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Comparison of the protective effects of betamethasone, dexamethasone and methylprednisolone in ischemia/reperfusion injury of rat ovary

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ABSTRACT

Our aim is to evaluate the protective effect of dexamethasone, methylprednisolone and betamethasone treatment against ischemia-reperfusion damage created experimentally in rat ovaries. For this study, 30 female Wistar albino rats were used and the rats were separated randomly into five groups consisting of six rats each: Normal ovary, torsion-detorsion, torsion-detorsion + betamethasone 3 mg/kg, torsion-detorsion + dexamethasone 4 mg/kg and torsion-detorsion + methylprednisolone 10 mg/kg. Except for the normal group, an ovarian torsion procedure was implemented in all other groups for three hours. Then, a detorsion procedure was implemented to the groups for three hours. Medications were given intraperitoneally, 30 minutes before the detorsion procedure. Ovaries of all rats were removed and anti-mullerian hormone (AMH) levels were examined. The methylprednisolone treatment seems to be protective for the damage in terms of vascular congestion (p=0.238), inflammation (p=0.575), edema (p=0.118) and cellular degeneration (p=0.523) by preventing the meaningful increase. The dexamethasone and betamethasone treatment seems to be protective for tissue damage in inflammation (p=0.575, 0.299), cellular degeneration (p=0.575, 0.368) and edema (p=0.212, 0.162). For all steroid groups, preantral+antral follicle decrease and atretic follicle increase were prevented. AMH decline was prevented and levels were similar to normal group (methylprednisolone, betamethasone and dexamethasone p values, respectively; 0.872, 0.064, 0.335). In ischemia / reperfusion injury due to ovarian torsion, steroid use reduces damage and protects ovarian reserves. There was no significant difference between dexamethasone, betamethasone and methylprednisolone in terms of success.

1. Introduction

Ovarian torsion refers to a complete or partial rotation of the infindibulopelvic or uteroovarian ligament, resulting in ischemic changes in the ovary. Ovarian

torsion accounts for 2.7% of all the gynecologic emergencies (Aslan et al., 2017). Torsion could occur in all ages, but it is more common in reproductive ages, especially early 20s and mid-30s (Pinar et al., 2017). Early diagnosis and treatment is essential for protecting ovarian injury and fertility (Huang et al., 2017). In case of delayed diagnosis and intervention, it may cause deterioration or loss of fertility (Sintim-Damoa et al., 2017). Ovarian damage can decrease the reserves, cause infertility and also early menopause risk increases in later periods (Oktem and Oktay, 2007). Ovarian reserves can be assessed by serum markers or follicle counts (Yeral et al., 2019).

The process of ovarian torsion / detorsion is called ischemia / reperfusion (I/R) damage (Behroozi-Lak et al., 2017). I/R injury cause inflammation, including migration of leukocytes and macrophages, and production of proinflammatory cytokines. I/R induced hypoxia damages the vascular endothelial cells, which may consequently lead to leukocyte-endothelial cell adhesion and neutrophil migration through the endothelial barrier results (Auphan et al., 1995; Luscinskas et al., 2002; Uchiyama et al., 2016). Glucocorticosteroids primarily acts by binding to the steroid receptor and regulating the activity, promoter sequences and gene expression as a nuclear transcription factor. Inducible nitric oxide synthase inhibits the transcription of selectins and adhesion molecules and neutralize transcription factors activator protein-1 and nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) (De Bosscher and Haegeman, 2009; Wystrychowski et al., 2018). Corticosteroids are potent anti-inflammatory and immunosuppressive agents. They inhibit the synthesis of almost all known cytokines and cell surface molecules (Uchiyama et al., 2016).

