Thymoquinone Effect (an Active Metabolite of the Plant *Nigella sativa*) on Ischemia-Reperfusion Injury in Rat Ovary

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Introduction

Ovarian torsion is an emergent gynecological consultation, with a prevalence of 2.5% to 7.4% [1]. It is mostly seen in the population of young women of reproductive age, and if a quick diagnosis is not achieved, the ovary may be lost, which carries an importance related with the likely future fertility problems [2]. Excluding the irreversibly damaged and necrotic ovaries, the suggested treatment route in young, fertile women is the laparoscopic detorsion and evaluating the tissue reperfusion [3].

During reperfusion following the ischemic state, hypoxic and/or necrotic cells exposed to the new oxygen (O₂) supply produce radical oxygen species (ROS) which causes injury to the post-ischemic cells and tissues [4]. Reperfusion creates the oxidative stress that refers to an imbalance between the production and manifestation of ROS and antioxidants.

A balance is needed between ROS and the antioxidants in order to establish a healthy status for the intracellular and intercellular interactions. The mechanism in the cellular damage is mainly based on reacting with and disturbing the intracellular components. The most important of these are the lipids, proteins and nucleic acids. Reaction with the lipids in the cell membrane causes lipid peroxidation and disturbs the membrane integrity, permeability and ion transport. Free radicals act on the lysosome membrane, and disturbed organ lets the various oxidative enzymes free into the cytoplasm, at which point the main cellular destruction starts [5]. Malondealdehyde (MDA), a stable metabolite of the ROS-mediated lipid peroxidation cascade, is a highly toxic molecule and significantly increases in ischemia/reperfusion (I/R).

Injury, suggesting damaged cell wall integrity and permeability. Moreover, it can exacerbate I/R injury through its ability to react with DNA and proteins [6].

Abstract

Objectives: To determine the antioxidant effects of thymoquinone (one of the active components of the plant *Nigella sativa*) on ischemia-reperfusion injury in rat ovary.

Study design: Twenty four rats were divided into four groups: control (n=6), ischemia (n=6), ischemia (n=6), ischemia-reperfusion+thymoquinone (n=6). Ovarian torsion was surgically reperfusion+saline induced by vascular clamps. The ovary was clamped for 3 hours and 30 minutes before reperfusion 10 mg/kg thymoquinone was injected intraperitoneally. Following three hours of reperfusion, right oophorectomy was done and the rats were sacrificed.

Main outcome measures: Histological disturbances of the ovarian tissues were scored and tissue levels of catalase (CAT), malondealdehyde (MDA) and xanthine oxidase (XO) were measured.

Results: In the thymoquinone treated group, tissue CAT levels were significantly higher (p=0.02) and MDA levels were significantly lower (p=0.042). XO levels were similar among the groups. Histological examinations of thymoquinone group revealed a better preserved ovarian tissue compared to the control, ischemic and the saline treated ischemia-reperfusion groups.

Conclusion: Thymoquinone at dose of 10 mg has a potent antioxidant and tissue preserving effect against the ischemia-reperfusion injury in the rat ovary prone to torsion/detorsion.

Keywords: Ischemia/Reperfusion; Thymoquinone; Ovarian torsion; MDA
Another member of the oxidative stress is the enzyme xanthine oxidase (XO). Under some circumstances, it produces superoxide ion (O$_2^-$ and H$_2$O$_2$), which has a significant oxidative potential that disturbs the cell integrity [7].

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes that are the only chemicals having compensatory action on the ROS load, and their activity may prevent cell damage. Some of the enzymatic antioxidants are superoxide dismutase, catalase (CAT) and peroxiredoxins whereas non-enzymatic forms are also present like vitamin C, vitamin E, N-acetylcysteine and melatonin [8].

Prevention or minimization of the reperfusion injury in the ischemic ovaries is the most important point in the function of the post-ischemic tissue and the future fertility [9]. Few reports exist concerning ovarian reperfusion injury and its prevention. Disulfiram, vitamin E, pentoxifylline, melatonin, tadalafil, erythropoetin, dimethyl sulfoxide, atorvastatin, genistein, L-carnitine, N-acetyl cysteine, famotidine, vitamin C were found to have beneficial effects [8,10-17].

Thymoquinone (TQ, 2-isopropyl-5-methyl-1,4-benzoquinone) is one of the active components of the plant *Nigella sativa* Linn. (Ranunculaceae), which is also known as black seed or black cumin. This plant is a traditional drug used for medical purposes [18]. This herbal medication has been reported to have immune stimulatory, anti-inflammatory, hypoglycemic, antihypertensive, antiasthmatic, antimicrobial, antiparastic, antioxidant, and anticancer effects [19-23]. The antioxidant effect of TQ has been shown on the I/R injury in various organs such as the brain, kidney, testis, and gastric mucosa [21,22,24-26].

Although several antioxidant enzymes and drugs have been used to decrease ovarian IR injury to date [10,11-13,16-18,27-29] only one study has investigated the protective effect of thymoquinone in ovarian IR injury [30]. In present study, we aimed to show the antioxidant and tissue protective effect of TQ at a lower dose of 10 mg in a rat model of ovarian I/R, which was investigated via histopathological examinations and analyses of tissue CAT, XO and MDA.

