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Research Article
Biochemical and Histopathological Evaluation of Sunitinib Effect on Ovarian Injuries by Ischemia-Reperfusion in Rats

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Abstract

Background and Objective: Interleukin 1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α) as the proinflammatory cytokines, whose production increases during reperfusion after ischemia (I/R), were reported to produce the Reactive Oxygen Species (ROS). It was reported that sunitinib has proinflammatory cytokine expression suppression and antioxidant effects. The study aims to investigate the effects of sunitinib on ovarian injuries created by I/R in albino Wistar female rats biochemically and histopathologically. **Materials and Methods:** Animals divided into 3 groups as sunitinib+ovarian tissues (SOIR), I/R ovarian tissues (OIR) and Sham operation (SG) groups. There were ischemia and reperfusion procedures for 2 hrs on the right ovaries of the OIR and SOIR group rats. **Results:** The Nuclear Factor kappa B (NF-κB), Tumour Necrosis Factor-alpha (TNF-α), Malondialdehyde (MDA) and Interleukin 1 beta (IL-1β) levels in the OIR group's ovarian tissue were high whereas their total glutathione (tGSH) level was found to be low compared to the SOIR and SG group. While distinct histopathologic injuries were seen in the ovarian tissue of the OIR group, histopathologic injuries in the SOIR group were mild. **Conclusion:** Sunitinib prevented the oxidant and proinflammatory cytokine increase and antioxidant decrease related to I/R. It is concluded that sunitinib might be used for the treatment of ovarian I/R injuries.

Key words: Sunitinib, cytokine, ischemia, reperfusion, histopathologic injuries, SOIR group, malondialdehyde

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

As it is known, while ischemic injuries are seen in ovarian torsion, reperfusion injuries occur after the detorsion procedure, which is performed to restore blood supplies in torsionedovaries[1]. This is known as the injury of ischemia-reperfusion (I/R) in medicine. Ovarian torsion is a serious surgical gynaecological emergency that can be seen at any age in women and can result in losing the ovary[2,3]. Thus, the first intervention is to ensure reperfusion with ovarian detorsion in the torsion ovaries. However, the reperfusion of the ovarian tissue after the detorsion procedure causes more severe injuries in the tissue than the injuries created by ischemia related to torsion[3]. During reperfusion, the O_2 given with a large volume of blood to the tissue with ischemia induces the hypoxanthine's metabolism with xanthine oxidase, excessively accumulating in the ischemia period. This event causes the overproduction of byproducts such as ROS[4,5]. In the literature, the ROS's produced in the reperfusion period is known as reperfusion mediators. ROS's cause further cell damage from lipids by oxidizing lipids of the cell membrane and creating toxic products such as MDA, which causes the death of cell[6]. In the ischemia after the reperfusion period, proinflammatory cytokines with increased production including Interleukin 1 beta (IL-1β) and Tumour Necrosis Factor-alpha (TNF-α) were reported to increase ROS production[7,8].

The effects of sunitinib will be examined on protection against ovarian I/R injuries, as an inhibitor of tyrosine kinase, which is the anticancer medicine curing gastrointestinal and pancreas neuroendocrine tumours resistant to imatinib and metastatic renal carcinoma[9]. It has been noted that sunitinib decreases reduced glutathione (GSH), which is an endogen antioxidant related to cisplatin and prevents MDA increase[10]. It has been shown that sunitinib distinctly inhibits TNF-α production and IL-1β expression[11,12]. Moreover, it has been stated that sunitinib suppresses the basal activity of the pathway of the Nuclear Factor kappa B (NF-κB) 13. Data obtained from the literature suggests that sunitinib may be useful for the treatment of ovarian I/R injuries. There were no studies in the literature, investigating the effects of sunitinib on the injury of ovarian I/R.

This study aims to study the effects of sunitinib on ovarian injuries created with I/R in rats biochemically and histopathologically.

MATERIALS AND METHODS

Study area: The study was carried out at the Medical Experimental Application and Research Center of Ataturk University, Turkey from January-March, 2020.

