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Eucalyptol attenuates testicular ischemia-reperfusion injury in rats

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Abstract

The efficacy of eucalyptol on testicular ischemia-reperfusion (I/R) injury remains uncertain. This study aimed to investigate the protective effects of eucalyptol on I/R injury induced by testicular torsion/detorsion (T/D) in rats. A total of 32 rats were divided into four groups, including control, T/D, E100 (T/D + 100 mg kg^{-1} eucalyptol administered 30 min prior to torsion), and E200 (T/D + 200 mg kg^{-1} eucalyptol administered 30 min prior to torsion). Testicular T/D was induced in the left testis *via* a 720° clockwise torsion for 3 hr, followed by 3 hr of detorsion. Testicular tissues were harvested for histopathological, immunohistochemical including cleaved caspase-3, B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), Bcl-2, and proliferating cell nuclear antigen (PCNA), and biochemical parameter as glutathione peroxidase (GPx), reduced glutathione (rGSH), glucose-6-phosphate dehydrogenase (G6PD), vitamin C, and malondialdehyde (MDA) analyses. In the T/D group, significant reductions in GPx, rGSH, G6PD, and vitamin C levels were observed, alongside increased MDA levels compared to the control group. Immuno-histochemically, the T/D group exhibited increased expression of cleaved caspase-3 and Bax, along with decreased expression of Bcl-2 and PCNA, compared to the control group. Histologically, Johnsen and Cosentino scores were irregular in the T/D group. Pre-treatment with eucalyptol resulted in reduced MDA, cleaved caspase-3, and Bax levels, while GPx, rGSH, vitamin C, G6PD, PCNA, and Bcl-2 levels increased. Additionally, improvements in Cosentino and Johnsen scores and histopathological damage were observed. These findings suggest that eucalyptol may exert a protective effect against I/R injury caused by testicular T/D, likely due to its anti-oxidant and anti-apoptotic properties.

Introduction

Testicular torsion/detorsion (T/D)-induced testicular ischemia-reperfusion (I/R) injury is one of the leading causes of subfertility in males.[1,2] Testicular torsion is more frequently observed in children, adolescents, and young adults, and it is considered a urological emergency.[3,4] The condition occurs when the spermatic cord twists upon itself, obstructing blood flow and leading to testicular ischemia. If not treated within 4–6 hr, infarction of the testicular tissue ensues.[4,5] Intra-vaginal torsion, where the spermatic cord and testis twist within the tunica vaginalis, is the most common form; however, extra-vaginal torsion, involving the twisting of the tunica vaginalis, can also occur. Surgical intervention is essential for detorsion and restoration of blood flow. Even with successful detorsion, irreversible damage to the testis may occur. Reports indicate that the rates of testicular atrophy and infertility following successful surgery range between 40.00 and 60.00%.[3,5,6] Recent clinical and experimental researches have shown that ipsilateral testicular atrophy can occur even after detorsion and orchiopexy.[5,7] This condition is exacerbated by disruptions in testicular histo-architecture, decreased testosterone levels, impaired spermatogenesis, and germ cell loss. Numerous researches have provided evidence linking oxidative stress-sensitive signaling pathways to testicular I/R injury.[6,8,9]

Ischemia-reperfusion injury arises when blood flow is restored following an acute ischemic event. During T/D, testicular injury progresses through two stages, including the ischemic phase, where disrupted blood flow damages metabolically active tissues, and the reperfusion phase,

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where the restoration of blood flow paradoxically triggers a cascade of events causing further cellular and tissue damage.[5,6,10] The underlying mechanisms of this injury include the generation of free radicals, lipid peroxidation, pro-inflammatory cytokine release, apoptosis, hypoxia, and alterations in micro-vascular blood flow.[6,10,11] Additionally, hypoxia has been shown to induce the production of reactive oxygen species (ROS) through various pathways.[6,10] Hypoxia-induced excessive ROS generation leads to lipid peroxidation, DNA damage, and apoptosis in testicular cells and sperms. Furthermore, hypoxia stimulates prostaglandin E1 production, contributing to collagen accumulation and vascular dysfunction, and upregulates vascular endothelial growth factor, inhibiting germ cell proliferation.[10,11] In this context, anti-oxidants may mitigate such damages by neutralizing free radicals or preventing their formation within testicular cells.

Eucalyptol (1.8-cineole), an oxygenated monoterpene, derived from eucalyptus species, constitutes a major component of eucalyptus essential oils and is also found in the extracts of several other plants, such as sage, camphor, and rosemary.[12,13] Eucalyptus has a long history of use in traditional medicine and is widely used in foods, fragrances, and cosmetics due to its flavor and aroma.[12] Eucalyptol has been investigated in various cellular and animal models, as well as patients with chronic diseases. It has demonstrated anti-oxidant and anti-inflammatory bioactivities in conditions, such as respiratory diseases, pancreatitis, liver and kidney disorders, and colon injury.[13-16] Researches have highlighted eucalyptol's potent free radical-scavenging activity and its ability to protect cells from oxidative damage by neutralizing ROS.[13,17] Eucalyptol has been reported to exhibit a range of biological and pharmacological activities, primarily through the modulation of nuclear factor kappa B and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways.[13,14]

