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Experimental Research Article

The effect of Safranal on histological damages and oxidative stress induced by ischemia-reperfusion in adult rat ovaries

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ABSTRACT

Introduction: Ovarian torsion is an emergency condition that occurs when the adnexa undergoes complete or partial rotation. Following ovarian torsion, damage caused by ischemia/reperfusion can impact fertility and sex hormone secretion. Our study aims to investigate the role of safranal in preventing ovarian ischemia-reperfusion injury in rats.

Methods: Animal subgroups included: sham, ovarian torsion/detorsion (OT), ovarian torsion/detorsion with the treatment of safranal (OTS 0.1, 0.5), and safranal group (OS 0.1, 0.5). Ovarian torsion was induced in the left ovary of rats for 2 hours. Intraperitoneal treatment with safranal at 0.1 and 0.5 mg/kg doses was performed 30 minutes before the detorsion operation. 48 h after detorsion, ovarian tissue was collected to evaluate the histopathological scores and apoptosis gene expression of Bcl-2, caspase 3, and Bax. Blood samples were collected to measure plasma estradiol levels and oxidative stress parameters.

Results: Analyses demonstrated that ovarian follicular damage was accompanied by increased serum malondialdehyde (MDA) levels and increased expression of Bcl2 and caspase-3 genes. Additionally, serum levels of glutathione peroxidase (GPX), estrogen, and superoxide dismutase (SOD) decreased in the OT group. However, treatment with safranal 0.5 mg/kg was accompanied by an improvement in the histopathological score of the ovarian tissue and a reduction in apoptosis and oxidative stress.

Conclusion: Safranal exhibits antioxidant properties in a rat model of ovarian torsion and could be considered a cost-effective therapeutic option for ovarian detorsion in gynecological clinics.

Keywords:
Safranal
Oxidative stress
Ischemia-reperfusion
Ovaries

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Introduction

According to the studies, ovarian torsion has been introduced as one of the scarce and emerging surgical conditions, which are more prevalent in women of childbearing age (Hibbard 1985). Twisting or torsion of the ovary occurs around the axis of its supporting ligaments. Following ovarian torsion, the arteries and veins of the ovary are blocked, and finally, ovarian ischemia occurs (Becker et al., 2009). This disorder causes damage to ovarian tissue, leading to necrosis, degeneration, and destruction of developing follicles (Ersoy et al., 2016). Female infertility could be caused by any of these conditions. Notably, nonspecific symptoms and clinical findings of such pathological conditions could delay the diagnosis and treatment process. After a diagnosis of torsion, detorsion or untwisting is performed as the first line of treatment. Detorsion is associated with a reperfusion injury, including reactive oxygen species (ROS) released during reperfusion (Aslan et al., 2017). In addition, researchers attempted several anti-inflammatory and anti-oxidant agents for preventing damage to the ovary tissues induced by the ischemia/reperfusion (I/R) process (Abali et al., 2013; Akdemir et al., 2014; Yurtcu et al., 2015). Massive oxidative stress-induced ROS generation could disrupt the mitochondrial membrane and trigger the release of cytochrome c in the subsequent apoptotic event. Components with antioxidant properties could prevent the potential subsequent damages (Agarwal et al., 2012). However, regarding the unfavorable consequences of chemical medicines, researchers further emphasized the utilization of conventional medicine, especially plant therapy. Saffron (stigma of the Crocus sativus flower) has been largely utilized in the food industry as a natural color, natural therapeutic product, and spice for many centuries (Hosseinzadeh et al., 2008; Melnyk et al., 2010). Some studies implied the fact that the antitumor as well as anti-oxidant characteristics of the saffron extract are caused by the activities of the secondary metabolites and active derivatives like crocin, safranal, dimethylcrocetin, and crocetin (Kanakis et al., 2007a; Tsafrir et al., 2012). Based on several studies in the field, dimethylcrocetin and crocin naturally occur in saffron, and safranal has been considered to be a key element of the saffron oil (Becker et al., 2009; Kanakis et al., 2007b). Traditionally, saffron is regarded as an essential herb for supporting the reproductive system and promoting overall menstrual health. Considering the histopathological examinations, saffron ingredients attenuate the testicular cell damage caused by genotoxins (Koul and Abraham 2018). Other studies also pointed to the correlation of the use of saffron to lower risks of several disorders like cardiovascular diseases, depression, gastric distress, anxiety, premenstrual syndrome, and insomnia (Hosseinzadeh et al., 2008; Melnyk et al., 2010). Moreover, evidence indicated numerous dietary health merits, including protection against cataracts, oxidative stress in the brain, diabetes, kidneys, and livers for saffron and specifically safranal. In addition, safranal has anti-cancer, anti-bacterial, and anti-oxidant effects (Samarghandian et al., 2014). Our previous study showed that safranal may protect testicular tissue from I/R injury through antioxidant and antiapoptotic pathways (Ebrahimi et al., 2023). However, fewer data are available on the effects of saffron on female reproductive system; Thus, this research investigated the possible impacts of Safranal as a main component of saffron, on the histological damage, oxidative stress, and evaluation of apoptosis induced by the ovarian I/R in the rat model.

