Combining tadalafil and hyperbaric oxygen therapy protects against ovarian ischemia-reperfusion damage in rats

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Abstract. A frequently occurring surgical emergency in gynecology is ovarian torsion, which occurs when the ovary twists on its ligamentous supports, which obstructs blood flow. The aim of the present study was to evaluate the protective effects of hyperbaric oxygen therapy (HBOT) and tadalafil against ischemia-reperfusion (IR) injury in a rat model of ovarian torsion. Female Wistar albino rats were randomly assigned to five groups (n=8/group): Control, IR, IR + tadalafil, IR + HBOT and IR + tadalafil + HBOT. Ovarian torsion was induced under anesthesia for 2 h, followed by daily post-operative treatments with tadalafil (10 mg/kg) and/or HBOT (2.4 atmospheres absolute for 1 h) for 7 days. Blood and ovarian tissue specimens were collected for analysis at the end of the treatment period. IR-induced ovarian tissue injury significantly decreased the counts of primordial, primary and preantral follicles compared with those in the control group. In addition, serum ELISA and immunohistochemical analysis revealed that IR injury reduced anti-Müllerian hormone (AMH) levels in serum and the granulosa cells of primary, preantral and secondary follicles. HBOT alone resulted in a significant increase in the counts of primordial, primary and preantral cells, as did the combination of HBOT with tadalafil. In addition, AMH immunoreactivity significantly increased in primary, preantral and secondary follicles following treatment with HBOT and tadalafil. Furthermore, all therapeutic interventions elevated serum AMH levels in the IR model rats. These findings suggest that tadalafil treatment combined with HBOT may help protect ovarian reserve and mitigate IR-induced tissue damage in rat ovaries.

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Introduction

Ovarian torsion is a rare but serious gynecological emergency that can occur in women of all ages. The prevalence of ovarian torsion is 2-15% among patients undergoing surgical treatment for adnexal masses, and 10-22% of all ovarian torsion cases occur in pregnant women (1). Torsion typically involves both the ovary and fallopian tube, with torsion affecting only one of these structures being less common (2-4). Ovarian torsion involves the total or partial rotation of the adnexa around its supporting structures. Torsion of the ovarian vascular pedicle along its axis leads to diminished arterial blood flow and obstructed venous and lymphatic drainage, ultimately leading to ischemia (5), which has been widely recognized to induce cellular damage (6).

Since the extent of ischemic injury is strongly time-dependent, rapid intervention to reduce the duration of hypoperfusion is critical for preventing cell damage. Rapid diagnosis and prompt surgical intervention are therefore essential for reducing the hypoperfusion period and preserving ovarian and tubal function (7,8). Surgery is the main method of treating ovarian torsion, involving either laparoscopy or laparotomy. After surgery, medications are typically administered to reduce the risk of recurrence (8). However, the restoration of blood flow during reperfusion can trigger events that worsen cellular damage (9). The reperfusion of ischemic tissue triggers an influx of molecular oxygen that activates xanthine oxidase and NADPH oxidase, generating highly reactive oxygen species (ROS) (10). Excessive quantities of ROS induce lipid peroxidation, leading to the synthesis and systemic release of proinflammatory eicosanoids, which decrease membrane permeability and ultimately lead to cell death through autophagy, necroptosis or apoptosis (11). In parallel, lactate

dehydrogenase and other anaerobic metabolites contribute to oxidative tissue damage and necrosis (12). Under physiological conditions, ROS are neutralized by natural antioxidant mechanisms. However, under conditions of ischemia-reperfusion (IR), the radical-scavenging ability of these antioxidants is overwhelmed (13).