Currently, numerous experimental researches are being conducted to prevent and/or treat T/D-induced I/R injury.[9,11,18] However, there are no studies on the effects of eucalyptol on testicular I/R injury. In this research, we established an experimental testicular T/D model in rats to evaluate whether eucalyptol provides protection against testicular damage in I/R injury and whether this effect is linked to oxidative stress and apoptosis. The study aimed to demonstrate the effects of eucalyptol on experimental testicular T/D-induced I/R injury in rats through changes in biochemical parameters (glutathione peroxidase [GPx], glucose-6-phosphate dehydrogenase [G6PD], reduced glutathione [rGSH], malondialdehyde [MDA], and vitamin [Vit] C) and histopathological and immunohistochemical (cleaved caspase-3, B-cell lymphoma 2 [Bcl-2]-associated X protein [Bax], Bcl-2, and proliferating cell nuclear antigen [PCNA]) evaluations.

Materials and Methods

Chemicals. In this study, eucalyptol (99.00% purity; Sigma-Aldrich, Darmstadt, Germany), ketamine (Ege-vet, Izmir, Türkiye), and xylazine (Ege-Vet) were used. All other chemicals used were of analytical grade and supplied by Sigma-Aldrich, Thermo, and Merck.

Experimental animals. Wistar albino rats (8 - 10 weeks) weighing 250 – 300 g were used in the research. The animals were procured 1 week prior to the commencement of the experimental protocol, and their health statuses were monitored. They were housed in standard plastic cages maintained at 20.00 - 22.00 ˚C with a 12-hr light/dark cycle and provided with *ad libitum* access to food. Present study was approved by Hatay Mustafa Kemal University Local Ethical Committee of Experimental Animal Ethics granted ethical permission for the investigation (Approval Number: 2022/02-07, 28 February 2022).

Testicular ischemia-reperfusion injury induction and experimental design. All procedures were performed under sterile conditions. To induce testicular T/D-associated I/R injury, general anesthesia was administered *via* intra-peritoneal (IP) injection of 10.00 mg kg^{-1} xylazine and 50.00 mg kg^{-1} ketamine. A vertical scrotal incision was made to access the left testis of the experimental animals. For T/D-induced I/R injury, the left testis was rotated 720° clockwise and affixed to the inner scrotal wall using cross mattress sutures with 4/0 thread (Polypropylene; Dogsan, Trabzon, Türkiye). Torsion was maintained for 3 hr to induce ischemia, followed by 3 hr of reperfusion upon detorsion.[9,19] The vehicle solution used for the T/D group contained physiological saline with 3.00% ethanol and 2.00% Tween 80. Immediately before application, the eucalyptol solution (10.00%) was prepared with 3.00% ethanol, 2.00% Tween 80, and 85.00% physiological saline.[15,16] The rats were divided into four groups (N = 32), including control, T/D, T/D + eucalyptol 100 (E100), and T/D + eucalyptol 200 (E200). In the control group, the animals were given 0.50 mL of vehicle solution (IP). Under general anesthesia, after 30 min, only a skin incision was made (without testicular T/D), the scrotum was opened and closed, and the rats were sacrificed within the following 6 hr and their testicular tissues were removed. In the T/D group, 0.50 mL of vehicle solution was given IP to the animals. After 30 min, under general anesthesia, torsion was created by exteriorizing the testicle, rotating it 720° clockwise, and maintaining the torsion for 3 hr. Then, detorsion was performed, and reperfusion was allowed for 3 hr. In the E100 group, 100 mg kg^{-1} eucalyptol solution was administered IP to the animals.[9,15] After 30 min, under general anesthesia, torsion was created by exteriorizing the testicle, rotating it 720° clockwise, and maintaining the torsion for 3 hr. Then, detorsion was performed, and reperfusion was allowed for 3 hr. In the E200 group, 200 mg kg^{-1} eucalyptol solution was administered IP to the animals.[17] After 30 min, under general anesthesia, torsion was created by exteriorizing the testicle, rotating it 720° clockwise, and maintaining the torsion for 3 hr. Then, detorsion was performed, and reperfusion was allowed for 3 hr. At the conclusion of each experiment, testicular tissues were excised under general anesthesia (10.00 mg kg^{-1} xylazine and 100 mg kg^{-1} ketamine hydrochloride). Rats were then euthanized *via* cervical dislocation. The collected testicular samples were subjected to biochemical, histopathological, and immunohistochemical analyses.

Histopathological examination. Testicular tissue samples were fixed in 10.00% neutral formaldehyde solution for 24 - 48 hr and subsequently processed through alcohol and xylene series before embedding in paraffin. Paraffin blocks were sectioned at 4.00 - 5.00 µm thickness, stained with Hematoxylin and Eosin, and examined under a light microscope (BX51; Olympus, Tokyo, Japan).[20] Images were taken using an Olympus EP50 to assess mean seminiferous tubule diameter (MSTD) and germinal epithelium thickness (GET). Johnsen's scoring system was used to evaluate the stages of spermatogenesis,[21] and the degree of tubular damage and necrosis was assessed using the Cosentino scoring method.[22] A blinded pathologist examined all relevant sections at 20 × magnification, evaluating seminiferous tubules in 20 different regions, and averages were calculated for each section. Additionally, findings of severe deterioration in seminiferous tubules, degeneration, disorganization, and desquamation of the germinal epithelium, coagulation necrosis, congestion, hemorrhage, and inter-tubular edema were scored semi-quantitatively as none (-), mild (+), moderate (++), severe (+++).

