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The effect of a natural molecule in ovary ischemia reperfusion damage: does lycopene protect ovary?

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Abstract: Ovarian ischemia is a gynecological emergency case that occurs as a result of ovarian torsion. Oxidative stress plays a central role in the development of ischemia/reperfusion (IR) injuries. Lycopene (LYC) is a lipophilic, natural carotenoid well known for its antioxidant properties. This study provides information on the potential applications of lycopene. The Wistar Albino rats were distributed into six groups: Sham group (only a laparotomy was performed), Control group [laparotomy and intraperitoneal dissolvent (olive oil)], IR group, IR+olive oil group, IR+LYC 2.5 mg/kg/dose, intraperitoneal group, IR+LYC 5 mg/kg/dose intraperitoneal group. Evaluated in terms of histopathological changes, tissue malondialdehyde levels (MDA), ovarian expressions of phosphorylated nuclear factor-kappa B (p-NF-κB) and the TUNEL method was utilized to show apoptosis of ovarian tissue. There was a significant decrease in MDA, p-NF-κB values and the proportion of apoptotic cells assessed by TUNEL compared to the group that did not receive intraperitoneal LYC in rat injury with IR damage (P<0.05). In histopathological damage scoring, it was observed that the cell damage was significantly reduced in LYC-administered groups. LYC showed significant ameliorative effects on ovary injury caused by IR through acting as an antioxidant, antiinflammatory, and antiapoptotic agent.
Key words: ischemia-reperfusion, lycopene, ovary, rat

Introduction

Ovarian torsion is a gynecological urgent pathology with an incidence of 2.7–3% leading to ovarian ischemia [1]. Ovarian torsion is seen more commonly in the reproductive age. With the increase in assisted reproductive techniques, the incidence increases up to 16% due to ovarian hyperstimulation [2]. The torsion of the ovarian vascular pedicle on its axis results in decreased arterial blood flow and the obstruction of venous and lymphatic drainage [3]. It has been known for many years that ischemia causes cell damage [4]. The first method required to prevent cell damage is to shorten the hypoperfusion time and provide reperfusion. However, recent studies have reported that a series of incidents, called "Ischemia /Reperfusion (IR) injury", with reperfusion, can occur, which may lead to the impairment in organ functions [5]. The basic mechanism in IR damage begins with the induction of anaerobic metabolism with ischemia, resulting in lower ATP production which leads to the disruption of ion exchange channels. In spite of the cessation of ATP production, ATP, adenosine, inosine, and hypoxanthin are formed from ATP due to the continued use of it. In reperfusion, XO (xanthine oxidase),

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which forms during ischemia by restoring oxygen flow, converts the accumulated hypoxanthin to xanthine and causes excessive free oxygen radical formation. reactive oxygen species (ROSs) cause autophagy, mitoptosis, necrosis, necroptosis, apoptosis, resulting in cell death [5].

Contrary to popular belief, in ischemic ovarian damage, total ovarian necrosis does take place [6]. In long-term ischemic cases, in spite of the blue-black discoloration in the ovary, the presence of live follicles has been shown. Therefore, the current treatment approach is the detortion, regardless of the ovarian appearance [7]. The question here is whether detortion will cause IR damage in the ovary. Studies have shown that varying degrees of ovarian follicles decrease with detorsion [6, 8]. However, the common belief is that follicular viability is still ongoing. Therefore, to prevent ovarian damage, as in all organ ischemia, the ischemia duration must be primarily shortened and then the reduction of reperfusion injury in the detorsioned ovary should be targeted. However, early diagnostic methods that will shorten the duration of hypoperfusion in clinical practice may not always be available to clinicians or there may be delays in diagnosis due to some other factors. Therefore, studies focused on the reduction of reperfusion injury. Accordingly, the efficacy of many antioxidant agents in preventing oxidative damage was investigated [9, 10]. The use of antioxidants can help support cells antioxidant defense mechanisms. LYC is a carotenoid with antioxidant [11], anti-apoptotic [12], anti-inflammatory [13], and anti-ischemic [14] properties that protect cells from lipid peroxidation and oxidative DNA damage. Its efficacy has been shown in IR damage in organs such as kidney, myocardium, and testicles [14–16].

