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The effect of adenosine triphosphate on bevacizumab-induced ovarian damage and reproductive dysfunction in rats

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Abstract. We investigated the effect of ATP's protection against possible bevacizumab-induced ovarian damage and reproductive dysfunction in female albino Wistar rats. A total of 42 rats, 36 females, and 6 males were used in the experiment. Normal saline (0.9% NaCl) was injected as a solvent to the Bevacizumab (BVZ; $n = 12$) and Control ($n = 6$) groups. 25 mg/kg ATP was injected *intraperitoneally* (*i.p.*) to the ATP + bevacizumab (ABZ; $n = 12$) group. One hour after ATP and solvent administration, 10 mg/kg bevacizumab was *i.p.* injected to the ABZ and BVZ groups. Bevacizumab was administered once a day every two weeks; ATP was administered one a day for 30 days. At the end of this period, six rats from each group were sacrificed with high dose of anesthesia (thiopental sodium 50 mg/kg) and biochemical and histopathological examinations were performed in ovarian tissues. Mature male rats were kept in the laboratory for two months to breed the remaining female animals. The values showed that the oxidant parameters increased in the ovarian tissue of the BVZ group compared to the healthy controls and the ABZ group, while antioxidant parameters decreased. The number of breeding animals was significantly decreased in the BVZ group compared to the Control and the ABZ groups. This result suggests that ATP may be effective in preventing oxidative damage to the ovaries and infertility induced by bevacizumab.

Key words: ATP — Bevacizumab — Ovarian damage — Reproductive dysfunction — VEGF-A

Introduction

One of the main goals of cancer treatment is the development of novel drugs with reduced ovarian and reproductive toxicity. Accordingly, new targeted agents, including monoclonal antibodies, have been developed (Atsushi et al. 2017). However, long-term use of these targeted drugs has led to the emergence of new toxic effects (Ferrara et al. 2004). As is known, bevacizumab is a targeted monoclonal antibody acting on all isoforms of human vascular endothelial growth factor A (VEGF-A). Bevacizumab is the first anti-angiogenic agent approved for the use in the treatment of a wide range of malignancies, including metastatic colorectal, breast, ovarian, lung, and cervical cancer (Pham et al. 2016). Although the most common adverse effects caused by bevacizumab are considered to be temporary, there are some statements in the literature emphasizing that basic studies are required to generate more evidence-based application guidelines (Ferrara et al. 2004). Furthermore, the effects of bevacizumab treatment on reproductive function have not been clearly established;

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however, it is emphasized that studies at the molecular level may be beneficial in terms of explaining the toxicity (Stone et al. 2010). As VEGF is highly important in folliculogenesis and oocyte maturation, its suppression is very likely to induce side effects in the ovary (Atsushi et al. 2017). Angiogenesis in ovaries is important in follicular growth and corpus luteum formation (Stouffer et al. 2001; Tamanini and De Ambrogi 2004). Considering the functions of anti-angiogenic agents on normal physiological processes, female fertility may be affected temporarily or permanently (Stone et al. 2010).

Studies have revealed that bevacizumab toxicity is caused by VEGF inhibition (Ng et al. 2006; Hanna et al. 2019). VEGF increases intracellular ATP levels and decreases ROS production (Guo et al. 2017). Bevacizumab lowering intracellular ATP levels (Cehofski et al. 2018) indicates VEGF inhibition. Decreased ATP levels lead to the inhibition of the Na^+/K^+-ATPase pump and increase intracellular Na^+ and Ca^{2+} concentrations (McCord 1985). This causes stimulation of ROS production and cellular damage (Orrenius et al. 1992; Gordeeva et al. 2003). In the past study, the effect of ATP against possible bevacizumab-induced ovarian damage and reproductive dysfunctions will be investigated in female rats. ATP is a molecule continuously produced by oxidative phosphorylation in mitochondria under aerobic conditions (Bulanova and Bulfone-Paus 2010). Chiang et al. (2007) have reported that ATP provides wound healing by increasing VEGF levels. The aim of the present study is to investigate the effect of ATP against possible bevacizumab-induced ovarian damage and reproductive dysfunction in female rats and to examine ovarian tissues biochemically and histopathologically.

