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Original Article

Protective Effects of Rosmarinic Acid on Testicular Torsion Detorsion in an Animal Model

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ABSTRACT

Testicular torsion is a disorder that may cause infertility or subfertility in males. Rosmarinic acid (RA) is a natural polyphenol with potent antioxidant effects that its protective effects in ischemia/reperfusion (I/R) injury in the hepatic, renal, cerebral, and myocardial tissues was demonstrated. The aim of this study was to evaluate the protective effects of rosmarinic acid on testicular torsion/detorsion. 20 healthy male Wistar rats were randomly divided into four groups: sham-operated, torsion/detorsion, rosmarinic acid 50 mg/kg, and rosmarinic acid 70 mg/kg. testicular torsion was performed for two hours and detorsion was maintained for two hours. Administration of rosmarinic acid was performed 30 min before detorsion in treatment groups. Sperm parameters (sperm motility and sperm concentration), oxidative stress biomarkers (MDA, GPx, and CAT), and histopathological damage were examined in all groups. Results indicated that testicular I/R significantly reduced sperm motility and sperm concentration compared to the sham group, while treatment with RA significantly increased motility. Testicular I/R significantly increased MDA level and reduced GPx and CAT levels in the T/D group compared to the sham group, whereas treatment with RA reduced MDA level and increased GPx and CAT levels. Histopathological damages in the T/D group were significantly higher than those in the sham group. RA treatment reduced pathological damages. In conclusion, rosmarinic acid improved sperm parameters, increased antioxidant activity, and reduced histopathological damages. Therefore, it is recommended for attenuating testicular damages.

Introduction

Infertility is a well-known problem that may affect up to 15% of couples. 50% of infertility disorders are related to male reproductive disorders. Testicular torsion is one of the disorders that may lead to infertility or subfertility in males.[1] Approximately 0.5% of emergency cases are represented due to acute scrotal pain. Several factors may lead to acute scrotal pain including, orchitis, scrotal hernia, epididymitis, trauma, and testicular torsion. Of these factors testicular torsion triggers the most acute pain due to the vascular disturbance and testicular ischemia, which reinforces prompt surgical intervention to correct it.[2] Injury in the

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twisted testis is caused by the perfusion impairment and subsequent reperfusion.[3] Several previous studies demonstrated that injury caused by the reperfusion and blood flow to the ischemic testicle is more deteriorating than that caused by perfusion impairment.[4] In the ischemic condition, xanthine oxidase system is activated and reactive oxygen species (ROS) including superoxide, hydroxyl, and hydrogen peroxide are overproduced and the antioxidant defense is not capable to neutralize the excessive free radicals [3]. Consequently, oxidative damage triggers injury to the cell membrane, DNA, dysfunction of proteins, germinal cell apoptosis and decreased spermatozoa.[5]

Natural polyphenols are potent antioxidants and several studies have been conducted regarding their antioxidant activities.[6] Rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, is a famous polyphenol with a wide usage in the pharmacology and nutrition. This compound is found in various plants such as *Perilla frutescens*, *Rosmarinus officinalis*, *Coleus blumei*, *Salvia officinalis*, *Prunella vulgaris*, *Ocimum basilicum* and other herbs.[7,8] Different beneficial properties such as antioxidant, anti-inflammatory, anticancer, and antimicrobial effects have all been demonstrated for Rosmarinic acid.[9] Effects of RA in the ischemia/reperfusion (I/R) condition of various tissues including liver,[9] kidney,[10], hippocampal neurons from cerebrum,[11] and myocardium,[12] have been studied and its protective effects have been proven.

protective effects of different agents on testicular torsion/detorsion such as anaesthetics, antioxidants, calcium channel blockers, interaction with neutrophils, modulators of inflammation, phytotherapeutic, platelet inhibitors, and vasodilator agents were examined in previous studies.[13]

Since Rosmarinic acid is considered as a potent antioxidant, we hypothesized that RA could also protect the testis from I/R damage. In order to evaluate this hypothesis, this study was designed to examine the protective effects of Rosmarinic acid on testicular torsion detorsion damage in rats.