Recently, the beneficial effects of steroids on I/R injury had been shown in many different tissues. Corticosteroids had been shown to be effective in the prevention of renal tissue, brain tissue, also of myocardial tissue after cardiopulmonary bypass and of liver tissue during the hepatic ischemic events (Wang et al., 2001; Glanemann et al., 2004; Saidi et al., 2007; Wang et al., 2008; Subhas et al., 2010; Uchiyama et al., 2016). In a study, oxidative stress markers in I/R injury were investigated. High-dose methylprednisolone was found to improve the histopathological damage. They stated that the effect of I/R injury on rats was effective but there is need for future studies for the clarification of the protective mechanisms (Osmanagaoglu et al., 2012).

To the best of our knowledge, in the literature, there is not any research investigating the protective effects of steroids on ovary follicles against ischemia-reperfusion damage and the effects on the level of AMH. Our aim in this study is to evaluate the benefits of steroids on I/R injury and to compare the effects of the agents.

2. Materials and methods

This study was conducted at the Animal Testing Laboratory of Marmara University after the approval of the Ethics Committee (dated on November 5, 2018; protocol No. 101.2018.mar).

Laboratory animals and the care of animals in research

Female Wistar albino (Rattus Norvegicus spp.) rats, 12-weeks-old, were used and the rats weighed 200 to 250 grams. The rats were exposed to light for 12 hours per day (08:00 to 20:00), access to food and drinking water without restriction (standard rodent pellet, tap water), and held in rooms with a humidity of between 40 - 50% with room temperature of 21 to 23°C, and 4 or 5 per cage. The number of rats was selected based on previous studies (Celik et al., 2014;Tokgoz et al., 2018; Yildirim et al., 2018). The rats were randomly divided into five groups with 6 mice per group. The rats were not fed for 6 hours prior to laparotomy to empty the intestines and ease the surgery, but they had access to drinking water.

Groups

For this study, 30 female Wistar albino rats were used, and the rats were separated randomly into five groups consisting of six rats each: Normal, torsion-detorsion, torsion-detorsion + betamethasone, torsion-detorsion + dexamethasone and torsion-detorsion + methylprednisolone.

Group 1 (normal ovary group): This group of rats underwent laparotomy once. During the laparotomy, one of the ovaries was removed and fixed in 10% formaldehyde. And at least 1 ml of blood sample was taken for AMH test.

Group 2 (torsion ovary group): Laparotomy was performed and one of the ovaries was twisted 720 degrees and untwisted 3 hours later and the surgical wound was closed without administering any medicine. A second surgery was performed 3 hours later and both ovaries were removed. And at least 1 ml of blood sample was taken for AMH test.

Group 3 (betamethasone group): At the first laparotomy one of the ovaries was twisted 720 degrees. Betamethasone 3 mg kg (Celestone® ampule, Schering Plough Tibbi Urunler Ticaret A.S., Istanbul, Turkey) was administered intraperitoneally 30 minutes before detorsion. At the second laparotomy, the ovaries were detorsioned and reperfusion was maintained for 3 hours. At the third laparotomy rats were sacrificed and at least 1 ml of blood was taken for AMH testing and both ovaries were removed by laparotomy.

Group 4 (dexamethasone group): At the first laparotomy one of the ovaries was twisted 720 degrees. Dexamethasone 4 mg/kg (Dekort® ampule, DEVA Holding, Istanbul, Turkey) was administered intraperitoneally 30 minutes before detorsion. At the

second laparotomy, the ovaries were detorsioned and reperfusion was maintained for 3 hours. At the third laparotomy rats were sacrificed and at least 1 ml of blood was taken for AMH testing and both ovaries were removed by laparotomy.

Group 5 (methylprednisolone group): At the first laparotomy one of the ovaries was twisted 720 degrees. Methylprednisolone 10 mg/kg (Prednol® ampule, Mustafa Nevzat, Istanbul, Turkey) was administered intraperitoneally 30 minutes before detorsion. At the second laparotomy, the ovaries were detorsioned and reperfusion was maintained for 3 hours. At the third laparotomy rats were sacrificed and at least 1 ml of blood was taken for AMH testing and both ovaries were removed by laparotomy.

