Taiwanese Journal of Obstetrics & Gynecology 60 (2021) 45–50

Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Metformin reduces ovarian ischemia reperfusion injury in rats by improving oxidative/nitrosative stress

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ARTICLE INFO

Article history:
Accepted 17 August 2020

Keywords:
Ischemia reperfusion injury
Rat ovarian torsion
Metformin
Oxidative stress index
Peroxynitrite

ABSTRACT

Objective: To assess the preventive role of metformin on rat ovarian ischemia reperfusion injury.
Materials and methods: Forty rats were divided equally into five groups; Group 1: sham, Group 2: surgical control with 3-hr torsion and detorsion, Group 3: 50 mg/kg p.o. metformin 30 min before 3-hr torsion, Group 4; metformin just after detorsion, Group 5; metformin 30 min before torsion and just after detorsion. Bilateral ovaries and blood sample were obtained seven days after detorsion for biochemical and histopathological evaluation.
Results: Ovarian tissue total anti-oxidant status (TAS) levels were significantly increased in group 4 when compared to group 1, 2 and 3 (all $p < 0.01$). In addition, there was a significant decrease in tissue oxidative stress index (OSI) level in group 4 with respect to group 2 ($p < 0.01$). Moreover, serum levels of OSI were significantly higher in group 2 with respect to group 1 and 5 (both $p < 0.05$). Similarly, there was significant increase in serum levels of peroxynitrite in group 2 as compared to serum levels in group 3 and 5 ($p < 0.01$ and 0.05, respectively). Furthermore, there were significant decrease in histopathological scores metformin and sham groups when compared to rats in the control group (Group 2).
Conclusion: Metformin reduces ischemia reperfusion injury in rat torsion detorsion model by improving histopathological and biochemical findings including TAS, OSI and peroxynitrite.

Introduction

When the ovary alone or both the ovary and fallopian tube twists on the axis created between the infundibulopelvic ligament and the utero-ovarian ligament, ovarian or adnexal torsion occurs, respectively [1]. Ovarian torsion usually presents as acute lower abdominal pain and observed in all ages, particularly in reproductive aged women. It is one of the most common gynecological emergencies and accounts for 15% of surgically treated adnexal masses [2]. Unless diagnosed and treated promptly, ovarian torsion will result in loss of ovarian blood supply and eventually necrosis and irreversible damage in the ovarian tissue.

Factors affecting the decision of the surgeon include age, future fertility, menopausal status of the patient, and any evidence of malignancy. Conservative treatment includes only detorsion of the twisted ovary using the laparoscopy as the gold standart surgical technique, since the gross necrotic appearance of the twisted ovary alone is not a sufficient finding to perform oophorectomy. Moreover, residual blood supply of the twisted ovary allows ovarian follicles and parenchyma to survive [3].

However, ovarian viability and function is related not only to the period of ischemia, but also to reperfusion injury. Although, reestablishment of the blood supply by detorsion of the twisted ovary prevent ischemia, tissue damage at a cellular level continues due to oxygen-derived free radicals (ROS) and neutrophil infiltration, called as ischemia reperfusion injury [4]. Lots of pharmacologic agents with antioxidants and anti-inflammatory features have been

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https://doi.org/10.1016/j.tjog.2020.10.004

recently studied to treat ischemia reperfusion injury in an animal model [5–7].

Metformin, which is a first-line antidiabetic agent, is the recommended first-line treatment for type 2 diabetes mellitus and is related with a decreluased risk of cardiovascular disorders and death [8]. In addition, metformin is a pleiotropic agent, which has anti-inflammatory, antioxidant and antiproliferative properties besides its anti-diabetic action [9–12]. Moreover, a number of animal studies have shown that metformin administration protects brain, heart and testis from ischemia reperfusion injury by its antioxidant activity [13–15]. Nonetheless, there are only two studies in the literature presenting protective effects of metformin on ischemia reperfusion injury in the rat ovarian torsion detorsion model [16,17].

