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The Protective Effect of Letrozole In A Rat Ovarian Ischemia-Reperfusion Injury Model

Rat Overi Iskemi-Reperfüzyon Hasarı Modelinde Letrozolün Koruyucu Etkisi

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ABSTRACT

Objective: Torsion of the ovary is a surgical emergency. Future fertility is an important question for choosing the most appropriate treatment strategy as radical or conservative. Conservative treatment includes detorsion of the twisted ovary and after detorsion, the ischemic injury in ovary increases with reperfusion. During the detorsion process, abundant amounts of reactive oxygen species (ROS) are produced. ROS causes cellular injury by attacking cellular membranes through the peroxidation of polyunsaturated fatty acids and causing cellular death. Letrozole is a nonsteroid aromatase inhibitor that blocks estrogen production in all tissues, increases gonadotropin secretion and induces follicular development.

Materials and Methods: In this study, rats are divided into 5 groups including 8 rats in each group; control group, ischemia group, ischemia and letrozole group, ischemia-reperfusion group, ischemia-reperfusion and letrozole group. For each group Malondialdehyde (MDA) levels were measured, degree of ischemia and number of follicles were recorded by histopathological examination. Endometrial thicknesses were also measured.

Results: In the ischemia and ischemia-reperfusion groups, MDA levels and grade of ischemia were significantly decreased with letrozole. Ovarian follicle numbers were higher and endometrial thickness was lower in the letrozole used groups.

Conclusion: Letrozole can be protective on ovarian ischemia-reperfusion injury and this effect is due to hypoestrogenic environment by inhibition of aromatase activity, antiinflammatory effects and increased blood flow to the ovary by letrozole.

Key Words: Ovary, torsion, ischemia-reperfusion injury, letrozole

ÖZET

Amaç: Over torsiyonu cerrahi acil bir durumdur. Fertilite kaygısı tedavinin konservatif mi yoksa radikal mi olacağını belirleyen çok önemli bir sorudur. Konservatif tedavi torsiyone overin detorsiyonunu içerir ve overde oluşan iskemik hasar detorsiyon sonrası reperfüzyon ile artar. Detorsiyon sırasında büyük miktarda reaktif oksijen radikalleri-substratları (ROS, Reactive Oxygen Species) oluşur. ROS ise hücre membranında bulunan poliansature yağ asitlerinin peroksidasyonuna yol açarak hücre hasarına ve hücre ölümüne neden olur. Nonsteroid tipte bir aromataz inhibitörü olan letrozol tüm dokulardaki östrojen üretimini bloke eder, gonadotropin sekresyonunda artışa sebep olur ve foliküler gelişimi uyarır.

Gereç ve Yöntem: Bu çalışmada dişi ratlar her gurupta 8 rat olacak şekilde 5 gruba ayrıldı; kontrol grubu, iskemi gurubu, iskemi ve letrozol gurubu, iskemi-reperfüzyon gurubu, iskemi-reperfüzyon ve letrozol gurubu. Overlerdeki Malondialdehit (MDA) seviyeleri tespit edildi. İskemiden etkilenme dereceleri ve folikül sayıları histopatolojik olarak değerlendirilip not edildi. Endometrial kalınlık ölçümü yapıldı.

Bulgular: Letrozol hem iskemi grubunda hem de iskemi-reperfüzyon grubunda MDA seviyelerini ve histolojik grade'i anlamlı olarak azalttı. Letrozol verilen guruplarda ovaryan folikül sayıları daha fazla, endometrium kalınlıkları ise daha az bulundu.

Sonuç: Letrozolun over iskemi-reperfuzyon hasarında koruyucu olabilir ve bu etkisi aromataz inhibisyonu sonucu gelişen hipoöstrojenik ortama, antiinflamatuar etkilerine ve artmış ovaryan kan akımına bağlıdır.

Anahtar Kelimeler: Over, torsiyon, iskemi-reperfüzyon hasarı, letrozol

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Introduction

Ovarian torsion is a surgical emergency condition observed in approximately 3% of gynecologic emergency practice (1). Normal ovaries may be torsioned; however, a mass in the ovary frequently accompanies the condition. Benign cysts, abscess or malignant neoplasm may accompany torsion. An ovary may twist around its vascular pedicle, and the tube may also be torsioned with the ovary.