Surgical procedures

Sterile, powder-free latex gloves were preferred for surgery. For laparotomy anesthesia, a dose of 10% ketamine hydrochloride at 80 mg/kg per rat and 2% hydrochloride xylazine (Rompun, Bayer Health Care LCC, Kansas City, KS) at a dose of 15 mg / kg (Ketalar; ECZACIBAŞI) Warner Lambert, Istanbul, Turkey) were used. The procedure was performed while rats were lying in a supine position. 10% povidone-iodine solution (Batticon; Adeka Laboratories, Istanbul, Turkey) was used for shaving before the procedure. To enter the abdominal cavity, a median (approximately 5 cm, on the line between the xiphoid process and pubis) incision was applied, and the right ovary was twisted 720 degrees along with tubo-ovarian blood vessels (Fig. 1). To fix the ovary to the abdominal muscles, 5/0 silk sutures were used and the abdominal wall (peritoneum, fascia and skin) was closed in two layers using running locking sutures with 2/0 polyglactin 910, following bleeding control. Each surgical procedure lasted 15 to 20 minutes to protect the drying effect of the room air and the rats were allowed to wake up.

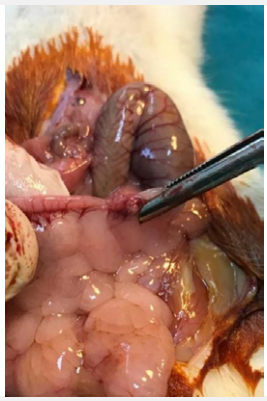

Fig. 1. Torsion of ovaries.

Histopathological examinations

Surgically excised ovaries were fixed in 10% formalin. Paraffin blocks were prepared 24 hours after the oophorectomy procedure. Tissue sections of 5 micrometers were taken and follicular activity was assessed in 5 randomly selected samples from each ovary. Slides were stained with hematoxylin eosin and examined under the light microscope. The paraffin blocks were sectioned using a microtome blade (Leica, Nussloch, Germany). Every slide was blindly assessed by the same pathologist. A light microscope (Olympus Clinical Microscope, Tokyo, Japan) was used to analyze the sections.

Edema, vascular congestion, inflammation, cellular degeneration and hemorrhage were examined as histopathological injury scores (Fig. 2). The scores were evaluated as described by Celik et al. (Celik et al., 2014). Pathological findings were rated. Grade 0 indicated normal alterations, no abnormal findings; Grade 1 indicated mild edema, mild vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 2 indicated moderate edema, moderate vascular congestion, absence of hemorrhage or leukocyte infiltration; Grade 3 indicated severe edema, severe vascular occlusion, minimal hemorrhage and minimal leukocyte infiltration, Grade 4 indicated severe edema, severe vascular occlusion, hemorrhage and leukocyte infiltration.

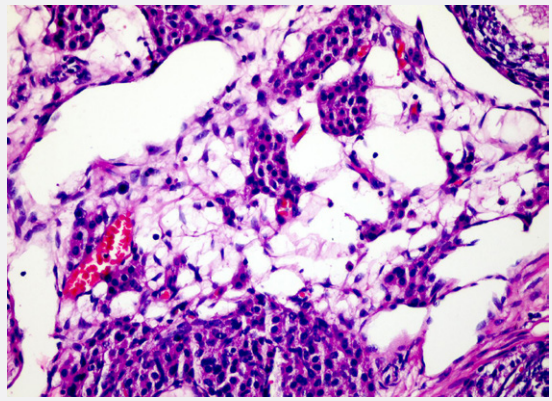

Fig. 2. Moderate edema x400 hematoxylin eosin.

All follicles were counted to assess ovarian reserve. Primordial, primary, secondary (pre-antral) and tertiary (antral) follicles were counted. Follicles were evaluated as described by Parlakgumus et al. (Parlakgumus et al., 2014). Primordial, primary, secondary (pre-antral) and tertiary (antral) follicles were counted. Primordial follicle is described as an oocyte with surrounded only one layer of epithelial cell layer, primer follicle is surrounded with one or more layer of cuboidal granulosa cells. Secondary/pre-antral follicle is surrounded with more than two cell layers and consists of antrum folliculi and zona pellucida. Tertiary follicle is defined as possessing antrum, stratum granulosum and surrounding cumulus oophorus layers (Fig. 3, 4).

