Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2023.70033

Turk J Obstet Gynecol 2023;20:227-33

Protective and/or therapeutic effects of berberine in a model of premature ovarian failure induced by cyclophosphamide in rats

Sıçanlarda siklofosfomid ile indüklenen prematüre ovaryan yetmezlik modelinde berberinin koruyucu ve/veya tedavi edici etkileri

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Abstract

Objective: We conducted a biochemical and histological evaluation of whether berberine has a protective and/or therapeutic effect in a cyclophosphamide-induced premature ovarian failure (POF) model.

Materials and Methods: We divided 28 Wistar albino female rats into 4 groups [control group, POF group, cyclophosphamide (CP)+berberine (Bb) group, and POF+Bb group]. The POF model was established by intraperitoneal administration of 50 mg/kg CP on day 1 followed by 8 mg/kg/day CP dissolved in saline for the following 14 days. The CP+Bb group received Bb concurrently for two weeks with CP. The POF+Bb group received berberine for two weeks following the completion of CP administration. Left ovaries were used for histopathological evaluation and right ovaries were used for biochemical analysis [tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-6 levels in tissue].

Results: Ovarian damage scoring was significantly higher in the POF group than in the other groups ($p<0.005$). In the POF group, primordial and primary follicle counts were the lowest, while secondary and corpus luteum counts were the highest ($p<0.005$). There was no significant difference between the other groups. The POF group had significantly elevated levels of TNF-α, IL-1, and IL-6 in the biochemistry results ($p<0.005$).

Conclusion: We demonstrated that berberine could be effective in the protection and treatment of POF by reducing proinflammatory cytokines. We believe that our study can make a considerable contribution to the literature in terms of POF protection and/or treatment.

Keywords: POF, ovarian damage, TNF-α, follicle count, histopathologic evaluation

Öz

Amaç: Çalışmamızda siklofosfamid ile oluşturulan prematüre ovaryan yetmezlik (POY) modelinde berberinin koruyucu ve/veya tedavi edici etkisi olup olmadığı biyokimyasal ve histolojik olarak değerlendirdik.

Gereç ve Yöntemler: Çalışmada toplam 28 adet Wistar albino cinsi 180-220 g ağırlıklarında dişi ratlar 4 gruba [kontrol grubu, POY grubu, siklofosfomid (CP)+berberin (Bb) grubu ve POY+Bb grubu)] ayrıldı. POY modeli, 1. gün 50 mg/kg CP ve sonraki 14 gün 8 mg/kg/gün CP tuzlu su ile çözülerek intraperitoneal uygulanarak oluşturuldu. CP+Bb grubuna CP ile eşzamanlı 2 hafta Bb uygulandı. POY+Bb grubuna ise CP uygulamaları bittikten sonraki 2 hafta berberin uygulandı. Denekler sakrifiye edilirken, sağ ve sol overler eksize edildi. Sol ovaryumlar histopatolojik değerlenmede ve sağ ovaryumlar ise biyokimyasal analizde (dokuda tümör nekroz faktörü-alfa [TNF-α, interlökin (IL)-1, IL-6 düzeyleri] kullanıldı.

Bulgular: Overlerin hasar skorlaması, POY grubunda diğerler gruplara göre istatistiksel anlamlı yüksek bulundu ($p<0,005$). POY grubunda, primordial ve primer folikül sayımı en düşük, sekonder folikül ve korpus luteum sayımı en yüksek bulundu ($p<0,005$). Diğer gruplar arasında

PRECIS: We showed that Berberine had anti-inflammatory effects by decreasing IL-1, IL-6, TNF-alpha levels and thus prevented ovarian damage.

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Received/Geliş Tarihi: 13.06.2023 **Accepted/Kabul Tarihi:** 06.08.2023

istatistiksel farklılık gözlenmedi. Biyokimya sonuçlarında, POY grubunda TNF-α, IL-1, IL-6 düzey yüksekliği istatistiksel olarak anlamlı bulundu (p<0,005).

Sonuç: Berberinin proenflamatuvar sitokinleri azaltarak POY profilaksisinde ve tedavisinde etkili olabileceğini gösterdik. Çalışmamızın POY profilaksisi ve/veya tedavisi açısından literatüre önemli katkısı olacağını düşünmekteyiz.

