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Investigation of the Therapeutic Effects of Epigallocatechin Gallate in the Ovarian Ischemia Reperfusion Model in Rats

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ABSTRACT

In this study, we investigated the healing effects of Epigallocatechin gallate (EGCG) on ischemia reperfusion injury in rat ovaries. We used 32 female rats in the study. We randomly divided the animals into 4 groups. The first group was the control group and was anesthetized only. In the second group (IR), experimental ischemia (60 min) and reperfusion (2 hrs) were performed. Animals in the third group (IR+EGCG) were first exposed to ischemia for 60 min. Then, EGCG at a dose of 100 mg kg^{-1} was administered into the peritoneal cavity. Afterwards, the ovarian tissue was reperfused for 2 hrs. Rats in the fourth group (EGCG) were given EGCG at a dose of 100 mg kg^{-1} intraperitoneally. Blood and tissue samples were collected under anesthesia at the end of the reperfusion period. We measured TAS and TOS in serum to determine antioxidant capacity and oxidative stress. Histopathological analyzes were performed to examine the ovarian tissue. Additionally, TNF-immunoreactivity was investigated in the sections taken to determine inflammation. In addition, we demonstrated tissue caspase-3 levels immunohistochemically to assess apoptosis. No difference was observed between control group and IR group in terms of TAS level ($p>0.05$). Compared to the control and IR group, TAS level was significantly higher in EGCG treated groups ($p<0.05$). When TOS levels were compared between groups, the difference was significant and the highest value was in the 3rd group. The oxidative stress index were significantly higher in the IR group compared to the control ($p<0.05$). EGCG reduced pathological changes such as edema, congestion, vasoconstriction, hemorrhage and necrosis caused by IR. Additionally, the treatment reduced the occurrence of IR-related inflammation. Polymorphous nuclear cell infiltration was less in rats given EGCG. TNF-α staining was severe in group 2 and moderate in group 3. Caspase 3 immunoreactivity was very severe in the IR group. In the treatment group, the ovaries were moderate caspase 3 positive. Our findings showed that EGCG has antioxidant, antiapoptotic and anti-inflammatory effects and reduces ovarian IR damage. As a result, it was concluded that EGCG treatment can be used in IR injury.

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Key Words
Ischemia, reperfusion, ovary, rat, Epigallocatechin gallate

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INTRODUCTION

Ovarian torsion is a clinical case that can be seen in women of all ages[1]. This condition, called adnexal torsion, is characterized by the twisting of the ovary around its supporting ligaments. As a result of torsion, ovarian arteries and veins are blocked, blood flow is blocked and ischemia occurs[2]. The main reason for torsion is the mobility of the ligaments of the ovary. However, the cause of ovary torsion has not been clearly revealed. Its causes include pregnancy, ectopic pregnancy, adhesions, congenital malformations, paraovarian cysts, ovarian hyperstimulation, ovarian cysts and benign tumors. In particular, pregnancy is seen as an important risk factor[3-6].

In cases of adnexal torsion, which manifest as acute abdominal pain, there is usually a sudden onset of sharp, blunt and continuous right lower quadrant pain[7]. In this case, early diagnosis and intervention are of great importance[8]. Diagnosis can be detected by ultrasound, magnetic resonance or computed tomography methods[9]. Doppler ultrasonography is an important method in preoperative diagnosis[10]. Definitive diagnosis is made by explorative laparotomy[11]. As a result of torsion, blood and lymph flow are blocked, resulting in ischemia, gangrene and necrosis of the ovarian tissue, which can lead to a serious condition[12]. Therefore, failure to diagnose early and delay in intervention; It causes risky clinical problems such as ovarian necrosis, peritonitis, abscess or sepsis[13]. Delay in diagnosis and failure to detect the condition is a common condition. As a result of this situation, loss of the ovary and oviduct or both is observed[9,14]. With early diagnosis and intervention, it is possible to prevent irreversible damage[15]. Following the diagnosis, urgent surgical intervention is required[16].

