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ORIGINAL ARTICLE

Protective effect of propolis on myocardial ischemia/reperfusion injury in males and ovariectomized females but not in intact females

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Abstract

The aim of this study is to investigate the effect of propolis, which may have estrogenic effects, on myocardial ischemia/reperfusion (mI/R) injury not only in male rats but also in intact and ovariectomized (ovx) female rats. Six groups were formed: untreated males ($n = 8$), treated males ($n = 9$), untreated intact females ($n = 9$), treated intact females ($n = 10$), untreated ovx females ($n = 10$), and treated ovx females ($n = 8$). An alcoholic extract of a single dose of propolis (200 mg/kg) was administered orally daily for 14 days. Thirty minutes of ischemia and 120 min of reperfusion were performed. Blood pressure, heart rate, arrhythmias (ventricular premature contraction [VPC], ventricular tachycardia [VT], ventricular fibrillation [VF]), and myocardial infarct size were evaluated. Total antioxidant status (TAS), total oxidant status (TOS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and 17 beta-estradiol (E2) were measured. The untreated females showed more resistance to mI/R injury than the untreated males, as evidenced by lower duration, incidence, and score of arrhythmias, and smaller infarct size ($p < .05$). After ovx, this resistance disappeared. Propolis improved these values in treated males and treated ovx females ($p < .05$). Propolis increased TAS in treated males and decreased TOS in treated ovx females as well as elevated SOD in all treated groups ($p < .05$). Propolis decreased E2 level in treated intact females; however, it increased E2 level in treated ovx females ($p < .05$). The results revealed that propolis could protect the heart against mI/R injury in males and ovx females.

Practical applications

It is known that the female heart has an increased sensitivity to myocardial ischemia/reperfusion (mI/R) injury due to estrogen deficiency and/or estrogen deprivation following menopause or surgical removal of the ovaries. Propolis has the potential to mimic estrogen under physiological and pathophysiological conditions, as well as its antioxidant property. The results indicated that propolis decreased myocardial infarct size, arrhythmia score, arrhythmia duration, and incidence in ovariectomized female rats and male rats. In addition, the present results demonstrated that an alcoholic

extract of propolis as a natural product can effectively maintain the resistance of female heart to mI/R injury after estrogen deficiency.

KEYWORDS
arrhythmia, female, estrogen, infarction, male, propolis

1 | INTRODUCTION

Cardiovascular diseases, including cardiomyopathy, heart attack, and myocardial ischemia/reperfusion (mI/R) injury are still leading cause of mortality and morbidity all over the world (Sattler et al., 2019; Virani et al., 2021). Ventricular arrhythmias caused by acute myocardial infarction are one of the most common causes of death in humans. Fatal ventricular arrhythmias have been observed in acute myocardial infarction, and the survival rate is known to be low (Sattler et al., 2019). It is well known that male and female hearts show different susceptibility to mI/R injury (Ruiz-Meana et al., 2020). Mortality from ischemic heart disease was higher in men than in women, suggesting a possible protective role of estrogen (Sivasinprasasn et al., 2016). However, this advantage disappears with menopause, that is, due to a deficiency or absence of estrogen. After menopause, women showed less resistance to mI/R injury, as indicated by a higher incidence of acute myocardial infarction, ventricular arrhythmias, and an increase in mortality from cardiovascular diseases (Benjamin et al., 2019; Sivasinprasasn et al., 2016).

Propolis is a natural compound produced by honeybees to protect their colonies from invaders such as insects, bacteria, and viruses, and to keep temperature and humidity in the hive stable. Propolis contains more than 300 active chemical ingredients and has been used by many civilizations from ancient times to the present as a traditional remedy to improve health and prevent disease (Rivera-Yanez et al., 2020). Several studies have indicated that propolis and/or its active compounds have a protective effect on diabetic heart (Ibrahim et al., 2019), in the mobile phone-induced myocardial oxidative stress (Ozguner et al., 2005), in the mI/R injury (Ahmed et al., 2017; Wang et al., 2018), and in the hypertension (Ozdemir et al., 2021) as well as in the cardiac hypertrophy (Sun et al., 2016). Recently, limited but precious studies have suggested that propolis may have estrogenic effects (Camargo et al., 2013; Jung et al., 2010; Okamoto et al., 2015; Song et al., 2002; Zingue et al., 2017). In vivo and in vitro studies have proposed that propolis may exert estrogenic effects via modulating estrogen receptors (Song et al., 2002), alleviating hot flashes (Zingue et al., 2017), and increasing uterine wet weight and epithelial thickness in ovariectomized rats (Okamoto et al., 2015). However, to the best of our knowledge, no studies have been conducted to indicate that propolis is directly effective in the improvement of myocardial infarct size, arrhythmia score, and incidence of ventricular arrhythmias in intact and/or ovariectomized female rats to mimic the postmenopausal situation or estrogen deficiency.

Oxidative stress is implicated in mI/R injury (Kurian et al., 2016). It was proposed that estrogen could ameliorate oxidative stress via acting as an antioxidant by scavenging free radicals and/or enhancing the activity of antioxidant enzymes (Barp et al., 2002). In addition, a previous study indicated that estrogen decreased myocardial infarct size via regulating manganese superoxide dismutase (Luo et al., 2016). So, one of the reasons why the female heart is more resistant to mI/R injury than the male heart may be due to the protective role of estrogen in oxidative stress, and this also explains why estrogen deprivation due to menopause and/or oophorectomy makes the female heart more vulnerable to mI/R injury. The antioxidant features of propolis due to the presence of various active compounds such as flavonoids and phenolic acids are well documented (Diniz et al., 2020). Based on this information, propolis may be suggested as an effective agent to improve mI/R injury or preserve the heart resistance to ischemia after estrogen deficiency both by antioxidant and possible estrogenic effects.

In the light of the above information, it appears that propolis may be beneficial in the protection of heart against mI/R injury due to its antioxidant properties in estrogen deficiency. To the best of our knowledge, this is the first study to investigate the effect of propolis on the intact and ovariectomized female heart in an experimental model of mI/R injury in vivo. Hence, the present study was designed to investigate the potential protective role of propolis against mI/R-induced arrhythmias and myocardial infarction not only in male rats but also in intact and ovariectomized female rats.

2 | MATERIALS AND METHODS

2.1 | Animals

In the present study, a total of 24 male *Sprague Dawley* rats, 300–350 g (12 weeks old) and 48 female *Sprague Dawley* rats, 200–250 g (12 weeks old) from the Experimental Animal Application and Research Center of Duzce University were used. The experimental animals were housed in polypropylene cages with four rats in each on wood shavings. Animals were kept at an average temperature of 22°C and humidity on a 12-h light–dark cycle in a controlled environment with free access to food and water ad libitum. The Animal Research Ethics Committee of Duzce University approved the present study (protocol number: 2017/4/4). The experiments 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).

2.2 | Propolis preparation

Propolis samples were obtained from honeybee colonies of the Beekeeping Research, Development, and Application Centre of (DAGEM), Duzce University, Yıgılca, Turkey. Raw propolis was harvested and collected during the 2019 summer season. Propolis samples were stored at −80°C and protected from light until analysis. In the current study, we also investigated the effects of four different solvents, including water, olive oil, propylene glycol, and ethanol on the total phenolic and flavonoid compounds, as well as the antioxidant capacity of propolis to decide which one to use in the study (see the Supporting Information for details). Briefly, the ethanolic extract of propolis had the highest antioxidant capacity compared to others. Moreover, the total amount of flavonoids and phenolic compounds of propolis was also highest in the extract prepared with alcohol. Therefore, given the results of this and previous studies (Okamoto et al., 2015; Zingue et al., 2017), we decided to treat the experimental animals with the ethanolic extract of propolis.