Materials and Methods

Animals

In the present study, 36 female and 6 male albino Wistar rats 135–146 days old (4.5–4.9 months old) weighing 228–240 g rats were used. All animals were obtained from Ataturk University Medical Experimental Application and Research Center. Prior to the experiment, rats were housed and fed at normal room temperature (22°C). The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 27.02.2020 meeting no:1/28).

Chemicals

Bevacizumab (100 mg/4 ml of Altuz) was supplied from Roche Switzerland, thiopental sodium was supplied from İ.E ULAGAY (Turkey), and ATP was supplied from Zdorove Narodu (Ukraine).

Experimental procedures

Rats were divided into Control, Bevacizumab (BVZ) and ATP + bevacizumab (ABZ) groups. Normal saline (0.9% NaCl) was injected as a solvent to the BVZ (n = 12) and Control (n = 6) groups. 25 mg/kg ATP was injected *intraperitoneally* (*i.p.*) to the ABZ (n = 12) group. One hour after ATP and solvent administration, 10 mg/kg bevacizumab was injected *i.p.*to the ABZ and BVZ groups. Bevacizumab was administered once a day every two weeks, and ATP was administered once a day for 30 days. During the experiment, every morning between 8:00–9:00 a.m. each animal cage was carried to the experimental room. Vaginal smear was collected with a plastic pipette filled with 10 µl of normal saline by inserting the tip into the rat vagina, but not deeply. Vaginal smear samples taken from each rat were taken on a different slide and examined under a light microscope, without the use of the condenser lens, with 10× and 40× magnification. In the microscopic examination, three types of cells could be recognized in the vaginal smear samples: round and nucleated ones are epithelial cells; irregular ones without a nucleus are the cornified cells, and the little round ones are the leukocytes. The proportion among them was used for the determination of the estrous cycle phases (Evans and Long 1922; Marcondes et al. 2002). At the end of this period, six rats from each group were sacrificed with high dose of anesthesia (thiopental sodium 50 mg/kg) and ovarian tissues were removed. Biochemical and histopathological examinations were performed in extracted ovarian tissues. Mature male rats were kept in the laboratory for two months to breed the remaining animals (6 female rats from each group). Rats that became pregnant during this period were placed in separate cages and kept in a suitable environment. Rats that did not become pregnant and did not give birth within two months were considered infertile. All biochemical and histopathological findings obtained from the ABZ and Control groups were compared with the results from the BVZ group.

Biochemical analyses

Prior to dissection, all tissue was rinsed with phosphate-buffered saline solution. The ovarian tissues were homogenized in ice-cold phosphate buffers (50 mM, pH 7.4) that were appropriate for the variable to be measured (Weydert and Cullen 2010). The tissue homogenates were centrifuged at 5,000 rpm for 20 min at 4°C, and the supernatants were extracted to analyze, MDA, tGSH, TAS, TOS, and protein concentration. Protein concentration of the supernatant was determined spectrophotometrically at 595 nm according to the Bradford method using bovine serum albumin as the standard (Bradford 1976). All spectrophotometric measurements were performed *via* a microplate reader (Bio-Tek, USA).

Malondialdehyde (MDA) analysis

MDA measurements were based on the method used by Ohkawa et al. (1979), involving spectrophotometrical measurement of absorbance of the pink-colored complex formed by thiobarbituric acid (TBA) and MDA. The tissue-homogenate sample (25 µl) was added to a solution containing 25 µl of 80 g/l sodium dodesil sulfate and 1 ml mixture solution (200 g/l acetic acid + 1.5 ml of 8 g/l 2-thiobarbiturate. The mixture was incubated at 95°C for 1 h. Upon cooling, 1 ml of n-butanol:pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 10 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetra methoxy propane (Ohkawa et al. 1979).

