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Research Article

Granulocyte colony stimulating factor (GCSF) protected in ovarian tissues against ischemia-reperfusion injury

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Article Info:



Article History:

Received 18 May 2022
Reviewed 16 June 2022
Accepted 21 June 2022
Published 15 July 2022

Cite this article as:

Aktaş A, Aşır F, Başaran SO, Kaplan O, Ermiş IS, Deveci E, Granulocyte colony stimulating factor (GCSF) protected in ovarian tissues against ischemia-reperfusion injury, Journal of Drug Delivery and Therapeutics. 2022; 12(4):26-30

DOI: <http://dx.doi.org/10.22270/jddt.v12i4.5538>

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Abstract

Ovarian torsion is an important reproductive gynecological emergency in daily life and physiology. Granulocyte colony stimulating factor (G-CSF) is a glycoprotein capable of hematopoietic development and successfully used in the treatment of congenital granulocytopenia. In our study, we aimed to investigate protective effects of G-CSF on ovarian ischemia reperfusion (IR) immunohistochemically. Four groups were used. In control group, abdomen was opened and closed with the surgical protocol. The rats in the G-CSF group were given 100 µg/kg G-CSF subcutaneously before the IR and the abdomen was surgically opened and closed. 3-h of ischemia was and then 3-h reperfusion was induced in the ischemia-reperfusion (I/R) group. In the IR+G-CSF group, 100 µg/kg G-CSF was given before the procedure and IR was performed. At the end of the experiment, ovarian tissues were fixed in 10% formalin and then processed for routine paraffin tissue protocol. Normal ovarian histology was observed in the control and G-CSF groups. In the IR group, vascular dilatations, hemorrhage and increased inflammation were observed. In the I/R+G-CSF group, pathology in seen IR was decreased. IL-6 expression was mainly negative in control and G-CSF groups. Positive IL-6 immune reaction was observed in the granulosa cells and stromal area. In the I/R+G-CSF group, IL-6 expression was significantly decreased in ovarian follicular structures and in the stromal area compared to the I/R group. In conclusion, G-CSF reduced vascular dilatation and inflammation in the ovarian IR model and promoted ovarian folliculogenesis.

Keywords: Ovary, Ischemia/reperfusion, G-CSF, Azan, IL-6, Immunohistochemistry

1. Introduction

Ovarian torsion is the rotation of the peduncle, which contains the arterial and venous vessels of the ovary, on its axis in a partial or full turn, preventing blood flow. As a result of torsion, ischemia develops in tissues and tissue damage occurs. Ovarian torsion is seen in 2.7% of gynecological emergencies. Although it affects all age groups from female fetuses to menopause, it is observed at a higher rate in women of reproductive age ^{1,2}. The etiology of ovarian torsion is not known exactly, but most cases have functional cysts or neoplasia of the ovary. Delay in the diagnosis may cause the patient to lose the ovary or decrease the follicle reserve ³. When clinical signs of acute abdomen (peritoneal irritation) are present, most cases go to diagnostic laparotomy or laparoscopy. However, in most cases of torsion, the clinical findings are indistinct. As a result, delays occur in diagnosis and treatment ⁴.

Granulocyte colony stimulating factor (G-CSF) is in glycoprotein structure and is currently actively used in the treatment of congenital neutropenia (abnormal low level of neutrophils in the blood). It is also an effective drug in the treatment of neutropenic complications in chemotherapy patients⁵. G-CSF has growth factor and cytokine functions as well as proliferation of hematopoietic cells, antiapoptotic and anti-inflammatory effects. G-CSF has also been proven to have a protective effect against ischemia-reperfusion (I/R) injury in

some cell types such as neural, muscle, cardiovascular and cerebral cell models ^{6,7}.

IL-6 is a low molecular weight proinflammatory cytokine associated with many cell types including neutrophils, macrophages, monocytes, fibroblasts, endothelial cells, and some tumor cells ⁸. Moreover, the IL-6 receptor is also expressed by the blastocyst, trophoblast, and endometrium, and in patients with endometriosis. IL-6 response is impaired in endometrial stromal cells and peritoneal and peripheral macrophages ⁹. IL-6 is secreted as an acute phase reactant in many tissues secondary to infection and acts as an immunomodulator ¹⁰.

In this study, the effect of subcutaneously administered GCSF on IL-6 in ovarian ischemia-reperfusion injury was investigated.