The treatment of ovarian torsion is done surgically[17]. However, another problem occurs in the detorsioned ovary. This condition, called reperfusion injury, causes more damage to the tissue than ischemia[18]. To prevent ischemia-reperfusion (IR) injury, different antioxidant substances are used and their protective effects have been investigated by researchers[19-21].

Tea grown contains many phenolic substances and antioxidants. Among these, the substance that is abundant in green tea and has the most antioxidant properties is Epigallocatechin gallate (EGCG)[2]. As a result of scientific studies conducted with EGCG, it has been determined that this substance has beneficial effects on renal, hepatic, myocardial, testicular and cerebral IR damage[22]. However, in the literature review, there are no studies using EGCG in ovarian IR damage. We hypothesized that EGCG may protect the ovary from IR damage. So, we aimed to investigate effects of EGCG on ovarian IR injury.

MATERIAL AND METHOD

Material: Wistar albino rats were used in this study. Animals were obtained from KSU Faculty of Medicine Experimental Animal Research Center. EGCG derived from green tea was purchased from Sigma-Aldrich as a ready-to-use commercial product.

Method: This research was approved with the permission of Sütçü İmam University, Faculty of Medicine, Experimental Animals Local Ethics Committee, protocol number 2022-12 and decision number 02. Thirty two Wistar albino adult female rats, weighing approximately 200 g, were used in the study. One week before starting the research, the rats were taken into the adaptation process. They were fed in a cage at constant room temperature and humidity, under standard laboratory conditions of 12 hrs of light and 12 hrs of darkness. There were no feed or water restrictions before the experiment. All operative procedures were performed under general anesthesia and sterile conditions.

Experimental groups were formed as follows:

- **Group 1 (Control group) (n = 8):** No medication was administered to the rats in the group.
- **Group 2 (IR group) (n = 8):** Sixty minutes of ischemia and 2 hours of reperfusion were applied to the ovaries.
- **Group 3 (IR+EGCG group) (n = 8):** Sixty minutes of ischemia, single-dose EGCG was administered intraperitoneally (100 mg kg^{-1}) immediately before reperfusion and reperfusion were applied for 2 hrs.
- **Group 4 (EGCG group) (n = 8):** Only laparotomy was performed and EGCG was given intraperitoneally as a single dose (100 mg kg^{-1}) to the rats

Rats were anesthetized with xylazine (Control 10%, Doğa İlaç, İstanbul) and ketamine (Brema, Bremer Pharma, Germany) protocol. The operation was performed with dorsal laparotomy technique. No drug was administered to the control group and blood and ovarian tissue were taken immediately after anesthesia. In the second and third groups, firstly the skin was shaved and disinfected with povidone-iodine. Afterwards, the abdominal wall was incised and ovary was detected in the animals. Then, clamps were placed on the ovarian vessels, taking into account the relevant anastomoses and ischemia was created for 60 min. Subsequently abdomen was temporarily closed and surgical site was covered with sterile gauze soaked in physiological saline. Finally, the clamp was removed and the organ was reperfused for 2 hrs. In the fourth group, the abdominal cavity was entered and EGCG was administered directly. In this last group, blood and

tissue samples were taken 2 hrs later after anesthesia. In the 2nd and 3rd groups, samples were collected at the end of the reperfusion period. Vital signs were monitorized during experiment in all rats. At the end of the study, the rats were anesthetized again and then they were sacrificed by exsanguinations.

Previous studies were taken as basis in determining the dose of EGCG[20] and IR durations[21].

Collection of blood and tissue samples: An average of 4-5 mL of blood was taken from anesthetized rats by intracardial injection. Serum was obtained by centrifuging the blood samples at 3000 rpm for 10 min. These collected samples were stored at -80°C until the study was carried out. The ovaries were removed and washed with physiological saline. Then, they were placed in 10% formaldehyde solution for histopathological analysis.

Biochemical analysis

Measurement of serum total antioxidant status (TAS) levels: Total Antioxidant Status (TAS) measurement in serum was performed with the Rel Assay Diagnostics brand TAS measurement kit (LOT: AK21123A) (Rel Assay Diagnostics, Gaziantep, Turkey). Absorbance reading was made on Chromate 4300 brand elisa reader device (Awareness Technology, Inc. Martin Hwy. Palm City, USA). The measurement was made at 630 nm. Data were calculated with the formulation suggested by the kit. The results were given as mmol/L.

