Cardiovascular protective effect of pioglitazone on oxidative stress in rats with metabolic syndrome

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Abstract
Background: Although cardiovascular oxidative stress is examined in type 2 diabetes, there is relatively limited number of reports about the effect of pioglitazone, an insulin sensitizer, on cardiovascular oxidative stress in sucrose diet-induced metabolic syndrome (MetS). As a regulator of cardiovascular homeostasis, thioredoxin (TRX) has an important role in defense against oxidative stress in cardiovascular diseases. The purpose of this study is to investigate the role of pioglitazone on oxidative stress markers and TRX1 level in tissues of both heart and aorta from MetS rats.

Methods: Male Wistar rats (200 to 250 g in weight) were divided into three groups: control group, MetS group receiving drinking water including 935 mM sucrose, and pioglitazone-treated MetS (MetS-P) group. Aspartate aminotransferase (AST), lactate dehydrogenase (LDH), total oxidant status (TOS), and total antioxidant status (TAS) levels were measured in serum and tissues using commercial kits. Malondialdehyde (MDA) and superoxide dismutase (SOD) were measured in serum and tissues for experimental groups. TRX1 protein level was measured by western blot.

Results: The sucrose-fed rats exhibited several characteristics of MetS. In MetS group, AST, LDH, TOS, and MDA levels of heart and aorta tissues increased, whereas TAS and SOD levels of these tissues decreased. TRX1 protein level of heart and aorta tissues increased, whereas TAS and SOD levels of these tissues decreased. TRX1 protein level was measured by western blot.

Conclusion: Pioglitazone treatment significantly increased TRX1 protein level in heart and aorta tissues in MetS group. Pioglitazone affected the TRX1 protein level via regulation of reactive oxygen intermediates. Pioglitazone reduced the elevated oxidative stress in heart and aorta of MetS rats.

Keywords: Cardiovascular disease; Insulin resistance; Metabolic syndrome; Oxidative stress; Pioglitazone

1. INTRODUCTION
Insulin resistance (IR), diabetes mellitus, hyperglycemia, abdominal obesity, atherogenic dyslipidemia, hypertension are the symptoms of metabolic syndrome (MetS). These symptoms are the significant risk factors for the development of vascular dysfunction and cardiovascular disease.1 Hyperglycemia exacerbates these cardiovascular dysfunctions.

Pioglitazone is an emerging class of antidiabetic drug that enhances insulin sensitivity in a peroxisome proliferator-activated receptor (PPAR)-γ dependent fashion.2 The activation of PPARγ suppresses lipolysis, decreases the plasma free fatty acids and leptin, and increases adiponectin level.3,4 Furthermore, activation of PPARγ also inhibits detrimental vascular inflammatory events. It has a distinctive role in upregulating the expression of endothelial nitric oxide synthase, thus resulting in the enhanced generation of vascular nitric oxide.1 By blocking Ca2+ channels, pioglitazone causes acute dilation of peripheral blood vessels, and this effect may partly contribute to their antihypertensive actions in type 2 diabetes.5 Besides, pioglitazone has been shown to heal endothelial dysfunction in diabetes6,7 in the aorta of fructose-fed diabetic rats.8

Reactive oxygen species (ROS) are generated physiologically by cellular metabolism. They have been implicated in cardiac functional damage.9 Possibly as a result in part of the ROS-induced production of adipocytokines, the increased oxidative stress is also thought to contribute to MetS.10 The oxidative stress is also a common factor linked separately to each of the components of MetS.11

The hypothesis of this study is that MetS has an augmenter effect on oxidative stress in the cardiovascular system. The current study, therefore, investigated the effect of pioglitazone on oxidative stress markers and TRX1 protein level in isolated heart and aorta from sucrose-fed induced MetS rat model.