Tadalafil, a phosphodiesterase type 5 (PDE-5) inhibitor, is commonly used for the treatment of erectile dysfunction. PDE-5 inhibitors increase 3',5'-cyclic guanosine monophosphate (cGMP) levels by inhibiting the enzymatic degradation of cGMP (14). The tadalafil-induced elevation of cGMP levels has been shown to diminish lipid peroxidation and suppress NADP oxidase activity, which are the primary ROS-producing systems during oxidative stress (15). Tadalafil exhibits a dual role in tissues undergoing ischemia and reperfusion, exerting both vasodilatory and antioxidant effects (16). Previous studies suggest that PDE-5 inhibitors may mitigate the negative consequences of IR injury on the brain, kidneys and heart (17-19).

Hyperbaric oxygen therapy (HBOT) increases the partial pressure of oxygen in the circulatory system and tissues by administering 100% oxygen at pressures >1 atmosphere absolute (ATA). HBOT can mitigate IR injury by reducing inflammation, promoting microcirculation, preserving the metabolic function of injured tissues, and limiting the generation of ROS and the resulting oxidative tissue damage (20,21).

To the best of our knowledge, no information is currently available regarding the combined effects of tadalafil and HBOT on IR-induced ovarian damage. Therefore, the present study aims to evaluate the protective efficacy of HBOT and tadalafil treatment against IR injury in an experimental model of ovarian torsion in rats.

Materials and methods

Animals. The study was conducted at the Experimental Research Application and Research Center (DEKAM) of Erciyes University. The rats were obtained from DEKAM. The Experimental Animal Ethics Committee of Erciyes University (Kayseri, Turkey) approved the study protocol (approval no. 21/124). A total of 40 female Wistar albino rats (body weight, 250-300 g; age, 6-8 weeks) were used in the study. The rats were divided into five groups (n=8/group) as follows: Control group; IR group, subjected to 2 h ischemia; IR + tadalafil group, subjected to 2 h ischemia followed by treatment with 10 mg/kg tadalafil daily for 7 days; IR + HBOT group, subjected to 2 h ischemia followed by treatment with 2.4 ATA/1 h HBOT daily for 7 days; and IR + tadalafil + HBOT, subjected to 2 h ischemia followed by treatment with 10 mg/kg tadalafil and 2.4 ATA/1 h HBOT daily for 7 days. The rats were housed away from stressful situations in a well-ventilated environment under controlled conditions (22-25˚C, 40-70% relative humidity and a 12-h light/dark cycle). Animals had free access to water and food, and were fed a standard rodent diet.

Experimental design. Prior to surgery, the rats were anesthetized under sterile conditions with xylazine hydrochloride (Rompun; 10 mg/kg; Bayer AG) and ketamine hydrochloride (Ketalar; 80 mg/kg; Pfizer, Inc.). A 2-cm midline incision was performed for laparotomy in the lower abdomen of the rats, allowing for examination of the uterine horns, adnexa and ovaries. The left adnexa was sutured with 4/0 Vicryl, rendering it ischemic for 2 h. The ischemia was terminated by removal of the suture. Following the procedure, the surgical site was closed in two layers using 4/0 silk sutures; the muscular and subcutaneous connective tissues were closed first, followed by the skin. In the control group (Sham), the left side of the abdominal wall was opened and closed 1 min later with 4/0 silk sutures.

For HBOT, the cages containing the rats of the IR + HBOT and IR + tadalafil + HBOT groups were placed in a hyperbaric chamber. The chamber was filled with 100% oxygen (GAZSAN Sanayi Gazlari Ltd. Sti.) at a rate of 0.1 ATA/min for ~15 min until the pressure reached 2.4 ATA. The rats then underwent HBOT for 1 h. The HBOT was initially administered 4 h post-surgery, then performed once daily and terminated after 7 days. In the tadalafil and IR + tadalafil + HBOT groups, the rats received tadalafil (10 mg/kg; Nuvomed Pharmaceuticals) via oral gavage (22) 4 h post-surgery and once daily for a total of 7 days. At the end of the treatment period, ovarian tissue was harvested from the rats under anesthesia Thereafter, the thoracic cavity was accessed, intracardiac blood was extracted and the rats were euthanized by cervical dislocation.

