TÜBİTAK

Turkish Journal of Zoology

http://journals.tubitak.gov.tr/zoology/

Research Article

Turk J Zool
(2021) 45: 267-276
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doi:10.3906/zoo-2103-5

Investigation of the effect of silymarin on oxidative DNA damage and inflammatory markers in ischemia/reperfusion injury following experimental testicular torsion/detorsion in rats

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Received: 06.03.2020 • **Accepted/Published Online:** 01.06.2021 • **Final Version:** 14.07.2021

Abstract: The aim of the present study was to clarify the effects of silymarin on experimental testicular ischemia / reperfusion injury. A total of 40 Wistar albino rats (10–12 weeks of age, weighing 280–300 g) were randomly divided into five groups. Control group: No surgical procedures were performed. Torsion 3 h / detorsion 3 h group; torsion 3 h / detorsion 24 h group; torsion 3 h / detorsion 3 h + silymarin (250 mg/kg) group; and torsion 3 h / detorsion 24 h + silymarin (250 mg/kg) group. In the study, 720° torsion was applied to the left testicle. At the end of the study, blood was collected from the rats, and an orchiectomy was performed on the left testicles. It was found that tumor necrosis factor alpha (Tnf-α) and 8-hydroxy-2-deoxyguanosine (8-OHdG) expression levels in testicular tissue increased significantly in torsion/detorsion groups, and the expression levels decreased significantly with silymarin administration. In addition, in the testicular tissue of the torsion/detorsion groups, glutathione (GSH) levels, superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities decreased, while malondialdehyde (MDA) levels increased. It was found that the parameters specified were reversed with the administration of silymarin. Based on our findings, we can say that silymarin reduces testicular injury by activating antioxidant mechanisms in ischemia/reperfusion injury and minimizing the inflammatory response.

Key words: Rat, silymarin, testicular ischemia / reperfusion, tnf-α, 8-OHdG

1. Introduction

Testicular torsion can lead to infertility caused by necrosis in male germ cells; this is a urological condition requiring urgent treatment (Gezici et al., 2006; Wei et al., 2011). The immediate intervention required is to perform detorsion to restore blood flow to the testis. However, reperfusion during detorsion causes damage as large amounts of free oxygen radicals are released in reperfusion (Akgür et al., 1993; Akgür et al., 1994). The increase in free oxygen radicals leads to deterioration of cell membrane permeability and lipid peroxidation (Sikka, 2004). Maintaining the viability of testicular tissue is possible by relieving the reperfusion event created. For this purpose, antioxidants have been used to prevent the detrimental effects of reactive oxygen radicals in testicular torsion (Dilber et al., 2016).

In recent years, many chemical agents of herbal origin have been used in the treatment of various diseases with good results (Abdel-Daim et al., 2020; Manzoni et al., 2020; Muhammad et al., 2020). These include silymarin (*Silybum marianum*), a medicinal plant also known as milk thistle, a member of the Asteraceae family. Silymarin is widely used in traditional medicine (Morazzoni et al., 1995). It has been reported that silymarin's biological activities include hepatoprotective (Kheiripour et al., 2019), anti-cancer (Won et al., 2018), anti-inflammatory (Jin et al., 2016), and antioxidant (Nouri et al., 2019) properties. Its antioxidant power is high due to the methoxy group on the phenolic ring (Kidd, 2009). Positive effects on reproductive performance and increased testosterone levels in diabetic rats have been reported (Khoei et al., 2019). It has also been shown to reduce apoptosis and improve spermatogenesis in rats with varicocele (Mazhari et al., 2018) and to have ameliorating effects on lipid peroxidation and inflammatory processes in lung ischemia/reperfusion injury (Jin et al., 2016).

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Studies investigating the effect of silymarin on ischemia/reperfusion have determined that silibinin, the major active constituent of silymarin, has a protective effect on vital organs such as the liver, kidney, lung, and heart after ischemia/reperfusion (Chen et al., 2020; Jin et al., 2016; Qajari et al., 2020; Tsaroucha et al., 2018; Tsaroucha et al., 2020). However, no previous data could be found on its effect on ischemia/reperfusion in the testicles, which is critical for the continuity of fertility. Therefore, the aim of the present study was to investigate the effects of silymarin on ischemia/reperfusion injury after testicular torsion/detorsion in rats.