2. METHODS

2.1. Experimental model
Three-month-old male Wistar Albino rats (200 to 250g) were used and held under standardized conditions (12-hour light/dark cycle, 24°C ± 2°C, 35% to 60% humidity). Rats were fed with standard laboratory chow and they had free access to water. The animals were randomly divided into the three groups consisting of eight rats each. Control group (Con) received standard laboratory diet and drinking water. MetS-induced group received drinking water including 32% sucrose (935 mM) for 20 weeks.11,12 Pioglitazone-treated MetS (MetS-P) group received
pioglitazone treatment (30 mg/kg/day, via gavage) for 2 weeks at the end of the 18th week of MetS group. After this period, the rats were anesthetized with pentobarbital sodium (30 mg/kg body weight, intraperitoneally), and then whole blood samples were taken from the abdominal aorta. The heart and thoracic aortas were removed and then used for homogenization. All animal-related procedures and experiments described in this study were approved by the Animal Ethics Committee of Ankara University Faculty of Medicine (2015-2-37).

2.2. Measurement of the homeostatic model assessment
Homeostatic model assessment (HOMA) is a method for assessing β-cell function and IR from basal (fasting) glucose and insulin concentrations. HOMA-IR is calculated using the following formula: HOMA-IR = fasting blood glucose (mmol/L) × fasting insulin (mU/L)/22.5. HOMA-β is calculated using the following formula: HOMA-β = (20 × fasting insulin [mU/L])/(fasting glucose [mmol/L] − 3.5). Insulin and triglyceride were measured using commercial kits (Cayman).

2.3. Tissue homogenization
Heart and aortas were homogenized with a motor-driven Teflon to glass homogenizer in cold (mM) TrisHCl 20 (pH 7.4), NaCl 150, KCl 2, EDTA 2, DTT 0.5, protease inhibitor cocktail 100, PMSF 0.4, and 2% NP-40. And then centrifugation process was performed to separate the cell membrane and cytosol. Protein content of cytosol was used in biochemical assays and western blot measurement.

2.4. Biochemical assays
After homogenization of heart and aorta tissues, protein content was analyzed using the Bradford method (Bio-Rad), and the bovine serum albumin was used as the standard. Then, the important cardiac enzymes such as lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured in tissues and serum via using commercial kits (Casabio, Biovision, respectively). As the markers of oxidative stress, total oxidant status (TOS) and total antioxidant status (TAS) were determined in tissues and serum using commercial kits (Rel assay diagnostics). Malondialdehyde (MDA) levels were determined by the thiobarbituric acid method and the pink color produced by these reactions was measured spectrophotometrically at 532 nm to determine the levels. Determination of the superoxide dismutase (SOD) enzyme activity was based on the production of hydrogen peroxide (H₂O₂) from xanthine oxidase and reduction of nitro blue tetrazolium. A spectrophotometric evaluation was performed at 560 nm.

2.5. Western blot
Protein level of thioredoxin 1 (TRX1) was determined by Western blot. Shortly, equal amount of proteins (20 µg) from samples were loaded and spread on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis (150 V, 1.5 hours), the samples were electroblotted onto a PVDF membrane (20 V, 2 hours). TRX1 contents in the samples were identified using anti-TRX1 (1/1000, rabbit, Abcam) antibody. Using the ECL plus detection system, immunoreactive protein bands were visualized.

2.6. Statistical methods
All parameters were expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (One-way ANOVA) followed by Bonferroni post-hoc analysis. p < 0.05 was considered as statistically significant level.

3. RESULTS
3.1. General parameters of experimental groups
Compared with Con group, at the end of the 20th-week experimental period, MetS group had significantly high glucose levels (Table 1) and they gain weight. The serum insulin level of MetS group increased approximately 90% and triglyceride content increased approximately 30% compared with the Con group. The HOMA (homeostasis model of assessment) index, which is the other marker of MetS, for measuring IR increased 190% compared with the Con group. There was a decrease in body weight in MetS-P group compared with MetS group. In MetS group, the pioglitazone treatment significantly decreased the elevated blood glucose level. The serum insulin level of MetS-P group increased compared with Con group, whereas it decreased significantly when compared with MetS group (Table 1).

3.2. Effects of pioglitazone on biochemical parameters in serum of MetS rats
The levels of LDH, AST, TOs, TAS, MDA, and SOD in the serum of experimental groups were represented in Table 2. Comparing with the Con group, while the level of AST, the level of LDH, TOs level, and MDA level increased significantly (p < 0.05) in MetS group, TAS level and SOD level of MetS group significantly decreased (p < 0.05). Pioglitazone treatment significantly decreased TOS level and significantly increased TAS level in MetS group. Compared with the MetS group, SOD level significantly increased (p < 0.05) and MDA level significantly decreased (p < 0.05) in the MetS-P group.

