Rom J Morphol Embryol 2021, 62(1):179–190 ISSN (print) 1220–0522, ISSN (online) 2066–8279 doi: 10.47162/RJME.62.1.17

ORIGINAL PAPER

RJME Romanian Journal of Morphology & Embryology http://www.rjme.ro/

Carvacrol treatment opens Kir6.2 ATP-dependent potassium channels and prevents apoptosis on rat testis following ischemia–reperfusion injury model

CEMRE NUR BALCI[1)], TULIN FIRAT[2)], NURAY ACAR[1)], AYSEL KUKNER[3)]

[1)]*Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, Antalya, Turkey*

[2)]*Department of Histology and Embryology, Faculty of Medicine, Bolu Abant Izzet Baysal University, Bolu, Turkey*

[3)]*Department of Histology and Embryology, Faculty of Medicine, Near East University, Nicosia, Cyprus*

Abstract

Testicular torsion is a urological problem that causes subfertility and testicular damage in males. Testis torsion and detorsion lead to ischemia–reperfusion (IR) injury in the testis. Testicular IR injury causes the increase of reactive oxygen species (ROS), oxidative stress (OS) and germ cell-specific apoptosis. In this study, we aimed to investigate whether Carvacrol has a protective effect on testicular IR injury and its effects on Kir6.2 channels, which is a member of adenosine triphosphate (ATP)-dependent potassium channels. In the study, 2–4 months old 36 albino Wistar rats were used. For experimental testicular IR model, the left testis was rotated counterclockwise at 720° for two hours, and after two hours following torsion, detorsion was performed. Carvacrol was dissolved in 5% Dimethyl Sulfoxide (DMSO) at a dose of 73 mg/kg and half an hour before detorsion, 0.2 mL was administered intraperitoneally. In testicular tissues, caspase 3 and Kir6.2 immunoexpressions were examined. Serum malondialdehyde (MDA) and testosterone levels were measured. Apoptotic cells and serum MDA levels were significantly decreased and Kir6.2 activation was significantly increased in Carvacrol-administrated IR group. As a result of our study, Carvacrol may activates Kir6.2 channels and inhibits apoptosis and may have a protective effect on testicular IR injury.

Keywords: Carvacrol, testis, ischemia–reperfusion injury, Kir6.2, caspase 3.

Introduction

Testicular torsion is a urological problem that causes testicular damage and subfertility by rotating around the testicular vessels and spermatic cord of the testicle [1]. Testicular torsion or spermatic cord torsion is one of the most serious urological emergencies and it is usually seen in newborns and young men. If testicular torsion is misdiagnosed or improperly treated, it leads to the development of male subfertility [2]. The testicle suddenly rotates in the scrotum, bending the blood vessels inward and blood flow is stopped. If intervention is not performed to testicular torsion more than 4–8 hours; it causes severe pain and can lead to testicular necrosis. Testicular torsion incidence is approximately 5/100 000 men under the age of 25 [3]. Testicular torsion in animal models, such as rat, represents a functional model of ischemia–reperfusion (IR) in humans [4]. In testicular torsion, there are two events that cause testicular necrosis. The first is ischemic damage during torsion, the second is reperfusion damage during detorsion. With the cessation of blood flow by testicular torsion, ischemia occurs, energy-rich phosphates are depleted and the level of products, such as hypoxanthine, increases [5, 6]. Following testicular detorsion, reperfusion occurs in testicular tissue which causes more serious damage than ischemia [7]. Reperfusion damage occurs with the increase of free oxygen species and neutrophils [8].

IR injury causes cellular damage by the formation of free radical species, proinflammatory cytokines, adhesion molecules, lipid peroxidation, apoptosis, anoxia, and alteration of microvascular blood flow. This chain of pathological events causes atrophy in the testicle [8]. Oxidative stress (OS) associated with overproduction of reactive oxygen species (ROS) forms the basic patho-physiological process in IR injury [9, 10]. ROS attack polyunsaturated fatty acids in the cell membrane, which causes a series of chain chemical reactions called lipid peroxidation [11]. Lipid peroxidation in the cell membrane damages the cells by disrupting the fluidity and permeability of the cell membrane [12]. In other words, when the cell membrane is damaged by free radicals, cell death may occur. Malondialdehyde (MDA) is released during lipid peroxidation and used as a marker of peroxidative damage; it can be detected *via* various biochemical assays [13–15]. MDA molecules penetrate cell membrane structures and leads to asymmetric disorders in lipid membrane components [12]. Lipid peroxidation damage on testicular dysfunction has been known for a long time [16]. This damage impairs mid part of sperm and reduces acrosome capacitation during fertilization [12]. Testicular IR causes germ cell apoptosis [17]. During IR high ROS levels induce caspase activation and apoptosis by disrupting the internal and external mitochondrial membranes and it causes the release of cytochrome *c* protein. There is a correlation

between increased ROS levels and high levels of cytochrome *c*, caspase 9 and caspase 3 which indicate apoptosis in infertile male patients [18].

Adenosine triphosphate (ATP)-sensitive potassium (KATP) channels are hetero-octameric proteins consisting of KIR 6.x-type subunits and Sulfonylurea receptor (SUR) subunits [19]. It was shown that KATP channel subunits are found in the cell membrane, organelles of spermatogenic cells and Sertoli cells in mouse and rat testis [20, 21]. Potassium channels are responsible for maintaining the membrane potential of germ cells. Different potassium channels are localized in germ cells at different stages [22]. ATP-dependent potassium channels are formed by two types of molecules, a pore-forming subunit molecule Kir6.x (Kir6.1 and Kir6.2) and a SUR regulatory subunit molecule [23, 24]. It is shown that KATP channel subunits Kir6.1, Kir6.2 and SUR2B are found highly in round spermatids and SUR2A in long spermatids [20]. Kir6.2 is known to be localized in spermatids together with SUR2B. These channels may play a role in capacity-related sperm hyperpolarization [21]. Also, it has been determined that opening of the KATP channels can protect the tissue against IR damage [19]. Many testicular IR model studies showed that spermatogenesis activation was decreased. This loss in spermatogenesis has been defined as germ cell-specific apoptosis [17]. It has been reported that KATP channels were opened in testicular tissue during IR injury, and it prevented caspase-dependent apoptosis [25].

Carvacrol [2-methyl-5-(1-methylethyl)-phenol] is a monoterpenoid compound with a characteristic Oregano smell, found in essential oils of aromatic plants, such as Thyme, Pepper, and Wild Bergamot [26]. *In vitro* and *in vivo* studies described that Carvacrol has different bioactivities, such as antibacterial, antioxidant, antiseptic, antispasmodic, growth regulator, antifungal, antiviral, anti-inflammatory, expectorant, antitussive, immunomodulator and chemical inhibitor, rumen microbial fermentation modifier and reduction of Methane emission [27–29]. Studies have shown that antioxidants protect spermatozoa from ROS, preventing abnormal spermatozoa production. Antioxidants reduce ROS produced by leukocytes, prevent deoxyribonucleic acid (DNA) fragmentation, improve sperm quality, prevent premature sperm maturation [30]. Carvacrol plays a critical role as a natural antioxidant in reducing lipid peroxidation [31].

Aim

Based on the literature, we hypothesized that Carvacrol has a protective role against testicular IR damage. Therefore, we aimed to investigate the effects of Carvacrol administration on testicular IR injury morphologically, immunohistochemically, and biochemically.

Materials and Methods

Animals

Two–four months old 36 male Wistar albino rats, weighing between 200–250 g, were placed in a temperature (21±2°C) and humidity (60±5%) controlled room in which a 12-hour light–dark cycle was maintained. Animals were fed with standard laboratory food and tap water in type 3H cages. Experimental animals were procured from Experimental Animals Research Center, Bolu Abant Izzet Baysal University Bolu, Turkey. This study was approved by the Ethics Committee of Experimental Animal Studies, Bolu Abant Izzet Baysal University (Approval No. 2017/08) and were in accordance with the *Declaration of Helsinki* and *International Association for the Study of Pain* Guidelines. In this study, rats were randomly divided into six groups, each containing six rats. Experimental groups were determined as Sham, Dimethyl Sulfoxide (DMSO), Carvacrol, IR, IR+DMSO and IR+Carvacrol. Before experiment, animals were anesthetized with an intraperitoneal (i.p.) injection of 0.25 mL Ketamine hydrochloride (75 mg/kg) and 0.1 mL Xylazine (8 mg/kg).

Experimental design and testicular IR model

Testicular IR model in rats were performed in Experimental Animals Unit, Faculty of Medicine, Bolu Abant Izzet Baysal University. We used power analysis to determine sample size and included six rats in each experimental group. There was no animal death due to experimental procedure.

Sham group (n=6)

A Sham operation (rats that underwent only scrotal incision and suturing) was performed on rats. The scrotum of the rats was opened under anesthesia and the left testicle was taken out. Without any experiment, the testicle was put into the scrotum and the scrotum was sutured. After waiting for four hours based on IR time, left testicle tissue was taken.

DMSO group (n=6)

0.2 mL of 5% DMSO (Sigma D8418, 50 mL) was injected i.p. under anesthesia. Left testicular tissue was taken at the end of 2.5 hours after i.p. injection.

Carvacrol group (n=6)

73 mg/kg Carvacrol [32] (Sigma W224511-100G-K) dissolved in 5% DMSO was injected 0.2 mL i.p. Left testicular tissue was taken at the end of 2.5 hours.

IR group (n=6)

Torsion was created by rotating the left testis 720° counterclockwise and then two hours later for detorsion, the testis was counter-rotated to the natural position and replaced into the scrotum. Left testicle tissue was removed after two hours of reperfusion injury.

