Physiology and Pharmacology 27 (2023) 403-416
Original Article

Safranal Ameliorates Ischemic/Reperfusion Injury Induced by Testicular Torsion in Rat

Marzieh Ebrahimi[1], Seyed-Hosein Abtahi-Eivary[2], Balal Brazvan[3], Majid Shokoohi[4], Malihe Soltani[3], Morteza Rostamian[5], Masoumeh Fani[3], Maryam Moghimian[6*]

1. Student Research Committee, Gonabad University of Medical Sciences, Gonabad, Iran
2. Department of Biochemistry, School of Medicine, Gonabad University of Medical Sciences, Gonabad, Iran
3. Department of anatomy, School of Medicine, Gonabad University of Medical Sciences, Gonabad, Iran
4. Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
5. English department, School of Medicine,Gonabad University of Medical Sciences, Gonabad, Iran
6. Department of Physiology, School of Medicine, Gonabad University of Medical Sciences, Gonabad, Iran

ABSTRACT

Introduction: Testicular torsion is very common in urological emergencies, which damages testicular tissue and reproductive function. Safranal, known for its robust antioxidant properties, has demonstrated effective inhibition of ischemia/reperfusion injury (IRI) in various tissues such as the hippocampus, cerebral, and skeletal muscles. Therefore, this study aimed to evaluate the effect of Safranal on testicular tissue following IRI.

Methods: This research involved 48 male adult Wistar rats. They were randomly divided into six groups: control, testicular torsion/detorsion (TD), torsion and detorsion/safranal (0.1, 0.5 mg/kg, ip), and safranal control groups (0.1, 0.5 mg/kg, ip). Under anesthesia, the left testicular torsion was induced for four hours, 30 minutes before detorsion, a single dose of safranal was injected. After 24 hours of reperfusion, assessments encompassing oxidative markers, estradiol, testosterone, LH hormone, sperm parameters, testicular histopathology, and gene expression were conducted on blood and tissue samples.

Results: Heightened seminiferous epithelia (HE) was observed in the TD groups receiving safranal (TD+Sa 0.1, 0.5). There was a significant increase in sperm count and a notable reduction in abnormal sperm count compared to the TD group. Also, the expression of the Bax gene significantly decreased in comparison to the TD group. In rats receiving 0.1 mg/kg of safranal, there was an improvement in superoxide dismutase (SOD) and glutathione peroxidase (GPx). Although not statistically significant, the TD+Sa groups exhibited slightly enhanced levels of estradiol, testosterone, and LH compared to the TD group.

Conclusion: These findings suggest that safranal may protect testicular tissue from IRI through antioxidant and antiapoptotic pathways.

Keywords:
Torsion-Detorsion
Safranal
Oxidative Markers
Bax/Bcl-2
Apoptosis

Introduction

Among urological emergencies, torsion of the testis is very common and causes the vascular pedicle of the testicles to rotate (Quintaes et al., 2013). This rotation

* Corresponding author: Maryam Moghimian, moghimian.m@gmu.ac.ir
Received 26 June 2022; Revised from 2 January 2023; Accepted 11 January 2023

Citation: Ebrahimi M, Abtahi-Eivary S.H., Brazvan B, Shokoohi M, Soltani M, Rostamian M, Fani M, Moghimian M. Safranal Ameliorates Ischemic/Reperfusion Injury Induced by Testicular Torsion in Rat. Physiology and Pharmacology 2023; 27: 403-416. http://dx.doi.org/10.61186/phypha.27.4.403

interrupts the blood flow and causes ischemia, necrosis, and edema in the testes. The severity of injury depends on two important factors: the duration and the degree of testicular (Turkmen et al., 2012). In the case of testicular pain, a differential diagnosis must be made to rule out other possibilities because delay in diagnosis and treatment can lead to loss of the testicles (Sung et al., 2012). Torsion of the testicles can happen at any age, but it is most prevalent among young pediatric individuals. The age distribution is bimodal, with the first peak occuring in the first year of life and the second peak between 13 and 16 years of age (Pogorelic et al., 2019). Manual or surgical detorsion is the standard emergency treatment for these patients. According to the literature, detorsion has a relationship with high rates of testis tissue preservation within the first six hours (Ta et al., 2016).

Furthermore, patients who are successfully treated for testicular torsion may still experience significant pathologies, including disrupted sperm quality, infertility, and testicular atrophy for the rest of their lives (Moghimian et al., 2017). Reactive oxygen species (ROS) emerge as a consequence of torsion-induced ischemia-reperfusion injury (IRI), inflicting damage upon DNA, endothelial, and germ cells within the testis (Karaguzel et al., 2014). The human body's array of enzymatic (catalase, superoxide dismutase) and non-enzymatic natural antioxidants (glutathione peroxidase and melatonin, cytochrome C, zinc, vitamin C, E,) can protect the testicles against tissue damage (Aitken & Roman, 2008). Nucleic acids, carbohydrates, proteins, lipids, and all cellular components can be affected by oxidative stress. It is noteworthy that the amount of produced ROS and the length of exposure determine the rate of injury (Agarwal et al., 2008). Previous studies have indicated that increased lipid peroxide content and oxidative damage decrease sperm motility, harm DNA in germ cells and spermatozoa, and induce tissue apoptosis in the testis (Aitken & Baker, 2006; Sawyer et al., 2003; Wilhelm et al., 2004).

Results of previous research have indicated the role of exogenous antioxidants or ROS scavenger agents in reducing or preventing oxidative damage to rat testicular tissues due to IRI. In addition, plant-derived antioxidants stand as potent medicinal agents in managing oxidative stress (Moradi-Ozarlou et al., 2020). Nevertheless, the therapeutic properties of traditional medicinal plants remain unfamiliar to clinicians and patients. Over the past decade, there has been substantial emphasis on utilizing the medicinal qualities of saffron, an ancient spice, and its constituents (Abdullaev, 1993; Farahmand, et al., 2013).