Animals: The 18 albino Wistar female rats (265-277 g) were used in the experiment and they were collected from the Medical Experimental Application and Research Center of Ataturk University, Turkey. Before experimenting, the rats were included and fed in groups at normal room temperature (22°C). The local Animal Experimentation Ethics Committee (Date: 16.01.2020 meeting no: 88012460-804.01-E.2740) approved the procedures and protocols.

Chemical substances: The ketamine was supplied by Pfizer medicine Co. Ltd., (Turkey) and sunitinib (Sutent) was supplied by Pfizer to be used in the experiment (USA).

Animal groups: There was a division of the albino Wistar female rats in our study into groups as, the ones who will undergo Sham Operation (SG), I/R to be applied to their ovarian tissues (OIR) and the I/R procedure to be applied to their sunitinib+ovarian tissues (SOIR).

Experimental procedure: There were surgical interventions in a suitable laboratory environment under sterile conditions, by making them inhale 60 mg kg^{-1} IP ketamine anaesthesia and xylazine (inhaler) at proper intervals. The rats were put on standby at the appropriate time for the surgical intervention after injection of the ketamine. The suitable anaesthesia period for surgical intervention was the period during which the animals were motionless in the supine position. Oral administration of a sunitinib dosage of 25 mg kg^{-1} in the SOIR group (n-6) of animals was done with a probe to the stomach before the anaesthesia. Distilled water with equal volume was given as a solvent to the OIR (n-6) and SG (n-6) group rats in the same way. During the anaesthesia, the ovaries of all rat groups were reached by vertically opening the lower part of the abdomen at a 2-2.5 cm length. Then, ischemia was created for 2 hrs by applying vessel clips to the right ovaries of the OIR and SOIR group rats on the lower part (the ovaries of the SG group were closed with surgical thread without creating ischemia). Once this period was finished, the vessel clips were removed to ensure two hrs of reperfusion. After reperfusion, the ovaries of the animals killed with high dosage ketamine (120 mg kg^{-1}) anaesthesia were removed. Biochemical analyses were performed on one part of the removed ovarian tissue and there were histopathologic examinations on the

other. The biochemical and histopathologic results obtained from the SOIR and OIR groups were evaluated by comparing them with SG.

Biochemical analysis

Preparing the samples: During this phase of the study, 0.2 g were weighed from each removed tissue. For the MDA assignment, a 1.15% potassium chloride solution was completed to 2 mL in a phosphate buffer that was pH = 7.5 for the tGSH measurement and homogenized in an icy environment.

Then, it was centrifuged for 15 min at +4°C, 10000 rpm the analysis sample was the supernatant part.

Malondialdehyde (MDA) determination: MDA measurement is based on the spectrophotometric measurement (at 532 nm) of absorbance of a pink coloured complex formed by thiobarbituric acid (TBA) and MDA at high temperature (95°C)[14].

Total glutathione (tGSH) determination: DTNB [5,5'-Dithiobis (2-nitrobenzoic acid)] in the measurement medium is a disulfide chromogen and is readily reduced by compounds with sulfhydryl groups. The resulting yellow colour was measured spectrophotometrically at 412 nm[15].

Analysis of NF-κB, IL-1β and TNF-α: The rat specific sandwich enzyme-linked immunosorbent test was used to measure the tissue-homogenate NF-B and TNF-α concentrations. Rat NF-κB ELISA immunoassay kits (Cat. No.: 201-11-0288, SunRed). Rat TNF-α and Rat IL-1β ELISA kits (Cat no.: YHB1098Ra, Shanghai LZ).

Histopathological examination: Light microscope assessment required the identification of all of the tissue samples in a 10% formaldehyde solution. After the identification process, tap water in cassettes was used to wash the tissue samples for 24 hrs. the water within tissues was removed by treating the samples with a conventional grade of alcohol (80, 90, 70 and 100%). Through xylol, the tissues were then passed and fixed in paraffin. Sections with four to five microns were cut from the blocks of paraffin and hematoxylin-eosin staining was applied. The Olympus DP2-SAL firmware program was used to take their photos (Olympus® Inc. Tokyo, Japan). The pathologist blinded to the study groups conducted the histopathological assessment.

