# The Protective Effects of Hypericum Perforatum and Nigella sativa on Testicular Tissue After Testicular Torsion and Detorsion

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## ABSTRACT

The aim of this study was to investigate the antioxadant effects of Hypericum perforatum (HP) and Nigella sativa (NS) in protecting against biochemical and histopathological changes in rat testis injured by experimental ischemia/reperfusion (I/R). This research study was conducted on 40 adult male Wistar albino rats. Rats were randomly separated into four groups of 10 animals each: the sham (control) group in which only the scrotal area opened and closed without torsion and detorsion (T/D); the I/R group, which was T/D applied but not treated by any agent; the HP+I/R, and NS+I/R groups, HP and NS were given intraperitoneally 30 min before T / D 25 mg / kg groups. In the I/R group with HP+I/R and NS+I/R treatment groups, torsion was created by rotating only left testis 720 degrees clockwise for 2 hours. At the end of the 2 hours torsion, the left testis was detorsioned and replaced in the scrotum for 4 hours. Along with the completion of the experiment, the left testis was rapidly removed to measure oxidative stress markers and to examine it histopathologically. In the HP+I/R and NS+I/R groups, the malondialdeyhde (MDA) level was significantly lower than in the I/R group only, and the superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), calase (Cat) activities were higher than the I/R group. Also our histopathological results supported these biochemical findings. In conclusion, our results suggest that both HP and NS protect against injury of left testis induced by I/R, however the protective effects of NS were more pronounced than those the HP.

## INTRODUCTION

Testicular torsion is the rotation of the

spermatic cord and its components araound itself, characterized by the interruption of venous and arterial blood flow the testes.[1-3] Since reperfusion is provided as soon as possible to maintain ischemic tissue viability, surgery is recommended to correct the torsion with minimal time passing.[4] However, after correcting testicular torsion, reperfusion injury often adds to the clinical status.[4-6] Testicular torsion lead to ischemic injury and detorsion create reperfusion injuries that cause structural and biochemical changes in the tissue. Numerious research show that free oxygen radicals are responsible for ischemia-reperfusion injury in tissues.[1,3,6] Free oxygen radicals, increase membrane permeability by peroxidation lipids in the mitochondria, and cell membranes, causing protein denaturation, DNA damage and cell injury. This ischemia further increases germ cell injury.[3,4] Antioxidant defense systems eliminate reactive oxygen species (ROS) produced in the tissues.[7] Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (Cat) are antioxidant enzymes in tissues that rapidly remove ROS under normal circumstances. During I/R, the antioxidant defense system becomes inadequate, resulting in oxidative injury to the tissues.[1] This lead to cell injury by lipid peroxidation.

Previous studies have shown that the effects of ischemia-reperfusion injury in many organs and tissues can be reducted by various antioxidant treatments.[1,7] Hypericum perforatum (HP) L., locally known as "Kantaron" in Turkey, and St. John's Wort in English, is a perennial plant from the Hypericaceae family grown in North Africa, Asia, Europe, and the United States and has been used as a medication for approximately 2,000 years.[8,9] Recent studies have demonstrated that HP influences cancer, wound healing, inflammation, and bacterial and viral diseases, and has neuroprotective effect and antioxidant properties.[7,8,10] It is also used for many deseases in Turkish folk medicine[9] and demonstrated to have protective and antioxidant effects againist lipid peroxidation in tissues exposed to I/R injury.[7-12] Nigella sativa (NS) extract and its oil have anticarcinogenic, anti-inflammatory, antimicrobial, antifungal, anthelmintic, antidiabetic, analgesic, and antioxidant effects and stimulate the immune system. These effects probably come from active compounds in the NS. Pharmacological and toxicological studies have demonstrated that crude extracts of the seeds and some of its active compounds (volatile oil and thymoquinone) may have antioxidant effects and prevent membrane lipid peroxidation in tissues.[13-16]

In this empirical study, we investigated the protective effects of HP and NS, that are used commonly as herbal medication in the World, and which have antioxidant and anti-inflammatory properties, against tissues I/R injury.

## MATERIAL AND METHODS

### Experimental Animals

Forty male Wistar-Albino rats weighing between 220-250 g were obtained from The Experimental Animal Laboratory at Çukurova University Experimental Research and Application Centre. Prior to the experiment, rats were given food and water ad libitum (Feed Institution, Standard Rat Hatch). The animals were housed and fed in the laboratory at room temperature (22 °C) under a 12-hour day/night diurnal cycle. The study was approved by the Experimental Animal Local Ethics Committee of Çukurova University, Turkey (Protocol date and No: 2017/08).