"Does LYC have a protective effect on ovarian IR?" There are no previous studies in the literature on ovarian IR damage. Accordingly, the present study aimed to evaluate the efficacy of two different doses via the intraperitoneal route in the reperfusion stage without applying pretreatment.

Materials and Methods

Animals

The local Animal Ethics Committee approved the experimental procedures employed in the present study (via KOBAY D.H.L. A.Ş.Registry No: 309). All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals, as confirmed by the National Institute of Health (Washington, DC, USA).

Accordingly, 48 sexually mature female Wistar Albino rats, weighing 180–240 g, were used in the study. Rats were put in stainless steel cages until the time of the experiment and were fed with standard rodent food and water under appropriate ambient temperature (24–25°C), humid environment (55–60%) and controlled photo-period (12:12 h light:dark).

Surgical and experimental technique

As an anesthetic drug for surgical interventions, 50 mg/kg Ketamine (Ketalar; Parke Davis, Eczacıbaşı, İstanbul, Turkey) and 10 mg/kg Xylazine (Rompin, Bayer AG, Leverkusen, Germany) were used in combination. After anesthesia, rats were kept in a supine position and 2% iodine alcohol was used for antisepsis of the lower abdominal region. Then, 2.5 cm longitudinal incision was made in the lower abdominal region and the right ovary was visualized. For the induction of ischemia, vascular clamps were applied to the right ovarian vessels. At the end of the three-hour ischemia period, the vascular clamps were removed and a three-hour reperfusion was continued. The vital signs of all rats remained stable throughout the surgical procedure. At the end of the study, rats were sacrificed by decapitation in accordance with the ethical committee principles.

The experimental groups were formed as follows; Sham group (n=8) (only a laparotomy was performed), control group (n=8) [laparotomy and intraperitoneal dissolvent (olive oil)], IR group (n=8), IR + olive oil group (n=8), IR+LYC 2.5 mg/kg/dose, intraperitoneal group (n=8), IR+LYC 5 mg/kg/dose intraperitoneal group (n=8).

Drug protocol

The LYC dose administered in the experiment was determined according to a study previously performed in another organ ischemia [17]. LYC (Sigma Aldrich, St. Louis, MO, USA) was dissolved in olive oil. The prepared LYC was administered intraperitoneally in two different doses one hour before the reperfusion.

The groups created were recorded by the researchers who conducted the experiment. However, the researchers who carried out biochemical and histopathological examination were unaware of these groups formed until the end of the study.

Histological analysis

Ovarian tissues taken at the end of the experiment were fixed in a 10% formaldehyde solution. After fixation, the tissues were dehydrated by passingthe samples through alcohol series with increasing alcohol content (50%, 70%, 80%, 96%, and 100%). Tissues that made transparent with xylene were embedded in paraffin. Hematoxylin-Eosin (H&E) staining was applied to 5μm

thick sections taken from paraffin blocks and sealed with concealing solution (Entellan®, Merck, Darmstadt, Germany). Histopathological damage scoring was performed in terms of the degeneration of follicles in the ovarial cortical region, vascular congestion, edema and infiltration of inflammatory cells [18]. Each criterion was evaluated according to the classifications as normal (0), mild (1), moderate (2), and severe (3). The preparations were examined under the Olympus BX53 light microscope.

