

D-Carvone Attenuates Testicular Ischemia-Reperfusion Injury in Rats Through Anti-Inflammatory, Antioxidant, and Antiapoptotic Mechanisms

D-Karvon, Sıçanlarda Testiküler İskemi-Reperfüzyon Hasarını Anti-İnflamatuvar, Antioksidan ve Antiapoptotik Mekanizmalar Aracılığıyla Hafifletir

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ABSTRACT

Background: Testicular torsion is a urological emergency that can cause infertility and irreversible damage resulting from testicular ischemia-reperfusion (T/IR). The aim of this study was to investigate how D-carvone protects against T/IR injury.

Materials and Methods: Wistar-Albino male rats were randomly divided into SHAM, T/IR, and T/IR + D-carvone groups (8 animals in each group). The T/IR + D-carvone group received 20 mg/kg D-carvone for 15 days, with the last dose administered 15 minutes before reperfusion. T/IR was applied as 2 hours of ischemia followed by 2 hours of reperfusion.

Results: D-carvone markedly reduced oxidative stress indicators including malondialdehyde, myeloperoxidase, total oxidant status, oxidative stress index, ischemia-modified albumin, and neutrophil gelatinase-associated lipocalin, as well as proinflammatory cytokines such as tumor necrosis factor-alpha, interleukin-1 (IL-1) beta, and IL-6 compared with the T/IR group, while enhancing antioxidants including total antioxidant status, glutathione, and catalase, as well as the anti-inflammatory cytokine IL-10. Histopathological analysis revealed the preservation of seminiferous tubule architecture and an improvement in Johnsen scores in the D-carvone-treated group. Immunohistochemical analysis showed that D-carvone treatment decreased the expression of nuclear factor kappa B, Caspase-3, and 8-hydroxy-2'-deoxyguanosine.

Conclusion: D-carvone exerts significant protective effects against T/IR injury by modulating inflammation, oxidative stress, and apoptosis. The findings suggest that D-carvone might protect against testicular damage; however, additional research is necessary to assess its effects on infertility.

Keywords: Testicular torsion, ischemia-reperfusion injury, D-carvone, oxidative stress, apoptosis, inflammation

ÖZ

Amaç: Testis torsiyonu, infertiliteye ve testis iskemi-reperfüzyonu (T/IR) nedeniyle geri dönüşümsüz hasara yol açabilen ürolojik bir acil durumdur. Bu çalışmanın amacı, D-karvonun T/IR hasarına karşı nasıl koruma sağladığını araştırmaktır.

Gereç ve Yöntemler: Wistar-Albino erkek sıçanlar rastgele SHAM, T/IR ve T/IR + D-karvon gruplarına ayrıldı, her grupta 8 hayvan vardı; tedavi grubuna 15 gün boyunca 20 mg/kg D-karvon verildi, son doz reperfüzyondan 15 dakika önceydi. Testis iskemi-reperfüzyonu 2 saat iskemi ve 2 saat reperfüzyon şeklinde uygulandı.

Bulgular: D-karvon, T/IR grubuna kıyasla oksidatif stres göstergelerini (malondialdehit, miyeloperoksidaz, total oksidan statü, oksidatif stres indeksi, iskemi modifiye albümin ve nötrofil jelatinaz ilişkili lipokalın) ve proinflatuar sitokinleri [tümör nekroz



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faktörü-alfa, interlökin-1 beta (IL-1 β), IL-6] önemli ölçüde azaltırken, antioksidanları [total antioksidan statü, glutatyon, katalaz] ve antiinflamatuvar sitokin IL-10'u artırdı. Histopatolojik analiz, D-karvon ile tedavi edilen grupta seminifer tübül mimarisinin korunduğunu ve Johnsen skorlarında iyileşme olduğunu ortaya koydu. İmmünohistokimyasal analiz, D-karvon tedavisinin nükleer faktör kapp B, Kaspaz-3 ve 8-hidroksi-2'-deoksiguanosin ekspresyonunu azalttığını gösterdi.

Sonuç: D-karvonun, inflamasyon, oksidatif stres ve apoptozu düzenleyerek T/IR hasarına karşı önemli koruyucu etkileri vardır. Bu bulgular, D-karvonun testiküler hasara karşı potansiyel bir koruyucu ajan olabileceğini düşündürmektedir; infertilite üzerindeki etkileri için ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Testis torsiyonu, iskemi-reperfüzyon hasarı, D-karvon, oksidatif stres, apoptoz, inflamasyon

Introduction

Twisting of the spermatic cord around its axis is a sign of testicular torsion, a urological emergency. There are basically two kinds of torsion: intravaginal torsion, which happens inside the tunica vaginalis, and extravaginal torsion, which affects the tunica vaginalis itself (1).

