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GYNECOLOGIC ONCOLOGY

A new approach to prevent ischemia/reperfusion injury in a rat model: remote ischemic conditioning

Mehmet Sipahi[1] · Mucahit Gunaydin[2] · Tugrul Kesicioglu[3] · Murat Usta[4] · Berna Tezcan Yavuz[5] · Canberk Tomruk[6]

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Abstract

Purpose To evaluate the effect of remote ischemic conditioning (RIC) on ovarian ischemia/reperfusion injury in a rat model.

Methods A total of 36 Wistar albino rats with a body weight of 220–250 g were used for this study. Right adnexal torsion was performed for 180 min, and at the end of the period, the adnex was released and the abdomen was reclosed for 180 min for reperfusion. Torsion and detorsion procedures were applied to all rats except group 1 (sham, control). The right lower extremity was tied to perform remote tissue ischemia in groups 3, 4, 5, and 6. The goal of the procedure, which was purplish discoloration and pulselessness of the extremity, was maintained. After 5 min of ischemia, reperfusion was achieved for 5 min. Repeating this procedure 3 times was defined as hypoxia attacks (RIC). Retrieved ovaries were examined for tissue injury with biochemical, histopathologic, and immunohistochemical analysis.

Results Unlike the control group, vascular congestion, hemorrhage, edema, and inflammatory cell infiltration were observed in group 2 (only I/R [ischemia/reperfusion]). In groups 3 (I/R + RIC), 4 (I/R + RIC), 5 (I/R + RIC), and 6 (I/R + RIC), edema and inflammatory cell infiltration were not observed. However, vascular congestion and hemorrhage that were detected in these groups were higher than in group 1 (Control) and less than in group 2 (I/R). The Caspase-3 Index was found to be increased in all groups compared to group 1 ($P < .001$). However, the increase in the RIC-performed groups was significantly less than in group 2. The apoptotic index, which was determined by the TUNEL, was also found to be increased in all groups compared to group 1 ($P < .001$). When the comparison was made in relation to group 2, the decrease of AI in RIC-performed groups was statistically significant, except the decrease in group 6 ($P = .29$).

Conclusions It is not clinically conceivable to prepare the tissue for pre-ischemia in ovarian torsion. However, the RIC application, which will be initiated if torsion is suspected when arrangements are made for surgery, might be a simple, effective, and inexpensive approach to prevent I/R injury in the clinic.

Keywords Ischemia · Remote ischemia · Ovarian torsion · Reperfusion injury · Rat model

✉ Mehmet Sipahi
mehmetsipahi@hotmail.com

[1] Department of Obstetrics and Gynecology, Giresun University, Ataturk Bulvari, Teyyareduzu Mahallesi, Giresun 28200, Turkey

[2] Department of Emergency Medicine, Giresun University, Giresun, Turkey

[3] Department of General Surgery, Giresun University, Giresun, Turkey

[4] Department of Medical Biochemistry, Giresun University, Giresun, Turkey

[5] Biology Department of Council of Forensic Medicine, Izmir, Turkey

[6] Department of Histology and Embryology, Ege University, Izmir, Turkey

Introduction

Adnexal torsion is defined as the enhanced resistance or cessation of blood flow as a consequence of the rotation of the ovary around a suspensory ligament [1]. Since venous pressure is lower, venous return discontinues after torsion, while arterial blood flow keeps going regularly and edema develops in the ovarian tissue. As a result of edema, the blood supply to the ovary stops and ischemic damage occurs due to increased ovarian pressure [2]. Consequently, prolongation of this period causes necrosis and irreversible damage in the ovarian tissue. Ovarian torsion is the most common gynecological emergency, with a prevalence of 2.7% [3]. While previously the main treatment modality was adnexal detorsion and oophorectomy, nowadays, the main treatment

is cystectomy and detorsion of the ovary [4]. Although the ovarian tissue seems necrotic, the parenchyma remains largely intact [5]. However, the tissue damage increases after the detorsion due to the free oxygen radicals which are formed as a result of the abundant oxygen intake of the tissue [6]. Lots of antioxidants and anti-inflammatory drugs have been tried to minimize this damage; however, no consensus has been achieved.

Remote ischemic preconditioning (RIPC), which is frequently used in liver and cardiac surgeries, induces the body's own antioxidant mechanisms by artificially creating temporary hypoxia attacks in a remote tissue (often in the leg) [7, 8]. This method has been tried in various cardiac and neurological diseases, but there is no study about the ovary [9, 10]. The aim of this study was to compare the effectiveness of natural antioxidant mechanisms in preventing ovarian ischemia/reperfusion (I/R) injury by biochemical, histopathological, and immunohistochemical features with the control group.

