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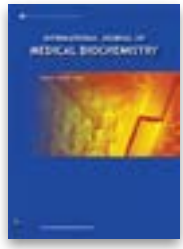

Research Article

A histological and biochemical study of the protective role of hesperidin in testicular ischemia-reperfusion injury

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Abstract

Objectives: This study aimed to investigate the possible effects of hesperidin on ischemia-reperfusion (IR) injury applied to rat testis.

Methods: Twenty-eight Wistar albino rats were used and divided into four groups of seven each. Group 1: Sham surgery was performed on the right testis. Group 2: Hesperidin 100 mg/kg was administered intraperitoneally to rats. Group 3: After 1 h of ischemia and 4 h of reperfusion, the testicles were removed. Group 4: 100 mg/kg hesperidin was given 30 min before reperfusion. Biochemical, immunohistochemical, and histopathological analyzes were performed on testicles obtained from each group.

Results: Total oxidant status (TOS) and oxidative stress index (OSI) levels increased significantly in the IR and IR-He groups (respectively, p=0.016, p=0.041; p=0.01, and p=0.024). TOS and OSI values in the hesperidin group decreased, although not statistically significant, compared to the IR group. Tumor necrosis factor-alpha (TNF-α) and nuclear factor kappa B (NF-kB) values were decreased in the hesperidin group compared to the IR group, although it was not statistically significant. Caspase-3 levels in testicular tissue were significantly increased in the IR group compared to the hesperidin group (p<0.05). While there were degenerative changes in the testicular tissue in the IR groups, a decrease in bleeding, congestion, edema, and degenerative changes was observed in the hesperidin-administered groups.

Conclusion: Hesperidin reduced oxidative stress (decreased total oxidant level and OSI), inflammation (TNF-α), and apoptosis (NF-kB and caspase-3). According to these results, it was observed that hesperidin application had a protective effect on IR injury.

Keywords: Apoptosis, hesperidin, ischemia-reperfusion injury, testis

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Testicular torsion is a urological disorder seen in newborns, children, and adolescents [1]. It is defined as the obstruction of blood flow to the testis due to the rotation of the spermatic cord around its axis [2]. Immediate treatment and surgery are required to prevent permanent urological damage. Despite the successful surgical operation, testicular torsion and detorsion may cause ischemia-reperfusion (IR) injury. This causes necrotic and biochemical changes in the tissue [3]. The main cause of testicular torsion-detorsion is testicular IR damage, resulting in oxidative stress, DNA damage, and cell dysfunction, which ultimately leads to cell death [4]. Increased oxidative stress as a result of torsion-detorsion is accompanied by inflammation [5]. Studies in the literature have shown that total oxidant status (TOS) and total antioxidant status (TAS) markers reveal the degree of oxidative stress, caspase-3 enzyme apoptotic response, tumor necrosis factor-alpha (TNF-α), and nuclear factor kappa B (NF-kB) factors which reveal inflammatory processes [6, 7]. In IR injury, besides surgical repair of the testis, the definition of free radical scavengers and pharmacological agents used in the treatment are important

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clinical targets. Hesperidin, which is recommended for treatment and claimed to have an antioxidant effect, is abundant in fruits, especially lemon and orange peels. Some studies have shown the antioxidant, antiapoptotic, and anti-inflammatory effects of hesperidin [8]. The previous studies have shown that hesperidin has a protective effect against damage to certain tissues in rats through suppression of inflammatory and apoptotic pathways, including NF-κB and caspase-3. The previous studies have shown that hesperidin has a protective effect against damage to certain tissues in rats through inhibition of inflammatory and apoptotic pathways, including NF-κB and caspase-3 [9, 10].

Despite this, the precise role and mechanisms of hesperidin in testicular IR injury are unknown. Considering the antioxidant, antiapoptotic, and anti-inflammatory effects of hesperidin and the role of oxidative stress in ischemia-reperfusion pathogenesis, the present study aimed to examine the protective effect of administration of this compound on testicular tissue damage in male rats. The effect of the active substance on testicular tissue will be explained using histological, biochemical, and immunohistochemical methods.

Materials and Methods

Animals

Twenty-eight Wistar albino male rats weighing 200–240 g were obtained from Erciyes University Experimental Research Application and Research Center. The rats were kept in an environment at 22±1°C, 12 light/12 dark cycles, and humidified by 50–60%. Animals were fed a standard diet and provided ad libitum access to tap water. Aksaray University Animal Experiments Ethics Committee reviewed and approved the experimental protocol (Approval No. 2020/03).