2. Materials and methods

2.1. Chemicals

The chemicals used in our study are as follows:
Silymarin (S0292, Sigma-Aldrich Merck KGaA, Darmstadt, Germany), Tnf-α antibody (Catalog No: sc52746, 1/50 dilution, Santa Cruz, Texas, USA), 8-OHdG antibody (Catalog No: sc-66036, 1/50 dilution, Santa Cruz, Texas, USA), GSH (Catalog No: SG-20391, SinoGeneClon Biotech Co., Ltd, Hangzhou, China), SOD (Catalog No: SG-10188, SinoGeneClon Biotech Co., Ltd, Hangzhou, China), GPx (Catalog No: SG-20976, SinoGeneClon Biotech Co., Ltd, Hangzhou, China), and 8-OHdG (Catalog No: YLA0061RA, Ylbiont, China).

2.2. Test animals and experimental design

Rats were obtained from Van Yüzüncü Yıl University Experimental Research Center. Body weight was measured, and clinical examinations were performed. Rats with scrotal distress or any other pathology and those with unsuitable body weight or poor general appearance were excluded. A total of 40 rats meeting these criteria were selected. They were housed in standard laboratory conditions (temperature 24 ± 3°C, humidity 50%–60%, 12 h light / 12 h dark). Experimental procedures were carried out in the same center.

All procedures were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the institutional ethical review committee (Van Yüzüncü Yıl University Local Ethics Committee of Animal Experiments, Van, Turkey, Approval number: 2019/11). The study was performed on male Wistar albino rats (10–12 weeks of age, weighing 280–300 g). A total of 40 rats were divided randomly into 5 groups of equal numbers. Torsion and detorsion were applied to the left testis of the rats under anesthesia. The left testes and blood were collected at the end of the study. Previous studies were taken as reference for the dose of silymarin used in our study (Jin et al., 2016; Rao and Viswanath, 2007).

Groups were treated as follows:

Group 1: Control group (n = 8). Approximately 2 cm incision was made to the left scrotal sac and the left testis was displaced from the scrotal sac and replaced again. The scrotal opening was closed with 4-0 silk thread.

Group 2: Torsion 3 h / detorsion 3 h group (n = 8). A small incision was made in the scrotum, and the left testis was exposed with the spermatic cord and the tunica vaginalis. The extracted testis was rotated 720° clockwise and fixed to the inside of the scrotum with 5-0 silk thread. Thus, testicular torsion was maintained for 3 h, after which the testis was exposed, detorsion performed, and it was returned to the scrotal sac. After 3 h of detorsion, blood samples were collected and an orchiectomy was performed.

Group 3: Torsion 3 h / detorsion 24 h group (n = 8). Similar procedures as in group 2 but with a detorsion time of 24 h.

Group 4: Torsion 3 h / detorsion 3 h + 250 mg/kg IP silymarin group (n = 8). In this group, 250 mg / kg IP silymarin was administered 2.5 hours after detorsion. At 0.5 h after silymarin administration, blood samples were collected and an orchiectomy was performed.

Group 5: Torsion 3 h / detorsion 24 h + 250 mg/kg IP silymarin group (n = 8). In this group, 250 mg / kg silymarin IP was administered 23.5 hours after detorsion. At 0.5 h after silymarin administration, blood samples were collected and an orchiectomy was performed.

Rats were anesthetized before torsion, again before detorsion, and again before blood collection. That is, each rat was anesthetized three times throughout the study. After the surgical procedure and silymarin applications, the rats undergoing anesthesia (xylazine 10 mg/kg IP and ketamine 75 mg/kg IP) were placed on the table in the dorso-ventral position, and blood samples were drawn in tubes without anticoagulant. The blood samples were centrifuged at 4000 rpm for 10 min, and the serum was removed. These serum samples were stored at –20°C through the work day. At the end of the study, the animals were euthanized with an overdose of dissociative anesthetic agents.

2.3. Histopathological examination

To perform histopathological evaluation after necropsy, testicular tissue samples were left in a 10% formalin solution for 48 h. Tissue tracking was performed and embedded in paraffin blocks. Sections were excised at 4 μm thickness. The prepared tissue samples were stained with hematoxylin-eosin (HE) and examined under light microscopy (Leica DM 1000, Leica Microsystems GmbH, Wetzlar, Germany). Sections were classified according to histopathological findings as none (–), mild (+), moderate (++), and severe (+++) (Yildirim et al., 2020).