Materials and methods

A total of 36 Wistar albino rats with body weights of 220–250 g were used for this study. All animals were fed with ad libitum for 1 week in separate cages under a constant temperature and humidity environment and a 12-h day/night cycle.

Surgery was performed after anesthesia with intraperitoneal administration of ketamine hydrochloride (50 mg/kg Ketalar; Eczacibasi, Istanbul, Turkey) and xylazine hydrochloride (10 mg/kg Rompun; Bayer Turk Ilac Ltd., Istanbul, Turkey). Lower abdominal laparotomy was performed via 25 mm vertical incision after applying an antiseptic solution (Baticon; Drogsan, Turkey) to the surgical site.

Right adnexal torsion was performed by rotating the ovarian and tubal vessels 720° counterclockwise via a microsurgical clamp and fixing them to the abdominal peritoneum with a 4-0 vicryl suture (Dogsan; Turkey) for 180 min. Abdominal layers were closed anatomically following the procedure. Relaparotomy was performed at the end of the period, and the adnex was released and the abdomen reclosed conveniently to the anatomy for 180 min of reperfusion. Torsion and detorsion procedures were applied to all rats except group 1 (sham, control). The right lower extremity was tied with 0 silk suture (Dogsan; Turkey) to perform remote tissue ischemia. The goal of the procedure, which was purplish discoloration and pulselessness of the extremity, was maintained. After 5 min of ischemia, reperfusion was achieved for 5 min. Repeating this procedure 3 times was defined as hypoxia attacks (remote ischemic conditioning [RIC]).

Following RIC, 180 min of ovarian ischemia and then 180 min of reperfusion were applied to group 3 rats, and oophorectomy was performed on both sides. RIC was applied 30 min after the torsion was performed, and the torsion time was continued to 180 min in group 4. Then, reperfusion was performed for 180 min and the ovaries were excised. After 150 min of torsion, RIC was applied and torsion time was continued to 180 min in group 5. Then, 180 min of reperfusion were initiated with RIC again. Both the ovaries were excised when the time was up. RIC was applied 30 min after the torsion was performed, and the torsion time was continued to 180 min in group 6. Then, 180 min of reperfusion were initiated with RIC again. Both the ovaries were excised when the time was up. Surgical procedures and RIC were applied to groups, as summarized in Fig. 1. Six experimental groups are formed, where six rats were enrolled in each group, adding up to 36 rats that are involved in total. However, due to surgical procedures, three rats (one in each group of 1, 3, and 6) have died, and in result, these respective groups have finished the experiments with the remaining 5 rats in each. All rats were sacrificed by cervical dislocation following the completion of surgical procedures.

Blood samples were drawn by intracardiac approach after the reperfusion period was over, prior to sacrification, and transferred to heparin-containing tubes. These samples were then centrifuged at 1000×*g* and 4° C for 20 min. The obtained supernatants were aliquoted and stored at − 80 °C until the day of the study. To determine the oxidative stress superoxide dismutase (SOD), plasma signal peptide, complement C1r/C1s, Uegf, and Bmp1 (CUB), and epidermal growth factor-like

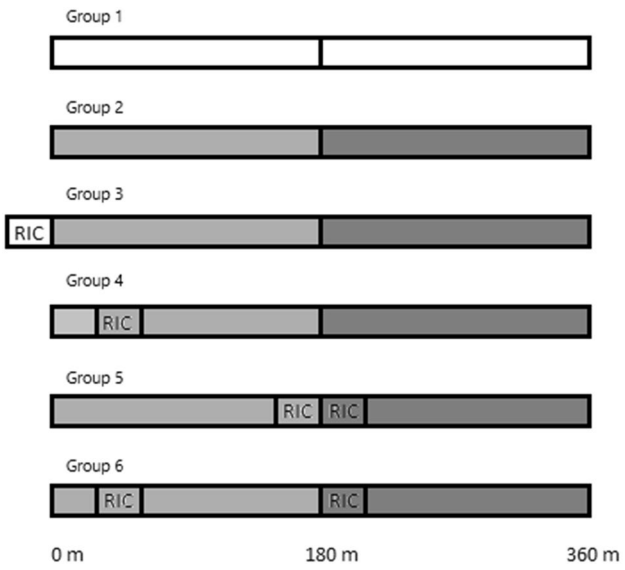

Fig. 1 Surgical procedures and RIC timing. Light grey zones are ischemia and dark grey zones are reperfusion