2.3 | Experimental groups and treatment protocols

The experimental animals were divided into two main groups: males and females. The male rats were then randomly separated into two groups: (1) male rats treated with the solvent of propolis (0.1 ml per 100 g of the animal) via oral gavage for 14 days (the untreated males, $n = 8$); (2) male rats treated with propolis (200 mg/kg in a volume of 0.1 ml per 100 g of the animal) via oral gavage for 14 days (the treated males, $n = 9$). The female rats were randomly separated into four experimental groups: (1) sham-operated female rats treated with the solvent propolis (0.1 ml per 100 g of the animals) via oral gavage for 14 days (the untreated intact females, $n = 9$); (2) sham-operated female rats treated with propolis (200 mg/kg in a volume of 0.1 ml per 100 g of the animal) via oral gavage for 14 days (the treated intact females, $n = 10$); (3) ovariectomized female rats treated with the solvent propolis (0.1 ml per 100 g of the animal) via oral gavage for 14 days (the untreated ovx females, $n = 10$); (4) ovariectomized female rats treated with propolis (200 mg/kg in a volume of 0.1 ml per 100 g of animal) via oral gavage for 14 days (the treated ovx females, $n = 8$). The decision to use a dose of 200 mg/kg propolis and a treatment duration of 14 days was based on the previous studies (Arabameri et al., 2017; Rivera-Yanez et al., 2018; Talas et al., 2014).

2.4 | A model of surgical menopause

In female rats, we performed ovariectomy (surgical removal of the ovaries) in order to evaluate the effect of estrogen deprivation on mI/R injury as well as the effect of propolis on mI/R injury under the condition of estrogen deficiency. For bilateral ovariectomy referring to surgical removal of both ovaries, 8-week-old female *Sprague-Dawley* rats were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). The fur over the abdominal area was shaved and the skin was disinfected with Betadine. Then, a longitudinal incision in the abdomen was performed. After dissection of the skin and muscles, each ovary was gently exposed, and then the distal uterine horns were ligated and tied with absorbable sutures. Then, each ovary was removed. After controlling any bleeding, the muscle, and skin layer were closed by using 3/0 sterile absorbable sutures. The rats in the sham group (the intact females) underwent the same surgical procedures without removing the ovaries. Successful ovariectomy was confirmed by the cessation of estrus cycles and a decreased plasma E2 level. The time necessary for endogenous hormonal decline ranges from one to 6 weeks (Chung et al., 2010; Melo et al., 2020). In addition, the vulnerability of the female rat hearts to mI/R injury was observed 4 weeks after ovariectomy (Ek et al., 2008). Depending on this information, the treatment of experimental animals with vehicle or propolis was started 4 weeks after ovariectomy in order to observe the effect of estrogen deficiency on the heart.

2.5 | Animal model of mI/R injury

Two weeks after treatment with propolis or vehicle, a 30-min ischemia, and 120-min reperfusion model was performed to induce mI/R injury in the rats. Rats were anesthetized with urethane (1.2 g/kg, i.p). Additional doses (0.6 g/kg, i.p) were administrated as needed to maintain anesthesia. A tracheotomy was performed for artificial respiration. The experimental animals were ventilated with an animal ventilator (R407 Small Animal Respirator, RWD Life Sciences, Inc.) using room air at a rate of 60 strokes/min and a tidal volume of 1 ml/100 g. The right carotid artery was cannulated with a polyethylene tubing filled with heparinized physiological saline to measure arterial blood pressure via a pressure transducer connected to the MP36 system (Biopac Systems Inc., Goleta, CA; Turkey Distributor Commat, Ankara, Turkey). The chest was opened by left thoracotomy and a retractor was used to expose the heart. After removal of the pericardium, a 5/0 silk suture was placed around the left coronary artery approximately 2 mm away from its origin. ECG was recorded by using subcutaneous standard needle lead II electrodes connected to a data acquisition system (MP36, Biopac Systems Inc., Goleta, CA; Turkey Distributor Commat, Ankara, Turkey) to monitor electrical activity and arrhythmias. Thereafter, a 20-min stabilization period was applied to regulate ventilation, restore normal sinus rhythm, and stabilize basic hemodynamics. Next, a snare was created around the left coronary artery using two shortened pipette tips to induce regional myocardial ischemia. Both ends of the silk thread were passed through one of the pipette tips and the pipette tip was pulled toward the epicardium by holding the silk thread and then, it was fixed by placing the second pipette tip into the first to induce 30 min of ischemia. If the animal survived within the 30-min period of coronary artery ligation, the ligature was carefully removed to allow blood to flow again from the left coronary artery and the 120-min period of reperfusion was initiated (Karwi et al., 2017).

2.6 | Measurement of area at risk and infarct size

At the end of the 120-min reperfusion, the heart was rapidly removed and perfused with physiological saline through the aorta via an aortic cannula. The left coronary artery was re-occluded at the same site of ligation. The heart was then perfused with 1% Evan's blue dye to delineate the area at risk of ischemia or the area of occluded artery. Thereafter, the heart was quickly frozen in a freezer at −20°C for 24 hr. The heart was then cut transversely into 5–6 sections of 2 mm thickness. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 20 min at a temperature of 37°C. The slices were then fixed with 4% formalin in phosphate buffer (pH 7.4) for 24 hr to increase contrast. Next, the slices were photographed. The normal ventricular area (non-ischemic area) was seen in blue color, which was stained with Evan's blue dye. The ischemic ventricular area was seen in red color, which was stained with TTC. The infarcted ventricular area was not stained with TTC and was seen in pale white color. Area at risk (AAR, TTC-stained) and infarcted area (I, TTC-negative) were analyzed by the Image J version 1.6 program (National Institutes of Health, Bethesda, MD, USA). Infarct size (IS) was expressed as a percentage of AAR (Karwi et al., 2017).

2.7 | Evaluation of ECG and arrhythmias

ECG and blood pressure recordings were analyzed to determine baseline, ischemic, and reperfusion arterial blood pressure and heart rate parameters. Ischemia-induced ventricular arrhythmias, including VPC, VF, VT, salvo, and bigeminy were identified based on the Lambeth Conventions (Curtis et al., 2013). The duration and incidence of arrhythmias in all experimental groups were measured. According to the incidence and duration of arrhythmia, each animal was given an arrhythmia score to indicate the severity of arrhythmia as 0: no arrhythmias; 1:10 s VT or other arrhythmias (VPC, salvo, bigeminy); 2:11–30 s VT or other arrhythmias; 3:31–90 s VT or other arrhythmias; 4:91–180 s VT or other arrhythmias and/or < 10 s reversible VF; 5: ≥ 180 s VT or other arrhythmias and/or > 10 s reversible VF; 6: irreversible VF (Curtis & Walker, 1988).

2.8 | The exclusion criteria

In the current study, we followed several criteria for exclusion in the model of mI/R injury to ensure reproducibility of the data and to avoid any bias between experimental groups. Before ligation of the left coronary artery, experimental animals with any sign of ischemia, arrhythmia, and blood pressure ≤ 70 mmHg were excluded from evaluation (one rat in the untreated males, two rats in the untreated ovx females, one rat in the treated ovx females). Successful ischemia was confirmed by a color change of the left ventricle from red to deep red or pale color, ST segment elevation, increased QRS amplitude, and a decrease in blood pressure. Animals were also excluded if there was no evidence of a successful coronary artery ligation (one rat in the untreated males, two rats in the treated males, one rat in the untreated intact females, and one rat in the treated intact females). In addition, experimental animals with bleeding after the ligature placed on the left coronary artery were excluded from the evaluation (one rat in the treated males, one rat in the untreated intact females, two rats in the treated ovx females). Another criterion for exclusion is the size of the ischemic risk area. If the area at risk is less than 40%, the heart was not evaluated (two rats in the untreated males, one rat in the untreated intact females, one rat in the treated intact females, one rat in the treated ovx females). Hence, at the beginning of the experimental study, a total of 24 male rats and 48 female rats had been planned to undergo mI/R injury. However, a total of 17 male rats and 37 female rats were used in the analysis, based on the criteria and reasons stated.

