INTRODUCTION

Ovarian torsion (OT) is the primary and most common reason for the pathological condition leading to ischemia reperfusion (I/R) in the ovaries. The situations such as ovarian cysts, pregnancy, polycystic ovary syndrome are classified among the causes of OT. 1, 2 The inability of the tissue feeding due to the restriction of the tissue blood flow is called ischemia, and reperfusion is the condition of restoring the blood flow. The reperfusion phase damages the tissues more than ischemia 3. The physiopathology of the ovarian damage has not been fully clarified yet 4. Besides, it is possible that oxygen-derived radicals are responsible for the I/R injury 3. Early and rapid intervention to ischemia may prevent ovarian injury and infertility. Nowadays, physiopathology of I/R injury and the different treatment methods are being improved and researched as experimental 5-7.

Hesperidin (HES), a flavonoid, is found in various fruits such as lemon and orange 8. It has several biological properties such as anti-allergic, anti-carcinogenic, anti-inflammatory, and strong antioxidant effects. One of the basic properties of HES is scavenging the radicals. Thus, HES applied cells indicated less oxygen-derived radicals and developed the antioxidant system 9, 11. Different agents with anti-inflammatory, antioxidant, and radical scavenging features performed beneficial effects in the alleviation or elimination of I/R injuries 5, 6. It was planned to find out the potential protective effects of HES, an antioxidant, autophagic and ant apoptotic agent against ovarian damage induced by I/R.

MATERIAL and METHODS

Abstract; In the current study, it was examined the possible protective properties of hesperidin (HES), an antioxidant, antiautophagic, and antiapoptotic molecule, against ovarian injury caused by ischemia reperfusion. 24 healthy rats were grouped as the group I (sham), group II (ischemia reperfusion (I/R)) and group III (I/R+HES) (n=8). In the sham group, the only abdominal incision was performed and closed. In the I/R group, following the incision, the I/R model was carried out. In the I/R+HES group, HES was administered intraperitoneally to the animals at the dose of 100 mg/kg approximately 1 hour before reperfusion. All the experimental procedures were performed under anesthesia. Following reperfusion, ovarian tissues were removed, and then some antioxidant, oxidant, autophagic and apoptotic parameters were evaluated. The oxidant parameters elevated, and antioxidant parameters declined in the I/R group. On the other side, antioxidant parameters were raised and oxidant parameters diminished in the I/R+HES group. Besides, it was observed that Light chain 3 (LC3) B, caspase-3, and Nuclear factor-kappa Bp50 (NF-kBp50) immunopositivity were fairly intensive in the I/R group while it was less in the I/R+HES group. A single dose of HES is quite efficient against I/R-induced oxidative ovarian injury.
University Experimental Animals Research and Application Center (ATADEM). Wistar type female rats, weighing 220–230 g, acquired from ATADEM. Rats were housed in regular cages with laboratory mediums including 12 light/12 darkness, the temperature of 22±2°C and humidity of %55±5. They were supplied standard rat feed and tap water. All animals were deprived of food 12 hours prior to the experiment but were allowed to drink water.

**Groups and experimental design**

All rats were fixed in the supine position. The lower abdominal region was shaved and disinfected with povidone-iodine. All surgical procedures were performed under anesthesia. 60 mg/kg intraperitoneal (i.p.) ketamine (Ketalar®, Pfizer, Istanbul) and 10 mg/kg i.p. xylazine hydrochloride (Rompun®, Bayer, Istanbul) were preferred as anesthetic. HES was obtained from Sigma-Aldrich Co, USA. HES was prepared by dissolving in 0.5% sodium carboxymethylcellulose as described in a previous study. 24 female, Wistar-Albino rats were separated into 3 groups (n=8): sham group; 2 cm incision was performed in the midline of the lower abdominal region and then, the incision was repaired with 3/0 silk suture. I/R group; Following the incision, as described in previous studies, uterine horns and adnexa were fixed with a microvascular clamp for 3 hours (ischemic phase). After ischemia, 3 hours of reperfusion was applied by releasing the clamps. I/R+HES group; All procedures in group II was performed and in addition, 100 mg/kg i.p. HES was administered one hour prior to reperfusion stage. The dose of HES was adjusted according to a previous I/R study.

The ovaries were cleaned in cold saline and then kept at -80 °C for biochemical measurements. A part of the ovarian tissue samples was also stored in 10% formaldehyde for immunohistochemical assessments.

**Biochemical assessments**

Total antioxidant status (TAS) level was detected with the commercial kit (Rel Assay Diagnostics, Product Code: RL0017). Total oxidant status (TOS) value was gauged with an appropriate kit (Rel Assay Diagnostics, Product Code: RL024). 16, 17. TOS to TAS ratio represents the oxidative stress index (OSI). The evaluation of superoxide dismutase (SOD) depends on the production of superoxide radicals. 18 Lipid peroxidation in ovarian tissue was measured by assessing malondialdehyde (MDA) level. 19 Myeloperoxidase (MPO) activity in the ovarian tissue was determined through the methods described by Bradley et al. 20.