Immunohistochemical analysis

The immuno-reactivity of the phosphorylated nuclear factor-kappa B (p-NF-κB) (Cat: MCA1292-MFG:20190827, Bioassay Technology Lab., Shanghai, China) protein was determined in the ovarian tissues of the rat model by IR using the Avidin-Biotin peroxidase method [19]. Briefly, after the deparaffinization of 5 μm thick sections, citrate buffer was used to open the epitopes (pH: 6.0). The slides were then taken into a 3% hydrogen peroxide solution in methanol to prevent endogenous peroxidase activity. A UV-block solution was applied to prevent non-specific staining. Sections were then incubated overnight at 4°C with p-NF-κB primary antibody. Biotinylated secondary, streptavidin-HRP (TP-125-HL, LOT: PHL181115, ThermoScientific, Waltham, USA) and DAB chromogens were applied respectively and then the slides were counterstained with Gill Hematoxylin. Then, the slides were dehydrated by passing them through alcohol series with increasing alcohol levels and mounted in entellan. The sections were examined using an Olympus BX53 light microscope. The evaluation of the immunoreactivity levels was conducted using the Image J program. Accordingly, 10 different areas were evaluated for each slide.

Biochemical analysis

Tissue samples from rats were used for the biochemical analysis. For the MDA testing in ovarian tissue the rat MDA (Cat. No: 201-11-0157, Sunred Bio, Shanghai, China) protocol in the manufacturer's kits was adopted and the concentration was measured at 450 nm with the ELISA reader. Results are given in nmol/ml for MDA.

The TUNEL method

Apoptotic cells were determined in the sections taken from the subjects using a Roche In Situ Cell Detection Apoptosis Fluorescein Kit (Cat No: 11684795910, Molten Biotechnology Lab., Roche Inc., Basel, Switzerland). The staining was carried out according to the kit procedure. Ovarian sections taken in 5μm thickness were deparaffinized and rehydrated andthen washed twice with PBS for 5 min. Then, for antigen recovery, the tissues were kept in 0.01 M 5% sodium citrate buffer in the microwave oven at 350 W for 5 min, then allowed to cool at room temperature for 10 min. The tissues that were washed two times for 5 min with PBS were placed in the humidity chamber at 37°C with the TUNEL reaction mixture in the kit and incubated in the incubator for 60 min. The tissues that werewashed two times for 5 min with PBS were contrasted with 4',6-diamidino-2- phenylindole (DAPI). Tissues sealed with glycerol closure solution were observed on the Olympus BX53 model fluorescent microscope at 450–500 nm wave length. For the apoptotic index, 50 different areas of apoptotic cells were counted in 20× lenses in total.

Statistical analysis of data

The SPSS version 22 package program was used for the statistical analyses. The results are given as the mean ± SD. The One-way ANOVA test was used to compare the groups. The Post-Hoc Tukey test was used for binary comparisons. The statistical value $P<0.05$ was taken as the significance level.

Results

Histopathological evaluation

Examining the ovarian sections of the Control and Sham group, follicles were observed at various developmental stages in normal histology, as shown in Fig. 1. In sections of IR-induced ovarian tissues, degeneration, vascular congestion, edema and infiltration of inflammatory cells were observed in follicular cells. Vascular congestion was more common in ovarian samples of ischemia/reperfusion and IR+olive oil group (Fig. 1). In the histopathological scoring of the ovarian tissue samples, it was observed that the groups treated with IR +LYC had statistically significant improvements ($P<0.05$) (Table 1).

Immunohistochemical findings

Evaluating the immunohistochemical results, p-NF-κB expression statistically increased in the ovarian tissues only in the IR group compared to those of all other groups ($P<0.05$). In ovarian tissues that received 2.5 and 5 mg/kg LYC, p-NF-κB expression significantly decreased compared to the IR group. It was observed that p-NF-κB expression was similar in these two groups (Fig. 2).

Biochemical findings

When the MDA results were evaluated, a significant increase was observed in the IR group (3.47 ± 0.27 nmol/

