Experimental Diabetes Research

Autonomic Nervous System, Inflammation, and Diabetes: Mechanisms and Possible Interventions

Guest Editors: M. C. Irigoyen, Dulce Elena Casarini, Mariana Morris, and Nicola Montano

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Editorial Board

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Editorial

Autonomic Nervous System, Inflammation, and Diabetes: Mechanisms and Possible Interventions

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It is well known that cardiac autonomic neuropathy increases morbidity and mortality and is associated with prognosis of cardiovascular events in diabetes. Indeed, autonomic imbalance between the sympathetic and parasympathetic nervous system regulation of cardiovascular function is markedly associated with mortality among patients with both type 1 and type 2 diabetes [1].

The published evidence supports a common pathogenesis for IHD, hypertension, and diabetes based on a sympathetic homeostatic shift, and the usefulness of prevention based on improving the risk/prevention balance by using standard pharmaceutical and lifestyle preventative measures [2].

In addition, autonomic nervous system has been indicated as an important element in the bidirectional communication between the brain and the immune system, allowing the central control of immune status and inflammation [3].

This special issue includes 9 papers on autonomic mechanisms, inflammation, and interventions being one of them a review. In fact, J. Petrofsky et al. examine the influence of autonomic dysfunction associated with aging and type 2 diabetes on daily life activities concentrating on how autonomic impairment alters normal daily activities. Impairments include the response of the blood vessels to heat, sweating, heat transfer, whole body heating, orthostatic intolerance, balance, and gait. In addition, the effects of ageing were examined.

In the submitted research papers, D. Senador et al. demonstrate that the effects of high-fructose diet in producing cardiovascular and metabolic pathologies depend on the timing of fructose intake, while G. Garruti and colleagues in a clinical study examine the links between metabolic syndrome and cardiovascular autonomic dysfunction. The authors suggest that metabolic syndrome not only increases the cardiovascular risk of relatively young subjects with T2D but is also associated with impaired cardiovascular autonomic function. In a very interesting research paper, D. C. Lieb et al. concluded that cardiac autonomic imbalance and adipose tissue-derived inflammation in newly diagnosed and established type 2 diabetes are interrelated.

In the following papers, F. G. Shiraishi et al. have shown that in patients with diabetes and chronic kidney disease, aerobic capacity was associated with inflammatory state independently of diabetes presence. On the other hand, L. Jorge and colleagues demonstrate that a single bout of dynamics aerobic exercise was able to improve hemodynamic and autonomic function as expressed by baroreflex sensitivity control of heart rate in experimental diabetes. In other interventional research paper, P. Fiorino et al. examined cardiac autonomic modulation and metabolic response in streptozotocin diabetic rats treated with green tea. The authors concluded that the green tea reduced hyperglycemia and prevented renal injury and autonomic dysfunction in experimental diabetes.

Finally, S. N. Xue et al. have shown that MMP9 deters the healing of diabetic foot ulcers by inhibiting the biological behaviors of skin fibroblasts, while M. Zhang et al. have suggested that *LYR motif containing* 1 gene may be an important mediator in the development of obesity-related insulin resistance since the expression of LYRM1 mRNA is

affected by a variety of factors that are related to insulin sensitivity.

The purpose of the present special issue was to discuss the role of autonomic nervous system not only in cardiovascular control but also in other pathophysiological mechanisms associated with inflammation and tissue damage, believing that sympathetic and parasympathetic balance may influence the risk and prevention equilibrium in diabetes. In conclusion, restoration of autonomic balance must be the aim of the prevention programs including therapeutic lifestyle changes and other pharmacologic approaches.

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Research Article

The Biological Behaviors of Rat Dermal Fibroblasts Can Be Inhibited by High Levels of MMP9

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Aims. To explore the effects of the high expression of MMP9 on biological behaviors of fibroblasts. *Methods*. High glucose and hyperhomocysteine were used to induce MMP9 expression in skin fibroblasts. Cell proliferation was detected by flow cytometry and cell viability by CCK-8. ELISA assay was used to detect collagen (hydroxyproline) secretion. Scratch test was employed to evaluate horizontal migration of cells and transwell method to evaluate vertical migration of cells. *Results*. The mRNA and protein expressions of MMP9 and its protease activity were significantly higher in cells treated with high glucose and hyperhomocysteine than those in control group. At the same time, the S-phase cell ratio, proliferation index, cell viability, collagen (hydroxyproline) secretion, horizontal migration rate, and the number of vertical migration cells decreased in high-glucose and hyperhomocysteine-treated group. Tissue inhibitor of metalloproteinase 1 (TIMP1), which inhibits the activity of MMP9, recovered the above biological behaviors. *Conclusions*. High expression of MMP9 in skin fibroblasts could be induced by cultureing in high glucose and hyperhomocysteine medium, which inhibited cell biological behaviors. Inhibitions could be reversed by TIMP1. The findings suggested that MMP9 deters the healing of diabetic foot ulcers by inhibiting the biological behaviors of fibroblasts.

1. Introduction

It is well known that ulcers of diabetic foot are refractory and can cause considerable morbidity and mortality [1]. In recent years, domestic and international studies have found that increased matrix metalloproteinases (MMPs) expression would contribute to the vulnerability of diabetic skin and the refractory nature of diabetic foot ulcers, especially MMP9 [2, 3]. MMPs are proteinases that participate in extracellular matrix macromolecule degradation. MMP9 is one of this growing family. An important mechanism for the regulation of the activity of MMPs is via binding to members of the family of proteins referred to as tissue inhibitor of metalloproteinases (TIMPs). Our previous studies have shown that local abnormal expression of MMP9 in the skin was correlated with skin damage. As compared with nondiabetic rats, the process of wound healing in the skin slowed down in diabetic rat and, at the same time, levels of MMP9 increased significantly, while TIMP1, the tissue-specific inhibitor of MMP9, decreased in the diabetic group [4–6].

It is widely recognized that high levels of MMP9 slow down the healing of diabetic foot ulcers by excessive degradation of extracellular matrix, growth factors, growth factor receptors, integrins, and their receptors, as well as increasing the local inflammatory response in the wound [7–9]. It is still unknown whether MMP9 can influence the biological behaviors of skin fibroblasts and affect wound healing. Skin fibroblasts play important roles in wound repairing. Anything that can affect their biological properties will ultimately affect wound healing. To investigate the mechanisms of MMP9 in diabetic foot wound healing, rat skin fibroblasts were cultured in high glucose and hyperhomocysteine medium [10–12]. Changes of biological behaviors of skin fibroblasts were observed before and after using the exogenous TIMP1.

2. Methods

2.1. Cell Culture and Grouping. Fibroblasts (CRL-1213, ATCC, USA) were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA) in a CO_2 incubator (37°C, 5% CO_2). After 24-hour serum deprivation (0.5% FBS), fibroblasts were cultured in DMEM containing 10% FBS at three different conditions (according to the grouping) for 6 hours in a CO_2 incubator (37°C, 5% CO_2).

Grouping: (1) Control group was cultured in DMEM containing normal glucose (5.5 mmol/L). (2) Model group was cultured in DMEM containing glucose (22.0 mmol/L) and homocysteine (100 μmol/L) [10–12]. (3) TIMP1 group was cultured in DMEM containing glucose (22.0 mmol/L), homocysteine (100 μmol/L), and TIMP1 (100 μg/L, R and D Systems, Minneapolis, USA).

2.2. Determination of MMP9 Levels. MMP9 mRNA expression was determined by real-time polymerase chain reaction (real-time-PCR) [13]. Total RNA was extracted by Trizol (Invitrogen, Carlsbad, CA, USA) from cells. To quantify MMP9 mRNA levels by real-time-PCR, GAPDH was used as the internal control. Primers were designed by Invitrogen Corporation (Carlsbad, CA, USA) according to the sequences of rat MMP9 and GAPDH in GeneBank. Rat MMP9 primers were sense, 5′-TCCAGTAGACAATCCTTG-CAATGTG-3′; anti-sense, 5′-CTCCGTGATTCGAGAACT-TCCAATA-3′. Rat GAPDH primers were sense, 5′-GGC-ACAGTCAAGGCTGAGAATG-3′; anti-sense, 5′-ATGGTG-GTGAAGACGCCAGTA-3′. Amplification was performed according to the instructions of the SYBR PrimeScript PCR Kit (Takara, Kyoto, Japan). Briefly, SYBR Premix Ex TaqTM (2×) 10 μL, PCR Forward Primer (10 μM) 0.4 μL, PCR Reverse Primer (10 μM) 0.4 μL, ROX Reference Dye (50×) 0.4 μL, and cDNA template 2.0 μL were added into a microfuge tube along with distilled water to make a total volume of 20.0 μL. The PCR reactions were performed in a LightCycler 480 real-time-PCR system (Roche, Basel, Switzerland), with denaturation at 95°C for 30 sec, then 40 cycles of 95°C for 5 sec followed by elongation at 60°C for 20 sec. In order to ensure equal sample sizes, the real-time-PCR for GADPH was performed at the same time. Melting curve analysis was conducted to ensure the specificity of amplification, the products were quantitated by the $2^{-\Delta\Delta CT}$ method, and the difference between the Ct values of MMP9 and the corresponding value of GADPH in each sample was used as the relative MMP9 Ct value.

MMP9 protein was measured by ELISA assay. At the end of cells culture, supernatants were collected. Double-antibody sandwich ABC-ELISA was used to detect protein expression of MMP9. Operation was performed according to the instructions of the rat MMP9 ELISA kit (Uscn Life Science Inc., Wuhan, China).

MMP9 protease activity in cell culture supernatants were assessed by gelatin zymography [14]. Equal aliquots of conditioned culture media from an equal number of cells were fractionated using precast zymogram gels containing gelatin, according to the manufacturer protocol (GuangDong Chemicalreagent Inc., Guangzhou, China). After electrophoresis, gels were incubated in renaturing buffer (50 mmol/L Tris-HCl (pH 7.4), 2% (vol/vol) Triton X-100) for 30 min at room temperature and then incubated in developing buffer (50 mmol/L Tris-HCl (pH 8.0), 2.5 mmol/L $CaCl_2$, 0.02% (wt/vol) Brij-35) for 72 h at 37°C. Lytic bands corresponding to the latent form of MMP9 (92 kDa) were analyzed as total activity and visualized by staining with 0.5% (wt/vol) Coomassie Brilliant Blue solution. The gels were photographed, followed by analysis using Image Quant 5.2 software.

2.3. Proliferation of Cells. Proliferation of cells was assessed by flow cytometry [15]. Cells were collected into the dedicated tube for flow cytometry and centrifuged for 6 min (2500 r/min), and supernatant was discarded. Cells were resuspended with 1 mL PBS. Then 300 μL of cell suspension was added into 700 μL ice-cold ethanol drop by drop, fixed overnight at 4°C in the dark. On the next day, suspension was centrifuged for 10 min (2500 r/min) and supernatant was discarded. Cells were resuspended again with 500 μL PBS containing RNase A (100 U/mL), incubated for 30 min at 37°C. Then ethidium bromide (2 mg/mL) was added to a final concentration of 50 μg/mL and incubated for 30 min in the dark. Cells cycle was detected by standard procedures of flow cytometry, and the S-phase cell ratio and proliferation index were calculated at the same time. S-phase cell ratio is S/(G0/G1 + S + G2/M); proliferation index is (S + G2/M)/(G0/G1 + S + G2/M).

2.4. Viability of Cells. Viability of cells was assessed by CCK-8 (Dojindo Laboratories, Kumamoto, Japan) [16]. Cells were inoculated to 96-well plates according to the density of 2.0-3.0 × 10^3 cells for each well. Premixed CCK-8 and medium (10 μL : 100 μL) were added into 96-well plates, and cells were then incubated for 0.5–1 h at 37°C. The values of A450 were obtained with the 3,550 automatic detector from Beckman (Brea, CA).

2.5. Collagen (Hydroxyproline) Secretion. Collagen (hydroxyproline) secretion was detected by ELISA. Hydroxyproline was measured according to the instructions of the rat hydroxyproline ELISA kit (Xinqidi Biological Technology Co., Wuhan, China). The concentration of hydroxyproline was calculated according to the A450 value obtained with the 3,550 automatic detector from Beckman (Brea, CA).

2.6. Horizontal Migration of Cells. Horizontal migration was assessed by scratch test [17]. Cells cultured in 6-well plates were scratched with a small tip along the ruler. Washing the scratch area was repeated with PBS until the cells in this area were removed thoroughly. Adding the purpose medium and culturing 6 h, we chose five different horizons under the inverted microscope and measured the distance between cells at 0 h and 6 h after scratching, taking the average for horizontal migration rate. Horizontal migration rate is (width at 0 h − width at 6 h)/width at 0 h × 100%.

2.7. Vertical Migration of Cells. Transwell was used to evaluate the vertical migration of cells [18]. Cells in the

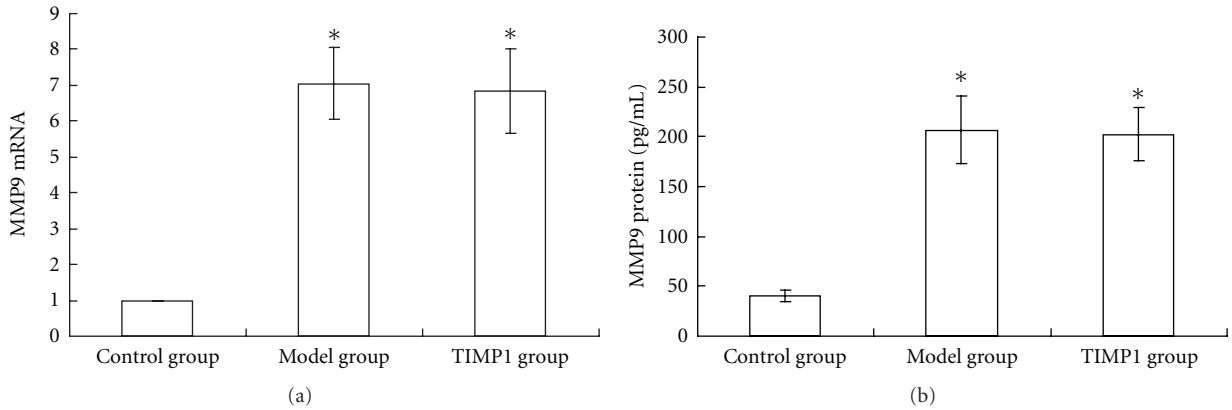

Figure 1: Establishment of the fibroblasts cell model of high matrix metalloproteinase 9 (MMP9) expression. The expressions of MMP9 mRNA, protein levels, and protease activity in model group were 6.05-, 4.12-, and 1.62-fold, respectively, greater than those in control group, which suggested that high expression of MMP9 was induced. When the cells were treated with tissue inhibitor of metalloproteinase 1 (TIMP1), the expression of MMP9 mRNA and protein had no statistical differences compared with model group. $^{*}P < 0.01$ versus control group. Data are mean ± SEM, $n = 5$.

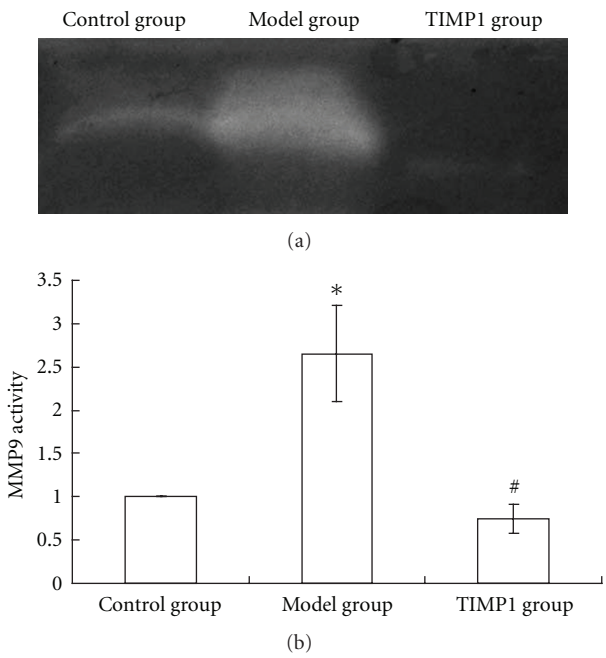

Figure 2: Matrix metalloproteinase 9 (MMP9) activity detected by Gelatin zymography. The activity of MMP9 in model group was 1.62-fold greater than that in control group. When the cells were treated with tissue inhibitor of metalloproteinase 1 (TIMP1), the activity of MMP9 decreased by 70.7% compared with model group. $^{*}P < 0.01$ versus control group; $^{\#}P < 0.01$ versus model group. Data are mean ± SEM, $n = 5$.

logarithmic growth phase were suspended by purpose medium containing 0.5% FBS after conventional digestion. 100 μL cell suspensions (1.0×10^5/mL) were added into the upper chamber, and 500 μL purpose medium (according to the grouping) containing 10% FBS was added into the lower chamber. After 16 h of culture, the upper chamber was removed, fixed for 30 min with 4% paraformaldehyde, and stained for 15 min with crystalline violet. We selected four visions randomly to count the number of cells moved to the lower of the membrane under inverted microscope, taking the average for the number of vertical migration cells.

2.8. Statistical Analysis. Data are presented as mean ± SEM. Comparisons were made by one-way ANOVA followed by Student-Newman-Keuls post hoc analysis. Data were analyzed with Microsoft Excel 2003 (Microsoft Inc., Seattle, WA, USA) and SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed using the average results of three repeated experiments under identical conditions. A P value <0.05 was considered statistically significant. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Establishment of the Fibroblast Cell Model of High MMP9 Expression. High glucose and high homocysteine induced MMP9 expression obviously. mRNA and protein expression and protease activity of MMP9 in model group were 6.05-, 4.12-, and 1.62-fold, respectively, higher than those in control group ($P < 0.01$). This suggested that the fibroblasts cell model of high MMP9 expression was created successfully. When the cells were treated with TIMP1 (100 μg/L), MMP9 protease activity decreased by 70.7% significantly ($P < 0.01$), while MMP9 mRNA and protein expression had no statistical differences compared with those in model group ($P > 0.05$) (Figures 1 and 2).

3.2. Inhibition of Fibroblasts Proliferation, Viability, and Collagen Secretion by High MMP9 Expression. Compared with those in control group, fibroblasts proliferation, viability, and collagen secretion in model group were inhibited with 29.8% of S-phase cell ratio, 18.1% of proliferation index, 23.3% of cell viability, and 68.7% of collagen secretion ($P < 0.01$,

Table 1: Proliferation, viability, and collagen secretion of fibroblasts.

Group	S-phase cell ratio (%)	Proliferation index (%)	CCK-8 OD value	Hydroxyproline (pg/mL)
Control group	9.31 ± 0.24	13.8 ± 0.5	1.76 ± 0.13	1126.4 ± 62.7
Model group	6.54 ± 0.29*	11.3 ± 0.6*	1.35 ± 0.12*	352.8 ± 60.6*
TIMP1 group	7.75 ± 0.27*#	12.5 ± 0.5*§	1.64 ± 0.14#	828.6 ± 58.9*#

TIMP1: tissue inhibitor of metalloproteinase 1; *$P < 0.01$ versus control group; §$P < 0.05$; #$P < 0.01$ versus model group. Data are mean ± SEM, $n = 5$.

resp.). After treatment with TIMP1, the inhibition reduced compared with those in model group ($P < 0.05$) (Table 1).

3.3. Inhibition of Fibroblasts Horizontal Migration by High MMP9 Expression. The horizontal migration rate of fibroblasts in model group (21.3 ± 2.1)% was lower than that in control group (38.7 ± 2.6)% with the inhibition rate being 45.0% ($P < 0.01$). After treatment with TIMP1, the horizontal migration rate (31.5±2.7)% increased compared with that in model group but failed to recover to the level of control group ($P < 0.01$, resp.) (Figure 3).

3.4. Inhibition of Fibroblasts Vertical Migration by High MMP9 Expression. Compared with control group (90.6 ± 3.8), the vertical migration of cells decreased by 21.4% in model group (71.2 ± 3.8) ($P < 0.01$). After treatment with TIMP1, the vertical migration of cells (85.3 ± 3.7) increased compared with that in model group but failed to recover to the level of control group ($P < 0.01$) (Figure 4).

4. Discussion

Fibroblasts are major repair cells in the skin, which account for 40% to 60% of total cells. The biological effects of fibroblasts play vital roles in wound healing [19]. Scholars have found that the DNA synthesis of skin fibroblasts significantly decreased, while apoptosis increased, in patients with diabetes, which indicates that the proliferation of fibroblasts was inhibited in the pathological statement of diabetes [20]. Studies in rats demonstrated similar results to those in humans [21]. However, whether the changes in biological behaviors of fibroblasts are related to high MMP9 expression in the diabetes is still unknown.

To avoid the complicating factors in vivo, high concentrations of glucose and homocysteine were used to mimic the local environment of diabetic skin [12]. Results of this study showed that MMP9 expressions of fibroblasts increased when cultured in high glucose and high homocysteine medium. The expressions of MMP9 mRNA, protein levels, and protease activity in high-glucose and high-homocysteine-treated group were 6.05-, 4.12-, and 1.62-fold, respectively, higher than those in control group, which suggested that high expression of MMP9 was induced.

At the same time, the S-phase cell ratio, proliferation index and cell viability in high-glucose and high-homocysteine-treated group decreased significantly compared with those in control group. After treatment by TIMP1, expression of MMP9 mRNA and protein levels had no statistical change, but activity of MMP9 protein was inhibited significantly, together with the increase of S-phase cell ratio, proliferation index, and cell viability. These results suggested that the inhibition of fibroblasts proliferation and viability were likely related with the high activity of MMP9.

Wound healing depends on the fibroblasts proliferation, migration, granulation tissue formation, collagen secretion, and collagen-based scar formation, which need the participation of many different cells [22]. As fibroblasts are the most important cells of collagen synthesis [23], barriers of proliferation and vitality inevitably have negative effects on new collagen synthesis and wound healing. The present study found that, compared with that in control group, the amount of hydroxyproline in model group decreased by 68.7%. After treatment by TIMP1, the hydroxyproline synthesis increased. The result suggested that in the diabetic status, not only did high protease activity of MMP9 increase the degradation of extracellular matrix [24] but also the collagen synthesis was decreased because of the decrease in the number and activity of fibroblasts. Thus the collagen metabolism was kept in negative balances and will ultimately delay the healing of diabetic wounds.

Migration is another biological property of fibroblasts [25]. It is generally accepted that there is a positive correlation between expression of MMP9 and migration behavior of cells [26, 27]. In our study, it is very interesting that both the horizontal and vertical migration abilities of fibroblasts were significantly decreased in model group. The possible mechanisms of this phenomenon may be as follows. Firstly, high expression of MMP9 may result in excessive matrix protein degradation, which is necessary to support the migration of fibroblasts. Our previous data showed that the expression level of MMP9 of the skin was significantly enhanced during wound healing in diabetic rats [4–6]. Reiss et al. [28] further demonstrated that exogenous MMP9 directly delayed wound healing in a mouse model. Secondly, increased activity of MMP9 is capable of cleaving nonmatrix proteins [29], which are essential to the migration of cells [30]. Kyriakides et al. [31] found that antibody-based blockade of MMP9 function or MMP9 deficiency retarded migration, and the rate of reepithelialization was significantly delayed in MMP9 knockout mice when compared with wide-type mice. In this study, the migration ability of fibroblasts in model group decreased, which will prevent fibroblasts from crawling to the site of wound in time to play their roles. Because the inhibition of fibroblasts proliferation, viability, and collagen synthesis, even if they migrate to the site of wound, they still cannot function normally—they cannot proliferate efficiently to make up adequate cells and they can not produce enough extracellular matrix and cell factors, so that the normal cycle of wound healing is broken, and this will cause the disorder of wound healing.

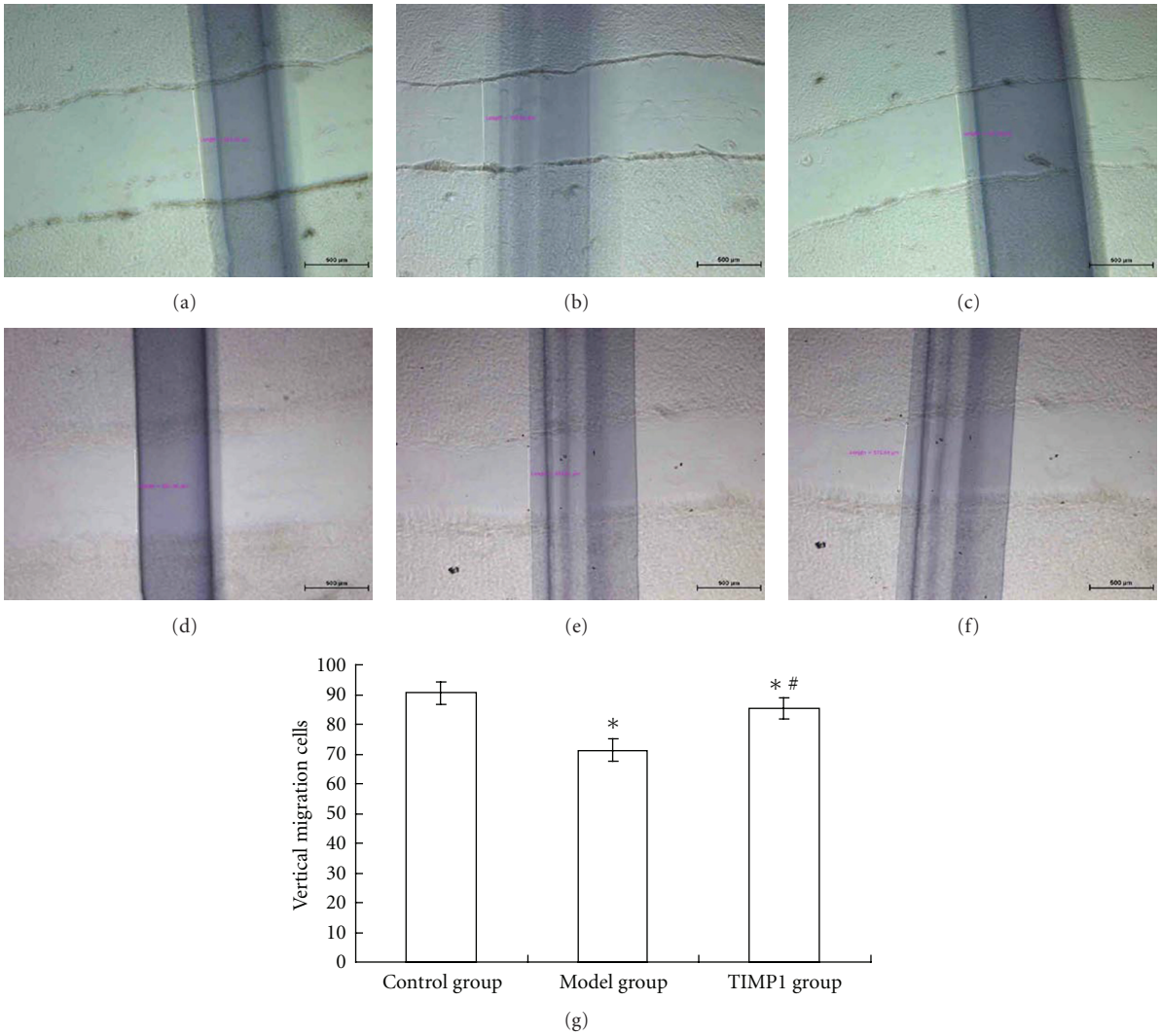

Figure 3: Scratch test of each group. (a) Control group 0 h (×40); (b) model group 0 h (×40); (c) tissue inhibitor of metalloproteinase 1 (TIMP1) group 0 h (×40); (d) control group 6 h (×40); (e) model group 6 h (×40); (f) TIMP1 group 6 h (×40); (g) histogram. The horizontal migration rate of fibroblasts in model group was lower than that in control group with the inhibition rate being 45.0%. After treatment with TIMP1, the horizontal migration rate increased compared with that in model group, but failed to recover to the level of control group. $^{*}P < 0.01$ versus control group; $^{\#}P < 0.01$ versus model group. Data are mean ± SEM, $n = 5$.

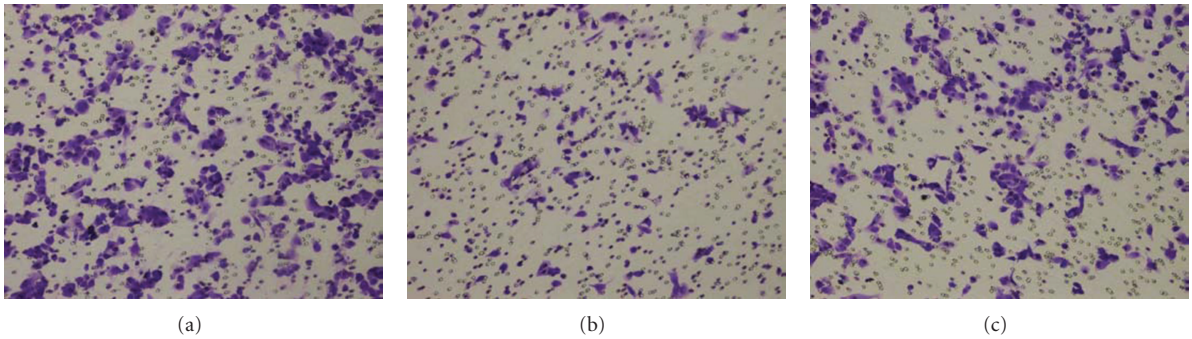

Figure 4: Vertical migration of fibroblasts. (a) Control group (×100); (b) model group (×100); (c) tissue inhibitor of metalloproteinase 1 (TIMP1) group (×100). Compared with control group, the vertical migration of cells decreased by 21.4% in model group. After treatment with TIMP1, the vertical migration of cells increased compared with that in model group but failed to recover to the level of control group.

