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PREVENTION OF ISCHEMIA-REPERFUSION INJURY IN RAT OVARIAN TISSUE WITH THE ON-OFF METHOD

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Ischemia is defined as cell death caused by insufficient perfusion of the tissue due to reduction in arterial or venous blood flow, depletion of cellular energy storages, and accumulation of toxic metabolites. The positive effects of controlled reperfusion are known and are used clinically. But the positive effects of controlled reperfusion on ovarian tissue have not been seen in the literature yet. The biochemical and histopathological comparative investigation of rat ovaries that were experimentally exposed to ischemia (IG), ischemia-reperfusion (I/R), and ischemia-controlled reperfusion (ICR) was aimed. Forthy rats were divided into four groups (10 rats per group). First group: 3 h ischemia by vascular clips on ovarian tissue. Second group: 3 h ischemia + 1 h reperfusion. Third group: 3 h ischemia + 1 h controlled reperfusion (on-off method: controlled reperfusion by opening and closing the clips (on/off) in 10-second intervals, for 5 times for a total of 100 seconds). Fourth group: healthy rats. Biochemical (tGSH, MDA, and DNA damage level and SOD activity) and histopathological analysis were performed. The highest glutathione and superoxide dismutase measurements were found in ischemia/controlled reperfusion group among the ischemia or ischemia/reperfusion groups. Similarly the damage indicators (malondialdehyde, DNA damage level and histopathological damage grade) were the lowest in ischemia/controlled reperfusion group. These results indicate that controlled reperfusion can be helpful in minimizing ischemia-reperfusion injury in ovarian tissue exposed to ischemia for various reasons (ovarian torsion, tumor, *etc.*).

Key words: *ischemia/reperfusion, on/off method, ovarian tissue, oxidant/antioxidant parameters, rat*

INTRODUCTION

Ischemia/reperfusion damage is accepted as one of the important problem clinically. Ischemia/reperfusion damage composes the basic of some clinical pictures such as liver resection, serious abdominal bleeding, various shock types, liver transplantation, and ovarian torsion/ detorsion. It was shown that the source of these clinical pictures was originated from free oxygen radicals (1-3). Ischemia is defined as cell death caused by insufficient perfusion of the tissue due to reduction in arterial or venous blood flow, depletion of cellular energy storages, and accumulation of toxic metabolites. Ischemic tissues need to recover blood supply for regeneration of cells and disposal of toxic metabolites. However, reperfusion of the ischemic tissue paradoxically leads to much more serious damage to the tissue than the damage caused by ischemia (4). Reperfusion-related damage in the cell is created by many factors, mostly including oxygen-derived free radicals, which are rapidly generated in the tissue as a result of reperfusion (5). Membrane lipids, proteins, nucleic acids, and deoxyribonucleic acid molecules are the cell structures most sensitive to reperfusion-related damage (6). Although intracellular mechanisms that produce oxygen radicals are fully active in ischemic tissues, they are unable to function due to lack of oxygen in the environment. Following restoration of blood flow and oxygen supply, large amounts of free-oxygen radicals are generated, leading to reperfusion damage (7). The most important and harmful effects of free radicals is lipid peroxidation in the cell, which causes reduction in the membrane potential and thus, cell injury. In addition to these, malondialdehyde (MDA), one of the end products of lipid peroxidation, leads to serious cell damage by causing polymerization and cross-linking of membrane components (8). Free oxygen radicals react with DNA and cause formation of 8-hydroxyguanine (8-OHGua), one of the damage products of DNA (9, 10). Despite the fact that generation of free oxygen radicals takes place in cells continuously, the presence of endogenous antioxidant defense systems preserves tissues from the harmful effects of free oxygen radicals (11). As a result of physiological or pathological events, oxidative damage may occur with changes in this balance in favor of the oxidation process (12). Although early diagnosis is thought to reduce ischemic injury, reperfusion injury and its results are still inevitable with this approach. As such, studies on preventing reperfusion injury have become more important.

Various controlled reperfusion methods with different time tables were tried on heart, liver, kidney, lung and striated muscle tissues to prevent ischemic damage and these methods had positive effects (13-17). In another words, different controlled reperfusion techniques with different time tables were much more useful than normal reperfusion techniques after ischemic conditions. In addition, not to prolong the time of the operation, it might be useful to follow reperfusion process with short intervals. The positive effects of controlled reperfusion are known in heart and other organs and are used clinically (13, 14). But the positive effects of controlled reperfusion on ovarian tissue have not been seen in the literature yet. Ovarian torsion is a serious reason of gynecological emergency surgical with 2.7% prevalence clinically (18).

