RABMS Journal of Research in Applied and Basic Medical Sciences
Urmia University of Medical Sciences

Biochemical assessments of effect of nefidipne on ischemia-reperfusion injury in rat ovaries via systemic administration

Mohammad Velayati[1], Hakim Rahimzadeh[1], Mozhgan Malekolkalami[1], Hamid Shamsi[1], Gholamreza Khanbaba[2]*

[1]*Department of Clinical Studies, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*

[2]*Department of Clinical Sciences. Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran*

***Corresponding authors:** Gholamreza Khanbaba, **Address:** Department of Clinical Sciences. Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran, **Email**: gholamreza.khanbaba@gmail.com, **Tel**: +984432258760

Abstract

***Background & Aims*:** Different conditions like long mesovarium and adnexal venous congestion could result in torsion of ovary and subsequently obstruction of the ovarian vessels. The aim of the present study was to investigate effects of systemic administration of nefidipine on ischemia-reperfusion injury in ovaries.

***Materials and Methods**:* Twenty-five healthy female Wistar rats about ~250g were randomized into five 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/N: A 3-hour ischemia only and 100 mg/kg intraperitoneal administration (IP) of nefidipine 2.5 hours after induction of ischemia. Group I/R/N: A 3-hour ischemia, a 3-hour reperfusion and 100 mg/kg IP of nefidipine 2.5 hours after induction of ischemia.

Results: Animals treated with nefidipine showed significantly ameliorated development of ischemia and reperfusion tissue injury compared to those of other groups ($P<0.05$). The significant higher values of SOD, tGSH, GPO, GSHRd and GST were observed in I/R/NC animals compared to those of other groups ($P<0.05$). Damage indicators (NOS, MDA, MPO and DNA damage level) were significantly lower in I/R/NC animal compared to those of other groups ($P<0.05$).

***Discussion*:** Intraperitoneal administration of nefidipine could be helpful in minimizing ischemia-reperfusion injury in ovarian tissue exposed to ischemia.

***Keywords*:** Ischemia-reperfusion; nefidipine; interaperitoneal; ovary

Received 25 September 2019; accepted for publication 18 December 2019

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).

Various agents, anti-inflammatory and antioxidant free radical scavengers have been reported with promising beneficial effects on prevention of ischemic/reperfusion injuries in tissues (14). In most of these studies the protective agents have been administered orally. In search of an alternative method, the authors were encouraged to evaluate interaperitoneal administration of nimodipine in prevention of ischemia reperfusion injury in ovary torsion in rats. Nimodipine, a calcium channel blocker, is a US Food and Drug Administration approved drug used to reduce the morbidity and mortality associated with delayed ischemic deficits in patients with subarachnoid hemorrhage. Nimodipine inhibits calcium ion transfer into these cells and thus inhibits contractions of vascular smooth muscle (15). The major tissue damage that occurs during ischemia-reperfusion injury is secondary to calcium influx into the cell. Hence, a calcium channel blocker might protect tissues against I–R injury by reducing calcium influx into the cell (16).

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

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).

Twenty-five healthy female Wistar rats weighing approximately 250 g were randomized into five 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/N: A 3-hour ischemia only and 100 mg/kg intraperitoneal administration of nefidipine (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) 2.5 hours after induction of ischemia. Group I/R/N: A 3-hour ischemia, a 3-hour reperfusion and 100 mg/kg intraperitoneal administration of nefidipine 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 ºC 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 biochemical assessments.

Biochemical assessments

Tissue processing for Biochemical assessments of ovary:

The tissue samples of ovaries were kept at - 80 ºC 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 PBS buffer *p*h 7.2. 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 ºC. 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 (NOS) 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 °C. For the total NOS (tNOS) assay, the incubation medium contained 1.6 mmol/L oxyhemoglobin, 200 mmol/L CaCl2, 1 mmol/L MgCl2, 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°C for 1 hr. n-butanol:pyridine 5 ml (ratio:15:l) was then added. The mixture was vortexed for 1 minute and centrifuged for 10 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°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 H2O2 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 tGSH 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 tGSH 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 KH2PO4, EDTA, GSH, B-NADPH, NaN3, and GR, the mixture was incubated. As soon H_2O_2 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:

Glutathione reductase 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 measurement of GSHRd activity by change at OD of NADPH + H to $NADP^+$ at 340 nm.

