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ORIGINAL RESEARCH

Carvacrol attenuates lipopolysaccharide-induced testicular injury in rats through anti-apoptotic mechanisms

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Abstract

Background: Carvacrol, a bioactive compound derived from the herb *Origanum vulgare*, is recognized for its ability to reduce inflammation, oxidative stress, and prevent apoptosis. Lipopolysaccharide (LPS) is a potent trigger that initiates an inflammatory process, oxidative stress, and damage to germ cells within the testes. The purpose of this research was to explore the protective ability of carvacrol against LPS-induced testicular damage in rats. **Methods**: The study was conducted over a two-week period, during which 32 adult Wistar rats were randomly assigned to four groups: Control (saline), LPS (1 mg/kg/day), and C25 and C50 (receiving carvacrol (25 and 50 mg/kg/day) 30 min before LPS administration). Histopathology, sperm parameters and the expression of apoptosis-related genes were assessed. The results were analyzed with Statistical Package for Social Science (SPSS). **Results**: Findings revealed that LPS significantly decreased sperm ($p < 0.001$) and the normal sperm proportion ($p = 0.02$) relative to the Control. Carvacrol treatment notably increased sperm count ($p < 0.001$) and improved sperm morphology in both C25 ($p = 0.006$) and C50 ($p < 0.001$). Furthermore, Johnson's score ($p = 0.008$ and $p < 0.001$), epithelial thickness ($p = 0.002$ and $p < 0.001$), and seminiferous tubule diameter (both $p < 0.001$) were significantly improved in C25 and C50 compared with LPS. Moreover, in C25 and C50, compared with LPS, carvacrol significantly reduced the gene expression of *Bax* ($p = 0.01$ and $p = 0.02$; in that order) and *caspase-3* (both, $p < 0.001$). **Conclusions**: The findings indicate that carvacrol can effectively mitigate LPS-induced testicular dysfunction, potentially through its anti-apoptotic properties. These results highlight the therapeutic capacity of carvacrol in ameliorating testicular injury caused by various pathological conditions. However, additional studies are needed to verify its efficacy and safety in other models and to elucidate molecular pathways involved before carvacrol can be considered a therapeutic agent in clinical settings.

Keywords

Apoptosis; Carvacrol; Lipopolysaccharides; Sperm parameters; Testicular injuries

Carvacrol atenúa la lesión testicular inducida por lipopolisacárido en ratas a través de mecanismos antiapoptóticos

Resumen

Antecedentes: El carvacrol, un compuesto bioactivo derivado de la hierba *Origanum vulgare*, es reconocido por su capacidad para reducir la inflamación, defender contra el estrés oxidativo y prevenir la apoptosis. El lipopolisacárido (LPS) es un potente desencadenante que provoca procesos inflamatorios, carga oxidativa y daño a las células germinales dentro de los testículos. El objetivo de esta investigación fue explorar la capacidad protectora del carvacrol frente al daño testicular inducido por LPS en ratas. **Métodos**: El estudio se llevó a cabo durante un período de dos semanas, en el cual 32 ratas Wistar adultas fueron asignadas aleatoriamente a cuatro grupos: Control (solución salina), LPS (1 mg/kg/día) y C25 y C50 (que recibieron carvacrol (25 y 50 mg/kg/día) 30 minutos antes de la administración de LPS). Se evaluaron la histopatología, los parámetros espermáticos y la expresión de genes relacionados con la apoptosis. Los resultados fueron analizados con el programa Paquete estadístico para ciencias sociales (SPSS). **Resultados**: Los hallazgos revelaron que el LPS disminuyó significativamente el conteo espermático ($p < 0.001$) y la proporción de espermatozoides normales ($p = 0.02$) en comparación con el grupo control. El tratamiento con carvacrol aumentó notablemente el conteo espermático ($p < 0.001$) y mejoró la morfología espermática en ambos grupos C25 ($p = 0.006$) y C50 ($p < 0.001$). Además, la puntuación de Johnson ($p = 0.008$ y $p < 0.001$), el grosor epitelial ($p = 0.002$ y $p < 0.001$) y el diámetro de los túbulos seminíferos (ambos $p < 0.001$) mejoraron significativamente en C25 y C50 en comparación con el grupo LPS. Asimismo, en los grupos C25 y C50 respecto al LPS, el carvacrol redujo de manera significativa la expresión génica de *Bax* ($p = 0.01$ y $p = 0.02$, respectivamente) y *caspasa-3* (ambos $p < 0.001$). **Conclusiones**: Los resultados indican que el carvacrol puede mitigar eficazmente la disfunción testicular inducida por LPS, posiblemente a través de sus propiedades anti-apoptóticas. Estos hallazgos destacan la capacidad terapéutica del carvacrol para mejorar las lesiones testiculares causadas por diversas condiciones patológicas. Sin embargo, se necesitan estudios adicionales para verificar su eficacia y seguridad en otros modelos y dilucidar las vías moleculares involucradas antes de que el carvacrol pueda considerarse un agente terapéutico en entornos clínicos.

Palabras Clave

Apoptosis; Carvacrol; Lipopolisacáridos; Parámetros espermáticos; Lesiones testiculares

1. Introduction

Certain environmental factors and diseases can disrupt testicular tissue, potentially leading to male infertility [1]. One notable cause is infection by Gram-negative bacteria, including species such as Escherichia and Salmonella. The cell walls of these bacteria contain a glycolipid component called lipopolysaccharide (LPS). When introduced into the host, LPS is known as an endotoxin due to its biological activities [2]. LPS, as a bacterial pathogen, is a potent inducer of testicular injury, leading to inflammation, oxidative stress, immune dysfunction, and germ cell damage and apoptosis [3, 4]. The etiology of LPS-induced testicular injury is complex and involves multiple mechanisms, including immune cell activation, increased release of pro-inflammatory cytokines, production of oxidative stress, reactive oxygen species (ROS), and oxidized lipids, as well as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and impairment of the body's antioxidant defenses [4–6]. On one hand, research involving rodent models has demonstrated that LPS exposure causes production of ROS, oxidative stress, pro-inflammatory cytokines, hypoxia, mitochondrial dysfunction, and apoptosis in the testes [4, 7–10]. Oxidative stress, in particular, is recognized as a key factor in impairing both spermatogenesis and steroid hormone production [11]. On the other hand, several studies have shown that compounds such as olibanum, lycopene, lipoic acid, oxytocin, and melatonin can mitigate the detrimental effects of LPS on the male reproductive system due to their various bioactivities, including antioxidant, anti-inflammatory, anti-apoptotic, and immunomodulatory properties [4, 12].