Material and Methods

The animal experiment protocol and ethical considerations in the present study were approved and supervised by the animal ethics committee of Gonabad University of Medical Science (IR.GMU.REC.1398.021).

In this experiment, 48 adult female Wistar rats (225±25g) were provided by the animal house of Gonabad University of Medical Sciences and kept under the standard conditions. All the animals were assigned to groups of 3-4 in a cage at 21-23 °C and 12-h light/dark cycle with free access to water and rat special food.

Experimental groups

Based on Safranal treatment, 48 female rats were randomly grouped into 6 experimental groups:

1. Sham: Without inducing torsion/detorsion, the midline of the abdominal wall has been cut and then sutured.

2. Ovarian torsion/detorsion (OT group): Induction of torsion in the rats' left ovary for 2 h. In the next stage, detorsion was done for 24 h. 30 min before the detorsion operation, animals were injected with normal saline intraperitoneally.

3. Torsion/detorsion/safranal at the dose of 0.1 mg/kg (OTS 0.1 group): Induction of ovarian torsion/detorsion similar to OT group was performed. The animals have

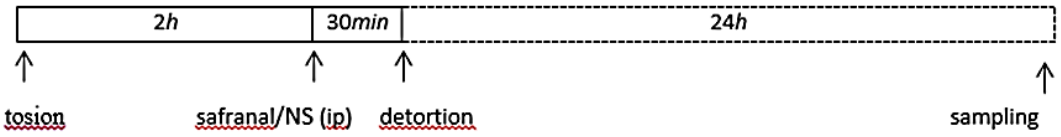

FIGURE 1. Time-line diagram showing the experimental protocol used in sham, OT, OTS 0.1, 0.5 and OS 0.1, 0.5 groups. OT; ovarian torsion, S; safranal.

been given safranal (0.1 mg/kg) 30 min prior to the detorsion operation.

4. Torsion/detorsion/safranal at the dose of 0.5 mg/kg (OTS 0.5 group): Induction of ovarian torsion/detorsion similar to OT group was performed. The animals have been given safranal (0.5 mg/kg) 30 min before the detorsion operation.

5. Safranal at the dose of 0.1 mg/kg (S 0.1 group): No operations were performed. Animals were treated with 0.1 mg/kg safranal.

6. Safranal at the dose of 0.5 mg/kg: No operations were performed. Animals were treated with 0.5 mg/kg safranal (S 0.5 group).

The animals were in the same estrous cycle in all groups, and safranal was injected intraperitoneally.

Surgical procedures and sampling method

To induce ovarian torsion, the animals were first anesthetized by ketamine and xylazine (50 and 10 mg/kg, respectively). After that, a 2.5 cm long incision was done in the midsagittal line of the abdominal wall. After cutting the peritoneum, the ovary and uterine horn were exposed. The left ovary and the ligaments connected to it were rotated 720 clockwise around its longitudinal axis. To prevent untwisting, the ovaries were connected to the abdominal walls via a 5/0 suture. Then the abdominal wall was stitched by 4/0 sutures. After surgery, ovarian torsion induction continued for 2 h.