IR+DMSO group (n=6)

Torsion was created by rotating the left testis 720° counterclockwise for two hours ischemic injury. Half an hour before detorsion, 0.2 mL of 5% DMSO was injected i.p. Then two hours later from torsion beginning, the testis was counter-rotated to the natural position and replaced into the scrotum for detorsion. Left testicle was removed after two hours of reperfusion injury.

IR+Carvacrol group (n=6)

Torsion was created by rotating the left testis 720° counterclockwise for two hours ischemic injury. Half an hour before detorsion, 73 mg/kg Carvacrol was dissolved in 5% DMSO and injected i.p. Then two hours later from torsion beginning, the testis was counter-rotated to the natural position and replaced into the scrotum for detorsion. Left testicle was removed after two hours of reperfusion injury.

All testicular tissues were placed in 10% neutral buffered formalin. Left epididymis was taken into phosphate-buffered saline (PBS) solution for spermiogram. Approximately 3 mL of blood was taken from tail vein of each animal and placed in blood tubes. After obtaining tissue and blood samples, rats were sacrificed *via* application of high-dose anesthetics. Blood tubes were centrifuged at 9000 rpm for 10 minutes. The serum obtained were put into tubes and kept at -80°C for biochemical analysis.

Histological analysis

The collected tissue samples were fixed in 10% neutral buffered formalin for 24 hours. Following fixation, tissues washed with water for 30 minutes to remove fixation solution and tissues were dehydrated in solution containing an increasing percentage of ethanol. Following dehydration, tissues were passed through xylene to remove ethanol and clear tissues. After tissue processing, tissues were embedded in paraffin. Formalin-fixed paraffin-embedded (FFPE) tissues were cut into 5 μm sections and placed on slides. Sections were stained with Hematoxylin–Eosin (HE) and Masson's trichrome for histological analysis. After stainings, testicular tissues of all groups were investigated for histopathological (HP) changes, such as interstitial edema, interstitial bleeding, and hemorrhagic infarction.

The seminiferous tubules were graded according to the Johnsen's score [33]. Tubuli in 20 consecutive 200× field areas were scored, and mean values were determined. The seminiferous tubule diameters were measured in all groups. Johnsen's score is based on the premise that with testicular damage there is successive disappearance of the most mature cell type; progressive degeneration of germinal epithelium; and with disappearance of sperm and spermatids, then spermatocytes, and finally Sertoli cells (in that order). A score of 1 indicates no seminiferous epithelial cells and tubular sclerosis. Score 2: no germ cells, only Sertoli cells. Score 3: spermatogonia only. Score 4: no spermatids, few spermatocytes and arrest of spermatogenesis at the primary spermatocyte stage. Score 5: no spermatids and many spermatocytes. Score 6: no late spermatids, few early spermatids, arrest of spermatogenesis at the spermatid stage, and disturbance of spermatid differentiation. Score 7: no late spermatids and many early spermatids. Score 8: few late spermatids. Score 9: many late spermatids and disorganized tubular epithelium. Score 10: full spermatogenesis.

Immunohistochemistry (IHC)

FFPE samples were cut into 5 μm sections and placed on slides coated with poly-L-Lysine. Sections were incubated with primary antibodies anti-caspase 3 (Thermo Scientific™ caspase 3 (CPP32) Ab-4, rabbit polyclonal antibody) (1:200) and anti-Kir6.2 (Santa Cruz Kir6.2 (B-9) sc-390104, mouse monoclonal antibody), overnight, at 4°C. The slides were incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibody (Thermo Scientific™ Lab Vision™ UltraVision™ Detection System (TP-015-HD), anti-polyvalent antibody) for 40 minutes. Peroxidase activity was visualized with 3,3'-Diaminobenzidine (DAB) (Catalogue #D4168 – SIGMAFAST™ DAB tablets), and counterstained with Hematoxylin. Sections were evaluated and photographs were taken using a light microscope (Nikon Eclipse i80, Tokyo, Japan). The images were then analyzed using NIS-Elements Microscope Imaging Software (Nikon, Tokyo, Japan). The number of positively stained cells on seminiferous tubule was counted by a blinded manner by two independent observers in five random fields in the slides from each tissue samples.

Terminal deoxynucleotidyl transferase (Tdt) deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay

Apoptotic cells were determined by TUNEL assay. This assay was performed on testicular tissue sections using ApopTag® plus peroxidase *in situ* apoptosis detection kit (Catalogue #S7101, Millipore, Billerica, MA, USA) as per manufacturer's instruction. Reactions were developed with DAB detection system, and sections were counter-stained with Hematoxylin. Stained sections were visualized under light microscope (Nikon Eclipse i80, Tokyo, Japan). Images captured at 200× magnification were used for quantification and are presented in results.

H-Score

The evaluation of the immunohistochemical labeling of Kir6.2, caspase 3 and TUNEL in samples from experimental groups was performed using *H*-Score. Briefly, sections were evaluated using a light microscope (Nikon Eclipse i80, Tokyo, Japan) with a special ocular scale. Five different fields from all slides at 200× magnification were evaluated for immunohistochemical labeling of Kir6.2, caspase 3 and TUNEL. The labeling was scored in a semi-quantitative fashion that included the intensity of specific labeling in sections. The evaluations were recorded as percentages of labeled cells of all types in each of four intensity categories, denoted as 0 (no labeling), 1+ (weak labeling but detectable above control), 2+ (distinct labeling), and 3+ (intense labeling). For each tissue, an *H*-Score value was derived by summing the percentages of cells that were labeled at each intensity multiplied by the weighted intensity of the labeling: $H\text{-Score} = \sum Pi\ (i+1)$, where i is the intensity score and Pi is the corresponding percentage of the cells. Two observers blinded to the experimental groups performed the *H*-Score evaluations, and the average score was used.

Spermiogram analysis

The cauda part of the left epididymis was cut into small pieces in PBS and the mobilized sperm were collected. The obtained sperm samples were counted

with Makler counting chamber under light microscope (Olympus CX21). Sperm motilities were classified as: (*i*) moving forward; (*ii*) slow motion; (*iii*) mobile in place; (*iv*) immobile.

Biochemical analysis

Blood serums in -80ºC were taken to -20ºC and waited overnight. Then, samples were taken to +4ºC and the temperature was gradually increased. Biochemical analyzes were performed in Biochemistry Research Laboratory, Faculty of Medicine, Bolu Abant Izzet Baysal University. For measuring MDA and testosterone levels, enzyme-linked immunosorbent assay (ELISA) method was used. Reference ranges of 0.1–20 ng/mL for testosterone (Elabscience E-EL-0072) and 31.25–2000 ng/mL for MDA (Elabscience E-EL-0060) were used. Measurements were done on BioRad ELISA reader. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Statistics 20 program. Groups were compared by using one-way analysis of variance (ANOVA) tests followed by *post hoc* Tukey's honestly significant difference (HSD) test. The significance value was accepted as $p \leq 0.05$.

Results

Morphological assessment of testicular tissues

According to our HE-staining, no HP changes were observed in testicular tissues of Sham group (Figure 1a). Similarly, there was no HP defect in testicular tissues of DMSO and Carvacrol groups (Figure 1, b and c). In testicular tissue sections of IR and IR+DMSO groups, there were congestion, hemorrhage, thickening, edema, disruption in seminiferous tubule forms, irregularity in germinal epithelium and cell debris in seminiferous tubule lumen (Figure 1, d and e). Structural defects and congestion in IR+Carvacrol group was less compared to IR and IR+DMSO groups (Figure 1f).

According to Masson's trichrome staining, there were not any HP changes in testicular tissues of Sham, DMSO and Carvacrol groups (Figure 2, a–c). Congestion, thickening, edema, disruption in seminiferous tubule forms, irregularity in germinal epithelium and cell debris in seminiferous tubule lumen were seen clearly in IR group (Figure 2d). Also, hemorrhage was seen in both IR and IR+DMSO groups (Figure 2, d and e). Structural defects and congestion in IR+Carvacrol group was less compared to IR and IR+DMSO groups (Figure 2f).

Seminiferous tubule diameter measurements showed that, there was no significant difference between Sham, DMSO and IR groups ($p>0.05$). Likewise, there was no significant difference between IR, IR+DMSO and IR+Carvacrol groups ($p>0.05$). The tubule diameter was significantly decreased in IR and IR+DMSO groups compared to Carvacrol group ($p<0.05$) (Figure 3).

According to data obtained from Johnsen's scoring, scores were significantly decreased in IR, IR+DMSO and IR+Carvacrol groups compared to Sham, DMSO and Carvacrol groups ($p<0.0001$). This statistically significant difference showed that IR injury caused seminiferous tubules disruption. There was also statistically significant difference between IR and IR+Carvacrol groups ($p<0.001$). In IR+Carvacrol group, scores were significantly increased compared to IR and IR+DMSO group's scores. The increase of Johnsen's score in IR+Carvacrol group compared to IR and IR+DMSO group may suggest that Carvacrol could prevent damage in seminiferous tubules (Figure 4).