### Chemicals

HP and NS oil were used as antioxidant agents. Commercial formulations of HP oil (Aksu,Vital, 20 mL, Istanbul, Turkey) and NS oil (Hel-Kim, 50mL, Mersin, Turkey) were obtained from a pharmacy. Both drugs were administered intraperitoenally (i.p) at a dose dose of 25 mg/kg. General anaesthesia was administered i.p at 10 mg/kg xylazine hydrochloride (Alfazyne® 2%, Alfasan International, 3440, AB, Worden, Holland), and 50 mg/kg ketamine (Ketalar®, Pfizer Pharma GMBH, Germany).

### Experimental Design

Wistar-Albino male rats weighing 220-250

g were used in the study. The rats fasted for 8 h before the study and were randomly divided into four groups of 10 animals each. Prior to surgery, general anaesthesia was administered i.p to all rats by a 10 mg/kg xylazine hydrochloride and 50 mg/kg ketamine cocktail. The left scrotal area was shaved, cleaned, and disinfected with 10% povidone-iodine solution, and an ilioinguinal incision was made. In the scrotal space, the left testis was removed by blunt dissection from the gubernaculum with the tunica vaginalis and spermatic cord. The left testis was then rotated 720° clockwise with the cord structures to create an experimental testis torsion model and placed on the inner scrotum surface with 4/0 propylene sutures.

**Groups**

For the the sham (control) group (n=10), only their scrotal area was opened and closed again without applying a torsion/detorsion pattern. The I/R group (n=10) were subjected to the torsion/detorsion procedure. The left testis of each rat was rotated 720° clockwise by the cord to create an experimental testis torsion pattern, then placed on the inner scrotum surface with 4/0 propylene sutures. After the 2 hours torsion period, the stures were opened, the seams were removed, and the left testis was rotated counter-clockwise to correct the torsion. The incision was closed again with a 4/0 propylene suture and reperfusion was allowed for 4 hours. HP+I/R group (n=10) were given 25 mg/kg HP oil, NS+I/R group (n=10) were given 25 mg/kg NS oil via i.p before 30 minutes from the torsion/detorsion pattern. In the both groups, then 30 minutes administration of the antioxidant agent, an experimental testis torsion pattern was generated by rotating the left testis of each rat 720° clockwise by the cord, and the testis was fixed to scrotum with a 4/0 propylene suture from the tunica albuginea to maintain the torsion. At the end of the 2 hours torsion period, the sutures were opened, fixation seams were removed, and the left testis tortion was corrected by counter-clocwise rotation. The incision was closed again with a 4/0 propylene suture and reperfusion was allowed for 4 hours.

All rats were sacrificed by administering high dose anaesthesia after the experiment. The left testes were collected from all animals. The appropriate amount of tissue was removed from the testes for biochemical analysis and stored at -80 °C until assayed for MDA level and SOD, GSH-Px, and Cat enzyme activities. The remaining tissue was fixed by placing it in a 10% neutral formaldehyde solution for histopathological examination.

**Biochemical Assay**

*Malondialdehyde (MDA) Assay*

Tissue MDA levels were measured spectrophotometrically per the method of Ohkawa et al. [17] Briefly, the tissue MDA assay was performed under aerobic conditions at pH 3 and 5. The tissue was incubated for 1 hour in a boiling water bath with the homogenate. The secondary product of lipid peroxidation, MDA, is pink based on the spectrophotometric measurement of the complex at 532 nm. The results are given as nmol/mg protein.

*Determination of SOD Activity*

SOD enzyme activity was measured spectrophotometrically according to the method of Sun et al.,[18] in which SOD activity is measured by the xanthine/xanthine oxidase system, and the produced superoxide is based on nitro blue tetrazolium (NBT) reduction. The coloured formazan formed by the NBT reduction of the superoxide radicals is measured spectrophotometrically. This complex gives a maximum absorbance at 560 nm. If no enzyme exists, it is reduced to blue-purple. When SOD is present in the medium, there is no reduction, but a blue-purple colour and a clearer colour are present depending on enzyme activity. SOD activity is expressed as U/g protein.

