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Insulin Resistance, Type 1 and Type 2 Diabetes, and Related Complications: Current Status and Future Perspective

Guest Editors: Joseph Fomusi Ndisang, Sharad Rastogi, and Alfredo Vannacci

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Editorial Board

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Editorial

Insulin Resistance, Type 1 and Type 2 Diabetes, and Related Complications: Current Status and Future Perspective

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The global escalation of obesity and diabetes in developed and developing nations poses a great health challenge. Obesity is one of the major causes of type 2 diabetes [1, 2]. Type 1 diabetes is primarily due to the autoimmune-mediated destruction of pancreatic beta-cell leading to insulin deficiency [3, 4]. This is usually accompanied by alterations in lipid metabolism, enhanced hyperglycemia-mediated oxidative stress, endothelial cell dysfunction, and apoptosis [3, 4]. Similarly, in type 2 diabetes, increased glucotoxicity, lipotoxicity, endoplasmic reticulum-induced stress, and apoptosis lead to the progressive loss of beta-cells [3, 4]. While type 1 diabetes is characterized by the presence of beta-cell autoantibodies, a combination of peripheral insulin resistance and dysfunctional insulin secretion by pancreatic beta-cells is implicated in the pathogenesis of type 2 diabetes [3, 4]. Although insulin resistance has traditionally been associated with type 2 diabetes, mounting evidence indicates that the incidence of insulin resistance in type 1 diabetes is increasing [5, 6]; therefore novel mechanistic approaches deciphering insulin resistance are needed. Many pathophysiological factors are implicated in insulin resistance. Although the exact nature of these factors is not completely understood, it is becoming increasingly clear that oxidative stress, inflammation, genetic, habitual, environmental, and epigenetic factors may be involved [7–14].

In the past decade, significant strides have been made in elucidating important mechanisms associated with insulin resistance, overt diabetes, and related cardiometabolic diseases. However, more intense research is still needed for more comprehensive understanding of the pathophysiological profile of insulin resistance in diabetes, and especially in situations where diabetes is comorbid with other chronic diseases.

In this special issue research and review papers that address a broad range of mechanisms associated with insulin resistance, type 1 diabetes, type 2 diabetes, and related cardiometabolic complications are discussed. Accordingly, in a review article by X. Jiang et al. the impact of habitual and environmental factors on the development of diabetes is discussed. Particularly, X. Jiang et al. showed that nutritional, environmental, and physiological factors at prenatal age are correlated to the manifestation of insulin resistance and type 2 diabetes in later stages of adult life. On the other hand, healthy habits including exercise may prevent diabetes. Accordingly, C.-H. Hunget al. wrote an article showing the benefits of exercise on attenuating inflammation in diabetes and related complications. To further elucidate the pathophysiological role of inflammation on diabetes, Y. I. Sánchez-Zamora and M. Rodriguez-Sosa wrote a review article on the effects of macrophage migration inhibitory factor in type 1 and type 2 diabetes. In another related article, A. Zhu et al. reviewed the effects of abdominal adiposity on diabetes, while X. Zhang et al. reported the effects of fluctuating glucose levels on carotid artery intima-media thickness and

the development of coronary artery disease. Besides vascular complications, many diabetic patients have increased risk of thrombosis due to insulin resistance and elevated inflammation [15]. This notion was further investigated in an article featuring in this special issue written by I. Isordia-Salas et al.

Among the major microvascular complications in diabetes are diabetic retinopathy and diabetic nephropathy. To shed more lights on these diabetic complications, X. Li et al. investigated the effects of oxidative stress on retinal neuron apoptosis, while G. Wu et al. reported the renoprotective effects of benidipine in streptozotocin-induced diabetic rats.

Taken together, the articles featuring in this special issue cover a broad range of themes of considerable interest and would benefit a wide audience.

Joseph Fomusi Ndisang
Sharad Rastogi
Alfredo Vannacci

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Research Article

Proinflammatory and Prothrombotic State in Subjects with Different Glucose Tolerance Status before Cardiovascular Disease

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Background. Inflammation has been associated with insulin resistance, type 2 diabetes mellitus (T2DM), and atherothrombosis. *Aim*. To determine differences in levels of proinflammatory and prothrombotic markers such as high sensitivity C-reactive protein (hs-CRP) and fibrinogen in subjects with normal glucose tolerance (NGT), prediabetes, and T2DM and to establish their relationship with other cardiovascular risk factors before clinical manifestations of cardiovascular disease. *Methods*. We conducted a nonrandomized, cross-sectional assay in a hospital at México City. The levels of hs-CRP and fibrinogen were measured and compared according to glucose tolerance status. *Results*. We enrolled 1047 individuals and they were distributed into NGT $n = 473$, pre-DM $n = 250$, and T2DM $n = 216$. There was a statistical difference between NGT and T2DM groups for fibrinogen ($P = 0.01$) and hs-CRP ($P = 0.05$). Fibrinogen and hs-CRP showed a significant positive correlation coefficient ($r = 0.53$, $P < 0.0001$). In a multiple stepwise regression analysis, the variability in fibrinogen levels was explained by age, HbA1c, and hs-CRP (adjusted $R^2 = 0.31$, $P < 0.0001$), and for hs-CRP it was explained by BMI and fibrinogen (adjusted $R^2 = 0.33$, $P < 0.0001$). *Conclusion*. Inflammation and prothrombotic state are present in people with T2DM lacking cardiovascular disease. Fibrinogen and Hs-CRP are positively correlated. Fibrinogen and hs-CRP concentrations are predominantly determined by BMI rather than glucose levels.

1. Introduction

Cardiovascular disease (CVD) is the first cause of death all over the world, accounting for 17.3 million deaths per year, a number that is expected to grow more than 23.6 million by 2030 [1]. Atherosclerosis is the commonest cause of CVD. Currently, atherosclerosis is considered an inflammatory disease triggered by cholesterol-rich lipoproteins and other

noxious factors such as cigarette smoke, diabetes mellitus, and hypertension [2]. Increasing evidence supports the concept that atherosclerosis is initiated through developmental in utero processes beginning before birth [3]. There is a long time delay between the start of atherosclerosis and the first clinical manifestation many decades later, supporting the hypothesis that subclinical atherosclerosis represents the vessel memory of lifetime risk factor exposure [4]. In addition, some studies have shown a correlation between subclinical atherosclerosis and selected proinflammatory factors such as C-reactive protein (CRP), fibrinogen, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [5]. Furthermore, these selected inflammatory markers seem to be associated with destabilization of atherosclerotic plaque, rather than degree of artery stenosis [6]. Both fibrinogen and CRP are acute-phase reactants produced by the liver. Fibrinogen is a precursor of fibrin, a protein essential for blood clotting and one of the main factors determining blood viscosity and platelet aggregation [7], and elevated levels of CRP have been associated with an elevated risk of adverse ischaemic events and hypercoagulability [8].

The increased prevalence of CVD morbidity and mortality is being followed by an increasing prevalence of diabetes [9]. Worldwide estimation by 2030 predicts more than 470 and 439 million adults with prediabetes (pre-DM) and diabetes mellitus, respectively [10, 11]. Pre-DM is the intermediate stage between normal glucose tolerance (NGT) and diabetic glucose thresholds and occurs 5 to 10 years before the diagnosis of type 2 diabetes mellitus (T2DM) [12, 13]. Even though all individuals with T2DM passed through a pre-DM phase, not all individuals with pre-DM will become T2DM [14]. Approximately, 5 to 10% of people per year with pre-DM will progress to T2DM, with the same proportion converting back to NGT [10]. Two different types of pre-DM have been described: those individuals with hepatic insulin resistance and impaired suppression of hepatic glucose production manifested by hyperglycemia at fast and those individuals with muscle insulin resistance and impaired glucose uptake manifested by postprandial hyperglycemia; both conditions are known as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), respectively [12]. There is evidence to support an inflammatory contribution for the development of pre-DM and T2DM. Increased levels of high sensitivity C-reactive protein (hs-CRP) and plasminogen activator inhibitor type-1 (PAI-1) are associated with insulin resistance rather than impaired pancreatic β-cell function in prediabetic individuals [15]. Also, hs-CRP and PAI-1 have shown to be predictors of incipient diabetes [16].

Although the risk of CVD already exists before the onset of diabetes mellitus since other cardiovascular risk factors are still prevalent in prediabetic subjects, a gradual increment of independent risk of CVD has been reported as blood glucose levels up even under prediabetic glucose thresholds [17]. However, whether IFG or IGT differs in the magnitude of the risk of CVD is still not well established yet [18]. How glucose tolerance status affects the inflammatory and thrombotic response before the development of clinical manifestations of CVD is poorly understood.

Because inflammation is a common feature between atherosclerosis, insulin resistance, and T2DM, and atherothrombosis might exhibit many years before the onset of T2DM, the objective of the present study was to evaluate serum levels of some proinflammatory and prothrombotic markers such as hs-CRP and fibrinogen in subjects with NGT, pre-DM, and T2DM, and to establish their relationship with other traditional cardiovascular risk factors before clinical manifestations of CVD.

2. Materials and Methods

We conducted an observational, analytic, and nonrandomized cross-sectional assay in a secondary care level hospital at México City from May 2010 to May 2011. We screened apparently healthy members of the medical staff and relatives of outpatients who came to medical consultation, as well as those who were in routine follow-up for diabetes mellitus. The recruitment was made by invitation through printed announcements and personal appeal to people to participate in the survey if they were interested to know their glucose tolerance status and cardiovascular risk factors. We included all individuals $\geq$ 20 years old who accepted to participate. Informed written consent was obtained from all subjects before enrollment. The study protocol was reviewed and approved by the Human Ethical Committee and Medical Research Council of the Instituto Mexicano del Seguro Social and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

The exclusion criteria were subjects with previous or current diagnosis of CVD (i.e., myocardial infarction, angina, stroke, transient ischemic attack, and peripheral artery disease), cancer, autoimmune disorders, acute and chronic infectious diseases, and hepatic or renal failure and those under immunosuppressive therapy and transplant receivers.

Demographic and clinical data, anthropometric measures, and a fasting blood sample were taken from each subject. The same physician interviewed all the participants in private using a questionnaire and collected the following information: sex, age, smoking status, previous diseases, and familial history of diabetes and CVD. The subjects were considered smokers if they were currently smoking or stopped smoking at least one year before the examination. A positive familial history of diabetes was defined as parents or siblings having T2DM. A familial history of CVD disease was defined as acute myocardial infarction, stroke, or sudden death in a first-degree male relative younger than 55 years of age or a female relative younger than 65 years of age. Same physician performed the anthropometric measurements. Waist circumference (WC) was measured at the midpoint between the last rib and the iliac crest. Body weight was measured by precision scale while subjects were minimally clothed without shoes. Height was measured in a standing position without shoes using measuring tape mater while the shoulders were in a normal state. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Patients were defined as overweight with a BMI of 25.0–29.9 kg/m^2 and obese with a BMI $\geq$ 30 kg/m^2. Hypertension

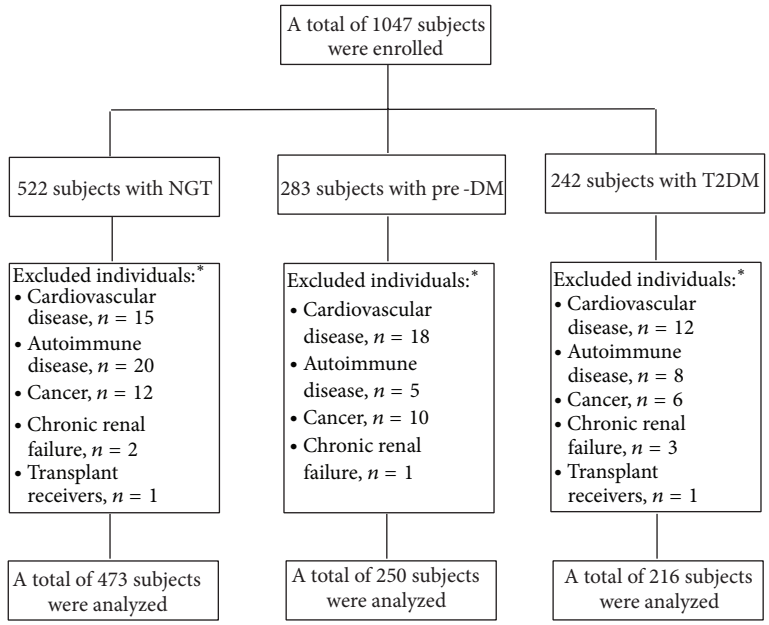

Figure 1: Enrollment. * Some individuals had two or more conditions.

was defined as a mean systolic blood pressure of ≥ 140 mmHg or a mean diastolic blood pressure of ≥ 90 mmHg resulted from two separate measures at rest, or previous diagnosis.

After an overnight fast of at least eight hours, venous blood was sampled for measurement of serum glucose, glycosylated hemoglobin (HbA1c), hs-CRP, fibrinogen, triglycerides, and high-density lipoprotein cholesterol (HDL-C). The hs-CRP was measured with a high-sensitivity assay with an interassay coefficient of variation (CV) of 8.9% [19]. Fibrinogen was measured in citrated plasma with a modified clot-rate assay using the Diagnostica STAGO ST4 instrument [20] and based on the original method of Clauss [21] with a CV of 3.0%.

The overall sample was categorized according to glucose tolerance status assessed by fasting plasma glucose (FPG) and HbA1c levels as [22]: (1) group with NGT defined as a FPG < 100 mg/dL and HbA1c < 5.7%; (2) group with Pre-DM defined as a FPG concentration of 100 to 125 mg/dL or HbA1c of 5.7 to 6.4%; and (3) group with T2DM defined as a FPG ≥ 126 mg/dL or previous diagnosis. The pre-DM group was stratified as follows: (1) subjects with impaired fasting glucose (IFG) defined as a FPG of 100–125 mg/dL plus HbA1c < 5.7% and (2) subjects with impaired glucose tolerance (IGT) defined as a FPG < 100 mg/dL plus HbA1c 5.7 to 6.4%.

Statistical Analysis. The overall sample was stratified according to glucose tolerance status. Demographic and clinical data between subjects with NGT, pre-DM, and T2DM were compared using one-way analysis of variance (ANOVA) for continuous variables, whereas the chi-squared test (χ^2) was used to compare the categorical parameters. Continuous data were expressed as mean ± standard deviation (SD) and median (interquartile range). Categorical data were expressed as total number plus percentage. A Pearson correlation analysis was performed to the overall sample by standard methods to evaluate the association between proinflammatory and prothrombotic markers with traditional cardiovascular risk factors. A multiple linear stepwise regression analysis was performed to develop a model including all the variables significantly associated with hs-CRP and fibrinogen in the correlation analysis as independent variables to estimate contribution of each independent feature to variation in hs-CRP and fibrinogen levels. Data analysis was performed after natural logarithmic transformation. A P value ≤ 0.05 (two tailed) was considered as statistically significant. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) statistical software package (version 15: SPSS Inc., Chicago, IL, USA).

3. Results

A total of 1047 individuals were enrolled, Figure 1. One hundred and eight subjects were excluded from the analysis because the presence of exclusion criteria; some of them had two or more conditions: cardiovascular disease (n = 45), autoimmune disorders (n = 33), cancer (n = 28), renal failure (n = 6), and transplant receivers (n = 2). Demographic and clinical characteristics of 939 subjects are present in Table 1. Individuals were distributed according to glucose tolerance status into three groups: NGT 50.4% (n = 473), pre-DM 26.6% (n = 250), and T2DM 23% (n = 216). There were no differences regardless of gender, smoking status, and familial history of CVD between all groups. However, age, BMI, and WC showed a statistical difference between NGT and pre-DM, pre-DM and T2DM, and NGT versus T2DM groups, with an older age and higher values of abdominal perimeter and BMI in the diabetes mellitus group. Hypertension cases were more frequent in subjects with T2DM compared with pre-DM and NGT, but cases with familial history of T2DM

Table 1: Demographic and clinical characteristics of the overall sample categorized according to glucose tolerance status.

Parameter	Total = 939 individuals		
	NGT $n = 473$ (50.4%)	Pre-DM $n = 250$ (26.6%)	T2DM $n = 216$ (23%)
Age (years)			
Mean ± SD	48.5 ± 12.9	52 ± 10.6*	55.8 ± 10.4*†
Median [IQR]	50 [40–58]	52 [45–59]	57 [49–62]
Women, *n* (%)	328 (69.3)	168 (67.2)	142 (65.7)
BMI (kg/m^2)			
Mean ± SD	28 ± 4.6	29.3 ± 4.4*	30.4 ± 5.2*†
Median [IQR]	27.5 [24.7–31]	29 [26.1–31.7]	29.6 [26.8–32.8]
BMI ≥ 30 kg/m^2, *n* (%)	150 (31.7)	99 (39.6)	102 (47.2)*
WC (cm)			
Mean ± SD	91.3 ± 12.2	95.6 ± 9.9*	98.3 ± 11.2*†
Median [IQR]	91 [82–99]	95 [89–102]	98 [91-105]
Hypertension, *n* (%)	137 (28.9)	77 (30.8)	117 (54.1)*†
Currents smoking, *n* (%)	107 (22.6)	48 (19.2)	48 (22.2)
FH of T2DM, *n* (%)	234 (49.4)	146 (58.4)	147 (68)*
FH of CVD, *n* (%)	71 (15)	38 (15.2)	34 (15.7)

Data are means ± standard deviation, median [interquartile range], and percentages; * P value ≤ 0.05 versus NGT; † P value ≤ 0.05 versus pre-DM; NGT: normal glucose tolerance; pre-DM: pre-diabetes mellitus; T2DM: type 2 diabetes mellitus; BMI: body mass index; WC: waist circumference; FH of T2DM: familial history of type 2 diabetes mellitus; FH of CVD: familial history of cardiovascular disease.

were higher only in diabetic subjects when compared with NGT. Table 2 shows the biochemical characteristics of 939 subjects according to glucose tolerance status. There was a statistically significant difference of FPG and HbA1c plasma levels between the three groups with a progressive increase from NGT to pre-DM group and a highest value in the T2DM group. Plasma triglyceride levels were higher in T2DM and pre-DM groups when contrasted with NGT, and HDL-C concentrations were significantly lower in T2DM group compared with NGT. Figure 2 shows the plasma levels of fibrinogen and hs-CRP according to glucose tolerance status. There was a significant difference between NGT and T2DM groups for fibrinogen (369.8 ± 69.1 versus 385.9 ± 77.8, $P = 0.01$) and between NGT and T2DM for hs-CRP (0.4 ± 0.5 versus 0.5 ± 0.6, $P = 0.05$). But, there was not a statistical difference between NGT and pre-DM groups for fibrinogen (369.8 ± 69.1 versus 372 ± 69.8, $P = 0.71$) as well as for hs-CRP (0.4 ± 0.5 versus 0.4 ± 0.7, $P = 0.89$) concentrations, or between pre-DM and T2DM subjects. In a subgroup analysis, when we stratified pre-DM cases assessed by FPG and HbA1c, 95.2% ($n = 238$) accomplished with IFG criteria and 2.8% ($n = 7$) with IGT, and 2% ($n = 5$) shared an IFG plus IGT state (data not shown).

Table 3 shows a correlation analysis among proinflammatory and prothrombotic markers with traditional cardiovascular risk factors of the overall sample. Fibrinogen showed a significant positive correlation coefficient with BMI ($r = 0.25$, $P < 0.0001$), WC ($r = 0.18$, $P = 0.000$), age ($r = 0.14$, $P = 0.005$), HbA1c ($r = 0.14$, $P = 0.004$), and FPG ($r = 0.10$, $P = 0.03$). In turn hs-CRP was positively associated with BMI ($r = 0.33$, $P < 0.0001$), WC ($r = 0.27$, $P < 0.0001$), FPG ($r = 0.14$, $P = 0.004$), and HbA1c ($r = 0.12$, $P = 0.013$). Both proinflammatory (hs-CRP) and prothrombotic (fibrinogen) markers were moderately correlated ($r = 0.53$, $P < 0.0001$). Glucose and HbA1c ($r = 0.77$, $P < 0.0001$) as well as BMI and WC ($r = 0.82$, $P < 0.0001$) showed the strongest correlation coefficients (data not shown). A multiple linear stepwise regression analysis for fibrinogen and hs-CRP as dependent variables is shown in Table 4. The variability of serum levels of fibrinogen was explained only in 31% by a model including age, BMI, WC, FPG, HbA1c, and hs-CRP (adjusted $R^2 = 0.31$, $P < 0.0001$). The significant determinants of serum fibrinogen levels were age ($\beta = 0.14$, 95% CI [0.06–0.23], $P = 0.001$), HbA1c ($\beta = 0.12$, 95% CI [–0.003–0.25], $P = 0.05$), and hs-CRP ($\beta = 0.51$, 95% CI [0.42–0.60], $P < 0.0001$). The variability of serum levels of hs-CRP was explained only in 33% by a model including BMI, FPG, HbA1c, and fibrinogen (adjusted $R^2 = 0.33$, $P < 0.0001$). The significant determinants of serum hs-CRP levels were BMI ($\beta = 0.19$, 95% CI [0.04–0.34], and $P = 0.01$) and fibrinogen ($\beta = 0.48$, 95% CI [0.39–0.56], $P < 0.0001$).

4. Discussion

According to our results, subjects with T2DM without obvious cardiovascular complications showed statistically significant higher concentrations of fibrinogen ($P = 0.01$) and hs-CRP ($P = 0.05$) compared with apparently healthy individuals with NGT. However, subjects with pre-DM assessed by FPG and HbA1c tests, whose 95.2% coursed with IFG, had slightly increased concentrations of fibrinogen ($P = 0.71$) and hs-CRP ($P = 0.89$) compared with individuals with NGT without reaching statistical significance. Similar

Table 2: Biochemical characteristics of the overall sample categorized according to glucose tolerance status.

Parameter	Total = 939 individuals		
	NGT $n = 473$ (50.4%)	Pre-DM $n = 250$ (26.6%)	T2DM $n = 216$ (23%)
FPG (mg/dL)			
Mean ± SD	89.6 ± 6.4	107 ± 5.9*	158.1 ± 68.1*†
Median [IQR]	90 [86–95]	105 [102–111]	135 [111–181]
HbA1c (%)			
Mean ± SD	4.0 ± 0.5	4.2 ± 0.8*	5.9 ± 2.1*†
Median [IQR]	3.9 [3.7–4.2]	4.1 [3.9–4.5]	5.2 [4.4–7.2]
HDL-C (mg/dL)			
Mean ± SD	40.8 ± 12.7	39 ± 10.3	37.4 ± 10.2*
Median [IQR]	39.2 [31.6–48.5]	37.1 [31.4–46]	35.9 [30.5–42.4]
Triglycerides (mg/dL)	184.1 ± 117.2	222.3 ± 154.8*	231.8 ± 301.2*
Median [IQR]	159 [109–219]	182 [129–266]	191 [127–264]
Fibrinogen (mg/dL)			
Mean ± SD	369.8 ± 69.1	372 ± 69.8	385.9 ± 77.8*
Median [IQR]	365 [322–408]	370 [323–418]	377 [330–438]
hs-CRP (mg/L)			
Mean ± SD	0.4 ± 0.5	0.4 ± 0.7	0.5 ± 0.6*
Median [IQR]	0.3 [0.1–0.4]	0.2 [0.1–0.5]	0.3 [0.1–0.6]

Data are means ± standard deviation, median [interquartile range] and percentages; * P value ≤ 0.05 versus NGT; † P value ≤ 0.05 versus pre-DM; NGT: normal glucose tolerance; preDM: pre-diabetes mellitus; T2DM: type 2 diabetes mellitus; FPG: fasting plasma glucose; HbA1c: glycosylated hemoglobin; HDL-C: high-density lipoprotein colesterol; hs-CRP: high sensitivity C-reactive protein.

to our results, in the Third National Health and Nutrition Examination Survey (NHANES-III) participants with IFG, newly diagnosed diabetes and previously diagnosed diabetes had 0.9 (0.72–1.37), 1.84 (1.25–2.71), and 1.59 (1.25–2.01) odds of having an elevated CRP concentration after adjustment for age, sex, ethnicity, and BMI [23, 24]. Lucas-Luciardi et al. in a case-control study founded significant increased levels of fibrinogen, PAI-1, and D-dimer, but did not for hs-CRP, in a small sample of subjects with IGT assessed by oral glucose tolerance test (OGTT) without documented CVD [25]. In addition, Gui et al. reported a gradual increase of hs-CRP levels in patients with angiographically documented coronary artery disease as glucose tolerance status changes from NGT to IGT to T2DM, but only with a statistical difference between subjects with NGT and T2DM [26]. In turn, Xiang et al. founded higher levels of hs-CRP in T2DM versus NGT in Chinese population [27]. In contrast, The Insulin Resistance Atherosclerosis Study (IRAS) reported an independent negative association between hs-CRP and insulin sensitivity in healthy nondiabetic subjects [28]. Festa et al. showed higher levels of hs-CRP and PAI-1 in individuals with increased insulin resistance who converted to T2DM in a mean period of 5.2 years versus converters with decreased first-phase insulin secretion, suggesting that insulin resistance rather than impaired β-cell function contributes to the proinflammatory state in prediabetic individuals [15]. Evidence suggests that a proinflammatory state is associated with insulin resistance, and an inflammatory profile of people with pre-DM might help to differentiate those individuals with an increased risk to develop T2DM.

In our study, despite the increased levels of fibrinogen and hs-CRP observed in subjects with pre-DM and T2DM when compared to subjects with NGT, the mean thresholds of fibrinogen and hs-CRP into all three groups were inside ranges considered as normal (200–400 mg/dL and < 3 mg/L, resp.) [29]. Recently, Samaropoulos et al. reported an association between intensive glycemic control and a reduction in hs-CRP levels in subjects with T2DM [30]. Also, Bembde et al. observed a positive correlation between HbA1c and fibrinogen ($r = 0.49$) in diabetic patients assuming that the poorer the glycemic status, the higher the fibrinogen levels [31]. The apparently correct glycemic control according to mean levels of FPG (158±68.1 mg/dL) and HbA1c (5.9±2.1%) showed in the T2DM group might explain, at least partially, the mean normal ranges of fibrinogen and hs-CRP observed in our sample. Although we did not compare fibrinogen and hs-CRP levels in diabetic subjects with versus without an optimal glycemic control, we founded the highest plasma levels of proinflammatory and prothrombotic markers in subjects with T2DM. The use of different medications such as statins, fibrates, salicylic acid, and thiazolidinediones reduce serum hs-CRP levels. The Multi-Ethnic Study of Atherosclerosis (MESA) showed lower plasma levels of hs-CRP in subjects free of clinical CVD who were using statins than nonusers but did not for fibrinogen and PAI-1 concentrations [32]. According to our results, at the time of the blood sample we founded 54.9% ($n = 260$) subjects with dyslipidemia

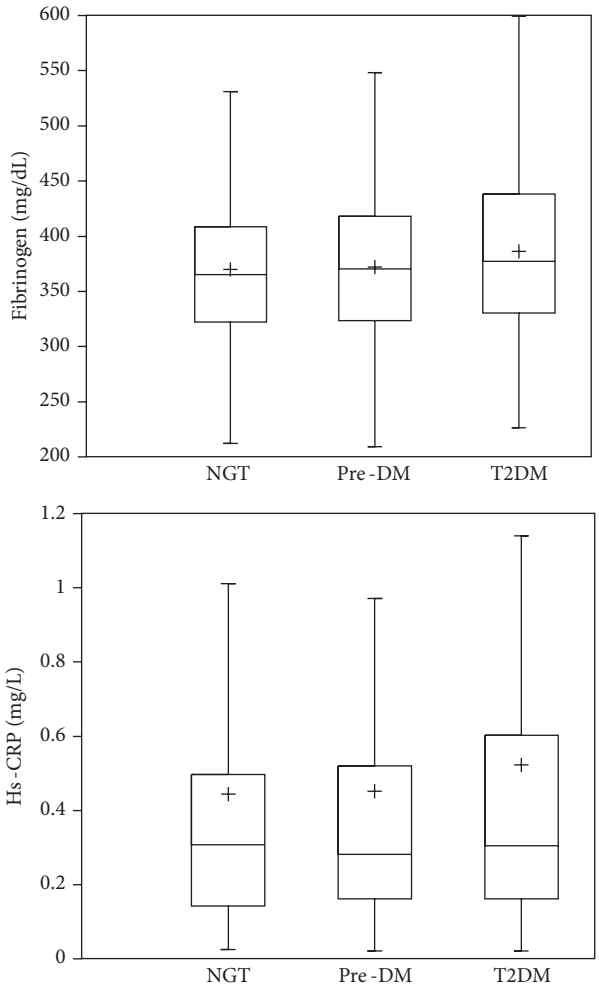

Figure 2: Box plots showing plasma levels of fibrinogen and hs-CRP according to glucose tolerance status. Fibrinogen showed significant difference between NGT and T2DM ($P = 0.01$), but it was not between NGT and pre-DM ($P = 0.71$). The hs-CRP concentration was statistically different between NGT and T2DM ($P = 0.05$), but it was not between NGT and preDM ($P = 0.89$). + = mean.

in the group of NGT, 65.2% ($n = 163$) in the group with pre-DM, and 65.7% ($n = 142$) in the group with T2DM. Each group was receiving medical treatment with statins or fibrates in only 13.9% ($n = 66$), 19.6% ($n = 49$), and 35.6% ($n = 77$) of cases for NGT, pre-DM, and T2DM, respectively. It will be interesting to determine the effect of glycemic control and medication employed in the proinflammatory and prothrombotic markers in future studies.

Another finding in our study was a positive correlation between hs-CRP and fibrinogen of $r = 0.53$ ($P < 0.0001$). This might suggest a crosstalk between coagulation and inflammation, by which an inflammatory response shifts the hemostatic system toward a prothrombotic state [33]. In summary, there are three mechanisms by which inflammation regulates coagulation: (1) inflammatory mediators initiate the coagulation system by promotion of tissue factor expression on the cell surface of monocytes, elevating the platelet count and platelet reactivity, and enhancing the expression of adhesion molecules, reactive oxygen species (ROS), and fibrinogen; (2) inflammation decreases the activity of natural anticoagulant mechanisms, such as tissue factor pathway inhibitor (TFPI), the heparin-antithrombin pathway, and the protein C anticoagulant pathway; and (3) inflammation impairs the fibrinolytic system promoting the formation of PAI-1 [34, 35].

