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EXPERIMENTAL STUDY

# The effect of protocatechuic acid on ovarian histopathology and reserve in rat ovarian torsion model

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**ABSTRACT**

OBJECTIVES: The aim of the study is to investigate the effects of Protocatechuic Acid (PCA), which is an antioxidant, anti-inflammatory and anti-apoptotic agent, on ovarian tissue and ovarian reserve against ischemia-reperfusion (IR) injury in a rat ovarian torsion model.

BACKGROUND: Reactive oxygen radicals cause histopathological changes in the ovarian tissue during the reperfusion phase. PCA may have protective effects on ovarian tissue and reserve due to its antioxidant and antiapoptotic properties.

METHODS: A total of 24 Wistar adult female rats were divided into 3 groups as the control (sham operation, n = 8), IR (Ischemia-Reperfusion, n = 8), and IR+PCA (Ischemia-Reperfusion + 80 mg/kg protocatechuic acid, n = 8). The IR and IR + PCA groups underwent 3 hours of ischemia followed by 3 hours of ovarian reperfusion. Protocatechuic acid (80 mg/kg) was administered to the IR+PCA group 30 minutes before reperfusion. After reperfusion, the ovaries were removed for histopathological and biochemical examination.

RESULTS: Histopathological score and TUNEL+ cell count were significantly lower and AMH expression level was significantly higher in the IR+PCA group when compared to the IR group ($p < 0.05$). However, in the comparison of the follicle counts, there was no statistically significant difference between all groups. Due to the increase in antioxidant activity, the MDA levels were found to be significantly lower in the IR+PCA group compared to the IR group ($p < 0.05$).

CONCLUSION: Protocatechuic acid may be an effective antioxidant in protecting ovarian tissue and follicle reserve against IR injury of the ovary *(Tab. 1, Fig. 4, Ref. 36).* Text in PDF *www.elis.sk*

KEY WORDS: protocatechuic acid, ischemia reperfusion, detorsion, ovarian reserve, AMH.

## Introduction

Ovarian torsion is a gynecological emergency, associated with infertility and is more common in women of reproductive age, with a prevalence of 2.7 % (1). It refers to a complete or partial rotation around the ligament support on the abdominal wall, resulting in reduced or obstructed arterial blood flow within the ligaments (2). Detorsion can be effective in treatment with early diagnosis, but diagnosis and surgical intervention are usually delayed because the clinical picture is nonspecific except for a few distinctive physical findings. Ovarian ischemia and necrosis, tubal obstruction, arterial thrombosis, and infarction can be seen in most patients due to delays in diagnosis and treatment, and as a result, infertility may develop (3–5).

The detorsion treatment and the evaluation of tissue reperfusion is advocated as a preventive approach for the maintenance of fertility (6, 7). During the re-blood supply of the ovaries, reactive oxygen radicals (ROS), which increase ischemic damage, are released into the tissue. The ovarian torsion injury continues during the reperfusion phase, concomitant with a typical oxidative stress condition. Endothelial cell dysfunction, inflammation and increased release of Reactive Oxygen Radicals (ROS) cause cellular damage and apoptosis in the ovarian tissue (8). This shows that the reperfusion phase decreases ovarian reserve in a short period of time (9, 10). In addition, it has been reported that there is a decrease in the number of follicles due to IR damage (11, 12).

Therefore, the effect of detorsion alone is insufficient to protect ovarian tissue and its reserve (13, 14). It has been shown that detorsion is more successful when applied together with supportive treatments. Some antioxidants have been reported to reduce damage in animal studies to investigate ischemia and reperfusion injury of ovarian tissue (15–17). Among the substances showing antioxidant and protective activity in the studies; vitamin C, mannitol, verapamil, lutein and N-acetylcysteine can be counted (17–20).