Materials and Methods

Study Design

Twenty healthy male Wistar albino rats (aged 2-2.5 months, 250 g) were utilized in the present study. Rats were kept in the standard conditions of room temperature (25 ± 1° C), humidity of 54 ± 5%, and photoperiod of 12-h light 12-h dark. Animals had free access to pellet diet and tap water. Ethical considerations for animal usage and experiments were based on regulations of the Urmia University Animal Ethics Committee. Rosmarinic acid (SIGMA-ALDRICH, CO.,3050 Spruce Street, St. Louis, MO 63178 USA 314-771-5765) was used in the treatment groups. Rats were randomly divided in to 4 groups of 5 rats each as follow:

1- Sham group: In this experimental group, the surgical incision was made in the scrotum of the left testicle and no other interventions were done and the incision was sutured.

2- Testicular torsion/detorsion group (T/D): In this group, testicular torsion was made and maintained for 2 hours. Subsequently, detorsion was performed and the rats were euthanized 2 hours after reperfusion.

3- Rosmarinic acid 50 mg/kg (RA50): In this treatment group, all procedures were performed such as the T/D group; however, 30 min prior to detorsion RA (50 mg/kg, IP) was administered.[14,15]

4- Rosmarinic acid 70 mg/kg (RA70): In this group all surgical procedures to induce torsion and detorsion were performed the same as T/D group. Animals were treated with RA (70 mg/kg, IP) 30 min before detorsion.[16]

Surgical Procedure

The surgical procedures for making testicular ischemia were performed in sterile condition after general anesthesia by intraperitoneal injection of xylazine and ketamine combination (5 mg/kg and 60 mg/kg, Alfasan, Woerden, The Netherlands).[17,18] Briefly, after shaving of scrotum and disinfecting of the surgical area, the left testis was exposed by incision on the midline of the scrotum. Then the left testis was twisted 720 degrees clockwise, and tunica albuginea was sutured to the scrotum in order to stabilize it. Testicular torsion was maintained for two hours to induce ischemia.

Also, reperfusion was performed by rotating the left testis 720 degrees counterclockwise. [4] The reperfused testicle was placed anatomically normal into the scrotum and the scrotal incision was sutured. Ultimately, left testicles of the rats were sampled and fixed in the 10% buffered formalin solution. Epididymis of the left testicle was separated and the caudal section of the epididymis was utilized for the sperm parameters evaluation.

Sperm Parameters

Sperm motility and sperm concentration were assessed in the various experimental groups. Initially, posterior part of the epididymis was cut and placed in to the 4 ml of RPMI medium to facilitate sperm extraction.[19] Then the plate containing RPMI medium was placed in to the incubator (37° C) for 15 min. Subsequently, a sperm sample from the medium was removed and placed on a microscopic slide. A phase-contrast microscope was employed to evaluate sperm motility. Sperm cells were divided in to the progressive, non-progressive, and immotile groups.

To examine sperm concentration, a fixative (0.5 g sodium bicarbonate ($NaHCO_3$) and 1 ml of 35% formalin solution dissolved in 100 ml of purified water) was used to fix the spermatozoa on the slide and stop movements and facilitate counting. A Neubauer hemocytometer was utilized to count sperm cells. The final concentration was reported as sperm/ml. Full details of sperm parameters assessment are presented in our previous study.[4]

Oxidative Damage

Oxidative stress parameters including malondialdehyde (MDA), catalase (CAT), and glutathione peroxidase (GPx) were evaluated using Navand Salamat biochemical kits (NS-15053, Urmia, Iran). The kit protocols were followed for each parameter and oxidative damage was assessed in the testicular tissue of all groups.

Histopathological Evaluation

hematoxylin and eosin (H&E) staining protocol was used in the present study. Briefly, after fixation of the samples in the 10% buffered formalin solution, they were removed from the solution, dehydrated by ascending levels of ethyl alcohol, and elucidated using Xylene.

Subsequently, samples were embedded in the paraffin and blocks were provided. 4–5 µm thick sections were prepared from the blocks and mounted on slides. Eventually, the slides were stained using hematoxylin and eosin. Slides were evaluated by an expert pathologist of the RASTA Special Research Institute, Urmia, Iran.

Statistical Analysis

All data within this study were analysed using SPSS software (version 25 for windows, SPSS Inc. Chicago IL USA). One-way ANOVA with Tukey post hoc test was applied to analyze data in various groups of the study. The results were considered significant at the p-value < 0.05 level.