Measurement of serum total oxidant status (TOS) levels: Total Oxidant Status (TOS) measurement in serum was performed with the Rel Assay Diagnostics brand TAS measurement kit (LOT: AK211360) (Rel Assay Diagnostics, Gaziantep, Turkey). Absorbance reading was made on Chromate 4300 brand elisa reader device (Awareness Technology, Inc. Martin Hwy. Palm City, USA). The measurement was made at 492 nm. Data were calculated with the formulation suggested by the kit. Results are given as µmol/L.

Calculation of oxidative stress index (OSI): Oxidative stress index (OSI) was calculated using the following formula[24].

OSI (Arbitrary Unit) = TOS (µmol H2O2 equiv./L) / TAS x 10 (mmol Trolox equiv./L)

Histopathological analyzes: The fixed ovarian tissues were washed in running water overnight to remove formalin. Afterwards, routine pathological tissue was monitored and passed through graded alcohol (50, 75, 96 and 100%) and xylol series. Then it was blocked in paraffin. Five micro thick sections were taken from the obtained paraffin blocks with a Rotary microtome (Leica RM 2125 RT). The first three sections and every tenth sections were taken on slides. The prepared slides were passed through alcohol and xylol series and stained with Hematoxylin Eosin (HE). All samples were examined under a high-resolution light microscope (Olympus DP-73 camera, Olympus BX53-DIC microscope; Tokyo, Japan).

Scoring: All changes detected in tissue samples were noted and scored according to the presence and severity of any findings (0: absent, 1: mild, 2: moderate, 3: severe), as in some preliminary studies[25].

Immunohistochemical analyzes: Four micron thick sections were cut from previously prepared paraffin blocks onto Poly-L-lysine coated glass slides. These sections were stained according to the streptavidin-biotin-peroxidase complex (ABC) technique after routine deparaffinization and rehydration methods (Zymed, Histostain Plus Kit, California, USA). For antigen retrieval purposes, heat treatment was applied with citrate buffer (pH 6.0) in the microwave for 20 min. Endogenous peroxidase activation was blocked with 0.3% hydrogen peroxide in methanol in 0.01 M PBS for 10 min. Tissues were incubated with 5% normal goat serum for 30 min for protein blocking. Sections were labeled with TNF-α (1:50, ab6671; Abcam, Cambridge, USA) and Caspase 3 (10 µg mL^{-1}, Invitrogen, (MA USA)) primary antibodies by incubating for 60 min at room temperature. Afterwards, it was incubated in rabbit anti-mouse biotinylated secondary antibody for 30 min and then incubated with streptavidin-peroxidase conjugate for 30 min. In order to make the reaction visible, diaminobenzidine (DAB, Dako/Denmark) chromogen was dropped onto the sections and left for 10 min. Finally, the sections were stained with hematoxylin for 1-2 min for backstaining and then washed in running water. Tissues were passed through a graded alcohol and xylol series and glued with Entellan. The entire staining procedure was performed at room temperature and in humid chambers. During the staining stages, phosphate buffered saline (PBS) solution was used to wash the sections. The slides were scored by counting the identified immunopositive cells at X40 and X100 magnifications for each sample under a high-resolution microscope (Olympus DP 73 camera, Olympus BX53-DIC microscope; Tokyo, Japan).

Scoring: (Staining indices were calculated based on the percentages of nuclei stained for these three markers as follows; negative: 0 (<1% positive); weak: 1 (1-25% positive); moderate: 2 (>25-75% positive) and strong: 3 (>75% positive)[25].

Statistical analysis: The data were statistically analyzed with the Graphpad Prism 6 program (GraphPad Software, USA). First, One way ANOVA parametric test was applied to detect differences in groups. Afterwards, Tukey multiple comparison test was applied. $p<0.05$ was considered statistically significant.