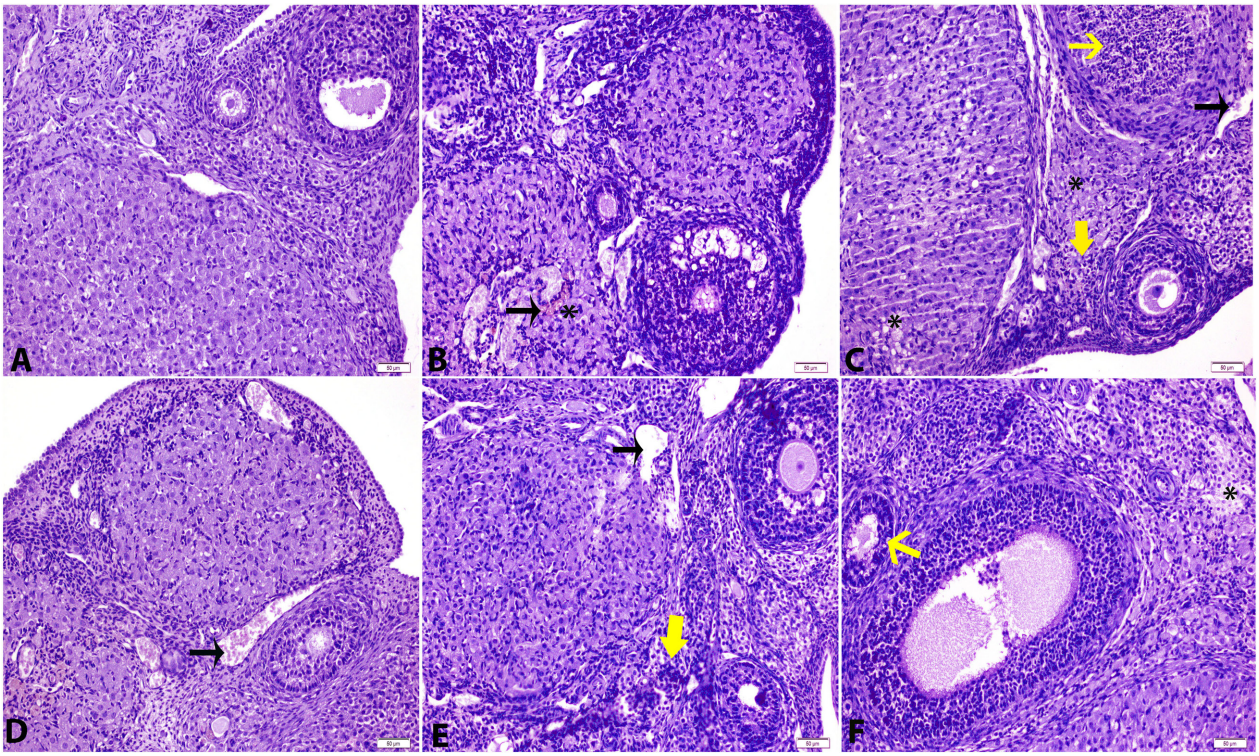


Fig. 1. The H&E staining images of ovarian tissues belonging to Control (A), Sham (B), IR (C), IR +olive oil (D), IR+2.5 mg/kg Lycopene (E), IR+5 mg/kg lycopene (F) experimental groups (Yellow thick arrow indicates the infiltration of inflammatory cells while yellow thin arrow indicates degeneration of follicular cells, and black arrow indicates vascular congestion; *edema) (×200).

Table 1. Histopathological damage scores in the ovarian samples belonging to the experimental groups

	Degeneration of follicles	Vascular congestion	Edema	Infiltration of inflammatory cells
Control	0.375 ± 0.51	0.375 ± 0.51	0.375 ± 0.51	0.625 ± 0.51
Sham	0.625 ± 0.51	0.375 ± 0.51[a]	0.625 ± 0.51	0.625 ± 0.51
IR	2 ± 0.5[ab]	2.375 ± 0.48[ab]	2.625 ± 0.48[ab]	2 ± 0.71[ab]
IR+Olive oil	1.875 ± 0.83[ab]	2 ± 0.75[ab]	1.625 ± 0.74[abc]	2.375 ± 0.52[ab]
IR+LYC 2.5 mg/kg	1.375 ± 1.06[a]	1 ± 0.82[cd]	1.375 ± 0.74[ac]	1.625 ± 0.74[ab]
IR+LYC 5 mg/kg	1.375 ± 0.74[a]	1.375 ± 0.92[abc]	1.5 ± 0.76[ac]	0.625 ± 0.74[cde]