On the other hand, we observed a variability of plasma fibrinogen concentrations of 31% (adjusted $R^2 = 0.31$, $P < 0.0001$) determined by hs-CRP ($\beta = 0.51$ [0.42–0.60], $P < 0.0001$), age ($\beta = 0.14$ [0.06–0.23], $P = 0.001$), and HbA1c ($\beta = 0.12$ [−0.003–0.25], $P = 0.05$). Fibrinogen is determined by several modifiable and nonmodifiable factors like age, gender, smoking, BMI, glycemic control, and lipid profile [31]. Also, estimates of the heritability of plasma fibrinogen concentrations range from 34% to 50% [36]. Interestingly, fibrinogen was predominantly determined by hs-CRP in our sample, which is considered an acute phase reactant that promotes an increase of fibrinogen concentrations [34]. In turn, T2DM alters fibrinogen and fibrin structure by glycation diminishing the susceptibility to degradation and promotes their accumulation [37]. Regardless of hs-CRP, we reported a variability of 33% (adjusted $R^2 = 0.33$, $P < 0.0001$) determined by fibrinogen ($\beta = 0.48$ [0.39–0.56], $P < 0.0001$) and BMI ($\beta = 0.19$ [0.04–0.34], $P = 0.01$). A preceding study in Mexican population showed higher mean serum hs-CRP levels above 5 mg/L in obese subjects and obese plus T2DM individuals, compared with a mean hs-CRP levels of 1.8 (0.9–3.9) mg/L in diabetic individuals without obesity [38]. Adipose tissue synthesizes proinflammatory cytokines such as TNF-α, interleukins, and cytokine-like proteins known as adipokines [39], and CRP (an acute phase protein) is primarily derived from IL-6 hepatic biosynthesis [40].

This low-grade proinflammatory and prothrombotic state present by T2DM and obesity is an inseparable condition of atherosclerosis and might confer an increased propensity to accelerated atherogenesis and macrovascular complications [23]. Previously, Mita et al. reported an independent association between hs-CRP and subclinical atherosclerosis in early-state T2DM [41]. Also, Schulze Horn et al. reported an independent relationship between hs-CRP and subclinical atherosclerosis in a population-based sample [42]. Despite the lack of clinical manifestations of CVD in our sample, we cannot dismiss the presence of subclinical atherosclerosis.

Biochemical markers, such as hs-CRP and fibrinogen have been used for evaluating the cardiovascular risk and incipient diabetes [18, 43, 44]. Although none of those biomarkers improve diabetes prediction and the magnitude of the risk varies according to the type of CVD, the presence of higher levels of proinflammatory and prothrombotic markers in our study must encourage us for maintenance of an adequate control of the traditional cardiovascular risk factors to prevent the deterioration of atherosclerosis and consequently the development of CVD.

There are strengths in our study. Although this is a cross-sectional nonrandomized assay, the gender and smoking status distribution were similar between all three groups

Table 3: Correlation analysis of proinflammatory and prothrombotic markers with traditional cardiovascular risk factors.

	Total = 939 individuals								
	Age	BMI	WC	FPG	HbA1c	HDL-C	Tg	hs-CRP	Fibrinogen
Fibrinogen	**0.14**	**0.25**	**0.18**	**0.10**	**0.14**	0.04	0.005	**0.53**	—
hs-CRP	−0.01	**0.33**	**0.27**	**0.14**	**0.12**	−0.09	0.07	—	**0.53**

Dependent variables are listed vertically, and covariates are listed horizontally. Cells show univariate correlation coefficients (significant in bold = $P \leq 0.05$). BMI: body mass index; WC: waist circumference; FPG: fasting plasma glucose; HbA1c: glycosylated hemoglobin; HDL-C: high-density lipoprotein cholesterol; Tg: triglycerides; hs-CRP: high sensitivity C-reactive protein.

Table 4: Multiple linear stepwise regression analysis for fibrinogen and hs-CRP as dependent variables.

Independent variables	Total = 939 individuals			
	Fibrinogen		Hs-CRP	
	β (95% CI)	P value	β (95% CI)	P value
Age	0.14 (0.06–0.23)	**0.001**	—	—
BMI	0.14 (−0.008–0.29)	*0.06*	0.19 (0.04–0.34)	**0.010**
WC	−0.08 (−0.23–0.06)	0.24	—	—
FPG	−0.10 (−0.23–0.03)	0.14	0.11 (−0.02–0.24)	0.10
HbA1c	0.12 (−0.003–0.25)	**0.05**	−0.06 (−0.19–0.06)	0.32
Fibrinogen	—	—	0.48 (0.39–0.56)	<**0.0001**
Hs-CRP	0.51 (0.42–0.60)	<**0.0001**	—	—
R^2	**0.31**		**0.33**	

Dependent variables are listed horizontally, and independent variables are listed vertically; β: standardized correlation coefficients (95% confidence interval); R^2: adjusted determination coefficient (significant in bold = $P \leq 0.05$); BMI: body mass index; WC: waist circumference; FPG: fasting plasma glucose; HbA1c: glycosylated hemoglobin; hs-CRP: high sensitivity C-reactive protein.

and did not act as cofounder variables. A low grade of obesity assessed by BMI was present in the T2DM group, diminishing the effects of obesity in serum hs-CRP levels. Also, individuals with T2DM were in optimal glucose control assessed by FPG and HbA1c concentrations. Our study had some limitations. Even though all individuals did not have clinical manifestations of CVD at the time of the enrollment, we cannot discharge the influence of subclinical atherosclerosis in the results. Furthermore, some potential cofounders such as physical activity and alcohol intake were not incorporated into the analysis. In addition, cases of IGT assessed by FPG plus HbA1c were limited and could not been evaluated. Also, this research was conducted in one center only. Therefore, more studies are needed to confirm our results.

Numerous publications indicate a significant contribution of inflammatory and procoagulant factors to the development of atherosclerosis and diabetes. Also, inflammatory and procoagulant markers, as well as hyperglycemia, increase the cardiovascular risk. Previous studies had demonstrated that an increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. Our findings suggest a subclinical proinflammatory and prothrombotic state in individuals with T2DM without clinical manifestations of cardiovascular disease. We consider that those markers could be a useful tool to screen subjects with higher cardiovascular risk, in order to prevent an atherothrombotic disease such as myocardial infarction or stroke.

5. Conclusions

Proinflammatory and prothrombotic state are present in people with T2DM lacking clinical history of CVD. Fibrinogen and hs-CRP serum concentrations are correlated and predominantly determined by BMI. The increased concentration of fibrinogen and hs-CRP added to other cardiovascular risk factors in a same individual might accelerate the development and severity of CVD, and preventive measures should be encouraged to improve glycemic control and reduce BMI.

Conflict of Interests

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

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Review Article

The Role of MIF in Type 1 and Type 2 Diabetes Mellitus

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Autoimmunity and chronic low-grade inflammation are hallmarks of diabetes mellitus type one (T1DM) and type two (T2DM), respectively. Both processes are orchestrated by inflammatory cytokines, including the macrophage migration inhibitory factor (MIF). To date, MIF has been implicated in both types of diabetes; therefore, understanding the role of MIF could affect our understanding of the autoimmune or inflammatory responses that influence diabetic pathology. This review highlights our current knowledge about the involvement of MIF in both types of diabetes in the clinical environment and in experimental disease models.

1. Introduction

MIF was originally reported in 1966 by two different groups and was described as a T cell derived cytokine that inhibited the random migration of macrophages *in vitro* and promoted macrophage accumulation during delayed-type hypersensitivity reactions [1, 2]. Human and mouse MIF genes are 90% homologous; MIF protein has a molecular weight of 12.5 kDa [3]. MIF is an evolutionarily conserved molecule that is constitutively expressed in many tissues and cells (Figure 1).

Moreover, MIF is stored in intracellular pools and therefore does not require immediate synthesis before secretion. MIF lacks an aminoterminal leader sequence; this indicates that MIF is released from cells through a nonconventional protein-secretion pathway [3].

After the discovery of MIF, several studies were conducted to establish its role in the immune response [4–6]. However, not until 1990 was MIF recognized as the first molecule to arrive at the inflammation site and the factor that likely determines the degree of cellular inflammation [7]. Different experimental strategies, including anti-MIF antibodies and knockout (KO) and transgenic MIF mice (MIF-Tg), have been used to establish that MIF counterregulates the immunosuppressive effects of steroids and to implicate MIF in tumor necrosis factor (TNFα) and nitric oxide (NO) production [8]; additionally, MIF was found to possess growth factor activity [9], overregulate the expression of Toll-like receptor (TLR)-4 on antigen-presenting cells [10], sustain macrophage proinflammatory abilities by inhibiting p53, and also possess tautomerase and oxidoreductase activities [11].

All the above-described inherent properties permitted the recognition of MIF as a critical molecule in proinflammatory innate immune responses and the restriction of certain parasite infections [12–14]. Additionally, MIF involvement has been demonstrated in immunological and inflammatory diseases [15, 16] such as septic shock [17], cancer [18], and chronic diseases including bowel disease [19], rheumatoid arthritis [20–22], colitis [23], obesity [24–26], and diabetes [25, 27, 28]. More recently, MIF was proposed as a diagnostic biomarker for autoimmune diseases such as arthritis, ulcerative colitis, and diabetes [23, 29, 30].

In this review, we will focus on some of the properties that have been conferred upon MIF with regard to the development and maintenance of T1DM and T2DM. We will discuss the data that have been collected in clinical studies and studies of MIF-KO mice and other protocols in which MIF has been proposed as a therapeutic diabetes mellitus pathological target.

2. MIF and Diabetes

2.1. Diabetes. This disease comprises a group of metabolic diseases that are characterized by hyperglycemia, which is

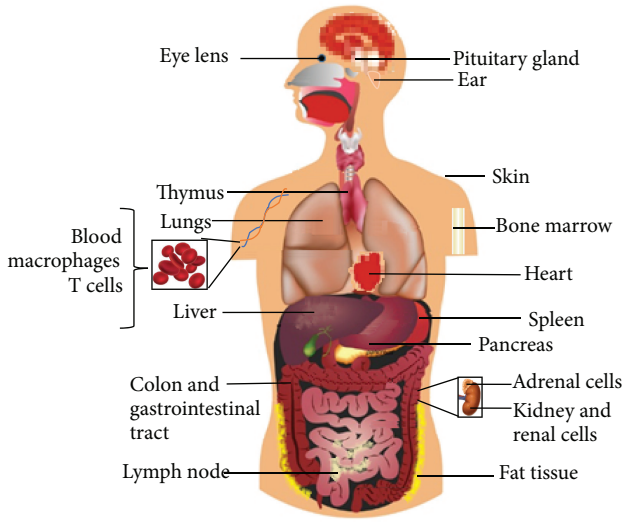

Figure 1: MIF expression pattern. MIF, a cytokine, is distributed throughout almost the entire body. This is because MIF is part of the innate immune system or first line of immune defense.

associated with damage to and/or malfunction or failure of various organs, including the eyes, kidneys, nerves, heart, and blood vessels, among others. The causes of this disease range from autoimmune or metabolic abnormalities to deficiencies in insulin activity and secretion [31]. Currently, there is no cure for this chronic degenerative disease; however, the constant development of knowledge helps us to better understand the disease etiology and potential therapeutical targets that, when combined, could lead to good symptom and disease control [31].

2.2. MIF and T1DM or Insulin-Dependent Diabetes Mellitus (IDDM). According to the American Diabetes Association, T1DM patients comprise only 5–10% of those with diabetes [26]. This disease is a multifactorial, organ-specific autoimmune disease that occurs in genetically susceptible individuals [32]. T1DM is the result of the autoimmune destruction of pancreatic islet β cells by infiltrating immune cells (insulitis); this occurs due to a failure in immune tolerance because the organism has had contact with specific viruses [33] such as cytomegalovirus [34] or with food molecules that caused molecular mimicry [35]. The common autoantigens recognized in this disease are insulin, glutamate decarboxylase 65 (GAD65), and the islet antigens IA-2 and IA-2β [36, 37]. During insulitis, high levels of proinflammatory cytokines, including IL-1β, TNFα, IL-12, MIF, and IFNγ, are secreted by effector T cells to trigger the β cell destruction process [32]. MIF is considered one of the most common factors in autoimmunity [38]. In humans with T1DM, blood MIF concentrations were found to be high, compared to those in healthy controls [39]; normal plasma MIF concentrations in healthy humans range from 2.3 to 8.4 ng/mL [40]. In contrast, plasma MIF concentrations dramatically change from 5 ng/mL to 1 ng/mL after islet transplantation [41]. Also high MIF concentrations are associated with a subsequent loss of islet graft function [41]. IL-1β and TNF-α are expressed at high levels along with advanced type one diabetes complications such as ketoacidosis [42], and thus it is possible that high levels of MIF are also expressed at this point in the disease. MIF studies were facilitated by the development of MIF-KO mice in 1999 [43]. Using these mice as an efficient tool, MIF was shown to be an important molecule in early syngeneic islet transplantation function, and blocking of MIF resulted in transplant success [44]. Additionally, we know that MIF participates in T1DM by controlling the functional activities of monocytes/macrophages and T cells and modulating their abilities to secrete proinflammatory molecules [45]. Furthermore, MIF has been recognized as important molecule to the development of T1DM complications such as cardiac dysfunction, which is associated with AMPK signaling [46], and diabetic foot disease [47] and is known to promote inflammatory cytokine and palmitic acid-induced pancreatic islet apoptosis [48, 49]. After successful antibody and pharmacological inhibitor-mediated MIF neutralization, MIF was proposed as a new target strategy for the treatment of T1DM [45, 50].

The involvement of MIF in T1DM is summarized in Figure 2. With the above-outlined information, we can conclude that the participation of MIF in the pathology of T1DM is a well-documented fact; however, we do not know the exact point in disease development at which MIF exerts the most influence. Considering that the insulitis process marks the beginning of the disease and is an autoimmune inflammatory process, we propose the hypothesis that MIF plays an important role in insulitis onset or development. This hypothesis is supported by studies in which MIF was found to play important roles in the processes of antigen presentation and inflammatory cell activation [13, 51]. However, additional studies should be performed to establish the mechanism related to the role of MIF in T1DM.

2.3. MIF and T2DM or Noninsulin-Dependent Diabetes Mellitus (NIDDM). T2DM patients account for 90–95% of all diabetic patients, and this disease is characterized by the presence of insulin resistance and, usually, relative insulin deficiency. The most common risk factors for this type of diabetes are genetic conditions, obesity, lifestyle, and eating habits. Therefore involvement of the inflammatory response is equally important in disease development, hence the reason why the role of MIF has been studied most in T2DM [31].

Several clinical studies have shown that the serum MIF levels are elevated in patients with T2DM [52]. In 2006, high blood MIF levels were suggested to precede the onset of T2DM [40]. More recently, both patients with T2DM and those with impaired glucose tolerance were shown to have significantly elevated MIF serum levels [25, 27, 53]. Some data report normal plasma MIF concentrations in healthy humans to range from 2.3 to 8.4 ng/mL [35]. In contrast, plasma MIF concentrations from T2DM subjects range from 7.3 to 15.8 ng/mL. As in T1DM, MIF is highly expressed in T2DM complications such as myocardial damage [54], coronary artery disease [53], diabetic retinopathy [55], obesity [56],

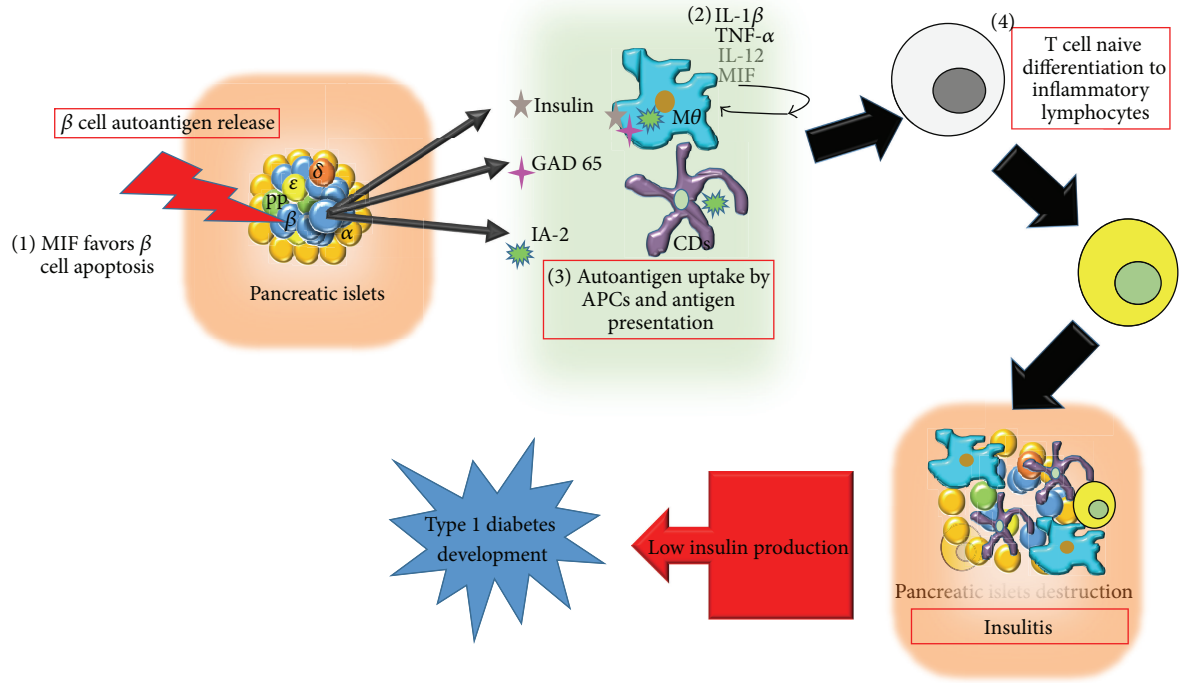

Figure 2: MIF involvement in T1DM development. (1) MIF promotes pancreatic β cell apoptosis. (2) MIF promotes the production of inflammatory cytokines such as IL-1β, TNF-α, and IL-12. (3) MIF favors autoantigen presentation. (4) MIF promotes the activation of an inflammatory response, leading to insulitis.

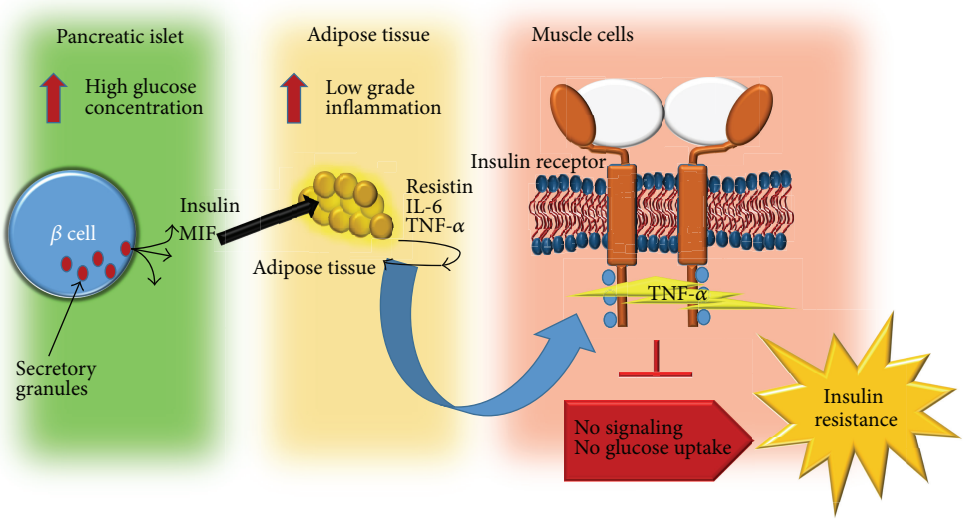

Figure 3: MIF plays an indirect role in T2DM development by promoting the production of proinflammatory cytokines and adipocytokines that are involved in insulin receptor signaling, leading to insulin resistance.

and metabolic syndrome [57]. The role of MIF in T2DM has been studied in murine models. Using db/db mice, MIF was suggested as a factor that could initiate the onset of microalbuminuria in diabetic nephropathy [58]. MIF plays an important role in the chronic, obesity-associated adipose tissue inflammation that leads to the development of insulin resistance in MIF-KO mice [59]. Additionally, we previously demonstrated in a NIDDM mice model that MIF could be a therapeutic target for disease treatment. Indeed, two candidate drugs (synthetic inhibitors for MIF) for diabetes treatment were described as very effective in the control of the systemic inflammation and the control of some diabetes symptoms [60]. Additionally, we showed that MIF is important to the production of some adipocytokines such as

resistin, which is the most important adipocytokine in the development of insulin resistance [60]. In this type of diabetes, MIF has been suggested to contribute to the disease in an indirect form due to its ability to stimulate the production of other inflammatory cytokines that directly cause damage to muscle cells. This is true for TNF-α, which acts on the insulin receptor and prevents the dephosphorylation of insulin receptor substrates, thus blocking receptor signaling and preventing glucose entry into the cell (insulin resistance). Furthermore, MIF also stimulates the production of certain inflammatory adipocytokines, such as resistin and IL-6, which are key molecules in the development of insulin resistance. As demonstrated, MIF influences T2DM development at different levels. In the pancreas, adipose tissue, and muscle cells, the pleiotropic characteristics of MIF are reflected in the different routes that lead to insulin resistance (Figure 3).

3. MIF and Pancreatic Beta Cells (β Cells)

MIF was shown to colocalize in secretory insulin granules within β cells and to be released during both phases of insulin secretion. Most importantly, in this regard, MIF appears to have an autocrine, glucose-dependent regulatory effect on insulin secretion [45].

While MIF is related to insulin secretion under homeostatic conditions, altered homeostasis in an organism (such as the presence of inflammation) apparently induces MIF to act differently and become a destructive molecule that can lead to β cell apoptosis [48, 61]. Apparently, MIF is not a foreign molecule in the pancreatic β cell microenvironment and can act in response to the concentration of glucose and the presence of inflammation.

4. Conclusions

Previously, different models were used to show that MIF is a pleiotropic molecule [62–64], and this property is very evident in pancreatic islets. Due to the large number of studies that support this idea, we can conclude that MIF is a proinflammatory cytokine with great importance not only during the course of diabetes but also before the establishment of diabetes and in the risk factors of disease such as obesity. We support the statement that MIF is a therapeutic target and propose that it is necessary to design synthetic MIF inhibitors that could interact with the existing therapies used to treat diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

The Morphological Features and Mitochondrial Oxidative Stress Mechanism of the Retinal Neurons Apoptosis in Early Diabetic Rats

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This paper aims to explore the relationship of retinal neuron apoptosis and manganese superoxidase dismutase (MnSOD) at early phase of diabetic retinopathy. Sprague-Dawley rats were grouped into normal controls and diabetics. Data were collected after 4, 8, and 12 weeks ($n = 12$). The pathological changes and ultrastructure of the retina, the apoptosis rate of retinal neurons by TdT-mediated dUTP nick end label (TUNEL), mRNA expressions of MnSOD and copper-zinc superoxide dismutase (Cu–Zn SOD), and the activities of total SOD (T-SOD) and subtypes of SOD were tested. For the controls, there was no abnormal structure or apoptosis of retinal neurons at any time. There was no change of structure for rats with diabetes at 4 or 8 weeks, but there was a decrease of retinal ganglion cells (RGCs) number and thinner inner nuclear layer (INL) at 12 weeks. The apoptosis ratio of RGCs was higher than that of the controls at 8 and 12 weeks ($P < 0.001$). The activity and mRNA levels of MnSOD were lower in diabetics at 4, 8, and 12 weeks ($P < 0.05$). In summary, the apoptosis of the retinal neurons occurred at 8 weeks after the onset of diabetes. Retinal neuron apoptosis in early diabetic rats may be associated with the decreased activity and mRNA of MnSOD.

1. Introduction

Diabetic retinopathy (DR) is a chronic complication of diabetes mellitus (DM), the leading cause of blindness in adults, affecting 51 million people all over the world [1, 2]. The prevalence of diabetic retinopathy is about 20.5%~46.9% [3, 4]. DR is not just a microvascular complication of DM [5–7]. All types of cells within the retina are involved in an array of pathophysiological processes even at the early stages of diabetes. A growing number of studies suggested that DR is also the lesion of the retinal neurons [8, 9]. The newly onset clinical features of DR, breakdown of the blood-retinal barrier (BRB), and various visual deficits all support this concept in human studies [10–13].

Because of gradual and accelerating deterioration, intervention should be implemented before irreversible DR occurs [14, 15]. Although the mechanism remains unclear, it is generally believed that excessive reactive oxygen species (ROS) from mitochondria induce apoptosis. With the consequent oxidative damages, it contributed to the development of diabetes and DR [16–19]. The importance of mitochondria in regulating apoptosis has been well documented. Under hyperglycemic conditions, inflammation [20] and excessive ROS damage mitochondria and thus promote the release of apoptosis-inducing factors such as cytochrome C, which further activates the neuronal apoptosis process [21–23]. Therefore, alleviation of mitochondrial oxidative damage may reduce apoptosis of retinal neurons and thus provide a new strategy for treatment of newly onset DR. One effective method to reduce mitochondrial oxidative damage is lowering ROS. SOD, an important antioxidant enzyme, combines and disproportionates superoxide anion and thus reduces their effects on lowering SOD activity [24]. Therefore, it is very important to understand and restore SOD activity to protect mitochondria. There were two types of SODs: Mn

SOD and Cu-Zn SOD [25]. It remains unknown which one plays a key role in preventing retinal neurons apoptosis.

In this study, by examining the activity and mRNA expressions of Mn SOD and Cu-Zn SOD in retinal neuron of rats with diabetes, we hope to demonstrate the relationship of retinal neurons apoptosis and expression/activity of Mn SOD in rats at the early phase of diabetes.

2. Materials and Methods

2.1. Animals and Diabetic Models. A total of 72 male healthy Sprague-Dawley (SD) rats (8 weeks old, weighted at 230–250 g) were provided by PLA Academy of Military Medical Experimental Animal Center, grouping into normal controls and diabetics. STZ 60 mg/kg body weight was injected intraperitoneally to establish diabetic models [26]. A blood glucose >16.7 mmol/L after 48 hours was regarded as diabetic.

The rats were kept at 12 hr day-night cycle room, with free access to chaw diet and water. Data were collected at 4, 8, and 12 weeks. The left or right eyeballs from 6 rats in each group were used for histopathology HE staining, TdT-mediated DNA nick end dUTP label (TdT-mediated dUTP nick end labeling, TUNEL) detection, SOD activity detection, and tissue RNA extraction and detection respectively.

2.2. Chemicals. STZ and TUNEL detection kits were purchased from Sigma, USA. Xanthine oxidase enzymatic detection kit for SOD detection was purchased from Nanjing Jiancheng Bioengineering Institute. Primers used were as follows: for Cu-Zn SOD [27], forward: 5′-GTT CCG AGG CCG CGC GT-3′ and reverse: 5′-GTC CCC ATA TTG ATG GAC-3′; for Mn SOD [28], forward: 5′-GGC CAA GGG AGA TGT TAC AA-3′ and reverse: 5′-GCT TGA TAG CCT CCA GCA AC-3′; and for Rat β-actin [29], forward: 5′-CCT GCT TGC TGA TCC ACA-3′ and reverse: 5′-CTG ACC GAG CGT GGC TAC-3′. RNA extraction kit was from Tiandz, Inc., Reverse-transcription kit was from Promega, USA, and Real Master Mix (SYBR Green) was from Tiangen Biotech (Beijing) Co., Ltd.

2.3. Paraffin Section Preparation. Left eyes were quickly removed and fixed in 4% paraformaldehyde for 15–20 min. Sections were made at the front corneal limbus. The vitreous were then carefully removed and the remaining "eye cup" was fixed for 2 more hours and then followed by graded ethanol dehydration, xylene, and embedded wax. Starting at 2 mm from the optic nerve, the sections were made (4 μm thick) and stained with HE.

2.4. TUNEL Detection of the Apoptosis Rate of Neurons in Retinal Ganglion Cell Layer. As described in our previous study [30], the paraffin was dewaxed, and then the tissues were treated with 3% H_2O_2 at room temperature for 10 min, followed by proteinase K treatment at 37°C for 10 min and then with TdT and DIG-d-UTP. The biotinylated antidigoxin antibody was added and incubated at 37°C for 30 min and then 1 : 100 diluted streptavidin-biotin complex reagents were added; the sample was further processed with 3, 3′-diaminobenzidine coloration, slightly redyed with Mayer hematoxylin, then washing, dehydration, transparent and mounting. Three slices from each sample were used to count cell number with 5 independent fields for each. The average was used to calculate apoptotic index.

The apoptosis index of neurons in retinal ganglion cell layer was calculated as follows:

$$\text{Apoptotic index} = \frac{(\text{apoptotic cell number})}{(\text{total cell number within the same field})} * 100\%. \quad (1)$$

2.5. Detection of Retinal Total SOD (T-SOD) and Subtype SOD. Retina tissue was homogenized in lysis buffer. After being centrifuged at 2,000 rpm for 15 min, the supernatant was collected. The protein concentration was measured by use of Bradford method; T-SOD and Cu-Zn SOD activities were detected by use of xanthine oxidase enzymatic detection according to the instruction manual. Mn SOD activity was calculated by subtracting Cu-Zn SOD activity from T-SOD activity.

2.6. mRNA Expression of Retinal Mn SOD, Cu-Zn SOD. Total retina RNA were extracted from retina tissues with TRIzol. Reverse transcription was performed according to the instruction manual (SYBR Green Master Mix, ABI). A 7500 quantitative PCR machine was used and the relative quantitative (RQ) values were used for analysis.

2.7. Statistical Analysis. SPSS 13.0 statistical software was used for data analysis. The data was presented as mean ± standard deviation (mean ± SD). The differences among groups were analyzed with ANOVA. Pearson correlation was used to detect the relationship of mRNA levels of Cu-Zn and Mn SOD with apoptosis of the retina neurons. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Values of Blood Glucose Levels and Weight. The results were summarized in Table 1.

In the controls, body weights of the rats were about 230–250 g after week 8. The coat is smooth and supple. Fasting blood glucose was between 4.2 and 6.3 mmol/L and urine sugar test was negative. Eye cornea and lens were transparent. Daily water intake and urine output were normal. There was an upward trend for body weight with time, not statistically significant. In the diabetics, fasting blood glucose was between 19.7 and 35.5 mmol/L. There was no self-normalization of blood glucose. The daily water intake and urine output were higher than those of the controls. Body weight decreased gradually and the rats were emaciated finally. The lens was cloudy, which was worsening with time.

3.2. The Morphology of the Retinal Structure under Light Microscope. In the controls, at week 4 (Figure 1(a)), week 8

Table 1: Values of rat blood glucose and weight at 4, 8, and 12 weeks.

Group	Rat number	Values of blood glucose (mmol/L)			Weight of rats (gram)		
		4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Control	12	5.1 ± 0.5	4.942 ± 0.664	5.017 ± 0.681	263 ± 6.179	360 ± 9.391	433.083 ± 10.561
Diabetic	12	28.9 ± 2.2[a]	30.233 ± 2.053[a]	28.375 ± 1.573[a]	257.333 ± 4.849[b]	249.333 ± 5.914[a]	239.5 ± 6.516[a]
P value			<0.001			<0.001	

Compared with the control: a = 0.000; b = 0.07.

