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# The propolis and boric acid can be highly suitable, alone/or as a combinatory approach on ovary ischemia-reperfusion injury

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## Abstract

**Purpose** Ovarian ischemia-reperfusion (IR) damage continues to be a serious infertility problem. The oxidative stress plays central role in the development of IR injuries. Activation of antioxidants decreases IR injuries; however, the efficacy of antioxidant agents remains controversial. Unfortunately, there has been no evidence for medicinal use of boric acid (BA) and propolis (Prop) on ovarian IR injury on rats so far. This study will provide to reveal the potential applications of the Prop and BA in ovarian IR therapy.

**Methods** The Sprague–Dawley rats were randomized into five groups: I—control, II—IR, 3 h of ischemia and 3 h of reperfusion, III and IV—a signal dose of oral BA (7 mg/kg) and Prop (100 mg/kg) alone 1 h before induction of IR, V—Prop and BA together 1 h before induction of IR. SOD (superoxide dismutase), CAT (catalase), GSH (glutathione), MPO (myeloperoxidase), MDA (malondialdehyde), and IL-6 (interleukin-6) levels were quantified by ELISA and the TNF-α (tumor necrosis factor-α), 8-OHdG (8-hydroxylo-2′-deoxyguanosin) and Caspase-3 expressions were performed by immunohistochemical analyses.

**Results** BA and Prop pretreatment significantly reduced MPO, MDA, and IL-6 levels and pathologic score in IR rats, with no effects in control group. These agents used in therapy also decreased TNF-α, 8-OHdG and Caspase-3 protein expressions increased by IR. Furthermore, BA and Prop combination showed significant ameliorative effects on ovary injury caused by IR through acting as an antioxidant, anti-inflammatory and antiapoptotic agent.

**Conclusion** BA and Prop alone and especially in combination could be developed as therapeutic agents against ovary IR injury.

**Keywords** Ischemia-reperfusion · Ovary · Propolis · Boric acid · Oxidative stress/antioxidant activity · Inflammation

## Introduction

Ischemia process cuts off blood supply to the affected tissues, resulting in ischemic damage that is often irreversible. Reperfusion following ischemia causes cellular damage and organ dysfunction known as reperfusion injury. Especially in adolescents and young adults, ovarian IR injury can become a risk factor for the loss of ovarian function. Timely diagnosis and intervention are keys to saving the affected ovary and to prevent future infertility [1]. Although there are many studies in the literature, ischemia-reperfusion (IR) damage continues to be a serious problem clinically [2, 3].

Propolis (Prop), a natural bioactive resin produced by the bees (*Apis mellifera*), with anti-neurotoxic, anti-viral, antibacterial, anti-cancer and antioxidant effects has been used to treat heart disease, diabetes, and cancer diseases [4–6]. This natural compound would have a promising role in future medicine [7]; however, the mechanisms by which Prop mediates protection to improve health or prevent diseases are still unclear [8].

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Boron-containing compounds have received tremendously attention from the pharmaceutical industry and academia over the last decades. They have shown interesting and useful biological properties including antiparasitic, antibacterial, antifungal and antioxidant activities [9, 10]. Boron is an essential and multifunctional microelement for animals and human [11]. It has important roles in the inflammatory process, the central nervous system, endocrine system, and mineral metabolism [12, 13]. Several lines of evidence indicate that boron is important for normal reproduction function in plants [14]. On the other hand, the toxic doses of boron can significantly inhibit the development of the reproductive organs in humans and animals [15, 16]. However, the detailed action of boron on reproductive systems remains obscure. Therefore, an appropriate dose of boron is necessary for the health of body.

Post-ischemic reperfusion causes enhanced production of free radicals and the development of inflammatory response within a few hours after onset of the insult [17]. While oxidative stress plays central roles in the development of IR injuries, the efficacy of antioxidant therapy remains controversial [18]. Further investigation should explore the Prop' targets and actions to obtain new drugs. In view of the use of the antioxidants in combination with bee products for treatments of disorders and promotion of health, we think that these pharmacological materials will represent a remedy to be used for severe IR damages now. Up until now, the protective effects of pre-ischemic treatment with boron compounds remain unexplored. The aim is to justify the consideration of Prop and boric acid (BA) as effective remedies for the treatment of ovarian IR injury. In addition, the aim of this research is to profoundly analyze the impact of the combined therapy of Prop and BA on ovary IR damage in rats.