Anahtar Kelimeler: POY, ovaryan hasar, TNF-α, folikül sayımı, histopatolojik değerlendirme

Introduction

Premature ovarian failure (POF), also known as primary ovarian failure, was coined by Fuller Albright in 1942 to describe hypergonadotropic hypogonadism in women younger than 40 years of age. In women under 40 years of age, amenorrhea is defined as the presence of amenorrhea lasting for at least four months and follicle-stimulating hormone levels greater than 40 mIU/L at two different intervals. Primary ovarian insufficiency occurs because of decreased follicular reserve and accelerated follicular atresia.

Currently, there is no clear understanding of the exact mechanism of POF. However, programed cell death, also known as apoptosis, has been closely associated with ovarian granulosa cell quality and condition. Ovarian granulosa cells play a promoting and estrogen-mediated regulatory role in the maturation and development of oocytes, maintaining hormonal balance in the ovarian niche to promote oocyte maturation through autocrine and paracrine mechanisms. It has been well documented that the sensitivity of ovarian granulosa cells and the decline in reserves and reduced function that occurs as a result of apoptosis, stimulation of ovarian atresia and early insufficiency in ovarian function as well as a significant reduction in the follicle pool result in POF. Thus, high-quality ovarian granulosa cells are needed to maintain ovarian function, oocyte viability, and normal ovulation[1,2].

Alkylating chemotherapeutics such as cyclophosphamide (CP) may cause POF by affecting nondividing oocytes and primordial follicles. It is common for women with POF to experience infertility, and a small proportion may still have the ability to conceive. Therefore, there is increasing interest in the preservation and/or treatment of fertility in cancer patients. In the literature, research on the etiology and/or treatment of POF involves creating a POF model with CP in rats or mice[3,4].

Berberine (Bb) is a nonbasic herbal quaternary benzylisoquinoline alkaloids with proven medicinal history in Ayurveda and Chinese medicine. It is present in the roots, rhizomes, and bark of a number of medicinally important plants, including hydrastis canadensis, coptis chinensis, berberis aquifolium, berberis vulgaris, and berberis aristata (tree turmeric, family berberidaceae). It is a shrub that can reach heights of up to 3 meters, growing at altitudes of 2000 to 3000 meters, with a wide distribution in the Himalayan region and the Nilgiri hills in southern India[5]. Its active components are Bb, Berbamine and Palmatine. Bb is currently also produced by chemical synthesis. The chloride or sulfate salt of Bb is often used for clinical purposes.

It is an odorless, dense yellow powder with characteristic alkaloidal bitter taste. The use of Bb in Ayurvedic and Chinese medicine dates back at least 3000 years due to its potent antimicrobial, antiprotozoal and antidiarrheal properties. Furthermore, various clinical trials over the years have revealed that Bb has a broad range of pharmacological effects. Many studies have suggested that it has significant antihypertensive, antiarrhythmic, antihyperglycemic, anticancer, antidepressant, anxiolytic, neuroprotective, antioxidant, anti-inflammatory, analgesic and hypolipidemic activities[6,7]. Furthermore, various studies have shown that Bb possesses nephroprotective[8], hepatoprotective[9], cardioprotective[10] and cerebroprotective[7] properties.

The potent antioxidant, anti-inflammatory and antiapoptotic effects of Bb may suppress ovarian damage and prevent or ameliorate POF by impeding the reduction in ovarian reserve. Hence, in our study, we aimed to investigate the protective and therapeutic effects of Bb in a CP-induced POF model by biochemically evaluating interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) levels in tissue and histologically by ovarian damage scoring and follicle counting.

Materials and Methods

The experimental procedure was approved by the Dokuz Eylül University Local Ethics Committee (protocol no: 09/2021, date: 10.03.2021). This study was conducted at the Dokuz Eylül University Experimental Animal Laboratory in January 2022.

The study employed 28 Wistar albino female rats weighing 180-220 g. Throughout the experimental period, the rats were followed under normal environmental conditions and dietary habits (21±2 °C, water and food were provided ad libitum). To equalize the effects of sex hormones on female rats, vaginal smears were performed at the beginning of the study and estrus rats were included in the study.