Torsion of normal ovaries is more frequently detected in children and adolescents, the preservation of fertility in the future life in this patient group causes an important question in treatment, whether to choose conservative or radical treatment methods. Conservative treatment involves detorsion of the ovary, but ischemic damage in the ovary increases with reperfusion after the detorsion procedure.

A significant amount of oxygen is delivered to tissues during detorsion procedures, and thus reactive oxygen species (ROS) are formed. ROS cause cellular damage and cell death by causing the peroxidation of polyunsaturated fatty acids in the cell membrane (2). Reperfusion and neutrophil infiltration, and the condition that emerges with the formation of ROS is described as ischemia/reperfusion injury.

Various agents have been studied for prevention of ischemia/reperfusion damage in the ovary in the literature, some of which were sildenafil (3), colchicine (4), metformin (5), and human interleukin-1 receptor antagonist (6). Letrozole is a non-steroidal aromatase inhibitor, which has been used in the treatment of postmenopausal breast cancer for years. In addition, letrozole has been used for ovulation induction in infertility treatment and in the treatment of endometriosis (7). Letrozole decreases the production of Estradiol (E2) and decreases the production of prostaglandins and inflammatory cytokines by preserving physiologic hormonal pathways (8).

Letrozole can increase blood flow to genital organs while stimulating follicle development because it reduces estrogen production without disrupting the central-pathways. Ibrahim M. examined doppler flow indices in the uterine and sub-endometrial artery on two hundred and seventy women using letrozole for infertility (9). Blood flow indices were decreased significantly in letrozole-treated patients and that denotes an increase in blood flow to genital organs. Estrogen application increases vascular density and increases Vascular Endothelial Growth Factor (VEGF) expression in rats (10). VEGF and vascular permeability were decreased by letrozole use in a rat ovarian hyperstimulation study performed by Şahin et al. (11).

In the present study, based on the hypothesis that letrozole decreases E2 and increases gonadotropin levels, causes stimulation in the ovary and increases blood flow into the ovary, causing a decrease in prostaglandins and inflammatory cytokines with the decrease of E2, we investigated whether letrozole had a cytoprotective effect in the ovary in the event of ischemia and reperfusion by evaluating the histopathologic results of rat ovaries, ovarian follicle numbers, tissue Malondialdehyde (MDA) levels, and endometrial thickness.

Materials and methods

Animals: The study was performed in accordance with international guidelines (ARRIVE guidelines, U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, European Union Directive 2010/63/EU for animal experiments) with a total of 40 adult female Wistar Albino rats weighing 250-300 grams aged 12 weeks. The rats were kept in cages, which were cleaned daily, maintained at a temperature of 22 ± 2°C, and photoperiods organized as 12 hours' light, and 12 hours' dark during the study. The rats were given standard pellet feed and tap water ad libitum. The study was approved by the ethics board council of Experimental Animals Research Unit, (local ethics committee no: 2008-02-5).

Groups: Animals were randomly divided into five groups as follows:

Group 1 (sham group, n=8): Only laparotomy and bilateral oophorectomy was performed, and no ovarian torsion was performed.

Group 2 (ischemia group, n=8): Bilateral adnexal torsion was performed, and 3 h after torsion, bilateral oophorectomy was performed.

Group 3 (ischemia + letrozole group, n=8): Daily 1 mg/kg letrozole was administered to rats using the gavage technique for 7 days by diluting with 0.9% saline. On day 8, bilateral adnexal torsion was performed, and 3 h after torsion, bilateral oophorectomy was performed.

Group 4 (ischemia-reperfusion group, n=8): Bilateral adnexal torsion was developed, and 3 h after torsion, detorsion was performed. Bilateral oophorectomy was performed 3 h after reperfusion.