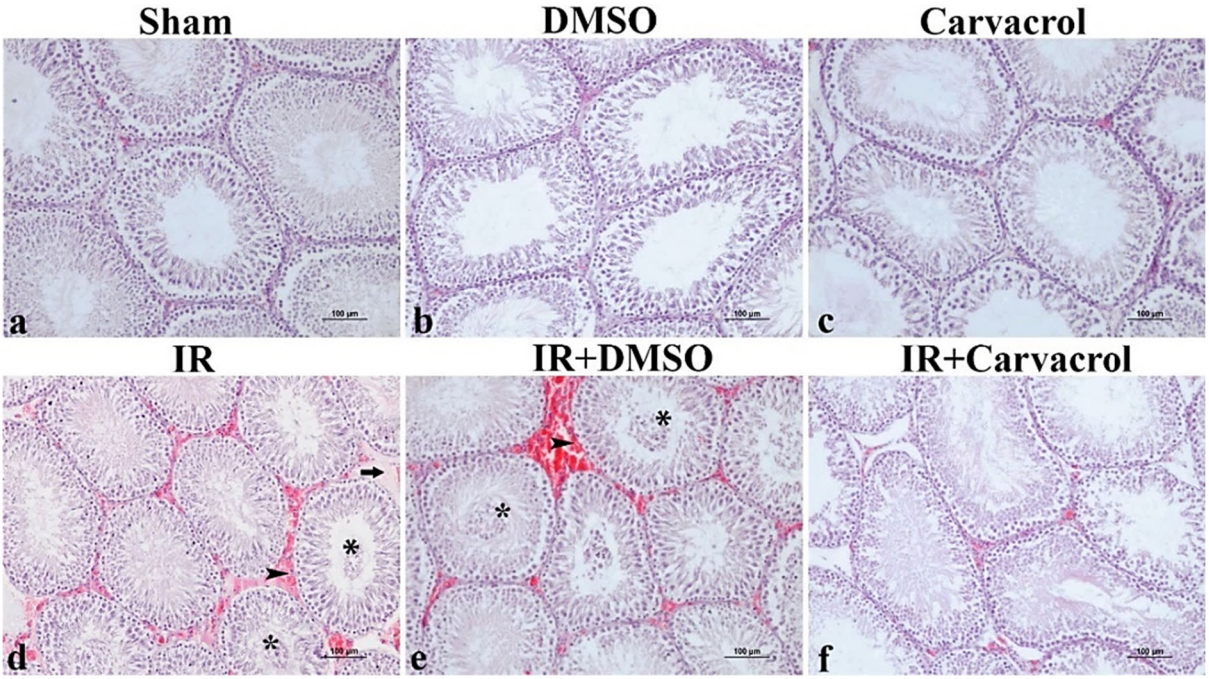

Figure 1 – ***HE-staining of all groups: (a) Sham; (b) DMSO; (c) Carvacrol; (d) IR; (e) IR+DMSO; (f) IR+Carvacrol. Cell debris is shown with asterisks (*), edema is shown with arrows and hemorrhage is shown with arrowheads. Scale bar: 100 µm. DMSO: Dimethyl Sulfoxide; HE: Hematoxylin–Eosin; IR: Ischemia–reperfusion.***

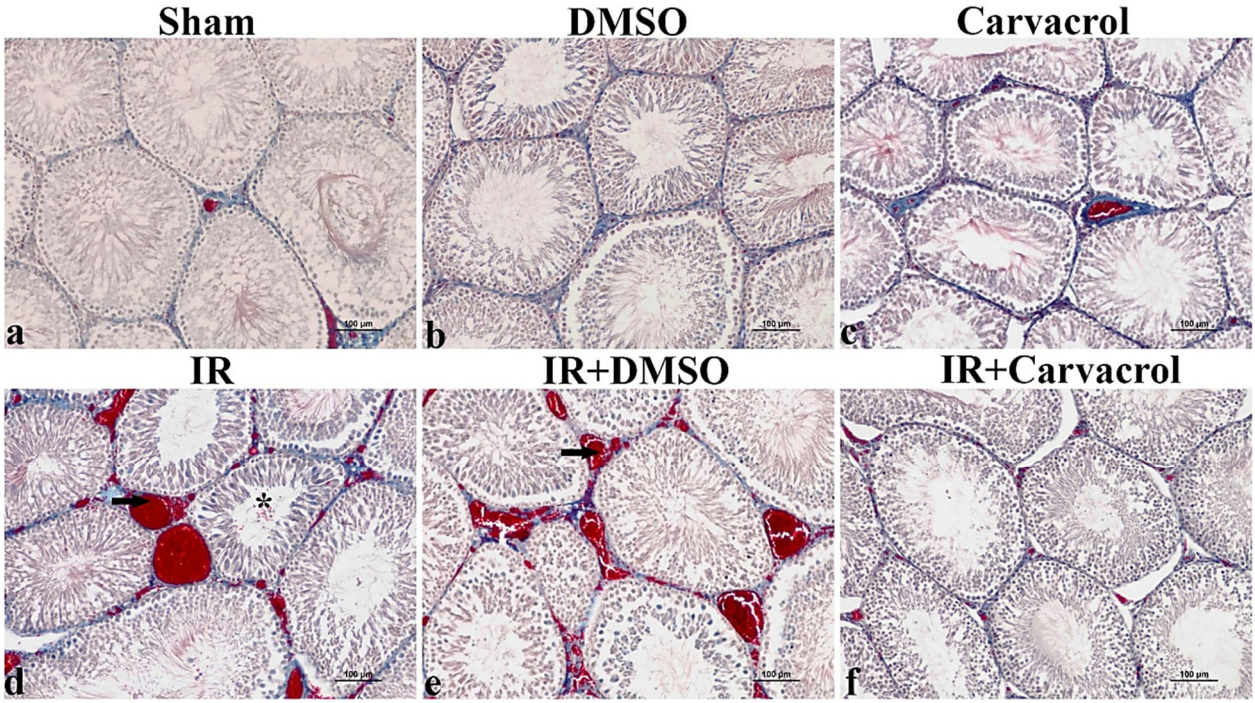

Figure 2 – ***Masson's trichrome staining of all groups: (a) Sham; (b) DMSO; (c) Carvacrol; (d) IR; (e) IR+DMSO; (f) IR+Carvacrol. Cell debris is shown with asterisks (*), hemorrhage is shown with arrows. Scale bar: 100 µm. DMSO: Dimethyl Sulfoxide; HE: Hematoxylin–Eosin; IR: Ischemia–reperfusion.***

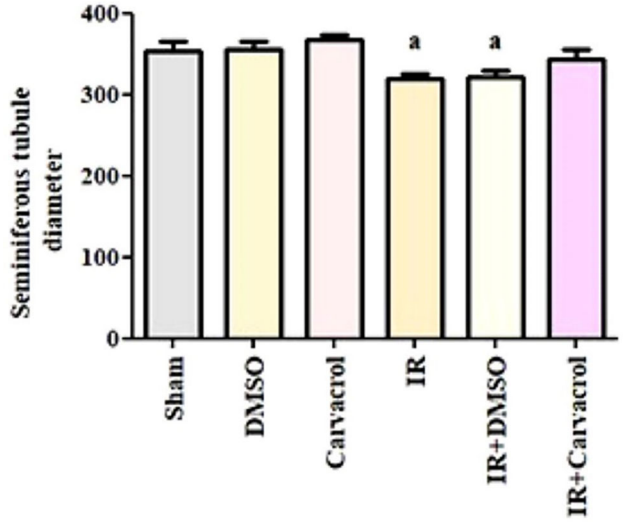

Figure 3 – ***Mean seminiferous tubule diameters of all groups. Significant difference was accepted as p<0.05. [a]Significant difference compared to Carvacrol group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion.***

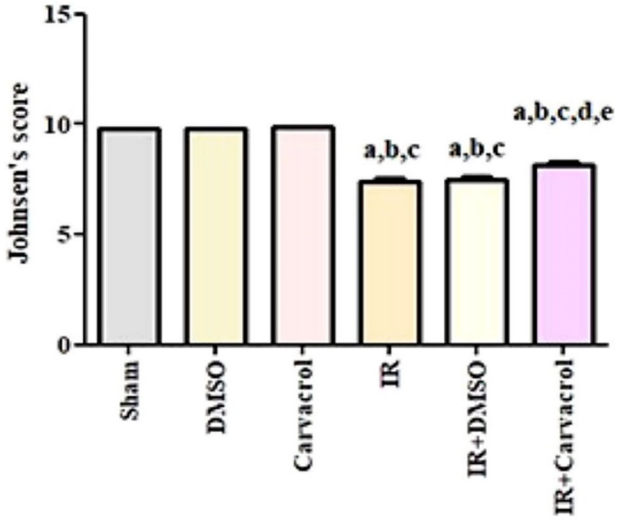

Figure 4 – ***Mean Johnsen's scores of all groups. Significant difference was accepted as p<0.05. Significant difference compared to: [a]Sham group; [b]DMSO group; [c]Carvacrol group; [d]IR group; [e]IR+DMSO group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion.***

Spermiogram results

According to our spermiogram results there was no significant difference in total sperm concentration between groups (Figure 5a). Our total sperm motility results showed that, there was a statistically significant difference between Sham, DMSO, Carvacrol and IR groups (p<0.0001). Total sperm motility was increased in Carvacrol group compared to Sham group (p<0.05). In IR, IR+DMSO and IR+Carvacrol groups, total number of motile sperm was decreased compared to Sham, DMSO and Carvacrol groups. This data may show that IR injury could cause decrease of motile sperm count. There was no statistically significant difference between IR and IR+Carvacrol groups (p>0.05) also IR+DMSO group (p>0.05) (Figure 5b).