Total glutathione (tGSH) analysis

The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay with some modifications (Sedlak and Lindsay 1968). DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) disulfite is chromogenic in the medium, and DTNB is reduced easily by sulfhydryl groups. The yellow color produced during the reduction is measured by spectrophotometry at 412 nm. For measurement, a cocktail solution (5.85 ml 100 mM Na-phosphate buffer, 2.8 ml 1 mM DTNB 3.75 ml 1 mM NADPH, and 80 µl 625 U/l glutathione reductase was prepared. Past measurement, 0.1 ml meta-phosphoric acid was added to 0.1 ml tissue-homogenate and centrifuged for 2 min at 2000 rpm for deproteinization. The 0.15 ml cocktail solution was added to 50 µl of supernatant. The standard curve was obtained by using GSSG (Sedlak and Lindsay 1968).

Measurements of total oxidant status (TOS) and total antioxidant status (TAS)

TOS and TAS levels of tissue homogenates were determined using a novel automated measurement method and commercially available kits (Rel Assay Diagnostics, Turkey), both developed by Erel (Erel 2004, 2005). The TAS method is based on the bleaching of the characteristic color of a more stable ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation by antioxidants and, measurements are performed at 660 nm. The results are expressed as nmol hydrogen peroxide (H_2O_2) equivalent/l. In the TOS method, the oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured at 530 nm spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The results are expressed as µmol Trolox equivalent/l. The percentage ratio of TOS to TAS was used as the oxidative stress index (OSI). OSI was calculated as TOS divided by 100×TAS.

Histopathological examination

Necropsy was performed on the rats and ovarian tissues were placed in 10% buffered formalin solution. The samples were then subjected to routine follow-up procedures and embedded in paraffin blocks. 5 µm-thick sections taken from the blocks were stained with Hematoxylin-Eosin and examined for histopathological findings under light microscopy. The tissue sections were evaluated semi-quantitatively in terms of vacuolization, degeneration of primordial follicles, necrotic changes, and hyperemia. The severity of histopathological findings in each section was scored between 0 and 3:0 was normal tissue, 1 was mild damage, 2 was moderate damage and 3 was severe damage. The data obtained were analyzed with the SPSS 20.00 program. Intergroup differences were determined by the nonparametric Kruskal Wallis test, and the group causing the difference was determined by the Mann-Whitney U-test ($p < 0.05$).

Statistical analyses

Results were expressed as mean ± standard deviation (SD) and percentage. The significance level of the difference between the groups was determined by one-way ANOVA test. Then Fisher's *post hoc* LSD (least significant differences) test was performed. All statistical procedures were performed in the statistical program "SPSS for Windows, 22.0" and $p < 0.05$ was considered significant.

Results

Biochemical analyses

MDA and tGSH analysis

Bevacizumab administration increased MDA levels in the ovarian tissue of animals. MDA level was significantly higher in the BVZ group compared to both ABZ and Control groups. ATP significantly inhibited the bevacizumab-induced increase of MDA. There was no significant difference between the ABZ group and the Control group in terms of the MDA level. Bevacizumab increasing MDA levels caused a significant decrease in tGSH in the ovarian tissue compared to ABZ and Control groups. ATP significantly prevented the bevacizumab-induced decrease in tGSH from and kept tGSH levels similar to Control group (Figure 1).