Results

Sperm Parameters

Table 1 presents sperm motility and sperm concentration in experimental groups. For all measured parameters, a significant difference was observed between the sham-operated group and the T/D and RA50 groups ($p < 0.05$). Additionally, in the RA70 group progressive motile sperm decreased and immotile sperm increased significantly compared to the sham-operated group ($p < 0.05$). Significant difference was observed between the RA50 and RA70 groups and T/D group for progressive motile and immotile sperm parameters ($p < 0.05$). Significant reduce was found for the non-progressive sperm motility in the RA70 group when compared to the T/D group ($p < 0.05$). No significant difference was found in sperm concentration and non-progressive sperm motility parameters between RA70 group and sham-operated group ($p > 0.05$).

Table 1. Sperm parameters in different experimental groups.

	Sperm motility			
Groups	**Progressive (%)**	**Non-progressive (%)**	**Immotile (%)**	**Sperm concentration ×10⁶ sperm/ml**
Sham	75.00 ± 4.7	5.96 ± 2.14	19.93 ± 5.22	65.56 ± 3.56
T/D	41.45 ± 2.60a	19.07 ± 4.61a	40.56 ± 2.44a	54.62 ± 3.74a
RA50	54.39 ± 4.27a,b	14.87 ± 3.30a	31.75 ± 5.97a,b	55.56 ± 4.64a
RA70	59.60 ± 2.32a,b	10.85 ± 2.88b	31.18 ± 1.20a,b	59.90 ± 3.57

Data are expreesed as Mean ± SD. a: $p < 0.05$ compared to the sham group. b: $p < 0.05$ compared to the TD group.

Oxidative Stress Biomarkers

The results of the MDA evaluation are presented in Figure 1. MDA concentration of testicular tissue in the T/D group was remarkably higher than that in the other experimental groups ($p < 0.05$). Furthermore, this parameter was significantly higher in the RA50 and RA70 groups than the sham group ($p < 0.05$). MDA concentration in RA50 group was significantly higher

than that in the RA70 group ($p < 0.05$). RA treatment significantly reduced MDA levels in both treated groups compared to the T/D group ($p < 0.05$).

The experimental data on catalase enzyme activity is shown in Figure 2. The CAT level was remarkably lower in T/D group than that in the sham, RA50 and RA70 groups ($p < 0.05$). A significant difference in level of CAT was observed between sham group and RA50 and RA70 groups ($p < 0.05$). RA50 and RA70 groups significantly increased the CAT level compared to the T/D group ($p < 0.05$).

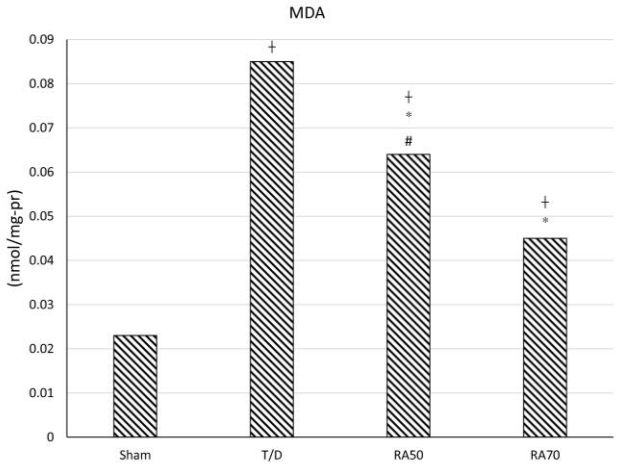

Figure 1. Levels of malondialdehyde (MDA) in different study groups (nmol/mg-pr). † Indicates a significant difference with the sham group ($p < 0.05$). * Indicates a significant difference with the T/D group ($p < 0.05$). # Indicates a significant difference with the RA70 group.

