ORIGINAL ARTICLE

https://doi.org/10.1590/1806-9282.20220385

Effect of protection of enoxaparin against experimental ischemia/reperfusion injury in the rat ovary on in vitro fertilization outcomes

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SUMMARY

OBJECTIVE: The study aimed to investigate the protection of enoxaparin (E) against experimental ischemic (I) and ischemic-reperfusion (I/R) injury in rat ovaries on in vitro fertilization outcomes.

METHODS: In total, 56 adult female Sprague-Dawley albino rats were randomly assigned to 6 groups of 8 animals each: Sham, Ischemia, I/R, Sham+E, I+E, and I/R+E. Ischemia groups were subjected to bilateral adnexal torsion for 3 h. In contrast, I/R and I/R+E groups received subsequent detorsion for 3 h. Enoxaparin (0.5 mg/kg s.c.) was administered 30 min prior to ischemia (I+platelet-rich plasma) or reperfusion (I/R+I+platelet-rich plasma). Ovaries were stimulated through intraperitoneal injection of 150–300 internal units IU/kg pregnant mare serum gonadotropin. Anti-Müllerian hormone levels were measured before and after surgery in all groups.

RESULTS: When the number of metaphase II oocytes was evaluated, statistically significant differences were observed between the I and I+E (p=0.001) and I/R and I/R+E (p=0.000) groups. When both I and I+E groups and I/R and I/R+E groups were compared, it was found that E application increased the number of fertilized oocytes. The number of embryos on the second day was higher in the I/R+E group than that in the I/R group. Statistically significant differences were found in the number of grade 1 embryos between the I/R and I/R+E groups (p=0.003). In comparing anti-Müllerian hormone values within the group, the highest decrease was observed in the I and I/R groups.

CONCLUSION: Enoxaparin effectively minimizes ovarian damage and preserves ovarian reserve following ovarian torsion.

KEYWORDS: Fertilization in vitro. Reperfusion injury. Ovarian torsion. Enoxaparin.

INTRODUCTION

Adnexal torsion is a surgical emergency resulting from partial or complete rotation of the ovary, fallopian tube, or both[1]. It has an annual prevalence of approximately 2–6%, commonly in women of reproductive age[2].

It may occur in normal ovaries, with the conditions such as ovarian and/or paratubal cysts, hyperlaxity of the utero-ovarian or infundibulopelvic ligaments, as well as ovulation induction[3]. Timely diagnosis and subsequent surgical intervention for detorsion are crucial to preserving ovarian function and future fertility[3]. The most successful strategy for preserving ovarian tissue is detorsion of the twisted tissues with early surgical intervention[4]. While the ischemic injury is the possible cause of adnexal damage that occurs as the initial result of torsion, ischemia/reperfusion (I/R) injury[5]. Oxidative stress causes tissue damage due to the imbalance between the free radicals formed and the antioxidant defense mechanisms[6]. For this reason, it was determined that the treatment performed as detorsion alone was insufficient, and it was stated that an effective antioxidant and anti-inflammatory treatment could be effective[7].

Enoxaparin (E) sodium, a low-molecular-weight heparin (LMWH), is an anticoagulant agent and has been shown to protect ovarian reserve against ovarian I/R injury by evaluating histopathological damage scores in a rat ovarian torsion model[8].

The protective effects of many agents against I- and I/R-related damage to the ovarian tissue due to ovarian torsion have been demonstrated by histopathological or serum biochemical markers[6,8].

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Conflicts of interest: the authors declare there is no conflicts of interest. Funding: none.
Received on July 10, 2022. Accepted on July 10, 2022.

Although histopathological and biochemical results were obtained in these studies, they do not provide sufficient information about the number of oocytes, embryo number, and quality that result in reproductive physiology.

Therefore, we planned to evaluate the number and quality of embryos obtained by oocyte retrieval and in vitro fertilization (IVF) to predict reproductive outcomes. We aimed to support the situation by evaluating the serum anti-Müllerian hormone (AMH) level.

