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Journal of Pediatric Surgery xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Protective effects of intraperitoneal administration of nimodipine on ischemia–reperfusion injury in ovaries: Histological and biochemical assessments in a rat model

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ARTICLE INFO

Article history:
Received 5 June 2016
Received in revised form 31 August 2016
Accepted 29 September 2016
Available online xxxx

Key words:
Ischemia–reperfusion
Nimodipine
Intraperitoneal
Ovary
Biochemical
Histopathology
Rat

ABSTRACT

Purpose: Ovarian torsion must be diagnosed and treated as much early as possible. The aim of the present study was to investigate effects of intraperitoneal administration of nimodipine on ischemia–reperfusion injury in ovaries.
Methods: Thirty healthy male Wistar rats weighing approximately 250 g were randomized into six experimental groups (n = 5): Group Sham: The rats underwent only laparotomy. Group I: A 3-h ischemia only. Group I/R: A 3-h ischemia and a 3-h reperfusion. Group I/Nimodipine: A 3-h ischemia only and 1 mg/kg intraperitoneal administration of nimodipine 2.5 h after induction of ischemia. Group I/R/Nimodipine: A 3-h ischemia, a 3-h reperfusion and 1 mg/kg intraperitoneal administration of nimodipine 2.5 h after induction of ischemia.
Results: Nimodipine treated animals 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/Nimodipine animals compared to those of other groups ($P < 0.05$). The damage indicators (NOS, MDA, MPO and DNA damage level) were significantly lower in I/R/Nimodipine animal compared to those of other groups ($P < 0.05$).
Conclusions: Intraperitoneal administration of nimodipine could be helpful in minimizing ischemia–reperfusion injury in ovarian tissue exposed to ischemia.

There are various conditions like long mesovarium and adnexal venous congestion that 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 are 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]. Owing 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 is 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 [13–15]. In most of these studies the protective agents have been administered orally. In search of

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http://dx.doi.org/10.1016/j.jpedsurg.2016.09.067
0022-3468/

an alternative method, the authors were encouraged to evaluate intraperitoneal administration of nimodipine in prevention of ischemia reperfusion injury in ovary 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 [16]. 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 [17].

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 [18]. 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. 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 [19].

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

1. Materials and methods

1.1. 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 °C ± 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 Oral et al., 2011. Thirty healthy male Wistar rats weighing approximately 250 g were randomized into six experimental groups (n = 5): Group Sham: The rats underwent only laparotomy. Group I: A 3-h ischemia only. Group I/R: A 3-h ischemia and a 3-h reperfusion. Group I/Nimodipine: A 3-h ischemia only and 1 mg/kg intraperitoneal administration of nimodipine (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) 2.5 h after induction of ischemia. Group I/R/Nimodipine: A 3-h ischemia, a 3-h reperfusion and 1 mg/kg intraperitoneal administration of nimodipine 2.5 h 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 °C for biochemical assessments.

1.2. Surgical procedure

Animals were anesthetized by intraperitoneal administration of ketamine–xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain [20]. 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-h 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-h period, the vascular clamps were chosen, removed and a 3-h reperfusion was continued. Then, the ovaries were dissected out for histopathological and biochemical assessments.

1.3. Histopathological assessments

Ovaries were fixed in 10% buffered formalin for 24 h. The tissue samples were then processed and embedded in paraffin. A 5-μm semithin 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 in2.5% buffered glutaraldehyde and postfixed in 2% OsO4 for 2 h, dehydrated through an ethanol series and embedded in Epon. Semithin transverse sections (5 μm) were next stained with toluidine blue and examined under a light microscope.

1.4. Biochemical assessments

1.4.1. Tissue processing for biochemical assessments of ovary

The tissue samples of ovaries were kept at −80 °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 min. Homogenates were filtered and centrifuged using a refrigerator centrifuge at 4 °C. Then the supernatants were used to determine the enzymatic activities. All assays were carried out at room temperature.

1.4.2. 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 [21]. Superoxide dismutase activity was then measured at 560 nm by the degree of inhibition of this reaction and expressed as millimoles per minute per milligram of tissue.

1.4.3. 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 [22]. 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 [23].

1.4.4. Malondialdehyde (MDA) analysis

Concentrations of ovarian lipid peroxidation were determined by estimating MDA using the thiobarbituric acid test [24]. 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 h. 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 using 1,1,3,3-tetramethoxypropane.

