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The Ovario-Protective Effect of Erythropoietin against Oxidative Damage Associated with Reperfusion Following Ovarian Torsion in Rat

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Abstract: Problem statement: To show the effect of recombinant Erythropoietin (rhEPO) on the ovarian viability and histology in the twisted ischemic ovaries in rats, followed by detorsion. **Approach:** An experimental study was conducted in primate clinic of the School of Medicine, Tabriz University of Medical Sciences from Dec. 2008 to Apr. 2009. Forty, 4 month old Wistar rats are cased in the present study divided into 4 groups. Ovarian ischemia was performed by torsion which was kept stable by using a vascular microclip for 4 h. In group 1, the ovary were surgically removed, fixed and analyzed histochemically. In group 2, the same procedure was repeated after 3 h reperfusion. In the next two groups, the same was performed rhEPO was administrated 400 u kg^{-1} 1 h after torsion of the ovaries. **Results:** Thirty-two out of 40 rats were followed. There was a significant difference between groups in the levels of Malondialdehyde (MDA), Total Antioxidants (TA), Superoxidase (SOD), Nitric Oxide (NO) glutathione ($p<0.001$). Addition of rhEPO maintained the normal histologic appearance in interventional groups compared with controls. **Conclusion:** Administration of rhEPO was effective in reducing the ischemic effect and free radicals damages of ovarian torsion in rats.

Key words: Ovarian torsion, Analysis Of Variance (ANOVA), Malondialdehyde (MDA), oxidative, cytokine, Superoxidase (SOD), ischemic effect

INTRODUCTION

Ovarian Torsion (OT) is one of the most encountered gynecological emergency requiring surgeries with an incidence of 3% in a series of acute gynecological complaints (Becker *et al*., 2009). To avoid complications and preserve future fertility, OT should be suspected at an early stage (Galinier *et al*., 2008). The preoperative findings of ovarian blood flow on Doppler sonography in cases with surgically proven adnexal torsion may be helpful in prediction of OT, but the accuracy remains low (Nizar *et al*., 2009). In routine practice, tends to remove adnexa to prevent embolism. However, it seems that this intervention is not a good option for young women especially during the reproductive years. Galinier *et al*. (2009) showed that when the ovary was preserved after detorsion, the outcome is good conservative approach after detorsion of black-bluish ovaries is safe and effective. Regarding these observations even after immediate surgical intervention, however, when encountering with a twisted adnexa, the best intervention to save ovarian function has not been elucidated yet. Several antioxidant supplementations have been investigated in reducing the ischemic effect of OT on reperfusion injury in a controlled experimental rat torsion-

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detorsion model, including Melatonin (Turkoz *et al*., 2004; Labibzadeh and Sadrnejad, 2007a), Aprotinin (Sahin *et al*., 2008), α-lipoic acid (Cosar *et al*., 2007), Genistein (Yazici *et al*., 2007), Iloprost (Ozat *et al*., 2009) Marrubium cordatum extract (Cigremis *et al*., 2009). Natural antioxidants have also been examined in testicular torsion. According to Unsal *et al*. (2006), dietary supplementation with Garlic Extract (GE) seems to attenuate the generation of toxic free radicals, as evidenced indirectly by low tissue MDA levels. EPO has been proved to be a multiple functional cytokine to attenuate Ischemia-Reperfusion (I/R) injury in various organs. In addition, the antioxidant effect of EPO has been demonstrated in a number of tissues (Sharples *et al*., 2004; Calvillo *et al*., 2003; Wu *et al*., 2006; Labibzadeh and Sadrnejad, 2007b). EPO and its Receptors (EPOR) have also been recognized to be essential for the survival, proliferation differentiation of erythroid progenitor cells (Calvillo *et al*., 2003; Labibzadeh *et al*., 2008). Based on these findings and other observations, we designed the present study to investigate the anti-oxidative effects of rhEPO and its possible usefulness for the ovarian protection at torsion in rat ovarian tissue. The histopathological changes together with oxidant status of rat ovaries before and after administration of EPO will also be covered.