Statistical analysis: Experimental results were shown as "mean value±standard deviation" (x±SD). Normality assumption for all measurements was checked with the Kolmogorov Smirnov test and all parameters were normally distributed. A one-way ANOVA test was used to determine the significance level of the difference between groups. According to the homogeneity of variances Tukey's HSD or Games-Howell test was used as a *post hoc* test for comparisons of the groups. the "IBM SPSS 22" program (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used for all statistical procedures. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

Biochemical results

Results of MDA and tGSH analysis: The data in Fig. 1a shows that the ovarian tissue had a significant increase in the

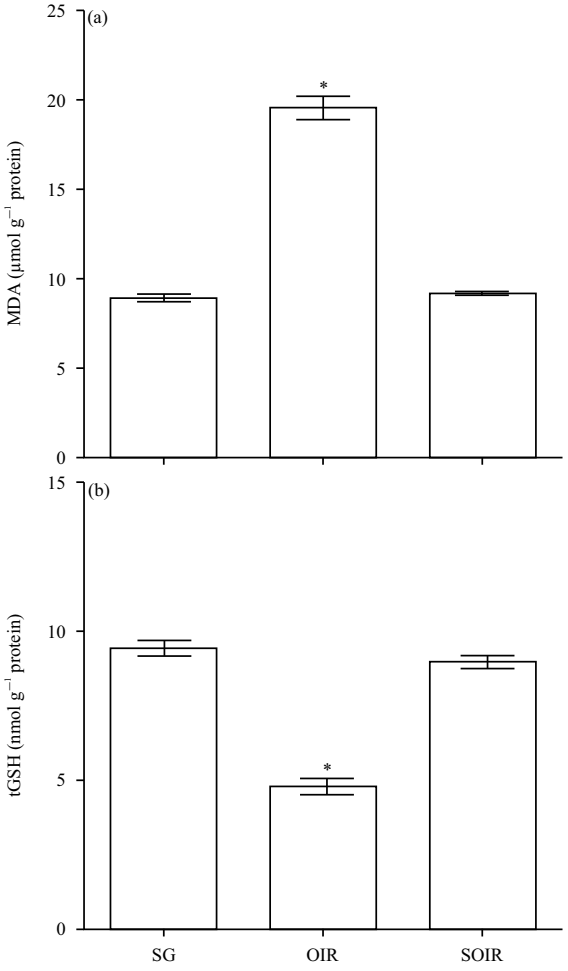

Fig. 1(a-b): Malondialdehyde (MDA) and total intracellular glutathione (tGSH) levels in the ovarian tissue of study groups

*p<0.001 according to OIR group (n = 6). Sham Operation (SG), I/R to be applied to their ovarian tissues (OIR) and the I/R procedure to be applied to their sunitinib+ovarian tissues (SOIR)