RESULT

Biochemical findings: According to the results of TAS analysis performed on serum, a significant difference was detected between the groups ($p<0.0001$). There was no significant difference between the IR and control groups in terms of TAS level ($p>0.05$). A statistically significant difference was found between the IR group and the IR+EGCG group. EGCG treatment significantly increased TAS values in rats with IR ($p<0.05$). When the IR group was compared with the EGCG group, antioxidant capacity was higher in the 4th group given EGCG ($p<0.05$) (Fig. 1).

When serum TOS levels were compared, the difference between the groups was found to be significant ($p<0.0001$). There was no significant difference between the control group and the IR group in terms of TOS value. But the TOS value of the 3rd group was significantly higher than the IR group ($p<0.05$). Compared to the IR group, although the TOS value was higher in the rats given only EGCG, the difference was statistically insignificant ($p>0.05$) (Fig. 2).

OSI was given in Fig. 3. Compared to the control group, the highest OSI value was in the IR group ($p<0.05$). When the OSI values of the 3rd and 4th groups given EGCG were compared with the IR group, the difference was insignificant ($p>0.05$) (Fig. 3).

Histopathological findings: The histopathological score of the ovaries in the experimental groups is given in Table 1. Light microscope images of ovarian tissue sections are shown in Fig. 4. The ovarian tissue of the control group and EGCG were found to have a normal histological structure (Fig. 4a,d, Table 1). According to the analysis results, in the ovarian tissues of the rats for which the IR model was created; Significant histopathological findings such as edema, dilatation, congestion, hemorrhage, necrosis, degeneration and polymorph nuclear leukocyte (PMN) infiltration were observed (Fig. 4b and Table 1). In the third group of rats, there were mild degenerative changes in the ovary (Table 1 and Fig. 4c).

Immunohistochemical findings: The immunohistochemical score of the ovaries in the experimental groups is given in Table 2. The slides stained with TNF-α are shown in Fig. 5. In the control group, normal ovarian tissue was observed (Fig. 5a). TNF-α immunoreactivity was severe in the ovary of the IR group (Table 2, Fig. 5b). However, this reactivity was

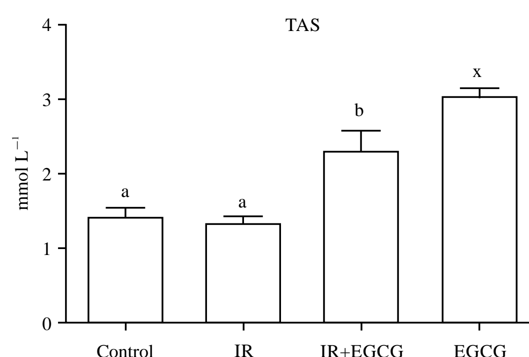

Fig. 1: TAS values of the experimental groups

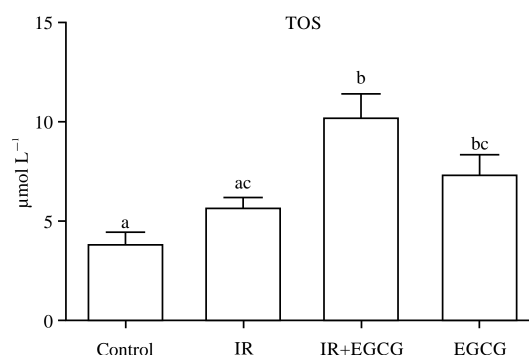

Fig. 2: TOS values of the experimental groups

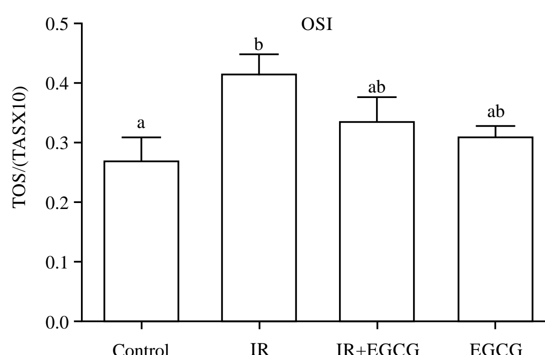

Fig. 3: OSI values of experimental groups

Table 1: Ovarian histopathological scoring in experimental groups

Groups	n	Edema	Dilation	Congestion	Hemorrhage	Necrosis	Degeneration	PMN
Control	8	-	-	-	-	-	-	-
IR	8	+++	++	++	++	++	+++	++
IR+EGCG	8	++	+	+	-	-	+	-
EGCG	8	-	-	-	-	-	-	-