In this study, the rat model of torsion and detorsion was used to test whether metformin could prevent ovarian ischemia reperfusion injury by biochemical and histopathological analysis.

Materials and methods

Animal care

A total of 40 female adult Wistar Albino rats weighing between 150 and 250 g and aging between 10 and 15 weeks were included in this study. All experimental procedures were carried out at the Hacettepe University, Department of Physiology, Animal Research Laboratory. The animals were fed ad libitum and kept in a temperature and humidity controlled environment (22 ± 2 °C) with 12 h light/dark cycles. The study protocol was approved by the Hacettepe University, Ethical Committee on Animal Research (Approval No. 2019/08-02) with a commentary advising that both sham and surgical control group (16 rats totally) should be same (to decrease the number of rats used) with our another similar study (unpublished) at the same time period.

Surgical procedures and groups

Animals were randomly allocated into five groups as follows: Group 1 (n = 8, sham group): Abdominal wall was kept open for 3 h and then closed. Ovarian torsion and detorsion was not applied. The rats were followed-up for one week after the first operation without any medication. Group 2 (n = 8, torsion/detorsion + control group): Bilateral ovarian torsion was performed, and detorsion was applied after the 3 h of ischemia. No medication was given to the rats during follow-up period of one week. Group 3 (n = 8, torsion/detorsion + metformin prophylaxis only group): Thirty minutes before the start of ovarian torsion operation, the rats were administered only single dose of 50 mg/kg peroral (p.o.) metformin (Glucophage; Merck Sante S.A.S., Istanbul, Turkey) via orogastric tubes after dissolving in 2 mL of saline as in our previous studies [18,19]. No medication was given during the one week period of follow-up. Group 4 (n = 8, torsion/detorsion + metformin prophylaxis plus maintenance group): A dosage of 50 mg/kg p.o. metformin was given to each rats 30 min before the commencement of ovarian torsion and repeated just after detorsion proceeding 3 h of ischemia. Same dosage of metformin p.o. was administered daily for one week. Group 5 (n = 8, torsion/detorsion + metformin maintenance only group): The rats received a single dose of 50 mg/kg metformin p.o. just after the ovarian detorsion proceeding 3 h of ischemia and the treatment was repeated daily with the same dosage during the postoperative period. Study flow chart depicts treatment groups and study design more clearly in Fig. 1.

All rats were anaesthetized using the combination of ketamine hydrochloride (50 mg/kg Ketalar; Eczacibasi, Istanbul, Turkey) and xylazine hydrochloride (10 mg/kg Rompun; Bayer Turk Ilac Ltd., Istanbul, Turkey). Then, the abdomen was shaved and prepared with a povidone-iodine scrub. A laparotomy was performed by making a 20 mm longitudinal incision in the midline area of the lower abdomen, and the uterine horns and adnexa were viewed. Ischemia was induced for 3 h using a torsion model conducted by applying and rotating atraumatic vascular clips 360° clockwise to the vascular pedicle 1 cm above and below the each ovary bilaterally as in previous studies [7,8]. Abdominal wall (muscle–aponeurotic plane and skin) was sutured with 3-0 vicrly (polyglactin 910, Ethicon, Somerville, New Jersey). Bilateral oophorectomy was carried out one week after the first surgery in all groups for histological scoring and biochemical evaluation. A 1-mL blood sample was drawn from the vena cava of each rat for the measurement of biochemical markers. The rats were sacrificed by an overdose of anesthetic usage.

Histopathological examinations

Ovarian tissues were fixed in 10% formalin for 48 h. After fixation, the tissues were prepared using standard procedures and embedded in paraffin. Serial 5-μm sections were cut from ovary blocks with a microtome and stained with hematoxylin–eosin. For each specimen, all the sections with 10× magnification were analyzed using a light microscope (Olympus BX46; Olympus Corporation). Ovarian damage, including follicular cell degeneration, vascular congestion, hemorrhage, and inflammation (neutrophil infiltration), was scored histologically using a graduated scale (0 = none; 1 = mild; 2 = moderate; and 3 = severe).Total scores were calculated according to these parameters. Evaluation was performed by a pathologist who was blind to the study groups.