The rats (n=28) were randomly assigned to four groups. Group I (n=7): 1 mL saline (SF) was given by oral gavage to the control (sham) group. Group II (n=7): The POF group was administered 50 mg/kg CP (Endoxan, EIP Eczacibasi, Istanbul, Turkey) intraperitoneally on the first day, followed by 8 mg/kg/day CP SF for the subsequent 14 days, and the POF model was created[3,4]. Group III (n=7): The CP+Bb group was administered CP intraperitoneally by dissolving 50 mg/kg CP on the first day, followed by 8 mg/kg/day CP SF on the following 14 days. A single dose of 200 mg/kg/day Bb (Bb chloride, Sigma-Aldrich, cas. no: 633-65-8) prepared in 1 mL SF was administered by oral gavage for two weeks accompanied by CP administration[11,12].

In this group, the protective (prophylactic) effects of Bb against CP damage were studied[13,14]. Group IV (n=7): The POF+Bb group was administered 50 mg/kg CP intraperitoneally on the first day, followed by 8 mg/kg/day CP SF on the subsequent 14 days, and a POF model was created. For a duration of two weeks following the administration of the POF model, a single-dose oral gavage of 200 mg/kg/day Bb prepared in 1 mL SF was administered. In this group, the therapeutic effects of Bb on CP injury were studied.

At the end of the experiment, the subjects were sacrificed and the right and left ovarian tissues were collected. The left ovaries were fixed in formalin for histological evaluation, and the right ovaries were collected for biochemical analysis.

Histopathologic Evaluation

The left ovaries were fixed with formalin, dehydrated with different concentrations of alcohol, and embedded in paraffin. The maximum number of sections (4 µm thick) was collected from the ovaries and stained with hematoxylin and eosin. Follicle count and damage scoring were conducted in the histopathological evaluation of ovarian tissue[15]. Follicle counting included counting and averaging the primordial follicle, primary follicle, secondary follicle, tertiary follicle, and corpus luteum[16]. Damage scoring, on the other hand, assessed follicular cell degeneration, vascular congestion, hemorrhage, and inflammation parameters. Minimum five microscopic domains were examined to assign semiquantitative scores to the samples. Each sample was scored for each parameter using a scale ranging from 0 to 3 (0=absent; 1=mild; 2=moderate; 3=severe)[17,18]. Periodic acid-Schiff (PAS) staining was used to examine the zona pellucida structure[19].

Biochemical Evaluation

The biochemical evaluation included measurement of IL-1β, IL-6, and TNF-α levels in tissue. IL-1β, IL-6, and TNF-α (BTLAB, catalog numbers E0119Ra, E0135Ra, and E0764Ra) levels were analyzed by ELISA in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analysis of the study data was conducted using SPSS (Statistical Package for Social Sciences) 26.0 software. For analysis, the mean and standard deviation of the data were calculated. The difference between the groups was analyzed by the Kruskal-Wallis test, while the Mann-Whitney U test was utilized to determine which group caused the difference.

Results

There were no pathologic changes in the control group (Figure 1). The follicular degeneration, vascular congestion, edema, hemorrhage, and inflammation scores of the POF group were significantly higher than those of the other groups (control, CP+Bb, POF+Bb) ($p<0.005$). However, there was no significant difference between the control group, CP+Bb group, and

