RESEARCH ARTICLE

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Protective Effect of Adenosine Triphosphate against 5-Fluorouracil-Induced Oxidative Ovarian Damage *in vivo*

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Abstract

Objective: The aim of our study was to analyze the effect of ATP on possible ovarian damage of 5-FU in rats. **Methods:** The animals were divided to three groups; healthy group (HG), 5-FU alone group (FUG) and ATP+5-FU administered group (AFU). The ATP 4 mg/kg was injected intraperitoneally (IP) into the AFU group. The same volume of saline (0.9% NaCl) as the solvent was administered intraperitoneally to the HG and FUG groups. One hour after administering ATP and solvent, 5-FU 100 mg/kg was injected intraperitoneally to the animals in the AFU and FUG groups. ATP was administered to the animals once a day for 10 days. On the 1st, 3rd and 5th days of 5-FU, one dose (total of 3 doses) was administered. On day 10, the animals were euthanasia with high-dose anaesthesia and ovarian tissues were removed. The removed ovaries were analyzed biochemically andhistopathological. **Result:** ATP significantly suppressed both the increase in MDA and IL-6 levels, and the decrease in tGSH, SOD and CAT levels. Treatment with ATP significantly suppressed the severe vacuolization and primordial follicle degeneration induced by 5-FU in our study. **Conclusion:** ATP was possible to be useful for the treatment of 5-FU-induced ovarian damage.

Keywords: 5-fluorouracil- adenosine triphosphate- inflammation- ovarian- rat- reproductive

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Introduction

5-Fluorouracil (5-FU) is an antimetabolite chemotherapeutic drug that inhibits DNA synthesis with the inactivation of thymidylate synthase (Dos Santos et al., 2022; Zhang et al., 2008). It also inhibits protein synthesis affecting the RNA (Longley et al., 2003). The anticancer effect of 5-FU appears during the "S" phase of the cell cycle (Zhang et al., 2008). 5-FU is used in patients with gastric(Arjmandi et al., 2022), breast, head, neck and other cancers (Grem, 2000). 5-FU has still remained the cornerstone for colorectal cancer chemotherapy (Kobuchi et al., 2020). However, serious side effects and drug resistance phenomenon have limited its clinical use (Ciaffaglione et al., 2021). Serious cardiac side effects including cardiomyopathy, angina pectoris, ventricular tachycardia, heart failure, acute myocardial infarction and cardiogenic shock have been reported in patients treated with 5-FU (Zhang et al., 2018) . Nausea, diarrhea, vomiting, oral and intestinal mucositis, mouth ulcers, anorexia, neutropenia and thrombocytopenia are the most frequent side effects induced by the use of 5-FU (Thomas et al., 2016). Previous studies have revealed the negative effects of 5-FU upon reproductive function. The reproductive toxicity of 5-FU has been expressed to be commonly associated with ovarian damage in females (Naren et al., 2022). The cytotoxicity of 5-FU contributes upon ovarian dysfunction and causes risk of infertility (Marhhom et al., 2007). No study revealing the toxic effect of 5-FU on ovarian cells to be associated with oxidative stress has been found in the literature. However, it has been revealed that 5-FU causes oxidative damage increasing malondialdehyde (MDA) levels in heart, liver and kidney tissues and decreasing antioxidant levels such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) (Fukuno et al., 2016; Gelen et al., 2021; Refaie et al., 2022). It has been expressed in the study reports of Zhang et al. that the intracellular adenosine triphosphate (ATP) level of 5-FU decreased(Zhang et al., 2018). ATP is a nucleoside triphosphate including adenine, ribose sugar and three phosphate groups (Dunn et al., 2022). It has also been reported that ATP is associated with

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the synthesis of ROS scavenging antioxidants (Saquet et al., 1999). Moreover, ATP has been emphasized to be an energy source for the synthesis of low molecular weight antioxidants (Yi et al., 2010). This information obtained from the literature suggests that ATP is possible to be useful for the treatment of 5-FU-induced organ and tissue toxicity. No information investigating the protective effect of ATP against 5-FU-induced ovarian damage and infertility in rats has been noticed in the literature. The Aim of this study was to analyze the effect of ATP on possible ovarian damage of 5-FU in rats.