Biochemical analysis

Total anti-oxidant status (TAS) and total oxidant status (TOS) levels were measured by a colorimetric method applied by Erel [20]. The results are expressed in millimoles Trolox equivalent/L (mmol Trolox equivalent/g wet tissue) for TAS and micromolar hydrogen peroxide (H_2O_2) equivalent per liter of TOS (μmol H_2O_2 equivalent/g wet tissue) [21]. The ratio of TOS to TAS was accepted as oxidative stress index OSI. For calculation, the millimoles TAS unit was first converted to micro mole unit. OSI value was calculated according to the formula below. OSI (arbitrary unit): TOS (micromol H_2O_2 equivalent/gr protein)/TAS (micromol Trolox equivalent/gr protein) × 10. The peroxynitrite assay and protein content were determined, as previously described [22,23]. The results were expressed as micromole/L for serum and micromole/g for wet tissue for tissue, respectively. Biochemical measurements were carried out using a spectrophotometer (Shimadu U 1601, Japan).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS), Version 24.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis. A two-tailed p value of <0.05 was considered as significant. The mean, standard deviation and sample size values were presented in the tables for the continuous variables. The normality of the distribution of the variables was tested with the Shapiro–Wilk test. The homogeneity of the variances of the groups was tested with the Levene's test. Parametric variables were compared by one-way ANOVA, followed by Tukey's HSD test. The multi-group comparison of non-parametric variables was conducted by Kruskal Wallis test and pairwise comparisons were conducted by using Mann–Whitney-U test with Bonferroni correction. The Bonferroni correction was carried out by multiplying uncorrected p values

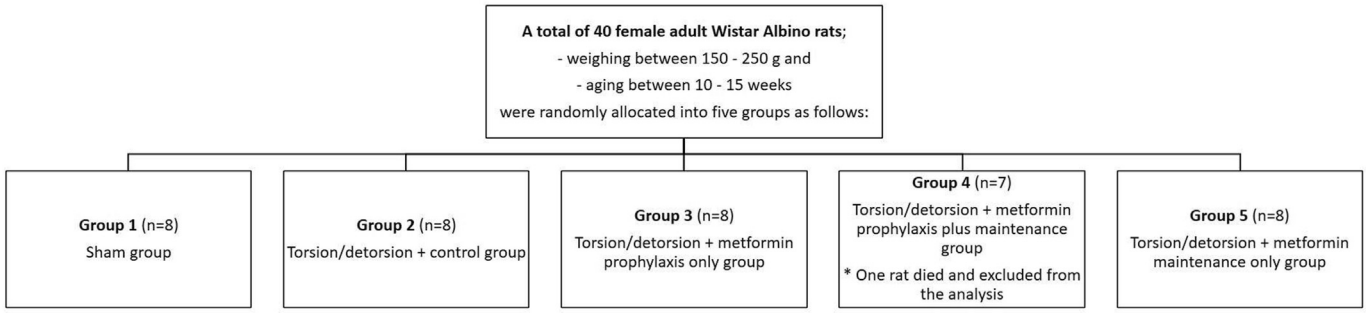

Fig. 1. Study flow chart.

with the total comparison number of the groups. For these adjusted *p* values, two-tailed *p*-value significance threshold was taken as 0.05.

Results

A total of 40 rats, with 8 rats in each group, were included in the study. One rat in group 4 was died and excluded from the analysis. Macroscopic appearance of surgical procedures of rat ovarian torsion detorsion model was as presented in Fig. 2: Ovaries just before (A) and after torsion (B), and just before (C) and one week after (D) detorsion.