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1.4.5. Myeloperoxidase (MPO) analysis

The activity of MPO in the total homogenate was measured according to previously described methods [25]. 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.

1.4.6. Total glutathione (tGSH) analysis

The amount of GSH in the total homogenate was measured according to the previously described methods with some modifications [26]. 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 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.

1.4.7. Glutathione peroxidase (GPO) analysis

GPO activity was determined according to the method of Lawrence and Burk [27]. 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 as H_2O_2 was added, the chronometer was turned on and the absorbance at 340 nm was recorded for 5 min every 15 s.

1.4.8. 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 [28]. After tissue homogenization, supernatant was used for GR measurement. After the NADPH and GSSG addition, chronometer was on and absorbance was measured for 5 min in 30 min intervals at 340 nm spectrophotometrically.

1.4.9. Glutathione S-transferase (GST) activity

GST activity was determined based on Habig and Jakoby [29]. 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.

1.4.10. 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 resuspended and rehomogenized in 1 ml of extraction buffer (0.1 M Tris, pH 8.0, 0.1 M NaCl, 20 mM EDTA) and recentrifuged as above for 2 min. The washed pellet was resuspended in 300 μl of extraction buffer with a wide orifice 200 μl Pipetman tip. The resuspended pellet was subsequently incubated at 65 °C for 1 h 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 microcentrifuge 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.

1.4.11. 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 microvials, 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 redissolved in the eluent, final volume of 200 μl.

1.4.12. 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 an HPLC system equipped with an electrochemical detector, HP Agilent 1100 module series and E.C.D. HP 1049A. 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 vol acetonitrile and a 3 vol 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.

1.5. 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 $P < 0.05$.

2. Results

2.1. 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/Nimodipine group general histologic and cellular structures of the tissues were not normal in appearance, however, moderate vascular congestion and edema were observed. In I/R/Nimodipine group only a 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 mild inflammation, vascular congestion and edema (Fig. 1).

The numerical densities of neutrophils were also estimated, which were 14 × 10–6/μm3, 10 × 10–6/μm3, 16 × 10–6/μm3, 15 × 10–6/μm3 and 12 × 10–6/μm3 in Sham, I, I/R, I/Nimodipine and I/R/Nimodipine groups, respectively.

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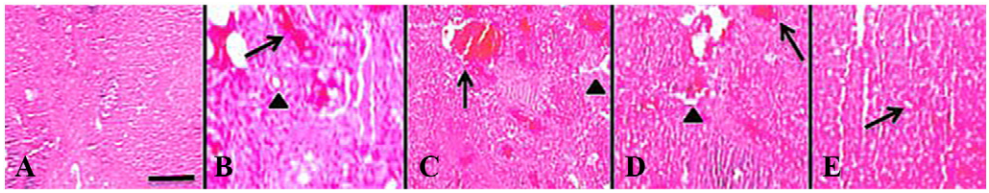

Fig. 1. Histologic micrographs of the ovarian tissue in Sham (A), I (B), I/R (C), I/Nimodipine (D) and I/R/Nimodipine (E) groups. Micrograph B shows condensed hemorrhage and severe vascular congestion (arrow), and severe edema (arrowhead). Micrograph C shows condensed hemorrhage and vascular congestion (arrow), and edema (arrowhead). Micrograph D shows moderate vascular congestion (arrow), and moderate edema (arrowhead). Micrograph E shows mild vascular congestion and edema (arrow). Scale bar: 200 μm.

2.2. Biomedical findings

2.2.1. Superoxide dismutase (SOD) analysis

The value of SOD activity was 68.4 ± 0.55 mmol/min/mg tissue in the sham group. The values of SOD were decreased to 34.4 ± 0.25 and 56.3 ± 0.19 mmol/min/mg tissue in I and I/R groups, respectively. However, intraperitoneal administration of 1 mg/kg of nimodipine inverted the trend and increased the activity of SOD to 74.5 ± 0.28 mmol/min/mg tissue in the ovarian tissue in I/R/Nimodipine groups. The value of SOD activity in I/R/Nimodipine group was significantly higher than those of the other experimental groups ($P < 0.05$) (Table 1).

2.2.2. Nitric oxide synthase (NOS) activity

The values of tNOS activities were increased in the I and I/R groups, which were significantly higher than those of Sham group ($P < 0.05$). However, intraperitoneal administration of 1 mg/kg of nimodipine inverted the trend and decreased tNOS activity in the rat's ovary. In I/R/Nimodipine group the value of tNOS activity was significantly lower than those of the other experimental groups ($P < 0.05$) (Table 1).

