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Research Article

Investigation of the Effect of Quercetin in Experimental Ischemia-Reperfusion Injury Model in Rat Testicle

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Abstract



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Testicular torsion (TT) is the most important cause of acute scrotum in childhood. It is an emergency surgical problem that can lead to loss of organ if left untreated. The aim of treatment is to reduce or eliminate the torsion/detorsion damage. The affect of antioxidants is one of the methods to be used for this reason. In this study, a special bioflavonoid and quercetin, an antioxidant known to protect against oxidative damage, were used to avoid experimentally caused torsion/detorsion damage in rats.

32 male Sprague Dawley rats, 12 weeks old, weighing 250–300 g, were used in the study. Rats were randomized into 4 groups. No surgical procedure was performed in the control group. 5-hour ischemia was achieved by applying torsion to the left testicle in the torsion group. After 5 hours of ischemia, a 2-hour reperfusion was applied in the torsion/detorsion group. In the torsion/detorsion+quercetin group, 2 hours of reperfusion was applied after 5 hours of ischemia and i.p. quercetin was injected.

A significant decrease was observed in the quercetin-administered group in terms of spermatocyte degeneration and inflammation. There was a decrease in dilatation data in the quercetin group, but it was not statistically significant.

Quercetin, which has a strong antioxidant effect, has been shown to have positive effects on experimentally induced torsion/detorsion damage.

Keywords: Testicular torsion, ischemia-reperfusion, antioxidant, quercetin, Inflammation

INTRODUCTION

Testicular torsion is defined as a urological emergency that can lead to permanent ischemic damage to the testicles as a result of the rotation of the "funiculus spermatic" on its axis and the disruption of vascular flow ¹⁻³. Although it is seen in adults, most cases are said to occur in the first years of life and between 12-18 years of age. The most common symptom is unilateral scrotal pain, with nausea and vomiting. The diagnosis of the presence of torsion as a result of the examinations requires both early and correct treatment. Otherwise, testicular losses that are difficult to heal may occur. Manual detorsion can be applied while waiting for transfer or urology consultation in the emergency department ². Rapid diagnosis and treatment can prevent further damage to the tormented testis. For this, the duration of the symptoms, the degree of bending and the homogeneity of the testicular parenchyma together with advanced ultrasound findings are important. More than 4-8 hours after the onset of symptoms can lead to cell necrosis and, as a result, cell loss in the testicular tissue ⁴. It has been stated in experimental studies that germ cells, particularly spermatogonia and spermatocytes, are most vulnerable type of cells to testicular ischemia ⁵. Additionally, it is said that in instances where early

intervention is not made, the likelihood of conducting an orchiectomy rises ⁴. It has been reported that using Testicular Torsion Risk Score will be appropriate to evaluate acute scrotal pain in early diagnosis. The parameters of this test are testicular swelling (2 points), hard testis (2 points), elevated testicles (1 point), absence of cremaster reflex (1 point), and nausea/vomiting (1 point) with a total score of 7 points ⁶. Testicular torsion may cause ischemic injury, while detorsion may cause reperfusion injury. Ischemic reperfusion injury can cause testicular problems such as oxidative stress (OS), inflammation and germ cell death ⁷. Although surgery fixation is required before departing the hospital, external manual reduction can be used as starting treatment ^{1,3}. In addition, prophylactic fixation should be performed to prevent torsion at the contralateral testis ¹. Orchiectomy is performed if the affected testis has lost its vitality and cell degeneration has developed ⁸.

Antioxidant agents have been used in experimental studies to prevent this situation. These antioxidants consist of various natural and pharmaceutical agents that can prevent or ameliorate the harmful effects of I/R injury ³. Studies using molecular assays have demonstrated that testicular torsion-induced ischemia/reperfusion increases the production of free

oxygen radicals⁹⁻¹¹. It has been shown that the use of different antioxidant agents can reduce free oxygen radicals¹⁰⁻¹². Quercetin is a natural flavonoid found in many vegetables and fruits with immunomodulatory, anti-inflammatory and antioxidant properties¹³. Studies have shown that quercetin is an important antioxidant that has a protective role against oxidative damage¹³⁻¹⁵. In a recent study, it was reported that quercetin, which also contributes to the maintenance of the endogenous cellular antioxidant defense system, has beneficial properties on various parameters of fresh and post-thawed sperm in different species¹⁶.