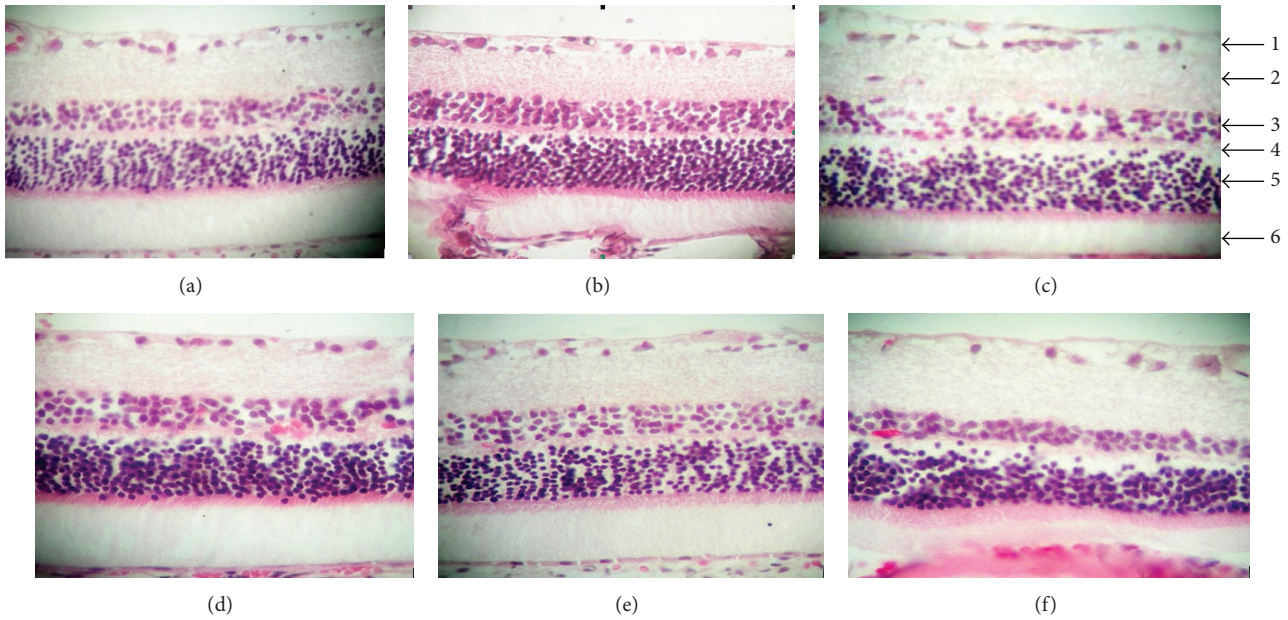


Figure 1: The morphology of retinal structure under light microscope (hematoxylin-eosin staining ×400). (a–c) Control group; (d–f) diabetic group. Numbers 1–6 represent layers of ganglion cell, inner plexiform, inner nuclear, outer plexiform, outer nuclear, rods, and cones.

(Figure 1(b)), and week 12 (Figure 1(c)), all the retinal layers were clear and arranged in order; the retinal ganglion cells were arranged in a layer; the nucleus of RGCs was big, circle- or oval-shaped with light staining; the inner plexiform layer was net-shaped with loose structure; the inner nuclear cell layer consisted of 3 to 5 layers of cells; the nucleus was big with dark staining; the outer plexiform layer was thinner than the inner plexiform layer; the outer nuclear layer was thicker and arranged tightly with 8 to 10 layers of cells; the nucleus was small with dark staining; and the rods and cones layers were thicker. There is no difference in the diabetics at week 4 (Figure 1(d)) or week 8 (Figure 1(e)). At week 12 (Figure 1(f)), the intercellular space increased and the number of the cells decreased in retinal ganglion cells layer, and the inner nuclear layer was thinner.

3.3. The Ultrastructure of the Retina under EM. In the controls, RGCs (Figure 2(a)) showed a big but low electronic density nucleus, with abundant organelles including mitochondria (with normal structure and clear mitochondrial ridge, without swollen), endoplasmic reticulum, and ribosome. The cells of the INL were circle- or oval-shaped with big nucleus and more organelles (Figure 2(b)) and were arranged tightly (Figure 2(c)); the outer nuclear layer of retina composed of the nucleus of the photoreceptor and arranged tightly and trimly, with uniform nuclear chromatin distribution (Figure 2(d)).

In the diabetics at week 4, there was mitochondrial swollen in some RGCs (Figure 2(e)), with undefined ridge, shorter than normal or diminished; some chromatin of INL cells was distributed asymmetrically (Figure 2(f)) or condensed as crescent (Figure 2(g)) with undefined cell membrane (Figure 2(h)). There was no significant difference in the outer nuclear layer of retina compared with the controls.

In the diabetics at week 8, more mitochondrial RGCs were swollen (Figure 2(i)), and some RGCs were shrunk with condensed cytoplasm and decreased organelles (Figure 2(j)). Some inner nuclear cells became smaller with undistinguishable nuclear membrane but with more vacuolization in cytoplasm (Figure 2(k)). There was no significant difference at the outer nuclear layer compared with controls.

In the diabetic at week 12, some retinal ganglion cells became smaller (Figure 2(l)) with barely defined nuclear membrane (Figure 2(m)), even with nucleus collaps (Figure 2(m)); there were more vacuolization in some fragments of the nucleus in the inner nuclear cells (Figure 2(n));

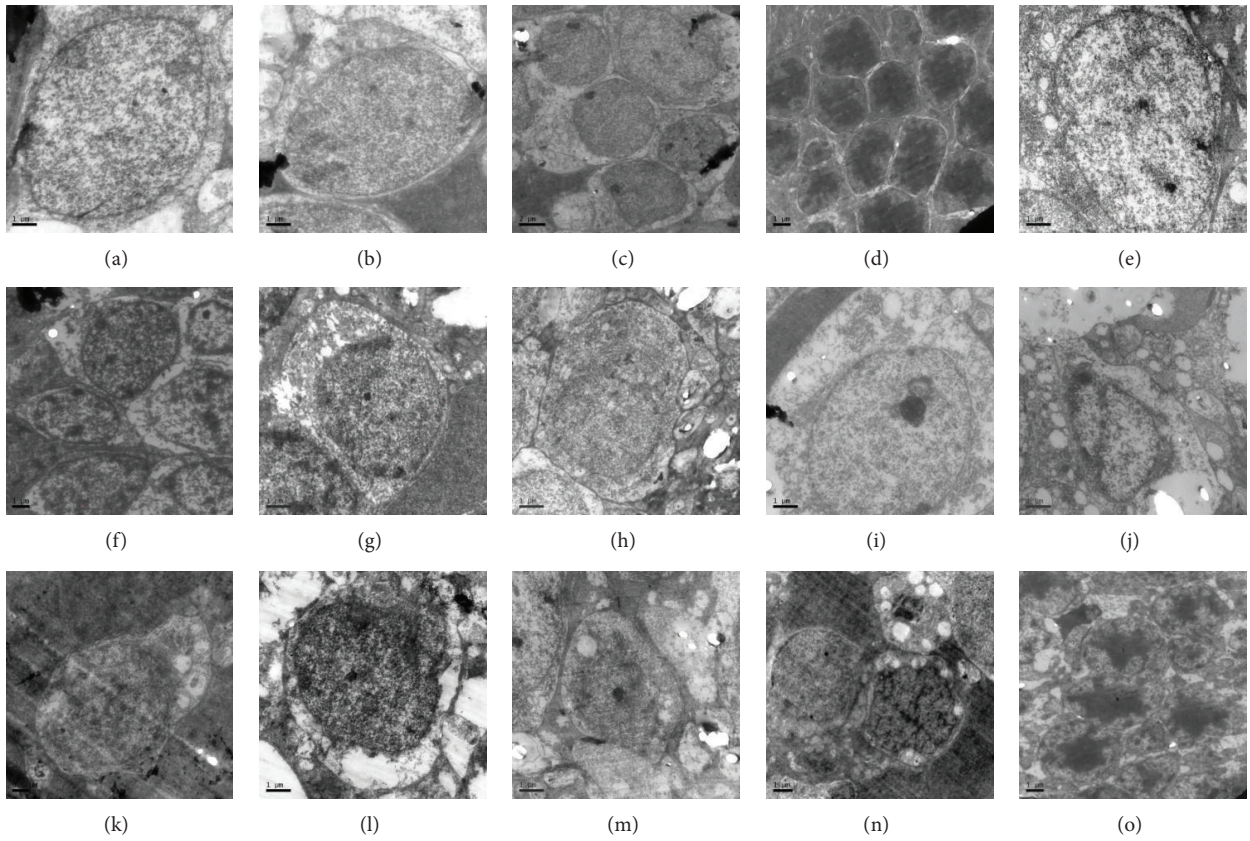


Figure 2: The ultrastructure of retina under electron microscope. (a–d) Control group; (e–h) diabetic group at week 4; (i–k) diabetic group at week 8; (l–o) diabetic group at week 12. In control group, RGC (a ×15 K); INL (b ×15 K), (c ×8000); ONL (d ×10 K). In diabetic group at week 4, RGC (e ×15 K); INL (f ×15 K), (g ×15 K), and (h ×15 K). In diabetic group at week 8, RGC (i ×15 K), (j ×15 K); INL (k ×15 K). In diabetic group at week 12, RGC (l ×15 K), (m ×15 K); INL (n ×15 K); ONL (o ×15 K).

the electrical density of the cytomembrane was asymmetrical and condensed with asymmetrical chromatin in ONL (Figure 2(o)).

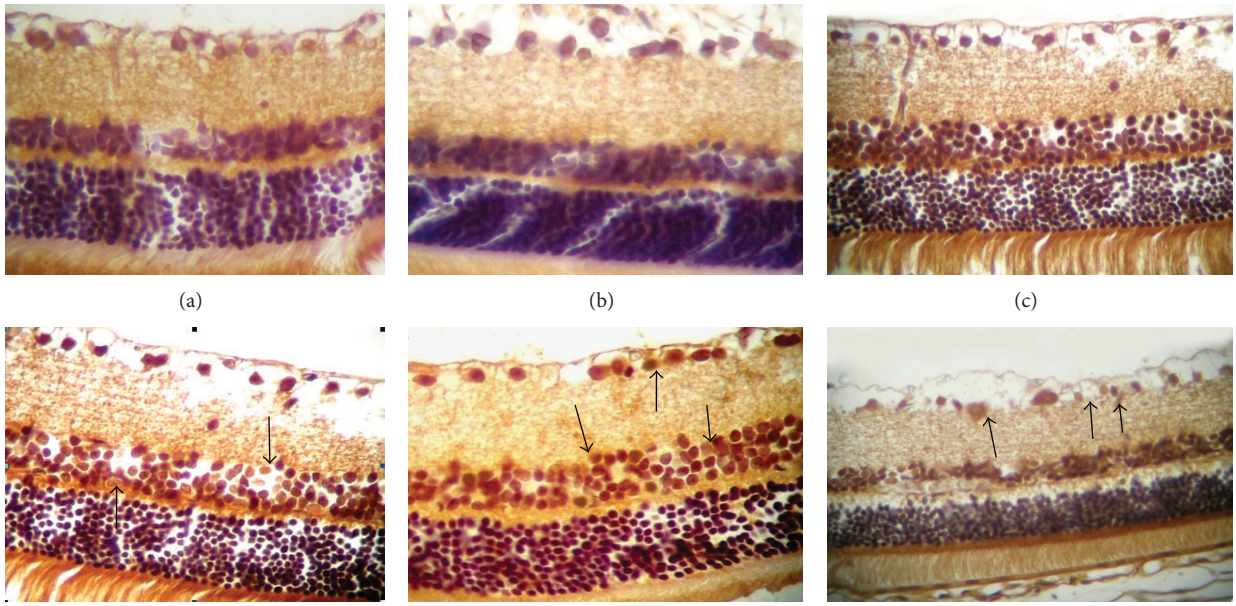
3.4. Apoptotic Index of Neurons in Retinal Ganglion Cell Layer. For the controls, at week 4, 8, and 12, there was no apoptosis of neurons in retinal ganglion cell layer (Figures 3(a)–3(c)). At week 4 of the diabetics, scattered apoptotic cells appeared and distributed at the inner nuclear layer of the retina. No apoptotic cells appeared in ganglion cell layer or outer nuclear layer cells (Figure 3(d)) with an apoptosis index of (0.48 ± 0.53)%. After week 8, there were more apoptotic cells within the inner nuclear layer of retina ganglion cells. There were apoptotic cells within the neurons of retinal ganglion cell layer (Figure 3(e)), with an apoptotic index of (5.66 ± 2.1)%. At week 12, there was dramatic increase of apoptotic cells within neurons of retinal ganglion cell layer and inner nuclear layer (Figure 3(f)), with an apoptotic index of (11.83 ± 1.58)%. The difference of apoptosis index between the controls and diabetics was not significant at week 4 ($P = 0.405$) but was statistically significant at weeks 8 and 12 ($F = 112.896$, $P < 0.001$).

3.5. Total SOD (T-SOD), Cu-Zn SOD, and Mn SOD Activity Test. There were significant differences for the activities of T-SOD, Cu-Zn SOD, and Mn SOD, between the controls and the diabetics (T-SOD: $F = 40.907$, $P < 0.05$; Cu-Zn SOD: $F = 15.735$, $P < 0.05$; Mn SOD: $F = 17.636$, $P < 0.05$). As shown in Table 2, there was no significant change of the activities of Cu-Zn SOD or Mn SOD in the controls at all-time points. While the activities of T-SOD, Cu-Zn SOD, and Mn SOD decreased with time in the diabetics, which mainly occurred at weeks 4 and 8 for Mn SOD and weeks 8 and 12 for Cu-Zn SOD.

3.6. mRNA Expression of Mn SOD and Cu-Zn SOD. As shown in Table 3, there was no significant difference for mRNA expressions (RQ values) of Cu-Zn SOD and Mn SOD in the controls at any time point. While in the diabetics, both mRNA expressions of Cu-Zn SOD and Mn SOD decreased gradually with time. There was significant difference between controls and diabetics (Cu-Zn SOD: $F = 10.917$, $P < 0.05$; Mn SOD: $F = 75.579$, $P < 0.05$). The mRNA expression of Mn SOD decreased at week 4, 8 and 12, while that of Cu-Zn SOD decreased at week 8 and 12. There was a negative correlation between Mn SOD mRNA and apoptosis of the retina neurons but not with Cu-Zn SOD mRNA ($P < 0.001$, $r = -0.89$).

4. Discussion

Diabetic retinopathy includes retinal neuropathy and microvascular pathology, both causing retinal lesion and visual



(a) (b) (c)

(d) (e) (f)

Figure 3: Microscopic detection of retina neuron apoptosis (×400). (a–c) Control group; (d–f) diabetic group. (Apoptosis is labeled with arrow.)

Table 2: Activity comparison of T-SOD, Cu–Zn, and Mn SOD at 4, 8, and 12 weeks.

Group	Weeks	Rat number	Activity of SOD (U/mg)		
			T-SOD	Cu–Zn SOD	Mn SOD
Control	4	6	161.002 ± 9.996	113.884 ± 9.07	47.118 ± 5.018
	8	6	158.334 ± 7.788	112.301 ± 5.24	46.033 ± 6.835
	12	6	163.333 ± 5.369	117.52 ± 7.982	45.813 ± 6.858
Diabetic	4	6	143.656 ± 4.981[a]	109.793 ± 7.468[c]	33.863 ± 6.909[b]
	8	6	121.465 ± 11.203[b]	98.588 ± 9.212[d]	22.877 ± 7.875[b]
	12	6	98.202 ± 11.813[b]	78.168 ± 12.180[b]	20.034 ± 6.796[b]
F value			40.907	15.735	17.636
P value			0.000	<0.05	<0.05

Compared with the control group: a = 0.001; b = 0.000; c = 0.36; d = 0.003.

Table 3: RQ value of Cu–Zn and Mn SOD mRNA expression at 4, 8, and 12 weeks.

Group	Weeks	Rat number	RQ value of Cu–Zn and Mn SOD mRNA expression	
			Cu–Zn SOD mRNA	Mn SOD mRNA
Control	4	6	1.066 ± 0.111	0.973 ± 0.123
	8	6	1.055 ± 0.119	0.974 ± 0.085
	12	6	1.092 ± 0.180	0.994 ± 0.074
Diabetic	4	6	0.976 ± 0.108[a]	0.627 ± 0.083[c]
	8	6	0.829 ± 0.048[b]	0.333 ± 0.080[c]
	12	6	0.621 ± 0.033[c]	0.256 ± 0.057[c]
P value			0.000	0.000
R value to apoptosis			−0.27	−0.89

Compared with the control group: a = 0.157; b = 0.001; c = 0.000.

function defect. In our study, we investigated the onset, the morphological features of retinal neuron apoptosis, and the mitochondrial structure features along with the development of diabetes and provided an evidence of retinal neuron apoptosis in newly onset diabetic rats.

Our study showed that there were increased intercellular space, decreased cell number in retinal ganglion cell layer with time, and thinner inner nuclear layer at week 12 in the diabetic rats. Apoptosis of retinal neurons is the initial presentation in diabetic retinopathy. In previous studies [31, 32], retinal ganglion cells were labeled by injection of 3% fluorogold into both sides of superior colliculus and the numbers and distribution of RGC were observed. This is a complicated but indirect method. With EM, we found multistage nonsynchronous apoptosis of retinal neurons along with time. Our study confirmed that there was a series of ultrastructure changes of retinal neurons in newly onset diabetic rats, varying in mitochondrial swollen, cytoplasm condensation, chromatin margination, and apoptosis.

Although EM provides direct vision of apoptosis, it can not be used to quantify apoptosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) is an in situ method for detecting the $3'$-OH ends of DNA exposed during the internucleosomal cleavage during apoptosis [33]. It is a combination of molecular biology and morphology. The incorporation of biotinylated dUTP allows detection by immunohistochemistry. The labeled apoptotic cells may be visualized under light microscope. It can be used to quantify apoptotic degree sensitively and specifically, especially for newly onset apoptosis. Our research demonstrated that apoptotic cells were mainly located at retinal ganglion cell layer and inner nuclear layer, getting more significant with the development of diabetes. Barber et al. [34] showed there were 22% and 14% reductions in the thickness of the inner plexiform and inner nuclear layers in rats with 7.5 months of diabetes. It was believed that these reductions were related to apoptosis of retinal neurons. Our consistent results from TUNEL and EM further support this concept.

Apoptosis of retinal neurons affected visual function in patients with diabetes. If the mechanism of apoptosis can be identified, it may be very helpful for preventing or alleviating diabetic retinopathy. Two classical cellular apoptotic pathways were identified: extracellular (cell surface death receptor) and intracellular (mitochondrial-mediated) pathways. Mitochondria play a key role in regulating apoptosis. The reactive oxidant intermediates can trigger the release of cytochrome C from mitochondria, a key event in activation of caspase-3 and a downstream pivotal step in the initiation of apoptosis [35, 36]. The mitochondrial electron transport chain is a major source of superoxide, converting up to 5% of molecular O_2 to superoxide normally but even more under pathologic conditions [37, 38]. Studies suggested that reactive oxygen species (ROS) in nmol level improve cell proliferation, cause apoptosis in pmol level, and induce cell necrosis in mmol level [39]. At low density, it is a normal physiological process. At high density, excessive ROS leads to irreversible cell damage [40]. Hyperglycemia increases oxidative stress and plays an important role in the onset and development of diabetic complications [41]. Mitochondrion is vulnerable to oxidative stress and thus releases more ROS, which accelerates oxidative stress and leads to vicious circle. Excessive ROS causes mitochondrial swollen and excessive mitochondrial permeability thus induces release of cytochrome C and activates the mitochondrial-mediated apoptosis. Under hyperglycemic conditions, oxidative stress is increased in retina and isolated retinal capillary cells (both endothelial cells and pericytes) [42]. In another rat model of DR induced by galactose, it was reported that long term administration of antioxidants inhibits the development of DR [41, 43]. This further supported the importance of oxidative stress in the development of DR. Under normal condition, the excessive ROS can be scavenged by antioxidative defense system. Whereas, in diabetes, the activity and defense system of the antioxidative enzymes were impaired significantly, and thus the damage of ROS was worsened. SOD was considered as an important antioxidative enzyme, which combines superoxide anion and converts them into nonreactive products. Consistent with our results, it is shown that the activity of SOD is decreased in diabetic retina [44, 45]. Overexpression of SOD reduces oxidative stress, decreases release of cytochrome C and apoptosis of neurons, and prevents diabetes-induced glomerular injury, suggesting its major role of regulating apoptosis [46–49]. There were 2 types of SODs: Mn SOD and Cu-Zn SOD. Due to its location, Mn SOD is considered as the first line to prevent oxidative damage. Thus, it is very important to investigate the change of Mn SOD to clarify the apoptosis pathway. However, in previous studies, the focus has been placed on total SOD or Cu-Zn SOD. Our analysis demonstrated that both activities of Cu-Zn and Mn SOD of rat retina decreased with extended course of diabetes, suggesting that the longer the course of diabetes, the worse oxidative damage of the retina and the less the anti-oxidative capacity of SOD [50]. Although the content of Cu-Zn SOD in T-SOD was higher than that of Mn SOD, its activity decreased at a quite late stage. In contrast, there was significant decrease for the activity of Mn SOD even at week 4, further confirming its frontier activity against oxidative stress under diabetic conditions. More interestingly, this is consistent with the reduction of its mRNA expression, suggesting the possibility cause of decreased activity. Our study showed that mRNA overexpression of Mn SOD could enhance its activity and antioxidative capacity. Study has reported that Mn SOD activity in nontransfected retinal endothelial cells was 20% of the total SOD activity and increased to 60% in Mn SOD-transfected cells. Overexpression of Mn SOD prevented glucose-induced increase of oxidative stress and apoptosis of retinal endothelial cells [27, 51]. Cu-Zn SOD was located in the cytoplasm. It also plays an important role in preventing oxidative damage but may not influence the newly onset mitochondrial-mediated apoptosis because its changes only occurred at a rather late stage. Thus, in newly onset DR, overexpression or increased activity of Mn SOD is more significant than that of Cn-Zn SOD and T-SOD. It may challenge the concept that Cu-Zn SOD plays an irreplaceable role in scavenging ROS because of its large proportion in T-SOD [52]. There are other studies on antioxidants, such as vitamin C and vitamin E. However, their antioxidative effects were not promising. This may be due to the fact that they only

change Cu-Zn SOD activity and are short of protection for mitochondria.

In summary, our study suggested a protective role for Mn SOD in the apoptosis of retinal neurons and, ultimately, in the pathogenesis of diabetic retinopathy. It may elucidate a better understanding of Mn SOD in modifying the course of diabetic retinopathy and suggest an important molecular target for future pharmacological interventions.

Conflict of Interests

The authors declared that there is no conflict of interest.

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Review Article

Early Life Factors and Type 2 Diabetes Mellitus

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Type 2 diabetes mellitus (T2DM) is a multifactorial disease, and its aetiology involves a complex interplay between genetic, epigenetic, and environmental factors. In recent years, evidences from both human and animal experiments have correlated early life factors with programming diabetes risk in adult life. Fetal and neonatal period is crucial for organ development. Many maternal factors during pregnancy may increase the risk of diabetes of offsprings in later life, which include malnutrition, healthy (hyperglycemia and obesity), behavior (smoking, drinking, and junk food diet), hormone administration, and even stress. In neonates, catch-up growth, lactation, glucocorticoids administration, and stress have all been found to increase the risk of insulin resistance or T2DM. Unfavorable environments (socioeconomic situation and famine) or obesity also has long-term negative effects on children by causing increased susceptibility to T2DM in adults. We also address the potential mechanisms that may underlie the developmental programming of T2DM. Therefore, it might be possible to prevent or delay the risk for T2DM by improving pre- and/or postnatal factors.

1. Introduction

Type 2 diabetes (T2DM) is a metabolic disease caused by genetic and multiple environmental factors. Epidemical and experimental studies have found that detrimental early life factors may predispose high incidence of cardiovascular disease and metabolic diseases in later life, which is also termed as "barker hypothesis." Organs are under development and functional maturation from fetal stage to childhood; disturbance of the homeostasis during crucial periods might predispose increased risk of insulin resistance and even T2DM in late life.

2. Part I: Prenatal Factors (Figure 1)

2.1. Diet and Nutrition. It has been suggested that the quality and quantity of the nutrition during pregnancy may cause strong and permanent effects on the fetus. The altered structure of chromosome during this procedure might be the cause of cell dysfunction and increased susceptibility to diseases through altered gene expression [1].

2.1.1. Malnutrition and Low Protein Diet. The associations between maternal malnutrition, low protein diet, and T2DM have been widely studied. Typical epidemical studies from the population born during the Dutch famine period [2] or in some poor countries [3] have found that those who had been exposed to maternal malnutrition may have increased morbidity of metabolic diseases including T2DM in adult life.

The mechanisms responsible for the prenatal malnutrition programming insulin resistance or T2DM remain unclear. Orozco-Solís et al. [4] have found that low protein diet during pregnancy and lactating may cause permanent altered hypothalamic expression of genes in rat offspring involved in insulin signaling and lipid and glucose metabolism, which may programme metabolic diseases.

In addition, the effect of low protein diet during pregnancy on postnatal β cell has also been noticed recently.

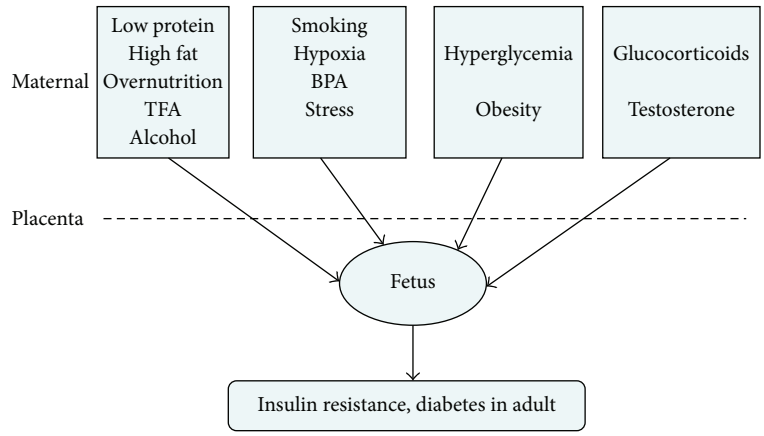

Figure 1: Prenatal factors mentioned in recent years that might correlate with insulin resistance and/or T2DM. Data from human and animal studies have shown that malnutrition or overnutrition, metabolic disorders, exposure to hypoxia, some chemicals and hormones, and unhealthy lifestyle such as smoking and alcohol drinking during pregnancy might predispose detrimental long-term effects on offspring, leading to increased risk of insulin resistance or T2DM. TFA: transfatty acids; BPA: biophenol A.

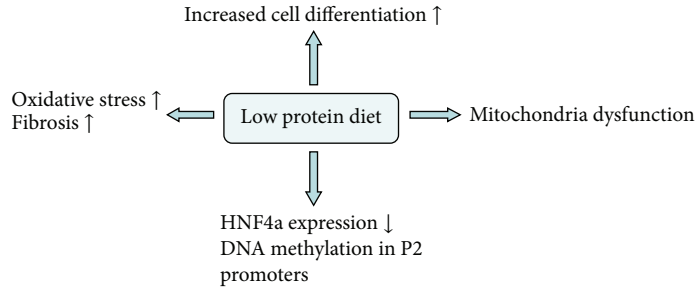

Figure 2: The effect of low protein diet during pregnancy on postnatal β cell. Low protein diet during pregnancy may lead to increased oxidative stress, fibrosis, decreased HNF4a expression, defected mitochondriogenesis, and mitochondria dysfunction, and increased cell differentiation instead of proliferation was found in β cell of adult animal offspring, which may participate in β-cell dysfunction and consequently increase the incidence of T2DM.

Increased oxidative stress and fibrosis [5], decreased HNF4a expression with increased DNA methylation in P2 promoters [6], defected mitochondriogenesis and mitochondria dysfunction [7], and increased cell differentiation instead of proliferation [8] were found in β cell of adult animal offspring whose mothers were under low protein diet during pregnancy. These may cause β-cell dysfunction and consequently increase the incidence of T2DM in postnatal life (Figure 2).

2.1.2. Overnutrition

High Protein. A study from Maurer and Reimer [9] in Wistar rats has found that high protein diet during pregnancy and lactating may cause increased resistin and IL-6 mRNA levels in brown fat tissue in 35-day-old offspring; both factors were included in the pathogenesis of insulin resistance [10, 11].

High Fat Diet. Both human and animal studies have identified that fat diet may cause obesity and insulin resistance [12, 13]. Intriguingly, the effect of high fat diet on metabolic disorders seemed to be programmative. The prenatal period is a key developmental window for nutrition status. Masuyama and Hiramatsu [14] found that mice offspring exposed to high fat diet during pregnancy developed insulin resistance and hyperlipidemia at 24 wks of age, which was associated with altered levels of leptin in adipose tissue. The experiment conducted in C57BL/6 mice by Liang et al. [15] has also showed that high saturated fatty acids diet during pregnancy led to insulin resistance, hyperglycemia in adult offspring under normal diet condition. The mechanisms underlying are still under investigation. Evidences from animal study have indicated that overexposure to high fat diet in utero may lead to elevated mRNA level of hypothalamic signal transducer and activator of transcription-3 and suppressor of cytokine signalling-3 in the offspring [16]. Both of these two factors are found to participate in obese and insulin resistance cases [17]. In addition, prenatal exposure to high saturated fats may cause increased hepatic phosphoenolpyruvate carboxykinase expression, fatty liver, reduced basal acetyl CoA carboxylase phosphorylation, and insulin signalling [18]. Impaired Wnt/β-catenin signaling pathway in skeletal muscle has also been found [19], which may also participate in pathogenesis of insulin resistance in adult life, since the insulin sensitivity can be improved by activating Wnt/β-catenin [20].

2.1.3. Transfatty Acids and Junk Food. Transfatty acids are unsaturated fatty acids that contain nonconjugated double bond in the trans-configuration. So far, data that correlated transfatty acids diet with insulin resistance or diabetes is weak and inconsistent [21–23]. However, it is still worth noting that prenatal exposure to transfatty acids might cause impaired insulin resistance and increased content of abdominal fat after birth [24]. No similar effects can be observed in mice exposed to transfatty acid during lactating.

Data about long-term effects of prenatal junk food taking is quite limited; experiment from Bayol et al. [25] has indicated that junk food taking during prenatal and lactating period may cause reduced insulin sensitivity in female offspring rats. More intensive studies still need to be performed for the convincing conclusion.