2.4. Immunohistochemical examination

All sections taken on adhesive (Poly-L-Lysin) slides were first passed through a xylol and alcohol series to perform immunoperoxidase examination. They were then washed with PBS and kept at 3% H_2O_2 (10 min) to ensure endogenous peroxidase inactivation. To expose

the antigen in the tissues, they were treated with antigen retrieval solution (at 500 watts for 2–5 min) and allowed to cool. Tissues were incubated (30 min at 37 °C) with Tnf-α and 8-OHdG antibody (Catalog No: sc52746, dilution 1/50; sc-66036, dilution 1/50; Santa Cruz, USA). Immunohistochemistry was carried out according to the manufacturer's instructions (AbcamHRP / DAB Detection IHC kit). 3-3 'Diaminobenzidine (DAB) was used as a chromogen. Floor painting was done with hematoxylin. Sections were evaluated according to their immune positivity as (–), mild (+), moderate (++), severe (+++), and very severe (++++) (Yildirim et al., 2020).

2.5. Biochemical examination

MDA was determined by spectrometric method. An Abbott kit was used for testosterone detection (Dubovsky et al., 2008). Measurement was done with an ARCHITECT c1616200 autoanalyser. It was assessed by the ELISA method using GSH (Catalog No: SG-20391), SOD (Catalog No: SG-10188), GPx (Catalog No: SG-20976), and 8-OHdG (Catalog No: YLA0061RA) commercial kits. The researchers conducting these analyses were blinded to the identity of the groups.

2.6. Statistical analysis

SPSS (Statistical Package for the Social Sciences) software program (version 20.00, IBM Inc., Chicago, IL, USA) was used for statistical analysis. In histopathological examination, the Kruskal–Wallis (non-parametric) test was used to determine the differences between groups. A Mann–Whitney U test was used to compare the binary groups. One-way ANOVA and then a post hoc Tukey test were used in the analysis of biochemical data. Data are presented as mean ± standard error. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Histopathological findings

Control group: The histological appearance of testicular tissues was observed to be normal (Figure 1A). Torsion 3 h / detorsion 3 h group: While congestion and hemorrhage were detected at a severe level in the intertubular intervals, edema was detected at a mild level. The decrease in the number of spermatids in the tubulus lumen was severe (Figure 1B). Torsion 3 h / detorsion 24 h group: Edema detected at intertubular intervals was severe. In addition, there was a severe decrease in the number of spermatocytes in the tubule lumen and a severe thinning in the tubulus wall (Figure 1C). Torsion 3 h / detorsion 3 h + 250 mg/kg IP silymarin administered group: Congestion in the interstitial spaces, active spermatozoa in the tubulus lumen, and thinning in the tubulus walls were mild (Figure 1D). When this group was compared with the torsion 3 h / detorsion 3 h group, there was a statistically significant difference ($p < 0.05$). Torsion 3 h / detorsion 24 h + 250 mg/kg IP silymarin administered group: While there was mild edema and congestion in the intertubular intervals, a slight decrease in the number of spermatozoa was determined in the tubule lumen (Figure 1E). When this group was compared with the torsion 3 h / detorsion 3 h group, there was a statistically significant difference ($p < 0.05$). Histopathological findings are summarized in Table 1.

3.2. Immunohistochemical findings

Control group: Tnf-α and 8-OHdG expressions were determined to be negative in testicular tissues of this group of rats (Figures 2–3A). Torsion 3 h / detorsion 3 h group: Expression of Tnf-α at the intertubular intervals and around the vessels was very severe. In addition, cytoplasmic 8-OHdG in spermatocytes was severe (Figures 2–3B). Torsion 3 h / detorsion 24 h group: Expression of Tnf-α in the intertubular intervals and around the vessels was severe. Cytoplasmic 8-OHdG was detected at a severe level in spermatocytes (Figures 2–3C). Torsion 3 h / detorsion 3 h + 250 mg/kg IP silymarin administered group: Moderate expression of Tnf-α in intertubular intervals and vascular circumference, and moderate cytoplasmic 8-OHdG expression in spermatocytes were detected (Figures 2–3D). When this group was compared with the torsion 3 h / detorsion 3 h group, there was a statistically significant difference ($p < 0.05$). Torsion 3 h / detorsion 24 h + 250 mg/kg IP silymarin administered group: Mild expression of Tnf-α in intertubular intervals and vascular circumference and mild cytoplasmic 8-OHdG expression in spermatocytes were observed (Figures 2–3E). When this group was compared with the torsion 3 h / detorsion 24 h group, there was a statistically significant difference ($p < 0.05$). Immunohistochemical findings are summarized in Table 1.