## Materials and methods

### Boric acid (BA) and Prop preparation

The water-soluble Prop extract (Aksuvital Natural Products Food Industry Trade Inc., Turkey) was dissolved in distilled water and then diluted at 100 mg/kg concentrations. BA (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) was dissolved in distilled water and then diluted at 7 mg/kg concentrations. The doses of them were selected according to the literature data and our preliminary studies [19, 20].

### Animals

This study was carried out in Atatürk University Experimental Research Center in Erzurum, Turkey. The rats were housed in polypropylene cages and given ad libitum access to food and water with a natural day/night cycle. All animal manipulations were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

### Experimental design and ovarian IR procedure

The female Sprague–Dawley rats (250–300 g) were randomized into five groups ($n=7$):

Group I: Sham (control), the rats underwent laparotomy operation;
Group II: Ischemia/reperfusion (IR), 3 h of ischemia and following 3 h of reperfusion was created;
Groups III and IV: The rats received a signal dose of oral BA (7 mg/kg) and Prop (100 mg/kg) alone 1 h before induction of IR;
Group V: The rats received BA and Prop together 1 h before this procedure.

Each rat was anesthetized via intraperitoneal (i.p.) injection of a combination of ketamine (75 mg/kg, i.p) and xylazine (10 mg/kg, i.p.). And then ovaries were visualized by a 2–2.5 incision in the lower abdomen under anesthesia. Vascular clips were applied on the lower part of the ovaries of the rats in IR and treatment groups. Then, 3 h of ischemia and following 3 h of reperfusion were created [21]. At the end of procedure, the animals were killed.

### Biochemical analysis

The ovarian tissues were homogenized, and the supernatants were used to determine the antioxidant enzyme profile, oxidative status and cytokine levels. The levels of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), myeloperoxidase (MPO), lipid peroxidation (LPO), and interleukin-6 (IL-6) in the tissue were measured with the Clinical Automatic Biochemistry Analyzer 7600 (Hitachi, Japan) employing enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All experiments were performed with triplicate samples and repeated three times.

### Immunohistochemical and histopathological assessments

The ovarian tissues were fixed in 10% neutral buffered formalin overnight, dehydrated, and embedded in paraffin for hematoxylin and eosin (H&E) staining or immunostaining. The ovarian tissues were sectioned at 5 µm. Sections were stained with H&E and analyzed using a light microscope (Leica DM 1000, Germany). Analysis of the sections was performed by the same pathologist blindly.

Tumour necrosis factor (TNF-α), Caspase-3, and 8-hydroxylo-2′-deoxyguanosin (8-OHdG) were detected by specific monoclonal antibodies. The ovarian tissues were cut into 4-μm sections and the sections were deparaffinized. After diaminobenzidine (DAB) was applied as chromogen, slides were counterstained with hematoxylin, dehydrated, and covered by coverslips. The expression of TNF-α was determined by goat monoclonal anti-TNF-α (1:300 dilution; Sigma, USA). Caspase-3 immunostaining was performed using polyclonal rabbit-anti-human (rabbit, 1:1000, Cell Signaling Technology, Beverly, MA, USA). Immunohistochemical staining of 8-OHdG was performed using anti-8-hydroxydeoxyguanosine (8-OHdG) antibody (Santa Cruz; 1:2500 dilution) with a Novolink Polymer Detection kit (Leica Microsystems Pte Ltd., Taipei, Taiwan), following the manufacturer's instructions. The pathologists continuously observed at least 10 high-power fields (×200) for each slice. 21 different ovarian sections of 7 animals per experimental group were quantified. The pathologists counted the number of positive cells in each high-power field, and calculated the average number of positive cells to reflect the intensity of positive expression. The sections were evaluated as none (−), weak (+), mild (++), moderate (+++) and severe (++++) according to their immunity positivity [22].