Previous studies have shown that carvacrol is capable of inhibiting the generation of pro-inflammatory cytokines and decreasing oxidative stress in various cell types [13–15]. This compound, a phenolic constituent of *Origanum vulgare* essential oil, has well-documented antioxidant, anti-inflammatory, and anti-apoptotic effects [16]. Its antioxidant activity is attributed to the presence of the hydroxyl (OH) group and phenol structure [17]. Studies indicate that carvacrol can reduce the number of abnormal and dead sperm by mitigating oxidative stress, inflammation, apoptosis, and autophagy induced by sodium arsenite in rat testicular tissue [18]. Additionally, carvacrol has been shown to protect against reproductive damage induced by cisplatin in male rat testes [19]. Further research by Shoorei *et al.* [20] suggested that carvacrol alleviates testicular damage in diabetic rats, likely due to its antioxidant effects. Nonetheless, the impacts of carvacrol on testicular detriment and germ cell apoptosis triggered by LPS exposure have not yet been explored. The main objective of this study was to investigate the effects of carvacrol on LPS-induced testicular damage and its possible protection against this damage. The study focused on sperm parameters (including number and morphology), morphological-histological changes in testicular tissue, and apoptosis genes. The ultimate goal was to determine the possible protective mechanisms of carvacrol and its role in improving reproductive function and reducing inflammatory and oxidative damage caused by LPS, in order to investigate the potential use of carvacrol as an adjunct therapeutic agent in maintaining testicular tissue health and

sperm quality. The novelty of this study lies in the integration of molecular, cellular, and morphological analyses to better understand the protective mechanisms of carvacrol on testicular health and sperm quality in an inflammatory model.

2. Materials and methods

2.1 Animals and experimental setup

Thirty-two adults male Wistar rats, weighing between 250 and 300 grams, were sourced from the animal room of Gonabad University of Medical Sciences (GUMS). Rats were kept in appropriate cages and maintained under consistent environmental and dietary conditions. The housing environment was controlled with a temperature of 22 ± 2 °C, humidity maintained at 40–50%, and a 12-hour light/dark cycle. The rats were allowed free access to food and water during the entire duration of the study. All investigational protocols were approved by the Ethical Committee of Gonabad University of Medical Sciences (No: IR.GMU.REC.1398.129), and the rats care procedures as well as experiment execution were validated by the Committee. The start and end date of the tests of this project was from February 2020 to May 2021. The rats were randomly allocated into four groups of eight animals each:

(a) Control group: rats received 0.9% saline solution injections.

(b) LPS group: rats received daily intraperitoneal (i.p.) injections of lipopolysaccharide at a dosage of 1 mg/kg [21].

(c) C25 group: carvacrol was given i.p. at a dose of 25 mg/kg daily, 30 min prior to LPS injection [22].

(d) C50 group: carvacrol was given i.p. at a dose of 50 mg/kg daily, 30 min prior to LPS injection [22].

The treatment regimen continued for two weeks as illustrated in Fig. 1.

LPS (Escherichia coli 055:B5, Chemical Abstracts Service 93572-42-0) and carvacrol (purity ≥98%, Chemical Abstracts Service Number: 499-75-2) were bought from Sigma (Sigma Aldrich Chemical Co., Louis, MO, USA).

2.2 Collection of samples

After completing the two-week treatment, rats were anesthetized with an i.p. injection of ketamine/xylazine at doses of 100/10 mg/kg, respectively. Under deep anesthesia, euthanasia was performed through exsanguination and cardiac removal, followed by the collection of testicular tissue. The left testis from each animal was immersed in Bouin's fixative for 48 hours to prepare for histopathological analysis, while the right testis was rapidly frozen and stored at −80 °C for subsequent evaluation of *Bax* and *Bcl-2* protein expression.

2.3 Assessment of testicular injury

2.3.1 Histopathological assessment

After fixing the left testes, they were embedded in paraffin blocks. The blocks were subsequently cut into sections measuring 5 μm in thickness, followed by deparaffinization and staining with hematoxylin and eosin (H&E). From each testicular sample, three sections were taken from the upper, middle, and lower regions. The slides were carefully evaluated under a Nikon light microscope (Nikon Eclipse E200 light microscope, Nikon Instruments Inc., Tokyo, Japan) by two independent observers who were unaware of the group assignments. To determine the mean diameter of the seminiferous tubules (STD) and the germinal epithelium height (HE or HST), ten randomly chosen round seminiferous tubules were evaluated on each slide. Testicular damage was evaluated by assessing spermatogenesis through the mean Johnson's score (MJS), which ranges from 1–10 for each seminiferous tubule, following criteria established in previous research [23, 24].

2.3.2 Weight and volume of testis

The testis weight index (Ti) was determined by the formula below (Eqn. 1):

$$Ti = \frac{Testis\ weight\ (g)}{animal\ Body\ weight\ (g)} \times 100 \qquad (1)$$

Testicular volume (Tv) was determined by the following

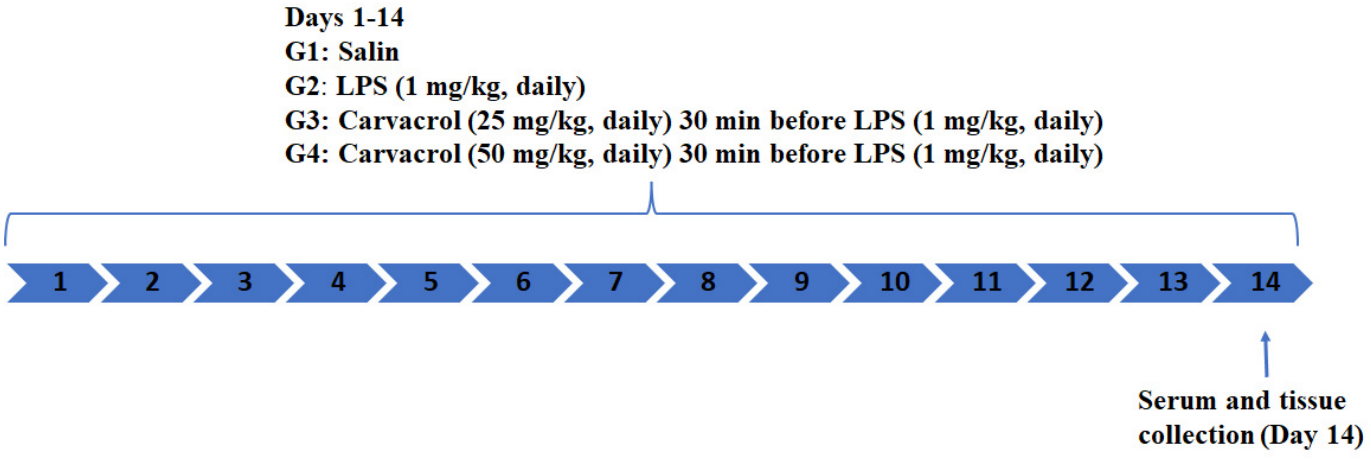

FIGURE 1. Timeline illustrating the key experimental procedures and assessments conducted throughout the study period. LPS: Lipopolysaccharide.

specific formula (Eqn. 2):

$$Tv = \frac{(w2 - w1)}{\delta} \quad (2)$$

W1 = primary weight (beaker + 0.9% saline solution + basket);

W2 = secondary weight (beaker + 0.9% saline solution + basket + testis);

δ = density of 0.9% saline solution (1.0048) [25, 26].