3.3. Biochemical findings

The GSH level of testicular tissue was significantly lower in the torsion 3h / detorsion 3 h and the torsion 3 h / detorsion 24 h groups compared to the control group. In the torsion 3 h / detorsion 3 h + silymarin group, the testicular tissue GSH level was the same as the control group, while, in the torsion 3 h / detorsion 24 h + silymarin group, it was significantly higher than the control group. In torsion 3 h / detorsion 3 h and torsion 3 h / detorsion 24 h groups, testicular tissue SOD and GPx activities were significantly lower than in the control group. Testicular tissue SOD and GPx activities were significantly higher in the torsion 3 h / detorsion 24 h group than in the torsion 3 h / detorsion 3 h group. In the torsion 3 h / detorsion 3 h and torsion 3 h / detorsion 24 h groups, testicular tissue 8-OHdG level was found to be significantly higher than in the control. In torsion/detorsion groups given silymarin, the 8-OHdG level of testicular tissue was found to be significantly lower than in the torsion 3 h / detorsion 24

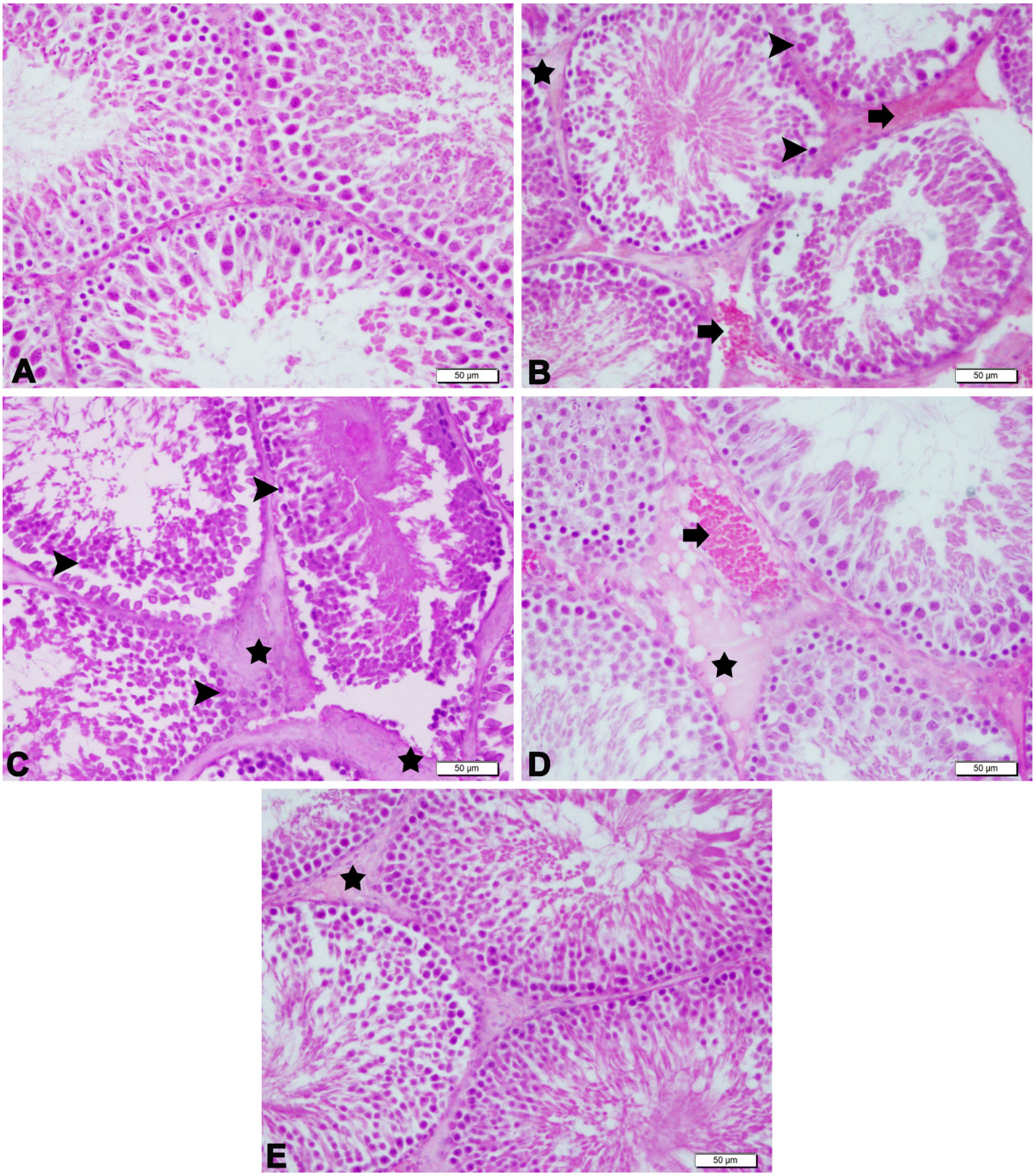

Figure 1. Testicular tissue, control group, normal histological view (A). T 3 h / D 3 h group: necrosis in spermatocytes (arrowheads); severe congestion in intertubular intervals (thick arrows); hemorrhage, thinning in the tubulus wall (B). T 3 h / D 24 h: necrosis in spermatocytes (arrowheads); severe edema in the intertubular space (stars); thinning of the tubulus wall (C). T 3 h / D 3 h + silymarin group: moderate edema in the intertubular intervals (star); congestion (thick arrow) (D). T 3 h / D 24 h + silymarin group: mild edema at intertubular intervals (star) (E); H and E, bar: 50 µm.

h group, while it was significantly higher than the control group. Testicular tissue MDA level was significantly higher in torsion 3 h / detorsion 3 h and torsion 3 h / detorsion 24 h groups compared to the silymarin applied torsion/detorsion groups and the control group. It was determined that the serum testosterone level decreased significantly in the torsion/detorsion groups compared to the control group. With silymarin administration, it was determined

Table 1. Scoring histopathological and immunohistochemical findings in testicular tissues.

	Control	T 3 h / D 3 h	T 3 h / D 24 h	T 3 h / D 3 h + Slymarin	T 3 h / D 24 h + Slymarin
Congestion-hemorrhage in intertubular intervals	-	+++	+	++	+
Edema at intertubular intervals	-	+	+++	++	+
Necrosis in spermatocytes	-	++	+++	+	-
Decrease in the number of spermatogonium	-	+++	+++	+	-
Tnf-α	-	++++	+++	++	+
8 OHdG	-	+++	+++	++	+