Safranal, a primarily active constituent of saffron renowned for its antioxidant properties (Farahmand et al., 2013; Samarghandian et al., 2015), exihibits capabilities in mitigating cell membrane damage, scavenging intracellular ROS, and reducing membrane lipid peroxidation. This suggests its potential therapeutic effects in scenarios involving radical sifting activities (Samarghandian et al., 2015). Moreover, safranal protects the skeletal muscle (Hosseinzadeh et al., 2009), and cerebral tissues (Ahmad et al., 2017; Hosseinzadeh & Sadeghnia, 2005) in ischemia rats against oxidative damage. Also, it has exhibited anticonvulsant effects in mice experiencing persistent seizures, resulting in reduced seizure duration, delayed tonic phases, and increased survival rates (Hosseinzadeh & Talebzadeh, 2005). It has been found that Saffron revitalizes the reproductive system in males, which may help to treat diseases like early ejaculation, erectile dysfunction, and decreased sperm concentration, motility, and morphology (Szafrańska, et al., 2002). However, the precise mechanisms underlying its actions remain unknown.

Since oxidative stress and apoptosis are considered responsible for torsion/detorsion pathogenesis, this research aimed to examine the impacts of different doses of safranal on IRI after testis torsion treatment using objective histological, hormonal, and gene expression assessments, and evaluates oxidative stress caused by torsion/detorsion in the testes of adult male rats.

Materials and Methods

Codes of Ethics

All procedures and protocols for this experiment received approval from the Animal Ethics Committee at Gonabad University of Medical Sciences (IR.GMU.REC.1398.130). The experiments adhered to the guidelines outlined by the National Ethics Committee of the Ministry of Health and Medical Education concerning the care and utilization of laboratory animals. Wistar rats weighing 260 to 290 g were obtained from Gonabad University of Medical Sciences in Khorasan Razavi Province, Iran. Prior to the experiments, all rats underwent a two-week acclimatization period in the

testing environment. They were housed in an animal home maintained at 25±2 °C with 30-70% humidity and subjected to a twelve-hour light/dark cycle. There were no restrictions on food and water intake, except during experiment trials.

Experimental Groups

The number of rats allocated to each group was determined using power analysis set between 80 and 90% to detect an effect at a significance level of 5%, using Statmate™ version 1 (GraphPad Software Inc, San Diego, CA, USA). In total, forty-eight adult male rats were divided into six groups, each comprising eight rats, according to the study design:

1. Control group: Underwent all procedural steps except testicular torsion.

2. Torsion/Detorsion group (TD): Underwent testicular torsion surgery for 4 hrs followed by detorsion. 3 and 4. Torsion/Detorsion/Safranal groups (TD+Sa 0.1 and TD+Sa 0.5): Subjected to testicular torsion/detorsion for 4 hrs and received a single intraperitoneal dose of safranal at concentrations of 0.1 or 0.5 mg/kg (1ml/kg) (Hosseinzadeh et al., 2009), administrated 30 min before testicular detorsion. 5 and 6: Safranal control groups (Sa 0.1 and Sa 0.5): Received safranal at concentrations of 0.1 or 0.5 mg/kg (1ml/kg) without undergoing any surgical procedures.

Surgical Procedure and Sampling

The procedure adhered to previous experimental methodologies (Ameli et al., 2018; Shokoohi et al., 2018). To induce effective anesthesia, rats received a combination of xylazine (10mg/kg) and low doses of ketamine (50 mg/kg) intraperitoneally. Anesthesia was considered adequate when rats did not show somatic motor reflexes upon tail pinching or blinking in response to corneal stimulation. The next step involved a longitudinal skin incision in the scrotum to expose the testis. The left testicle was then twisted 720 degrees counterclockwise and affixed to the scrotum at the ischemic site using 6/0 non-absorbable silk sutures (Elmimehr et al., 2021), in line with prior research protocols. Testicular torsion was maintained in the TD groups for 4 hrs (Ameli et al., 2018; Danarto, et al., 2019), followed by detorsion for 24 hrs (Moghimian et al., 2017). To alleviate postoperative discomfort, all rats recieved buprenorphine (0.02 mg/kg) as an analgesic. After 24 hrs of treatment, the animals were sedated with ketamine-xylazine, and heart blood was drawn for antioxidant enzyme level assessment. The blood samples were centrifuged at 3000 rpm for 10 min, and the serum was extracted and stored at -70 °C for further analysis. In addition, orchiectomy of the left testicular and epididymis was performed to examine gene expression, sperm analysis, and histological tests (Elmimehr et al., 2021).

Sperm Count and Morphology

According to the previous studies, the left epididymis was placed in one ml of normal saline (Elmimehr et al., 2021), dissected using scissors, and gently compressed with forceps. Afterward, 4 ml of normal saline was added, and the mixture was incubated in a CO_2 incubator at 37 °C for 10 min. Next, the solution was thoroughly homogenized by shaking and a droplet was placed on a Neubauer chamber for sperm counting using an optical microscope with a 40x objective lens. Moreover, smears from this solution were stained usig the Papanicolaou method to analyze sperm morphology. Aberrant sperm, including tail-free, coiled, or bent tails, were identified and quantified under an optical microscope (Jalilvand et al., 2019; Vafaei et al., 2020).

Tissue Fixation, Sample Preparation, and Histopathological Analysis

Testicular tissues were fixed in 10% formalin for six days post-surgery. Subsequently, the testicular tissues underwent a gradual ethanol dehydration prosess before embedding in paraffin. Afterward, the paraffin-embedded samples were sectioned at 5 µm thickness and deparaffinized. Hematoxylin-eosin (H&E) staining was performed, and the slides were examined using an optical microscope (NIKON) (Ameli et al., 2018). Thirty seminiferous tubules with a curved structure were randomly selected per slide. The mean seminiferous tubule diameter (STD) was measured at 100x magnification, from one side of the tubule′s basement membrane to the other. In addition, the height of the germinal epithelium (HE) was measured in micrometers (µm) (Shokoohi et al., 2018; Wilhelm et al., 2004). Furthermore, the Johnson's score method was used to histopathologically assess spermatogenesis (Johnsen, 1970).

