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Protective Effects of Ellagic Acid on Testicular Ischemia-Reperfusion Injury in Rats

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

In experimental studies, many agents have been studied to prevent ischemia-reperfusion injury in testicular torsion. Ellagic acid appears to have protective effects against experimental testicular ischemia-reperfusion injury in rats.

Abstract

Objective: This study aimed to investigate the protective effects of ellagic acid on testicular ischemia/reperfusion injury in rats.

Materials and Methods: Twenty-one Sprague-Dawley rats were randomly divided into three groups: sham, ischemia/reperfusion (I/R), I/R + ellagic acid (EA). All animals underwent left scrotal exploration. In all groups except the sham group, the left testes were rotated 720 degrees clockwise for 3 h and 3 h reperfusion. 10 mg/kg ellagic acid was administered intraperitoneally to the I/R+E group before reperfusion. Then, the left orchiectomy was performed. The testes underwent biochemical and histological examination.

Results: There was a significant difference between the sham and the I/R, I/R+EA groups according to the Cosentino system ($p<0.001$, $p=0.036$), and there was no difference between the I/R and IR+EA groups ($p=0.319$). A significant difference was found between sham and I/R groups according to Johnsen spermatogenesis score ($p<0.001$), but there was no significant difference between sham and I/R+EA groups ($p=0.063$). Superoxide dismutase, catalase, malondialdehyde, total oxidant status values were statistically different between I/R and I/R+EA groups ($p=0.001$, 0.002, 0.002, 0.001 respectively).

Conclusion: Ellagic acid has a protective effect against testicular ischemia/reperfusion injury in rats.

Keywords: Ellagic acid, ischemia/reperfusion injury, testis

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Introduction

Testicular torsion is a surgical emergency that can cause loss of testicular function and infertility. The incidence of torsion is estimated at 3.8 per 100.000 (0.004%) for boys under the age of 18 (1). Testicular damage development is directly related to the duration of the torsion. Therefore, surgical detorsion should be applied as soon as possible and the first 4 to 8 h are defined as the golden windows for testicular salvage (2). However, 27% testicular atrophy and 36-39% subfertility were reported in long-term follow-up after torsion (3,4). Testicular damage may occur due to the direct effect of interruption of blood flow during torsion or due to the formation of oxygen-derived free radicals by ischemia/reperfusion (I/R) injury (4). Experimental studies have been conducted with many potential agents to prevent reperfusion injury. Antioxidant drugs (Vitamin E, taurine, apocynin, quercetin, alpha lipoic acid, selenium, ascorbic acid, etc.), non-steroid anti-inflammatory drugs (ibuprofen, dexetoprofen), phosphodiesterase type 5 inhibitors (sildenafil, tadalafil), nitric oxide, neutrophil elastase inhibitors, platelet-rich plasma are some agents used in these studies (5,6). Ellagic acid (EA) is a potent polyphenol antioxidant found in fruits and natural sources such as grapes, nuts, strawberries, raspberries, honey, green tea (7). It has chemo preventive, antiapoptotic, radical scavenging properties in the previous studies (8). EA has been experimentally shown to have protective effects on testicular damage induced by chemotherapeutic agents such as cisplatin, adriamycin, doxorubicin (8-10). Furthermore, it has been reported that EA has protective effects against kidney and ovarian I/R injury in rats (11,12). However, there is no study in the literature investigating the effects of EA on experimental testicular I/R injuries. In this study, we evaluated the protective effects of EA on testicular I/R injury in rats.

Materials and Methods

Twenty-one male Sprague-Dawley female rats (12 weeks old, weight 250-300 g) were obtained from the Karadeniz Technical University Laboratory Animals Research Centre (Trabzon, Turkiye). This study was approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University (Trabzon, Turkiye) (approval number/ID: 2018/20). The same environment and nutritional conditions were provided for all the animals. Rats were entrained under a 12:12 h dark: Light cycle (lights on 6 am-6 pm) with stable temperature (21±2 °C) and humidity (60±5%). The rats had sterile water and food available ad libitum. The surgical protocol was performed at Karadeniz Technical University, Faculty of Medicine, Surgical Applications Center. A biochemical was examined in the Biochemistry Department of Karadeniz Technical University, Faculty of Medicine, histological was examined in the Pathology Department of University of Health Sciences Turkiye, Trabzon Kanuni Training and Research Hospital.

