



# Protective Effect of Hydroalcoholic Extract of Orange Peel on PCNA and FSH-R Gene Expression in Histological Damage and Oxidative Stress Due to Ovarian Torsion in Adult Rats

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## Abstract

**Objectives:** The aim of this study was to evaluate the protective effect of the hydroalcoholic extract of orange peel on proliferating cell nuclear antigen (PCNA) and follicle-stimulating hormone receptor (FSH-R) gene expression in histological injuries and acid stress caused by ovarian torsion in adult rats.

**Materials and Methods:** In this experimental study, 32 adult female rats were randomly divided into 4 groups. In group 1 (Sham), the abdominal wall was cut without applying torsion and in group 2, ovarian torsion was performed for 2 hours, followed by detorsion for 2 weeks. The hydro-alcoholic extract of orange peel was added to their diet for two weeks in group 3, followed by ovarian torsion for 2 hours and detorsion for 2 hours. Group 4 received the orange peel extract for two weeks and after then ovarian resection for the evaluation of histological damage and blood sampling to examine the serum level of antioxidant enzymes, as well as the expression of PCNA and FSH-R genes in the ovarian tissue.

**Results:** Histological changes in the ovary tissue of rats showed that torsion and detorsion have destructive effects on the ovarian tissue, and torsion/detorsion led to a reduction in the expression of PCNA and FSH-R ( $P < 0.05$ ). Based on biochemical and hormonal results, the ovarian torsion resulted in an imbalance in the oxidative stress markers and hormone profile of rats. Finally, the administration of the hydroalcoholic extract of orange peel due to its high antioxidant properties improves these effects.

**Conclusions:** In general, administering an appropriate dose of the hydroalcoholic extract of orange peel for two consecutive weeks in the diet had a protective effect on the ovarian tissue at the risk of torsion/detorsion

**Keywords:** Orange peel extract, Torsion, Detorsion, PCNA, FSH-R, Rat

## Introduction

Ovarian torsion is a gynecological emergency in all age groups and is about 3% of women's emergencies. The incidence of ovarian rotations in women of all ages and women of childbearing age (15-45 years) is 5.9 and 9.9 per 100 000 women, respectively (1,2). Early diagnosis and management of ovarian torsion treatment are especially important for women in the reproductive age group. Detorsion is one of the interventions that is performed to prevent the loss of the ovarian tissue. Torsion followed by ovarian detorsion or ovarian ischemia/reperfusion is a pathophysiological event (2, 3). Because of the histological damage caused by ovarian torsion with reduced blood flow and subsequent lack of oxygen (ischemia) is observed in the ovarian tissue, and following the inflammatory response caused by detorsion (restoration of blood flow) causes damage to vascular endothelial cells and disorders. It is a microcirculation injury, which is largely responsible for damage to ovarian tissue. Injuries from this ischemia/reperfusion lead to the overproduction of reactive oxygen species such as free radical superoxide ( $O_2^-$ ), hydrogen

peroxide ( $H_2O_2$ ), and free radical hydroxyl species (2,4,5).

Today, natural products such as plant extracts are a good alternative to the applied drugs in oxidative damage to various tissues of the body (1,3). Although many studies exist on the antioxidant and antibacterial effects of orange peel, there are conflicting articles on the wastes of citrus fruits, lemons, and oranges of various types. The citrus extract is rich in nutrients and contains many components. Citrus peel is rich in flavonoids, glycosides, coumarins,  $\beta$  and  $\gamma$ -cystostols, glycosides, and volatile oils and can be effectively used as drugs or dietary supplements (6-8). Orange belongs to the order Sapindales and the family Rutaceae, which is cultivated in the temperate regions of northern and southern Iran, including Gilan and Mazandaran provinces. The composition of orange peel is widely used to aromatize syrups, leucines, and lemonades (9,10). Some studies stated that phenolic compounds at low concentrations affect the activity of enzymes involved in energy production. In this context, several studies showed the antioxidant feature of orange peel that maintains any tissue damage related to oxidative stress (11,12). According

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## Key Messages

- ▶ Orange peel can protect the ovarian tissue against oxidative stress.
- ▶ Orange peel can improve the expression of PCNA and FSH-R genes.

to the above-mentioned explanations, the present study sought to investigate the anti-oxidant effect of orange peel on proliferating cell nuclear antigen (PCNA) and follicle-stimulating hormone receptor (FSH-R) gene expression in histological damage and oxidative stress due to ovarian torsion in adult rats.

