Indian Journal of Experimental Biology
Vol. 61, November 2023, pp. 871-880
DOI: 10.56042/ijeb.v61i09.3638

Protective effect of *Diospyros kaki* L. leaves extract against oxidative damage and inflammation in the testicular ischemia-reperfusion model in rats

Ziya Yurtal*[1], Kadri Kulualp[2], Hüseyin Özkan[3], Tuncer Kutlu[4], Halil Alakuş[1], Abdullah Aslan[5], Muhammed E. Altuğ[1], Mehmet Güvenç[6] & Ufuk Kaya[7]

[1]Department of Surgery; [3]Department of Genetics; [4]Department of Pathology; [6]Department of Physiology; [7]Department of Biostatistics; Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, Tayfur Sökmen Campus 31060, Hatay, Turkey

[2]Department of Surgery, Faculty of Veterinary Medicine, Dokuz Eylul University, İzmir, Turkey

[5]Department of Molecular Biology and Genetics, Faculty of Science, Firat University, Elazig, Turkey

Received 03 December 2022; *revised 26 May 2023*

The Oriental persimmon, *Diospyros kaki* L., native of China, with its bioactive compounds from leaves, flavonoids and polyphenols in particular, possess antibacterial, antiallergic, anti-inflammatory, neuroprotective and antihypertensive properties. Though, the anti-inflammatory and antioxidative effects of the *D. kaki* leaves have beneficial effects in I/R injury in the brain, the same has not been investigated in testicular I/R injury until now. Hence, in the present study, we investigated its leaf extract against oxidative damage and inflammation in the testicular ischemia-reperfusion model. Thirty-two male rats were randomly and equally divided into four groups. While saline (SF) was given to the control and ischemia/reperfusion (I/R) groups for 5 days, *Diospyros kaki* (DK) and I/R+DK were given 200 mg/kg of DK leaf extract. Afterwards, left orchiectomy was performed in control and DK, and testicular torsion was performed in I/R and I/R+DK for 2 hours. All rats were sacrificed 24 hours after procedures and samples were collected. Compared to the control, I/R had high MDA levels, low GSH and GSH-Px levels; compared to the I/R, MDA levels of the I/R+DK were lower, and the GSH and GSH-Px levels were higher. In I/R, the presence of congestion and hemorrhage were determined and the deterioration in seminiferous tubule structure and spermatogenesis were less in the I/R+DK. *TNF-α*, *COX-2*, *BCL-2*, and *BAX* genes expressions were significantly lower in the I/R+DK group. TNF-α and COX-2 proteins were higher in I/R and I/R+DK groups, while NRF2 was higher in I/R+DK. These results show that DK leaves extract has crucial protective effect against oxidative damage and inflammation in the I/R model of rats.

Keywords: Chinese persimmon, Japanese persimmon, Oriental persimmon, Reactive oxygen species (ROS), Testicular torsion

Testicular torsion is a condition that can be observed in all age groups, especially in newborns and adolescents, develops due to the rotation of the spermatic cord structures around its own axis and results in decreased blood flow[1,2]. The ischemic state, which is formed because of decreased blood flow in the testicles, may lead to necrosis in germinal cells and subsequently to infertility[3,4]. If there is no early intervention, permanent loss of function will occur in the testicles, and reperfusion should be performed by detorsion without wasting time[5]. Although blood flow in the testicles returns to normal after reperfusion, non-toxic reactive oxygen species (ROS) such as nitric oxide (NO−), superoxide anions (O2−), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH−) accumulate excessively. Excessive accumulation of ROS causes tissue damage due to protein denaturation and lipid peroxidation in cells and suppresses endogenous antioxidant activities such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)[6-9]. As a result, sperm production and quality deteriorate, the release of various proinflammatory (TNF-α and IL-1β) and inflammatory cytokines with strong chemotactic potential is stimulated and increases in neutrophils and leukocytes occur in endothelial cells[6,10-12]. Structural defects in the seminiferous tubules that develop in this process may cause irreversible apoptosis of germ cells[13,14]. Therefore, inhibition of oxidative stress and inflammation, which play a role in the pathophysiological process, is as important as surgical interventions in reducing testicular ischemia/reperfusion injury[15]. Based on this strategy, the therapeutic effects of many antioxidative and anti-

*Correspondence:
Phone: +90 0505 311 08 42
E-mail: ziyayurtal@mku.edu.tr; ziyayurtal@gmail.com

inflammatory agents in the treatment of testicular ischemia/reperfusion injury have been investigated in recent years[12,15-21].