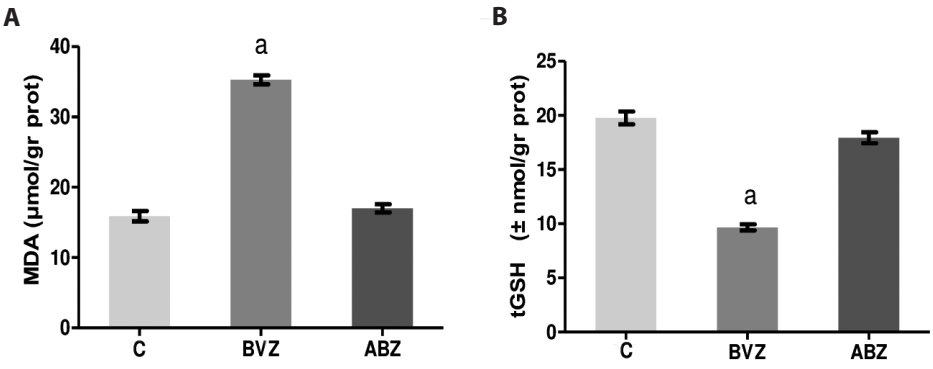

Figure 1. Malondialdehyde (MDA; **A**) and total glutathione (tGSH; **B**) levels in blood serum of the study groups. [a] $p < 0.001$ *vs.* Control (C) and ATP + bevacizumab (ABZ) groups.

TOS and TAS analysis

As can be seen in Figure 2, bevacizumab increased TOS level in ovarian tissue compared to ABZ and Control groups. ATP significantly suppressed the bevacizumab-induced increase in TOS. No significant difference was found between the ABZ and Control groups in terms of TOS levels. Furthermore, bevacizumab caused a decrease in TAS level in ovarian tissue. Statistical analyses showed that TAS level was significantly different between the BVZ and ABZ groups. However, there was no significant difference between the ABZ and Control groups in terms of TAS levels.

Breeding results

As can be seen in Table 1, all six female rats in Control group reserved for breeding gave birth within two months. However, only two of the bevacizumab-treated rats gave birth within this period. In the ABZ group, the number of breeding animals was two times higher compared to BVZ group.

Histopathological findings

There was a statistically significant difference ($p < 0.05$) between the groups (Table 2). Ovarian tissues of the rats in Control group had a normal histological appearance (Fig. 3). Severe (Grade 3) vacuolization was observed in the primary and secondary follicles of the ovaries in BVZ group (Fig. 4A), whereas moderate (Grade 2) degeneration was observed in the primordial follicles (Fig. 4B). In the ABZ group, the severity of vacuolization decreased to moderate (Grade 2) (Fig. 4C), while degeneration in the primordial follicles also decreased (Grade 1) (Fig. 4D). The vacuolizations in BVZ group were large and mostly formed close to the follicular center, but the vacuolizations in ABZ group were both smaller and localized towards the periphery of the follicle. In BVZ group, degenerations were detected in all cells (3–5 cells) that make up the primordial follicles. Accordingly, it was determined that the primordial follicles were erased from the eye. In the ovaries in ABZ group, degenerations were observed in the

Table 1. Effect of ATP on bevacizumab-related reproductive dysfunctions

Group	Breeding animals *n* (%)	Non-breeding animals *n* (%)
Control	6 (100)	–
BVC	2 (33.33)	4 (66.66)
ABZ	4 (66.66)	2 (33.33)

BVZ, Bevacizumab group; ABZ, ATP + bevacizumab group.

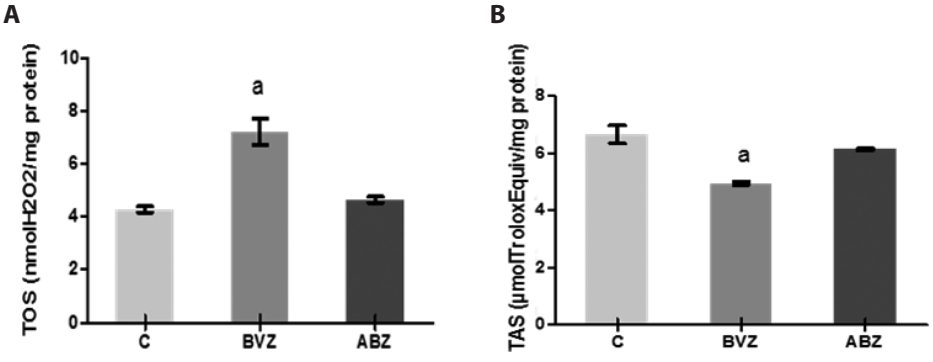

Figure 2. Total oxidant status (TOS; **A**) and total antioxidant status (TAS; **B**) levels in blood serum of the study groups. [a] $p < 0.001$ *vs.* Control (C) and ATP + bevacizumab (ABZ) groups.