MATERIALS AND METHODS

Animals

After dividing the rats into 4 equal groups (n:10), general anesthesia was administered to rats by using 90 mg/kg ketamine hydrochloride and 8 mg/kg xylazine (intramuscular) after 6 hours of fasting before the operation. After shaving the skin of the rats and providing antisepsis with 10% povidone iodine solution, the midline of the scrotum was incised for about 2.5 cm. The following procedures were applied to the experimental groups.

Experimental groups:

1. Group (control): The animals did not undergo any surgical operation and were sacrificed at the end of the experiment.

2. Group (torsion): An incision was made in the midline of the scrotum of the rats under general anesthesia. The left testicles was rotated 720° clockwise and fixed on the tunica dartos. In the last 30 minutes of the 5 hour ischemia, i.p. physiological saline was administered to the animals in this group. At the end of the fifth hour, animals were sacrificed.

3. Group (torsion/detorsion): Animals were anesthetized, an incision was made from the midline of the scrotum and the left testicles was rotated 720° clockwise and fixed on the tunica dartos. In the last 30 minutes of the 5 hour ischemia, i.p. physiological saline was administered to the animals in this group. At the end of the fifth hour, suture was opened and the left testicles were repositioned. Scrotum was closed again and the testicles was reperfused for 2 hours. At the end of the reperfusion period, the animals were sacrificed.

4. Group (torsion/detorsion+quercetin): An incision was made in the midline of the scrotum of the rats under general anesthesia. The left testicles was rotated 720° clockwise and fixed on the tunica dartos. In the last 30 minutes of the 5 hour ischemia, i.p. 30 mg/kg quercetin was administered to the animals in this group. At the end of the fifth hour, suture was opened, and the left testicles were repositioned. Scrotum was closed again, and the testicles was reperfused for 2 hours. At the end of the reperfusion period, the animals were sacrificed.

Histopathological method

At the end of the experimental protocol, testicular tissue samples were taken into 10% neutral buffered formalin solution and routine paraffin tissue follow-up was applied. After the fixation process (24 hours) the tissues were infiltrated with paraffin at 58°C by the processes with washing (1 night) and transparentizing (3x30 minutes in xylene) with increased alcohol series (50%, 70%, 80%, 90%, 96% and absolute ethyl alcohol). Then, the tissues were embedded in paraffin blocks and 4-6 µm thick sections were prepared with the help of a microtome (catalog no: Leica RM2265, Wetzlar, Germany) for immunohistochemical staining with Hematoxylin-Eosin. For Hematoxylin-Eosin Staining, testicular tissue sections prepared from paraffin blocks were placed in a bain-marie which was set at 37°C. Sections were kept in an

oven at 58-62°C for 6 hours to dissolve the excess paraffin on the slide.

Sections were deparaffinized in xylene for 3x15 minutes. The sections were passed through the reduced alcohol series (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes and kept in distilled water for 5 minutes. After waiting for 8 minutes in Harris Hematoxylin stain, the sections were washed under running water for 5 minutes. After rinsing the sections, they were kept in alcoholic eosin stain for 6 minutes. Sections were rapidly immersed in increasing alcohol series (through 80%, 90%, 96% ethyl alcohol series) and kept in absolute alcohol for 2 minutes. Finally, the sections were kept in xylene for 3x15 minutes and covered with a coverslip by dripping Entellan on the tissue¹⁷.

Immunohistochemical method

Sections prepared from paraffin blocks for immunohistochemical staining were allowed to be opened in a bain-marie set at 37°C and then transferred to polylysine slides. The sections were kept in an oven at 58-62°C for 6 hours in order to dissolve excess paraffin on the slide. The sections were deparaffinized in xylene for 3x15 minutes. The sections were passed through the decreasing alcohol series (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes and kept in distilled water for 5 minutes. Sections were washed 3x5 minutes in phosphate buffer solution (PBS). Sections were taken in EDTA buffer solution (pH: 8.0, catalog number: ab93680, Abcam, Cambridge, USA) and heat-induced epitope retrieval process was performed. Sections left at room temperature for 20 minutes were taken back into PBS. Sections were arranged on an immunohistochemistry bar, and the humidity and temperature of the bar was controlled.