Diospyros kaki (Persimmon) is a fruit plant originating from China[22]. The leaf of this plant contains many bioactive compounds such as phenols, tannins, flavonoid oligomers, natural acids, ascorbic acid, caffeine and chlorophyll[23,24]. Strong antioxidant of flavonoids such as kaempferol, quercetin and catechin found in the leaf; polyphenols such as vasorelaxan and proanthocyanidin were reported to have antiallergic, anti-inflammatory, antibacterial and antihypertensive properties[25]. Bei *et al.*[26] reported that DK leaf extract showed a neuroprotective effect in the acute I/R model of the forebrain of rats. Similarly, Miao *et al.*[27] reported that this extract increases ischemic tolerance by reducing inflammatory reactions and vascular endothelial damage in the cerebral I/R model, thus preventing I/R injury. The therapeutic effect of DK leaf extract, which is known to have anti-inflammatory and antioxidative effects and has been reported to have beneficial effects in I/R injury in the brain, has not yet been investigated in testicular I/R injury.

In this context, here, we have investigated the potential protective effect of the extract obtained from DK leaves in the testicular I/R model of rats at the molecular and histopathological levels.

Material and Methods

This study was carried out with the approval of Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Decision no:2022/02-05). About 32 adults male Wistar Albino rats weighing 250-300 g were used. Rats had free access to food and water and were housed in clean plastic cages in a temperature and humidity-controlled environment of 20-22°C with a 12 h light/dark cycle. The rats were randomly and equally divided into 4 groups before the study (n=8) and the details of the groups were as follows. (i) Control group: Rats were first given 1 mL of saline by oral gavage for 5 days, and simple scrotal left orchiectomy was performed, and the rats were sacrificed 24 h after this procedure. (ii) DK group: Rats were first given DK at 200 mg/kg for 5 days by oral gavage, simple scrotal left orchiectomy was performed, and the rats were sacrificed 24 h after this procedure. (iii) I/R group: Rats were first given 1 mL of physiological saline by oral gavage for 5 days, testicular torsion-detorsion procedure was performed afterwards, and the subjects were sacrificed after 24 h. (iv) I/R+DK group: Rats were first given 200 mg/kg DK extract by oral gavage for 5 days followed by torsion-detorsion procedure, and were sacrificed 24 h after this procedure.

Preparation of DK leaves extract

The persimmon leaves (*Diospyros kaki* L.) were obtained from the city of Elazig (Turkiye). DK leaves were dried in the laboratory in dry air and then turned into powder using an herb grinder. 800 g of the powder sample was taken, and this sample was extracted in a sterile shaker with 7 L of ethanol for 3 h at room temperature (24°C). After extraction, filtration was done with Whatman 4 filter paper. After repeating this process thrice, the obtained extracts were concentrated by evaporation under reduced pressure in a rotary evaporator at 40°C. Finally, an ethanol extract of 33 g of DK leaves was obtained[28]. This extract was kept in the freezer until used in the experimental stage and its dose (200 mg/kg) was formulated by dissolving in distilled water.

Surgical procedure

All surgical procedures were performed under sterile conditions. A combination of xylazine/ketamine HCL (10/50 mg/kg, i.p.) (Basilazine, Bavet, Turkiye/ Ketasol, Interhas, Turkiye) was used to provide the desired depth of anesthesia. After providing asepsis and antisepsis, the testes of the subjects in the (I/R) and (I/R+DK) groups were removed from the left inguinoscrotal incision. The left testis was rotated 720 degrees clockwise and sutured to the scrotum with 5/0 polyglycolic acid (P.G.A, Çetin Kimya, TURKIYE) suture material and waited for 2 h in this way (torsion position) for ischemia to occur. At the end of the period, the testis was detorsion and the testis was left in its normal anatomical position for 24 h to evaluate the reperfusion injury[29]. At the end of the period, testicles were collected by performing orchiectomy to be used in histopathological and molecular evaluation. Finally, blood was collectedby intracardiac route, and the rats were sacrificed.

Biochemical analyzes

To determine the oxidative damage and antioxidative activity in tissues MDA, GSH, CAT and GPx levels were measured spectrophotometrically. First, the samples collected from testicular tissues were homogenized with 1.15% KCl at a ratio of 1/10. MDA analysis was performed on half of the

homogenate. The other half was centrifuged at 5000 g for 1 h (+4°C) and separated from their supernatants, and then GSH level, GPx and CAT enzyme activity analyzes were performed. Protein analyses in the homogenate and supernatant were determined by the Lowry method and the results were adapted to the protein[30].

