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Original Research

The Effect of Granulocyte Colony Stimulating Factor (G-CSF) on Ischemia/Reperfusion Injury in an Ovarian Torsion Rat Model: A Prospective Randomized Study

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Abstract

Background: Ovarian torsion is an important gynecological emergency and various agents are used in the experimental phase to prevent ovarian injury. The aim of this study was to determine the effect of granulocyte-colony-stimulating factor (G-CSF) use on ischemia/reperfusion injury in torsion/detorsion model of rat ovary. **Methods**: Twenty-eight Wistar-albino rats were randomly divided into four groups. The first group was designated as the sham group, and torsion/detorsion was applied to the remaining three groups. The third group was given saline and the fourth group was given G-CSF before detorsion. The total antioxidant and oxidant status, oxidative stress index, catalase, malondialdehyde and total thiol values were measured in rat ovaries, and after torsion/detorsion the follicles numbers of rat ovary were determined by histopathological examination. **Results**: There was no significant difference between groups in oxidative stress markers. However, the primary and secondary follicle numbers in the G-CSF group were significantly higher than the other torsion/detorsion groups ($p < 0.01$). **Conclusions**: Although the use of G-CSF in the rat ovary torsion/detorsion model was histopathologically protective in terms of the number of primary and secondary follicles, no difference was found in biochemical markers associated with reperfusion injury.

Keywords: ischemia/reperfusion; rat ovary; G-CSF; TAS; TOS; MDA

1. Introduction

Adnexal torsion is an important cause of gynecological emergency surgery with a prevalence of 2.7%, and it is more common after menarche, although it can be seen at any age [1–3]. Today, preserving the adnexa is essential, but salpingo-oophorectomy or oophorectomy was previously performed in cases of ovarian torsion [3]. Therefore, for the preservation of ovarian function, early diagnosis and treatment of torsion is very important. However, the goal of ischemia therapy is not only to restore blood flow but also to improve tissue perfusion. When detorsion is applied to the adnexa, some local and systemic effects occur due to the reperfusion of the ovaries [4].

Paradoxically, the reperfusion of ischemic tissue causes much more serious damage to the tissue than damage caused by ischemia alone [5,6]. The restoration of circulation and reperfusion following ischemia presents a new physiopathological process called "reperfusion injury", which itself results in various degrees of tissue damage. Total tissue damage is accepted as damage caused by both ischemia and reperfusion [7–10]. Therefore, the prevention of reperfusion injury will further increase the success of ischemia treatment [7,8].

Total antioxidant status (TAS) represents the whole effect of all antioxidants, while total oxidant status (TOS) expresses the total effect of all oxidants in body fluids and plasma [11]. The oxidative stress index (OSI) is considered a more proper index of oxidative stress and is calculated with the TOS/TAS formula. Malondialdehyde (MDA) and total thiol (t-SH) are other biomarkers that measure the level of oxidative stress [12]. Catalase is also a major indicator of the antioxidant defense system [13]. These substances can be a marker of reperfusion damage in various organs. Many antioxidant and anti-inflammatory treatments have been examined in terms of their efficacy in preventing ischemia/reperfusion (I/R) injury [14–16].

Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein that mediates the production, differentiation and function of macrophages and neutrophils. It is used to provide peripheral stem cell mobilization in allogeneic or autologous stem cell transplantation and to increase the neutrophil count in neutropenic patients [17]. G-CSF also

has protecting effects against I/R in certain tissues, such as cerebral injury [18], myocardial infarction [19,20], kidney injury [21], and retinopathy [22].

Since rat experiments, at least to some extent, do indeed reflect human ovarian torsion and I/R injury, many studies have been conducted in this regard. In this study, we aimed to investigate the role of G-CSF as an antioxidant in the prevention of ovarian I/R injury and to examine its histo-pathological effects on rat ovarian tissue.

2. Materials and Methods

After obtaining approval from the Animal Experiments Local Ethics Committee of Health Sciences University Ankara Health Research Application Center (approval number: 552/2019), the study was conducted in accordance with the international guidelines for the ethical use of animals. The sample consisted of a total of 28 healthy adult non-pregnant female Wistar rats (weighs 220–260 g). The rats were housed in cages of 3–4 animals under optimum conditions (50–60% humidity, 20–24 °C and 12-hour light/dark cycle) and during the experimental period, rats were fed ad libitum cubes and tap water. No feed and water restrictions were applied, and all the rats were allowed to acclimate to their environment one week before the experiment. Four groups of seven rats each were formed: Group 1, sham; Group 2, torsion/detorsion; Group 3, torsion/detorsion + saline; and Group 4, torsion/detorsion + G-CSF.