Extravaginal torsion typically manifests perinatally as the testicle descends into the scrotum, but intravaginal torsion is more common in teenage males, especially those with the bell-clapper deformity. Males of all ages may be affected, although newborns, children, and adolescents are more likely to experience the condition. The estimated yearly incidence of this condition is 4.5 per 100,000 males, while regional variations may occur (2,3). Orchiectomy is required in 42% of patients with testicular torsion, which accounts for 10–15% of acute scrotal diseases. Acute unilateral scrotal discomfort, nausea, and vomiting are the hallmarks of this urological emergency. On physical examination, the cremasteric reflex is usually absent. Ischemia-reperfusion (IR) injury may occur in testicular tissue if it is not identified promptly and treated with an appropriate surgical procedure. This could result in decreased fertility or require an orchiectomy (4).

Testicular torsion results in two-phase damage: ischemia due to interrupted blood supply, followed by reperfusion after surgical detorsion. Reduced oxygen delivery during ischemia causes hypoxia, adenosine triphosphate depletion, acidosis, and calcium overload, all of which initiate damage to testicular cells. Additionally, hypoxia increases oxidative stress and inflammatory mediators. Reperfusion paradoxically exacerbates damage due to increased formation of reactive oxygen species (ROS), infiltration of inflammatory cells (particularly neutrophils and CD4 T cells), release of cytokines, and further calcium accumulation. Together, these occurrences may harm germ cells, impede mitochondrial activity, and result in infertility or testicular loss. Lipid peroxidation, DNA damage, edema, and inflammatory cascades are characteristics of reperfusion injury that worsen testicular damage caused by ischemia (5-8).

In general, the length of ischemia and the extent of the resulting IR injury determine the prognosis of testicular torsion. Many studies have indicated that prompt diagnosis and timely surgical treatment can help minimize testicular damage and its most serious consequences, such as infertility. To avoid treatment-related problems and diagnostic delays, more sophisticated therapeutic research is required.

Dietary seeds, especially cumin seeds, contain a substance called D-carvone in their essential oil, which has antitumoral, antiproliferative, and antihypertensive properties. Additionally, D-carvone's antioxidant, anti-inflammatory, and antiapoptotic qualities have been demonstrated to diminish infarct size and show protective effects against cerebral IR damage in a rat model of the injury (9,10). The main component of *Carum carvi* essential oil is D-carvone, a monoterpene with a distinct caraway-like aroma; D-carvone is also present in the seed oil of *Anethum graveolens* (dill) (11). Its pharmacological advantages have been validated by numerous research (12-14). The development of pre-neoplastic lesions, oxidative stress, and the activity of biotransformation enzymes in colon carcinogenesis have all been shown to be modulated by D-carvone (15). Additionally, D-carvone has been shown to reduce insulin resistance, hepatic steatosis, and obesity in animal models. Besides, it has been demonstrated to lower hyperglycemia in diabetic rats by modulating the activity of essential enzymes involved in carbohydrate metabolism (16).

The present investigation aims to examine the protective effects of D-carvone, a potential agent against ischemia-reperfusion injury caused by testicular torsion, a condition associated with impaired reproductive function and representing a significant public health concern, by evaluating its effects in a testicular ischemia-reperfusion (T/IR) model using enzyme-linked immunosorbent assay and histopathological techniques.

Several phytochemicals have been shown to confer protective effects in animal models by blocking the pathophysiological pathways associated with testicular torsion. This study examined the preventive effects of

D-carvone on the complex pathophysiology linked to the reperfusion process, which has not been previously examined in the literature (16).

Materials and Methods

Ethics Committee Approval and Animals

The Atatürk University Experimental Animal Ethics Committee approved the experimental procedure (approval no: 2022/11-260, dated 08.11.2022). The study involved 24 male Wistar-Albino rats, aged between 12 and 16 weeks and weighing 200–250 grams. The Atatürk University Experimental Animal Research and Application Center conducted all experiments. Animals were kept in controlled environments with a 12-hour light/dark cycle for the duration of the study. The night before the IR procedures, tap water was provided for hydration, while food was withheld from the rats. Because this study did not involve human subjects, patient consent was not required.

Experimental Procedure, Drugs, and Groups

Thermo Scientific Chemicals provided D-carvone (D(+)-Carvone, 96 natural) (CAS #2244-16-8). D-carvone was given intraperitoneally at a dose of 20 mg/kg, as was the case in previous studies (3,17). D-carvone is liquid at room temperature and is dissolved in 1% dimethyl sulfoxide to obtain the appropriate dose prior to administration.

The investigation utilized twenty-four rats, which were randomly assigned to three groups of eight rats each. Prior to surgery, the weight of each rat was recorded, and intraperitoneal doses of xylazine (8 mg/kg) and ketamine (75 mg/kg) were used to produce anesthesia (18,19).

Group I (SHAM): A 1–2 cm incision was made to access the peritoneum, and the site was closed with sutures. Four hours later, the rats were subjected to general anesthesia, their testicles were removed, and they were sacrificed.

