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Original Research

The effect of carvacrol on oxido-inflammatory ovarian injury and infertility induced by ischemia-reperfusion in rats

Nurullah Sahin[1], Ilhan Bahri Delibas[2], Unal Isaoglu[3], Bahadir Suleyman[4], Gulce Naz Yazici[5], Taha Abdulkadir Coban[6], Kemine Uzel[7,*], Halis Suleyman[4], Veysel Arslan[8]

[1]Department of Gynecology and Obstetrics, Private Kastamonu Anadolu Hospital, 37200 Kastamonu, Turkey
[2]Department of Gynecology and Obstetrics, Faculty of Medicine, Tokat Gaziosmanpasa University, 60250 Tokat, Turkey
[3]Department of Gynecology and Obstetrics, Private Adatıp İstanbul Hospital, 34912 İstanbul, Turkey
[4]Department of Pharmacology, Faculty of Medicine, Erzincan Binali Yildirim University, 24100 Erzincan, Turkey
[5]Department of Pathology, Faculty of Medicine, Erzincan Binali Yildirim University, 24100 Erzincan, Turkey
[6]Department of Biochemistry, Faculty of Medicine, Erzincan Binali Yildirim University, 24100 Erzincan, Turkey
[7]Department of Obstetrics and Gynecology, Erzincan Binali Yıldırım University Mengucek Gazi Training and Research Hospital, 24000 Erzincan, Turkey
[8]Department of Bayburt Vocational High School of Health Services, Bayburt University, 69000 Bayburt, Turkey
*Correspondence: kemineuzel@hotmail.com (Kemine Uzel)

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Abstract

Background: Increased oxidants and proinflammatory cytokines play a role in the pathogenesis of ovarian ischemia-reperfusion (OIR) injury and related infertility. Carvacrol has antioxidant, antibacterial and anti-inflammatory properties. **Methods**: Protective effect of carvacrol against ischemia-reperfusion (IR)-related ovarian damage and infertility was investigated. IR process were applied to the ovaries of rats, which were divided into the following groups (n = 12): OIR, IR +50 mg/kg carvacrol (IRC-50), IR +100 mg/kg carvacrol (IRC-100) and sham group (SG). After the reperfusion process, six rats from each group were killed and the removed ovaries were examined biochemically and histopathologically. The remaining animals were kept two months with mature male rats to reproduce. **Results**: At a dose of 50 mg/kg, carvacrol suppressed the oxidant parameter increase and antioxidant decrease caused by IR in ovarian tissue. At a dose of 100 mg/kg, carvacrol antagonized both oxidant and proinflammatory cytokine increase and antioxidant decrease. Histopathologically, severe degeneration of follicles in the ovaries of the OIR group, necrotic cell accumulations, hemorrhage in the corpus luteum, edema in the interstitial tissue, polymorphous nuclear leukocyte (PNL) infiltration, and congestion and dilation of blood vessels were detected. Inflammatory symptoms such as edema in the ovarian tissue, congested dilated blood vessels and PNL infiltration were observed at a dose of 50 mg/kg of carvacrol, but these histopathological findings were not observed at a dose of 100 mg/kg. **Conclusion**: A dose of 100 mg/kg carvacrol, which eliminated inflammatory damage, significantly prevented the development of IR-induced infertility. Carvacrol may be beneficial in the treatment of IR-related ovarian damage and infertility.

Keywords: Carvacrol; Oxido-inflammatory; Infertility; Rat

1. Introduction

Ovarian ischemia is an emergency resulting from a sprain (torsion) of the ovaries [1]. Ovarian torsion is more common in patients with ovulation induction, pregnant women, women of reproductive age, patients with a benign or malignant ovarian mass greater than 5 cm, and women who have experienced a previous ovarian torsion [2,3]. Delays in the diagnosis and treatment of ovarian torsion may result in permanent tissue damage and organ loss [3,4]. Therefore, it is recommended that patients receive reperfusion of the torsioned ovaries with detorsion [5]. However, detorsion of torsioned ovaries can cause more severe tissue damage. This event is called ischemia-reperfusion (IR) injury [6]. Studies show that oxidative stress is significant in IR damage. As is known, nicotinamide adenine dinucleotide is used in the metabolism of hypoxanthine (HX) with xanthine dehydrogenase (XDH) in an anaerobic environment. Therefore, reactive oxygen species (ROS) are not produced as intermediates [7]. However, XDH produced in an aerobic environment is converted to xanthine oxidase (XO) in an anaerobic environment [8]. Since molecular oxygen (O_2) is used in the metabolism of HX with XO, ROS are produced as an intermediate product. Therefore, the metabolism of HX by the XO enzyme cannot occur unless there is reperfusion in ischemic tissue [9]. Providing reoxygenation in reperfusion causes the metabolism of HX and excessive ROS formation [10]. These ROS, known as reperfusion mediators, cause the formation of toxic products such as aldehyde and malondialdehyde (MDA) from lipids by oxidizing cell membrane lipids [11]. It is known that reduced glutathione (GSH), known as an endogenous antioxidant, decreases in ovarian tissue with high MDA content [12].