Hydrogen peroxide 3% solution (catalog no: TA-015-HP, ThermoFischer, Fremont, CA, USA) was dripped onto the sections and incubated for 20 minutes. Then sections were washed with PBS for 3x5 minutes and kept in Ultra V Block (catalog no: TA-015-UB, ThermoFischer, Fremont, CA, USA) solution for 7 minutes. Sections were kept overnight at +4°C with antibodies against caspase-9 (catalog no: PA5-16358, ThermoFischer, Fremont, CA, USA) and TNF-α (catalog no: PHC3015, ThermoFischer, Fremont, CA, USA). The next day, the sections were left at room temperature for 30-60 minutes. Biotin-containing secondary antibody (catalog no: TP-015-BN, ThermoFischer, Fremont, CA, USA) was dripped onto the sections washed with PBS and incubated for 14 minutes. Then, streptavidin-peroxidase (catalog no: TS-015-HR, ThermoFischer, Fremont, CA, USA) was dripped and waited for 15 minutes and then washed with PBS. Diaminobenzidine (DAB) (catalog no: TA-001-HCX, ThermoFischer, Fremont, CA, USA) was dropped onto the washed sections and the reaction was monitored under a microscope and stopped with PBS. After counterstaining with Harris hematoxylin, the sections were covered with entellan (catalog #107961, Sigma-Aldrich, St. Louis, MO, United States) and visualized by evaluation under a Zeiss Imager A2 photomicroscope¹⁸.

Statistical analysis

IBM SPSS Statistics 23.0 (IBM Inc, Chicago, IL, USA) computer program was used for statistical analysis. One-way analysis of variance (One-Way ANOVA) test was used to compare the mean value of 2 or more independent groups. The homogeneity of the groups was examined by Levene's test. Tukey HSD, a Post-hoc test, was used to analyze the differences between the groups in the presence of normally and homogeneous distributed variables. In the descriptive statistics of the data, "mean, standard deviation, median, lowest and highest values" were used. In all tests, P<0.05 error was considered statistically significant.

RESULTS

Histopathological findings

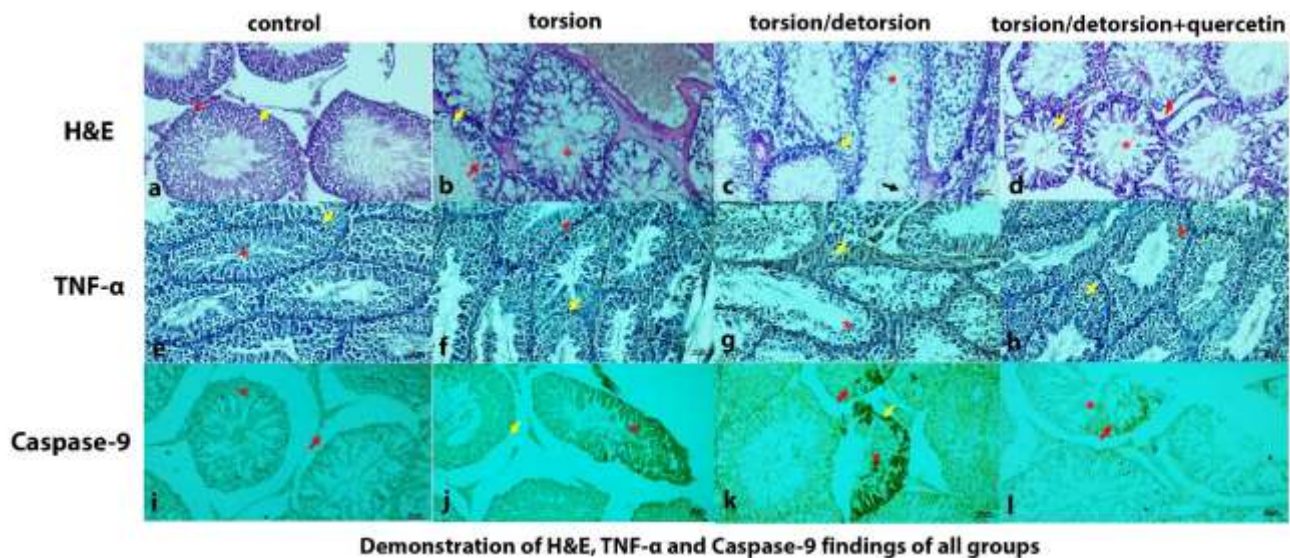


Figure 1(a-l): Cross-sections of histological and immunohistopathological findings of all groups.

H&E findings

In control group: In the transversal section of the seminiferous tubules, spermatogonia cells (red asteriks) werelined up along the basal membrane (yellow arrow), andsertoli cells of the spermatocytes, especially in the chromatin-rich nucleus, a mitotic broad base regionwas present close to the basal membrane and their ends werepointed. Although there was a strong mitotic activity in the spermatogonia at the seminiferous tubule, the spermium formations showed a regular alignment towards the lumen (Figure 1a).