5. Conclusion

In our previous studies, fibroblasts exhibited an upregulation of MMP9 as a result of high glucose and high homocysteine incubation [10, 11]. The present study further proved that the biological behaviors of rat dermal fibroblasts can be inhibited by high level of MMP9, and the inhibited effect can be reduced by TIMP1, which can inhibit the activity of MMP9. So we believe that inhibiting the activity of MMP9 may be a feasible way to accelerate the healing of diabetes skin ulcers. However, the delayed wound healing is a complicated procedure, and the potential involvement of other MMP and TIMP family members and related cytokines still needs further investigation.

Deficiencies: The present study is performed in vitro, and the results of this research remain to be further confirmed in future animal experiments.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

S.-N. Xue, J. Lei, C. Yang, and L. Yan designed and coordinated the experiments and prepared the paper. S.-N. Xue and D.-Z. Lin performed the research/study. J. Lei managed the literature searches and undertook the statistical analysis.

Acknowledgments

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Clinical Study

Inflammation, Diabetes, and Chronic Kidney Disease: Role of Aerobic Capacity

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The persistent inflammatory state is common in diabetes and chronic kidney disease (CKD). These patients present exercise intolerance and increased arterial stiffness. Long-term aerobic exercise has been associated with better arterial compliance, antidiabetic and antiinflammatory benefits. We assessed the hypothesis that in patients with diabetes and CKD, better aerobic capacity is associated with less inflammatory state and arterial stiffness. Thirty-nine CKD patients (17 in hemodialysis) were evaluated. According to CKD etiology two patient groups were obtained: group of diabetics (GD) was formed by 11 patients and nondiabetics (GND) formed by 28 patients. Central blood pressure and arterial stiffness were evaluated by Sphygmocor device. Carotida intima-media thickness (CA-IMT) was evaluated by ultrasonography. Aerobic capacity was measured by estimated VO_2max according to treadmill test by Bruce protocol. The GD showed a higher frequency of C-reactive protein above laboratory cutoff ($P = 0.044$), higher frequency of male gender, and a non significant higher value of VO_2max ($P = 0.099$). The CA-IMT was similar. Only better aerobic capacity was associated with lower frequency of high C-reactive protein when adjusted to diabetes and gender in a logistic regression model. In conclusion, aerobic capacity was associated with inflammatory state, in CKD patients, independently of diabetes presence.

1. Introduction

Chronic kidney disease (CKD), characterized by irreversible loss of renal function [1], is a major public health problem in the world. The prevalence of CKD increases with age and reaches around 17% of individuals over 60 years [2]. Cardiovascular (CV) disease is the leader cause of morbidity and mortality in these patients.

One of the major risk factors for development of CKD is diabetes, which causes, in addition to kidney damage, several cardiovascular comorbidities and visual and peripheral vascular complications. Persistent chronic microinflammatory state is very common in diabetic and CKD patients. It is associated with malnutrition and cardiovascular disease and is a potent predictor of mortality [3].

Increased arterial stiffness has been recognized as important predictors of CV diseases in patients with CKD and diabetes patients and is associated with chronic inflammatory state [4]. Like pulse wave velocity (PWV), central blood pressure (CBP), and augmentation index (AIx), carotid intima-media wall thickness (CA-IMT) is also a predictor of CV mortality in elderly patients with ischemic heart disease, hypertension, diabetes, and CKD [5].

CKD patients usually manifest symptoms of exercise intolerance such as muscle weakness and fatigue and are less active, exhibiting muscle atrophy even when compared

to inactive normal subjects [6]. CKD patients substantially improve their strength, power, and muscle endurance, evaluated by exercise capacity and physical function tests after training [7]. Others have reported that training improve quality of life, maximum oxygen uptake (VO_2max), muscle gain in mass and capillary density, velocity of nerve conduction, and blood pressure (BP) [8–11].

Recent evidence had shown that physical activity can ameliorate inflammatory state in CKD [12]. Two interventional studies showed a reduction of inflammatory markers with aerobic training [13, 14]. Another two studies, one interventional [15] and one transversal [16] have not demonstrated any effect in inflammatory state. Thus, the benefits of physical activity and its importance were recognized in diabetic patients from the general population, and physical training is fully recommended [17]. However, the effectiveness of physical activity in the prevention or improvement of inflammatory state in diabetic CKD patients had not yet been proved [15].

Therefore, the aim of this study is to evaluate the association between aerobic capacity and presence of diabetes, inflammation, arterial stiffness, and CA-IMT in chronic kidney disease patients.

2. Materials and Methods

Were included hemodialysis patients for at least six months or CKD predialysis patients. All subjects were aged more than 18 years. Subjects unable to perform exercise testing, with coronary heart disease or previous coronary artery bypass, active infection, cancer, or liver cirrhosis were excluded. Informed consent was obtained from each patient. This study was approved by Research Ethics Committee of the Botucatu Medical School with the protocol 3083/2009.

Clinical data evaluated were age, gender, body mass index, and kidney disease etiology. The following laboratorial data was evaluated: hemoglobin, calcium, phosphorus, parathyroid hormone, albumin, iron, ferritin, transferrin saturation, C-reactive protein, creatinine, and creatinine clearance. All laboratorial evaluations were performed in the morning while fasting. The hemodialysis patients were evaluated in the morning while fasting in the interdialytic day.

The C-reactive protein was evaluated by dry chemistry (VITROS kit, Ortho-Clinical Diagnostics, Rochester, NY, USA), and test sensitivity was 0.1 mg/dL. Elevated CRP was defined when a patient had values superior to 1 mg/dL, as defined by superior limit of our laboratory.

Patients who met the inclusion criteria underwent Sphygmocor CPV (AtCor Medical, Australia) evaluation for CBP, PWV, and AIx. The measurements were performed in supine position. The BP stabilization was considered when the difference between three consecutive measurements in five-minute interval was not greater than 5 mm Hg. Average and maximum of left and right CA-IMT by ultrasonography according to Mannheim carotid intima-media consensus [18] were evaluated.

For the estimation of VO_2max, the patients were submitted to a treadmill test (Bruce protocol [19]), and the appropriate formula for nonathletes was used to calculate this variable as follows: VO_2max = (time $\times$ 3.29) + 4.07 for men, and VO_2max= (time $\times$ 3.36) + 1.06 for women. All evaluations were performed on the same day in the same sequence. Hemodialysis patients underwent their examinations on interdialytic days.

According to the kidney disease etiology, two patient groups were obtained. Group D (GD) formed by diabetic patients and group ND (GND) formed by nondiabetic patients. The groups were compared regarding age, body mass index, and laboratorial data, systolic, diastolic and pulse CBP, PWV, Aix, and CA-IMT.

For comparison between groups, *t*-test or Mann-Whitney was used when appropriate. Multiple logistic regressions were performed to identify variables independently associated with inflammation and selected variables with statistical probability of <0.1 between groups. Values were expressed as mean $\pm$ standard deviation to parametric variables or median (first–third quartile) when appropriate. A $P < 0.05$ was considered statistically significant.

3. Results

There were included 39 patients (17 in hemodialysis). Clinical and laboratory characteristics are shown in Table 1. The GD was formed by 11 diabetic patients and the GND by 28 nondiabetic patients, there were 13 (46%) patients in hemodialysis in GND and four (36%) in GD ($P = 0.725$). Other clinical and laboratorial variables of the groups are shown in Tables 2 and 3, respectively. The number of male patients was higher in GD compared to GND, with $P = 0.011$.

Diabetic group had a CRP value of 1.50 (0.32–2.72) mg/dL and GND 0.5 (0.20–0.75) mg/dL. When patients were divided according to the inflammatory status (CRP > 1 mg/dL or CRP $\leq$ 1 mg/dL) a significantly greater number of chronic inflammatory state patients was found in GD ($P = 0.044$; Table 3). The values of creatinine, creatinine clearance, hemoglobin, calcium, phosphorus, parathyroid hormone, albumin, ferritin, and transferrin saturation were not different between groups (Table 3).

Was observed a trend of higher VO_2max values in the patients without diabetes compared to diabetic patients (27.9 $\pm$ 8.09 mL/kg/min in GND and 23.3 $\pm$ 7.08 mL/kg/min in GD, $P = 0.099$; Table 2). Reanalyzing VO_2max by gender, that is, excluding female sex from analysis, we have VO_2max of 30.9 $\pm$ 8.58 mL/kg/min in nondiabetic group ($n = 9$ nondiabetic men) and 23.5 $\pm$ 7.44 mL/kg/min ($P = 0.042$) in diabetic group ($n = 13$ diabetic men), so, reinforcing the difference in VO_2max between diabetics and nondiabetics. Thickness of the intimae-media right carotid artery showed a trend of higher values in diabetic patients (0.9 $\pm$ 0.29 mm in GD and 0.76 $\pm$ 0.216, $P = 0.081$), other indexes of carotid thickness were similar between groups. Comparing the values of arterial stiffness indexes, GD had similar values of GND.

In multiple logistic regression, with CRP as dependent variable (Table 4), the main determinant of C-reactive

Table 1: Clinical variables and laboratorial characteristics of patients.

Patients	39
Hemodialysis	17
Predialysis	22
Age (years)	54.9 ± 14.16
Kidney disease etiology	
Hypertension	16
Diabetes	11
Glomerulopathies	7
Others	5
BMI (Kg/m^2)	26.5 ± 5.25
Central SBP (mm Hg)	122 ± 19.3
Central DBP (mm Hg)	83 ± 12.8
Central PP (mm Hg)	39 ± 13.4
Pulse wave velocity (m/s)	8.3 ± 1.26
Augmentation index (%)	26.3 ± 14.02
VO_2max (mL/kg/min)	26.6 ± 8.02
CA-IMT L—m (mm)	0.79 ± 0.194
CA-IMT L—mx (mm)	0.96 ± 0.234
CA-IMT R—m (mm)	0.81 ± 0.247
CA-IMT R—mx (mm)	0.97 ± 0.273
Presence of atherosclerotic plaque (%)	41%
Creatinine (mg/dL)	6.0 ± 4.29
Clearance of creatinine (mL/min)	32 ± 20.4
Hemoglobin (g/dL)	12 ± 1.7
Calcium (mg/dL)	9.3 ± 0.79
Phosphorus (mg/dL)	4.8 ± 1.48
CRP (mg/dL)	0.9 ± 1.06
PTH (pg/mL)	493 ± 553.3
Albumin (g/dL)	4 ± 0.45
Ferritin (g/dL)	635 ± 568.1
Transferrin saturation (%)	33.5 ± 15.77

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; PWV: pulse wave velocity; AIx: augmentation index; CA-IMT L—m: mean of left carotid intima-media thickness; CA-IMT L—mx: maximal of left carotid intima-media thickness; CA-IMT R—m: mean of right carotid intima-media thickness; CA-IMT R—mx: maximal of right carotid intima-media thickness; CRP: C-reactive protein; PTH: parathyroid hormone.

protein was VO_2max ($P = 0.024$). Gender and diabetes were not independently associated with inflammatory state ($P = 0.210$ and $P = 0.107$, resp.). Figure 1 shows the negative correlation between C-reactive protein and VO_2max, in all patients, where the higher the aerobic capacity, the lower their inflammatory state ($R = -0.514$, $P < 0.001$).

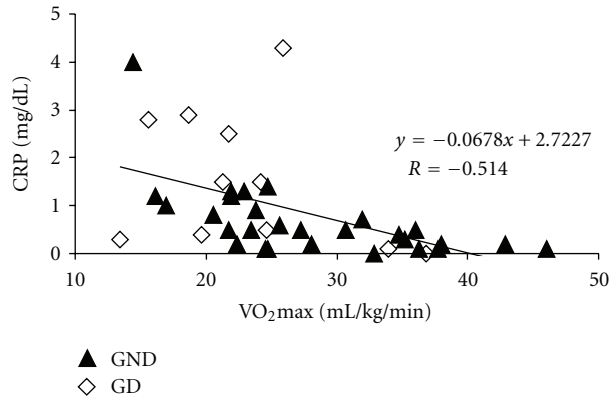

Figure 1: Correlation between C-reactive protein and VO_2max. GND: nondiabetic group; GD: diabetic group.

4. Discussion

The C-reactive protein is a sensitive marker of inflammation, and an increase in its levels has been associated with higher risk of vascular disease [20]. Diabetic CKD patients' have high concentration of CRP, and improvement of aerobic capacity could affect that variable. The main result of this work is that CRP was not independently associated with diabetes when we take into account the VO_2 level and that the VO_2 level had a strong association with CRP independently of diabetes. We can speculate that it is possible that the inflammatory state of chronic kidney disease diabetic patients would be caused, in grand part or at least in part, by physical deconditioning. This speculative possibility must be confirmed in other studies with a great number of individuals and especially in longitudinal and interventional studies.

The long-term exercise training increases the nitric oxide availability and diminishes the oxidative stress, inflammation and blood pressure, which improves the endothelial function and consequently cardiovascular mortality [21–23]. Patients with coronary artery disease submitted to adequate chronic training with increase in the VO_2max have lower levels of CRP compared with basal values [24]. Nitric oxide, a potent anti-inflammatory molecule, plays a role in this mechanism [24].

In the elderly, training also decreased the concentration of CRP [20, 25]. These data are consistent with our study, since individuals with higher aerobic capacity also had a lower inflammatory state. In diabetes, some recent studies have demonstrated the role of physical training in reducing CRP [26–29]. Experimental studies have also demonstrated the benefits of physical activity in reducing inflammatory markers and oxidative stress in animals with CKD [30, 31].

According to the American College of Sports Medicine and American Heart Association, to reduce risk of cardiovascular events, individuals with chronic illnesses should perform moderate-intensity physical activity, 30 min, 5 times weekly [32].

Physical capacity proved to be an important predictor of survival. In a study that evaluated patients with CKD and followed them over three years, patients with a VO_2max superior to 17.5 mL/kg/min had a higher survival compared to individuals with lower peak VO_2 [33]. The mean of VO_2max of the current study was 26.6 ± 8.02 mL/kg/min.

Although the benefits of physical training have been demonstrated in CKD patients [11, 34–36], the results are

Table 2: Clinical Variables between groups.

	Diabetics ($n = 11$)	Nondiabetics ($n = 28$)	P
Age (years)	60 ± 13.7	53 ± 13.9	0.126
Gender: male/female	10 M/1 F	13 M/15 F	0.011
BMI (Kg/m^2)	28 ± 3.4	26 ± 5.8	0.298
Central SBP (mm Hg)	117 ± 19.5	124 ± 19.3	0.344
Central DBP (mm Hg)	74 ± 10.4	87 ± 12.1	0.005
Central PP (mm Hg)	43 ± 15.1	37 ± 12.5	0.205
PWV (m/seg)	8.3 ± 1.36	8.3 ± 1.24	0.884
AIx (%)	21 ± 14.1	28 ± 13.6	0.124
VO_2max (mL/kg/min)	23.3 ± 7.08	27.9 ± 8.09	0.099
CA-IMT L—m (mm)	0.8 ± 0.17	0.78 ± 0.202	0.328
CA-IMT L—mx (mm)	1.0 ± 0.20	0.94 ± 0.248	0.459
CA-IMT R—m (mm)	0.9 ± 0.29	0.76 ± 0.216	0.081
CA-IMT R—mx (mm)	1.1 ± 0.28	0.92 ± 0.258	0.112
Presence of atherosclerotic plaque (%)	54.5	35.7	0.383

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; PP: pulse pressure; PWV: pulse wave velocity; AIx: augmentation index; CA-IMT L—m: mean of left carotid intima-media thickness; CA-IMT L—mx: maximal of left carotid intima-media thickness; CA-IMT R—m: mean of right carotid intima-media thickness; CA-IMT R—mx: maximal of right carotid intima-media thickness.

Table 3: Laboratorial data.

	Diabetics ($n = 11$)	Nondiabetics ($n = 28$)	P
Creatinine (mg/dL)	5.8 ± 4.14	6.1 ± 4.42	0.863
Creatinine clearance (mL/min)	23 ± 9.6	36 ± 22.8	0.206
Hemoglobin (g/dL)	11.4 ± 1.51	12.3 ± 1.71	0.159
Calcium (mg/dL)	8.9 ± 0.69	9.4 ± 0.80	0.109
Phosphorus (mg/dL)	4.9 ± 1.40	4.7 ± 1.52	0.633
CRP (mg/dL)	1.50 (0.32–2.72)	0.5 (0.20–0.75)	0.021
CRP > 1.0 mg/dL	6	6	0.044
CRP ≤ 1.0 mg/dL	5	22	
PTH (pg/mL)	454 ± 473.2	509 ± 591.9	0,787
Albumin (g/dL)	3.9 ± 0.46	4.0 ± 0.44	0.465
Ferritin (g/dL)	740 ± 792.2	571 ± 388.7	0.448
Transferrin saturation (%)	40 ± 13.4	30 ± 16.23	0.142

CRP: C-reactive protein; PTH: parathyroid hormone.

Table 4: Multiple linear regression: C-reactive protein as dependent variable.

	P	RR	95.0% C.I.	
			Lower	Upper
Gender	0.210	0.221	0.021	2.335
Diabetes	0.107	7.487	0.646	86.803
VO_2max (mL/Kg/min)	0.024	0.827	0.701	0.976

controversially related to inflammatory markers, and few studies have demonstrated the effects of a physical activity program on CRP in these patients. A recent pilot study of obese diabetic CKD patients did not show any change in CRP after a period of aerobic training [15]. In the current study, a multiple logistic regression identified VO_2max as a principal determinant of C-reactive protein. Our results were in accordance with previous studies [37–39] and reinforce the possibility that diabetic CKD patients would be benefited engaging in a physical activity program.

In the present study, measures of intima-media thickness of carotid arteries and arterial stiffness did not correlate with VO_2max. So, better physical aerobic capacity was associated only with the metabolic marker of inflammation and not with structural or functional properties of arteries. However, these data do not exclude that long-term assisted physical

training could have an impact on those parameters not observed in this transversal study.

In the current study, some limitations must be recognized. First, a small number of subjects was evaluated, nevertheless this number was sufficient to detect statistically significant correlations. This is a transversal study, so it submits to limitations inherent to this design and must be confirmed in a longitudinal and interventional study. However, we evaluated the confounding variables and took into account those in multiple analysis. Finally, the VO_2max was indirectly measured.

In conclusion, better VO_2max was associated with lower frequency of elevated CRP levels in subjects with chronic kidney disease. The current study strengthens previous findings and reinforces the hypothesis of cardiovascular benefits with a better aerobic capacity in CKD diabetic patients.

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Review Article

The Influence of Autonomic Dysfunction Associated with Aging and Type 2 Diabetes on Daily Life Activities

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Type 2 diabetes (T2D) and ageing have well documented effects on every organ in the body. In T2D the autonomic nervous system is impaired due to damage to neurons, sensory receptors, synapses and the blood vessels. This paper will concentrate on how autonomic impairment alters normal daily activities. Impairments include the response of the blood vessels to heat, sweating, heat transfer, whole body heating, orthostatic intolerance, balance, and gait. Because diabetes is more prevalent in older individuals, the effects of ageing will be examined. Beginning with endothelial dysfunction, blood vessels have impairment in their ability to vasodilate. With this and synaptic damage, the autonomic nervous system cannot compensate for effectors such as pressure on and heating of the skin. This and reduced ability of the heart to respond to stress, reduces autonomic orthostatic compensation. Diminished sweating causes the skin and core temperature to be high during whole body heating. Impaired orthostatic tolerance, impaired vision and vestibular sensing, causes poor balance and impaired gait. Overall, people with T2D must be made aware and counseled relative to the potential consequence of these impairments.

1. Introduction

There is a natural senescence of the nervous system with aging [1, 2]. This has a direct impact on autonomic function that has been observed in a number of different ways. For example, there is a reduction in endothelial cell function due to impairment in the release of vasodilator substances such as nitric oxide and prostacyclin [3–5]. This has the overall effect of reducing blood flow to all tissues in the body such as skin [6, 7], synapses [8], and neurons [9, 10]. Impaired circulation and high levels of free radicals found in older people and people with diabetes [11–13] and glycosylation end products in the cells [14] cause reduced synaptic activity [8], neuronal death [3], and decreased baroreceptor response with age but little change in baroreceptor sensitivity [15]. When coupled with hardening of the arteries, this accounts for an increase in blood pressure, reduced orthostatic tolerance, and a general sluggishness in the autonomic nervous system to respond to environmental stressors associated with aging [15].

Type 2 diabetes exacerbates the normal senescence of the cardiovascular system with aging [16, 17]. For example, when looking at the response to vascular occlusion, in relation to age, with greater age there is a reduction in the response of the circulation to vascular occlusion [18–20]. For individuals with diabetes, the reduction in blood flow with each year of age is the same but at a much lower baseline level. Thus, in many ways, the effect of aging and diabetes ar synergistic in reducing cardiovascular function with aging. However, there are also differences which will be discussed below. This paper will illustrate how autonomic dysfunction associated with aging and diabetes affects activities of daily life. The topics that will be reviewed are the response to local pressure, contrast baths, global heating, local heating, exercise, orthostatic tolerance, balance, and gait and how these are influenced by diabetes. All of these activities are common activities accomplished by individuals in their normal daily lives.

2. The Vascular Endothelial Cell and Age and Diabetes

At its simplest level, ageing and diabetes both cause damage to vascular endothelial cells in the body. Since blood vessels

provide nutrients for neurons, synapses, and other tissues in the body, endothelial dysfunction has a major impact on the autonomic nervous system [21–24]. A major complicating factor is free radicals which also damage blood flow to tissues and increase both with ageing and diabetes [25]. Thus it is appropriate to start any discussion of autonomic damage and its effects on the body with a discussion of endothelial dysfunction.

In early stages of diabetes, the insulin receptor becomes defective on cells [26]. The defect is believed to be in the transduction after the binding of insulin to the activation of Phosphatidylinositol 3-kinase (PI3K) [27]. This signaling pathway is responsible for mediating the effect of insulin on the cell. In the vascular endothelial cell there are 2 competing pathways, both activated by insulin binding. The predominant pathway is the PI3K pathway [28]. This pathway activates the enzyme endothelial nitric oxide synthetase (ENOS). ENOS catalyzes the conversion of the amino acid L-Arginine to L-Citrulline producing, as a byproduct, nitric oxide [29]. Nitric oxide, in itself, is a free radical and causes relaxation in vascular smooth muscle. This causes an increase in blood flow in the skin and other organs as needed for metabolism, or as commonly seen in the skin, increasing heat loss from the body [30, 31].

In diabetes, the damage to the PI3K pathway is believed to be due to elevated blood glucose concentrations above 120 mg/dL [26, 32, 33]. Evidence shows that spikes in glucose during a given 24-hour period also cause significant damage to the insulin transduction mechanism in the cells and may be more important than the average blood glucose [34, 35]. The exact relationship between these variations in blood glucose and endothelial dysfunction has not been clearly shown [32, 36–39]. Tissue studies have shown that, if 2 individual populations of cells are exposed to glucose, where the average glucose is the same but in one set of cells glucose fluctuates from highs to lows, there is more death of cells than in the population where glucose is just maintained high [35, 40–42]. Further, oxidative stress is much higher in the population subjected to spikes [42].

This mechanism itself reduces the transport of glucose into the cells under the control of insulin. Thus, cells shift to metabolism of free fatty acids to provide energy from metabolism for the cell. As free fatty acid metabolism increases, so do free radicals and the release of inflammatory cytokines [43, 44]. These cytokines and free radicals damage the cell even further by both increasing insulin resistance through glycosylation end products, damaging the insulin receptor even further, and reducing the bioavailability of nitric oxide as a mediator of vasodilation [45]. High concentrations of free radicals in the cell have been shown to oxidize nitric oxide released from endothelial cells into a superoxide, peroxynitrite. This superoxide has no biological effect on vascular smooth muscle [37, 46, 47]. Further, in addition to nitric oxide eliciting vasodilation, a second vasodilator pathway in blood vessels, prostacyclin (prostaglandin I_2) is also damaged by free radicals [48]. The reduced vasodilators in tissues cause vasoconstriction of the blood vessels to tissue, making the cells anoxic and releasing more free radicals [48]. This cyclic process accelerates and increases damage to cells over time until severe damage to the endothelial cells is seen.

A second pathway contributes to vasoconstriction well. As cited above, the main effect of insulin binding to the cells is activation of the PI3K pathway [49]. This pathway catalyzes the release of nitric oxide from cells so that as more glucose metabolism can take place, blood vessel dilation increases oxygen delivery and removes carbon dioxide from the increased metabolism in the cell. But, a competing pathway is also seen in the cell. Normally a minor pathway, the mitogen-activated protein kinase (MAPK) signaling pathway is activated in diabetes [50]. Whereas insulin causes vasodilation due to the PI3K pathway, it causes vasoconstriction in the MAPK pathway. The impairment in the PI3K pathways in diabetes shifts the effect of insulin, rather than causing vasodilation of blood vessels, to vasoconstriction, making the cell even more anoxic [50].

In addition to the increased vasoconstriction and increased free radicals in the cells associated with damage to the PI3K pathway and activation of the MAPK pathway, there are morphological changes in the endothelial cell. Normally, vascular endothelial cells have electrotonic connections to the surrounding vascular smooth muscles [46]. When endothelial cells increase their potassium permeability they hyperpolarize. As a result, these electrotonic connections to smooth muscle contribute to the relaxation of vascular smooth muscle [51, 52]. This electrical connection aids in the hyperpolarization and relaxation of vascular smooth muscle to increase vasodilation. However, in Type 2 diabetes, these electrical connections are impaired [17, 51, 53]. Studies on rat retinal preparations of these gap junctions show that the principal gap junction protein, connexin 43 but not 37 and 40 is downregulated by incubation of these cells with high glucose media [54]. This may or may not pertain to vascular endothelial cells in the skin, but poses an interesting possibility.

Partially through glycosylation end products, and partly through impaired circulation (enhanced vasoconstriction), there is damage to the sympathetic nervous system that leads to a reduced blood flow response to stressors in organs such as the skin. Autonomic damage occurs to the sympathetic ganglia and neurons even at the time of the clinical diagnosis of diabetes [18, 37, 55–60]. A common clinical measure of autonomic nervous system impairment is heart rate variability with the subject at rest. Normally, vasomotor rhythm in the sympathetic and parasympathetic systems causes the heart rate to vary continuously at rest [6, 37, 61]. These variations in heart rate can be seen by a frequency analysis of the EKG. As diabetes progresses, heart rate variability is reduced such that finally, sympathetic damage and parasympathetic damage have occurred to the extent that there is very little variation in heart rate with normal respiration or even a change in body position [37, 57, 61]. In addition, damage to tactile sensory nerves as well as to autonomic nerves in the skin contributes even more to the reduction in function seen in the autonomic nervous system [62].

3. The Effect on Local Pressure

The predominance of vasoconstrictors over vasodilators released from vascular endothelial cells in people with diabetes and older individuals causes skin blood flow to be lower at rest in people with diabetes than in age-matched controls compared to younger people [29]. Numerous studies have shown resting skin blood flow to be as little as one third (1/3) that of age-matched controls [56, 63–66]. Making matters worse, various protective mechanisms in the body at the level of the vascular endothelial cell are also damaged in diabetes. One of these is the response to local pressure. When standing on the feet, the pressure on the skin tends to impair the circulation. To prevent this, skin vertical pressure receptors are involved in eliciting vasodilation of the skin. When light pressure (up to 4 Kpa) is applied to the skin, there is an increase in skin blood flow [67–70]. When pressures as high as 22 Kpa are applied to the skin, blood flow initially increases as pressure increases and then eventually by pressures of 22 Kpa the blood flow is occluded [67, 70]. For a normal weight person standing on their feet, the pressure on the feet is approximately 15 Kpa [42]. Thus, for the average person, skin blood flow increases as they stand increasing perfusion and protecting the skin of the feet from damage. The mechanism is largely related to nitric oxide release. It is not surprising, then, with impairment in nitric oxide pathways due to free radicals and damaged endothelial nitric oxide synthetase, that the pressure response is also reduced. In people with diabetes, pressures of only 4 Kpa occlude most blood flow, and pressures of only 2 Kpa cause a mild decrease in skin blood flow. Thus, by the time pressure reaches 7 Kpa, blood flow is totally occluded [71, 72]. Thus, for the average individual of normal weight, what this means is that when standing, skin blood flow is occluded. For someone with diabetes, due to the usual higher incidence of obesity, pressures are even higher and can range to over 30 Kpa on the skin of the feet and blood flow is absent from the skin during standing. This occlusion of the circulation during quite standing as well as during movement adds to possible circulatory damage, and, when lesions occur to the skin, healing is severely impaired [73].