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Protocatechuic Acid (3,4-dihydroxybenzoic acid, PCA) is a simple phenolic acid with structural similarities to well-known antioxidant compounds such as caffeic acid, gallic acid and vanillic acid (21). PCA, which is found in many fruits such as green tea, grapes, and plums, has strong antioxidant activity as well as various pharmacological effects such as antiapoptotic, antifibrotic and anti-inflammatory (22). It was reported that PCA supplementation has a reducing effect on induced oxidative stress (23). In some studies conducted on the reproductive system, it was observed that PCA treatment has positive effects on histopathological, hormonal and oxidative parameters (24). It was also reported that PCA used in in vitro culture medium has positive effects on the growth of ovarian follicles (25, 26).

However, there are no studies on the effects of PCA on ovarian IR injury. Therefore, we aimed to evaluate the protective effect of PCA on ovarian histology and follicle reserve in female rats with ischemia-reperfusion injury due to ovarian torsion.

## Materials and methods

*Surgical procedure and experimental groups*

24 healthy female Wistar albino rats aged 3–4 months (200–250 g) were obtained from Bolu Abant Izzet Baysal University, Experimental Animals Research Center. The experiment was approved by the Ethics Committee of the Bolu Abant Izzet Baysal University, Bolu, Turkey, and performed according to the National Health and Medical Research Council Guideline (approval number: 2017/54). The rats were placed in polypropylene cages with a 12-hour light at 22 °C ambient temperature given a dark cycle and moistened at 50–60 %. Tap water was used with a standard pellet until the day of the experiment.

The rats were randomly divided into three groups of 8 animals each. The control group (sham operation, n = 8), the IR (ischemia-reperfusion, n = 8), and the IR+PCA (ischemia- reperfusion+80 mg/kg PCA, n = 8). The rats were anesthetized with ketamine hydrochloride (50 mg/k, Ketalar, Eczacibaşi, Istanbul, Turkey) and xylazine hydrochloride (10 mg/kg Rompun, Bayer, Leverkusen, Germany) intraperitoneally. Rats were placed in a dorsal recumbent position, the incision area was disinfected and a 2.5 cm midline subcutaneous incision was performed for the laparotomy. The control group underwent a sham operation and a unilateral ovariectomy was performed at the end of the experiment. Rats, except the control group, had unilateral adnexal torsion, including 36 °C clockwise rotation of the ovarian vessels (3 hours ischemia). After this period, detorsion was performed (3 hours reperfusion) (13). For the I/R+PCA group, PCA 0.5 % isotonic solution (80 mg/kg) was administered intraperitoneally 30 minutes before detorsion. At the end of the detorsion period, re-aparotomy was performed, and the right ovaries were surgically excised. Subsequently, the rats were sacrificed with a high dose of anesthetic.

*Histopathological examination*

The ovarian tissues were fixed in 10 % neutral buffered formalin. A routine histological procedure was performed, and the tissues were embedded in paraffin. The cross-sections (5 μm) were stained with hematoxylin and eosin (H&E). A light microscope (Leica DM 1000, Germany) was used for evaluations and Leica DMC 2900 (CH-9435 Heerbrugg, Germany) was used to photograph. The whole microscopic area was evaluated in three sections for each rat. All ovarian tissue sections were examined in a blinded fashion by a single histologist. The largest diameter of the ovary sections was examined to investigate ovarian cortex characteristics and total tissue damage. The scoring system was used for congestion, hemorrhage, stromal edema, polymorphonuclear leukocyte infiltration, and follicular degeneration. According to their severity, each parameter was scored from 0 to +3 (0 = no pathological change, 1 = <33 %, 2 = 33– 66 %, and 3 = >66 % of the ovary, respectively).

The scores for all parameters were summed to calculate the total histopathological score value of the ovary as described previously (17). Follicles were counted in three sections for each rat to evaluate ovarian reserve. Follicles were defined as primordial follicles (smaller 20 μm diameter), preantral follicles (20-220 μm), the antral follicle (221-370 μm) and large antral follicle (311–μm) (27).

