

# Pioglitazone Eases Testicular Torsion/Detorsion-Induced Ischemia-Reperfusion Injury in Rats

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## What's known on the subject? and What does the study add?

Ischemia-reperfusion-induced changes in testicular histological parameters have been shown to be improved by pioglitazone 6 mg/kg therapy. Pioglitazone can be used in emergencies such as torsion and other chronic diseases to improve testicular functions.

## Abstract

**Objective:** This study demonstrated the protective effects of pioglitazone (Pio) on testicular torsion/detorsion-induced ischemia-reperfusion (I/R) injury.

**Materials and Methods:** A total of 48 male rats were randomly divided into six experimental groups of eight rats each; Control group, I/R group, Pio 3 mg/kg group, I/R treated with Pio 3 mg/kg group, Pio 6 mg/kg group, and I/R treated with Pio 6 mg/kg group. Testicular torsion was induced by twisting the left testis 720 °C in a clockwise direction (I/R groups). Both ischemia and reperfusion periods were 4 h. Single-dose 3 mg/kg Pio or 6 mg/kg Pio was administered orally two hours before reperfusion (Pio groups). Left orchiectomy was performed at the end of the protocol.

**Results:** In the I/R within-group analysis for mean seminiferous tubule diameter and epithelial lengths, a statistically significant difference was found only in the Pio 6 group ( $p=0.005$ ;  $p=0.005$ ). But Pio treatment failed to improve the levels of malondialdehyde and glutathione. Also, it did not cause any change in the non-I/R groups.

**Conclusion:** Considering the findings of Pio, it may be used in emergencies such as torsion and other chronic diseases.

**Keywords:** Testicular torsion, ischemia, reperfusion

## Introduction

Torsion of the spermatic cord is a urological emergency that usually requires surgical intervention (1). Permanent damage to the testis and spermatogenesis may occur despite early surgery (2). Ischemia-reperfusion (I/R) injury due to interruption of blood flow is seen in end organs such as the testis and kidney (3). Both ischemia and reperfusion phases cause the accumulation of reactive oxygen species (ROS) after induction of lipid peroxidation and oxidative stress (4). Pharmacological agents

that protect the testis in the I/R period by reducing oxidative stress have been the subject of many studies (5).

Peroxisome proliferator-activated receptors (PPARs) are subfamily nuclear hormone receptors, which regulate the transcription of several genes related to lipid metabolism, energy expenditure, atherosclerosis, infertility and inflammation (6). Pioglitazone (Pio) is a synthetic agonist of PPAR- $\gamma$  that was used to reduce insulin resistance in type 2 diabetes mellitus patients (7). Several studies have revealed the protective effects of Pio on I/R damage (8-10). There is strong evidence

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that it decreases injury by increasing the antioxidant capacity, especially in the kidneys (11). In this study, we investigated the possible protective effects of Pio on testicular I/R injury by measuring malondialdehyde (MDA), which is a marker of lipid peroxidation in tissues, and reduced glutathione (GSH) levels that show intense exposure to oxidative stress, and assessment of the histological pattern of testicular tissue.

## Materials and Methods

### Pio's Biochemical Properties

Thiazolidinediones have beneficial effects on lipid metabolism, endothelial function, oxidative stress, and vascular inflammation in addition to their antihyperglycemic effects (12).

Thiazolidinediones have intracellular antioxidant activity (13). This property reflects its anti-oxidant effects. These compounds work as antioxidants by preventing multiple pathways that induce oxidative stress in hyperglycemic conditions, rather than by releasing free radicals. Thiazolidinediones, in particular Pio, have been discovered to be a potent glycation and protein cross-linking inhibitor as well as a strong antioxidant (14).

### Animals

All experimental procedures were managed according to international ethical guidelines and were approved by the Local Ethical Committee of Trakya University (reference code

2020.02.01). A total of 48 healthy male Wistar Albino rats aged 16-20 weeks (400-500 g) were obtained from the Medical Faculty Experimental Animals and Research Laboratory. Rats were housed at the Animal Care and Research Unit under standard laboratory conditions at a relative humidity of 60%, temperature of  $22\pm 2$  °C, 12-hr light-dark cycle, and fed with dry rodent chow and water ad libitum.