### Statistical analysis

The differences in variance were analyzed statistically using a one-way analysis of variance (ANOVA) test by Graphpad prism 5.0 statistics software (GraphPad, La Jolla, CA, USA). Tukey's test was used as a post hoc. The non-parametric Kruskal–Wallis test was used to analyze variations among data obtained using the semi-quantitative method at histopathological examination. Analyses between two groups were performed using the Mann–Whitney *U* test. $p < 0.05$ was regarded as statistically significant.

## Results

### Effect of BA and Prop on biochemical parameters in ovarian IR

As shown in Table 1, ovarian IR caused increases in the MPO and LPO levels and decreases in the SOD, CAT, and GSH levels in rats compared with sham group ($p < 0.0001$). However, pretreatment with BA 7 mg/kg significantly increased the SOD and GSH, and decreased in the LPO ($p < 0.0001$) level in ovary compared with the IR group. Nevertheless, BA did not affect the level of MPO induced by IR in ovary. Similarly, pretreatment with Prop 100 mg/kg significantly increased the SOD, CAT and GSH and decreased in the MPO ($p < 0.001$) and LPO ($p < 0.0001$) levels in ovary compared with the IR group. On the other hand, pretreatment of the combination with Prop and BA had a more positive effect on these biochemical levels than those of the other experimental groups in IR model ($p < 0.0001$).

The level of IL-6 was increased in IR group ($p < 0.0001$) (Table 1). However, oral administration of 100 mg/kg prop decreased the level of IL-6 in the rat's ovary ($p < 0.05$). Moreover, 7 mg/kg BA did not alter the IL-6 level when compared with IR group. The level of IL-6 in BA+Prop+IR group was significantly lower than Prop+IR and control groups ($p < 0.0001$).

### Effect of BA and Prop on TNF-α, Caspase-3, and 8-OHdG immunoreactivity in ovarian IR

The representative images of immunoreactivities for TNF-α, Caspase-3, and 8-OHdG are depicted on Fig. 1. There was no immunoreactive TNF-α, Caspase-3, and 8-OHdG in ovary of sham-operated group, while it became present in the IR group. Pretreatment with alone BA or Prop

**Table 1** The effects of BA and Prop on ovary SOD, CAT, GSH, MPO, LPO, and IL-6 levels after IR

| Groups | SOD mmol/min/mg tissue | CAT μmol/min/mg tissue | GSH mmol/mg tissue | MPO EU/mg tissue | LPO nmol MDA/g tissue | IL-6 pg/mg tissue |
|---|---|---|---|---|---|---|
| Sham | $2.23 \pm 0.07$ | $1120 \pm 16.9$ | $3.51 \pm 0.06$ | $12.9 \pm 0.45$ | $45.85 \pm 0.26$ | $947.2 \pm 31.48$ |
| IR | $0.30 \pm 0.04^{a4}$ | $767.3 \pm 23^{a4}$ | $2.58 \pm 0.03^{a4}$ | $126.7 \pm 3.27^{a4}$ | $67.56 \pm 1^{a4}$ | $1714 \pm 37.65^{a4}$ |
| BA+IR | $3.76 \pm 0.16^{a4,\ b4}$ | $720 \pm 23.9^{a4}$ | $3.16 \pm 0.04^{a4,b4}$ | $125.4 \pm 2.48^{a4}$ | $59.24 \pm 0.48^{a4,b4}$ | $1667 \pm 57.9^{a4}$ |
| Prop+IR | $2.57 \pm 0.06^{b4,\ c4}$ | $865 \pm 25.6^{a4,c2}$ | $3.09 \pm 0.03^{a4,b4}$ | $109.2 \pm 3.71^{a4,b3,c3}$ | $57.33 \pm 0.55^{a4,b4}$ | $1537 \pm 45.1^{a4,b1}$ |
| BA+Prop+IR | $2.19 \pm 0.08^{b4,\ c4}$ | $923 \pm 39.9^{a3,b2,c3}$ | $4.62 \pm 0.04^{a4,b4,c4,d4}$ | $1.91 \pm 0.22^{a1,b4,c4,d4}$ | $48.15 \pm 0.74^{b4,c4,d4}$ | $1117 \pm 38.4^{b4,c4,d4}$ |