Experimental Protocol and Surgical Procedure

Animals were randomly divided into three groups: Sham, I/R, I/R+EA. Rats were anesthetized with xylazine (20 mg/kg) and ketamine hydrochloride (50 mg/kg). All animals underwent left scrotal exploration during the first procedure. In all groups except the sham group, the left testes were rotated 720 degrees clockwise for 3 h and sutured to the scrotum with a 4/0 prolene through the tunica albuginea and subcutaneous tissue. The incision was closed with a 4/0 prolene suture. After 3 h, using the same incision, the testis was turned round to its natural position and 10 mg/kg EA was administered intraperitoneally to the I/R+EA group before reperfusion. The testis was left for 3 h to evaluate results of I/R injury. After 3 h, the experiment was terminated, and orchiectomy was performed. The testes were divided into two transverse planes for biochemical and histological examination.

Biochemical Analysis

Tissues were washed with saline and stored at -80 °C until evaluation. In the biochemical analysis, first they were homogenized in cold phosphate-buffered saline (PBS) (0.05 M, pH 7.4), and were centrifuged at 3000 rpm for 10 min to remove debris and to obtain a clear supernatant fraction. Then, the analyses were performed in this fraction. Malondialdehyde (MDA), total oxidant status (TOS), as well as enzyme activities of superoxide dismutase (SOD) and Catalase (Cat) were measured in this fraction. MDA levels in tissue samples were determined using the method described by Uchiyama and Mihara (13). Tetramethoxypropane was used as a standard, and tissue MDA levels were calculated as nmol/10 g wet tissue. TOS levels were determined using a colorimetric TOS kitas previously described by Erel (14). Cat activity was measured by modifying the method based on the measurement of the absorbance of ammonium molybdate with H_2O_2 at 405 nm. Cat standard (Sigma C9322) was used as a standard, and tissue Cat activity was calculated as nmol/g protein (15). The SOD enzyme activity was determined by the method of Sun et al. (16). This method is based on the measurement of the absorbance of the purple-colored formazan molecule at 560 nm resulting from the reduction of nitroblue tetrazolium of $O_2^{\cdot-}$ formed by the xanthine-xanthine oxidase system. The tissue SOD activity was calculated as nmol/g protein by using the SOD standard (Sigma S8160) (16).

Histological Analysis

Testicular tissue samples were detected in 10% formaldehyde for 48 h and then underwent routine histological follow-up. 5 µm thick sections were prepared from paraffin embedded tissues and stained with hematoxylin-eosin. Then, the changes caused

by I/R were examined by light microscopy. Four-grade scale defined by Cosentino et al. (17) was used to assess histological changes (Table 1). The Johnsen (18) scoring system was used for evaluating spermatogenesis (Table 2). Histological changes and spermatogenesis scoring were evaluated randomly by a pathologist blinded to the groups.

Statistical Analysis

Data are expressed as the median (min-max). The Kolmogorov-Smirnov test was used to test normality, and the groups were compared using the Mann-Whitney U test. Statistical significance was set at $p<0.05$ (IBM SPSS Statistics 22.0). In our study, data have not followed a normal distribution.

Results

The biochemical analysis results are shown in Table 3. SOD in the I/R group decreased significantly compared with the I/R+EA group (p=0.001) but was similar in the sham and I/R+EA groups (p=0.209). The cat in the I/R group was significantly lower than the sham and I/R+EA groups (p=0.001, p=0.002). MDA and TOS in the I/R group increased significantly compared in the I/R+EA group (p=0.001, p=0.001). Although TOS was similar in the sham and I/R+EA groups (p=0.805), MDA was significantly higher in the I/R+EA group than in the sham group (p=0.01) (Figure 1).