MATERIALS AND METHODS

An experimental study was conducted from December 2008 to April 2009. Forty, 4 month old Wistar rats are cased in the study divided into 4 groups by simple randomization. All rats were anesthetized with Ketamine (5 mg kg^{-1}) and 0.2 ml diazepam anesthesia was maintained by supplementary intraperitoneally Ketamin. The anesthetized rats were placed onto a thermostatically controlled heating mat and body temperature maintained at 37 ± 0.5°C by means of a rectal probe attached to a homoeothermic blanket. A midline laparotomy was performed in order to carefully expose the ovaries.

The blood supply of the right ovaries, both artery and vein circulation, was interrupted by its torsion and the torsion was kept stable by using a vascular microclip. The ovary was fixed on the abdominal wall. The wound was closed by clips. Occlusion was verified visually by changing in the color of the ovary to a paler shade and reperfusion by a blush. At the end of all experiments, rats were sacrificed by an overdose of anesthetic.

Rats were housed and fed at the Animal Center of School of Medicine, before surgery for adaptation with the environment. All animals' procedures complied with an approved Tabriz University of Medical Sciences' animal care and use committee. The rhEPO (PD poetin) was purchased from Pooyesh Darou Pharmaceutical Co Iran. The study protocol was reviewed and approved by the Tabriz Research Affair Review Board.

Animals were randomly allocated into four groups as follow: In group 1: rats were subjected to the surgical procedures described above. Following an ischemic period of 4 h, the occluded adnexa was surgically removed and the ovaries were harvested for histopathological and biochemical studies. Then the rats were sacrificed. In group 2, the same procedure was repeated. The clips were removed and the ovaries detorsioned and allowed for reperfusion for 3 h, then surgically were removed. In the next two groups, the same procedure was performed and rhEPO (400 u kg^{-1}) was administrated intraperitoneally 1 h after occlusion of the ovarian vessels. The dose of rhEPO was chosen based on the findings of Villa *et al*., 2003; Bonakdar *et al*., 2007).

Measurement of biochemical parameters: After performing surgery, at the end of ischemic period and also at the end of the reperfusion period, the ovaries surgically have been removed in each group and fixed for biochemical examination. A part was fixed in 10% formalin neutral buffered for histological examination. Samples were transported in liquid nitrogen and kept frozen at -70°C.

Malondialdehyde (MDA) level as an index of lipid peroxidation was measured by the Thiobarbituric Acid (TBA) method (26). Superoxidase activities (SOD) were measured spectrophotometrically with RANSOD test kits (obtained from Randox Laboratories Ltd). Total Antioxidants (TA) was determined with the RANDOX kit. Plasma glutathione levels were measured by Cayman Immuno enzyme assay kit (LOT. 7032002). NO was measured spectrophotometrically by Miranda K.M method. In this method at first NO produce nitrate and nitrite. At second step simultaneous evaluation of nitrate and nitrite concentrations involves reduction of nitrate by vanadium (III) and detection with Griess reagents (Miranda *et al*., 2001).

Histopathological analysis: At the end of each experiment, a part of ovary fixed in 10% buffered formalin, embedded in paraffin, cut into 4 μm sections

stained with haematoxylin-eosin for histopathological analyzes vascular dilatation, interstitial edema, hemorrhage, PMN infiltration were examined and photomicrograph were taken.

All data are presented as means ± SD. Intragroup comparison was carried out using the Turkey Post Hoc test and One-way Analysis Of Variance (ANOVA). A 95% Confidence Interval (CI) for independent variables was calculated. For all statistical analyses, the differences were considered statistically significant at $p<0.05$. To evaluate the distributions, box-and whisker plots and the Kolmogorov-Smirnow (K-S) test of normality were used. The statistical analysis was performed by Statistical Package for Social Sciences program (SPSS version 15.0 for Windows).