*AMH immunohistochemistry*

Anti-Müllerian hormone is one of the predictive markers for ovarian reserve (28). In the study, immunohistochemical AMH staining was performed to evaluate ovarian reserve. The ovarian tissues were sectioned into 5 μm slices and after routine laboratory methods, blocked in paraffin. The sections were retrieved in 0.001 M citrate buffer for 15 min and immersed in 1 % $H_2O_2$ for 10 min. The sections were pretreated with blocking serum (Histostatin plus kit broad spectrum; Invitrogen, California, USA) to eliminate non-specific binding. The goat polyclonal antibody against anti-Müllerian hormone (AMH) (SC-6886, Santa Cruz Biotechnology, Inc.) was incubated at 40 °C overnight. After washing with PBS, the biotinylated secondary antibodies (Mouse and Rabbit Specific HRP Plus (ABC) Detection IHC kit (ab93697; Abcam) were applied for 30 min at room temperature. 3,3-diaminobenzidine (DAB kit 88-2014, Invitrogen, California, USA) was used for visualization.

Then Mayer's Hematoxylin (Thermo Scientific, USA) was used as a counterstain and Entellan (Merck, Darmstadt, Germany) was used for mounting on glass slides. Follicular AMH expression was evaluated semi-quantitatively. The brown cytoplasmic staining of follicle cells was noted as positive for AMH expression. The expression level was assessed on a scale of 0–2, as; 0 = no staining, 1 = weak staining, and 2 = strong cytoplasmic staining. The mean expression level of the follicles was determined for 3 ovary sections of each animal.

*TUNEL staining*

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining with the In Situ Cell Death Detection Kit (Merck Millipore, Darmstadt, Germany) technique was used to determine apoptotic cells as previously reported (20). To determine the numerical distribution of TUNEL+ cells in the ovaries samples stained with a TUNEL kit, cells were examined under light microscopy (40X). In each section, the number of positive cells was counted to determine follicular apoptosis. Apoptosis is

evaluated semi-quantitatively as TUNEL+ cells on the follicles; 0, <5 % of the cells; 1, 5–25 % of the cells; 2, 26–50 % of the cells; 3, >50 % of the cells.

*Tissue MDA measurement*

Ovarian tissue samples were used for biochemical analysis of malondialdehyde (MDA). Ovarian tissue samples taken from each rat were homogenized, and the MDA measurements were done at 532 nm by using the lipid peroxidation (MDA) colorimetric/fluorometric assay kit (Cat. No: K739-100, Biovision, Milpitas, USA) in line with the manufacturer's protocol. Then, MDA levels are given as nmol/mg.

*Statistical analysis*

The data were examined using the Shapiro-Wilk test to determine whether or not variables were normally distributed. The data determined to be normally distributed were analyzed with one-way ANOVA test. When a significant difference was observed between the means as a result of ANOVA, the group that made the significant difference was determined by using Tukey's-b test in homogeneous subjects and Dunnett's C test in non-homogeneous subjects. The descriptive data were expressed as mean± standard deviation, minimum and maximum values. Statistical analysis was performed with SPSS 22.0 statistical software. A p value < 0.05 was considered to be statistically significant.

