ORIGINAL ARTICLE

Hyperbaric oxygen and metformin treatment in ovarian torsion preserves ovarian reserve

El tratamiento con oxígeno hiperbárico y metformina en la torsión ovárica preserva la reserva ovárica

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Abstract

Objective: *Ovarian torsion is a gynecological emergency that reduces ovarian reserve in reproductive-aged women. This study aimed to evaluate the effects of metformin and hyperbaric oxygen therapy (HBOT) on ovarian reserve after ovarian torsion.* **Methods:** *Forty female Wistar-Albino rats were divided into five groups: control, torsion/detorsion (T), torsion/detorsion + metformin (TM), torsion/detorsion + HBOT (THBO), and torsion/detorsion + metformin + HBOT (TMHBO). Rats in the experimental groups underwent 2 h of unilateral ovarian torsion followed by detorsion. Metformin (50 mg/kg/day) and HBOT (100% oxygen at 2.4 atm for 2 h/day) were administered for 14 days post-detorsion. Serum AMH levels, tissue AMH expression, and ovarian follicle counts were evaluated.* **Results:** *In the torsion group, ovarian histology was disrupted, follicle numbers decreased, TUNEL-positive cells increased, and both serum and tissue AMH levels were reduced. The TM, THBO, and TMHBO groups demonstrated improvements in follicle numbers, TUNEL-positive cells, and AMH levels compared to the torsion group. Among them, TMHBO exhibited the best numerical outcomes, but no significant superiority was observed among TM, THBO, and TMHBO groups.* **Conclusions:** *Both metformin and HBOT were effective in preserving ovarian reserve following ovarian torsion. These therapies may have potential as protective treatments in gynecological emergencies involving ovarian torsion.*

Keywords: *Ovarian torsion. Metformin. Hyperbaric oxygen therapy. Ovarian reserve.*

Resumen

Objetivo: *La torsión ovárica es una emergencia ginecológica que afecta la reserva ovárica en mujeres en edad reproductiva. Este estudio evaluó los efectos de la metformina y la terapia de oxígeno hiperbárico (TOH) en la reserva ovárica tras una torsión ovárica.* **Métodos:** *Cuarenta ratas Wistar-Albino hembras se dividieron en cinco grupos: control, torsión/destorsión (T), torsión/destorsión + metformina (TM), torsión/destorsión + TOH (TTOH) y torsión/destorsión + metformina + TOH (TMTOH). Los grupos experimentales fueron sometidos a torsión ovárica unilateral durante 2 horas, seguida de destorsión. Se administraron metformina (50 mg/kg/día) y TOH (oxígeno al 100%, 2.4 atm, 2 h/día) durante 14 días. Se midieron los niveles séricos y tisulares de hormona antimulleriana y el recuento de folículos ováricos.* **Resultados:** *En el grupo de torsión, la histología ovárica estaba alterada, el número de folículos disminuyó, las células TUNEL positivas aumentaron y los niveles de hormona antimulleriana se redujeron.*

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TM, TTOH y TMTOH mejoraron estos parámetros en comparación con el grupo T. Aunque TMTOH mostró los mejores resultados numéricos, no se observó superioridad significativa entre los grupos tratados. **Conclusiones:** *La metformina y la TOH demostraron ser eficaces para preservar la reserva ovárica tras una torsión ovárica, lo que destaca su potencial terapéutico en estas emergencias ginecológicas.*

Palabras clave: *Torsión ovárica. Metformina. Terapia de oxígeno hiperbárico. Reserva ovárica.*

Introduction

Gynecological emergencies such as ovarian torsion, which occurs when the ovary, fallopian tube, or both rotate completely or partially around the vascular axis, can occur at rates ranging from 2-5% to 7-4%[1,2]. Risk factors for ovarian torsion include ovarian cysts, polycystic ovary syndrome, previous ovarian torsion episodes, and previous pelvic surgeries[3]. Since there is no specific clinical, laboratory, or radiological finding of ovarian torsion and patients with the condition are typically admitted late, the torsion observed during surgery is used to make a definitive diagnosis[4]. Immediate adnexal detorsion is the recognized treatment for ovarian torsion.

Detorsion should be performed as soon as possible before ovarian torsion causes a serious decrease in follicle reserve or loss of the ovary[5]. However, this intervention causes reperfusion injury after detorsion in ovaries that have suffered ischemic damage due to torsion. Thus, ovarian torsion and detorsion give rise to ischemia-reperfusion (I/R) damage, which leads to biochemical, histological, and morphological changes in the ovarian tissue[6]. During the reperfusion period, the entry of molecular oxygen into the cell rapidly causes the formation of reactive oxygen species (ROS). Excessive production of ROS causes lipid peroxidation, DNA damage, apoptosis, and protein dysfunction[7]. Therefore, in addition to surgical treatment in ovarian torsion, antioxidant and anti-inflammatory treatment is also recommended to prevent the resulting I/R damage[8]. Numerous pharmacological interventions (such as curcumin, lipoic acid, melatonin, vitamin C, lycopene, erdosteine, methylprednisolone, and verapamil) have been studied to prevent I/R injury in ovarian torsion[9-16]. However, new treatments that provide good protection against inflammation and oxidative stress and minimize adverse effects are desired.