Materials and Methods

Animals

Totally 18 albino Wistar female rats weighing between 246-260 grams were used in the experiment. The animals were procured from Erzincan Binali Yıldırım University Medical Experimental Application and Research Center. Before the experiment, animals were housed in the suitable laboratory environment at normal room temperature (22°C) and the rats were fed ad libitum.

Chemical substances

The chemical substances used in the experiment: The thiopental sodium used in the experiments was procured from IE Ulagay (Turkey), ATP from Zdorove Narodu (Ukraine), Fluorouracil (1000 mg 20 ml, solution for i.v. injection) from Training and Research Hospital (Turkey) affiliated to the Ministry of Health.

Experimental groups

The rats to be used in the experiment were categorized into three groups as healthy group (HG), 5-FU alone group (FUG) and ATP+5-FU administered group (AFU).

Experimental procedure

The ATP 4 mg/kg was injected intraperitoneally (IP) into the AFU group animals (n=6) (Erdem et al., 2021). The same volume of saline (0.9% NaCl) as the solvent was administered IP to the HG (n=6) and FUG (n=6) groups. One hour after administering ATP and solvent, 5-FU 100 mg/kg was injected IP to the animals in the AFU and FUG groups (Safarpour et al., 2022). ATP was administered to the animals once a day for 10 days. On the 1st, 3rd and 5th days of 5-FU, one dose (total of 3 doses) was administered. On day 10, the animals were euthanasia with high-dose anaesthesia (50 mg/kg thiopental sodium) and ovarian tissues were removed. The removed ovaries were analyzed biochemically and histopathological. All experimental results obtained from HG and AFU groups were analyzed comparing with the FUG group.

Biochemical analyses

Sample preparation

After washing the tissue samples with physiological saline, they were placed in petri dishes, and the tissues were ground into powder in liquid nitrogen. The tissue samples were homogenized to determine malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and protein levels. The supernatants were used for MDA, GSH, SOD and CAT protein analyses.

MDA, GSH, SOD, CAT and Protein determination in tissue

MDA, GSH, and SOD determination in tissue samples was measured with commercial enzyme-linked immunosorbent assay (ELISA) kits for experimental animals, and each analysis was performed according to the kit instructions (Cayman Chemical Company, product numbers 706002, 703002 and 10009055, respectively). CAT determination was performed according to the method suggested by Goth (Goth, 1991). Protein determination was determined spectrophotometrically at 595 nm according to Bradford method (Bradford, 1976).

IL-6 analysis in tissue

The weight of the samples was measured, and all tissues were cut and rapidly frozen with liquid nitrogen and homogenized with pestle and mortar; the samples were kept at 2-8^{o}C after melting. We added Phosphate-Buffered Saline (PBS) (pH 7.4), 1/10 (w/v), after then vortex for 10 seconds, centrifuge 20 min at 10,000 xg collected carefully. The levels of Interleukin 6 (IL-6; ng/L) were measured using a commercial kit procured from Eastbiopharm Co Ltd ELISA kit, China.

Histopathologic processes

Hematoxylin-Eosin Method: Necropsies of the rats were performed and ovarian tissues were placed in 10% buffered formalin solution. The samples were subsequently processed in routine follow-up processes and embedded in paraffin blocks. For the histopathological findings, 5 μm sections taken from the blocks to the slides were analyzed under a light microscope with Hematoxylin-Eosin. The histopathological findings determined semi-quantitatively were analyzed as absent (0), mild (1), moderate (2), and severe (3).

Statistical Analyses

The results obtained from the experiments were expressed as "mean value ± standard deviation" (x ± SD). The Shapiro wilk test was used to determine whether the groups were normally distributed. The significance level of the difference between the groups was determined using one-way ANOVA test. Then, Fisher's post-hoc LSD (least significant differences) was performed. All statistical analyses were performed in "SPSS for Windows 25.0" statistical software, and $p<0.05$ level was regarded to be significant statistically.

Results

Biochemical findings

Ovarian MDA and tGSH analysis in tissue

As could be noticed from Figure 1, 5-FU significantly increased the MDA level in the ovarian tissue of animals but decreased the tGSH level. Whereas ATP suppressed ($p < 0.001$) the increase of MDA with 5-FU, it significantly ($p < 0.001$) suppressed the decrease of tGSH (Table 1).

