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The long-term protective effect of hypothermia and gradual detorsion on ovarian tissue in adnexal torsion/detorsion model in rats

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ABSTRACT

Introduction: Ischemia in ovarian torsion and subsequent reperfusion has significant effects on fertility in the long term. The most important reason for these changes is thought to be a reperfusion injury rather than ischemia. We aimed to evaluate whether the reperfusion injury following ovarian detorsion could be reduced by hypothermia and intermittent reperfusion.

Materials and Methods: Forty adult female rats were divided into five groups as follows: Sham (Sh) (n = 8), torsion detorsion (control TD) (n = 8), progressive reperfusion "gradual detorsion" (GD) (n = 8), hypothermia (H) (n = 8) and the progressive reperfusion + hypothermia (GD + H) (n = 8). In all rats, except for the Sh group, the left ovary was rotated counter clockwise 1080° and fixed to the abdominal wall by three 5–0 non-absorbable sutures followed by the closure of the laparotomy. After 30 h, reperfusion was achieved following the detorsion of the ovaries. In both the control TD and H groups, the torsed ovaries were detorsed. H group, however, was subjected to hypothermia with ice packs 30 min before and during the detorsion. Tissue temperature was kept constant at 4 °C, controlled by a digital thermometer. In the GD group, the torsed ovary and pedicle were detorsed by 360°, followed by a 5 min pause. This procedure was repeated twice until a complete detorsion was achieved. GD + H group underwent hypothermia with ice packs 30 min before the procedure and the torsed ovary and pedicle were detorsed by 360°. After a 5 min pause, we repeated this process twice to provide full detorsion. The tissue temperature was constantly held at 4 °C. In the hypothermia groups, we applied hypothermia for an additional 30 min after detorsion and then left the rats at normal body temperature during reperfusion. We followed the rats in all groups for 60 days. Then we excised the left ovaries of all rats through laparotomy and spared some of the ovaries for biochemical and pathological examination. Intracardiac blood was taken at the end of the procedure and it was sent to the biochemical laboratory to assess oxidative stress markers. Finally, all the animals were sacrificed with high-dose of anesthesia.

Results: Evaluation of the results revealed that oxidative stress markers were significantly lower, and antioxidant parameters were higher in the experimental groups compared with the control TD group ($p < 0.05$). Histopathologically, we found that tissues were preserved in GD, H, GD + H groups ($p < 0.05$). When we compared the groups among each other, both biochemical and histopathological values in GD + H group showed that the tissue was preserved from oxidative damage, albeit the difference did not reach a level of significance.

Discussion: Several studies have shown that both hypothermia and intermittent reperfusion protect tissue from IR damage in the early period. However, as far as we know there is no study on long-term outcomes of both practices. Our study showed that both hypothermia and intermittent reperfusion alone protect tissue from IR damage in the long term. However, it did not show the superiority of the combination of both methods compared to that of individual application. The advantages of these methods lie in their easy application and cost-effectiveness. We believe that our study will serve as a base for future studies on the subject.

Ovarian torsion is rare in young children and in the prepubertal period. Only about 15% of cases occur in this age group[1,2]. Previously, this patient group underwent oophorectomy due to the concern of malignancy. However, recent studies have shown that ovarian detorsion is an appropriate treatment approach even if the ovary has a blackish-blue color. In the late period after treatment, the ovary may become functional and even regain the function close to normal [3–5].

In ovarian torsion, blood flow to the tissue decreases and ischemia develops. This leads to an increase in lipid peroxidation products as

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well as in lactic acid levels in the tissue. Reperfusion after detorsion leads to the release of too many free oxygen radicals. These oxygen radicals are too high to be eliminated by antioxidative defense mechanisms in the tissue. They cause fatal and permanent damages such as DNA damage. This paradoxical injury mechanism during reperfusion in ischemic tissue is referred to as "Reperfusion Injury" [6–8]. Many techniques and medications such as ozone therapy, various medications, intermittent reperfusion, and hypothermia have been studied to reduce the reperfusion injury in ovarian tissue [2,9–19]. However, these studies were not reflected in clinical practice. Hypothermia is routinely used to prevent ischemia–reperfusion injuries in clinical practice in some tissues such as brain, kidney, heart, and liver [20–23]. It has also been shown that hypothermia experimentally protects against ischemia–reperfusion injuries in many tissues [24–27].