**Materials and Methods**

**Animals and experimental design**

In this study, we followed the recommendations of the Declaration of Helsinki for animal care and complied with the ”Principles of Laboratory Animal Care (National Institutes of Health [NIH] publication No. 86-23, revised 1985). The study was approved by the Gazi University Ethics Committee for Animals (Report no. B.30.2.GUN.0.05.06.00/137-12279).

A total of 24 Wistar-albino female rats were used in the present research. All of the rats were young and in cycling reproductive age, with weights ranging from 250-300 g. Animals were divided into four randomized groups as follows:

C (Control group; n=6); I (Ischemia group; n=6); I/R + saline (Ischemia/reperfusion + saline group; n=6); I/R + TQ (Ischemia/reperfusion + thymoquinone group; n=6).

The surgical procedures were performed while each rat was under anesthesia with ketamine (Eczacibasi, Istanbul, Turkey,50 mg/kg i.m.) and xylazine (9 mg/kg i.m.). Rats were placed in dorsal recumbent position, and presurgical sterilization procedures were done with skin preparation, cleaning and covering with sterile drapes. Laparotomy was performed with a mid-line 2.5 cm incision. Ovarian torsion was induced by vascular clamps, which were applied just below the right ovary in the junction area of the uterine horn [9]. At the end of the reperfusion period, ovaries were excised and samples were used for the biochemical and histological examinations. Rats were sacrificed by exsanguination through the abdominal aortic artery.

Rats in the control group were sham operated and oophorectomized (group I). In ischemia group, unilateral right ovary was clamped for 3 hours and then oophorectomy was performed (group II). In ischaemia/reperfusion+saline and ischaemia/reperfusion+thymoquinone groups (Group III and IV), right ovary was prone to 3 hour of ischemia and 1 ml saline and 10 mg/kg thymoquinone (MP Biomedicals, LLC, Illkirch, France, LOT No: 45521) that was diluted in 1 ml of saline were injected i.p., respectively 30 minutes before 3 hour of reperfusion (24). In all of the excised ovaries, a small part was fixed for histological examinations and the rest was freezeed at -80°C.

**Histopathology**

The ovarian tissue samples collected from all the groups were blindly sent to the laboratory. Tissues were fixed in 10% phosphate-buffered formalin and then embedded in paraffin. The paraffin block sections were 5 μm in thickness and routinely stained with hematoxylin and eosin (H & E). The microscopic examination of the sections with regard to the degenerative changes was also done blindly.

Tissue damage scoring was assessed in the histopathological evaluation. The parameters were the congestion, hemorrhage, edema, and perivascular lymphocyte infiltration. The severity of the histological changes were scored from 0 to +3 (0=no pathological finding; 1, 2 and 3 ≤ 33%, 33-66% and >66% affected ovary, respectively) [16].

**CAT activity determination**

The CAT activity was assessed according to the method of Aebi [31] and was evaluated in whole ovary homogenates. The principle of the method was based on the determination of the rate constant k (s$^{-1}$, k) of the H$_2$O$_2$ decomposition rate at 240 nm. Tissue samples were homogenized in ice-cold isotonic 0.9% sodium chloride solution and centrifuged for 5 min at 10,000 g at 4°C. The H$_2$O$_2$ added to the assessment medium is decomposed by the catalyzing activity of the CAT enzyme, and reduction in absorbance at 240 nm is monitored with a spectrophotometer. The reduction in absorbance is directly proportional to the enzyme activity. The results were expressed as IU/mg protein.
XO activity determination

The XO activity was assessed according to the method of Hashimoto [32]. The enzyme activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm. This method is based on the amount of uric acid produced by XO from the xanthine, added to the medium. Tissue homogenate was incubated (100 μL) for 24 h at 25°C in 2.80 mL of medium containing phosphate buffer (pH 7.5, 50 mM) and xanthine (0.067 mmol final concentration in each tube). The absorption at 293 nm of the resultant clear supernatant was measured against blank. The activity was expressed in units per gram protein (mIU/mg protein).

MDA determination

The tissue MDA levels were assessed according to the method of Dahle [33]. This method is based on the reactivity with thiobarbituric acid (TBA) at 90-100°C. The sample was mixed with 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of TBA in a boiling water bath for 30 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nmol/mg protein.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences software (SPSS, version 15.0). The data were presented as median (minimum-maximum) values. The MDA, XO and CAT levels were compared with Kruskal-Wallis test, and Bonferroni-corrected Mann-Whitney U test was used for post-hoc analysis when Kruskal-Wallis test revealed a statistical significance between the four groups. A p value <0.05 was considered statistically significant.