Another event that is thought to contribute to the exacerbation of IR injury is inflammation. Inflammation mediators induce an inflammatory response. Recently, proinflammatory interleukin 1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) have been shown to increase in tissues exposed to IR [13]. In addition, ROS is known to stimulate the expression of nuclear factor kappa B (NF-κB), which controls the production of proinflammatory cytokines. Fatma *et al*. [14] indicated that the rising in oxidative stress parameters in ovarian IR was associated with a considerable rising the expression of NF-κB and TNF-α. Unlubilgin *et al*. [15] reported in their study that the increase of oxidant and proinflammatory cytokines in IR-treated ovarian tissue was associated with infertility. This information obtained from the literature reveals the importance of the early diagnosis and treatment of ovarian torsion in preserving ovarian function and preventing future infertility [1]. As mentioned above, detorsion of the torsioned ovaries causes more severe damage to the ovarian tissue. Therefore, a traditional operation option with detorsion is mainly suggested [16].

Carvacrol is a monoterpene phenol produced from *Origanum vulgare* and *Thymus vulgaris* [17]. Research has been conducted on the clinical use of carvacrol. *In vitro* and *in vivo* researches have indicated that carvacrol has antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory and spasmolytic effects [18]. It has also been reported that carvacrol has an inhibitory effect on proinflammatory cytokine and MDA production and a stimulant effect on total glutathione (tGSH) production [19]. This information suggests that carvacrol may protect ovarian tissue from the oxidative and inflammatory damage of IR. The goal of our research is to analyze the impact of carvacrol against IR-induced ovarian damage and reproductive dysfunction in female rats.

2. Materials and methods

2.1 Animals

A total of 48 female Albino Wistar rats, 8 months old and weighing 248–256 grams were used in our study. The animals were obtained from Atatürk University Medical Experimental Application and Research Center. To allow the animals to adapt to the environment, they were fed with animal feed and tap water for seven days in the laboratory (22 °C) in a 12 hours light/12 hours dark conditions. To make sure that the female rats to be used in the experiment were not pregnant, they were kept in cages without male rats for one month. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 23.03.2021, meeting no: 236643897-000).

2.2 Chemicals

The carvacrol used in the assay was purchased from Sigma (Sigma Chemical Co., Saint Louis, MO, USA), and ketamine was purchased from Pfizer (İstanbul, Turkey).

2.3 Experimental groups

Two hours of ischemia and two hours of reperfusion (IR) were applied to the ovaries of Albino Wistar female rats, and they were split into the following groups (with each group containing 12 rats): OIR, IR +50 mg/kg carvacrol (IRC-50), IR +100 mg/kg carvacrol (IRC-100), and a control group that underwent a sham operation (SG).

2.4 Surgical procedures

The 50 and 100 mg/kg carvacrol doses were injected intraperitoneally (i.p.) to the rats in the IRC-50 and IRC-100 groups, respectively, which had a unilateral ovariectomy two weeks previously. Unilateral ovariectomy was also performed in the OIR and SG groups. As a solvent, 25% dimethyl sulfoxide in normal saline was applicated to the OIR and SG groups in the same way. One hour after administration of carvacrol and solvent, ketamine was administered i.p. at a dose of 60 mg/kg to all of the rats to provide anesthesia. In the previous study, antioxidants were applied before IR treatment [20]. However, administration of antioxidations one hour before or after I/R has been shown to provide beneficial treatment [21,22]. The period when animals are immobilized in the supine position is considered an appropriate period for surgical operation [23]. During this time, the lower abdomens of all the rats were opened 2–2.5 cm vertically to reach the ovaries. Vascular clips were applied to the lower part of the right ovary in the OIR, IRC-50, and IRC-100 groups, and two hours of ischemia and two hours of reperfusion were performed [24]. The reason for our application of ischemia for two hours and reperfusion for two hours is that the parameters that cause oxidative and inflammatory damage of the ovary increase significantly during this period [15]. The ovaries of the SG group were closed without any procedure. At the end of the reperfusion process, six rats from each group were killed with high-dose anesthesia (ketamine 120 mg/kg). The right ovaries of the killed rats were removed, and biochemical and histopathological examinations were performed on the ovarian tissue. The results obtained from the IRC-50, IRC-100 and SG groups were compared with the results of the OIR group. The remaining animals (six in each group) were kept in the laboratory environment with mature male rats for reproduction. The rats that became pregnant during this period were taken to divided cages and kept alone in an appropriated condition. Pregnancy times of animals SG: 25, OIR: 32, IRC-50: 30, IRC-100: 28 days, respectively. The rats that did not give birth within two months were considered sterile.

3. Biochemical analysis

3.1 Preparation of samples

At this stage, for biochemical examination, 0.2 g from each removed tissue was weighed. Tissues were homogenized with a high-speed homogenizer in an ice-cold phos-

phate buffers (50 mM, pH 7.4), which was appropriate for the variable to be measured. The tissue homogenates were centrifuged at 5000 rpm for 20 min at 4 °C, and the supernatants were extracted to analyse for total glutathione (tGSH), total oxidant status (TOS), total antioxidant status (TAS), nuclear factor kappa B (NF- κB) IL-1β, and total protein. All tissue results were expressed as mg/g total protein. All spectrophotometric measurements were performed using a microplate reader (Bio-Tek, Winooski, VT, USA).