2.2.3. Malondialdehyde (MDA) analysis

The results of the present study showed that concentration of MDA in sham group was 4.7 ± 0.19 μmol/g protein in ovarian tissue. The MDA level I/R group was significantly increased to 10.6 ± 0.25 μmol/g protein ($P < 0.01$). Intraperitoneal administration of nimodipine significantly decreased level of MDA in ovarian tissues of I/R/Nimodipine animals ($P < 0.05$) (Table 1).

2.2.4. Myeloperoxidase (MPO) analysis

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

2.2.5. Total glutathione (tGSH) analysis

The values for tGSH levels were 9.6 ± .25 and 4.6 ± 0.21 nmol/g protein in Sham and I/R animals, respectively. Intraperitoneal administration of nimodipine significantly increased level of GSH in ovarian tissues of I/R/Nimodipine animals ($P < 0.05$) (Table 1).

2.2.6. Glutathione peroxidase (GPO) analysis

The values for GPO levels were 38.7 ± 2.25 and 17.8 ± 1.35 U/g protein in Sham and I/R animals, respectively. Intraperitoneal administration of nimodipine significantly increased level of GPO in ovarian tissues of I/R/Nimodipine animals ($P < 0.05$) (Table 1).

2.2.7. Glutathione reductase (GSHRd) analysis

The GSHRd activities in ovarian tissue in the Sham and I/R animals were 33.4 ± 3.23 and 16.8 ± 1.14 U/g protein, respectively. Intraperitoneal administration of nimodipine significantly increased level of GSHRd in ovarian tissues of I/R/Nimodipine animals ($P < 0.05$) (Table 1).

2.2.8. Glutathione S-transferase (GST) activity

The GST activities in ovarian tissue in the Sham and I/R animals were 20.7 ± 1.15 and 14.6 ± 1.45 U/g protein, respectively. Intraperitoneal

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.

Variables	Group Sham	Group I	Group I/R	Group I/Nimodipine	Group I/R/Nimodipine
SOD (mmol/min/mg)	68.4 ± 0.55	34.4 ± 0.25	56.3 ± 0.19	49.7 ± 0.34	74.5 ± 0.28*
NOS (nmol/min/mg)	3.6 ± 0.15	3.7 ± 0.12	3.7 ± 0.16	3.4 ± 0.19	3.1 ± 0.24*
MDA (μmol/g protein)	4.7 ± 0.19	12.3 ± 0.15	10.6 ± 0.25	9.4 ± 0.33	5.8 ± 16*
MPO (U/g protein)	6. 2 ± 0.14	16. 6 ± 0.32	13. 5 ± 0.23	12. 4 ± 0.18	8. 2 ± 0.45*
tGSH (nmol/g protein)	9.6 ± 0.25	2.7 ± 0.42	4.6 ± 0.21	5.4 ± 0.32	7.3 ± 0.19*
GPO (U/g protein)	38.7 ± 2.25	13.5 ± 2.18	17.8 ± 1.35	18.9 ± 1.36	28.4 ± 2.45*
GSHRd (U/g protein)	33.4 ± 2.23	9.7 ± 1.33	16.8 ± 1.14	19.7 ± 1.28	24.3 ± 2.35*
GST (U/g protein)	20.7 ± 1.15	10.5 ± 1.22	14.6 ± 1.45	15.4 ± 1.32	18.9 ± 1.15*
8-OHGua/Gua (pmol/L)	1.2 ± 0.11	2.4 ± 0.12	2.1 ± 0.13	1.8 ± 0.15	1.4 ± 0.31*

I: Ischemia, I/R: Ischemia–reperfusion, I/Nimodipine: Ischemia plus intraperitoneal administration of nimodipine, I/R/Nimodipine: Ischemia plus reperfusion plus intraperitoneal administration of nimodipine. 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 group.

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administration of nimodipine significantly increased level of GST in ovarian tissues of I/R/Nimodipine animals ($P < 0.05$) (Table 1).

2.2.9. Measurement of 8-hydroxy-2 deoxyguanine (8-OH gua)

The levels of 8-OHGual/Gua, a DNA damage product, were1.2 ± 0.2 and 2.1 ± 0.2 pmol/L in Sham and I/R animals, respectively. Intraperitoneal administration of nimodipine significantly decreased level of GSHRd in ovarian tissues of I/R/Nimodipine animals ($P < 0.05$) (Table 1).