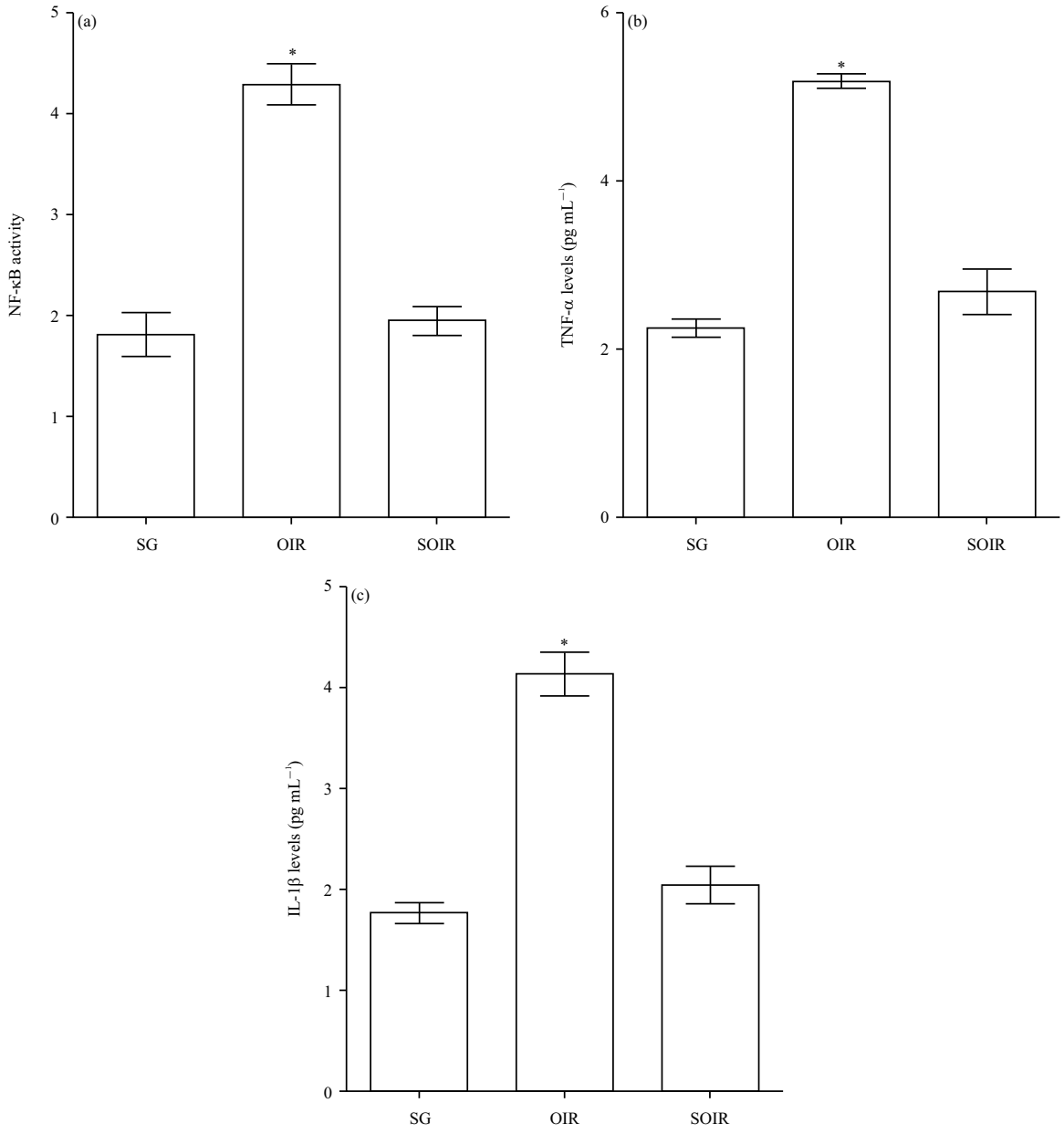

Fig. 2(a-b): NF-κB, TNF-α and IL-1β levels in the ovarian tissue of study groups

*p<0.001 according to OIR group (n = 6). Sham Operation (SG), I/R to be applied to their ovarian tissues (OIR) and the I/R procedure to be applied to their sunitinib+ovarian tissues (SOIR)

amount of MDA due to the I/R procedure as compared to the group treated with sunitinib (p<0.001) and the sham group (p<0.001). It was found that the MDA amount was very close in the sham group and group treated with sunitinib. Also, it was found that the tGSH amount in the ovarian tissue that underwent the I/R procedure was significantly low compared to the sham group (p<0.001) and the group with sunitinib (p<0.001). Sunitinib prevented tGSH decrease related to I/R and ensured that it stayed at an almost close level to the sham group (Fig. 1b).