Serum anti-Müllerian hormone (AMH) ELISA. AMH levels were analyzed in serum samples from the rats using a commercially available ELISA kit (cat. no. 201-11-0457; Shanghai Sunred Biological Technology Co., Ltd.). Briefly, micro-ELISA strip plates pre-coated with an AMH-specific antibody were incubated with standards and serum samples. A horseradish peroxidase-conjugated AMH-specific antibody was then added to each well. Following incubation, a substrate solution was added to induce a color change reaction (23). Optical density was measured using a BioTek Synergy HT microplate reader (Agilent Technologies, Inc.).

Immunohistochemistry. AMH immunoreactivity in primordial, primary, preantral, secondary and tertiary ovarian follicles was evaluated using a streptavidin-biotin immunoenzymatic antigen detection kit (UltraVision Large Volume Detection System; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The ovarian tissues were fixed in 10% formaldehyde at 20˚C for 72 h. In brief, the 5-μm paraffin-embedded slices were deparaffinized at 67˚C for 2 h and subsequently rehydrated. Xylene solutions were employed in the purifying process of paraffin. Then, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 10 min at room temperature. The sections were subsequently incubated with a serum-blocking reagent for 10 min at room temperature (10% goat serum; cat. no. ab138478; Abcam) to prevent non-specific staining, and then treated overnight at 4˚C with goat polyclonal anti-AMH antibody (1:150; cat. no. sc-166752; Santa Cruz Biotechnology, Inc.). After incubation with the primary antibody, the section was washed and then incubated with the secondary antibody at room temperature for 15 min. Finally, the sections were treated with 3,3'-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin at

room temperature for 5 min. Images were acquired using an Olympus BX51 microscope (Olympus Corporation). The images were evaluated using the ImageJ program (version 1.54 k; National Institutes of Health).

Histopathological evaluation. Ovarian tissues were fixed in % 10 formalin at 20˚C for 72 h and dehydrated using graded alcohols, cleared with xylene, and embedded in paraffin. Serial 5-μm sections were cut with a microtome, and stained with either hematoxylin (5 min at room temperature) and eosin (3 min at room temperature) (H&E) or Masson's trichrome (MT; hematoxylin, 5 min; acid fuchsin, 30 sec; aniline blue, 3 min; all at room temperature). The stained sections were examined under an Olympus BX51 microscope. Primordial, primary, preantral, secondary follicles were counted on the slides to assess the follicular reserve.

TUNEL assay. Apoptosis was assessed using the ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit (MilliporeSigma) according to the manufacturer's protocol. The nuclei were then stained with DAPI for 15 min at 37˚C. (Sigma-Aldrich), and evaluated using an Olympus BX51 fluorescence microscope. Images were acquired from ≥10 independent fields in each tissue section at 40x magnification, and the number of TUNEL-positive apoptotic cells was quantified.

Statistical analysis. All statistical analyses were conducted using SPSS for Windows, version 22.0 (IBM Corp.). Comparisons among the control and experimental groups for normally distributed variables were conducted using one-way analysis of variance (ANOVA), and multiple comparisons were performed using Tukey's test when the ANOVA indicated a significant difference. The Kruskal-Wallis test was used for the comparison of non-normally distributed data, and multiple comparisons were performed using Dunn's test when the Kruskal-Wallis analysis indicated a significant difference. Results are presented as the mean ± standard deviation, with $P<0.05$ considered to indicate a statistically significant difference.

Results

Serum AMH levels. AMH is a well-established marker of ovarian reserve during the early follicular phase and is associated with natural fertility (24). The rat ovarian torsion model resulted in a significant reduction of serum AMH levels in the IR group compared with those in the control group, consistent with a reduction of ovarian reserve. Treatment with either tadalafil or HBOT significantly elevated serum AMH levels in the IR model rats. In addition, compared to the IR group, the combined administration of tadalafil and HBOT resulted in a notable elevation of the serum AMH levels (Fig. 1).