RESULTS

Forty rats included, 8 of them demised and 32 of them (8 in each group) were eligible to follow-up. The results of biochemical analysis are shown in Table 1. The results of one-way ANOVA test show significant differences between 4 groups in the mean levels of MDA [$(F_{3,27}) = 65.54$, $p<0.001$)], TA [$(F_{3,27}) = 17.91$, $p<0.001$)], SOD [$(F_{3,27}) = 13.96$, $p<0.001$)], NO [$(F_{3,27}) = 18.35$, $p<0.001$)] glutathione [$(F_{3,27}) = 77.89$, $p<0.001$)]. According to the results of Turkey Post Hoc test analysis, there were significant differences between group 1 and other groups ($p<0.001$) at the levels of MDA. In addition, the same test shows significant difference between mean MDA of group 2 with other groups ($p<0.03$), but the mean difference between group 3 with group 4 which both had been received EPO, was not significant ($p = 0.99$) (Fig. 1). The data from this comparison for TA levels showed significant difference between group 1 and other groups ($p<0.027$), no significant difference between group 2 with group 3 ($p = 0.13$), significant difference between group 2 with other groups ($p<0.027$) no significant difference between group 3 with groups 2 and 4 ($p>0.13$). For SOD, there was no significant difference between group 1 and 2 ($p = 0.49$), no significant difference between group 3 with group 2 and 4 ($p>0.13$) also no significant difference between group 2 with group 1 and 3 ($p>0.08$). But the mean difference between group 2 with group 4 was statistically significant ($p = 0.01$).

In addition, according to our data for NO, there was no significant difference between group 1 and 2 ($p = 0.93$), with group 3 and group 4 ($p = 0.17$), but it was significant between group 3 and 4 ($p<0.001$) group 2 with group 3 and 4 ($p<0.004$) (Fig. 2).

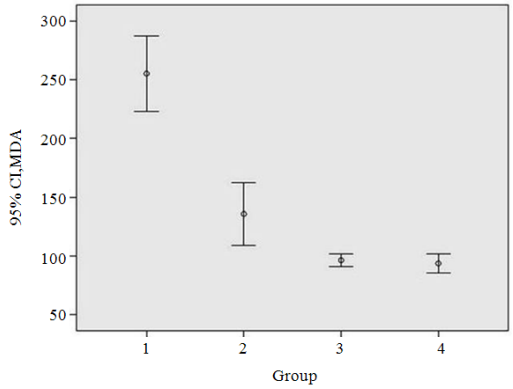

Fig. 1: The changes at the mean levels of MDA in the right ovary of studied groups. MDA: Malondialdehyde, 95% CI: 95% confidence interval.1: Group 1: The ovary was removed 4 h after occlusion. 2: Group 2: The ovary was allowed for reperfusion unit 3 h after 4 h ischemic period, then removed. Group 3: rhEPO (400 μ kg^{-1}) was administrated intraperitoneally 1 h after occlusion of the ovary. The ovary was removed 4 h after occlusion. Group 4: rhEPO (400 μ kg^{-1}) was administrated intraperitoneally 1 h after occlusion of the ovary. The ovary was allowed for reperfusion until 3 h after 4 h ischemic period, then removed