Group II (T/IR): A 1–2 cm incision was made in the lower abdomen. Both testicles were subjected to 2 hours of ischemia using atraumatic bulldog clamps; after removing the clamps, reperfusion was applied for 2 hours. After reperfusion was completed, blood samples were collected by cardiac puncture, and the animals were sacrificed to harvest testicular tissues.

Group III (T/IR + 20 mg/kg D-carvone): Rats received D-carvone (20 mg/kg) intraperitoneally for 15 consecutive days. On day 16, a 1–2-cm lower abdominal incision was made to expose the peritoneum, and both testes were subjected to ischemia for 2 hours using bulldog clamps. The final dose of D-carvone was administered intraperitoneally 15 minutes before the reperfusion period. A 2-hour reperfusion period followed. Following reperfusion, both

testes were removed and blood samples were collected by cardiac puncture.

Blood was collected from the hearts of each set of rats while they were sedated prior to euthanasia.

Statistical Analysis

IBM SPSS Statistics 20 (USA) for Windows was used to conduct the statistical analyses. The Tukey Honestly Significant Difference test was employed for multiple group comparisons following a one-way analysis of variance. The mean \pm standard deviation was applied to all results. The statistical analysis of the pathological results was performed using California-based GraphPad Prism software, version 7.0. The Kruskal-Wallis and Mann-Whitney U tests were used to assess differences in non-parametric data, and a p-value of less than 0.05 was regarded as statistically significant.

Tissue Homogenization and Biochemical Analysis

Testicular tissues were combined with phosphate buffer to create a 10% homogenate, which was then centrifuged at 12,000 rpm on ice for one to two minutes for biochemical tests (IKA, Germany). Homogenized tissue samples were centrifuged for 30 minutes at +4°C and 5000 rpm to extract the supernatant. The levels of interleukin-1 beta (IL-1 β) (Cat No: E-EL-R0012, Elabscience), IL-6 (Cat No: E-EL-R0015, Elabscience), IL-10 (Cat No: E-EL-R0016, Elabscience), tumor necrosis factor-alpha (TNF- α) (Cat No: E-EL-R0019, Elabscience), ischemia-modified albumin (IMA) (Cat No: EA0053Ra, BT LAB), and neutrophil gelatinase-associated lipocalin (NGAL) (Cat No: E0762Ra, BT LAB) were determined in the supernatants during the biochemical evaluation of the groups. The kits' instructions were followed when taking the measurements. IMA concentrations were expressed as U/mg protein, whereas testis tissue levels of IL-1 β , IL-6, TNF α , IL-10, and NGAL were expressed as pg/mg protein. The resultant supernatants were also used for further analyses. All chemicals and enzymes used in the measurement of catalase (CAT), malondialdehyde (MDA), GSH, and myeloperoxidase (MPO) were obtained from Sigma (St. Louis, USA). The reagents were of analytical grade.

Oxidative Stress and Antioxidant Parameters

The method described by Aebi (20) was used to measure CAT activity. The results are expressed in terms of protein kg⁻¹. Each sample underwent two examinations. MDA levels, a marker of lipid peroxidation, were measured using the Ohkawa et al. (21) method. The data are displayed as μ mol/g of protein. Glutathione (GSH) levels were measured using the Ellman method, and the results are displayed as μ mol/mg protein. MPO activity was measured in U/mg protein

using the Hillegas et al. (22) approach. Using appropriate kits, total antioxidant status (TAS) and total oxidant status (TOS) levels were determined and reported in mmol/L and $\mu\text{mol/L}$, respectively. The TOS/TAS ratio was used to calculate the oxidative stress index (OSI).

Protein Determination

Tissue protein was determined using spectrophotometry in accordance with Lowry et al. (23). Every sample was tested twice.

Histopathology

Testicular tissues were fixed in 10% neutral buffered formalin for 24 hours. The standard approach was employed to process each tissue. Samples were stained with hematoxylin and eosin (H&E), cut into 5 μm slices, and examined under a light microscope (Leica DM750, Flexicam i5). Tissue samples were checked for necrotic changes, cell death, and swelling. The mean testicular biopsy score was calculated using the Johnsen score which gave a number between 1 and 10 to each tubule (Table 4).

Immunohistochemistry

Oxidative DNA damage, inflammation, and apoptosis were evaluated using immunohistochemistry. The primary antibodies employed were anti-nuclear factor kappa B (NF- κB) (Cat. No. sc-8414, Dilution: 1/100, Santa Cruz Biotechnology), anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) (Cat. No. sc-66036, Dilution: 1/100, Santa Cruz Biotechnology), and anti-Caspase-3 (Cat. No. sc-56053, Dilution: 1/100, Santa Cruz Biotechnology). The sections were treated with the appropriate secondary antibody (Labeled streptavidin-biotin immunoenzymatic antigen detection system, TP-125-HL, Thermo) after an overnight incubation at 4 °C with the primary antibody. The immunological reaction was visualized using hematoxylin counterstaining and 3,3'-diaminobenzidine-tetrahydrochloride. Positive cells were examined using ImageJ®.