After the ovarian torsion period, the ovarian twist was opened (detorsion) and the ovaries were set aside for 24 h for re-perfusion. The Safranal extract was intraperitoneally injected at 30 min before opening the ovarian torsions. After the period of detorsion, the animals were anesthetized with ketamine/ xylazine to collect ovarian tissues and blood samples (Fig. 1). In this study, half of the rats in each group (4 rats) were randomly selected for real-time PCR, while the other half (4 rats) were chosen for histological preparations. The ovarian tissues were removed to evaluate the histological and gene expression alterations. Blood samples were taken from the animals' hearts to assess the hormones levels and oxidative balance. The blood samples were centrifuged at 4000 RPM, for 5 min, and the serum was seperated. Serum samples were kept at -70 °C until the assays (Samarghandian et al., 2014).

Histopathological evaluation

Upon the ovariectomy, ovary samples were fixed in a ten percent formalin solution for 3 days. The samples were placed in the tissue processor. Subsequently, paraffin molds were created from the samples. 5-µm-thick sections of paraffin mold containing ovary samples were prepared using a microtome. Then H&E staining technique was performed on the slides. The entire tissue sample from the cortex to the ovarian medulla was used for histometric evaluations. Thus, the number of different follicle types was counted during ovarian folliculogenesis for each slide. The counts of the various follicle types were compared among the studied groups. (Samare-Najaf et al., 2020).

Apoptotic cell detection

We used TUNEL staining for evaluating the apoptotic oocyte in the follicle ovaries. Paraffin-embedded sections of ovaries were placed on poly-l-lysine coated slides. A solution of 3% hydrogen peroxide in ethanol was applied to block the internal peroxidase enzymes for 15 min. After washing with PBS, the samples were incubated with proteinase K at room temperature for 20 min, and with the reaction solution according to instruction provided be the TUNEL staining kit. In the next step, after washing, the samples were exposed to diaminobenzidine solution for 15 min. After washing again, hematoxylin was used to stain the nuclei of the unreacted cells. Finally, the slides were observed using a light microscope. The brown color of the cell nucleus was considered as a positive TUNEL reaction (Jalilvand et al., 2019).

Real-time PCR

The isolated ovarian samples were homogenized, and total RNA was extracted using the Total RNA Mini Kit (Favorgen, Taiwan, Cat. No.: FABRK000). cDNA synthesis was carried out with the YT4500 cDNA Synthesis Kit (Yekta Tajhiz Azma®, Iran). Subsequently, real-time PCR was performed using the YTA Super SYBR Green qPCR MasterMix: The real-time PCR was performed following the kit's protocol: initial heating at 94°C for 3 min, then cycling through 95°C for 10 sec, 60°C for 10 sec, and 72°C for 20 sec. The β-actin gene was used as an internal control gene to calculate ΔCT values. Also, the relative measurement of gene expression changes was using the $2^{-\Delta\Delta CT}$ formula. Oligomer sequence (5'-3') of primers used in real-time-PCR were as: ***β-actin*** (F primer: GTCGTGCTTGCCATTCAG/ R primer: GGTATCTTCTTTCCATTCTTCAGTAG), ***Bax*** (F primer: TTTGCTACAGGGTTTCATCCAG / R primer: GTTGTCCAGTTCATCGCC), ***Bcl$_2$*** (F primer: TGTGGATGACTGACTACCTGAACC / R primer: CAGCCAGGAGAAATCAAACAGAGG), ***Caspase3*** (F primer: GTGGAACTGACGATGATATGGC / R primer: CGCAAAGTGACTGGATGAACC),

Evaluating the oxidative stress parameters (MDA, SOD, and GPX)

After the test period, 0.20 ml of serum were transferred into a microtube containing 3.0 ml of glacial acetic acid. Then, 1% thiobarbituric acid (TBA) in 2% sodium hydroxide (NaOH) was added to the microtube. The mixture was then immersed in boiling water for 15 min. Once cooled,

the absorbance of the pink-colored solution was measured at 532 nm using the ABER-2 AccuBioTech spectrophotometer, comparing test and standard solutions.

