wave velocity (PWV) in 37 CKD patients with DM (CKD-DM group) and in 37 without DM (CKD group). *Results*. CKD-DM group had a higher prevalence of obesity ($P < 0.01$), previous myocardial infarction ($P = 0.02$), myocardial revascularization ($P = 0.04$), and a trend for more peripheral artery disease ($P = 0.07$). Additionally, CKD-DM group had higher EPC ($P = 0.001$) and PWV ($P < 0.001$) values. On the other hand, no difference in SDF-1α and serum or urinary NO and FMD was observed between the groups. *Conclusions*. Endothelial dysfunction is frequent in CKD patients, and an additive effect of diabetes cannot be implicated, suggesting the predominant role of uremia in this condition.

1. Introduction

The Global Burden Disease 2010 study had highlighted chronic kidney disease (CKD) as an important cause for global mortality [1]. It is estimated that 10–15% of the adult population has CKD at various stages of severity [2]. This rate has grown [3] in parallel with the increasing incidence and prevalence of type 2 diabetes mellitus (DM) [3, 4], one of the main causes of CKD [4].

It is well known that patients with CKD and DM have higher mortality rates compared to their counterparts without DM [2, 5]. Cardiovascular disease (CVD) is the most important cause of mortality in CKD as well as in DM patients [6, 7]. Endothelial dysfunction, the initial lesion of atherosclerosis [8, 9], is an early marker of CVD frequently observed both in CKD [10, 11] and DM patients [12]. Several factors are associated with endothelial dysfunction in these populations [13, 14], such as uremic toxins and hyperglycemia, that are related to the depletion of endothelial nitric oxide (NO) [12, 14–16]. Moreover, uremic toxins stimulate the expression of adhesion molecules, which are also associated with endothelial dysfunction [14].

The evaluation of endothelial function includes the measurement of endothelial progenitor cells (EPCs), which have been shown to take part in the endogenous vascular repair system. The EPC count is considered to be a predictor of endothelial dysfunction and cardiovascular outcomes [17, 18] in populations with known high cardiovascular risk, who

have reduced number or impaired function of EPC. Several studies have been demonstrated that EPC number was reduced in patients with isolated CKD and DM, compared to healthy controls [19, 20]. Ozuyaman et al. [21] demonstrated that EPC mobilization and function require NO. Among other factors and chemokines, stromal cell-derived factor-1 alpha (SDF-1α) is the most potent chemokine that mobilizes EPC from bone marrow to the injured vessel sites [22, 23]. The levels of SDF-1α are associated with increased CVD risk, both in general [24] and CKD patients [25].

Endothelial dysfunction can also be quantified by the degree of flow-mediated dilation (FMD) of the brachial artery, a widely used noninvasive technique [26]. The reduction of FMD occurs early in the development of atherosclerosis [27]. Several studies have shown that FMD is impaired in CKD [28, 29] as well as in DM patients [20, 30].

Data regarding endothelial dysfunction in patients with concomitant DM and CKD is scarce. Therefore, we aimed to evaluate the impact of DM on the endothelial function of patients with CKD.

2. Materials and Methods

2.1. Study Subjects. In this case-control study, 74 patients with CKD were recruited: 37 patients with DM (CKD-DM group) and 37 patients without DM (CKD or control group), from the outpatients CKD clinic of the Federal University of São Paulo, Brazil.

The inclusion criteria were age ≥ 18 years and CKD stages 3a–4. Regarding diabetic patients, only those on insulin therapy were included. Exclusion criteria were type 1 or secondary forms of DM; use of oral hypoglycemic agents, erythropoietin, or estrogen supplementation; malignancy in the last 5 years; hepatic insufficiency, New York Heart Association class III/IV heart failure, acute myocardial infarction, or peripheral arterial disease, decompensated in the last 6 months; acute infectious disease in the last 30 days; and pregnant or breastfeeding and regular smokers. Regular smokers were considered to be those consumers of at least one daily cigarette for at least six months.

All patients underwent an assessment of their medical history, physical examination, laboratory tests, and endothelial evaluation, including EPC number, SDF-1α, serum and urinary NO levels, and FMD.

The study was reviewed and approved by the Ethics Advisory Committee of the Federal University of São Paulo (approval number: 569.458). All patients gave written informed consent.

2.2. Laboratory Tests. After a 12-hour overnight fast, blood samples were collected to measure serum creatinine, glucose, glycosylated hemoglobin (HbA1c), total HDL and LDL cholesterol, triglycerides, potassium, sodium, intact parathyroid hormone (iPTH—chemiluminescent microparticle immunoassay performed at ARCHITECT i4000, Abbott), ionized calcium, phosphate, alkaline phosphatase, bicarbonate, and blood count. Serum SDF-1α was determined by enzyme immunoassay (ELISA, Elabscience, Wuhan, Hubei, China). Nitric oxide was quantified in serum and 24-hour urine sample by chemiluminescence, using nitric oxide analyzer (NOATM 280, Sievers Instruments Inc., Boulder, CO, USA). Albuminuria was measured in 24-hour urine sample. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.

2.3. Flow Cytometry Analysis of Circulating EPCs. Ten milliliters of peripheral blood was collected in an EDTA tube for EPC analysis. The blood samples were processed within 4 hours after collection. Mononuclear cells were separated using Ficoll–Hypaque density gradient centrifugation (Sigma-Aldrich, St. Louis, USA) and washed using phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, USA). An automatic cell counter was used to ensure that in each analyzed tube there were 1,000,000 cells. Subsequently, the samples were exposed to the following antibodies: CD45-PE-Cy7 (BD Biosciences, San Diego, USA), CD34-FITC (BD Biosciences, San Diego, USA), and VEGFR2-PE (BD Biosciences, San Diego, USA). Isotype-stained samples were used as negative control. After incubation in the dark, excess antibody was removed by washing with PBS. Lastly, the cells were washed with PBS buffered with sodium azide and analyzed by flow cytometry. To facilitate lymphocyte gate demarcation, CD3-PerCP or CD3-APC lymphocyte markers (BD Biosciences, San Diego, USA) were used in most samples. After demarcation of this gate, EPCs were identified by the low expression of CD45 and by CD45-dim and by the double expression of CD34 and VEGFR2 (Figure 1), as previously described [20, 31, 32].

The detection of all antibodies was performed by a flow cytometer (FacsCanto I, BD Biosciences, San Diego, CA, USA). The gated data of $CD3^+$ (T lymphocytes), CD45-PE-Cy7, CD34-FITC, and VEGFR2PE were presented as cells per 900,000 events.

2.4. Measurement of Brachial Artery FMD. Ultrasound-based measurements of brachial artery reactivity were performed according to the guidelines of the International Brachial Artery Reactivity Task Force [33]. The assessment of vascular reactivity was always carried out by the same examiner who was blinded to the group allocation. The brachial artery was assessed and measured in longitudinal section just above the antecubital fossa using a high-resolution ultrasound system (Sequoia Echocardiography System, version 6.0, Acuson, Siemens, Vernon, CA, USA) equipped with a multifrequency linear transducer (7–12 MHz) to produce two-dimensional images. The techniques used to evaluate the changes of FMD and nitrate-mediated dilation (NMD) after physical and pharmacological stimulation, respectively, are described elsewhere [34].

2.5. Pulse Wave Velocity (PWV). As a surrogate marker of subclinical atherosclerosis, arterial stiffness was noninvasively measured by the PWV of the carotid and femoral arteries. PWV was carried out by the same examiner who was blinded to the group allocation using the Complior SP equipment (Artech Medical, Pantin, France) and then analyzed by appropriate software.

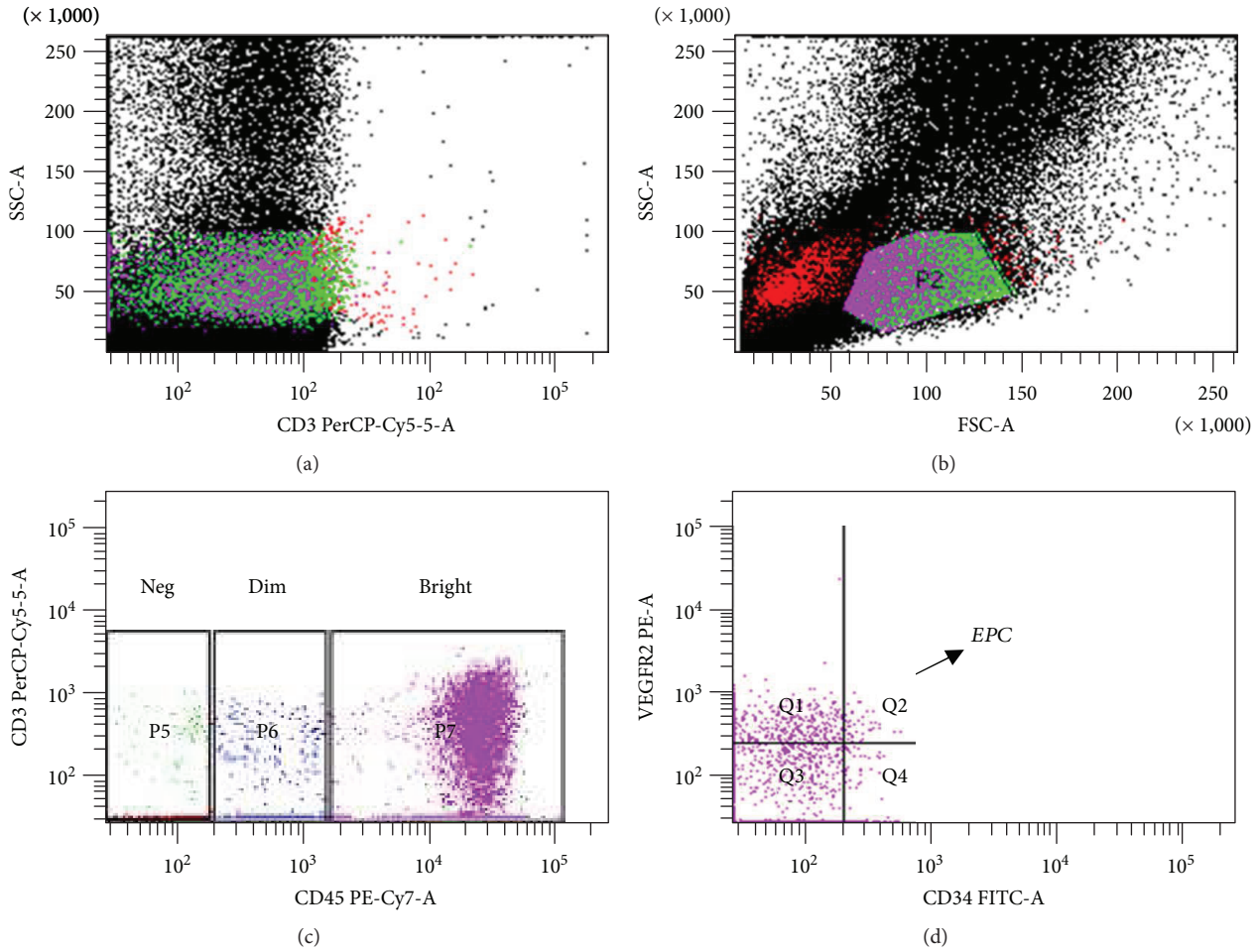

Figure 1: Analysis of EPC by flow cytometry: (a) labeling with CD3-PerCP for identification of lymphocytes, (b) lymphocytic gate, (c) identification of cells with CD45-dim, and (d) after identification of item (c), selection of those with labeling for CD34 and VEGFR2 (Q2).

2.6. Statistical Analysis. Mean and standard deviation, median, and interquartile range or frequencies (proportion) were calculated for each variable, as appropriate. The Kolmogorov-Smirnov statistical test was used to investigate the normal distribution of data. Comparisons of continuous variables were performed using Student's t-test and the Mann-Whitney U test, for normal and skewed data, respectively. Comparisons of proportions were performed using chi-squared analysis or Fisher's exact test, as appropriate. Among the variables that evaluated endothelial function, FMD was the only one that showed normal distribution. Thus, generalized linear models (GLMs) were performed with normal or gamma distribution, according to the variable characteristics. For the assessment of FMD, the model was adjusted to the following variables: age, gender, peripheral artery disease, and use of acetylsalicylic acid and antihypertensive drugs; for the evaluation of SDF-1α: age, gender, and use of acetylsalicylic acid; and for the EPC assessment: use of acetylsalicylic acid, lipid-lowering agents, and CD3 type. P values < 0.05 were considered statistically significant. All statistical analysis was performed using the SPSS for Windows (SPSS 19.0, Chicago, IL, USA). The sample size was calculated based on previous study by Wong et al. [35]. For this calculation, a conservative approach was adopted and was performed using the Gpower 3.1.2 software. Assuming a difference in EPCs and FMD of 50%, a total of 74 subjects, 37 in each group, were required to reach a level of significance of 5% and a power of 80%.

3. Results

Characteristics of the CKD patients according to the presence (CKD-DM group) or absence (CKD group) of diabetes are listed in Tables 1 and 2. There was a predominance of elderly hypertensive men in both groups.

When compared to the CKD group, patients with CKD-DM showed a higher prevalence of previous myocardial infarction, myocardial revascularization, and a trend for more peripheral artery disease (Figure 2). Diabetic patients received more diuretic and acetylsalicylic acid but less calcium channel blocker. There was no difference in the use of ACEI/ARB or lipid-lowering drugs. The CKD-DM group had higher proportion of obese individuals (21 (57%) vs. 9 (24%); $P = 0.004$). Of note, only 2 patients in the CKD group had waist-hip circumference ratio within normal range. A higher prevalence of patient with uncontrolled systolic blood

Table 1: Clinic characteristics of the study population.

	CKD group ($n = 37$)	CKD-DM group ($n = 37$)	P
Age, years	65.9 ± 13.9	64.1 ± 9.9	0.54
Male, *n* (%)	21 (56.8%)	22 (59.5%)	0.814
Hypertension, *n* (%)	35 (97.2%)	34 (94.4%)	0.555
Chronic kidney disease etiology, *n* (%)			<0.001
Diabetes	0 (0%)	33 (89.2%)	
Undetermined	19 (51.4%)	1 (2.7%)	
Hypertension	7 (18.9%)	0	
Glomerulopathy	5 (13.5%)	0	
Others	6 (16.2%)	3 (8.1%)	
Cardiovascular disease, *n* (%)	12 (32.4%)	19 (51.4%)	0.099
Myocardial infarction	1 (2.7%)	7 (18.9%)	0.025
Myocardial revascularization	0 (0%)	4 (10.8%)	0.04
Cerebrovascular accident	5 (13.5%)	4 (10.8%)	0.722
Peripheral artery disease	7 (18.9%)	14 (37.8%)	0.071
Drugs, *n* (%)			
ACEI/ARB	27 (75%)	33 (91.7%)	0.058
Calcium channel blockers	27 (75%)	17 (47.2%)	0.016
Diuretics	25 (69.4%)	34 (94.4%)	0.006
Vasodilator	2 (5.6%)	7 (19.4%)	0.075
Lipid-lowering agents	24 (68.6%)	30 (85.7%)	0.088
Acetylsalicylic acid	14 (38.9%)	27 (73%)	0.003
Systolic blood pressure, mmHg	130 (125–140)	140 (123.5–158.5)	0.214
Diastolic blood pressure, mmHg	80 (70–90)	77 (70–84)	0.343
Body mass index, kg/m^2	27.7 ± 4.7	31.4 ± 5.7	0.004
Waist-hip circumference ratio	0.97 ± 0.07	1.00 ± 0.06	0.032

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker.

Table 2: Laboratorial characteristics of the study population.

	CKD group ($n = 37$)	CKD-DM group ($n = 37$)	P
Creatinine, mg/dl	2.33 ± 0.65	2.22 ± 0.65	0.442
CKD-EPI, ml/min/1.73 m^2	24 (21–34.5)	28 (23.5–35.5)	0.267
Albuminuria, μg/min (24 h)	42.1 (11.5–131.7)	132.3 (39.5–767.4)	0.014
Glucose, mg/dl	88 (85–92)	142 (80–206)	0.003
Hemoglobin A1c, %	5.6 (5.3–5.9)	8.4 (7.2–9.9)	<0.001
Total cholesterol, mg/dl	163 (151–187)	183 (141–217)	0.141
HDL cholesterol, mg/dl	52 (42–61)	46 (40–53)	0.074
LDL cholesterol, mg/dl	90 (70–105)	103 (71–123)	0.113
Triglycerides, mg/dl	128 (90–183)	176 (126–305)	0.002
Ionized calcium, mmol/l	1.31 ± 0.06	1.29 ± 0.06	0.095
Phosphate, mg/dl	3.5 ± 0.6	3.6 ± 0.6	0.338
Alkaline phosphatase, U/l	67 (60–86)	80 (66–95)	0.036
Bicarbonate, mmol/l	24.8 ± 2.8	26.9 ± 3.4	0.005
Parathyroid hormone, pg/ml	163 (96–240)	167 (117–210)	0.948
Hemoglobin, g/dl	13.5 ± 1.6	13.7 ± 1.6	0.607
Pulse wave velocity, m/s	8.5 ± 1.8	10.3 ± 1.7	<0.001

HDL = high-density lipoprotein; LDL = low-density lipoprotein.

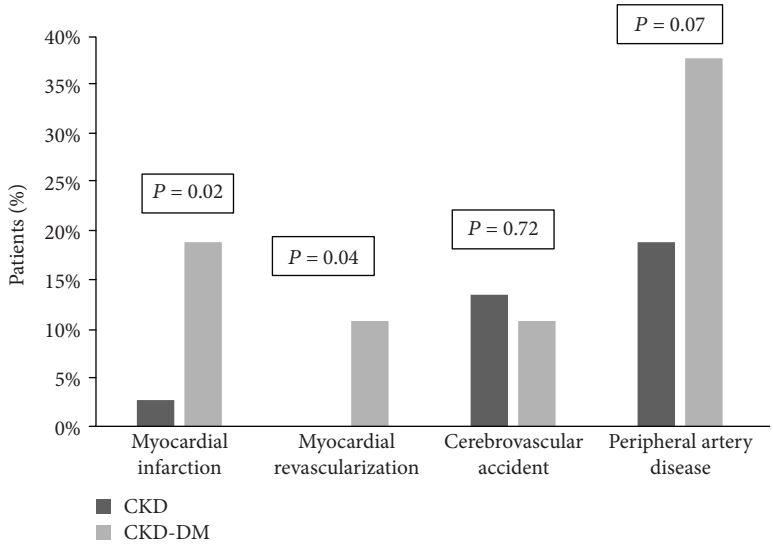

Figure 2: Previous cardiovascular disease according to the groups.

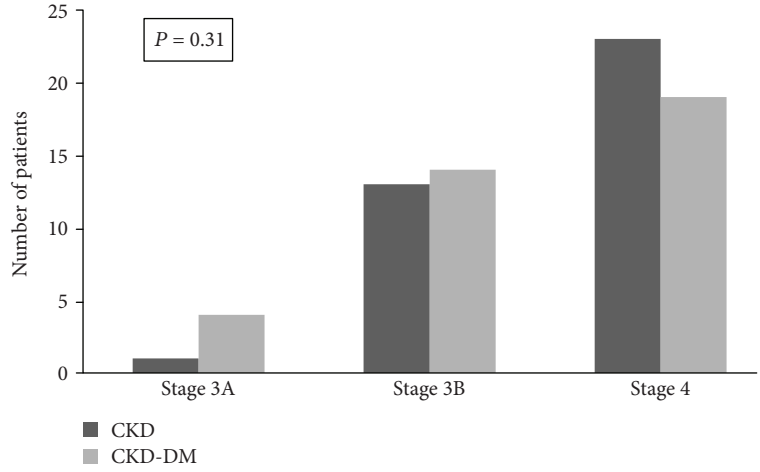

Figure 3: Distribution of patients based on stages of CKD.

pressure was observed among CKD-DM patients (20 (54) vs. 11 (30) %; $P = 0.034$).

Renal function as well as the distribution of patients according to CKD stages did not differ between groups (Figure 3). As expected, the CKD-DM group had higher albuminuria, glucose, HbA1c, and triglyceride levels. There was a trend towards lower HDL in this group. Bicarbonate was significantly higher in the CKD-DM group, although the supplementation of bicarbonate was similar in both groups. Alkaline phosphatase was significantly higher in the CKD-DM group; however, only 3 patients (2 of CKD-DM group) presented serum levels above the normal range. There was no difference in hemoglobin (Hb) concentration between the groups, and all patients had Hb greater than 10 mg/dl. The PWV was higher in the CKD-DM group as well as the proportion of patients with increased values (21 (60) vs. 8 (22) %; $P = 0.001$).

EPC number was higher in the CKD-DM group compared to CKD. The FMD was similar, showing low values in both groups. Of note, 10% of the patients in each group failed to display any dilation during the test. No difference in SDF-1α and serum or urinary NO between the groups was observed (Table 3).

When the sample was divided based on CKD stages, there was no difference in endothelial parameters (EPC, SDF-1α, serum, and urinary NO and FMD) (Table 4).

4. Discussion

The present study has demonstrated a high prevalence of endothelium dysfunction in CKD patients regardless the presence of diabetes. All the endothelial dysfunction markers, but EPC number, were similar in CKD patients with and without diabetes.

Table 3: Endothelial dysfunction markers in CKD and CKD-DM groups.

	CKD group ($n = 37$)	CKD-DM group ($n = 37$)	P
FMD, %	2.68 ± 3.11	2.95 ± 3.69	0.737
NMD, %	11.51 ± 6.05	9.26 ± 5.47	0.104
EPC, %	0.25 (0.1–0.6)	0.60 (0.3–0.9)	0.009
SDF-1α, pg/ml	3730 (2915–4830)	3430 (2695–4770)	0.699
Serum nitric oxide, μmol/l	390.5 (296.5–568.8)	387.5 (241.5–613.8)	0.641
24 h urinary nitric oxide, μmol	3432 (1593–5521)	3336 (1213–5896)	0.734

FMD = flow-mediated dilation; NMD = nitrate-mediated dilation; EPC = endothelial progenitor cell; SDF-1α = stromal cell-derived factor 1.

Table 4: Endothelial dysfunction markers based on CKD stages.

	CKD 3	CKD 4	P
FMD, %	2.78 ± 3.26	2.85 ± 3.55	0.930
EPC, %	0.4 (0.2–1.0)	0.3 (0.1–0.7)	0.180
SDF-1α, pg/ml	3535 (2935–4927)	3800 (2697–4472)	0.739
Serum nitric oxide, μmol/l	414.0 (294.0–581.2)	347.0 (260.0–586.9)	0.659
24 h urinary nitric oxide, μmol	3121 (2224–6348)	3149 (1010–4976)	0.252

CKD 3 = chronic kidney disease stage 3; CKD 4 = chronic kidney disease stage 4; FMD = flow-mediated dilation; EPC = endothelial progenitor cell; SDF-1α = stromal cell-derived factor 1.

Few studies, including dialysis and nondialysis patients, demonstrated a similar number of EPCs in CKD patients with and without DM [36–38]. In contrast with these studies, our results showed that CKD-DM patients had higher EPC number. This unexpected finding could be related to the fact that all diabetic patients were using insulin, which is known to increase the EPC number [39, 40]. Moreover, one could hypothesize that this elevated number of EPC reflects a better activity of the endogenous vascular repair system. However, our CKD-DM patients had high prevalence of cardiovascular disease and an inadequate arterial stiffness, revealed by the increased PWV. Based on that, we could speculate that the EPC could be dysfunctional or the increased number might be insufficient to repair the vessels.

It is well known that diabetic patients have EPC dysfunction [20, 41] mainly due to hyperglycemia [41, 42]. High glucose level leads to an increasing of advanced glycation end products, reactive oxygen species, and inflammatory cytokines, factors that could induce EPC dysfunction [12, 20, 43]. Likewise, there are substantial data indicating EPC dysfunction in CKD patients [16, 44]. Uremic environment causes a deficient NO production, which leads to a decreased EPC mobilization [38]. Additionally, uremic toxins were found to cause EPC dysfunction by inhibiting migratory activity, adhesion to matrix proteins and to endothelial cells [38, 45]. Corroborating with that, studies have suggested that the reduction of uremic toxins by kidney transplantation improves EPC function [46, 47]. Unfortunately, we did not evaluate EPC function in the present study.

Other factor that could be related to the endothelial dysfunction observed in our CKD patients is the EPC resistance. Herbrig et al. observed that chronically elevated SDF-1α levels result in impair EPC homing to sites of vascular damage, indicating EPC resistance [46]. Of note, Jie et al. demonstrated that SDF-1α is increased in CKD patients due to its reduced renal clearance [19]. On the other hand, in diabetic patients, SDF-1α concentration has been shown to be decreased. The diminished concentration of SDF-1α, observed in that diabetic population, was associated to the reduction of EPC releasing to the damaged vessels [48]. In the present study, SDF-1α levels were high in both CKD and CKD-DM groups, which may suggest that uremia effect on SDF-1α overcomes that of diabetes. Supporting this hypothesis, serum and urinary NO and FMD values were similar in both groups. In agreement with this finding, previous studies, including nondialysis [49] and dialysis [50] patients, were not able to demonstrate that the presence of diabetes had influenced FMD, even after adjustments for confounding factors.

Some limitations of this study should be acknowledged, such as the relative small sample size, the absence of a healthy control group, and its cross-sectional design. Nevertheless, to the best of our knowledge, this is the first study designed to investigate the additive effect of diabetes on the endothelial function of CKD patients.

5. Conclusion

Endothelial dysfunction is frequent in CKD patients, and an additive effect of diabetes cannot be implicated, suggesting the predominant role of uremia in this condition.

Data Availability

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

Conflicts of Interest

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

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Research Article

Artificially Cultivated *Ophiocordyceps sinensis* Alleviates Diabetic Nephropathy and Its Podocyte Injury via Inhibiting P2X7R Expression and NLRP3 Inflammasome Activation

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Background/Aims. It is known that chronic low-grade inflammation contributes to the initiation and development of both diabetes and diabetic nephropathy (DN), so we designed this study to investigate the role of P2X7R and NLRP3 inflammasome in DN pathogenesis and the antagonistic effects of artificially cultivated *Ophiocordyceps sinensis* (ACOS). *Methods*. A rat model of DN caused by high-fat-diet feeding and low-dose streptozotocin injection and a mouse podocyte injury model induced by high-glucose (HG) stimulation were established, and the intervention effects of ACOS on them were observed. The biological parameters of serum and urine and the pathological manifestations of kidney tissue were examined. The expression of mRNA and protein of P2X7R and NLRP3 inflammasome (NLRP3, ASC, and caspase-1) and downstream effectors (IL-1β and IL-18), as well as podocyte-associated molecules, was determined by real-time quantitative PCR and Western blot assay, respectively. *Results*. The DN rats showed to have developed insulin resistance, elevated fasting blood glucose, increased urinary protein excretion, and serum creatinine level as well as corresponding glomerular pathological alterations including podocyte damages. ACOS significantly antagonized the above changes. The experiments *in vivo* and *in vitro* both displayed that the mRNA and protein expression of P2X7R, NLRP3, ASC, caspase1 (procaspase-1 mRNA in the gene level and active caspase-1 subunit P10 in the protein level), IL-1β, and IL-18 was significantly upregulated and the mRNA and protein expression of podocyte-associated molecules was significantly changed (downregulation of nephrin, podocin, and WT-1 expression and upregulation of desmin expression) indicating podocyte injury in the kidney tissue of DN rats and in the HG-stressed mouse podocytes, respectively. ACOS also significantly antagonized all the above changes. *Conclusion*. Our research work suggests that P2X7R and NLRP3 inflammasome are involved in the pathogenesis of DN, and ACOS can effectively inhibit the high expression of P2X7R and the activation of NLRP3 inflammasome, which may contribute to the therapeutic effects of *Ophiocordyceps sinensis*.

1. Introduction

The global prevalence of diabetes has dramatically increased over the recent decades. The International Diabetes Federation reported that the overall prevalence of diabetes was estimated to be 8.8% in people aged 20~79 years worldwide in 2015, which was predicted to rise to 10.4% in 2040 [1]. The China Noncommunicable Disease Surveillance Group reported that the overall prevalence of diabetes was estimated to be 11.6% and the prevalence of prediabetes in Chinese adult population aged 18 years or older was estimated to be 50.1% in 2010 [2]. Diabetic nephropathy (DN), also known as diabetic kidney disease (DKD), is one of the most common chronic microvascular complications of diabetes, which is the leading cause of end-stage renal disease (ESRD) in developed countries and the second cause of ESRD in China [3]. So, diabetes with its kidney complication, DN, has become a worldwide public health problem that seriously threatens human health.

DN is a glomerular disease. Now, it has been recognized that podocyte injury is a pivotal event in the pathogenesis of DN [4]. However, the underlying mechanism of podocyte injury in DN has not been fully clarified. A growing number of studies show that diabetes is characterized by chronic low-grade inflammation, referred to as "metaflammation," which is a relevant factor contributing to the initiation and development of both diabetes and its complications including DN [5–7]. In the past years, it was discovered that the expression of purinergic 2X7 receptor (P2X7R) was significantly upregulated in glomerular cells, mainly in podocytes, in diabetic rat models [8]. It was also observed that the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome activation in glomerular cells including podocytes was associated with the onset of DN, while NLRP3 inflammasome deficiency or inhibition could ameliorate DN in mouse models [7, 9]. The above studies have drawn our attention to the pathogenic role of P2X7R and NLRP3 inflammasome in DN.

P2X7R is a member of the P2XR family. It is characterized by the response to extracellular ATP (eATP), with a rapid opening of a ligand-gated cation channel, resulting in potassium ion (K^+) efflux and intracellular potassium depletion. When the level of intracellular potassium reduces below the threshold of 90 mM, the assembly and activation of NLRP3 inflammasome are initiated [10–12]. The NLRP3 inflammasome is an intracellular multiprotein complex consisting of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC, adaptor protein), and cysteinyl aspartate specific protease-1 (caspase-1, effect protein). Caspase-1 usually exists as an inactive precursor procaspase-1, which can be self-enzymatically hydrolyzed to its active forms, P10 and P20 subunits, during the assembly and activation of NLRP3 inflammasome. The active P10/P20 subunits can turn the inactive precursors of interleukin 1 beta (IL-1β) and IL-18 into the mature forms to induce inflammation [13–15]. In this study, we investigated the relationship between P2X7R with its downstream NLRP3 inflammasome and DN and inspected the effects of *Ophiocordyceps sinensis* on them.

Ophiocordyceps sinensis (once called *Cordyceps sinensis*), a fungus-caterpillar complex formed after the fungus infects the larva of the moth that belongs to Hepialidae, is a well-known traditional Chinese medicine and has been used for centuries in China and Asian countries. Clinical practice and experimental studies have shown that it is effective in the treatment of many kinds of kidney diseases including DN [16–18]. However, so far, there have been no reports of the effects of *Ophiocordyceps sinensis* on P2X7R and NLRP3 inflammasome, as we did in this study. Wild *Ophiocordyceps sinensis* is now too scarce to meet the medical needs in China. So, artificially cultivated *Ophiocordyceps sinensis* (ACOS) has been highly expected for a long time. Fortunately, it has finally succeeded in recent years (Figure 1) [19–21]. In this study, we utilized the ACOS instead of wild *Ophiocordyceps sinensis* for the experiments *in vivo* and *in vitro*, which is the first experimental study of ACOS in the treatment of diseases.

Figure 1: Artificially cultivated *Ophiocordyceps sinensis* (ACOS). *Ophiocordyceps sinensis* is a fungus-caterpillar complex formed after the fungus infects the larva of the moth that belongs to Hepialidae. The black part of the complex is the fungal part that is called the fruiting body and consists of stromatophore and stroma; the yellowish-brown part is the dead larva body that is filled with mycelia, called the sclerotium. The *Ophiocordyceps sinensis* in this photo is the ACOS, which has been produced through industrialized artificial cultivation in China now.

In this study, we established a rat model of DN caused by type 2 DM and a mouse podocyte injury model induced by high-glucose (HG) stress and then studied the role of P2X7R and NLRP3 inflammasome in the pathogenesis of DN and the antagonistic effects of ACOS by using these models.

2. Materials and Methods

2.1. Animals and Grouping. Thirty-two male Sprague-Dawley rats weighing 180–200 g at the age of 6 weeks were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China) and were housed in an animal room of specific-pathogen-free cleanliness grade with 50–60% humidity at temperature 20–26°C. Rats were randomly and equally divided into the following 4 groups: control group, DN model group, intervention group with a low dose of ACOS, and intervention group with a high dose of ACOS (HEC Pharm Co., China). The rats in the control group were fed with ordinary chow (energy ratio: fat—12.11%, protein—22.47%, and carbohydrates—65.42%), while the rats in the other three groups were fed with high-fat chow (energy ratio: fat—45.65%, protein—16.46%, and carbohydrates—37.89%). At the end of the 4th week, the insulin resistance index (IRI) was measured with the HOMA-IR formula in the rats fed with high-fat chow. After insulin resistance was confirmed, the rats in the DN model group and two intervention groups were intraperitoneally injected with streptozotocin (Sigma, USA) in a single dose of 35 mg/kg, while the rats in the control group were only injected with an equivalent volume of buffer. 72 h after the injection, the fasting blood glucose (FBG) of each rat was tested and rats are considered to have type 2 DM when their FBG level is >11.1 mmol/L. From the 5th week, the rats in the low- and high-dose intervention groups were given ACOS by gavage in a dose of 2.5 g/kg (LD-ACOS group) and 5.0 g/kg (HD-ACOS group), respectively, every day for 8 weeks, while the rats in the control and model groups were given the equal volume of tap water by gavage every day for 8 weeks.

2.2. Biological Parameters. Body weight was measured at baseline and at the 4th and 13th week. Kidney weight was

measured after the rat was sacrificed, and then the ratio of kidney weight/body weight (KW/BW) of each rat was calculated. Urinary protein excretion of 24 h urine sample was tested at baseline and the 13th week. Serum creatinine (SCr) was detected at the 13th week. FBG was detected at the 4th and 13th week and also at 72 h after streptozotocin injection. Glycated hemoglobin (HbA1c) was measured at the 13th week. Fasting insulin was detected at the 4th and 13th week, and then IRI was calculated using the HOMA-IR formula: IRI = fasting blood glucose (mmol/L) × fasting insulin (mIU/L)/22.5 [22, 23].

2.3. Pathological Examination. After the renal tissue was conventionally processed, the 3 μm thick sections were stained with a periodic acid-Schiff reagent for light microscopy. Twenty images of glomerular maximal profiles with a vascular pole and/or urinary pole were taken under a high-power microscope (×400, Olympus, Japan) and were analyzed by Nikon NIS-Elements BR image analysis software (Nikon, Japan). The length (μm) of the two longest perpendicular diameters in every glomerular capillary tuft without Bowman's space was measured, and then the mean value was calculated. The areas of the glomerular mesangial region and capillary tuft were also measured, and then the relative area of the mesangial region (%) was calculated according to the formula: area of the mesangial region/area of the capillary tuft × 100%.

After the renal tissue was processed according to standard techniques. The ultrathin sections were stained with uranium acetate-lead citrate for electron microscopy. For each specimen, ten photographs (×20,000 magnification) covering different regions in the glomerular cross section were taken separately. The length (μm) of the peripheral GBM was measured, and the number of slit pores overlying this GBM length was counted by Nikon NIS-Elements BR image analysis software (Nikon, Japan). The mean of the foot process width ($\bar{W}_{\mathrm{FP}}$) was calculated as follows [24]:

$$\bar{W}_{\mathrm{FP}} = \frac{\pi}{4} \cdot \frac{\sum \mathrm{GBM\,length}}{\sum \mathrm{slits}}, \tag{1}$$

where $\sum$slits is the total number of slits counted and $\sum$GBM length is the total GBM length measured in one glomerulus.

2.4. Double Immunofluorescence Staining. For the double staining of an indirect immunofluorescence assay of P2X7R/NLRP3 and podocyte marker synaptopodin, frozen renal tissues of rats were cut into 5 μm thick sections. A rabbit anti-P2X7R polyclonal antibody (1 : 100 dilution, Alomone) or rabbit anti-NLRP3 polyclonal antibody (1 : 100 dilution, Novus) and mouse anti-synaptopodin monoclonal antibody (1 : 50 dilution, Santa Cruz) were used as primary antibodies. Rhodamine-labeled goat anti-rabbit immunoglobulins (Beijing Zhongshan) and FITC-labeled goat anti-mouse immunoglobulins (Beijing Zhongshan) were used as secondary antibodies, respectively. After staining, the tissue sections were observed with a fluorescent microscope (Nikon, Japan).

2.5. Podocyte Culture and Grouping. The conditionally immortalized mouse podocyte cell line was kindly provided by Professor Maria Pia Rastaldi (S. Carlo Hospital, University of Milan). Podocytes were incubated in RPMI-1640 medium (Thermo Fisher Scientific) containing 10% inactivated fetal bovine serum (FBS, Thermo Fisher Scientific) and 10 μ/mL interferon-γ (IFN-γ, Cell Signaling Technology) at 33°C in a humidified air with 5% CO_2. When the cells reached 80–90% confluence, they were transferred to RPMI-1640 medium containing 10% inactivated FBS without IFN-γ and incubated at 37°C in a humidified air with 5% CO_2 for 10–14 days to allow differentiation.

Well-differentiated podocytes were used for experiments. The podocytes were incubated in the RPMI-1640 medium with 5% inactivated FBS and grouped as follows: medium alone (control group), medium containing 30 mM glucose (Sigma) (HG group), medium containing 50 μg/mL ACOS extract (HEC Pharm Co., China) (ACOS control group), and medium containing both 30 mM glucose and 50 μg/mL ACOS extract (HG + ACOS group). According to the LDH release test, 30 mM glucose had no cytotoxic effects on cellular viability (Table 1S, see Supplementary Materials).

2.6. Reverse Transcription and Real-Time Quantitative PCR. Total RNA was extracted from rat renal cortex tissue or cultured podocytes using TRIzol reagent (Invitrogen) following the manufacturer's instructions. 2 μg total RNA from each sample was reverse-transcribed to cDNA with EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). The gene-specific primers (SBS Genetech) are listed in Tables 1 and 2. RT-PCR was performed using SYBR Green RT-PCR Master Mix (TransGen Biotech) according to the manufacturer's instruction. The GAPDH was set as the internal control gene in the animal and cellular experiments. The relative quantity of mRNA expression was calculated according to the formula: $2^{-(\mathrm{targetgeneCt} - \mathrm{GAPDHCt})} \times 10^3$, in which Ct was the threshold cycle number. All assays were repeated at least in triplicate independently.

2.7. Western Blot Assay. Total protein lysates were extracted from rat renal cortex tissue or cultured podocytes using RIPA lysis buffer (ComWin Biotech). Protein samples were sonicated five times for 1 s each, centrifuged at 12000 rpm for 10 min at 4°C, and then boiled for 5 min. Protein samples were separated by 10–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (General Electric Co.). After being blocked with 5% skim milk in phosphate-buffered saline with 0.1% Tween 20 for 1 h, the membranes were incubated with a primary antibody at 4°C overnight and then incubated with a secondary antibody at room temperature for 1 h. Details regarding primary and secondary antibodies are listed in Table 3. The blotted proteins were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). β-Actin was set as an internal control. The relative expression level of each target protein was displayed as a ratio of target protein/β-actin protein. All the assays were performed at least in triplicate independently.

Table 1: Primer sequences for PCR analysis in animal experiments.