Kir6.2 IHC

Kir6.2 immunoexpression was cytoplasmic in all experimental groups. Kir6.2 immunoexpressions were more frequently observed in round spermatids. There was Kir6.2 immunoexpression in primary spermatocytes but intensity was less compared to round spermatids. In Sham, DMSO and Carvacrol groups, Kir6.2 immunoexpressions were low (Figure 6, a–c). Kir6.2 immunoexpression was increased in both IR and IR+DMSO groups compared to Sham, DMSO and Carvacrol groups but it

was less than IR+Carvacrol group (Figure 6, d and e). We observed that Kir6.2 immunoexpression was higher in IR+Carvacrol group compared to Sham, DMSO and Carvacrol groups (Figure 6f). *H*-Score for Kir6.2 IHC is shown in Figure 6g. Kir6.2 immunoexpression was increased in IR, IR+DMSO and IR+Carvacrol groups compared to Sham, DMSO and Carvacrol groups (p<0.0001). This result may show that IR injury can cause the opening of ATP-dependent potassium channels in testis. In IR+DMSO group, Kir6.2 immunoexpression was significantly increased compared to IR group (p<0.05). Kir6.2 immunoexpression was significantly increased in IR+Carvacrol group compared to IR (p<0.0001) and IR+DMSO groups (p<0.001) (Figure 6g). This observation suggests that Carvacrol may have a protective role against IR injury *via* opening Kir6.2 channels.

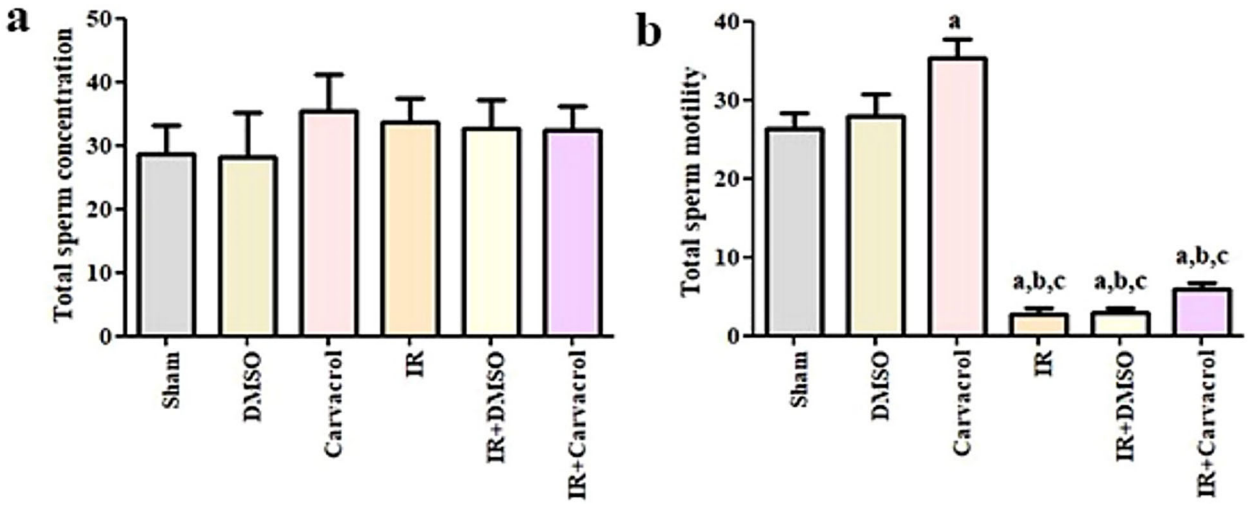

Figure 5 – ***(a) Mean total sperm concentration of all groups. There was no statistically significant difference between groups, p<0.05; (b) Total sperm motility of all groups. Significant difference was accepted as p<0.05. Significant difference compared to: [a]Sham group; [b]DMSO group; [c]Carvacrol group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion.***

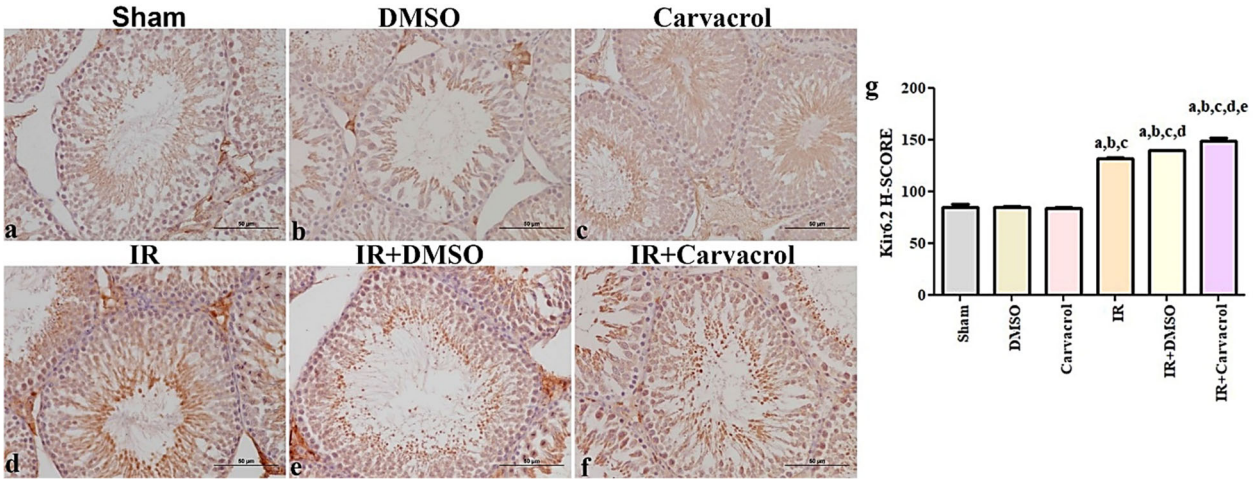

Figure 6 – ***Kir6.2 immunohistochemistry in seminiferous tubules of all groups. Scale bar: 50 µm. (a) Sham; (b) DMSO; (c) Carvacrol; (d) IR; (e) IR+DMSO; (f) IR+Carvacrol; (g) Ki6.2 H-Score. Significant difference was accepted as p<0.05. Significant difference compared to: [a]Sham group; [b]DMSO group; [c]Carvacrol group; [d]IR group; [e]IR+DMSO group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion.***

Caspase 3 IHC

Apoptotic cells in testicular tissue were positively immunostained with caspase 3. Caspase 3 exhibited mostly cytoplasmic immunoexpression and a little nuclear immunoexpression in all groups. We observed caspase 3 immunoexpressions especially in cell debris, round spermatids, and primary spermatocytes (Figure 7). In Sham, DMSO and Carvacrol groups, caspase 3 immunoexpressions were low (Figure 7, a–c). Caspase 3 immunoexpressions were increased in IR and IR+DMSO groups compared to Sham, DMSO and Carvacrol groups (Figure 7, d and e). In IR+Carvacrol group, caspase 3 immunoexpression was higher than Sham, DMSO and Carvacrol groups but lower than IR and IR+DMSO groups (Figure 7f). *H*-Score for caspase 3 IHC is shown in Figure 7g. Caspase 3-positive cells were statistically significantly increased in IR, IR+DMSO and IR+Carvacrol groups compared to Sham, DMSO, and Carvacrol groups (p<0.0001). A statistically significant increase of stained cell number in IR group may show that IR injury could induce apoptosis in seminiferous tubules. In IR+DMSO group, caspase 3 immunoexpression was decreased compared to IR group (p<0.05). Apoptotic cells in IR+Carvacrol group were decreased compared to

IR and IR+DMSO groups (p<0.0001) (Figure 7g). These results suggest that Carvacrol could prevent apoptosis during IR injury.

TUNEL assay results

TUNEL assay results were compatible with caspase 3 IHC. In Sham, DMSO and Carvacrol groups, TUNEL-positive cells were low compared to IR and IR+DMSO groups (Figure 8, a–c). TUNEL-positive cell numbers were increased in IR and IR+DMSO groups compared to IR+Carvacrol groups (Figure 8, d–f). *H*-Score for TUNEL assay is shown in Figure 8g. TUNEL-positive cells were decreased significantly in IR+DMSO and IR+Carvacrol groups compared to IR group (p<0.0001). A significant decrease of apoptotic cell number in IR+Carvacrol group may show that Carvacrol could prevent apoptosis during IR injury.

Serum MDA and testosterone levels

Serum MDA and testosterone levels were determined by ELISA method in all groups, and the results are shown in Figures 9 and 10. Serum MDA levels were highest in IR group. In Sham, DMSO and Carvacrol groups, MDA levels were lowest. MDA levels also decreased in IR+Carvacrol group. Serum MDA levels were increased in IR (p<0.001) and IR+DMSO (p<0.05) groups compared to Sham, DMSO and Carvacrol groups. These data showed that IR injury caused lipid peroxidation. MDA level was decreased in IR+Carvacrol group compared to IR (p<0.001) and IR+DMSO (p<0.05) groups. The decrease of MDA values in IR+Carvacrol group showed that treatment with Carvacrol reduced lipid peroxidation because of its antioxidant role (Figure 9).

Serum testosterone level was highest in Carvacrol group. Also, serum testosterone level was lowest in IR group. Serum testosterone level was significantly decreased in IR group compared to Carvacrol group (p<0.05). In IR+DMSO group, serum testosterone level was decreased compared to Carvacrol group (p<0.05). There was no significant difference between IR, IR+DMSO and IR+Carvacrol groups (p>0.05). The decrease in testosterone levels in all IR groups has shown that IR injury could reduce testosterone level (Figure 10).