Serum levels of GPX and SOD were measured according to the protocols provided by the kits' manufacturers (Randox and Ransod, UK). GPX levels were measured using the Paglia-Valentine method, which assesses enzyme activity based on the catalyzed oxidation of glutathione (GSH) by cumene hydroperoxide. SOD levels were determined by generating superoxide radicals through xanthine and xanthine oxidase; these radicals react with iodonitrotetrazolium chloride to produce a red formazan dye, allowing spectrophotometric quantification.

Measurement of estrogen level

Serum estrogen levels were measured using the Demeditec Estradiol rat ELISA Kit (Germany). The kit utilizes a solid-phase enzyme-linked immunosorbent assay (ELISA) that functions based on the principle of competitive binding. Microtiter wells are coated with an anti-estradiol antibody. Once the substrate solution is added, the intensity of the resulting color is also inversely proportional to the concentration of free estradiol in the sample. With a Sorbance, readings were taken at 405 nm. (Soltani et al., 2017).

Data analysis

The statistical analysis was conducted using SPSS 22 (IBM, USA). The Kolmogorov-Smirnov test was employed to ascertain the normal distribution of the data. Each data point was presented as the mean ± standard error (SE). ANOVA (one-way analysis of variance) and Tukey post hoc tests were utilized to compare histopathological variables and oxidative stress values. The significance level was set at $P<0.05$.

Results

Histological variables of the ovarian tissues

Considering the count of follicles, results indicated a significant decline in the quantities of preantral, antral, and graafian follicles, along with a notable increase in atretic follicles in the OT group when compared with the sham group (*$p<0.05$). Treatment with safranal enhanced follicle numbers in the OTS 0.5 group compared to the OT group. Additionally, both OTS (0.5, 0.1) groups exhibited a marked reduction in etheric bodies compared to the OT group (#$p<0.05$) (Table1) (Fig. 2).

The results obtained from the TUNEL staining showed in the group treated with ovarian torsion (OT), there was a significant increase in positive TUNEL reaction in pre-antral, antral, and graafian follicles when compared to the sham group. However, treatment with safranal in the OTS 0.5 group, significantly decreased the apoptosis index compared to the OT group ($p<0.05$, Table 1 and Fig. 2).

Relative mRNA levels of Bcl-2, Bax, and Caspase3

Regarding the relative expression of Bcl-2, Bax, and Caspase-3, the examination unveiled that in the OT group, the expression levels of these genes were significantly elevated compared to the sham group. However,

TABLE 1. Mean number of the pre-antral follicles, graafian follicles, antral follicles, atretic bodies as well as the apoptosis index in the rats' ovaries in sham and experimental groups.

Group	Pre-antral follicles	Antral follicles	Graafian follicles	Atretic bodies	Apoptosis index
sham	10± 0.44	2.4±0.24	2.6±0.4	1.4±0.21	4.89±0.31
OT	5.4±0.24*	0.8±0.2*	0.8±0.37*	5.4±0.24*	19.65±0.11*
OTS 0.1	6.1±0.92	1.7±0.31	1.2±0.37	2.9±0.2#	15.76±0.27
OTS 0.5	8.8±0.48 #	1.9±0.44 #	2.1±0.73 #	2.1±0.3 #	10.84±0.1 #
OS 0.1	9.5±0.8	2.2±0.38	2.4±0.37	1±0.7	4.32±0.12
OS 0.5	9.8±1.4	2.8±0.48	2.8±0.2	1.1±0.37	4.11±0.21

Evaluation of the number of follicles and apoptosis index following the safranal treatment in ovarian torsion. All data have been shown as the mean± SEM. $p<0.05$ are significant, *$p< 0.05$ vs sham, #$p < 0.05$ vs OT. OT; ovarian torsion, S; safranal.