domain-containing protein-1 (SCUBE-1), total antioxidant status (TAS) and plasma malondialdehyde (MDA) for the evaluation of lipid peroxidation were studied. Plasmas were placed on ice and studied with a commercial kit (Superoxide Dismutase Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA, Batch: 0525723) as described to determine SOD activity, and the results were expressed in U/mL. MDA levels were studied as recommended in the commercial kit (TBARS Assay Kit, Cayman Chemical, Ann Arbor, MI, USA, Batch: 0525498) based on the formation of thiobarbituric acid reactive substances (TBARS) in a high-temperature and acidic medium, and results were expressed in mmol/L. SCUBE-1 levels were analyzed using the enzyme-linked immunosorbent assay reagent kit (Cloud-Clone Corp., 23603 W.Fernhurst Dr., Unit 2201, Katy, TX 77494, USA, Lot: L180307290) and results were expressed in ng/mL. TAS was evaluated on the basis of the conversion of dark blue–green 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS* +) of the antioxidants in the plasma sample to the colorless reduced ABTS form [11]. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox Equivalent) was used as a vitamin E analog for standardization, and results were expressed in mmol Trolox Equiv./L.

The removed right-hand-side ovarian tissues were quickly fixed in 10% neutral formol solution. Following histologic tissue preparation and paraffin embedding, 5-µm-thick sections were taken from paraffin blocks. Hematoxylin–eosin (H–E) staining was first applied to the sections to examine the histological structure of the tissues. The tissues were evaluated histologically for vascular congestion, hemorrhage, edema, and inflammatory cell infiltration under light microscopy.

Immunofluorescent staining of terminal deoxynucleotidyl transferase-mediated d-UTP Nick-end Labeling (TUNEL) (Merck, Germany) and Caspase 3 (Santa Cruz Biotechnology, Inc., USA) were performed in accordance with the manufacturer's protocol to determine apoptosis differences between the experimental and control groups. Immunopositive stained and unstained cells in each section were counted by two independent investigators (BT and CT) at different times under a fluorescence microscope with a random selection of 5 areas at 40× magnification at Ege University, Department of Histology and Embryology. The ratio of immunopositive stained cells to total cells in the counting area (Apoptotic index and Caspase 3 index) was calculated according to the following formulas: Apoptotic index (AI) = the number of apoptotic cells/the number of total cells × 100. Caspase 3 index (CI) = the number of Caspase 3 immunopositive cells/the number of total cells × 100.

Statistical analyses

Statistical analyses were performed using SPSS version 17 (SPSS, Chicago, Illinois). The comparison of scores between the groups was performed using a Kruskal–Wallis test. Post hoc analysis was performed using either a Tamhane test or a Tukey test to calculate the difference between groups according to the homogeneity of variables. Values were given as means (standard deviation), and $P < .05$ was accepted as statistically significant.

Ethical approval

The study was initiated after the approval of Giresun University, Ethical Committee on Animal Research (2017/7). All experimental procedures were in accordance with the principles of the Declaration of Helsinki.

Results

Biochemical results

Serum SOD, SCUBE-1, TAS, and MDA results are presented in Table 1. Although the value of TAS, which is an antioxidant, decreased in group 2 (I/R) compared to the control group, it was found to be increased in RIC-performed groups. However, this difference was not statistically significant. The values of the other antioxidant marker SOD were found to be decreased, except for group 6 (I/R + RIC). An oxidative marker, MDA, was found to be increased in all groups except group 3 (I/R + RIC) when compared to the control group. This difference was also statistically significant between group 3 (I/R + RIC) and group 5 (I/R + RIC) ($P = .021$). There was no significant difference between the groups for values of SCUBE-1, which is an oxidative marker and increases in thrombotic events and in acute ischemic conditions due to increasing platelet activity. However, SCUBE-1 values were found to be higher in groups 5 and 6, which would be due to performing RIC twice.

Histopathologic results

H–E-stained sections from paraffin blocks of the control and the experimental groups were histologically evaluated and compared to each other under a light microscope. Unlike in the control group, vascular congestion, hemorrhage, edema, and inflammatory cell infiltration were observed in group 2 (I/R). In groups 3 (I/R + RIC), 4 (I/R + RIC), 5 (I/R + RIC), and 6 (I/R + RIC), edema and inflammatory cell infiltration were not observed. However, vascular congestion and hemorrhage that were detected in these groups were higher than in group 1 (Control) and less than in group 2 (I/R) (Fig. 2).