3.2 Tissue MDA and tGSH determinations

MDA measurement is based on the method used by Ohkawa *et al.* [25], which includes spectrophotometric measurement of the absorbance of thiobarbituric acid (TBA) and the pink-colored complex formed by MDA. The absorbance of the supernatant was measured at 532 nm. The tGSH was measured using the method defined by Sedlak and Lindsay RH [26].

3.3 Total Oxidant Status (TOS) and Total Antioxidant Status (TAS) determinations

TOS and TAS levels of tissue homogenates were determined using a novel automated measurement method and commercially available kits (Rel Assay Diagnostics, (İstanbul, Turkey), both developed by Erel [27,28]. The TAS method was based on the bleaching of characteristic colour of a more stable ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation by antioxidants and, measurements were performed at 660 nm. The results were expressed as nmol hydrogen peroxide (H_2O_2) equivalent/L. In TOS method, the oxidants presented in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction was enhanced by glycerol molecules, which had been abundantly present in the reaction medium. The ferric ion produced a coloured complex with xylenol orange in an acidic medium. The colour intensity, which could be measured at 530 nm spectrophotometrically, was related to the total amount of oxidant molecules presented in the sample. The results were expressed as μmol Trolox equivalent/L.

3.4 NF-κB, TNF-α and IL-1β analysis

Tissue homogenate NF-κB and TNF-α concentrations were measured using sandwich enzyme-linked immunosorbent assays (Rat NF- NF-κB **B** ELISA immunoassay kits, cat. No. 201-11-0288; SunRed and Rat TNF-α and Rat IL-1β ELISA kits, cat no. YHB1098Ra, Shanghai LZ, Shanghai, China). Analyses were performed according to the manufacturers' instructions.

3.5 Histopathological examination

The tissue specimens were immersed in formaldehyde solution (10%) for 72 hours. After fixation, the tissues were placed in a cassette and washed in running water for 24 hours, and then were successively passed through increasing strengths of alcohol (70%, 80%, 90%, and 100%). Ovarian tissues, which were made transparent in xylol, were embedded in paraffin blocks, and 4- to 5-micron-thick sections were cut. The sections were stained with hematoxylin-eosin dual staining and evaluated and photographed using the Olympus DP2-SAL firmware program (Olympus® Inc. Tokyo, Japan). In the serial sections taken, one center and five peripheral areas were selected at $\times$100 magnification for a total of six sections for each experimental group, and a score was assigned to each section for the amount of degeneration, dilatation/congestion in the vessels, interstitial edema, hemorrhage, and polymorphonuclear cell infiltration. For histopathological criteria, a score was assigned between 0–3 points: 0 = not found, 1 = mild, 2 = moderate, and 3 = severe. Since there was a significant difference in the number of follicles developing in the ovarian tissue among the experimental groups, the developing follicles were classified and counted at 100$\times$ magnification in serial sections taken from each experimental group. Blind histopathological evaluation of the groups was conducted by a histologist.

4. Statistical analysis

The experimental results were expressed as mean value $\pm$ standard deviation ($\bar{x} \pm$ SD). The significance of the differences between groups was determined using the one-way ANOVA test followed by post-hoc Tukey tests. Histopathological grading and the number of offspring are presented as the median (min–max) value. While comparing fertility between groups Chi Square was used. While comparing the number of offspring in groups, Kruskal Wallis test and then Dunn post hoc test were used and adjusted p values were presented. All statistical processes were performed using SPSS for Windows version 18.0 (IBM Corp., Chicago, IL, USA), and $p < 0.05$ value was considered significant. While performing histopathological scoring, the median of the values was calculated.

5. Biochemical findings

5.1 MDA and tGSH analysis results

Fig. 1 shows that the amount of MDA in the ovarian tissue of the OIR group, to which only the IR procedure was applied, was found to be significantly higher than the amount of MDA in the ovarian tissue of the SG group ($p < 0.0001$). The amount of MDA in the IRC-50 and IRC-100 groups was found to be significantly lower than the OIR group ($p < 0.0001$). The same figure (Fig. 1) shows that the amount of tGSH in the ovarian tissue of the OIR group was lower than in the SG group ($p < 0.0001$).