2.3.3 Spermatozoa analysis

The left epididymis was separated and immersed in 1 mL of 0.9% saline solution, followed by dissection and gentle compression. Following this, 4 mL of 0.9% saline solution was combined to dilute the sample, and the mixture was maintained at 37 °C in a CO_2 incubator lasting 10 min. The sample was then thoroughly homogenized by shaking, and a drop was loaded onto a Neubauer slide counting chamber. Spermatozoa were counted under a light microscope at 40× magnification across four squares. The quantity of spermatozoa per milliliter was determined by applying the formula below (Eqn. 3) [26]:

$$N = \frac{Mean\ of\ sperms\ in\ four\ square\ \times\ 2.5}{Volume\ of\ one\ square\ (100\ nL)} \quad (3)$$

Additionally, smears were prepared from the solution to evaluate sperm morphology by applying Papanicolaou staining. The sperm on all slides were then examined microscopically to identify and quantify abnormal forms. Specifically, sperm with coiled tails, those lacking tails, and sperm with bent tails were counted using a light microscope. The formula below (Eqn. 4) was applied to determine the proportion of abnormal spermatozoa [25, 26]:

$$Percentage\ of\ abnormal\ sperm = \frac{Number\ of\ abnormal\ sperms}{Number\ of\ total\ sperms} \times 100 \quad (4)$$

2.3.4 RNA extraction and quantitative real-time PCR analysis

Total RNA extraction from rat testicular tissue was performed utilizing the Favor Prep Blood/Cultured Cell Total RNA Mini Kit (v202505, Favorgen, Taipei, Taiwan, Catalog No: FABRK000). The extracted RNA was eluted in 50 μL of RNase-free water and stored at −80 °C until further use. The RNA purity was evaluated by determining the absorbance ratios at wavelengths of 260/280 nm and 260/230 nm, utilizing a NanoDrop Epoch 2 Microplate Spectrophotometer (Biotech, Winooski, VT, USA). Samples exhibiting an absorbance ratio near 2.0 at 260/280 nm and a 260/230 nm ratio ranging from 2.0 to 2.2 were deemed to be of high purity. Integrity of RNA samples was verified through electrophoretic analysis on a 1.5% agarose gel [27]. An amount greater than 500 ng of total RNA was converted into cDNA using the cDNA Synthesis Kit (Catalog No: YT4500; Yekta Tajhiz Azma®, Tehran, Iran), following the instructions provided by the manufacturer and employing an 18-mer oligo(dT) primer. For each batch, a negative control lacking RevertAid™ M-MuLV reverse transcriptase (RT reaction) was included to detect any genomic DNA contamination. The generated cDNA was stored at −20 °C for up to one week; for longer storage, samples were transferred to −70 °C. Quantitative real-time polymerase chain reaction (PCR) analyses were carried out using an ABI 7500 Real-Time PCR-Fast 7498 system (Foster City, CA, USA) in a total reaction volume of 20 μL. The mixture included BioFact™ 2X Real-Time PCR Smart Mix SYBR Green (batch number: DQ383-40h; BioFact, Daejeon, Korea), cDNA at a concentration of 20 ng/μL, and primers each at 0.4 μM, with nuclease-free water added to complete the volume. Primers were designed using PerlPrimer software version 1.1.20, with sequences listed in Table 1. Each reaction was performed in triplicate. *β-actin* was used as the internal control gene. The thermal cycling protocol included an initial denaturation at 95 °C lasting 5 min, followed by 45 cycles of 95 °C lasting 15 seconds and 61 °C lasting 1 min. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, where ΔCT represents the difference between the cycle threshold (CT) values of the target gene and *β-actin* [28].

2.4 Statistical analysis

The data are presented as mean ± standard error (SE) and were evaluated using SPSS software version 22 (SPSS Inc., Chicago, IL, USA). A one-way Analysis of variance (ANOVA) was conducted, followed by Tukey's *post-hoc* test to assess multiple group differences. Statistical significance was set at *p*-values below 0.05.

3. Results

3.1 Histological examination

The ANOVA revealed a significant effect of treatment on the mean Johnson's score (MJS) ($F_{3,\ 21} = 144.12, p < 0.001$). *Post hoc* comparisons revealed that the MJS was notably lower in the LPS rats relative to the Control rats ($p < 0.001$). In contrast, treatment groups C25 and C50 showed a significant increase in MJS relative to the LPS rats ($p = 0.008$ and $p < 0.001$, respectively). Similarly, carvacrol significantly affected the seminiferous tubule diameter (STD) ($F_{3,\ 21} = 189.63$, $p < 0.001$), with the LPS group exhibiting a significantly reduced STD compared with Controls ($p < 0.001$). Both C25 and C50 groups expressed a significant increase in STD relative to LPS ($p < 0.001$ for both). HE was markedly diminished in the LPS rats compared with the Control rats ($p < 0.001$), whereas treatment with carvacrol at both doses (25 and 50) significantly enhanced HE relative to the LPS rats ($p = 0.002$, $p < 0.001$). Additionally, significant differences were found in MJS, STD, and HE between the C25 and C50 groups (all $p < 0.001$) (Fig. 2, Table 2).

3.2 Weight and volume of testis

Comparison of the testis weight index among the groups indicated a reduction in the LPS rats compared with the Control,

TABLE 1. Oligonucleotide sequences used for quantitative real-time RT-PCR.

Gene	Oligomer sequence (5′-3′)	Amplicon size (bp)
β-actin	Fwd primer: GTCGTGCTTGCCATTCAG Rev primer: GGTATCTTCTTTCCATTCTTCAGTAG	309
Bax	Fwd primer: TTTGCTACAGGGTTTCATCCAG Rev primer: GTTGTCCAGTTCATCGCC	145
Bcl-2	Fwd primer: TGTGGATGACTGACTACCTGAACC Rev primer: CAGCCAGGAGAAATCAAACAGAGG	122
Caspase-3	Fwd primer: GTGGAACTGACGATGATATGGC Rev primer: CGCAAAGTGACTGGATGAACC	135

Fwd: Forward primer; Rev: Reverse primer; bp: base pair.