An additional mechanism is that when pressure is removed from the skin, there is a large reactive hyperemia that washes out metabolites and restores oxygen supply [74]. This reactive hyperemia is not present in people with diabetes. Even when the pressure is removed recovery from the anoxia takes a much longer period of time [74, 75].

4. Response to Occlusion

Occlusion of the circulation occurs during normal daily activities. For example, at night, rolling on an arm or leg often results in occlusion of the circulation. This also occurs when changing body positions while sitting in a chair and leaning at various angles, or even, to some extent during exercise. Isometric exercise for example (to be discussed later) results in occlusion of the circulation in muscle during the exercise. Normally, as shown in Figure 1, the response to occlusion is, depending on the length of the occlusion, a hyperemia after the blood flow is restored. The magnitude of the hyperemia in younger individuals can be very pronounced.

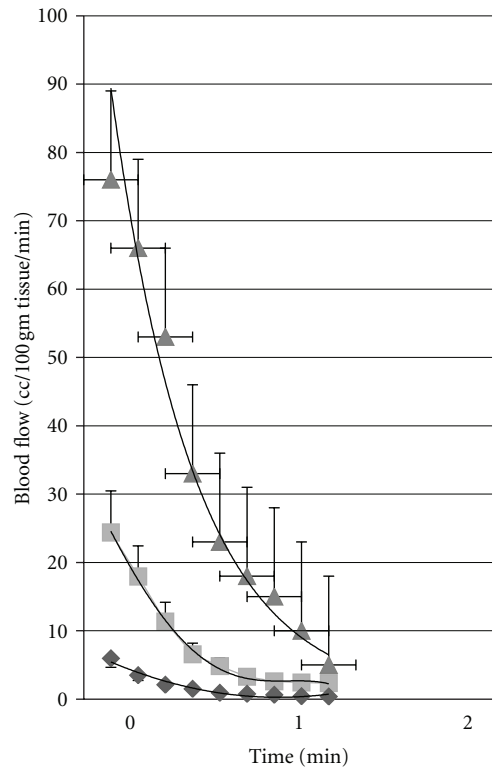

Figure 1: This figure shows the blood flow recorded for 2 minutes following the release of an arterial occlusion cuff on the brachial artery in young controls (triangle), nondiabetic age-matched controls (squares), and subjects with type 2 diabetes (diamonds). Illustrated here are the average results for 15 subjects in each group ± standard deviation. Blood flows are expressed in cc/100 mL muscle per minute and the time scale on the bottom is in minutes. Blood flows are recorded every 12 seconds starting at 3 seconds after occlusion (from [56]).

For example, for whole arm blood flows measured by volume plethysmography, resting blood flow normally averages 3 or 4 cc's per 100 grams tissue per minute. If occlusion is maintained for 4 minutes (a standard measure of vascular endothelial function used clinically) and then the occlusion is released, blood flow in a younger individual can increase to well over 75 cc's per 100 grams tissue per minute in the first few seconds, and then after 2 minutes return back to normal [65, 76]. This exponential decrease in blood flow, as shown in Figure 1, is characteristic of younger individuals and, to a larger extent, older individuals. During the period that blood flow remains high, metabolites are washed from tissue and oxygen is rapidly restored protecting the tissue from damage and preparing it to return to normal activity [19, 26]. In older individuals, as also shown in Figure 1, the magnitude of the reactive hyperemia is only 25 cc's per 100 grams tissue per minute. The hyperemia then, as was the case for younger individuals, returns within a few minutes back towards the normal resting blood flow. However, for

people with diabetes, as shown in this figure, blood flow is barely above rest after the occlusion is removed even after 4 minutes of occlusion. After 2 minutes after occlusion, blood flow is restored once again to a level at about 25 or 30% that of the normal age-matched controlled individuals. These differences between the 3 groups of subjects was significant at all times from rest to 2 minutes postocclusive hyperemia (ANOVA $P < 0.05$). Recent evidence shows that nitric oxide is only a minor contributor to the mechanism for postocclusive hyperemia [77]. Thus, the reason it is impaired with ageing and diabetes is also only poorly understood. However, the clear effect of aging and diabetes can easily be seen in Figure 2. As shown in Figure 2, if the area under the entire 2 minute postocclusive curve in Figure 1 is calculated as a single number (excess blood flow needed after occlusion) and is plotted on a graph in relation to age, the top line in the figure shows the reduction in postocclusive hyperemia associated with the aging process. The second line on the figure, the squares, shows an equivalent line for people with diabetes starting at age 20 to age 80. Over this age range, while the line is parallel to that of age-matched controls, it is at several levels of magnitude lower showing the additional damage to occlusion caused in people with diabetes. However, if shear response is related to post-occlusive hyperemia, it maybe mediated by a prostaglan-din-mediated mechanism.

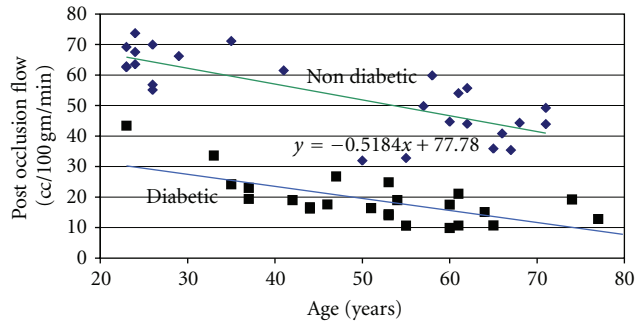

Figure 2: This figure illustrates the excess blood flow above rest during a two-minute period after the release of an occlusion cuff on the brachial artery of age-matched control subjects and subjects with diabetes. Individual data points are shown for control subjects (diamonds) and subjects with diabetes. For the control subjects the regression line was Blood Flow = −0.518 age + 77.78. For the subjects with diabetes the regression equation was Blood Flow = −0.253 age + 31.01 [65].

5. Local Heat

The reaction of tissue to an increase in tissue temperature, like the reaction to skin pressure, is mediated by the vascular endothelial cell [26, 29, 30]. The blood flow increase associated with local heat is a biphasic response. There are 2 pathways involved. When heat above 42°C is applied to the skin, it immediately (phase 1) responds with a rapid increase in circulation. This increase in circulation is mediated by skin sensory nerves. The sensory nerves release substance-P and Calcitonin-gene-related peptide (CGRP) [78–82]. These substances diffuse laterally from the sensory nerves causing an increase in circulation by relaxing vascular smooth muscle around blood vessels. This protects the skin from rapid changes in temperature that might cause damage [30, 83]. In skin tactile sensory nerves, TRPV1 voltage-gated calcium channels are responsible for releasing these substances [84]. However, this is short lived. Skin sensory receptors accommodate and soon lose their ability to sustain vasodilation [50]. As they do, the skin vascular endothelial cells begin to release nitric oxide. Endothelial nitric oxide synthetase has a calcium-binding domain [29, 33, 35, 85]. Intracellular calcium, released by temperature sensitive ion channels in the cell membrane (TRPV4), activates the enzyme ENOS and thus elicits the production of nitric oxide in vascular endothelial cells [26, 86, 87]. In people with diabetes, as cited above, resting blood flow is much lower than in age-matched controls. Also, with the nitric oxide pathway being damaged through oxidation of nitric oxide [88] or lack of production of nitric oxide, or in some cases under bioavailability of L-Arginine as the precursor to nitric oxide in people with diabetes, the skin blood flow response is greatly diminished with heat [89–91]. Associated with diabetes are elevated levels of asymmetrical dimethylarginine (ADMA). This competes with l-arginine for a binding site on ENOS reducing production of nitric oxide form vascular endothelial cells [92]. Arginine supplements can compete with ADMA reducing endothelial dysfunction [92]. The impairment in skin blood flow with local heat in people with diabetes can be seen in Figure 3.

In Figure 3, when the skin blood flow response to heat at 42°C is examined, the blood flow response in people with diabetes is substantially lower than age-matched controls [93, 94].

This occurs in both the first and second phase of the blood flow response to heat. Even more interesting is the calories transferred through the skin. When the relationship between skin blood flow and heat gained by the skin in young, older, and subjects with type 2 diabetes is examined, it can be clearly seen that to warm the skin to the same temperature, people with diabetes take a fraction of the calories to warm the skin as is seen in either young subjects or age-matched controls [63, 64, 89, 96]. One of the contributors to this is the fact that skin structure is also different in people with diabetes [97]. Thinner skin and more subcutaneous insulation impair the conductive heat loss through the skin to an applied thermal load. Skin thickness varies in different parts of the body. The thermal coefficient of the skin is much lower than that seen in age-matched controls without diabetes [97, 98]. The skin temperature rises faster in people with diabetes when a constant heat source is applied due to circulatory impairment. This, in turn, allows the skin to overheat. It is not surprising then that in Figure 4, it takes fewer calories in older people and people with diabetes to heat the skin.

The overall effect of this is that older people and people with diabetes are more susceptible to burns. Since the principal means of removing heat from the skin is the circulation when a warm heat source is applied [97], it is no surprise that people with diabetes are more susceptible to

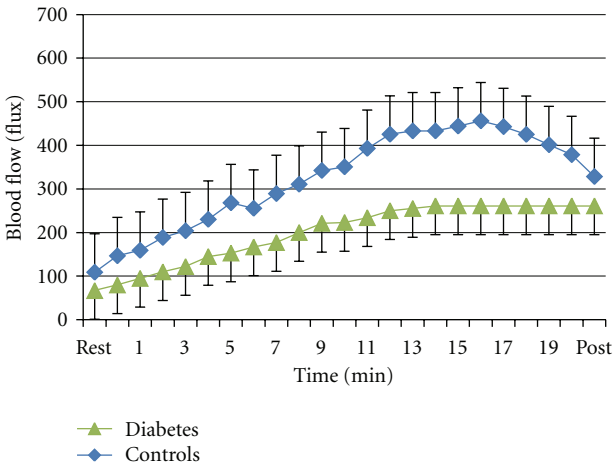

Figure 3: The blood flow response to a local heat source applied to the forearm in 10 subjects with diabetes and 10 age-matched controls over a 20-minute period. Each point is the mean do 10 subjects ± the SD (from [71, 95]).

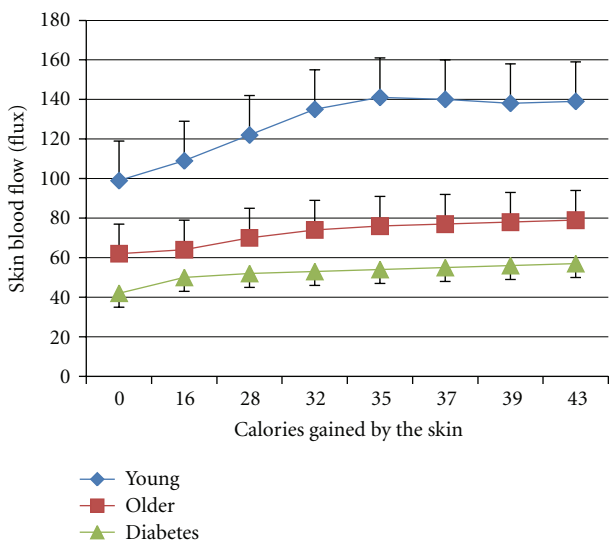

Figure 4: Illustrating the relationship between skin blood flow and heat gained by the skin in young subjects, older subjects, and subjects with type 2 diabetes. Average ± the SD for each group is shown ($n = 15$). Experiments involved adding calories of heat to the skin by applying a brass 50 gram heated block at different temperatures and measuring the change in skin blood flow for a given caloric load [99, 100]. The difference in heat absorbance between the 3 groups was significant (ANOVA $P < 0.05$).

burns than are age-matched control subjects [101]. Another contributor to the poor thermal response of the skin in people with diabetes is drier skin. Skin moisture content is about half that of age-matched controls in people with diabetes [98]. When the skin is dry, the blood flow response to heat is less, presumably due to TRPV-4 osmotic receptor interaction with normal ENOS activation pathways [97, 98]. The same TRPV-4 channels that sense heat on endothelial cells also sense blood and skin osmolarity. When skin is dry, the channels show a diminished response to heat [102]. This is worse in people with diabetes. Even when a moist heat source is used on people with diabetes, the heat tolerance is still less for hot packs compared to age-matched controls [97, 98].

Another contributor to the diminished response of the skin to local heat is damage to sweat glands. Associated with diabetes, and to a much lesser extent aging, sweat glands have a reduced output and, in diabetes, are eventually destroyed [59]. This impaired pseudomotor response starts usually in the periphery such as the feet and then spreads throughout the body [18]. Since sweat glands are both apocrine and eccrine, it is not just the lack of sweat that becomes an issue for diabetes in that without adequate sweat the skin does not cool as fast, but since apocrine sweat glands provide oil for lubrication of the skin, the skin is even drier than that associated with aging [103].

6. Contrast Baths

Contrast baths are a good example of how impaired endothelial function impairs the response to heat. Beginning with the Greeks and Romans, contrast baths have been used in therapy [6, 104, 105]. The theory behind contrast baths is that by alternating hot and cold using a ratio of approximately 3 minutes in a hot bath to 1 minute in a cold bath, that there will be a greater increase in skin circulation than with a warm bath alone [6, 104]. The enhanced blood flow response is alleged to cause greater removal of waste products from the skin than simple placement of the skin in a local warm bath [105]. When young subjects place their legs in contrast baths with a ratio of 3 minutes of warm to 1 minute of cold, the overall result is a much greater increase in skin blood flow than can be achieved by just leaving the leg in a contrast bath of the same temperature continuously. The oscillation in skin blood flow associated with placing the limb in alternating hot and cold baths compared to the sustained increase in blood flow with emersion of constant heat [6, 104]. However, while this same phenomenon holds to a lesser extent in older individuals, in people with diabetes, contrast baths cause a smaller overall blood flow response in the skin than with a continuous warm bath. Thus for people with diabetes they provide a negative therapeutic effect. This is illustrated in Figure 5 (controls) and people with diabetes (Figure 6). Note the higher average blood flow above that of continuous heat in the age-matched controls but not in the subjects with diabetes [6].

7. Global Heat

The endothelial response to pressure, occlusion, and local heat shows a clear defect in the control of circulation. It is of no surprise then that when looking at more grandiose control where the sympathetic nervous system is needed to coordinate the body, the effects of age and diabetes are more pronounced. The response to global heat is mediated by the circulation and sweat. When subjects are exposed to a hot room, the sweat response is greatly diminished in older subjects compared to younger individuals [18]. The sweat rate is approximately 70% of that in age-matched controls.

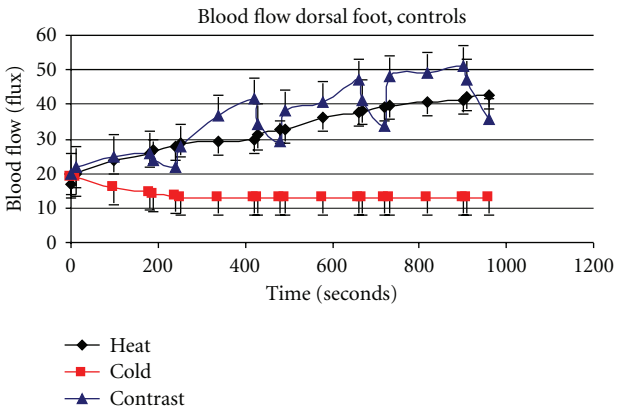

Figure 5: This figure illustrates the blood flow in the skin (flux) measured over the experimental period in control subjects during immersion in contrast baths (triangles), continuous heating (diamonds), and continuous cold immersion (squares). All data is the mean ± the SD [106, 107].

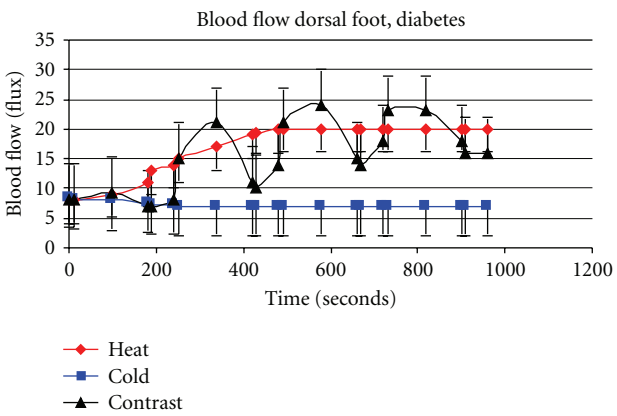

Figure 6: This figure illustrates the blood flow in the skin (flux) in subjects with diabetes during immersion in contrast baths (triangle), continuous hot (diamond), and cold immersion (squares) [106, 107].

It has been known for many decades that the sweat rate is lower and sweat gland density is lower in older compared to younger individuals [108]. When subjects are exposed to a hot room, the blood flow response is also greatly diminished compared to age-matched controls or younger individuals [18]. The lower resting skin blood flow due to age and lower blood flow response to heat, as was described above in the response to local heat, allows skin temperature to become elevated as is central body temperature in response to heat [108–111]. Plasma volume percent decrease during heat exposure was also greater in older than younger people [110]. It is not surprising that older individuals are more susceptible to hyperthermia as well [112]. Further, total sweat sodium loss increases with age [112].

Autonomic impairment, common in diabetes [24], alters both the blood flow response to global heat and the sweat response to global heat. Numerous studies have shown that people who have diabetes have impaired response to whole body (global) heating [59, 113, 114]. At rest, the lower resting skin blood flow lowers skin temperature compared to age-matched controls [115]. But when subjects are exposed to a hot room, the blood flow response and sweat response are greatly diminished compared to age-matched controls or younger individuals [18, 26]. The reduced sweat rate in people with diabetes is related to low nitric oxide production in sweat glands and high concentrations of free radicals in the blood [116]. This same study related autonomic nerve dysfunction to this same concentration of high free radicals [116]. These investigators show that Glutathione, a potent antioxidant in cells, is generally depleted in people with diabetes [117] and may allow enhanced nitric oxide production to increase free radicals in the cells and lead to autonomic neuropathy. Because of impairment in sweating, skin temperature is elevated far above that seen in younger or older individuals as is rectal temperature [118].

8. Orthostatic Tolerance

Blood pressure is normally well regulated in the body. Changes in posture usually cause a small and transient change in blood pressure [119, 120]. The most common test is the head up tilt test to 70°C [121]. If blood pressure drops more than 20 mmHg for 1 minute after going from a seated to standing position, the World Health Organization classifies this as orthostatic intolerance [122]. Orthostatic tolerance can also be measured by measuring the blood pressure response to standing from a seated position or from a lying position. A more radical stress is by having someone squat and then stand [119, 120]. Another laboratory-based measure is to use lower body negative pressure to induce a change in blood pressure [123]. Aside from the obvious risk of falling in the elderly, orthostatic hypotension is associated with cardiovascular mortality and all-cause mortality [124]. Orthostatic tolerance decreases with both age and diabetes [125–128].

Normally when standing upright there is decreased blood pressure, and as the autonomic nervous system compensates, the blood pressure comes back towards normal [122, 129]. This is achieved by an increase in peripheral vasoconstriction and an increase in heart rate. In older people and people with diabetes, there is a reduction in heart rate variability caused by reduced control of both the sympathetic and parasympathetic nervous systems [130, 131]. It is of no surprise then that orthostatic tolerance is also reduced in older people and people with diabetes due to loss of autonomic control. In many people with diabetes, we have observed no change in heart rate when changing body position [132, 133]. The reduced heart rate variability shows impairment in autonomic function in these groups [130]. Some of the deficit in autonomic function has been linked to ganglionic damage [134]. Autonomic neuropathy is also common with age and diabetes [135]. There is some evidence that there is reduced baroreceptor sensitivity with ageing and diabetes possibly due to increased angiotensin II activity due to increased renin release form the kidney [136].

In people with diabetes, in many studies, there is conventionally about 25% of the population that have orthostatic tolerance defined by the World Health Organization. However, in recent studies, we found that if the room is first warmed to 39°C and subjects are warmed in the room for 20 minutes, upon standing, 100% of the people had orthostatic intolerance [122]. This has been confirmed in other studies [123, 137]. Thus, it appears that when stressors are combined, the autonomic nervous system cannot handle the combined load of a thermal stress and an orthostatic stress together, and all people with diabetes seem to have orthostatic intolerance. In younger controls, there is enough reserve in the cardiovascular system to accommodate 2 simultaneous stressors.

9. Isometric Exercise

Isometric exercise is a type of exercise where force is exerted by the muscle but the muscle does not change length [138, 139]. With impaired neurogenic control of the circulation in muscle, impaired local control of the circulation and impaired sweating, the cardiovascular response to isometric exercise is also impaired in people with type 2 diabetes compared to age-matched controls [59, 140]. For example, looking at endurance and recovery of endurance, if 2 isometric contractions are accomplished 10 minutes apart, the endurance for the first contractions is similar in an age-matched control group than in people with diabetes [141, 142]. However, for the second contraction the endurance in the group of diabetes is much shorter showing a prolonged recovery time, and when blood flow is measured lower blood flows are found during and after exercise in people with diabetes compared to age-matched controls [59, 140]. During the exercise, traditionally blood pressure increases (both systolic and diastolic) driven by high sympathetic outflow. In people with diabetes, resting blood pressure is higher as is the blood pressure response to the exercise. Since the heart rate change and heart rate variability are both reduced with ageing and diabetes, the higher blood pressure response is driven by higher total peripheral resistance, increasing stroke work considerably [142–144]. Sympathetic impairment also reduces the sweating response to isometric exercise [59]. For dynamic exercise, strength is generally less and endurance is less for either kinetic or dynamic exercise. Thus, for people with diabetes, exercise performance and the cardiovascular responses are both affected by diabetes.

10. Balance

One of the most pronounced effects of diabetes is on balance and gait. Balance and gait are both contributed to by the vestibular system, the eyes, and proprioceptive system in the legs [57, 145]. All 3 are important in allowing someone to balance themselves and to walk properly. With diabetes, with diabetic polyneuropathies, there is impaired vestibular function, impaired vision due to retinopathies, and impaired somatosensory input into the vestibular nuclei [57]. It is of no surprise then that there are balance impairments in people with diabetes. During quite standing and during movement balance is impaired [146, 147]. Incorrect visual cues simply make matters worse. Thus, in dim light, poor visual cues make balance even worse in people with diabetes [148]. People with diabetes have better balance in a totally dark room because they do not confuse incorrect visual cues and hamper their ability to balance. Further, the color of light seen by the eyes shows the greatest impairment with diabetes in the blue range, the very color that most night lights are [148]. In another study, the autonomic response to balance is also impaired in people with diabetes. Thus, for people with diabetes, there is an abnormally low heart rate and blood pressure response to allow the body to be maintained stable during balancing attempts, making balance even worse [26].

These all have a pronounced impact on gait. In general, people with diabetes, because of lack of feeling and proprioceptive sense and poor balance, have slower gait and maintain a wider balance for postural support during gait. Further, during gait they circumduct thus having the legs further apart to be able to catch their balance if the trip or feel unsteady during gait [149]. This slows gait down about 30% in people with diabetes compared to age-matched controls subjects.

11. Summary

In summary, for older individuals and people with diabetes there are multiple impairments in the autonomic nervous system that affect activities of daily living from very simple ones, such as response to local heat or the pressure on the feet during standing leading to increased possibilities of burns and skin damage, to more complex movements such as balance and gait which are substantially impaired in people with diabetes. Patients must be counseled during therapy, relative to these impairments, so as to protect them as they carry out their normal daily life activities.

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Research Article

Effects of Restricted Fructose Access on Body Weight and Blood Pressure Circadian Rhythms

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High-fructose diet is known to produce cardiovascular and metabolic pathologies. The objective was to determine whether the timing of high fructose (10% liquid solution) intake affect the metabolic and cardiovascular outcomes. Male C57BL mice with radiotelemetric probes were divided into four groups: (1) 24 h water (control); (2) 24 h fructose (F24); (3) 12 h fructose during the light phase (F12L); (4) 12 h fructose during the dark phase (F12D). All fructose groups had higher fluid intake. Body weight was increased in mice on restricted access with no difference in total caloric intake. Fasting glycemia was higher in groups with restricted access. F24 mice showed a fructose-induced blood pressure increase during the dark period. Blood pressure circadian rhythms were absent in F12L mice. Results suggest that the timing of fructose intake is an important variable in the etiology of cardiovascular and metabolic pathologies produced by high fructose consumption.

1. Introduction

Given the substantial levels of fructose consumption in our everyday diet, it is important to delineate its consequences [1, 2]. There is compelling evidence that increased fructose intake has metabolic and cardiovascular effects in both human and animals [3–8]. Our group showed that a high-fructose diet in mice produced glucose intolerance and increased blood pressure [4, 6]. Evidence also showed that sympathetic activation occurred rapidly before fructose induction of metabolic dysfunction [9]. A clinical study showed that ingestion of water containing 60 g of fructose acutely elevates blood pressure in healthy human subjects [3].

In addition to the global detrimental effects of high fructose intake, the timing of consumption might also play a causative role in the metabolic and cardiovascular pathologies. There are many examples of work and lifestyle patterns that require alternative intake cycles. This is seen in public service work: police and fire protection, transportation, and utilities, all of which rely on 24 hr around the clock attention. Likewise, shift work as required in health care or manufacturing industries forces people to be active in the normal sleeping phase of the light-dark (L/D) cycle. Obesity and metabolic syndrome are often associated with the nocturnal eating syndrome and shift work, demonstrating the connection between L/D cycle and metabolic pathologies [10–15]. Circadian misalignment has also been shown to produce changes in glucose and insulin levels, as well as increases in blood pressure, in both mice and humans [16–18]. Although the mechanisms underlying these adverse cardiometabolic changes remain unknown, it is possible that disturbances in the sleep/wake and eating schedule, could, in turn, influence appetite, food intake, and energy balance. This could have important implications for the increased obesity, diabetes, and cardiovascular disease in the shift work population [15].

In the context of the worldwide epidemic of obesity and diabetes [19], the increase in sugar consumption in our contemporary western diets [20], and the increase in sedentary lifestyles [21], we conducted studies in mice to evaluate the effects of 24 h access to fructose as well as

the influence of the timing of consumption (fructose access provided only in the light or dark period). The parameters of interest focused on cardiovascular and metabolic function: 24 hr L/D rhythm in BP, glucose tolerance, plasma insulin, and the pattern and amount of fructose intake.

2. Methods

2.1. Animals, Surgery, and General Procedures. Eight-week-old male C57BL mice (~25 gm, Harlan Inc, Indianapolis, IN) were given water or fructose (10%) for 8 weeks. Groups are (1) 24 h water (Control, $n = 6$); (2) 24 h fructose (F24, $n = 6$); (3) 12 h fructose during the light phase (F12L, $n = 6$); (4) 12 h fructose during the dark phase (F12D, $n = 6$). Animals were housed at 22°C under a 12/12 light/dark cycle with *ad libitum* access to standard pellet chow and water or fructose. Telemetric probes (model TA11PA-C10, Data Sciences International, St. Paul, MN) were inserted into the left common carotid artery at 6 weeks in mice anesthetized with ketamine/xylazine (120 : 20 mg/kg, im). The transmitter body was positioned subcutaneously on the right flank [22, 23]. 24 h cardiovascular recordings, fluid, and food consumption measurements were made at 8 wks. Body weight was measured during the 2nd, 4th, and 8th weeks of the experimental protocol.

2.2. Glucose Tolerance Test (GTT). GTT was performed at the beginning of the 8th week (last week) of the experimental protocol. Mice were fasted, with animals receiving only water, for 6 h. Blood samples were taken from a tail cut at 0, 15, 30, 60, and 90 min after i.p. glucose load (1.5 g/kg). Blood glucose was determined by Accu-Chek Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, Indianapolis, IN).

2.3. Plasma Measurements. At the end of the 8th week of treatment, mice were sacrificed by decapitation and trunk blood was collected in ice-chilled heparinized tubes. Total plasma cholesterol and triglycerides were determined by colorimetric enzymatic assays (Thermo Fisher Scientific Inc., Waltham, MA). Plasma insulin levels were measured using a multianalytic profiling beads assay (Linco Research Inc., St Charles, MO).

2.4. Urinary Corticosterone Measurements. Spot urine samples (100~100 uL) were collected during the last hour of the light phase. Urinary corticosterone levels were measured by radioimmunoassay (MP Biomedical, Orangeburg, NY, USA). Urinary creatinine was measured by spectrophotometry (Microvue Creatinine Assay Kit, Quidel Corporation, San Diego, USA). The corticosterone : creatinine ratio was calculated for each urine specimen.

2.5. Statistics. Values were expressed as mean ± SEM. Data were analyzed using ANOVA two-way or repeated measures when applicable and followed by the Newman-Keuls test. Differences were considered to be statistically significant at $P < 0.05$.