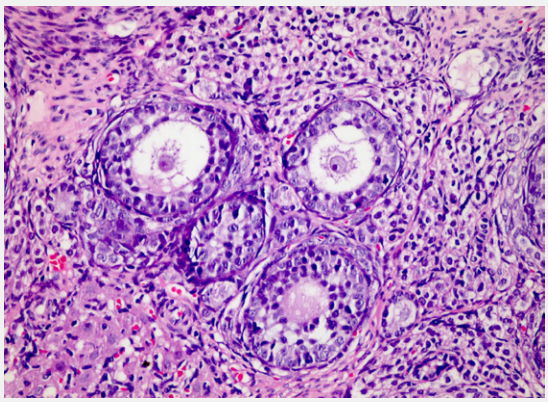

Fig. 3. Preantral follicle x400 hematoxylin eosin.

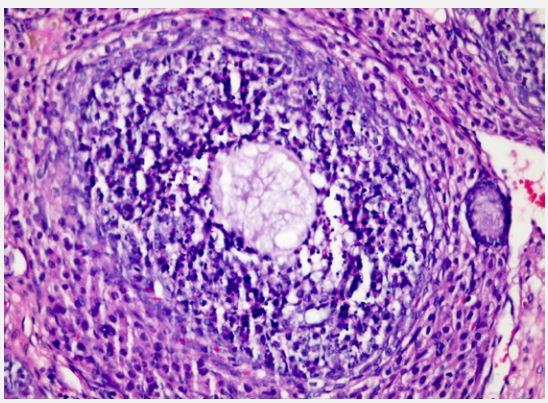

Fig. 4. Degenerated follicle x400 hematoxylin eosin.

AMH assays

Blood samples were collected into tubes containing lithium heparin (BD Vacutainer Plasma tubes®, Manchester, England). The concentration of the Lithium Heparin additive in these tubes is 17 international units of heparin/ml of blood. Blood samples were centrifuged within 30 minutes of sampling. After 15 minutes of centrifugation at 1000xg, serum was removed and remaining plasma was transferred into an eppendorf tube and stored frozen at -20°C until the time of analysis AMH concentrations were measured in "ng/ml" plasma using ELISA method. The rat AMH kit used in study had a sensitivity of 0.10 g/mL, a detection range of 0.16 to 10 ng/mL and a coefficient of variation less than 10% (Elabscience®, Rat AMH kit; Houston, Texas, ABD). The laboratory technician of the laboratory of the university hospital was blinded to the study groups and unaware of which samples belonged to which rat. All samples were analyzed in the same assay.

Statistical analysis

Statistical analyses were performed using the SSPS Version 15.0. The Kolmogorov-Smirnov test and histograms were used to assess the normality of the distribution of variables. The mean ± standard deviation or median [interquartile range] were used to present descriptive analyses. One-way ANOVA test was used to analyze normally distributed numerical data and the Kruskal-Wallis test was used to analyze non-normally distributed numerical data. The limit of statistical significance was set at p-values <0.05.

3. Results

Histopathological damage scores

According to the histopathological damage scores, minimum damage was seen in the normal group and maximum damage was seen in the torsion-detorsion group. There was an increase in histopathological damage scores (edema, cellular degeneration, hemorrhage) in the torsion group which was not given any drug (p scores, respectively; 0.011, 0.011, 0.003). The methylprednisolone treatment seems to be protective for the damage in terms of vascular congestion (p=0.238), inflammation (p=0.575), edema (p=0.118) and cellular degeneration (p= 0.523) by preventing the meaningful increase (Table 1). Its protection was not sufficient only in case of hemorrhage. Four of the five parameters were found to be successful (4/5). Dexamethasone and betamethasone treatment seems to be protective for tissue damage in inflammation (p=0.575, 0.299), cellular degeneration (p=0.575, 0.368) and edema (p=0.212, 0.162). Three of the five parameters were found to be successful (3/5). Dexamethasone and betamethasone treatment could not provide successful protection in vascular congestion

Table 1. Comparison of normal ovarian tissue and histopathological damage scores of torsion and torsion + steroid treated groups.