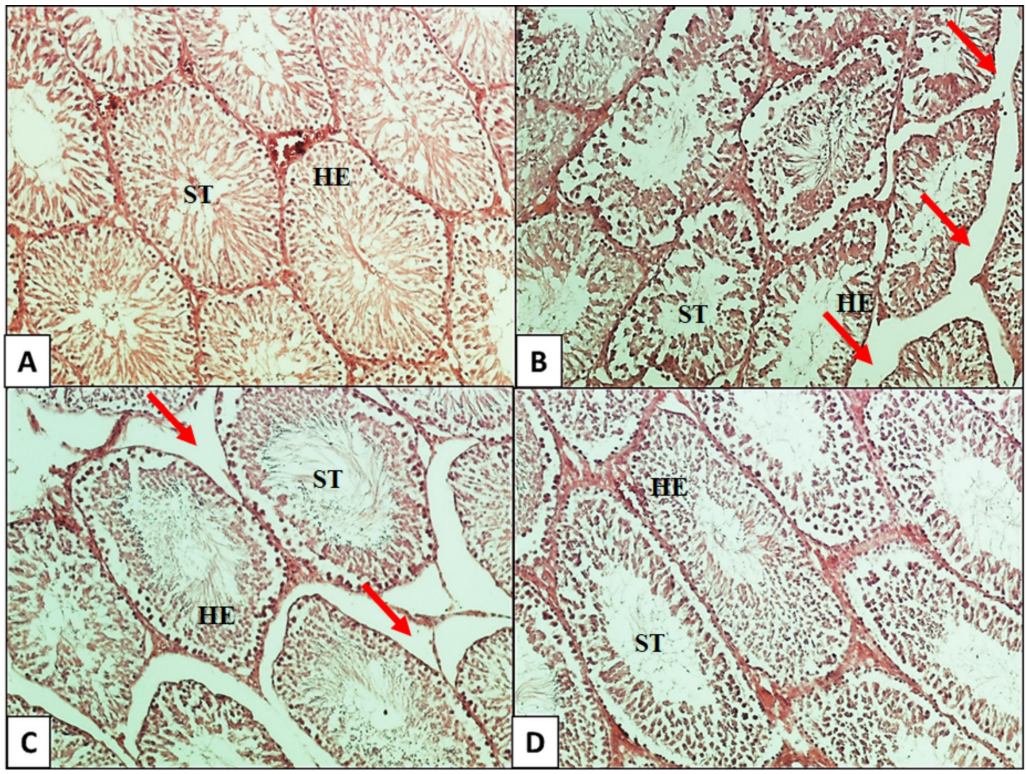

FIGURE 2. Testicular histology of adult rats following LPS administration and Carvacrol treatment. (A) Control group showing normal seminiferous tubules (ST) and epithelial height (HE) without oedema; (B) LPS group with markedly reduced epithelial height with oedema. Red arrows indicate edema in testicular tissue; (C) C25 group (LPS + Carvacrol 25 mg/kg) with near-normal epithelial height and reduced oedema; (D) C50 group (LPS + Carvacrol 50 mg/kg) with irregular tubular lumen but near-normal epithelial height. C: Carvacrol; LPS: Lipopolysaccharide; C25: LPS + Carvacrol 25 mg/kg; C50: LPS + Carvacrol 50 mg/kg. H&E. Scale bar = 50 μm.

TABLE 2. Comparison of the seminiferous histological marker in Control, LPS, C25, C50 groups.

Groups	Mean of Johnsen's Score	STD	HE
Control	9.2833 ± 0.12072	279.7567 ± 0.91657	132.1933 ± 2.34597
LPS	6.2200 ± 0.08828*	186.5400 ± 3.09757*	85.0340 ± 1.64486*
C25	6.7460 ± 0.13785$^{\#}$	205.4040 ± 2.05497$^{\#}$	95.8040 ± 1.32610$^{\#}$
C50	7.7620 ± 0.05659$^{\#,\&}$	234.1080 ± 3.24688$^{\#,\&}$	123.5220 ± 2.19020$^{\#,\&}$

*STD: seminiferous tubule diameter; HE: height of epithelium; C: Carvacrol; LPS: Lipopolysaccharide; C25: LPS + Carvacrol 25 mg/kg; C50: LPS + Carvacrol 50 mg/kg. * Compared with Control group, $^{\#}$ compared with LPS group, and $^{\&}$ compared with C25 group. *$p < 0.001$ compared with Control group, $^{\#}p < 0.01$ compared with LPS group, $^{\&}p < 0.001$ compared with C25 group. All outputs have been reported as mean ± SE (n = 8 per group).*

C25, and C50 rats; however, this decrease was not statistically significant (Fig. 3a). Additionally, the testis volume was higher in the C50 group relative to the others, though this variation was not statistically significant (Fig. 3b).

3.3 Sperm analysis: count and morphology

The findings revealed a marked decrease in the average sperm count in the LPS rats relative to the Control rats ($p < 0.001$). Treatment with carvacrol resulted in a notable rise in sperm count compared with the LPS rats ($p < 0.001$); however, no statistically significant difference was observed between the two treatment groups (Figs. 4,5a).

Regarding sperm morphology, the percentage of abnormal sperm was notably higher in the LPS rats than in the Control rats ($p = 0.02$). Both carvacrol-treated groups (C25 and C50) showed a notable decrease in abnormal sperm percentages relative to the LPS group, with p-values of 0.006 and 0.001, respectively (Figs. 4,5b).

3.4 *Bax*, *Bcl-2* and *Caspase-3* gene expression

The treatment had a significant effect on the levels of the *Bax* gene ($F_{3,\ 8} = 11.36$, $p = 0.003$). The level of the *Bax* gene was elevated in the LPS rats relative to the Control rats, but the difference was not significant ($p = 0.06$). Carvacrol administration at both 25 and 50 mg/kg doses resulted in a remarkable reduction in *Bax* expression than the LPS rats ($p = 0.01$ and $p = 0.02$, respectively) (Fig. 6a).

The carvacrol significantly affected *Bcl-2* expression ($F_{3,\ 10} = 11.36$, $p = 0.004$). A significant decrease in *Bcl-2* expression was observed in the C25 and C50 treatment groups compared with the LPS group ($p = 0.006$ and $p = 0.005$, respectively). However, no significant variation was found when comparing the Control and LPS rats ($p = 0.8$) (Fig. 6b).