Data are expressed as mean ± SD. Two-way ANOVA variance analysis and Tukey post hoc multiple comparison test were applied ([a]P<0.05 different from control group; [b]P<0.05 different from sham group; [c]P<0.05 different from ischemia/reperfusion (IR) group; [d]P<0.05 different from IR/olive oil group; [e]P<0.05 different from IR+ 2.5 mg/kg lycopene group). Lycopene; LYC.

ml) compared to the control group (2.63 ± 0.18 nmol/ml). There was a statistically significant decrease in the groups treated with 2.5 mg (2.65 ± 0.47 nmol/ml) and 5 mg (2.44 ± 0.38 nmol/ml) LYC compared to those in the IR group (P<0.001) (Table 2).

TUNEL results

Evaluating the number of apoptotic cells, it was observed that the number of apoptotic cells increased in the IR group compared to the control group whereas there was a significant decrease in the groups treated with LYC compared to the IR group (Table 3, Fig. 3).

Discussion

The present study showed that LYC, which is a natural molecule, improved the histopathological, immunohistochemical and biochemical parameters in ovarian IR damage. It was found that there was a significant decrease in MDA, p-NF-κB values and the rate of apoptotic cells in the groups that received intraperitoneal LYC evaluated by TUNEL compared to the group that did not receive intraperitoneal LYC in the rat ovary with IR damage.

Evaluating the basic mechanism of cell damage, regardless of the causing factor (hypoxia, physical and chemical agents, aging), free radicals were the main factors dominating the process. Therefore, the efficacy