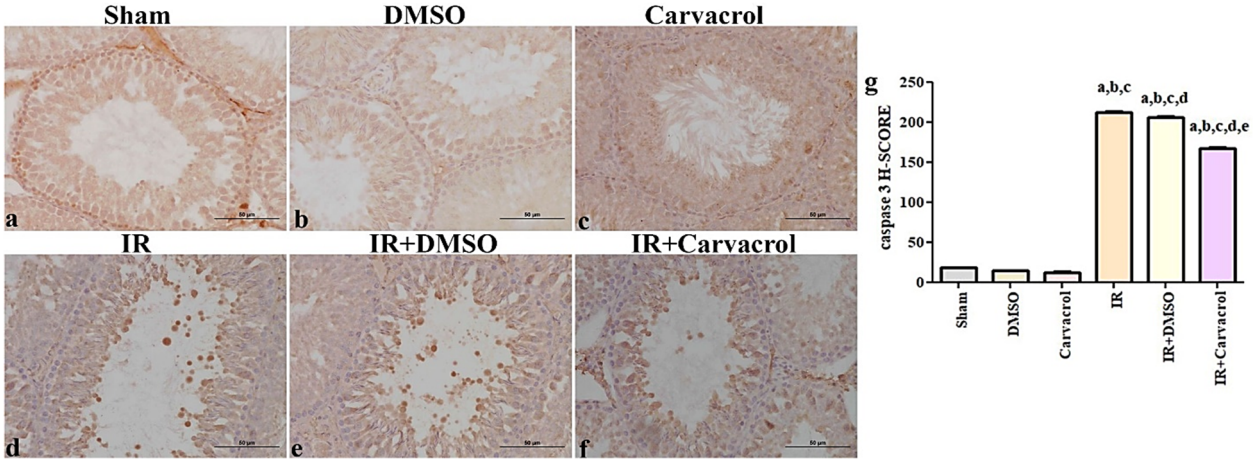

Figure 7 – ***Caspase 3 immunohistochemistry in seminiferous tubules of all groups. Scale bar: 50 µm. (a) Sham; (b) DMSO; (c) Carvacrol; (d) IR; (e) IR+DMSO; (f) IR+Carvacrol; (g) Caspase 3 H-Score. Significant difference was accepted as p<0.05. Significant difference compared to: [a]Sham group; [b]DMSO group; [c]Carvacrol group; [d]IR group; [e]IR+DMSO group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion.***

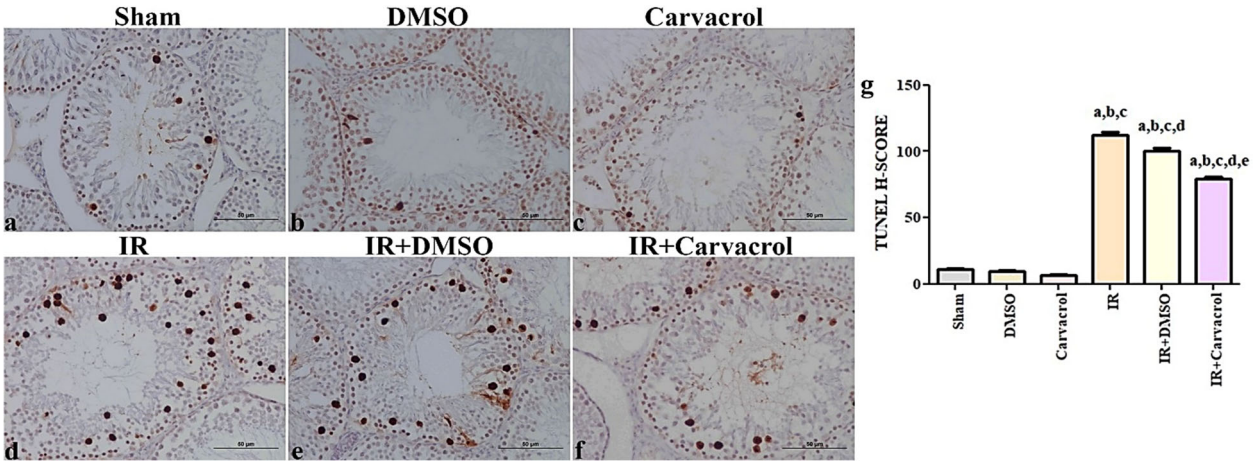

Figure 8 – ***Determination apoptotic cells with TUNEL assay on seminiferous tubules of all groups. Scale bar: 50 µm. (a) Sham; (b) DMSO; (c) Carvacrol; (d) IR; (e) IR+DMSO; (f) IR+Carvacrol; (g) TUNEL H-Score. Significant difference was accepted as p<0.05. Significant difference compared to: [a]Sham group; [b]DMSO group; [c]Carvacrol group; [d]IR group; [e]IR+DMSO group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion; TUNEL: Terminal deoxynucleotidyl transferase (Tdt) deoxyuridine triphosphate (dUTP) nick end labeling.***

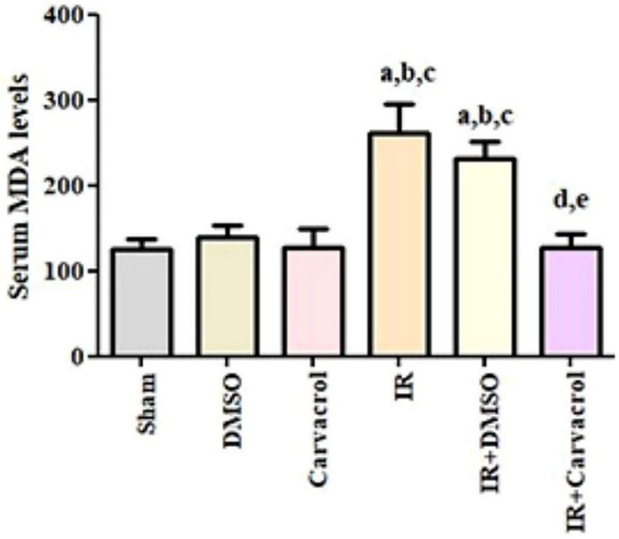

Figure 9 – ***Mean serum MDA levels in all groups. Significant difference was accepted as p<0.05. Significant difference compared to: [a]Sham group; [b]DMSO group; [c]Carvacrol group; [d]IR group; [e]IR+DMSO group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion; MDA: Malondialdehyde.***

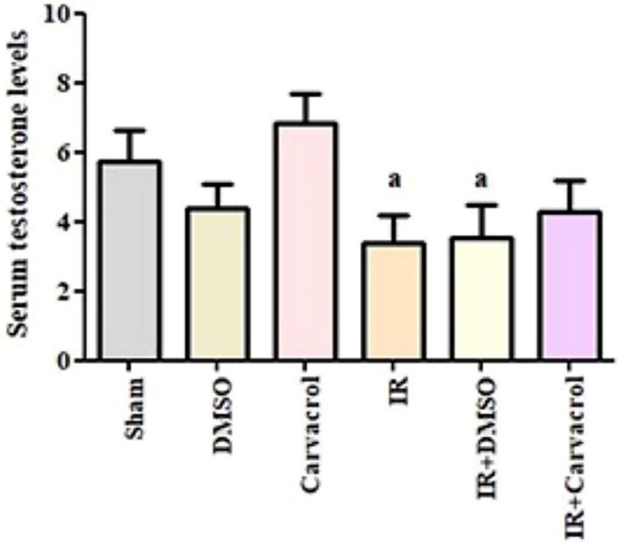

Figure 10 – ***Mean serum testosterone levels in all groups. Significant difference was accepted as p<0.05. [a]Significant difference compared to Carvacrol group. DMSO: Dimethyl Sulfoxide; IR: Ischemia–reperfusion.***

Discussions

Testicular torsion is a urological problem that causes tissue damage and subfertility. When the testis tissue rotates around the spermatic cord and veins, blood flow stops, and tissue damage begins [1]. Testicular torsion–detorsion causes testicular damage because torsion leads to ischemia and detorsion causes reperfusion injury [34]. IR injury leads to increase of ROS, lipid peroxidation and apoptosis in tissues [8]. ROS initiates lipid peroxidation in the cell membrane by attacking polyunsaturated fatty acids [11]. Lipid peroxidation causes testicular dysfunction, sperm anomalies and decreasing capacitation of sperms [12, 16]. ROS, which causes OS, accelerates the release of cytochrome *c* by disrupting the mitochondrial membranes. With the release of cytochrome *c*, caspase activation begins, and apoptosis occurs [35–37]. KATP channels were first discovered in cardiomyocytes [38]. The opening of these channels is associated with the metabolic status of the cell and the level of ATP [39]. Kir6.2, which is subunit of the KATP, is found in spermatogonium, spermatocyte and late-stage sperm [20]. Opening of KATP protects tissue against IR injury [19]. Carvacrol is a monoterpenoid compound found in essential oils of various aromatic plants [26]. It has antioxidant, antiseptic, antibacterial and antifungal activities [27–29]. Carvacrol reduces lipid peroxidation *via* its antioxidant role [31].

Romeo *et al.* reported dilatation, edema, and hemorrhage in the intertubular areas of the testicle of IR injury-induced rats [40]. In another study of rat IR injury model, irregularity in the testicular tissue, thinning of the germinal epithelial layers, degeneration of germ cells in the tubules was observed [41]. Aydıner *et al.* also reported damage in seminiferous tubules and intertubular areas of testicular tissues of rats that IR was induced [42]. Our results are compatible with the literature; we observed that testicular IR injury caused congestion, thickening, edema, impairment of seminiferous tubule structures, abnormalities in germinal epithelium and cell debris in the lumen. All these findings show that IR injury causes HP damage of testicular tissue.

We used Johnsen's score to evaluate the morphological damage of the seminiferous tubules [33]. When we checked the literature, we found that different research groups found significant decrease in Johnsen's score of the testicular IR injury group compared to the control group [43–46]. In our study, there was a significant decrease of Johnsen's score in IR group compared to Sham group as in other studies. There was a significant increase of Johnsen's score in IR+Carvacrol group compared to IR group. This result shows that Carvacrol may protect spermatogenic cells and seminiferous tubule structure against IR damage.