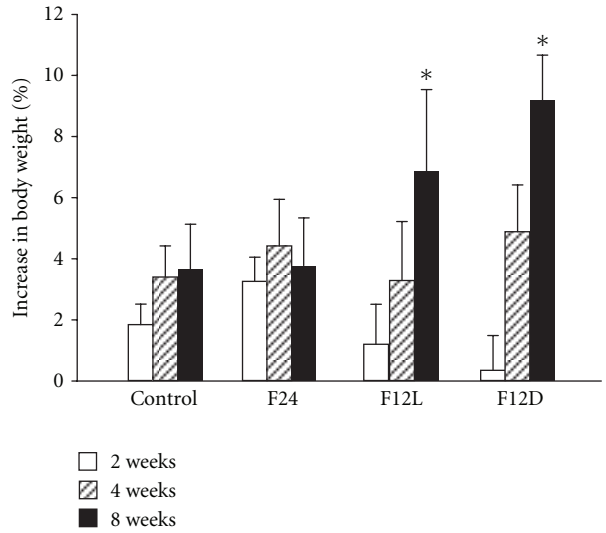

Figure 1: Body weight gain as percentage increase from day 0 in control and fructose-treated groups. ANOVA showed main effect of time (F (2.6156) = 8.97.23, $P < 0.0012$). $^*P < 0.015$ vs. 2 wks.

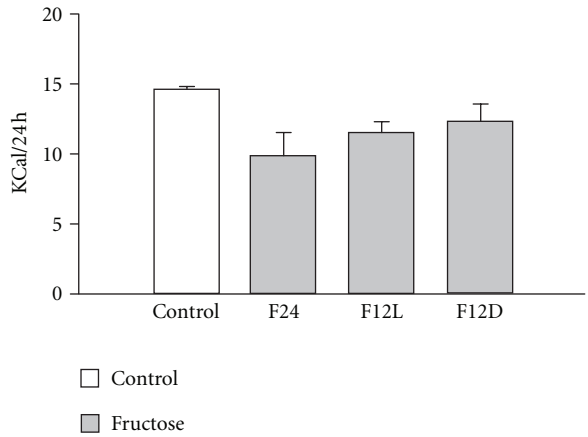

Figure 2: Daily total caloric intake and fructose percentage caloric intake in control and fructose-treated groups.

3. Results

Baseline body weights (BWs) ranged from 26 to 28 g. The percentage increase in BW over the 8 wk period was significantly higher in the restricted access groups (Figure 1). The F12L and F12D groups showed significant increases in BW gain after 8 wks of treatment as compared to 2 wks ($P < 0.05$). Total caloric intake was not different between groups (Figure 2).

Fluid intake was measured during the light and dark phases with significant effects of diet, light/dark, and interaction between diet and light/dark (Figure 3). 24h fluid intake was higher in F24 and F12D mice when compared to Control group ($P < 0.01$). There were light/dark differences in intake in Control, F24, and F12D groups. In the F12L group, there was no difference in fluid intake between the light and dark phases. In the restricted access groups, fluid intake was

Table 1: Plasma insulin, triglycerides, and cholesterol and urinary corticosterone.

	Control	F24	F12 L	F12 D
Insulin (ng/mL)	0.6 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	1.2 ± 0.3
TGL (mg/dL)	104 ± 18	129 ± 13	85 ± 1#	89 ± 11#
Cholesterol (mg/dL)	95 ± 13	94 ± 11	94 ± 11	81 ± 13
Corti (mg/mmol/L)	0.24 ± 0.1	0.36 ± 0.1	0.36 ± 0.1	0.23 ± 0.1

Plasma insulin, triglycerides, and cholesterol levels were measured in nonfasted mice at 8-9 wk. ANOVA showed a significant effect of diet for insulin (F (3.17) = 3.45, $P < 0.05$) and triglycerides (F (3.17) = 2.95, $P < 0.05$). #$P < 0.01$ vs. F24.

Table 2: Effect of restricted fructose access on fluid intake.

	Water (Control)	F24	F12L	F12D
24 h volume (mL)	11.5 ± 0.7	20.2 ± 2.4*	11.5 ± 1.6	19.8 ± 1.5*
Light phase (%)	30.4 ± 6.0	32.8 ± 3.9	44.0 ± 1.8*	25.4 ± 5.6
Dark phase (%)	69.6 ± 6.0#	67.2 ± 3.9#	56.0 ± 1.8	74.6 ± 5.6#

ANOVA showed a significant effect of diet for 24 h volume intake [F (3.21) = 8.9, $P < 0.001$]. ANOVA showed main effect of light/dark [F (1.42) = 100.6, $P < 0.00001$] and interaction between light/dark and diet [F (3.42) = 5.6, $P < 0.003$]. *$P < 0.01$ vs. Control. #$P < 0.01$ vs. light.

greatest during the fructose consuming period, light for F12L and dark for F12D ($P < 0.01$ vs. Control). With regard to the percentage fluid intake during light and dark phases, mice consuming fructose during the dark period drank a greater percentage of fluid, 67–74%, during this phase (Table 2). Mice given fructose during the light period drank almost equal amounts during the light and dark (44 and 56% resp.).

Fasting glucose was higher in groups under restricted fructose access as compared to Control (Figure 4(A), $P < 0.01$) with higher levels in F12L as compared to F12D (Figure 4(A), $P < 0.01$). GTT showed impaired glucose tolerance in F24 and F12L groups as compared to control (Figure 4(B), $P < 0.01$).

There were no significant changes in insulin levels among groups (Table 1). Triglyceride levels were lower in restricted fructose access groups (Table 1, $P < 0.01$). Both plasma cholesterol and urinary corticosterone levels showed no difference among the groups (Table 1).

Light/dark differences in BP were present in Control, F24, and F12D groups (Figure 5(A), $P < 0.05$). The circadian BP rhythm was not seen in the F12L (Figure 5(A)). Fructose consumption produced an increase in BP in the F24 group during the dark phase when compared to the Control group during the same phase (Figure 5(A), $P < 0.05$) and also when compared to F24 during the light phase (Figure 5(A), $P < 0.05$). This fructose effect was not observed in the F12D group (Figure 5(A)). All groups showed elevated HR during the dark as compared to the light phase (Figure 5(B), $P < 0.05$) with no effect of the fructose regimen (Figure 5(B)).

4. Discussion

The increase in fructose consumption, due to its widespread presence in the modern diet, has become a major public health concern. This is related to evidence that excessive fructose intake may have detrimental effects, including the promotion of obesity and its cardiovascular and metabolic complications [1, 2]. Studies focused on the direct effects of fructose intake in animals and humans showed increased BP, dyslipidemia, insulin resistance, and glucose intolerance [3–8]. In addition to the detrimental effects of a high-fructose diet, the timing of consumption might have additional effects. Changes in circadian feeding patterns, such as the night eating syndrome (NES), can promote weight gain, hormonal changes, and psychological disorders in either in animals or humans [10–14, 24]. In this study, we evaluated the time effect (light or dark) of fructose consumption on BP, body weight, and metabolic and hormonal parameters. We observed that day or night restricted fructose consumption increased body weight gain and glycemia. Further changes were observed in mice submitted to light-restricted fructose access: glucose intolerance, exacerbated hyperglycemia, and a lack of circadian BP oscillation. These additional changes were observed after 8 weeks of treatment, suggesting that the chronic aspect of the restricted regimen itself might be responsible, at least partially, for these results.

The circadian BP rhythm refers to the daily variation in BP that in humans shows higher levels during day vs. night. Healthy subjects usually present a 10–20% decline in arterial BP during nighttime intervals which is called the "dipper" pattern [25]. Impaired nocturnal BP decline (nondipping) is a BP abnormality that is frequently seen among patients with diabetes [26–30]. In order to follow the L/D BP rhythm in our study, we used radiotelemetry for BP monitoring in conscious, undisturbed mice. 24 hr BP recordings showed, as expected, that control mice exhibited light/dark circadian oscillations with lower BP seen during the light period, the inactive period for mice [4, 31–33]. When fructose access was restricted to the light phase (F12L), the light/dark BP rhythm was nonexistent. This could be related, at least partly, to the drinking pattern since the F12L group also showed a lack of circadian oscillation in fluid intake, that is, similar levels during the light and dark periods. This indicates that animals spent more time, during the light phase, drinking and therefore awake and possibly active. Blood pressure circadian rhythm disruption may also be due to changes in sleep since it has been observed in diabetic patients, who wake up more frequently during the night (for instance, due to nocturia) [34]. Although sleep disruption may also be associated with stress [35], stress as documented by unchanged urinary corticosterone does not seem to be responsible for the observed cardiovascular and metabolic effects. Therefore, the disturbance in circadian BP pattern in the F12L group might be due to the timing of fructose ingestion (during the light phase) itself. Possibly, a higher fructose load or longer treatment could produce a shift in the BP circadian

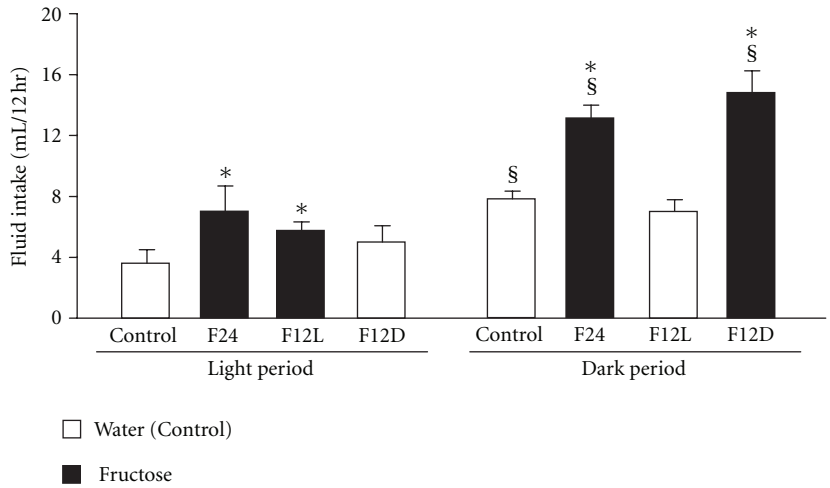

Figure 3: 12 h fluid intake in control and fructose groups. ANOVA showed main effect of fructose diet [F (3.20) = 8.4, $P < 0.001$], light/dark (F (1.20) = 50.2, $P < 0.00001$) and interaction between diet and light/dark (F (3.20) = 5.7, $P < 0.01$). $^{\S}P < 0.01$ light vs. dark. $^{*}P < 0.01$ versus Control.

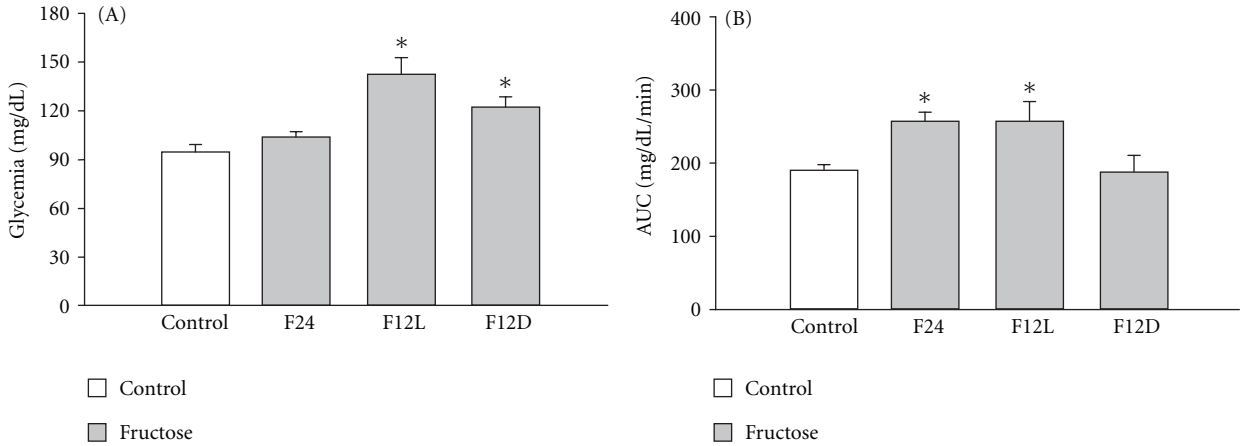

Figure 4: (A) Fasting glycemia in control and fructose groups. F12L and F12D mice showed hyperglicemia ANOVA treatment (F (1.18) = 12.3, $P < 0.0003$). $^{*}P < 0.01$ versus control. (B) Glucose tolerance test estimated by area under the time curve (AUC) in control and fructose-treated groups. F24 h and F12L mice showed impaired glucose tolerance. ANOVA treatment (F (1.16) = 4.9, $P < 0.05$). $^{*}P < 0.01$ versus Control.

pattern in the F12L. However, these speculations do not affect the conclusion drawn from this group: fructose access restricted to the light phase produces disruption in BP rhythms.

The circadian pattern on HR light/dark oscillations, showing a rhythmic pattern of variation in HR during 24 hours characterized by higher HR in the dark (awake period) over the light period (sleep period), was not affected by any of the treatments. As previously shown by our group [4, 22], although nocturnal BP was increased in F24 when compared to controls, no further changes due to fructose were observed in HR.

Circadian changes, such as the nondipping BP pattern, are associated with diabetic micro- and macrovascular [36–41] complications and end-organ damage [42]. The circadian changes in BP and fluid intake observed in F12L could also be implicated in the impaired glucose metabolism (exacerbated hyperglycemia and glucose intolerance), exclusively observed in this group (F12L). Preliminary information suggests derangements in renal histology in mice with fructose access restricted to the light phase (Morris et al., unpublished data). Changes in circadian pattern of food consumption can lead to weight gain, metabolic dysfunction [10–14, 43], and increased risk of obesity and diabetes [44] in humans. The light phase-restricted fructose drinking regimen could be a parallel for human NES, which is associated with hyperglycemia, glucose intolerance [45], and weight gain without changes in total caloric consumption [12, 46], similar to our observations in mice. Although the mechanism behind light-fed weight gain in mice is unknown, the study of Arble et al., showed that there is causal evidence that feeding at the "wrong" time can lead to weight gain [10].

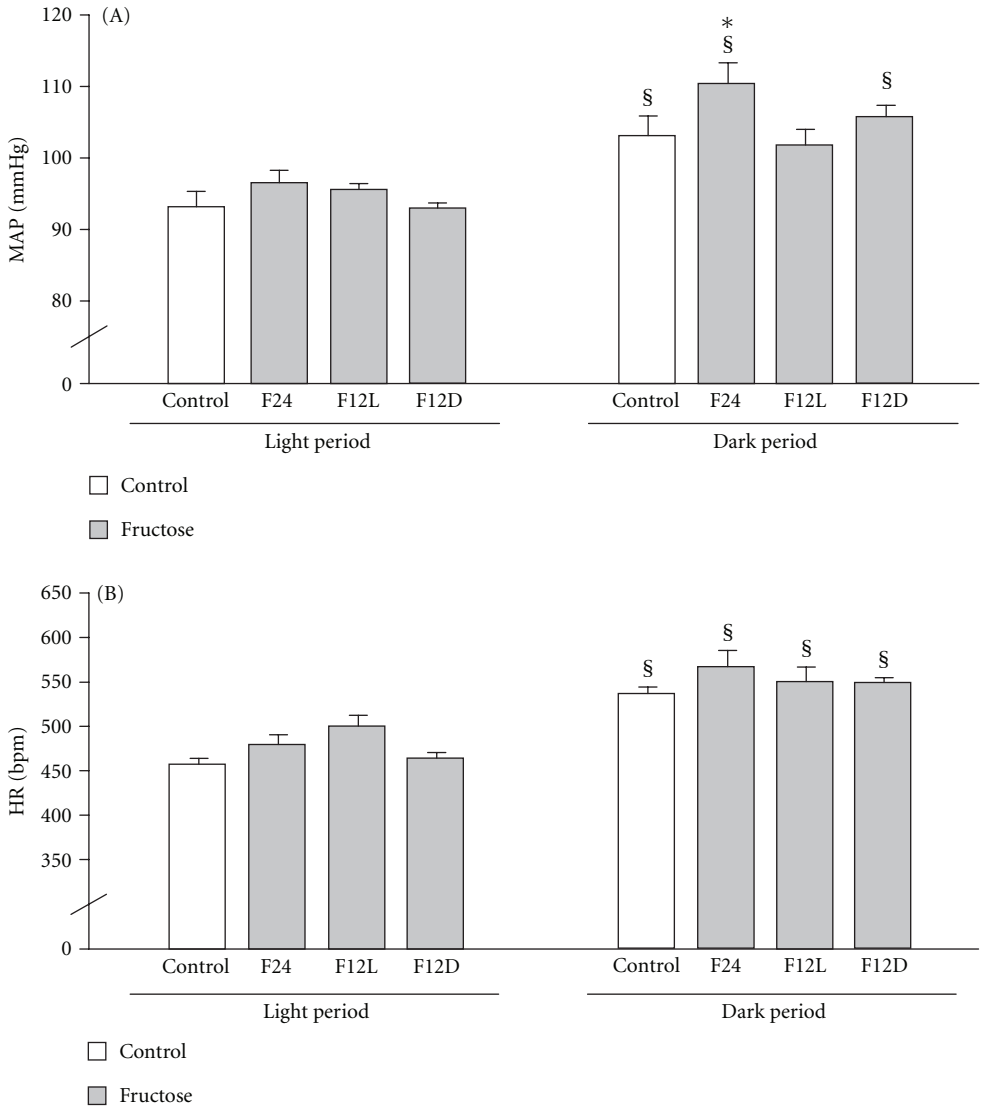

Figure 5: MAP (A) and HR (B) were recorded for 24 h and analyzed during 24 h light and dark phases. ANOVA showed main effect of light/dark for MAP (F (1.32) = 11.2, $P < 0.005$) and HR (F (1.32) = 16.62, $P < 0.0003$). $^{\S}P < 0.05$ light versus dark. $^{*}P < 0.05$ versus Control.

Although F12L mice showed greater impairment in glucose handling, both groups on restricted fructose access (F12L and F12D) showed increased BW gain and hyperglycemia, not observed in the F24 group. Chronic misalignments between endogenous circadian timing system and behavioral cycles have adverse metabolic effects in humans [43], including increased risk of obesity and diabetes [44]. As shown by our group [4, 6], 24 h access to fructose produces glucose intolerance without increases in BW. Therefore, the circadian restriction aspect of the drinking regimen itself could contribute to the increase in weight gain and blood glucose levels observed in F12L and F12D.

A high-fructose diet in rodents has been associated with cardiovascular dysregulation and insulin resistance [4, 6, 47–49], More specifically, increased fructose intake, in both, animals and humans, increases blood pressure and causes dyslipidemia and changes in glucose handling [3–8]. As expected, mice receiving fructose 24 h a day exhibited nocturnal hypertension [4, 6]. However, BP was not increased in groups on restricted fructose access (F12L and F12D) despite the circadian disruption observed in the F12L group. Diet concentration and length of treatment are important factors for hypertension development in the rodent fructose model [50]. Therefore, the restricted aspect of the fructose access regimen itself could be a possible explanation for the absence of BP increase in the F12L and F12D groups.

As mentioned previously, fructose consumption is often associated with dyslipidemia. Chronic consumption of fructose led to hypercholesterolemia without changes in plasma triglycerides [4, 6, 51, 52]. However, in this study, the F24 group showed no changes in cholesterol. This could be due to the nature of the fructose source, since in previous studies [4, 6] fructose was given in the chow (pellet diet containing 67% carbohydrate—98% fructose, 13% fat, and

20% protein) instead of as a fluid (10% fructose solution). Both groups on restricted access (F12L and F12D) had lower levels of triglycerides with no changes in cholesterol. Since circulating factors such as glucose and triglycerides can be modulated by time-restricted food intake access [13, 53], the restricted nature of the fructose drinking regimen itself could explain the results observed in these groups.

5. Conclusion

In conclusion, we demonstrated that circadian phase restriction of fructose access leads to changes in glucose homeostasis, body weight gain, and light/dark BP rhythms. The data suggests that even moderate circadian changes might contribute to the onset or development of the metabolic syndrome symptoms, which might be exacerbated by the timing of fructose consumption. The results have clinical implications since time of day and intake may be considered in the overall treatment regimen.

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Clinical Study

Links between Metabolic Syndrome and Cardiovascular Autonomic Dysfunction

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Background. Type 2 diabetes (T2D) might occur within metabolic syndrome (MbS). One of the complications of T2D is an impaired (imp) cardiovascular autonomic function (CAF). *Aims*. In subjects with T2D and age ≤ 55 years, the prevalence of impCAF and its relationship with BMI, waist, HbA_{1c} values, MbS, hypertension, and family history of T2D and/or hypertension were analysed. *Methods*. 180 subjects consecutively undergoing a day hospital for T2D were studied. The IDF criteria were used to diagnose MbS. To detect impCAF, 5 tests for the evaluation of CAF were performed with Cardionomic (Meteda, Italy). Univariate and multivariate analyses were performed. *Results*. The prevalence of impCAF and MbS were 33.9% and 67.8%, respectively. Among diabetics with impCAF, 86.9% had MbS. ImpCAF was significantly associated with MbS, overweight, and $HbA_{1c} > 7\%$. Both logistic ($P = 0.0009$) and Poisson ($P = 0.0113$) models showed a positive association between impCAF and MbS. The degree of ImpCAF showed a positive linear correlation with BMI and HbA_{1c} values. *Conclusions*. The study demonstrates that glycaemic control and overweight influence CAF and that T2D + MbS is more strongly associated with impCAF than isolated T2D. We suggest that MbS not only increases the cardiovascular risk of relatively young subjects with T2D but is also associated with impCAF.

1. Introduction

Epidemiological studies demonstrated that diabetics display a cardiovascular risk which is twice that of sex- and age-matched nondiabetic population. In line with the high cardiovascular risk of subjects with diabetes mellitus (DM) are their frequent silent myocardial infarctions (MIs) [1, 2]. Clinically unrecognized MIs might be due to impaired cardiovascular autonomic function (impCAF) which finally evolves to cardiovascular autonomic neuropathy (CAN), a chronic complication of both type 1 and type 2 DM. In the Rochester diabetic neuropathy study concerning subjects with T2D, no correlation was found between autonomic symptoms and autonomic cardiovascular tests [3]. Therefore, an analysis of cardiovascular reflexes with tests which are sensitive and noninvasive allows to suspect diabetic CAN.

In subjects with DM, cardiovascular risk is known to be higher when clinical features of the metabolic syndrome (MbS) are present along with DM [4]. Several reports show that a higher cardiovascular risk is present in subjects displaying a cluster of factors predisposing to the atherosclerotic cardiovascular disease and included in the syndrome named MbS (Table 1) [5–7]. Subjects with T2D always have one of the diagnostic criteria of MbS (glycaemia ≥ 110 mg/dL), but do not obligatorily show other diagnostic features for MbS. In the present study we tried to assess whether MbS is more frequently associated with ImpCAF in relatively young type 2 diabetics.

2. Aims

Our study evaluated the association, if any, between an early deficit of CAF and the presence of MbS defined on the criteria

Table 1: Diagnostic criteria for the metabolic syndrome.

Any 3 of the following conditions		
(1) Central obesity		
According to NCEPIII*		
Country/ethnic group	Sex	Waist in cm
Any	Male	≥102 cm
	Female	≥88 cm
Or according to IDF†		
Country/ethnic group	Sex	Waist in cm
Europids	Male	≥94
	Female	≥80
South Asians, Chinese	Male	≥90
	Female	≥80
Japanese	Male	≥85
	Female	≥90
(2) Elevated triglyceridemia (≥150 mg/dL).		
(3) Decreased HDL cholesterolemia (<40 mg/dL in males, <50 mg/dL in females).		
(4) Elevated arterial blood pressure (≥130/85 mmHg).		
(5) Elevated fasting blood glucose (≥110 mg/dL or ≥100 mg/dL according to IDF†).		

*Adapted from the third report of the National Cholesterol Education Program (NCEPIII) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, ATP III).
†International Diabetes Federetion.

of the International Diabetes Federation (IDF) [6] and whether any correlation existed between the detection of an early deficit of CAF and HbA_{1c}, the duration of T2D and/or hypertension (HBP), the occurrence of a positive family history of DM, HBP, or both, and the nutritional habits in a cohort of type 2 diabetics not older than 55 years.

3. Methods

The study included subjects with T2D and age ≤ 55 years consecutively undergoing a day hospital (DH) for chronic complications of DM at the Unit of Endocrinology of the University Hospital of Bari from October 2004 to September 2006. We screened 210 subjects. Thirty subjects out of 210 were excluded because they could not be screened for cardiovascular reflexes (11 experienced acute MI less than 6 months before DH and the remaining 19 showed arrhythmias at the basal ECG at DH admission). 180 type 2 diabetics (117 males and 63 females) with mean age of 48.62 ± 6.12 years (48.18 ± 7.26 and 48.86 ± 5.46 for female and males, resp.) were recruited and underwent 5 different tests for cardiovascular reflexes. At DH admission, all subjects gave their written informed consent. The tests included beat-to-beat heart rate variation (DB), heart rate response to standing (lying to standing, LS), heart rate response to Valsalva maneuver (Vs), heart rate response to cough (cough test, CT), and systolic blood pressure response to standing (PH) [1, 8–11]. All tests were performed with Cardionomic [8, 9], which is a portable computerised system that is used for step-by-step performance of several cardiovascular tests for autonomic neuropathy. All tests were performed after an overnight fast but never after overnight hypoglycaemia. Each subject was instructed to refrain from smoking and drinking coffee at least 8 h before tests. Before the tests, patients were lying in the supine position for 30 minutes and a basal ECG was performed. As far as DB is concerned, it evaluates the physiologic arrhythmia induced by respiration and is an index of the vagus nerve function. Inspiration induces pulmonary expansion which stimulates stretch receptors in the lungs, in the atrium, and in the chest wall. The above-mentioned receptors stimulate the nucleus solitarius and the bulbar cardioinhibitory center through afferent vagal fibers. The final effect is the inhibition of the vagus which is followed by the heart rate increase. During expiration, opposite mechanisms occur which induce heart rate deceleration. Therefore, respiratory arrhythmia is mainly due to the prevailing effect of the parasympathetic nervous system. When parasympathetic autonomic dysfunction occurs, the respiration-induced heart rate variation is decreased or abolished.

For DB, a parasympathetic test function, a 1 min ECG was performed when the subject was lying supine and deeply breathed 6 times per minute. The expiration/inspiration R-R ratio was calculated. For LS, a parasympathetic test function, the patient was invited to stand suddenly and the R-R interval was measured at beats 15 and 30 after standing and the 30/15 ratio was calculated.

VS simultaneously evaluates parasympathetic, sympathetic, and baro receptor functions. For VS, the patient exhales for 15 min into the mouthpiece of a manometer exerting a pressure of 40 mmHg. The ratio of longest-to-shortest R-R interval was measured. For HP assessment, supine systolic blood pressure was measured after the patient was lying down for 30 min and orthostatic blood pressure after the patient was standing for 2 minutes. Orthostatic hypotension was diagnosed when the fall in systolic blood pressure (SBP) levels was ≥30 mmHg or that of diastolic BP (DBP) was >10 mmHg in response to a postural change from supine to upright position [12]. Orthostatic hypotension is known to reflect sympathetic dysfunction [13]. CT, a parasympathetic test function, evaluates the cough-mediated increase in heart rate. During the test, the patient was in the supine position and ECG was performed when patient breathed for 15 seconds (basal) and again when he coughed 3 times. The R-R ratio between the shortest R-R interval after the last cough and the mean R-R interval during regular respiration was calculated [10, 11].

Since for each test the range of normal values was changing with age, we elaborated a score grading from 0 (normal response to all performed tests) to 5 (impaired response to all performed tests). Normal values for tests were according to Vespasiani et al. [8] but were also confirmed in a cohort of age- and sex-matched control subjects selected in our region ($n = 130$). Part of this cohort of controls was already used to validate CAF tests in a cohort of subjects with β-thalassemia [9]. Control subjects showed normal glucose tolerance (normal fasting glucose circulating levels and HbA_{1c} levels < 5.9%), they did not display any of the diagnostic features

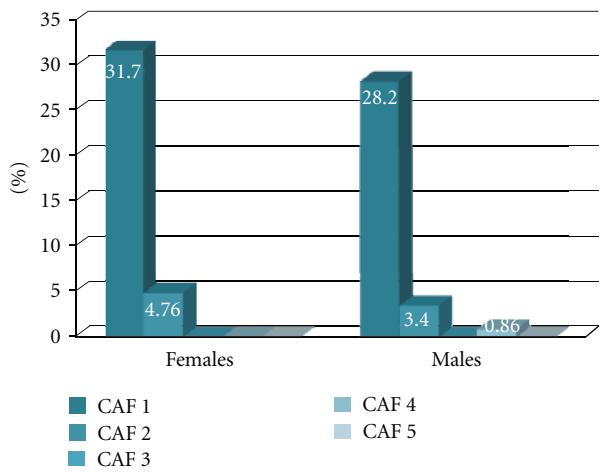

Figure 1: Distribution of different scores of impaired CAF in the cohort.

of the MbS, and they were not taking any pharmacological treatment.

We also analysed whether any relationship existed between the detection of different degrees of impCAF and the presence of MbS according to the International Diabetes Federation [6]. The nutritional habits were also assessed by a dietician through a dietary interview. HbA_{1c} was measured in HPLC (Menarini Diagnostics). Total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides circulating levels were measured with specific Dimension clinical chemistry systems (Siemens Healthcare Diagnostics Ltd.) which are *in vitro* tests intended for the quantitative determination in human serum or plasma.