Immunohistochemical and TUNEL findings

Caspase 3 proteins were immunohistochemically stained and CI scores are given in Table 2. CI was found to be increased in all other groups compared to group 1 ($P < .001$). However, the increases in RIC-performed groups were less than in group 2. The apoptotic index was determined by the TUNEL assay and the results are given in Table 2. AI was also found to be increased in all groups compared to group 1 ($P < .001$). Similar to CI, the increases in RIC-performed groups were less than in group 2.

Immunofluorescence staining of the control and experimental groups by Caspase-3 and TUNEL are given in Figs. 3 and 4, respectively. Immunopositive stained cell counts were

Table 1 Serum SOD, SCUBE-1, TAS, and MDA results according to groups

Serum	Group 1 (control)	Group 2 (I/R)	Group 3 (I/R + RIC)	Group 4 (I/R + RIC)	Group 5 (I/R + RIC)	Group 6 (I/R + RIC)
SOD (U/mL)	6.24 ± 1.69	5.61 ± 1.03	5.15 ± 1.15	5.32 ± .64	5.70 ± .78	6.26 ± .86
SCUBE-1 (ng/mL)	1851.88 ± 381.96	1517.80 ± 371.29	1761.02 ± 516.90	2005.56 ± 380.86	2214.91 ± 988.49	2346.32 ± 630.34
TAS (mmol Trolox Equiv./L)	1.15 ± .30	.91 ± .04	1.00 ± .33	.89 ± .10	.98 ± .20	1.05 ± .32
MDA (mmol/L)	18.62 ± 6.38	21.10 ± 6.33	14.18 ± 5.15*	30.62 ± 11.72	36.37 ± 18.99	25.08 ± 5.24

*$P = .021$, group 3 vs 5. SOD: superoxide dismutase; SCUBE-1: plasma signal peptide, complement C1r/C1s, Uegf, and Bmp1 (CUB), and epidermal growth factor-like domain-containing protein-1; TAS: total antioxidant status; MDA: malondialdehyde; I/R: ischemia/reperfusion; RIC: remote ischemic conditioning

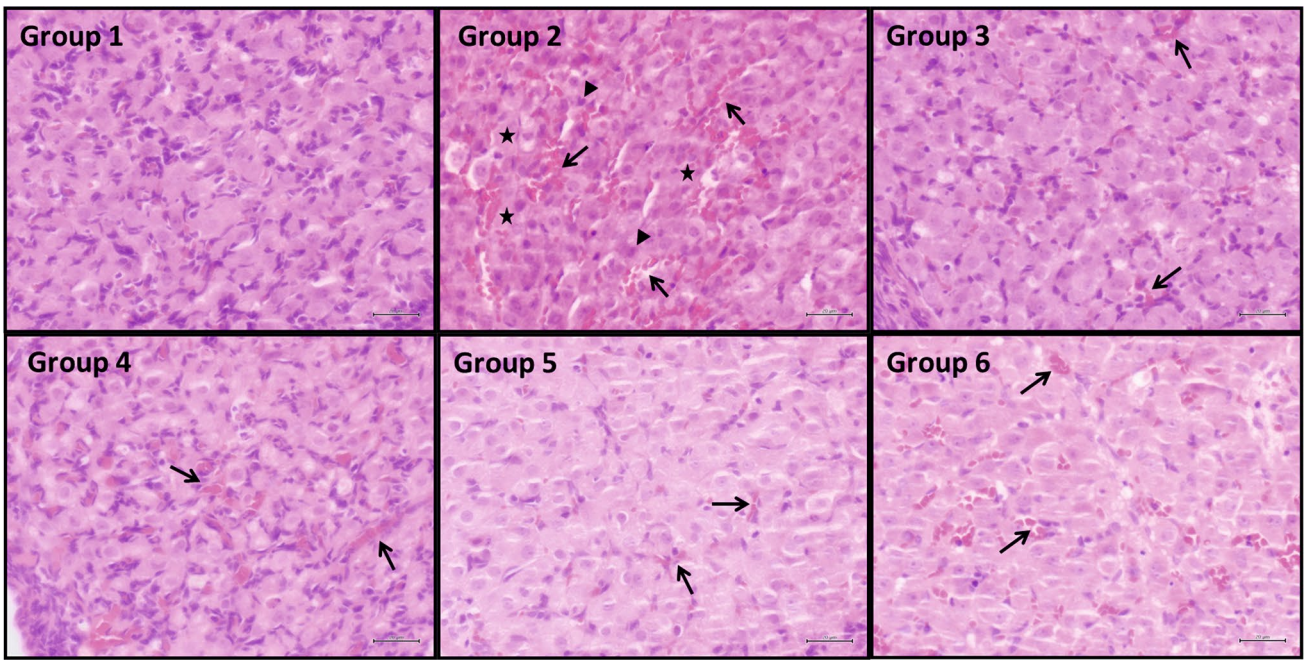

Fig. 2 H-E staining of ovarian tissues from control and experimental groups, light microscopy, 40× magnification. Arrows indicate areas of vascular congestion and hemorrhage; arrowheads indicate inflammatory cell infiltration; stars indicate edematous areas