3.3. Effects of pioglitazone on AST and LDH levels in MetS tissues of heart and aorta
Figure 1A shows the effects of MetS and pioglitazone treatment on nonspecific cardiac enzymes (especially AST) in tissues of heart and aorta. Compared with the Con group, the level of AST significantly increased (p < 0.05) in heart and aorta tissues of MetS-P group. In terms of AST levels of both heart and aorta tissues, any statistically significant change was not observed in MetS-P group compared with the Con group. LDH levels of MetS and MetS-P groups in heart and aorta tissues are shown in Fig. 1B. As compared with the Con group, the LDH levels in heart and aorta tissues significantly increased (p < 0.05) in MetS group. Pioglitazone treatment significantly decreased the activities of LDH in heart in MetS-P group when compared with the MetS group.

Table 1
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<th>General characteristics of animals</th>
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<tr>
<td><strong>Body weight, g</strong></td>
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<td>Con (n = 8)</td>
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<td>MetS (n = 8)</td>
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<td>MetS-P (n = 8)</td>
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All parameters were expressed as mean ± SEM.
* p < 0.05 vs Con; ** p < 0.05 vs MetS.
Con = control; HOMA-IR = homeostatic model assessment insulin resistance; MetS = metabolic syndrome; MetS-P = pioglitazone treated metabolic syndrome group; n = number of rats.

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This study was conducted to assess the effect of pioglitazone on induced oxidative stress in the cardiovascular system of sucrose-fed rats. A previous report demonstrated that pioglitazone decreased body mass and blood glucose in a model of high-fat streptozotocin-induced diabetic rats. Several studies related to the MetS have shown that fructose and high-fat diet intake are associated with the risk of cardiovascular disease. Our results indicated that pioglitazone may play an important role in the regulation of oxidative stress in induced MetS in sucrose-fed rats.

In agreement with the previous studies that investigated the effect of high sucrose diets on rodents, we found that Wistar albino rats receiving the drinking water with 32% sucrose had higher daily water intake, higher glycemia and triglyceridemia, higher Ir, and higher insulin values than those in the control group. Pioglitazone restored these parameters to near the Con group levels.

Myocardium contains high concentrations of AST, which are released into the blood when heart tissue is damaged. Therefore, AST may be used to evaluate cardiovascular disease. Our research showed that compared with the Con group, AST increased significantly in serum and in both heart and aorta tissues of MetS group. This increase may be due to the high sucrose-induced damage in heart and aorta tissues. The LDH enzyme activity can reflect the degree of the tissue damage, which is mainly present in myocardium, skeletal muscle, liver, and kidney. The high concentrations of LDH are the indicator of cellular injury and inflammatory changes in tissues, particularly in the heart. Therefore, LDH is usually used to diagnose cardiovascular disease. In the present study, compared with the Con group, LDH levels increased significantly in serum and in both heart and aorta tissues of MetS group. The results showed that pioglitazone reduced the increased AST and LDH levels in heart and aorta tissues of MetS group.

Measuring plasma TAS is a sensitive and reliable marker in evaluation of the effects of different treatments such as exercise on plasma redox status. In the present study, compared with the Con group, there was a significant increase in TAS level in serum and in both heart and aorta tissues of MetS group, whereas there was a significant decrease in TAS level in serum and in both heart and aorta tissues of MetS group. These results showed that there was a level of oxidative stress in high sucrose-induced MetS. A previous study showed that SOD decreased and MDA increased in high-fat high-sucrose diet rats. Similarly, in the present study, it was observed that MDA increased and SOD decreased in serum, heart, and aorta tissues of MetS group. It was also shown that pioglitazone treatment caused a significant increase in the level of TAS in serum of MetS-P group compared with the Con group.