The histological grade of testicular damage and spermatogenesis scores of all animals are shown in Table 4. Different findings were observed in different areas in each testicle on histopathological examination, the dominant one was recorded. In the I/R group, it was observed that one testicle was damaged enough to not be histologically evaluated, and therefore it was excluded from the study. There were generally nearly normal findings in the sham group. In four cases, signs of mild interstitial edema-bleeding and germ cell detachment were observed in some seminiferous tubules were observed. In the I/R group, severe interstitial edema-hemorrhage, Leydig cell detachment, contraction in tubules, intratubular edema, and germ cell detachment were observed. In the I/R+EA group, mild interstitial edema-hemorrhage, germ cell detachment, and irregularity in some tubules were observed (Figure 2). There was a statistically significant difference between the sham group and the I/R, I/R+EA groups according

Table 1. The Cosentino histological grading system

Grade	Characteristics
I	Normal testicular architecture with an orderly arrangement of germinal cells
II	Injury showed less orderly, noncohesive germinal cells and closely packed seminiferous tubules
III	Injury exhibited disordered sloughed germinal cells, with reduced size of pyknotic nuclei and less distinct seminiferous tubule borders
IV	Injury exhibited seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells

Table 2. Spermatogenesis scoring system proposed by Johnsen (18)

Score	Characteristics
10	Complete spermatogenesis and normally organized tubules
9	Numerous spermatozoa present, but the germinal epithelium is disorganized
8	Only a few spermatozoa present in the section
7	No spermatozoa, but numerous spermatids present
6	Only a few spermatids present
5	No spermatozoa or spermatids, but numerous spermatocytes present
4	Only a few spermatocytes present
3	Only spermatogonia present
2	No germ cells, but only Sertoli cells present
1	No germ cells and no Sertoli cells present

Table 3. Results of superoxide dismutase (SOD), catalase (Cat), malondialdehyde (MDA), total oxidant capacity (TOC) of groups

Median (min-max)	Sham group (n=7)	I/R group (n=7)	I/R+EA group (n=7)	p-value
SOD (U/Gprotein)	42.8 (28.8-40.7)	9.94 (7.35-12.7)	34.4 (29.1-53.4)	**0.001*** 0.209& **0.001#**
Cat (U/Gprotein)	11.8 (11.6-12)	4.35 (2.97-5.09)	11.3 (11.2-11.5)	**0.002*** **0.001&** **0.001#**
MDA (nmol/Gtissue)	23.4 (21.4-27)	67.9 (66.3-68.6)	36.4 (33.9-40.2)	**0.001*** **0.001&** **0.001#**
TOS (µmol/L)	23.6 (19.9-29.4)	39.4 (35.6-43.1)	23.1 (17.4-34.6)	**0.001*** 0.805& **0.001#**

*I/R vs I/R+EA, &sham vs I/R+EA, #sham vs I/R

to the Cosentino grading system (p<0.001, p=0.036), and there was no difference between the I/R and I/R+EA groups (p=0.319). A statistically significant difference was found between sham and I/R groups according to Johnsen spermatogenesis scoring system (p<0.001), but there was no statistically significant difference between the sham and I/R + EA groups (p=0.063) (Table 5).

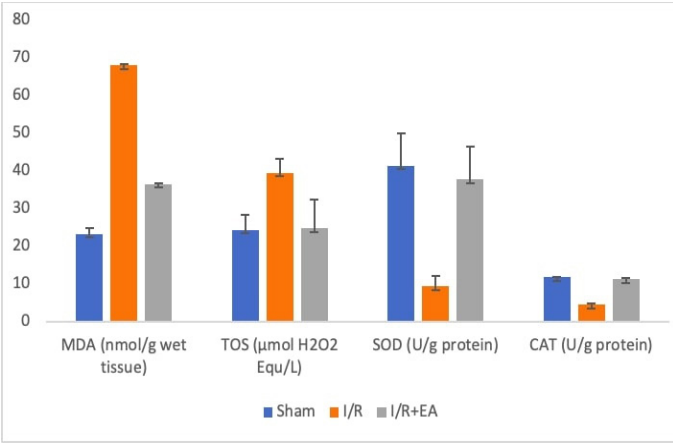


Figure 1. Results of biochemical analyses in all groups (Control: Sham group, I/R: Ischemia/Reperfusion, Ellagic Acid + T/D: Ellagic Acid+Torsion/Detorsion)

Discussion

Testicular torsion is an emergency that is frequently encountered in childhood and can cause testicular damage, infertility, and hypogonadism if detorsion is not performed within a short time (3). Although reperfusion is essential for the salvage of the testicle, it induces the formation of reactive oxygen radicals in the tissue (19). When the balance between ROS and antioxidative defense mechanisms is damaged, the amount of ROS can increase. ROS may provoke tissue damage with the development of an inflammatory response and activation of some mediators. This process causes membrane dysfunction and potential cell death by peroxidation of the phospholipid structure in cell membranes (20). Experimental testicular torsion has been shown in previous studies to cause tissue reperfusion damage (6,19). Although many agents have been studied to prevent I/R damage in the literature, there is currently no recommended agent for clinical use. This study showed that EA has protective effects on rat testicular tissue against I/R damage.