Results of NF-κB, TNF-α and IL-1β analysis: The data in Fig. 2a-c shows that the NF-κB, TNF-α and IL-1β levels in the ovarian tissues that underwent the I/R procedure showed a significant increase compared to the sham group (p<0.001, p<0.001, p<0.001, respectively) and the group with sunitinib (all p<0.001, p<0.001, p<0.001, respectively). Sunitinib significantly prevented the increase of NF-κB, TNF-α and IL-1β levels in the ovarian tissues that underwent I/R The NF-κB, TNF-α and IL-1β levels between the sunitinib and sham groups were found to be very close to each other.

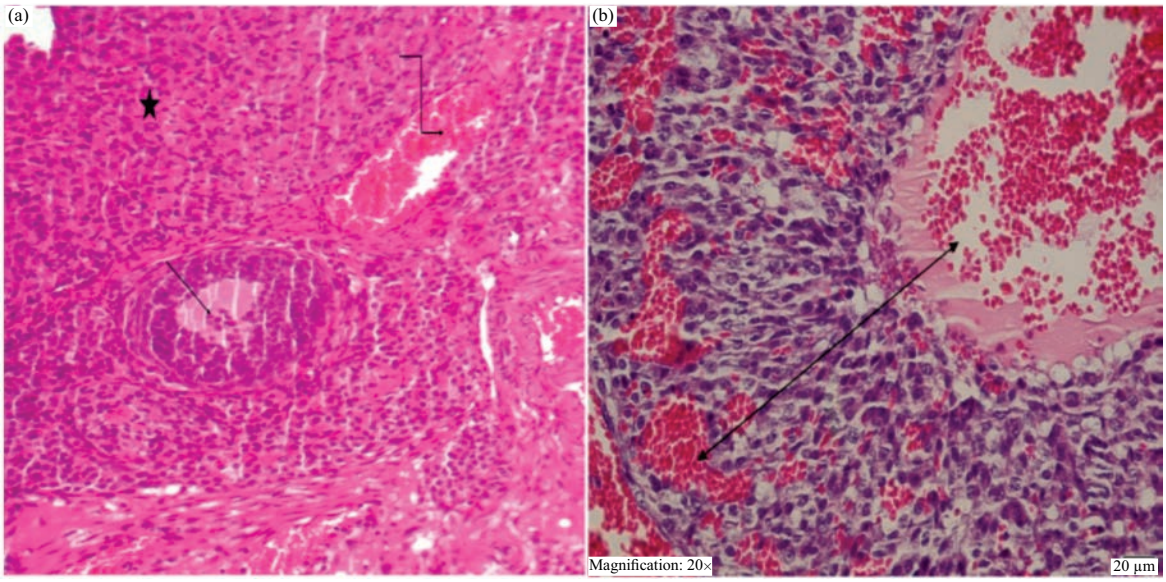

Fig. 3(a-b): Histopathological representation of ovarian tissue of sham and OIR groups

(a) In the ovarian tissue of the Sham Group (SG), corpus luteum (star), developing follicle structure (arrow), mild congestion (zigzag arrow) (H and E ×200) and (b) In the ovarian tissue of the group that underwent I/R (OIR); histopathology of degenerated secondary follicles containing haemorrhage areas (double-sided arrow) (HE ×400)

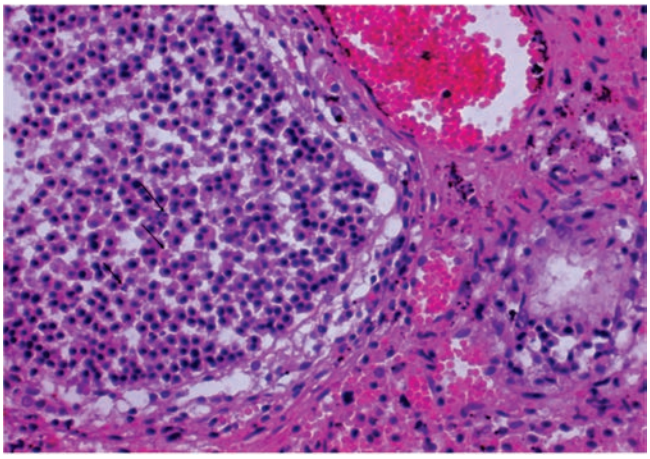

Fig. 4: Histopathological representation of apoptosis in granulosa cells in OIR group