	Steroids	Normal	Torsion	P****	Detortion+ Dexamethasone	P*	Detortion+ Betamethasone	P**	Detortion+ Methylprednisolone	P***
Edema	Mean SD	1.33±0.52	2.50±0.55	**0.011**	1.83±0.75	**0.212**	2.00±.89	**0.162**	0.67±0.82	**0.118**
	Median- IQR	1.00 (1.00-2.00)	2.50 (2.00-3.00)		2.0 (1.0-2.0)		2.0 (1.0-3.0)		0.5 (0.0-1.0)	
Vascular congestion	Mean SD	0.83±0.98	1.33±1.03	**0.403**	2.17±0.75	**0.036**	3.00±0.00	**0.002**	1.50±0.84	**0.238**
	Median- IQR	0.50 (0.00-2.00)	1.00 (1.00-2.00)		2.0 (2.0-3.0)		3.0 (3.0-3.0)		1.0 (1.0-2.0)	
Inflammation	Mean SD	0.50±0.55	1.00±0.00	**0.056**	0.33±0.52	**0.575**	1.00±0.89	**0.299**	0.33±0.52	**0.575**
	Median- IQR	0.50 (0.00-1.00)	1.00 (1.00-1.00)		0.0 (0.0-1.0)		1.0 (0.0-2.0)		0.0 (0.0-1.0)	
Cellular degeneration	Mean SD	0.33±0.52	1.83±0.98	**0.011**	0.50±0.55	**0.575**	1.00±1.26	**0.368**	0.17±0.41	**0.523**
	Median- IQR	0.00 (0.00-1.00)	1.50 (1.00-3.00)		0.5 (0.0-1.0)		0.5 (0.0-2.0)		0.0 (0.0-0.0)	
Hemorrhage	Mean SD	0.33±0.52	2.50±0.55	**0.003**	2.00±1.26	**0.028**	2.83±0.41	**0.002**	1.67±1.21	**0.042**
	Median- IQR	0.00 (0.00-1.00)	2.50 (2.00-3.00)		2.5 (1.0-3.0)		3.0 (3.0-3.0)		1.5 (1.0-3.0)	

*p **p ***p ****p Mann Whitney U Test

and hemorrhage. Methylprednisolone was the only effective agent in vascular congestion. It is noteworthy that no steroid can provide successful protection in hemorrhage.

Ovarian follicle counts

Primordial and primer follicle counts were statistically different between normal and the torsion group (p=0.010). Primordial follicles were significantly decreased in all steroid groups, (dexamethasone p= 0.004, betamethasone p=0.004, methylprednisolone p=0.004) highest decrease in median and median values was in the group 5. Primary follicles were also found to be significantly decreased in all steroid groups (dexamethasone p=0.006, betamethasone p= 0.010, methylprednisolone p=0.003), highest decrease in mean and median values was also in the group 5. Secondary (preantral) follicles decreased significantly only in group 5 (p=0.033), it was observed that follicles were similar to group 1 in other steroid groups (Table 2). Tertiary (antral) follicles were found to be in similar rates with group 1 in all steroid groups. Atretic follicle counts were statistically different between normal and torsion groups (p=0.002). On the other hand, methylprednisolone (p=0.317), dexamethasone (p=0.056) and betamethasone (p=0.140) group did not statistically differ from the normal group. In the group 1, no atresic follicle was detected, whereas the most atresic follicle was in the group 2. It was determined that atresic follicle increase was prevented in all steroid groups and was similar to normal group.