Similarly, carvacrol significantly affected *caspase-3* expression ($F_{3,\ 10} = 39.09$, $p < 0.001$). The levels of *caspase-3* expression were notably reduced in the C25 and C50 groups relative to the LPS group (both $p < 0.001$), while no notable difference was detected when comparing the Control and LPS rats ($p = 0.37$) (Fig. 6c).

4. Discussion

This research focused on examining how carvacrol might protect against testicular damage caused by LPS in rats. Outcomes illustrated that carvacrol attenuates LPS-induced testicular injury in a rat model by modulating the expression of apoptosis-associated genes, including *Bax*, *Bcl-2*, and *caspase-3*.

LPS, a component found in the cell walls of Gram-negative bacteria, is recognized for triggering strong inflammatory responses associated with disease [2]. Such inflammation in male reproductive organs can negatively affect fertility by lowering sperm motility and reducing the production of male hormones like androgens, and the epididymis [29]. Additionally, the infection and inflammation disrupt key processes, like steroid hormone production and sperm development inside the testes [30]. It is shown that LPS leads to the production of inflammatory responses through the dysregulation of lipid metabolism in the adult rat testes, both in living animals and in laboratory conditions [6]. For example, LPS exposure reduces the activity of enzymes responsible for steroid production in Leydig cells in male mice [31]. A study in 2024 showed that LPS increases inflammatory mediators, including Tumor Necrosis Factor-alpha (TNF-α), Interleukin 1 beta (IL-1β), and IL-6, elevates oxidative stress markers such as malondialdehyde (MDA), and decreases antioxidants like glutathione (GSH), thereby activating the oxidative stress cascade in testicular tissue. It has also been reported to cause an increase in inflammatory markers, such as Toll-like Receptor 4 (TLR4), Myeloid differentiation primary response 88 (MyD88), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and Prokineticin Receptor 1 (PK2/PKR1). The combination of these conditions induced by LPS is able to disrupt sperm count, morphology, and testicular structure [4]. In line

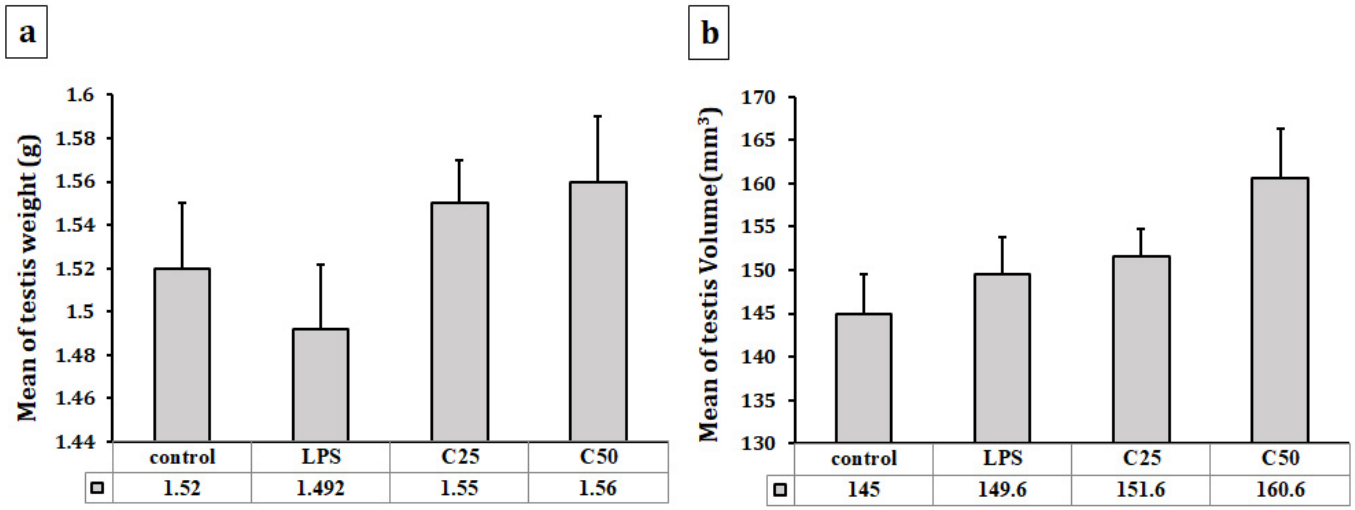

FIGURE 3. Comparison of testicular weight and volume among experimental groups. (a) Average testicular weight and (b) volume measured in Control, LPS, C25, and C50 groups. C: Carvacrol; LPS: Lipopolysaccharide; C25: LPS + Carvacrol 25 mg/kg; C50: LPS + Carvacrol 50 mg/kg. Data are expressed as mean ± SE (n = 8 per group).