However, there is only one experimental study reporting that hypothermia protects the tissue after ovarian torsion [19]. The effect of gradual reperfusion has been demonstrated in our previous study. We thought that we could achieve more effective results in minimizing reperfusion injuries by combining progressive reperfusion and hypothermia and planned this experimental study accordingly[17,28].

1.1. Material and methods

The study was carried out in the Electrophysiology Research Laboratory following the approval of the Animal Experiments Local Ethics Committee, Adnan Menderes University. The study involved 40 healthy mature female Wistar-Albino rats weighing 250–300 g. The rats were kept in the pre-test wire cages, in the circadian rhythm (12-h light and 12-h dark cycles) and between 20 and 25 °C.

Before starting the surgical procedure, we administered 50 mg/kg intramuscular ketamine to each rat. We divided all rats into five groups: sham (Sh) (n = 8), torsion-detorsion (control TD) (n = 8), intermittent reperfusion (GD) (n = 8), hypothermia (H) (n = 8) and intermittent reperfusion + hypothermia (GD + H) (n = 8). We made a midline abdominal incision. In control TD, H, GD, GD + H groups, we rotated the left ovarian and tuba vessels in the clockwise direction three times (1080°) and fixed them to the abdominal wall. The abdominal wall was closed using a 5–0 non-absorbable suture. After a waiting period of 30 h, rats were anesthetized and a laparotomy was reperformed. Detorsion was applied to ovarian mesentery in control TD group. In GD group, detorsion was performed for a single 360° turn in the ovarian mesentery, followed by a 5 min waiting period. In our previous experimental studies, we showed that with short interval controlled reperfusions in the ovarian torsion model, the ovarian tissue was protected from reperfusion damage in the short term and the most effective reperfusion time was 5 s [17]. Therefore, in this study we used 5 s, the most effective time and when we compared the control TD group and the PC group (interval controlled reperfusions), we once again confirmed that the ischemia–reperfusion injury was low. This procedure was repeated until complete detorsion was obtained (twice). H group was subjected to hypothermia 30 min before and during the procedure. Left ovaries were placed in ice bag and cold application was started. With the help of a digital thermometer (Calibrated TFA 30.1018-Digital Probe Thermometer POCKET-DIGITEMP) placed in the parenchyma, the temperature of the parenchyma of ovarian tissue was measured and remained constant at 4 °C. It was ensured that there was no hypothermia in the rats, but only hypothermia in the tissue. We applied detorsion on the ovarian mesentery and then waited for 30 min under the effect of hypothermia. GD + H group underwent hypothermia with an ice pack 30 min before the procedure, while detorsion was performed in the ovarian mesentery for a single turn (360°) followed by a waiting period (5 min). This procedure was repeated with 5 min waiting periods until complete detorsion was obtained (twice). Hypothermia was started 30 min before the procedure and applied for 30 min during and 30 min after the procedure. After hypothermia, the ovaries were left to normal body temperature during the reperfusion. In Sh group, we waited for 30 h following the torsionless mobilization of the ovary under anesthesia. Rats were sacrificed 60 days after the procedure. There is no experimental study on long-term results of ovarian torsion. They evaluated long-term results of study on male gonads "testis" [28,29]. So we decided to wait for 60 days to evaluate long-term results of our experimental study on female rats. At the end of 60 days, oxidative stress parameters were evaluated separately in both blood and tissue. In order to evaluate long-term outcomes, we took intracardiac blood samples from all rats 60 days after the detorsion to see malonyldialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities. We removed the left ovary and separated the tissue to look into MDA, MPO, SOD, GPx, CAT levels. We sent some of them to pathology for pathological examination. At the end of the procedure, all animals were sacrificed with high-dose of anesthesia.