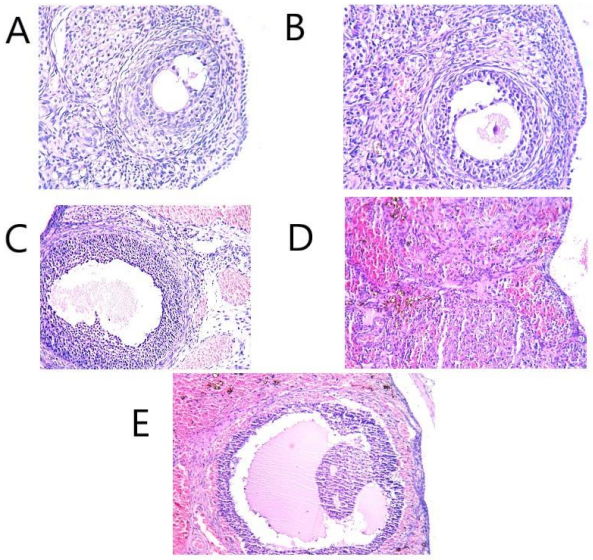

Fig. 1. Histological changes in ovaries: grade 0 (A); Normal , grade 1 (B);
Edema, mild congestion, no haemorrhage, no leukocyte, grade 2 (C);
Moderate edema, moderate congestion, no haemorrhage, no leukocyte, grade 3 (D);
Severe edema, severe congestion, mild hemorrhage, minimal leukocyte , grade 4 (E); Severe edema, severe congestion, severe hemorrhage, modarate leukocyte

Group 5 (ischemia-reperfusion + letrozole group n=8): Daily 1 mg/kg letrozole was administered using the gavage technique to rats for 7 days by diluting with 0.9% saline. On day 8, bilateral adnexal torsion was developed, and 3 h after torsion, detorsion was performed. Bilateral oophorectomy was performed 3 h after reperfusion.

Anesthesia and Surgical Methods: All surgical procedures were performed under sterile conditions under appropriate laboratory settings. Animals were anesthetized using 50 mg/kg of intraperitoneal ketamine (Ketasol; Richter Pharma AG), then a 2-cm midline vertical incision was made. The uterine horns and adnexa were located. Rats in group 1 were excised before the ovaries were torsioned. The ovaries of rats in groups 2 and 3 were torsioned clockwise 360 degrees involving the tube and ovarian vessels, and fixated to the abdominal wall using a 5/0 silk; the ovaries were excised 3 hours later. The ovaries of rats in groups 4 and 5 were torsioned clockwise 360 degrees involving the tube and ovarian vessels, and fixated to the abdominal wall using a 5/0 silk; the torsion was opened 3 hours later, and the ovaries were excised after allowing 3 hours more reperfusion. After these processes, all rats were euthanized and their ovaries and uteri were removed for biochemical and histopathologic examination. The results were compared between the groups.

Histopathologic Evaluation: The ovary and uterus samples excised from the 40 rats were embedded in paraffin wax after 48 hours fixation in 10% neutral formalin solution. Four-micrometer-thick uterus sections involving ovary and endometrial surfaces were taken using a Leica RM 2155 microtome and stained with hematoxylin and eosin (H&E). The sections obtained from the ovaries were histologically evaluated for interstitial edema, vascular dilatation, hemorrhage, and polymorphonuclear leukocyte infiltration using a Leica DMSL microscope, and the observed changes were divided into grades as demonstrated in Figure1.

Mature follicles were counted with microscopic investigation on the widest diameter tissue section of each ovary stained using H&E. The microscopy images of H&E-stained uterine tissue sections involving endometrial surfaces of each rat were transferred to a computer using a video camera (Leica DFC 280) from the microscope (Leica DMLB 100S); 3 areas where the thickest endometrium was observed in these sections were manually measured using an image analysis program (Leica QWIN Plus v. 3.1.0).

Laboratory Evaluation: Some of the ovary sections of rats in all groups were stored at -80°C for biochemical research. The evaluation of lipid peroxidation was performed based on the reaction of MDA with thiobarbituric acid (TBA) (12). The ovary tissues were weighed, and 10% tissue homogenate was obtained after they were homogenized using a 1.5% potassium chloride (KCI) solution. The volume was completed to 4 mL using distilled water with the inclusion of 0.2 mL 8.1% sodium dodecyl sulfate (SDS), 1.5 mL sodium hydroxide (NaOH), and 20% acetic acid with the pH adjusted to 3.5, and 1.5mL 0.8% TBA on 0.2 mL homogenate. The volume was cooled with water, and 1 mL distilled water was added after incubation for 60 minutes at 95°C in a water bath. Five milliliters of n-Butanol pyridine (15/1, V/V) mixture were included and mixed. After 10 minutes of centrifugation at 4000 rpm, the absorbance of the organic layer was measured at 532 nm using a Shimadzu Ultraviolet 1600 spectrophotometer. The standard 1, 1, 3, 3 tetra ethoxy propane was used. The tissue MDA concentrations were evaluated as nmoL/g tissue weight.