5.2 The results of TOS and TAS analysis

Our experimental results revealed that the TOS level was significantly higher and the TAS level significantly

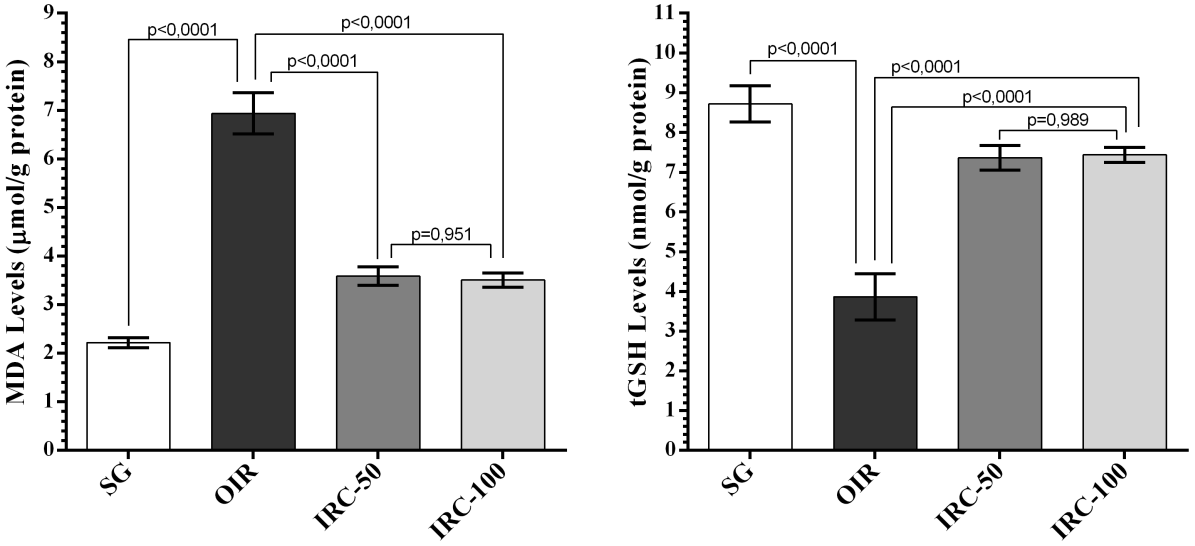

Fig. 1. MDA and tGSH levels in the ovarian tissue. IR, Ischemia-reperfusion; MDA, Malondialdehyde; tGSH, Total glutathione; OIR, Ovary ischemia-reperfusion; IRC-50, IR +50 mg/kg carvacrol; IRC-100, IR +100 mg/kg carvacrol; SG, Sham operation group.

lower in the ovarian tissue of the OIR group compared to the SG ($p < 0.0001$). The TOS and TAS levels in the IRC-50 and IRC-100 groups were significantly different from those of the OIR group (Fig. 2) ($p < 0.0001$).

5.3 The results of NF-κB, TNF-α and IL-1β analysis

As presented in Fig. 3, the levels of NF-κB, TNF-α and IL-1β in the ovarian tissue of the IR group increased significantly compared to the SG group ($p < 0.0001$). The levels of NF-κB, TNF-α and IL-1β in the IRC-100 group significantly decreased compared to OIR group ($p < 0.0001$). However, there was no significant difference in NF-κB and TNF-α levels between the OIR and IRC-50 groups ($p > 0.05$). The levels of IL-1β are statistically different between the OIR and IRC-50 groups ($p < 0.05$).

5.4 Reproduction test results

As seen in Table 1, reproductive test results differ between groups ($p = 0.009$). One of the six rats in the SG group taken for breeding was considered infertile. On the other hand, in the IR group, none of the six rats taken for breeding gave birth. In the IRC-50 group, one of the six rats gave birth, and the remaining five rats were recorded as infertile. In the IRC-100 group, four out of six rats gave birth, and two remained infertile. When the median number of offspring born in the SG and OIR groups was compared, a statistically significant difference was found ($p = 0.007$). When the median number of offspring in the IRC-100 group was compared with the SG, the difference was statistically insignificant ($p = 0.932$), while a significant difference was found between the number of offspring in the SG and IRC-50 groups ($p = 0.027$). Atretic follicles were significantly reduced in the OIR-100 group compared to the OIR group. The numbers of primordial and developing follicle of SG, OIR-50, and OIR-100 groups were found significantly higher than the OIR group (Table 2).

5.5 Histopathological findings

In histopathological examination, the ovary tissue of the SG group was evaluated as grade 0. The appearance of the cortex and medulla in this group was normal; the cortex, follicles, interstitial connective tissue, and blood vessels at different stages of development were normal (Fig. 4A). Degeneration in the follicles and follicle cells, necrotic cell accumulations around the follicle, hemorrhage in the tissue and corpus luteum, edema in the interstitial tissue, and congestion and dilatation in the blood vessels were found to be grade 3 in the follicles that developed in the ovary sections of the IR group (Fig. 4B). Dilatation of blood vessels, congestion, and PNL infiltration in surrounding connective tissue were detected as grade 3 in large magnification images (Fig. 4C). In the IRC-50 group treated with low-dose carvacrol, degeneration was observed in some of the follicles at grade 2 severity, while necrotic cell debris was observed in the lumen of grade 1 degenerated follicles and around the follicle (Fig. 4D). Moderate PNL infiltration, dilatation and congestion in the blood vessels were observed in the IRC-50 group (Fig. 4E). Ovarian tissue samples belonging to the IRC-100 group treated with high-dose carvacrol revealed that the developing follicles generally had a normal structure and morphology (grade 0), the interstitial connective tissue was partially edematous (grade 1), and mild congestion in the blood vessels (grade 1) was present (Table 3). In addition, the rarely seen PNL infiltration in the tissues belonging to the IRC-100 group and the presence of a small amount of necrotic cell debris in some follicle lumen were calculated as grade 0 (Fig. 4F).