2.9 | Biochemical analyses

At the end of 120-min reperfusion, blood samples were collected into heparin-containing tubes via a cannula inserted into the right carotid artery. Subsequently, the samples were centrifuged at 7500 rpm for 15 min at 4°C to separate plasma. Then, the plasma samples were aliquoted and were stored at −80°C until used for measurement of biochemical parameters, including TAS, TOS, CAT, SOD, GPx, and E2 levels. TAS, TOS, CAT, and SOD were measured using commercially available kits (Rel Assay Diagnostic, Ankara, Turkey) with Mindray's BS-300 auto chemistry analyzer according to the manufacturer's instructions. Similarly, GPx and E2 levels were measured using commercially available kits (Rel Assay Diagnostic, Ankara, Turkey) with Biotek ELx800 microplate reader according to the manufacturer's instructions.

2.10 | Statistical analyses

Data were analyzed using the Statistical Package for Social Sciences (SPSS: version 21.0; SPSS Inc., IL, USA). Two-way ANOVA was used to explore the effects of sex (male vs. intact female), treatment (control vs. propolis), and the interactions between them (sex × treatment) on dependent variables including arterial blood pressure, heart rate, infarct size, durations of ventricular arrhythmias, arrhythmia score, TAS, TOS, CAT, SOD, GPx, and E2 levels. In addition, two-way ANOVA was performed to investigate the effects of surgery (intact female vs. ovx female), treatment (control vs. propolis), and the interactions between them (surgery × treatment) to explore the potential role of estrogen deficiency in the dependent variables. Pair-wise comparisons were performed using the Bonferroni correction when the effect of factor(s) and/or interactions on the dependent variables were significant. The incidences of arrhythmias were analyzed using Fisher's exact test. Results were expressed as mean ± standard error (mean ± *SE*). *p* values less than .05 were considered as statistically significant.

3 | RESULTS

The results of the two-way analysis with sex (male vs. intact female) and treatment (control vs. propolis) as well as surgery (intact female vs. ovx female) and treatment (control vs. propolis) as main factors are shown in Table 1.

3.1 | Hemodynamic variables

We examined the effect of propolis on hemodynamic parameters before ischemia (basal value), at the end of ischemia and reperfusion. Changes in the heart rate and the arterial blood pressure in response to 30 min of ischemia and 120 min of reperfusion are illustrated in Table 2.

In the case of sex and treatment as main factors, only treatment had a significant effect on basal arterial blood pressure and arterial blood pressure at the end of ischemia (Table 1, $p < .05$). Treatment with propolis significantly increased basal arterial blood pressure by 18% in the treated males and arterial blood pressure at the end of ischemia by 21% in the treated intact females when compared to their corresponding controls ($p < .05$).

For the effect of estrogen deficiency and treatment, a significant effect of surgery was observed on basal arterial blood pressure, arterial blood pressure at the end of ischemia, as well as heart rate recorded before ischemia, at the end of ischemia and reperfusion (Table 1, $p < .05$). In addition, only treatment did significantly affect heart rate at the end of ischemia and reperfusion ($p < .05$). Basal heart rate, heart rate at the end of ischemia, and basal arterial blood pressure were significantly lower in the untreated ovx females than in the untreated intact females ($p < .05$). Treatment with propolis significantly decreased heart rate at the end of reperfusion by 43% in the treated intact females as well as lowered heart rate at the end of ischemia and reperfusion by 47% and 48% in the treated ovx females compared with their respective controls ($p < .05$).

TABLE 1 Two-way ANOVA analysis of the main factors on the parameters

	Male vs. female			Intact female vs. Ovx female		
Parameters	Sex	Treatment	Sex × treatment	Surgery	Treatment	Surgery × treatment
BBP	$p = .433$	**$p = .044$**	$p = .198$	**$p = .001$**	$p = .208$	$p = .759$
IBP	$p = .423$	**$p = .028$**	$p = .397$	**$p = .005$**	$p = .091$	$p = .050$
RBP	$p = .743$	$p = .466$	$p = .735$	$p = .177$	$p = .920$	$p = .731$
BHR	$p = .618$	$p = .656$	$p = .054$	**$p = .004$**	$p = .099$	$p = .416$
IHR	$p = .710$	$p = .520$	$p = .066$	**$p = .001$**	**$p = .005$**	$p = .615$
RHR	$p = .213$	$p = .184$	$p = .211$	**$p = .009$**	**$p = .002$**	$p = .868$
AAR	$p = .735$	$p = .571$	$p = .620$	$p = .650$	$p = .831$	$p = .243$
IS	**$p = .030$**	**$p = .000$**	**$p = .006$**	**$p = .000$**	**$p = .000$**	**$p = .000$**
VT duration	**$p = .000$**	**$p = .004$**	**$p = .007$**	**$p = .000$**	**$p = .023$**	$p = .089$
VF duration	$p = .068$	$p = .188$	$p = .188$	$p = .178$	$p = .318$	$p = .318$
VPC duration	**$p = .002$**	**$p = .001$**	**$p = .003$**	**$p = .001$**	$p = .232$	$p = .332$
Total duration of arrhythmias	**$p = .000$**	**$p = .000$**	**$p = .000$**	**$p = .000$**	**$p = .048$**	$p = .124$
ASC	**$p = .000$**	**$p = .013$**	**$p = .006$**	**$p = .000$**	$p = .232$	$p = .147$
TAS	$p = .515$	$p = .133$	**$p = .012$**	$p = .595$	$p = .572$	$p = .092$
TOS	$p = .982$	$p = .311$	$p = .917$	$p = .624$	**$p = .048$**	$p = .408$
OSI	$p = .639$	$p = .086$	$p = .233$	$p = .510$	$p = .064$	$p = .219$
CAT	**$p = .046$**	$p = .570$	$p = .488$	$p = .152$	$p = .068$	$p = .570$
SOD	**$p = .484$**	**$p = .000$**	**$p = .013$**	$p = .060$	**$p = .000$**	**$p = .034$**
GPx	$p = .840$	$p = .682$	$p = .991$	$p = .555$	$p = .283$	$p = .156$
E2	**$p = .000$**	**$p = .008$**	**$p = .000$**	**$p = .000$**	$p = .127$	**$p = .000$**

Note: Bold values denote significance at $p < .05$.

Abbreviations: AAR, area at risk; ASC, arrhythmia score; BBP, basal arterial blood pressure; BHR, basal heart rate; CAT, catalase; E2, 17 beta-estradiol; GPx, glutathione peroxidase; IBP, arterial blood pressure at the end of ischemia; IHR, heart rate at the end of ischemia; IS, infarct size; OSI, oxidative stress index; RBP, arterial blood pressure at the end of reperfusion; RHR, heart rate at the end of reperfusion; SOD, superoxide dismutase; TAS, total antioxidant status; TOS, total oxidant status; VF, ventricular fibrillation; VPC, ventricular premature contraction; VT, ventricular tachycardia.