2.1.4. Alcohol. Studies have already correlated chronic alcohol intake with insulin resistance even T2DM [26, 27]. In the series of studies performed by Chen and Nyomba they have found that SD rats with alcohol intake (4 g/kg/day) during pregnancy may have hyperglycemia and reduced glucose transporter type 4 (GLUT4) content in muscle in adult offspring after a reduced birth weight and then catch up growth [28]. In addition, in this animal model, impaired inhibition effects of insulin on hepatic gene expression of phosphoenolpyruvate carboxykinase and peroxisome proliferator activated receptor gamma coactivator-1 mRNA [29] and reduced phosphorylation of protein kinase C zeta isoform [30] were exhibited in the offspring. Yao et al. [31] have found prenatal alcohol intake elevated expression of Tribbles 3 and phosphatase and tensin homolog deleted on chromosome 10 in both liver and muscle [32, 33], leading to impaired insulin sensitivity [34, 35]. In addition, increased 11beta-hydroxysteroid dehydrogenase type-1 level in liver and adipose tissue [36] may also partly contribute to the insulin resistance caused by prenatal alcohol taking though elevating local glucocorticoid levels.

2.2. Environmental Factors

2.2.1. Biophenol A. Biophenol A, a biochemical material used in plastic containers that are widely used in daily life [37], has been found that it may achieve similar effects with estrogen [38, 39]. Studies have supported that biophenol A might be correlated with the pathogenesis of T2DM [40, 41]. A human study performed by Lang et al. [42] has shown that the biophenol A concentration in urine positively correlated with cardiovascular diseases and diabetes.

In rats, 50 μg/kg·d biophenol A intake during pregnancy and lactating period may lead to insulin resistance in adult offspring, and this effect can be largely enhanced by high fat diet after birth [43]. Similar results have also been described by Alonso-Magdalena et al. [44] who further found that the altered Ca2+ signaling pathway and reduced cell numbers in pancreas might contribute to reduced insulin sensitivity. However, controversial conclusions have also been raised out by Ryan et al. [45] in CD-1 mice, which indicate that perinatal exposure to ecologically relevant dose of BPA could not impair the glucose tolerance in the offspring. Therefore, different biophenol A dosages applied in different animal models may vary the conclusion.

2.2.2. Maternal Hypoxia. Data from animal experiment has found that exposure to hypoxia during pregnancy leads to insulin resistance, impaired glucose homeostasis, and altered expression of genes involved in insulin-signaling pathways in the offspring [46]. Mechanisms underlying this relationship are unclear since intrauterine hypoxia may partly correlate with undernutrition. However, Camm et al. [47] found that, compared to prenatal undernutrition, prenatal hypoxia may cause different gene expression patterns in the liver and muscle in adult offspring, including reduced expression of hepatic insulin receptor substrate 1, phospho-Akt, and muscle Akt2, indicating that prenatal hypoxia may promote markers of insulin resistance independent of undernutrition.

2.2.3. Maternal Smoking. Studies have reported the unfavorable effects of smoking on diabetes in adult [48, 49]. However, a clearly causal relationship has only been found between maternal smoking and increased risk of T2DM in the offspring. A human study performed by Thiering et al. [50] had found increased insulin levels in 10-year-old children after prenatal smoking, and breast milk feeding made this alteration even more magnificent. This finding is consistent with the study performed previously by Bruin et al. [51] in animals which indicated that both conception and lactation periods were needed for nicotine exposure that may result in permanent β-cell loss and subsequent impaired glucose tolerance. In addition, Holloway et al. [52] found that fetal and neonatal exposure to nicotine has transgenerational effects and insulin resistance can be found in the F2 offspring. Exact mechanism still remains largely unknown; reduced pancreas cell numbers and size and reduced expression of β-cell marker genes such as pdx-1, Pax-1, and Nkx6.1 [53] all have been addressed. In addition, data from Chen et al. have indicated that nicotine may also downregulate gene expression of appetite regulators neuropeptide Y and pro-opiomelanocortin in the arcuate nucleus of the hypothalamus in fetal brain, which may consequently lead to unhealthy eating habits in the offspring and predispose high risk of obesity or diabetes [54].

2.3. Prenatal Psychological Stress. It is already known that exposure to high levels of maternal stress hormones during pregnancy may produce detrimental effects on the offspring [55]. The effect of prenatal stress in programming T2DM has been found in both human and animal studies [56–58]. A retrospective study has shown that children exposed to stress caused by bereavement during their prenatal life had more risk to T2DM later in life [57]. Another human data from Entringer et al. [58] found that maternal stressful life experiences may cause significantly elevated 2-hour insulin and C-peptide levels under glucose tolerance test in young adult offspring, indicating insulin resistance, which is independent of birth weight and family history of diabetes. The elucidation of the mechanism underlying this relationship is still not clear. A finding from a human study has shown that

prenatal stress leads to shorter leukocyte telomere length in adult offspring [59], which has also been found to positively correlate with the pathogenesis diabetes [60] and children obese [61].

2.4. The Metabolic Situation during Pregnancy

2.4.1. Obesity. Maternal obesity has risen dramatically over the past 20 years. Evidences from human and animal studies suggest that maternal obesity in pregnancy predisposes hyperinsulinemia, insulin resistance, and T2DM in the offspring [62–65]. Shankar et al. [66] have found in mice that the male offspring with overweight mother may exhibit magnificent increase in body weight and adipose tissue content, which also combined with insulin resistance and increased levels of insulin, leptin, and resistin. The precise underlying mechanisms that contribute to increased susceptibility of offspring to develop insulin resistance in later life remain poorly understood. Both increased number of apoptosis of the fetal pancreas β cell [67] and accelerated fetal β-cell growth and cell proliferation (which was regarded as overload working and consequently end up to β cell failure) [68] were observed in animal offsprings with obese mother, which may all contribute to the increased blood glucose level after birth. In addition, increased hepatic lipogenesis and fatty liver disease [69, 70] found in the offspring exposed to maternal obesity also contribute to hepatic insulin resistance.

2.4.2. High Gestational Glucose Concentration. Exposure to elevated intrauterine glucose environment has been found to cause alterations in fetal growth patterns, which predispose these infants to developing obesity, insulin resistance, and diabetes later in life. So far the effects of intrauterine hyperglycemia on the offspring have been studied in human in pregnant mothers with T2DM or with gestational diabetes and in diabetic animal models mainly caused by streptozotocin treatment. Data accumulated from theses studies uniformly show glucose intolerance in the offspring. Human study performed by Boerschmann et al. [71] has indicated that, compared with those children with T1DM and normal glycemia mothers, children with mothers with gestational diabetes mellitus exhibit overweight and increased HOMA-IR. Another study from Bush et al. [72] in 5–10-year-old children also found that maternal gestational glucose concentration was inversely associated with offspring insulin sensitivity. Insulin resistance was also observed in rodent offspring prenatally under hyperglycemia environment caused by streptozotocin injection [73, 74]. It seems that in utero "diabetic" environment in which the fetus develops can increase the risk of diabetes in the child. In addition to genetic susceptibility, blunted insulin sensitivity in the offspring might largely contribute to this correlation. Relative gene expression was only explored in animal models which indicated that intrauterine hyperglycemia induced by streptozotocin injection resulted in increased hepatic gluconeogenic gene expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in the offspring [74] and the adult offspring of this cohort are prone to develop insulin resistance under high fat diet [73]. A human study found that maternal diabetes might cause an inherent defect in β-cell glucose sensitivity in the adult offspring [75].

2.5. Maternal Hormone Levels during Pregnancy

2.5.1. Prenatal Testosterone. Prenatal testosterone overexposure has been considered to be correlated with polycystic ovary syndrome in adult female and was widely studied [76, 77]. Animal experiments performed in sheep [78, 79], rodents [80–82], and even monkeys [83] have all confirmed that prenatal testosterone overexposure leads to insulin resistance in the offspring. Testosterone overexposure during fetal development may impair insulin sensitivity pathways in both liver and muscle [79], increase hepatic gluconeogenesis [84], and impair pancreas islet response to glucose [80] in the offspring.

2.5.2. Prenatal Glucocorticoids. Synthetic glucocorticoids have been used in pregnant women who are at risk of preterm delivery to promote fetal lung maturation. However, concerns have already emerged about the metabolic disorders caused by prenatal glucocorticoids excess. Studies from animal models have found that prenatal glucocorticoids treatment leads to increased hepatic gene expression of hepatocyte nuclear factor 4 alpha [85], phosphoenolpyruvate carboxykinase [86], and glucose-6-phosphatase [87] in the offspring, indicating elevated hepatic gluconeogenesis and hepatic insulin resistance. Nyirenda et al. also found that prenatal dexamethasone administration during late gestation may result in elevated 11 beta-HSD1 [88] and glucocorticoids receptor [86] expression in the liver, which may cause insulin resistance by increasing local glucocorticoids level [89] or activity.

Similar phenomenon has also been found when increased endogenous glucocorticoids pass through maternal to fetus. So far, maternal nicotine [90], food or energy restriction [91], and alcohol intake [92] have all been found to impair placental barrier and consequently cause increased endogenous glucocorticoids in utero.

3. Part II: Postnatal Factors

3.1. New Born

3.1.1. Catch-Up Growth. Catch-up growth, which appeared after lower birth body weight, is the issue that has been studied for years. Accumulated data suggest that low birth weight and catch-up growth are strongly associated with increased risk of insulin resistance and type 2 diabetes [93–96]. Intriguingly, different periods of catch-up growth seem to cause different effects on glucose tolerance and insulin sensitivity. Catch-up growth only in the first year after birth seems to have no effect on insulin sensitivity in 7-year-old child [97], while sustained catch-up growth (more than 1 year after birth) leads to higher insulin levels in 7-year-old child [97] or insulin resistance in 8-year-old child [98]. Compelling evidences raise the thrifty "catch-up fat" mechanisms, indicating that this growth trajectory is

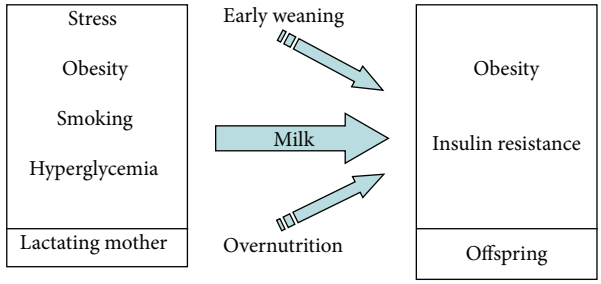

Figure 3: Lactation and insulin resistance. It has been found that both early weaning and overfeeding by more milk intake may lead to insulin resistance in later life. Maternal stress, obesity, hyperglycemia, and even smoking during lactation might also cause reduced insulin sensitivity in the offspring, which suggest that the breast milk can be the "agent," transferring altered levels of hormones, insulin, or fatty acid contents from maternal circulation to neonate.

characterized by a disproportionately higher rate of fat gain and redistribution of glucose from skeletal muscle to adipose tissue, contributing to insulin resistance in skeletal muscle while hyperresponsiveness to insulin in adipose tissue [99–101].

3.1.2. Lactation. Lactation is also a sensitive period for the programming of later metabolic disorders (Figure 3).

Early Weaning. Early weaning may lead to undernutrition, which consequently program metabolic disorders later in life. Hyperglycaemia, higher insulin resistance index, and hyperleptinemia were observed in 6-month-old rats that were weaned early, which were accompanied with central leptin resistance [102].

Overnutrition. Overnutrition during lactation period is associated with metabolic disorders in later life. An experiment conducted in male mice by Pentinat et al. [103] has found that overgrowth mice caused by reduced pups per dams during lactation may develop metabolic disorders at the age of 4 months, including obesity, insulin resistance, and glucose intolerance. Similar results have also been found by Plagemann et al. in their serial experiments performed on rats [104, 105]. Strikingly, the effect of neonatal overnutrition on diabetes risk can be "inherent" to subsequent generations. Impaired glucose tolerance was found in the adult male mice offspring with the father overfed neonatally, and peripheral insulin resistance was found in the grand offspring, although these two generations of animals were not exposed to overnutrition during the neonatal time [103]. Increased oxidative stress in liver and reduced hepatic insulin signaling pathways [106] may underlie effects of neonatal overfeeding. Moreover, early overfeeding leads to permanent dysregulation of hypothalamic circuits in animal models, including reduced negative feedback to the satiety signal insulin on medial arcuate neurons in juvenile as well as adult rats [104] and increased hypothalamic insulin receptor promoter methylation ratio [105]. This may lead to functional resistance to insulin and leptin, which may underlie permanently an increase in food intake, overweight, and insulin resistance.

Maternal Situation during Lactation. Maternal physical or pathological situation during lactation may imprint elevated risk of metabolic diseases in the offspring. Experiments in animals have showed that mother under stress [107], with obesity [108], and exposed to nicotine [109] during lactating period may lead to obesity and insulin resistance in adult offspring, which implicates that the postnatal maternal environment is a major effecter of metabolic outcome in the offspring. It is also found that fostering nondiabetic offspring to diabetic dams may produce smaller offspring with altered arcuate nucleus neuropeptide Y, agouti-related peptide, and pro-opiomelanocortin expression [108]. The underlying mechanism is still far more conclusive. Therefore, the breast milk can be the "agent"; altered levels of hormones, insulin, or fatty acid contents may enter the milk from maternal circulation and then can be transferred to neonate.

3.1.3. Neonatal Stress. Limited data from animal studies have found that stress caused by handling during the neonatal period may also be detrimental. Studies have found that neonatal mice, which were under maternal separation plus subcutaneous sham injection during the lactation period, developed hyperglycemia, hyperinsulinemia, hyperleptinemia, and hyperlipidemia in adult under fasting [110, 111]. Increased plasma corticosterone and adrenocorticotropin were found in these animals [110, 111] which might be responsible for the "diabetic" alteration.

3.1.4. Neonatal Hormone Exposure. There are evidences indicated that exposure to some hormones during neonatal life may predispose metabolic disorders in adult life. Glucocorticoids treatment in neonatal rats caused increased fasting and postprandial blood glucose, which is combined with magnificent insulin resistance and lipid disorder in later life [112]. In addition, in newborn female rats, one subcutaneous injection with 0.35 mg oestradiol benzoate led to reduced insulin sensitivity in adult life by inducing inflammation and disturbance glucose metabolism in skeletal muscle [113], while 1 mg testosterone injection to female neonatal rats caused insulin resistance and increased mesenteric adipose tissue content in adult life [114].

3.1.5. Neonatal Monosodium Glutamate Intake. Monosodium glutamate (MSG) is the sodium salt of glutamic acid, and it is a flavor enhancer that is widely used in Chinese food. The study of neonatal MSG treatment on neonatal animals has been performed since the 1970s and so far there are plenty of animal experiments that have evidenced detrimental effects of MSG administration during early life time, including growth retardation, retinal degeneration, and increased proinflammation in hippocampus [115–117]. In 1997, Hirata et al. found that MSG-treated animals developed central obesity, altered glucose tolerance, and hyperinsulinaemia [118]. More similar evidences have been documented in later experiments [119–121], indicating that neonatal MSG

treatment may lead to increased risk of diabetes in adult life. MSG may cause obesity and nonfatty liver [120, 122] and increased mRNA level of IL-6, TNFα, resistin, and leptin in visceral fat tissue [119], which might all predispose insulin resistance in later life.

3.2. Childhood

3.2.1. Low Socioeconomic Status. Socioeconomic status has a notable impact on health disparities, including type 2 diabetes risk. Low childhood socioeconomic status was found linked to type 2 diabetes in some studies [123, 124] and the association remained even after being adjusted for adult socioeconomic status and obesity. Low childhood socioeconomic status was considered to be a robust independent factor of incidence of type 2 diabetes in adulthood and the risk was found greater when childhood socioeconomic status combined with adult obesity. Poor nutrition, unhealthy behaviors, and limited access to material goods and limited socioeconomic opportunities may contribute to altered body composition in later life, which might explain the relationship between childhood socioeconomic position and metabolic disorders in adult.

3.2.2. Famine. Undernutrition during childhood has been found to be associated with an increased type 2 diabetes risk in adulthood. Study in women who had experienced Dutch famine has shown that short period of moderate or severe undernutrition during childhood increases type 2 diabetes risk in adulthood [125].

3.2.3. Obesity. Childhood obesity is an issue of serious medical and social concern. Many studies have demonstrated the positive correlations between childhood obesity and adult metabolic disorders, including type 2 diabetes [61, 126, 127]. Obesity, which mostly caused by high caloric food intake, may always combine with insulin resistance [128]. An unfavorable programming of body composition could be one mechanism linking early childhood growth with later increased risk for type 2 diabetes. In addition, a study performed in 793 French children aged 2–17 yr has suggested that obese children have significantly shorter leukocyte telomeres than their nonobese counterparts [61]. Leukocyte telomere length (LTL), a marker of biological age, is associated with age-related conditions including cardiovascular disease and type 2 diabetes which highlights a potentially deleterious impact of early onset obesity on future health.

4. Conclusion

There is increasing recognition that the risk of type 2 diabetes can be influenced by prenatal, neonatal, and childhood exposures. In the present studies, we have reviewed nutritional, environmental, and physiological factors from prenatal to postnatal periods, which have been documented in studies that may correlate with insulin resistance or type 2 diabetes in adult life. Further investigations are still required. However, relative knowledge education might be successful in women of child-bearing age and ultimate to reduce the disease risk in their potential offspring.

Acknowledgments

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Research Article

Treadmill Exercise Preconditioning Attenuates Lung Damage Caused by Systemic Endotoxemia in Type 1 Diabetic Rats

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Endotoxemia induces a series of inflammatory responses that may result in lung injury. However, heat shock protein72 (HSP72) has the potential to protect the lungs from damage. The objective of this study was to determine whether prior exercise conditioning could increase the expression of HSP72 in the lungs and attenuate lung damage in diabetic rats receiving lipopolysaccharide (LPS). Streptozotocin was used to induce diabetes in adult male Wistar rats. Rats were randomly assigned to sedentary or exercise groups. Rats in the exercise condition ran on a treadmill 5 days/week, 30–60 min/day, with an intensity of 1.0 mile/hour over a 3-week period. Rats received an intravenous infusion of LPS after 24 hrs from the last training session. Elevated lavage tumor necrosis factor-alpha (TNF-α) level in response to LPS was more marked in diabetic rats. HSP72 expression in lungs was significantly increased after exercise conditioning, but less pronounced in diabetic rats. After administration of LPS, exercised rats displayed higher survival rate as well as decreased lavage TNF-α level and lung edema in comparison to sedentary rats. Our findings suggest that exercise conditioning could attenuate the occurrence of inflammatory responses and lung damage, thereby reducing mortality rate in diabetic rats during endotoxemia.

1. Introduction

Pulmonary edema is a common complication of diabetes mellitus because of the increased capillary permeability [1]. Severely uncontrolled diabetic state may initiate pathologic events leading to the capillary leak of acute respiratory distress syndrome [2]. Due to the lower level and impaired binding activity of cell-surface receptors on monocytes [3], a poorly controlled diabetic state increases susceptibility to infections such as endotoxemia [4]. Additionally, infection can be more serious and difficult to eradicate in diabetic patients [5]. The highest incidence of acute respiratory distress syndrome occurred in endotoxemic patients and resulted in lung injury [6]. However, the severity of lung injury during systemic endotoxemiain type 1 diabetes remains unclear.

Endotoxemia is mainly caused by anendotoxin (lipopolysaccharide; LPS) from gram-negative bacteria [7]. Endotoxins induce a great amount of alveolar monocytes and macrophages to release tumor necrosis factor-alpha (TNF-α), which subsequently damages pulmonary vessels and increases lung vascular/epithelial permeability. This change allows more albumins to pass from the vessels to the alveolar space. Consequently, albumin content in the brochoalveolar

lavage fluid increases significantly [8], contributing to lung edema and poor lung compliance. In unison, the alveolar distortion, atelectasis, and a thick alveolar-capillary membrane produce low efficiency of gas exchange, often resulting in increased mortality.

It is well known that regular exercise enhances cardiopulmonary function [9]. Prior research has demonstrated that heat shock protein72 (HSP72) can be detected in the lungs of exercise trained rats [10], and that its induction by heat can protect respiratory systems from systemic inflammation [11]. Moreover, HSP72 facilitates the action of an anti-inflammatory cytokine, interleukin (IL)-10, and alleviates TNF-α induced lung damage [12]. In nondiabetic rats, exercise training attenuated septic responses and abrogated pulmonary pathological change [13]. Swimming trained rats also showed a lower pulmonary edema index after LPS challenge [14]. In diabetic rats, exercise upregulated HSP72 expression in the heart and alleviated circulatory dysfunction after LPS injection [15]. Nevertheless, it remains unknown whether aerobic conditioning via treadmill exercise can attenuate pulmonary injury in diabetic rats receiving endotoxin. Hence, the purpose of this study is to reveal whether endotoxemia-induced lung injury is more marked in type 1 diabetes and to determine whether treadmill exercise conditioning could induce HSP72 overexpression in the lung, alleviate lung injury and proinflammatory cytokines overproduction in endotoxemia, and thus reduce the mortality rate.

2. Materials and Methods

2.1. Experimental Animals. Male Wistar rats ($n = 144$; 320 ± 20 g; aged 10 weeks) were purchased from the Animal Resource Center of the National Cheng Kung University in Taiwan. Rats were housed in groups of four at an ambient temperature of $24 \pm 1^{\circ}$C. Pelleted rat chow and tap water were allowed *ad libitum*. All experimental procedures were conducted in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Streptozotocin (STZ)-induced type 1 diabetic rats were prepared by intravenous injection with STZ (Sigma, St. Louis, MO, USA) (60 mg/kg) after a 72-hour fast. This method has been shown to irreversibly destroy pancreatic beta cells, as previously described [16]. After 1 week of STZ administration, rats with blood glucose levels higher than 300 mg/dL along with symptoms of polyuria were considered diabetic. Animals were separated into six groups ($n = 24$ in each group): (a) nondiabetic control rats receiving 0.9% normal saline administration (NS), (b) nondiabetic control rats receiving LPS administration (NL), (c) STZ-induced diabetic rats received normal saline administration (SS), (d) STZ-induced diabetic rats received LPS administration (SL), (e) STZ-induced diabetic rats subjected to exercise training before the injection of normal saline (SES), (f) STZ-induced diabetic rats subjected to exercise training before the injection of LPS (SEL).

2.2. Exercise Training Protocol. The exercise training protocol was performed according to a previously described method with some modification [17]. Specifically, 1 week after STZ injection, the trained rats ran gradually on a treadmill (treadmill exerciser T510, Diagnostic & Research Instruments, Singa) for 5 days/week with intensity of 1.0 mile/hr for 3 weeks at room temperature. Before the initiation of exercise training, the rats were acclimated to run 15 min at a time for 3 days. A minor electrical shock (1.0 mA) was used in the beginning to encourage rats to run forward. Subsequently, the animals ran without electrical stimulation. The duration of the exercise was progressively increased, with the rats running for 30 min/day during the first 2 weeks, and 60 min/day during the last week of training. The intensity of exercise was maintained throughout the training period at approximately 75–80% of maximal oxygen consumption [15, 18]. Same exercise training protocol is used in all training groups, close to 90% of the trained rats were able to finish the training program. Rats which could not achieve the intensity and duration of this training protocol were withdrawn from this experiment. We observed that the diabetic rats have lower body weight and lower endurance capacity. However, the withdrawal rate was indistinguishable between nondiabetic and diabetic group. At the 24th hr after the last training session, the rats received an intravenous injection of lipopolysaccharide (LPS, 15 mg/Kg) or saline. LPS (from *Escherichia coli* 0111:B4, Sigma, St. Louis, MO) was used as a fresh solution in phosphate buffered saline (pH 7.40) at a concentration of 10 mg/mL. After administration of LPS, the survival time of each rat was continuously monitored.

Different parts of the animals were used for three experiments ($n = 8$ for each part in each group): (I) determination ofsurvival time and survival rate in sedentary and exercised rats after receiving an injection of LPS; (II) determination of HSP72 expression in sedentary and exercised rats; and (III) determination of arterial blood gas, lung injury, and TNF-α, IL-6, and IL-10 levels in bronchoalveolar lavage at 240 min after normal saline or LPS injection.

2.3. Detection of Lung Vascular/Epithelial Permeability. The albumin content in bronchoalveolar lavage fluid was determined to assess the damage of endothelial cells within the lung capillaries. The rat's trachea was intubated and a bronchoalveolar lavage sample was collected by perfusing saline (15 mL) from the endotracheal tube at 240 min after the administration of LPS or saline. The lavage samples were centrifuged for 20 min (15000 rpm, 4°C) to remove cells. The supernatant of lavage was analyzed by a Bio-Rad protein assay system (Bio-Rad Hercules, CA) with bovine serum albumin as the standard. The albumin content in lavage was calculated by dividing albumin by the dried weight of the lung to evaluate pulmonary capillary/endothelial cell permeability.

2.4. Determination of Cytokine Concentration in Bronchoalveolar Lavage Fluid. The concentrations of TNF-α, IL-6, and IL-10 in lavage fluid were assayed using double-antibody sandwich ELISA (R&D Systems, Minneapolis, MN, USA)

according to the manufacturer's instructions. The optical density of each well was determined by a microplate photometer (Multiskan EX, Thermo Fisher Scientific Inc, Waltham, MA, USA).

2.5. Measurement of Lung Edema. At 240 min after administration of LPS, both whole lungs were removed, wiped clean, and weighed as the wet weight. Dry weight was measured after the lungs were dried at 63°C for 48 hrs. In order to assess degree of lung edema, the wet/dry weight ratios of lung were calculated by dividing the wet weight by dry weight.

2.6. Histopathology of Lung. At 240 min after injection of LPS, the rats received 3 mL of air via tracheotomy with syringe. The systemic circulation was perfused with 250 mL of 0.9% saline (163.2 cm-H_2O) while the pulmonary circulation was perfused with 50 mL of 0.9% saline (40.8 cm-H_2O). Subsequently, the same amounts of 4% formalin were instilled into systemic and pulmonary circulation. Lung tissues were harvested and stored in 10% formalin for 3 days. Right upper lobes were embedded in paraffin blocks and serial sections (4 μm in thickness) were stained with hematoxylin-eosin for histopathologic evaluation. The characteristics of lung damage include vascular congestion, hemorrhage, polymorphonuclear leukocytes (PMN) infiltration, and edematous changes of alveolar wall [19]. Each characteristic was scored (0: normal; 1: mild; 2: moderate; 3: severe) by a pathologist and overall lung injury was further calculated according to the sum of the scores.

2.7. Analysis of Arterial Blood Gas. In order to determine the arterial pH, arterial partial pressure of O_2 (PaO_2), CO_2 ($PaCO_2$), and O_2 saturation (SO_2) of rats, 0.4 mL of arterial blood was sampled from the femoral artery by a heparin-rinsed syringe at 240 min after administration of LPS. The sample was analyzed by a blood gas analyzer (Synthesis1725, Diamond Diagnostics Inc, Hollinton, MA, USA).

2.8. Western Blotting Analysis of HSP72. For purposes of quantifying HSP72 expression in the lungs, rats with or without exercise preconditioning were sacrificed 240 min after LPS injection. The rats' lungs were removed and stored at −70°C until analysis. Lung tissue was homogenized and denatured in a SDS sample buffer (0.5 M Tris-HCl (pH 6.8), 10% SDS, 0.1% bromphenol blue, 2-mercaptoethanol, and glycerol). Protein contents were assayed using the Bio-Rad kit (Bio-Rad, Hercules, CA, USA) and an ELISA reader (Multskan EX, Thermo, MA, USA) at 630 nm. After equal amounts of protein extract were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was incubated with a mouse monoclonal anti-HSP72 antibody (SPA 810; StressGen Biotechonologies, Victoria, BC, Canada). Immunodetection for HSP72 was performed using the enhanced chemiluminescence protocol using a Renaissance reagent (NEN Life Science Products, Boston, MA, USA). Mouse anti-actin monoclonal antibody was used as aninternal control. Quantification of blot band was performed using an optical scanner and ImageMaster TotalLab 1D Elite software (version 2.01; Amersham Pharmacia, Piscataway, NJ, USA).

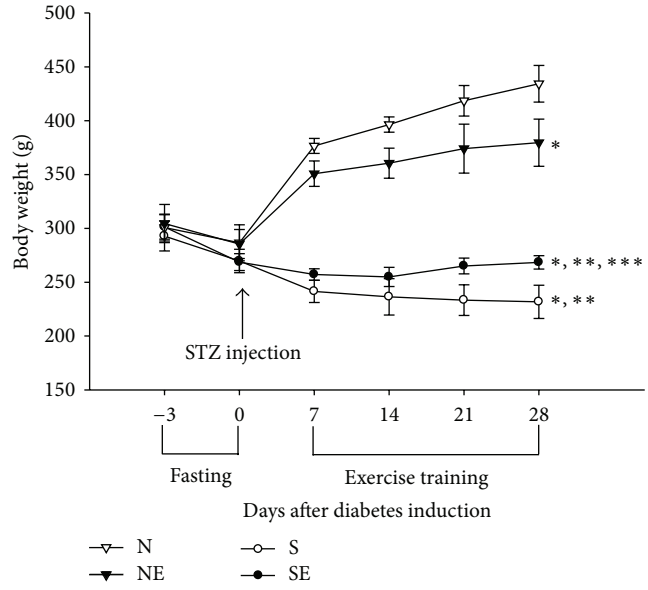

Figure 1: The changes of rats' body weight after STZ injection and 3-week exercise training. N: nondiabetic rats; NE: nondiabetic rats with exercise; S: STZ-induced diabetic rats; SE: STZ-induced diabetic rats with exercise ($n = 8$ per group). $^{*}P < 0.05$, compared with the N group; $^{**}P < 0.05$, compared with NE group, and $^{***}P < 0.05$, compared with the S group; (one-way repeat measurement ANOVA).

2.9. Statistical Analysis. All values are presented as mean ± S.E.M. for each point. Statistical analysis was conducted using ananalysis of variance (ANOVA) for factorial experiments. The Wilcoxon rank-sum test was used for the analysis of survival time, while the Kaplan-Meier test was used for survival rates. Differences between groups were considered to be significant at values of $P < 0.05$.

3. Results

After induction of diabetes, the body weight of diabetic rats was lower than nondiabetic ones (Figure 1). Exercise training decreased the body weight of nondiabetic rats and increased the body weight of diabetic rats. In other words, exercise moves the body weight of rats toward a healthier status. After LPS administration, we found that the colonic temperature is slightly elevated within 10 mins and it returned to baseline level at 20 minutes (Figure 2). There are no significant difference within NL, SL, and SEL group. Therefore, LPS-induced fever is not altered by exercise training. The survival time of the SL group was markedly shorter than that of the NL group (316.4 ± 39.6 min versus 420.78 ± 38.99 min, resp.; $P < 0.05$). Additionally, survival time of the SEL group was significantly longer than that of the SL group (489.2±23.5 min

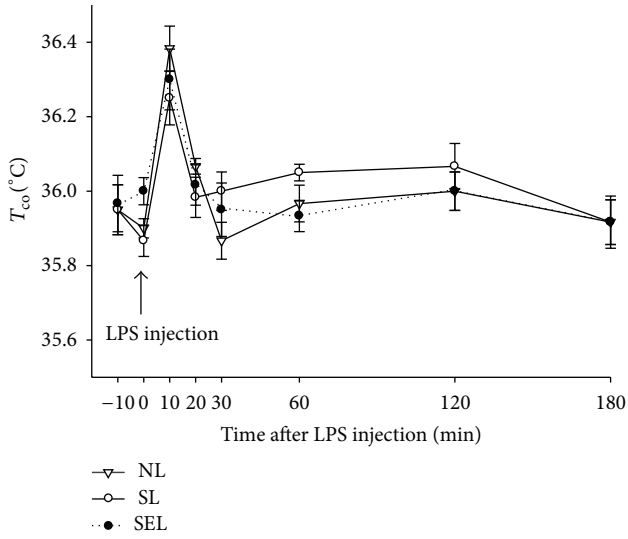

Figure 2: The colonic temperature (T_{co}) of the animals in response to LPS challenge. NL: nondiabetic rats receiving LPS administration; SL: STZ-induced diabetic rats injected with LPS; SEL: STZ-induced diabetic rats with exercise injected with LPS (n = 8 per group).

