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Original Article

Protective effect of Vaccinium myrtillus on ischemia- reperfusion injury in rat ovary

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ABSTRACT

Objective: It is aimed to evaluate the protective effect of bilberry on I/R injury in rat ovary.
Materials and methods: A total 48 female Wistar–Albino rats were utilized to form five groups: Group 1 (control group) (n = 8), neither drug was given and nor procedure was performed. Group 2 (bilberry control group) (n = 10), single dose 200 mg/kg bilberry was administered by gavage and no procedure was performed. Group 3 (I/R group) (n = 10), no drug was given, 1-h ischemia and 2-h reperfusion was performed. Group 4 (bilberry before I/R group) (n = 10), single dose 200 mg/kg bilberry was administered by gavage before ischemia. Then 1-h ischemia and 2-h reperfusion was performed. Group 5 (bilberry after I/R group) (n = 10), first 1-h ischemia was performed. Single dose 200 mg/kg bilberry was administered by gavage and then 2-h reperfusion was performed. Right ovaries were surgically extirpated in all groups. In ovarian tissue samples, malondialdehyde (MDA) levels and enzymatic activities of superoxide dismutase (SOD), catalase (CAT) were studied. In ovarian tissue samples, DNA damage and apoptosis were assessed by using TUNEL method. Histopathologic examination was performed by light microscopic findings.
Results: When group 3 was compared with another groups, MDA levels were significantly higher, enzymatic activities of SOD and CAT were found to be as significantly lower in ovarian tissue and blood ($p < 0.001$). In histopathologic examination, ovarian tissue damage in the group 3 were significantly higher than other groups ($p < 0.001$). Also, DNA damage and apoptosis were significantly higher in group 3 than other groups ($p < 0.001$).
Conclusion: Biochemical findings were lower and histopathologic damage was less especially in bilberry before I/R group (group 4). In conclusion, bilberry seems to be effective in prevention of ovarian I/R injury and short-term treatment.

Introduction

Ovarian torsion is the fifth most common gynecological emergency and its incidence has been reported as 2.7% [1]. The exact reason of the torsion is the rotation of the adnexa. The degree of the clinical prognosis is depended on the time and grade of the rotation. Ovarian torsion leads to ischemia and if the treatment is not addressed energetically, necrosis occurs. The patho-physiology of the ovarian injury still remains unclear [2]. Abramov et al. reported that, oxidative damage due to reperfusion could be paradoxically much more than ischemic damage [3]. Ischemia-reperfusion (I/R) injury causes an increase in reactive oxygen species (ROS) and malondialdehyde (MDA) production. Ovarian torsion leads to neutrophil adhesion and consequently ROS secretion from these increased neutrophils [4]. The enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) rise to diminish the detrimental effects of these toxic substances. These enzymatic and nonenzymatic defense mechanisms balance the homeostasis and protect the cells against oxidative damage [5].

Bilberry (Vaccinium myrtillus) is a natural antioxidant that is found in colored fruits and vegetables [6]. Structurally, it is an anthocyanoside derivative and a novel free-radical scavenger. It has been utilized as an antioxidant in many tissues such as vascular tissues, heart and intestine [7]. Ziberna et al. reported that usage of anthocyanins in ischemia-reperfusion injury due to cardiac toxicity

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could be useful [8]. It was demonstrated that the antioxidant effect of bilberry was dependant to the phenolic content. Bornsek et al. claimed that intracellular antioxidant activity was observed even if very low doses of the compound [9]. Jang et al. reported that anthocyanins exhibited antioxidant activity and their usage could be useful to prevent light-induced damage to the cell [10]. Furthermore, it has been demonstrated that bilberry could serve as an excellent free-radical scavenger in the treatment of heart diseases and cancer [11]. Jakesevic et al. investigated the effects of bilberry in combination with lactic acid bacteria on intestinal oxidative stress induced by ischemia-reperfusion in mouse. They concluded that a food supplement of bilberry protected small intestine against oxidative stress and inflammation induced by ischemia-reperfusion [12]. Therefore, we thought that bilberry would prevent I-R damage of ovary. Also, there is no information about the effects of bilberry on I/R damage of the rat ovary. As such, this study was aimed to assess the effects of bilberry on I/R injury of the ovaries.