All the rats were anesthetized by administering 40 mg/kg ketamine hydrochloride (Ketalar, Pfizer, Istanbul, Turkey) and 7 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey) under sterile conditions. The abdominal hair of the rats was shaved and then 10% povidone iodine solution was used for antiseptic. A longitudinal incision was made in the midline region of the lower abdomen by placing the rats in the supine position. Then, the uterine horns and adnexa were detected by making a peritoneal incision. In Group 1 (sham), after the uterus and adnexa were observed, the incision was repaired using 3/0 silk sutures. Three hours later, both ovaries were excised. In group 2, the ovarian pedicles on both sides were rotated 360° clockwise and 3/0 silk sutures were used for fixation to the abdominal wall. After three hours of torsion, bilateral adnexal detorsion was performed. After three more hours of reperfusion period after suturing the surgical fields, both ovaries were removed. According to the protocol applied by Bostancı [16], intraperitoneal saline and G-CSF (100 IU/kg, Leucostim 30 MIU; Dem İlaç, Istanbul, Turkey) were administered in Groups 3 and 4 after 2.5 hours of torsion, respectively. After 30 minutes, bilateral adnexal detorsion was performed. Both ovaries were removed at the end of a total period of six hours, one for the analysis of biochemical markers and the other for histopathological evaluation.

2.1 Histopathological Examination

After the ovaries stored in 10% formaldehyde were dehydrated and embedded in paraffin blocks, sections of 4–6 millimeters were prepared and stained with hematoxylin-eosin. To determine follicular activity, the samples were analyzed under a light microscope by two experienced histopathologists. Follicles were histologically classified according to the epithelial cells surrounding the oocyte (primordial, primary, secondary, tertiary and Graafian) and the number of follicles was recorded.

2.2 Biochemical Analysis

Commercially available kits (Rel Assay, Gaziantep, Turkey) were used to measure the levels of the TAS (mmol Trolox equivalent/L) and TOS (μmol H_2O_2 Equivalent/L). OSI was calculated as the ratio of TOS to TAS [23]. Catalase activity was measured with a spectrophotometer using the method described by Aebi [24]. The MDA level was measured according to Wasowicz *et al*. [25], and t-SH measurements were made with a spectrophotometer according to Sedlak and Lindsay [26].

2.3 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). The distribution pattern of the data was evaluated using the Shapiro-Wilk test. The descriptive data of the variables were presented as mean rank and mean ($\pm$ standard deviation). Kruskal-Wallis test was used to compare continuous independent variables between the groups, and one-way analysis of variance was used in those with a normal distribution. Mann-Whitney U test was conducted for the comparison between the groups. A p value of <0.05 was considered statistically significant.

3. Results

Although TOS was lower in the G-CSF group (11.1 μmol H_2O_2 equivalent/L) compared to the torsion/detorsion group (13.76 μmol H_2O_2 equivalent/L), it was not statistically significant ($p > 0.05$). OSI was also lower in the G-CSF group (37.0 ± 17.2 vs 28.8 ± 11.7), but this was also not significant ($p > 0.05$). There was no statistically significant difference between the groups in terms of the TAS, catalase, MDA and t-SH values. Biochemical parameters are shown in Tables 1,2.

No primary follicle was observed in any of the rats in the torsion/detorsion group. In the G-CSF group, the number of primary follicles was similar to the sham group (3.14 vs 3.43) but significantly higher compared to the torsion/detorsion group ($p = 0.001$). The number of secondary follicles was also significantly higher in the G-CSF group than in the torsion/detorsion group (2.29 vs 0.14, $p = 0.001$). There was no statistically significant difference between the groups in terms of the numbers of tertiary and Graafian follicles ($p > 0.05$) (Table 3) (Fig. 1).

Table 1. Catalase, TAS and TOS levels of the groups.

Groups		n	Mean rank	Kruskal Wallis H	SD	*p*
Catalase U/mg protein	Group 1	7	18.14	3.777	3	0.287
	Group 2	7	10.57			
	Group 3	7	12.79			
	Group 4	7	16.50			
TAS mmol Trolox equivalent/L	Group 1	7	17.57	2.427	3	0.489
	Group 2	7	10.79			
	Group 3	7	11.93			
	Group 4	7	14.71			
TOS μmol H_2O_2 equivalent/L	Group 1	7	11.29	1.809	3	0.613
	Group 2	7	15.43			
	Group 3	7	13.00			
	Group 4	7	14.29			

TAS, total antioxidant status; TOS, total oxidant status; SD, standard deviation.