Statistical Analysis: The Statistical Package for the Social Sciences (SPSS) 16.0 for Windows

(SPSS Inc., IL, USA) was used to record the data and for the statistical evaluations. Kruskal-Wallis variance analysis was used in the investigation of the difference between the groups. The Mann-Whitney U test was used with Bonferroni correction for post hoc paired comparisons after results were found significant. In addition, Chi-square analysis was performed, and the likelihood ratio value was used in the analysis. Minimum, maximum, and median values were used in the descriptive statistics of the variables.

The results were evaluated at 95% confidence intervals. Statistical significance was accepted as $p<0.05$ in all analyses.

Results

A significant difference was found according to MDA levels in ovarion tissues between the five groups ($p<0.001$). Paired comparisons were performed for MDA, histological grades, follicle numbers and endometrial thicknesses between the groups (Table 1). The MDA level was found significantly higher in the comparison of the ischemia group and the control group ($p=0.01$). The MDA level was found significantly higher in the ischemia-reperfusion group compared with the control group ($p=0.01$); however, no significant difference was detected in the comparison of the ischemia-reperfusion group and ischemia only group ($p>0.05$). The MDA levels in the ischemia and letrozole groups were found higher than the control group; however, the result was not statistically significant ($p>0.05$). No significant increase was observed in the ischemia-reperfusion and letrozole group compared with the control group ($p>0.05$). The MDA levels in the ischemia and letrozole group were significantly lower than the ischemia only group ($p=0.05$). However, the MDA levels in ischemia-reperfusion + letrozole group were found significantly lower than the ischemia-reperfusion group ($p=0.01$).

All ovaries ($n=8$) in the control group were found at grade 0 in the investigation of histologic changes after ischemia and reperfusion. The difference was found significant in the comparison of the groups ($p=0.003$). The grades of ovaries in the ischemia + letrozole group were found significantly lower than in the ischemia only group in the paired comparison of the groups ($p=0.02$). The grades of ovaries in ischemia-reperfusion + letrozole group were found significantly lower than the ischemia-reperfusion group ($p=0.03$).

A significant difference was detected regarding the number of follicles between the groups ($p<0.001$). The paired comparisons of follicles showed significantly increased follicle numbers in the ischemia + letrozole group compared with the control group, ischemia group, and ischemia-reperfusion group ($p=0.01$). Similarly, the follicle numbers were significantly higher in the ischemia-reperfusion and letrozole group than the control group, ischemia group, and ischemia-reperfusion group ($p=0.01$).

Endometrial thickness measurements were found significantly different between the groups ($p<0.001$). Paired intergroup comparisons demonstrated that the endometrial thickness was significantly decreased in the ischemia + letrozole group compared with the control group, ischemia group, and ischemia-reperfusion group ($p=0.01$). Similarly, the endometrial thickness in the ischemia-reperfusion and letrozole groups was found significantly lower than the control group, ischemia group, and ischemia-reperfusion group ($p=0.01$).

Discussion

Ovarian torsion is an emergency surgical condition. The most common pathologies causing adnexial torsion are benign cystic teratomas, tubal and para tubal cysts, follicular cysts, and serous and mucinous cystadenomas (13). The diagnosis of ovarian torsion is often delayed, and sometimes the diagnosis is made after adnexial structures are necrotized because the clinical presentation is atypical and differential diagnosis is hard.

Color Doppler sonography is useful in the diagnosis; however, there may be limitations in its clinical use because blood is provided from two different sources to the ovary, venous thrombosis develops before the arterial thrombosis, and torsion may develop intermittently. Decisions taken based only on Doppler sonography may cause misdiagnosis and organ loss (14).