Material and methods

The study protocol was accepted by the Erciyes University's Experimental Animal and Local Ethics' Committee (no: 15/104/2015). This study was supported by the Erciyes University Scientific Research Projects' Unit with the number of TYL-6251. Forty-eight female adult Wistar–Albino rats weighing between 150 and 220 g were taken from the Erciyes University's Experimental Animal Laboratory. The ages of the animals were between 8 and 10 weeks. The animals were housed between 20 and 22 °C under a 12 h light/12 h dark cycle and were fed by ad libitum.

Animals were inserted randomly into five groups. In group 1 (n = 8, control group), right oophorectomy was performed without torsion or any administered drug. In group 2 (n = 10, bilberry control group), single dose 200 mg/kg bilberry was given via gavage and ovary torsion was not performed. In group 1 and 2 oopherectomy was performed after 3 h lasting anesthesia. In group 3 (n = 10, I/R group), no drug was given and subsequently 1-h ischemia and 2-h reperfusion was performed. In group 4 (n = 10, bilberry before I/R group), single dose 200 mg/kg bilberry was given via gavage before ischemia and then torsion/detorsion was performed as 1-h ischemia and 2-h reperfusion. In group 5 (n = 10, bilberry after I/R group), first 1-h ischemia was performed. Single dose 200 mg/kg bilberry was administered by gavage and then 2-h reperfusion was performed.

All rats were anesthetized a combination with i.p ketamine hydrochloride (45 mg/kg, Ketalar, Eczacibasi, Istanbul, Turkey) and xylazin hydrochloride (5 mg/kg, Rompun, Bayer, Leverkusen, Germany). After the completion of the drug doses, all rats were sacrificed. The surgical procedure was performed by using a 2-cm laparotomic incision. Atraumatic vascular clamps were used to produce right adnexal torsion. The skin was closed with 3/0 silk sutures. Right ovarian ischemia was lasted 1-h. Then relaparotomy was performed in previous incision cite. Reperfusion was terminated after 2 h. At the end of the procedure, right ovaries were surgically removed and were fixed in 10% formalin. Cervical dislocation was performed by using a forceps for sacrification. For light microscopic examination, ovarium tissue samples were fixed for 48 h and were dehydrated by using alcohol trays. Then these samples were embedded in parafin blocks. Cutting process were performed as 5 μm thickness and these sections were stained with hematoxylin and eosin (H&E). The sections were examined and photographed with a Olympus BX 50 light microscope (Olympus Corp., Tokyo, Japan). Histopathological changes scanned to detect the presence and severity of tissue damage were defined as congestion, hemorrhage, leukocyte infiltration, edema, and follicular degeneration. Scoring was done between 0 and 3 according to severity of damage. 0 was accepted as having no pathology, and 1, 2 and 3 represented pathologic findings of less than 33%, 33%–66%, and more than 66% of the examined areas, respectively. The total score was calculated by summing the scores obtained for each parameter.

Paraffin blocks were stained with poly-L-Lysine coated slides for 2 h at 69 °C to remove paraffin. Tissue specimens which were thoroughly paraffinized were kept in the gradually decreasing alcohol series of xylene I, xylene II, and xylene III series for 5 min (100%, 96%, 80%, 70%). In Situ Cell Death Detection Kit, Fluorescein Cat No: 11684795910 (Roche, Inc., Molten Biotechnology Lab.) was used. After washing twice with PBS for 5 min each, the antigen was allowed to stand for 5 min in a microwave oven at 350 W in 0.01 M 5% sodium citrate buffer for recovery and allowed to cool to room temperature for 10 min. Tissue samples washed with PBS for 5 min were incubated with the TUNEL reaction mixture in a humidity chamber for 60 min at 37 °C. Contrast staining with 4 ′, 6-diamidino-2-phenylindole (DAPI) was performed on tissue samples washed 5 min with PBS. Photographs were taken with an Olympus® BX51 fluorescent microscope at a wavelength of 450–500 nm. For the apoptotic index, apoptotic cells were counted by scanning ten different areas from each section on a 40X objective.

Tissues were fixed with 150 mM KCl and centrifuged at 10,000 rpm for 30 min. The supernatants were used for analyses of MDA levels and SOD and CAT activities. Rat malondialdehyde (MDA) ELISA Kit Cat No: YHB0708Ra (Molgen Biotech., Roche®, Istanbul, Turkey) was utilized for assessing MDA levels. Rat Super Oxide Dismutase (SOD) ELISA Kit Cat No: YHB1021Ra (Molgen Biotech., Roche®, Istanbul, Turkey) and Rat Catalase (CAT) ELISA Kit Cat No:YHB0207Ra (Molgen Biotech., Roche®, Istanbul, Turkey) were used to measure the SOD and CAT activities, respectively.