### Materials and Methods

This study was conducted after receiving approval from the Ethics Committee of the Research Committee of Tabriz University of Medical Sciences. In general, 32 adult female rats (180-220 g) were studied in this experimental study. All rats were selected from one breed and sex (female) and weighed before any action. All rats received a normal diet and were monitored for two weeks. After two weeks, all rats were weighed again. The rats were randomly divided into 4 groups and 8 rats tested in each group as follows:

1. Group 1: Cutting the abdominal wall without applying torsion (Sham);
2. Group 2: Receiving a normal diet for two weeks and then ovarian torsion for 2 hours and ovarian detorsion for 2 hours. Next, the ovarian resection and evaluation of histological damage and blood sampling to assess the serum level of antioxidant enzymes;
3. Group 3: Adding a hydroalcoholic extract of orange peel (100 mg/kg) to their diet for two weeks, and then receiving ovarian torsion for 2 hours and ovarian detorsion for 2 hours (13, 14), respectively.
4. Group 4: Adding the hydroalcoholic extract of the prepared orange peel (100 mg/kg) to their diet for two weeks (13,14).

### Preparation of the Hydroalcoholic Extract

To prepare the whole plant orange peel extract, half a kilogram of orange peel was dried at 25°C and protected from direct sunlight. For extraction after grinding the dried plants, the peels were dissolved in 2 liters of alcohol 96% and then kept at room temperature for 48 hours. Over this period, the solution was filtered after frequently shaking. Then, the solution was centrifuged at 3000 rpm for 5 minutes. At the end of the process, the resulting solution was poured into an open-top container and the solvent was evaporated, and finally, 90 g of a semisolid extract was obtained from 500 g of chamomile powder.

### Surgical Procedure

In the ovarian torsion method, rats were anesthetized with xylazine ketamine, and a longitudinal incision was made in the abdominal wall. Then, the left ovary was

turned 360 degrees clockwise and fixed to the abdominal wall with a suture of 0.6. Next, the torsion was kept for 2 hours and one hour before opening the ovary, the hydroalcoholic extract of orange peel was injected to the face intraperitoneally. After the end of the ischemic period (2 hours), the ovaries were opened and allowed to reperfuse for 2 hours. Next, xylazine ketamine was used for anesthesia and blood sampling to examine metabolic changes, and eventually, the ovarian tissue was removed for histological changes (1).

### Histological Study

To accomplish histological and histometric studies, the tissue sections of each ovarian sample from the cortex to the medulla were auscultated in a spirally and clockwise direction. The number of preantral, antral, and graafian follicles, as well as atretic and yellow bodies was enumerated in each slide and then compared between study groups (15).

### Hormone Analyses

To separate the sera, blood samples were collected through the penetration of a syringe into the hearts of the rats, and then the sample was centrifuged at 3000 rpm for 10 minutes. The serum concentrations of luteinizing hormone (LH), FSH, testosterone, and estradiol were measured using existing ELISA kits (Demeditec Diagnostics, Germany), LH, and FSH (Cusabio kit China).