Histopathological examinations

Rats were sacrificed under anesthesia. The testicles tissues were fixed in a 10% buffered formalin. The fixed tissues were washed overnight to remove formalin under tap water. The routine tissue process was followed. Then, tissues were embedded in paraffin, sectioned at 5 μm thickness from each block and deparaffinized in xylol, then passed through a series of 100, 96, 80 and 70% alcohol, respectively. After the tissues were stained with Hematoxylin and Eosin (H&E)[31] and evaluated under a light microscope (Olympus CX31) and microphotographed (Olympus DP12). Testicular parenchyma damage, such as hemorrhage, edema, and congestion, was scored as normal (0), mild (1), moderate (2), and severe (3)[32]. Seminiferous tubule structure, spermatogenesis, and sperm cell maturation were calculated using Johnsen's score (MTBS: Mean testicular biopsy score) according to the table below[33,34]. Spermatogenesis was scored between 0-10 according to epithelial maturity (Table 1).

Immunohistochemical analyses

To demonstrate proliferating cell nuclear antigen (PCNA) expression in tissues, the Avidin Biotin Peroxidase Complex (ABC) technique was performed according to the standard procedure prescribed in the commercial kit (SensiTek HRP, ScyTek Laboratories, Logan, UT). Anti-PCNA antibodies (STJ96933) (overnight/4°) (dilution ratio 1/100) were used as primary antibody. 3% $H_2O_2$was used to block endogenous peroxidase activity; Endogenous Avidin/Biotin blocking kit (ab64212) was used for endogenous biotin blocking. Heat was applied (3×5 min) as antigen retrieval; PBS (Phosphate buffered saline) was used on the tissues as a negative control. 3,3'-diaminobenzidine tetrahydrochloride (DAB, ScyTek Laboratories, Logan, UT) was used as chromogen. For background staining, Harris hematoxylin was applied. In immunohistochemical grading, 10 seminiferous tubules were counted in each testis for PCNA positivity. Both stained and unstained germ cells were counted and the ratio of stained cells to the total germ cell number, the "PCNA index" was calculated for each seminiferous tubule[35].

Table 1 —Mean testicular biopsy scores (MTBS)[33,34]

Score	Description
1	No cells
2	Sertoli cells without germ cells
3	Only spermatogonia
4	Only a few spermatocytes
5	Many spermatocytes
6	Only a few early spermatids
7	Many early spermatids without differentiation
8	Few late spermatids
9	Many late spermatids
10	Full spermatogenesis

Molecular analyses

Collected tissues were rapidly placed in liquid nitrogen and stored at −86°C until analysis. Total RNA isolation from samples was performed with a commercial kit according to the modified Trizol method[36]. For this purpose, tissue samples were homogenized under cold conditions in 1 mL of Hybrizol (Hibrigen, Turkiye). After homogenization, the samples, which were kept at room temperature for 10 min, were passed through the stages of chloroform, isopropyl alcohol and ethyl alcohol, respectively[37]. After the obtained RNA pellets were left to dry for about 10 min at room temperature, they were diluted with 20-40 μL of nuclease-free water according to their size. Then, the purity and concentration values of the samples were determined by a nucleic acid meter (SMA-1000 Spectrophotometer, Merinton, CHINA). Integrity control of the samples with appropriate purity and concentration values was evaluated by 1% agarose gel electrophoresis.

cDNA synthesis and RT-qPCR analysis

Following RNA isolation, samples were treated with DNase I enzyme (EN0521, Thermo Scientific, USA) to eliminate possible DNA contamination. Afterwards, cDNA synthesis was performed with the Onescript Plus cDNA synthesis kit (G236, ABM, CANADA) via conventional thermal cycler (BioRad T100, USA) in accordance with the kit protocol. Following the reaction, the final volumes of the samples were made up to 200 μL with nuclease-free water and the samples were stored at −86°C until gene expression analysis. Amplifications of *TNF-α*, *COX-2*, *NRF2*, *BAX* and *BCL-2* target genes and *ACTB* housekeeping gene in the samples were determined by Real Time PCR (Rotorgene Q, Qiagen, USA) via commercial kit containing SYBR Green dye (EnTurbo™ SYBR Green PCR SuperMix, EQ014, ELK Biotechnology, CHINA). The reaction protocol was arranged as 40 cycles at 95°C for 15 s, at 60°C