The use of 100% oxygen at pressures greater than one atmosphere is known as hyperbaric oxygen therapy (HBOT), and it has been used in medicine for more than 30 years[17]. Increasing the partial oxygen pressure in the tissues is the goal of HBOT. Thus, by boosting the synthesis of numerous growth factors and cytokines, elevated tissue oxygen levels promote angiogenesis. Due to this effect, HBOT is used in the treatment of decompression sickness, carbon monoxide poisoning, diabetic foot, osteomyelitis, and ischemic wounds[18]. HBOT is known to be an effective treatment method in ischemia-reperfusion injury and has anti-inflammatory and antioxidant effects[19].

A biguanide agent, metformin, is used as a first-line drug in the treatment of type 2 diabetes[20]. In addition to its antidiabetic effect, metformin has been shown to have antioxidant, anti-inflammatory, and antiproliferative properties[21,22]. Metformin protects the heart, brain, and testes from I/R damage[23-25]. It has also been reported that metformin can increase ovulation in women[26], and improve ovarian I/R damage[27].

In the clinical evaluation of fertility in women, the number of follicles in the ovaries and the levels of anti-Müllerian hormone (AMH) are important. Ovarian reserve is measured using AMH secreted from preantral ovarian follicles[28]. Detorsion surgery has been shown to reduce AMH[29]. This situation shows that conservative treatment of ovarian torsion alone cannot protect the ovarian reserve. In this context, new treatment approaches aimed at overcoming I/R damage in ovarian torsion are needed. The aim of this study was to evaluate the effects of HBOT and metformin treatment on the preservation of ovarian reserve in I/R damage resulting from ovarian torsion and detorsion in rats.

Methods

Animals

This experimental study was conducted at the Department of Gynecology and also at the Department of Histology and Embryology, Faculty of Medicine, Erciyes University, between December 2021 and June 2022. Ethical approval was obtained from the ethics committee of the Faculty of Medicine, Erciyes University, and the study was conducted in accordance with the World Animal Rights Declaration. The G power program was used to calculate the sample size of the study. According to the one-way ANOVA

test analysis with 95% confidence (1-α), 95% test power (1-β), and d = 0.5 effect size, the number of samples to be taken in each group was determined as seven. Considering the possibility of losing 10% of the rats during surgical interventions, eight rats per group were included in the study. Eight-week-old female Wistar-Albino rats weighing 180-260 g, obtained from the Erciyes University Experimental Applications and Research Center, were used in the study. The rats were kept in a room with 12 h of light and 12 h of darkness, 25 ± 2 °C, and a humidity rate of around 40-50%, and were fed a balanced diet and unlimited water.

The 40 female Wistar-Albino rats included in the study were randomly divided into five groups, each containing eight rats. Control Group: No procedure was applied to this group. Torsion/detorsion group (T): The rats in this group were subjected to unilateral ovarian torsion for 2 h, and then the ovary was detorsioned and returned to its anatomical position. Torsion/detorsion + metformin group (TM): The rats in this group were subjected to unilateral ovarian torsion for 2 h, and then the ovary was detorsioned and returned to its anatomical position. Four hours after the detorsion procedure and daily for the following 13 days, the rats were treated with 50 mg/kg oral metformin (glucophage 50 mg tablet; Merck Pharmaceutical Industry Inc., Istanbul, Turkey). Torsion/detorsion + hyperbaric oxygen therapy group (THBO): The rats in this group were subjected to unilateral ovarian torsion for 2 h, and then the ovary was detorsioned and returned to its anatomical position. Four hours after the detorsion procedure and daily for the following 13 days, the rats were treated with HBOT by breathing 100% O_2 at 2.4 atmospheres pressure for 2 h in a hyperbaric chamber. Torsion/detorsion + metformin + hyperbaric oxygen therapy group (TMHBO): The rats in this group were subjected to unilateral ovarian torsion for 2 h, and then the ovary was detorsioned and returned to its anatomical position. HBOT and metformin were administered to rats 4 h after the detorsion procedure and for the next 13 days. HBOT was applied in a hyperbaric chamber at 2.4 atmospheres of pressure with 100% O_2 inhalation for 2 h. Metformin was administered by oral gavage at 50 mg/kg/day. On the 14th day of the study, all rats were sacrificed, and tissue samples and blood were collected.

Surgical procedure

Before the operation, each rat was given 50 mg/kg cefazolin sodium intramuscularly as a prophylactic. All rats were given 50 mg/kg ketamine (Ketalar; Parke Davis, Eczacibasi, Istanbul, Turkey) and 7 mg/kg xylazine hydrochloride (Rompun; Bayer AG, Leverkusen, Germany) intraperitoneally to induce general anesthesia. The rat was placed in the dorsal position. The operation area was shaved, cleaned, and sterilized with 10% povidone-iodine. The surgical field was covered with sterile compresses. After opening the abdomen by making a 2.5 cm midline laparotomy incision under sterile conditions, the large intestines were gently separated, and the left ovary was made visible. The ovary was rotated 720 degrees and fixed to the abdominal side wall with 4.0 Vicryl sutures. After 2 h of waiting, the ovary was separated from the abdominal side wall, and detorsion was carried out. The abdominal incision was closed with 3.0 Vicryl suture. Following this procedure, cefazolin sodium was administered intramuscularly to the rats at a dose of 50 mg/kg daily for 3 days. None of the rats died following the surgery procedure[30].

Histopathological examination

Ovarian tissues were fixed in 10% formaldehyde solution for 48 h and then embedded in paraffin blocks after routine histological procedures. Serial sections of 5 μm thickness were taken with a microtome. Sections were stained with Hematoxylin and Eosin (H&E) and Masson Trichrome (MT) and evaluated histopathologically. Furthermore, primordial, primary, preantral, secondary, and tertiary follicles were counted under a light microscope (Olympus® Inc. Tokyo, Japan)[30].