### Measurement of Serum Oxidative Stress Markers

The plasma levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured by an ELISA device based on the kit protocol (Randox, Ransod). The serum levels of malondialdehyde (MDA) were measured by the following method. First, 0.20 mL of the serum was added to a micro-tube containing 3.0 mL of "glacial acetic acid", and then, 1% thiobarbituric acid (in 2% NaOH) was added to the micro-tube. The tube was then placed in boiling water for 15 minutes. After cooling, the adsorption of the resulting solution was read as pink at 532 nm. The calibration curve was made by MDA from tetrabutylammonium salt (Sigma, USA).

### Evaluation of PCNA and FSH-R Gene Expression in the Ovarian Tissue With Real-time Polymerase Chain Reaction (RT-PCR) Method

Left ovaries were utilized for the assessment of PCNA and FSH-R, gene expression. In the beginning, ovaries were frozen into the liquid nitrogen at -196 and tissue RNA was extracted according to the kit protocol (Thermo Scientific, Waltham, MA). Then, the RNA concentration was assigned by NanoDrop, and cDNA was synthesized through it. RNA concentration was 500 ng/mL. The primer sequence of genes (Table 1) is described as follows (2,16).

**Table 1.** The Applied Primer Sequence for the Expression Analysis of Genes

Genes	Primer Sequence (5'→3')
FSH-R	Forward: GGAATCTGTGGAAGTTTTCC Reverse: ATGGCCTGCTCTTCAGAAGG
PCNA	Forward: GTGATTCCACCACCATGTTTC Reverse: TGAGACGAGTCCATGCTCTG
GAPDH	Forward: GCAGCTCCTTCGTTGCCGGT Reverse: CCCGCCATGGTGCCGTTTC

Note. FSH-R: Follicle-stimulating hormone receptor; PCNA: Proliferating cell nuclear antigen; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

### Real-time Polymerase Chain Reaction

The RT qPCRs were accomplished in a 48-well plate at the volumes of 20 microliters comprising 1  $\mu$ L cDNA, 2  $\mu$ L of forward and reverse primers mix, 7 microliters deionized water, and 10  $\mu$ L "SYBER GREEN" PCR master mix. The one-step RT-PCR was accomplished in the Applied Biosystems 7500 Fast RT-PCR System (Applied Biosystems Deutschland GmbH). The cycling and melting conditions included one cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and a final extension step (the melt curve step) at 95°C, 60°C, and 95°C for 15, 15, and 60 seconds, respectively. Finally, the quantitative analysis was calculated by the Pfaffl method shown as ratios ( $2^{-\Delta\Delta CT}$  target:2  $-\Delta\Delta CT$  reference) according to (2,17,18).

### Statistical Analysis

All statistical analyzes were performed using SPSS software, version 11.5, and all values were expressed as the mean ( $\pm$  standard error). Finally, the data were analyzed using one-way ANOVA and Tukey's tests, and a  $P \geq 0.05$  was considered statistically significant.

## Results

### MDA Serum Level

The statistical analysis represented that the serum level of MDA in the torsion-detorsion (TD) group was significantly different from that of the sham group ( $P = 0.000$ ). Serum MDA levels in the treatment group receiving the hydroalcoholic extract of orange peel (TDOP) were significantly different in comparison with the TD group ( $P = 0.001$ ). Based on the results (Figure 1), serum MDA levels significantly differed in the healthy group receiving the hydroalcoholic extract of orange peel compared to the TD group ( $P = 0.019$ ).

### Serum SOD Level

According to the statistical analysis, the serum level of SOD in the TD represented a significant difference compared to the sham group ( $P = 0.004$ ). Further, serum SOD levels in the treatment group receiving the hydroalcoholic extract of orange peel TDO significantly differed from those of the TD group ( $P = 0.003$ ). Finally, the serum levels of SOD (Figure 1) demonstrated a significant difference from those of the TD group when receiving the hydroalcoholic