The most important question in the treatment of ovarian torsion is whether to preserve future fertility. Conservative treatments are particularly preferred when the fertility concern is the priority. The diagnosis must be made before necrosis develops in the ovary to enable conservative treatment. Data obtained from animal experiments showed that histologic recovery could not be accomplished when the ischemic period exceeded 36 hours and the ovary was necrotized (15). Although the data of animal models can not be

Table 1. Comparision of Ovarian Tissue MDA, Histological Grades, Ovarian Follicles and Endometrial Thicknesses (Mean±SD)

	Group 1 (sham)	Group 2 (ischemia)	Group 3 (ischemia & letrozole)	Group 4 (ischemia-reperfusion)	Group 5 (ischemia-reperfusion & letrozole)	P value
MDA (nmol/μg protein)	11,77 ± 5,50	85,38 ± 7,15	20,88 ± 1,38	129,43 ± 4,36	21,24± 9,95	<0.001 †
Grade	0 ± 0	3,5 ± 0,5	2,3 ± 0,5	3,2 ± 0,7	2,3± 0,7	0,003 ‡
Follicles	2,1 ± 0,8	1,8 ± 0,8	5,2 ± 0,7	2,0 ± 0,7	5,6 ± 0,5	<0,001 §
Endometrial thickness (nm)	710,8 ± 156,5	603,3 ± 119,3	415,5 ± 41,1	715,8 ± 98,1	294,7 ± 51,5	<0,001 ¶

†: Comparison of Group 1/Group 2, Group 1/Group 4, Group 2/Group 3, Group 2/Group 5, Group 3/Group 4, and Group 4/Group 5 (P = 0.01, P = 0.01, P = 0.05, P = 0.05, P = 0.01 and P = 0.01, respectively)
‡: Comparison of Group 2/Group 3, and Group 4/Group 5, (P = 0.02, P = 0.03, respectively)
§: Comparison of Group 1/Group 3, Group 1/Group 5, Group 2/Group 3, Group 2/Group 5, Group 3/Group 4, and Group 4/Group 5 (P = 0.01, P = 0.01, P = 0.01, P = 0.01, P = 0.01 and P = 0.01, respectively)
¶: Comparison of Group 1/Group 3, Group 1/Group 5, Group 2/Group 3, Group 2/Group 5, Group 3/Group 4, Group 3/Group 5 and Group 4/Group 5 (P = 0.01, P = 0.01, P = 0.01, P = 0.01, P = 0.01, P = 0.01 and P = 0.01, respectively)
MDA: malondialdehyde, SD: standart deviation

completely used in humans, they emphasize the necessity of diagnosis and intervention before irreversible histologic changes develop in the ovary. In addition, the macroscopic appearance of the ovary mostly does not correspond with microscopic observations; morphologic damage may not be obvious in ovaries with a black and edematous appearance. This appearance is due to the maintenance of arterial blood flow even though the venous blood flow is interrupted.

The time from pain onset until the operation must be instructive in the decision of the physician. Any medical therapy could not replace the surgery in ovarian torsion but can help us to prevent ischemia and reperfusion injury. The loss of blood flow in the torsion may not disappear completely and sometimes can develop intermittently. The use of letrozole from the onset of pain to the surgery may prevent complete necrosis of the ovary and protect against reperfusion damage after detorsion.

Tissues that are exposed to ischemia meet with high amounts of oxygen with the return of blood flow with the detorsion of the torsioned ovary, and an excessive amount of ROS is formed. The immigration of the activated neutrophils and emergence of ROS have a role in the pathogenesis of the tissue damage (16). Activated neutrophils release mediators such as tumor necrosis factor and interleukin-1 (6).

Lipid peroxidation is a chemical process initiated by free radicals and results with the oxidation of unsaturated fatty acids in the membrane structure. Free oxygen radicals such as superoxide anion, hydrogen peroxide, and hydroxyl damage the cell membrane structure. Lipid in the cell membrane is mostly affected by free radicals, and the damage in the cell membrane causes cell death (17). The generated ROS is cleared by various enzymatic and nonenzymatic antioxidant systems such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). The excessive increase of ROS in the hypoxic environment causes cell death (17).

Letrozole, which is a nonsteroidal aromatase inhibitor, blocks estrogen production in all tissues, causes the increase of gonadotropin secretion, and stimulates follicular development in the ovaries.

Although MDA is not a specific or quantitative indicator of fatty acid oxidation, it has a good correlation with the degree of lipid peroxidation. MDA is a reliable indicator due to being the end product of lipid peroxidation (18). In our study, we found significantly higher MDA levels in the ischemia group and ischemia-reperfusion groups compared with the control group. These results were similar to studies that were conducted using agents with antioxidant characteristics (3,5). The MDA levels in the ischemia + letrozole group were significantly lower compared with the

ischemia only group. The MDA level in the ischemia-reperfusion + letrozole group was found significantly lower compared with the ischemia-reperfusion group.