Results

Biochemical Results

Impacts on Inflammation

Pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 were considerably lower in the D-carvone-treated group than in the T/IR group. Relative to the control group, these cytokine levels were considerably higher in the T/IR group. Conversely, levels of the anti-inflammatory cytokine IL-10 were much lower in the T/IR group than in the control group and noticeably higher in the D-carvone-treated group than in the T/IR group (Table 1, Figure 1).

Impact on Oxidant-Antioxidant System

Oxidative stress markers (MDA, MPO, TOS, OSI, and IMA) were noticeably higher in the T/IR group than in the control group. However, the administration of D-carvone markedly reduced the levels of these markers, bringing them close

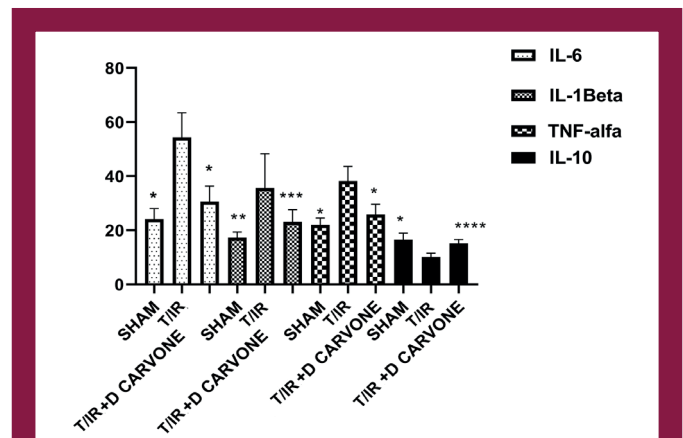


Figure 1. Pro- and anti-inflammatory cytokine levels between groups. * $p < 0.001$ comparison with T/IR group; ** $p = 0.003$ comparison with T/IR group; *** $p = 0.038$ comparison with T/IR group; **** $p = 0.001$ comparison with T/IR group. IL, interleukin; T/IR, testicular ischemia/reperfusion; TNF, tumor necrosis factor.

Table 1. Comparison of pro- and anti-inflammatory cytokine levels between groups.

	IL-6	IL-1 β	TNF- α	IL-10
SHAM (Group I)	24.13 \pm 3.90	17.32 \pm 2.06	22.02 \pm 2.52	16.58 \pm 2.42
T/IR(Group II)	54.38 \pm 9.06	35.62 \pm 12.70	38.25 \pm 5.38	10.17 \pm 1.39
T/IR+D CARVONE (Group III)	30.67 \pm 5.72	23.19 \pm 4.42	25.85 \pm 3.75	15.17 \pm 1.49
I vs. II	$p < 0.001$	$p = 0.003$	$p < 0.001$	$p < 0.001$
I vs. III	$p = 0.231$	$p = 0.421$	$p = 0.263$	$p = 0.402$
II vs. III	$P < 0.001$	$p = 0.038$	$p < 0.001$	$p = 0.001$

Values are presented as mean \pm standard deviation. IL, interleukin; TNF, tumor necrosis factor-alpha.

to control values (Table 2, Figure 2). Conversely, antioxidant parameters such as TAS, GSH, and CAT were significantly diminished in the T/IR group relative to controls; treatment with D-carvone significantly increased these levels, restoring them to values comparable to those in the control group. Notably, IMA levels, markedly elevated in the T/IR group, were considerably lower in the D-carvone-treated group (Table 2, Figure 2).

Effect on NGAL

Compared with the control group, the T/IR group had higher NGAL levels. However, NGAL levels were comparable to those in the healthy control group and were much lower in the D-carvone-treated group (Table 3, Figure 3).

Histopathology Results

Histopathological examination (Figure 4a) of testicular tissue sections from the SHAM group showed intact seminiferous tubules with the typical histologic architecture of the germinal epithelium. Conversely, the T/IR group exhibited significant disruption of the seminiferous tubules, resulting in degeneration and exfoliation of the tubular epithelial cell lining. Germ cells were likewise severely vacuolated. Furthermore, as seen in Figure 4b, tissue sections of this group exhibited edema and congestion. As shown in Figure 4c, D-carvone therapy markedly reduced these histological changes.