Table 2 — Forward/Reverse primer sequences used in the study[37-40]

Gene	Forward and Reverse Primer Sequences
ACTB[37]	F: 5′-GCAGGAGTACGATGAGTCCG-3′
	R: 5′-ACGCAGCTCAGTAACAGTCC-3′
TNF-α[38]	F: 5′-GGCATGGATCTCAAAGACAACC-3′
	R: 5′-CAAATCGGCTGACGGTGTG-3′
COX-2[38]	F: 5′-TGTATGCTACCATCTGGCTTCGG-3′
	R: 5′-GTTTGGAACAGTCGCTCGTCATC-3′
NRF2[38]	F: 5′-TTGTAGATGACCATGAGTCGC-3′
	R: 5′-TGTCCTGCTGTATGCTGCTT-3′
BAX[39]	F: 5′-TGGCGATGAACTGGACAACAA-3′
	R: 5′-GGGAGTCTGTATCCACATCAGCA-3′
BCL-2[40]	F: 5′-TGGCCTTCTTTGAGTTCGGT-3′
	R: 5′-GATGCCGGTTCAGGTACTCA-3′

for 60 s, and at 72°C for 30 s after denaturation at 95°C for 10 min. Samples were studiedin duplicate and primer sequences used for amplification of target genes were checked by Primary BLAST (NCBI) (Table 2).

ELISA analysis

The levels of the proteins encoded by the target genes in the relevant tissue were determined by ELISA method. For this purpose, with rat specific ELISA kits, *TNF-α* (E-EL-R2856_ELABSCIENCE, USA), *COX-2* (E-EL-R0792-ELABSCIENCE, USA), *NRF2* (E-EL-R1052-ELABSCIENCE, USA), *BAX* (ER0512-FineTEST, CHINA) and *BCL-2* (ER762-FineTEST, CHINA) proteins were determined. The total protein levels in the samples were determined with the BCA Assay Kit (23227, Thermo Fisher, USA) and the target protein levels were determined with the total protein levels.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics Software Version 23.0. The sample size was calculated with G*Power software (Version 3.1.9.2). Result of the sample size calculation showed that the minimum number of Wistar Albino rat was 32, considering an effect size of 0.65, an alpha value of 0.05, and a power of 0.80. The variables were examined as parametric test assumptions. To test the differences in biochemical parameters and protein levels between groups, one-way analysis of variance (ANOVA) was used. Tukey's test was used as a post-hoc test for the variables that were found statistically significant. Among the histopathological and immunohistochemical parameters, congestion, hemorrhage, edema and PCNA were evaluated with the Kruskal-Wallis test and MTBS was evaluated with ANOVA. When significant differences were observed, multiple Dunn test were used as post hoc procedures for congestion, hemorrhage, edema and PCNA parameters. Moreover, Tukey's test was used as a post hoc test for MTBS parameter. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The results were determined by comparing to "Control group" and presented as fold changes[41]. In addition, gene expression levels were presented as a figure. Results of parametric and non-parametric variables were calculated as "Mean ± Standard Error of Mean" and "Median (Min-Max)", respectively. Differences with P <0.05 were considered statistically significant.

Table 3 — Mean ± SE Values of malondialdehyde (MDA), glutathione (GSH) levels and glutathione peroxidase (GSH.Px) and catalase (CAT) in testis

Groups	MDA (nmol/mL)	GSH (nmol/mL)	GPx (IU/gr prot)	CAT (kU/L)
Control	13.821±0.50^{c}	4.144±0.35^{a}	68.359±3.13^{ab}	24.847±0.75^{ab}
DK	11.250±0.45^{c}	3.588±0.09^{ab}	71.618±2.79^{a}	28.252±1.57^{a}
I/R	25.121±1.29^{a}	2.694±0.23^{b}	49.559±2.42^{c}	21.394±1.13^{b}
I/R+DK	18.656±0.99^{b}	4.090±0.32^{a}	60.368±1.52^{b}	25.393±1.19^{ab}
P Value	<0.001	0.003	<0.001	0.006

[Different letters in the same column represent statistical significance (P <0.05)]

Results

Biochemical evaluations

Results related to oxidative damage and antioxidant activity parameters are given in Table 3. According to this evaluation, while MDA levels of the I/R group increased significantly (P <0.001) compared to the control group, GSH (P=0.003) and GPx levels decreased significantly (P <0.001). Although the CAT activity of the I/R group decreased compared to the control group, this decrease was not statistically significant. The MDA levels of the I/R+DK group were significantly decreased (P <0.001), while the GSH (P =0.003) and GPx levels (P <0.001) were significantly increased compared to the I/R group. Although the CAT activity of the I/R+DK group increased compared to the I/R group, this increase was not significant. As a result, the decrease in MDA, which is one of the important markers of oxidative stress, and the increase in the levels of GSH, GPx and CAT, which are indicators of antioxidant activity, in the I/R+DK group indicate that DK has higher antioxidant activity in testicular ischemia.