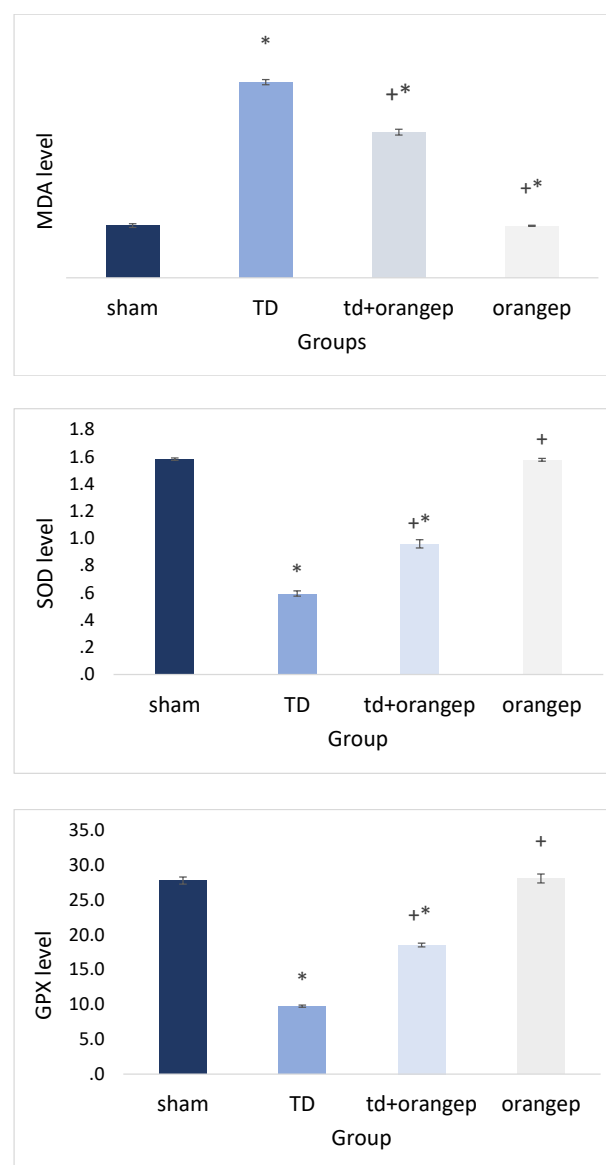
extract of orange peel ( $P = 0.018$ ).

### Serum GPx Level

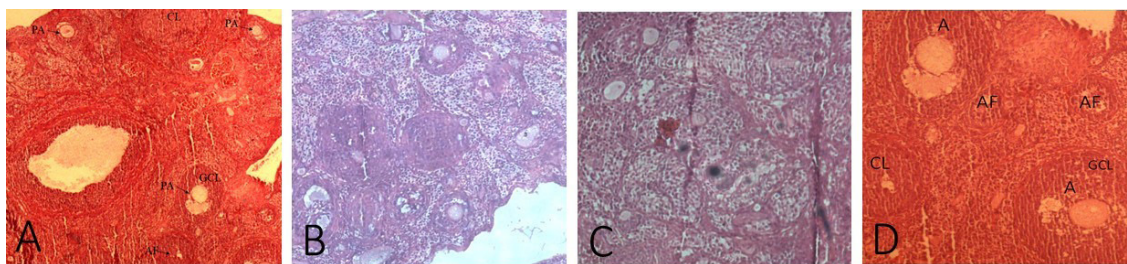
Based on the findings, the serum level of glutathione peroxidase (GPx) was significantly different in the T/D group in comparison with the sham group ( $P = 0.004$ ). Serum GPx levels in the treatment group receiving the hydroalcoholic extract of orange peel T/DO significantly differed from those of the T/D group ( $P = 0.003$ ). Eventually, the serum levels of SOD (Figure 1) indicated a significant difference compared to the T/D group when receiving the hydroalcoholic extract of orange peel ( $P = 0.018$ ).

### Serum Estrogen Levels

Statistical analysis demonstrated that serum estrogen levels



**Figure 1.** Serum Oxidative Stress Markers. Note. TD: Torsion-detorsion. Sign (\*) indicates that groups with \* have a significant difference compared to the sham group and sign (+) represents a significant difference with the TD group.