Immunohistochemical analysis

AMH staining was performed immunohistochemically to evaluate ovarian reserve. Sections taken on slides with Poly-L-lysine were stained according to the procedure recommended by the Avidin-Biotin Peroxidase Complex (ABC) (TP-125- HL, Thermo Ultravision Detection System, Fremont, USA) kit manufacturer. Deparaffinized sections were washed with phosphate buffered solution (PBS, pH 7.4) (Afg Bio-science 729350) for 3 × 5 min. To block tissue endogenous peroxidase activity, sections were incubated with 3% H_2O_2 prepared in methanol for 12 min. The sections were incubated in 10% normal goat serum at room temperature for 10 min to prevent non-specific antigenic binding and were then incubated with AMH primary antibody (1:150; anti-AMH Antibody, sc-1667529,

Santa Cruz Biotechnology, Oregon, ABD) at 4°C overnight. The slides were then incubated with ready-to-use biotinylated secondary antibody at room temperature for 15 min. Following this, the sections were incubated with Streptavidin Peroxidase conjugate at room temperature for 15 min, and the staining process was terminated after applying DAB (3,3'-diaminobenzidinetetrahydrochloride) (DAB Plus substrate system, Thermo Scientific, Fremont, USA) substrate for 5 min. Sections counterstained with Gill hematoxylin were covered with a coverslip. To evaluate AMH expression in ovarian tissue, images obtained from an Olympus BX 51 light microscope were analyzed using the Image J software program[30].

TUNEL assay for apoptosis

Terminal Deoxynucleotide-Transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) method was applied for apoptosis. ApopTag® Fluoresce In Situ Apoptosis Detection Kit (EMD Millipore, Darmstadt, Germany) was used for staining, and staining steps were performed according to the kit procedure. A 5 μm sections taken on polylysine-coated slides were deparaffinized and washed 3 times with PBS. Slides were incubated with proteinase K for 15 min and then washed with distilled water. Slides were treated with 3% H_2O_2 for 10 min to minimize endogenous peroxidase activity. Then, the slides were washed with PBS 3 times for 5 min and were then incubated with TdT at 37 °C in a humid and dark environment for 1 h. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. All procedures were performed in a humidity chamber. The TdT step was omitted for the negative control. Slides were evaluated using a fluorescent microscope (Olympus BX51, Tokyo, Japan). After the immunofluorescent staining procedure, positively stained apoptotic cells in the obtained slides were counted using the Image J software program[31].

Enzyme-linked immunosorbent assay (ELISA)

Serum AMH levels were analyzed by the ELISA method. Serum AMH levels were measured using an ELISA kit (Rat ELISA kit, 201-11-1246, Baoshan District, Shanghai, China) according to the manufacturer's instructions. The quantities were determined at 450 nm in a micro ELISA reader (BioTek ELx800)[32].

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) for Windows 24.0 program. One-way Analysis of Variance (ANOVA) was used for comparisons between control and experimental groups in normally distributed variables, and the Tukey test was used for *post hoc* analysis. For statistical analysis results, a $p < 0.05$ was considered significant[32].

Results

Histological findings

The ovaries in the control group showed normal histological structure. In the torsion group, the general structure of the ovary was irregular, and there was hemorrhage, edema, and inflammation. Irregularity in the structures of follicles at different maturation stages, an increase in granulosa cells with pyknotic nuclei in secondary and tertiary follicles, and an increase in the number of atretic follicles were observed in the torsioned ovary. There was a significant improvement in the follicular structures of the ovary in the TM, THBO, and TMHBO groups compared to the torsion group (Fig. 1).

The mean follicle numbers are given in table 1. It was determined that the primordial, primary, preantral, secondary, and tertiary follicle numbers in the torsioned ovarian tissue were significantly lower than the follicle numbers in the control group. The follicle numbers in the TM, THBO, and TMHBO groups were significantly improved compared to the follicle numbers in the torsion group. However, no significant difference was found between the follicle numbers in the TM, THBO, and TMHBO groups.

Immunohistochemical findings

AMH expression in ovarian tissue is given in table 2 and figure 2. AMH expression was observed in primary, preantral, secondary, and tertiary follicles in both control and experimental groups. In addition, AMH expression in tertiary follicles was lower than in other follicles. AMH expression in all follicles in the