Therefore, the aim of our study is to investigate the advantage of controlled reperfusion in the prevention of damage in ovarian tissue ischemia and to examine biochemically and histo-pathologically.

MATERIAL AND METHOD

Animals

Fourty albino Wistar female rats weighing between 200 and 210 grams, 8 month-old and provided by Ataturk University Medical Experimental Application and Research Center, were used in this study. These rats were born at the same generation. Rats were divided into four groups as (10 rats per group):

Group 1: ischemia group (IG)

Group 2: ischemia/reperfusion group (I/R)

Group 3: ischemia/controlled reperfusion group (ICR)

Group 4: healthy group (HG)

These animals were kept and fed at room temperature (22°C) in groups. Animal experiments were performed in accordance with the national guidelines for the use and care of laboratory animals and were approved by the Local Ethical Committee of Experimental Animals of Ataturk University.

Surgical procedures

The surgical procedures on rats were performed under anesthesia with 25 mg/kg thiopental sodium and sterile, appropriate laboratory conditions. Thiopental sodium was injected intraperitoneally (i.p.), and we waited for the appropriate time to perform the surgical intervention, which was considered to be when the animals remained motionless in a supine position. At that point, the ovaries of 30 rats were obtained by performing a 2-2.5 cm lower abdominal vertical incision. Ischemia was then created by applying vascular clips to the lower part of the right ovary (the region where the ovary is connected to the uterus). The first group of animals (IG: ovarian ischemia group) was killed with an overdose of anesthesia three hours after the application of the clips. Just after the euthanasia, first clips and then ovarian tissue were removed and were sent to the Pathology and Biochemistry departments for histopathological and biochemical analysis. The second group of rats received reperfusion (I/R: ischemia-reperfusion group), while controlled reperfusion was conducted on the third group of rats (ICR: ischemia-controlled reperfusion) by opening and closing the clips (on/off) in 10-second intervals five times for a total of 100 seconds. At the end of this time (100 seconds), the vascular clips were completely removed and regular reperfusion was achieved. Both of these groups underwent reperfusion for one hour. At the end of this period, the animals were killed with an overdose of anesthesia and their ovaries were removed and sent for histopathological and biochemical analysis. Pathological and biochemical results were compared among the IG, I/R, and ICR groups, as well as with the intact healthy group (HG).

Biochemical analysis of ovarian tissue

In this part, 0.2 g of whole ovarian tissue was weighed for each ovary. The samples were homogenized in ice with 2 ml buffers consisting of 1.15% potassium chloride solution for malondialdehyde analysis and pH=7.5 phosphate buffer for the other analyses. Then, they were centrifuged at 4°C, 10,000 rpm for 15 minutes. The supernatant part was used as the analysis sample. For all the measurements the tissue-protein estimation was performed according to Bradford's method (19).

1. Total glutathione (tGSH) analysis

The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay with some modifications (20). The sample was weighed and homogenized in 2 ml of 50 mM Tris-HCl buffer containing 20 mM EDTA and 0.2 mM sucrose at pH 7.5. The homogenate was immediately precipitated with 0.1 ml of 25% trichloroacetic acid, and the precipitate was removed after centrifugation at 4200 rpm for 40 min at 4°C and the supernatant was used to determine GSH level. 1500 µl of measurement buffer (200 mM Tris-HCl buffer containing 0.2 mM EDTA at pH 7.5), 500 µl supernatant, 100 µl DTNB (10 mM) and 7900 µl methanol were added to a tube and vortexed and incubated for 30 min in 37°C. 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) was used as an chromogen and it formed a yellow-colored complex with SH groups. The absorbance was measured at 412 nm using a spectrophotometer. The standard curve was obtained by using reduced glutathione.

2. Superoxide dismutase (SOD) analysis

Measurements were performed according to Sun *et al.* (21). When xanthine is converted into uric acid by xanthine oxidase, SOD forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT and a purple colored-formazan dye occurs. The sample was weighed and homogenized in 2 ml of 20 mM phosphate buffer containing 10 mM EDTA at pH 7.8. The sample was centrifuged at 6000 rpm for 10 minutes and then the brilliant supernatant was used as assay sample. The measurement mixture containing 2450 µl measurement mixture (0.3 mM xanthine, 0.6 mM EDTA, 150 µM NBT, 0.4 M Na_2CO_3, 1 g/L bovine serum albumin), 500 µl supernatant and 50 µl xanthine oxidase (167 U/L) was vortexed. Then it was incubated for 10 minute. At the end of the reaction, formazan occurs. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme exists, the least $O2^-$radical that reacts with NBT occurs.