In the ovarian tissue of the group that underwent I/R (OIR), apoptosis (black arrows), apoptosis in granulosa cells inside of follicles (H and E ×400)

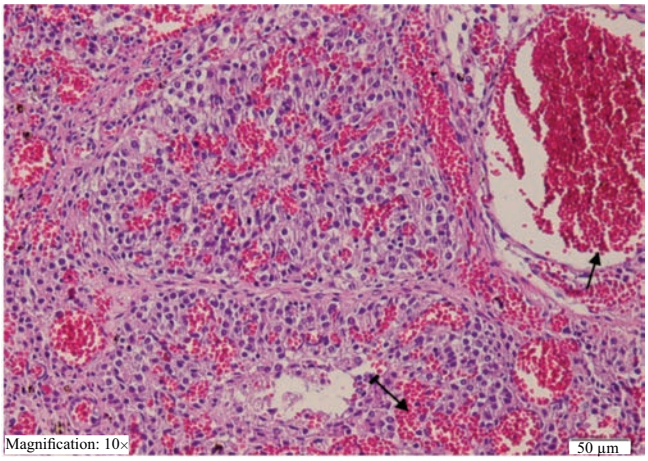

Fig. 5: Histopathological representation of ovarian tissue of SOIR group

In the ovarian tissues of the group who was administered sunitinib (SOIR); mildly dilated, congested blood vessels (straight arrow) and histopathology of haemorrhage (striped line) (H and E ×200)

Histopathological results: In Fig. 3a, the ovarian tissues of SG animals had healthy corpus luteum, developing follicle structures and mild congestion. In the ovarian tissue of the group that underwent I/R (OIR), severe haemorrhage areas and degenerated secondary follicles can be seen (Fig. 3b). Furthermore, the OIR group's ovarian tissue had apoptosis in the granulosa cells in the follicle (Fig. 4). However, there were no histopathologic findings other than congested and mildly dilated blood vessels and mild haemorrhage in the ovarian tissue of the group with sunitinib (SOIR) (Fig. 5).

DISCUSSION

This study examined the effects of sunitinib on ovarian injuries created by I/R in rats biochemically and histopathologically. Results of our biochemical experiment showed a significant increase in the MDA amount in the ovarian tissue in all of the animals in the group that underwent the I/R procedure compared to the sham group and the group with sunitinib. As it is known, during the ischemia period, the lack of oxygen in the tissue causes excessive accumulation of Xanthine Oxidase (XO) and hypoxanthine. By providing reoxygenation in reperfusion, XO metabolizes hypoxanthine xanthine and causes the creation of ROS as a by-product[16]. The reperfusion mediators or ROSs make lipids of the cell membrane oxidize and ensure that MDA as one of the toxic products is created from the lipids[17]. MDA, the last product of Lipid Peroxidation (LPO), is used as a bioindicator of oxidative stress and LPO[18]. MDA is also commonly used in evaluating oxidative ovarian injuries induced by I/R19 because MDA can create serious damage in cells by crosslinking membrane components and causing their polymerization[20]. The information obtained from the literature showed that the ROS production increased in the ovarian tissues that we applied I/R and this increase was suppressed with sunitinib.