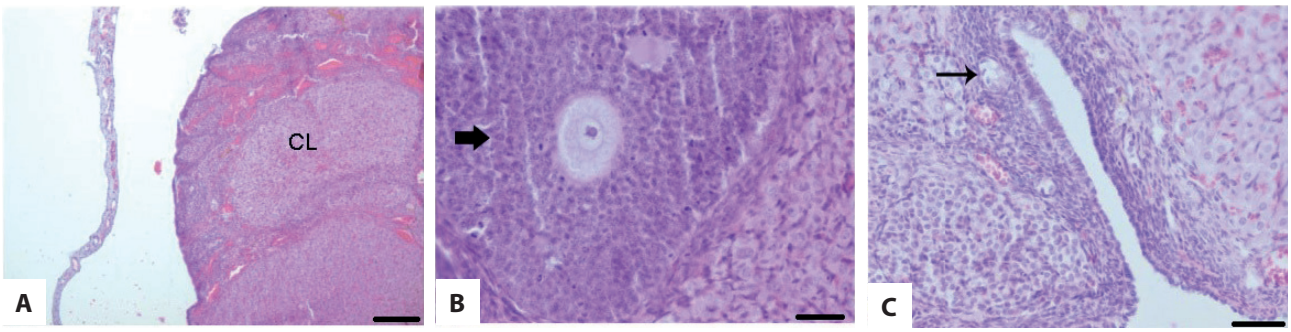

Figure 3. Normal histological view of the corpus luteum (CL) (**A**; H&E ×10), of the primary follicle (arrow) (**B**; H&E ×20) and of the primordial follicle (thin arrow) (**C**; H&E ×20) in Control group.

primordial follicles, but these were not all primordial follicle cells, so it was not completely erased from the eye as in BVZ group. Necrotic changes in the ovaries in the study groups were not yet formed. Therefore, statistically, no significant difference was found in terms of necrotic changes. Also, it was determined that hyperemic vessels were of physiological origin ($p < 0.05$).

Table 2. Histopathological findings of groups

Group	Vacuolization	Primordial follicules degeneration
Control	0.16 ± 0.51^{a}	0.16 ± 0.40^{a}
BVC	2.83 ± 0.40^{b}	2.16 ± 0.40^{b}
ABZ	2.16 ± 0.40^{c}	1.33 ± 0.51^{c}

^{a,b,c} $p < 0.05$. BVZ, Bevacizumab group; ABZ, ATP + bevacizumab group.

Discussion

In the present study, the effect of ATP against possible bevacizumab-induced ovarian damage and reproductive dysfunction was investigated in rats. Ovarian tissues were also examined biochemically and histopathologically. Our biochemical results showed that oxidant/antioxidant levels were different in the ovarian tissues of healthy, ATP- and bevacizumab-treated animals. In the ovarian tissue of BVZ group, MDA and TOS levels were higher than the healthy and