### Study Design

A total of 48 male rats were divided into six experimental groups;

1. The control group (n=8): Rats treated only with orchietomy.
2. I/R (n=8): Rats were subjected to four hours of ischemia then four hours of reperfusion and treated with a 0.9% saline solution orally two hours before reperfusion.
3. Pio 3 (n=8): Rats were treated with 3 mg/kg Pio (Sanovel, Istanbul, Turkiye) orally six hours before orchietomy.
4. I/R + Pio 3 (n=8): Rats were subjected to four hours of ischemia then four hours of reperfusion and treated with 3 mg/kg Pio orally two hours before reperfusion.
5. Pio 6 (n=8): Rats were treated with 6 mg/kg Pio orally six hours before orchietomy.
6. I/R + Pio 6 (n=8): Rats were subjected to four hours of ischemia then four hours of reperfusion and treated with 6 mg/kg Pio orally two hours before reperfusion (Figure 1).

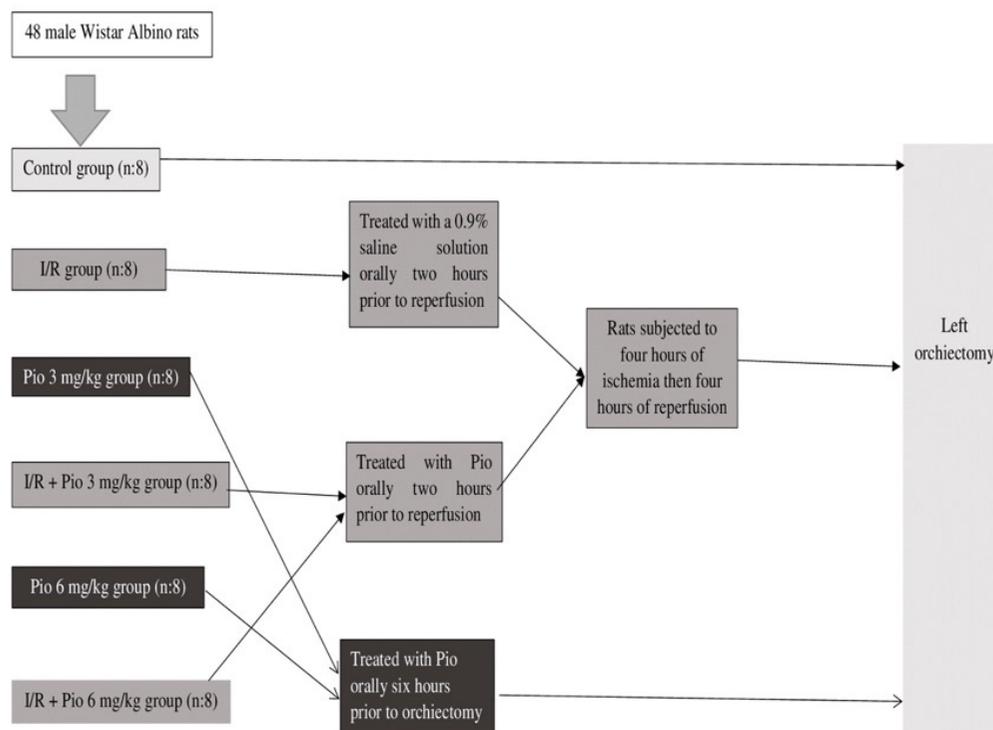


Figure 1. Study design

## Surgical Procedure

All surgical operations were performed under IM administration of xylazine (10 mg/kg, Alfasan International, Woerden, Holland) and ketamine (75 mg/kg, Pfizer Pharma GMBH, Germany). The skin of the scrotum was disinfected with 10% povidone-iodine solution. The scrotum was entered with a midline incision. After the opening of the tunica vaginalis, the left testis was identified. Torsion was then induced by twisting it 720 °C in a clockwise direction (I/R groups). The torsion position was maintained by fixing the testicle to the scrotum with a 4-0 silk suture (Figure 2). Single-dose 3 mg/kg Pio or 6 mg/kg Pio was administered orally two hours before reperfusion (Pio groups). After 4 h of ischemia, the testis was returned to the normal position for a 4-hour reperfusion period. Then left orchietomy was performed for all groups. Testes tissue samples were taken for histopathological investigation. Rats were killed by cervical dislocation at the end of the protocol.