Statistical Package for the Social Sciences (17.00SPSS Inc., Chicago, IL) was used for statistical analyses. One-way ANOVA test with Bonferroni correction for tissue and blood MDA, SOD and CAT levels, chi square test for histologic damage scores were used to compare variables. Pearson correlation analysis was also used to measure the correlation coefficient between tissue and blood MDA, SOD and CAT levels. p value < 0.05 was accepted as statistically significant.

Results

When the experimental groups were macroscopically evaluated, it was observed that except for the control and bilberry control groups (Group 1 and 2) others had necrotic appearance and were hemorrhagic. In medulla, developing follicles and ovarian stroma were observed in normal histological features. Moreover, hilus cells and veins were normal (Fig. 1A). In the bilberry control group, two secondary follicles were seen in the cortex of the ovary. One of the secondary follicles was in the inferior follicular phase. Germinal epithelium, tunica albuginea and cortical connective tissue showed no histological degeneration (Fig. 1B). In group 3, hemorrhage and edema were seen in the sections stained with Hematoxylin & Eosin. All the tissue contained the fields of intensive hemorrhage. Furthermore, the normal morphologic structures and the integrity of the blood vessels were impaired (Fig. 1C). It was noted that the damage decreased significantly in Group 4 than Group 3. Germinal epithelium had normal fine structure. However, the areas of connective tissue near the corpus luteum showed small hemorrhagic foci (Fig. 1D). In group 5, small haemorrhagic foci were observed between the corpus luteum and connective tissue, in the connective tissue and some follicles (Fig. 1E).

In group 1, fluorescence microscopy at 450–500 nm wavelength showed some apoptotic cells. However, since these apoptotic cells