According to histopathologic investigation; the grades of the ovaries in the ischemia letrozole group were significantly lower compared with the grades of the only ischemia group. The grades of the ovaries in the ischemia-reperfusion letrozole group were found significantly lower than the only ischemia-reperfusion group. Yapca et al. reported severe hemorrhage, severe edema, numerous congested vascular vessels and a number of degenerative cells formed in ovarian tissue subjected to the ischemia-reperfusion process (19). Authors noted that histopathological assessments were consistent with the molecular and biochemical findings and cyclooxygenase-2 (COX-2) activity plays a role in ischemia-reperfusion injury. Also, controlled reperfusion may attenuate the effects of ischemia-reperfusion injury. Sayan et al reported beneficial effects of high dose metformin in terms of ovarian tissue damage scores (5).

Letrozole was studied on ischemia and reperfusion models of few brain studies. The experimental cerebral ischemia model of letrozole showed that striatal infarction and increased seizure volume. The combination of flutamide with letrozole reversed the beneficial effects of flutamide (20). Zhang et al. found decreased neurological scores and the number of intact neurons detected via Nissl staining and increased infarct volume with letrozole after ischemia and reperfusion of middle cerebral artery (21).

The decrease in the MDA levels and the difference in the histopathologic evaluations were compatible with the suggestion that letrozole has a protective effect against ischemia-reperfusion damage. The source of this cytoprotective effect is associated with the inhibition of aromatase. Prostaglandin E2 (PGE2) was demonstrated to be the strongest stimulator of aromatase in endometriosis studies. COX-2, which has a role in the synthesis of PGE2, is stimulated by E2, VGEF, and PGE2 itself. The hypoestrogenic environment that emerges with aromatase inhibition brakes this vicious circle and suppresses inflammation (22). In addition, researchers reported that one other COX-2 activator, interleukin (IL)-1β increased in ischemia-reperfusion damage (6). The cytoprotective effect in ischemia-reperfusion damage is associated with the decrease in inflammatory cytokines. In addition, increased follicle-stimulating hormone (FSH) levels might have a role in the protective mechanism by increasing the blood flow into the ovary.

The inhibition of aromatization blocks the estrogen production in all tissues and saves the hypothalamic-pituitary axis from the negative feedback effect of estrogen, and the increase in the gonadotropin secretion stimulates development in ovarian follicles. The decrease of estrogen in the brain causes an increase of FSH production by increasing activins in many tissues such as the pituitary gland (23). Different than clomiphene citrate, aromatase inhibitors do not block estrogen receptors, thus the normal central feedback mechanisms are not affected.

In the induction of ovulation using letrozole, ovarian E2 production is suppressed, which increases FSH and results with the emergence of multiple follicles in the ovaries (8). It has been reported that E2 levels were suppressed in 80% of rats, and the weights of ovaries increased approximately 35% after treatment with the administration of letrozole to female rats with the aim of ovulation induction (24). Therefore, the ovarian follicle numbers were expected to increase in the letrozole administered groups. We found the follicle numbers higher in both the ischemia and letrozole groups, and the ischemia-reperfusion + letrozole group compared with the other groups. In addition, in our study, the thickness of the endometrium was found thinner in the ischemia + letrozole group, and the ischemia-reperfusion and letrozole groups compared with the other groups. The presence of most thin endometrium in ischemia-reperfusion and letrozole group may be due to the toxic effect of ROS released after reperfusion on the endometrium. Similarly Oner G. found increased follicle numbers and decreased endometrial thickness with letrozole compared to metformin and control groups in rats (25).

Various antioxidant and anti-inflammatory agents were studied in ovarian torsion-detorsion, and the results were found protective in ischemia-reperfusion damage; letrozole is a new drug being studied on this issue.

Taken together, it may be suggested that letrozole has a protective effect against ischemia-reperfusion damage in the ovary. This effect may be associated with the deficiency of estrogen, and due to developed anti-inflammatory and cytoprotective mechanisms. The limitations of the present study are that we only used MDA levels, which is the endproduct of lipid peroxidation in

the biochemical evaluation of the damage in tissues, and confirmed findings with histopathologic results. The joint evaluation of markers such as serum catalase activity, SOD, and GPx may be useful for future studies. There is a need for clinical studies that evaluate the effects of letrozole in ischemia-reperfusion damage in tissues within and beyond the ovary because animal model studies cannot be generalized for humans.

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