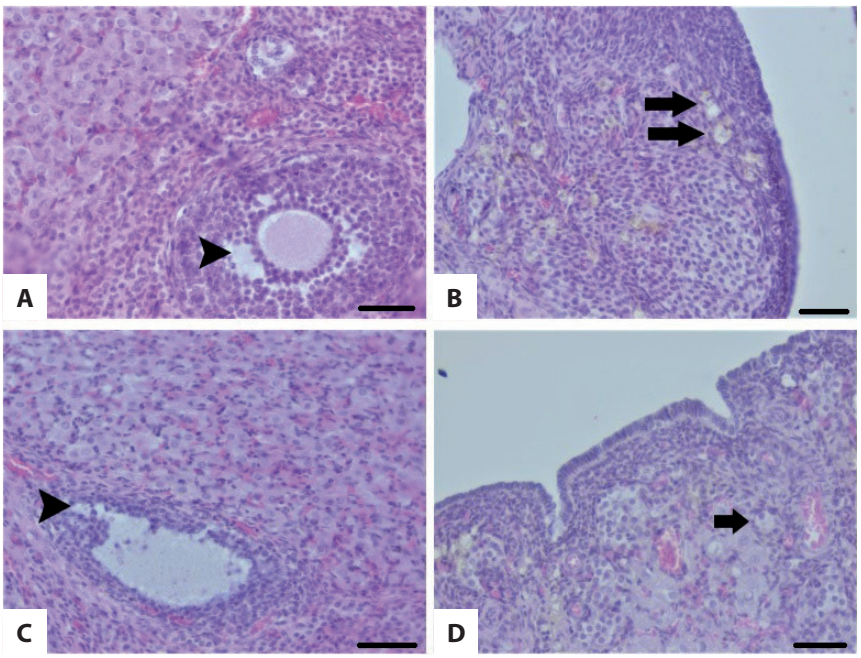

Figure 4. A. Severe vacuolization in primary and primordial follicles (arrowhead) in BVZ (Bevacizumab) group. Vacuoles are large and follicular centrally located. **B.** Moderate degeneration in primordial follicles (arrows) in BVZ group. Follicular cells are obscured. **C.** Moderate vacuolization in primary and primordial follicles (arrowhead) in ABZ (ATP + bevacizumab) group. Small diameter and located in the follicular periphery. **D.** Mild degeneration in primordial follicles (arrow) in ABZ group. Prominent cells are present in the follicles. (H&E ×20).

ABZ group, whereas tGSH and TAS levels were lower. As is known, MDA elevation indicates increased ROS production, because MDA is a ROS product. ROS oxidize cell membrane lipids (lipid peroxidation, LPO), generating toxic products such as MDA from the lipids (Kisaoglu et al. 2013). In many studies, MDA is commonly used as an oxidant parameter for the evaluation of oxidative ovarian damage of anticancer drugs (Chinwe et al. 2018; Ata et al. 2019). TOS levels were measured in the ovarian tissue of the bevacizumab-treated group to demonstrate that other oxidant parameters in addition to MDA also increased. TOS was used to determine the cumulative oxidative effects of all oxidants in biological systems (Erel 2005).

ROS overproduced in healthy tissues are neutralized by GSH and other enzymatic and non-enzymatic endogenous antioxidants. When endogenous antioxidants fail to neutralize oxidants, the oxidant/antioxidant balance is disrupted in favor of the oxidants (Urso and Clarkson 2003). Decreased tGSH level in BVZ group indicates that GSH is depleted on neutralizing overproduced ROS. Catalyzed by glutathione peroxidase, GSH is an antioxidant molecule reacting with hydrogen peroxides and detoxifies them and protects the cells from ROS damage (Murray et al. 2000). Previous studies have reported that the administration of anticancer drugs increases MDA levels and decreases tGSH levels in ovarian tissue compared to healthy tissue (Soyman et al. 2018). Our experimental results show that in BVZ group the oxidant/antioxidant balance is disrupted in favor of oxidants due to ROS overproduction. Disruption of oxidant/antioxidant balance in favor of oxidants is called as oxidative stress in the literature (Yeum et al. 2004). The fact that bevacizumab reduces TAS level indicates that it decreases all antioxidant parameters in ovarian tissue. The reason is that TAS is used to determine the cumulative antioxidative effects of antioxidants in biological systems (Erel 2004).