1.2. Biochemical analysis

We used the Myeloperoxidase Activity Colorimetric Assay Kit (Bio Vision, K744–100) for MPO assessment in accordance with the appropriate procedure according to the manufacturer's instructions [30]. MDA was measured by the Lipid Peroxidation Colorimetric/Fluorometric Assay Kit (BioVision, K739–100, USA) in accordance with the procedure according to the manufacturer's instructions [31]. We kept it at −20 °C until the samples were taken. We also used a catalase (CAT) kit (Catalase Activity Colorimetric/ Fluorometric Assay Kit, BioVision, K773–100). We kept the kit at +4 °C until the samples were taken. We followed the appropriate procedure according to the manufacturer's instructions [32]. GSHPx- Kit (Glutathione Peroxidase Activity Colorimetric Assay Kit, BioVision, K762–100) was preferred for GPx levels. Samples were stored at −20 °C until the day of collection. Measurements were performed following the appropriate procedure according to the manufacturer's instructions [33]. SOD activity was measured by Superoxide Dismutase (SOD) Activity Colorimetric Assay Kit −100 assays (K335–100, Bio Vision). Measurements were performed using the appropriate procedure according to the manufacturer's instructions [34]. All parameters were read in the ELISA plate reader at an appropriate wavelength, a calibration graph was drawn using the standards, and the results were calculated.

1.3. Histopathologic examinations

The specimens were fixed in 10% formalin solution and then embedded in paraffin. The tissue samples were stained with hematoxylin and eosin (H&E). Specimens were evaluated by a single pathologist specialized in the fields of gynecology and genitourinary system in a blinded fashion. Ovarian tissue damage was graded as Celik et al. [11] graded: Grade 0, normal; Grade 1, mild edema and vascular congestion, no hemorrhage and leukocytic infiltration; Grade 2, moderate edema and vascular congestion, no hemorrhage and leukocytic infiltration; Grade 3, severe edema and vascular congestion, minimal hemorrhage and leukocytic infiltration; Grade 4, severe edema, and vascular congestion, presence of hemorrhage and leukocytic infiltration.

1.4. Statistical analysis

All data were compared statistically using the One-way ANOVA and Tukey's multiple comparison test (SPSS 15.0; SPSS, Chicago, IL). $p < 0.05$ was considered statistically significant.

2. Results

All rats were maintained during the experiment. Sham group values were compared with values of other groups. The oxidative stress parameters in tissue and serum are shown in Table 1. We found that

Table 1
Comparison of all parameters in the tissue and serum with the control TD group. Biochemical evaluation of (experimental and control) ovary and serum samples with mean and standard deviation scores.

	SOD (%)		MPO (mU/mL)		MDA (nmol/g tissue)		CAT (mU/mL)		GPx (mU/mL)	
	OVARY	SERUM	OVARY	SERUM	OVARY	SERUM	OVARY	SERUM	OVARY	SERUM
Control TD	62.23 ± 3.94	43.43 ± 6.58	556.63 ± 36.19	80.13 ± 8.81	163.63 ± 12.15	51.38 ± 5.10	274.19 ± 11.51	93.91 ± 9.92	824.25 ± 43.94	303.62 ± 10.68
Sham	10.98 ± 1.99**	7.70 ± 2.09**	142.25 ± 13.77**	34.00 ± 6.39**	61.38 ± 9.32**	14.63 ± 3.96**	59.85 ± 4.11**	41.21 ± 3.27**	538.12 ± 29.40**	175.75 ± 13.31**
GD	55.08 ± 5.74*	38.91 ± 5.09ns	501.38 ± 57.34*	73.88 ± 14.99ns	151.63 ± 14.42ns	42.38 ± 4.98*	258.1 ± 10.67*	84.19 ± 7.30*	741.25 ± 35.52*	281.25 ± 11.11*
H	39.88 ± 5.00**	25.44 ± 3.61**	235.63 ± 27.17**	55.25 ± 5.82**	140.75 ± 21.73*	42.00 ± 5.01*	255.21 ± 14.65*	73.2 ± 6.11**	750.37 ± 50.07*	246.75 ± 22.75**
GD + H	33.71 ± 5.64**	17.46 ± 3.00**	231 ± 22.34**	51.75 ± 8.21**	127.50 ± 10.98**	34.13 ± 9.13**	232.12 ± 15.51**	69.31 ± 7.24**	685.12 ± 89.76**	252.12 ± 12.25**

*p < 0.05 Compared with Control group, **p < 0.01 Compared with Control group.
ns p > 0.05 Compared with Control group.(All data obtained after comparison of all groups with control TD group in both tissue and blood. If p < 0.05 *, if p < 0.01 ** symbols were usEd.)

oxidative stress parameters such as SOD, MPO, and MDA values were significantly lower in all groups compared to the control TD group. In the combined group, this value was below p < 0.01. The comparison among the groups revealed no statistically significant difference.