Ovarian follicular AMH immunohistochemistry. Semiquantitative immunohistochemistry was performed to assess the expression of AMH in ovarian follicles (Fig. 2). AMH immunoreactivity was detected in the cytoplasm of granulosa cells in the control and experimental groups, whereas no nuclear staining was observed. No AMH expression was detected in primordial follicles, whereas AMH labeling was prominent in primary, preantral and secondary follicles but absent from tertiary follicles. AMH labeling in the IR group was significantly reduced compared with that in the control group. This reduction was significantly attenuated by the combination of tadalafil and HBOT, but not by tadalafil or HBOT alone (Fig. 2).

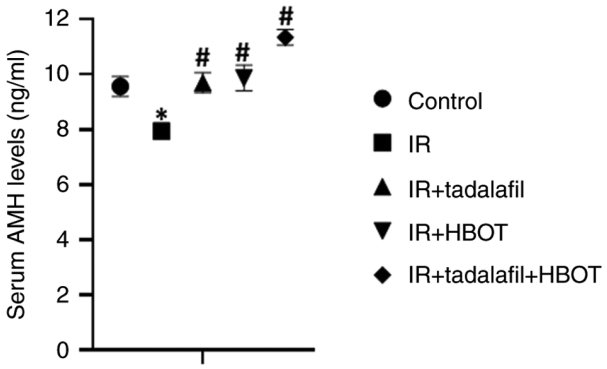

Figure 1. Serum AMH levels in five groups of rats. Data presented as mean ± SD. *P<0.05 vs. the control group and #P<0.05 vs. the IR group. AMH, anti-Müllerian hormone; IR, ischemia-reperfusion; HBOT, hyperbaric oxygen therapy.

Histopathological evaluation. Ovarian tissues stained with H&E or MT were examined under a light microscope. The ovarian tissues of the control group exhibited a normal histological structure. The surface of the ovary was covered by a single layer of flat or cuboidal germinal epithelium, beneath which lay the tunica albuginea, a dense connective tissue layer separating it from the cortex. The cortical layer contained primordial, primary, preantral, secondary, tertiary and atretic follicles from the preceding cycle, exhibiting diverse sizes and morphologies. By contrast, the ovarian histological architecture was disrupted in the IR group (Fig. 3). The IR group exhibited morphological abnormalities and a significant reduction in primordial, primary and preantral follicle counts compared with those in the control group. In addition, blood vessels in the medulla were markedly dilated. Follicular architecture in the IR + tadalafil and IR + HBOT groups was more regular than that in the IR group. A significant increase in primordial, primary and preantral follicle counts was observed in the IR + HBOT group but not the IR + tadalafil group. In the IR model rats treated with a combination of tadalafil and HBOT, ovarian histology was nearly identical to that of the control group, and a significant increase in primordial, primary and preantral follicle counts compared with those in the IR group was observed (Fig. 3).

TUNEL assay. A TUNEL immunofluorescent staining assay was performed on ovarian tissues from all groups (Fig. 4). The IR group exhibited the greatest number of TUNEL-positive cells, which was significantly higher than that in the control group (Fig. 4). Although the treatment groups exhibited a reduction in the number of TUNEL-positive cells compared with that in the IR group, these changes were not statistically significant.