Table 2 CI and AI scores according to groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Caspase-3 index	1.16 ± .25	7.22 ± 1.18	4.18 ± .36	4.96 ± .88	5.04 ± .81	4.94 ± .24
TUNEL apoptotic index	1.56 ± .26	6.27 ± 1.59	3.73 ± .84	3.88 ± 1.0	4.41 ± .35	5.08 ± .6

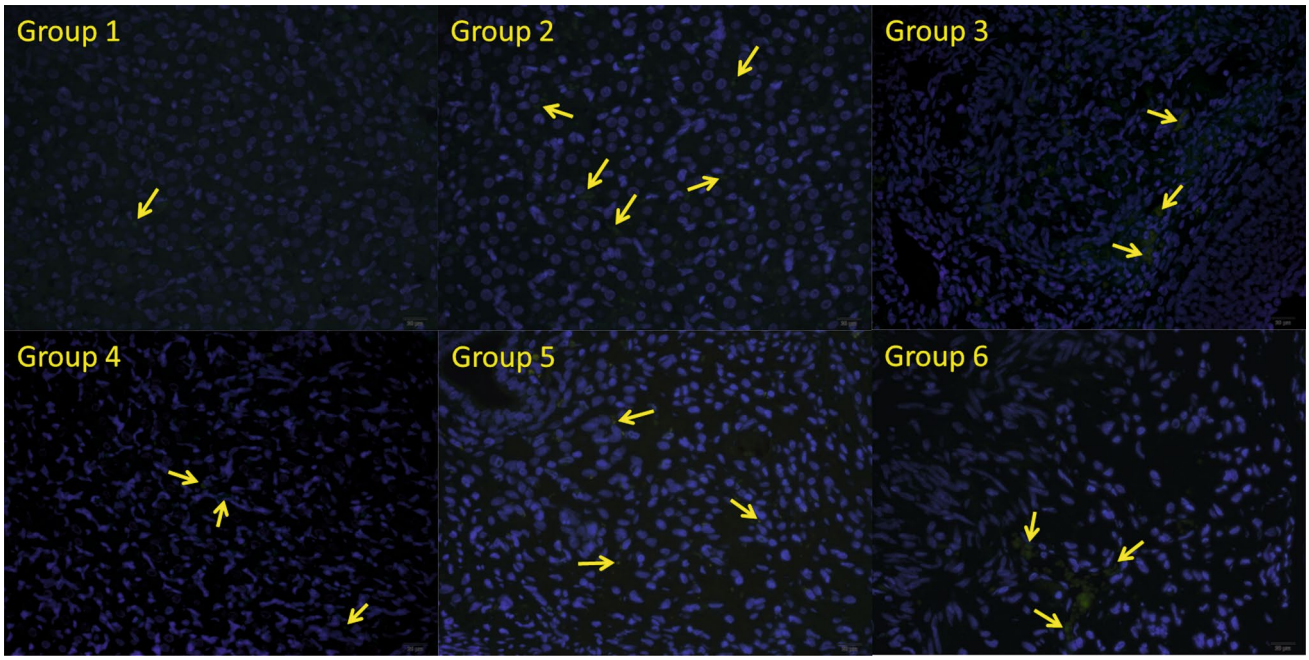

Fig. 3 Caspase 3 immunofluorescence staining of ovarian tissues from control and experimental groups, fluorescence microscope, 40× magnification. Arrows indicate immunopositive stained cells