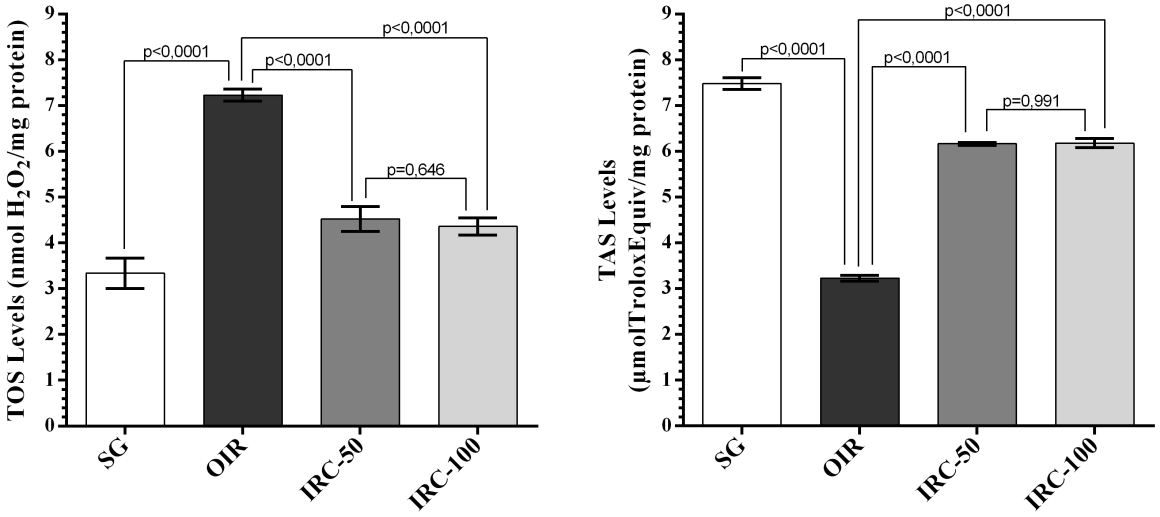

Fig. 2. TOS and TAS levels in the ovarian tissue. IR, Ischemia-reperfusion; TOS, Total Oxidant Status; TAS, Total Antioxidant Status; OIR, Ovary ischemia-reperfusion; IRC-50, IR +50 mg/kg carvacrol; IRC-100, IR +100 mg/kg carvacrol; SG, Sham operation group.

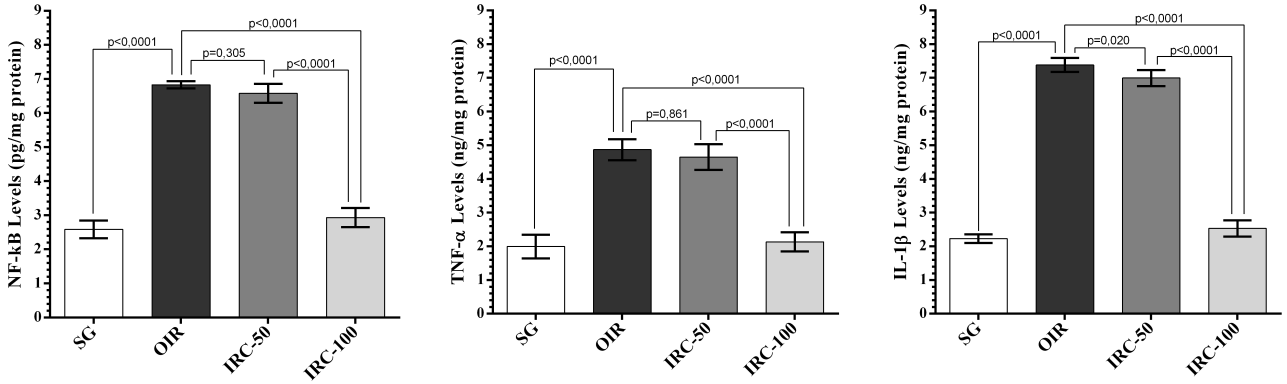

Fig. 3. NF-κB, TNF-α, and IL-1β levels in the ovarian tissue. IR, Ischemia-reperfusion; IL-1β, Interleukin 1 beta; TNF-α, Tumor necrosis factor-alpha; NF-κB, Nuclear factor kappa B; OIR, Ovary ischemia-reperfusion; IRC-50, IR +50 mg/kg carvacrol; IRC-100, IR +100 mg/kg carvacrol; SG, Sham operation group.