We found that MDA, a lipid peroxidation product, was significantly increased in the control TD group. Tissue MDA levels were significantly decreased in both H and GD groups (p < 0.05). As for GD + H group, a more significant decrease was observed in MDA levels in both tissue and serum samples (p < 0.05) (Fig. 4). However, we found no statistically significant difference between the groups.

Both the tissue and serum levels of SOD and MPO (Fig. 2-3), effective oxidant parameters to evaluate IR damage, Cat and GPx, (Fig. 5-6), effective antioxidant parameters to evaluate IR damage were significantly lower in all groups compared with that of the control TD group. When the tissue and serum samples of all these parameters were compared, tissue levels were lower. However, no statistically significant difference was found.

Table 2 shows the histopathological scoring values. In terms of tissue damage scores, all groups were maintained according to the control TD group. (p < 0.05) It was found that hypothermia and combined application provided higher protection. (p < 0.01) (Fig. 1-7).

3. Discussion

Tissue changes occurred in reperfusion injury after detorsion of ovarian torsion manifested itself in the late period. Although the tissue looks black, it can maintain its vigor and fertility in the long term after detorsion[4,5]. We aimed to protect the ovary from reperfusion injuries and evaluated the late findings in order to see its long-term effects on tissue. In our study, we statistically demonstrated the superiority of gradual reperfusion (p < 0.05), hypothermia (p < 0.01), and the combination of both measures (p < 0.01) against the control TD group. However, we did not find a statistical difference among the groups.

In ovarian torsion, blood flow to the tissue ceases completely or partially, resulting in tissue damage. This depends on the degree and duration of torsion of the vascular pedicle. The most important parameters to prevent late ovarian damage are the timing of emergency surgery and the degree of torsion [35]. Earlier studies have shown that there is partial blood flow in the ovary by Doppler USG even when rotated 360 degrees. Therefore, we rotated the ovaries three times to ensure that the blood flow was completely discontinued [36]. In a study by Taskin et al., the ovaries were left ischemic for 4–8–12–18–24–36 h, and the most severe injury was found in ovaries that were left ischemic for ≥24 h. Therefore, we left the ovaries ischemic for 30 h in our study [37].

Torsion causes the tissue to remain ischemic. When the blood flow is restored to the tissue by detorsion, free oxygen radicals are formed in the tissue in large amounts. As a result, tissue damage increases. Preventing or reducing the production of free oxygen radicals may be

Table 2
The histopathological scoring values. Pathology scores of tissue with mean and standard deviation scores.

	Scores
Control TD	3.14 ± 0.90
Sham	0 ± 0**ns
GD	2.14 ± 0.69*
H	0.29 ± 0.49**
GD + H	0.86 ± 0.90**

(Mean data obtained from all histopathological evaluations result. If p < 0.05 *, if p < 0.01 ** symbols were usEd.)
* p < 0.05 Compared with Control TD group.
** p < 0.01 Compared with Control TD group.
ns p > 0.05 Compared with Control TD group.

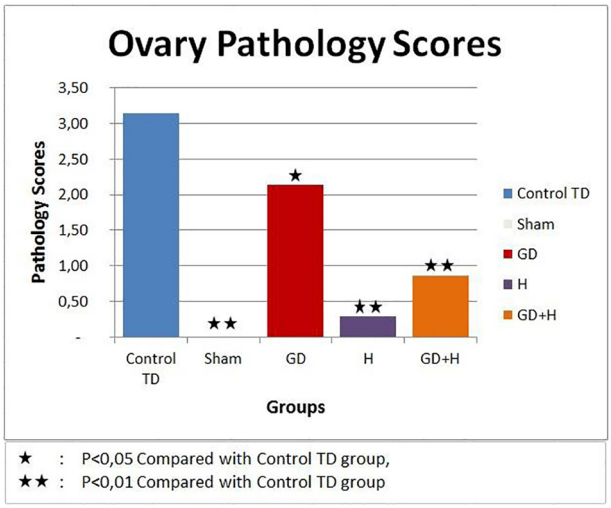

Fig. 1. The histopathological scoring values. (Histopathologically generated histogram graph according to control TD and sham groups. If $p < 0.05$ *, if $p < 0.01$ ** symbols were usEd.)