Experimental design

Operation procedure and medication administration

Group 1 (Sham): Sham-operated,

Group 2 (He): 100 mg/kg hesperidin was given as i.p. without IR.

Group 3 (IR): The testicles of the rats were torsioned 720° counterclockwise by the right scrotal incision and the testis was removed. It was, then, fixed to the scrotal skin with 6/0 silk and the scrotum was stured. After 1 h of torsion, the scrotum was opened, then the testicles were detorted and reperfused for 4 h.

Group 4 (IR-He): Ischemia was induced and hesperidin (100 mg/kg) i.p. was given 30 min before reperfusion [8]. After 4 hours of detorsion, the testis was removed for histological and biochemical examination.

Biochemical analysis

Serums were obtained by taking blood samples from animals. Serums were stored at −80°C for analysis. For biochemical examination, TAS (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey) and TOS (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey) in blood serums were analyzed using commercial kits. TNF-α (BT, cat no: E0764Ra) and NF-κB (BT, cat no: E0287Ra) levels were measured in blood using ELISA kits. The analysis was performed following the instructions of the commercial kits. Oxidative stress index (OSI) was calculated as the ratio of TOS levels to TAS levels as a percentage [1].

Histopathological procedures

Testicular tissues were fixed in 10% formalin solution and histopathological studies were performed. Then, the tissues were applied to routinely used procedures. Then, 3 micron thick sections were taken from the blocked tissues for immunohistochemical and histological staining. Some of the sections were stained with routinely used hematoxylin/eosin. For immunohistochemical staining, caspase-3 antibodies were used and Ventana BENCHMARK GX automatic immunohistochemistry staining system was used. Then, the stained sections were photographed. Cells showing Caspase-3 positive reaction were counted in 100 tubules and evaluated.

Statistical analysis

SPSS 15.0 package program was used to evaluate the data obtained in the study. The normal distribution of the data was evaluated with Shapiro–Wilk test. In the evaluation of the comparison between the groups, the data showing normal distribution were evaluated with the ANOVA test, and those that did not show normal distribution were evaluated with the Kruskal–Wallis test. Normally distributed data were analyzed by independent t-test and non-distributed data were analyzed with a Mann–Whitney U-test. The obtained results are summarized with descriptive statistics such as mean± standard deviation and (median [min-max]). The statistical significance level of $p<0.05$ was chosen in the study.

Results

Biochemical results

When TAS levels were examined between the groups, there was no statistical significance. TOS and OSI levels were found to be statistically significant within the groups compared to each other (Table 1). A significant increase was observed in TOS levels in the IR and IR-He groups compared to the Sham group (respectively, p=0.016 and p=0.041). Although not significant, a decrease was observed in the IR-He group compared to the IR group. OSI levels increased significantly in the IR and IR-He groups compared to the Sham group (p=0.01 and p=0.024, respectively). Although not significant, there was a decrease in the IR-He group compared to the IR group.

NF-kB Elisa level did not show a statistically significant difference between groups. Hesperidin administered following ischemia administration decreased the increased NF-κB level due to ischemia-reperfusion, although it was not significant (Table 2). TNF-α Elisa level was not statistically significant between groups. Similarly, hesperidin administered following ischemia administration decreased the increased TNF-α level due to ischemia-reperfusion, although it was not significant (Table 2).

Table 1. Distribution of oxidative stress results within the groups

	Sham		He		IR		IR-He		p
TAS	1.1±0.82		0.72±0.35		1.34±0.87		1.29±0.36		0.083
TOS	9.68±3.07	0.73 (0.41–2.65)	7.3±3.03	0.62 (0.3–1.4)	21.41±7.67	1.24 (0.68–3.2)	30.42±19.32	20.62 (12.96–33.46)	**<0.001***
OSI	1104.62±434.14	8.4 (7.04–14.82)	1200.92±736.07	6.91 (4.69–13.58)	1862.72±625.47	1899.87 (993.44–3604.28)	2224.48±933.71	2014.22 (702.73–2699.26)	**0.025***