Table 1 shows the comparison of tissue TAS, TOS, OSI and peroxynitrite levels among the five groups including sham (Group 1), surgical control (Group 2) and metformin treated (Group 3, 4 and 5) rats. Tissue levels of TOS and peroxynitrite were similar between

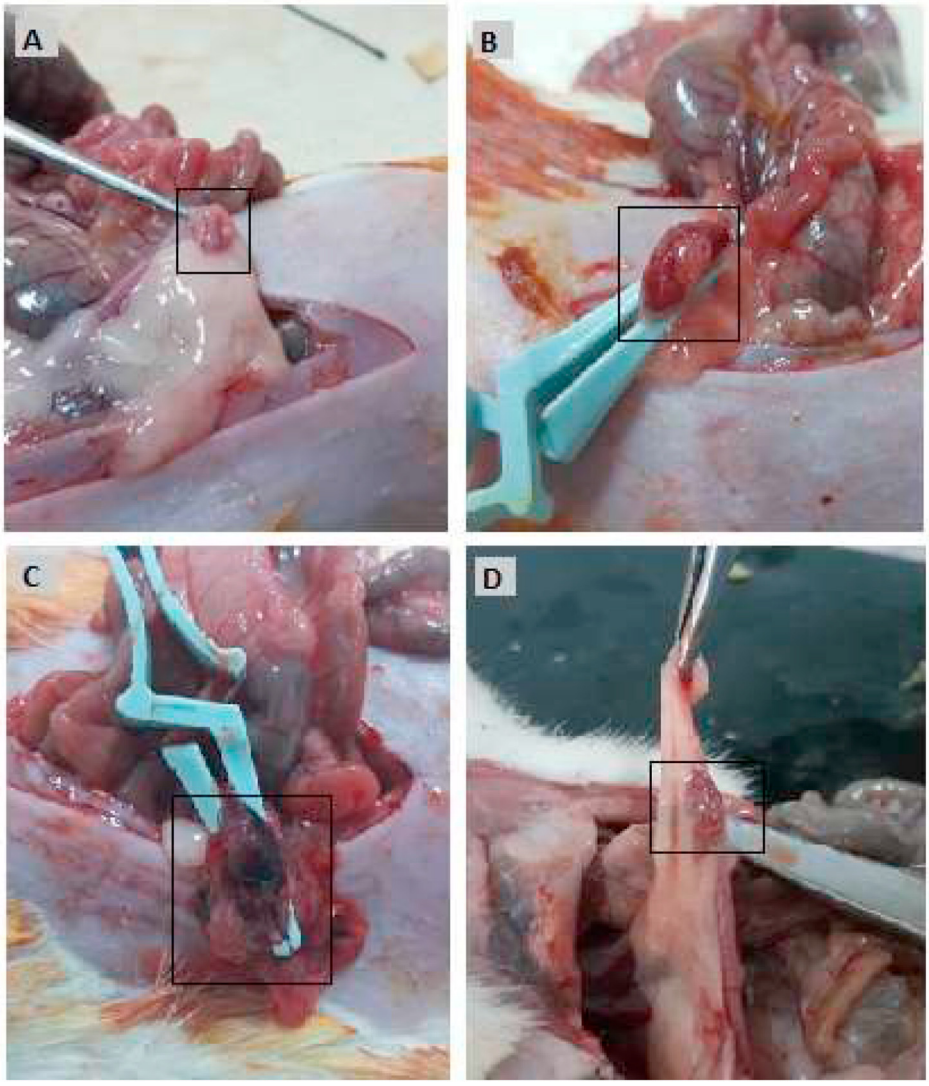

Fig. 2. Macroscopic appearance of rat ovaries before and after twisting: A; rat ovary just before torsion, B; rat ovary just after torsion by bulldog vascular clamps, C; black-bluish and dark appearing rat ovary just before detorsion proceeding 3 h of ischemia, D; rat ovary in the second laparotomy one week after the first laparotomy.

Table 1
Comparison of tissue levels of TAS, TOS, OSI, and peroxynitrite between metformin treated and control groups.