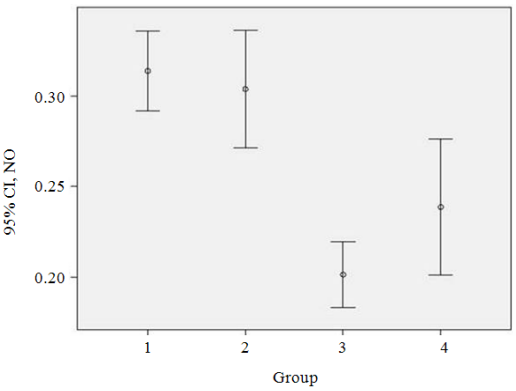

Fig. 2: The changes at the mean levels of NO in the right ovary of studied groups. NO: Nitric Oxide, 95% CI: 95% confidence interval.1: Group 1: the ovary was removed 4 h after occlusion. 2: Group 2: The ovary was allowed for reperfusion unit 3 h after 4 h ischemic period, then removed. Group 3: rhEPO (400 μ kg^{-1}) was administrated intraperitoneally 1 h after occlusion of the ovary. The ovary was removed 4 h after occlusion. Group 4: rhEPO (400 μ kg^{-1}) was administrated intraperitoneally 1 h after occlusion of the ovary. The ovary was allowed for reperfusion until 3 h after 4 h ischemic period, then removed

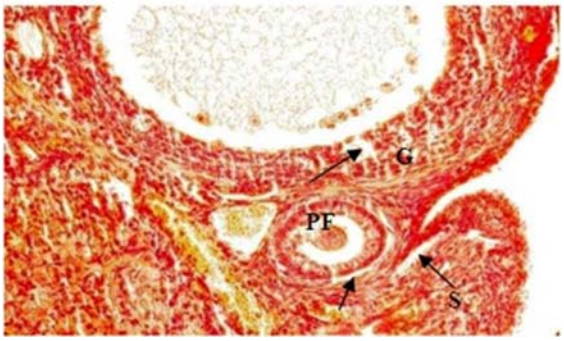

Fig. 3: A section from ovary 4 h after torsion showing parts of an antenatal follicle and a late Primary Follicle (PF). As the figure shows the Granulose (G) and Stormal (S) cells are separated by accumulation of extra cellular fluid, in the oocyte, perivitelline space is enlarged and oocyte is in a degenerating starus. (haematoxyli-eosin, original magnification 3100)

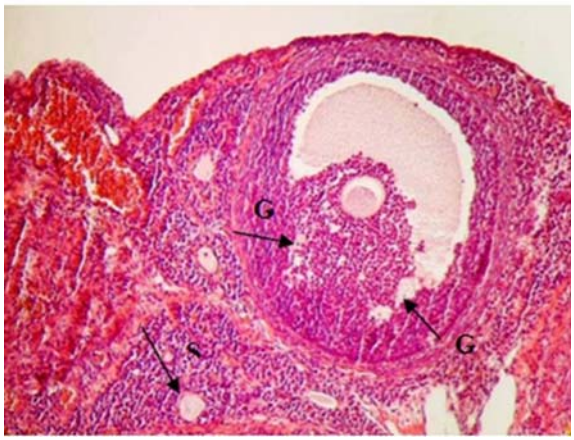

Fig. 4: A section from ovary torsion 4 h and removed after 3 h. Postdetorsioning anantenatal follicle is present in the figure with the ovarian stromal cells (S) and follicular granulose (G). The granulose and strromal cells are separated by extra cellular fluid. The oocyte has a degenerating appearance and zona pellucid looks very thin. (haematoxyli-eosin, original magnification 3100)

There was also significant difference between mean levels of glutathione between group 1 and other groups ($p<0.001$), but there were no significant differences between mean levels of glutathione between group 3 and group 4 ($p = 0.97$). Histopathological changes of ovarian stroma and follicles are shown in Fig. 3-6.