*: p<0.05, Data were tested using Mann–Whitney U-test. Results are shown as median±SD. Sham: Sham-operated; He: Hesperidin; IR: Ischemia Reperfusion; IR-He: Ischemia reperfusion-hesperidin; TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

Table 2. Distribution of NF-kB and TNF-α elisa results within the groups

	Sham		He		IR		IR-He		p
NF-kB (ng/ml)	0.74±0.08		0.64± 0.11		0.73±0.16		0.78±0.1		0.238
TNF-a (ng/L)	60.17± 11.22	0.74 (0.64–0.87) 60.06 (47.63–72.18)	58.33±4.07	0.61 (0.51–0.81) 58.59 (52.39–63)	68.84±7.63	0.79 (0.6–0.91) 66.27 (55.59–96.81)	71.61±14.69	0.73 (0.48–0.98) 66.75 (61.37–80.58)	**0.097***

*p<0.05, Data were tested using Mann–Whitney U-test. Results are shown as median±SD. NF-kB: Nuclear Factor kappa B; TNF-α: Tumor necrosis factor-alpha; Sham: Sham-operated; He: Hesperidin; IR: Ischemia reperfusion; IR-He: Ischemia reperfusion-hesperidin.

IHC results

Considering the results of immunohistochemistry staining count, no statistical significance was found between group 1 and group 2 for caspase-3 immunopositivity in the seminiferous tubules (Fig. 1a, b). Caspase-3 expression in testicular tissue was statistically increased in the IR group compared to the other groups (p<0.05) (Figs. 1a-d and 2). It was observed that the expression of caspase-3 in the IR-He group treated with hesperidin was significantly reduced compared to the IR group (Fig. 2).

Light microscopic results

When the sections of the Sham group were examined histologically, it was observed that the seminiferous tubules were in normal morphology. The spermatogenic cell lines in the tubules were normally distributed and tailed spermatids are observed in the lumens. Leydig cells in the interstitial area were observed in normal morphology. Germinal epithelium and basement membrane were found to be normal (Fig. 3a).

Seminiferous tubules and germinal epithelium were found to be normal in the sections belonging to the hesperidin group. Spermatogenic serial cells were observed to be normal in the basal and abdominal compartments. It was observed that the testicular tissue of the rats was in normal morphology, there was no pathological appearance (Fig. 3b).

Considering the histological features of the sections belonging to the IR group, the borders of the seminiferous tubule have distorted and a thickening of the basement membrane was observed. There are signs of hyalinization and edema in the interstitium. Bleeding was also observed in this area. There were defects and ruptures in the series forming the abdominal compartment. Some ruptures were observed in the germinal epithelium and its cells. Sections in this group had neutrophil infiltration. Some thickening was also observed in the interstitial connective tissue (Fig. 3c).

When the IR-He group preparations were examined, a decrease was observed in the germinal epithelial thickness compared to the IR group. Among the spermatogenic cells, edemas have been noted in some places. It was observed that the seminiferous tubules were in better morphology compared to the IR group. Neutrophil infiltration was greatly reduced. Structural disorder and congestion in the treatment group were more minimal than in the IR group. When the preparations were examined, it was observed that there was decreased vacuolization and some areas of the germ cell arrangement in the seminiferous tubules were disrupted (Fig. 3d).

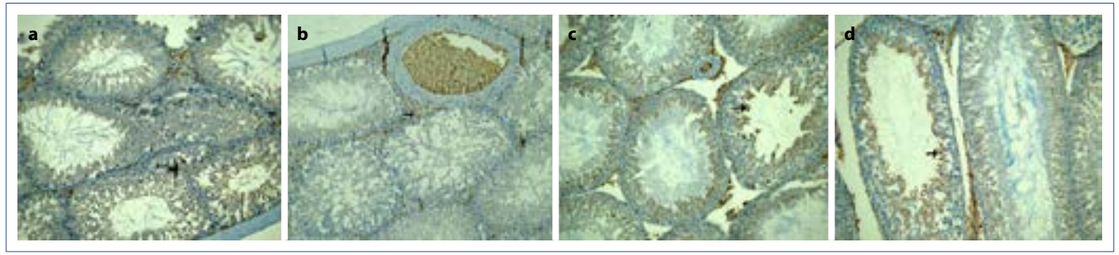

Figure 1. Caspase-3 immunohistochemistry in seminiferous tubules of all groups. (a) Sham; arrow: positive cell (b) He; Hesperidin (c) IR; Ischemia Reperfusion, and (d) IR-He; Ischemia Reperfusion-Hesperidin.