**Immunohistochemical assessments**

Upon completion of the experiment, the ovarian samples were taken into 10% neutral formalin solution. Then, they were washed in tap water and embedded in paraffin blocks following tissue processing procedures. Following the deparaffinization, they were kept in 3% H2O2 for 10 min to inactivate the endogenous peroxidase and then washed in phosphate-buffered saline (PBS). The tissues were heated for 10 min at 500W into an antigen retrieval solution (citrate buffer, pH 6.0) to retrieve antigens and washed again in PBS. Protein block solution was used to avoid nonspecific binding and washed in PBS. NF-kB (Abcam, Cat. No: ab7971, Dilution:1/150), caspase-3 (Novus Biological, Cat. No: NB600-1235, Dilution:1/100), and LC3B (Abcam, Cat. No: ab48394 Dilution:1/200) primary antibodies were used for 1 hour at room temperature to the sections. Finally, it was followed by the procedure described by the expose mouse and rabbit specific HRP/DAB detection IHC kit (abcam: ab80436). The 3,3'-diaminobenzidine chromogen was used and counterstained with hematoxylin. The intensity of immunopositivity was evaluated as no (-), mild (+), moderate (++) and severe (+++). 21

**Statistical analysis**

For the biochemical evaluation, all results were presented as Means±SD analyzed using One-way ANOVA. Tukey test was preferred for pairwise comparisons of the groups. The differences were accepted as significant when p<0.05. SPSS 16.0 program was preferred for statistical evaluation in immunohistochemistry results. The difference between groups was determined by the Kruskal Wallis test and Mann–Whitney U-test followed it. p<0.05 value was accepted significant statistically. Immunohistochemistry data were expressed as Means±SEM.

**RESULTS**

**Biochemical results**

TAS, TOS, OSI, SOD, MDA, and MPO data were presented (figure 1). TAS and OSI levels elevated in the I/R group when it was compared to the sham group. MDA level and MPO...
activity raised significantly in the I/R group compared to the sham group (p<0.05). HES treatment diminished TOS, MDA, OSI levels and MPO activity significantly in the I/R+HES group when it was compared to the I/R group (p<0.05). TAS value and SOD activity elevated significantly in the I/R+HES group compared to the I/R group (p<0.05).

**Figure 1.** Effect of HES on (a) TAS, (b) TOS, (c) OSI, (d) SOD, (e) MDA and (f) MPO levels in ovarian tissues. *p<0.05 compared to sham group, #p<0.05 compared to I/R group

**Immunohistochemical results**

NF-kBp50 immunopositivity was not found in the sham group (figure 2a). In the I/R group, intensive immunopositivity was observed in the interstitial area (figure 2b). In the I/R+HES group, there was mild immunopositivity in the interstitial tissue and luteal cells (figure 2c).

In the sham group, caspase-3 immunopositivity did not occur (figure 3a), but in the I/R group, intense caspase-3 immunopositivity was observed in the interstitial area and luteal cells (figure 3b). In the I/R+HES group, the severity of immunopositivity decreased in both areas (figure 3c).

LC3B immunopositivity was not found in the sham group (figure 4a). Conversely, in the I/R group, intense LC3B immunopositivity was detected in the interstitial area and luteal cells (figure 4b). In the I/R+HES group, there was mild immunopositivity in the luteal cells (figure 4c).

NF-kBp50, caspase 3, and LC3B positivity were immunohistochemically different between groups (p<0.05, table 1). There was a difference between the sham group and the other groups. A difference also occurred between the I/R and I/R+HES groups. (P<0.05, table 1).
Figure 2. A) Sham group B) I/R group, intensive NF-kB immunopositivity in interstitial region (arrow) C) I/R+HES group, mild NF-kB immunopositivity (arrow) in luteal cells

Figure 3: A) Sham group B) I/R group, in luteal cells (arrow) and interstitial area (star) intense caspase-3 immunopositivity C) I/R+HES group, mild caspase-3 immunopositivity in luteal cells (arrow) and interstitial area (arrowhead)

Figure 4. a) Sham group b) I/R group, intensive LC3B immunopositivity in luteal cells (arrow) and interstitial area (arrowheads) c) I/R+HES group, mild LC3B (arrow) immunopositivity in luteal cells

Table 1. NF-kB, Caspase-3, and LC3B immunopositivity among different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>NF-kB</th>
<th>caspase-3</th>
<th>LC3B</th>
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<tbody>
<tr>
<td>Sham</td>
<td>0.25±0.16</td>
<td>0.25±0.25</td>
<td>0.50±0.26</td>
</tr>
<tr>
<td>I/R</td>
<td>2.75±0.16</td>
<td>2.62±0.18</td>
<td>2.87±0.12</td>
</tr>
<tr>
<td>I/R+HES</td>
<td>1.50±0.42</td>
<td>1.75±0.25</td>
<td>1.37±0.18</td>
</tr>
</tbody>
</table>