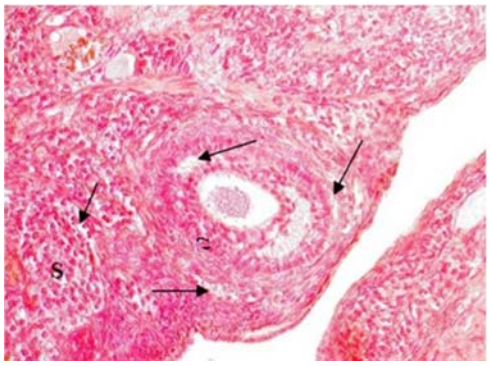

Fig. 5: Received erythropoietin 1 h after torsion. Ovary removed 4 h after torsion. The granulose (G) and Stormal cells (S) are separated from each other. The cells in Storma (S) and Granulose layer (G) are normal in size but intercellular space are enlarged (arrow). (haematoxyli-eosin, original magnification 3100)

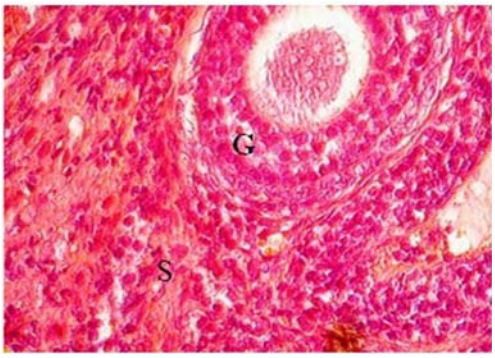

Fig. 6: A section from ovary 3 h after detorsion, which received EPO 1 h after torsion. The Stromal (S) and Granulosa cells (G) and ECM appears almost normal, indicating protective effect of erythropoietin injection. Zona pellucid has normal width and oocyte looks normal. (haematoxyli-eosin, original magnification 3100)

DISCUSSION

Although considerable progress has been made in the diagnosis and treatment of ovarian torsion, the problem remains as a challenging issue, often leading to delayed operative intervention and resultant ovarian loss (Oltmann *et al*., 2009). To preserve the ovarian function, treatment of torsion should be as conservative as possible (Rousseau *et al*., 2008). RhEPO which was used in this study has several beneficial effects in ischemic organs (Sharples *et al*., 2004; Bonakdar *et al*., 2007; Calvillo *et al*., 2003; Wu *et al*., 2006). EPO is a

potent antioxidant (Asli-Ardeh and Abbaspour-Gilandeh, 2008). From the findings of this study, rhEPO exerted an antioxidant effect in reducing ischemia/reperfusion induced tissue damage in ovary. The data also showed an increase in the tissue levels of MDA after ovarian torsion which, in turn, probably resulted in ovarian tissue damage by means of lipid peroxidation (Fig. 3 and 4). According to Celik *et al*. (2004), MDA is a marker of tissue injury increased levels of it reflect the destruction of unsaturated fatty acids in the cell membrane. In our study, administration of rhEPO significantly reduced the oxidant levels within the ovary (Table 1) a reduction at the levels of MDA, prevented post-ischemic ovarian injury and maintained the ovarian morphology (Fig. 5 and 6). Therefore, based on these results, the beneficial effects of rhEPO could be attributed, at least in part, to a reduction in the oxidative injury to ovarian cellular tissue and a stimulatory effect on SOD, NO, TA and glutathione (Table 1). Histopathologically, we determined that EPO group was better than controls with normal histopathologic appearance (Fig. 5 and 6). One possible explanation for these findings is the ability of rhEPO to protect the tissues from damages associated with hypoxia. The histological and biochemical results in the present study are in agreement with those reports which suggest administration of EPO for reduction of oxidative stress in other organs (Sharples *et al*., 2004; Calvillo *et al*., 2003; Wu *et al*., 2006; Nejat *et al*., 2009; Karami *et al*., 2009).