Table 1. Biochemical Analysis Results in the Ovarian Tissue

Biochemical parameters	Mean ± Standard Deviation		
	HG	FUG*	AFU
MDA	3.63 ± 0.17	5.84 ± 0.08	3.81 ± 0.22
tGSH	6.16 ± 0.10	3.68 ± 0.08	5.72 ± 0.15
SOD	7.17 ± 0.07	4.52 ± 0.14	6.87 ± 0.07
CAT	9.24 ± 0.09	4.59 ± 0.10	8.40 ± 0.16
IL-6	2.42 ± 0.15	4.87 ± 0.06	3.42 ± 0.12

* p < 0.001 compared with HG and AFU groups. MDA, malondialdehyde; tGSH, total glutathione; SOD, superoxide dismutase; CAT, catalase; IL-6, interleukin 6; HG, healthy group; FUG, 5-FU alone group; AFU, ATP+5-FU administered group; N=6.

Ovarian SOD and CAT analysis in tissue

Both SOD and CAT activities were determined to be decreased ($p < 0.001$) in ovarian tissues of animals treated with 5-FU. ATP was noticed to significantly inhibit the statistical ($p<0.001$) decrease of SOD and CAT activities with 5-FU (Figure 2, Table 1).

Ovarian IL-6 analysis in tissue

As could be seen in Figure 3, 5-FU caused increase ($p < 0.001$) at IL-6 in ovarian tissue of animals. ATP significantly ($p<0.001$) inhibited the increase of IL-6 with 5-FU (Table 1).

Table 2. Histopathological Analysis Findings in Ovarian Tissue

Groups	Mean± Standart Deviation	
	Follicular vacuolization	Degeneration of primordial follicles
HG	0.16± 0.40[a]	0.16± 0.40[a]
FUG	2.66± 0.40[b]	2.83± 0.51[b]
AFU	1.00± 0.00[c]	1.33± 0.40[c]

[a,b,c], show differences between groups ($p < 0.05$). HG; healthy group, FUG; 5-FU alone group; AFU, ATP+5-FU administered group; N=6.

Histopathologic Findings

Hematoxylin-Eosin Staining

A statistically significant difference was found between the groups (Table 2, $p < 0.05$). The ovarian tissues of rats in the healthy group had normal histological appearance (Figure 4). Severe follicle vacuolization and primordial follicle degeneration were observed in the ovaries of rats in the animal group treated with 5-FU alone (Figure 5,

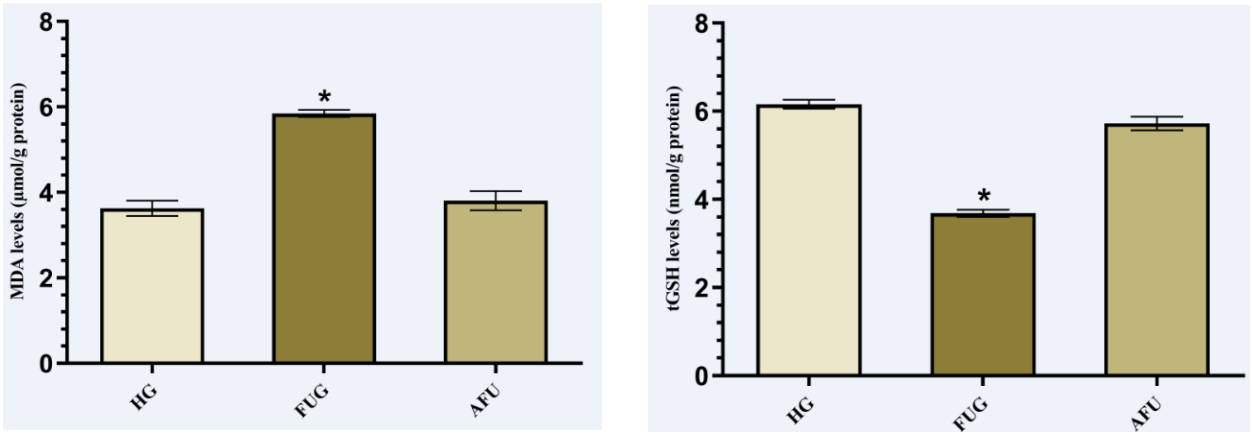

Figure 1. MDA and tGSH Analysis of Ovarian Tissue in Groups. *p < 0.001, according to HG and AFU groups. MDA; malondialdehyde, tGSH; total glutathione, HG; healthy group, FUG; 5-FU alone group, AFU; ATP+5-FU administered group.