RNA and PCR Analysis

To extract RNA from rat testicles, the Favor Prep

TABLE 1: Primers 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

Blood/Cultured Cell Total RNA Mini Kit (Favorgen, Taiwan, Cat no: FABRK000) was employed (Elmimehr et al., 2021). The extracted RNA was eluted in 50 µl of RNase-free water and stored at -80 ºC. Assessment of RNA quantity was perfomed using Nanodrop Epoch two microplate spectrophotometer, measuring absorbance ratios of 260/280 and 260/230 (Biotech, USA). RNA purity was confirmed with a 260/280 ratio of 2.0 and a 260/230 ratio ranging from 2.0 to 2.2. The integrity of the RNA samples was assessed using 1.5% agarose gel electrophoresis (Rodrigues & de Brito, 2017; Saadatian et al., 2019). For cDNA synthesis, total RNA (>500 ng) was transformed to cDNA using a cDNA synthesis kit (YT4500, Yekta Tajhiz Azma®, Iran) (Elmimehr et al., 2021). The cDNA samples were generated based on the instructions of the company by an 18-meer oligo (dT) primer (Elmimehr et al., 2021). One RNA sample was obtained without RevertAidTM M-MuLV reverse transcriptase (RT reaction) for each reaction set, serving as a negative control in the following PCR experiments (Elmimehr et al., 2021). The cDNA generated from the RT reaction was stored at -20 °C for one week before long-term storage at -70 °C (Rodrigues & de Brito, 2017).

For real-time PCR analysis, BioFact™ 2X Real-Time PCR Smart mix Syber green (BioFact, Korea), cDNA (20 ng/µl), primer set (0.4 µM of each primer), and nuclease-free water were employed with an ABI 7500 real-time PCR-fast 7498 (USA). Primers were designed using the per primer software (version 1.1.20) (Elmimehr et al., 2021). The list of the sequences is summarized in Table 1. Amplification was carried out in triplicate, using β-actin as the endogenous housekeeping gene. The thermal cycling conditions consisted of an initial denaturation step of 5 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C and one minute at 61°C. Afterward, Consistent with previous research methodologies, the $2^{-\Delta\Delta CT}$ method was used to calculate delta CT (cycle threshold) values. ΔCt represents the difference between the CT value of β-actin and the CT values of the targeted genes (Elmimehr et al., 2021; Saadatian et al., 2019).

Lipid Peroxidation and Antioxidant Enzymes Activity

According to the prior research (Danarto et al., 2019; Shokoohi et al., 2018), the Malondialdehyde (MDA) levels in the blood were assessed using 0.20 cm^3 of serum combined with 3.0 cm^3 glacial acetic acid and 3.0 cm^3 of 1% TBA in 2% NaOH in a micro-tube. The mixture was then heated in hot water for 15 min, and the absorbance of the resultant pink-colored solution was measured at 532 nm after cooling. An MDA standard curve was generated using MDA tetra-butylammonium salt from Sigma (USA). Serum superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) activity were determined according to the provided instructions using commercial test kits (Randox: UK).

Measuring the Level of Catalase Activity

Catalyze activity was determined by incubating the enzyme sample for three minutes in a 1.0 ml substrate containing 65 mol/ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer (pH 7.4) at 37 °C. The reaction was halted using ammonium molybdate, and the absorbance of the yellow peroxomolybdate complex was measured at 374 nm and compared to a blank (Hadwan & Abed, 2016).

Testosterone, Estradiol, and LH Serum Levels

ELISA kits (Demeditec, Germany) were utilized for testosterone and estradiol assays, with absorbance reading at 405 nm. Similarly, an ELISA kit (Cusabio, China) was employed to measure serum luteinizing hormone

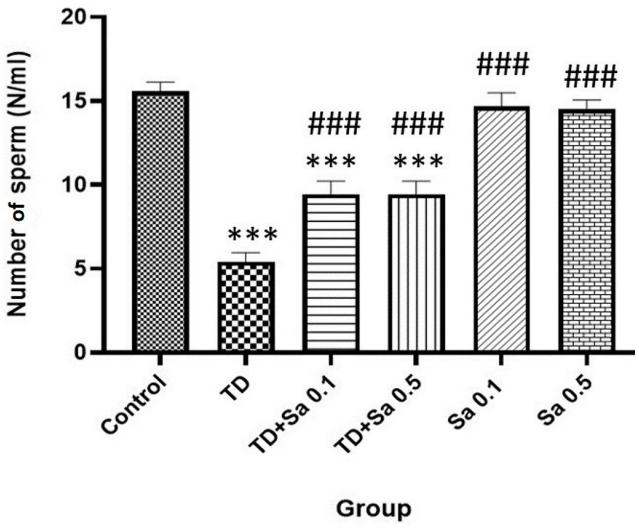

FIGURE 1. Sperm count comparison among different groups. 1. control group, 2. torsion/detorsion group (TD), 3. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), 4. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), 5. Safranal 0.1 (Sa 0.1), and 6. Safranal 0.5 (Sa 0.5). The symbol * represents significant differences with the control and # stands for significant differences with TD group ($^{***}P < 0.001$, $^{###}P < 0.001$).

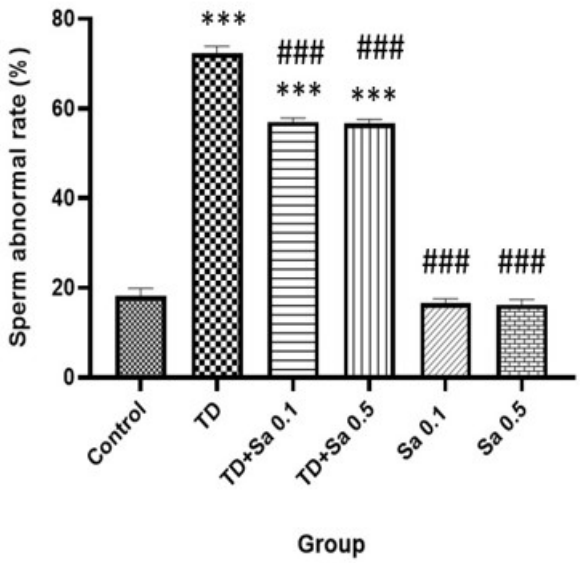

FIGURE 2. The percentage comparison of abnormal sperm count among different groups. 1. control group, 2. torsion/detorsion group (TD), 3. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), 4. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), 5. Safranal 0.1 (Sa 0.1), and 6. Safranal 0.5 (Sa 0.5). The symbol * represents significant differences with the control and # stands for significant differences with TD group ($^{***}P < 0.001$, $^{###}P < 0.001$).

(LH) levels (Shokoohi et al., 2018).

Statistical Analyses

The SPSS 22 statistical software was used for data analysis. Normal distribution of data was assessed using the Kolmogorov– Smirnov test. One-way ANOVA and Tukey's post hoc test were performed for parametric data, while the Kruskal-Wallis test was used for non-parametric data. Results were expressed as mean ± standard error of the mean (SEM).