3.1. Statistical Analysis. Data are expressed as mean ± SD or %. Two-sided P values refer to the Mann-Whitney U test for continuous variables and Pearson's χ^2 for categorical variables. Univariate and multivariate analyses for at least one positive CAF test versus none and mean number of positive CAF tests were, respectively, assessed with logistic and Poisson's regression models. Results are expressed as odds ratios (ORs) or rate ratios (RRs) and their 95% confidence intervals (CIs). Two-sided P values less than 0.05 were considered significant. All the analyses were performed using SAS (Release 9.1, SAS Institute, Cary, NC, USA, 2002-2003).

4. Results

4.1. Prevalence of Impaired CAF. Patients' characteristics of the sample by number of positive tests for impaired cardiovascular autonomic function (CAF) are reported in Tables 2(a), 2(b), and 2(c). In our cohort 33.9% subjects (61 out of 180) showed at least one pathologic test for CAF. Among female patients, 4.76% showed a pathological response to DB, 20.6% and 9.5% showed pathological responses to Vs and CT, respectively. Among male patients, 4.3% showed a pathological response to DB, 6.84% to LS, 18% to VS, and 6.84% to CT. Among females, no subject showed a score ranging from 3 to 5. Score 2 was found in 4.76% and score 1 in 31.7% of female subjects (Figure 1). Among male subjects, nobody showed score 3 or 5 and less than 1% (0.86%) had a score 4. The distribution of scores 1 and 2 was comparable to that found in the female cohort since 28.2% and 3.4% of male subjects showed scores 1 and 2, respectively (Figure 1).

4.2. Impaired CAF and Anthropometric and Metabolic Variables. When female diabetics were stratified for BMI classes, we found 23.8% normal-weight, 30.16% overweight, 38.1% obese (class 1 and 2), and 7.94% severe obese women (BMI $\geq$ 40 Kg/m^2) (Figure 2). Among male subjects, 25.64% were normal-weight, 41.03% overweight, 30.77% obese (class 1 and 2), and 2.56% severely obese (BMI $\geq$ 40 Kg/m^2) (Fig-ure 2). The prevalence of MbS in the presence of impCAF was significantly higher than that in the absence of impCAF in both sexes (Figure 3). The distribution of the different components of the MbS in the male and female cohort in the presence or in the absence of impCAF was similar (Figures 4 and 5).

When subjects were stratified for both CAF score and BMI classes, the presence of at least one pathologic test for CAF showed a significant positive correlation with BMI > 25 Kg/m^2 (P = 0.0227, Table 3). Subjects with at least one pathologic test had BMI (P = 0.0032), waist circumferences (P = 0.0146), triglycerides (P = 0.0089), and HbA_{1c} (P = 0.0292) levels significantly higher than those of subjects with normal tests. The occurrence either of one or more abnormal tests was not significantly associated with a positive FH for DM and/or HBP or with a duration of T2D longer than 5 years. By contrast, the occurrence of at least one pathological test was positively associated to the occurrence of HBP (P = 0.0061), a waist value >94 cm (according to IDF) (P = 0.0146), and triglycerides ≥150 (P = 0.0089). In the multivariate Poisson model, we considered the association between the mean number of tests positive for impaired CAF and age, sex, classes of BMI, FH of DM, and duration of DM longer than 5 years (Table 4). A statistically significant association with the mean number of positive tests for impaired CAF was found when MbS was considered as dichotomous (P = 0.0018). Significant associations were also found between the mean number of tests positive for impaired CAF and the occurrence of overweight (BMI between 25 and 30 Kg/m^2) and HbA_{1c} > 7% (Table 4). With adjustment for BMI classes, FH of diabetes, and/or hypertension, there was still a significant association between the mean number of tests positive for impaired CAF and HbA_{1c} > 7% (Table 4). With additional adjustment for sex, significant associations of the mean number of tests positive for impaired CAF with HbA_{1c} > 7% and the occurrence of MbS were confirmed (Table 4).

4.3. Impaired CAF, MbS, and Nutrient Intake. Significant associations were found between the mean number of tests positive for impCAF and a lipid intake >30% (Table 3).

When patients were stratified according to a daily lipid intake >30%, the occurrence of at least one pathologic

Table 2: (a) Patients' characteristics by number of positive CAF tests. (b) Patients' characteristics by number of positive CAF tests. (c) Patients' characteristics by antidiabetic therapy.

(a)

Variable	Category	Number of positive CAF tests		All	*P* value
		At least one	None		
N		61	119	180	
Age		48.9 ± 5.3	48.5 ± 6.5	48.6 ± 6.1	0.9372
BMI		31.6 ± 7.3	28.0 ± 5.0	29.2 ± 6.1	0.0032
BMI class	<25	10 (16.4)	35 (29.4)	45 (25.0)	0.0227
	25–30	21 (34.4)	49 (41.2)	70 (38.9)	
	≥30	30 (49.2)	35 (29.4)	65 (36.1)	
Duration		6.0 ± 5.9	6.4 ± 6.5	6.3 ± 6.3	0.9071
Duration ≥5 years	Not assessed	3 (·)	5 (·)	8 (·)	0.4557
	No	34 (58.6)	60 (52.6)	94 (54.7)	
	Yes	24 (41.4)	54 (47.4)	78 (45.3)	
Family diabetes	No	11 (18.0)	23 (19.3)	34 (18.9)	0.8336
	Yes	50 (82.0)	96 (80.7)	146 (81.1)	
Family hypertension	No	38 (62.3)	71 (59.7)	109 (60.6)	0.7324
	Yes	23 (37.7)	48 (40.3)	71 (39.4)	
Fibers		27.6 ± 4.2	27.8 ± 4.0	27.7 ± 4.1	0.9747
HDL-cholesterol		39.1 ± 10.1	42.5 ± 11.3	41.3 ± 11.0	0.0685
HbA_{1c}		7.7 ± 1.4	7.3 ± 1.5	7.4 ± 1.5	0.0292
HbA_{1c} > 6.5%	Not assessed	1 (·)	0 (·)	1 (·)	0.0864
	No	15 (25.0)	45 (37.8)	60 (33.5)	
	Yes	45 (75.0)	74 (62.2)	119 (66.5)	
HbA_{1c} > 7%	Not assessed	1 (·)	0 (·)	1 (·)	0.0299
	No	25 (41.7)	70 (58.8)	95 (53.1)	
	Yes	35 (58.3)	49 (41.2)	84 (46.9)	
Height		1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	0.0429
Fibers >30 g/diet	Not assessed	13 (·)	22 (·)	35 (·)	0.8568
	No	26 (54.2)	51 (52.6)	77 (53.1)	
	Yes	22 (45.8)	46 (47.4)	68 (46.9)	
Hypertension	No	27 (44.3)	78 (65.5)	105 (58.3)	0.0061
	Yes	34 (55.7)	41 (34.5)	75 (41.7)	
Lipids >30%	Not assessed	13 (·)	22 (·)	35 (·)	0.0048
	No	28 (58.3)	78 (80.4)	106 (73.1)	
	Yes	20 (41.7)	19 (19.6)	39 (26.9)	
Metabolic syndrome	No	8 (13.1)	50 (42.0)	58 (32.2)	<0.0001
	Yes	53 (86.9)	69 (58.0)	122 (67.8)	

Data are expressed as mean ± SD or %. Two-sided *P* values refer to the Mann-Whitney *U* test for continuous variables and Pearson's χ^2 for categorical variables.

(b)

Variable	Category	Number of positive CAF tests		All	*P* value
		At least one	None		
Metabolic syndrome score	1	3 (4.9)	13 (10.9)	16 (8.9)	0.0001
	2	5 (8.2)	37 (31.1)	42 (23.3)	
	3	18 (29.5)	27 (22.7)	45 (25.0)	
	4	23 (37.7)	34 (28.6)	57 (31.7)	
	5	12 (19.7)	8 (6.7)	20 (11.1)	
Proteins >15%	Not assessed	13 (·)	22 (·)	35 (·)	0.0838
	No	24 (50.0)	34 (35.1)	58 (40.0)	
	Yes	24 (50.0)	63 (64.9)	87 (60.0)	

(b) Continued.

Variable	Category	Number of positive CAF tests		All	P value
		At least one	None		
Glucides >55%	Not assessed	13 (·)	22 (·)	35 (·)	0.4146
	No	6 (12.5)	8 (8.2)	14 (9.7)	
	Yes	42 (87.5)	89 (91.8)	131 (90.3)	
Sex	Female	23 (37.7)	40 (33.6)	63 (35.0)	0.5859
	Male	38 (62.3)	79 (66.4)	117 (65.0)	
Triglycerides		167.3 ± 92.1	140.9 ± 95.8	149.9 ± 95.1	0.0089
Waist circumference		104.9 ± 17.9	97.8 ± 12.6	100.2 ± 15.0	0.0146
Weight		85.0 ± 21.3	78.1 ± 14.7	80.4 ± 17.5	0.0597

Data are expressed as mean ± SD or %. Two-sided P values refer to the Mann-Whitney U test for continuous variables and Pearson's χ^2 for categorical variables.

(c)

Antidiabetic therapy	Sex	Patients/total
Diet alone	Male	34/117
	Female	17/63
Diet + metformin	Male	26/117
	Female	25/63
Diet + sulphonylureas or glinides	Male	15/117
	Female	3/63
Diet + metformin+ sulphonylureas or glinides	Male	27/117
	Female	8/63
Diet + insulin (basal/bolus)	Male	6/117
	Female	6/63

Notes. (1) The remaining 4 female subjects out of 63 were treated either with Insulin (basal/bolus) plus metformin or with insulin (basal/bolus) plus sulphonylureas or glinides plus metformin).
(2) The remaining 9 male subjects out of 117 were treated either with insulin basal plus glitazones and metformin or with insulin basal plus sulphonylurea and metformin.

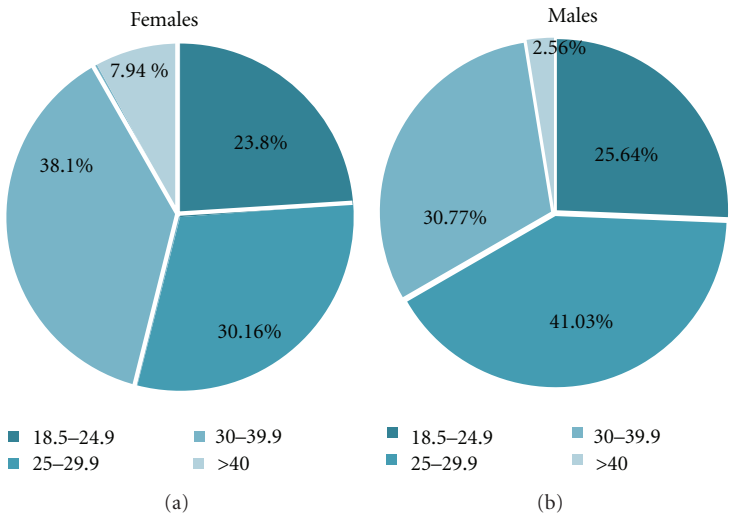

Figure 2: Distribution of BMI classes in the cohort.

CAF test was significantly associated to a lipid intake >30% (41.7% at least one versus 19.6% none $P = 0.0048$) (Table 3).

In a univariate analysis, a significant correlation was found between the mean number of tests positive for impaired CAF and a protein intake <15% and a lipid intake >30%. However, in the multivariate analysis protein and lipid content does not predict impCAF (Table 4).

5. Discussion

The study of both micro- and macroangiopathic complications of DM is crucial for both prognosis and therapeutic strategy. Among chronic microangiopathic complications of DM, CAN involves the cardiovascular branch of the autonomic nervous system (ANS) [14–17]. Because of CAN,

Table 3: Association between impaired CAF and anthropomentric and metabolic variables.

BMI ≥ 24.9 Kg/m^2	$P = 0,0032$
Hypertension	$P = 0,0061$
Waist > 94/80 cm	$P = 0,0146$
Tryglycerides > 150 mg/dL	$P = 0,0089$
$HbA_{1c} \geq 7\%$	$P = 0,0299$
Protein intake ≤15%/day	$P = 0,0838$
Lipid intake > 30%	$P = 0,0048$
Metabolic syndrome	$P < 0,0001$

BMI: Body mass index was calculated as Kg/m^2.

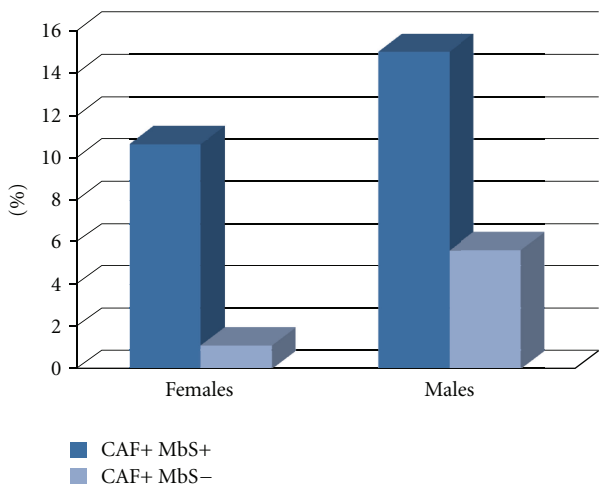

Figure 3: Prevalence of metabolic syndrome in the cohort in the presence or in the absence of impaired CAF. Abbreviations: CAF+, presence of impaired CAF; MbS+, presence of Metabolic syndrome; MbS−, absence of metabolic syndrome. Notes. Sixty patients out of 180 had an impaired CAF.

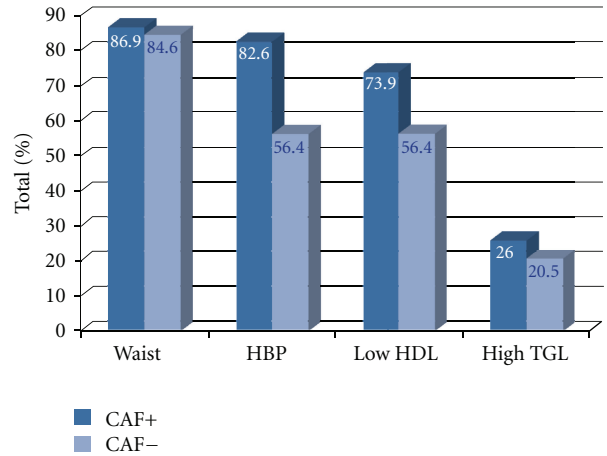

Figure 4: Distribution of different components of the metabolic syndrome in the female cohort in the presence or in the absence of impaired CAF. Abbreviations: CAF+: presence of impaired CAF; CAF−: absence of impaired CAF.

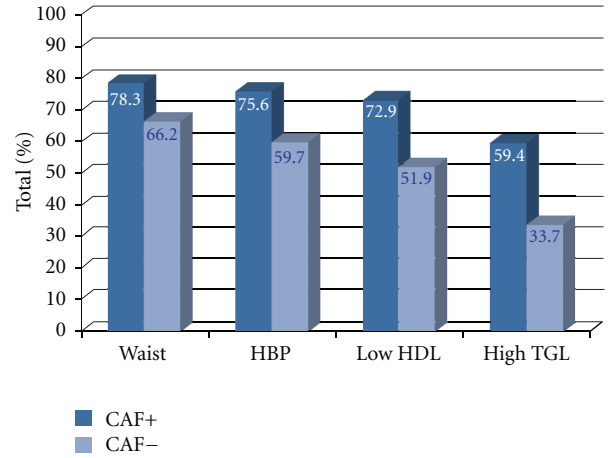

Figure 5: Distribution of different components of the metabolic syndrome in the male cohort in the presence or in the absence of impaired CAF. Abbreviations: CAF+: presence of impaired CAF; CAF−: absence of impaired CAF.

diabetics might experience silent MI, silent hypoglycaemia, and a high ASA risk during major surgery. ANS is anatomically poorly accessible, and few direct physiological tests are available to study CAF. Therefore, some indirect clinical tests are used as screening tests which detect deficits of CAF on the basis of heart responses to a simple stimulus [18]. In subjects with pathologic screening tests, the diagnosis might be completed with more sensitive techniques, but indirect screening tests help to select candidate subjects for more sophisticated analyses [18]. The diagnosis of CAN is usually done when at least 2 screening tests display pathologivc responses [18], but often when more than one test is already impaired and the diagnosis of CAN is made is not possible to reverse the situation. Vice versa sometimes early parasympathetic neuropathy may improve. In a longitudinal study Gottsäter et al. [19] demonstrated that after 7–10 years some subjects with parasympathetic neuropathy did not fulfill the criteria for the diagnosis anymore. Therefore we thought to consider as an early deficit of CAF the detection of at least one pathological test (score 1). CAF was analysed by utilizing five different tests. To each pathologic test, we gave score 1 to establish a grading of severity of impCAF. In our cohort of relatively young subjects with T2D, a score of impCAF higher than 2 was luckily rare, but the prevalence of at least one pathologic test was 33.9%. In two multicenter studies and a population study of type 2 diabetics, the prevalence of CAN was 16–22% [16, 20, 21]. The prevalence we found was slightly higher. However, in the above-mentioned studies, 2 screening tests (DB, LS) or 3 (DB, LS, PH) were used. By contrast, in our small cohort, 5 tests were always performed in triplicate thus increasing the sensitivity of tests. Concerning MbS, in our young cohort, 65% subjects had MbS according to IDF, but the prevalence of MbS among the subjects showing at least one pathologic test of CAF was more than 85%. A significant positive correlation between impaired CAF and MbS was confirmed with two different models of multivariate analysis. It was previously assessed

Table 4

	Variable	Age	BMI class			Family DM2	Duration > 5 yrs	Fibers > 30 g/die	Glucides 55%	HbA_{1c} > 7%	Family hypertension	Lipids > 30%	Metabolic syndrome	Proteins > 15%	Sex
	Category		*<25*	*25–30*	*≥30*										
Univariate	IRR	0.99	0.68	0.51	1.00	1.14	0.79	1.02	0.87	1.91	0.89	2.10	2.90	0.60	0.93
	IRR 95% CI	0.96–1.03	0.41–1.14	0.26–0.98	1.00-1.00	0.62–2.13	0.49–1.29	0.60–1.72	0.37–2.04	1.18–3.11	0.55–1.44	1.23–3.59	1.49–5.66	0.35–1.01	0.58–1.51
	P value	0.7451	0.1475	**0.0448**	—	0.669	0.351	0.9554	0.7531	**0.0087**	0.6265	**0.0062**	**0.0018**	***0.0567***	0.7748
Multivariate	IRR	0.98	1.45	2.32	1.00	1.18	0.71	1.44	0.71	2.02	0.92	1.93	2.53	0.65	0.93
	IRR 95% CI	0.93–1.04	0.69–3.02	0.82–6.53	1.00-1.00	0.53–2.64	0.39–1.29	0.76–2.72	0.25–2.03	1.09–3.73	0.52–1.63	0.82–4.50	1.08–5.90	0.30–1.39	0.51–1.69
	P value	0.4908	0.3265	0.1115	—	0.683	0.2609	0.2682	0.5197	**0.0256**	0.7664	0.13	**0.0319**	0.2649	0.802
Multivariate	IRR	0.98	1.4	2.45	1.00		0.7			1.92		1.97	2.50	0.76	1.00
	IRR 95% CI	0.94–1.03	0.68–2.91	0.88–6.85	1.00-1.00		0.39–1.25			1.07–3.46		0.87–4.47	1.08–5.78	0.38–1.52	0.55–1.80
	P value	0.5131	0.3614	***0.0863***	—		0.2241			**0.0297**		0.106	**0.0318**	0.4357	0.9944
Multivariate	IRR	0.98					0.72			1.95		1.47	1.88	0.85	1.08
	IRR 95% CI	0.94–1.03					0.40–1.29			1.08–3.53		0.73–2.93	0.90–3.96	0.43–1.66	0.60–1.92
	P value	0.5243					0.263			**0.0268**		0.2775	***0.0948***	0.6309	0.8003
Multivariate	IRR	0.98					0.72			1.96		1.62	1.90		1.06
	IRR 95% CI	0.94–1.03					0.40–1.30			1.08–3.54		0.92–2.85	0.90–3.99		0.60–1.89
	P value	0.5333					0.2797			**0.0261**		***0.097***	***0.0909***		0.8385
Multivariate	IRR	0.98								1.77		1.60	2.08		0.97
	IRR 95% CI	0.93–1.03								0.99–3.15		0.91–2.80	0.99–4.34		0.55–1.70
	P value	0.3911								***0.0529***		0.1021	***0.052***		0.9098
Multivariate	IRR	0.98								1.97			2.91		0.93
	IRR 95% CI	0.94–1.02								1.20–3.24			1.48–5.71		0.57–1.52
	P value	0.3205								**0.0075**			**0.0019**		0.7838
Multivariate	IRR	0.98								1.96			2.91		
	IRR 95% CI	0.94–1.02								1.19–3.22			1.48–5.71		
	P value	0.3223								**0.0078**			**0.0019**		
Multivariate	IRR									1.87			2.82		
	IRR 95% CI									1.15–3.04			1.44–5.51		
	P value									**0.0113**			**0.0024**		

Statistical analysis was performed with Poisson's model.

an association between parasympathetic dysfunction (pathologic cardiac response to DB) and some features of the MbS according to the WHO [22–24]. However, to our knowledge this is the first report stating that MbS, according to the criteria of IDF, is associated with a higher occurrence of an early deficit of CAF in a relatively young cohort of type 2 diabetics. In the same cohort, we also analysed the possible associations between the single components of MbS and the detection of an early deficit of CAF. However, score 1 was strongly associated with most of the components of the MbS.

We found a significant correlation between the occurrence of at least one pathologic test of CAF and a BMI > 25, which supports the negative role played by overweight on cardiovascular risk. The link between the high cardiovascular risk of T2D and overweight might be explained considering the negative effect played by overweight on glycaemic control. In this line of evidence in our cohort, a significant association was found between high HbA_{1c} values and CAF score. When subjects were stratified on HbA_{1c} values higher or lower than 7, a significant association was found between $HbA_{1c} > 7$ and the occurrence of at least one pathologic test. Many studies have already demonstrated that either an acute or a chronic poor glycaemic control might help the appearance of CAN [25–27]. From different meta-analyses the median value of mortality after 5 years was around 25% in diabetics with CAN and 4% in diabetics without CAN. If the diagnosis of CAN was based on the occurrence of 2 pathological tests, the relative risk of mortality was 3.5 [28, 29]. By contrast, an improvement in glycaemic control improves an early deficit of CAF or stops its progression [25]. In studies utilizing heart rate variability as an index of CAF, mild CAF abnormalities improved if HbA_{1c} values decreased from 9.5% to 8.4% [26].

Interestingly subjects showing impaired CAF also show different dietary habits as compared with subjects with normal CAF since they consume a higher daily fat intake (increased consumption of saturated fat derived from cheese and meet) as compared with diabetics with normal CAF. In some reports it has already been stated [30] that in subjects with MbS a Mediterranean-style diet (high content of whole grains, fruits, vegetables, nuts, and olive oil) improves chronic low-grade inflammatory state (reduction in serum concentrations of C-reactive protein, interleukin 6, insulin resistance, and improved endothelial function score) as compared to a balanced low-fat diet. Our data suggest that subjects with impaired CAF and MbS chose a wrong diet even if they were living in a Mediterranean area.

Unexpectedly no association was found between CAF score and the duration of diabetes or a positive family history of DM and/or HBP. In other papers a strong association was found between the duration of diabetes and CAN [23]. In several studies both PH and decreased heart rate variability are more frequent and evident 5 years after the diagnosis of Diabetes [23]. However, the subjects of our cohort were younger than those considered in previous studies and they experienced a program of education to healthy life style together with drugs (glitazone or insulin analogues) of last generation since the onset of diabetes, thus showing a metabolic memory better than that of subjects from previous studies.

The lack of association between any deficit of CAF and a positive family history of DM probably suggest that genetic and familiar factors might play a minor influence in compromising CAF as compared with environmental factors such as glycaemic control.

In conclusion, our data strongly suggest the role played by glycaemic control (assessed on the basis of HbA_{1c} values) and overweight on an early deficit of CAF. The more significant association between MbS and impaired CAF as compared with isolated T2D might suggest that the presence of MbS not only increases the global cardiovascular risk of diabetics not older than 55 years but also accelerates the appearance of a deficit of CAF which additionally increase cardiovascular risk.

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Research Article

The Effects of Green Tea Consumption on Cardiometabolic Alterations Induced by Experimental Diabetes

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We evaluated cardiac autonomic modulation by heart rate (HRV), and arterial pressure variability (APV), and metabolic response in streptozotocin diabetic rats treated with green tea. Male Wistar rats were separated in groups: control, drinking tap water (C), green tea-treated (GT) group, diabetic, drinking tap water (D), and diabetic, treated with green tea (DGT). Kidney mass was greater in D and DGT than in C and GT, but reduced in DGT compared to D. Green tea prevented the increase in creatinine clearance and reduced hyperglycemia in DGT compared to D. Arterial pressure was increased in GT and decreased in D compared to C. HRV was reduced in D compared with all groups. APV was decreased in D compared to C and recovery in DGT. Sympathetic modulation of APV was decreased in D compared with all groups. Green tea reduced hyperglycemia, prevented renal injury and autonomic dysfunction, suggesting reduced cardiovascular risk and target organ damage in diabetes.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by an impairment of carbohydrate, fat, and protein metabolism caused by either lack of insulin secretion or decreased sensitivity of the tissues to insulin [1]. This disease has reached epidemic levels in the United States and threatens a worldwide epidemic. The prevalence of diabetes is increasing rapidly, and the disease incidence in 2010 was about 285 million people worldwide, and is projected to increase to 438 million in 2030 [2].

A number of studies have been published concerning the association between chronic hyperglycemia and cardiovascular complications in DM, including systemic hypertension, atherosclerosis, nephropathy, coronary ischemia, vascular disease, and stroke [3–7]. Dysfunction of autonomic neural control is a frequent complication of diabetes which is associated with high morbidity and mortality [8], and has been found in clinical and animal models of DM [9, 10]. Methods to detect early abnormalities in cardiac autonomic modulation in DM have been applied such as heart rate and arterial pressure variability analysis.

Heart rate and blood pressure variability (HRV and BPV), estimated in time or frequency (spectral analysis) domain, have been widely used to evaluate the cardiac autonomic modulation in both clinical and experimental studies [11–16]. Heart rate as well as blood pressure oscillations at low frequency (LF: 0.2 to 0.75 Hz) is an index of cardiac sympathetic modulation, while high-frequency (HF: 0.75 to 3 Hz) oscillations of HR reflect parasympathetic modulation of the heart [17]. The importance of HRV and BPV was established with reports that showed a reduction in HRV and/or increased in BPV is associated with a large number of pathologies like diabetes and cardiovascular diseases [12, 16, 18, 19]. Moreover, there is evidence showing that decrease in HRV is an independent predictor of sudden cardiac death as well as increased BPV was associated with end-organ damage [20–22].

Green tea (leaves of *Camellia sinensis*, Theaceae) is a popular beverage in east Asia, and in recent years green tea has been widely studied to assess its beneficial effects in the treatment and prevention of human disease [23, 24]. Several epidemiological studies and clinical trials suggest that green tea consumption reduces the risk of many chronic diseases, including cardiovascular diseases [23, 24]. Moreover, green tea consumption improved glucose metabolism implicated in DM [25, 26], revealing that green tea consumption could be beneficial in the management of DM. Additional published studies showed that the administration of green tea to STZ diabetic rats promoted blood glucose reduction, hypolipidemic response, antioxidant effect, improved kidney function, and cardiac protection [27–29]. However, only partial protection of green tea against STZ-induced hyperglycemia and oxidative stress has also been reported [30], suggesting that these contradictory results could be due to variations in severity, duration, and treatment of the disease.

Although the literature provides evidence that green tea is useful to DM treatment, no studies to date have tested the potential of green tea to prevent autonomic dysfunction associated with DM. Specifically, the present study evaluates the autonomic modulation of cardiovascular function as measured by heart rate, arterial pressure variability, and the metabolic response in STZ diabetic rats subject to green tea consumption since diabetes induction.

2. Materials and Methods

2.1. Animals. Experiments were performed on adult male (eight-week-old) Wistar rats weighing 260–300 g. The animals were randomly separated in four experimental groups: control, drinking tap water (C, $n = 8$), green tea-treated group (GT, $n = 8$), diabetic, drinking tap water (D, $n = 9$), and diabetic, treated with green tea (DGT, $n = 10$). The animals were housed in cages with free access to water and food, at a constant temperature of 23°C, on a 12-hour light/dark cycle. All experimental protocols were in accordance with the Guidelines for Ethical Care of Experimental Animals and were approved by the Institutional Animal Care and Use Committee (Protocol: 025/09/2008).

After the experimental period, rats were killed by over dose of sodium pentobarbital anesthesia (50 mg/Kg i.p.) and blood glucose, and creatinine concentrations were determined in nonfasting animals using colorimetric enzyme kits (LABTEST, Brazil). The kidneys were quickly removed and the ratios of kidney: body weight (kidney mass index) were determined.

2.2. Diabetes Induction. Diabetes was induced by a single injection of STZ (50 mg/kg, i.v., Sigma Chemical Co, St Louis, MO, USA) dissolved in 0.05 M citrate buffer, pH 4.5, administered 21 days before the treatment. Controls (C and GT) were injected with the vehicle (0.05 M citrate buffer, pH 4.5) alone. Animals were fasted for 4 h before STZ or vehicle injection. During the 24-hr after STZ induction (D and DGT) or vehicle injection (C and GT), the animals were fed with glucose solution (12.5 g/L) to avoid ketoacidosis.