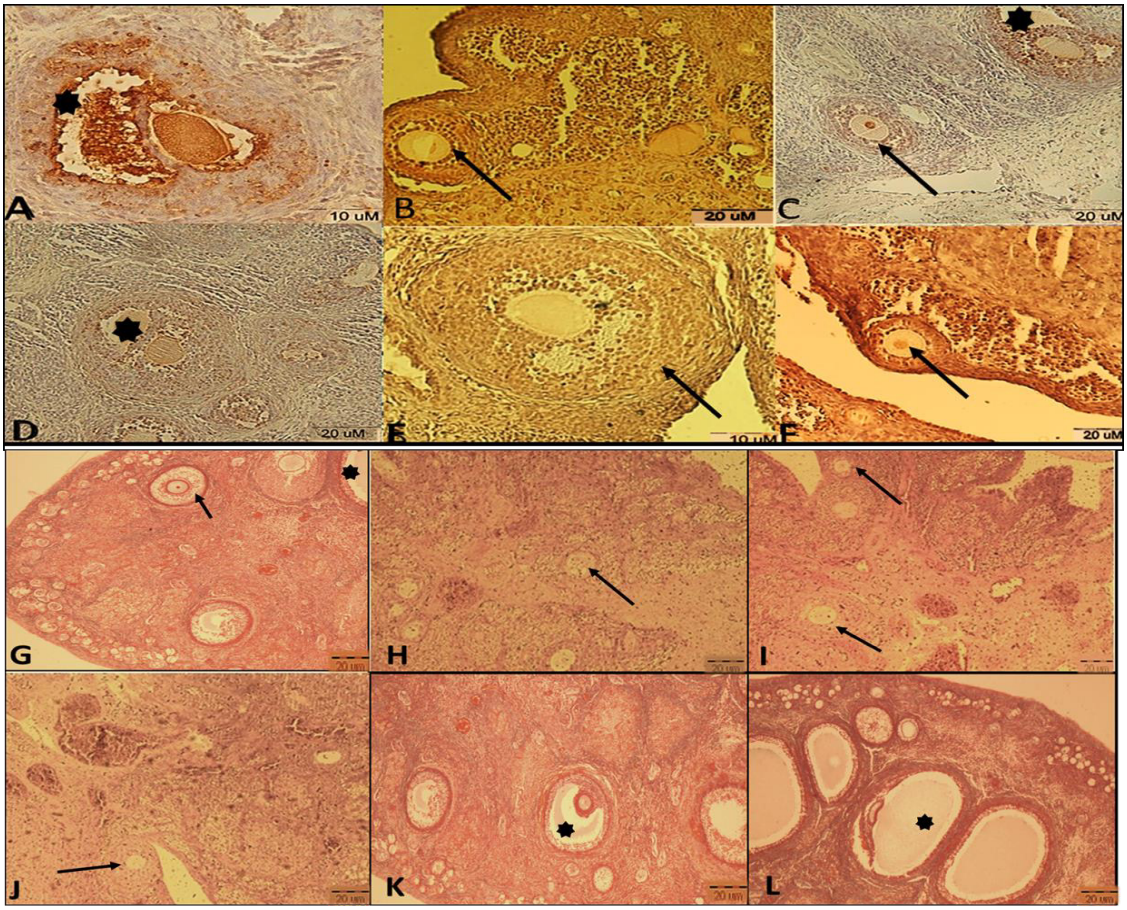

FIGURE 2. Histological findings of study groups with the H&E staining: A: Sham group, B: OT group, C: OTS 0.1 group, D: OTS 0.5 group, E: OS 0.1 group, F: OS 0.5 group. Histological findings of study groups with TUNEL staining: G: Sham group, H: OT group, I: OTS 0.1 group, J: OTS 0.5 group, K: OS 0.1 group, L: OS 0.5 group. OT; ovarian torsion, S; safranal. The arrows indicate the primary follicle, and asterisks indicate the antral follicle in the ovary tissue of all groups.

safranal treatment led to a decrease in their expression levels, with significance observed only in the OTS 0.5 group ($p<0.05$). Notably, a decrease in Bcl2 gene expression was noted in the OT group compared to the sham group, while safranal treatment in the OTS 0.5 group resulted in an enhancement of Bcl2 gene expression ($p<0.05$, Fig. 3).