AMH levels

Besides AMH levels were statistically different between torsion and detorsion, whereas methylprednisolone, betamethasone and dexamethasone groups were similar to normal group (p values respectively 0.872, 0.064, 0.335) (Table 2). It was determined that decrease in AMH was also prevented in all steroid groups and was similar to normal group.

4. Discussion

In this experimental study, it is seen that ischemia / reperfusion injury was successfully achieved and caused a significant increase in histopathological damage scores in the torsion group, a significant decrease in primordial and primary follicles, an increase in atresic follicles and a decrease in AMH levels.

All of the steroids used to prevent this damage provided significant protection against edema, inflammation and cellular degeneration damage. While methylprednisolone was the only one that is successful in vascular congestion injury, no steroid agent was found to provide significant protection in hemorrhage injury.

I/R injury due to torsion of ovarian tissue can be detected by histopathological examination. Edema, vascular congestion, inflammation, cellular degeneration and hemorrhage may be seen in the damaged tissue. It is possible to come across numerous studies in the literature investigating these outcomes. In different studies, it has been determined that there are different degrees of damage at different damage scores. In some studies, I/R resulted in an increase in all damage scores in ovaries, while in some studies an increase in only one or several damage scores was observed (Celik et al., 2014; Parlakgumus et al., 2014; Aslan et al., 2017; Behroozi-Lak et al., 2017; Pinar et al., 2017; Tokgoz et al., 2018; Yildirim et al., 2018; Yeral et al., 2019).

The exact mechanisms of corticosteroids have not yet been elucidated. Inhibition of inflammatory cytokines, improvement of blood flow, modulation of immune/inflammatory cells, prevention of extracellular and intracellular Ca^2 + flow and cell degeneration have all been proposed (Kahraman et al., 2007). The anti-inflammatory effects of glucocorticoids are primarily via the glucocorticoid receptors. This effect may be related with the inhibition of the expression of phospholipase A2 and cyclo-oxygenase-2 that leads to reduction of inflammation-induced prostaglandin production or can be related with the direct effect on

Table 2. Comparison of normal rat AMH levels and ovarian follicle counts with torsion and torsion + steroid treated groups.

		Normal ovary	Torsion ovary	P*	Detortion+ Dexamethasone	P**	Detortion+ Betamethasone	P***	Detortion+ Methylprednisolone	P****
Primordial follicle count	Mean SD	14.83±3.54	5.50±3.99	**0.010**	4.2±3.5	**0.004**	4.2±2.6	**0.004**	3.0±1.4	**0.004**
	Median- IQR	15.50 (11.00-18.00)	4.50 (2.00-8.00)		3.0 (1.0-8.0)		4.00 (2.00-7.00)		3.0 (2.0-4.0)	
Primer follicle count	Mean SD	16.83±3.19	9.33±3.27	**0.010**	8.8±4.0	**0.006**	9.8±4.6	**0.010**	5.0±2.4	**0.003**
	Median- IQR	18.00 (13.00-18.00)	9.00 (6.00-12.00)		9.5 (5.0-12.0)		9.5 (8.0-12.0)		4.0 (3.0-8.0)	
Secondary (preantral) follicle count	Mean SD	7.83±3.92	9.17±2.93	**0.466**	5.8±3.7	**0,569**	5.2±2.6	**0.196**	3.5±1.6	**0.033**
	Median- IQR	7.50 (4.00-12.00)	8.50 (8.00-12,.00)		5.5 (4.0-7.0)		5.0 (3.0-7.0)		3.5 (3.0-4.0)	
Tersier (antral) follicle count	Mean SD	4.17±1.33	4.67±2.16	**0.739**	5.5±3.1	**0.616**	5.5±2.1	**0.211**	5.0±2.4	**0.513**
	Median- IQR	5.00 (3.00-5.00)	4.50 (3.00-6.00)		4.5 (3.0-7.0)		5.5 (4.0-6.0)		5.0 (3.0-7.0)	
Athresic follicle count	Mean SD	0.00±0.00	2.50±0.55	**0.002**	0.5±0.5	**0.056**	1.0±1.7	**0.140**	0.2±0.4	**0.317**
	Median- IQR	0.00 (0.00-0.00)	2.50 (2.00-3.00)		0.5 (0.0-1.0)		0.0 (0.0-2.0)		0.0 (0.0-0.0)	
AMH level (ng/ml)	Mean SD	2.64±0.95	0.84±0.25	**0.004**	1.95±0.57	**0.335**	1.82±0.95	**0.064**	2.77±0.98	**0.872**
	Median- IQR	2.59 (1.64-3.70)	0.92 (0.67-1.00)		1.85 (1.74-1.98)		1.39 (1.25-2.25)		2.98 (2.22-3.50)	