3. Discussion

The present study investigated whether intraperitoneal administration of nimodipine 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 IP administration of nimodipine 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 nimodipine 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 [30]. Edema, vascular congestion, hemorrhage, and leukocyte infiltration in the I/R/Nimodipine animals were much milder than in the I and I/R groups. This was in agreement with reports of others on protective effects of amlodipine, montelukast, curcumin and CoQ_{10} on ischemia reperfusion injury of rat ovary [13,15,31,32].

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 IR/Nimodipine 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 [33]. It has been reported that oral administration of amlodipine (a calcium channel blocker), montelukast (an antioxidant agent) and Tadalafil (a phosphodiesterase type 5 inhibitor) increased SOD level in experimental I/R injuries [13,15,34]. Our results showed that in the I/R/Nimodipine animals, SOD was increased compared to those in I and I/R groups and intraperitoneal administration of nimodipine, 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 [35]. iNOS is increased after cellular stimulation via cytokines in macrophages, neutrophils, and microglia and may also contribute to late-stage tissue injury [36]. The iNOS is derived primarily from the polymorphonuclear neutrophilic leukocytes during reperfusion and downregulation of iNOS could limit cell injury caused by hypoxia [37,38]. 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. Downregulation of iNOS could limit cell injury caused by hypoxia. In another study amlodipine treated animals showed decreased level of iNOS when administered orally [15]. Our results showed that in the I/R/Nimodipine animals, iNOS was downregulated compared to those in I and I/R group. Thus, intraperitoneal administration of nimodipine protected ovarian tissue against ischemia–reperfusion injury.

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 [39,40]. MDA levels in the present study were found to be much lower in the I/R/Nimodipine animals compared to those in other experimental groups. This could protect the tissues against ischemia–reperfusion injury in nimodipine treated animals of our study, which was in agreement with report of others on the effect of lacidipine, tadalafil and CQ_{10} on ischemia–reperfusion induced oxidative damage in ovaries of female rats [32,34,41].

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 [42,43]. It has been demonstrated that MPO activity is increased in ischemia–reperfusion induced ovarian tissue using nimesulide, a relatively COX-2 selective, nonsteroidal anti-inflammatory drug [44]. This finding was in agreement with results of the present study. MPO activity was suppressed in nimodipine 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 [30]. 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 [45]. 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 [45]. In a study using oral administration of montelukast, the amount of GSH was increased [13]. These findings were in agreement with our results. We found that oxidative stress was minimized and the severe damage owing to sudden reperfusion was prevented in nimodipine treated animals.

GPO activity is significantly reduced in tissues undergoing oxidative stress-related conditions like ischemia–reperfusion injury [46]. 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 [47]. An investigation on the effect of vardenafil on ischemia–reperfusion (I/R) injury in rat ovary demonstrated that vardenafil treated animals showed decreased activity of GPO compared to control animals [48]. Consistently, in the present study a significant decrease in GPO activity was observed in ovarian tissues of I/R/Nimodipine animals.

GSH is oxidized during the detoxification of hydrogen peroxide radical. GSHRd is an NADPH-dependent enzyme that converts oxidized glutathione to reduced glutathione [49]. GSHRd is reported to show higher activity in healthy tissue and in parallel with tissue damage its activity is decreased [50]. In our study activity of GSHRd was significantly increased in nimodipine treated animals compared to those of I and I/R groups. This was in agreement with findings of others that investigated the effects of melatonin, famotidine, mirtazapine, and thiamine pyrophosphate on ischemia/reperfusion (I/R) injury in diabetic rats and evaluated oxidant and antioxidant marker measurement results [51].

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 nimodipine treated animals was significantly lower than those in I and I/R groups.

DNA molecules are damaged if free radicals are in a close proximity to the DNA molecules [53,54]. 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 [55]. 8-OH Gua is considered an important marker of DNA oxidation [56]. Prevention of ischemia–reperfusion injury in rat ovarian tissue with the on–off method resulted in higher levels of 8-OHGua in ischemic group [8]. Our findings showed that the ovarian tissues of the I and I/R animals had higher levels of 8-OHGua than those of the Sham animals. However, our results showed that there were no significant difference

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between Sham and nimodipine 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 play an important role to provide urgent protection against life-threatening complications from ischemia and to prevent future infertility [57].