Target	Primer sequence (5′–3′)	Length (bp)
Nephrin	Forward CTGGGGGACAGTGGATTGAC Reverse CCACCAACTGCAAAGAGCAC	158
Podocin	Forward GCTGTCTGCTACTACCGCAT Reverse GTGAGGGATCGATGTGCCAA	125
WT-1	Forward TGCGTCCTATCAGGTTTGCC Reverse CCCAGCGAAATGAGCACAAG	188
Desmin	Forward CTGAGCAAAGGGGTTCCGA Reverse GTGTGACATCCGAGAGTGGA	185
P2X7R	Forward TAACCACTGAGCCGTCCCTA Reverse AGGTCCTCATGCTTGTGCTC	127
NLRP3	Forward TAGCTTCTGCCGAGGTCTCT Reverse ATTGATGGGTCAGTCCGCAG	176
ASC	Forward ACAGTACCAGGCAGTTCGTG Reverse GGTCTGTCACCAAGTAGGGC	143
Procaspase-1	Forward CCGGGGATCCCTCTTCATTG Reverse ACCCTTTCAGTGGTTGGCAT	144
IL-1β	Forward AGGCTGACAGACCCCAAAAG Reverse CTCCACGGGCAAGACATAGG	178
IL-18	Forward ACCGCAGTAATACGGAGCAT Reverse GTCTGGGATTCGTTGGCTGT	110
GAPDH	Forward TGGGTGTGAACCACGAGAA Reverse GGCATGGACTGTGGTCATGA	143

Table 2: Primer sequences for PCR analysis in cellular experiments.

Target	Primer sequence (5′–3′)	Length (bp)
Nephrin	Forward GTCTGGGGACCCCTCTATGA Reverse CAGGTCTTCTCCAAGGCTGT	209
Podocin	Forward CAGAAGGGGAAAAGGCTGCT Reverse GATGCTCCCTTGTGCTCTGT	200
WT-1	Forward TCCGGTCAGCATCTGAAACC Reverse GAGCTGGTCTGAGCGAGAAA	179
Desmin	Forward GTTTCAGACTTGACTCAGGCAG Reverse TCTCGCAGGTGTAGGACTGG	106
P2X7R	Forward CACCGTGCTTACAGGTGCTA Reverse CGGTCTTGGGGAACTCCTTC	115
NLRP3	Forward TCTGCACCCGGACTGTAAAC Reverse CATTGTTGCCCAGGTTCAGC	131
ASC	Forward GACAGTGCAACTGCGAGAAG Reverse CGACTCCAGATAGTAGCTGACAA	106
Procaspase-1	Forward ACAAGGCACGGGACCTATG Reverse TCCCAGTCAGTCCTGGAAATG	237
IL-1β	Forward CGCAGCAGCACATCAACAAG Reverse GTGCTCATGTCCTCATCCTG	118
IL-18	Forward ACTTTGGCCGACTTCACTGT Reverse GTCTGGTCTGGGGTTCACTG	135
GAPDH	Forward TGTGAACGGATTTGGCCGTA Reverse GATGGGCTTCCCGTTGATGA	202

Table 3: Primary and secondary antibodies for Western blot assays.

Primary antibody	Secondary antibody
Rabbit anti-nephrin pAb (Abcam)	IRDye 800-conjugated goat anti-rabbit IgG antibody (LI-COR)
Rabbit anti-podocin pAb (Sigma)	Ditto
Rabbit anti-WT-1 pAb (Abcam)	Ditto
Rabbit anti-desmin pAb (Abcam)	Ditto
Rabbit anti-P2X7R pAb (Alomone)	Ditto
Rabbit anti-NLRP3 pAb (Novus)	Ditto
Rabbit anti-ASC pAb (Santa Cruz)	Ditto
Rabbit anti-caspase-P10 pAb (Santa Cruz)	Ditto
Rabbit anti-IL-1β pAb (Abcam)	Ditto
Mouse anti-IL-18 mAb (Santa Cruz)	IRDye 680-conjugated goat anti-mouse IgG antibody (LI-COR)
Mouse anti-β-actin mAb (Sigma)	Ditto

Table 4: Biological parameters in the different groups at the 13th week ($\bar{x} \pm s$).

Group	BW (g)	KW/BW (mg/g)	FBG (mmol/L)	HbA1c (%)	SCr (μmol/L)	Upro (mg/d)
Control	526.9 ± 51.5	6.1 ± 0.3	6.95 ± 0.53	11.8 ± 0.7	24.9 ± 2.6	6.0 ± 1.5
Model	$452.9 \pm 41.1^{*}$	$8.8 \pm 1.5^{*}$	$32.58 \pm 1.31^{**}$	$20.3 \pm 0.9^{**}$	$34.8 \pm 4.9^{*}$	$33.8 \pm 9.7^{*}$
LD-ACOS	$438.7 \pm 50.1^{*}$	$8.5 \pm 1.1^{*}$	$30.96 \pm 4.03^{**}$	$20.0 \pm 1.1^{**}$	$32.8 \pm 4.2^{*}$	$24.6 \pm 8.1^{*\#}$
HD-ACOS	466.1 ± 105.3	$7.4 \pm 1.1^{*\#}$	$20.25 \pm 7.86^{**\#\#}$	$18.4 \pm 3.2^{**}$	$28.9 \pm 2.5^{*\#}$	$18.4 \pm 4.6^{*\#}$

$^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. model group. BW: body weight; KW: kidney weight; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin A1c; SCr: serum creatinine; Upro: urinary protein.

2.8. Statistical Analysis. All the data of continuous variables were represented as the mean ± SD and analyzed by using SPSS 21.0 statistical software. One-way ANOVA was used to test the differences among groups. Statistical significance was defined as $P < 0.05$.

3. Results

In the course of the experiment, each one rat died in the model group, LD-ACOS group, and HD-ACOS group. Therefore, at the end of the experiment, only the data of the remaining seven rats in the 3 groups were statistically analyzed.

3.1. Effects of ACOS on the Body Weight and the Ratio of KW/BW of the Rat DN Model. Body weight at baseline among the four animal groups had no statistical difference (247.4 ± 5.9 g, 254.6 ± 9.3 g, 277.8 ± 9.8 g, and 252.6 ± 8.4 g in the DN, LD-ACOS, HD-ACOS, and control groups, respectively, $P > 0.05$). At the 4th week, the body weight of rats in the three groups fed with high-fat chow (412.7 ± 30.7 g, 422.3 ± 27.3 g, and 409.7 ± 31.6 g in the DN, LD-ACOS, and HD-ACOS groups, respectively) was significantly higher than that in the control group (374.4 ± 25.0 g) ($P < 0.05$). At the 13th week, the body weight in the DN group and LD-ACOS group was significantly lower than that in the control group ($P < 0.05$), and the body weight in the HD-ACOS group was higher than that in the DN group, but the difference between them was not yet statistically significant ($P > 0.05$) (Table 4).

At the 13th week, the ratio of KW/BW in the DN group and two intervention groups was significantly higher than that in the control group ($P < 0.05$), while in the HD-ACOS group, the ratio was significantly lower than that in the DN group ($P < 0.05$) (Table 4).

3.2. Effects of ACOS on the Blood Glucose and Insulin Resistance of the Rat DN Model. At the 4th week, the FBG levels among the four groups had no statistical difference (6.11 ± 0.65 mmol/L, 6.03 ± 1.11 mmol/L, 5.91 ± 1.08 mmol/L, and 6.14 ± 0.79 mmol/L in the DN, LD-ACOS, HD-ACOS, and control groups, respectively, $P > 0.05$). The FBG levels of rats in the DN group and two intervention groups were all beyond 11.1 mmol/L at 72 h after streptozotocin injection. At the 13th week, the FBG levels in the DN group and two intervention groups were significantly higher than those in the control group ($P < 0.01$), while in the HD-ACOS group, the level was significantly lower than that in the DN group ($P < 0.01$) (Table 4).

In addition, at the 13th week, the HbA1c levels in the DN group and two intervention groups were significantly higher than those in the control group ($P < 0.01$), and the HbA1c level in the HD-ACOS group was lower than that in the DN group, but the difference between them was not yet statistically significant ($P > 0.05$) (Table 4).

At the 4th week, the levels of blood insulin and IRI in the three groups fed with high-fat chow were significantly higher than those in the control group ($P < 0.05$). At the 13th week, the blood insulin level in the DN group was significantly lower than that in the control group ($P < 0.05$) and the level

Table 5: Serum insulin and insulin resistance index in different groups ($\bar{x} \pm s$).

Group	4th week		13th week	
	Serum insulin (mIU/L)	IRI	Serum insulin (mIU/L)	IRI
Control	18.6 ± 1.5	5.03 ± 0.56	20.7 ± 2.2	6.43 ± 1.99
Model	$23.9 \pm 2.6^{*}$	$6.52 \pm 1.29^{*}$	$17.2 \pm 1.4^{*}$	$24.89 \pm 2.81^{**}$
LD-ACOS	$24.5 \pm 4.2^{*}$	$6.49 \pm 0.85^{*}$	19.4 ± 2.2	$24.47 \pm 2.55^{**}$
HD-ACOS	$24.3 \pm 3.0^{*}$	$6.48 \pm 2.11^{*}$	$20.6 \pm 2.3^{\#}$	$18.49 \pm 7.33^{**\#\#}$

$^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. model group. IRI: insulin resistance index.

in the HD-ACOS group was significantly higher than that in the model group ($P < 0.05$). The IRI levels in the DN group and two intervention groups were significantly higher than those in the control group ($P < 0.01$), while the level in the HD-ACOS group was significantly lower than that in the model group ($P < 0.01$) (Table 5).

3.3. Effects of ACOS on the Proteinuria and Renal Function of the Rat DN Model. Urinary protein excretion among the four groups was not statistically different at baseline (5.4 ± 1.6 mg/d, 4.3 ± 0.9 mg/d, 4.4 ± 1.0 mg/d, and 4.9 ± 1.2 mg/d in the DN, LD-ACOS, HD-ACOS, and control groups, respectively, $P > 0.05$). At the 13th week, the urinary protein excretion in the DN group and two intervention groups was significantly higher than that in the control group ($P < 0.05$), while the excretion in the two intervention groups was significantly lower than that in the DN model group ($P < 0.05$) (Table 4).

At the 13th week, the SCr levels in the DN group and two intervention groups were significantly higher than those in the control group ($P < 0.05$), while the level in the HD-ACOS group was significantly lower than that in the DN group ($P < 0.05$) (Table 4).

3.4. Effects of ACOS on the Renal Pathological Parameters of the Rat DN Model. Light microscopy of kidney tissue showed that the average glomerular size, which was represented as an average glomerular diameter, in the DN group and LD-ACOS group was significantly larger than that in the control group ($P < 0.05$), while the size in the two intervention groups was significantly smaller than that in the DN group ($P < 0.05$). The relative area of the mesangial region in the DN group was significantly larger than that in the control group ($P < 0.05$), while the area in the two intervention groups was significantly smaller than that in the DN group ($P < 0.05$) (Figure 2).

Electron microscopy of kidney tissue indicated that the foot processes of podocytes appeared to experience segmental effacement and the average width of foot processes in the DN group and two intervention groups was significantly larger than that in the control group ($P < 0.05$), while the width in the two intervention groups was significantly smaller than that in the DN group ($P < 0.05$) (Figure 2).

3.5. Effects of ACOS on the Expression of Podocyte-Associated Molecules in the Renal Cortex of the Rat DN Model. The results showed that the mRNA and protein expression of nephrin, podocin, and WT-1 was significantly downregulated in the DN group compared with the control group ($P < 0.01$) and was significantly upregulated in the two intervention groups compared with the DN group ($P < 0.01$ or 0.05) (Figure 3).

In contrast, the mRNA and protein expression of desmin was significantly upregulated in the DN group compared with the control group ($P < 0.01$) and was significantly downregulated in the two intervention groups compared with the DN group ($P < 0.01$) (Figure 3).

The above results suggest that podocyte injury occurs in the DN model and ACOS can alleviate the podocyte injury.

3.6. Effects of ACOS on the Expression of P2X7R/NLRP3 Inflammasome and IL-1β/IL-18 in the Renal Cortex of the Rat DN Model. The results showed that the mRNA and protein expression of P2X7R, NLRP3, ASC, and caspase-1 (procaspase-1 mRNA in the gene level and active caspase-1 subunit P10 in the protein level) was significantly upregulated in the DN group compared with the control group ($P < 0.01$), and the above expression was significantly downregulated in the HD-ACOS group compared with the DN group ($P < 0.01$) (Figure 4).

The mRNA and protein expression of downstream effectors of NLRP3 inflammasome, IL-1β and IL-18, was also significantly upregulated in the DN group compared with the control group ($P < 0.01$), and the above expression was significantly downregulated in the HD-ACOS group compared with the DN group ($P < 0.01$) (Figure 5).

The above results suggest that there are enhanced expression and activation of P2X7R/NLRP3 inflammasome and IL-1β/IL-18 in the DN model and ACOS can attenuate the changes.

3.7. The Localization of P2X7R/NLRP3 in Podocytes of the Rat Kidney in the DN Model. As shown in Figure 6, double immunofluorescence staining analysis revealed that the P2X7R (Figure 6(a), red spots) and NLRP3 (Figure 6(b), red spots) both were colocalized with synaptopodin (a podocyte marker; green spots indicate alone localization and yellow spots indicate colocalization) in glomeruli of DN rats. Outside of glomeruli, there was hardly any expression of P2X7R and NLRP3. Furthermore, the expression levels of P2X7R/NLRP3 in podocytes were obviously decreased in the HD-ACOS and LD-ACOS groups compared with the DN group.

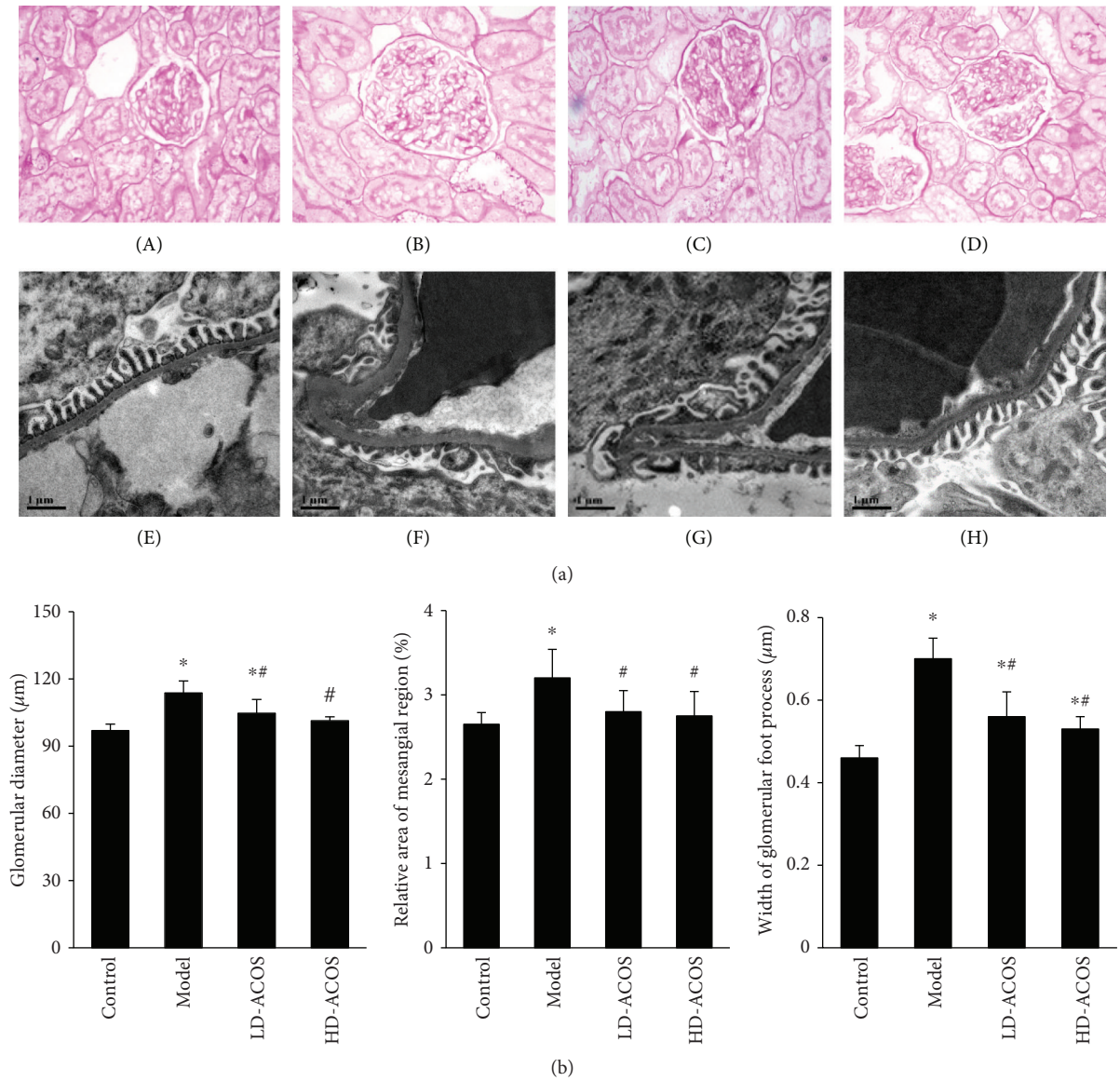

Figure 2: Effects of ACOS on the pathological parameters of the rat DN model. (a) Light microscopic images of glomerular size (PAS staining ×400) and electron microscopic images of foot processes of podocytes (×20,000). (A, E) Control group. (B, F) DN model group. (C, G) LD-ACOS group. (D, H) HD-ACOS group. (b) Histograms of the glomerular diameter, relative area of the mesangial region, and foot process width. Values are represented as mean ± SD. $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. DN model group.

The above results suggest that P2X7R and NLRP3 both are expressed on podocytes and ACOS can weaken their expression.

3.8. Effects of ACOS on the HG-Induced Expression of Podocyte-Associated Molecules in Cultured Podocytes. The results showed that the mRNA and protein expression of nephrin, podocin, and WT-1 was significantly downregulated in the HG group compared with the control group ($P < 0.01$) and was significantly upregulated in the HG + ACOS group compared with the HG group ($P < 0.01$ or 0.05) (Figure 7).

On the contrary, the mRNA and protein expression of desmin was significantly upregulated in the HG group compared with the control group ($P < 0.01$) and was significantly downregulated in the HG + ACOS group compared with the HG group ($P < 0.01$ or 0.05) (Figure 7).

We performed an experiment of parallel controls with mannitol and did not find podocyte injury being caused by HG-related high osmotic pressure (Figure 1S, see Supplementary Materials). The results of the cell experiment are consistent with the results of the animal experiment, and both suggest that ACOS can alleviate the HG-induced podocyte injury.

3.9. Effects of ACOS on the HG-Induced Expression of P2X7R/NLRP3 Inflammasome and IL-1β/IL-18 in Cultured Podocytes. The results showed that the mRNA and protein expression of P2X7R, NLRP3, ASC, and caspase-1 (procaspase-1 mRNA in the gene level and active caspase-1

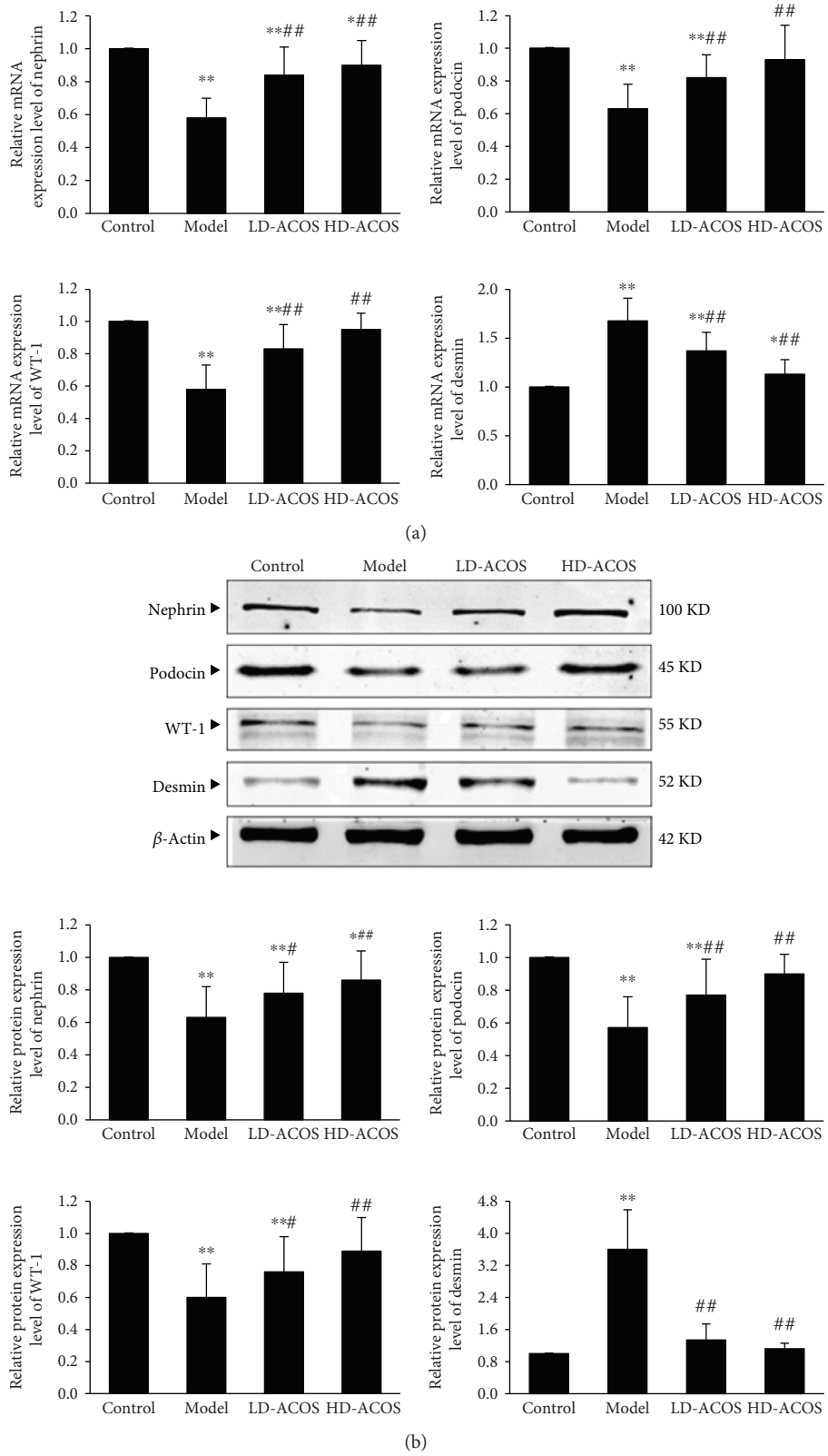

Figure 3: Effects of ACOS on the expression of podocyte-associated molecules in the rat DN model. (a) Total RNA was extracted from renal cortex tissue, and the relative mRNA expression levels of nephrin, podocin, WT-1, and desmin were measured by real-time quantitative PCR. (b) Renal cortex tissue was lysed, and total lysates were analyzed by the Western blot assay with antibodies against nephrin, podocin, WT-1, desmin, and β-actin, respectively. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. DN model group.

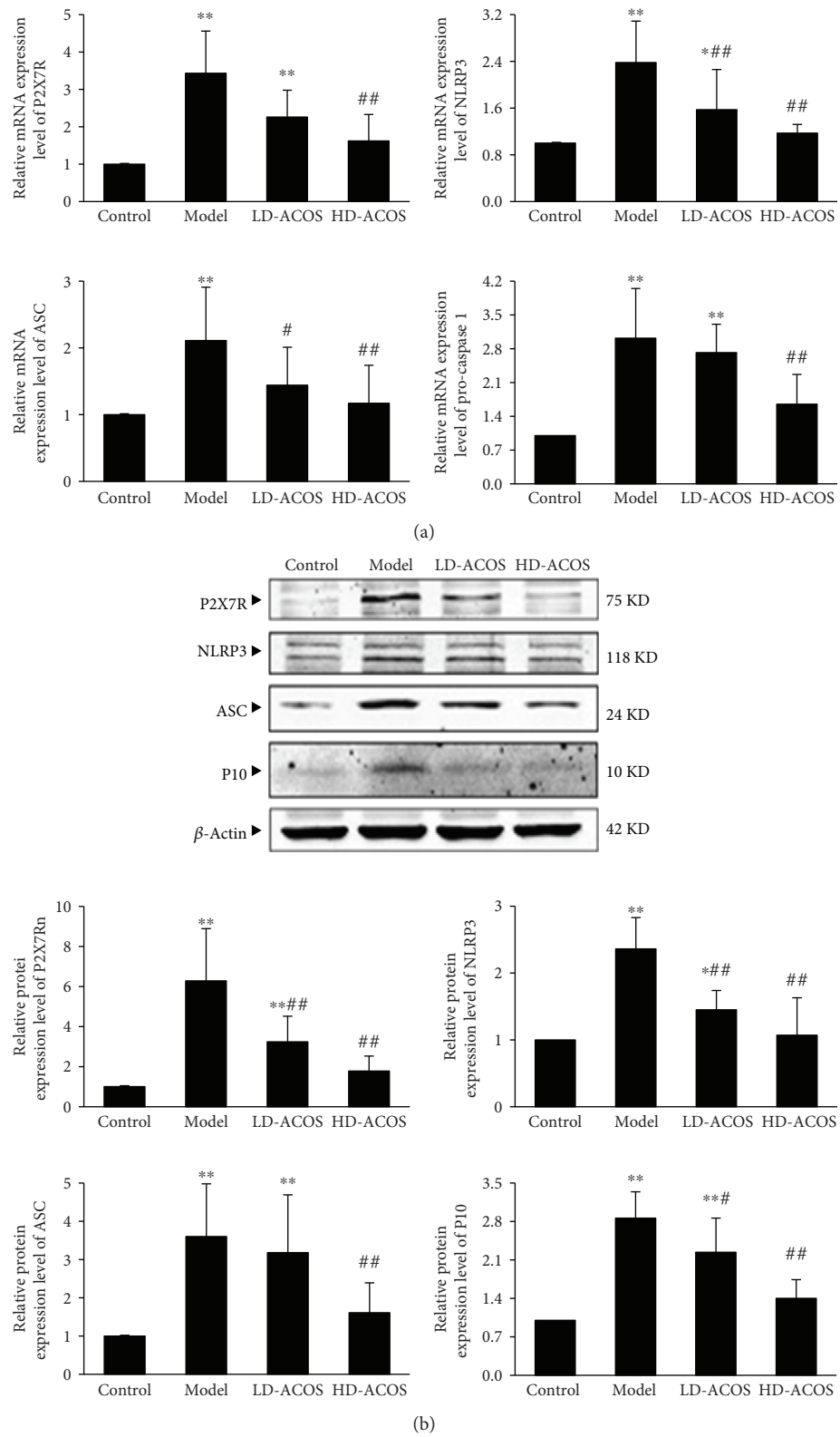

Figure 4: Effects of ACOS on the expression of P2X7R/NLRP3 inflammasome in the rat DN model. (a) Total RNA was extracted from renal cortex tissue, and the relative mRNA expression levels of P2X7R, NLRP3, ASC, and procaspase-1 were measured by real-time quantitative PCR. (b) Renal cortex tissue was lysed, and total lysates were analyzed by the Western blot assay with antibodies against P2X7R, NLRP3, ASC, active caspase-1 subunit P10, and β-actin, respectively. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. DN model group.

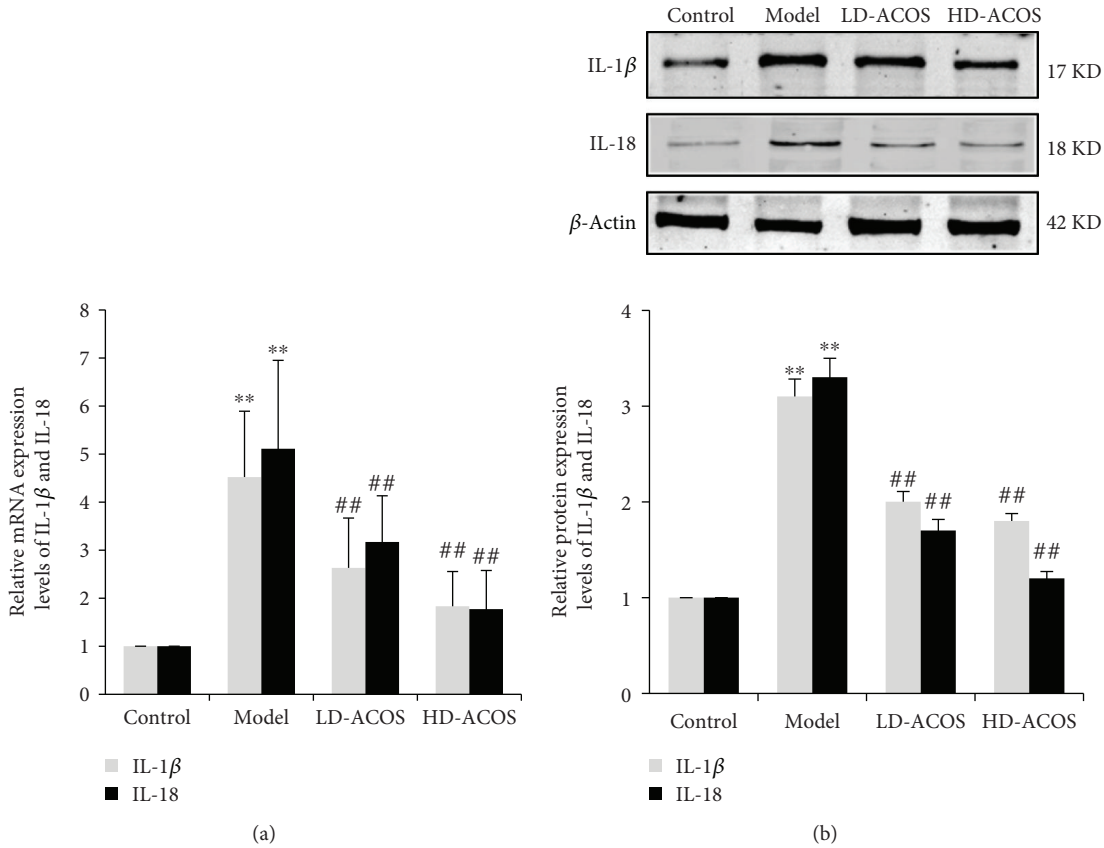

Figure 5: Effects of ACOS on the expression of IL-1β/IL-18 in the rat DN model. (a) Total RNA was extracted from renal cortex tissue, and the relative mRNA expression levels of IL-1β and IL-18 were measured by real-time quantitative PCR. (b) Renal cortex tissue was lysed, and total lysates were analyzed by the Western blot assay with antibodies against IL-1β, IL-18, and β-actin, respectively. The relative protein expression level was expressed as the target protein/β-actin ratio. Values are represented as mean ± SD. $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. DN model group.

subunit P10 in the protein level) was significantly upregulated in the HG group compared with the control group ($P < 0.01$), and the above expression was significantly downregulated in the HG + ACOS group compared with the HG group ($P < 0.01$ or 0.05) (Figure 8).

Accordingly, the mRNA and protein expression of IL-1β and IL-18 was significantly upregulated in the HG group compared with the control group ($P < 0.01$ or $P < 0.05$), and the above expression was significantly downregulated in the HG + ACOS group compared with the HG group ($P < 0.01$ or 0.05) (Figure 9).

The results of the cell experiment are consistent with the results of the animal experiment, and both suggest that ACOS can attenuate the HG-induced inflammatory reaction in podocytes.

4. Discussion

In this study, we successfully established a rat model of DN caused by type 2 DM, which was induced by high-fat-diet feeding and low-dose streptozotocin injection and characterized by insulin resistance and hyperglycemia [25, 26]. In the model, the rats of DN developed obvious proteinuria, elevated serum creatinine, and podocyte injury showing as foot process segmental effacement with an increased width and significant alteration of the expression of podocyte-associated molecules (downregulation of nephrin, podocin, and WT-1 and upregulation of desmin). In addition, we also successfully established a cell model of podocyte injury, which was produced with the stimulation of HG [27]. In the cell model, the expression of podocyte-associated molecules was also significantly changed, which was similar to what is seen in the rat DN model, suggesting podocyte injury. Both the animal and cell models provide good platforms for us to study *in vivo* and *in vitro* the mechanism of podocyte injury of DN and the intervention effects of ACOS.

It has been recognized that podocyte injury is a pivotal event in the pathogenesis of DN, which contributes to the occurrence and progression of DN [4]. The podocyte injury in DN includes decreased podocyte density and number, foot process effacement, detachment and disruption of the slit diaphragm, podocyte loss and its resulting hypertrophy and/or dedifferentiation, and podocyte death [4]. The above changes of podocytes will cause proteinuria, while the proteinuria itself can further aggravate the podocyte injury to form a vicious cycle, eventually leading to glomerulosclerosis and renal dysfunction [4]. Therefore, in this study,

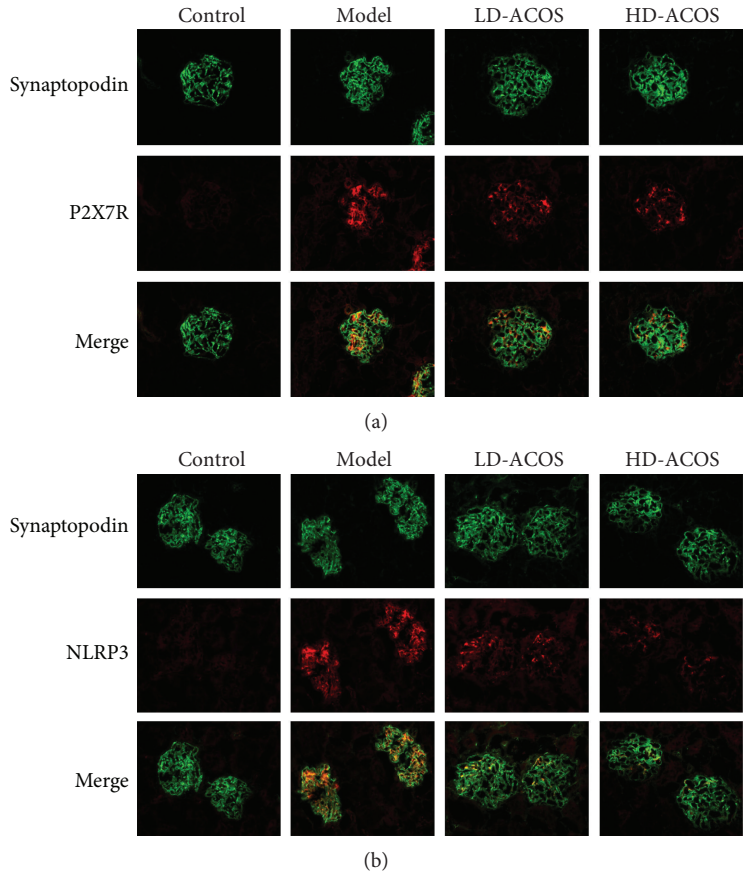

Figure 6: Double immunofluorescence staining of the podocyte marker and P2X7R/NLRP3 in renal tissues of the rat DN model. The colocalization of P2X7R (a, red spots) or NLRP3 (b, red spots) and synaptopodin (a podocyte marker; green spots indicate alone localization and yellow spots indicate colocalization) in the frozen renal tissue section of the rat DN model (immunofluorescence microscopy ×400).

we focused on watching the changes of podocytes in the rat DN model and in the HG-stressed cell model.

In the past years, the pathogenic role of P2X7R and NLRP3 inflammasome in kidney diseases was highly noticed [28, 29], and the expression of P2X7R and all the components of NLRP3 inflammasome in podocytes was also affirmed [8, 30, 31]. In 2004, Vonend et al. [8] first reported that the expression of P2X7R in glomeruli, mainly in podocytes, was significantly upregulated in a diabetic rat model. In 2014, Shahzad et al. [7] observed that the NLRP3 inflammasome was activated in the podocytes of the patients with DN and the mouse models of DN and in the glucose-stressed podocytes in culture. Our studies *in vivo* and *in vitro* further confirm their previous findings. Furthermore, because P2X7R is a potent activator of the NLRP3 inflammasome [32], we put them together to watch their changes in the studies *in vivo* and *in vitro* and found that the upregulation of P2X7R expression was always synchronized with the activation of NLRP3 inflammasome and its downstream effectors IL-1β and IL-18. So, our study extends, to a certain extent, the previous observations.

Ophiocordyceps sinensis and its anamorph *Hirsutella sinensis* are widely used in the treatment of kidney diseases in China and have been proven to be effective in the treatment of DN both in clinical practice and in animal experiments [16]. In this study, our experiments with ACOS also affirmed its effectiveness. Moreover, through the experiments *in vivo* and *in vitro*, we found that *Ophiocordyceps sinensis* could significantly inhibit the high expression of P2X7R and the activation of NLRP3 inflammasome in podocytes, which may be one of the important mechanisms for the therapeutic effects of *Ophiocordyceps sinensis* on DN. To our knowledge, no similar research results have been published so far. Our study also showed that ACOS could improve insulin resistance and reduce blood glucose level in a diabetic rat model, which is consistent with previous studies [16, 18, 33]. Undoubtedly, the effects can also contribute to the prevention and treatment of DN.

Wild *Ophiocordyceps sinensis* only grows in China's Qinghai-Tibet Plateau, and its resource is quite limited. The predatory digging and collecting every year not only exhaust the resources but also destroy the fragile ecological environment on the local plateau. Now, wild *Ophiocordyceps sinensis* has been listed by the Chinese government as "National Grade II Protected Species" and has been restricted from picking [19, 20]. So, artificial cultivation of *Ophiocordyceps*

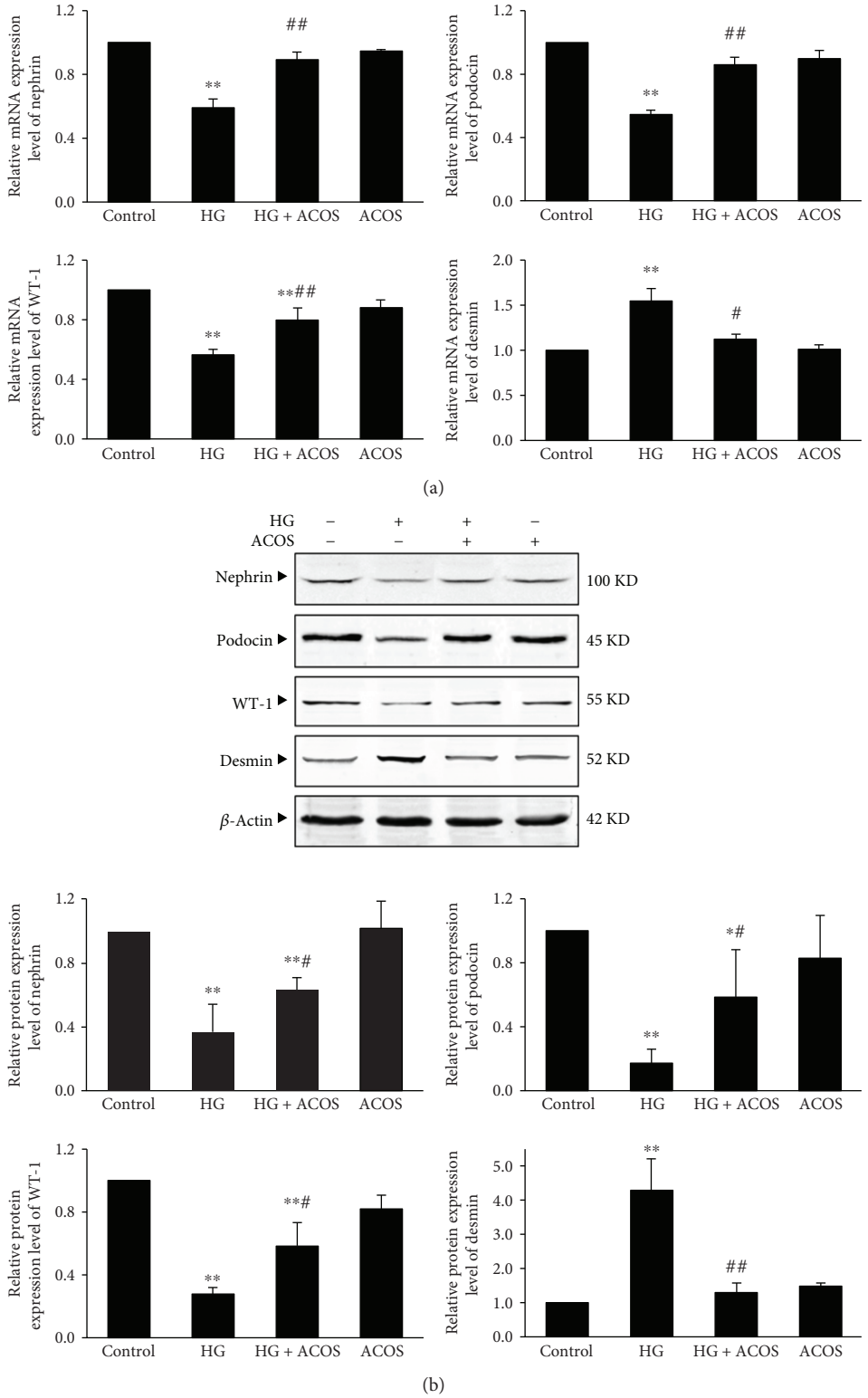

Figure 7: Effects of ACOS on the high-glucose-induced expression of podocyte-associated molecules in cultured podocytes. Podocytes were incubated in medium and medium containing 30 mM glucose and/or 50 μg/mL ACOS, respectively. (a) After 6 h of incubation, cells were harvested. Then, the total RNA was extracted, and the relative mRNA expression levels of nephrin, podocin, WT-1, and desmin of podocytes were measured by real-time quantitative PCR. (b) After 24 h of incubation, cells were lysed and the total lysates were used to determine the protein expression levels of nephrin, podocin, WT-1, desmin, and β-actin by the Western blot assay. The relative protein expression level was expressed as the target protein/β-actin protein ratio. Values are represented as mean ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. HG group.