METHODS

Ethics and animals

The study was conducted in Sakarya University's SÜDETAM laboratory under the authority of Sakarya University's experimental animal ethics committee on 05/05/2021 under decision No. 25. Applications for all research animals were carried out according to the "The European Commission Directive 86/609/ECC guideline" protocol.

The study consisted of a total of 36 virgin Sprague-Dawley albino rats (weighing 220–260 g) and 1 male Sprague-Dawley albino rat (weighing 300 g). Animals were fed ad libitum and tap water in 13 separate cages, using controlled ambient conditions of 20–24°C and 50–60% humidity, with a 12-h light/dark cycle.

Surgical procedures and experimental protocol

The rats were arbitrarily categorized into six groups of six animals each: Sham (S) operation, Ischemia (I) (3 h), I/R (3 h ischemia plus 3 h reperfusion), S+E (0.5 mg/kg enoxaparin s.c. 30 min before surgery), I+E (0.5 mg/kg enoxaparin s.c. 30 min before surgery, 3 h ischemia), and I/R+E (3 h ischemia and 3 h reperfusion with 0.5 mg/kg enoxaparin s.c. before reperfusion).

Ketamine hydrochloride (30 mg/kg Ketalar; Eczacıbaşı, İstanbul, Turkey) and xylazine hydrochloride (15 mg/kg Rompun; Bayer Türk_Ilaç Ltd., İstanbul, Turkey) were used for anesthesia applied to rats[9]. A preoperative blood sample (AMH1) was taken from each rat. Uterine horns and adnexa were observed after a 2-cm longitudinal midline incision. The abdominal wall was closed with 3/0 silk sutures in the S group after 2 min observation. In group I, the ovarian pedicles were rotated 360° clockwise and fixed to the abdominal wall. In the I/R group, the 3 h ischemia period. In the S+E group, enoxaparin 0.5 mg/kg (s.c.) was administered intraperitoneally (i.p.) 30 min before the sham operation. In the I+E group, adnexal torsion was performed, as described, 30 min after 0.5 mg/kg enoxaparin (s.c.) administration. In the I/R+E group, 0.5 mg/kg enoxaparin (s.c.) was followed 30 min later by sequential bilateral adnexal torsion and detorsion.

Rats were prepared for ovulation induction and IVF after waiting for three consecutive estrous cycles. On the day of stimulation, female rats were sacrificed, and their oocytes were collected (Figure 1). Oocytes were classified as germinal vesicle, metaphase I (MI), and metaphase II (MII) stages. To compare the meiotic progression in the maturation process of oocytes in different systems, the mean times taken by each stage of nuclear progression as previously described by Sirard et al. were used[10].