2. Materials and Methods

Ethics committee approval of this study was obtained from Dicle University Animal Experiments Local Ethics Committee (protocol no: 2020/44). This study was supported by the Dicle University Scientific Research Platform (project no: TIP.21.019).

The estrus cycles of the rats were detected by vaginal smear taken at 6-12 hour intervals. After cell examination under the microscope, 24 female rats in oestrus cycle were included in the experiment. Before starting the experimental procedure, 90 mg/kg intramuscular ketamine hydrochloride (Ketalar;

Pfizer, Istanbul, Turkey) and 8 mg/kg xylazine (Rompun; Bayer, Istanbul, Turkey) were given general anesthesia. Rats were divided into four groups with eight rats in each group.

Control group: No treatment was applied to the animals. Only the abdomen was opened with the surgical protocol and the abdominal layers were closed without any other intervention.

G-CSF group: 100 µg/kg G-CSF (Neupogen, Roche, Basel, Switzerland) was given subcutaneously 60 minutes before starting the protocol. Under general anesthesia, the abdomen was opened with a 2 cm lower midline incision. Abdominal layers were closed without any further intervention.

Ischemia/reperfusion I/R group: The abdomen was opened with a 2 cm lower midline incision under general anesthesia. Ovarian adnexa containing ovarian tissues were taken out and ischemia was created for 3 hours with a disposable Bulldog clamp. Then, the ovaries were placed back in their anatomical position and reperfused for 3 hours.

IR+G-CSF group: 100 µg/kg G-CSF (Neupogen, Roche, Basel, Switzerland) was given subcutaneously 60 minutes before starting the protocol. The abdomen was opened with a 2 cm lower midline incision under general anesthesia. Ovarian adnexa containing ovarian tissues were taken out and ischemia was created for 3 hours with a disposable Bulldog clamp. Then, the ovaries were placed back in their anatomical position and reperfused for 3 hours.

At the end of the experimental procedure, animals were sacrificed under general anesthesia. The ovaries of the rats were fixed in 10% neutral zinc-formalin solution for histological tissue protocol. After 72 hours of fixation, ovarian tissues were passed through ascending alcohol and washed in xylene. 5 micron sections were taken from the paraffin blocks with a rotary microtome and Azan staining was performed to examine the tissue histopathology. IL-6 protein expression levels were examined for immunohistochemical analysis.

Azan Staining Protocol

Sections were brought to distilled water. They were stained with Azocarmine G for 30 minutes in a 60-degree oven. Sections were allowed to cool for 5 minutes and washed in distilled water. 10 drops of Reagent B solution were dropped and waited for 1 minute. Then 10 drops of Reagent C were dropped and waited for 1 minute. Without washing the sections, the D reagent was dropped and waited for 30 minutes. 10 drops of the E reagent were added and stained for 30 minutes. After this step, the sections were washed in 95% ethanol. They were passed through a descending alcohol series. After clearing in xylene, the sections were closed with mounting medium and evaluated under a Zeiss Imager A2 photomicroscope.

Immunohistochemistry Staining Protocol

Immunohistochemical protocol was performed according to Ozkorkmaz et al¹¹. 5 µm thick sections taken from paraffin blocks on slide were kept in an oven at 58-62°C for 6 hours to dissolve excess paraffin. Afterwards, the sections were kept in xylene for 3x15 minutes and dewaxed. Sections were passed

through decreasing alcohol series (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes and kept in distilled water for 5 minutes. Sections were washed 3x5 minutes in phosphate buffer solution (PBS). Hydrogen peroxide solution (catalog no: TA-015-HP, ThermoFischer, Fremont, CA, USA) was dripped onto the sections and repeated after 10 minutes and waited for a total of 20 minutes. Then, it was washed again with PBS for 3X5 minutes and kept in Ultra V Block (catalog no: TA-015-U.B, ThermoFischer, Fremont, CA, USA) solution for 7 minutes. Sections were overnight at +4°C with IL-6 antibody. The next day, the sections were left at room temperature for 1 hour and washed with PBS. Secondary antibody with biotin (catalog no: TP-015-BN, ThermoFischer, Fremont, CA, USA) was dripped onto the sections and incubated for 14 minutes. Then, streptavidin-peroxidase (catalog no: TS-015-HR, ThermoFischer, Fremont, CA, USA) was dripped and left for 15 minutes. Diaminobenzidine (DAB) (catalog no: TA-001-HCX, ThermoFischer, Fremont, CA, USA) was dripped onto the sections. After soaking the tissues in PBS for 15 minutes, counterstaining was performed with Harris hematoxylin. Afterwards, the sections were covered with entellan (catalog no: 107961, Sigma-Aldrich, St. Louis, MO, United States), evaluated under a Zeiss Imager A2 photomicroscope and visualized.