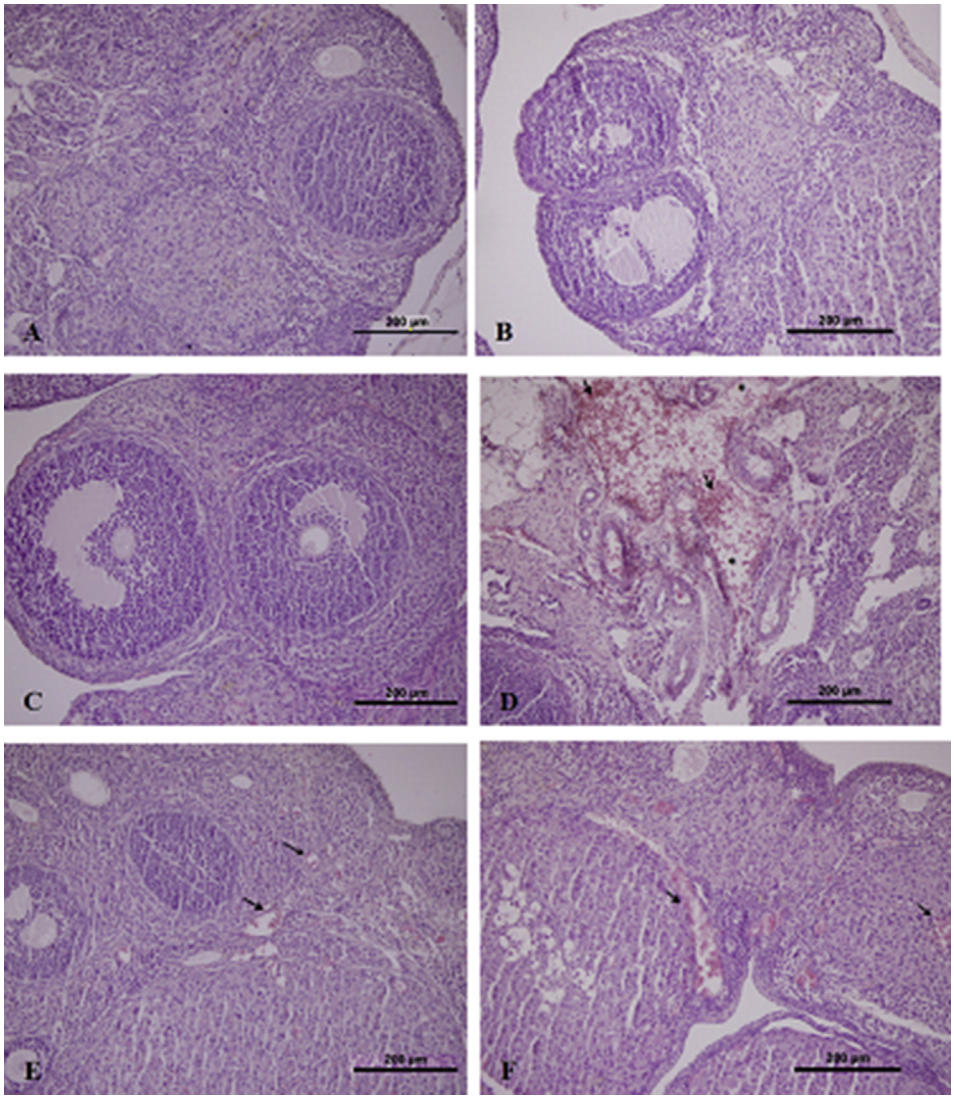

Fig. 1. A, B) Ovarian tissue of the control group (Group 1). **C)** Bilberry control group (Group 2). **D)** Hemorrhage (→) and edema (*) in hematoxylin & eosin-stained sections in I/R group (Group 3). **E)** Hemorrhagic foci (→) were observed in bilberry before I/R group (Group 4). **F)** Hemorrhagic foci (→) were observed in bilberry after I/R group (Group 5) ×20.

are not seen in these follicles, they are thought to be follicles that go to natural atresia (Fig. 2A). In group 2, apoptotic cells were found in secondary and tertiary follicles. The apoptotic cell in secondary follicle was next to the lumen. Lesser numbers of apoptotic cells were observed in theca cells and connective tissue adjacent to them (Fig. 2B). In group 3, apoptotic cells were detected in the connective tissue areas adjacent to the tertiary follicles. Although a small number of apoptotic cells were observed in connective tissue adjacent to the tertiary follicle, a large number of apoptotic cells were observed in the medullar region where the bleeding foci were located (Fig. 2C). In group 4, very few apoptotic cells were observed in the tertiary and secondary follicles and in the connective tissue areas near the corpus luteum (Fig. 2D). In group 5, less apoptotic cells were observed in the outer layers of the primary follicle which was adjacent to the corpus luteum (Fig. 2E).

Ovarian tissue damage was scored according to vascular congestion, hemorrhage, leucocyte infiltration, edema and follicular degeneration (Table 1). When all of these damage scores and biochemical parameters were compared with each other, the worst ones were in group 3. Ovarian tissue damage was significantly lower in group 4 and 5 than group 3 ($p < 0.001$).

The mean blood and tissue SOD and CAT levels were significantly lower in group 3, 4 and 5. While the mean SOD and CAT levels were significantly higher in group 4 when compared to group 3 (Table 2, Table 3). Tissue and blood MDA levels were significantly higher in group 3 and 5. There was strong correlation between blood and tissue SOD, MDA and CAT levels. ($p < 0.001$) Pearson correlation coefficient were 0.781, 0.694 and 0.641 respectively.

Discussion

We aimed to evaluate the protective effect of bilberry on I/R injury in rat ovary in this prospective randomized controlled trial. We found that tissue and blood MDA levels seem to be increased and tissue and blood SOD and CAT activities seem to be decreased on I/R injury in rat ovary. Bilberry could be useful to diminish the tissue and blood MDA levels and to elevate the tissue and blood SOD and CAT activities. Our results indicated that bilberry decreased the harmful biochemical, histopathological and immuno-histochemical changes due to ovarian torsion. To the best of our knowledge, this is the first trial about the preservative effect of bilberry on ovarian torsion in rats.

