



## Interaperitoneal Administration of Alpha-Tocopherol Loaded Nanoparticles Improves Ischemia-Reperfusion Injury in Rat Ovaries Torsion and Detorsion Model

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### ABSTRACT

**Objective:** To investigate effects of intraperitoneally administration of  $\alpha$ -tocopherol loaded nanoparticles (TNP) on ischemia-reperfusion injury in ovaries.

**Methods:** Thirty-five healthy female Wistar rats ~250g were randomized into seven experimental groups (n=5): Group SHAM: The rats underwent only laparotomy. Group Ischemia: A 3-hour ischemia only. Group I/R: A 3-hour ischemia and a 3-hour reperfusion. Group I/T: A 3-hour ischemia only and 100 mg/kg intraperitoneal administration (IP) of  $\alpha$ -tocopherol 2.5 hours after induction of ischemia. Group I/R/T: A 3-hour ischemia, a 3-hour reperfusion and 100 mg/kg IP of  $\alpha$ -tocopherol 2.5 hours after induction of ischemia. Group I/TNP: A 3-hour ischemia only and 1 mg/kg IP of TNP 2.5 hours after induction of ischemia. Group I/R/TNP: A 3-hour ischemia, a 3-hour reperfusion and 1 mg/kg IP of TNP 2.5 hours after induction of ischemia.

**Results:** Animals treated with  $\alpha$ TNP showed significantly ameliorated development of ischemia and reperfusion tissue injury compared to those of other groups ( $p=0.001$ ). The significant higher values of SOD, tGSH, GPO, GSHRd and GST were observed in I/R/NC animals compared to those of other groups ( $p=0.001$ ). Damage indicators (NOS, MDA, MPO and DNA damage level) were significantly lower in I/R/NC animal compared to those of other groups ( $p=0.001$ ). **Conclusion:** Intraperitoneal administration of TNP could be helpful in minimizing ischemia-reperfusion injury in ovarian tissue exposed to ischemia.

**Keywords:** Ischemia-reperfusion;  $\alpha$ -tocopherol nanoparticles; Intraperitoneal; Ovary.

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### Introduction

Different conditions like long mesovarium and adnexal venous congestion could result in

torsion of ovary and subsequently obstruction of the ovarian vessels. This causes a life-threatening reduction in tissue blood flow and permanent tissue damage [1]. Therefore, ovarian torsion must be

diagnosed and treated as much early as possible to preserve ovarian functions and prevent future infertility [2]. Upon detection of ovarian torsion, detorsion of the twisted adnexa and evaluation the tissue reperfusion is proposed to prevent future infertility even in case of cyanotic tissues [2, 3]. This ovarian torsion-detorsion process is named as ischemia-reperfusion injury [4].

Reperfusion of the ischemic tissue leads to much more serious damage to the tissue than the damage caused by ischemia [5]. Reperfusion-related damage in the cell is created by many factors, mostly including oxygen-derived free radicals, which are rapidly generated in the tissue as a result of reperfusion [6]. Due to physiological or pathological alterations, oxidative damage takes place with changes in favor of the oxidation process [7]. Prompt diagnosis to reduce ischemic and reperfusion injury, and its consequents are still inevitable with this approach. Therefore, studies on preventing reperfusion injury seem very important [8].

A proposed pathogenesis of tissue injury during reperfusion is accumulation of the activated neutrophils that release reactive oxygen species [9]. Lipid peroxidation in the cell is the most deleterious effects of free radicals that end up reduction in the membrane potential and subsequently, cell injury. Malondialdehyde (MDA), one of the end products of lipid peroxidation, also results in serious cell damage through induction of polymerization and cross linking in membrane components [10]. Free oxygen radicals react with DNA and form 8-hydroxyguanine (8-OHGua) that is one of the damage products of DNA [11]. In spite of the fact that generation of free oxygen radicals occurs continuously in cells, the presence of endogenous antioxidant defense systems preserves tissues from the harmful effects of free oxygen radicals [12]. Various agents, anti-inflammatory and antioxidant free radical scavengers have been reported with promising beneficial effects on prevention of ischemic/reperfusion injuries in tissues [13-16].

$\alpha$ -tocopherol, the most studied and bioavailable component of vitamin E, presents antioxidant activity, its consumption is widely considered to help to reduce risk of many chronic diseases associated to oxidative stress [17]. Thus,  $\alpha$ -tocopherol is a widely used component in functional food, cosmetic and pharmaceutical industries. However, the design of proper dosage forms is still challenging due to its hydrophobicity and well-known sensitivity to oxygen and light.

In order to overcome its environmental susceptibility, and improve its solubility in aqueous medium, encapsulation has been recently exploited by several authors [18-20]. The use of nanoparticles besides improving solubility in water, also provides controlled releasing. Some materials such as gliadin nanoparticles, self-assembled nanoparticles of tocopheryl monoesters modified-chitosan

conjugates, and chitosan nanoparticles coated with zein have been reported [21-23]. The physiologic characteristic of the peritoneal cavity which helps remove toxic metabolites from the body has been successfully exploited to provide peritoneal dialysis in end stage renal disease patients [24]. The same characteristics of the peritoneal membrane also provide a useful portal of entry in the body for several pharmacological agents. One advantage would be that the drug achieves therapeutic efficacy in the region of interest while minimizing the systemic toxicities. Interaperitoneal administration seems more effective and available where oral administration of an agent may cause difficulties. It is clear that transperitoneal absorption of the agent is far faster than oral administration [25].