Immunohistochemical examination. Immunohistochemical staining was performed according to the previously reported method,[15] using UltraVision Detection System anti-polyvalent, horseradish peroxidase (HRP; Thermo Scientific, Waltham, USA). Primary antibodies included anti-Bax (1:200; Elabscience, Houston, USA), anti-Bcl-2 (1:200; Thermo Fisher Scientific, Waltham, USA), anti-cleaved caspase-3 (1:100; Merck Millipore, Hayward, USA), and anti-PCNA (Santa Cruz Biotechnology, Dallas, USA). In negative controls, sections were incubated with phosphate-buffered saline instead of primary antibodies. The 3.3-diaminobenzidine was used as a chromogen, and sections were counterstained with Mayer's Hematoxylin. Immunopositivity in seminiferous tubules was semi-quantitatively evaluated in 40 different fields at 20× by a blinded pathologist (0: No staining, 1: Mild staining, 2: Moderate staining, and 3: Severe staining).[15]

Tissue homogenization for biochemical analysis. For biochemical analyses, 100 mg of testicular tissue was homogenized in 1.00 mL of phosphate buffer by ultra-sonic homogenizer. The homogenates were centrifuged at 4,000 rpm and 4.00 ˚C for 40 min, and the supernatants were stored at – 80.00 ˚C until biochemical analysis.[9]

Determination of malondialdehyde, anti-oxidant status, and total protein levels in testis. The MDA levels in testicular supernatants were determined using the method reported by Ohkawa *et al*.,[23] and results were expressed as nmol/g tissue. Vitamin C levels were measured according to the Haag,[24] and optical densities were read at 520 nm using the enzyme-linked immunosorbent assay reader (µQuant; BioTek Instruments, Winooski, USA). The rGSH levels were measured using Ellman method,[25] with absorbance read at 412 nm and results expressed as µmol per g protein. The GPx activity was determined following the method of Beutler,[26] and expressed as U per g protein. The G6PD activity was assessed using the method described by Beutler,[27] and expressed as U per g protein. Total protein concentrations were also measured using the method of Lowry *et al*.,[28] and utilized to calculate rGSH levels, as well as GPx and G6PD activities.

Statistical analysis. The G*Power software (version 3.1; UCLA, California, USA) was used for the sample size calculation. It was resulted that the minimum number of rats was 32, considering an effect size of 0.63, an alpha value of 0.05, and a power of 0.80. Data were analyzed using SPSS Software (version 25.0; IBM Corp., Armonk, USA). One-way analysis of variance and *post-hoc* Duncan tests were applied to compare biochemical parameters across groups. Histopathological and immunohistochemical analyses were evaluated using the Kruskal-Wallis test, with inter-group comparisons conducted *via* the Mann-Whitney U test. A significance level of $p < 0.05$ was considered statistically significant.

Results

Effect of eucalyptol on Cosentino and Johnsen scores and histopathological findings. The histological and histopathological statistical comparisons between groups are shown in Figure 1 and Table 1. Testicular tissue sections from the control group were observed to have normal architectural structures (Fig. 2A). In the T/D group, severe disruption of seminiferous tubules, degeneration, disorganization, and desquamation of the germinal epithelium, coagulation necrosis, congestion, hemorrhage, and inter-tubular edema were identified (Figs. 2B and 2C). The E100 and E200 groups exhibited less severe and widespread histopathological changes compared to the T/D group (Figs. 2D and 2E and Table 1). The MSTD and GET values were found to be significantly lower in the T/D group compared to the control group ($p < 0.001$). However, the E100 and E200 groups showed improvements in MSTD and GET values compared to the T/D group (Figs. 2D and 2E).

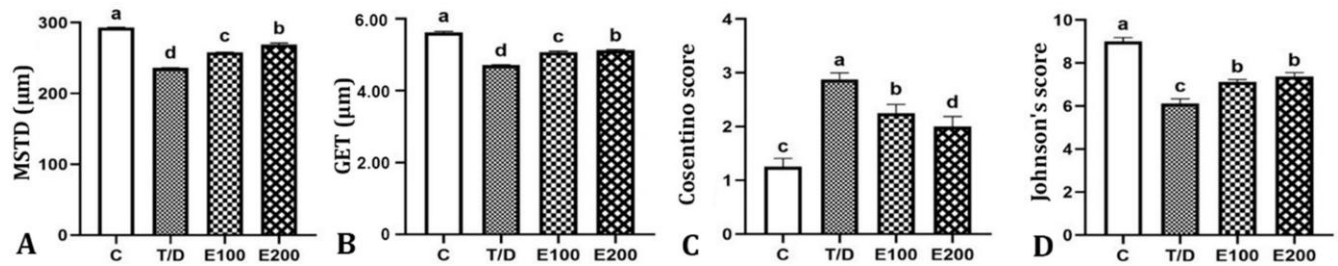

Fig. 1. The effect of eucalyptol on **A)** mean seminiferous tubule diameter (MSTD), **B)** germinal epithelium thickness (GET), **C)** Cosentino and **D)** Johnson's scores. Data are presented as mean ± standard error (n = 8). C: Control; T/D: Torsion/detorsion; E100: T/D + 100 mg kg^{-1} eucalyptol; E200: T/D + 200 mg kg^{-1} eucalyptol.