3. Results

Histochemical analysis of ovarian tissues is shown in Figure 1 with Azan staining. In the control group sections, the germinal epithelium was cylindric and ciliated. Ovarian follicles were and collagen fiber in the stroma were regular (Figure 1a). Development of ovarian follicles was regular in the GCSF group. No pathology was found in the stromal area and a histology similar to the control group was observed (Figure 1b). In the IR group, fibrosis, vascular dilatation and congestion were observed in the theca follicles and stromal area. Dense inflammatory cell was observed in the mesovarium (Figure 1c). In the IR+GCSF group, folliculogenesis and corpus luteum development were observed. A decrease in vascular dilatation, stromal fibrosis and inflammation cells was observed (Figure 1d).

IL-6 immune reactivity of ovarian tissues in the groups is shown in Figure 2. In the control group sections, mostly negative IL-6 expression was observed in the granulosa cells and stromal cells of preantral and degenerated follicles. IL-6 expression was positive in some cells in the stroma. IL-6 expression was negative in the germinal epithelium (Figure 2a). In the GCSF group, mostly negative IL-6 expression was observed in the granulosa cells and stromal cells of preantral, antral and degenerated follicles. IL-6 expression was negative in the germinal epithelium (Figure 2b). In IR group ovarian sections, IL-6 expression was very intense in connective tissue cells and inflammatory cells. IL-6 expression was intense in granulosa cells of preantral follicles (Figure 2c). Compared to the IR group, IL-6 expression was decreased in the follicles, stromal area and germinal epithelium, and IL6 immune reactivity was generally negative in the IR+GCSF group (Figure 2d).