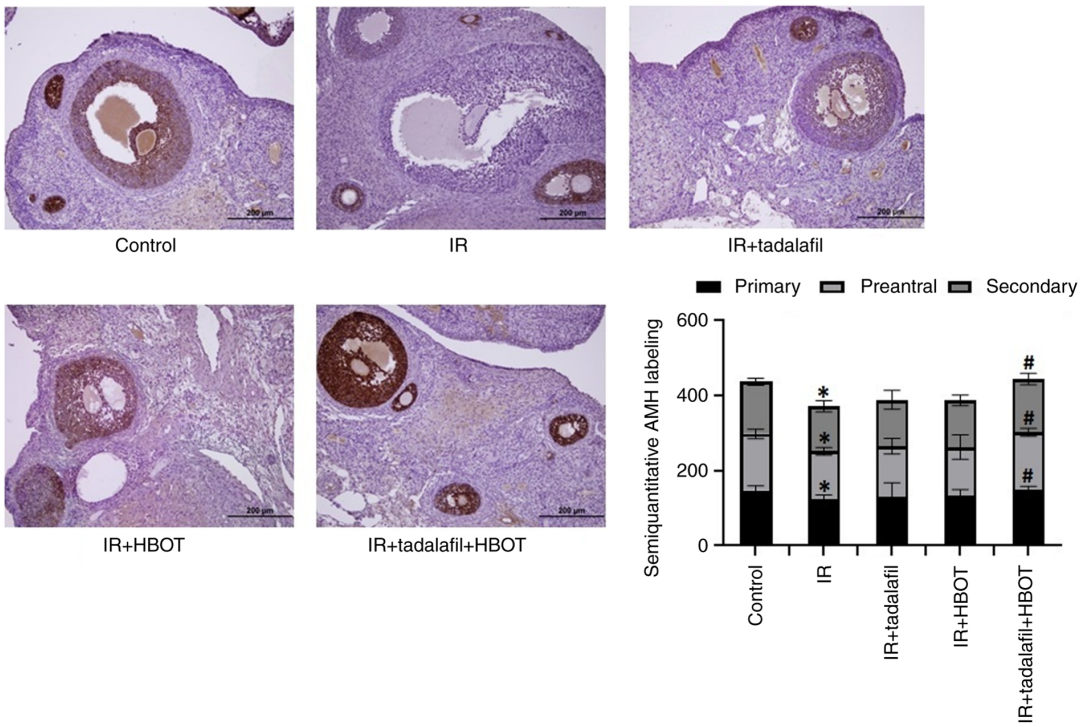

Figure 2. Immunostaining of AMH in ovarian tissue and the semiquantitative analysis of AMH expression in various types of ovarian follicles. Scale bar, 200 μm. Data presented as mean ± SD. *P<0.05 vs. the control group and #P<0.05 vs. the IR group. AMH, anti-Müllerian hormone; IR, ischemia-reperfusion; HBOT, hyperbaric oxygen therapy.