*Determination of GSH-Px Activity*

The GSH-Px activity was measured spectrophotometrically per the method of Paglia et al.[19] Briefly, GSH-Px is an enzyme that converts $H_2O_2$ into water and is mediated by oxidizing the reduced glutathione. This

requires oxidization of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADP), which occurs with NADPH in the medium. Spectrophotometric measurement of the change in absorbance by adding $H_2O_2$ to the medium containing NADPH, reduced glutathione, sodium azide, and glutathione reductase at 340 nm reflects the GSH-Px activity, which is expressed as U/mg protein.

*Determination of Cat Activity*

Catalase converts water and oxygen by decomposing $H_2O_2$ with catalytic activities. Cat enzyme activity was measured spectrophotometrically per the method of Aebi,20 in which hydrogen peroxide shows a maximum absorbance at 240 nm. When Cat is added to the test medium, disruption is followed by an absorbance reduction in the ultraviolet spectrum. This decreased absorbance is directly proportional to the enzyme activity. The results are expressed as k/g protein.

*Histopathological Evaluation*

In all testes exposed to ischemia during experimental torsion, edema and colour changes due to venous stasis were observed macroscopically. Testicular tissue samples were fixed in 10% formalin and embedded in paraffin to conduct light microscopy. Embedded samples were cut into 5 µm-thick sections, then mounted onto slides and stained with haematoxylin and eosin (H&E). Sections were examined for changes in seminiferous tubules, edema, vascular congestion, and coagulation necrosis.

**Statistical Analyses**

The one-way analysis of variance (ANOVA) and Duncan's post hoc tests were performed on the data to examine the differences among groups using the SPSS statistical programe. The results are presented as the Mean±SD. The statistically significant was contucted as $p<0.05$.

## RESULTS

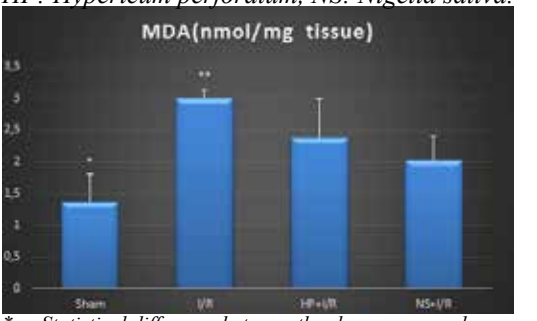
**Results of the Biochemical Analyses**

In the I/R group, the tissue MDA levels were higher than the sham, HP+I/R, and NS+I/R groups. The tissue MDA levels decreased significantly in the HP+I/R and NS+I/R groups compared to the I/R group ($p<0.05$). However, there was no significant difference between the HP+I/R and NS+I/R groups ($p>0.05$) (Fig 1).

SOD activity decreased significantly in the I/R group compared to the sham group. SOD activity was significantly increased in the HP+I/R and NS+I/R groups compared to the I/R group ($p<0.05$). The HP+I/R and NS+I/R groups also differed significantly ($p<0.05$) (Fig. 2).

Differences in GSH-Px enzyme activity in the testis tissue between all groups were statistically significant ($p<0.05$). In

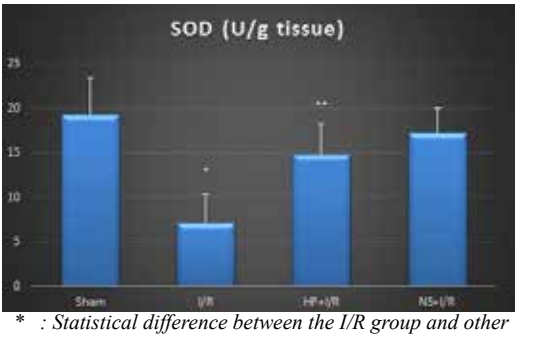
***Figure 1:*** *Malondialdehyde (MDA) levels in the left testis tissue. I/R: Ischemia/Reperfusion, HP: Hypericum perforatum, NS: Nigella sativa.*




** : Statistical difference between the sham group and other all groups*

*** : Statistical difference between the I/R group and other all groups*

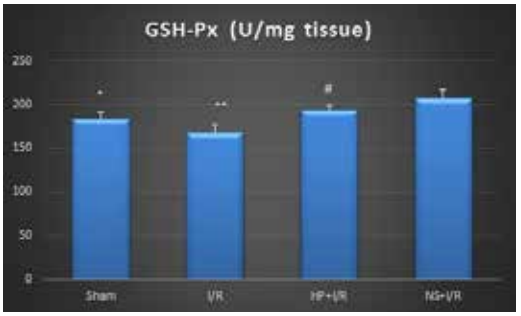
***Figure 2:*** *Superoxide dismutase (SOD) activity in the left testis tissue. I/R: Ischemia/Reperfusion, HP: Hypericum perforatum, NS: Nigella sativa*