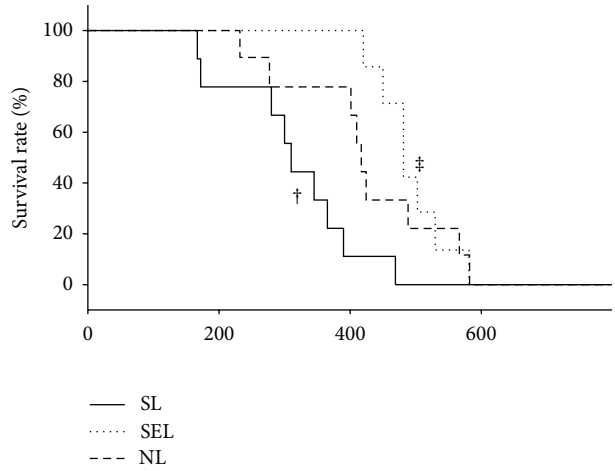

Figure 3: The survival rate of rats with or without exercise after LPS administration (15 mg/kg, i.v.). NL: nondiabetic rats receiving LPS administration; SL: STZ-induced diabetic rats injected with LPS; SEL: STZ-induced diabetic rats with exercise injected with LPS (n = 8 per group). $^{\dagger}P < 0.05$, compared with the NL group; $^{\ddagger}P < 0.05$, compared with the SL group (Kaplan-Meier test).

versus 316.4 ± 39.6 min, resp.; $P < 0.05$), suggesting that exercise preconditioning significantly improved survival rate of diabetic rats during endotoxemia (Figure 3). Presented in another way, by 420 min after administration of LPS, 100% of the SEL group was still alive in contrast to only 11.1% survival among the SL group.

Figure 4 shows that all groups treated with LPS displayed significantly raised TNF-α (120.34 ± 14.11 pg/mL in NL group and 197.43 ± 30.11 pg/mL in SL group) and IL-6 (142.11 ± 124.83 pg/mL in NL group and 319.11 ± 106.52 pg/mL in SL group) levels in lavage fluid at 240 min after treatment. However, lavage TNF-α level was statistically higher in the SL group as compared to the NL group ($P < 0.05$). Additionally, the increases of TNF-α and IL-6 were attenuated significantly by exercise preconditioning (25.5 ± 3.72 pg/mL in TNF-α and 97.52 ± 58.40 pg/mL in IL-6), while levels of IL-10 were actually higher in exercise-treated groups (68.50 ± 23.14 pg/mL).

The lung wet/dry weight ratios and albumin content of the lungs increased significantly after administration of LPS (Figure 5). These parameters were not significantly different between the NL and SL groups, but the values were significantly decreased in rats with exercise preconditioning. Even though the albumin content in the SEL group was significantly lower than that in the SL group (0.76 ± 0.07 mg · mL^{-1} · g^{-1} versus 1.64 ± 0.22 mg · mL^{-1} · g^{-1}, resp.; $P < 0.05$), the value of the SEL group was still significantly higher than that reported among the SES groups (0.76 ± 0.07 mg · mL^{-1} · g^{-1} versus 0.20 ± 0.05 mg · mL^{-1} · g^{-1}, resp.; $P < 0.05$). Histopathologic examination of the lungs and quantitative analyses of histology were shown in Figures 6 and 7. The lung injury score was significantly increased in NL, SL, and SEL groups compared with that of NS groups. Although exercise preconditioning diminished these changes, the values of lung injury score are indistinguishable between SL and SEL groups. After administration of LPS, the values of $PaCO_2$ decreased significantly, whereas the value of PaO_2 and SaO_2 increased significantly, indicating that hyperventilation had occurred. Nevertheless, values of $PaCO_2$, PaO_2, and SaO_2 were indistinguishable between the SL and SEL groups (Table 1). Hence, the protective effect of exercise on blood gas changes is considered minor.

While levels of HSP72 expression in the lung were increased in rats with exercise as compared to those without exercise, the increase of expression was less pronounced in the diabetic groups (Figure 8). A negative correlation between lung HSP72 level and lavage TNF-α level was also demonstrated ($r = -0.758$, $P < 0.05$).

4. Discussion

In this study, we demonstrated that after LPS challenge, the TNF-α level in lung lavage fluid is higher in diabetic rats than that in nondiabetic ones. We also found overexpression of HSP72 in the lung and higher IL-10 level in lavage among exercised diabetic rats. Thus, our data suggest that preconditioning with treadmill exercise reduces lung edema, albumin content, TNF-α and IL-6 levels, and consequently, the mortality rate, after administration of LPS.

Lung injury is associated with elevated cytokines [20]. TNF-α is believed to play an important role in the pathogenesis of endotoxin-induced multiple organ failure [21]. Previous findings showed that LPS treatment resulted in higher lung TNF-α levels, as well as increased lung edema and pulmonary albumin [22]. However, HSP72 could facilitate the action of an anti-inflammatory cytokine, IL-10, thereby decreasing TNF-α-induced lung damage [12]. Several studies also demonstrated that HSP72 could actually inhibit

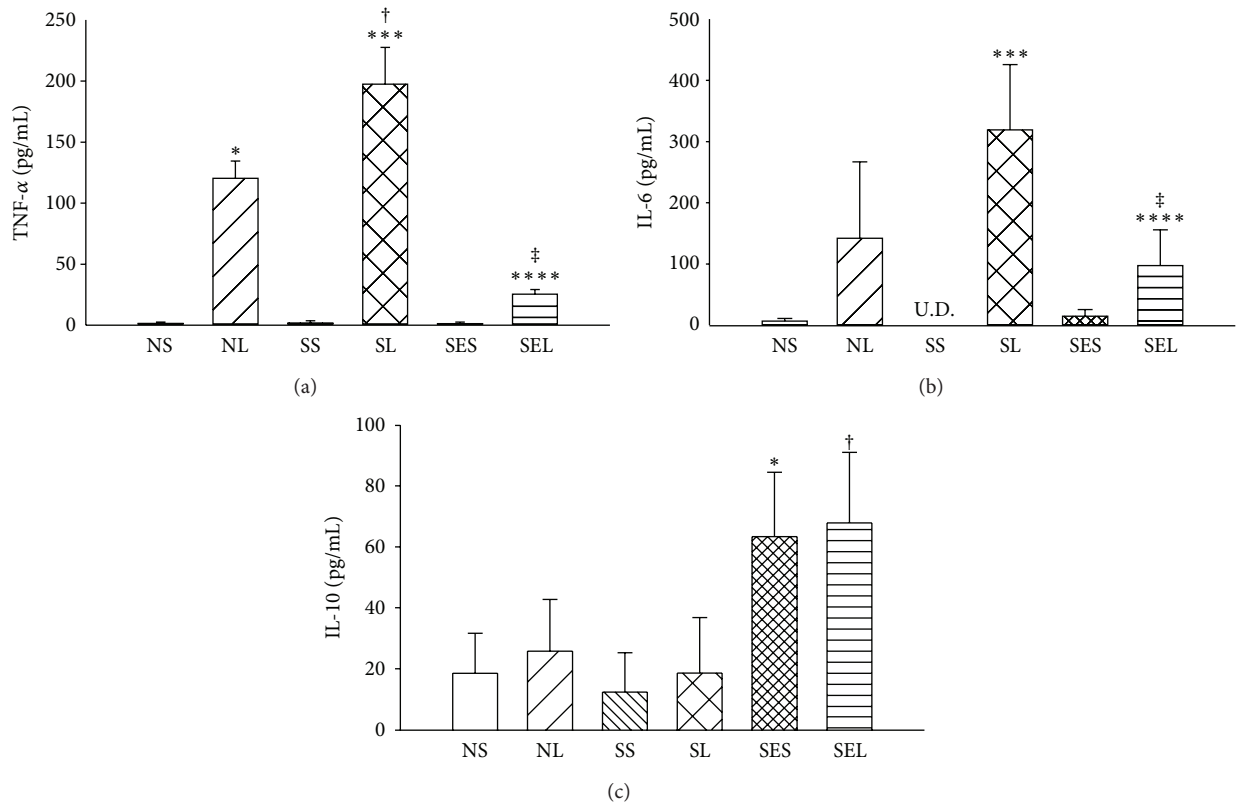

Figure 4: Levels of TNF-α, IL-6, and IL-10 in lavage fluid at 240 min after saline or LPS administration. NS: nondiabetic rats injected with saline; NL: nondiabetic rats injected with LPS; SS: STZ-induced diabetic rats injected with saline; SL: STZ-induced diabetic rats injected with LPS; SES: STZ-induced diabetic rats with exercise injected with saline; SEL: STZ-induced diabetic rats with exercise injected with LPS. Data are expressed as the mean ± SEM of eight rats per group. $^{*}P < 0.05$, compared with the NS group; $^{\dagger}P < 0.05$, compared with the NL group; $^{***}P < 0.05$, compared with the SS group; $^{\ddagger}P < 0.05$, compared with the SL group; $^{***}P < 0.05$, compared with the SES group (one-way ANOVA).

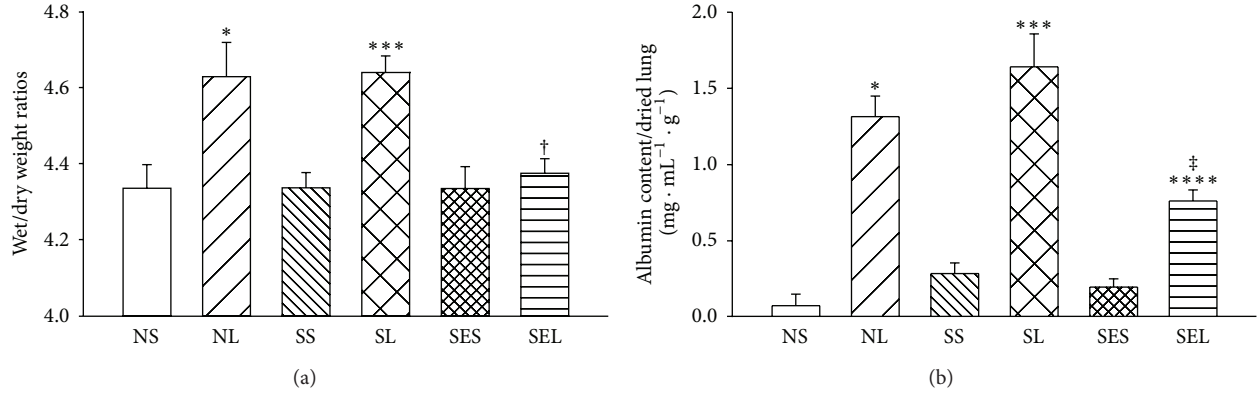

Figure 5: The lung wet/dry weight ratios and albumin content in lung lavage at 240 min after administration of saline or LPS. Albumin content in the brochoalveolar lavage fluid was calculated as [(mg of albumin)/(mL of lavage fluid)]/(g of dried lung weight). NS: nondiabetic rats injected with saline; NL: nondiabetic rats injected with LPS; SS: STZ-induced diabetic rats injected with saline; SL: STZ-induced diabetic rats injected with LPS; SES: STZ-induced diabetic rats with exercise injected with saline; SEL: STZ-induced diabetic rats with exercise injected with LPS. Data are expressed as the mean ± SEM of eight rats per group. $^{*}P < 0.05$, compared with the NS group; $^{\dagger}P < 0.05$, compared with the NL group; $^{***}P < 0.05$, compared with the SS group; $^{\ddagger}P < 0.05$, compared with the SL group; $^{****}P < 0.05$, compared with the SES group (one-way ANOVA).

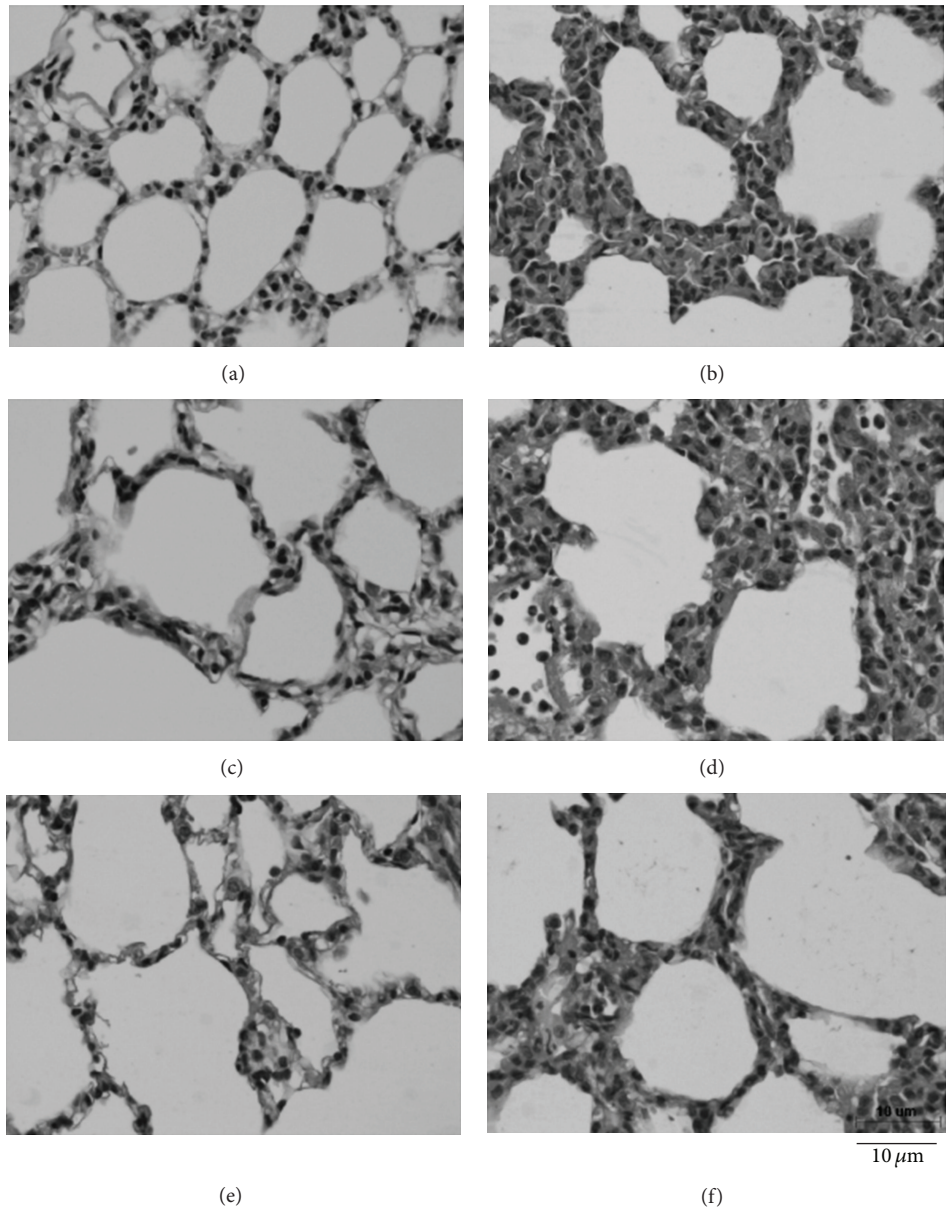

Figure 6: Histological examination of right upper lobe taken after 240 min of saline or LPS administration. (a) NS: nondiabetic rats injected with saline; (b) NL: nondiabetic rats injected with LPS; (c) SS: STZ-induced diabetic rats injected with saline; (d) SL: STZ-induced diabetic rats injected with LPS; (e) SES: STZ-induced diabetic rats with exercise injected with saline; (f) SEL: STZ-induced diabetic rats with exercise injected with LPS. The interstitial spaces of alveoli became wider after LPS administration, due to polymorphonuclear cell infiltration and edematous changes of alveolar walls (b), (d). Scale bar: 10 μm.

Table 1: Effects of LPS administration (15 mg/kg, i.v.) on arterial blood gas in rats with or without exercise.

	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	SaO_2 (%)
NS	7.38 ± 0.01	42.03 ± 1.08	106.95 ± 2.28	97.82 ± 0.17
NL	7.40 ± 0.02	30.66 ± 1.99*	122.09 ± 5.93*	98.77 ± 0.21*
SS	7.41 ± 0.01	41.96 ± 0.92	91.40 ± 2.64	96.93 ± 0.37
SL	7.43 ± 0.01	30.63 ± 1.58***	106.50 ± 3.55***	97.96 ± 0.23***
SES	7.39 ± 0.02	40.05 ± 1.35	89.92 ± 3.94	97.18 ± 0.21
SEL	7.41 ± 0.01	34.70 ± 0.92****	104.29 ± 4.83****	98.11 ± 0.25****

$PaCO_2$: arterial carbon dioxide tension; PaO_2: arterial oxygen pressure tension; SaO_2: oxygen saturation. Data are expressed as means ± SEM of eight rats per group obtained 240 min after injection of LPS or saline. $^{*}P < 0.05$, compared with the NS group; $^{***}P < 0.05$, compared with the SS group; $^{****}P < 0.05$, compared with the SES group (one-way ANOVA).

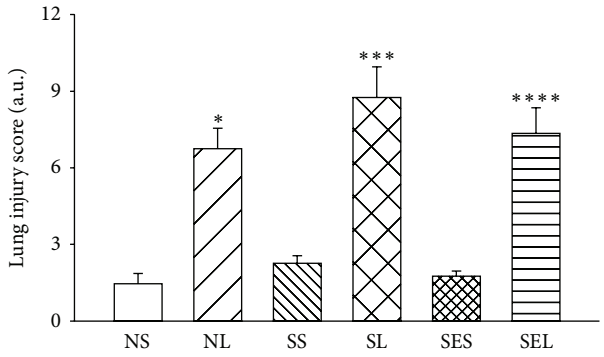

FIGURE 7: The lung injury score in different groups after LPS administration. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, compared with the NS group; $^{***}P < 0.05$, compared with the SS group; $^{****}P < 0.05$, compared with the SES group (one-way ANOVA).

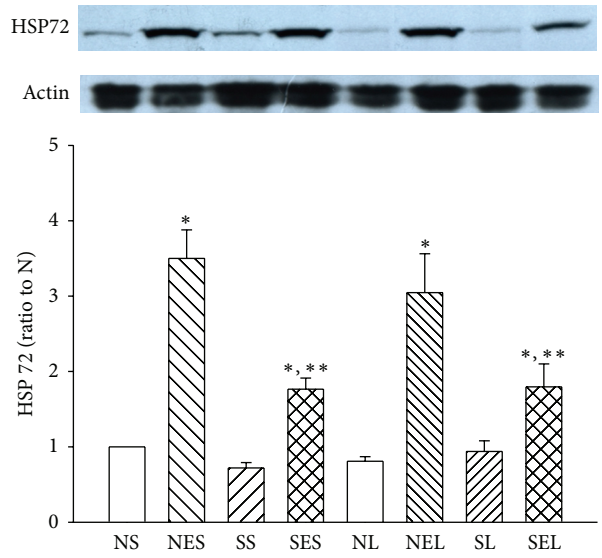

FIGURE 8: The expression of HSP72 in lungs in the rats with or without exercise after saline or LPS administration. NS: nondiabetic rats injected with saline; NES: nondiabetic rats with exercise injected with saline; SS: STZ-induced diabetic rats injected with saline; SES: STZ-induced diabetic rats with exercise injected with saline; NL: nondiabetic rats injected with LPS; NEL: nondiabetic rats with exercise injected with LPS; SL: STZ-induced diabetic rats injected with LPS; SEL: STZ-induced diabetic rats with exercise injected with LPS. Data are expressed as the mean ± SEM of eight rats per group. Protein levels are expressed as a ratio to the NS group. Below each column is a representative Western blot of HSP72 protein. Data are expressed as the mean ± SEM of eight rats per group. $^{*}P < 0.05$, compared with the N group; $^{**}P < 0.05$, compared with NE group (one-way ANOVA).

the production of TNF-α [23] and TNF-α-induced inflammatory responses [24]. After LPS injection, we found that the colonic temperature is slightly elevated within 10 mins and it returned to baseline level at 20 minutes. Some evidence indicated that HSP72 is fundamental for survival at normal and raised temperatures. HSP72 also plays an important role in thermotolerance and cytoprotection against damage from stresses such as ischemia and cytokines [10, 17, 25]. In the present study, LPS-induced fever is not altered by exercise training. Nevertheless, exercise-induced HSP72 may provide cellular protection during the LPS-induced raised temperature period and lessen tissue inflammation and damage.

HSP72 is induced in multiple organs by heat stress or exercise [10, 26] and plays a critical role in the protection of cellular damage from such stressors [27]. More specifically, after exercise activates its generation, HSP72 interacts with denatured proteins causing refolding activity that intensifies the structure of normal proteins to prevent their degradation in response to a subsequent stress [25]. Additionally, HSP72 induced by heat was found to protect respiratory systems from systemic inflammation [28]. Previous study has demonstrated that the levels of other heat shock proteins, such as heat shock protein90 and heme oxygenase-1, are also changed in STZ-induced diabetic rats [29]. Induction of diabetes increased heme oxygenase-1 levels. Heat shock protein90 levels were increased in heart and decreased in liver of STZ-induced diabetic rats. However, exercise training significantly increased the expression of HSP72, but not heat shock protein90 and heme oxygenase-1, in heart, liver, and muscles of STZ-induced diabetic rats. Therefore, we focus on whether exercise training increases the expression of HSP72 in lung of STZ-induced diabetic rats in the present study.

LPS can induce proinflammatory cytokines, such as TNF-α or IL-6 [30]. Our data indicated that exercise training suppresses the LPS-induced inflammatory status. A prior study has reported that long-term exercise has an anti-inflammatory effect that is partially mediated by IL-6 [31]. After long-term exercise, IL-10 is stimulated by IL-6 in systemic circulation, which then causes a reduction in the TNF-α level. Furthermore, after 12 weeks of aerobic exercise, IL-6 level was found to be decreased while IL-10 level was increased in type 2 diabetic patients [32] and the patients with coronary heart disease [33].

In nondiabetic rats, recent studies documented that treadmill exercise attenuates septic responses and protects the lungs from damage [13]. Swimming trained rats also showed a lower pulmonary edema index after LPS challenge [14]. In the present study of diabetic rats, treadmill exercise increased the lung expression of HSP72, but this induction was less pronounced than in the nondiabetic controls. These data are consistent with prior reports indicating that induction of diabetes decreased HSP72 expression in heart, liver, and muscles [29]. Also, we found that exercised diabetic rats had lower inflammatory response during endotoxemia as well as a negative correlation between lavage TNF-α level and the HSP72 level in the lungs. A prior study has shown that activation of stress protein response caused intracellular expression of HSP72 in lung endothelial, epithelial, and macrophage cells and that this activation has immunomodulatory effects [34]. Hence, the attenuation of lavage cytokine levels during endotoxemia may be, in part, related to the

higher expression of HSP72 in the lungs. Hence, we have demonstrated that LPS-induced pulmonary dysfunction is more marked in type I diabetic rats, but endurance training may upregulate lung HSP72 expression and counterbalance some of the negative effects of diabetes. However, since LPS injections affect multiple organs, the effect on survival time may not only be explained by the reduced severity of acute lung injury. In fact, our previous study has indicated that exercise could increase HSP72 expression in heart and NTS as well and attenuate cardiovascular dysfunction in diabetic rats during endotoxemia [15].

The present results showed that $PaCO_2$ decreased and PaO_2 increased significantly in diabetic rats during endotoxemia. LPS can induce many mediators, such as nitric oxide, reactive oxygen species, and TNF-α, all of which are associated with respiratory muscular dysfunction [35]. Furthermore, pulmonary morphology alterations can be involved in the changes of blood gas levels [36]. Our results are similar to those of Fakioglu et al. [37], who documented that hyperventilation occurred during endotoxemia, but inconsistent with findings from another study [38]. The controversial findings may be explained by different dosage of LPS injection in each study. In addition, some studies have demonstrated that alloxan-induced diabetes may be associated with a reduced risk of acute lung injury in response to intratracheal instillation of endotoxin [39]. The conflicting outcome in our study may result from the lung injury being induced by intravenous infusion of endotoxin and the use of a different animal model of diabetes.

Our results suggest that regular exercise could prevent the occurrence of a rapidly lethal infection or endotoxemia even among those with poorly-controlled diabetes. This finding may help the treatment of poorly controlled type 1 diabetes. Although exercise prolonged the survival time only by hours in this acute septic shock model, it could provide more opportunities for providing enough emergency treatments and critical care within these hours for the diabetic patients and reduce the higher mortality. Therefore, regular exercise is recommended for type 1 diabetic patient.

There are some limitations to this study. In present study, diabetic status is induced 1 week before the exercise protocol started. Therefore, exercise training is started from acute/young diabetic state. The tissue responses to exercise training may be different in chronic/prolonged diabetic state. A direct relationship between lung HSP72 content and related damage cannot be inferred since this study assessed the consequences separately. Such a question may be addressed by injecting HSP72 antisense or shRNA into the exercising rats to further clarify the effect of exercise-induced HSP72 expression on endotoxemia. Although the increase of TNF-α and IL-6 levels in exercised rats was alleviated after LPS challenge, a much broader panel of pro- and anti-inflammatory cytokines, chemokines, inflammatory mediators, and oxidative stress bio-markers also need to be measured in the lavage fluid as well as in other tissues and in systemic circulation. Since these data are derived from a rapidly lethal model of endotoxemia, the present results may not be extrapolated to less severe models.

In conclusion, LPS-induced lung inflammation and damage are more marked in type 1 diabetic rats. Exercise could alleviate lung damage and confer significant protection against the high mortality risk in diabetic rats during endotoxemia. This protective effect may be correlated with HSP72 overexpression in the lungs.

Nomenclature

HSP72: Heat shock protein72
IL-6: Interleukin-6
IL-10: Interleukin-10
LPS: Lipopolysaccharide
NEL: Nondiabetic rats with exercise injected with LPS
NES: Nondiabetic rats with exercise injected with saline
NL: Nondiabetic control rats injected with LPS
NS: Nondiabetic control rats injected with saline
SEL: STZ-induced diabetic rats with exercise preconditioning injected with LPS
SES: STZ-induced diabetic rats with exercise preconditioning injected with saline
SL: STZ-induced diabetic rats injected with LPS
SS: STZ-induced diabetic rats injected with saline
STZ: Streptozotocin
TNF-α: Tumor necrosis factor-alpha.

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Research Article

Benidipine Protects Kidney through Inhibiting ROCK1 Activity and Reducing the Epithelium-Mesenchymal Transdifferentiation in Type 1 Diabetic Rats

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We investigated the protective effect of benidipine, by testing the changes of the activity of Rho kinase and transdifferentiation of renal tubular epithelium cells *in vivo*. Wistar rats were randomly divided into two groups: normal (N) and diabetes. STZ were used to make the rats type 1 diabetic and were randomly assigned as diabetes without treatment (D), diabetes treated with benidipine (B), and diabetes treated with fasudil (F) and treated for 3 months. Immunohistochemistry and western blotting were for protein expressions of ROCK1, α-SMA, and E-cadherin and real-time PCR for the mRNA quantification of ROCK1. Compared with N group, D group had significant proliferation of glomerular mesangial matrix, increased cell number, thickened basement membrane, widely infiltrated by inflammatory cells and fibrosis in the renal interstitial, and dilated tubular. Those presentations in F and B groups were milder. Compared with N group, D group showed elevated MYPT1 phosphorylation, increased expression of ROCK1, α-SMA protein, and ROCK1 mRNA and decreased expression of E-cadherin protein. B group showed attenuated MYPT1 phosphorylation, decreased ROCK1, α-SMA protein, and ROCK1 mRNA expression and increased expression of E-cadherin protein. In conclusion, benidipine reduces the epithelium-mesenchymal transdifferentiation and renal interstitial fibrosis in diabetic kidney by inhibiting ROCK1 activity.

1. Introduction

Benidipine is a triple calcium channel blocker, simultaneously blocking L, T, and N type channels. It is reported that the effect on T channel is stronger than that on L channel [1], making it a great potential protection for kidney. A number of studies explored the Rho signaling pathway, renal interstitial fibrosis, and tubular epithelium cell transdifferentiation (EMT) [2–4]. The blocking of T calcium channel (TCC) was reported to inhibit the activity of Rho kinase [5], and this is essential in podocyte effacement in immune complex-mediated glomerular disease and other kidney injuries [6]. Furthermore, under cellular stress, Rho kinase activation results in cytoskeletal rearrangement, stress fiber formation, and loss of cellular integrity and function [7]. Rho kinase inhibition prevented these changes and enhanced process formation [8]. These suggested that blocking T channel may have a protective effect on diabetic kidney and reduce epithelium-mesenchymal transdifferentiation and fibrosis via inhibiting ROCK1 (Rho kinase 1) activity.

It was suggested that fasudil, a Rho kinase inhibitor, may attenuate EMT through reduced activation of RhoA/ROCK signaling and be a renoprotective agent for the treatment of DN [9].

Based on that, in this study, we proposed that by inhibiting Rho kinase activity, benidipine reduces epithelium-mesenchymal transdifferentiation and protects kidney in rats with type 1 diabetes (T1DM). By treating type 1 diabetic rats

with benidipine and using Rho kinase inhibitor fasudil as positive control, we studied the effects of benidipine on the activity of Rho kinase and EMT in diabetic nephropathy *in vivo*.