6. Discussion

Our study investigated the protective effect of two different doses of carvacrol against IR-induced ovarian damage and reproductive dysfunction in female rats. Our biochemical test results showed that carvacrol at 50 and 100 mg/kg doses equally prevented the increase of MDA in ovarian tissue by IR. As is known, lipids are the biomolecules most affected by oxidative stress [29]. ROSs, by lipid peroxidation (LPO), lead to the formation of toxic aldehydes that exacerbate a variety of oxidative damage [30]. MDA is one of the toxic products of the LPO reaction, which is most commonly used in determining oxidative stress [31]. MDA creates a cytotoxic effect by causing cross-linking and polymerization of cell membrane components and inactivation of receptor and membrane-bound enzymes [32]. Meeran *et al.* [33] reported that carvacrol and its isomer, thymol, inhibited LPO; they also found that the inhibitory effect of thymol on LPO was stronger than that of carvacrol. It has been suggested that this is due to the greater steric hindrance of thymol. Moreover, the high potential of phenolic compounds to scavenge radicals may be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups [34]. From the literature, it is understood that the protective effects of carvacrol and thymol on ovarian tissue are due to their common antioxidant and anti-inflammatory properties [35]. Another common feature of thymol, carvacrol and other plant-derived terpenes in their biological activities is the inhibition of voltage-dependent Na^+ ion channels [36]. Ingec *et al.* [37] reported that the increase in the amount of MDA was associated with the severity of the ovarian tissue injury. Kurt *et al.* [38], on the other hand, argued that suppressing the increase in the amount of MDA is important in reducing IR-related ovarian damage. Dursun *et al.* [39] showed that carvacrol suppressed the increase of MDA in testicular tissue after torsion detorsion in rats, they also stated that can prevent

Table 1. The effect of carvacrol on IR-related infertility in female rats.

Groups (n = 24)	Number of births	Number of infertile animals*	The average andmedian number of offspring born	*p*
SG (n = 6)	5	1	$4/6_{(0–6)}$	0.009
OIR (n = 6)	0	6	$0/0_{(0–0)}$	
IRC-50 (n = 6)	1	5	$3/3_{(0–3)}$	
IRC-100 (n = 6)	4	2	$3/3_{(0–6)}$	

SG, Sham group; OIR, Over ischemia-reperfusion; IRC-50, Ischemia-reperfusion +50 mg/kg carvacrol; IRC-100, Ischemia-reperfusion +100 mg/kg carvacrol.

Number of offspring borns were presented as median (min–max) value for groups.

*While comparing fertility between groups Chi Square was used.

Table 2. Follicle staging in fertile rat groups.

Groups (n = 24)	Primordial follicle	Developing follicle	Atretic follicle	Corpus luteum
SG	14	23	3.1	13
OIR	12	19	6.6	12
OIR-50	13	22	4.5	13.1
OIR-100	14	23	3.3	13.5

SG, Sham group; OIR, Over ischemia-reperfusion; IRC-50, Ischemia-reperfusion +50 mg/kg carvacrol; IRC-100, Ischemia-reperfusion +100 mg/kg carvacrol.

Table 3. Histopathological damage grading in rat ovary tissue.

Findings	Groups and median score			
	Sham	OIR	IRC-50	IRC-100
Degeneration	0	$3_{(2–3)}$	$2_{(1–2)}$	$0_{(0–1)}$
Necrosis	0	$3_{(2–3)}$	$2_{(0–2)}$	$1_{(0–1)}$
Vascular dilatation/congestion	0	$3_{(2–3)}$	$2_{(1–2)}$	$1_{(0–1)}$
Interstitial edema	0	$3_{(2–3)}$	$2_{(1–2)}$	$1_{(0–1)}$
Hemorrhage	0	$3_{(2–3)}$	$0_{(0–1)}$	$0_{(0–1)}$
PNL infiltration	0	$3_{(2–3)}$	$2_{(0–2)}$	$0_{(0–1)}$

0 = Normal, 1 = Mild damage, 2 = Moderate damage, 3 = Severe damage.

PNL, Polymorphous nuclear leukocyte.

apoptotic development and signaling of spermatogenic cells with strong mitotic activity in the basement membranes of seminiferous tubules. Taxifolin, another antioxidant that prevents the increase of oxidants and the decrease of antioxidants in testicular tissue applied torsion/detorsion, has been shown to alleviate histopathological damage. Taxifolin has been proven to prevent the reduction in germinal epithelial thickness caused by the torsion/detorsion, detached germinal cell lines, cell necrosis of the seminiferous tubule, and the development of degenerated germinal giant cells [40]. The present study shows that the IR procedure caused a decrease in tGSH in the ovarian tissue. GSH is known to be a tripeptide antioxidant consisting of L-glutamate, L-cysteine and glycine [41]. GSH has been proven to play a critical role in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles while maintaining redox homeostasis [42]. Isaoglu *et al.* [43] demonstrated that an IR event causes a decrease in the amount of tGSH in the ovarian tissue. Another study found that drugs that prevent the decrease of tGSH levels protect ovarian tissue from IR damage [44]. Kadioglu *et al.* [12] emphasized the importance of keeping MDA low as well as keeping tGSH high in reducing IR-related damage. It has been reported that carvacrol significantly prevents the depletion of cellular GSH content by hydrogen peroxide (H_2O_2) [45]. It has also been reported that carvacrol is a good scavenger of peroxyl radicals and inhibits the peroxidation of phospholipid liposomes in the presence of iron (III) and ascorbate [46].