Results

Count of Sperm

The data analysis revealed notable differences in sperm count among the groups. The TD group exihinbited a significantly lower average sperm count compared to the control group ($^{***}P$<0.001). In the torsion/ detorsion groups receiving safranal (TD+Sa 0.1,0.5), the sperm count was notably lower than the control group ($^{***}P$<0.001). However, a significant increase in sperm count was observed in these groups compared compared to the TD group ($^{###}P$<0.001). Additionally, the groups adminstrated only safranal (Sa 0.1, 0.5), exihibited a significantly higher sperm count than the TD group ($^{###}P$<0.001) (Figure 1).

Sperm Morphology

The analysis of sperm morphology revealed noteworthy findings. The TD group exhibited a significant increase in the proportion of abnormal sperm compared to the control rats ($^{***}P$<0.001). Moreover, both TD+Sa 0.1 and TD+Sa 0.5 groups showed a significant increase in the number of abnormal sperms in comparison with the control group (***P<0.001). However, there was a notable reduction in aberrant sperm count in the TD+Sa 0.1 and TD+Sa 0.5 groups compared to the TD group ($^{###}P$<0.001). Moreover, the groups adminstrated only safranal (Sa 0.1, 0.5) displayed a significant decrease in abnormal sperm count compared to the TD group ($^{###}P$<0.001)(Figure 2).

Testicular Histological Variables

Based on the findings, the mean Johnson's score (MJS) underwent a substantial decrease in the TD and TD+Sa (0.1,0.5) groups in comparison with the control group ($^{***}P$< 0.001, $^{**}P$< 0.01). However, no significant changes in MJS were observed in the TD+Sa 0.1, 0.5 groups in comparison with the TD group, despite an increase. The seminiferous tubular diameter (STD) analysis demonstrated a substantial reduction in tube diameter in the TD group compared to the control group ($^{***}P$< 0.001).

TABLE 2: Comparison of seminiferous tubule diameter, epithelium height, and testicular mean Johnson's score in control, TD, TD+Sa 0.1&0.5, Sa 0.1&0.5 groups.

Group	Mean Johnsen's Score	STD	HE
Control	8.8± 0.25	127.6±0.67	19±0.44
TD	6.5±0.28***	110±0.81***	9.7±0.47***
TD+Sa0.1	7.3±0.12**	120.6±0.78$^{\#}$	16.1±0.9$^{\#\#\#}$
TD+Sa0.5	7.2±0.14***	118.7±1.1	14.8±0.6$^{**/\#\#}$
Sa0.1	8.5±0.2$^{\#\#\#}$	126.3±5.9$^{\#\#}$	18.6±1.3$^{\#\#\#}$
Sa0.5	8.2±0.14$^{\#\#\#}$	123.6±2.3$^{\#\#}$	18.8±0.5$^{\#\#\#}$

Notes: All outputs have been written as Mean ± SE. STD: seminiferous tubule diameter; HE: height of epithelium. "1. control group, 2. torsion/detorsion group (TD), 3. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), 4. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), 5. Safranal 0.1 (Sa 0.1), and 6. Safranal 0.5 (Sa 0.5)." The symbol "* represents significant differences with the control and # stands for significant differences with TD group ($^{***}P$<0.001, $^{**}P$< 0.01, $^{\#\#\#}$P<0.001, $^{\#\#}$P<0.01, $^{\#}$P< 0.05)."

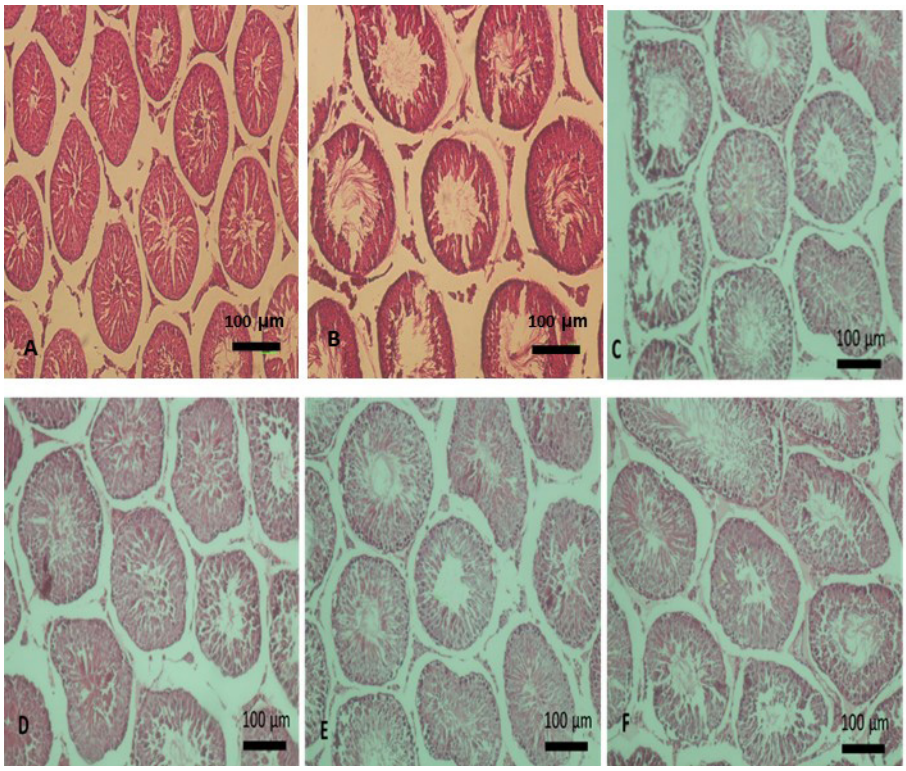

FIGURE 3. Comparison of the histological findings among different groups. A. control group, B. torsion/detorsion group (TD), C. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), D. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), E. Safranal 0.1 (Sa 0.1), and F. Safranal 0.5 (Sa 0.5).