Studies showed that sperm motility and sperm concentrations were decreased in IR group rats compared to control group rats [44, 47, 48]. In our study, like other studies, there was a significant decrease in the percentage of total sperm motility in IR group compared to control group, but no significant difference was found between groups for sperm concentration. We did not observe any difference in total motile sperm percentage; therefore, we think that if Carvacrol treatment duration is extended, positive effects on sperm motility and concentration could be seen.

Teshima *et al.* reported that mitochondrial (mito)KATP channels opened with Diazoxide plays a neuroprotective role by preventing apoptosis [49]. Similarly, Tsounapi *et al.* reported that Kir6.2 was opened after Cromakalim administration and prevented apoptosis in rat testicular IR model [25]. In another study, sarcolemmal (sarc)KATP channel was opened *via* P-1075 and it reduced lipopolysaccharide-induced apoptosis, while blocking sarcKATP channel with HMR-1098 increased lipopolysaccharide-induced apoptosis in cultured neonatal rat cardiomyocyte cells [50]. Oliveira *et al.* described that Carvacrol mediates the release of endogenous prostaglandins on an experimental

gastric lesion model in rodent, which has a gastroprotective role by causing increased mucus production, opening of KATP channels, activation of nitric oxide (NO) synthase [51]. Our results support the findings of Tsounapi *et al.* and Oliveira *et al.* [25, 51]. It is presumed that Kir6.2 KATP channels are opened in IR injury and Carvacrol administration could help prevent IR injury by further activating opening of these channels.

Different research groups reported the increase of TUNEL-positive cells and caspase 3 immunoexpressions in testicular IR injury-induced rats compared to control and Sham group rats [52, 53]. Similarly, in traumatic neuronal injury *in vitro* model, Carvacrol treatment significantly decreased TUNEL-positive cell number and caspase 3 immunoexpressions compared to injury group [54]. Suo *et al.* reported that Carvacrol treatment decreased TUNEL-positive cell number and caspase 3 activation in liver IR injury model induced rats [55]. In another study, Carvacrol treatment significantly reduced the number of TUNEL-positive cells in kidney tissues of mice that were treated with Cisplatin [56]. We obtained similar results in our study as well. We observed caspase 3 immuno-expressions especially in cell debris in the lumen, round spermatids, and primary spermatocytes. Apoptotic cells had a nuclear staining with TUNEL assay. Our results may show that testicular IR injury initiates apoptosis, while Carvacrol prevents apoptosis during IR injury.

The increase of MDA levels as a marker of lipid peroxidation in testicular IR injury-induced rats compared to control group rats was shown in different studies [57–59]. Yu *et al.* indicated significant increase of MDA levels in acute myocardial infarction model induced rats compared to Sham group rats, while Carvacrol administration decreased MDA levels [60]. Samarghandian *et al.* reported significant increase of MDA levels in age-related OS damaged livers of rats and significant decrease of MDA levels in Carvacrol treatment group [61]. In liver of IR, injury model induced rats MDA levels were significantly increased compared to control group, and MDA levels were significantly decreased in Carvacrol-treated group [55]. Cetik *et al.* reported a significant decrease in MDA levels after Carvacrol administration in their cardiotoxicity model [62]. In our study, our findings were similar to previous studies; we found a significant increase in MDA levels in IR group. We also observed a significant decrease of MDA levels in Carvacrol administrated IR group. We can comment that testicular IR injury causes lipid peroxidation and Carvacrol treatment may protect against IR damage and reduce lipid peroxidation.

In the literature, effects of DMSO on MDA levels were shown. Sahin *et al.* reported that DMSO caused to decrease MDA levels in liver of IR injury-induced rabbits compared to untreated IR group rabbits [63]. Tamjidipoor *et al.* showed MDA levels were increased after ovariectomy and decreased after DMSO application in rats [64]. Li *et al.* indicated that Zymosan injection increased MDA levels and DMSO suppressed the increase of MDA in Zymosan-induced intestinal inflammation model in rats [65]. We observed that DMSO, which we used as a solvent, decreased MDA levels. All these studies may show that Carvacrol and DMSO prevent lipid peroxidation.

There are studies showing decrease of serum testosterone levels after IR in testicular tissue [66, 67]. Some studies also show increase of testosterone levels after different treatment agents. Gholami *et al.* reported a significant decrease of testosterone levels in IR group and a significant increase in honey administrated testicular IR model group, 50 days after reperfusion [68]. Araghi *et al.* reported increase of serum testosterone levels after Carvacrol administration in mouse with Ketamine-induced testicular damage [69]. Other studies and our study may show that IR injury reduces testosterone levels. Despite two hours of ischemia and two hours of reperfusion and Carvacrol administration, serum testosterone levels did not change. To see whether Carvacrol influences testosterone levels, hormone levels can be measured after long-term Carvacrol treatment.

Conclusions

Taken together, IR injury causes HP damage of testicular tissue and spermatogonial cells. There was a significant decrease in total motile sperm percentage in IR group. There was no statistically significant difference for total motile sperm percentage between IR and IR+Carvacrol group, but Carvacrol treatment led to an increase of approximately 3% in the rate of motile sperm in IR+Carvacrol group, which has an important value in terms of assisted reproduction techniques. There was no significant difference for sperm concentration between groups. To better understand the effect of Carvacrol on sperm concentration and motility, Carvacrol administration time should be extended. A significant increase of Kir6.2 activation was detected in IR group. In IR+Carvacrol group, Kir6.2 activation was significantly increased compared to IR group. As a result, it is concluded that Carvacrol has a KATP channel opening effect. Caspase 3 immuno-expression was increased in testicular IR injury-induced group, and it was decreased in Carvacrol-treated group. This suggests that Carvacrol may have an anti-apoptotic effect. There was a significant increase in TUNEL-positive cell number in IR group, and a significant decrease in IR+Carvacrol group. Carvacrol may have protective properties against germ cell death. In testicular IR injury model, it has been observed that Carvacrol has an antioxidant role and significantly reduces MDA levels and prevents OS. A minimal increase in serum testosterone values was observed in the IR+Carvacrol group as a treatment, but no statistically significant difference was found. To understand the effect of Carvacrol on testosterone level, the hormone level should be measured in the tissue or at the end of long-term treatment and administration. Studies have shown that Carvacrol has various bioactivities, such as antiviral, antifungal, antioxidant. Our study showed that Carvacrol, as an antioxidant, reduces lipid peroxidation by inhibiting ROS in testicular IR damage, inhibits apoptosis and acts as a KATP channel opener, thus reducing ischemic damage.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors would like to express their gratitude to Associate Professor Buket Kin Tekce, who is in an eternal sleep, for her great help. This study was supported by Bolu Abant Izzet Baysal University Research Foundation, Bolu, Turkey, project number 2017.08.03.1175.

References

[1] Saba M, Morales CR, De Lamirande E, Gagnon C. Morphological and biochemical changes following acute unilateral testicular torsion in prepubertal rats. J Urol, 1997, 157(3):1149–1154. https://doi.org/10.1016/S0022-5347(01)65158-9 PMID: 9072560

[2] Ringdahl E, Teague L. Testicular torsion. Am Fam Physician, 2006, 74(10):1739–1743. PMID: 17137004

[3] Mansbach JM, Forbes P, Peters C. Testicular torsion and risk factors for orchiectomy. Arch Pediatr Adolesc Med, 2005, 159(12):1167–1171. https://doi.org/10.1001/archpedi.159.12.1167 PMID: 16330742

[4] Greenberg JI, Suliman A, Barillas S, Angle N. Chapter 7. Mouse models of ischemic angiogenesis and ischemia–reperfusion injury. Methods Enzymol, 2008, 444:159–174. https://doi.org/10.1016/S0076-6879(08)02807-3 PMID: 19007664

[5] Werns SW, Lucchesi BR. Free radicals and ischemic tissue injury. Trends Pharmacol Sci, 1990, 11(4):161–166. https://doi.org/10.1016/0165-6147(90)90068-J PMID: 2185587

[6] Elshaari FA, Elfagih RI, Sheriff DS, Barassi IF. Oxidative and antioxidative defense system in testicular torsion/detorsion. Indian J Urol, 2011, 27(4):479–484. https://doi.org/10.4103/0970-1591.91436 PMID: 22279313 PMCID: PMC3263215

[7] Unsal A, Eroglu M, Avci A, Cimentepe E, Guven C, Derya Balbay M, Durak I. Protective role of natural antioxidant supplementation on testicular tissue after testicular torsion and detorsion. Scand J Urol Nephrol, 2006, 40(1):17–22. https://doi.org/10.1080/00365590500407514 PMID: 16452050

[8] Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev, 2001, 53(1):135–159. PMID: 11171943

[9] Akbas H, Ozden M, Kanko M, Maral H, Bulbul S, Yavuz S, Ozker E, Berki T. Protective antioxidant effects of carvedilol in a rat model of ischaemia–reperfusion injury. J Int Med Res, 2005, 33(5):528–536. https://doi.org/10.1177/147323000503300508 PMID: 16222886

[10] Cay A, Alver A, Küçük M, Işik O, Eminağaoğlu MS, Karahan SC, Değer O. The effects of *N*-acetylcysteine on antioxidant enzyme activities in experimental testicular torsion. J Surg Res, 2006, 131(2):199–203. https://doi.org/10.1016/j.jss.2005.11.572 PMID: 16412470

[11] Su LJ, Zhang JH, Gomez H, Murugan R, Hong X, Xu D, Jiang F, Peng ZY. Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. Oxid Med Cell Longev, 2019, 2019:5080843. https://doi.org/10.1155/2019/5080843 PMID: 31737171 PMCID: PMC6815535