*p **p ***p ****p Mann Whitney U Test

vascular permeability and edema. Inhibition of lipid peroxidation was assumed to be the most protective effect of glucocorticoids, and methylprednisolone appears to be particularly effective when compared to other glucocorticoids (Osmanagaoglu et al., 2012).

In our study, methylprednisolone was successful in four of the five parameters (4/5) of the histopathological damage scores. Methylprednisolone was the only one that is successful in vascular congestion injury, no steroid agent was found to provide significant protection in hemorrhage injury. All of the steroids used to prevent the damages provided significant protection against edema, inflammation and cellular degeneration damage. Their direct effect on edema appears that it helped to prevent this damage in all steroid groups successfully.

Anti-mullerian hormone is produced by granulosa cells of preantral follicles and small antral follicles. AMH is used as a marker in the assessment of responsiveness of ovarian follicles. It is known as an indicator of the size of growing follicles pool. The level of AMH reaches the maximum in preantral follicles and small antral follicles. However, the production of AMH cannot be detected in follicles in response to FSH and disappears in atresia follicles (Yuan et al., 2014). In our study, we observed that there were significant decreases due to I/R damage in primordial and primary follicles and these decreases could not be prevented by steroid use. Decreases occurred in all steroid groups.

However, it was determined that preantral and antral follicles were significantly protected in all steroid groups (except methylprednisolone preantral follicle group) and follicular reduction was prevented. In addition, atretic follicle increase was also prevented in all steroid groups. As a result of all follicle examinations, it can be concluded that all steroid agents are effective in protecting follicle damage with the preservation of preantral + antral follicles and the prevention of atresic follicle increase. With the prevention of follicle damage, the decrease in AMH level was prevented in all groups and the AMH levels were found to be similar to the normal ovarian group.

In the literature, in different studies, it was found that AMH values decreased after 3 hours, 24 hours or 7 days of reperfusion following 3 or 6 hours of ovarian torsion. AMH decrease seems to be not primarily dependent with the torsion time and reperfusion time. If I/R damage occur, AMH value decreases. An effective therapeutic agent should be used to reduce this damage (Ozler et al., 2013; Kaya et al., 2014; Parlakgumus et al., 2014; Sahin Ersoy et al., 2016). Kaya et al. found significant difference in AMH values in all groups after 3 hours of reperfusion following 3 hours of ischemia (Kaya et al., 2014). They stated that in the control group there was no significant difference in preoperative and postoperative AMH values of detorsion and detorsion+enoxaparin groups, but AMH level decrease was less in the enoxaparin group. In another similar study, significant decrease in preantral + antral follicle count and AMH value was observed after 3 hours of ischemia followed by 3 hours of reperfusion. It has been stated that the decrease in AMH can be reduced by the use of N-acetyl cysteine and enoxaparin (Ersoy et al., 2016).

This is the first comprehensive study on the protective effects of steroids on ovarian I/R injury. All the agents were found to be effective in reducing I/R damage with similar efficacy and they did not show a significant difference in success.

In conclusion, in ischemia / reperfusion injury due to ovarian torsion, steroid use reduces damage and protects ovarian reserves. There was no significant difference between dexamethasone, betamethasone and methylprednisolone in terms of success.

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