## Histopathological Study and Spermatogenesis Evaluation

The tissue samples of each rat were fixed in 10% neutral formaldehyde and then the testis was sampled by the pathologist, including the largest surface from the middle. Each tissue was prepared as 5-µm sections from each paraffin block and stained with hematoxylin-eosin (H-E). Seminiferous tubule diameters, epithelial lengths (EL) and Johnsen testicular biopsy scores were calculated. Mean seminiferous tubule diameter

(MSTD) was measured in micrometers. Spermatogenesis was defined using Johnsen's mean testicular biopsy score (MTBS) criteria (15). Twenty tubules from each sample were randomly selected and scored from 1 to 10. Due to oxidative stress, EL and MSTD become shorter. These parameters are expected to deteriorate during I/R damage.

## Biochemical Analysis

A solution of 0.15 M KCl was used for the determination of MDA and GSH levels. Homogenates of 10% (w:v) were prepared from tissue samples and 0.15 M KCl solution. The supernatants were obtained by centrifuging the homogenates at 1500xg for 10 min at + 4 °C. Supernatants were used for spectrophotometric measurements of MDA and GSH levels. The pink resulting from the reaction of MDA with thiobarbituric acid (TBA) in a hot and acidic environment was measured using the spectrophotometric method (16). The GSH level was determined according to the Ellman (17) method. The color of free sulfhydryl groups in tissue homogenates was determined spectrophotometrically by Ellman (17) reagent. Tissue protein levels were determined by the Lowry method, which is based on the principle that the alkali copper tartrate reagent complexes with peptide bonds (18). When the phenol reagent is added to the mixture treated with copper, it creates a blue-purple color and which was measured spectrophotometrically at 660 nm.

## Statistical Analysis

All statistical analyses were performed using SPSS version 22.0 (SPSS, Chicago, IL, USA). All data are presented as mean ± standard deviation. One-Way analysis of variance (ANOVA) followed by post-hoc Tukey multiple comparison test was used to define statistical significance in multiple group comparisons. For the comparison of quantitative data, the Kruskal-Wallis test was used for intergroup comparisons of parameters that did not have a normal distribution, and the Mann-Whitney U test was used to identify the group that caused the difference. A  $p < 0.05$  value was considered statistically significant.

## Results

### Histopathological Changes

The MSTD was significantly shorter in the groups with I/R than those without I/R ( $p < 0.05$ ). In the I/R within-group analysis, eased MSTD was seen only in the Pio 6 group. Furthermore, a statistically significant difference was found only in the Pio 6 group ( $p = 0.005$ ;  $p = 0.005$ ). The EL was significantly shorter in the groups with I/R than those without I/R ( $p < 0.05$ ). In the I/R within-group analysis, eased EL was seen only in the Pio 6 group. Moreover, a statistically significant difference was found only in the Pio 6 group ( $p < 0.05$ ;  $p < 0.05$ ). The MTBS was significantly



**Figure 2.** Surgical Procedure A. Midline scrotal incision, B. The left testicle was released from the gubernaculum, C. Twisting testicular cord 720 °C in a clockwise direction, D. The torsion position was maintained by fixing the testicle to the scrotum with a 4-0 silk suture

lower in the groups with I/R compared with those without I/R ( $p < 0.05$ ). The differences in the levels of MSTD, EL and MTBS were not significant between the non-I/R within-group analysis ( $p = 0.591$ ,  $p = 0.674$ ,  $p = 0.767$ ) (Figure 3). In the I/R within-group analysis, little ease was observed in the Pio 6 group, but it was not statistically significant ( $p = 0.767$ ) (Table 1).