Tnf-α = tumor necrosis factor alpha; 8-OHdG = 8-hydroxy-2-deoxyguanosine; T = torsion; D = detorsion.

that testosterone levels increased significantly compared to torsion/detorsion groups without silymarin. Biochemical findings are presented in Table 2.

4. Discussion

Early diagnosis and treatment is very important in testicular torsion, because it can lead to infertility by causing hypoxia and necrosis of germ cells in the testicular tissue (Melekos et al., 1988). Reestablishing blood flow as soon as possible is critical to prevent permanent damage. This is possible by detorsion of the torsioned testicle. However, many free oxygen radicals invade the environment in the reperfusion event caused by detorsion (Akgür et al., 1993; Akgür et al., 1994). The increase in free oxygen radicals leads to peroxidation and DNA damage of lipids in the cell membrane (Akgür et al., 1993; Akgür et al., 1994; Hamed et al., 2011). In addition, although detorsion performed immediately can protect testicular tissue from damage, it is estimated that an average of 25% of men with testicular torsion will experience infertility (Krarup, 1978). In order to minimize the damage inflicted in this way, it may be effective to use appropriate chemical agents with the detorsion event and evaluate the results.

MDA, the end product of lipid peroxidation, which is used to determine oxidative stress in post ischemic tissue, was shown to increase in our study in torsion/detorsion groups, but the increase diminished in groups administered silymarin. Especially in the 24 h detorsion + silymarin group, the MDA level approached the control group values. Silymarin's antioxidant effect may be attributed to the decrease in the level of MDA. Our conclusion regarding MDA in torsion/detorsion groups supports previous studies (Ganjani et al., 2020; Javanmardi and Khordadmehr, 2017; Shokoohi et al., 2018).

Tnf-α produced in the primary phase of inflammation has been reported to increase excessively in testicular tissue in an ischemia/reperfusion event (Dinarello et al., 2000; Minutoli et al., 2007; Refaie et al., 2017; Turner et al., 2004). Supporting these findings, our study found immunohistochemically that the level of Tnf-α expression increased significantly to a severe level after torsion/detorsion. This result was in parallel with the Tnf-α expression result evaluated immunohistochemically in an ovarian ischemia/reperfusion study (Geyikoglu et al., 2019). In our study, the decline of the level of Tnf-α expression in groups administered silymarin compared to the untreated torsion/detorsion groups may have resulted from silymarin's anti-inflammatory effect.