$^{a-d}$ Means with different superscripts are statistically significant ($p < 0.001$).

Table 1. Histopathological scores between groups.

Histopathological lesions	Control	T/D	E100	E200
Severe disruption of seminiferous tubules	-	+++	++	++
Degeneration, disorganization, and desquamation of the germinal epithelium	-	+++	++	+
Coagulation necrosis	-	+++	++	++
Congestion	+	+++	++	++
Hemorrhage	-	++	+	+
Inter-tubular edema	+	+++	++	++

T/D: Torsion/detorsion; E100: T/D + 100.00 mg kg^{-1} eucalyptol; E200: T/D + 200 mg kg^{-1} eucalyptol; (-): None; (+): Mild; (++): Moderate; (+++): Severe.

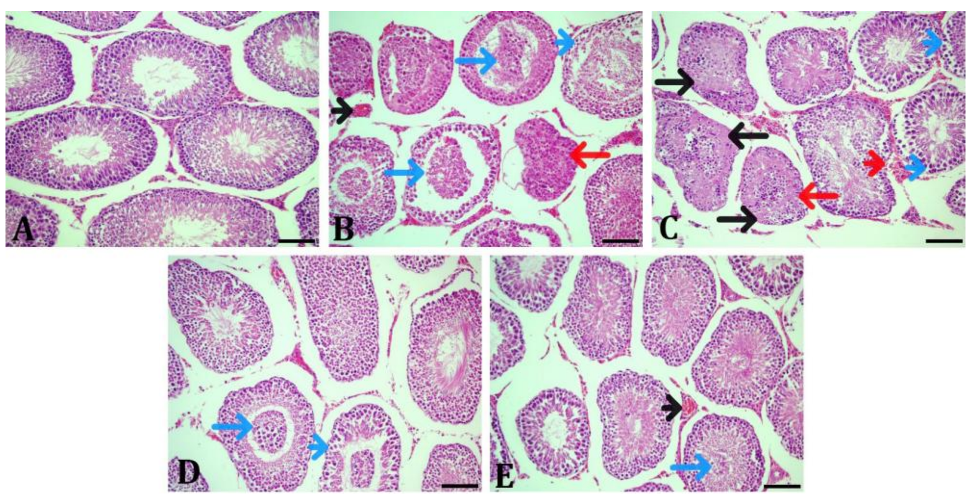

Fig. 2. Microscopic examination of testicular tissues between groups. **A)** Histological appearance of the testicles in the control group with normal structure; **B)** Torsion/detorsion (T/D) group, severe disarray of seminiferous tubules, atrophy of seminiferous tubule (red arrow), disarray of germinal epithelium, degenerated (blue arrowhead) and sloughed and packed cells (blue arrows), and congestion (black arrowhead) are evident; **C)** Coagulation necrosis (black arrows), atrophic seminiferous tubule (red arrow), degeneration (blue arrowheads), and bleeding (red arrowhead) in the T/D group; **D)** Degeneration (blue arrowhead), and shed and packed cells (blue arrow) in the germinal epithelium in the T/D + 100 mg kg^{-1} eucalyptol group; **E)** In the T/D + 200 mg kg^{-1} eucalyptol group, sloughed and packed cells (blue arrow) and congestion (black arrowhead) are observed (Hematoxylin and Eosin staining; bars = 100 µm).

When assessing the degree of tubular injury and necrosis using the Cosentino scoring system, the T/D group demonstrated statistically higher scores than the control group ($p < 0.001$). The E100 and E200 groups significantly reduced these scores relative to the T/D group. In the evaluation of spermatogenesis stages using the Johnsen scoring system, the T/D group exhibited significantly lower scores compared to the control group ($p < 0.001$). The E100 and E200 groups appeared to restore these scores to a more regular state compared to the T/D group.

Effect of eucalyptol on cleaved caspase-3, Bax, Bcl-2, and PCNA levels. Immunohistochemical scores are presented in Figure 3. No immunoreactivity was detected in negative control staining. Cleaved caspase-3 and PCNA showed nuclear staining, while Bax and Bcl-2 showed cytoplasmic staining. In the T/D group, Bcl-2 and PCNA expressions decreased, while cleaved caspase-3 and Bax expressions increased compared to the control group ($p < 0.001$). Pre-treatment with eucalyptol decreased cleaved caspase-3 and Bax expressions while increasing Bcl-2 and PCNA expressions (Fig. 4).