TABLE 2 The effect of propolis on hemodynamic parameters

Groups		N	Heart rate (beat/min)			Arterial blood pressure (mmHg)		
			0	30	120	0	30	120
Male	Control	8	284 ± 16	255 ± 18	256 ± 18	123 ± 9	93 ± 9	87 ± 7
	Propolis	9	310 ± 13	272 ± 10	255 ± 19	141 ± 4$^{\#}$	102 ± 4	95 ± 7
Intact female	Control	9	309 ± 20	276 ± 12	256 ± 12	126 ± 4	92 ± 8	87 ± 8
	Propolis	10	268 ± 15	242 ± 12	213 ± 16$^{£}$	130 ± 3	113 ± 4$^{£}$	90 ± 7
Ovx female	Control	10	245 ± 13$^{£}$	233 ± 14$^{£}$	221 ± 11	109 ± 5$^{£}$	87 ± 4	81 ± 3
	Propolis	8	231 ± 14	186 ± 16*	173 ± 13*	116 ± 4	86 ± 3	79 ± 7

Notes: 0, 20 min before ischemia; 30, 30 min after ischemia; 120, 120 min after reperfusion; Ovx, ovariectomize. The data are represented as mean ± *SE*. $^{\#}p < .05$ compared with male treated with control or vehicle, $^{£}p < .05$ compared with intact female treated with control or vehicle, $^{*}p < .05$ compared with ovx female treated with control or vehicle.

3.2 | Cardiac arrhythmias and myocardial infarction

mI/R injury induces ventricular arrhythmias in the animal model of myocardial infarction. Thus, we investigated the effect of propolis on ventricular arrhythmias afterward (Figure 1a).

A significant effect of sex and treatment on the duration of VPC, VT, and total arrhythmias was obtained (Table 1, $p < .05$). As shown in Figure 1a–e, the duration of VPC, VT, and total arrhythmias were significantly lower by 65.83%, 94.79%, and 78.50%, respectively, in the untreated females than in the untreated males ($p < .05$). Treatment with propolis significantly decreased the duration of VPC, VT, and total arrhythmias in the treated males compared to untreated males ($p < .05$).

In the case of estrogen deficiency and treatment as main factors, a significant effect of surgery and treatment on the duration of VT and total arrhythmias was also observed (Table 1, $p < .05$). The durations of VPC, VT, and total arrhythmias were significantly higher by 63.13%, 86.03%, and 70.11% in the untreated ovx females than in the intact females ($p < .01$). Treatment with propolis significantly decreased the duration of VT and total arrhythmias in the treated ovx females compared to the untreated ovx females ($p < .01$). However, treatment with propolis did not affect the duration of VPC, VT, VF, and total arrhythmias in the treated intact females.

To assess the incidence of arrhythmias is an important parameter in understanding the severity of arrhythmias induced by mI/R injury. Therefore, we evaluated the incidence of arrhythmias in all groups. No difference was observed among the groups in the incidence of VPC and VF (Table 3). The incidence of VT was significantly higher in the untreated males (87.5%) than in the untreated females (33.3%, $p < .05$). In addition, the incidence of VT was higher in the untreated ovx females (100%) than in the untreated females (33.3%, $p < .05$). Treatment with propolis did not influence the incidence of VT, VF, and VPC in the treated males and the treated intact females. In contrast, propolis significantly reduced the incidence of VT by 50% in the treated ovx females compared to the untreated ovx females ($p < .05$).

Another important parameter for evaluating the severity of arrhythmias is the arrhythmia score which is determined by the duration, type, and incidences of arrhythmias. There was a significant effect of sex and treatment on the arrhythmia score ($p < .01$). The arrhythmia score was significantly lower by 43.43% in the untreated females than in the untreated males (Figure 1f, $p < .01$). In the case of estrogen deficiency and the treatment as main factors, a significant effect of surgery on the arrhythmia score was observed ($p < .01$). The arrhythmia score was significantly higher by 38.60% in the untreated ovx females than in the untreated females ($p < .01$). Propolis significantly reduced the arrhythmia score in the treated males; however, it had no effect on the arrhythmia score in the treated ovx females or in the treated intact females when compared to their untreated control groups.

Determination of AAR is also important to determine the region where the ligated coronary artery supplies blood and confirm that the correct site is ligated between groups each time (Figure 2a). In addition, myocardial infarct size is one of the key predictors of the severity of mI/R injury. Therefore, we determined myocardial infarct size with TTC staining in the current study (Figure 2b). As depicted in Figure 2c, there were no significant differences in AAR among the groups. A significant effect of sex and treatment on infarct size was obtained ($p < .05$). Besides, a significant effect of surgery and treatment on infarct size was observed ($p < .05$). The infarct size was significantly lower by 18% in the untreated females than in the treated males (Figure 2d, $p < .01$). In addition, the infarct size was significantly higher by 22.14% in the untreated ovx female than in the untreated intact female ($p < .01$). Treatment with propolis significantly decreased infarct size in the treated males and the treated ovx females. However, propolis did not affect infarct size in the treated intact females.

3.3 | Biochemical analysis

In order to investigate the role of oxidative stress in mI/R injury between the males and the females, as well as in the possible protective effect of propolis on the arrhythmias and myocardial infarction induced by mI/R injury, we evaluated the parameters related to oxidative stress in the present study. Figure 3 displays the experimental data on oxidative stress parameters.