Immunohistochemistry Results

Immunohistochemical staining revealed that NF-κB, Caspase-3, and 8-OHdG expression levels were considerably elevated in the T/IR group ($p < 0.0001$) compared with the

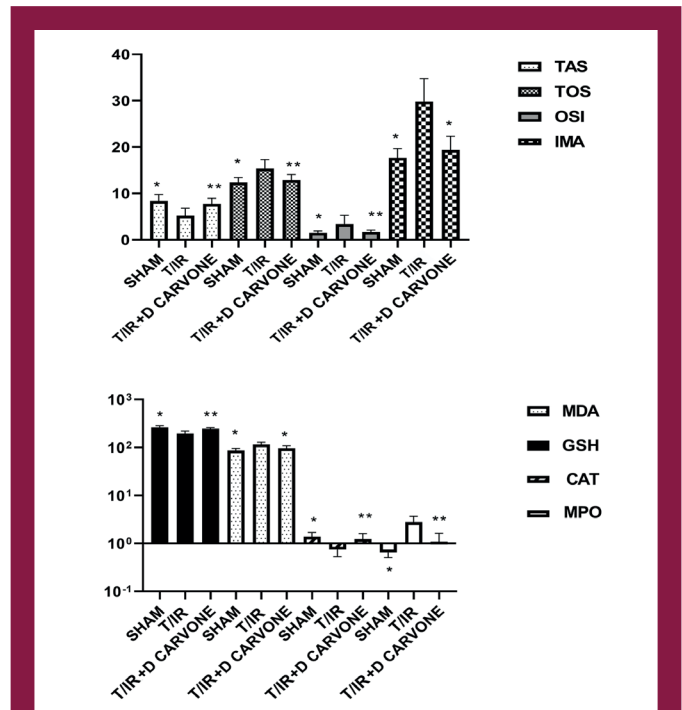


Figure 2. Oxidant and antioxidant levels.

For TAS: * $p < 0.004$ vs. T/IR group; ** $p = 0.02$ vs. T/IR group.
 For TOS: * $p = 0.006$ vs. T/IR group; ** $p = 0.024$ vs. T/IR group.
 For OSI: * $p = 0.027$ vs. T/IR group; ** $p = 0.047$ vs. T/IR group.
 For GSH: * $p < 0.001$ vs. T/IR group.
 For CAT: * $p = 0.005$ vs. T/IR group; ** $p = 0.03$ vs. T/IR group.
 For MDA: * $p = 0.001$ vs. T/IR group; ** $p = 0.015$ vs. T/IR group.
 For MPO: * $p < 0.001$ vs. T/IR group; ** $p = 0.001$ vs. T/IR group.
 For IMA: * $p < 0.001$ vs. T/IR group.

TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; GSH, glutathione; CAT, catalase; MDA, malondialdehyde; MPO, myeloperoxidase; IMA, ischemia-modified albumin.

Table 2. Comparison of oxidant and antioxidant levels between groups.

	TAS	TOS	OSI	GSH	CAT	MDA	MPO	IMA
SHAM (Group I)	8.38 ± 1.41	12.37 ± 1.06	1.53 ± 0.39	262.59 ± 21.89	1.39 ± 0.29	86.64 ± 9.37	0.65 ± 0.14	17.69 ± 1.98
T/IR (Group II)	5.21 ± 1.62	15.37 ± 1.91	3.43 ± 1.87	197.78 ± 21.23	0.74 ± 0.21	116.58 ± 11.29	2.79 ± 0.88	29.81 ± 4.95
T/IR+D CARVONE (Group III)	7.74 ± 1.19	12.93 ± 1.16	1.72 ± 0.38	245.81 ± 12.69	1.24 ± 0.36	96.50 ± 11.78	1.09 ± 0.53	19.39 ± 2.92
I vs. II	p = 0.004	p = 0.006	p = 0.027	p < 0.001	p = 0.005	p = 0.001	p < 0.001	p < 0.001
I vs. III	p = 0.716	p = 0.777	p = 0.956	p = 0.308	p = 0.646	p = 0.287	p = 0.422	p = 0.685
II vs. III	p = 0.002	p = 0.024	p = 0.047	p < 0.001	p = 0.03	p = 0.015	p = 0.001	p < 0.001

Values were given as mean ± standard deviation. TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; GSH, glutathione; CAT, catalase; MDA, malondialdehyde; MPO, myeloperoxidase; IMA, ischemia-modified albumin.

Table 3. Comparison of NGAL levels among the groups.

	SHAM (Group I)	T/IR (Group II)	T/IR+D CARVONE (Group III)	p-value
NGAL	200.27 ± 12.11	267.71 ± 31.72	226.89 ± 15.13	<0.001^a 0.113 ^b 0.013^c

Values were given as mean ± standard deviation. ^agroup 1 vs. group 2; ^bgroup 1 vs. group 3; ^cgroup 2 vs. group 3. T/IR, testicular ischemia/reperfusion; NGAL, neutrophil gelatinase-associated lipocalin.