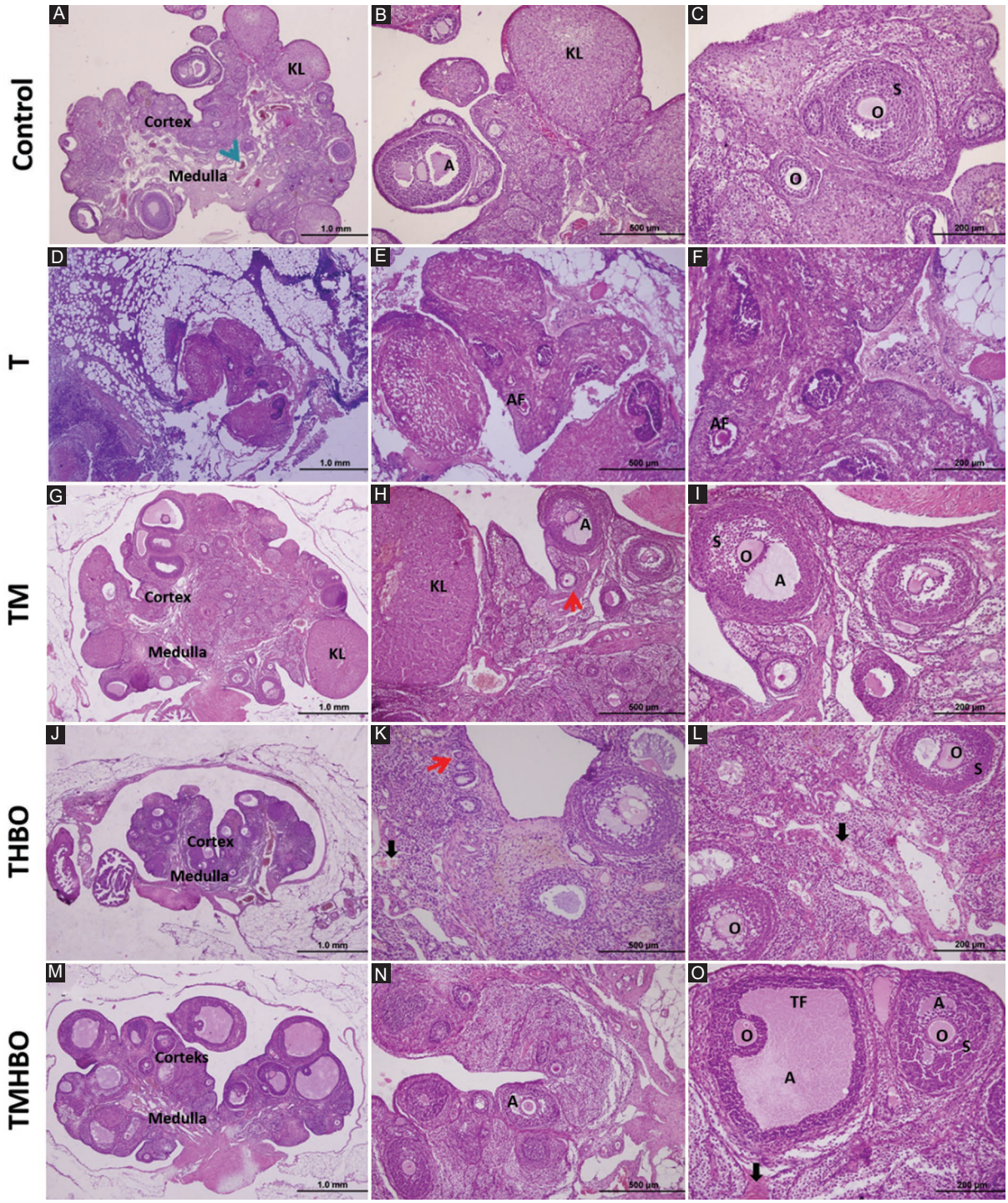

Figure 1. *Ovarian section view of control and experimental groups. Control group* **(A-C)**, *T: torsion/detorsion group* **(D-F)**; *TM: torsion/detorsion+metformin group* **(G-I)**; *THBO: torsion/detorsion+hyperbaric oxygen therapy group* **(J-L)**; *TMHBO: torsion/detorsion+metformin+hyperbaric oxygen therapy group* **(M-O)**. *S: secondary follicle; TF: tertiary follicle; PA: preantral follicle; primary follicle (red arrow), oocyte* **(O)**, *atretic follicle* **(AF)**, *antrum* **(A)**, *zona pellucida (green arrow), hemorrhage (black arrow). Hematoxylin & Eosin staining. Scale bars: 1.0 mm* **(A, D, G, J, M)**; *500 µm* **(B, E, H, K, N)**; *200 µm* **(C, F, I, L, O)**.

torsion group was significantly lower than in the control group. AMH expression in TM, THBO, and TMHBO groups was significantly higher than in the torsion group. Although the greatest numerical improvement was in the TMHBO group, it was not significantly different from the TM and THBO groups.

Table 1. Comparison of follicle numbers of control and experimental groups

Follicle types	Control	T	TM	THBO	TMHBO	p
Primordial Follicle	154.25 ± 18.40[a]	41.00 ± 9.44[b]	78.00 ± 12.60[c]	80.50 ± 8.29[c]	94.25 ± 10.68[c]	0.001
Primary Follicle	77.63 ± 7.59[a]	27.63 ± 10.96[b]	61.75 ± 12.36[c]	67.25 ± 11.74[ac]	71.38 ± 6.02[ac]	0.001
Preantral Follicle	50.88 ± 7.60[a]	26.00 ± 7.19[b]	45.00 ± 10.82[a]	49.63 ± 9.25[a]	49.75 ± 10.55[a]	0.001
Secondary Follicle	27.13 ± 5.22[a]	8.50 ± 2.77[b]	20.88 ± 3.56[c]	19.63 ± 3.58[c]	22.13 ± 5.46[ac]	0.001
Tertiary Follicle	12.50 ± 2.44[a]	4.50 ± 2.33[b]	7.38 ± 1.92[bc]	7.75 ± 1.90[c]	9.38 ± 1.84[c]	0.001

One way ANOVA. Different letters in the row indicate statistically significant difference.
T: torsion/detorsion group; TM: torsion/detorsion + metformin group; THBO: torsion/detorsion + hyperbaric oxygen therapy group; TMHBO: torsion/detorsion + metformin + hyperbaric oxygen therapy group. "The same letters on the same line indicate similarity between groups and different letters indicate the difference between groups."

