

# Effect of pioglitazone on oxidative stress of skeletal muscle in the insulin resistance rat model induced by high sucrose diet

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## Abstract

**Background:** Metabolic syndrome is associated with some medical disorders such as central obesity, being overweight, insulin resistance and hypertension. This study was designed to determine the effect of pioglitazone on oxidative stress in the insulin resistance rat model.

**Methods:** In this study, the model was induced by high sucrose (935 mm) diet for 20 weeks. Three groups were used in the experiment. Control group received standard laboratory diet and drinking water. Metabolic syndrome induced group received 32% sucrose containing drinking water for 20 weeks. Pioglitazone-treated metabolic syndrome group has received pioglitazone treatment (30 mg/kg/day, via oral gavage) for two weeks at the end of the 18th week of metabolic syndrome group. After experimental period, skeletal muscle tissues were homogenized to measure important enzymes such as aspartate aminotransferase, lactate dehydrogenase and as the marker of oxidative stress; total-antioxidant-status, total-oxidant-status and malondialdehyde. Western blot technique was used to determine protein level of thioredoxin1.

**Results:** Aspartate aminotransferase and lactate dehydrogenase levels increased in metabolic syndrome group but pioglitazone treatment decreased these levels. In metabolic syndrome group the oxidative stress status increased but the treatment of pioglitazone decreased the level of oxidative stress in the skeletal muscle. In addition, thioredoxin1 decreased in metabolic syndrome group but administration of pioglitazone increased this level.

**Conclusions:** There was an elevated effect of oxidative stress in high sucrose fed rats but the treatment of pioglitazone improved glucose tolerance and insulin sensitivity.

**Keywords:** Metabolic Syndrome X, Oxidative Stress, Pioglitazone, Wistar Rat, TRX1 Protein.

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## INTRODUCTION

Insulin resistance (IR) comprises a common prevalent metabolic disorder, which seems to govern the pathophysiology of diabetes mellitus, metabolic syndrome (MS), and obesity (1). It is a central feature of MS, showing a strong association with most components of the syndrome (2). The metabolic consequences, hyperinsulinemia, hyperglycemia, lipid, and lipoprotein dysregulation act in synergy to sustain the pathologic state of insulin resistance (3). With the evolution of insulin resistance, endothelial dysfunction, inflammation, and atherosclerosis worsen progressively (4).

Skeletal muscle tissue is a primary tissue responsible for 85% of total body insulin-stimulated glucose uptake and oxidative metabolism (5). It is well established that skeletal muscle insulin resistance and impaired glucose metabolism are both due in part to reduced insulin action and glucose uptake (6). It plays a central role in the whole-body insulin resistance, as well as in the subsequent development of metabolic syndrome, type 2 diabetes (7).

Thiazolidinediones (TZDs) are a class of oral antidiabetic agents that improve insulin sensitivity and glucose homeostasis in type 2 diabetic patients (8,9). TZDs activate the transcription factor peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ), which is predominantly expressed in adipose tissue (10). Intramyocellular lipid (IMCL) is predominantly stored as intramuscular triglyceride in lipid droplets and is utilized as metabolic fuel during physical exercise. Pioglitazone is one of the members of TZDs; reduce IMCL content in insulin-resistant states (11), which is the most likely mechanism for TZDs to improve peripheral insulin sensitivity (12).

Impaired thioredoxin1 (TRX1) responses enforce a negative impact on antioxidant defense and tissue protection in experimental diabetes (13). An early study showed that serum TRX levels were higher in type 2 diabetics compared to controls (14).

The aim of the present study was to see whether oxidative stress occurs in skeletal muscles and to examine the possible ameliorative effect of pioglitazone on oxidative stress if oxidative stress has occurred in a skeletal muscle.