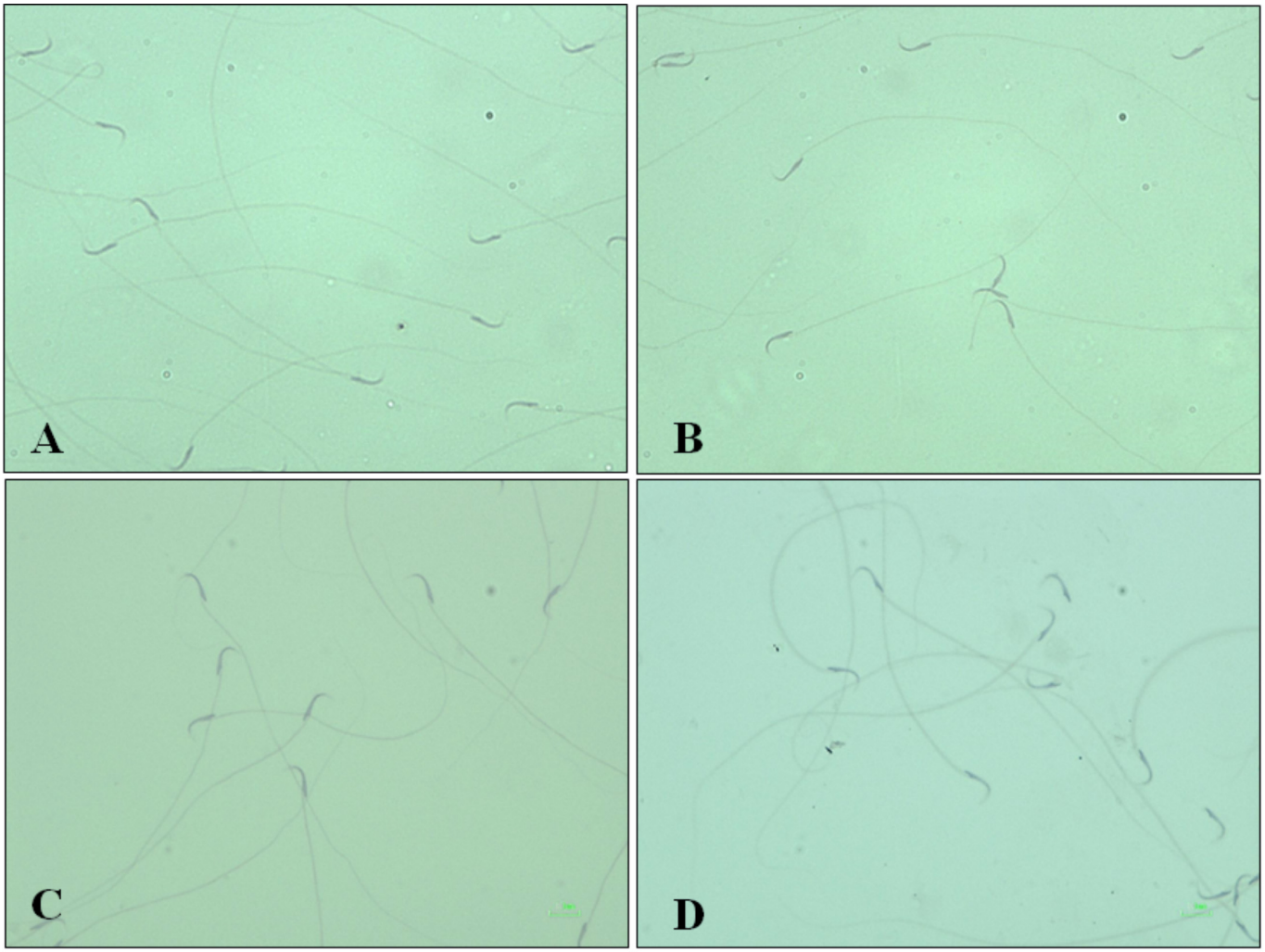

FIGURE 4. Microscopic comparison of sperm morphology across experimental groups. Representative photomicrographs illustrating sperm morphology in (A) Control, (B) LPS, (C) C25 (LPS + Carvacrol 25 mg/kg), and (D) C50 (LPS + Carvacrol 50 mg/kg) groups. The LPS group exhibited a significant reduction in sperm count and increased abnormal sperm percentage, whereas carvacrol treatment partially restored sperm count and decreased the percentage of abnormal sperm in both C25 and C50 groups, as shown in (C) and (D). Papanicolaou staining. Scale bars = 10 μm.

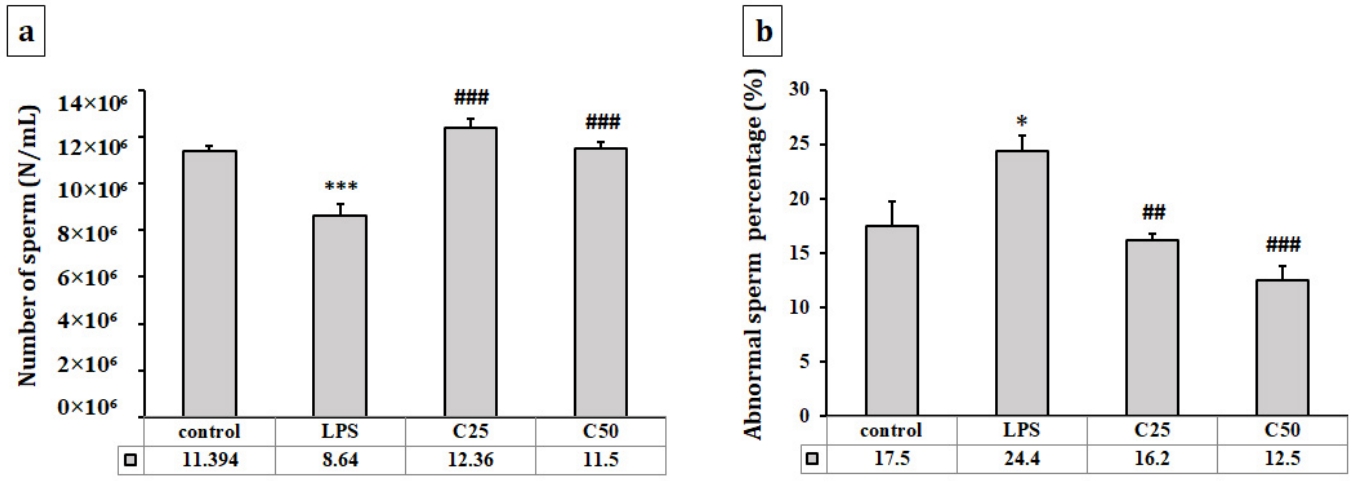

FIGURE 5. Comparison of sperm count and abnormal sperm percentage across experimental groups. (a) Mean sperm count and (b) percentage of abnormal sperm in Control, LPS, C25, and C50 groups. C: Carvacrol; LPS: Lipopolysaccharide; C25: LPS + Carvacrol 25 mg/kg; C50: LPS + Carvacrol 50 mg/kg. * Compared with Control group, # compared with LPS group. *$p < 0.05$, ***$p < 0.001$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. Data are presented as mean ± SE (n = 8 per group).