3. Malondialdehyde (MDA) analysis

The concentrations of ovarian lipid peroxidation were determined by estimating MDA using the thio barbituric acid test (22). The rat ovaries were rinsed with cold saline. The corpus mucosa was scraped, weighed, and homogenized in 10 ml of 100 g/L KCl. The homogenate (0.5 ml) was added to a solution containing 0.2 ml of 80 g/l sodium lauryl sulfate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/L 2-thiobarbiturate, and 0.3 ml distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 ml of n-butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane.

4. Isolation of DNA from ovarian tissue

Ovarian tissue was drawn and DNA isolated using Shigenaga *et al.*'s modified method (23). Samples (for ovarian tissue 50 mg) were homogenized at 4°C in 1 ml of homogenization buffer (0.1 M NaCl, 30 mM Tris, pH 8.0, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.5% (v/v) Triton X-100) with 6 passes of a Teflon-glass homogenizer at 200 rpm. The samples were centrifuged at 4°C for 10 min at 1000 g to pellet nuclei. The supernatant was discarded, and the crude nuclear pellet re-suspended and re-homogenized in 1 ml of extraction buffer (0.1 M Tris, pH 8.0, 0.1 M NaCl, 20 mM EDTA) and re-centrifuged as above for 2 min. The washed pellet was re-suspended in 300 µl of extraction buffer with a wide-orifice 200 µl Pipetman tip. The re-suspended pellet was subsequently incubated at 65°C for 1 hour with the presence of 0.1 ml of 10% SDS, 40 µl proteinase K, and 1.9-ml leukocyte lysis buffer. Then, ammonium acetate was added to the crude DNA sample to give a final concentration of 2.5 mol/L, and centrifuged in a micro centrifuge for 5 min. The supernatant was removed and mixed with two volumes of ethanol to precipitate the DNA fraction. After centrifugation, the pellet was dried under reduced pressure and dissolved in sterile water. The absorbance of this fraction was measured at 260 and 280 nm. Purification of DNA was determined as A 260/280 ratio 1.8.

5. DNA hydrolysis with formic acid

Approximately 50 mg of DNA was hydrolyzed with 0.5 ml of formic acid (60%, v/v) for 45 min at 150°C (24). The tubes were allowed to cool. The contents were then transferred to Pierce micro-vials, covered with Kleenex tissues cut to size (secured in place using a rubber band), and cooled in liquid nitrogen. Formic acid was then removed by freeze-drying. Before analysis by HPLC, they were re-dissolved in the eluent (final volume 200 µl).

6. Measurement of 8-hydroxy-2 deoxyguanine (8-OH Gua) with high performance liquid chromatography (HPLC) system

The amount of 8-OH gua and guanine (Gua) was measured by using a HPLC system equipped with an electrochemical detector (HP Agilent 1100 module series, E.C.D. HP 1049 A), as described previously (24, 25). The amount of 8-OH gua and Gua was analyzed on a 250 4.6 mm Supelco LC-18-S reverse-phase column. The mobile phase was 50 mM potassium phosphate, pH 5.5, with acetonitrile (97 volume acetonitrile and 3 volume potassium phosphate), and the flow rate was 1.0 ml/min. The detector potential was set at 0.80 V for measuring the oxidized base. Gua and 8-OH Gua (25 pmol) were used as standards. The 8-OH gua levels were expressed as the number of 8-OH gua molecules/10^5 Gua molecules (26).

Histological examination

At the end of each experiment the ovaries were removed and fixed in 10% neutral buffered formalin solution and then embedded in paraffin as usual. Serial sections were cut using a microtome to a thickness of 4 µm and stained with hematoxylin and eosin. The histologic sections were examined for the presence of interstitial edema, vascular dilatation, hemorrhage, and polymorphonuclear neutrophilic (PMN) infiltrations using a microscope Olympus BX-50 with a microscope and photographed. The slides were coded and semiquantitative analysis of the ovary sections was performed without knowledge of the treatment protocol. The changes observed were graded as follows: grade 0, normal; grade I, mild edema, mild vascular congestion, no hemorrhage, and no leukocytic infiltration; grade II, moderate edema, moderate vascular congestion, no hemorrhage, and no leukocytic infiltration; grade III, severe edema, severe vascular congestion, minimal hemorrhage, and minimal leukocytic infiltration; grade IV, severe edema, severe vascular congestion, hemorrhage, and leukocytic infiltration (27).