The present study was different from the other studies in the literature for using  $\alpha$ -tocopherol on ischemia/reperfusion injury. Aimed to study peritoneal effects of  $\alpha$ -tocopherol loaded nanoparticles on ischemia/reperfusion injury, a study was designed to determine if  $\alpha$ -tocopherol loaded nanoparticles could in fact protect against ischemia/reperfusion induced ovarian damage. The assessments were based on histopathological and biochemical parameters.

## Materials and Methods

### Study Design and Animals

Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of (23±3) °C, stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. All measurements were made by two blinded observers unaware of the analyzed groups. The present study was designed and modified based on a method described by others [13]. Thirty-five healthy female Wistar rats weighing approximately 250 g were randomized into seven experimental groups (n=5): Group SHAM: The rats underwent only laparotomy. Group Ischemia: A 3-hour ischemia only. Group I/R: A 3-hour ischemia and a 3-hour reperfusion. Group I/T: A 3-hour ischemia only and 100 mg/kg intraperitoneal administration of  $\alpha$ -tocopherol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) 2.5 hours after induction of ischemia. Group I/R/T: A 3-hour ischemia, a 3-hour reperfusion and 100 mg/kg intraperitoneal administration of  $\alpha$ -tocopherol 2.5 hours after induction of ischemia. Group I/TNP: A 3-hour ischemia only and 1 mg/kg intraperitoneal administration of TNP (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) 2.5 hours after induction of ischemia. Group I/R/TNP: A 3-hour ischemia, a 3-hour reperfusion and 1 mg/kg intraperitoneal administration of TNP 2.5 hours after induction of ischemia. The right ovaries were transferred to a 10% formaldehyde solution for

histopathological assessments and the left ovaries were cleaned of surrounding soft tissues and then stored in a freezer at  $-80^{\circ}\text{C}$  for biochemical assessments.

#### *Surgical Procedure*

Animals were anesthetized by interaperitoneal administration of ketamine-xylazine (ketamine 5%, 90mg/kg and xylazine 2%, 5mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain [26]. The ethical Committee of the Urmia University of Medical Sciences approved all the experiments. A longitudinal midline incision was made in the lower abdomen and the uterine horns and adnexa were exposed. For induction of ischemia, a vascular clamp was applied on vessels of the ovaries in rats. After a 3-hour period of ischemia, both ovaries were surgically dissected out for histopathological and biochemical assessments. For induction of ischemia/reperfusion, both ovaries underwent ischemia the same way and at the end of a 3-hour period, the vascular clamps were chosen, removed and a 3-hour reperfusion was continued. Then, the ovaries were dissected out for histopathological and biochemical assessments.

#### *Histopathological Assessments*

Ovaries were fixed in 10% buffered formalin for 24 hours. The tissue samples were then processed and embedded in paraffin. A 5- $\mu\text{m}$  semi-thin section was paraffin-embedded. The samples were then dewaxed, rehydrated and stained routinely with hematoxylin and eosin. The sections were then observed under a light photomicroscope. For semithin sections, ovaries were fixed in 2.5% buffered glutaraldehyde and post fixed in 2% OsO<sub>4</sub> for 2 h, dehydrated through an ethanol series and embedded in epon. Semithin transverse sections (5  $\mu\text{m}$ ) were next stained with toluidine blue and examined under a light microscope.

#### *Biochemical Assessments*

##### *Tissue Processing for Biochemical Assessments of Ovary*

The tissue samples of ovaries were kept at  $-80^{\circ}\text{C}$  for 3 days, and then enzyme activities were determined in rat ovary tissues. The ovary tissues were ground with liquid nitrogen in a mortar. One half gram was weighed for each group and then treated with 4.5 mL of an appropriate buffer. This mixture was homogenized on ice with use of an ultra-turrax homogenizer (IKA, Werke, Germany) for 15 minutes. Homogenates were filtered and centrifuged by using a refrigerator centrifuge at  $4^{\circ}\text{C}$ . Then the supernatants were used to determine the enzymatic activities. All assays were carried out at room temperature.

##### *Superoxide Dismutase (SOD) Analysis*

Superoxide dismutase estimation was based on

the generation of superoxide radicals produced by xanthine and the xanthine oxidase system, which reacts with nitroblue tetrazolium to form formazan dye [27]. Superoxide dismutase activity was then measured at 560 nm by the degree of inhibition of this reaction and is expressed as millimoles per minute per milligram of tissue.

##### *Nitric Oxide Synthase (tNOS) Activity*

Nitric oxide synthase activity of rat ovaries was measured spectrophotometrically using the oxidation of oxyhemoglobin to methemoglobin by NO as described by others [28]. The absorption difference between 401 and 421 nm was continuously monitored with a dual wave length recording spectrophotometer at  $37^{\circ}\text{C}$ . For the total NOS (tNOS) assay, the incubation medium contained 1.6 mmol/L oxyhemoglobin, 200 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, 100 mmol/L L-arginine, 100 mmol/L of the reduced form of nicotinamide-adenine dinucleotide phosphate, 40 mmol/L potassium phosphate (pH 7.2), 1 mmol/L NG-nitro-L-arginine, and 10% (vol/vol) tissue extract with 50 mmol/L L-valine to inhibit arginase [29].