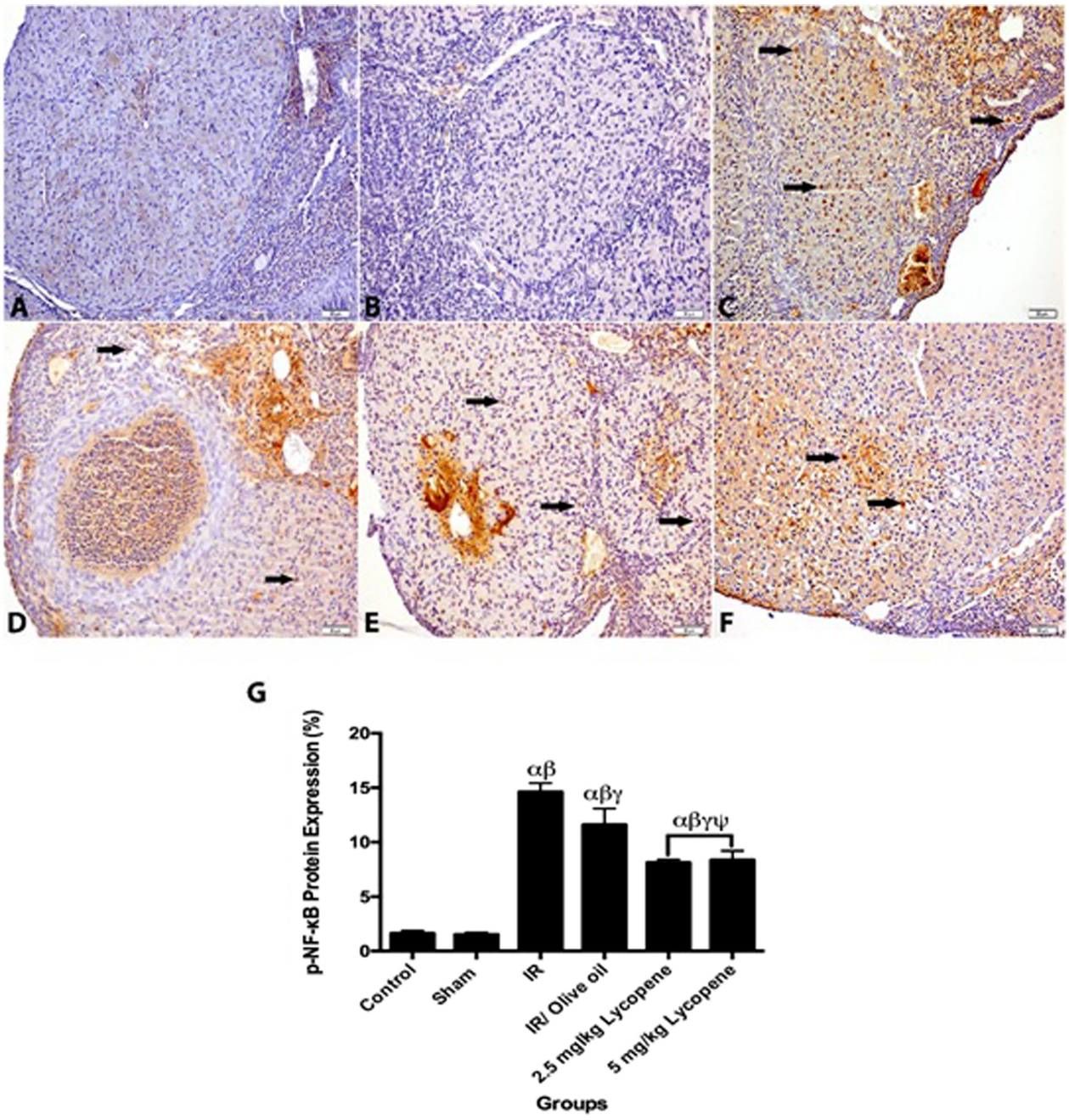


Fig. 2. The p-NF-κB immune staining of the ovarian tissues of Control (A), Sham (B), IR (C), IR+olive oil (D), 2.5 mg/kg lycopene (E), 5 mg/kg lycopene (F) (the black arrow shows the immune reactive areas) (G). The p-NF-κB immunoreactivity data shown on the histogram graph are expressed as mean ± SD (α P<0.05 different from the control group; β P<0.05 different from the Sham group; γ P<0.05 different from the IR group; Ψ P<0.05 different from the IR+olive oil group; δ P<0.05 2.5 mg/kg different from the lycopene group) (×200).

of many different agents that support protective mechanisms for all organs, such as 'antioxidant', 'antiinflammatory', etc. were studied to be shown experimentally. Carotenoids are micronutrients that have strong antioxidant effects but cannot be *de novo* synthesized by humans and should be taken by diet [20, 21]. LYC is one of more than 600 carotenoids found in nature [20]. LYC content in frequently consumed fruits and vegetables such as fresh ripe tomatoes, grapefruit, guava, papaya, and watermelon attracts interest in the study results on this carotenoid [20]. In the literature, there are clinical studies showing that the diet rich in foods with high LYC content (especially tomatoes) has protective effects against many diseases such as cancer, coronary artery and neuro-degenerative diseases and male infertility [20, 21]. Also, experimental studies were carried out in recent years to determine the effects of preventing cell damage and protecting organ functions. Studies showed that the

Table 2. MDA analysis results in ovarian tissue

Groups	Control	Sham	IR	IR+Olive Oil	IR+2.5 mg LYC	IR+5 mg LYC	*P*
MDA nmol/ml	2.63 ± 0.18[a]	2.62 ± 0.16[a]	3.47 ± 0.27[b]	3.28 ± 0.74[b]	2.65 ± 0.47[a]	2.44 ± 0.38[a]	<0.001

Data are shown as mean ± SD. $P<0.05$ was considered significant. There is no significant difference between groups containing the same letter (a and b). Ischemia/reperfusion; IR, lycopene; LYC.

Table 3. TUNEL results in ovarian tissue

Groups	Control	Sham	IR	IR+Olive Oil	IR+2.5 mg LYC	IR+5 mg LYC	*P*
TUNEL Positive cell	0.83 ± 0.37[a]	0.8 ± 0.48[a]	1.76 ± 0.89[c]	1.75 ± 1.03[c]	1.03 ± 0.18[ab]	1.36 ± 0.96[bc]	<0.001

Data are shown as mean ± SD. $P<0.05$ was considered significant. There is no significant difference between groups containing the same letter (a, b and c). Ischemia/reperfusion; IR, lycopene; LYC.