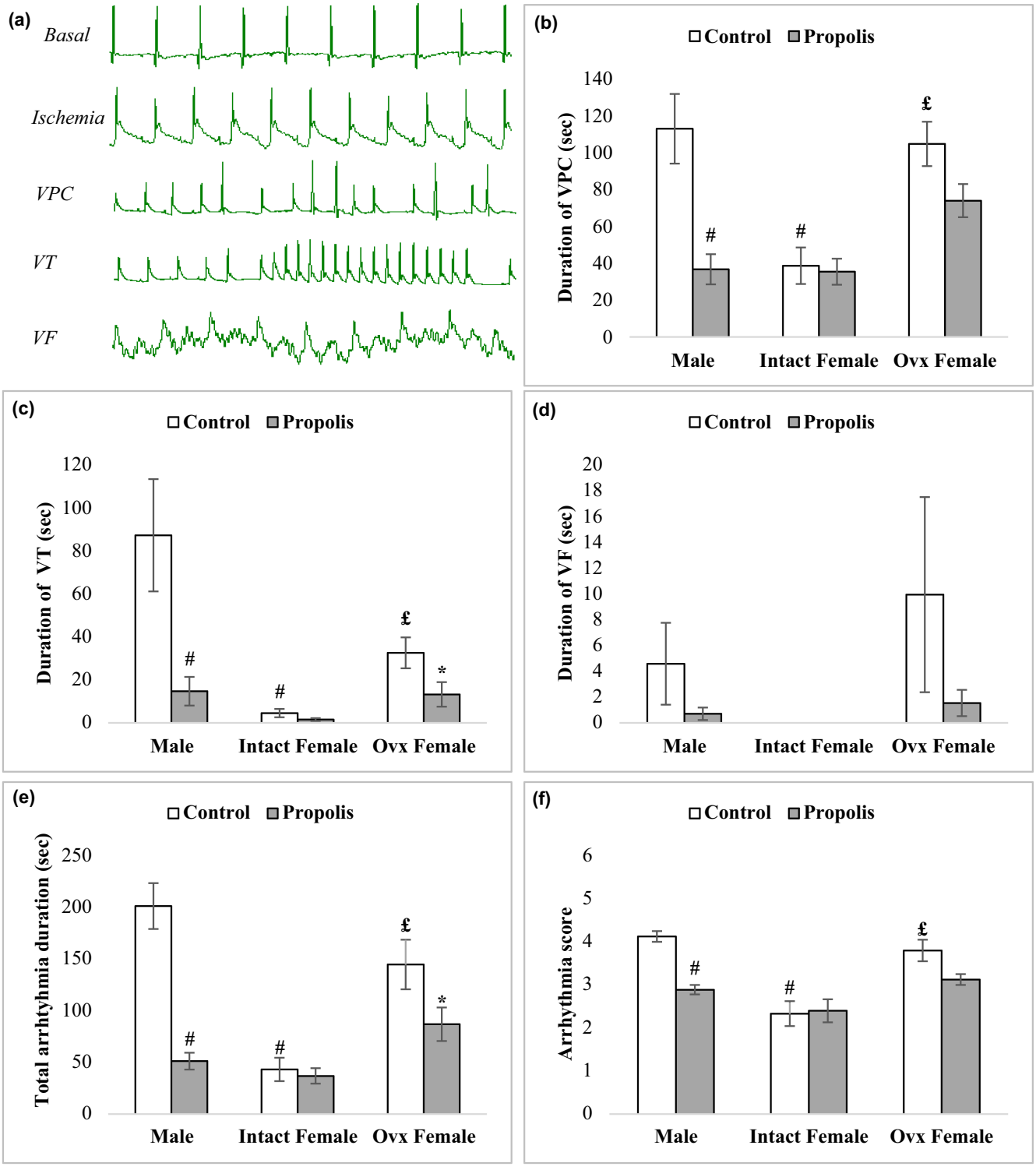

FIGURE 1 The effect of propolis treatment on ventricular arrhythmias. (a) Typical examples of ECG traces showing ventricular arrhythmias, (b) Duration of VPC (sec), (c) Duration of VT (sec), (d) Duration of VF (sec), (e) Total Duration of arrhythmias (sec), (f) Arrhythmia score. VPC, ventricular premature contraction; VT, ventricular tachycardia; VF, ventricular fibrillation; Ovx, Ovariectomize. The data are represented as mean ± *SE*. $^{\#}p < .01$ compared with male treated with control or vehicle, $^{*}p < .01$ compared ovx female treated with vehicle or control. $^{£}p < .01$ compared to intact female treated with vehicle or control. Untreated male, $n = 8$; treated male, $n = 9$; untreated intact female, $n = 9$; treated intact female, $n = 10$; untreated ovx female, $n = 10$; treated ovx female, $n = 8$

A significant effect of sex on CAT, SOD, and E2 levels was obtained, and there was a significant effect of treatment on SOD and E2 levels (Table 1, $p < .05$). Furthermore, there was a significant effect of sex and treatment interaction on TAS, SOD, and E2 levels. When compared to the untreated females, TAS, TOS, and OSI, calculated by the ratio of TOS to TAS, did not show a significant difference in

TABLE 3 The effect of propolis on the incidence of ventricular arrhythmias

Groups		N	Incidence of arrhythmias (n/%)		
			VT	VF	VPC
Male	Control	8	7/87.5	3/37.5	8/100
	Propolis	9	6/66.7	2/22.2	9/100
Intact female	Control	9	3/33.3$^{\#}$	0/0	9/100
	Propolis	10	4/40	0/0	10/100
Ovx female	Control	10	10/100$^{£}$	4/40	10/100
	Propolis	8	4/50*	2/25	8/100

$^{\#}p < .05$ compared with male treated with control or vehicle, $^{£}p < .05$ compared with intact female treated with control or vehicle, $^{*}p < .05$ compared with ovx female treated with control or vehicle.
Abbreviations: *N*, the number of animal; *n*, the number of animals having arrhythmias; Ovx, Ovariectomize; VF, ventricular fibrillation; VPC, ventricular premature contraction; VT, ventricular tachycardia.

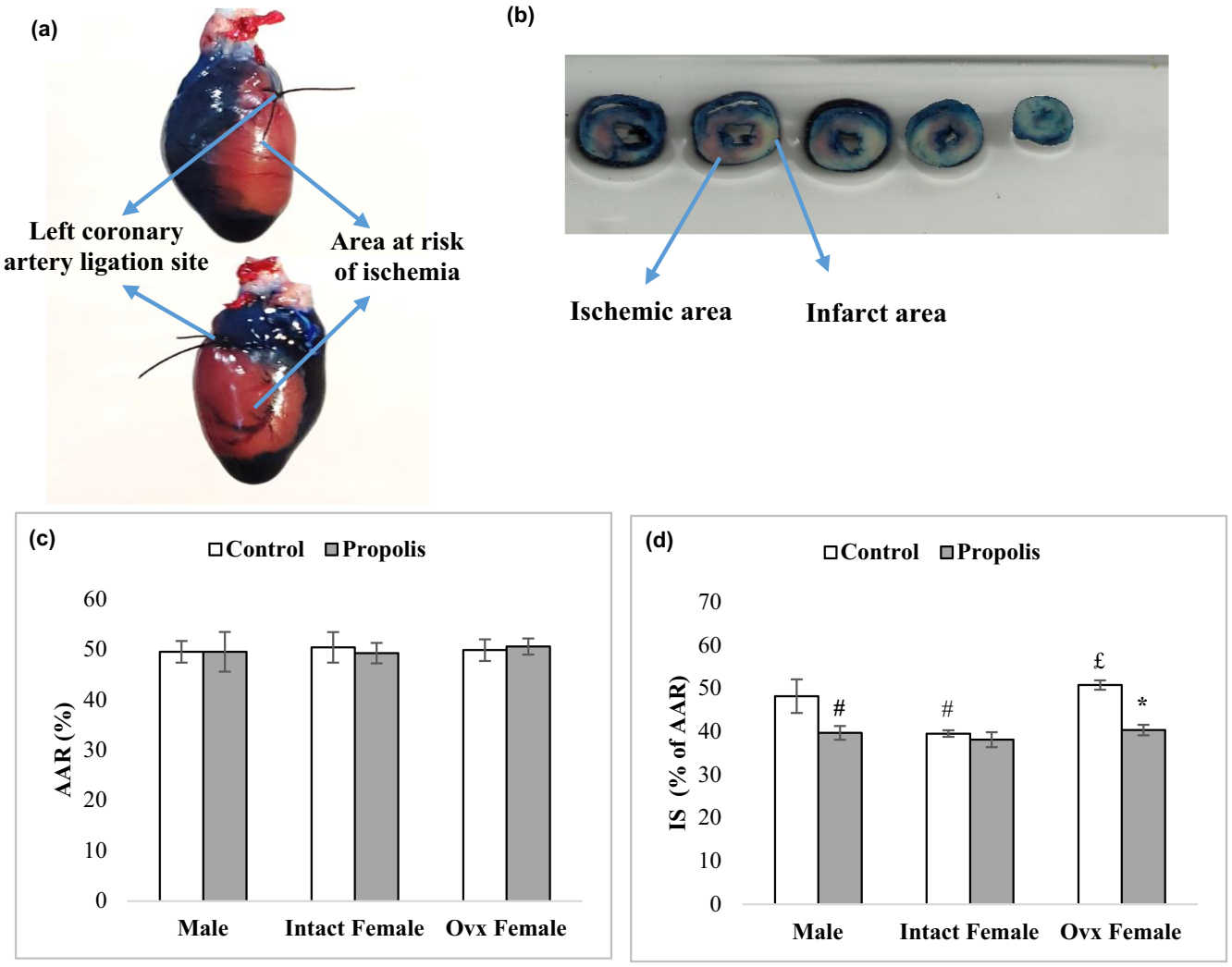

FIGURE 2 The effect of propolis on myocardial infarct size. (a) Representative images of heart with Evans blue staining, (b) Representative images of cardiac slices with Evans blue staining and TTC staining (Blue area: non-ischemic, Red area: area at ischemic risk, whitish, or pale area: infarct area), (c) Area at risk (%), (d) Infarct size (%), The data are represented as mean ± *SE*. $^{\#}p < .01$ compared with male treated with control or vehicle, $^{*}p < .01$ compared ovx female treated with vehicle or control. $^{£}p < .01$ compared intact female treated with vehicle or control. Untreated male, $n = 8$; treated male, $n = 9$; untreated intact female, $n = 9$; treated intact female, $n = 10$; untreated ovx female, $n = 10$; treated ovx female, $n = 8$