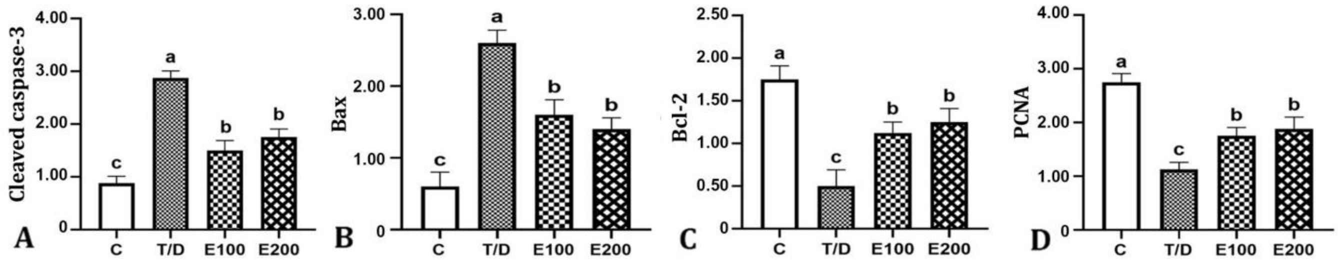

Fig. 3. Statistical scores of the effects of eucalyptol on **A)** cleaved caspase-3, **B)** B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), **C)** Bcl-2, and **D)** proliferating cell nuclear antigen (PCNA) expression in testicular tissue. Data are presented as mean ± standard error (n = 8). C: Control; T/D: Torsion/detorsion; E100: T/D + 100 mg kg^{-1} eucalyptol; E200: T/D + 200 mg kg^{-1} eucalyptol.
^{abc} Means with different superscripts are statistically significant ($p < 0.001$).

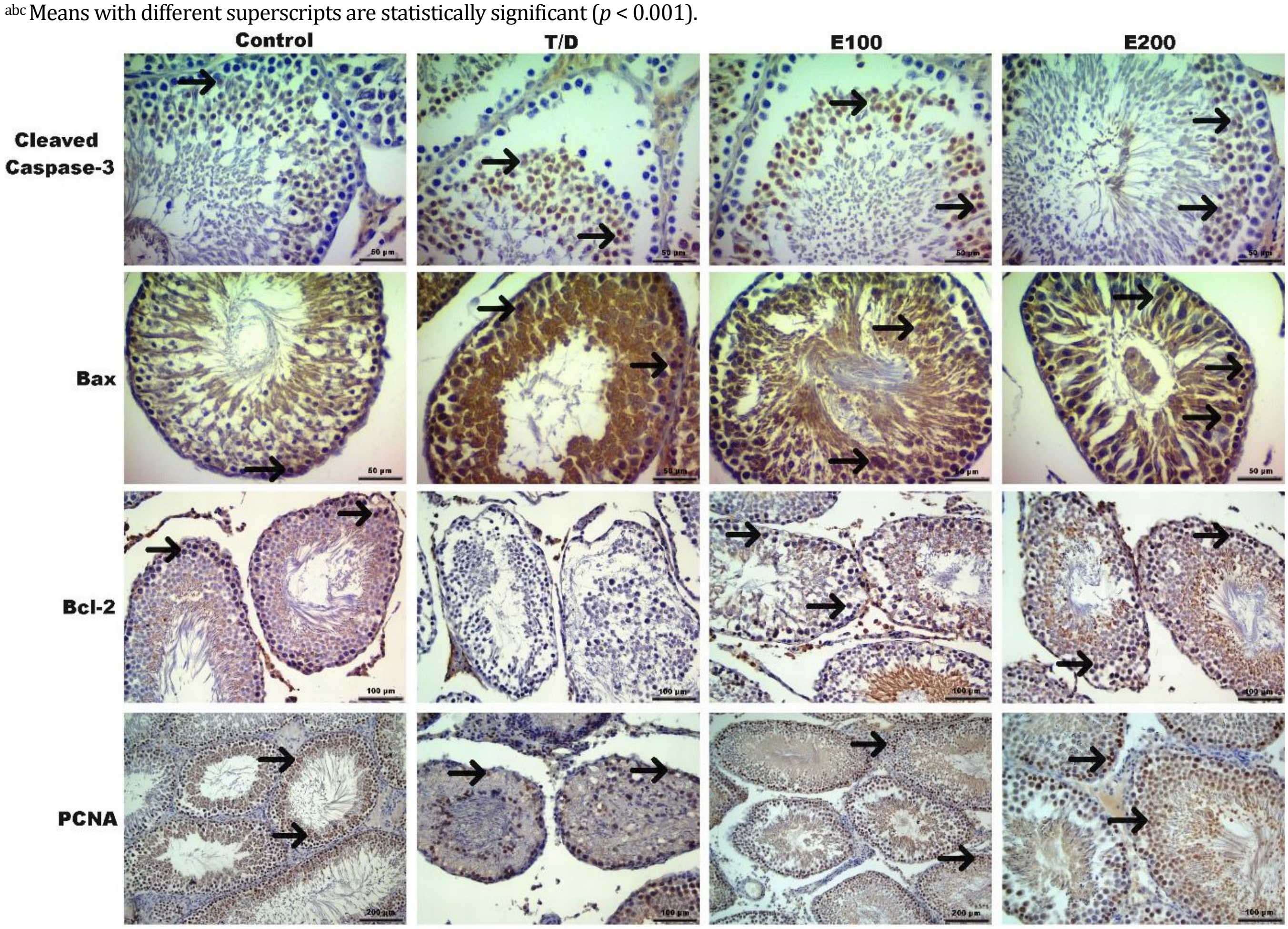

Fig. 4. Immunohistochemical microscopic appearance of cleaved caspase-3, B-cell lymphoma 2 (Bcl-2)-associated X protein Bax, Bcl-2 and proliferating cell nuclear antigen (PCNA) protein expressions among groups. Arrows indicate cytoplasmic immunoreactivity for Bax and Bcl-2, as well as nuclear immunoreactivity for cleaved caspase-3 and PCNA. T/D: Torsion/detorsion; E100: T/D + 100 mg kg^{-1} eucalyptol; E200: T/D + 200 mg kg^{-1} eucalyptol.