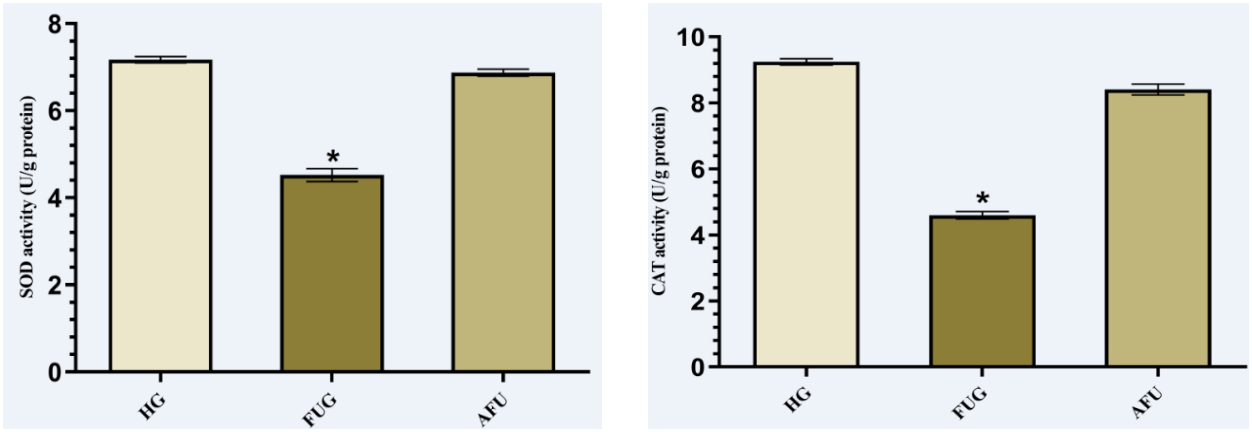

Figure 2. SOD and CAT Analysis of Ovarian Tissue in Groups. *p < 0.001, according to HG and AFU groups. SOD; superoxide dismutase, CAT; catalase, HG; healthy group, FUG; 5-FU alone group, AFU; ATP+5-FU administered group.

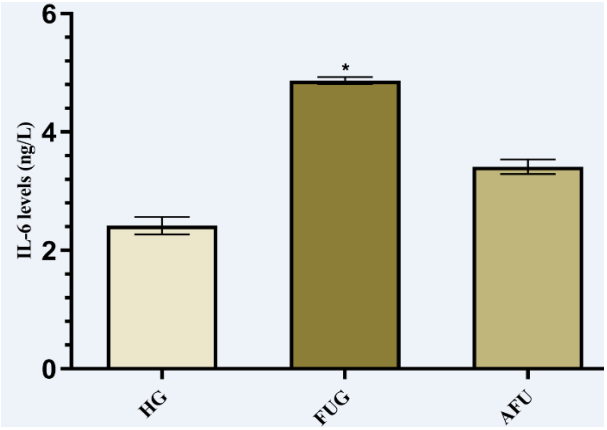

Figure 3. IL-6 Analysis of Ovarian Tissue in Groups. *p < 0.001, according to HG and AFU groups. IL-6; Interleukin 6, HG; healthy group, FUG; 5-FU alone group, AFU; ATP+5-FU administered group.

A-B). Vacuolization and primordial follicle degeneration were determined to be at mild level in the group treated with ATP (Figure 5, C-D).