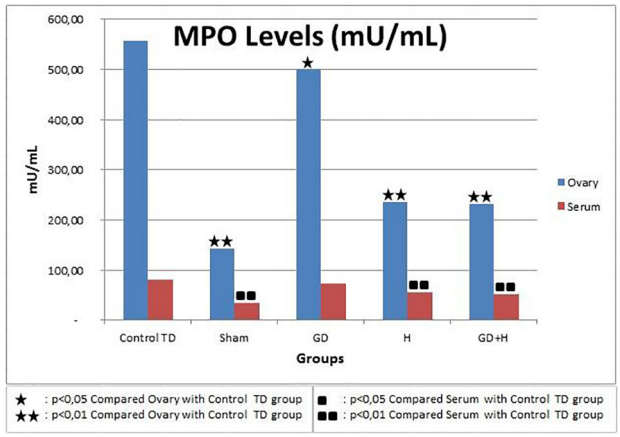

Fig. 3. MPO average levels of tissue and serum. (Comparison of MPO values in tissue and serum with control TD group. Tissue and serum samples were evaluated among themselves. If $p < 0.05$★, if $p < 0.01$ ★★ symbols were used in histogram for tissue. For blood samples, if $p < 0.05$, $p < 0.01$ symbols were usEd.)

a method for the control of tissue damage. In an ovarian-torsion model we previously created, short-term outcomes revealed that more controlled oxygen flow to the tissue could be achieved by gradual detorsion of the ovarian pedicle, resulting in reduced free radical production and prevented tissue damage [17]. It remains the only study showing the long-term outcomes of the effect of gradual reperfusion on protecting the ovary from reperfusion injury. In this study, we think it would also be valuable to evaluate the long-term outcomes of gradual detorsion and could shed light on clinical applications. Long-term outcomes of our study showed that the GD group protected the tissue both biochemically and histopathologically compared to the control TD group ($p < 0.05$) (Fig. 1).

We evaluated the free oxygen radicals separately in tissue and blood samples and found no statistical difference. We think that the products formed in the tissue are reflected in the blood serum samples as a result of the ongoing regeneration process after reperfusion. Likewise, late outcomes revealed no statistical difference in the oxidative parameters between tissue and blood samples. However, blood and tissue values were consistent between the groups. In other words, both blood and tissue values were parallel for each group. (Table 1).

Free oxygen radicals after reperfusion in tissue may cause DNA damage, protein denaturation, and peroxidation of lipids, which are primarily responsible for tissue damage.

Malonyldialdehyde, the final product of lipid peroxidation, is a good biochemical parameter to determine increased free oxygen radicals in post-ischemic tissue [38]. MDA levels were significantly higher in tissue and blood samples in all study groups than those of the SH group, but lower than those of the control TD group. Comparison of H, GD and GD + H groups revealed no superiority to each other statistically. (Fig. 4).

We see that hypothermia has both experimental and clinical use in various fields in the prevention of ischemia–reperfusion injuries in tissue [20–27]. It is known that hypothermia reduces both the amount of free oxygen radicals formed in tissue and the tissue damage histopathologically. Another experimental study showed that vasoconstriction in the blood vessels leading to locally

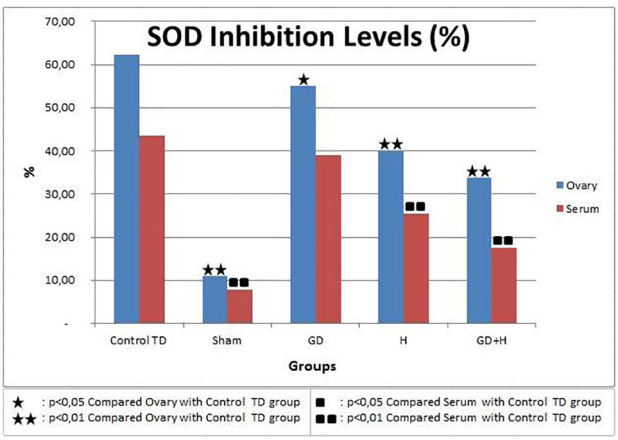

Fig. 2. SOD average levels of tissue and serum. (Comparison of SOD values in tissue and serum with control TD group. Tissue and serum samples were evaluated among themselves. If $p < 0.05$★, if $p < 0.01$ ★★ symbols were used in histogram for tissue. For blood samples, if $p < 0.05$, $p < 0.01$ symbols were usEd.)