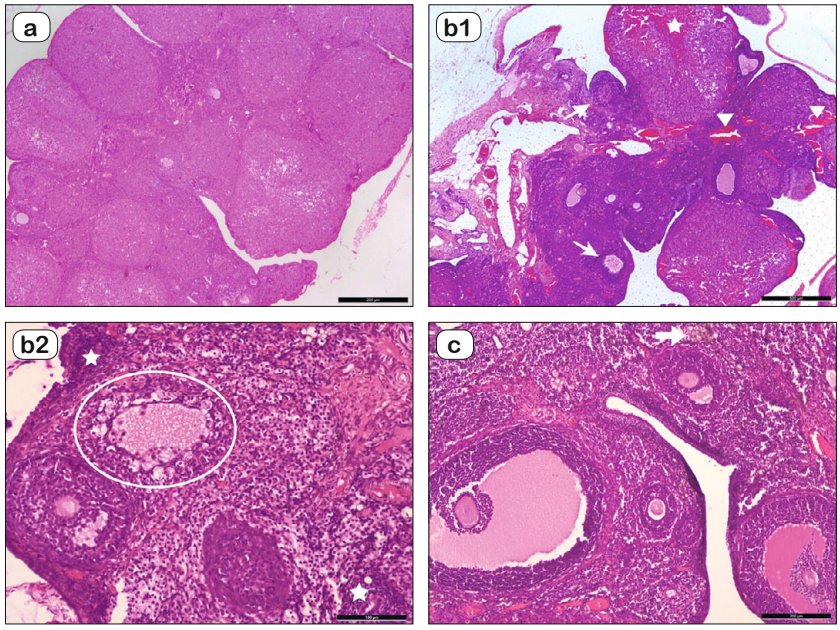


**Fig. 1. The histopathological appearances in rat ovarian tissue, hematoxylin and eosin (H&E). (a) Control group showing normal ovarian cortex characteristics and follicles, X10. (b1) Ischemia and reperfusion (IR) group showing hemorrhage (star), X10, vascular congestion (arrow heads), (b2) polymorphonuclear leukocyte infiltration (star), and follicular degeneration (circular lined), X20. (c) IR+PCA treatment group shows improved histological structure with minimal vascular dilatation, and brown hemosiderin-pigmented area (arrow) X20.**

## Results

*Histopathological evaluation*

The total histopathological scores were significantly different between all groups ($p < 0.05$) (Fig. 3, Tab. 1). The lowest score was observed in the control group with normal histological appearance of follicle and stromal cells (Fig. 1a). Also, the total histopathological score including bleeding, stromal edema, inflammatory cell infiltration and follicle degeneration was higher in the IR and IR+PCA groups compared to the control group (Fig. 1). Moreover, in the PCA treatment group, a statistically significant decreased vascular congestion, bleeding, and follicular degeneration were observed compared to the IR group (Fig. 1 b).

However, when the groups were compared in terms of number of follicles (primordial, preantral, antral, and large antral follicle), no statistically significant difference was detected (Tab. 1).

**Tab. 1. The histopathological score, TUNEL score, AMH expression, MDA levels and follicle counts of the groups.**

| | Control | IR | IR+PCA | p |
|---|---|---|---|---|
| Histopathologic Score | 0.21±0.15 | 2.4±0.20 | 1.25±0.43 | 0.00 |
| TUNEL | 0.47±0.35 | 2.59±0.38 | 1.57±0.31 | 0.00 |
| AMH | 1.52±0.42 | 0.75±0.05 | 1.04±0.1 | 0.00 |
| Primordial | 40.93±8 | 38.61±8.71 | 40.82±8.18 | 0.82 |
| Preantral | 5.7±1.55 | 4.62±1.62 | 4.63±2.05 | 0.06 |
| Antral | 8.13±2.46 | 8.88±1.94 | 8.54±1.56 | 0.76 |
| Large Antral | 7.97±1.89 | 7.7±1.66 | 6.18±1.66 | 0.11 |
| MDA | 84.57±6.24 | 161.52±17.38 | 78.97±6.61 | 0.00 |

Data are shown as mean ± standard deviation, p values in the column for comparison of the three groups. IR – ischaemia-reperfusion, AMH – anti-Müllerian hormone, TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, MDA; malondialdehyde

*Immunohistochemical findings*

In the comparison of the AMH expression levels, there was a significant difference between all groups, and the highest expression level was in the control group ($p<0.05$). Also, it was seen that the AMH expression levels were higher in the IR+PCA group compared to the IR group ($p<0.05$) (Figs 2 and 3, Tab. 1).

*TUNEL results*

According to evaluation of TUNEL assay, a significant decrease in apoptosis score was observed in the IR+PCA group compared to the IR group ($p<0.05$). In addition, the highest TUNEL+ staining was seen in the IR group, and the lowest TUNEL+ staining was seen in the control group ($p<0.05$) (Figs 2 and 3, Tab. 1).