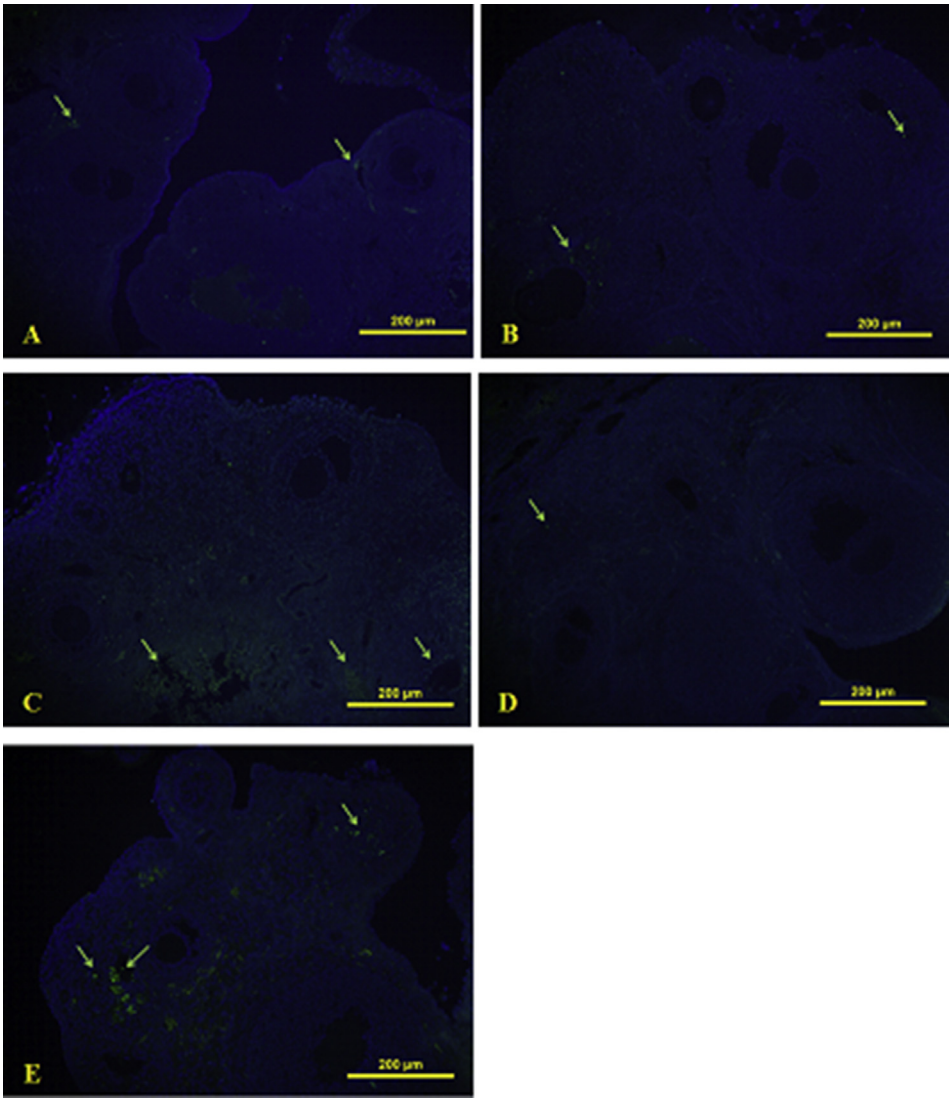

Fig. 2. A) Apoptotic cells (→) were seen in control group (Group 1). **B)** Apoptotic cells (→) in bilberry control group (Group 2). **C)** Apoptotic cells (→) in I/R group (Group 3). **D)** Apoptotic cells (→) in bilberry before I/R group (Group 4). **E)** Apoptotic cells (→) in bilberry after I/R group (Group 5), TUNEL ×20.

Table 1
Distribution of histological damage according to groups.

	Group 1 (n = 8)	Group 2 (n = 10)	Group 3 (n = 10)	Group 4 (n = 10)	Group 5 (n = 10)
Vascular congestion	0.00	0.00	3.00	2.30 ± 0.7*	2.44 ± 1.1
Hemorrhage	0.00	0.00	3.00	2.20 ± 0.7*	2.44 ± 0.8
Leucocyte infiltration	0.00	0.00	3.00	1.50 ± 0.6*	2.04 ± 0.9
Edema	0.00	0.00	3.00	1.70 ± 0.6*	2.16 ± 0.7
Follicular degeneration	0.60 ± 0.4	0.60 ± 0.5	3.00	1.25 ± 0.6*	1.88 ± 0.7

*Chi square test, the difference between Group 3 and Group 4 was statistically significant ($p < 0.001$).

Table 2
[a]Comparisons of the mean values of superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) levels in tissue samples.

	Group 1 (Control group, n = 8)	Group 2 (Bilberry, n = 10)	Group 3 (Torsion + detorsion, n = 10)	Group 4 (Bilberry + torsion + detorsion n = 10)	Group 5 (Torsion + bilberry + detorsion, n = 10)	p
SOD(unit/mg)	8.7 ± 0.5	8.5 ± 0.6	7.1 ± 0.7	8.0 ± 0.7	7.6 ± 0.4	0.001
MDA(nanomole/mg)	2.7 ± 0.3	2.9 ± 0.3	3.4 ± 0.3	3.0 ± 0.2	3.2 ± 0.2	0.001
CAT(unit/mg)	107.0 ± 5.8	98.0 ± 4.1	83.2 ± 9.3	94.6 ± 4.2	90.8 ± 5.6	0.001

[a] One-way ANOVA with Bonferroni correction.