Discussion

In this study, the effect of ATP on 5-FU-induced ovarian damage in rats was analyzed biochemically and histopathologically. The biochemical findings shown that 5-FU increased MDA and IL-1β levels in the ovarian tissue and decreased the activities of enzymatic and nonenzymatic antioxidants such as tGSH, SOD and CAT. As known, MDA was a cytotoxic product occurred as result of oxidation of lipids with ROS and was one of the important indicators of oxidative damage (Kisaoglu et al., 2013). There was found no information in the literature associating 5-Fu-induced ovarian damage with oxidative stress. However, it has been known that ROS was the major component for the pathogenesis of organ toxicities such as liver and cardiac induced by 5-FU (Barary et al., 2022; Fukuno et al., 2016). The increase at ROS induced by 5-FU caused LPO and MDA formation in cells and resulted in oxidative tissue damage (Fukuno et al., 2016; Refaie et al., 2022). In this study, it was found that the MDA level increased in the ovarian tissue of rats as result of administering 5-FU, but the increase of MDA induced by 5-FU was found to be significantly decreased after administering ATP. Ince et al. indicated that the increase in the amount of MDA in bevacizumab-induced ovarian damage was significantly inhibited as result of administering ATP (Ince et al., 2021). Our experimental results and the information obtained from the literature suggested that ATP created an antioxidant effect upon ROS.

It has been known from the literature that the impaired redox balance plays an important role in the pathogenesis of ovarian damage (Ince et al., 2021; Ingec et al., 2012). Therefore, the levels of antioxidants such as tGSH in the ovarian tissue of rats treated with 5-FU were analyzed in this study. Our experimental results revealed that tGSH level decreased in the 5-FU group. GSH as an endogenous antioxidant has been known to maintain the oxidation-reduction balance and protect cells from the harmful effects of oxidants (Kurutas, 2016). In the literature, there have been no studies analyzing the GSH activity in 5-FU-induced ovarian damage. However, there have recently been experimental studies that associated the toxicity of anticancer drugs on the ovary with a decrease in GSH (Algandaby, 2021; Kaygusuzoglu et al., 2018). Moreover, Kulhan et al.,2019 reported that tGSH level decreased and caused oxidative damage in cisplatin-related ovarian toxicity in rats. Low tGSH level in the ovarian tissue of rats treated with 5-FU in our study indicated that our

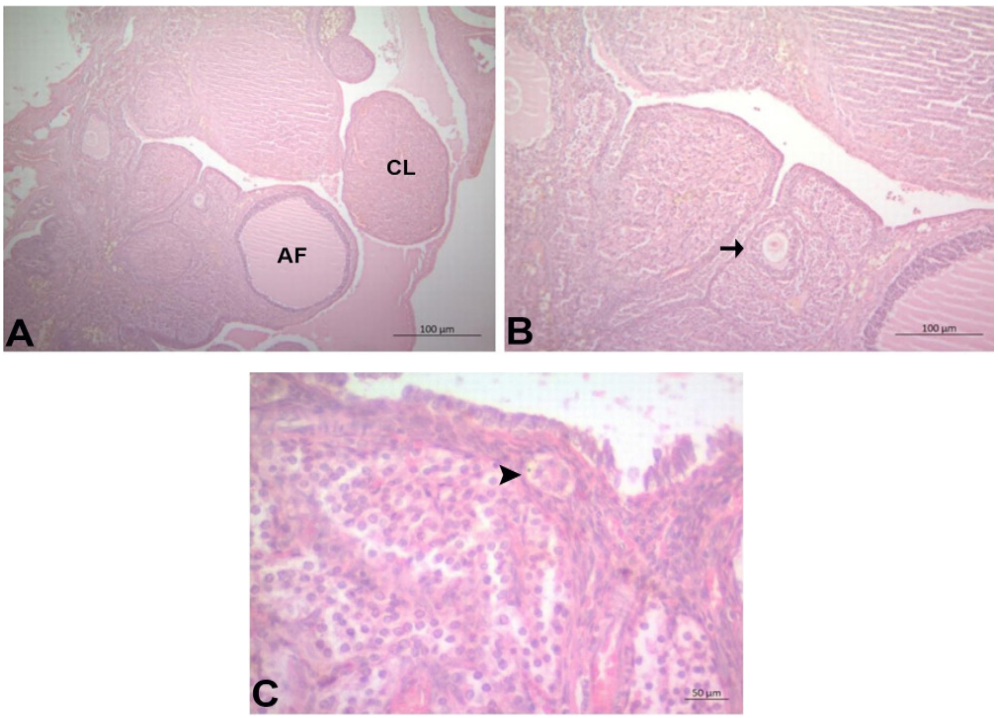

Figure 4. Normal Histological Appearance of Ovarian Tissue the Rats in the Healthy Group