	TAS	TOS	OSI	Peroxynitrite
Group 1 (n: 8)	8.04 ± 1.36	83.59 ± 14.61	1.08 ± 0.34	38.18 ± 8.07
Group 2 (n: 8)	8.43 ± 1.83	114.80 ± 27.07	1.41 ± 0.38	45.34 ± 5.70
Group 3 (n: 8)	9.58 ± 1.54	100.31 ± 24.82	1.04 ± 0.14	36.39 ± 7.19
Group 4 (n: 7)	14.43 ± 4.21	121.44 ± 38.87	0.87 ± 0.24	36.14 ± 7.22
Group 5 (n: 8)	11.46 ± 3.27	107.81 ± 21.76	1.00 ± 0.28	34.60 ± 9.80
p-value	<0.001	0.070	0.013	0.071

Values are presented as mean ± SD, T; tissue, TAS; total antioxidant status, TOS; total oxidant status; OSI; oxidative stress index, n; number.
Units of ovarian tissue TAS; milimol Trolox Eq/gr, TOS; micromol H_2O_2 Eq/gr, OSI; arbitrary unit and peroxynitrite; micromol/gr.
p-values were calculated with One-way ANOVA. The significant pairwise group comparison results were shown below (Post Hoc test: Tukey HSD); For TAS: 1 vs 4: $p < 0.001$, 2 vs 4: $p = 0.001$, 3 vs 4: $p = 0.009$ For OSI: 2 vs 4: $p = 0.008$.

Table 2
Comparison of serum levels of TAS, TOS, OSI, and peroxynitrite between metformin treated and control groups.

	TAS	TOS	OSI	Peroxynitrite
Group 1 (n: 8)	1.12 ± 0.13	10.37 ± 6.80	0.96 ± 0.67	0.85 ± 0.20
Group 2 (n: 8)	1.04 ± 0.07	26.64 ± 10.12	2.56 ± 1.01	1.28 ± 0.24
Group 3 (n: 8)	1.18 ± 0.23	12.87 ± 3.22	1.10 ± 0.29	0.63 ± 0.16
Group 4 (n: 7)	1.12 ± 0.03	13.12 ± 4.69	1.17 ± 0.41	0.75 ± 0.28
Group 5 (n: 8)	1.13 ± 0.06	9.46 ± 3.20	0.84 ± 0.31	0.63 ± 0.26
p-value	0.303[a]	0.006[b]	0.004[b]	0.001[b]

Values are presented as mean ± SD, T; tissue, TAS; total antioxidant status, TOS; total oxidant status; OSI; oxidative stress index, n; number.
Units of serum TAS; milimol Trolox Eq/L, TOS; micromol H_2O_2 Eq/L, OSI; arbitrary unit and peroxynitrite; micromol/L.
p-values were calculated with.
[a] One-way ANOVA or.
[b] Kruskal Wallis. The significant pairwise group comparison results were shown below (by Mann–Whitney-U test with Bonferroni correction); for TOS: 1 vs 2: $p = 0.023$; for OSI: 1 vs 2: $p = 0.023$, 2 vs 5: $p = 0.045$; for peroxynitrite: 2 vs 3: $p = 0.008$, 2 vs 5: $p = 0.015$.

groups. However, tissue TAS levels were significantly increased in group 4 when compared to group 1, 2 and 3 (all $p < 0.01$). In addition, there was a significant decrease in serum OSI level in group 4 with respect to group 2 ($p < 0.01$).

Comparison of serum levels of these markers are presented in Table 2. Although serum TAS levels were did not differ between the groups, TOS levels was significantly increased in group 2 when compared to group 1 ($p < 0.05$). Moreover, serum levels of OSI were significantly higher in group 2 with respect to group 1 and 5 (both $p < 0.05$). Similarly, there was significant increase in serum levels of peroxynitrite in group 2 as compared to serum levels in group 3 and 5 ($p < 0.01$ and 0.05, respectively).