EA is a natural antioxidant substance, and its chemical name is 2,3,7,8-tetrahydroxy-chromeno [5,4,3-cde] -chromene-5,10-dione. EA is a weak acid that ionizes at physiological pH. It has two pairs of hydroxyl groups and this structure makes

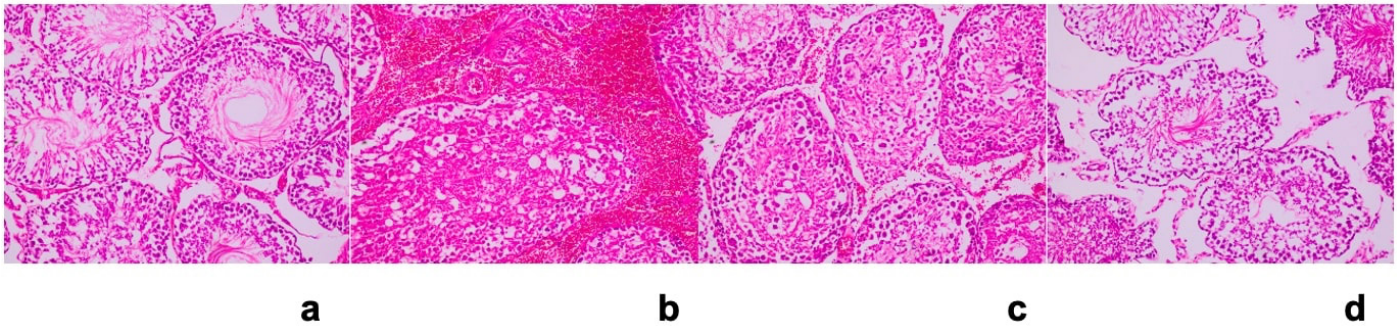


Figure 2. Histological images of rat testis sections. a) Normal testis appearance in sham group (HEx40), b) Grade IV changes in the I/R group (Interstitial hemorrhage, uncertainty in the seminiferous tubule margins, coagulation necrosis (HEx40)), c) Grade III changes in the I/R+EA group (interstitial edema-hemorrhage, seminiferous tubules detachment, border ambiguity, germ cell detachment, intercellular edema, dead germ cells, and multinucleated cells and (HEx40)), d) Grade II changes in the I/R+EA group (less regular germ cells)

Table 4. Histological examination results of testicular tissues

Animal number	Sham group (n=7)		I/R group (n=6)		I/R+ EA group (n=7)	
	Cosentino grading	Johnsen score	Cosentino grading	Johnsen score	Cosentino grading	Johnsen score
1	1	9.5	NA	NA	3	8
2	1	9.5	3	6	2	9
3	1	10	3	6	2	8
4	1	10	3	6	2	8
5	1	9.5	3.5	5.5	2	7
6	1	9.5	4	5.5	3	7
7	1	10	3.5	5.5	3	6

Table 5. Comparison of the histological scores of the groups				
	Sham (n=7)	I/R (n=6)	I/R + EA (n=7)	p-value
Cosentino grading system [Median (min-max)]	1 (1-1)*^	3.25 (3-4)*	2 (2-3)^	<0.001
Johnsen score [Median (min-max)]	9.5 (9.5-10)*	5.75 (5.5-6)*	8 (6-9)	<0.001
Kruskal-Wallis test (pair wise analysis, meaningfulness is indicated as follows: *Sham group and I/R group difference statistically significant; ^Sham group and I/R+EA group difference statistically significant)				

EA a potent antioxidant (21). In previous studies, EA has anticarcinogenic, neuroprotective, cardio-liver-skin protective and, angiogenic effects (22). EA exerts these effects by activating specific antioxidant enzymes and suppressing genes responsible for inflammation. In this process, the amount and duration of EA play an important role for treating oxidative stress (22). Daily administration of EA with diet significantly decreases the expression of cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) and prevents the production of excessive inflammatory mediators in the tissue (23). Additionally, it has been revealed that EA may be a potential agent against human diseases due to its antiobesity, antimicrobial and antioxidant properties (24).