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 [58]. 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 [19]. It seems that time saving is very important in emergency conditions like ovarian torsion.

In conclusion, histopathological results obtained from all the experimental groups were consistent with the results of the biochemical analyses indicating that intraperitoneal administration of nimodipine could be helpful in minimizing ischemia–reperfusion injury in ovarian tissue exposed to ischemia. Regarding the transperitoneal absorption of the nimodipine that is far faster than its oral administration, it could be considered in clinical practice wherein 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 nimodipine could improve ischemia–reperfusion injury in ovarian tissue exposed to ischemia. Thus, dose–response studies should be conducted for nimodipine to determine its maximal efficacy in minimizing ischemia–reperfusion injury in ovarian tissue.

Acknowledgments

The authors would like to thank Dr. Keyvan Amini, Department of Veterinary Pathology, University of Saskatchewan and Mr. Jaafary, Urmia Pathobiology Center, for their technical expertise.

References

[1] Oelsner G, Shashar D. Adnexal torsion. Clin Obstet Gynecol 2006;49:459–63.
[2] Geimanaite L, Trainavicius K. Ovarian torsion in children: management and outcomes. J Pediatr Surg 2013;48:1946–53.
[3] Celik A, Ergün O, Aldemir H, et al. Long-term results of conservative management of adnexal torsion in children. J Pediatr Surg 2005;40:704–8.
[4] Carden DL, Granger DN. Pathophysiology of ischaemia–reperfusion injury. J Pathol 2000;190:255–66.
[5] Zimmerman BJ, Granger DN. Reperfusion injury. Surg Clin North Am 1992;72:65–83.
[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;61:171–9.
[7] Halliwell B, Gutteridge JM. Free radicals in biology and medicine. London: Oxford University Press; 1999.
[8] Ingec M, Isaoglu U, Yilmaz M, et al. Prevention of ischemia–reperfusion injury in rat ovarian tissue with the on–off method. J Physiol Pharmacol 2011;62:575–82.
[9] Filho DW, Torres MA, Bordin AL, et al. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia- reperfusion injury. Mol Aspects Med 2004;25:199–210.
[10] Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 1998;39:1529–42.
[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;9:647–52.
[12] Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 1993;90:7915–22.
[13] Oral A, Odabasoglu F, Halici Z, 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;95:1360–6.
[14] Mogilner JG, Lurie M, Coran AG, et al. Effect of diclofenac on germ cell apoptosis following testicular ischemia–reperfusion injury in a rat. Pediatr Surg Int 2006;22:99–105.
[15] Halici Z, Karaca M, Keles ON, et al. Protective effects of amlodipine on ischemia/reperfusion injury of rat ovary: biochemical and histopathologic evaluation. Fertil Steril 2008;90:2408–15.
[16] Lindsay RW, Heaton JT, Edwards C, et al. Nimodipine and acceleration of functional recovery of the facial nerve after crush injury. Arch Facial Plast Surg 2010;12:49–52.
[17] Kulah B, Besler HT, Akdag M, et al. The effects of verapamil vs. allopurinol on intestinal ischemia/reperfusion injury in rats. An experimental study. Hepatogastroenterology 2004;51:401–7.
[18] Popovich R, Moncreif J, Nolph K. Continuous ambulatory peritoneal dialysis. Artif Organs 1978;2:84–6.
[19] Chaudhary K, Haddadin S, Nistala R, et al. Intraperitoneal drug therapy: an advantage. Curr Clin Pharmacol 2010;5:82–8.
[20] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.
[21] Sun Y, Larry WO, Ying L. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988;34:497–500.
[22] Feelisch M, Noack EA. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol 1987;139:19–30.
[23] Knowles RG, Merrett M, Salter M, et al. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. Biochem J 1990;270:833–6.
[24] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.
[25] Wei H, Frenkel K. In vivo formation of oxidized DNA bases in tumor promoter-treated mouse skin. Cancer Res 1991;51:4443–9.
[26] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25:192–205.
[27] Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 1976;71:952–8.
[28] Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985;113:484–90.
[29] Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. Methods Enzymol 1981;77:398–405.
[30] Celik O, Turkoz Y, Hascalik S, et al. The protective effect of caffeic acid phenethyl ester on ischemia–reperfusion injury in rat ovary. Eur J Obstet Gynecol Reprod Biol 2004;117:183–8.
[31] Sak ME, Soydinc HE, Sak S, et al. The protective effect of curcumin on ischemia–reperfusion injury in rat ovary. Int J Surg 2013;11(9):967–70.
[32] Ozler A, Turgut A, Görük NY, et al. Evaluation of the protective effects of CoQ10 on ovarian I/R injury: an experimental study. Gynecol Obstet Invest 2013;76(2):100–6.
[33] Arosio B, Gagliano N, Fusaro LM, et al. Aloe-Emodin quinone pretreatment reduces acute liver injury induced by carbon tetrachloride. Pharmacol Toxicol 2000;87:229–33.
[34] Arikan DC, Bakan V, Kurutas EB, et al. Protective effect of tadalafil on ischemia/reperfusion injury of rat ovary. J Pediatr Surg 2010;45(11):2203–9.
[35] Chatterjee PK, Patel NS, Kvale EO, et al. Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury. Kidney Int 2002;61:862–71.
[36] Jugdutt BI. Nitric oxide and cardioprotection during ischemia–reperfusion. Heart Fail Rev 2002;7:391–405.
[37] Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia–reperfusion injury and preconditioning. Br J Pharmacol 2003;138:532–43.
[38] Kiang JG, Tsen KT. Biology of hypoxia. Chin J Physiol 2006;49:223–33.
[39] Wilhelm J. Metabolic aspects of membrane lipid peroxidation. Acta Univ Carol Med Monogr 1990;137:1–53.
[40] Niki E, Yoshida Y, Saito Y, et al. Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Commun 2005;338:668–76.
[41] Kumbasar S, Yapca OE, Bilen H. The effect of lacidipine on ischemia–reperfusion induced oxidative damage in ovaries of female rats. Biomed Res-India 2012;23(4):495–500.
[42] Ximenes VF, Paino IM, Faria-Oliveira OM, et al. Indole ring oxidation by activated leukocytes prevents the production of hypochlorous acid. Braz J Med Biol Res 2005;38:1575–83.
[43] Van Antwerpen P, Boudjeltia KZ, Babar S, et al. Thiol-containing molecules interact with the myeloperoxidase/H2O2/chloride system to inhibit LDL oxidation. Biochem Biophys Res Commun 2005;337:82–8.
[44] Isaoglu U, Yılmaz M, Sener E, 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;31:1481–8.
[45] Meister A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. Pharmacol Ther 1991;51:155–94.
[46] Celebi F, Akbas A, Saglam MB. Effect of sertraline in indomethacin-induced gastric mucosal damage. Asian J Chem 2012;24:1966–70.
[47] Valko M, Rhodes CJ, Moncol J, et al. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006;160:1–40.
[48] Yurtcu E, Togrul C, Ozyer S. Dose dependent protective effects of vardenafil on ischemia–reperfusion injury with biochemical and histopathologic evaluation in rat ovary. J Pediatr Surg 2015;50(7):1205–9.