##### *Malondialdehyde (MDA) Analysis*

Concentrations of ovarian lipid peroxidation were determined by estimating MDA using the thiobarbituric acid test [30]. The rat ovaries were rinsed with cold saline. The corpus mucosa was scraped, weighed, and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added to a solution containing 2-thiobarbiturate (1.5 ml of 8 g/l), acetic acid (1.5 ml of 200 g/l), sodium lauryl sulfate (0.2 ml of 80 g/l), and distilled water (0.3 ml). The mixture was incubated at  $98^{\circ}\text{C}$  for 1 hr. n-butanol:pyridine 5 ml (ratio:15:1) was then added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The standard curve was obtained by using 1,1,3,3- tetramethoxypropane.

##### *Myeloperoxidase (MPO) Analysis*

The activity of MPO in the total homogenate was measured according to previously described methods [31]. The sample was weighed and homogenized in 2 ml of 50 mmol/l phosphate buffer containing 0.5% hexadecyltrimethyl ammonium bromide (HDTMAB) and centrifuged at 3500 rpm for 60 min at  $4^{\circ}\text{C}$ . The supernatant was used to determine MPO activity using 1.3 ml 4-aminoantipyrine-2% phenol (25 mM) solution. 25 mmol/l 4-aminoantipyrine-2% phenol solution and 0.0005% 1.5 ml H<sub>2</sub>O<sub>2</sub> were added and equilibrated for 3–4 min. After establishing the basal rate, a sample suspension (0.2 ml) was added and mixed. Increases in absorbance at 510 nm for 4 min at 0.1-min intervals were recorded. Absorbance was measured at 412 nm.

### Total Glutathione (tGSH) Analysis

The amount of GSH in the total homogenate was measured according to the previously described methods with some modifications [32]. The sample was homogenized at pH 7.5, in Tris-HCl buffer (2 ml of 50 mmol/l). The homogenate was precipitated with trichloroacetic acid (0.1 ml of 25%), and the precipitate was removed after centrifugation at 4200 rpm at 4°C for 40 min, and the supernatant was used to measure GSH level. A total of 1500 µl of measurement buffer (200 mmol/l Tris-HCl buffer containing 0.2 mmol/L EDTA at pH 7.5), 500 µl supernatant, 100 µl DTNB (10 mmol/l) and 7900 µl methanol were added to a tube and vortexed and incubated for 30 min in 37°C. 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) was used as a chromogen; it formed a yellow-colored complex with sulfhydryl groups. The absorbance was measured at 412 nm using a spectrophotometer (Beckman DU 500, USA). The standard curve was obtained using reduced glutathione.

### Glutathione Peroxidase (GPO) Analysis

GPO activity was determined according to the method of Lawrence and Burk [33]. After tissue homogenization, supernatant was used for GPO measurement. Following the addition of KH<sub>2</sub>PO<sub>4</sub>, EDTA, GSH, B-NADPH, NaN<sub>3</sub>, and GR, the mixture was incubated. As soon H<sub>2</sub>O<sub>2</sub> was added the chronometer was turned on and the absorbance at 340 nm was recorded for 5 min every 15 sec.

### Glutathione Reductase (GSHRd) Analysis

GR activity was determined spectrophotometrically by measuring the rate of NADPH oxidation at 340 nm according to Carlberg and Mannervik method [34]. After tissue homogenization, supernatant was used for GR measurement. After the NADPH and GSHAM addition, chronometer was on and absorbance was measured for 5 min in 30 min intervals at 340 nm spectrophotometrically.

### Glutathione S-Transferase (GST) Activity

GST activity was determined by Habig and Jakoby [35]. Enzyme activity was determined in a 4-ml cuvette containing 30 mM GSH, 30 mM 1-chloro-2,6-dinitrobenzene, 0.1 M PBS (pH: 6.5), and tissue homogenate at 340 nm using a spectrophotometer.

### Isolation of DNA from Ovarian Tissue

The isolation of DNA was performed based on a method described by others [8]. In brief, the tissue samples were homogenized at 4°C in 1 ml of homogenization buffer (0.1 M NaCl, 30 mM Tris, pH 8.0, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.5% (v/v) Triton X-100) with 6 passes of a Teflon-glass homogenizer at 200 rpm. The samples were centrifuged at 4°C for 10 min at 1000 g to pellet nuclei. The supernatant was discarded, and the crude nuclear pellet re-suspended and re-homogenized in

1 ml of extraction buffer (0.1 M Tris, pH 8.0, 0.1 M NaCl, 20 mM EDTA) and re-centrifuged as above for 2 min. The washed pellet was re-suspended in 300 µl of extraction buffer with a wide-orifice 200 µl Pipetman tip. The re-suspended pellet was subsequently incubated at 65°C for 1 hour with the presence of 0.1 ml of 10% SDS, 40 µl proteinase K, and 1.9-ml leukocyte lysis buffer. Then, ammonium acetate was added to the crude DNA sample to give a final concentration of 2.5 mol/L, and centrifuged in a micro centrifuge for 5 min. The supernatant was removed and mixed with two volumes of ethanol to precipitate the DNA fraction. After centrifugation, the pellet was dried under reduced pressure and dissolved in sterile water. The absorbance of this fraction was measured at 260 and 280 nm. Purification of DNA was determined as A<sub>260</sub>/A<sub>280</sub> ratio 1.8.