Statistical analysis

All data were subjected to one-way ANOVA using SPSS 18.0 software. Differences among groups were attained using the Scheffe option and significance was declared at $P<0.05$. Results are means ±S.E.M.

RESULTS

tGSH, SOD, MDA tests

As shown in *Fig. 1* and *2*, while the levels of tGSH, SOD, and MDA in the ovarian tissue with ischemia (IG) were 1.82 nmol/g protein, 3.6 u/g, and 13.6 mol/g protein respectively, these levels in the ischemia-reperfusion (I/R) group were 0.78 nmol/g protein, 1.8 u/g, and 20.4 mol/g protein, respectively. tGSH, SOD, and MDA values in the ischemia-controlled reperfusion (ICR) group were 4.6 nmol/g protein, 7.1 u/g, and 8.3 mol/g protein, respectively. The healthy intact group (HG) had tGSH 5.0 nmol/g, SOD 8.1 u/g, and MDA 7.5 mol/g.

8-OHGua/Gua amount

As shown in *Fig. 3*, the levels of 8-OHGual/Gua, a DNA damage product, were 1.72±0.2 pmol/L, 1.98±0.4 pmol/L, 1.14±0.1 pmol/L, and 1.13±0.2 pmol/L in the ovaries of the IG group, I/R group, ICR group, and HG group, respectively.

Histopathological findings

The ovarian tissue of the healthy intact group (HG) was considered to be normal ovarian tissue (Grade 0) (*Fig. 4*). Middle (Grade II) and severe (Grade III) edema, vascular congestion, and hemorrhage were observed in the ovaries of the IG group. Some of the tissues from this group did not contain any leukocyte infiltration (*Fig. 5*), and some of the others had mild leukocyte infiltration (*Fig. 6*). A much more severe edema

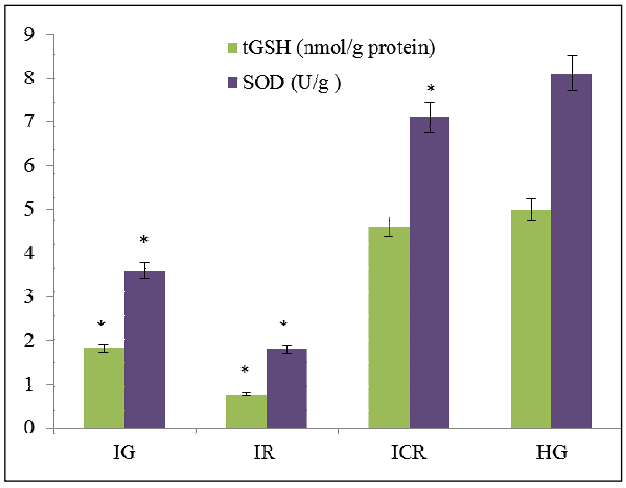

Fig. 1. tGSH level and SOD activity in ischemia group (IG), ischemia /reperfusion group (IR), ischemia/controlled reperfusion group (ICR) and healthy group (HG). * means $p<0.05$, N=10.

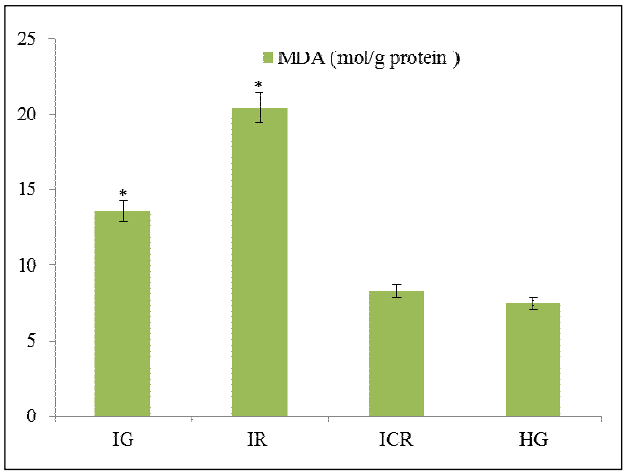

Fig. 2. MDA level in ischemia group (IG), ischemia/reperfusion group (IR), ischemia/controlled reperfusion group (ICR) and healthy group (HG). * means $p<0.05$, N=10.