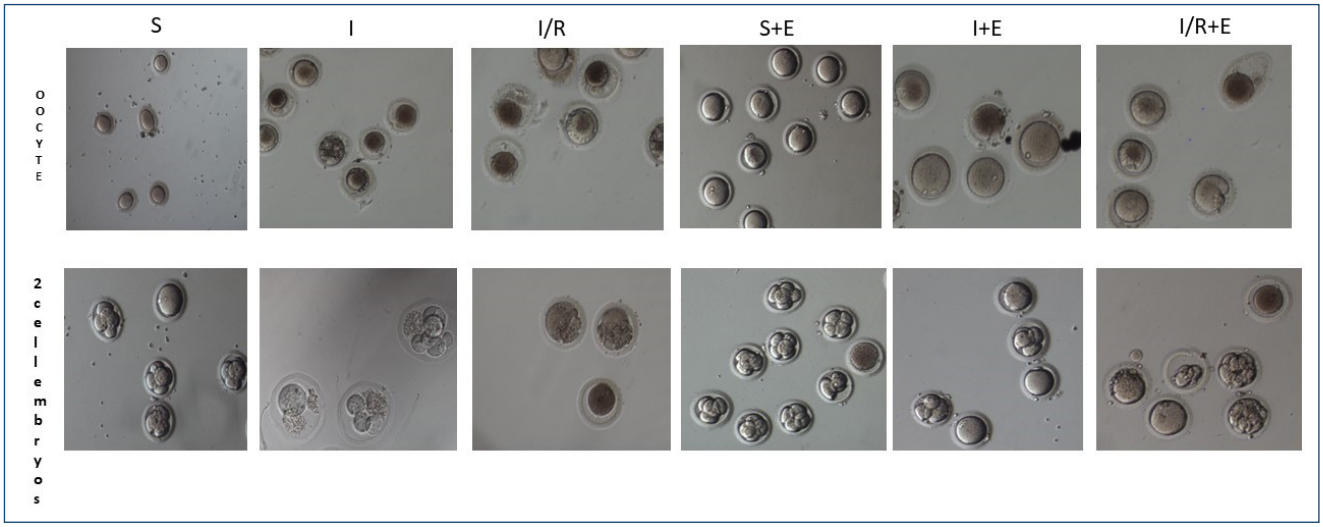

Figure 1. Oocytes and second-day embryos of the groups are seen; 100× magnification. The second-day embryo counts, oocytes maturation and embryo quality in the S and S+E groups were quite good compared to the other groups. It is seen that the quality and number of oocytes and embryos on the second day are significantly better in the I+E and I/R+E groups compared to the I and I/R groups, respectively. S: Sham operation; I: Ischemia; I/R: Ischemia and reperfusion; S+E: Sham+Enoxaparin; I+E: Ischemia+Enoxaparin; I/R+E: Ischemia and reperfusion+Enoxaparin.

Stimulation and collection of oocytes

For ovarian stimulation, 150–300 IU/kg pregnant mare serum gonadotropin (Chronogest/PMSG, Intervet, Istanbul, Turkey) was administered using i.p. injection, followed by 150–300 IU/kg human chorionic gonadotropin (hCG; Gonatropin, Chorulon ® Intervet, Istanbul, Turkey) approximately 48 h later. Notably, 15 IU Pregnant Mare Serum Gonadotropin (PMSG) was administered 17–19 h after hCG administration[11]. After anesthesia was applied to all rats, they were immobilized on a standard operating board, blood samples (AMH2) were taken, ovaries were removed, and the oocytes to be used in the study were collected from the ovaries. A human tubal fluid medium (Cat. No. 90166, Irvine Scientific, USA) was used for sperm pre-incubation, fertilization, and embryo transfer. After fertilization control, fertilized embryos were washed and transferred to culture drops, and the resulting embryos were followed up to the two-cell stage[11]. Before the oocyte collection, male rat testicles were excised under appropriate anesthesia. The epididymis was carefully peeled off using forceps, and the sperm were transferred into petri dishes and incubated at 37°C for 30 min before IVF[12]. After pre-incubating the sperm for 15–60 min, the final sperm concentration was found to be approximately 4.5 to 6×10^5 mL.

After transferring them to culture medium containing sperm in cumulus-oocyte complexes, they were incubated in culture medium at 37°C and 5% CO_2 for 10 h. Oocytes with two pronuclei (2PN) and at least one sperm tail in the ooplasm under the inverted microscope used for fertilization control were considered fertilized[13]. Total cell count was counted as described by Ahumada as an embryo evaluation criterion[14]. Embryo grade assessment was performed based on Veeck's cleavage-stage embryo score using blastomere symmetry and fragmentation rate[15].

Hormonal assays

Serum concentrations of AMH were quantified using enzyme-linked immunosorbent assay (ELISA) AMH kit according to the manufacturer's instructions (BT LAB Biotech Co. Ltd., Shanghai, Cat. No. E0456Ra). The sensitivity of the AMH ELISA was 0.1–40 ng/mL.