[12] Asadi N, Bahmani M, Kheradmand A, Rafieian-Kopaei M. The impact of oxidative stress on testicular function and the role of antioxidants in improving it: a review. J Clin Diagn Res, 2017, 11(5):IE01–IE05. https://doi.org/10.7860/JCDR/2017/23927.9886 PMID: 28658802 PMCID: PMC5483704

[13] Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril, 1979, 31(5):531–537. https://doi.org/10.1016/s0015-0282(16)43999-3 PMID: 446777

[14] Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl, 1987, 8(5):338–348. https://doi.org/10.1002/j.1939-4640.1987.tb00973.x PMID: 2822642

[15] Fraczek M, Wiland E, Piasecka M, Boksa M, Gaczarzewicz D, Szumala-Kakol A, Kolanowski T, Beutin L, Kurpisz M. Fertilizing potential of ejaculated human spermatozoa during *in vitro* semen bacterial infection. Fertil Steril, 2014, 102(3):711–719e1. https://doi.org/10.1016/j.fertnstert.2014.06.002 PMID: 25044081

[16] Peltola V, Mäntylä E, Huhtaniemi I, Ahotupa M. Lipid peroxidation and antioxidant enzyme activities in the rat testis after cigarette smoke inhalation or administration of polychlorinated biphenyls or polychlorinated naphthalenes. J Androl, 1994, 15(4):353–361. https://doi.org/10.1002/j.1939-4640.1994.tb00463.x PMID: 7982804

[17] Turner TT, Tung KS, Tomomasa H, Wilson LW. Acute testicular ischemia results in germ cell-specific apoptosis in the rat. Biol Reprod, 1997, 57(6):1267–1274. https://doi.org/10.1095/biolreprod57.6.1267 PMID: 9408230

[18] Wang X, Sharma RK, Sikka SC, Thomas AJ Jr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil Steril, 2003, 80(3):531–535. https://doi.org/10.1016/s0015-0282(03)00756-8 PMID: 12969693

[19] Zhuo ML, Huang Y, Liu DP, Liang CC. K_{ATP} channel: relation with cell metabolism and role in the cardiovascular system. Int J Biochem Cell Biol, 2005, 37(4):751–764. https://doi.org/10.1016/j.biocel.2004.10.008 PMID: 15694835

[20] Zhou M, He HJ, Tanaka O, Sekiguchi M, Kawahara K, Abe H. Different localization of ATP sensitive K^+ channel subunits in rat testis. Anat Rec (Hoboken), 2011, 294(4):729–737. https://doi.org/10.1002/ar.21348 PMID: 21328565

[21] Acevedo JJ, Mendoza-Lujambio I, de la Vega-Beltrán JL, Treviño CL, Felix R, Darszon A. K_{ATP} channels in mouse spermatogenic cells and sperm, and their role in capacitation. Dev Biol, 2006, 289(2):395–405. https://doi.org/10.1016/j.ydbio.2005.11.002 PMID: 16343479

[22] Gong XD, Li JCH, Leung GPH, Cheung KH, Wong PYD. A BK(Ca) to K(*v*) switch during spermatogenesis in the rat seminiferous tubules. Biol Reprod, 2002, 67(1):46–54. https://doi.org/10.1095/biolreprod67.1.46 PMID: 12079998

[23] Inagaki N, Gonoi T, Clement JP 4th, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science, 1995, 270(5239):1166–1170. https://doi.org/10.1126/science.270.5239.1166 PMID: 7502040

[24] Clement JP 4th, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, Bryan J. Association and stoichiometry of K_{ATP} channel subunits. Neuron, 1997, 18(5):827–838. https://doi.org/10.1016/s0896-6273(00)80321-9 PMID: 9182806

[25] Tsounapi P, Saito M, Dimitriadis F, Kitatani K, Kinoshita Y, Shomori K, Takenaka A, Satoh K. The role of K_{ATP} channels on ischemia–reperfusion injury in the rat testis. Life Sci, 2012, 90(17–18):649–656. https://doi.org/10.1016/j.lfs.2012.03.006 PMID: 22480512

[26] Suntres ZE, Coccimiglio J, Alipour M. The bioactivity and toxicological actions of carvacrol. Crit Rev Food Sci Nutr, 2015, 55(3):304–318. https://doi.org/10.1080/10408398.2011.653458 PMID: 24915411

[27] Hashemipour H, Kermanshahi H, Golian A, Veldkamp T. Effect of thymol and carvacrol feed supplementation on performance, antioxidant enzyme activities, fatty acid composition, digestive enzyme activities, and immune response in broiler chickens. Poult Sci, 2013, 92(8):2059–2069. https://doi.org/10.3382/ps.2012-02685 PMID: 23873553

[28] Luna A, Lábaque MC, Zygadlo JA, Marin RH. Effects of thymol and carvacrol feed supplementation on lipid oxidation in broiler meat. Poult Sci, 2010, 89(2):366–370. https://doi.org/10.3382/ps.2009-00130 PMID: 20075292

[29] Youssefi MR, Moghaddas E, Tabari MA, Moghadamnia AA, Hosseini SM, Farash BRH, Ebrahimi MA, Mousavi NN, Fata A, Maggi F, Petrelli R, Dall'Acqua S, Benelli G, Sut S. *In vitro* and *in vivo* effectiveness of carvacrol, thymol and linalool against *Leishmania infantum*. Molecules, 2019, 24(11):2072. https://doi.org/10.3390/molecules24112072 PMID: 31151304 PMCID: PMC6600403

[30] Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol, 2008, 59(1):2–11. https://doi.org/10.1111/j.1600-0897.2007.00559.x PMID: 18154591

[31] Yanishlieva N, Marinova EM, Gordon MH, Raneva VG. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem, 1999, 64(1):59–66. https://doi.org/10.1016/S0308-8146(98)00086-7

[32] Canbek M, Uyanoglu M, Bayramoglu G, Senturk H, Erkasap N, Koken T, Uslu S, Demirustu C, Aral E, Husnu Can Baser K.

Effects of carvacrol on defects of ischemia–reperfusion in the rat liver. Phytomedicine, 2008, 15(6–7):447–452. https://doi.org/10.1016/j.phymed.2007.11.022 PMID: 18222668

[33] Johnsen SG. Testicular biopsy score count – a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones, 1970, 1(1): 2–25. https://doi.org/10.1159/000178170 PMID: 5527187

[34] Celik E, Oguzturk H, Sahin N, Turtay MG, Oguz F, Ciftci O. Protective effects of hesperidin in experimental testicular ischemia/reperfusion injury in rats. Arch Med Sci, 2016, 12(5): 928–934. https://doi.org/10.5114/aoms.2015.47697 PMID: 27695481 PMCID: PMC5016569

[35] Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol, 2013, 87(7):1157–1180. https://doi.org/10.1007/s00204-013-1034-4 PMID: 23543009

[36] Cheng MH, Pan CY, Chen NF, Yang SN, Hsieh S, Wen ZH, Chen WF, Wang JW, Lu WH, Kuo HM. Piscidin-1 induces apoptosis *via* mitochondrial reactive oxygen species-regulated mitochondrial dysfunction in human osteosarcoma cells. Sci Rep, 2020, 10(1):5045. https://doi.org/10.1038/s41598-020-61876-5 PMID: 32193508 PMCID: PMC7081333

[37] Smaili SS, Hsu YT, Carvalho AC, Rosenstock TR, Sharpe JC, Youle RJ. Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling. Braz J Med Biol Res, 2003, 36(2):183–190. https://doi.org/10.1590/s0100-879x2003000200004 PMID: 12563519

[38] Noma A. ATP-regulated K^+ channels in cardiac muscle. Nature, 1983, 305(5930):147–148. https://doi.org/10.1038/305147a0 PMID: 6310409

[39] Seino S. Physiology and pathophysiology of K_{ATP} channels in the pancreas and cardiovascular system: a review. J Diabetes Complications, 2003, 17(2 Suppl):2–5. https://doi.org/10.1016/s1056-8727(02)00274-x PMID: 12623161

[40] Romeo C, Antonuccio P, Esposito M, Marini H, Impellizzeri P, Turiaco N, Altavilla D, Bitto A, Zuccarello B, Squadrito F. Raxofelast, a hydrophilic vitamin E-like antioxidant, reduces testicular ischemia–reperfusion injury. Urol Res, 2004, 32(5): 367–371. https://doi.org/10.1007/s00240-004-0436-4 PMID: 15316698

[41] Aydos TR, Başar MM, Kul O, Atmaca HT, Uzunalıoğlu T, Kisa Ü, Efe OE. Effects of ozone therapy and taurine on ischemia/reperfusion-induced testicular injury in a rat testicular torsion model. Turk J Med Sci, 2014, 44(5):749–755. https://doi.org/10.3906/sag-1308-20 PMID: 25539540

[42] Aydıner CY, Pul M, İnan M, Bilgi S, Çakır E. Can *N*-acetylcysteine play a role on preventing tissue damage on experimental testicular torsion. Cumhuriyet Med J, 2012, 34(4):462–471. https://dergipark.org.tr/en/pub/cmj/issue/4223/56130

[43] Ozcan L, Otunctemur A, Polat EC, Ozbek E, Kirecci SL, Somay A. Selective nuclear factor kappa B (NFκB) inhibitor, pyrrolidium dithiocarbamate prevents, long-term histologic damage in ischemia–reperfusion injuries after delayed testicular torsion. Urol J, 2016, 13(3):2702–2706. PMID: 27351326

[44] Azizollahi S, Aflatoonian R, Sadighi Gilani MA, Behnam B, Tajik N, Asghari-Jafarabadi M, Asgari HR, Koruji M. Alteration of spermatogenesis following spermatogonial stem cells transplantation in testicular torsion–detorsion mice. J Assist Reprod Genet, 2016, 33(6):771–781. https://doi.org/10.1007/s10815-016-0708-2 PMID: 27052833 PMCID: PMC4889484