Data are presented as mean ± SEM ($n = 7$)

*IR* ischemia/reperfusion, *BA* boric acid, *Prop* propolis

[a]Significant differences between other studied groups and sham ($^{a1}p < 0.05$, $^{a3}p < 0.001$, $^{a4}p < 0.0001$)

[b]Significant differences between other studied groups and IR group ($^{b1}p < 0.05$, $^{b2}p < 0.01$, $^{b3}p < .0.001$, $^{b4}p < 0.0001$)

[c]Significant differences between other studied groups and BA+IR group ($^{c2}p < 0.01$, $^{c3}p < .0.001$, $^{c4}p < 0.0001$)

[d]Significant differences between other studied groups and Prop+IR ($^{d4}p < 0.0001$) by Tukey's multiple range tests

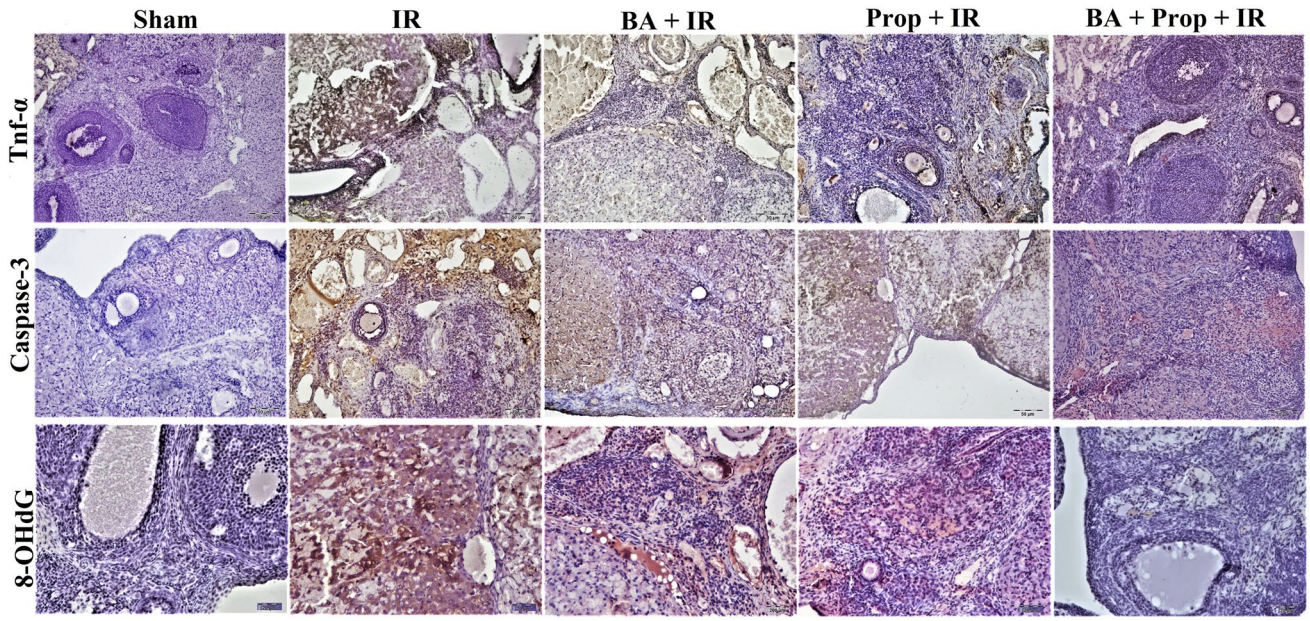


**Fig. 1** Immunohistochemical staining of the TNF-α, Caspase-3, and 8-OHdG in the rat ovarian tissue. Sham—TNF-α, Caspase-3, and 8-OHdG negative, IR—severe TNF-α, Caspase-3, and 8-OHdG expression, BA+IR—moderate TNF-α, Caspase-3, and 8-OHdG expression, Prop+IR group—mild TNF-α, Caspase-3, and 8-OHdG expression, BA+Prop+IR group—weak TNF-α, Caspase-3, and 8-OHdG expression (bar 20 µm). *IR* ischemia/reperfusion, *BA* boric acid, *Prop* propolis