Two days following STZ injection, diabetes induction was confirmed by blood glucose level (10 μL of blood from tail vein) (Accu-Check Advantage Glucose Monitor, Roche Diagnostic Corporation, Indianapolis, IN, USA). Animals with blood glucose levels less than 250 mg/dL or greater than 400 mg/dL were excluded from the study to avoid great discrepancies in our data due to changes in glucose levels since it is well known the influence of glucose levels on metabolic and cardiovascular functions.

2.3. Green Tea Preparation, Analysis, and Treatment. Green tea was prepared daily by adding 3.0 g of dry green tea (Farmanatural, Natural Pharma, São Paulo, Brazil) to 1000 mL of boiled water cooled to 90°C. The solution was filtered after 15 min, cooled to room temperature, and dispensed into clean drinking bottles. The total content of caffeine and polyphenols in the green tea solution was 0.07% and 422.80 μg/mL, respectively. Green tea solution was administered for 21 days to the GT and DGT groups.

2.4. Metabolic Cages. In a subgroup of rats randomly selected (C, $n = 7$; GT, $n = 6$; D, $n = 6$; DGT, $n = 8$), at the end of the second week (day 14), animals were housed separately for 48 h in metabolic cages (Andrade's, São Paulo, Brazil) with free access to food and tap water or green tea solution. Hydric consumption, urinary excretion, and food intake during the last 24 h between 9:00 am and 10:00 am. Urine glucose and creatinine were measured using a colorimetric enzyme assay (LABTEST, Brazil).

2.5. Glucose Tolerance Test (GTT). At the end of the study, in a subgroup of randomly selected rats (C, $n = 7$; GT, $n = 5$; D, $n = 7$; DGT, $n = 5$), glucose was measured using an Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, Indianapolis, IN, USA). Animals were fasted for 4 h, given an intraperitoneal glucose load (1.5 g/kg, i.p.), and blood samples were taken at baseline and 15, 30, 60, and 90 min from a cut made on the tip of the tail.

2.6. Cardiovascular Measurements. One day prior to arterial pressure (AP) recordings, an arterial catheter was placed in the right femoral artery of study animals under ketamine-xylazine anesthesia (50 : 10 mg/kg i.p.), for the direct measurement of AP in all groups (C, $n = 8$; GT, $n = 8$; D, $n = 9$; DGT, $n = 10$). The catheter was exteriorized through the back of the neck.

During the study, AP was recorded during 30 minutes via a transducer (Hewlett-Packard 1280, USA) connected to the arterial catheter. Animals were conscious and moved freely during recording. The AP signal was fed into an amplifier (GPA-4 model 2, Stemtech Inc.) connected to a 16-channel analog-to-digital interface, and continuously sampled (2 kHz) on an IBM/PC (T23, IBM Thinkpad, Inc). Beat-to-beat values of systolic, diastolic, and mean AP (SAP, DAP and MAP, resp.) were determined, and heart rate (HR) or pulse interval (PI) were calculated as the interval between successive systolic pressure values using the software application WinDaq (DataQ Instruments, Inc., USA).

Table 1: Body weight, kidney mass index, and parameters obtained of metabolic cage.

	C	GT	D	DGT
Initial body weight (g)	280 ± 6	270 ± 9	265 ± 4	260 ± 8
Final body weight (g)	335 ± 14.0	320 ± 11	245 ± 14*	235 ± 18*
Left kidney mass index (mg/g)	3.7 ± 0.2	4 ± 0.2	6.3 ± 0.2*	5.0 ± 0.2*#
Right kidney mass index (mg/g)	3.6 ± 0.1	4.3 ± 0.2	6.5 ± 0.2*	5.2 ± 0.2*#
Food intake (g)	15 ± 1.6	11 ± 2.5	23 ± 6.6*	31 ± 4*
Hydric consumption (mL/24 h)	19 ± 1.7	21 ± 2.6	73 ± 4.6*	90 ± 15*
Urinary excretion (mL/24 h)	6 ± 0.6	8 ± 0.7	61 ± 12*	77 ± 12*

C: control, drinking tap water group; GT: green tea-treated group; D: diabetic, drinking tap water; DGT: diabetic, treated with green tea group. Initial and final body weight (g) and kidney mass index (mg/g): C ($n = 8$), GT ($n = 8$), D ($n = 9$), and DGT ($n = 10$). Metabolic cage: C ($n = 7$), GT ($n = 6$), D ($n = 6$) and DGT ($n = 8$). $^{*}P < 0.05$ versus C and GT; $^{\#}P < 0.05$ versus D.

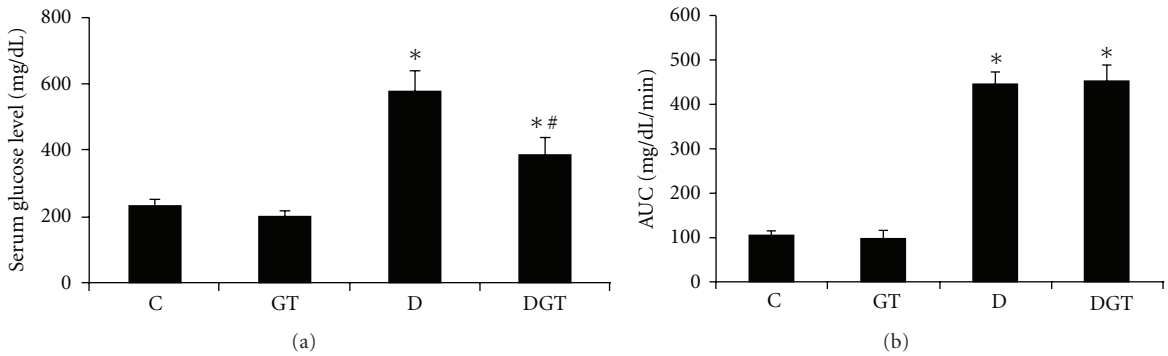

Figure 1: Serum glucose concentration (a) and area under curve (AUC) of blood glucose (b) during glucose tolerance test. C: control, drinking tap water group; GT: green tea-treated group; D: diabetic, drinking tap water; DGT: diabetic, treated with green tea group. Serum glucose: C ($n = 8$), GT ($n = 8$), D ($n = 9$), and DGT ($n = 10$). Glucose tolerance test: C ($n = 7$), GT ($n = 5$), D ($n = 7$), and DGT ($n = 5$). GTT: C ($n = 7$), GT ($n = 5$), D ($n = 7$) and DGT ($n = 5$). $^{*}P < 0.05$ versus C and GT; $^{\#}P < 0.05$ versus D.

PI and SAP fluctuations were assessed in the time and frequency domains using autoregressive spectral analysis, as described elsewhere [12, 15, 31, 32]. The theoretical and analytical procedures for autoregressive modeling of oscillatory components have been fully described previously [12, 13, 17]. Briefly, the PI and SAP series derived from each recording were divided into 300-beat segments with a 50% overlap. The spectra of each segment were calculated via the Levinson-Durbin recursion, and the order of the model chosen according to Akaike's criterion, with the oscillatory components quantified in low-(LF: 0.2 to 0.6 Hz) and high-frequency (HF: 0.6 to 3.0 Hz) ranges.

2.7. Statistical Analyses. Data are reported as mean ± SEM. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by the Bonferroni test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. The Effect of Green Tea on Metabolic Measurements. Table 1 shows the effect of diabetes and green tea consumption on body weight and kidney mass index. No differences in body weight were observed among groups in the beginning of the experiment. However, both diabetic groups (D and DGT animals) showed lower body weight compared to nondiabetic control groups (C and GT animals) after the period of 21 days ($P < 0.05$). There was no significant difference between the mean body weights of the D group compared with DGT showing that green tea consumption did not prevent weight loss in diabetic animals. In fact, while the groups C and GT showed a significant increase in body weight (20% and 19%, resp., $P < 0.05$), a significant reduction in body weight was observed in D (7.5%) and DGT (9.6%) groups at the end of the study. The kidney mass index was greater in D and DGT than in C and GT animals ($P < 0.05$). However, green tea intake reduced the kidney mass index in DGT animals compared with D (5.0 ± 0.2 versus 6.3 ± 0.2 mg/g, resp., $P < 0.05$).

The induction of diabetes mellitus in the experimental animals was confirmed by blood glucose value above 250 mg/dL 48 h after STZ induction. D and DGT animals developed hyperglycemia (577 ± 62 mg/dL and 384 ± 52 mg/dL, resp.), their serum glucose concentrations being significantly higher than nondiabetic animals (C, 232 ± 16 mg/dL and GT, 202 ± 16 mg/dL). Diabetic rats treated with green tea (DGT) showed lower hyperglycemia than diabetic rats not treated with green tea (D), suggesting that green tea consumption protects from severe hyperglycemia in diabetic rats. It is important to note that green tea did not change the serum glucose level in GT animals (Figure 1(a)). Despite

Table 2: Changes in urine, serum glucose, and creatinine clearance after 21 days of treatment with green tea in diabetic rats induced by streptozotocin.

Groups	C	GT	D	DGT
Urine glicose (mg/dL)	3 ± 1.7	14 ± 3.2	845 ± 94*	637 ± 58*#
Serum glicose (mg/dL)	232 ± 16	202 ± 16	577 ± 62*	384 ± 52*#
Creatinine clearance (mL/min/Kg)	0.21 ± 0.03	0.43 ± 0.09	1.1 ± 0.3*	0.75 ± 0.1*#

C: control, drinking tap water group; GT: green tea-treated group; D: diabetic, drinking tap water; DGT: diabetic, treated with green tea group. Serum glucose: C (n=8), GT (n=8), D ($n = 9$), and DGT ($n = 10$). Urine glucose: C ($n = 7$), GT ($n = 6$), D ($n = 6$), and DGT ($n = 8$). $^*P < 0.05$ versus C and GT; $^\#P < 0.05$ versus D.

Table 3: Mean arterial pressure, heart rate, and respective variabilities in time and frequency domains in diabetic rats subject to green tea treatment.

	C ($n = 8$)	GT ($n = 8$)	D ($n = 9$)	DGT ($n = 10$)
MAP(mmHg)	106 ± 2	128 ± 3#	99 ± 1.5*	111 ± 4
HR (bpm)	360 ± 7	341 ± 8	295 ± 11*	328 ± 14
HRV (ms^2)	18 ± 1.3	16 ± 1.1	8.8 ± 0.7*	15 ± 1.6
APV ($mmHg^2$)	23 ± 3.2	31 ± 7.5	7.5 ± 0.2*	15 ± 4.4
LF ($mmHg^2$)	2.5 ± 0.36	3.8 ± 0.6	0.9 ± 0.4*	3.1 ± 0.8

C: control, drinking tap water group; GT: green tea-treated group; D: diabetic, drinking tap water; DGT: diabetic, treated with green tea group. MAP: mean arterial pressure; HR: heart rate; HRV: heart rate variability; APV: arterial pressure variability; LF: low-frequency domain of APV. $^*P < 0.05$ versus all groups; $^\#P < 0.05$ versus C.

hyperglycemia suppression by green tea, we observed no differences in the area under curve (AUC) of the glucose tolerance tests between D (446 ± 29 mg/dL/min) and DGT (454 ± 39 mg/dL/min), nor between C (109 ± 9 mg/dL/min) and GT (101 ± 18 mg/dL/min) groups, confirming that green tea did not change glucose tolerance in STZ-induced diabetic rats (Figure 1(b)).

As shown in Table 1, diabetic animals (D and DGT) showed signs of polydipsia, polyphagia, and polyuria. No significant differences between diabetic groups (D versus DGT) nor between control animals (C versus GT) were observed. The administration of green tea did not exert a significant effect on water or food intake as well as in urine volume collected in metabolic cages over 24 hours. However, it is important to note that DGT animals ingested higher amount of green tea (0.3 ± 0.05 g/day) than GT animals (0.06 ± 0.01 g/day). Glomerular filtration rate assessed by creatinine clearance (Table 2) was increased in diabetic (D and DGT) compared with nondiabetic rats C and GT ($P < 0.0001$). Green tea consumption prevented the strong increase in creatinine clearance in the DGT group (0.75 ± 0.1 mL/min/Kg) compared with D group (1.1 ± 0.3 mL/min/Kg). However, in nondiabetic rats there was no statistically significant effect of green tea on creatinine clearance (0.21 ± 0.03 versus 0.43 ± 0.09 mL/min/Kg, C and GT resp.). Finally, diabetic rats treated with green tea showed a significant reduction in urine glucose compared with D. Although, there was a difference between C and GT in urine glucose, it was not statistically significant (Table 2).

3.2. The Effect of Green Tea on Cardiovascular Measurements. The values obtained for MAP and HR are presented in Table 3. Diabetes induced by STZ promoted attenuation in MAP (∼10% of reduction) as well as in HR (∼20% of reduction) in D group. Green tea intake prevented the reduction of both parameters, MAP and HR in diabetic animals. However, in nondiabetic animals green tea increased MAP (∼20% of increase) with no alterations in HR. The total power of the HRV was reduced in D in comparison with all groups. The APV decrease in D compared to C and recovery in DTG. Sympathetic modulation of the arterial pressure variability (LF) decreased in D compared with groups C, GT, and DGT.

4. Discussion

The present study was conducted to evaluate the role of green tea consumption in metabolic response and cardiovascular autonomic modulation in STZ-induced diabetic rats. Experimental diabetes induced by STZ is a well-established method used to evaluate the mechanisms involved in the alterations of physiopathology observed in diabetic patients. STZ destroys pancreatic b cells, resulting in a diabetic syndrome in animals, similar to that seen in human type 1 diabetes and characterized by hyperglycemia, hypoinsulinemia, glucosuria, and loss in body weight [33–35].

Although the nondiabetic rats registered approximately 20% growth in body weight, diabetic rats showed reduced body weight and green tea extract treatments did not improve weight gain in STZ-treated animals. These responses were also observed by Renno et al. [29] and Juśkiewicz et al. [30]. In other studies, the consumption of green tea prevented the loss in body weight in STZ-treated rats [27, 28]. However, the contradictory results could be due to the differences in dosage and methods used in dietary treatment. Despite green tea plays an important role in the regulation of body weight [36], our results showed that the body weight of nondiabetic rats, C and GT, was similar. This response may be associated with the lower content of caffeine in the green

tea extract (0.07%) used in the present study, if we considered that the caffeine is the main factor involved in thermogenesis, fat oxidation, and sparing fat-free mass [37].

In the present study, diabetic-treated (DGT) and untreated (D) animals showed serum, and urine glucose levels elevated compared with C group. However, we observed a significant reduction in both serum and urine glucose levels in the DGT group as compared to the D group. Considering that hyperglycemia is the principal factor responsible for kidney and cardiovascular damage [7], the antihyperglycemic effect of green tea observed in the DGT group suggests an important clinical relevance to diabetes treatment. In support of the present results, recent reports have shown that green tea administration caused similar antihyperglycemic effect in STZ induced rats [28, 29], in diabetic db/db mice [25] and in diabetes type 2 patients [38]. On the other hand, the effect of green tea on glucose tolerance was not observed in both nondiabetic (GT) and diabetic (DGT) groups. Similar results with glucose tolerance were shown by Wu et al. [39].

With regard to metabolic parameters, STZ rats showed polydipsia, polyphagia, and polyuria [27]. However, the administration of green tea in this study did not exert a significant effect on liquid intake or food intake as well as in urine volume since no significant differences among groups were observed. Despite our results were similar to that showed by Tomlinson et al. [34], Babu et al. [27] observed decreased food and water intake in diabetic rats treated with green tea. The differences may be associated with the period and moment that green tea treatment was introduced in STZ rats. In the present study, green tea was administrated for 21 days following the first day of diabetes identification while in the other study green tea was administrated for 4 weeks and the treatment was begun 6 weeks after the onset of diabetes [27].

Glomerular filtration rate assessed by creatinine clearance was measured in our study to provide information about renal function. Diabetic rats (D and DGT) increased creatinine clearance compared with nondiabetic rats C and GT. It is interesting that green tea consumption resulted in lower creatinine clearance in DGT compared with D, but did not affect nondiabetic rats (C versus GT), thus, our data suggests that green tea prevents glomerular hyperfiltration in diabetes. In support with our data, it has been reported that in rats with streptozotocin-induced diabetes, green tea consumption showed a significant reduction in renal injury associated with hyperglycemia [29].

Diabetes caused an abnormal increase in kidney mass index in both diabetic groups D and DGT. Interestingly, green tea consumption by DGT group significantly reduced diabetes-induced hypertrophy of kidney by 20% as compared to the D group. These results suggest the importance of green tea in reducing kidney hypertrophy and corroborate another study with STZ rats [30]. Creatinine clearance as well as kidney mass are important markers of nephropathy and our data are in agreement with Ribaldo et al. [40] that showed a decrease in the markers of nephropathy in diabetic hypertensive rats following the consumption of green tea.

In the present study, we observed in rats with STZ-induced diabetes a reduction in AP and HR associated with a decrease in HRV and APV as previously described [10, 41, 42]. On the other hand, there are reports of increased blood pressure [43–45] or no change in STZ-induced diabetic rats [46]. Some of discrepancies in the literature regarding BP changes in STZ rats may be due to differences in age and time of experimentation as well as the methodology for BP measurement. However, the new data is that green tea consumption was able to prevent all of these cardiovascular alterations. Since the HRV and APV are useful tools to evaluate autonomic modulation of the cardiovascular system in humans and experimental models, our data suggests that green tea consumption was able to reduce the autonomic neuropathy observed in diabetes. It is well accepted that diabetes is associated with elevated oxidative stress [47, 48], which is correlated with alterations in the autonomic control of the circulation [14, 49].

The favorable effects attributed to the green tea extract in the prevention of cardiovascular diseases are correlated with the antioxidant properties of the catequins, which are the major components of green tea [50]. Experimental studies have been demonstrated the increase in total plasma antioxidant activity determined by green tea catequins [51, 52]. Moreover, *in vitro* studies have demonstrated that green tea extracts and tannin mixtures have a direct scavenging activity against nitric oxide and superoxides [53]. Thus, we can hypothesize that antioxidants, such as green tea may have beneficial effects on the cardiovascular autonomic nervous system. In fact, previous studies showed that the administration of antioxidants, like vitamin E, exerts beneficial effects on the cardiac control by rebalancing autonomic nervous system in diabetes [54]. Therefore, when taken together, these data support the conclusion that other studies need to be carried out to better understand the mechanisms involved in green tea pharmacological action and also reveal that indiscriminate chronic consumption of green tea might be a risk for health individuals, since in our study we have observed that the green tea ingestion determined an increase in blood pressure in GT.

In summary, the major findings of this study are that green tea decreased hyperglycemia and prevented renal injury by improvement of glomerular filtration and kidney hypertrophy observed in diabetic rats. Moreover, green tea was able to prevent the autonomic dysfunction in diabetic rats by blocking the alterations in arterial pressure variability. In conclusion, our data suggest that green tea consumption has important effects in reducing the cardiovascular risk and in targeting organ damage observed in diabetes.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Dynamic Aerobic Exercise Induces Baroreflex Improvement in Diabetic Rats

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The objective of the present study was to investigate the effects of an acute aerobic exercise on arterial pressure (AP), heart rate (HR), and baroreflex sensitivity (BRS) in STZ-induced diabetic rats. Male Wistar rats were divided into control ($n = 8$) and diabetic ($n = 8$) groups. AP, HR, and BRS, which were measured by tachycardic and bradycardic (BR) responses to AP changes, were evaluated at rest (R) and postexercise session (PE) on a treadmill. At rest, STZ diabetes induced AP and HR reductions, associated with BR impairment. Attenuation in resting diabetes-induced AP (R: 103 ± 2 versus PE: 111 ± 3 mmHg) and HR (R: 290±7 versus PE: 328±10 bpm) reductions and BR dysfunction (R: −0.70±0.06 versus PE: −1.21±0.09 bpm/mmHg) was observed in the postexercise period. In conclusion, the hemodynamic and arterial baro-mediated control of circulation improvement in the postexercise period reinforces the role of exercise in the management of cardiovascular risk in diabetes.

1. Introduction

Diabetes mellitus is commonly associated with a large number of complications. Patients with diabetes are particularly prone to disorders affecting the control of the cardiovascular system, including microangiopathy, atherosclerosis, hypertension, and autonomic neuropathy. It has been frequently reported that diabetes can affect both somatic and autonomic nerves. Autonomic neuropathy is the most serious complication of diabetes in terms of morbidity and mortality [1–3].

Baroreflex dysfunction observed in diabetic subjects has important clinical implications, because the arterial baroreceptors constitute an important system that acts against wide oscillations in arterial pressure (AP), acting on both the sympathetic and parasympathetic limbs of the autonomic nervous system. Additionally, clinical trials have shown an association between baroreflex dysfunction and morbidity and mortality [3–5].

Studies using experimental models have been conducted to investigate the mechanisms of autonomic cardiovascular reflex dysfunction in diabetes [6–13]. We have demonstrated that, in the course of streptozotocin-(STZ-) induced experimental diabetes, baroreflex control of circulation was impaired [7, 9, 11–13]. Also, we have previously demonstrated the benefits of exercise training in diabetes-induced cardiovascular, tonic and reflex autonomic dysfunction in rats [10–13]. Furthermore, Loimaala et al. [14] have demonstrated that 12 months of exercise training applied to type 2 diabetic patients without autonomic neuropathy-induced improvement in baroreflex sensitivity (BRS). In fact, studies have demonstrated that physical activity delays or improves the hemodynamic and metabolic dysfunction observed in diabetes and should be considered in prevention and treatment of this disease [15, 16]. However, there is little data on the effects of acute exercise (a single exercise bout) on diabetics, especially on hemodynamics and BRS.

Although cardiovascular effects of acute exercise have been studied in nondiabetic and hypertensive rats [17–19], they have not been investigated in diabetic rats. Hence, a more complete understanding of acute exercise and the

Table 1: Hemodynamic evaluations in control and diabetic rats at rest and postdynamic aerobic exercise.

	Control at rest	Diabetic at rest	Control postexercise	Diabetic postexercise
SAP (mmHg)	126 ± 2	115 ± 3*	126 ± 2	124 ± 3†
DAP (mmHg)	98 ± 3	86 ± 3*	95 ± 2	96 ± 3†
MAP (mmHg)	113 ± 2	102 ± 2*	112 ± 2	111 ± 3†
HR (bpm)	350 ± 10	290 ± 7*	378 ± 12	328 ± 10*†

Values are means ± SEM. *$P < 0.05$ versus controls in similar state; †$P < 0.05$ versus diabetics at rest. Systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), heart rate (HR).

health benefits that it may promote, for example, in the postexercise period, could provide useful information to help prevention, management, and treatment of the diabetes mellitus. Therefore, the objective of the present study was to investigate the effects of a single bout of dynamic aerobic exercise on HR, AP, and BRS in STZ-induced diabetic rats.

2. Methods

Male Wistar rats (3 mo, 200–300 g) were obtained from the breeding facility of the University of Sao Judas Tadeu (São Paulo, Brazil). Rats received standard laboratory chow and tap water ad libitum and were housed in temperature-controlled rooms (22°C) under a 12:12 h dark-light cycle. All animal protocols were approved by the Experimental Animal Use Committee of the University of Sao Judas Tadeu and were conducted in accordance with the National Research Council's guide for the care and use of laboratory animals. Rats were randomly assigned to control (C, $n = 7$) or diabetic (D, $n = 7$) groups.

Diabetes was induced by a single intravenous injection of STZ (50 mg/kg; Sigma, St. Louis, MO, USA) after an overnight fast (8–10 h). Control rats received only vehicle (10 mM citrate buffer, pH 4.5) after a similar fasting period. Blood glucose was measured to confirm the diabetic-induced hyperglycemia 29 days after STZ injection (Accu-Check Instant test, Roche, Brazil). The resting and acute exercise hemodynamic evaluations started 30 days after either STZ or citrate buffer administration [6, 7, 9–13].

All animals were progressively adapted to exercise (10 min/day at 0.3 km/h) for 5 days (23 to 27 days after STZ or buffer injection) on a treadmill before the start of the acute exercise protocol. After adaptation, both control and diabetic rats were submitted to a dynamic aerobic exercise session on a treadmill with a gradual 0.3 km/h speed increase, that is, from 0.3 km/h to 0.9 km/h (three minutes at each load stage) [19].

Twenty-eight days after STZ-diabetes induction, rats were anaesthetized (ketamine-xylazine 80 : 40 mg/kg ip), and a polyethylene-tipped Tygon cannulas (4 cm of PE-08 connected to 2 cm of PE-50) filled with heparinised saline solution were inserted into the common carotid artery and jugular vein for direct measurements of arterial pressure and drug administration, respectively. Hemodynamic measurements were carried out in conscious and active rats 48 hours after catheters implantation (30 days after STZ or buffer injection). The arterial cannula was connected to a transducer (Kent Instrumental, USA) and AP signals were recorded using a microcomputer equipped with an analog-to-digital converter (CODAS, 2-kHz sampling frequency, Dataq Instruments, USA). The AP and the heart rate (HR) were recorded at rest (20 min) [6, 7, 9–13, 19]. Immediately after, increasing doses of phenylephrine and sodium nitroprusside were given sequentially as bolus injections to produce at least four pressure responses, ranging from 5 to 40 mmHg at rest. Peak increases or decreases in MAP after phenylephrine or sodium nitroprusside injections and the corresponding peak reflex changes in HR were recorded for each dose of the drug. A time interval between doses was necessary for blood pressure and heart rate to return to baseline. Beat-to-beat analysis was performed to quantify changes in MAP and HR as previously described. BRS was evaluated by two methods: linear regression and mean index. The linear regression method reported values derived from fitting sensitivity indices to through points corresponding to all changes in HR related to the induced changes in MAP [12, 13]. The mean index method related maximum changes in HR to the maximum changes in MAP after each dose of vasoactive drugs [11].

At least 2 hour after AP and BRS resting evaluations, the animals were submitted to acute exercise protocol on a treadmill. The AP was recorded during the postexercise period (5–25 minutes after the exercise ended) and the BRS was evaluated as described above in the postexercise period (started 30 min after exercise).

Data are expressed as means ± SEM. Student's unpaired t-test (glycemia and body weight) and two-way ANOVA (hemodynamic and BRS evaluations) were used to compare groups, followed by the Student-Newman-Keuls test. Significance level was established at $P < 0.05$.

3. Results

STZ-induced diabetic rats presented hyperglycemia (C: 97 ± 12 versus D: 362 ± 24 mg/dL, $P < 0.001$) and reduced body weight (C: 303 ± 5 versus D: 246 ± 12 g, $P < 0.001$).

STZ diabetes significantly reduced systolic AP, diastolic AP, MAP ($P < 0.05$), and HR ($P < 0.001$) when compared with control rats (Table 1).

As can be seen in Table 1, acute dynamic aerobic exercise induced attenuation in diabetic resting hypotension and bradycardia ($P < 0.05$) during the recovery period (5–25 min after exercise). No differences in AP were observed between diabetic rats and control groups during the postexercise period ($P > 0.05$). However, HR remained reduced in

Table 2: Baroreflex sensitivity evaluated by mean index method or by linear regression method in control and diabetic rats at rest and postdynamic aerobic exercise.

	Control at rest	Diabetic at rest	Control postexercise	Diabetic postexercise
Mean index method				
TR (bpm/mmHg)	3.69 ± 0.10	3.42 ± 0.32	3.50 ± 0.10	4.51 ± 0.28
BR (bpm/mmHg)	−1.28 ± 0.10	−0.70 ± 0.06*	−1.52 ± 0.10	−1.21 ± 0.09†
Linear regression method				
TR (bpm/mmHg)	3.56 ± 0.38	3.27 ± 0.29	3.57 ± 0.32	4.15 ± 0.37
BR (bpm/mmHg)	−1.54 ± 0.27	−0.74 ± 0.31*	−1.95 ± 0.25	−1.19 ± 0.34†

Values are means ± SEM. *$P < 0.05$ versus controls in similar state; †$P < 0.05$ versus diabetics at rest. Bradycardic reflex response (BR), tachycardic reflex response (TR).

diabetic rats in relation to control rats in the period ($P < 0.05$).

The BRS evaluation demonstrated that the baroreflex tachycardic responses elicited by sodium nitroprusside were not significantly reduced in diabetic rats in relation to controls at rest ($P > 0.05$). However, the baroreflex bradycardic responses evaluated by linear regression ($P < 0.05$) or mean index methods ($P < 0.01$) were significantly reduced in diabetic animals as compared to controls at rest (Table 2).

Acute dynamic aerobic exercise did not alter baroreflex tachycardic responses in diabetic rats ($P > 0.05$). However, exercise induced a significant increase in bradycardic reflex responses evaluated by linear regression ($P < 0.05$) or mean index methods ($P < 0.01$) in diabetic rats in the postexercise period as compared to diabetic resting values. In control rats, BRS did not significantly differ between rest and postexercise ($P > 0.05$) (Table 2).

4. Discussion

The major new insight of the present investigation is that a single bout of dynamic aerobic exercise improves diabetes-induced BRS impairment and attenuated resting hemodynamic dysfunctions. Furthermore, this study also confirms our preliminary findings that STZ-diabetes induces hypotension, bradycardia, and BRS impairment [6, 7, 9–13].