*Biochemical results*

In terms of MDA results, a significant increase was found in the IR and IR+ PCA groups compared to the control group ($p<0.05$)

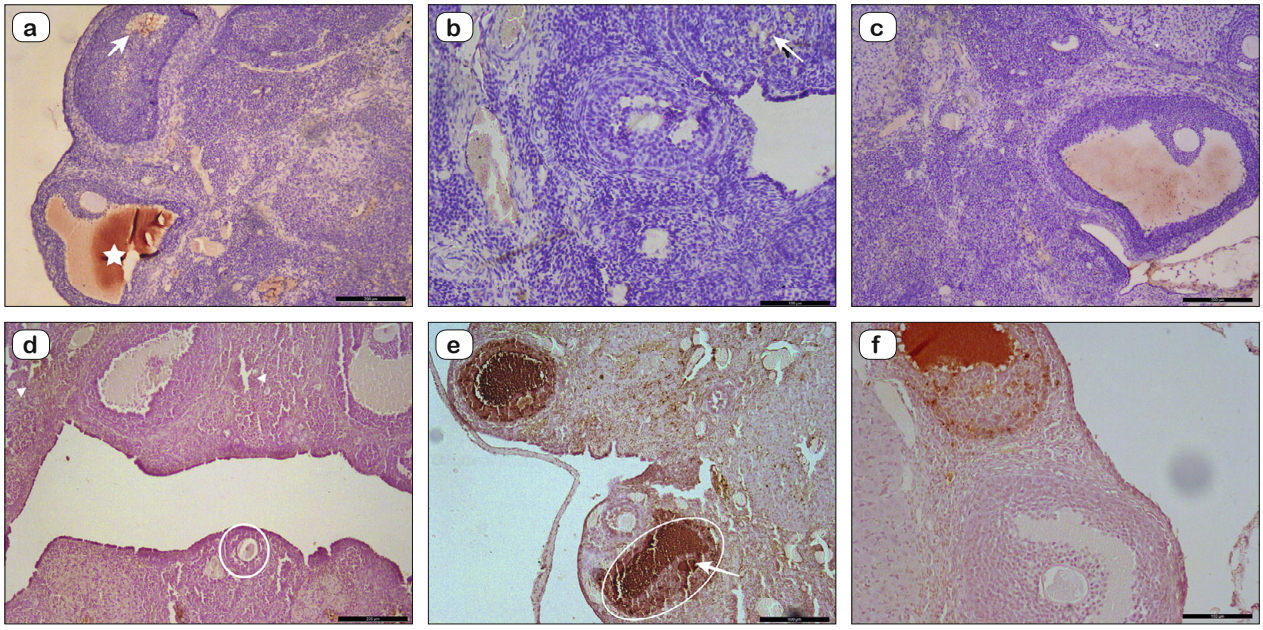


**Fig. 2. The histological appearances of AMH and TUNEL staining in experimental ovarian tissues, X20. (a) Control group shows strong staining in the follicle fluid (star) (arrow) of large antral follicles and primordial follicles. (b) Ischemia and reperfusion (IR) group shows no staining in the stroma and follicles, weak staining in antral follicle (arrow) (c) IR+PCA treatment group demonstrates more AMH staining in follicles than IR only group. (d) Control group shows no apoptotic cells in follicles; large antral, antral, preantral (circular lined) and primordial (arrowhead) follicles, only few apoptotic cells in stroma. (e) IR group shows numerous TUNEL+ cells in large antral follicles (circular lined), primer oocytes (arrow) and stroma. (f) IR+PCA treatment group shows fewer apoptotic cells than IR only group or no apoptotic cells in large antral follicles, X40.**