Nevertheless, the control group showed no significant difference from the other groups. In addition, a comparison between the TD group and safranal-treated groups revealed a significant increase in diameter in the TD+Sa 0.1and Sa 0.1, 0.5 rats ($^{\#\#}P$< 0.01, $^{\#}P$< 0.05). In the TD and TD+Sa 0.1 group, the epithelium height of the seminiferous tubules (HE) underwent a decrease which was significant compared to the control group ($^{***}P$< 0.001, $^{**}P$< 0.01). Conversely, significant increases were noted in the safranal-treated groups ($^{\#\#\#}P$< 0.001, $^{\#\#}P$< 0.01) compared to the TD group (Table 2, Figure 3).

Effect of Safranal on Apoptosis in TD Rats

The assessment of Bax expression revealed a notable

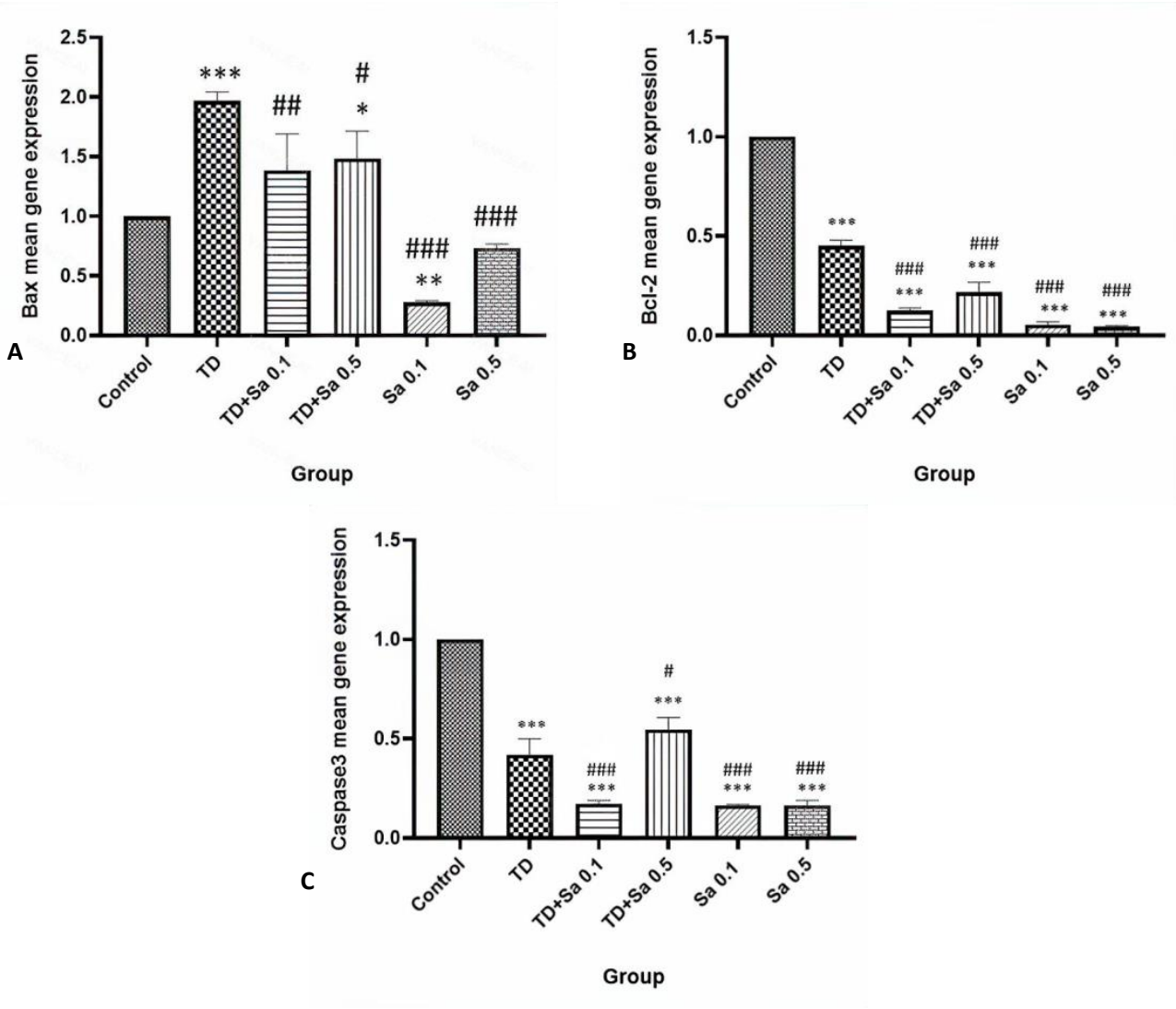

FIGURE 4. (A,B,C): Comparison of expression of Bax, Bcl-2 mRNA and caspase-3 ratio among different groups. 1. control group, 2. torsion/ detorsion group (TD), 3. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), 4. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), 5. Safranal 0.1 (Sa 0.1), and 6. Safranal 0.5 (Sa 0.5). The symbol * marks significant differences with the control and # represents significant differences with the TD group ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$).

increase in the TD group compared to the control group ($^{***}P< 0.001$). In addition, in the torsion/detorsion groups receiving safranal, Bax levels also shoewed an increase ; nevertheless, this rise was significant only in the TD+-Sa 0.5 group when compared to the control group ($^{*}P< 0.05$). Besides, a reduction in Bax levels was observed in groups treated solely with safranal compared to the control group, with a significant decrease ($^{**}P<0.01$) noted in the Sa 0.1 group. Notably, all treatment groups showed a significant decrease in Bax levels compared to the TD group ($^{###}P<0.001$, $^{##}P$ 0.01, $^{#}P<0.05$) (Figure 4-A).

Regarding Bcl-2 analysis, a substantial decrease in expression was observed in all groups compared to the control group ($^{***}P < 0.001$). Specifically, the TD group displayed a significant decrease in Bcl-2 levels compared to all other groups ($^{###}P < 0.001$) (Figure 4-B).

The results of caspase-3 showed significantly reduced expression in all experimental groups in comparison with the rats in the control group ($^{***}P < 0.001$). Moreover, a significant reduction in caspase-3 level was found in the TD group compared to the other groups ($^{###}P < 0.001$, $^{#}P < 0.05$) (Figure 4-C).