In torsion group: When the seminiferous tubules were examined transversely, it was found that the tubule of the basal membrane structure was especially disrupted (yellow arrow), the spermatogonia and spermatocytes on the basal membrane were degenerated (red asteriks), their nuclei were pycnotic and had an apoptotic appearance. It was determined between the 2 seminiferous tubules that, the vacuole structures were increased, the connective tissue areas were expanded (red arrow), and the leydig cells showed degenerative changes. Pyknotic nuclei were also present. Excessive dilatation and congestion was detected at the blood vessels, especially at the tunica vasculosa area (Figure 1b).

In torsion/detorsion group: It was determined that the integrity of the basal membrane (yellow arrow) was impaired at the seminiferous tubules.An increase was observed especially in the hyalinized areas (black arrow), excessive expansion of the blood vessels between 2 tubules and degeneration in the leydig cells. When the seminiferous tubule was examined, it was discovered that spermatogonia (red asteriks), particularly in the basal region, increased in the form of cells that appeared to be in the process of apoptosis and in regions that had been hyalinized, and that the structural integrity had completely disappeared (Figure 1c).

In torsion/detorsion+quercetin group: In the transversal section of the testicular seminiferous tubules, it was observed that especially the basal membrane structure began to become evident (yellow arrow), and the mitotic activities of spermatogonia increased locally at certain tubules (red arrow), and spermium began to become evident towards the

lumen. While local enlargements and hypertrophies were observed in the interlobular area atleydig cells (red arrow), it was seen that extracellular matrix areas were prominent within the connective tissue areas (Figure 1d).

TNF-α findings

In control group: AlthoughTNF-αexpression was positive in a small number of plasma cells (yellow arrow), in general TNF-αexpression in seminiferous tubules, sertoli cells and spermatogonia (red asteriks) was evaluated as negative (Figure 1e).

In torsion group: An increase in TNF-αexpression was also observed in the parts of spermatogonia (red asterisk) close to the basal membrane, in leydig cells (yellow arrow) and sertoli cells. This indicated damage due to degenerative changes. Occasionally, inflammation and TNF-αexpression positivity were seen around the vessel (Figure 1f).

In torsion/detorsion group: TNF-αwas increased in intertubular areas and abundant macrophage cells (yellow arrow). TNF-αpositivity was demonstrated in the cells along the peritubular area of the basal membrane. Spermatogonia is impaired (red asteriks). Here, both sertoli and spermatogonia TNF-αare positive. It is noteworthy that the inside of some sertoli cells are empty (Figure 1g).

In torsion/detorsion+quercetin group: spermatogonia have entered the recovery process, but degenerative changes still continue in some of them. TNF-αpositivity was observed especially in degenerated spermatogonia (red asteriks) and in some sertoli cells. TNF-αpositivity is mostly found in the range of basal membrane and leydig cells (yellow arrow). This indicated an increase in inflammation. Negative expression was observed in the vast majority of spermatogonia (Figure 1h).

Caspase-9 findings

In control group: It was seen that caspase-9 expression was positive in a small number of spermatogonia (red asteriks). However, when looked in general, a small amount of positivity was detected in spermatogonia (red arrow) (Figure 1i).

In torsion group: At the transversal section of the seminiferous tubules, caspase-9 cells in the apoptotic state were positive, especially in the degenerated spermatogonia (red asteriks) (both sertoli and spermatogonia cells attached to the basal membrane). Caspase-9 was evaluated positive in some leydig cells (yellow arrow) (Figure 1j).

In torsion/detorsion group: Intense degenerative changes were observed especially in some of the seminiferous tubules and increased caspase-9 reaction was found in sertoli and degenerated cells (red asteriks). Again, caspase-9 expression was positive in the cells lined up along the basal membrane (red arrow), and caspase-9 reaction was positive especially in the peripherally directed parts of leydig cells (yellow arrow) (Figure 1k).

In torsion/detorsion+quercetin group: Spermatocytes lined up along the basal membrane (red arrow) especially in some seminiferous tubules, at an area that we call 1/3 of the tubule, in the drug group. Primary spermatocytes (asterisk) especially in degenerative apoptotic appearance were evaluated as caspase-9 positive (Figure 1l).