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[49] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–10.
[50] Polat B, Suleyman H, Alp HH. Adaptation of rat gastric tissue against indomethacin toxicity. Chem Biol Interact 2010;186:82–9.
[51] Yapca OE, Turan MI, Borekci B, et al. Bilateral ovarian ischemia/reperfusion injury and treatment options in rats with an induced model of diabetes. Iran J Basic Med Sci 2014;17(4):294–302.
[52] Pickett CB, Lu AY. Glutathione S-transferases: gene structure, regulation, and biological function. Annu Rev Biochem 1989;58:743–64.
[53] Mansoorali KP, Prakash T, Kotresha D, et al. Cerebroprotective effect of *Eclipta alba* against global model of cerebral ischemia induced oxidative stress in rats. Phytomedicine 2012;19:1108–16.
[54] Kayal R, Cakatay U. Basic mechanisms of protein oxidation. Cerrahpasa J Med 2004; 35:83–9.
[55] Milligan JR, Ward JF. Yield of single-strand breaks due to attack on DNA by scavenger-derived radicals. Radiat Res 1994;137:295–9.
[56] Grollman AP, Moriya M. Mutagenesis by 8-oxoguanine: an enemy within. Trends Genet 1993;9:246–9.
[57] Aksoy AN. Ovarian ischemia–reperfusion injury: a brief review. SM J Gynecol Obstet 2015;1:1008–111.
[58] Turner PV, Brabb T, Pekow C, et al. Administration of substances to laboratory animals: routes of administration and factors to consider. J Am Assoc Lab Anim Sci 2011;50:600–13.

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