In the present study, it is clearly seen that ATP inhibits bevacizumab-induced MDA increase and tGSH decrease. These results indicate that ATP suppresses bevacizumab-induced ROS increase. There are no studies in the literature showing that bevacizumab directly increases ROS production. However, it has been proven that bevacizumab toxicity is caused by VEGF inhibition (Ng et al. 2006). VEGF is known to increase intracellular ATP levels and decrease ROS production (Guo et al. 2017). This shows that bevacizumab increases ROS production by decreasing ATP levels through VEGF inhibition. A decrease in intracellular ATP leads to Na^+/K^+-ATPase pump inhibition and increases intracellular Na^+ and Ca^{2+} concentrations (Cord 1985). This event leads to the stimulation of ROS production and oxidative cell damage (Orrenius et al. 1992; Gordeeva et al. 2003). There are experimental studies reporting that ATP inhibits the targeted drug-induced increase of MDA and decrease of tGSH (Aldemir et al. 2020). No information was found regarding the direct effect of ATP on TOS and TAS levels. However, ATP content was found to decrease in the animal group with high mitochondrial TOS level and low TAS level (Taskin and Dursun 2012). Recovery of lost mitochondrial ATP level caused TOS levels to decrease, TAS levels to increase, and the elimination of oxidative stress (Dursun et al. 2011).

In the present study, it was also observed that infertility developed in most of the animals treated with bevacizumab. It was found that ATP was highly effective in preventing bevacizumab-induced infertility. Chemotherapy has harmful effects on ovarian function and may cause infertility in women (Imai et al. 2018). However, the effects of bevacizumab treatment on reproductive function have not been clearly determined in the literature (Stone et al. 2010). As mentioned above, bevacizumab is a targeted monoclonal antibody acting on all isoforms of human VEGF-A. It has been suggested that VEGF-A plays an important role in regulating angiogenesis in the ovary (Stouffer et al. 2001). Therefore, ovarian dysfunction is seen as an important adverse effect of drugs suppressing angiogenesis (Atsushi et al. 2017). This suggests that stimulating angiogenesis either directly or *via* VEGF may be effective in terms of preventing the development of infertility due to ovarian dysfunction. There are studies in the literature reporting that ATP increases VEGF levels (Chiang et al. 2007). The biochemical results obtained in the present study were supported by histopathological findings. In BVZ group, where oxidant/antioxidant balance was disrupted in favor of oxidants and a significant level of infertility was observed, histopathological damage in ovarian tissues was more severe than ABZ group. Vacuolization in primary and secondary follicles of the ovaries was more pronounced in the bevacizumab group compared to ABZ group. In addition, in BVZ group, degeneration in the primordial follicles was more severe than ABZ group. Turkler et al. (2020) have shown that oxidative stress can cause infertility. Unlubilgin et al. (2017) reported infertility in animals with histopathological damage such as hemorrhage, congestion, follicle degeneration, neutrophil infiltration, and necrosis. However, Kerr et al. (1999) reported that the primary mechanism of infertility was due to follicular degeneration and to the significant reduction in primordial follicles. Our histopathological test results show that chronic damage has developed in the ovarian tissue. In our study, no animals died during the experiment from the animals who received 10 mg/kg *i.p.* bevacizumab every 15 days. These results indicate that bevacizumab did not cause a significant acute toxic effect at this dose. In the literature, acute toxicity is evaluated according to the number of animals that die within 24 hours after drug administration (Suleyman et al. 2003). Some clinical studies have shown that bevacizumab causes fatal acute heart damage within 12–48 hours (Gruenberg et al. 2016).

In conclusion, oxidant parameters increased in the ovarian tissue of the BVZ group compared to Control and the

ABZ groups, while antioxidant parameters decreased. In addition, the number of breeding animals significantly decreased in BVZ group compared to Control and ABZ groups. Histopathologically, the damage in the ovarian follicle cells of BVZ group was more severe than Control and ABZ groups. This suggests that bevacizumab-induced infertility may have been caused by the toxic effect on the follicles and ATP may be effective in terms of preventing bevacizumab-induced oxidative ovarian damage and infertility.

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Conflict of interest. No potential conflict of interest was reported by the author(s).

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