Table 3
[a]Comparisons of the mean values of superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) levels in blood samples.

	Group 1 (Control group, n = 8)	Group 2 (Bilberry, n = 10)	Group 3 (Torsion + detorsion, n = 10)	Group 4 (Bilbery + torsion + detorsion n = 10)	Group 5 (Torsion + bilberry + detorsion, n = 10)	p
SOD(unit/mg)	6.3 ± 1.9	5.6 ± 0.8	2.2 ± 1.5	3.6 ± 1.9[a]	2.9 ± 1.2	0.001
MDA(nanomole/mg)	0.8 ± 0.3	1.2 ± 0.4	2.3 ± 0.5	1.4 ± 0.3[a]	2.0 ± 0.4	0.001
CAT(unit/mg)	89.9 ± 6.0	82.0 ± 11.8	32.2 ± 11.4	62.2 ± 11.0[a]	46.5 ± 11.3	0.001

[a] One-way ANOVA with Bonferroni correction.

Ovarian torsion causes a decrease in blood vessels due to the rotation of the adnexa. The ischemic process leads to an increase in the levels of toxic substances such as ROS and an elevation in the lipid peroxidation products such as MDA. The ischemic cell reacts by rising the enzymatic activities of SOD and CAT to prevent the harmful effects of toxic molecules [13]. In our study, bilberry led to a significant decrease in MDA levels and a significant increase in the activities of SOD and CAT. There are many studies investigating the ovarian torsion. Taskin et al. reported that conservative treatment of ovarian torsion was important for fertility preservation [14]. Elevated MDA levels were shown due to ovarian I/R injury and SOD and CAT activities were found to be essential to prevent the toxic effects of MDA [15]. In the present study, MDA levels accepted as the final product of lipid peroxidation was found to be increased.

Halici et al. claimed that amlodipine could be useful against ovarian torsion [16]. There are many reports demonstrating the effects of some chemicals [17–19]. Hascalik et al. assessed the possible protective role of resveratrol on I/R damage of the ovaries in a rat model [20].

Bilberry was used as a free radical scavenger for superoxide anions and hydroxyl radicals. Ichiyanagi et al. demonstrated that the anthocyanins in bilberry extracts could be responsible from the protective effect towards reactive nitrogen species [21]. Milbury et al. claimed that bilberry could upregulates the oxidative stress related enzymes such as heme oxygenase-1 and glutathioneS-transferase [22]. Pandir et al. reported that bilberry administration seems to reduce the cisplatin induced ovarian toxicity [23]. However, there are no data about the effect of bilberry against ovarian torsion. Therefore, we hypothesized that as a free radical scavenger, bilberry would preserve I/R injury of the ovaries.

In our study, bilberry was used in prevention of ovarian I/R injury. When group 3 was compared with another groups, MDA levels were significantly higher, enzymatic activities of SOD and CAT were found to be as significantly lower in ovarian tissue and blood ($p < 0.001$). In histopathologic examination, ovarian tissue damage in the group 3 were significantly higher than other groups ($p < 0.001$). Also, DNA damage and apoptosis were significantly higher in group 3 than other groups ($p < 0.001$). Biochemical findings were lower and histopathologic damage was less especially in bilberry before I/R group (group 4). Group 5 was designed as feeding with bilberry after the ovarian torsion. In gynecology practice, the medical treatment is given after the diagnosis of ovarian torsion. However, when the ovarian torsion occurred the protective effect of the treatment would be limited. Therefore, we aimed to compare the different effect of bilberry before and after the ovarian torsion.

In conclusion, bilberry seems to be effective in prevention of ovarian I/R injury and short-term treatment. However, large prospective and randomized clinical studies are necessary to evaluate the protective effect of bilberry on ovarian torsion in rats.

Author contributions

O Kara: Project development, Data Collection, Manuscript writing

B Yakan: Project development, Data management, Manuscript writing

M Kara: Project development, Data analysis

E Kaymak: Data Collection

Compliance with ethical standards

Conflict of interest

This study was supported by the Erciyes University Scientific Research Projects' Unit with the number of TYL-6251.

Ethical approval

The study protocol was accepted by the Erciyes University's Experimental Animal and Local Ethics' Committee (no: 15/104/2015).

Informed consent

No informed consent was obtained. Because it was an experimental animal study.

Research involving human and animal rights

Institutional and national guidelines were used for the animals. All animals were handled in accordance with these criteria.

Acknowledgement

None declared.

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