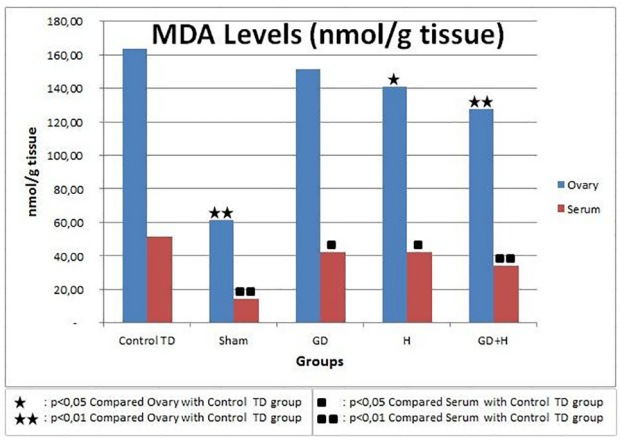

Fig. 4. MDA average levels of tissue and serum. (Comparison of MDA values in tissue and serum with control TD group. Tissue and serum samples were evaluated among themselves. If $p < 0.05$★, if $p < 0.01$ ★★ symbols were used in histogram for tissue. For blood samples, if $p < 0.05$, if $p < 0.01$ symbols were used.

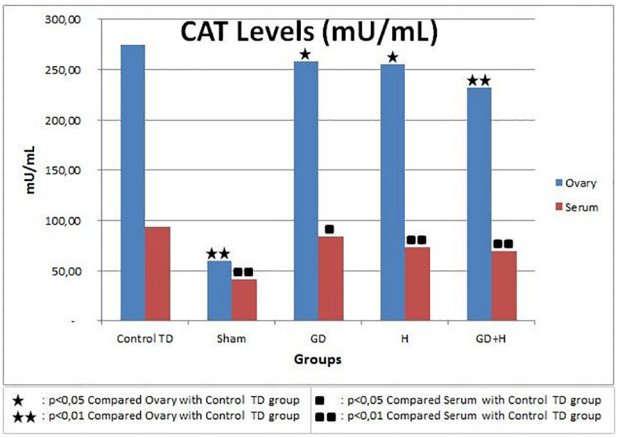

Fig. 5. CAT average levels of tissue and serum. (Comparison of CAT values in tissue and serum with control TD group. Tissue and serum samples were evaluated among themselves. If $p < 0.05$★, if $p < 0.01$ ★★ symbols were used in histogram for tissue. For blood samples, if $p < 0.05$, $p < 0.01$ symbols were usEd.)

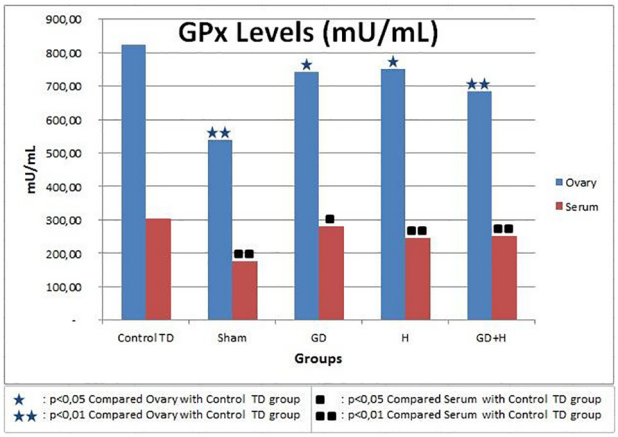

Fig. 6. GPx average levels of tissue and serum. (Comparison of GPx values in tissue and serum with control TD group. Tissue and serum samples were evaluated among themselves. If $p < 0.05$★, if $p < 0.01$ ★★ symbols were used in histogram for tissue. For blood samples, if $p < 0.05$, if $p < 0.01$ symbols were usEd.)