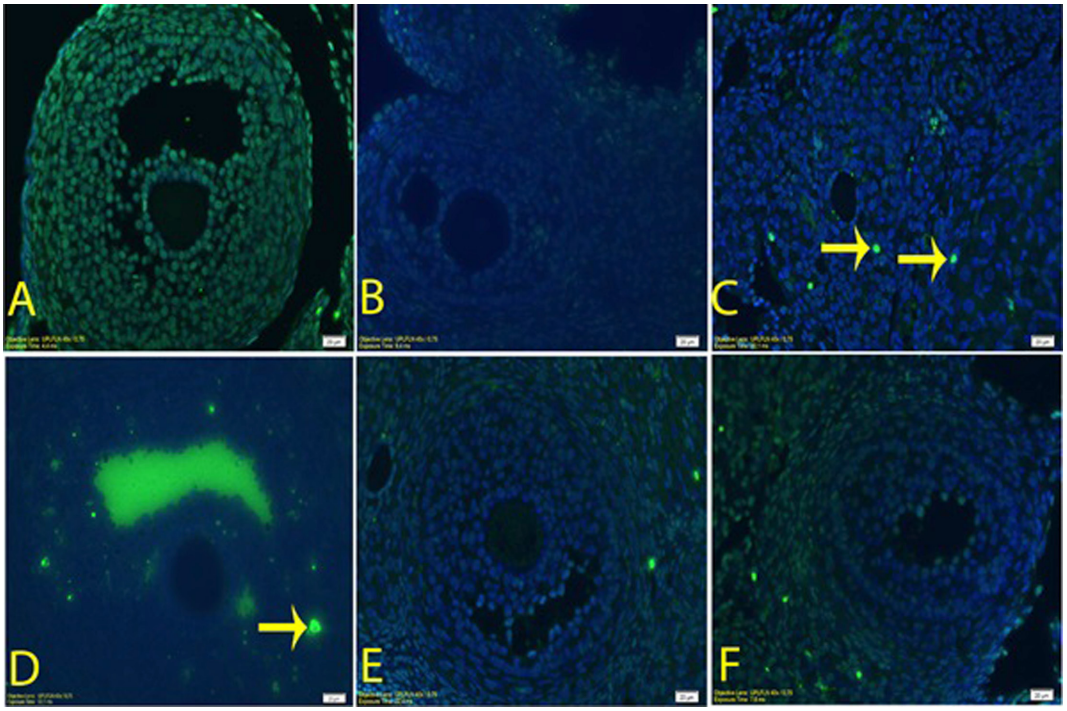


Fig. 3. Control (A), Sham (B), IR (C), IR+olive oil (D), 2.5 mg/kg lycopene (E), 5 mg/kg lycopene (F) Apoptotic cells are indicated with a yellow arrow ×200. 46 × 30 mm (300 × 300 DPI).

toxicodynamic properties of LYC were the same in rats and humans [22]. Therefore, rats can be regarded as a useful and suitable animal model to evaluate the potential effect and toxicity of LYC in humans [22].

Examining the studies on the effects of LYC on ovarian damage in the literature, it was seen that, in rats with Cisplatin-induced ovarian injury, pretreatment with oral LYC inhibited the increase in Cisplatin-induced MDA and increased the levels of antioxidants such as total glutathione, glutathione reductase and superoxide dismutase [23]. MDA is a major reactive aldehyde that forms during the peroxidation of polyunsaturated fatty acids in the biological membrane. It has been reported that LYC significantly reduced MDA in IR damage caused by liver, myocardium, neuron and renal cells with its strong antioxidant effects [14–17, 24]. In a similar vein, in the present study, there was an increase in MDA with IR damage whereas there was a significant decrease in MDA in groups treated with intraperitoneal LYC. In previous studies, LYC has been shown to reduce oxidative damage in a dose-dependent manner [25, 26]. In the present study, 5 mg/kg LYC application group, the decrease in MDA was higher than that in the 2.5 mg/kg LYC application group, however, this decrease was statistically not significant. The effect of LCY on cell damage was clearly seen in the histopathological evaluation. In the sections of IR ovarian tissues, degeneration, vascular congestion, edema and infiltration of inflammatory cells were observed in follicle cells, while the presence of these pathologies decreased in the groups that