Although there are studies reporting the effects of EA on rat testis toxicity induced by the chemotherapeutic agents, arsenic, and monosodium glutamate, there is no study examining the effect of EA on rat testis I/R damage in literature. In a study investigating the effect of two separate EA doses (10 mg/kg, 30 mg/kg) in rats developing testicular toxicity with 10 mg/kg sodium arsenite daily, SOD and Cat were found to be significantly higher and MDA lower in EA groups compared to the toxicity group (7). In a recent study, it was revealed that EA could be a potential agent against MDA with its torsion-detorsion in rat ovaries (12). Additionally, EA was reported to lead to decreasing MDA levels in a study on cerebral ischemia (25). Ekinci Akdemir et al. (26) reported the protective effect of EA against I/R injury created in skeletal muscle. While MDA increased in the I/R group compared to S and EA+I/R group, SOD, Cat activities decreased.

On histological examination, while there were no abnormal findings in the EA groups, reduction and destruction of germinal epithelium cells, and irregularity in the arrangement of seminiferous tubule epithelium were observed in the sodium arsenite group. Testosterone values were reported to be significantly higher in the EA groups than in the sodium arsenic group. In our study, biochemical and histology results were similar, but testosterone measurement was not performed.

It has been shown that gallic acid, a monomer of EA, affects the hypothalamus-pituitary-gonadal axis and increases FSH, LH concentration, and testosterone level. In an experimental study that investigated gonadal toxicity by giving cyclophosphamide to rats, it was stated that epididymis degeneration was prevented, and a medium-normal level of sperm maturation was provided by gallic acid treatment (27). In a similar study, the effectiveness of EA in reducing the testicular toxicity of doxorubicin, which is widely used in cancer treatment, was examined. In this study, it was determined that EA significantly improved sperm parameters, serum testosterone levels, glutathione, MDA, TNF-alpha, sialic acid, and testicular cholinesterase levels in testicular tissue (10). Additionally, EA is effective in reducing the degenerative effects of doxorubicin in histopathological examinations. Similarly, in this study, the median value of the Johnsen score, in which spermatogenesis was evaluated, was 5.75 (5.5-6) in the EA+I/R group and was significantly lower than that in the I/R group [8(6-9)] ($p<0.05$). Also, there was no statistical difference between the Sham and the EA+I/R groups in terms of Johnsen score. However, there are some publications that EA has not shown positive effects on sperm parameters. In the studies of Çeribaşı et al. (9), protective effects of EA on lipid peroxidation and apoptosis on experimental adriamycin toxicity in rat testes were reported. However, in the same study, it was stated that EA had no significant protective effect on reproductive organ weight and sperm quality parameters. In this study, it was shown that EA has a more protective effect in histological evaluation than the study by Çeribaşı et al. (9). This situation can be explained by chronic adriamycin exposure (8 weeks) and different experimental models in studies.

Hypoxia that develops following testicular torsion causes some pathological changes in the tissue. The interruption of blood flow leads to venous congestion, edema, hemorrhage in the seminiferous tubules, and eventually germ cell death (28). In this study, a contraction in seminiferous tubules and necrosis of germ cells were more pronounced in the I/R group.

In the I/R study performed with torsion-detorsion in rat ovaries, the improvement was observed in MDA, SOD, glutathione reductase, and Cat enzyme activities with EA (12). In the same study, it was shown that EA is also effective in reducing tissue damage. Although biochemical results of the present study were similar to those in this study, the histopathological examination did not show a statistically significant difference in tissue

damage between the I/R and EA+I/R groups. However, in this study, the ischemia period was shorter and the EA dose was higher than this study.

Study Limitations

This study has some limitations. Firstly, testosterone levels were not measured because the experiment was terminated at the third hour after reperfusion. Secondly, apoptosis was not evaluated. Thirdly, a single dose (10 mg/kg) EA group was formed.

Conclusion

Intraperitoneal Ellagic Acid administration supports the endogenous antioxidant defense system and reduce oxidative stress in testis I/R injuries in rats. Also, it has a protective effect on spermatogenesis but has no significant protective effect on histological examination against experimental I/R injury in the rat testis.

Ethics

Ethics Committee Approval: This study was approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University (Trabzon, Turkiye) (approval number/ID: 2018/20).

Informed Consent: Not necessary.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

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

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