## MATERIALS AND METHODS

### *Animals and Experimental Model*

Three-months-old male Wistar Albino rats (200-250g) were used. Animals were kept under standard conditions (12-hour (h) light/ dark cycle,  $24\pm 2^\circ\text{C}$ , 35-60% humidity). Rats were fed both with standard laboratory chow which includes (as percentage) torula yeast 30.0, corn oil 2.0, sucrose 59.0, DL-methionine 0.3 and AIN-76 TM mineral mixture 5.0 and AIN-76 TM vitamin mixture 1.0 with digestible energy 12.59 MJ/kg from Horland Tekland, Madison, WI, USA and had free access to water. The animals were randomly divided into the three groups consisting of 8 rats each. Control group (Con) received standard laboratory diet and drinking water. MS group received 32% sucrose (935 mm) containing drinking water for 20 weeks (15). Pioglitazone-treated MS group (MS-PGZ) was given pioglitazone (30 mg/kg/day, via oral gavage) for two weeks. At the end of the experimental period, animals were anesthetized after being deprived of food for 12 h. Skeletal muscles were removed and used for measurements. In each experimental group, body weight (g), blood glucose level (mg/dl), insulin (ng/ml), triglyceride (mg/dl), homeostatic model assessment-insulin resistance (HOMA-IR), and HOMA- $\beta$  scores were measured as previously described in our study (16). All animal procedures and experiments described in the present study were approved by the Animal Ethics Committee of Ankara University Faculty of Medicine (Date: 02/02/2015, Number: 2015-2-37).

### *Tissue Homogenization*

Skeletal muscles were homogenized with a motor-driven Teflon to glass homogenizer in cold buffer: (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.

### *Biochemical Assays*

After homogenization of skeletal muscle tissues, protein content was analyzed using the Bradford method (Bio-

Rad), and bovine serum albumin was used as the standard. Then, the important enzymes such as AST and LDH (Biovision, Cusabio; respectively) were measured in tissues using commercial kits. As the marker of oxidative stress, TAS and TOS (Rel assay diagnostics) were determined in tissues using commercial kits. MDA levels were determined by the thiobarbituric acid (TBA) method and the pink color produced by these reactions was measured spectrophotometrically at 532 nm.

#### Western Blot

Protein level of TRX1 was determined by Western blot. Shortly, equal quantities of proteins (20  $\mu$ g) from samples were loaded and separated spread on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis (150 V, 1.5 h), the samples were electro-blotted onto a polyvinylidene difluoride (PVDF) membrane (20 V, 2 h). TRX1 contents in the samples were identified using anti-TRX1 (12 kd, 1/1000, rabbit, Abcam) antibody. Using the ECL plus detection system, immunoreactive protein bands were visualized. TRX1 protein levels in skeletal muscle tissues normalized according to the  $\beta$ -actin levels in skeletal muscle tissues from experimental group.

#### Chemicals

Unless specified, the reagents used were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Molecular weight markers and PVDF membranes were supplied from Bio-Rad. To detect proteins with Western blotting, enhanced chemiluminescence (ECL) plus reagents were used and supplied from GE Healthcare.

#### Statistical Analysis

All parameters were expressed as mean  $\pm$  standard error of mean (S.E.M.). Statistical analyses were performed using one-way analysis of variance followed by Bonferroni post-hoc analysis. The p values less than 0.05 were considered to be statistically significant.

## RESULTS

#### General parameters of experimental groups

The sucrose-fed rats exhibited several characteristics of MS, including central obesity, adiposity, and insulin

resistance. MS animals had significantly high blood glucose, insulin, triglyceride, and HOMA-IR scores compared with control animals (data not shown).

#### Aspartate aminotransferase and lactate dehydrogenase levels

Table 1 represents the effects of metabolic syndrome and pioglitazone treatment on AST enzymes in tissues of skeletal muscle. The activities of AST were significantly increased ( $p<0.05$ ) in MS as compared to the Con group. In MS group, the LDH level significantly increased as compared to the Con group. Pioglitazone treatment reduced significantly the LDH level in MS group as compared with both MS group and Con group.

**Table 1. Biochemical parameters in skeletal muscle of experimental groups.**

	Con (n=8)	MS (n=8)	MS-PGZ (n=8)
AST (mU/mL)	0.3 $\pm$ 0.05	0.6 $\pm$ 0.10 <sup>*</sup>	0.4 $\pm$ 0.08
LDH (mU/mL)	0.4 $\pm$ 0.03	0.9 $\pm$ 0.05 <sup>*</sup>	0.6 $\pm$ 0.03 <sup>**</sup>

All parameters were expressed as mean  $\pm$  standard error of mean (S.E.M.).

Con: control, MS: metabolic syndrome, MS-PGZ: pioglitazone-treated metabolic syndrome group, n number of rats. \* $p<0.05$  versus Con, \*\* $p<0.05$  versus MS.