Table 2. Anti-mullerian hormone (AMH) expression in follicles of control and experimental groups

Follicle types	Control	T	TM	THBO	TMHBO	p
Primary Follicle	157.70 ± 22.79[a]d	123.84 ± 23.47[bc]	144.11 ± 22.74d[c]	137.15 ± 16.27[c]	158.85 ± 26.53[a]	0.001
Preantral Follicle	159.89 ± 23.31[ac]	129.10 ± 24.6[b]	172.18 ± 14.09[c]	142.05 ± 13.86[ab]	172.71 ± 24.58[c]	0.001
Secondary Follicle	154.10 ± 24.40[a]	122.41 ± 23.42[b]	131.94 ± 24.92[b]	129.37 ± 25.00[b]	164.11 ± 24.17[a]	0.001
Tertiary Follicle	121.09 ± 18.55[a]	115.48 ± 14.96[a]	127.46 ± 29.76[a]	119.61 ± 18.34[a]	154.82 ± 28.02[b]	0.001

One-way ANOVA. Different letters in the row indicate a statistically significant difference.
AMH: anti-mullerian Hormone; T: torsion/detorsion group; TM: torsion/detorsion + metformin group; THBO: torsion/detorsion + hyperbaric oxygen therapy group; TMHBO: torsion/detorsion + metformin + hyperbaric oxygen therapy group. "The same letters on the same line indicate similarity between groups and different letters indicate the difference between groups."

TUNEL findings

TUNEL-positive cells were observed in the cortex and medulla layers of the ovary of all control and experimental groups. There was also an increase in TUNEL-positive cells in tertiary and secondary follicles. The highest number of apoptotic cells among all experimental groups was in the T group. The number of TUNEL-positive cells in the ovary tissues of the torsion group was statistically significant when compared with the number of TUNEL-positive cells obtained from the control, TM, and TMHBO groups. However, there was no statistical difference in the number of TUNEL-positive cells between the T and THBO groups (Table 3 and Fig. 3).

Biochemical findings

The mean AMH level of the torsion group was 7.89 ± 0.30 pg/mL, which was significantly lower than the mean AMH level of the control group of 9.65 ± 1.06 pg/mL. The mean AMH level of the TM group was 10.77 ± 0.55 pg/mL, the mean AMH level of the THBO group was 9.95 ± 0.41 pg/mL, and the mean AMH level of the TMHBO group was 11.73 ± 0.94 pg/mL, which were significantly improved compared to the T group. In addition, the mean AMH levels of the TM and TMHBO groups were significantly higher than the mean AMH levels of the control group (Table 4).

Discussion

This experimental study focused on the effects of metformin and HBOT on damage to torsioned ovarian tissue. In the torsion group, it was determined that the histology of follicular structures was impaired, the number of follicles decreased, the number of TUNEL-positive cells increased, and the serum AMH level and tissue AMH expression decreased. Metformin and HBOT given for treatment purposes improved all these damages. Metformin and HBOT were not superior to each other, but the best results were obtained in the TMHBO group.

Ovarian torsion is one of the emergency gynecological surgeries that causes ischemic cell damage in the ovary. Reversing torsion is the current treatment used to preserve fertility in young patients. However, reperfusion of ischemic tissue causes excessive ROS production, thus developing reperfusion damage and eventually I/R damage occurs[33]. I/R damage causes