Statistical analysis

Statistical analyses were performed using the SPSS version 24.0 package program (SPSS Inc. and Lead Tech. Inc., Chicago, USA). The Shapiro-Wilk test was used for the normal distribution of the data. The Kruskal-Wallis test compared more than two variables that did not show normal distribution. The Kruskal-Wallis comparison was performed using the Mann-Whitney U test for pairwise comparisons between groups in the parameters that differed. Since AMH1 and AMH2 values showed normal distribution, dependent groups were compared using the paired-sample t-test. All results are presented as mean±SD. Results with $p<0.05$ were considered significant.

RESULTS

The group with the highest oocyte collection had an average of 10.13±0.64 in the S group, while the lowest number of oocytes was seen in the I/R group with 2.38±0.51. With the effect of enoxaparin, it was observed that the number of collected oocytes increased in the I and I+E ($p=0.000$) and I/R and I/R+E ($p=0.001$) groups (Figure 2).

When the number of MII oocytes was evaluated, statistically significant differences were observed between the I and I+E ($p=0.001$) and I/R and I/R+E ($p=0.000$) groups (Figure 2). When we compared the I and I+E and I/R and I/R+E groups, statistically significant differences were observed between the fertilized oocytes numbers. The order of p-values was 0.021 when I and I+E groups were compared and 0.011 when I/R and I/R+E groups were compared. Statistically significant differences were also observed between the groups in the number of two-cell embryos ($p=0.000$). While the number of two-cell embryos did not show statistically significant differences between S and S+E and I and I+E groups ($p>0.05$), the number of embryos on the second day was higher in the I/R+E group than in the I/R group (Figure 2).

While there was no statistically significant difference between the number of grade 2, grade 3, and grade 4 embryos between the I and I+E groups ($p>0.05$), there was a significant difference between the number of grade 1 embryos ($p=0.003$). Statistically significant differences were found in the number of grade 1 embryos between the I/R and I/R+E groups ($p=0.003$). When the number of grade 2, grade 3, and grade 4 embryos of the same groups were compared, no significant differences were observed ($p>0.05$).

To compare the effects of E application on AMH values, we examined the correlation between AMH1 and AMH2 values of the S+E, I+E, and I/R+E groups. It was observed that E application had positive effects on AMH concentration in the S+E, I+E, and I/R+E groups. A high degree of correlation was observed between AMH1 and AMH2 values in all three groups. The p-values were the same in all three groups ($p=0.000$ for both groups) (Figure 3).

DISCUSSION

The current management of ovarian torsion is to perform a surgical detorsion procedure to preserve ovarian reserve for