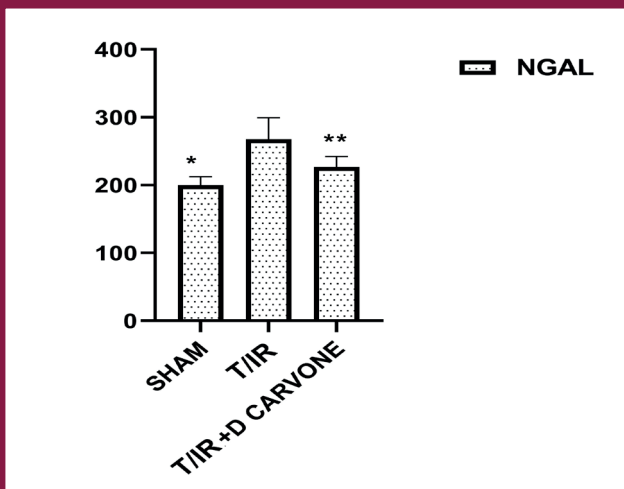


Figure 3. Comparison of NGAL levels among experimental groups. * $p < 0.001$ comparison with T/IR group; ** $p=0.013$ comparison with T/IR group. T/IR, testicular ischemia/reperfusion; NGAL, neutrophil gelatinase-associated lipocalin.

Table 4. Johnsen score.

Groups	Mean \pm standard error
SHAM	9.88 \pm 0.13 ^a
T/IR	5.75 \pm 0.37 ^b
T/IR+D carvone	7.50 \pm 0.42 ^c

Different superscripts (^{a,b,c}) show the difference between groups.

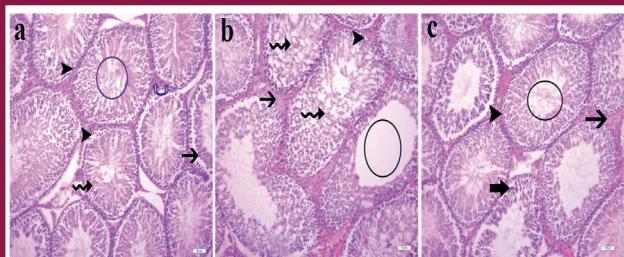


Figure 4. Testicular tissue histopathological alterations (H&E stain). Arrowheads: Deterioration of the structure of seminiferous tubules. Thin arrows: Degeneration of germ cells. Wavy arrows: Luminal shedding and loss of germinal epithelium. Edema in the intertubular region is represented by circles (o). (a) SHAM group: The seminiferous tubules have a regular germinal epithelial layer and normal histological structure. (b) T/IR group: Interstitial edema, germ cell loss, epithelium shedding, and noticeable structural degradation. (c) Compared to the T/IR group, the T/IR + D-carvone group exhibits less edema and cellular degradation, while maintaining some seminiferous tubule structure. The scale bar is 100 μ m. T/IR, testicular ischemia/reperfusion.

SHAM group, and that these levels were reduced after D-carvone treatment (Figure 5, $p < 0.0001$). While expression of 8-OHdG was shown as dark brown nuclear staining in testicular sections, expressions of NF- κ B and Caspase-3 were observed as brown cytoplasmic staining.

Discussion

Because IR damage develops, testicular torsion remains a urological emergency with serious consequences for male fertility. Oxidative stress, inflammation, and apoptosis are pathogenic pathways associated with T/IR that, if not addressed, result in irreparable tissue damage. In the T/IR injury model, we examined the potential protective effects of D-carvone, a naturally occurring monoterpene with established anti-inflammatory and antioxidant properties.

T/IR injury was shown to substantially increase levels of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6), oxidative stress markers (MDA, MPO, TOS, OSI, IMA, NGAL), and apoptotic and immunohistochemical markers (NF- κ B, Caspase-3, 8-OHdG) while decreasing levels of the anti-inflammatory cytokine IL-10 and antioxidant parameters (TAS, GSH, CAT). The restoration of cytokine and antioxidant levels toward normal levels, a decrease in tissue injury biomarkers, an improvement in the histological architecture of the seminiferous tubules, and a decrease in the immunohistochemical expression of oxidative, inflammatory, and apoptotic markers all demonstrated that treatment with D-carvone substantially reversed these pathological alterations. The results presented suggest that D-carvone provides significant protection against T/IR-induced testicular damage.

Inflammatory activity that developed post-T/IR was mostly reduced by D-carvone treatment, indicating an immunomodulatory effect rather than a metabolic one. Oxidative stress occurs when ROS exceed the capacity of the body's defense mechanisms, and it has been described as one of the major contributors to reperfusion-induced tissue damage. In this study, T/IR exposed animals showed a marked decrease in endogenous antioxidants TAS, GSH, and CAT and an increase in pro-oxidative markers MDA, MPO, TOS, OSI, and IMA corresponding biochemical observations have been reported previously (3,7). After D-carvone administration, the imbalance shifted significantly toward increased antioxidant reserves and decreased excess oxidants, though not completely reversed. Such shifts strongly indicate enhanced antioxidant defense by D-carvone toward normalization of cellular redox status.