Proinflammatory cytokines produced in excessive amounts with ischemia/reperfusion injury increase the movement of neutrophils and leukocytes toward the testicular tissue. This causes excessive production of reactive oxygen species (Kostakis et al., 2017). Physiological levels of free oxygen radicals play an important role in many biological mechanisms, such as sperm capacitation and acrosome reaction. However, excessive free oxygen radicals can impair sperm function (Saalu, 2010). In the literature, it has been reported that testicular ischemia/reperfusion events cause a decrease in SOD value (Abdelzaher et al., 2020; Dejban et al., 2019; Ganjani et al., 2020; Shakoohi et al., 2018; Un et al., 2015). In our study, the SOD value decreased significantly in the torsion/detorsion groups compared to the control group. The increase of SOD value in groups administered silymarin demonstrates the strength of antioxidant therapy.

The expression of 8-OHdG increased significantly in the torsion/detorsion groups compared to the control and silymarin administered groups in our study. Our conclusion is in agreement with previous studies (Geyikoglu et al., 2019; Tamer et al., 2018). In our study, this increase was detected both biochemically and immunohistochemically. While the 8-OHdG value decreased significantly in the 24 h detorsion group administered silymarin, the decrease in the 3 h detorsion group was not significant.

Testosterone is known to play an important role in testicles (Ramaswamy and Weinbauer, 2015). Serum testosterone levels in the torsion/detorsion groups were