### Biochemical Analysis

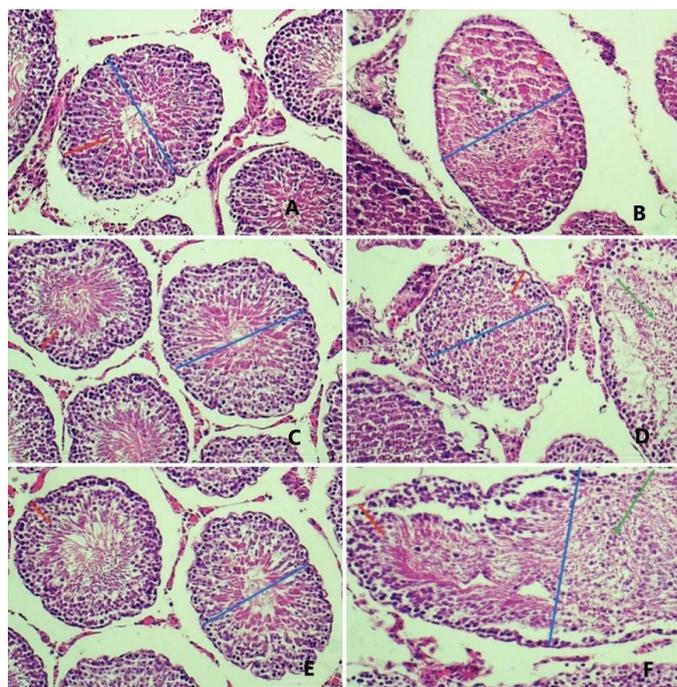
The MDA was significantly higher in the groups with I/R than those without I/R ( $p < 0.05$ ). The differences in the levels of MDA were not significant between the non-I/R within-group

analysis ( $p = 0.812$ ). In the I/R within-group analysis, little ease was observed in the Pio 6 group, but it was not statistically significant ( $p = 0.492$ ). GSH was significantly lower in the groups with I/R than those without I/R ( $p < 0.05$ ). The differences in the levels of GSH were not significant between the non-I/R within-group analysis ( $p = 0.996$ ). In the I/R within-group analysis, little ease was observed in the Pio 6 group, but it was not statistically significant ( $p = 0.845$ ) (Table 2).

### Discussion

Testicular torsion is a urological emergency that mostly affects young men (19). Despite early and successful surgical intervention, testicular functions are damaged by oxidative stress during I/R periods (20). The burst of mitochondrial ROS generation consumes natural antioxidants and leads to oxidative stress. Identifying medical agents to protect the testes from I/R injury would be potentially useful. Until now, many medications like oxygen radical scavengers have been successfully studied to reduce oxidative stress in animal models with I/R injury (21). But most of them are not currently in clinical use because of severe adverse effects.

I/R prompts the activation of neutrophils, increased thrombogenicity and inflammatory cytokines, the release of intracellular Ca, and the production of oxygen-derived free radicals (22). Pio was shown to reduce I/R damage by increasing the antioxidant capacity in many tissues (11,23). In a previous study, it was shown that Pio eased histopathological findings at both 1 and 3 mg/kg in testicular ischemia in rats and increased the levels of GSH and decreased levels of MDA (24). It's possible that some variables may cause this study's results to differ from ours. First unlike us, Pio was given intraperitoneally in that study. Also, pio was administered 30 min before detorsion, whereas it was administered 2 h before detorsion in our study. Another study revealed, Pio increased the levels of GSH at 10



**Figure 3.** Histological images of hematoxylin and eosin (H & E, 100X) staining of testicular tissue, A: Control, B: I/R, C: Pio 3, D: I/R + Pio 3, E: Pio 6, F: I/R + Pio 6. I/R: Ischemia-reperfusion, Pio: Pioglitazone, Red line: Epithelial length, Blue line: Seminiferous tubule diameter, Green arrow in B, D, F: Necrosis accumulation

Groups	MSTD ( $\mu\text{m}$ )	p-value	EL ( $\mu\text{m}$ )	p-value	MTBS	p-value
Control vs Pio 3	165-170	0.591	44.38-43.75	0.674	9.75-9.88	0.591
Control vs Pio 6	165-167.5	0.591	44.38-45.62	0.674	9.75-9.88	0.591
Pio 3 vs Pio 6	170-167.5	0.591	43.75-45.62	0.674	9.88-9.88	0.591
Control vs I/R	165-113.75	0.001	44.38-32.5	0.001	9.75-6.25	0.001
Pio 3 vs I/R+Pio 3	170-113.75	0.001	43.75-32.5	0.001	9.88-6.25	0.001
Pio 6 vs I/R+Pio 6	167.5-128.75	0.001	45.62-38.75	0.001	9.88-6.62	0.001
I/R vs I/R+Pio 3	113.75-113.75	0.002	32.5-32.5	0.001	6.25-6.25	0.767
I/R vs I/R+Pio 6	113.75-128.75	0.002	32.5-38.75	0.001	6.25-6.62	0.767
I/R+Pio 3 vs I/R+Pio 6	113.75-128.75	0.002	32.5-38.75	0.001	6.25-6.62	0.767