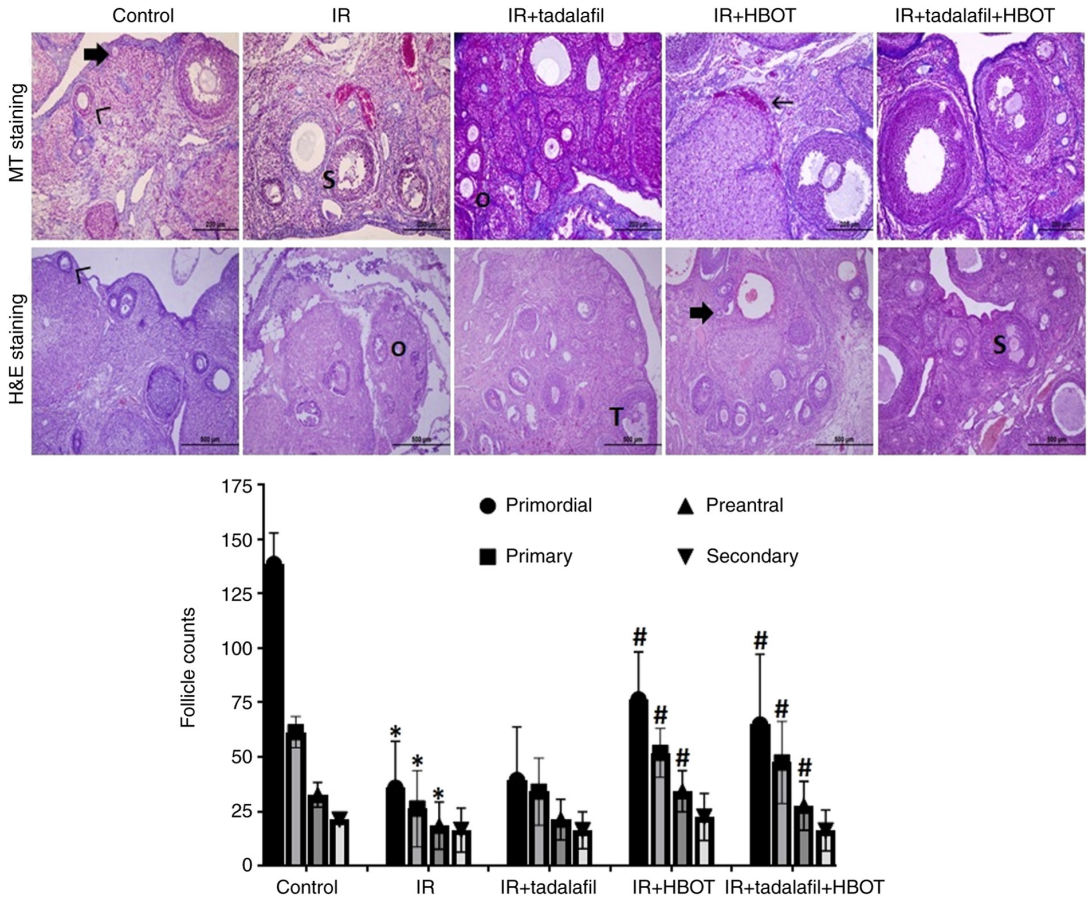

Figure 3. Representative MT and H&E staining images and follicle counts of the ovarian tissues from five groups of rats. A thick arrow indicates a primary follicle, S indicates a secondary follicle, T indicates a tertiary follicle, O indicates an oocyte, the thin arrow indicates a blood vessel and an arrowhead indicates a preantral follicle. Scale bar, 200 μm. MT, Masson's trichrome; H&E, hematoxylin and eosin; IR, ischemia-reperfusion; HBOT, hyperbaric oxygen therapy. Data presented as mean ± SD. *P<0.05 vs. the control group and #P<0.05 vs. the IR group.