**Figure 2.** Histopathological Results. Note. TD: Torsion-detorsion. (A) Sham (cutting the abdominal wall without applying torsion); (B) T/D ovarian torsion for 2 hours (T2h) and then ovarian detorsion; (C) TD + orange peel ovarian torsion for 2 hours, receiving orange peel extract; (D) The orange peel healthy group receiving the orange peel hydroalcoholic extract.

were significantly different in the TD group compared to the sham group ( $P=0.041$ ). The results further revealed that serum estrogen levels in the treatment group receiving the TDOP hydroalcoholic extract of orange peel significantly varied from those of the TD group ( $P=0.002$ ). Eventually, the healthy group receiving the hydroalcoholic extract of orange peel demonstrated significantly different serum estrogen levels in comparison to the TD group ( $P=0.004$ ).

#### Serum LH and FSH Levels

Based on the obtained data, the serum levels of LH and FSH in the TD group significantly reduced compared to the sham group ( $P=0.001$ ). The serum levels of LH and FSH in the treatment group receiving the hydroalcoholic extract of orange peel TDOP were significantly different from those of the TD group ( $P=0.002$ ). In addition, the serum levels of LH and FSH in the healthy group receiving the hydroalcoholic extract of orange peel highly differed compared to the TD group ( $P=0.004$ ).

#### Serum Testosterone Levels

The results indicated that serum testosterone levels

in the TD group increased significantly compared to the sham group ( $P=0.041$ ). According to the findings, serum testosterone levels in the treatment group receiving the hydroalcoholic extract of orange peel TDOP were significantly lower in comparison with the TD group ( $P=0.002$ ). Ultimately, serum estrogen levels in the healthy group receiving the hydroalcoholic extract of orange peel significantly differed from those of the TD group ( $P=0.004$ ).

#### Evaluation of Ovarian Histomorphometric Results

Counting of primary follicles, preantral, antral, and cystic follicles, as well as the number of corpus luteum in the ovarian tissue of healthy control groups and TD control and treatment groups (in Table 2-4) showed that the number of primary follicles in the control group with TD significantly reduced compared to the healthy control group. Additionally, their number increased in all treatment groups with the orange peel extract in comparison to the TD control group ( $P<0.05$ ) (Figure 2).

The number of preantral follicles in the control group decreased significantly to TD compared to the healthy

**Table 2.** Comparison of the Serum Levels of Hormonal Profiles

Groups	LH (ng/mL)	FSH (ng/mL)	Testosterone (ng/mL)	Estrogen (pg/mL)
Sham	1.97 ± 0.35	2.57 ± 0.045	0.48 ± 0.050	52.25 ± 1.75
TD	1.09 ± 0.21 <sup>a</sup>	1.28 ± 0.032	2.94 ± 0.078 <sup>a</sup>	33.50 ± 1.19 <sup>a</sup>
TD + Orange peel	1.48 ± 0.014 <sup>b</sup>	1.77 ± 0.022	1.59 ± 0.064 <sup>b</sup>	44.75 ± 1.31 <sup>b</sup>
Orange peel	2.18 ± 0.022 <sup>b</sup>	2.63 ± 0.038	0.49 ± 0.030 <sup>b</sup>	56.25 ± 0.85 <sup>b</sup>

Note. TD: Torsion-detorsion; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone. Sign (a) indicates that TD groups have a significant difference compared to the sham group and sign (b) demonstrates a significant difference with the TD group. Sham (cutting the abdominal wall without applying torsion); T/D ovarian torsion for 2 hours (T2h) and then ovarian detorsion; TD + orange peel ovarian torsion for 2 hours, receiving orange peel extract; The orange peel healthy group receiving the orange peel hydroalcoholic extract.