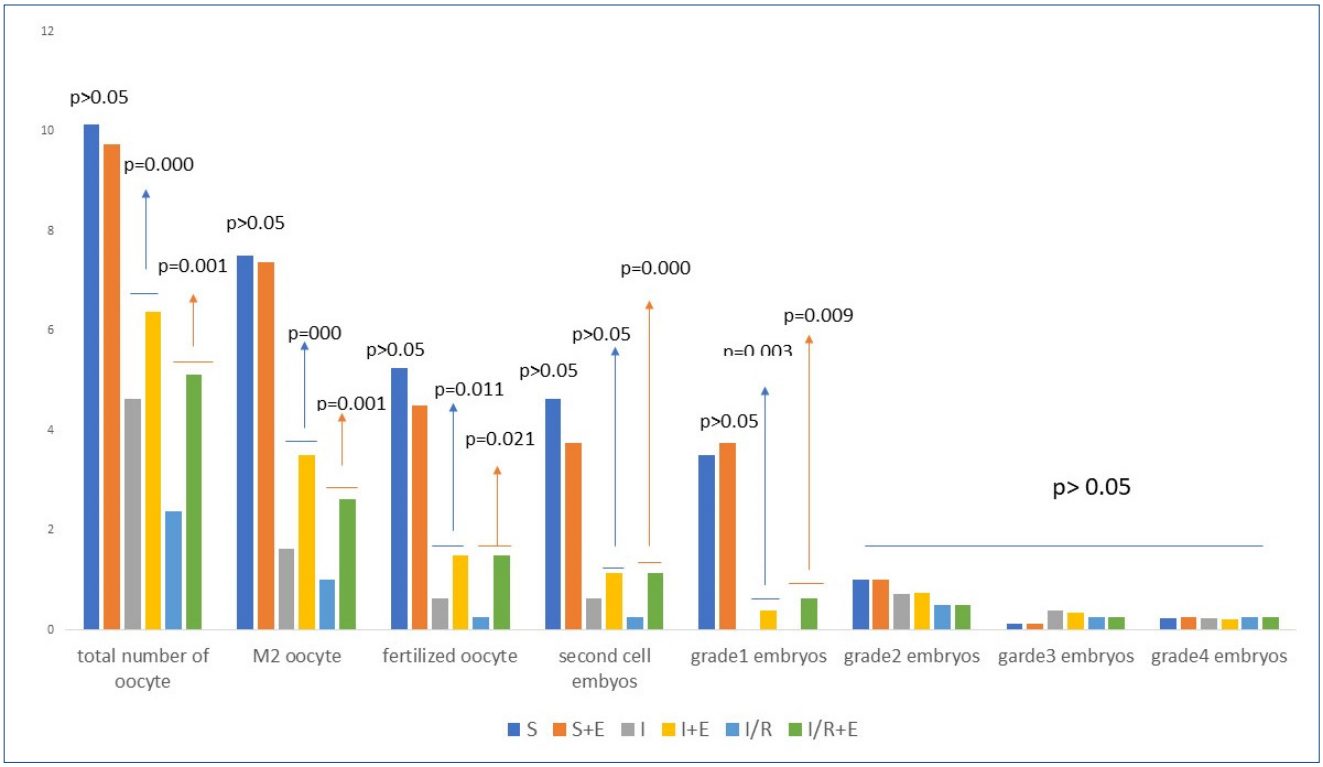

Figure 2. Comparison of the study groups' total oocyte count, metaphase II, fertilized oocyte counts, two-cell embryo, and embryo grade score. Statistical analysis between all groups was performed with the Kruskal-Wallis test. Pairwise comparisons were made with the Mann-Whitney U test (p<0.05 was considered statistically significant). All parameters were statistically significant between groups (p=0.000). No significant difference was observed in all parameters in pairwise comparisons between S and S+E groups. While the embryo Grade 1 score showed statistical differences between I-I/E and I/R-I/R+E groups, there was no statistical difference between grade 2, grade 3, and grade 4 embryos. S: Sham operation; I: Ischemia; I/R: Ischemia and reperfusion; S+E: Sham+Enoxaparin; I+E: Ischemia+Enoxaparin; I/R+E: Ischemia and reperfusion+Enoxaparin; M2: metaphase II.