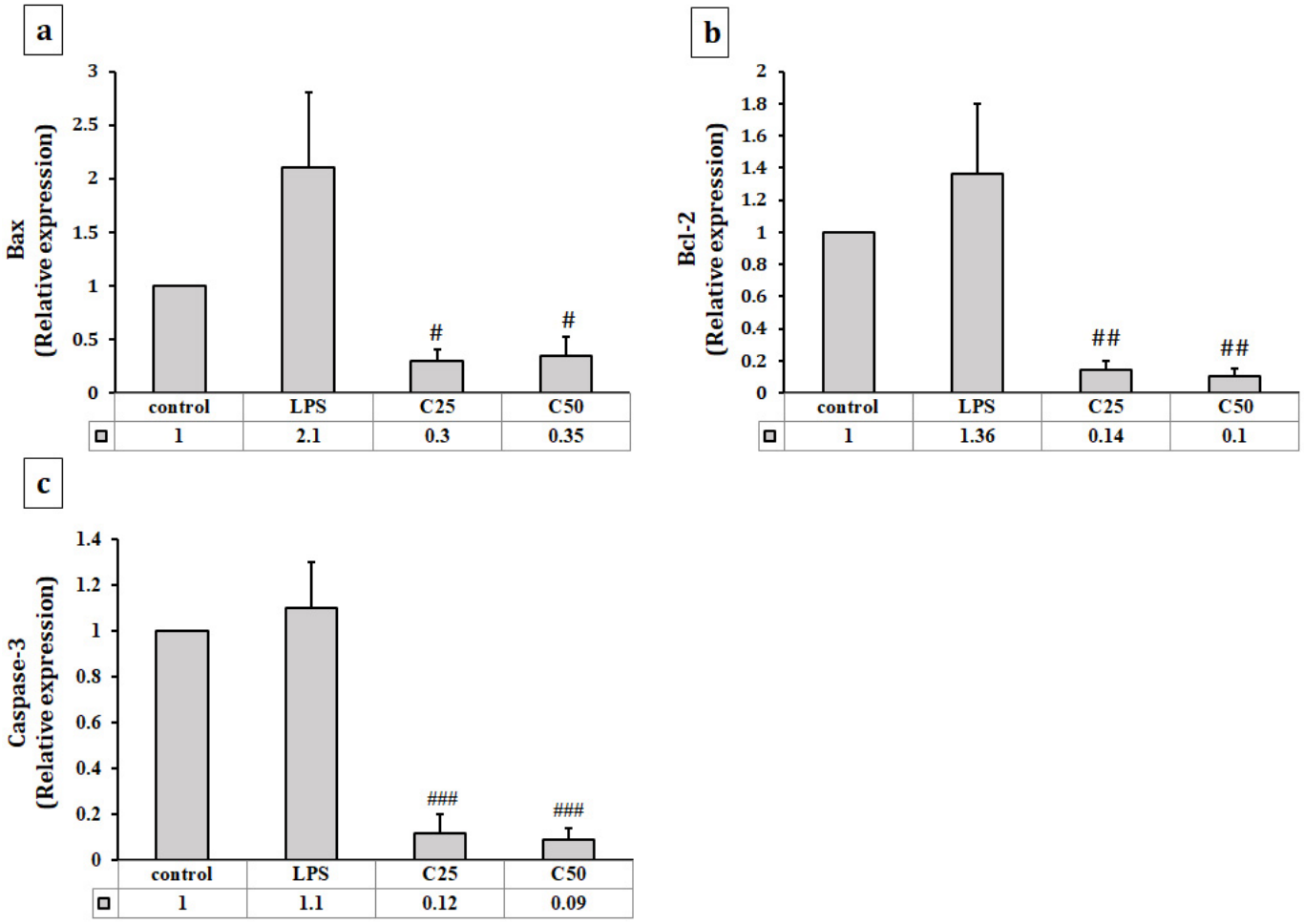

FIGURE 6. Comparison of gene expression levels of apoptosis-related markers across groups. (a) Relative mRNA expression level of *Bax*, (b) *Bcl-2*, and (c) *Caspase-3* in Control, LPS, C25, and C50 groups. C: Carvacrol; LPS: Lipopolysaccharide; C25: LPS + Carvacrol 25 mg/kg; C50: LPS + Carvacrol 50 mg/kg. $^{\#}$ Compared with LPS group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. Data are presented as mean ± SE (n = 8 per group).

with El-Sherbiny *et al.* [4] 2024 study, the results of the present research indicated that LPS administration for 14 days led to degeneration in testicular tissue, including damage to the seminiferous tubules and reduction in sperm count and normal morphology. The oxidative stress caused by LPS harms both steroid hormone production by Leydig cells and sperm formation, which are likely the main causes of testicular problems seen with LPS or infections [29]. In addition, the outcomes of the current study indicate an increment in apoptosis, supporting previous reports that LPS exposure harms spermatogenesis and testicular structure mainly through oxidative stress and apoptosis [12, 32]. Moreover, earlier research has shown that LPS causes apoptosis in cells lining the seminiferous tubules, degeneration of early sperm cells, and early pachytene spermatocytes [4, 12].

The outcomes of the present research demonstrated that carvacrol can mitigate the detrimental effects of LPS on testicular tissue. It has been proven that carvacrol, as an antioxidant and anti-inflammatory compound, can reduce testicular damage caused by testicular torsion, cisplatin, and arsenic [18, 19, 33]. In accordance with our study's findings, the research by Gur *et al.* [18] in 2023 demonstrated the protective role of carvacrol against testicular damage caused by arsenic. The outcomes of this study illustrated improvement in the structure of seminiferous tubules, morphology, and sperm motility. Inflammatory factors, oxidative stress, and autophagy were also reduced. Carvacrol was also able to reduce *Bax* and *caspase-3* in the testes and increase the *Bcl-2* expression [18]. Balci *et al.* [33] in 2021 revealed the influence of carvacrol in preventing testicular destruction following torsion and ischemia. They showed that carvacrol improved the diameter of the seminiferous tubules, sperm motility, and the score of Johnson. Carvacrol also notably diminished the expression of *caspase-3* and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells after testicular torsion-induced ischemia [33]. Aksu *et al.* [19] (2016) investigated the efficacy of carvacrol on cisplatin-induced reproductive system impairment in male rats. They demonstrated that carvacrol treatment prevented the loss of testicular weight caused by cisplatin-induced reproductive toxicity in rats [19]. In our study, although testicular weights varied between the Control group and rats exposed to LPS, the difference was not statistically significant. However, exposure to LPS resulted in a significant decline in the diameter of the seminiferous tubules and caused noticeable structural damage. This was accompanied by a substantial decline in average Johnson's scores,

which reflect impaired testicular function. Importantly, administering carvacrol to LPS-treated rats considerably improved these histological alterations. Overall, these findings indicate that carvacrol mitigates testicular injury and reduces apoptosis triggered by LPS exposure. Our observations align well with those of Aksu and colleagues, who reported that carvacrol reduces the degeneration of seminiferous tubules and counteracts reproductive harm induced by cisplatin [19]. Additionally, oxidative stress driven by ROS affects the phosphorylation of *Bcl-2* family proteins, shifting the ratio towards apoptosis-promoting proteins like *Bax*, which promotes programmed cell death [34].

A limitation of this study is that oxidative and inflammatory markers were not examined in testicular tissue. However, previous studies have shown that carvacrol reduces LPS-induced oxidative stress and inflammatory cytokines in other models and tissues [22, 35, 36]. For example, Yan *et al.* [35] (2023) documented that carvacrol reduces LPS-induced production of the inflammatory cytokine IL-6 *in vivo* and *in vitro* via the Extracellular signal-Regulated Kinase 1/2 (ERK1/2) signaling pathway in macrophages. Somensi *et al.* [36] (2019) demonstrated that carvacrol suppresses LPS-induced pro-inflammatory activation by inhibiting ERK1/2 and NF-κB pathways through a cellular mechanism, which may be linked to its antioxidant characteristics as a phenolic compound. Additionally, Mortazavi *et al.* [22] (2022) showed that LPS injection increased malondialdehyde and IL-1β while decreasing total thiol and the activities of catalase and superoxide dismutase enzymes in kidney tissue. Treatment with carvacrol not only reduced the levels of malondialdehyde and IL-1β but also increased total thiol, catalase activity, and superoxide dismutase activity.