Histopathological scores of ovarian damage are as demonstrated in Table 3 and Fig. 3. Group 1 had significantly lower scores than almost all other groups. In addition, group 2 showed significantly higher scores of hemorrhage (both $p < 0.05$ vs group 3 vs 4), congestion (both $p < 0.05$ vs group 3 vs 5), full degeneration ($p < 0.05$ vs group 5), inflammation ($p < 0.01$ vs group 3 and $p < 0.05$ vs group 5) and total damage score ($p < 0.05$ vs group 4 and both $p < 0.01$ vs group 3 and 5) with respect to treatment groups.

Discussion

The present study was carried out to evaluate the protective effects of metformin on the ischemia-reperfusion injury of the rat ovary in surgically induced torsion and detorsion model by its action on oxidant and antioxidant status. Resulting findings of the study suggested that metformin significantly decreased the ischemia-reperfusion damage of the ovary as shown by biochemical and histopathological parameters. Metformin significantly decreased both tissue and serum OSI levels and serum levels of peroxynitrite while causing significant increase in tissue levels of TAS as compared to rats in control group. Moreover, histopathological evaluation of the rat ovaries has shown that hemorrhage, congestion, follicular degeneration, inflammation and total score of tissue damage were significantly lower in metformin treated group as compared to control rats.

Ovarian torsion is one of the important gynecological emergency which causes severe pelvic pain in women. Immediate detorsion is the preferred choice of the treatment. However, reperfusion after surgery by detorsion may cause oxidative stress by excessive release of ROS, which is responsible for the reperfusion injury of the detorsed ovary. Therefore, prevention of ovarian damage due to ischemia reperfusion injury became important in the area of gynecology practice.

Metformin is raises the scavenging ability of free radicals and their metabolites by enhancing antioxidant enzyme activity and decreasing mitochondrial ROS production [11,12]. We have reported that metformin was effective for prevention of adhesion formation and regression of endometriotic implants in rats due its antioxidant property [18,19]. Moreover, it is protective against ischemia reperfusion injury in various rat models as demonstrated in previous studies [14,15].

TOS and TAS show the total effects of all oxidants and antioxidants, respectively [20]. OSI is calculated with the formula of TOS/TAS and is assumed as a more appropriate index of oxidative stress on the plasma or tissue. Several antioxidant free radical scavengers have been shown to protect ovary against ischemia reperfusion injury in rats by improving TAS, TOS and OSI [24,25]. Peroxynitrite (ONOO−), the primary component of nitroxidative stress, is generated with nitricoxide (NO) and superoxide anion (O2•−) in the case of reperfusion after ischemia. As peroxynitrite exerts its cytotoxic action thru mitophagy, ihibiting peroxynitrite-mediated mitophagy activation improves ischemia

Table 3
Comparison of histopathological scores of ovarian tissue damage between metformin treated and control groups.

	Hemorrhage	Congestion	Full degeneration	Inflammation	Total score
Group 1 (n: 8)	0.00 ± 0.00	1.00 ± 0.00	0.13 ± 0.35	0.13 ± 0.35	1.25 ± 0.46
Group 2 (n: 8)	1.88 ± 0.35	1.88 ± 0.64	2.38 ± 0.74	2.75 ± 0.46	8.88 ± 1.25
Group 3 (n: 8)	1.13 ± 0.35	1.00 ± 0.00	1.38 ± 0.52	1.25 ± 0.46	4.75 ± 1.16
Group 4 (n: 7)	1.00 ± 0.00	1.00 ± 0.00	1.29 ± 0.49	2.14 ± 0.69	5.29 ± 0.76
Group 5 (n: 8)	1.25 ± 0.46	1.00 ± 0.00	0.63 ± 0.52	1.38 ± 0.52	4.13 ± 0.83
p-value	<0.001	<0.001	<0.001	<0.001	<0.001