received LYC. The protective effects of LYC on histopathological findings were also observed in Cisplatin-damaged ovarian tissue. This effect was evaluated together with the decrease in MDA levels and was associated with the reduction of oocyte and follicle damage by the antioxidative activity of LYC [23].

Cellular abnormal ROS accumulation and I/R damage not only directly damages cell proteins and membranes, but also causes damage by activating pro-apoptotic pathways. Apoptosis is also a process associated with inflammation. NF-kB-dependent transcription controls inflammatory processes, cytokines, chemokines, cell adhesion molecules, complement cascade and acute phase proteins. Also, this pathway has been also thought to be the primary oxidative stress-response pathway. NF-κB signal pathway can increase inflammation and apoptosis [27]. Studies showed that beta carotenes and LYC suppress NF-κB [25, 28]. With this suppression, cyclins, growth factors which are effective in cell growth and prophylaxis which are directly related to NF-κB, and anti-apoptotic Bcl family members and inhibitor of apoptosis proteins associated with and apoptosis are also affected. Thus, in addition to the anti-inflammatory effect, an anti-apoptotic effect takes place. It has been reported that LYC reduced apoptosis in studies on testicular tissue, neuron and heart in rats [12, 15]. However, the functioning of these mechanisms have not been completely revealed. Redox mechanisms have been also thought to be effective in processes associated with LYC and apoptosis. Palozzo *et al.* have associated the anti-inflammatory activity of LYC, which has strong antioxidant effects, with its ability to regulate the activity of transcription factors and redox sensitive kinases by the lipophilic interaction in the cell membrane [29]. Similarly, Yue *et al.* have reported that the anti-apoptotic activity was particularly associated with the anti-oxidant activity [14]. In the present study, p-NF-κB activity was higher in the IR group. Also, p-NF-κB activity was significantly suppressed in both 2.5 and 5 mg/kg LYC applications. Also, in the present study, a clear ovarian apoptotic response was observed in the IR group in the apoptotic evaluation with TUNEL and in LYC-treated groups, and the number of surviving cells was found to increase significantly.

LYC is found in varying levels in all tissues including ovaries, especially in the testicles and adrenal, but it is not stored [20]. The efficacy of LYC in varying doses was shown in IR damage in different organs. For example, it has been known that LYC has significant effects between 4 and 20 mg/kg in the testicle, 2.5 and 5mg/kg in the liver and 2.5 and 40 mg/kg in the myocardium [14, 15, 17]. Tissue and sex differences, usage patterns, and the variability of IR time may cause different doses to be effective against the same damage. In the present study, significant antiapoptotic, anti-inflammatory and antioxidative activity was observed at 2.5 and 5 mg/kg doses.

Since LYC is not stored, the intraoperative use may be reasonable in acute conditions such as ovarian torsion. This study was designed on this idea. However, the inability to evaluate the ovarian reserve after reperfusion is an important limitation of the study. Another limitation was that the study was performed on a relatively small number of experiment animals due to ethical issues. Also, the results of animal experiments could not be directly applied to human models and cannot be directly interpreted. The evaluation of the ovarian reserve by creating IR in varying periods with LYC application at different doses can reveal the efficacy of LYC more clearly.

In conclusion, this study is the first in the literature to evaluate the effect of LYC on ovarian IR damage. It was seen that LYC prevented oxidative and inflammatory pathways that cause tissue damage with its anti-oxidative, anti-apoptotoic and anti-inflammatory activity. It was seen that, in ovarian tissues, these effects were not dose-dependent and occured even at low doses. This natural antioxidant, which is not stored in the body, can be a promising pharmaceutical preparation that protects the ovary in ovarian torsion cases.

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