Effect of eucalyptol on oxidative stress and anti-oxidant parameters. Data regarding MDA, rGSH, and Vit C levels, and G6PD and GPx activities in testicular tissue are presented in Figure 5. When MDA levels were compared with control group rats, it was determined that there was an important increase in the testicular tissues of T/D group rats ($p < 0.001$). A significant decrease was found in rGSH and Vit C levels, as well as GPx and G6PD activities in the testicular tissues of T/D group rats ($p < 0.001$). Compared to T/D group, decreased MDA levels, and increased rGSH and Vit C levels, as well as GPx and G6PD activities were detected in E100 and E200 groups ($p < 0.001$). Also, the MDA and rGSH levels of E200 group rats were lower than those of control group rats ($p < 0.001$). The Vit C levels, and G6PD and GPx activities were found to be statistically like to control, E100 and E200 groups ($p > 0.05$).

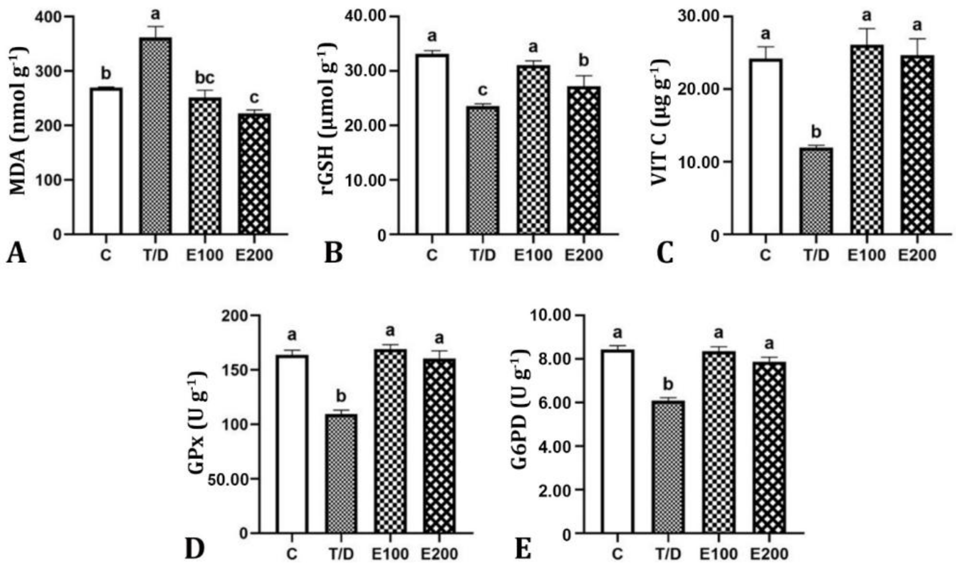

Fig. 5. Effects of eucalyptol on **A)** Malondialdehyde (MDA), **B)** Reduced glutathione (rGSH), **C)** Vitamin C (VIT C), **D)** Glutathione peroxidase (GPx), and **E)** Glucose-6-phosphate dehydrogenase (G6PD) levels in testicular tissue. Data are presented as mean ± standard error (n = 8). C: Control; T/D: Torsion/detorsion; E100: T/D + 100 mg kg^{-1} eucalyptol; E200: T/D + 200 mg kg^{-1} eucalyptol.
[abc] Means with different superscripts are statistically significant ($p < 0.001$).

Discussion

The present study demonstrated that testicular I/R injury induced by T/D adversely affected testicular tissue integrity, oxidative status, and apoptosis-related protein expressions in rats. This was evident through histo-pathological lesions, decreased levels of GSH, GPx, G6PD, and PCNA, increased MDA levels, and heightened apoptosis protein expressions. However, the study revealed that eucalyptol pre-treatment effectively mitigated most of these side effects.

Animal testicular T/D serves as a valuable model for human I/R injury. In recent years, a significant number of researches on testicular I/R have been conducted to explore the protective and/or therapeutic effects of natural compounds with anti-oxidant properties, aiming to translate findings from animal researches to clinical trials and ultimately to clinical practice.[11,18] Additionally, recent research has provided substantial evidence supporting the protective effects of eucalyptol against ischemic damage.[29] In the current study, the protective efficacy of eucalyptol against induced I/R injury was investigated by evaluating oxidative stress and apoptosis processes. To the best of our knowledge, this is the first study to evaluate the effects of eucalyptol (100 and 200 mg kg^{-1}) on testicular T/D-induced I/R injury.