** : Statistical difference between the I/R group and other all groups*

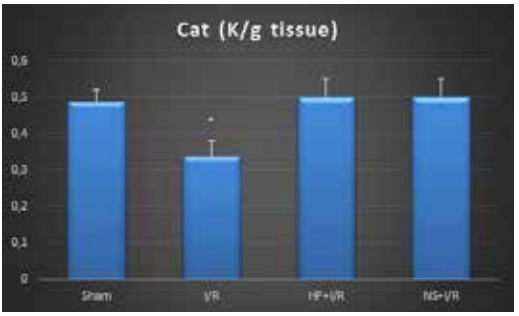
*** : Statistical difference between the HP+I/R and NS+I/R groups*

***Figure 3:*** *Glutathione peroxidase (GSH-Px) enzyme activity in the left testis tissue. I/R: Ischemia/Reperfusion, HP: Hypericum perforatum, NS: Nigella sativa.*




** : Statistical difference between the I/R group and other all groups*
*** : Statistical difference between the HP+I/R and NS+I/R groups*

***Figure 4.*** *Catalase (Cat) enzyme activity in the left test tissue. I/R: Ischemia/Reperfusion, HP: Hypericum perforatum, NS: Nigella sativa.*




** : Statistical difference between the I/R group and other all groups.*

the NS+I/R group, the GSH-Px activity was greater than the other groups (Fig. 3).

The testis tissue Cat activity in the I/R group was lower than the sham group ($p<0.05$). Cat activity in the HP+I/R and NS+I/R groups was significantly higher compared with the I/R group ($p<0.05$). No significant difference was found in Cat activity between the HP+I/R and NS+ I/R groups ($p>0.05$) (Fig. 4).

**Result of the Histopathological Examination**

In testis sections revealed from the sham group, the seminiferous tubule structure was histopathologically normal and tails of mature spermatozoid observed in the lumen of the seminiferous tubules (Fig. 5A, B). Spermatogonial cells in the tubule walls could be distinguished at larger magnifications. Spermatagonia in the basal membrane, spermatozoids in the adluminal compartment, and mature spermatozoids in the nearest part of the lumen were seen in the normal structure (Fig. 5B, C).

In the I/R group sections, the seminiferous tubules revealed irregular boundaries with reduced thickness of the tubule germinal epithelium (Fig. 6A). Excessive blood in the interstitial tissue was remarkable (Fig. 6A, B) and coagulation necrosis was observed in some seminiferous tubules (Fig. 6C, D).

The border irregularity in the seminiferous tubules, and the basal membrane thickness of the tubules was improved in the HP+I/R group (Fig. 7A). Basal membrane improvement was more pronounced when compared with the I/R group. Haemorrhaging in the interstitial tissue was decreased (Fig. 7A, B).

Seminiferous tubule structure was assessed histologically in the NS+I/R group. Thickness tubule basal membranes were near to sham (Fig. 8A). Slight vascular congestion and mild vascular edema were observed between the tubules (Fig. 8B).

## DISCUSSION

Ischemia and reperfusion (I/R) due to testicular torsion and detorsion (T/D) causes testicular injury.[21,22] The standard procedure for ischemic tissues is to provide reperfusion to restore viability. Thus, testicular torsion should be intervered immediately after diagnosis without losing time. Testicular torsion causes tissue hypoxia, which leads to infertility or decreased fertility with germinal cell necrosis.[22] Testicular T/D leads to detoriorated testicular function and consequent atrophy based on the rotation number and duration of torsion of the same side of the testis.[21-23] An experimental study has shown that testicular necrosis results in arterial occlusion for within 2 hours and venous occlusion within 6 hours.[22]

***Figure 5.*** *Cross-sectional views of the sham group. Sections were stained with haematoxylin and eosin (H&E; magnification × 40). Right arrow: Adluminal compartment, Arrowhead: Basal compartment, Sg: Spermatogonia, St: Spermatosit, Sp: Spermatid.*