Table 2. OSI, MDA and t-SH levels of the groups.

		n	Mean $\pm$ SD	F	*p*
OSI	Group 1	7	23.41 $\pm$ 8.3	1.242	0.316
	Group 2	7	37.00 $\pm$ 17.2		
	Group 3	7	34.32 $\pm$ 14.3		
	Group 4	7	28.80 $\pm$ 11.7		
MDA nmol/μg protein	Group 1	7	85.67 $\pm$ 31.0	0.045	0.987
	Group 2	7	78.18 $\pm$ 9.7		
	Group 3	7	79.73 $\pm$ 58.0		
	Group 4	7	84.96 $\pm$ 58.0		
t-SH mmol/g protein	Group 1	7	81.56 $\pm$ 16.1	0.381	0.768
	Group 2	7	77.21 $\pm$ 13.3		
	Group 3	7	76.66 $\pm$ 42.7		
	Group 4	7	85.32 $\pm$ 42.5		

OSI, oxidative stress index (TOS/TAS); MDA, malondialdehyde; t-SH, total thiol; SD, standard deviation.

4. Discussion

Ovarian torsion treatment should be aimed at protecting the ovarian reserve, as well as relieving symptoms. The duration of ischemia in patients with ovarian torsion is an important factor to reduce possible ovarian damage. In this study, 3 hours of I/R was performed. Previous studies showed morphological and biochemical changes after I/R injury at different time intervals and revealed that a 3-hour torsion-3-hour reperfusion time is sufficient for I/R injury to occur [27,28]. It is known that ovarian tissue can be seriously damaged by not only ischemia but also reperfusion. After reperfusion in tissue, free oxygen radicals can cause protein denaturation, lipid peroxidation and DNA damage, which are mainly causing tissue damage. Therefore, I/R in ovarian torsion is an important issue that has often been the subject of many studies, and various substances have been used to reduce reperfusion injury in rat ovaries [29–32]. The presence of free radicals produced due to reperfusion suggests that the effects of most agents used in I/R models are due to their antioxidant properties.

G-CSF is thought to have a protective effect in reperfusion after ischemia due to its inhibitory effect on inflammation and oxidative stress. For this reason, it has been used for treatment and protection in I/R injury of many organs [18–22]. However, G-CSF using does not ameliorate in all types of injury and all tissues [33]. Although the antioxidant effect of G-CSF has also been demonstrated in I/R in rats both biochemically and histopathologically [16,34], we found no significant difference between the groups in relation to biochemical markers evaluated in our study. However, the primary and secondary follicle counts were significantly higher in the histopathological examination in the group that received G-CSF compared to the rats that underwent torsion/detorsion alone.

In cases of ovarian torsion treated with detorsion, histopathological changes are first observed in the Graafian follicles, while primary follicles are affected last [35]. In the current study, the use of G-CSF before detorsion prevented the reduction of primary and secondary follicles numbers in the rat ovary, similar to previous studies indicating histopathological improvement in the rat ovary follow-

Table 3. Histopathological evaluation of the groups and comparison of the follicle numbers.

Groups		n	Mean rank	Kruskal- Wallis H	SD	*p*
Primordial follicle	Group 1	7	18.3	4.397	3	0.222
	Group 2	7	11.00			
	Group 3	7	12.00			
	Group 4	7	16.07			
Primary follicle	Group 1	7	17.00	14.910	3	0.002*
	Group 2	7	4.50			
	Group 3	7	7.29			
	Group 4	7	17.21			
Secondary follicle	Group 1	7	16.36	12.007	3	0.007*
	Group 2	7	5.57			
	Group 3	7	8.86			
	Group 4	7	17.21			
Tertiary follicle	Group 1	7	17.71	3.187	3	0.364
	Group 2	7	10.50			
	Group 3	7	11.11			
	Group 4	7	16.07			
Graafian follicle	Group 1	7	16.29	2.358	3	0.502
	Group 2	7	10.50			
	Group 3	7	12.43			
	Group 4	7	15.79			

*$p < 0.01$.