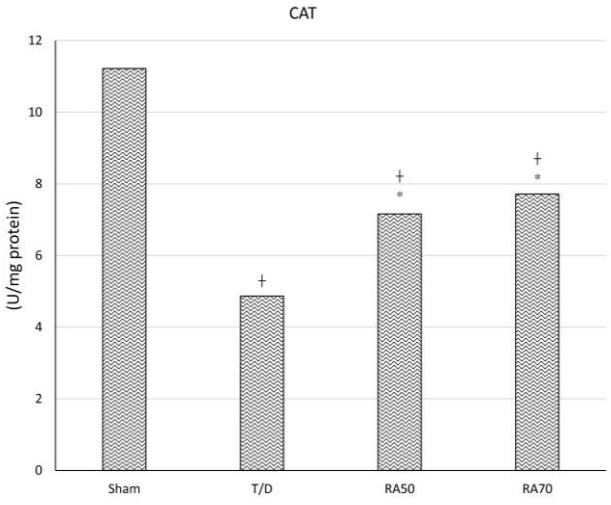

Figure 2. Levels of catalase (CAT) in different study groups (U/mg-pr). † Indicates a significant difference with the sham group ($p < 0.05$). * Indicates a significant difference with the T/D group ($p < 0.05$). # Indicates a significant difference with the RA70 group.

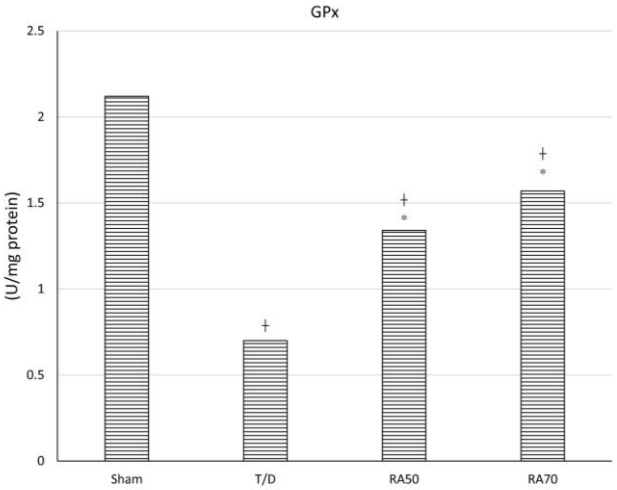

Figure 3. Levels of glutathione peroxidase (GPx) in different study groups (U/mg-pr). † Indicates a significant difference with the sham group ($p < 0.05$). * Indicates a significant difference with the T/D group ($p < 0.05$). # Indicates a significant difference with the RA70 group.

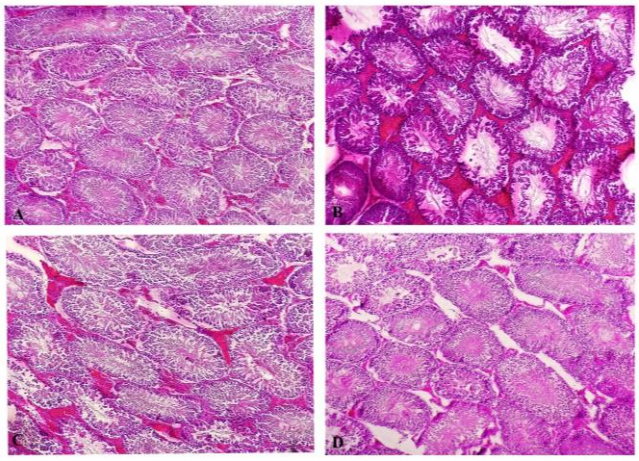

Figure 4. Hematoxylin and eosin (H&E) staining of testicular tissue. A: sham-operated group, B: T/D group, C: RA50 group, D: RA70 group. magnification of slides is 100×.

Testicular tissue level of GPx was significantly reduced in T/D, RA50 and RA70 groups compared to the sham-operated group (Figure 3) ($p < 0.05$). GPx concentration in RA50 and RA70 groups was significantly higher than the T/D group ($p < 0.05$).

Histopathology Findings

Histopathological slides are shown in Figure 4. In the sham group no testicular damage was detected and seminiferous tubules were healthy. Ischemia/reperfusion in the T/D group induced significant damage to testicular tissue and inter tubular-hemorrhage, depletion of cells into the tubule, vacuolization, and necrosis were observed. Pre-treatment with RA in both 50 and 70 doses caused

significant improvement and only low damage to the seminiferous and hemorrhage between tubules was found.

Discussion

Testicular torsion is considered as an emergency and requires fast intervention (manual detorsion or surgical correction).[20] The incidence of this disorder is 1 in 4000 males and the most prevalent age is under the 25 years.[21] Several previous studies have focused on the effects of testicular torsion on the contralateral testicle; However, this is still debating.[21]

Four to six hours is the golden time for successful testicular rescue as described in the literature. As time goes on, the prognosis of testicular tissue survival decreases. The intensity of testicular torsion and the elapsed time are two critical factors in preserving the testicle.[22] One of the principal factors involved in the testicular damage is overproduction of the reactive oxygen species.[23] Therefore, several previous studies have focused on the protective role of the antioxidants on the testicular torsion/detorsion.[13] In the present study effects of RA was assessed as a potent antioxidant.