Table 1: P values of the tissue damage scores between the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I-II</td>
<td>0.005</td>
</tr>
<tr>
<td>Group I-III</td>
<td>0.003</td>
</tr>
<tr>
<td>Group I-IV</td>
<td>0.075</td>
</tr>
<tr>
<td>Group II-III</td>
<td>0.212</td>
</tr>
<tr>
<td>Group II-IV</td>
<td>0.022</td>
</tr>
<tr>
<td>Group III-IV</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Results

Histopathologic examination

Total tissue damage scores are shown in Figure 1. The difference between the four groups were statistically significant; p=0.001. Tissue damage was significantly higher in the ischemia and the ischemia/reperfusion+saline groups when compared with the control group; p=0.05 and p=0.03, respectively (Table 1).

Figure 1: Tissue damage scores of the ovaries in the control (Group I), ischemia (GroupII), ischemia/reperfusion+saline (Group III) and ischemia/reperfusion +Thymoquinone (Group VI) groups.

Histologic examination of the ovarian sections revealed the followings:

Group I, control group: The results showed normal-in-appearance ovaries (Figure 2).

Group II, ischemia group: Moderate congestion, hemorrhage and perivascular lymphocyte infiltration were observed (Figure 2).

Group III, ischemia/reperfusion group+saline: Severe hemorrhage together with severe edema and perivascular lymphocyte infiltration were observed (Figure 2). Developing follicles were disrupted together with granulosa cell desquamations. Vascularization in the ovarian medulla was increased.
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Histological appearances of the ovaries of Group I/R+TQ were close to the control group. XO levels of the groups I, II, III and IV were 1.67 (1.43-2.46); 1.36 (0.59-2.92); 2.70 (1.54-3.30) and 1.99 (1.20-2.35), respectively and the difference was insignificant (p=0.165). CAT levels were significantly higher in group IV compared to groups II and III; 993.04 (755.36-1234.79) vs. 365.23 (157.35-470.23) and 291.28 (206.93-767.83), respectively (p<0.02) and MDA levels were significantly lower in group IV compared to groups II and III 30.62 (15.87-149.41) vs. 84.04 (5.07-165.75) and 84.04 (5.07-165.75), respectively (p=0.042).

Discussion

The antioxidant effect of TQ was demonstrated in various studies. In a hypercholesterolemic rat model, TQ increased the antioxidant enzyme glutathione peroxidase and decreased the levels of thiobarbituric acid reactive substances. These effects counteracted the formation of atherosclerosis [34]. In another study TQ-administration reduced tissue MDA levels in the ischemic rat brain and prevented lipid peroxidation and cell death. The antioxidant effect of TQ was examined in an in vitro study and microsomal lipid peroxidation was inhibited via the potent superoxide anion scavenger action of TQ [19]. Doxorubicin induced cardiotoxicity was also prevented by TQ action via inhibiting lipid peroxidation and scavanging superoxide radicals.

In this present study, TQ significantly reduced the histological damage and the oxidative stress in the rat ovaries following I/R. Related to the histological damage after I/R injury, TQ-administered ovarian tissues were close to the control ovaries. Minimal hemorrhage, lesser edema and minimal perivascular inflammatory cells demonstrated the tissue protective effect of TQ.

Related with the antioxidant action of TQ, tissue MDA levels were significantly lower in TQ-administered rat ovaries. Since MDA is an oxidant chemical resulting in cell membrane lipolysis leading to cell death, decreased MDA levels due to TQ effect protected the ovarian cells from membrane damage. XO being one of the enzymes producing toxic oxidative metabolites was decreased in the TQ-administered ovaries even if it was not significant.

With respect to the antioxidant enzyme CAT, increased levels in the I/R ovaries showed that TQ is fairly effective in stabilizing the antioxidant levels in the protection of tissues from oxidative stress during reperfusion. The enzyme CAT was thought to be decreased because of its consumption along the I/R pathways, but detection of still higher levels even after these chemical pathways of the reperfusion demonstrated how effective TQ was as an antioxidant.

A recent study by Ural et al. has evaluated the antioxidant effects of TQ at doses of 20 and 40 mg and investigated the biochemical, histopathological, and immuno-histochemical changes in ovarian torsion model [30]. Similar to our results, they showed that TQ reduced ischemic and I/R-induced injury by increasing CAT level and by decreasing the MDA and reduced the tissue damage.

Table 2 shows the comparison of CAT, XO and MDA levels of the ovaries between the groups. XO levels of the groups I, II, III and IV were 1.67 (1.43-2.46); 1.36 (0.59-2.92); 2.70 (1.54-3.30) and 1.99 (1.20-2.35), respectively and the difference was insignificant (p=0.165). CAT levels were significantly higher in group IV compared to groups II and III; 993.04 (755.36-1234.79) vs. 365.23 (157.35-470.23) and 291.28 (206.93-767.83), respectively (p<0.02) and MDA levels were significantly lower in group IV compared to groups II and III 30.62 (15.87-149.41) vs. 84.04 (5.07-165.75) and 84.04 (5.07-165.75), respectively (p=0.042).

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In conclusion, the results of the biochemical and histopathological parameters in our study suggest that administration of TQ even at the dose of 10 mg has beneficial effects in the prevention of I/R injury in the rat ovary prone to torsion/detorsion. Even in this smaller dose, TQ administration has a potent antioxidant action.

References