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 wideorifice 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 260/280 ratio 1.8.

cDNA hydrolysis with formic acid:

DNA hydrolysis with formic acid was performed based on a modified method described by others (8). Breifly, 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 is 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 (8). Briefly, the amount of 8-OH gua and guanine (Gua) was measured using a HPLC system equipped with an electrochemical detector , HPLC 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:

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. The differences were considered significant when $P < 0.05$.

Results

Biochmical Findings

Superoxide dismutase (SOD) analysis:

The value of SOD activity was 69.1 ± 0.53 mmol/min/mg tissue in the SHAM group. The values of SOD were decreased to 35.3 ± 0.27 and 57.6 ± 0.24 mmol/min/mg tissue in I and I/R groups, respectively. However, interaperitoneal administration of 1 mg/kg of nefidipine loaded nanoparticles inverted the trend and increased the activity of SOD to 78.9 ± 0.37 mmol/min/mg tissue in the ovarian tissue in I/R/NEFIDIPINE group. The value of SOD activity in I/R/NEFIDIPINE group was significantly higher than those of the other experimental groups (P<0.05) (Table 1).

Nitric oxide synthase (NOS) activity:

The value of NOS activities was increased in I and I/R groups that were significantly higher than those of SHAM group (P<0.05). However, interaperitoneal administration of 1 mg/kg of nefidipine loaded nanoparticles inverted the trend and decreased NOS activity in the rats ovary. In I/R/NC group the value of NOS activity was significantly lower than those of the other experimental groups (P<0.05) (Table 1).

Malondialdehyde (MDA) analysis:

The results of the present study showed that concentration of MDA in SHAM group was 5.7 ± 0.19 µmol/g protein in ovarian tissue. The MDA level I/R group was significantly increased to 11.6 ± 0.23 µmol/g protein (P<0.01). Intraperitoneal administration of nefidipine loaded nanoparticles significantly decreased level of MDA in ovarian tissues of I/R/NEFIDIPINE animals (P<0.05) (Table 1).

Myeloperoxidase (MPO) analysis:

The level of MPO was significantly increased in I and I/R groups (P<0.05). Intraperitoneal administration of nefidipine loaded nanoparticles reversed the trend and significantly decreased level of MPO in ovarian tissues of I/R/NEFIDIPINE animals (P<0.05) (Table 1).

Total glutathione (tGSH) analysis:

The values for tGSH levels were 9.7 ± 0.34 and 4.8 ± 0.32 nmol/g protein in SHAM and I/R animals, respectively. Intraperitoneal administration of nefidipine loaded nanoparticles significantly increased level of tGSH in ovarian tissues of I/R/NEFIDIPINE animals (P<0.05) (Table 1).

Glutathione peroxidase (GPO) analysis:

The values for GPO levels were 38.4 ± 2.63 and 17.3 ± 1.45 u/g protein in SHAM and I/R animals, respectively. Intraperitoneal administration of nefidipine loaded nanoparticles significantly increased

level of GPO in ovarian tissues of I/R/NEFIDIPINE animals (P<0.05) (Table 1).

Glutathione reductase (GSHRd) analysis:

The GSHRd activities in ovarian tissue in the SHAM and I/R animals were 33.6 ± 2.25 and 16.7 ± 1.24 u/g protein, respectively. Intraperitoneal administration of nefidipine loaded nanoparticles significantly increased level of GSHRd in ovarian tissues of I/R /NC animals (P<0.05) (Table 1).

Glutathione S-transferase (GST) activity:

The GST activities in ovarian tissue in the SHAM and I/R animals were 21.6± 1.21 and 14.7 ± 1.38 u/g protein, respectively. Intraperitoneal administration of nefidipine loaded nanoparticles significantly increased level of GST in ovarian tissues of I/R /NC animals (P<0.05) (Table 1).

Measurement of 8-hydroxy-2 deoxyguanine (8-OH Gua):

The levels of 8-OHGual/Gua, a DNA damage product, were1.3±0.14 and 2.3±0.15 pmol/L in SHAM and I/R animals, respectively. Intraperitoneal administration of nefidipine loaded nanoparticles significantly decreased level of GSHRd in ovarian tissues of I/R/NEFIDIPINE animals (P<0.05) (Table 1).

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±SD. I: Ischemia, I/R: Ischemia-reperfusion, I/T: Ischemia plus interaperitoneal administration of nefidipine, I/R/N: Ischemia plus reperfusion plus interaperitoneal administration of nefidipine, SOD: Superoxide dismutase, NOS: Nitric oxide synthase, MDA: Malondialdehyde, MPO: Myeloperoxidase, tGSH: Total glutathione, GPO: Glutathione peroxidase, GSHRd: Glutathione reductase, GST: Glutathione S-transferase and 8-OHGua/Gua: 8-hydroxy-2 deoxyguanine. * P<0.0 *vs.* other experimental groups.