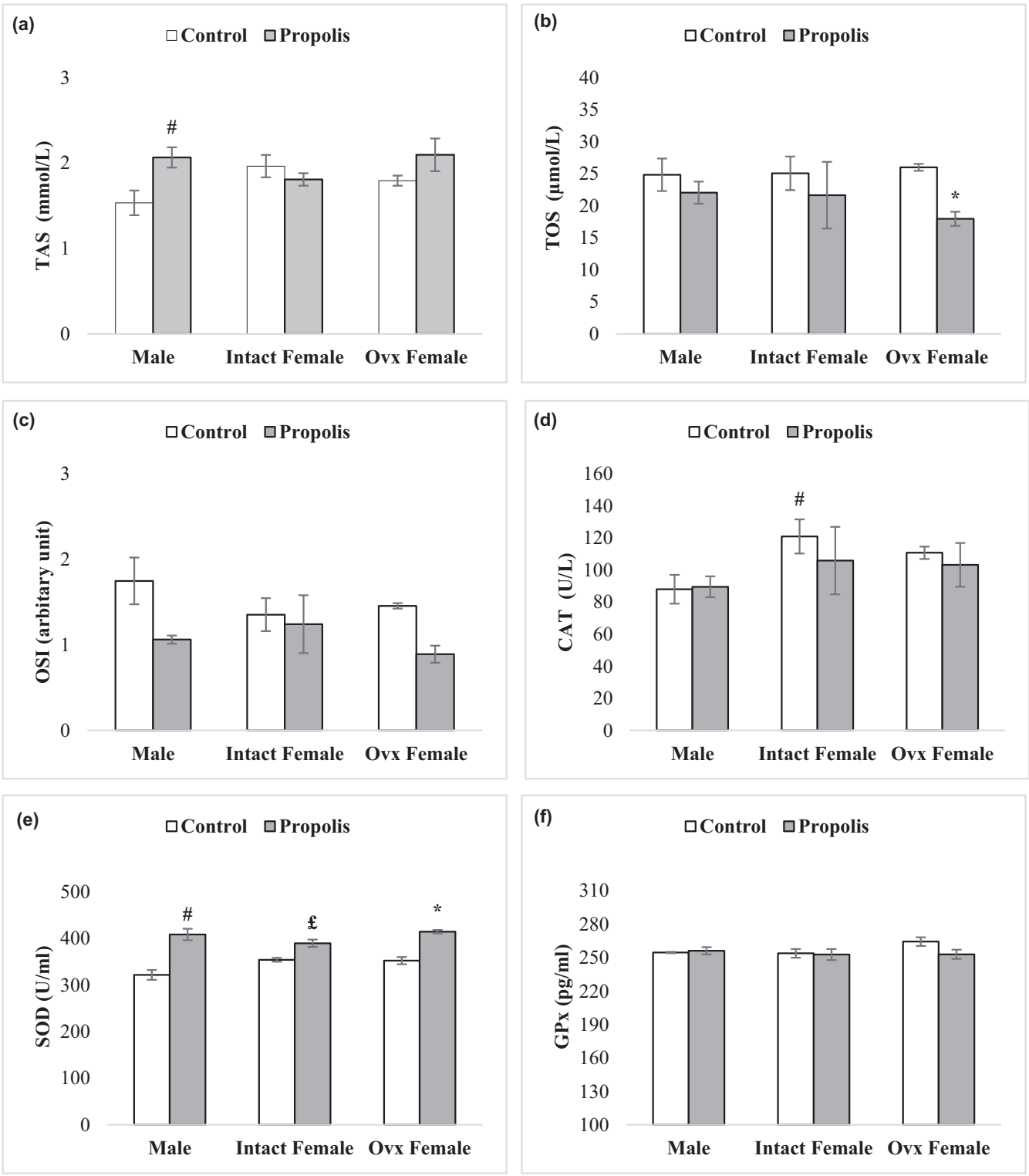

FIGURE 3 The effect of propolis treatment on oxidative stress parameters. (a) TAS (mmol/L), (b) TOS (µmol/L), (c) OSI (Arbitrary unit), (d) CAT (U/L), (e) SOD (U/ml), and (f) GPx (pg/ml). TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase. The data are represented as mean ± *SE*. $^{*}p < .05$ compared with male treated with control or vehicle, $^{*}p < .05$ compared with ovx female treated with control or vehicle, $^{£}p < .05$ compared with intact female treated with control or vehicle, untreated male, $n = 8$; treated male, $n = 8$; untreated intact female, $n = 8$; treated intact female, $n = 6$; untreated ovx female, $n = 8$; treated ovx female, $n = 6$

the untreated males (Figure 3a–c). Furthermore, SOD and GPx levels did not significantly differ between the untreated males and the untreated females; however, CAT levels were significantly higher in the untreated females than in untreated males (Figure 3d–f, $p < .05$).

In the case of estrogen deficiency and treatment as main factors, a significant effect of treatment on TOS and SOD levels was obtained ($p < .05$). In addition, there was a significant effect of surgery on the E2 level. For SOD and E2 levels, there was a significant interaction between surgery and treatment (Table 1, $p < .05$). There were no significant differences in TAS, TOS, and OSI between the untreated intact females and the untreated ovx females. When compared to the untreated females, there were no significant differences in CAT, SOD, and GPx levels in the untreated ovx females. Treatment with propolis significantly increased TAS and SOD levels in the treated males compared to the untreated males ($p < .05$). In addition, propolis significantly increased SOD levels in the treated females and the treated ovx females when compared to their corresponding controls ($p < .05$). When compared to the untreated ovx females, propolis significantly decreased TOS in the treated ovx females ($p < .05$). On the other hand, treatment with propolis did not affect the CAT, GPx, OSI in all treated groups compared to their corresponding controls.

As expected, the plasma E2 levels were significantly higher in the untreated female than in the untreated males (Figure 4, $p < .01$). After ovx, the plasma E2 levels were significantly decreased in the untreated ovx females when compared to the untreated intact females ($p < .01$). Propolis significantly decreased the plasma E2 level in the treated intact females compared to the untreated intact females ($p < .01$). On the other hand, propolis significantly increased the plasma E2 level in the treated ovx females in comparison to the untreated ovx females ($p < .01$). In the treated males, propolis tended to increase the plasma E2 levels, but it did not reach a significant level.

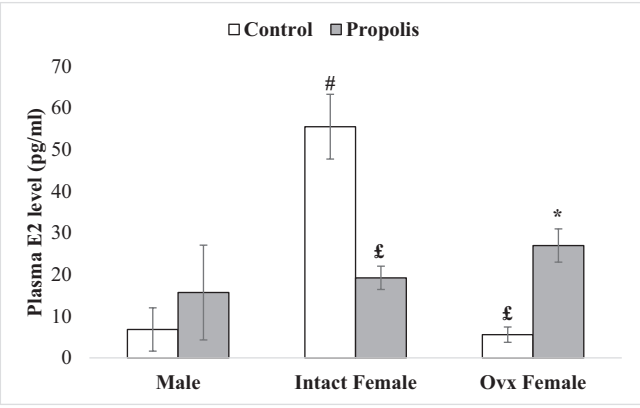

FIGURE 4 The effect of propolis on plasma 17 beta-estradiol. The data are represented as mean ± *SE*. $^{\#}p < .01$ compared with male treated with control or vehicle, $^{*}p < .01$ compared with ovx female treated with control or vehicle, untreated male, $^{£}p < .01$ compared with intact female treated with control or vehicle, $n = 8$; treated male, $n = 8$; untreated intact female, $n = 8$; treated intact female, $n = 6$; untreated ovx female, $n = 8$; treated ovx female, $n = 6$

4 | DISCUSSION

The major findings of the present study: (1) The female hearts exerted more resistance to mI/R injury than the male hearts, as indicated by lower arrhythmia scores, infarct size, duration, and incidence of arrhythmias in the untreated females. (2) Ovx increased the sensitivity of female hearts to mI/R injury, as shown by higher arrhythmia scores, infarct size, duration, and incidence of arrhythmias in the untreated ovx females. (3) Propolis increased the resistance of the male hearts and ovx female hearts to mI/R injury, as evidenced by the improvement of arrhythmia's duration and incidence, arrhythmia scores, and infarct size in the treated males and treated ovx females. (4) Propolis decreased TOS in the ovx females and increased TAS in the males, as well as increased SOD in all treated groups. (5) Propolis decreased plasma E2 levels in the intact females; however, it increased plasma E2 levels in the ovx females. (6) Propolis increased basal blood pressure in the treated males and alleviated the blood pressure at the end of ischemia in the treated females. In addition, propolis decreased the heart rate at the end of ischemia and reperfusion in the treated ovx females.