Effect of Safranal on Redox Status in TD Rats

The MDA levels in the TD group significantly increased compared to the control group ($^{*}P < 0.05$), while no substantial changes were noted in the other groups. Although MDA levels decreased in other groups compared to TD, these changes did not reach substantial sig-

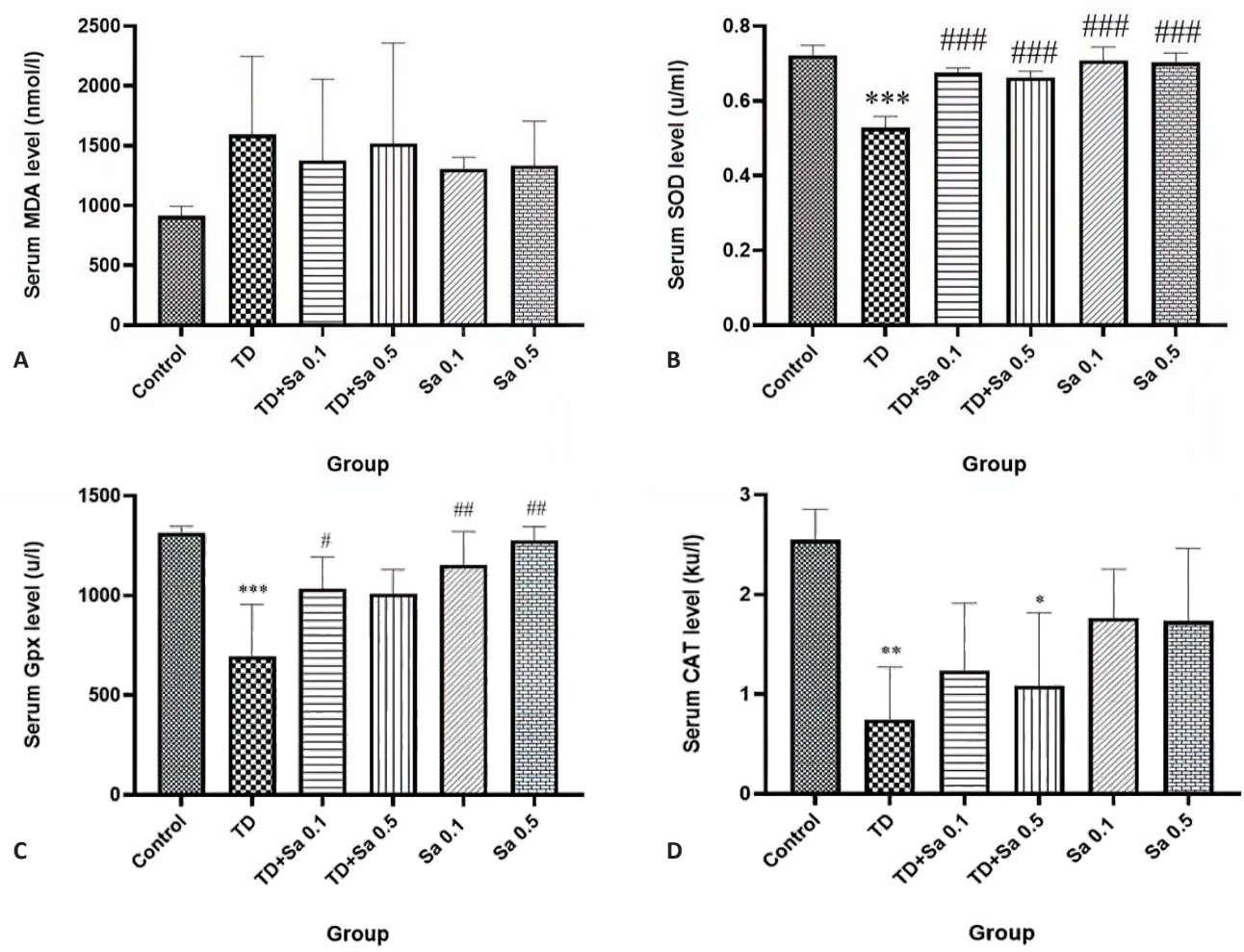

FIGURE 5. (A,B,C,D): Comparison of antioxidant capacity among different groups. 1. control group, 2. torsion/detorsion group (TD), 3. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), 4. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), 5. Safranal 0.1 (Sa 0.1), and 6. Safranal 0.5 (Sa 0.5). The symbol * marks significant differences with the control and # represents significant differences with the TD group ($^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$).

nificance (Figure 5-A).

In terms of SOD levels, the TD group displayed a significant decrease compared to the control group ($^{***}P<0.001$). However, this change was not statistically significant in the other groups compared to the control. The TD group exhibited significantly lower SOD levels than all other groups ($^{###}P <0.001$) (Figure 5-B).

GPx analysis revealed a significant reduction in the TD group ($^{***}P<0.001$), while other groups did not show significant changes. Moreover, when compared to the TD group, the TD+Sa 0.1, Sa 0.1, and 0.5 groups disclosed a significant increase in GPx levels ($^{##}P<0.01$, $^{#}P<0.05$) (Figure 5-C).

Analysis of CAT showed a significant reduction in the TD group compared to the control group ($^{**}P< 0.01$). In addition, the TD+Sa 0.5 group indicated significantly lower CAT levels than the controls ($^{*}P < 0.05$). Although an increase in CAT levels was observed compared to TD and other groups, these differences were statistically significant (Figure 5-D).

The Serum Level of Testosterone

Testosterone analysis revealed a significant decrease in the TD group compared to the control group ($^{**}P< 0.01$). However, no significant differences were observed in the other groups compared to the control. Additionally, compared to the TD group, there was an insignificant increase in testosterone levels in the other groups (Figure 6-A).

The Serum Level of LH

A significant decrease in the serum level of LH hormone was observed in the TD group ($^{*}P<0.05$), but no statistical difference was found when compared to the other experimental groups. Likewise, compared to the TD group, LH levels increased in the other groups. No-