Parameters	Group SHAM	Group I	Group I/R	Group I/N	Group I/R/N
SOD (mmol/min/mg)	67.5 ± 0.57	36.7 ± 0.22	55.5 ± 0.21	62.3 ± 0.40	77.9 ± 0.33*
NOS (nmol/min/mg)	3.5 ± 0.10	3.6 ± 0.10	3.5 ± 0.15	3.7 ± 0.13	3.3 ± 0.11*
MDA (µmol/g protein)	5.5 ± 0.19	12.3 ± 0.21	11.5 ± 0.25	8.8 ± 0.60	5.5 ± 15*
MPO (U/g protein)	6.4 ± 0.15	17.5 ± 0.35	14.1 ± 0.14	10.8 ± 0.17	7.5 ± 0.35*
tGSH (nmol/g protein)	9.9 ± 0.30	2.9± 0.20	4.5 ± 0.30	6.5 ± 0.25	8.7 ± 0.18*
GPO (U/g protein)	35.4 ± 2.60	12.5 ± 2.31	17.7 ± 1.55	22.8 ± 1.39	3.57 ± 2.75*
GSHRd (U/g protein)	33.9± 2.28	9.5 ± 1.88	16.0 ± 1.28	21.0 ± 1.11	28.0 ± 2.50*
GST (U/g protein)	21.5 ± 1.20	10.0 ± 1.20	14.2 ± 1.15	17.0 ± 1.00	20.0± 1.29*
8-OHGua/Gua (pmol/L)	1.7 ± 0.19	2.7 ± 0.15	2.8± 0.11	1.8 ± 0.10	1.4 ± 0.15*

Discussion

The present study it was investigated whether intraperitoneally administration of nefidipine 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. 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 nefidipine 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/N 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/N 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/Nanimals, SOD was increased compared to those in I/T and I/R/T groups and intraperitoneal administration of nefidipine 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/NEFIDIPINE animals, iNOS was down-regulated compared to those in I/T and I/R/T group. Thus, interaperitoneal administration of 1 mg/kg nefidipine loaded nanoparticles protected ovarian tissue against ischemia-reperfusion injury more than 100 mg nefidipine.

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/N animals compared to those in other experimental groups. This could protect the tissues against ischemia-reperfusion injury in nefidipine 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 nefidipine 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 nefidipine 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/NEFIDIPINE 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 ita activity is decreased (51). In our study activity of GSHRd was significantly increased in nefidipine 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 nefidipine 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 955). 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 nefidipine loaded nanoparticles treated animals regarding the levels of DNA damage.

There are many studies in the literature on 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).

On nefidipine 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). Interaperitoneal administration seems more effective and available where oral administration of an agent may cause difficulties. It is clear that tran-speritoneal absorption of the agent is far faster than oral administration.[59] It seems time saving is very important in emergency conditions like ovarian torsion.

In conclusion, Regarding the transperitoneal absorption of the 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 nefidipine loaded nanoparticles could improve ischemia-reperfusion injury in ovarian tissue exposed to ischemia. Thus, dose-response studies should be conducted for nefidipine loaded nanoparticles to determine its maximal efficacy in minimizing ischemia-reperfusion injury in ovarian tissue.

References

1. Oelsner G, Shashar D. Adnexal torsion. Clin Obstet Gynecol 2006;49(3):459-63.
2. Geimanaite L, Trainavicius K. Ovarian torsion in children: management and outcomes. J Pediatr Surg 2013;48(9):1946-53.