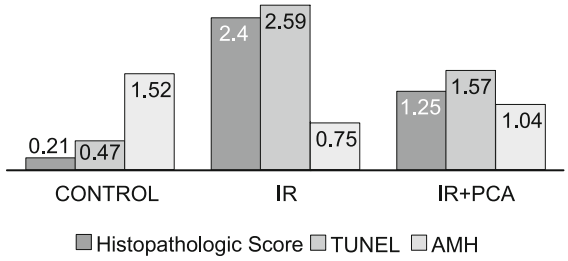


**Fig. 3. Comparison of the mean values of histopathological score, TUNEL score and AMH expression level between the three groups. Data are shown as mean; n = 8 rats/group, a significant difference shown between the means as a result of ANOVA, in all groups, p < 0.05.**

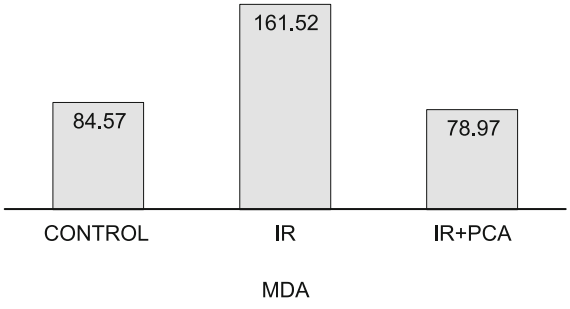


**Fig. 4. Comparison of the mean values of MDA level between the three groups. Data are shown as mean; n = 8 rats/group, a significant difference shown between the means as a result of ANOVA, in all groups, p < 0.05.**

(Tab. 1). In the IR+PCA group, there was a significant decrease in MDA levels due to the increased antioxidant activity compared to the IR group ($p<0.05$) (Fig. 4).

## Discussion

Over torsion is an acute condition associated with infertility and is seen especially in women of reproductive age. The most successful treatment is detorsion surgery as quickly as possible (7, 29, 30). However, detorsion-only is not sufficient to protect the ovarian tissue against adnexal torsion During detorsion of the ovaries, a pathological condition called reperfusion injury occurs. In ttis condition, free radical production increases with the activation of leukocytes in the re-blooded tissue. More ROS production leads to the peroxidation of lipids in cell membranes and also apoptosis (10, 13). Antioxidant additional treatments are required to reduce reperfusion damage with detorsion. In the studies, it has been reported that antioxidant and cytoprotective drug treatments are sufficient to protect ovarian tissue (8, 20, 31). Protocatechuic acid is a phenol compound that has antioxidant, antibacterial, and anti-inflammatory pharmacological activities and is naturally found in green tea, many fruits, nuts, olive oil, and medicinal plants (22). Also, PCA protects against histopathological statuses such as hemorrhage and necrosis (24). However, there are no studies investigating the protective effects of PCA on the female reproductive system against oxidative damage. In our study, we created an experimental ovarian torsion model and investigated PCA

treatment with detorsion. For the additional treatment, 80 mg/kg PCA was used as a treatment dose, similar to the therapeutic dose (50–100 mg/kg) stated in the literature (32).

The histopathological, hormonal, and biochemical changes occurring as a result of oxidative damage in the ovarian tissue and the positive effects of PCA against them were investigated in this study. Firstly, the antioxidant effect of PCA against the ROS products that will accumulate in the tissue after detorsion was evaluated as the total histopathological score value. For evaluation, the presence of congestion, bleeding, stromal edema, infiltration and follicular degeneration was examined in each group separately. Although hemorrhage and follicular degeneration were mild in rats treated with PCA, all histopathologic statuses were observed in both ischemia and reperfusion groups. With the antioxidant effect of PCA, it was found that tissue damage caused by increased blood flow after detorsion decreased in the treatment group, especially vascular congestion and follicular cell degeneration.

As in previous studies, it was seen that only reperfusion therapy is not sufficient to protect the ovarian tissue reserve (10,13). In our study, it was found that the addition of PCA treatment was significant in terms of ovarian tissue histopathology. In some studies, an increase in the number of follicles, which supports histopathological evaluation, was also reported (31, 12). In our study, preantral follicle numbers were higher in the treatment group than in the detorsion group but there was no significant difference between the groups ($p = 0.06$).