NGAL, recognized as a responsive biomarker of both oxidative and inflammatory stress (24,25), increased sharply following IR injury. After administration of D-carvone, NGAL levels returned to near-normal levels, suggesting that it

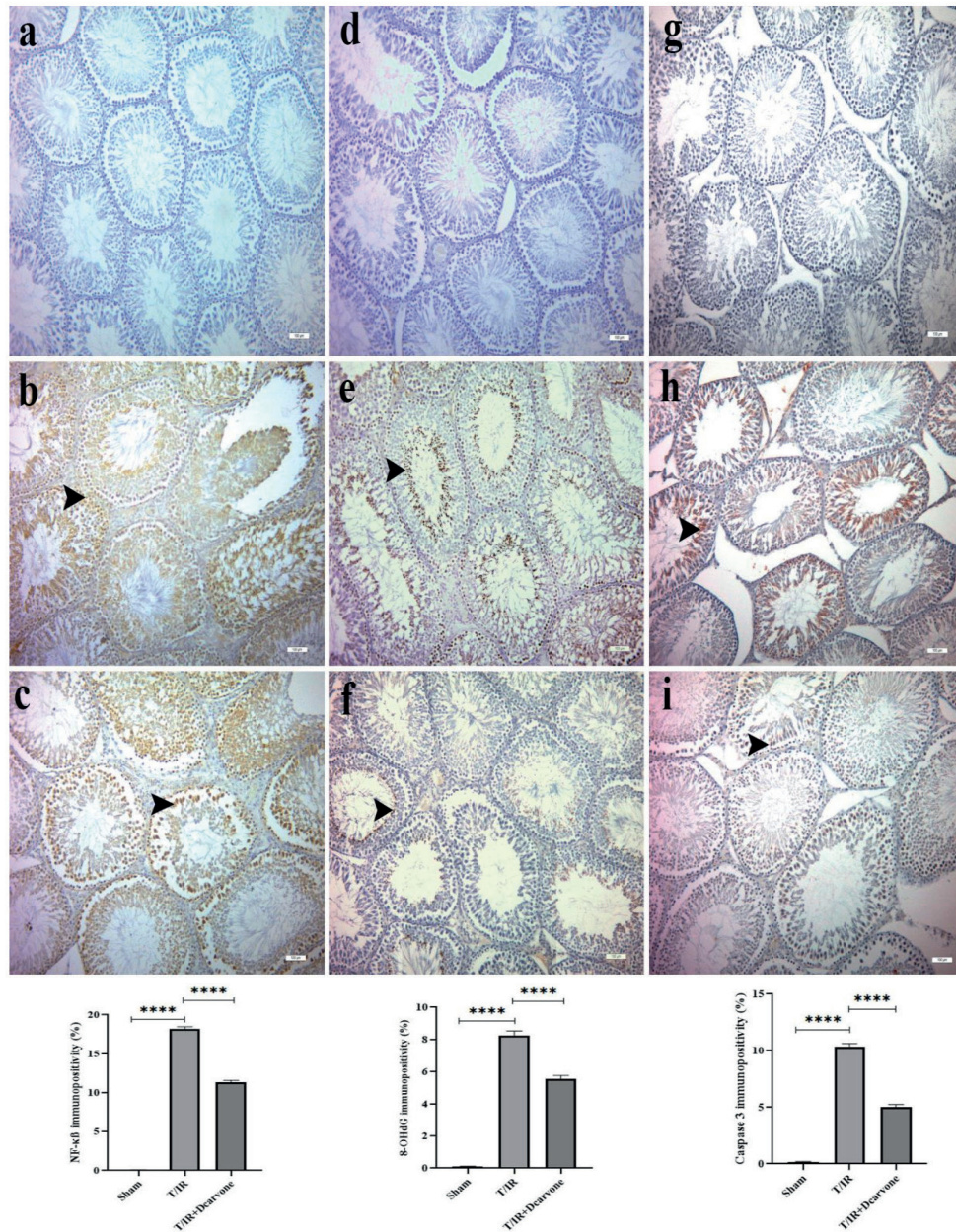


Figure 5. D-carvone treatment attenuates apoptosis, oxidative DNA damage, and inflammation in T/IR. Immunohistochemical staining was used to compare levels of NF-κB, 8-OHdG, and Caspase-3 in testicular tissues of the SHAM group (a-d-g), the T/IR group (b-e-h), and the T/IR+D carvone group (c-f-i), respectively. Arrowheads indicate positively stained cells. Scale bar 100 μm. The image analysis for evaluating **nuclear factor kappa B, 8-hydroxy-2'-deoxyguanosine**, and Caspase-3 in testis tissue. ****p < 0.0001 vs. SHAM and T/IR group. T/IR, testicular ischemia/reperfusion.

significantly attenuates the inflammatory response typically observed during reperfusion.