Apoptosis may be initiated by stimuli originating outside the cell. When ligands like TNF-α and Fas Ligand (FasL) bind to their corresponding death receptors, TNF-R1 and Fas respectively, this interaction triggers the activation of initiator *caspases*, primarily *caspase-8* and *caspase-10*. These enzymes activate *caspase-3*, *-6*, and *-7*, thereby triggering a cascade of biochemical events that ultimately result in cell death [37]. In addition to this external pathway, germ cell programming death may be prompted internally through what is known as the intrinsic apoptosis pathway. Here, internal stress signals, including ROS, trigger mitochondrial mechanisms that enable the discharge of cytochrome c from the inner mitochondrial membrane, further promoting programmed cell death [37]. Our data revealed that rats treated with both carvacrol and LPS exhibited significantly lower *Bax* and *caspase-3* expression compared with those treated with LPS alone. Previous research indicates that carvacrol modulates the balance of pro- and anti-apoptotic proteins, reducing mitochondrial pathway-mediated apoptosis by downregulating *Bax* and *caspase* activity while upregulating *Bcl-2* expression [36]. Although we did not specifically measure Fas–FasL interactions or *Caspase-9* expression, the observed changes in these apoptotic indicators suggest that carvacrol primarily mitigates mitochondrial pathway activation. The apoptosis-inhibiting properties of carvacrol, in the role of a supplementary treatment, have been explored in prior research by Aksu and colleagues, who reported that carvacrol decreased *caspase-3* activity in rat testes damaged by cisplatin exposure [19]. *Caspase-3* is a key mediator of the *caspase*-dependent apoptotic pathway, leading to DNA degradation and ultimately cell death [38]. Another study demonstrated that administering carvacrol at 50 mg/kg over two weeks significantly lowered *caspase-3* expression and reduced *Bax* protein levels in rat testes subjected to arsenic toxicity, likely owing to its antioxidant and anti-apoptotic capabilities [18]. Interestingly, *Bcl-2* mRNA expression also decreased markedly in the carvacrol-treated rats relative to the untreated LPS rats. The current outcomes indicate that carvacrol was able to protect against apoptosis through the *caspase* and extracellular pathways. Furthermore, research has indicated that *Bcl-2* may play a role in regulating antioxidant defenses or preventing lipid peroxidation without directly changing intracellular ROS concentrations. This suggests that *Bcl-2* possesses antioxidant properties, which contribute to cellular protection [39]. The reason for this discrepancy can be explained by the notion that the reduction in *Bcl-2* transcript levels in the carvacrol-treated groups may reflect post-transcriptional regulation, rather than decreased protein expression. Previous evidence suggests that *Bcl-2* activity can be modulated through phosphorylation, localization changes, or interaction with *Bax* and other mitochondrial proteins, rather than mRNA abundance alone [40]. It is also possible that the carvacrol treatment influenced *Bcl-2* family gene balance toward functional stabilization of the protein, rather than transcriptional upregulation. This point is now suggested as a limitation and an area for further exploration in future protein-level studies.

In our investigation, it appears that extracellular apoptotic mechanisms may contribute to the harmful effects induced by LPS injection. Consequently, we propose that carvacrol's protective effects against testicular dysfunction are largely related to its ability to inhibit apoptosis. According to this evidence, the protective efficacy of carvacrol led to improved sperm parameters, reduced apoptosis, decreased *caspase* activity, and preserved testicular architecture. Taken together, these outcomes highlight carvacrol as a promising natural compound for the prevention and management of testicular damage caused by LPS.

Inflammatory programmed cell death pathways, such as pyroptosis and necroptosis, play complex dual roles in cancer by both suppressing tumor growth through the elimination of malignant cells and promoting tumor progression via sustained inflammation. These mechanisms exemplify the intricate interplay between apoptotic and inflammatory pathways in regulating cell fate and tissue responses to injury. Our findings on carvacrol's modulation of apoptotic signaling may contribute to a deeper understanding of such interactions and support the therapeutic potential of targeting regulated cell death to protect against tissue damage [41]. Also, PANoptosis represents a highly integrated form of programmed cell death that combines key molecular features of apoptosis, pyroptosis, and necroptosis. This coordinated pathway allows cells to respond more effectively to diverse stress signals by engaging multiple death mechanisms simultaneously. PANoptosis can regulate cell fate during inflammation and tissue injury, revealing new therapeutic targets for diseases involving dysregulated cell death [42]. These studies help demonstrate that the anti-

apoptotic signaling observed in our study shares mechanistic similarities with pathways modulated in other pathological conditions, thus reinforcing the broader biomedical relevance of our work.

Oxidative stress, on hand, arises from an imbalance between ROS generation and antioxidant defenses, leading to cellular damage and initiation of apoptosis. Excess ROS can injure DNA and mitochondria, activating pro-apoptotic proteins like p53 and *Bcl-2* family members, which facilitate mitochondrial membrane permeabilization and *caspase* activation. Moreover, oxidative stress triggers inflammatory signaling by increasing cytokines such as TNF-α and IL-1β, which in turn enhance apoptosis via death receptor pathways [43]. This interconnected cycle of oxidative stress, inflammation, and apoptosis contributes significantly to tissue injury in various diseases, including LPS-induced testicular damage. On the other hand, carvacrol, with its antioxidant and anti-inflammatory effects, has been able to modulate apoptosis and testicular tissue damage.

It is suggested that future studies use the sertoli cells and germ cells marker staining technique combined with TUNEL to determine which types of germ cells undergo apoptosis after LPS-induced testicular injury.

5. Conclusions

In conclusion, our study demonstrates that carvacrol exhibits anti-apoptotic properties that can protect against LPS-induced testicular injury in a rat model. The current study highlights that carvacrol can be an effective natural agent in the prevention and treatment of LPS-induced testicular injury. However, additional studies are needed to verify its efficacy and safety in other models and to elucidate molecular pathways involved before carvacrol can be considered a therapeutic agent in clinical settings.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

MM and MH—designed the study and supervised the overall project, they had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. NJ, MS, BB and MF—performed the animal experiments and data collection. AEB, MS and RB—conducted the biochemical and histological analyses. MM, MH and AEB—carried out the statistical analysis and interpretation of results. MS, NJ, BB, MF and RB—drafted the manuscript and prepared the figures. All authors contributed to the discussion of the results, reviewed the manuscript critically for important intellectual content, and approved the final version for publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the ethical committee of Gonabad University of Medical Sciences, Gonabad, Iran (IR.GMU.REC.1398.129).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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