### cDNA Hydrolysis with Formic Acid

DNA hydrolysis with formic acid was performed based on a modified method described by others [8]. Briefly, 50 mg of DNA was hydrolyzed with 0.5 ml of formic acid (60%, v/v) for 45 min at 150°C. The tubes were allowed to cool. The contents were then transferred to Pierce micro-vials, covered with Kleenex tissues cut to size, secured in place using a rubber band, and cooled in liquid nitrogen. Formic acid was removed by freeze-drying and prior to analysis by HPLC they were re-dissolved in the eluent, final volume 200 µl.

### Measurement of 8-Hydroxy-2 Deoxyguanine (8-OH Gua)

Measurement of 8-hydroxy-2 deoxyguanine (8-OH Gua) was performed based on a modified method described by others [8]. Briefly, the amount of 8-OH gua and guanine (Gua) was measured using a HPLC system equipped with an electrochemical detector, HP Agilent 1100 module series and E.C.D. HP 1049 A. The amount of 8-OH gua and Gua was analyzed on a 250 4.6 mm Supelco LC-18-S reverse-phase column. The mobile phase was 50 mM potassium phosphate, pH 5.5, with acetonitrile, a 97 volume acetonitrile and a 3 volume potassium phosphate, and the flow rate was 1.0 ml/min. The detector potential was set at 0.80 V for measuring the oxidized base. Gua and 8-OH Gua (25 pmol) were used as standards. The 8-OH gua levels were expressed as the number of 8-OH gua molecules/105 Gua molecules.

### Statistical Analysis

The sample size calculation formula was based on following formula that is used for animal studies. In this method a value E is calculated based on decided sample size.

$$E = \text{Total number of animals} - \text{Total number of groups}$$

In our animal study 7 groups of animal with

5 animals each were considered for different interventions. Then the total animals were 35 (7×5). Hence E was:

$$E=35-7=28$$

This gives enough power for statistical analysis. Experimental results were expressed as means± SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using repeated measures and a factorial ANOVA with two between-subject factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. A 2-sided p-value of less than 0.05 was considered statistically significant.

## Results

### Histopathological Findings

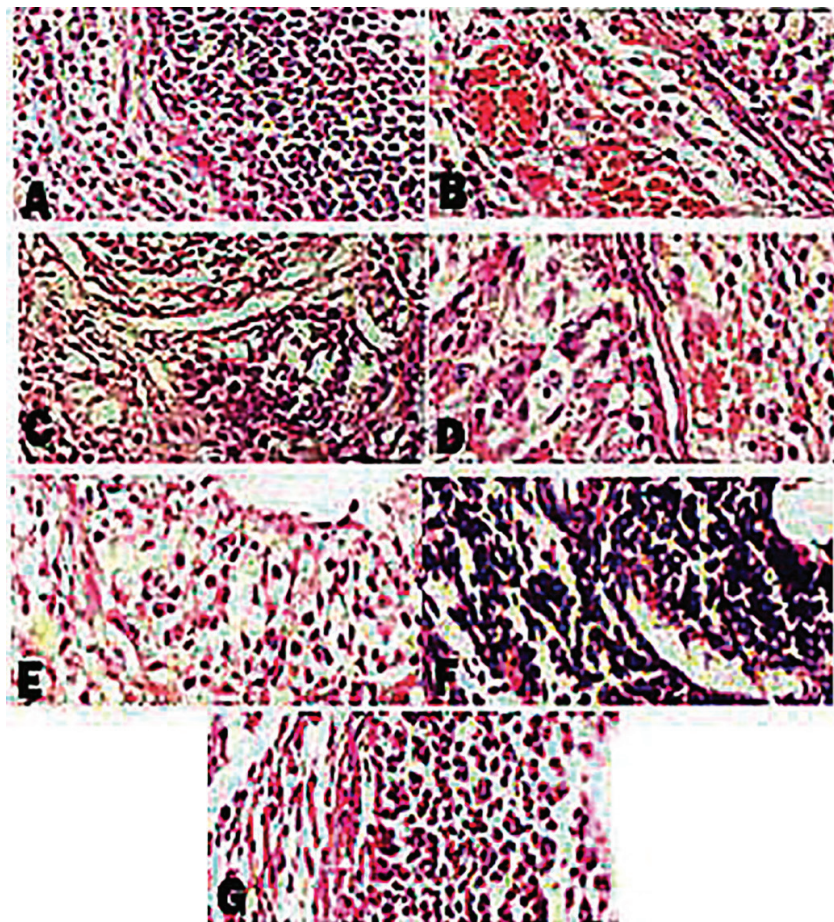
The histologic design of the ovarian tissue in the SHAM animals was normal. Ovarian tissues in the ischemia group showed condensed hemorrhage and severe vascular congestion along with degenerative and necrotic changes in many of the cells. The tissues