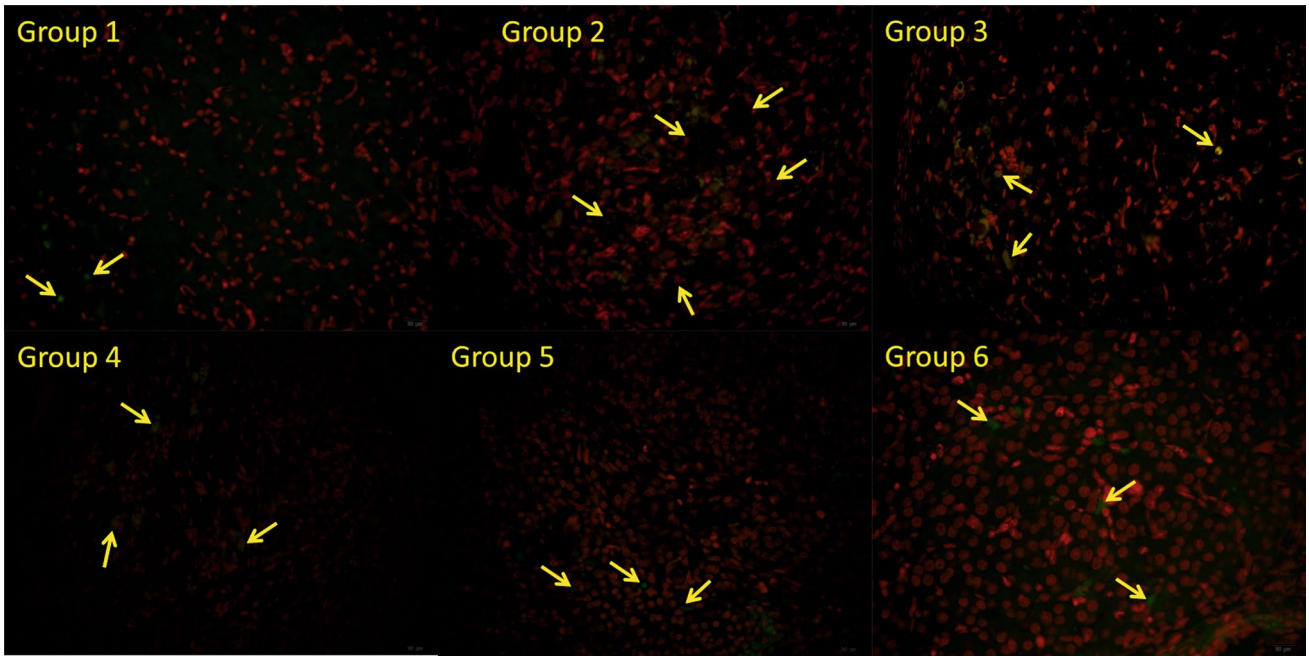

Fig. 4 Staining with immunofluorescence by the TUNEL method of ovarian tissues from control and experimental groups, fluorescence microscope, 40× magnification. Arrows indicate immunopositive stained cells

significantly higher in group 2 compared to group 1. However, immunopositive cell counts were less than group 2 in all RIC-performed groups.

The statistical relationships between CI and AI scores are given in Figs. 5 and 6 according to the groups. Although the AI and CI scores increased significantly in the I/R group, the protective effect of RIC determined in all scores of groups, except for the AI score of group 6 ($P = .29$).