DISCUSSION

Testicular torsion in boys during childhood and adolescence is a urological emergency that should be treated with acute surgery^{19, 20}. It is defined as the obstruction of blood flow to the testis and its appendages due to the rotation of the spermatic cord around its axis. The incidence in male individuals under the age of 25 is approximately 1/4000¹⁹. In testicular torsion, rotation and subsequent arterial narrowing cause ischemia, which causes damage to the testicular tissue^{4, 20}. In most people, the testis rotates between 90-180 degrees and the blood flow cannot be provided at the desired level to nourish the tissues. Complete torsion is very rare. If it does, it quickly reduces the vitality of the tissues. If the torsion lasts less than 8 hours, salvage of the tissues is possible, but if it has lasted more than 24 hours, the probability of tissue salvage is severely reduced²¹. The affected testis can either be fully removed via orchiectomy or manually detorted and attached to the scrotum via orchiopexy^{4, 20}. It has been demonstrated that TT reduces sperm motility and sperm concentration. Ischemic damage and reperfusion injury are thought to lead to decreased semen quality²⁰. Restoration of blood flow causes accumulation of ROS⁹. Excessive amounts of ROS can lead to disruption of the cellular reduction-oxidation balance and cell damage^{10, 11}. Additionally, due to an imbalance in the antioxidant systems, this increase causes OS^{3, 10}. Antioxidant agents are used in experimental studies to minimize this damage. In our study, we used quercetin, which is found in many vegetables and fruits and has immunomodulatory, anti-inflammatory and antioxidant properties.

In a study in which the protective effect of serum oxide on testicular function and OS was reported, coagulative necrosis characterized by loss of cohesion of germinal cells, shed germinal cells in the seminiferous tubules and coagulation, and severe degenerative changes in the testis were reported in rats in the TS/TD group. In addition, it has been reported that a diffuse structuring in germ cells is shown, together with loss of seminiferous tubule epithelium and uncertain borders of some tubules. In the interstitial region, hemorrhage and edema were prominent. It was found that the seminiferous tubules were more robust and the germinal cells more cohesive in the cerium oxide-administered group with TS/DT. Degenerated, shed germinal cells and multinucleated giant cells were detected in some tubules. It is said that the atrophy of the tubules is reduced and only a few tubules are irregular in their borders. Bleeding and vascular edema are also said to be reduced²¹. In another study, disruption of the basal membranes of the seminiferous tubules, deterioration in

spermatogenic cells, increase in apoptotic cells and significant deformation in Sertoli cells were observed in the TS/DT group. In the TS/DT group, deformation was increased in spermatogenic cells with hyperplastic nuclei and degenerated Sertoli cells separating from the basal membrane towards the lumen²². In another study, it was reported that the seminiferous tubular diameter was significantly lower in the TS/DT group compared to the sham group, and there was an increase in germ cell apoptosis as a result of impaired blood flow to the testis²³. In their study, Doğan and İpek observed that the basal membrane thickness of the tubules increased in the TS/DT group, pycnosis of spermatogenic cells and deterioration in their nuclei, attenuated maturation in sperm cells and the presence of degenerated Sertoli cells. They stated that there was bleeding and dilatation, increase in connective tissue and degenerated leydig cells in the blood vessels of the interstitial region. A decrease in the basal membrane thickness of the tubules, increased mitosis, and degenerated and impaired spermatic cells in some tubules were detected in the TS/DT+ *Ganoderma lucidum* group, compared to the TS/DT group. It is also said that there is very little bleeding in the blood vessels of interstitial regular connective tissue cells and heterochromatin leydig cells²⁴.

In our study, it was found that the tubule of the basal membrane structure was disrupted in the torsion group, the spermatogonia and spermatocytes on it were degenerated, the nuclei were pycnotic, and there were connective tissue areas between the apoptotic cells and the seminiferous tubules. In the tubule, especially vacuoles increased and Leydig cells showed degenerative changes in places. Especially in the tunica vasculosa region, excessive dilation and occlusion of the blood vessels were observed. In the TS/DT group, the integrity of the basal membrane was disrupted in the seminiferous tubules. An increase in the hyalinized areas, excessive expansion of the blood vessels between the tubules and degeneration in the Leydig cells were observed. After the application of quercetin, it was observed that there was no deterioration in the integrity of the basal membrane structure in the cross-section of the testicular seminiferous tubules. Additionally, the mitotic activities of the spermatogonia have increased and the spermium began to become evident towards the lumen. In Leydig cells, local enlargements and hypertrophies were observed in the interlobular area, while extracellular matrix areas were observed in the connective tissue areas. This demonstrates how quercetin treatment reduces testicular damage brought on by testicular torsion.