in the I/R group showed histopathological changes of condensed hemorrhage, infiltration of inflammatory cells along with degenerative and apoptotic cells. Polymorphonuclear leukocytes (neutrophils) were dominant cell types. In I/R/T group general histologic and cellular structures of the tissues were not normal in appearance, however, mild vascular congestion and edema were observed. In I/R/TNP group only a slightly mild hemorrhage was around ovarian follicles. The general histologic structure of the ovarian tissue in this group was normal and no important pathologic findings in the structural level were observed except for only a slightly mild inflammation, vascular congestion and edema (Figure 1). The numerical densities of neutrophils were also estimated that were  $14 \times 10^{-6} / \mu\text{m}^3$ ,  $12 \times 10^{-6} / \mu\text{m}^3$ ,  $18 \times 10^{-6} / \mu\text{m}^3$ ,  $17 \times 10^{-6} / \mu\text{m}^3$ ,  $13 \times 10^{-6} / \mu\text{m}^3$ ,  $16 \times 10^{-6} / \mu\text{m}^3$  and  $11 \times 10^{-6} / \mu\text{m}^3$  in SHAM, I, I/R, I/T, I/R/T, I/TNP and I/R/TP groups, respectively.

### Biochemical Findings

#### Superoxide Dismutase (SOD) Analysis

The value of SOD activity was  $69.1 \pm 0.53$  mmol/min/



**Fig. 1.** Histologic micrographs of the ovarian tissue in SHAM (A), I (B), I/R (C), I/T (D), I/R/T (E), I/TNP (F) and I/R/TP (G) groups. (A) Shows normal secondary follicles with compact stroma between them. (B) Many follicles at different stages of development are observed. Edematous ovarian stroma and multiple dilated congested blood vessels with some areas of hemorrhage are shown. (C) Ovarian follicles at different stages of development with edema in the stroma and hemorrhage are observed. (D) Many follicles at different stages of development are seen. There is edematous ovarian stroma and multiple dilated congested blood vessels with some areas of haemorrhage. (E) Preserved follicles at different stages of development with the presence of mild edema, congested blood vessels, and hemorrhage are seen. (F) Shows persistent marked edema among follicles at different stages of development. (G) Normally appearing ovarian tissue with preserved healthy follicles at different stages of development with slight edema or hemorrhage is observed.

mg tissue in the SHAM group. The values of SOD were decreased to  $35.3 \pm 0.27$  and  $57.6 \pm 0.24$  mmol/min/mg tissue in I and I/R groups, respectively. However, intraperitoneal administration of 1 mg/kg of  $\alpha$ -tocopherol loaded nanoparticles inverted the trend and increased the activity of SOD to  $78.9 \pm 0.37$  mmol/min/mg tissue in the ovarian tissue in I/R/TNP group. The value of SOD activity in I/R/TNP group was significantly higher than those of the other experimental groups ( $p=0.001$ ). (Table 1).

#### Nitric Oxide Synthase (NOS) Activity

The value of tNOS activities was increased in I and I/R groups that were significantly higher than those of SHAM group ( $p=0.001$ ). However, intraperitoneal administration of 1 mg/kg of  $\alpha$ -tocopherol loaded nanoparticles inverted the trend and decreased tNOS activity in the rats ovary. In I/R/NC group the value of tNOS activity was significantly lower than those of the other experimental groups ( $p=0.001$ ) (Table 1).

#### Malondialdehyde (MDA) Analysis

The results of the present study showed that concentration of MDA in SHAM group was  $5.7 \pm 0.19$   $\mu$ mol/g protein in ovarian tissue. The MDA level I/R group was significantly increased to  $11.6 \pm 0.23$   $\mu$ mol/g protein ( $p=0.001$ ). Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles significantly decreased level of MDA in ovarian tissues of I/R/TNP animals ( $p=0.032$ ) (Table 1).

#### Myeloperoxidase (MPO) Analysis

The level of MPO was significantly increased

in I and I/R groups ( $p=0.001$ ). Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles reversed the trend and significantly decreased level of MPO in ovarian tissues of I/R/TNP animals ( $p=0.001$ ) (Table 1).

#### Total Glutathione (tGSH) Analysis

The values for tGSH levels were  $9.7 \pm 0.34$  and  $4.8 \pm 0.32$  nmol/g protein in SHAM and I/R animals, respectively. Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles significantly increased level of GSH in ovarian tissues of I/R/TNP animals ( $p=0.032$ ) (Table 1).

#### Glutathione Peroxidase (GPO) Analysis

The values for GPO levels were  $38.4 \pm 2.63$  and  $17.3 \pm 1.45$  u/g protein in SHAM and I/R animals, respectively. Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles significantly increased level of GPO in ovarian tissues of I/R/TNP animals ( $p=0.001$ ) (Table 1).

#### Glutathione Reductase (GSHRd) Analysis

The GSHRd activities in ovarian tissue in the SHAM and I/R animals were  $33.6 \pm 2.25$  and  $16.7 \pm 1.24$  u/g protein, respectively. Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles significantly increased level of GSHRd in ovarian tissues of I/R/NC animals ( $p=0.001$ ) (Table 1).