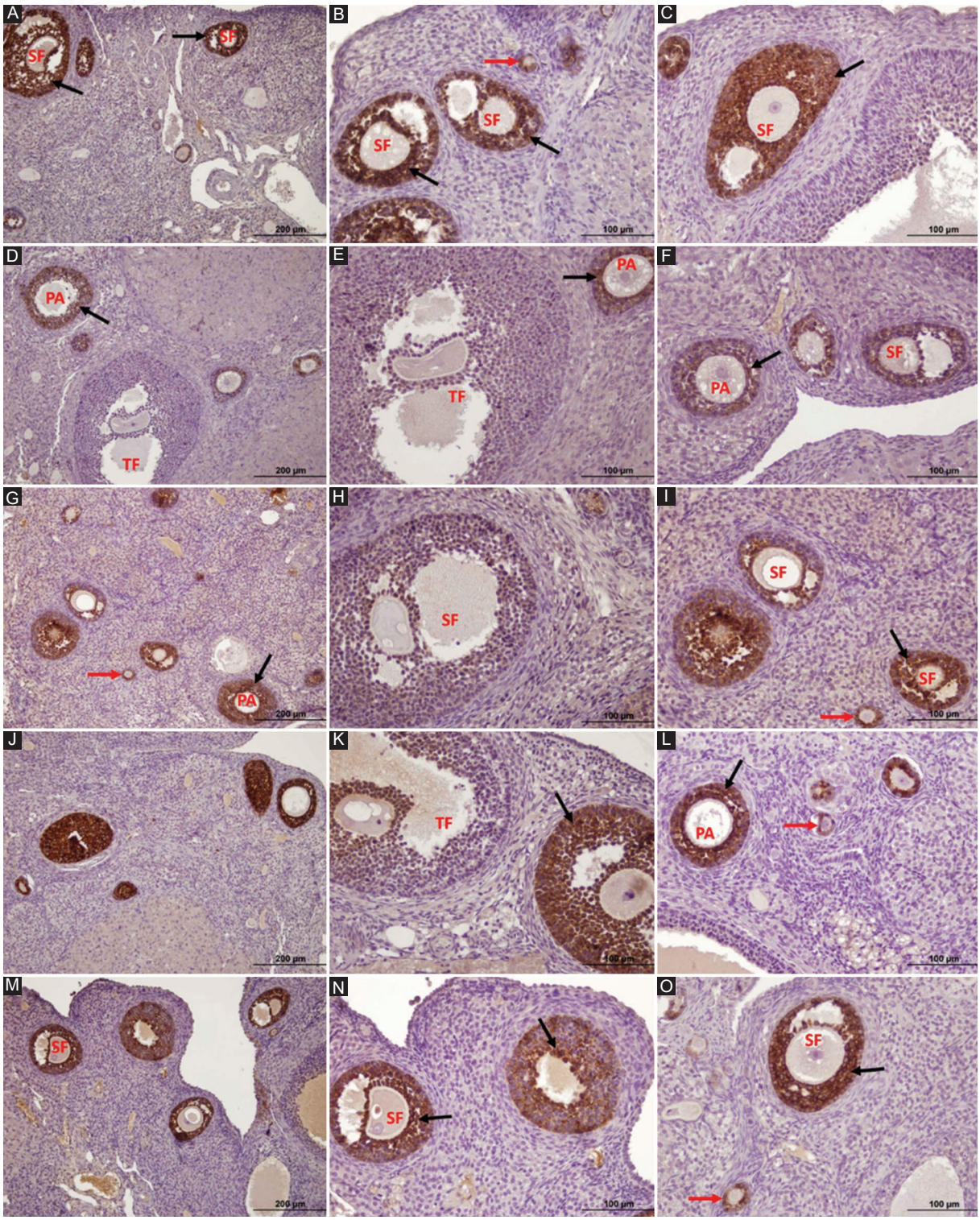

Figure 2. *Immunohistochemical staining for anti-Mullerian hormone in ovarian sections of control and experimental groups. Control group* **(A-C)***; T: torsion/detorsion group* **(D-F)***, TM; torsion/detorsion+metformin group* **(G-I),** *THBO; torsion/detorsion+hyperbaric oxygen therapy group* **(J-L)***; TMHBO; torsion/detorsion+metformin+hyperbaric oxygen therapy group* **(M-O)***. SF: secondary follicle; TF: tertiary follicle; PA: preantral follicle; primary follicle (red arrow). Black arrow: AMH-positive cells. Scale bars: 200 µm* **(A, D, G, J, M)***; 100 µm* **(B, C, E, F, H, I, K, L, N, O)**.

morphological and biochemical changes in the ovarian tissue. Moreover, different I/R techniques have been used to better understand the damage that develops as a result of ovarian torsion. It has been reported that 2 h of ischemia time is sufficient to induce experimental I/R injury in the ovaries[34]. In our

Table 3. Number of TUNEL positive cells in the control and experimental groups

Positivity of the cells	Control	T	TM	THBO	TMHBO	p
Number of TUNEL (+) cells	0.69 ± 0.11[a]	1.69 ± 1.23[b]	1.00 ± 0.93[a]	1.09 ± 1.01[ab]	0.83 ± 0.78[a]	0.001

One-way ANOVA. Different letters in the row indicate a statistically significant difference.
T: torsion/detorsion group: TM: torsion/detorsion + metformin group; THBO: torsion/detorsion + hyperbaric oxygen therapy group; TMHBO: torsion/detorsion + metformin + hyperbaric oxygen therapy group. "The same letters on the same line indicate similarity between groups and different letters indicate the difference between groups."