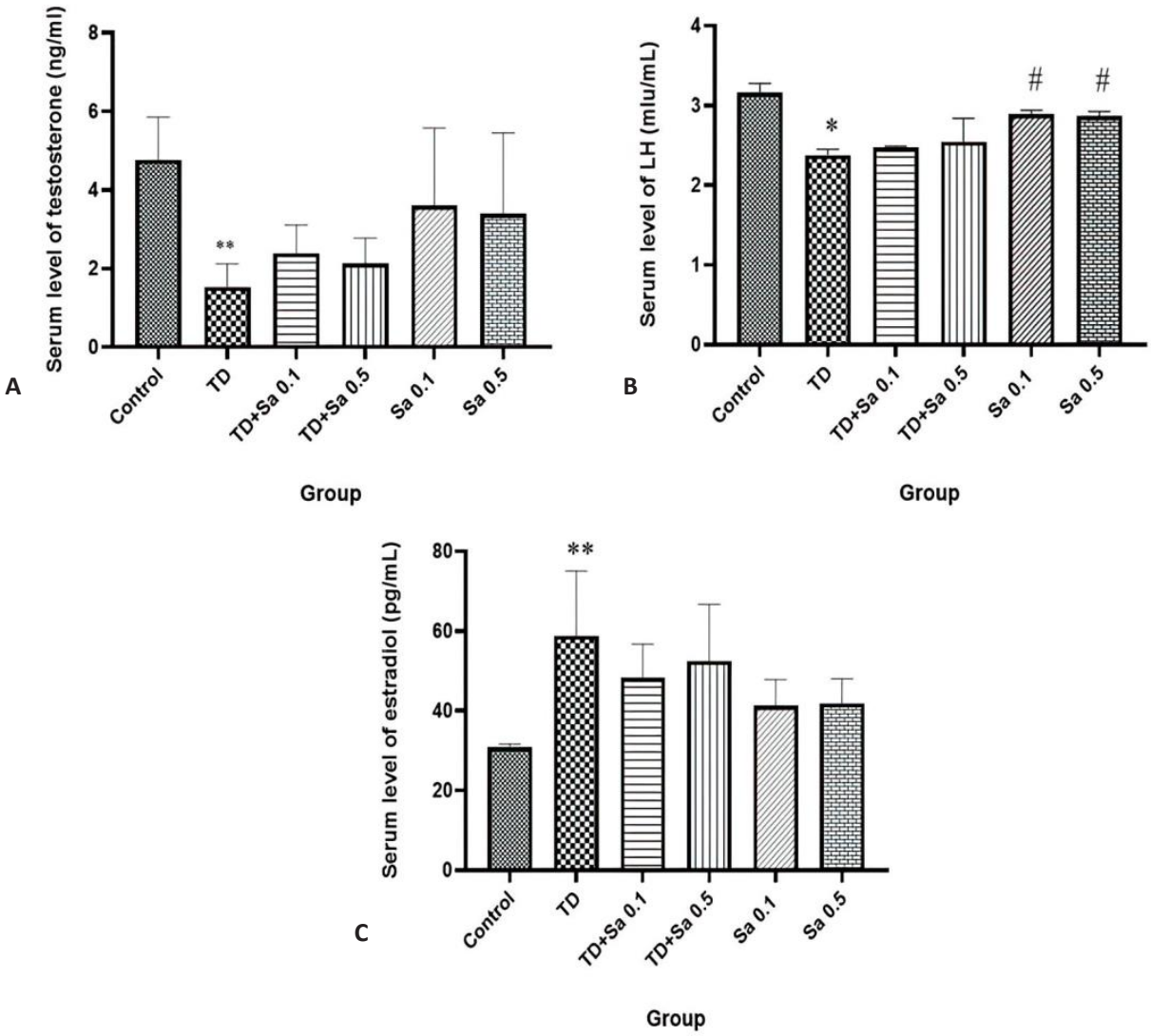

FIGURE 6. (A,B,C): Comparison of the serum level of testosterone, LH, and estradiol among different groups. 1. control group, 2. torsion/ detorsion group (TD), 3. torsion/ detorsion/ safranal (0.1) group (TD+Sa 01), 4. torsion/ detorsion/ safranal (0.5) group (TD+Sa 0.5), 5. Safranal 0.1 (Sa 0.1), and 6. Safranal 0.5 (Sa 0.5). The symbol * marks significant differences with the control and # represents significant differences with TD group ($^{**}P < 0.01$, $^{*}P < 0.05$, $^{\#}P < 0.05$).

tably, this increase was only significant in the groups receiving safranal alone (Sa 0.1, 0.5) ($^{\#}P < 0.05$) (Figure 6-B).

The Serum level of estradiol

Estradiol hormone underwent a significant increase in the TD group ($^{**}P < 0.01$), while there was no significant difference in the other groups. Moreover, compared to the TD group, estradiol levels also decreased in the other groups, although the decrease was not statistically significant (Figure 6-C).

Discussion

The present investigation highlights safranal's potential in improving various parameters associated with testicular injury. It notably increased the HE of the seminiferous tubules, elevated sperm count, reduced abnormal sperm count, lowered Bax (apoptosis Regulator) gene expression, and increased levels of SOD and GPx enzymes. These findings align with prior research indicating that TD induces progressive histopathologic alterations, including reduced Johnson's score, seminiferous tubule epithelium thickness, and diameter corroborating earlier studies (Elmimehr et al., 2021; Moghimian et al., 2017; Shokoohi et al., 2018). Also, testosterone concentration decreased in the TD group, potentially due to injury in the testis' Leydig cells. Cinsistant with our previous research, detox-torsion caused a reduction in blood testosterone levels (Shokoohi et al., 2018). Testosterone usually suppresses LH secretion through negative feed-

back. Despite this, the appropriate rise in LH levels did not occur, possibly indicating a lowered set-point for negative feedback, a functional disorder in the hypothalamic-pituitary-gonadal (HPG) axis (Cicero et al., 1989; Gabriel et al., 1985), or an increase in oxidative stress. Additionally, testicular torsion increased serum estrogen concentrations. Adult testicular Leydig cells express aromatase (P450 aromatase), which actively synthesizes estradiol at a notably higher rate compared to adult Sertoli cells (Carreau et al., 1999; Carreau et al., 2003; Hess 2003; Payne et al., 1987). Furthermore, sperm counts in the TD groups were lower than the sham group, aligning with previous studies (Ameli et al., 2018; Sertkaya et al., 2014).

Moreover, this study revealed that oxidative stress markers significantly impaced parameters related to biochemistry and histology. Testicular IRI resulted in increased lipid peroxidation concentrations and reduced serum activity in SOD, GPx, and CAT. These findings parallel our earlier research (Shokoohi et al., 2018; Shokoohi et al., 2018).