#### Oxidative damage markers

The level of TAS in skeletal muscle tissue from experimental group was shown in Figure 1A. TAS level was significantly decreased ( $p<0.05$ ) in MS as compared to the Con group. But the treatment of pioglitazone significantly restored ( $p<0.05$ ) these changed TAS levels to that of Con group. TOS level of MS group was significantly increased ( $p<0.05$ ) as compared to the Con group. Furthermore, administration of pioglitazone decreased the level of TOS in MS group as compared to the MS group. MDA level of MS group was significantly increased ( $p<0.05$ ) as compared to the Con group. The level of MDA was significantly decreased ( $p<0.05$ ) in pioglitazone-treated MS group as compared to the MS group.

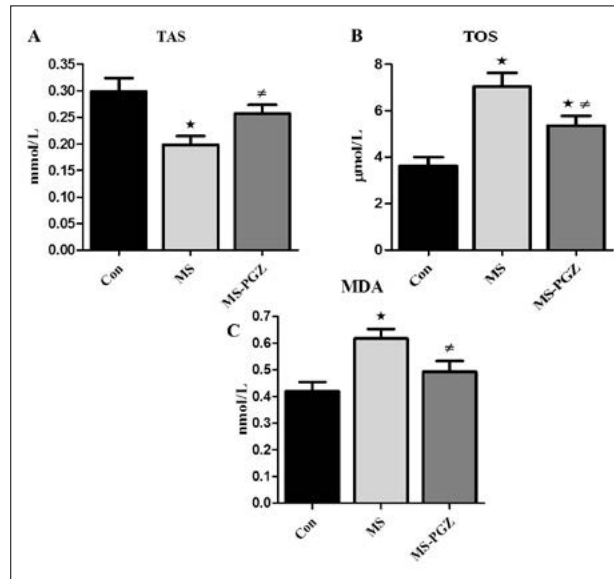


Figure 1. Changes of total antioxidant status (TAS) (A), changes of total oxidant status (TOS) (B) and malondialdehyde (MDA) (C) in skeletal muscles of experimental groups. Bar graph was expressed as mean ± standard error of mean (S.E.M.) from control group (Con, n=8), metabolic syndrome group (MS, n=8), pioglitazone-treated metabolic syndrome group (MS-PGZ, n=8). \* p<0.05 versus Con, ≠p<0.05 versus MS.

**Thioredoxin1 protein**

Figure 2 showed TRX1 protein level in experimental groups. TRX1 protein level in MS group significantly

decreased (p<0.05) as compared to the Con group. Pioglitazone treatment increased TRX1 protein level in MS group, but not significantly.

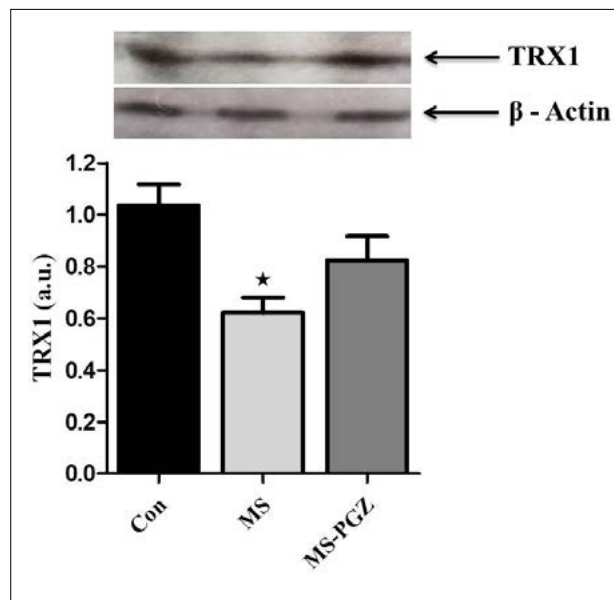


Figure 2: Representative western blots of thioredoxin1 (TRX1) for skeletal muscle in experimental groups is given at the top of the bar graphs. Densitometric results are expressed as a percentage of the band obtained with control in each of the experiments. Bar graphs were expressed as mean ± standard error of mean (S.E.M.) from control group (Con, n=8), metabolic syndrome group (MS, n=8), pioglitazone-treated metabolic syndrome group (MS-PGZ, n=8) \* p<0.05 versus Con.