2. Materials and Methods

2.1. Materials. Eight-week old male Wistar rats weighed at 180–200 g (SPF class) were supplied by The Center for Animal Experiment of Wuhan University (Produce Permission no. SCXK (Yu) 2003-0004, Environment Permission no. SYXK (Yu) 2004-0027). Rabbit antibody p-MYPT1 (p853) and E-cadherin antibody were purchased from Bioworld Technology, USA, ROCK1 antibody was purchased from Santa Cruz, USA, rabbit antibody α-SMA from Sigma, USA, secondary antibody for internal control protein from Santa Cruz, USA, enzyme-labeling secondary antibody from Sigma USA, Streptozocin (STZ) from Sigma USA, hydrochloride fasudil injection from Tianjin Hongri Pharmaceutical Inc. (lot: 070525), Benidipine from Japanese Kyowa Hakko Kogyo Co., Ltd. (lot: 119AFI), anti-rabbit/rat universal immunohistochemistry kit from Denmark (DAKO), protein extraction buffer from Shanghai Xinghan (DBI), real-time PCR Master Mix from Japanese TOYOBO Biotech, real-time fluorescence PCR equipment from BioRad USA, and the analysis software for fluorescence quantitation was purchased from icycler (version 3.1.7050).

2.2. Rat Model Preparation. Fifty-four SPF male Wistar rats were fed with normal chow diet, had free access to water, with room temperature of 20~25°C and relative humidity of 40%~70%, and were in the 12 h light-dark cycle. The rats were randomly assigned into normal group ($n = 8$) and diabetic model group ($n = 46$). After a one-week adaption, the model group was injected intraperitoneally with a single dose of streptozocin (STZ) 60 mg/kg (dissolved in 10 mmol/L citrate buffer, pH 4.5), after a 12-hour fasting. Seventy-two hours after the injection, blood glucose was tested with the samples from tail vein for 3 consecutive days. The criteria for diabetic models were as follows: nonfasting blood glucose is ≥11.1 mmol/L (all was ≥16.7 mmol/L in this study), urine output exceeds the controls over 50%, and urine glucose is strongly positive. During the procedure, 3 rats died and 6 did not meet the criteria. Thirty-seven diabetic rats were randomly assigned into three groups: diabetic without treatment (D, $n = 13$), diabetic treated with fasudil (F, $n = 12$), and diabetic treated with benidipine (B, $n = 12$). Fasudil was injected intraperitoneally with 10 mg/kg/d; benidipine was dissolved in 0.3% carboxymethyl cellulose solution and given via gastric tubing with 3 mg/kg/d. Rats in normal group were injected with citrate buffer. After three months, 8 rats in N group, 9 in D group, 9 in F group, and 8 in B group survived and were sacrificed accordingly.

2.3. Samples Collection. One day prior to the sacrifice, 24-hour urine was collected in metabolic chamber. On the same day of sacrifice, tail artery blood pressure was measured with noninvasive blood pressure meter and blood samples were collected. After rinsing with normal saline, some of the kidney tissues were fixed with 10% neutral formalin, embedded with paraffin, made into 3 μm slides, and stained with HE for pathological analysis. The rest of the kidney tissues were stored at −70°C.

2.4. The Testing of Biochemistry Parameters. Twenty-four-hour urine protein quantification was measured with sulfosalicylic acid method; serum creatinine (Scr) was tested with picric acid method; blood glucose was tested with glucose oxidase method; and NAG activity was measured with colorimetry as described previously [10].

2.5. Immunohistochemistry. Deparaffin the slides routinely, heat repair with microwave, incubate in 3% peroxide at room temperature for 15 minutes, and rinse with PBS (pH 7.4) for three times, 5 minutes for each. Add rabbit antibody p-MYPT1 (1 : 50), α-SMA (1 : 50), and E-cadherin (1 : 100) antibody, respectively, and incubate at 4°C overnight. Incubate with horseradish peroxidase-labeled ChemMateTMEnVision secondary antibody at room temperature for 45 minutes, detect with DAB, repeat staining with HE, dehydrate, and seal the film with transparent plastic membrane.

2.6. Western Blotting. The total proteins of kidney tissues were extracted with total protein extraction kit. The protein concentration was analyzed with UV spectrophotometry at 260 nm wavelength. Thirty μg of total protein was loaded for SDS-PAGE electrophoresis, then was transferred to nitrocellulose membrane, and then was observed with ponceau S staining. Block with TTBS containing 5% fat-free milk at 4°C for 2 hours. Wash with PBS, then add rabbit anti-rat p-MYPT1 (1 : 1000), ROCK1 (1 : 400), α-SMA (1 : 400), E-cadherin (1 : 1000), and β-actin (1 : 1000), respectively, and incubate overnight. And then incubate with 1 : 2000 HRP-labeled goat anti-rabbit IgG. Detect with chromogenic agent and expose the film. Scan the image and analyze absorbance with computer software.

2.7. Real-Time PCR. Take the kidney cortex tissue 0.1 g from each rat, extract total RNA with TRIzol, and remove genomic DNA with DNase I. Reverse RNA and obtain cDNA. The fluorescence PCR quantification of cDNA was performed with SYBR Green, with triplets for each sample per protocol. The total volume of each reaction was 30 μL, with the following condition: 95°C for 3 min for predenature, then 95°C for 20 s, 60°C for 20 s, and 72°C for 30 s and repeat for 35 cycles, and then 72°C for 5 min. Make a standard curve under 55°C–95°C and save its cycle threshold (CT). Take the CT ratio of each sample to internal control as the relative value of the gene expression of this sample. The primers for ROCK1 and β-actin were shown in Table 1.

2.8. Statistical Analysis. SPSS (version14) was used for data analysis. Normal distributed quantitative variables were presented as mean ± SD. Comparison among groups was performed with ANOVA, SNK, and LSD tests. Abnormally

Table 1: Primer sequences for ROCK1 and β-actin.

Primers	Primer sequences	Product	Denature T
Rock1	Forward: AAGAGAGTGATATTGAGCAGTTGCG Reverse: TTCCTCTATTTGGTACAGAAAGCCA	192 bp	61°C
β-Actin	Forward: AAGATGACCCAGATCATGTTTGAG Reverse: TAGATGGGCACAGTGTGGGTG	146 bp	60°C

Table 2: The changes of UTP/24 h, urine NAG activity, Ccr, and Scr.

Group	n	Urine NAG (u/L)	UTP/24 h (mg/24 h)	Scr (μmol/L)	Ccr (mL/min)
N	8	14.86 ± 4.79	5.62 ± 2.38	59.17 ± 7.87	4.92 ± 1.03
D	9	26.48 ± 6.49*	27.38 ± 7.13*	69.08 ± 5.03*	2.49 ± 0.66*
F	9	19.39 ± 3.57$^{\triangle}$	20.43 ± 4.69$^{\triangle}$	60.93 ± 7.76$^{\#}$	3.05 ± 0.77*
B	8	21.08 ± 5.26$^{\triangle}$	22.47 ± 3.1$^{\triangle}$	61.24 ± 6.35$^{\#}$	2.96 ± 0.83*

Compared with N group, $^{\star}P < 0.01$, compared with D group, $^{\triangle}P < 0.01$; $^{\#}P < 0.05$.

distributed variables were log-transformed into normal distributed variables and then analyzed thereafter; data were presented with median. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. The Changes of 24-Hour Urine Protein (UTP/24 h), Urine N-Acetyl-β-glucosaminase (NAG) Activity, Creatinine Clearance (Ccr), Serum Creatinine (Scr), Blood Pressure, and Glucose in Each Group Rats. As shown in Table 2, at 12 weeks, compared with N group, D group had elevated 24-hour urine protein, NAG activity, Scr ($P < 0.05$), and decreased Ccr ($P < 0.05$). Compared with D group, F group had decreased 24-hour urine protein, NAG activity, and Scr ($P < 0.05$). There was no significant difference between F and B groups. There was no significant difference for blood pressure or glucose among groups, as shown in Table 3.

3.2. The Pathological Changes of Kidney in Each Group. Compared with N and F groups, D group had significant expanded glomerular mesangial matrix, increased cell number, thickened basement membrane, with multiple inflammatory cells infiltrated in interstitial space, dilated renal tubular, and fibrosis in interstitial space. There were mild proliferation of glomerular mesangial matrix, inflammatory infiltration, tubular dilatation, and fibrosis in F and B groups, as shown in Figure 1.

3.3. The Changes of Protein Expressions of ROCK1, α-SMA, and E-Cadherin in Renal Cortex of the Rats by Immunohistochemistry. The expression of ROCK1 showed trace in tubular epithelium cells in N group, was enhanced in D group which mainly distributed in dilated renal tubular, and was reduced in F and B groups. α-SMA was presented in the smooth muscle cells of renal small artery in N group and visible in epithelium of renal tubular in D group with majority expressed in medullar area but no expression in F or D group. E-Cadherin was mainly presented in the epithelium cells of renal tubular in N group, especially in the cell conjunction area, with partial expression for F and B groups which was enhanced at the cell junction area but no expression on tubular epithelium in D group, as shown in Figure 2.

Table 3: The changes of blood pressure and glucose.

Group	n	BP (mmHg)	Blood glucose (mmol/L)
N	8	86.37 ± 11.24	6.37 ± 0.85
D	9	87.72 ± 10.03	27.84 ± 4.56*
B	9	85.26 ± 12.45	28.91 ± 5.39
F	8	86.95 ± 10.27	26.91 ± 3.37

Compared with N group, $^{\star}P < 0.01$.

3.4. The Protein Expression of p-MYPT1, ROCK1, α-SMA, and E-Cadherin with Western Blotting. As shown in Figure 3, compared with N group, the rats in D group had enhanced protein expressions of p-MYPT1, ROCK1, and α-SMA in renal cortex but reduced E-cadherin. Compared with D group, F group and B group had reduced protein expressions for p-MYPT1, ROCK1, and α-SMA and enhanced E-cadherin which was still less than that of the normal group. There was no significant difference between F and B groups, as shown in Figure 3 and Table 4.

3.5. Real-Time PCR Showed Changes of mRNA Expression for ROCK1. Compared with N group, mRNA expression of ROCK1 in renal cortex was increased in D group. Compared with D group, there was less mRNA expression of ROCK1 in F and B groups, lower than normal, as shown in Figure 4.

4. Discussion

In this study, by treating rats with type 1 diabetic nephropathy with benidipine, a triple channel blocker, and fasudil, a Rho kinase inhibitor, we successfully investigated the effect of

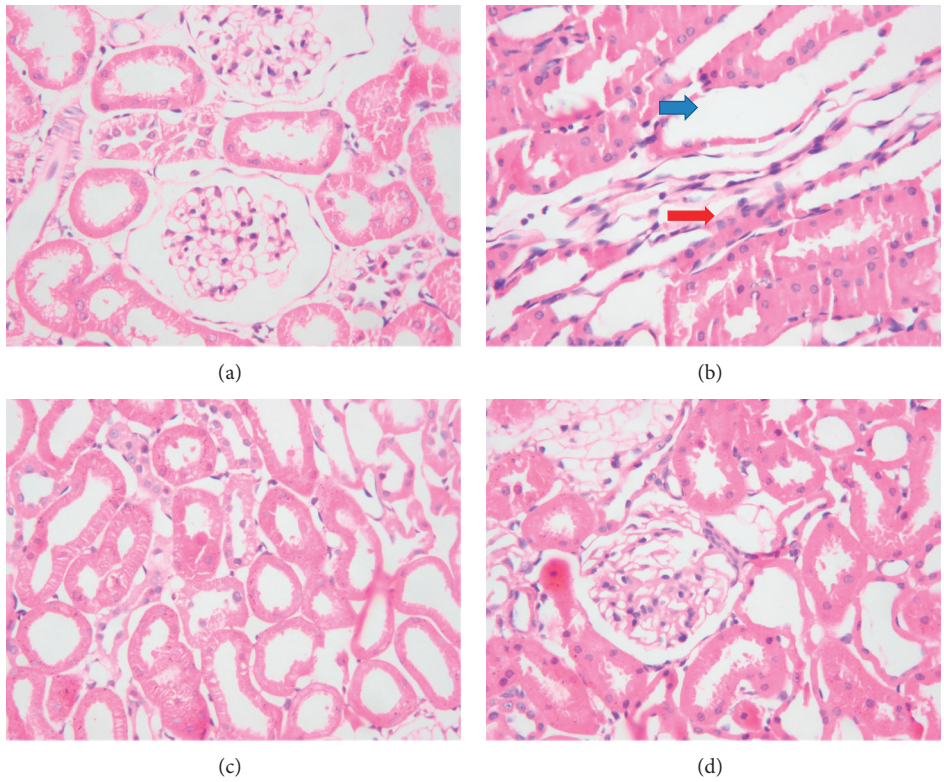

Figure 1: The pathological changes of renal glomerular and interstitial space (×400). (a), (b), (c), and (d) represent N group, D group, F group, and B group, respectively, with HE staining. (a) showed normal tubular; (b) showed tubular dilated and distorted with inflammatory infiltration and fibrosis in renal interstitial space; (c) and (d) showed that the distorted tubular with inflammatory infiltration and fibrosis in renal interstitial were significantly reduced. Blue arrow: tubular dilation; red arrow: inflammatory cell infiltration.

benidipine on epithelium-mesenchymal transdifferentiation and its possible mechanism via inhibiting Rho kinase activity. These results were consistent with some previous studies [11].

Rho protein is a small molecular guanylate binding protein. Rho kinase (ROCK) is a widely studied downstream signaling molecule of RhoA. ROCK directly affects myosin light chain (MLC) or indirectly affects the target subunit of myosin phosphatase (MYPT1) and thus increases the phosphorylation of MLC in plasma and controls the attachment, chemoattractant, contraction, and so forth. Phosphorylated MYPT1 level can be used as a marker of ROCK activation. Fasudil is a ROCK-specific inhibitor and inhibits ROCK activity by competitively combining ATP sites of ROCK catalytic domain [12]. ROCKI and ROCKII were both reported. In kidney tissues, ROCKI is the major one presented.

Recent studies revealed that abnormal activation of ROCK signaling pathway played a very important role in the pathophysiology of all kinds of complications of diabetes [13, 14]. Our study confirmed that there was ROCK activation in renal tubular epithelium cells of 12 weeks of diabetic rats, and the effects of ROCK on diabetic renal interstitial space were through ROCK1. Further study found that the protein expression of E-cadherin, the marker protein of renal tubular epithelium cells, was downregulated in diabetic rats, while the protein expression of α-SMA, the marker protein of myofibrillar cells, was upregulated, indicating that there was EMT in diabetic nephropathy of rats. Benidipine or fasudil can significantly inhibit NAG activity, reduce urine protein and Scr level, decrease the expression of p-MYPT1, ROCK1, and α-SMA, and increase the expression of E-cadherin without affecting blood glucose or pressure. This suggested that benidipine inhibited the activity of ROCK1 and thus partially blocked EMT. Benidipine has protective effect on diabetic nephropathy of rats, independent of its effect of lowering blood pressure.

TCC is a low-voltage activated channel, mainly located in renal efferent arterioles and pacing cells of the heart. Through its strong blocking effects on TCC, benidipine dilates renal afferent and efferent arterioles equally and thus effectively reduces the resistance of renal vessels and intrarenal pressure [15]. It is showed that, besides its effects on adjusting capillary pressure of glomerular, TCC has many nonhemodynamic effects [16–19]. It regulates the activity of NF-kβ and thus inhibits inflammation, promotes the secretion and release of aldosterone, improves the remodeling of the heart and kidney, and anti-oxygenize and anti-proliferate [20–23]. It is found that in the subremoval kidney models, by inhibiting Rho kinase activity, selective T channel blocker improves renal interstitial fibrosis and EMT. Our study demonstrated that benidipine inhibited Rho kinase activity and thus partially blocked EMT. So far, there is few studies done and the results all supported ours. However, there are limitations of

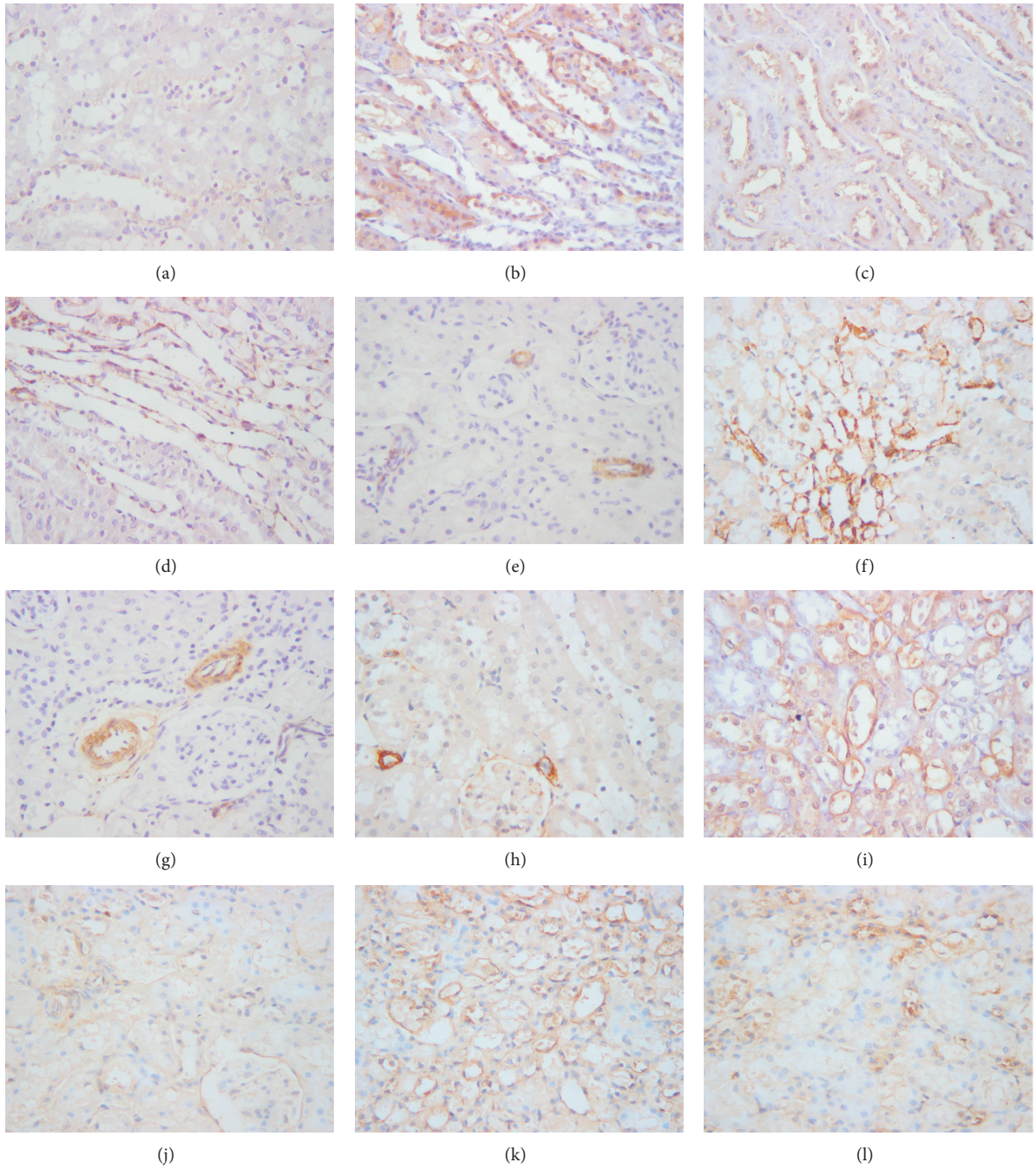

Figure 2: Immunohistochemistry expression (envision ×400). (a), (e), and (i) are for N group; (b), (f), and (j) are for D group; (c), (g), and (k) are for F group; and (d), (h), and (l) are for B group. (a), (b), (c), and (d) are the expression of ROCK1; (e), (f), (g), and (h) are the expression of α-SMA; (i), (j), (k), and (l) are the expression of E-cadherin. The brown color in plasma, membrane represented positive expression. (a) showed little expression of ROCK1 in renal tubular epithelium cells; (b) showed widely expressed ROCK1 in renal tubular epithelium cells; (c) and (d) showed the range and degree of staining slighter than those of B group. (e) showed that α-SMA is only expressed in the smooth muscle cells of renal small artery; (f) showed that α-SMA is expressed in renal tubular epithelium cells; (g) and (h) showed no expression in renal epithelium cells; (i) showed the expression of E-cadherin in renal tubular epithelium membrane; (j) showed no expression; (k) and (l) showed partial expression of E-cadherin on the surface of the cell membrane.

our study; the sample size was rather small, and it is a study in animals instead of human being.

In summary, our study is the first study suggesting that benidipine protects kidney in rats with type 1 diabetes, possibly through its effect of inhibiting Rho kinase activity and thus reducing epithelium-mesenchymal transdifferentiation (EMT). This may guide further animal studies, clinical trials on the importance of benidipine in diabetic EMT

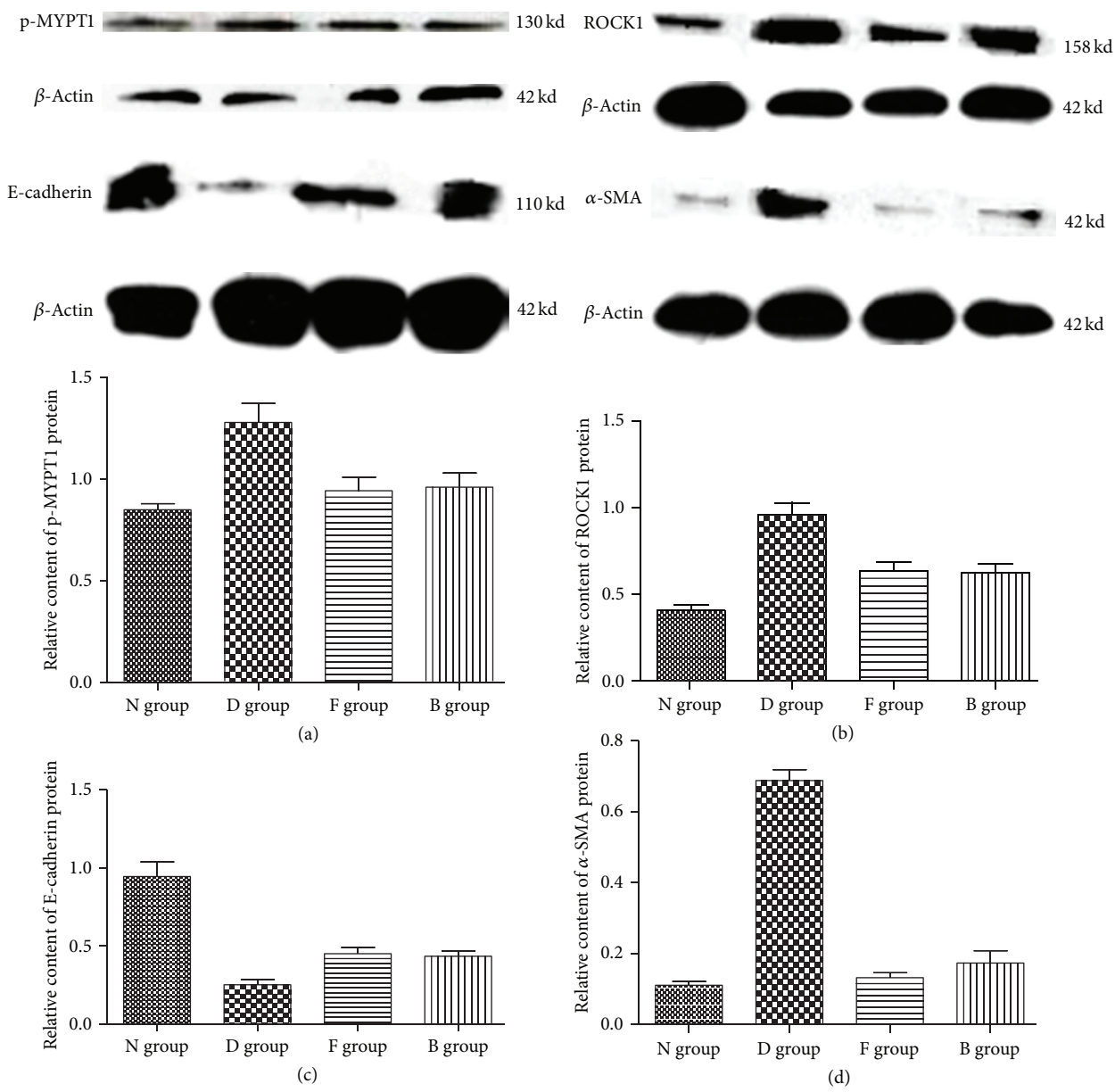

Figure 3: The protein expression changes of p-MYPT1, ROCK1, E-cadherin, and α-SMA.

Table 4: The protein expressions of p-MYPT1, ROCK1, α-SMA, and E-cadherin.

Group	n	p-MYPT1	ROCK1	α-SMA	E-Cadherin
N	8	0.87 (0.72~0.95)	0.39 (0.23~0.55)	0.11 (0.06~0.18)	0.95 ± 0.27
D	9	1.32 (0.93~1.62)*	0.93 (0.64~1.28)*	0.68 (0.57~0.82)*	0.25 ± 0.09*
F	9	0.86 (0.73~1.25)$^{\triangle}$	0.62 (0.51~0.8)$^{\triangle}$	0.14 (0.08~0.19)$^{\triangle}$	0.45 ± 0.11$^{\#}$
B	8	0.93 (0.74~1.39)$^{\triangle}$	0.61 (0.52~0.9)$^{\triangle}$	0.13 (0.08~0.32)$^{\triangle}$	0.44 ± 0.10$^{\#}$
F value		7.37	20.94	125.26	28.15
P value		<0.01	<0.01	<0.01	<0.05

Note: compared with N group, $^{\star}P < 0.01$; compared with D group, $^{\triangle}P < 0.01$, $^{\#}P < 0.05$.

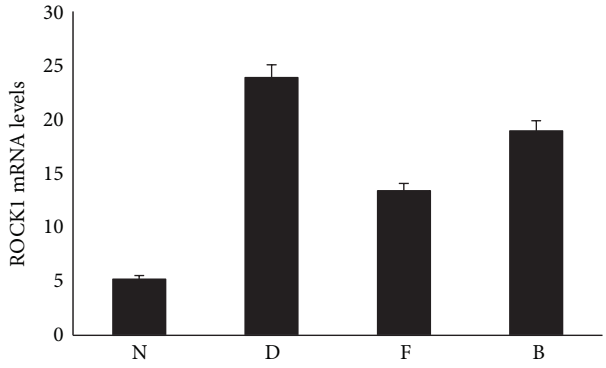

Figure 4: The mRNA expression of ROCK1. Compared with N group, $P < 0.01$; compared with D group, $P < 0.01$.

development especially in type 1 diabetic, and the possible mechanism involved. From the long run, it may direct the clinical use of benidipine in treating patients with diabetic nephropathy especially those in T1DM.

Conflict of Interests

The authors declared no conflict of interests.

Acknowledgment

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Research Article

Skeletal Muscle-Specific CPT1 Deficiency Elevates Lipotoxic Intermediates but Preserves Insulin Sensitivity

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Objective. By specific knockout of carnitine palmitoyl transferase 1b (CPT1b) in skeletal muscles, we explored the effect of CPT1b deficiency on lipids and insulin sensitivity. *Methods*. Mice with specific knockout of CPT1b in skeletal muscles (CPT1b M−/−) were used for the experiment group, with littermate C57BL/6 as controls (CPT1b). General and metabolic profiles were measured and compared between groups. mRNA expression and CPT1 activity were measured in skeletal muscle tissues and compared between groups. Mitochondrial fatty acid oxidation (FAO), triglycerides (TAGs), diglycerides (DAGs), and ceramides were examined in skeletal muscles in two groups. Phosphorylated AKT (pAkt) and glucose transporter 4 (Glut4) were determined with real-time polymerase chain reaction (RT-PCR). Insulin tolerance test, glucose tolerance test, and pyruvate oxidation were performed in both groups. *Results*. CPT1b M−/− model was successfully established, with impaired muscle CPT1 activity. Compared with CPT1b mice, CPT1b M−/− mice had similar food intake but lower body weight or fat mass and higher lipids but similar glucose or insulin levels. Their mitochondrial FAO of skeletal muscles was impaired. There were lipids accumulations (TAGs, DAGs, and ceramides) in skeletal muscle. However, pAkt and Glut4, insulin sensitivity, glucose tolerance, and pyruvate oxidation were preserved. *Conclusion*. Skeletal muscle-specific CPT1 deficiency elevates lipotoxic intermediates but preserves insulin sensitivity.

1. Introduction

Obesity and diabetes have become such big worldwide health problems. The incidences were getting higher and higher [1]. Increased intramyocellular lipid accumulation plays a very important role in obesity and diabetes, as well as their complications [2, 3]. This attracted great interest in the effect of accumulated lipid intermediates on insulin resistance [4, 5]. Lipotoxicity was regarded as the link of high levels of lipids with impaired insulin signaling [6, 7]. This sparked the interest in how excessive lipid affects β-oxidation and mitochondrial biogenesis, which may have a great impact on glucose utilization and insulin resistance [7–10].

Carnitine palmitoyl transferase 1 (CPT1) is an important rate-limiting enzyme of mitochondrial β-oxidation, by controlling the mitochondrial uptake of long-chain acyl-CoAs. Its muscle isoform, CPT1b, is the predominant isoform rich in the heart and skeletal muscles [11]. It was suggested that inhibiting CPT1 activity by specific CPT1 inhibitors alleviates insulin resistance in diet-induced obese mice [12]. However, previous studies have shown controversial results [13–15]. This brought up great concerns on the safety of this type of medications. Studies using genetic animal models may provide a better understanding. However, there is no study investigating if specific CPT1b deficiency in skeletal muscles had significant impact on lipids and insulin sensitivity.

Therefore, in this study, by establishing a mouse model, we directly explored this issue. With specific knockout of carnitine palmitoyl transferase-1b (CPT1b) in skeletal muscles of mice, we tested the following aspects: mitochondrial FAO, lipids, and insulin sensitivity.

2. Materials and Methods

2.1. Model Establishment. CPT1b M−/− mouse model was established as per previously described [12, 16, 17] by using male C57/BL6 mice (12 weeks old; 20–25 g) with Cre-lox technology [17, 18]. Mice were genotyped by standard PCR

of tail DNA with PCR Master Mix (Applied Biosystems Inc.). Age-matched male littermates were used as controls (CPT1b). Twenty mice from each group (CPT1band CPT1b M−/−) were kept on a 12 h/12 h light/dark cycle, in temperature-steady rooms, and had *ad libitum* access to water and chow diet. All experimental procedures were conducted according to the Care and Use Guide of Laboratory Animals and were approved by the IAEC of the Huzhou Central Hospital.

2.2. General and Metabolic Profiles. General and metabolic profiles were measured at 12, 18, 24, and 30 weeks of age, including the following: body weight, lipids in serum, food intake, body mass, and fat mass, as per related protocols [19, 20].