Oxidative damage of testicular torsion/detorsion possesses two stages. First stage begins promptly after detorsion of the ischemic testicle and lasts a few hours. The second stage occurs when testicular torsion continues for several days and damage to the testicle is irreversible.[24] In the present study, oxidative stress was examined in the first stage of the damage and according to previous studies reperfusion time was considered to be two hours.[25,26]

Ramalho *et al*. (2014) investigated the effects of Rosmarinic acid on hepatic I/R damage in rats. They found that I/R damage significantly reduced the TAC and GSH levels compared to the sham group. Treatment with RA significantly increased the levels of TAC and GSH compared to the I/R group. Moreover, histopathological damages to the liver tissue were reduced in the treatment group compared to the I/R group.[9] In another study effects Rosmarinic acid against renal ischemia/reperfusion was evaluated. Results of this study revealed that I/R of the kidney significantly increased the MDA and MPO concentrations and reduced GPx and SOD levels compared to the sham group. RA treated group significantly increased GPx and SOD levels and decreased MDA and MPO levels in comparison to the I/R group. Furthermore, tissue injury was reduced in the RA treated group compared to the I/R group.[10] Değer and Çavuş (2020) have examined role of the RA in the ovarian torsion/detorsion and found that MDA level in the I/R group was significantly higher than that in the sham group and levels CAT, SOD, and GSH were significantly lower in the I/R group than that in the sham group. Treatment with RA significantly reduced oxidative stress biomarkers compared to the I/R group. Additionally, ovarian tissue injury was reduced in the treatment group compared to the I/R group.[27] In accordance with previous studies, our findings revealed that MDA level in the T/D group significantly increased compared to the sham group and both treatment group significantly reduced level of MDA compared to the T/D group. Moreover, levels of CAT and GPx were significantly lower in the T/D group compared to the sham group and both RA treated groups remarkably increased the CAT and GPx levels compared to the T/D group. Totally, treatment groups decreased oxidative stress.

Protective effects of curcumin on testicular torsion/detorsion were assessed and sperm parameters were evaluated. Findings of the mentioned study revealed that a significant difference was observed between the sham and I/R group for sperm parameters including progressive motility and immotile. Sperm progressive motility significantly increased in the treatment group compared to the I/R group. Also, sperm count in the treatment group was significantly increased compared to the I/R group.[1] Sperm parameters were evaluated in another study on the effects of G-CSF in testicular torsion/detorsion. Results indicated that testicular torsion/detorsion significantly reduced sperm counts and motility compared to the sham group. Treatment with G-CSF remarkably increased both sperm count and motility compared to the I/R group.[28] Rosmarinic acid is the main antioxidant agent of the Salvia Miltiorrhiza plant. Davoodi *et al.* (2020) evaluated effects of Salvia Miltiorrhiza hydroalcoholic extract on sperm parameters following testicular ischemia/reperfusion. In all various motility categories such as progressive, non-progressive, and immotile a significant difference was observed between the sham and torsion/detorsion group. Treatment group significantly increased progressive motility and reduced non-progressive motility compared to the I/R group. For sperm concentration no significant difference was observed between the sham and the I/R groups. However, treatment group significantly increased the sperm

concentration.[4] In agreement with previous studies, our results revealed that for all sperm parameters there was a significant difference between the sham and the T/D group. RA significantly increased progressive motility and reduced non-progressive and immotile spermatozoa compared to the T/D group.

The purpose of the current study was to determine the protective effects of Rosmarinic acid against testicular torsion/detorsion in rats. This study has found that generally Rosmarinic acid reduced oxidative stress biomarkers, improved sperm parameters, and decreased tissue damages induced by ischemia/reperfusion. Taken together, these results suggest that Rosmarinic acid as a potent antioxidant can be used to alleviate damages triggered by testicular ischemia reperfusion. Although RA treatment in both doses has beneficial effects on testicular tissue, RA with high dose was superior in some measured parameters.

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Conflict of Interest

The authors declare that they have no competing interests.

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