3. Celik A, Ergün O, Aldemir H, Ozcan C, Ozok G, Erdener A, et al. Long-term results of conservative management of adnexal torsion in children. J Pediatr Surg 2005;40(4):704-8.
4. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol 2000;190(3):255-66.
5. Zimmerman BJ, Granger DN. Reperfusion injury. Surg Clin North Am 1992;72(1):65-83.
6. Nakagiri A, Sunamoto M, Takeuchi K. Evidence for the involvement of NADPH oxidase in ischemia/reperfusion-induced gastric damage via angiotensin II. J Physiol Pharmacol 2010;61(2):171-9.
7. Javanmardi S, Khordadmehr M. Benidipine reduces ischemia/reperfusion injury following testicular torsion/detorsion in rats. Iranian J Vet Surg 2017;12(2):21-7.
8. Ingec M, Isaoglu U, Yilmaz M, Calik M, Polat B, Alp HH, et al. Prevention of ischemia-reperfusion injury in rat ovarian. J Physiol Pharmacol 2011;62(5):575-82.
9. Filho DW, Torres MA, Bordin AL, Crezcynski-Pasa TB, Boveris A. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia- reperfusion injury. Mol Aspects Med 2004;25(1-2):199-210.
10. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 1998;39(8):1529-42.
11. Huang HY, Helzlsouer KJ, Appel LJ. The effects of vitamin C and vitamin E on oxidative DNA damage: results from a randomized controlled trial. Cancer Epidemiol Biomarkers Prev 2000;9(7):647-52.
12. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993;90(17):7915-22.
13. Oral A, Odabasoglu F, Halici Z, Keles ON, Unal B, Coskun AK, et al. Protective effects of montelukast on ischemia-reperfusion injury in rat ovaries subjected to torsion and detorsion: biochemical and histopathologic evaluation. Fertil Steril 2011;95(4):1360-6.
14. Halici Z, Karaca M, Keles ON, Borekci B, Odabasoglu F. Protective effects of amlodipine on ischemiareperfusion injury of rat ovary: biochemical and histopathologic evaluation. Fertil Steril 2008;90:2408-15.
15. Lindsay RW, Heaton JT, Edwards C, Smitson C, Hadlock TA. Nimodipine and Acceleration of Functional Recovery of the Facial Nerve After Crush Injury. Arch Facial Plast Surg 2010;12:49-52.
16. Kulah B, Besler HT, Akdag M, Oruc T, Altinok G, et al. The effects of verapamil vs. allopurinol on intestinal ischemia/reperfusion injury in rats. An experimental study. Hepatogastroenterology 2004;51:401-7.
17. Bramley PM, Elmadfa I, Kafatos A, Kelly FJ, Manios Y, Roxborough HE, et al. Vitamin E. J Sci Food Agric 2000;80(7):913-38.
18. Farias MC, Moura ML, Andrade L, Leão MH. Encapsulation of the alpha-tocopherol in a glassy food model matrix. Mate Res 2007;10(1):57-62.
19. Hategekimana J, Masamba KG, Ma J, Zhong F. Encapsulation of vitamin E: effect of physicochemical properties of wall material on retention and stability. Carbohydr Polym 2015;124(1):172-9.
20. Trombino S, Cassano R, Muzzalupo R, Pingitore A, Cione E, Picci N. Stearyl ferulate-based solid lipid nanoparticles for the encapsulation and stabilization of β-carotene and nefidipine. Colloids Surf B Biointerfaces 2009;72(2): 181-7.
21. Duclairoir C, Orecchioni A, Depraetere P, Nakache E. nefidipine encapsulation and in vitro release from wheat gliadin nanoparticles. J Microencapsul 2002;19(1):53-60.
22. Quiñones JP, Gothelf KV, Kjems J, Yang C, Caballero AMH, Schmidt C, et al. Self-assembled nanoparticles of modified-chitosan conjugates for the sustained release of dl-nefidipine. Carbohydr Polym 2013;92(1):856-64.
23. Luo Y, Zhang B, Whent M, Yu L, Wang Q. Preparation and characterization of zein/chitosan complex for encapsulation of nefidipine, and its in vitro controlled release study. Colloids Surf B Biointerfaces 2011;85(2):145-52.
24. Cortés-Sanabria L, Paredes-Ceseña CA, Herrera-Llamas RM, Cruz-Bueno Y, Soto-Molina H, Pazarín L, et al. Comparison of cost-utility between automated peritoneal dialysis and continuous ambulatory peritoneal dialysis. Arch Med Res 2013;44(8):655-61.
25. Chaudhary K, Haddadin S, Nistala R, Papageorgio C. Intraperitoneal Drug Therapy: An Advantage. Curr Clin Pharmacol 2010;5(2):82-8.

26. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109-10.
27. Sun Y, Larry WO, Ying L. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988;34(3):497-500.
28. Feelisch M, Noack EA. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol 1987;139(1):19-30.
29. Knowles RG, Merrett M, Salter M, Moncada S. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem J 1990;270(3):833-6.
30. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95(2):351-8.
31. Wei H, Frenkel K. In vivo formation of oxidized DNA bases in tumor promoter-treated mouse skin. Cancer Res 1991;51(16):4443-9.
32. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25(1):192-205.
33. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. 1976. Biochem Biophys Res Commun 2012;425(3):503-9.
34. Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985;113:484-90.
35. Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. Methods Enzymol. 1981;77:398-405.
36. Celik O, Turkoz Y, Hascalik S, Hascalik M, Cigremis Y, Mizrak B, et al. The protective effect of caffeic acid phenethyl ester on ischemia-reperfusion injury in rat ovary. Eur J Obstet Gynecol Reprod Biol 2004;117(2):183-8.
37. Arosio B, Gagliano N, Fusaro LM, Parmeggiani L, Tagliabue J, Galetti P, et al. Aloe-Emodin quinone pretreatment reduces acute liver injury induced by carbon tetrachloride. Pharmacol Toxicol 2000;87(5):229-33.
38. Chatterjee PK, Patel NS, Kvale EO, Cuzzocrea S, Brown PA, Stewart KN, et al. Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury. Kidney Int 2002;61(3):862-71.
39. Jeddi S, Zaman J, Zadeh-Vakili A, Zarkesh M, Ghasemi A. Involvement of inducible nitric oxide synthase in the loss of cardioprotection by ischemic postconditioning in hypothyroid rats. Gene 2016;580(2):169-76.
40. Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. Br J Pharmacol 2003;138(4):532-43.
41. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem 2005;12(10):1161-208.
42. Niki E, Yoshida Y, Saito Y, Noguchi N. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun 2005;338(1):668-76.
43. Ximenes VF, Paino IM, Faria-Oliveira OM, Fonseca LM, Brunetti IL. Indole ring oxidation by activated leukocytes prevents the production of hypochlorous acid. Braz J Med Biol Res 2005;38(11):1575-83.
44. Van Antwerpen P, Boudjeltia KZ, Babar S, Legssyer I, Moreau P, Moguilevsky N, et al. Thiol-containing molecules interact with the myeloperoxidase/H2O2/chloride system to inhibit LDL oxidation. Biochem Biophys Res Commun 2005;337(1):82 -8.
45. Isaoglu U, Yılmaz M, Sener E, Cetin N, Altuner D,Bilen H, et al. The impaired balances of oxidant/antioxidant and COX-1/COX-2 in ovarian ischemia-reperfusion injury and prevention by nimesulide. Lat Am J Pharm 2012;31(10):1481-8.
46. Meister A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. Pharmacol Ther 1991;51(2):155-94.
47. Celebi F, Akbas A, Saglam MB. Effect of sertraline in indomethacin-induced gastric mucosal damage. Asian J Chem 2012;24(5):1966-70.
48. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006;160(1):1-40.
49. Sharma H, Zhang X, Dwivedi C.The effect of ghee (clarified butter) on serum lipid levels and microsomal lipid peroxidation. Ayu 2010;31(2):134-40.

50. Polat B, Suleyman H, Alp HH. Adaptation of rat gastric tissue against indomethacin toxicity. Chem Biol Interact 2010;186(1):82-9.
51. Shi HY, Li ZH, Zhang YX, Chen L, Xiang DY, Zhang YF. Two pear glutathione S-transferases genes are regulated during fruit development and involved in response to salicylic acid, auxin, and glucose signaling. PLoS One 2014;9(2):e89926.
52. Mansoorali KP, Prakash T, Kotresha D, Prabhu K, Rama Rao N. Cerebroprotective effect of Eclipta alba against global model of cerebral ischemia induced oxidative stress in rats. Phytomedicine 2012;19(12):1108-16.
53. Milligan JR, Aguilera JA, Nguyen TT, Ward JF, Kow YW, He B, et al. Yield of DNA strand breaks after base oxidation of plasmid DNA. Radiat Res 1999;151(3):334-42.
54. Grollman AP, Moriya M. Mutagenesis by 8-oxoguanine: an enemy within. Trends Genet 1993;9(7):246-9.
55. Aksoy AN. Ovarian Ischemia-Reperfusion Injury: A Brief Review. SM J Gynecol Obstet 2015;1:1008-111.
56. Srivastava G, Mehta JL. Currying the heart: curcumin and cardioprotection. J Cardiovasc Pharmacol Ther 2009; 14(1):22e7.
57. Uguralp S, Bay Karabulut A, Mizrak B. Effects of pentoxifylline and vitamin E on the bilateral ovary after experimental ovarian ischemia. Eur J Pediatr Surg 2005;15(2):107-13.
58. Nugent D, Newton H, Gallivan L, Gosden RG. Protective effect of vitamin E on ischaemia-reperfusion injury in ovarian grafts. J Reprod Fertil 1998;114:341-6.
59. Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. J Am Assoc Lab Anim Sci 2011;50(5): 600–13.