#### Glutathione S-Transferase (GST) Activity

The GST activities in ovarian tissue in the SHAM and I/R animals were  $21.6 \pm 1.21$  and  $14.7 \pm 1.38$  u/g

**Table 1.** Comparison of the activities of SOD, NOS, MDA, MPO, GSH, GPO, GSHRd, GST and a DNA damage product of 8-OHGua/Gua in the ovarian tissues of the animals of the all experimental groups. Data are expressed as Mean $\pm$ SD.

Variables	Group SHAM	Group I <sup>a</sup>	Group I/R <sup>b</sup>	Group I/T <sup>c</sup>	Group I/R/T <sup>d</sup>	Group I/TNP <sup>e</sup>	Group I/R/TNP <sup>f</sup>
SOD <sup>g</sup> (mmol/min/mg)	69.1 $\pm$ 0.53	35.3 $\pm$ 0.27	57.6 $\pm$ 0.24	53.9 $\pm$ 0.34	73.8 $\pm$ 0.29	61.7 $\pm$ 0.45	78.9 $\pm$ 0.37 <sup>p</sup>
NOS <sup>h</sup> (nmol/min/mg)	3.9 $\pm$ 0.15	3.7 $\pm$ 0.12	3.6 $\pm$ 0.50	3.3 $\pm$ 0.17	3.3 $\pm$ 0.21	3.4 $\pm$ 0.15	3.2 $\pm$ 0.17 <sup>p</sup>
MDA <sup>i</sup> ( $\mu$ mol/g protein)	5.8 $\pm$ 0.15	12.6 $\pm$ 0.20	11.3 $\pm$ 0.21	9.5 $\pm$ 0.31	6.9 $\pm$ 17	8.5 $\pm$ 0.66	5.3 $\pm$ 18 <sup>p</sup>
MPO <sup>j</sup> (U/g protein)	6.6 $\pm$ 0.12	16.5 $\pm$ 0.45	13.8 $\pm$ 0.24	12.5 $\pm$ 0.23	8.9 $\pm$ 0.49	10.4 $\pm$ 0.15	7.2 $\pm$ 0.38 <sup>p</sup>
tGSH <sup>k</sup> (nmol/g protein)	9.7 $\pm$ 0.34	2.8 $\pm$ 0.25	4.8 $\pm$ 0.32	5.4 $\pm$ 0.10	7.1 $\pm$ 0.17	6.3 $\pm$ 0.21	8.3 $\pm$ 0.14 <sup>p</sup>
GPO <sup>l</sup> (U/g protein)	38.4 $\pm$ 2.63	12.9 $\pm$ 2.41	17.3 $\pm$ 1.45	20.3 $\pm$ 1.57	29.4 $\pm$ 2.55	22.5 $\pm$ 1.33	3.27 $\pm$ 2.69 <sup>p</sup>
GSHRd <sup>m</sup> (U/g protein)	33.6 $\pm$ 2.25	9.3 $\pm$ 1.85	16.7 $\pm$ 1.24	19.8 $\pm$ 1.85	24.2 $\pm$ 2.33	21.5 $\pm$ 1.15	28.6 $\pm$ 2.55 <sup>p</sup>
GST <sup>n</sup> (U/g protein)	21.7 $\pm$ 1.23	10.5 $\pm$ 1.24	14.8 $\pm$ 1.35	16.3 $\pm$ 1.24	19.5 $\pm$ 1.12	17.6 $\pm$ 1.07	20.3 $\pm$ 1.24 <sup>p</sup>
8-OHGua/Gua <sup>o</sup> (pmol/L)	1.2 $\pm$ 0.15	2.6 $\pm$ 0.12	2.2 $\pm$ 0.18	1.6 $\pm$ 0.17	1.6 $\pm$ 0.14	1.6 $\pm$ 0.10	1.3 $\pm$ 0.17 <sup>p</sup>

<sup>a</sup>I: Ischemia; <sup>b</sup>I/R: Ischemia-reperfusion; <sup>c</sup>I/T: Ischemia plus intraperitoneal administration of  $\alpha$ -tocopherol; <sup>d</sup>I/R/T: Ischemia plus reperfusion plus intraperitoneal administration of  $\alpha$ -tocopherol; <sup>e</sup>I/TNP: Ischemia plus intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles; <sup>f</sup>I/R/TNP: Ischemia plus reperfusion plus intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles; <sup>g</sup>SOD: Superoxide dismutase; <sup>h</sup>NOS: Nitric oxide synthase; <sup>i</sup>MDA: Malondialdehyde; <sup>j</sup>MPO: Myeloperoxidase; <sup>k</sup>tGSH: Total glutathione; <sup>l</sup>GPO: Glutathione peroxidase; <sup>m</sup>GSHRd: Glutathione reductase; <sup>n</sup>GST: Glutathione S-transferase; <sup>o</sup>8-OHGua/Gua: 8-hydroxy-2 deoxyguanine; <sup>p</sup>P<0.0 vs. other experimental groups.

protein, respectively. Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles significantly increased level of GST in ovarian tissues of I/R/NC animals ( $p=0.001$ ) (Table 1).