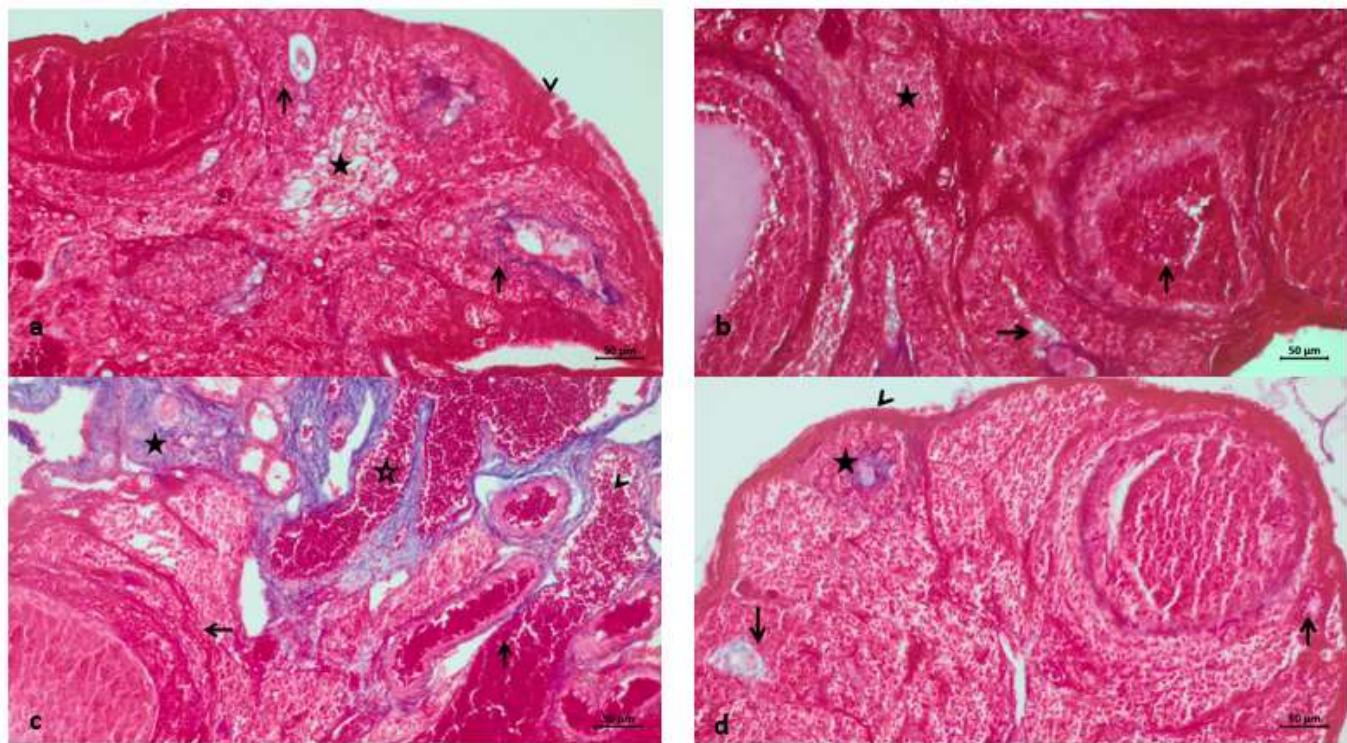


Figure 1: Azan Staining **a)** control group: Germinal epithelium (arrowhead), ovarian follicles (black arrow), collagen fiber in the stroma (star); **b)** G-CSF group: Ovarian follicles (arrow), stromal area (star); **c)** IR group: Follicles (arrow), fibrosis in the stromal area (star), vascular dilatation and congestion (empty star), dense inflammatory cell in the mesovarium (arrowhead); **d)** IR+G-CSF group: Germinal epithelium (arrowhead), folliculogenesis (arrow) and corpus luteum (star). Azan Staining, Bar: 50 μ m