Testicular tissue is particularly susceptible to oxidative stress due to its abundance of highly unsaturated fatty acids, especially 20:4 and 22:6.[30] Excessive ROS production is considered a key factor in testicular I/R injury.[8,18] The MDA, a primary and stable by-product of lipid peroxidation, serves as an indirect biomarker of ROS and is elevated in testicular I/R injury.[6,30,31] Anti-oxidant defense systems, including rGSH, GPx, Vit C, and G6PD, play a crucial role in protecting testicular tissue against ROS and oxidative damage.[30,32] Furthermore, GPx has been identified as the primary ROS scavenger and anti-oxidant enzyme in the male reproductive system, particularly in protecting sperms within testicular and epididymal tissues, and it serves as a first line of defense against oxidative stress in injured testes during I/R.[1,8,33] Previous researches have shown that MDA levels significantly increase in the testes of rats subjected to T/D compared to the controls.[8,34] Abdel-Kader *et al*.,[8] have reported significant increases in testicular MDA levels and decreases in rGSH levels and GPx activities in T/D group rats compared to the sham group ones. Similarly, Davoodi *et al*.,[34] have observed significant increases in MDA levels and reductions in GPx activities in rats subjected to experimental testicular I/R injury. During T/D, the oxidant-antioxidant balance in testicular tissue shifts, leading to changes in G6PD activity, a key enzyme in the hexose monophosphate shunt, and reductions in rGSH content. In this study, consistent with the literature, the T/D group exhibited significantly increased testicular MDA levels and decreased rGSH levels and GPx activities compared to the control group, indicating oxidative damage. Consequently, significant reductions in rGSH and Vit C levels, as well as GPx and G6PD activities, were observed in T/D rats, suggesting diminished free radical scavenging capacity and increased oxidative stress in testicular tissue.

Researches employing testicular I/R models have reported decreases in anti-oxidant parameter values and increases in MDA levels, with protective and therapeutic agents reversing these changes to mitigate ischemic injury.[8,34] In this study, rats pre-treated with eucalyptol (E100 and E200 groups) exhibited significant increases in testicular rGSH and Vitamin C levels, as well as GPx and G6PD activities compared to the T/D group. Moreover, MDA levels were significantly lower in the eucalyptol-treated groups than T/D group. These findings suggest that eucalyptol possesses anti-oxidant properties, protecting testicular tissue by reducing the detrimental effects of free radicals.

Testicular I/R injury has been shown to initiate oxidative and inflammatory processes, contributing to histological disruption, increased apoptosis, and inflammation.[1,6,8] Excessive ROS production during testicular reperfusion damages cellular genomes, induces oxidative stress, and activates caspases, leading to cell death.[4,5,35] In the present study, histopathological findings indicated that the T/D group exhibited adverse effects regarding spermatogenesis, consistent with previous reports.[9,18,34] In the present study, MSTD and GET measurements were found to be significantly reduced in the T/D group compared to the control group. Additionally, statistical scores based on the Johnsen and Cosentino scoring systems were observed to be irregular in the T/D group compared to the control group. These findings are consistent with the results reported in numerous researches in the literature regarding the T/D group.[36,37] Eucalyptol pre-treatment relatively preserved MSTD and GET values, reduced Cosentino scores, and increased Johnsen scores. The results of our study indicate less evidence of I/R injury in the testicular tissue of the T/D group following eucalyptol pre-treatment. Furthermore, when evaluated in conjunction with histopathological findings, it can be suggested that T/D causes severe destruction of testicular tissue, and this damage can be mitigated through pre-treatment with eucalyptol.

Previous researches have reported down-regulation of the Nrf2/HO-1 pathway and up-regulation of the nuclear factor kappa B pathway in the T/D group compared to the controls.[8,37] Many testicular T/D researches have targeted the Nrf2/HO-1 pathway to demonstrate the protective or therapeutic efficacy of anti-oxidant compounds.[8,38] The Nrf2 activation may serve as a promising therapeutic target for testicular dysfunction.[39] In a study conducted by Akcakavak *et al*.,[14] on gentamicin-induced kidney injury in rats, eucalyptol was reported to provide protection against gentamicin-induced kidney damage by up-regulating Nrf-2 pathway and down-regulating nuclear factor kappa B pathway. In another study, eucalyptol was shown to mitigate early brain injury following sub-arachnoid hemorrhage through the up-regulation of Nrf-2 and HO-1.[40] In the present study, the mitigation of testicular T/D histopathological changes by eucalyptol may be attributable to its influence on the Nrf2/HO-1 pathway. In addition, the present results revealed that eucalyptol, being previously reported to reduce damage to kidney and liver tissues, could also alleviate testicular damage following T/D.[14-16]

Apoptosis is defined as programmed cell death and serves as a response to developmental signals or cellular stress. The Bcl-2 family proteins regulate the mitochondrial pathway of apoptosis by controlling the permeability of the outer mitochondrial membrane. Among the members of the Bcl-2 protein family are pro-apoptotic proteins (especially Bax) and anti-apoptotic proteins (especially Bcl-2).[41,42] The balance between Bax and Bcl-2 expression influences the sensitivity to apoptotic cell death by regulating the release of cytochrome c from the mitochondria. Caspase-3, known as the major effector caspase, interacts with caspase-8 and caspase-9, and plays an important role in the apoptosis process through the degradation of nuclear polymerase.[42-44] Spermatogenic cells apoptosis is a frequently observed physiological event in normal homeostasis.[11,43] Furthermore, it is well established that widespread spermatogenic cells death is typically a consequence of the pathogenic process of testicular I/R injury. Recent evidence has shown that apoptosis plays a significant role in the pathogenesis of I/R damage caused by T/D in the testes.[41,42] Shokoohi *et al*., [41] have reported that excessive ROS released after T/D injury led to oxidative stress in testicular parenchyma, causing destructive effects on the cellular genome and inducing apoptosis by activating caspase cascades. Kazak *et al*.,[9] have observed increased levels of cleaved caspase-3 and Bax, and a decrease in Bcl-2 expression in rats after testicular T/D compared to the sham group. It has been reported that ROS induce apoptosis through the intrinsic pathway *via* various mechanisms, primarily by affecting mitochondrial permeability. In the apoptotic mechanisms induced by ROS, the permeable pores of the mitochondrial double-layer membrane are opened, releasing cytochrome c, apoptosis-inducing factor, and Smac/DIABLO proteins, leading to the activation of pro-caspases and caspases.[41-44] The current study demonstrated an increase in cleaved caspase-3 and Bax expressions and a decrease in Bcl-2 expression compared to the control group after T/D, thereby inducing the apoptotic process.