Discussion

Testicular torsion is an important factor in male infertility [11]. The torsion process is an IR injury induced by reperfusion as a result of bending of the spermatic cord followed by relaxation of the twisted cord. The importance of testicular damage is often suggested to be related to the duration and degree of testicular torsion [12]. With the detorsion of the testis after torsion, some biochemical and morphological changes occur in the testis [13]. In addition, after IR, neutrophil accumulation, proinflammatory, and inflammatory cytokines, necrotic and apoptotic processes in the tissue also accelerate [14, 15].

Many agents have been used to reduce IR damage. Due to the low efficacy, safety concerns, and doses of these agents, many of them cannot be used routinely in the clinic. Especially in recent years, it has been shown that hesperidin has effects such as anti-inflammatory, antioxidant, anticancer, and by multiple mechanisms [16, 17]. However, the mechanism of testicular IR injury has not been fully known. Studies have shown that hesperidin inhibits the increased production of reactive oxygen species after IR and reduces the destructive effects of reactive oxygen species [18]. Therefore, this study aims to determine the protective effect of hesperidin against testicular IR damage by looking at oxidative stress markers, inflammatory processes, apoptotic, and histopathological data.

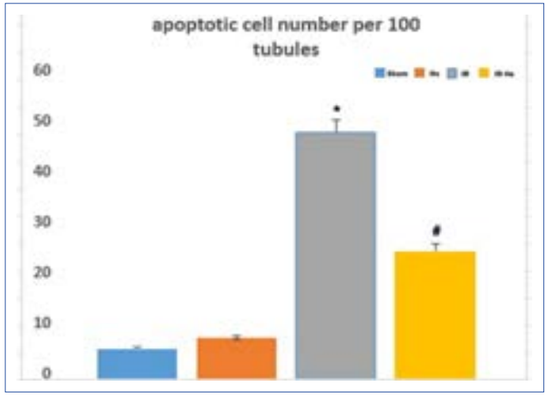

Figure 2. Caspase-3 H-Score.

*: p<0.05, compared with the Sham group; #: p<0.05, compared with the IR group. Data were tested using ANOVA followed by Tukey test. Values are mean±SD. Sham: Sham-operated; He: Hesperidin; IR: Ischemia Reperfusion; IR-He: Ischemia Reperfusion-Hesperidin.

The main underlying cause of reperfusion injury is oxidative stress. Measurement of values in TAS, TOS and OSI is often used as an index of oxidative stress [19, 20]. We used these markers in this study to assess oxidative stress. We measured oxidative stress with the resulting OSI. Our study observed a significant increase in TOS values in the IR group compared to the Sham group. Although not significant, a decrease was observed in the treatment group compared to the IR group. When the TAS levels were examined, no significant difference was found between the groups. According to the results, we determined that the hesperidin dose we applied did not have sufficient protective effect against oxidative stress due to testicular IR damage.

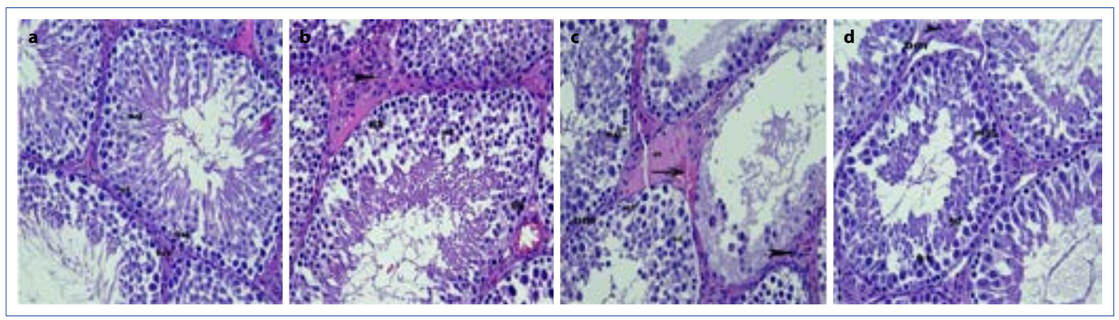

Figure 3. Light microscopy of testicular tissue in different groups. (a) Sham; sg: Spermatogonia cell, st: primary spermatocyte cell, sd: Spermatid cell, ld: leydig cell, normal testicular architecture, (b) He; Hesperidin, arrowhead: İnterstitial space, (c) IR; Ischemia Reperfusion. (d) IR-He; Ischemia Reperfusion-Hesperidin.

sg*: Spermatogonia cell loss; sd: Spermatid cell; st: Primary spermatocyte cell; ld: leydig cell; arrow: Testicular hemorrhage; arrowhead: İnterstitial space; bm: Basement membrane damage; e; Interstitial edema.