The TOS levels in heart and aorta tissues from MetS and MetS-P group are shown in Fig. 2A. TOS level in aorta tissues of MetS group significantly increased (p < 0.05) when compared to the MetS group. It was also shown that pioglitazone treatment caused a significant reduction (p < 0.05) in the TOS levels in aorta tissues of MetS-P group. The level of TAS is shown as a graph in Fig. 2B for tissues of both heart and aorta. Heart tissues of MetS group showed a significant reduction (p < 0.05) in TAS levels compared to the Con group. Also, there was a significant decrease (p < 0.05) in the TAS level of aorta tissues of MetS group compared with the Con group. Compared with the Con group, the MDA level significantly increased (p < 0.05) in heart tissues of MetS group, whereas there was a significant decrease in TAS level in serum and in both heart and aorta tissues of MetS group. This increase may be due to the high sucrose-induced damage in heart and aorta tissues. The LDH enzyme activity can reflect the degree of the tissue damage, which is mainly present in myocardium, skeletal muscle, liver, and kidney. The high concentrations of LDH are the indicator of cellular injury and inflammatory changes in tissues, particularly in the heart. Therefore, LDH is usually used to diagnose cardiovascular disease. In the present study, compared with the Con group, LDH levels increased significantly in serum and in both heart and aorta tissues of MetS group. The results showed that pioglitazone reduced the increased AST and LDH levels in heart and aorta tissues of MetS group.

3.5. Pioglitazone restores MetS-induced altered TRX1 protein level of heart and aorta

As shown in Fig. 3A, B, TRX1 protein level significantly decreased (p < 0.05) in heart and aorta tissues of the MetS group compared with the Con group. On the contrary, the TRX1 protein level in heart tissues of MetS-P group significantly increased compared to the MetS group.
protective effect in the cardiovascular system against MetS damage in rats. This effect was thought to be related to the improvement of the cardiovascular antioxidant defense.\textsuperscript{16}

TRXs are ubiquitous antioxidant enzymes that play important roles in many health-related cellular processes. TRX1 is an oxidoreductase that plays an important role in maintaining intracellular thiols in a reduced state.\textsuperscript{26} A previous study showed that overexpression of TRX1 attenuated the α-adrenergic receptor-stimulated decrease in free thiols in adult rat ventricular myocytes.\textsuperscript{27} Serum concentrations of TRX protein level raise in congestive heart failure.\textsuperscript{28} However, our results indicated that compared with Con group, TRX1 protein level significantly decreased in both heart and aorta tissues of MetS group. Pioglitazone treatment significantly restored these TRX1

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**Fig. 2** Changes of total oxidant status (TOS) in heart and aorta (A), changes of total antioxidant status (TAS) in heart and aorta (B), malondialdehyde (MDA) in heart and aorta (C), and superoxide dismutase (SOD) in heart and aorta (D) in experimental groups. Bar graph was expressed as mean ± SEM from control group (Con, n = 8), metabolic syndrome group (MetS, n = 8), pioglitazone-treated metabolic syndrome group (MetS-P, n = 8). *p < 0.05 vs Con; **p < 0.05 vs MetS.

**Fig. 3** Representative western blots of thioredoxin1 (TRX1) for heart (A) and aorta (B) in experimental groups. Bar graphs were expressed as mean ± SEM from Control, (n = 8); MetS, (n = 8); MetS-P, (n = 8); *p < 0.05 vs control; **p < 0.05 vs MetS.
protein levels in both heart and aorta tissues when compared with MetS group. Uproregation of TRX1 protein is affected by pioglitazone.

In conclusion, the results of the current study demonstrated that pioglitazone application significantly reduced the observed level of blood glucose, triglyceride, IR, and the other markers of MetS when compared with Con group. In addition, it was also observed that pioglitazone restored the changed levels of AST, LDH, TOS, TAS, MDA, and SOD in serum and in both heart and aorta tissues of MetS group. Furthermore, TRX1 protein level increased in MetS-P group. This aspect may be crucial for the maintenance of redox control. The results of this study showed that pioglitazone has protective effect against the MetS-induced oxidative stress in the cardiovascular system of rats. Our results may provide a basis for future research on the role of clinical application of pioglitazone to protect against oxidative stress in MetS and its complications.

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