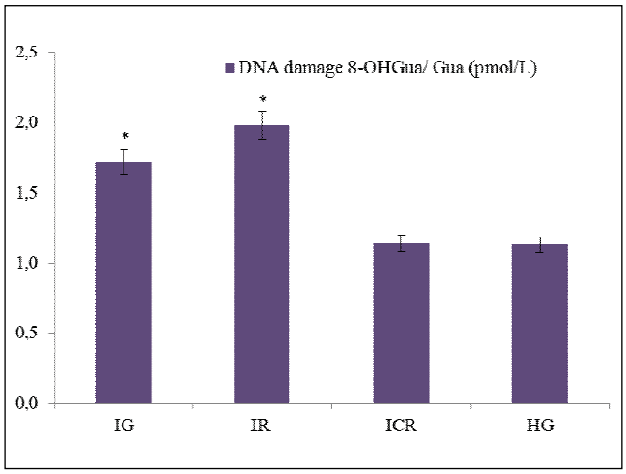

Fig. 3. DNA damage level in ischemia group (IG), ischemia/reperfusion group (IR), ischemia/controlled reperfusion group (ICR) and healthy group (HG). * means $p<0.05$, N=10.

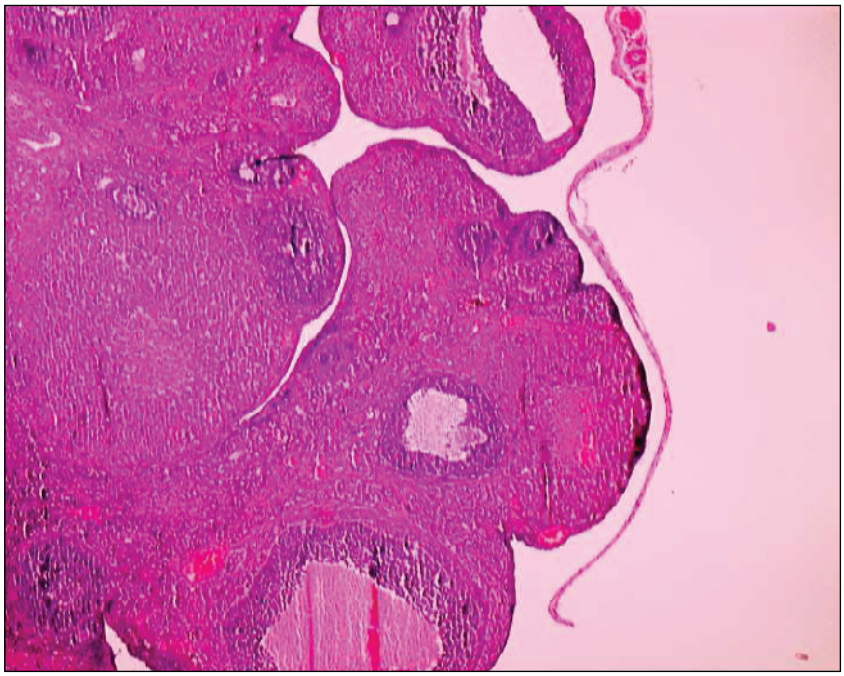

Fig. 4. The histo-pathological examination of healthy intact rats. (Grade 0).

(Grade IV), vascular congestion, hemorrhage, and leukocyte infiltration were observed in the I/R group (*Fig. 7*). Mild edema, mild vascular congestion, and hemorrhage (Grade 1) were detected in the ICR group. Leukocyte infiltration was not observed in this group (*Fig. 8*).

DISCUSSION

In this study it was investigated whether controlled reperfusion is useful or not in the prevention of ovarian damage in ischemic conditions. In addition, biochemical and histopathological examinations were performed in ischemia, ischemia/reperfusion, ischemia/controlled reperfusion groups. Biochemically, the amounts of tGSH, SOD, MDA, and a DNA damage product of 8-OHGua/Gua were investigated in the ovarian tissues of all the rats. Histo-pathologically, edema, vascular congestion, hemorrhage and leukocyte infiltration parameters were used.

Our experimental results showed that the levels of tGSH and SOD in the IG group were lower, but the MDA levels were higher than in the HG group. The highest levels of MDA and the lowest levels of tGSH and SOD were in the I/R group. The levels of MDA, tGSH, and SOD in the ICR group were similar to those in the HG group.