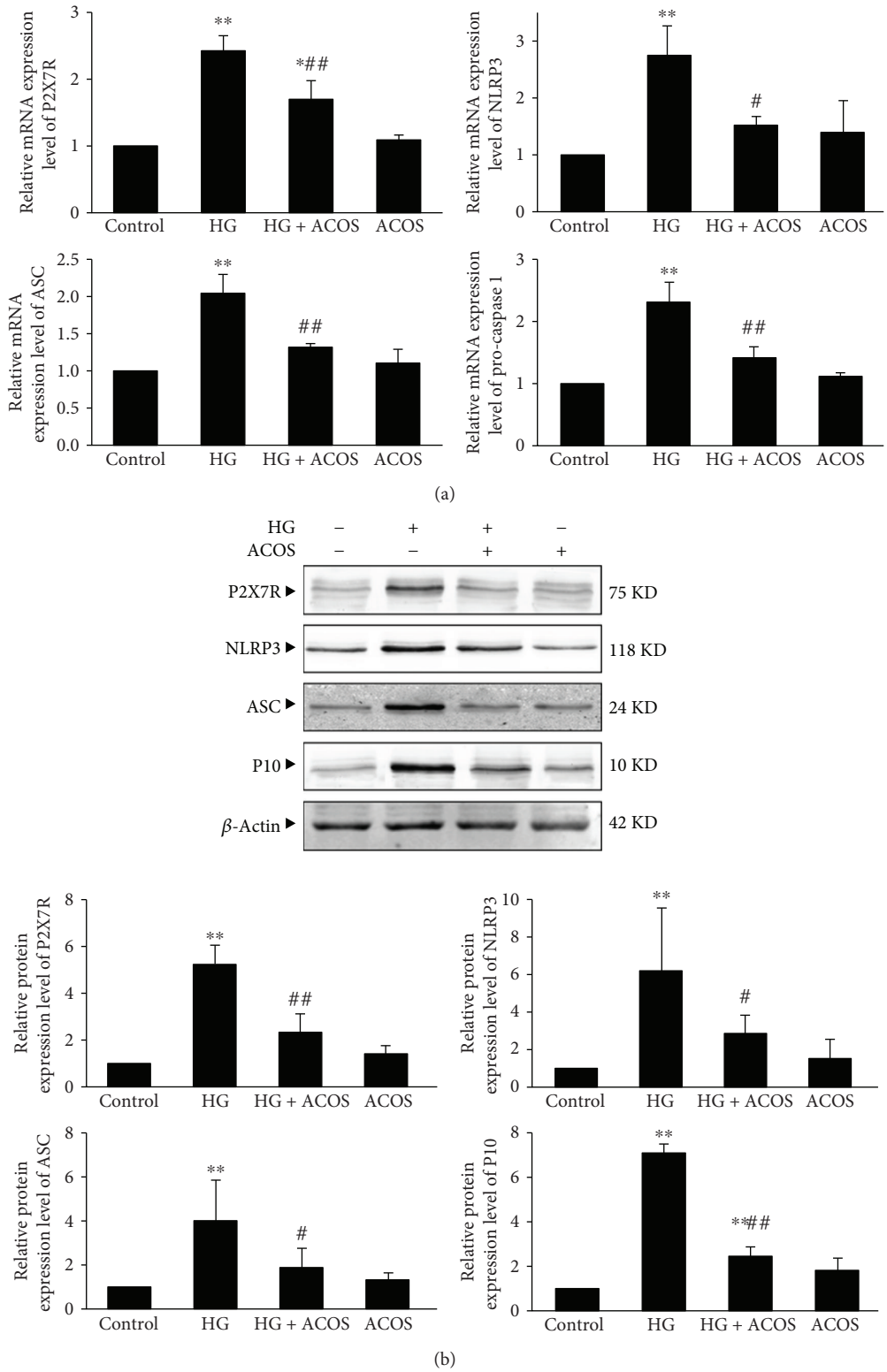

Figure 8: Effects of ACOS on the high-glucose-induced expression of P2X7R/NLRP3 inflammasome in cultured podocytes. Podocytes were incubated in medium and medium containing 30 mM glucose and/or 50 μg/mL ACOS, respectively. (a) After 6 h of incubation, cells were harvested. Then, the total RNA was extracted, and the relative mRNA expression levels of P2X7R, NLRP3, ASC, and procaspase-1 were measured by real-time quantitative PCR. (b) After 24 h of incubation, cells were lysed and the total lysates were used to determine the protein expression levels of P2X7R, NLRP3, ASC, active caspase-1 subunit P10, and β-actin by the Western blot assay. The relative protein expression level was expressed as the target protein/β-actin protein ratio. Values are represented as mean ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. HG group.

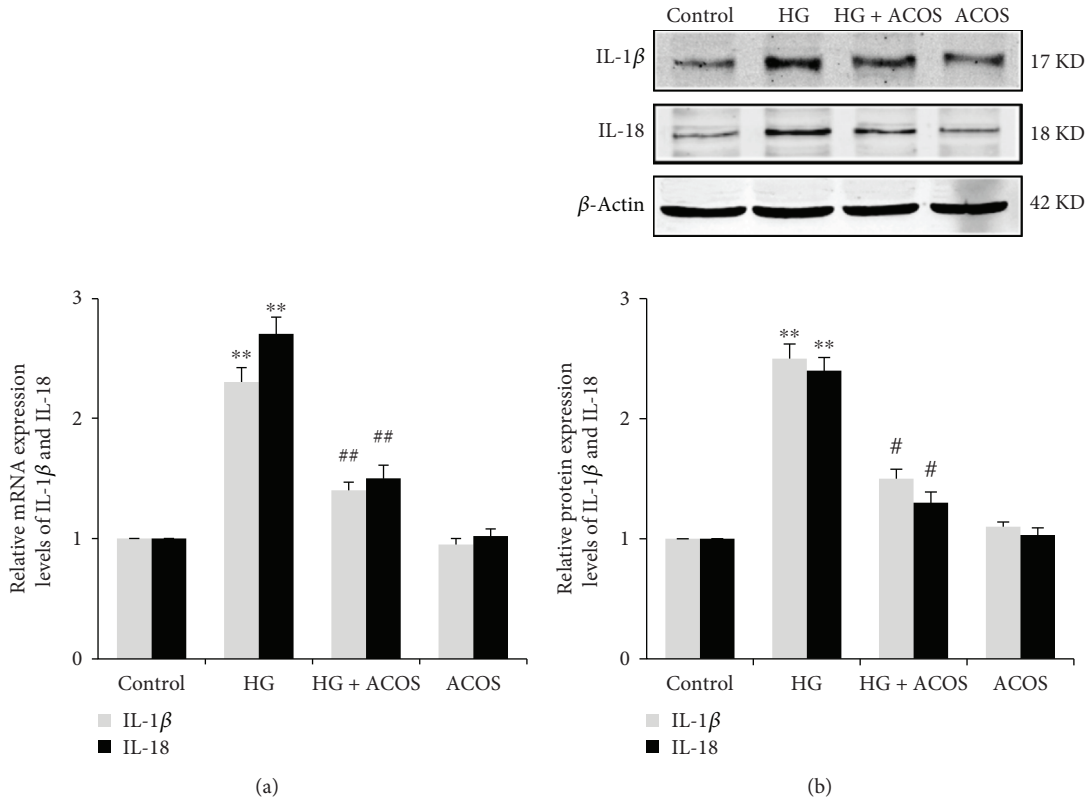

Figure 9: Effects of ACOS on the high-glucose-induced expression of IL-1β/IL-18 in cultured podocytes. Podocytes were incubated in medium and medium containing 30 mM glucose and/or 50 μg/mL ACOS, respectively. (a) After 6 h of incubation, cells were harvested. Then, the total RNA was extracted, and the relative mRNA expression levels of IL-1β and IL-18 were measured by real-time quantitative PCR. (b) After 24 h of incubation, cells were lysed and the total lysates were used to determine the protein expression levels of IL-1β, IL-18, and β-actin by the Western blot assay. The relative protein expression level was expressed as the target protein/β-actin protein ratio. Values are represented as mean ± SD ($n = 3$). $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. HG group.

sinensis is urgently needed. After more than 30 years of hard work to explore and overcome a lot of technical difficulties, artificial cultivation and industrialized production of *Ophiocordyceps sinensis* were finally successful in 2015 [20], and the ACOS has been verified by the Chinese Academy of Sciences [21]. After molecular systematic analysis [21], nuclear magnetic resonance fingerprint analysis [34], and chemical compound analysis [35, 36], it has been confirmed that the ACOS has no significant difference with wild *Ophiocordyceps sinensis* and its quality fully complies with the standard of Chinese pharmacopoeia. Our study is the first experimental research to treat the disease with ACOS and has gained good results, which clearly displays that the ACOS is effective for the treatment of DN.

In conclusion, our experiments *in vivo* and *in vitro* conducted in the DN rat model and HG-stressed podocyte model suggest that P2X7R and NLRP3 inflammasome are involved in the pathogenesis of DN including its podocyte injury and *Ophiocordyceps sinensis* including ACOS can effectively alleviate the podocyte injury of DN. The therapeutic efficacy may be related to its antagonistic effects on the P2X7R/NLRP3 inflammasome axis. The above research results provide a valuable experimental basis for the clinical application of ACOS in the prevention and treatment of DN.

Data Availability

The original data of the current study are available in the following website: https://pan.baidu.com/s/16Np4-nJjQjtovUhCdYdzcQ.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Authors' Contributions

Chao Wang and Xiao-xia Hou contributed equally to this work.

Acknowledgments

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Supplementary Materials

Table 1S: the cytotoxicity of high glucose (HG) on cultured podocytes. Figure 1S: effects of mannitol or high glucose on the expression of podocyte-associated molecules in cultured podocytes. *(Supplementary Materials)*

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Research Article

Polymorphisms in the Glucagon-Like Peptide 1 Receptor (*GLP-1R*) Gene Are Associated with the Risk of Coronary Artery Disease in Chinese Han Patients with Type 2 Diabetes Mellitus: A Case-Control Study

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Background. Glucagon-like peptide 1 (GLP-1) bestows protective effects upon the cardiovascular system through direct cardiovascular interactions or by improvements to metabolic function. Both these effects are thought to be at least partly mediated by the GLP-1 receptor (GLP-1R). This case-controlled study investigated whether polymorphisms in the *GLP-1R* gene affect the risk of cardiovascular disease in type 2 diabetic patients in the Chinese Han population. *Methods*. Eleven haplotype-tagging single nucleotide polymorphisms (SNPs), distributed across 22 kb of the 39 kb *GLP-1R* gene, were selected and genotyped in diabetic patients from a Chinese Han population. Patients were classified based on the severity of coronary artery stenosis. Coronary artery stenosis was ≥50% in 394 patients (coronary artery disease- (CAD-) positive group), and coronary artery stenosis was <50% in 217 patients (control group). Allele and genotype frequencies were compared between the two groups at all 11 SNPs. *Results*. When considered in recessive inheritance mode, patients with the GG genotype at rs4714210 had a lower CAD risk than patients with other genotypes (OR = 0.442, 95% CI = 0.258–0.757, $p = 0.002$), even when other known CAD risk factors were taken into account ($OR_a = 0.440$, 95% $CI_a = 0.225$–0.863, $p_a = 0.017$). In additive inheritance mode, GG genotype carriers at rs4714210 exhibited a lower risk of CAD than AA carriers ($OR_a = 0.475$, $CI_a = 0.232$–0.970, $p_a = 0.041$). *Conclusion*. In type 2 diabetic patients from a Han Chinese population, some variations in the *GLP-1R* gene were associated with a lower risk of developing CAD.

1. Introduction

Coronary artery disease (CAD) is a life-threatening condition that is a frequently occurring complication in patients with type 2 diabetes mellitus (T2DM), with diabetic patients being 2–4 times more likely to develop CAD than nondiabetics [1]. Determination of genetic variants associated with CAD development in T2DM patients may assist in the identification of at-risk individuals and allow targeting of primary prevention and early intervention measures. In recent years, glucagon-like peptide 1 receptor (GLP-1R) agonists such as exenatide and liraglutide have been widely studied because of their glucose-dependent insulinotropic effects [2] and their other physiological effects such as decrease in fatty acid absorption, increase in satiety, and reduction in body weight [3]. GLP-1Rs are widely expressed in the cardiovascular system [4], and a number of beneficial effects that protect against coronary heart disease are associated with the GLP-1/GLP-1R signal pathway and its agonist interactions. Previous studies demonstrated that GLP-1 agonists could reduce the rate of the first occurrence of death from cardiovascular causes and nonfatal myocardial infarction among patients with T2DM [5, 6]. GLP-1 agonists were also found to improve heart function, decrease the size of

infarct areas in ischemia-reperfusion heart models (pig and mouse) [7], increase coronary blood flow in isolated mouse heart [4], and reduce monocyte adhesion and atherosclerotic lesions in apoE$^{-/-}$ mice [8]. Consequently, we reasoned that genetic variation in the *GLP-1R* gene might affect CAD risk in patients with T2DM. This hypothesis was investigated by examining CAD-positive and CAD-negative patients with T2DM in a Chinese Han population.

2. Materials and Methods

2.1. Ethics Statement. The study protocol and informed consent procedures were approved by the Research Ethics Committees of Peking University First Hospital. Written informed consents were acquired from all subjects participating in this study, in agreement with the 1975 Helsinki Declaration.

2.2. Subjects. Diabetes mellitus was diagnosed according to World Health Organization criteria (1999) [9] as follows: fasting plasma glucose ≥ 7.0 mmol/l, and/or 2 h plasma glucose ≥ 11.1 mmol/l, or casual plasma glucose (random blood sugar) ≥ 11.1 mmol/l. Patients with type 1 diabetes and subjects with active inflammatory conditions, autoimmune diseases, malignancies, usage of immunosuppressive drugs, and known hematological disorders were excluded. In total, 611 unrelated Chinese Han subjects with T2DM were included in the study: 394 with coronary artery stenosis (CAS) ≥ 50% (CAD-positive group) and 217 with CAS < 50% (control group). Diagnostic procedures were carried out at Peking University First Hospital. CAS ≥ 50% individuals were defined as those who exhibited ≥ 50% stenosis in at least one of the major coronary arteries or their main branches upon cardiac catheterization. Control individuals had <50% coronary stenosis in all main coronary arteries and main branches as determined by cardiac catheterization or high specificity spiral computed tomography (CT) scan [10]. Demographic data and patient cardiovascular risk factor data were collected for all subjects from medical records. These data comprised gender, age, body mass index (BMI), fasting plasma glucose (FPG), history of dyslipidemia, hypertension (blood pressure ≥ 140/90 mmHg or receiving any antihypertensive therapies), and smoking history ("ever" or "never," with "ever" defined as having smoked more than one cigarette per day for more than 6 months, as per World Health Organization criteria).

2.3. Single Nucleotide Polymorphism Genotyping. Genomic DNA was extracted from peripheral blood using a Whole Blood DNA Extraction Kit (BioTeke).

The GLP-1R gene is located at chromosome 6p21, is 38.9 kb in length, and includes 13 exons. In total, 33 haplotype-tagging single nucleotide polymorphisms (SNPs) were identified at the GLP-1R locus in the CHB (Han Chinese from Beijing) population from the HapMap Phase II database (http://www.hapmap.org) (R#27, $r^2 < 0.8$, MAF ≥ 0.05). Eleven of these SNPs, dispersed across 22,058 bp of the total 38,964 bp of *GLP-1R*, were selected for further study: rs761387 (T>C), rs2268635 (G>A), rs7769547 (G>A), rs910162 (T>A), rs3765468 (G>A), rs3765467 (G>A), rs3765466 (A>T), rs10305456 (C>T), rs10305518 (T>G), rs1820 (T>A), and rs4714210 (A>G). Target regions were amplified by PCR. Direct DNA sequencing was used for 8 of the 11 selected SNPs, using a MassARRAY system (Sequenom iPLEX assay, San Diego, CA, USA) [11], and the remaining three SNPs were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Genotyping success rates were 95–100%, and repeatability rates were 98–100%. To validate PCR-RFLP assays, 5% of amplicons were directly sequenced to confirm the genotypes for each SNP. Concordance rates between RFLP and DNA-sequencing results were 98–100%.

2.4. Data Analysis. Clinical and laboratory data were expressed as means ± SD or percentages. Genotype distributions described departure from Hardy-Weinberg equilibrium at each polymorphic locus. Linkage disequilibrium (LD) and haplotype analysis were performed using Haploview 4.2, with haplotypes estimated using an accelerated expectation-maximization algorithm.

Allele frequencies were determined by gene counting. SNP association with risk of CAD was assessed using the SPSS statistical package (SPSS version 19.0, USA). Qualitative variables were compared using a χ^2 test, and quantitative variables were compared using an independent samples t-test or a Mann–Whitney U test. Associations between CAD and genotype were analyzed using multiple logistic regression with adjustment for the following potential confounders: age, gender, BMI, smoking status, positive histories of dyslipidemia and hypertension, and diabetic duration. As a descriptive measure of association between genotypes and outcomes, $p < 0.05$ was considered to be statistically significant and odds ratios (ORs) were calculated with 95% confidence intervals (CIs). Bonferroni correction was used to correct for multiple comparisons. Power and Sample Size Calculation software (version 3.1.2, 2014) was used for power calculations [12].

3. Results

3.1. Characteristics of Study Subjects. Males were more likely to be in the CAD-positive T2DM group than in the control T2DM group ($p < 0.05$); otherwise, no significant differences in phenotypic characteristics were found between groups (Table 1). HbA1c and FPG measurements were acquired after antidiabetic treatment. Genotype distributions at all 11 loci were in agreement with Hardy-Weinberg equilibrium (data not shown). Statistical power was 0.99.

3.2. Allele and Genotype Analysis. The minor allele G at rs4714210, in the 3′ untranslated region (UTR), was found more frequently in the control group than in the CAD-positive group, and carriers of the G allele displayed a lower risk of CAD when compared with noncarriers (OR = 0.783, 95% CI = 0.613–1.002, $p = 0.051$). In dominant inheritance mode, no significant difference in genotype distribution was found between the CAD-positive and control groups (Supplementary Material 1).

Table 1: Clinical characteristics of CAD-positive and control patients with T2DM.

	CAD-pos.	Controls	p
N	394	217	
Male, n (%)	274 (69.5)	97 (44.7)	<0.001
Age (y)	61.38 ± 10.13	62.19 ± 10.28	0.384
T2DM duration (y)	6.0 (1.0–12.0)	6.0 (2.0–10.0)	0.912
BMI (kg/m^2)	26.1 ± 3.55	25.9 ± 3.65	0.521
HbA1c (%)	7.28 ± 1.45	7.09 ± 1.64	0.275
FPG (mmol/l)	7.3 ± 2.7	6.9 ± 2.1	0.164
eGFR (ml/min/1.73m^2)	70.0 (58.94–81.72)	77.8 (57.9–85.0)	0.279
Positive dyslipidemia history (%)	76.5	78.1	0.697
Positive hypertension history (%)	79.3	72.8	0.106
Positive smoking history (%)	45.1	37.1	0.099

Data are presented as mean ± SD, n (%). BMI: body mass index; FPG: fasting plasma glucose; CAD: coronary artery disease; T2DM: type 2 diabetes mellitus. Independent t-test was used to compare BMI, HbA1c, FPG, and T2DM duration between groups; Mann–Whitney U test was used to compare the difference in T2DM duration between two groups. Age was compared by t-test. Other phenotypic characteristics were compared by χ^2 test between groups.

The protective effect of the homozygous GG minor allele genotype at rs4714210 was also observed in additive (codominant) inheritance mode using logistic regression analysis (Table 2). GG genotype carriers at rs4714210 exhibited a lower risk of CAD than AA carriers ($OR_a = 0.475$, $CI_a = 0.232–0.970$, $p_a = 0.041$, after adjustment for confounders as above), while no such protective effect was observed in heterozygote carriers.

In recessive inheritance mode, the carriers of genotype GG at rs4714210 had a decreased risk of CAD (OR = 0.442, 95% CI = 0.258–0.757, $p = 0.002$; $OR_a = 0.440$, 95% $CI_a = 0.225–0.863$, $p_a = 0.017$), after adjusting for other known CAD risk factors (gender, age, BMI, smoking status, dyslipidemia history, hypertension history, and diabetic duration) (Table 3).

The other ten SNPs tested in this study displayed similar allele frequencies between the CAD-positive and control groups, and no significant associations were noted between genotype and CAD risk (Tables 2 and 3).

3.3. Haplotype Analysis. Haploview plotting was used to construct haplotypes depending on the physical position and the value of D' ($D' > 0.5$) between each pair of SNPs in one block. Three blocks were delineated as follows: LD block 1 (rs910162, rs3765468, rs3765467, rs765466, and rs10305456), block 2 (rs761387 and rs7769547), and block 3 (rs10305518, rs1820, and rs4714210). The SNP distributions in the three haplotype blocks did not differ significantly between the CAD-positive and control groups (Supplementary Material 2).

4. Discussion

GLP-1R is a 463 amino acid member of the class B GPCR secretin family. GLP-1R is a classic seven-transmembrane protein; the C-terminus of GLP-1R interacts with a signaling G protein, and the large N-terminal extracellular domain plays an important role in ligand binding [13, 14]. After binding to the GLP-1 ligand, GLP-1R transmits a signal through a Gas-coupled subunit. This induces an increase in cAMP (cyclic adenosine monophosphate) levels and consequently activates the PKA pathway. The effect of the GLP-1/GLP-1R pathway in the myocardial ischemia-reperfusion model was previously summarized by Ravassa et al. [15]. In particular, GLP-1/GLP-1R can activate the PKA, PI3K, MEK1/2, and eNOS pathways, resulting in cardiovascular protective effects such as reduced apoptosis, improved energy metabolism, reduced inflammation in myocardial cells, and vasodilation in myocardial arteries. Systematically, GLP-1R transmits signals that prompt insulin secretion increases, appetite reduction, metabolism improvement, and lower blood pressure and, as a result, decreases the severity of atherosclerotic lesions [5]. The wide-ranging effects of GLP-1/GLP-1R suggest that variations in the *GLP-1R* gene may contribute to the risk of CAD.

Here, we found an association between rs4714210 in the *GLP-1R* gene and CAD risk in T2DM patients in the Chinese Han population. Patients homozygous for the minor allele G at rs4714210 exhibited a 50% lower risk of CAD than other genotype carriers. The mechanisms through which this allele confers protection are unclear. The rs4714210 locus is in the 3′ UTR of the *GLP-1R* gene, and 3′ UTRs are thought to play important roles in gene regulation. For example, 3′ UTRs can influence chromosome structure, regulate transcription, stabilize mRNA, and modulate translation, thus affecting the stability and transport of the encoded proteins [16]. We therefore speculate that variations at rs4714210 may differentially affect the function of GLP-1R through one or more of these mechanisms, but this remains to be confirmed. It is also likely that the rs4714210 SNP is in strong LD with other SNPs that have biological effects.

GLP-1R SNPs have been confirmed in the association with obesity [17], pancreatic beta-cell function [18, 19], and T2DM [20] in different populations. However, there are few studies about the variations of *GLP-1R* with CAD. In 2016, Scott et al. first observed an effect of GLP-1R genetic variation in Caucasian CAD patients with or without T2DM [21], identifying an association between *GLP-1R* rs10305492 and

Table 2: Distribution of SNP genotype frequencies in CAD-positive and control groups in additive inheritance mode.

SNP	Genotype	CAD-pos. $n = 394$ (%)	Controls $n = 217$ (%)	p	OR_a	CI_a	p_a
	TT	268 (68.0)	142 (65.4)		1		
rs761387	TC	113 (28.7)	69 (31.8)	0.696	0.855	0.530–1.382	0.523
	CC	13 (3.3)	6 (2.8)		1.065	0.277–4.102	0.927
	GG	168 (42.6)	109 (50.2)		1		
rs2268635	GA	186 (47.2)	88 (40.6)	0.193	1.064	0.666–1.699	0.795
	AA	40 (10.2)	20 (9.2)		1.105	0.496–2.458	0.807
	GG	102 (25.9)	54 (24.9)		1		
rs7769547	GA	208 (52.8)	107 (49.3)	0.446	0.829	0.476–1.445	0.509
	AA	84 (21.3)	56 (25.8)		0.750	0.390–1.442	0.388
	TT	91 (23.1)	57 (26.3)		1		
rs910162	TA	220 (55.8)	105 (48.4)	0.204	1.127	0.653–1.945	0.668
	AA	83 (21.1)	55 (25.3)		0.920	0.480–1.764	0.801
	GG	268 (68.0)	143 (65.9)		1		
rs3765468	GA	112 (28.4)	68 (31.3)	0.683	0888	0.548–1.441	0.632
	AA	14 (3.6)	6 (2.8)		1.201	0.317–4.546	0.788
	GG	240 (60.9)	143 (65.9)		1		
rs3765467	GA	134 (34.0)	65 (30.0)	0.469	0.883	0.553–1.411	0.603
	AA	20 (5.1)	9 (4.1)		2.546	0.557–11.64	0.228
	AA	67 (17.0)	35 (16.1)		1		
rs3765466	AT	204 (51.8)	108 (49.8)	0.766	0.681	0.349–1.330	0.261
	TT	123 (31.2)	74 (34.1)		0.833	0.401–1.731	0.625
	CC	330 (83.8)	177 (81.6)		1		
rs10305456	CT	63 (16.0)	39 (18.0)	0.743	1.114	0.611–2.030	0.725
	TT	1 (0.3)	1 (0.5)		/[a]	/	/
	TT	281 (71.3)	155 (71.4)		1		
rs10305518	TG	102 (25.9)	57 (26.3)	0.935	0.812	0.487–1.354	0.425
	GG	11 (2.8)	5 (2.3)		0.985	0.250–3.887	0.983
	TT	341 (86.5)	189 (87.1)		1		
rs1820	TA	49 (12.4)	28 (12.9)	0.170	1.165	0.584–2.324	0.665
	AA	4 (1.0)	0 (0.0)		/	/	/
	AA	173 (43.9)	88 (40.6)		1		
rs4714210	AG	193 (49.0)	97 (44.7)	**0.01**	1.159	0.715–1.878	0.550
	GG	28 (7.1)	32 (14.7)		0.475	0.232–0.970	**0.041**

p for the chi-square test using crosstabulation. ORs are odds ratios of each genotype as compared with homozygous for the major allele. Logistical regression was used to calculate ORs, CIs (95% confidence intervals of ORs), and corresponding p values (p_a), and all three values are presented after adjustment for gender, age, BMI, smoking status, hyperlipidemia history, hypertension history, and diabetic duration. [a]OR_a, CI_a, or p_a could not be acquired because allele frequencies were too small.

CAD in Caucasians ($p < 0.05$). Although SNP rs10305492 is not found in the Chinese Han population, SNPs rs10305492 and rs4714210 are in complete LD in Caucasians ($r^2 = 1$), indicating that our results are concordant with Scott's.

We acknowledge some limitations of this study. Sample size was relatively small, for in the cases were only 26 type 2 diabetes patients with one CAD vessel affected, 83 and 285 for two and three CAD vessels affected, respectively, so we did not stratify the cases and analyze the association with the number of affected vessels. And clinical features were not perfectly matched, and urine albumin creatinine ratio (ACR) was not collected, between the case and control groups. Both of them may introduce bias. Moreover, further functional studies on genetic variations at the *GLP-1R* locus would be beneficial. If our findings were confirmed through prospective studies, *GLP-1R* polymorphisms could be used as predictors of CAD risk in patients with T2DM in the Chinese Han population.

5. Conclusions

In T2DM patients from a Han Chinese population, some variations in the *GLP-1R* gene were associated with a lower risk of developing CAD.

Table 3: Distribution of SNP genotype frequencies in CAD-positive and control groups in recessive inheritance mode.

SNPs	CAD-pos. $n = 394$ (%)	Controls $n = 217$ (%)	OR	95% CI	p	OR_a	95% CI_a	p_a
rs761387								
CC	13 (3.3)	6 (2.8)	1.200	0.450–3.203	0.716	1.123	0.295–4.283	0.865
TX	381 (96.7)	211 (97.2)	1			1		
rs2268635								
AA	40 (10.2)	20 (9.2)	1.113	0.633–1.957	0.710	1.070	0.499–2.295	0.862
GX	354 (89.8)	197 (90.8)	1			1		
rs7769547								
AA	84 (21.3)	56 (25.8)	0.779	0.528–1.148	0.207	0.851	0.500–1.447	0.551
GX	310 (78.7)	161 (74.2)	1			1		
rs910162								
AA	83 (21.1)	55 (25.3)	0.786	0.532–1.161	0.226	0.847	0.497–1.446	0.544
TX	311 (78.9)	162 (74.7)	1			1		
rs3765468								
AA	14 (3.6)	6 (2.8)	1.296	0.491–3.421	0.600	1.249	0.333–4.682	0.742
GX	380 (96.4)	211 (97.2)	1			1		
rs3765467								
AA	20 (5.1)	9 (4.1)	1.236	0.553–2.764	0.605	2.662	0.588–12.04	0.204
GX	374 (94.9)	208 (95.9)	1			1		
rs3765466								
TT	123 (31.2)	74 (34.1)	0.877	0.617–1.248	0.466	1.130	0.691–1.848	0.627
AX	271 (68.8)	143 (65.9)	1			1		
rs10305456								
TT	1 (0.3)	1 (0.5)	0.550	0.034–8.831	1.000	/[a]	/	/
AX	393 (99.7)	216 (99.5)	1			1		
rs10305518								
GG	11 (2.8)	5 (2.3)	1.218	0.418–3.551	0.718	1.044	0.267–4.083	0.950
TX	383 (97.2)	212 (97.7)	1			1		
rs1820								
AA	4 (1)	0 (0)	0.990	0.980–1.000	0.303	/	/	0.999
TX	390 (99)	217 (100)	1			1		
rs4714210								
GG	28 (7.1)	32 (14.7)	0.442	0.258–0.757	**0.002**	0.440	0.225–0.863	**0.017**
AX	366 (92.9)	185 (85.3)				1		

CAD: coronary artery disease; OR: odds ratio; CI: confidence interval. OR_a, CI_a, and p_a represent OR, CI, and p after adjustment for gender, age, BMI, smoking status, hyperlipidemia history, hypertension history, and diabetic duration. OR, 95% CI, and p values were compared using chi-square analysis. OR_a, 95% CI_a, and p_a were assessed using multiple logistic regression analysis. [a]OR_a, CI_a, or p_a could not be acquired because allele frequencies were too small.

Ethical Approval

The study protocol and informed consent procedures were approved by the Research Ethics Committees of Peking University First Hospital (no. 2007-026).

Consent

Written informed consents were acquired from all subjects participating in this study, in agreement with the 1975 Helsinki Declaration.

Conflicts of Interest

We declare that we have no conflict of interest.

Authors' Contributions

Ran Lu analyzed data and wrote the manuscript. Xiaowei Ma edited the manuscript. Xiaowei Wei, Ge Bai, Jianwei Zhang, Ruifen Deng, Nan Gu, and Nan Feng analyzed data and contributed to the discussion. Xiaohui Guo reviewed the manuscript and contributed to the discussion. Xiaowei Ma accepts responsibility for the article.

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Supplementary Materials

Supplementary Material 1: distribution of SNP genotype frequencies in CAD-positive and control groups in dominant inheritance mode. Supplementary Material 2: association of common haplotypes with CAD risk. *(Supplementary Materials)*

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Research Article

Distribution of Microbes and Drug Susceptibility in Patients with Diabetic Foot Infections in Southwest China

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Objective. To investigate the microbial distribution and drug susceptibility among diabetic foot ulcers (DFUs) with different Wagner grades and between acute and chronic DFUs. *Methods*. We enrolled 428 DFU patients who were hospitalized and treated in the Southwest Hospital. We collected deep ulcer secretion for microbial culture and drug susceptibility tests and analyzed the results. We reexamined 67 patients with poor anti-infection efficacy and analyzed microbial species. *Results*: The 354 positive samples included 201 cases (56.8%) of single-pathogen infections and 153 cases (43.2%) of multiple-pathogen infections before antibiotic therapy. A total of 555 strains were cultivated, including 205 (36.9%) strains of gram-positive organisms (GPOs), 283 (51.0%) gram-negative bacilli (GNB), and 67 (12.1%) fungal strains. In terms of distribution, patients with different Wagner grades had different bacterial composition ratios ($P < 0.01$). Patients with Wagner grades 3–5 mainly had GNB. The specimens from chronic ulcer wounds were primarily GNB (54.2%), whereas fungi accounted for 14.4% of the infections; the distribution was significantly different from that of acute ulcers ($P < 0.01$). The susceptibility tests showed that the *Staphylococcus* genus was more susceptible to vancomycin, linezolid, and tigecycline. Tobramycin was the most effective drug (97%) for the treatment of *Escherichia coli*, followed by ertapenem (96.4%), imipenem (93.5%), and cefotetan (90%). Most of the remaining GNB were susceptible to antibiotics such as carbapenems, aminoglycosides, fluoroquinolones, ceftazidime, cefepime, and piperacillin-tazobactam (>63.2%). After antibiotic therapy, the positive rate of microbial culture was 52.2%, and the proportion of GNB and fungi increased to 68.9% and 20%. *Conclusion*. The distribution and types of bacteria in diabetic foot infection (DFI) patients varied with the different Wagner classification grades, courses of the ulcers, and antibiotic therapy. Multidrug resistance were increased, and the clinical treatment of DFIs should select the most suitable antibiotics based on the pathogen culture and drug susceptibility test results.

1. Introduction

Diabetes is prevalent worldwide. Diabetic foot disease is one of the most difficult to treat complications of diabetes and has become an important cause of nontraumatic amputation. The probability of diabetic patients suffering from diabetic foot ulcers (DFUs) during their lifetime can reach 25% [1], and the amputation rate for China's DFU patients is up to 21.5% [2]. The risk of a DFU complicated by a diabetic foot infection (DFI) is high [3]. DFIs not only extend the average length of the hospital stay, resulting in a huge economic burden [4] but also increase the risk of amputation [5], which seriously affects the quality of life and life expectancy of patients with diabetes. Control of DFIs in a timely and effectively manner has become an urgent problem for clinicians. Studies from different countries have revealed different DFI-related microbial compositions and drug susceptibilities [6–8], and the ratios of patients associated with multidrug resistance (MDR), methicillin-resistant *Staphylococcus* (MRS), and extended-spectrum β-lactamase (ESBL) bacterial infections have increased every year, suggesting that administration of empirical anti-infective regimens will increase the chances of treatment failure. China has a large population of DFI patients with a vast geographical distribution and significant variations in the types of bacterial infections found in DFI wounds from different regions. However, studies on this

aspect are rare. In this study, we aimed to retrospectively analyze the pathogen culture and drug susceptibility test results for DFI patients in southwest China to help clinicians choose a more appropriate standard antibiotic treatment for DFIs.

2. Patients and Methods

A total of 428 DFI patients who were hospitalized from January 2014 to June 2017 in the Diabetic Foot Center at the Southwest Hospital of the Third Military Medical University, which is a large tertiary grade A hospital in southwest China, were enrolled in this study. Diabetic foot secretion samples were collected for the microbial culture and drug susceptibility tests. Before DFI patients were treated with antibiotics, they should undergo debridement with normal saline. After removing surface carrion and exudate, deep ulcer secretion should be taken with sterile cotton swab, kept by sterile tube, and sent to microbiology lab of laboratory medicine quickly for anaerobic bacteria, aerobic bacteria, fungal culture, and drug susceptibility test. Bacterial drug resistance was determined based on the antimicrobial susceptibility test guidelines published by the Clinical and Laboratory Standards Institute (CLSI). MDR strains were determined according to the interim standard definition of acquired resistance published by Magiorakos et al. [9]. We also collected basic information, diabetes-related complications, and other information from the DFI patients. In some patients, there was no significant improvement or continuous aggravation in the wound after >7days of antibiotic therapy. The above method was used again to examine the deep ulcer secretion and to count the species of bacteria. The DFUs were graded using the Wagner classification system. The ulcer disease course was staged based on acute and chronic wound staging; chronic ulcers referred to those with no improvement after 4 weeks of treatment or those not cured within 8 weeks [10].

All data were analyzed using the SPSS 19.0 software. The measurement data were expressed as the mean ± standard deviation ($\bar{x} \pm s$) and analyzed using an independent t-test. The counting data were analyzed with the chi-square test with a significance level of $\alpha = 0.05$.

3. Results

3.1. Patient Information. In this study, we included the tissue culture results from a total of 428 DFI cases, including 273 (63.8%) male patients and 155 (36.2%) female patients. The patients were aged between 25 and 94 years, with an average age of 65.1 ± 11.9 years, and 292 (68.3%) patients were aged 60 years and older. The average length of hospital stay varied with the different Wagner grades and wound stages. The duration of hospitalization was significantly longer for patients with chronic ulcer wounds than for patients with acute ulcer wounds ($t = -2.917$, $P = 0.004$). There were 354 (82.7%) DFI patients with vascular disease, 346 (80.8%) with peripheral neuropathy, 180 (42.1%) with renal lesions, and 200 (46.7%) with retinopathy. Of the 388 patients, 90 (23.2%) had good glycemic control (HbA1c ≤ 7%), 59 (15.2%) had fair glycemic control, and 239 (62.6%) had poor glycemic control (Table 1).

Table 1: Clinical and demographical variables.

Parameter	Values	Values (range or n (%))
Gender	Male	273 (63.8)
	Female	155 (36.2)
Age	<40 years	4 (0.9)
	40–50 years	39 (9.1)
	50–60 years	93 (21.7)
	60–70 years	122 (28.5)
	>70 years	170 (39.8)
Hospital stays (days)	Wagner grade 1	11.5 ± 6.1
	Wagner grade 2	19.3 ± 14.9
	Wagner grade 3	22.1 ± 17.4
	Wagner grade4	21.0 ± 17.3
	Wagner grade 5	22.3 ± 13.4
	Duration of ulcer ≤ 4 weeks	17.6 ± 12.6
	Duration of ulcer > 4 weeks	21.8 ± 17.8
Complication	Vascular diseases	354 (82.7)
	Neuropathy	346 (80.8)
	Nephropathy	180 (42.1)
	Retinopathy	200 (46.7)
HbA1c (%)	≤7% (good control)	90 (23.2)
	7.1–8% (fair control)	59 (15.2)
	8.1–10% (poor control)	109 (28.1)
	>10% (very poor control)	130 (33.5)

3.2. Microbial Culture. Before antibiotic therapy, a total of 354 of the 428 samples applied for testing were positive cases, for a positive rate of 82.7%. The positive rate of Wagner grades 2–5 was significantly higher than that of Wagner grade 1 ($X^2 = 33.911$, $P \leq 0.001$). There were 201 cases (56.8%) with single-pathogen infections and 153 cases (43.2%) with multiple-pathogen infections (microbial strain numbers ≥ 2). Samples from Wagner grade 2-3 ulcers mainly had single-pathogen infections, whereas those from Wagner grade 4-5 ulcers mainly had multiple-pathogen infections; additionally, differences in the microbial distribution were observed between the different Wagner grades ($X^2 = 11.101$, $P = 0.025$). A total of 555 strains were cultivated, including 205 (36.9%) gram-positive organisms (GPOs), 283 (51.0%) gram-negative bacilli (GNB), and 67 (12.1%) fungal strains. Samples from Wagner grade 3–5 ulcers mainly had GNB, with differences in the microbial distribution between different Wagner grades ($X^2 = 25.278$, $P = 0.001$). *Staphylococcus aureus* was the most common GPO in the ulcers with different Wagner grades. Ulcers with Wagner grade 3 had the highest incidence rates of MDR, MRS, and ESBL at 32.4%, 47.1%, and 40%, respectively. The proportion of *Enterococcus* increased gradually with the higher Wagner grades. The most common gram-negative bacteria in Wagner

Table 2: The distribution of pathogenic bacteria was detected in DFI with different wagner grades.