Data were expressed as means±SEM. Groups with different letters (a,b,c) in columns are significantly different from each other (p<0.05). Sham group performed significant difference compared to I/R and I/R+HES groups (a,b and a,c, p<0.05). On the other side, a difference also occurred between the I/R and I/R+HES groups (b,c, P<0.05).
DISCUSSION

Ischemia is defined as the loss of oxygen and lack of nutrition as a result of mechanical reasons or clot. Adenine triphosphate (ATP) production ceases in cells due to hypoxic genesis resulting from ischemia. Only the current ATP continues to be used, and the adenosine breaks down. Adenosine diffuses out of the cell, the formation of inosine and hypoxanthine is shaped 22, 23. In oxygen deficiency, xanthine dehydrogenase enzyme cannot be activated, so hypoxanthine is metabolized by xanthine oxidase, and xanthine occurs. Free oxygen radicals are released as a result of these reactions 24, 25. The recovery of blood flow is called reperfusion. The damage after the reperfusion is a pathophysiological process that has not yet been fully understood. In this process as a result of the various mechanisms such as the increase of nitric oxide synthase levels, free oxygen radicals, lipid peroxidation, activation of leukocytes and cellular signal pathways, elevation in intracellular Ca$^{2+}$ ion level, impairment of Na$^+$-K$^+$ pump in the membrane, activation of protein kinase C (PKC) and mitogenic activating protein kinase may cause pathologic conditions that result in necrosis or apoptosis in cells 26-29.

During I/R injury, free oxygen radicals occur and may lead to oxidative damage in various cellular biomolecules containing proteins, lipids, and DNA 30. SOD and CAT, other similar antioxidant enzymes, convert the superoxide radicals to hydrogen peroxide and scavenger lipid peroxides 31, 32. Derogating the activities of SOD has been connected with free oxygen-mediated pathologies 33. Different agents with various features, including anti-inflammatory, antioxidant, and radical scavenging properties, have been found beneficial in the alleviation or elimination of I/R injuries 3, 5-7. HES has vasodilating activities, antioxidant, anti-inflammatory, anticarcinogenic, and antimicrobial properties 10, 34. Numerous studies have been conducted to reduce oxidative stress by HES administration in different experimental models 9, 35, 36. It has been shown by Banji et al. that HES performed protective effects against D-galactose-induced apoptosis in rat brain by decreasing the MDA level and increasing the antioxidant activities 37. HES performed hepato-amlerorative effect in rats intoxicated by the mercuric chloride 38. In addition, HES protected the liver against valproate-caused toxicity 39. In another study, it was determined by Iskender et al. that HES demonstrated protective effects against oxidative injury in STZ-induced diabetic model 40. In a previous ovarian I/R study, HES was effective in alleviating ovarian I/R injury 41.

One of the experimental ovarian I/R studies showed that lipid peroxidation significantly increased, but the antioxidant defense system was inadequate 42. In another study about I/R injury in ovaries, lipid peroxidation increased dramatically due to I/R, whereas antioxidant enzyme level decreased 43. TOS and OSI values elevated, and TAS level declined in the I/R group in a previous ovarian I/R study 44. MPO activity is an essential marker for the neutrophil infiltration in tissues 45. In an ovarian I/R injury study, it was detected an increase in MPO activity and a significant decrease in SOD antioxidant enzyme activity 46. Consistent with all these literature studies, in this study, MDA level, MPO activity, TOS, and OSI values increased in the I/R group. In the HES treatment group, these parameters returned to normal levels.

Autophagy is a physiological process in living organisms. Uncontrolled autophagy is initiated in various situations, including chemotherapeutics, reactive oxygen species (ROS), hypoxia, starvation, intracellular pathogens, growth factor deprivation, and DNA damage 47. Hitherto, microtubule-related protein 1 LC3 has been detected on the autophagosomal inner membrane. LC3B immunoblotting is a method and commonly used for the determination of autophagic activity 48. Programmed cell death or apoptosis is a physiologic process that is known as a staminal ingredient of many biologic procedures of the organism 49, 50. NF-kBp50 is a nuclear transcription factor and takes a role in differentiation, tumorigenesis, neurodegeneration, immunity, apoptosis, inflammation, and cell growth 51. ROS formation induces the activation of NF-kBp50. Previous studies demonstrated a strong correlation between autophagic response and NF-kBp50transcription 52, 53. Here, the immunopositivity of LC3B, NF-kBp50, and caspase-3 were determined intensively in the I/R group when it was compared to sham group. But all the immunopositivity were less observed in the I/R+HES group compared to the I/R group.

In the current study, ovarian I/R injury led to dramatic increases of MDA level, MPO activity, TOS, and OSI values while it causes a decrease in SOD activity and TAS value. HES treatment effectuated in the positive direction the changes of MDA, TOS, OSI, and MPO, stimulated an overproduction of enzymatic antioxidant SOD activity, and increased TAS value. Thereby, a single dose of HES significantly derogated ovarian tissue injury.
**Conflict of interest**

The authors declare that they have no conflict of interest.

**REFERENCES**