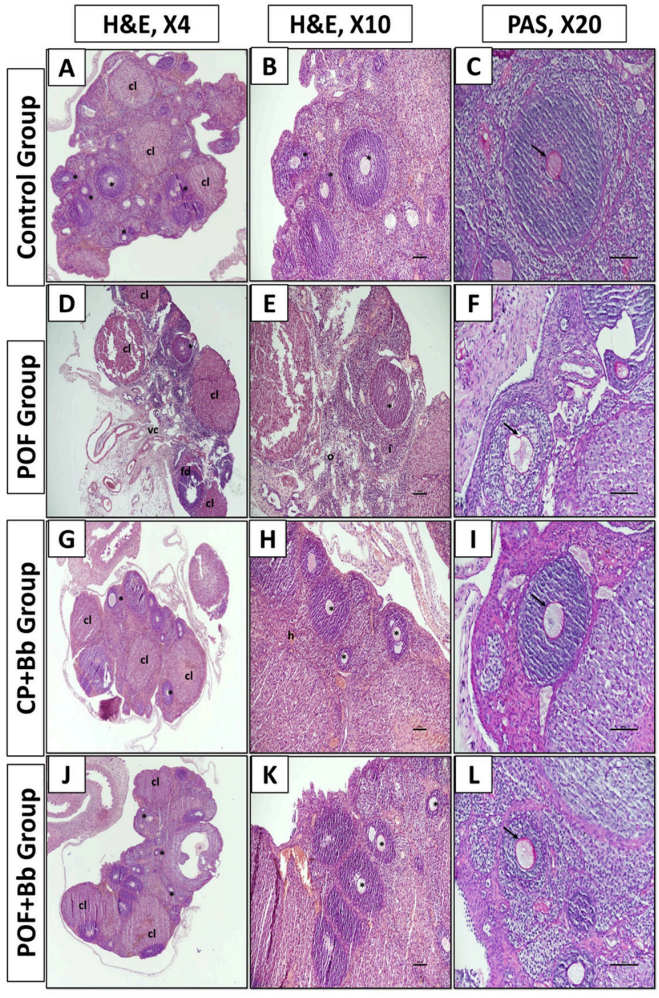


Figure 1. Photomicrographs of the ovarium tissue. *Follicles at different stages of development, corpus luteum (cl), vascular congestion (vc), hemorrhaging (h), edema (o), follicular degeneration (fd), inflammation (i). Black arrows show the zona pellucida. A, B, C: Control group, D, E, F: POF group, G, H, I: CP+Bb group, J, K, L: POY+Bb group, Scale bar: 100 µm

H&E: Hematoxylin and eosin staining, PAS: Periodic acid-Schiff staining, POF: Premature ovarian failure

POF+Bb group. Additionally, there was no significant difference between the CP+Bb group and POF+Bb group. Figure 2 shows the histopathologic damage scoring. In PAS staining, thinning of the zona pellucida was observed in the POF group.

Figure 2 summarizes the follicle numbers and damage scoring of the groups. The POF group had the lowest mean number of primordial follicles, whereas the number of primordial follicles in the other groups was significantly higher than that in the POF group ($p<0.005$). However, there was no difference between the control, CP+Bb and POF+Bb groups. The mean number of primary follicles was similar to the mean number of primordial follicles. Conversely, the highest numbers of secondary and tertiary follicles and corpus luteum were observed in the POF group. Secondary and tertiary follicles and the corpus luteum

counts of the other groups were significantly lower than those in the POF group (p<0.005). However, the number of secondary and tertiary follicles and corpus luteum did not differ between the other groups.

Figure 3 presents biochemical results of TNF-α, IL-1, and IL-6 levels in tissue. TNF-α, IL-1, and IL-6 levels in the tissue of the POF group were significantly higher than those in the other groups (control, CP+Bb and POF+Bb) (p<0.005). However, there was no significant difference between the control group, CP+Bb group, and POF+Bb group.3

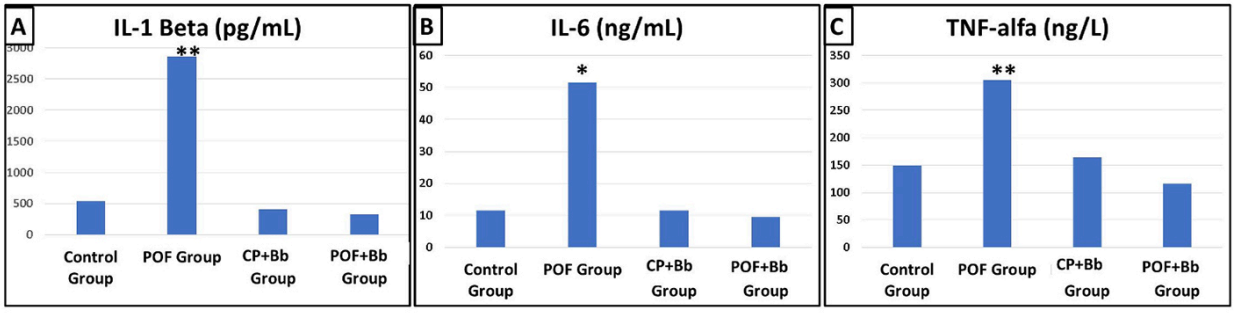
Discussion

Infertility is one of the most significant factors affecting the health of women today. POF, one of the leading causes of infertility, particularly in women under 40 years of age, is a multifactorial disease with an unclear etiology that results in ovarian dysfunction and early ovarian reserve depletion. Ongoing studies elucidate the etiology of infertility caused by POF and to develop protective and/or treatment protocols. To achieve this, appropriate experimental models are utilized, which can reproduce the clinical symptoms of the disease and