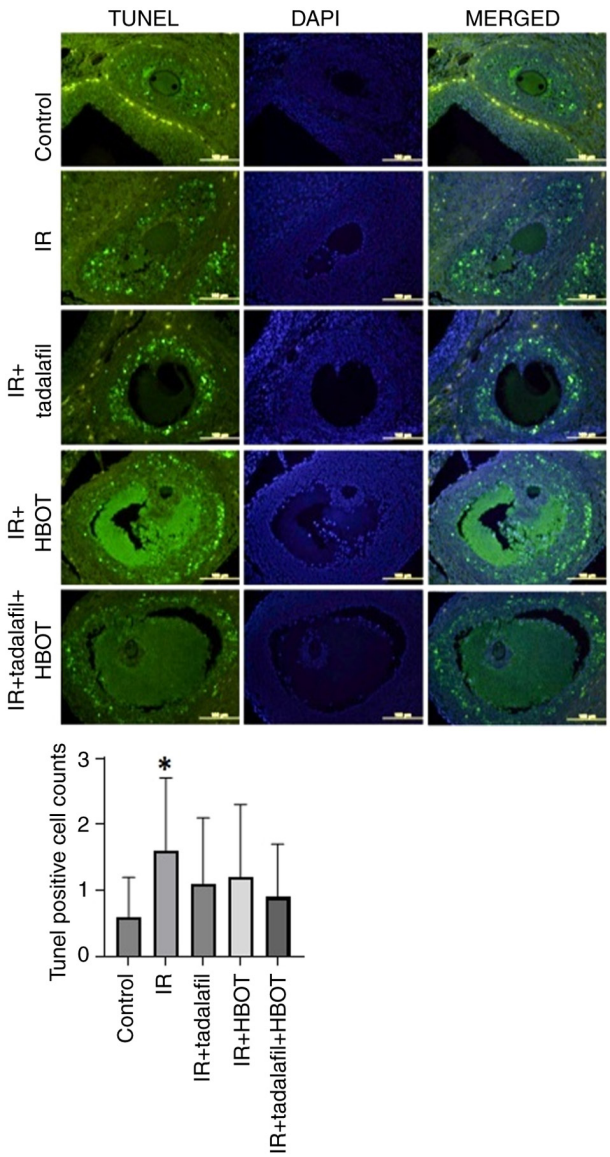

Figure 4. Representative TUNEL assay images and TUNEL-positive cell counts of the ovarian tissues from five groups of rats. Scale bar, 100 μm. IR, ischemia-reperfusion; HBOT, hyperbaric oxygen therapy. The assay was repeated three times. Data presented as mean ± SD. *P<0.05 vs. the control group.

Discussion

In the present study, the histopathological and biochemical effects of tadalafil, HBOT and their combination on a rat model of ovarian IR injury were examined. The results demonstrate that while damaged histological architecture was observed in the IR group, the combined administration of tadalafil and HBOT preserved ovarian histological architecture. In the IR + tadalafil + HBOT group, IR injury was diminished, evidenced by increased follicle counts. As well increasing follicle counts, the combination therapy also elevated serum and follicular AMH levels, indicating enhanced ovarian reserve compared with that in the IR group. Although tadalafil and HBOT, individually and in combination, decreased the number of apoptotic cells in the ovaries compared with that in the IR group, these reductions were not statistically significant. Overall, these findings suggest that a combination of tadalafil and HBOT can mitigate some of the negative consequences of IR, and may provide a greater protective effect than either tadalafil or HBOT alone. This highlights the potential benefit of combining tadalafil and HBOT in the ovarian IR model.

Ovarian torsion obstructs arterial blood flow to the ovaries, leading to ischemia and cellular damage. The surgical reversal of ovarian torsion, an urgent gynecological procedure, is the standard intervention to preserve fertility in young women (25). The reperfusion of ischemic tissue triggers a complex cascade of cellular and humoral responses, including mitochondrial dysfunction in immune cells that leads to elevated levels of ROS production, thereby exacerbating tissue injury. During IR injury, oxidative stress is accompanied by several critical pathological processes, including apoptosis, ferroptosis, inflammatory responses and extracellular matrix remodeling (6). In addition, intracellular enzymes are released during cell necrosis or in response to inflammatory mediators such as TNF (26). These elevated ROS levels and other pathological alterations can impair oocyte quality and ovarian reserve, thereby negatively impacting future fertility (27).

Supplementary treatments to mitigate surgical IR damage in the ovary have long been explored. Natural products (28,29), vitamins (30,31) and antioxidants (32,33) have been used in experimental models of ovarian IR injury to preserve ovarian function and enhance ovarian reserve. However, no standardized therapy plan for the conservative management of this condition exists in the literature. In the current study, a combination of tadalafil and HBOT was employed for the first time, to the best of our knowledge, in a rat model of ovarian IR injury. Tadalafil is a more selective PDE-5 inhibitor than sildenafil with a longer half-life (34). It enhances nitric oxide (NO) production by inhibiting the enzymatic degradation of cGMP (35). NO acts as an efficient antioxidant, inhibiting lipid oxidation by rapidly neutralizing peroxyl radicals (36), and protecting against cell death induced by hydrogen peroxide, alkyl-hydroperoxides and xanthine oxidase (37).

The present study aimed to evaluate the combined efficacy of the antioxidant tadalafil and HBOT in mitigating oxidative tissue damage in a model of ovarian torsion. The results revealed that 7 days of treatment with 10 mg/kg tadalafil and HBOT at 2.4 ATA for 60 min exhibited a beneficial effect by preserving ovarian reserves and ameliorating the histological architecture of ovaries compromised by IR injury. Previous studies on PDE-5 inhibition in ovarian torsion have been limited to single-dose experiments. For example, in one study, the administration of 20 mg/kg tadalafil to rats immediately before torsion diminished vascular congestion and hemorrhage in the ovaries. In addition, the tadalafil treatment restored the histological architecture following reperfusion, and helped to preserve the ovarian reserve (16). Other single-dose studies have also demonstrated the protective effects of PDE-5 inhibitors on ovarian torsion (38,39).