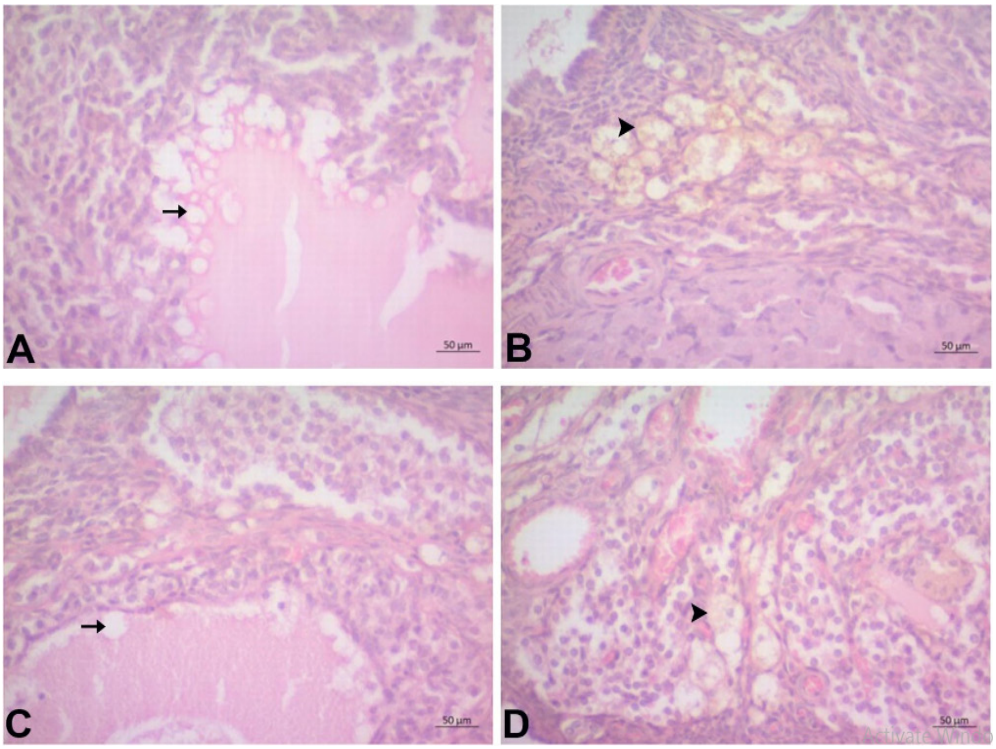

Figure 5. A-B, Ovarian tissue belonging to the FUG group. Severe follicle vacuolization and primordial follicle degeneration were observed; C-D, ovarian tissue belonging to the AFU group. Vacuolization and primordial follicle degeneration were determined to be at mild level in the group treated with ATP.

experimental results were compatible with the ones in the literature.

In this study, SOD was another endogenous antioxidant with decreased level in the ovarian tissue of animals treated with 5-FU. SOD was the most important antioxidant defense system against reactive oxygen species (ROS) and superoxide anion radicals (Wang et al., 2018). SOD increased one superoxide radical to O_2 molecule and catalyzed the reduction of another superoxide radical to hydrogen peroxide, as a less reactive molecule (Buettner, 2011; Wang et al., 2018). Hydrogen peroxide appeared as result of this reaction was one of the toxic types of oxygen, and CAT prevented its accumulation (Ighodaro et al., 2018). Therefore, it was considered in our study that the SOD enzyme should be analyzed together with the CAT enzyme to determine the oxidative damage induced by 5-FU. Our study findings revealed that CAT activity was also low in the 5-FU group with low SOD activity. No studies were found in the literature that associated ovarian damage induced by 5-FU with endogenous antioxidant enzyme activities. However, there were many studies reporting that anticancer drugs induced reactive oxygen species production in ovarian tissue causing a decrease in endogenous antioxidants such as SOD and CAT (Ata et al., 2019; Demir et al., 2021). In the study carried out by Ata et al.,2019 endogenous SOD levels were found to be low in the ovarian tissue of rats treated with methotrexate. Demir et al.,2021 reported that cisplatin caused a decrease in CAT level increasing oxidative stress in ovarian tissue.