	Control Group	POF Group	CP+Bb Group	POF+Bb Group
Follicular Degeneration	0,2±0,4	2,5±0,5**[a]	1,2±0,5**[b]	1,0±0,5**[c]
Vascular Congestion	0,6±0,5	2,4±0,5**[a]	2,1±0,9	0,8±0,7**[c]
Haemorrhage	0,5±0,5	2,5±0,5**[a]	1,4±0,5*[b]	1,2±0,7*[c]
Inflammatory Cell	0,4±0,5	2,5±0,5**[a]	1,2±0,5**[b]	1,1±0,7**[c]
Primordial Follicles	6,0±1,1	1,2±0,5**[a]	4,1±1,0**[b]	6,0±0,8**[c]
Primary Follicles	4,8±0,7	1,7±0,5**[a]	4,0±0,8**[b]	4,4±1,2**[c]
Secondary Follicles	2,4±0,9	3,1±0,9**[a]	1,7±0,7**[b]	1,4±0,5**[c]
Tertiary Follicles	1,5±0,5	3,0±0,8*[a]	1,5±0,5*[b]	1,4±0,5**[c]
Corpus Luteum	1,4±0,5	4,8±2,1**[a]	2,1±0,9**[b]	1,2±0,4**[c]

Figure 2. Histopathological scores of the ovaries between groups, follicular cell degeneration, vascular congestion, hemorrhage, and inflammation parameters (0=none, 1=mild, 2=moderate, 3=severe). Primordial, primary, secondary, tertiary follicles and the corpus luteum were counted, and their averages were calculated. Data are expressed as the mean ± standard error of the mean. *p<0.05 **p<0.005. [a]Group POF vs. other groups, [b]group POF vs group CP+Bb, [c]group POF vs group POF+Bb. POF: Premature ovarian failure (n=7 for each group)

POF: Premature ovarian failure, CP: Cyclophosphamide, Bb: Berberine



D	IL-1 Beta (pg/mL)	IL-6 (ng/mL)	TNF-alfa (ng/L)
Control Group	541,3±413,8	11,5±2,9	149,1±41,4
POF Group	2856,5±1391,2**	51,64±63,9*	305,5±136,4**
CP+Bb Group	411,3±279,7	11,5±3,5	164,0±17,4
POF+Bb Group	330,0±313,3	9,5±1,9	116,6±19,5

Figure 3. The results of biochemical evaluation, A: IL-1, B: IL-6, C: TNF- alfa (TNF-α) levels in tissue. D: Comparison of IL-1, IL-6, and TNF-alpha (TNF-α) levels. *p<0.05, **p<0.005, Group POF vs. other groups

POF: Premature ovarian failure, IL: Interleukin, TNF-α: Tumor necrosis factor-alpha

induce ovarian damage, allowing histopathological evaluations to be performed. In our study, we created a POF model in rats with CP and formed four groups to investigate the role of Bb in both treatment and protection of POF. We administered Bb simultaneously with CP to investigate the prophylactic effect[13,14]. To evaluate the treatment process, we scrutinized the effects of Bb following CP administration (after the POF pattern was induced).

Known for its cytotoxic effects on ovaries, CP is an agent widely used in cancer treatment. The development of POF occurs as a secondary disease, particularly in women undergoing cancer treatment. Follicular atresia is a process in which multiple cell death processes are involved in ovarian granulosa cells. It is well known that apoptosis and autophagy play a primary role in this process[20]. Song et al.[21] administered 200 mg/kg CP on day 1 followed by 8 mg/kg/day for the subsequent 15 days in POF modeling. We did not prefer 200 mg/kg dose because of its high and strong toxic effect. Kilic et al.[22] preferred a dose of 200 mg/kg in their study, but they aimed to investigate the ovarian damage caused by ovarian cancers and medications used in cancer treatments. However, in our study, we administered a single dose of 50 mg/kg CP followed by 8 mg/kg/day CP for the following 14 days.

The process of declining the number and quality of oocytes and the failure of oocytes to regenerate is also called female reproductive aging. The term ovarian reserve refers to the quantity and quality of oocytes capable of forming dominant follicles during the late follicular phase of the menstrual cycle. Most women with decreased ovarian reserve have regular menstruation and a reduced number of ovarian follicles. Due to this condition, a limited response and decreased fecundity have been revealed in response to ovarian stimulation in the treatment of infertility[23]. Studies on POF have examined follicle numbers. In their POF model study, Özel et al.[19] demonstrated a decrease in the number of primordial and primary follicles, while indicating an increase in the number of corpus luteum and secondary follicles. Similarly, we observed a decrease in the number of primordial and primary follicles and an increase in the number of corpus luteum and secondary follicles in the POF group. The data we have obtained clearly indicate that we have successfully created a POF model fit for purpose.