	Before antibiotic therapy, *n* (%)						After antibiotic therapy, *n* (%)
	Wagner grading						
	1	2	3	4	5	Total	
Total samples	36 (8.4)	114 (26.6)	155 (36.2)	105 (24.5)	18 (4.3)	428	67
Positive samples	20 (55.6)	101 (88.6)	119 (76.8)	98 (93.3)	16 (88.9)	354 (82.7)	35 (52.2)
Total strains	28	156	176	165	30	555	45
Monomicrobial infection	17 (47.2)	59 (58.4)	73 (61.3)	46 (46.9)	7 (38.9)	201 (56.8)	24 (68.6)
Polymicrobial infection	4 (11.1)	42 (41.6)	46 (38.7)	52 (53.1)	9 (61.1)	153 (43.2)	11 (31.4)
MDR	7 (3.8)	50 (27.5)	59 (32.4)	58 (31.9)	8 (4.4)	182 (32.8)	20 (57.4)
Gram-positive bacteria	18 (64.3)	73 (46.8)	62 (35.2)	45 (27.3)	7 (23.3)	205 (36.9)	5 (11.1)
Staphylococcus aureus	11 (61.1)	29 (39.7)	29 (46.8)	15 (33.3)	1 (14.3)	85 (41.5)	4 (80.0)
Other *Staphylococcus*	6 (33.3)	26 (35.6)	7 (11.3)	9 (20.0)	1 (14.3)	49 (23.9)	1 (20.0)
MRSA	1 (5.9)	5 (29.4)	8 (47.1)	3 (17.6)	0 (0)	17	4
MRS	2 (6.5)	17 (54.8)	2 (6.5)	9 (29.0)	1 (3.2)	31	0
Streptococcus	1 (5.6)	6 (3.8)	13 (21.0)	6 (13.3)	3 (42.9)	29 (14.1)	0
Enterococcus	0 (0)	10 (8.2)	11 (17.7)	13 (28.9)	2 (28.6)	36 (17.6)	0
Gram-negative bacteria	9 (32.1)	68 (43.6)	91 (51.7)	96 (58.2)	19 (63.3)	283 (51.0)	31 (68.9)
Escherichia coli	1 (11.1)	4 (5.9)	17 (18.7)	10 (10.4)	1 (5.3)	33 (11.7)	6 (19.4)
Klebsiella	1 (11.1)	10 (14.7)	12 (13.2)	9 (9.4)	3 (15.8)	35 (12.4)	4 (12.9)
Product ESBL	2 (6.7)	3 (10.0)	12 (40.0)	11 (36.6)	2 (6.7)	30	5
Enterobacter cloacae	1 (11.1)	5 (7.4)	5 (5.5)	11 (11.5)	1 (5.3)	23 (8.1)	1 (3.2)
Proteusbacillus vulgaris	0 (0)	2 (2.9)	11 (12.1)	13 (13.5)	6 (31.6)	32 (11.3)	5 (16.1)
Citrobacter amalonaticus	0 (0)	2 (2.9)	6 (6.6)	3 (3.1)	1 (5.3)	12 (4.2)	0
Serratia marcescens	1 (22.2)	2 (2.9)	5 (5.5)	10 (10.4)	1 (5.3)	19 (6.7)	1 (3.2)
Pseudomonas aeruginosa	2 (22.2)	8 (11.8)	9 (9.9)	17 (17.7)	3 (15.8)	39 (13.8)	6 (19.4)
Acinetobacter baumannii	1 (11.1)	5 (7.4)	3 (3.3)	6 (6.3)	0 (0)	15 (5.3)	1 (3.2)
Morganella	1 (11.1)	6 (8.8)	12 (13.2)	7 (7.3)	0 (0)	26 (9.2)	2 (6.4)
Fungus	1 (3.6)	15 (9.6)	23 (13.1)	24 (14.5)	4 (13.3)	67 (12.1)	9 (20.0)

grade 2–5 ulcers were *Klebsiella* (14.7%), *Escherichia coli* (18.7%), *Pseudomonas aeruginosa* (17.7%), and *Proteus* (31.6%) (Table 2).

The positive rates for the GPO or GNB culture results in the acute ulcer wounds were both 45.2%. *Staphylococcus aureus* was still the most common GPO (46.6%), and the GNB mainly consisted of *Escherichia coli* (12.6%). The specimens from chronic ulcer wounds mainly had GNB (54.2%), and fungi accounted for 14.4% of the infections. Comparing the acute ulcer wounds with the chronic ulcer wounds showed significant differences in the microbial composition ($X^2 = 184.449$, $P \leq 0.001$), with *Pseudomonas aeruginosa* (16.6%) the most common GNB (Figure 1).

We reexamined the deep ulcer secretions of 67 patients with poor anti-infection efficacy (>7 days). 35 specimens training result was positive, positive rate of 52.2%. A total of 45 strains were cultured, mainly with monomicrobial infection (68.4%), among which 11.1%, 68.9%, and 20% were gram-positive coccus, gram-negative bacilli and fungus, respectively. *Escherichia coli* (19.4%), *Pseudomonas aeruginosa* (19.4%), and *Proteusbacillus vulgaris* (16.1%) were the main gram-negative bacilli (Table 2).

3.3. Drug Susceptibility Test. The *Staphylococcus* genus was more susceptible to vancomycin, linezolid, and tigecycline, with only 1 case of vancomycin-resistant *Staphylococcus epidermidis*. A total of 48 MRS strains of the *Staphylococcus* genus were identified from the culture and drug susceptibility tests. The MRS strains were more susceptible to linezolid (100%), tigecycline (100%), and vancomycin (97.9%), followed by moxifloxacin (79.2%), and showed poor susceptibility to clindamycin (8.3%) and erythromycin (10.4%) (Figure 2). There was 1 case of vancomycin-resistant *Enterococcus faecalis* among the *Enterococcus* strains, and *Enterococcus faecalis* was most susceptible to tigecycline (100%) and ampicillin (100%), followed by vancomycin (96.6%), penicillin G (96.6%), and linezolid (86.2%). The susceptibilities of *Enterococcus faecium* to vancomycin, linezolid, and tigecycline were all 100% (Table 3).

Tobramycin was the most effective drug (97%) for the treatment of *Escherichia coli*, followed by ertapenem (96.4%), imipenem (93.5%), and cefotetan (90%), but *Escherichia coli* had poor susceptibility to amikacin, gentamicin, and levofloxacin (all <50%). Most of the remaining GNB were susceptible to antibiotics such as carbapenems,

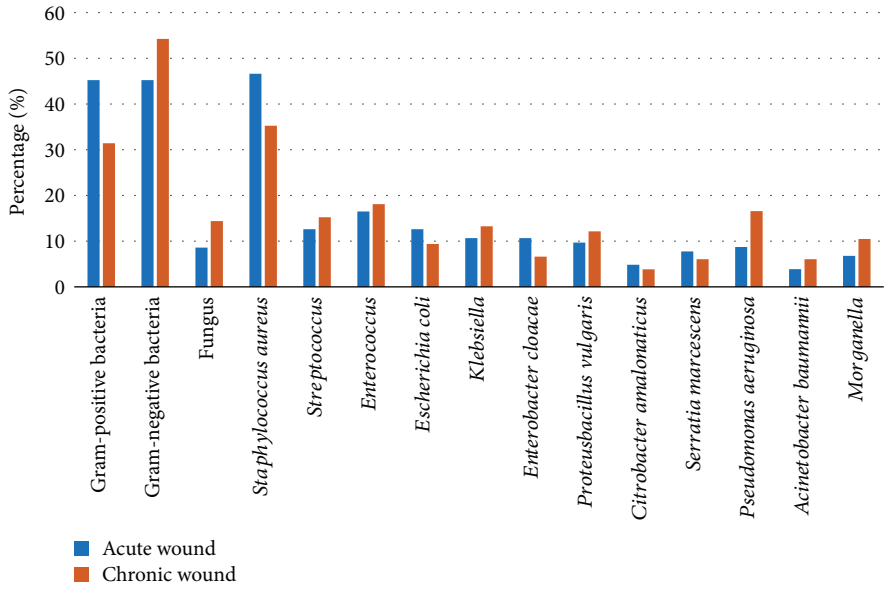

Figure 1: The distribution of pathogenic bacteria was detected in DFI with different duration of ulcer.

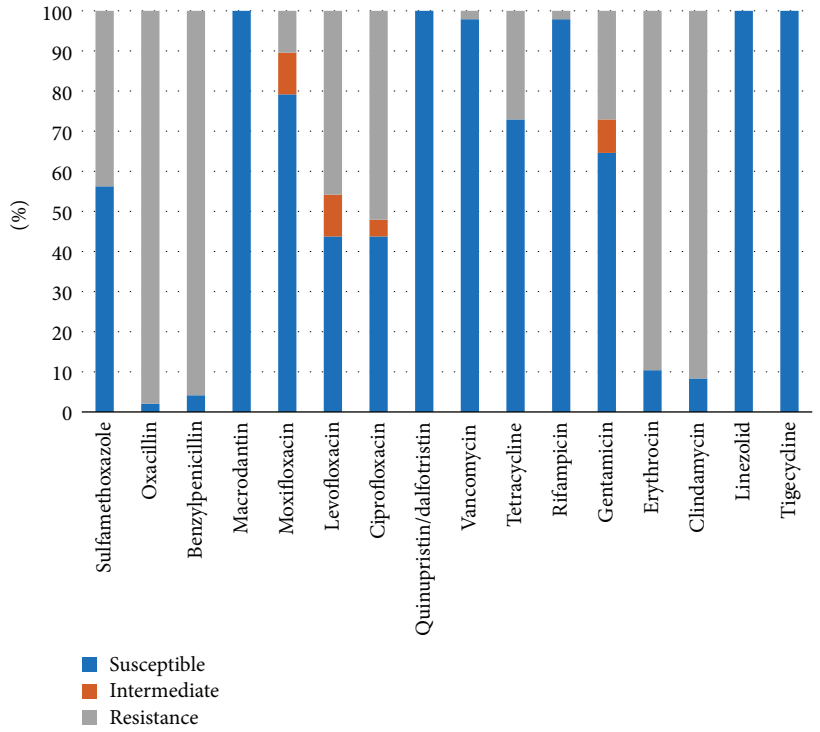

Figure 2: The susceptible pattern of MRS from diabetic foot patients.

aminoglycosides, fluoroquinolones, ceftazidime, cefepime, and piperacillin-tazobactam (>63.2%) (Table 4). In addition, this study cultured 30 ESBL strains, which showed high susceptibilities (100%) to imipenem and ertapenem, followed by amikacin (90%), cefotetan (83.3%), and piperacillin-tazobactam (76.7%), and were less susceptible to levofloxacin (36.7%) and ciprofloxacin (26.7%) (Figure 3).

The proportion of fungi was only 12.1%. The proportion of fungi was increased in Wagner grade 3–5 ulcers, but the difference was not significant ($X^2 = 3.954$, $P = 0.412$). The susceptibilities of the fungi to voriconazole, amphotericin B, itraconazole, 5-fluorocytidine, and fluconazole were 98.3%, 100%, 62.1%, 84.7%, and 96.8%, respectively (Figure 4).

4. Discussion

Consistent with most studies [11, 12], the DFI patients were often elderly males with multiple complications, possibly

Table 3: The susceptible pattern of gram-positive bacteria from diabetic foot patients.

	S. aureus	*S. haemolyticus*	*S. epidermidis*	*Enterococcus faecalis*	*Enterococcs faecium*	*Streptococcus*
Total strains	85	14	13	29	5	31
Sulfamethoxazole (%)	81.2	21.4	38.5	—	—	—
Oxacillin (%)	78.9	0	23.1	—	—	—
Ampicillin (%)	—	—	—	100	20	—
Benzylpenicillin (%)	5.9	0	0	96.2	—	—
Macrodantin (%)	98.8	100	100	88.9	0	—
Moxifloxacin (%)	95.3	57.1	84.6	69.6	0	—
Levofloxacin (%)	82.4	0	38.5	69.2	20	80.6
Ciprofloxacin (%)	81.2	0	23.1	57.1	0	—
Quinupristin/dalfotristin (%)	100	100	100	5	100	—
Vancomycin (%)	100	100	92.3	96.6	100	100
Tetracycline (%)	75.3	78.6	46.2	10.7	40	—
Rifampicin (%)	98.8	100	100	—	—	—
Gentamicin (%)	82.4	28.6	61.5	—	—	—
Erythrocin (%)	48.2	7.1	23.1	—	20	21
Clindamycin (%)	60	20	15.4	5.6	0	25.8
Linezolid (%)	100	100	100	86.2	100	100
Tigecycline (%)	100	100	100	100	100	—

Table 4: The susceptible pattern of gram-negative bacteria from diabetic foot patients.

	E. coli	*Serratia*	*Klebsiella*	*Enterobacter*	Proteusbacillus	*Pseudomonas*	*baumannii*	*Morganella*
Macrodantin (%)	79.3	10	35.1	55.6	—	2.6	14.3	0
Ampicillin (%)	21.9	—	5.4	0	55.6	0	0	0
Ampicillin/sulbactam (%)	25.8	—	48.6	0	73.7	5.9	50	0
Piperacillin-tazobactam (%)	87.9	100	94.6	84.2	100	74.4	80	96
Sulfamethoxazole (%)	31.3	94.7	67.6	61.9	52.6	5.3	64.3	40
Cefazolin (%)	22.2	5.3	27	18.8	—	0	0	3.8
Cefoxitin (%)	—	—	—	—	100	—	—	—
Ceftriaxone (%)	32.3	100	70.3	71.4	—	0	0	76.9
Ceftazidime (%)	62.5	100	89.2	81	100	87.2	66.7	88.5
Cefotetan (%)	90	—	94.6	9.1	—	0	0	100
Cefepime (%)	66.7	94.7	94.6	90.5	73.7	89.7	71.4	90.5
Levofloxacin (%)	39.4	94.7	83.8	75	73.7	89.5	73.3	92.3
Ciprofloxacin (%)	30.3	94.7	81.1	70	57.9	87.2	73.3	65.4
Amikacin (%)	46.9	78.9	75.7	76.2	63.2	87.2	93.3	53.8
Tobramycin (%)	97	100	94.6	93.3	88.9	89.5	—	88.5
Gentamicin (%)	46.9	94.7	78.4	95.2	68.4	84.6	93.3	53.8
Meropenem (%)	—	—	—	—	100	94.3	—	100
Imipenem (%)	93.5	89.5	100	100	68.4	82.1	73.3	—
Ertapenem (%)	96.4	100	100	94.7	100	—	—	100
Aztreonam (%)	48.5	84.2	83.8	76.2	84.2	—	22.2	100

due to the burdens of life and exercise habits. Most of the patients had poor glycemic control. The patients with Wagner grades 2–5 had significantly longer hospital stays than did the grade 1 patients, and patients with chronic DFUs had longer hospital stays than those with acute DFUs ($t = -2.704$, $P < 0.05$).

Our results indicated that the DFI-causing bacteria were dominated by GNB (51%), which differed from the results of the survey performed in southern China from 2009 to 2014 [13] in which GPOs accounted for 54% of the infections. This finding suggests that different regions may have different dominant DFI pathogens or that

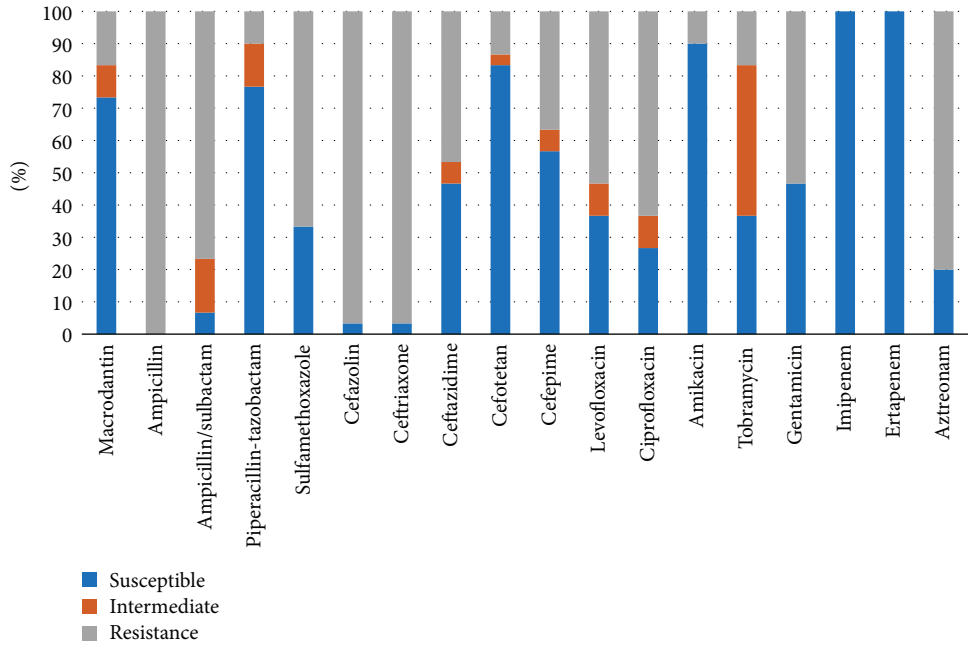

Figure 3: The susceptible pattern of product ESBL bacteria from diabetic foot patients.

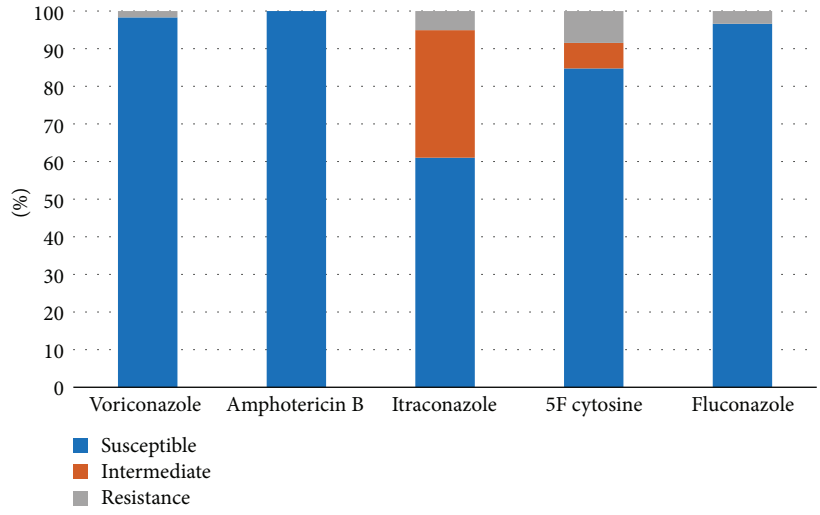

Figure 4: The susceptible pattern of Fungus from diabetic foot patients.

GNB may have replaced GPOs as the main pathogens in Chinese DFI patients. We also noted that different Wagner grades and changes in the ulcer course led to different bacterial distributions and types. Patients with Wagner grades 1-2 mostly had GPOs, whereas those with grades 3–5 mostly had GNB; additionally, acute wounds had similar ratios of GPOs and GNB, but chronic infections mostly had GNB. After antibiotic therapy, the positive rate of bacterial culture decreased significantly, and the proportion of GNB and fungi increased significantly. Studies have shown that GNB infections are positively correlated with amputation and negatively correlated with DFU healing [14], suggesting that GNB infections are a serious DFI warning. A study from Pakistan from 2013-2014 [15]showed that mixed infections accounted for the majority (56.9%), whereas the results of our study showed predominantly single-pathogen infections (56.8%). Further analysis showed that patients with Wagner grades 1–3 mainly had single-pathogen infections, whereas those with Wagner grades 4-5 mainly had multiple-pathogen infections, indicating that multiple-pathogen infections were also a sign of severe DFIs.

Staphylococcus aureus is the most common GPO. However, the percentage of *Staphylococcus aureus* decreased with the increased Wagner level and prolonged duration of the ulcer, whereas the proportion of *Enterococcus* gradually increased. Agudelo Higuita and Huycke indicated that enterococci often appeared in patients with low immunity [16] and could participate in the formation of biofilms [17].

Biofilms can act as a virulence factor to cause treatment failure, suggesting that *Enterococcus* infection should be taken seriously. Studies from Mexico [18] showed that the resistance rate of *Staphylococcus aureus* to vancomycin was as high as 49%. Our results suggested that most GPOs, including *Staphylococcus aureus*, were susceptible to vancomycin, linezolid, and tigecycline and were resistant to penicillin G, erythromycin, and clindamycin. These findings were different from the observations from Bravo-Molina et al. [19], which showed that fluoroquinolone antibiotics were the most susceptible antibiotics for GPOs. In this study, only *Staphylococcus aureus* showed good susceptibility to fluoroquinolone antibiotics (>80%), whereas the other GPOs showed low susceptibility.

In 1961, the first methicillin-resistant *Staphylococcus aureus* (MRSA) was found in the UK [20]. Today, various MDR strains have become epidemic strains worldwide. We identified 182 (32.8%) MDR strains from the 558 strains, including 51.1% of the GNB, which slightly differed from the results of a study from Tianjin, China [21]. We cultured 31 MRS strains, of which 17 were MRSA strains, accounting for 20% of the *Staphylococcus aureus* strains. Our result is consistent with the results from studies in Pakistan [15] and differs from the 78% of strains found by a study in Iran [22]. The MRS susceptibility test showed that the MRS strains were still highly susceptible to vancomycin, linezolid, and tigecycline but showed significantly reduced susceptibility to levofloxacin and ciprofloxacin, which are frequently used in clinical practices. This finding suggests that the occurrence of MRS will increase the risk of anti-infective treatment failure. Long-term (over 6 months) use of antibiotics, a long ulcer course, high blood pressure, anemia, and chronic osteomyelitis are all MRSA risk factors [23]. Clinicians should be alerted to the possibility of associated MRSA infections in patients with the above conditions and can select antibiotics capable of treating this type of pathogen. In addition, studies have shown that *Staphylococcus aureus* infection is more likely to cause the formation of bacterial biofilms in diabetic foot wounds [24], which reduces the susceptibility of bacteria to antibiotics. Genetic testing can determine whether *Staphylococcus aureus* is invasive [25]. Therefore, methods such as biofilm detection and genetic testing may be used as a new means of detection in the future to improve DFI assessment in clinical practice.

In contrast to the results of Gadepalli et al. [11], *Pseudomonas aeruginosa* accounted for the highest percentage of the GNB, followed by *Klebsiella* and *Escherichia coli*. Further analysis showed that the most common GNB types among patients with chronic wounds were *Pseudomonas aeruginosa*, *Klebsiella*, and *Proteus*, which accounted for 16.6%, 13.3%, and 12.2%, respectively; these results were similar to the findings of de Vries et al. [14]. The Enterobacteriaceae family showed the highest susceptibility to ertapenem and imipenem. Sugandhi and Prasanth [26] suggested that amikacin was the most susceptible antibiotic for the treatment of GNB. However, we found that the susceptibility of *Escherichia coli* to amikacin was only 46.9%. The susceptibilities of *Escherichia coli* to the commonly used levofloxacin and ciprofloxacin were also low (<40%), whereas the susceptibility of this bacterium to piperacillin-tazobactam, cefotetine, and tobramycin was higher (>87.9%). In contrast, *Klebsiella* was more susceptible to fluoroquinolones (>81.1%). Partially consistent with the results of studies on *Pseudomonas aeruginosa* from Pakistan [27], we found that this bacterium was less susceptible to ampicillin and was more susceptible to quinolone antibiotics. In China, *Pseudomonas aeruginosa* is fairly susceptible to aminoglycoside antibiotics (>84.6%), possibly because these antibiotics are less frequently used in China at present.

In this study, we found 30 strains of ESBL-producing *Enterobacter*, which accounted for 10.6% of the GNB. Most of these strains were derived from *Escherichia coli* and were resistant to most antibiotics; these strains showed the highest susceptibility to carbapenems (100%), followed by amikacin (90%), cefotetan (83.3%), and piperacillin-tazobactam (76.7%), and lower susceptibility to fluoroquinolones (<36.7%), which was consistent with the results from Bangladesh and Nepal [28, 29]. This study did not find carbapenem-resistant ESBL-producing bacteria. ESBL-producing bacterial infections increase the hospitalization rate of DFI patients and further reduce the choice of antibiotics [30]. For example, fluoroquinolone antibiotics, which are frequently used in our hospital, show a significantly reduced susceptibility to ESBL-producing bacteria, suggesting that we should perform drug susceptibility testing to select susceptible antibiotics for treatment.

In summary, different Wagner grades and changes in the course of ulcers led to different distributions of bacteria and different bacterial species. Wagner grades 4-5 and chronic ulcer wounds had high ratios of GNB infections and mixed infections. For patients at risk of infections with MDR, MRS, and ESBL-producing bacteria, clinicians should focus on the use of antibiotics for the treatment of these types of bacteria when conducting empiric therapy and should adjust the drugs according to the results of the drug susceptibility test and clinical treatment efficacy. In addition to actively applying appropriate antibiotic treatment, multidisciplinary management combined with foot pressure reduction, timely debridement, and lower extremity vascular intervention should be applied to increase the success rate of anti-infection treatment and to reduce the amputation rate.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Mingxia Wu and Hang Pan contributed to this work.

Acknowledgments

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Research Article

Renal Ischemia-Reperfusion Injury in a Diabetic Monkey Model and Therapeutic Testing of Human Bone Marrow-Derived Mesenchymal Stem Cells

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Clinically, acute kidney injury (AKI) episodes in diabetes mellitus (DM) patients are associated with a cumulative risk of developing end-stage renal disease. In this study, we asked whether the severity of AKI induced by renal ischemia-reperfusion injury (IRI) is more prominent in DM than in non-DM control using a cynomolgus monkey (*Macaca fascicularis)* model. We also investigated whether human bone marrow-derived mesenchymal stem cells (hBM-MSCs) infused via the renal artery could ameliorate renal IRI in DM monkeys. The experimental data, including mortality rate, histologic findings, and urinary albumin secretion indicate that the severity of AKI was greater in DM monkeys than in control animals. Moreover, histological findings and qRT-PCR analysis of *Ngal* mRNA in renal biopsy tissue showed that hBM-MSC promoted the recovery of tubular damage caused by AKI. Serum analysis also revealed that the level of albumin and ALT was increased 24 and 48 hours after AKI, respectively, suggesting that AKI induced acute liver injury. We suggest that this nonhuman primate model could provide essential information about the renal and nonrenal impairment related to DM and help determine the clinical usefulness of MSCs in AKI.

1. Introduction

Prolonged hyperglycemia causes various renal stress responses, and it is well reported that diabetes is the single largest contributor to the growing prevalence of chronic kidney disease (CKD) [1, 2]. On the other hand, acute kidney injury (AKI) is a disorder characterized by a rapid decrease in renal function, and it is understood to be associated with renal and systemic inflammation [3]. Although diabetes mellitus (DM) and AKI are separate diseases with distinct pathophysiologies, recent studies have demonstrated their interdependent relationship [4, 5]. Indeed, episodes of AKI in diabetic patients are associated with a cumulative risk of developing end-stage CKD [6]. Specifically, a meta-analysis showed that preadmission DM status, arterial hypertension, and proteinuria are independent AKI risk factors [7]. Also, preclinical studies showed that diabetes can increase the susceptibility of AKI [8–11].

During the last several decades, mesenchymal stem cells (MSCs) have emerged as an innovative tool for tissue regeneration therapy, mainly due to its unique secretome profile and capacity for self-renewal and differentiation [12]. Their reparative ability on damaged tissue are mainly attributed to paracrine effects, transdifferentiation, and the release of

extracellular vesicles [13], and numerous evidence in rodent models have revealed the therapeutic potential of human MSCs in various models of tissue repair including AKI [14].

Many studies have shown that inflammatory responses, including those involved in AKI, differ between humans and mice [15–17], and a recent finding demonstrated that the renal structure also differs between humans and mice [18]. Thus, using animal models other than rodents could better translate preclinical findings into the human setting. In this study, we developed a nonhuman primate model of renal ischemia-reperfusion injury (IRI), which is the most common cause of AKI in human [19]. We used this model to confirm whether diabetes can increase the susceptibility to AKI and also to evaluate the efficacy of human bone marrow-derived MSCs (hBM-MSCs) in reducing renal IRI.

2. Materials and Methods

2.1. Animals. Male, 5- to 8-year old cynomolgus monkeys (*Macaca fascicularis*) weighing between 2.8 and 5 kg were used in this study. All animals were originated from Cambodia. The animal procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* [20] and the Animal Welfare Act [21] in the animal facility of the Primate Organ Transplantation Research Center at Genia (Sung-nam City, Korea). Animals were individually housed indoors on a 12 : 12 h, light–dark cycle and were fed standard macaque biscuits (Certified Primate Diet 5048*, LabDiet, St. Louis, MO, USA) and fresh fruits twice daily. Animal rooms were maintained at 23 to 25°C and 40% to 60% relative humidity, with 15 changes of conditioned air hourly. Chlorinated, filtered fresh water was provided without restriction. Tuberculosis was tested every six months with immunochromatographic test kit (SD Bioline TB Ag MPT64 RAPID, Standard Diagnostics, Yongin-si, South Korea), and all were negative during the study period. Tests on herpes B virus, simian T-cell leukemia virus, simian retrovirus, simian immunodeficiency virus, measles, cynomolgus cytomegalovirus, and simian varicella virus were conducted at a diagnostic laboratory (Zoologix Inc., CA), and all were diagnosed negative. This animal study was approved by the Institutional Animal Care and Use Committee of Orient Bio Laboratories (ORIENT–IACUC–16317).

2.2. Induction and Management of Diabetes Mellitus. DM was induced by subtotal pancreatectomy and a 60 mg/kg injection of streptozotocin (STZ, Sigma, St. Louis, MO, USA). Subjects did not receive food or liquid for 12 h prior to surgery. After intramuscular injection of 10 mg/kg ketamine, subjects were intubated with 4.0 to 4.5 Fr endotracheal tubes, and general anesthesia was induced with 3–5% isoflurane. Subjects were kept under anesthesia with 1-2% isoflurane, nitrous oxide, and oxygen. Prophylactic antibiotics (20 mg/kg cefazolin sodium intravenously) were given at the time of skin incision. STZ was given intravenously immediately after the pancreatectomy.

Type 1 DM (T1DM) was diagnosed upon satisfaction of all of the following criteria: (1) sustained hyperglycemia (blood glucose level > 250 mg/dl), (2) fasting NHP C-peptide level below 0.5 ng/ml or less than one-third of preinduction levels, and (3) absence of stimulated C-peptide response with intravenous glucose tolerance test (IVGTT). We measured serum C-peptide using a radioimmunoassay kit developed for human plasma (C-peptide IRMA kit; IMMUNOTECH, Beckman Coulter Inc., Prague, Czech Republic), which shows 90% cross-reactivity with cynomolgus monkeys. IVGTT was measured after 12 hours of fasting. After sedation with ketamine, three blood samples were drawn for C-peptide and blood glucose measurements. Then 0.5 g/kg dextrose was given intravenously, and blood samples were drawn 1, 3, 5, 7, and 10 min thereafter. Blood samples were drawn at 15, 20, 25, 30, and 60 min to measure the glucose disappearance rate. Acute C-peptide response (ACR) was calculated as the difference between mean C-peptide after glucose infusion and C-peptide at baseline. The glucose disappearance rate (K_G, %/min) was calculated as the slope of the decline in the log-transformed value of blood glucose between 10 and 30 min.

After successful induction of T1DM, blood glucose levels were checked 3 to 4 times daily and maintained between 200 and 300 mg/dl with insulin (glargine (Lantus®, Sanofi-Aventis, Bridgewater, NJ, USA) and glulisine (Apidra®, Sanofi-Aventis)). Body weight measurements and physical examinations were performed at regular intervals. The following blood hematological parameters were also checked regularly: white blood cell count (WBC) with differential count, hemoglobin (Hb), hematocrit and platelet count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, blood urea nitrogen (BUN), creatinine, albumin, globulin, sodium, potassium, chloride, calcium, inorganic phosphorus, cholesterol, triglyceride, amylase, and C-reactive protein (CRP). The fasting glucose levels of non-DM monkeys were less than 100 mg/dl in all cases and those of DM monkeys were more than 250 mg/dl in all cases at least for 5 months prior to this experiment.

2.3. Human Bone Marrow-Derived Mesenchymal Stem Cell Preparation. HBM-MSCs were obtained from the Catholic Institute of Cell Therapy (CIC; Seoul, Korea). Human bone marrow aspirates were obtained from the iliac crest of healthy donors aged 20 to 55 years after approval by the Institutional Review Board of Seoul St. Mary's Hospital. Bone marrow aspirates were obtained from healthy donors after written informed consent and sent to the Good Manufacturing Practice-compliant facility of the CIC. The detailed procedures for the isolation, expansion, and quality control of MSCs, including differentiation potential (adipogenic, chondrogenic, and osteogenic differentiation) and cell surface marker analyses, are described previously [22].

2.4. Renal Ischemia-Reperfusion Injury Model and Experimental Design. We allocated eight monkeys into three groups ($N = 3, 3,$ and 2 in Groups 1, 2, and 3, resp.). Three normal animals (Group 1) and five DM animals were subjected to renal IRI for one hour, and 2 of the latter 5 received 5×10^6 cell/kg of hBM-MSCs by injection (Group 3). To induce IRI, the monkeys were anesthetized, and midline incisions were made. The renal pedicles were exposed and

subsequently clamped with bulldog clamps for one hour (Supplementary Figure 1). In Group 3, 5×10^6 cell/kg of hBM-MSCs was injected into the suprarenal aorta after reperfusion while the aorta was clamped just below the renal arteries to allow blood flow only to the kidneys. Blood and urine samples were obtained, and a kidney gun biopsy from each kidney was performed every 24 hours after reperfusion.

2.5. Assessment of Renal Injury. We performed serum biochemical analysis of Cr, BUN, CRP, and total protein levels (FUJI DRI-CHEM 4000i, Tokyo, Japan). The concentration of interferon gamma (IFN-γ) and tumor growth factor alpha (TGF-α) in serum were detected using Luminex assay kits (EMD Millipore, Billerica, MA) and then normalized against the amount of urine creatinine. Albumin secretion in urine was measured by monkey albumin ELISA kit (Abcam, Cambridge, UK). The concentration of urinary neutrophil gelatinase associated lipocalin (NGAL) at each designated time point was analyzed using a monkey NGAL ELISA kit (Bioporto, Hellerup, Denmark).

At designated time points after renal IRI (0, 24 and 48 hours), renal biopsy slices were obtained from the right kidney, fixed in 10% formalin, and embedded in paraffin wax. Tissue sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and examined under a light microscope to discern any histological changes. To morphologically assess the extent of tubular injury and necrosis, H&E stained sections were reviewed by a renal pathologist who was blinded to the experimental groups. The proportion of areas with tubular injury or necrosis to areas with relatively intact tubules was visually estimated. Tubular injury was identified in areas with cytoplasmic vacuolization, loss of brush border, flattening of epithelium, or evidence of tubular necrosis as manifested by granular casts and necrotic debris.

Total RNA was isolated using the TRIzol® method from snap-frozen renal biopsy tissues collected from the left kidney (Sigma Aldrich, St. Louis, MO). cDNA synthesis and quantitative real time reverse transcription PCR (qRT-PCR) were conducted as previously described [23]. Primer sequences were as follows: *Gapdh*, forward: 5′-CGGAGCTCTCCAGA ACATCA-3′, reverse: 5′-ggtcaggtccaccactgaca-3′; *Ngal*, forward: 5′-ctgtcagggaatgcagttgg-3′, reverse: 5′-caggatggaggtcacgttgt-3′.

2.6. Statistical Analysis. Differences among the three groups at specific time points were analyzed using the Kruskal-Wallis test. Repeated ANOVA was applied to repeated measurements of parameters. The statistical significance between two groups was determined using two-tailed unpaired *t*-tests (tubular injury extent between non-DM and DM animals and qRT-PCR of Ngal mRNA). P values < 0.05 were considered significant and were corrected using Bonferroni's method in post hoc analyses. Statistical analyses were performed using SPSS version 22.0 (IBM, Armonk, NY, USA). The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Table 1: Allocation of animals and survival rate in each group.

Group	Without (−) or with (+) DM	MSC treatment	Number of animals	Hours survived by each animal
1	(−)	(−)	3	>96, >96, >96
2	(+)	(−)	3	60, 53, 49
3	(+)	(+)	2	72, 53

3. Results and Discussion

In this study, we investigated whether diabetes aggravates the severity of renal IRI-induced AKI in cynomolgus monkeys and assessed the therapeutic potential of intra-arterially injected hBM-MSCs. Although renal IRI models normally require a pilot study to determine the ischemia duration [24], we used a one-hour bilateral warm ischemic injury model based on the results of our pilot studies for renal transplantation in monkeys (data not included). The number of MSCs for intra-arterial injection was based on the previous study that had assessed the effect of MSCs in an interstitial fibrosis model using rhesus monkeys [25]. To ensure successful modeling, we monitored changes in the kidneys during the ischemia period (Supplementary Figure 1). Table 1 shows that this protocol was tolerable in normal monkeys but not in DM monkeys; all the non-DM monkeys (Group 1) survived until day 4 after AKI, whereas the DM animals (Group 2) died within 60 hours. Of the two DM monkeys that received hBM-MSCs (Group 3), one monkey died after 72 hours and the other 53 hours. In Groups 2 and 3, the monkeys showed respiratory distress symptoms before they died. When they were examined by autopsy, pleural effusion was detected in common, while their kidneys were not ischemic in gross appearance. Microscopically, we found a large extent of necrosis and fluid retention in the tubules, while liver tissues showed hepatocyte swelling and fatty change. Also, alveolar capillary interstitial edema was detected in the lung (Supplementary Figures 2 and 3). From these results, we suspected that fluid overloading due to severe AKI as well as multiorgan (i.e., the lung and liver) damage secondary to AKI may have contributed to early death in DM animals. In group 3, we could not see any clinical evidence of rejection response against hBM-MSCs such as hypotension or fever immediate after injection.

Figure 1(a) shows that the BUN level of the control animals peaked at 24 hours and then decreased thereafter, whereas that of the DM monkeys kept increasing for 48 hours. MSC treatment of the DM animals led to a partial decrease at 48 hours. The serum creatinine level tended to increase more abruptly in the DM animals than in normal animals, while MSC treatment partly contributed to lowering its level in DM monkeys (Figure 1(b)). Thus, our data indicate that DM compromised the monkey's ability to recover from AKI, and that the effect of MSCs on AKI recovery was not clear based on the results of the renal function marker during the study period.