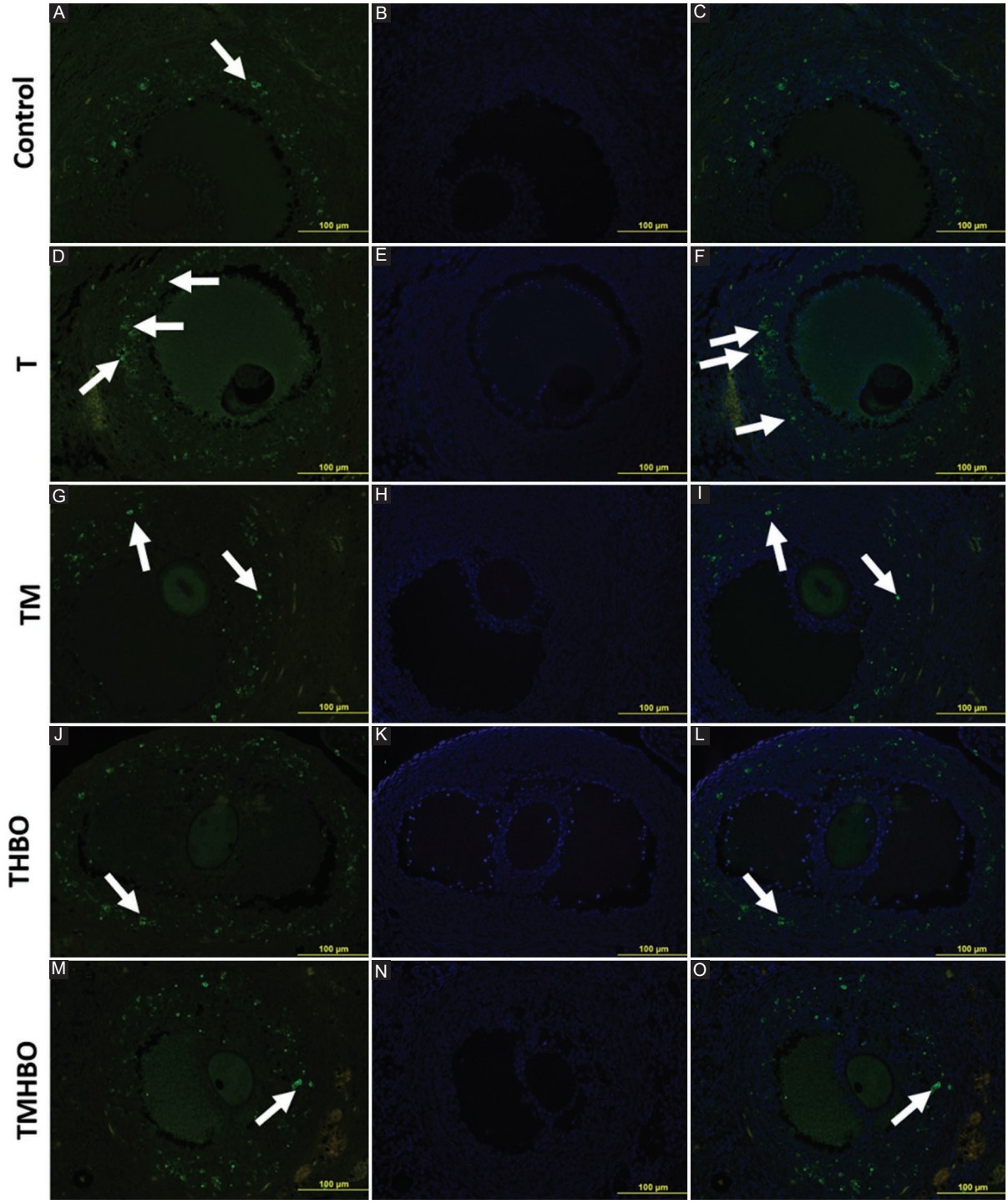

Figure 3. *TUNEL staining in the ovary sections of the control and experimental groups. Control group* **(A-C)***; T: torsion/detorsion group* **(D-F)***; TM: torsion/detorsion+metformin group* **(G-I);** *THBO: torsion/detorsion+hyperbaric oxygen therapy group* **(J-L)***; TMHBO: torsion/detorsion+metformin+hyperbaric oxygen therapy group* **(M-O)***. Arrows: TUNEL-positive cells. Scale bars: 100 µm.*

Table 4. Comparison of serum Anti-mullerian hormone (AMH) levels in the control and experimental groups

Serum AMH levels	Control	T	TM	THBO	TMHBO	p
AMH, pg/ml	9.65 ± 1.06[a]	7.89 ± 0.30[b]	10.77 ± 0.55[cd]	9.95 ± 0.41[ac]	11.73 ± 0.94[d]	0.001

One-way ANOVA. Different letters in the row indicate a statistically significant difference.
T: torsion/detorsion group; TM: torsion/detorsion + metformin group; THBO: torsion/detorsion + hyperbaric oxygen therapy group; TMHBO: torsion/detorsion + metformin + hyperbaric oxygen therapy group. "The same letters on the same line indicate similarity between groups and different letters indicate the difference between groups."

study, 2 h of ischemia were created. Different reperfusion times were studied to reveal the tissue effects of I/R damage. Yurtcu et al.[34] used a 2-h reperfusion period, Hortu et al.[35] used a 3-h reperfusion period, Sahin Ersoy et al.[36] used a 24-h reperfusion period, and Karakas et al.[37] used 14 14-day reperfusion period. We believed that a longer reperfusion period would be more appropriate because the purpose of our study was to assess the impact of I/R injury on ovarian reserve. Therefore, the reperfusion period in our study was 14 days.

The pool of follicles and serum AMH levels are used to assess ovarian function and reserve during the fertile period. AMH secreted from the granulosa cells of preantral follicles is an indirect marker reflecting the growing follicle pool[38]. The levels of serum AMH rise during childhood and adolescence, peaking at age 18, and then fall as the ovarian reserve gradually runs out[28]. As a measure of ovarian reserve, serum AMH levels are superior to those of age, LH, FSH, and estradiol[29,39,40]. Eken et al.[41] observed a significant decrease in the number of primordial, preantral, and antral follicles after ovarian torsion. Mohammadi et al.[42] determined that torsion-detorsion decreased the number of follicles at each stage, such as antral, graafian, and preantral, and also increased the number of atretic bodies. Sakin et al.[43] observed that after 3 h of ischemia and reperfusion, the number of primordial, primary, secondary (pre-antral), and tertiary (antral) follicles decreased significantly compared to the control group. The researchers also determined that the number of atretic follicles increased and serum AMH levels decreased in the torsion group. Erimsah and Cetinkaya[44] showed that I/R injury in the ovary decreased AMH expression in follicles. In our previous study, hemorrhage, edema, and inflammation, an increase in histopathological score, a decrease in follicle numbers, and a decrease in AMH expression were observed in the ovarian tissue after 2 h of torsion. In addition, an increase in Hsp70, NF-κß, CD31, COX-2, Beclin-1, LC-3, and p62 expressions was determined after ovarian torsion[30]. In the current study, AMH expression was observed in primary, preantral, secondary, and tertiary follicles, but AMH expression in tertiary follicles was lower than in other follicles. AMH expression in all follicles and serum AMH levels in the torsion group were significantly lower than in the control group. It was also determined that the number of primordial, primary, preantral, secondary, and tertiary follicles decreased, and the number of atretic follicles and TUNEL-positive cells increased in the T group of rats. Our study results reaffirm previous study results showing that ovarian torsion negatively affects ovarian reserve.