Ischemia-reperfusion of the testis causes germ cell apoptosis. It is said that OS products accumulate in the testicles following ischemia-reperfusion and that the apoptosis observed in the germ cell is related to mitochondrial Caspase-9²⁵. A crucial enzyme in the intrinsic or mitochondrial route, caspase-9 is activated by a number of triggers, such as radiation, stressors, and chemotherapies²⁶. Apoptosis has a crucial role in cancer, brain disease, aging and heart disease. Caspase-9 is an important therapeutic target of various apoptosis-related diseases, as it is an apoptosis-initiating Caspase. Regulators of Caspase-9 are important²⁷. TNF-, IFN-, IL-6, IL-12, IL-17, and IL-23 are proinflammatory cytokines secreted by immune cells that alter the typical testicular immunosuppressive microenvironment²⁸. TNF- is a pleiotropic cytokine that generates numerous stimuli under various normal and pathological circumstances. In addition, it has strong anti-inflammatory and apoptosis-inducing properties²⁹. TNF- α is used as an inflammatory biomarker³⁰. In a study investigating the effects of gallic acid on I/R-induced testicular damage, it has been shown that TNF- α expression is positive in degenerative spermatogonia, Sertoli cells, Leydig cells and interstitial macrophages due to increased inflammation in the torsion and detorsion group, TS/DT damage is present, and it

induces apoptotic cell formation with increased TNF - α signals. In addition, it has been shown that a new process in cell remodeling started with the decrease of TNF- α activity in degenerative spermatocytes in the group to whom gallic acid was administered, and inflammation was decreased in seminiferous tubule cells, which was also observed in Sertoli and Leydig cells ³¹.

When the TNF- α activity in the torsion group of our study was examined, an increase in TNF- α expression in Leydig cells and Sertoli cells in the parts of spermatogonia close to the basal membrane, inflammation around the vessel in the inter tubular area and positivity in TNF- α expression were observed. Spermatogonia cells in the TS/DT group exhibited TNF-positive cells along the basement membrane peritubular area, and they were dysfunctional. TNF- α reaction in cells increases in parallel with inflammation.

In the quercetin administered group, negative expression was observed in the majority of Spermatogonias, while TNF- α expression due to inflammation was observed in some degenerated Sertoli and Leydig cells. When the Caspase-9 activity of our study was examined, degenerated spermatogonia in the torsion group (in both sertoli and spermatogonia cells attached to the basement membrane) were positive in apoptotic Caspase-9 cells, and Caspase-9 expression was positive in some Leydig cells in accordance with the TNF- α signal reaction. The apoptotic process was hastened in some of the seminiferous tubules in the TS/DT group, and Sertoli cells that had undergone severe degeneration showed an increase in the Caspase-9 response. Positive Caspase-9 expression, particularly in cells arranged in a row along the basal membrane, was believed to denote mitotic suppression. In the examination of the quercetin applied group, it was observed that the primary spermatocytes lined up along the basal membrane were in a degenerative state and Caspase-9 positive cells were found in apoptotic appearance. However, in the examination of seminiferous tubules, it was observed that the effect of Quercetin was effective in spermatogonia cells and in reducing the apoptotic process.

Positive Caspase-9 expression was believed to suggest mitotic inhibition, particularly in cells arranged in a row along the basal membrane. This reduction in apoptosis is beneficial on spermatogenesis and indicates less spermatocyte degeneration. The inhibitory and protective effect of quercetin against the morphological damage of ROS in testicular torsion may be due to its antioxidant and anti-inflammatory effects. Quercetin may be beneficial in the prevention of testicular torsion and the preservation of germ cell mass and fertility.

CONCLUSION

- Restriction of blood flow in the testicular tissue due to ischemia and the increase in OS due to reperfusion process, inflammation and cell degeneration adversely affected the development of spermatogenesis.
- Increased MDA, MPO and decreased CAT values as a result of tissue damage were regulated to normal levels as a result of quercetin administration.
- Quercetin administration has a protective and healing effect against the damage caused by testicular torsion in the testicular tissue.
- It was concluded that the protective effect of quercetin is mainly due to its antioxidant properties and induces the angiogenic effect. In addition, it was concluded that quercetin acts to stop apoptotic development by reducing

the inflammation signal and can inhibit germ cell degeneration due to infertility.

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