The serum level of MDA, SOD and GPX

In terms of the serum level of MDA, a substantial increase was detected in the OT group relative to the sham group. Nonetheless, administration of safranal

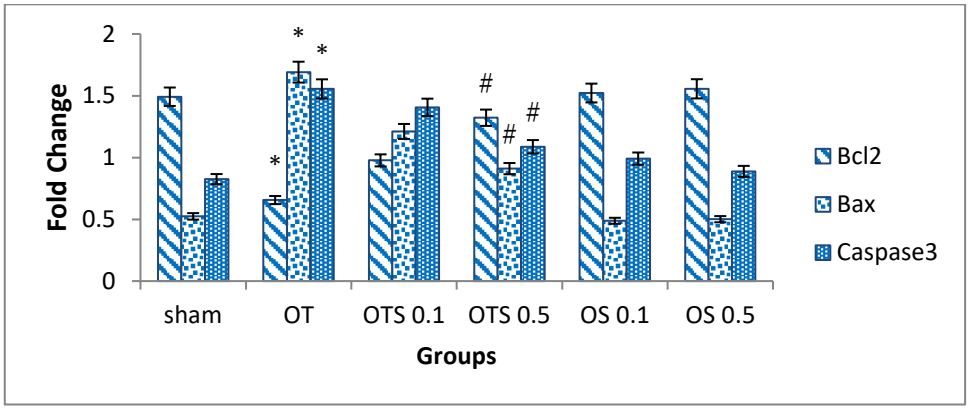

FIGURE 3. Comparisons of the Bcl2, Bax, and Caspase3 gene fold change following the safranal treatment in ovarian torsion. All data have been shown as the mean ±SEM. p<0.05 are significant,* p < 0.05 vs sham, # P < 0.05 vs OT. OT; ovarian torsion, S; safranal.

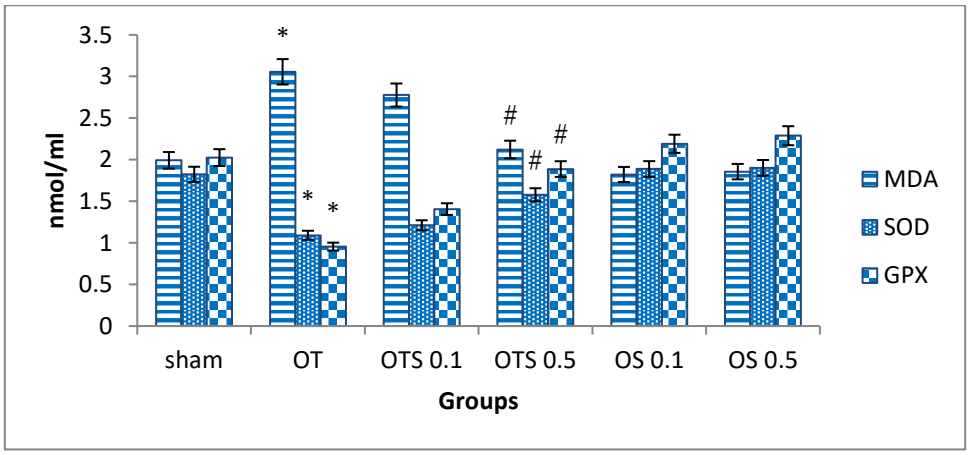

FIGURE 4. Comparison of the level of MDA (nmol/mL), SOD (U/ml), and GPX (U/ml) following the safranal treatment in ovarian torsion. All data have been shown as the mean ±SEM. p<0.05 are significant,* p< 0.05 vs sham, # p < 0.05 vs OT. OT; ovarian torsion, S; safranal.

at 0.5 mg/kg led to a notable decrease in MDA levels (p<0.05), with no significant variance between the OTS 0.5 and OTS 0.1 groups (Fig. 4).