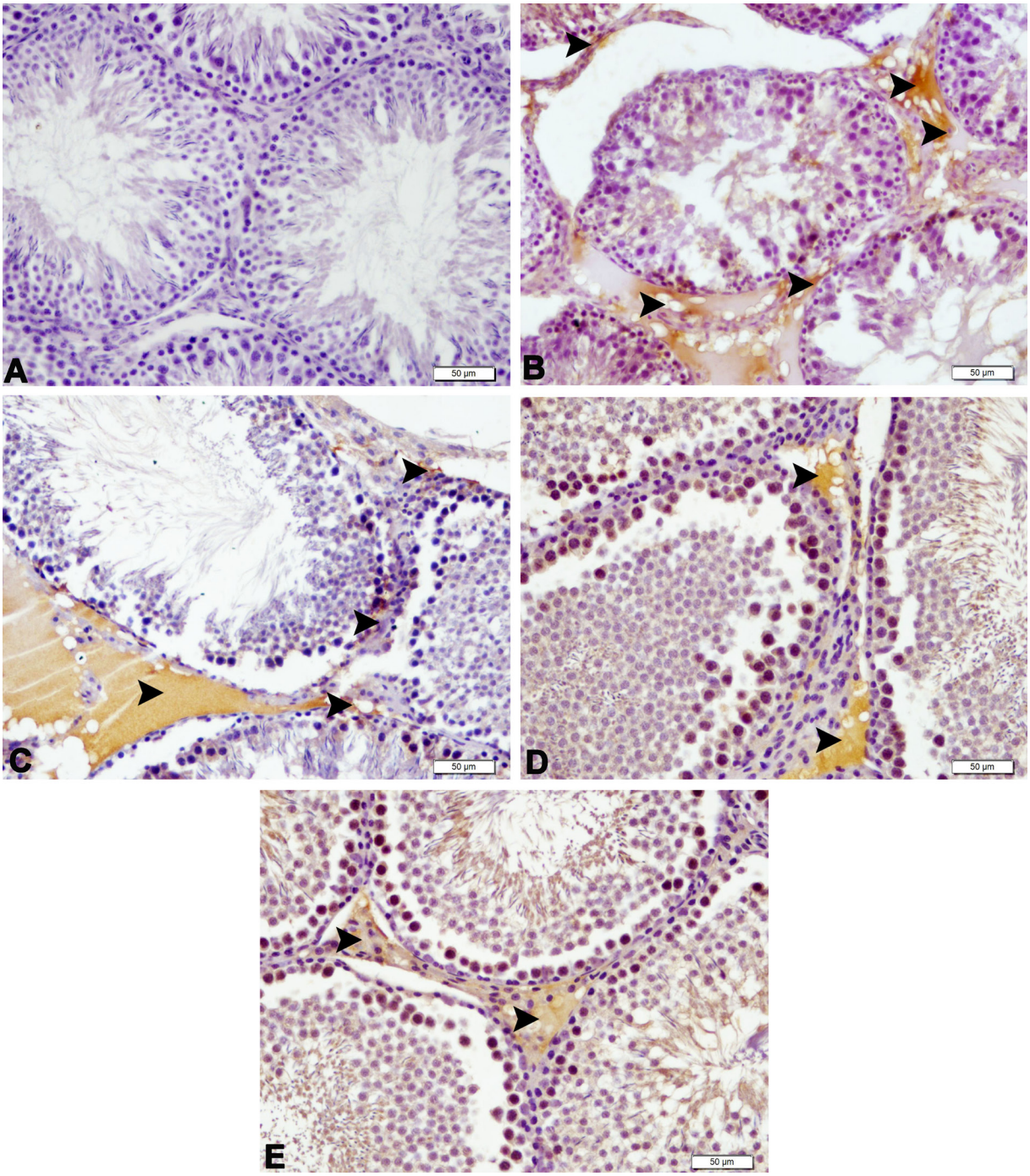

Figure 2. Testicular tissue, control group, negative Tnf-α expression (A). T 3 h / D 3 h group: very severe Tnf-α expression in the perivascular and intertubular intervals (arrowheads) (B). T 3 h / D 24 h group: severe Tnf-α expression in the perivascular and intertubular intervals (arrowheads) (C). T 3 h / D 3 h + silymarin group: medium level Tnf-α expression in the perivascular and intertubular intervals (arrowheads) (D). T 3 h / D 24 h + silymarin group: mild Tnf-α expression in the perivascular and intertubular intervals (arrowheads) (E). IHC-P, bar: 50 µm.

found to be significantly decreased when compared to the control group. The decrease in testosterone has been similarly reported in previous testicular ischemia studies (Abdelzaher et al., 2020; Kheirollahi et al., 2018; Shakoohi et al., 2018). The decrease in testosterone may be associated with damage to the Leydig cells (Benny et al., 2019).

In our study, the significant decrease in GSH value in the torsion/detorsion groups compared to the control