**Table 2** Immunohistochemical findings and their scores in ovarian tissue

| Groups | Tnf-α | Caspase-3 | 8-OHdG |
|---|---|---|---|
| Control | – | – | – |
| IR | ++++ | ++++ | ++++ |
| BA+IR | +++ | +++ | +++ |
| Prop+IR | ++ | ++ | ++ |
| BA+Prop+IR | + | + | + |

According to immunohistochemical findings: none (−), weak (+), mild (++), moderate (+++) and severe (++++)

*IR* ischemia/reperfusion, *BA* boric acid, *Prop* propolis

moderately attenuated expression of TNF-α, Caspase-3, and 8-OHdG in the ovary with IR (Table 2). In contrast, pretreatment of the combination with them showed mild immunoreactivity for TNF-α, Caspase-3, and 8-OHdG.

## Histopathological effects of BA and Prop in ovarian IR

Ovary histology was also assessed by H&E staining. As shown in Fig. 2, sham rat had normal ovary histology. Ovarian tissues in the IR group showed severe vascular congestion, hemorrhage, edema, cell degeneration, necrosis, and

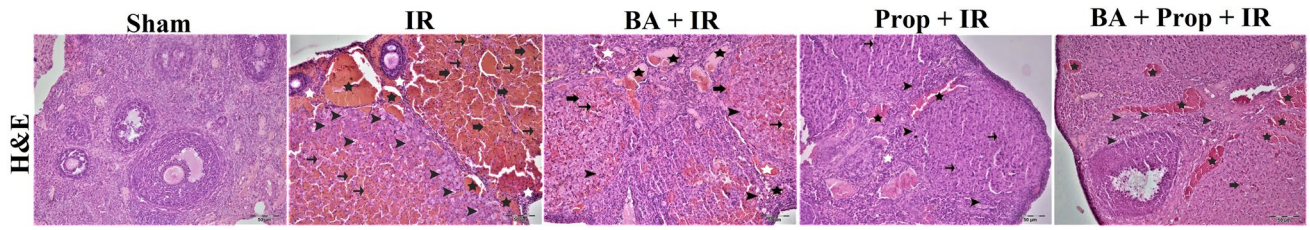


**Fig. 2** H&E staining of rat ovary. Sham—Sham group with normal ovary histology, IR group—arrow heads: degenerated parenchyma cell, thick arrows: hemorrhage, thin arrows: necrosis, black asterisk: congestion, white asterisk: neutrophil infiltration, BA+IR—decreased histological changes, Prop+IR—decreased histological changes, BA+Prop+IR—the group protected by previous administrations of BA and Prop (bar: 20 µm). *IR* ischemia/reperfusion, *BA* boric acid, *Prop* propolis

neutrophil infiltration. In contrast, BA or Prop pretreatment caused mild reduction of these histological changes induced by IR in ovarian tissues. On the other hand, BA+ Prop+ IR pretreatment group markedly ameliorated the ovarian damage induced by IR. Particularly, the group treated with combination of BA and Prop showed no hemorrhage and necrosis.

## Discussion

Many researchers have investigated pharmacological strategies that can make the ovarian more resistant to ischemic death so-called "novel theraphies" interventions [23–25]. It was also emphasized that further in vivo studies are needed to confirm the beneficial effects of novel therapies in prevention of vascular diseases [26]. A recent study reported that intraperitoneal administration of alpha-tocopherol loaded nanoparticles could be helpful in minimizing IR injury in ovarian tissue exposed to ischemia [27]. To our knowledge, this is the first study that provides insights on the protective effect of BA and Prop against ovary IR injury in rats. In this paper, we are focusing on IR-induced ovary damage and beyond, the role of antioxidant factors, inflammatory and apoptotic alterations, ovarian's pathology, and also the DNA damage marker, 8-OHdG.