Ischemic damage occurs when the final substances of anaerobic metabolism accumulates related to the decrease in blood stream and insufficient oxygen delivery. On the other hand reperfusion damage is composed by free oxygen radicals directly or by the depression of cellular antioxidant systems (28). When the blood stream stopped in ischemic term in tissue, ATP synthesis decreased (29). Because the use of ATP continues despite the ATP synthesis comes to the end, AMP, adenosine, inosine and hypoxanthine composes from ATP respectively. If there is no reperfusion, cumulative hypoxanthine never converts into xanthine (30). Thanks to re-oxygenation during reperfusion, xanthine oxidase enzyme which composes during ischemia (31), causes the

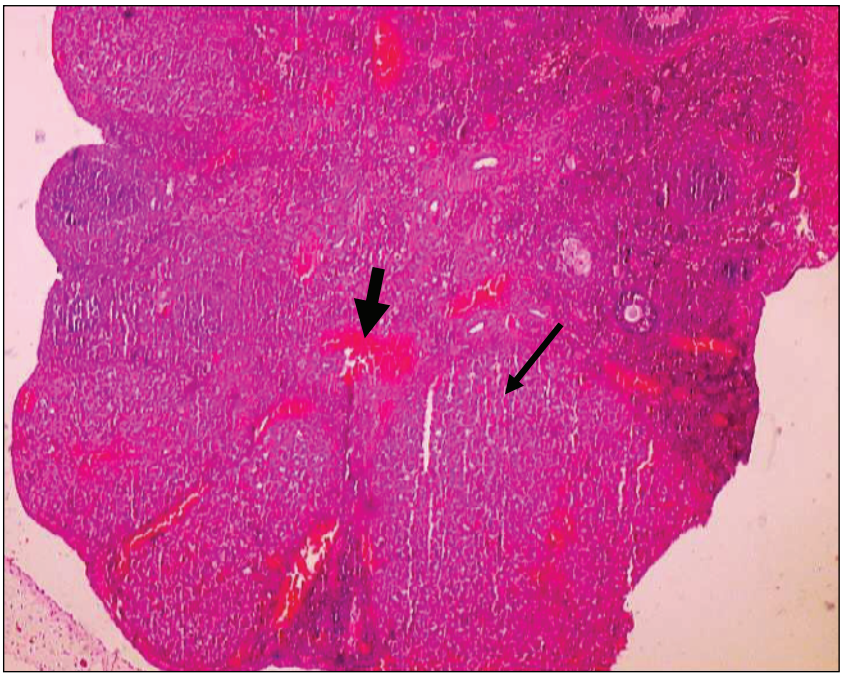

Fig. 5. The histo-pathological examination of ischemia rats. Grade 2 moderate edema (thin arrow), moderate vascular congestion (thick arrow).

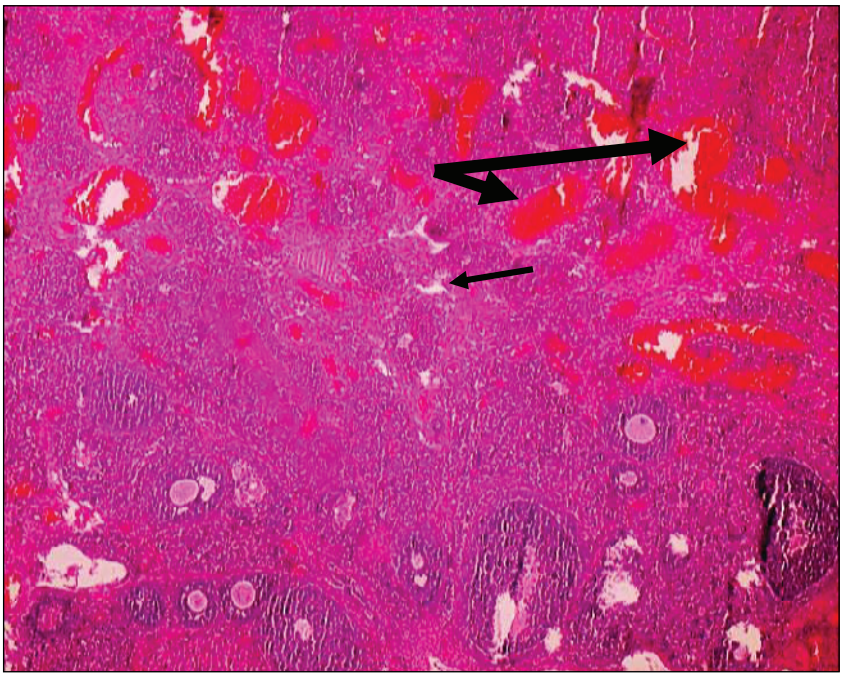

Fig. 6. The histo-pathological examination of ischemia rats. Grade 3 severe vascular congestion (thick arrow), severe edema (thin arrow).