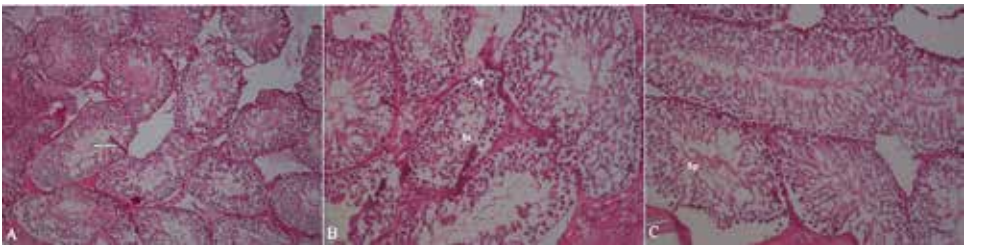


***Figure 6.*** *Cross-sectional views of the I/R group. Sections were stained with haematoxylin and eosin (H&E; magnification × 40). (A) Vascular congestion (blue star), coagulation necrosis in some seminiferous tubules (blue arrows). (B) Vascular congestion (blue star), dense haemorrhage (blue arrow). (C) Dense haemorrhage (blue star), coagulation necrosis in some seminiferous tubules (blue arrows). (D) Vascular congestion (star), dense haemorrhage (blue arrow), coagulation necrosis in seminiferous tubules (black arrows).*

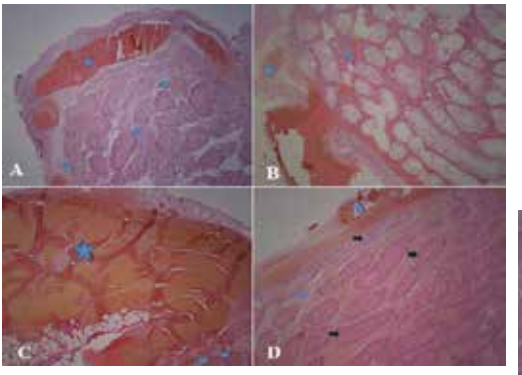


***Figure 7.*** *Cross-sectional views of the HP+I/R group. Sections were stained with haematoxylin and eosin (H&E; magnification × 40). (A) Vascular congestion (stars). (B) Stromal edema (blue arrows), vascular congestion (blue star).*

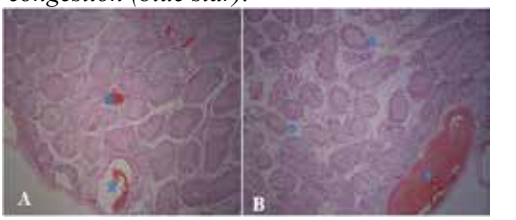


***Figure 8.*** *Cross-sectional views of the NS+I/R group. Sections were stained with haematoxylin and eosin (H&E; magnification × 40). (A) Coagulation necrosis in some seminiferous tubules (blue stars). (B) Slight vascular congestion (blue star), mild vascular edema (blue arrow).*

Testicular loss can occur in cases not treated within the first 12 hours after the onset of the symtoms.[22,24] Studies on ischemic injury resulting from testicular torsion associated with testicular rotation number and torsion found that blood flow was irreversibily stopped and complete ischemia occured from 720° testis torsion. In addition, left testicular torsion is more frequent because the left testis has a longer spermatic cord than the right.[22] Testicular torsion leads to many complications based on the time of formation and the side on which it occurs. Because the only currently avaliable treatment is a surgery, studies are beign conducted to reduce the surgery wait time and duration to prevent further tissue injury. We investigated the effects of HP and NS by studying left testicular torsion after 2 hours of ischemia and 4 hours of detorsion in left testis in rat, which is most smiliar to clinical testicular torsion.

Studies have shown correlation between decreased antioxidants and tissue injury, particulary due to ischemia reperfusion injury as antioxidant eliminate increased free radicals during ischemia and reperfusion.[7,22,25]

Our study evaluated the possible effects of HP and NS, which have antioxidant effects, on testicular ischemia reperfusion injury. MDA easily diffuses from the cell membrane and accumulates in the cytoplasm as lipoxin in the cell. MDA affects the membrane surface state and membrane lipids, enzyme activity and ion transport in ischemia and reperfusion.[22] Studies have reported that MDA increased by ischemia reperfusion in tissue injury.[8,26-28] Süzen[11] and Doğan[22] found that MDA levels in experimental groups were lower than testes of the T/D or I/R groups. These studies show that free radicals are formed in excess amount causing tissue damage in ischemia and reperfusion, and thus, MDA level is an indicater of ischemia reperfusion injury. Smiliar to various studies,[8,11,22,26-28] we found that MDA levels in the I/R group were increased significantly compared to the sham group, and they were decreased in the HP+I/R and NS+I/R groups, which were the therapeutic antioxidant groups.