Indeed, as basic and clinical research continues to support effectiveness and reliability of therapeutic interventions against ischemia-reperfusion injury in acute ovary injury, our information suggests that potent activities of BA and Prop can be highly suitable, alone/or as a combinatory approach. In the presented study, oxidative stress was evident in IR group which had lower antioxidant enzyme activities with higher MDA and lower GSH levels. Lipid metabolism disorders are known to increase oxidative stress, which correlates with increased reactive oxygen species (ROS) production. This mechanism has been shown to exacerbate the damage produced by IR injury [28]. Compared to control rats, pre-ischemic treatment in our study was associated with reductions in IR-induced oxidative stress and ovarian damage. According to our in vivo findings, 100 mg/kg Prop or 7 mg/kg BA alone significantly restored the MDA levels in ovary tissue as well as SOD and CAT activities. Furthermore, the significant higher values of GSH were observed in treatment groups compared to IR group ($p < 0.0001$). Increased ROS production and impaired antioxidant capacity have been proposed as major contributory factors to ovary dysfunction during ovarian IR injury [18]. The antioxidant activities of p BA and Prop were shown in several physiological and cellular processes leading to therapeutic effects against different diseases [29, 30]. The biological functions, including antioxidant properties of BA, were mainly associated with its composition. The previous reports indicated that BA with its electron-withdrawing groups may play important role in maintaining protective effects against tissue damage [31, 32].

In fact, Prop was used alone or in combination with other natural products, not only as a nutraceutical supplement, but also as a natural antioxidant in food and related products [33]. A research has been carried out to examine the antioxidant and anti-inflammatory effects of the combination of honey and Prop [34]. It was suggested that polyphenols contained in the Prop induce the enzyme activity, leading to increase in GSH levels [35]. Since boron deficiency influences the metabolism of phenolic compounds in plants [36, 37], the present study focused on the antioxidant response of the combination BA and Prop against ovarian IR injury. These were the first observations that indicated BA and Prop together could modulate oxidative stress and antioxidant status, suggesting the potential of the combined therapy for IR injury of ovarian. It was reported that oxidative stress causes irreversible damage in DNA and other macromolecules (lipids, proteins, kinases, etc.); however, the endogenous antioxidant defence systems could preserve cells from the harmful effects of free oxygen radicals [38].

Regarding IR injury, the degree of inflammation may have deleterious consequences for ovary with important implications in a variety of pathophysiological conditions, including ovary IR injury. Studies demonstrated that the agents with anti-inflammatory activities may be beneficial in reducing ovarian IR injury [39]. In our study, BA and Prop inhibited ovary inflammation through the levels of IL-6. Moreover, BA and Prop led to a decline in TNF-α expression supporting their anti-inflammatory effects. A study reported that polyphenol-rich Prop exhibited significant in vitro anti-inflammatory effects by modulating key inflammatory mediators of mRNA transcription, inhibiting the production of specific inflammatory cytokines [40]. A recent report indicated that the mechanisms of Prop on modulating inflammatory diseases are still not fully elucidated [41]. Boron compounds might have anti-inflammatory effects in the case of increased inflammation of the metabolism due to various reasons in the literature [42, 43]. Our study supported the fact that the negative tableau incurring in ovary with IR can be significantly remedied by the anti-inflammatory effects of the BA and Prop combination.

The IL-6, TNF-α and ROS interactions result in a complex inflammatory response and facilitate inflammatory cells entry into the vessel wall following reperfusion [44]. Prop was shown to have immunomodulating effects on inflammatory cells as mastocytes and macrophages in a new sponge implant hamster model [45]. Noteworthy, IR induces MPO release from neutrophils, aggravates tissue damage. Thus, MPO can be used for studying tissue-damaging neutrophilia in IR [46]. Our results were confirmed by measuring MPO release in ELISA. The BA and Prop together preserved

ovarian tissue in vivo much better than BA and Prop alone in IR model. The significant suppressive effects on MPO levels and on neutrophil infiltration in the ovarian tissue were found. This translates into a significant decrease in acute damage due to inflammation of ovary tissue.