HBOT is a complementary therapy designed to mitigate ischemic tissue damage. It has been reported to diminish IR injury during the acute phase by modulating inflammation, enhancing the microcirculation and preserving metabolic function in the affected tissues (13,21,40). HBOT may also alleviate IR-induced oxidative tissue damage by reducing the production of ROS (13). Although HBOT has been shown to provide preventive benefits against IR damage, further research is needed to determine the

optimal timing of HBOT initiation, treatment pressure, duration and number of treatments required. In a previous study, HBOT was administered to rats for 1 week, at a pressure of 60 kPa three times daily. This treatment alleviated the histopathological and biochemical alterations associated with IR. It also reduced the levels of 8-hydroxy-2'-deoxyguanosine and malondialdehyde, and exhibited a protective effect on the ovaries by minimizing edema, vascular blockage, neutrophilic infiltration and follicular cell damage (41). Testicular torsion involves a similar type of rotation as ovarian torsion, and both testicular and ovarian torsion involve IR damage in their pathophysiology. Notably, rat testicular IR models subjected to HBOT during ischemia or reperfusion have shown markedly diminished testicular damage (42), supporting the protective effects of HBOT against IR injury. Consequently, HBOT has emerged as a potential supplementary treatment for male infertility, addressing sperm defects at the molecular and membrane levels, and enhancing reproductive outcomes (43).

A previous study investigating the impact of progesterone on ovarian IR damage revealed that the number of TUNEL-positive cells in the ovaries was higher in rats subjected to IR than in those subjected to a sham procedure, indicating increased apoptosis (44). Consistent with this, the present study found that IR injury resulted in an elevation in the number of TUNEL-positive cells in the ovaries, reflecting cellular apoptosis following IR. In a separate study, rats with spinal cord injury were treated with HBOT at 2.0 ATA twice daily for 3 days and once daily thereafter, which resulted in a substantial reduction in TUNEL-positive cells by day 7 (45). By contrast, the present study did not find HBOT alone to be effective in reducing the number of TUNEL-positive cells in the ovaries. However, the HBOT treatment in the present study differs from that in the previous study regarding pressure levels and duration. The pressure level, duration of treatment and frequency of administration may influence the efficacy of HBOT.

To the best of our knowledge, the present study provided the first evidence that using tadalafil with HBOT was more successful when compared with the use of the two treatments alone in treating ovarian torsion. However, it has certain limitations. These include the lack of evaluation of oxidative parameters and apoptosis markers, including Bax, Bcl2 and caspase 3, the absence of experimental groups to assess the effects of tadalafil and/or HBOT without IR injury, and the small sample size. In addition to addressing these factors, future studies should also explore varying dosages, treatment durations and administration routes of HBOT and tadalafil. Comprehensive clinical trials are also necessary to clarify the findings further.

To the best of our knowledge, the combined effect of tadalafil and HBOT on ovarian torsion has not been investigated prior to the present study, despite the use of various medications to protect the ovaries from IR damage. However, the present study demonstrated that HBOT, an anti-ischemic therapy, has protective effects against ovarian IR injury in rats, and these beneficial effects are augmented when HBOT is combined with tadalafil.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MAB was responsible for conceptualization and methodology, particularly HBOT administration. ME and EK was responsible for the methodology of the surgical procedure. EB, BY and AC performed the formal analysis. MAB and AC wrote the original draft of the manuscript. MAB and AC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethical approval was provided by the Ethics Committee of Erciyes University (approval no. 21/124; Kayseri, Turkey).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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