Histopathological and immunohistochemical evaluations

Histopathological and immunohistochemical changes in the testicular I/R model and the effect of DK treatment on these changes is presented in Table 4. In the I/R group, congestion, hemorrhage and edema were observed in the testicular stromal region

Table 4 — Congestion, hemorrhage, edema, MTBS and PCNA scores (Mean ± SE (Median; Min-Max)

Groups	Congestion	Hemorrhage	Edema	MTBS	PCNA
Control	0.13±0.13^{b}(0; 0-1)	0.00±0.00^{b}(0; 0-0)	0.00±0.00^{b}(0; 0-0)	8.37±0.50^{a}(8,50; 6-10)	1.86±0.34(2; 1-3)
DK	0.00±0.00^{b}(0; 0-0)	0.00±0.00^{b}(0; 0-0)	0.00±0.00^{b}(0; 0-0)	8.38±0.42^{a}(8,50; 6-10)	1.71±0.29(2; 1-3)
I/R	1.00±0.19^{a}(1; 0-2)	1.13±0.23^{a}(1; 0-2)	0.63±0.32^{ab}(0; 0-2)	4.38±0.46^{c}(4,50; 3-6)	1.07±0.17(1; 0,50-2)
I/R+DK	0.75±0.25^{ab}(1; 0-2)	0.25±0.16^{b}(0; 0-1)	1.38±0.26^{a}(1,50; 0-2)	6.00±0.57^{b}(6; 4-8)	1.29±0.18(1; 1-2)
P Value	0.001*	<0.001*	0.001*	<0.001$^{\#}$	0.143*

[*: Kruskal-Wallis test; $^{\#}$: One-Way ANOVA. Different letters in the same column represent statistical significance (P <0.05)]

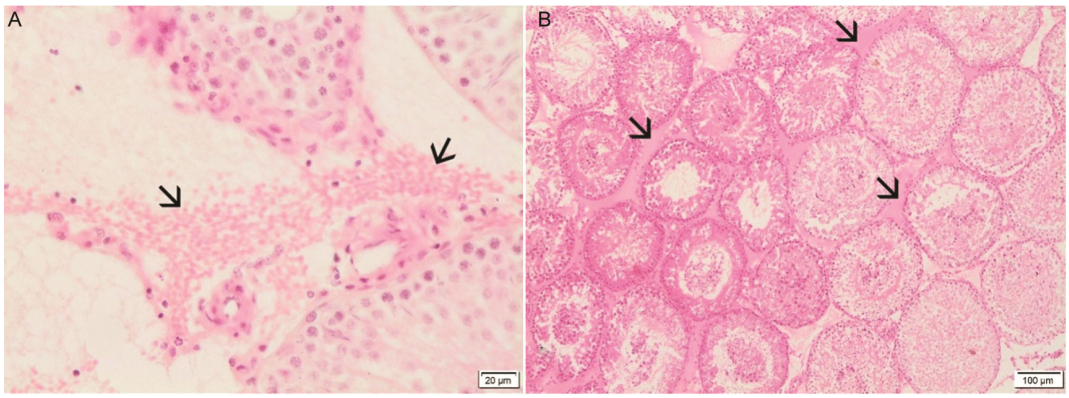


Fig. 1 — Histopathological findings observed in testicles (A) Hemorrhage in the stromal region (arrows), I/R group, HE; and (B) Stromal edema (arrows), I/R+DK group, HE.