IR: Ischemia-reperfusion, IR+EGCG: Ischemia-reperfusion+EGCG

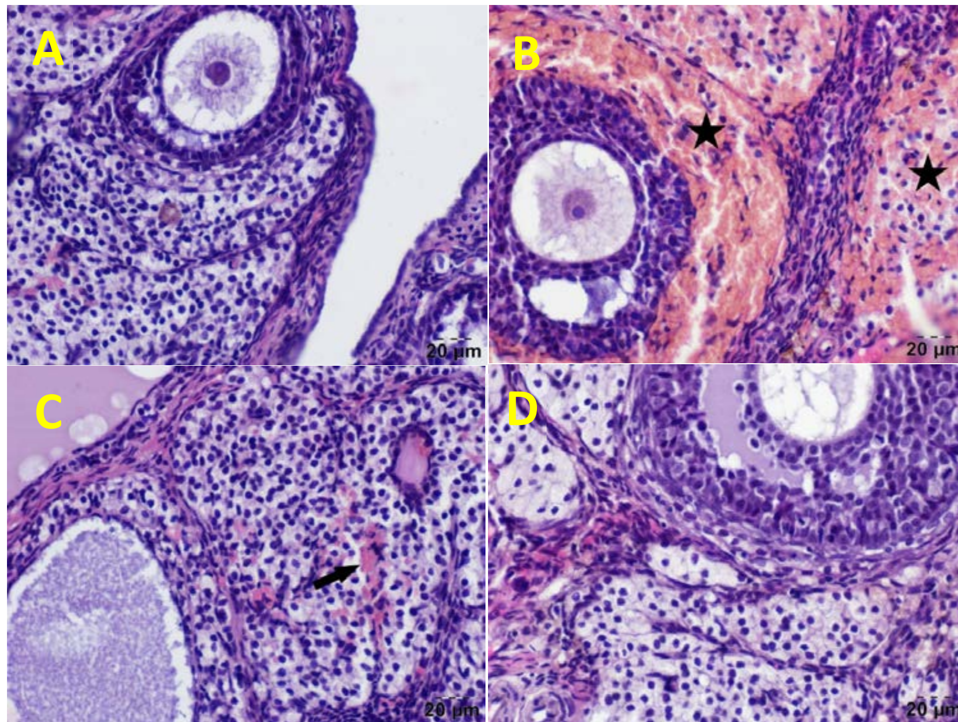

Fig. 4(A-D): Histopathological appearance of the ovary (scale bar = 20 µm), Representative light microscopic image of HE stained ovarian tissue sections. (A) Control group (B) IR group ovarian tissue: severe bleeding and necrosis are observed in the areas marked with asterisks, (C) IR+EGCG group ovarian tissue: slight bleeding is observed in the area indicated by the thick arrow and (D) EGCG group