Analysis of the serum levels of SOD and GPX revealed a significant decrease in the OT group compared to the sham group. Treatment with safranal at 0.5 mg/kg resulted in a noteworthy increase in SOD and GPX serum levels in comparison to the OT group (p<0.05), while no significant difference was observed between the OTS 0.1 group and the OT group. (Fig. 4).

The Serum level of estrogen

Evaluation of estrogen levels in serum displayed a significant decrease in the OT group compared to the sham group. However, post-ovarian torsion treatment with safranal significantly boosted estrogen levels in the OTS 0.1 and 0.5 groups relative to the OT group (p<0.05, Fig. 5).

Discussion

The present study was designed to assess the effects of safranal against ovarian I/R injuries. Our study shows safranal treatment was significantly associated with the improvement of ovarian tissue and antioxidant defense enzymes, as well as the reduction of pro-apoptosis indices. As expected, following I/R induction, various damages were observed in ovarian tissue. ROS metabolism leads to DNA damage and lipid peroxidation in mitochondrial and cell membranes and disrupting ion channels. ROS accumulation induces cell death processes such as autophagy, apoptosis, necroptosis, and necrosis (Kalogeris et al., 2014; Zhu et al., 2016). Apoptosis is a programmed cell death that targets ovarian follicles, leading to impaired fertility (Luan et al., 2019). In this

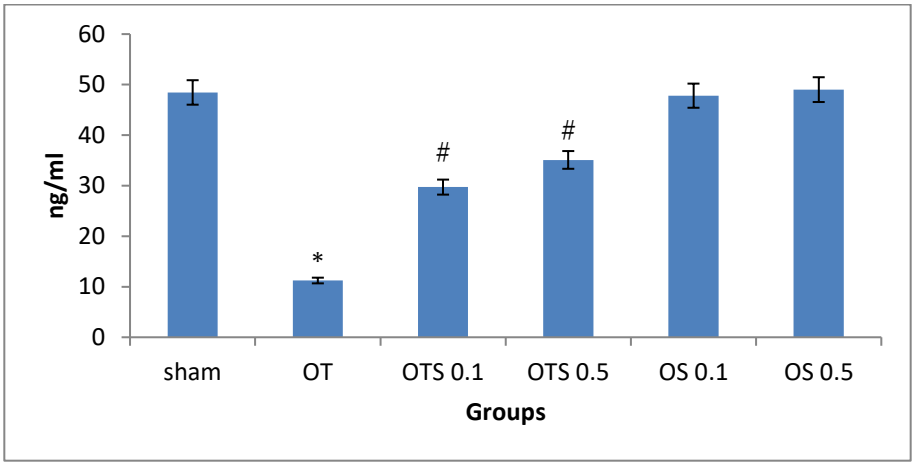

FIGURE 5. Comparison of the level of estrogen following the safranal treatment in ovarian torsion. All data have been shown as the mean± SEM. p<0.05 are significant,* p < 0.05 vs sham, # p< 0.05 vs OT. OT; ovarian torsion, S; safranal.

study, ovarian I/R was induced by ovarian torsion/detorsion. Following ovarian ischemia, remarkable increase of the apoptosis markers Bax and caspase3 was observed, along with reduced Bcl-2 gene expression and decreased antioxidant enzyme levels. It seems that during I/R, the increase of free radicals over the ovarian defense capacity was the main reason for the impaired antioxidant defense system (GPX, SOD) against the appearance of apoptosis (Basini et al., 2008). In this regard, Beyazit F et al. stated that excessive production of ROS during ovarian torsion leads to lipid peroxidation. The result of these events is the production of MDA and impairing antioxidant defense systems. It has also been shown that an increase in MDA was associated with an increase in the number of apoptotic cells in ovarian I/R models (Beyazit et al., 2019).