To further investigate the effect of DM on AKI progression, a renal pathologist who was blinded to the experimental groups conducted histological analyses on renal biopsies

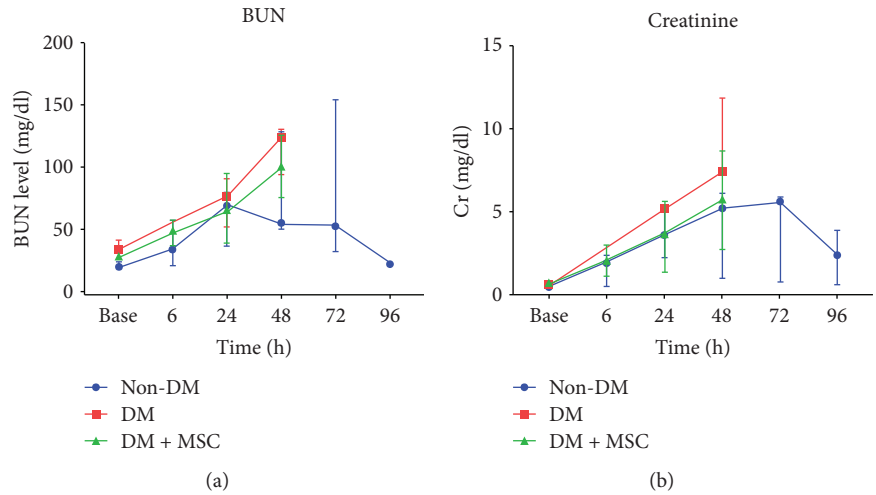

Figure 1: The survival rate and changes in renal function markers. DM monkeys that experienced renal ischemia-reperfusion injury (IRI) were subsequently injected with MSC (DM + MSC) or not (DM). Non-DM animals were used as the control. The serum levels of creatinine (a) and blood urea nitrogen (BUN) (b) were measured at designated study points. Data are median with range. $n = 3, 3$, and 2 in the non-DM, DM, and DM + MSC groups, respectively.

from the animals. As shown in Figure 2(a), a diverse pattern of tubular injury, such as cytoplasmic vacuolization, loss of brush border, flattening of epithelium, and luminal cell debris, was seen after IRI. Histologic findings showed preexisting tubular injury before IRI in DM animals ($P < 0.001$, Figure 2(b)), while the degree of tubular injury at 24 and 48 hours in DM animals was comparable to those of non-DM monkeys after ischemic insult (Figures 2(a) and 2(b)). The coagulation necrosis of renal tubules and prominent hyaline casts was also more evident postischemia in the DM group compared to the control group (Figure 2(c)). In DM animals treated with MSCs, the gross morphologies at 48 hours appeared to resolve better than those seen in the noninjected DM animals (Figure 2(a)). Similarly, among DM animals, a reduction in tubular necrosis was found in the MSC-treated group at 24 ($P < 0.05$) and 48 hours ($P < 0.001$, Figure 2(c)). Thus, hBM-MSC treatment apparently led to tubular regeneration within 24–48 hours, and those changes might have preceded functional recovery as shown by the serum markers (Figures 1(a) and 1(b)).

The cytokine analysis shows that the serum concentrations of IFN-γ and TNF-α, which are known mediators of AKI progression [26, 27], were not different among groups at 24 hours. Unlike the tissue recovery by MSC treatment at 48 hours, their level seems to be even higher than those from nontreated animals. These finding possibly suggests that the utilization of these two early inflammatory markers may not be an ideal option for evaluating early progress of AKI in monkeys. (Figure 3(a)). Urine AKI markers were also analyzed and found a significant increase of albumin at 24 and 48 hours in DM compared with non-DM monkeys ($P < 0.05$, Figure 3(b)). We also found out that the level of urinary NGAL protein, which is also an AKI marker, was similarly detected among groups at 24 and 48 hours (Figure 3(b)). However, qRT-PCR showed that the expression of *Ngal* mRNA was significantly higher in the renal tissue of DM animals 24 hours after IRI compared with non-DM monkeys, and hBM-MSC treatment decreased its expression to a level comparable to that seen in the non-DM control ($P < 0.05$). We also measured the level of GST-alpha and TIMP-1, which are potential AKI biomarkers in urine [28, 29], and found out that the increase of GST-alpha was more evident in DM animals than in non-DM animals at 24 and 48 hours. A similar pattern was observed in the TIMP-1 level only at 48 hours. From these three urinary proteins, we could not observe their remarkable changes after MSC treatment. Based on these findings, we suggest that DM animals tend to be more susceptible to early ischemic injury as shown by urinary AKI marker secretion, and that MSC treatment was not effective in renal recovery at least during 48 hours after AKI.

Recently, the mechanism by which AKI triggers more severe diseases in DM has been studied in rodent models. Consistent with our results, Gao et al. [9] demonstrated that the AKI sensitivity of diabetic mice could be suppressed using a TNF-α neutralizing antibody, thereby supporting the role of the TNF-α-related inflammatory response in AKI sensitivity. Another study suggested that renal failure manifested by chronic inflammation and vasculopathy could be accelerated by postischemic inflammation in obese-diabetic rats [8].

The serum levels of albumin and total protein declined as AKI progressed, and a significant decrease in albumin was found in DM animals at 48 hours, compared with non-DM monkeys (Figure 4(a), $P < 0.05$). A similar pattern of downshift was detected in the total protein level, though that difference was not statistically significant (Figure 4(b)). These results indirectly suggest that AKI might have caused an injury to the livers of DM animals, because the liver is the major organ that synthesizes and secretes albumin and immunoglobulins [30]. In line with this, the ALT serum level was significantly higher in the DM animals than in the DM + MSC and non-DM animals at 24 hours (Figure 4(c), $P < 0.05$), though no difference was observed at 48 hours. In contrast, no significant difference was found

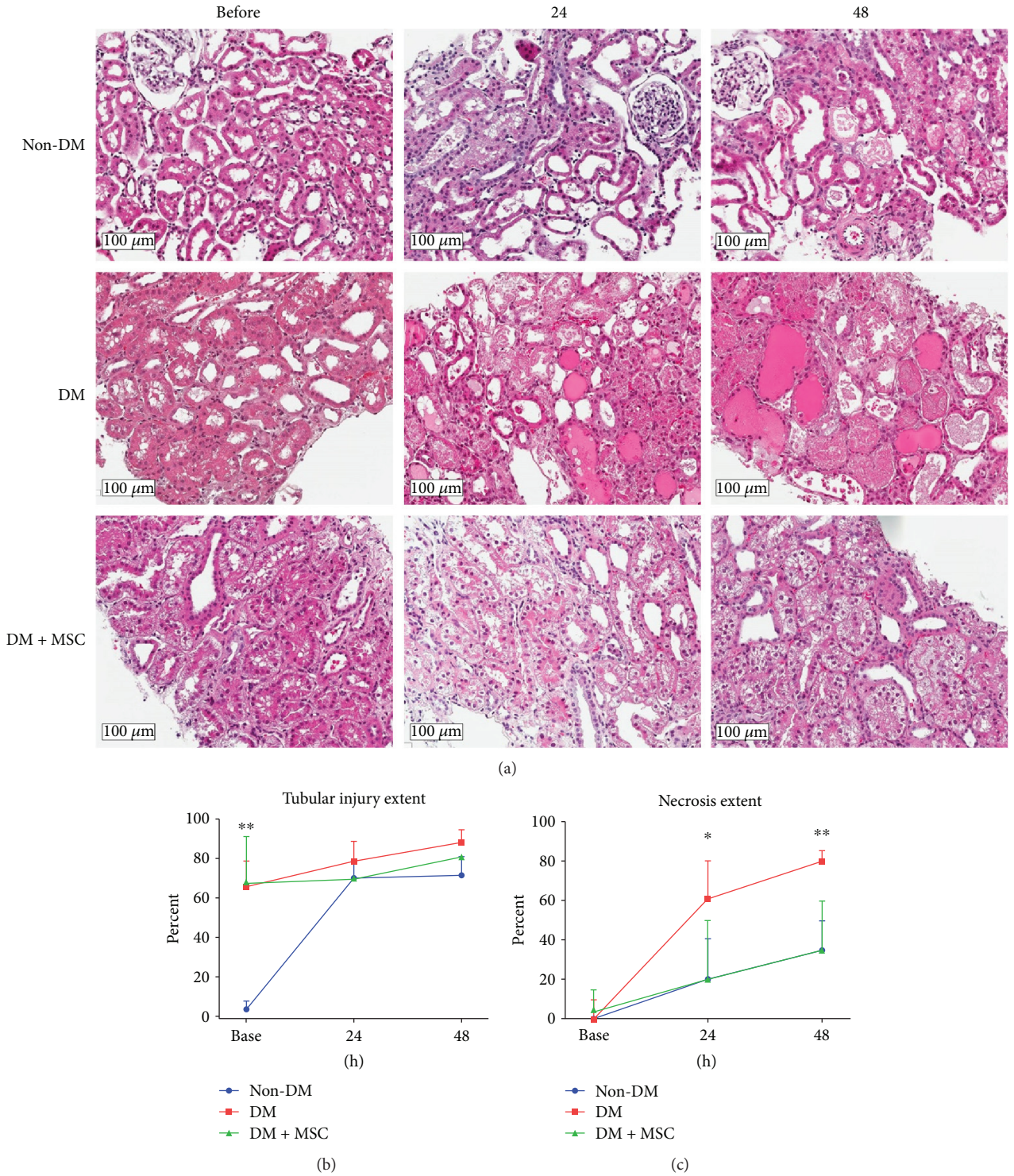

Figure 2: Histological examination of renal tissues. At designated time points, renal biopsy specimens were collected and subjected to H&E staining (a) to assess their tissue morphology. Scale bar = 100 μm. Magnification: ×200. The extent of tubular injury (b) and necrosis (c) were also calculated. $n = 3, 3$, and 2 in the non-DM, DM, and DM + MSC groups, respectively. $^{*}P < 0.05$ and $^{**}P < 0.001$.

in AST levels among the groups, possibly due to the large within-group variances caused by the small number of animals (Figure 4(c)). Thus, we suggest that AKI might have led to an abrupt injury to the liver. Liver tissues at the end of the experiment showed hepatocyte swelling and fatty change. Those findings were observed more severely in the DM animals (Supplementary Figure 2). Also, inflammatory cell infiltration was observed in the lung tissue of Groups 1 and 2 animals during necropsy (Supplementary Figure 3). Indeed, previous studies have shown that AKI can induce

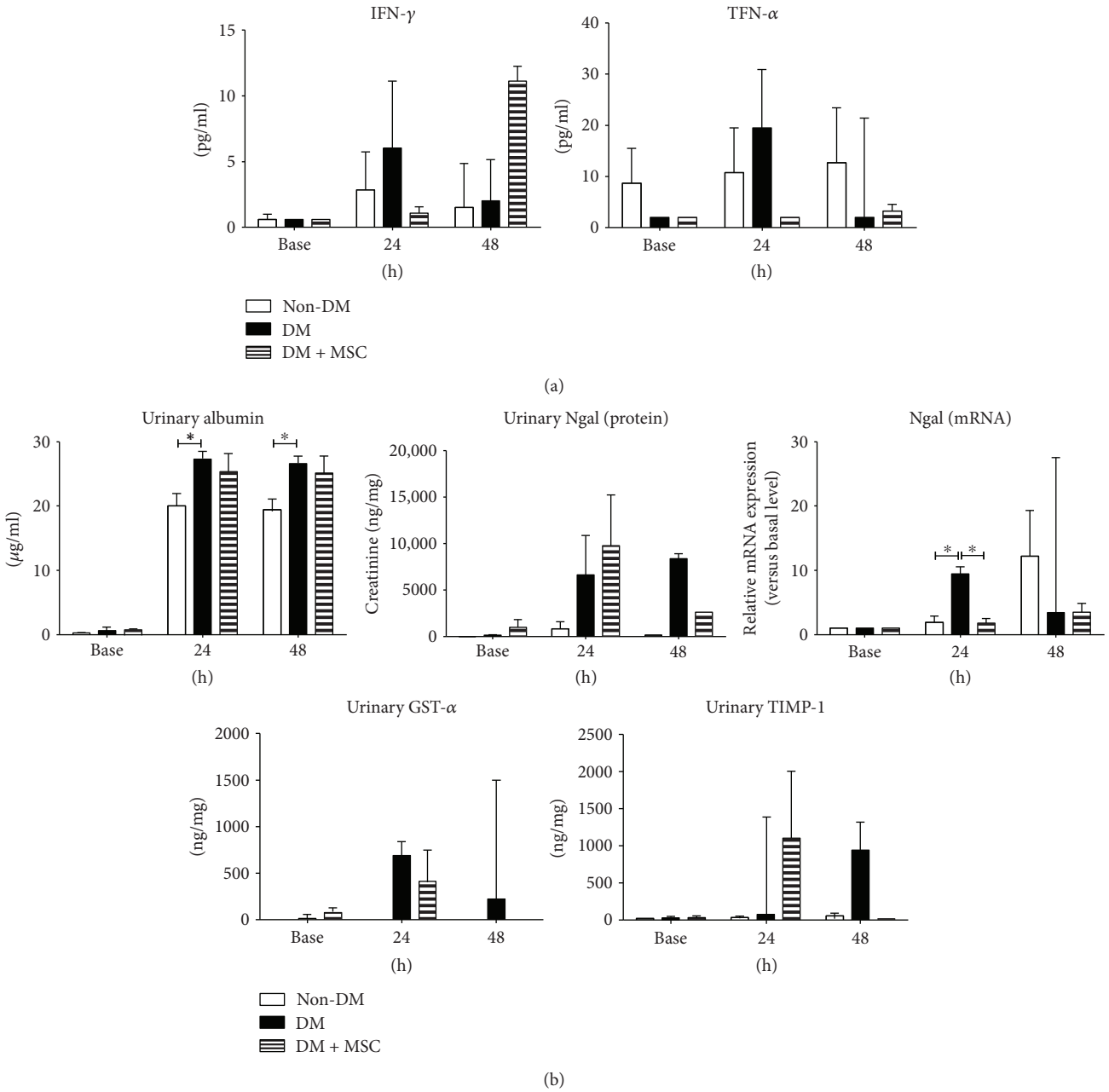

Figure 3: Analysis of proinflammatory markers following renal IRI and MSC treatment. (a) Blood was collected every 24 hours, and the serum levels of each cytokine were measured by a multiplex bead assay. Data are median with range. (b) Analysis of urinary markers for AKI. The concentration of urinary albumin and NGAL was measured by ELISA, and *Ngal* mRNA in renal tissue was analyzed by qRT-PCR. The level of GST-alpha and TIMP-1 in urine was obtained by a multiplex bead assay. Data are the median with range. $n = 3, 3,$ and 2 in the non-DM, DM, and DM + MSC groups, respectively. $^*P < 0.05$.

secondary injuries to other organs, including the liver and lungs—the so-called organ cross talk [31, 32].

Autologous or allogenic MSC-based therapy has been suggested as an alternative treatment for AKI [33], and numerous rodent studies reported that systemic injection of human MSCs contributed to renal function recovery by various processes such as regulating immune cell trafficking, reducing oxidative stress, stimulating tubular proliferation, or differentiating into tubular epithelial cells [25, 34–39]. In most cases of rodent studies, systemic delivery of human BM-MSCs has been widely used to study their therapeutic potential mainly due to the easiness in cell injection and future clinical application. However, this method has several disadvantages in that injected cells can be accumulated in the organs including the lung and liver [40]. Also, the cells can be diluted via systemic circulation. Accordingly, the intra-arterial injection has been attempted in several kidney diseases using large animal models including ewe and rhesus monkeys [25, 41]. Based on the methods that these studies had used, we adopted an alternative route whereby the

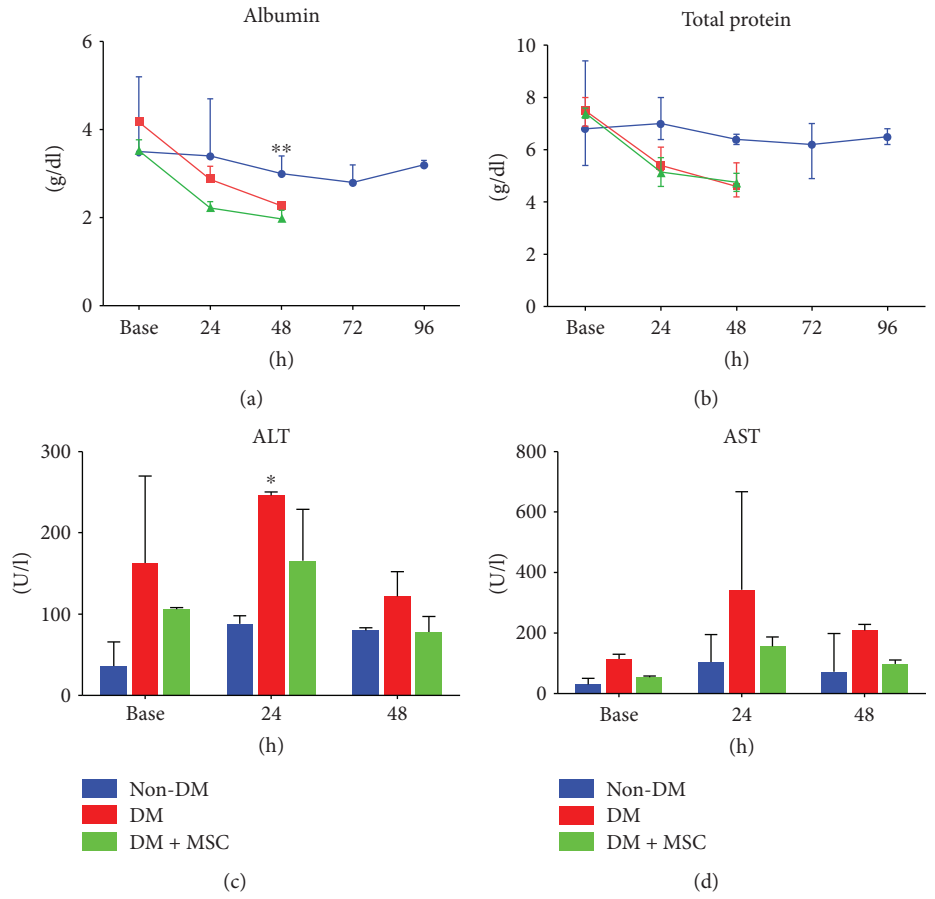

FIGURE 4: Serum biochemical analysis of markers indicative of liver function. Blood was collected every 24 hours, and the serum levels of total protein (a), albumin (b), ALT (c), and AST (d) were measured. $n = 3, 3,$ and 2 in the non-DM, DM, and DM + MSC groups, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$. Data are the mean with range.

suprarenal aorta was used while the infra-renal aorta was occluded to allow the MSCs to naturally go into the kidneys via downstream of the renal artery because direct injection into the renal artery was unavailable due to its small diameter (<1 mm) and the risk of bleeding. We consider that the renal or systemic effect of occlusion of the infra-renal aorta was minimal because the overall time for the cell administration took less than a minute.

Our renal IRI protocol in DM monkeys might not be optimal. Apparently, the duration of renal pedicle clamping in our protocol provoked severe AKI for DM monkeys; therefore, the duration should be reduced so that long-term follow-up data with less variance can be obtained. Although histological studies supported our hypothesis, mortality as early as 49–60 hours in DM animals did not enable us to further monitor decreases in the BUN or creatinine of DM animals. Also, it should be noted that we used only a few monkeys in our experiments, which might have contributed to significant intra- or intergroup variations. The potential effects of other intrinsic/extrinsic factors, such as differences in diabetic condition or subtle changes in procedures during AKI induction, should also be noted. Nonetheless, our finding will provide important aspects while conducting experiments that therapeutic cell or biomolecules are tested for reducing AKI using nonhuman primates. This model will also be beneficial in developing new therapies to ameliorate AKI that occurs during organ transplant.

4. Conclusions

Using a nonhuman primate model, we showed that DM increases susceptibility to renal ischemia-reperfusion injury, and that hBM-MSCs have the potential to attenuate renal injuries.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interests.

Authors' Contributions

Kyo Won Lee and Tae Min Kim contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: representative images of inducing renal ischemia and reperfusion injury by the exposure and occlusion of the renal pedicles. A uniform dusky color appeared in both the right (a) and left (b) kidneys after bulldog clamps were applied to the renal pedicles. After 1 hour, the clamps were removed, and the reversal of the dusky appearance was confirmed in both the right (c) and left (d) kidneys. Supplementary Figure 2: histologic examination of liver tissues with H&E staining. Non-DM monkeys (a), DM monkeys (b), and DM + MSC monkeys (c) at the end of the experiment. Hepatocyte swelling and fatty change were observed in one of three non-DM monkeys (a). However, those findings were observed more severely in all of DM monkeys (b) and MD + MSC monkeys (c). Scale bar = 100 μm. Magnification: ×200. Supplementary Figure 3: histologic examination of lung tissues with H&E staining. Non-DM monkeys (a), DM monkeys (b), and DM + MSC monkeys (c) at the end of the experiment. Interstitial inflammatory cell infiltration was observed in one of three non-DM monkeys (a). Alveolar capillary interstitial edema and interstitial inflammatory cell infiltration were observed in one of three DM monkeys (b). Alveolar capillary interstitial edema was observed in one of two DM + MSC monkeys (c). Scale bar = 100 μm. Magnification: ×200. *(Supplementary Materials)*

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Research Article

Insoles Treated with Bacteria-Killing Nanotechnology Bio-Kil Reduce Bacterial Burden in Diabetic Patients and Healthy Controls

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Our study investigated the effectiveness of bacteria-killing nanotechnology Bio-Kil socks on bacterial burden reduction in diabetic patients and healthy individuals. Four strains of *S. aureus* and four strains of *E. coli* were cultured and dropped on Bio-Kil socks and control socks for 0 h, 8 h, and 48 h of incubation. Diluted samples were inoculated and bacterial counts were recorded. Additionally, 31 patients with type 2 diabetes and 31 healthy controls were assigned to wear one Bio-Kil sock on one foot and a control sock on the other for four hours, and then they were told to exchange socks from one foot to the other for four hours. The socks were sampled and diluted and then inoculated to record bacterial counts. Bacterial counts were reduced in Bio-Kil socks compared with control socks in all *S. aureus* strains after 0 h, 8 h, and 48 h of incubation. In *E. coli* strains, bacterial counts declined in Bio-Kil socks comparing with control socks in most of the experiments with ESBL-negative *E. coli* and ATCC35218 at 0 h and 48 h of incubation. In all participants, the mean bacterial counts significantly decreased in Bio-Kil socks in comparison with control socks both at 0 h and at 40 h of incubation ($p = 0.003$ at 0 h and $p = 0.006$ at 40 h). Bio-Kil socks from diabetic patients showed significantly lessened bacterial count at 40 h of incubation ($p = 0.003$). In healthy individuals, Bio-Kil socks reflected a significantly smaller mean bacterial count than control socks ($p = 0.016$). Socks using Bio-Kil nanotechnology efficiently reduce bacterial counts in both diabetic patients and healthy individuals and might exert stronger efficacy in Gram-positive bacteria.

1. Introduction

A diabetic foot is a severe complication of diabetes, which brings about high disability and mortality rates, as well as social-economic burden on patients with diabetes. The associated foot problems include ulceration, infection, and amputation and have a tremendous impact on health-related quality of life for diabetic patients. Yearly incidence of foot ulcers in diabetic patients is estimated as 2%, and its recurrence rate is as high as 30% to 40% in the first year after successfully healing from a foot ulcer [1]. Among all diabetes-associated foot problems, 80% of them are preventable [2]. Since the prevention of diabetic foot plays a critical role in management of foot problems, new methods for foot ulcer prevention such as insoles are emerging and proved their efficacy in clinical trials. In a randomized controlled trial (RCT), custom-made foot orthoses showed a significant reduction in plantar pressure [3]. Another custom-made footwear focused on peak pressure reduction, but the primary end-point ulcer recurrence was not significantly declined [4]. A nanotechnology sock, which was designed to increase skin moisture content, displayed protective function in subjects with diabetes [5].

Bio-Kil (Cargico Group, Taiwan) is a bacteria-killing nanotechnology agent comprising inorganic metal components and organic quaternary ammonium compounds (QACs) [6]. Bio-Kil molecules could attract pathogens due to a high-affinity structure and electric field and damage the membrane structure of microorganisms with its electrical charge to kill the pathogens. Furthermore, the bacterial killing efficacy of Bio-Kil agent remains even after over fifty times of washing as a result of the permanent, covalent bond between

Bio-Kil agents and the surface of the textile fibers [7]. The Bio-Kil antibacterial catalyst was applied to the ICU environment and surface of instruments such as bed sheets, pillows, computer keyboards, nursing station desktops, and surfaces close to patients, where it was proved to maintain a long-term bactericidal function [8]. However, it was the first time that Bio-Kil nanotechnology was being applied to footwear for patients with diabetes and healthy individuals. The possible bactericidal effect on the local foot area was investigated in the following study that we conducted, thereby providing a choice for patients with at-risk diabetic foot or foot ulcer to suppress bacterial burden in their feet.

2. Material and Methods

2.1. Bacterial Strain Experiments. Four clinical separated bacterial strains and four standard bacterial strains were provided by the Institute of Clinical Pharmacology, Peking University First Hospital: two strains of methicillin-resistant *Staphylococcus aureus* (MRSA) (15B183 and ATCC43300), two strains of methicillin-sensitive *Staphylococcus aureus* (MSSA) (15B190 and ATCC29213), two strains of extended spectrum β-lactamase- (ESBL-) positive *Escherichia coli* (E. *coli*) (15B254 and ATCC35218), and two strains of ESBL-negative E. *coli* (15B253 and ATCC25922). S. *aureus* strains were cultured in Tryptone Soya Broth (TSB) (Oxoid, UK) and E. *coli* strains were cultured in Luria Broth (LB) (Oxoid, UK) at 35°C for 16 hours. Bio-Kil nanotechnology socks (Cargico Group, Taiwan) in grey color and custom-made control socks in white color, which were designed to be similar in appearance and textile with Bio-Kil socks, were cut into 1 cm × 1 cm squares. 100 μl of each bacterial strain culture was dropped on Bio-Kil sock and control sock squares. We planned to evaluate immediately, after 8 hours of contacting (imitating 8 hours of working), and a lasting contacting time of 48 hours, and sock squares were incubated separately for 0 h, 8 h, and 48 h. After incubation, sock squares were steeped in 5 ml Mueller-Hinton Broth (MHB) (Oxoid, UK) and vibrated for 2 minutes and then diluted with MHB for 10^2, 10^3, and 10^4 times. 100 μl of each diluted samples was inoculated into Mueller-Hinton agar (Oxoid, UK) with blood for S. *aureus* strains or China blue agar for *E. coli* strains and then placed in a 35°C incubator for 24 hours. The number of colonies was recorded after incubation as colony-forming units (CFUs)/cm^2.

2.2. Experiments in Diabetic Patients and Healthy Participants

2.2.1. Settings. The following study was conducted during the period of 20 September to 20 October 2017 in the Department of Endocrinology, Peking University First Hospital in Beijing, China. The Research Ethics Committee of Peking University First Hospital (PUFH) approved the protocol for the purpose of our study in this manuscript. Written informed consent with signatures was obtained from all participants enrolled in this study. Thirty-one patients who were diagnosed with type 2 diabetes based on the World Health Organization criteria, which included fasting plasma glucose ≥ 7.0 mmol/l, and/or 2-hour plasma glucose ≥ 11.1 mmol/l or random plasma glucose ≥ 11.1 mmol/l. Healthy individuals were defined as no diagnosis of any diseases, for instance, type 2 diabetes, hypertension, and coronary artery diseases. Participants were excluded in the study when subject had existing foot ulcer or history of foot ulcer, foot infection, or was incapable of wearing socks or fully understanding the research protocol. Anthropometric data was collected after enrollment, which consisted of age, sex category, history of diabetes, history of peripheral artery disease, and history of diabetic peripheral neuropathy.

2.2.2. Embedding and Sampling of Socks. Thirty-one diabetic patients and thirty-one healthy individuals were assigned to wear one Bio-Kil sock on one foot and wear a control sock on the other for 4 hours and exchanged bilaterally and wear the socks for another 4 hours. During the process, participants were unaware to which sock Bio-Kil nanotechnology was applied, which was to say a single-blinded study design. After eight hours of wearing, socks were cut into 1 cm × 1 cm squares and were incubated separately for 0 h and 40 h to compare with the incubating time in bacterial strain experiments. Sock samples were steeped in 5 ml MHB after incubation and vibrated for 2 minutes and then diluted with MHB for 10 and 10^2 times. 100 μl of each samples and diluted samples was inoculated into Trypticase Soy Agar and incubated at 35°C for 24 hours. After incubation, the number of colonies was recorded as colony-forming units (CFUs)/cm^2.

2.3. Statistical Analysis. Data were analyzed using the Statistical Package for the Social Sciences for Windows (SPSS version 16.0, Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables were as percentage. Student's paired *t*-test was used for quantitative variables, and non-Gaussian variables were compared using Mann–Whitney *U* test. A p value of <0.05 was considered to be of statistical significance.

3. Results

3.1. Bacterial Strain Experiments. In the experiments with bacterial strains, bacterial counts were reduced in Bio-Kil socks compared with control socks in all *S. aureus* strains and in 0 h, 8 h, and 48 h of incubation, indicating an effective and continuous bacterial killing activity in socks treated with Bio-Kil (Table 1). However, in *E. coli* strains, bacterial counts declined in Bio-Kil socks comparing with control socks in most of the experiments with ESBL-negative *E. coli* and ATCC35218 at 0 h and 48 h of incubation (Table 1). These results indicated that the bactericidal nanotechnology Bio-Kil might exert a stronger bacterial killing effect on Gram-positive bacteria.

3.2. Experiments on Diabetic Patients and Healthy Participants. The baseline data of the thirty-one patients with type 2 diabetes and thirty-one healthy individuals was listed in Table 2. There were more female participants in both diabetes group and healthy individual group. The mean age in the two groups was unequal (diabetes versus healthy subjects, 56.6 ± 13.1 versus 28.0 ± 4.5). The duration of diabetes

Table 1: Bacterial counts in Bio-Kil socks and control socks in bacterial strain experiments.

Bacterial counts (5×10^5 CFU/cm^2)	Bio-Kil socks (0 h)	Control socks (0 h)	Bio-Kil socks (8 h)	Control socks (8 h)	Bio-Kil socks (48 h)	Control socks (48 h)
15B253 (ESBL− *E. coli*)	25*	26	45*	221	22*	60
ATCC25922 (ESBL− *E. coli*)	8*	26	18*	26	2.3	1.1
15B254 (ESBLs+ *E. coli*)	33	13	58	47	13	8.3
ATCC35218 (ESBLs− *E. coli*)	35*	44	44	30	0.06*	14
15B190 (MSSA)	9*	28	2.6*	140	2*	57
ATCC29213 (MSSA)	36*	41	0.79*	14	0.23*	28.4
15B183 (MRSA)	14*	28	4.4*	142	0.4*	4.9
ATCC43300 (MRSA)	59*	68	4.8*	132	0.3*	34

*Bacterial counts were reduced in Bio-Kil socks compared to control socks.

Table 2: Baseline data of diabetic patients and healthy individuals.

	Diabetes group ($n = 31$)	Healthy individual group ($n = 31$)
Sex category (male/female)	11/20	14/17
Age (y)	56.6 ± 13.1	28.0 ± 4.5
History of diabetes (y)	9.5 ± 6.7	None
History of diabetic peripheral neuropathy (n/%)	13 (41.9%)	None
History of peripheral artery disease (n/%)	4 (12.9%)	None

history was 9.5 ± 6.7 years in the diabetes group. Thirteen of the thirty-one diabetic patients (41.9%) were diagnosed with diabetic peripheral neuropathy, and four of them (12.9%) were diagnosed with peripheral artery disease.

In all participants, the mean bacterial counts significantly decreased in Bio-Kil socks in comparison with control socks both at 0 h and at 40 h of incubation (Figures 1, 30.61 ± 9.77 versus 97.15 ± 27.80, $p = 0.003$ at 0 h and 8.21 ± 1.92 versus 39.57 ± 11.86, $p = 0.006$ at 40 h) (the unit of bacterial counts was 5×10^2 CFU/cm^2). Meanwhile, Bio-Kil socks from diabetic patients showed significantly lessened bacterial count at 40 h of incubation (Figures 2 and 3 0.76 ± 0.99 versus 25.05 ± 6.99, $p = 0.003$) and revealed a reducing trend of bacterial count in Bio-Kil socks than control socks (4.98 ± 1.89 versus 72.75 ± 37.26, $p = 0.068$). Similarly, Bio-Kil socks from healthy individuals reflected a significantly smaller mean bacterial count than control socks (Figures 3, 56.24 ± 18.45 versus 121.56 ± 41.42, $p = 0.016$), and a declining trend of mean bacterial count was discovered comparing with control socks at 40 h of incubation (12.67 ± 3.56 versus 54.09 ± 22.56, $p = 0.060$).

3.3. Safety and Side Effects. There were no reports of contact dermatitis or other adverse effects during the period of research. Furthermore, none of the participants who wore Bio-Kil socks complained with any discomfort.

4. Discussion

Diabetic ulcer is a severe complication of diabetes and has always been a problem in real-world practice. The prevention of diabetic foot complications requires a great deal of effort [9]. Numerous studies focused on the improvement of footwear for the prevention of diabetic foot. A variety of socks was designed to significantly reduce peak plantar pressure [10–12]. In some RCTs, the therapeutic footwear could lessen the rate of recurrent ulcer over a one-year period of follow-up [13]. Nevertheless, the possibility of having a footwear with antibacterial activity had not yet been developed for patients with diabetes on the purpose of prevention of diabetic foot.

The Bio-Kil nanotechnology was a promising bactericidal technology that could be applied to any textiles for environment with hygienic needs. The Bio-Kil nanotechnology has been successfully induced to ICU environment or clothes for ICU nurses, and its bactericidal efficacy has been proved after certain investigation [14]. However, this nano-based technology had never been used in footwear for patients with diabetes until our research. In addition, the antibacterial spectrum of Bio-Kil-treated textiles has not yet been studied.

The part of experiments with bacterial strains was designed on the base of clinical practice. In diabetic foot with clinically infected wounds, a tissue specimen for culture was routinely obtained. Superficial and limited foot ulcer infections are usually caused by aerobic Gram-positive cocci, especially *S. aureus*. Chronic and severe foot ulcer infections are often polymicrobially caused, with aerobic Gram-negative rods and anaerobes accompanying the Gram-positive cocci [15–18]. Therefore, we chose *S. aureus* and *E. coli* to be representative of Gram-positive and Gram-negative bacteria, respectively.

Since it was the first time that socks were innovatively treated with Bio-Kil nanotechnology, the bactericide activity was required to be evaluated. In the experiments with

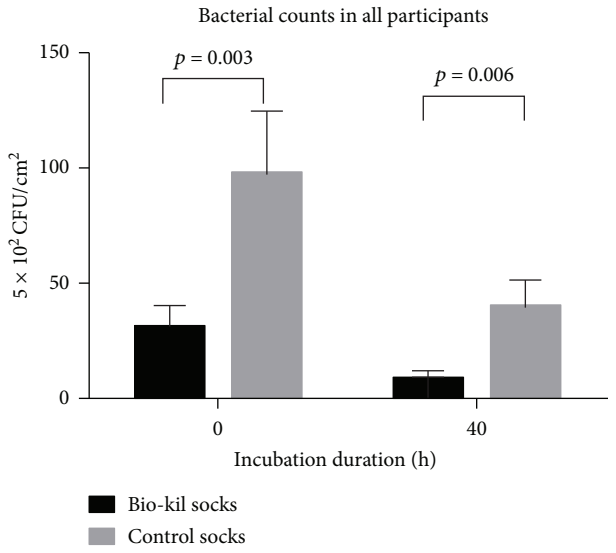

Figure 1: Bacterial counts in Bio-Kil-treated socks and control socks in all participants.

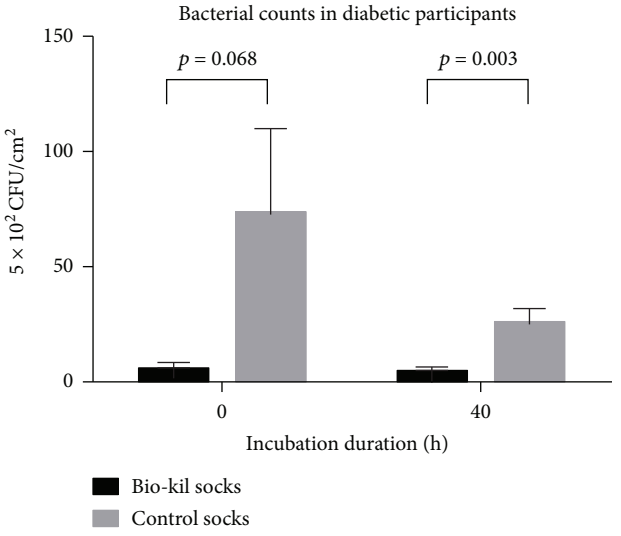

Figure 2: Bacterial counts in Bio-Kil-treated socks and control socks in diabetic patients.

bacterial strains, we compared the bacterial counts in socks treated with Bio-Kil and standard shop-bought socks as a true control condition. Bacterial counts diminished in Bio-Kil-treated socks comparing with control socks in all *S. aureus* strains at all incubating durations. In *E. coli* strains, bacterial counts declined in socks treated with Bio-Kil than control socks in five out of six pairs of samples inoculated with ESBL-negative *E. coli* strains, and two out of six pairs of ESBL-positive *E. coli* strain inoculating samples showed lower bacterial counts in Bio-Kil-treated socks than control socks. These results confirmed that Bio-Kil treatment in textiles could result in a self-disinfecting surface with antimicrobial activity, but the activity was stronger for Gram-positive bacterial strains compared with Gram-negative bacterial

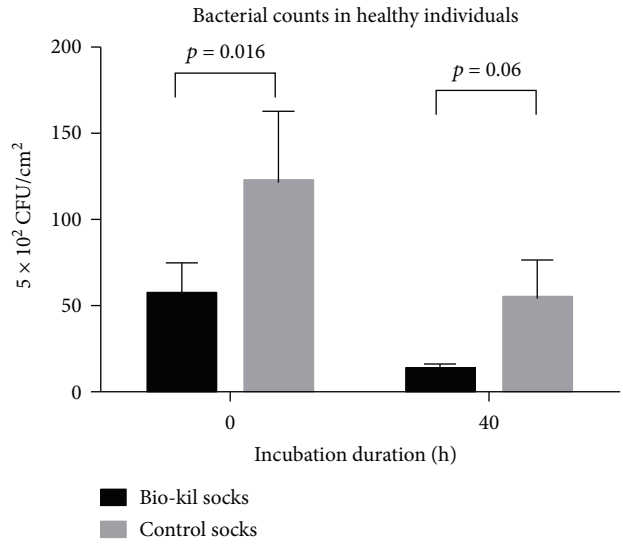

Figure 3: Bacterial counts in Bio-Kil-treated socks and control socks in healthy individuals.

strains, which indicated that the antibacterial function was not broad-spectrum.

The reason of the phenomenon that Bio-Kil-treated textiles had stronger bactericidal efficacy in Gram-positive bacteria might be associated with the difference of cell wall structure for Gram-positive and Gram-negative bacteria. Gram-positive bacteria has a single-lipid membrane surrounded by a thick layer of cell wall (30–100 nm) composed of peptidoglycan, while the cell wall of Gram-negative bacteria consists of a thin layer of peptidoglycan (2–10 nm) and an outer membrane which contains lipid bilayers and lipopolysaccharides on its outer surface [19]. Since Bio-Kil molecules implement its bactericidal function through damaging the membrane structure of microorganisms with its electrical charge, it could be postulated that the out membrane structure of Gram-negative bacteria might produce certain impairment in the bacterial killing activity of Bio-Kil nanotechnology. Further study that concentrated on the bactericidal mechanism of Bio-Kil molecules could confirm this assumption.

In the part of experiments that volunteers are involved, thirty-one patients with type 2 diabetes and thirty-one healthy participants were enrolled. The study was designed in a single blind, control manner using custom-made control socks in a different color and the same textile with Bio-Kil socks but without treatment of Bio-Kil. Our results demonstrated a significant reduction in bacterial counts in Bio-Kil-treated socks comparing with control socks in the statistical analysis of all participants. The tendency of bacterial count decline was also observed in both diabetes group and healthy participant group, which illustrated that the bacterial killing activity was efficient for both diabetic patients and healthy individuals and indicated that Bio-Kil socks may play a part in prevention and multifaceted treatment of diabetic foot. The footwear showed its feasibility in the prevention of diabetic foot along with other kinds of treatment.

