Antioxidant and antiapoptotic effects of erdosteine in a rat model of ovarian ischemia-reperfusion injury

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Objective(s): To evaluate the protective effect of erdosteine, an antiapoptotic and antioxidant agent, on reperfusion injury in experimental ovarian ischemia-reperfusion (IR) injury.

Materials and Methods: Eighteen female Wistar albino rats were used in control, IR, and IR+Edosteine (IR-E) groups, (n=6 in each). The IR-E group received the erdosteine for seven days before the induction of torsion/retorsion, (10 mg/kg/days). The IR and IR-E groups were exposed to right unilateral adnexal torsion for 3 h. Three hours later, re-laparotomy was performed, and the right ovaries were surgically excised. Oxidant and antioxidants levels were determined in serum. The ovarian tissue samples were received and fixed with 10% neutral buffered formalin. The sections were stained with H&E, anti-PCNA, and TUNEL.

Results: The IR group showed severe acute inflammation, polymorphonuclear leukocytes and macrophages, stromal oedema and haemorrhage. Treatment with erdosteine in rats significantly retained degenerative changes in the ovary PCNA (+) cell numbers were significantly increased in the IR and IR-E groups unlike the control group. However, its numbers were significantly increased in the IR-E group unlike the IR group. TUNEL (+) cell numbers were significantly increased in the IR group unlike the control and the IR-E groups. In erdosteine treated group, TUNEL (+) cells were detected significantly less than the IR group (P<0.05).

Conclusion: In conclusion, erdosteine maybe a protective agent for ovarian damage and decreasing lipid peroxidation products and leukocytes aggregation after adnexal torsion in animals.

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Introduction

Adnexal torsion is an infrequent gynaecologic emergency with a prevalence of 2.7%, which denotes the bending of the ovary and fallopian tube around the broad ligament. Because 70% of the cases are women of reproductive age, the early diagnosis and treatment is necessary for the preservation of the affected ovary, and hence that of fertility (1).

Symptoms are nonspecific, and the diagnosis is not always made until the surgical exploration of the adnexa. It is not always possible to determine the extent of necrosis intraoperatively, and subsequent pregnancies are possible even in patients with morphologically necrotic appearing ovaries; so, conservative surgical management with detorsion of the pedicle is preferred over adnexectomy (2).

Adnexal torsion related pathology can be divided into two phases: a) The time elapsed until the detorsion operation, as the ischemia period, b) The creation of reactive oxygen species (ROS) after detorsion, as the reperfusion period (3).

While exhaustion of cellular energy reservoirs leads to dysfunction of Na+/K+-ATP pumps in the cell membranes and results in swelling of the cell during ischemia period, increased production of hydroxyl free radicals and hydrogen peroxide by conversion of hypoxanthine to xanthine, through xanthine oxidase enzyme, occurs only after adequate oxygenation by reperfusion of the ischemic tissue. Thus, reperfusion followed by ischemia causes more severe tissue damage. Ovarian torsion followed by detorsion leads to biochemical and histological changes in ovarian tissue. Hypoxia by weakening oxidative phosphorylation incites catabolism of ATP with accumulation of ADP (4).
In healthy conditions, ROS levels are retained under control by enzymes such as catalase (CAT) and glutathione peroxidase (GPX) (5). However, in case of oxidative stress, it is likely that uncontrolled production of ROS damages the cell structure and several biomolecules such as protein, DNA, RNA, and lipids (6).

Current research interest is directed to the agent that possibly prevents the ischemia-reperfusion (IR) related cellular damage by antioxidant effects. Erdosteine [S-(2-(N-3-(2-oxo-tetrahydrothienyl) acetamido) thioglycolic acid], originally a mucolytic drug, contains two sulphhydryl groups which are released after hepatic first-pass metabolism to be active and to act as free oxygen radical scavengers that these groups calculus for the antioxidant activity of erdosteine (7).

The aim of this experimental study is to investigate the protective effect of erdosteine on rat ovarian IR injury model, by determining the levels of antioxidant enzymes (CAT and glutathione (GSH)), antioxidant vitamins (ascorbic acid, β-carotene, retinol, and α-tocopherol), lipid peroxidation products (malondialdehyde (MDA)) in serum, and changes in the numbers of apoptotic and proliferative cells.