AMH is a glycoprotein belonging to the transforming growth factor-β superfamily secreted by granulosa cells of secondary, preantral, and early antral follicles and it acts as a factor in follicular growth and differentiation. AMH secretion occurs independently of the ovarian cycle. For this reason, AMH is a marker that is easily used to determine ovarian reserve and the best predictive hormonal marker of ovarian function (33, 34). AMH expression was observed in many follicles, including primordial follicles, in all immunohistochemical AMH staining applied preparations. Also, the expression was observed in the follicular fluid with granulosa cells in the antral follicles and the connective tissue of the vascular ovarian medulla. In our study, a decrease in AMH expression has been observed in ovaries with ovarian torsion, likely due to the decreased blood flow and oxygen supply to the ovary, which can lead to damage in the growth and differentiation of the follicles. AMH expression was significantly lower in the IR and IR+PCA groups compared to the control group, also it was significantly higher in the treatment group compared to the IR group as a result of the positive effects of PCA. The decreased expression levels of AMH in the IR group indicate that this group may be affected negatively by ovarian reserve. However, in terms of follicle numbers, there was no difference between all groups. This may depend on the short experimental period that was not enough to affect the number of follicles. The study of Ersoy et al. reported that applying antioxidant treatment for 24 hours after detorsion can significantly increase the number of primordial and preantral follicles (20). Similarly, in our study, a notable increase in the number of preantral follicles was observed in the PCA treatment group, but it was not statistically significant. This result can be interpreted as one of the limitations of our study.

Reperfusion damage leads to increased reactive oxygen radicals which react with DNA, resulting in the appearance of 8-hydroxyguanine (8-OHdG). 8-OHdG is a good biomarker of oxidative damage in DNA which is one of the most stable DNA bases (35). In this study, 8-OHdG concentrations were measured by TUNEL assay. TUNEL+ cells were observed in reperfused ovarian tissue due to increased reactive oxygen species, especially in the detorsion-only group. As a result of the measurements, a significant difference was detected between the groups in terms of TUNEL staining scores. Also, similar to the histopathological evaluation scores, TUNEL staining scores were significantly higher in the IR group than in the IR+PCA group. As a result of the antioxidant and antiapoptotic properties of PCA, the ovarian tissues were protected against free radicals in the PCA treatment group, similar to many studies (16, 17, 20). This result shows the decrease of AMH expression and the increase in TUNEL+ cells in ovarian follicles in the IR group due to an insufficient treatment of detorsion.

The tissue MDA level increases as an indicator of the increased lipid peroxidation. MDA level shows the cellular damage caused by Reactive Oxygen Radicals. It was reported in previous studies that PCA application, which has antioxidant and anti-inflammatory properties, significantly reduces ROS and MDA levels (23, 32, 36). According to the results of this study, it was found that MDA levels increased in the detorsion groups due to ischemia and reperfusion injury, and decreased in the detorsion+PCA treatment group. This result also supported the reduction in the number of apoptotic cells in the treatment group of the study. Based on the data obtained from previous studies, the present study was planned with the knowledge that histological changes in the ovaries may occur in a similar 3-hour ischemia and 3-hour reperfusion period. A limitation of this study was the period of the torsion and detorsion process. Immunohistochemical AMH expression levels were also evaluated as indicators of fertility in the study. In further studies, the long-period effect of PCA treatment on IR damaged ovaries can be evaluated, and the ROS parameter can be added to the assessment. Also, the preventive treatment applied 30 minutes before the detorsion of the ovaries can be optimized with different PCA doses.

**Learning point**

PCA has a protective effect on ovarian tissue and reserve due to its antioxidant and antiapoptotic properties in the ischemia and reperfusion phases of ovarian torsion.

**Conclusion**

In conclusion, PCA protects ovarian tissue against oxidative damage after detorsion.

This study shows that PCA treatment may have more protective effects on ovarian reserve than only-detorsion treatment due to its antioxidant and antiapoptotic properties. According to the results obtained from the study, PCA can provide a new approach to clinical preventive treatments against ovarian torsion damage.

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