Still, our study has limitations. A main limitation is the lack of assessment of the effect of Bio-Kil on other diabetic foot-associated pathogens, including anaerobes and fungi (e.g., *Candida albicans*) [20, 21]. Furthermore, the sample size was relatively low. Theoretically speaking, an RCT will provide a strong evidence for the bacterial killing efficacy of the sock. However, in terms of availability, the socks used as part of a comprehensive prevention package would be more feasible, which could be further studied. Further work is also required to validate that Bio-Kil-treated textiles has stronger bactericidal efficacy in Gram-positive bacteria due to the different cell wall structure of Gram-positive and Gram-negative bacteria.

In conclusion, foot socks using Bio-Kil bactericidal nanotechnology efficiently reduce bacterial counts in both diabetic patients and healthy individuals and might exert stronger efficacy in Gram-positive bacteria.

Data Availability

Data of the manuscript is repeatable and is available after the permission of the corresponding author.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The Research Ethics Committee of Peking University First Hospital (PUFH) approved the protocol for the purpose of our study in this manuscript.

Consent

Written informed consent with signatures was obtained from all participants enrolled in this study.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Review Article

Addressing Stem Cell Therapeutic Approaches in Pathobiology of Diabetes and Its Complications

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High morbidity and mortality of diabetes mellitus (DM) throughout the human population is a serious threat which needs to be addressed cautiously. Type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are most prevalent forms. Disruption in insulin regulation and resistance leads to increased formation and accumulation of advanced end products (AGEs), which further enhance oxidative and nitrosative stress leading to microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular complications. These complications affect the normal function of organ and tissues and may cause life-threatening disorders, if hyperglycemia persists and improperly controlled. Current and traditional treatment procedures are only focused on to regulate the insulin level and do not cure the diabetic complications. Pancreatic transplantation seemed a viable alternative; however, it is limited due to lack of donors. Cell-based therapy such as stem cells is considered as a promising therapeutic agent against DM and diabetic complications owing to their multilineage differentiation and regeneration potential. Previous studies have demonstrated the various impacts of both pluripotent and multipotent stem cells on DM and its micro- and macrovascular complications. Therefore, this review summarizes the potential of stem cells to treat DM and its related complications.

1. Introduction

The diabetes mellitus (DM), one of the most prevalent noncommunicable disease, is characterized by hyperglycemia leading to the development of severe life-threatening complications [1, 2]. Recent decades have witnessed a sudden increase of diabetes throughout the world, in spite of numerous efforts made to control to outspread of this metabolic disorder. Currently, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are the most prevalent type of diabetes. The T1DM, which is also known as insulin-dependent DM, is caused due to impairment in regulation of blood glucose by absolute destruction of insulin-producing β-cells, whereas insufficient or no response to insulin is attributed to the pathogenesis of T2DM. The International Diabetes Federation (IDF) reported that the number

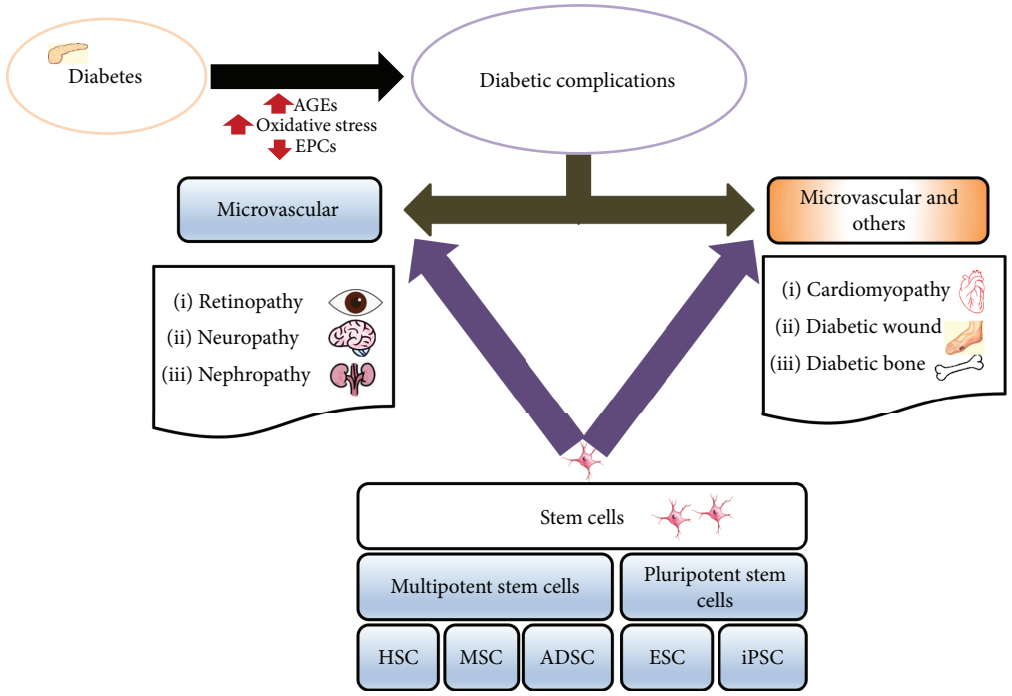

Figure 1: Schematic overview of stem cell therapy in diabetic complications. AGEs: advanced glycated end products; EPCs: epithelial progenitor cells; MSC: mesenchymal stromal cells; HSCs: hematopoietic stem cells; ADSC: adipose-derived stem cells; ESCs: embryonic stem cells; iPSCs: induced pluripotent stem cells.

of diabetic population will increase from 415 million in 2015 to 642 million by 2040 [2]. Of note, any defect in insulin regulation in blood triggers the in metabolic disorders of carbohydrate, fat, and protein leading to a condition of hyperglycemia [3]. Insulin secretion is mainly stimulated by glucose; however, other factors such as amino acids, fatty acids, acetylcholine, pituitary adenylate cyclase-activating polypeptide (PACAP), glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) also participate in regulating the metabolism of their respective biomolecules [4]. The thirst, polydipsia, weight loss, polyuria, and blurred vision are some common symptoms of diabetes; in severe cases, hyperglycemia along with ketoacidosis or nonketotic hyperosmolar conditions are prevalent [4].

Currently, diabetic retinopathy, nephropathy, and neuropathy are the major reported complications. The other complication also includes foot ulcer [3, 5]. These complications have been reported to mediate via advanced glycations end products (AGEs), which mainly are the posttranscriptional modified proteins or lipids, and might be excessively synthesized during hyperglycemic conditions or present in the diet. These high levels of AGE also disrupt the defense mechanisms and assist in the destruction of β-cells [6]. 1Specifically, AGEs bind to their multiligands, known as a receptor of advanced glycation end products (RAGE), which activates different kinase and NADPH oxidase leading increased levels of ROS and further promotes the synthesis of more AGEs, thereby triggering cell-damaging mechanisms [7–9]. Notably, the AGEs not only destroy insulin-producing cells but also develop insulin resistance, a major symptom of T2DM [10].

It is well-known that the exercise and diet control are helpful to manage glucose level at initial stage [11]. The use of therapeutic insulin and other external hypoglycemic agents have also been employed to control the glucose level in blood, yet they are not capable enough to mimic the natural activity of endogenous insulin and may result in a hypoglycemic coma [12, 13]. The other therapeutic approach is transplantation of pancreas or islet cells; however, this approach is limited due to the lack of donors and surgical and postsurgical complexities associated with therapy [14].

In general, stem cell is a population of cells defined by its ability to indefinitely expand, self-renew, and undergo asymmetric divisions to produce progeny cells committed to specific differentiation lineages [15]. Embryonic stem cells, a pluripotent cell derived from the inner cell mass of a blastocyst, are capable of generating almost every cell types of the body but are unable to form an entire organism. Multipotent stem cells reside within various niches in the body and are limited to differentiating into specialized cell types of their tissue of origin such as mesenchymal stem cells and hematopoietic stem cells [16]. Stem cells are important for living organisms due to their functions of homeostatic tissue maintenance and replacing dysfunctional and senescent cells. Given their remarkable regenerative capacities, stem cells are being applied in treatments for various diseases as a novel potential therapeutic intervention, which is also referred to as regenerative medicine (Figure 1). In previous years, the role of stem cells has been extensively studied for their therapeutic potential to treat diabetic pathology and related complications. Therefore, this article reviewed the possibilities of stem cell therapies in diabetes and its associated complications.

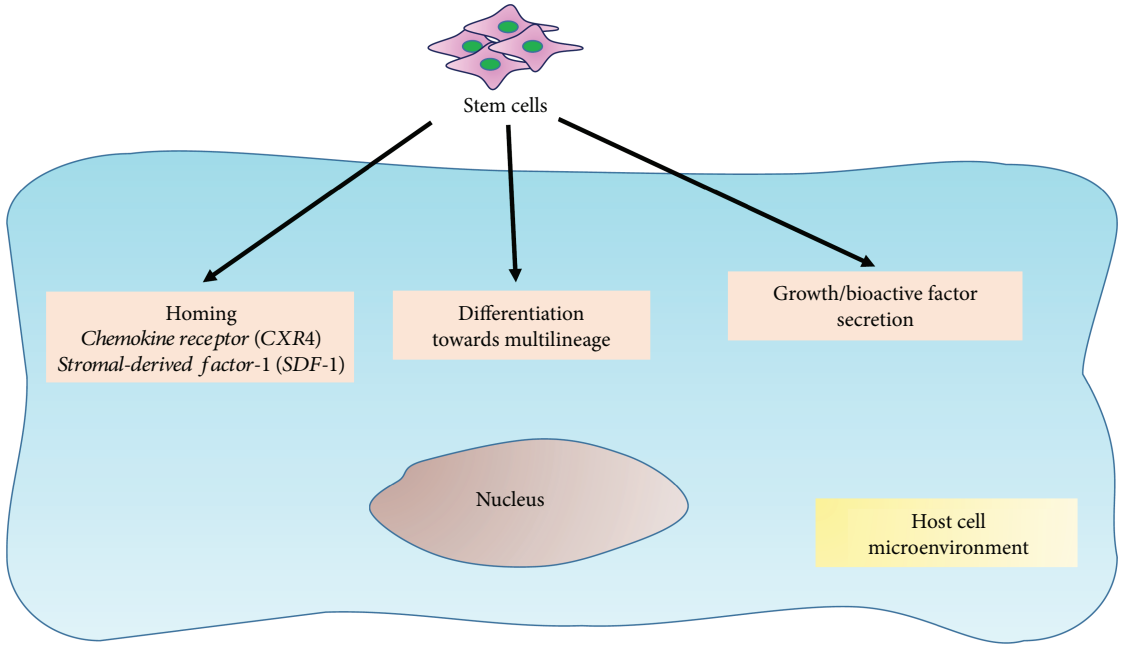

Figure 2: The possible mechanistic insight of therapeutic action of stem cells. During repair and regeneration, the transplanted MSC exhibit three modes of action, including homing, multilineage differentiation, and secretion of growth/bioactive factors.

2. Stem Cells in Treatment of Diabetes

Transplantation of insulin-producing cells [17] has paved the path to stem cell-based regeneration of insulin-secreting pancreatic β-cells [18]. Stem cells are unspecialized having the potential to regenerate and differentiate into specialized cells such as myocyte, hepatocyte, leukocyte, lymphocytes, erythrocytes, muscles, and nerve cells under proper environmental condition and signal [19]. On the basis of cell source, stem cells are generally classified as embryonic stem cells (ESCs) or adult stem cells (ASC). However, stem cells are also classified on the basis of origin, potential methods of derivations, and so on [19]. ESCs or pluripotent stem cells are isolated from inner cell mass of the blastocyst and have the potential to differentiate in different germ cell lines. However, the ethical issues make it very difficult to explore its potential to regenerate insulin-secreting cells. Notably, ASCs are multipotent stem cells and have the capacity to differentiate into only fewer cell types [17, 19]. ASC such as hematopoietic stem cell (HSC) not only multiply itself but also develop into blood cells, whereas mesenchymal stem cells (MSCs) trigger the generation of fat, bone, and cartilage. ASC also helps in repair and replacement of damaged tissues along with developments of the central nervous system and muscle cells. The therapeutic potential of stem cells may be ascribed to three major embodied mechanisms of action (Figure 2). First, the systemically administered stem cells undergo "homing" which further migrate to the site of injury possibly due to chemoattraction mediated by cell surface receptors such as the chemokine receptors. Although the exact mechanism of stem cells and endothelial interaction at the target site is not well established, the integrins and selectins have been suggested to mediate such interactions [20, 21]. The stem cell transmigration to the focal point of injury occurs across the endothelium through vascular cell adhesion molecule 1 (VCAM-1) and G-protein-coupled receptor signaling [22]. Secondly, the transplanted stem cell may undergo differentiation into multiple cell types, which after local engraftment can replace damaged tissues and induce restoration of their function [23, 24]. Thirdly, stem cell may also secrete growth/bioactive factors, which may potentially positively influence both local as well as systemic physiological processes [25].

3. Stem Cell-Derived Secretome in Organ Repair and Regeneration

Regeneration and repair activities of stem cells depend on their differentiation potential to replace the damaged or injured tissues [26]. Recent in vivo studies have established the fact that most of the transplanted MSCs are cleared rapidly from the in vivo microenvironment, thus limiting the regenerative therapeutic potential of stem cell differentiation to direct organ repair [20]. Therefore, their paracrine and immunomodulatory function of MSCs seems more effective through cellular communication without physical contact between cells, along with secreted trophic factors, extracellular RNAs, and miRNA which leads to cellular modulation, thereby triggering change in the microenvironment [21]. Various studies have documented the role of secretory factors of MSCs in tissue repair and regeneration via regulating inflammatory and allogenic immune response [23–25, 27]. It is clearly evident from recent reports that MSCs release soluble paracrine factors which regulate cellular proliferation, migration, differentiation, immunomodulation, and anti-inflammatory response through p38 MAPK, Akt, STAT-3, and TNF receptor pathways [28]. Stem cell-specific secretome includes the extracellular molecules such

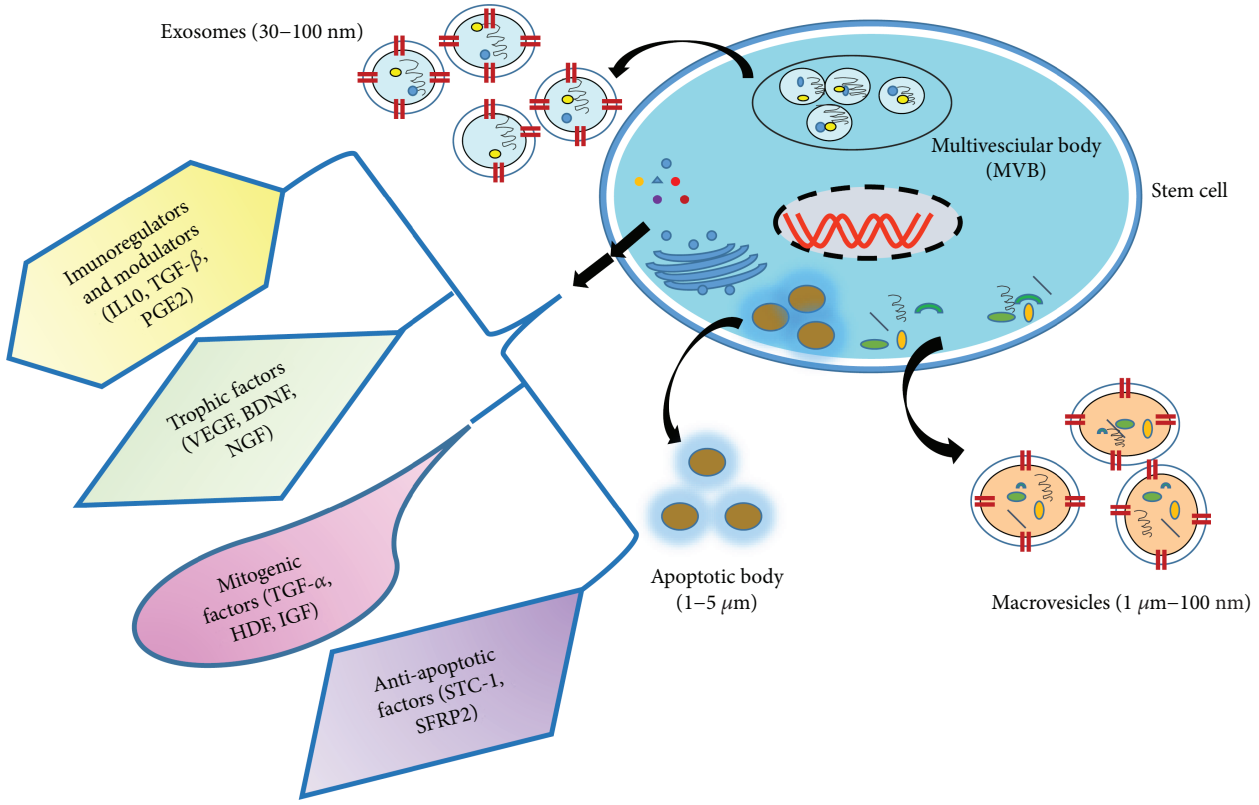

Figure 3: Mesenchymal stem cell-derived secretome and extracellular vesicles. IL: interleukin; TGF-β: transforming growth factor beta; PGE2: prostaglandins E2; VEGF: vascular endothelial growth factor; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; HGF: hepatocyte growth factor; IGF: insulin-derived growth factor; STC-1: stanniocalcin-1; SFRP2: secreted frizzled-related protein 2.

as extracellular vesicles (EVs), soluble proteins (e.g., chemokines, cytokines, and growth factors), lipids, and free nucleic acids [29, 30]. These EVs are produced by internal budding and when released into cellular microenvironment promotes regeneration of injured/damaged cells similar to stem cells after endocytosis; this regeneration procedure is mediated by receptor-ligand interaction, fusion or transfer of proteins, and nucleic acids or miRNA [31–34]. Based on their physical characteristics, EVs are further categorized among exosomes, apoptotic bodies, and microvesicles (MVs) (Figure 3) [35]. Exosomes are made up of spherical bi-lipid layer ranging from 30–100 nm in size. These membrane vesicles are released by various cells and considered as critical component for cellular communications, and in altering cellular signaling has rendered it an interesting candidate in regenerative therapy [36]. Exosomes promote specific interaction with targeted tissues/cells along with the disposal of unwanted proteins, antigen presentation, genetic exchange, immune responses, angiogenesis, inflammation, tumor metastasis, and spreading of pathogens or oncogenes [28, 37, 38]. Furthermore, apoptotic bodies are released from cells undergoing programmed death as blebs of 1–5 μm in diameter [39]. Besides these secretomes, the expression of factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), insulin-derived growth factor-1 (IGF-1), and thymosin B4 (TB4) is also released and is regulated by Akt signaling [40]. Interestingly, the increase in expression level of these factors has been observed under hypoxic conditioned medium. The increased production of VEGF in MSCs under normoxia or hypoxia has been found to be associated with STAT3 and p38 MAPK signaling pathways [28, 41], whereas in adult rat bone marrow multipotent progenitor cells (rMAPCs), JAK2/STAT3 signaling pathways have been ascribed [42]. Moreover, another study suggests that transforming growth factor-α- (TGF-α-) induced VEGF production is associated with MEK and PI3-K signaling pathways in homogenous human BM-MSCs [43]. These observations indicate the varying signaling pathways are associated with VEGF production in different species [28]. Besides, the expression of TGF-β1 in rat MAPCs has also been linked with STAT3 pathway [42]. TNF receptor (TNFR) and associated signaling pathways also plays a critical role in expression of paracrine factors such as VEGF, TNF, cytokines, and IL-6 [28, 44]. It has also been reported that the production of HGF in human MSCs is closely associated with TNF and TGF-α/epidermal growth factor (EGF) receptors and MEK, p38, and PI-3K signaling pathways [45], where the TNF receptor 1 played role in decrease of HGF, when stimulated with TGF-α and TNF-α. A comparative study of paracrine factor profile of swine and human bone marrow MSCs showed that both cell cultures produced similar factors including VEGF and endothelin, along with other different paracrine factors under various conditions, which indicate that secretion of paracrine factors varies according to the species [28, 46]. Apart from this, the age also impacts proliferation rate of

MSCs and their secretome level of paracrine factors. In a recent study, p38 and ERK signaling pathways seemed to be associated with cytokine and growth factors in neonatal BM-MSCs [47]. Along with the abovementioned factors, the gender [48, 49], disease status [50, 51], and environmental factors also significantly influence the type and level of secretory factors of MSCs [28].

Homeostasis, cell development, and cell repair/regeneration/survival are mediated by membrane protein and cell adhesion biomolecules (integrins, tetraspanins, and cadherins) which direct receptor-mediated cellular communication [52, 53], whereas coupling of cellular cytoplasm is mediated by gap junctions [21, 54, 55]. Stem cells lack gap junction; however, differentiated cells may communicate through gap junctions. This was evidenced in a report in which BM-MSCs were able to differentiate into cardiac cells via their communication to near myocytes through gap junction [55, 56]. Besides, tunneling nanotubes (TNTs) are a newly explored actin-based elements involved in long distance-based cellular communication [57, 58], leading to tissue developments and regeneration [21, 59].

4. Stem Cells Therapy in T1DM

Insulin-secreting β-cells become nonfunctional in T1DM, and this condition primarily arises due to autoimmune destruction of cells causing hyperglycemia. Traditional insulin therapy assists to control blood glucose level; however, it has proven ineffective in the long-term. Islet transplantation therapy is limited due to the availability of pancreatic cells, cell rejection, use of immunosuppressive drugs, and other complexities [17, 60]. These limitations could be avoided through stem cell therapies, owing to their very low immunogenic potential, immune-privileged, and immunomodulating properties [61–66]. Stem cells are also prone to genetic modification, through which the desired MHC complex may be introduced to control chance of immune rejections [67]. Furthermore, MSC has also been reported for their role in inhibition of T-cell proliferation, development of dendritic cells (DCs), and B-cell proliferation [63, 64, 68]. These reports are indicative of the immunosuppressive role of stem cells in transplantation therapy; however, more studies are required to establish their clinical significance.

In recent years, stem cells are emerging as a potential candidate for efficacious treatment for T1DM as these cells are capable to differentiate into mature β-cells in presence of required signals [12, 69]. The immunomodulation properties of stem cells can be helpful to control a balance between β-cell destruction and their regeneration [70]. Mouse ESCs (mESCs) have been widely studied and reported to promote the differentiation of insulin-producing cells under induced conditions to avoid ethical conflicts. ESC controls self-renewal by regulating the expression of different transcription factors such as Oct4, Sox2, and Nanog in presence of suitable medium [71]; germ cell nuclear factor (GCNF) and phosphoinositide kinase inhibitors catalyze the differentiation of specific functional cells. The designed media and transcription factors (Pax4 or Pdx-1) are reported for their potential to generate insulin-secreting cells [71–75]. Human ESC (hESC) has been demonstrated to differentiate into functional β-cells in vivo [76]. However, the regulation of differentiation, teratoma formation, risk of viral infection, transplantation rejection, and ethical issues are still major bottlenecks to utilize it as a potential therapy.

iPSCs are the new alternatives of ESCs to avoid ethical concerns. iPSCs are mainly somatic cells which are reprogrammed to pluripotency. Though the traditional method of generating iPSCs are controversial, the iPSCs developed by Takahashi and Yamanaka have accelerated their use for generation of functional cells; in particular, the mouse and human fibroblasts have already been reprogrammed into pluripotent cells by using Oct3/4, Sox2, c-MYC/Lin28, and Nanog/Klf4 transcription factors [77, 78]. Miyazaki et al. also reprogrammed cancerous cells into induced pluripotent cells using the same transcription factors [79]. Kim et al. suggested that somatic cells which express any of the transcription factors required for induction of pluripotency will reduce the requirement of complete transcription factors [80]. For insulin regulation, mouse fibroblast cells have also been induced into pluripotent stem cells, which were further triggered to differentiate into insulin-producing cells for insulin regulation [81]. The potential of iPSCs in diabetes treatment is promising; however, the chances of tumor formation and immune response to transplantation need to be critically evaluated [70].

Adult stem cells such as hepatic stem cells, bone marrow-hematopoietic stem cells (BM-HSCs), and mesenchymal stromal cells (MSCs) derived from the bone marrow and umbilical cord blood (UCB) and adipose tissue-derived MSCs (ADSCs) have been explored for their potential to generate insulin-producing cells. The endodermal nature of pancreatic cells makes hepatic stem cells a prospective stem cell source for therapeutic use. In various studies study, Pdx-1 was used to induce growth of β-cell precursors from hepatic tissues [69, 72, 82, 83]. Mouse and human hepatic stem cells were differentiated into insulin-secreting β-like cells and used to overcome the condition of hyperglycemia [84]. The application of hepatic stem cells to induce the regeneration of insulin-producing cells is promising; however, further extensive research is required to establish the protocols for clinical application. Since MSCs have the potential to differentiate into pancreatic cells as well as to heal damaged cells, these have been exploited in treatment of T1DM [85]. BM-MSCs are also able to promote graft acceptance and reduce autoimmunity [70, 86–88]. However, BM-MSCs' potential for stem cell therapy is limited by lack of standardized methods, difficulty in in vivo differentiation, and the possibility of tumor induction [70]. ADSCs are closely similar to the BMSCs and clinically accepted for their therapeutic potential due to ease of isolation with abundant cell numbers. The ADSCs have also been successfully used to counter type 1 diabetes in mice, and its potential to counteract the graft rejection response enhances the chance of success of T1DM therapy [70, 89–91].

5. Stem Cell Therapy in T2DM

Insulin resistance and a decrease in insulin production are the characteristics of T2DM. Conventional treatment

approach includes using external insulin and use of oral antidiabetic drugs [92]. However, the regular use of in vitro insulin makes T2DM patients insulin resistant and contemporary therapy does not address this complication [93]. Transplantation of islet cells was once considered as a promising therapeutic approach; however, this approach is not common due to lack of donors, ethical conflict, and risk of immunogenicity. Regeneration and multipotent potential of stem cells make it an integral candidate for cell-based therapy. Stem cells such as BMSCs, ADSCs, ESCs, and iPSCs are able to differentiate into insulin-producing cells resulting in an increase in insulin level in patients under defined conditions and well-established procedures [94, 95]. Intrapancreatic autologous stem cell injection under hyperbaric oxygen condition regulates glycemic condition and insulin level [96]. Similar results were also reported when autologous bone marrow-derived stem cells were intra-arterially injected [97]. MSCs have improved islet function and controlled insulin resistance in T2DM. Various trials are under clinical phase I and II, however, only a few of them are based on random and placebo-controlled [92]. Moreover, the establishment of the exact pathway in stem cell-based treatment of T2DM still needs to be well established.

6. Stem Cells in Diabetic Complications

Diabetes not only disrupts the blood glucose regulations but also alters the metabolism in long run if poorly managed. As a result, micro- and macrovascular complications occur [98–100]. The microvascular complications arise due to impairment in small blood vessels under chronic hyperglycemic milieu. Some of these complications are diabetic retinopathy, neuropathy, and nephropathy, whereas the macrovascular complication is caused by damage to arteries leading to cardiovascular disease (CVD), coronary artery disease (CAD), peripheral arterial disease, myocardial infarction (MI), and stroke. Diabetes-associated disorders like osteoporosis, osteoarthritis, foot ulcers, and diabetic cardiomyopathy are some other secondary complications [101–104]. Regeneration and differentiation capability of stem cells make it possible to explore their therapeutic potential to treat and control diabetic complications. Specifically, the multipotent stem cells such as MSCs/HSCs, progenitor stem cells, tissue-specific stem cells, and pluripotent stem cells (ESCs and iPSCs) are considered to counter the diabetes-associated disorders [98, 100]. Therefore, the selection of the suitable source of stem cells is critical to ensure the differentiation of stem cells into both endothelial and perivascular cells to repair diabetic complications [105]. In the further sections, we have discussed the role of stem cell therapy in several diabetic complications.

7. Microvascular Diabetic Complication and Stem Cells

7.1. Stem Cells and Diabetic Retinopathy. Abnormal ocular vascularity and retinal lesions lead to the development of blindness in retinopathy. The diabetic retinopathy (DR) is more prevalent in T1DM patients; however, it is hard to differentiate its incidence between T1DM and T2DM [106, 107]. DR is classed as either nonproliferative diabetic retinopathy (NPDR) or as proliferative diabetic retinopathy (PDR) [108]. Microvascular alterations cause retinal ischemia in NPDR, whereas PDR is caused by disruption of the ocular vitreous cavity due to the generation of abnormal blood cells leading to blindness [106, 109, 110]. Contemporary therapies such as vitrectomy and laser photocoagulation do not address the root cause of the disease [111]. Thus, stem cells seem as the most effective long-term treatment option for DR. In previous studies, MSCs and HSCs have been reported for their potential to differentiate into ocular cells to repair retinal damages [104]. In a seminal study in a rat model, it has been evidenced that MSCs are capable enough to mitigate and recover the loss of visual impairments [112, 113]. Scalinci et al. found that neuroprotective growth factors such as brain-derived neurotrophic factor (BDNF), ciliary-derived neurotrophic factor (CTNF), nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), and basic fibroblast growth factor (bFGF) were significantly increased in DR rata injected with hMSCs [114]. However, inferior homing capacity of intravitreally administered MSCs and increased level of vascular endothelial growth factor (VEGF), a factor responsible for vascular lesion, were found. In another study, atorvastatin, a reductase inhibitor enzyme, had also reduced VEGF when MSCs were injected and hypoxic condition was maintained subsequently [115]. Siqueira et al. also demonstrated that BM-HSCs led to an improved visual activity [116]. Further, in animal models, the injected EPCs derived from murine BMSCs and hUCB promoted neovascularization and ameliorated DR [117–119].

7.2. Stem Cell in Diabetic Neuropathy. Diabetic neuropathy (DN) is one of the most prevalent complications among T1DM and T2DM patients, which may lead to foot ulcers and limb amputation [120]. DN becomes more chronic with an increase in the level of hyperglycemia and with the passage of time [121, 122]. Microvascular factors, metabolic regulations, unregulated glucose level, increased glycated hemoglobin level, oxidative and nitrosative stress, and reduced blood flow rate (due to the accumulation of ROS) are some factors which are attributed to the incidence of DN [121, 123]. ROS and reactive nitrogen species reduce blood flow leading to microvascular ischemia, which finally disrupts the function of the nerve [124]. Prolonged hyperglycemia also promotes the production of AGEs which after binding to RAGEs trigger an inflammatory response and enhance oxidative stress, leading to degeneration of Schwann cells. These cells not only insulate neuron but also regulate nerve regeneration, and any oxidation-mediated loss in their function promotes DN among diabetic patients [124, 125].

To develop an efficient therapy against DN, the treatment procedure should address both neurotrophic and angiogenic requirements simultaneously. Considering these requirements, stem cells seem viable and efficient, as they are capable to synthesize neurotrophic, angiogenic, and other essential factors required for regeneration of neuronal and vascular cells. The multilineage potential and adherent nature of MSCs cells helps it to secrete factors which are essential for

neurotrophic and angiogenic effects. Different studies have revealed that MSCs improved DN symptoms in streptozotocin- (STZ-) induced diabetic rats. Though this treatment, VEGF and fibroblast growth factor-2 (FGF2) were increased and the capillary number to muscle fiber ratio in soleus muscles and sural nerve morphometry were improved [126]. In a multiple intravenous MSC treatment in STZ-induced T2DM rats, a controlled hyperglycemia with enhanced serum insulin and C-peptide was found at 9 weeks [127]. Motor and sensory nerve function restored in BMSC-treated STZ-induced diabetic rat [128]. Nerve regeneration has also been demonstrated with combined treatment of human MSCs and poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) in Sprague-Dawley albino rats [129]. These animal-based studies strongly indicate that MSCs should have essential elements to address DN complications. However, lack of established clinical procedures, risk of tumor formation, and lack of understanding of clear mechanism are posing challenges to MSCs' candidacy as a therapeutic agent for DN [120].

7.3. Stem Cells in Diabetic Nephropathy. Diabetic nephropathy (DNP) is responsible for high mortality and a major contributor in end-stage chronic renal disease [130, 131]. Podocytes, the matrix molecule-synthesizing elements in the glomerular basement membrane, are injured and lost in DNP, leading to proteinuria and fibrosis and finally to renal failure. The regeneration capacities of podocytes are limited when injured, and it will adversely affect the glomerular barrier, further aggravating proteinuria [132]. Proteinuria promotes the dysfunction of proximal tubular epithelial cells (PTECs) by increasing fibrosis and tubulointerstitial inflammation, resulting in decreased renal activity [133]. Increase in immune cells in the interstitium is a characteristic feature of DNP [131]. Prolong hyperglycemia, AGEs, and glycated albumin enhance the inflammatory and fibrotic properties of PTECs [134]. AGEs also activate the renin-angiotensin system (RAS), triggering the secretion of ROS thereby increasing the formation of cytokine and growth factors [135]. In an important study, an enhanced DNP symptom in mice was revealed through an increased level of carboxymethyl-lysine (CML) an advanced glycation end product [136]. However, the ESCs, under the presence of required growth factors, including retinoic acid, activin A, BMP-2, BMP-7, and FGF-7, can be differentiated into renal cells [137, 138]. Various studies have also successfully differentiated iPSCs into renal cells to improvise the DNP characteristics [139, 140]. MSCs have also been introduced into an STZ-induced diabetic rat to repair renal damage and regenerate insulin-secreting cells [141, 142], whereas the stromal cell-derived factor (SDF-1) promoted homing of MSCs when released in the kidneys [143]. Nagaishi et al. demonstrated that BM-MSCs inhibited the proinflammatory cytokine, TGF-β1, and fibrosis in tubular interstitium They further revealed exosome-assisted antiapoptotic effect in tight junction structures of tubular interstitial cells indicating improved DNP [130]. The MSCs also exerted regenerative and protective effects in DNP by improvement in fibrosis and glomerulosclerosis, possibly via reducing the loss of podocytes and increased the secretion of BMP-7 [144]. BM-MSC treatment has regulated the serum level of insulin, hemeoxygenase-1, AGEs, and glucose with recovery in renal function [145]. Overall, the role of MSCs in the treatment of DNP is prospective, however, it is limited due to previously discussed hurdles.

8. Stem Cells in Macrovascular and Other Complications

DM patients are prone to atherosclerosis in large arteries finally developing macrovascular complication in the artery. Prolong hyperglycemia and atherosclerosis enhance the risk of myocardial infarction, artery disease, and stroke [98, 146]. CD 133 and CD34 are potent markers of cardiovascular diseases (CVD), and reduction in EPCs is used as an indicator of peripheral artery disease (PAD) [147–149]. Vascular stem cells (VSCs) are capable to differentiate EPCs and are a potential target for treatment of diabetic macrovascular complications. Vascular progenitor cell isolated from human vascular smooth muscle cells under proper condition was able to grow into vascular networks [150]. In a report, Keats and Khan proposed a hypothesis to develop vascular network from CD133+ VSC due to its ability to differentiate into EPCs and MPCs [105]. Further, the interaction between AGEs and RAGEs plays a critical role in the development of macroangiopathy and macrovascular complications [105].

8.1. Stem Cells in Diabetic Cardiomyopathy. Diabetic cardiomyopathy (DCM) is mainly developed due to cellular apoptosis. DCM reduces tissue-specific stem cells, intensifies fibrosis, and decreases perfusion in the capillaries [151, 152]. This complication is characterized by the reduced activity of metalloproteases-2 (MMP-2), high collagen in specific tissue, and upregulated activity of apoptotic factor MMP-9 [98]. However, MSCs have also been implicated in regenerating myocardial cells for restoring normal function of the heart. Specifically, administration of BM-MSCs has shown to improve diabetic myocardium in the T1DM rat by reducing collagen level and activity of MMP-9 [153]. Other stem cells such as ESCs, iPSCs, and cardiac stem cells had also been explored to recover myocardial infarction in animal models [154–156].

Besides, MSCs also induce myogenesis and angiogenesis by releasing various angiogenic, mitogenic, and antiapoptotic factors, including vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), adrenomedullin (AM), and hepatocyte growth factor (HGF) [20]. This was demonstrated using a rat model of DCM [20], wherein intravenously administered rat BM-MSCs improved cardiac function via differentiating into cardiomyocytes and improved myogenesis and angiogenesis. In addition, the activity of MMP-2 was significantly increased, while MMP-9 increased, which led to enhanced myocardial arteriolar density and reduced collagen volume. MSCs also promoted the secretion of Bcl-2, hypoxia-related HOM-1, HSP-20, stromal cell-derived growth factor, and VEGF under hypoxic condition and stimulated neovascularization and restored myocardial function [157–159]. Notably,

the site of injection and cell load has also been considered as determinants for improvement in myocardial infarction during MSC therapy [160].

8.2. Stem Cells in Diabetic Bone. T1DM and T2DM both interfere with normal osteogenic pathways, resulting in elevated risk of bone fractures and reduced ability of fracture healing. Bone-associated complications, affecting osteoblasts and osteoclasts, are mainly attributed to increased levels of AGEs, inflammation, and ROS [161]. AGEs not only block the osteoblastic differentiation and formation of mineralized matrix but also promote apoptosis of osteoblast, leading to impaired bone formation [162, 163]. Interaction between blood vessels and bone cells promotes regeneration and repair of the bone, which is disrupted in a hyperglycemic microenvironment, thereby hindering the repair of bone fracture [164]. Increase in secretion of TNF-α, IFN-γ-inducible protein 10 (IP-10), IL-1β, IL-6, and high-sensitivity C-reactive protein (hsCRP) was also reported after bone fracture in T2DM patients [165]. Current grafting procedures for treatments are limited due to rejection, difficulty in integration, long-term relief, and cost [166]. To overcome these challenges, the tissue engineering approaches have been used in MSCs are considered as leading therapeutic candidates [164]. MSCs are capable to differentiate into osteoblasts and also secrete factors such as VEGF and BMP-4 to promote bone cell regeneration [167, 168]. Studies have also used immortalized BMSCs in osteoarthritic recovery [169]. These studies showed the potential of MSC therapy in bone-associated disorders. However, further studies are still needed to establish a definite role of MSCs in the treatment of these disorders. Furthermore, the role of pluripotent and other adult stem cells in regeneration and repair of bone is also needed to be extensively explored.

8.3. Stem Cells in Wound Healing. Persistent and long-term hyperglycemia disrupts the wound healing capacity of T1DM and T2DM patients leading to chronic wound [170] and increases the risk of opportunistic infections. This chronic condition is developed due to impaired angiogenesis, uncontrolled release of growth factors, and incoherence in the accumulation of collagen matrix [98]. The increased rate of apoptosis of EPC and their numbers among DM patients have already been observed [171]. Additionally, the high level of inflammatory cytokines like TNF-α, CRP, and IL-8 are also found to be associated with poor wound healing capacity. Other factors related to collagen metabolism such as keratinocyte growth factor (KGF), transforming growth factor β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and VEGF are also associated with chronic diabetic wound [172]. However, studies have demonstrated that both the MSCs and EPCs were recruited at the injury site and exerted the healing effect [98]. In a study, the iPSCs showed wound healing in diabetic patients by increasing the level of proangiogenic factors and controlled the activity of protein kinase C delta (PKC-δ) [173]. Another study demonstrated increased collagen accumulation in diabetic fascial wounds of rats, when treated with BM-MSCs which have been ascribed to the secretome of growth factors such as TGF-β, KGF, EGF, PDGF, and VEGF, essential to healing efficacy [174]. These factors also improve cell adhesion and promote an increase in secretion of chemokines at wound site [157, 158]. In various previous studies on animal models, MSC therapy has already been evidenced with an improved wound healing, for which different mechanisms have been explained [104, 174–177].