2.3. RNA Extraction and Quantization. Skeletal muscle mRNA and protein were extracted and purified with standard protocols as per our previous protocols [7, 21]. RT-PCR was performed for mRNA quantization per standard protocols with the reagents from ABI system Inc. Primers were designed with Primer Express and made by Invitrogen Inc. Quantitative real-time RT-PCR analyses were carried out by using Step one real-time PCR system (Applied Biosystems Inc.). Cycles were 50°C for 2 min, a 2 min 95°C denaturing step, followed by 50 cycles of 95°C denaturation, incubated at 60°C for 2 min, and denatured at 95°C for 1 sec for the final step. Results were normalized to β-actin. The sequences of the primers are as follows: for CPT1b: forward: GAC CAT AGAGGC ACT TCT CAGCAT GG, reverse: GCAGCA GCTTCA GGG TTTGT; β-actin: forward: GTC CTC TCC CAA GTC CAC AC, reverse: GGG AGA CCA AAA GCC TTC AT; pAkt forward: TAA TAC GAC TCA CTA TAG GGC CAA GGAGATCATGC, reverse: GATTTAGGT-GACACTATAGCTCCAAGCTATCGTCC; Glut4 forward: ACCGTGGTCCTTIGCTGTGTT, reverse: ACC CCA ATG TTG TAC CCA AAC T.

2.4. CPT1 Activity Measurement. As described previously [16, 22, 23], a modified mitochondrial CPT1 assay was performed to measure CPT1 activity. Briefly, quantified mitochondria (100 μg protein) from skeletal muscles were incubated with reaction buffer (117 Mm Tris-HCl, 0.28 mM reduced glutathione, 4.4 mM ATP, 4.4 mM $MgCl_2$, 16.7 mM KCl, 2.2 mM KCN, 40 mg/l rotenone, 0.1% BSA, and 50 mM palmitoyl-CoA) at 37°C for 5 min. Initiate the reaction by adding 2 mM [^{14}C]-carnitine (0.1 μCi) and queued it 10 min later with 50 μL 1.2 mM ice cold HCl. [^{14}C]-palmitoyl carnitine was extracted with water saturated butanol and measured via liquid scintillation counting.

2.5. Fatty Acid Oxidation (FAO) Measurement. As previously described [24, 25], FAO was examined with radiolabeled palmitate FAO assay. Skeletal muscle tissues were homogenized, and mitochondria were isolated and quantified (100 μg proteins). Palmitate oxidation assay was performed with reaction buffer (75.5 mM sucrose, 12.5 mM K_2HPO_4, 100 mM KCl, 1.75 mM $MgCl_2$-$6H_2O$, 1.75 mM L-carnitine, 0.125 mM L(-) malic acid, 1.75 mM DTT, 0.07 mM NAD+, 2 mM ATP, 10 Mm Tris-HCl, and 0.07 mM Coenzyme A). The reaction started when 200μM [^{14}C]-palmitate-15% BSA (1 : 6) complex (0.04 μCi/reaction mixture) was added and stopped by 3.5 M perchloric acid after incubation at 37°C for 30 min. CO_2-trapping medium (NaOH, 0.1 M) for ^{14}C radioactivity was measured by liquid scintillation to calculate palmitate oxidation rate. After incubation, $^{14}CO_2$ and ^{14}C-ASPs were measured (LS 6500; Beckman Coulter). All assays were performed in triplicate, and data were normalized to protein content. Calculations were then performed accordingly.

2.6. TAGs, DAGs, and Ceramides Measurement. As previously reported [26–28], TAGs, DAGs, and ceramides were tested with KC-ESI-MS and gas chromatography (Applied Biosystems Inc.). Skeletal muscle tissue homogenates were extracted according to the protocol. Samples for ceramides were spiked with 50 ng C17:0. After the solution was evaporated dry and reconstituted, the samples were analyzed with mass spectrometry (MS). The analysis was performed in positive ion mode electrospray ion (ESI-MS) source and precursor ion scans m/z 264 and 282 (ceramides). Ceramides were quantified by taking the ratios of the integrated intensity for each subspecies to the intensity of C17:0.

2.7. Insulin Tolerance Test and Glucose Intolerance Test. As described [29–31], insulin tolerance test and glucose intolerance test were performed at 31 weeks of age. An intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT) were performed on nonanaesthetized mice after 8 hours' fasting. Blood glucose was measured with lancet glucometer (Johnson and Johnson). For iGTT, 20% glucose (2.0 g/kg body weight) was administered intraperitoneally. Blood samples were collected from tail vein at 30, 60, 90, and 120 min for glucose levels. For ITT, glucose blood levels were sampled at 5, 10, 15, 20, 25, and 30 min following intraperitoneal injection of human insulin (0.75 U 4.5 nmol/kg; Novolin R, Novo Nordisk).

2.8. Glucose Oxidation (Pyruvate Oxidation). As described previously [32, 33], skeletal muscles were homogenized in ice-cold buffer (250 mM sucrose, 10 mM Tris-HCl, 2 mM EDTA, and 1 mM ATP (pH 7.4)). For glucose oxidation, fresh skeletal muscle tissues were homogenized in ice-cold buffer (5 mM KCl, 2 mM Tris-HCl, 0.5 mM Tris base, 0.25 mM $MgCl_2$-$6H_2O$, 0.05 mM EDTA, and 0.05 mM ATP (pH 7.4)), 400 μL homogenate was used and the reaction started when 200 μM [^{14}C]-glucose (0.1 μCi/reaction mixture) was added. The reaction buffer and condition are the same for palmitate oxidation as described above. CO_2-trapping medium (NaOH, 0.1 M) for ^{14}C radioactivity was measured by liquid scintillation to calculate glucose oxidation and quantified by weight of the skeletal muscle tissue homogenate.

2.9. Statistical Analysis. SAS 9.3 (SAS Institute Inc.) was used for data analysis. All data are presented as the mean ± SD. Student's t-test was used to evaluate the statistical significance of differences between knockout and controls. $P < 0.05$ was regarded as significant.

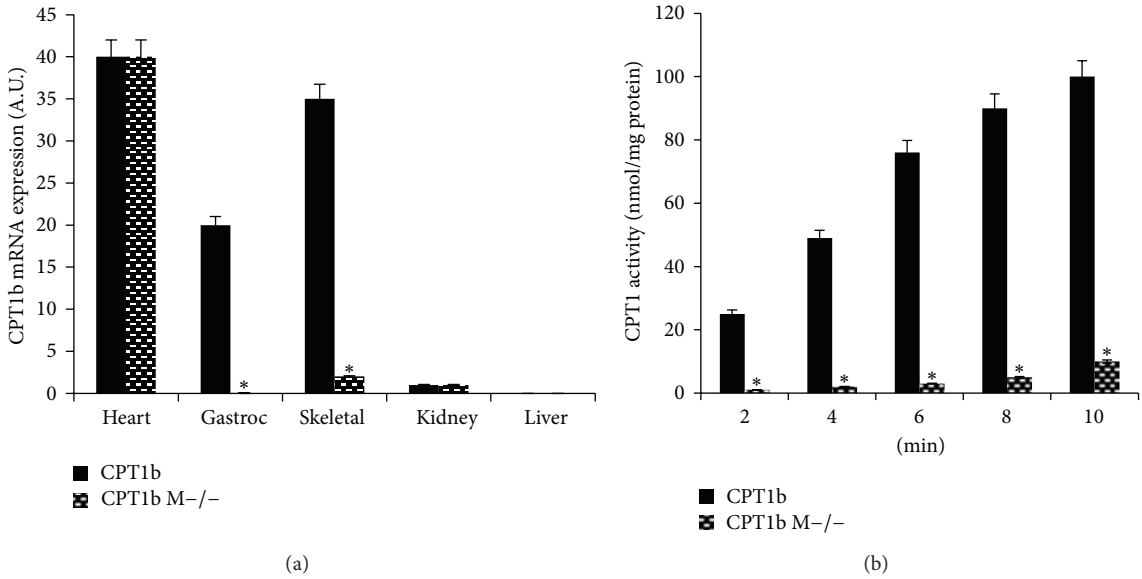

Figure 1: (a) CPT mRNA expression in different tissues, compared between wild type with CPT1b and knockout mice with CPT1b M−/−. It showed that the expression of CPT1b was significantly reduced in gastric and skeletal muscles ($^*P < 0.05$). (b) CPT1 activities were reduced in knockout mice compared with wild type, significant at 2, 4, 6, 8, and 10 minutes ($^*P < 0.05$). $n = 20$ for each group.

Table 1: General and metabolic profiles at 30 weeks of age.

Profiles	CPT1b	CPT1b M−/−	P value (CPT1b versus CPT1b M−/−)
Body weight (gram)	35.1 ± 2.2	26.3 ± 1.1	<0.05
Fat mass (gram)	15.2 ± 1.0	5.1 ± 0.4	<0.05
TAG in serum (ng/dL)	100.3 ± 2.1	150.1 ± 2.9	<0.05
DAG in serum (ng/dL)	10.3 ± 0.1	14.1 ± 0.2	<0.05
Fasting glucose (mg/dL)	105.1 ± 5.2	102.5 ± 4.3	NS
Fasting insulin (ng/mL)	2.0 ± 0.1	2.3 ± 0.1	NS

3. Results

3.1. CPT1b M−/− Model with Impaired Muscle FAO. As shown in Figure 1(a), the mRNA expression of CPT1b decreased specifically in skeletal muscles (RT-PCR) but not in the heart muscle in CPT1b M−/− mice. It is barely expressed in kidney or liver regardless of knockout or not. CPT1 activity is barely detectable (DTNB assay at 412 nm) in mitochondria (Figure 1(b)) for the knockout mice, even up to 10 minutes. This indicated the successful establishment of this model.

3.2. General and Metabolic Profiles. Compared to the mice in CPT1b group, those mice in CPT1b M−/− group had similar daily food intake. However, as shown in Table 1, their body weights were lower, starting about 12 weeks of age, along with lower fat mass ($P < 0.05$, resp.). Their lipids levels were higher ($P < 0.05$), but their glucose and insulin levels were similar.

3.3. Impaired Mitochondrial FAO and Lipid Accumulation. As shown in Figure 2(a), FAO was decreased in isolated mitochondria (radiolabeled palmitate) in skeletal muscles of CPT1b M−/− mice. This was accompanied by elevation of ceramides, TAGs, and DAGs (Figures 2(b) and 2(c)). Interestingly, for the ceramides, C16, C18, and C18:1 had significant increases for CPT1b M−/−, but not for C24 or C24:1. Both DAGs and TAGs are dramatically elevated in CPT1b M−/− mice.

3.4. Preserved Insulin and Enhanced Glucose Sensitivity. As shown in Figure 3(a), CPT1b M−/− mice maintain insulin sensitivity (insulin tolerance test). Compared with wild type, glucose tolerance test of CPT1b M−/− mice had improved significantly (as shown in Figure 3(b)). Pyruvate oxidation went up (Figure 3(c)) and so did insulin-stimulated pAkt and GLUT4 (Figure 4). This suggested that insulin sensitivity and glucose homeostasis were improved in CPT1b M−/− mice.

4. Discussion

The relationship of fatty acid oxidation, lipids accumulation, and insulin sensitivity in skeletal muscles has been quite interesting. Imbalanced fatty acid uptake and fatty acid oxidation (FAO) have been linked to insulin resistance in muscles, which in turn worsens and complicates obesity

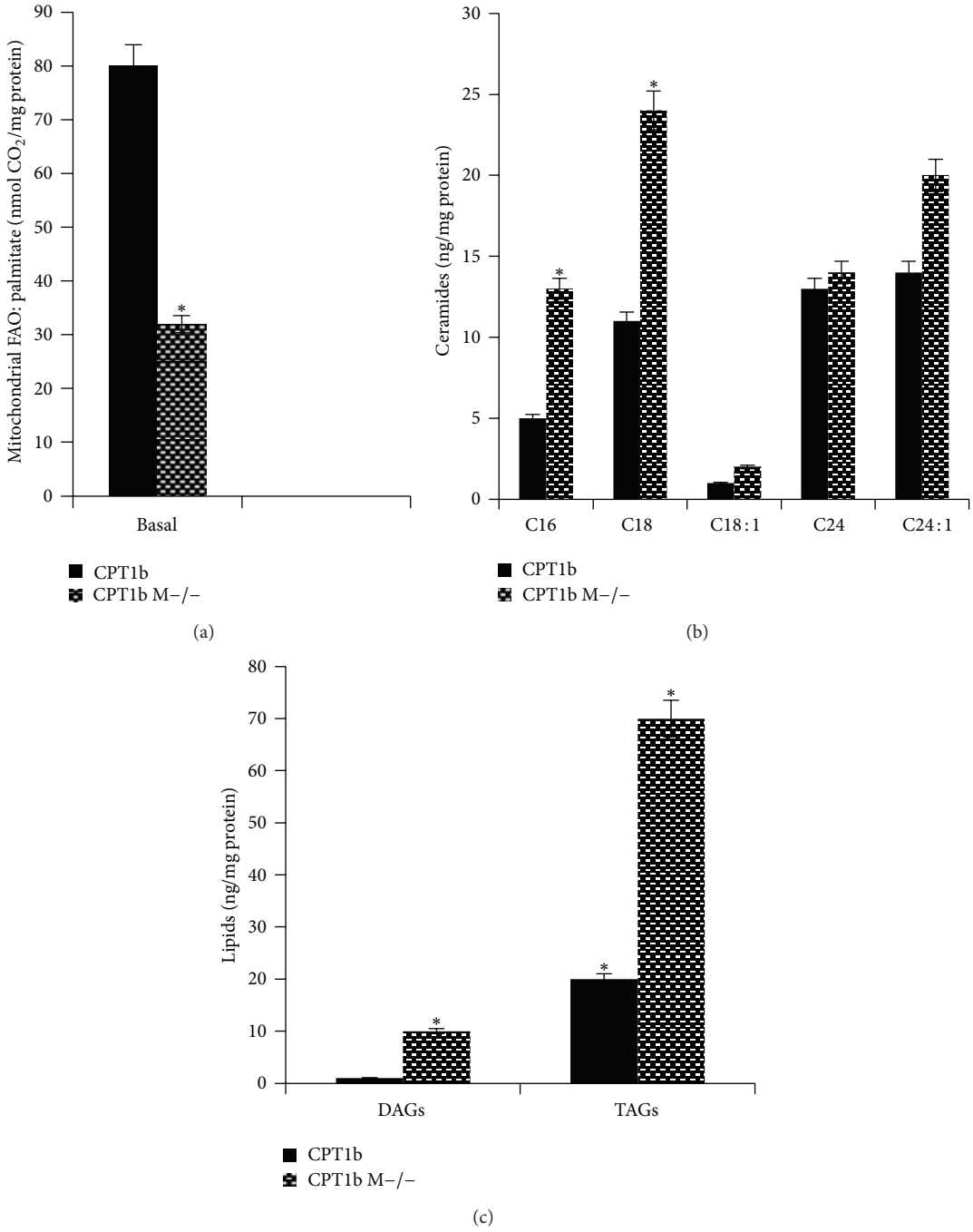

FIGURE 2: (a) Basal mitochondrial FAO was reduced in the skeletal muscle of knockout mice, compared with wild type ($^*P < 0.05$). (b) Ceramides were accumulated in skeletal muscles of knockout mice, especially for C16 and C18 ($^*P < 0.05$) but not for C18:1 or C24 or C24:1. (c) DAGs and TAGs were accumulated in skeletal muscles of knockout mice ($^*P < 0.05$). $n = 20$ for each group.

and diabetes, as well as their complications [7, 34, 35]. The findings have been quite controversial.

Some studies showed that increasing fatty acid oxidation alleviated insulin resistant [36]. Our study demonstrated that in mice with conditional knockout CPT1b in skeletal muscles, mitochondrial fatty acid oxidation was depressed dramatically. This was accompanied by increased accumulation of lipids in skeletal muscles, such as DAGs, TAGs, and ceramides. Regardless of these changes, insulin tolerance test, glucose tolerance test, and pyruvate oxidation all proved better insulin sensitivity for the knockout mice. This is partially consistent with the recent results in high-fat diet-induced obese mice, when given CPT1 inhibitor oxfenicine [10], as far as the major findings are concerned. But for the lipids accumulation, they showed that fatty acid intermediates decreased. Another study done with different CPT1 inhibitor,

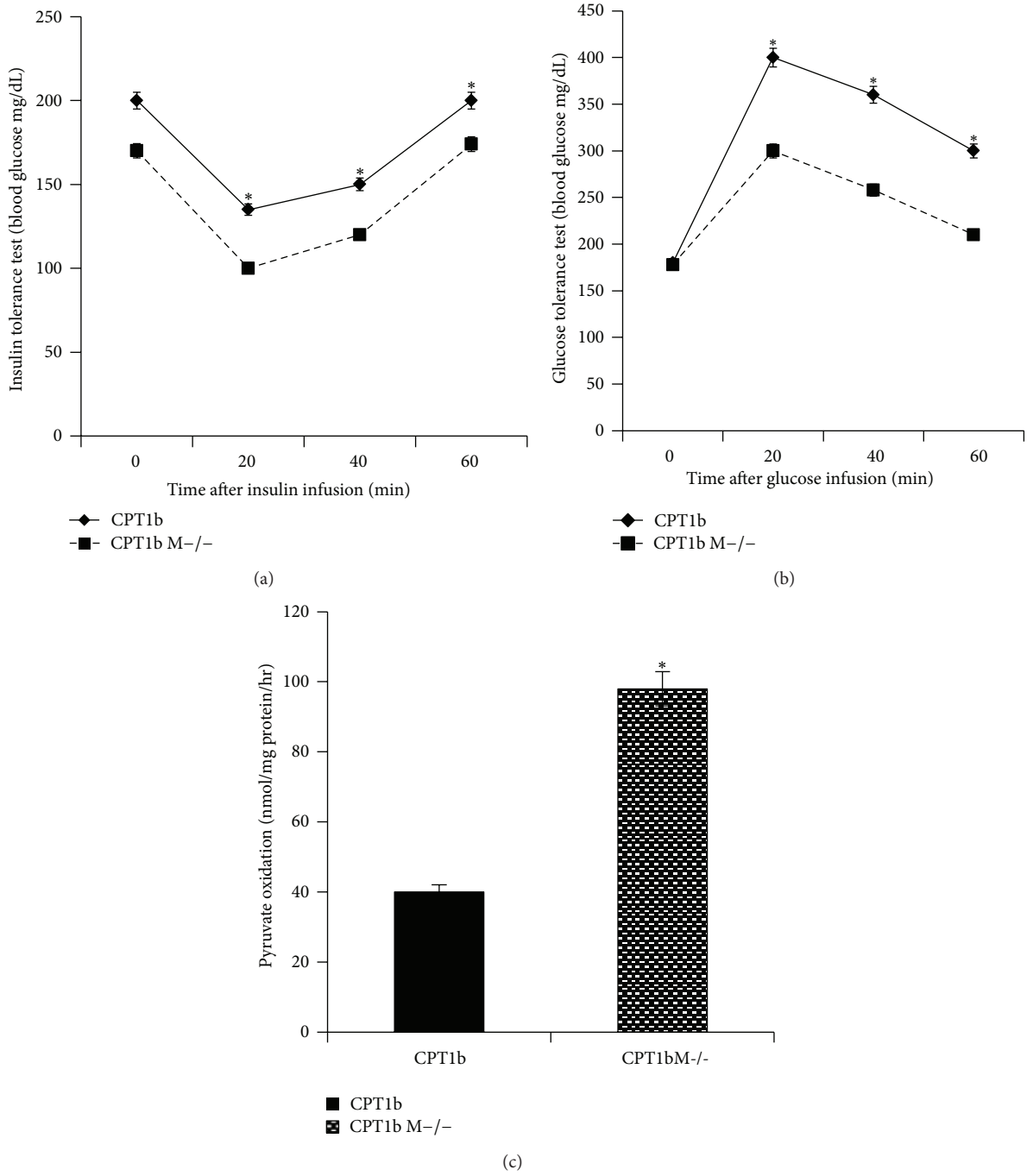

Figure 3: Insulin tolerance test showed the knockout mice preserved insulin sensitivity (a) with better glucose tolerance test (b) and improved glucose metabolism (c). $n = 20$ for each group. $^{*}P < 0.05$.

etomoxir, suggested that lipids intermediates increased [37]. Interestingly, this was correlated with impaired glucose disposal from skeletal muscles. Since about 70% of total insulin-induced glucose disposal occurs in skeletal muscles, this may indicate that increased fatty acid intermediates in skeletal muscles could damage insulin sensitivity. This is not the case shown in our study. On the contrary, our study showed that regardless of the characteristics of lipids accumulation, the mice are still insulin sensitive.

This is very exciting. There may be many pathways involved. First, due to the knockout of CPT1b in skeletal muscles, the major fuel sources were in deficit. This may suggest that signaling pathways related to energy were impaired or injured such as AMPK or mTOR [38]. Second, abnormal mitochondrial biogenesis and mitochondrial dysfunction may contribute to insulin resistance and diabetes [39, 40]. We explored from these aspects the enzymes involved in mitochondrial activity such as β-HAD and citrate synthase,

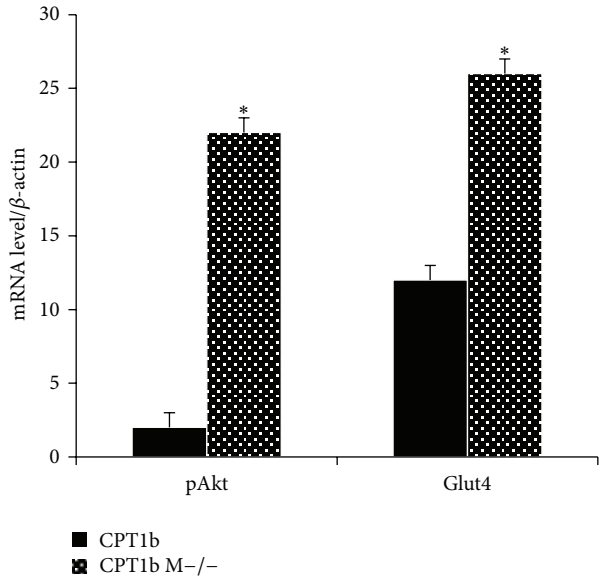

Figure 4: RT-PCR for pAkt and Glut4 showed preserved expression with CTP1b specific knockout. $n = 20$ for each group. $^*P < 0.05$.

genes related to transcriptions such as NRF (nuclear respiratory factor), and the major nuclear receptor activator PGC1α [7]. Thirdly, considering the correlation of adaptation of peroxisomal and amino acid to mitochondria, we further explored these changes. We will report these results in our next paper and further discuss the mechanism involved.

In summary, our study finding is very unique. It suggested that with specific knockout of CPT1b in skeletal muscles, although there were lipids accumulations in skeletal muscles, the insulin sensitivity still remains. This may point towards a great direction for pharmaceutical development.

Conflict of Interests

The authors declared no conflict of interests.

Authors' Contribution

Drs. Wanchun Shi and Siping Hu contributed equally to this paper and both were regarded as the first authors.

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Research Article

Correlation of Abdominal Fat Distribution with Different Types of Diabetes in a Chinese Population

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To investigate abdominal fat distribution in Chinese subjects with diabetes and its correlation with different types of diabetes. A total of 176 diabetic subjects were enrolled, 92 with type 1 and 84 with type 2, with a mean age of 27.41 and 49.3 yrs. No subject has history of severe diseases. Multi-slice CT was used to measure total abdominal adipose (TA) and visceral adipose (VA) tissues. Subcutaneous adipose (SA) tissue was obtained by subtracting VA from TA. There were differences between subjects with T1DM and T2DM for TA, VA, SA, VA/SA, body mass index (BMI), triglyceride (TG) and high density lipoprotein, but not total Cholesterol or low density lipoprotein. There were positive correlations between TA, VA, SA, VA/SA and T1DM and T2DM ($P < 0.05$ and $r > 0.86$). In subjects with T1DM, VA was negatively correlated with HDL, positively with BMI and age, and SA was positively correlated with BMI and sex ($P < 0.05$ and $r > 0.86$ for all). In subjects with T2DM, VA was positively correlated to BMI, TG and age, and SA was positively correlated to TG and sex ($P < 0.05$ and $r > 0.86$ for all). Abdominal fat content was positively correlated to diabetes in Chinese, which differs in different types of diabetes.

1. Introduction

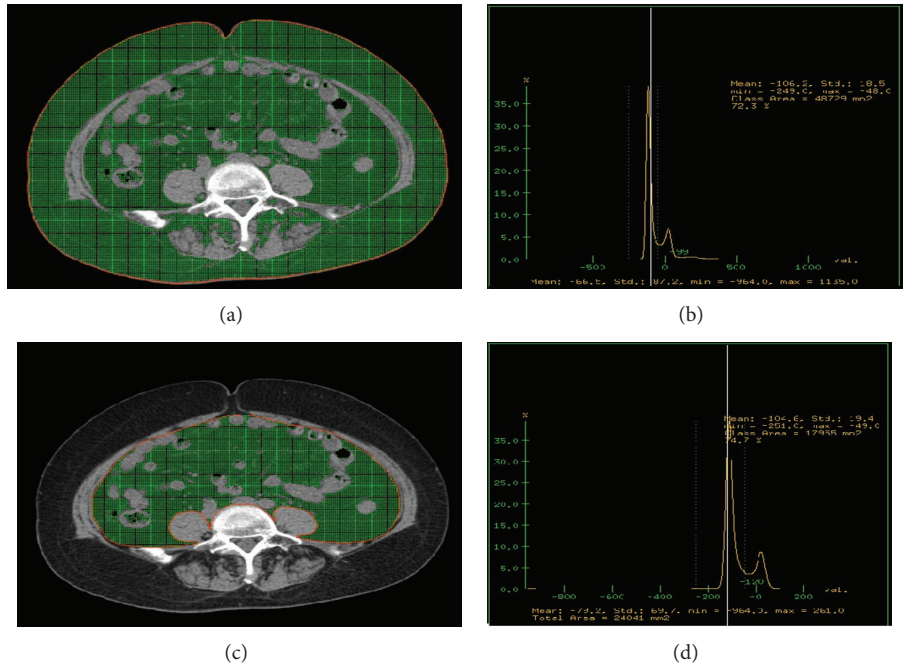
Glucose and lipid metabolism are closely interacted with each other. When lipid metabolism is in disorder, glucose metabolism would be interfered. On the other hand, fat is deposited in different regions of the body, and people become obese. It is well known that obesity is highly associated with diabetes. There are many methods to evaluate obesity, such as BMI and waist circumference (WC) [1, 2]. Although these parameters are helpful for diagnosis of obesity, they do not demonstrate the correlation of obesity and other diseases [3]. With the development of medical imaging techniques, there are more and more techniques used for measuring body fat distribution in human being, such as CT and magnetic resolution imaging (MRI). Especially MSCT, whose exact value can be used to determine the nature of tissues, combined with the process of imaging, can measure the area of fat precisely, with minor error. MSCT has been regarded as the gold standard for measuring fat distribution [4–6].

There was a study that compared abdominal fat distribution in different types of diabetes and their correlations [7]. However, it is done for different regions of the body such as fat in skeletal muscle and thigh, and it was performed in Canadians. Fat distribution varied in different ethnic groups [8] and thus had different contribution to the onset of diabetes [9]. As per our knowledge, there is no study investigating the abdominal fat distribution in different types of diabetes or its contributions in Chinese population.

Therefore, in this study, by using MSCT, we measured the abdominal fat of Chinese subjects with diabete, and explored the characteristics of abdominal fat distribution in Chinese subjects with different types of diabetes, as well as its correlation with diabetes in Chinese population.

2. Materials and Methods

2.1. Study Population. This study was approved by ethics board and institutional review board of our hospital. All the subjects signed the written consent form prior to the study initiation. All the procedures meet the requirement of clinical research and health insurance portability and



(a) (b)

(c) (d)

Figure 1: (a)–(d) are the measurement for abdominal fat. (a) is the line along abdominal skin at the axial position of CT image (red line); (b) is the area within adipose tissue of this area with histogram calculation (green area), that is, the total abdominal adipose tissue (TA) (mm^2); (c) is the line along the abdominal wall and the internal edges of bilateral psoas major (red line); (d) is the area of adipose tissue within this area with histogram calculation (green area), the visceral adipose tissue (VA) (mm^2).

accountability act compliance. From those who visited our department during Jan of 2010 to Jan of 2013, 176 subjects with diabetes were randomly enrolled into this study, including 92 with T1DM and 84 with T2DM. There were 51 males and 41 females in T1DM group, aged 14~59 yrs, with a mean age of 27.41 ± 11.03 yrs. There were 60 males and 24 females in T2DM group, aged 23~64 yrs, with a mean age of 49.3 ± 10.11 yrs. Total abdominal adipose tissue (TA) and visceral adipose tissue (VA) were measured with MSCT and histogram method. Subcutaneous adipose tissue (SA) area was obtained by subtracting VA from TA. The ratio of visceral adipose tissue and subcutaneous adipose tissue (VA/SA) was calculated. Other characteristics such as BMI, TG, HDL, total cholesterol (Tch), and LDL were collected in the whole population enrolled. All the measurements were performed per manufacturer protocol.

2.2. Methods

2.2.1. CT Scanning. GE LightSpeed 16 spiral CT was used. Scan was performed at the umbilicus level (L4), with the axial view, a voltage of 120 Kv, a current of 200 mA, and by Axial/2i, for 5 mm of thickness and an interval of 5 mm. The imaging was rebuilt in stand mode.

2.2.2. Measurement of Abdominal Adipose Content. Transfer the imaging to ADW4.4 workstation for process, set the CT value of adipose tissue attenuating region as −50~−250 Hu with histogram method, and draw a line along the abdominal skin. Within this area of adipose tissue, the total abdominal adipose tissue (TA) (mm^2) (Figures 1(a) and 1(b)) was measured. Draw another line along the abdominal muscles and the internal edges of bilateral psoas, and measure adipose content within this area, which is the visceral adipose tissue area (VA) (mm^2) (Figures 1(c) and 1(d)). The difference between TA and VA was then calculated, by which the subcutaneous adipose area (SA) (mm^2) was obtained. The ratio of VA/SA was then calculated. The measurements were conducted by 2 experienced CT who were radiologists blinded to subjects' information. Data was then reviewed by the third radiologist. The mean of the measurements was used as the final value for analysis.

2.2.3. Statistical Analysis. SPSS13.0 was used for data analysis. Data consistency between two measurements by two radiologists was tested with *Kapper* test. The basic parameters among subjects with different types of diabetes were analyzed with student t-test. The correlations were performed with linear regression analysis (stepwise). The data were presented as mean ± SD. $P < 0.05$ was regarded significant.

3. Results

3.1. Data Consistency by Two Radiologists Was Tested with Kapper Test. The *Kapper* value for these two radiologists was >0.8, suggesting a good consistency between radiologists. The mean of these two measurements was used for analysis.

3.2. Characteristics of Abdominal Fat Distribution in Subjects with Different Types of Diabetes. As shown in Table 1, there were significant differences for TA, VA, SA, VA/SA, BMI, TG, and HDL between subjects with T1DM and those with T2DM

Table 1: Comparison of parameters in subjects with type 1 and type 2 diabetes.