damaged tissue protected the tissue from sudden reperfusion injury [39]. Locally applied hypothermia slows down the metabolism in the region and reduces the oxygen consumption of the tissue and the amount of carbon dioxide to be formed. It suppresses inflammatory mechanisms and reduces free oxygen radical production. As a result, we think that the damage to the tissue after ischemia–reperfusion can be reduced by local hypothermia application. In their study on early effects of hypothermia on the ovary, Turk et al. observed a decrease in free oxygen radicals in the tissue but did not find a statistical difference histopathologically [19]. We found a difference between H and GD + H groups in terms of both biochemical and histopathological preservation of tissue from damage compared to the control TD group. ($p < 0.01$) (Table 1) In our opinion, the reason for this difference between the two studies is that they examined early and late responses in the tissue. As we looked at the late outcomes, our study revealed the reflections of the tissue more clearly. In other words, we have seen that the protective effect of hypothermia on the tissue is more clearly reflected in the late results.

The histopathological results obtained with hypothermia were significant in H, GD + H, and GD groups. However, they were more significant in H and GD + H groups ($p < 0.01$) than the GD group ($p < 0.05$) (Fig. 1). Biochemical data indicated that SOD, MPO and MDA values in serum and tissue samples were more significant in the combined group. Thus, our experimental study demonstrated that hypothermia, especially the combined therapy (GD + H), provides more effective protection in tissue. Both of the methods we practiced are actually based on the same logical principle where the sudden blood flow reaching the tissue during reperfusion and the free oxygen radicals responsible for the main tissue damage in this process can be reduced and thus the tissue can be preserved with less damage. Both methods are easy to implement and can easily be integrated into the clinic. In addition, if they enter clinical applications, there is no additional financial burden and no additional risk to the operations to be performed. Whether it is open or laparoscopic surgery, it is very easy to apply and cost-free.

In conclusion, especially the late outcomes in our study revealed that gradual detorsion, cold application, and combined application can protect the tissue from reperfusion injury. We think that our study may provide insight into clinical applications for the use of these very easy and low-cost methods.

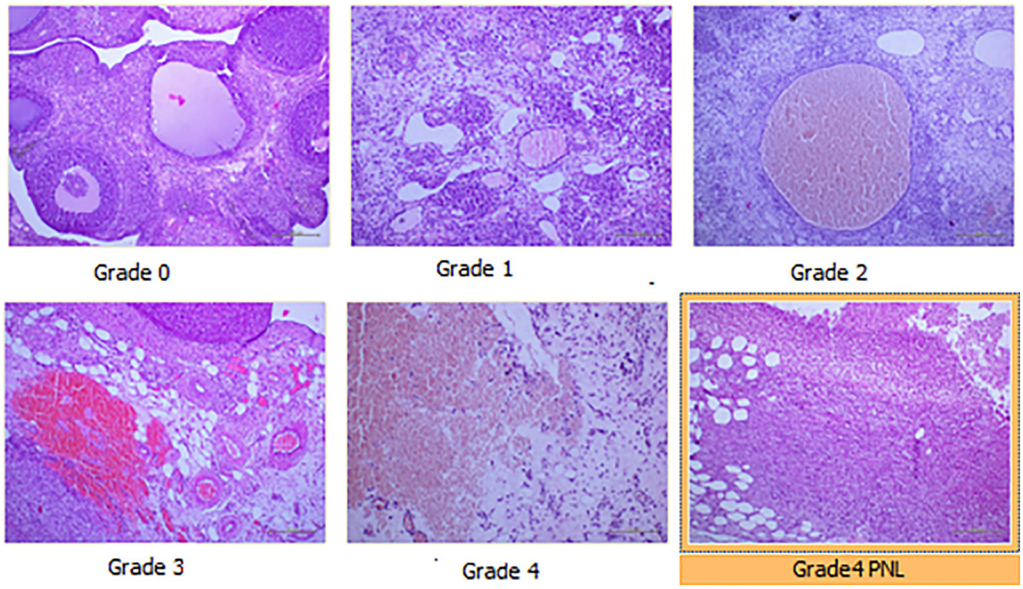

Fig. 7. Images of ovary tissue damage H&E, ×200. Grade 0 normal Grade 1 Mild edema vascular congestion Grade 2 Edema vascular congestion Grade 3 Edema vascular congestion hemorrhage Grade 4 Edema vascular congestion hemorrhage Grade 5 Edema vascular congestion hemorrhage leukocyte infiltration.

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