9. Combinatorial and Coculture Approaches in Stem Cell-Based Therapy of Diabetes and Its Complications

Therapeutic potency of stem cells is still in developmental phase for diabetic treatment, and the interactive effect of other chemical molecules on stem cell-based therapy is needed to be widely screened to improve their efficacy and safety. The pathological state such as diabetic wound healing have limited therapeutic options; however, a therapeutic combinational approach using ADSCs and exendin-4 (Ex-4) significantly improved the wound healing than singleton treatment in diabetic mice [178]. This effect was exhibited through proliferation and migration of endothelial cells and keratinocytes. Another combinatorial effect of MSCs and obestatin significantly improved the pancreatic damage in the T2DM rat model [179]. This was achieved through obestatin-mediated promotion of proliferation of active β-cells or islet-like cell clusters in vitro. Similarly, a study demonstrated the cumulative therapeutic effect of icariin and MSCs towards diabetes-induced erectile dysfunction, where icariin enhanced the therapeutic potential of ADSCs through its antioxidative and antiapoptotic activities [180]. In an interesting study, murine ESCs differentiated rapidly into pancreatic β-cells by using activin A, all-trans retinoic acid and some other factors such as Matrigel [181]. These differentiated cells were able to control the blood glucose level in vivo in the diabetic murine model; however, tumor formation in the kidney limited the use of transplanted cells. Besides, the impaired endothelial progenitor cell (EPC) homing reduce the wound healing ability in the diabetic microenvironment, which is associated with reduced expression of stromal cell-derived factor-1α (SDF-1α). However, the homing of EPCs can be improved at wound site under hyperoxia and via administration of SDF-1α [182]. In a clinical study, the synergistic administration of hyperbaric oxygen and intrapancreatic autologous stem cell was effective in controlling the metabolic level of insulin in T2DM patients [96]. It has also been shown that the preconditioning of the stem cell might improve the efficacy of cell-based therapy. MSCs harvested from diabetic mice were preconditioned in presence of insulin-like growth factor-1 (IGF-1) and fibroblast growth factor-2 (FGF-2) in medium and were further acclimatized under hypoxia and high glucose condition. After implantation of conditioned MSCs, the improvement in heart condition of diabetic mice was observed, indicating stem cell-based strategies to treat diabetic heart failure [183].

Recently, coculture techniques have also been used to improvise the efficacy of stem cells through enhancing their differentiation potential. In a study, the ESCs were cocultured

with hepatocytes and induced to differentiate into endodermal cells, which were further induced to differentiate into pancreatic islet cells in presence of Matrigel and retinoid [184]. Another experimental study showed that differentiated islet cell clusters from human Wharton's jelly-derived mesenchymal stem cells in the presence of rat pancreatic cells could suppress blood glucose level [185]. Cotransplantation of kidney-derived MSCs with islets in diabetic mice has also remodelled islet organization and vascularization and reduced hyperglycemia [186]. Similarly, a seminal study pointed out that the viability of isolated islet was improved, when cocultured with collagen mixed hydrogel (collagen type I, collagen type III, and laminin) [187]. It is of note that the coculture system is used not only in improving therapeutic efficacy of stem cells but also to contemplate the pathogenesis of diabetes. In a conclusive study, a coculture system of BMSCs and macrophage helped to understand that association between local inflammation and immune response promotes diabetic periodontitis, particularly by upregulating the expression of chemokine (C-C motif) ligand 2 (CCL2) and TNF-α in periodontal tissues [188].

10. Gene Editing in Stem Cell for Treatment of Diabetes and Its Complications

Recent developments in gene targeting, editing, and delivery have made it feasible to develop an effective and long-term therapy for the treatment of genetic disorders. Adult stem cells, such as HSCs and MSCs are considered as promising candidates for exploiting gene modification techniques in cell-based regenerative therapy [189–191]. Vectors derived from retroviruses and adenoviruses are most commonly used to transfer the genes in stem cells; however, the chances of random integration might be deleterious. The other limiting factor associated with gene editing is no retaining of the edited gene by stem cells during their ex vivo proliferation. To overcome the limitations of viral vectors genetic control elements such as scaffold attachment region (SAR) and chicken beta-globin locus are added into the vectors to effectively control the gene expression in stem cells [192]. In diabetic mice, the transplanted BM-MSC expressing pancreatic duodenal homeobox 1 (Pdx1) gene differentiated into insulin-releasing β-cell and controlled the glucose level [193]. Similarly, a seminal study showed that the transfected MSCs with vascular endothelial growth factor (VEGF) gene improved the erectile dysfunction in diabetic rats [194]. Though this stem cell-mediated gene therapy demonstrated successful results in rats, it possesses a few limitations as it was carried out only in the T1DM animal model and used adenovirus vector is not considered as a robust gene expression system. In a recent interesting study, the genetically modified human urine-derived stem cells with FGF2 gene significantly improved ED in T2DM SD-rat model [195].

Recent gene editing techniques such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats-associated Cas protein system (CRISPR/Cas) seems promising to understand the role of specific genes in beta cell development and to manipulate the stem cell differentiation into insulin-producing cells [196]. The CRISPR/Cas9 system is currently favoured due to its modularity, flexibility, specificity, reduced toxicity, ease of designing target single-guide RNA (sgRNA) and reduced side effects. Gene-editing techniques have clearly established the role of transcription factor, neurogenin 3 in development of endocrine cells of pancreas, and demonstrated that even low expression of this factor is sufficient to promote the stem cell differentiation into insulin-producing beta cells [197]. Further, the CRISPR/Cas9 mediated deletion of CDKAL1, KCNJ11, and KCNQ1 genes in hESCs disrupted the regulated production of insulin in differentiated beta cells. These recent studies imply that human pluripotent stem cells can be exploited as an effective model to understand molecular development of insulin-producing pancreatic beta cells [196]. Furthermore, the clear understanding of genetic regulation will help in developing and controlling the differentiation of functional beta cells. Notably, gene editing in stem cells also help to escape immune response during transplantation of differentiated cells. This was evidenced in a study in which complete knock out of human leukocyte antigens (HLAs) class-I through disrupting beta 2-microglobulin (β2m) in hESCs maintained the cellular pluripotency level with significantly reduced immunogenicity [198].

11. Conclusions

The diabetic complications are the most prominent reason for high mortality among diabetic patients; therefore, due to proven repair and regeneration potential, the cell-based therapies, including pluripotent and multipotent adult stem cells are currently being considered. This therapeutic approach will not only be helpful to overcome the limitations of contemporary therapy but also provide a long-term cure for diabetes and its complications. However, extensive studies are needed to establish standard procedures for stem cell treatment in diabetic complications.

Conflicts of Interest

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

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Research Article

Effects of High Glucose on the Expression of LAMA1 and Biological Behavior of Choroid Retinal Endothelial Cells

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Hyperglycemia is one of the main causes of proliferative diabetic retinopathy (PDR) characterized by thickening of the vascular basement membrane. Laminin alpha 1 (LAMA1) is a primary component of laminin, a major protein constituent of the basement membrane. In this study, we investigated the role of LAMA1 in the development of PDR. Retinal choroidal vascular endothelial cells (RF/6A line) were exposed to glucose at different concentrations (5 mM, 15 mM, 25 mM, and 35 mM) and analyzed for cell growth, migration, proliferation, and adhesion. LAMA1 expression was examined 24 and 48 h following glucose treatment using Western blotting, RT-PCR, and immunofluorescence. The results showed that the proliferation, migration, and adhesion of RF/6A cells were increased by high glucose, whereas LAMA1 expression was slightly higher at 15 mM but decreased at 25 mM and 35 mM glucose compared to control. Thus, the changes in the biological behavior of high glucose-exposed retinal vascular endothelial cells correspond to variations in LAMA1 expression, indicating a possibility for LAMA1 involvement in PDR development. Our findings suggest that LAMA1 may play a role in PDR and, thus, may serve as a potential target for DR diagnosis and/or treatment.

1. Introduction

Diabetic retinopathy (DR) is one of the major microvascular complications caused by long-term hyperglycemia, and is also one of the most serious causes of blindness worldwide [1]. It is estimated that about one third of diabetic patients can develop DR [2] as a result of long-term accumulation of damaged small retinal vessels due to chronically poor blood glucose control [3]. Hyperglycemia is associated with the formation of advanced glycation end products, oxidative stress, inflammation, and neovascularization. These processes induce changes in the microvascular system, such as increased synthesis of extracellular matrix (ECM) proteins and thickening of the capillary basement membrane, which are the main pathological features of diabetic microangiopathy [4, 5].

DR is characterized by endothelial cell dysfunction, basement membrane thickening, increased fibrogenesis, and retinal detachment caused by fiber membrane retraction; in the advanced or proliferative (PDR) stage, blood vessels proliferate, resulting in neovascularization [6]. The thickened basement membrane is considered to be the consequence of upregulated expression of vascular basement membrane components such as type IV collagen, fibronectin, and laminin [5]. Oxidative damage is an important factor in endothelial cell dysfunction, and high glucose can cause excessive generation of reactive oxygen species (ROS) and activation of the protein kinase C (PKC)-beta pathway, leading to increased expression of ECM proteins and vascular endothelial growth factor (VEGF), and changes in endothelial cell permeability [7]. The ECM provides a scaffold required for vascular endothelial cells to form blood vessels, whereas adhesion to specific ECM components via integrins triggers intracellular signaling critically required for endothelial cell proliferation, migration, survival, and tube morphogenesis [8]. Thus, changes in the ECM are

involved in endothelial dysfunction, basement membrane thickening, and neovascularization.

Laminin is one of the major ECM components participating in basement membrane formation. By binding to cell surface receptors, laminin induces different signal transduction pathways, which influence cell behavior such as adhesion, differentiation, migration, phenotype stability, and resistance to anoikis [9]. A previous study has shown that serum laminin can be used as a biomarker of DR associated with vascular endothelial dysfunction [10]. Laminin is a trimeric glycoprotein consisting of three chains, alpha, beta, and gamma and has been found in at least 16 different isoforms [11]. It was reported that the expression of laminin chains is regulated both spatially and temporally [12], suggesting that different laminin isoforms have distinct functional roles. A number of laminin isoforms are expressed in the basement membrane of retinal blood vessels and where they could influence vascular development [13].

Laminin α 1 (LAMA1), the most conserved subunit in all laminin isoforms, plays an essential role in embryonic development and promotes neurite outgrowth [14]. In mice, LAMA1 was shown to be expressed in the internal limiting membrane (ILM) between the retina and the vitreous body and in Bruch's membrane of the choroid, and it was reported that LAMA1 deficiency could cause abnormal cell adhesion and migration, leading to altered retinal angiogenesis, persistent vitreal fibroplasias, epiretinal membrane formation, and peripheral retinal degeneration [15, 16]. Epiretinal membranes were described in both of these mutants (LAMA1 knockout mice) which had characteristics similar to human persistent fetal vasculature (PFV) and proliferative vitreoretinopathy (PVR).

The aim of this study was to examine the role of LAMA1 in the development of PDR. As vascular endothelial cells play an important role in DR development [6], we used retinal choroidal vascular endothelial cells, which were exposed to different glucose concentrations and analyzed for cell behavior and LAMA1 expression.

2. Materials and Methods

2.1. Reagents and Antibodies. Rabbit anti-human LAMA1 antibody (sc-5582) was from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse anti-human GAPDH antibody, goat anti-rabbit IgG H&L (ab150077), and goat anti-mouse IgG H&L were purchased from Abcam (Cambridge, MA, USA). TRIzol reagent and DAPI (4′,6-diamidino-2-phenylindole) were obtained from Invitrogen (Carlsbad, CA, USA). The PrimeScript RT reagent kit with gDNA Eraser and SYBR Premix Ex Taq™ II (Tli RNaseH Plus) kit were from Takara Clontech (Kyoto, Japan). Cell Counting Kit-8 (CCK-8) was from Dojindo (Kumamoto, Japan), and 4-well culture inserts were from Ibidi (cat. number 80469; Martinsried, Germany).

2.2. Cell Culture. Rhesus choroid retinal endothelial cells (RF/6A line; Chi Scientific, Jiangsu, China) were cultured in DMEM (Gibco, Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified incubator with 95% air-5% CO_2. Cells reaching at least 80% confluence were lifted by treatment with 0.25% trypsin/0.02% EDTA and passaged. To model hyperglycemic conditions, cells were grown in medium supplemented with 15 mmol/L, 25 mmol/L, and 35 mmol/L glucose for different times; control cells were grown at 5 mmol/L glucose.

2.3. Western Blotting. LAMA1 protein expression was assessed by Western blotting. RF/6A cells were washed twice with PBS and lysed with cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Shanghai Beyotime Biotech Co., China). Lysed cells were collected using a cell scraper, transferred to Eppendorf tubes, and centrifuged for 30 min at 12,000 rpm (4°C) to obtain supernatants. A BCA protein assay kit (Shanghai Beyotime Biotech Co.) was used to determine protein concentrations. Protein samples (20 μg protein/well) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% BSA for 2 h at room temperature. After that, the membranes were incubated with antibodies to LAMA1 (1 : 1000) overnight, washed three times with Tris-buffered saline containing 0.05% Tween 20 for 30 min each, and incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. Specific bands were visualized using Immobilon Western Chemiluminescent HRP Substrate reagents (Millipore Corporation, MA, USA) in a trans-illuminator (ChemiDoc; Bio-Rad, Hercules, CA, USA). Band densities were quantified using the Image-Pro Plus software.

2.4. Real-Time PCR. LAMA1 mRNA expression in glucose-treated RF/6A cells was detected by real-time PCR. Total RNA was extracted using TRIzol according to the manufacturer's instructions, and its concentration was assessed by spectrophotometry; an $A_{260}/_{280}$ ratio of 1.8–2.0 was considered acceptable. cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's protocol and used as a template for PCR amplification. LAMA1 primers were synthesized by Sangon Corporation (Shanghai, China). Real-time PCR (SYBR Green) was performed according to the manufacturer's instructions in a LightCycler 480 system (Roche, Basel, Switzerland) in a volume of 20 μL containing SYBR Premix Ex Taq II (Takara RR820A), cDNA, and specific primers. Relative mRNA expression of LAMA1 was quantified by the comparative $2^{-\Delta\Delta ct}$ method after normalization to that of GAPDH. Primer sequences are shown in Table 1.

2.5. Immunofluorescence Staining. RF/6A cells were seeded onto glass coverslips, incubated at 37°C and 5% CO_2 until 80% confluence, fixed with 4% paraformaldehyde for 15 min at room temperature, washed three times with PBS, permeabilized with 0.1% Triton X-100 for 4 min, blocked in 3% BSA for 1 h, and incubated with anti-LAMA1 antibodies (1 : 50) overnight at 4°C. Cells were washed three times in PBS and incubated with goat anti-rabbit secondary antibodies for 1 h at 37°C followed by

Table 1: Primer sequences used for PCR.

Gene	Sequence	Length
LAMA1 (forward)	5′- GTT TCG AAC CTC CTC GCA GA-3′	88 bp
LAMA1 (reverse)	5′- CTT GCC GTC CAC AAG CTC TAG T-3′	
GAPDH (forward)	5′- GAT TCC ACC CAT GGC AAA TT-3′	103 bp
GAPDH (reverse)	5′- TCT CGC TCC TGG AAG ATG GT-3′	

DAPI staining for 10 min to visualize nuclei. Fluorescence images were obtained under a fluorescence microscope (model DM6000; Leica, Wetzlar, Germany).

2.6. Cell Growth Assay. RF/6A cells were cultured in normal glucose medium (5 mM) for 24 h to allow cell adherence; then, the medium was removed, and cell monolayers were washed once with PBS and incubated in medium supplemented with different concentrations of glucose. The bottom was marked to determine the position of observed cells; the growth of cells at the same position was observed at different time points (0, 1, 2, 3, and 4 days) under a microscope (Axio Observer A1; Carl Zeiss, Oberkochen, Germany).

2.7. Cell Proliferation Assay. Cell proliferation was measured using the CCK-8 method in accordance with the manufacturer's protocol. Briefly, RF/6A cells (7×10^3 cells/100 μL/well) were seeded into 96-well plates overnight and then cultured at different glucose concentrations for 96 h; normal glucose (5 mM) served as control. Cell proliferation was assessed every 24 h after addition of 10 μL of the CCK-8 solution for 90 min at 37°C and subsequent measuring of absorbance at 450 nm using a microplate reader (Synergy H1; BioTek, Winooski, VT, USA). The optical density reflected the proliferation of RF/6A cells. Each experiment was performed in five replicates, and detection was repeated three times per group to ensure accuracy of measurements. The experiment was repeated independently three times, and the mean values obtained in each experiment were used for statistical analysis.

2.8. Wound Healing Assay. Cell migration ability was assessed using wound healing assay by 4 Well Insert (Ibidi cat. number 80469). The Culture-Insert 4 Well consists of four wells, representing the four quarters of the round Culture-Insert. The wells are separated by a wall of 500 μm. When the wells are filled with adherent cells, a cell-free gap of approx. 500 μm is created between the adjacent wells after removing the Culture–Insert 4 Well. RF/6A cells (4×10^5 cells/mL) were plated into the four wells of a culture insert located at the center of a 35 mm culture dish. After 24 h, when the cells reached 90% confluence, the culture insert was removed to reveal the wound gap, and micrographs (baseline time 0) were taken at ×10 magnification using a digital camera attached to an inverted microscope (Axio Observer A1). Then, cells were washed with PBS to remove floating cells, fresh medium containing different glucose concentrations (5 mM, 15 mM, 25 mM, or 35 mM) was added, and cells were cultured for 72 h. Wound healing was monitored by taking micrographs at the same coordinates every 24 h. Quantitative analysis was performed by measuring the gap size of the wound using the Image-Pro Plus software and the results were presented as the percentage of the wound coverage; complete coverage was defined as 100%. Experiments were repeated three times.

2.9. Cell Adhesion Assay. Cell adhesion ability was measured using the modified CCK-8 method. Cell suspension (7×10^4 cells/mL) was prepared in medium containing different concentrations of glucose, and 100 μL of cell suspension was placed into each well of 96-well plates, which were then placed in 37°C incubator for 2, 4, 6, and 8 h. Then, cell monolayers were washed twice with PBS to remove unattached cells, and 100 μL of fresh culture medium containing 10 μL CCK-8 solution was added for 2 h. The assay was performed with five replicates. The absorbance was measured in a microplate reader; higher absorbance values reflected higher number of adhered cells.

2.10. Statistical Analysis. Each experiment was repeated at least three times and the data are presented as the mean ± standard deviation (SD). To assess the significance of differences between groups in multiple comparisons, one-way ANOVA was used; two-sided P values less than <0.05 indicated statistical significance. All statistical analyses were performed using the Prism 6.0 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effects of High Glucose on the Growth of RF/6A Cells. RF/6A cells were cultured with different glucose concentrations and their growth was analyzed at 1, 2, 3, and 4 days after treatment. After 1 day of culture, no significant difference in cell growth was observed compared with control (normal glucose concentration, 5 mM); however, further culture in hyperglycemic conditions induced cell growth in a glucose concentration-dependent manner (Figure 1).

3.2. Effects of High Glucose on LAMA1 Expression in RF/6A Cells

3.2.1. Localization of LAMA1 Expression. Immunocytochemistry analysis indicated that LAMA1 was expressed mainly in the cytoplasm. LAMA1 expression was not affected by the treatment with 15 mM glucose for 24 h and 48 h, but was reduced by that with 25 mM and 35 mM glucose compared with control (5 mM) (Figure 2).

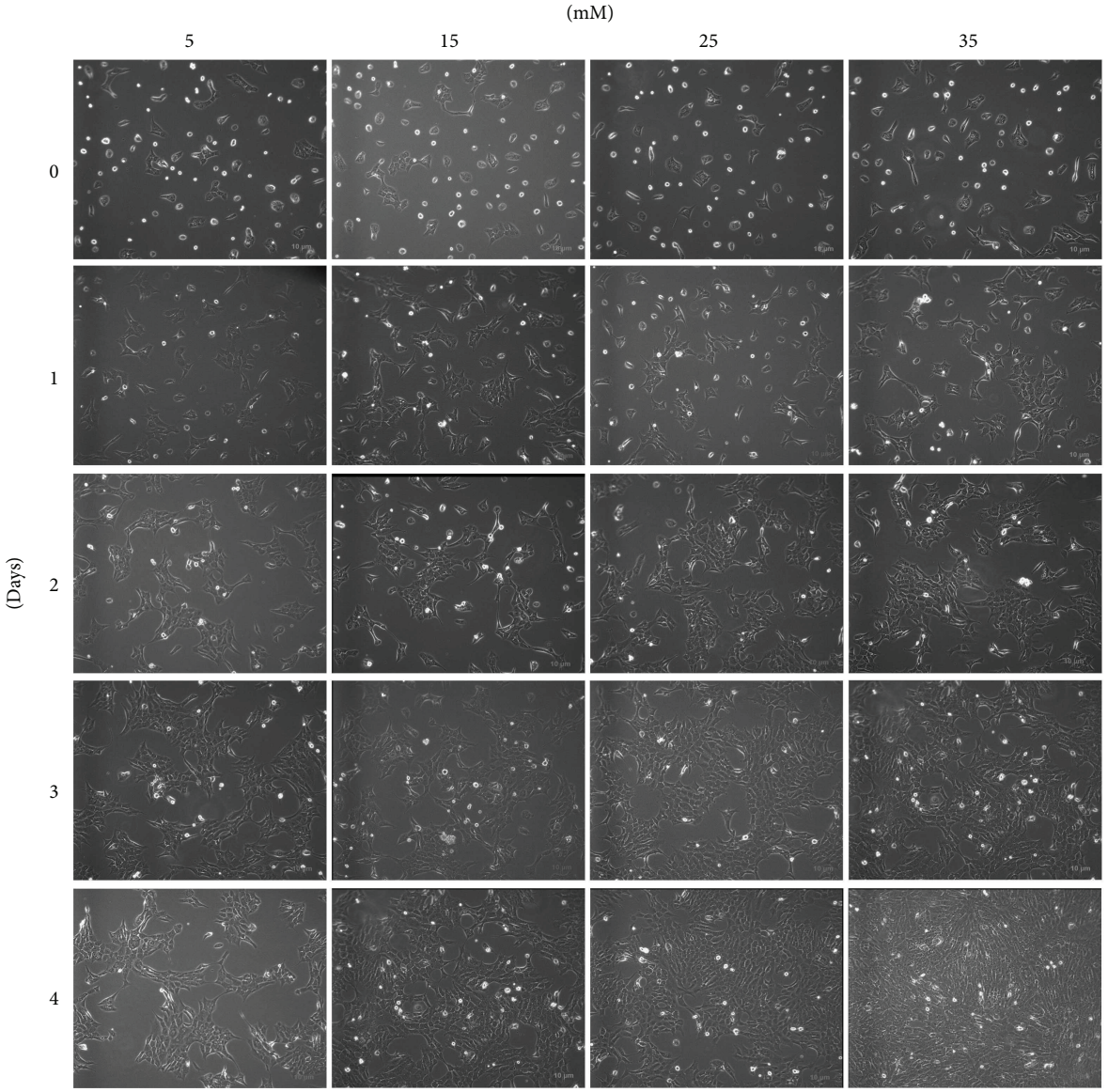

Figure 1: Effects of high glucose on the growth of RF/6A cells. RF/6A cells were grown in medium supplemented with different concentrations of glucose for the indicated times.

3.2.2. LAMA1 Protein Expression. The expression of LAMA1 protein was examined by Western blotting, which showed that 15 mM glucose slightly induced LAMA1 expression, but the difference with control (5 mM) was not statistically significant ($P > 0.05$). However, the increase in glucose concentration significantly downregulated LAMA1 protein expression, especially in the 35 mM group ($P < 0.05$), and the effect was more pronounced after 48 h exposure to high glucose (Figure 3).

3.2.3. LAMA1 mRNA Expression. Real-time PCR analysis of LAMA1 transcription in RF/6A cells showed that the exposure to 35 mM glucose significantly decreased LAMA1 mRNA levels compared to control (5 mM) ($P < 0.05$). There was also a slight decrease by 25 mM glucose, but the difference with control did not reach statistical significance ($P > 0.05$). These results are consistent with LAMA1 protein expression detected by Western blotting (Figure 4).

3.3. Effects of High Glucose on the Migration Ability of RF/6A Cells. The effect of high glucose concentration on retinal endothelial cell migration ability was analyzed by the wound healing assay. The results showed that wound healing was accelerated by high glucose in a concentration-dependent manner, indicating the induction of cell migration ability. At 24 h, the healed area was increased to $37.12 \pm 4.83\%$ (15 mM), $45.52 \pm 4.37\%$ (25 mM), and $49.77 \pm 2.35\%$ (35 mM) compared to $26.4 \pm 5.91\%$ in the control group (5 mM), and the difference was statistically significant ($P < 0.05$). At 48 h, the healed area was increased to $63.42 \pm 2.98\%$ (15 mM), $75.01 \pm 3.49\%$ (25 mM), and $80.79 \pm 5.53\%$ (35 mM) compared to $44.96 \pm 4.56\%$ in the control

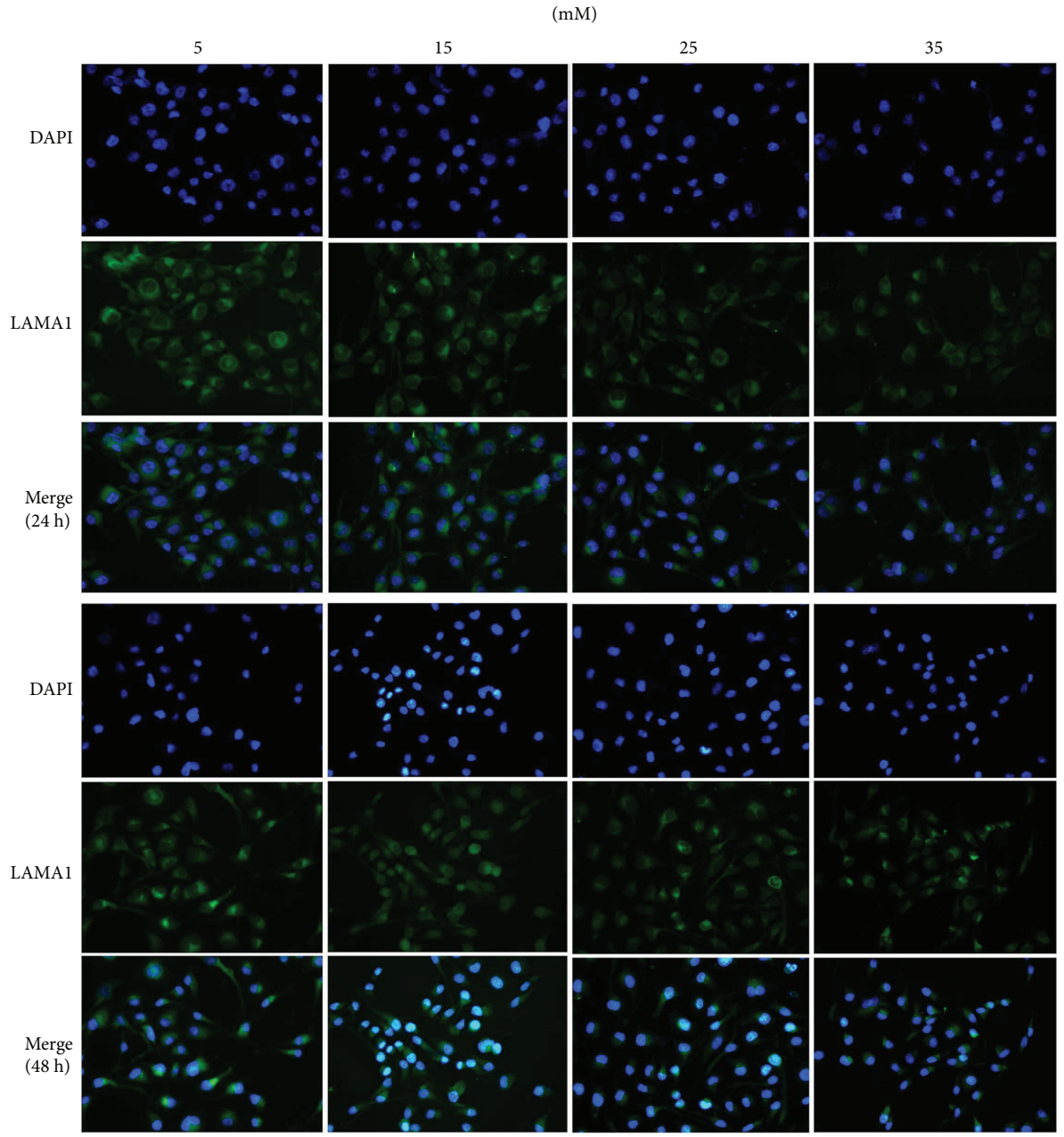

Figure 2: Immunocytochemistry analysis of LAMA1 expression in RF/6A cells treated with different concentrations of glucose for 24 and 48 h.

group (5 mM), and the difference was statistically significant ($P < 0.05$). After 72 h, the healed area in the control group was 71.44 ± 5.29, which was significantly lower ($P < 0.05$) than that in high glucose groups (100%) (Figure 5).

3.4. Effects of High Glucose on the Proliferation of RF/6A Cells. The CCK-8 assay showed that RF/6A cell proliferation in high glucose-supplemented medium was induced compared to control (5 mM), which was consistent with microscopic observations of cell growth. At 24 h, there was no statistically significant difference between the groups. However, at 48 h, an increase in cell proliferation compared to control was detected in the 25 mM group ($P < 0.05$), whereas at 72 h, it was revealed in the 25 mM and 35 mM groups ($P < 0.05$), and at 96 h, it was observed in all cells exposed to high glucose ($P < 0.05$) (Figure 6).

3.5. Effects of High Glucose on the Adhesion Ability of RF/6A Cells. The adhesion of cells cultured at the elevated glucose concentrations showed a trend for increase compared to control, and the difference between control and 25 mM and 35 mM glucose-treated cells was statistically significant at 6 and 8 h ($P < 0.05$; Figure 7).

4. Discussion

DR is one of the leading complications of diabetes worldwide and the primary cause of blindness in developed countries

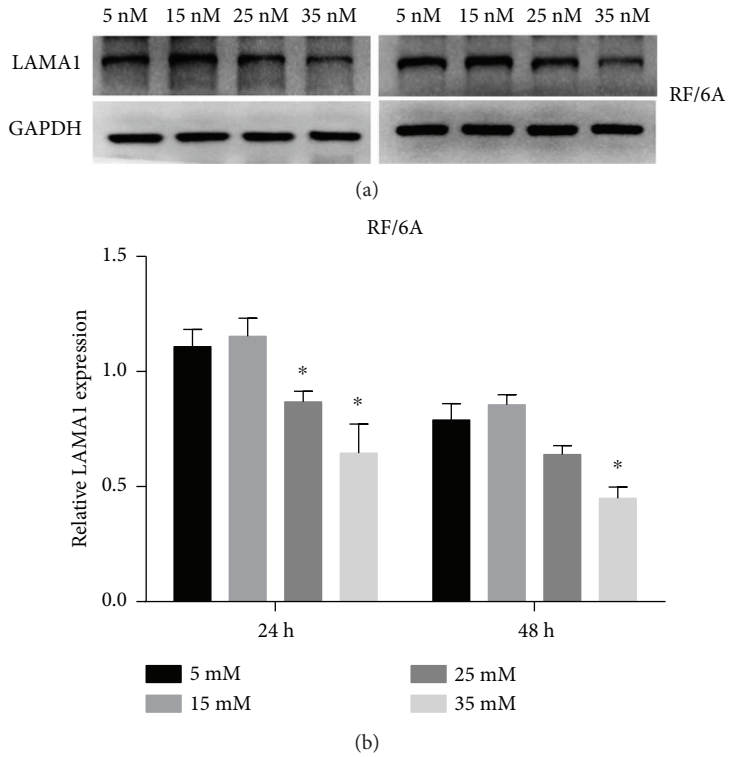

Figure 3: Expression of LAMA1 protein in RF/6A cells. RF/6A cells were treated with different concentrations of glucose for 24 and 48 h and LAMA1 expression was analyzed by Western blotting; GAPDH was used as loading control. (a) Representative gel images. Left, 24 h; right, 48 h. (b) Quantitative analysis of LAMA1 expression performed using the ImageJ software. The data are presented as the mean ± SD of three independent experiments; $^*P < 0.05$.

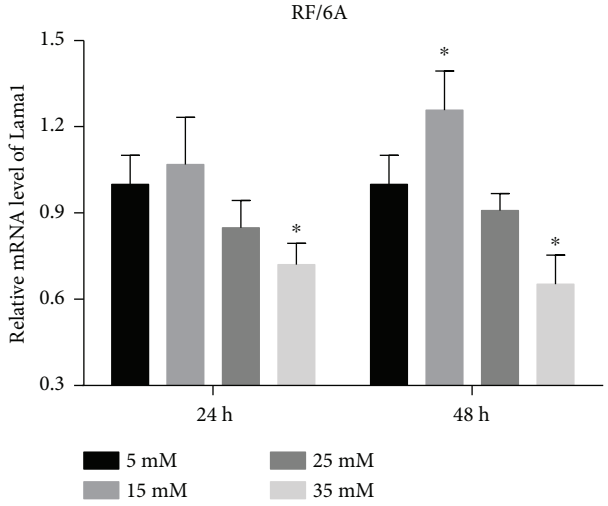

Figure 4: LAMA1 mRNA expression. RF/6A cells were treated with different concentrations of glucose for 24 and 48 h and analyzed for LAMA1 mRNA levels by real-time PCR. There was a significant decrease of LAMA1 mRNA expression in cells exposed to 35 mM glucose both at 24 and 48 h. The data are presented as the mean ± SD of three independent experiments; $^*P < 0.05$.

[17]. DR incidence is expected to grow in the next few years, and the disease is predicted to affect nearly 600 million people by the year 2035 [18]. However, the exact pathogenesis of DR is still unclear and is considered to be induced through several mechanisms: (1) polyol pathway, (2) advanced glycation end products, (3) PKC activation, (4) genetic factors, (5) inflammation, and (6) oxidative stress [6, 19]. Each mechanism can cause endothelial cell dysfunction, resulting in a series of physiological and biochemical abnormalities such as retinal ischemia, vascular permeability change, macular edema, VEGF upregulation, and neovascularization, which eventually lead to PDR [20]. Therefore, it is important to study the reaction of retinal endothelial cells to hyperglycemic condition characteristic for diabetes.

In recent years, research on PDR has been focused on the role of growth factors, especially VEGF-A, in ocular angiogenesis [21, 22], whereas less attention has been paid to the ECM, which is important for the development of retinal vessels and pathological neovascularization [8, 23]. Endothelial cells secrete proteases that degrade the original vascular basement membrane and cause its components to diffuse from the blood vessels to the surrounding ECM [24]. Laminin is one of the important ECM components that, together with collagen, form the basement membrane. In 1992, Ingber et al. [25] found that interactions between endothelial cells through basement membrane components play a critical role in angiogenesis. Among ECM proteins, LAMA1 is highly expressed in the eye structures, especially in retinal vessels and the lens [26]. Interestingly, several studies indicate that high glucose can induce the secretion of ECM proteins (collagen IV, fibronectin, and laminin) through signaling pathways such as TGF-β/Smad and PI3K/AKT [27–29];

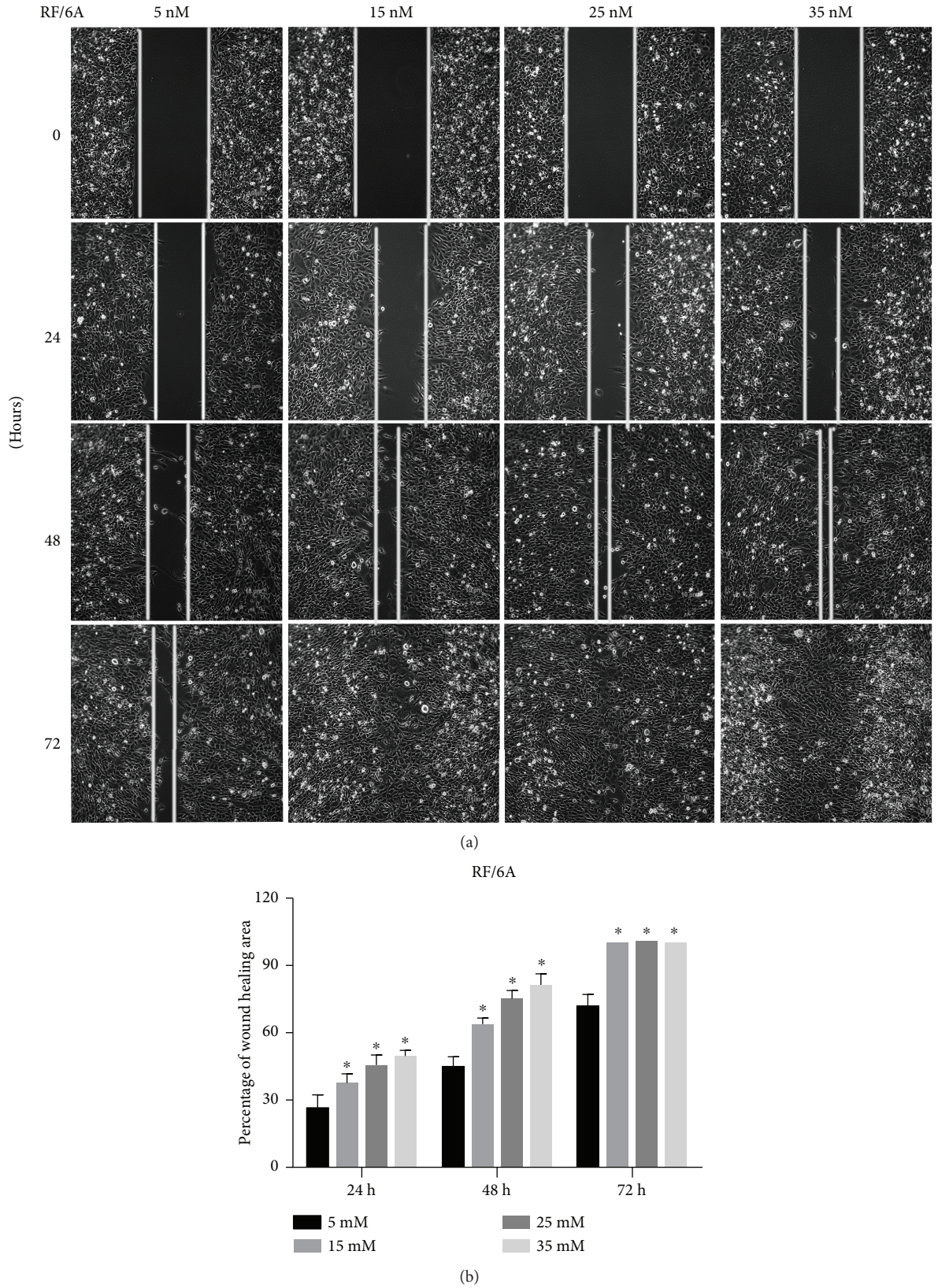

Figure 5: Cell migration ability. RF/6A cells grown at different concentrations of glucose for 24, 48, and 72 h were analyzed by the wound healing assay. (a) Representative microscopic images of wound healing. (b) Quantitative analysis of the gap size measured using the Image-Pro Plus software; $^{*}P < 0.05$.

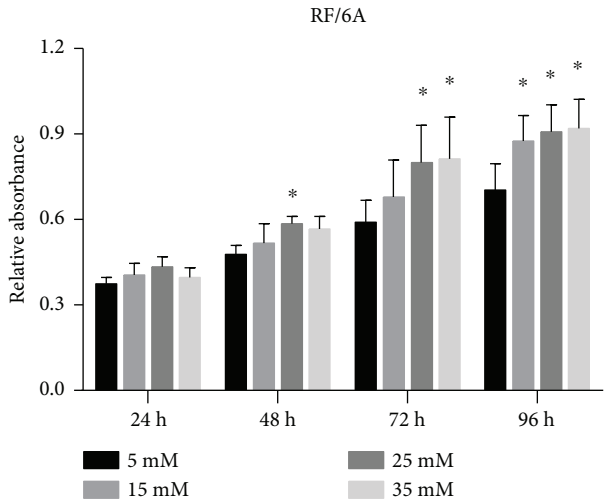

Figure 6: Proliferation of RF/6A cells exposed to high glucose concentrations. RF/6A cells grown at different concentrations of glucose for 96 h were analyzed for cell proliferation every 24 h using the CCK-8 assay. The data are presented as the mean ± SD of three independent experiments; $^*P < 0.05$.