#### Measurement of 8-Hydroxy-2 Deoxyguanine (8-OH Gua)

The levels of 8-OHGua/Gua, a DNA damage product, were  $1.3\pm 0.14$  and  $2.3\pm 0.15$  pmol/L in SHAM and I/R animals, respectively. Intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles significantly decreased level of GSHRd in ovarian tissues of I/R/TNP animals ( $p=0.001$ ) (Table 1).

## Discussion

The present study it was investigated whether intraperitoneally administration of  $\alpha$ -tocopherol loaded nanoparticles is useful or not in the prevention of ovarian damage in ischemia/reperfusion conditions in rat ovaries and it was found to have beneficial effects. Histopathological and biochemical assessments were performed in SHAM, ischemia, ischemia-reperfusion, ischemia-controlled plus intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles groups. Histopathological, edema, vascular congestion, hemorrhages and leukocyte infiltration parameters were used. Biochemically, the activities of SOD, NOS, MDA, MPO, GSH, GPO, GSHRd, GST and a DNA damage product of 8-OHGua/Gua were assessed in the ovarian tissues of the animals of the all experimental groups.

Ischemia, ischemia-reperfusion and intraperitoneal  $\alpha$ -tocopherol loaded nanoparticles applied to tissues were analyzed histopathologically. Results showed that oxidative stress level followed a parallelism with the tissue damage. Edema, vascular congestion, hemorrhages, and leukocyte infiltration have been used as histopathological parameters in the evaluation of the condition of the cell [36]. Edema, vascular congestion, hemorrhage, and leukocyte infiltration in the I/R/TNP animals were milder than in the I/R/T group.

In the present study, levels of SOD in ovarian tissue were assessed and compared in all the experimental groups. The SOD activity in SHAM and I/R/TNP showed no significance difference. SOD is an antioxidant enzyme that catalyzes the conversion of superoxide free radical into hydrogen peroxide and molecular oxygen. SOD and endogenous antioxidant enzymes neutralize free radicals and protect tissues from the harmful effects of free radicals and active oxygen species [37]. Our results showed that in the I/R/TNP animals, SOD was increased compared to those in I/T and I/R/T groups and intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles, secured ovarian tissue against ischemia-reperfusion injury.

It has been demonstrated that hypoxia causes iNOSs that play an important damaging role in I/R injury

[38]. iNOS is increased after cellular stimulation via cytokines in macrophages, neutrophils, and microglia and may also contribute to late-stage tissue injury [39]. The iNOS is derived primarily from the polymorphonuclear neutrophilic leukocytes during reperfusion and down-regulation of iNOS could limit cell injury caused by hypoxia [40, 41]. Findings of the present study showed that the iNOS levels in I and I/R groups of rats' ovarian tissue were increased compared to those of the SHAM animals. Down-regulation of iNOS could limit cell injury caused by hypoxia. Our results showed that in the I/R/TNP animals, iNOS was down-regulated compared to those in I/T and I/R/T group. Thus, interaperitoneal administration of 1 mg/kg  $\alpha$ -tocopherol loaded nanoparticles protected ovarian tissue against ischemia-reperfusion injury more than 100 mg  $\alpha$ -tocopherol.

MDA is a lipid peroxidation product and occurs as a result of the peroxidation of fatty acids that contain three or more double bonds. MDA causes cross-linking of membrane components and leads to negative consequences like changes in ion permeability and enzyme activity via affecting the ion exchange through the cell membranes [42, 43]. MDA levels in the present study were found to be much lower in the I/R/TNP animals compared to those in other experimental groups. This could protect the tissues against ischemia-reperfusion injury in  $\alpha$ -tocopherol loaded nanoparticles treated animals.

MPO is produced by neutrophils and macrophages, catalyzes the reaction between hydrogen peroxide and chlorine and results in the toxic compound hypochlorous acid. Hypochlorous acid is involved in the formation of the hydroxyl radical [44, 45]. It has been demonstrated that MPO activity is increased in ischemia-reperfusion induced ovarian tissue [46]. This finding was in agreement with results of the present study. MPO activity was suppressed in  $\alpha$ -tocopherol loaded nanoparticles treated animals of our study.

GSH is an antioxidant used to measure oxidative stress. Reperfusion after ischemia is reported to cause severe damage to ovarian tissue and suppress the GSH levels [36]. GSH plays a role in the protection of the cell against oxidative stress and toxic compounds as well as the metabolic processing of many endogenous compounds like estrogen, prostaglandin, and leukotrienes [47]. GSH, as an antioxidant, reacts with peroxides and free radicals and converts them into harmless products and subsequently protects the cells against the potential oxidative damage of free radicals. These findings were in agreement with our results. We found that oxidative stress was minimized and the severe damage due to sudden reperfusion was prevented in  $\alpha$ -tocopherol loaded nanoparticles treated animals more than those in curcumin treated animals.

GPO activity is significantly reduced in tissues

undergoing oxidative stress-related conditions like ischemia-reperfusion injury [48]. GPO detoxifies the hydrogen peroxide radical that forms in the cell by converting it to water and prevents the formation of more toxic products from hydrogen peroxide radical [49]. In the present study a significant decrease in GPO activity was observed in ovarian tissues of I/R/TNP animals. GSH is oxidized during the detoxification of hydrogen peroxide radical. GSHRd is a NADPH-dependent enzyme that converts oxidized glutathione to reduced glutathione [50]. GSHRd is reported to show higher activity in healthy tissue and in parallel with tissue damage its activity is decreased [51]. In our study activity of GSHRd was significantly increased in  $\alpha$ -tocopherol loaded nanoparticles treated animals compared to those of I/T and I/R/T groups.