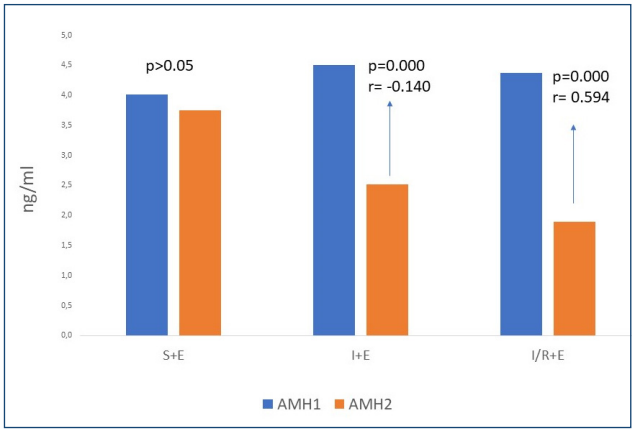

Figure 3. Anti-Müllerian hormone correlation graph between S+E, I+E, and I/R+E groups. There was no correlation between AMH1 and AMH2 values in the S+E group (p>0.05). A high level of correlation was observed between AMH1 and AMH2 concentrations in the I+E and I/R+E groups. AMH: anti-Müllerian hormone (ng/mL). Analysis was performed with the paired sample test (p<0.05 was considered statistically significant). S+E: Sham+Enoxaparin; I+E: Ischemia+Enoxaparin; I/R+E: Ischemia and reperfusion+Enoxaparin; AMH1: preoperative serum anti-Müllerian hormone level; AMH2: postoperative serum anti-Müllerian hormone level.

the continuation of fertility[4]. Reperfusion injury, called I/R injury, that occurs after surgical correction provided by detorsion can also cause serious problems on ovarian reserve. After detorsion, the reestablishment of oxygen in the ischemic cellular environment can trigger tissue injury by the activation of the proapoptotic signaling cascade and complement systems[16]. There are also studies on LMWHs and other molecules, in which the protective effect against oxidative stress in ovarian torsion is demonstrated by changes in histopathological and serum AMH levels[8,17,18].

In earlier tissue I/R studies, such as in cerebral I/R injury, and cardiac and hepatic toxicity models, LMWHs helped prevent cellular damage[19,20]. Enoxaparin has an anti-inflammatory effect in addition to acting as an anticoagulant[21]. This effect is mainly due to its ability to bind proteins such as chemokines, growth factors, enzymes, and adhesion molecules involved in the inflammatory process[21].

In the literature, it has been shown by vascular congestion and hemorrhage scores and histopathological and serum biochemical markers that LMWHs have a protective effect

in ovarian torsion cases[8,22]. These results do not include the number of oocytes obtained later and the number and status of embryos after fertilization. In our study, it is seen that the number of MII oocytes is lower in the I and I/R groups when compared to the I+E and I/R+E groups. It shows that E effectively prevents the effects of torsion and detorsion.

In our study, when I and I/R groups and I+E and I/R+E groups are compared, the number of grade 1 embryos is higher in E group. It shows that E treatment is protective against ovarian reserve damage, and this situation also affects the embryo quality. Evaluating the number of oocytes, the number of fertilized embryos, and their quality instead of histopathological evaluation in predicting fertility is the strength of our study. However, the most critical limitation of the study is that it does not show implantation rates and birth rates after embryo transfer, and there is no human study, although it is an animal study that provides usefulness in predicting clinical outcomes.

The decline of AMH concentrations in experimental ovarian torsion/detorsion injury has been reported in previous studies[8,23]. In our study, the decrease in AMH values was significantly higher in the I and I/R groups than in the I+E and I/R+E groups. To the best of our knowledge, this study reveals more meaningful results in terms of long-term effects, as it includes ovulation induction and IVF with three estrus cycles, rather than the early results provided by the histopathological evaluation performed in other torsion.

CONCLUSION

This is the first experimental study investigating the effects of enoxaparin therapy on ovarian reserve with IVF results. Enoxaparin has a protective effect against ovarian reserve damage caused by torsion and subsequent detorsion.

AUTHORS' CONTRIBUTIONS

MSB: Conceptualization (lead), Data curation (equal), Formal Analysis (equal), Funding acquisition (equal), Investigation (lead), Methodology (lead), Project administration (lead), Resources (equal), Software (equal), Supervision (equal), Validation (lead), Writing – original draft (lead), Writing – review & editing (lead). **ÖB:** Conceptualization (equal), Investigation (equal), Methodology (equal), Writing – original draft (equal), Writing – review & editing (equal). **HÇ:** Investigation (equal), Methodology (equal), Resources (equal). **OK:** Methodology (equal), Software (equal), Supervision (equal), Validation (equal), Visualization (equal). **ÖD:** Investigation (equal), Software (equal). **EÇ:** Methodology (equal), Software (equal), Supervision (equal), Validation (equal).

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