Microscopic evaluation confirmed these biochemical findings. The testes in the T/IR group without D-carvone treatment manifested significant distortion of the seminiferous tubules, accompanied by germ-cell

degeneration, edema, and cytoplasmic vacuoles. Comparable degenerative features—reduced tubule diameter, diminished testicular volume, and impaired spermatogenesis—have been described by others, and the present results follow the same pattern (3,26). D-carvone treatment maintained the testicular histoarchitecture, consistent with higher Johnsen

scores. A previous study on hepatic IR showed extensive necrosis, parenchymal disarray, and sinusoidal dilatation in tissues of reperfused rats. D-carvone treatment reduced necrosis while preserving the histological integrity of the liver. Likewise, in the present study's T/IR group, severe degeneration of seminiferous tubules, along with germ cell death, edema, and vacuolization, was noted; these changes improved following treatment with D-carvone, as indicated by preserved testicular histoarchitecture and significantly improved Johnsen scores. These findings support the notion that D-carvone exerts similar protective effects against IR-induced damage in different organs (27).

Immunohistochemical studies showed that NF- κ B, Caspase-3, and 8-OHdG were highly expressed in the T/IR group, suggesting oxidative DNA damage, inflammation, and activation of apoptotic pathways. D-carvone treatment significantly reduced the expression of these markers, suggesting protective effects against cellular stress induced by T/IR. Similarly, Ulaş et al. (28) reported that 20 mg/kg D-carvone significantly reduced Caspase-3 levels in an LPS-induced lung injury model, while Mohamed and Younis reported that D-carvone treatment significantly suppressed the NF- κ B signaling pathway in a hepatic IR model (27). The current study's results are in line with research showing that D-carvone effectively protects against IR injury by decreasing apoptosis and inflammation, regulating oxidative stress, and preserving antioxidant defense capacity. This defense mechanism is thought to act by regulating two major pathways: NF- κ B signaling and the response to oxidative DNA damage.

D-carvone has been reported to exert a neuroprotective effect through the reduction of inflammation in the brain IR (29). Additionally, it has protective effects from the doxorubicin cardiotoxicity (30). Moreover, D-carvone has been demonstrated to lessen liver fibrosis caused by carbon tetrachloride by blocking oxidative stress (31). Recent investigations have shown multiple pharmacological actions of D-carvone, including anti-fibrosis and antioxidant (31), anti-inflammatory (32), anti-arthritic (11), anticancer (33), antinociceptive (34) effects. The protective influence of D-carvone against T/IR injury has not been widely explored. In the present work, D-carvone showed antioxidant and anti-inflammatory effects that contributed to limiting T/IR-associated tissue damage. Its apparent anti-apoptotic activity suggests that the compound may help preserve testicular structure and spermatogenic function after torsion-detorsion injury, supporting its potential use as a preventive agent. More comprehensive and long-term studies are needed for clinical use.

Study Limitations

There are several limitations to the study. First, only short-term outcomes were studied; hence, the long-term effects of D-carvone on fertility-related parameters and general reproductive performance remain unknown. Second, the study was limited to a single, relatively high dose of D-carvone—the maximum dose used in previous studies—and hence it cannot be used to assess possible dose-dependent effects. Third, direct fertility outcomes such as sperm parameters, hormonal profiles, and functional assessments of reproduction were not included in this study. Therefore, findings that may protect against testicular ischemia-reperfusion injury at the tissue and molecular levels should be viewed with caution regarding any conclusions about infertility until further experimental and clinical studies clarify the role of D-carvone in reproductive function and fertility.

Conclusion

Within the limitations of this study, D-carvone appeared to lower oxidative stress by reducing oxidant load and reinforcing antioxidant defenses. It also displayed anti-inflammatory potential, as reflected by a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory mediators. Immunohistochemical results indicated decreases in oxidative DNA damage, inflammation, and apoptosis. These findings indicate that D-carvone has potential as a protective agent against T/IR-induced testicular injury.

Ethics

Ethics Committee Approval: The Atatürk University Experimental Animal Ethics Committee approved the experimental procedure (approval no: 2022/11-260, dated 08.11.2022).

Informed Consent: Not required.

Footnotes

Authorship Contributions

Surgical and Medical Practices: S.Ö.Ş., Ş.Y.T., A.T., B.G., M.C.G., E.L., M.Y., Concept: S.Ö.Ş., Ş.Y.T., A.T., S.Ç., E.L., N.Y., M.Y., Design: S.Ö.Ş., Ş.Y.T., A.T., B.G., M.C.G., N.Y., M.Y., Data Collection or Processing: S.Ö.Ş., Ş.Y.T., B.G., M.C.G., S.Ç., E.L., N.Y., Analysis or Interpretation: S.Ö.Ş., B.G., S.Ç., N.Y., M.Y., Literature Search: S.Ö.Ş., Ş.Y.T., A.T., B.G., M.C.G., S.Ç., E.L., N.Y., M.Y., Writing: S.Ö.Ş., Ş.Y.T., A.T., S.Ç., N.Y., M.Y.

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