MSTD: Mean seminiferous tubule diameter, EL: Epithelial lengths, MTBS: Johnsen's mean testicular biopsy score, Pio: Pioglitazone, I/R: Ischemia-reperfusion, values are mean  $\pm$  standard deviation  $p < 0.05$  was considered statistically significant  
\*Statistical analyses were performed between treatment groups.

**Table 2. Biochemical analysis between groups (n=8 for each group)**

Groups	MDA (nmol/mg)	p-value	GSH (nmol/mg)	p-value
Control vs Pio 3	0.78-0.75	0.812	891.8-895.3	0.996
Control vs Pio 6	0.78-0.76	0.812	891.8-905.6	0.996
Pio 3 vs Pio 6	0.75-0.76	0.812	895.3-905.6	0.996
Control vs I/R	0.78-1.00	<b>0.001</b>	891.8-694.4	<b>0.001</b>
Pio 3 vs I/R+Pio 3	0.75-0.99	<b>0.001</b>	895.3-679.0	<b>0.001</b>
Pio 6 vs I/R+Pio 6	0.76-0.90	<b>0.001</b>	905.6-746.0	<b>0.001</b>
I/R vs I/R+Pio 3	1.00-0.99	0.492	694.4-679.0	0.845
I/R vs I/R+Pio 6	1.00-0.90	0.492	694.4-746.0	0.845
I/R+Pio 3 vs I/R+Pio 6	0.99-0.99	0.492	679.0-746.0	0.845

MDA: Malondialdehyde; GSH, glutathione; Pio, Pioglitazone; I/R, Ischemia-reperfusion; values are mean ± SD. p<0.05 was considered statistically significant  
\*Statistical analyses were performed between treatment groups.

mg/kg during renal ischemia in rats (11). Pio increased the levels of GSH at both 20 and 40 mg/kg for renal ischemia in rats in a other study (25). In this study, Pio showed improvement in histopathological findings at a dose of 6 mg/kg, but failed to improve the level of MDA and GSH. Also, it did not cause any change in the non-I/R groups. These findings may be related to the beneficial effects of spermatic cell proliferation on the seminiferous tubules.

Consensus has not yet been reached on the dose and duration of Pio for the most effective antioxidant treatment. Furthermore, randomized controlled studies on large human samples are needed in terms of the side effect profile with this effective dose.

### Study Limitations

The fact that our study is an animal study is an important limitation. Studies on the effects of Pio on humans should be conducted to strengthen these findings.

### Conclusion

I/R of 4 h causes severe damage in the testis. The administration of Pio improved the histopathological parameters. Moreover, further investigations with higher doses must demonstrate the potential effects of Pio in I/R injury and other diseases that affect spermatogenesis.

### Ethics

**Ethics Committee Approval:** All experimental procedures were managed according to international ethical guidelines and were approved by the Local Ethical Committee of Trakya University (reference code 2020.02.01).

**Informed Consent:** Informed consent was obtained.

**Peer-review:** Externally and internally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: İ.Y.Ş., M.D.D., M.A.A., S.M., O.K., E.T., T.A., Concept: İ.Y.Ş., M.A.A., S.M., O.K., E.T., T.A., Design: İ.Y.Ş., M.D.D., M.A.A., S.M., O.K., E.T., T.A., Data Collection or Processing: İ.Y.Ş., M.D.D., M.A.A., S.M., O.K., E.T., T.A., Analysis or Interpretation: İ.Y.Ş., M.D.D., M.A.A., S.M., O.K., E.T., T.A., Literature Search: İ.Y.Ş., M.D.D., M.A.A., S.M., O.K., E.T., T.A., Writing: İ.Y.Ş., M.D.D., M.A.A., S.M., O.K., E.T., T.A.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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