Reperfusion of ischemic ovary initiates death of parenchymal cells due to a combination of apoptosis and necrosis. For this reason, the limitation apoptosis and/or necrosis represent a major unmet clinical need [47]. At this point, our study can exhibit a new therapeutic potential for Prop on down-regulation of caspase-3 expression. Similarly, BA significantly decreases Caspase-3 protein expression increased by IR and can lead to considerably an increase in viable cells. The main issue to be clarified here is the extent to which Caspase-3 has been activated with which mechanisms. As is the case in the presented study, agents used in therapy can inhibit some mechanisms that activate Caspase-3. Oxidative stress and inflammation are used to explain the mechanisms involved in apoptosis formation [48]. And the last step in the mechanism of apoptotic damage is the activation of Caspase-3 [49]. Therefore, a general interpretation of the results of the apoptotic process can only be made based on Caspase-3 mRNA expression levels. In our study, ovarian IR injury is characterized by increased oxidative stress, inflammation and apoptosis so optimal utility of BA and Prop in IR model is important. From these data, we conclude that BA and Prop play an important role to protect ovary tissue, and the combination of them provides a significant decrease in necrotic cells.

From a biological standpoint, IR was found to induce the DNA strand breaks, oxidative DNA damage and mutations [50, 51]. However, genetic evidence supporting the key role of 8-OHdG formations in oxidative stress and ovary IR injury is still missing. Previous results suggested that Prop might decrease products of oxidative processes (4-hydroxy-2-nonenal and 8-OHdG) and prevent the development of pulmonary and renal cancers [52, 53]. It was noteworthy that DNA strand breakage can be induced by hydroxyl radical (HO•) via formation of 8-hydroxylo-2′-deoxyguanosin (8-OHdG) DNA adduct [54]. A recent study showed that BA may reduce the formation of DNA double-strand breaks due to oxidative stress and accelerate wound healing in human epithelial cell line [55]. Our findings revealed that treatment with BA and Prop greatly attenuated the deleterious effects of IR through suppression of 8-OHdG. However, it was reported that high boron levels are toxic to plants and animals and new studies on the effects of different doses of boron compounds in laboratory animals with regard to antioxidant and metabolic effects may be helpful for the understanding of the subject [56]. Our results showed that there was no significant difference between control and BA-treated animals regarding the levels of DNA damage. Data above indicated that BA with a dose 7 mg/kg was safe and had a significant influence against IR-induced ovary injury in rats. Furthermore, 8-OHdG expressions in our study were markedly mitigated after the combine BA and Prop treatments. Moreover, BA and Prop together improved the histological features of the ovary as represented by slight edema and slight degeneration, no hemorrhage and necrosis. These effects have been related to the highest antioxidant values and free radical scavenging properties of BA and Prop.

## Conclusions

This is the first study that shows that BA and Prop alone can ameliorate ovary injury and this outcome is essentially due to the antioxidant, anti-inflammatory and anti-apoptotic effects of them. Furthermore, therapeutics such as BA and Prop for ovary IR damage have significant impacts on oxidative DNA damage and lesions of the ovarian. Especially, the combined therapy could be a useful dietary supplement or may become the new medicines in the future which can also relieve ischemic injury.

**Author contributions** FG and KK: performed the experiments, collected and analyzed the data and wrote the manuscript. HSE and SC: performed the experiments, and analyzed the data and performed the quality control of data and algorithms. HA and SAJ: collected the data. GE and MBD: participated in animal studies and related experiments. YSS: analyzed the data and revised the manuscript. All the authors read and approved the final manuscript.

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### Compliance with ethical standards

**Conflict of interest** All the authors declare that there is no conflict of interest.

**Ethics approval** This study was approved by the Atatürk University Local Ethics Committee for Animal Experiments (No. 129, 11.07.2016).

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