GST binds foreign substances to the -SH group of cysteine in glutathione, neutralizes the electrophilic regions and protects the cells from the harmful effects of foreign substance regions [52]. Activity of GST has been reported to be suppressed in oxidative tissue injury induced by ischemia [52]. Consistently, our findings showed that GST activity in ovarian tissue of  $\alpha$ -tocopherol loaded nanoparticles treated animals was significantly lower than those in I/T and I/R/T groups.

DNA molecules are damaged if free radicals are in a close proximity to the DNA molecules [53]. Hydroxyl radical reacts very easily with deoxyribose and the bases and causes DNA damage through extracting hydrogen from nucleic acids or reacting with double bonds [54]. 8-OH Gua is considered an important marker of DNA oxidation [55]. Our findings showed that the ovarian tissues of I/C and I/R/C animals had higher levels of 8-OHGua than those of the SHAM animals. However, our results showed that there was no significant difference between SHAM and  $\alpha$ -tocopherol loaded nanoparticles treated animals regarding the levels of DNA damage.

There are many studies in the literature about the improvement of ischemia reperfusion injury. Studies demonstrated that the agents with antioxidant or anti-inflammatory activities may be beneficial in reducing ovarian ischemia reperfusion injury. Also, studies revealed the beneficial effect of controlled reperfusion in the prevention of ovarian tissue damage. Although there are many studies in the literature; ischemia/reperfusion damage continues to be a serious problem clinically. Essentially, early diagnosis and treatment of ovarian torsion plays an important role to provide urgent protection against life-threatening complications from ischemia and to prevent future infertility [56].

Vitamin E has been reported as a useful agent both for the prevention and treatment of I/R injury in many organs. These protective effects are mainly believed to be based on inhibitory actions of curcumin on disease-mediated induction of inflammatory transcription factors, protein kinases, adhesion molecules, oxidative

stress and inflammation [56]. The administration of vitamin E has reported to reduce the generation of reactive oxygen species (ROS), monocyte adhesion, phosphorylation of c-Jun N-terminal kinase (JNK), p38 MAP kinase, and signal transducer and activator of transcription (STAT)-3 in TNF- $\alpha$ -stimulated cells [57]. It has also been documented that the administration of vitamin E prior to the conservative surgery (detorsion) provides a significant decrease for the oxidative stress markers in the ovarian tissues [57]. The comparison between the oxidative status and antioxidative status is clear enough to suggest that the administration of vitamin E, as reported previously, leads to a decrease in the oxidative stress and an increase in the antioxidation [58].

Nano-sized particles ranging below several 10 nm are of great interest, because of the chemical and physical behavior of the particles arising from the quantum size effect which are remarkably different from those in bulk form giving the great potential for use in practice [59]. The findings of the present study showed that  $\alpha$ -tocopherol loaded nanoparticles in very low concentrations, 1 mg/kg  $\alpha$ -tocopherol loaded nanoparticles versus 100 mg  $\alpha$ -tocopherol, gave rise to significant improvements compared to native  $\alpha$ -tocopherol.

Substances are administered by a wide variety of routes. A key factor determining the route selected is whether the agent is being administered for a local or systemic (either enteral or parenteral) effect. Parenteral administration methods typically produce the highest bioavailability of substances because these methods avoid the first-pass effect of hepatic metabolism, which occurs commonly with orally administered chemicals and therapeutics [59]. Intraperitoneal administration seems more effective and available where oral administration of an agent may cause difficulties. It is clear that transperitoneal absorption of the agent is far faster than oral administration [59]. It seems time saving is very important in emergency conditions like ovarian torsion.

The most important advantage of nanomaterials is the ability to synthesize them in required size, shape and biocompatibility in relation to biological systems. Nanoscale devices and components are of the same basic size as biological entities. They are smaller than human cells and organelles and similar in size to large biological macromolecules and enzymes like lipid bilayers. They can transit through blood vessel walls and can also penetrate the blood brain barriers, testis barrier [60].

In conclusion, histopathological results obtained from all the experimental groups were consistent with the results of the biochemical analyses indicating that intraperitoneal administration of  $\alpha$ -tocopherol loaded nanoparticles could be helpful in minimizing ischemia-reperfusion injury in ovarian tissue exposed to ischemia. Regarding the transperitoneal absorption of the  $\alpha$ -tocopherol loaded nanoparticles that is far faster than its oral



administration, it could be considered in clinical practice where that ovarian torsion is the case and ovarian functions must be resumed as early as possible to preserve and prevent future infertility. The present study demonstrated that intraperitoneal administration of 1 mg/kg  $\alpha$ -tocopherol loaded nanoparticles could improve ischemia-reperfusion injury in ovarian tissue exposed to ischemia. Thus, dose-response studies should be conducted for  $\alpha$ -tocopherol loaded nanoparticles to determine

its maximal efficacy in minimizing ischemia-reperfusion injury in ovarian tissue.

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