**Table 2.** Comparison of the Serum Levels of Hormonal Profiles

Groups	Primary Follicles	Pre-antral Follicles	Antral Follicles	Yellow Body
Sham	18.4 ± 1.14	27.8 ± 1.92	16.2 ± 1.30	9.4 ± 1.83
TD	7.4 ± 1.14 <sup>a</sup>	7.2 ± 1.30 <sup>a</sup>	3.8 ± 1.14 <sup>a</sup>	1.8 ± 0.89 <sup>a</sup>
TD + Orange peel	13.8 ± 1.30 <sup>b</sup>	18 ± 1.58 <sup>b</sup>	12 ± 1.58 <sup>b</sup>	6.8 ± 0.83 <sup>b</sup>
Orange peel	18.2 ± 2.38 <sup>b</sup>	25.8 ± 2.48 <sup>b</sup>	14.6 ± 2.07 <sup>b</sup>	7.5 ± 1.89 <sup>b</sup>

Note. TD: Torsion-detorsion. Sign (a) denotes that TD groups have a significant difference compared to the sham group and sign (b) indicates a significant difference with the TD group. Sham (cutting the abdominal wall without applying torsion); T/D ovarian torsion for 2 hours (T2h) and then ovarian detorsion; TD + orange peel ovarian torsion for 2 hours, receiving orange peel extract; The orange peel Healthy group receiving the orange peel hydroalcoholic extract.

Table 4. Comparison of PCNA and FSH-R Gene Expression in Study Groups

Group	FSH-R	PCNA
Sham	1.38 ± 0.034 <sup>b</sup>	1.42 ± 0.022
TD	0.41 ± 0.038 <sup>a</sup>	0.38 ± 0.032 <sup>a</sup>
TD + Orangep	0.64 ± 0.014 <sup>b</sup>	1.01 ± 0.04 <sup>b</sup>
Orangep	1.43 ± 0.028 <sup>b</sup>	1.45 ± 0.014 <sup>b</sup>

Note. Torsion-detorsion. Sign (<sup>a</sup>) indicates that \* groups have a significant difference in comparison to the sham group and sign (<sup>b</sup>) demonstrates a significant difference with the TD group. Sham (cutting the abdominal wall without applying torsion); T/D ovarian torsion for 2 hours (T2h) and then ovarian detorsion; TD + orangep ovarian torsion for 2 hours, receiving orange peel extract; The orangep healthy group receiving the orange peel hydroalcoholic extract.

control group ( $P < 0.05$ ) while it was significantly higher in all treatment groups as compared to the control group to TD ( $P < 0.05$ ).

The number of antral follicles in the control group decreased significantly to TD compared to the healthy control group ( $P < 0.05$ ). However, the count of antral follicles significantly increased in all treatment groups with the orange peel extract compared with the group with TD ( $P < 0.05$ ).

Conversely, the number of corpus luteum in the control group with TD significantly reduced compared to the healthy control group ( $P < 0.05$ ). Finally, the number of corpus luteum increased significantly in all treatment groups with orange peel extracts in comparison with the TD control group ( $P < 0.05$ ).

#### Expression of PCNA and FSH-R Genes in Study Groups

The analysis of FSH-R gene expression in the study groups revealed that the expression of these genes in the TD group significantly reduced compared to the sham group. On the other hand, the expression of these genes in the groups treated with the orange peel extract showed a significant increase compared to the TD group.

Based on the data analysis of PCNA gene expression in the study groups, the expression of this gene significantly reduced in the TD group compared to the sham group. Conversely, PCNA gene expression represented a significant increase in the groups treated with the orange peel extract compared to the TD group.