In the present study, we observed an increase in AP and HR in the postexercise period in diabetic rats. Jackson and Carrier [20] have suggested that the decrease in AP in STZ-induced diabetic rats at rest may be the result of a decreased cardiac output due to hypovolemia caused by hyperglycemic osmotic diuresis. However, Cohen et al. [21] have observed that these animals were polyuric with a high urine flow, reflecting the osmotic diuretic effects of glucose. Despite the mechanism, several studies have demonstrated impaired cardiac function in STZ-diabetic rats [3, 22–24]. In this aspect, the AP postexercise normalization in comparison to control rats in the present study could be ascribed to a better ventricular contractility and to an enhanced resting HR, as observed previously in trained diabetic rats [3, 22, 23]. Moreover, the reduction in HR in diabetic animals at rest has been attributed to changes in the sinoatrial node [3, 6, 13], although functional alterations in the cholinergic mechanism cannot be excluded as a causal factor. In this regard, the attenuation of resting bradycardia in the postexercise period in the present study may be related to changes in intrinsic HR or sympathovagal cardiac balance as previously observed in trained diabetic rats [3, 12, 13]. Regarding the physiological importance, the AP and HR changes during the recovery period in diabetic rats may reflect a transitory improvement in autonomic control of circulation and can represent a better perfusion pressure to the tissues.

Regarding BRS, it is well known that exercise training improves baroreflex control of circulation in normotensive and diabetic in animals and humans [11–14, 25–28]. Figueiroa et al. [29] have demonstrated that endurance exercise training reduced blood pressure without changes in heart rate variability (HRV) and BRS at rest, but training increased HRV and BRS during the recovery of acute endurance exercise, indicating an improved postexercise autonomic modulation of HR, which was similar in obese women with and without type 2 diabetes. In the present paper we demonstrated, for the first time, that a single bout of exercise restores previously impaired baroreflex-mediated bradycardic responses in sedentary diabetic rats. In this regard, it is worth emphasizing that baroreceptor cardiac reflex sensitivity abnormalities in diabetic patients increase the risk of sudden cardiac death [1, 2]; BRS improvement after each exercise session can favorably modify long-term survival as demonstrated in postmyocardial infarction patients [4, 5].

Baroreflex improvement induced by acute exercise in the present study may be associated with transitory changes in baroreceptors, in central nervous system, or in efferent fibers to the effectors organs. Although in the present paper we have not investigated these pathways, vagal function impairment in diabetic rats has been previously described by our group [6, 10, 12, 13]. Moreover, exercise training in diabetic male rats induced a 40% increase in vagal tonus as compared to sedentary STZ-rats [10]. A similar increase in vagal function could also occur after each exercise session in sedentary diabetic rats in the present investigation, which can represent an increase in the vagal reserve used during HR responses evoked by the baroreceptors. Additionally, an increase in vascular compliance [30] and/or an enhancement in shear stress during exercise may induce the release of endothelial factors [31], increasing the arterial baroreceptor afferent sensitivity [26].

Diabetic autonomic neuropathy is a serious complication found in one-fourth of type 1 and one-third of type 2 diabetic patients [2, 32]. The effects of diabetic autonomic dysfunction are seen as changes in autonomic modulation of the cardiac sinus node, resulting in reduced heart rate variability, which is strongly (i.e., relative risk is doubled) correlated with an increased risk of silent myocardial ischemia and resultant mortality [2, 33]. Furthermore, reduced BRS is a well-documented indicator of increased risk for mortality and morbidity in nondiabetics and diabetics [1–5]. Given these findings, the arterial baro-mediated control of circulation improvement in the postexercise period demonstrated in the present study reinforces the role of exercise in the management of cardiovascular risk in diabetic individuals.

5. Conclusion

In conclusion, a single aerobic exercise session induced attenuation of hemodynamic impairment associated with baroreflex improvement in STZ-induced diabetic rats.

Conflict of Interests

The authors have no conflicts of interest to disclose.

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Research Article

Cardiac Autonomic Imbalance in Newly Diagnosed and Established Diabetes Is Associated with Markers of Adipose Tissue Inflammation

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Introduction. Diabetics die from cardiovascular disease at a much greater rate than nondiabetics. Cardiac autonomic imbalance predicts increased cardiovascular risk and mortality. We studied the relationship between cardiac autonomic imbalance and adipose tissue-derived inflammation in newly diagnosed and established type 2 diabetes. *Materials and Methods*. Non-diabetics, newly diagnosed diabetics, and established diabetics were included. Anthropomorphic and biochemical measurements were obtained, and insulin resistance was approximated. Cardiac autonomic function was assessed using conventional measures and with power spectral analysis of heart rate. *Results and Discussion*. Heart rate variability was reduced in all diabetics. Interleukin-6 was higher in diabetics, as was the high molecular weight adiponectin-to-leptin ratio. Interleukin-6 correlated negatively with measures of autonomic balance. Ratios of adiponectin to leptin correlated positively with measures of autonomic balance. Cardiac autonomic imbalance and inflammation occur early in diabetes and are interrelated. *Conclusions*. Cardiac autonomic imbalance correlates with the adipose tissue-derived inflammation seen early in type 2 diabetes.

1. Introduction

Greater than 50% of adults with type 2 diabetes (T2D) have coronary artery disease (CAD), and death from CAD is two- to five-times more likely in a diabetic than in a nondiabetic patient [1]. Many individuals with diabetes have silent myocardial ischemia, and may succumb to cardiovascular disease (CVD) before presenting for further evaluation and treatment. As demonstrated by the Detection of Silent Myocardial Ischemia in Asymptomatic Diabetic Subjects (DIAD) study, traditional risk factors for CVD such as dyslipidemia and hypertension do not accurately predict all individuals with silent myocardial ischemia [2]. Forty percent of patients with silent ischemia are missed using only these traditional risk factors. Clearly, better predictors of myocardial ischemia are needed.

Cardiovascular autonomic neuropathy (CAN), defined as abnormalities of the cardiovascular system as a result of damage to the autonomic fibers innervating the heart and vasculature, is associated with increased major cardiovascular events, as well as increased mortality after a myocardial infarction, in diabetic patients [3–5]. In the DIAD study, a diminished Valsalva heart rate ratio (a measure of CAN) was strongly associated with silent myocardial ischemia, independent of more traditional risk factors including gender, age, hypertension, and smoking [2]. Symptoms of more generalized autonomic neuropathy were also associated with myocardial ischemia, including orthostatic hypotension, erectile dysfunction, and postmeal bloating. In the European Epidemiology and Prevention of Diabetes (EURODIAB) study, autonomic dysfunction was present in one-third of type 1 diabetic (T1D) subjects, and was strongly associated with coexisting CVD after adjustment for age, hemoglobin A1c (HbA1c), and duration of diabetes [6]. Results from the recently reported Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial, which showed an increased

mortality in T2Ds at increased risk for CVD randomized to receive intensive glycemic control, demonstrated that individuals with CAN were 1.55- to 2.14-times as likely to die (all-cause mortality) during the study when compared to those without CAN [7]. Death from CVD (including that related to arrhythmia, myocardial infarction, heart failure, cardiovascular interventions, and stroke) was even more likely (1.94–2.95-times) in subjects with CAN. Together, these data suggest that factors indicating autonomic dysfunction may be better predictors of cardiac events than more traditional risk factors, and their early identification could prove to be salutary for CVD prevention [8].

Inflammation plays a key role in the development of both T2D and atherosclerosis. Obesity, a well-known risk factor for both diabetes and CVD, is an inflammatory condition, and adipose tissue, especially abdominal or visceral fat, produces a variety of proinflammatory cytokines involved in the development of insulin resistance and atherosclerosis [9]. These proinflammatory "adipokines," including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), lead to increased release of free fatty acids (FFA) from adipose tissue that stimulate increased hepatic production of proatherogenic lipids such as very-low-density lipoprotein (VLDL), and prothrombotic proteins such as plasminogen activator inhibitor-1 (PAI-1) [10]. The adipocyte-derived hormones leptin and adiponectin are also involved in energy balance, and adiponectin in particular may have anti-inflammatory properties that are lost or reduced in the obese, diabetic patient [11].

The relationship between autonomic balance and adipose tissue-derived inflammation in T2D is not entirely clear, though both play important roles in the CVD that affects a majority of individuals with this disease, and cardiac autonomic imbalance predicts cardiovascular risk in diabetic patients [12]. In our case-control study involving nondiabetic, newly diagnosed T2D, and established T2D obese subjects, we measured cardiac autonomic function and circulating adipose tissue-derived inflammatory cytokines to determine if there was indeed such a relationship. We hypothesized that there would be significant differences between our groups in regard to both autonomic function and markers of inflammation, and that autonomic imbalance would be associated with "sick," or inflammatory adipose tissue. We report here a number of exciting differences between people with newly diagnosed diabetes, established diabetes, and healthy controls and significant correlations between inflammation and autonomic balance in early and established T2D that have implications for both the determination of CVD risk in T2D patients, and for the treatment and reduction of that risk.

2. Materials and Methods

2.1. Study Participants. Subjects were recruited from a population in Norfolk, Virginia through the Strelitz Diabetes Center at Eastern Virginia Medical School. Three groups were recruited: healthy controls with no prior history of diabetes, individuals with newly diagnosed diabetes (duration less than 6 months), and those with established diabetes (duration greater than 6 months, with a mean duration of 11 years). Diabetes was defined using current American Diabetes Association criteria and/or the need for anti-diabetic medications to control blood glucose. Exclusion criteria included the presence of type 1 diabetes, diabetic retinopathy, diabetic kidney disease, untreated hypothyroidism, liver disease, congestive heart failure, a history of major macrovascular events (including stroke and myocardial infarction within the previous 3 months), significant nondiabetic neuropathy, and/or lower extremity ulcerations or amputations. Anthropomorphic measurements were obtained for each subject, including body mass index (BMI), percent (%) body fat, waist circumference and hip circumference (for calculation of waist-to-hip ratio), systolic and diastolic blood pressure, and heart rate (HR).

2.2. Laboratory Assessment and Homeostasis Model 2 Assessment (HOMA2) Calculations. Each subject had a fasting laboratory evaluation, including serum glucose, triglycerides, total cholesterol, high-density and low-density lipoprotein cholesterol, C-peptide, HbA1c, and insulin. Markers of inflammation including IL-6, TNF-α, and PAI-1 were measured, as were the adipokines leptin and adiponectin (both total (TA) and high-molecular weight forms (HMW)). Analyte specific reagents (ASR) were obtained for IL-6, TNF-α, PAI-I, leptin, and adiponectin (total and high molecular weight). The specific analytes were validated at Inter Science Institute Laboratory, in accordance with standard protocol for validations. EIA, Elisa, and RIA kits that were commercially available for these analytes presented a more challenging task for validation than the ASR approach. Adiponectin-to-leptin ratios were then calculated [13]. Determinations of beta cell function and insulin resistance were made using the homeostasis model assessment 2 (HOMA2) [14] (calculator available from the University of Oxford Diabetes Trials Unit; http://www.dtu.ox.ac.uk/homacalculator/index.php). The study protocol was approved by the institutional review board at Eastern Virginia Medical School.

2.3. Autonomic Nervous System Function Assessment. Autonomic nervous system (ANS) function can be assessed by the measurement of heart rate variability (HRV), which is reduced early in the development of CAN in diabetic patients, and has been associated with increased risk for death after myocardial infarction [3, 15, 16]. Time- and frequency-domain analyses are techniques for assessing HRV that are used in both the research and clinical settings. Time-domain analysis of HRV provides a measure of the sympathetic and parasympathetic control of the heart beat (the R-R interval on an electrocardiogram) recorded with maneuvers including deep breathing, the Valsalva maneuver, and standing from the supine position while frequency-domain analysis is performed under resting conditions [3, 15]. Given their complementary nature both time- and frequency-domain analyses were utilized in this study.

Subjects were instructed to sit with their feet flat on the floor, and a blood pressure cuff was placed on the upper left arm and electrodes were placed on the chest. Subjects were instructed not to speak during the examination, and

were instructed in how to perform a Valsalva maneuver prior to starting. Subjects were told to breathe normally (relaxed breathing) for 5 minutes, followed by 1 minute of slow, deep breaths (inspiration and expiration for approximately 5 seconds each). Subjects took regular breaths for 1 minute, and then performed 5 consecutive Valsalva maneuvers (each lasting approximately 10 seconds), followed by 2 minutes of regular breathing. Finally, the subjects were asked to stand while taking regular breaths. Heart rate and blood pressure were monitored during the examination. Measures of autonomic function, including responses in HRV to deep breathing (expiratory/inspiratory ratio; E/I), Valsalva maneuver (Valsalva ratio), and standing (postural ratio), were recorded. These ratios have been described in detail [15, 17, 18].

Power spectral analysis of HRV was performed under resting conditions (ANSAR; ANX 3.0 software; ANSAR Group, Inc., Philadelphia, PA) with demonstration of low-frequency (LF) and high-frequency (HF) components. Data using ANSAR were normalized. The LF component of the power spectrum of HRV primarily reflects sympathetic activity while the HF component (also termed the respiratory-frequency, RF) primarily reflects parasympathetic activity [3]. LF/RF ratios were calculated providing a measure of sympathetic/parasympathetic activity. The total spectral power (TSP) was calculated, as was the standard deviation of all normal R-R intervals (sdNN), a measure of both sympathetic and parasympathetic action on HRV, and the root-mean square of the difference of successive R-R intervals (rmSSD), a measure primarily of parasympathetic activity [15].

2.4. Statistical Analysis. All data are presented as mean ± SEM. Analysis of variance (ANOVA) was used to compare differences in baseline characteristics, autonomic function, adipokines, β-cell function, and insulin sensitivity results between groups. If significance was observed, a Tukey-Kramer post hoc analysis was performed to determine which groups differed. To determine correlations between autonomic function and adipokines, a Spearman's rank correlation test was used. In cases where the data were not normally distributed, the Wilcoxon signed-rank test was used to determine differences between study groups. Comparison of ANS assessment variables was performed after age adjustment since age has been shown to affect HRV [19]. JMP 8.0 software (SAS Institute, Inc., Cary, North Carolina) was used for all statistical analyses. Significance was accepted at the $P < 0.05$ level for all statistical analyses.

3. Results and Discussion

3.1. Baseline Characteristics. Baseline characteristics are shown in Table 1. Fifteen subjects were recruited to each group (nondiabetic controls, newly diagnosed diabetics, and established diabetics). As expected the diabetic subjects had higher HbA1c values (established 7.9 ± 0.3%; newly diagnosed 7 ± 0.4%; controls 5.3 ± 0.2%; $P < 0.0001$). The diabetic subjects had a greater percent body fat when compared with those in the control group (established 38.7 ± 2.1%; newly diagnosed 37.1 ± 2.0%; controls 26.4 ± 2.9%; $P < 0.01$). LDL cholesterol was significantly higher in the control subjects (control 3.34 ± 0.17; newly diagnosed 2.59 ± 0.31; established 2.39 ± 0.21 mmol/L; $P < 0.05$).

3.2. Measures of Autonomic Nervous System Function. Measures of ANS function in each of the three groups are shown in Table 2. The newly diagnosed and established diabetics had reduced HRV with deep breathing when compared with the controls (1.20 ± 0.03 and 1.17 ± 0.05 versus 1.26 ± 0.04, $P < 0.05$). The established diabetic subjects had a significantly lower Valsalva ratio when compared with the newly diagnosed and control subjects (1.24 ± 0.05 versus 1.37 ± 0.06 and 1.58 ± 0.21, $P < 0.05$).

Spectral analysis of the HRV in the three groups revealed several significant differences. Total spectral power (TSP) was significantly lower (indicative of reduced HRV) in the established diabetic subjects (416.95 ± 142.61 versus 950.88 ± 191.95 for controls and 772.94 ± 290.95 for newly diagnosed T2D; $P < 0.01$). Baseline sdNN was also reduced in the established diabetic subjects (28.92 ± 4.65 versus 47.66 ± 4.87 for controls and 41.04 ± 3.92 for newly diagnosed T2D; $P < 0.001$). Baseline rmSSD was significantly lower in the newly diagnosed and established T2D compared with the control subjects (28.77 ± 6.97 for the newly diagnosed T2D and 18.97 ± 3.38 for the established T2D versus 30.18 ± 3.76 for the controls; $P < 0.05$).

Together, these data validate previous studies showing reductions in HRV in established diabetes [20]. They also suggest specific measurements of HRV (R-R ratio with deep breathing and rmSSD) that demonstrate abnormalities in autonomic function within 6 months of diabetes diagnosis, and presumably, earlier in the course of the disease.

3.3. Adipose Tissue-Derived Cytokines and Adipokines. Concentrations for various adipokines are given in Table 3. IL-6 concentrations were significantly higher in the diabetic subjects (newly diagnosed and established) compared with the control, nondiabetic subjects (11.6 ± 2.8 pg/mL for newly diagnosed T2D and 12.0 ± 1.2 pg/mL for established T2D versus 2.8 ± 0.7 pg/mL for controls, $P < 0.0001$). PAI-1 concentrations were significantly higher in the established diabetics compared with the newly diagnosed and control subjects (6.41 ± 1.36 ng/mL for established T2D versus 5.23 ± 0.76 ng/mL for newly diagnosed T2D and 3.05 ± 0.56 ng/mL for controls, $P < 0.05$).

When analyzed using the Wilcoxon signed-rank test, the total adiponectin-to-leptin ratio (TA/L) was significantly higher in the established T2D when compared with the newly diagnosed diabetics and the controls (0.53 ± 0.32 for the established T2D versus 0.24 ± 0.05 for the newly diagnosed T2D and 1.17 ± 0.76 for the controls, $P < 0.05$). The high-molecular weight adiponectin-to-leptin ratio (HMWA/L) was significantly higher in the established and newly diagnosed diabetics compared with the control subjects (0.07 ± 0.02 for newly diagnosed T2D and 0.26 ± 0.22 for established T2D versus 0.49 ± 0.40 for controls, $P < 0.05$).

3.4. Measures of Insulin Sensitivity/Resistance and Pancreatic Beta Cell Function. HOMA IR values were not significantly different between the groups and are provided in Table 4.

Table 1: Baseline characteristics of study subjects.

Characteristics	Controls ($n = 15$)	Newly diagnosed T2D ($n = 15$)	Established T2D ($n = 15$)	P value
Age (years)	51.47 ± 2.63	53.53 ± 3.80	55.87 ± 1.99	NS
Diabetes duration (months)	NA	2.80 ± 0.45	132.0 ± 18.55	<0.0001*
Systolic blood pressure (mmHg)	126.73 ± 3.13	129.40 ± 4.9	131.93 ± 3.32	NS
Diastolic blood pressure (mmHg)	77.53 ± 2.01	77.73 ± 2.23	77.6 ± 1.81	NS
Resting heart rate (bpm)	73.67 ± 2.97	66.60 ± 2.74	76.6 ± 3.44	NS
BMI (kg/m^2)	30.16 ± 2.09	35.27 ± 2.22	35.39 ± 1.68	NS
Waist circumference (cm)	39.10 ± 2.08	44.53 ± 1.87	43.43 ± 1.63	NS
Waist-hip ratio	0.91 ± 0.03	0.95 ± 0.02	0.92 ± 0.02	NS
Percent body fat	26.39 ± 2.91	37.05 ± 2.03	38.71 ± 2.07	0.0012#
Fasting plasma glucose (mmol/L)	5.32 ± 0.13	6.12 ± 0.34	7.87 ± 0.84	0.0045*
Fasting plasma insulin (pmol/L)	74.93 ± 11.1	133.3 ± 27.48	115.47 ± 31.06	NS
Fasting C-peptide (nmol/L)	0.70 ± 0.07	1.14 ± 0.15	1.03 ± 0.17	NS
A1C (%)	5.29 ± 0.19	6.95 ± 0.42	7.93 ± 0.33	<0.0001#
Fasting total cholesterol (mmol/L)	5.15 ± 0.20	4.87 ± 0.31	4.37 ± 0.24	NS
Fasting triglycerides (mmol/L)	1.02 ± 0.15	1.13 ± 0.17	1.39 ± 0.24	NS
HDL cholesterol (mmol/L)	1.33 ± 0.08	1.41 ± 0.09	1.34 ± 0.13	NS
LDL cholesterol (mmol/L)	3.34 ± 0.17	2.59 ± 0.31	2.39 ± 0.21	0.0157#
C-reactive protein (nmol/L)	45.33 ± 18.29	13.91 ± 4.00	43.72 ± 20.76	NS

Data are presented as means ± SE. NA: not applicable; NS: not significant. *Established T2D versus controls/newly diagnosed T2D. #Newly diagnosed/established T2D versus controls.

Table 2: Measures of autonomic function.

Measure	Controls ($n = 15$)	Newly diagnosed T2D ($n = 15$)	Established T2D ($n = 15$)	P value
R-R ratio (deep breathing)	1.26 ± 0.04	1.20 ± 0.03	1.17 ± 0.05	0.0325#
R-R ratio (Valsalva)	1.58 ± 0.21	1.37 ± 0.06	1.24 ± 0.05	0.0270*
R-R ratio (standing)	1.29 ± 0.04	1.26 ± 0.05	1.18 ± 0.02	NS
LFa	1.60 ± 0.43	1.90 ± 0.39	1.37 ± 0.37	NS
RFa	1.21 ± 0.27	1.89 ± 0.79	0.73 ± 0.29	NS
LFa/RFa ratio	1.53 ± 0.24	2.51 ± 0.51	2.88 ± 0.56	NS
TSP baseline	950.88 ± 191.95	772.94 ± 290.95	416.95 ± 142.61	0.0023*
sdNN baseline	47.66 ± 4.87	41.04 ± 3.92	28.92 ± 4.65	0.0008*
rmSSD baseline	30.18 ± 3.76	28.77 ± 6.97	18.97 ± 3.38	0.0356#

Data are presented as means ± SE. NS: not significant. *Established T2D versus controls/newly diagnosed T2D. #Newly diagnosed/established T2D versus controls. TSP: total spectral power; SDNN: standard deviation of all normal R-R intervals; rmSSD: root mean square of the difference of successive R-R intervals.

Using HOMA 2%B as a marker, β-cell function was significantly increased in the newly diagnosed T2D when compared with the established diabetics and control subjects and significantly impaired in established type 2 diabetes (118.02 ± 13.30 versus 68.31 ± 11.00 for established T2D and 105.16 ± 10.63 for controls, $P < 0.05$). Similar differences were seen when HOMA 2%B was assessed using fasting C-peptide concentrations in place of fasting insulin concentrations (120.74 ± 10.31 versus 85.15 ± 12.57 for established T2D and 111.42 ± 6.85 for controls, $P < 0.05$).

3.5. Correlations between Autonomic Function and Inflammation. We found a number of very interesting correlations between measures of autonomic function and adipose tissue-derived inflammatory products in our study (see Table 5). IL-6 correlated negatively with sdNN at baseline. This suggests that elevated levels of this adipocytokine are associated with reductions in HRV seen in patients with diabetes. Of note, IL-6 was significantly elevated in newly diagnosed as well as established diabetics in our study, suggesting that this cytokine may play a role in the autonomic dysfunction that is seen early in the course of diabetes. HMW adiponectin correlated negatively with the LFA/RFA ratio, and an elevated TA/L ratio (and also an elevated HMWA/L ratio) correlated positively with total spectral power and rmSSD. These latter findings demonstrate that the balance of two significant adipokines involved in insulin sensitivity (adiponectin and leptin) may be related to autonomic function.

Table 3: Adipokines in study subjects.

Measurement	Controls ($n = 15$)	Newly diagnosed T2D ($n = 15$)	Established T2D ($n = 15$)	P value
IL-6 (pg/mL)	2.84 ± 0.68 ($n = 14$)	11.58 ± 2.83	12.04 ± 1.20 ($n = 12$)	<0.0001#
TNF-α (pg/mL)	9.32 ± 2.18 ($n = 14$)	9.14 ± 1.15	27.93 ± 15.4 ($n = 12$)	NS
PAI-1 (ng/mL)	3.05 ± 0.56	5.23 ± 0.76	6.41 ± 1.36	0.0305*
Total adiponectin (mg/mL)	6.76 ± 0.78	7.48 ± 1.11	8.91 ± 2.3	NS
High molecular weight adiponectin (μg/mL)	1.78 ± 0.26	2.33 ± 0.60	2.71 ± 1.54	NS
Leptin (ng/mL)	28.66 ± 7.93	46.87 ± 7.96	55.93 ± 11.39	NS
TA/L ratio	1.17 ± 0.76	0.24 ± 0.05	0.53 ± 0.32	0.0340*
HMWA/L ratio	0.49 ± 0.40	0.07 ± 0.02	0.26 ± 0.22	0.0442#

Data are presented as means ± SE. NS, not significant. *Established T2D versus controls/newly diagnosed T2D. #Newly diagnosed/established T2D versus controls. IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; PAI: plasminogen activator inhibitor 1; TA/L: total adiponectin/leptin ratio; HMWA/L: high molecular weight adiponectin/leptin ratio.

Table 4: Measures of β-cell function and insulin sensitivity as per HOMA2 IR.

Measurement	Controls ($n = 15$)	Newly diagnosed T2D ($n = 15$)	Established T2D ($n = 15$)	P value
HOMA2 %B	105.16 ± 10.63	118.02 ± 13.30	68.31 ± 11.0	0.0126$
HOMA2 %S	84.93 ± 8.19	60.03 ± 10.22	135.09 ± 37.85	NS
HOMA2 IR	1.41 ± 0.20	2.55 ± 0.52	2.67 ± 0.88	NS
HOMA2 %B C-peptide	111.42 ± 6.85	120.74 ± 10.31	85.15 ± 12.57	0.0473$
HOMA2 %S C-peptide	77.13 ± 9.81	135.79 ± 87.13	68.09 ± 17.06	NS
HOMA2 IR C-peptide	1.57 ± 0.17	2.71 ± 0.38	2.92 ± 0.75	NS

Data are presented as means ± SE. $Newly diagnosed T2D versus controls/established T2D.

4. Discussion

This study reveals a number of novel relationships in regard to the autonomic nervous system, adipose tissue-derived inflammation, and the onset and progression of diabetes. We demonstrate ANS dysfunction in newly diagnosed diabetic subjects, as measured by a reduction in R-R variability with deep breathing, as well as by a reduction in rmSSD as measured by HRV through time-domain analysis. Established diabetics also had a reduction in their R-R variability during the Valsalva maneuver, as well as reductions in total spectral power, sdNN, and rmSSD. Newly diagnosed diabetics had higher concentrations of the inflammatory adipokine IL-6, and had low HMW adiponectin-to-leptin ratios compared with control subjects. Established diabetics also had significantly higher concentrations of PAI-1. We found significant correlations between an inflammatory adipokine (IL-6) and measures of autonomic function in our established and newly diagnosed diabetics (sdNN at baseline). We also noted correlations between the HMWA/L ratio and various measures of autonomic function. Our findings suggest that newly diagnosed diabetics have measurable abnormalities in their ANS, and that these changes may be in part regulated through the adipokines IL-6, leptin, and adiponectin but cannot rule out that the effects could be primarily due to autonomic dysfunction with its consequences upon adipose tissue cytokine release.

Others have reported abnormal autonomic function in newly diagnosed diabetics. Thirty years ago Fraser et al. reported abnormal ANS function in a group of 10 newly diagnosed diabetics (six of whom were being treated with insulin) [21]. Pfeifer et al. reported abnormal autonomic responses in a group of 33 newly diagnosed diabetic subjects (duration of diabetes less than 12 months), and suggested that abnormal carbohydrate metabolism was a cause of their ANS abnormalities [22]. McDaid et al. described abnormal vasoconstrictor responses to temperature changes and deep-breathing maneuvers in newly diagnosed T2Ds when compared with control subjects [23].

The etiology of ANS dysfunction seen early after the diagnosis of diabetes is not well understood. Some have suggested elevated concentrations of nitric oxide as well as chronic hyperglycemia as causative factors [24, 25]. A recent study from Chang et al. demonstrated that cardiac autonomic dysfunction (as measured by spectral analysis and expiratory/inspiratory ratio) may occur prior to the development of insulin resistance in individuals with 1 or 2 components of the metabolic syndrome [26]. Thus, it is not surprising to find abnormalities in autonomic function, but we add the dimension of change in autonomic balance without necessarily autonomic neuropathy suggesting a potential reversibility at the stage of newly diagnosed diabetes.

It has been suggested that adipose tissue becomes "sick" (adiposopathy) in states of obesity, leading to various

Table 5: Significant correlations between adipokines and measures of autonomic function.