In the current study, the stereometric data showed a decrease in the types of follicles along with an increase in the TUNEL index in the remaining follicles following I/R. Also, the biochemical data showed a decreased level of estrogen (E2). Reducing estrogen levels can either be due to the direct oxidative stress interference in the apoptosis of estrogen-secreting follicles or due to the hypothalamic-pituitary-gonadal axis dysfunction (Stanley et al., 2014). In line with our findings, it has been shown that the decrease in ovarian E2 levels along with the lack of hormone-secreting follicles can be signs of oxidative stress damage during ovarian I/R (Kolusari et al., 2018). Also, it was shown that I/R damage by increasing oxidative stress causes a significant decrease in ovarian reserve, disruption of sex hormone levels, and histopathological damages (Calis et al., 2015).

The present study recommends pretreatment with the antioxidant and anti-apoptotic safranal to mitigate the damage observed during ovarian ischemia. Safranal is a monoterpene aldehyde and a key component of saffron oil. It has long been considered an antioxidant, anti-apoptotic, anti-tumor, and anti-inflammatory (Nanda and Madan 2021). Safranal has high effectiveness in inhibiting free radicals and membrane-lipid peroxidation during various types of tissue damage (Lei et al., 2022; Samarghandian et al., 2015; Samarghandian et al., 2017). Many experimental studies have reported the effect of safranal in modulating oxidative and apoptosis responses. In this regard, Ahmad N et al. found that treatment with safranal following cerebral ischemia was associated with antioxidant and neurobehavioral activity improvement. In another study, safranal therapy was analyzed during the process of apoptosis on the PC12 cell line, it was observed that safranal leads to a decrease in the ratio of bax/bcl-2 and active caspase-3 (Forouzanfar et al., 2021).

In our previous study, we showed that the protective effect of safranal in testicular I/R, may be primarily due to its ability to inhibit different apoptotic pathways in germ and Leydig cells. In the present study, we found that safranal preserves primary and graft follicles, and reduces apoptosis of rescued follicles, revealed by decreased levels of Bax and caspase 3, increased Bcl-2 gene expression, alongside reduced number of TUNEL-positive cells. The Safranal may have led to the preservation of ovarian follicles by inhibiting apopto-

sis signaling pathways (Zhang et al., 2018), However, enhancement of the antioxidant defense system (GPX, SOD) following safranal treatment can be also responsible for ovarian follicle preservation (Cerdá-Bernad et al., 2022). In this study, the increase in all types of follicles and the decrease in atresia follicles in the safranal-treated groups indicate the preservation of ovarian tissue. In line with our results, Bharti S et al. in a study on myocardial ischemia, showed that safranal has anti-inflammatory and anti-apoptotic potential. They also showed that the effect of safranal is exerted through the IKK-b/NF-jB/Bax/caspase3 signaling pathway inhibition or the positive regulation of Bcl2 expression (Bharti et al., 2012). Also, it was shown that safranal could increase antioxidant capacity and decrease MDA levels in the lower limb and hippocampuss(Hosseinzadeh et al., 2009; Hosseinzadeh and Sadeghnia 2005). It should be noted that ischemia in the present study, treatment with safranal only at a high dose (0.5mg/kg) could prevent apoptotic cell death. In this regard, Bharti S et al.'s study on cardiac ischemia showed that safranal regulates antioxidant levels, TNF-α, and other markers of cardiac damage in a dose-dependent manner and from doses of 1, 2, 3 and 4, only dose 3 was effective (Bharti et al., 2012).

Conclusions

Based on the findings of the present study it may be concluded that safranal treatment regulates the level of sex hormones and tissue damage through the antioxidant defense system after ovarian torsion/detorsion. It also inhibits apoptosis pathways resulting from ovarian ischemia/reperfusion. However, further investigation is needed to better understand and confirm the beneficial effects of safranal in animal models and clinical cases of ovarian damage.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval

The Animal Ethics Committee of Gonabad University of Medical Sciences verified each experimental procedure (IR.GMU.REC.1398.021).

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