production of lots of free oxygen radicals while cumulative hypoxanthine is converting into xanthine (30, 32). The revelation of lots of free oxygen radical in reperfusion causes endothelial damage directly or increases oxidative damage anymore by causing neutrophil infiltration to the post-ischemic tissues; but on the contrary a decrease occurs in antioxidant enzyme (33, 34). This knowledge are in accordance with our results.

Potassium-ATP channel (KATP) activation is responsible for the protective effect of controlled reperfusion (35). When KATP channel activates, thanks to decrease in neutrophil accumulation, endothelial dysfunction, oxidative stress and mitochondrial Ca^{2+} accumulation, cell death may be prevented (36). It was reported that the protective effect of controlled reperfusion is *via* adenosine receptors (A1) (37). When adenosine A1 activates, KATP channels open and Ca^{2+} flux decreases (38).

It has been experimentally shown that GSH levels are suppressed in ischemic ovarian tissue (39). GSH is an antioxidant that is used to measure oxidative stress. It has been reported that reperfusion following ischemia causes more severe damage to ovarian tissue and suppresses the GSH levels further (27). One of the many factors leading to oxidative damage is ischemia (40). GSH plays a role in the protection of the cell against oxidative stress and toxic compounds as well as the metabolic processing of many endogenous compounds such as estrogen, prostaglandin, and leukotrienes (41). GSH, as an antioxidant, reacts with peroxides and free radicals and converts them into harmless products. By this mechanism, GSH protects the cells against the potential oxidative damage of free radicals. GSH keeps -SH groups in proteins at the reduced state and prevents them from being oxidized (42). Our study has also

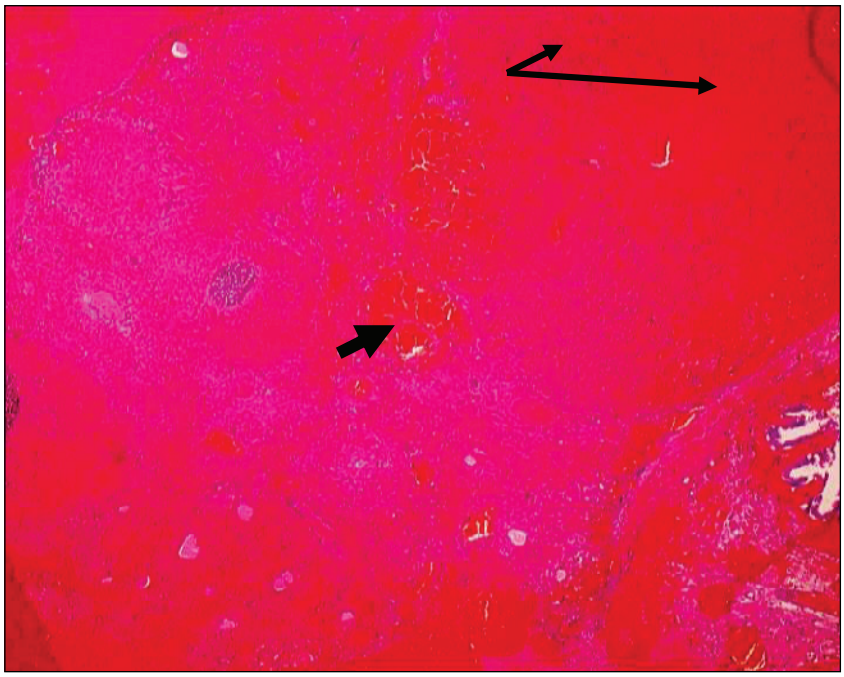

Fig. 7. The histo-pathological examination of ischemia/reperfusion rats. Grade 4 severe vascular congestion (thick arrow), hemorrhage and leukocytic infiltration (thin arrow).