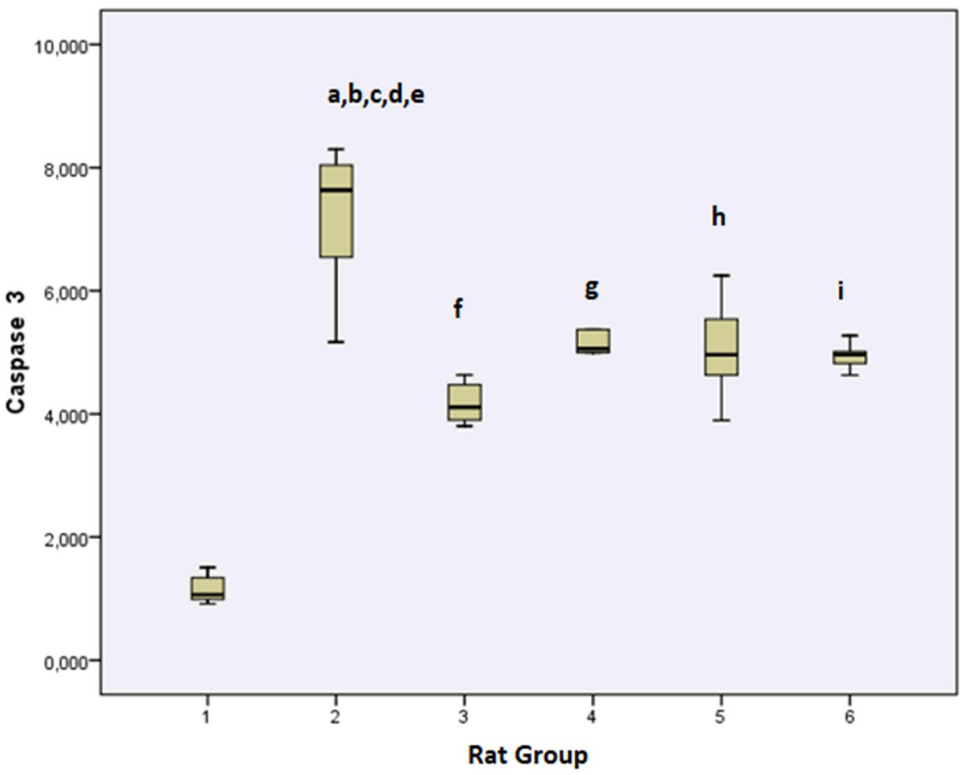

Fig. 5 Caspase 3 index scores of groups. **a** $P < .001$, compared with control; **b** $P < .001$, compared with group 3; **c** $P < .001$, compared with group 4; **d** $P < .001$, compared with group 5; **e** $P < .001$ compared with group 6, **f–i** $P < .001$ compared with group 1

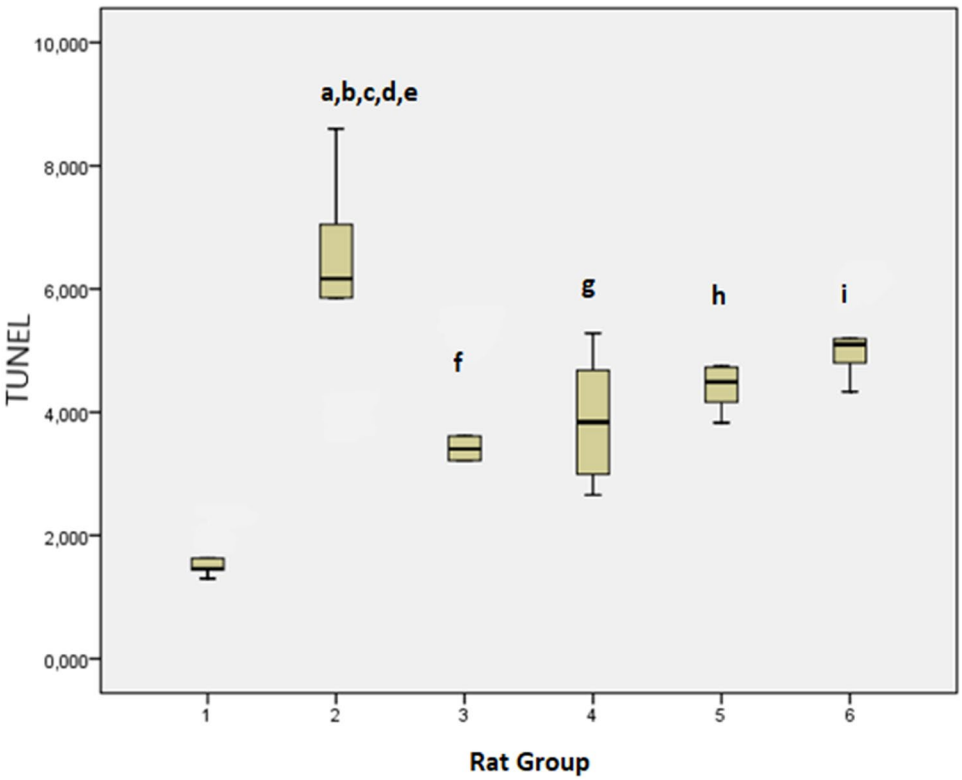

Fig. 6 TUNEL AI scores of groups. **a** $P < .001$, compared with control; **b** $P = .001$, compared with group 3; **c** $P = .001$, compared with group 4; **d** $P = .018$, compared with group 5; **e** $P = .299$ compared with group 6; **f** $P = .010$ compared with group 1; **g** $P = .003$ compared with group 1; **h**, **i** $P < .001$ compared with group 1

Discussion

Ovarian torsion is a rare condition. However, it is a significant cause of acute pelvic pain in women [1]. Although the vitality expectation of the ovarian tissue decreased when it took on a dark-blue appearance due to ischemia, the main treatment is detorsion of the ovary. Viable ovarian parenchyma has been documented by a study from Kruger et al. [12] at oophorectomy materials of adnexal torsion. Calis et al. [13] also compared the number of primordial and primary follicles in I/R-performed ovaries with intact ovaries and did not find any significant difference. A valuable question in these cases is whether or not the detorsion of necrotic-looking tissue will result in a thromboembolic event. While minimal complications such as fever were reported in a study of 214 cases, there was no thromboembolism [14].