Furthermore, we evaluated apoptosis, which has been extensively shown as one of the main methods of controlled death caused by cell damage or external stress (Nikoletopoulou et al, 2013). There are two (non-exclusive) caspase-dependent pathways of apoptosis that can be mediated by several mechanisms (Jan, 2019). According to the first principle, an internal death signal would be changed into an "external" and/or receiver-mediated pathway, recognized as one of the externally identified signals/stimuli. The other relies on proteins such as Bax, initiating the "intrinsic" route and the caspase machinary (Wolter et al., 1997). Likewise, anti-apoptotic Bcl-2 family proteins respond to cell survival signals and regulate apoptosis. Increased expression of Bax protein could lead to elevated apoptosis rates (Danarto et al., 2019). Consequently, caspase-3 is one of the major molecules in caspase-dependent apoptosis, as it establishes biochemical pathways that lead to DNA breakage and cell death (Bell & Megeney, 2017). In this study, the TD group exihibited elevated Bax and caspase-3 expressions while Bcl-2 expression dramatically decreased. The heightened apoptotis in the TD group correlated with increased histological alterations, elevated malondialdehyde (MDA) activity, and decreased SOD, GPx, and CAT. This indicates that Bcl-2 may indirectly regulate antioxidant defense or inhibit lipid peroxidation, serving as a potent antioxidant (Hockenbery, Oltvai, Yin, Milliman, & Korsmeyer, 1993). These findings align with previous studies (Koji, Hishikawa, Ando, Nakanishi, & Kobayashi, 2001; Lee et al., 2012; Mertoğlu et al., 2016).

Previous research has used different medications and chemical mediators to mitigate germ cell injury post-detorsion operation following testicular torsion. However, few of these studies have been widely used in medical practice due to their after effect profiles (Beheshtian et al., 2008; Üstün et al., 2008). Currently, treating diseases with herbal medications is gaining attention. Safranal, a monoterpene aldehyde found in saffron oil (Assimopoulou, Sinakos, & Papageorgiou, 2005), exhibits antioxidant effects by stabilizing membranes, suppressing ROS, and reducing membrane lipid peroxidation (Hosseinzadeh & Sadeghnia, 2005). Its preventive effect have been established in experimental IRI studies (Hosseinzadeh & Sadeghnia, 2005; Sadeghnia, Shaterzadeh, Forouzanfar, & Hosseinzadeh, 2017). Despite numerous studies on the effects of safranal, its mechanism of action remains elusive. This research aimed to investigate different doses of safranal's impacts on IRI post-testis torsion treatment, examining the expression of Bax, Bcl-2, and caspase-3 genes in an adult rat testicular IRI model. According to the findings of the present research, safranal at both administered doses can elevate the "Mane Johnson score," seminiferous tubule diameters, and thickness of the seminiferous tubule epithelium compared to the TD group. This could be because of safranal's antioxidant properties and its capacity to suppress ROS generation (Kulkarni & Patil, 2004; Sertkaya et al., 2014). The findings also showed higher serum testosterone and LH levels in the treatment groups compared to the TD group, with lower estrogen levels in the safranal groups. This potential prevention of Leydig cell damage by safranal in testicular tissue might explain the estrigen leve reduction. Safranal has demonstrated antioxidant activities in vitro (Delkhosh-Kasmaie, Farshid, Tamaddonfard, & Imani, 2018), reducing oxidative-stress-related brain damage following cerebral ischemia/reperfusion (Ahmad et al., 2017). In other studies, safranal improved learning and memory impairments in diabetic rats, decreased MDA levels, and elevated serum SOD levels (Dokmeci et al., 2007). In this study, safranal consumption notably reduced MDA levels while remarkably increasing GPx, CAT, and SOD levels in plas-

ma compared to the TD group, whith the 0.1 mg/kg dose showing more improvement than the 0.5 mg/kg dose.

Due to the high polyunsaturated fatty acid content in their cell membranes, Spermatozoa are more sensitive to ROS deleterious effects than other cells (Hekimoglu et al., 2009). Testicular IRI can lead to DNA damage, spermatogenesis arrest, and inhibited protein synthesis, resulting in decreased sperm generation (Majzoub & Agarwal, 2018). Previous studies have highlighted a correlation between the use of antioxidants and an increase in sperm concentration (Asadi et al., 2014; Belhan et al., 2020). In the current research, safranal at doses of 0.1 and 0.5 mg/kg improved sperm count and mitigated abnormalities caused by testicular IRI. These results are in line with prior animal studies that adminstrated safranal (Asadi et al., 2014; Mardani, Vaez, & Razavi, 2014).

Additionally, this study demonstrated that safranal significantly decreased Bax and caspase-3 expression, potentially indicating a connection with reduced oxidative stress. Safranal may exert its protective effect by inhibiting apoptosis, due to the modulation of both pro-apoptotic protein Bax expression and caspase-3 activation. Notably, the 0.1 mg/kg safranal dose exhibited a more pronounced increase in Bax and Caspase expression. Previous studies have showcased safranal's decreasing effect on caspase-3 and Bax expression in ischemic liver injury (Ozkececi et al., 2016). Another study showed that the anti-apoptotic and anti-inflammatory effects of safranal on myocardial ischemia/reperfusion damage involved obstructing the IKK-β/NF-κB/Bax/caspase-3/TNF-α signaling pathway while increasing Bcl-2 expression (Bharti, Golechha, Kumari, Siddiqui, & Arya, 2012). Likewise, safranal enhanced recovery in rats with spinal cord injuries by upregulating Bcl-2 and suppressing Bax in spinal tissue (Zhang et al., 2015).

Conclusion

These data suggest that safranal may protect testicles against IRI, significantly reduce apoptosis, and exhibit antioxidant properties, particularly evident with the 0.1 safranal dose. These medications hold promise in reducing male infertility due to IRI and its associated treatment costs. The protective effect of saffron seems multifaceted, prompting the need for further extensive studies to unravel its intricate mechanisms concerning testicular IRI. Hence, comprehensive research involving varying time intervals, mating studies, and repeated doses of safranal is needed to apply a clinical application of safranal.

Conflict of interest

The authors disclosed no proprietary or commercial interests associated with any of the products or concepts examined in this study.

Acknowledgments

This article draws upon a research project identified by the code (IR.GMU.REC.1398.130). We would like to thank the Research and Technology Deputy and the Student Research Committee of Gonabad University of Medical Sciences for funding our study.

Ethical Approval

The Animal Ethics Committee of Gonabad University of Medical Sciences verified each experimental procedure (IR.GMU.REC.1398.130).

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