In our study, TOS and TAS levels were measured to gain an understanding of the way that IR in ovarian tissue changes the oxidant-antioxidant balance in favor of oxidants. MDA, tGSH and other oxidant-antioxidant parameters are known to be used to determine the oxidant-antioxidant balance. TOS and TAS reflect the total effects of all antioxidants and oxidants in tissues [25,26]. Therefore, TOS levels are used for practical measurement of ROS, and TAS levels are used to evaluate total antioxidant status. In a study supporting our TOS and TAS findings, it was observed that the IR procedure increased TOS levels in ovarian tissue and decreased TAS levels [47].

Our study showed that proinflammatory cytokine levels such as NF-κB, TNF-α and IL-1β were increased in the IR-applied ovarian tissue. As noted in the literature, the production of NF-κB, TNF-α, IL-1β and oxidant is increased in tissues exposed to IR [48]. Keskin *et al.* [49] showed that histopathological damage developed in IR-induced testicular tissue with high NF-κB, TNF-α and oxidant levels and low antioxidants. NF-κB plays an important

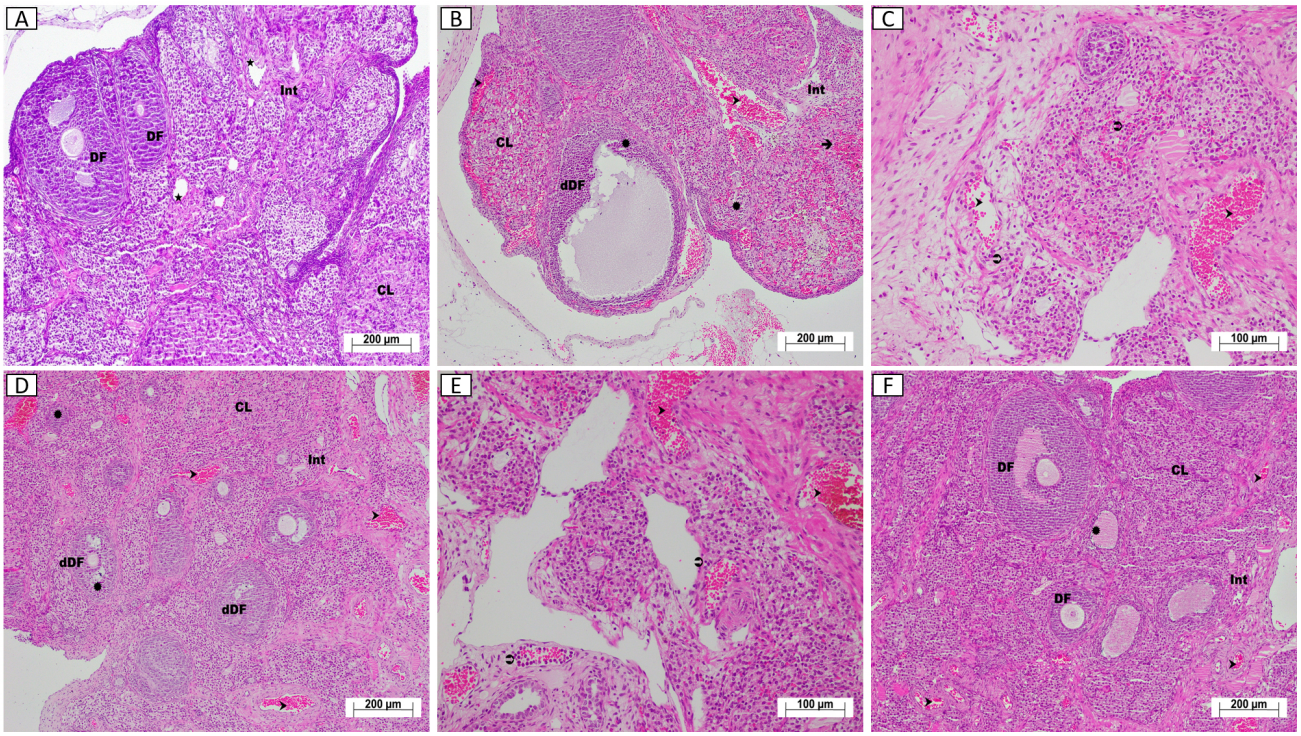

Fig. 4. Histopathological examination of ovary tissue. (A) Ovarian tissue of SG group stained with hematoxylin-eosin; DF, developing follicle; Int, interstitial area; CL, corpus luteum; ★, blood vessel, ×100. (B) Ovarian tissue of the IR group stained with hematoxylin-eosin; dDF, degeneration in developing follicles; ✹, accumulation of necrotized cells in the follicle and surrounding connective tissue; Int, edema in the interstitial area; CL, hemorrhage in the corpus luteum; ➔, hemorrhagic areas; ➤, densely dilated and congested blood vessel, ×100. (C) Ovary tissue of the OIR group stained with hematoxylin-eosin; at large magnification, ➤, dense dilated and congested blood vessel; ➲, polymorphonuclear cell infiltration, ×200. (D) Ovarian tissue of the IRC-50 group stained with hematoxylin-eosin; locally degenerated developing follicle (dDF), Int, edema in the interstitial area (Int); CL, corpus luteum; ✹, necrotic cell debris in the lumen of the follicles; ★, dilated and congested blood vessels, ×100. (E) Ovarian tissue of the IRC-50 group stained with hematoxylin-eosin; at large magnification, ➤, dilated and congested blood vessel and ➲, PNL infiltration, ×200. (F) Ovarian tissue of the IRC-100 group stained with hematoxylin-eosin; normal structure DF, developing follicle; Int, edema in the interstitial area (Int); CL, corpus luteum; ✹, small amount of necrotic cell debris in the lumen of some follicles; ➤, congested blood vessels in patches, ×100.