The role of Erythropoietin-Erythropoietin-Receptor (EPO-EPO-R) system in the cellular survival pathway has also been well recognized (Aydin *et al*., 2007). EPO is a tissue-protective cytokine this seems to be true for all organs expressing the EPO receptor. The antioxidative effects of EPO have also demonstrated by Katavetin *et al*. (2007). According to these authors, the antioxidative properties of EPO may arise from its direct effect on intracellular antioxidative mechanisms such as heme oxygenase-1 and glutathione peroxidase. It is also possible that EPO induces iron depletion and thereby inhibiting iron-dependent oxidative injury. In addition, it has been suggested that EPO can increase red blood cells leading to a reduction in cellular oxidative stress. According to the literature, EPO also exerts its beneficial effect in ischemic organs by prevention of apoptosis, probably by increasing Bcl2 and HSP70 expression (Celik *et al*., 2004; Bonakdar *et al*., 2007). The time which the tissue exposing to ischemia is critical. Calvillo *et al*. (2003; Bonakdar *et al*., 2007), in an experimental study on myocardial cells showed that rhEPO prevents apoptosis when the cells exposed to extreme and prolonged hypoxia. In their study reduction of medium oxygen content to <1% triggered a majority of cells to enter in apoptosis. It appears that the ovaries have high resistance to ischemia (Ming *et al*., 2001; Bonakdar *et al*., 2007). EPO is a protective tissue cytokine it's receptors have been shown to exist in lots of tissues. The anti-inflammatory effect, angiogenesis anti-apoptosis pleiotropic effects of EPO make possible the clinical application of it. As a result, rhEPO mediated resistance of tissues to damage may help to account for the increased cell survival. In addition, when dealing with I/R, a potential role for rhEPO is the recruitment of stem cells into the region of injury (Shringo *et al*., 2001). Its safety has also been demonstrated in orthopedic surgeries (Shringo *et al*., 2001) shock management (Calvillo *et al*., 2003; Nejat *et al*., 2009). Tissue protective properties of erythropoietin as a hematopoietic growth factor are also recognized in preterm infants and term neonates by inhibition the production of various cytokines (Santoro *et al*., 2007). The main concept that emerges from our study and above observations is that rhEPO induces a tolerance of the ovary and other organs to a subsequent insult with I/R. However, these findings are not ignoring the importance of early diagnosis, conservative surgical and medical management in OT. More studies are necessary to be conducted to show the effect of erythropoietin at a longer time from occlusion. In addition, to implement administration of EPO against oxidative damage associated with reperfusion, clinical studies will be necessary to examine the therapeutic properties of rhEPO in patients with ovarian torsion.

Table 1: The results of biochemical analysis

Group	MDA mean±SD† (95% CI)‡ (p<0.001)*	TAO mean±SD (95% CI) (p=<0.001)	SOD mean±SD (95% CI) (p<0.001)	NO mean±SD (95% CI) p<0.001)	Glutathione mean±SD (95% CI) (p<0.001)
1	255.19±38.62	0.53±0.13	0.45±0.02	0.31±0.02	198.63±15.80
	(222.90-287.48)	(0.42-0.64)	(0.43-0.47)	(0.29-0.33)	(185.41-211.84)
2	135.75±32.15	0.99±0.20	0.49±0.01	0.30±0.03	277.55±43.07
	(108.87-162.63)	(0.82-1.16)	(0.47-0.51)	(0.27-0.33)	(241.74-313.76)
3	96.43±5.99	1.35±0.35	0.57±0.05	0.20±0.01	451.86±46.96
	(90.88-101.97)	(1.02-1.67)	(0.52-0.62)	(0.18-0.21)	(408.67-495.04)
4	93.63±9.91	1.58±0.43	0.53±0.8	0.23±0.04	443.88±45.25
	(85.34-101.91)	(1.22-1.95)	(0.54-0.70)	(0.20-0.27)	(406.05-481.70)

The results showed that the erythropoietin was effective in reducing the ischemic effect of ovarian torsion in female rats. Histochemical changes show that adnexal integrity is maintained at the presence of prompt surgical and medical intervention. To bring this entity and its great variability in clinical presentation into focus once again, we are highlighting the importance of subject. Taken together, the data presented in this report provide important information in regard to the possible effect of EPO. Further researches to examine the effect of rhEPO in human ovarian torsion are recommended.

CONCLUSION

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