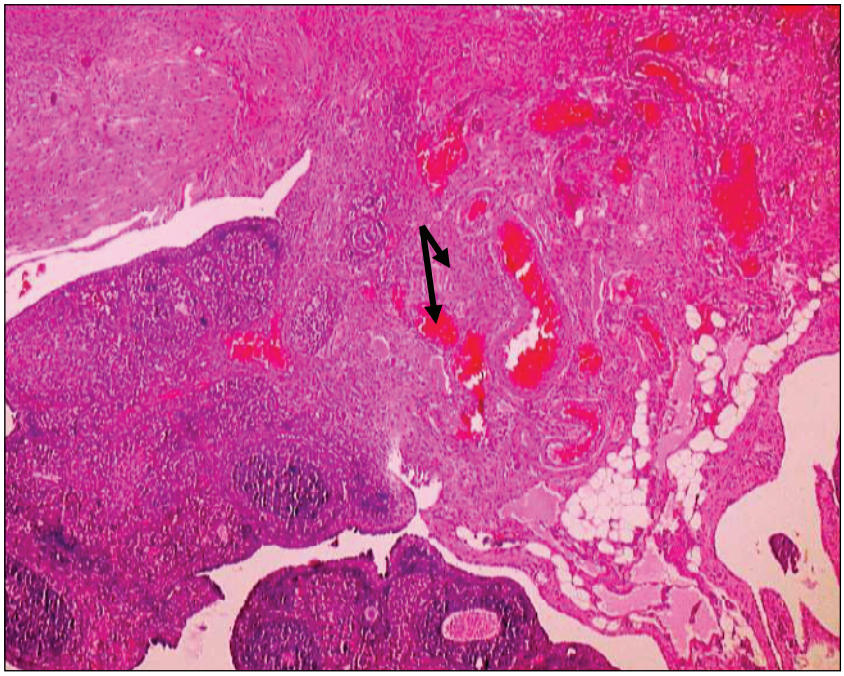

Fig. 8. The histo-pathological examination of ischemia/controlled reperfusion rats. Grade 1 mild edema and congestion (arrow).

shown that oxidative stress occurs in ischemic ovarian tissue and is aggravated with reperfusion. However, it was found that oxidative stress was minimized and the severe damage due to sudden reperfusion was prevented in the controlled reperfusion group (ICR). Similar conclusion was found by Munakata *et al.* in brain tissue (43).

In our study, SOD activities in ovarian tissue were also measured and compared among all the animal groups. The closest SOD activity was between ICR and HG groups. Superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the conversion of superoxide free radical (O_2-•) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD and endogenous antioxidant enzymes neutralize free radicals and protect tissues from the harmful effects of free radicals and active oxygen species (44).

In addition, MDA was found to be much lower in the ICR group than in the IG and I/R groups in our study. MDA occurs as a result of the peroxidation of fatty acids that contain three or more double bonds. That is, MDA is a lipid peroxidation product. By affecting the ion exchange through the cell membranes, MDA causes cross-linking of membrane components and leads to negative consequences, such as changes in ion permeability and enzyme activity (6, 45). It was shown in these animal experiments that MDA levels are increased in parallel with the increase in damage (27).

While membrane lipids are sensitive to the effects of free radicals, proteins and nucleic acids are more resistant to these detrimental effects. However, DNA molecules can be easily damaged if free radicals are located in an area very close to the DNA molecules (46, 47). Hydroxyl radical reacts very easily

with deoxyribose and the bases. Hydroxyl radical causes DNA damage by extracting hydrogen from nucleic acids or reacting with double bonds (48). As mentioned in the introduction, 8-OH Gua is considered an important marker of DNA oxidation (49). In our study, it was observed that the ovaries of the IG and I/R rat groups had higher levels of 8-OHGua than the healthy rat ovaries. However, the levels of DNA damage product were similar in the ICR and HG groups. Results have showed that, oxidative stress level showed a parallelism to the tissue damage.

Ischemia, ischemia-reperfusion, and ischemia-controlled reperfusion applied to tissues were also analyzed histopathologically. Edema, vascular congestion, hemorrhages, and leukocyte infiltration have been used as histopathological parameters in the evaluation of the condition of the cell (27). It was observed that histopathological results obtained from all the experimental groups were in concordance with the results of the biochemical analysis. Edema, vascular congestion, hemorrhage, and leukocyte infiltration in the ICR group were much milder than in the IG and I/R groups. In summary, these results indicate that controlled reperfusion can be helpful in minimizing ischemia-reperfusion injury in ovarian tissue exposed to ischemia for various reasons (ovarian torsion, tumor, *etc*.).

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Conflict of interests: None declared.

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