	Age	TA	VA	SA	VA/SA	BMI	TG	Tch	HDL	LDL
T1DM	27.4 ± 11.0	12562 ± 7557	3246 ± 2280	9315 ± 5905	0.41 ± 0.25	21.0 ± 3.6	0.830 ± 0.435	4.48 ± 1.21	1.2 ± 0.30	2.6 ± 0.9
T2DM	49.3 ± 10.1	34722 ± 34799	13767 ± 6711	20955 ± 33686	0.93 ± 0.46	25.4 ± 3.5	1.993 ± 1.329	4.63 ± 0.91	0.9 ± 0.23	2.8 ± 0.8
T value	−13.7	−5.96	−14.17	−3.26	−9.31	−8.3	−7.94	−0.90	7.44	−1.7
P value	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.369	<0.001	0.1
95% CI	−25.0	−29501	−11986	−18685	−0.623	−5.5	−1.453	−0.461	0.218	−0.5
	−18.7	−14818	−9055	−4593	−0.405	−3.4	−0.874	0.172	0.376	0.04

T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; TA: total adipose; VA: visceral adipose; SA: subcutaneous adipose; BMI: body mass index; TG: triglyceride; Tch: total cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein.

Table 2: Correlation of abdominal fat in patients with type 1 diabetes.

	TA	VA	SA	VA/SA
Correlation equation	−9201.700 +970.632BMI +6689.191SEX +138.576AGE −4563.910HDL	−3492.221 +291.358BMI +79.431AGE −1299.145HDL	−9674.451 +5735.256SEX +779.121BMI	0.411 −0.249SEX +0.004AGE
R Square	0.551	0.489	0.512	0.299
F value	26.646	28.051	46.622	18.963
P value	<0.001	<0.001	<0.001	<0.001

($P < 0.05$), all but HDL higher in T2DM, but not for Tch or LDL.

3.3. Correlation Analysis for Abdominal Fat Content with Different Types of Diabetes

3.3.1. Correlation Analysis for Abdominal Fat Content with Type 1 Diabetes. As shown in Table 2, TA, VA, SA, and VA/SA are positively correlated to the onset and duration of T1DM ($P < 0.05$), and the correlation coefficients of TA and SA were relatively higher. According to the correlation equation, TA and VA were negatively correlated with HDL, TA was positively correlated with BMI, AGE, and SEX, VA was positively correlated with BMI and AGE, and SA was positively correlated with BMI and SEX ($P < 0.05$).

3.3.2. Correlation Analysis for Abdominal Fat Content with Type 2 Diabetes. TA, VA, SA, and VA/SA are positively correlated to the onset of type 2 diabetes, and the correlation coefficient with SA was relatively high. According to the correlation equation, TA and VA were positively correlated with TG or SEX, and VA was positively correlated with BMI, AGE, and TG (Table 3).

4. Discussion

Currently, fat distribution has been widely used for the studies in metabolic disease in many institutes. It is confirmed that the bigger the area with subcutaneous and visceral adipose tissue, the higher the incidence of related diseases, such as intolerant glucose test, diabetes mellitus, insulin resistance, and lipids metabolic disorders [10–17]. In this study, it is found that TA, VA, SA, and VA/SA are positively correlated to both type 1 and type 2 diabetes. The subjects with VA/SA >1 account for 40.48% (34/84) of those with type 2 diabetes, whereas the number is only 2.18% (2/92) for subjects with type 1 diabetes, statistically significant. This suggested that compared with subjects with type 2 diabetes, the subjects with type 1 diabetes have more subcutaneous fat than visceral fat. This seems not to be reported previously and may be a novel finding.

Wagenknecht et al. [10] showed that abdominal fat distribution is closely associated with insulin resistance and insulin sensitivity. Hayashi et al. [15] revealed that abdominal fat distribution was significantly correlated with glucose tolerance abnormality. There were very few studies in Chinese population. It is shown [3] that abdominal fat content is positively correlated to insulin resistance. While Li et al. [18] demonstrated that abdominal fat is an independent factor for insulin sensitivity. However, these studies were for subjects with type 2 diabetes. So far, there is no report for subjects with T1DM. Furthermore, most the subjects in those studies were middle aged or the elders. There is no large study for younger aged. In this study, the mean age of the subjects was below 60 yrs old. With the comparison between T1DM and T2DM, it is shown that there were significant differences for TA, VA, SA, VA/SA, BMI, TG, and HDL between T1DM and T2DM in a Chinese population. The levels of TA, VA, SA, VA/SA, BMI, and TG were higher in subjects with T2DM than those with T1DM, but lower for HDL. Furthermore, there is no difference for Tch or LDL between T1DM and T2DM, both close to normal or only slightly higher than the normal range. This may be due to the different state of obesity. In this study, the subjects with type 1 diabetes had a BMI of 21.01 ± 3.6, while that for subjects with type 2 diabetes was 25.42 ± 3.47, statistically significant. In another word, the

Table 3: Correlation of abdominal fat in subjects with type 2 diabetes.

	TA	VA	SA	VA/SA
Correlation equation	11907.740 +8451.085TG +20055.980SEX	−20541.600 +1017.048BMI +133.967AGE +924.560TG	1003.820 +22210.083SEX +6693.090TG	0.544 −0.449SEX +0.010AGE
R Square	0.174	0.398	0.161	0.221
F value	8.539	17.616	7.794	11.478
P value	<0.001	<0.001	0.001	<0.001

TA: total adipose; VA: visceral adipose; SA: subcutaneous adipose.

subjects with T2DM were fatter than those with T1DM. It was reported that obesity was closely associated with T2DM and lipids disorders. Moreover, due to insulin resistance in type 2 diabetes, there is significant lipid disorders, presented as elevated TG and decreased HDL [19].

It was confirmed in [17] that abdominal fat distribution was correlated with the elder subjects with type 2 diabetes (≥65 yrs). There is no report in subjects with T1DM. In this study, we showed that TA and VA were positively correlated to BMI and negatively with HDL in subjects with T1DM. TA was positively correlated to TG, while VA was positively correlated to BMI, TG, and age in subjects with T2DM. It can be assumed that the correlated factors of abdominal fat content in different types of diabetes are different. This may guide the individual treatment strategy in the clinic.

Another unique aspect of this study is that it is done with Chinese population. Compared with other studies done with other population [7, 20, 21], there was unique finding from our study. First, it is found that more subcutaneous fat in subjects with T1DM, and this is correlated to the state of diabetes. Secondly, different types of diabetes had different factors impacting the total abdominal fat and visceral fat.

With the development of radiology, we now can measure abdominal fat distribution with MRI and CT in human being. MRI has no radiation and has a high resolution for tissues; thus it determine the abdominal fat accumulation very well. However, it takes longer time to perform and is complicated and difficult to be used widely. On the other hand, multislice spiral CT (MSCT) is convenient and rapid [22]. It is now regarded as the gold standard to measure fat distribution. There were limitations, such as the computer software which has to be matched up, there is radiation, and it is limited for some specific populations. Nonetheless, its benefit weight over its limitations for fat measurement [23]. Another drawback of our study was that we did not compare with the normal controls for each group, as we intended to take a look at the difference between T1DM and T2DM. This will be included in our ongoing study, and the data will possibly be reported in our next paper.

In summary, MSCT is a simple, convenient, and reliable method to measure abdominal adipose tissue in human being. Per the best of our knowledge, this is the first study investigating the correlation of T1DM and abdominal fat distribution. It is also the first study evaluating the difference of abdominal fat content between T1DM and T2DM in Chinese population. It is suggested that abdominal fat content is positively correlated to the development of T1DM and T2DM in Chinese. Subcutaneous fat seems to play an important role in the onset of T1DM. Additionally, we showed that the impact factors affecting abdominal fat content in different types of diabetes are different. This may become a potential guide for individualized medicine in the clinic.

Conflict of Interests

The authors declared no conflict of interests.

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Clinical Study

The Effects of Glucose Fluctuation on the Severity of Coronary Artery Disease in Type 2 Diabetes Mellitus

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Objectives. To explore the difference of glucose fluctuations between the normal subjects and diabetes mellitus (DM) patients and explore their impact on the development of CAD in type 2 DM patients. *Methods*. The subjects were divided into 3 groups: normal control (group A, $n = 40$), type 2 DM patients without cardiovascular complications (group B, $n = 56$), and type 2 DM patients with cardiovascular complications (group C, $n = 92$). The SYNTAX scores were collected in group C. CGMS for 72 h was applied on all the subjects. The indexes such as MBG and the LAGE were calculated. Glycemic excursions were compared between groups A, B, and C, respectively. *Results*. The tested indexes had significant differences among the three groups. SYNTAX scores are related to systolic blood pressure, CRP, MAGE, and HbA1c and are significantly correlated at 6:00–8:00 and 11:00–13:00 time points in group C. *Conclusions*. Compared with normal subjects, T2DM patients have greater blood glucose fluctuations; T2DM patients with CAD have larger glucose fluctuations than T2DM patients without CAD. Blood glucose fluctuations are positively correlated with carotid artery intima-media thickness in T2DM patients and have a significant influence on the development of coronary artery.

1. Introduction

The incidence of type 2 diabetes mellitus (T2DM) is increasing these years with the improvement of people's living standard, the changes of life style, and the increasing aging population. Yang et al. reported that the prevalence of diabetes in adults over 20 years old was 9.7% and the prevalence of prediabetes (impaired fasting glycaemia and impaired glucose tolerance) has reached 15.5% [1]. The complications of T2DM almost involved each organ of the body; 60%–80% of the patients died of vascular disease [2]. Large vascular disease affects the aorta, coronary artery, cerebral artery, renal artery, and peripheral artery mainly, which is hard to ignore; many researchers have studied the effect of blood glucose fluctuation on the vascular complications of T2DM [3–6]. Quagliaro et al. confirmed that the blood vessel endothelium was damaged greater by blood glucose fluctuation than by chronic persistent hyperglycemia [6]; recent studies have demonstrated that acute and chronic fluctuations in blood glucose levels can increase oxidative stress in type 2 diabetes mellitus patients [7], which results in cell dysfunction and tissue injury [8]. Therefore, it is important to evaluate the relationship between the blood glucose fluctuation and the coronary artery disease by dynamic glucose monitoring. Su et al. [9] have reported that the intraday glycemic variability is associated with the presence and severity of CAD in patients with T2DM, and effects of glycemic excursions on vascular complications should not be neglected in diabetes. However, the significance and value of this study were limited by small population and less correlation analysis of some important medical indexes, such as mean blood glucose (MBG), glucose standard deviation (SD), the largest amplitude of glycemic excursions (LAGE), the average amplitude of glycemic excursions (MAGE), the number of effective blood glucose excursions (NEGE), and postprandial glucose excursions of 3 dinners (PPGE1, PPGE2, and PPGE3).

Aiming to evaluate the coronary artery disease, coronary angiography and new complexity of coronary artery disease scoring method (SYNTAX scores) were used in the current study [10]. Coronary angiography is accepted as a golden standard for the diagnosis of coronary heart disease. The SYNTAX score is a complete angiography scoring system and

can be used for the comprehensive evaluation of coronary lesion. The higher the score is, the more severe it may be; following treatment may be more difficult and the prognosis may be worse [11].

In this study, we explored the effect of chronic blood glucose fluctuation on coronary artery disease through studying T2DM patients with or without cardiovascular complications. Compared to previous similar studies [9], SYNTAX score system was used to estimate the severity of coronary lesion through coronary angiography findings, which is significant for the diagnosis of large vascular complications in patients with T2DM. We also provided more adequate indexes, such as MBG, SD, LAGE, MAGE, NEGE, PPGE1, PPGE2, and PPGE3. We also analyzed the correlation of HOMA-IR, HOMA-β function index (HBCI), and IMT with glucose fluctuations.

2. Patients and Methods

2.1. Subjects. The study has been approved and registered by our hospital's Ethics Committee in January 2012; the Ethics Committee approved related screening, treatment, and data collection of these patients; written informed consent was obtained from each patient for the use of their blood sample and clinical information. All works were undertaken following the provisions of the Declaration of Helsinki.

The inclusion criteria of DM patients were 50 to 69 years old; gender not limited; type two diabetes mellitus diagnosed according to the WHO diagnostic criteria in 1999 [12], admission glucose <16.7 mmol/L, and without diabetic ketosis or nonketotic hyperosmolar coma; cardiovascular risk equal or higher than 10% according to continuous metabolic syndrome risk score [13]. They were excluded if they have known coronary artery disease; symptomatic heart failure; objective inability to perform treadmill exercise; known or active malignancy, advanced renal failure (serum creatine clearance <25 mL/mi/1.73 m^2), and liver cirrhosis (child Pugh III); stroke within the past 30 days; presence of left bundle branch block or ST depression at rest greater than 0.9 mm.

A total of 148 patients diagnosed with T2DM were enrolled in this study; these patients were suspected with coronary artery disease. The coronary angiography was conducted to get the information of their coronary lesion in General Hospital of Beijing Military Region, PLA. Healthy individuals without history of diabetes mellitus and coronary artery disease were recruited from volunteers of community inhabitants in Beijing (matched with age range); they were set as normal control group (group A, $n = 40$).

3. Coronary Angiography

Coronary artery angiography was performed by using standard Judkins techniques or a radical approach. During cardiac catheterization, nitroglycerine was administrated routinely in all cases suspected of having coronary spasm. Angiographic analysis was carried out by two experienced interventional cardiologists who were blinded to the study protocol. Angiography results were divided into CAD (≥50% obstruction in ≥1 coronary artery) group and non-CAD group. According to coronary angiography results, 148 patients were divided into 2 groups: patients without cardiovascular complications (group B, $n = 56$) and with cardiovascular complications (group C, $n = 92$).

4. Routine and Biochemical Examinations

General clinical data such as the gender, age, body mass index (BMI), antihypertension drug, systolic pressure (SBP), and diastolic pressure (DBP) were recorded by routine medical examination.

Blood samples were obtained under overnight fasting conditions from these patients, and high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), triglyceride (TG), total cholesterol (TC), and C reactive protein (CRP) of all subjects were measured by routine blood examination. Serum concentration of hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatographic method using automatic HbA1c analyzer (Tosoh HLC-723G7, Japan). The SYNTAX scores in group C were calculated with the help of professional website tool: http://www.syntaxscore.com/.

Meanwhile, we tested the plasma-reduced glutathione (GSH) level in each group to evaluate the oxidative stress; GSH was determined with a colorimetric assay using Bioxytech GSH-400 kit (Oxis International, Portland, OR, USA) based on a two-step reaction: thioethers formation followed by a β-elimination under alkaline conditions. Thioethers obtained are transformed into chromophoric thiones, which have a maximal absorbance wavelength at 400 nm.

4.1. Dynamic Blood Glucose Monitoring. The HOMA-IR and HBCI were calculated with the homeostasis model assessment. HOMA-IR = fasting blood glucose (FBG) × fasting insulin (FINS)/22.5, HBCI = 20 × FINS/(FBG-3.5) [14].

Continuous glucose monitoring system (CGMS) for 72 h was applied for all the subjects. A CGMS sensor was inserted into the subcutaneous abdominal fat tissue, calibrated according to the standard Medtronic MiniMed operating guidelines. The indexes of mean blood glucose (MBG), glucose standard deviation (SD), the largest amplitude of glycemic excursions (LAGE), the average amplitude of glycemic excursions (MAGE), the number of effective blood glucose excursions (NEGE), and postprandial glucose excursions of 3 dinners (PPGE1, PPGE2, and PPGE3) were recorded separately by CGMS.

The MAGE was calculated by measuring the arithmetic mean of the differences between consecutive peaks and nadirs, provided that the differences are greater than one standard deviation of the mean glucose value. The MODD was calculated as the mean of the absolute differences between glucose values at the same time of two consecutive days. The PPGE was obtained by calculating the postbreakfast increments in blood glucose.

Glycemic excursions were compared between groups A, B, and C. The key factors impacting SYNTAX scores were analyzed in group C by multiple linear regression analysis. Blood glucose fluctuation was recorded at 8 time sessions,

Table 1: Comparison of the indexes in the three groups.

	Group A	Group B	Group C
Sex (M/F)	24/16	24/32	48/44
Age (years)	56.3 ± 6.1	56.1 ± 6.6	61.7 ± 7.2$^{*\#}$
BMI (Kg/m^2)	23.8 ± 2.5	27.0 ± 3.6*	24.5 ± 1.7
CD (year)	—	6.10 (4.54, 8.11)	4.73 (2.77, 7.70)
Antihypertension drug user (%)	4 (10%)	31 (55.4%)**	55 (59.8%)**
SBP (mmHg)	114 ± 13	124 ± 9	136 ± 18$^{**\#}$
DBP (mmHg)	70 (65–75)	72 (66–79)	80 (80-81)$^{*\#}$
HbA1c (%)	5.3 ± 0.3	6.6 ± 1.2**	7.5 ± 1.4$^{**\#}$
HDL-c (mmol/L)	0.88 (0.83–0.92)	0.96 (0.92–1.27)*	0.91 (0.85–1.13)
LDL-c (mmol/L)	2.16 (2.05–2.20)	2.51 (1.78–2.92)	2.17 (2.07–3.23)
TG (mmol/L)	1.63 (1.58–1.66)	2.12 (1.21–4.11)	1.70 (1.42–2.23)
TC (mmol/L)	4.14 (3.93–4.25)	4.74 (4.05–4.79)	4.11 (3.57–4.46)
GSH (mmol/L)	4.23 ± 0.64	3.18 ± 0.86	2.24 ± 0.73
MBG (mmol/L)	6.1 ± 0.6	8.1 ± 2.1*	8.0 ± 1.4**
MAGE (mmol/L)	2.2 (1.8, 2.8)	2.6 (1.9, 3.5)**	4.0 (3.3, 4.8)$^{**\#\#}$
NEGE (times)	6.8 (6.0, 7.7)	4.1 (3.5, 4.7)**	4.0 (3.4, 4.8)**
SDBG (mmol/L)	0.8 ± 0.3	1.5 ± 0.4**	2.0 ± 0.8$^{**\#}$
LAGE (mmol/L)	2.9 (2.6–4.5)	6.6 (5.8–12.4)**	7.8 (6.1–9.9)**
PPGE1 (mmol/L)	2.7 (1.6–4.3)	3.2 (1.7–9.8)	4.6 (3.2–8.1)**
PPGE2 (mmol/L)	2.3 (1.7–3.5)	2.4 (2.2–8.6)	3.3 (2.6–5.4)*
PPGE3 (mmol/L)	2.2 ± 0.4	2.7 ± 1.1	4.7 ± 2.5$^{**\#\#}$
IMT (mm)	0.6 ± 0.1	0.9 ± 0.3*	1.1 ± 0.3$^{**\#}$
CRP (mg/L)	1.0 (0.9–1.1)	1.1 (1.0–2.8)*	3.8 (2.8–5.4)$^{**\#\#}$
HOMA-IR	1.58 (1.52–1.69)	2.3 (1.9–9.1)**	4.3 (3.0–4.9)**
HBCI	87 (82–103)	45 (28–67)**	80 (48–139)$^{\#\#}$
SYNTAX score	—	0	23.5 ± 5.9

CD: course of disease. Compared with group A, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with group B, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

which were 0:00–3:00, 3:00–6:00, 6:00–8:00, 8:00–11:00, 11:00–13:00, 13:00–17:00, 17:00–19:00, and 19:00–24:00; the correlations with SYNTAX scores were analyzed in every section.

4.2. Statistical Analysis. If the data were normal distribution data, *t*-test was used in comparison of two groups and single factor analysis of variance was used in comparison of three groups. If the data were nonnormal distribution data, rank-sum test was used in comparison between two groups and the Kruskal-Wallis analysis was used in comparison of three groups. The Chi-square test was used for qualitative data test; multiple factors were analyzed by multiple linear regression analysis. All analyses were performed using SPSS software program, version 16.0, for Windows (SPSS Institute Inc.) and $P < 0.05$ was considered statistically significant.

5. Results

5.1. The Comparison of the Indexes among the Three Groups. After coronary angiography, DM patients were divided into group B and group C. Table 1 showed the detailed data of the three groups. Compared with group A (healthy control), patients from group B have significantly higher BMI (27.0 ± 3.6 versus 23.8 ± 2.5), antihypertension drug using rate (55.4% versus 10%), HbA1c (6.6 ± 1.2 versus 5.3 ± 0.3%), HDL-c (0.96 versus 0.88 mmol/L), MBG (8.1 ± 2.1 versus 6.1 ± 0.6 mmol/L), MAGE (2.6 versus 2.2 mmol/L), SDBG (1.5 ± 0.4 versus 0.8 ± 0.3 mmol/L), LAGE (6.6 versus 2.9 mmol/L), IMT (0.9 ± 0.3 versus 0.6 ± 0.1 mm), and CRP (1.1 versus1.0 mg/L) and significantly lower NEGE (4.1 versus 6.8 times), HBCI (45 versus 87), and GSH level (3.18 ± 0.86 versus 4.23 ± 0.64 mmol/L); $P < 0.05$ or 0.01.

Consistent with group B, as shown in Table 1, and compared with group A, group C had the same trend with significantly higher age, antihypertension drug using rate, SBP, DBP, HbA1c, MBG, MAGE, SDBG, LAGE, PPGE1, PPGE 2, PPGE3, IMT, CRP, HOMA-IR, and GSH and lower NEGE value ($P < 0.05$ or $P < 0.01$).

Compared with group B patients, group C patients have significantly higher age (61.7 ± 7.2 versus 56.1 ± 6.6 yr), SBP (136 ± 18 versus 124 ± 9 mmHg), DBP (80 versus 72 mmHg), MAGE (4.0 versus 2.6 mmol/L), SDBG (2.0 ± 0.8 versus 1.5 ± 0.4 mmol/L), PPGE3 (4.7 ± 2.5 versus 2.7 ± 1.1 mmol/L), IMT (1.1 ± 0.3 versus 0.9 ± 0.3 mm), CRP (3.8 versus 1.1 mg/L), and HBCI (80 versus 45) and lower NEGE (4.0 versus 6.8 times) and GSH level (2.24 ± 0.73 versus 4.23 ± 0.64 mmmol/L). $P < 0.05$ or $P < 0.01$.

Table 2: Linear correlation analysis between SYNTAX scores and relative factors in group C.

SYNTAX scores	Age▼ (years)	BMI▼ (Kg/m^2)	SBP▼ (mmHg)	DBP▲ (mmHg)	CD▼ (year)	CRP▼ (mg/L)	HbA1c▼ (%)
R	−0.115	0.046	0.551	−0.015	−0.298	0.435	0.488
P	0.602	0.836	0.006	0.947	0.167	0.038	0.018
SYNTAX scores	MAGE▼ (mmol/L)	HOMA-IR▲	HBCI▲	HDL-c▲ (mmol/L)	LDL-c▲ (mmol/L)	TC▲ (mmol/L)	TG▲ (mmol/L)
R	0.518	−0.199	−0.040	0.329	0.183	−0.069	−0.059
P	0.011	0.363	0.855	0.125	0.403	0.754	0.789

CD: course of disease. ▼Pearson's correlation analysis. ▲Spearman's correlation analysis.

Table 3: Correlation analysis between SYNTAX scores and the blood glucose excursion of different time sessions in group C.

SYNTAX scores	0:00–3:00 (mmol/L)	3:00–6:00 (mmol/L)	6:00–8:00 (mmol/L)	8:00–11:00 (mmol/L)
R	−0.442	−0.208	0.678	0.115
P	0.035	0.340	0.000	0.600
SYNTAX scores	11:00–13:00 (mmol/L)	13:00–17:00 (mmol/L)	17:00–19:00 (mmol/L)	19:00–24:00 (mmol/L)
R	0.523	0.257	0.358	−0.018
P	0.011	0.237	0.094	0.933

Pearson correlation analysis was used in 0:00–3:00 and Spearman's correlation analysis was used in other's time sessions.

Table 4: Linear correlation analysis between MAGE and the related factors in groups B and C.

MAGE	Age (years)	BMI (Kg/m^2)	CD (year)	CRP (mg/L)	HbA1c (%)	HOMA-IR	HBCI	IMT (mm)
R	0.383	−0.193	−0.065	0.599	0.595	0.498	−0.297	0.460
P	0.008	0.193	0.702	0.000	0.000	0.000	0.042	0.001
MAGE	NEGE (time)	SD (mmol/L)	MBG (mmol/L)	LAGE (mmol/L)		PPGE1 (mmol/L)	PPGE2 (mmol/L)	PPGE3 (mmol/L)
R	−0.712	0.928	0.576	0.862		0.764	0.631	0.672
P	0.000	0.000	0.000	0.000		0.000	0.000	0.000

CD: course of disease. All are analyzed with Spearman's correlation analysis.

5.2. The Linear Correlation Analysis between SYNTAX Scores and Relative Factors in Group C. Multiple linear regression analysis showed the SYNTAX scores were significantly correlated with CRP, MAGE, and HbA1c in group C ($P < 0.05$) and were significantly correlated with SBP ($P < 0.01$) (Table 2).

5.3. The Correlation Analysis between SYNTAX Scores and the Blood Glucose Excursion of Different Time Sessions in Group C. In our study, a day was divided into eight sessions. Significant correlations were found in 6:00–8:00 ($P < 0.01$) and 11:00–13:00 ($P < 0.05$) between the SYNTAX scores and blood glucose excursion in group C (Table 3).

5.4. The Linear Correlation Analysis between MAGE and the Related Factors in Groups B and C. After analysis, MAGE was positively correlated with age, CRP, HbA1c, HOMA-IR, IMT, SD, MBG, LAGE, and glucose excursions before and after meals ($P < 0.01$) and was negatively correlated with HBCI ($P < 0.05$) and NEGE ($P < 0.01$) both in groups B and C (Table 4).

6. Discussion

The risk of coronary heart disease (CHD) mortality in type 2 diabetic patients is more than twofold higher compared with that in age-matched healthy subjects. The incidence of stroke events and all manifestations of CHD, myocardial infarction (MI), sudden death, and angina pectoris are at least twofold higher in patients with type 2 diabetes than in nondiabetic individuals [15]. Therefore, it is important to investigate the relevant affecting factors which cause such high risk.

Brownlee found that too much mitochondrial reactive oxygen species (ROS) may be a common mechanism of diabetic complications [16]. The oxidative stress was enhanced by the blood sugar disorder in T2DM patients; the uncompensated antioxidant capacity in vivo leads to endothelial damage, thus causing macrovascular complications. In the current study, the lowest CGH level in group C also proved this point. CRP, a biomarker of cardiovascular diseases [17], was highly related to MAGE in our study. Glucose excursions in subjects with impaired glucose regulation and T2DM trigger the activation of oxidative stress [18]; MAGE was correlated with HOMA-IR positively and negatively correlated with HBCI,

which suggests MAGE could affect the insulin resistance and the function of pancreatic islets.

Recently, large-scale clinical studies have suggested that only using HbA1c for strict glycemic control is not sufficient to reduce the risk of macrovascular complications [19, 20]. The effects of blood glucose fluctuation on vascular complications in T2DM have been researched by many scientists. Hanefeld M. et al. found that postprandial blood glucose peak can predict myocardial infarction better than fasting glucose [21]; Ceriello et al. demonstrated that accelerated oxidative stress accompanying fluctuations in blood glucose levels could worsen endothelial dysfunction more than constant hyperglycemia [22]; Torimoto et al. found that fluctuations in blood glucose levels play a significant role in vascular endothelial dysfunction in T2DM [23]; Su et al. found that the glucose variability was closely associated with the severity of cardiovascular disease in T2DM; the effect of MAGE on coronary artery was greater than that of HbA1c [9]; Colette and Monnier suggested that the MAGE can serve as the gold standard to measure the blood glucose fluctuation [24]. We found that MAGE in T2DM patients with coronary artery disease was higher than that in T2DM patients without coronary artery disease. Multiple linear regression analysis suggested that both HbAlc and MAGE were important factors affecting the cardiovascular complications of T2DM, but MAGE was more predictive than HbAlc. This study also showed that there was significant correlation between MAGE and IMT. IMT can be used as the index of early atherosclerosis.

In this study, MAGE was positively correlated with age, CRP, HbA1c, HOMA-IR, IMT, SD, MBG, LAGE, and glucose excursions before and after meal and negatively correlated with HBCI and NEGE. It suggested that MAGE may be influenced by the above factors.

The shortage of this research lies in that the impact of other factors such as blood pressure, age, and HbA1c cannot be completely ruled out although the multiple linear regression analysis has been used; these previous factors also can influence progression of CAD. Group C patients have higher age and antihypertension drug using rate; 85% of the blood pressure of them is below or near the critical range 140/90 mm Hg. Previous studies have reported that DM patients with pressure below 140 mm Hg can benefit from aggressive antihypertensive treatment [25]. Besides that, other few limitations of this study should be mentioned. Firstly, the sample size was relatively small in this study, so some subgroup comparisons may have lacked power to detect significant differences for selected variables. Secondly, although we had maintained the patients antihyperglycemic therapy as usual and avoided glucose infusion during CGMS monitoring period, some factors, such as different diets and physical and emotional stress, which may affect levels of admission glucose fluctuations could not be all prevented. Thirdly, lack of microvascular complications data in another limitation; we did not include those risk factors in study.

Currently, although there is still an extensive debate about glucose fluctuation as a risk factor for complications independent of HbA1c in diabetes [26, 27], by this present study, we provide some evidence to suggest that, at least, glucose fluctuation has potential to be a risk factor for predicting the occurrence and progression of CAD; it can be helpful to test this index in clinical treatment for DM patients.

7. Conclusions

Compared with normal subjects, T2DM patients have greater blood glucose fluctuations and higher average blood glucose. T2DM patients with larger glucose fluctuations could have higher risk for coronary artery disease compared with patients having smaller glucose fluctuations. Compared with high blood glucose, blood glucose fluctuations may be more importantly influential on the development of coronary artery disease in patients with T2DM. Blood glucose fluctuation is significantly related to carotid artery intima-media thickness in T2DM.

Abbreviations

BMI:	Body mass index
CD:	Course of disease
CGMS:	Continuous glucose monitoring system
CRP:	C reactive protein
DBP:	Diastolic pressure
DM:	Diabetes mellitus
HbA1c:	Hemoglobin A1c
HBCI:	HOMA β-cell function index
HDL-c:	High-density lipoprotein cholesterol
HOMA-IR:	HOMA insulin resistance index
LAGE:	Largest amplitude of glycemic excursions
LDL-c:	Low-density lipoprotein cholesterol
MAGE:	Mean amplitude of glycemic excursions
MBG:	Mean blood glucose
NEGE:	Number of effective glucose excursions
PPGE1:	Postprandial glucose excursions of breakfast
PPGE2:	Postprandial glucose excursions of lunch
PPGE3:	Postprandial glucose excursions of supper
SBP:	Systolic pressure
SDBG:	Standard deviation of blood glucose
TC:	Total cholesterol
TG:	Triglyceride
SYNTAX:	Synergy between PCI with taxus and cardiac surgery
CAD:	Coronary artery disease.

Conflict of Interests

The authors have no conflict of interests to declare.

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