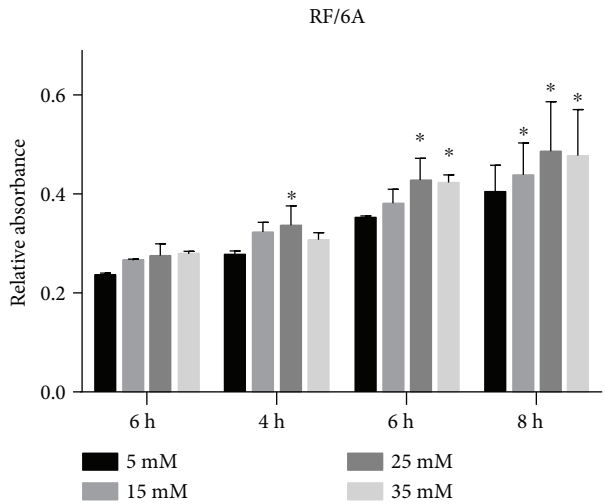

Figure 7: Adhesion ability of RF/6A cells exposed to high glucose. RF/6A cells suspended in medium containing different concentrations of glucose were seeded in 96-well plates and analyzed for adhesion after indicated times using the modified CCK-8 method. The data are presented as the mean ± SD of three independent experiments; $^*P < 0.05$.

however, our results showed that at the increase of glucose concentration, LAMA1 expression was only slightly upregulated and then showed a marked downward trend. Ning et al. [30] found that LAMA1 plays a critical role in the function and aging of the kidneys by regulating the mesangial cell population and mesangial matrix deposition through TGF-β/Smad signaling. TGF-β is a secreted signaling molecule with a fibrogenic effect that regulates diverse cellular processes, including proliferation, differentiation, migration, and apoptosis, and is being recognized as an important contributor to DR pathogenesis [31]. As in vitro and in vivo studies indicate that LAMA1 deficiency promotes mesangial cell proliferation and that this pathological change is very similar to diabetic nephropathy [30], we hypothesize that hyperglycemia can influence the proliferation of retinal endothelial cells through the LAMA1-TGF-β axis. LAMA1 expression could be first increased by high glucose through a compensatory mechanism and then start to decline, which would negatively regulate TGF-β signaling, inducing cell proliferation and migration. However, our present findings cannot confirm this hypothesis, which should be tested in further studies. The decrease of LAMA1 expression in RF/6A cells under high glucose conditions suggests that LAMA1 may serve as a protective factor in DR. Studies in mice indicate that mutations in the Lama1 gene promoted the formation of vitreoretinal blood vessels and the epiretinal membrane, supported persistence of fetal vasculature, and could cause vitreal fibroplasia and abnormal vascular development [15, 32]. Taken together, these and our data indicate that LAMA1 deficiency plays a role in DR pathogenesis.

Our study shows that high glucose promotes the growth and proliferation of endothelial cells and accelerates their migration, providing a material basis for angiogenesis and fiber proliferation. These results are consistent with a report by Beltramo et al. [33] that in the early stages of DR, hyperglycemia can cause a loss of pericytes, reducing their association with endothelial cells and increasing vascular permeability and endothelial cell proliferation, which ultimately leads to neovascularization. In contrast, other studies showed that high glucose inhibited endothelial cell proliferation and promoted apoptosis, causing endothelial damage, which results in diabetes-related vascular complications [34, 35]. However, it is well known that hyperglycemia is the main cause of PDR characterized by pathological neovascularization due to cell proliferation. Therefore, diverse effects of high glucose on endothelial cells suggest the existence of multiple molecular mechanisms underlying the development of PDR.

In summary, our study indicates that glucose at high concentration downregulates the expression of LAMA1, which corresponds to increased adhesion, proliferation, and migration of choroid retinal endothelial cells, suggesting that LAMA1 may exert a protective effect against DR. Thus, LAMA1 may be used as a new target for diagnosis and/or treatment of DR, providing a new direction for DR clinical research. Further studies are required to address specific molecular mechanisms and signaling pathways underlying LAMA1 role in DR development.

Data Availability

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

Conflicts of Interest

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

Authors' Contributions

Guangwei Song and Da Lin contributed equally to this work.

Acknowledgments

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Research Article

Prevalence of Chronic Complications, Their Risk Factors, and the Cardiovascular Risk Factors among Patients with Type 2 Diabetes Attending the Diabetic Clinic at a Tertiary Care Hospital in Sri Lanka

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Diabetes incurs heavy burden to patients and the healthcare system. Assessment of disease burden is important in taking necessary precautions and management decisions. We aimed to determine the prevalence of macro- and microvascular complications, their risk factors, and coronary artery disease (CAD) risk factors among patients with type 2 diabetes mellitus (T2DM). A descriptive cross-sectional single-centre study was carried out among 3000 patients with T2DM attending the diabetic clinic at the National Hospital of Sri Lanka from January to July 2016. The study population had 72.7% females and 27.3% males. Mean age and disease duration were 58.3 ± 10.3 and 10.8 ± 7 years, respectively. Prevalence of CAD, stroke, and peripheral vascular disease were 10.6%, 1.1%, and 4.7% while diabetic retinopathy, neuropathy, nephropathy, diabetic foot, and lower extremity amputation (LEA) were 26.1%, 62.6%, 50.8%, 2.6%, and 1.3%, respectively. Prevalence of overweight/obesity, hypertension, dyslipidemia, and smoking were 80%, 77.6%, 76.7%, and 11%, respectively. Increased age, disease duration, and HBA1c were risk factors for microvascular disease and diabetic foot while age was the only risk factor for macrovascular complications. Occurrence of CAD, peripheral neuropathy, diabetic foot, and LEA was significantly higher among males than when compared to females. This study highlights the major burden of chronic complications and high prevalence of CAD risk factors in this population.

1. Introduction

The global epidemic of diabetes has become one of the biggest challenges to mankind in the 21st century. The International Diabetes Federation in a recent report estimated that in 2011, 366 million people worldwide had diabetes, and if the trend continues by 2030, 552 million people or one in ten adults will suffer from diabetes [1]. The Western Pacific region was shown to have the largest number of people with diabetes (132 million) followed by the Southeast Asian region (71.4 million) [1]. The unprecedented rise in diabetes in the South Asian region has had its toll in Sri Lanka as well. According to statistics in 2006, one in five adults in Sri Lanka was diabetic or prediabetic with one-third of those with diabetes being undiagnosed [2]. South Asians are at risk of increased visceral adiposity, insulin resistance, and impaired

β cell function and are genetically predisposed to diabetes [3–5]. Additionally, economic development following the ending of the 30-year-old war in Sri Lanka has led to rapid urbanization causing decreased physical activity and consumption of fast food causing amplification of this risk and increasing the incidence of this disease.

Chronic hyperglycemia results in multisystemic complications of the eyes, nerves, kidneys, heart, and blood vessels. The disease burden of diabetes is mainly attributed to the morbidity and mortality associated with microvascular and macrovascular complications. The UK prospective study done among patients with type 2 diabetes showed that intensive glycemic control reduces the risk of development of micro- and macrovascular complications [6]. However, a significant number of patients harbor these complications as well as other metabolic risk factors even prior to the diagnosis of diabetes [7]. Newly diagnosed South Asians with diabetes have a higher prevalence of these vascular complications at the time of diagnosis when compared to European macrovascular complications (15.7% versus 9.4%) and microvascular complications (27.3% versus 16.5%) [8]. Poor glycemic control and duration of diabetes seem to be the strongest risk factors for the development of vascular complications while other factors such as hypertension, dyslipidemia, obesity, smoking, age, and genetic factors all contribute. It is also notable that the incidence of CAD among South Asians is higher when compared to that among Europeans [9]. In a study done among diabetic patients in the United Kingdom (UK), South Asians were 3.8 times more likely to develop myocardial infarction [10]. In contrast, the UK prospective study 32 did not detect a significant difference in the rates of CAD among South Asians and white Europeans [11]. However, according to the study authors, this finding could be due to potential biases. In another large study done in Northern California, there was similar incidence between South Asians and Whites but much higher than the other ethnic groups [12]. Larger and more long-term studies are required to determine if the actual CAD risk is higher among South Asians. Several other studies have demonstrated that South Asians with diabetes have a higher mortality rate of CAD when compared to other ethnic groups [10, 13–15]. However, on the other hand, a large study done in Canada reported that South Asians had a lower mortality rate compared to their Canadian counterparts [16]. In a large multinational study on the prevalence of diabetes complications by Litwak et al., macrovascular complications were reported in 23.3% and microvascular complications in 39% among the patients in South Asia [17]. Surprisingly, Russia had the highest prevalence of 72% for macrovascular and 89% for microvascular complications. European and North American countries were not taken into consideration in this study. A large study done in Sri Lanka in 2012 among patients with diabetes attending an outpatient clinic in the Western Province reported prevalence of CAD (5.6%), peripheral vascular disease (PVD) (0.5%), neuropathy (28.4%), nephropathy (20.4%), and retinopathy (25.7%) [14]. Another small study done in Sri Lanka among 147 inward patients in 2012 reported prevalence of CAD, stroke, PVD in 52.6%, 6.2%, and 4.1% and neuropathy, nephropathy, and retinopathy in 31%, 19%, and 28.7%, respectively [18]. However, the difference in prevalence could be due to different patient populations and sample sizes. Patients who are inward are probably more likely to have a higher disease burden from chronic complications. In another study done in Jaffna (Northern Province) among 8400 outpatients, CAD was present in 21.1%, stroke in 3.9%, peripheral neuropathy in 34.1%, and nephropathy in 39.5% [19]. In a study done in the Eastern Province, the prevalence for heart disease, renal impairment, and eye disease among patients with diabetes was 22.5%, 22.5%, and 56%, respectively [20]. However, the variables have not been clearly defined in this study.

Micro- and macrovascular complications cause many disabilities to the patient leading to reduction of quality of life and incur a heavy burden on the free healthcare system. Diabetes is a major global cause of premature mortality that is widely underestimated. CAD is the biggest killer and is the cause for mortality in more than 70% of diabetics [21]. This is the reason for the change in focus from managing only hyperglycemia to managing hyperglycemia along with the other cardiovascular risk factors. It is apparent that evidence on the prevalence of diabetes-related complications is essential for the adjustment of policies and practices in diabetic care management. Screening for macro- and microvascular complications will have important implications for understanding the need of vigorous screening and for planning out effective preventive and management strategies in order to lessen the burden of this chronic debilitating disease on healthcare resources and expenditure. Therefore, it is important that such prevalence studies are done from time to time to detect the changing trends in order to plan out the course of action. Even though many such studies have been done in different parts of the country, a comprehensive study has not been done recently among the outpatients in the diabetic clinic at the National Hospital of Sri Lanka, which caters to a large patient population in the capital city and its suburban areas. Therefore, the aim of this study was to assess the prevalence of macro- and microvascular complications and their association with possible risk factors and to describe the cardiovascular risk factors in a large cohort of patients with type 2 diabetes.

2. Methods

This was a descriptive cross-sectional single-centre study carried out at the National Hospital of Sri Lanka during the time period of 1 January 2016 to 31 July 2016. The National Hospital is the biggest hospital in Sri Lanka with a bed strength of more than 3000. Around 16,000 patients are registered in the diabetes clinic, and around 400 patients a day attend the clinic to seek care. It is a tertiary care referral centre and caters mainly to diabetic patients in Colombo and its suburbs. A total of 3000 patients with type 2 diabetes (T2DM) were systematically sampled. Pregnant patients, patients with gestational diabetes, and patients with type 1 diabetes were excluded. All patients included in the study were screened for vascular complications.

Sociodemographic data was recorded by specially trained data collectors. Height, weight, and blood pressure were

Table 1: Metabolic profile and its significance to the study population by gender.

Variable	Females ($n = 2180$) Mean ± (SD)	Males ($n = 820$) Mean ± (SD)	Total ($n = 3000$) Mean ± (SD)	T value	p value
Basal metabolic index (BMI)	26.5 ± (4.5)	24.8 ± (4.5)	26.3 ± (4.6)	1.06	0.289
HBA1c (%)	8.46 ± (1.8)	8.0 ± (3.7)	8.3 ± (2.5)	2.85	0.04
Fasting blood sugar (mg/dl)	159.6 ± (35.5)	127.9 ± (35.5)	137 ± (40.1)	2.80	0.005
Postprandial blood sugar (mg/dl)	162.6 ± (30.1)	156.0 ± (29.2)	161.1 ± (32.5)	0.92	0.35
Systolic blood pressure (mmHg)	131.5 ± (20.1)	128 ± (10.1)	130 ± (19.9)	3.92	0.001
Diastolic blood pressure (mmHg)	79.4 ± (10.2)	79.1 ± (11.8)	79.4 ± (10.7)	0.29	0.77
Low-density lipoprotein (mg/dl)	99.7 ± (9.1)	98.8 ± (18.7)	99.78 ± (14.10)	1.83	0.06
High-density lipoprotein (mg/dl)	47.8 ± (10.8)	45.8 ± (10.5)	46.93 ± (10.5)	1.35	0.17
Triglyceride (mg/dl)	127.8 ± (35.3)	118. 1 ± (29.5)	127.92 ± (35.0)	1.61	0.10

measured and recorded by trained health staff. Screening for diabetic complications and extracting disease-related data from the health records were performed by specially trained doctors in the diabetes clinic.

2.1. Baseline Data Definitions

2.1.1. Type 2 Diabetes. Patients were diagnosed with diabetes according to the American Diabetes Association criteria of fasting blood glucose of over 126 mg/dl or a value of >200 mg/dl in the 2 hr value in the oral glucose tolerance test. Two values were used if the patient was asymptomatic, and one value was used if the patient was symptomatic for diagnosis. Patients who had been diagnosed for at least 3 months were included. Patients with other forms of diabetes such as type 1 diabetes, maturity-onset diabetes (MODY), and latent autoimmune diabetes of adults (LADA) were excluded if they already carried such a diagnosis or on clinical criteria.

2.1.2. Complications. Presence of ischemic heart disease and stroke/transient ischemic attack was considered if the patient had such evidence in the medical records. Peripheral vascular disease was considered if the ankle brachial pressure index was less than 0.9.

Neuropathy was diagnosed if either touch sensation detection using 10 g monofilament test showed a score of less than 7 out of 10 or vibration sensation detection using biothesiometer was abnormal. Retinopathy was diagnosed by detailed fundus examination by retinoscope and classified into diabetic maculopathy, nonproliferative retinopathy, and proliferative retinopathy.

Nephropathy was diagnosed based on the urine albumin/creatinine ratio (UACR) of a single urine spot sample. A ratio of >2.5 mg/mol in males and >3.5 mg/mol in females was considered abnormal.

Diabetic foot disease was defined if the patient had an amputation or current or past history of foot ulcer which took 2 weeks or more to heal in the presence of peripheral vascular disease and peripheral neuropathy.

Staging of chronic kidney disease was calculated by using the Cockcroft-Gault equation.

2.1.3. Ethical Issues. Ethical clearance was obtained from the ethical review committee of the University of Colombo prior to the initiation of the study. Administrative approval was obtained from the hospital representatives. Participation was entirely voluntary and written informed consent was obtained from the participants. The patients had the full liberty to exit the study at any given point of time if they wished to do so.

2.1.4. Statistical Analysis. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20. Data was reported as mean ± SD and percentages. Differences of means between males and females were calculated using independent sample t-test for skewed variables with Wilcoxon rank test. Categorical variables were analysed by using chi-square and Fisher's exact tests. Significant level was set at 5%. Results were expressed as odds ratios (OR) and 95% confidence intervals (CI). Associations for predictor variables with each chronic complications were examined initially with bivariate analysis and then with binary logistic regression separately. Backward stepwise method was used to do the regression modeling. The main model consists of the following variables: duration of the illness, HBA1C level, fasting blood sugar values (FBS), postprandial blood sugar levels (PPBS), systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), and gender.

3. Results

Out of the 3000 patients studied, 2180 were females (72.7%) and 820 (27.3%) were males. Mean age ± SD was 58.3 ± 10.3 years with 68.3% of the total population falling within the age group of 50–70. Only 4.5% of patients were below the age of 40 years. Mean duration of diabetes ± SD was 10.8 ± 7.3 years with 42.9% of patients having disease duration of over 10 years. The metabolic profile of the study population is given in Table 1. It is also noteworthy that 75.7% of the total population were either overweight or obese with a basal metabolic index (BMI) of >23. Females had a higher BMI compared to males (26.5 versus 24.8). However, this difference was not significant. All parameters of glycemic, blood pressure, and lipid control were higher in females than in males but the observed difference was significant only for

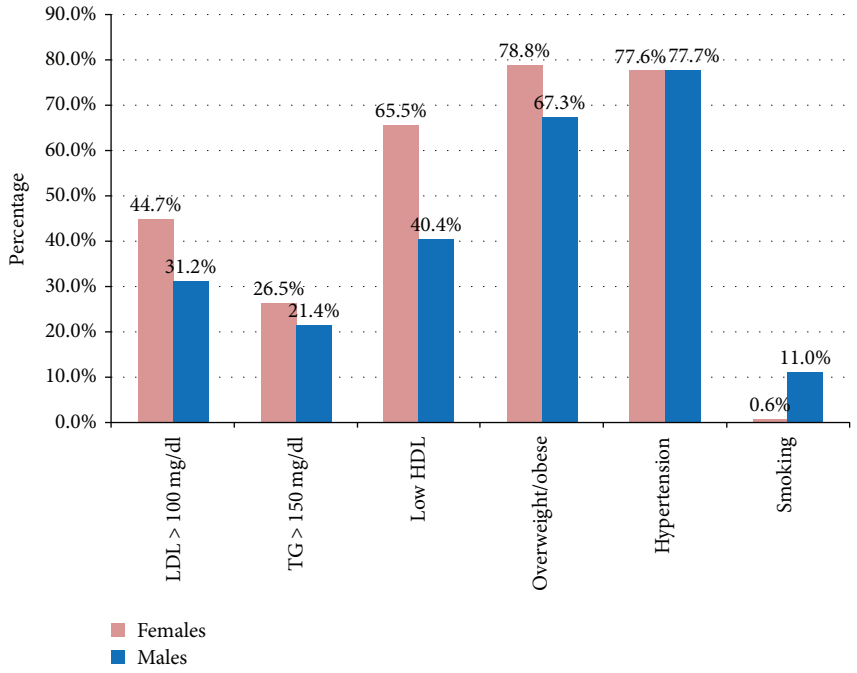

Figure 1: Prevalence of cardiovascular risk factors in the study population by gender (females $n = 2180$, males $n = 820$).

Table 2: Vascular complications by gender.

Disease complication	Overall prevalence $n = 3000$	Females $n = 2180$	Males $n = 820$	X^2 value	Significance
Cardiovascular diseases	318 (10.6%)	211 (9.7%)	107 (13.0%)	7.15	**0.007**
Stroke	33 (1.1%)	19 (0.8%)	14 (1.8%)	3.81	**0.05**
PVD	140 (4.7%)	105 (4.8%)	35 (4.2%)	0.81	0.36
Retinopathy	783 (26.1%)	545 (25%)	238 (29%)	2.62	0.10
Neuropathy	1879 (62.6%)	1332 (61.1%)	547 (68.5%)	20.3	**0.001**
Nephropathy	446/878 (50.8%)	306/631 (51.6%)	136/247 (55%)	2.77	0.96
Diabetic foot	78 (2.6%)	40 (1.8%)	38 (4.7%)	19.45	**0.001**
Lower extremity amputation	54 (1.3%)	26 (1.2%)	28 (3.4%)	15.09	**0.001**

HBA1c ($p = 0.04$), fasting blood sugar (FBS) ($p = 0.005$), and systolic blood pressure (SBP) ($p < 0.001$). Figure 1 illustrates the cardiovascular risk factors in the study population by gender. This reiterates again the pressing issue of obesity with more than three-fourths of the population falling into the overweight/obese category. Low high-density lipoprotein cholesterol (HDL) levels were seen among 65.5% of the females and 40.4% of the males. High low-density lipoprotein (LDL) cholesterol levels were seen in 41% of the population, and 25% had high triglyceride levels. These lipid derangements were in spite of 88.6% of the patients who had been on statin therapy. The prevalence of hypertension in the study population was 77.6% with no significant difference between the genders. However, blood pressure was not under control in 50.3% of the population in spite of treatment. The prescription of angiotensin-converting enzyme inhibitors (ACEI)/angiotensin receptor blockers (ARB), calcium channel blockers (CCB), thiazide diuretics, beta blockers, alpha blockers, and methyldopa was 70%, 15.9%, 16.5%, 15.3%, 5.2%, and 0.1%, respectively. Among the males in the study population, 11% were current smokers while 48.4% were ex-smokers, and 40.6% said they have never smoked before. Almost all the females (98.4%) said they have never smoked before.

The prevalence of vascular complications is described in Table 2. CAD was seen among 10.5% of the population with 0.6% and 1.9% having undergone angioplasty and coronary artery bypass grafting, respectively. Stroke and peripheral vascular disease was seen in 1.1% and 4.7% of the total population, respectively. The prevalence of diabetic retinopathy was 26.1% in the total study population with nonproliferative retinopathy, proliferative retinopathy, and diabetic maculopathy seen in 21.3%, 1.6%, and 6.2%, respectively. Neuropathy, nephropathy, and diabetic foot were seen in

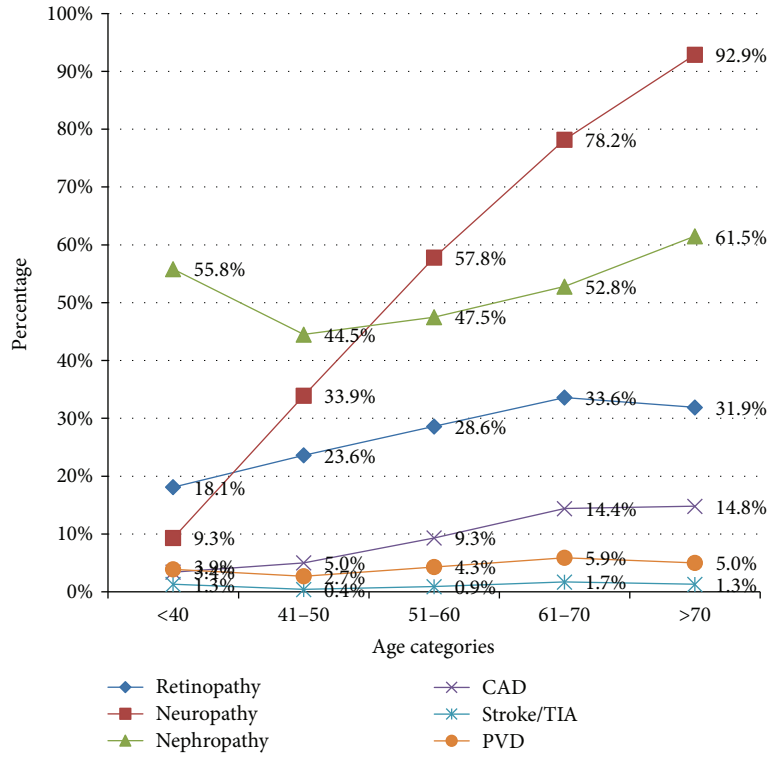

Figure 2: Variation of chronic complications with age among 3000 patients with type 2 diabetes.

62.6%, 50.8%, and 2.6%, respectively. Out of the total patient population, 41.1% had chronic kidney disease (CKD) staging of 2, and 27.1% had a CKD staging of 3. Only 3.2% of patients belonged to CKD stages of 4 and 5. In the study sample, 13.7% had at least one macrovascular complication, and 74% had at least one microvascular complication while 10.5% had both macro- and microvascular complications. All vascular complications except peripheral vascular disease were more prevalent among males than females. However, statistically significant difference was observed with CAD ($p = 0.007$), neuropathy ($p = 0.001$), diabetic foot ($p = 0.001$), and lower extremity amputation (LEA) ($p = 0.001$) only.

The variation of chronic complications with age of the patient and duration of diabetes is demonstrated in Figures 2 and 3, respectively. According to these graphs, it is evident that all the chronic complications in diabetes increase with age and the duration of the illness. Out of all the complications, neuropathy is the commonest complication followed by nephropathy and retinopathy. Among the patients who had diabetes for more than 15 years, 80.5% were having neuropathy while retinopathy and nephropathy were seen among 55.9% and 41.4%, respectively. Logistic regression analysis was conducted with stepwise method using backward selection. As shown in Table 3 microvascular complications were significantly associated with age > 60 years (OR 1.92, 95% CI 1.41–2.73; $p < 0.001$), duration of diabetes > 10 years (OR 2.14, 95% CI 1.58–2.88; $p < 0.001$), and HBA1c > 7% (OR 1.37, 95% CI 1.01–1.87; $p < 0.04$) while macrovascular complications were associated with age > 60 years (OR 1.82, 95% CI 1.36–2.44; $p < 0.001$) only.

A comparison of vascular complications with other regions is demonstrated in Table 4. Accordingly, macrovascular complications are lower and microvascular complications higher than the other regions in Asia.

4. Discussion

Sri Lanka is currently witnessing an unprecedented rise in the prevalence of diabetes mainly due to the cultural, demographical, behavioral, and environmental changes brought on by rapid urbanization and globalization. Abdel Omran's theory on the "epidemiological transition" where victory over infectious disease is allowing people to live longer and hence develop chronic noncommunicable disease perhaps may be another contributory factor [23]. According to the WHO 2014 update, the three highest causes of death in Sri Lanka were reported as CVD, stroke, and diabetes. A study done on trends in Sri Lankan cause-specific adult mortality concluded that chronic diseases are the greatest cause of morbidity and mortality and highlighted the importance in adaptation of the health sector to this alarming trend [22].

This study was carried out at the National Hospital in Colombo, which is the largest hospital and referral centre in Sri Lanka, catering to diabetic patients in Colombo and its suburbs as well as the complex diabetic patients referred by other units in the hospital. The prevalence of chronic complications was high among these patients with 84.4% suffering from at least one vascular complication. Retinopathy was seen in more than one-fourth of the population with sight-threatening retinopathy in 8%. Nephropathy was seen

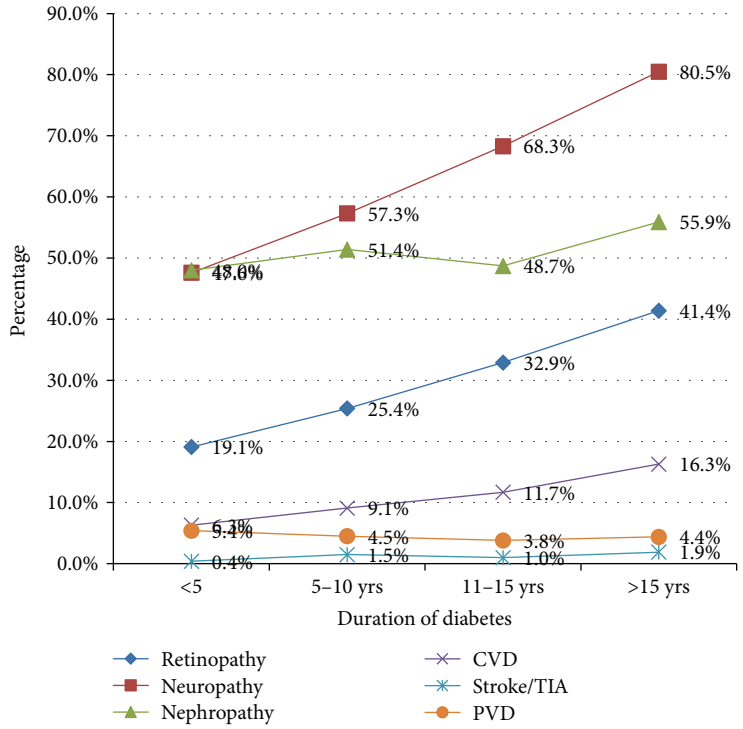

Figure 3: Variation of chronic complications with disease duration among 3000 patients with type 2 diabetes.

Table 3: Logistic regression analysis showing risk factors which were significantly associated with chronic complications.

Complication	Risk factor	Odds ratio (95% confidence interval)	p value
Microvascular disease	Age (<60 yrs versus >60)	1.92 (1.41–2.73)	0.001
	Duration of diabetes (<10 yrs versus >10 yrs)	2.14 (1.58–2.88)	0.001
	HbA1c (<7% versus >7%)	1.37 (1.01–1.87)	0.04
Macrovascular disease	Age (<60 yrs versus >60)	1.82 (1.36–2.44)	0.001
Diabetic foot	Age (<60 yrs versus >60)	2.07 (1.13–3.80)	0.01
	Duration of diabetes (<10 yrs versus >10 yrs)	1.96 (1.05–3.65)	0.03
	HbA1c (<7% versus >7%)	2.20 (1.00–4.83)	0.04

Table 4: Comparison of chronic complications with other regions in Asia.

Complication	Sri Lanka 2016	Sri Lanka 2012	South Asia	East Asia	China	Middle East
Macrovascular complications	13.4%	—	23.3%	26.8%	21.3%	28.7%
CVD	10.5%	5.6%				
PVD	4.2%	0.5%				
Stroke	1.1%	—				
Microvascular complications	74%	—	39%	56%	49.6%	65.8%
Retinopathy	26.1%	25.7%	16.3%	23.7%	22.1%	33.9%
Nephropathy	50.8%	20.4%	20.3%	28.4%	22.3%	40.8%
Neuropathy	62.6%	28.4%	24.6%	36.95	33.3%	53.4%
Diabetic foot	2.6%	—	4.9%	5.3%	2.5%	8.6%

Data from other regions taken from a multinational study done from 2009-2010 [22].

in half of the patient population. However, nephropathy in this setting was defined based on a single UACR. According to the 2017 "Standards of medical care in diabetes" by the American Diabetes Association (ADA), two out of three reports done within 3–6 months should be positive to de diagnosed with nephropathy leaving allowance for biological

variability. Due to the limited availability of this test, we had to rely on a single value, and thus the actual percentage of patients with nephropathy would have been lower than this.

According to Table 4, the prevalence of macrovascular complications seems to be lower, and microvascular complications seem to be higher in Sri Lanka, when compared to other Asian countries. However, this data was from a multinational study carried out in 2009-2010, and the current prevalence may be different [17]. Compared to Sri Lankan data from a study done in 2012 at the National Diabetic Centre in Colombo, both macro- and microvascular disease burdens have increased, with the prevalence of CVD almost double the prevalence reported in 2012 [14]. The National Diabetic Centre is a nonprofitable organization which caters to patients with diabetes in Colombo and from the suburban areas. The present study was carried out among the outpatients in the largest tertiary care hospital in Colombo. The increase in disease burden over the years could actually be due to the rising trend of chronic complications. However, this would have been further compounded by the concentration of patients with complex disease in this tertiary care referral centre. Differences in defining various vascular complications would also have contributed to this discrepancy. A higher prevalence of vascular complications among a small sample of inward patients with diabetes in the same tertiary care hospital was reported by Perera et al. [18]. However, this is probably due to higher disease burden seen among patients with diabetes who require admission. It is also interesting to note that two recent studies done in the Northern and Eastern Provinces of Sri Lanka reported a much higher prevalence of CAD [19, 20]. Future research may be warranted to determine if there is a significant difference in prevalence of CAD among various provinces and of any possible contributory risk factors.

Determining the risk factors for the development of micro- and macrovascular angiopathy is important in order to attempt to reduce the burden from disease complications. Previous studies have shown that age, duration of diabetes, and age at diagnosis have varying effects on the risk of angiopathy. The UK Prospective Diabetes Study (UKPDS) reported an increase in the prevalence of myocardial infarction but not in retinopathy or nephropathy with old age in patients newly diagnosed with diabetes. In contrast, after follow-up of 6 years, younger age at diagnosis was associated with an increased risk of retinopathy and nephropathy but not so in risk of myocardial infarction or nephropathy [24]. This suggests that interrelation between age, age at diagnosis, and duration of diabetes is complex with regard to angiopathy in different organ systems and further complicated by the unquantified time period of hyperglycemia prior to diagnosis. A general inference is that macrovascular events are more common among the elderly even among nondiabetics, and this is further exacerbated with the setting in of diabetes. Microvascular complications however are more closely related to diabetes, and the risk of these is closely related to disease duration [25]. In this study, microvascular complications were significantly associated with age, disease duration, and glycemic control while the only factor significantly associated with macrovascular complications was age. Many prospective randomized controlled trials have shown that persistent intensive glycemic control reduces the risk of mainly microangiopathy as well as macroangiopathy in the long term. In our study, poor glycemic control was significantly associated with microvascular disease but not so with macrovascular disease. Diabetic foot is a complication which arises due to combination of macro- and microangiopathy in the background of poor glycemic control. According to our findings, age, duration of diabetes, and poor glycemic control were all associated with diabetic foot. Previous studies have also shown that hypertension and smoking play a role in microangiopathy [26, 27]. In this study, high systolic blood pressure was associated only with retinopathy, and none of the chronic complications were associated with smoking. It is important to keep in mind that this study was a descriptive cross-sectional study looking at data at a given point of time. The effect of these risk factors on vascular complications cannot be determined by such a methodology and would require long-term prospective randomized control trials.

Abundant evidence shows that diabetes is a risk factor for CAD, and it is now the leading cause of diabetes-related morbidity and mortality. Patients with T2DM with insulin resistance have a proatherogenic cardiovascular risk profile, which includes risk factors such as poor glycemic control, hypertension, abdominal obesity, microalbuminuria, smoking, and atherogenic lipid profile with reduced LDL cholesterol, increased TG, and reduced HDL cholesterol levels. However, we were unable to find any significant association between these risk factors and the occurrence of CAD in this population. This is not unusual as such variations have been reported previously [27, 28]. The descriptive cross-sectional nature of the study design would have also contributed to this as we have looked at these risk factors at only one given point of time. Nevertheless, it is important to note that the prevalence of these well-documented CVD risk factors in this population is high (Figure 1).

Obesity seems to be a major problem with almost 75.7% of the patients with diabetes being overweight or obese. Most of these patients are invariably suffering from metabolic syndrome. Obesity is an emerging global problem giving rise to increase in diabetes, hypertension, dyslipidemia, metabolic syndrome, CVD, stroke, cancer, and reproductive and psychosocial diseases. Prospective studies which have followed up patients for more than two decades have documented that obesity is an independent risk factor for CVD [29]. However, it is a heterogenous condition, and it is mainly the abdominal obesity that is associated with excess cardiovascular risk [30]. Research evaluating the association with BMI and risk of death among patients with diabetes has shown inconsistent results with many studies showing a U-shaped association with BMI and all-cause mortality [31]. Katulanda et al. reported a high prevalence of overweight (25.2%), obese (9.2%), and centrally obese (26.2%) among the general population of Sri Lanka [32]. The prevalence of overweight and obese individuals in our study population was alarmingly high at 75.7%. However, central obesity was not measured in this study.

Hypertension in diabetics is an important issue as the combination often coexists. It affects around 30% of the

European diabetic population [33]. Several studies have shown the close association of diabetes with hypertension. Hypertension is significantly more prevalent among patients with type 2 diabetes. This link is mainly attributed to hyperinsulinemia [34]. The prevalence of hypertension is 1.5 to 2 times more in patients with diabetes than in those without diabetes, while almost one-third of patients with hypertension develop diabetes later [35]. The presence of hypertension will increase the risk of CAD, stroke, retinopathy, and nephropathy. In this study, the prevalence of hypertension was 77.6%. This was significantly higher than neighboring India where a study reported hypertension among only 25% of the patients with T2DM [36]. The American Diabetes Association recommends patients with diabetes to achieve a blood pressure goal of less than 140/90 mmHg [37]. Treatment of hypertension in these patients should include an agent which has shown to reduce cardiovascular events (ACEI/ARB, thiazide-like diuretics, and dihydropyridine CCB). Multiple drugs are usually needed to achieve blood pressure targets. In our study, 70% of patients were on ACEI/ARB followed by 16.5% on thiazide-like diuretics and 15.9% on CCB. The superiority of ACE/ARBs over other antihypertensive agents for the prevention of CAD has not been consistently proven [38, 39]. In a recent meta-analysis, thiazide-like diuretics or dihydropyridine CCB have shown cardiovascular benefits similar to ACEI/ARB among patients without albuminuria [38]. It is important to note that blood pressure was inadequately controlled in almost half of the patients on treatment for hypertension in this study. More focus should be upon achieving targets using multiple drug combinations to alleviate the risk CAD.

Dyslipidemia is one of the key risk factors for CVD among diabetic patients. The characteristic features in diabetic dyslipidemia is a high TG and low HDL concentrations. Low LDL levels may not be significantly different from LDL levels among nondiabetics. However, all these promote atherogenesis. In this study population, the prevalence of dyslipidemia was 76.7%, with high LDL, high TG, and low HDL seen in 41%, 25%, and 54.7%, respectively. Out of the patients with dyslipidemia, 88.6% of the patients were on statin therapy. The most commonly deranged component of the lipid profile was HDL. Low HDL was commoner among females (61.2%) than among males (37.4%). Such gender-based predisposition in low HDL has been observed by Weerarathna et al. as well [40]. This may be due to the fact that most females in this study sample were menopausal and thus have lost the favorable increase in HDL due to estrogen. Furthermore, higher level of inactivity among this population may also contribute to the low HDL levels. As of present, guidelines do not recommend specific pharmacotherapy for increasing HDL apart from statins. According to the American Diabetes Association 2017 standards of care, men with TG levels of >204 mg/dl and HDL levels of <30 mg/dl who are already on statin can be commenced on a fibrate. High prevalence of dyslipidemia among these patients while on treatment suggests that intensive medical therapy and lifestyle modification need to be emphasized.

Among the males in the study population, 11% were current smokers. This is self rated, and more objective methods of assessing smoking would have been appropriate and yielded a higher prevalence. Smoking is an independent risk factor for all-cause mortality mainly due to CVD [41]. This should be reemphasized during the consultations with the patients.

It is interesting to note the gender-based discrepancy among the metabolic parameters and vascular complications in this study population. All metabolic parameters such as BMI, FBS, PPBS, HBA1c, blood pressure, LDL, and TG levels were higher in females than in men. However, statistical difference was observed for FBS, HBA1c, and systolic blood pressure. This was in contrast to vascular complications, where almost all the complications were more prevalent in men than in women with significant difference observed in CAD, neuropathy diabetic foot, and lower limb amputation. Data from the World Health Organization shows that men have twice as higher risk of developing CAD than women [42]. However, diabetes seems to erase this gender-based advantage that women have over men. Women with diabetes are said to have a high risk for CAD than men [43]. However, this fact is somewhat controversial. Some studies have shown that diabetes definitely eliminates this female advantage over men while other studies have not shown a significant difference [44, 45]. More studies would be required to determine the relationship between gender and CAD among patients with diabetes. Major complex gender differences exist between diabetes-related LEA. Evidence suggests that men are more liable to experience LEA than women [46, 47]. Men are more likely to have independent risk factors such as diabetic foot, PVD, cigarette use, and peripheral neuropathy leading to amputation [48, 49]. Sensory neuropathy is the most common neuropathy associated with amputation, and men have twice the risk of developing this than women. This gender-based predisposition was evident in our study population as well.

4.1. Limitations. There are several limitations of this study. The target population of patients attending the clinic in a tertiary referral centre reflects a population with more complex disease burden. Therefore, the prevalence reported may be an overestimation of the actual disease burden. Thus, to generalize the findings of this study to the entire population of patients with T2DM may not be quite appropriate. The diagnosis of CAD was based on medical records without considering if the patient had any current ischemic symptoms. The diagnosis of neuropathy was based only on the results of vibration and monofilament tests without taking the patients' symptoms into consideration. Due to the scarcity of laboratory resources, the diagnosis of nephropathy was based on a single UACR. Limiting the evaluation of obesity to general obesity without taking central obesity into consideration was another important limiting factor.

5. Conclusion

This study is evidence for the high prevalence of chronic vascular complications with increased disease burden. Age, duration of diabetes, and HBA1c were significantly associated with microvascular complications and diabetic foot

while only age was associated with macrovascular complications. Furthermore, this population was at high risk of CVD with high prevalence of hypertension, dyslipidemia, obesity, and microalbuminuria. Men had a higher prevalence of CAD, peripheral neuropathy, diabetic foot, and LEA. Appropriate measures to intensify medical therapy and lifestyle measures to control modifiable risk factors and routine screening for the detection of new complications need to be emphasized in order to prevent morbidity and mortality.

Conflicts of Interest

The authors declare that they have no competing interests.

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