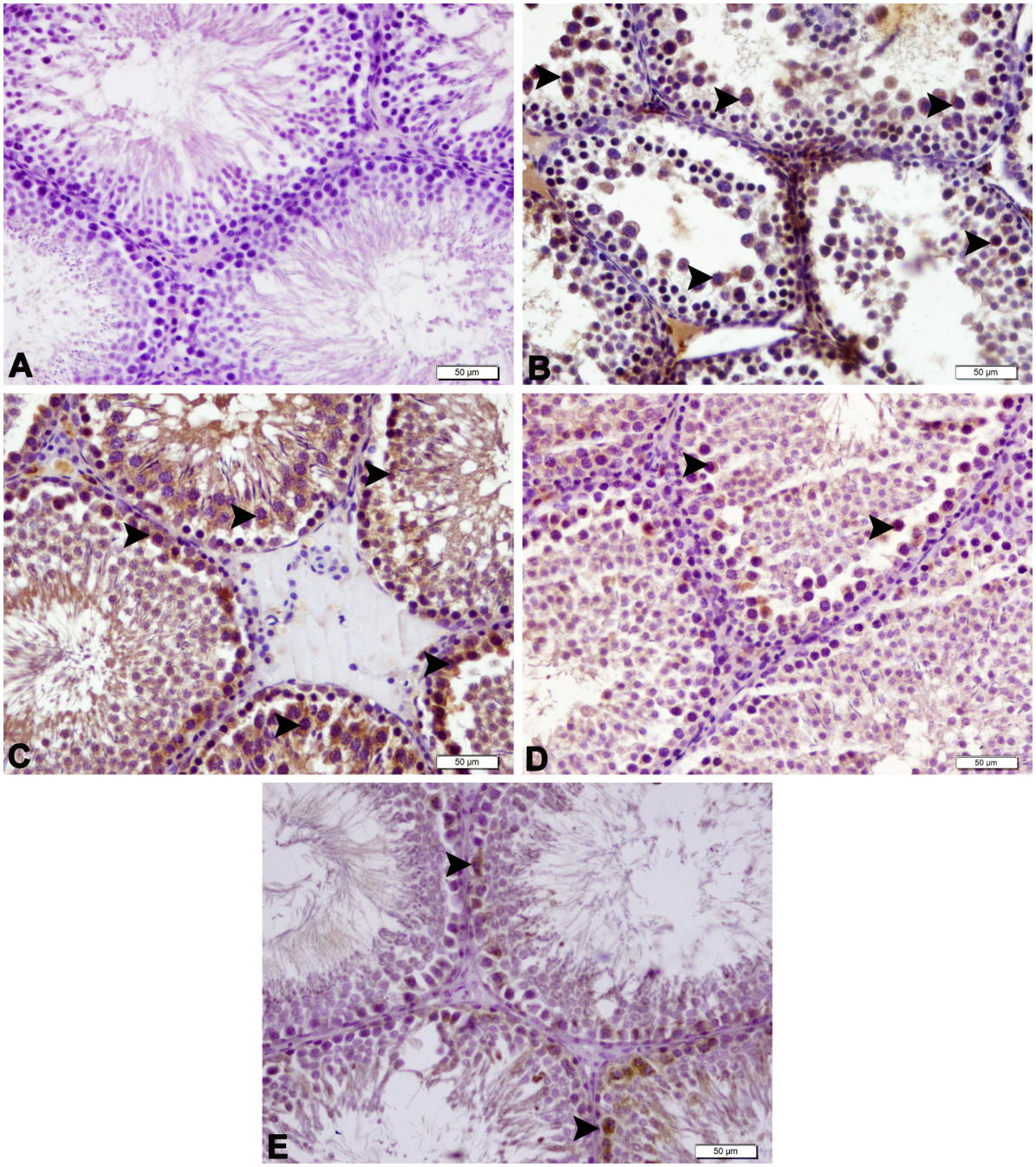

Figure 3. Testicular tissue, control group, negative 8-OHdG expression (A). T 3 h / D 3 h group: severe cytoplasmic 8-OHdG expression in spermatocytes (arrowheads) (B). T 3 h / D 24 h group: severe cytoplasmic 8-OHdG expression in spermatocytes (arrowheads) (C). T 3 h / D 3 h + silymarin group: moderate cytoplasmic 8-OHdG expression in spermatocytes (arrowheads) (D). T 3 h / D 24 h + silymarin group: mild cytoplasmic 8-OHdG expression in spermatocytes (E), IHC-P, bar: 50 µm.

group was comparable to previous research (Un et al., 2015). Although the reductions in torsion groups differ from each other, this difference is not significant. While increases in the GSH value of the groups to which we administered silymarin were similar to the control group in the 3 h detorsion group, it was significantly higher in the 24 h detorsion group compared to all groups, including the control group.

In the literature, it has been reported that GPx value decreased significantly in testicular torsion/detorsion groups compared to a control group (Ganjani et al., 2020; Shokoohi et al., 2018). A similar condition was detected

Table 2. Testicular tissue GSH, SOD, GPx, 8-OHdG, MDA levels, and serum testosterone levels.

	Control	T 3 h / D 3 h	T 3 h / D 24 h	T 3 h / D 3 h + Slymarin	T 3 h / D 24 h + Slymarin
GSH (ng/L)	137.30±3.30[b]	114.16±2.37[c]	118.31±4.35[c]	137.78±3.31[b]	147.36±5.06[a]
SOD (pg/ml)	240.07±5.76[a]	204.88±7.26[c]	230.49±6.68[b]	209.76±7.59[c]	232.07±8.27[a, b]
GPx (IU/ml)	27.38±1.83[a]	23.68±1.68[b]	22.10±1.20[b]	21.88±1.57[b]	28.07±1.94[a]
8-OHdG (ng/ml)	1.66±0.12[a]	1.87±0.05[b]	2.00±0.06[c]	1.84±0.02[b]	1.90±0.05[b]
MDA	0.31±0.009[a]	0.38±0.01[c]	0.37±0.02[c]	0.035±0.02[b]	0.033±0.01[a]
Testosterone (mmol/L)	7.12±0.22[a]	1.76±0.11[d]	1.73±0.16[d]	2.18±0.30[c]	3.16±0.07[b]

Different letters in the same line represent statistical significance ($p < 0.05$).
GSH = glutathione; SOD = superoxide dismutase; GPx = glutathione peroxidase; 8-OHdG = 8-hydroxy-2-deoxyguanosine; MDA = malondialdehyde.

in our study. After silymarin administration, a significant increase in GPx value was detected only in the 24 h detorsion group compared to other torsion groups. This increase is even higher than the control group value.

There are no studies on the use of silymarin in testicular ischemia/reperfusion injury. In the current study, in groups to which silymarin was applied, it was determined that GSH, SOD, and testosterone levels increased, 8-OHdG and MDA levels decreased, and the immunohistochemical intensity of Tnf-α and 8-OHdG expressions decreased. Therefore, it was concluded that silymarin reduces testicular damage by activating antioxidant mechanisms and decreasing inflammatory response in testicular ischemia/reperfusion injury. Further clinical studies using different treatment protocols and different doses may be useful to evaluate the effects of silymarin on other complications in testicular ischemia/reperfusion injury and to reveal other underlying mechanisms.

Conflict of interest

There are no conflicts of interest in the current study.

Acknowledgment

This research has not received a special grant from funding organizations in the public, commercial, or non-profit sectors.

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