Variable	Variable	Spearman	*P* value
IL-6	sdNN baseline	−0.3619	0.0217
TA/L ratio	TSP baseline	0.3519	0.0191
	sdNN baseline	0.2943	0.0525
	rmSSD baseline	0.2958	0.0512
HMWA/L ratio	LFA/RFA ratio	−0.4185	0.0042
	TSP baseline	0.3934	0.0082
	rmSSD baseline	0.3218	0.0332
HMW adiponectin	LFA/RFA	−0.5192	0.0003

components of the metabolic syndrome [27]. In our study we build upon this hypothesis, and suggest that adipokines play a role in the development of ANS dysfunction in obese subjects with type 2 diabetes. We demonstrated increased concentrations of the inflammatory protein IL-6 in both newly diagnosed and established diabetic subjects. IL-6 correlated negatively with sdNN, suggesting an association between IL-6 and abnormalities in both the sympathetic and parasympathetic components of the reduced HRV seen in patients with diabetes. März et al. demonstrated that rat sympathetic neurons were able to produce and respond to IL-6, and suggested a possible paracrine role for the interleukin in regulating the sympathetic nervous system (SNS) [28]. Sloan et al. demonstrated a strong inverse relationship between HRV and serum concentrations of IL-6 and CRP in young men and women in the CARDIA study, which looked at risk factors for the development of atherosclerosis [29]. In a group of 264 middle-aged men, Lampert et al. found that IL-6 was inversely associated with various measures of HRV. IL-6 was also associated with multiple cardiovascular risk factors (including BMI, hypertension, and smoking) [30]. The authors suggested that autonomic dysfunction might lead to inflammation, which may then play a role in the development of CAD. Further studies should explore this relationship, and the regulation of IL-6 by both the parasympathetic and sympathetic nervous systems is amenable to examination by determining the impact of adrenergic and cholinergic blockade on IL-6 concentrations. Interestingly, we did not see a significant difference in C-reactive protein (CRP) concentrations between our three groups. CRP has been shown to predict an increased risk for CVD, as well as stroke and non-insulin-dependent diabetes [31]. As CRP production is primarily regulated by IL-6, much of which comes from adipose tissue, it may be that an elevated IL-6 concentration is an earlier predictor of CVD risk than CRP [32]. Our data suggest that measures of cardiac autonomic imbalance, which correlate with IL-6 concentrations, are also early predictors of CVD risk, and may be clinically relevant before more well-known predictors of risk including CRP.

We found that newly diagnosed diabetic subjects had significantly lower HMW adiponectin/leptin ratios when compared with control subjects, and saw significant correlations between the HMWA/leptin ratio and multiple measures of autonomic function (LFA/RFA ratio, TSP baseline, and rmSSD baseline). Intravenous leptin infusion has been shown to stimulate SNS activity in animal models [33]. In a study of 120 nonobese humans without diabetes, Paolisso et al. demonstrated that increasing plasma leptin concentrations were associated with increasing LFa/HFa ratios, suggesting greater SNS activity as compared with PS activity [34]. Studies have also shown interesting correlations between the adiponectin and autonomic function. In mice, Imai et al. demonstrated that activation of the SNS (via cold temperatures) suppressed the production of adiponectin from white adipose tissue [35]. In humans, Wakabayashi and Aso studied 105 men and women with T2D and found that adiponectin concentrations correlated negatively with the LFa/RFa ratio [36]. Of note, changes in the adiponectin/leptin balance in the course of diabetes may first be seen by utilizing measures of high-molecular weight adiponectin rather than total adiponectin, as demonstrated by our findings (Table 3). These findings and the results in animal studies again suggest that the effects on adipose tissue cytokine release may be a consequence of autonomic dysfunction as opposed to the corollary.

Tracey and others have described a neural reflex arc whose afferent arm responds to markers of inflammation, such as cytokines, by eliciting a cholinergic anti-inflammatory effector response, mediated through the parasympathetic nervous system [37]. In animal models of inflammation, inhibition of the afferent arm of this reflex arc (e.g., by vagotomy) leads to increased inflammation and disease severity [38]. Activation of the efferent arm of the reflex arc (through the administration of an acetylcholine receptor agonist) causes a decrease in proinflammatory cytokine production, and a reduction in disease severity [39, 40]. In his review Tracey points out a number of important clinical studies that show correlations between vagal nerve activity and inflammatory human diseases such as rheumatoid arthritis and lupus [37, 41, 42]. Our results are in keeping with this research, and suggest that such a reflex arc may be involved in the inflammation seen early in T2D.

There are limitations to our study. This is a case-control study with small numbers of subjects. This limits the power of the findings, especially considering that HRV can vary significantly between individuals. However, the differences and correlations demonstrated by the data are highly significant. Also, there were not specific restrictions on whether patients could take medications that might affect ANS function (such as beta-blockers) or anti-inflammatories and future studies would include these exclusionary criteria. Previous studies have demonstrated significant differences

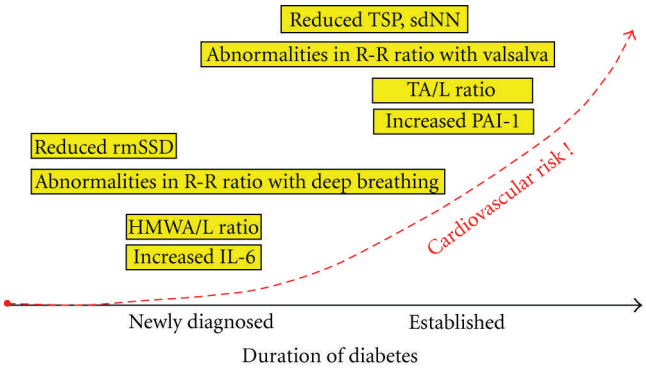

Figure 1: Cardiac autonomic imbalance and inflammation in the progression of diabetes.

in cholesterol concentrations and HOMA IR values between diabetics and nondiabetics [43]. The diabetic subjects in our study had lower cholesterol concentrations than the nondiabetic subjects. We suspect that many of these subjects were taking cholesterol-lowering agents such as statins. Surprisingly HOMA IR did not differ significantly between the diabetic and nondiabetic subjects in our study. This may be a reflection of our small sample size, as the HOMA IR values do differ between the groups, but not significantly. Both adiponectin and leptin increased with progression of diabetes in this study. This might be a result of the increased percent of body fat that these individuals had when compared with the control group. The established diabetic subjects had been diagnosed as having diabetes for an average of 11 ± 1.5 years. It will be important to evaluate subjects with varying durations of diabetes in the future, including a group of prediabetic individuals with impaired glucose tolerance and/or impaired fasting glucose, to determine if they fit with our hypothesis that early hyperglycemia is associated with autonomic dysfunction and changes in adipokine secretion that promote an inflammatory milieu.

Our study suggests that autonomic dysfunction is seen early in the course of diabetes, and that this occurs alongside alterations in various adipokines, including adiponectin and leptin, and various inflammatory adipocytokines, including IL-6. Future studies could evaluate differences in various inflammatory markers in both subcutaneous and visceral fat in nondiabetic, diabetic, and newly diagnosed diabetic subjects, and seek correlations with autonomic balance and risk factors for CVD in these patients. Future therapies might be directed at reducing the early sympathetic activation seen in newly diagnosed diabetics, with a goal of reducing the impact of the inflammatory response upon the ANS before it leads to the development of CVD thereby reducing CVD and mortality (Figure 1).

5. Conclusions

Here we show that there is strong evidence of inflammation with activation of inflammatory cytokines such as IL-6 and leptin in newly diagnosed type 2 diabetes. These changes correlate with abnormalities in sympathetic-vagal balance. Autonomic dysfunction has been shown to be a predictor of cardiovascular risk and sudden death in patients with type 2 diabetes. A better understanding of the autonomic dysfunction and adipose tissue inflammation seen early in the development of diabetes will lead to further measures for determining which individuals are at the highest risk for cardiovascular disease and mortality, and will also lead to the development of new therapies for reducing that risk.

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Research Article

Regulation of *LYRM1* Gene Expression by Free Fatty Acids, Adipokines, and Rosiglitazone in 3T3-L1 Adipocytes

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LYR motif containing 1 (*LYRM1*) is a novel gene that is abundantly expressed in the adipose tissue of obese subjects and is involved in insulin resistance. In this study, free fatty acids (FFAs) and tumor necrosis factor-α (TNF-α) are shown to upregulate *LYRM1* mRNA expression in 3T3-L1 adipocytes. Conversely, resistin and rosiglitazone exert an inhibitory effect on *LYRM1* mRNA expression. These results suggest that the expression of *LYRM1* mRNA is affected by a variety of factors that are related to insulin sensitivity. *LYRM1* may be an important mediator in the development of obesity-related insulin resistance.

1. Introduction

Obesity has become a global public health problem in recent decades [1]. Type 2 diabetes is characterized by an inadequate beta-cell response to progressive insulin resistance, which is typically accompanied by weight gain [2]. The increasing global prevalence of type 2 diabetes is tied to rising rates of obesity [3]. Common obesity (complex polygenic obesity) results from interactions between genetic, environmental, and psychosocial factors [4]. However, the mechanisms underlying individual differences that lead to a predisposition to obesity remain obscure.

In our earlier studies, we isolated and characterized LYR motif containing 1 (*LYRM1*), a novel human gene that was expressed at a high level in the omental adipose tissue of obese patients. *LYRM1* promotes preadipocyte proliferation and inhibits apoptosis of preadipocytes [5, 6]. Overexpression of *LYRM1* in 3T3-L1 adipocytes resulted in a reduction of insulin-stimulated glucose uptake, an abnormal mitochondrial morphology, decreased intracellular ATP synthesis, and decreased mitochondrial membrane potentials. In addition, *LYRM1* overexpression led to an excessive production of intracellular reactive oxygen species [7]. Our findings indicate that *LYRM1* may be a new candidate gene related to obesity-associated insulin resistance.

Several studies have shown that adipose tissue in obese patients releases large amounts of free fatty acids (FFAs) and several adipokines, including tumor necrosis factor-α (TNF-α) and resistin [8–11]. All of these factors have been identified as major regulators of insulin activity. A synthetic activator of peroxisome proliferator-activated receptor-γ (PPAR-γ) called rosiglitazone (BRL49653) is part of the thiazolidinedione (TZD) class of drugs. Thiazolidinedione is one of a few classes of drugs that acts primarily as an insulin sensitizer by repressing, in mature adipocytes, the expression and secretion of adipokines [12]. However, the underlying molecular mechanisms of how these factors affect insulin sensitivity have not been clarified.

In this study, we show that *LYRM1* is a novel gene related to obesity-associated insulin resistance. We hypothesize that these factors (FFAs, TNF-α, and resistin) and drug (rosiglitazone) may have a potential regulatory mechanism in

Table 1: Nucleotide sequences for primer and probe sets used in qPCR.

Gene	Forward primer (5′–3′)	Probe	Reverse primer (5′–3′)
LYRM1	CAGATGGATAGGGCGTGGATAAGG	TGGTAATGCAGTCCAATCTCAATCCG	GACAGCAGCAACCCGACAAGAAGT
β-actin	CCTGAGGCTCTTTTCCAGCC	TCCTTCTTGGGTATGGAATCCTGTGGC	TAGAGGTCTTTACGGATGTCAACGT

obesity through the regulation of *LYRM1* mRNA expression, thereby affecting insulin sensitivity. The purpose of this study was to investigate the effects of FFAs, TNF-α, resistin, and rosiglitazone on *LYRM1* mRNA expression in 3T3-L1 adipocytes.

2. Materials and Methods

2.1. 3T3-L1 Cell Culture and Treatment. 3T3-L1 cells were cultured, maintained, and differentiated as previously described [13]. Briefly, after confluence was achieved, the cells were grown for 2 days in DMEM/high-glucose medium (Gibco, Carlsbad, Calif, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, Calif, USA), in a 5% CO_2 environment. Differentiation was subsequently induced by incubation in a similar medium that was supplemented with 0.5 mmol/L 3-isobuty-1-methylxanthine (MIX; Sigma, St. Louis, Mo, USA), 1 μmol/L dexamethasone (Sigma, St. Louis, Mo, USA), and 10 μg/mL insulin (Sigma, St. Louis, Mo, USA), for 2 days. The cells were then placed in a medium containing 10 μg/mL insulin for another 2 days. Afterwards, the medium was replaced with DMEM containing only 10% FBS, every 2 days.

On the eighth day after differentiation was induced, if more than 90% of the cells showed the morphological and biochemical properties of adipocytes, the cells were used for experiments. After overnight incubation in serum-free DMEM, the 3T3-L1 adipocytes were treated with either 10 ng/mL TNF-α (T7539), 60 ng/mL resistin (SRP4560), 0.5 μM rosiglitazone (375004), which were all dissolved in DMSO, or a 1 mM FFA cocktail composed of palmitic acid (p5585), oleic acid (O1008), and linoleic acid (L1376; Sigma, St. Louis, Mo, USA). The high FFA solution was prepared according to previously published methods [14, 15]. Briefly, the fatty acids were dissolved in 2% (w/v) fatty acid-free bovine serum albumin (BSA), with a stock concentration of 100 mM or an equivalent volume of vehicle. The stock solution was diluted 1 : 100 in DMEM to a final concentration of 1 mM. After 12 h or 24 h of incubation in the TNF-α, resistin rosiglitazone and high FFA solution, the adipocytes were collected for subsequent experiments.

2.2. Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from 3T3-L1 adipocytes using Trizol reagent (Invitrogen, Carlsbad, Calif, USA). The extracted RNA was quantified by spectrophotometry at 260 nm. cDNA was synthesized from 1 μg of total RNA using an AMV Reverse Transcriptase Kit (Promega A3500; Promega, Madison, Wis, USA), according to the manufacturer's instructions. Real-time RT-PCR was performed on an Applied Biosystems 7500 Sequence Detection System (ABI 7500 SDS; Foster City, Calif, USA) by following the manufacturer's protocol.

Two primer sets were used for PCR analysis. A 259-bp DNA fragment within the *LYRM1* gene was used for the quantification of *LYRM1* mRNA. The PCR product had previously been cloned into the plasmid pMD-T 18 and verified by DNA sequencing. Plasmid standards of known copy numbers were used to generate a log-linear standard curve, from which the copy numbers of *LYRM1* could be determined by real-time qPCR. A 110-bp region of the *β-actin* gene was used to normalize the results. A standard curve was generated from plasmids containing the *β-actin* fragment. This standard curve was used to determine the copy numbers of *β-actin*. Briefly, the samples were incubated at 95°C for 10 min for an initial denaturation, followed by 40 PCR cycles. Each cycle consisted of an incubation at 95°C for 15 s and annealing at 60°C for 1 min. The concentration ratio of *LYRM1* to *β-actin* reflected the expression level of *LYRM1* mRNA per cell. Primer and Taqman probe (Invitrogen, Shanghai, China) sequences are shown in Table 1.

2.3. Statistical Analysis. Each experiment was performed at least three times. All data was expressed as means ± SD. Statistical analysis was performed using one-way ANOVA using the SPSS 12.0 statistical software package (SPSS Inc., Chicago, Ill, USA). For all tests, P-values less than 0.05 were considered statistically significant.

3. Results

3.1. The Expression of LYRM1 mRNA during the Conversion of 3T3-L1 Preadipocytes into Adipocytes. *LYRM1* mRNAs were expressed at very low levels In the 3T3-L1 preadipocytes. During the conversion of 3T3-L1 cells to adipocytes, the expression of the *LYRM1* gene was gradually increased to reach a stable level after the 10th day (Figure 1). More than 90% of the cells exhibited typical adipocyte morphology on the 10th day.

3.2. The Effect of FFAs on the Expression of LYRM1 mRNA in 3T3-L1 Adipocytes. To assess the effect of FFAs on *LYRM1* mRNA levels, we examined the expression of *LYRM1* mRNA in 3T3-L1 adipocytes treated with 1 mM FFAs. Treatment durations were for either 12 or 24 h, 10 days after differentiation was stimulated. We found that FFAs concentrations of 1 mM led to a time-dependent increase in *LYRM1* mRNA expression. *LYRM1* mRNA expression dramatically increased after 12 h of exposure (Figure 2) and continued to increase after a 24 h exposure. At this time point, the expression of *LYRM1* mRNA was approximately 2-fold greater than the control mRNA ($P < 0.001$). This result shows that FFAs

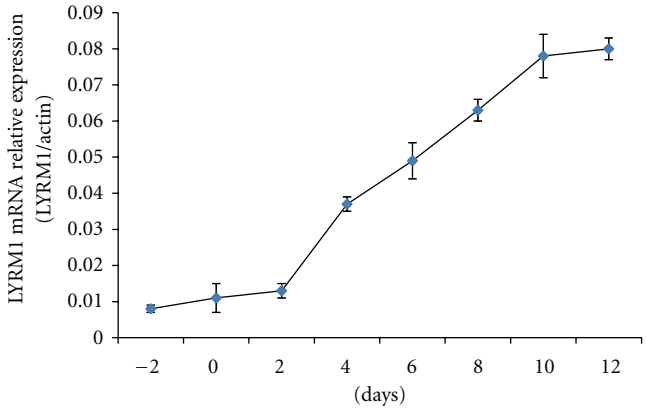

Figure 1: The expression of the *LYRM1* mRNA during the conversion of 3T3-L1 cells to adipocytes. 3T3-L1 cells were induced to differentiate, as described in "Materials and Methods" section. Total RNA was harvested from the 3T3-L1 cells on alternate days before (day −2, day 0) and after (day 2, day 4, day 6, day 8, day 10, and day 12) the switch from growth medium to differentiation medium. *LYRM1* mRNA levels were analyzed using quantitative real-time RT-PCR and normalized to *β-actin* levels. The results are presented as the means ± SE of six experiments.

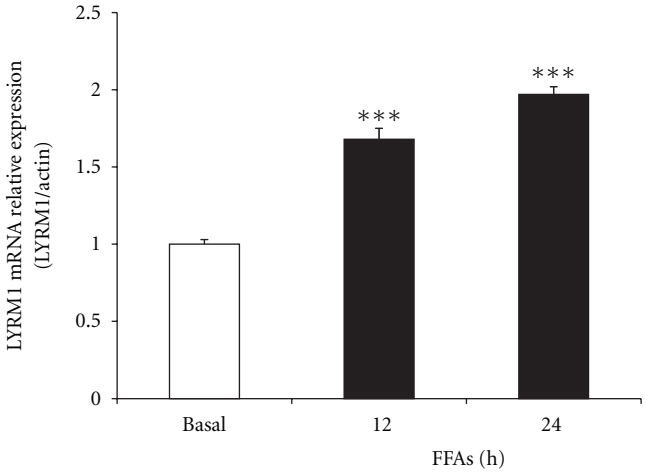

Figure 2: The effect of FFAs on the expression of *LYRM1* mRNA in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 1 mM FFAs for the indicated periods (up to 24 h). *LYRM1* mRNA levels were analyzed using quantitative real-time RT-PCR and normalized to *β-actin* levels. Results are presented as mean ± SE of six experiments. $^{***}P < 0.001$ in comparison with basal levels (untreated cells).

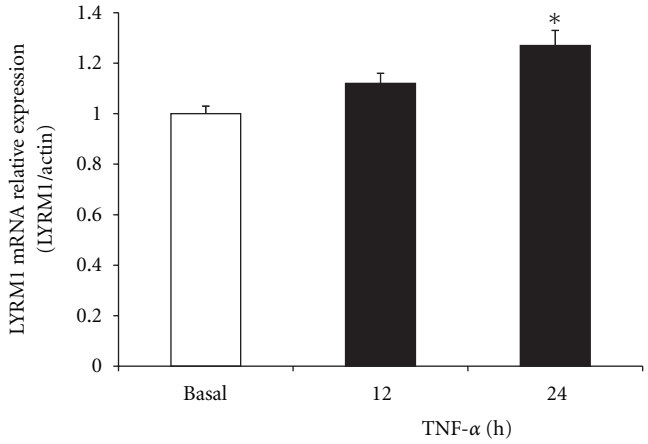

Figure 3: The effect of TNF-α on the expression of *LYRM1* mRNA in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 10 ng/mL TNF-α for the indicated periods (up to 24 h). *LYRM1* mRNA levels were analyzed using quantitative real-time RT-PCR and normalized to *β-actin* levels. Results are presented as mean ± SE of six experiments. $^{*}P < 0.05$ in comparison with basal levels (untreated cells).

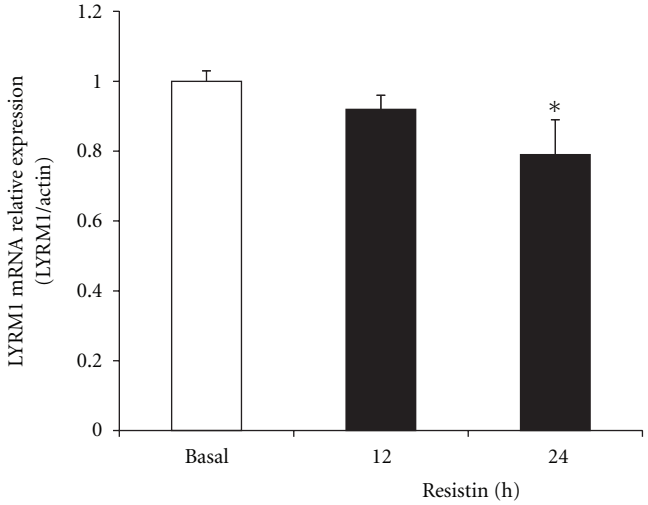

Figure 4: The effect of resistin on the expression of *LYRM1* mRNA in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 60 ng/mL resistin for the indicated periods (up to 24 h). *LYRM1* mRNA levels were analyzed using quantitative real-time RT-PCR and normalized to *β-actin* levels. Results are presented as mean ± SE of six experiments. $^{*}P < 0.05$ in comparison with basal levels (untreated cells).

dramatically increased the mRNA expression level of the *LYRM1* gene.

3.3. The Effects of TNF-α and Resistin on the Expression of LYRM1 mRNA in 3T3-L1 Adipocytes. We examined *LYRM1* mRNA expression 10 days after differentiation was stimulated in 3T3-L1 adipocytes, which had been treated with 10 ng/mL TNF-α or 60 ng/mL resistin. TNF-α slightly increased *LYRM1* mRNA expression in 3T3-L1 adipocytes after 12 h. mRNA expression continued to increase 24 h after treatment ($P < 0.05$; Figure 3). Resistin showed a moderate inhibitory effect on *LYRM1* gene expression at 12 h; however, expression was significantly diminished 24 h after resistin treatment ($P < 0.05$; Figure 4).

3.4. The Effect of Rosiglitazone on the Expression of LYRM1 mRNA in 3T3-L1 Adipocytes. To study the relationship between *LYRM1* expression and a PPAR-γ agonist, we

examined the effect of rosiglitazone at 60 ng/mL on 3T3-L1 adipocytes. Twelve hours after treatment, *LYRM1* mRNA expression in 3T3-L1 adipocytes decreased. After 24 h mRNA expression had significantly diminished to approximately half that of the control ($P < 0.001$; Figure 5).

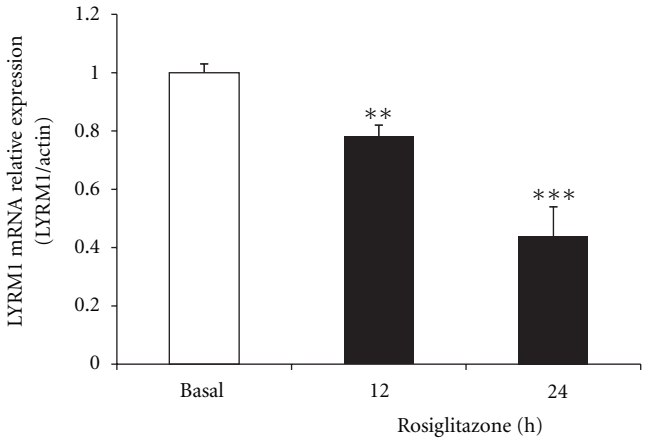

Figure 5: The effect of rosiglitazone on the expression of *LYRM1* mRNA in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 0.5 μM rosiglitazone for the indicated periods (up to 24 h). *LYRM1* mRNA levels were analyzed using quantitative real-time RT-PCR and normalized to *β-actin* levels. Results are presented as mean ± SE of six experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ in comparison with basal levels (untreated cells).

4. Discussion

The World Health Organization reports that at least one billion adults are overweight and 300 million are obese. In the absence of intervention, these numbers are expected to rise [16]. Most obese individuals are insulin resistant, which is an important etiological factor for type 2 diabetes mellitus. Adipocytes are known to secrete a variety of mediators, including FFA, TNF-α, and resistin, all of which regulate insulin signaling and glucose uptake. *LYRM1* is a recently discovered gene that is involved in obesity-associated insulin resistance [5, 7]. *LYRM1* mRNA expression is upregulated during conversion of 3T3-L1 cells to adipocytes, indicating that the expression of the *LYRM1* gene is involved in adipocyte differentiation. From the 10th day after induction of differentiation, the *LYRM1* mRNA expression remained at a stable high level, indicating that this clonal cell line can be used to investigate the regulation of *LYRM1* gene expression. To elucidate the mechanisms by which *LYRM1* is involved in the pathogenesis of obesity-associated insulin resistance, we characterized how this gene is regulated by factors that modulate insulin sensitivity. Furthermore, we also investigated the effects of rosiglitazone, which is a PPAR-γ agonist, on *LYRM1* mRNA expression in 3T3-L1 adipocytes.

Elevated concentrations of circulating free fatty acids are characteristic of type 2 diabetes and are implicated in the etiology of insulin resistance [17]. Insulin resistance is thought to arise from impaired insulin signaling in target tissues. Signaling is impaired due to augmentation of the serine/threonine phosphorylation sites of insulin receptor substrates (IRS-1 and IRS-2). In addition, insulin resistance is compounded by a reduction of activated PI3-kinase (PI3K) and an inhibition in the translocation of insulin-stimulated glucose transporter 4 (GLUT4) [18, 19]. An excess of FFAs causes the intracellular accumulation of metabolic products such as ceramides, diacylglycerol, or acyl-CoA. These FFA-derived products may lead to defects in insulin signaling and glucose transport through the PI3K-dependent pathway [20, 21]. However, the underlying mechanisms of these phenomena have not been clarified. In this study, we observed that FFAs added exogenously upregulated *LYRM1* mRNA expression in 3T3-L1 adipocytes. We had previously shown that *LYRM1* overexpression can inhibit insulin-stimulated glucose transport in adipocytes [7]. We observed that an excess of FFAs might induce insulin resistance. Resistance could partly be induced through the upregulation of *LYRM1* expression, which would inhibit glucose uptake in adipocytes. These findings support and extend other results in the literature that investigate the effects of FFAs on insulin signaling.

As one of the most widely studied cytokines, TNF-α is reported to modulate insulin resistance [10]. A key role for TNF-α in obesity-related insulin resistance was identified when TNF-α or TNF-α receptors were deleted in both diet-induced obese mice and leptin-deficient ob/ob mice, which resulted in significantly improved insulin sensitivity [22]. However, the infusion of TNF-α-neutralizing antibodies into obese, insulin-resistant subjects, or type 2 diabetic patients, did not improve insulin sensitivity [23, 24]. In this study, we observed that TNF-α slightly upregulates *LYRM1* mRNA expression in 3T3-L1 adipocytes. There is a need for further studies in human adipocytes. Currently, we suggest that TNF-α-induced insulin resistance is only indirectly involved in increased *LYRM1* expression.

Resistin was identified as a gene that was downregulated by TZD in mouse adipocytes [11]. In rodents, the circulating levels of resistin increased in obesity [25]. Furthermore, an increase in serum resistin levels induced insulin resistance in several rat and mouse models, including after acute administration [26]. Recombinant *resistin* caused severe hepatic insulin resistance in rodents [26]. However, a study observed a decrease in fasting glucose, improved glucose tolerance and enhanced insulin sensitivity in resistin knockout mice [27]. In humans, there is considerable controversy surrounding the role of *resistin*. We showed that resistin exerts a moderate inhibitory effect on *LYRM1* gene expression in 3T3-L1 adipocytes. This data suggests that *LYRM1* and resistin interact during the development of obesity-associated insulin resistance.

In this study, we observed that rosiglitazone inhibits *LYRM1* gene expression in 3T3-L1 adipocytes. Rosiglitazone is part of the TZD class of drugs, which act as insulin sensitizers and agonists for the transcription factor PPAR-γ. PPAR-γ is a member of three nuclear receptor isoforms (the other two are PPAR-α and PPAR-δ), which are encoded

by different genes. PPAR-γ is the master regulator of adipogenesis, being both essential and sufficient for adipocyte differentiation [28]. It also upregulates the expression of fatty acid transporter proteins (FATP-1 and D036) [29]. Rosiglitazone suppresses TNF-α mediated inhibition of adipocyte differentiation, whilst TNF-α decreased the expression of PPAR-γ [30]. TZDs inhibit resistin gene expression in human macrophages [31, 32] and lower serum resistin levels in humans as well as rodents [33–35]. We deduced that rosiglitazone inhibits *LYRM1* gene expression most likely through PPAR-γ.

Our results demonstrate that *LYRM1* mRNA expression is greatly affected by rosiglitazone, FFAs, and two adipokines, TNF-α and resistin. These two adipokines are involved in the regulation of insulin sensitivity. The upregulation or downregulation of *LYRM1* expression may be strongly linked to FFA or rosiglitazone-related insulin resistance. Recently, *LYRM1* in rat myoblasts has been shown to negatively regulate the function of IRS-1 and PI3K/Akt, whilst decreasing GLUT4 translocation and glucose uptake in response to insulin (L6) [36]. However, a more precise characterization of the physiological activities of *LYRM1* is required to fully understand these processes.

Conflict of Interests

Relevant to this paper, no potential conflict of interests is declared by the authors.

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