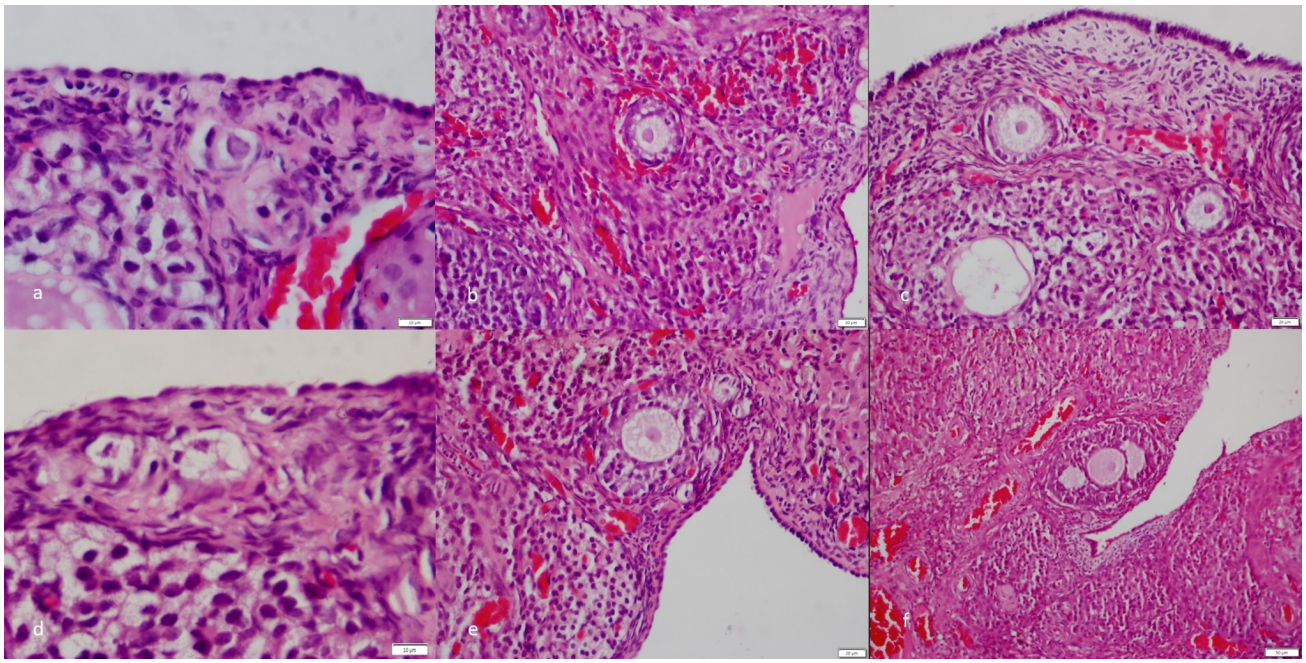

Fig. 1. Images of histopathological examination obtained by light microscope. (a) Primordial follicle in the ovary of a rat in the sham group. (b) A normal primary follicle in the ovary of a rat in the torsion/detorsion + G-CSF group. (c) A normal secondary follicle in the ovary of a rat in the torsion/detorsion + G-CSF group. (d) A degenerated primary follicle in the ovary of a rat in the torsion/detorsion group. (e) A degenerated secondary follicle in the ovary of a rat in the torsion/detorsion + saline group. (f) A degenerated Graafian follicle in the ovary of a rat in the torsion/detorsion + saline group.

ing detorsion with the use of G-CSF [16,34]. It has been previously shown that G-CSF has an anti-apoptotic effect on vascular endothelial cells, and it was suggested to be the most effective mechanism to reduce reperfusion injury [22].

In the literature, the presence of G-CSF receptors has been demonstrated in ovarian and endometrial tissue [36, 37]. However, there is no study that detected receptors in tissue in an I/R model established with G-CSF, and we did

not measure them in our study either. We speculate that differences in tissue response across studies may be related to the number of these receptors. The number of receptors can affect the antiapoptotic response in tissue, thus reducing tissue reperfusion injury and resulting in a lower level of biochemical markers.

Reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical and superoxide are essential components of oxidative damage in I/R. A large number of biochemical markers are used to identify the overproduction of ROS and reveal the degree of associated damage. Although we investigated several markers in this study, we found no significant difference between the groups with and without torsion/detorsion. The main mechanism in torsion is the complete or partial cessation of blood flow to the tissue, causing damage. The main determining factors in this damage are the degree and duration of torsion of the vascular pedicle. Blood flow has been observed in some cases of ovarian torsion even torsion was applied up to 360 degrees and it has also been shown that the damage increases as the duration of torsion increases [38]. Therefore, retrospectively reviewing the technique we applied in our model, we consider that 360-degree torsion may not be sufficient.

As a limitation of our study, we did not investigate different doses of G-CSF or examine the effect of G-CSF on healthy rat ovarian tissue. In addition, although we used many markers were used to determine antioxidant activity, unlike previous studies on the subject, the use of G-CSF in the I/R model did not result in a significant difference in relation to these markers. However, there was significant histopathological improvement in the group where G-CSF was administered.

5. Conclusions

In conclusion, although oxidative stress markers did not significantly differ between the groups, primary and secondary follicle numbers in rat ovaries were significantly higher in those using G-CSF.

Author Contributions

SAyd—Protocol/project development, data analysis, manuscript writing/editing. MG—Protocol/project development, data analysis, manuscript writing/editing. MCA—Data collection/management. SAyh—Data collection/management. MES—Protocol/project development, data collection management. MK—Data collection/management. MÇ—Data collection management. MŞ—Data collection management. YÜ—Protocol/project development, data analysis. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

After obtaining approval from the Animal Experiments Local Ethics Committee of Health Sciences University Ankara Health Research Application Center (approval number: 552/2019), the study was conducted in accordance with the international guidelines for the ethical use of animals.

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Conflict of Interest

The authors declare no conflict of interest.

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