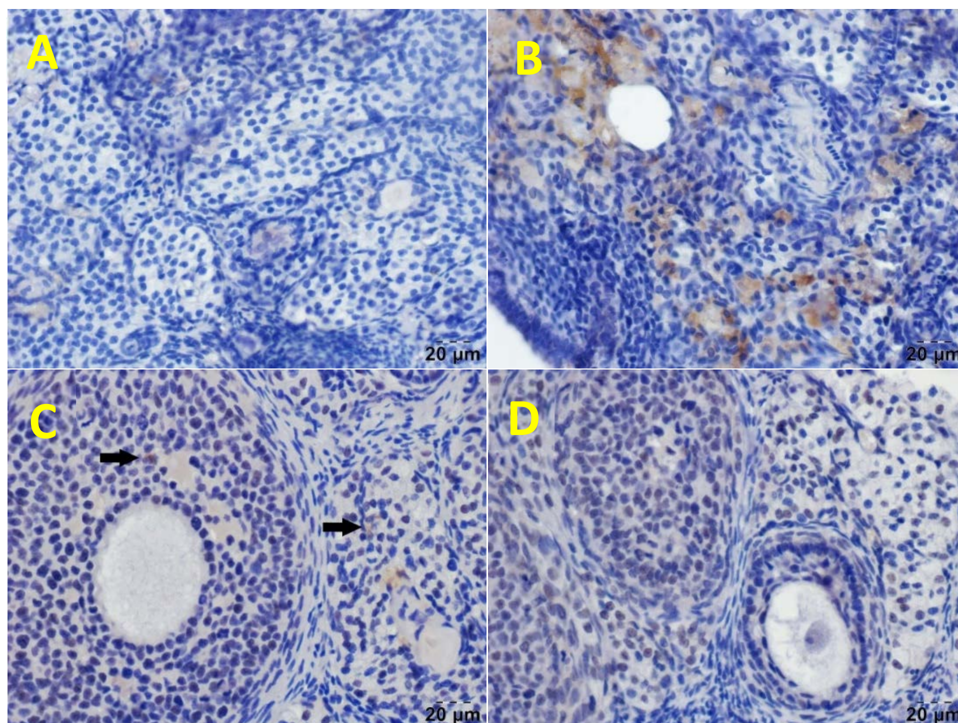

Fig. 5(A-D): Image of sections of ovarian tissue stained with TNF-α (scale bar = 20 µm), (A) Normal ovarian tissue is observed in the control group, (B) Severe TNF-α immunoreactivity in the ovarian tissue of the IR group, (C) Mild TNF-α positivity in the ovarian tissue of the IR+EGCG group (thick arrows) and (D) ECGG group ovarian tissue (AEC, DAB chromogen)

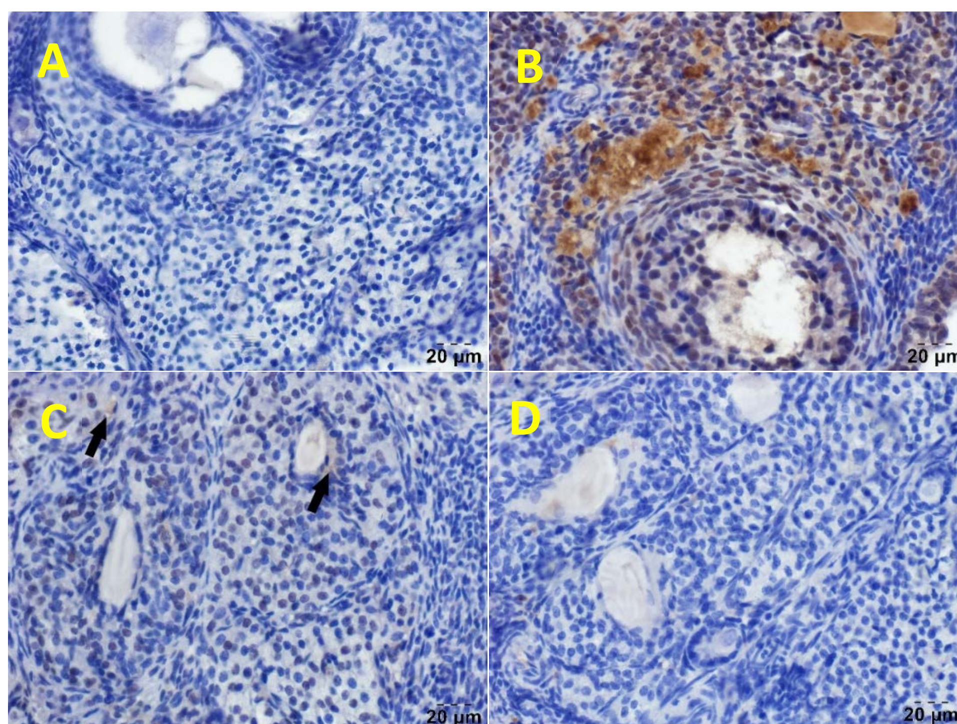

Fig. 6(A-D): Microscopic image of sections of ovarian tissue stained with caspase 3 (scale bar = 20 µm), (A) Control group ovarian tissue: Normal, (B) IR group ovarian tissue: Severe Caspase 3 immunoreactivity is observed, (C) IR+EGCG group ovarian tissue: Mild Caspase 3 immunopositive cells (thick arrows) are observed and (D) EGCG group ovarian tissue: immunonegative ovarian tissue is observed (AEC, DAB chromogen)