Both clinical and experimental studies have demonstrated that there is a difference in the susceptibility to mI/R injury between males and females (Benjamin et al., 2019; Chen et al., 2013). In the present study, the arrhythmias score, myocardial infarct size, the duration of total arrhythmias, VPC, VT, and the incidence of VPC were lower in the intact females than in the males, indicating that the female hearts could exert more resistance to mI/R injury than the male hearts. Our results are consistent with data obtained in previous studies (Bozdogan et al., 2013; Brown et al., 2005; Chen et al., 2013). In females, the protective effect against mI/R injury can disappear over time due to estrogen deprivation. Previous studies indicated that the sensitivity to ventricular arrhythmias, cardiac injury, and mortality due to lethal arrhythmias increased in the ovx female rats (Hou et al., 2018; Zhang et al., 2013), as implied by the increased VPC, VT, VF, and arrhythmia score. Our study also supports the results of the mentioned studies. The underlying mechanisms of differences in the sensitivity to mI/R injury between male and female hearts have not been fully elucidated. After menopause, the resistance of female hearts to mI/R injury disappears, which suggests that estrogen may be one of the important factors in the protective mechanism against mI/R injury (Sivasinprasasn et al., 2016). Evidence from the available data supports this hypothesis. In a previous study, the treatment of ovx mice with E2 resulted in a reduction in myocardial infarct size (Luo et al., 2016). In our study, E2 levels were decreased in the ovx females when compared to the intact females, which supports the results of previous studies (Luo et al., 2016). As mentioned before, the mI/R injury was more profound in the ovx females than the intact females in the present study, suggesting that estrogen may underlie the resistance of the female hearts to mI/R injury.

Several cellular and molecular pathways are known to be involved in preventing or reducing myocardial ischemia and reperfusion injury. One of them may be the role of estrogen in antioxidant activity (Iorga et al., 2017). Oxidative stress plays an important role

in myocardial damage and the occurrence of ventricular arrhythmias, as well as the incidence and duration of arrhythmias in response to mI/R injury via modulating automaticity, excitability, and conductivity of the heart (Adameova et al., 2020). SOD, CAT, and GPx are important enzymes to fight reactive oxygen species in cells. Under stress conditions such as mI/R injury, the females, and the males are likely to give different responses to oxidative stress due to the differences in these enzymes. However, studies indicate conflicting results on this issue (Barp et al., 2002; Chen et al., 2011; Dhote & Balaraman, 2007). A previous study reported that there was no difference in CAT between males and females; however, females have higher SOD activity and lower GPx than males (Barp et al., 2002). On the other hand, the results of another study indicated that SOD and CAT activity were not different between the male heart and the female heart, but with a lower activity of GPx in the female hearts (Chen et al., 2011). There was no significant difference in oxidative stress parameters between males and females rats in a model of mI/R injury, as indicated by no difference in SOD and CAT levels between males and females rats (Dhote & Balaraman, 2007). In the present study, although the females had higher levels of CAT than the males, there were no significant differences in TAS, TOS, OSI, SOD, and GPx between them. In addition, our results demonstrated that estrogen withdrawal by ovx did not alter antioxidant and oxidant status, as indicated by no differences in TAS, TOS, OSI, CAT, SOD, and GPx between the intact female rats and the ovx female rats. A previous study revealed that there were no significant differences in SOD and CAT levels between intact females and ovx females (Dhote & Balaraman, 2007). Besides, another study reported that ovx did not change GPX and CAT activity (Barp et al., 2002). The results of the mentioned studies were consistent with our results. Taken together, it may be concluded that the presence or absence of estrogen may not affect the balance between antioxidants and oxidants. Since there was no significant difference in the antioxidants between the intact female and the ovx females, which may suggest that the role of E2 in oxidative stress is not likely to be a factor in the loss of resistance to mI/R injury in the ovx females. Rather than the beneficial role of estrogen on oxidative stress, it can be proposed that the response to mI/R injury involves a number of complex molecular pathways, including genomic and non-genomic effects of estrogen via binding to estrogen receptors including ER-alpha, ER-beta, and GPR30, which are likely responsible for the observed differences depending on sex and the estrogen deficiency (Puglisi et al., 2019).

The use of exogenous estrogen as a therapeutic agent in reducing the sensitivity of the heart to cardiovascular diseases induced by the absence and/or decrease of estrogen after menopause or after surgical procedures in women is still controversial because of the increased risk of possible thrombotic events, cancers, and arrhythmias (Iorga et al., 2017). Therefore, one of the aims of this study is to test the hypothesis of whether propolis has a protective effect against mI/R injury in the deprivation of estrogen in female rats. In the current literature, there is very limited information about the effect of propolis on ventricular arrhythmias and infarct area induced by mI/R injury in rats. To the best of our knowledge, this study was the first one to demonstrate the effect of propolis on ventricular arrhythmias and myocardial infarct size induced by mI/R injury in intact female rats and ovx female rats. Thus, it is of great significance to explore the effect of propolis on mI/R injury after estrogen deprivation. A recent in vivo study showed that oral administration of propolis might be protective against ISO-induced myocardial infarction in rats (Ahmed et al., 2017). Propolis was indicated to improve the serum levels of CK-MB, LDH, AST, ALT, TC, TGs, VLDL-C, and HDL-C. In addition, propolis improved the levels of antioxidant enzymes such as SOD, GRx, GPx, and GST in the heart, which underlies the cardioprotective effects of propolis in the model of ISO-induced cardiac damage in rats. In a model of mI/R injury, administration of CAPE, an active compound of propolis, reduced apoptosis and improved oxidative stress in myocardial infarction (Cagli et al., 2005). Pinocembrin, also an active compound of propolis, decreased the incidence, and duration of arrhythmias and improved arrhythmia score and myocardial infarct size by decreasing the ratio of Bax/BCL-2 and increasing the expression levels of Connexin-43 (Lungkaphin et al., 2015). A recent study showed that propolis improved cardiac fibrosis induced by 4-week ligation of the left coronary artery in rats via activating silent information regulator 1 (Wang et al., 2018). Our results were consistent with the above-mentioned reports. The results of our study indicated that propolis increased the resistance of the heart to mI/R injury in the males and the ovx females, evidenced by the improvement in the duration of total arrhythmias, VPC, VT, arrhythmia score, and myocardial infarct size. A striking finding in the present study was that propolis did not affect the sensitivity to mI/R injury in intact females.

Propolis is known to reveal antioxidant capacity due to its active compounds, such as flavonoids and phenolic acid, via free radical scavenging ability (Kocot et al., 2018). In a clinical study, it was reported that 30 days of propolis treatment resulted in increased SOD activity in healthy men, but there was no change in the SOD activity in healthy women. In addition, propolis had no effect on the CAT and GPx activities in both men and women (Jasprica et al., 2007). Propolis exerted antioxidant features in mI/R injury, as indicated by a decrease in ROS production (Wang et al., 2018). A recent study also indicated that propolis exerted antioxidant effects in L-name-treated rats via increasing TAS and decreasing TOS (Ozdemir et al., 2021). Consistent with the results of the mentioned studies, our results indicated that propolis did not affect the CAT and GPx levels in either the male or the intact female rats. In addition, propolis did not affect the TAS, TOS, and OSI in the intact female rats; however, propolis increased TAS in the male rats and decreased TOS in the ovx female rats. Taken together, our findings suggest that the protective effect of propolis on mI/R injury in males and ovx females might be likely due, at least in part, to its antioxidant features.