Materials and Methods

Animals

In the current study, we used a total of 18 female Wistar albino rats, which were 9–10 weeks old and 200–230 g. The experiment was conducted in the Experimental Research Center of Trakya University. The animals were preserved under specific pathogen free and optimum laboratory conditions. All animals were fed a standard laboratory diet and had access to tap water ad libitum. All animals were treated humanely and in compliance with the recommendations of the Animal Ethical Committee of Trakya University (Permission No: TUHDYEK-2014/37).

Animals were randomly separated into three groups: the control (sham operation, n=6), the IR (torsion-retorsion, n=6), and the IR-E (torsion/retorsion plus erdosteine, n=6). The IR-E group was treated with erdosteine for seven days (orally, 10 mg/kg/days) before the induction of torsion/retorsion.

The rats were anesthetized with 75 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey), and IP injection of xylazine hydrochloride (10 mg/kg, Rompun, Bayer, Leverkusen, Germany).

Rats were placed in a dorsal recumbent position, the incision area was cleaned and dressed. A 2.5 cm midline incision was performed for laparotomy, the uterine horns and adnexa were located in the control group. Rats, except the control group, were exposed to right unilateral adnexal torsion for 3 hr. The bended adnexa were fixed to abdominal muscles by a 3/0 silk suture in the IR and the IR-E groups. The skin was sutured with 5/0 silk. Through this procedure, the right ovary, its vessels, and the right cornu of the uterus were rotated by 360 ° in a clockwise direction. Three hours later, re-laparotomy was performed, and the right ovaries were surgically excised. Subsequently, the rats were scarified by a high dose of anaesthetic (8).

Chemicals

Methylene blue, hydrogen peroxide, reduced glutathione, thiobarbituric acid, phosphate buffer, butylated hydroxytoluene, trichloroacetic acid, 5,5-dithiobis-(2-nitrobenzoic acid) [EDTA], disodium hydrogen phosphate, hexane, ethanol, phenylendiamine, sodium nitrite, ethylenediamine dihydrochloride, and sodium nitrate were purchased from Sigma Aldrich (MA, USA). The chemicals and reagents used in this study were of analytical grade. Ultra-distilled water was used as the solvent.

Biochemical analyses

Fasting blood samples were drawn into heparinized and heparin-free tubes during routine blood sampling for biochemical analyses. After immediate centrifugation (4000 g for 7 min at +4 °C), the serum was stored in a polystyrene plastic tube at −80 °C, until the time of analysis. The red blood cells were washed with isotonic saline (0.89% NaCl), and further processed for the preparation of hemolysate. Whole blood was collected into heparinized tubes and whole blood MDA and GSH levels were studied on the same day of admission.

Whole blood MDA levels were determined using the method described by Jain et al (9) and based on the thiobarbituric acid reactivity. The optical density was measured at 532–600 nm in a spectrophotometer (Jenway 6305 UV/VIS). The results were calculated by the absorbance coefficient of this complex as nmol/ml. Blood GSH concentration was measured using the method described by Beutler et al (10). The results were expressed in mg/dl.

Ascorbic acid (vitamin C) serum concentration was measured by spectrophotometric method (12). The levels of β-carotene at 425 nm and retinol (vitamin A) at 325 nm were detected after the reaction of serum:ethanol:hexane at the ratio of 1:1:3, respectively (13). The level of α-tocopherol (vitamin E) was determined with 2,4,6-triprydyl-s-triazin and ferric chloride, after the extraction (14).

CAT activity was calculated according to the constant rate of hydrogen peroxide decomposition, by catalase enzyme at 240 nm in erythrocytes (15).

Histopathological examination

The ovarian tissues were collected and fixed with 10% neutral buffered formalin, and blocked in paraffin. Sections (5 μm) were obtained, and then stained with hematoxylin and eosin for photo microscopic observations.
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Table 1. Body and ovary weights of groups

|                      | Control      | IR           | IR+Erdosteine |
|----------------------|--------------|--------------|---------------|
| First body weight (g) | 206.2±25     | 208.1±21     | 209.7±18      |
| Final body weight (g) | 212.0±18     | 210.9±12     | 207.5±23      |
| Ovary weights (g)    | 0.07±0.01    | 0.20±0.05    | 0.21±±0.07*   |

*: P<0.05; Compared with Control group

PCNA immunohistochemistry

Ovaries were removed and fixed in 10% neutral buffered formalin solution, and following the routine laboratory methods, blocked in paraffin. Immunocytochemical stains were performed based on the avidin-biotin complex (ABC) technique described by Oguz et al (16). The samples were incubated with specific monoclonal antiproliferative cell nuclear antigen (anti-PCNA, 1:100, ab2426-1; Abcam, USA).