It has been reported that ovarian reserves decrease after ovarian torsion/detorsion and that surgical detorsion alone is ineffective in preserving ovarian reserves[45]. To avoid I/R damage and maintain ovarian reserves, antioxidants and anti-inflammatory were applied in this experimental study. Clinical approval for the use of these agents has not yet been granted, despite their demonstrated experimental benefits. Consequently, research efforts persist in the pursuit of safe and effective medications aimed at mitigating I/R damage in the ovary. Metformin, one of the primary drugs used in the treatment of patients with type 2 diabetes, has been shown to have anti-inflammatory and antioxidant effects[46]. Asghari et al.[47] showed that metformin protects testicular I/R injury in rats. Wang et al.[48] reported that metformin reduces inflammation and prevents apoptosis of renal tubular epithelial cells in a renal I/R rat model study. Palomba et al.[26] observed that metformin use in patients with polycystic ovaries improves ovulation capacity. Topcu et al.[2] documented that metformin improves estradiol levels, tissue oxidative system parameters, and histopathological score in their studies where they applied 250 mg/kg and 500 mg/kg of metformin against ovarian I/R injury. Karakas et al.[37] showed that 50 mg/kg metformin administration for 14 days protects ovarian reserve. Dayangan Sayan et al.[27] showed that 500 mg/kg metformin applied after ovarian torsion improved histopathological score, apoptosis levels, and biochemical oxidant/antioxidant levels. In our

study, 50 mg/kg metformin was applied for 14 days against I/R injury in the ovaries. After metformin administration, improvements were observed in the number of follicles, TUNEL positive cell count, and AMH expression in the ovarian tissue and serum AMH levels. Our results show that metformin has a protective effect on ovarian reserve in I/R injury.

HBOT provides 100% oxygen at environmental pressures greater than 1 atmosphere. HBOT has anti-inflammatory and antioxidant effects and is used for the treatment of ischemia-reperfusion injury. Yu et al.[49] applied HBOT to patients with ovarian cysts after laparoscopic ovarian cystectomy. At the end of the study, it was determined that the serum AMH, estradiol levels, and antral follicle count of patients in the HBOT group were higher than the control group, and serum FSH and LH levels were lower. Ma et al.[19] showed that HBOT reduced follicular apoptosis, improved oocyte maturation, fertilization, and blastocyst formation in aged mice, and improved age-related serum AMH levels. In the study of Cagli et al.[50] evaluating premature ovarian failure, determined that primodial, primary, secondary, and tertiary follicle counts and serum AMH levels decreased compared to control after Cyclophosphamide application. Researchers observed that HBOT provided improvement in all these parameters. Bulutlar et al.[51] documented that HBOT applied to rats after ovarian torsion improved 8-hydroxy-2'-deoxyguanosine, malondialdehyde, AMH, neutrophilic infiltration, vascular occlusion, follicular cell damage, and edema. HBOT, which has been clinically approved, can last up to 120 min at a pressure of three atmospheres. However, for typical therapeutic uses, 1.8-2.8 atmosphere pressure is typically applied for 60-90 min[18,52]. In the current study, HBOT was applied in a hyperbaric chamber at 2.4 atmospheres pressure for 2 h/day for 14 days, with 100% O_2 inhalation. HBOT applied after torsion provided improvement in follicle numbers, AMH expression in follicles, and serum AMH levels. Our results show that HBOT is a treatment protocol that can be successfully applied in preserving ovarian reserve.

To our knowledge, there is no study in which HBOT and metformin were applied together in ovarian torsion. However, combined steroid and HBOT significantly improves hearing thresholds in patients with idiopathic sudden sensorineural hearing loss[53], demonstrating the potential of combining therapies to enhance clinical outcomes. Our study is the first study in which HBOT and metformin were applied together in ovarian torsion. According to this study, metformin and HBOT improved the damage caused by torsion both histologically and biochemically, but the individual effect of any of them is not better than using both of them. In the TMHBO group, where metformin and HBOT were applied together, there was a numerically greater improvement in follicle counts, follicular AMH expression, and TUNEL-positive cell count, but they were not significantly different from the groups that received only metformin and only HBOT. The highest serum AMH levels were in the TMHBO group and were significantly higher than the control, torsion, and THBO groups.

The strength of our study is the evaluation of the effects of HBOT and metformin on ovarian reserve. There are some limitations in our study, in that which sample size was small. We think that our results are promising for larger sample clinical studies. Another limitation is the lack of studies on metformin and HBOT to determine the optimum dose.

Conclusion

In this research, the impact of metformin and HBOT administered following ovarian torsion on ovarian reserve was assessed. The findings indicated that both metformin and HBOT, whether utilized independently or in combination, enhanced the follicular pool within the ovaries as well as serum levels of AMH. Given that this investigation was conducted on animal models, it is essential for our results to be validated through clinical studies involving larger sample sizes.

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Conflicts of interest

The authors declare no conflicts of interest.

Ethıcal consıderatıons

Protection of humans and animals. The authors declare that the procedures followed complied with the ethical standards of the responsible human experimentation committee and adhered to the World Medical Association and the Declaration of Helsinki. The

procedures were approved by the institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. The authors have followed their institution's confidentiality protocols, obtained informed consent from patients, and received approval from the Ethics Committee. The SAGER guidelines were followed according to the nature of the study. This study was approved by the Erciyes University Animal Research Ethics Committee (Date: November 03, 2021, No: 21/224).

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing of this manuscript.

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