Values are presented as mean ± SD, n; number.
p-values were calculated with Kruskal Wallis. The significant pairwise group comparison results by Mann–Whitney-U test with Bonferroni correction, were shown below; for hemorrhage: 1 vs 2: $p = 0.002$, 1 vs 3: $p = 0.002$, 1 vs 4: $p = 0.002$, 1 vs 5: $p = 0.002$, 2 vs 3: $p = 0.037$, 2 vs 4: $p = 0.011$; for congestion: 1 vs 2: $p = 0.031$, 2 vs 3: $p = 0.031$, 2 vs 5: $p = 0.031$; for full degeneration: 1 vs 2: $p = 0.005$, 1 vs 3: $p = 0.008$, 1 vs 4: $p = 0.012$, 2 vs 5: $p = 0.013$; for inflammation: 1 vs 2: $p = 0.003$, 1 vs 3: $p = 0.009$, 1 vs 4: $p = 0.007$, 1 vs 5: $p = 0.008$, 2 vs 3: $p = 0.008$, 2 vs 5: $p = 0.012$; for total score: 1 vs 2: $p = 0.004$, 1 vs 3: $p = 0.005$, 1 vs 4: $p = 0.007$, 1 vs 5: $p = 0.005$, 2 vs 3: $p = 0.009$, 2 vs 4: $p = 0.015$, 2 vs 5: $p = 0.006$.