Terminal dUTP nick-end labeling (TUNEL) staining

Apoptotic cells were determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique using an apoptosis detection kit (Calbiochem, San Diego, CA, USA), as previously reported (16). To determine the numerical distribution of PCNA and TUNEL (+) cells in the ovaries samples stained with anti-PCNA antibody and TUNEL kit, cells were examined under light microscopy (400X). In each section, the numbers of positive cells in 10 different enlarged areas selected at random, were counted in random high-power sections using a light microscope (Olympus BX51, Japan) and incorporating a software analysis system (Argenit Kameram, ver. 2.11.5.1, Istanbul, Turkey). Finally, all the counts were converted to number of PCNA and TUNEL (+) cells per mm² area.

Statistical analyses

A statistical comparison of differences between experimental groups was performed by means of analysis of variance (ANOVA) and Tukeys Post hoc test, and a value of P<0.05 was considered statistically significant. All values were expressed as mean standard deviation (SD), and statistical tests were performed using SPSS version 12.0 PL for Windows.

Results

Biochemical findings

The oxidant and antioxidant levels in the study and control groups are summarized in Table 1. The highest MDA level was observed in the IR group and the lowest in the control group (P<0.01). Comparison among the groups revealed that the MDA level in the IR group was higher than those in the control and IR-E groups (P<0.01). The GSH and CAT levels in the IR group were lower than in the control and IR-E groups (P<0.01 and P<0.05, respectively). We also detected the lowest level of ascorbic acid, β-carotene, and α-tocopherol in the IR group, and the highest level in the control group (P<0.05).

Histopathological findings

The body and ovaries weight changes and histopathological results are summarized in Table 1 and Figure 1, respectively. In the control group, the ovaries have normal appearance of the cortex and the medulla. The IR group showed severe acute inflammation, polymuclear leukocytes, and macrophages, as well as degenerative cells, stromal oedema, and haemorrhage. Erdosteine treatment, significantly retained degenerative changes in the ovary. Histopathological changes were significantly decreased in IR-E group compared with IR group. The IR and IR-E groups showed significant increase in ovaries weight compared with the control group (P<0.05; Table 1).

Immunohistochemical and TUNEL findings

Results of PCNA staining of the ovary are summarised in Figure 2. PCNA (+) cells were detected in the oocytes, follicle epithelium, theca follicle, and stromal cells of all groups. PCNA (+) cell numbers were significantly decreased in the IR and IR-E groups compared with control group. However, PCNA (+) cell numbers were significantly increased in the IR-E group compared with the IR group (Figure 2).

Ovaries tissues were rarely stained for TUNEL (+) cells in the control rats. TUNEL (+) cell numbers were significantly increased in the IR group compared with the control and the IR-E groups. In the erdosteine
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Discussion

The previous studies suggested that ovarian torsion/detorsion (IR injury) causes morphological and biochemical variation in ovarian tissue (18). The aim of this study was to investigate the antioxidant effect as well as DNA protective effect of erdosteine on torsion/detorsion induced ovarian injury in rats. To our knowledge, this is the first study demonstrating the protective effect of erdosteine on DNA damage, and

**Figure 2.** PCNA staining. (a) Control group, (b) Ischemia–reperfusion group; decreased PCNA (+) cells were seen in the ovary, (c) Ischemia–reperfusion plus erdosteine-treated group; (Immunoperoxidase, hematoxylin counterstain; Scale bar =50 μm). (d) PCNA (+) cells counts per mm² in groups

treated group, TUNEL (+) cells were detected significantly less than the IR group (P<0.05) (Figure 3).

**Figure 3.** TUNEL staining. (a) Control group, (b) Ischemia-reperfusion group; increased TUNEL (+) cells were seen in the ovary, (c) Ischemia-reperfusion plus erdosteine-treated group. (Scale bar =50 μm), (d) TUNEL (+) cells counts per mm² in groups
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