Table 2: Ovarian immunohistochemical scoring in experimental groups

Groups	n	Caspase 3	TNF alpha
Control	8	-	-
IR	8	+++	+++
IR+EGCG	8	+	+
EGCG	8	-	-

IR: Ischemia-reperfusion, IR+EGCG: Ischemia-reperfusion+EGCG

determined to be weak in the IR+EGCG treatment group (Table 2, Fig. 5c). In the group given only EGCG, TNF-α was immunonegative in the ovarian tissue (Table 2, Fig. 5d).

Caspase 3 stained slides were shown in Fig. 6. This enzyme was found to be immunonegative in the ovarian tissue of the rats in the control group (Table 2, Fig. 6a). Caspase 3 was observed to be severely immunopositive in the ovarian tissue of the IR group (Table 2, Fig. 6b). Weak immunoreactivity was detected in the IR+EGCG group (Table 2, Fig. 6c). Ovaries were immunonegative in the group given EGCG (Table 2, Fig. 6d).

DISCUSSION

Adnexal torsion is a pathological condition that causes ischemia in the ovary and occurs when the ligaments and vessels of the organ rotate around their own axis. Its incidence is 2.7% among acute abdominal pain cases[26]. This problem causes ischemia damage in the ovary[9]. Until it is diagnosed and treated, hypoxia occurs in the ovarian tissue. With treatment, the organ can be restored to its former position. However, this time, with the onset of reperfusion, the oxygen coming to the ovary causes the formation of reactive oxygen species in the tissue[27]. Additionally, the arrival of PMN cells to the scene causes inflammation. Thus, more damage occurs compared to ischemia[27,29]. As a result, IR damage can lead to organ necrosis depending on the ischemia duration[26]. Therefore, new strategies are needed to prevent tissue death. For this purpose, researchers are investigating the use of antioxidant molecules in the treatment of IR damage[19,21,23]. At this point, the substance EGCG attracts attention[20].

EGCG is an antioxidant found abundantly in green tea. It is used to reduce oxidative stress in experimental protocols[22]. The effects of EGCG, especially on IR injury, are being investigated in detail. The effects of EGCG were examined by creating IR models in many organs such as brain, skeletal muscle, heart, intestine, liver, kidney, retina, pancreas, umbilical cord and testis. Application of this antioxidant in IR prevents damage to cells and tissues and has healing effects. This catechin reduces oxidative stress and increases antioxidant capacity. It protects

against neutrophil infiltration and prevents inflammation. EGCG also showed beneficial effects through antiapoptotic and antifibrotic mechanisms[23]. IR damage produces various histopathological effects in tissues and organs[28]. When the ovarian tissue of rats is injured in this way; Significant disorders are observed in severe degrees, including severe hemorrhage, congestion, neutrophil infiltration, edema, follicular cell damage, loss of cohesion, degeneration and necrosis[29,30]. In this study, severe degenerative changes were detected in the ovarian tissues of the IR group (Fig. 4b). These findings are harmony with previous studies[29,30]. In the IR+EGCG group (Fig. 4c), EGCG treatment improved pathological conditions such as edema, dilatation, congestion, hemorrhage, neutrophil infiltration, degeneration and necrosis. These effects of EGCG are similar to previous studies[31]. These results suggest that EGCG treatment ameliorates IR-induced inflammation.