The processes of inflammation, apoptosis, oxidative stress, and aging are considered interconnected. For instance, an increase in pro-inflammatory cytokines has been observed in aging vessels[24]. Another study noted an increase in inflammatory cytokines in ovarian failure, also called ovarian aging. The expression levels of pro-inflammatory cytokines such as TNF-α, TGF-β, IL-8, IL-6, IL-1β, and increased interferon-gamma increased by PCR in mice in the ovarian failure model induced by CP administration, and pro-inflammatory cytokines decreased after the administration of skin-derived mesenchymal stem cells (SMSCs). Thus, SMCs may play a role in restoring the function of the damaged ovary[25]. Park et al.[26] observed increased apoptosis and inflammation in mice treated with CP. With the increase in TNF-α and IL-6, it was hypothesized that oogenesis and ovarian function could be restored with an anti-inflammatory effect. Similarly, in our study where we created a POF model, we observed elevated pro-inflammatory cytokines in rat ovaries. These data suggest that proinflammatory cytokines may play a role in the decrease in the number and quality of oocytes.

Bb has a very wide range of pharmacological action profiles, ranging from antioxidant action to affect neurotransmitters, enzymes, molecular targets, and immunomodulation. Various clinical studies have best demonstrated the antioxidant and anti-apoptotic effects of Bb in a variety of diseases ranging from diabetes to hypercholesterolemia, Alzheimer's disease and cerebral ischemia[27,28]. Song et al.[11] investigated the potential therapeutic effect of Bb on diabetes-induced testicular damage in rats. They administered 200 mg/kg/day Bb for 28 days. The results showed that the testicular tissue improved histomorphological terms and the TUNEL assay revealed a decrease in apoptosis[11]. Zhang et al.[12] examined the effect of Bb in the PCOS model induced in rats. Twenty-eight days of 200 mg/kg and 400 mg/kg Bb administration improved the histomorphologic damage scoring of the ovary and increased follicle reserve. Another study examined the effect of 150 mg/kg Bb in the PCOS model created in rats, but no improvement findings were observed[29]. This might be due to the inadequate dosage of Bb. Zhu et al.[30] showed an anti-inflammatory effect by decreasing IL-1, IL-6 and TNF-α levels by administering 50 mg/kg Bb for 4 weeks in an ulcerative colitis model. Huang et al.[31] demonstrated the anti-inflammatory and antioxidant effects of Bb in head trauma. In our study, we observed the anti-inflammatory effect of Bb and its healing effects on ovarian damage.

A similar study by Xue et al.[32] examined the effects of Bb at three different dosages for the treatment of POF and found that it was effective. In our study, we investigated and compared the preventive and therapeutic effects of Bb. We also showed that it has protective and therapeutic effects. Our study has contributed to the literature by demonstrating that it can be used in POF prophylaxis.

Study Limitations

Our study has some limitations. First, we did not assess oxidative stress markers and apoptosis parameters in serum or tissue samples. Second, the subjects were rats and clinical data are needed to confirm its efficacy and dose estimation in humans. These limitations should be considered in future studies.

Conclusion

In conclusion, we have revealed that Bb use may have POF protective and therapeutic effects. We suggest that the anti-inflammatory effect of Bb (by decreasing proinflammatory cytokines) and its effects on follicle reserve may preserve

fertility. We believe that our study will make a significant contribution to the literature regarding the prophylactic and/or therapeutic use of POFs.

Ethics

Ethics Committee Approval: The experimental procedure was approved by the Dokuz Eylül University Local Ethics Committee (protocol no: 09/2021, date: 10.03.2021). This study was conducted at the Dokuz Eylül University Experimental Animal Laboratory in January 2022.
Informed Consent: Not necessary.
Peer-review: Internally and externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: O.İ., F.Y., S.K., Concept: O.İ., F.Y., S.K., Design: O.İ., F.Y., S.K., Data Collection or Processing: O.İ., F.Y., S.K., Analysis or Interpretation: O.İ., F.Y., S.K., Literature Search: O.İ., F.Y., S.K., Writing: O.İ., F.Y., S.K.
Conflict of Interest: No conflict of interest was declared by the authors.
Financial Disclosure: The authors declared that this study received no financial support.

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