#### Discussion

Ovarian torsion is one of the rare types of emergency surgery during pregnancy. Its incidence rate is 1 in every 5000 pregnancies and includes 3% of gynecological emergencies. For in vitro fertilization, pregnancy replacement in the fallopian tube and ovarian mass increases by up to 5 times. Ovarian cyst or tumor is associated with it. Ovarian torsion is more prevalent on the right side because the left side of the ovary is less likely to rotate due to the presence of the sigmoid colon (1,19,20). The results of the present study showed that ovarian TD

causes tissue, hormonal, and biochemical damage, and an appropriate dose of the orange peel extract due to its anti-inflammatory and antioxidant properties can improve these effects. Ovarian torsion caused damage to the rat ovarian tissue, reducing the thickness and number of follicles in the transverse section of the ovarian tissue (21). It also increased the corpus luteum. Atresia follicles were also observed in the cross-section of the ovarian tissue (2,22), which is in line with the results of Mostajeran et al (23). However, hormonal and biochemical damage was not investigated in their study (23). In the current study, it was found that ovarian ischemia and reperfusion in rats cause oxidative stress and oxidative damage in the rat ovarian tissue. Considering that oxidative stress has degenerative effects on the ovary, the use of antioxidants such as medical plants can improve these damages (21). Orange peel is found to be a rich source of vitamin C in terms of chemical compositions. In orange peel, the essential oil is a cyclic aldehyde and C10 H16 contains di limonene and other substances such as Aurantin, carotenoids, and coumarins - Vitamin E (Nobiletin) that has antioxidant and terpeneol properties. Ferrari essential oil, which is also pectin, is derived from its flowers. It is known as the Neroli essential oil, which is obtained from the essential oils of the leaves and stachydrine, glycoside, and petitgrain. Its leaves contain the substance L-hesperidin (13, 24). Accordingly, orange peel has many antioxidant properties based on previous evidence. Shokoohi et al also used this plant due to its antioxidant properties, demonstrating that hesperidin in orange peel protected the testicular tissue against damage caused by varicocele by improving its antioxidant mechanism in addition to improved oxidative stress markers and a reduced apoptosis index in the testis (17).

In this regard, Hozayen et al evaluated the effect of hesperidin and rutin on doxorubicin-induced elliptical poisoning and concluded that hesperidin and rutin can protect the testicle against doxorubicin poisoning and reduce oxidative stress (25). In another study, Arafa et al focused on the effects of hesperidin on benzopyrene poisoning, testicular protection and reported that hesperidin can protect the testicular tissue from benzopyrene poisoning and damage to seminiferous tubules and improve epididymal function (26). All these studies showed that orange peel has many antioxidant properties, showing the consistency of our results with those of other studies on the use of orange peel (13, 24-26). Similarly, the hydroalcoholic extract of orange peel increased the level of gonadotropins in rats treated with the extract, which corroborates with the result of Ghosh et al, indicating that the orange peel extract improved serum testosterone levels, sperm, and histological changes in diabetic rats (27). Other results of the study demonstrated that ovarian torsion followed by detoxification reduced the expression of FSH-R and PCNA genes, which could

be due to ovarian tissue damage that destroys hormone receptors and treatment with the orange peel extract. The increased expression of these genes could be due to the antioxidant properties of the compound and reductions in tissue damage (28,29). In this regard, the findings of other studies showed that oxidative stress reduces the expression of FSH-R and PCNA genes, and the use of antioxidants can increase the expression of these genes (29,30).

### Conclusions

Overall, administering an appropriate dose of the hydroalcoholic extract of orange peel as a single dose had a protective effect on histological damage and oxidative stress caused by TD in rat ovaries.

### Study Limitations

This study had some limitations. The researchers were unable to properly evaluate the expression of genes and proteins of the folliculogenesis pathway due to the lack of time and resources.

### Authors' Contribution

RT, Ak, and SA planned and designed the experiments. Furthermore, RT and AK performed the experiments. AK, RT, and SA analyzed the data. Finally, AK, RT, and SA reviewed and discussed the data.

### Conflict of Interests

Authors declare that they have no conflict of interests.

### Ethical Issues

This study was approved by the Ethics Committee of the Research Committee of Tabriz University of Medical Sciences (Ethics No. IR.TBZMED.VCR..REC.1398.277).

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