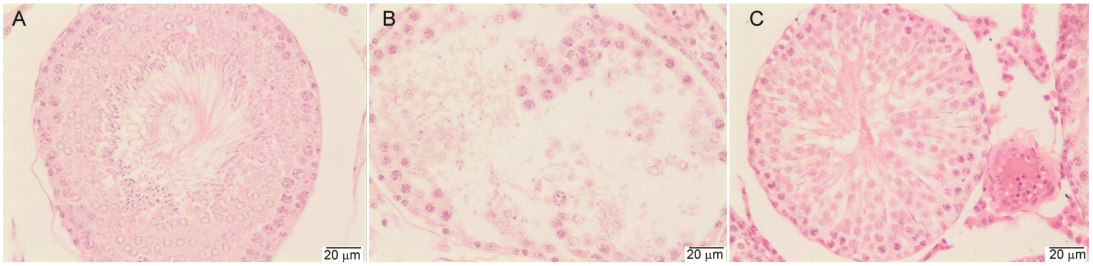


Fig. 2 — Tubular damage and spermatogenesis in testicles (A) Seminiferous tubule in normal histological structure, control group, HE; (B) Severe damage to the seminiferous tubule, group I/R, HE; and (C) Seminiferous tubule is close to normal histological structure, I/R+DK group, HE.

(Fig. 1A). Accordingly, testis and seminiferous tubules were normal histologically in the control and DK groups (Fig. 2A). According to Johnsen's scoring method, it was determined that the seminiferous tubule structure and spermatogenesis of this group was negatively affected (Fig. 2B). While edema was also observed in the I/R+DK group; it was determined that both congestion and hemorrhage in the testicles decreased significantly. Seminiferous tubule structure and spermatogenesis was improved in the I/R+DK group (Fig. 1 B and C). In immunohistochemical examination, no significant difference was observed between the groups in terms of PCNA positivity (Fig. 3 A-C).

Molecular findings

Gene expression results

It was determined that the expression levels of target genes *TNF-α*, *COX-2* and *BCL-2* were significantly increased in the I/R group compared to the control group. *TNF-α*, *COX-2*, *NRF2* expression levels were found to be close to the control group in the I/R+DK group, while the expression levels of *BAX* and *BCL-2* genes were significantly decreased (Fig. 4).

ELISA analysis

According to the results obtained, it was determined that TNF-α protein levels increased approximately 3 folds in the I/R group, as in the gene expression results, however, they were at similar levels to the control in the DK group. In terms of COX-2 protein levels, sham and IR+DK groups were found to be significantly higher than control and DK groups. NRF2, whose gene expression levels were similar between the groups, was found to be significantly higher in the DK group in terms of protein levels (Table 5).

Table 5 — Protein levels of TNF-α, COX-2, NRF2, BAX and BCL-2 in Testis (Mean ± SE)

Groups	TNF-α (pg/mg protein)	COX-2(pg/mg protein)	NRF2(pg/mg protein)	BAX(ng/mg protein)	BCL-2(ng/mg protein)
Control	8,96±1,53[b]	79,40±7,57[b]	9,60±0,92[b]	0,79±0,08	2,24±0,28
I/R	24,33±4,51[a]	553,95±92,88[a]	14,36±3,15[ab]	0,98±0,24	2,91±0,90
DK	7,31±1,69[b]	83,10±18,53[b]	9,55±1,29[b]	0,79±0,06	1,92±0,18
I/R+DK	17,24±2,78[ab]	346,45±52,72[a]	22,70±6,35[a]	0,98±0,24	2,71±0,74
P Value	0,003	0,001	0,026	0,978	0,876

[Different letters on the same line indicate significance]

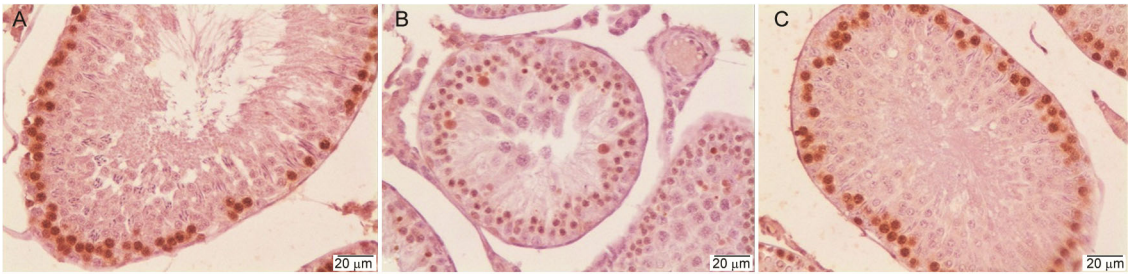


Fig. 3 — PCNA immunohistochemical findings in testicles (A) PCNA positivity in seminiferous tubule, control group; (B) Slight PCNA positivity in the seminiferous tubule, I/R group; and (C) PCNA positivity like the control group in the seminiferous tubule, I/R+DK group.