Cells are protected against harmful superoxide radical effects by the physiological function of SOD, which reduces superoxide radicals to hydrogen peroxide.[22,29] Studies have shown that the SOD level decreases in testicular damage.[22,30] Other studies [22,31,32] found that SOD levels decreased in the testis I/R groups, but increased in the experimental groups and did not return to initial values. Similar to the results of,[22,31,32] we found that the SOD level in the I/R group decreased significantly compared to the sham group, whereas the SOD level increased in the HP and I/R and NS and I/R groups. When comparing the HP and I/R group with the NS and I/R group, the increased SOD level was higher in the NS and I/R group.

We also investigated GSH-Px in our study. GSH-Px is the most effective enzyme in the antioxidant defence system in ischaemia-reperfusion injuries to the testes.[33,34] It destroys intracellular hydroperoxides and converts $H_2O_2$ to water to prevent methaemoglobin formation and protects cell membrane integrity by protecting membrane lipids against peroxide anions.[35] GSH-Px levels in experimental testis torsion and detorsion studies are different. Çay et.al.[32] studied the effects of N-acetylcysteine on experimental testicular torsion and found that GSH-Px activities were increased significantly in all groups compared with the sham surgery group ($p<0.001$) and were significantly higher in the T/D+ N-acetylcysteine group ($P<0.001$) compared with all untreated groups. Öner-İyidoğan et.al.[34] found that GSH-Px activity was higher in the three experimental groups than the controls. Another study[36] reported a statistically significant decrease in GSH-Px activity in the torsion group ($P<0.0001$) and an increase in the T/D+saline group ($P<0.0001$) compared with the sham group. When caffeic acid phenethyl ester (CAPE) was applied, testicular tissues showed significantly increased GSH-Px activity compared to the torsion group ($P<0.0001$) and a significant

decrease compared to the T/D+saline group (P<0.0001). In the same study, no differences in GSH-Px activity were found between the T/D+CAPE and sham groups. Our study also found that the GSH-Px level in the I/R group decreased significantly compared to the sham (control) group and increased in the HP and NS groups. This increase was larger in the NS group.

We also investigated the level of Cat, an intracellular antioxidant enzyme. Cat is found in hepatocellular mitochondria and is located in the peroxisomes of other cells. It neutralizes $H_2O_2$ by converting water and oxygen.[35] Cat enzyme activity in the testis tissue behaved differently in testis torsion.[33] Cat activity was significantly increased by 1.3-2.1 times in all torsion-reperfusion situations reported by Filho et al.[33] Other studies reported that Cat activity decreased after 6 h but not after 2 h of torsion followed by long periods of ischaemia.[37] Cat activity was also reported to decrease in the ipsilateral, but not in the contralateral, testis after torsion-reperfusion.[38] We found that the I/R group had significantly lower Cat levels than the sham group, but the Cat level was increased in the HP and NS groups, with a greater increase in the NS group. Other studies also found that Cat levels fell in the I/R groups, which were similar but for different tissues. These results revealed that testicular injury caused by I/R can benefit from the corrective effects of HP and NS in terms of oxidant and antioxidant balance. By comparing the antioxidant effects of HP and NS, we concluded that NS is more protective than HP on testicular injury due to I/R and is associated with lower MDA levels and higher Cat levels in the NS group.

In our study, we also histopathologically evaluated the testis injury due to I/R. We determined that the seminiferous tubule boundaries were deteriorated and the germinal epithelium thickness decreased in the I/R group compared to the sham group. Blood was also noted in the interstitial tissues from the sham group. Coagulation necrosis was present in some seminiferous tubules. In the HP and I/R group, the seminiferous tubule boundaries were improved over the I/R group, and the basal membrane thickness of the tubules was almost normal. In the I/R group, haemorrhaging in the interstitial tissue was decreased. In the NS and HP groups, the seminiferous tubule structures were considered histologically near normal. The basal membrane thickness of the tubules was also near normal. Slight vascular congestion was seen between the tubules.

In conclusion, HP and NS were shown to have protective effects on testicular torsion injury in our study. In experimentally induced testicular injury, HP and NS reduced the levels of free radicals against this injury, as well as SOD, GSH-Px, Cat, which are caused by increased antioxidants and positively affect the oxidant/antioxidant balance. These results were supported histopathologically, demonstrating that the tissue injury was improved. Antioxidant parameters and histopathological findings suggested that NS was more protective than HP against testicular tissue injury. Based on these results, we believe that Nigella sativa and Hypericum perforatum can be used in addition to surgery to treat testicular torsion.

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