role in regulating the expression of other proinflammatory cytokine genes [50]. In addition, it has been reported that NF-κB activation is induced by ROS, and oxidative stress triggers inflammation [51,52]. However, in our study, although carvacrol inhibited the increase of IR-related oxidants at 50 and 100 mg/kg doses to the same extent, it was able to inhibit the increase of NF-κB, TNF-α and IL-1β only at a dose of 100 mg/kg. No information was found in the literature to support these findings. However, carvacrol is known to protect lung tissue from damage due to oxidative stress and proinflammatory cytokine increase [53], and carvacrol has been shown to have a stronger inhibitory effect on oxidant and proinflammatory cytokines at high doses [53,54]. Li *et al*. [55] suggested that the anti-inflammatory effect of carvacrol is due to inhibition of the pro-inflammatory NF-κB pathway. This information indicates that the IR process causes damage in different tissues with a similar mechanism.

Carvacrol at a dose of 100 mg/kg, which significantly inhibited the overproduction of NF-κB, TNF-α and IL-1β, reduced ovarian IR injury histopathologically better than the 50 mg/kg dose. In addition, inflammatory symptoms such as edema, congested and dilated blood vessels and PNL infiltration were observed in the ovarian tissue of the carvacrol group administered a dose of 50 mg/kg, while these histopathological findings were not observed in the rats that received a dose of 100 mg/kg. Previous studies have found that IR caused histopathologically dilated and congested blood vessels, PNL infiltration, hemorrhage, edema, follicular degeneration and necrosis in the ovarian tissue [56,57]. Unlubilgin *et al*. [15] explained that this IR-related histopathological damage leads to infertility. In our study, proinflammatory cytokine production was significantly suppressed, and the number of infertile animals decreased significantly in the carvacrol group (at a dose of 100 mg/kg), in which, histopathologically, no inflammatory symptoms were observed. Bhandari *et al*. [58] found

that the development of infertility was associated with an increase in proinflammatory cytokines and a decrease in anti-inflammatory cytokines. which supports our experimental results. As a result, the IR event caused an increase in oxidant and proinflammatory cytokines and a decrease in antioxidants in ovarian tissue. Ovarian IR caused tissue degeneration, necrosis, vascular damage and inflammatory events histopathologically. At a dose of 100 mg/kg, carvacrol, which inhibits the overproduction of oxidant and proinflammatory cytokines increased by IR in ovarian tissue, prevented the development of inflammation in the tissue. The 50 mg/kg dose of carvacrol, which inhibited the IR-related oxidant release in ovarian tissue to the same extent, did not inhibit the increase of proinflammatory cytokines and the development of inflammation. Carvacrol significantly prevented IR-related infertility at a dose of 100 mg/kg but not at a dose of 50 mg/kg. This indicates that the anti-inflammatory effect of carvacrol at a dose of 100 mg/kg is not related to its antioxidant activity. It also shows that inflammation is an important factor in the pathogenesis of ovarian IR-related infertility. Induction of inflammation in IR-related ovarian tissue suggests that it is not limited to oxidants.

7. Conclusions

Our experimental results suggest that effective doses of antioxidants and anti-inflammatory drugs may be beneficial in the clinical treatment of ovarian IR injury due to torsion and detorsion and in preventing the development of infertility. In addition, the information obtained from our study and literature indicates that it may be beneficial to apply antioxidant and anti-inflammatory treatment before and after reperfusion.

Abbreviations

IR, Ischemia-reperfusion; HX, Hypoxanthine; XDH, Xanthine dehydrogenase; ROS, Reactive oxygen species; XO, Xanthine oxidase; MDA, Malondialdehyde; tGSH, Total glutathione; TOS, Total Oxidant Status; TAS, Total Antioxidant Status; IL-1β, Interleukin 1 beta; TNF-α, Tumor necrosis factor-alpha; NF-κB, Nuclear factor kappa B; PNL, Polymorphous nuclear leukocyte; OIR, Ovary ischemia-reperfusion; IRC-50, IR +50 mg/kg carvacrol; IRC-100, IR +100 mg/kg carvacrol; SG, Sham operation group.

Author contributions

NS, IBD and UI designed the research study. BS performed the research. HS provided help and advice on the experiments. GNY and TAC, VA analyzed the data. BS, HS and KU wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Atatürk University Animal Experiments Local Ethics Committee, Erzurum, Turkey (Date: 23.03.2021, meeting no: 236643897-000). All animals received care in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

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Conflict of interest

The authors declare no conflict of interest.

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