The balance between oxidant and antioxidant in healthy tissues is kept under the dominance of antioxidants. Any factor that can result in tissue damage ensures that the balance between oxidant and antioxidant is disrupted in favour of the oxidants. It has been reported that this situation develops due to the excessive use of antioxidant systems[21]. In our study, the I/R procedure caused to increase in the MDA in the ovarian tissues and a decrease in tGSH. According to the results of the experimental results, it could be noted that the balance between oxidant and antioxidant favoured the oxidants in the I/R group and that this balance resulted against oxidants in the sham group and the group with sunitinib. Turkler *et al.*[22] have also reported that tGSH significantly decreased the ovarian tissue injuries related to I/R compared to the sham group and the group using antioxidants. The decrease in tGSH in the ovarian tissue of the group that underwent the I/R procedure indicates that the GSH endogen is insufficient in neutralizing oxidants. In this study, it was determined that the ovarian tissue that underwent I/R and had high MDA, low tGSH, displayed a significant increase in proinflammatory cytokine levels such as TNF-α, NF-κB and IL-1β compared to the sham group and group with sunitinib. As it is known, which is one of the members of the pleiotropic transcription factors family, plays an important role in regulating gene expressions taking place during inflammation periods[23]. It has been shown that activation can be induced by different molecules such as ROS[24]. This information supports the increase seen in NF-κB levels in ovarian tissues having high MDA in our study. In the literature, information about inducing TNF-α, IL-1β and other proinflammatory cytokine expressions can be found[25]. Once again, it was determined that the increase in NF-κB levels in the ovarian tissue that underwent I/R showed similarities with the increase in TNF-α and IL-1β. These results indicated consistency with the information in the literature. Previous study reported an increase of TNF-α in ovarian tissues that underwent I/R[26]. In the study by Aksak Karamese *et al.*[27], it can be seen that NF-κB and IL-1β expression was associated with MDA and GSG levels. The information obtained from the literature and our experimental results showed that the I/R case was a complicated pathological process starting with the tissue lacking oxygen continues ROS production and expands with an inflammatory response. As can be seen from our experimental results, severe haemorrhage areas, severely degenerated secondary follicles and apoptosis in follicle granulosa cells were seen in the ovarian tissue of the I/R group with high MDA, TNF-α, IL-1β levels and low tGSH levels. However, no histopathological findings were found except for mildly dilated, congested blood vessels and mild haemorrhage in the sunitinib group being close to the sham group in terms of oxidant, cytokine and antioxidant levels. Turkler *et al.*[22] also explained that histopathological symptoms such as congestion, haemorrhage and degeneration were seen in ovarian tissues they performed I/R on. Demiryilmaz *et al.*[28] reported that apoptotic cells were also seen in ovarian injuries related to I/R. Unlubilgin *et al.*[29] also expressed that histopathological symptoms were more severe in ovarian tissues with increased levels of cytokine and oxidant and decreased levels of antioxidant. On the other hand, they showed that the number of histopathological results and their severity decreased in the group being treated, with MDA and cytokine levels close to the sham group. As stated above, it has been reported that sunitinib prevents excessive MDA production and GSH consumption[10]. Furthermore, it has been noted that sunitinib suppresses the basal activity of the NF-κB pathway[13]. It has been shown that sunitinib distinctly inhibits TNF-α production and IL-1β expression[11,12]. Zhao *et al.*[11] have stated that sunitinib suppresses cytokine production. The most important implication of this study is that sunitinib significantly prevented the damage in ovarian ischemia and may be one of the most effective drugs. Its clinical applicability can be increased with the support of other studies on this subject. Since ovarian torsion is a serious gynaecological

surgery emergency that can be seen at any age in women and can result in losing the ovary, we recommended further studies in this area. Studies on sunitinib should not be limited to this.

CONCLUSION

In conclusion, the IR procedure caused oxidative and inflammatory injuries in the animal's ovarian tissues. Sunitinib inhibited the oxidant and proinflammatory cytokine increase and antioxidant decrease related to I/R. It has been histopathologically shown that sunitinib minimizes ovarian injuries related to I/R. According to the results of our experiment, sunitinib can be useful for curing the treatment of ovarian I/R injury.

SIGNIFICANCE STATEMENT

This study discovers the sunitinib can be useful for curing the treatment of ovarian I/R injury. It has been noted that sunitinib decreases reduced glutathione (GSH), which is an endogen antioxidant related to cisplatin and prevents MDA increase. Thus, a new theory on sunitinib may be useful in reducing tissue damage in ovarian detorsion.

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