Reperfusion injury caused by reactive oxygen species and free radicals, in addition to ischemic injury, also causes serious damage to tissue [15]. To prevent I/R damage, many molecules have been tested in the literature, such as carvedilol, ursodeoxycholic acid, and vitamin D [16–18]. However, remote ischemic conditioning to prevent I/R injury in ovarian torsion, which is the subject of this study, is being examined for the first time in the literature. In patients admitted to clinic with ovarian torsion, RIPC does not seem practically possible because of the onset of ischemic events in the ovary. However, in patients undergoing surgery for torsion, RIPC can be performed until the operation and during the operation period. Therefore, group 3 has an experimental quality; however, other groups are clinically feasible.

Initial studies on preparing the tissue for a longer duration of hypoxia with ischemic attacks were reported by Murry et al. [19]. They concluded that the attacks of angina may be protective for myocardial infarction in an experimental animal study. Ischemic attacks have also been utilized in the areas of general surgery, neurology, and urology. Ischemic attacks are widely used in liver surgery, because clamping the portal triad to reduce blood loss in surgeries involving liver parenchyma causes extra damage to the post-operative ischemic liver [20]. The most common surgical techniques are ischemic preconditioning and intermittent clamping [7, 21]. In addition, the approach of using RIPC has recently attracted attention. Unlike other techniques, in this application, it is aimed to protect the target tissue by creating natural antioxidants with short-term consecutive ischemia–reperfusion attacks in a tissue away from the surgical field (usually in an extremity) [22]. It has been shown that ischemia in the remote organ increases serotonin release from platelets, which increases endothelial Vegf release and provides protection over the target tissue through Il10 and Mmp8 [8].

In this study, the effect of RIC at different times on ovarian I/R damage is investigated. While vascular congestion, hemorrhage, edema, and inflammatory cell infiltration were observed together only in the I/R group at histopathological evaluation, edema and inflammatory cell infiltration were not observed in RIC-performed groups at any time, except for vascular congestion and hemorrhage. Vascular congestion and hemorrhage also occurred less in RIC-performed groups than in the control group. This shows that RIC administration provides protection against I/R injury. In response to remote tissue ischemia, protective factors are released into the bloodstream to reach the target tissue. Although these factors have not been fully elucidated, neuronal (adenosine, bradykinine, cyclic guanosine monophosphate, and calcitonin), humoral (opioids, endocannabinoids, and angiotensin-2 receptors), and systemic (suppression of proinflammatory genes, expression of leucocytes, and reduced neutrophilic adhesion) mechanisms are known to be involved in this role [23, 24]. In a meta-analysis that presented data from 32 studies, it was shown that RIC could lead to rhabdomyolysis in the applied muscle and that renal damage could develop due to striated muscle damage and I/R injury in the muscle as side effects [9]. However, there are studies that highlighted the protective effects on kidneys [23]. In accordance with this information, RIC is generally considered as a safe and non-harmful method.

Apoptosis, a type of cell death, has been shown to be involved following I/R injury in many studies [16]. Thus, the reduction of oxidative stress reduces the apoptosis as expected [25]. According to Caspase 3 and TUNEL results, the CI and AI scores were lower in all RIC-administered groups when compared to the only I/R-performed group. According to the TUNEL results, the AI score of group 6 was found to be significantly increased when compared to group 1; however, the decrease compared to group 2 was not significant. This suggests that the RIC timing in group 6 is not effective in preventing I/R damage.

Negative effects of iatrogenic ischemia on the serum markers are also a possible cause of discordance between the biochemical data of serum and the histopathological data. Similarly, Wang et al. [9] argued that acute renal injury was more likely after RIPC, which was administered for cardioprotective effect; theoretically, it could be due to plasma catalytic iron and rhabdomyolysis after I/R. If biochemical markers were studied with tissue homogenization, the results could be consistent with histopathological findings. Lack of these results could be considered as a limitation of this study. On the other hand, it is a positive aspect of this study that it was tried to prevent ovarian I/R damage by taking advantage of natural antioxidants for the first time in the literature.

Conclusion

It is not clinically conceivable to prepare the tissue for pre-ischemia in ovarian torsion. However, the RIC application, which will be initiated if torsion is suspected when arrangements are made for surgery, might be a simple, effective and inexpensive approach to prevent I/R injury in the clinic.

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Authors' contributions MS: 65%: Hypothesis, development of project, surgery, and statistical analyses. MG: 10%: Surgery. TK: 10%: Surgery. Murat Usta: 5%: Biochemistry. BT: 5%: Histology. CT: 5%: Histology

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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