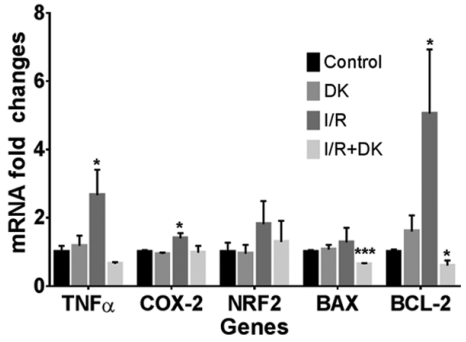


Fig. 4 — Gene expression findings. [*: P <0.05; ***: P <0.001]

Discussion

Testicular torsion, which is defined as rotation of the spermatic cord, causes ischemia by obstructing testicular vessels and impairing tissue perfusion[12]. This may lead to apoptosis of germ cells, testicular atrophy, disruption of spermatogenesis and eventually infertility[42]. The severity of ischemic injury varies according to the degree and duration of rotation[12]. Although reperfusion of the cellular structures exposed to ischemia normalizes the impaired blood flow, the production of ROS after reperfusion and their excessive accumulation cause protein denaturation, lipid peroxidation and cell genome damage in the cells, and oxidative stress develops[12,42]. The interaction of endothelial cells with inflammatory cells also plays an important role in the excessive accumulation of ROS. Developing neutrophil activation and proinflammatory cytokines such as TNF-α, IL-1β and IL-6 released from these cells increase the existing oxidative stress. Germ cell apoptosis continues due to excessive ROS accumulation during the detorsion process, in which oxidative stress and inflammation play a key role, as after torsion[42]. In many experimental studies, it has been reported that the testicular I/R model causes an increase in the level of MDA, one of the end products of lipid peroxidation, and a decrease in the levels of endogenous antioxidant enzymes such as CAT, GSH and GSH-Px[6-9,12]. In the current study, when compared to the control group, the MDA levels of the I/R group, which underwent 4 h of torsion and 24 h of detorsion, were significantly higher, and GSH and GSH-Px levels were low. No significant difference in CAT levels was noted. In model studies investigating the therapeutic effect of DK leaves extract, this extract reduces the levels of MDA, which is one of the oxidative stress markers[22,43], GSH[43], GSH-PX[22] and it has been reported to increase CAT[22,43] levels, and based on these data, it was stated that DK is an effective antioxidant[22,25,43]. In the current study, MDA levels, which increased in the I/R group compared to healthy subjects, were significantly lower in the DK leaves extract group. Compared to the control group, GSH, GSH-PX and CAT levels, which were found to be low in the I/R group, were measured at high levels in the I/R+DK group. These effects of DK on oxidative stress markers confirm that this agent has an antioxidative property, as previously revealed by other model studies[22,25,43].

TNF-α and other cytokines play a role not only in the proinflammatory process, but also in testicular pathologies such as immunoregulatory, apoptosis and I/R[44]. These and other cytokines form the basis for the normal homeostasis of the testicles and may cause dysfunction in the testicles if they are overproduced[45]. In the I/R model studies, it was noted that the TNF-α level of disease groups was measured high due to torsion/detorsion[44,46]. Minutoli *et al.*[46] also stated that the TNF-α level increases as the reperfusion time increases. In addition, it has been reported that prostaglandins as well as proinflammatory cytokines play crucial roles in I/R related inflammation and isoenzymes such as COX-1 and COX-2 are responsible for their synthesis. Some researchers have indicated that especially COX-2 plays a mediating role in the development of I/R injury[44]. As a matter of fact, in the studies conducted by these researchers, it is seen that COX-2 was measured high in I/R groups[44]. In the current study, TNF-α and COX-2 levels were found to be higher in I/R group cases compared to the control group, according to both ELISA and RT-qPCR analyses. Kim *et al.*[47] reported that DK leaves extract, which they used in the treatment of allergic inflammation, and Miao *et al.*[27] in the cerebral I/R model, reduced proinflammatory cytokine levels and inflammatory reactions, respectively. Sun *et al.*[48] reported that DK significantly reduced *COX-2* expression. In the RT-qPCR analyzes of our study, both *TNF-α* and *COX-2* levels of the I/R group were significantly upregulated compared to the control group in line with the results obtained by many researchers[44,46]. In addition, while the gene expression levels of *NRF2*, an important factor involved in the antioxidant response, were similar between the groups, it was determined that the NRF2 protein encoded by this gene was relatively high in the IR+DK group[49]. However, it was determined that the gene expression levels of *BAX* and *BCL-2*, which are involved in the apoptosis pathway, were significantly decreased in the I/R+DK group compared to the control group, and the protein levels encoded by these genes were similar between the groups. Although no significant difference was found in protein levels, DK extract is thought to suppress the apoptosis pathway. Because it caused significant changes in the expression levels of *BAX* and *BCL-2* genes, which have a key role in the apoptosis pathway. It is thought that the difference in gene expression levels and protein levels may be regulated by some non-coding RNAs such as miRNAs with post-transcriptional regulation[50].