The estrogenic effects of propolis in vivo and in vitro were first reported by Song et al. (2002). Both ethanol and ether extract of propolis competed with E2 to bind hERα; however, as mentioned by the authors, the binding affinity of propolis was lower than that of E2 and there was no direct data to indicate the activation of

estrogen receptors as well. As mentioned previously, propolis contains several active compounds in its ingredients. One of them, CAPE has an especially higher binding affinity for ERβ than ERα and interestingly downregulate the expression levels of ERα in vitro. In contrast to previous studies, CAPE does not reveal estrogenic action in cell culture (Jung et al., 2010). In cell culture, propolis acts as an agonist for human estrogen receptors and upregulates the expression levels of estrogen-responsive genes, which proves the estrogenic action of propolis. Furthermore, in vivo data support the estrogen-like effects of propolis because the beneficial effects of oral administration of propolis, such as improvement of uterine weight and luminal epithelium thickness, are disappeared by pretreatment with an ER antagonist (Okamoto et al., 2015). In vitro, estrogen receptors such as ERα and ERβ have been activated by propolis; however, interestingly a high dosage of propolis inhibits the activation of receptors by estradiol, indicating its anti-estrogenic activity at higher levels (Zingue et al., 2017). Another result of the mentioned study was that even 3 days of oral administration of propolis ameliorates the higher core temperature, the duration, the number, and the frequency of hot flushes observed in ovariectomized rats. These results suggest that propolis may be used as a phytoestrogen for reliving post-menopausal symptoms. Hence, in view of the above-mentioned actions of propolis, the present study was also designed to investigate the effect of propolis on the female hearts after ovx in rats. In the ovx female rats, propolis resulted in an increase in serum E2 level; however, it is important to state that propolis led to a decrease in serum E2 level in the intact female rats. A previous study demonstrated that the serum E2 level in mice exposed to maternal separation stress decreased, which was improved by propolis administration (Arabameri et al., 2017). In pre-menopausal females, the main structure in which estrogen is produced and released is the ovaries. However, estrogen can also be produced in adipose tissue, brain, bone, vascular endothelium, and aortic smooth muscles. Among them, adipose tissue is one of the main tissues contributing to circulatory estrogen levels in women after menopause (Nelson & Bulun, 2001). It is also known that aromatase activity becomes the primary source of estrogen synthesis in postmenopausal women (Iorga et al., 2017). The main function of aromatase is to catalyze locally and systemically the synthesis of estrogen from steroids in the body. Taken together, it is possible to suggest that propolis might regulate plasma E2 levels via modulating the aromatase enzyme in the present study. In addition, propolis can interact with ERs as mentioned earlier (Song et al., 2002). Therefore, it is likely to affect hormonal signaling within the hypothalamic–pituitary–gonadal axis (Dominguez-Lopez et al., 2020), by which propolis may differently modulate the secretion of circulatory estrogen in the intact female and the ovx female. However, such contributions need further studies, and additional studies on its role in circulating estrogen are warranted.

In our experiment, heart rate and blood pressure decreased after ovx, which supports the result of a previous study that indicated that heart rates were lower in the anesthetized ovx female rat (da Silva et al., 2017). However, there are conflicting data on the changes in hemodynamic parameters, which indicate that there were no significant changes in arterial blood pressure and heart rate after the ovx (Castardo-de-Paula et al., 2018; Jankowski et al., 2001; Lam et al., 2002). The changes in hemodynamic parameters may be related to how much time has passed since the loss of estrogen that induced cellular changes (Fortepiani et al., 2003), which is likely to be one reason for this discrepancy in the literature. Another reason may be related to invasive or non-invasive measurement of hemodynamics, type of anesthesia, and age. In models of experimental hypertension in rats, propolis has been shown to improve hemodynamic parameters via several mechanisms, including antioxidant and anti-inflammatory properties as well as modulating nitric oxide synthase (Ozdemir et al., 2021; Zhou et al., 2020). However, in our study, propolis increased basal arterial blood pressure in the male and raised arterial blood pressure at the end of ischemia in the intact female. In addition, propolis decreased heart rate at the end of ischemia and reperfusion in the ovx females as well as heart rate at the end of reperfusion in the intact females. Heart rate is linked to the occurrence and incidence of arrhythmias induced by mI/R injury. A decrease in heart rate might exert an anti-arrhythmic action (Ng et al., 2013). Therefore, the anti-arrhythmic action of propolis in the treated ovx females may be due to its heart rate-lowering effect. Although the underlying mechanism(s) of propolis's effect on the hemodynamic parameters was not investigated in the present study, it may be stated that propolis may exert a negative chronotropic effect in the ovx female via modulating parasympathetic outflow. A previous study indicated that CAPE, an active compound of propolis, decreased heart rate. In the mentioned study, the authors stated that acetylcholine release might be involved in the effect of CAPE (Iraz et al., 2005). It is also important to state that the dose of propolis may be an important factor in the effect of propolis on hemodynamic parameters. In addition, it is possible that the effects of chronic or acute administration of propolis on the modulation of blood pressure and heart rate as well as the effect of propolis on the hemodynamic parameters in response to mI/R injury may be different. In our study, there are limitations that should be considered. Cellular signaling mechanism(s) underlying the effect of propolis on the hemodynamic parameters, ventricular arrhythmias, and myocardial infarct size could not be evaluated. Future studies of the cardioprotective effects of propolis via estrogen receptors, including ERα, ERβ, and GPER as well as up- and downstream signaling pathways involved in its effects, would be of interest. Notwithstanding these limitations, the present study provides the first comprehensive assessment of the effects of propolis on mI/R injury depending on sex. In addition, the present research explored, for the first time, the effects of propolis on mI/R injury in the presence and/or estrogen deficiency in females. These findings draw our attention to the importance of considering sex and estrogen status in the effect of propolis as a traditional remedy on mI/R injury. Therefore, this study lays the groundwork for future research on propolis as a

traditional remedy in the field of complementary and traditional medicine.

5 | CONCLUSION

Propolis as an apitherapy product is of interest due to several biological activities. Our findings clearly demonstrate the efficacy of propolis in preserving resistance to mI/R injury in female rats with estrogen deficiency. Our findings also show that the effect of propolis on mI/R injury varies depending on sexes and estrogen status. Within the clinical setting, propolis may be suggested as an adjuvant to maintain the resistance of the female heart to mI/R injury after menopause and/or surgical removal of the ovaries due to its potential estrogenic and antioxidant action, except for those with an allergy to propolis. In addition, the results of the current study indicate that propolis may be consumed as supplementary food to improve the health of the heart and/or to decrease the adverse effects of mI/R injury.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Salih Tunc Kaya: Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; validation; visualization; writing – original draft; writing – review and editing. **Kagan Agan:** Investigation; methodology; writing – review and editing. **Aydan Fulden-Agan:** Investigation; methodology; writing – review and editing. **Pinar Agyar-Yoldas:** Investigation; methodology; writing – review and editing. **Talat Ogulcan Ozarslan:** Investigation; methodology; writing – review and editing. **Meral Kekecoglu:** Funding acquisition; investigation; methodology; writing – review and editing. **Adnan Kaya:** Investigation; methodology; supervision; writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL

The Animal Research Ethics Committee of Duzce University approved the present study (Protocol number: 2017/4/4). Experiments 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).

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