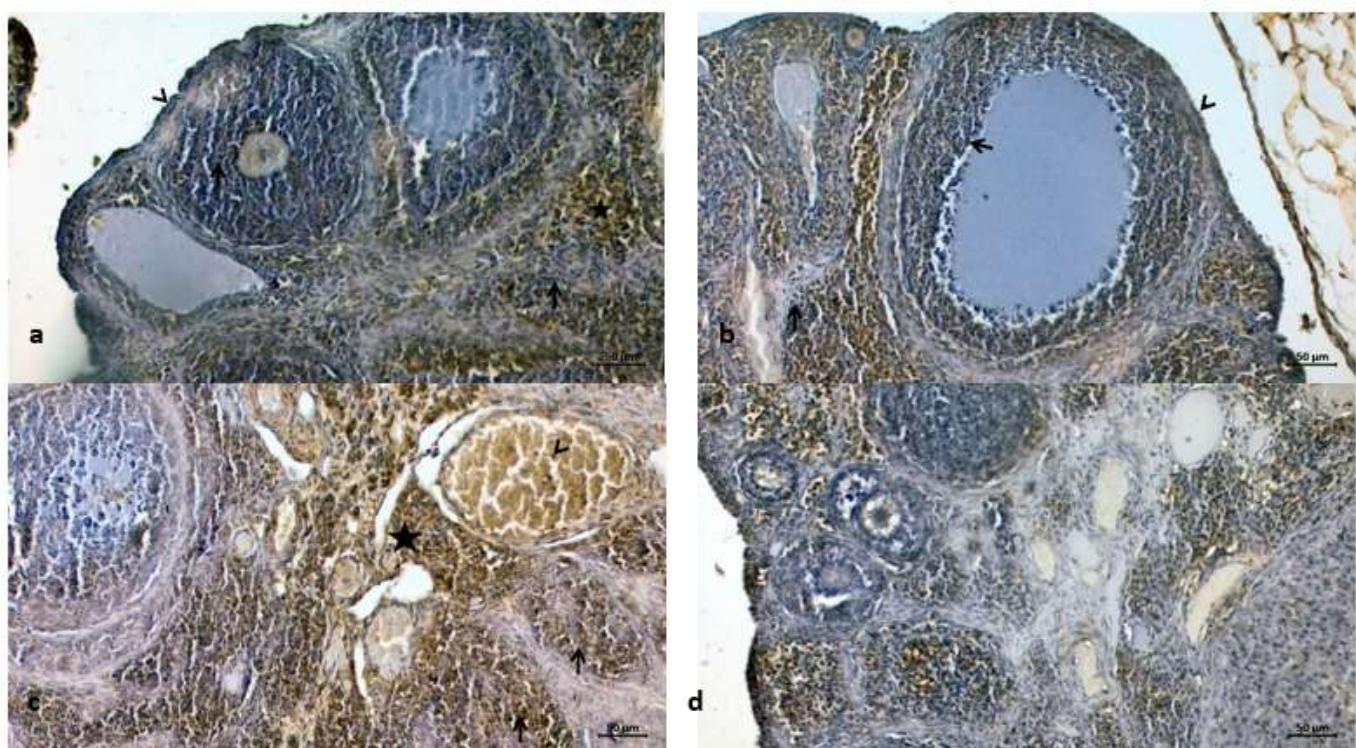


Figure 2: IL-6 immunostaining **a)** control group: negative expression in germinal epithelium (arrowhead), granulosa cells and stromal cells (arrow); **b)** G-CSF group: negative expression in granulosa cells, stromal cells (arrow) and germinal epithelium; **c)** IR group: positive expression in preantral follicles, connective tissue cells (star) and inflammatory cells (arrowhead); **d)** IR+G-CSF group: Decreased expression in follicles (arrow), stromal area (star) and germinal epithelium (arrowhead). IL-6 immunostain, Bar: 50 μ m

4. Discussion

Reduction or absence of blood flow to tissues or organs is called ischemia (I), and re-blooded of this tissue or organ is called reperfusion (R). Normally, the ischemia process damages the tissues as a result of the production of many free radicals, but this damage may increase after the reperfusion procedure. This phenomena is called ischemia-reperfusion (I/R) injury. Ovarian IR injury occurs as a result of the rotation of the ovarian adnexa around itself. Ovarian IR occurs especially in women of reproductive age and may cause infertility if treatment is delayed¹².

Bostancı et al. applied 3 hours of ischemia and 3 hours of reperfusion in their ovarian ischemia reperfusion study in Sprague Dawley rats. The authors stated edema, vascular congestion, bleeding, and ovarian tissue damage in the histopathological results. They said that GCSF treatment after ovarian IR preserved ovarian histology¹³. Hortu et al. investigated the protective effect of GCSF in the ovarian IR study. Similarly, they applied 3 hours of ischemia and 3 hours of reperfusion and recorded edema, vascular congestion and hemorrhage in their histopathological findings. The authors stated that GCSF application reduced edema, and no hemorrhage and follicular degeneration were observed. They stated that GCSF has a protective effect on ovarian IR damage¹⁴. Akdemir et al. investigated the protective effect of GCSF in cisplatin-induced ovarian damage. They stated that the number of ovarian follicles decreased significantly in the cisplatin-administered groups, but GCSF treatment after cisplatin preserved the ovarian follicle reserve and returned the number of follicles to its previous level¹⁵. Asir et al studied effects of Momordica charantia on ovarian IR. They found that IR cause degenerated follicles, vascular dilatation, inflammation and fibrin accumulation in ovarian tissues¹⁶. In an ovarian IR study performed by Peker et al, the author investigated effects of carvacrol on ovarian tissue by immunohistochemical method. In the ovarian sections of IR group, the authors found edema, inflammation, congestion, degenerated follicles, and cells with pyknotic nuclei¹⁷. In the AZAN staining of our study, it was observed that ovarian histology was normal in the control and GCSF groups, ovarian follicle development continued, and the stromal area and germinal epithelium were seen regular. In the ischemia-reperfusion (IR) group, intense fibrosis, vascular dilatation and congestion, and inflammation were observed in the stromal area. It was observed that after IR GCSF treatment, folliculogenesis and corpus luteum development continued and IR-induced pathology decreased (Figure 1).

IL-6 is a molecule that belongs to the interleukin protein family and has pro- and anti-inflammatory properties. IL-6 is secreted immediately in tissue damage and inflammation and takes a role in host defense as a result of immune reactions¹⁸. IL-6 level was investigated in the retinal ischemia reperfusion study of Ruben et al. The authors stated that IL-6 level increases rapidly after retinal IR¹⁹. Wang et al. investigated the expression levels of IL-6 in retinal IR injury. The authors stated that IL-6 levels increase in phagocytic cells after ischemia¹²⁰. In our study, generally negative IL-6 expression was observed in the control and GCSF groups. After ovarian IR, an intense IL-6 expression was observed in the ovarian follicles and stromal area. After IR, GCSF treatment suppressed IL-6 expression, and mostly negative expression was observed in the ovarian follicles and stroma (Figure 2).

In conclusion, ischemic reperfusion injury has a degenerative effect on ovarian follicles and germinal epithelium, leading to defects in oocyte 2 development. G-CSF downregulated the inflammation signal and could contribute to follicular development and oocyte formation by reducing the degeneration and apoptotic effect.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

All animal procedures in this study were performed with the permission of the Dicle University Animal Experiments Local Ethics Committee with protocol number 2020/44. Dicle University Scientific Research Platform was financed by the project numbered TIP.21.019 for this study

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