Spermatogenesis is one of the most important parameters affected by testicular torsion. Atrophy of the seminiferous tubules and decreases in the number of spermatogenic cells indicate impaired spermatogenesis[42]. MTBS is the most frequently cited scoring system in this field, which uses morphometric and cellular parameters to determine whether there is a degenerative condition in the germinal epithelium of testicular specimens, and thus evaluates spermatogenesis[51]. According to this method, in which Johnsen scoring[33] is used, the lower the scores, the higher the degeneration of spermatozoa, spermatids, spermatocyte and Sertoli cells, respectively. As a matter of fact, Davoodi *et al.*[52] determined that the MTBS of cases in the torsion/detorsion group in I/R models was lower than that of healthy subjects. Similarly, there are many studies reporting that MTBS is low in I/R cases, that is, spermatogenesis is impaired[42,45]. In our study, MTBS averages were found to be significantly lower in the I/R group subjects compared to the control group, and higher in the I/R+DK group compared to the I/R group. These results show that the I/R model causes degeneration in the germinal epithelium of the seminiferous tubules and that DK has beneficial effects on the recovery of this degeneration and impaired spermatogenesis. It has been reported that parameters such as edema, congestion and hemorrhage, which are included in the histopathological procedure and reveal the state of the testicular parenchyma, are important in the evaluation of the I/R process[12] and that these symptoms increase due to torsion and detorsion[53]. In the current study, it is seen that hemorrhage and congestion are prominent in I/R cases, and symptoms that develop after I/R model, especially hemorrhage, are observed less frequently in the I/R+DK group. These data obtained from histopathological evaluations show that I/R causes a degeneration in testicular parenchyma and germinal epithelium, and DK treatment has beneficial effects on all these parameters.

The efficiency of spermatogenesis also depends on the loss of germinal cells in spermiocytogenesis and meiosis, and on the proliferative activity of spermatogonia[42]. PCNA is an antigen used for imaging the cellular proliferation phase and detecting germinal cell loss[54,55]. DK is known to inhibit

apoptosis[56,57]. The absence or low levels of PCNA indicate that cells have entered apoptosis[54,55]. The high level of PCNA is evidence that it shows anti-apoptotic activity[58]. Aktas *et al.*[42] noted in their study that the PCNA-positive cell count of cases with I/R was significantly lower than that of healthy subjects, and that spermatogenesis and proliferative activity decreased in relation to these data. Demir and Altındağ[59] stated in their study that the number of PCNA positive cells is directly related to spermatogenesis. In another study, the number of PCNA positive cells was found to be high in the early spermatocyte and spermatogonia of testes of healthy subjects, and low in the seminiferous tubules of cases with I/R[54]. Although the mean number of PCNA positive cells obtained in our study was not statistically significant, it was found to be lower in the I/R group compared to the control group and higher in the I/R+DK group compared to the I/R group. These results show that our I/R model created by applying 4 h torsion/24 h detorsion causes germinal cell loss and reduces the proliferative activity of spermatogonia, while DK treatment has a protective effect in terms of the specified parameters.

Conclusion

In this study we investigated the protective effect of the extract obtained from the Persimmon, *Diospyros kaki* (DK) leaves on the oxidative damage and anti-inflammatory response developed in the experimental I/R model created by applying testicular torsion/detorsion procedure in rats, by molecular, biochemical, histopathological and immuno-histochemical analyses. The results have demonstrated the protective effects on testicular parenchyma and germinal epithelium by regulating mainly oxidative and inflammatory pathways. Thus, it indicates that the DK leaves have curative effect on spermatogenesis by preventing germ cell loss in I/R state.

Funding

This study was supported by the Scientific Research Projects Coordination Unit of Hatay Mustafa Kemal University (Project No. 21.GAP.053).

Conflict of interest

Authors declare no competing interests.

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