There are many antioxidant substances in cells and tissues. Different methods can be used to determine the amount of these endogenous antioxidants. In this study, we investigated the TAS level, which is a method that evaluates all of these[32]. In our study, TAS level was highest in the EGCG group (Fig. 1). This finding shows that EGCG is an antioxidant substance. TAS values of the control and IR groups were no different from each other and the lowest values were in these two groups. Compared with the IR group, the TAS value of the 3rd group was significantly higher. This result shows that antioxidant capacity increased when EGCG was administered to the animals in the third group after ischemia. Our findings are parallel to previous studies[33]. As a conclusion, EGCG strengthens the antioxidant defense system and reduces oxidative stress.

Oxidative stress is a condition in which reactive oxygen species (ROS) increase in the body and antioxidants cannot cope with ROS[34]. To detect this situation, the amount of many enzymes and radicals in the body, such as MDA, MPO, COX, peroxynitrite, nitrate, nitrite, are investigated[35-38]. TOS provides the opportunity to look at all of these at once[32]. For this reason, we investigated TOS values as an oxidative stress indicator in our study (Fig. 2). In this study, there was no significant difference between the control and IR groups in terms of TOS level ($p>0.05$). However, TOS levels were significantly higher in the 3rd group (IR+ EGCG) compared to the IR group ($p<0.05$). In previous studies, it is known that antioxidant substances reduce the TOS level occurring in ovarian IR damage[39]. However, in our study, TOS level was decreased significantly in IR group compared to the IR+EGCG group. This situation may have occurred due to strong activation of endogenous antioxidant mechanisms in animals with IR. But, it would be useful to clarify these effects of EGCG with new studies. Oxidative stress can also be determined by dividing TOS by TAS[40]. When we calculated the OSI with this method, we found that our data was more meaningful (Fig. 3). Compared with the control group, the IR group had significantly higher OSI values. When compared with the rats in the third group receiving EGCG treatment, it was determined that the OSI values of the rats with IR in the second group were higher, although not significant.

IR injury causes the inflammation in the ovarian tissue. The reason for this is that ischemia is treated and reperfusion is achieved. Thus, plenty of proinflammatory cells come to the scene from the bloodstream[41]. Cytokines are subsequently secreted by both tissue and inflammatory cells. These molecules are specific for inflammation and are used as markers of inflammation. In previous studies, we see that molecules such as Interleukin-1beta, Interleukin-6 and TNF-α are used as indicators in the detection of inflammation[23,42,43]. In this study, sections obtained from the ovaries were stained immunohistochemically with TNF-α stain. In the control and EGCG groups, the ovaries were immunonegative (Fig. 5a,d). In the IR group, severe TNF-α immunoreactivity was observed (Fig. 5b). With EGCG application, TNF-α reactivity caused by IR decreased and moderate immunopositivity was detected in the ovaries. Our findings are consistent with previous studies[42,43]. In this study, IR damage caused inflammation in the ovary, while EGCG treatment reduced inflammation in the tissue (Fig. 5c). In this case, it can be interpreted that EGCG exerts its effect through anti-inflammatory pathways.

The programmed cell death is called apoptosis[44]. Physiologically, it occurs in some tissues and organs in the body. However, it may also occur as a result of pathological disorders. It is known that tissue apoptosis occurs in ovarian IR damage[45]. Many enzymes and molecules play a role in the occurrence of the event. Caspase enzymes are the main mediators. Especially Caspase 3 enzyme level is used to detect apoptotic events[46]. In our study, we investigated Caspase-3 immunoreactivity to detect IR damage. No reactivity was detected in the ovaries of the control and EGCG groups (Fig. 6a,d). Severe immunopositivity was observed in the ovarian tissues of the IR group (Fig. 6b). Caspase-3 immunoreactivity decreased in IR rats receiving EGCG treatment (Fig. 6c). This result shows that EGCG prevents apoptosis. This finding is compatible with previous studies[27,47]. Ultimately, EGCG treatment protected the ovary from damage.

As a result, this study showed that EGCG has a protective effect against IR injury in ovaries of rats. EGCG treatment reduced the ovarian damage

following IR. Also TNF-α was expressed less in injured ovaries treated with EGCG. This finding indicates that this catechin protects ovarian tissue against inflammation. Moreover the decrease in Caspase 3 immunoreactivity depicts that it also prevents apoptosis. We concluded that EGCG could be used against ovarian IR injury in female rats.

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