[45] Mete F, Tarhan H, Celik O, Akarken I, Vural K, Ekin RG, Aydemir I, Ilbey YO. Comparison of intraperitoneal and intratesticular ozone therapy for the treatment of testicular ischemia–reperfusion injury in rats. Asian J Androl, 2017, 19(1):43–46. https://doi.org/10.4103/1008-682X.171570 PMID: 26732112 PMCID: PMC5227672

[46] Kemahli E, Yildiz M, Firat T, Özyalvaçli ME, Üyetürk U, Yilmaz B, Gücük A. An experimental study on effects of pyrrolidine dithiocarbamate on ischemia–reperfusion injury in testis. Can Urol Assoc J, 2016, 10(3–4):E104–E109. https://doi.org/10.5489/cuaj.3160 PMID: 27330576 PMCID: PMC4907771

[47] Hekimoglu A, Kurcer Z, Aral F, Baba F, Sahna E, Atessahin A. Lycopene, an antioxidant carotenoid, attenuates testicular injury caused by ischemia/reperfusion in rats. Tohoku J Exp Med, 2009, 218(2):141–147. https://doi.org/10.1620/tjem.218.141 PMID: 19478470

[48] Taati M, Moghadasi M, Dezfoulian O, Asadian P, Kheradmand A, Abbasi M, Zendehdel M. The effect of ghrelin pretreatment on epididymal sperm quality and tissue antioxidant enzyme activities after testicular ischemia/reperfusion in rats. J Physiol Biochem, 2012, 68(1):91–97. https://doi.org/10.1007/s13105-011-0122-2 PMID: 21994044

[49] Teshima Y, Akao M, Li RA, Chong TH, Baumgartner WA, Johnston MV, Marbán E. Mitochondrial ATP-sensitive potassium channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress. Stroke, 2003, 34(7): 1796–1802. https://doi.org/10.1161/01.STR.0000077017.60947.AE PMID: 12791941

[50] Zhang X, Zhang X, Xiong Y, Xu C, Liu X, Lin J, Mu G, Xu S, Liu W. Sarcolemmal ATP-sensitive potassium channel protects cardiac myocytes against lipopolysaccharide-induced apoptosis. Int J Mol Med, 2016, 38(3):758–766. https://doi.org/10.3892/ijmm.2016.2664 PMID: 27430376 PMCID: PMC4990318

[51] Oliveira IS, da Silva FV, Viana AFSC, dos Santos MRV, Quintans-Júnior LJ, Martins MCC, Nunes PHM, Oliveira FA, Oliveira RCM. Gastroprotective activity of carvacrol on experimentally induced gastric lesions in rodents. Naunyn Schmiedebergs Arch Pharmacol, 2012, 385(9):899–908. https://doi.org/10.1007/s00210-012-0771-x PMID: 22739789

[52] Al-Maghrebi M, Renno WM. Genistein alleviates testicular ischemia and reperfusion injury-induced spermatogenic damage and oxidative stress by suppressing abnormal testicular matrix metalloproteinase system *via* the Notch 2/Jagged 1/Hes-1 and caspase-8 pathways. J Physiol Pharmacol, 2016, 67(1):129–137. PMID: 27010902

[53] Ayan M, Tas U, Sogut E, Caylı S, Kaya H, Esen M, Erdemir F, Uysal M. Protective effect of thymoquinone against testicular torsion induced oxidative injury. Andrologia, 2016, 48(2):143–151. https://doi.org/10.1111/and.12424 PMID: 25906970

[54] Li WT, Zhang SY, Zhou YF, Zhang BF, Liang ZQ, Liu YH, Wei Y, Li CK, Meng XJ, Xia M, Dan Y, Song JN. Carvacrol attenuates traumatic neuronal injury through store-operated Ca^{2+} entry-independent regulation of intracellular Ca^{2+} homeostasis. Neurochem Int, 2015, 90:107–113. https://doi.org/10.1016/j.neuint.2015.07.020 PMID: 26220904

[55] Suo L, Kang K, Wang X, Cao Y, Zhao H, Sun X, Tong L, Zhang F. Carvacrol alleviates ischemia reperfusion injury by regulating the PI3K-Akt pathway in rats. PLoS One, 2014, 9(8):e104043. https://doi.org/10.1371/journal.pone.0104043 PMID: 25083879 PMCID: PMC4118998

[56] Potočnjak I, Domitrović R. Carvacrol attenuates acute kidney injury induced by cisplatin through suppression of ERK and PI3K/Akt activation. Food Chem Toxicol, 2016, 98(Pt B):251–261. https://doi.org/10.1016/j.fct.2016.11.004 PMID: 27825756

[57] Ghasemnejad-Berenji M, Ghazi-Khansari M, Yazdani I, Saravi SSS, Nobakht M, Abdollahi A, Ansari JM, Ghasemnejad-Berenji H, Pashapour S, Dehpour AR. Rapamycin protects testes against germ cell apoptosis and oxidative stress induced by testicular ischemia–reperfusion. Iran J Basic Med Sci, 2017, 20(8):905–911. https://doi.org/310.22038/IJBMS.2017.9112 PMID: 29085582 PMCID: PMC5651476

[58] Jiang D, Wu D, Zhang Y, Xu B, Sun X, Li Z. Protective effects of hydrogen rich saline solution on experimental testicular ischemia–reperfusion injury in rats. J Urol, 2012, 187(6):2249–2253. https://doi.org/10.1016/j.juro.2012.01.029 PMID: 22503049

[59] Cvetkovic T, Stankovic J, Najman S, Pavlovic D, Stokanovic D, Vlajkovic S, Dakovic-Bjelakovic M, Cukuranovic J, Zivkovic V, Stefanovic V. Oxidant and antioxidant status in experimental rat testis after testicular torsion/detorsion. Int J Fertil Steril, 2015, 9(1):121–128. https://doi.org/10.22074/ijfs.2015.4216 PMID: 25918600 PMCID: PMC4410030

[60] Yu W, Liu Q, Zhu S. Carvacrol protects against acute myocardial infarction of rats *via* anti-oxidative and anti-apoptotic pathways. Biol Pharm Bull, 2013, 36(4):579–584. https://doi.org/10.1248/bpb.b12-00948 PMID: 23386194

[61] Samarghandian S, Azimi-Nezhad M, Farkhondeh T. Preventive effect of carvacrol against oxidative damage in aged rat liver. Int J Vitam Nutr Res, 2017, 87(1–2):59–65. https://doi.org/10.1024/0300-9831/a000393 PMID: 27866466

[62] Cetik S, Ayhanci A, Sahinturk V. Protective effect of carvacrol against oxidative stress and heart injury in cyclophosphamide-induced cardiotoxicity in rat. Braz Arch Biol Technol, 2015, 58(4):569–576. https://doi.org/10.1590/S1516-8913201500022

[63] Sahin M, Avsar FM, Ozel H, Topaloglu S, Yilmaz B, Pasaoglu H, Avunduk MC, Erikoglu M, Hengirmen S. The effects of dimethyl sulfoxide on liver damage caused by ischemia–reperfusion. Transplant Proc, 2004, 36(9):2590–2592. https://doi.org/10.1016/j.transproceed.2004.09.057 PMID: 15621097

[64] Tamjidipoor A, Tavafi M, Ahmadvand H. Effect of dimethyl sulfoxide on inhibition of post-ovariectomy osteopenia in rats. Connect Tissue Res, 2013, 54(6):426–431. https://doi.org/10.3109/03008207.2013.841678 PMID: 24020358

[65] Li YM, Wang HB, Zheng JG, Bai XD, Zhao ZK, Li JY, Hu S. Dimethyl sulfoxide inhibits zymosan-induced intestinal inflammation and barrier dysfunction. World J Gastroenterol, 2015, 21(38):10853–10865. https://doi.org/10.3748/wjg.v21.i38.10853 PMID: 26478676 PMCID: PMC4600586

[66] Zhang X, Lv F, Tang J. Protection from ischemia by preconditioning, postconditioning, and combined treatment in rabbit testicular ischemia reperfusion injury. Arch Biochem Biophys, 2016, 608:1–7. https://doi.org/10.1016/j.abb.2016.08.020 PMID: 27586089

[67] Tsounapi P, Saito M, Dimitriadis F, Shimizu S, Kinoshita Y, Shomori K, Satoh I, Satoh K. Protective effect of sivelestat, a neutrophil elastase inhibitor, on ipsilateral and contralateral testes after unilateral testicular ischaemia–reperfusion injury in rats. BJU Int, 2010, 107(2):329–336. https://doi.org/10.1111/j.1464-410X.2010.09481.x PMID: 20575981

[68] Gholami M, Abbaszadeh A, Khanipour Khayat Z, Anbari K, Baharvand P, Gharravi AM. Honey improves spermatogenesis and hormone secretion in testicular ischaemia–reperfusion-induced injury in rats. Andrologia, 2018, 50(1):e12804. https://doi.org/10.1111/and.12804 PMID: 28370451

[69] Araghi A, Tabari MA, Golshahi H. Protective effect of carvacrol on ketamine induced testicular damage in mouse model of schizophrenia. J Herbmed Pharmacol, 2017, 6(3):100–106. http://herbmedpharmacol.com/Article/JHP_4077_20170221211626

Corresponding author

Cemre Nur Balci, MSc, Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, 07070 Antalya, Turkey; Phone +905073889024, Fax +902422496903, e-mail: balcicemrenur@gmail.com

Received: December 9, 2020

Accepted: September 16, 2021