Inflammatory processes together with oxidative stress resulting from IR and apoptosis may be triggered as a result. The previous studies have reported that proinflammatory cytokines and NF-kB increased as a result of IR. Proinflammatory cytokines are important for testicular function [21, 7]. TNF-α, one of these mediators, plays an important role in germ cell differentiation and proliferation and steroidogenesis [22] After reperfusion, the increased ROS level stimulates the formation of inflammatory cytokines, followed by increased apoptosis in cells and atrophy in germ cell division and tissue [22–24] Although TNF-α levels were not statistically significant between the groups in our study, TNF-α levels that increased in the IR group decreased in the treatment group. It has been shown that increased ROS as a result of IR also increases the activation of NF-kB. ROS increase increases NF-kB activation due to 1kBβ phosphorylation. As a result of increased ROS, the accumulation of TNF-α and IL-1β, which are known activators of NF-kB, may cause an increase in cytokines [7, 25].

In our study, NF-kB Elisa level did not differ significantly between the groups. Hesperidin, given following ischemia administration, decreased the increased NF-kB level due to ischemia-reperfusion, although it was not significant. With these data, it was seen that the protective effect on hesperidin against inflammation was not sufficient.

Caspase-3, one of the apoptosis markers, can be highly activated due to inflammation and oxidative stress. Apoptosis is an important marker of tissue damage [26, 27]. In this study, caspase-3 expression was evaluated immunohistochemically. This study marked cells showing caspase-3 positivity in testicular tissue found in sections. It was observed that the number of caspase-3 positive cells increased significantly in the IR group. The outcome of these data was similar to the results of other studies showing that reperfusion increases apoptosis in germ and interstitial cells [28, 29]. The positive cell density of the treatment group was lower than that of the IR group and it was seen that the treatment had a positive effecl/t. Based on this result, it was demonstrated that hesperidin has an antiapoptotic effect against testicular ischemia-reperfusion injury and causes a significant reduction in caspase-3-positive cells.

H-E staining was performed to examine the histopathological changes in the testis tissue. When the testicular structure of the Sham group and only the treatment group was examined, it was observed that it was normal. As a result of testicular histopathological evaluation in the IR group, disruptions in tubule borders, edema in interstitial areas, hemorrhages and congestion in capillaries, neutrophil infiltration, and deterioration in spermatogenic cell lines were quite remarkable. Consistent with our result Ghasemnejad-Berenji et al. showed that the testicular IR result causes damage and degenerative changes in the testicular tissue [30].

When the testis of the treated group was examined, there was a decrease in interstitial edema, more regular spermatogenic cells, and less congestion. Less severe vacuolization and regular tubules were also observed. These results suggest that hesperidin may protect in a testicular IR injury.

Conclusion

In this study, we found that hesperidin decreased caspase-3 activation, although its potential to protect from increased oxidative stress after testicular IR was low.

Thus, it was revealed that spermatogenic cell organization was improved, the borders of the seminiferous tubules were preserved, and degenerative changes were healed in rats treated with hesperidin.

Considering these results, we found that hesperidin reduces testicular IR-induced cell death and, thus, is therapeutic on testicular tissue damage. We believe that the hesperidin agent can improve testicular functions, including IR-dependent impaired steroidogenesis and spermatogenesis.

Although there are limited studies in the literature and its mechanism has not been fully elucidated, the results obtained with hesperidin application after testicular IR in this study may be a new approach to prevent testicular damage. However, it would be beneficial to elucidate the mechanisms from different aspects by conducting more studies. In additiona, due to limited resources, the parameters we used to prove the antioxidant and anti-inflammatory properties of hesperidin were limited. Therefore, further studies are needed.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethics Committee Approval: The study was approved by The Aksaray University Animal Experiments Ethics Committee (No: 2020/03-08, Date: 30/12/2020).

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