One of the protective mechanisms of eucalyptol against I/R injury may be associated with apoptosis inhibition. In testicular I/R injury, the ischemic phase of testicular tissue leads to necrosis, while the reperfusion phase induces apoptosis. Kim *et al*.,[45] have reported that eucalyptol exhibits therapeutic potential by inhibiting retinal apoptosis through the down-regulation of the pro-apoptotic marker Bax and up-regulation of the anti-apoptotic marker Bcl-2 in diabetic eyes. Wang *et al*.,[46] have found that eucalyptol alleviates isoprenaline-induced apoptosis in H9C2 cardiomyocytes by reducing the Bax/Bcl-2 ratio and expression of cleaved caspase-3.

Additionally, it was reported by Sun *et al.*,[47] that eucalyptol down-regulated cytochrome c and caspase-9 levels in grass carp hepatocytes stimulated by tetrabromo-bisphenol A. In the present study, eucalyptol demonstrated potential for preventing apoptosis by inhibiting the up-regulation of cleaved caspase-3 and Bax, while enhancing Bcl-2 activity in testicular I/R injury induced by T/D. Indeed, the biochemical findings in this study support these relevant results. It is hypothesized that eucalyptol pre-treatment led to a reduction in the elevated ROS and associated oxidative stress levels following T/D, thus having beneficial effects on the expressions of caspase-3, Bax, and Bcl-2. Furthermore, the effects of eucalyptol pre-treatment (E100 and E200 groups) on Bax and Bcl-2 expressions suggest that it may prevent cell apoptosis in testicular I/R injury through the mitochondrial pathway.

The PCNA is a 36-kDa nuclear protein expressed in the G1 and S phases. The PCNA is involved in various processes, including DNA replication, cell cycle control, DNA excision repair, and chromatin assembly.[48] It serves as a marker for the proliferation of spermatogenic cells, and its decreased expression indicates a reduction in the rate of spermatogenesis. In the present study, PCNA expression was observed to decrease significantly in the T/D group compared to the control group, being consistent with previous study findings.[9] Eucalyptol pre-treatment increased PCNA expression compared to the T/D group, and this was thought to be due to its positive effect on spermatogenesis and testicular tissue integrity.

Recently, parallel to pre-clinical studies, interest in the use of eucalyptol has been steadily increasing in the fields of medicine, agriculture, cosmetics, and food. Eucalyptol has been studied in both pre-clinical and clinical settings for its various pharmacological activities. Studies on the pharmacological activities of eucalyptol have revealed surprising potential in the treatment and management of respiratory disorders, COVID-19, pain, oral health, infectious diseases, and cancer.[13,49] Thaker and Nelson,[50] reported that in a literature review published from 2000 to 2020, clinical and laboratory studies focusing on adjuvant pharmacological techniques mitigating torsion-related injury in animal models and humans were evaluated. Fifty-four Food and Drug Administration-approved agents and 52 herbal/investigational drugs were reported in animal models of testicular torsion, with each study demonstrating beneficial effects at measured endpoints of the investigational agents compared to the controls, and despite these universally promising animal findings, no pharmacological studies in humans were reported.[50] The present study is encouraging for the clinical use of eucalyptol for human treatment as it demonstrates protective effects in experimental testicular torsion. However, there is no literature on clinical studies regarding the clinical treatment of eucalyptol or how eucalyptol can be applied clinically for testicular torsion patients. In addition, numerous pharmacologic agents show promise in animal studies, but none have been studied in humans.[50] It is suggested that eucalyptol with its various therapeutic applications may lead to a paradigm shift in the treatment regimen of various diseases in this era of modern science.[49] In conclusion, the potential of a treatment paradigm for how eucalyptol can be applied clinically in patients with testicular torsion remains to be evaluated. This study suggests that a carefully planned clinical trial design using eucalyptol treatment in patients with testicular torsion may lead to significant advances in the field of testicular torsion treatment.

This study shows that eucalyptol (100 and 200 mg kg^{-1}) can effectively reduce histopathological damage, apoptosis, and oxidative stress in testicular T/D-induced I/R injury. The beneficial effects of eucalyptol can be attributed to its ability to support anti-oxidant systems, block oxidative stress, prevent apoptosis, and protect testicular integrity. Moreover, eucalyptol was shown to be an effective protective agent against I/R injury caused by testicular T/D and has the potential for future drug development.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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