Furthermore, ATP administration proved that 5-FU-induced MDA increase and decrease in tGSH, SOD and CAT enzyme activities were significantly suppressed in the ovarian tissue of rats. It was understood from our experimental results that whereas the oxidant-antioxidant balance changed in favor of oxidants in the 5-FU group, this balance was maintained with the predominance of antioxidants in the ATP group. No information was found in the literature upon the protective effect of ATP against 5-FU-induced oxidative damage in ovarian tissue. Ince et al., (2021) reported that ATP protected the ovarian tissue against oxidative damage suppressing LPO and strengthening the antioxidant system. Our study findings and the information obtained from the literature proved that ATP created an antioxidant effect.

It was stated in several previous experimental studies that overproduction of reactive oxygen species in ovarian damage caused the release of proinflammatory cytokines in cells (Chen et al., 2021). LPO and increased oxidative stress accelerated the inflammatory process triggering the release of pro-inflammatory cytokines from the damaged tissue (Kaygusuzoglu et al., 2018). It was reported that increased levels of IL-6 as an important pro-inflammatory cytokine were associated with increased inflammation and oxidative stress (Algandaby, 2021). However, no information fully explaining the inflammation mechanism in 5-FU-induced ovarian damage was found in the literature. In the study carried out by Logan et al., (2008) increase in serum IL-6 level was reported as result of 5-FU treatment in rats. In our study, as well, it was determined that there was an increase in IL-6 proinflammatory cytokine level together with the oxidative damage that increased with 5-FU in the ovarian tissue. However, ATP treatment significantly decreased the increase of IL-6 with 5-FU in the ovarian tissue of rats. These findings

indicated that ATP protected the ovarian tissue from the toxic effect of 5-FU. The information obtained from the literature revealed that the decrease in intracellular ATP level was associated with the increase in inflammation and oxidative stress (Hasko et al., 2013; Yi et al., 2010). Moreover, it was found that inflammatory symptoms decreased in individuals with rheumatoid arthritis and the increase in the levels of proinflammatory parameters such as TNF-α was significantly suppressed with ATP treatment (Bours et al., 2010).

The biochemical findings in our study were supported with the histological findings. In our histological findings, severe follicle vacuolization and primordial follicular degeneration were observed in the ovarian tissue of 5-FU group. Stringer et al., (2018) supporting our histopathological findings, a large decrease in ovarian volume was found due to progressive atresia in the growing follicles and decreasing corpus luteum numbers of mice treated with 5-FU. Furthermore, Wang et al., (2014) reported that 5-FU significantly decreased ovarian degeneration and primary follicle numbers in rats. However, treatment with ATP significantly suppressed the severe vacuolization and primordial follicle degeneration induced by 5-FU in our study. These findings were consistent with the ones in the study of Ince et al., (2021) revealing that ATP prevented ovarian damage induced by bevacizumab.

In conclusion, in this study, oxidative damage and increased inflammation were determined in the ovarian tissue of rats treated with 5-FU. It was observed that the general morphology of the follicles was close to normal in the ATP group with low oxidant and inflammatory parameters and high antioxidant parameters. To the best of our knowledge, this study first revealed that 5-FU-induced ovarian damage was associated with increased oxidative stress in rat model. Treatment with ATP also reversed oxidative stress and inflammation and prevented tissue damage. Our experimental results suggested that ATP was possible to be useful for the treatment of 5-FU-induced ovarian damage. Further studies on molecular basis have been needed to clarify the mechanism.

Author Contribution Statement

Ozer M. writing, Ince S. literature research, Altuner D. conceptualization and editing, Suleyman Z. experiment, Cicek B. writing, Gulaboglu M. data analysis, Mokhtare B. data analysis, Gursul C. investigation, Suleyman H. writing, review and editing. The authors report no conflict of interest in this work. The procedures were approved by the Local Animal Experimentation Ethics Committee (Date: 25.08.2022, meeting no: 08/41)..

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