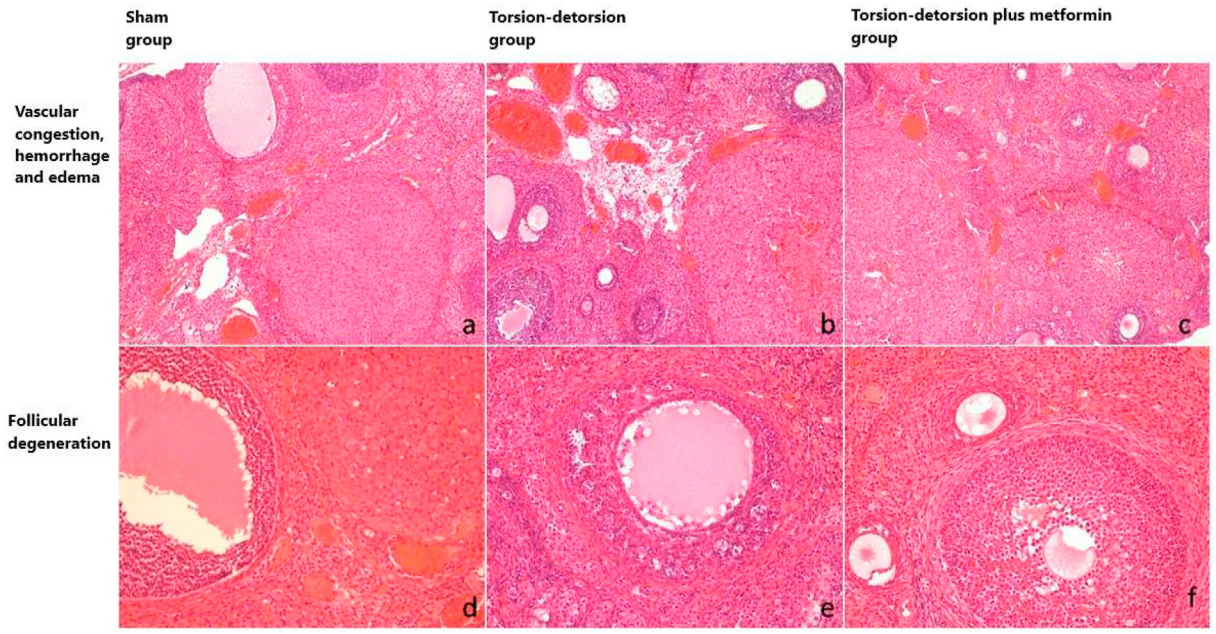

Fig. 3. Histological sections of rat ovaries stained with hematoxylin and eosin (H&E): a; normal ovarian morphology with normal ovarian cortical and follicular architecture in the Sham group, H&E ×100, b; vascular congestion, hemorrhage and edema increased in the torsion–detorsion group, H&E ×100, c; mild vascular congestion hemorrhage and edema in the torsion–detorsion + metformin group H&E ×100, d; ovarian follicle without follicular degeneration in sham group, H&E ×200, e; ovarian follicle with prominent follicular degeneration in torsion–detorsion group, H&E ×200, f; ovarian follicle with mild follicular degeneration in torsion–detorsion + metformin group, H&E ×200.

reperfusion injury [26]. In addition, metformin improves TOS, TAS, OSI and peroxynitrite by its antioxidation action as shown in previous studies [27–30].

As far as is known, the effect of metformin on the prevention of rat ovary from ischemia reperfusion injury has been investigated in only two studies up to date. In the first study, authors suggested that low and high metformin (100 and 500 mg/kg p.o., respectively) attenuated ovarian ischemia-reperfusion damage due to its antioxidant property by improving malondialdehyde and reduced-glutathione-to-oxidized-glutathione ratio [16]. Similarly, metformin (250 and 500 mg/kg p.o., respectively) was found to protect ovarian damage by improving tissue levels of malondialdehyde, glutathione, tumor necrosis factor-alpha, caspase-3 and nuclear factor kappa B (NF-Kb) in the second study [17].

In our present study, rats were divided into five groups: the sham group, the torsion–detorsion group, three metformin (50 mg/kg p.o.) groups (firstly, only before torsion as single dose in "prophylaxis group"; secondly, only after detorsion daily for one week in "the maintenance group"; and thirdly, both before torsion and after detorsion daily for one week in the "prophylaxis plus maintenance group", respectively).s.

The rationale in behind designing our treatment groups as the "prophylaxis group", "maintenance group" and "prophylaxis plus maintenance group" in our current study was to mimic real clinical scenario that if the patient attends with the suspicion of adnexal torsion, (i) the physician can give therapeutic agent before detorsion for "prophylactic treatment", (ii) the physician can give therapeutic agent only after detorsion for a while as daily for "maintenance treatment" or (iii) the physician can give therapeutic agent not only before detorsion for prophylactic treatment but also after detorsion for a while as daily for maintenance treatment (prophylaxis plus maintenance treatment).

The results of this study have demonstrated that metformin attenuated ischemia reperfusion injury in rat ovary by histopathological and biochemical findings as the second time in the literature. However, our study was first in the literature that metformin improved oxidative stress by improving both tissue and serum levels of OSI, and serum levels of TAS and peroxynitrite in rat ovarian torsion detorsion model. In addition, our study has shown that metformin was effective for preventing ischemia reperfusion injury with lower dose than the dose administered in the previous studies [16,17].

ROS induces cellular and mitochondrial membrane disruption and DNA injury due to lipid peroxidation, developing eventually apoptosis [31]. NF-Kb is a transcription factor that regulates the expression of multiple inflammatory and immune genes [32]. It is activated by ROS and may cause to the apoptosis [33]. It could be better to test inhibitory effect of metformin on NF-Kb to see the apoptosis status in rat ovaries in this study as metformin has been found to prevent NF-Kb activation by decreasing ROS production [17,34].

Experimental rat model of ovarian torsion detorsion cannot exactly represent the classical pathophysiology in clinical practice in human that is a limitation of this study. Therefore, large-scale prospective studies in humans are needed to determine the value of metformin for preventing of ovaries from ischemia reperfusion injury after detorsion.

In conclusion, results of this study show that metformin is effective for prevention of ischemia reperfusion injury in rat torsion detorsion model by improving histopathological and biochemical findings including tissue and serum levels of TAS, OSI and peroxynitrite.

Funding

This research received no grant.

Declaration of competing interest

Authors report no conflict of interest.

Acknowledgment

None.

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