## DISCUSSION

The present study demonstrated that pioglitazone has a favorable effect on oxidative stress of skeletal muscles in the insulin resistance rat model induced by high sucrose diet. This investigation provided new information about the oxidative stress markers and TRX-1 of skeletal muscles in a rat model of high sucrose diet. Additionally, the treatment of pioglitazone reduced the high sucrose induced elevated levels of AST and LDH in skeletal muscles of the MS group.

The defect is situated in peripheral tissues such as skeletal muscles and adipose tissue resulting in impaired insulin stimulated glucose utilization. Non-insulin dependent diabetes mellitus (NIDDM) is associated with insulin resistance and often accompanies hypertension, coronary heart disease or obesity (17). Association of these disorders with an atherogenic lipid profile, procoagulant state and other defects is usually referred to as metabolic syndrome (18). In the present study, the rats receiving 32% sucrose in the drinking water had higher daily water intake, higher glycemia, triglyceridemia, higher insulin resistance, and higher insulin values than those observed in control animals. As sucrose feeding altered total body weight, the observation of heavier fat depots suggests that the treatment increased the body adipose tissue mass, probably due to hypertrophy of the adipocytes. Treatment of pioglitazone significantly decreased the body weight compared to the MS group due to the decrement of both insulin resistance and daily uptake of calorie of rats as previously shown in our study (16).

AST and LDH, which are used as biomarkers for myotoxicity in both humans and animals (19), were elevated in patients with skeletal muscle injury due to various etiologies in the documented absence of liver disease in the study of Nathwani et al. (20). In accordance with this information, in the present study AST and LDH levels of MS groups were increased in skeletal muscles. This elevation may be caused by a defect of the skeletal muscle. The decrease in these elevated levels of AST and LDH via application of pioglitazone supports this information.

TAS shows the antioxidant levels in serum or related tissues (21). In a study, it was observed that the antioxidant levels of the alpha-lipoic acid group were significantly

higher in the hind limb than that of the control group (22). In the present study, TAS level significantly decreased in the skeletal muscles in MS group. Treatment of pioglitazone significantly increased the TAS levels of the skeletal muscles when compared to the MS group. TOS shows the oxidant levels in serum or related tissues. Kartal et al. observed that the alpha-lipoic acid reduces the TOS levels of the hind limb in ischemia reperfusion group. In our study, the TOS level of the MS group was significantly higher than Con group. Also, the TOS level of the MS-PGZ group was significantly lower than that of the MS and Con groups. These results show that pioglitazone reduces the oxidative stress status in skeletal muscles.

MDA increases in proportion to the severity of lipid peroxidation, but it is not specific (23). In a previous study, fibromyalgia rats showed significantly elevated muscle levels of MDA. PGZ administration resulted in a decrease of MDA levels, which were similar to levels of normal healthy rats (24). Our findings come in agreement with this study; PGZ treatment decreased the elevated MDA levels of MS compared to the Con group levels. This result may explain that PGZ treatment confers protection against metabolic syndrome-induced alterations.

In previous studies, serum, plasma, and lymphocyte levels of TRX1 were significantly elevated in type 2 diabetes when compared to the healthy controls (14, 25). Miyamoto et al. (26) showed that the levels of plasma TRX were higher in diabetes mellitus and impaired glucose tolerance groups than in normal glucose tolerance group. In contrast to these reports, it is found that the TRX1 level significantly decreased in skeletal muscle of MS group. Treatment of PGZ increased this level to the control level but not significantly. The decrease of TRX1 may be responsible for the enhanced oxidative damage in skeletal muscle.

These observations in the present study may be interpreted as that both insulin resistance and hyperglycemia increase oxidative stress and may be one reason behind the changing's levels of oxidative stress markers and TRX1 expression in MS group. Our findings of beneficial effects of PGZ on biomarkers, oxidative markers and TRX1 levels are important, since changes in levels of biomarkers and oxidative stress are considered to be among the most important molecular mechanisms leading to complications in MS group.

## Declarations

This study was supported by TUBITAK-SBAG-115S827 and Ankara Yıldırım Beyazıt University Projects Office-2864.

This study was approved by the clinical research Ethics Committee of the Animal Ethics Committee of Ankara University. (Date: 02/02/2015, Number: 2015-2-37)

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